Inhibitory effect of desflurane on degranulation of mast cells induced by lateral ventricular injection of stimulator-C48/80 in C57BL/6 male mice

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Abstract
Inhalation of anesthetic agents have been observed to confer neuroprotection for decades. The present study was intended to determine whether desflurane (DES) prohibits mast cells (MCs) from degranulation induced by lateral ventricular injection (LVC) with Compound 48/80 (C48/80) in C57BL/6. Total 100 mice were recruited to this study, but only 88 male mice (20–24 weeks) were survived from the procedure, and randomized and allocated into four groups: (A) the saline group; (B) the C48/80 group; (C) the sodium cromoglycate (CRO + C48/80) group; (D) 7.5% DES preconditioning for 2 h + C48/80 lateral ventricular injection (DES + C48/80) group. The slices of mice brain thalamus were performed for toluidine blue staining (MCs) and immunochemistry (fluorescence of Iba1 and GFAP, respectively), and brain tissues were extracted to probe IL-6, TNF-α, NF-κB (p65), and TLR4 against GAPDH by western blotting. Our results demonstrated that administration of C48/80 provoked degranulation of mast cells at thalamus, increasing the fluorescence intensities of Iba1 and GFAP, and over-expressing IL-6, TNF-α, NF-κB(p65), as well as TLR4. However, preconditioning inhalation of DES prohibited MCs from degranulation, diminishing the fluorescent intensities of Iba1 and GFAP, decreasing expressed levels of IL-6, TNF-α, NF-κB(p65), as well as TLR4. It suggests inhalation DES could inhibit the neuroinflammation and deactivate glial and astrocytes via direct prohibiting degranulation of MCs at thalamus in the central nervous system (CNS).

Keywords
compound 48/80, desflurane, mast cells, neuroinflammation

Date received: 21 October 2020; accepted: 11 February 2021

Introduction
There are three types of distinctive mature MCs in human body classified as serosal, mucosal, and brain MCs with unique biochemistry, morphology, and function.1 Alternatively, MCs can be classified based on their granule content as tryptase (MCT) or tryptase chymase (MCTC).2 Each kind of MCs contains various sizes of mature granules.3 A plethora of mediators (effectors) are degranulated from MCs primed and provoked by a variety of inducers, which includes biogenic amines, cytokines,
enzymes, neuropeptides, growth factors, and nitric oxide by virtue of their biochemical properties. In terms of their functions, those mediators from granules can be characterized as direct activators (histamine, serotonin, serineproteases), cytokines, and growth factors.

The process of degranulation from MCs can be distinct by two stages: an acute immediate releasing stage and a later releasing stage (a slower, de novo synthesis of cytokines and chemokines). In addition, MCs also play roles in phagocytosis, serving as antigen presenting cells (APCs) as dendritic cells (DCs) to present MHC (major histocompatibility complex) I or II with antigen to CD8 or CD4 that are responsible for leading neuro-inflammation down to neurodegeneration. MCs seem to act as an accomplice with microglia and astrocytes in promoting the pathogenesis of neuro-degenerative diseases. An increase of absolute number of MCs was observed in autoimmune demyelination, concurring with degranulation of MCs in the central nervous system (CNS). Other effectors from MCs directly resulted in demyelination and induced apoptosis of oligodendrocytes. Most MCs are usually resided along the side of the blood vessel in the brain, where they were co-localized and communicated with microglia, and astrocytes around neurons. By utilizing cell adhesion molecule-1 (CADM1), Nectin-3, or histamine receptors docking on the surface of astrocytes, microglia, and astrocytes directly responded to pro-inflammatory mediators released from MCs. In addition, released mediators from MCs degranulation might enhanced activation of MCs via positive feedback. Indeed, activated MCs shed impact on components of the neurovascular unit (including intravascular, microvascular, and perivascular structures), and extended it to neurons, microglia, and astrocytes. Some reagents were reported to either stabilize or destabilize the membrane of MCs, like C48/80 (MCs degranulator), sodium cromoglycate (MCs stabilizer), respectively. However, a paucity of attention has been paid to inhalation anesthetized agents on MCs that mediated the acute inflammation in the CNS, though the bulk of evidence indicated that several anesthetic agents such as isoflurane, sevoflurane, and DES were able to render from mild to moderate neuroprotection through different mechanisms, particularly pre or postconditioning. Given that sevoflurane rendered neuroprotective effects on various pharmacological models both in vivo and in vitro; likewise, DES may hold similar potency and even safer than sevoflurane, and isoflurane in some respects. The focus of the present study has been placed on the potency of DES, and determine whether it could be stronger enough to prohibit MCs from degranulation in comparison with CRO in the acute inflammation of CNS model induced by the lateral ventricular injection with C48/80 in C57BL/6 male mice, thereby exploring some underlying mechanisms.

Materials and methods

Animals

Mice were aged 20–24 weeks with body weights from 25 to 30 g. All mice were group-housed at constant room temperature (20–25°C), at the moisture of 40%–70% under 12:12 light-dark cycle with ad libitum access to feed and water. This experimental study protocol was approved by the Ethics Committee for the Care and Use of Laboratory Animals of Nanjing Jinling Hospital (IACUC No. YYZD 201400, 01/Jul/2019) before C57BL/6 SPF mice were purchased from the Animal Center of Jinling Hospital.

Experimental protocols

Total 100 male mice were randomly allocated into 4 groups but average 88% of tested mice were survived from the procedure, and 22 mice per each group were acquired with integrated data, as some mice died before injection, or some mice died after injection but did not go through the procedure and failed to collect the integrated data. On average, the mortality rate was around 12%.

The diagram of experimental procedure was depicted as below. The upper row was listed with different treatments of different groups, whereas the lower row was labeled with different time points, and the mice between two rows went through it upon different treatments (seen in the Table 1).
All the mice were anesthetized with sodium pentobarbital at dosage of 60 mg/kg before being placed on the stereo-positioned platform. After the fur being removed, animals received an antiseptic treatment with 75% alcohol, and then locally injected with 0.5% bupivacaine 0.1 ml. The lateral ventricular injection co-ordinates relative to bregma were posterior 0.2 mm, lateral 1 mm, and ventral 2.5 mm for mice. The listed different reagents were injected into the right lateral ventricle. The micro-syringe was left in the injection site for 3 min to facilitate diffusion of reagents. EMLA cream (2.5% lidocaine and 2.5% prilocaine) was smeared around the injection site for pain-killer per every 8 h. The brain tissues (thalamus) of the mice were harvested at 24 h after the lateral ventricular injection.

Reagents
Routine reagents were purchased from the market, and the primary antibodies were purchased from the following companies as in Table 2.

Mast cell staining and analysis
Six mice per group (n=6) were euthanized by overdosed with sodium pentobarbital for brain slicing. The 2-dimensional area of each mast cell from the same magnification was calculated. Since image data can’t be accurately, quantitatively analyzed but randomly picked-up from at least 10 fields per animal under the microscope, and semi-quantitatively analyzed with a statistical software. So six animals per group were able to provide sufficient and integrated data and representative. Slides were stained with 0.05% toluidine blue (Sigma, St. Louis, USA) for sectioning thalamus. The thalamus is the key in the integration of sensory and motor information, whereas MCs are predominantly resided in the thalamus in rats, and respond to nociceptive signaling by degranulation. Toluidine blue staining for MCs was employed as described by Dong and Yu. Briefly, a 1% stock solution in 70% ethanol was dissolved in 0.9% Sodium chloride injection (pH 2), and the slides were immersed in the above solution for 30 min, then washed twice with distilled water, and followed by dehydration with a series of escalated concentrations of ethanol, and eventually hyalinized in butyl acetate ester. Slides were cover-slipped by using Eukitt® mounting medium, and were allowed to dry overnight. For “hot-slices” of thalamic sections, images from the entire surface area of the ipsilateral and contralateral thalamus was manually

### Table 1

Twenty-two mice per group were completed and integrated data were collected. Six of twenty-two mice per group were assigned for mast cell staining, and eight of twenty mice per group were deployed for immunofluorescent histochemistry, and the rest eight mice per group were employed for western blotting, respectively (n=22).

| Group                              | Treatment                                                                 |
|------------------------------------|---------------------------------------------------------------------------|
| Control group (Saline)             | LVC with 0.9% saline 1 µL                                                  |
| Model group (C48/80)               | LVC with C48/80 1 µL (1 µg/µL)                                             |
| CRO Pretreated group (CRO + C48/80) | First LVC with CRO 1 µL (15 µg/µL), and LVC with C48/80 1 µL (1 µg/µL) half an hour later |
| DES Pretreated group (DES + C48/80) | First inhalation with the mixture gases (100% oxygen + 7.5% DES) for 2 h, and then receiving LVC with C48/80 1 µL (1 µg/µL) |

### Table 2

Source of primary antibodies listed.

| Antibody name | Company                  | Catalog no. | Molecular weight (KDa) | Animal species | Dilution of 1st Ab | Dilution of 2nd Ab |
|---------------|--------------------------|-------------|------------------------|----------------|--------------------|--------------------|
| GAPDH         | Servicebio               | GB1202      | 37                     | Mouse          | 1:25000            | 1:3000             |
| IL-6          | Servicebio               | GB11117     | 26                     | Rabbit         | 1:1000             | 1:3000             |
| NF-κB (p65)   | Santa Cruz Biotechnology | Sc-8008     | 77                     | Mouse          | 1:1000             | 1:3000             |
| TLR4          | Servicebio               | GB11519     | 95                     | Rabbit         | 1:1000             | 1:3000             |
| TNF-α         | Proteintech Group Inc.   | 17590-1-ap  | 26                     | Rabbit         | 1:1000             | 1:3000             |
| Iba1          | ABCAM                    | Ab178846    | Fluorescein isothiocyanate (FITC) | Rabbit | 1:500             | 1:200              |
| GFAP          | CST                      | Ab80788     | Fluorescein isothiocyanate (FITC) | Rabbit | 1:500             | 1:200              |

GAPDH: glyceraldehyde-3-phosphate dehydrogenase; GFAP: glial fibrillary acid protein; Iba1: ionized calcium binding molecule-1; IL-6: Interleukin -6; NF-κB: Nuclear factor-kappa-B; TLR4: Toll-like receptors-4; TNF-α: Tumor necrosis factor-α.
scanned under light microscopy at 80 × magnification. MCs were calculated with the assistance of the Cell D software (Olympus). Criteria for degranulation of MCs were determined mainly based on morphology: large cell bodies with loss of blue staining, fuzzy appearance, distorted shape, or broken membrane and debris left, or multiple granules visible in the vicinity of the cell. Instead, without degranulation, intact MCs displayed with round bodies but concentrated blue staining particles around the edge of cell membrane. The 2-dimension area of individual MCs was calculated by using digital slice reading software (K-viewer software, Ningbo Konfoong Bioinformation Tech., LTD, China).

Immunofluorescent histochemistry
As compared to light images of MCs staining, the images of immunohistochemistry often contain more uncertain factors that may compromise data. Eight mice per group was an appropriate sample size so that statistical significance would be measured in a semi-quantitative manner. The activation of microglial cell was represented by Iba1 positive with the immunochemical staining, whereas the activation of astrocytes was represented with GFAP positive staining.8,9 Cell nuclei was stained concurrently with DAPI (Beyotime, China). Eight mice completed different treatments were perfused with cold saline followed by 0.1 M phosphate buffered saline (PBS, pH 7.3) containing 4% paraformaldehyde under anesthesia with sodium pentobarbital (60 mg/kg intraperitoneal injection) as described by Yu et al.9 The brain was evacuated and post-fixed in the same fixative for 4 h, and then cryopreserved for 24 h at 4°C in 0.1 M PBS containing 30% sucrose. A serial coronal sections (30 μM) per brain were sequentially sliced via a Leica cryostat (Leica CM1800, Heidelberg, Germany), and collected into 3 separate dishes, each of which contained a set of complete serial sections. “Hot” sections were rinsed in 0.01 M PBS, (pH 7.3) 3 times (10 min each), and blocked with 2% goat serum in 0.01 M PBS containing 0.3% Triton X-100 for 1 h at room temperature (RT). The sections were incubated overnight at 4°C with the primary antibodies Iba1 and GFAP upon different dilutions (seen in the Table 2). The sections were then washed 3 times in 0.01 M PBS (10 min each) and incubated with corresponding secondary antibodies for 2 h at RT. Fluorescent images were captured under a confocal microscope (LSM700), and fluorescent intensities from fluorescent images were semi-quantitatively calculated by using a software (Image-Pro Plus, Media Cybernetics).

Western blotting
The gray density extracted from the membrane of western blotting can be calculated after the bands have been scanned. Seven samples from eight mice per group were an appropriate sample size that were sufficient to render statistical significance if the difference between groups were present. Eight mice per group (n = 8) from the different treatments were euthanized by cervical dislocation (seen in the Table 1). After mice brains were taken out, both sides of thalamus were dissected and homogenized in sodium dodecyl sulfate sample buffer with a mixture of proteinase and phosphatase inhibitors cocktail (Sigma-Aldrich, USA). The protein concentration of the supernatant was determined by using BCA protein determination kit (G2026, Servi-cebio). The rest routine procedures were employed as described by previous reports.12 In the present study, several inflammatory factors as IL-6, TNF-α, NF-κB(p65), and TLR4 were probed with different individual antibodies under different conditions (seen in the Table 2). The colometry of each band on the transferred membrane was scanned and quantitatively measured by the software of Image J (NIH).

Statistical analysis
All the quantitative or semi-quantitative data were expressed as mean ± standard error. The statistical differences among different groups were determined with one-way analysis of variance (ANOVA) followed by post hoc Duncan test on SPSS 18.0 software. The differences were considered as statistical significance if P value was less than 0.05.

Results
Pretreatment of DES prohibited degranulation from MCs induced by C48/80 in vivo
As shown in Figure 1(a), a single MCs was illustrated from quiescent, commencing to degranulate, thoroughly degranulated and the status of post-degranulation. Granules in quiescent MCs were evenly stained with toluidine blue and the boundary were clear and distinguishable. Highly concentrated
granules were packaged within cells, and outlines of cells were round shaped. When MCs were activated and started to degranulate, intracellular granules, or particles moved to the rim of cell boundary. After granules released or emptied, the 1-dimensional sizes, or 2-dimensional area of MCs

Figure 1. MCs on the slides of mice brain thalamus are stained with 0.05% toluidine blue by microscopy (a: 80× in high magnification, b and c: 40× in high magnification). Total six samples per group were processed. One of those pictures was chosen for representation from the Saline group, the C48/80 group, the CRO + C48/80 group and the DES + C48/80 group. (Scale bar: 10 μm). CRO: sodium cromoglycate; DES: desflurane. (a) The dynamic process of degranulation from MCs. (1) A normal and quiescent MCs; (2) a mast cell started to liberate the granule, and granules moved to the rim of cell membrane; (3) a degranulated and depleted MCs had one granule left; (4) a complete degranulated MCs. (b) The number of activated MCs in the C48/80 group were increased and more than the number in the Saline (**p < 0.01), but the sum were decreased and lower in both of the CRO + C48/80 group and the DES + C48/80 group (###p < 0.01) than in the C48/80 group. However, the number of activated MCs in the DES + C48/80 group were slightly more than in the Saline group (*p < 0.05). (c) The area of individual MCs was calculated by using digital slice reading software (K-viewer software). Fifty MCs per group were selected from ten random fields, and an area of each cell was calculated twice and averaged. Total areas of 50 mast cells in the C48/80 group were summed up and larger than total areas of the same quantity of MCs in the Saline group (***p < 0.01), but total areas of 50MCs in both of the CRO + C48/80 group and the DES + C48/80 group were decreased and smaller than in the C48/80 group (###p < 0.01). However, the total areas of 50 mast cells in the DES + C48/80 group were slightly larger than in the Saline group (**p < 0.05).
were bulged and enlarged twice bigger, and blue staining of toluidine seemed blanched as if the dye poured out or washed away due to degranulation. After completely degranulated, only cell debris were left or linked with broken cell membrane. (seen in Figure 1(b) and (c)), After the lateral ventricle injected with C48/80, the number of MCs were increased and more in the C48/80 group than in the saline group, and the 2-dimensional area of individual activated MCs became bigger and bigger in the C48/80 group than in the saline group ($p < 0.01$). However, the sum of activated MCs was decreased and fewer in both of the CRO + C48/80 group and the DES + C48/80 group ($p < 0.01$). The number of activated MCs in the DES + C48/80 group was slightly more than in the saline group ($p < 0.05$). Likewise, total areas of 50 MCs in the C48/80 group were summed up and larger than total areas of the same quantity of MCs in the saline group ($p < 0.01$), but total areas of 50 MCs in both of the CRO + C48/80 group and the DES + C48/80 group were decreased and smaller than in the C48/80 group ($p < 0.01$). However, the total areas of 50 MCs in the DES + C48/80 group were slightly larger than in the saline group ($p < 0.05$).

**Pretreatment of DES prohibited both microglial cells and astrocytes from activation induced by degranulation of MCs**

Iba1 represents activation of microglial cells, whereas GFAP represented activation of astrocytes, which were probed with the primary antibodies, respectively and emitted out green fluorescence with FITC secondary antibody. The higher fluorescent intensity, the more microglial cells were activated. As shown in Figure 2, the stronger fluorescent intensities of Iba1 and GFAP were shown in the C48/80 group than in the saline group ($p < 0.01$). However, after pretreated with either CRO or DES, both fluorescent intensities of Iba1 and GFAP were greatly decreased and weaker than in the C48/80 group ($**p < 0.01$).
reduced, which meant that fewer of microglial cells and astrocytes were activated in both the CRO + C48/80 group and the DES + C48/80 group than in the C48/80 group (p < 0.01), though the number of activated microglial cells and astrocytes were slightly more than those in the saline group (seen in Figure 2(a) and (b)). A high magnification of Iba1 and GFAP was illustrated in Figure 2(c).

**Pretreatment of DES inhibited expressions of inflammatory factors (IL-6, TNF-α, and NF-κB(p65)) induced by degranulation of MCs**

In order to evaluate effect of DES pretreatment on inflammatory responses induced by mediators from degranulation, two important factors (IL-6 and TNF-α) and the inflammatory axis-NF-κB (p65, one of activated elements) were determined by western blotting (shown in Figure 3), though electromobility shift analysis (EMSA) was not applied to study the binding of NF-κB. As compared to expression levels of IL-6 and TNF-α in the saline group, these protein expressions, particularly NF-κB(p65), were significantly increased in the C48/80 group. The grey densities of NF-κB(p65) were quantitatively measured, and the grey densities from individual bands in the C48/80 group were statistically higher than in the saline group (p < 0.01). IL-6 and TNF-α were higher in higher in the C48/80 group than in the saline group (p < 0.05), whereas expressions of IL-6 and NF-κB(p65) were both decreased and lower in the CRO + C48/80 group and the DES + C48/80 group than in the C48/80 group (##p < 0.01). However, the expression level of TNF-α in both of the DES + CRO group and DES + C48/80 group were slightly lower than in the C48/80 group (#p < 0.05).

**Pretreatment of DES inhibited expression of TLR4 induced by degranulation of MCs**

As shown in Figure 4, the expression level of TLR4 in the C48/80 group was significantly increased and higher than in the saline group (p < 0.01). Pretreated with either CRO and DES, the protein expression of TLR4 was both decreased and lower in the CRO + C48/80 group and the DES + C48/80 group than in the C48/80 group (both p < 0.01).
Discussion

Several anesthetic gases confer various kinds of neuroprotection in different disease models, particular more attention has been attained to study neuroprotective and cardiac protective effect of sevoflurane. Although DES was much safer and more potent than sevoflurane, very few studies have been shed light on desflurane. In the present study, we first reported that inhalation of DES renders neuroprotection by decreasing inflammatory factors (IL-6, TNF-α, and NF-κB) and disabling inflammation regulatory factor-TLR4, which may go through directly stabilizing MCs in thalamus, deactivating MCs, and prohibiting degranulation from MCs, thereby inactivating microglial cells and astrocytes in the CNS inflammation mice model induced by brain LVC with C48/80.

Figure 4. Expression of TLR4 was determined by western blotting at the protein level. (a) The Saline group; (b) the C48/80 group; (c) the CRO + C48/80 group; (d) the DES + C48/80 group (n = 8). Individual protein expression level was calculated based on a colometry density of individual band. Expression of TLR4 was increased and higher in the C48/80 group than in the Saline group (**p < 0.01), whereas the protein expression of TLR4 was both decreased and lower in the CRO + C48/80 group and the DES + C48/80 group than in the C48/80 group (##p < 0.01).

In general, underlying neuroprotection mechanisms of these gases can be approximately classified into: (1) astrocytic and microglial activations, and neutrophil infiltration are inhibited; (2) excitatory amino acids and receptors (AMPA, NMDA receptors) are suppressed, and ECM (extracellular matrix) breakdown is diminished by inhibiting MMP-2 (Matrix Metalloproteinase) and MMP-9 activities; (3) anti-oxidation, anti-inflammation, maintaining ion homeostasis and anti-apoptosis. However, the role of MCs in the CNS is oblivious. As a matter of fact, MCs has been playing a more important role in mediating chronic inflammation in the CNS and aged neurodegeneration through direct liberating granules and depleting contents, or indirect activating microglial cells and astrocytes, and cross-talking with each other. Sevoflurane has been reported to confer protections against cerebral ischemia, cardiac ischemia, intestinal ischemia-reperfusion induced lung injury through pre-treatment, concurrent treatment and post-treatment, but pretreatment was more frequently and dominantly applied. Nevertheless, a paucity of research on DES has led us to studying its effect on mast cell.

The present study was intended to employ inhalation of DES for pretreatment to procure unique efficacy of stabilizing granule membrane and prohibiting degranulation from MCs. DES was safer than sevoflurane in pre and postconditioning against myocardial infarction in mice, as 9% DES for 2 h daily for consecutive 3 days had no impact on cognitive impairment and neuroinflammation, but 3% sevoflurane for 2 h daily for 3 days would
elicit cognitive detriment and neuroinflammation. About 7.5% DES was demonstrated as an appropriate concentration to provide protection of preconditioning.11,16 In the present experiment, 7.5% DES inhaled for 2 h was able to provide sufficient and beneficial effect. Different lengths of DES inhalation were also tested at 1, 2, and 4 h, and the potency of neuroprotection was enhanced over the time course (data not shown here). Our results demonstrated that pretreatment of DES, like sodium cromoglycate, clearly prohibited degranulation from MCs induced by C48/80 brain LVC in mice, and diminished activation of microglial cells and astrocytes that might be stemmed from mediators released from degranulation, and interrupted cross-talking among three kinds of cells, which was in line with the previous reports.3,17

It seemed DES has direct effect on inhibiting inflammatory responses.10,15 Alternatively, such an inhibition of DES may come from stabilizing MCs and prohibiting MCs from degranulation, which is validated as decreasing IL-6, TNF-α, NF-κB(p65), and TLR4. Toll-like receptors (TLRs), which is respond to invading pathogens, pro-inflammatory cytokines, and environmental stresses, playing a key role in the innate immune system. TLR-4, among the 13 known TLR subtypes, is activated in the brain after cerebral ischemia-reperfusion, and recognize a relative narrow spectrum of LPS-like pathogens, including endogenous Heat shock protein 60 (HSP60). TLR signaling activates NF-κB, which triggers the production of pro-inflammatory cytokines, including IL-6, TNF-α.17–19 However, the current study still has some shortcomings and pitfalls. It will be more convincible that demonstrate the effect of DES on stabilizing mast cell membrane in vitro, and the several major contents such as histamine liberated from MCs can be quantitatively assayed. In addition, in the methods, there was no software to calculate the power of statistics and justify the size of sample for different experiments in the present study.

Conclusion

In conclusion, our results demonstrate that DES directly stabilize MCs and prohibits degranulation from MCs at thalamus in the brain, whose potency is equivalent to sodium cromoglycate in mice model induced by LVC with C48/80, inhibiting inflammatory responses, diminishing activation of microglial cells and astrocytes. Thus, our study reveals a novel mechanism for DES to fight against chronic inflammation in the CNS and associated neurodegenerative diseases in the future. Practically, it is propitious for elder patients who are sic ill with Alzheimer’s disease, Parkinson’s disease, or other neurodegenerative diseases to choose DES as an anesthetic agent before surgery.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

Ethical application for this study was approved by the Ethics Committee for the Care and Use of Laboratory Animals of Nanjing Jinling Hospital (IACUC No. YYZD 201400, 01/Jul/2019).

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the grants from the Jinling Hospital (Nos. 12MA091).

Animal welfare

The present study followed the Animal Care and Use Committee of Nanjing Medical University for humane animal treatment and complied with relevant legislation.

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