InncRNA uc.48+ regulates immune and inflammatory reactions mediated by the P2X7 receptor in type 2 diabetic mice

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Abstract. Diabetes and non-coding RNAs are receiving increasing attention in contemporary medical research. The present study aimed to explore the role of the long non-coding RNA uc.48+ in the pathological changes of type 2 diabetes mellitus (T2DM) by observing the effects of uc.48+ small interfering RNA (siRNA) on the abdominal cells of a mouse model of T2DM. Mice with T2DM (DM group) were established by feeding with a high-sugar and -fat diet combined with intraperitoneal injections of low-dose streptozotocin. An intraperitoneal injection of uc.48+ siRNA was administered to the diabetic mice, and the serum levels of cytokines together with other clinical parameters, namely blood pressure, heart rate, mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were examined. Following the collection and identification of abdominal cells from the mice, the mRNA levels of uc.48+, mRNA and protein levels of the P2X7 receptor, and phosphorylation levels of ERK1/2 were evaluated by reverse transcription-PCR and western blotting, respectively. The MWT and TWL were significantly decreased in the DM group compared with the non-diabetic control group. However, the reductions in MWT and TWL were significantly attenuated following uc.48+ siRNA injection. The systolic and diastolic blood pressure, as well as the serum levels of tumor necrosis factor α and interleukin 1β of mice in the DM group were significantly increased compared with those in the control group, whereas these changes were significantly attenuated following the injection of uc.48+ siRNA. In addition, the expression levels of P2X7 receptor mRNA and protein, and the degree of phosphorylation of ERK1/2 in the abdominal cells were significantly increased in the DM group compared with the control group. These changes were also significantly attenuated following transfection with uc.48+ siRNA in vivo. In conclusion, these data suggest that uc.48+ may play an important role in the pathological changes of blood pressure, neurology and abdominal cell function in T2DM via interaction with the P2X7 receptor.

Introduction

The global prevalence of diabetes has increased markedly. Due to the aging population and continued increase in obesity rates, the prevalence is expected to rise to 592 million by 2035 (1). Concomitantly, the incidence of type 2 diabetes mellitus (T2DM) has increased rapidly, and patients with diabetes often experience a wide range of complications. T2DM is associated with low-grade inflammation, and this immune inflammatory response can be influenced and regulated by monocytes and macrophages via the secretion of cytokines and antigen-presenting cells (2-4). Previous studies have shown that the tissue macrophage status regulates the development and progression of T2DM (4,5). In addition, other studies have shown that peritoneal macrophages modulate the immune response by regulating cytokines and nitric oxide production in diabetic rats (6,7). Our previous study demonstrated that the P2X7 receptor (P2X7r) receptor of the mononuclear phagocyte system is involved in the pathology of diabetes (8). As expression of the P2X7 receptor may be associated with inflammation (9), it would be interesting to investigate whether the P2X7 receptor is able to ameliorate the chronic inflammatory state of T2DM.

Long non-coding RNAs (lncRNAs) are generally RNA transcripts >200 nucleotides in length that lack the ability to encode proteins (10). Previous studies have shown that lncRNAs may contribute to the regulation of cell apoptosis, proliferation and differentiation at the RNA epigenetic level via the induction of changes in gene transcription and post-transcriptional modifications (11). uc.48+ is an lncRNA that has been observed to be expressed at increased levels, along with the P2X7r receptor, in the superior cervical ganglia of a rat model of T2DM, and is associated with cardiac autonomic dysfunction (12). In our previous study, uc.48+ small interfering RNA (siRNA) was found to influence immune and inflammatory responses in a diabetic mononuclear phagocyte system...
through the P2X<sub>3</sub> receptor in vitro (13). A previous study revealed that macrophages may exist in large numbers in the peritoneum of mice (14). Therefore, the aim of the present study was to investigate whether uc.48+ exerts an effect on mouse abdominal cells through P2X<sub>3</sub> receptors in vivo. The ERK signaling pathway is the key pathway by which signals are transmitted from surface receptors to the nucleus (15). Decreased ERK phosphorylation appears to be associated with decreased P2X<sub>3</sub> receptor expression in RAW264.7 macrophages (13). In addition, uc.48+ siRNA has been shown to ameliorate diabetic sympathetic neuropathy in a rat model of T2DM (12). However, the exact mechanism by which uc.48+ affects the abdominal cells and neuropathological changes in mouse models of T2DM requires elucidation.

The present study aimed to investigate if uc.48+ siRNA has a beneficial effect on the function of abdominal cells, and whether it ameliorates the neuropathological changes associated with T2DM through the P2X<sub>3</sub> receptor and ERK signaling pathway. Therefore, the role of uc.48+ and the mechanism underlying its effects on the pathological changes in T2DM were evaluated. This was achieved by monitoring and assessing the effects of uc.48+ siRNA on mice with T2DM and their abdominal cells in which P2X<sub>3</sub> receptor expression was upregulated.

Materials and methods

Animals and animal groups. A total of 24 male Kunming mice of clean grade (32-42 g) were purchased from the Center of Laboratory Animal Science of Nanchang University at 8 weeks of age. They were acclimatized for 2 weeks at room temperature with 40-60% relative humidity and 12-hour light/dark cycles and given standard feed with free access to drinking water, and randomly divided into four groups (n=6 in each group), comprising the control group, the DM group, DM treated with uc.48+ siRNA group (DM + uc.48+ si) and DM treated with scrambled siRNA group (DM + NCsi). All procedures involving animals were approved by the Animal Care and Use Committee of the Medical College of Nanchang University (approval no. 2018 16).

Mice in the DM group were fed with a high-sugar and -fat diet (consisting of 22% fat, 48% carbohydrate and 20% protein with a total calorific value of 44.3 kJ/kg) for 4 weeks and subsequently injected intraperitoneally (i.p.) with streptozotocin (STZ; 80 mg/kg). Control mice were fed with normal diet (consisting of 5% fat, 53% carbohydrate and 23% protein, with a total calorific value of 25 kJ/kg) for 4 weeks and subsequently injected i.p. with the same concentration of saline. On the third day after STZ injection (the last day of week 6), the blood glucose levels of the mice were measured. The successful establishment of the DM model was confirmed by measuring the concentrations of each mouse were detected in the serum at the end of the 8-week period following animal sacrifice. The inflammatory state of the body can be evaluated by the determination of cytokines. The cytokine concentrations of each mouse were measured in the serum at the end of the 8-week period following animal sacrifice. The

uc.48+ siRNA treatment. The siRNA sequences specific for uc.48+ were purchased from Invitrogen (Thermo Fisher Scientific, Inc.). The following target sequence was used: sense, 5'-GGCACUCUACUUCAGAATT-3' and antisense, 5'-UUUCUGAAGUGCCGTCCT-3'. The uc.48+ siRNA was injected i.p. into DM model mice at the end of week 7 along with Entranster<sup>TM</sup>-in vivo Transfection Reagent (Engreen Biosystem Co., Ltd.) to establish the DM + uc.48+ si group. Similarly, DM model mice were injected with scrambled siRNA: sense, 5'-UUUCUGGACGUGUCCAGUTT-3' and anti-sense, 5'-ACGUGACGCUCCGAGATT-3' (Invitrogen; Thermo Fisher Scientific, Inc.) and transfection reagent to establish the DM + NCsi group. According to the transfection reagent manufacturer's protocol, a mixture of 1 µg uc.48+ or scrambled siRNA in diluent (100 µl) and 0.5 µl transfection reagent in diluent (100 µl) was injected i.p. The control and DM groups were injected with the same volume of saline.

At the end of week 8, the animals were sacrificed with CO<sub>2</sub> using a chamber displacement rate of 10-30%/min. Subsequently, 0.6-1.0 ml blood was collected by cardiac puncture and abdominal cells were harvested from the mice by intra-abdominal lavage.

Nociceptive behavior assays. Since uc.48+ siRNA has been shown to ameliorate diabetic sympathetic neuropathy in type 2 diabetic rats (8), the ability of uc.48+ siRNA to alleviate neuropathological changes in diabetic mice was investigated through behavioral assays in the present study. The behavioral assays comprised the assessment of mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) after 6 weeks (prior to STZ injection), 7 weeks (prior to siRNA injection) and 8 weeks (following siRNA injection), respectively.

Measurement of MWT. Noxious-pressure stimulation was used to evaluate mechanical hyperalgesia. The experimental protocol of Liu et al (18) was used.

Measurement of TWL. Noxious heat stimulation was applied using the Thermal Paw Stimulation System (BME-410C; Boerni Science and Technology Co., Ltd.) and hyperalgesia was assessed using thermal stimulation by Hargreaves’ test. The experimental protocol published by Liu et al (18) was followed, except the difference in animal strains used.

Measurement of heart rate (HR) and blood pressure. HR and blood pressure are indicators that reflect the health of the cardiovascular system. Each mouse was assessed by measuring its HR and blood pressure, including systolic blood pressure (SBP) and diastolic blood pressure (DBP), after 6 weeks (prior to STZ injection), 7 weeks (prior to siRNA injection) and 8 weeks (following siRNA injection). HR and blood pressure were assessed through an indirect tail cuff method (Softron BP-98A; Softron Co., Ltd.). A tail cuff 1.5 cm in diameter and 3.2 cm in length was used. Systolic pulsation was detected using an electrocardiogram coupler (ZH45-Z, MD3000; Anhui Zhenghua Biological Apparatus Facilities Co., Ltd.). In brief, the experimental protocol published by Wu et al (12) was followed, except the difference in animal strains used. The blood pressure of the mice was measured five times in the morning at each time point by one person.

Cytokine assays. The inflammatory state of the body can be evaluated by the determination of cytokines. The cytokine concentrations of each mouse were detected in the serum at the end of the 8-week period following animal sacrifice. The
cytokines assessed comprised tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-10. The concentration levels of IL-10 (cat. no. EK0417), IL-1β (cat. no. EK0394) and TNF-α (cat. no. EK0527) were determined using ELISA kits (Wuhan Boster Biological Technology, Ltd.) according to the manufacturer's protocol.

Isolation of abdominal cells. The effect of uc.48+ siRNA on P2X7 receptors in a diabetic mononuclear phagocyte system has previously been demonstrated in vitro (13). Therefore, the present study aimed to determine whether uc.48+ affects mouse abdominal cells through P2X7 receptors in vivo. Abdominal cells were harvested from the mice at the end of the 8-week period by intra-abdominal lavage, cultured overnight in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Hyclone; GE Healthcare Life Sciences), 100 U/ml penicillin and 100 mg/ml streptomycin sulphate at 37˚C in a humidified atmosphere containing 5% CO2, and enriched for abdominal cells by washing away non-adherent peritoneal cells with lukewarm serum-free culture medium. Wright-Giemsa dye was used for staining at room temperature for 5 min and samples were observed using SZ61 Olympus microscope. The cell seeding density was 5x10^6/ml for the Wright-Giemsa and trypan blue staining. The viability of the purified adherent abdominal cells was determined by trypan blue exclusion assay. A total of 0.1 ml trypan blue stock solution was added to 1 ml of cells at room temperature for 5 min. The number of stained and total cells was counted. Healthy log-phase cultures exhibit cell viability of ≥95% (19). The following equation was used in the present study: % Viable cells=[1.00-(number of blue-stained cells/number of all cells)] x100%.

Total RNA isolation and reverse transcription-PCR (RT-PCR) analysis. Total RNA was isolated from the abdominal cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The quality assessment of the RNA and synthesized cDNA was performed according to our previously published method (13). The PCR amplification of the P2X7 receptor and β-actin (internal standard for quantification) genes was performed according to our previously published method (20). The amplification system included 2 µl cDNA, 12.5 µl PCR mixture (Tiangen Biotech Co., Ltd.), 2 µl primers (1 µl each of sense and anti-sense primers) and 8.5 µl nuclease-free water. The sequences of the primers used for RT-PCR analysis were as follows: P2X7 sense primers) and 8.5 µl nuclease-free water. The sequences of the primers used for RT-PCR analysis were as follows: P2X7-sense primer (5'-CAC GAT GGA GGG GCC GGA CTC ATC-3') and antisense, 5'-TAA AGA CCT CTA TGC CAA CAC AGT-3'; β-actin (240 bp) sense, 5'-GCA AACTGGATGAGGAT-3' and antisense, 5'-GTAGTGCCACAAGAGA-3'; P2X7-uc.48+ (231 bp): sense, 5'-GCA AACTGGATGAGGAT-3' and antisense, 5'-CAAGCATGAGGAGGCCCCGACATC-3'. The PCR conditions used to detect these genes and analyze the PCR products were as described in the study by Wu et al (13). The steps of the western blot analysis for P2X7 receptor and (p-)ERK1/2 were performed as described previously in the study of Wu et al (13).

Statistical analysis. All experiments were carried out in triplicate to confirm the accuracy of the results and data are presented as the mean ± standard deviation. The data of the abdominal cell experiments were normalized to those of the control group. Statistical analyses were carried out using SPSS 11.5 software (SPSS, Inc.). Statistical significance was determined by one-way analysis of variance. The Tukey's test was used for comparison between groups. P<0.05 was considered to indicate a statistically significant difference.

Results
Effects of uc.48+ siRNA on the nociceptive behavior of DM mice. The MWTs and TWLs of the mice were measured. Prior to the STZ injection (at the end of week 6), no significant differences in MWT were detected among the four groups, while the TWLs of the DM, DM + NCsi and DM + uc.48+ si groups were lower than that of the control group. At 7W, the MWTs and TWLs of the DM + NCsi and DM + uc.48+ si groups were lower compared with those of the control group. At 8W, the MWT and TWL of the DM + uc.48+ si group were higher than those of the DM and DM + NCsi groups, n=6/group. P<0.05, **P<0.01 vs. the control group; ***P<0.001 vs. the DM group. siRNA, small interfering RNA; uc.48+, si.48+ siRNA; NCsi, scrambled control siRNA; siRNA, small interfering RNA; uc.48+, si.48+ siRNA; NCsi, scrambled control siRNA; MWT, mechanical withdrawal threshold; TWL, thermal withdrawal latency; 6W, 6 weeks (prior to STZ injection); 7W, 7 weeks (following STZ injection); 8W, 8 weeks (after siRNA injection); DM, diabetes mellitus; STZ, streptozotocin.
in the DM + uc.48+ si group were higher than those of the DM group.

These results reveal that the MWTs and TWLs in the DM group were significantly decreased compared with those of the control group. However, such effects were significantly attenuated following the injection of uc.48+ siRNA. Moreover, the TWL appeared to be significantly affected by a high-sugar and high-fat diet while the MTL was not.

**Effects of uc.48+ siRNA on the HR and blood pressure of DM mice.** The effects of uc.48+ siRNA treatment on HR and blood pressure are shown in Table I. The HR of the DM group was significantly increased compared with that of the control group. However, no significant differences were noted in HR between the DM, DM + NCsi and DM + uc.48+ siRNA groups.
following uc.48+ siRNA injection. The SBP and DBP in the DM group were significantly increased compared with those of the control group. However, these changes were significantly diminished following uc.48+ siRNA injection (Table I).

**Effects of uc.48+ siRNA on the cytokine levels of DM mice.** The serum TNF-α and IL-1β concentrations of the DM group were significantly higher compared with those of the control group, and these increases were significantly attenuated following the injection of uc.48+ siRNA. The serum IL-10 concentration of the DM group was significantly decreased compared with the corresponding concentration in the control group, whereas uc.48+ siRNA injection restored the IL-10 concentration to its initial levels (Fig. 2).

**Morphological identification and viability of abdominal cells.** The scattered distribution, variable size, irregular shape and strong refractive index of the adherent abdominal cells were observed by microscopy (Fig. 3A). Following Wright-Giemsa staining, these cells exhibited the morphological features of macrophages. They were oval, round or irregularly shaped. They possessed abundant cytoplasm with irregular margins and pseudopodia and often exhibited single eccentric nuclei (Fig. 3B). No marked changes were noted with regard to the size, morphology and number of abdominal cells following transfection with uc.48+ siRNA.

The viabilities of the adherent abdominal cells in all four groups were >95% as determined by the trypan blue exclusion assay, and no significant difference was detected among the groups (Fig. 3C).

**Changes in the uc.48+ expression levels in the abdominal cells of DM mice following uc.48+ siRNA treatment.** The expression levels of uc.48+ in the abdominal cells were significantly reduced following treatment with uc.48+ siRNA (1.20±0.10) compared with the DM group, whereas no significant differences were noted between the DM (1.50±0.15) and the DM + NCsi (1.49±0.14) groups (Fig. 4). These results indicate that the targeting of uc.48+ with siRNA effectively suppressed the expression of uc.48+ in the abdominal cells of DM model mice.

**Changes in the expression levels of P2X7 receptor mRNA and protein in the abdominal cells of DM mice following uc.48+ siRNA treatment.** As shown in Fig. 5, the upregulated mRNA and protein levels of the P2X7 receptor were significantly decreased following uc.48+ siRNA transfection in vivo (1.46±0.19 and 2.46±0.38, respectively) compared with those in the DM (2.29±0.29 and 4.41±0.63, respectively) group. No significant differences were detected between the DM and the DM + NCsi groups.

**Changes in phosphorylated (p-)ERK1/2 levels in the abdominal cells of DM mice following uc.48+ siRNA treatment.** The levels of p-ERK1/2 were normalized to the total ERK1/2 protein levels. The normalized p-ERK1/2 levels were significantly increased in the DM group (4.57±0.52) compared with the control group (1.00±0.00; Fig. 6). The knockdown of uc.48+ with uc.48+ siRNA (2.47±0.44) significantly decreased the ratio of p-ERK1/2 to total ERK1/2 compared with the DM group, while scrambled siRNA (4.99±0.85) exhibited no significant effects on p-ERK levels (Fig. 6).

**Discussion**

Diabetes is a considerable global health problem and has been classified as a major disease that requires prevention and control by the World Health Organization (21). The etiology of T2DM has not been fully clarified. Previous studies have shown that diabetic autonomic neuropathy, a complication of T2DM, can lead to cardiac dysfunction (22), while a chronic low-grade inflammatory response serves an important role in the occurrence and development of T2DM (2). In the present study, it was observed that the MWT and TWL of mice in the DM group were significantly lower than those in the control group, indicating that DM damages autonomic nerves and causes autonomic dysfunction, resulting in significant increases in blood pressure and HR. Concomitantly, the DM...
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The mRNAs and protein levels (top panel and bottom panel, respectively) of the P2X7 receptor were downregulated in the uc.48+ siRNA group (1.46±0.19 and 2.46±0.38, respectively) as determined by RT-PCR and western blotting, respectively, following transfection of uc.48+ siRNA iit vivo, while no significant differences were detected between the DM (2.29±0.29 and 4.41±0.63, respectively) and the DM + NCsi groups (2.30±0.24 and 4.27±0.44, respectively), n=6/group. **P<0.01 vs. the control group; ##P<0.01 vs. the DM group. DM, diabetes mellitus; NCsi, scrambled control siRNA; uc.48+ si, uc.48+ siRNA; siRNA, small interfering RNA; RT-PCR, reverse transcription PCR.

The RT-PCR results of the present study indicate that the expression levels of uc.48+ in the abdominal cells of mice were significantly higher in the DM group than in the control group. The results further revealed that the MWT and TWL of mice with DM were significantly reduced following uc.48+ siRNA injection, suggesting that uc.48+ siRNA treatment may relieve diabetic neuropathic pain. Concomitantly, the blood pressure and expression levels of the anti-inflammatory cytokines IL-1β and TNF-α were also significantly decreased in DM model mice following uc.48+ siRNA injection, demonstrating that the downregulation of uc.48+ is associated with changes that relieve the diabetic inflammatory state and cardiovascular disease.

Figure 5. Changes in the expression levels of P2X7 receptor mRNA and protein in the abdominal cells of type 2 diabetic mice following uc.48+ siRNA treatment. The mRNA and protein levels (top panel and bottom panel, respectively) of the P2X7 receptor were downregulated in the uc.48+ siRNA group (1.46±0.19 and 2.46±0.38, respectively) as determined by RT-PCR and western blotting, respectively, following transfection of uc.48+ siRNA iit vivo, while no significant differences were detected between the DM (2.29±0.29 and 4.41±0.63, respectively) and the DM + NCsi groups (2.30±0.24 and 4.27±0.44, respectively), n=6/group. **P<0.01 vs. the control group; ##P<0.01 vs. the DM group. DM, diabetes mellitus; NCsi, scrambled control siRNA; uc.48+ si, uc.48+ siRNA; siRNA, small interfering RNA; RT-PCR, reverse transcription PCR.

Figure 6. Changes of p-ERK1/2 levels in the abdominal cells of type 2 diabetic mice following uc.48+ siRNA treatment. The ratio of p-ERK1/2 to total ERK1/2 (4.57±0.52) was increased in the DM group compared with the control group, and uc.48+ siRNA (2.47±0.44) significantly decreased the proportion of p-ERK, while scrambled siRNA (4.99±0.85) did not significantly affect it. n=6/group. **P<0.01 vs. the control group; ##P<0.01 vs. the DM group. DM, diabetes mellitus; NCsi, scrambled control siRNA; uc.48+ si, uc.48+ siRNA; siRNA, small interfering RNA; p, phosphorylated.

In vitro studies have shown that IncRNAs exhibit important cellular functions (23-25). Experiments using knockout animal models have confirmed that multiple IncRNAs serve roles in disease pathogenesis (26,27). A number of specific IncRNAs have been shown to participate in pathological processes of the endocrine system, including DM (28,29).

In vivo studies have shown that IncRNAs exhibit important cellular functions (23-25). Experiments using knockout animal models have confirmed that multiple IncRNAs serve roles in disease pathogenesis (26,27). A number of specific IncRNAs have been shown to participate in pathological processes of the endocrine system, including DM (28,29).
Monocytes/macrophages play an important role in the occurrence and development of T2DM, which is regarded as a type of low-grade inflammation (4,5,30,31). The in vitro results of our previous study using RAW264.7 macrophages revealed that uc.48+ siRNA is able to regulate immune and inflammatory responses, thus influencing the course and outcome of these effects, which are mediated by the P2X7 receptor (13). In the present study, the role of uc.48+ in the pathological changes of T2DM were investigated by monitoring the effects of uc.48+ siRNA on the abdominal cells of a mouse model of T2DM. The results indicated that the mRNA and protein expression levels of the P2X7 receptor and p-ERK1/2 level in the abdominal cells were significantly increased in DM model mice compared with the control group. However, these changes were significantly attenuated following transfection with uc.48+ siRNA in vivo. The experimental results indicate that uc.48+ serves an important role in the pathological changes of T2DM via regulation of the function of abdominal cells, while uc.48+ siRNA treatment may influence the ERK signaling pathway via the P2X7 receptor. However, it is unclear whether uc.48+ regulates the expression of cytokines through the ERK signaling pathway, or the expression of cytokines activates the ERK signaling pathway. Therefore, the specific mechanism requires further study. In particular, more experiments to verify that the effects of uc.48+ siRNA therapy are mediated by the P2X7 receptor and ERK signaling are necessary. In future studies, rescue experiments in which P2X7 receptor functions or ERK activity are blocked will be conducted to elucidate the mechanism of uc.48+ siRNA treatment.

In conclusion, the present study demonstrated that the uc.48+ expression levels of abdominal cells were significantly increased in a mouse model of DM compared with those in non-diabetic controls. Treatment of the mice with uc.48+ siRNA ameliorated the blood pressure and neuropathological changes associated with T2DM, and also downregulated the expression levels of the P2X7 receptor. In addition, uc.48+ siRNA regulated the inflammatory response and ERK signaling pathway in the abdominal cells of the diabetic mice. These effects may be mediated by the P2X7 receptor in T2DM. It is suggested that uc.48+ may serve an important role in T2DM via regulation of the P2X7 receptor, and thereby exert an effect on pathological changes in blood pressure, neuropathological changes and abdominal cells function.

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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

YN and HW were responsible for the conception and design of the study. YN, HW, FW, MJ and QL acquired the data. HW drafted the manuscript and YN revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving animals were approved by the Animal Care and Use Committee of the Medical College of Nanchang University (approval no. 2018 16).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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