Original Article

The Effect of Aqueous Extract of *Malva neglecta* on Expression of Inflammatory Biomarkers Involved in Pain in Synoviocytes and THP-1 Cells as a Model of Monocyte/Macrophage and Human Cartilage Cells in Osteoarthritis

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Abstract

Background: Osteoarthritis is now considered as an active complex of biomechanical, biochemical and cellular processes, not a mere degenerative disorder. Considering the complications of common treatments of osteoarthritis, including non-steroidal anti-inflammatory drug (NSAIDs) and corticosteroids, establishment of new treatments is crucial. This study aimed to explore the effect of *Malva neglecta* extract on the main inflammatory biomarkers in osteoarthritis.

Materials and Methods: Aqueous extract of *Malva neglecta*, ibuprofen and betamethasone were prepared to investigate their effects on inflammatory biomarkers. Synoviocytes were obtained from the healthy radiocarpal joint cartilage of an 8-month-old Holstein cow. Human monocyte/macrophage (THP-1) cells were also obtained to investigate the effect of *Malva neglecta* extract on inflammatory agents. Lipopolysaccharide (LPS) was used to induce production of inflammatory cytokines in both cells. Real-time PCR was used to investigate the effect of *Malva neglecta* extract on expression profile of TNF-α, IL-1β, COX-2, IL-18 and iNOS. Production of NO and PGE2 was also investigated in THP-1 cells.

Results: *Malva neglecta* extract reduced TNF-α, IL-1β, iNOS, IL-18 and COX-2 expression in synoviocytes. Expression of all of these factors was also reduced by the extract in THP-1 cells. Moreover, production of PGE2 and NO in the LPS-induced THP-1 cells was reduced by Malva neglecta extract. Ibuprofen and betamethasone were more effective in reducing inflammatory agents than the extract.

Conclusion: According to Malva's ability to reduce the pro-inflammatory cytokines in the synoviocytes and THP-1 cells, its potential role as a supplement method to common NSAIDs and corticosteroids was confirmed.

Keywords: Osteoarthritis, *Malva neglecta*, synoviocyte, monocyte/macrophage, cytokine

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Introduction

Osteoarthritis (OA) is one of the most common joint diseases worldwide and has a strong association with aging. Based on the report of Davatchi et.al, the prevalence of OA is about 16% in adults aged between 15-85 in urban areas of Iran (1). OA is beyond a mere degenerative disorder and is now considered as an active complex of biomechanical, biochemical and cellular processes (2). This complex includes activation of macrophages and synoviocytes and also synovial inflammation which is considered as the main cause of joint pain in OA (3, 4). These activated cells contribute to the release of inflammatory cytokines such as TNFα and IL-1β and also up-regulation of iNOS and COX-2 enzymes that produce NO and PGE2, respectively. Mentioned pro-inflammatory factors induce chondrocyte apoptosis and inhibit proteoglycans synthesis which leads to articular cartilage degeneration (5, 6). Although current treatments of OA, including NSAIDs and corticosteroids, can partially relieve the sign and symptoms of this disease, they are often associated with some side effects such as peptic ulcers and gastrointestinal bleeding. Because of the degree of inflammation associated with pain in patients with OA, there is an increasing trend on the plants with anti-inflammatory and analgesic properties. Compared to NSAIDs and corticosteroids, the side effects of such plants are low; hence, these plants can make a substantial contribution to the patients with osteoarthritis.

Malva neglecta (English name Common mallow) is an annual herbaceous species of genus Malva. This plant is widespread in all regions of Iran. Leaf and other parts of this plant are traditionally used in different conditions such as wound healing, stomach, menstrual aches and muscle pain and contains hydroxycinnamic acids, flavonoids, flavonols, proanthocyanidins and anthocyanins (7). Recent researches have focused on the different medical benefits of this plant. Previous studies have reported the antioxidant (7, 8), anti-ulcerogenic (9) and antibacterial activity (10) of *Malva neglecta* extract. Moreover, a recent study reported that *Malva neglecta* aqueous extract has beneficial effects on preventing and treating nephrolithiasis and decreasing tubulointerstitial damage in a dosage-dependent manner (11). Although the effect of this extract on inflammatory agents in chondrocytes was investigated before (11), its effect on synoviocytes, which their involvement in OA is recognized in recent years, is less studied. In this study, we investigated the effect of *Malva neglecta* aqueous extract on joint inflammation through analyzing expression and production of pro-inflammatory factors in synoviocytes and THP-1 cells.

Methods

Malva neglecta extract, corticosteroid and NSAID preparation

Malva neglecta was initially obtained from Iran's center of genetic resources, and then its aqueous extract was prepared according to the available protocols. Betamethasone, a corticosteroid, was applied as an aqueous solution of betamethasone 21-phosphate disodium with a concentration of 1.30 mg/mL, equivalent to betamethasone-base 1.00mg/mL. Ibuprofen, a NSAID, was dissolved in water to a concentration of 1.00 mg/mL at 100˚C for five minutes.

Synoviocyte and THP-1 cell culture

Synovial fluid was punctured from the radiocarpal joint cartilage of an 8-month-old Holstein cow and washed out three times by 1 Molar PBS buffer (pH=7.2). Then, it was incubated in collagenase type II at 37ºC for 16h. After incubation, it was filtered through 1mm Wire Strainer Screen, which was sterilized, and the wastes resulted from the effect of the collagenase type II were isolated from synoviocyte cells. The tube was centrifuged for 3 min, the supernatant was discarded and the pellet cells were washed four times with HBSS. The supernatant was removed with a pipette and finally deposited cells were incubated in the medium containing DMEM-F12 supplemented with FBS, 50 µg/ml ascorbic acid, 100 u/ml penicillin and 0.25 µg/ml streptomycin, with a density of 5×105 cell in the 22.2 cm plates at a temperature of 37ºC, the humidity of 90% and 5% CO_2 to reach cell density of 80-85%. THP-1 cells were obtained from the Pasteur Institute of Iran and were amplified in a sterile medium to the extent necessary. The next steps were accomplished completely identical and separately in the two groups of synoviocyte cells and THP-1 cells.

Cell viability assay

MTT assay and trypan blue were used to investigate the cytotoxic effect of aqueous extract of *Malva neglecta* and also the LC50 of it. MTT, a yellow tetrazole, is reduced to purple formazan by succinate dehydrogenase (SDH) in the mitochondria of living cells. To dissolve the insoluble purple formazan product into a colored solution, Dimethyl sulfoxide (DMSO) was added. The absorbance of this colored solution was measured by a spectrophotometer at a wavelength between 500 and 600 nm.

LPS treatment

To induce inflammatory cytokines, the cells were treated with LPS. 6×10^6 cells were cultured in the medium. After 72h, 100ng LPS was added to each plate. Then, the plates were divided into two group. Group one was incubated for 24h at a temperature of 37°C, the humidity of 90% and 5% CO_2 to investigate the expression of pro-inflammatory cytokines. Group two was incubated for 1h at the same conditions to investigate PGE2 and NO production.

Expression analysis

RNA was isolated and RNA concentration was determined using spectrophotometry method. DNase enzyme was used to isolate RNA. Then, isolated RNA was employed to produce cDNA using RT-PCR method. PCR was used to amplify cDNA and finally, Real-Time PCR was used to determine the expression levels of IL-18, IL-1B, TNF-α, COX-2 and iNOS genes by specific primers.

PGE2 production analysis

ELISA system was used to determine the amount of prostaglandins in the required cells. The production of nitric oxide was determined using biochemical methods.

Grouping of cell cultures

Cultured synoviocyte and THP-1 cells were divided into 5 groups and treated according to table 1.

Statistical analysis

Data are presented as mean±SD. ANOVA analysis was done by REST software version.20 and SPSS software version 20. $P<0.05$ was considered statistically significant.

Ethical considerations

No study with human or animal subjects is performed by any of the authors of this article. The ethical committee of "Research and Technology Chancellor" in Shahid Beheshti University of Medical Sciences approved this study.

Results

Real Time-PCR results were analyed using the Pfaffi or ΔΔC method,

$2^{-\Delta\Delta}$ = Expression Ratio

Ct= ΔΔC Calibrater Δ-C main cytokine Δ

Ct= (ΔΔCt main cytokine – Ct GADPH) – (Ct sample positive – Ct GADPH)

Effect of Malva neglecta extract on COX-2 gene expression in synoviocytes

The results of the expression of inflammatory factors in synoviocytes are depicted in figure 1. COX-2 expression was increased in group 3 (LPS+Cell) up to 100%.

In group 4 (LPS+Extract+Cell), COX-2 expression increased about 51% which was significantly lower than group 3. 18% and 21% upregulation of the COX-2 expression was observed in group 6 (LPS+Corticosteroid+Cell) and 7 (LPS+NSAID+Cell), respectively. Upregulation of the expression of COX-2 in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of COX-2.

Effect of Malva neglecta extract on IL-1β gene expression in synoviocytes

IL-1β expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), IL-1β expression was increased about 47% which was significantly lower than group 3. Upregulation of the IL-1β expression was about 19% in group 6 and 23% in group 7. Upregulation of the expression of IL-1β in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of IL-1β.

Effect of Malva neglecta extract on TNF-α gene expression in synoviocytes

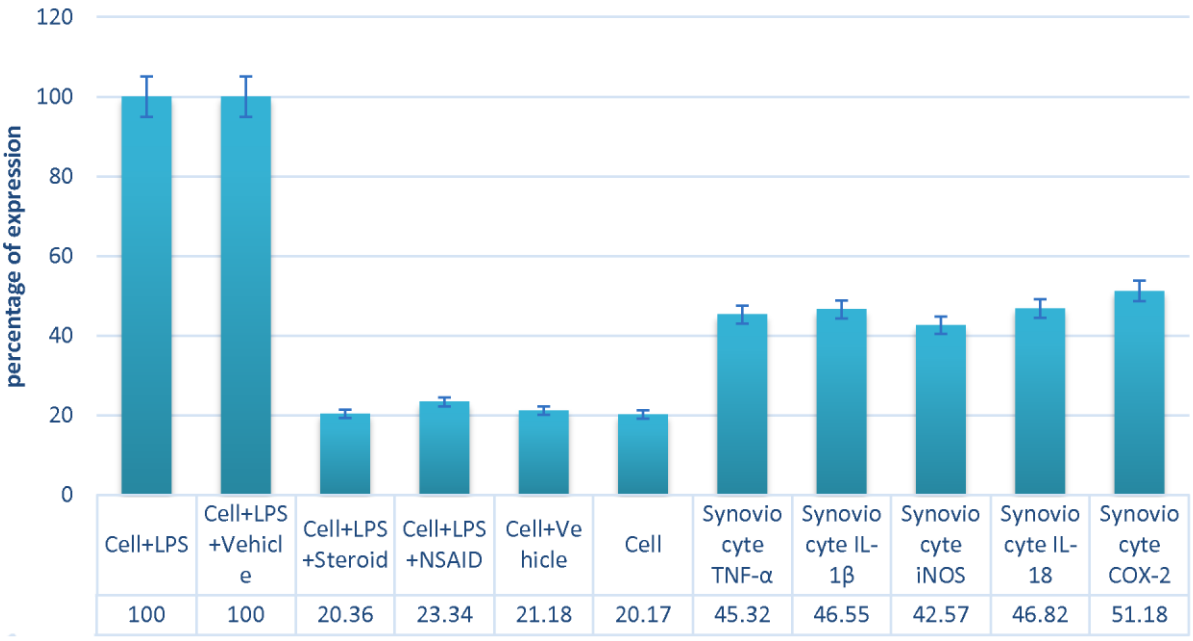

Figure 1. Relative gene expression analysis of proinflamatory cytokines in synoviocytes with Pfaffi method. The data represented as the mean±S.RE.M of three different experiments run in duplicate. The comparisons were done by one-way repeated ANOVA.

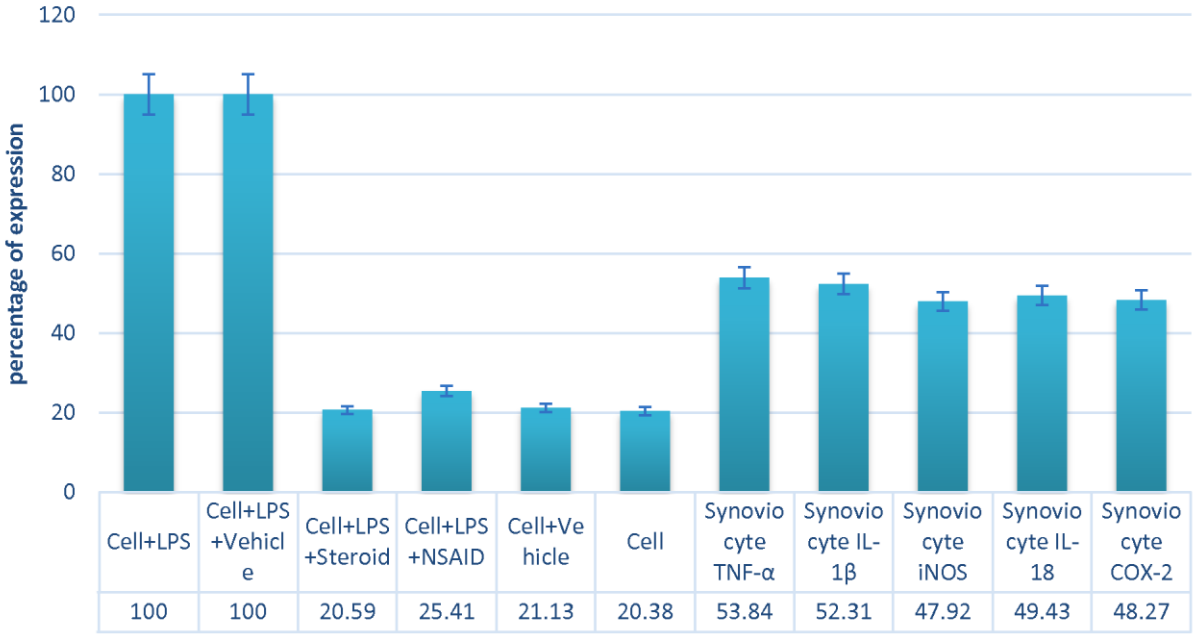

Figure 2. Relative gene expression analysis of proinflamatory cytokines in THP-1 cells with Pfaffi method. The data represented as the mean±S.RE.M of three different experiments run in duplicate. The comparisons were done by one-way repeated ANOVA.

TNF-α expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), TNF-α expression was increased about 45% which was significantly lower than group 3. Upregulation of the TNF-α expression was about 21% in group 6 and 24% in group 7. Upregulation of the expression of TNF-α in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of TNF-α.

Table 1: Studied groups.

Group	Synoviocyte/THP-1	Malva neglecta extract	Corticosteroid	NSAID	LPS	Placebo (PBS)
1	+	-	-	-	-	-
2	+	+	-	-	-	-
3	+	-	-	-	+	-
4	+	+	-	-	+	-
5	+	-	-	-	+	+
6	+	-	+	-	+	-
7	+	-	-	+	+	-

Studied groups according to receiving different treatments. NSAID: non-steroidal anti-inflammatory drug, LPS: Lipopolysaccharide, PBS: Phosphate-buffered saline.

Table 2: Production of prostaglandin E2 in THP-1 cells.

Group	Logit (%B/B0)	Picogram/ml PGE2
1	1.1	4
2	1.15	4.1
3	2.2	11.8
4	1.9	8.5
5	2.2	11.8
6	1.3	5.5
7	1.5	6.2

Production of prostaglandin E2 in THP-1 cells. PGE2: Prostaglandin E2.

Table 3: Production of nitric oxide in THP-1 cells.

Group	Optical density	Nanomol nitrite
1	0.5	800
2	0.7	1000
3	2.4	2500
4	1.4	1500
5	2.4	2500
6	0.8	1200
7	1.0	1100

Effect of *Malva neglecta* extract on iNOS gene expression in synoviocytes

iNOS expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), iNOS expression was increased about 43% which was significantly lower than group 3. Upregulation of the iNOS expression was about 19% in group 6 and 23% in group 7. Upregulation of the expression of iNOS in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of iNOS.

Effect of *Malva neglecta* extract on IL-18 gene expression in synoviocytes

IL-18 expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), IL-18 expression was increased about 47% which was significantly lower than group 3. Upregulation of the IL-18 expression was about 22% in group 6 and 25% in group 7. Upregulation of the expression of IL-18 in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of IL-18.

Effect of *Malva neglecta* extract on COX-2 gene expression in THP-1 cells

The results of the expression of inflammatory factors in THP-1 cells are depicted in figure 2. COX-2 expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), COX-2

expression increased about 48% which was significantly lower than group 3. 17% and 19% upregulation of the COX-2 expression was observed in group 6 and 7, respectively. Upregulation of the expression of COX-2 in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of COX-2.

Effect of *Malva neglecta* extract on IL-1β gene expression in THP-1 cells

IL-1β expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), IL-1β expression was increased about 52% which was significantly lower than group 3. Upregulation of the IL-1β expression was about 21% in group 6 and 26% in group 7. Upregulation of the expression of IL-1β in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of IL-1β.

Effect of *Malva neglecta* extract on TNF-α gene Expression in THP-1 cells

Although *Malva neglecta* extract did not change TNF-α expression in group 2, it increased TNF-α expression in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), TNF-α expression was increased about 54% which was significantly lower than group 3. Upregulation of the TNF-α expression was about 22% in group 6 and 27% in group 7. Upregulation of the expression of TNF-α in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of TNF-α

Effect of *Malva neglecta* extract on iNOS gene expression in THP-1 cells

iNOS expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), iNOS expression was increased about 48% which was significantly lower than group 3. Upregulation of the iNOS expression was about 19% in group 6 and 26% in group 7. Upregulation of the expression of iNOS in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of iNOS.

Effect of *Malva neglecta* extract on IL-18 gene expression in THP-1 cells

IL-18 expression was increased in group 3 (LPS+Cell) up to 100%. In group 4 (LPS+Extract+Cell), IL-18 expression was increased about 49% which was significantly lower than group 3. Upregulation of the IL-18 expression was about 20% in group 6 and 27% in group 7. Upregulation of the expression of IL-18 in group 4 was significantly higher than groups 6 and 7. In group 5, the placebo was administrated and no change was observed in the expression of IL-18.

Effect of *Malva neglecta* extract on PGE2 production in THP-1 cells

Production of PGE2 was assessed using ELIZA method, then a standard curve was drawn using different concentrations of PGE2 and the equation proposed by Invitrogen Corporation. Based on the results of this analysis, PGE2 production was significantly higher in groups 3 and 5 compared to other groups (table 2). Production of PGE2 was significantly reduced in group 4 (LPS+Extract+Cell) compared to group 3 about 28%. The reduction in PGE2 production was 53% and 47% in groups 6 and 7 respectively which was significantly higher than the reduction observed in group 4.

Effect of *Malva neglecta* extract on NO production in THP-1 cells

Production of NO was assessed using spectrophotometry, then a standard curve was drawn using different concentrations of standard nitric oxide. Production of NO was significantly higher in groups 3 and 5 compared to other groups (Table 3). Production of NO was significantly reduced in group 4 (LPS+Extract+Cell) compared to group 3 about 40%. The reduction in NO production was 52% and 56% in groups 6 and 7 respectively which was significantly higher than the reduction observed in group 4.

Discussion

According to our knowledge, this is the first study investigating the effects of *Malva neglecta* extract on reducing pro-inflammatory cytokines in synoviocytes. We showed that *Malva neglecta* extract reduces TNF-α gene expression in both LPS-induced THP-1 cells and synoviocytes. Moreover, it can reduce IL-1β, COX-2, IL-18 and iNOS genes

expression in synoviocyte cells. These results further confirm the effect of *Malva neglecta* on the reduction of pain and inflammation in OA (12). However, compared to ibuprofen and betamethasone, *Malva neglecta* extract was less effective in reduction of inflammation.

Previous studies have investigated the adverse effects of synovial fluid macrophages in OA. Blom et.al. reported that synovial macrophages induce fibrosis and osteophyte formation in rat models of OA (13). There is a strong association between macrophage count in synovial space and severity and duration of OA (14). These results suggest the role of macrophages in induction and maintaining inflammatory processes in joints involved in OA. It is well known that OA is not exclusively a disease of cartilage. Indeed, studies in humans and animals have led to growing evidence that the synovium also is involved in the disease and its role starts relatively early in OA (4). Several cytokines are produced in OA synovium and are implicated in OA pathology. IL-1β and TNF-α are the primary cytokines in OA pathogenesis, thus suppression of their expression in synoviocytes and macrophages is essential for reducing the cartilage damage (15). Both IL-1β and TNF-α induce production of PGs, NO and matrix metalloproteinases such as collagenase, gelatinase, proteoglycanase etc. leading to cartilage destruction (6, 16). Accordingly, reduction in the production of prostaglandin and nitric oxide is reported in the pain relief and inflammation of OA (17-19). Given that corticosteroids and NSAIDs reduce the expression of genes involved in this matter, they are commonly used as the treatment options in OA.

In this study, we investigated the effect of *Malva neglecta* aqueous extract on bovine synoviocyte and also THP-1 cells. Human THP-1 cells are a proper model for acting as a replacement model for monocytes/ macrophages and now is commonly used in the experimental researches on the inflammatory agents (20). In this study, we showed that *Malva neglecta* extract reduces expression of IL-1β, TNF-α, iNOS, IL-18 and COX-2 in synoviocytes and THP-1 cells which may subsequently lead to a reduction in synovitis and cartilage degeneration. Suppression of the expression of these pro-inflammatory factors, also lead to suppression of COX-2 and iNOS enzymes activity; subsequently leading to a reduction in the NO and PGE2. Compared to groups treated with ibuprofen or betamethasone, reduction in expression or production of inflammatory agents was less when *Malva neglecta* extract was administrated. Therefore, *Malva neglecta* extract has anti-inflammatory properties in OA but it should be used as a supplementary treatment. This extract is reported to be effective in preventing and treating other diseases associated with inflammation and tissue damage such as nephrolithiasis (21). The exact chemical constituents of *Malva neglecta* extract involved in such effects are not fully recognized yet. Studies reveal that *Malva neglecta* contains alkaloids, flavonoids, saponins (22) phenolic compounds (7) and mucilage content (23). Different properties of these components are well recognized; for example, Saponins have antiviral, antifungal and antioxidant effects (24). Thus, anti-inflammatory effect of *Malva neglecta* extract may be due to saponins content presented in this plant.

Conclusion

Malva neglecta extract is effective in reducing expression of inflammatory agents in synoviocytes and monocyte/macrophage implying that this effect can be extended to different cells involved in the pathogenesis of OA. However, this extract cannot be accounted as a replacement of common treatments of OA. Indeed, it may be used as a supplementary treatment to prevent complications of common treatments of OA. Further in-vivo researches should be done to elucidate the effect of *Malva neglecta* extract in treating OA patients.

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Conflicts of Interest

The authors declare that they have no conflict of interest.

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