Research Paper

Cardioprotective Effect of Coenzyme Q_{10} on Apoptotic Myocardial Cell Death by Regulation of Bcl-2 Gene Expression

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Abstract

Objectives: To investigate the effect of coenzyme Q10 (CoQ10) on apoptotic myocardial cell death in rat model of heart ischemia and reperfusion I/R injury. **Materials and Methods:** Eighteen rats (200–250 g) were divided into three groups of 6 rats in each. Group I (sham-operated control group): this is the control group. The animals received the surgical procedure without IR injury or any drug treatment. Group II (I/R group): ischemia was accomplished by the occlusion of coronary artery for 30 min followed by reperfusion for 45 min and Group-III (Coenzyme Q_{10} treated group): Treated with CoQ_{10} at a dose of 1 mg/kg, postoperative for 7 days before induction of IR injury. **Results:** The study revealed that pretreatment with CoQ_{10} has shown protective effect on apoptotic rat heart and agreed with earlier reports that CoQ_{10} significantly protects from oxidative stress and cytopathological changes caused by cardiac ischemia followed by reperfusion and attenuated decrease of antioxidant enzymes. Nitric oxide production in the heart of ischemic rats was significantly increased by the pretreatment with CoQ_{10} in comparison with IR group. **Conclusions:** CoQ_{10} protects against cardiac apoptosis induced by IR injury by significantly decreasing the apoptotic DNA and regulating the expression of *Bcl-2* gene.

Keywords: Apoptosis, coenzyme Q10, free radicals, myocardial infarction, necrosis, nitric oxide, oxidative stress

Introduction

Cardiovascular diseases (CVDs) are the major cause of morbidity and mortality worldwide, especially in the developing countries for both male and female. If the current trends of death due to CVDs continue, the global deaths from CVD could rise globally in next decade (approximately 7.8 million/year by 2025). This is reported that the United Nations' member states have set an objective of a 25% decline in the impulsive CVD death rate by the year 2025.[1] Continuous efforts have been made by medical community/researchers to improve the management of CVDs to decrease the morbidity and mortality.[2] Myocardial infarction (MI) is a common fatal CVD where some part of myocardium gets damage due to inadequate oxygen supply for short period followed by reperfusion causing irreversible damage to the part of the myocardium. MI is an ischemic injury due to blockage of a coronary artery.[3] The coronary artery blockage leads to an inadequate blood supply to the heart muscles which results MI and also causes permanent damage to cardiomyocytes along with the deterioration of contractility of the heart muscles and causes arrhythmias. Balanced pharmacological interventions are required to protect the myocardium due to ischemia. There has been a continuous search of the rational therapeutic interventions for the past 30 years.[4] The myocardial ischemia leads to the development of arrhythmias and necrosis of the

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myocardium.[5] The oxidative stress due to free radicals generation and apoptotic damage are major factors in myocardium ischemia which plays an important role in the development of ischemic heart diseases such as MI. A variety of qualitative and quantitative changes in the myocardium takes place due to the myocardial damage.[6-10]

Mitochondria are mainly affected in case of ischemia-reperfusion (IR)-induced injury. The free radicals cause oxidative damage to the proteins, lipids, and nucleic acids. The reactive oxygen species have played a significant role in the reperfusion injury.The tissue injury and/or death initially occurs by ischemia due to disruption in the blood supply, ultimately oxygen level. Subsequently, reperfusion restores oxygen levels but abundant generation of reactive oxygen species takes place. Therefore, reperfusion further causes myocardial tissue damage. This damage might be due to opening of the mitochondrial permeability transition pore (mPTP), that results to the imbalance of ionic homeostasis and then, finally cell death due to necrosis.[11] Apoptosis is defined as the physiological mechanism of programmed cell death in multicellular organisms. It is an essential mechanism for the normal development, the homeostasis, and to prevent the generation of tumors. Various human diseases are also linked to the apoptosis. Many therapeutic approaches are used to control the rate of apoptosis include inhibition or activation of caspases and to suppress the anti-apoptotic factors such as Bcl-2 and activation of proapoptotic receptors such as DR4 and DR5.[12-14] Coenzyme Q_{10} (CoQ_{10}) is an oil-soluble substance, well known as ubiquinone. CoQ_{10} is an essential element of the electron transport chain in the mitochondria and it contributes in the ATP production. CoQ_{10} occurs in very high concentrations in the liver, heart, kidney, and pancreas. Heart is metabolically very active and vital organ of the body. Heart needs a lot of energy for continuous work load; therefore, it contains abundant mitochondria.[15] CoQ_{10} has valuable use in the treatment and prevention of arrhythmias, hypertension, cardiovascular surgery, chronic heart failure, ischemic heart disease, arteriosclerosis, valvular heart diseases, toxin, and drug-induced cardiomyopathy such as Meniere's disease.[16-18] Nitric oxide (NO) is a free radical and is an essential cellular signaling molecule, especially act as a cardiovascular signaling molecule. NO participates in diverse physiological and pathological processes of the body. It also called as endothelium-derived relaxing factor. It is endogenously synthesized from L-arginine by the action of NO synthase enzymes family. NO plays a vital role in vascular tone and in the maintenance of blood pressure.[19] The research interest on CoQ_{10} has been increased in recent years for both basic experimental research and clinical application. Many research studies have reported the protective potential of CoQ_{10} against a variety of tissue injury. The current study was designed to evaluate the effects of CoQ_{10} administration on vasodilatation in coronary artery by NO and generation of free radicals and *Bcl-2* gene expression in apoptotic rat model of heart I/R injury.

Materials and Methods

Drugs and chemicals

The CoQ_{10} was received as generous gift sample by Tishcon Corporation, New York, USA. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). All chemicals used in this study were of analytical grade.

Animals and experimental design

Male Wistar rats weighing ranging from 200 g to 250 g were selected to undertake the current study. All animals were kept under standard laboratory conditions of temperature (25°C ± 2°C), humidity. The animals were provided 12 h light and 12 h dark of light cycle. Twenty-four rats were selected for the current research protocol and equally divided into three experimental groups having eight animals in each group. All animals were acclimatized for 1 week before commencement of the experiment. The animals were given standard pellet diet and water *ad libitum* during whole experiment. The approval for the experimental protocol was obtained from the Institutional Animal Ethics Committee (Permission No: 837/ac/04/CPCSEA). The ethical norms of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), the Government of India, were followed throughout the study.

- Group I (sham-operated control group): This is the normal control group. The animals received the surgical procedure without IR injury or any drug treatment
- Group II (I/R group): This is the disease control group. Ischemia was induced by occlusion of the coronary artery for 30 min followed by reperfusion for 45 min
- Group III (Coenzyme Q_{10}-treated group): This is the therapeutic group. All rats in this group were pretreated with CoQ_{10} at a dose of 1 mg/kg, postoperative for 7 days before induction of IR injury.

In group II (I/R group) and group III (Coenzyme Q10-treated group); the left coronary artery was ligated 2-3 mm from its origin with a suture for 15-30 min and then the ligation was released for reperfusion. The left portion of the heart (left ventricle) had taken for TEM studies and other histopathological studies.

The determination of Infarct size

The heart was removed after ischemia followed by reperfusion. The cardiac ischemic zone was determined as per the method described by Joshi *et al*., 2004 using computer-assisted plainimetry.[20]

Estimation of nitric oxide production in cardiocytes by measuring nitrite (NO_2) production

The spectrophotometric assay method of nitrite/nitrate detection based on the Griess reagent is the most popular method. In this method, a single cell suspension from the heart tissue samples was obtained immediately after the removal by mechanical disaggregation with a 50 μm filtered Medimachine system (BD Bioscience, CA, USA). The cardiocytes were washed with cold

phosphate buffer saline (PBS) to remove cell debris. The cells were transferred into petri dishes containing phenol red free culture medium (RPMI 1640) with additional 10% fetal calf serum. The culture were incubated at a temperature of 37°C in 5% CO_2 for 4 h. After 48 h, the Griess reagent (5 mL) was added to this mixture and again incubated in the dark at 30°C for 1 hour. Griess reagent was prepared by mixing equal volume (1:1) of sulfanilamide (1% in phosphoric acid) and N-[1-naphthyl] ethylenediamine-HCl (0.1% in water). Finally, the absorbance of the samples at 546 nm was measured and the concentration of nitrite was calculated using a standard curve of sodium nitrite (NaNO2).[21]

Polymerase chain reaction for amplification of Bcl-2 gene

Many factors plays important role in the choice of primers. The length of the primer and Primer melting temperature (Tm) plays vital role in the quality of polymerase chain reaction (PCR). The selection of primers and determination of primer melting temperature (Tm) are important factors to find an "ideal" primer pair. It is very difficult to choose primers for efficient amplification of elongated fragments of DNA. Primers were synthesized by integrated DNA technology. Several rules and parameters were considered for the selection of primers. The length of the primer from 18 to 25 bases long and primer' melting temperature (Tm) in between 57°C and 65°C, optimal Tm 60°C -62°C; amplification product length ranges from 200 to 500 bp were among selected parameters. The primers were manually selected by considering various criteria such as C + G content, absent; repetitive bases, repetitive sequences, >60%; stretches of >3 identical bases were not included. The sense primer was a 21-mer with sequences of CGT-CAT-AAC-TAA-AGA-CAC-CCC and the reverse primer was also a 21-mer with sequences of TTC-ATC-TCC-AGT-ATC-CGA-CTC. The product length was 234 base pair Tm of 52.3°C.

RNA isolation, cDNA formation, and amplification Bcl-2 gene

RNA isolation kit (Sigma) was used to isolate RNA. The cardiac tissue samples were treated with total RNA isolation reagent. The spectrophotometric method was used to determine the purity and concentration of the extracted RNA.

Agarose gel electrophoresis assay for apoptosis

The electrophoresis assay of DNA on agarose gel was used to find the DNA fragmentation pattern in apoptotic cells. This qualitative apoptosis assay was used to detect apoptotic DNA as per the method described by Patel.[22]

Fluorescence microscopy

Fluorescence microscopy systems are significantly more advance and accurate as compared to the light microscopy. In this method, heart sample cells from the heart were cleaned and washed with PBS, and these cells were fixed with formaldehyde (4%), PBS-buffered (pH 7), and methanol solution (1.5%) at 4°C for 15 min. The staining procedure was carried out using fluorescent substances. The sample cells (25 μL) were transferred in a PCR Tube. The acridine orange (100 μL/mL) and ethidium bromide (EB) (5 μL) were added to it. After this, the cover slips were placed on the slides coated with buffered mounting medium which consists of PBS (10%) with 0.1% NaN_3, glycerol (90%), and 3% DABCO (triethylenediamine). The examination of slides was carried out with the help of inverted Olympus microscope. Maximum excitation was carried out at 543 nm and fluorescence emissions were examined more than 570 nm.

Transmission electron microscopy of cardiac tissue

Transmission electron microscopy (TEM) is an advance technique which accomplished the imaging of objects at a significantly high resolution as compared with light microscopes. The washing of sample tissues with sodium cacodylate buffer at 4°C was done throughout the night. After washing, the specimens of the tissues were fixed with osmium tetroxide (1%) in 0.1M phosphate buffer at pH 7.4. The samples were dehydrated by ethyl alcohol and then, they were embedded in Araldite resin. Ultrathin sections of the samples (varies from 40 to 60 nm thickness) were kept on the copper mesh grids with 200 mesh size and then double stained with lead citrate and uranyl acetate and these stained sections of specimens were carefully examined using transmission electron microscope (Moragagni 268D by Netherlands). The photographs were also taken for the study and inference.

Statistical analysis

The results were presented as mean ± standard deviation; all statistical analysis were carried out by one-way ANOVA using SPSS 11.0 by one-way ANOVA followed by Bonferroni test and Student-Newman-Keuls test. The level of statistically significance of the results was accepted at the value of $P < 0.05$.

Results

The infarct size in different groups is given in Table 1 and shown in Figures 1 and 2. The Infarct size in the sham-operated group was found to be 5.71% of the total heart surface [Figure 2a].

The Infarcts size in IR injury group was increased and found to be 65% of the total heart surface as shown in Figure 2b. The Infarct size in the CoQ_{10}-treated rat heart was decreased to 26.13% and NO production significantly increased after 15 min

Table 1: Effect of coenzyme Q_{10} on Infarct size and nitric oxide production in apoptotic myocardial cell death in rats

Groups	Treatment	Infarct size (%)	NO production
Group I	Sham operated	5.71±0.81	13.34±5.64
Group II	IR injury	65.60±7.34**	9.07±2.54*
Group III	CoQ_{10} treated	26.13±4.23##	11.45±4.13##

Results are expressed as mean±SD (n=6). Significantly different (*P<0.05, **P<0.01) from sham operated rats. Significantly different (##P<0.01) from IR injury group. Production of NO in ischemic rat heart increased significantly (P< 0.01) by CoQ10 as compared to I/R group and ‡Expressed as nmol/min/mg of heart tissues. CoQ10 = Coenzyme Q10, NO = Nitric oxide, SD = Standard deviation, IR = Ischemia-reperfusion

of ischemia, followed by reperfusion for 45 min [Figure 2c] in comparison with IR injury group.

Morphological changes in physical appearances of heart were observed at the different phases of ischemia followed by reperfusion. After 30 min, complete ischemic heart turned to complete black due to prevention of circulation of blood by coronary artery ligation and after reperfusion (opening ligation), blood forcibly entered the cardiac circulation and discoloration of cardiac muscle was observed; but 45 min after reperfusion a few permanent ischemic zones were observed; but after 45 min reperfusion in some portion of heart, permanent ischemic zone was observed (black coloration).

The amplification of antiapoptotic *Bcl-2* gene and GPDH gene housekeeping was carried out using RT-PCR and separation was done by electrophoresis by staining with EB. The length of RT-PCR product of *Bcl-2* was 234 bp. The *Bcl-2* gene expression in sham-operated group was maximum as compared to other group. There was a significant increase in the Bcl-2 gene expression in the animal treated with CoQ_{10} as compared with IR injury group [Figure 3].

The apoptosis level in the heart of IR injury rats was also determined. Apoptotic DNA level was increased in IR injury rats as compared to sham-operated control rat. The significantly increased apoptotic DNA level that occurred after ischemia followed by reperfusion in the vehicle treated rats was significantly decreased by the administration of CoQ_{10}. The apoptotic DNA level of CoQ_{10}-treated rats was found to be nearly to the sham-operated control rats [Figure 4].

In electron microscopic studies, the results revealed the protective effect of CoQ10 on ischemic-reperfused rat's heart [Figure 5]. The morphology of cardiomyocytes in sham-operated group was seen as normal with intact and abundant cell organelles [Figure 5a]. The IR injury group showed swollen mitochondria, increased endoplasmic reticulum (ER), and dilated lysosome (L). The matrix cleared out, cristae disappeared, and nucleus were not clearly noticeable in IR rats (Figure 5b). CoQ10-treated group showed prominent nucleus (N), healthy mitochondria (M), and endoplasmic reticulum (ER) (Figure 5c).

The results of fluorescence microscopy studies showed the effects of CoQ10 on apoptosis cells death in rat heart induced by ischemic and reperfusion injury [Figure 6]. In sham-operated rats group, green fluorescence means that viable cells were found maximum in number and red fluorescence means that apoptotic cells were found to be lowest in number as compared to other groups [Figure 6a]. Cell death in heart induced by IR injury in control rat group were revealed by decreased green nuclear fluorescence (viable cells) and increased red nucleus (apoptotic cells) [Figure 6b]. Green fluorescence (viable cells) was shown to be increased in number and red fluorescence (apoptotic cells) was significantly decreased in CoQ10-treated group as compared to sham-operated group [Figure 6c].

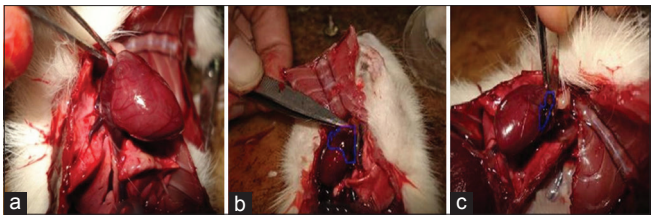

Figure 1: (a): Sham-operated rat heart. (b) Ischemia and reperfused rat heart. (c): Coenzyme Q_{10}-treated rat heart

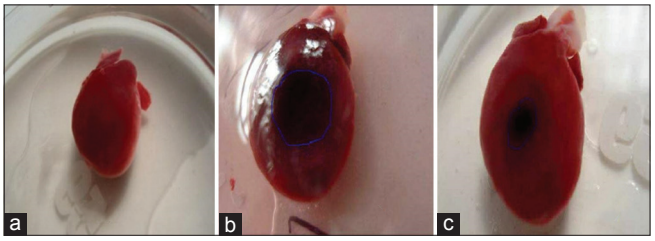

Figure 2: The Infarct size in different groups: (a) Sham-operated rat heart. (b) Ischemia and reperfused rat heart, (c) coenzyme Q_{10}-treated rat's heart

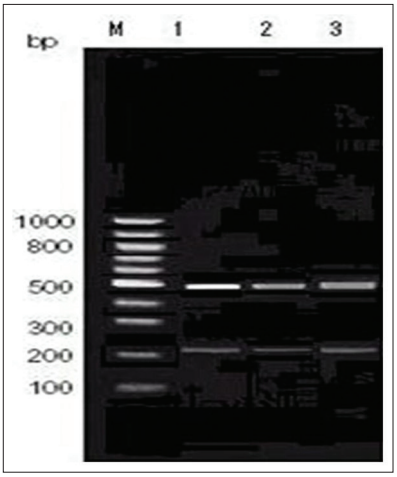

Figure 3: Shows *Bcl-2* mRNA expression using real-time-polymerase chain reaction method. Lane M: Marker, Lane 1: mRNA of sham-operated rat's heart, Lane 2: mRNA of coenzyme Q_{10} rat's heart, Lane 3: mRNA of ischemic and reperfused--treated rat's heart

Discussion

In present investigations, the cardioprotective effect of CoQ_{10} on apoptotic myocardial cell death in experimental rats and study of the regulation of Bcl-2 gene expression in the cardiac cells were evaluated. The results indicated the cardioprotective effect of CoQ_{10} against apoptotic myocardial cell death in rat model of heart IR injury. Ischemia of the myocardium occurs as result of an inadequate blood supply to the heart muscles, which leads to arrhythmias and cardiac necrosis.[3] MI is the irreversible damage due to lack of oxygen supply when blood flow stops to a part of the myocardium for some time followed by reperfusion causing irreversible damage to the heart muscle.[23] The necrosis plays its role during ischemia, while apoptosis plays its role during reperfusion. In fact, reperfusion followed by ischemia causes further damage as compared to the ischemia alone.

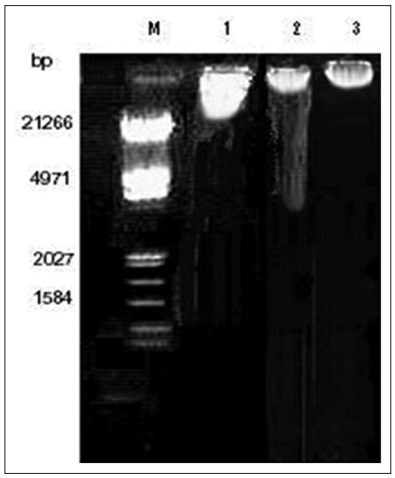

Figure 4: DNA levels in different studied groups

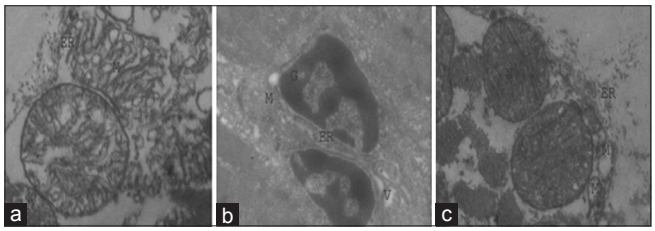

Figure 5: Transmission electron microscopic studies of left ventricle showing the effect of coenzyme Q_{10} on ischemic-reperfused rat's heart. (a) Sham-operated group; (b) IR injury group (c) CoQ10-treated group

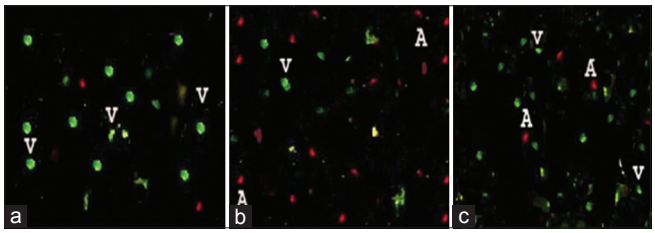

Figure 6: Effects of coenzyme Q_{10} on apoptosis cells death in rat's heart induced by ischemic and reperfusion injury (fluorescence microscopy). (a) Represents sham-operated group; (b) represents IR group; (c) c represents coenzyme Q_{10}-treated group

In the present study, ischemia produced by coronary artery occlusion for 30 min followed by reperfusion for 45 min as described by Kato *et al.,* 2004.[24] Morphological changes in physical appearances of heart were observed at the different phase of ischemia followed by reperfusion. After 30 min, complete ischemic heart turned to complete black due to prevention of circulation of blood by coronary artery ligation and after reperfusion (opening ligation) blood forcibly entered cardiac circulation and discoloration of cardiac was observed; but after 45 min reperfusion in some portion of heart, permanent ischemic zone was observed (black coloration). In our study, it was observed that the Infarct size was significantly ($P < 0.01$) reduced in the CoQ_{10}-treated group as compared to the IR group. It may be due to potent antioxidant activity of CoQ_{10}, which prevents both the initiation and propagation of lipid peroxidation.[25] Apoptosis is believed to be controlled by a *Bcl-2* family of proteins. The Bcl-2 gene family has proapoptotic or antiapoptotic actions. The Bcl-2 gene can also help in the survival of the cells by antiapoptotic action The changes in bcl-2 gene expression studied after the IR injury. This is also reported that overexpression of *Bcl-2* gene may decrease the cardiac apoptosis after reperfusion and cause protection of heart tissues against IR injury.[26] In this study, apoptosis is believed to be controlled by a *Bcl-2* family of proteins. In undertaken study, the pretreated rats with CoQ_{10} showed the upregulation of the *Bcl-2* gene expression against IR injury. It indicates the cardioprotective potential of CoQ_{10} against IR injury-induced cardiac apoptosis by upregulation of the *Bcl-2* gene expression. The cardioprotective mechanism by reduction in the apoptotic cell death by upregulation of the *Bcl*-2 gene might be due to the antioxidants property of CoQ_{10}.[27] Determination of apoptosis level in IR-injured heart was evaluated by agarose gel electrophoresis. In our study, apoptotic DNA level was increased in IR injury rats as compare to sham-operated control rat. The administration of CoQ_{10} significantly decreases the increased level of apoptotic DNA level due to ischemia followed by reperfusion (IR group). DNA level of CoQ_{10}-treated rats exhibited nearly to sham-operated control rats. It was reported that release of cytochrome-C into cytoplasm and caspase-9 activation triggers caspase cascade that culminates apoptotic DNA laddering. All these apoptosis execution-related events occurred in experimental model and were substantially prevented by treatment with CoQ_{10}.[28] Apoptosis was also measured by florescence microscopy, showing reduction in apoptotic cells in CoQ_{10}-pretreated group as compared to rats IR injury. Green fluorescence, i.e., viable cells, was increased and red fluorescence, i.e., apoptotic cells, was significantly decreased in CoQ_{10} pretreated group as compared to IR-treated group. The cardioprotective effect of CoQ_{10} was also demonstrated by changes of cell morphology detected by light microscopy and ultramicroscopy, quantification of living and apoptotic cells, and analysis of ATP cellular levels significantly lowers the number of cumulative apoptotic events and enhances the number of living cells.[29] In TEM studies, the results revealed the protective effect of CoQ_{10} on ischemic-reperfused rat's heart. The present study showed the protective effect of CoQ_{10} against IR injury-induced cardiac apoptosis by decreasing the apoptotic DNA, protective antioxidant activity, increased NO production, regulation of the *Bcl-2* gene expression.

Conclusions

The pretreatment of rats with CoQ_{10} exhibited good protection against IR injury. This study revealed that CoQ_{10} might protect against cardiac apoptosis induced by IR injury by decreasing the apoptotic DNA and have role in the regulation of *Bcl-2* gene expression in the cardiac cells and evidently indicated that the CoQ_{10} had cardioprotective effect.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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