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SHORT COMMUNICATION

Oral Administration of *Aloe vera* and Honey Reduces Walker Tumour Growth by Decreasing Cell Proliferation and Increasing Apoptosis in Tumour Tissue

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Cancer is diagnosed in approximately 11 million people and is responsible for almost 8 million deaths worldwide every year. Research in cancer control has shown the importance of co-adjuvant therapies. *Aloe vera* may reduce tumour mass and metastasis rates, while honey may inhibit tumour growth. This study verified the influence of *Aloe vera* and honey on tumour growth and in the apoptosis process by assessing tumour size, the cell proliferation rate (Ki67-LI) and Bax/Bcl-2 expression at 7, 14 and 20 days after Walker 256 carcinoma implant in Wistar rats distributed into two groups: the WA group – tumour-bearing rats that received a gavage with a 670 μL/kg dose of *Aloe vera* and honey solution daily, and the CW group – tumour-bearing rats which received only a 0.9% NaCl solution. The effect of *Aloe vera* and honey against tumour growth was observed through a decrease in relative weight (%) and Ki67-LI in tumours from the WA group compared with those from the CW group. The Bax/Bcl-2 ratio increased in tumours from the WA group at all tested timepoints. These data suggest *Aloe vera* and honey can modulate tumour growth by reducing cell proliferation and increasing apoptosis susceptibility. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: Aloe vera; honey; cancer; proliferation; apoptosis.

INTRODUCTION

Cancer is diagnosed in approximately 11 million people and is responsible for almost 8 million deaths worldwide every year. Starting from a single cell that is transformed by a multistage process, the genesis of cancer is mainly characterized by the rapid proliferation of abnormal cells, invasion of adjacent tissues and spread to distant organs (metastasis) (World Health Organization, 2009).

Cancer growth could be linked to two major abnormal cell characteristics: unrestrained cell proliferation and insufficient apoptotic turnover (Nicholson, 2000).

The nuclear protein Ki-67 is expressed during the cell cycle in all the proliferation phases (G1, S, G2 and mitosis) but is absent in the resting phase (G0). These expression patterns make Ki-67 an excellent and commonly used marker to identify the 'growth fraction' of a cell population. According to several studies of some types of tumours, the Ki-67 labelling index (Ki67-LI), expressed as the percentage of immuno-reactive tumour cells among the total counted tumour cells, seems to have a prognostic value for survival and tumour recurrence (Scholzen and Gerdes, 2000).

Cell survival is dependent on the complex interplay between proapoptotic and antiapoptotic proteins. Perhaps the main class of proteins involved in this process is the Bcl-2 family, which includes a large number of proteins. Among them, Bax and Bcl-2 are proapoptotic and antiapoptotic factors, respectively.

Under normal conditions, Bax is localized in the cytoplasm as a soluble monomeric protein, but upon stimulation (e.g. DNA damage), Bax undergoes a conformational change to an activated form and translocates to the mitochondrial membrane. Therefore, this protein associates as homo-oligomers that promote the permeabilization of the outer mitochondrial membrane. This allows water and small molecules to pass through, leading to swelling of the intermembrane space and rupture of the outer mitochondrial membrane. Consequently, toxic proteins such as cytochrome c are released from the intermembrane space of the mitochondria; this essential component of the respiratory chain, when released in the cytoplasm and in the presence of ATP, together with Apaf-1 and procaspase 9 forms the 'apoptosome'. This activates caspase 9 and triggers the classic apoptotic cascade, leading to apoptotic cell death (Bröker *et al.*, 2005; Dai and Grant, 2007).

In contrast, the antiapoptotic Bcl-2 protein prevents cell death by hetero-dimerizing with Bax-like proteins, blocking its function and consequently, the progress of the apoptotic cascade (Adams and Cory, 2007).

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Recent studies have shown that conventional cancer therapies, such as chemotherapy, radiotherapy and surgery, could be administered in association with co-adjuvant therapies. Together, these can improve the prognosis and the quality of life of cancer patients (Montbriand, 2004). Studies suggest that *Aloe vera* (Linne) (Liliaceae) has some pharmacological activities, including immunomodulation and anticancer properties. The components of *Aloe vera* might inhibit tumour growth, reduce tumour mass and inhibit metastasis (Akev *et al*., 2007; He *et al*., 2008). Concurrently, honey has also been shown to have anticancer properties, including the inhibition of tumour cell transformation and proliferation and induction of apoptosis (Swellam *et al*., 2003). As based on ethnopharmacological studies, the combination of honey with *Aloe vera* is a common practical in alternative medicine, especially used in Brazil/South America, but there are few scientific results in the literature showing the real benefits of this association. Knowing these previous facts, the main purpose of this research was to analyse the modulating effects of *Aloe vera* and honey solution on tumour growth, tumour cell proliferation and apoptosis induction during cancer development in an *in vivo* model.

MATERIALS AND METHODS

***Aloe vera* and honey solution.** Mature and healthy leaves of *Aloe vera* (500 g) collected from the greenhouse at the State University of Campinas/SP Brazil were homogenized with honey (Lambertucci Ltda, Rio Claro, Brazil) (500 g) and ethanol (30 mL) in aseptic conditions and kept in dark vials and at a low temperature (4°C) (Zago, 2004).

Experimental protocol. Adult male Wistar rats (90 days old, body weight approximately 300 g) were implanted subcutaneously with a Walker 256 carcinoma cell suspension (1×10^6 viable tumour cells). Treatment was distributed in two groups of rats: the WA group ($n = 15$), received a gavage with a 670 µL/kg dose of the *Aloe vera* and honey solution daily, while the CW group ($n = 15$) was gavaged with 670 µL/kg of 0.9% NaCl solution.

At timepoints of 7, 14 and 20 days after tumour implantation, five rats from each group (CW and WA) were randomly chosen to be killed, and their tumours were dissected, weighed and aliquoted for further analyses.

The general guidelines of the United Kingdom Coordinating Committee on Cancer Research for animal welfare were followed (Vale *et al*., 2005) and the experimental protocols were approved by the Institutional Committee for Ethics in Animal Research (CEEA.IB/UNICAMP, protocol # 1400-1).

Immunohistochemistry. Once the tumour weights were determined, appropriately sized samples were fixed in a 4% para-formaldehyde solution for 24 h and then buried in paraffin blocks following routine procedures. Cross sections (5 µm) taken from these blocks were mounted on poly-L-lysine-coated slides, de-paraffinized, rehydrated through a series of incubations from alcohol to distilled water, treated with 3% hydrogen peroxide in PBS for 30 min (to block endogenous peroxidase activity) and microwaved on high for 10 min in 0.01 N sodium citrate buffer, pH 6.4. After blocking with horse serum at room temperature for 30 min, the primary antibodies specific to Ki-67 (AnaSpec, Inc., San Jose, CA, USA), Bax and Bcl-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), were applied at appropriate dilutions for incubation at 4°C in a humidified chamber overnight. The sections were washed five times for 5 min each in PBS and linked with the appropriate host avidin biotinylated horseradish peroxidase-tagged secondary antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 60 min. The ABC kit (Easy Path) was used to obtain better visualization. Colour development was obtained with 30-30-diaminobenzidine, and counterstaining was performed with Harris haematoxylin. Immunostaining of a tissue section in the absence of the primary antibody was used as a negative control. Visualization and documentation were accomplished with a LEICA microscope supporting a video camera, and analyses were done on Image Pro-Plus software (1.0 version, Media Cybernetics, Silver Spring, MD, USA).

To determine the stained cell percentages, about 300 cells from at least three random fields (approximately 12×10^3 µm^2) were counted from each tissue section.

Statistical analysis. The results were expressed as the mean ± SEM. Comparisons between the untreated tumour-bearing group (CW) and *Aloe vera*- and honey-treated tumour-bearing group (WA) were performed using the unpaired *t*-test. The p values < 0.05 were considered statistically significant. Statistical analysis was performed using the Graph Pad Prism software (v3.00 for Windows 98, USA) (Gad and Weil, 1994).

RESULTS

Tumour growth was modulated by the *Aloe vera* and honey solution

The effect of the *Aloe vera* and honey solution on tumour growth was evident in the treated group (WA) compared with the untreated group (CW) (Fig. 1A). Upon tumour growth analysis, the tumours in the WA group were smaller in mass (represented by a decrease in relative weight (%)) compared with the tumours in the CW group, and this difference continued to increase at later timepoints, especially on day 20 after the original tumour implantation (Fig. 1A).

To investigate and verify the difference in tumour growth, the cell proliferative rates (Ki-67 labelling index, Ki67-LI) in the CW and WA tumour tissue samples (Fig. 1B and C) were obtained by the immunohistochemistry assay for Ki-67. The Ki67-LI in WA tumours was markedly decreased at all timepoints, especially on days 14 and 20 (Fig. 1B).

The *Aloe vera* and honey solution affected the Bax and Bcl-2 expression in tumour cells

In comparing the treated and untreated groups, it was found that the proteins related to the apoptotic pathway (Bax and Bcl-2) also exhibited differences in expression.

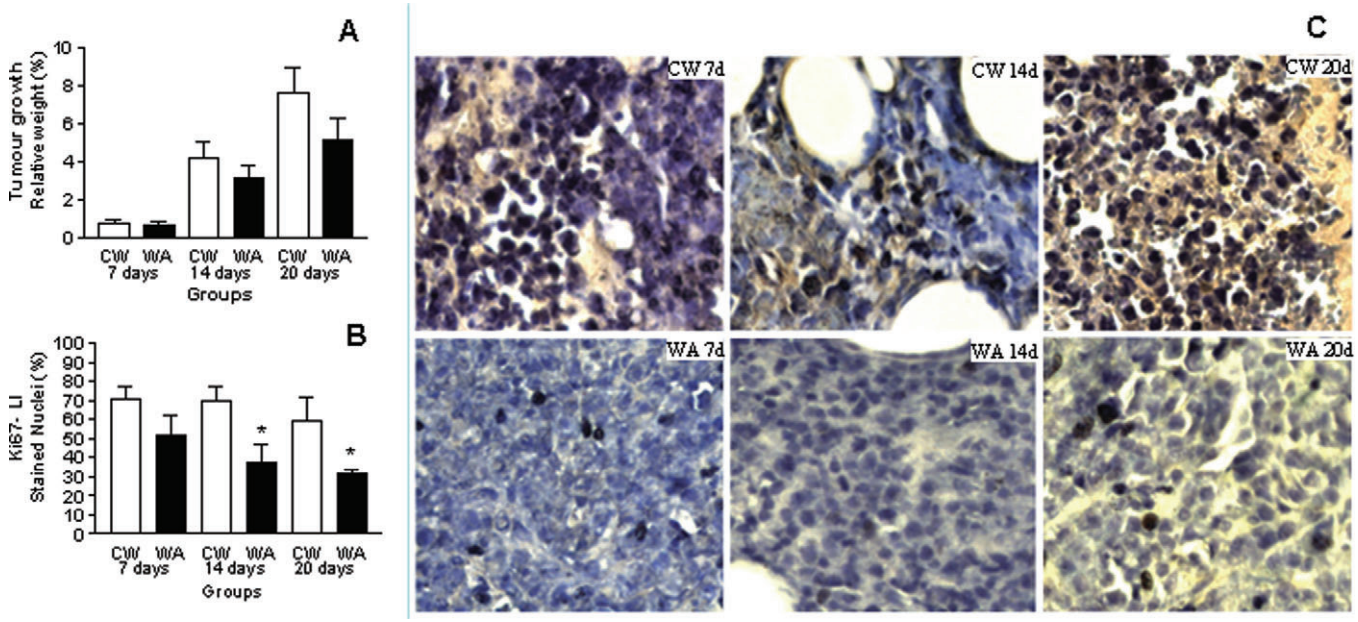

Figure 1. Tumour growth evolution was affected by *Aloe vera* and honey solution. (A) Tumour size (%, relative weight). (B) Ki-67 expression in tumour samples from untreated rats (CW) and *Aloe vera*-and honey-treated rats (WA) at 7, 14 and 20 days after tumour implantation. (C) Immunohistochemistry images for Ki-67-stained cells in tumours from CW and WA groups on different days after tumour induction (magnification 1000×). CW 7d, tumour-bearing control group (CW) on day 7 of tumour development; CW 14d, CW group on day 14 of tumour development; CW 20d, CW group on day 20 of tumour development; WA 7d, *Aloe vera*- and honey-treated tumour-bearing group (WA) on day 7 of tumour development; WA 14d, WA group on day 14 of tumour development; WA 20d, WA group on day 20 of tumour development. The results are expressed as the mean ± SEM. * $p < 0.05$ indicates significant differences within each column between the CW and WA groups on the different tested timepoints. This figure is available in colour online at http://wileyonlinelibrary.com/journal/ptr

The percentage of tumour cells expressing the proapoptotic protein Bax significantly decreased in the untreated group (CW) on day 20 after tumour implantation compared with the preceding days (7 and 14). On the other hand, compared with the control group, there was an increase in the percentage of Bax-positive cells found in the tumours of the *Aloe vera*- and honey-treated group (WA) on day 20 (Fig. 2A and 2D).

Compared with expression found in the tumours from the untreated group (CW), the expression of anti-apoptotic protein Bcl-2 was lower in the tumours from the *Aloe vera*- and honey-treated group (WA) at all examined timepoints (Fig. 2B). This difference was larger, especially on days 7 and 14 of tumour development (Fig. 2E).

Indeed, the Bax/Bcl-2 ratio analysis remarkably showed that in comparison with the tumours from the untreated group (CW), tumours from the *Aloe vera*- and honey-treated group (WA) tended to show bigger indexes throughout the experimental period (Fig. 2C).

DISCUSSION

Natural compounds have been used as therapeutic products by humans for hundreds of years. It has been shown that approximately 89% of patients with cancer or other chronic diseases use alternative therapies, often herbal or natural products (Montbriand, 2004). Studies have suggested the effectiveness and applicability of herbs and natural products to prevent or treat diseases such as cancer (Montbriand, 2004; Surh, 2003).

The present study demonstrated that oral administration of an *Aloe vera* and honey solution to tumour-bearing rats could inhibit tumour growth; the decrease in tumour mass could be observed by comparing the tumour weights of treated rats (WA) and untreated rats (CW) throughout the experiment. There are several lines of evidence that *Aloe vera* and their components may reduce tumour size or inhibit tumour growth *in vivo* and *in vitro* (Surh, 2003; Acevedo-Duncan *et al.*, 2004; Lissoni *et al.*, 2009) and these effects have also been seen with honey (Swellam *et al.*, 2003). There is a need to investigate and further elucidate the active components within the *Aloe vera* and honey solution so that they may be used as co-adjuvants to combat tumour cells.

The understanding of the action and target mechanisms of natural or naturally derived compounds as cancer-preventative or cancer-therapeutic agents is essential for their application in modern science. Additionally, they could represent a very simple but promising strategy in the treatment of human cancer, once uncontrolled proliferation and abnormal apoptosis leads to the occurrence and development of neoplasic cells, and some natural products that can promote the apoptosis of cancer cells or decrease their proliferation. Therefore, in the present work, it is worth a mention that some events occurring in the tumours, among them, changes in cell cycle progression and cell death promotion caused by *Aloe vera* and honey (Acevedo-Duncan *et al.*, 2004; Swellam *et al.*, 2003), could lead to the smaller tumour sizes observed.

The concomitant decrease of Ki67-LI observed in the treated tumour-bearing rats (WA), suggested that administration of the *Aloe vera* and honey solution can also lead to lower tumour cell proliferation. A high level of Ki67-LI implies that a tumour has a rapid growth rate and, in some cases, indicates aggressiveness and poor prognosis (Scholzen and Gerdes, 2000; Bush *et al.*,

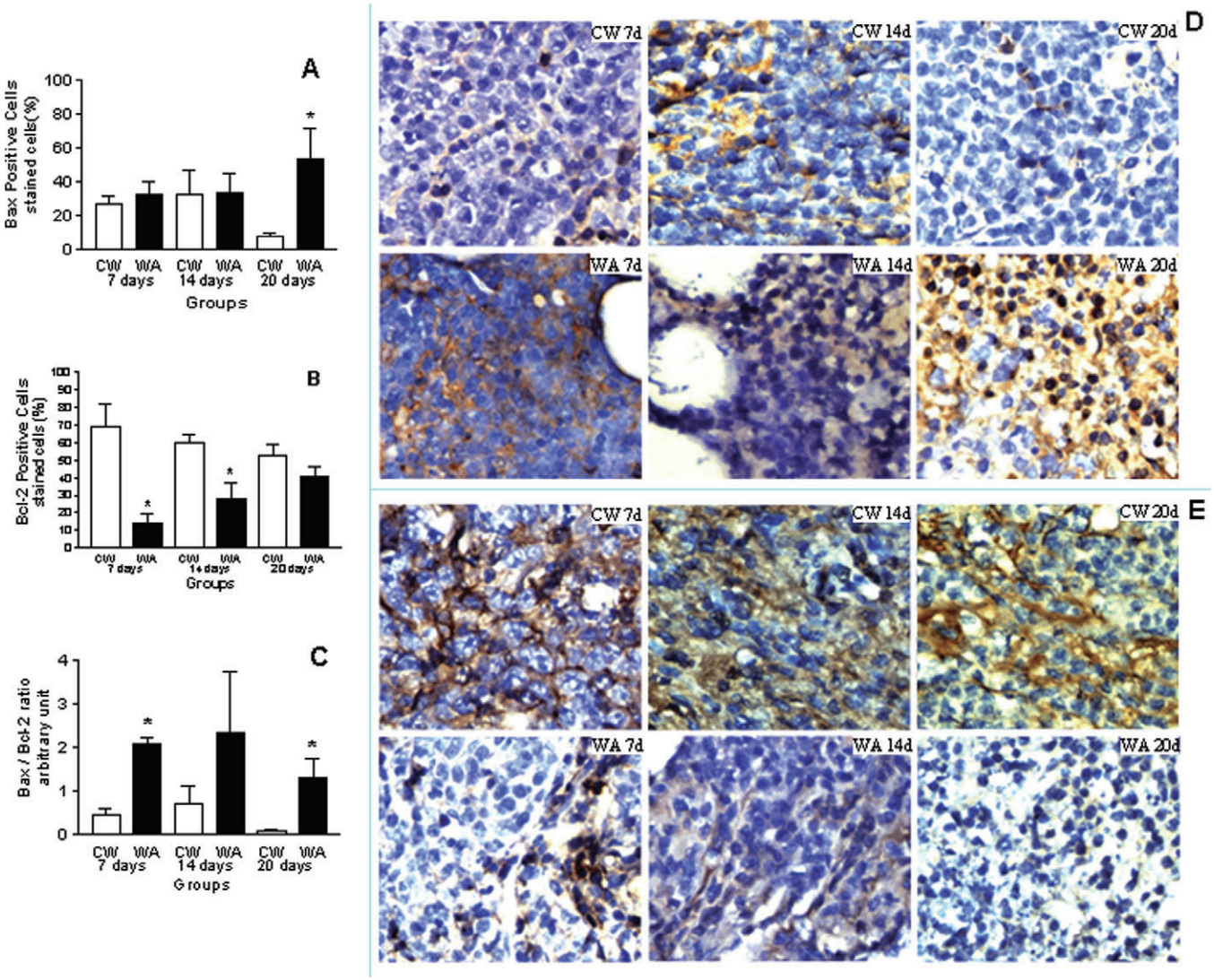

Figure 2. The *Aloe vera* and honey solution changed the expression of Bax and Bcl-2 in tumour cells. (A) % of Bax-positive cell expression. (B) % of Bcl-2-positive cell expression. (C) the Bax/Bcl-2 ratio (arbitrary unit) in tumour samples from untreated rats (CW) and *Aloe vera*-and honey-treated rats (WA). (D) Immunohistochemistry images of cells stained for immunohistochemical detection of Bax protein in CW and WA tumours on different days after tumour induction (magnification 1000×). (E) Immunohistochemistry detection for Bcl-2 expression in tumours from the CW and WA groups on different days after tumour induction (magnification 1000×). CW 7d, tumour-bearing control group (CW) on day 7 of tumour development; CW 14d, CW group on day 14 of tumour development; CW 20d, CW group on day 20 of tumour development; WA 7d, *Aloe vera*- and honey-treated tumour-bearing (WA) group on day 7 of tumour development; WA 14d, WA group on day 14 of tumour development; WA 20d, WA group on day 20 of tumour development. * $p < 0.05$ indicates significant differences within each column between the CW and WA groups among the different tested timepoints. This figure is available in colour online at http://wileyonlinelibrary.com/journal/ptr

1991). Therefore, the reduction of Ki67-LI observed in the WA group might indicate that the treatment with *Aloe vera* and honey could modulate tumour growth and progression.

Another mechanism usually involved in tumour control is cell death, especially apoptosis. It is known that Bax and Bcl-2 regulate apoptosis. Bax has a proapoptotic effect which can be blocked by Bcl-2 (Vaux *et al*., 1988; Oltvai *et al*., 1993). In consequence, the balance between Bax and Bcl-2 expression in the cell could determine the apoptosis occurrence.

The results obtained in the present work showed that compared with the Bax expression examined on day 7 and 14 after tumour implantation, the Bax expression decreased on day 20 in the tumours from the CW group. In contrast, Bax expression increased in the tumours from the WA group. These data suggested that without treatment the apoptosis process could be inhibited through tumour development. The treatment with *Aloe vera* and honey could have stimulated the increased expression of the proapoptotic factor Bax and increased the possibility of cell death.

Bcl-2 is overexpressed in many types of tumours, especially in relapsed or chemoresistant malignancies (Reed *et al*., 1996; Krajewska *et al*., 1996; Borner *et al*., 1999; Villar *et al*., 2001; Tothova *et al*., 2002), and is an important target for a selective new cancer therapy (An *et al*., 2004).

Bcl-2 expression was significantly lower in the tumours from the WA group compared with the expression in tumours from the CW group, especially during an early timepoint (day 7 after implantation). Although the tumour expression levels of Bcl-2 tended to equalize between the WA and CW groups at a later time in tumour development (day 20 after implantation), the Bax/Bcl-2 ratio was significantly larger in the tumours

from the WA group than in the tumours from the CW group at all tested timepoints. It has been proposed that the Bax/Bcl-2 ratio may manage the sensitivity of cells to apoptotic stimuli (Gazzaniga *et al*., 1996; Thomas *et al*., 1996), thus, increasing the apoptosis propensity of tumours in rats receiving the *Aloe vera* and honey treatment. In support of these data, several researchers have shown that *Aloe* or honey components can induce apoptosis through a large number of mechanisms, including Bax/Bcl-2 regulation (Lin *et al*., 2006; Choi *et al*., 2008). Studies have also shown the importance of the synergistic activity of *Aloe* components (Kametani *et al*., 2007).

These data suggested that *Aloe vera* and honey can modulate tumour growth by reducing cell proliferation and reducing tumour weight. The Bax/Bcl-2 ratio increased in the tumours from the WA group at all tested timepoints, suggesting an increase in apoptosis susceptibility. Further studies are underway in our laboratory to determine what and how the active components within the *Aloe vera* and honey solution could affect the tumour growth, and we are also investigating whether this combination could be better therapeutically than the individual therapy. Certainly, a large number of complex mechanisms may be involved in the tumour growth modulation effects of *Aloe vera* and honey that result in changes in proliferative capacity and apoptosis induction.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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