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Research of Decoctions and Granules of Aconite in Combination with Pinellia's Toxic and Effect Transformation Mechanism Based on Differences in Antioxidant Capacity

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Abstract: *This project focuses on investigating the differences in toxicity and antioxidant activity between traditional decoction pieces and granule preparations of Aconiti Radix (Chuanwu) and Pinelliae Rhizoma (Banxia), aiming to elucidate the toxicological and efficacy relationship of their incompatible combination according to the "Eighteen Incompatible Medicaments" (Shiba Fan).We employed the MTT (thiazolyl blue tetrazolium bromide) assay to evaluate the effects of Aconiti Radix and Pinelliae Rhizoma on the proliferation of HL-7702 hepatocytes in vitro. Additionally, we measured protein content using the Bradford method and assessed oxidative stress indices, including superoxide dismutase (SOD), total antioxidant capacity (T-AOC), malondialdehyde (MDA), and glutathione (GSH).The results demonstrated that compared to the untreated control group, both Aconiti Radix and Pinelliae Rhizoma decoction pieces significantly enhanced cell viability relative to their granule counterparts at equivalent concentrations. Furthermore, granule preparations of Aconiti Radix and Pinelliae Rhizoma markedly increased MDA levels while reducing T-AOC, SOD, and GSH levels compared to their respective decoction pieces.In contrast, the combination groups—Aconiti Radix and Pinelliae Rhizoma granule mixture, co-decocted liquid of their traditional pieces, and mixed liquid of individually decocted pieces—showed decreased intracellular MDA levels and elevated T-AOC, SOD, and GSH levels.Thus, within a certain concentration range, Aconiti Radix granules exhibited higher toxicity than their decoction piece counterparts, whereas Pinelliae Rhizoma decoction pieces promoted greater cell proliferation than equivalent granule preparations. Notably, the combination of Aconiti Radix and Pinelliae Rhizoma enhanced cell proliferation and reversed the oxidative stress induced by their individual administration.*

Keywords: *Chuanwu, Pinellia, Chinese Herbal Medicine Pieces, Chinese Herbal Granules, Toxicity, Antioxidant Activity*

1. Introduction

Chinese herbal medicine slices refer to Chinese herbal medicines that have been processed and prepared according to the theory and processing methods of traditional Chinese medicine and can be directly used in clinical practice. They are a special part of the Chinese medicine system for clinical practice and are currently widely used in the field of disease prevention and treatment in clinical practice, playing an important role in maintaining people's health[1] . Chinese herbal medicine granules are Chinese herbal medicine dosage forms made from Chinese herbal medicine slices through extraction, separation, drying and other means. They have the advantages of being easy to carry and take, having a large market capacity and being rapidly absorbed[2]. However, there are few reports on the research on the "toxicity-effect" relationship of Chinese herbal medicine slice granules by domestic and foreign scholars. The "Eighteen Antidotes" are the core content of the incompatibility of Chinese medicines and have been used in China for a long time. However, there is no clear record in the medical books of all dynasties on the conditions under which the conclusions were drawn. Among the prescriptions containing various groups of incompatibility drugs, the internal prescriptions of Aconitum are the most widely used, and the incompatibility of Aconitum is the most representative. Aconitum and Pinellia are one of the "Eighteen Antidotes". Based on the records of Aconitum in Compendium of Materia Medica, it can be basically assumed that the Aconitum used in the Song Dynasty and later

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should be Chuanwu[3]. Chuanwu (Aconiti Radix) is the mother root of the Ranunculaceae plant Aconitum carmichaeli Debx. It was first recorded in Hou Ningji's "Pharmacopoeia", which said that it is hot in nature, highly toxic, and has the effects of dispelling wind and dampness, warming the meridians and relieving pain[4]. Pinellia (Pinellia ternata) is the dried tuber of the Araceae plant Pinellia ternata (Thunb.) Breit. It is warm in nature and has the effects of warming the middle and resolving phlegm, relieving adverse reactions and relieving vomiting. Its irritation can be reduced when used with ginger[5,6]. However, there are also some ancient books such as "Golden Chamber Synopsis", "Hejiju Fang" and "Puji Fang" that record the use of the two together. However, whether the toxicity of the two is enhanced when they are combined has been a focus of debate among doctors of all generations.

In this study, typical representatives of counter-drugs such as Chuanwu and Pinellia were selected, and HL-7702 hepatocytes were used as the research carriers. The toxicity differences of Chuanwu granules and their traditional decoction pieces at the same raw drug concentration from the same batch of medicinal materials, the toxicity differences of Pinellia granules and their traditional decoction pieces at the same raw drug concentration, and the toxicity differences of the combined solution of Chuanwu and Pinellia granules, the combined decoction of Chuanwu and Pinellia traditional decoction pieces, and the combined solution of Chuanwu and Pinellia traditional decoction pieces were compared. While revealing the toxicity differences between Chinese herbal granules and traditional decoction pieces, the toxicity -effect relationship of the counter-drugs Chuanwu and Pinellia was clarified, providing a reference for safe clinical drug use.

2. Material

2.1 Experimental samples

Chuanwu Formula Granules (Batch No.: K2108135): Weigh 2g (Each gram of formula particles is equivalent to 6g crude drug), add deionized water 10mL, the concentration is 1.2g/mL, add deionized water to dilute to 1g/mL. Chuanwu decoction (batch number: 22061103): weigh 50g Chuanwu slices, put them into a 1000mL round-bottom flask, add 500 Soak in deionized water for 60min, heat to reflux and boil for 1.5h, filter to obtain filtrate 1; add 400mL of deionized water to the filtered residue and heat to reflux and boil for 1.5h, filter to obtain filtrate 2, combine filtrates 1 and 2, put into a round-bottom flask and concentrate to 50mL, then the concentration of Chuanwu medicinal solution is 1g/mL , solution 1000rpm/min centrifugation 30min, and the supernatant was stored at -20℃. Ginger and Pinellia Formula Granules (Batch No.: K2109093): Weigh 2g (Each gram of formula particles is equivalent to 12g crude drug), add deionized water to 20mL, the concentration is 1.2g/mL, add deionized water to dilute to 1g/mL; Ginger and Pinellia decoction slices decoction (batch number: 22041301): the preparation method is the same as that of Chuanwu decoction slices decoction.

2.2 Experimental reagents

1640 culture medium (Punosai, batch number: WH0022D301), fetal bovine serum (Bio Channel, batch number: 20230111), trypsin (Gibico, batch number: 2666516), penicillin-streptomycin solution (Biyuntian, number: C0222), DMSO (Sigma, batch number: RNBK5742), MTT (Sigma , batch number: MKCR0748), Coomassie Brilliant Blue G250 (McLean, batch number: C14003064), superoxide dismutase (SOD) activity detection kit (Solarbio, catalog number: BC0175), total antioxidant capacity (T-AOC) detection kit (Solarbio, catalog number: BC1315), malondialdehyde (MDA) content detection kit (Solarbio, catalog number: BC0025), reduced glutathione (GSH) content detection kit (Solarbio, catalog number: BC1175) .

2.3 Experimental equipment

Precision balance (ME104E, Mettler), full-wavelength microplate reader (Multis, MD), carbon dioxide incubator (3111, Thermo), clean bench (HCB-1300V, Haier), water purifier (Milli-Q Direct 8, Millipore), cell technology instrument (CountessII, Life), desktop high-speed refrigerated centrifuge (ST16R, Thermo).

2.4 Cell

HL-7702 cells were purchased from the Cell Bank of the Chinese Academy of Sciences.

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3. Method

3.1 Cell culture

HL-7702 cells were cultured in DMEM containing 10% FBS and 1% double antibody in an incubator at 37°C and 5% CO2 under saturated humidity. The color change of the culture medium and the cell growth were observed.

3.2 MTT assay

HL-7702 cells in the logarithmic growth phase were taken and the cell density was adjusted to 10×104/mL. They were inoculated on a 96-well plate with 100μL per well. A blank group, a control group and 28 drug-treated groups were set up, with 6 parallel control wells in each group. The cells were placed in an incubator and cultured for 24 hours. After the cells adhered to the wall, replace with fresh culture medium for 200μL, different concentrations of drug solution were added to the experimental groups as shown in Table 1; the final concentration of MCPG group, DCPS group and CCPS group was 1g/mL, the specific dosing ratio is shown in Table 1. After dosing, the 96-well plate was placed in a constant temperature incubator and cultured for 48h, aspirate the solution in the wells and add freshly prepared MTT solution (5g/L) and continue to culture in a constant temperature incubator for 4h. Aspirate the supernatant and add 150μL of DMSO, shake 10min, measure at 490nm and calculate the cell viability.

Table 1 Final concentration of drug in experimental group

Experimental Group	Dosing concentration (μg / mL)			
CDS	800	667	500	333
CGS	800	667	500	333
PDS	200	333	500	667
PGS	200	333	500	667
MCPG	4:1	2:1	1:1	1:2
DCPS	4:1	2:1	1:1	1:2
CCPS	4:1	2:1	1:1	1:2

Note: CDS: Chuanwu Decoction Solution; CGS: Chuanwu Granule solution; PDS: Pinellia Decoction Solution; PGS: Pinellia Granule solution; MCPG: Mixture of Chuanwu Pinellia Granules; DCPS: Decoction of Chuanwu and Pinellia slices; CCPS: Combined liquid of single decoction of Chuanwu and Pinellia Slices

3.3 Protein content was determined by Coomassie Brilliant Blue method[7]

3.3.1 Bovine serum albumin standard solution

Accurately weigh 100mg bovine serum albumin, dissolved in 100mL PBS , that is 1000 Prepare a standard solution of 12.5, 25, 50, 100, 200, 400, 800, and 1000 μg/mL.

3.3.2 Preparation of Coomassie Brilliant Blue G-250

Weigh 10mg Coomassie Brilliant Blue G-250, dissolved in 5mL 95% ethanol, add 85% (W/V) phosphoric acid 10mL, and finally dilute to 100mL.

Use a 96-well plate, each well added to 10μL of diluted BSA standard solution of different concentrations, and 200μL of G-250 was added to each well. 10μL PBS and 200μL G-250 were added to the control wells. Three replicate wells were set for each concentration, and the control wells were used as zero wells. After shaking for 2 min, the OD value of each well was measured at 595 nm using an ELISA reader, and the standard curve was drawn with protein concentration (μg/ml) as the horizontal axis and OD value as the vertical axis.

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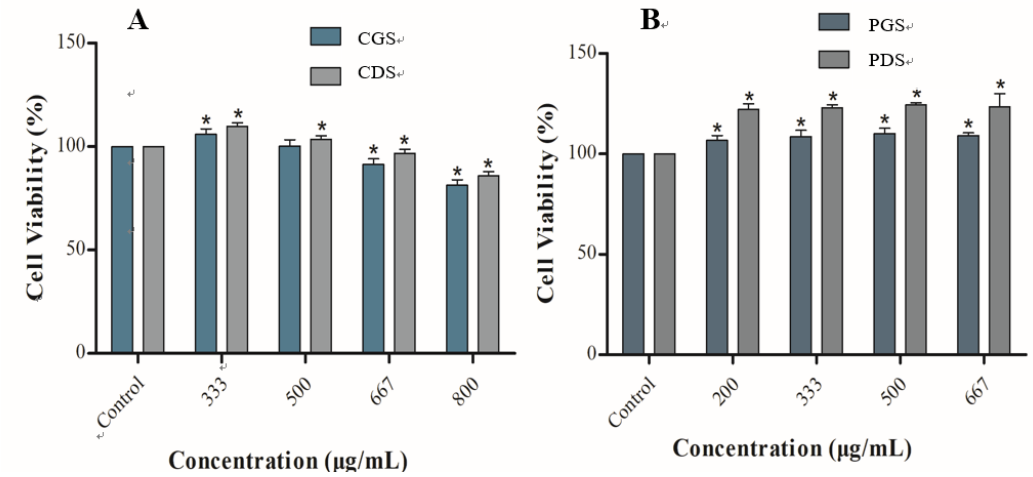
3.4 Determination of oxidative stress index

The cells were seeded in 6-well plates and the density was adjusted to 30×104cells. A control group and 7 drug-treated groups were cultured for 24h. Fresh culture medium was replaced in the control group, and different volumes of drug solution (10mg/mL), so that the final mass concentration of CDS and CGS is 800μg/mL, the final mass concentration of PDS and PGS is 200μg/mL, and the final mass concentration of MCPG, DCPS, and CCPS mixed in a ratio of 4:1 is 1g/mL , and after culturing for 48h, the protein content was determined by the Coomassie Brilliant Blue method, and the levels of SOD, T-AOC, MDA, and GSH were determined using their respective detection kits.

4. Result

4.1 Radix Aconiti Lateralis and Rhizoma Pinelliae on HL-7702 Hepatocytes

The experimental results showed that compared with the control group, CGS and CDS (concentrations of 333 and 500μg/mL) were negatively correlated with the viability of HL-7702 cells as the concentration increased. When the concentration increased to 667μg/mL, cell growth was inhibited. When the concentration increased to 800μg/mL, there was stable cytotoxicity to HL-7702 cells (Figure 1A). Therefore, the subsequent experimental drug administration concentration was 800μg/mL; MCPG, DCPS, CCPS (4:1, 2:1, 1:1, 1:2), as the concentration of Pinellia in the mixed solution gradually increased, the proliferation effect of HL-7702 cells gradually increased, and when the ratio of the mixed solution was 4:1, Pinellia could weaken the toxicity of HL-7702 cells induced by Chuanwu (800μg/mL) (Figure 1C). In order to be able to match the selected Chuanwu concentration (800μg/mL) to form a control, and to better study the toxicity conversion mechanism of Chuanwu and Pinellia after mixing, the subsequent experiments selected 4:1 (Chuanwu 800μg/mL , Pinellia 200μg/mL); MTT results showed that when the concentrations of PGS and PDS were 200, 333, and 500μg/mL, the viability of HL-7702 cells gradually increased, and when the concentration increased to 667μg/mL, the cell viability began to weaken (Figure 1B). According to the selected ratio of Chuanwu and Pinellia mixed solution (4:1), subsequent experiments were carried out at a dosing concentration of 200μg/mL. In addition, from the results, we can also observe that the cell viability of the decoction group of Chuanwu and Pinellia was greater than that of the granule group at the corresponding concentration, whether it was Chuanwu or Pinellia.



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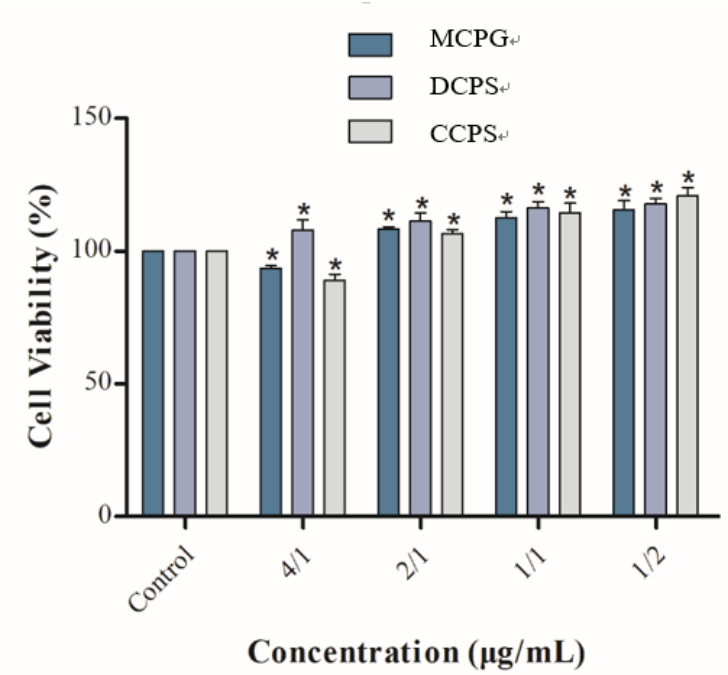

Figure 1 The blocking effect of the combination of Chuanwu and Pinellia on the cytotoxicity induced by Chuanwu and Pinellia alone.(A) The difference in toxicity between CGS and CDS at the same crude drug concentration . (B) The difference in toxicity between PGS and PDS at the same crude drug concentration . (C) The difference in toxicity between MCPG, DCPS and CCPS at different ratios of Chuanwu and Pinellia .

Note: * p <0.05 compared with the control group, n =6.

4.2 Coomassie brilliant blue method to determine protein content

Table 2 OD values of proteins at different concentrations

Protein concentration (μg / ml)	OD value ($\bar{x}$±SD)
12.5	0.514±0.001
25	0.536±0.001
50	0.560±0.003
100	0.614±0.009
200	0.717±0.007
400	0.865±0.016
800	1.128±0.005
1000	1.281±0.013

After processing with Graphpad Prism software, the linear equation was obtained: Y=0.0008x+0.5312, R2 = 0.9944. The standard curve of the conventional Bradford method is nonlinear. The experimental results of the method used in this experiment are shown in Table 2. The BSA protein content has a good linear relationship with the OD value between 12.5 and 1000 μg/mL, and the operation is convenient and fast, and the sample amount required is small, which can meet the needs of protein content determination of various biological samples. Therefore, this method is used in subsequent experiments to determine the protein content of samples.

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4.3 Effects of Radix Aconiti Lateralis and Rhizoma Pinelliae on oxidative stress in HL-7702 cells

High levels of malondialdehyde (MDA) and low levels of T-AOC, SOD, and GSH are indicators of oxidative stress and apoptosis. The results of this experiment showed that compared with the untreated control group, CGS and CDS, PGS and PDS all significantly increased the MDA level, while reducing the T-AOC, SOD, and GSH levels (Figure 2). The intracellular MDA level in the MCPG, DCPS, and CCPS administration groups decreased, while the T-AOC, SOD, and GSH levels increased. These results indicate that the combination of Chuanwu and Banxia can block the oxidative stress induced by the administration of Chuanwu and Banxia alone.

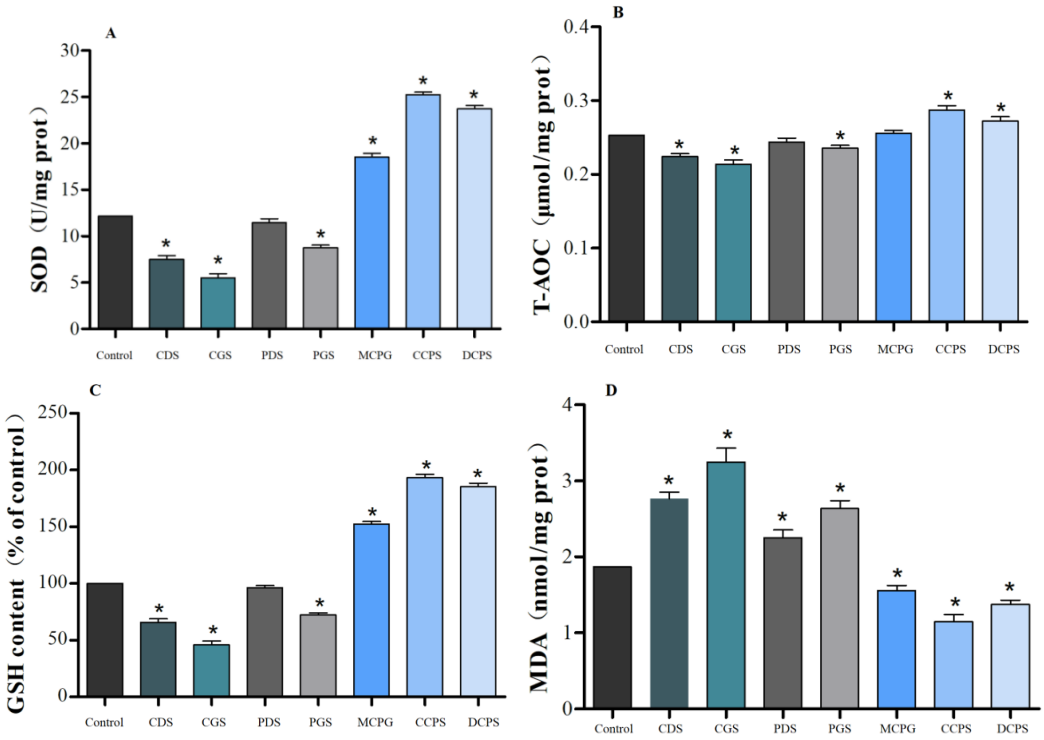

Figure 2 Effects of the combination of Chuanwu and Banxia on oxidative stress induced by Chuanwu and Banxia alone.(A) Effect on T-AOC level. (B) Effect on SOD level. (C) Effect on GSH. (D) Effect on MDA.

Note: * $p < 0.05$ compared with the control group, $n = 3$.

5. Discuss

With China's emphasis on and vigorous promotion of the traditional Chinese medicine industry, Chinese herbal medicine pieces and formula granules have developed rapidly. However, there has been no clear conclusion on whether the effectiveness and safety of Chinese herbal medicine pieces and formula granules, especially toxic medicinal materials, compound preparations or the compatibility of anti-drugs in the "Eighteen Anti-drugs", are consistent.

This study explored the toxicity and antioxidant stress resistance of the toxic Chinese herbal medicines Chuanwu formula granules and their decoctions, Jiang Banxia formula granules and their decoctions, Chuanwu Banxia granules combined solution, Chuanwu Banxia traditional decoctions, and Chuanwu Banxia traditional decoctions combined solution at the cellular level. The results showed that within the concentration range selected in this experiment, the decoction of Chuanwu (667, 800μg/mL) was less toxic to HL-7702 hepatocytes than the Chinese herbal medicine formula granules under the same crude drug concentration conditions. The reason may be that the highly toxic components of Chuanwu, diester-type diterpenoid alkaloids, decompose into less toxic aconitine, mesaconitine, and mesaconitine. These components are chemically unstable and are easily hydrolyzed by heating in water to form less toxic monoester-type alkaloids. Further hydrolysis can generate less toxic aconitine[8] ; Pinellia (200, 333, 500, 600μg/mL) Under the same crude drug concentration conditions, the single decoction of Chinese herbal medicine slices has a greater effect on the proliferation activity of

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HL-7702 hepatocytes than the Chinese herbal medicine granules. The study found that the polysaccharides in the components of Pinellia ternata have a scavenging effect on oxygen free radicals and DPPH, and can significantly increase the levels of antioxidant enzymes such as SOD and GSH, reduce the content of MDA, enhance the body's immune function, and improve the quality of immune organs. After sufficient boiling, the extract of the single decoction of Pinellia ternata slices is rich in polysaccharides and has a stronger ability to scavenge excessive free radicals; the combination of Chuanwu and Pinellia ternata can improve cell viability. This study result is consistent with the results of Yang Kunbao et al.[9] who believed that raw Pinellia ternata has an antagonistic effect on the hepatotoxicity of raw Chuanwu. At the same time, the combination of the two can block the oxidative stress induced by the administration of Chuanwu and Pinellia ternata alone. The reason may be that the combination of Pinellia ternata and Chuanwu can reverse the inhibitory effect of Chuanwu on CYP3A[10]. Therefore, from the perspective of the effect on CYP3A, the combination of Pinellia ternata and Chuanwu will not increase the toxicity of Chuanwu, but will reduce the toxicity of Chuanwu.

There are abundant plant resources of Chuanwu and Pinellia. In-depth research on their effective ingredients and their mechanisms of action can lay the foundation for studying their pharmacological effects and safe use of drugs. In view of the complexity of the traditional Chinese medicine system, the effects of drugs are affected by various physiological and pathological conditions. The effects of the combined use of Chuanwu and Pinellia need to be explored from multiple angles. In the later stage, the author will combine the analysis of medicinal ingredients, the changes in ingredients in the drug preparation process, and the in vivo and in vitro efficacy evaluation system to conduct more in-depth research and verification on the specific mechanisms of action of the toxic Chinese medicine Chuanwu and Pinellia slices and their formula granules, so as to provide a theoretical basis for giving full play to their medicinal value and rational development and utilization.

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Authors' contributions

MHJ.Paper writing guidance, paper revision, ZLQ.Paper writing, data statistical analysis and submission, All authors reviewed the manuscript.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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