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Research Article

Different Mechanisms Underlying Swallowing Initiated by Optogenetic Activation and Water Administration Methods in Mice

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ABSTRACT

To compare the mechanisms underlying swallowing initiated by optogenetic activation and water administration methods using electromyography (EMG), twelve adult C57 mice were used and equally grouped into a light group and a water group. Optogenetic activation and water administration were used to induce a swallowing response in the two groups. The swallowing latency and area under the curve (AUC) of the EMG response in mylohyoid muscle, the interval between swallows and each swallow were compared between the two groups. The pharyngeal pressure was detected to reflect the swallowing response using a homemade balloon. There was a significant difference in the latency of first swallowing response between the light and water groups (0.216±0.278 vs. 1.427±0.136, $P<0.001$). However, there were no significant differences in the AUC of the EMG response between the two groups. The intervals between consecutive swallows in the light group were all shorter than those in the water group ($P<0.01$). Differences in latency may indicate different regulatory mechanisms for swallowing initiated by different methods. The activation of the swallowing motor cortex can induce swallowing movement more directly, which may be related to the efferent neural mechanisms of swallowing.

INTRODUCTION

Swallowing is a complex function that requires a combination of sensory and motor integration. It results from a series of complex and highly coordinated nerve impulses and muscle movements [1]. Precise neuromuscular coordination enables the mouth, pharynx and esophagus to complete the push of the food bolus, including three interactive processes [2]. Voluntary swallowing occurs when humans or animals have a desire to eat or drink during the awake and conscious state [3]. Previous studies have demonstrated that voluntary and involuntary methods can induce swallowing, which is then controlled by central outputs or peripheral inputs to the swallowing CPG [4].

A report found that voluntary water swallowing activated more of the cortex than swallowing induced by water supplied to the pharynx [5]. However, some researchers have proposed that chemical sensory stimulation, such as water stimulation, could promote voluntary swallowing through the supramedullary nerve mechanism and reduce the difficulty of initiating voluntary swallowing [6,7], which indicating the influence of peripheral stimulation on voluntary swallowing. As a chemical stimulus, water is innervated by the water fibers of the superior laryngeal nerve and is

considered the most effective stimulus to activate chemoreceptors in the pharynx and larynx. Water stimulation in the pharyngeal mucosa transmits sensory signals to the nucleus of the solitary tract, which is a common way to induce the swallowing reflex [8].

Our previous study confirmed that the activation of excitatory neurons in primary motor cortex (M1) Layer 5 (L5) by optogenetic techniques could induce the activity of the pharyngeal swallowing response [9], which was thought to mimic voluntary swallowing. Another study also confirmed the role of M1 in the regulation of voluntary swallowing in animals [10]. Studies have stated that the initiation of voluntary swallowing requires the drive of the motor cortex to the tongue and the submental muscles, including the mylohyoid muscle [3]. The activities of the mylohyoid muscle were detected after activation of M1 by optogenetics, in which 8 mW and 50 Hz showed maximum muscle response when compared with 8 mW, 20 Hz or 8 mW, 100 Hz and 50 Hz, 4 mW, or 50 Hz, 6 mW.

Optogenetic activation of M1 neurons provided central stimulation, while water delivery to the pharynx provided peripheral stimulation. The central control of swallowing mainly involves two centers: the cortical swallowing center and the brainstem swallowing center. Voluntary swallowing can be induced by the activation of the cortical motor area or stimulation of the movement auxiliary area, while the swallowing reflex is controlled by the bilateral brainstem swallowing center [11]. Therefore, the initiation of voluntary swallowing mainly depends on the regulation of higher cortical centers [12]. The swallowing reflex primarily depends on the integration and regulation of the brainstem-network system [13], but the cortical swallowing center also plays a regulatory role. Related studies have suggested that cortical descending fibers mainly project to the nucleus tractus solitarii (NTS) of the brainstem swallowing center [14], which is an essential part of the swallowing central pattern generator (CPG). We also confirmed that the NTS plays an important role in the regulatory function of voluntary swallowing in specific neuron-associated circuits of the M1. Considering that the NTS plays a role in both voluntary and reflexive swallowing, we will continue to explore the mechanism behind two different swallowing initiations.

Therefore, the optogenetic method was used to activate excitatory neurons in M1, which mimicked voluntary swallowing. For the initiation of involuntary swallowing, also called reflexive swallowing, water was delivered to the pharynx, which mimicked involuntary swallowing without initial movements of the mouth [15,16].

Notably, the differences between these two methods of swallowing initiation will be explored in this study. C57 mice were used, and the swallowing latency, the area under the curve (AUC) and the interval time between swallows of electromyography (EMG) in the mylohyoid muscle were compared. These results might elucidate a further mechanism for swallowing initiation and its regulation.

METHODS

Animals

C57BL/6J mice from the Laboratory Animal Center of Guangzhou University of Chinese Medicine were used. Twelve adult C57 mice were randomly and equally divided into two groups. Mice in both groups were male, aged 5–6 weeks, and weighed approximately 20-30 g. They lived in a 12 h day/night cycle environment with free access to food and water. Mice implanted with optical fibers were individually housed. All the experimental procedures in our study were performed following the guidelines of the Committee for Care and Use of Research Animals of Guangzhou University of Chinese Medicine (No. 201703303). The mice were randomly assigned into two groups for the entirety of the study.

Virus injection

The optogenetic activation virus (rAAV2/9-CaMKIIa-hChR2 (E123T/T159C)-mCherry-WPRE-hGH-pA) was selected and injected into M1 L5 (AP: −0.12 mm, ML: −1.03 mm, DV: −1.10 mm) (Figure. 5B). Mice were anesthetized with 25% tribromoethanol and fixed to the adaptor in a prone position. The hair on their skull was removed, and a line was cut along the midline of the scalp to expose the skull, followed by clearing of the tissue. The target area was selected, and a hole was drilled. After the dura was opened, a microinjection needle fixed on the stereotaxic instrument was filled with the target virus and injected slowly into the target area at a speed of 30 nl/min. After 10 min, the needle was lifted slowly, and an optical fiber was implanted above the virus injection site and fixed with dental cement. Afterward, mice were fed

individually for 21 days in their cages before any other experiment was carried out.

Optogenetic activation

A rAAV2/9 virus with the CaMKIIa promotor, which could specifically infect excitatory neurons, was used to transfect M1 neurons. This virus, encoding the light photosensitive channel ChR2, was injected into M1 L5. ChR2 can open cation channels and induce action potentials when blue light is administered. An optical fiber (NEWDOON, China) was implanted 2.5 mm above the virus injection site. After the viruses were adequately expressed, 473 nm with 50 Hz, 8 mW blue light was administered for 5 s by a stimulus modulation generator (Tinker Tech, China). Light was administered during the EMG recording.

***In vivo* EMG recording**

Mice were first anesthetized with isoflurane in the anesthesia box and then quickly fixed to a mouse adaptor in the supine position on a homemade foam board. Two EMG stainless steel wire recording electrodes (-15 mm in diameter) were inserted into the left mylohyoid muscle to record the swallowing response. The location of the mylohyoid muscle is below the digastric muscle and geniohyoid muscle, which are superficial pharyngeal muscles [9]. A ground electrode was inserted into one masseter muscle. Electrodes were connected to the signal acquisition system with recording wires to acquire the EMG signals (1902, CED, UK). Throughout the whole process, an anesthetic mask connected to isoflurane was available to the mouse's nostrils to keep the mice from struggling.

In the light group, the swallowing response was detected by EMG recording during optogenetic activation. EMG was recorded before and during blue light administration.

In the water group, a microinjection pump (HARVARD, USA) was used to deliver the water into the pharynx of mice through a soft tube[14]. The EMG was recorded while the water was administered, with a total amount of 20 μl of water at a rate of 2 μl per second. The mice were stimulated at least 5 times, with at least 1 minute between each stimulation to reduce the influence of residual water. After the experiment, every effective stimulus that induced swallowing was included in the calculation.

After recording, the area under the curve (AUC) of the mylohyoid muscle data collected during the water or light administration was analyzed according to the method previously used to detect the swallowing response in a previous study [9], in which data were filtered at 0.1-1 kHz with a 1902 amplifier (CED, UK). For the latency, the onset time of light stimulation or water supply was marked, and the latency was calculated as the time between this mark and the onset of the first EMG signal. On the basis of the EMG baseline, the start of the swallowing event was defined as the appearance of the EMG burst, and the fall of the EMG was the end of one swallow [17]. For the analysis of AUC, the activities of all swallows during light or water stimulation were calculated and averaged. Each swallow during the stimulation was also analyzed. The swallowing interval time was defined as the duration between two swallows and was calculated between the first and second swallows (first interval), the second and third swallows (second interval), and the third and fourth swallows (third interval).

Balloon pressure detection

The swallowing response can be reflected by pharyngeal pressure measured by balloon pressure. First, the balloon, with a diameter of 3 mm, was fixed against the root of the tongue and near the hard palate and connected to a pharyngeal pressure transducer called a baroreceptor. The Powerlab data acquisition device (ADInstruments, Germany) was connected to the baroreceptor, which was linked to a three-way valve. A syringe filled with water was connected to the three-way valve after adjusting the pressure of the baroreceptor by a sphygmomanometer. The pressure of the balloon lumen was detected during swallowing induced by water administration, which was considered to reflect the pressure of the pharynx when swallowing [18]. A reference mark was recorded at the time the water was administered. The AUC of the balloon pressure was analyzed (the unit is millimeters of mercury)

Immunofluorescence for Nuclear Staining

The location of optogenetics virus in the light group was confirmed via nuclear staining with DAPI. After the experiment, mice were transcardially perfused, and their brain tissue was extracted, followed by posterior fixation with 4% paraformaldehyde (PFA). After gradual dehydration in 15% and 30% sucrose in PBS, the brain tissue of the virus injection site was sectioned to a thickness of 40 μm with a freezing microtome (Thermo, Germany). After free-floating washing in PBS three times, brain sections were incubated with 4,6-

diamidino-2-phenylindole (DAPI, 1 μg/5 ml) and mounted on glass slides. Next, confocal fluorescence images were acquired on a Nikon scanning laser microscope using 10x and 40x air objectives. Neurons that have been successfully transfected by the virus will appear purple when combined with the virus carrying mCherry.

Statistical Analysis

Independent two-sample t tests were used to analyze the differences between the two groups, including the results of the AUC analysis, balloon recording of pharyngeal pressure, and swallowing interval calculation. Two-way analysis of variance was used to compare the first three swallowing intervals in the light and water groups. *P<0.05* indicated a significant difference. A total of 12 animals were analyzed for latency, AUC, pharyngeal pressure and swallowing interval time. All statistical analyses were performed with GraphPad Prism 8.0 software. All figures were made in SigmaPlot 14.

RESULTS

Table 1: The means and standard deviations of the latency, AUC and interval time between swallows.

Groups	Latency	AUC	First interval	Second interval	Third interval
Light	0.216± 0.278	0.318± 0.018	0.378± 0.085	0.614± 0.144	0.864± 0.290
Water	1.427± 0.136	0.335± 0.042	1.451±0 .440	1.542± 0.360	1.839± 0.494
P	0.0001	0.4172	0.0073	0.0039	0.0153

The EMG swallowing responses induced by optogenetics were compared to those induced by water administration. Our results showed that the latency of the first EMG response induced by optogenetics (light group) was 0.216±0.278, and the first EMG response induced by water administration had a latency of 1.427±0.136 (water group) (Table 1). There was a longer latency in the first swallow in the water group than in the light group (Figure 1B, 1C). A representative example is shown in Figure 1A and B. For the area under the curve (AUC) analysis, there was a trend toward a larger AUC in the water group (0.335±0.042) compared with 0.318±0.018 in the light group (Table 1), but there were no significant differences between the groups (Figure 2B). A representative example is shown in Figure 2A.

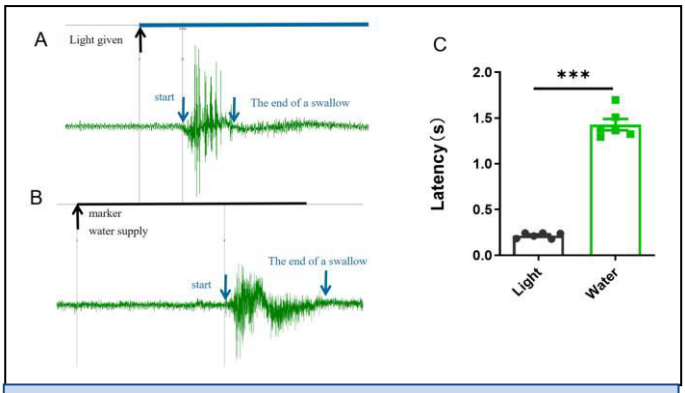

Figure 1: Latency of the first EMG in the light and water groups was shown.

(A) The black arrow indicates the onset of light administration, the two blue arrows indicate the beginning and end of the first swallow induced by optogenetics, and the blue line represents the duration of light administration. (B) The latency in the water group. The black arrow indicates the onset of water administration, the first blue arrow shows the beginning of the first swallow, the second arrow indicates the end of the first swallow, and the black line represents the duration of water supplied. (C) The analysis results of A and B. The dots represent the number of swallows in each group (two-tailed unpaired Student's t test, n=6 per group, t=19.21, ***$P<0.001$).

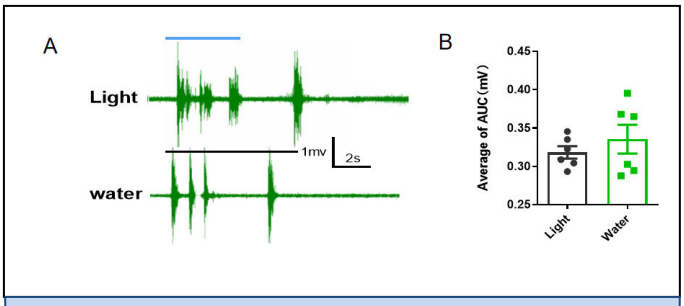

Figure 2: The AUCs of EMG in the mylohyoid muscle are shown for the light and water groups.

(A) Representative example figure of the two groups. Above: the blue line represents the 5 s duration of light administration. Below: the black light represents the 10 s duration of the water administration. The scale bars are 1 mV and 2 s. (B) The statistical results of AUC in the two groups (two-tailed unpaired Student's t test, n=6 per group, t=0.846, $P>0.05$).

The result of pharyngeal pressure was consistent with the results of EMG. The sample figure is shown in Figure 3A. The correlation analysis between the EMG and pharyngeal pressure is shown in Figure 3B. The average amplitudes were 0.425±0.097 in EMG and 32.139±7.362 in pharyngeal pressure, and the correlation coefficient was R=0.868. These results indicated that the swallowing response could also be detected by the pharyngeal pressure. The swallowing response measured by EMG was consistent with pharyngeal pressure.

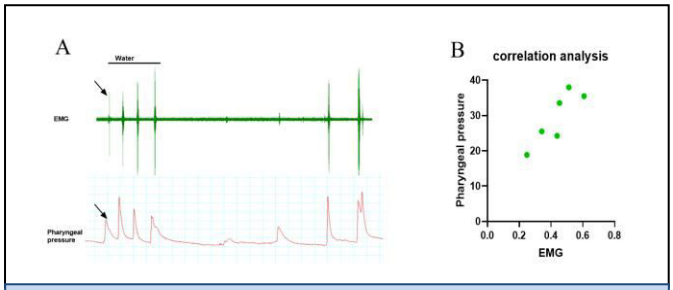

Figure 3. The correlation of EMG induced by water administration and pharyngeal pressure was shown.

(A) Above: EMG of swallowing response induced by water administration. The black light indicates the duration of water delivery, and the black arrow represents a swallow. Below: the pharyngeal pressure is shown. The black arrow indicates a swallow. (B) Correlation analysis of the EMG and pharyngeal pressure (r=0,868). The horizontal coordinates indicate the EMG, while the vertical axis indicates the pharyngeal pressure.

Furthermore, to determine the characteristics of swallowing intervals, the intervals between two consecutive swallows during the stimulation were measured. Representative examples of the swallow and interval time are shown in Figure 4A. The first swallowing interval was 0.378±0.085 in the light group and 1.451±0.440 in the water group, showing a significant difference between groups (*P<0.001*) (Figure 4B). Then, the second swallowing interval was 0.614±0.144 in the light group and 1.542±0.360 in the water group, and this interval significantly differed between groups (*P<0.01*). So was the third swallowing interval (light: 0.864±0.290 vs. water: 1.839±0.494, *P<0.05*) (Figure 4B). Furthermore, the first three intervals gradually became longer in both the light group and the water group. To further observe whether the swallowing response decreased over time, each swallow during the light or water stimulation was detected. The results showed that there was no significant difference between each swallow (*P<0.05*) (Figure 4C).

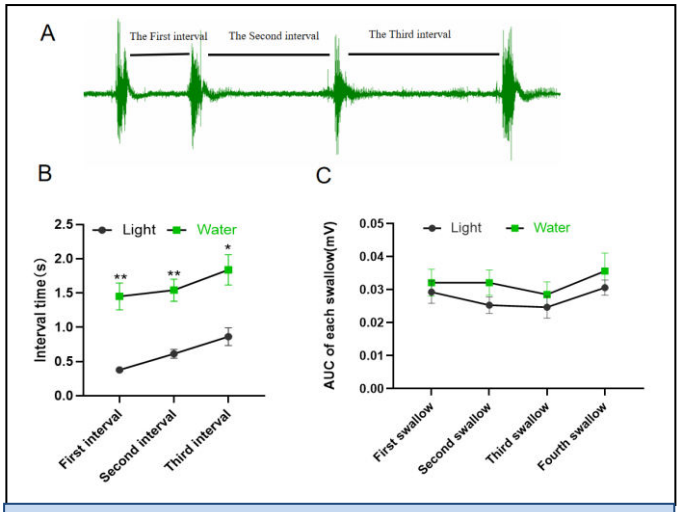

Figure 4: The intervals between consecutive swallows in the two groups.

The swallowing interval was specified as the time difference between the beginning and end of Each EMG response. (A) A representative figure of the three intervals. (B) Comparison of the first three interval times in the light group and the water group. (Two-way ANOVA, Sidak's multiple comparisons test, ***P<0.01*, first interval, second interval, **P<0.05*, third interval). (C) The statistical results of the AUC in each swallow during the light or water stimulation.

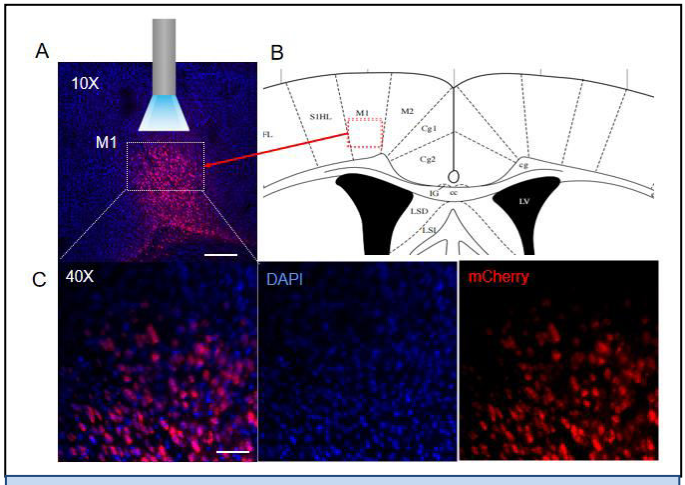

Figure 5: The injection location of the virus encoding ChR2 was verified through immunofluorescence.

the light administered to activate the excitatory neurons in M1. (B) The location of M1 in the mouse atlas is marked in the red box. (C) An enlarged version of panel A, from left to right, shows the merged figure, DAPI staining, and mCherry immunofluorescence. Scale bar: 10X: 100 mm, 40X: 50 mm.

The location of the optogenetic virus was verified by immunofluorescence after the swallowing experiment was

completed. As shown in Figure 5A, blue light was given to M1 L5, 2.5 mm above the virus injection site. Figure 5B shows the mouse brain atlas, and the red box shows the location of M1 L5. Figure 5C is an enlarged version of the panel shown in Figure 5A, indicating mCherry and DAPI staining in M1 L5.

DISCUSSION

In this study, different methods for the initiation of a swallowing response were used, and their characteristics were demonstrated. We found a shorter swallowing latency in the light group than in the water group. However, no significant differences were found in the AUC between the two groups. The interval between two consecutive swallows was analyzed, with a shorter interval in the light group than in the water group.

The role of the cerebral cortex in swallowing, controlling the initiation of swallowing, has been confirmed for more than a century [11,19] and has even been demonstrated by cortical stimulation. Recently, we published a study in *Nature Communications* that identified a cluster of excitatory neurons in M1 L5 that induces a swallowing response [9]. Here, we used this method and found that it has a shorter latency to induce swallowing than water administration, which indicated the precise control of swallowing achieved by optogenetically activating M1 neurons. Compared with the light group, peripheral water stimulation needs to activate the sensory organ in the pharynx, and then the sensory signals must ascend to the neural center to initiate swallowing movement, especially in the pharyngeal phase. [20]. As a chemical stimulus, water-induced swallowing is indeed innervated by the water fibers of the superior laryngeal nerve, which could initiate swallowing by electrical stimulation [8]. Nevertheless, the latency was lower than that of the light group. After consulting the literature, the latency of swallowing induced by stimulation of the unilateral superior laryngeal nerve is 2.71 s, and the latency of swallowing induced by stimulation of the bilateral superior laryngeal nerve is 0.72s [21]. Another study showed that the latency of water-induced swallowing was 5.8 s when water was supplied into the pharyngeal cavity in rats [22]. The latency of light-induced swallowing was 0.216 s, which was calculated in our results (Figure 1). Therefore, the latency of the water group was longer than that of the light group. In the water group, water was first delivered to the hard palate that is controlled by the surrounding nerves, such as the vagus nerve and glossopharyngeal nerve, which could also induce reflexive swallowing regulated by the swallowing CPG [23]. Moreover, this process is also affected by higher cortical descending pathways, a finding that was confirmed in our previous study [9]. However, earlier studies considered that the corticobulbar system is not an essential component of the basic pattern of reflexive swallowing. It may only play a role in the mechanism of initiating reflexive swallowing and not in the full execution of a swallowing event [13]. Therefore, the precise control of the excitatory neurons in M1 by optogenetics directly induced pharyngeal swallowing, resulting in a shorter latency when compared with the water supplied group.

The activation of the anterior cingulate cortex, as well as the lateral cortex of the insula, may be related to the activation of the swallowing CPG in the brainstem [24]. Our previous study also demonstrated that the swallowing process is influenced by both the cortex and the swallowing CPG, especially at the NTS, which might relay information about subsequent swallows [9]. In our study, the three intervals of the light group were all shorter than those of the water group, which might be attributed to the precise activation of neurons in M1 that induced each swallow. These results indicated that the swallowing movement was initiated quickly after the activation of M1 excitatory neurons, and the EMG response induced by water administration might be affected by the pharyngeal sensory and swallowing reflex centers simultaneously. A previous study considered that the agonist of transient receptor potential (TRP) channels induced a larger number of swallows and shorter intervals of swallowing reflex than water administration [25]. These results might be related to the improvement of pharyngeal sensory function by an agonist, which further supported the role of sensory transmission in the water group. The same comparison of voluntary swallowing and reflexive swallowing was reported in an older study that recorded the onset of submental electromyographic activity (SM-EMG) and the upward movement of the larynx. However, they reported a longer latency for voluntary swallowing than for reflexive swallowing [13]. Indeed, this study recorded from a different muscle compared with the method we used in this study. Additionally, water was used to induce voluntary swallowing, which involved

water in the oral cavity to initiate swallowing and might prolong the swallowing interval.

However, when the AUCs of the total swallows or each swallow of the mylohyoid muscle in the two groups were compared, no significant differences were found (Figure 2, 4). These results might indicate that the same intensity of muscle activity was induced by the two approaches. The optogenetic method mimicked the voluntary pharyngeal swallow response but without food or a bolus to initiate the response, which might influence the AUC results. More importantly, this process was regulated by the motor output pathway, including the NTS, which modulates reflexive swallowing.

There was a limitation in this paper; the difference between light delivery and water supply was compared only in normal mice. Whether this change also occurs in model mice is not clear and needs further research.

CONCLUSION

The latency and the swallowing interval of EMG were different between the light group and the water group. The difference in latency suggests that activation of the swallowing motor cortex can induce swallowing movement more directly, which may be related to the efferent neural mechanisms of swallowing. These results might motivate the use of more specific methods in future research on the mechanism of dysphagia.

AUTHOR CONTRIBUTIONS

Zulin Dou, Hongmei Wen and Qiuping Ye designed this study. Qiuping Ye, Yong Dai and Jiahui Hu completed the whole experiment. Yong Dai and Jiahui Hu analyzed the data. Yan Liu, Chao Li and Nenggui Xu made significant modifications to the manuscript. Qiuping Ye finished writing the article. All authors contributed to the article and approved the submitted version.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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