An *In Vitro* Study on the Mechanism of Formation of Rounded Melamine Cyanurate Crystals with Special Reference to Melamine-Triggered Urolithiasis in the Kidney

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Abbreviation

ABO: antibiotic solution

ABS: acrylonitrile butadiene styrene

BCA: bicichoninic acid

BGG: bovine gamma-globulin

B2M: beta2-microglobulin

BSA: bovine serum albumin

C: cyanuric acid

°C: Celsius

CO_2: carbon dioxide

DW: distilled water

FBS: fetal bovine serum

GI: gastrointestinal tract

GNP: gold nanoparticle

HBSS: Hanks' buffered salt solution

L-Glu: L-Glutamine

M-C: melamine-cyanurate

min: minute (s)

M: melamine

MW: molecular weight

RIPA: radioimmunoprecipitation assay

PVP: polyvinylpyrrolidone

SEM: scanning electron microscopy

Preface

In September 2008, a melamine contamination scandal came to public attention. More than 47,000 infants and children suffered long-term kidney damage, and three infants died, due to accumulation of intra-renal urolites after consuming melamine-tainted milk, and the incident was reported by the World Health Organization (WHO) (Bhalla et al., 2009; WHO, 2008). Several other melamine adulteration incidents involving pet food were also reported. In 2007, melamine and its related compound triazine were detected in wheat gluten and rice protein extract used for producing pet food. Such adulterated pet food products are believed to have caused the deaths of more than a thousand pets due to kidney impairment leading to renal failure (Brown et al., 2007; Reimsschuessel et al., 2008; Tolleson, 2008) (Fig. 1).

Melamine, a colorless crystalline substance belonging to the family of heterocyclic organic compounds, is used primarily in the synthesis of melamine-formaldehyde resins for the manufacture of plastics, coatings, glues and kitchenware (Bizzari and Yokose, 2008) (Fig. 1). Melamine has a high nitrogen content (66.6% by weight), which makes it attractive as an adulteration ingredient for falsely increasing the level of protein in feedstuffs, food and dairy products. Adulteration is tempting because commonly used methods for protein analysis cannot distinguish between nitrogen from protein and that from non-protein sources, and thus measurement of protein in products containing non-

protein sources such as melamine appear to be high, thus providing an economic incentive for illegal adulteration. New, simple, specific, rapid and cost-effective methods for protein quantification should therefore be developed to discourage adulteration.

Cyanuric acid is an oxytriazine melamine analogue that may be produced as a byproduct in melamine synthesis (Fig. 1). It is accepted for use as a water disinfectant in swimming pools and a ruminant feed additive.

Humans are exposed to melamine and its analogues from a number of different sources, both dietary and environmental. Sources include breakdown of the pesticide cyromazine, which is approved for use in many countries, migration from approved food packaging material, as well as adulteration of specific foods (Bradley et al., 2005; Ishiwata et al., 1986; Ishiwata et al., 1987; Lund and Petersen, 2006).

Although melamine can be degraded by some organisms (Jutzi et al., 1982; Shelton et al., 1997), it is not metabolized by animals and is rapidly absorbed from the gastrointestinal (GI) tract and eliminated in the urine. More than 90% of ingested melamine is excreted within 24 h through the kidney. However, it can pass through the placenta of humans and animals if melamine-contaminated food has been consumed during gestation (Jingbin et al., 2010; Partanen et al. 2012). Normally, melamine alone has low acute toxicity, but daily simultaneous exposure to melamine and its derivative cyanuric acid has an effect that is more toxic than exposure to each compound individually.

Melamine and cyanuric acid are able to form a melamine-cyanurate (M-C) complex through interaction between diaminopyridine and diimide moieties, exhibiting three complementary NH-O and N-NH hydrogen bonds (Fig. 2), and this complex precipitates in renal tubule as urolites, thus leading to severe occlusion and renal failure (Chen et al., 2009; Hau et al., 2009; NPT, 1983; OECD, 1999; Puschner et al., 2007; Ren et al., 2009; Report of a WHO Expert Meeting, 2009; Wang et al., 1990; Xu et al., 2007; Yagai, 2006).

A urolite, also known as a renal calculus (plural, calculi), is a solid crystal aggregation formed in the urinary system. Formation of calculi is known as lithiasis. Several types of lithiasis can occur in the human body, including precipitation of uric acid calculi in the kidneys or joints, a condition known as gout (Eggebeen, 2007).

Gout is a type of arthritis where calculi of monosodium urate form inside and around the joints. It is caused by a build-up of serum uric acid concentration (hyperuricemia) to values exceeding 6.5 mg/dl. Uric acid is a byproduct that forms daily when the body breaks down chemicals known as purine in cells. Normally, uric acid dissolves in blood and mostly passes through the kidney into urine, where it is eliminated. Sometimes, however, the body either produces too much uric acid or the kidneys excrete too little uric acid. When this happens, hyperuricemia can occur, forming sharp, needle-like urate calculi in a joint or surrounding tissue, causing pain, inflammation and swelling (Eggebeen, 2007).

It is, however, of interest that *in vivo* formation of M-C urolite differs from that of urate calculi reported in gout, being restricted to the urinary system in the former case but usually accumulating at other sites in the body, such as joints and soft tissues, in the latter. The reason for this difference remains unknown.

Therefore, the purpose of this *in vitro* study was to investigate the possible mechanism of M-C-triggered lithiasis in the kidney and to provide a potential explanation for the mechanism responsible for the formation of M-C urolites *in vivo*.

The present thesis consists of three chapters. In brief, first chapter describes evaluation of the threshold concentrations of melamine and cyanuric acid necessary for *in vitro* formation of visible M-C crystals using a modification of the GNP assay. The second chapter describes the effects of melamine, cyanuric acid and M-C compounds on expressions of nephrin and podocin in mouse podocytes using Western blotting analysis. Finally, the third chapter describes the effects of possible intra-renal factors, such as pH and serum and urinary proteins, and an artificial macromolecule PVP, whose physico-chemical properties are comparable to those of high-molecular-weight serum proteins, on the M-C crystal formation, investigated using SEM.

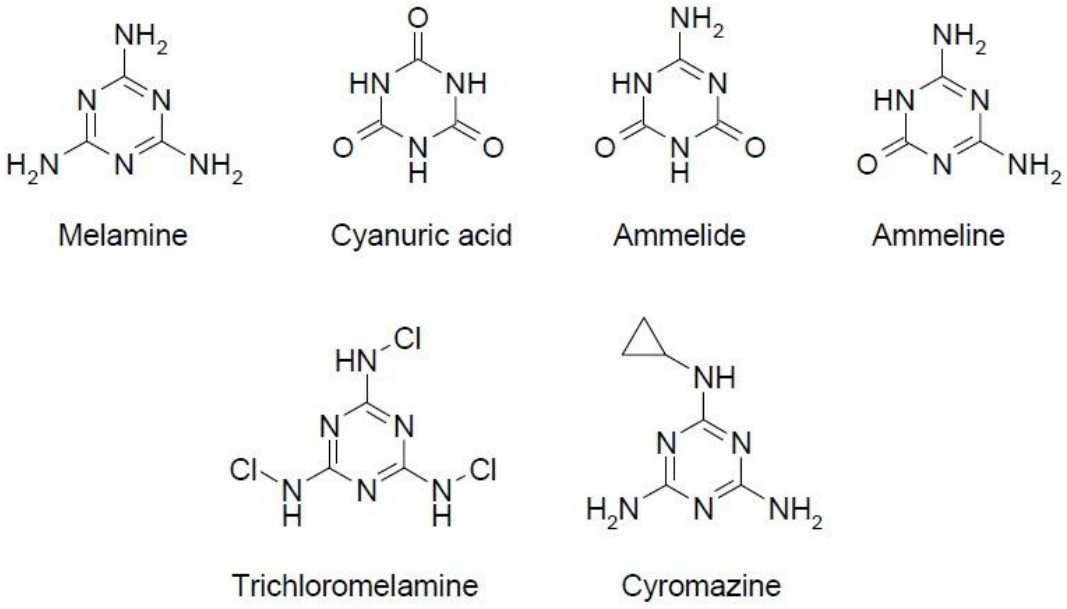

Fig. 1 Structures of melamine and related triazine compounds (WHO, 2008)

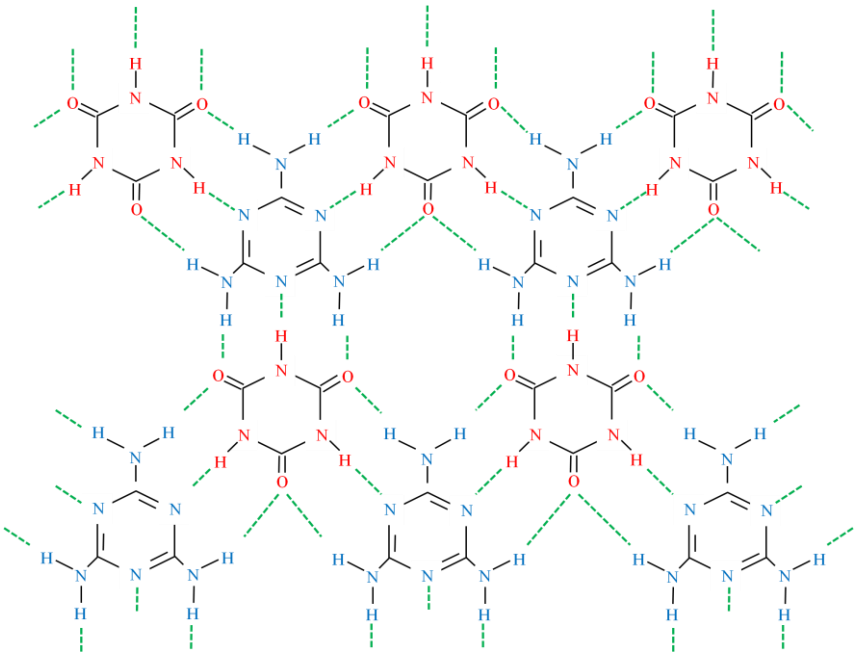

Fig. 2 Schematic representation of the melamine-cyanuric acid lattice (heteromolecular interactions) stabilized by O···H and N···H hydrogen bonds (dotted green line).

Chapter I

An *in vitro* investigation of the threshold concentrations of melamine and cyanuric acid for formation of melamine-cyanurate crystals

Introduction

Melamine-induced nephrotoxicity has been extensively described in cases of simultaneous exposure to melamine and its derivative cyanuric acid, triggering intra-renal urolite formation and tubule cell injury. Melamine-cyanurate (M-C) urolites, formed by melamine and cyanuric acid accumulate in the urinary tract where they mainly induce occlusion leading to renal failure (Kobayashi et al., 2010; Lu et al., 2012).

When ingested, melamine and cyanuric acid are assumed to pass into the gastrointestinal (GI) tract, bloodstream and kidney. M-C crystal formation is restricted to the urinary system as urolites, and does not occur at other sites in the body including the GI tract and blood. Although the reason for this phenomenon remains unknown, increased water intake is believed to reduce the potential for formation of kidney stones by melamine and cyanuric acid (Peng et al., 2012). Therefore I hypothesized that the presence of abundant water leading to low concentrations of melamine and cyanuric acid might help to prevent the formation of M-C crystals outside the urinary tract. To examine this hypothesis, I conducted this preliminary *in vitro* study to determine the threshold concentrations of melamine and cyanuric acid necessary for formation of visible M-C crystals, using a modification of the gold nanoparticle (GNP) assay (Li et al., 2010).

Materials and Methods

Briefly, solutions of melamine ($C_6H_6N_6$, MW=126.12) and cyanuric acid ($C_3H_3N_3O_3$, MW=129.08) were prepared at concentrations of 20-1000 μg/ml using 10% (v/v) fetal bovine serum (FBS, Gibco, Invitrogen, Grand Island, NY, U.S.A.) in Hanks' buffered salt solution [HBSS (-), Nacalai Tesque Inc., Kyoto, Japan] (10% FBS-HBSS), based on previous studies (Taksinoros and Murata, 2012; Taksinoros and Murata, 2013). The melamine or cyanuric acid solutions were passed through an ultrafiltration unit with a 10-kDa cut-off (Amicon Ultra-0.5 m*l* 10K, Millipore Ireland Ltd., Cork, Ireland) (ultrafiltration treatment) or left unfiltered (non-treated). The ultrafiltration was performed to simulate the selective filtration that occurs in the glomerulus, where macromolecules are separated from urine, which therefore predominantly contains low-molecular-mass material. For both procedures, the M-C crystals were formed by mixing the same volumes [ratio 1:1 (v/v)] of melamine and cyanuric acid solutions, followed by two washes with 10 kDa-ultrafiltrates of 10% FBS-HBSS containing no large molecules. The GNP assay used here has recently been developed to allow detection of free melamine in food samples with the naked eye (Li et al., 2010). Prior to the present study, I had found that this assay is also applicable to the detection of M-C crystals under a microscope after blue staining of the crystal surface. The GNP solution, prepared by the hydroborate reduction method (Zhang et al., 2006), was then added to the melamine and

cyanuric acid mixture in a 5:1 ratio (on a volume basis) in order to verify the presence of M-C crystal formation based on the degree of opacity judged by the naked eye and the change in morphology viewed with a digital light microscope (BA210EINT, Shimadzu, Kyoto, Japan). The crystal morphology and opacity were then compared between the ultra-filtration and non-treatment groups.

Results

Figure I-1 shows the difference in opacity of the M-C crystals obtained by ultra-filtration treatment. Opacity was evident with the naked eye in the melamine and cyanuric acid mixtures at concentrations ranging from 1,000 to 200 μg/ml. The degree of opacity lessened as the concentration of the mixture decreased, and the mixture became apparently transparent when the concentrations were less than 200 μg/ml. Similar results were obtained in the non-treatment group.

Microscopic observation with GNP staining supported the findings obtained with the naked eye. Table I-1 shows the data for formation of M-C crystals in both treatments. The M-C crystals obtained after ultrafiltration had a sharp needle-like shape, outlined by the surrounding blue staining (short arrows, Fig. I-2A). The crystals became smaller and blunter as the concentration of the mixture decreased. On the other hand, in the absence of filtration treatment, the rounded M-C crystals were clearly detectable at concentrations higher than 200 μg/ml, whereas the blue staining on them was less obvious (Fig. I-2B). It remains unexplained why the staining of the rounded crystals was weaker. In both treatments, M-C crystals were not found at concentrations of less than 200 μg/ml. The difference in crystal shape between the treatments was the same as that noted in my previous study (Taksinoros and Murata, 2012), but the precise reason is unclear.

Table I-1. M-C crystal formation after ultrafiltration treatment and no treatment.

Treatment	Microscopic appearance			
	Melamine and cyanuric acid concentrations (μg/ml)	Crystal formation	Crystal morphology	GNP recognition (blue staining)
Ultrafiltration	1000	√	Sharp needle-like	P
	800	√	Round needle-like	P
	600	√	Long-round	P
	400	√	Short-elongated	P
	200	√	Short-elongated	P
	100	×	-	-
	80	×	-	-
	60	×	-	-
	40	×	-	-
	20	×	-	-
Non-filtered	1000	√	Spherical-round	PW
	800	√	Spherical-round	PW
	600	√	Spherical-round	PW
	400	√	Spherical-round	PW
	200	√	Spherical-round	PW
	100	×	-	-
	80	×	-	-
	60	×	-	-
	40	×	-	-
	20	×	-	-

√: Formed, ×: Not formed, -: Not found, P: Positive, PW: Positive but weak.

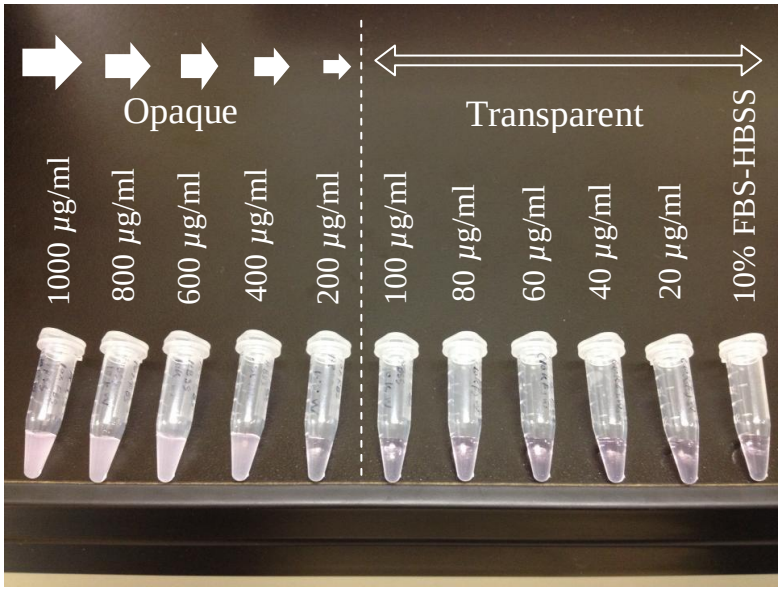

Fig.I-1. Difference in opacity of M-C crystals after ultra-filtration treatment (opaque to transparent: from left to right). 10%FBS-HBSS: No M-C crystals (control).

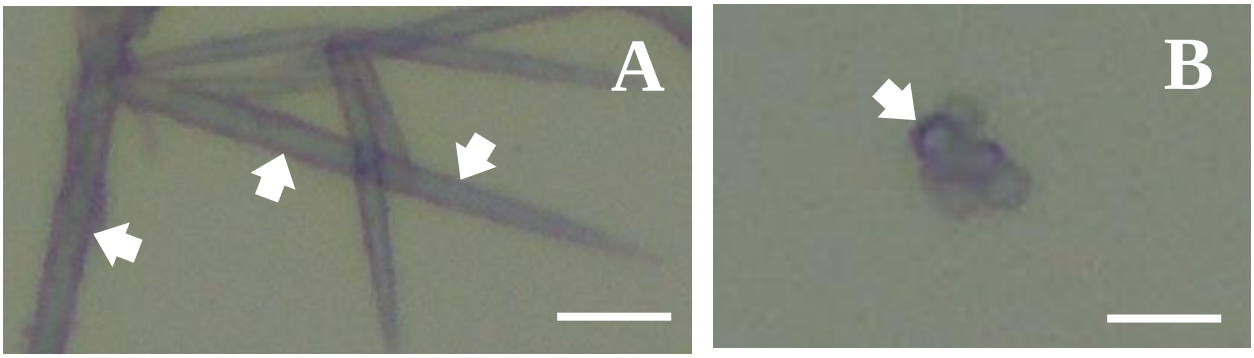

Fig. I- 2. Microscopic morphology of M-C crystals in suspension of 1000 μg/ml M-C mixture after ultrafiltration (Fig 2A) and no treatment (Fig 2B) (short arrows: blue staining). Bar=2.5 μm.

Discussion

Although previous reports have revealed that various *in vitro* and *in vivo* factors can affect M-C crystal formation (Peng et al., 2012; Taksinoros and Murata, 2012; Taksinoros and Murata, 2013), the actual mechanism responsible for melamine-triggered urolithiasis still remains unknown. The present *in vitro* study showed that a low concentration (<200 μg/ml) of a mixture of melamine and cyanuric acid [1:1 ratio (v/v)] did not contribute to the formation of M-C crystals, regardless of the conditions (with or without macromolecules) and shape (needle-like or rounded). It has been reported that the *in vivo* concentration of melamine in plasma is 0.79-23.17 μg/ml, while cyanuric acid is assumed to exist at the same level in the blood (Baynes et al., 2008; Baynes et al., 2010; Cruywagen et al., 2011; Liu et al., 2010; Zheng et al., 2011). Taking these clinical values into consideration, the present *in vitro* results suggest that the plasma melamine and cyanuric acid concentrations would be too low for formation and/or accumulation of M-C crystals in the bloodstream.

The abundance of water molecules in blood and their multiple hydrogen-bonding sites might be a major factor involved in preventing free melamine and cyanuric acid residues from reacting and forming intravascular M-C crystals (Li et al., 2006; Zhang, 2008). However, such effects of water molecules on both compounds may become weakened when they are reabsorbed within the kidney. If so, an increase of intra-renal melamine and cyanuric acid concentrations

could occur, thus eventually increasing the chance of M-C urolite formation (Neumann and Rector, 1976; Prior et al., 2013).

Although further details of the mechanism involved need to be clarified, my present *in vitro* study seems to provide a potential explanation for why M-C crystals are not formed outside the urinary system, unlike urate calculi formation reported in cases of gout, accumulating in joints and soft tissue (Eggebeen, 2007).

Summary

Melamine-cyanurate (M-C) crystals can cause nephrotoxicity in melamine-exposed humans and animals. The crystals accumulate as urolites but are not found outside the urinary system. In this *in vitro* study I found that M-C crystals were not formed in a mixture of melamine and cyanuric acid at low concentration (<200 μg/ml). These results suggest that the plasma melamine and cyanuric acid concentrations reported in clinical cases would be too low for formation and/or accumulation of M-C crystals in the bloodstream, thus explaining why the crystals are not detected outside the urinary organs in affected humans and animals.

Chapter II

Effects of melamine, cyanuric acid and melamine-cyanurate on expression of nephrin and podocin by mouse podocytes

Introduction

Recently, melamine-induced nephropathy has been widely confirmed both experimentally and in clinical cases of melamine ingestion. In the urinary system, melamine alone or its byproduct cyanuric acid has a less pronounced effect, but exposure to both simultaneously leads to tubule cell damage and intra-renal deposition of melamine-cyanurate (M-C) urolites, inducing kidney malfunction and thus leading to renal failure (Bhalla et al., 2009; Brown et al., 2007; Chen et al., 2009; Chen et al. 2014; Dobson et al., 2008; Hau et al., 2009; Lu et al. 2012; Puschner et al., 2007; Reimschuessel et al., 2010). The mechanism of M-C-triggered urolithiasis still remains unclear. However, the previous *in vitro* study (Chapter I) suggested that significant formation of M-C crystals would not occur in the bloodstream because of the low concentration of melamine and/or cyanuric acid, and would be restricted to the renal system as urolites.

Although M-C urolites induce mainly tubule blockage, proteinuria also occurs in cases of melamine disorder (Lam et al., 2008; Zhu et al., 2009). The reason for this leakage of protein into urine is not clear. However, previous clinical studies have shown that decreased expression of nephrin and podocin, major slit diaphragm proteins of podocytes, which are glomerular epithelial cells, causes proteinuria in several forms of nephrotic syndrome (Asanuma and

Mundel, 2003; Nakatsue et al., 2005; Shankland, 2006; Zhang and Huang, 2012).

The slit diaphragms of renal podocytes normally act as selective barrier to coordinate the filtration of cells and serum macromolecules, such as proteins (Fig. II-1) (Cheng and Harris, 2010; Kumagai et al., 2012).

Therefore I hypothesized that melamine and/or cyanuric acid might affect nephrin and podocin in podocytes. To test this hypothesis, I conducted an *in vitro* study to determine the effects of melamine, cyanuric acid and M-C compounds on nephrin and podocin expression in cultured mouse podocytes.

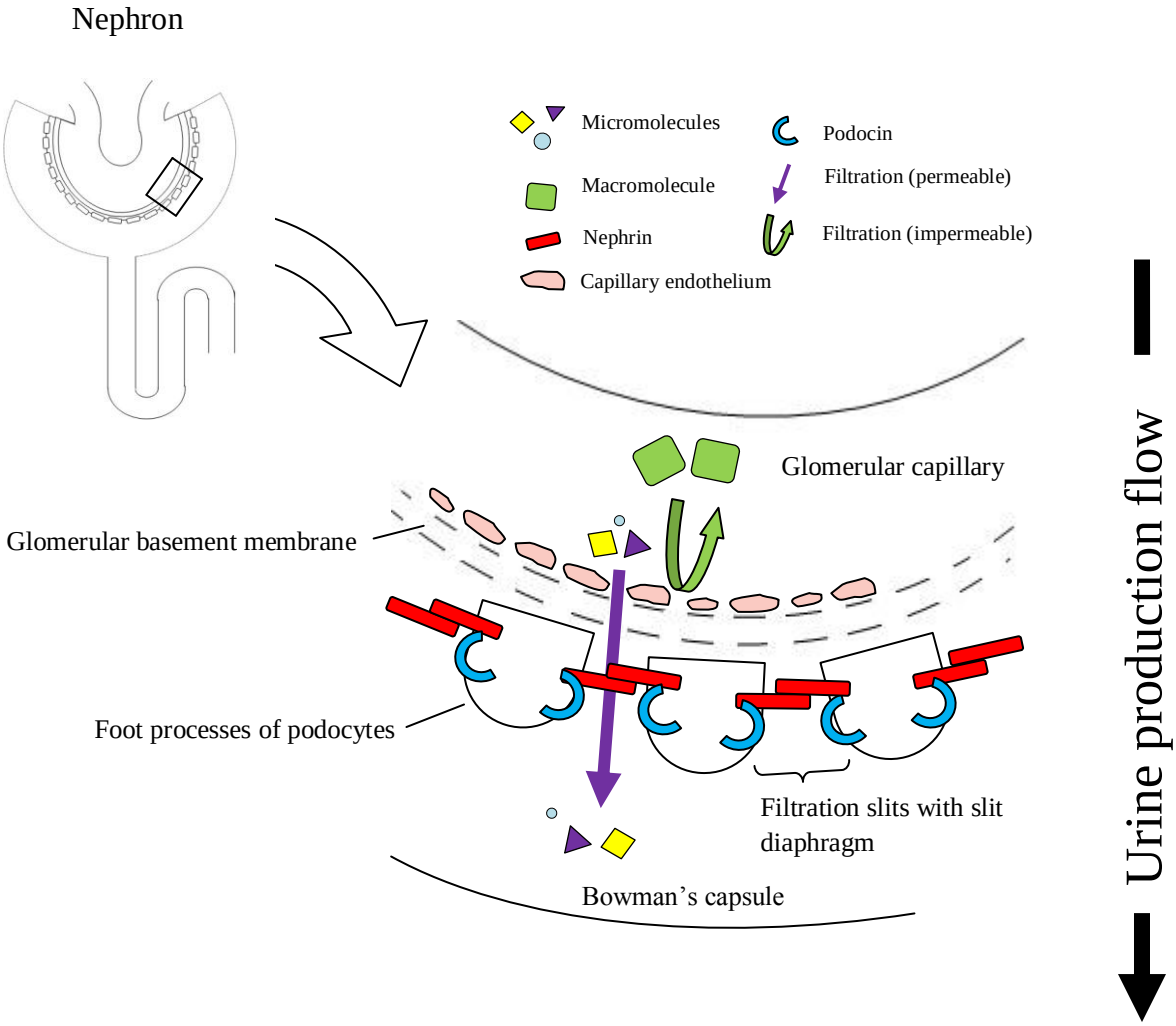

Fig. II-1 A cross-section of the filtration membrane. The micromolecules are filtered to pass first through a fenestra in the capillary endothelium (pink). Next, they pass across the glomerular basement membrane and finally through the filtration slits that are located between the interdigitating foot processes (purple arrow). Macromolecules cannot permeate (green arrow).

Materials and Methods

In brief, a mouse kidney podocyte cell line (a permanent cell line, CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in RPMI 1640 medium (RPMI 1640, Nacalai Tesque) containing 10% FBS (Gibco, Invitrogen) (FBS), 1% Antibiotic solution [Antibiotic-Antimycotic Mixed Stock Solution (100×), Nacalai Tesque] (ABO) and 1% L-glutamine [200 mM-L-Glutamine Stock Solution (100×), Nacalai Tesque] (L-Glu). Podocyte cells (5×10^5 cells/dish) were incubated at 33°C in 5% CO_2 until they reached 80% cell growth density, and then the cultured cells were starved in medium lacking FBS (RPMI 1640-1% L-Glu-1% ABO) for a further 24 hours. Solutions of melamine ($C_6H_6N_6$, MW=126.12) and cyanuric acid ($C_3H_3N_3O_3$, MW=129.08) were prepared at a concentration of 10^3 μg/ml using 1% ABO and 1% L-Glu in RPMI 1640 medium without FBS while the M-C compounds (with a needle-like shape) were formed by mixing the same volumes of melamine and cyanuric acid solutions (ratio 1:1). These solutions were gently added to the top of the podocyte layer and subsequently incubated without any shaking. The cell lysates were then extracted at 0, 24, 48, 72 and 96 hours using a lysis buffer (RIPA buffer, Nacalai Tesque) (RIPA buffer) and centrifugation (10,000×g, 4°C, 10 min). The protein concentration was measured with a BCA protein kit (Pierce®, BCA Protein Assay Kit, Thermo Scientific, U.S.A.) and the expressions of nephrin (MW=138 kDa), podocin (MW=42 kDa) and β-actin

(MW=42 kDa) were investigated using primary rabbit polyclonal antibodies (Anti-Nephrin antibody: ab58968, Anti-NPHS2: ab50339, Anti-beta Actin antibody-Loading Control: ab75186, Abcam®, Tokyo, Japan) and a goat polyclonal secondary antibody (Goat anti-rabbit IgG H&L (HRP) (ab6721), Abcam®). The immunocomplexes were visualized using Western blotting reagents (ImmunoStar®LD, Wako Pure Chemical Industries, Japan). Detection of chemiluminescent signals was performed using an ImageQuant LAS4000 Mini Luminescent Image Analyzer (GE Healthcare Biosciences). Visible changes in the morphology of cultured podocytes were observed by light microscopy (Nikon E200, Tokyo, Japan). Independent *t* test was performed for data analysis using Image-J software version 1.48 (National Institutes of Health, U.S.A.). Differences at $P<0.05$ were considered to be significant.

Results

Treatments with melamine or cyanuric acid alone did not induce any significant difference in the levels of nephrin and podocin expression between the treatment and control (non-treatment) groups at any incubation time point (Table II-1 and 2, and Fig. II-3, a-d). In contrast, melamine-cyanurate (needle type) supplementation significantly reduced the level of nephrin expression by 30% after 72 hours and that of podocin by 30-50% after 48 hours (Table II-1 and 2, Fig. II-3, e and f and Fig. II-4, a and b). However, no morphologic abnormalities of podocytes were evident after any of the treatments (Fig II-2). The control levels of expression of either nephrin or podocin became weaker as the period of exposure to the M-C combination increased.

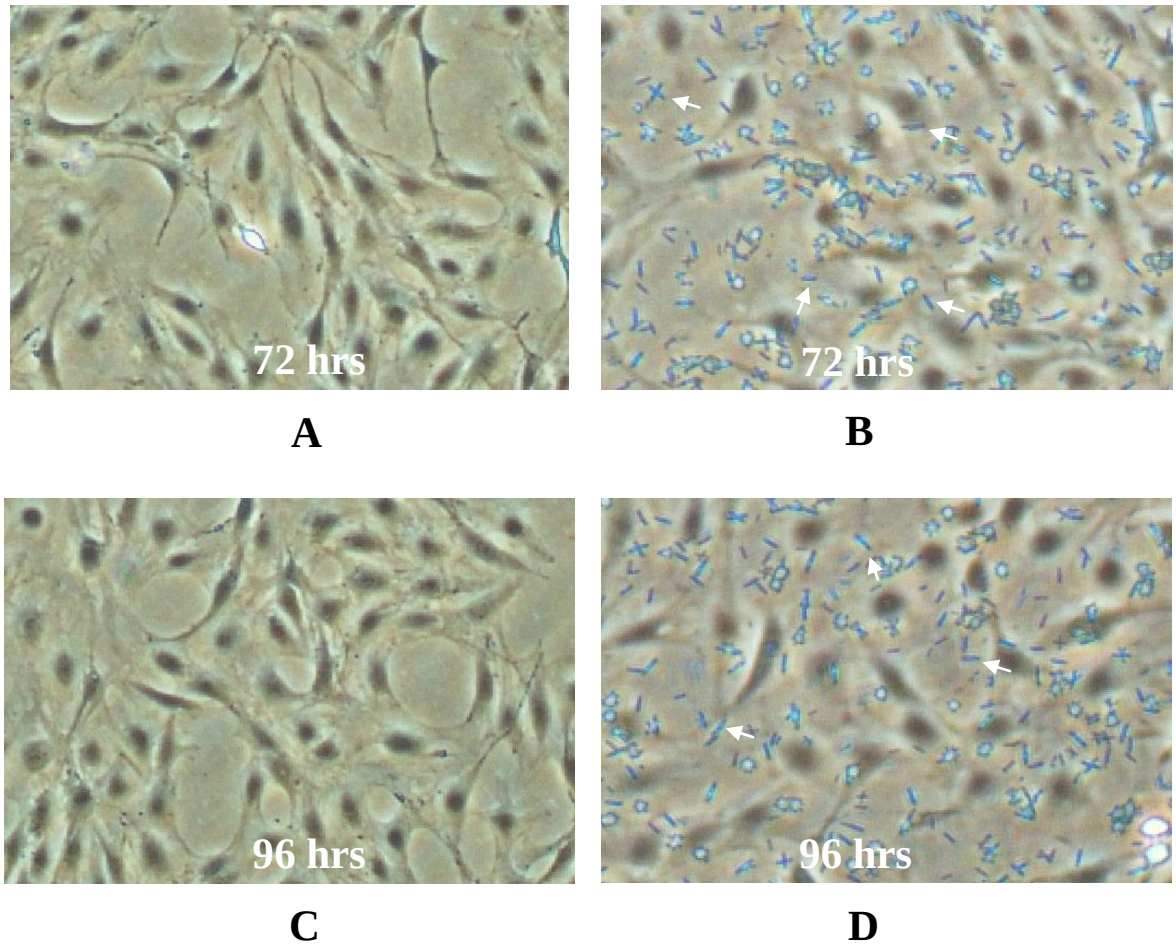

Fig. II-2 Microscopic morphology of podocyte cells supplemented with 1000 μg/ml melamine-cyanurate at 72 hours (B) and 96 hours (D) compared with the respective controls (A and C). Arrows; needle-like M-C crystals. Original magnification ×400.

Table II-1 Expression of nephrin after treatment with melamine, cyanuric acid and melamine-cyanurate.

Time	Treatment solutions	P-value	Relative values	Mean (n=3)	SD
0 hour	Melamine	0.20	1^{C}	17233.40	201.07
			0.99^{T}	17114.60	101.46
	Cyanuric acid	0.28	1^{C}	17989.40	105.05
			0.98^{T}	17574.20	415.01
	Melamine-cyanurate	0.35	1^{C}	18945.60	85.87
			0.99^{T}	18774.50	258.87
24 hours	Melamine	0.67	1^{C}	16985.48	1706.74
			1.04^{T}	17620.36	842.22
	Cyanuric acid	0.68	1^{C}	16606.50	795.13
			0.98^{T}	16224.27	659.49
	Melamine-cyanurate	0.22	1^{C}	17782.84	328.88
			0.97^{T}	17343.79	360.01
48 hours	Melamine	0.32	1^{C}	14500.58	984.98
			0.94^{T}	13536.87	753.51
	Cyanuric acid	0.68	1^{C}	14095.08	343.59
			0.99^{T}	13927.20	527.42
	Melamine-cyanurate	0.28	1^{C}	14794.08	530.53
			0.94^{T}	13850.30	657.67
72 hours	Melamine	0.15	1^{C}	14237.72	983.19
			0.95^{T}	13473.72	676.10
	Cyanuric acid	0.28	1^{C}	12215.25	251.83
			1.03^{T}	12546.97	329.34
	Melamine-cyanurate	< 0.05*	1^{C}	12260.52	174.81
			0.69^{T}	8516.15	211.61
96 hours	Melamine	0.31	1^{C}	12243.05	481.15
			1.06^{T}	12951.87	501.77
	Cyanuric acid	0.36	1^{C}	11551.13	632.46
			1.06^{T}	12233.37	394.89
	Melamine-cyanurate	< 0.05*	1^{C}	11730.64	125.85
			0.71^{T}	8384.45	108.76

^{C}: Control group, ^{T}: Treatment group and *: significant difference compared with the control. Values (chemiluminescence signals) are represented as relative values of means compared to the standard (Control), means and standard deviation (SD) (n=3).

Table II-2 Expression of podocin after treatment with melamine, cyanuric acid and melamine-cyanurate.

Time	Treatment solutions	P-value	Relative values	Mean (n=3)	SD
0 hour	Melamine	0.22	1 [C]	5128.40	100.72
			0.98 [T]	5011.70	111.96
	Cyanuric acid	0.72	1 [C]	5003.50	256.34
			0.98 [T]	4901.40	343.20
	Melamine-cyanurate	0.47	1 [C]	4954.60	61.59
			1 [T]	4934.10	63.57
24 hours	Melamine	0.25	1 [C]	4587.17	306.03
			0.93 [T]	4273.80	280.92
	Cyanuric acid	0.22	1 [C]	4698.37	303.33
			1.04 [T]	4898.68	254.66
	Melamine-cyanurate	0.22	1 [C]	4659.81	120.92
			1.04 [T]	4860.10	88.10
48 hours	Melamine	0.84	1 [C]	3878.68	544.56
			1.03 [T]	3994.78	435.23
	Cyanuric acid	0.49	1 [C]	4082.85	228.12
			0.97 [T]	3978.46	134.51
	Melamine-cyanurate	< 0.05*	1 [C]	4205.28	130.35
			0.50 [T]	2111.21	97.12
72 hours	Melamine	0.76	1 [C]	3230.63	166.52
			0.97 [T]	3143.39	273.99
	Cyanuric acid	0.62	1 [C]	3512.56	170.66
			0.97 [T]	3411.63	143.98
	Melamine-cyanurate	< 0.05*	1 [C]	3934.93	142.03
			0.50 [T]	1986.03	77.77
96 hours	Melamine	0.34	1 [C]	2941.08	302.30
			0.94 [T]	2752.39	469.52
	Cyanuric acid	0.72	1 [C]	3022.46	192.65
			1.03 [T]	3101.21	139.96
	Melamine-cyanurate	< 0.05*	1 [C]	2807.78	164.22
			0.69 [T]	1949.93	116.64

[C]: Control group, [T]: Treatment group and [*]: significant difference compared with the control. Values (chemiluminescence signals) are represented as relative values of means compared to the standard (Control), means and standard deviation (SD) (n=3).

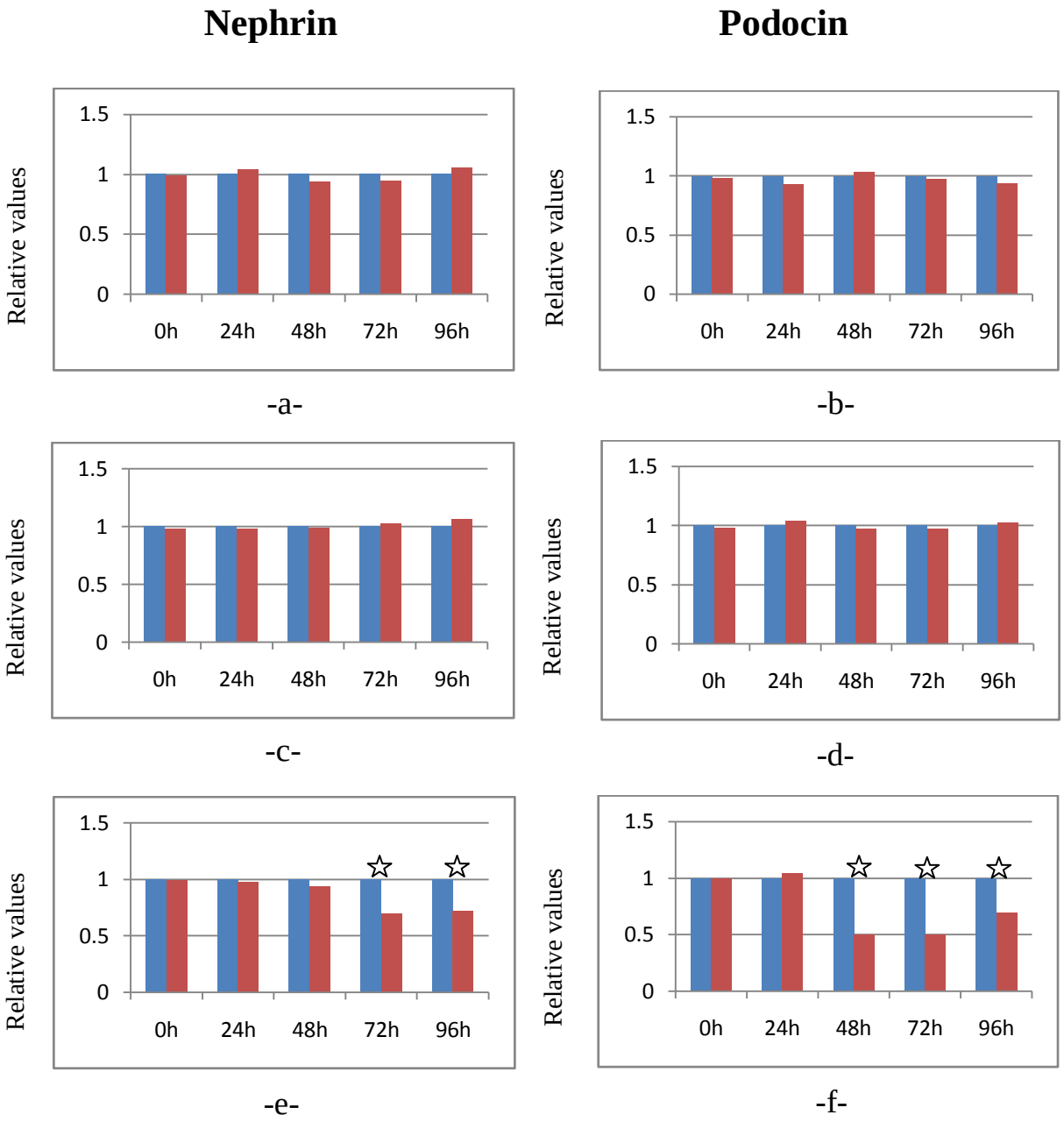

Fig. II-3. Expression of nephrin (left column) and podocin (right column) induced by melamine (a and b), cyanuric acid (c and d) and melamine-cyanurate (e and f) respectively. (control group=blue column chart and treatment group=red column chart).☆: Significant decrease compared with the control. Values (chemiluminescence signals) are represented as relative value of means compared to the standard (Control) (n=3).

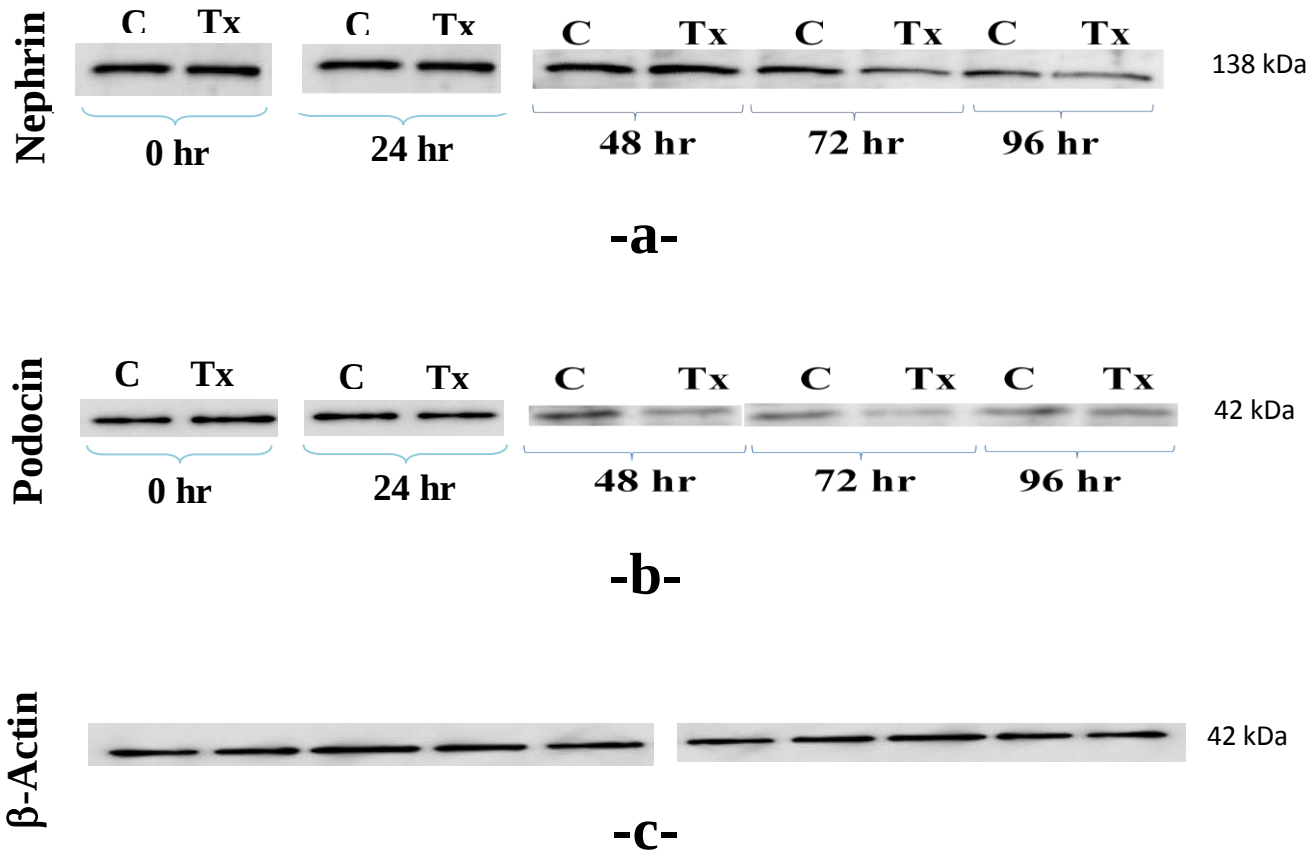

Fig. II-4 Effects of melamine-cyanurate on expression of nephrin (a) and podocin (b) in cultured podocytes at 0-96 h. C; Control. Tx; Treatment. β-actin was used as a loading control in Western blotting analysis (c).

Discussion

Although previous *in vivo* and *in vitro* studies have shown that melamine and its derivative cyanuric acid can induce nephrotoxicity (Brown et al. 2007; Dobson et al., 2008; Hau et al., 2009; WHO, 2008), the actual mechanism of this phenomenon remains unclear. In the kidney, the post-glomerular region starting from the proximal tubule is the main site where affected by M-C urolite deposition, tubule cell damage and/or renal obstruction, but no previous reports have detailed glomerular defects triggered by melamine (Chen et al. 2014; Lu et al. 2012). Podocyte cells work in the glomerulus as a selective filter, retaining large macromolecules such as albumin, which are essential for body function. Podocytes abnormality in several forms of nephrotoxic syndrome is often associated with proteinuria, as is the case for melamine disorder (Aaltonen and Holthöfer, 2007; Kerjashki, 1994; Lam et al., 2008; Mundel and Shankland, 2002; Pavenstädt, 2000; Zhu et al., 2009). To date, however, there has been no clear explanation for the induction of symptoms in cases of melamine-related toxicity. The present *in vitro* study revealed that melamine-cyanurate compounds (needle-like form) can decrease the expression levels of nephrin and podocin within two or three days after addition to cultured podocytes. It is also of interest that exposure to either melamine or cyanuric acid alone produces almost no effects, supporting previous evidence that each individual compound has low toxicity (NTP, 1983; WHO, 2008). Additionally, no visible

abnormalities in the morphology of podocytes were detected (Fig. II-2). The levels of nephrin and podocin expression in the control groups became weaker as the period of M-C exposure increased, which may have been due to the effect of FBS deprivation (Kulkarni et al., 1994).

The present *in vitro* results suggest that simultaneous accumulation of melamine and cyanuric acid, and thus the formation of M-C crystals, has an adverse effect on nephrin and podocin, the major slit diaphragm proteins of podocytes. Impairment of these proteins may lead to malfunction of glomerular filtration, thus allowing downstream leakage of proteins and contributing to proteinuria in clinical cases of melamine disorder (Lam et al., 2008; Zhu et al., 2009).

Summary

Apoptosis, injury and/or impairment of podocytes often underlines proteinuria in cases of renal disorder. Proteinuria has also been confirmed in animals with melamine-related disorder. However, the actual pathogenesis still remains unclear. The present *in vitro* study showed that although each of melamine and cyanuric acid alone seems to have no harmful effect on mouse podocytes, simultaneous co-exposure to both can affect the major slit diaphragm proteins, nephrin and podocin. These results suggest that melamine-cyanurate uroliths might exert micro-injury on the renal filtration slits, thereby inducing downward leakage of serum proteins.

Chapter III

Effects of serum proteins and polyvinylpyrrolidone on *in vitro* melamine-cyanurate crystal formation

Introduction

Melamine toxicity, a form of renal failure, has come to prominence following widely publicized cases in humans and other species (An et al., 2011; Brown et al., 2007; Kobayashi et al., 2010; Reimschuessel et al., 2010; Yang et al., 2011). Consumption of melamine-tainted foodstuffs, including powdered infant formulas, can cause renal tubule occlusion, finally leading to kidney dysfunction and renal failure (WHO, 2008).

Melamine, a nitrogen-rich heterocyclic triazine, is a chemical that has been widely used in many industries, primarily in the production of melamine-formaldehyde resin. However, adulteration of dairy products with melamine in order to increase their apparent protein content can be harmful if those products are ingested (Bizzari and Yokose, 2008; Dobson et al., 2008; Report of a WHO Expert Meeting, 2009; Suchy et al., 2009). In general, exposure to melamine alone has low acute toxicity, but when cyanuric acid is also present, the effect is more toxic than exposure to each substance alone (NTP, 1983; OECD, 1999; Report of a WHO Expert Meeting, 2009). A complex of melamine and cyanuric acid, melamine cyanurate, aggregates in the proximal and distal renal tubules (Chen et al., 2009), causing renal tubule obstruction and finally kidney dysfunction (Puschner et al., 2007).

In vitro, either melamine or cyanuric acid in water appears as a clear solution, but when they are mixed they form an opaque suspension of the

melamine cyanurate complex, which can be detected as needle-like crystals by light microscopy (Wang et al., 2010). *In vivo*, however, the crystals found in the kidney or urine of some species are rounded in shape and brown in color (Kobayashi et al., 2010). The reasons for the morphological difference in these crystals between *in vitro* and *in vivo* conditions remain obscure.

The results of previous *in vitro* and *in vivo* studies have suggested that M-C crystals can exert micro-injury on the renal filtration slits, inducing downstream leakage of serum proteins into post-glomerular regions where rounded M-C urolites have been reported in clinical cases of melamine disorder (Chapters II; Chen et al., 2009; Kobayashi et al., 2010; Puschner et al., 2007).

Therefore I hypothesized that some intra-renal factors, such as serum proteins, might alter the shape of M-C crystals, turning them into a spherical form. If so, then an artificial macromolecule, PVP, whose physico-chemical properties (i.e., molecular size, structure or electrical polarity) are comparable to those of proteins, might exert a similar influence on M-C morphology.

PVP, a synthetic macromolecule produced from acetylene products, is widely used in industry, cosmetics, pharmaceuticals and medicines (Folttmann and Quadir, 2008). In particular, PVP with a molecular mass of >30 kDa is a useful blood volume expander in the medical field (Bowman, 1953; Haaf et al., 1985; Kostrzewska, 1976; Shapiro, 1976).

In the present study, I investigated the *in vitro* effects of several possible intra-renal factors, i.e., pH, serum and urinary proteins, and PVP on the

morphology of melamine-cyanurate crystals in order to clarify what might be responsible for the formation of rounded urolites *in vivo*.

Materials and Methods

Melamine and cyanuric acid solutions at 10 mmol/*l* were prepared with 100 mmol/*l* sodium phosphate buffer (Na_2HPO_4-$NaHPO_4$, Wako Pure Chemical Industries) at pH 5.6, 7.0 and 8.1. The pH values were confirmed and monitored using a compact pH meter (Twin pH meter B-212, Horiba, Ltd., Kyoto, Japan).

Fetal bovine serum (FBS, Gibco), bovine serum albumin (BSA, MW=66 kDa, Wako), bovine gamma-globulin (BGG, MW=ca.150 kDa, MP Biomedicals, LLC, Illkirck, France) and beta2-microglobulin (B2M, MW=12 kDa, Sigma, St.Louis, MO, U.S.A.) were used for the preparation of serum and urinary protein solutions. All of them were added to each of the 10 mmol/*l* melamine or cyanuric acid solutions in Hanks' buffred salt solution [HBSS (–), Nacalai Tesque] at concentrations of 0.1%, 0.5%, 1%, 5% and 10% (w/v) based on the concentration of plasma proteins (6-8%) in several animal species (Anonymous, 1973).

To observe the process of melamine cyanurate (M-C) crystal formation, solutions of melamine and cyanuric acid were mixed in a ratio of 1:1, on a molar basis. The suspensions were observed using a light microscope (Nikon E200, Tokyo, Japan) for their physical properties, i.e., opacity, color and pH.

To simulate the selective glomerular filtration that occur *in vivo*, 10-kDa cut-off ultrafiltration was used to separate the serum proteins (FBS, BSA and BGG) from melamine or cyanuric acid solution. The solution of melamine or

cyanuric acid, containing each of the serum proteins at 5% concentration, was centrifuged (14,000× g at 20ºC for 10 min) using an ultrafiltration unit (Amicon Ultra-0.5 m*l* 10K, Millipore Ireland), and then M-C crystals in the filtrates were evaluated for their morphology in comparison with those in the non-filtered solutions.

To verify the effects of PVP on *in vitro* M-C formation, the macromolecules used in this study were PVP K30 (approximately 40 kDa, Wako) and BSA (66 kDa, Wako). PVP or BSA was added at a concentration of 10% (w/v) to the 10 mmol/*l* melamine or cyanuric acid solution in distilled water (DW). The crystal samples from the mixtures of the two solutions, (i.e., with BSA or PVP added), and the crystals formed in DW (macromolecule-free control solution), were rinsed three times with DW and dispersed on ABS (acrylonitrile butadiene strylene)-coated glass slides and air-dried. The samples were then coated with osmium at a thickness of 20 nm using an osmium plasma coater (OCP-80, Nihon Laser Electronics, Nagoya, Japan) with an electrical discharge voltage of 1.1-1.2 kV, an electrical discharge current of 2-4 mA and a vacuum of 6-8 Pa. The samples were then observed with a scanning electron microscope (JSM-6320F, JEOL Ltd., Tokyo, Japan) at 5 kV.

Results

At the various pH values, all of the M-C mixtures were opaque. The crystal morphology was needle-like, no rounded crystals were found (Table III-1). When the solutions contained serum proteins, the M-C crystals became rounded. Some differences in crystal morphology and the color of the fluid used as a rough pH indicator were found among the protein groups (Table III-2).

At a concentration of 1%, the crystal morphology of BSA-M-C and FBS-M-C was elongated, but a rounded surface and elongated shape were obtained from BGG-M-C at concentrations of both 0.5% and 1% (Fig. III-1). The presence of serum protein at high concentration induced rounded M-C crystals, whereas the presence of a low serum protein concentration changed the crystal shape to a larger and longer form. However, no rounded M-C crystals were found at any concentration of B2M. In addition, the changes in fluid color and pH appeared to depend on the serum protein concentration (Table III-2).

Similar results were obtained from the ultrafiltration treatments of the 5% BSA, 5% FBS and 5% BGG mixtures. The morphology of M-C crystals obtained from the control solution (without ultrafiltration) was rounded, while that of crystals derived from all of the filtrate mixtures was needle-like (Table III-3). Furthermore, rounded M-C crystals appeared when serum protein was mixed before combination with the melamine and cyanuric acid. The serum proteins failed to change the needle-like M-C crystals to a rounded form if they

were added after the melamine and cyanuric acid had been combined under protein-free conditions (Table III-4).

Figure III-3 shows the difference in morphology of M-C crystals formed *in vitro* in the presence of absence of macromolecules. All of the M-C compounds formed in the absence of macromolecules were rod-shaped crystals, including small shapeless debris attached to the rods. The width and length of the crystals varied (average 0.5 μm × 4.5 μm) (Fig. III-3A and 3B). In contrast, M-C crystals that formed in the presence of BSA (M-C-BSA) showed a uniform, spherical shape (diameter: approximately 2 μm) with a rough outer surface. The M-C-BSA crystals had bridge-like connections when closely gathered together (arrows, Fig. III-3C and 3D). The M-C crystals formed in the presence of PVP (M-C-PVP) were also spherical. The M-C-PVP compounds had a diameter of approximately 2.5 μm, and most of them were apparently much bigger than the M-C-BSA crystals. The outer surface of M-C-PVP was composed of several densely aggregated small particles (short arrows, Fig. III-3E and 3F). No bridge-like connections between neighboring crystals were seen. Although the general morphology of M-C-BSA and M-C-PVP crystals was spherical and the two resembled each other (Fig. III-3C and 3E), their size and surface structure apparently differed. Judging from the structural complexity of M-C crystals, PVP seemed to have a much more potent effect than BSA on crystal formation.

Table III-1. Effect of pH on M-C crystal morphology

pH	Physical appearances	
	Crystal morphology	Opacity
5.6	Needle-like	Opaque
7	Needle-like	Opaque
8.1	Needle-like	Opaque

Table III-2. Effects of serum and urinary protein concentration on M-C crystal morphology

Proteins	Physical appearances				
	Concentration (w/v %)	M-C crystal morphology	Liquid		
			Opacity	Color	pH
FBS (serum)	0.1	Round-Needle-like	√	Dark-Pink	9.5
	0.5	Long-round	√	Dark-Pink	9.3
	1	Short-elongated	√	Dark-Pink	9.3
	5	Rectangle-round	√	Dark-Pink	9.2
	10	Rectangle-round	√	Dark-Pink	8.8
BSA (serum)	0.1	Round-Needle-like	√	Dark-Pink	9.3
	0.5	Long-round	√	Dark-Pink	8.8
	1	Short-elongated	√	Pink	8.2
	5	Square-round	√	Dark-Yellow	7.3
	10	Square-round	√	Dark-Yellow	7.1
BGG (serum)	0.1	Short-elongated	√	Pink	8.8
	0.5	Round	√	Pink	8.6
	1	Sphere-round	√	Pink	8.6
	5	Sphere-round	√	Pink	7.6
	10	Sphere-round	√	Pink	7.2
B2M (urine)	0.1	Needle-like	√	Pink	-
	0.5	Round-Needle-like	√	Pink	-
	1	Thin-Long-round	√	Pink	-
	5	Thin-elongated	√	Pink	-
	10	Short-elongated	√	Pink	-

√: Opaque, -: Not determined due to the small volume of mixture.

Table III-3. Effect of ultra-filtration on M-C crystal morphology

Serum protein	Crystal morphology	
	Control	Filtrate
FBS	Rectangle-round	Needle-like
BGG	Round	Needle-like
BSA	Round	Needle-like

Table III-4. Effect of the order of combination on M-C crystal morphology

Combination order			Crystal morphology
1st	2nd	3rd	
M	C	-	Spike-like
M	C	BGG	Spike-like
M	BGG	-	Not found
M	BGG	C	Round
C	BGG	-	Not found
C	BGG	M	Round

M: Melamine (10 mmol/L), C: Cyanuric acid (10 mmol/L), BGG: bovine gamma globulin (5 w/v %). M, C and BGG were mixed in a 1:1:1 ratio (on a volume basis).

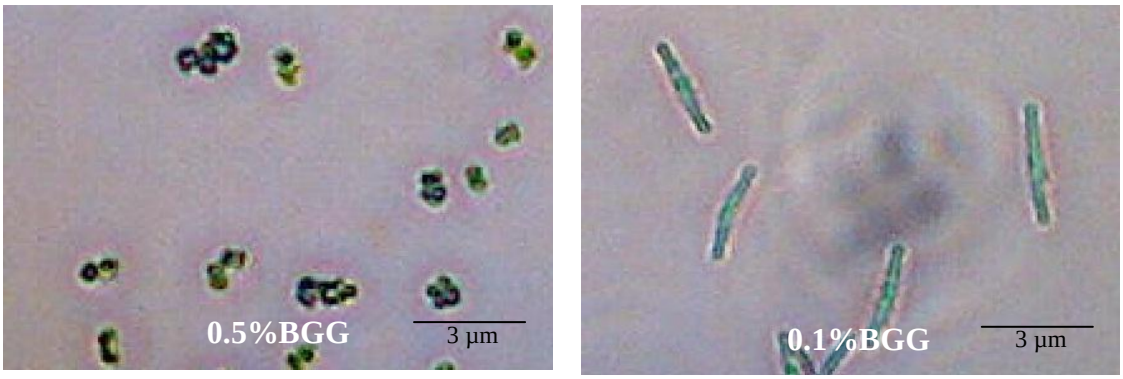

Fig. III-1 M-C crystal morphology at different concentrations of bovine gamma-globulin (BGG).

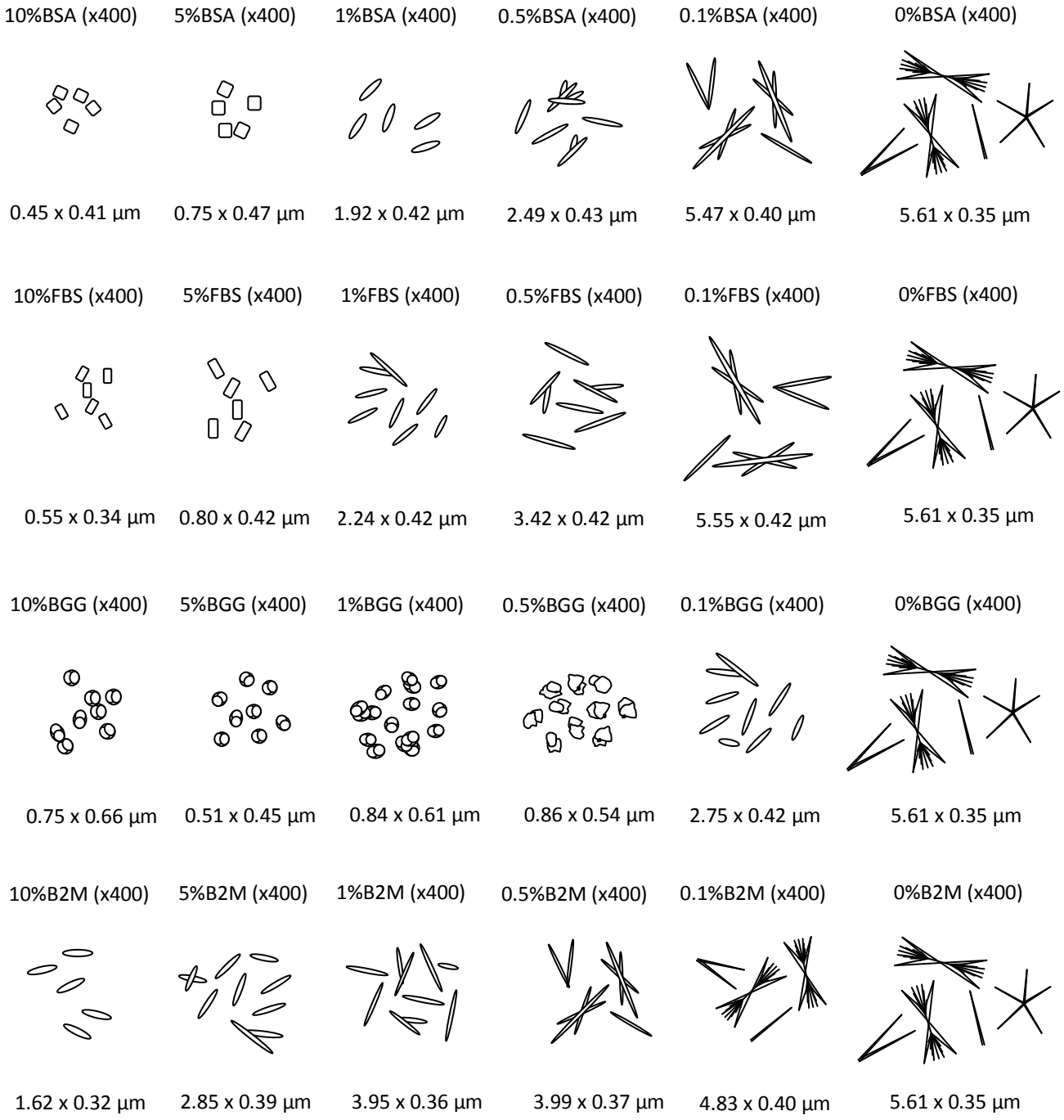

Fig. III-2 Effects of serum proteins and their concentrations on M-C crystal morphology (an illustration). Size (length × width): 10 randomly selected crystals were used for calculation.

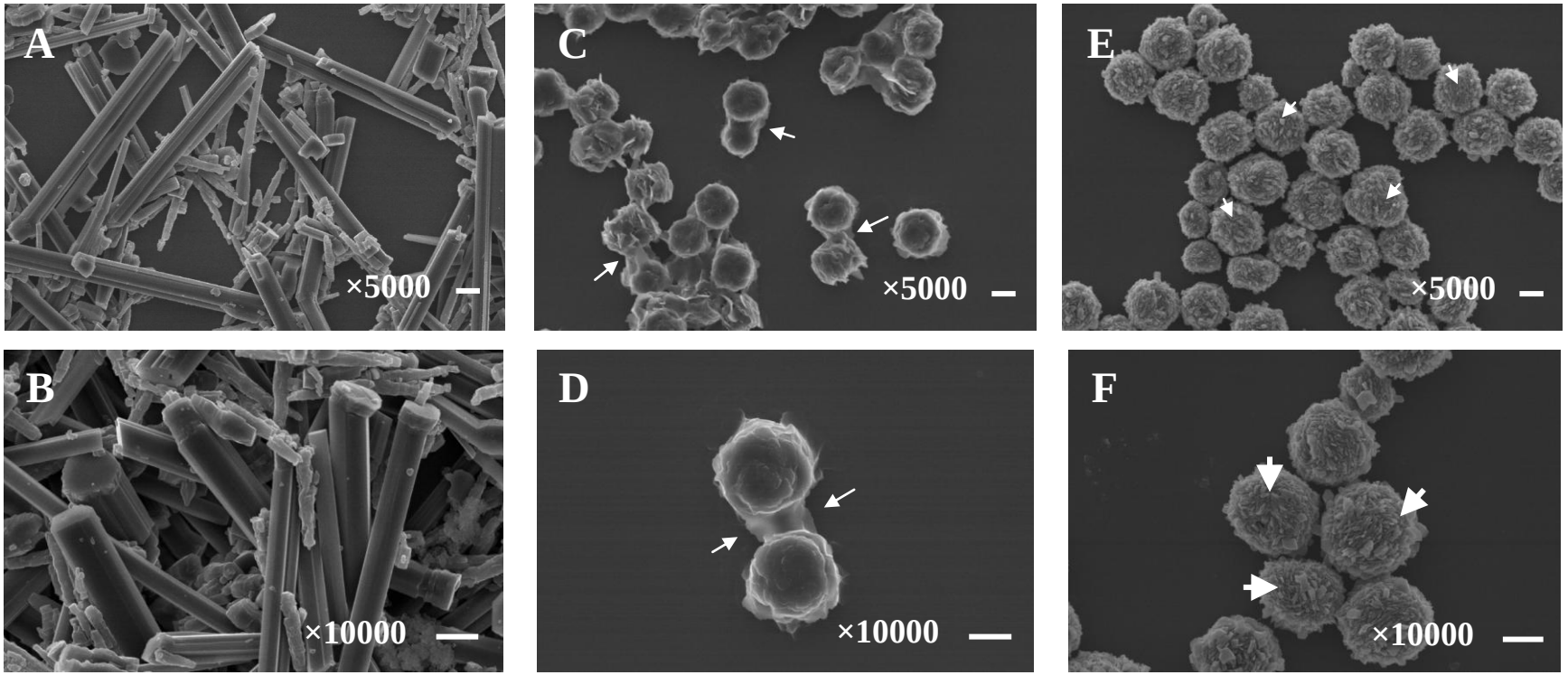

Fig. III-3. The morphology of M-C crystals formed *in vitro* without macromolecules (A and B), with BSA (C and D) (arrows; bridge-like structure) and with PVP (E and F) (Short arrow; aggregation of small particles). The scale bar indicates 1 μm.

Discussion

This study provides the first *in vitro* demonstration of serum protein as a factor involved in the formation of rounded M-C crystals, which are similar to the brown melamine crystals that occlude the renal tubules of patients with melamine disorders (Kobayashi et al., 2010).

Previous reports have suggested that pH is a factor possibly affecting M-C crystal production and morphology (Lu et al., 2011; Martin Hernández et al., 2001; Quaggin and Kreidberg, 2008). It is known that normal human urine contains excess water and electrolytes with a pH range of 4.6-8, and lacks blood and proteins (Lauridsen et al., 2007). However, the present study confirmed *in vitro* that needle-like M-C crystals appeared in suspensions containing no proteins, within a pH range of 5.6-8.1 (Table III-1). Furthermore, when some suspensions became alkaline, it did not seem to affect M-C crystal formation (Table III-2). These results suggest that pH is not important for formation of rounded M-C crystals. The present filtration results also strongly suggest that rounded crystals can be formed in the presence of serum proteins (Table III-3). As for B2M, its effect on the formation of rounded M-C crystals seems to be less significant.

Although the actual mechanism responsible for the involvement of proteins in urolites still remains unclear, it is possible that proteins might exert a

catalyst-like effect on the triple hydrogen bonding of the melamine-cyanurate complex, thus contributing to a change in crystal morphology (Yagai, 2006).

The results of the SEM study suggested that , like BSA, PVP can alter the morphology of M-C crystals to a spherical form. Especially, the M-C-BSA crystals resembled the *in vivo* urolites reported in cases of melamine disorders (Kobayashi et al., 2010). The rounded M-C-PVP crystals were a little larger and densely covered with small particles.

The reasons as to (1) why and how BSA and PVP can change the shape of M-C crystals, and (2) how the morphological difference between M-C-BSA and M-C-PVP compounds is induced still remain unclear. However, I suggest that the electrical charges of serum proteins might directly specify the hydrogen bonding sites of the M-C compound (See Fig. 2 in Preface), and thus induce the formation of rounded M-C crystals *in vitro*. As for PVP, the polymer is known to have two moieties, i.e., a dipolar imide group binding polar molecules, and a hydrophobic region acting on non-polar molecules, as well as interacting with several metal cations (Liu et al., 2000; Molyneux and Ahmed, 1973), thereby changing the morphology to a rounded shape (Yagai, 2006).

Summary

In this chapter, I investigated the *in vitro* effects of several possible intra-renal factors, i.e., pH and serum and urinary proteins, on the morphology of melamine-cyanurate crystals. Serum proteins, such as FBS, BSA and BGG, were found to effectively alter the morphology of melamine-cyanurate crystals, making them rounded. The urinary protein B2M had a less pronounced effect, and the crystal morphology was unaffected by pH.

The present SEM study confirmed for the first time that not only BSA, but also PVP, a synthetic macromolecule, can induce the formation of rounded M-C crystals *in vitro*. The BSA- and PVP- induced M-C compounds differed slightly in both size and surface structure. The reasons for these phenomena remain to be determined.

Conclusion

The pathogenesis of melamine disorder in the kidney still remains unsettled. Therefore, the main purpose of this thesis is to propose a preliminary model for the mechanism of intra-renal M-C-induced urolithiasis, on the basis of the anatomy of the glomerulus, existing clinical reports and the present *in vitro* findings.

The glomerulus, a capillary network enclosed by Bowman's capsule, is composed of three filtration barriers including podocytes, which play a role in renal excretion by filtering micromolecules, including sugars, electrolytes and small serum proteins such as B2M, through the glomerular capillary wall (Karnovsky and Ainsworth, 1972; See Fig. 3). Normally, the capillary wall is impermeable to macromolecules. However, in the process of melamine and/or cyanuric acid filtration, some yet unknown defects might occur in the glomerular barriers, thus leading to leakage of large protein molecules into Bowman's capsule and far beyond. A recent medical report has indicated that melamine might cause glomerulosclerosis and proteinuria in humans (Sun et al., 2010).

In the present thesis, I suggest a possible mechanism of melamine-cyanurate (M-C)-induced urolite formation in the kidney, based on the *in vitro* findings described in Chapters I to III.

1) M-C crystals may not be formed outside the urinary system (Chapter I).

2) An increase in the concentrations of intra-renal melamine and cyanuric acid may occur, thus triggering the formation of M-C compounds. M-C compounds, probably formed initially as needle-like crystals close to foot processes of podocytes, which wrap around the glomerular capillaries, may be involved in impairment of the filtration slits (Chapter II). Such a disorder could lead to leakage of serum macromolecules downward.

3) The leaked macromolecules (probably serum proteins) may affect the formation of M-C urolites, turning them into a rounded form (Chapter III). The urolites may accumulate in the urinary tubule lumen and cause occlusion in the long term, diagnosed as melamine-triggered urolithiasis.

These steps are illustrated in Fig. 3.

Although the proposed model does not consider the possible role of other sites in the nephron, another possibility is that altered conditions in the tubule lumen, including proteinuria, could arise through decreased re-absorption and/or unusual secretion of proteins from malfunctioning tubule cells (Dalal and Goldfarb, 2011; Waller et al., 1989). This might induce morphological transformation of M-C crystals and eventually contribute to their accumulation as urolites.

Although further details of the mechanism involved remain to be clarified, my proposed model for the involvement of proteins somewhere in the nephron seems to provide a potential explanation for the *in vivo* pathogenesis of melamine-induced urolithiasis.

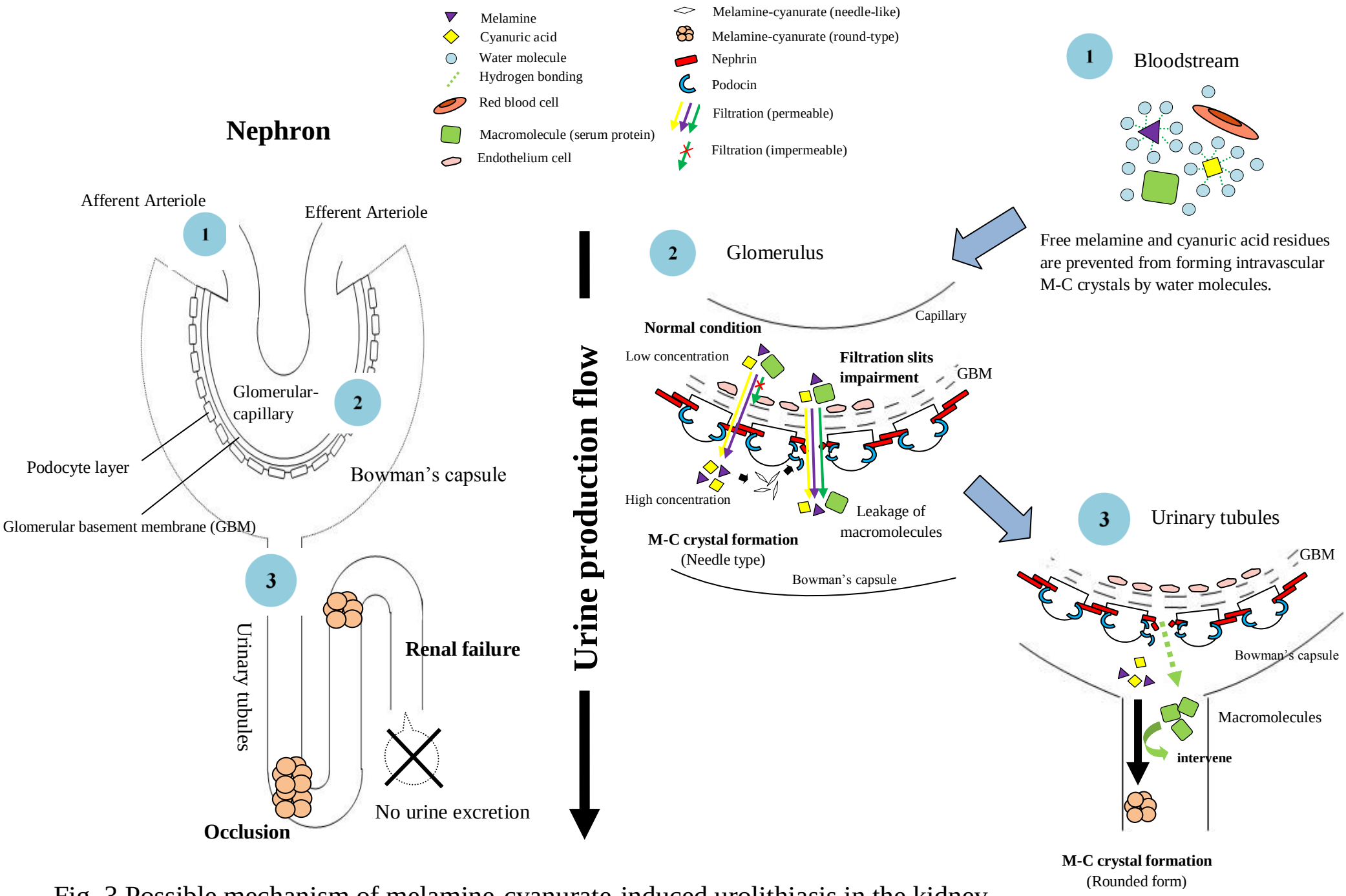

Fig. 3 Possible mechanism of melamine-cyanurate-induced urolithiasis in the kidney.

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