รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยผลของเยื่อหุ้มเซลล์ต่อการโตและ/หรือการรวมกลุ่ม ของผลึกแคลเซี่ยมอ๊อกซาเลท

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สนับสนุนโดยสำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

ABSTRACT

Calcium oxalate monohydrate (COM) kidney stone disease is one of the most common urological problems worldwide. Prevention of COM stone disease is still ineffective because of incomplete understanding in stone pathogenic mechanisms i.e., crystal growth and aggregation. Of particular, urinary cell membranes have been proposed as stone promoting factors. However, functional studies of cellular membranes on crystal growth and aggregation promotion have not been directly investigated and also required a pioneering study. This study then focused on examining lithogenic effects of urinary cell membranes. Three common urinary cells i.e., red blood cells (RBC), renal tubular cells using Madin Darby Canine kidney (MDCK) cell line as a model, and bacteria i.e., *E.coli* were prepared and tested at clinical related concentration. Crystal image analysis was performed to evaluate crystal morphological changes and to quantitatively measure crystal area and number of crystal aggregates. Spectrophotometric oxalate depletion assay was then performed to confirm growth lithogenic activity of urinary cell membranes. Interestingly, both fragmented and intact MDCK cells inhibited crystal growth but promoted crystal aggregation, whereas RBC membrane fragments, but not intact RBC, promoted growth and aggregation of COM crystals. *E.coli* showed crystal growth and aggregation promoting effects as same as those of fragmented RBC. This study provided direct evidence supported the hypothesis of urinary cell membranes as COM stone promoters and also successfully pioneered an approach to measure lithogenic promoting activity of substances of interest. Understanding the lithogenic roles of urinary cell membranes may lead to better management and prevention of COM stone formation and recurrence.

Keywords: Calcium oxalate; Cellular membranes; Crystal growth; Crystal aggregation; Stone promoter.

บทคัดย่อ

โรคนิ่วในไตชนิดแคลเซี่ยมอ๊อกซาเลทโมโนไฮเดรต (COM) เป็นโรคในระบบทางเดินปัสสาวะที่พบบ่อยที่สุดทั่วโลกโรคหนึ่ง แต่การป้องกันโรคนิ่วในไตชนิดนี้ยังไม่มีประสิทธิภาพมากนักเนื่องจากการขาดความเข้าใจกลไกการเกิดโรคนิ่ว เช่นกระบวนการโตและการรวมกลุ่มของตะกอนนิ่ว เยื่อหุ้มเซลล์ที่ปรากฏอยู่ในปัสสาวะถูกเสนอว่าอาจเป็นปัจจัยส่งเสริมให้เกิดโรคนิ่ว แต่ฤทธิ์ดังกล่าวของเยื่อหุ้มเซลล์ต่อการโตและการรวมกลุ่มของผลึกแคลเซี่ยมอ๊อกซาเลทยังไม่เคยมีการศึกษาและยังต้องการแนวทางการศึกษาวิจัยต้นแบบ การศึกษานี้จึงมุ่งวัดฤทธิ์ส่งเสริมการเกิดนิ่วของเยื่อหุ้มเซลล์ที่ปรากฏอยู่ในปัสสาวะของเซลล์สามชนิดคือ เซลล์เยื่อบุท่อไตชนิด Madin Darby Canine kidney (MDCK) เซลล์เม็ดเลือดแดง และเซลล์แบคทีเรียเช่น *E.coli* โดยทดสอบที่ความเข้มข้นเท่ากับในปัสสาวะของคนไข้ Crystal image analysis ถูกใช้ตรวจการเปลี่ยนแปลงรูปร่างและวัดขนาดของตะกอนนิ่ว รวมถึงนับจำนวนของตะกอนนิ่วที่มีการรวมกลุ่ม จากนั้นฤทธิ์ส่งเสริมการโตของตะกอนนิ่วจะถูกตรวจยืนยันโดย spectrophotometric oxalate depletion assay ผลการศึกษาพบว่าในเซลล์เยื่อบุท่อไตชนิด MDCK เซลล์ปกติและเศษเยื่อหุ้มเซลล์มีฤทธิ์เหมือนกันคือยับยั้งการโตแต่ส่งเสริมการรวมกลุ่มของตะกอนนิ่ว แต่ในเซลล์เม็ดเลือดแดงนั้น เศษเยื่อหุ้มเซลล์เม็ดเลือดแดงเท่านั้นที่ส่งเสริมการโตและการรวมกลุ่มของตะกอนนิ่วในขณะที่เซลล์เม็ดเลือดแดงปกติจะไม่มีฤทธิ์ดังกล่าว สำหรับเซลล์ *E.coli* พบว่ามีผลส่งเสริมการโตและการรวมกลุ่มของตะกอนนิ่วซึ่งคล้ายคลึงกับผลของเศษเยื่อหุ้มเซลล์เม็ดเลือดแดง การศึกษานี้สนับสนุนสมมติฐานเรื่องเยื่อหุ้มเซลล์ในปัสสาวะเป็นปัจจัยส่งเสริมการเกิดนิ่วในไตชนิด COM และประสบความสำเร็จในการบุกเบิกแนวทางการตรวจวัดฤทธิ์ส่งเสริมการเกิดนิ่วในไตด้วย ความเข้าใจบทบาทของเยื่อหุ้มเซลล์ต่อการส่งเสริมการเกิดนิ่วในไตอาจนำไปสู่แนวทางการดูแลคนไข้และการป้องกันการเกิดโรคนิ่วในไตชนิด COM ที่ดียิ่งขึ้นต่อไป

คำสำคัญ: แคลเซี่ยมอ๊อกซาเลท; เยื่อหุ้มเซลล์; การโตของผลึก; การรวมกลุ่มของผลึก; ตัวส่งเสริมการเกิดนิ่ว

EXECUTIVE SUMMARY

1. Background and Research Rational

Nephrolithiasis remains a public health problem around the world affecting 1 - 20% of the adult population and the prevalent is rising. The most common type is calcium oxalate (CaOx) kidney stones. Pathogenic mechanisms of stone formation remain unclear; however, these abnormal calcification processes may be involved by organic substances that incorporated into CaOx stones as same as those of biomineralization in other calcified tissues. Composition analysis of stone core matrix, that is the most inner part of kidney stones, showed several organic macromolecules including glycoproteins, carbohydrate, and membrane phospholipids. These substances also found in the urine and then were proposed as urinary stone modulators, which may function as inhibitors or promoting factors of CaOx crystallization including nucleation, growth, aggregation, and adhesion to renal tubular cell surface. According to theirs importance, stone research in the last decade were focused to study the effects of these modulators in CaOx stone formation. Although stone inhibitors and promoting factors of CaOx stones have been thought to play equally roles in stone pathogenesis, there were relatively less investigation and understanding of CaOx stone promoters. Most previous studies have been focused on stone inhibitors, for example, identification and characterization of citrate, nephrocalcin, osteopontin, urinary prothrombin fragment 1, bikunin, and urinary trefoil factor 1; whereas only a few numbers of promoters were well-identified, i.e. calcium and oxalate. It is necessary to identify additional stone promoters for better understanding of pathogenic mechanisms of stone disease.

Cellular membranes in the urine have long been hypothesized as a promoting factor of CaOx stones. Membrane degradation products from cell turnover and its phospholipid constituents could always be detected in human urine and has been thought to act as pre-existing nucleus for secondary nucleation of CaOx crystals. There were two evidences supported this hypothesis. First, lipids extracted from core matrices of CaOx stones are always detectable for phospholipids and lipid derivatives of cell membranes. Second, those lipids extracted and renal cell membrane vesicles isolated from kidney of Sprague-Dawley rats could promote nucleation of CaOx crystals. Both evidences suggested the potential roles

of cellular membranes on CaOx stone formation but restricted to nucleation process. Up to now, the functional studies of cellular membranes in the aggravation of crystal growth and aggregation have not been directly investigated and also required a pioneering study.

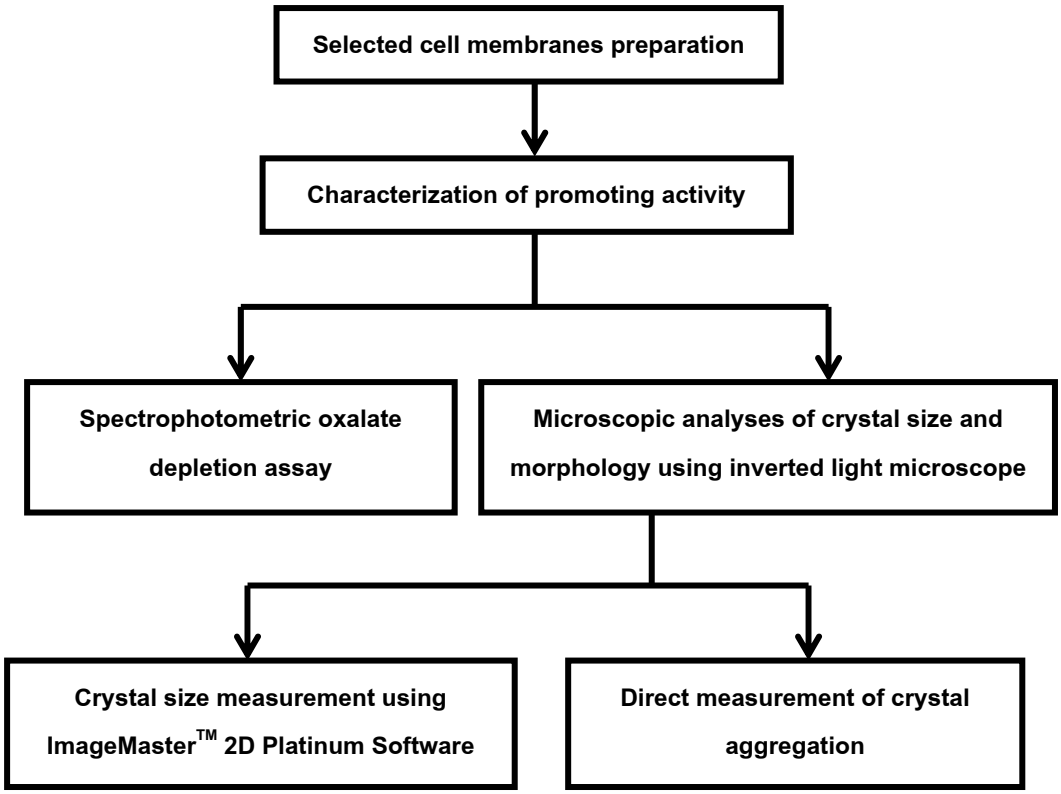
In this study, promoting activity of several cell membranes including red blood cells, renal tubular cells and bacteria on crystal growth and/or aggregation of CaOx monohydrate (COM) (the pathogenic form of CaOx) were examined. These selected urinary associated cells were collected and prepared for theirs fragmented membranes. Functional studies including spectrophotometric-based oxalate depletion assay and microscopic evaluation of COM crystal growth and aggregation using crystal image analysis were used to measure promoting activity of COM crystal growth and/or aggregation. Characterization of additional stone promoters and elucidating the COM crystal growth promoting activity of cellular membranes are beneficial for better understanding of the pathogenic mechanisms of COM stone formation, which may lead to better management and prevention of stone disease.

2. Objective

The specific aim of this proposed study is to examine whether the selected cell membranes including RBC, MDCK cells, and *E. coli* could promote COM crystal growth and/or aggregation or not.

3. Materials and Methods

Promoting activities of selected cell membranes were evaluated by various techniques as shown in this below figure.



4. Research Activities and Outputs

Period	Research activities	Outputs
Month 1-6	- Preparation of COM and CaOx dihydrate (COD) crystals - Morphological examination of COM and COD crystals by light microcopy and/or scanning electron microscopy - Culture of MDCK cells and *E. coli* - 1st Report	- Typical COM and COD crystals were successfully generated (confirmed by light and/or scanning electron microscopy) - MDCK cells and *E. coli* were successfully maintained
Month 7-12	- Harvest of MDCK cells and *E. coli* - Isolation of RBC from whole blood - Preparation of cell membranes - Establishment and optimization of	- All the required cells were ready for subsequent analytical steps - Cell membranes were successfully isolated - COM crystal growth promoting assay was established and optimized

	COM crystal growth promoting assay using positive and negative controls - 2nd Report	
Month 13-18	- Evaluation of promoting activity of various cell membranes on COM crystal growth and aggregation using the established assay - 3rd Report	- All analyses of promoting activity of various cell membranes on COM crystal growth and aggregation were done
Month 19-22	- Data analyses and summary - Manuscript preparation - Final report	- The data are summarized - The manuscripts are ready for submission or accepted for publication in international journals - Final report is submitted

5. Publications in International Journals/Manuscript in Submission

5.1. **Chutipongtanate S**, Thongboonkerd V.
RBC membrane fragments, but not intact RBC, promote growth and aggregation of calcium oxalate monohydrate crystals.
J Urol 2010; 184(2): 743-9
Impact factor 2009 = 4.016

5.2. **Chutipongtanate S**, Thongboonkerd V.
Renal tubular cell membranes inhibit growth but promote aggregation of calcium oxalate monohydrate crystals.
Chem Biol Interact 2010; 188(3): 421-6
Impact factor 2009 = 2.457

5.3. **Chutipongtanate S**, Suchitra Sutthimethakorn, Wararat Chiangjong and Thongboonkerd V.
Bacteria promote calcium oxalate crystal growth and aggregation.
(*Manuscript in submission*)

CONTENTS

CONTENTS (cont.)

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
°C	Degree Celsius
$CaCl_2$	Calcium chloride
$CaCl_2 \cdot 2H_2O$	Calcium chloride dihydrate
CaOx	Calcium oxalate
CBG-250	Coomassie blue G-250
CFU	Colony forming unit
$C_6H_5Na_3O_7 \cdot 2H_2O$	Trisodium citrate dihydrate
CO_2	Carbon dioxide
COM	Calcium oxalate monohydrate
COD	Calcium oxalate dihydrate
d	Day
E. coli	Escherichia coli
ESWL	Extracorporeal shockwave lithotripsy
et. al.	And others
FBS	Fetal bovine serum
g	Gram
h	Hour
HCl	Hydrochloric acid
HPF	High-power fields
H_3PO_4	Phosphoric acid
IVP	Intravenous pyelography
KCl	Potassium chloride
K. pneumoniae	Klebsella pneumoniae
λ	Lambda
l	Liter
LPS	Lipopolysaccharide

LIST OF ABBREVIATIONS (cont.)

MDCK	Madin Darby Canine Kidney
MEM	Complete Eagle's minimum essential medium
mg	Milligram
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
min	Minute
ml	Milliliter
mM	Millimolar
NaCl	Sodium chloride
$Na_2C_2O_4$	Sodium oxalate
$(NH_4)_2SO_4$	Ammonium sulfate
OD	Optical density
OPN	Osteopontin
PBS	Phosphate buffered saline
PNL	Percutaneous nephrolithotomy
R^2	Coefficient of determination
rpm	Revolutions per minute
S. aureus	Staphylococcus aureus
sec	Second
SD	Standard deviation
SEM	Standard error of the mean
S. pneumoniae	Streptococcus pneumoniae
RBC	Red blood cell
THP	Tamm-Horsfall protien
Tris	Hydroxymethyl aminomethane
µg	Microgram
µl	Microliter
µm	Micrometer
UTI	Urinary tract infection
x *g*	Times gravity

CHAPTER I
INTRODUCTION

Nephrolithiasis remains a public health problem around the world affecting 1 - 20% of the adult population (1) and the prevalent is rising (2). The most common type is COM kidney stones (3). Pathogenic mechanisms of stone formation remain unclear; however, these abnormal calcification processes may be involved by organic substances that incorporated into COM stones as same as those of biomineralization in other calcified tissues (4). Composition analysis of stone core matrix, that is the most inner part of kidney stones, showed several organic macromolecules including glycoproteins, carbohydrate, and membrane phospholipids (5, 6). These substances also found in the urine (6, 7) and then were proposed as urinary stone modulators, which may function as inhibitors or promoting factors of COM crystallization including nucleation, growth, aggregation, and adhesion to renal tubular cell surface (8, 9). According to theirs importance, stone research in the last decade were focused to study the effects of these modulators in COM stone formation. Although stone inhibitors and promoting factors of COM stones have been thought to play equally roles in stone pathogenesis, there were relatively less investigation and understanding of COM stone promoters. Most previous studies have been focused on stone inhibitors, for example, identification and characterization of citrate (10), nephrocalcin (11), osteopontin (12), urinary prothrombin fragment 1 (13), bikunin (14), and urinary trefoil factor 1 (15); whereas only a few numbers of promoters were well-identified, i.e. calcium and oxalate (16, 17). It is necessary to identify additional stone promoters for better understanding of pathogenic mechanisms of stone disease.

Cell membranes in the urine have been hypothesized to serve as promoter of COM kidney stone formation (6, 18). Membrane degradation products from cell turnover and their phospholipid constituents can be detected in human urine and has been thought to act as pre-existing nucleus for secondary nucleation of COM crystals (6). There are two lines of evidence supporting this hypothesis. First, extracts from core matrices of COM stones are always detectable for phospholipids and lipid derivatives originated from cell membranes (6). Second, the extracted lipids(6) and renal cell membrane vesicles isolated from the kidney of

Sprague-Dawley rats (19) can promote nucleation of CaOx crystals. Both evidences suggest the potential roles of cell membranes on COM kidney stone formation. However, the data are restricted only to effects of renal cell membranes in nucleation process, whereas functional studies on effects of membrane degradation products from other cells in other stone formation processes (i.e., crystal growth and aggregation) are also required.

Previous evidences showed that three urinary cell membranes including distal renal tubular cells, RBC and bacteria particularly *E. coli* may participate in COM stone disease as the promoting factors. Distal renal tubular cells have been known to play a crucial role in COM crystal-cell adhesion (9). It is possible that anionic molecules on renal tubular cell membranes may establish local negatively charged environment that may provide several modulatory effects on COM crystals; i.e., crystallization, growth, aggregation, and/or transformation. Lieske JC, *et al*. (20) have demonstrated that apical surface of renal tubular cells could induce transformation of COM to COD crystals; the crystalline type that generally occurs in high negatively charged environment such as normal human urine in the presence of COM stone modulators (21). Nonetheless, the modulatory effects of renal tubular cell membranes on COM crystals remain largely unknown. RBC is one of the most common cells found in the urine in many kidney diseases and related disorders. Several studies have demonstrated an association between CaOx nephrolithiasis and abnormalities in circulatory RBC (22, 23), while it is unclear (or even unknown) whether urinary RBC has any role in pathogenic mechanisms of COM stone formation. Interestingly, there is evidence demonstrating that crenate membrane fragments of RBC are found inside core matrices of CaOx stones (about 10% of all stones examined) (24). This data suggests that RBC membrane fragments may play some (still unknown) roles in the stone pathogenesis. *E. coli* is the most common cause of urinary tract infection. While most evidences indicated that bacteria were not an inducer of COM stone, few reports suggested that this metabolic stone sometimes had an infectious origin (25-27). This speculation was based on bacteriology and compositional analysis of the inside parts of stone (25-27). Of these, Sohshang HL, *et. al.*, (27) reported that about 47% of 100 calculi obtained from 100 stone patients had positive urine and stone nidus culture. *E. coli* was the most common organism isolated from the urine and stone nidus, while COM was the major stone nidus composition (27). It seemed that uropathogenic bacteria, i.e., *E. coli* could deposit inside the stone nidus and may play some roles in COM stone formation. Nevertheless, direct lithogenic activities of bacteria were not yet examined.

In this study, promoting activities of cell membranes on COM crystal growth and/or aggregation were verified. The selected urinary associated cells were collected and prepared for theirs fragmented membranes. Functional studies including spectrophotometric-based oxalate depletion assay (28) and microscopic evaluation of CaOx crystal growth and aggregation with image analysis using ImageMaster 2D-Platinum Software^{TM} (21) were used to measure promoting activity of COM crystal growth and/or aggregation. Characterization of additional stone promoters and elucidating the COM crystal growth promoting activity of cellular membranes will be beneficial for better understanding of the pathogenic mechanisms of COM stone formation, which may lead to better management and prevention of stone disease.

CHAPTER II
HYPOTHESIS AND OBJECTIVE

Hypothesis

It was hypothesized that the common urinary cell membranes including distal renal tubular cells, RBC, and *E. coli* could promote CaOx crystal growth and/or aggregation.

Objective

To examine lithogenic effects of Madin-Darby Canine Kidney cells (MDCK; a model of distal renal tubular cell), RBC and *E. coli* on COM crystal growth and aggregation using spectrophotometric oxalate depletion assay and microscopic image analysis of CaOx crystal growth and aggregation using ImageMaster 2D Platinum Software.

CHAPTER III
LITERATURE REVIEW

1. CaOx monohydrate (COM) kidney stone disease

1.1 General information

Kidney stone disease is a common public health problem over several regions of the world, particularly in the middle income countries (29-31). Recent epidemiologic data based on the national health and welfare survey 2003 also showed that kidney stone disease is one of the most common diseases affecting general Thai population (31), reflecting the burdens on socioeconomic costs and health care management (30, 31). The most common type of kidney stone disease is COM (3). COM stone has been sorted as multi-etiologic disease (3, 29). Numerous factors i.e., genetic, environment and metabolic impairment are proposed to involve in modulation (either aggravation or inhibition) of COM stone development (3, 29, 32). The symptoms of COM stones are mostly resulted from obstruction of renal collecting system. The intraluminal pressure, which is increased from obstruction, stretches nerve ending of mucosa and produces severe colicky flank pain. This pain is often accompanied by frequent urination, dysuria, hematuria, nausea, vomiting and psychogenic syncope. Chronic obstruction can eventually lead to a serious complication, particularly renal failure. Diagnosis of COM stone disease can be made upon clinical history, physical examination and several imaging techniques i.e., abdominal X-ray including kidney, ureter and bladder, intravenous pyelography (IVP), ultrasonography and computerized tomography (CT). COM stone disease can be cured by several modalities such as conservative treatment, extracorporeal shockwave lithotripsy (ESWL), percutaneous nephrolithotomy (PNL), ureteroscopic stone removal, and open surgery. However, stone patients frequently experienced the recurrent episode within 10-15 years following therapy, reflecting ineffective strategy of stone prevention which may be due to incomplete understanding in stone pathogenic mechanisms.

1.2 Pathogenic mechanisms

COM crystal growth, aggregation, transformation, and crystal-cell adhesion have been proposed as the basis pathogenic mechanisms of COM kidney stone disease. Although

the information of these mechanisms has still been incomplete, crystal growth and aggregation are two most initial processes of COM stone formation. Renal tubular fluid at distal part of nephron is supersaturated with respect to calcium and oxalate (approximately 100 times above solubility of CaOx in aqueous solution), so COM crystals are always spontaneously formed in the kidney (9). The newly formed COM crystals would subsequently consume free calcium and oxalate in the urine for crystal growth and aggregation, forming the larger crystalline complex that may occlude renal tubular lumens. Recent confocal microscopic study demonstrated that COM crystals generally grow by addition of substrates onto [121], [010] and [001] face of crystallographic direction (33). Once formed, these crystals may or may not retained in distal part of nephron depending on several factors including urinary calcium and oxalate levels, acidity (pH), fluid flowing and COM stone modulators (21, 34). COM stone modulators (including inhibitors and promoting factors) seem to be the most important factors determining renal crystal retention. Although both inhibitors and promoters are important for the pathogenesis of COM kidney stone disease, there are fewer investigations and less understanding of the stone promoters, as most of previous studies have focused mainly onto the stone inhibitors (e.g., citrate (10), nephrocalcin (11), osteopontin (12), urinary prothrombin fragment 1 (13), bikunin (14), and urinary trefoil factor 1 (15). Only few promoters are well documented (16, 17). It is therefore crucial to identify additional stone promoters for better understanding of the pathogenic mechanisms of COM kidney stone disease.

2. Cellular membranes and COM stone disease

Lipid residues are found as common components of organic matrices of normal calcified tissues and abnormal calcifications (35)Core organic matrices of all CaOx stones also contained some lipids (6, 35-37); approximately 10.15% by dry weight (35). Those lipid extracts were identified as phosphatidyl choline, phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl glycerol, sphingomyelin, cholesterol ester, triglyceride, and cardiolipin (35). Most of them especially acidic phospholipids were known as major components of cellular (35), except for cardiolipin which was a significant part of bacterial membranes (35). Function of lipids in core matrix was hypothesized as a nucleator of stones, which was supported by the evidence that lipid extracts from core matrix could catalyze CaOx crystallization in vitro (36). The presence of lipids and particularly phospholipids in the core matrix with promoting activity of COM crystallization indirectly suggested that cellular membranes, as a source of phospholipids, may involve in the

formation of CaOx stones.

Direct involvement of cellular membranes in COM stone disease may be expressed by 2 pathogenic pathways according to free and fixed theory of COM stones formation (38, 39). First, suspended cell membranes (free particle) may act as the pre-existed nucleus which aggravate heterogeneous nucleation of COM crystals and followed by crystal growth and aggregation (38, 39). Second, surface of intact renal epithelial cells (fixed particle) may act as the adhered sites for COM crystals that formed in tubular lumen. This adhesion then induces renal cell injury resulting in the formation of a fixed nucleus that followed by crystal growth and aggregation (38, 39). Both processes could lead to the obstruction in renal tubular lumen, followed by intratubular calcification, subsequently stone nucleus formation, and finally may result in COM stone disease (38, 39). In these pathogenic mechanisms, only cell-crystal adhesion was well approved. Lieske *et al.* demonstrated that COM crystals could rapidly adhere to surface of cultured renal tubular cells (9, 40). This adhesion was a selective process, that is, COM crystals were selective adhered to distal renal tubular cells but not proximal tubular cells (41, 42). The adhered crystals were then internalized (9, 40) and followed by several cellular responses (9, 43). These membrane-crystal adhesion and subsequent cellular response have been proposed as a crucial process in COM stone disease (9). Another mechanism that had been studied for promoting activity of membranes was crystal nucleation (19). Cell membrane vesicles isolated from kidney of kidney of Sprague-Dawley rats could promote crystallization of COM crystals (19) which partly reflected to the increase rate of crystal nucleation. Up to date, functional studies of cellular membranes in the activation of crystal growth and aggregation have not been investigated and required a pioneering study. At least, common cell membranes that existed in urine including distal renal tubular cells, red blood cells (RBC), and *E. coli* should be evaluated for their lithogenic activities.

2.1 Distal renal epithelial cells

Distal renal tubular cells locate at distal nephron where new CaOx crystals usually formed and play a crucial role in cell-crystal interaction (9). According to the current knowledge, this interaction was mediated by anionic sites of the intact distal tubular cell surface (9). Once COM crystals were adhered onto renal tubular cell surface, these crystals would be internalized via endocytosis (9), following by crystal degradation in endolyzosome (9). Several cellular responses were triggered during the interaction between renal tubular cell and COM crystals including altered gene expression (9, 43) cytoskeletal reorganization (9, 43), inflammatory cytokine production (44), oxidative stress (45) and renal tubular cell injury (46). Although the final outcome is still unclear, it is believed that these responses may

provoke detrimental effect leading to COM stone formation.

Despite advances in understanding of COM crystal-cell interaction, most previous studies have been focused on the mechanisms of crystal attachment and renal tubular cell responses (9, 44-48). In the other hand, the reverse effects of cell-crystal interaction on intraluminal COM crystals were rarely investigated. It is considerable that anionic molecules on renal tubular cell membranes generated local negatively charge environment affecting the nearby COM crystals. Beside crystal adhesion, this specific environment may also modulate COM crystallization, growth, aggregation, and/or transformation. Previous studies supported this hypothesis (19, 20). Fasano JM and Khan SR (19), demonstrated that renal cell membrane vesicles isolated from rat kidney could enhance rate of COM crystallization. In addition, Lieske JC, *et. al*., (20) demonstrated that apical surface of renal tubular cells could induce transformation of COM to COD crystals; the crystalline type that generally occurred in high negatively charge environment such as normal human urine in the presence of COM stone modulators (46). Nonetheless, the modulatory effects of renal tubular cell membranes on COM crystals have remained largely unknown.

2.2 RBC

RBC is one of the most common cells found in the urine in many kidney diseases and related disorders. Several studies have demonstrated an association between CaOx nephrolithiasis and abnormalities in circulatory RBC (22, 23, 49, 50). It is unclear (or even unknown) whether urinary RBC has any role in pathogenic mechanisms of CaOx stone formation. However, there is evidence demonstrating that crenate membrane fragments of RBC are found inside core matrices of CaOx stones (about 10% of all stones examined) (24). This data suggests that RBC membrane fragments may play some (still unknown) roles in the stone pathogenesis.

2.3 E. coli

E. coli is a gram negative bacterium and is the most common cause of urinary tract infection. Current knowledge indicates that urinary tract infection is not a cause of COM stone disease but only a complication due to accidental bacterial entrapment during stone formation (if UTI precedes stone formation) or the source of infection (if UTI follows stone formation) (51). However, few reports suggested COM stones sometimes had an infectious origin (25-27). This speculation was based on bacteriology and compositional analysis of the inside parts of stone (25-27). Of these, Sohshang HL, *et. al.* (27), reported that about 47% of 100 calculi obtained from 100 stone patients had positive urine and stone nidus culture. *E. coli* was the most common organism isolated from the urine and stone nidus, while COM was

the major stone nidus composition. (27) It seemed that *E. coli* could deposit inside the stone nidus and may play some roles in COM stone formation. To the best of our knowledge, at least two publications demonstrated lithogenic effects of bacteria indirectly (52, 53). Hirano S, *et. al.* (52), reported that bacteria including *E. coli* tended to aggregate crystalline and organic matters in the urine of stone formers and suggested these bacteria may participate actively in stone genesis as an adhesive agent. Venkatesan, N, *et. al.* (53), showed that *E. coli* and *P. mirabilis* aggravated CaOx encrustation onto the surface of polyurethane film, the same material as that of urinary stents. Investigators suggested that biofilm created by those bacteria might be responsible for CaOx encrustation. Nevertheless, direct lithogenic activities of those bacteria were not yet examined.

3. Assays of COM crystal growth and aggregation

Previously established functional assays in CaOx stone research were primarily aimed to measure inhibitory activity of urinary macromolecules; however, they could be applied to measure the promoting activity.

3.1 Spectrophotometric oxalate depletion assay

Oxalate depletion assay was first described in 1981 by Nakagawa et al. for evaluation of CaOx crystal growth inhibitory activity of urinary proteins (54). The principle of this assay is to measure the decrease of oxalate ions due to CaOx crystal seeds consumption. There were 2 detection systems for measurement of soluble oxalate ions levels for this assay; liquid scintillation counter to measure soluble ^{14}C-oxalate (54) and spectrophotometry with optical density of 214 nm monitoring to measure soluble oxalate concentration (28). According to the principle of this assay, it could be applied to measure crystal growth promoting activity by change the method of interpretation.

3.2 Crystal turbidity assay

Crystal turbidity assay could measure the levels of crystal aggregation by monitoring degree of light scattering of the solution containing CaOx crystals using spectrophotometer with optical density of 620-nm (14). The change of turbidity is directly correlated to levels of crystal aggregation. This assay is simple, reliable, and reproducible; however, it has some limitations. First, crystal turbidity assay could not apply to measure the activity of insoluble samples or suspensions such as cell membranes because those samples could disperse the light path which then interfere turbidity monitoring. Second, there is some degree of crystal growth in the reaction which interfere the turbidity values of crystal aggregation and lead to misinterpretation of the results.

3.3 Crystal image analysis

Microscopic evaluation combined with image analysis using ImageMaster 2D Platinum Software^{TM} was firstly established by Thongboonkerd *et. al.* to simultaneously measure CaOx crystal growth and aggregation under the presence of stone inhibitors including nephrocalcin and trefoil factor 1 (21). Inhibition of crystal growth can be directly examined by visual evaluation and quantitative measurement of crystal area, whereas number of aggregates were counted and averaged per high power field (21). This assay offers simplicity, reproducibility, and reliability and can be applied to measure promoting activity of crystal growth and also crystal aggregation.

CHAPTER IV
MATERIALS AND METHODS

1. Preparation of COM and COD crystals

COM crystals were prepared in a 24-well, polystyrene, disposable cell culture cluster (with lid) (Corning Inc., Corning, NY) by making a final concentration of 5 and 0.5 mM of calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) and sodium oxalate ($Na_2C_2O_4$), respectively, in 10 mM Tris-HCl buffer (pH 7.4) containing 90 mM sodium chloride (NaCl). This crystallization reaction was incubated without agitation at 25°C. After 1-h crystallization, the supernatant was discarded, whereas the crystals were gently washed with 100% methanol and then dried in room temperature. COD crystals were prepared in 24-well polystyrene plate (with lid) (Corning) by adding $CaCl_2$ and $Na_2C_2O_4$ to a final concentration of 6.27 and 1.6 mM, respectively, in a buffer (pH 6.5) containing 9.6 mM trisodium citrate dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$), 11.6 mM magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), and 63.7 mM potassium chloride (KCl). The solution was continuously gently mixed by a vertical shaker at 25°C for 1-h before harvesting.

2. Preparation of cellular membranes

2.1 MDCK membrane fragments

Approximately 1 x 10^5 MDCK cells were inoculated in each well of a 24-well, polystyrene, disposable cell culture cluster (with lid) (Corning Inc.; Corning, NY) containing complete Eagle's minimum essential medium (MEM) (GIBCO, Invitrogen Corporation; Green Island, NY) supplemented with 10% fetal bovine serum (FBS), 1.2% penicillinG/streptomycin and 2 mM glutamine. The cultured cells were maintained in a humidified incubator at 37°C with 5% CO_2 for 24 h. Thereafter, MDCK cells were harvested by scraping and then washed 5 times with an isotonic buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl to remove culture media and contaminations. MDCK cell numbers were determined using a hemacytometer and 1 x 10^4 cells were used for each assay. To prepare MDCK membrane fragments, equal number of MDCK cells (1 x 10^4 cells/assay) were sonicated in the isotonic buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl

using a probe sonicator (Bandelin Sonopuls HD 200; Bandelin electronic; Berlin, Germany) at MS 72/D (50 cycles) for 15 s in an icebox. MDCK membrane fragments were then isolated using a centrifugation at 10,000 x *g* for 5 min. Membrane fragmentation was confirmed by microscopic examination.

2.2 RBC membrane fragments

Packed RBC sample collected from a normal healthy donor was obtained from our institutional blood bank following the principles of the Helsinki Declaration on the study using human samples. The cells were then washed 5 times with an isotonic buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. Numbers of RBC were determined using a hemacytometer and 2 x 10^5 cells were used for each assay. To prepare RBC membrane fragments, equal number of cells (2 x 10^5 cells/assay) were resuspended in deionized (18 MΩ·cm) water to allow hypoosmotic-induced cell lysis. Microscopic examination showed that the cells were completely disrupted and membrane fragmentation occurred after hypoosmotic-induced cell lysis for 5 min. RBC membrane fragments were then isolated using a centrifugation at 10,000 x *g* for 5 min.

2.3 Bacterial cells

To prepare *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*, a single colony of each bacterium was inoculated from a freshly streak agar plate by a sterile metal loop and then transfer to 5 ml LB Broth (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) (BD Diagnostic Systems; Frankin Lakes, NJ) in a sterile glass culture tube with a plastic cap. After mixing, the culture tubes were incubated in a rotating incubator at 37°C overnight. Bacterial cultures were then harvested during logarithmic phase. After estimation of bacterial concentration, bacteria were harvested by centrifugation at 10,000 x g for 5 min and washed 3 times with sterile isotonic saline to eliminate media contaminations. Bacteria were freshly prepared and immediately used at 10^5 CFUs/ml for each assay. Bacterial concentrations were estimated by plate colony counting combined with turbidimetry at an optical density of 600 nm (OD_{600}). Briefly, 10 µl medium from 2-fold serial dilutions (1:1–1:1024) of each bacterial culture were plated on agar plates and incubated at 37°C overnight, counted the numbers of bacterial colony, and calculated for the concentrations as colony forming units per ml (CFUs/ml). Turbidity at OD_{600} of those bacterial suspensions was measured by a spectrophotometer at the time of bacterial plating. Linear regression analysis was performed to predict the correlation of bacterial concentrations and the measured OD_{600} values. Range of linearity was selected and validated by coefficient of determination (R^2). Linear regression equation, range of linearity at OD_{600} and R^2 were reported as followed (where x was OD_{600}

value and y was bacterial concentration x 10^6 CFUs/ml); *E. coli*, y = 647.3x + 42.007, OD_{600} of 0.015-0.960, R^2 = 0.9971; *K. pneumoniae*, y = 1111x + 1.4525, OD_{600} of 0.023-1.021, R^2 = 0.9996; *S. aureus*, y = 1753.7x + 35.848, OD_{600} of 0.034-0.944, R^2 = 0.9962; *S. pneumoniae*, y = 67.386x + 0.8032, OD_{600} of 0.016-0.198, R^2 = 0.9976. As a result, the clinical-related concentration of 10^5 CFUs/ml was accurately prepared by measuring OD_{600} values within the established linear range of each bacterium.

3. Staining of RBC membrane fragments by modified blue silver dye

Modified blue silver reagent (0.3% w/v Coomassie blue G-250 (CBG-250), 2% H_3PO_4, 10% $(NH_4)_2SO_4$, 20% methanol) was used to visualize and localize transparent RBC membrane fragments. This staining could be used also to demonstrate adhesion of RBC membrane fragments on surfaces of CaOx crystals. The reagent was prepared using the same protocol as described previously (55), but with a modest modification (by increasing the amount of CBG-250 dye from 0.12% to 0.3%) to enhance the sensitivity of cell membrane detection. To stain RBC membrane fragments that adhered onto surfaces of CaOx crystals, the modified blue silver reagent was added into the reaction well with a ratio of 1:20 v/v of dye reagent per crystal solution. After incubation at 25°C for 1 h, while crystals and RBC membrane fragments lied at the bottom of reaction well, the dye solution was gently removed. Crystals and RBC membrane fragments were washed several times with a buffer containing 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl until the discarded solution was colorless. Finally, the adhesion of RBC membrane fragments on surfaces of CaOx crystals was observed under the Olympus CKX41 inverted light microscope. The numbers of crystals with adherent RBC membrane fragments were counted from at least 100 crystals in individual samples.

4. COM crystal image analysis for growth, aggregation and transformation

The effects of cellular membranes on CaOx crystal morphology and aggregation were first evaluated by phase-contrast microscopic examination using an Olympus CKX41 inverted light microscope (Olympus Co. Ltd.; Tokyo, Japan). Thereafter, quantitative measurement of crystal size, aggregation and transformation were performed by image analysis using ImageMaster™ 2D Platinum software (GE Healthcare; Uppsala, Sweden), which can accurately measure crystal area (21). The crystal size was measured from at least 100 crystals in individual samples. COM crystal numbers (a representative of the rate of COM crystallization) and COD crystal number (a representative of COM-to-COD crystal

transformation) were counted in at least 10 high-power fields (HPF) and reported as their average numbers. COM crystal mass (the total products of the reaction of COM crystallization and growth) was calculated by the following equation;

Crystal mass (pixels/HPF) = Crystal area (pixels) x Crystal number (No./HPF)

A crystal aggregate was defined as an assembly of individual crystals (two or more crystals) tightly joined together. Numbers of aggregates were counted in at least 10 HPF (original magnification power of 400X) of individual samples.

5. Spectrophotometric oxalate-depletion assay to monitor COM crystal growth

Effects of cellular membranes on COM crystal growth were confirmed and monitored by using spectrophotometric oxalate-depletion assay (28). COM crystal seeds (160 μg) were added to 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Basically, the seeded crystals would grow further using excess calcium ions (from $CaCl_2$) and oxalate ions (from $Na_2C_2O_4$), leading to depletion of free oxalate, which is detectable by UV-visible spectrophotometry at λ214 nm. Oxalate depletion (which reflects crystal growth) was then monitored for up to 60 min during the reaction, with or without cellular membranes, using a Shimadzu UV-160A spectrophotometer (Shimadzu; Kyoto, Japan). Alternatively, rate of free oxalate depletion could be calculated using the baseline value and the value after 60-sec incubation with or without cellular membranes. The relative promoting activity was calculated using the following equation;

% CaOx crystal growth promotion = [(C-S)/C] x 100

Where C = Rate of free oxalate depletion without cellular membranes

S = Rate of free oxalate depletion with cellular membranes

6. COM crystal aggregation-sedimentation study

COM crystal aggregation-sedimentation study was performed as described previously by Atmani F and Khan SR (14) with a modification of $CaCl_2$ and $Na_2C_2O_4$ concentrations (from original concentrations of 3 mM $CaCl_2$ and 0.5 mM $Na_2C_2O_4$) (14) to prevent spontaneous COM nucleation. Individual COM crystals (100 μg) were added into 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 in a cuvette. The reaction was stirred at 800 rpm to prevent sedimentation of individual crystals but not the large aggregates. Cellular membranes were added before adding COM crystals. Crystal aggregation process would consume a number of individual

COM crystals to form larger particulates. Sedimentation of those aggregates was then resulted in reduction of solution turbidity, which can monitor by a Shimadzu UV-160A spectrophotometer (Shimadzu) at λ620 nm for 60-min. Aggregation promotion was evaluated by comparing the turbidity slope in the presence and absence of bacteria to be test at the end of the assay. In addition, morphology of the aggregates generated by this reaction was observed under a phase contrast microscope to reconfirm crystal aggregation potency of bacteria. The maximal diameter of the largest COM aggregate in each HPF (but not the smaller aggregates or individual crystals) was measured by Tarosoft® Image framework v.0.9.6 (Nikon Corp.; Tokyo, Japan). Each data point was derived from 5 individual samples and the data are reported as mean ± SEM.

7. Statistical analysis

All the quantitative data are reported as mean ± SEM. Multiple comparisons were performed using ANOVA with Tukey's post-hoc test (SPSS, version 11.5). *P* values < 0.05 were considered statistically significant.

CHAPTER V
RESULTS

Three types of cellular membranes were prepared successfully as described in "**Materials and Methods**" section. Effects of each membrane type on COM crystal growth and aggregation were reported as followed.

1. Lithogenic effects of MDCK cell membranes on COM crystal growth and aggregation

Figure 1 showed phase-contrast microscopic examination COM crystal size and morphology in the absence (control) or presence of intact MDCK cells or membrane fragments. Both intact cells and membrane fragments had similar effects on COM crystals – they reduced COM crystal size but aggravated crystal aggregation and induced COM-to-COD crystal transformation as compared to the control. However, the membrane fragments tended to have greater promoting activity on crystal aggregation than that of the intact cells.

Quantitative analyses were performed using ImageMaster^{TM} 2D Platinum software. **Figure 2A** clearly shows that MDCK intact cells and membrane fragments had significant inhibitory effects on COM crystal growth (approximately 22.6% and 25.2% decreases in crystal size, respectively). We also evaluated crystal numbers (which reflected COM crystallization) and total crystal mass (which reflected the final product of COM crystallization and growth). The results demonstrated that both intact cells and membrane fragments did not affect crystal numbers (**Figure 2B**) but significantly reduced COM total crystal mass (approximately 23.1% and 25.6% decreases in total crystal mass, respectively) (**Figure 2C**). However, both intact cells and membrane fragments had a significant promoting effect on COM crystal aggregation (approximately 1.9-fold and 3.2-fold increases in the number of COM crystal aggregates, respectively) (**Figure 2D**). Between both forms of MDCK membranes, the membrane fragments had a more potent promoting effect on COM crystal aggregation compared to intact MDCK cells. **Figure 2E** demonstrates that both intact cells and their membrane fragments could transform COM to COD crystals at a comparable degree, whereas there was no COM-to-COD crystal transformation observed in the control.

To confirm and monitor the COM crystal growth inhibitory effect of MDCK intact cells and membrane fragments, a spectrophotometric oxalate-depletion assay (the gold

standard method for assessment of COM crystal growth) was performed. **Figure 3** shows that both forms of MDCK membranes significantly inhibited COM crystal growth (as the rate of depletion of free oxalate ions was significantly less than that of the control) at comparable degrees from 15 min post-incubation through the end of the assay (60 min). These data strengthened our initial results and indicated that both forms of MDCK membranes had a significant inhibitory effect against COM crystal growth.

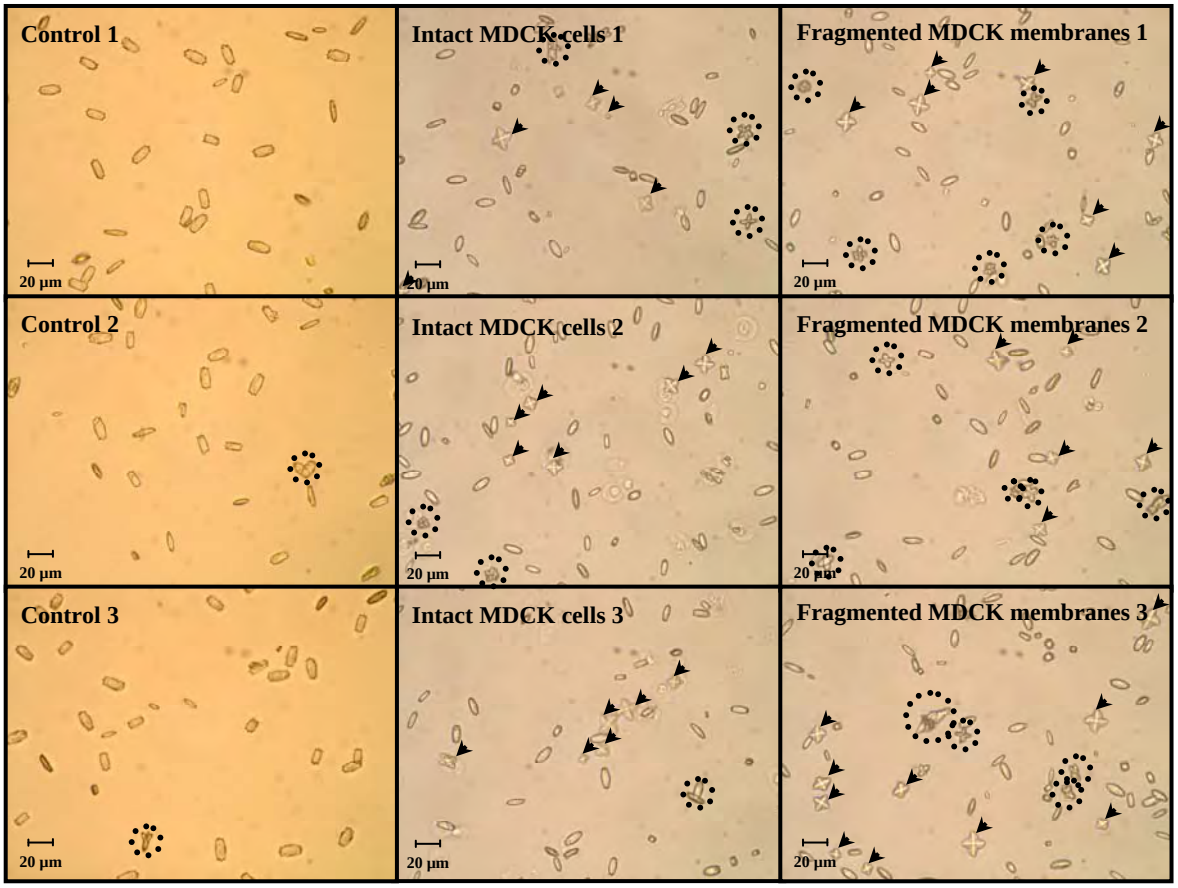


Figure 1. Morphological evaluation of modulatory effects of MDCK intact cells and membrane fragments on COM crystals. COM crystals were generated in absence (control) or presence of intact cells or membrane fragments (see details in "Materials and Methods"). Dashed circles indicate COM aggregates (which were defined as groups of two or more COM crystals tightly adhered together). Arrow heads indicate COM-to-COD crystal transformation. All experiments were done in triplicate. Original magnification = 400X for all panels.

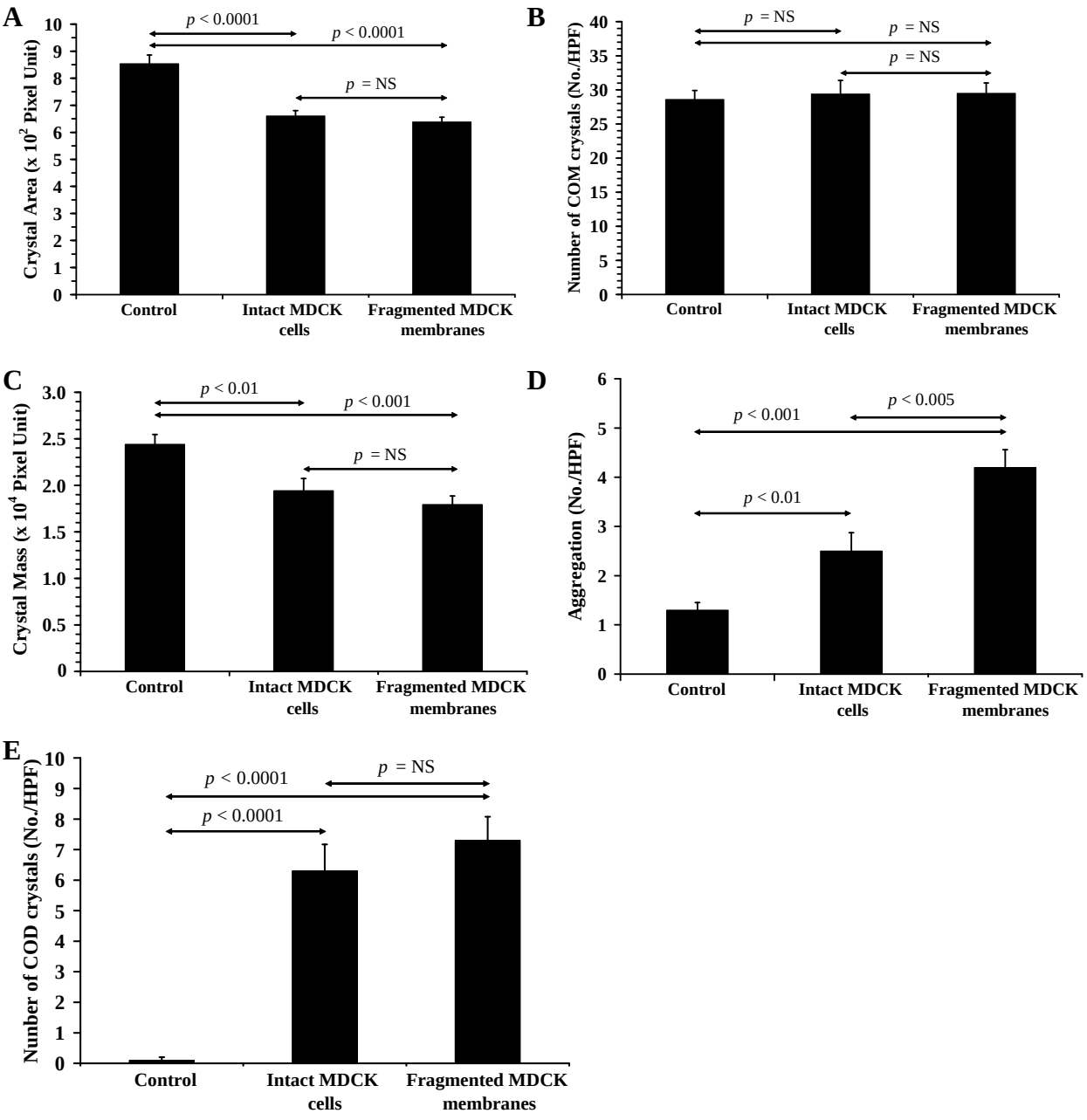


Figure 2. Crystal image analysis of lithogenic effects of intact and fragmented MDCK membranes. COM crystal size **(A)**, number of COM crystals **(B)**, COM total crystal mass **(C)**, number of COM crystal aggregates **(D)**, and number of COD crystals **(E)** affected by MDCK intact cells or membrane fragments were shown. Each bar was derived from 3 independent experiments and the data are reported as mean ± SEM. NS = not significant.

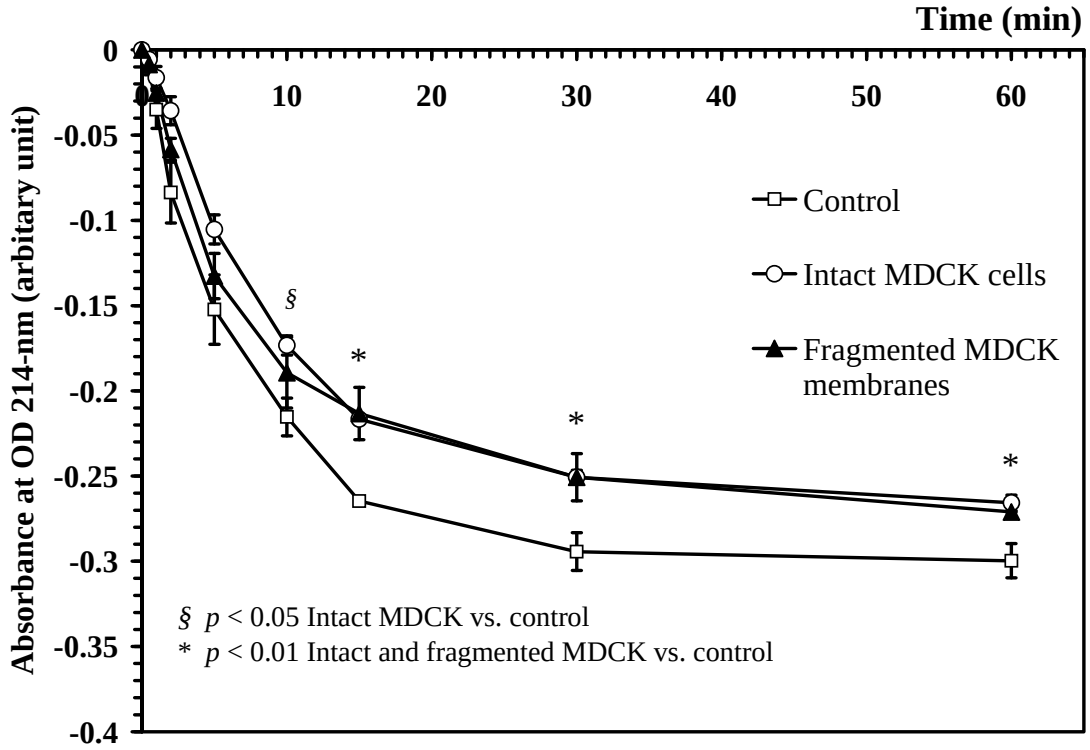


Figure 3. Spectrophotometric oxalate-depletion assay of lithogenic effects of intact and fragmented MDCK membranes. COM crystal seeds (160 μg) were added to 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Levels of free oxalate-depletion (which reflected crystal growth) was then monitored by measuring absorbance at λ214 nm for up to 60-min in the absence or presence of MDCK intact cells or membrane fragments (equivalent to 1 x 10^4 cells per assay). Each data point was derived from 3 independent experiments and the data are reported as mean ± SEM.

2. Lithogenic effects of RBC membranes on COM crystal growth and aggregation

Figure 4A showed phase-contrast microscopic examination COM crystal size and morphology in the absence (control) or presence of intact RBC or their membrane fragments. Intact RBC did not affect COM crystal size but could transform COM crystal (which has the most potent adsorptive capability) to COD crystal (which has the least adsorptive capability) (56). In contrast, RBC membrane fragments caused obvious enlargement of individual COM crystals and increased number of COM aggregates (groups of two or more COM crystals adhered together). **Figure 4B** shows that the modified blue silver could stain RBC membrane fragments (which were present as greenish blue-colored cell ghosts). The visualized membrane fragments were also present on COM crystal surfaces, indicating their adhesion with COM crystals. Approximately 50% of COM crystals were adhered by RBC membrane fragments. There was no adhesion between intact RBC and COM crystals observed (**Figure 4A**).

Quantitative crystal image analysis was further performed by ImageMaster^{TM} 2D Platinum software to measure COM crystal size, which reflects the crystal growth. **Figure 5A** shows that RBC membrane fragments significantly promoted COM crystal growth (approximately 75% increase in crystal size, compared to the control), whereas intact RBC had no effects on crystal growth. Additionally, **Figure 5B** demonstrates that RBC membrane fragments significantly promoted the formation of COM aggregates (approximately 2.5-fold increase in number of aggregates, compared to the control), whereas intact RBC had no effects on number of COM aggregates. **Figure 5C** shows that intact RBC could transform COM to COD crystals as the number of COD crystals were increased significantly. Approximately 14.5 % of initial COM crystals were transformed to COD crystals after incubation with intact RBC, whereas RBC membrane fragments had no effects on crystal transformation.

To further confirm the morphological data and to monitor effects of RBC membrane fragments on COM crystal growth, we performed spectrophotometric oxalate-depletion assay. **Figure 6** shows that RBC membrane fragments significantly aggravated COM crystal growth (as the depletion of free oxalate ions was significantly greater than the control) from 2 min of incubation through the end of the assay (60 min), consistent to the data obtained from morphological study. Although intact RBC showed a slight delay of crystal growth during 10-30 min of incubation, it had no significant effects on crystal growth at 60-min of incubation, consistent to the morphological data.

Effects of intact RBC and RBC membrane fragments on COD crystals were also evaluated. However, **Figure 7** shows that both morphological and quantitative data of COD crystals did not change significantly by intervention with intact RBC or RBC membrane fragments. Therefore, this result suggested that the crystal growth promoting activity of RBC membrane fragments was selectively to COM crystals.

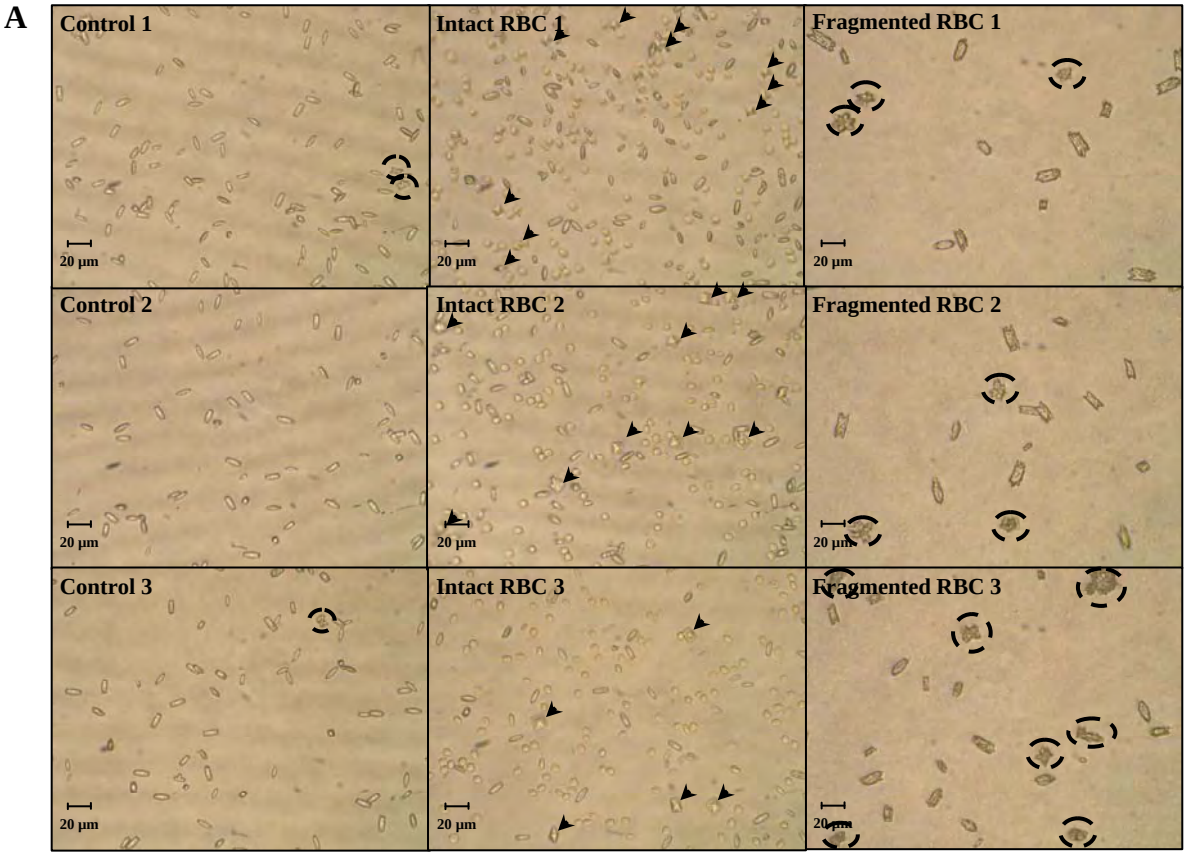


Figure 4. Effects of intact RBC and RBC membrane fragments on COM crystals. (**A**) COM crystals were generated in absence (control) or presence of intact RBC or RBC membrane fragments. The results clearly indicated that RBC membrane fragments could promote COM crystal growth and aggregation, whereas intact RBC had no effects on COM crystal growth and aggregation but could transform COM crystals to COD crystals. (**B**) Modified blue silver reagent was used to stain RBC membrane fragments, some of which adhered onto COM crystal surfaces (illustrated as zoom-in insets). Dashed circles indicate COM aggregates, which were defined as groups of two or more COM crystals adhered together. Arrow head indicates COD crystal transformation. All experiments were done in triplicate. Original magnification = 400X for all panels.

B

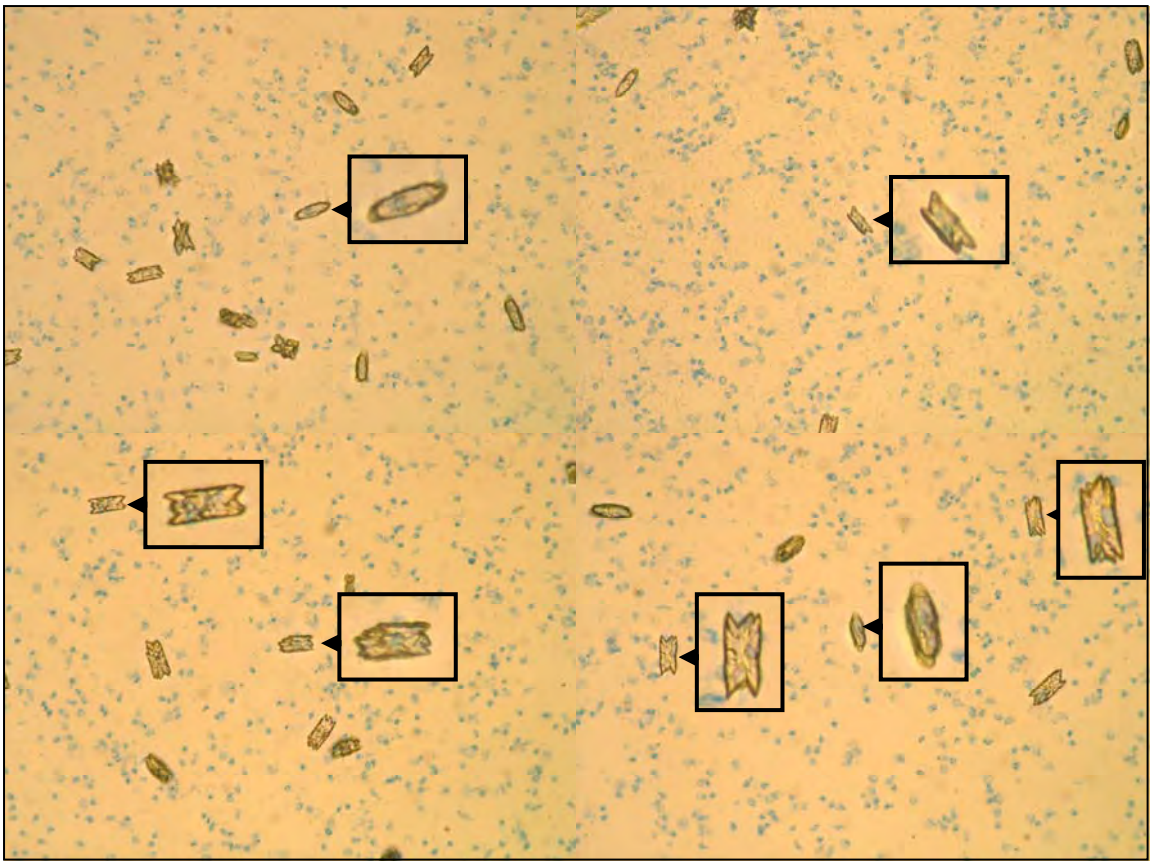


Figure 4. (continued)

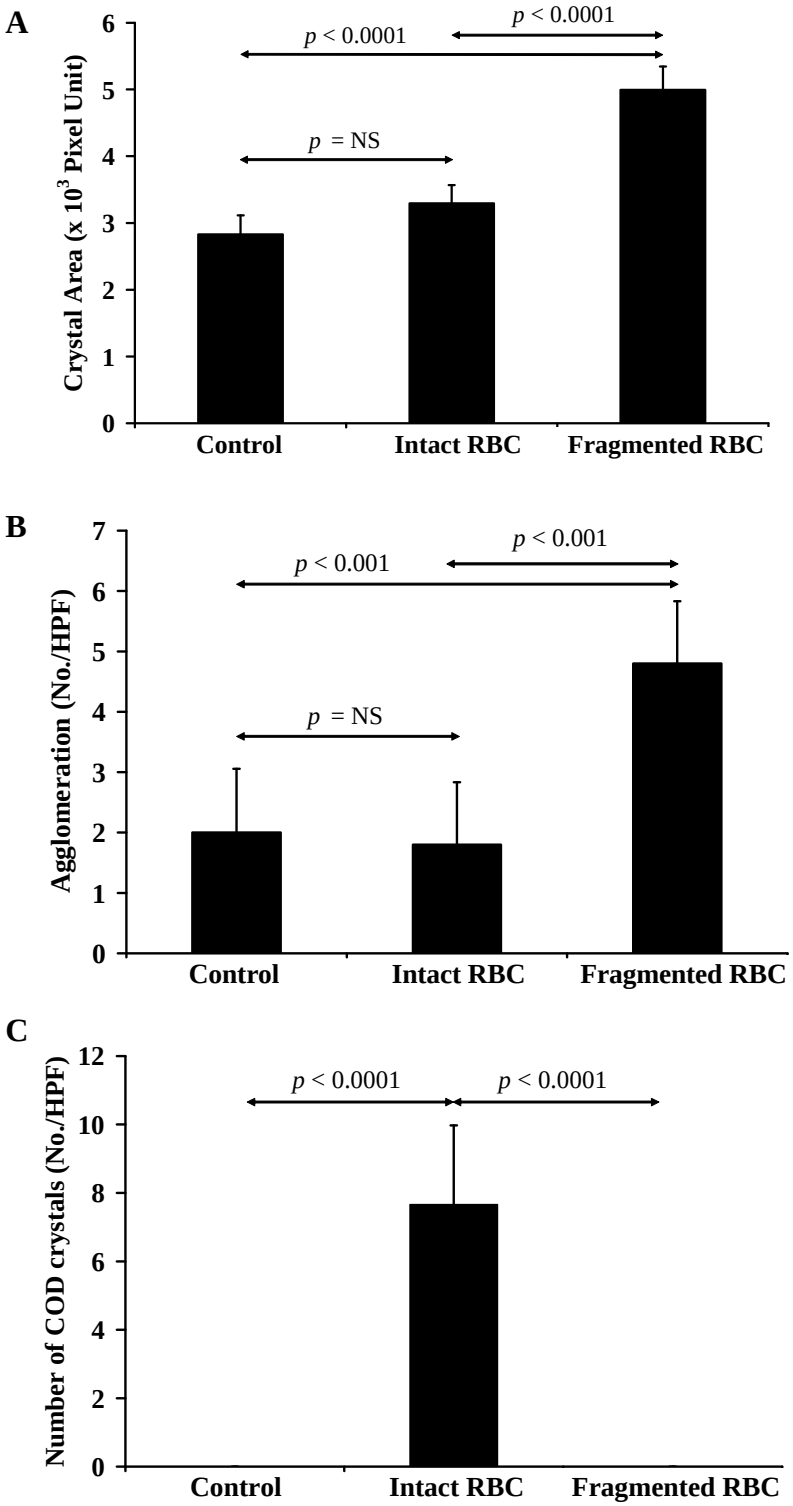


Figure 5. Crystal image analysis of lithogenic effects of intact and fragmented RBC. COM crystal size **(A)**, number of COM crystal aggregates **(B)**, and number of COD crystals **(C)** affected by RBC membrane fragments or intact RBC were shown. Each bar was derived from 3 independent experiments and the data are reported as mean ± SEM. NS = not significant.

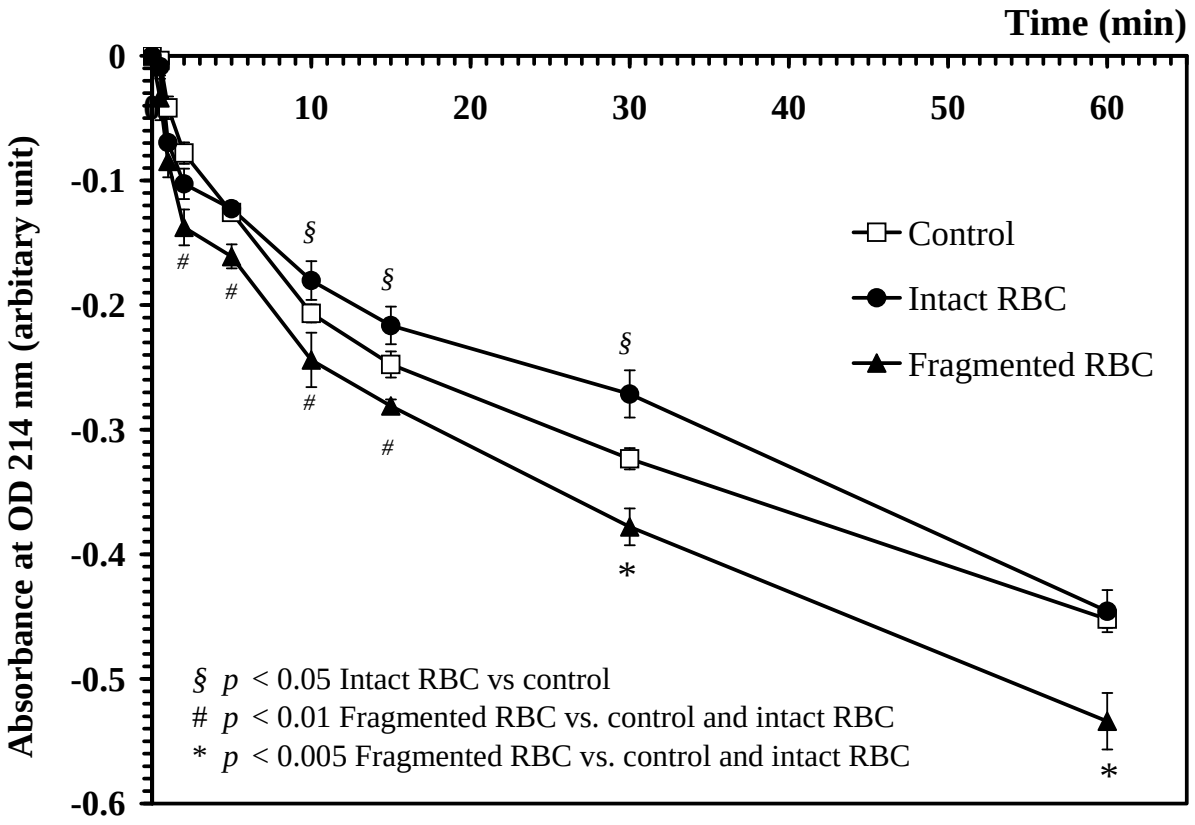


Figure 6. Spectrophotometric oxalate-depletion assay of lithogenic effects of intact and fragmented RBC. COM crystal seeds (160 μg) were added to 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Oxalate depletion (which reflects crystal growth) was then monitored by measuring absorbance at λ214 nm for up to 60 min during the reaction, with or without cells or their membrane fragments (equivalent to 2 x 10^5 cells per assay). Each data point was derived from 3 independent experiments and the data are reported as mean ± SEM.

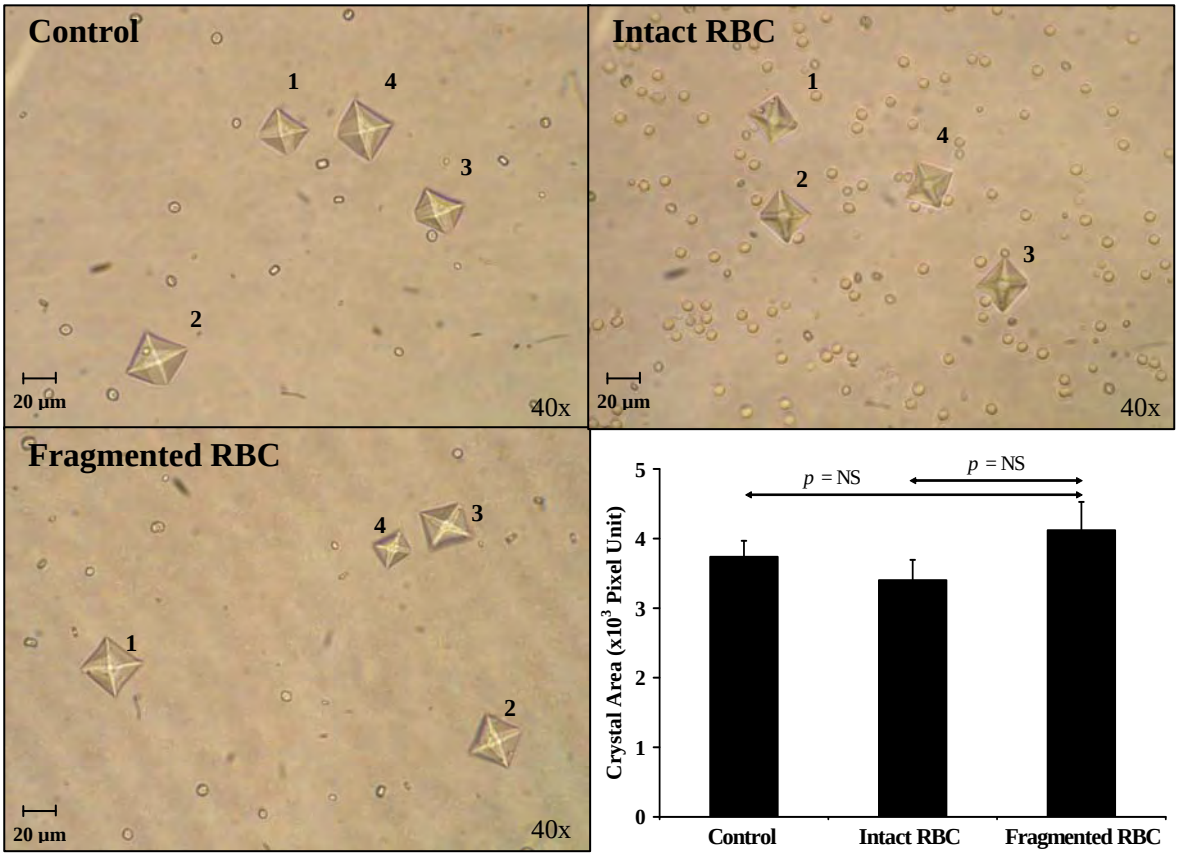


Figure 7. Effects of intact RBC and RBC membrane fragments on COD crystals. COD crystals were generated in absence (control) or presence of intact RBC or RBC membrane fragments (detailed in "Materials and Methods"). Original magnification = 400X for all panels. Each bar was derived from 3 independent experiments and the data are reported as mean ± SEM. NS = not significant.

3. Lithogenic effects of *E. coli* on COM crystal growth and aggregation

In addition to *E. coli*, other bacteria including *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were also examine lithogenic activities to determine whether stone promoting effects were confined to only uropathogenic bacteria or not. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were propagated in LB Broth before estimation of bacterial concentrations by plate colony counting and turbidimetry at OD_{600}. Correlations of bacterial concentrations from plate colony counting and OD_{600} values were analyzed by linear regression analysis (as presented in **"Materials and Methods"** section). By this method, a clinical relevant concentration of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* was accurately prepared for subsequent analyses.

COM lithogenic activities of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were initially screen by phase contrast microscopy. Fragmented RBC (which had promoting effects) and intact RBC (which had no modulating effect) were adopted as the positive and negative controls, respectively. Consistent with previous result, morphological data showed that fragmented RBC could promote COM crystal growth (as represented by increase in crystal size) and aggregation (as represented by increase in number of COM aggregates) as compared to the blank control, whereas intact RBC had no promoting activity [**Figure 8A**]. Interestingly, *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* also promoted COM crystal growth and aggregation [**Figure 8A**]. These observed effects of bacteria were clearly demonstrated by quantitative analyses of COM crystal area [**Figure 8B**] and number of crystal aggregates [**Figure 8C**]. The initial results suggested that *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* had potential lithogenic effects on COM growth and aggregation.

To confirm the promoting effect on COM crystal growth, spectrophotometric oxalate depletion assay was performed. At 60-sec of monitoring, fragmented RBC (the positive control) promoted 23.67±3.36% of crystal growth, whereas intact RBC (the negative control) had no promoting effect (0.85±2.86%) as comparable to that of blank control (0.42±1.12%) [**Figure 9**]. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* showed crystal growth promoting activity of 20.17±3.42, 17.55±2.27, 16.37±1.38 and 21.87±0.85%, respectively [**Figure 9**]. This result was consistent with the initial screening experiment [**Figure 8A** and **8B**] and confirmed that *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* had direct lithogenic activity on COM crystal growth.

COM crystal aggregation-sedimentation study was performed to validate promoting effect of bacteria on COM crystal aggregation. Reduction of λ_{620} was resulted from the large particle sedimentation, which in turn reflected the degree of crystal aggregation. At 60-min,

fragmented RBC (the positive control) aggravated 53.19±3.61% of crystal aggregation, whereas intact RBC (the negative control) had no significant promoting effect (6.38±5.51%) as compared to that of blank control (0.00±5.51%) [**Figure 10A**]. *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* exhibited crystal aggregation promotion of 57.45±2.08, 51.06±5.51, 55.32±2.08 and 46.81±3.61%, respectively [**Figure 10A**]. This result confirmed that *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* had the direct lithogenic effect on COM crystal aggregation and also supported Hirano's proposal (52), in which bacteria might actively participate in stone genesis by acting as the adhesive agent.

To reconfirm COM crystal aggregation promoting effect of bacteria, the aggregated particulates from the aggregation-sedimentation experiment were collected to observe the size and morphology of aggregates under phase-contrast light microscopy. As showed in **Figure 10B**, COM aggregates induced by *E. coli*, *K. pneumoniae*, *S. aureus*, *S. pneumoniae* and fragmented RBC were obviously larger than those of blank control and intact RBC. Some bacterial-induced COM aggregates formed as the huge, tightly packed, round shaped particulates which probably behaved as stone core nidus. Maximal diameter of COM aggregates induced by *E. coli, K. pneumoniae, S. aureus, S. pneumoniae* and fragmented RBC were 69.74±4.79, 62.99±2.88, 58.53±5.64, 57.33±7.57 and 63.49±4.39 μm, respectively, which were clearly larger than that of blank control and intact RBC (26.14±2.15 and 28.28±0.93 μm, respectively) [**Figure 10C**]. Moreover, the size of bacterial-induced COM aggregates also exceeded the intraluminal diameter of distal renal tubule (approximately 20-30 μm) (57), suggesting that tubular occlusion was possible. This direct observation, together with lithogenic functional studies of bacteria, may explain how bacteria presented inside the stone core nidus as reported previously (27).

A

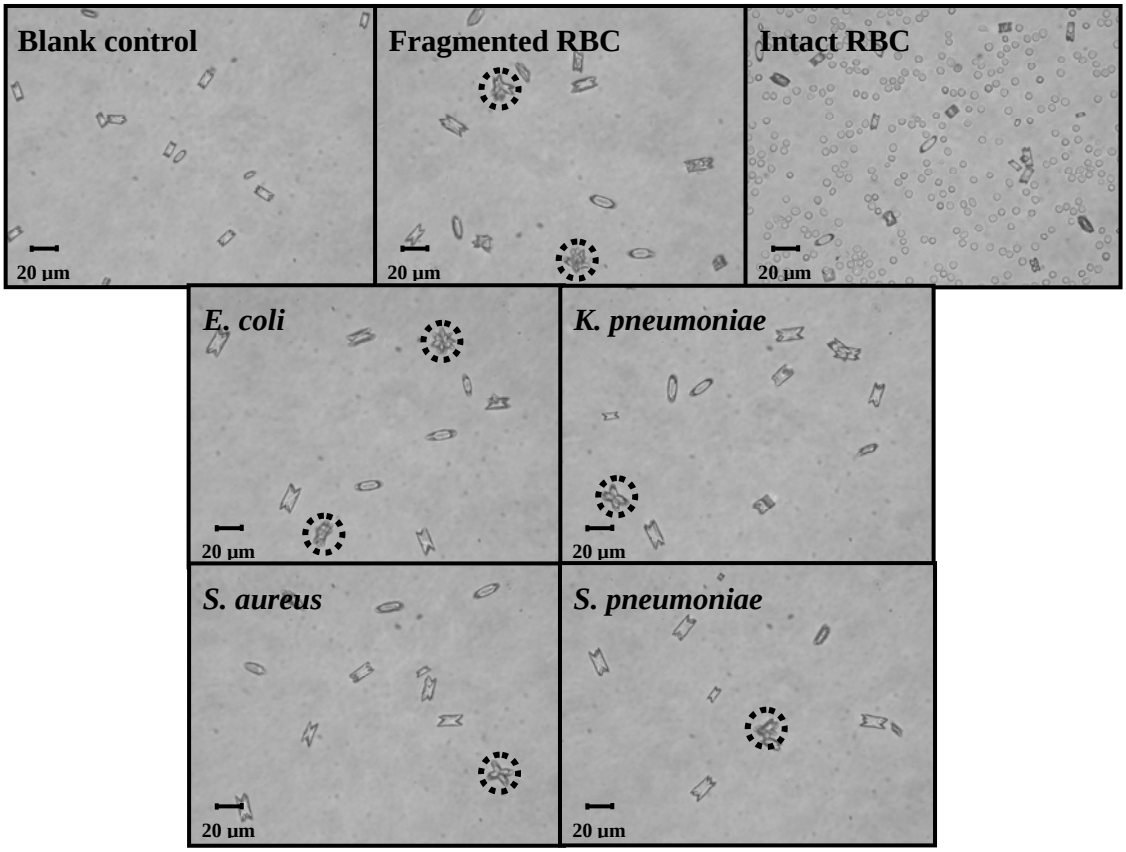
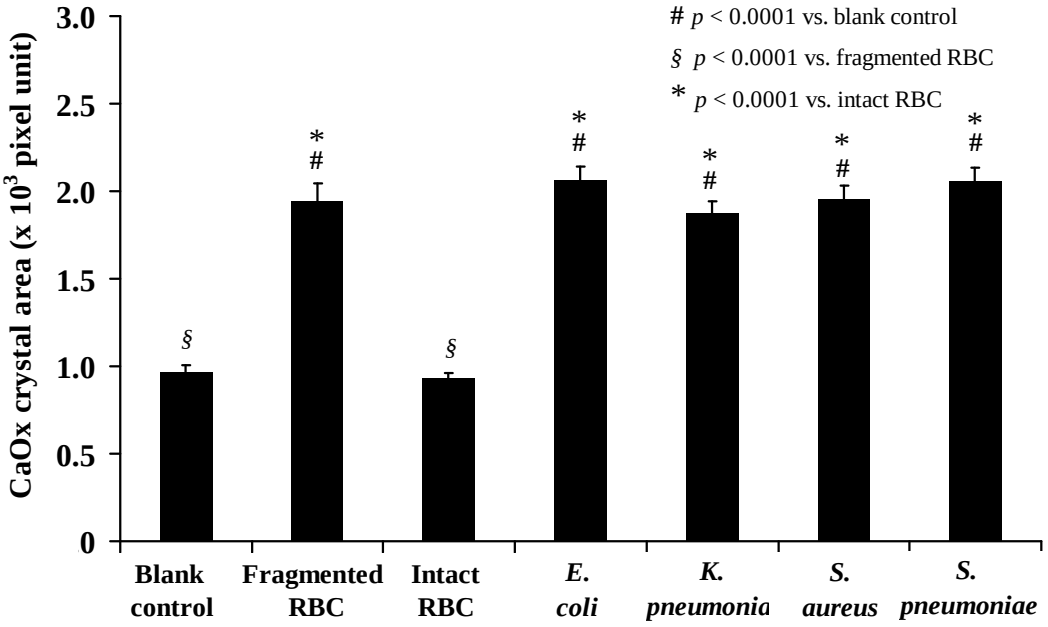


Figure 8. Crystal image analysis of lithogenic effects of bacteria. COM lithogenic effects of bacteria were firstly screened by morphological evaluation (**A**). COM crystals were generated in absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* (see details in "Materials and Methods"). Fragmented RBC and intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. Dashed circles indicate COM aggregates (which were defined as groups of two or more COM crystals tightly adhered together). Original magnification = 400X for all panels. Crystal images were captured by a digital camera and submitted into ImageMaster 2D Platinum Software for quantitative data of COM crystal area (**B**) and number of CaOx aggregates (**C**). Each bar was derived from 3 independent experiments and the data are reported as mean ± SEM.

B



C

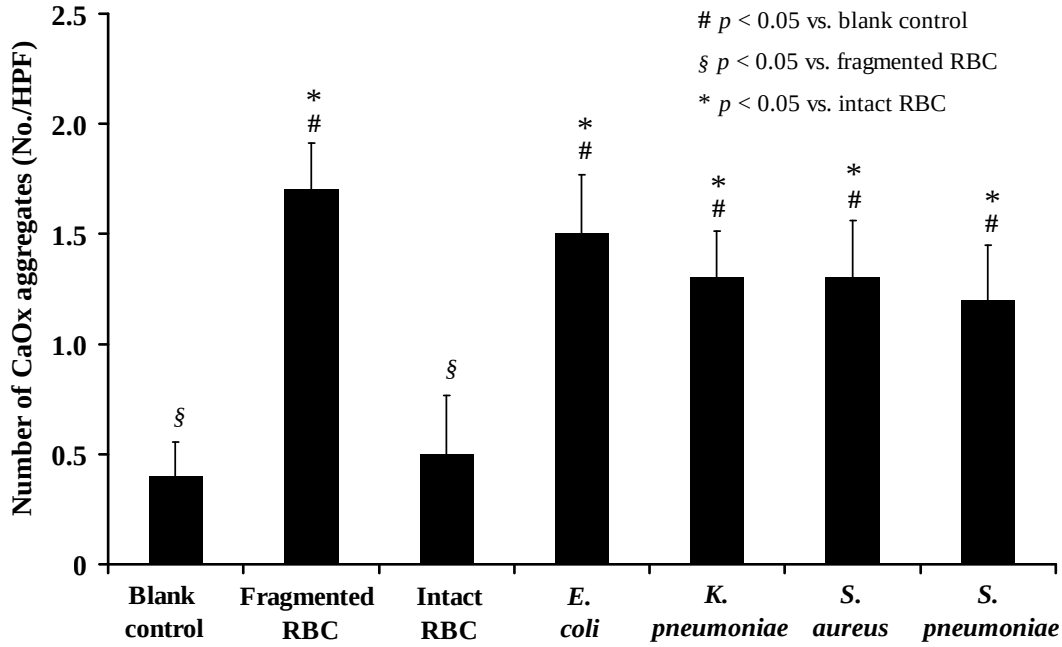


Figure 8. (continued)

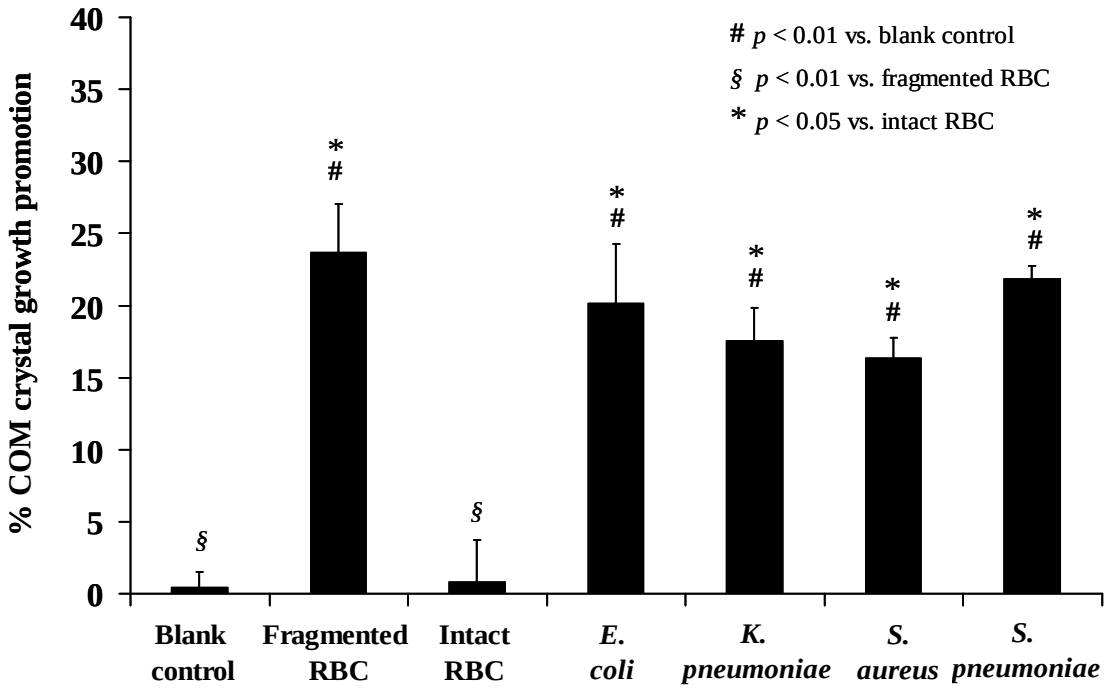


Figure 9. Spectrophotometric oxalate depletion assay of lithogenic effect of bacteria. COM crystal seeds (160 μg) were added to 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Depletion of free oxalate (which indicated crystal growth) was detectable by measuring absorbance at λ214 nm for up to 60-sec in the absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Fragmented RBC and intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. % COM crystal growth promotion = [(C-S)/C] x 100; where C = Rate of free oxalate depletion without bacteria and S = Rate of free oxalate depletion with bacteria to be test. Each data point was derived from 3 independent experiments and the data are reported as mean ± SEM.

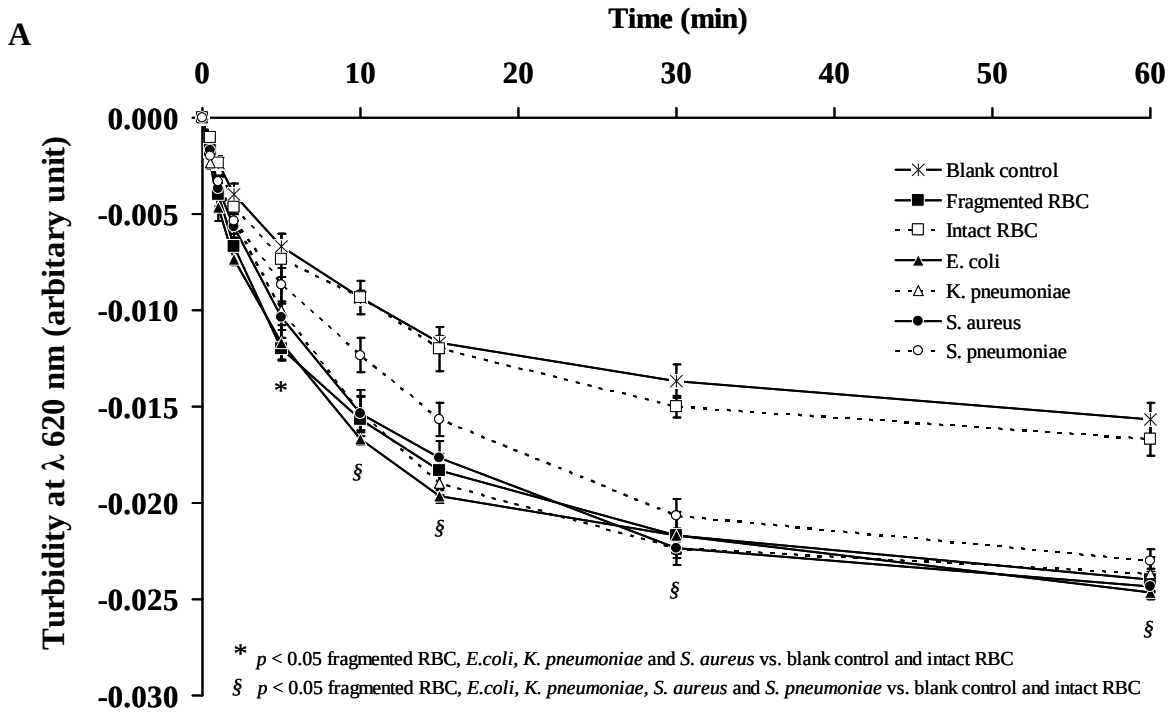


Figure 10. COM crystal aggregation-sedimentation study. (**A**) Individual COM crystals (100 μg) were added into 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $Na_2C_2O_4$, in a cuvette. The reaction was stirred at 800 rpm to prevent sedimentation of individual crystals but not the large aggregates. Sedimentation of those aggregates reduced solution turbidity, which was monitored at λ620 nm for 60-min in the absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Fragmented RBC and intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. Aggregated particulates generated by this reaction were morphologically observed under a phase contrast microscope **(B)**. The maximal diameter of the largest COM aggregate in each HPF (but not the smaller aggregates or individual crystals) was measured by Tarosoft® Image framework from 5 individual samples (**C**). Dash line indicates an intraluminal diameter of distal renal tubules (30 μm). Each data point was derived from 5 individual samples and the data are reported as mean ± SEM.

B

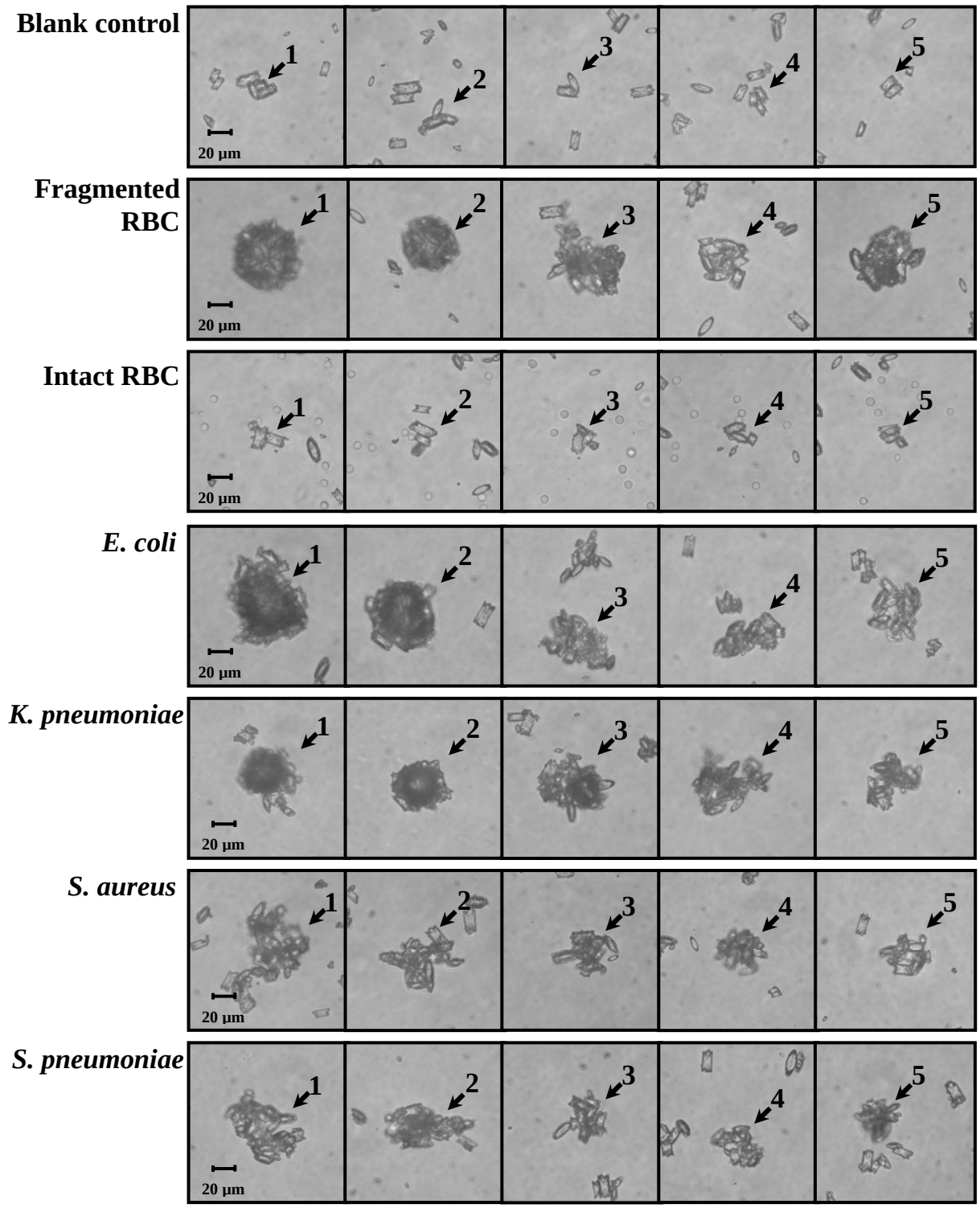


Figure 10. (continued)

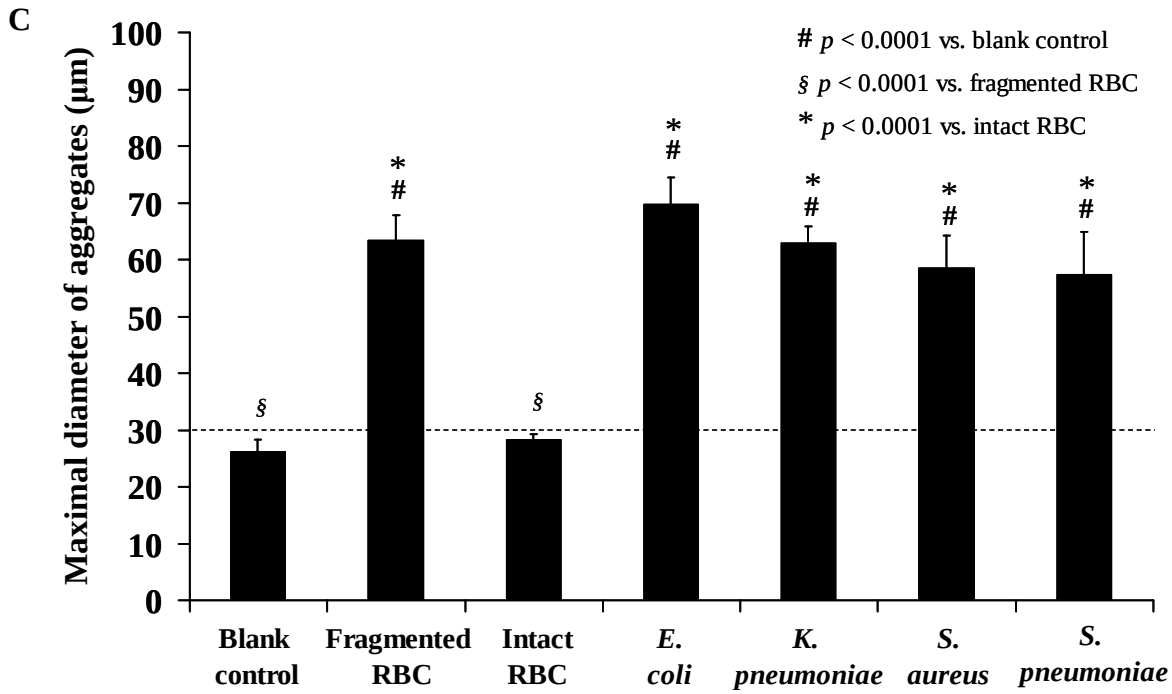


Figure 10. (continued)

CHAPTER VI
DISCUSSION

This study demonstrated that, for the first time, direct lithogenic effects of cellular membranes that commonly presented in the urine including renal tubular cells, RBC and *E. coli*. This study also pioneered an approach to define stone promoting factors by adapting the previously established assays for measuring inhibitory activities to determine promoting effects on COM crystal growth and aggregation. Since each cell type has involved in different circumstances, so the discussion would be focally made on each single type of cell as followed.

1. Renal tubular cells inhibit growth but promote COM crystal aggregation

Dual (counterbalance) effects on COM stone pathogenic mechanisms have been observed previously in some COM crystal modulators, including Tamm-Horsfall protein (THP) (58) and OPN (59). Levels of these dual modulators in the urine are clearly important to protect the kidney against COM stone disease as confirmed by THP or OPN knock-out animal models (60-62). However, dual functions of THP and OPN depend on their regulatory factors – THP is regulated by urinary parameters (e.g., pH, ionic strength, calcium and citrate levels) (63, 64), whereas OPN is regulated by protein localization (e.g., at the cell surface, in the extracellular matrix or urine) (65-67) and post-translational modifications, particularly phosphorylation (68, 69). These regulatory factors mostly cause alterations in molecular structure (aggregation of THP and phosphorylation/dephosphorylation of OPN), which determine the functions of both proteins and may be involved in COM stone formation (63-69).

Interestingly, our results demonstrated that both forms of MDCK cell membranes had dual (counterbalance) effects on COM crystals. Although the growth of individual COM crystals was inhibited (**Figures 1, 2A** and **3**), their aggregation was promoted (Figures 1 and 2D). In addition, both forms of MDCK cell membranes also induced COM-to-COD crystal transformation (**Figure 2E**), which has been considered as a protective mechanism against COM crystal retention in the kidney. (Note that COM has a much greater potent adhesive

capability as compared to COD (56, 70). Dual activities of distal renal tubular cell membranes, however, have totally been unknown for their regulation or how they affect on kidney stone disease *in vivo*.

This data may improve, at least in part, the understanding of the roles for cellular membranes in COM kidney stone formation. Cellular membranes are usually present in renal tubular fluid and urine because renal tubular cells can be sloughed up to 70,000 cells/h (19, 71). Intact cells are detached and shed into the urine by the biological process of epithelial turnover, whereas fragmented membranes in the urine may be derived from cell death/apoptosis (physiological processes) or renal tubular damage caused by noxious stimuli (pathophysiological processes). These membranes in renal tubular fluid and urine may aggravate COM stone formation by serving as pre-existing nuclei for secondary nucleation of stone nidus (6, 18). A part of our study supported this hypothesis by providing additional information that renal tubular cell membranes could promote COM crystal aggregation. Large COM aggregates may occlude the renal tubular lumen and be retained in the kidney, leading to the formation of stone nidus and ultimately to COM kidney stones. However, our study also demonstrated that renal tubular cell membranes had the counterbalance effect – they could also inhibit COM crystal growth and induce COM-to-COD crystal transformation. The compositions of cell membranes, particularly proteins, may differ among various cell types and lineages. As a result, the cell membranes derived from different parts of the nephron (e.g., proximal vs. distal) would exhibit differential modulatory activities on COM crystals (42, 72). Additionally, surface molecules on the same cell type can be altered dynamically in response to external stimuli (66, 73), which, in turn, may affect their inhibiting or promoting effects. Further studies are required to elucidate the regulatory mechanisms of the dual effects of renal tubular cell membranes on COM crystal modulation.

2. RBC membrane fragments, but not intact RBC, promote growth and aggregation of COM crystals

In addition to an evidence to demonstrate the presence of crenate membrane fragments of RBC inside the core matrices of CaOx stones by electron microscopy (24), RBC was selected as a model of urinary cellular membranes in the present study by several additional reasons. First, RBC is easily accessible and widely available. Second, RBC can be present in renal tubular fluid (upper tract) where COM crystals are originated. Third, RBC provides *ex vivo* cellular membranes that are more similar to the real clinical events than those derived from cell lines. Finally, RBC is a ghost cell that can be studied for their

membrane effects without any worrisome in contamination of intracellular organelles during sample preparation to generate membrane fragments, which have sizes and masses similar to those of some intracellular organelles.

Our results clearly demonstrated that RBC membrane fragments could significantly promote COM crystal growth and aggregation, as shown by microscopic evaluation with quantitative analyses (**Figures 1A, 2A and 2B**) and spectrophotometric oxalate-depletion assay (**Figure 3**). Moreover, we also found that RBC membrane fragments could adhere onto COM crystal surfaces (**Figure 1B**), while there was no adhesion observed for intact RBC. Interestingly, intact RBC could transform COM to COD crystals (**Figures 1A and 2C**), but had no effects on crystal growth and aggregation (**Figures 1A, 2A and 2B**). Crystal transformation affected by intact cells was also evidenced in a previous study by Lieske *et al*. (20), in which newly formed CaOx crystals found on the surface of living monkey (BSC-1) and canine (MDCK) renal tubular epithelial cells were all COD crystals. It seemed that those intact cells may share some surface properties responsible for COM-to-COD crystal transformation, a process that is presently considered as a protecting mechanism against crystal retention in the kidney (because COD crystal has the least adsorptive capability to renal tubular cell surface) (70). Indeed, spectrophotometric oxalate-depletion assay showed that intact RBC slightly delayed COM crystal growth during the first 10-30 min of incubation. This delay might be related to COM-to-COD crystal transformation. Taken together, intact RBC may play a role in counterbalance mechanism against promoting activity of the RBC membrane fragments.

This model experiment could be connected to hematuria, one of the most common presentations in renal and urological diseases. In the past, involvement of hematuria in the pathogenic mechanisms of kidney stone disease was under-investigated or even overlooked because hematuria is impressed as a result, not the cause, of kidney stone disease. This is not surprising as almost all forms of hematuria are associated with the presence of intact RBC in the urine and our data also show no promoting activity of intact RBC on growth and aggregation of COM crystals, which are the major crystalline component found in CaOx kidney stones (74). However, our present study revealed that fragmented RBC could promote COM crystal growth and aggregation; and thus may be involved in the pathogenic mechanisms of CaOx kidney stone disease. This less common form of hematuria is found in patients with underlying conditions, in which fragmented RBC is also found in the systemic blood circulation. Interestingly, there are some scattered reports on unexplained co-incidence of nephrolithiasis with hemolytic uremic syndrome (75) and microangiopathic hemolytic

anemia (76). We therefore hypothesize that, perhaps, RBC membrane fragments present in the urine of these hemolytic patients may be involved, at least in part, in the development of kidney stone formation.

3. Bacteria promote COM crystal growth and aggregation

This study demonstrated, for the first time, that bacteria could directly promote COM crystal growth and aggregation (14, 28). COM stone promoting activities of bacteria were comparable to those of RBC membrane fragments. This data supported a novel hypothesis of bacterial-induced metabolic stone and the significance of several (scattered) previous reports (25-27, 52, 53), and also suggested to reconsidering UTI as a possible cause of metabolic stone disease. Unexpected finding was that all bacterial strains used in this study showed COM lithogenic activities. At first, we assumed that only uropathogenic bacteria i.e., *E. coli* and *K. pneumoniae* would promote COM crystal growth and aggregation based on clinical microbiology and previous result of stone nidus culture(27). However, non-uropathogenic bacteria, including *S. aureus* and *S. pneumoniae*, also exhibited COM lithogenic effects. One explanation was that COM promoting activities of bacteria did not require a unique biological activity (unlike struvite stone which needed urease activity) (51, 77), but may depend on a common property among bacteria.

Most bacteria have an overall negative charge under physiologic pH (78). Ionic interaction between anionic bacteria and cationic COM crystals should then be suspected, since this interaction was reported as the adhesive force between stone modulating factors (such as anionic urinary molecules or epithelial cell surfaces) and COM crystals by atomic force microscopy (42, 70). Specific molecules on bacterial surface that responsible for this interaction have remained totally unknown. Teichoic acid (79) and lipopolysaccharide (LPS) (80), two abundant anionic molecules on bacterial cell walls which provide an overall negative charge to gram-positive and gram-negative bacteria, respectively, may be responsible as the key players in this setting. Further studies are required to elucidate the detailed mechanisms of lithogenic activities of bacteria. Noted that LPS and gram-negative uropathogenic bacteria may be more important in bacterial-induced metabolic stone due to clinical microbiology. Based on potential ionic interaction between bacteria and COM crystals, pathogenic mechanisms of bacterial-induced COM stone were anticipated as followed. Once bacteria interacted with COM crystals, anionic nature of bacteria may attract the free calcium ions from environment to increase local calcium concentrations, resulting in promotion of crystal growth. In addition, the interacted bacteria on COM surface may act as a

linker or adhesive agent (52) among individual COM crystals, leading to crystal aggregation. A huge, tightly impacted, round-shaped COM aggregates (as shown in **Figure 3B**) seemed to be a subsequent result of lithogenic effects of bacteria. Since the size of bacterial-induced COM aggregates was larger than the lumen of distal tubules and collecting ducts (57) (as shown in **Figure 3C**), these aggregates may be retained inside the kidney due to tubular occlusion and potentially act as the core nidus of stone development.

Currently, UTI is not recognized as a cause of metabolic stone disease. It might be due to the discordant correlation between UTI episode and metabolic stone formation (unlike UTI with urease-producing bacteria and struvite stone formation). However, COM stone development was relatively slow (when compared to that of struvite), so it may require several months or many years after UTI episode for stone growth into a clinical-relevant size (which may be out-of-range of observational period). Moreover, direct COM lithogenic activities of bacteria had never been examined. In contrast, the present study, together with previous reports (25-27, 52, 53), provided crucial information that fill the knowledge gap and may correct some misconceptions of infection-induced kidney stone. Our study proved that bacteria could directly promote COM crystal growth and aggregation, two important stone pathogenic mechanisms. This functional evidence was corresponding to microbiological evidences (25-27), reflecting that bacterial-nidus deposition may play some crucial roles in stone genesis. Further investigations to complete understanding of bacterial-induced metabolic stone may lead to better management and prevention of the first stone episode and the recurrent stone disease. At least, UTI should be reconsidered as a possible cause of metabolic stone disease, particularly COM type.

CHAPTER VII
CONCLUSION

Our present study provides the first direct evidence demonstrating that urinary cell membranes including distal renal tubular cells, RBC and *E. coli* have promoting effects on COM crystal growth and/or aggregation. In addition, functional-based approach pioneered in this study can also be applied to explore any other potential stone promoters. This study proved a long standing hypothesis of cellular membranes as stone promoters and provided novel information to elucidate some parts of the complex pathogenic mechanisms of COM stone disease. Clearly, presence of these cell membranes in the urine does not mean kidney stone will always be developed, since there are several other factors in the urinary microenvironment i.e., calcium, oxalate, phosphate, uric acid, citrate and stone modulatory proteins that interplay with COM crystals and cellular membranes and that determine whether the inhibitory or promoting effects would predominate. Thus, this interplay leads to stone prevention in normal healthy individuals but kidney stone formation in stone formers. Significance of urinary cell membranes therefore needs further studies, particularly clinical-related investigations. Once the exact roles of urinary cell membranes *in vivo* established, preventable strategies of COM stone disease targeting on this lithogenic factor will be possible.

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OUTPUTS

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1. **Chutipongtanate S**, Thongboonkerd V.
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   Impact factor 2009 = 4.016
2. **Chutipongtanate S**, Thongboonkerd V.
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3. **Chutipongtanate S**, Suchitra Sutthimethakorn, Wararat Chiangjong and Thongboonkerd V.
   Bacteria promote calcium oxalate crystal growth and aggregation.
   (*Manuscript in submission*)

Oral Presentation in International Conference

Dec 22-23, 2553	Mahidol-Kyoto Universities International Symposium: Integration of Bioscience for Innovative Medicine Topic: **"RBC membrane fragments, but not intact RBC, promote growth and aggregation of calcium oxalate monohydrate crystals"** Organized by: Mahidol and Kyoto Universities At: Prasong Tuchinda Conference Room, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, THAILAND

APPENDIX:

REPRINT AND MANUSCRIPT

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Renal tubular cell membranes inhibit growth but promote aggregation of calcium oxalate monohydrate crystals

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ABSTRACT

Cell membranes have been proposed to serve as promoters for calcium oxalate monohydrate (COM) kidney stone formation. However, direct evidence to demonstrate the modulatory effects of renal tubular cell membranes on COM crystals does not currently exist. We thus examined the effects of intact MDCK cells and their fragmented membranes on COM crystal growth, aggregation and transformation. COM crystals were generated in the absence (control) or presence of intact MDCK cells or their membrane fragments. Intact MDCK cells and their membrane fragments significantly inhibited COM crystal growth (22.6% and 25.2% decreases in size, respectively) and significantly reduced COM total crystal mass (23.1% and 25.6% decreases, respectively). In contrast, both of them markedly promoted crystal aggregation (1.9-fold and 3.2-fold increases, respectively). Moreover, both intact cells and membrane fragments could transform COM to calcium oxalate dihydrate (COD) crystals. Finally, COM crystal growth inhibitory activities of both membrane forms were successfully confirmed by a spectrophotometric oxalate-depletion assay. Our data provide the first direct evidence to demonstrate the dual modulatory effects of MDCK membranes on COM crystals. Although growth of individual COM crystals was inhibited, their aggregation was promoted. These findings provide additional insights into the mechanisms of COM kidney stone formation.

1. Introduction

Cell–crystal interaction is one of the major mechanisms of calcium oxalate monohydrate (COM) kidney stone formation and is initiated by COM crystal adhesion at anionic sites on the surfaces of renal tubular cells, particularly in the distal nephron [1–3]. Many lines of evidence have suggested that several surface anionic molecules, i.e., phosphatidylserine [4], hyaluronan [5], sialic acid [6] and annexin II [7] on renal tubular cell membranes may be responsible for this interaction. In addition, recent studies using atomic force microscopy have demonstrated that cell–crystal interaction may be mediated by adhesive force (i.e., ionic interaction and/or hydrogen bond) between the large hexagonal {1 0 0} face of COM crystals and the surface of distal renal tubular cells [8,9]. Moreover, the affinity between COM crystals and distal renal tubular cells is dramatically increased in the presence of a calcium ion [10,11]. Some specific anionic molecules in normal human urine such as citrate, nephrocalcin, and osteopontin (OPN) can inhibit this interaction by coating the COM crystal surface to prevent cell adhesion [1,12–14], whereas in the urine of COM stone formers, their inhibitory activities are decreased [15]. After COM crystals are adhered on the renal tubular cell surface, these crystals are then internalized via endocytosis and can be degraded by the endolysosome [1,16,17]. Interestingly, some evidence has suggested that the adherent COM crystals may be able to pass through the renal epithelial layer via transcytosis and/or exotubulosis, resulting in interstitial COM crystal deposition [18,19]. Several cellular responses are triggered upon the interaction between renal tubular cells and COM crystals, including altered gene expression [1], cytoskeletal reorganization [1], production of inflammatory cytokines [20], oxidative stress response [21] and renal tubular cell injury [22]. These cellular responses ultimately provoke detrimental effects leading to COM stone formation.

However, most of the previous studies on cell–crystal interaction in COM kidney stone disease have focused mainly on the effects of crystal attachment on renal tubular cells (cellular responses) [1–22], whereas the effects of renal tubular epithelial cells on the intratubular COM crystals (crystal responses) remain poorly characterized and under-investigated. It is possible that anionic molecules on the renal tubular cell membranes may establish a

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local negatively charged environment that can affect the nearby COM crystals. In addition to crystal adhesion, this specific environment may provide several modulatory effects on COM crystals, i.e., crystallization, growth, aggregation and/or transformation. Some previous studies have supported this hypothesis. For example, Fasano and Khan [23] have demonstrated that renal cell membrane vesicles isolated from rat kidney could enhance the rate of COM crystallization. In addition, Lieske et al. [24] have demonstrated that the apical surface of renal tubular cells could induce transformation of COM to calcium oxalate dihydrate (COD) crystals. (Note that COD crystals generally occur in a negatively charged environment, such as normal human urine, in the presence of COM stone inhibitors [25].) Nonetheless, the modulatory effects of renal tubular cell membranes on COM crystals remain largely unknown.

The present study therefore aimed to examine the modulatory effects of renal tubular cell membranes on COM crystals. Madin-Darby canine kidney (MDCK) cells, which were derived from distal nephron [26], were used as the source of cellular membranes (both intact and fragmented forms). Several effects of MDCK cell membranes on COM crystals (i.e., crystallization, growth, aggregation and COM-to-COD transformation) were initially screened by phase-contrast microscopy. Quantitative analyses were then performed to evaluate crystal size, number and total mass. A spectrophotometric oxalate-depletion assay was finally adopted to validate and monitor the COM crystal growth modulatory effects of MDCK cell membranes. The knowledge of modulatory effects of renal tubular cell membranes on COM crystals would lead to a better understanding of cell–crystal interaction, which is an important mechanism for COM kidney stone formation.

2. Materials and methods

2.1. MDCK cell culture and preparation of membrane fragments

MDCK cells (approximately 1×10^5 cells) were inoculated in each well of a 24-well, polystyrene, disposable cell culture cluster (with lid) (Corning Inc., Corning, NY) containing complete Eagle's minimum essential medium (MEM) (GIBCO, Invitrogen Corporation; Green Island, NY) supplemented with 10% fetal bovine serum (FBS) (GIBCO, Invitrogen Corporation; Catalog #10270098; Batch #41F0871K), 1.2% penicillin G/streptomycin and 2 mM glutamine. In this MEM/FBS medium, the final concentrations of calcium, oxalate and phosphate were 2.25 mM, 0.003 mM and 1.50 mM, respectively. The cells were maintained in a humidified incubator at 37 °C with 5% CO_2 for 24 h. Thereafter, the cells were harvested by scraping and then washed five times with an isotonic buffer containing 10 mM Tris–HCl (pH 7.4) and 150 mM sodium chloride (NaCl). A cell count was performed using a hemacytometer, and an equal number of 1×10^4 cells were used for each assay. To prepare MDCK membrane fragments, an equal number of MDCK cells (1×10^4 cells/assay) was sonicated in the aforementioned isotonic buffer using a probe sonicator (Bandelin Sonopuls HD 200; Bandelin electronic; Berlin, Germany) at MS 72/D (50 cycles) for 15 s in an icebox. MDCK membrane fragments were then collected by centrifugation at 10,000 × *g* for 5 min. Membrane fragmentation was confirmed by microscopic examination.

2.2. Crystal preparation and modulation by MDCK intact cells and membrane fragments

COM crystals were generated according to a protocol published previously [27]. Briefly, COM crystals were prepared in a 24-well, polystyrene, disposable cell culture cluster (with lid) (Corning Inc.) using calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium oxalate ($Na_2C_2O_4$) at final concentrations of 5.0 mM and 0.5 mM, respectively, in a buffer containing 10 mM Tris–HCl (pH 7.4) and 90 mM NaCl. The crystal samples without cells or their membrane fragments served as controls.

To examine the effects of intact cells and their membrane fragments on crystals, COM crystals were prepared as described above, but in the presence of intact MDCK cells (1×10^4 cells/ml crystal solution) or MDCK membrane fragments (equivalent to 1×10^4 cells/ml crystal solution). After a 1-h incubation, the effects of the presence of intact cells or their membrane fragments on COM crystals were evaluated as follows. All of these experiments were performed in triplicate.

2.3. Qualitative and quantitative analyses of COM crystal growth, aggregation, and transformation to COD

Effects of MDCK intact cells and membrane fragments on COM crystals were first screened by phase-contrast microscopic examination using an Olympus CKX41 inverted light microscope (Olympus Co. Ltd., Tokyo, Japan). Thereafter, quantitative measurements of crystal size, aggregation and transformation were performed by image analysis using ImageMaster™ 2D Platinum software (GE Healthcare, Uppsala, Sweden), which can accurately measure crystal area [25]. The crystal size was measured from at least 100 crystals in individual samples. Numbers of COM crystals (which reflected the rate of COM crystallization) and COD crystals (which reflected COM-to-COD crystal transformation) were counted in at least 10 high-power fields (HPF) (original magnification power of 400×). COM total crystal mass (the final product of COM crystallization and growth) was calculated using the following equation:

$$\text{Crystal mass (pixels/HPF)} = \text{Averaged crystal size (pixels)} \times \text{Crystal number (No./HPF)}$$

A crystal aggregate was defined as an assembly of individual crystals (two or more crystals) tightly joined together. Numbers of aggregates were counted in at least 10 HPF (original magnification power of 400×) of individual samples.

2.4. Spectrophotometric oxalate-depletion assay

Effects of MDCK intact cells and membrane fragments on COM crystal growth were confirmed and monitored by a spectrophotometric oxalate-depletion assay [28]. COM crystal seeds (160 μg) were added into a 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Basically, the seeded crystals would increase in size using excess calcium (from $CaCl_2$) and oxalate (from $Na_2C_2O_4$) ions, leading to the depletion of free oxalate, which is detectable by UV–visible spectrophotometry at $\lambda = 214$ nm. Oxalate depletion (which reflected crystal growth) was then monitored for up to 60 min during the reaction, with or without cells or their membrane fragments (equivalent to 1×10^4 cells/assay), using a Shimadzu UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

2.5. Statistical analysis

All the quantitative data are reported as the mean ± SEM. Multiple comparisons were performed using ANOVA with Tukey's post hoc test (SPSS, version 11.5). *P* values < 0.05 were considered statistically significant.

3. Results and discussion

It has been hypothesized for a long time that cellular membranes are the promoters of COM kidney stone formation [29,30]. How-

ever, their modulatory activities in stone pathogenic mechanisms have not been clearly elucidated. The MDCK cell line was selected as the source of cellular membranes in this study for the following reasons: first, this cell line originated from the distal part of the nephron, where COM crystallization usually initiates as a result of supersaturation of calcium and oxalate ions in the renal tubular fluid. Therefore, these cells directly interact and provide modulatory effects on newly formed COM crystals. Second, several lines of evidence have demonstrated that the plasma membranes of renal tubular cells may be directly involved in the pathogenic process of cell–crystal adhesion [1–3,10,11], suggesting that some surface molecules of renal tubular cell membranes may provide specific interactions with COM crystals [3–7]. Based on these hypotheses and background, our present study thus focused on the evaluation of the modulatory effects (either promotion or inhibition) of MDCK cells on COM crystals.

COM crystal size and morphology in the absence (control) or presence of intact MDCK cells or membrane fragments were initially screened by phase-contrast microscopic examination (Fig. 1). The results showed that both intact cells and membrane fragments had similar effects on COM crystals–they reduced COM crystal size but aggravated crystal aggregation and induced COM-to-COD crystal transformation as compared to the control. However, the membrane fragments tended to have greater promoting activity on crystal aggregation than that of the intact cells.

Quantitative analyses were performed using image analysis software (ImageMaster™ 2D Platinum software; GE Healthcare). Fig. 2A clearly shows that MDCK intact cells and membrane fragments had significant inhibitory effects on COM crystal growth (approximately 22.6% and 25.2% decreases in crystal size, respectively). We also evaluated crystal numbers (which reflected COM crystallization) and total crystal mass (which reflected the final product of COM crystallization and growth). The results demonstrated that both intact cells and membrane fragments did not affect crystal numbers (Fig. 2B) but significantly reduced COM total crystal mass (approximately 23.1% and 25.6% decreases in total crystal mass, respectively) (Fig. 2C). However, both intact cells and membrane fragments had a significant promoting effect on COM crystal aggregation (approximately 1.9-fold and 3.2-fold increases in the number of COM crystal aggregates, respectively) (Fig. 2D). Between both forms of MDCK membranes, the membrane fragments had a more potent promoting effect on COM crystal aggregation compared to intact MDCK cells. Fig. 2E demonstrates that both intact cells and their membrane fragments could transform COM to COD crystals at a comparable degree, whereas there was no COM-to-COD crystal transformation observed in the control. These findings were consistent with the data obtained from previous studies demonstrating that most of the newly formed calcium oxalate crystals in the presence of distal and collecting tubular cell membranes from the kidney of Sprague–Dawley rats [23] and MDCK cells [24] were COD, not COM.

A spectrophotometric oxalate-depletion assay (the gold standard method for assessment of COM crystal growth) was performed to confirm and monitor the COM crystal growth inhibitory effect of MDCK intact cells and membrane fragments. Fig. 3 shows that both forms of MDCK membranes significantly inhibited COM crystal growth (as the rate of depletion of free oxalate ions was significantly less than that of the control) at comparable degrees from 15 min post-incubation through the end of the assay (60 min). These data strengthened our initial results and indicated that both forms of MDCK membranes had a significant inhibitory effect against COM crystal growth.

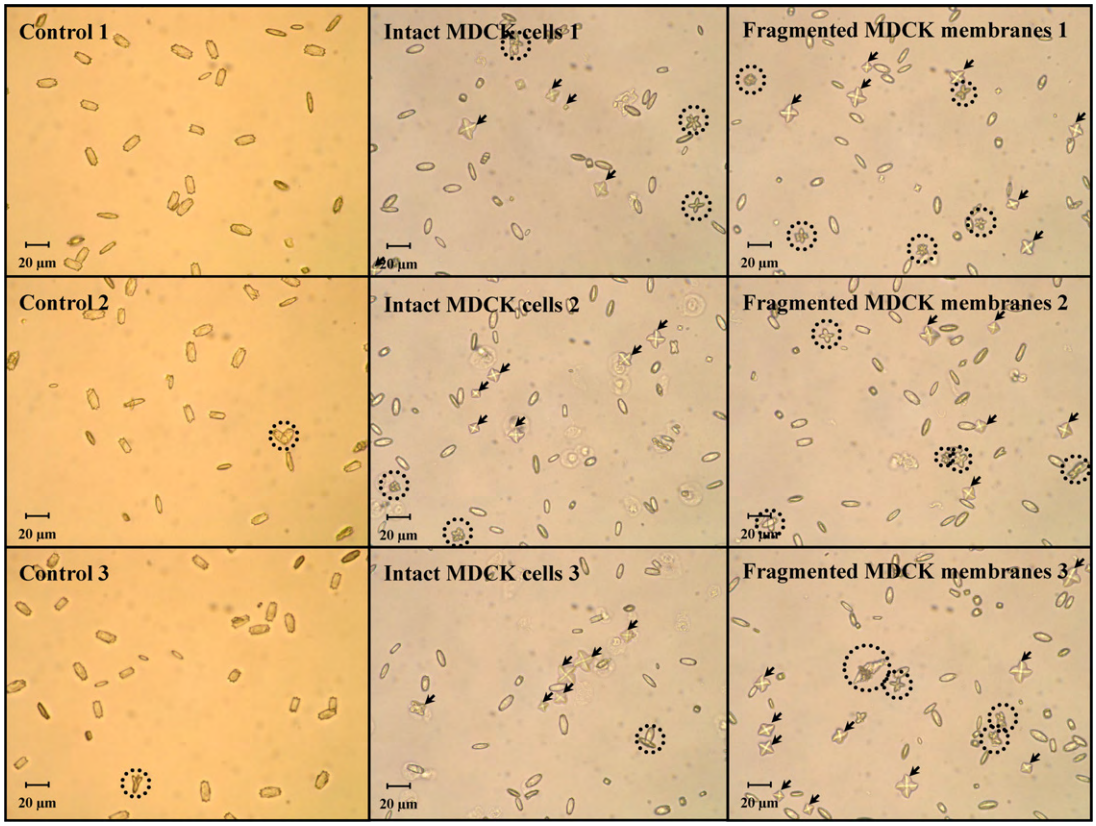

Fig. 1. Morphological evaluation of modulatory effects of MDCK intact cells and membrane fragments on COM crystals. COM crystals were generated in the absence (control) or presence of intact cells or membrane fragments (see details in Section 2). Dashed circles indicate COM aggregates (which were defined as groups of two or more COM crystals adhered tightly together). Arrowheads indicate COM-to-COD crystal transformation. All experiments were done in triplicate. Original magnification = 400× for all panels.

Dual (counterbalance) effects on COM stone pathogenic mechanisms have been observed previously in some COM crystal modulators, including Tamm–Horsfall protein (THP) [31] and OPN [32]. Levels of these dual modulators in the urine are clearly important to protect the kidney against COM stone disease as confirmed by THP or OPN knock-out animal models [33–35]. However, dual functions of THP and OPN depend on their regulatory factors – THP is regulated by urinary parameters (e.g., pH, ionic strength, calcium and citrate levels) [36,37], whereas OPN is regulated by protein localization (e.g., at the cell surface, in the extracellular matrix or urine) [38–40] and post-translational modifications, particularly phosphorylation [41,42]. These regulatory factors mostly cause alterations in molecular structure (aggregation of THP and phosphorylation/dephosphorylation of OPN), which determine the functions of both proteins and may be involved in COM stone formation [36–42]. Interestingly, our results demonstrated that both forms of MDCK cell membranes had dual (counterbalance) effects on COM crystals. Although the growth of individual COM crystals was inhibited (Figs. 1, 2A and 3), their aggregation was promoted (Figs. 1 and 2D). In addition, both forms of MDCK cell membranes also induced COM-to-COD crystal transformation (Fig. 2E), which has been considered a protective mechanism against COM crystal retention in the kidney. (Note that COM has a much greater potent adhesive capability as compared to COD [2,43].)

Our data may improve the understanding of the roles for cellular membranes in COM kidney stone formation. Cellular membranes are usually present in renal tubular fluid and urine because renal tubular cells can be sloughed up to 70,000 cells/h [23,44]. Intact cells are detached and shed into the urine by the biological process of epithelial turnover, whereas fragmented membranes in the urine

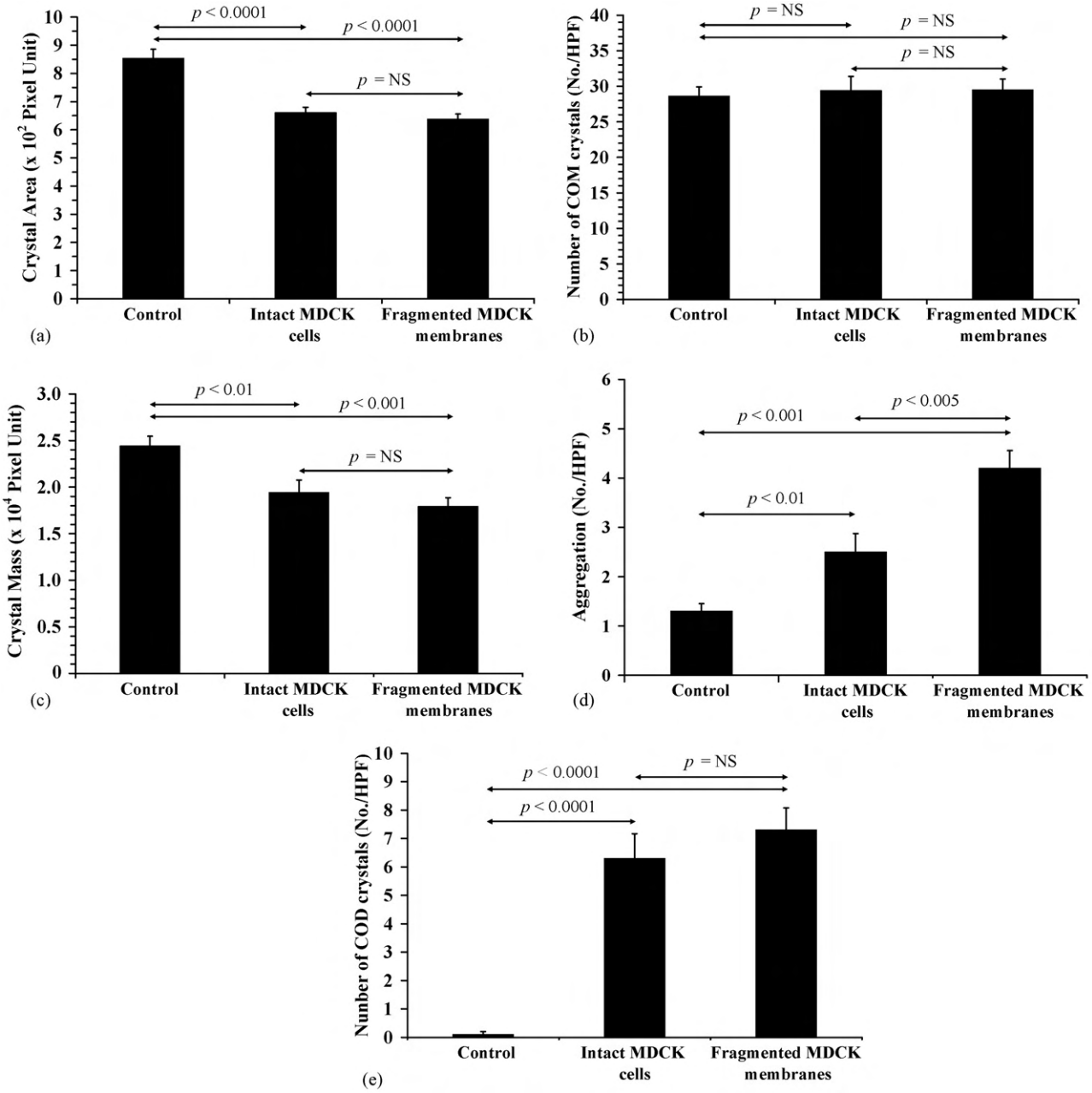

Fig. 2. Quantitative and statistical analyses of COM crystal size (A), number of COM crystals (B), COM total crystal mass (C), number of COM crystal aggregates (D), and number of COD crystals (E) affected by MDCK intact cells or membrane fragments. Each bar was derived from three independent experiments and the data are reported as mean ± SEM. NS = not significant.

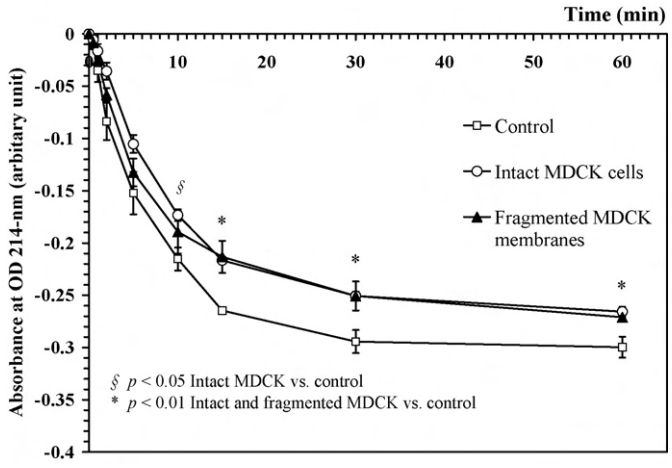

Fig. 3. Spectrophotometric oxalate-depletion assay. COM crystal seeds (160 μg) were added to a 1-ml solution containing 1 mM $CaCl_2{\cdot}2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. The depletion of free oxalate (which reflected crystal growth) was then monitored by measuring absorbance at λ = 214 nm for up to 60 min in the absence or presence of MDCK intact cells or membrane fragments (equivalent to 1×10^4 cells per assay). Each data point was derived from three independent experiments, and the data are reported as the mean ± SEM.

may be derived from cell death/apoptosis (physiological processes) or renal tubular damage caused by noxious stimuli (pathophysiological processes). These membranes in renal tubular fluid and urine may aggravate COM stone formation by serving as preexisting nuclei for secondary nucleation of stone nidus [29,30]. A part of our study supported this hypothesis by providing additional information that renal tubular cell membranes could promote COM crystal aggregation. Large COM aggregates may occlude the renal tubular lumen and be retained in the kidney, leading to the formation of stone nidus and ultimately to COM kidney stones. However, our study also demonstrated that renal tubular cell membranes had the counterbalance effect – they could also inhibit COM crystal growth and induce COM-to-COD crystal transformation.

The compositions of cell membranes, particularly proteins, may differ among various cell types and lineages. As a result, the cell membranes derived from different parts of the nephron (e.g., proximal vs. distal) would exhibit differential modulatory activities on COM crystals [3,45]. Additionally, surface molecules on the same cell type can be altered dynamically in response to external stimuli [6,39], which, in turn, may affect their inhibiting or promoting effects. Nevertheless, it is still unclear how the dual effects of MDCK cell membranes are regulated. Further studies are required to elucidate the regulatory mechanisms of the dual effects of renal tubular cell membranes on COM crystal modulation.

In summary, our present study provides the first direct evidence demonstrating that renal tubular cell membranes have dual (both inhibitory and promoting) effects on COM crystals. There are several other factors in the microenvironment of the distal nephron that interplay with COM crystals and renal tubular cells and that determine whether the inhibitory or promoting effects would predominate. Thus, this interplay leads to stone prevention in normal healthy individuals but kidney stone formation in stone formers.

Conflict of interest statement

All the authors declare no conflict of interest.

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Red Blood Cell Membrane Fragments but Not Intact Red Blood Cells Promote Calcium Oxalate Monohydrate Crystal Growth and Aggregation

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Purpose: Cell membranes are thought to promote calcium oxalate kidney stone formation but to our knowledge the modulating effect of red blood cell membranes on calcium oxalate crystals has not been previously investigated. Thus, we examined the effects of red blood cell membrane fragments on calcium oxalate monohydrate and calcium oxalate dihydrate crystal growth and aggregation.

Materials and Methods: Calcium oxalate monohydrate and calcium oxalate dihydrate crystals were treated with red blood cell membrane fragments or intact red blood cells from a healthy donor. Phase contrast microscopy was performed to evaluate crystal morphology and aggregation. We used ImageMaster™ 2D Platinum software to evaluate crystal size and spectrophotometric oxalate depletion assay to monitor crystal growth.

Results: Red blood cell membrane fragments had significant promoting activity on calcium oxalate monohydrate crystal growth with an approximately 75% increase in size and aggregation with an approximately 2.5-fold increase in aggregate number compared to the control without membrane fragments or cells. Approximately 50% of calcium oxalate monohydrate crystals were adhered by red blood cell membrane fragments. Intact red blood cells had no significant effect on calcium oxalate monohydrate crystal growth or aggregation but they could transform calcium oxalate monohydrate to calcium oxalate dihydrate crystals. Red blood cell membrane fragments and intact red blood cells had no effect on calcium oxalate dihydrate crystals. The promoting activity of red blood cell membrane fragments on calcium oxalate monohydrate crystal growth was successfully confirmed by spectrophotometric oxalate depletion assay.

Conclusions: To our knowledge our data provide the first direct evidence that red blood cell membrane fragments are a promoting factor for calcium oxalate monohydrate crystal growth and aggregation. Thus, they may aggravate calcium oxalate stone formation.

Key Words: kidney, kidney calculi, erythrocytes, calcium oxalate, crystallization

Abbreviations and Acronyms

CaOx = calcium oxalate

COD = calcium oxalate dihydrate

COM = calcium oxalate monohydrate

RBC = red blood cell

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URINARY organic macromolecules have important roles in CaOx kidney stone formation.[1–3] Composition analysis of stone core matrix reveals several organic macromolecules, including glycoproteins, carbohydrate and membrane phospholipids.[1,2] These substances are also found in urine[2,3] and, thus, can act as stone modulators (inhibitors or promoters) of CaOx crystal growth, aggregation and adhesion to the renal tubular cell surface.[4,5] According to importance, stone research in the last 2 decades has focused mainly on

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the effects of these modulators on CaOx stone formation. Inhibitors and promoters are important for CaOx kidney stone pathogenesis but there are few studies and less understanding of stone promoters. Most previous studies focused mainly on stone inhibitors, eg citrate,[6] nephrocalcin,[7] osteopontin,[8] urinary prothrombin fragment 1,[9] bikunin[10] and urinary trefoil factor 1,[11] while only a few promoters have been identified.[12,13] Thus, it is crucial to identify additional stone promoters to better understand the pathogenic mechanisms of CaOx kidney stone disease.

Cell membranes in urine are hypothesized to serve as CaOx kidney stone promoters.[2,14] Membrane degradation products from cell turnover and their phospholipid constituents can be detected in human urine and are thought to act as preexisting nuclei for secondary nucleation of CaOx crystals.[2] Two lines of evidence support this hypothesis. 1) Extracts from core matrices of CaOx stones always contain phospholipids and lipid derivatives originating from cell membranes.[2] 2) Extracted lipids[2] and renal cell membrane vesicles isolated from the kidney of Sprague-Dawley® rats[15] can promote CaOx crystal nucleation. This evidence suggests the potential roles of cell membranes on CaOx kidney stone formation. However, data are restricted only to the effects of renal cell membranes in the nucleation process. Functional studies of the effects of membrane degradation products from other cells in other stone formation processes, ie crystal growth and aggregation, are also required.

The RBC is one of the most common cells in urine in many kidney diseases and related disorders. Several studies have shown an association between CaOx nephrolithiasis and abnormalities in circulatory RBCs[16,17] but to our knowledge it is unclear or even unknown whether urinary RBCs have any role in the pathogenic mechanisms of CaOx stone formation. Evidence shows that RBC crenated membrane fragments are found inside core matrices of about 10% of all CaOx stones examined.[18] These data suggest that RBC membrane fragments may have a still unknown role in stone pathogenesis. Thus, we sought direct evidence of the pathogenic role of RBC membrane fragments in CaOx crystal growth and aggregation.

MATERIALS AND METHODS

RBC Membrane Fragment Preparation

A packed RBC sample from a normal healthy donor was obtained from our institutional blood bank according to Helsinki Declaration principles for the study using human samples. Cells were washed 5 times with isotonic buffer containing 10 mM tris HCl (pH 7.4) and 150 mM NaCl. The number of RBCs was determined using a hemocytometer and 2×10^5 cells were used per assay. To prepare RBC membrane fragments an equal number of cells (2×10^5 per assay) was resuspended in deionized (18 $M\Omega \cdot cm$) water to allow hypo-osmotic induced cell lysis. Microscopic examination showed that the cells were completely disrupted and membrane fragmentation occurred after 5-minute hypo-osmotic induced cell lysis. RBC membrane fragments were then isolated using centrifugation at 10,000 $\times$ gravity for 5 minutes.

Crystal Preparation and Intervention

COM and COD crystals were generated according to our previously published protocols.[19,20] Briefly, COM crystals were prepared in a 24-well polystyrene disposable cell culture cluster with a lid (Corning Inc., Corning, New York) using $CaCl_2 \cdot 2H_2O$ and $Na_2C_2O_4$ at a final concentration of 5.0 and 0.5 mM, respectively, in buffer containing 10 mM tris HCl (pH 7.4) and 90 mM NaCl. COD crystals were prepared in the 24-well plate using $CaCl_2 \cdot 2H_2O$ and $Na_2C_2O_4$ at a final concentration of 6.27 and 1.6 mM, respectively, in 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). These conditions (without cells or membrane fragments) served as controls.

To examine the effect of intact cells and membrane fragments on crystals we prepared COM and COD crystals as described but in the presence of intact RBCs (2×10^5 cells per 200 μl crystal solution) or RBC membrane fragments (equivalent to 2×10^5 cells per 200 μl crystal solution). After 1-hour incubation the effect of intact cells or membrane fragments on COM and COD crystals was evaluated. All experiments were done in triplicate.

Crystal Morphology, Size and Aggregation

The effect of intact RBC and RBC membrane fragments on CaOx crystal morphology and aggregation was first evaluated by phase contrast microscopy using an Olympus® CKX41 inverted light microscope. A crystal aggregate was defined as 2 or more crystals adhered together. The number of aggregates was counted in at least 10 high power fields (original magnification 400×) in individual samples. Crystal size was examined using ImageMaster 2D Platinum software, which can accurately measure crystal area.[20] Crystal size was measured in at least 100 crystals in individual samples.

RBC Membrane Fragment Staining

Modified blue silver reagent (0.3% weight per volume Coomassie blue G-250, 2% H_3PO_4, 10% $(NH_4)_2SO_4$ and 20% methanol) was used to visualize and localize transparent RBC membrane fragments. This staining could be used also to identify RBC membrane fragment adhesion on CaOx crystal surfaces. Reagent was prepared using the same protocol as described previously[21] but with a modest modification by increasing the amount of Coomassie blue G-250 dye from 0.12% to 0.3% to enhance cell membrane detection sensitivity. To stain RBC membrane fragments that adhered on CaOx crystal surfaces the modified blue silver reagent was added to the reaction well with a ratio of 1:20 volume per volume of dye reagent per crystal solution. After 1-hour incubation at 25C the dye solution was removed while crystals and RBC membrane fragments were at the bottom of the reaction well. Crystals and RBC membrane fragments were washed several

times with buffer containing 10 mM tris HCl (pH 7.4) and 90 mM NaCl until the discarded solution was colorless. Adhesion of RBC membrane fragments on CaOx crystal surfaces was observed under the CKX41 inverted light microscope. The number of crystals with adherent RBC membrane fragments was counted in at least 100 crystals in individual samples.

Spectrophotometric Oxalate Depletion Assay of COM Crystal Growth

After initial evaluation of crystal size with ImageMaster 2D Platinum software the effect of RBC membrane fragments on COM crystal growth was confirmed and monitored by spectrophotometric oxalate depletion assay.[22] COM crystal seeds (160 μg) were added to 1 ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Seeded crystals would grow further using excess calcium ions from $CaCl_2$ and oxalate ions from $Na_2C_2O_4$, leading to the depletion of free oxalate, which is detectable by ultraviolet visible spectrophotometry at λ214 nm. Oxalate depletion, which reflects crystal growth, was monitored for up to 60 minutes during the reaction with or without cells, or membrane fragments equivalent to 2×10^5 cells per assay using a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan).

Statistical Analysis

Quantitative data are shown as the mean ± SEM. Multiple comparisons were done using ANOVA with Tukey's post hoc test using SPSS®, version 11.5 with $p < 0.05$ considered statistically significant.

RESULTS

Effect of Intact RBCs and RBC Membrane Fragments

COM crystals. Intact RBCs did not affect COM crystal size but could transform COM crystals, which have the most potent adsorptive capability, to COD crystals, which have the least potent adsorptive capability (fig. 1).[23] In contrast, RBC membrane fragments caused obvious enlargement of individual COM crystals and increased the number of COM aggregates, defined as 2 or more COM crystals adhered together. Modified blue silver stained RBC membrane fragments, noted as greenish-blue ghost cells (fig. 2). Visualized membrane fragments were also present on COM crystal surfaces, indicating adhesion to COM crystals. Approximately 50% of COM crystals were adhered by RBC membrane fragments. No adhesion was noted between intact RBCs and COM crystals (fig. 1).

Microscopic crystal images were further analyzed by ImageMaster 2D Platinum software to measure COM crystal size, which reflects crystal growth.

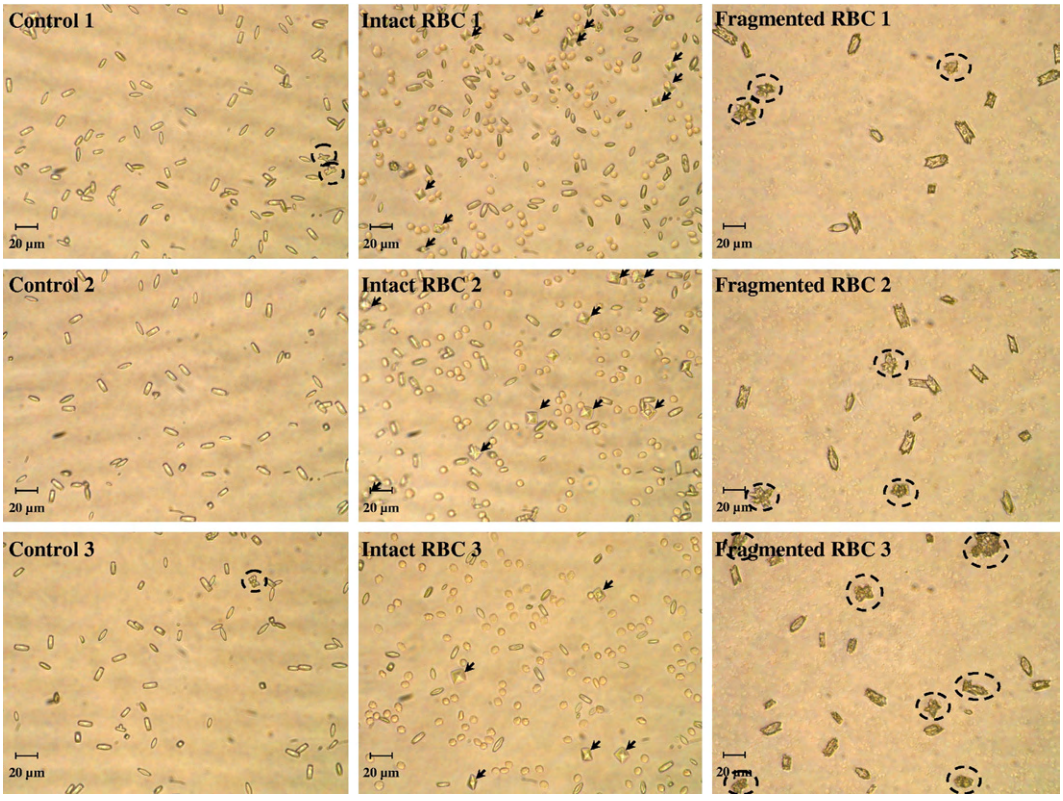

Figure 1. Intact RBC and RBC membrane fragment effect on COM crystals generated in absence (control) or presence of intact RBCs or RBC membrane fragments. RBC membrane fragments promoted COM crystal growth and aggregation. Intact RBCs had no effect on COM crystal growth and aggregation but transformed COM crystals to COD crystals (arrowheads). Circles indicate COM aggregates. Experiments were done in triplicate. Reduced from ×400.

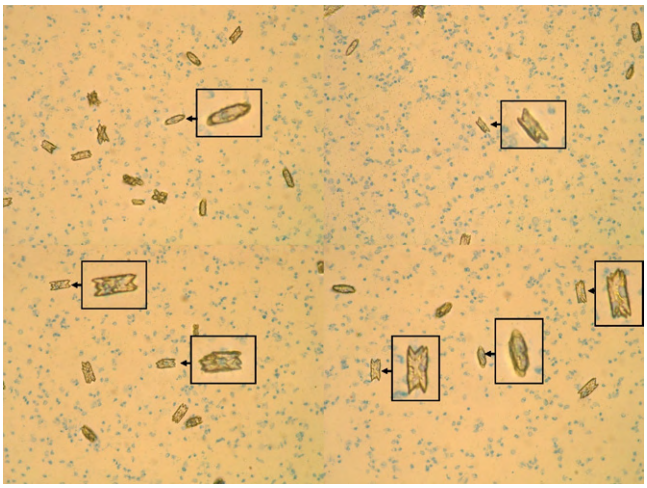

Figure 2. Intact RBC and RBC membrane fragment effect on COM crystals. Some RBC membrane fragments adhered to COM crystal surfaces (insets). Experiments were done in triplicate. Modified blue silver reagent stain, reduced from ×400.

RBC membrane fragments significantly promoted COM crystal growth (approximately 75% increase in crystal size vs control), whereas intact RBCs had no effect on crystal growth (fig. 3, *A*). RBC membrane fragments significantly promoted COM aggregate formation (approximately 2.5-fold increase in the number of aggregates vs control), whereas intact RBCs had no effect on the number of COM aggregates (fig. 3, *B*). Intact RBCs could transform COM to COD crystals since the number of COD crystals was significantly increased (fig. 3, *C*). Approximately 14.5% of initial COM crystals were transformed to COD crystals after incubation with intact RBCs while RBC membrane fragments had no effect on crystal transformation.

To further confirm morphological data and monitor the effect of RBC membrane fragments on COM crystal growth we performed spectrophotometric oxalate depletion assay. Since free oxalate ion depletion was significantly greater than in the control, RBC membrane fragments significantly aggravated COM crystal growth from 2 minutes of incubation to the end of the assay at 60 minutes (fig. 4, *A*), consistent with morphological data. Intact RBCs slightly delayed crystal growth during 10 to 30 minutes of incubation but had no significant effects on crystal growth at 60 minutes of incubation, consistent with morphological data.

COD crystals. We also evaluated the effect of intact RBC and RBC membrane fragments on COD crystals. However, morphological and quantitative data on COD crystals were not significantly changed by intervention with intact RBCs or RBC membrane fragments (fig. 5). Thus, this result suggests that the crystal growth promoting activity of RBC membrane fragments is selective for COM crystals.

RBC Membrane Fragment Effect on COM Crystal Growth

We performed a preliminary study to evaluate the effect of RBC membrane fragments on COM crystal growth in urine samples of 2 male and 1 female stone formers vs that in 2 male and 1 female healthy individuals (mean age 32.7 ± 6.8 vs 30.0 ± 2.6 years, respectively, p not significant) using spectrophotometric oxalate depletion assay. This experiment was done as described but with urine as a diluent instead of deionized water to make 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Other parameters, including crystal seeds and RBC membrane fragments, remained the same. Preliminary data showed that urine samples from stone formers had a significantly higher COM crystal growth rate than those from healthy individuals. RBC membrane fragments significantly aggravated COM crystal growth in each group, particularly in stone former urine samples (fig. 4, *B*).

DISCUSSION

Several cell membrane types are present in human urine, including those originated from RBCs, white

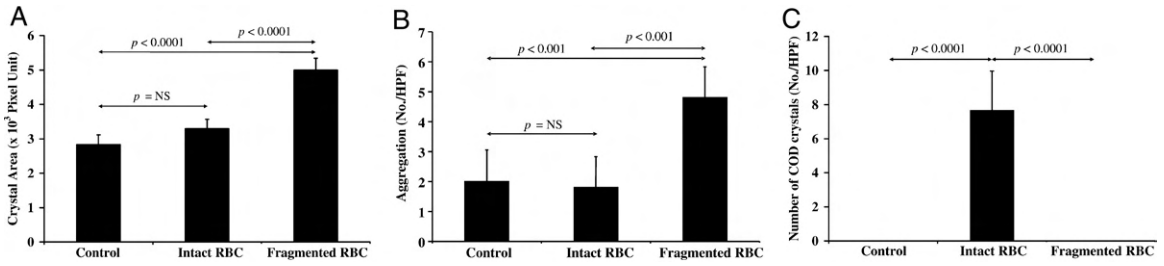

Figure 3. Quantitative and statistical analysis of effect of RBC membrane fragments and intact RBCs. *A*, COM crystal size. *B*, number of COM crystal aggregates. *C*, number of COD crystals. Bars represent mean ± SEM data from 3 independent experiments. *NS*, not significant.

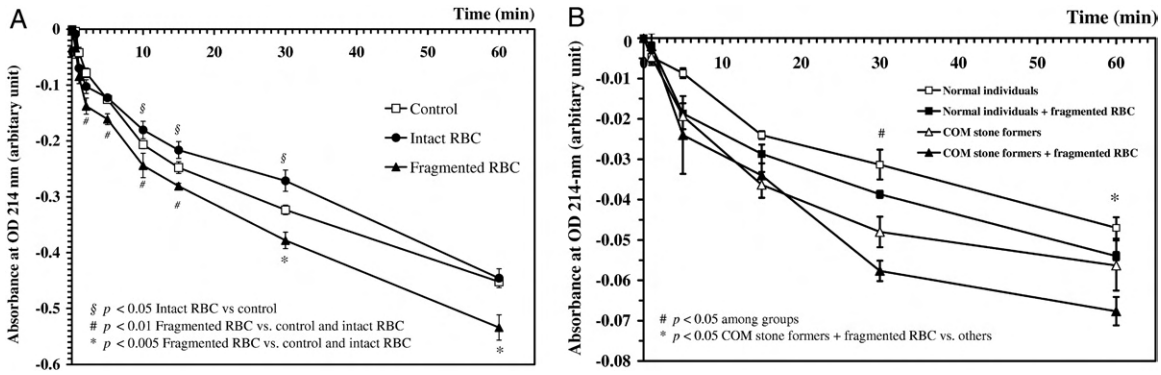

Figure 4. Spectrophotometric oxalate depletion assay data. *A*, COM crystal seeds were added to 1 ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$. Oxalate depletion, reflecting crystal growth, was monitored by measuring absorbance at λ214 nm for up to 60 minutes with or without RBCs, or RBC membrane fragments (equivalent to 2×10^5 cells per assay). Data points were derived from 3 independent experiments. *B*, preliminary study of RBC membrane fragment effect on COM crystal growth in urine samples of 3 stone formers vs 3 healthy individuals using urine instead of deionized water as diluent, as described. *OD*, optical density. Data are reported as mean ± SEM.

blood cells, proximal and distal tubules, the collecting duct and bacteria, eg Escherichia coli and Proteus mirabilis. In addition to evidence showing RBC crenated membrane fragments inside the core matrices of CaOx stones by electron microscopy,[18] we selected RBCs as a model of urinary cellular membranes for several other reasons. 1) RBCs are easily accessible and widely available. 2) RBCs are present in renal tubular fluid (upper tract), where COM crystals originate. 3) RBCs provide ex vivo cellular membranes that more closely resemble clinical events than those derived from cell lines. 4) RBCs are ghost cells that can be studied for membrane effects without worrisome contamination of intracel-

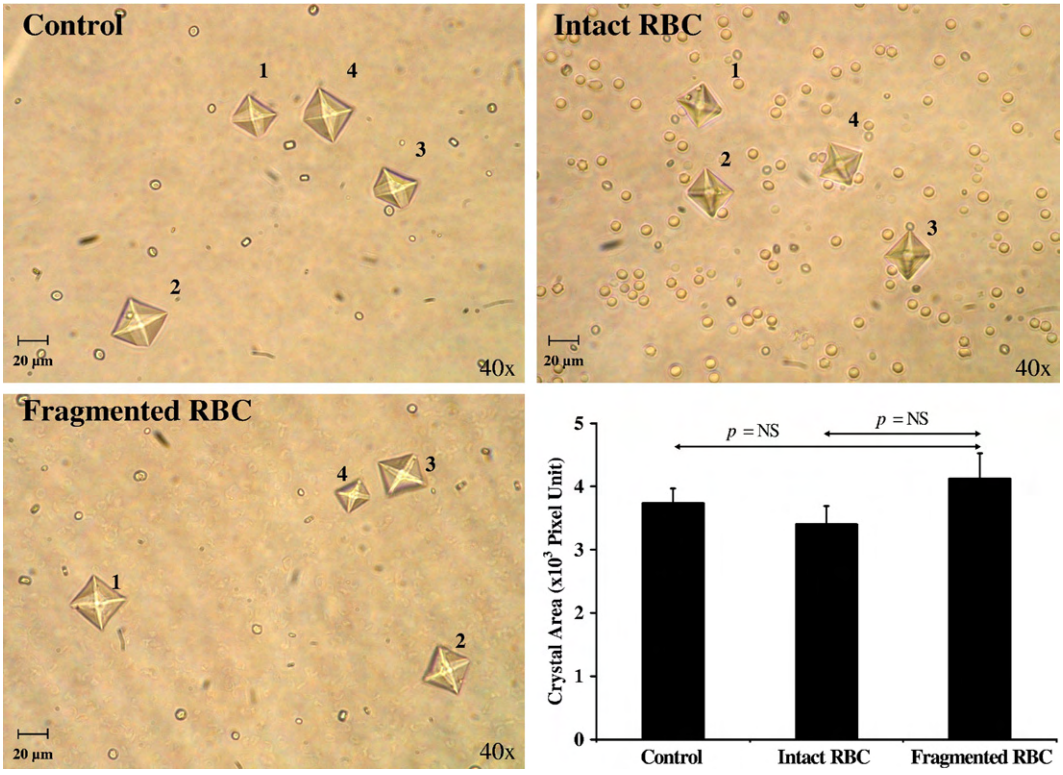

Figure 5. Intact RBC and RBC membrane fragment effect on COD crystals generated in absence (control) or presence of intact RBCs or RBC membrane fragments. Reduced from ×400. Bars represent mean ± SEM from 3 independent experiments. *NS*, not significant.

lular organelles during sample preparation to generate membrane fragments, which have a size and mass similar to those of some intracellular organelles.

Our results clearly reveal that RBC membrane fragments significantly promoted COM crystal growth and aggregation, as shown by microscopic evaluation with quantitative analysis (figs. 1 and 3, *A* and *B*) and spectrophotometric oxalate depletion assay (fig. 4, *A*). Also, RBC membrane fragments adhered to COM crystal surfaces (fig. 2), while no adhesion was observed for intact RBCs. Intact RBCs could transform COM to COD crystals (figs. 1 and 3, *C*) but had no effects on crystal growth and aggregation (figs. 1 and 3, *A* and *B*). Crystal transformation affected by intact cells was also noted in a study by Lieske et al, in which all newly formed CaOx crystals on the surface of living monkey (BSC-1) and canine (MDCK) renal tubular epithelial cells were COD crystals.[24] Those intact cells may share some surface properties responsible for COM-to-COD crystal transformation, a process that is currently considered a protective mechanism against crystal retention in the kidney since COD crystals have the least potent adsorptive capability to the renal tubular cell surface.[25] Spectrophotometric oxalate depletion assay showed that intact RBCs slightly delayed COM crystal growth during the first 10 to 30 minutes of incubation. This delay may be related to COM-to-COD crystal transformation. Intact RBCs may have a role as a counterbalance mechanism against promoting the activity of RBC membrane fragments.

The promoting effect of RBC membrane fragments on COM crystal aggregation may be mediated by calcium binding molecules, particularly transmembrane and membrane associated proteins on the inner surface of RBC membranes, which are denuded after RBC fragmentation. These calcium binding molecules may act as links among several COM crystals to form aggregates. RBC membrane fragments are not stagnant in the kidney. However, according to our data the role of RBC membrane fragments is to promote COM crystal growth and aggregation. These membrane-crystalline complexes may then stagnate in the kidney and become the core matrix or nidus of COM kidney stones.

Our model experiment may be associated with some specific types of hematuria, which is one of the most common manifestations of renal and urological disease. In the past hematuria involvement in kidney stone pathogenic mechanisms was under investigated or even overlooked since hematuria is considered an effect and not the cause of kidney stone disease. This is not surprising since almost all forms of hematuria are associated with intact RBCs in urine and our data also showed no promoting activity of intact RBCs on the growth and aggregation of COM crystals, which are the major crystalline components in CaOx kidney stones.[26] However, our study revealed that fragmented RBCs can promote COM crystal growth and aggregation. Thus, they may be involved in the pathogenic mechanisms of CaOx kidney stone disease. This less common form of hematuria is found in patients with underlying conditions, in which fragmented RBCs are also present in the systemic blood circulation.

Nevertheless, the onset of crystal modulation or promotion of COM crystal growth and aggregation by RBC membrane fragments must precede the clinical manifestations of COM kidney stone disease for years or decades. Thus, it is not surprising that to our knowledge no previous group has investigated whether RBC membrane fragments are present in the urine of stone formers. Some reports describe the unexplained coincidence of nephrolithiasis with hemolytic uremic syndrome[27] and microangiopathic hemolytic anemia.[28] Thus, we hypothesized that RBC membrane fragments in the urine of these hemolytic patients may be involved at least in part in kidney stone formation.

To address the clinical relevance of RBC membrane fragments we performed a preliminary study to evaluate the effect of RBC membrane fragments on COM crystal growth in urine samples of stone formers and healthy individuals (fig. 4, *B*). Data clearly showed that stone former urine samples had a significantly higher COM crystal growth rate than urine samples from healthy individuals. Furthermore, RBC membrane fragments significantly aggravated COM crystal growth in each group, although growth was still greater in stone former samples. However, it is generally known that the organic and inorganic composition of the urine of stone formers significantly differed from that of healthy individuals with greater levels of stone promoters but lower levels of stone inhibitors in stone formers. Thus, these factors should also be considered for significant differences between stone formers and healthy individuals. In conclusion, to our knowledge we report for the first time that RBC membrane fragments may serve as a promoting factor for COM crystal growth and aggregation, implying that RBC membrane fragments may be involved in CaOx kidney stone formation.

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Bacteria promote calcium oxalate crystal growth and aggregation

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ABSTRACT

Objective: Previous report showed that uropathogenic bacteria i.e., *E. coli* were commonly found inside the nidus of calcium oxalate (CaOx) kidney stone, thus probably involving stone genesis. The present study was conducted to prove this new hypothesis by direct examining CaOx lithogenic activities of bacteria.

Materials and Methods: Lithogenic effects of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were examined at 10^5 CFUs/ml. Crystal image analysis was performed to initially screen direct effects on CaOx crystal growth and aggregation. Validation of bacterial functions were performed by spectrophotometric oxalate depletion assay and CaOx crystal aggregation-sedimentation study. Bacterial-induced CaOx aggregates were also observed by phase-contrast microscopy. Fragmented RBC and intact RBC were used as positive and negative controls, respectively.

Results: Fragmented RBC, but not intact RBC, showed CaOx crystal growth and aggregation promotions, consistent to previous report. Functional analyses revealed *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* promoted crystal growth, approximately 20.17, 17.55, 16.37 and 21.87%, and aggravated crystal aggregation, approximately 57.45, 51.06, 55.32 and 46.81%, respectively. Bacterial-induced CaOx aggregates had the diameter larger than luminal size of distal tubules, suggested tubular occlusion was possible.

Conclusion: *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* at a clinical-relevant concentration had lithogenic effects on CaOx crystal growth and aggregation. This functional evidence supported the new hypothesis of bacterial-induced metabolic stone disease, particularly CaOx type.

INTRODUCTION

Infection-induced kidney stone is now referred to struvite,[1-3] but not metabolic stone. Struvite stones are mostly composed of magnesium ammonium phosphate. Since normal urine is usually undersaturated with ammonium phosphate, struvite stone then occurred only when patients have urinary tract infection (UTI) with a urease-producing organism such as *Proteus*.[1,2] Urease breaks down urinary urea into ammonia and ammonia subsequently combines with water, resulting in production of ammonium and hydroxide ion. Supersaturation of ammonia due to urease-producing bacteria and decreased solubility of phosphate due to elevated urine pH following hydroxide ion production finally drive struvite stone formation.[1,2]

In the other hand, metabolic stones have been characterized as the outcome of metabolic disturbances such as hypercalciuria, hyperoxaluria and hypocitraturia,[3,4] but not UTI. Alterations of urinary stone modulating factors including metabolic substrates (i.e., calcium and oxalate),[3,4] stone inhibitors (i.e, citrate and some urinary anionic proteins)[3-7] and promoting factors (i.e., cell membranes and lipid vesicles)[8-10] have much contribution on metabolic stone development. Once formed, metabolic stones might accidentally entrap some bacteria (if UTI precedes stone formation) and/or become the source of infection (if UTI follows stone formation).[2] According to these, UTI has only been understood as a complication, but not a cause, of metabolic stones.

While most evidences indicated that bacteria were not an inducer of metabolic stone, few reports suggested metabolic stone, particularly the most common calcium oxalate (CaOx), sometimes had an infectious origin.[11-13] This speculation was based on bacteriology and compositional analysis of the inside parts of stone.[11-13] Of these, Sohshang HL, *et. al.*,[13] reported that about 47% of 100 calculi obtained from 100 stone patients had positive urine and stone nidus culture. *E. coli* was the most common organism isolated from the urine and

stone nidus, while CaOx was the major stone nidus composition.[13] These evidences were in line with our recent report, in which CaOx stone cores obtained from stone patients commonly had a positive culture of *E. coli*, but not urease-producing bacteria. It seemed that uropathogenic bacteria, i.e., *E. coli* could deposit inside the stone nidus and may play some roles in CaOx stone formation.

Bacterial-nidus deposition is crucial in the respect of stone pathogenic mechanisms. The nidus locates at the innermost of each stone and acts as an initiator of stone formation.[14] It is now generally understood that organic components in stone nidus play a significant role in stone genesis.[14] Several organic substances which deposited inside CaOx nidus (such as anionic proteins, lipid and red blood cell (RBC) membranes)[8,9,14,15] have been demonstrated their lithogenic effects. Evidences of bacterial deposition inside CaOx stone nidus lead to a question whether uropathogenic bacteria (particularly *E. coli*) also had direct lithogenic activities or not. To the best of our knowledge, at least two publications demonstrated lithogenic effects of bacteria indirectly.[16,17] Hirano S, *et. al.*,[16] reported that bacteria tended to aggregate crystalline and organic matters in the urine of stone formers and suggested these bacteria may participate actively in stone genesis as an adhesive agent. Venkatesan, N, *et. al.*,[17] showed that *E. coli* and *P. mirabilis* aggravated CaOx encrustation onto the surface of polyurethane film, the same material as that of urinary stents. Investigators suggested that biofilm created by those bacteria might be responsible for CaOx encrustation. Nevertheless, direct lithogenic activities of those bacteria were not yet examined.

All above evidences,[11-13,16,17] together with our recent report, allowed us to propose a new hypothesis that UTI with uropathogenic bacteria possibly induced metabolic stone formation, particularly CaOx type. The present study was then conducted to prove this new hypothesis by direct examination of lithogenic activities of bacteria on CaOx crystals. Four bacterial strains i.e., *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* were included into

this study as the representatives of uropathogenic and non-uropathogenic organisms. Direct morphological evaluation and image analysis of CaOx crystals[18] were performed to initially screen promoting effects on crystal growth and aggregation. Lithogenic activities of bacteria were then validated by spectrophotometric oxalate depletion assay[5] and CaOx crystal aggregation-sedimentation study[6] (the gold-standard methods for analyzing CaOx crystal growth and aggregation, respectively). Size and morphology of bacteria-induced CaOx aggregates were also observed under phase-contrast microscopy to evaluate potential clinical significance.

MATERIALS AND METHODS

CaOx crystal preparation and intervention

CaOx crystals were generated according to our protocols published previously.[18,19] Briefly, CaOx crystals were prepared in 24-well, polystyrene, disposable cell culture cluster (with lid) (Corning Inc.; Corning, NY) by mixing calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium oxalate ($Na_2C_2O_4$) at final concentrations of 5.0 and 0.5 mM, respectively, in a buffer containing 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl. After 1-h of CaOx crystallization, the supernatant was discarded, whereas the crystals were gently washed with 100% methanol and then dried in room temperature.

To examine effects of bacteria on crystals, CaOx crystals were prepared as aforementioned, but in the presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* or *S. pneumoniae* (for bacterial preparation, please see "**Supplementary Materials and Methods**"). Fragmented RBC membranes (which previously reported as a promoter of CaOx crystal growth and aggregation)[9] and intact RBC (which had no promoting effect)[9] at 10^5 cells/ml were used as positive and negative controls, respectively. After 1-h incubation, effects of bacteria on CaOx crystals were evaluated by CaOx crystal image analysis (all these experiments were done in triplicate).

CaOx crystal image analysis

CaOx crystal growth and aggregation effect of each bacterium was initially screened by phase-contrast microscopic examination using an Olympus CKX41 inverted light microscope (Olympus Co. Ltd.; Tokyo, Japan). Crystal images were then captured using a digital camera and subjected to ImageMaster 2D Platinum software (GE Healthcare; Uppsala, Sweden) to quantitatively measure crystal area and number of CaOx aggregates.[18] The crystal area was measured and averaged from at least 100 crystals in individual samples. Number of

crystal aggregates, which defined as an assembly of two or more individual crystals tightly joined together,[9,10,20] was counted and averaged from 10 high-power fields (HPF).

Spectrophotometric oxalate depletion assay

CaOx crystal growth promotion was confirmed by spectrophotometric oxalate-depletion assay, as described previously by Nakagawa Y and colleagues.[5] CaOx crystal seeds (160 μg) were added to 1-ml 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 in a cuvette. Basically, the seeded crystals would grow further using excess calcium ions (from $CaCl_2$) and oxalate ions (from $Na_2C_2O_4$), leading to depletion of free oxalate, which is detectable by a Shimadzu UV-160A spectrophotometer (Shimadzu; Kyoto, Japan) at λ214 nm. The bacteria to be test were added into this solution to a final concentration of 10^5 CFUs/ml before adding the crystal seeds. Depletion of free oxalate ions would be accelerated if bacteria promote CaOx crystal growth. Rate of free oxalate depletion was calculated using the baseline value and the value after 60-sec incubation with or without bacteria. The relative promoting activity was calculated using the following equation;

% CaOx crystal growth promotion = [(C-S)/C] x 100

Where C = Rate of free oxalate depletion without bacteria

S = Rate of free oxalate depletion with bacteria to be test

CaOx crystal aggregation-sedimentation study

CaOx crystal aggregation promotion was validated by CaOx crystal aggregation-sedimentation study, as described previously by Atmani F and Khan SR[6] with a modification of $CaCl_2$ and $Na_2C_2O_4$ concentrations (from original concentrations of 3 mM $CaCl_2$ and 0.5 mM $Na_2C_2O_4$) [6] to prevent spontaneous CaOx nucleation. Individual CaOx crystals (100 μg)

were added into 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 in a cuvette. The reaction was stirred at 800 rpm to prevent sedimentation of individual crystals but not the large aggregates. Bacteria to be test were added into this solution to a final concentration of 10^5 CFUs/ml before adding CaOx crystals. Crystal aggregation process would consume a number of individual CaOx crystals to form larger particulates. Sedimentation of those aggregates was then resulted in reduction of solution turbidity, which can monitor by a Shimadzu UV-160A spectrophotometer (Shimadzu) at λ620 nm for 60-min. Aggregation promotion was evaluated by comparing the turbidity slope in the presence and absence of bacteria to be test at the end of the assay. In addition, morphology of the aggregates generated by this reaction was observed under a phase contrast microscope to reconfirm crystal aggregation potency of bacteria. The maximal diameter of the largest CaOx aggregate in each HPF (but not the smaller aggregates or individual crystals) was measured by Tarosoft® Image framework v.0.9.6 (Nikon Corp.; Tokyo, Japan). Each data point was derived from 5 individual samples and the data are reported as mean ± SEM.

Statistical analysis

All the quantitative data are reported as mean ± SEM. Multiple comparisons were performed using ANOVA with Tukey's post-hoc test (SPSS, version 11.5). P values < 0.05 were considered statistically significant.

RESULTS

Four bacterial strains including *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* were propagated in LB Broth before estimation of bacterial concentrations by plate colony counting and turbidimetry at an optical density of 600 nm (OD_{600}) (see "**Supplementary Materials and Methods**"). Correlations of bacterial concentrations from plate colony counting and OD_{600} values were analyzed by linear regression analysis (see "**Supplementary Figure S1**"). By this method, a clinical relevant concentration of 10^5 CFU/ml of *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* was accurately prepared for subsequent analyses.

Crystal image analysis was initially performed to screen CaOx lithogenic activities of *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae*. Fragmented RBC (which previously reported as a stone promoting factor)[9] and intact RBC (which had no modulating effect)[9] were adopted as the positive and negative controls, respectively. Consistent with previous report,[9] morphological data showed that fragmented RBC could promote CaOx crystal growth (as represented by increase in crystal size) and aggregation (as represented by increase in number of CaOx aggregates) as compared to the blank control, whereas intact RBC had no promoting activity [**Figure 1A**]. Interestingly, *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* also promoted CaOx crystal growth and aggregation [**Figure 1A**]. These observed effects of bacteria were clearly demonstrated by quantitative analyses of CaOx crystal area [**Figure 1B**] and number of crystal aggregates [**Figure 1C**]. The initial results suggested that *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* had potential lithogenic effects on CaOx growth and aggregation.

To confirm the promoting effect on CaOx crystal growth, spectrophotometric oxalate depletion assay was performed. At 60-sec of monitoring, fragmented RBC (the positive control) promoted 23.67±3.36% of crystal growth, whereas intact RBC (the negative control) had no promoting effect (0.85±2.86%) as comparable to that of blank control (0.42±1.12%)

[**Figure 2**]. *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* showed crystal growth promoting activity of 20.17±3.42, 17.55±2.27, 16.37±1.38 and 21.87±0.85%, respectively [**Figure 2**]. This result was consistent with the initial screening experiment [**Figure 1A** and **1B**] and confirmed that *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* had direct lithogenic activity on CaOx crystal growth.

CaOx crystal aggregation-sedimentation study was performed to validate promoting effect of bacteria on CaOx crystal aggregation. Reduction of λ_{620} was resulted from the large particle sedimentation, which in turn reflected the degree of crystal aggregation. At 60-min, fragmented RBC (the positive control) aggravated 53.19 ± 3.61% of crystal aggregation, whereas intact RBC (the negative control) had no significant promoting effect (6.38±5.51%) as compared to that of blank control (0.00±5.51%) [**Figure 3A**]. *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* exhibited crystal aggregation promotion of 57.45±2.08, 51.06±5.51, 55.32±2.08 and 46.81±3.61%, respectively [**Figure 3A**]. This result confirmed that *E. coli, K. pneumoniae, S. aureus* and *S. pneumoniae* had the direct lithogenic effect on CaOx crystal aggregation and also supported Hirano's proposal[16] in which bacteria might actively participate in stone genesis by acting as the adhesive agent.

To reconfirm CaOx crystal aggregation promoting effect of bacteria, the aggregated particulates from the aggregation-sedimentation experiment were collected to observe the size and morphology of aggregates under phase-contrast light microscopy. As showed in **Figure 3B**, CaOx aggregates induced by *E. coli, K. pneumoniae, S. aureus, S. pneumoniae* and fragmented RBC were obviously larger than those of blank control and intact RBC. Some bacterial-induced CaOx aggregates formed as the huge, tightly packed, round shaped particulates which probably behaved as stone core nidus. Maximal diameter of CaOx aggregates induced by *E. coli, K. pneumoniae, S. aureus, S. pneumoniae* and fragmented RBC were 69.74±4.79, 62.99±2.88, 58.53±5.64, 57.33±7.57 and 63.49±4.39 μm,

respectively, which were clearly larger than that of blank control and intact RBC (26.14±2.15 and 28.28±0.93 μm, respectively) [**Figure 3C**]. Moreover, the size of bacterial-induced CaOx aggregates also exceeded the intraluminal diameter of distal renal tubule (approximately 20-30 μm),[21] suggesting that tubular occlusion was possible. This direct observation, together with lithogenic functional studies of bacteria, may explain how bacteria presented inside the stone core nidus as reported previously.[13]

DISCUSSION

The present study demonstrated, for the first time, that bacteria could directly promote CaOx crystal growth and aggregation by the novel screening method[18] and the gold-standard assays.[5,6] CaOx stone promoting activities of bacteria were comparable to those of RBC membrane fragments, a newly proposed CaOx stone promoter.[9] This study supported our hypothesis of bacterial-induced metabolic stone and the significance of several (scattered) previous reports,[11-13,16,17] and also suggested to reconsidering UTI as a possible cause of metabolic stone disease.

Unexpected finding was that all bacterial strains used in this study showed CaOx lithogenic activities. At first, we assumed that only uropathogenic bacteria i.e., *E. coli* and *K. pneumoniae* would promote CaOx crystal growth and aggregation based on clinical microbiology and previous results of stone nidus culture.[13] However, non-uropathogenic bacteria, including *S. aureus* and *S. pneumoniae*, also exhibited CaOx lithogenic effects. One explanation was that CaOx promoting activities of bacteria did not require a unique biological activity (unlike struvite stone which needed urease activity),[1,2] but may depend on a common property among bacteria.

Most bacteria have an overall negative charge under physiologic pH.[22] Ionic interaction between anionic bacteria and cationic CaOx crystals should then be suspected, since this interaction was reported as the adhesive force between stone modulating factors (such as anionic urinary molecules or epithelial cell surfaces)[23,24] and CaOx crystals by atomic force microscopy. Specific molecules on bacterial surface that responsible for this interaction have remained totally unknown. Teichoic acid[25] and lipopolysaccharide (LPS),[26] two abundant anionic molecules on bacterial cell walls which provide an overall negative charge to gram-positive and gram-negative bacteria, respectively, may be responsible as the key players in this setting. Further studies are required to elucidate the detailed mechanisms

of lithogenic activities of bacteria. Noted that LPS and gram-negative uropathogenic bacteria may be more important in bacterial-induced metabolic stone due to clinical microbiology.

Based on potential ionic interaction between bacteria and CaOx crystals, pathogenic mechanisms of bacterial-induced CaOx stone were anticipated as followed. Once bacteria interacted with CaOx crystals, anionic nature of bacteria may attract the free calcium ions from environment to increase local calcium concentrations, resulting in promotion of crystal growth. In addition, the interacted bacteria on CaOx surface may act as a linker or adhesive agent[16] among individual CaOx crystals, leading to crystal aggregation. A huge, tightly impacted, round-shaped CaOx aggregates (as shown in **Figure 3B**) seemed to be a subsequent result of lithogenic effects of bacteria. Since the size of bacterial-induced CaOx aggregates was larger than the lumen of distal tubules and collecting ducts[21] (as shown in **Figure 3C**), these aggregates may be retained inside the kidney due to tubular occlusion and potentially act as the core nidus of stone development. Indeed, this proposed mechanism was similar to those of cell membrane fragments and lipid vesicles published previously,[8-10] reflecting that abnormal presentation of anionic charged particulates within urinary tract possibly lead to CaOx stone formation through a common pathway.

Currently, UTI is not recognized as a cause of metabolic stone disease. It might be due to the discordant correlation between UTI episode and metabolic stone formation (unlike UTI with urease-producing bacteria and struvite stone formation). However, CaOx stone development was relatively slow (when compared to that of struvite), so it may require several months or many years after UTI episode for stone growth into a clinical-relevant size (which may be out-of-range of observational period). Moreover, direct CaOx lithogenic activities of bacteria had never been examined. In contrast, the present study, together with previous reports,[11-13,16,17] provided crucial information that fill the knowledge gap and may correct some misconceptions of infection-induced kidney stone. Our study proved that

bacteria could directly promote CaOx crystal growth and aggregation, two important stone pathogenic mechanisms. This functional evidence was corresponding to microbiological evidences,[11-13] reflecting that bacterial-nidus deposition may play some crucial roles in stone genesis. Further investigations to complete understanding of bacterial-induced metabolic stone may lead to better management and prevention of the first stone episode and the recurrent stone disease. At least, UTI should be reconsidered as a possible cause of metabolic stone disease, particularly CaOx type.

We had to admit that *in vivo* study using animal model was required to confirm CaOx lithogenic roles of uropathogenic bacteria. However, UTI induction in animal models might generate a number of interfering factors e.g., altered stone inhibitors,[3-7] inflammatory reaction,[27] renal cell injury,[28] cell debris and membrane fragments,[9,10] and the presence of antibiotics for UTI treatment (if used) such as ceftriaxone.[29] These factors could modulate CaOx stone genesis[3-7,9,10,27-29] which may lead to misinterpretation. Therefore, all those factors need to be concerned during experimental designs in order to adjust the risk and evaluate the outcome of UTI on metabolic stone formation.

In conclusion, this study showed that, for the first time, bacteria including *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* at the clinical-relevant concentration could significantly promote CaOx crystal growth and aggregation *in vitro*. This functional evidence strongly supports the new hypothesis of bacterial-induced metabolic kidney stone disease.

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FIGURE LEGENDS

Figure 1. Crystal image analysis. CaOx lithogenic effects of bacteria were firstly screened by morphological evaluation (**A**). CaOx crystals were generated in absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae* (see details in "Materials and Methods"). Fragmented RBC and intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. Dashed circles indicate COM aggregates (which were defined as groups of two or more COM crystals tightly adhered together). Original magnification = 400X for all panels. Crystal images were captured by a digital camera and submitted into ImageMaster 2D Platinum Software for quantitative data of CaOx crystal area (**B**) and number of CaOx aggregates (**C**). Each bar was derived from 3 independent experiments and the data are reported as mean ± SEM.

Figure 2. Spectrophotometric oxalate depletion assay. CaOx crystal seeds (160 μg) were added to 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 1 mM $Na_2C_2O_4$ in a cuvette. Depletion of free oxalate (which indicated crystal growth) was detectable by measuring absorbance at λ214 nm for up to 60-sec in the absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Fragmented RBC and

intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. % CaOx crystal growth promotion = [(C-S)/C] x 100; where C = Rate of free oxalate depletion without bacteria and S = Rate of free oxalate depletion with bacteria to be test (see details in “Materials and Methods”). Each data point was derived from 3 independent experiments and the data are reported as mean ± SEM.

Figure 3. CaOx crystal aggregation-sedimentation study. (**A**) Individual CaOx crystals (100 μg) were added into 1-ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$ and 0.1 mM $Na_2C_2O_4$, in a cuvette. The reaction was stirred at 800 rpm to prevent sedimentation of individual crystals but not the large aggregates. Sedimentation of those aggregates reduced solution turbidity, which was monitored at λ620 nm for 60-min in the absence (blank control) or presence of 10^5 CFUs/ml of *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*. Fragmented RBC and intact RBC (equivalent to 10^5 cells per assay) were used as positive and negative controls, respectively. Aggregated particulates generated by this reaction were morphologically observed under a phase contrast microscope **(B)**. The maximal diameter of the largest CaOx aggregate in each HPF (but not the smaller aggregates or individual crystals) was measured by Tarosoft® Image framework from 5 individual samples (**C**). Dash line indicates an intraluminal diameter of distal renal tubules (30 μm). Each data point was derived from 5 individual samples and the data are reported as mean ± SEM.

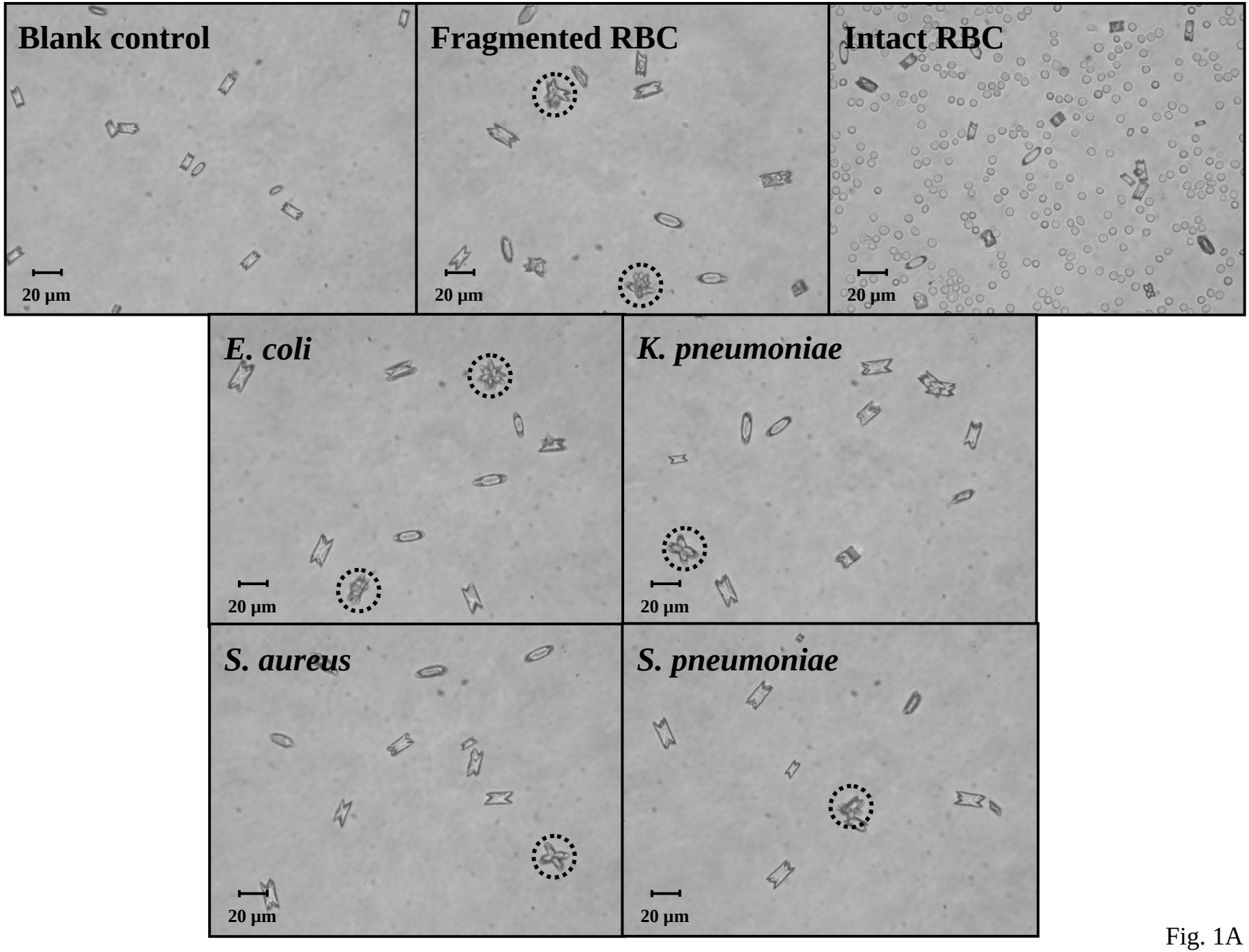

Blank control
20 µm
Fragmented RBC
20 µm
Intact RBC
20 µm
E. coli
20 µm
K. pneumoniae
20 µm
S. aureus
20 µm
S. pneumoniae
20 µm

Fig. 1A

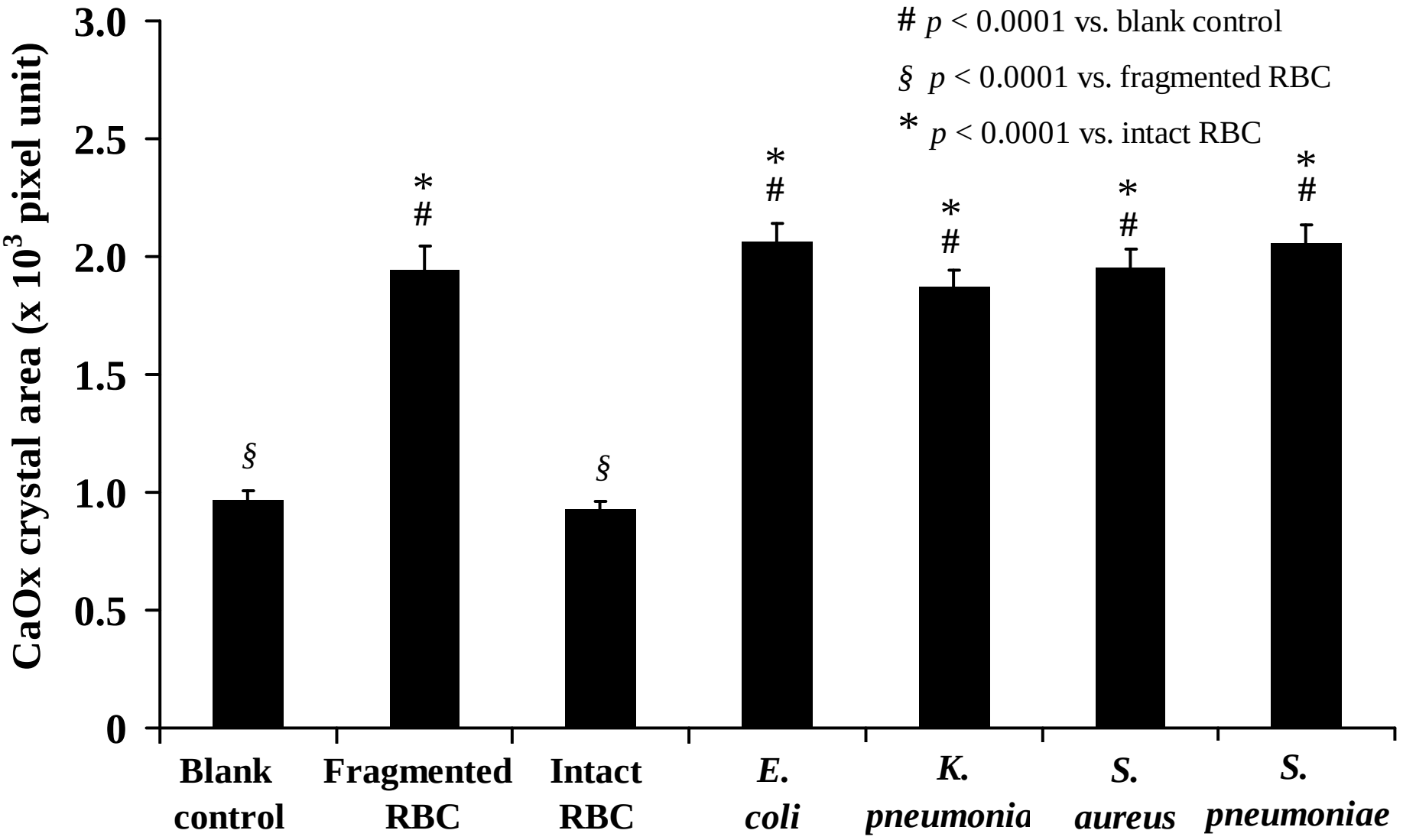

CaOx crystal area (x 10^3 pixel unit)
3.0
2.5
2.0
1.5
1.0
0.5
0
# p < 0.0001 vs. blank control
§ p < 0.0001 vs. fragmented RBC
* p < 0.0001 vs. intact RBC
Blank control
Fragmented RBC
Intact RBC
E. coli
K. pneumonia
S. aureus
S. pneumoniae

Fig. 1B

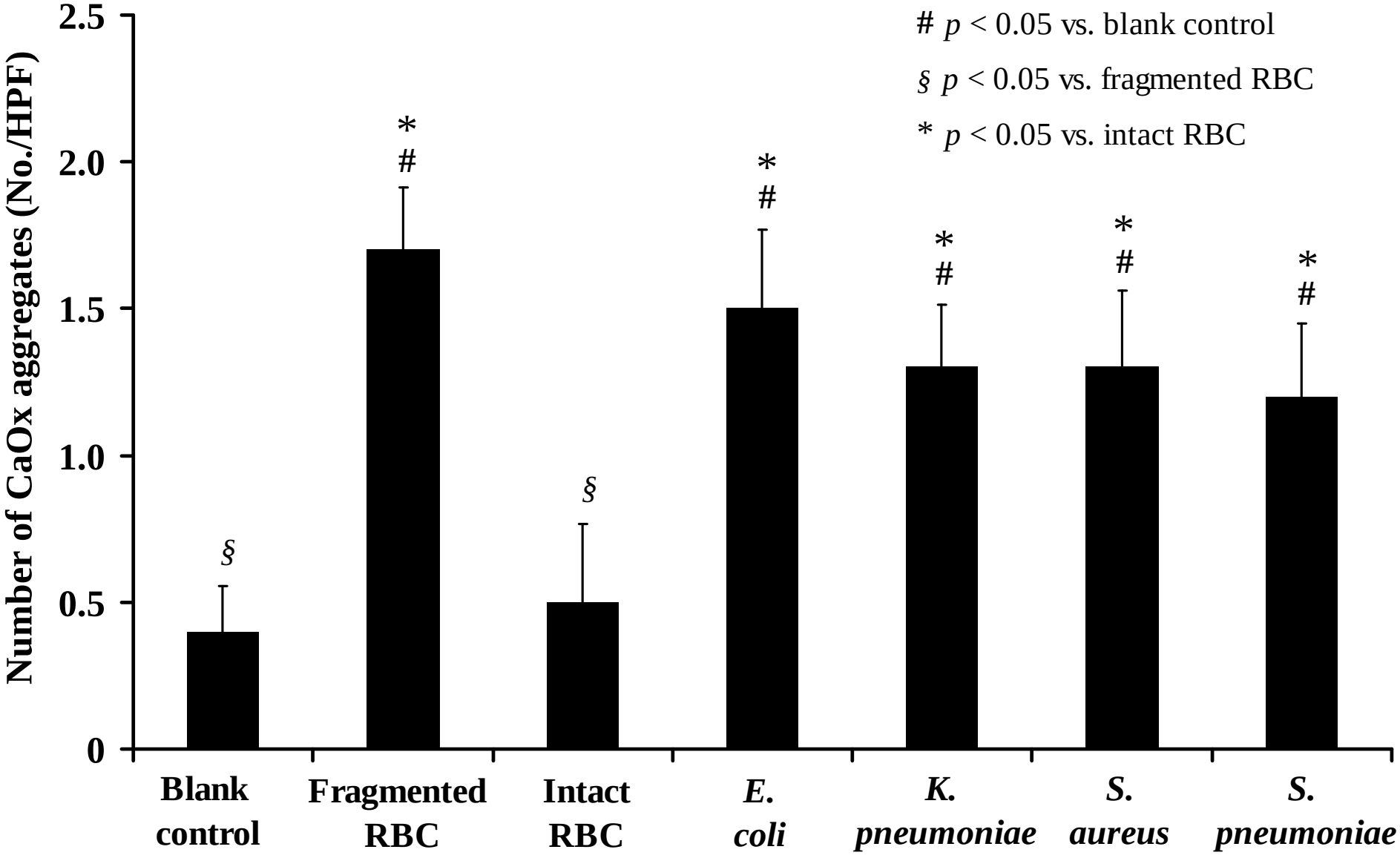

# $p < 0.05$ vs. blank control
§ $p < 0.05$ vs. fragmented RBC
* $p < 0.05$ vs. intact RBC
Number of CaOx aggregates (No./HPF)
2.5
2.0
1.5
1.0
0.5
0
Blank control
Fragmented RBC
Intact RBC
E. coli
K. pneumoniae
S. aureus
S. pneumoniae

Fig. 1C

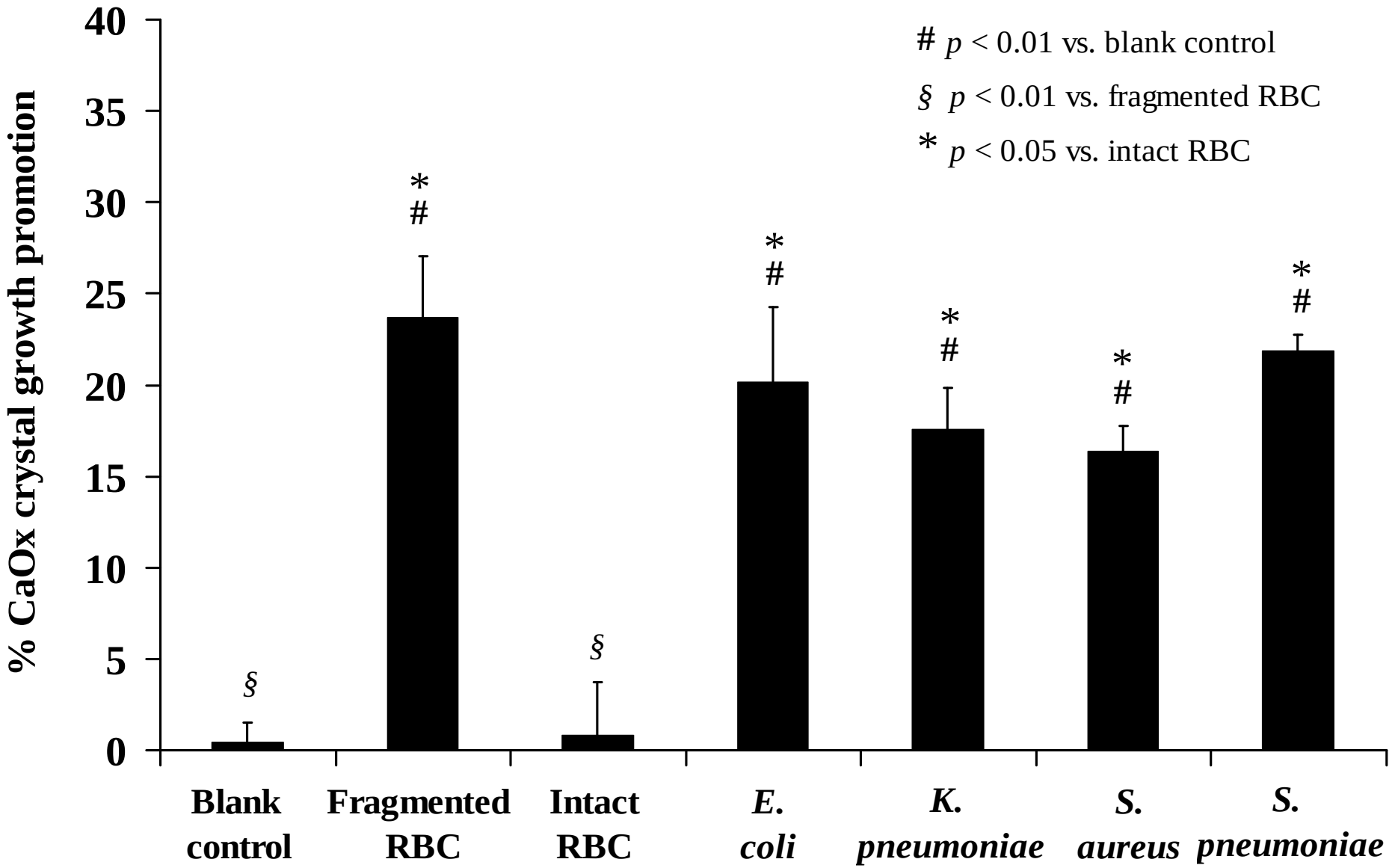

# $p < 0.01$ vs. blank control
§ $p < 0.01$ vs. fragmented RBC
* $p < 0.05$ vs. intact RBC
% CaOx crystal growth promotion
40
35
30
25
20
15
10
5
0
§
*
#
§
*
#
*
#
*
#
*
#
Blank control
Fragmented RBC
Intact RBC
E. coli
K. pneumoniae
S. aureus
S. pneumoniae

Fig. 2

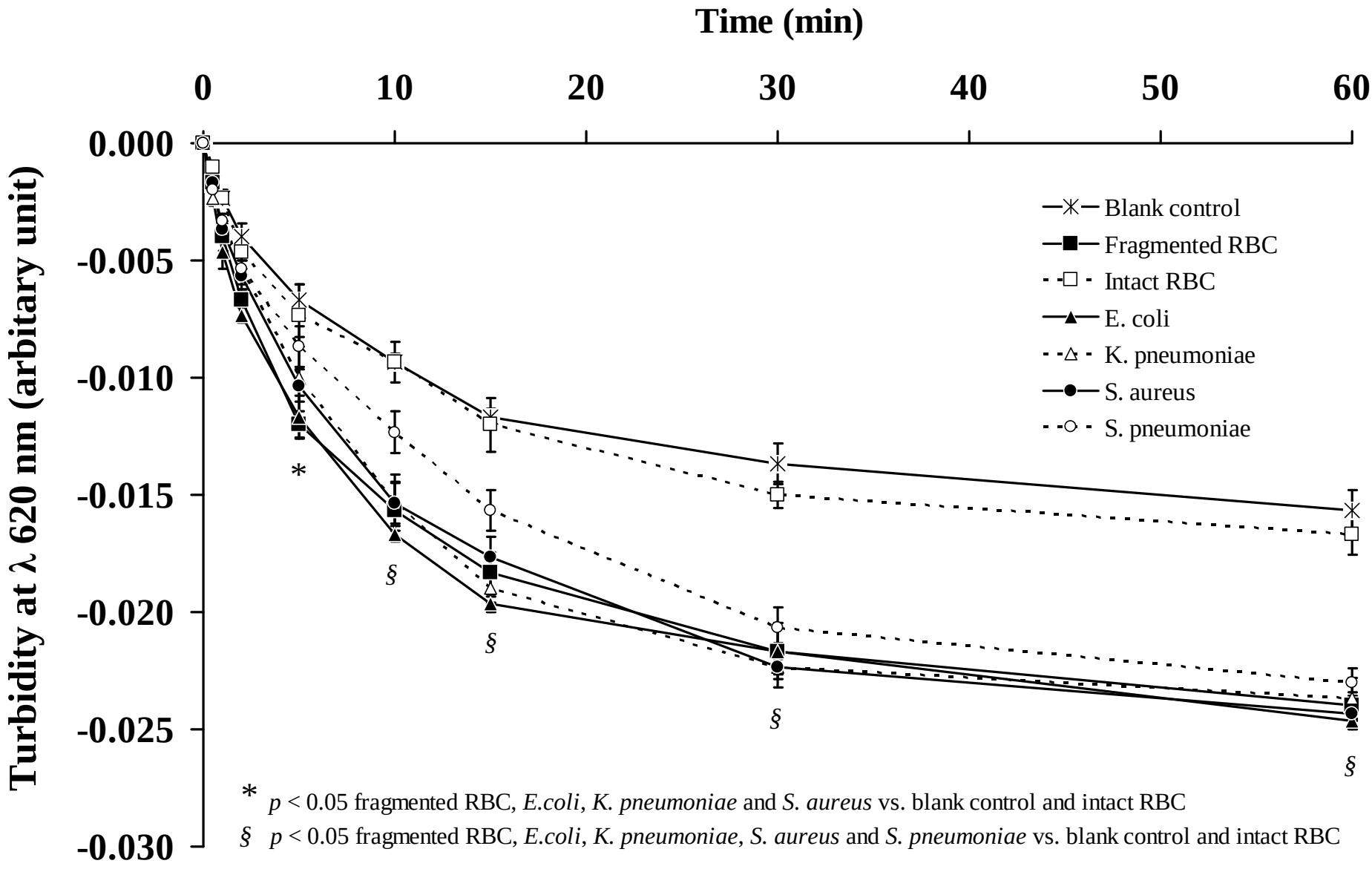

Time (min)
0
10
20
30
40
50
60
0.000
-0.005
-0.010
-0.015
-0.020
-0.025
-0.030
Turbidity at λ 620 nm (arbitary unit)
Blank control
Fragmented RBC
Intact RBC
E. coli
K. pneumoniae
S. aureus
S. pneumoniae
*
§
* p < 0.05 fragmented RBC, E.coli, K. pneumoniae and S. aureus vs. blank control and intact RBC
§ p < 0.05 fragmented RBC, E.coli, K. pneumoniae, S. aureus and S. pneumoniae vs. blank control and intact RBC

Fig. 3A

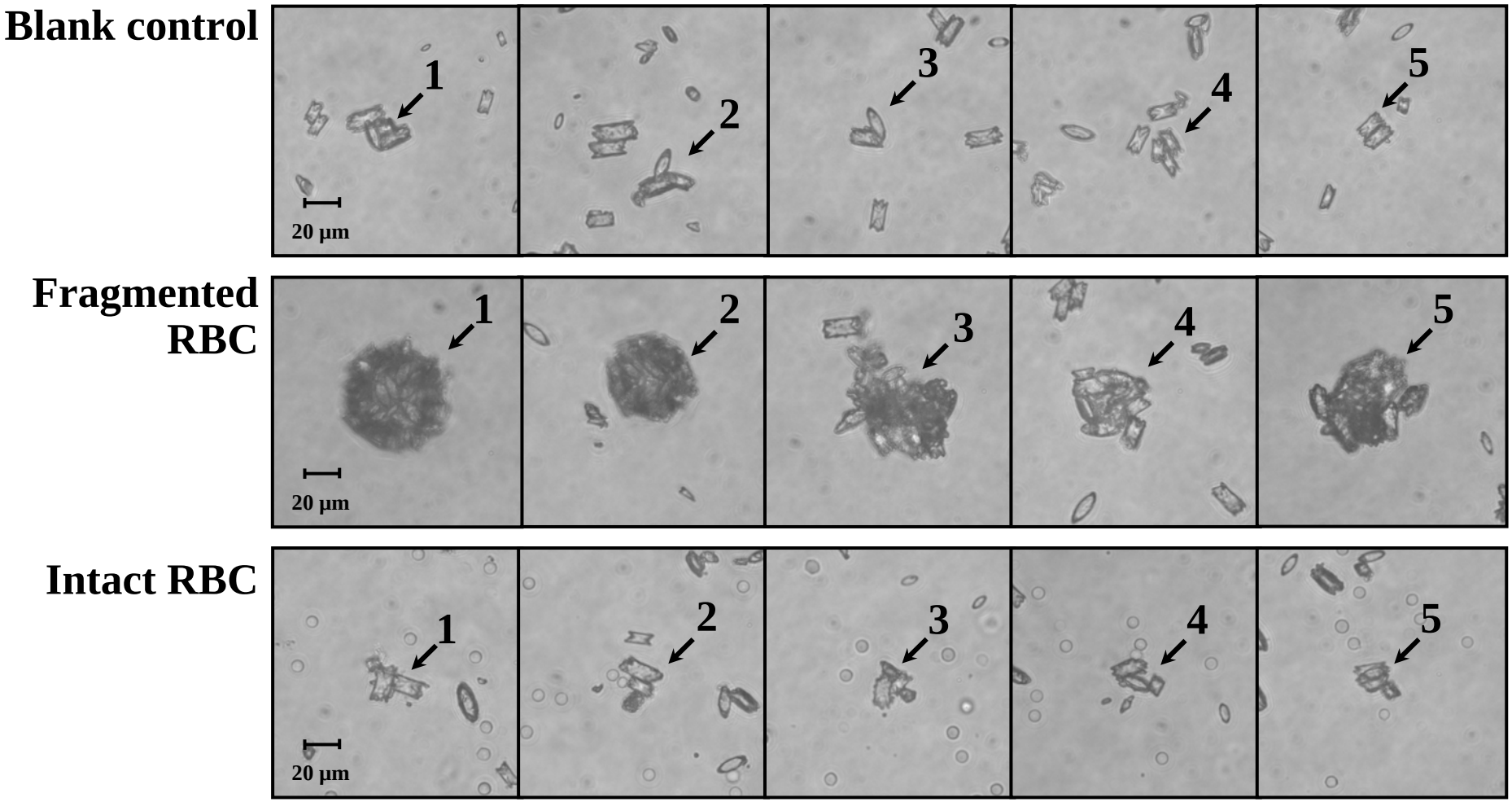


Fig. 3B

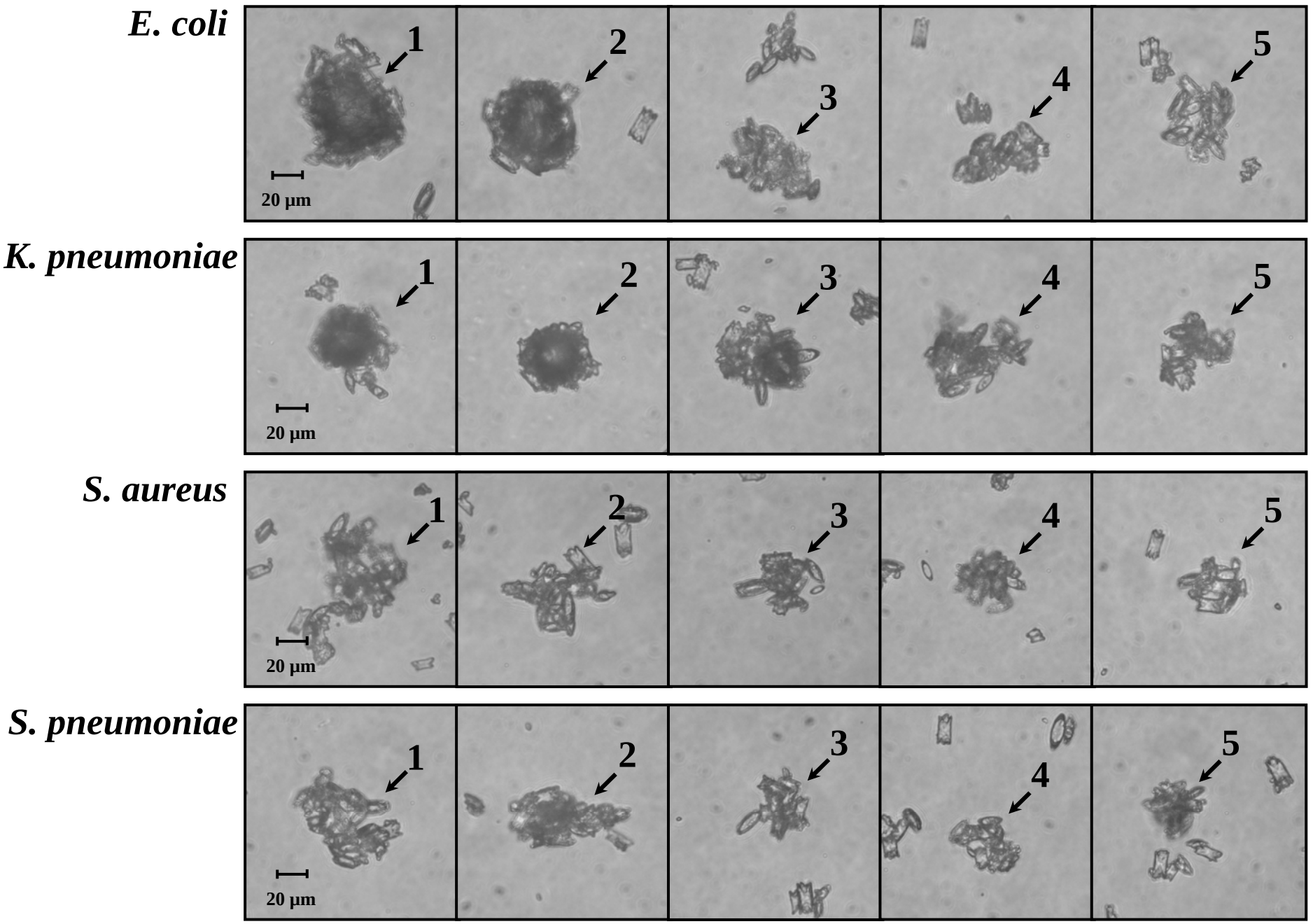


Fig. 3B (cont.)

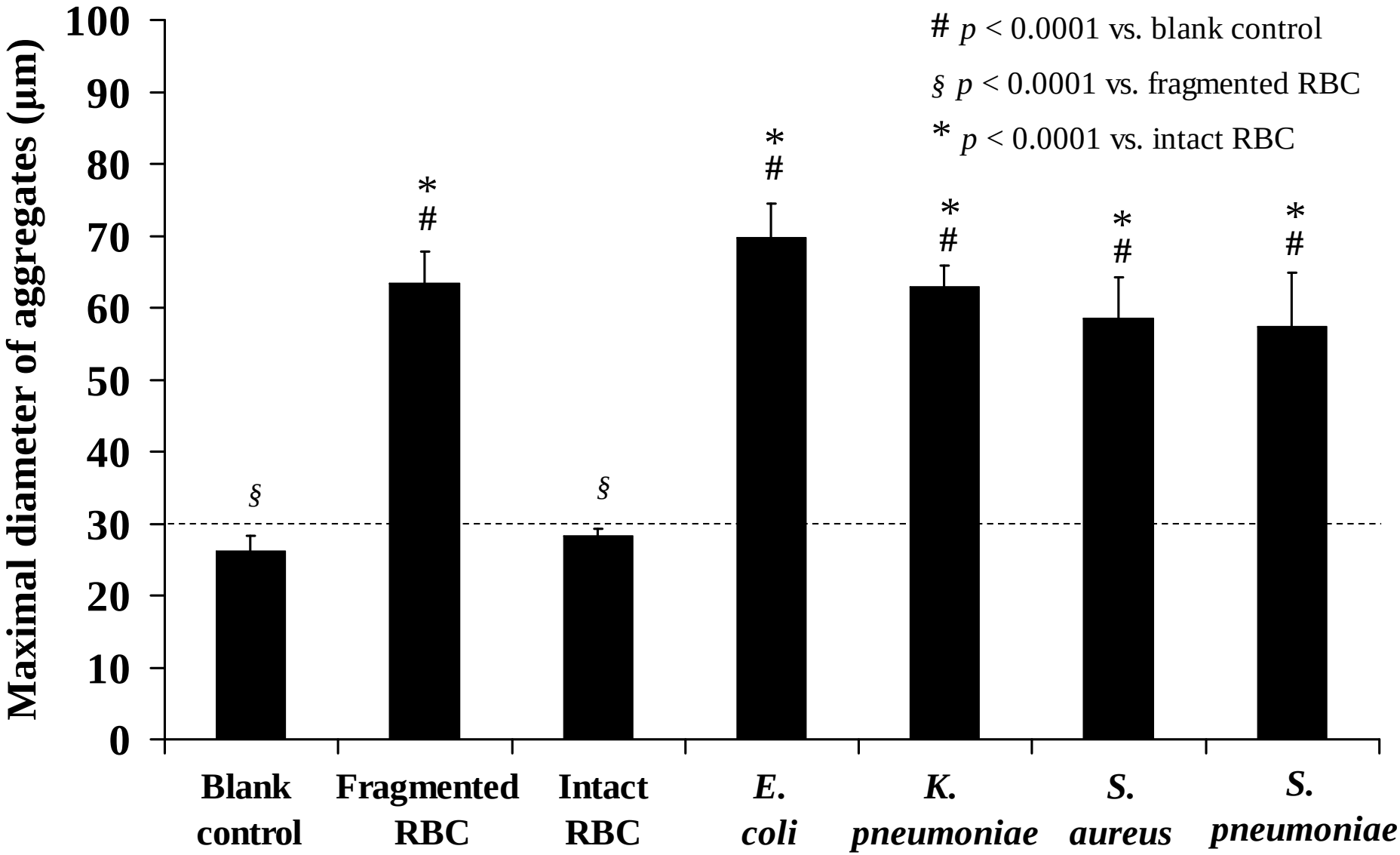

Maximal diameter of aggregates (µm)
100
90
80
70
60
50
40
30
20
10
0
# p < 0.0001 vs. blank control
§ p < 0.0001 vs. fragmented RBC
* p < 0.0001 vs. intact RBC
§
*
#
§
*
#
*
#
*
#
*
#
Blank control
Fragmented RBC
Intact RBC
E. coli
K. pneumoniae
S. aureus
S. pneumoniae

Fig. 3C

SUPPLEMENTARY METERIALS AND METHODS

Bacterial culture and estimation

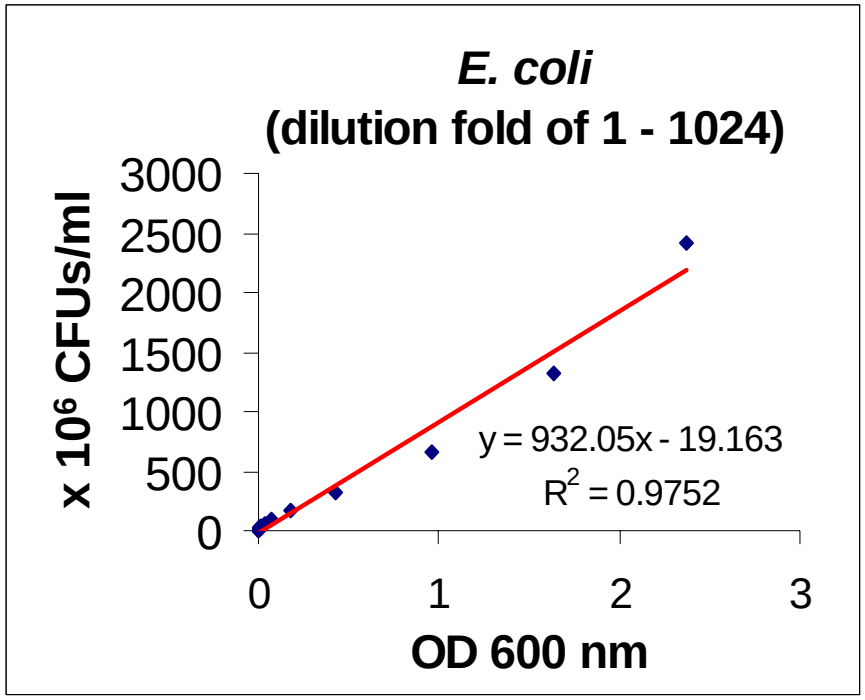
To prepare *E. coli*, *K. pneumoniae*, *S. aureus* and *S. pneumoniae*, a single colony of each bacterium was inoculated from a freshly streak agar plate by a sterile metal loop and then transfer to 5 ml LB Broth (1% tryptone, 0.5% yeast extract and 0.5% sodium chloride) (BD Diagnostic Systems; Frankin Lakes, NJ) in a sterile glass culture tube with a plastic cap. After mixing, the culture tubes were incubated in a rotating incubator at 37°C overnight. Bacterial cultures were then harvested during logarithmic phase. After estimation of bacterial concentration, bacteria were harvested by centrifugation at 10,000 x g for 5 min and washed 3 times with sterile isotonic saline to eliminate media contaminations. Bacteria were freshly prepared and immediately used for subsequent experiments.

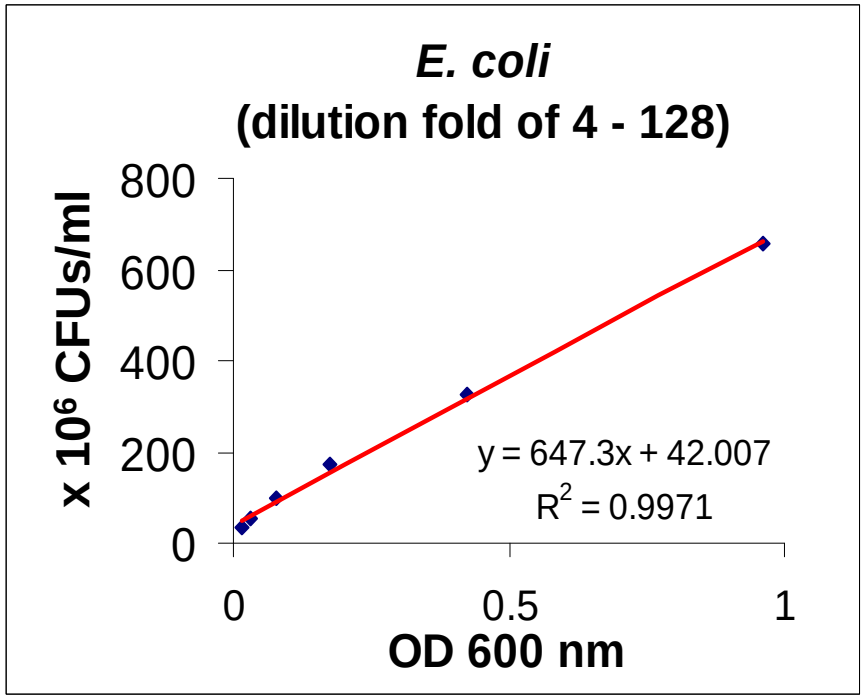
Bacterial concentrations were estimated by plate colony counting combined with turbidimetry at an optical density of 600 nm (OD_{600}). Briefly, 10 μl medium from 2-fold serial dilutions (1:1–1:1024) of each bacterial culture were plated on agar plates and incubated at 37°C overnight, counted the numbers of bacterial colony, and calculated for the concentrations as colony forming units per ml (CFUs/ml). Turbidity at OD_{600} of those bacterial suspensions was measured by a spectrophotometer at the time of bacterial plating. Linear regression analysis was performed to predict the correlation of bacterial concentrations and the measured OD_{600} values. Range of linearity was selected and validated by coefficient of determination (R^2). Linear regression equation, range of linearity at OD_{600} and R^2 were reported as followed (where x was OD_{600} value and y was bacterial concentration x 10^6 CFUs/ml); *E. coli*, $y = 647.3x + 42.007$, OD_{600} of 0.015-0.960, $R^2 = 0.9971$; *K. pneumoniae*, $y = 1111x + 1.4525$, OD_{600} of 0.023-1.021, $R^2 = 0.9996$; *S. aureus*, $y = 1753.7x + 35.848$, OD_{600} of 0.034-0.944, $R^2 = 0.9962$; *S. pneumoniae*, $y = 67.386x + 0.8032$, OD_{600} of 0.016-0.198, $R^2 = 0.9976$. As a result, the clinical-related concentration of

10^5 CFUs/ml was accurately prepared by measuring OD_{600} values within the established linear range of each bacterium.

Supplementary Figure

Supplementary Figure S1. Estimation of bacterial amounts. After propagation, amounts of E. coli (**S1A**), K. pneumoniae (**S1B**), S. aureus (**S1C**) and S. pneumoniae (**S1D**) were estimated by plate colony counting and turbidimetry at OD_{600}. Texts with red color labeled range of linearity at OD_{600} with corresponding bacterial amounts derived from plate colony counting. Linear regression equation of linear range and the measured OD_{600} value was then used to estimate bacterial amounts for subsequent analyses.



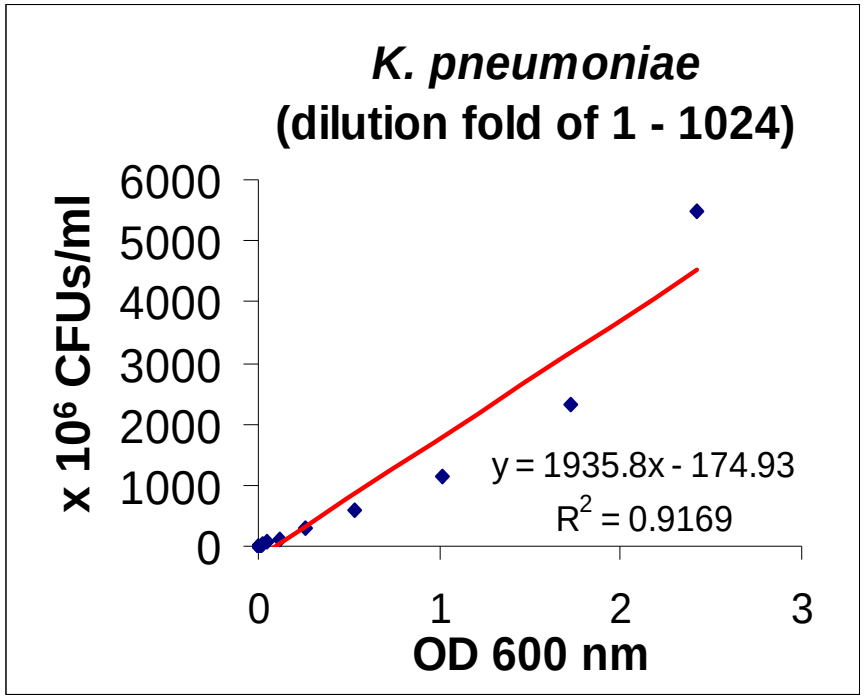


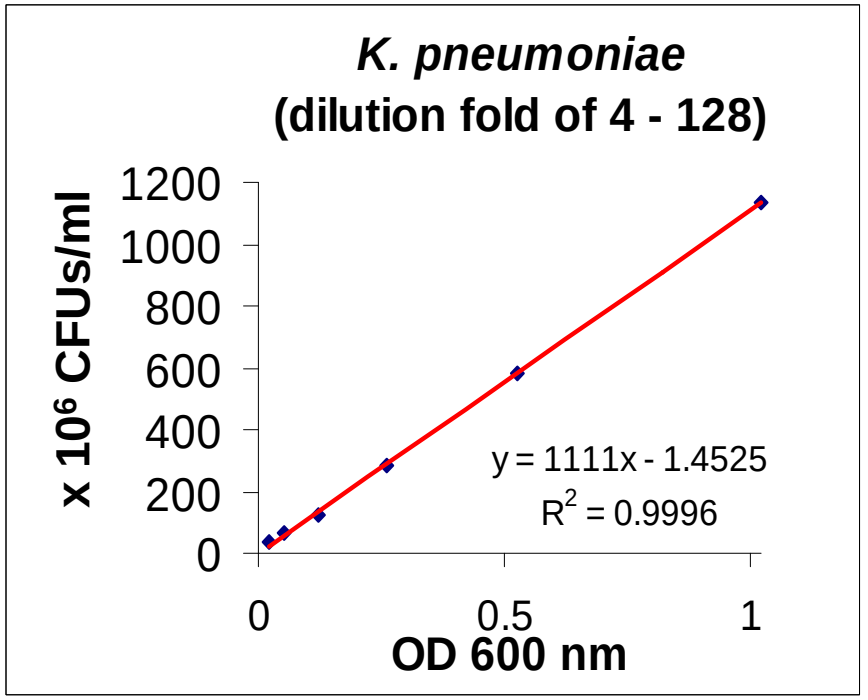
Dilutional fold	OD 600 nm	Number of bacteria (x 10^6 CFUs/ml)
1	2.374	2416.0
2	1.641	1323.2
4	**0.960**	**656.8**
8	**0.424**	**324.8**
16	**0.175**	**170.4**
32	**0.076**	**100.0**
64	**0.032**	**56.0**
128	**0.015**	**32.8**
256	0.008	16.8
512	0.004	9.6
1024	0.001	4.8

Linear range at OD_{600} of *E. coli*:

0.015-0.960

Supplementary Figure S1A

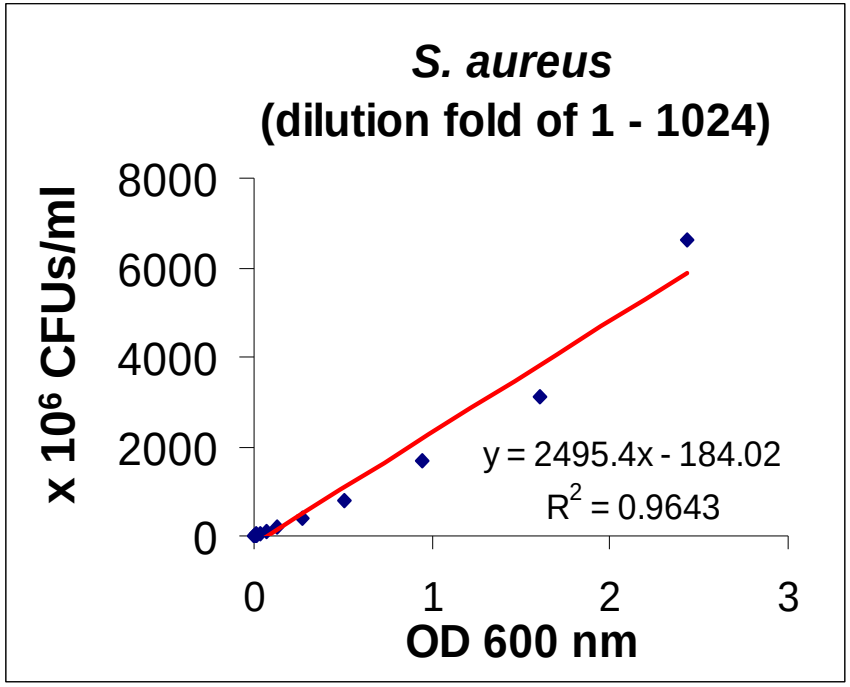


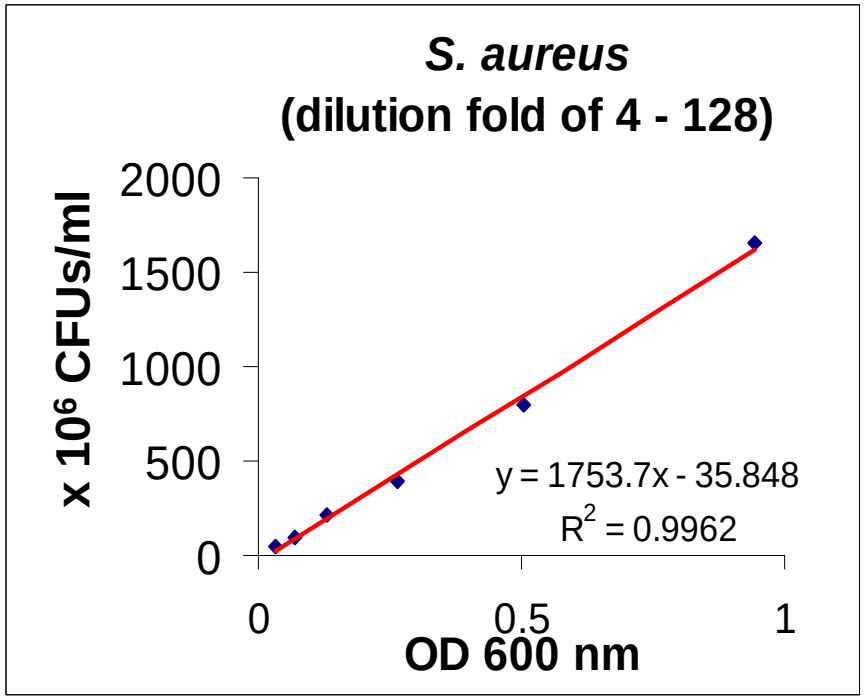


Dilutional fold	OD 600 nm	Number of bacteria (x 10^6 CFUs/ml)
1	2.427	5475.2
2	1.730	2306.4
4	**1.021**	**1137.6**
8	**0.527**	**658.4**
16	**0.261**	**285.6**
32	**0.122**	**120.8**
64	**0.052**	**62.4**
128	**0.023**	**35.2**
256	0.010	18.4
512	0.004	12.0
1024	0.001	3.2

Linear range at OD_{600} of *K. pneumoniae*: 0.023-1.021

Supplementary Figure S1B

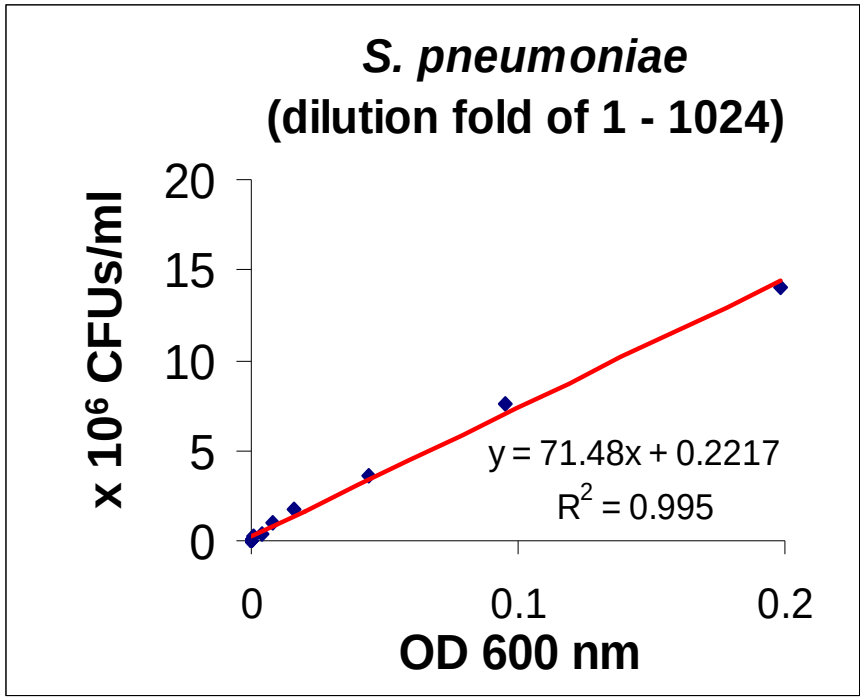


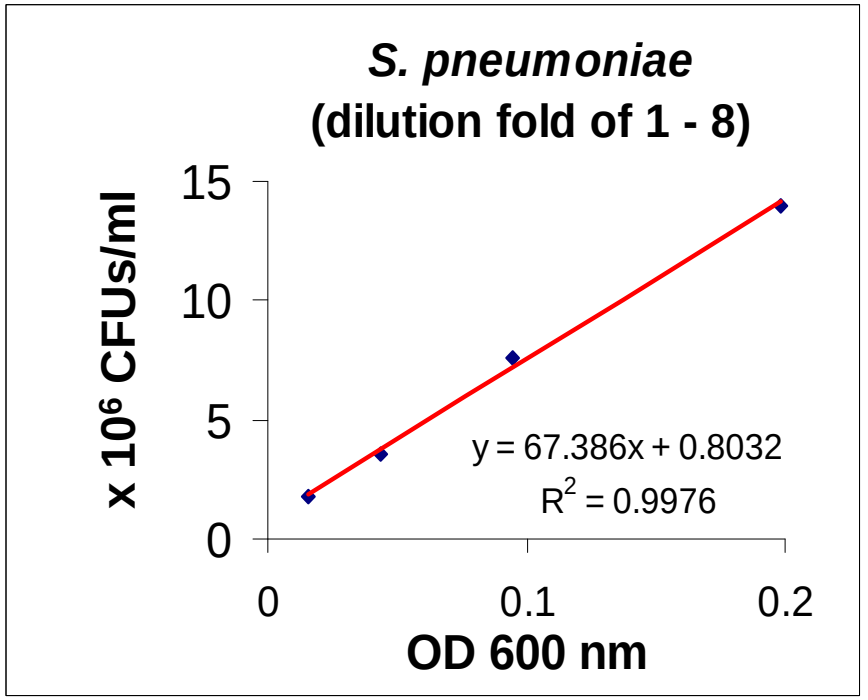


Dilutional fold	OD 600 nm	Number of bacteria (x 10^6 CFUs/ml)
1	2.434	6607.2
2	1.608	3132.0
4	**0.944**	**1656.0**
8	**0.505**	**793.6**
16	**0.266**	**392.0**
32	**0.129**	**210.4**
64	**0.068**	**96.0**
128	**0.034**	**49.6**
256	0.015	28.0
512	0.008	13.6
1024	0.004	7.2

Linear range at OD_{600} of *S. aureus*: 0.034-0.944

Supplementary Figure S1C





Dilutional fold	OD 600 nm	Number of bacteria (x 10^6 CFUs/ml)
1	**0.198**	**14.0**
2	**0.095**	**7.6**
4	**0.044**	**3.6**
8	**0.016**	**1.8**
16	0.008	1.0
32	0.004	0.4
64	0.001	0.2
128	0	-
256	0	-
512	0	-
1024	0	-

Linear range at OD_{600} of S. pneumoniae: 0.016-0.198

Supplementary Figure S1D