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**Note**

# Uremia toxin helps to induce inflammation in intestines by activating the ATM/NEMO/ NF-κB signalling pathway in human intestinal epithelial cells

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During progressive chronic kidney disease, toxic substances known as uremic toxins accumulate in body fluids. Uremia toxin has been documented to be involved in most inflammatory reactions, and indoxyl-sulfate (IS) a major serum metabolite of uremia is a key player in this. The mechanism by which uremia toxin establishes it inflammatory activity is scarcely known; however, researchers believes that a clear understanding of this process can serve as a guide to combat the situation. The study was designed to investigate the role played by uremia toxin in intestinal inflammation. SW480 was used as cell lines for this study. Luciferase assay was used to detect the cell viability of different concentrations of IS. RT-qPCR was used to detect the effect of IS on the expression of inflammatory factors. The comet assay was used as a tool to detect DNA damage. Western blot was used to detect the phosphorylation level of ATM/NEMO/NF-κB protein. The IS of 0.09 nM was determined to be the best experimental concentration by luciferase assay. Result showed that IS promotes the expression of inflammatory factors TNF-α and IL-6. In addition, IS led to enhanced DNA damage in cells. IS promoted ATM phosphorylation leading to phosphorylation of NEMO to activate the NF-κB signalling pathway. In conclusion, uremia toxin facilitates inflammation in intestines by activating the ATM/NEMO/ NF-κB signalling pathway in human intestinal epithelial cells.

**Keywords**: Chronic kidney disease, DNA damage, PCR, Phosphorylation, Toxin

## Introduction

Patients who suffer chronic kidney diseases (CKD) often are subjected to kidney clearance when they are receiving treatment. Kidney clearance is not guaranteed to be kept in the same level every time. In general, some solute remains in body fluids as the kidney clearance decreases. These solutes are called uremic toxins. On the basis of the behaviours of uremic toxins in the progression of dialysis, they have been divided into three categories by the European Uremic Toxin Work Group (EUTox)[1], which include first category as low-molecular-weight water-soluble molecules, second category as the middle molecules and third category are protein-bound molecules. Traditional haemodialysis treatments do not remove these solutes well enough, so they accumulate in the body[2]. Indoxyl-sulfate (IS) is a kind of protein-bound solutes, which is composited in liver from indoles[3]. Indoles are aroused *via* intestinal bacteria[4]. IS has attracted much attention since it is related to inflammation. For example, the uremic toxin indoxyl sulfate exacerbates reactive oxygen species production and inflammation in 3T3-L1 adipose cells[5], and the related research also included the crucial role of the AhR in indoxyl sulfate-Induced vascular inflammation[4]. Nevertheless, there are few studies on uremicin and intestinal inflammation. The major purpose of research is to investigate if IS can cause intestinal inflammation in human intestinal epithelial cells (HIECs). The study may offer new insights of the correlation of IS and intestinal inflammation.

## Materials and Methods

### Cell culture

SW480 was received from ATCC and selected to conduct the experiments. DMEM medium was utilized to deposit the cells with 10% FBS and 1% Penicillin-Streptomycin. The temperature for cultivating was 37°C and the air contained 5% $CO_2$.

### Transfection and NF-κB reporter assay

Opti-MEM, a Reduced-Serum Medium, was adopted to conduct transfection. On the basis of the protocols of suppliers, Lipofectamine 2000 was utilized for transfecting. With regard to NF-κB reporter assay, pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and pRL Renilla Luciferase Control Reporter Vector were utilized to transfect cells and accepted form Promega. Then the luciferase reporter assay system also came from Promega and it was adopted to estimate luciferase activity.

### Western blotting

Western blot analysis was performed as described by Zhaowei *et al.* with slight modification[6]. M-PER protein extraction reagent (Thermo Fisher Scientific, Waltham,

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MA, USA) was utilized to acquire the total cell lysate. Then protein lysate was loaded in precast gel. After electrophoresing, it was transferred to PVDF membranes. Membranes were blockaded in 5% bovine serum albumin (BSA; Abcam, UAS) at least 1 h. Following, they were incubated overnight at the temperature of 4°C with primary antibodies including: NF-κb (1:1000; Abcam, ab260004); ATM and p-ATM (1:1000; Cell Signalling Technology, Danvers, MA); NEMO and p-NEMO (1:1000; Abcam, ab230832 or ab206026); GAPDH (1:2000; Abcam, ab181602. The relevant second rabbit anti-mouse or anti-rabbit antibody (1:2000 diluted, Abcam, ab6728 or ab6721) incubated for 24 h. With the help of enhanced chemiluminescent substrate (Thermo, USA), protein bands were observed directly.

**RT-qPCR**

This was performed as described by Crookenden*et al.* with little modifications[7]. RNA Bee and Directzol RNA MiniPreps were utilized to abstract total RNA. Then transcriptor reverse transcriptase enzyme was adopted to conduct the reverse transcription. SYBR Premix Ex Taq was applied for qPCR. The adoption of ΔΔCt method was to calculate expression level. Primers used are:

IL-6:

Forward: CAGAGCTGTGCAGATGAGTACA
Reverse: GATGAGTTGTCATGTCCTGCAG

TNF-α:

Forward: GACGTGGAAGTGGCAGAAGAG
Reverse: TGCCACAAGCAGGAATGAGA

GAPDH:

Forward: TTCCAGGAGCGAGATCCCT
Reverse: CACCCATGACGAACATGGG

**Alkaline comet assay**

Cells were inoculated into 6 well plates at $1 \times 10^5$ cells per well. According to the protocols of suppliers, the Comet Assay Kit (Abcam, USA) was adopted to conduct alkaline comet assay. Simply put, after exposing to 100 μM IS for 24 h, SW480 cells were gathered suspended in low melt agarose (LM agarose). They were plated on slides in duplicate and then slides were soaked in lysis solution. After that, they were put in Comet Assay ES Tank for alkaline electrophoresis at least half an hour. Following, slides were subjected to fix and dry for a whole night. Then SYBR Gold was adopted to stain and M.C. Phillips was taken to photograph. At least 100 cells were picked out randomly for analysis and tail moment was utilized to present experimental data.

**Statistics**

Mean ± SD was utilized to express data. Shapiro-Wilk test was adopted to estimate normality. For the data confirmed as a parameter, the statistical significance was estimated by student *t*-test. With regard of the comparison of two means, ANOVA was used. For comparing more than two methods, Dunnett's test was utilized. The whole analyses were conducted in GraphPad Prism 7. *P* values were deemed statistically significant when it was less than 0.05.

## Results

**Free-radical scavenger blockades NF-κB activation**

In order to investigate the mechanisms of NF-κB that could induce inflammation, SW480 cells were used. Cells were subjected to transfection by NF-κB reporter plasmid, and it indicated that firefly luciferase was controlled by the transcription of NF-κB. The negative control was utilized to transfect cells for one day. After that, cells were subjected to exposure in IS or Vehicle for at least 3 h. As expected, luciferase activity was evidently increased in cells which were performed with IS at the dose of 0.01 μM, 0.09 μM and 0.17 μM in the contrast of the vehicle control (Fig. 1). For the sake of investigating if the existence of free radicals was associated with activating NF-κB in intestinal epithelium, the experiment was performed. First, N-acetyl cysteine, which served as a free radical scavenger, was picked out for pre-treating SW480 cells for 1 h. NAC was reported that it could effectually prevent oxidative damage. After cells were pre-treated by utilizing NAC, they were subjected to exposure in IS. NAC pre-treatment weakened the IS increase under the situation of activating NF-κB, and it prominently declined the rise of luciferase activity in cells (Fig. 1). It demonstrated that NAC could effectually prevent oxidative damage from IS in SW480 and free radicals

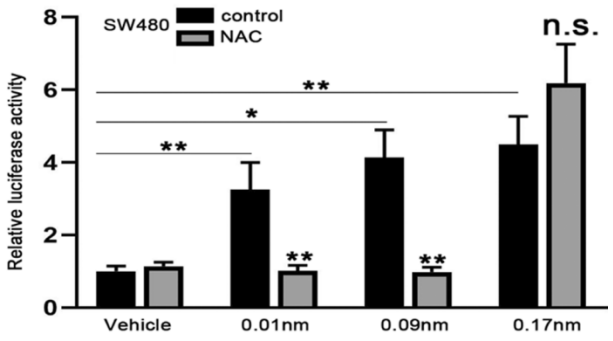


Fig. 1 — Free-radical scavenger blockades NF-κB activation. Luciferase reporter was utilized to test the luciferase activity of SW480 under dissimilar IS consistence

was the crucial drive for inflammatory effect of IS on intestinal epithelial cell and 0.09 μM IS was best for experiments.

**The effect of IS on the expression of inflammatory cytokines**

In order to test whether inflammatory cytokines could be affected by IS. We conducted RT-qPCR experiment. First of all, SW480 cells were subjected to exposure in IS or vehicle at least 3 h. Then RNA was collected and RT-qPCR was carried out for IL-6 and TNF-α. We discovered that the expressions of inflammatory cytokines were evidently increased in the comparison of vehicle, demonstrating that IS could stimulate the expression of inflammatory cytokines (Fig. 2A &B).

**IS causes genotoxic damage**

Although NF-κB could be activated *via* assorted pathways, our experimental data indicated that free radicals are responsible in activating NF-κB in the presence of IS. Hence, we envisioned that the genotoxic effect of IS may be associated with activation of NF-κB. To show that IS causes genotoxicity and damages DNA, we carried out the alkaline comet experiment (Fig. 3A). Cells were subjected to exposure in the control or IS. Then cells were collected and taken to make the assessment of DNA damage. We discovered that the tail moment was evidently increased in cell which were treated with IS in the comparison of the control. As shown in (Fig. 3B), the gap of tail moment was extremely significant.

**IS activates NF-κB by ATM/NEMO genotoxic response pathway**

In order to test whether NF-κB activation could be driven through genotoxic response pathway, we conducted experiments. Firstly, DNA damage led to phosphorylation of ATM. And NEMO was phosphorylated by ATM. After that, NEMO's responsibility was to activate NF-κB. Utilizing the identical exposure project which was previously described, proteins were abstracted from SW480 cells which were exposed to IS and Vehicle before. The outcomes of western blots indicated that the phosphorylated ATM was increased after exposing to IS (Fig. 4A). Following, NEMO that was phosphorylated was also subjected to measurement and we discovered that it was ascended in cells treated with IS (Fig. 4A). On the basis of these experimental outcomes, we found that ATM and NEMO were subjected to activation by IS, demonstrated that they were two crucial portions of genotoxic response pathway. In the end, for the sake of detecting that NF-κB could be activated by the activated ATM/NEMO *via* IS, we inhibited ATM by utilizing KU-55933. Then NF-κB reporter experiment was carried

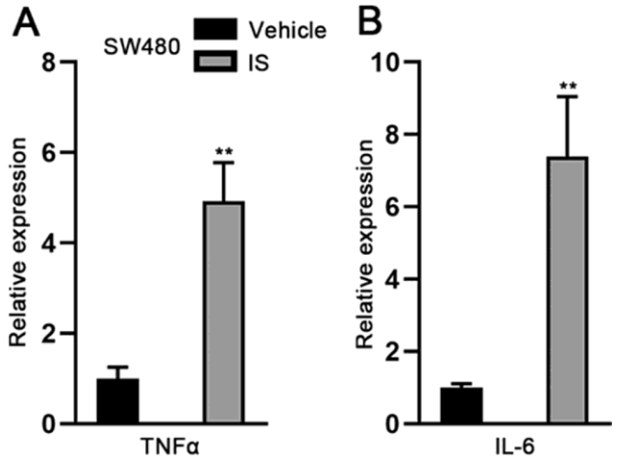


Fig. 2 — The effect of IS on the expression of inflammatory cytokines. (A & B) RT-qPCR was adopted to test the expression of TNF-α and IL-6 in SW480 cells

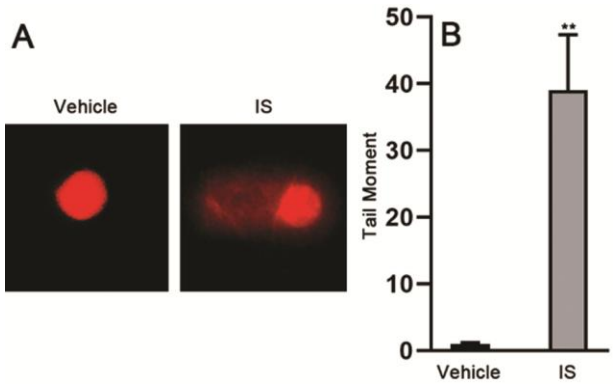


Fig. 3 — IS causes genotoxic damage. (A) Alkaline comet experiment was conducted to estimate genotoxicity; and (B) Tail Moment came from comet assay. Scale used for image was 25 μM

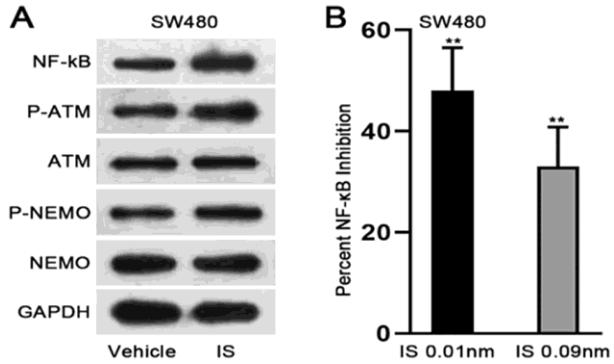


Fig. 4 — IS activates NF-κB by ATM/NEMO genotoxic response pathway. (A) Proteins were detected by western blots. Repeat each experiment three times; and (B) KU-55833 was utilized to pre-treat cells which were transfected by NF-κB plasmid. Following exposed to IS for 3 h

out. KU-55933 was adopted to pre-treat cells at least 1 h, and then cells were treated with the previous exposure project. We discovered that inhibiting ATM evidently restrained NF-κB activation in dissimilar IS consistence (Fig. 4B). All of the experimental outcomes demonstrated that IS could activate NF-κB by genotoxic response pathway using ATM/NEMO. And the activation could cause inflammation.

## Discussion

In this current study, it was demonstrated that IS could cause inflammation in intestinal epithelium and this confirms reports of earlier studies[8-11]. Result also proved that IS could activate ATM and NEMO, so as to activate NF-κB, and the inflammatory effect of IS requires the activation[12-13].

Intestinal inflammation may hurt mucosal immune system. Cells which were pre-treated with free radical scavenger and NAC showed reduced NF-κB activation, implying that free radicals are important for activating NF-κB that is mediated by IS. Actually, it has been reported that IS could produce free radicals, but further studies are required to validate this claim. Supplementing antioxidant may be the treatment direction against IS induced genotoxicity and inflammation.Previous studies have shown the protective role of antioxidants in wound healing and inflammation[14-15].

NF-κB is crucial for cell survival, but it is also driving the development of inflammation[14]. At the same time, IL-6 and TNF-α which serves as inflammatory cytokines were increased by IS[15]. Cytokines' transcription was mainly driven by NF-κB. After it was inhibited, the overexpression of IL-6 and TNF-α was subjected to amelioration. The overexpressed cytokines had been proved that they could associate with the outbreak of assorted inflammatory diseases. Meanwhile, inflammation which was mediated by NF-κB had been proved that it may associate with inflammatory bowel disease. The research uniquely investigates the activation of NF-κB that was induced by IS. This study tried to identify one way to study the correlation of IS and inflammation. The functions of IS may involve assorted mechanisms and various types of transcription factor.

This research further showed that IS could cause DNA damage and drive inflammation in the intestinal epithelium. Meanwhile, our outcomes demonstrated that NF-κB served as transcription factor to drive inflammation and it could be activated by ATM and NEMO, offering a new insight for curing IS-induced inflammation. Overall, uremia toxin could help to induce inflammation in intestines by activating the ATM/NEMO/NF-κB signalling pathway in human intestinal epithelial cells.

## Conclusion

Concluding, IS led to increased production of inflammatory cytokines and induced inflammation in intestinal epithelial cells *via* activating ATM/NEMO/ NF-κB signalling pathway. However, free radical scavengers are useful in reducing the NF-κB activation, which may be exploited in managing the disease condition

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## Conflicts of interest

All authors declare no conflict of interest.

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