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COMBINATION OF CURCUMIN AND PIPERINE IMPROVES OSTEOARTHRITIS IN AN ANIMAL MODEL

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ABSTRACT

In present study, the protective effects of curcumin - piperine combination against Mono-iodoacetate induced- osteoarthritis in rats was investigated. In experimental groups, 14 days after injection of Mono-iodoacetate, curcumin (200 mg/kg), piperine (25 mg/kg) and curcumin + piperine were gavaged respectively for 2 weeks. Then the rats were sacrificed and the right knee joints of them were removed, fixed in 10% formalin and decalcified for histological, immunohistochemical and Tunel assessments. Cellularity and matrix staining were significantly increased in articular cartilage of curcumin treated animals in compare to Mono-iodoacetate group. Expression of Matrix metalloproteinase-13 and apoptotic index was also significantly decreased in curcumin treated animals. These effects against osteoarthritis features were markedly improved in piperine + curcumin treated animals. The data demonstrates that combination of curcumin + piperine can effectively improve osteoarthritis - induced by MIA and might be clinically useful.

KEY WORDS

Articular cartilage, Curcumin, Piperine, Monoiodacetate, MMP-13 and Apoptosis.

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INTRODUCTION

Osteoarthritis (OA) is a typical slow and degenerative joint disease. It affects about 80% of individuals of both sexes over the age of 60 and nearly 15% of population. Although additional crucial factors have been described including mechanical and physical pressure, hereditary history, injury and hormonal alterations[1,2].

The joint is a complex organ composed of different tissues including the articular cartilage, the subchondral bone, the joint capsule, the synovial membrane and other soft tissue structures such as

ligaments, tendons and menisci[2]. Typically, the articular cartilage is regarded as the primary diseased tissue, and its loss of homeostasis with increased destruction and an insufficient tissue repair ultimately leads to end stage disease[3].

There are no commercially available drugs definitely proven to modify the natural progression of OA. Non-steroidal anti-inflammatory drugs are widely prescribed for the treatment of OA pain. But the long term use of such drugs may cause side effects such as suppression of platelet aggregation, erosions and ulcerations in upper gastrointestinal tract mucosa[4]. In fact, the current therapeutic interventions are only useful for controlling symptoms, especially pain.

Traditionally, plants have been used for centuries as a popular method for treating several health disorders[5]. Curcuma longa is one of the most studied plants. Curcumin (Cur) is the main component of the turmeric pigment of *Curcuma longa*. It possesses many beneficial effects including anti-inflammatory, antioxidant, anticancer, antimicrobial, hepatoprotective and anti-hyperlipidemic[6-11].

In spite of numerous therapeutic effects, the bioavailability of Cur is low due to a relatively low intestinal absorption[12], and rapid metabolism in the liver[13], followed by elimination through the gall bladder[14]. The bioavailability of Cur can be improved with piperine (Pip)[15]. Various drugs and nutrients bio-enhanced by Pip such as Tetracycline, Vitamine B6 and Propranolol[16-18].

Numerous studies have previously focused on the molecular characteristics of OA and articular cartilage. They have reported many different factors that drive the malformations to OA, such as cytokines, growth factors, matrix metalloproteinases (MMPs) and apoptosis that reviewed by Pulsatelli *et al.*[19]. MMP-13 or collagenase-3 has long been considered as the major enzyme involved in OA cartilage erosion. Suggestive evidence was obtained by transgenic postnatal overexpression of the enzyme in mice, resulting in focal OA cartilage pathology at load bearing sites[20]. Now, ultimate proof of its role is obtained by the demonstration of reduced OA cartilage erosions in MMP-13 deficient mice[21].

In the present study, we assessed the effects of Cur and Pip in combination on OA-induced by Mono-iodoacetate (MIA) in rats by using histopathology and immunohistochemical assessments. MIA is an inhibitor of glyceraldehyde-3 phosphate dehydrogenase activity, and therefore an inhibitor of glycolysis shown to induce chondrocyte death in vitro. Intra-articular injection of MIA induces chondrocyte death in the articular cartilage of rodent and non-rodent species. When used in rodents, the model reproduces cartilage lesions with loss of proteoglycan matrix and functional joint impairment similar to human OA[22].

MATERIALS AND METHODS

Animals

In this experimental study, 40 male Wistar rats weighing 250-300 g at the start of experiment were used. The animals were obtained from Ahvaz Jundishapur University of Medical Sciences, Experimental Research Center, and this study was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (AJUMS) and carried out in an ethically proper way by following the guidelines provided. The animals were housed two per cage in a room with controlled temperature conditions (21-22°C), a reversed light-dark cycle (12 h: 12 h) and free access to food and water.

Experimental design

The animals were randomly divided in to 5 groups. Fifty µl of saline was injected in right knee joints in control group. In order to induce OA, 1mg of MIA dissolved in 50 µl saline and injected in right knee joints. In experimental groups, 2 weeks after injection of MIA, 200 mg/kg Cur (Cur + MIA group), 25 mg/kg Pip (Pip + MIA group) and Cur plus Pip (Pip + Cur + MIA group) were gavaged respectively for 14 consecutive days. In the end of experiment, all rats were sacrificed. Right knee joints removed and fixed in formalin 10%. The fixed specimens were decalcified by using 5% formic acid for 6 days.

Histopathological staining

Paraffin-embedded blocks were cut in a 5-µm thickness. Sections were deparaffinized through xylene and graduated alcohol series to water and stained with hematoxyllin and eosin (H and E) for routine histological evaluation, Safranin O stain was also used to evaluate proteoglycans in the cartilage matrix. A modified Mankin grading was used to score cartilage change, as follows: the structure was scored on a scale of 0-6; where 0 = normal, 1 = irregular surface, including fissures into the radial layer, 2 = pannus, 3 = absence of superficial cartilage layers, 4 = slight disorganization (an absent cellular row and some small superficial clusrers), 5 = fissures into the calcified cartilage layer and 6 = disorganization (chaotic structure, clusters, and osteoclastic activity). Additionally, cellular abnormalities were scored on a scale of 0-3; where 0 = normal, 1 = hypercellularity, including small superficial clusters, 2 = clusters and 3 = hypocellularity. The matrix staining was also scored on a scale of 0-4; where 4= normal, slight reduction of staining, 3 = staining reduced in the radial layer, 2 = staining reduced in the interterritorial matrix, 1 = staining present only in the pericellular matrix and 0= staining absent [23]. Two observers, blinded to the control and experimental groups, analyzed the sections independently.

Immunohistochemistry (IHC)

The sections were deparaffinised and endogenous peroxidase activity was blocked by incubation in methanol containing 0.3% H2O2 for 15 min at room temperature. Antigen retrieval was performed with 10 Mm sodium citrate buffer (pH 6.0) at 98°C for 15 min. Sections were then incubated for 1 h at room temperature with a blocking solution consisting 5% normal goat serum in phosphate buffered saline (PBS). Thereafter, sections were incubated overnight at 4°C with primary antibody including the anti-mouse monoclonal antibody against MMP13 (ab39012) diluted 1: 100 in blocking solution. After three washes in PBS, sections were incubated for 30 min at room temperature with a biotinylated goat anti-rabbit IgG (sc-2018, Santa Cruz) as secondary antibody, washed in PBS and then incubated with the avidin -biotin complex (ABC Peroxidase kit, Santa Cruz, USA) for 30 min at room temperature. Following three 5 min PBS washes, the antigen was finally detected by treating the sections with a 3, 30-diaminobenzidine (DAB) substrate, after which positive immunoreactivity was revealed as brown staining. Sections were counterstained with haematoxylin, dehydrated in 100% ethanol, cleared in xylene and mounted with Entellan permanent mounting medium. Negative controls were generated by replacing the primary antibodies with the blocking solution. From each mouse, a minimum of four sections were examined.

TUNEL assay

For detection of apoptosis at a single cell level, based on labeling of DNA strand breaks, the In Situ Cell Death Detection Kit, POD (Boehringer Mannheim GmbH, Mannheim, Germany) was used. The paraffin sections were dewaxed and rehydrated by standard methods. Proteases were added and incubated with 5% of appropriate normal serum for 30 min at 37 °C. The slides were washed in phosphate buffered saline. The sections were permeabilized (2 min, on ice) and incubated with TUNEL reaction mixture (60 min, 37 °C). Anti-fluorescein-AP was added and incubated (30 min, 37 °C). Subsequently, the sections were washed in PBS and incubated for 20 min with substrate. They were analyzed by light microscopy. A cell was considered TUNEL-positive when the nuclear staining was intense, dark brown and homogenous. Apoptotic index was calculated by dividing the number of TUNEL-positive chondrocytes in a randomly focused field by the total number of chondrocytes that field, and the result was multiplied by 100. The apoptotic indexes of 10 randomly field for each slide were evaluated and the mean apoptotic index of each case was calculated [24]. Four slides/ animal were used in this study.

Statistical analysis

The data were analyzed using one-way ANOVA followed by post hoc LSD test and were presented as the mean ± SD. $p < 0.05$ was considered significant.

RESULTS

Histology

The joint from non-induced OA (control group) showed smooth articular cartilage surfaces with the underneath layer of flattened chondrocytes in the tangential zone and chondrocytes were normally distributed in parallel rows transitional and radial zones of the articular cartilage. Intercellular matrix deeply and uniformly stained with safranin O.

The joints of MIA-induced OA showed severe discontinuity, degeneration of the articular cartilage and disappearance of chondrocytes in the tangential, transitional and radial zones of the cartilage. The cellularity of articular cartilage was significantly decreased ($p < 0.05$). Sections stained with safranin O revealed severe reduction in their staining indicating proteoglycans loss.

Treatment with Pip showed little effects on the structural changes in the joints induced by MIA and Mankin's grading assessments showed no significant change ($p > 0.05$) compared to the MIA treated joints. Cellularity of articular cartilage in MIA + Pip treated group were slightly increased compared to MIA treatment. Safranin O staining showed a reduction in cartilage proteoglycans in MIA + Pip group.

In Cur + MIA group, histological changes were considerably reversed. Mankin's grading were significantly decreased in comparison to MIA treated animals. Cellularity and matrix staining of articular cartilage in MIA + Cur treatment were significantly increased ($p <0.05$) compared to MIA treatment.

Treatment with Cur + Pip effectively improved the structural changes in the joints induced by MIA. Cellularity and matrix staining of articular cartilage of MIA+ Cur + Pip group were significantly increased compared to MIA treated joints and slightly decreased in comparison to control group.

In MIA group vascularization in particular joints was observed. The vascularization of articular joints in MIA + Pip group was slightly less than MIA treatment. There were not any signs of vascularization in MI + Cur and MIA + Cur + Pip treated animals. These results are reported in Figure No.1 and 2.

IHC assessments

No expression of MMP-13 was observed in control group. Strong immunoreactivity was observed in extracellular matrix of MIA treated cartilages. In Pip group, expression pattern of MMP-13 was similar to MIA group. Moderate or weak immunostaining was observed in matrix of Cur group. In Cur + Pip group expression of MMP-13 was markedly decrease in compare to Cur group. These findings are shown in Figure No.3.

TUNEL assay

Spontaneous apoptosis was observed in some chondrocytes of normal cartilage from the control group. In the MIA group, apoptosis was observed in all parts of articular cartilage and apoptotic index was significantly increased compare to the control group ($p < 0.001$). In Pip+MIA group, Apoptotic index was slightly less than MIA group ($p > 0.05$). In Cur group, apoptotic index was significantly decreased compared to MIA treated animals ($p < 0.05$). In Cur+ Pip+MIA group apoptotic index was significantly decreased compared to Cur+MIA treated animals. The results of TUNEL assay are shown in Figure No.4 and Figure No.5.

DISCUSSION

This study has demonstrated that Co-administration of Pip can enhance the chondro-protective effects of Cur against MIA in rats. We found that the cellularity scores in knee OA cartilage were markedly reduced compared with those in nonarthritic control cartilage.

Our results revealed that Cur can reverse hypocellularity in MIA-induced OA, but the increase in cellularity was more pronounced when Pip was used as an adjuvant. Reduced cellularity is a characteristic feature of OA cartilage [25]. Actually, saving plausible number of cartilage cells in the joints articular structure is important in OA pathology and progression, because chondrocytes are the only component capable of controlling vital activities of the articular cartilage.

In addition to changes in the cellularity, the matrix staining was reduced in MIA treated joints. Proteoglycan depletion could be secondary to cell loss due to the OA Process. Combination of Cur and Pip was able to increase the cellularity and matrix protection, indicating that this combination can effectively suppress OA.

As mentioned in results MIA administration induces a significant increase in apoptotic index of chondrocytes.

Recent studies have shown a positive correlation between the degree of severity of OA and the number of apoptotic chondrocytes in both experimentally induced OA in rabbit cartilage and human OA cartilage [26, 27]. It is well known that the increase rate of apoptosis in cartilage can led to a reduction in cartilage cellularity, and thus, a reduced ability of the cartilage cells to replace any damaged cartilage of OA. Additionally, accumulation of apoptotic bodies and their removal from the tissue can cause further degradation of the cartilage. Cartilage is avascular and contains no mononuclear phagocytes capable of removing apoptotic bodies. Apoptotic bodies could release their contents (including proteases) into the matrix, promoting enzymatic degradation [28].

Ju *et al*. showed that Sinomenine, an alkaloid extracted from the stem of the Chinese medicinal plant sinomenium acutum, has protective effect on OA by reduction in MMP-13 expression and inhibition apoptosis [29].

This study demonstrated that Cur + Pip treatment could considerably decrease expression of MMP-13. It has been reported that MMPs play a role in the cartilage degradation that occurs in the MIA model [30]. MMP-13 or collagenase 3 has long been considered as the major enzyme involved in OA cartilage erosion. MMP-13 can cleave both type I and type II collagens [31]. Therefore, the inhibition of MMP-13 synthesis in OA chondrocytes by Cur or Cur+Pip could explain their positive effect of protecting the cartilage matrix macromolecules. Baragi *et al*. revealed that MMP-13 inhibitors exerted chondroprotective effects and they could potentially reduce joint pain in MIA-treated rats [32].

Vascularization in damaged cartilages was observed in MIA and MIA+ Pip treated animals. While, vascularization was not seen in MIA + Cur and MIA + Cur + Pip treated animals. It is well known that vascularization of the damaged articular cartilage is accompanied by innervations, which contributes to pain [33].

Previous studies have shown beneficial effects of Cur and Pip separately on OA. Bang *et al* have reported that Pip has antiarthritic effects in an arthritis animal model [34]. Ying *et al*. (2013) have demonstrated that Pip inhibits IL-β induced expression of inflammatory mediators in human OA chondrocyte. However, Pip showed poor anti-OA activity in present study [35].

Kulkarni *et al* have reported positive effects of Cur on pain and mobility in 42 patients with OA[36].

Belcaro *et al*. have demonstrated that Cur improves joint swelling, morning stifness, and walking time[37].

Present study has also demonstrated that Cur has chondro-protective effect, but this effect more pronounced when Pip was used as an adjuvant.

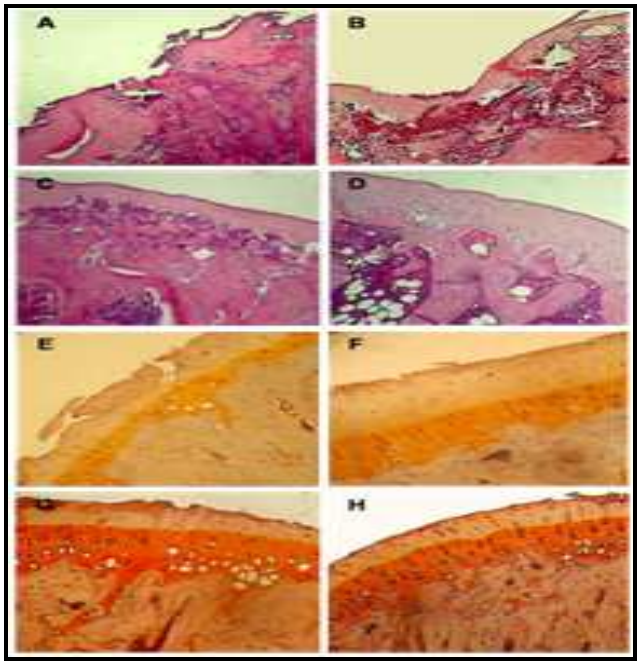

Figure No.1: Light microscopy of knee joints. A and E: MIA group, B and F: Pip+MIA treated joints, C and G: Cur+MIA group, D and H: Cur+Pip+MIA treated joints. A-D: H&E staining, E-H: Safranin O staining. Magnifications: ×200.

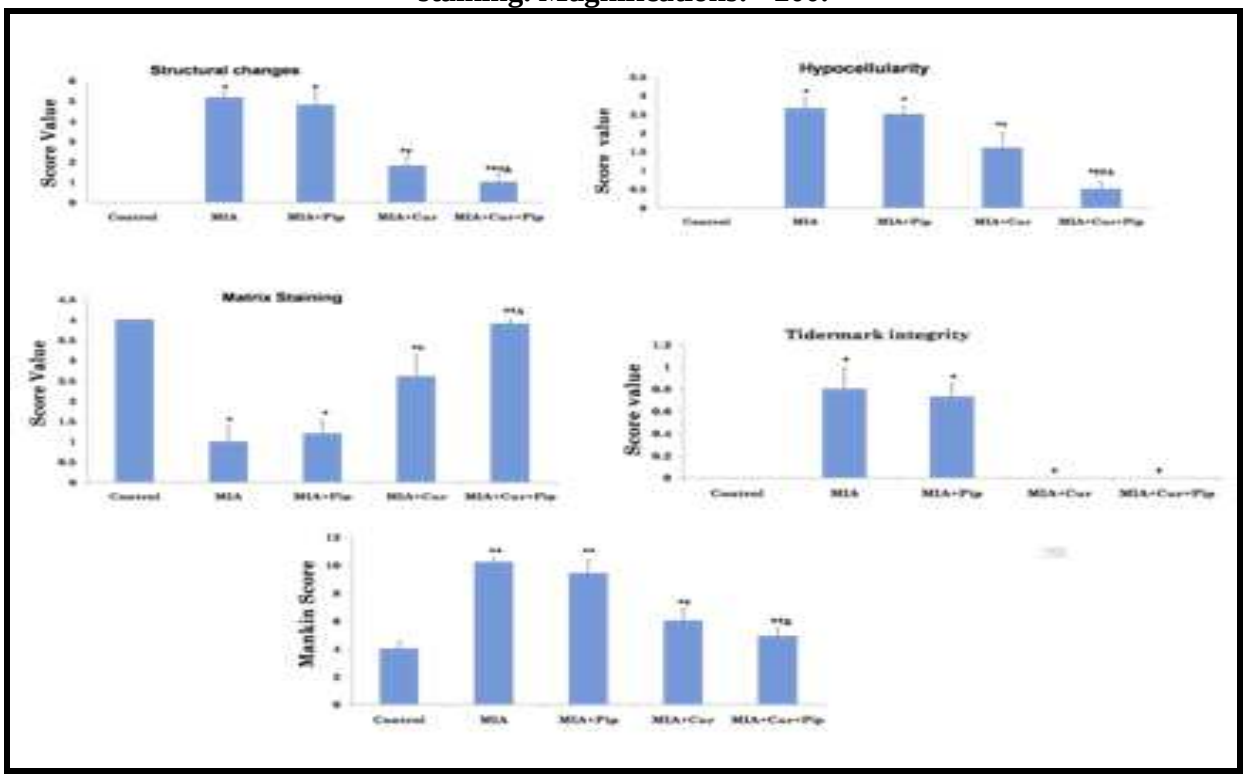

Figure No.2: Mankin grading of knee joints in control and experimental groups. Values expressed as mean ± SD for 8 mice. *p < 0.01, **p < 0.001, †p < 0.01, ††p < 0.001, $^{\Delta}$p < 0.05; *, † and Δ symbols respectively indicate comparison to control, MIA-induced OA and Cur group.

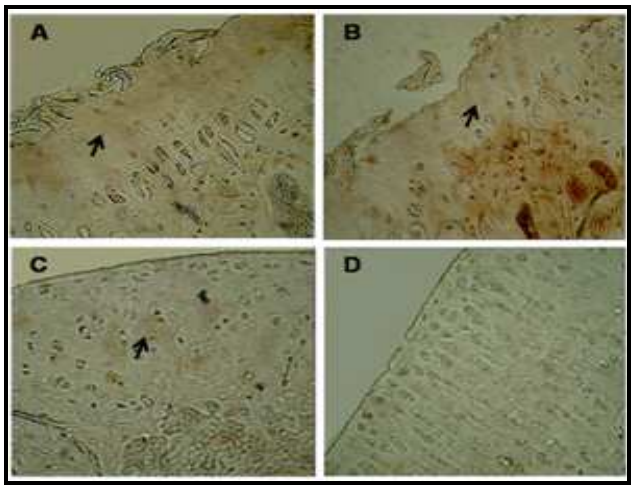

Figure No.3: immunohistochemistry staining in control and experimental groups. A: MIA group, B: Pip+MIA treated joints, C: Cur+MIA group, D: Cur+Pip+MIA treated joints. Magnifications: ×400.

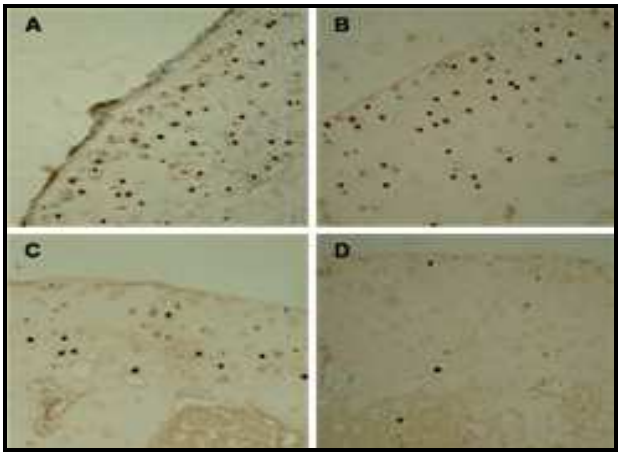

Figure No.4: TUNEL staining of knee joints. A: MIA group, B: Pip+MIA treated joints, C: Cur+MIA group, D: Cur+Pip+MIA treated joints. Magnifications: ×250.

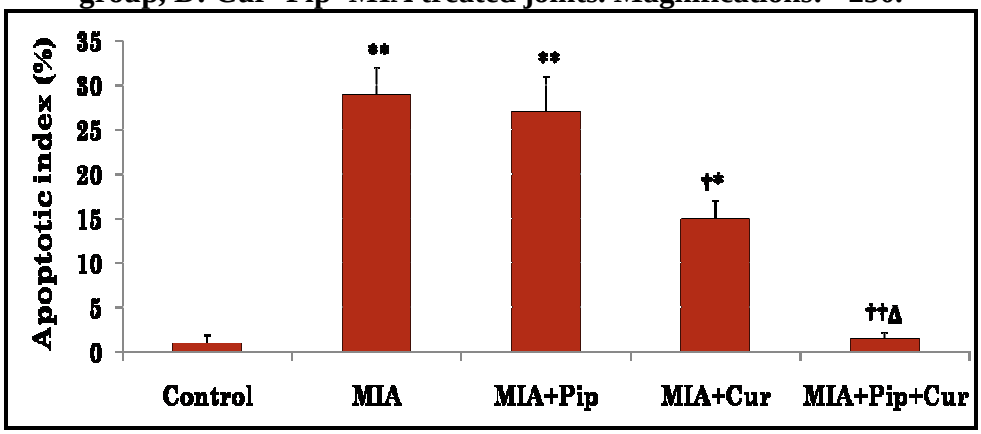

Figure No.5: Apoptotic index in control and experimental groups. Values expressed as mean ± SD for 8 mice. *p < 0.01, **p < 0.001, †p < 0.001, ††p < 0.001, Δp < 0.001; *, † and Δ symbols respectively indicate comparison to control, MIA-induced OA and Cur group.

CONCLUSION

Overall, the results of this study demonstrate that combination of Cur and Pip can effectively improve structural changes of articular cartilage in MIA induced OA. This effect appears to be mediated through the inhibition of apoptosis and MMP-13 expression, which are key mediators of the structural changes that take place in OA. These results suggest that combining Cur and Pip may have potential clinical applications for treating OA. Extrapolation of these data to the human situation is not appropriate. However, this information does provide a stimulus for true clinical investigations.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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