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Influence of roflumilast on sepsis mice through the JAK/STAT signaling pathway

X. CHANG[1], L.-F. HU[1], X.-J. MA[1], J. YIN[1], X.-Y. LIU[2], J.-B. LI[1]

[1]Department of Infectious Diseases, the First Affiliated Hospital of Anhui Medical University, Hefei, China.
[2]Anhui Medical University, Hefei, China

Abstract. – OBJECTIVE: The aim of this study was to explore the influence of roflumilast on sepsis mice through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway.

MATERIALS AND METHODS: A total of 36 Sprague-Dawley mice were randomly divided into normal group (n=12), model group (n=12) and roflumilast group (n=12). Mice in the normal group were fed normally. However, mice in the model group and roflumilast group were intraperitoneally injected with endotoxin to establish the sepsis mouse model. Furthermore, mice in the model group and roflumilast group were intraperitoneally injected with 0.9% sodium chloride and roflumilast once a day, respectively. After 7 d of intervention, mice were sampled. Lung tissue morphology was observed via hematoxylin-eosin (HE) staining, and the pathological score was given. The protein expression levels of JAK and STAT-3 were detected via Western blotting. The expression levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were detected *via* enzyme-linked immunosorbent assay (ELISA). Meanwhile, the mRNA expression levels of JAK, STAT-3, IL-6 and TNF-α were detected via quantitative Polymerase Chain Reaction (qPCR). The number of inflammatory cells in the lavage fluid was counted by a biochemical detector.

RESULTS: The survival rate of mice in the roflumilast group was significantly higher than that of the model group ($p<0.05$). The results of HE staining revealed that lung tissue morphology in roflumilast group was significantly improved when compared with the model group. Meanwhile, the pathological score in the roflumilast group was significantly lower than that of the model group ($p<0.05$). Western blotting showed that the protein expression levels of JAK and STAT-3 in the roflumilast group were markedly lower than those of the model group ($p<0.05$). According to the results of ELISA, the expression levels of IL-6 and TNF-α in the roflumilast group were remarkably lower than the model group ($p<0.05$). Further qPCR results manifested that the mRNA expression levels of JAK, STAT-3, IL-6 and TNF-α in the roflumilast group were significantly lower than those of the model group ($p<0.05$). Moreover, the number of neutrophils, monocytes and lymphocytes in the roflumilast group was significantly smaller than the model group.

CONCLUSIONS: Roflumilast can improve lung tissue morphology of sepsis mice by inhibiting the JAK/STAT signaling pathway.

Key Words
Sepsis, Inflammation, Roflumilast, JAK, STAT.

Introduction

Sepsis is a common critical disease in clinic. It is also a kind of systemic inflammatory response caused by infection and other injuries. Further development of sepsis will lead to multiple organ failure, shock, other pathological reactions and even death[1,2]. Therefore, sepsis has attracted worldwide attention from clinical researchers due to its high morbidity and mortality rates.

Studies have demonstrated that systemic inflammation is the major pathological reaction of sepsis. Meanwhile, systemic inflammation is also the main cause of multiple organ failure, shock and other secondary pathological injuries in sepsis patients. It has been demonstrated that systemic inflammation plays an important role in the pathogenesis of sepsis[3,4]. The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is an important cellular signal transduction pathway. It regulates various cellular physiological processes, including proliferation, differentiation, apoptosis and death[5]. Further studies have found that the JAK/STATA signaling pathway is involved in the regulation of inflammatory response in sepsis. After sepsis occurs, JAK and STAT3 in the JAK/STAT signaling pathway are activated to regulate the expression

Corresponding Author: Jiabin Li, Ph.D; e-mail: lijiabin@ahmu.edu.cn

of inflammatory factors [interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)]. This may eventually aggravate inflammation and lead to secondary pathological reactions of sepsis[6].

Roflumilast, as a new-generation phosphodiesterase-4 inhibitor, possesses a potent anti-inflammatory effect. Therefore, it has been widely applied in clinic[7]. However, the mechanism of its anti-inflammatory effect remains unclear. Therefore, the aim of this study was to clarify the underlying mechanism of roflumilast in the treatment of sepsis. Furthermore, we also explored whether it could inhibit inflammation and exert a better therapeutic effect on sepsis mice by regulating the JAK/STAT signaling pathway.

Materials and Methods

Experimental Animals and Grouping

36 Kunming mice (18 male and 18 female) weighing (18±2) g were purchased from the Shanghai SLAC Laboratory Animals Co., Ltd. (Shanghai, China). All mice were randomly divided into normal group (n=12), model group (n=12) and roflumilast group (n=12) using a random number table. This investigation was approved by the Animal Ethics Committee of Anhui Medical University Animal Center.

Experimental Reagents and Instruments

Anti-STAT-3 (Abcam, Cambridge, MA, USA) and anti-JAK primary antibodies (Abcam, Cambridge, MA, USA); AceQ quantitative polymerase chain reaction (qPCR) SYBR Green Master Mix kit (Vazyme, Nanjing, China); HiScript II Q RT SμperMix for qPCR (+gDNA wiper) kit (Vazyme, Nanjing, China); hematoxylin-eosin (HE) staining kit and enzyme-linked immunosorbent assay (ELISA) kit (Sigma-Aldrich, St. Louis, MO, USA); optical microscope (Leica DMI 4000B/DFC425C, Munich, Germany); and fluorescence qPCR instrument (ABI 7500, Applied Biosystems, Foster City, CA, USA).

Methods

Modeling

Mice were intraperitoneally injected with 7% chloral hydrate (5 mL/kg). After successful anesthesia, mice were treated with intraperitoneal injection of lipopolysaccharide (6 mg/kg) to establish the sepsis mouse model.

Treatment in Each Group

In the normal group, mice were fed normally without any treatment. In the model group, mice were intraperitoneally injected with normal saline once a day after the establishment of the sepsis model. In the roflumilast group, mice were intraperitoneally injected with roflumilast solution (1 mg/kg) once a day after the sepsis model was established. After 7 d of intervention, the mortality rate of mice in each group was detected.

Sampling

After successful anesthesia, bronchoalveolar lavage fluid was collected from each mouse and stored for subsequent experiments. 6 mice in each group were then perfused with paraformaldehyde. Next, the lung tissues were taken, fixed and sliced into paraffin-embedded tissue sections. The remaining 6 mice in each group were sampled directly, and the lung tissues were taken and stored for subsequent use.

HE Staining

After deparaffinization and rehydration, paraffin-embedded tissue sections were stained according to the instructions of the HE staining kit. Then, the sections were sealed with neutral balsam and photographed. The pathological score was given according to HE staining status.

Western Blotting

The tissues were added with lysis buffer, followed by ice bath for 60 min and centrifugation. The concentration of extracted protein was quantified using the bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA). After denaturation, the extracted protein sample was separated *via* gel electrophoresis and transferred onto membranes. After washing and sealing with blocking buffer for 1.5 h, the membranes were incubated with primary antibodies of anti-TLR-2 (1:1000) and anti-NF-κB (1:1000) overnight. Then the membranes were washed with Tris-Buffered Saline with Tween-20 (TBST), and the image was developed. The membranes were placed in chemiluminescence reagent for reaction for 1 min, followed by image development in the dark. Finally, the analysis was performed using a gel scanning imaging system.

ELISA

Stored lung tissues were ground, and the expression level of TNF-α in lung tissues was detected according to the instructions of the ELISA kit.

QPCR

Total RNA was extracted and reversely transcribed into cDNA using the reverse transcription kit (Vazyme, Nanjing, China). The reaction system was 20 μL. Specific reaction conditions were as follows: reaction at 51°C for 2 min, pre-denaturation at 96°C for 10 min, denaturation at 96°C for 10 s, annealing at 60°C for 30 s, for a total of 40 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The relative expression levels of mRNAs were calculated. The primer sequences were shown in Table I.

Detection of Lavage Fluid Cells

The lavage fluid stored was detected using a biochemical detector, and the number of inflammatory cells in the lavage fluid was counted.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 software (IBM, Armonk, NY, USA) was used for all statistical analyses. Enumeration data were expressed as mean ± standard deviation. The *t*-test was used for data in line with normal distribution and homogeneity of variance. A corrected *t*-test was used for data in line with normal distribution and heterogeneity of variance and a non-parametric test was performed for data not in line with normal distribution and homogeneity of variance. One-way ANOVA was applied to compare the differences among different groups, followed by Post-Hoc Test (Least Significant Difference). Rank sum test was adopted for ranked data, and chi-square test was adopted for enumeration data. *p*-values <0.05 were considered statistically significant.

Results

HE Staining

In the normal group, the tracheal wall structure was complete and normal. Meanwhile, the tissues were distributed orderly without congestion, edema and significant inflammatory cell infiltration. In the model group, the tracheal wall structure was incomplete and the alveolar space was wide. There were vascular congestion and edema, as well as significant inflammatory cell infiltration. In the roflumilast group, the tracheal wall structure was damaged to a certain extent with mild inflammatory cell infiltration. However, the tracheal wall structure was significantly improved compared with that of the model group (Figure 1). Subsequently, the pathological score was given for lung tissue morphology in each group. The results revealed that the pathological scores in the model and roflumilast groups were significantly higher than that of the normal group ($p<0.05$). However, the pathological score in the roflumilast group was markedly lower than that of the model group ($p<0.05$) (Figure 2).

Expression Levels of Related Proteins Detected Via Western Blotting

Western blotting indicated that the protein expression levels of JAK and STAT-3 were significantly lower in the normal group, whereas were markedly higher in the model group (Figure 3). The statistical analysis results were shown in Figure 4. Meanwhile, the protein expression levels of JAK and STAT-3 in model and roflumilast groups were significantly increased when compared with those of the normal group ($p<0.05$). However, the protein expression levels of JAK and STAT-3 were significantly declined in the roflumilast group when compared with the model group ($p<0.05$).

Table I. Primer sequences.

Name	Primer sequence
JAK	Forward: 5'-TCCACCAAGAAGCTGAGCGAG-3' Reverse: 5'-GTCCAGCCCATGATGGTTCT-3'
STAT-3	Forward: 5'-TTCTTTGAGTTCGGTGGGGTC-3' Reverse: 5'-TGCATATTTGTTTGGGGCAGG-3'
TNF-α	Forward: 5'-ATGAGCACTGAAAGCATGATC-3' Reverse: 5'-TCACAGGGCAATGATCCCAAAGTAGACCTGCCC-3'
IL-6	Forward: 5'-TGTCTTCCTCACCGATTCCT-3' Reverse: 5'-ACCACCCGAGCTCTGTCTTACTC-3'
GAPDH	Forward: 5'-ACGGCAAGTTCAACGGCACAG-3' Reverse: 5'-GAAGACGCCAGTAGACTCCACGAC-3'

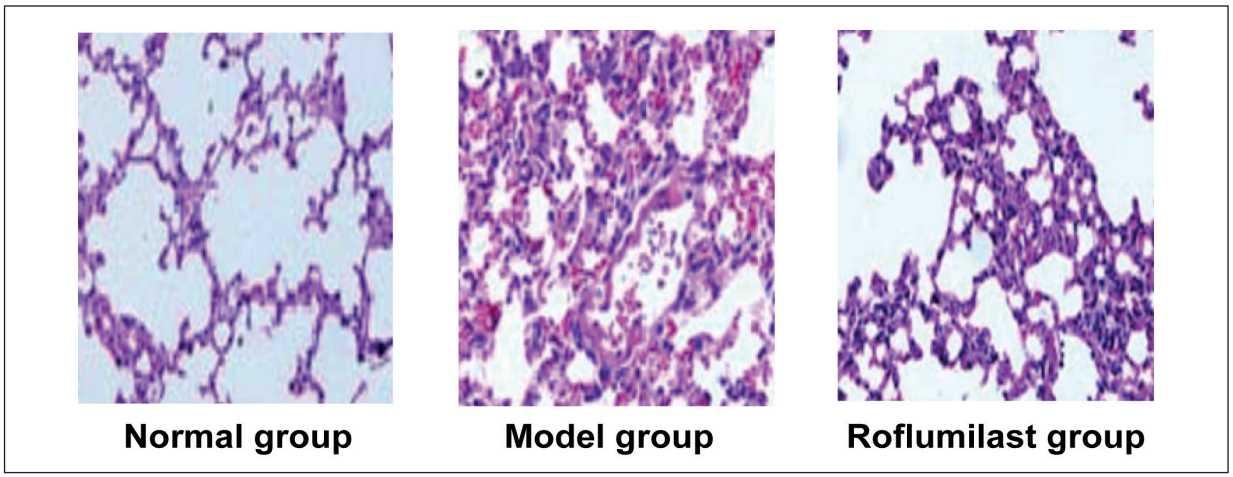

Figure 1. Figure 1. Observation of lung tissue morphology in each group via HE staining (×200).

Expression Levels of Related mRNAs Detected Via qPCR

Compared with the normal group, the mRNA expression levels of JAK, STAT-3, TNF-α and IL-6 in model and roflumilast groups were markedly increased (p<0.05). However, the mRNA expression levels of JAK, STAT-3, TNF-α and IL-6 in the roflumilast group were significantly decreased when compared with the model group (p<0.05) (Figure 5).

ELISA

ELISA results demonstrated that the expression levels of TNF-α and IL-6 were markedly lower in the normal group, whereas were remarkably higher in the model group. Compared with the normal group, the expression levels of TNF-α and IL-6 in the model and roflumilast groups were significantly increased (p<0.05). However, the expression levels of the above two molecules were markedly declined in the roflumilast group than the model group (p<0.05) (Figure 6).

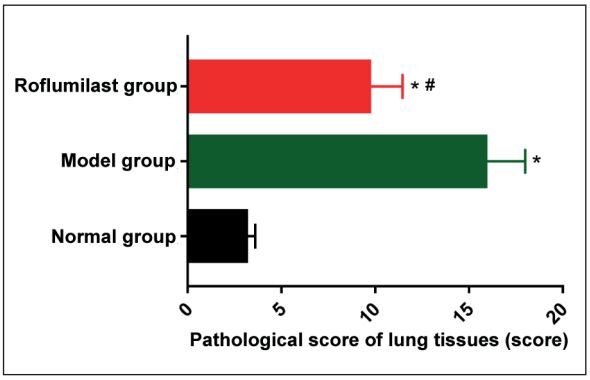

Figure 2. Pathological score of lung tissues in each group. Note: p*<0.05 vs. normal group, p#<0.05 vs. model group.

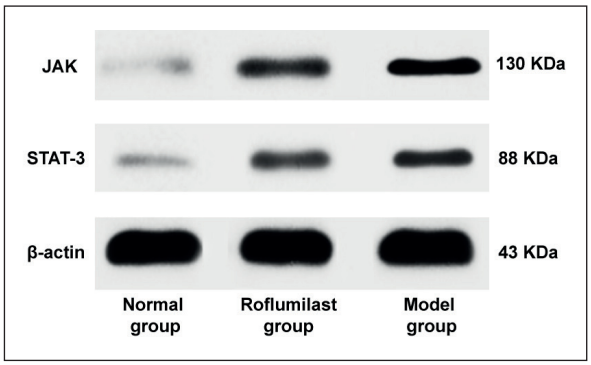

Figure 3. Expression of related proteins detected via Western blotting.

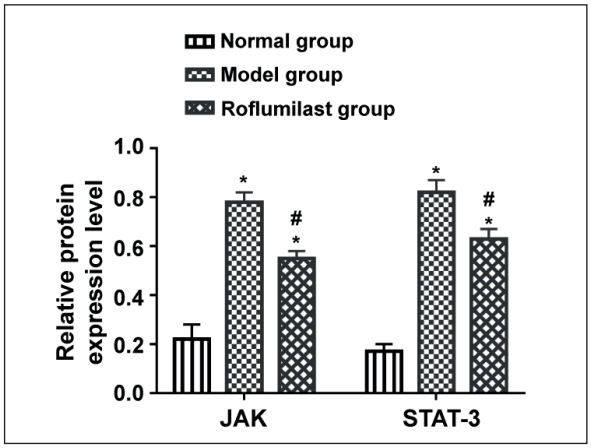

Figure 4. Relative protein expression level. Note: p*<0.05 vs. normal group, $p^{\#}$<0.05 vs. model group.

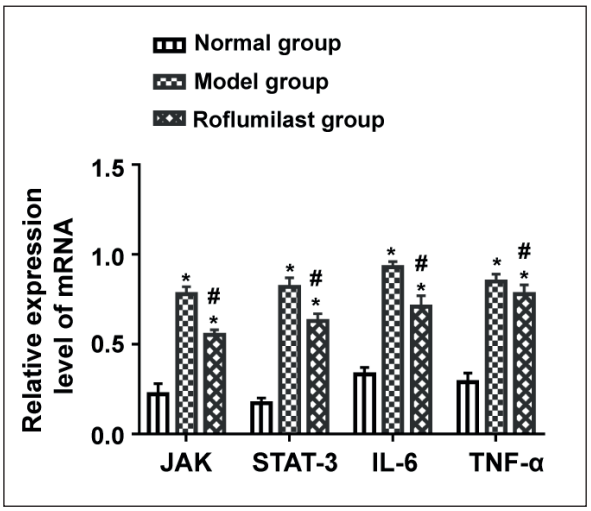

Figure 5. Relative mRNA expression level. Note: $p^*<0.05$ vs. normal group, $p^{\#}<0.05$ vs. model group.

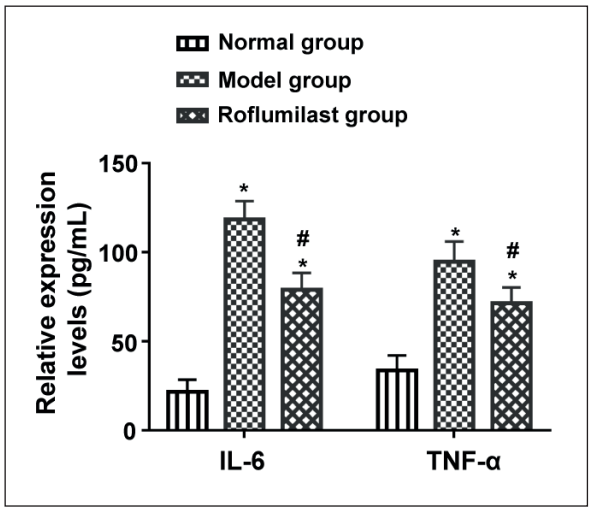

Figure 6. Relative expression levels of IL-6 and TNF-α detected via ELISA. Note: $p^*<0.05$ vs. normal group, $p^{\#}<0.05$ vs. model group.

Detection of Lavage Fluid Cells

The total counts of limphocytes, neutrophils and monocytes in the model and roflumilast groups were significantly increased when compared with those of the normal group ($p<0.05$). However, they were markedly decreased in the roflumilast group than the model group ($p<0.05$) (Figure 7).

Discussion

Sepsis is a systemic inflammatory disease caused by microbial invasion-induced infection. Its nature and pathological changes are inseparable from inflammatory response[8,9]. Sepsis is often accompanied by critical complications, such as multiple organ failure and shock. This can even lead to death in patients. Currently, the incidence of sepsis is on the rise, and the number of patients with sepsis can reach 19 million every year. Millions of sepsis patients will die from the disease. At the same time, the expenses of treatment for sepsis are extremely high. About 24 billion dollars are spent on the treatment of sepsis every year in the USA, bringing heavy economic burden to the patients' families and society. Therefore, it is of great significance to study the pathogenesis of sepsis and related therapeutic measures. After the onset of sepsis, a large number of early inflammatory factors, including IL-1, IL-6, and TNF-α, are released. It can activate a variety of cellular signal transduction pathways, including cellular signaling pathways (JAK/STAT signaling pathway). Meanwhile, it mediates the post-inflammatory cascade, thus forming a vicious circle of inflammation-inflammation pathological reaction, and amplifying inflammation. Eventually, uncontrolled inflammation, and even shock and multiple organ failure occurs[10,11]. The JAK/STAT signaling pathway is a key cellular signal transduction pathway in sepsis. It can mediate the transcription and expression of various inflammatory factors during sepsis[12,13]. There are many members in the JAK family, including JAK1, JAK2, and JAK3, all of which have different conserved regions. Currently, it is well known that two

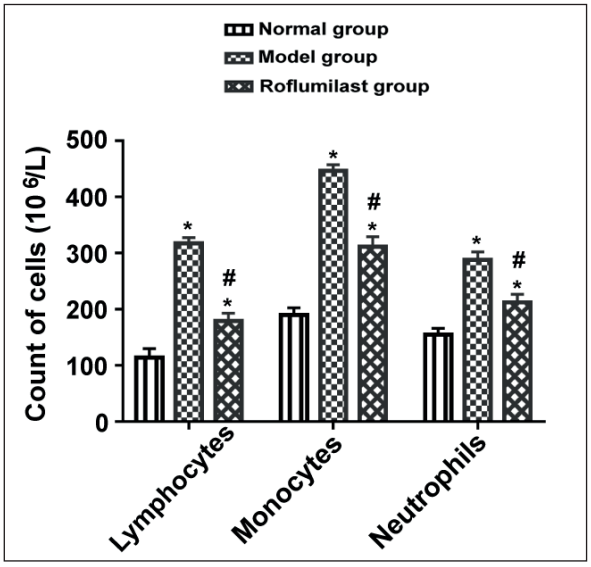

Figure 7. Number of lavage fluid cells. Note: $p^*<0.05$ vs. normal group, $p^{\#}<0.05$ vs. model group.

conserved regions of JAK are the most important, namely enzyme-activated structural region and pseudo-enzyme-activated structural region. The enzyme-activated structural region possesses the function of activating enzymes. However, the pseudo-enzyme-activated structural region possesses no related effect. Under the induction of cytokines, two kinds of JAK or multiple JAKs in the same kind are needed and activated. This may activate the signaling pathway and play an important role in signal transduction. STAT-3, an important member of the STAT family, is closely related to inflammation. It also exerts an important regulatory effect on IL-6 and TNF-α that play crucial roles in the pathological process of sepsis[14,15]. When sepsis occurs, inflammatory factors such as IL-6 and TNF-α released by early inflammation in the body may bind to STAT-3. As a result, STAT-3 is activated, and its ability to transcribe IL-6 and TNF-α is enhanced. Eventually, this can deliver inflammatory signals, mediate and exacerbate inflammation. A large number of resulting inflammatory factors can further activate the JAK/STAT-3 signaling pathway in turn, thereby producing a positive feedback signal loop. This may exacerbate inflammation, and ultimately lead to uncontrolled inflammation and systemic inflammation[16-19]. Therefore, effectively inhibiting inflammation is the key to the treatment of sepsis. In this study, the results proved that the JAK/STAT signaling pathway was highly expressed in sepsis mice. Meanwhile, the protein and mRNA expressions of JAK and STAT-3 were significantly up-regulated in the pathological process of sepsis. At the same time, the transcription and expression of inflammatory factors (IL-6 and TNF-α) were also increased. This indicated that the JAK/STAT signaling pathway was involved in the occurrence of sepsis and regulation of inflammation. It has been proved that roflumilast, a phosphodiesterase-4 inhibitor, can alter allergic reaction and inflammation, thereby promoting tissue injury repair[20,21]. In particular, recent studies have confirmed that roflumilast possesses good anti-inflammatory effect and immune-enhancing effect on chronic obstructive pneumonia, thus inhibiting chronic inflammation and promoting airway remodeling. According to clinical evidence-based medical researches, roflumilast can significantly improve many symptoms in patients with chronic obstructive pneumonia. This includes: improvement in forced expiratory volume in one second before and after relaxation, peak expiratory flow rate and forced vital capacity. Moreover, studies have also proved that the important mechanism of roflumilast in the above-mentioned roles is related to its regulation of extracellular regulated protein kinases and stress-activated protein kinases. However, the influence of roflumilast on the JAK/STAT signaling pathway remains unclear. In the present study, our findings revealed that the roflumilast intervention was conducive to improving lung tissue morphology of sepsis mice, inhibiting the protein and mRNA expressions of JAK and STAT-3, and suppressing inflammatory factors of IL-6 and TNF-α.

Conclusions

We found that roflumilast inhibited the JAK/STAT signaling pathway after the onset of sepsis, thereby inhibiting inflammation.

Conflict of Interests

The authors declare that they have no conflict of interest.

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