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SHORT COMMUNICATION

Comparisons of β2-microglobulin, apolipoprotein A1, and immunoglobulins (IgG and IgM) detected in the serum and urine from individual cats

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ABSTRACT. The detection of serum and urinary proteins is important for normal conditions, but comparisons of individual serum and urine proteins are rarely performed. The aim of this study was to examine β2-microglobulin (β2-MG), apolipoprotein A-I (ApoA-I), and immunoglobulins (IgG and IgM) in the serum and urine of cats with chronic kidney disease and lower urinary tract disease (LUTD), in addition to healthy cats. Serum and urine samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by immunoblotting for β2-MG, ApoA-I, IgG, and IgM. The molecular weight of serum β2-MG was greater than the predicted molecular weight (11,472 Da), and different types of modified β2-MGs were detected in the urine of healthy and diseased cats, including the original type, in addition to the grycocylated and partially digested types. Serum and urinary ApoA-I molecular weights were lower than the predicted molecular weight (28,943 Da), and high levels of urinary ApoA-I were detected in LUTD cats, although urinary ApoA-I was not detected in healthy cats. Under non-reducing conditions, the H-chains of urinary IgM pentamers and IgG monomers were detected in healthy cats. These results suggest that urinary β2-MG is modified in a different manner from serum β2-MG, urinary ApoA-I is a potential marker of LUTD, and urinary IgM pentamer, IgG monomer, and their H-chains are found after glomerular filtration, even in healthy conditions.

Keywords: apolipoprotein A-I, immunoglobulins, serum, urine, β2-microglobulin.

INTRODUCTION

In felines, detection of urinary proteins is important for the diagnosis of kidney disease. Although large amounts of albumin (67 kDa) are present in the plasma of cats, levels are negligible in the glomerular filtrate due to selective glomerular permeability, and the small quantity of albumin in the glomerular filtrate is almost completely reabsorbed by the proximal tubular epithelial cells, resulting in a detection level of < 1 mg/dL in normal urine (Kovarikova, 2015). β2-Microglobulin (β2-MG), composed of a single polypeptide with a molecular weight of 11.8 kDa, is associated with major histocompatibility complex I on the surface of all nucleated cells (Argyropoulos *et al*., 2017; Cobbin *et al*., 2013). Glomerular-filtered β2-MG is reabsorbed and catabolized in proximal convoluted tubules (Argyropoulos *et al*., 2017). Impairment of this system is used to diagnose kidney disease based on the presence of β2-MG in the urinary protein because of the lack of endocytosis mediated by the megalin-cubilin complex (Argyropoulos *et al*., 2017; Cobbin *et al*., 2013). Urinary β2-MG is thought to be glycosylated based on the detection of multiple proteins compared to recombinant feline β2-MG (Hoshi, 2014). However, the mechanism of circulating β2-MG glycosylation has not been fully characterized. Apolipoprotein A-I (ApoA-I), with a molecular weight of 28 kDa, is the primary high-density lipoprotein (HDL). ApoA-I is reabsorbed after glomerular filtration by tubular epithelial cells via Apo and HDL receptors and transporters (Clark *et al*., 2019; Nielsen *et al*., 2016). In humans, children with kidney disease have detectable levels of urinary ApoA-I depending on the underlying disease (Clark *et al*., 2019).

Immunoglobulin G (IgG), which has a high molecular weight (160 kDa), can enter the urine in some amounts via neonatal Fc receptor–mediated transcytosis that occurs in the glomerular basement membrane (Akilesh *et al*., 2008), but can be a marker of glomerular impairment (Kovarikova, 2015).

Kidney and lower urinary tract diseases (LUTDs) are common in cats; therefore, it is important to accurately diagnose acute and chronic renal diseases (Kovarikova, 2015; Gomes *et al*., 2018; Lekcharoensuk *et al*., 2001). Acute kidney injury (AKI) causes sudden renal dysfunction leading to acute renal failure, which is associated with high morbidity and mortality rates (Kovarikova, 2015). Chronic kidney disease (CKD) is diagnosed in 15-30% of geriatric cats, and its prevalence increases in cats over 15 years of age (Brown *et al*., 2016; Cobbin *et al*., 2013). A considerable amount of data is available regarding the detection of urinary proteins as a means of diagnosing common kidney

diseases in felines during the early stages of the disease, but there is relatively limited data concerning comparisons of serum and urinary proteins in individual normal cats. To further characterize the differences in specific proteins (β2-MG, ApoA-I, IgG, and IgM) between serum and urine, these proteins were characterized using serum and urinary samples from normal cats, including CKD and LUTD cats.

MATERIALS AND METHODS

Chemicals

Rabbit anti-human β2-MG and ApoA-I antibodies were purchased from Abcam (Cambridge, MA, USA) due to immunological cross-reactions between humans and cats and Proteintech Group Inc. (Chicago, IL, USA), respectively. Polyclonal goat antibodies specific for cat IgG Fc fragment and IgM were purchased from Bethyl Laboratories (Montgomery, TX, USA). Alkaline phosphatase (ALP)-conjugated rabbit and goat IgG antibodies were purchased from MP Biomedicals (Irvine, CA, USA) and Southern Biotechnology Associates, Inc. (Birmingham, AL, USA), respectively. Bovine serum albumin (BSA) was purchased from Roche Life Sciences, Inc. (San Francisco, CA, USA). All other reagents were of analytical grade.

Blood and urine

The following cat breeds were used in this study: American shorthair, Japanese cat (domestic short hair), Maine Coon, Siamese, and mixed. Serum and urine samples were collected by centrifugation after collecting blood and urine directly into sterilized tubes from 12 healthy cats, including five kittens, four cats with CKD, and three cats with LUTD, as diagnosed by veterinarians. The concentration of protein in the urine was determined using a Pierce BCA protein assay kit according to the microtiter method, with BSA as the standard. All experiments were conducted following the established guidelines for animal welfare and approved by the Committee on the Ethics of Animal Experiments of Kitasato University (permit No.:20-048).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Serum and urine samples were appropriately diluted with phosphate-buffered saline (150 mM NaCl, 20 mM sodium phosphate [pH 7.2]) and sample buffer with or without 40 mM dithiothreitol (DTT) was added. SDS-PAGE was performed according to the method described by Laemmli (1970) using a 4.5% stacking gel and a 12% running gel.

The amount of protein loaded on the SDS-PAGE gels varied from 4.8 μg/lane to 3.2 μg/lane for serum samples and from 4.0 μg/lane to 0.68 μg/lane for urine samples, which serum and urine samples were diluted finally 20-fold and 1.25-fold diluted.

Proteins separated by SDS-PAGE were transferred onto polyvinylidene fluoride membranes at 12 V for 12 min using an Invitrogen iBlot 2 Gel Transfer Device (Carlsbad, CA, USA). Briefly, the transferred proteins were incubated with rabbit anti-human β2-MG and ApoA-I antibodies, goat anti-cat IgG Fc fragment, and IgM, followed by ALP-conjugated polyclonal antibodies specific for rabbit or goat IgG (Fc) to detect primary antibodies. ALP-conjugated antibodies bound on the membrane were detected using 100 mM Tris-HCl (pH 7.6) containing 5 mM $MgCl_2$, 0.39 mM nitro blue tetrazolium, and 0.38 mM 5-bromo-4-chloro-3-indolylphosphate.

RESULTS AND DISCUSSION

Table 1 shows the results of the biochemical characterization of serum and urine samples from 12 healthy cats (Nos. 1-12), four cats (Nos. 13-16) with CKD, and three cats (Nos. 17-19) with LUTD. Except for cat No. 6, the healthy cats exhibited no azotemia based on either one or both of the following IRIS guideline criteria: serum creatinine ≤ 1.6 mg/dL and/or SDMA < 14 μg/dL (Hall *et al.*, 2018), the blood urea nitrogen (BUN) level of healthy cats was within the reference range (17-39 mg/dL) (Hall *et al.*, 2018). CKD was diagnosed based on serum creatinine levels of > 2.3 mg/dL (Boyd *et al.*, 2008; Ernst *et al.*, 2018).

β2-MG in the serum samples of 12 healthy cats was detected as a single band (17 kDa) by SDS-PAGE under reducing conditions via immunological cross-reaction with human β2-MG. The molecular weight of serum β2-MG was higher than that calculated based on its amino acid sequence (11,472 Da; accession no.: NM_001009876.1). In this study, the detection of β2-MG bands in Nos. 5 and 6 is shown as representative data, including all bands detected as described below (Figure 1A). In samples from five healthy cats (Nos. 2, 4-6, and 12), the molecular weight of β2-MG was slightly higher (18 kDa) than that observed in the other samples (Nos. 5 and 6). Urinary β2-MG was not detected in three healthy cats (Nos. 3, 7, and 11), but the protein was detected as a band that co-migrated with serum β2-MG in three healthy cats (Nos. 4, 10, and 12). An additional band (20 kDa) was also detected in three healthy cats (Nos. 1, 2, and 8), with two isoforms of serum β2-MG (17- and 18-kDa bands). Additionally, in three healthy cats (Nos. 5, 6, and 9), urinary β2-MG was detected at a lower molecular weight (15 kDa), with two or three more β2-MG bands, as previously described (Figure 1A). In cats with CKD, urinary β2-MG was observed as a 17- (No. 14) or 20-kDa (No. 13) band, or both bands (No. 16), in addition to a band (17 kDa) that co-migrated with serum β2-MG, except for cat No. 15, as described in healthy cats (Figure 1A). LUTD cats also demonstrated 18- and 20-kDa bands, with one band showing serum β2-MG, except for No. 18 (Figure 1A). In a previous study, urinary β2-MG exhibited a ladder pattern on immunoblotting with a monoclonal antibody against recombinant feline β2-MG, although only one band of purified recombinant feline β2-MG was detected, suggesting glycosylation of serum β2-MG (Hoshi, 2014). This study also revealed patterns of glycosylation in

Table 1.
Biochemical characterization of the serum and urine of healthy cats and cats with CKD or LUTD

N°	Breed	Diagnosis	Sex	Age (years)	TP (g/dL)	Alb (g/dL)	Ht (%)	BUN (mg/dL)	Cre (mg/dL)	SDMA (µg/dL)	GOT (U/L)	GPT (U/L)	Urinary protein (µg/mL)	UPC
1	D	Healthy	M (C)	0.5	6.8	3.3	31.4	27	0.7	ND	21	ND	613	ND
2	JC	Healthy	M	0.5	6.4	3	34.6	31	1.1	ND	36	116	215	ND
3	D	Healthy	M	0.6	6.1	3.1	40.7	24	1.5	ND	24	55	954	ND
4	MC	Healthy	M	0.5	6.6	3.3	36	28	1.1	ND	ND	ND	215	ND
5	BS	Healthy	M	0.6	6.3	3.3	39	20	1	ND	8	40	787	ND
6	MC	Healthy	M	2.6	6.8	2.8	38.4	27	2	ND	ND	ND	903	ND
7	D	Healthy	M(C)	12.6	7.9	3.3	ND	25	1.4	9	ND	ND	34	0.1
8	MC	Healthy	F	2	9.7	3.1	34.6	30	0.9	ND	29	ND	271	ND
9	JC	Healthy	M	14	ND	ND	35.7	25	1.5	11	16	25	572	<0.2
10	JC	Healthy	M	10	7.5	3.2	45	21	1.1	12	ND	30	516	<0.2
11	JC	Healthy	M	5	ND	ND	35.7	11	1.5	8	ND	ND	299	<0.2
12	JC	Healthy	F(S)	6	7.8	3	46.6	23	1.5	13	ND	71	253	<0.2
13	D	CKD	M(C)	6	7.5	3.3	24.5	<130	13.1	ND	22	31	89	0.18
14	JC	CKD	F(S)	17	7.7	3.3	38.4	<130	12.7	ND	50	48	206	0.12
15	D	CKD	M(C)	6	7.3	3.1	36.7	22	2.6	ND	ND	78	444	0.11
16	D	CKD	F(S)	9	7.4	3.5	39.4	38	3	13	ND	58	187	0.1
17	JC	Cystitis, CKD	M(C)	2	7.2	3.7	41.6	96	7.4	ND	21	ND	2,495	ND
18	D	Cystitis	M(C)	0.5	7.1	3.2	30.5	25	1	ND	36	179	283	ND
19	JC	Urolirhiasis, CKD	F(S)	15	7.9	3.4	30.7	56	3.3	ND	31	63	2,239	0.29

C: castrated; S: spayed; TP: total protein; Alb: albumin; Ht: hematocrit; BUN: blood urea nitrogen; Cre: serum creatinine; SDMA: serum symmetric dimethylarginine; GOT: glutamic-oxaloacetic transamidase; GPT: glutamic-pyruvic tranamidase; UPC: urine protein to creatinine ratio; ND: no data; JC: Japanese cat; MC: Maine Coon; BS: British shorthair; D: Domestic long- or short-hair cat.

serum and urinary β2-MG. Urinary β2-MG differs from serum β2-MG in some healthy cats (nos. 1, 2, 5, 6, 8, and 9). The lower molecular weight form of urinary β2-MG (15 kDa) found in only four healthy cats (Nos. 1, 5, 6, and 9) was expected to be the bona fide form of β2-MG. These unmodified and higher-molecular-weight β2-MG bands (18 and 20 kDa) may have been produced by glycosylation or de-glycosylation after the glomerular passage. Although β2-MG is a marker of damage to the proximal tubules, it was detected in healthy cats with SDMA levels within the normal range (Table 1). However, the presence of β2-MG is likely to be independent of age and/or urinary protein levels. Further studies are needed to examine more diseased cats regarding the lack of glomerular reabsorption of modified β2-MG; however, β2-MG appears to be a predictive marker of kidney dysfunction (Kovarikova, 2015).

Circulating feline ApoA-I was detected in all cats, including healthy cats and cats with kidney diseases, based on immunological cross-reactions with human ApoA-I, as shown in the sera of LUTD cats (Figure 1B). The apparent molecular weight of ApoA-I was 24 kDa, which is lower than that calculated from the predicted amino acid sequence (28,943 Da; ENSFCAG00000018483). However, ApoA-I was not detected in the urine of the healthy cats (Supplementary Data 1). ApoA-I was detected in the urine of the two cats with CKD (Supplementary Data 1), whereas abundant ApoA-I was strongly detected in LUTD cats. Circulating lipoproteins and their constituents, known as apolipoproteins, are urinary markers of CKD (Clark *et al.*, 2019; Nielsen *et al.*, 2016). Filtered ApoA-I is reabsorbed by the receptors of both megalin and cubilin in the proximal tubules (Nielsen *et al.*, 2016). ApoA-I was also detected in two cats with CKD, indicating that ApoA-I may be a marker of CKD in cats and humans (Clark *et al.*, 2019). Abundant ApoA-I was detected in the urine of three cats with LUTD with urolithiasis or cystitis, also indicating that urinary ApoA-I can be a marker of LUTD. However, the mechanism by which ApoA-I enters the urine of cats with LUTD remains unclear, although hematuria has not been examined.

Immunoblotting analysis of normal cat serum (C) using anti-IgM and anti-IgG Fc antibodies was performed under non-reducing conditions using SDS-PAGE (Figure 1C). Under non-reducing conditions, many bands were detected at 172, 155, 90, 82, 68, 49, 37, and 27 kDa, with the 85-kDa band strongly detected as the IgM H-chain, as indicated by the dotted arrow. Under non-reducing conditions, the IgM pentamer was strongly detected at a molecular weight > 250 kDa, although many protein bands were also observed (161, 148, 85, and 50 kDa). In the case of IgG, the H-chain was detected as a major band (50 kDa) in addition to a 31-kDa band under reducing conditions (Supplementary Data 2), and the IgG monomer was detected at a molecular weight of > 250 kDa in addition to a 50-kDa band under non-reducing conditions, as indicated by the dotted arrow. However, no bands were detected when the blot was probed with only the ALP-conjugated secondary antibody (data not shown), although many bands were detected for unknown reasons in the IgM and IgG analyses, as previously described. Identification of the H-chains of both IgM and IgG was enabled by comparing the analyses of IgM and IgG in normal feline serum under reducing and non-reducing conditions. Although urine samples from six healthy cats (Nos. 1-6) were used as representative data (Figure 1C), including normal feline serum (C), the IgM pentamer was identified as a faint band in the urine samples of eight healthy cats with the primary 45- and 33-kDa bands except for Nos. 1, 6, 7, and 9, and the IgM H-chain (85 kDa) was also detected in eight urine samples, except for Nos. 7-10, as indicated by dotted arrows under non-reducing conditions. In addition, under non-reducing conditions, IgG monomers were detected in the urine of seven healthy cats, except for Nos. 3-5, 7, and 12, and a 50-kDa band identified as the IgG H-chain was detected with a 31-kDa band in the urine of nine healthy cats, except Nos. 7 and 12. Under non-reducing conditions, each H-chain of IgM (μ-chain) and IgG (γ-chain) with molecular weights of 85 kDa and 50 kDa, respectively, migrated more rapidly than the H-chains of IgM and IgG under reducing conditions (Supplementary Data 2). In CKD cats (Nos. 13-16), a band corresponding to the IgM H-chain (90 kDa) was detected in addition to bands at 48 and 33 kDa, which were detected as major bands under reducing conditions. Under non-reducing and nonreducing conditions, IgM H chains and monomers were observed in cats with CKD and LUTD. Under reducing or nonreducing conditions, the IgG H-chain (55 kDa) and monomer were also observed in urine samples from cats with CKD and LUTD, but the IgG monomer was not detected in cat No. 19 (Supplementary Data 2).

Both IgG and IgM exhibited separated H-subunits, even in control serum samples and in the urine of diseased and healthy cats under non-reducing conditions. Filtered IgG is removed from the glomerular basement membrane to protect against accumulation in the glomerular barrier (Akilesh *et al.*, 2008; He *et al.*, 2020). Polymeric immunoglobulins (e.g., IgM and IgA) are likely to be secreted into the renal tubules as a result of kidney injury (Wei & Wang, 2021). In cats with CKD or LUTD, kidney injury is associated with the detection of immunoglobulins (IgM and IgA), rather than IgG reabsorption (Tencer *et al.*, 1998).

CONCLUSION

β2-MG, a marker of kidney tubular damage, was detected in healthy cats even with normal SDMA levels. Urinary β2-MG was detected as multiple bands on SDS-PAGE, probably due to glycosylation and protein cleavage. Although ApoA-I was not detected in the urine of healthy cats, it was strongly detected in cats with LUTD. In this study, detection of urinary IgG and IgM revealed the probability of detection in all cats even under normal conditions. These immunoglobulins can be separately divided into H- and L-chains from IgG and IgM molecules in circulation and/or after glomerular passage. This report provides preliminary data that yields new insights into the detection of urinary and serum proteins.

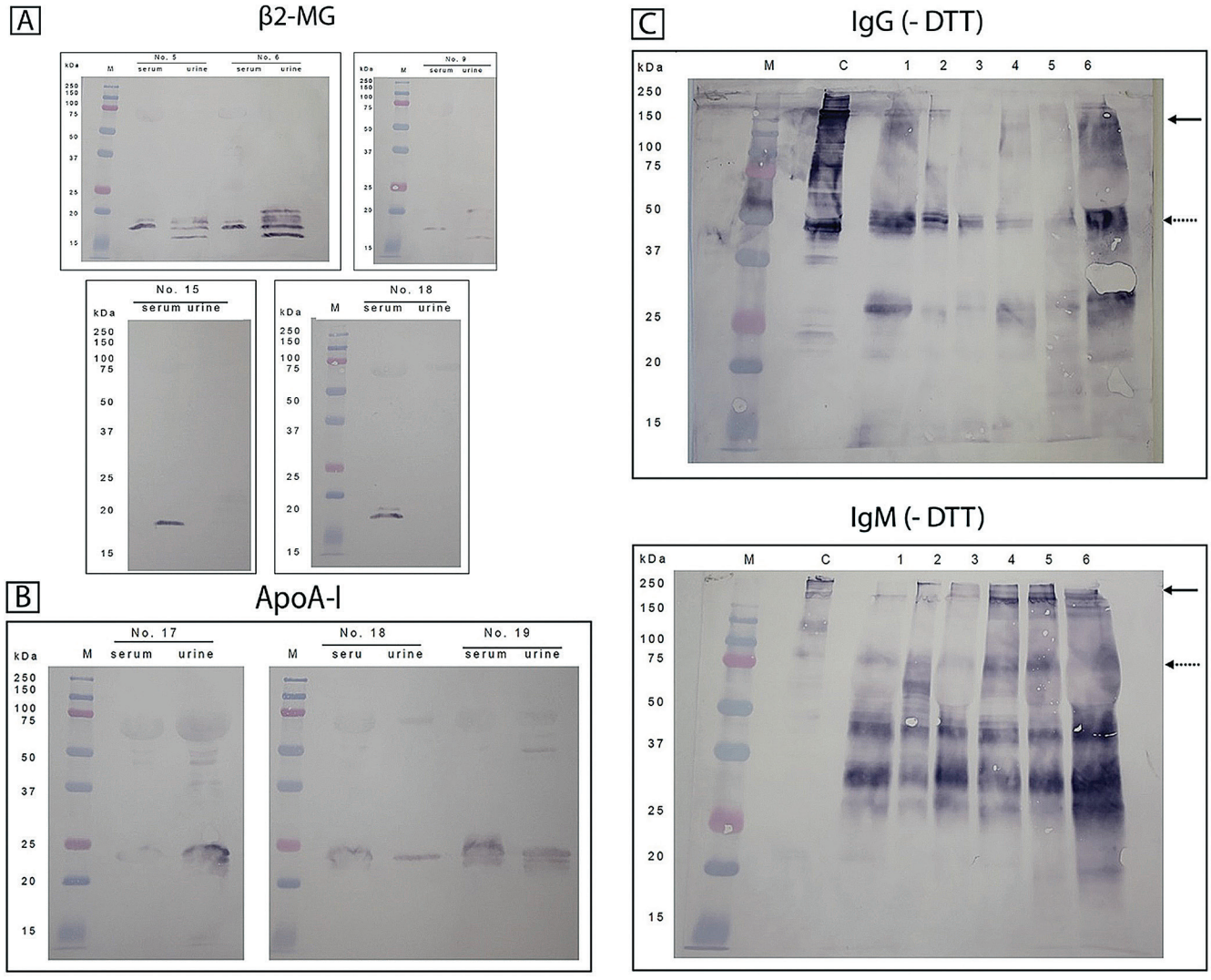

Figure 1.
Immunoblot analyses of the serum and urine of healthy and sick cats using antibody to human β2-MG antibody, human ApoA-I antibody or antibody to feline IgM μ-chain and IgG Fc fragment (γ-chain). A) and B) Serum and urine samples were diluted 40-fold and 1.25-fold with preparation buffer, respectively, and 20-μL aliquots were applied to SDS-PAGE gels followed by immunoblotting. C) Immunoblot analyses of the serum and urine of healthy cats (Nos. 1-6) using antibody to feline IgM μ-chain and IgG Fc fragment (γ chain). Urine samples of healthy cats were diluted 1.25-fold and with preparation buffer in the absence of DTT (-DTT), and 20-μL aliquots were applied to SDS-PAGE gels followed by immunoblotting as described in the "MATERIALS AND METHODS". The feline serum sample was used as a control sample (C). Arrows indicate H-chain of IgM or IgG and IgM pentamer or IgG monomer in the absence of DTT, and dotted arrows indicate H-chain of IgM or IgG detected. M represents marker proteins.

DECLARATIONS

Author contributions

Conceptualization, K. O., M. K., and Y. Y.; methodology, K. O. and M. K.; validation, K. O., M. K., and Y. Y.; formal analysis, K. O. and M. K.; investigation, K. K., M. K., H. I., and Y. T.; data curation, K. O., M. K., and H. I.; supervision, K. O.; writing-original draft, M. K.; writing-review and editing, K. O. All authors have read and agreed to the published version of the manuscript.

Ethics statement

All experiments were conducted following the established guidelines for animal welfare and approved by the Committee on the Ethics of Animal Experiments of Kitasato University (permit no.:20-048).

Conflict of interest

Authors declare no conflict of interests for this article.

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