

# Determination of the number of times for reuse of hemodialysis dialyzer through macrophage responses against dialyzer-eluted protein, a proposed method

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## Abstract

**Background:** Although the number of times dialyzer-reuse in hemodialysis is currently determined by the total volume of the dialyzer, the determination by macrophage activation using dialyzer-eluted protein might predict systemic inflammation.

**Objective:** The pro-inflammatory activities of the proteins from 5- and 15-times reused dialyzers were tested as a proof of concept experiment.

**Methods:** Accumulated proteins in dialyzers were eluted by the roller pump (the recirculation of 100 mL of buffer in a dialyzer with a roller pump at 15 mL/min for 2 h) or infusion procedures (infusion of 100 mL buffer in a dialyzer for 2 h) using chaotropic or potassium phosphate buffers (KPB) before the activation on macrophages cell lines (THP-1-derived human macrophages or RAW264.7 murine macrophages).

**Results:** The concentrations of dialyzer-eluted protein from both methods were not different and the infusion procedure was further used. The eluted proteins (by both buffers) from 15-times-reused dialyzers reduced cell viability, increased supernatant cytokines (TNF- $\alpha$  and IL-6), and upregulated pro-inflammatory genes (IL-1 $\beta$  and iNOS) in either THP-1-derived or RAW264.7 macrophages (higher responses in RAW264.7 cells) compared with the new dialyzer. Meanwhile, the 5-times-reused dialyzer protein did not reduce cell viability but enhanced some of these pro-inflammatory macrophage markers.

**Conclusion:** Due to the simpler preparation of KPB over chaotropic buffer with an easier protocol of RAW264.7 over THP-1-derived macrophages, the responses of RAW264.7 against dialyzer-eluted protein with infusion method using KPB buffer were proposed for determination of the number of times dialyzer reuse in hemodialysis.

**Key words:** Macrophages, dialyzer, reuse, inflammation, Proteins

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## Introduction

Hemodialysis (HD) is one of the important methods of renal replacement therapy for the treatment of patients with end-stage renal diseases (ESRD) which are a globally common healthcare problem caused mostly by diabetes mellitus, systemic lupus erythematosus, and others.<sup>1,2</sup> Among several factors, the effectiveness of toxin removal of the hemodialyzers is important for HD adequacy that needs to be monitored which allows the reuse of dialyzers in HD.<sup>3</sup> Indeed, dialyzer reuse in HD is a common practice worldwide, including in the United State, with several benefits, such as improved blood-membrane biocompatibility, reduced HD cost, and lessened environmental impacts.<sup>4</sup> However, the absorption of protein from the blood by the dialyzer in each use causes the reduction of the total dialyzer volume in the blood component of the dialyzer, referred to as “total cell volume (TCV; measurement of the volume of water being filled in a blood compartment of a dialyzer)”<sup>5</sup> While the absorbed proteins on the surface of the dialyzer reduce the hypersensitivity responses against the materials (the first-use syndrome), the accumulated proteins in the high concentrations and assembled germicides might be able to induce pro-inflammatory responses.<sup>6</sup> Although the number of times dialyzer-reuse in hemodialysis is currently determined by the TCV, the reuse determination by immune activation using dialyzer-eluted protein might be another concerning factor. Among several immune cells, monocytes and macrophages (the migrated monocytes from the bloodstream into any tissue in the body) are important cells for the recognition and responses against foreign molecules, especially for the innate immune responses.<sup>7-9</sup> As such, macrophages can be roughly categorized into M1 and M2 macrophage polarizations that are responsible for the pro-inflammation and alternative functions (anti-inflammation and healing processes) in responses against the different stimulations.<sup>10-12</sup> It is interesting to note that the defect on adaptive immunity (T cells, B cells, and plasma cells) with the profound innate immune responses (macrophages) are the well-known immune alteration of the chronic exposure against uremic toxins during chronic kidney disease (CKD) or ESRD.<sup>13</sup> Indeed, the lower responses to vaccination and profound pro-inflammation-induced atherosclerosis (and other complications) are commonly mentioned in CKD<sup>14-17</sup> and cardiovascular diseases are the common cause of death in patients with chronic HD.<sup>18</sup> Because i) several uremic toxins are able to induce macrophage hyper-inflammation and the microscopic pores on hemodialyzers allow an accumulation of uremic toxins which are a possible pro-inflammatory stimulator enhancing systemic inflammation and atherosclerosis in patients with HD<sup>19</sup> and ii) the possible chronic stimulation by the dialyzer-binding toxins due to the necessity of 2-3 times a week for hemodialysis, the use of overwhelming toxin-bound dialyzers might be harmful to the patients. Unfortunately, studies on the topic of immune responses against the reused dialyzers, including the protein-elution method, buffer, and proper representative cells to use, are still very less. Because RAW264.7 (macrophage murine cells) and the differentiated THP-1

(monocyte human cells) are common cell lines of macrophages that are currently frequently used in several publications,<sup>20-23</sup> both cell lines are interesting to adapt into clinical use. In parallel, the time of dialyzer reuse in the current clinical practice in Thailand is usually between 5 times to 15 (or 20) times depending on the hemodialysis unit<sup>5,24</sup> and the comparison of the eluted samples between 5 versus 15 times of reused dialyzers is interesting in the Thai situation. Hence, we used 2 protein-elution methods follow previous publications,<sup>25</sup> selected the reuse dialyzers in 2 time points (5 and 15 times of reuse), and stimulated 2 macrophage cell lines to test the pro-inflammatory activities of the proteins as a proof of concept experiment.

## Materials and Methods

### *Proteins elution from hemodialyzer*

The study was approved by the Ethical Committee for Research, Chulalongkorn University, Bangkok, Thailand (IRB No. 371/64). All patient received hemodialysis with ELISIO-21HX dialyzer (2.1 m<sup>2</sup> polyethersulfone membrane with ultrafiltration coefficient [KUF] 82 mL/h/mmHg, gamma irradiation sterilization: Nipro Corporation, Osaka, Japan). At the end of the hemodialysis session, dialyzers were flushed with 1 liter of saline to remove visible residual blood before protein elution by 100 mL of chaotropic buffer, a buffer with hydrogen bonding disruption property with 6 M urea, 2 M thiourea, 0.4% sodium dodecyl sulfate (SDS), and 1 mM Dithiothreitol (DTT), or 1 M potassium phosphate buffer (KPB or Gomori buffers; pH 6.5), a high capacity for water-soluble protein elution, using the roller pump (the recirculation of buffer within a dialyzer at a flow rate 15 mL/min for 2 h) or infusion methods (buffer instillation in a dialyzer for 2 h) before buffer collection,<sup>25</sup> the elution buffer was collected in a sterile tube. Each step was performed at room temperature, and collected samples were stored at -80°C until analysis. Then, protein precipitation (overnight -20°C cold acetone with 15 minutes of 13,000 rpm centrifugation), pellet collection, and phosphate buffer solution (PBS) resuspension (200 µL) was performed and measured total protein by the bicinchoninic acid assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Wilmington, DE, USA). Determination of the total cell volume (TCV) for the reuse of dialyzer (TCV higher than 80%) was performed following a routine hemodialysis protocol of the hemodialysis unit of the Nephrology department, King Chulalongkorn University Hospital. As such, the dialyzer reuse process is based on the Association for the Advancement of Medical Instrumentation (AAMI) standard using the reverse osmosis (RO) water through the dialyzer reprocessing machine (KIDNEY-KLEEN™ Dialyzer Reprocessor), Model Compact II (Meditop Co., Ltd., Thailand). To evaluate some uremic toxins from the eluted samples, the protein elution from dialyzer by 100 mL of chaotropic buffer with 2 h infusion method followed by acetone precipitation as mentioned above was performed. After that, 500 µL of PBS was used to dissolve the pellet before the measurement of severe possible toxins, including alpha-1-microglobulin (α1M), Beta-1-microglobulin (β2M),

**Table 1. List of primers.**

Primers	Forward	Reverse
Human $\beta$ -actin	5'-CCTGGCACCCAGCACAAT-3'	5'-GCCGATCCACACGGAGTACT-3'
Human interleukin-1 $\beta$ (IL-1 $\beta$ )	5'- CCACAGACCTTCCAGGAGAATG-3'	5'-GTGCAGTTTCAGTGATCGTACAGG-3'
Human inducible nitric oxide synthase (iNOS)	5'-ACCCACATCTGGCAGAATGAG-3'	5'-AGCCATGACCTTTCGCATTAG-3'
Human Transforming Growth Factor- $\beta$ (TGF- $\beta$ )	5'-CAGAGCTGCGCTTGCAGAG-3'	5'-GTCAGCAGCCGGTTACCAAG-3'
Human Arginase 1 (Arg-1)	5'-CTTGGCTTGCTTCGGAAGCTC-3'	5'-GGAGAAGGCGTTTGCTTAGTTTC-3'
Murine $\beta$ -actin	5'-CGGTTCCGATGCCCTGAGGCTCTT-3'	5'-CGTCACACTTCATGATGGAATTGA-3'
Murine IL-1 $\beta$	5'-GAAATGCCACCTTTTGACAGTG-3'	5'-TGGATGCTCTCATCAGGACAG-3'
Murine iNOS	5'-ACCCACATCTGGCAGAATGAG-3'	5'-AGCCATGACCTTTCGCATTAG-3'
Murine TGF- $\beta$	5'-CAGAGCTGCGCTTGCAGAG-3'	5'-GTCAGCAGCCGGTTACCAAG-3'
Murine Arg-1	5'-CTTGGCTTGCTTCGGAAGCTC-3'	5'-GGAGAAGGCGTTTGCTTAGTT-3'

lambda free light chain (FLC), indoxyl sulfate (IS), and lipopolysaccharide (LPS). The enzyme-linked immunosorbent assay (ELISA) for  $\alpha$ 1M and  $\beta$ 2M from Abcam (Cambridge, UK) and Thermo Fisher Scientific (Waltham, MA, USA), respectively, together with the Freelite assay (Binding Site, Birmingham, UK) for FLC was used. Indoxyl sulfate (a gut-derived uremic toxin) in the samples was determined by liquid chromatography-mass spectrometry (LC-MS/MS) using a silica column (Luna<sup>®</sup> silica; 00G-4274-E0, Phenomenex<sup>®</sup>, Torrance, CA, USA) plus high-performance liquid chromatography (HPLC Alliance<sup>®</sup> 2695; Waters, Zellik, Belgium), while LPS was evaluated by HEK-Blue LPS Detection Kit 2 (InvivoGen<sup>™</sup>, San Diego, CA, USA) as previously described.<sup>26,27</sup>

### ***In vitro experiments***

The samples were incubated with human monocytoid THP-1-derived macrophages (ATCC TIB- 202) or RAW 264.7 murine macrophages (ATCC-TIB-71) that were cultured in modified Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and Dulbecco's modified Eagle medium (DMEM) (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum and 1% Penicillin-Streptomycin, respectively.<sup>20,22</sup> The cells ( $1 \times 10^4$  cells/well in 96 well plates for determination of cell viability and  $1 \times 10^5$  cells/well in 6 well plates for cytokine measurement and gene expression) were incubated with the eluted protein using the whole pellet or the protein concentration-adjusted sample (1 mg/mL) for 24 h before the determination of cell viability with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay (Thermo Fisher Scientific), supernatant cytokines using ELISA (Invitrogen, Carlsbad, CA, USA), and the expression of several genes by quantitative polymerase chain reaction (qPCR). Total RNA and reverse transcription were prepared using TRIzol (Invitrogen) and a high capacity reverse transcription assay (Applied Biosystems, Warrington, UK), respectively.

Then, qPCR was performed using QuantStudio 6 Flex Real-Time PCR Systems (Thermo Scientific, Waltham, MA, USA) with PowerUp<sup>™</sup> SYBR<sup>™</sup> Green Master Mix (Thermo Scientific) and were demonstrated in terms of relative quantification using the comparative threshold ( $\Delta\Delta Ct$ ) method ( $2^{-\Delta\Delta Ct}$ ) as normalized by  $\beta$ -actin.<sup>28</sup> A list of primers for PCR is shown in **Table 1**. For the determination of cell death, the flow-cytometry analysis using fluorochrome-conjugated antibodies against annexin V and propidium iodide (PI) (BioLegend, San Diego, CA, USA) was used with FlowJo software in the stimulated macrophages as previously described.<sup>29</sup>

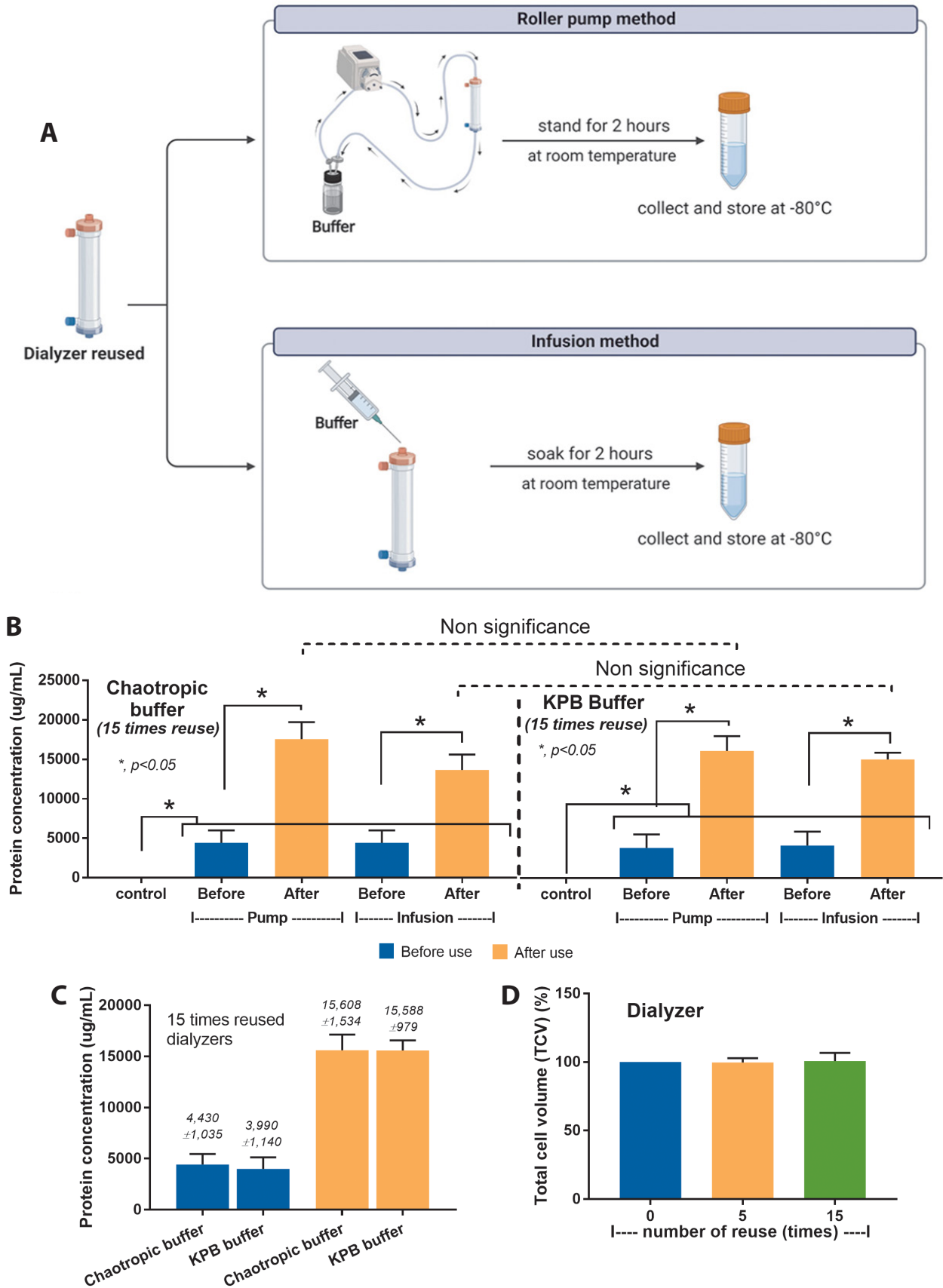
### ***Statistical analysis***

Data were presented with mean  $\pm$  standard error (SE) with one-way analysis of variance (ANOVA) with Tukey's analysis (multiple groups) or paired T-test (before-after) using SPSS 11.5 software (SPSS, IL, USA). A *p*-value of  $< 0.05$  was considered for statistical significance.

## **Results**

### ***The protein from 15-times reused dialyzer more profoundly enhanced macrophage inflammatory responses than the 5-times reused dialyzer***

The schema of the infusion procedure demonstrated the simpler process than the pump-based method that needs an infusion pump (**Figure 1A**). There were similar concentrations of protein from both methods of protein elution, regardless of the buffers (chaotropic versus KPb) as tested by the samples from 15-times reused dialyzers (**Figure 1B**). Protein concentration of the samples at "before use" in the hemodialysis session of the 15 times dialyzers using chaotic buffer was varied between 2,488 to 8,390  $\mu$ g/mL ( $4,430 \pm 1,035$ ) and by KPb buffer was 991 to 8,118  $\mu$ g/mL ( $3,990 \pm 1,140$ ), while the samples "after use" of these dialyzers demonstrated protein at 10,486 to 23,936  $\mu$ g/mL ( $15,608 \pm 1,534$ ) by chaotic buffer and at 11,120 to 19,803  $\mu$ g/mL ( $15,588 \pm 979$ ) by KPb buffer (**Figure 1C**). Notably, all of the dialyzers, either 5- or 15-times of the reuse,

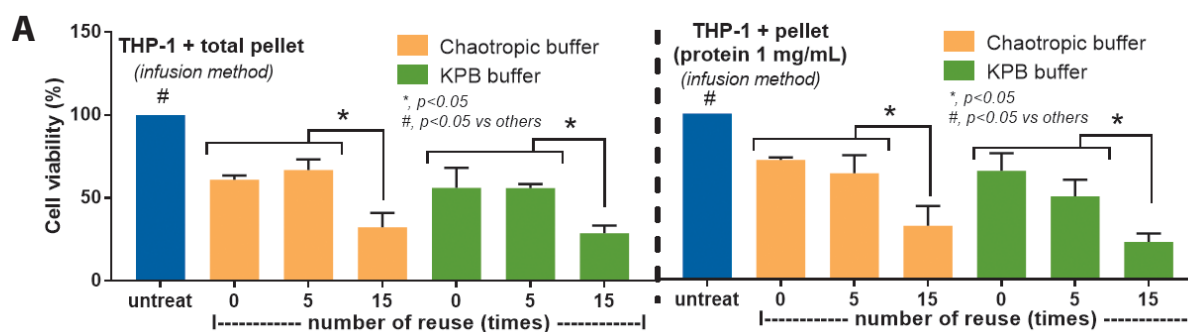


**Figure 1.** The picture of protein elution by pump-based and infusion methods (A) and the protein concentration before (before; buffer collection immediately after the complete infusion of 100 mL buffer) and after 2 h of the protein elution process (after) with chaotropic or potassium phosphate buffer (KPB) (B), and the combined data of both buffers at before use and after use (C) are demonstrated (n = 7 per group for B and n = 14 per group for C). The total cell volume of the dialyzers at 0, 5, and 15 times reuse (D) are also demonstrated (n = 7 per group).

were pass the criteria for further use in hemodialysis session as the total cell volume (TCV) of the dialyzers were still higher than 80% (Figure 1D). Then, only the infusion method was further tested in THP-1 cells with both chaotropic and KPB buffers (Figure 2A-J) because the infusion method was simpler than the pump-based method that was performed with an infusion pump. The eluted protein from the 15-times reused dialyzer (but not the 5-times reuse) reduced the cell viability in THP-1-derived macrophages (using all pellets and 1 mg/mL of the pellet) regardless of the buffer (Figure 2A). Because the concentration of the eluted protein from the reused dialyzer was vary from 1.1 to 23.9 mg/mL ( $9.9 \pm 1.2$ ) (Figure 1B, C) then the use of the minimum concentration (1 mg/mL) was selected allowing a better comparison among dialyzers. On the other hand, the use of all proteins retrieving from the dialyzers per sample (total pellet; mostly higher than 1 mg/mL) were parallely used to see if the higher protein concentration can enhance the responses which might increase the sensitivity of the test.

Interestingly, the reduced cell viability in the samples from new dialyzers implied the toxicity of both buffers toward THP-1-derived cells (Figure 2A). In parallel, both 5- and 15-times reused samples induced higher supernatant TNF- $\alpha$  with both buffers, regardless of total pellet or 1 mg/mL (Figure 2B). However, KPB buffer in 15-times reused dialyzers demonstrated the highest level of supernatant TNF- $\alpha$  (Figure 2B). Supernatant IL-6 was elevated after stimulation by 5 and 15-times reuse samples using all pellets with both buffers, while the 1 mg/mL samples induced higher IL-6 only with the 15-time reuse sample (Figure 2C), perhaps due to the higher protein concentration in the samples with all pellet compared with the 1 mg/mL samples.

Additionally, the protein from the 15-times reuse dialyzers using all pellets, but not the normalized sample (by 1 mg/mL protein), induced pro-inflammatory IL-1 $\beta$  and iNOS in THP-1-derived cells (Figure 2D-E) suggesting that the protein retrieved from all total pellet showed a more prominent pro-inflammation. There was no change in M2 macrophage polarization from all samples (Figure 2F, G) supporting a less impact of the eluted dialyzer proteins on M2 anti-inflammatory macrophages. For better visualization of the comparison, data of the activation by samples from the 15 times reused dialyzers with total pellet versus 1 mg/mL (combined data from both buffers) were demonstrated (Figure 2H-J). As such, the total pellet induced higher supernatant TNF- $\alpha$  and the expression of IL-1 $\beta$  and iNOS compared with the 1 mg/mL samples (Figure 2H-J) suggesting a better use of the whole protein eluted from the dialyzers. In comparison between 5 versus 15 times of reuse using the total pellet, the 15-times reuses decreased THP-1 cell viability with increased all inflammatory markers, while the 5-times reuses increased only supernatant TNF- $\alpha$  and IL-6 without the reduction of cell viability (Figure 2A-G), implying a less potent inflammatory activation in 5-time reuse compared with 15-time reuse. Notably, all 15-times reused dialyzers were allowable for further use in hemodialysis according to the higher than 80% TCV criteria (Figure 1D), indicating more rigid criteria to stop the reuse by the *in vitro* tests compared with the TCV procedure. Because a more complex procedure might lead to a higher human error in the clinical laboratory and the least complex procedure is probably more suitable for use in real clinical practice, the procedure using the total pellet without the process of protein measurement, but not 1 mg/mL of the protein preparation, was selected for further tests in RAW264.7 cells.



**Figure 2.** The characteristics of 24 h stimulated THP-1-mediated macrophages with all pellet or 1 mg/mL pellet by both types of buffers as indicated by cell viability (A), supernatant cytokines (TNF- $\alpha$  and IL-6) (B, C), pro-inflammatory M1 macrophage polarization genes (IL-1 $\beta$  and iNOS) (D, E), and M2 macrophage polarization genes (TGF- $\beta$  and Arg-1) (F, G) are demonstrated (n = 7 per group). For a further comparison, data from the 15 times reused samples (combined data from both buffers) using total pellet versus only 1 mg/mL in all parameters, including cell viability (H), supernatant cytokines (TNF- $\alpha$  and IL-6) (I), M1 and M2 macrophage polarization genes (IL-1 $\beta$ , iNOS, TGF- $\beta$ , and Arg-1) (J) are also demonstrated (n = 14 per group).

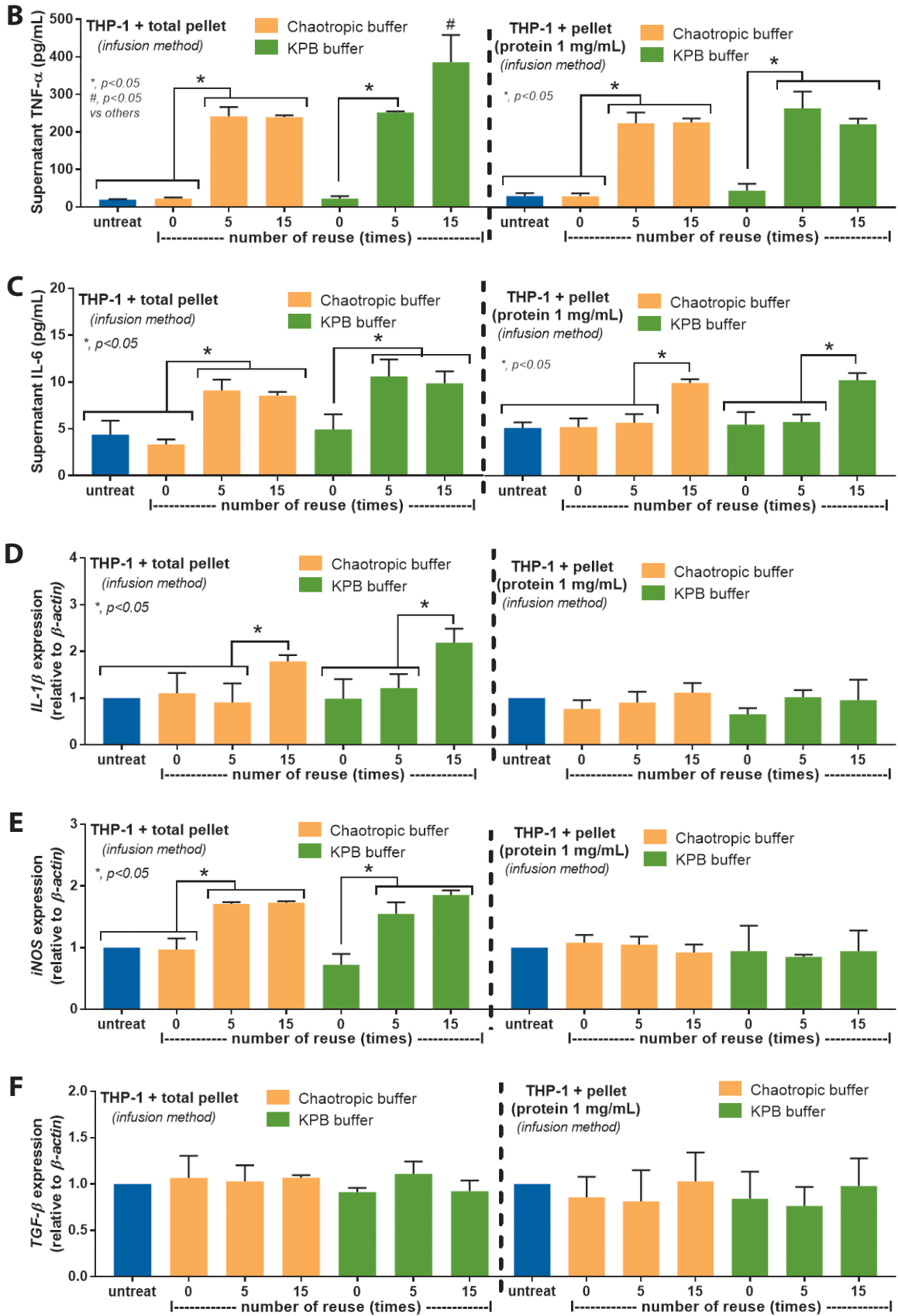


Figure 2. (Continued)

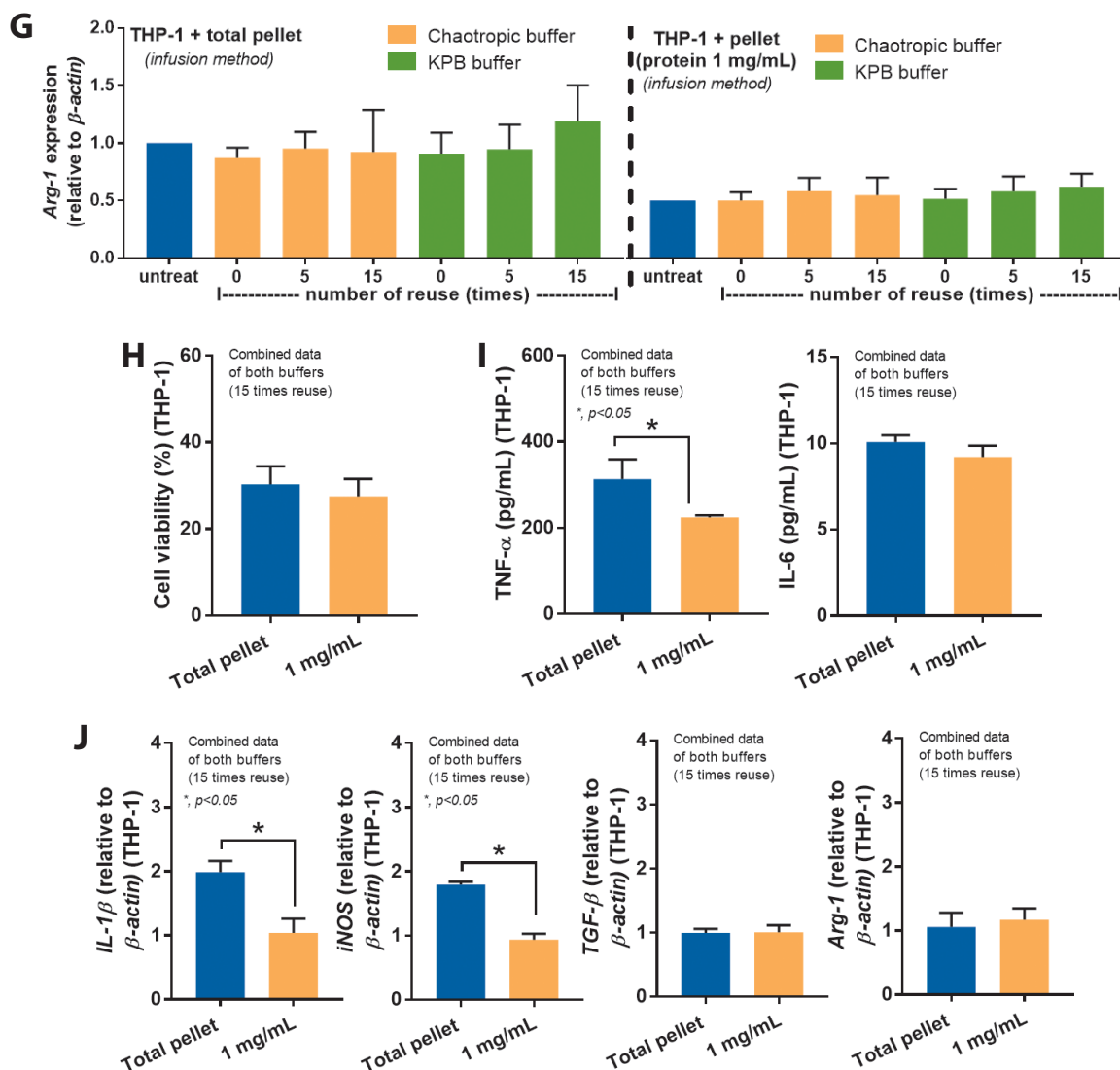
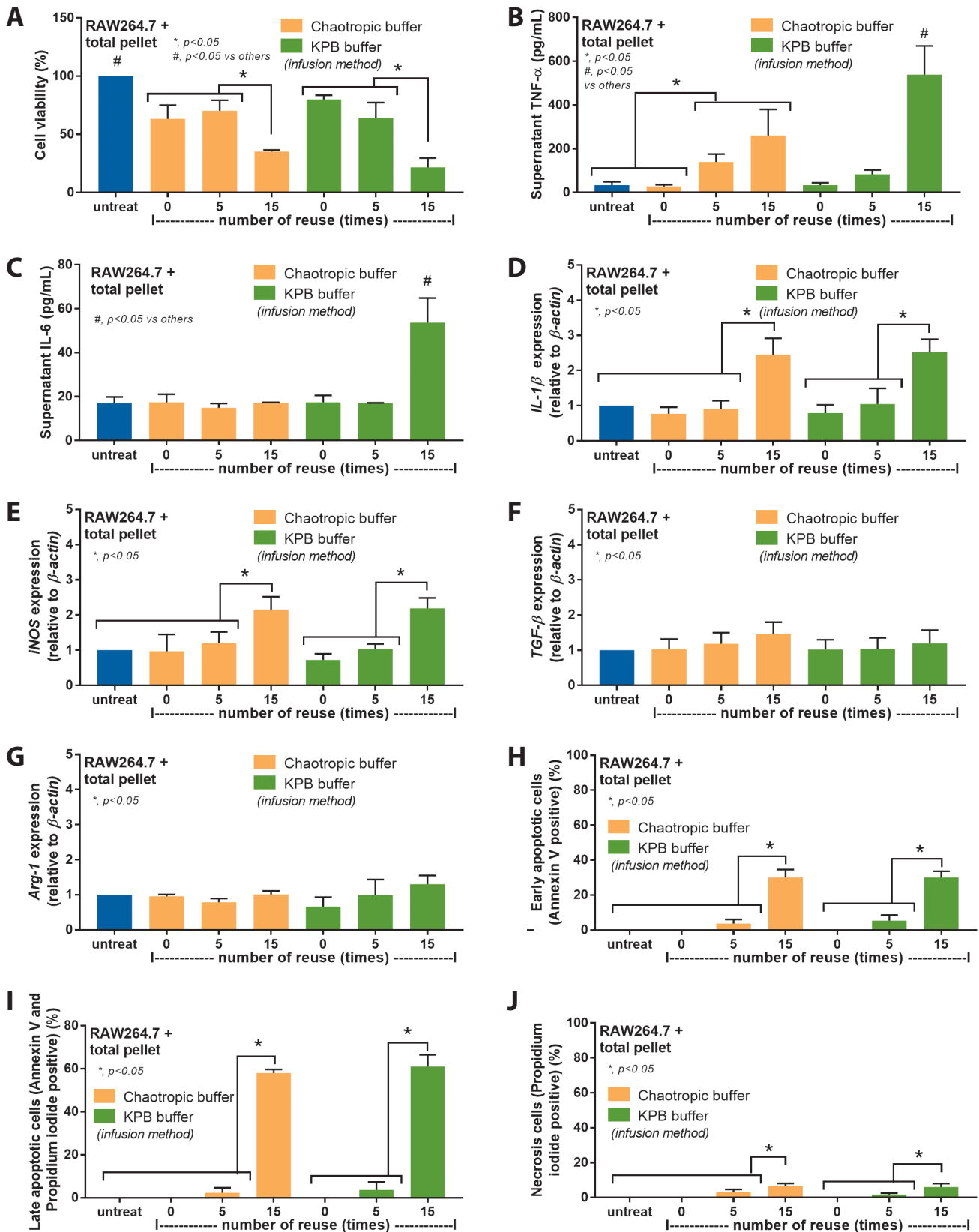


Figure 2. (Continued)

**The responses of RAW264.7 cells and the possible toxins that were responsible for macrophage activation**

Then, chaotropic or KPB buffers with a simple infusion method were used to retrieve the protein from all pellets and tested in RAW264.7 cells due to the frequent use of RAW264.7 (murine macrophages) in the scientific experiments and the possible differences between murine versus human cells.<sup>30</sup> Similar to THP-1-derived cells, the protein from the 15-times reused dialyzer, but not the 5-times reuse, induced more prominent death of RAW264.7 cells by both buffers (Figure 3A). Both 5- and 15-times reuse of chaotropic buffer increased supernatant TNF- $\alpha$ , while only 15-times reuse from KPB buffer elevated TNF- $\alpha$  (Figure 3B). Notably, the TNF- $\alpha$

responses from 15-times reuse with KPB buffer demonstrated the highest level among all samples (Figure 3B). Meanwhile, supernatant IL-6 was elevated only by 15-time reuse with KPB buffer, and upregulated IL-1 $\beta$  and iNOS were demonstrated in 15-times reuse by both buffers (Figure 3C-E). There was no alteration in M2 macrophage polarization genes (Figure 3F, G). Then, the cell death processes (apoptosis and necrosis) were determined by flow cytometry analysis. Interestingly, most of the cell death process by the 15-times reused dialyzer samples was late apoptosis with either chaotropic or KPB buffer, while there was no difference in cell death between samples of 0 and 5-times reuse dialyzers (Figure 3H-F).



**Figure 3.** The characteristics of 24 h stimulated RAW264.7 macrophages using all pellets by both types of buffers as indicated by cell viability (A), supernatant cytokines (TNF- $\alpha$  and IL-6) (B, C), pro-inflammatory M1 macrophage polarization genes (IL-1 $\beta$  and iNOS) (D, E), M2 macrophage polarization genes (TGF- $\beta$  and Arg-1) (F, G), and quantitative flow-cytometric analysis to determine early apoptosis (Annexin V positive), late apoptosis (Annexin V and propidium iodide positive), and necrosis (propidium iodide positive) (H-J) with the representative flow cytometry pattern using 5- and 15-times reused samples with KPB buffer (K, L) are demonstrated (n = 7 per group).



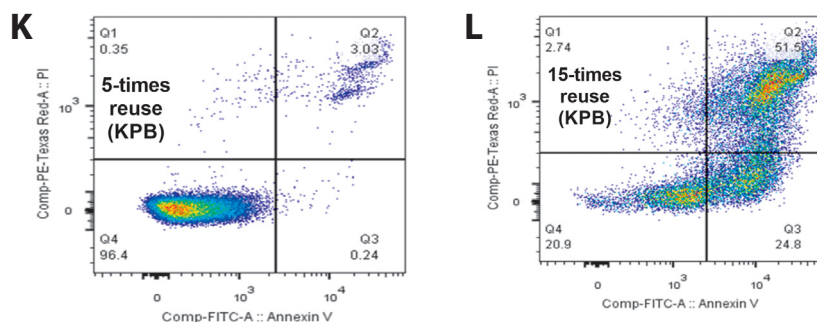


Figure 3. (Continued)

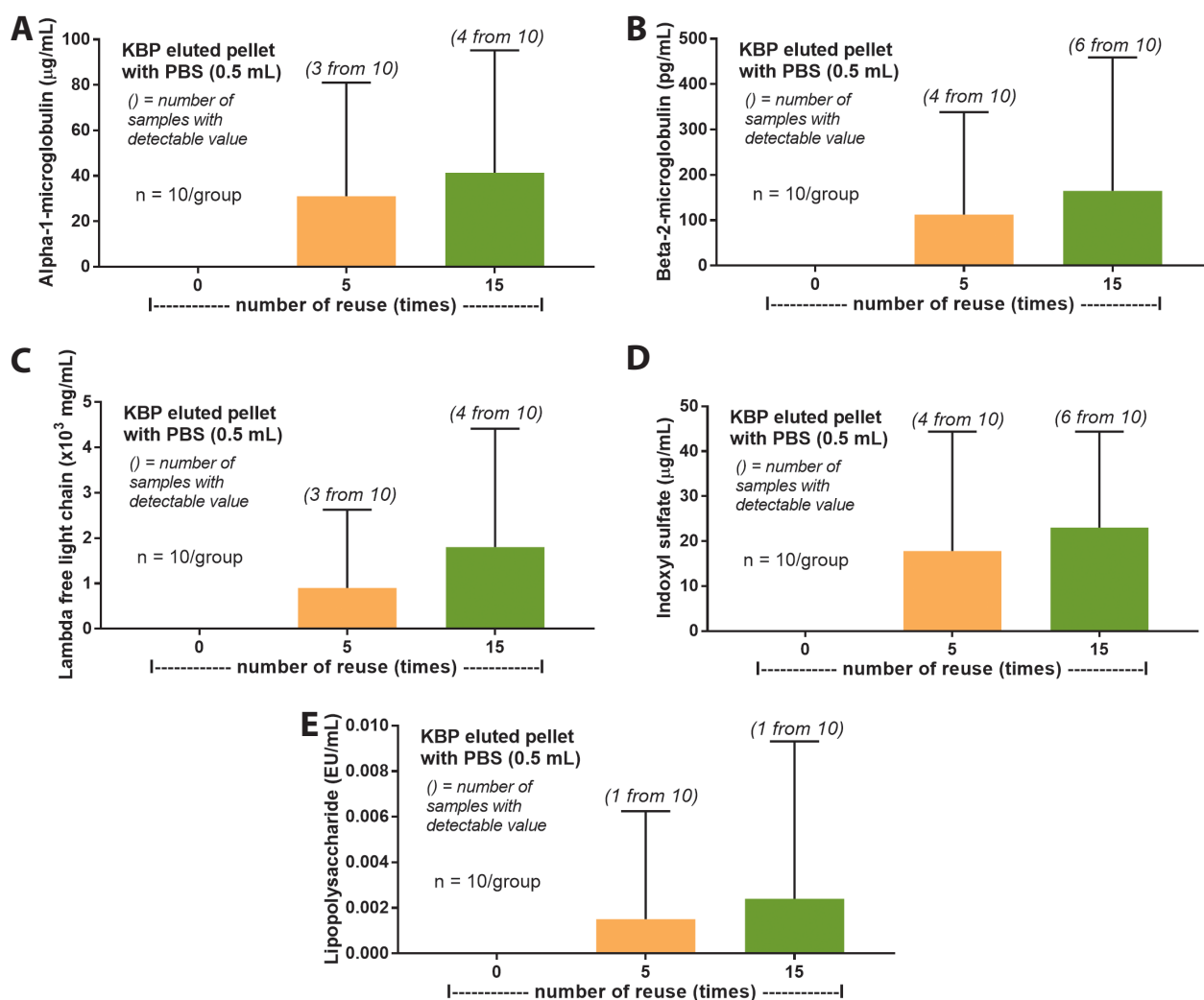


Figure 4. The analyses from the eluted pellet using potassium phosphate buffer (KPB) followed by acetone precipitation, and diluted with 1 mL phosphate buffer solution (PBS) as evaluated by several uremic toxins, including alpha-1-microglobulin (A), Beta-2-microglobulin (B), Lambda free light chain (C), and indoxyl sulfate (D), together with lipopolysaccharide (LPS) (E) are demonstrated (n = 10 per group).

Because of the well-known impacts of several uremic toxins and lipopolysaccharide (LPS) on macrophage responses and cell death,<sup>31,32</sup> some molecules were measured from the eluted samples using KPB buffer. Among the 5- and 15-times reused samples, there were 1-6 samples out of 10 specimens in the group with positive results of these molecules resulting in the non-significant difference from the new dialyzer (0-time reuse), as indicated by alpha-1-microglobulin, beta-2-microglobulin, lambda free light chain, indoxyl sulfate, and LPS (**Figure 4A-E**). Perhaps a better elution method might be necessary for the elution of the membrane-absorbed molecules. However, the detectable toxins in some eluted protein samples of the reused dialyzers supported a possible of toxin absorption into the dialyzer membrane<sup>25,33,34</sup> that might lead to systemic immune activation on several immune cells, including macrophages. Hence, our results suggested that the test of macrophage activation by the eluted proteins from the dialyzers might be beneficial for discarding the dialyzers that induced macrophage responses due to the possibility of enhanced systemic inflammatory complications in patients.

## Discussion

### *Protein elution from the reused dialyzers by different methods and buffers, a proposed protocol for the future use*

Our main objective was to adapt the use of common *in vitro* laboratory experiments on macrophage into the routine clinical practice to determine the times to stop using the dialyzers after reusing them in several hemodialysis sessions. Hence, the simplest procedure will be the most beneficial translation into a real clinical situation. Despite a very limited number of publications on dialyzer reuse, protocols for protein elution from literature, mostly consist of i) the roller pump method using the shearing stress from the fluid flow,<sup>25</sup> and ii) the infusion method with a simple infusion of the buffer, without a comparison between them. Here, the non-different effectiveness in protein elution from reduced dialyzers was demonstrated. Because of the simpler procedure of the infusion method compared with the roller pump method which needs the availability of machines to process, the infusion method was recommended for further use. Similarly, both chaotropic buffer (the buffer that can disrupt the hydrogen bonding network between water molecules) and potassium phosphate (KPB) buffers showed a similar amount of the eluted protein (**Figure 1B**) and KPB buffer might be a more practical buffer to use due to the need of less substances for the preparation (see method). Although both chaotropic and KPB buffers were toxic to macrophages as the eluted sample from the new dialyzers reduced cell viability into 50-60% (**Figure 2A**) possibly due to the impacts of surfactant (sodium dodecyl sulfate; SDS) in chaotropic buffer and high potassium in KPB buffer, both buffers were neither activated cytokines nor induced macrophage polarization.

Additionally, the supernatant TNF- $\alpha$  and expression of IL-1 $\beta$  and iNOS in THP-1 cells from the activation by total pellet was higher than 1 mg/mL samples (**Figure 2I, J**), despite a similar response in the cell viability by total pellet and 1 mg/mL samples (**Figure 2H**). Because the use of the total amount of eluted protein was easier than the preparation for 1 mg/mL samples due to no protein measurement process in the use of total protein from the pellet, the total pellet procedure might be more suitable for clinical use. Similarly, a differentiation process from monocyte into macrophage is necessary for the use of THP-1-derived macrophages (THP-1; a human monocytic cell line), while the use of RAW264.7 (murine macrophages) does not need any macrophage differentiation processes, then the preparation of RAW264.7 is easier than THP-1 cells and might be more suitable for clinical translation. Regarding an easier preparation between i) KPB versus chaotropic buffer, ii) the total pellet versus 1 mg/mL samples, and iii) RAW264.7 versus THP-1 protocol, the elution protocol by 2 h infusion method using KPB buffer with RAW264.7 activation by all eluted protein from the pellet is recommended use for further studies.

### *More prominent macrophage responses against the eluted protein from the dialyzers with longer reuse, a possible patient impact*

A possible adverse effect of the dialyzer with longer reuse was demonstrated by the reduced cell viability in both macrophages from THP-1 and RAW264.7 cells possibly due to the cytotoxicity of some toxins that are adsorbed (the compounds attach to the membrane surface) or absorbed (the toxins enter to the membrane material) into dialyzer membranes.<sup>35</sup> Indeed, the deposit of several molecules, such as immunoglobulin (Ig), beta-2-microglobulin ( $\beta$ 2M), complements, serotransferrin, and lipoproteins, on the damaged surface of dialyzers following continued use is nicely demonstrated by the electron microscope, X-ray spectrum analysis, and proteomic analysis.<sup>25,33,34</sup> Not only uremic toxins, but every molecule in the blood circulation might also deposit on pores of the dialyzer membranes, including lipopolysaccharide (LPS; a major cell wall component of Gram-negative bacteria), which could be demonstrated in the blood of some patients with chronic kidney disease and hemodialysis.<sup>36</sup> Hence, LPS might also be deposited on the dialyzer membrane. During renal insufficiency, accumulation of several molecules that are normally excreted through the kidney, including alpha-1-microglobulin ( $\alpha$ 1M; a housekeeping protein responsible for removal of and protection against harmful oxidants),  $\beta$ 2M (a component on all nucleated cells), free immunoglobulin light chain (small polypeptide subunit of an antibody), and indoxyl sulfate (a metabolite of dietary L-tryptophan producing from liver) are reported as uremic toxins with systemic inflammatory activities.<sup>37-39</sup> Unsurprisingly, these uremic toxins were detectable in some samples from the eluted proteins in both 5- and 15- times

reused dialyzers supporting the possible deposition of several pro-inflammatory molecules on the dialyzer membrane. On the other hand, LPS was detectable only in 1 sample from 5- and 15- times reused dialyzers possibly due to the patient's condition as the reported endotoxemia on dialysis cases with cirrhosis, infection, or malignancy.<sup>40</sup> However, the non-difference in levels of the toxins between 5- versus 15- times reuse and the non-detectable toxins in some samples might be due to the limitation of the protein elution protocol and the accumulated toxins in different patients might be different. For example, the production of the gut-derived uremic toxin, such as indoxyl sulfate, might partly be due to differences in the severity of abnormal microbiota in the gut (gut dysbiosis) among patients, resulting in a higher elevation of this toxin in some patients more than others that affect the amount of toxin eluted from dialyzers.

Nevertheless, the mixture of several uremic toxins, especially the protein-bound toxins, such as indoxyl sulfate,<sup>41</sup> might be responsible for macrophage activation and the cytotoxicity (mostly cell apoptosis). Also, the attached toxins on the dialyzer membrane might constantly activate immune cells during the hemodialysis sessions which might be harmful to the patients. Although only polyethersulfone dialyzer was used in our study because of the possible different adsorption properties of uremic toxins in various dialyzer membrane materials,<sup>42</sup> there was the difference in the eluted toxins from the reused dialyzers. Due to the possible different combinations of toxins deposited on the dialyzer membranes in individual patients, the use of only one toxin to determine time of reuse for the dialyzer might be not proper.

Here, we proposed the simplest method using KPB buffer on RAW264.7 cells and the use of 15-times reused dialyzers should be discouraged, despite the passing criteria on total cell volume (TCV) at more than 80%. The 15-times reuse samples prominently activated macrophages than 5-times reuse as indicated by all 3 aspects, including cell cytotoxicity, supernatant TNF- $\alpha$ , and M1-related macrophage polarization genes (IL-1 $\beta$  and iNOS) (**Figure 3A-E**), while the 5-times reuse induces only macrophage TNF- $\alpha$  (**Figure 3B**). Although the use of 5-times dialyzers might not be as good as a new dialyzer due to the possible macrophage cytokine activation, the economic burdens of fewer times of reuse should be explored. Also, the stop using reused dialyzers with complete all 3 criteria on macrophage activities or only 1 criterion with cell cytotoxicity or cytokine activation needed more studied before the clinical translation. The use of cytokine activation criterion alone to stop reuse might be more sensitive than the use of cell cytotoxicity as the inducible cytokine and cell cytotoxicity were demonstrated at 5- and 15- times reuse, respectively (**Figure 3A, B**). The macrophage cell death from 15-times reuse samples might be due to inflammation-induced cell injury<sup>29</sup> (increased reactive oxygen species and altered cell energy status).<sup>17,28,43,44</sup> The large randomized control trials and the debate on economic burdens in the use of sensitive macrophage activity criteria should be performed.

## Conclusion

Because of a limited number of studies on this topic, the activity of RAW264.7 against the dialyzer-eluted protein (using KPB buffer infusion) as indicated by cell cytotoxicity, cytokine responses, and pro-inflammatory macrophage polarization genes) is proposed. Although an indicator using supernatant cytokine elevation and cell cytotoxicity might result in fewer and longer times of dialyzer reuse, respectively, the stop reuse by these macrophage activation criteria seems to be earlier than the current indicator (TCV). Further studies on the clinical importance and economic burdens are interesting.

## Potential conflicts of interest

The authors declare that there are no conflicts of interest.

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