Original Article

Autophagy inhibitor regulates apoptosis and proliferation of synovial fibroblasts through the inhibition of PI3K/AKT pathway in collagen-induced arthritis rat model

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Abstract: Background and objective: Mounting studies have illustrated an important role of autophagy in various diseases, but few studies have reported its contribution to rheumatoid arthritis (RA) and the underlying mechanism was largely unknown. This study aimed to investigate whether autophagy inhibitors could regulate apoptosis and proliferation through PI3K/AKT pathway in RA. Methods: RA animal model was established by collagen induction. General observations and degree of joint swelling were observed. Inflammatory response, cell survival related factors and apoptosis were also detected in synovial fibroblasts. In addition, cultured rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) were subjected to TNF-α treatment in vitro, and TNF-α induced cell autophagy, synovial cell proliferation and apoptosis were detected. Moreover, cell cycle and cytokine secretion protein, along with the above parameters, were analyzed. Results: Results from the animal model showed that autophagy inhibitors attenuated inflammatory reaction and synovial hyperplasia, while promoted synovial fibroblasts apoptosis. Meanwhile, inhibition of autophagy promoted cell apoptosis and reversed cell proliferation in vitro, also blocked cell in the G2/M arrest and reduced the S phase cells. Furthermore, we observed that inhibition of PI3K/AKT pathway reversed TNF-α mediated autophagy and cytokine secretion. Conclusion: autophagy inhibitors could mitigate inflammation response, inhibiting RA-FLS cell proliferation while promoting cell apoptosis by the PI3K/AKT pathway.

Keywords: Rheumatoid arthritis, autophagy, fibroblast-like synoviocytes, apoptosis, PI3K/AKT pathway

Introduction

New treatment options and strategies have transformed the achievable outcomes in rheumatoid arthritis (RA) during the last 20 years [1]. RA was a complex autoimmune disease that primarily manifests as chronic inflammatory arthropathy [2], characterized by an abundant various cellular infiltration including neutrophils, lymphocytes and macrophages, causing the release of multiple inflammatory cytokines and matrix-degrading enzymes that promote the process of joint destruction [3], which producing a heavy burden for the affected individual and the whole society. Rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) played a pivotal role in the process of RA disease. RA-FLS were believed to actively contribute to the pathogenesis of RA and their proliferation was stimulated by inflammatory infiltration of RA hallmark [4]. Moreover, RA-FLS were capable of producing multiplex of factors involved in perpetuation of inflammation response including inflammatory cytokines, chemokines and matrix degrading enzymes, especially TNF-α and IL-1β, thereby strongly contribute to the inflammatory and joint destructive state in RA [5].

Autophagy was an evolutionarily conserved degradation process in response to metabolic stress or changing environment [6], and it had been implicated in a wide variety of physiological and pathological conditions. Autophagy was involved in a number of cellular homeostatic processes and played a central role in the
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innate and adaptive immune response by recycling and removing harmful protein aggregates and damaged cell organelles [7]. Emerging evidence suggested a crucial role of autophagy in the regulation of immunity and inflammation, and the dysregulation of autophagic had also been implicated in various disease processes, such as tumorigenesis and inflammation [8, 9]. In addition, a lot of studies have showed the correlation between autophagy and RA [10-12], however, its exact contribution to disease manifestation has not yet been fully illustrated.

Autophagy and apoptosis were two different types of programmed cell death and occurred in multi-cellular organisms, which acted an important role in maintaining metabolic homeostasis and organize activity [13]. Through “self-eating” or “self-killing”, autophagy and apoptosis caused by external stress can regulate cytokine, inflammatory factors and organelles to make damaged cells survive or program death. Disruption of cell apoptosis was one of the basic pathologies involved in the carcinogenesis of human carcinomas, including gastric cancer, liver cancer, lung cancer, RA [14-17] and so on. Huang and his colleagues found that niclosamide drug could inhibit cell proliferation and induce mitochondrial apoptosis of RA-FLS, which was associated with the modulation of AKT signaling pathways [18]. In addition, study showed that β-Elemene, a natural anticancer compound extracted from the Chinese medicinal herb Curcuma Wenyujin, and it induced apoptosis of human RA-FLS via reactive oxygen species-dependent activation of p38 mitogen-activated protein kinase [19]. Nowadays, many regulatory mechanisms were involved in the pathogenesis of RA, among them PI3K/AKT “cell survival” pathway was included. Studies have showed that over stimulation of PI3K/AKT/mTOR “cell survival” pathway were likely to be responsible for the high level of apoptosis-resistance [18, 19]. The activation of PI3K/AKT/mTOR appeared to be the critical driver of anti-apoptosis responses that was typically a characteristic of inflamed RA synovial tissue [20]. In addition, cytokines in RA-FLS led to activation of the PI3K/AKT pathway, thereby promoting cells migration and invasion. It had also been shown that the PI3K/AKT signaling pathway was involved in the pathogenesis of inflammation [21]. Therefore, modulation of the PI3K/AKT pathway may yield therapeutic benefits for RA disease [22].

In the present study, we aimed to explore whether and how autophagy inhibitors 3-Methyladenine (3-MA) and chloroquine (CQ) affected the proliferation and apoptosis of RA-FLS in vitro and in vivo. We also investigated the interaction between autophagy inhibition and the PI3K/AKT pathway to further elucidate the potential role of autophagy inhibitors in RA-FLS.

Materials and methods

CIA model establishment

A total of 40 newborn SD rats were chosen for the collagen-induced arthritis (CIA) model establishment. These rats were housed under controlled environmental conditions with free access to standard laboratory diet and water for acclimation. The model establishment process was as follows: The bovine collagen type 2 glacial acetic acid was mixed with the same volume of Freund’s adjuvant incomplete under ice bath to make type 2 emulsion, then these animals were subcutaneous injected with prepared emulsion at a dose of 0.2 ml per rat in the back and tail root. On the 7th day, rats were treated with the same concentration of emulsion once again. In addition, rats in the normal control group were injected with the same amount of normal saline. Total limb joint swelling grade score (Al value) was used to assess whether the modeling was successful or not. Limb joint swelling was graded according to 0-IV grade. 0: no swelling; I: slightly swollen toe joint; II: small toe joint and toe joint swelling; III: joint swelling below the ankle joint; IV: including ankle joint swelling, including all the feet. Al = the sum of the limb joint swelling grade score (I grade represented 1 point, total was 16 points). If Al value was more than 4 points, then the model was successful. Otherwise, rats can’t be selected for subsequent experiments. On day 12th, drugs capable of inhibiting autophagy (CQ, 3-MA) were used to intervene, in order to investigate the therapeutic effects on RA model rats. All animal experiments were approved by the Experimental Animal Ethics Committee of Xiang Ya Second Hospital, Central South University.

Experimental groups

After the model was successfully established (on day 12th), these animals were randomly divided into four groups: control group, model group, CQ treatment group and 3-MA treatment group.
group. There were 10 rats in each group. Drugs CQ and 3-MA were intraperitoneally injected at a concentration of 60 mg/kg/d and 500 nmol/kg/d, respectively. Samples were taken on day 28th and 42th after administration (n = 5 each time) and foot swelling was measured twice weekly during dosing period, including foot pad thickness, ankle circumference and foot volume. Pathological observations of anklebone joint’s synovium were performed following that, cell proliferation was observed by HE combined with electron microscopy methods, and apoptosis was detected by using TUNEL kit.

Histopathological examination

The synovial tissues were preserved in 10% buffered formalin for at least 24 h, dehydrated with a sequence of ethanol solutions and were processed for embedding in paraffin. Sections of 5~6 mm in thickness were cut, de-paraffinized, rehydrated, stained with haematoxylin and eosin (H&E) for the estimation of inflammatory, synovial fibroblast hyperplasia and vascular proliferation.

Flow cytometry

We determined the apoptosis and cell cycle of synovial fibroblast by flow cytometry according to the direction of manufacturer. Briefly, RA-FLS were collected and fixed in 75% ice ethylalcohol overnight at 4°C. After being centrifuged, these cells were separated from fixation fluid using ice PBS. Subsequently, with the addition of RNaseA, the cells were subjected to water bath in a dark condition for 30 min. Then propidio iodide (PI) was added for coloring. After being mixed evenly, cell cycle stages were detected by red fluorescence recording during flow cytometry and the proportions of cells in G0-G1, S, and G2/Mphase were calculated. Each experiment was performed in triplicate.

Western blotting

Synovial fibroblasts and synovial tissue were lysed in Laemmli buffer (62.5 mM TrisHCl, 2% SDS, 10% glycerol, 0.1% bromphenolblue, 5 mM β-mercaptoethanol). Whole lysates were separated on 10% SDS polyacrylamide gels and electro-blotted onto nitrocellulose membranes. Membranes were blocked with antibodies against p-AKT, AKT, LC3-I, LC3-II, CDK1, CyclinB1 and Cleaved-caspase3 or β-actin as an endogenous control. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse antibodies were used. Signals were detected using the ECL Western blotting detection reagents (GE Healthcare) and the Alpha Imager Software system. Expression analysis of specific proteins was performed by pixel quantification of the electronic image.

TUNEL assay

We determined the apoptosis synovial fibroblast by the terminal deoxynucleotidyl transferred-mediated dUTP nick end labeling (TUNEL) staining according to the direction of manufacturer. Briefly, following routine deparaffinization and treatment with 3% H2O2 for 20 min, the sections were digested with pepsin at 37°C for 30 min and incubated with the TUNEL reaction mixture at 37°C for 60 min. DAB was added and visualized using a Converter-POD. Haematoxylin dye was used for counterstaining. Apoptotic were quantified by counting TUNEL positive cell nucleus. For each sample, the number of TUNEL positive cells was counted under a magnification of ×400. Six representative fields were evaluated for each rat of the experimental groups.

Cell culture

Synovial tissues were obtained from patients with active RA (5 women, 1 men, aged 56-69 years), according to the revised criteria of the American College of Rheumatology [23], who were undergoing synovectomy or joint replacement. This study was approved by the Medical Ethics Committee of Xiang Ya Second Hospital, Central South University, and informed consent was obtained from all patients. Synovial tissues were cut into small pieces and digested with type I collagenase (4 mg/ml) in DMEM medium for 1 h and 0.25% trypase every 10 min at 37°C to isolate synoviocytes. The tissue was then fine tis using fine sterile gauze, washed and resuspended. Dissociated cells were then centrifuged at 800×g, following 1,000×g and then plated in 10 cm dishes. After overnight culture, the non-adherent cells were removed, and the adherent cells were maintained in the Dulbecco’s modified eagle medium (DMEM, Invitrogen, Karlsruhe, Germany) supplemented
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with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin under an atmosphere of 5% CO₂ and 95% air at 37°C.

**TNF-α treatment**

Synovial fibroblasts cells were plated to six well plates and cultured for 24 h. Then, these cells were incubated with TNF-α. After that, cells were washed and collected for further apoptosis, proliferation and cell cycle analysis.

**Analysis of caspase3 expression**

Following treatment, cells were detached with trypsin, re-suspended in cell culture medium at a concentration of 1×10⁶ cells/ml and incubated with cleaved-caspase3 substrate for 20 min at room temperature in the dark. Cleave-
caspase3 expression was analyzed by western blotting analysis.

**Analysis of cell proliferation**

After treatment with autophagy inhibitors, synovial fibroblasts cells were dissociated with Accutase® (Innovative Cell Technologies, USA), resuspended, and seeded in 96-well plates at 3000 cells per well. The CCK-8 assay was used to measure cells proliferation. CCK-8 solution (10 μl) was added to each well and the plate was incubated for 2 h at 37°C. The absorbance was recorded at 450 nm on a Spectra Max M5 micro-plate reader.

**Analysis of cytokines**

We determined the cytokines (IL-1β, MMP-3) secreted from synovial fibroblast by ELISA method according to the direction of manufacturer. Cell levels of IL-1β and MMP-3 were determined using the corresponding ELISA kit, respectively.

**Statistical analysis**

All experiments were performed in duplicate and repeated at least three times. Values are presented as mean ± SEM. Between-group differences were assessed by the t-test and \( P<0.05 \) were considered statistically significant.

**Results**

**Autophagy inhibitors CQ and 3-MA protected from collagen-induced joint swelling in CIA model rats**

The effects of autophagy inhibitors CQ and 3-MA on joint swelling in rats were evaluated. The CIA model was established successfully based on experimental procedures. As shown in Table 1, when compared with the control group, the arthritis scores (AI value) were
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significantly increased after collagen induction; while supplement with autophagy inhibitors CQ and 3-MA, it was decreased obviously as compared with the model group. In addition, we can see from Figure 1, there was hardly any swelling in the control group rats. However, joint swelling was observed obviously in other three groups. But the degree of joint swelling was mitigated in CQ and 3-MA treatment groups than collagen induction model rats. Moreover, compared with model group, foot pad thickness, ankle circumference and foot volume were significantly decreased during dosing period in CQ group and 3-MA group, along with a time-dependent manner. While no remarkable differences were detected between these two groups (Figure 2).

**Autophagy inhibitors CQ and 3-MA protected from collagen-induced inflammation in CIA model rats**

The effects of autophagy inhibitors CQ and 3-MA on inflammation response were investigated by H&E staining. As shown in Figure 3, synovial structure was normal and there was no fibrosis and inflammatory cell infiltration in the control group, while in the model group, obvious synovial endothelial hyperplasia, granulation formation, obvious fibroblast cells and vascular proliferation, accompanied by serve inflammatory cell infiltration were observed. However, compared with model group, synovial fibroblast proliferation and angiogenesis was apparently alleviated in autophagy inhibitors (CQ and 3-MA) groups, along with less inflammatory cell infiltration.

**Autophagy inhibitors CQ and 3-MA protected from collagen-induced apoptosis in CIA model rats**

The effects of autophagy inhibitors CQ and 3-MA on apoptosis were studied by TUNEL staining. Analysis showed increased TUNEL-positive cells (brown presented) in synovial fibroblasts compared with control group, espe-
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CQ and 3-MA inhibited synovial fibroblast survival through PI3K/AKT pathway in CIA model rats

A critical step in the autophagic process was the formation of autophagosomes and the conversion from LC3-I to LC3-II, indicating the activation of autophagy. 3-MA, a pharmacological inhibitor of autophagy, blocked autophago-
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some formation presented by reduced LC3-II expression. Another autophagy inhibition drugs CQ and 3-MA could block p-AKT phosphorylation, at the same time they further promoted the expression of apoptosis-related protein cleaved-caspase3 compared with control group. Therefore, autophagy inhibitors CQ and 3-MA may inhibit the phosphorylation of p-AKT.

Figure 6. Effects of autophagy inhibitors or PI3K inhibitor (LY294002) on the expression of p-AKT, LC3-I, LC3-II and cleaved-caspase3 in RA-FLS cells induced by TNF-α (A). Expression of β-actin acted as an internal reference. Apoptosis cells were evaluated by flow cytometry using annexin V/propidium iodide (PI) staining, and numbers in each compartment were the percentage of cells (B). Quantified histograms of apoptosis RA-FLS cells were shown in (C). *P<0.05: compared with control group. P values were calculated by independent-samples T test. Values are the mean ± SEM.

Figure 7. Effects of autophagy inhibitors or PI3K inhibitor (LY294002) on the synovial fibroblasts proliferation and cell cycle in RA-FLS cells treatment with TNF-α. Cell proliferation was detected by CCK-8 method (A). Cell cycle was detected by flow cytometry method (B). Blue column represented cell percentage of G0/G1 phase; Orange column meant cell percentage of S phase; Green column indicated cell percentage of G2/M phase. *P<0.05: compared with control group. #P<0.05: compared with TNF-α group. P values were calculated by independent-samples T test. Values are the mean ± SEM.
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Autophagy inhibitors CQ and 3-MA regulated TNF-α-induced synovial fibroblasts p-AKT expression and apoptosis in vitro

Next, in vitro study was performed in order to explore the potential mechanism. As shown in Figure 6, compared with control group, TNF-α could induce the concentration of p-AKT, AKT and LC3-II in RA-FLS, while following treated with autophagy inhibitors 3-MA and PI3K inhibitor (LY294002), obviously autophagy protein LC-3II level was down-regulated, along with inhibited p-AKT expression and activated apoptosis protein cleaved-caspase3 content, in further to promote apoptosis in RA-FLS in vitro (Figure 6A). Apoptosis analysis also indicated the same result as the above, the apoptosis degree was increased in TNF-α induction group, while supplemented with autophagy inhibitors (CQ and 3-MA) and PI3K inhibitor (LY294002), apoptosis of RA-FLS was significantly increased (Figure 6B and 6C). Taken together, these results showed that autophagy may inhibit the apoptosis of RA-FLS by regulating PI3K/AKT signaling pathway.

Autophagy inhibitors CQ and 3-MA restrained TNF-α-induced synovial fibroblasts proliferation and cell cycle in vitro

The effects of autophagy inhibitors CQ and 3-MA on cell proliferation and cell cycle were investigated by CCK8 and flow cytometry method. As shown in Figure 7, cell proliferation and cell cycle were strongly enhanced with TNF-α induction. However, when compared with TNF-α group, cells treated with autophagy inhibitors (CQ and 3-MA) or PI3K inhibitor (LY294002) presented significantly decreased the relative proliferation rate, making the cell cycle stagnated at G2/M phase and cells in S phase obviously reduced. In addition, CQ, 3-MA and PI3K inhibitor (LY294002) treatment could down-regulated the expression of cell cycle related proteins (CDK1 and cyclinB1) compared with the cells treated with TNF-α (Figure 8). All these results indicated that autophagy may regulate cell proliferation and cell cycle.

Autophagy inhibitors CQ and 3-MA affected TNF-α-induced synovial fibroblasts cytokine secretion in vitro

Cells were treated in order to evaluate the role of autophagy inhibitors (CQ and 3-MA) or PI3K

Figure 8. Effects of autophagy inhibitors or PI3K inhibitor (LY294002) on the cell cycle related protein in RA-FLS cells induced by TNF-α. The procedure was randomly divided into five groups, namely control, TNF-α group, TNF-α + CQ group, TNF-α + 3-MA group, TNF-α + LY294002 group. The expression of cyclin B1 and CDK1 were assessed by western blot method. The level of β-actin acted as an internal reference.

Figure 9. Effects of autophagy inhibitors or PI3K inhibitor (LY294002) on the synovial fibroblasts cytokine secretion in RA-FLS cells treated with TNF-α. The experiment was randomly divided into five groups, namely control, TNF-α group, TNF-α + CQ group, TNF-α + 3-MA group, TNF-α + LY294002 group. The expression of IL-1β (pg/ml) and MMP-3 (ng/ml) were detected by ELISA method. *P<0.05: compared with control group. #P<0.05: compared with TNF-α group. P values were calculated by independent-samples T test. Values are the mean ± SEM.
inhibitor (LY294002) on cytokine (IL-1β and MMP-3) secretion in synovial fibroblasts in vitro. As shown in Figure 9, the level of IL-1β and MMP-3 was significantly increased in TNF-α-treated groups comparing with control group. After adding autophagy inhibitors (CQ and 3-MA) or PI3K inhibitor (LY294002) to the cells, IL-1β and MMP-3 levels were obviously inhibited. The above results demonstrated that autophagy promoted TNF-α-induced cytokine secretion in synovial fibroblasts.

Discussion

In the present study, the final results consisted of two sections: in vivo and in vitro. Through in vivo experiments, it can concluded that the inhibition of autophagy with 3-MA and CQ could alleviate synovial inflammation and synovial endothelial hyperplasia, and promoted synovial cell apoptosis in CIA model rats through PI3K/AKT signaling pathway. Through in vitro experiments, we can concluded that autophagy inhibition by 3-MA and CQ suppressed TNF-α-induced cytokine secretion, and blocked G2/M phase through PI3K/AKT pathway, reducing the ratio of cells in S phase, decreasing RA-FLS cell proliferation, while promoting cell apoptosis.

RA was characterized by chronic arthritic inflammation and progressive destruction of cartilage and bone which led to severe joint pain and functional impairment [11]. The high rate of prevalence was confirmed to cause an increasing social and economic burden on societies. Recent advances in the identification of genetic causes of RA have provided new opportunities for finding therapeutic targets to prevent the degenerative process of the disease. To date, a number of animal models were applied in order to study the therapy of rheumatoid arthritis. Among them, CIA animal model in vivo was considered as the gold standard model of RA to evaluate therapeutic effects.

Although the induction of autophagy was primarily believed to be protective, it has also been reported that induction of autophagy was associated with autophagic cell death in a variety of tumor cells upon in vitro treatment with chemotherapeutic agents [24, 25]. At present, autophagy in RA in vitro experiments has focused mainly on osteoclast activity. Deletion of autophagy gene Atg7 in TNF-α transgenic mouse reduced the number of osteoclasts and mitigated joint damage [26]. Moreover, mounting studies have showed the correlation between autophagy and RA synovial fibroblast survival. Kato examined RA synovial fibroblasts (RASFs) ex vivo and found that RASFs were hypersensitive to autophagic cell death under conditions of severe endoplasmic reticulum (ER) stress [27]. However, the role of autophagy in the regulation of inflammation in RA and experimental arthritis has not been extensively studied. In our study, we observed that autophagy inhibitors could alleviate inflammatory characteristics, including synovial endothelial hyperplasia, granulation formation, obvious fibroblast cells and vascular proliferation, accompanied by inflammatory cell infiltration.

Except for studies of autophagy inhibition in RA, more and more studies have begun to explore the effect of proteasome inhibition on RA-LFS. Proteasome inhibition had pro-apoptotic and anti-inflammatory effects in experimental arthritis animal models [28, 29]. We showed that inhibition of autophagy made RA-FLS more susceptible to apoptosis that was may be associated with cleaved caspase-3. These data confirmed those from previous studies showing an apoptosis-resistant phenotype in RA-FLS [5]. Shin and his colleagues found that autophagy induction could promote survival of fibroblasts and increase cell resistance against ER stress-induced cell death in fibroblasts from RA patients [30]. Autophagy promoted a caspase3-independent induction of cell death under ER stress, autophagy had a protective role in apoptosis induced by proteasome inhibition [27]. Taken together, the regulation of autophagy activity on synovial fibroblasts apoptosis may provide new opportunities for the treatment of RA.

Additionally, we found increased PI3K and p-AKT levels in RA-FLS cells treated with TNF-α compared to controls, however, when added autophagy inhibitors 3-MA and CQ, the expression of PI3K and p-AKT was inhibited, which suggesting that autophagy inhibitors 3-MA and CQ could block PI3K expression, thereby regulating the PI3K/AKT signaling pathway. The decreased p-AKT protein was located across the cytoplasm or nucleus and regulated protein synthesis and gene transcription through a series of substrates [31]. As a serine/threonine protein kinase, AKT was involved in the regulation of cell growth, proliferation and apoptosis.

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PI3K was the most prominent upstream activator of AKT, and increased activities of PI3K and AKT influenced tumor development [32]. The PI3K/AKT signaling pathway normally restrained chondrogenesis [33], and the apoptosis modulated by PI3K pathway had been proposed as a potential therapy for immune-arthritis treatment [34]. Moreover, a previous study indicated that trichostatin A possessed anti-survival and anti-invasion activities in hypoxic RASFs, which was associated with PI3K/AKT signaling inactivation [35]. In our present study, PI3K/AKT pathway was involved in the proliferation and apoptosis in RA-FLS through in vivo and in vitro study.

In summary, our study showed that autophagy inhibitors could mitigate inflammation response, inhibiting RASFs cell proliferation while promoting cell apoptosis by PI3K/AKT pathway. This might add our understanding of the mechanisms in RA. However, further studies would be established to explore the deep mechanism underlying autophagy regulation in RA for new therapies.

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Disclosure of conflict of interest

None.

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