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Selective toxicity of Caspian cobra (*Naja oxiana*) venom on liver cancer cell mitochondria

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ABSTRACT

Objective: To explore the cytotoxicity effects of Caspian cobra (*Naja oxiana* or *N. oxiana*) venom on hepatocytes and mitochondria obtained from the liver of HCC rats.
Methods: In this study, HCC was induced by diethylnitrosamine (DEN), as an initiator, and 2-acetylaminofluorene (2-AAF), as a promoter. Rat liver hepatocytes and mitochondria for evaluation of the selective cytotoxic effect of *N. oxiana* venom were isolated and mitochondria and cellular parameters related to apoptosis signaling were then determined.
Results: Our results showed a raise in mitochondrial reactive oxygen species (ROS) level, swelling in mitochondria, mitochondrial membrane potential ($\Delta\psi$m) collapse and release of cytochrome c after exposure of mitochondria only isolated from the HCC group with the crude venom of the *N. oxiana* (12.5, 25, and 50 μg/mL). This crude venom also induced caspase-3 activation ($P < 0.001$) in the hepatocytes obtained only from the HCC rat liver.
Conclusions: Based on the over all results, we suggested that *N. oxiana* may be considered as a promising complementary therapeutic agent for the treatment of HCC.

1. Introduction

Cancer is a complex disease that is characterized by cell proliferation, cell transformation and disruption apoptotic process [1]. Studies have shown that this disease is one of the important causes of mortality and nearly 13 million cancer cases and 7.6 million cancer deaths are estimated to occur annually in the world [2]. Liver cancer represents one of the most common malignancy globally. Hepatocellular carcinoma (HCC) represents a major form of primary liver cancer in adults. It is the second and the sixth leading cause of cancer related death in males and females, respectively. The most important risk factors are hepatitis B and C infections. In patients for whom HCC could not be detected early, current treatments show poor tolerance and low efficacy. Also, the treatment of cancer is still a big challenge in medicine. So finding effective natural medicine, which has anti- HCC effect is of great significance undoubtedly [3–5]. The ideal anti-cancer candidates would have a tendency to kill cancer cells without affecting normal cells. Despite these efforts, anticancer drugs also have a side effects on normal cells [6].

Many studies have shown that snake venom is a complex mixture of proteins and peptides [1,7,8]. On the other hand, it is well known that these active components of snake venoms have a therapeutic potential [7,9]. In recent decades, snake venom has been used as a drug [1]. As previously described, there are several reports in the literature indicating the therapeutic value of *Naja* sp. venoms on different types of

cancer [1,10]. Previous studies suggest that the anticancer effects of snake toxins are through various mechanisms, including induction of apoptosis. The Caspian cobra (*Naja oxiana*), also called the Central Asian cobra, is a highly venomous species of cobra in the family Elapidae found in Central Asia. It has been shown that *Naja oxiana* have various functions and biological properties (anti-cancer) through apoptosis [2,11,12].

Several biochemical markers have been identified in the induction of apoptotic cell death, including an increase of reactive oxygen species (ROS), collapse of mitochondrial membrane potential (MMP) and changes in the level of Bcl-2 and Bax proteins in mitochondria. In the intrinsic apoptotic cell death pathway, ROS are potent inducers of oxidative damage and have been suggested as main regulators of apoptotic cell death. Remarkably, intracellular ROS increase prior to cytochrome c release from mitochondria during the activation of apoptotic process [12].

Studies have shown that, the genetic variations in the HCC disease lead to a lack of balance and regulation of the pro-apoptotic (such as the BH-3-only family) and anti-apoptotic (such as Bcl-XL and Mcl-1), members of the Bcl-2 family [13,14]. So that, over expression of several physiological pro-apoptotic proteins are decreased in HCC (such as, Bax and Bid); however, Bcl-XL as an anti-apoptotic protein is over expressed in the HCC. Also, it has been shown that levels of XIAP (X-linked inhibitor of apoptosis protein, as an inhibitor of caspases) in the patients with HCC were increased [13]. Hence, therapeutic methods to inhibit anti-apoptotic signals in HCC cells might have the potential to provide powerful tools in the future to treat in the patients with HCC [13,14].

The functions and the cytotoxicity mechanisms of crude venom of the *N. oxiana* on hepatocytes and mitochondria isolated from the HCC rat model by the DEN and 2-AAF were not completely reported till now. This study focused on the apoptotic effect of crude venom of the *N. oxiana* on hepatocytes and mitochondria obtained from the liver of HCC rats and the detailed mechanism.

2. Materials and methods

2.1. Crude venom

Crude venom of the *N. oxiana* was supplied from the Razi Vaccine and Serum Research Institute (Karaj, Iran).

2.2. Animals

The rats (male Sprague–Dawley) were acquired from the Institute Pasteur (Tehran, Iran). Then, all animals were kept in a temperature-controlled environment on a 12:12 h light/dark cycle with free access to food and water. All experiments were conducted according to the instructions of ethical standards and Institutional Animal Care and Use Committee (IACUC) of Shahid Beheshti University of Medical Sciences in Tehran, Iran.

2.3. The research model

In this study, the animals were placed in two separate groups (*n* = 10); group A as a normal group and group B as an HCC group. We induced HCC by DEN with a single injection (200 mg/kg body weight) through intraperitoneal (*i.p.*) route and

daily oral dose of 2-AAF (0.02%, w/w) for 14 days [15]. As per our previous published studies, HCC was confirmed through the histopathological evaluations, determinations of blood amounts of ALT, AST and ALP and finally alpha-fetoprotein (AFP) as a specific HCC marker [16–18].

2.4. Hepatocytes and mitochondria isolation

At Week 15, post HCC induction, the rats were anaesthetized with Ketamine (80 mg/kg, *i.p.*) and xylazine (5 mg/kg, *i.p.*) and then rat liver hepatocytes were isolated using the two-step collagenase liver perfusion technique. In order to evaluate cellular integrity (or viability), the trypan blue exclusion test was performed. Then, we prepared mitochondria from hepatocytes (30×10^6 cells). For the preparation of mitochondria, the hepatocytes were then pelleted ($300 \times g$ for 3 min) and re-suspended in 10 mL of Solution A [16–18]. Next, frozen at −80 °C for 10 min to break the plasma membrane, then centrifuged at $760 \times g$ for 5 min. The supernatant was kept while the pellet was homogenized for 10 min, followed by centrifugation at $760 \times g$ for 5 min. The supernatants from the previous two steps were combined and centrifuged for 20 min at $8\,000 \times g$. The final mitochondrial pellets were suspended in Tris buffer.

2.5. Succinate dehydrogenase (SDH) activity assay

The activity of SDH was measured by MTT test. To perform this test, the mitochondrial suspension from both groups were incubated with all applied concentrations of crude venom of the *N. oxiana* (0–100 μg/mL) at 37 °C for 30 min. In the next step, 50 μL of MTT (0.4%) was added to the medium and incubated at 37 °C for 30 min. Eventually, the product of formazan crystals was dissolved in 100 μL DMSO and the absorbance at 570 nm was measured with an ELISA reader (Tecan, Rainbow Thermo, Austria) [19].

2.6. ROS formation assay

In this study, isolated mitochondria from both groups were suspended in respiration assay buffer. Then, DCFH (Fluorescent probe) used for the ROS formation from mitochondria at the EXλ = 488 nm and EMλ = 527 nm [16,18].

2.7. MMP assay

At the first step, the mitochondria from both groups were isolated and then were suspended in the MMP assay buffer. Eventually, Rhodamine 123 (Rh 123), as a mitochondrial specific fluorescent probe, used for the MMP assay at the EXλ = 490 nm and EMλ = 535 nm [18].

2.8. Mitochondrial swelling assay

Briefly, isolated mitochondria from both groups were suspended in swelling assay buffer. In the next step, mitochondrial suspension was incubated with 12.5, 25 and 50 μg/mL of crude venom of the *N. oxiana*. Eventually, absorbance was measured at 540 nm. The decrease in absorbance represents increasing swelling in the mitochondria [16].

2.9. Release of cytochrome c

Briefly, the Quantikine Rat/Mouse Cytochrome c Immunoassay kit (R&D Systems, Inc., Minneapolis, MN, USA) was used for determination of cytochrome c release.

2.10. Caspase-3 activity assay

In this study, the level of caspase-3 activity in both groups was measured by using the Sigma's caspase-3 colorimetric assay kit (Sigma–Aldrich, Taufkirchen, Germany). The concentration of the p-nitroaniline released from the substrate at 405 nm used for assay caspase-3 activity.

2.11. Statistical analysis

The data were shown as the mean ± SD. The One- and Two-way ANOVA analysis (GraphPad Prism software, version 5) were used for the determination of differences between the mean values. $P < 0.05$ was considered to display a statistically significant difference.

3. Results

3.1. Effect of DEN/2-AAF on body weight and liver weight

As shown in Table 1, our results showed that the mean of body weight of the normal group (A) rats [258.10 ± 9.55) g] was considerably higher than that of the cancerous group (B) rats [203.17 ± 6.85) g]. Furthermore, in the cancerous group, the mean of liver weight of rats was considerably higher than that of the normal group rats (Table 1).

3.2. Effect of DEN/2-AAF on AFP, AST, ALT and ALP

Our results suggest that the levels of AST, ALT, ALP and AFP concentrations in the serum of cancerous group was considerably higher than of the normal group (Table 2).

Table 1
Effect of DEN/2-AAF regimen on final body and liver weights (g).

Group	Final body weight	Liver weight
Untreated control group (A)	258.10 ± 9.55	9.07 ± 0.81
HCC group (B)	203.17 ± 6.85[***]	12.25 ± 0.65[*]

[*]: $P < 0.05$, [***]: $P < 0.001$ compared with the untreated normal group.

Table 2
Effects of DEN/AAF regimen on the ALP, ALT, AST and AFP in serum.

Group	ALT (IU/L)	AST (IU/L)	ALP (IU/L)	AFP (IU/L)
Untreated control group (A)	82 ± 12	87 ± 8	590 ± 22	0.19 ± 0.09
HCC group (B)	714 ± 65[***]	600 ± 28[***]	781 ± 35[***]	3.08 ± 0.76[*]

All results were reported as mean ± SD. [*]: $P < 0.05$, [***]: $P < 0.001$ compared with the untreated normal group.

3.3. Effect of N. oxiana on SDH activity

In the normal group, the mentioned concentrations of N. oxiana venom did not cause a considerable decrease in the SDH activity (Figure 1A). As shown in Figure 1B, in the HCC group all applied concentration of N. oxiana venom (0–100 µg/mL) at a dose dependent manner induced significant ($P < 0.05$) decrease in the SDH activity.

3.4. Effect of N. oxiana on ROS level

In our study, results showed that the rate of ROS formation in the mitochondria from the HCC rat group significantly ($P < 0.05$) increased compared to the corresponding control in a time and concentration dependent manner after 1 h of incubation (Figure 2). However, in comparison with the HCC rat group, ROS formation did not significantly increase in the mitochondria from normal group compared with the corresponding control after 1 h of incubation.

3.5. Effects of N. oxiana on MMP

As shown in Figure 3, there was a significant difference ($P < 0.05$) in the MMP collapse after exposure to N. oxiana venom at all applied concentrations only in the mitochondria from the HCC rat group. While, there was no significant effect of N. oxiana on MMP levels in the mitochondria from the normal group as compared with corresponding control.

3.6. Effect of N. oxiana on mitochondrial swelling

In the HCC group, the addition of different concentrations of N. oxiana venom (25 and 50 µg/mL) to mitochondrial suspensions induced significant ($P < 0.05$) swelling in the mitochondria (Figure 4). Whereas, N. oxiana at venom lowest concentration (12.5 µg/mL) did not show this effect. Also, N. oxiana venom at all applied concentrations did not induce swelling in the mitochondria.

3.7. Effect of N. oxiana on cytochrome c release

As shown in Figure 5, N. oxiana (25 µg/mL) induced a significant ($P < 0.05$) release of cytochrome c only in the mitochondria isolated from the HCC group. Also, The results showed that pretreatment of N. oxiana (25 µg/mL)-treated mitochondria from the HCC group accompanied by cyclosporine A (CsA), as a MPT inhibitors like, and butylated hydroxyl toluene (BHT), as a antioxidants, inhibited cytochrome c release as compared with N. oxiana-treated HCC group.

3.8. Effects of N. oxiana on caspase-3 activation

Our result suggest that N. oxiana venom at a concentration of 25 µg/mL caused a considerable activity of caspase-3 only in the hepatocytes isolated from the HCC group (Figure 6).
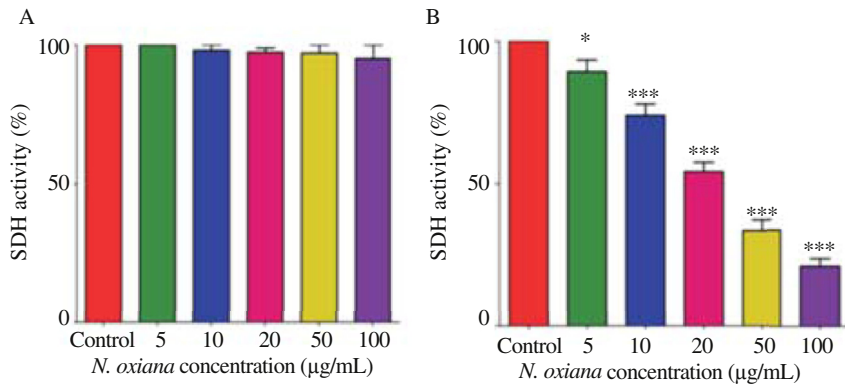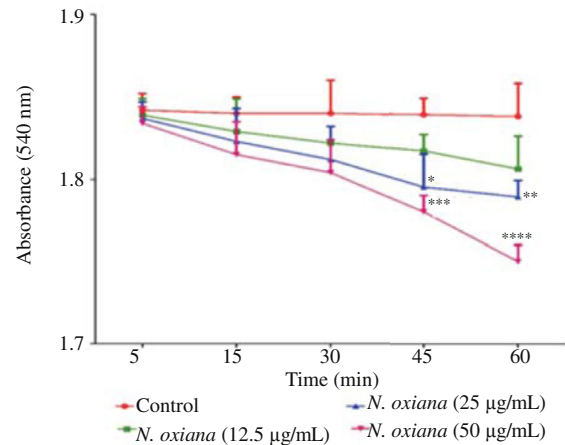
Figure 1. SDH activity assay.

The effect of different concentrations of *N. oxiana* venom on SDH activity in the liver mitochondria obtained from hepatocytes of the normal (A) and HCC (B) groups. Data are showed as mean ± SD ($n = 3$). *: $P < 0.05$, ***: $P < 0.001$ compared with the control group.
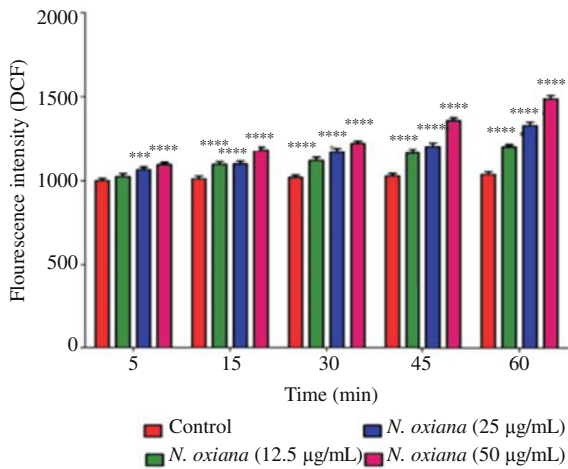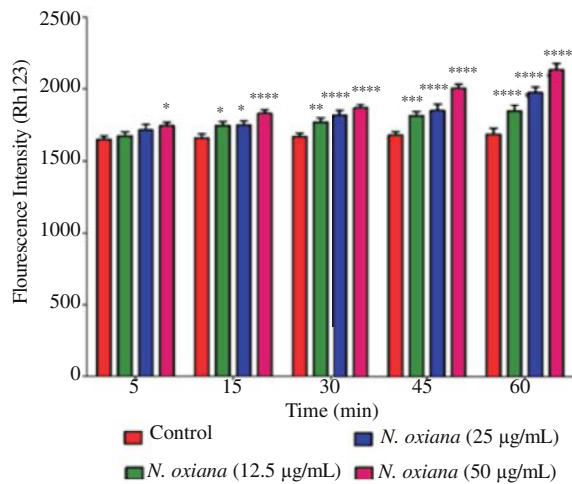

Figure 2. ROS formation assay.

The effect of different concentrations of *N. oxiana* venom (12.5, 25 and 50 μg/mL) on ROS formation in the liver mitochondria obtained from hepatocytes of the HCC group. Data are showed as mean ± SD ($n = 3$). ***: $P < 0.001$, ****: $P < 0.0001$ compared with the corresponding control.


Figure 4. Mitochondrial swelling assay.

The effect of different concentrations of *N. oxiana* venom (12.5, 25 and 50 μg/mL) on mitochondrial swelling level in the liver mitochondria obtained from hepatocytes of the HCC group. Data are showed as mean ± SD ($n = 3$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$ compared with the corresponding control.


Figure 3. Mitochondrial membrane potential (MMP) assay.

The effect of different concentrations of *N. oxiana* venom (12.5, 25 and 50 μg/mL) on MMP level in the liver mitochondria obtained from hepatocytes of the HCC group. Data are showed as mean ± SD ($n = 3$). *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, ****: $P < 0.0001$ compared with the corresponding control.
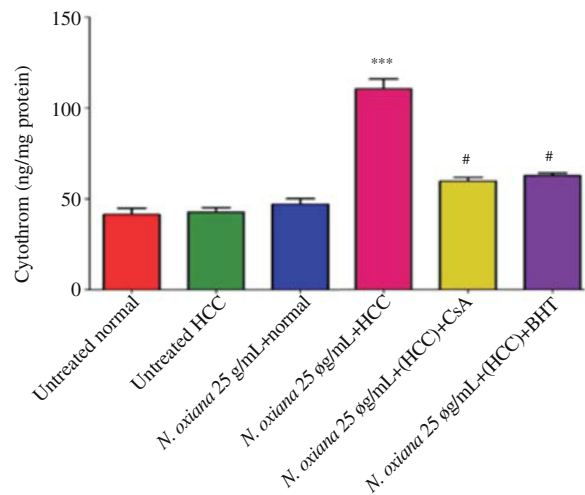
Figure 5. Cytochrome c release assay.

The amount of expelled cytochrome c from the mitochondrial fraction into the suspension buffer was determined using a rat/mouse cytochrome c ELISA kit. Data are presented as mean ± SD ($n = 3$). ***: $P < 0.001$ compared with the corresponding control. #: $P < 0.001$ compared with *N. oxiana* venom (25 μg/mL) treated HCC group.
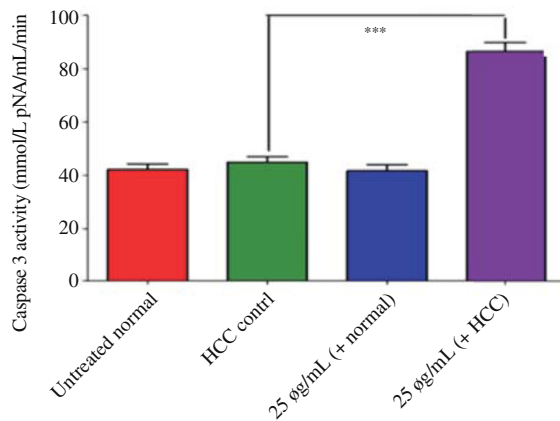
Figure 6. Evaluation of caspase-3 activity.
The caspase-3 activity was measured by using Sigma–Aldrich kit. Caspase-3 activation in the both HCC and untreated control rat hepatocytes following the exposure to *N. oxiana* venom (25 μg/mL). Data are presented as mean ± SD (*n* = 3). ***: *P* < 0.001 compared with corresponding control HCC group.

4. Discussion

Today, cancer is one of the causes leading to death globally and therefore there is a very urgent need for discovering a new therapy. Current techniques for cancer treatment included chemotherapy, surgery and radiation therapy [20]. Currently, many therapeutic techniques were used for the treatment of liver cancer, but these therapeutic techniques have not been successful [17]. Therefore, the designation of choice therapy with high potency and efficacy has led to the raised use of anticancer agent developed from natural resources [20].

In the last years, some researches have provided a document that bio-toxins present high potential as anti-cancer candidates, such as snake venom and so can be used as chemotherapeutic agents [21]. Snake venoms in general are highly attractive for researchers involved in the development of anti-cancer drugs. On the other hand, understanding of the molecular mechanisms of snake venoms has considerably contributed to the investigations. Snake venom is a complex of compounds with specific chemical and biological activities and used in the treatment of various types of cancer. It was reported that, snake venom is able to induce cell death in various cancer cells without effect on normal cells [22]. The mechanisms of cell death by snake venom are including: raising calcium ion influx, causing cytochrome c release from mitochondria to cytosol and cancer cell apoptosis [22]. HCC is one of the most common and deadly malignancies in the global [23]. It is recognized that, the genetic variations in this disease are leading to an imbalance in the pro-apoptotic and anti-apoptotic, members of the Bcl-2 family [13]. In the recent year, the considerable efforts have been made to prevent and treat HCC, and the induction of apoptosis has been measured as a promising therapeutic approach for patients with HCC [23].

It is well documented that, mitochondrial organelles play very important regulatory role in processes such as apoptosis. Due to the mitochondrial structural and functional differences between normal and cancer cells (such as liver cancer), these organelles could offer a unique potential for the designation of anticancer agents that deliver mitochondrial targeting drugs [16,18]. In our study it was shown that, *N. oxiana* induced a

significant decrease in the activity of complex II (SDH) only in the mitochondria isolated from the HCC rats.

The results of this study show that *N. oxiana* significantly raised ROS generation level only in mitochondria from the HCC group. A previous research showed that snake venom LAAOs could induce an increase H_2O_2 level. Our results are also in agreement with previous studies [22,24]. Some documents have indicated that ROS at physiological low levels play an important role (as "redox messengers") in intracellular signaling and regulatory pathways, but at higher levels cause the oxidation of cellular macromolecules and promote apoptotic cell death through the mitochondrial oxidative stress (OS) pathways [23].

Apoptosis process through the mitochondrial pathway induces MMP loss, cytochrome c release, and the activation of caspase-3 as an executioner caspases. A number of researches have shown that the activation of the apoptotic pathway in malignant cells is a main protective mechanism against the development and progression of cancer [23]. The following, results of our study showed that *N. oxiana* caused a significant decline in the level of MMP only in mitochondria from the HCC rat group. It is well known that $\Delta\psi$m is a vital variable toward the adjustment of mito-chondrial function, also its decline is the critical point of death signaling (especially apoptosis) [25,26]. Also, level of swelling in mitochondria as an index of MPT pore opening was furthermore evaluated in this research. *N. oxiana* extracts caused significant mitochondrial swelling only in the mitochondria the HCC rat group. The following, to prove the induction of apoptosis by *N. oxiana*, the release of cytochrome c from mitochondria to media buffer as subsequent events after mitochondrial swelling and collapse of MMP was also determined during this study. The most important result was that *N. oxiana* caused considerable release of cytochrome c only from the mitochondria cancerous. Also, cytochrome c release was prevented by BHT (a ROS scavenger) and Cs.A (an inhibitor of MPT pore). These events implies that ROS and also the changing of the MPT poreplay vital roles in the induction of apoptosis by *N. oxiana*.

Cellular apoptosis signaling could usually be initiated either through the cell death receptor-mediated extrinsic pathway or the mitochondrial-mediated intrinsic pathway [23]. Following the addition of crude venom from *N. oxiana*, to assess whether we could cause apoptosis only in the hepatocytes from HCC rat, caspase-3 activity was measured. The study showed that *N. oxiana* caused considerable caspase-3 activation only in hepatocytes from HCC group. Previous reports indicate that snake venom LAAOs could activate the proteolytic activity of caspases [22,24]. These results support the anticancer effects of snake venom.

Eventually, the results of our study showed that crude venom from *N. oxiana* raise the mitochondrial ROS level via the disruption of mitochondrial respiratory chain only in the mitochondrial from the liver of HCC rats. This process resulted in a decline of the MMP, alteration of mitochondrial swelling and release of cytochrome c, which can induce starting apoptosis signaling via caspase-3 activation in liver hepatocytes of HCC rats. Also, the results of our study show that raise of the ROS level proposed as important regulators of mitochondria-mediated apoptosis.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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