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# 6-shogaol suppresses oxidative damage in L6 muscle cells

Jinyoung Hur, Yeonmi Lee, Chang Jun Lee, Ho-Young Park and Sang Yoon Choi\* 

## Abstract

Ginger (*Zingiber Officinale Roscoe*) has been known reduce muscle pain after exercise, and 6-shogaol {(E)-1-(4-Hydroxy-3-methoxyphenyl)dec-4-en-3-one} is the major essential oil contained in ginger. In this study, the protective effect of 6-shogaol on L6 muscle cells against oxidative damage was measured. 6-shagol inhibited the damage of L6 cell induced by H<sub>2</sub>O<sub>2</sub>, and allowed the increase in mRNA and protein expression levels of intracellular HO-1 and NRF2. 6-shogaol also reduced the production of intracellular ROS. These results suggested that 6-shagol effectively inhibits oxidative damage of skeletal muscle cell.

**Keywords:** 6-shogaol, L6 cell, Antioxidant, Nrf2, HO-1

## Introduction

Active oxygen is produced in the normal metabolic process and it performs various biological functions; but active oxygen that is excessively generated causes damage to cells and tissues [1–3]. Regular and appropriate exercise help the prevention and mitigation of hypertension, stroke, cardiovascular disease, diabetes, hyperglycemia, and cancer [4, 5]. However, strenuous exercise causes generation of excessive active oxygen and consequently oxidative damage in muscle tissue [6, 7]. The human body protects itself by generating antioxidant enzymes as a defense system against such oxidative damage but the protection ability varies depending on the persons' age and health status.

The ginger is a zingiberaceae perennial plant of the subtropical or tropical regions, and its rhizome is edible. In recent years, it has been reported that if ingesting ginger reduces post-exercise muscle pain [8]. The typical essential oils of ginger are gingerol and shogaol (Fig. 1), and shogaol is gingerol that is converted by heat [9]. Shogaol has an antioxidant, anti-inflammatory, and the protective effect on the cranial nerve [10–14] and melanocytes [15], but studies of the effect on skeletal muscle have not been

reported. Nuclear factor erythroid 2- related foactor 2 (Nrf2) is a transcription factor that has been considered as the main regulator of antioxidant gene such as HO-1 [16]. Oxidative stress and inflammation are main mechanisms involved in muscle atrophy [17]. In this study, we show that that three types of gingerol and 6-shogaol exhibit protective effects against damage in L6 skeletal muscle cells; cell survival rate, the amount of ATP produced, and the amount of intracellular ROS generation were measured after treating the cells with hydrogen peroxide and these compounds. In addition, the effect on mRNA and protein expression of HO-1 and NRF2, which are the intracellular antioxidant factors, was measured.

## Materials and methods

### Materials

6-Shogaol, 6-gingerol, 8-gingerol, and 10-gingerol were purchased from Chromadex company (Irvine, CA, USA). Dulbecco's modification of Eagle medium (DMEM), fetal bovine serum (FBS), and Antibiotic- Antimycotic (AA) were purchased from Gibco-BRL (Carlsbad, CA, USA), and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) was obtained from Sigma Aldrich (St. Louis, MO, USA). Nucleospin was purchased from MACHEREY-NAGEL. (MACHEREY-NAGEL INC, PA, USA), iQ<sup>TM</sup> SYBR<sup>®</sup> Green Supermix, and iScript<sup>TM</sup> Reverse

\*Correspondence: sychoi@kfri.re.kr  
Korea Food Research Institute, Wanju 55365, Republic of Korea

Transcription Supermix for RT-qPCR were purchased from Bio-Rad (Hercules, CA, USA). HO-1 antibody was purchased from Enzo Life Science (Farmingdale, USA). NRF-2 antibody was purchased from Abcam (Cambridge, UK).

#### Cell culture

The L6 cell line was obtained from the Korean Cell Line Bank, and cultured by using DMEM containing 10% FBS, 1% AA at 37 °C, and 5% CO<sub>2</sub>. Once the cells reached confluence, the medium was changed to DMEM containing 2% horse serum and 1% AA for differentiation. Experiments were undertaken 6 days after fully differentiation.

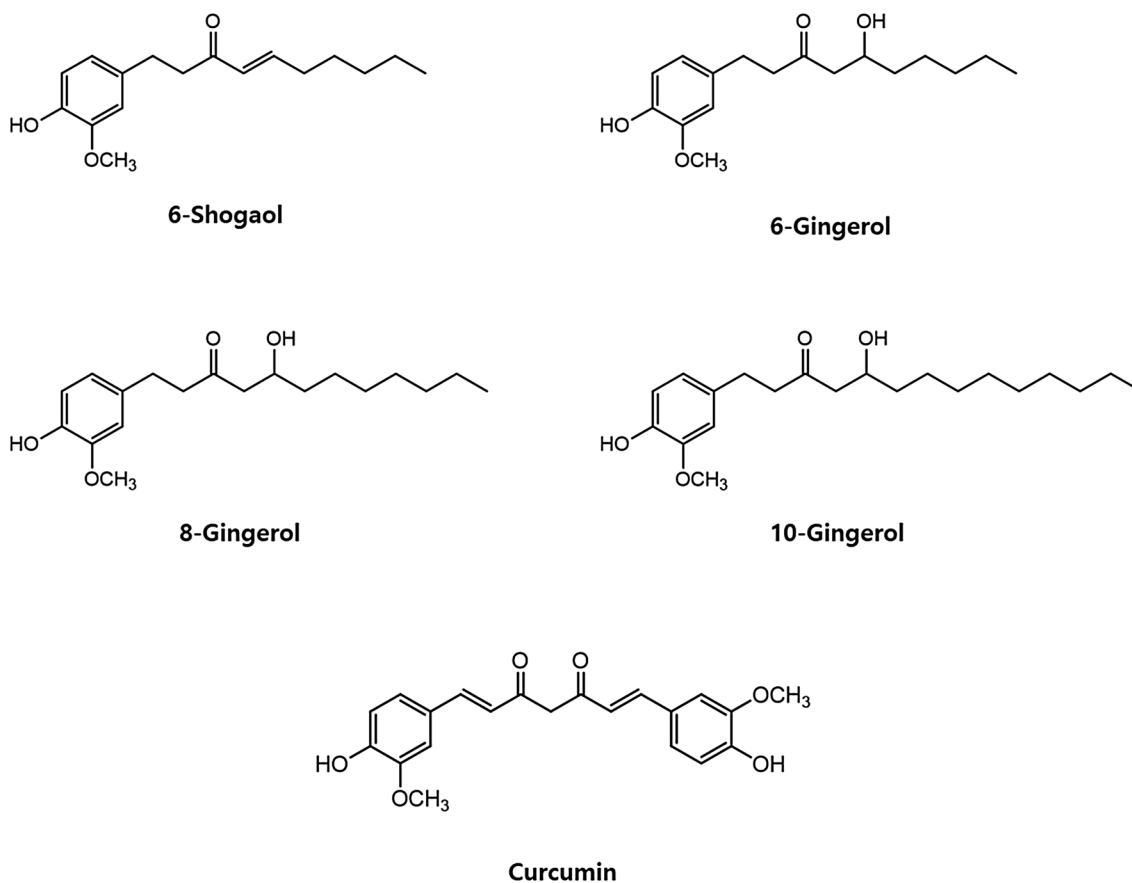
#### Measurement of cell viability

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay method was used to measure cell viability. To investigate the protective effect of the gingerol and shogaol on muscle cells, the cultured cells were treated with the sample and 1.5 mM H<sub>2</sub>O<sub>2</sub>, and cultured for another 24 h. And then the absorbance at 540 nm was measured by staining with MTT (0.5 mg/ml in PBS) solution. The protective effect was expressed

as a percentage by calculating the recovery ratio when treating with the sample versus the cell death ratio when treating with hydrogen peroxide.

#### Real-time PCR

Total RNA from each cell was extracted using NucleoSpin<sup>®</sup> RNA isolation kit (MACHEREY-NAGEL INC, PA, USA) and subjected to Real time-PCR using the following primers: HO-1 forward primer: AAGAGGCTA AGACCGCCTTC and reverse primer: 5'-GCAAGGCTA AGACCGCCTTC-3'; GAPDH forward primer: 5'-AGG TTGTCTCTGTGACTTC-3' and reverse primer; 5'-ctgttgctgtgacctatc-3'. The cDNAs were amplified by 30 cycles of 42 °C for 45 s on a real-time PCR using a iQ<sup>™</sup> SYBR<sup>®</sup> Green Supermix (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed according to CFX Manager software using C1000 thermo cycler system (Bio-Rad, Hercules, CA, USA) which allows for real-time quantitative detection of the PCR product based on an increase in SYBR green fluorescence caused by the binding of SYBR green to double stranded DNA. For sample analysis, the threshold was set based on the exponential phase of the products, and the CT value for the samples



**Fig. 1** Chemical structure of 6-shogaol, 6-gingerol, 8-gingerol, 10-gingerol and curcumin

was determined. The resulting data were analyzed using the comparative CT method for relative gene expression quantification against the housekeeping gene GAPDH.

**Western blot analyses**

Cells were washed and collected with PBS. After centrifugation, the cells were lysed with Protein Extraction Solution (Elpis Biotech, Daejeon, Korea). Protein concentrations in each fraction were measured using a protein assay dye reagent and bovine serum albumin as the standard. Equal amounts of protein were electrophoresed on a 10% sodium dodecyl sulphate–polyacrylamide gel and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% skim milk and incubated with primary antibodies against HO-1 (1:2000 dilution) and Nrf2 (1:1000 dilution). Subsequently, the membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. Antibody detection was performed using ECL reagent (Thermo Scientific, USA) and visualized with a ChemiDoc imaging system (Bio-Rad Laboratories, Hercules, CA, USA). Band intensities were analyzed using Bio-Rad Image Lad Software.

**Measurement of active oxygen generation**

Intracellular active oxygen was dyed by using fluorescence 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). The L6 cells were pretreated with 6-shogaol for 1 h and then treated with H<sub>2</sub>O<sub>2</sub> for 30 min. The cells were treated with 3 μM of DCF-DA and then supplementary cultured for 30 min. DCF-DA positive cells were confirmed by using a fluorescence microscope (Nikon, Eclips Ti microscopy, Japan).

**Measurement of adenosine triphosphate (ATP) generation**

The amount of ATP in L6 cells was measured by using ATP bioluminescence assay kit HS II (Roche, Germany). L6 cells were seeded on 96 well plates in the concentration of 1 × 10<sup>5</sup> cells/ml and cultured for 24 h. The cells were then simultaneously treated with 6-shogaol and 1.5 mM of H<sub>2</sub>O<sub>2</sub>. After additional culturing for 24 h, the cells were lysed and ATP bioluminescence assay was carried out. The absorbance was measured by using a luminometer (Molecular Devices, USA).

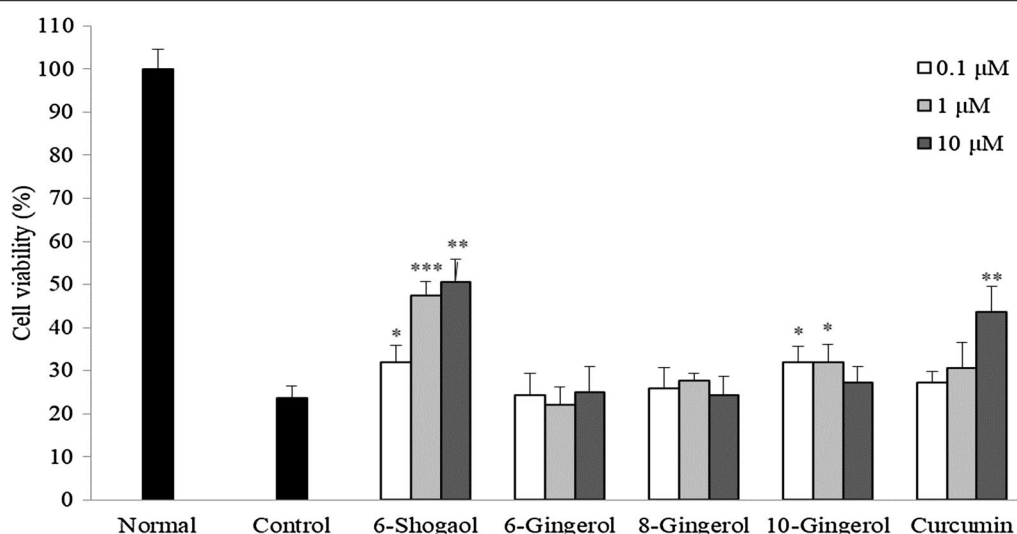
**Statistical analysis**

The data are indicated as mean ± standard deviation, and statistical significance was verified using one-way ANOVA followed by Tukey's post hoc test. P-values less than 0.05 were considered to be statistically significant.

**Results**

**Protective effects on L6 muscle cells**

Violent exercise generates excessive active oxygen, and as a result, the muscle tissues are subjected to oxidative damage [18]. In order to determine whether gingerol and shogaol exhibit protective effect on muscle cell against oxidative damage, the protective effect on L6 cells against hydrogen peroxide-induced damage was measured. After confirming that L6 cell death was not induced by 100 μM of 6-gingerol, 8-gingerol, 10-gingerol, and 6-shogaol, L6 cells were treated for 24 h with 1, 10, and 100 μM of these materials along with 2 mM of hydrogen peroxide. 76.4% of the cells died when treated with only hydrogen peroxide, but when the cells were simultaneously treated with 6-shogaol and hydrogen peroxide, cell death inhibition

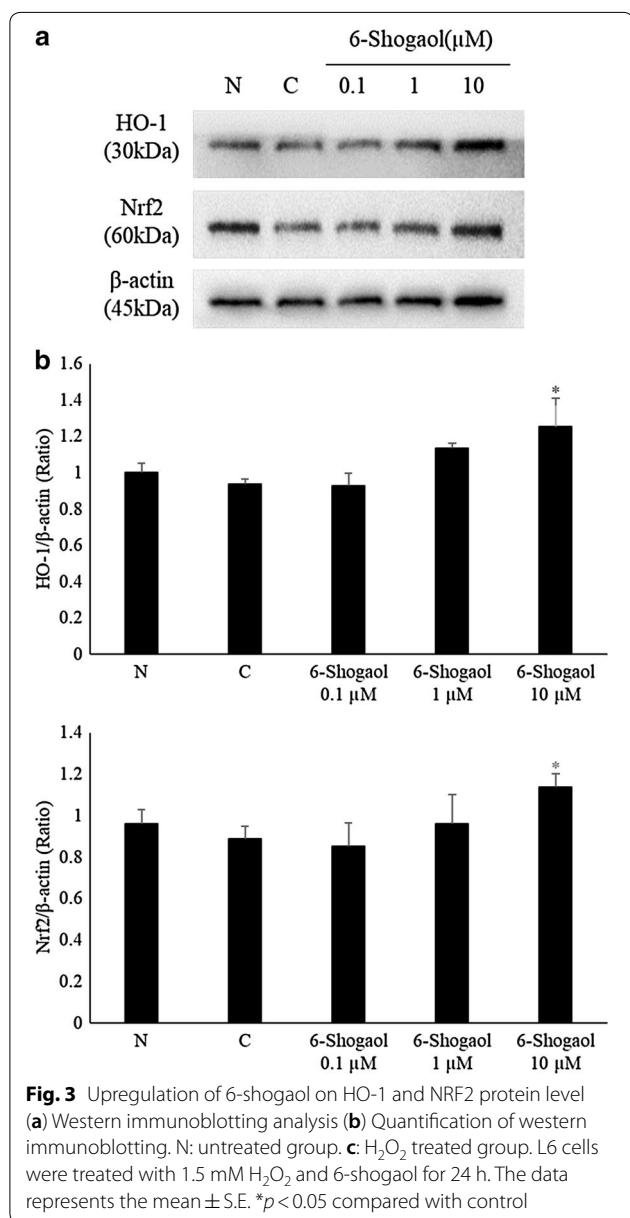


**Fig. 2** Protective effects of gingerols and 6-shogaol against H<sub>2</sub>O<sub>2</sub>-induced oxidative damage in L6 muscle cells Normal: untreated group. Control: H<sub>2</sub>O<sub>2</sub> treated group. L6 cells were treated with 1.5 mM H<sub>2</sub>O<sub>2</sub> and test samples for 24 h. The data represents the mean ± S.E. of three experiments. \*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001 compared with control

was dose dependently decreased. The cell death inhibition rate of 6-shogaol was 31.9, 47.5, and 50.7% at 1, 10, and 100 μM, respectively, and the inhibitory effect was higher than three kinds of gingerol (Fig. 2).

**Effect on HO-1 and NRF-2 expression in L6 cells**

HO-1 is an antioxidant transcription factor and NRF-2 helps the transcription of the gene related to antioxidant by entering in the nucleus because of an external stimulus [19, 20]. In order to measure the effect of 6-shogaol on the expression of HO-1 and Nrf2, the expression levels of the gene and protein were confirmed by western blot analysis and real time-PCR analysis. The protein levels of HO-1 and Nrf2 are shown in Fig. 3. As a result,



the expression of HO-1 and Nrf2 protein was increased compared to the control when treated with 6-shogaol. The expression of HO-1 mRNA increased approximately 2.5 times compared to the control with treatment with 10 μM of 6-shogaol. The expression of Nrf2 mRNA was increased 1.7 times compared to the control with 6-shogaol treatment (Fig. 4). These results suggested that the antioxidative effects of 6-shogaol mediated by upregulation of HO-1 and Nrf2.

**ROS measurement**

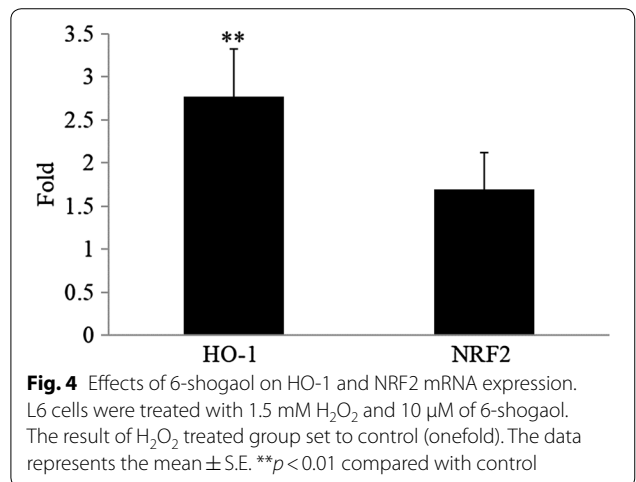
In order to confirm that 6-shogaol inhibits the production of ROS in L6 cell, the amount of intracellular ROS was measured using the fluorescent dye DCF-DA. L6 cells were simultaneously treated with 6-shogaol and H<sub>2</sub>O<sub>2</sub> for 1 h, and then were stained for 30 min by ROS-sensitive fluorescent dye 2',7'-dichlorofluorescein diacetate (DCF-DA, Sigma, USA) and observed by a fluorescence microscope. As a result, when treating with only H<sub>2</sub>O<sub>2</sub>, the production of intracellular ROS was significantly increased. However, when treating along with 6-shogaol, the production of intracellular ROS was decreased (Fig. 5).

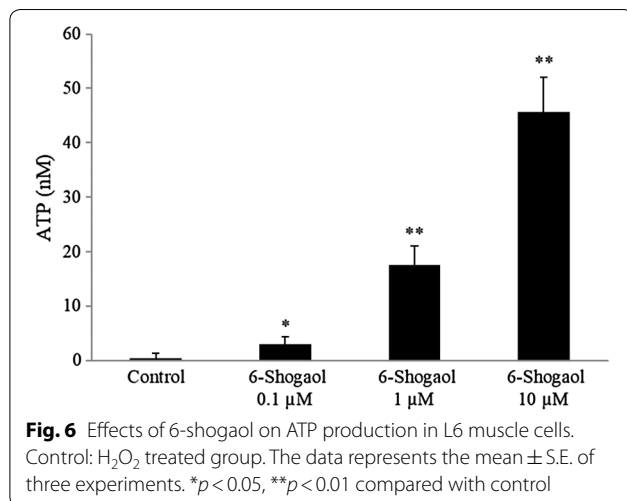
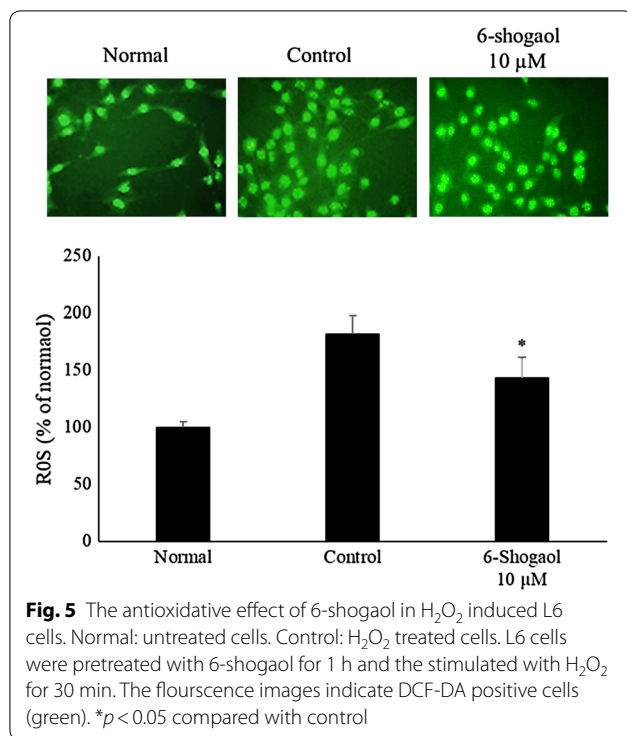
**Effect on ATP production in muscle cells**

ATP is necessary for the survival of cells [21]. The effect of 6-shogaol on production of ATP in L6 cells was measured and hydrogen peroxide-induced reduction of ATP production in L6 cells was effectively inhibited when treating with 6-shogaol (Fig. 6).

**Discussion**

Gingerol and shogaol are pungent component in ginger. Gingerol is the most abundant in fresh ginger and shogaol is produced by dehydrated from gingerol [9].





6-shogaol has been reported to have higher radical scavenging effect and anti-inflammatory effect than gingerol [22]. In addition, 6-shogaol acutely relaxes airway smooth muscle and attenuates human artery smooth muscle cells calcification [23, 24]. It has been reported that treatment of ginger extracts resulted in improvement of cellular differentiation of myoblast by cell cycle regulation [25]. However, effect of 6-shogaol in differentiated skeletal muscle cells has not been studied so far. Skeletal muscle can respond rapidly to other

environmental changes by modifying of genes involved muscle structure, energy metabolism. Changes of ROS and inflammation in skeletal muscle cell by exercise and energy metabolism affected muscle atrophy [26–28]. In this study, 6-shogaol significantly inhibited hydrogen peroxide induced cell death in L6 skeletal muscle cells, treatment with 6-gingerol, 8-gingerol and 10-gingerol had no inhibitory effect. Moreover, 6-shogaol inhibited the production of intracellular ROS by increasing mRNA and protein expression of HO-1 and Nrf2, which control the expression of the antioxidant enzyme. These results suggested that the 6-shogaol might be able to inhibit muscle atrophy in differentiated muscle damage as well as muscle regeneration which related muscle development. The in vivo study of 6-shogaol requires, and will be processed in further investigation.

In summary, 6-shogaol significantly inhibited hydrogen peroxide induced cell death in L6 skeletal muscle cells, treatment with 6-gingerol, 8-gingerol and 10-gingerol had no inhibitory effect. The 6-shogaol inhibited the production of intracellular ROS. Moreover, 6-shogaol increased mRNA and protein expression of HO-1 and Nrf2, which control the expression of the antioxidant enzyme. These results suggested that the 6-shogaol might be able to inhibit oxidative stress induced muscle damage.

#### Authors' contributions

Performed the experiments: JH, YL, CJL, Data analysis: JH, YL, HYP, SYC, Wrote the manuscript: SYC, JH, Designed and supervised the study: SYC. All authors read and approved the final manuscript.

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#### Availability of data and materials

The datasets used in this study are available from the corresponding author on reasonable request.

#### Competing interests

All authors declare that there is no competing interests.

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