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Original Article

Bcl-2/Bax switch was flipped during apoptosis of HCT116 colon cancer cells induced by a traditional Chinese medicine, BanxiaXiexin Decoction

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Abstract: BanxiaXiexin Decoction (BXD), a commonly-used natural Chinese herbal remedy for gastrointestinal disorders, was found to be capable of improving the symptoms of colorectal cancer alongside conventional modern treatments, but the specific molecular mechanism of this clinical significance remains unknown. Here, we investigated the effect of BXD on HCT116 colon cancer cells. HCT116 colon cancer cells were treated with serum samples containing BXD and BXD metabolites by intragastrically feeding Wistar rats with BXD solutions. We found that BXD- and its metabolites-containing serum could induce apoptosis of HCT116 cells by inhibiting Blc-2-regulated cell survival and activating Bax-induced cell death signal. In addition, the combined use of the BXD serum samples and 5-fluorouracil (5-Fu), an anti-cancer chemotherapy drug, has more significant cytotoxic effect on HCT116 cells compared to single use of BXD. Furthermore, the ratio of Bax/Bcl-2 was obviously increased in BXD, 5-Fu and BXD+5-Fu groups compared with controls, demonstrating BXD had an effective role in promoting apoptosis in HCT116 cells. These results suggested the antineoplastic potential role of BXD as supplemental treatments in colorectal cancer care.

Keywords: BanxiaXiexin Decoction, apoptosis, HCT116 colon cancer cells, Blc-2, BAX, colorectal cancer, traditional Chinese medicine

Introduction

A large body of human clinical studies has proven that traditional Chinese herbal medicines offer significant treatment in various types of cancer, such as lung cancer, liver cancer, breast cancer and colorectal cancer [1]. Specifically, herbal therapies have been successfully utilized in perioperative period after colorectal surgery to relieve intestinal obstruction and reduce postoperative ileus [2]. BXD and its modified forms, one kind of traditional Chinese medicine (TCM) formulated to harmonize the stomach, have been found to be effective in improving gastrointestinal functions during treating acute and chronic enteritis, gastric ulcers, peptic ulcers, and gastroesophageal reflux disease (GERD) [3]. As indicated in several animal studies, BXD could significantly protect against dextran sulfate sodium-induced chronic ulcerative colitis, which was possibly developed into colorectal cancer, due to its capacities in anti-inflammation and anti-oxidation [4]. Similarly, the intragastrical administration of the water-soluble extracts of BXD into BALB/c mice with oxazolone (OXA)-induced colitis was found to be remarkably lower the disease activity index (DAI) and histopathological inflammation score in colonic tissue [5]. The above research demonstrated that BXD had the potential role in preventing colorectal cancer, but few reports focused on therapeutic effect of BXD in treating colorectal cancer. In our previous clinical studies, we found the treatment of BXD in patients with colon cancer showed enhanced quality of life scores and improved symptoms compared to the ones without. However, the molecular studies seeking explanations of BXD-associated symptoms improvement in colorectal cancer are lacked and require further attention.

The molecular mechanism of antineoplastic drugs usually falls into the categories such as inducing apoptosis of tumor cells by activating pro-apoptotic factors or down-regulating cell death suppressors, inhibiting angiogenesis in tumor, and intervening pathways regulating cell

survival or cell cycle progression [6]. An important drug exerted antineoplastic effects through targeting on core apoptotic machinery, which was composed of the Caspases, members of the Bcl-2 family and Apaf-1. Two death signaling pathways are involved in this machinery, one is Bax-activated mitochondria apoptotic pathway, in which Bax punctures mitochondria outer membrane, leading to loss of membrane potential and release of the cytochrome C [7], the other one is the pathway that activates the Caspase proteases. The antiapoptotic factor Bcl-2 could interfere with the apoptotic machinery by controlling mitochondrial dynamics to promote cell survival [8]. Currently, there are few studies investigating the molecular mechanism of BXD in treating diseases. Recent evidence indicated that BXD could significantly reduce the level of inflammatory factors including TNF-α, IL-1β, IL-17, IL-23, COX-2 and p-p65 while increase SOD activity and transcription factor Nrf2 expression [4], which possessed powerful anti-inflammatory and anti-oxidant effects [9], respectively. However, whether BXD could interact with this core apoptotic machinery to kill tumor cells remains unknown. To determine the underlying mechanism of BXD in colorectal cancer treatment, we measured cell viability in HCT116 human colon carcinoma cells after treating active *in vivo* metabolites of BXD and focused on apoptosis-related pathways by measuring alteration in BAX and Bcl-2 activity. Our study provided scientific evidence for development of TCM-based colorectal cancer treatments.

Materials and methods

Cell culture

The colon cancer HCT116 cells (Peking University Center for Human Disease Genomics) were cultured in DMEM medium supplemented with 10% FBS (Hyclone, USA), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2. HCT116 cells were passaged using 0.25% trypsin (Hangzhou Genom, China) upon 70% to 80% confluence (2-3 days). The cell suspension was centrifuged and further splitted in fresh DMEM medium.

Animals

5-6 week-old Wistar rats were purchased from Vital River (Beijing, China) and maintained in animal facility from Beijing Shijitan Hospital. The rats were housed under standard conditions with 12 hrs light/dark cycles and constant room temperature of 22 ± 2°C. They were fed with standard lab diet and sterilized water ad libitum. All protocols were approved by animal use and care committee in Beijing Shijitan Hospital.

BXD preparation

To mimic the in vivo microenvironment where the interactions of active ingredients of BXD and tumor cells happened, we extracted blood samples containing BXD and BXD metabolites by feeding Wistar rats with BXD solutions. The herbal recipe of BXD was composed of 12 grams Rhizoma Pinelliae Preparata (Pharmaceutical Latin), 9 grams Radix Scutellariae, 3 grams Rhizoma Coptidis, 9 grams Glycyrrhizae Preparata, 9 grams Zingiberis, 9 grams Ginseng and 9 grams Jujube. All above ingredients were prepared by Beijing Shijitan Hospital and cooked with water. The water extract was collected and further condensed into 2 g/ml.

Wistar rats were randomly divided into two groups. One group was intragastrically treated with BXD at a dose of 10 ml/kg for 4 days; the other group was intragastrically treated with equal amount of saline. 1 hr after the 4th administration, the serum samples were obtained through drawing blood from abdominal aorta, which was centrifuged at low speed and conserved at -80°C.

MTT assay

To determine the cell growth of HCT116 cells, MTT cell proliferation assay was employed. Cells were placed into 96-well plates and incubated with 200 μl DMEM medium at 3000 cells per well. 10 μl 5 g/ml MTT reagent (Sigma, USA) was added to each well. The 96-well plates were incubated for 4 hrs until the purple precipitate was visible. 150 μl DMSO was added to the plates which would be subsequently left in the dark for 2 hrs at room temperature. Then the absorbance in each wells including the blanks was measured at 570 nm in a microtiter plate reader (Bio-TEK, USA). To measure the proliferation inhibitory activity of control serum (control group), serum containing BXD and its metabolites (BXD group), 5-Fu, and the combination of 5-Fu and serum containing BXD and

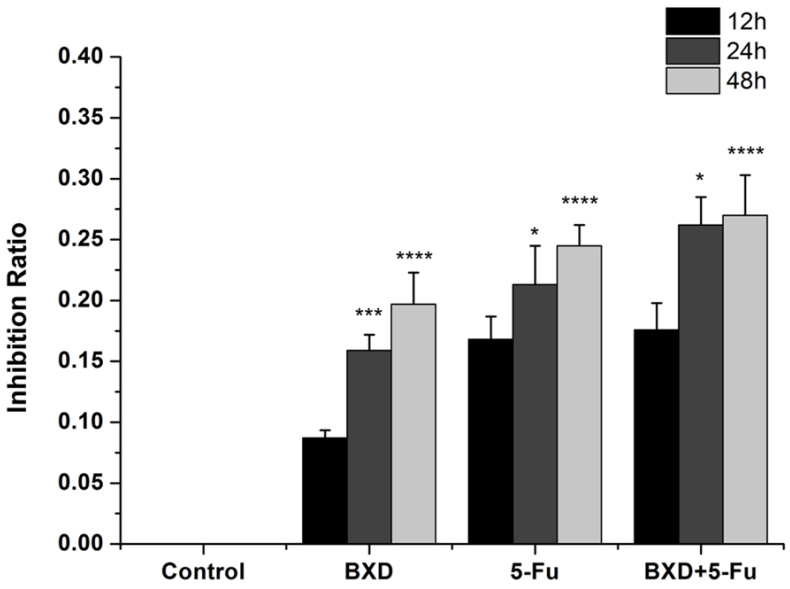

Figure 1. BXD significantly inhibited proliferation in HCT116 cells. Growth inhibitory ratios were compared among control, BXD, 5-Fu and BXD+5-Fu group with different incubation time (12, 24, 48 hrs). BXD, 5-Fu and BXD+5-Fu group showed significantly reduced cell viability in colon tumor cells compared to control group with all different incubation time (P<0.0001). Within the same time delay for incubation, BXD treatment displayed weaker inhibitory ability compared to 5-Fu group and BXD+5-Fu group, indicating that BXD and 5-Fu showed synergistic effect when in combined usage.

its metabolites (BXD+5-Fu group), the inhibition ratio was calculated by following equation after 12, 24 and 48 hrs incubation:

Inhibition ratio = $1-(OD_{treatment}-OD_{blank})/(OD_{control}-OD_{blank})$.

Flow cytometry

The detection of apoptosis in HCT116 cells was performed by Annexin V staining. Annexin V apoptosis kits (BD, USA) were used according to the protocol. HCT116 cells were cultured in DMEM medium containing 29.5 μmol/L 5-Fu, 10% serum without BXD, 10% serum containing BXD and its metabolites, the combination of 29.5 μmol/L 5-Fu and 10% serum containing BXD and its metabolites, respectively, for 48 hrs. The cells were harvested and washed with 1 × PBS and 1 × binding buffer. 5 μL of fluorochrome-conjugated FITC-Annexin V was added to 100 μL of the cell suspension which would be incubated for 10-15 minutes at room temperature without light. The cell suspensions were washed and resuspended again with 1 × binding buffer. 5 μL of propidium iodide staining solution was added. The above processed samples were further analyzed by FACS Calibur Flow Cytometer System (BD, USA).

Western blotting

HCT116 cells were seeded and incubated in DMEM medium for 24 hrs and then treated with 29.5 μmol/L 5-Fu, 10% serum containing no BXD, 10% serum containing BXD and its metabolites, the combination of 29.5 μmol/L 5-Fu and 10% serum containing BXD and its metabolites, respectively. Following 48 hrs of treatment, HCT116 cells were harvested and lysed through using RIPA solutions (APPLYGENE, China) containing protease inhibitors (1:99). The protein concentration of each sample was measured by using Lowry's method. Equal quantities of protein samples were loaded onto 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred to Immobilon-P transfer membranes (Millipore, USA). Transfer of proteins was assessed by ponceau-red staining. Non-specific binding membrane sites were blocked by incubation of blocking buffer [Tris-buffered saline (TBS) buffer containing 0.1% Tween 20 and 5% non-fat dry milk] for 1 hr at room temperature. The transfer membranes were subsequently incubated with primary antibody anti-β actin, anti-Bcl-2 (1:1000) and anti-Bax (1:1000) rabbit mAb (Cell signaling Technology, USA) at 4°C overnight. Thereafter, the membranes were washed in TBS, and then incubated with secondary antibodies goat anti-rabbit IgG (1:2000, Cell signaling Technology, USA) for 1 h at room temperature. The proteins were detected using enhanced chemiluminescence (ECL) detection system (Bio-RAD, USA). Intensity of each band was calculated by using the Image J software.

Statistical analysis

All data analyses were performed by SPSS 17.0 software (IBM, USA). The results were derived

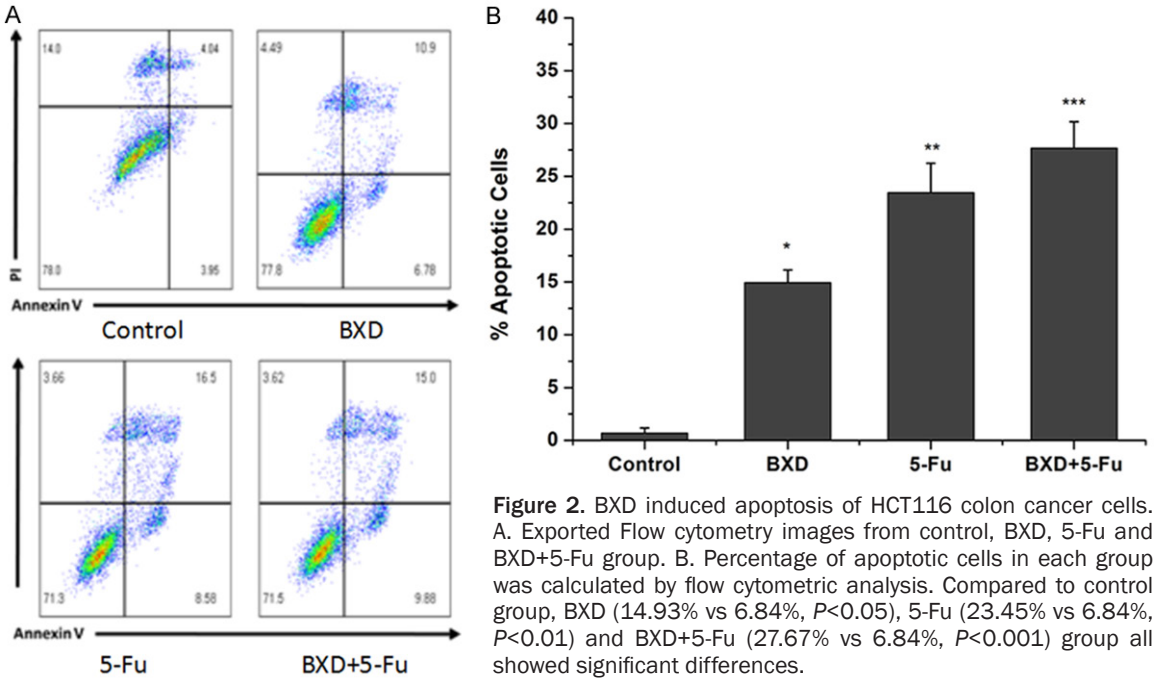

Figure 2. BXD induced apoptosis of HCT116 colon cancer cells. A. Exported Flow cytometry images from control, BXD, 5-Fu and BXD+5-Fu group. B. Percentage of apoptotic cells in each group was calculated by flow cytometric analysis. Compared to control group, BXD (14.93% vs 6.84%, *P*<0.05), 5-Fu (23.45% vs 6.84%, *P*<0.01) and BXD+5-Fu (27.67% vs 6.84%, *P*<0.001) group all showed significant differences.

from at least three independent experiments and presented as mean ± SD. Significant differences were determined using analysis of variance (ANOVA) or two-tailed t-tests. Post-hoc tests were used where appropriate. Statistical significance was set at *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Results

BXD inhibited cell proliferation in HCT116 colon cancer cells

Growth inhibition ratios of serum samples containing BXD and its metabolites, control serum samples, 5-Fu and the combination of 5-Fu and serum samples containing BXD and its metabolites were calculated by MTT assay. The result indicated that the proliferation activity of HCT116 cells was significantly reduced in the group with BXD treatment compared to the control group treated without any drug for 12 hrs (P<0.0001), 24 hrs (P<0.0001) and 48hrs (P<0.0001) incubation (**Figure 1**). Similarly, the commonly-used chemotherapy drug 5-Fu and the combination of the two drugs also potently inhibited growth of HCT116 cells following 12, 24 and 48 hrs incubation (P<0.0001). Turkey's multiple comparison tests were performed to compare the growth inhibitory effect among the different drug treatments but with same incubation time delay, and the data showed that the proliferation inhibitory activity of BXD was comparatively weaker than 5-Fu (12 hrs: P<0.001; 24 hrs: P<0.05; 48 hrs: P<0.05) and their combination (12 hrs: P<0.0001; 24 hrs: P<0.0001; 48 hrs: P<0.0001), suggesting a synergistic effect of BXD and 5-Fu on inhibiting cell proliferation when they were used together.

BXD induced apoptosis of HCT116 colon cancer cells

To investigate the circumstances of apoptosis induced by the different drugs, we further used flow cytometry by Annexin V staining (**Figure 2A**). Flow cytometric analysis was performed to present the percentage of apoptotic cells. The result demonstrated that the mean percentage of apoptotic cells in BXD group was 14.93% which was significantly higher than control group (P<0.05). Also, the percentage of apoptotic cells in 5-Fu (P<0.01) and BXD+5-Fu group (P<0.001) were significantly elevated compared to control group (**Figure 2B**).

BXD flipped Bcl-2/Bax switch to kill cancer cells by inhibiting expression of Bcl-2 and enhancing expression of Bax in HCT116 cells

An efficient mechanism of apoptosis in tumor cells involves in a process regulating cleavage of multiple intracellular proteins by Caspases [10]. The major switch that activates the pro-

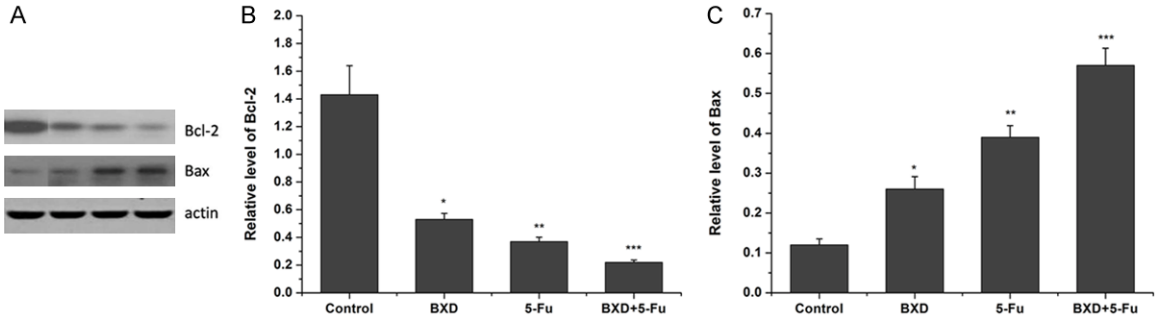

Figure 3. Inhibiting expression level of Bcl-2 and enhancing expression level of BAX during apoptosis of HCT116 cells. A: Western Blotting was used to detect the expression level of Bcl-2 and BAX in HCT116 cells. B: The level of Bcl-2 was significantly reduced in BXD (0.53 vs 1.43, $P<0.05$), 5-Fu (0.37 vs 1.43, $P<0.01$) and BXD+5-Fu group (0.22 vs 0.017, $P<0.001$) compared to controls. C: The level of BAX in HCT116 colon cancer cells was significantly elevated in BXD (0.26 vs 0.12, $P<0.05$), 5-Fu (0.39 vs 0.12, $P<0.001$) and BXD+5-Fu (0.57 vs 0.12, $P<0.001$) group compared to controls.

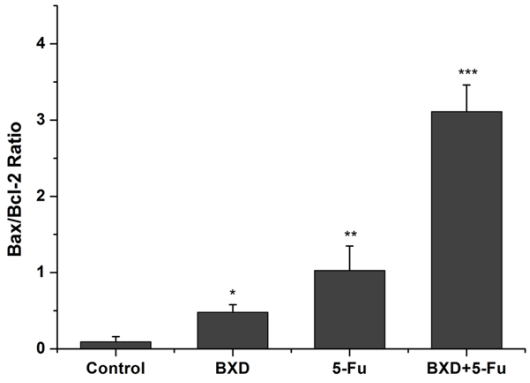

Figure 4. Bax/Bcl-2 ratio was assessed in control, BXD, 5-Fu and BXD+5-Fu groups. *$P<0.05$, **$P<0.01$, ***$P<0.001$, compared with controls.

cess is controlled by opposing components in Bcl-2 family. The functions of Bcl-2 were cell survival promotion, while Bax promoted cell suicide process. To determine whether this death-life switch for HCT116 cells was flipped by BXD to contribute to BXD-induced apoptosis, we employed western blotting to measure the expression level of Bcl-2 and Bax (**Figure 3A**). The result suggested that the protein level of Bcl-2 was significantly inhibited in BXD (P<0.05), 5-Fu (P<0.01) and BXD+5-Fu group (P<0.001) (**Figure 3B**). Meantime, the protein level of Bax in HCT116 cells was significantly elevated in BXD (P<0.05), 5-Fu (P<0.01) and BXD+5-Fu group (P<0.001) compared to controls (**Figure 3C**). Thus during the cytotoxic therapies on HCT116 cells by BXD and 5-Fu treatment, Bcl-2-regulated cell survival promotion was inhibited while Bax-mediated cell death signaling was activated.

BXD induced apoptosis of HCT116 cells by elevating Bax/Bcl-2 ratio

Previous studied have demonstrated that the Bax/Bcl-2 ratio determines cell survival or death following an apoptotic stimulus. Thus, we analyzed the ratio of Bax/Bcl-2 in control, BXD, 5-Fu and BXD+5-Fu groups. As shown in **Figure 4**, the ratio of Bax/Bcl-2 was obviously increased in BXD, 5-Fu and BXD+5-Fu groups compared with controls (**Figure 4**).

Discussions

Tumor cells have developed multiple mechanisms to become resistant to apoptosis. Accordingly, the cytotoxic drug therapy aimed at inducing tumor cells had become one of key strategies in anti-tumor drug development. TCMs have been used in treating various types of diseases for thousands of years. However, lacking of solid scientific evidence to explain their underlying mechanisms and complexity in composition of TCMs restricted them from modern clinical applications [11]. Our study provided a molecular basis for anti-tumor effect of BXD treatment, a TCM had long been used for treating gastrointestinal disorders, through demonstrating the inhibited proliferation and enhanced apoptosis of HCT116 colon cancer cells. By investigating activities of Bcl-2/Bax switch, one part of core machinery for cell programmed death, we found the expression of Bcl-2 was down-regulated and Bax was up-regulated in BXD-induced apoptosis of HCT116 cells. In addition, the combined treatment with 5-Fu and BXD has more significant cytotoxic

effect than single approach with BXD, suggesting a potential of BXD as an auxiliary medication in chemotherapy of colorectal cancer.

The bi-functional regulation of BXD in promoting Bax-mediated cell death signaling and inhibiting Bcl-2-enhanced cell proliferation in HCT116 cells was complied with a five-flavor theory which described TCMs mechanism of action. The theory divides the components of a TCM into five categories according to their flavors which imply certain properties and therapeutic action of a substance, for example, bitterness is downward-draining and precipitating while the spicy flavor is wind-dispelling and interior-warming [12]. Each compound with certain flavor would regulate disorders in body to achieve *in vivo* homeostasis. BXD has bitter and spicy flavors which could synergistically inhibit tumor growth by inhibiting cell proliferation and inducing apoptosis of tumor cells in which the components with other flavors would antagonize the above effects. This study casts light on spicy-and-bitter flavor TCMs in treating cancers, which may be helpful for developing anti-cancer medicines similar to BXD.

Even though we yielded some rudimentary conclusions on molecular basis of BXD-induced apoptosis in HCT116 cells, we realized there were some problems that remained unsettled. For instance, we used serum samples with BXD and its metabolites to simulate the *in vivo* tumor cell microenvironments, but still the pharmacological and molecular properties of active metabolites generated by intragastric administration of BXD in rats were unknown. Thus, in future, we plan to apply systematic approaches in metabolomics [13] to investigate exogenous factors and endogenous factors generated after digestion of BXD by rats, and analyze how BXD produces changes in the *in vivo* complex system that contributes to inhibition of tumor growth. Besides, in microenvironment of tumor cells, multiple signaling pathways such as NF-κB signaling [14], Notch signaling [15], and Wnt signaling [16] are involved in regulating the balance between cell proliferation and apoptosis, but the changes in Bcl-2/Bax switch interact with above signaling pathways to gate apoptosis are still needed further investigation.

In conclusion, our study contributed to the detailed mechanism of BXD-mediated apoptosis of HCT116 cells in colorectal cancer, and shed a new light on the exploration of targets against colorectal cancer through Blc-2/Bax-associated pathway. Understanding the key molecular of IDO mediating HCT116 cells apoptosis of colorectal cancer can pave the way for the development of novel and efficient TCM-based therapies of colorectal cancer treatment.

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Disclosure of conflict of interest

None.

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