Inhibitory effects of TGP on KGF-induced hyperproliferation of HaCaT cells via suppression of the p38 MAPK/NF-κB p65 pathway

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Abstract. Psoriasis is a chronic inflammatory skin disease, primarily caused by overgrowth and abnormal differentiation of epidermal keratinocytes. Studies have suggested that keratinocyte growth factor (KGF) may be involved in the regulation of differentiation and development of keratinocytes. Total glucosides of peony (TGP) have been widely used for the treatment of psoriasis. The present study aimed to determine whether the therapeutic effect of TGP on psoriasis is mediated by modulation of the p38 mitogen-activated protein kinase (p38 MAPK)/nuclear factor (NF)-κB p65 signaling pathways. Cell proliferation was evaluated by CCK-8 and cell cycle was assessed by flow cytometry assay. Protein and mRNA expression of genes were determined by western blot and reverse transcription-quantitative polymerase chain reaction, respectively. The results of the present study demonstrated that KGF can promote proliferation of HaCaT cells in a dose-dependent manner. In addition, it was demonstrated that TGP may suppress the hyperproliferation of HaCaT cells stimulated by KGF by inducing arrest of the cell cycle at the G1 phase. The expression levels of the proinflammatory cytokines interleukin (IL)-22 and vascular endothelial growth factor (VEGF) were markedly elevated in cells treated with KGF, whereas they were downregulated in cells treated with TGP. Furthermore, combination treatments with p38 MAPK inhibitor SB203580 and KGF, or TGP and KGF suppressed the mRNA and protein expression levels of IL-22 and VEGF, compared with cells treated with KGF alone. Furthermore, the expression profiles of phosphorylated-p38 MAPK and NF-κB p65 were similar to those of IL-22 and VEGF. The results of the present study suggested that the therapeutic effect of TGP on psoriasis may be mediated by modulation of the p38 MAPK/NF-κB p65 signaling pathway. The results of the present study contribute to the understanding of the role of TGP in the treatment of psoriasis. The present study provides insights suggesting that p38 MAPK may be a novel regulatory signaling pathway for the treatment of psoriasis.

Introduction

Psoriasis is a chronic inflammatory skin disease characterized by erythema, pimples, patches and silver scales (1). It has been demonstrated that psoriasis is a disorder resulting from a combination of mutations in multiple genes and interaction between numerous other factors, including trauma, infection and drugs (2). The primary pathological features of psoriasis include overgrowth and abnormal differentiation of epidermal keratinocytes, expansion of dermal papillary capillaries and infiltration of inflammatory cells, among which keratinocyte dysfunction serves a role in the pathogenesis of psoriasis (2,3).

Keratinocytes are able to secrete a number of cytokines, resulting in a local inflammatory response (4,5). Upregulation of cytokine expression further stimulates the keratinocytes and aggravates the inflammatory response of the skin (4,6). A complex immune network formed by keratinocytes and cytokines serves a role in psoriasis. Previous studies demonstrated that psoriasis is initiated when keratinocytes are subjected to a variety of stressors, including infection, pregnancy and trauma (4,7,8). These stressors result inactivation of the immune system, secretion of a number of immune-associated cytokines and chemokines, and induction of inflammatory responses (4). Cytokines are additionally produced by activated T cells and dendritic cells (9). Therefore, the formation of psoriatic lesions results from an interaction between the activation of skin T cells and stimulation of keratinocytes (10).

Keratinocyte growth factor (KGF) is a member of the fibroblast growth factor family, and is additionally termed fibroblast growth factor-7 (11). KGF is a paracrine growth factor secreted by various interstitial cells, including fibroblasts, endothelial cells, smooth muscle cells and dendritic epidermal T cells (11-13). KGF interacts with a specific receptor, fibroblast growth factor receptor 2 IIIb on epidermal cells (13). Although KGF is secreted by interstitial cells, it targets epidermal cells.
A previous study demonstrated that the expression of KGF was markedly increased following trauma in mice and humans (14). In addition, cytological studies have demonstrated that KGF is able to specifically promote the proliferation and migration of keratinocytes (14,15). These studies suggested that KGF may serve a role in wound healing and in promoting the proliferation of keratinocytes (15).

White peony is the dry root of plants in the Ranunculaceae (crowfoot family), and its primary therapeutic ingredient is a group of substances collectively termed total glucosides of peony (TGP), which include peony bitter, hydroxyl paeoniflorin, benzoyl and paeoniflorin, which accounts for >90% of all the ingredients (16,17). Clinical application and pharmacological studies have demonstrated that TGP exhibit anti-inflammatory, analgesic and immune regulatory effects, protect the liver and vascular endothelium against inflammation (17,18). TGP is used for the treatment of a variety of autoimmune diseases, including psoriasis (19), systemic lupus erythematosus (19,20) and ankylosing spondylitis (21).

A number of studies have suggested that TGP is involved in numerous aspects of autoimmune processes (1). The expression levels of interleukin (IL)-2 and -4 are reduced and elevated, respectively, in dinitrochlorobenzene-induced chronic dermatitis-eczema models (22). TGP are involved in the G protein-adenylate cyclase-cyclic adenosine monophosphate (cAMP) signal transduction pathway (23). It has additionally been demonstrated that TGP may downregulate the expression of inflammatory cytokines, including tumor necrosis factor-α (TNF-α), IL-6, IL-8 and interferon (IFN)-α (24).

A previous study reported that KGF may regulate the expression of the proinflammatory mediators TNF-α and IL-1 to stimulate inflammatory responses via activation of p38 mitogen-activated protein kinase (p38 MAPK) (25). The therapeutic effects of TGP may be associated with the regulation of p38 MAPK. Hyperproliferative HaCaT cells exhibit characteristics of keratinocytes in psoriatic lesions, and are therefore used as in vitro models of psoriasis in drug research (26). Therefore, in order elucidate the underlying mechanism of action of TGP in the treatment of psoriasis, the effects of TGP on the proliferation of HaCaT cells induced by KGF were determined. The p38 MAPK signaling pathway was selected to elucidate the molecular mechanism of TGP.

### Materials and methods

#### Cell culture.
The immortal human keratinocyte line HaCaT was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The HaCaT cells were cultured in Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin, and were incubated in a cell culture chamber at 37°C in a humidified atmosphere containing 5% CO2. Cells were grown to 80% confluence, the culture medium was removed and washed with PBS, and the cells were digested using 0.25% trypsin. Cells in the logarithmic growth phase were used for the subsequent experiments.

#### Cell experiments.
Confluent cultures of HaCaT cells were incubated with DMEM supplemented with 0, 1.25, 2.5, 5, 10, 20 and 40 ng/ml KGF (Prospect-Tany TechnoGene, Ltd.; East Brunswick, NJ, USA) for 24, 48 and 72 h at 37°C. The cell hyperproliferation model of psoriasis was established in HaCaT cells via treatment with KGF (10 ng/ml) for 24 h at 37°C and confirmed using a Cell Counting Kit-8 (CCK-8) assay. To investigate the effect of TGP on hyperproliferative HaCaT cells induced by KGF, cell proliferation and the cell cycle were detected for these cells following treatment with different concentrations of TGP: i) TGP-low (L; 10 mg/l); ii) TGP-medium (M; 50 mg/l); and iii) TGP-high (H; 120 mg/l) or 48 h at 37°C. Subsequently, to determine the role of TGP, cells were divided into five groups: i) Model, cells treated with 10 ng/ml KGF; ii) TGP, cells treated with 120 mg/ITGP; iii) control, cells with no treatment; iv) KGF-IN [cells treated with 10 ng/ml KGF+10 µm SB203580 (Calbiochem; Merck KGaA)]; and v) KGF-TGP (cells treated with 10 ng/ml KGF+120 mg/ITGP). Following incubation for 48 h at 37°C, cells were harvested and used for subsequent experimentation as described below.

#### Detection of cell proliferation activity by CCK-8 assay.
To determine alterations in cell proliferation, HaCaT cells from all groups were seeded in 96-well plates at a density of 1x10^4 cells/well. Cell proliferation was detected using a CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturer's instructions. A total of 20 µl CCK-8 was added and cells were incubated at 37°C for 4 h. The optical density values were measured at a wavelength of 450 nm using an ELISA reader (BioTek Instruments, Inc., Winooski, VT, USA), according to the manufacturer's protocol.

#### Cell cycle detection by flow cytometry assay.
HaCaT cells collected from all groups were washed with PBS and centrifuged at 500 x g for 5 min at 4°C. Cells were adjusted to a density of 1x10^6 and re-suspended. Propidium iodide (400 µl) and RNA-sea-A (100 µl; both from Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) were subsequently added to the cells. Following staining for 30 min at room temperature, the DNA content was determined using a FACSCalibur system and analyzed using CellQuest software (version 3.3; BD Biosciences, Franklin Lakes, NJ, USA).

#### Western blot analysis.
HaCaT cell lysates from all groups were extracted using lysis Triton X-100 buffer (250 mM HEPES, 50 mM MgCl2, 10 mM EGTA and 5% Triton X-100). Protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). Proteins (10 µg) were resolved on 10% SDS-PAGE gels and transferred onto polyvinylidene fluoride membranes. Membranes were blocked with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) in TBS with 0.1% Tween-20 for 1 h at room temperature, and incubated with anti-β-actin (ab8226; 1:2,000), anti-IL-22, anti-VEGF (ab9570; 1:1,000), anti-p38 MAPK (ab31828; 1:1,000), anti-phosphorylated (p)-p38 MAPK (ab4822; 1:1,000) and anti-NF-κB p65 (ab16502; 1:1,000) (all from Abcam) antibodies at 4°C overnight. β-actin was used as a loading control. Following washing with PBS, blots were incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).
at a concentration of 1:2,000-1:5,000 at room temperature for 1 h. Blots were visualized using chemiluminescence (GE Healthcare Life Sciences, Little Chalfont, UK) and developed on film (Kodak, Rochester, NY, USA), according to the manufacturer's instructions. ImageJ software version 1.42 (National Institutes of Health, Bethesda, MD, USA) was used for densitometry analysis.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was isolated using an RNeasy mini kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's instructions. A total of 1 µg total RNA was reverse transcribed to cDNA using a PrimeScriptH RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China). Reverse transcription reaction conditions were: 30˚C for 10 min, 42˚C for 30 min, 99˚C 5 min and 4˚C for 5 min. qPCR was performed using SYBR Green PCR master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) with a SYBR Green detection system [FS Universal SYBR Green Master (Rox); Roche Applied Science, Penzberg, Germany]. Thermocycling procedures consisted of 50˚C for 2 min, 95˚C for 10 min and 40 cycles of amplification reactions at 95˚C for 15 sec, and at 60˚C for 1 min. All samples were analyzed in triplicate and assessed by melting curve analysis, and the PCR products obtained were analyzed by 1% (w/v) agarose gel electrophoresis. The expression levels of IL‑22 and VEGF were normalized to β-actin and calculated using the 2^ΔΔCq method (27). The following primer sequences were used: IL‑22, 5’‑TGA GTG ACG CTG CAT CTA TG‑3’ (forward), 5’‑TGT GCT TAC GCT TGT GT‑3’ (reverse); VEGF, 5’‑CTG CTG ATG GCC CCT GGAG‑3’ (forward), 5’‑ACG GAG TGT CTC G‑3’ (reverse); β-actin, 5’‑GCA GGA TAT GAC G‑3’ (forward), 5’‑AAC AAG CAC CAT CAT CTT TGG AA‑3’ (reverse).

Statistical analysis. Data are presented as the mean ± standard deviation. All samples were analyzed in triplicate. Statistical comparisons between different groups were performed using SPSS software (version 20; IBM Corp., Armonk, NY, USA). One-way analysis of variance followed by Bonferroni post hoc pairwise comparison was used for the evaluation of differences between groups. P<0.05 was considered to indicate a statistically significant difference.

Results

HaCaT cell proliferation under different concentrations of KGF. To investigate the response of HaCaT cells to KGF, proliferation activity was determined in the presence of

Figure 1. Cell morphology and viability of HaCaT cells following treatment with different concentrations of KGF for 24, 48 and 72 h. Morphology of (A) control cells and cells treated with (B) 1.25, (C) 2.5, (D) 5, (E) 10, (F) 20, and (G) 40 ng/ml KGF. Cell morphology alterations included cell elongation following treatment with KGF (magnification, x400). (H) The Cell Counting Kit-8 assay demonstrated that the cell viability was markedly elevated in cells following exposure to KGF. KGF, keratinocyte growth factor. *P<0.05, >5 ng/ml KGF dosage group vs. control (0 ng/ml KGF); †P<0.05, 24, 48 and 72 h vs. 0 h.
different concentrations of KGF. Fig. 1 presents the alterations in cell morphology following treatment with 0-40 ng/ml KGF for 24 h. Cell densities markedly increased in cells treated with 0-10 ng/ml KGF, and marginally increased in the presence of KGF at 10-40 ng/ml. Cells treated with 5-20 ng/ml KGF appeared more elongated compared with cells treated with 0-2.5 ng/ml KGF. The viability of HaCaT cells in the presence of KGF was determined at different time-points (24, 48 and 72 h) and concentrations (0, 1.25, 2.5, 5, 10, 20 and 40 ng/ml). Cell viability was notably elevated following treatment with KGF in a time- and dose- dependent manner. Cell viability significantly increased in cells treated with a dose of >10 ng/ml of KGF, compared with the control.

Effect of TGP treatment on cell hyperproliferation triggered by KGF. One of the primary pathological characteristics of psoriasis is overgrowth and abnormal differentiation of epidermal keratinocytes (4,6). The present study investigated the inhibitory effect of treatment with TGP on the hyperproliferation of HaCaT cells. The cell viability of the model group treated with 10 ng/ml KGF (detected using a CCK-8 assay) significantly increased compared with the control group. However, additional treatment with TGP can reduce the excessive cell proliferation caused by KGF (Fig. 2). Cell hyperproliferation triggered by KGF was markedly inhibited in the TGP-H group, compared with the model group. The effect of TGP treatment on cell hyperproliferation induced by KGF was additionally validated in HaCaT cells obtained from CLS Cell Lines Service GmbH (data not shown).

Treatment with TGP suppresses the hyperproliferation of HaCaT cells stimulated by KGF by triggering cell cycle arrest at the G1 phase. To determine the mechanism underlying TGP-mediated inhibition of KGF-induced hyperproliferation, the cell cycle was investigated by flow cytometry. The effect of different concentrations of TGP on cells exposed to KGF was investigated (Fig. 3). The results demonstrated that among cells treated with KGF, fewer cells were in the G0/G1-phase and an increased percentage was in the S-phase, compared with the control group, indicating that KGF treatment stimulated the proliferation of HaCaT cells (Fig. 3A, B and F). However, the KGF-induced proliferation of HaCaT cells was inhibited by increasing concentrations of TGP. Among cells treated with TGP, an increased number of G0/G1-phase cells and a lower number of S-phase cells was determined, compared with the model group, suggesting that treatment with TGP suppressed the hyperproliferation of HaCaT cells stimulated by KGF by inducing G1 arrest (Fig. 3B-F).

Expression of inflammatory cytokines induced by KGF is suppressed by treatment with TGP. A previous study indicated that KGF can simulate the expression of cytokines to promote cell proliferation via activation of p38 MAPK (25). To confirm this function of KGF, the p38 MAPK inhibitor SB203580 was used to treat HaCaT cells. In addition, the effect of treatment with TGP on HaCaT cells was assessed. The results revealed that the expression levels of the inflammatory cytokines IL-22 and VEGF in HaCaT cells were markedly elevated in the model group, compared with the control (Fig. 4). The expression levels of these cytokines decreased following treatment with KGF and SB203580, and following treatment with TGP, compared with the control. Furthermore, the expression levels of IL-22 and VEGF were suppressed in cells treated with KGF and TGP, compared with the model group.

Activation of p38 MAPK is inhibited in cells treated with TGP. Based on the results of a previous study (25), the authors of the present study hypothesized that TGP may induce a similar inhibitory effect to SB203580 on p38 MAPK. Therefore, the expression of p38 MAPK and p-p38 MAPK was determined by western blot analysis. The expression level of p-p38 MAPK was significantly elevated following treatment with KGF compared with the control. By contrast, following treatment with TGP, the expression level of p-p38 MAPK was markedly inhibited compared with the control (Fig. 5). Furthermore, compared with the model group, the expression level of p-p38 MAPK was suppressed by combination treatment with KGF and SB203580, or KGF and TGP (Fig. 5B). However, the expression level of p38 MAPK was approximately equal in all groups. The ratio of p-p38 MAPK to p38 MAPK exhibited a similar pattern to the levels of p-p38 MAPK, since the expression of p38 MAPK was similar in all groups. The inhibitory effect of TGP on p38 MAPK activation was additionally validated in HaCaT cells obtained from CLS Cell Lines Service GmbH (data not shown).

Activation of NF-xB p65 in cells is suppressed by treatment with TGP. Expression of inflammatory cytokines regulated by p38 MAPK/NF-xB p65 pathway. Therefore, the present study further determined the expression levels of NF-xB p65 (Fig. 6). The NF-xB p65 protein expression level in cells treated with KGF was upregulated compared with the control. By contrast, treatment with TGP resulted in a downregulation of the expression levels of NF-xB p65. NF-xB p65 protein expression levels were downregulated in cells with combination treatment using KGF and SB203580 or KGF and TGP compared with the model group. The above results were additionally validated in HaCaT cells obtained from CLS Cell Lines Service GmbH (data not shown).
Discussion

The present study aimed to determine whether p38 MAPK may be involved in the therapeutic effect mediated by TGP in an in vitro model of psoriasis. Consistent with previous studies (24-26), the results of the present study demonstrated that KGF is associated with the activation of p38 MAPK, which was confirmed by the use of the p38 MAPK inhibitor SB203580. Similar to SB203580, TGP exhibited an apparent inhibitory effect on the activation of p38 MAPK. Furthermore, the expression of IL-22 and VEGF protein and mRNA was suppressed by treatment with TGP, which further supported the hypothesis that TGP may induce a similar inhibitory effect to SB203580 on p38 MAPK.

Figure 3. Treatment with TGP demonstrated the apparent suppression of KGF-induced hyperproliferation in HaCaT cells. Flow cytometry assays were performed to assess the effect of TGP on the hyperproliferation of HaCaT cells induced by KGF. Flow cytometry results for (A) the control, (B) model, (C) TGP-L, (D) TGP-M and (E) TGP-H groups (green, G0/G1, yellow, S blue, G2/M). (F) Groups treated with TGP demonstrated an increased percentage of G0/G1-phase cells and a decreased number of cells in the S-phase compared with the model group. TGP, total glucosides of peony; L, low; M, medium; H, high; KGF, keratinocyte growth factor. *P<0.05 vs. the control group; †P<0.05 vs. the model group.

TGP have been used to efficiently treat psoriasis (19). Even though the mechanism underlying the therapeutic effect of TGP on psoriasis has been previously investigated, and it has been demonstrated to involve the G protein-adenylate cyclase-cAMP signal transduction pathway, it remains to be completely elucidated (22,23). In addition, in a previous study, the proliferation of epidermal cells was affected by KGF, epidermal growth factor, nerve growth factor, TGF, TNF-α and other cytokines (28). However, KGF is the most potent and specific cytokine that promotes keratinocyte proliferation (14,15,29). Therefore, the present study aimed to determine the effect of TGP on the KGF-induced hyperproliferation of HaCaT cells. Consistent with other studies, in the present study, KGF enhanced the proliferation of HaCaT cells. However, this effect of KGF was suppressed following treatment with TGP. Cell cycle analysis demonstrated that among cells treated with KGF and TGP, an elevated number of cells was in the G0/G1-phase and a decreased number of cells was in the S-phase, compared with cells treated with KGF alone. The above results indicated that TGP may suppress the hyperproliferation of HaCaT cells stimulated by KGF, by triggering G1-phase arrest.

It has been hypothesized that the abnormal infiltration of T lymphocytes (primarily CD4+ T lymphocytes) serves
a role in the pathogenesis of psoriasis (4,10). Elevated levels of T helper (Th)1-type lymphocytes is the predominant response observed in patients with pancreatic diseases, and Th1 type cytokines, including TNF-α, interferon-γ and IL-2, contribute to the aggravation of psoriasis (30). Th2 type cytokines, including IL-4 and -10, serve a protective role instead (31). Previous studies suggested that psoriasis is a disease based on Th17/Th1 cell responses and hypothesized that these induced Th-17/IFN-γ cells may be the cause of psoriasis (32,33). However, psoriasis is a multi-gene genetic disease associated with a number of factors, including trauma, infection, and the underlying mechanisms of psoriasis remain to be completely elucidated (2,34). A number of previous studies demonstrated that the interaction between IL-23, -17 and -22, and other cytokines, serves a role in the development of psoriasis (35-37). Elevated mRNA and protein expression levels of IL-21, -22, -17 and -23 were detected in skin tissue with psoriatic lesions, and IL-23 may induce lesions similar to human psoriasis in mouse skin, including epidermal hyperplasia erythema, leukocyte infiltration and keratosis (35,36). Furthermore, inflammation and acanthosis of the dermal layer have been hypothesized to be associated with cytokine IL-22 and C-C chemokine receptor type 6 (38). The present study demonstrated that the mRNA and protein expression levels of IL-22 were upregulated in cells treated with KGF, whereas they were inhibited in model cells following treatment with TGP or the p38 MAPK inhibitor SB203580.

During the development of psoriasis, numerous vascular-derived cytokines secreted by epidermal cells are involved in angiogenesis, including VEGF (39). In psoriatic lesions, keratinocytes are the primary source of angiogenic cytokines (4,6). Previous studies demonstrated that the mRNA and protein expression levels of VEGF in patients with psoriatic lesions were markedly increased (40,41). Furthermore, it was demonstrated that serum levels of VEGF in patients with severe psoriasis were elevated and served a role in the progression of psoriasis (42). The results of the present study demonstrated that the protein and mRNA expression levels of VEGF were increased in cells treated with KGF. Similar to the levels of IL-22, the expression of VEGF protein and mRNA was suppressed following treatment with TGP. Consistent with these results, treatment of model cells with the p38 MAPK inhibitor SB203580 or TGP additionally inhibited the expression of VEGF.

A previous study demonstrated that KGF simulates p38 MAPK to trigger stress fiber formation in human prostate DU145 cells (43). In addition, a study revealed that the production of VEGF was upregulated following treatment with KGF, and fibroblast growth factor-1 and -2 via the MAPK and p38 MAPK pathways (44). Furthermore, the results of the present study revealed that the expression levels
of VEGF protein and mRNA were elevated in cells treated with KGF, while these levels were suppressed by combined treatment with KGF and p38 MAPK inhibitor SB203580, indicating that the expression of VEGF may be associated with the p38 MAPK signaling pathway. Therefore, it may be hypothesized that KGF induces p38 MAPK activation to promote the upregulation of VEGF, and the results of the present study are consistent with this hypothesis. The results of the present study additionally demonstrated that the levels of IL-22 and VEGF. In conclusion, TGP may suppress the KGF-induced hyperproliferation of HaCaT cells, which may be involved in the downregulation of the expression of proinflammatory factors, including IL-22 and VEGF. The results of the present study indicated that the p38 MAPK/NF-κB p65 signaling pathway may be associated with psoriasis, and may be effectively suppressed by treatment with TGP.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

WP and CC contributed equally to this study and analyzed and interpreted data relating to the study. XQ and SZ designed the study, implemented experiments and wrote the paper. All authors read and approved the final study.
Ethics approval and consent to participate
Not applicable.

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Competing interests
All authors declare that they have no conflict of interest.

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