Brucine inhibits TNF-α-induced HFLS-RA cell proliferation by activating the JNK signaling pathway

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Abstract. Rheumatoid arthritis (RA) is a diffuse connective tissue disease. Brucine selectively inhibits cell immunity, immune hypersensitivity and induces apoptosis. The current study aimed to investigate effects of brucine on human fibroblast-like synoviocytes (HFLS) of RA and to clarify associated molecular mechanisms. HFLS-RA were treated with tumor necrosis factor (TNF)-α prior to treatment with brucine at carrying concentrations. Cell Counting Kit-8 assays were performed to evaluate HFLS-RA proliferation. Western blot assays were employed to examine c-Jun N-terminal kinase (JNK) expression and phosphorylation in TNF-α-induced HFLS-RA. An association between brucine treatment and JNK phosphorylation was assessed by employing a linear regression analysis. The results suggested that low doses of brucine (0.125 and 0.25 mg/ml) significantly reversed proliferation effects induced by TNF-α, however, final cell viabilities were increased compared with the untreated control ($P>0.05$ and $P<0.05$, respectively). High brucine doses (≥0.5 mg/ml) significantly reversed TNF-α-induced proliferation and further inhibited viability compared with the untreated control ($P<0.05$). Regarding JNK expression, there were no significant differences among the brucine treatment, and between the Control and the TNF-α groups ($P>0.05$). Brucine treatment significantly decreased JNK phosphorylation compared with the TNF-α group ($P<0.05$). JNK specific inhibitor, SP600125, significantly inhibited brucine-induced cell viability enhancement compared with the brucine-treated groups without inhibitor ($P<0.05$). A linear regression analysis suggested that brucine was associated with JNK phosphorylation in TNF-α-treated HFLS-RA. In conclusion, brucine significantly inhibited TNF-α-induced HFLS-RA proliferation by activating the JNK signaling pathway. Therefore, brucine may have potential clinical applications in the treatment of RA.

Introduction

Rheumatoid arthritis (RA) is a diffuse connective tissue disease (1,2). RA is a chronic, systemic, autoimmune disorder that can affect multiple organ systems (2). RA affects 0.5-1% of the population and is most prevalent in older individuals, with an increased risk of developing RA in women (2). Due to structural damage and dysfunction of the joints caused by RA, patient mobility is limited, daily life is affected and the overall quality of life for patients and family members is reduced (3). It is recommended to begin treating patients with RA as early as possible (1-3). Traditional RA treatment is relatively affordable, but treatment efficacy is delayed and is associated with a number of side effects, which include neurotoxicity following long-term use (3). Novel bio-drugs, which include methotrexate (MTX), exhibit improved therapeutic effects, however, costs are increased and prolonged use can affect cognitive functions (3). In order to facilitate the discovery of novel anti-RA treatment is required without the side effects and costs associated with current treatment options, the underlying mechanisms of RA will need to be further examined.

In Traditional Chinese Medicine, various prescription medicines exhibit pain relieving and bone strengthening functions, including *Strychnosnux vomica* (4-6). *Strychnosnux vomica* has been used in Traditional Chinese Medicine for hundreds of years for the treatment of various diseases, including several types of cancer, orthopedic diseases and inflammatory disorders (4-6). Brucine is extracted from the seeds of *Strychnosnux vomica* (5) and it has been used as clinical treatment for several types of cancer, including multiple myeloma (6), diabetes mellitus (7) and inflammatory diseases (8). Brucine activates various signaling pathways, including the osteoprotegerin/receptor activator of nuclear factor κ-B (RANK)/RANK ligand (L), the Jagged1/Notch 1 and thevascular endothelial growth factor receptor 2 signaling pathways at cellular levels (5-8). A previous study reported that immuno-nanoparticles can selectively inhibit tumor cell growth in hepatocellular carcinoma (9). Brucine further inhibits fibroblast growth, including in synovial cells, serves analgesic effects and induces apoptosis *in vitro* (10,11). Therefore, the current study explored effects of brucine

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on human fibroblast-like synoviocytes (HFLS) of RA and investigated the associated molecular mechanisms.

Materials and methods

Instruments. FC microplate reader and Class 100 CO_2 gas incubator were purchased from Thermo Fisher Scientific, Inc. (Waltham, MA, USA). An HF safe 1200/c biosafety cabinet was purchased from Shanghai Lishen Scientific Equipment Co., Ltd. (Shanghai, China). The Mini-Protean Tetra system was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA).

Reagents preparation. Recombinant tumor necrosis factor-α (TNF-α; cat. no. 300-01A; PeproTech, Inc., Rocky Hill, NJ, USA) dry powder (0.5 g) was dissolved in PBS and centrifuged at 1,500 x g for 20 min at room temperature. TNF-α was then dissolved in Dulbecco's modified Eagle's medium (DMEM)/high glucose (10 μg/l; cat. no. SH30022.01B; HyClone; GE Healthcare Life Sciences, Logan, UT, USA). Brucine (cat. no. MUST12122812; Chengdu Must Bio-Technology Co., Ltd., Chengdu, China) dry powder (5 g) was dissolved in PBS and centrifuged at 1,500 x g for 20 min at room temperature. The supernatant was used to make a 20 mg/ml stock solution. c-Jun N-terminal kinase (JNK) specific inhibitor SP600125 (Cell Signaling Technology, Inc., Danvers, MA, USA) was added to the cells (at final concentration of 25 mol/l) to inhibit JNK phosphorylation.

Cell Counting Kit-8 (CCK-8) assay. HFLS-RA cells (cat. no. #408RA-05a; Cell Application, Inc., San Diego, CA, USA) were cultured in DMEM/high glucose and maintained at 37˚C in a 5% CO_2-humidified incubator. Cells were passaged to the fourth generation prior to subsequent experimentation. Cell viability was examined using the CCK-8 kit (QF0025; Shanghai Xiangsheng Biotechnology Co., Ltd., Shanghai, China), according to the manufacturer's protocol. In brief, HFLS-RA cells were seeded into 96-well plates ($5x10^5$/ml) and cultured for 24 h at 37˚C. Subsequently, cells were incubated with 10 μl TNF-α (10 μg/l) for 30 min at 37˚C followed by treatment with various concentrations (0.125, 0.25, 0.5 or 2 mg/ml) brucine or DMEM/high glucose (TNF-α control) for 24 h at 37˚C. For the control group, cells were treated with PBS only. Following 24-h incubation, CCK-8 solution (10 μl) was added to each well and further incubated at 37˚C for 3 h. Absorbance measurements at 450 nm were performed to evaluate the viability of HFLS-RA. Viability was reported as the percentage of optical density (OD) of treatment groups/OD of blank control (PBS only).

Western blotting. HFLS-RA were seeded into 96-well plates at a density of $5x10^4$ cells/well for 24 h at 37˚C. Subsequently, cells were incubated with 10 μl TNF-α (10 μg/l) for 30 min at 37˚C followed by treatment with various concentrations (0.125, 0.25, 0.5 and 2 mg/ml) of brucine or PBS (blank control group) for 24 h at 37˚C. Total protein was extracted from cells using the ProteoPrep® Total Extraction Sample kit (cat. no. PROTTOT-1KT; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), according to the manufacturer's protocol. Total protein was quantified using the bicinchoninic acid assay kit (cat. no. BCA1; Sigma-Aldrich; Merck KGaA)

Table I. HFLS-RA viability with TNF-α pretreatment followed by treatment with various concentrations of brucine.

Group	Repeats (n)	Cell viability (%)
0.125 mg/ml brucine + TNF-α	6	78.59±1.70[b,c]
0.25 mg/ml brucine + TNF-α	6	86.95±1.60[a-c]
0.5 mg/ml brucine + TNF-α	6	74.37±0.91[a-c]
2 mg/ml brucine + TNF-α	6	69.22±0.90[a-c]
TNF-α	6	109.1±1.10[a]
Control	6	77.25±2.01
Blank cell	6	100±0.00

Data are presented as the mean ± standard deviation. [a]P<0.05 vs. Control; [b]P<0.05 vs. TNF-α; [c]P<0.05 vs. Blank cells. TNF-α, tumor necrosis factor-α; HFLS-RA, human fibroblast-like synviocytes of rheumatoid arthritis.

and 0.2 μg protein/lane was separated via SDS-PAGE on a 15% gel. The separated proteins were transferred onto polyvinylidene difluoride membranes (Amersham; GE Healthcare, Chicago, IL, USA) and blocked for 2 h at 37˚C with 5% skimmed milk. The membranes were rinsed with PBST-ween-20 (PBST) prior to incubation with mouse anti-human JNK monoclonal antibody (1:3,000; cat. no. sc136205), mouse anti-human phosphorylated (p)-JNK monoclonal antibody (1:2,000; cat. no. sc6254) or mouse anti-human β-actin monoclonal antibody (1:3,000; cat. no. sc376421; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. Membranes were washed three times with PBST. The membranes were incubated with horseradish peroxidase-conjugated rabbit anti-mouse polyclonal antibody (1:2,000; cat. no. sc358914; Santa Cruz Biotechnology, Inc.) for 30 min at 37˚C. Membranes were washed six times with PBST. Protein bands were visualized using enhanced chemiluminescence (Amersham; GE Healthcare) and images were captured and analyzed using Quantity One (version 4.52; Bio-Rad Laboratories, Inc.).

Statistical analysis. Data presented as the mean ± standard deviation were and analyzed with SPSS (version 17.0; SPSS, Inc., Chicago, IL, USA). Data are representative of ≥6 independent tests. One-way analysis of variance followed by Tukey's post-hoc test was used in the comparison of multiple groups. The association between brucine dosage and JNK phosphorylation was analyzed using a linear regression analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

Brucine treatment reverses increased TNF-α-induced cell viability. Effect of brucine on cell growth was observed in TNF-α-treated HFLS-RA. TNF-α significantly improved cell viability compared with the untreated Control as determined by CCK-8 assay (P<0.05; Table I). Brucine treatment of TNF-α-treated HFLS-RA significantly reversed the observed viability increases induced by TNF-α (P<0.05; Table I). Low
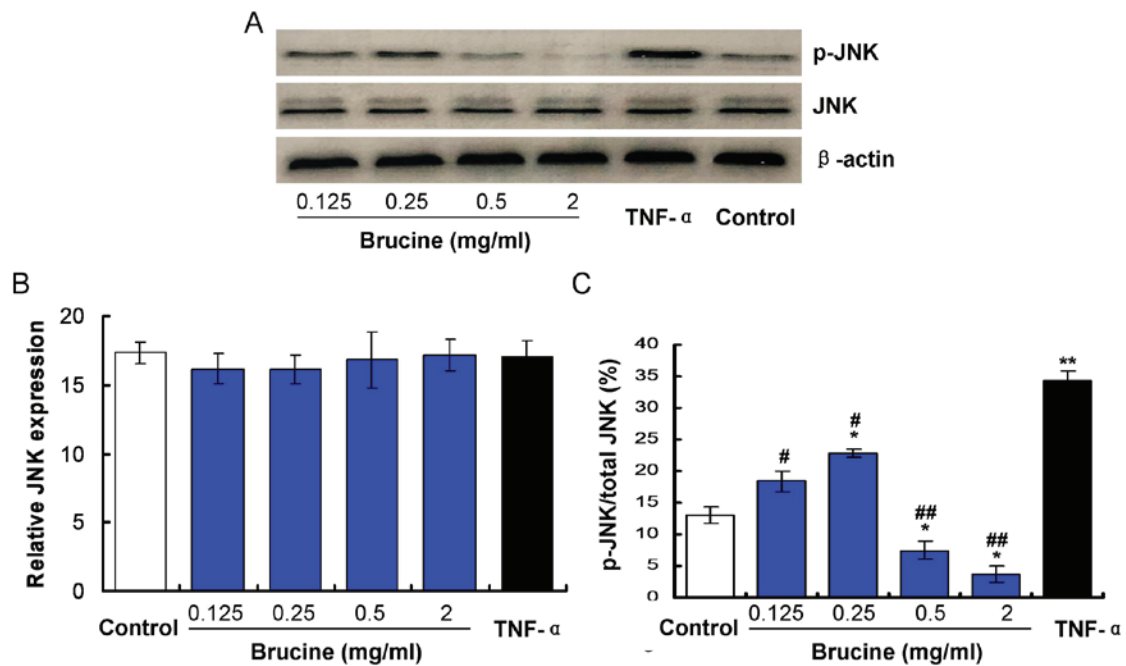
Figure 1. JNK expression and phosphorylation in TNF-α-induced HFLS-RA with brucine treatment. (A) Western blot analysis was performed to assess JNK and p-JNK levels in HFLS-RA pretreated with TNF-α and various concentration of brucine. (B) Evaluation of JNK expression. (C) Analysis of p-JNK/total JNK values. Data presented as the mean ± standard deviation of six experiments. $^*P<0.05$, $^{**}P<0.01$ vs. Control; $^#P<0.05$, $^{##}P<0.01$ vs. TNF-α. TNF-α, tumor necrosis factor α; JNK, c-Jun N-terminal kinase; p-, phosphorylated; HFLS-RA, human fibroblast-like synoviocytes of rheumatoid arthritis.

doses of brucine (0.125 and 0.25 mg/ml) reduced the cell viability of TNF-α-treated HFLS-RA to levels just above those determined for the untreated Control ($P>0.05$ and $P<0.05$, respectively; Table I). Higher doses of brucine (≥0.5 mg/ml) decreased the cell viability of TNF-α-treated HFLS-RA to levels significantly lower compared with the untreated control ($P<0.05$; Table I).

Brucine reduces JNK phosphorylation in TNF-α-treated HFLS-RA. Expression of JNK in TNF-α-induced HFLS-RA cells exposed to varying concentrations of brucine was evaluated. The results indicated no significant differences in JNK expression between the TNF-α and the Control groups, the brucine and the TNF-α groups, the brucine and the Control groups or among the brucine groups ($P>0.05$; Fig. 1). To investigate effects of brucine on JNK phosphorylation, p-JNK/JNK levels were examined by western blotting. Results indicated that the p-JNK/JNK value was significantly increased in TNF-α-treated HFLS-RA compared with the untreated Control ($P<0.05$; Fig. 1C). Significant decreases were observed in the brucine-treated groups compared with the TNF-α-treated HFLS-RA ($P<0.05$; Fig. 1C). At low brucine treatment doses (0.25 mg/ml) the p-JNK/JNK values were significantly increased compared with the untreated control ($P<0.05$) and at higher treatment doses (≥0.5 mg/ml) p-JNK/JNK values were decreased compared with the untreated Control ($P<0.05$; Fig. 1C).

Brucine reduces HFLS-RA viability by activating the JNK signaling pathway. To assess whether HFLS-RA viability was affected by the activation of the JNK signaling pathway, SP600125, a JNK specific inhibitor, was added to the various TNF-α-induced HFLS-RA treated with brucine. The result

indicated that SP600125 treatment increased cell viability in all TNF-α-induced HFLS-RA groups (Fig. 2). SP600125 induced a decrease in viability in the untreated Control group. Significant increases were observed in all groups treated with SP600125 and brucine compared with the groups only treated with brucine ($P<0.05$; Fig. 2). These results suggest that brucine reduced HFLS-RA viability by activating the JNK signaling pathway, however, as HFLS-RA viability did not fully recover, other pathways may also be involved.

Brucine is associated with JNK phosphorylation in TNF-α-treated HFLS-RA. Linear regression analysis was performed to assess the association between JNK expression and phosphorylation and brucine concentration. The results suggest that there was no association between JNK expression and brucine concentration (Fig. 3A). JNK phosphorylation exhibited an association with varying concentrations of brucine in TNF-α-induced HFLS-RA (Fig. 3B).

Discussion

In recent years, excessive proliferation and activity of FLS have been reported to be the major reason for joint injury in RA (12,13). Stimuli from inflammatory factors induce tumor-type unlimited proliferation in FLS (14). TNF-α is a proinflammatory factor produced by lymphomonocytes and macrophages with multiple roles, including the magnification of inflammatory signals and the induction of excessive FLS proliferation (15). Amplified inflammatory signals can trigger secretion of inflammatory factors, including interleukin (IL)-1 and IL-6, which aggravate synovitis and further damage the joints (16).

Brucine is a herbal compound extensively used in China, Thailand, East India and Northern Australia (17). Previous studies
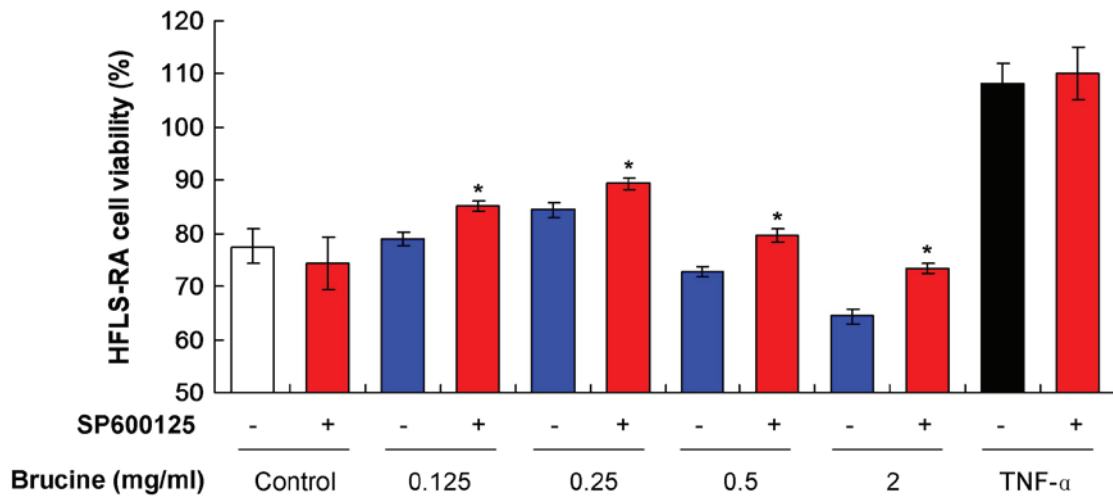
Figure 2. HFLS-RA viability in the presence of SP600125. Cell Counting Kit-8 assays were used to evaluate the effect of SP600125 on HFLS-RA viability in cells pretreated with TNF-α and various concentrations of brucine. *P<0.05 vs. SP600125 untreated. TNF-α, tumor necrosis factor-α; HFLS-RA, human fibroblast-like synoviocytes of rheumatoid arthritis.
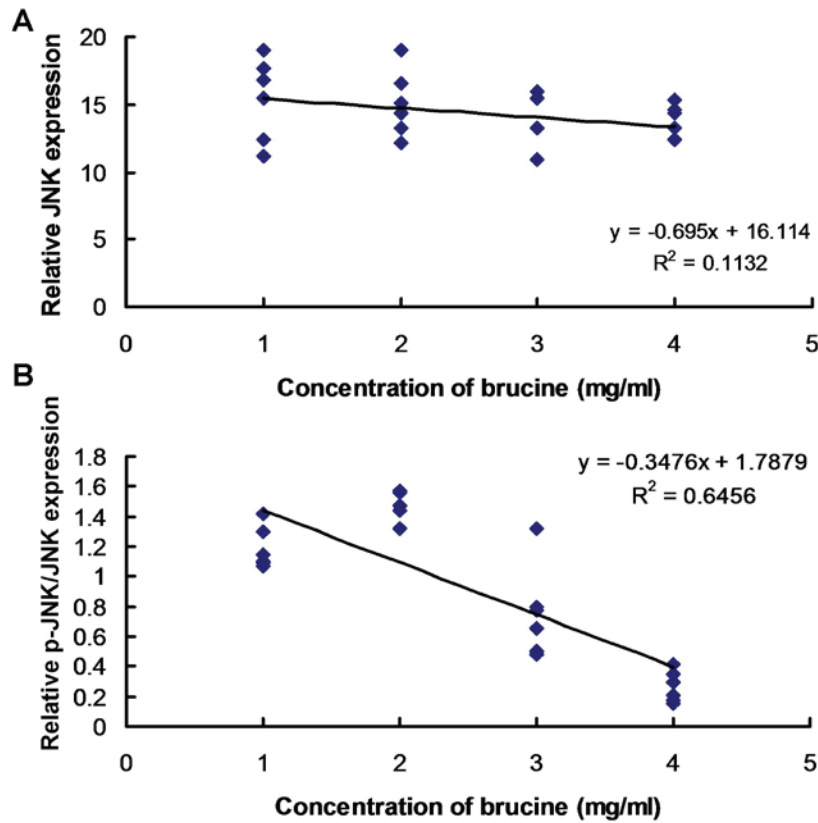

Figure 3. Brucine is associated with JNK phosphorylation. A linear regression analysis assessed an association between brucine and JNK (A) expression and (B) phosphorylation in TNF-α-treated HFLS-RA. JNK, c-Jun N-terminal kinase; p-, phosphorylated; TNF-α, tumor necrosis factor-α; HFLS-RA, human fibroblast-like synoviocytes of rheumatoid arthritis.

reported various functions of brucine, including anti-inflammation, antitumor, antianemia, antianalgesia, antidiabetes and antigonorrhea (18,19). Wu *et al* (8) recently reported that brucine inhibits arthritis symptoms and synoviocytes growth in an arthritis rat model. The current study investigated whether brucine acts as a TNF-α inhibitor and exerts antiproliferation effects in TNF-α-induced HFLS-RA. The present study illustrated that lower brucine doses (≤0.25 mg/ml) significantly

decreased TNF-α-induced viability changes to levels increased compared with the untreated Control, thus, not fully reversing the effects exerted by TNF-α. High brucine doses (≥0.5 mg/ml) fully reversed TNF-α-induced effects and further decreased cell viability compared with the untreated Control. The results of the present study are consistent with findings by Xin *et al* (20) regarding the induction of apoptosis inhuman monocytic leukemia cells by brucine.

A previous study reported that the mitogen-activated protein kinase (MAPK) signaling pathway induces expression of TNF-α in FLS (21). There are various enzymes associated with the MAPK signaling pathway, including JNK, p38 and extracellular signal-regulated kinase (21). Phosphorylation of these kinases triggers participation in pathogenic processes of synovitis (22). Therefore, the present study examined JNK expression and phosphorylation in TNF-α-induced HFLS-RA with brucine treatment. It was observed that JNK expression was not affected by brucine treatment, but phosphorylation levels were decreased in the brucine treatment groups compared with the TNF-α group. Only doses ≥0.5 mg/ml brucine fully reversed TNF-α-induced effects and further decreased phosphorylation levels compared with the untreated Control. A linear regression analysis suggested that brucine dose-dependently activated JNK phosphorylation in TNF-α-induced HFLS-RA. To further confirm the effects of brucine were mediated by the JNK signaling pathway, SP600125, a specific inhibitor, was employed in TNF-α-induced HFLS-RA. It was observed that the presence of the inhibitor alleviated the inhibitory effects exerted by brucine. However, viability levels did not fully recover to match the TNF-α-induced levels. The results indicated that brucine partially modulated cell viability of HFLS-RA by activating the JNK signaling pathway. The results indicated that TNF-α significantly activated JNK phosphorylation in HFLS-RA and triggered proliferation. The results are consistent with previous findings reporting that TNF-α promoted HFLS-RA proliferation through JNK activation of the MAPK signaling pathway (23).

A previous study demonstrated that brucine modulates physiological functions by regulating cell immune responses (9). Furthermore, brucine serves a role in several cellular processes, which include inhibiting tumor cell growth, conducting analgesic effects and inducing cell apoptosis (9-11). Based on a preliminary data (not shown), brucine has low bioavailability, however the current study used various concentrations of brucine ranging from 0.125-2 mg/ml, including optimal and higher brucine concentrations. However, a limitation associated with the current study is the lack of IC50 assay to measure the potency of brucine.

In conclusion, TNF-α promoted HFLS-RA proliferation and brucine significantly inhibited TNF-α-induced effects in HFLS-RA partially by activating the JNK signaling pathway. Brucine was further associated with JNK phosphorylation in TNF-α-treated HFLS-RA. Therefore, brucine may be physiologically relevant and have potential applications in the therapy of RA.

Availability of data and materials

All data generated and/or analyzed during the current study is available from the corresponding author on reasonable request.

Authors' contributions

MT, WJZ and ZCY performed the experiments. ZCY performed the statistical analysis. MT and CSH contributed to the design of the study and prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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