RESEARCH ARTICLE

The Effect of Xylazine Anesthesia on Goats Central NO/cGMP Pathway

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A B S T R A C T

Due to the unique physiological characteristics of ruminants, the process of their clinical anesthesia includes numerous adverse factors. In this study, we investigated the effects of xylazine anesthesia on the goat NO/cGMP signal transduction system. We explored changes of NOS activity, NO content, and cGMP concentrations in different encephalic regions of goats after xylazine anesthesia. Fifteen goats were divided into five groups: saline control group, induction period, anesthesia period, recovery period 1, and recovery period 2, respectively. Cerebrum, cerebellum, brainstem, hippocampus, and thalamus were collected. The results showed that the contents of NOS, NO, and cGMP in cerebrum, cerebellum, hippocampus, thalamus, and brainstem had significantly decreased during the period of anesthesia. The activity of NOS decreased by 51.0, 39.1, 39.7, 41.1 and 25.4%, respectively (P<0.01), the content of NO decreased by 39.0, 31.1, 17.1, 21.7 and 27.4%, respectively (P<0.01), and the concentrations of cGMP decreased by 56.4, 43.4, 40.9, 59.2 and 40.0% (P<0.01). The recovery period returned to normal level. The xylazine effect may be associated with the inhibition of the NO/cGMP signal transduction pathway in each encephalic region of goats.

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INTRODUCTION

Xylazine (Rompun) is an α2 receptor agonist that exerts a direct inhibitory effect on the central nervous system, inhibiting the nerve impulse transmission within neurons, which results in sedative and analgesic efficacy (Janssen et al., 2017). The field of animal anesthesia received increasing research attention and xylazine is of particular interest, since the central nervous system nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) signal transduction system has been reported to be involved in its mechanism of action (Romero et al., 2012). NO is an important transmitter between cells, which may play an important role in the central nervous system. As an information substance, its functions are related to neurotoxicity, consciousness, learning, and memory, mediated via excitatory amino acids (Hao et al., 2016). When anesthetics block the formation of NO, the excitatory nerve conduction decreases, while the inhibitory conduction is enhanced, revealing that the anesthesia sedation state is consistent with the overall enhancement of anesthesia (Abdulla et al., 2014). The contents of cGMP in the cerebrum, cerebellum, hippocampus, and caudate nucleus were significantly

reduced in mice, after injection with an α2-adrenergic receptor agonist, suggesting this to be a secondary mechanism for the inhibition of NO production (Chalimoniuk et al., 2015). The cGMP content reduction of neurons was happened mostly in the α2-adrenergic agonist concentration site, which is known to cause significant sedation, thus enhancing anesthesia (Turovsky et al., 2013).

The molecular mechanism of general anesthesia includes excitatory pathways of N-methyl-D-aspartic acid (NMDA) receptor and M cholinergic receptors in the central nervous system pathways that are involved in the NO/cGMP signaling system (Jin et al., 2000). Its inhibitory pathways have been reported to involve γ-aminobutyric acid (GABA) receptors and α2-adrenergic receptors (Gonzalez-Usano et al., 2014) and when anesthetics blocked NO production, excitability of nerve conduction was decreased. The inhibitory conduction was enhanced, thus presenting the anesthesia sedation state (Smith et al., 2016).

A study has shown that anesthetics can significantly affect the NO/cGMP signal transduction system (Liebenberg et al., 2015). At present, however, few studies focus on the mechanism of anesthesia in goats and

controversy still exists about the role of anesthetics in information transduction pathways and the specific effects of anesthetics. By measuring the activity of nitric oxide synthase (NOS), the content of NO, and the concentration of cGMP in five experimental groups, we explored the mechanism of xylazine anesthesia in goats.

MATERIALS AND METHODS

Animals and experimental groups: C57BL/6 mice (n=25) with a mean body weight of 20 ± 2 g was obtained from the laboratory animal center of the First Affiliated Hospital of the Harbin Medical University. These mice were divided into five groups and received xylazine injections with concentrations of 57.4, 67.5, 79.5, 93.5, and 110 mg/kg. Fifteen healthy goats aged eight months, and weighing 13 to 15 kg, were purchased from the Qingxi farms in HarBin. Goats were randomly divided into five groups (n=3 each): the normal saline control group (C group), the induction group (I group, standing unsteady, goats fell to the ground, the turning reflex was about to disappear), the anesthesia group (A group, placing the animal facing a ventral face, providing slight irritation, and the animal still could not turn back to the reflex), the recovery period 1 group (R1 group, on anastomosis after the reflection of the animals had disappeared, providing slight stimulus, animals were able to turn their own back, restoring the reflex), and the recovery period 2 group (R2 group, reflecting the reflection after a recovery period of 6 h, the animals were essentially awake, were free to walk, and took the initiative to drink water and to forage). All experiments were performed in accordance with the Ethical Committee for Animals.

Determination of ED$_{50}$: Intraperitoneal injection was used to correct the reflex as an effective standard for anesthesia: 10-15 min after injection, the reflex disappeared for more than 1 min, and neither clamp, needle tail, nor claw could reactivate the reflex. The Dixon-Mood method (ED$_{50}$=log^{-1}[x$_0$+i(A/N±0.5)] where "i" is the logarithmic dose group distance) was used to start the maximal effective dose D$_{max}$ at a dose ratio of 1: 0.85, which was injected intraperito-neally in a sequential manner. After administration, the anesthesia of mice was observed and the dose of the next mouse was determined via disappearance of the reflex after administration. The anesthetic doses of mice (pre-determined via experimentation) were 57.4, 67.5, 79.5, 93.5, and 110.0 mg/kg. The ED$_{50}$ of xylazine for mice anesthesia was calculated as 98 mg/kg. Different animal body surface areas could be used $A=K \cdot W^{2/3}$ (A represents the body surface area, K represents the body shape coefficient, and W represents the body weight) for the estimate. The drug dose was roughly proportional to the body surface area. The conversion coefficient of dose conversion among different animals could be deduced. $R_{ab}=(K_a/K_b)$. $(W_b/W_a)^{1/3}$ (R_{ab} is the conversion factor of dose conversions between animal a and animal b; K_a and K_b represent the body sizes of animal a and animal b; W_a, W_b represent the weights of animal a and animal b). The ED$_{50}$ of goat anesthesia was calculated according to the method of equivalent dose conversions between different animals. According to the formula, the conversion coefficient for

the dose conversion from mouse to goat (R_{ab}) was 0.131. To calculate xylazine anesthesia on goats an ED$_{50}$ of 12.8 mg/kg was used.

Sample collection: For the saline control group: intramuscular injection of saline solution was conducted and after 8 min, the goats were slaughtered along both interfrontal raphe self-nasal occipital condyles, cut with an electric plaster saw, and goats were sacrificed to retrieve their brain for further investigation. The cerebrum cerebellum, thalamus, hippocampus, and brainstem were quickly removed and stored on ice. All samples were immediately frozen in vials in liquid nitrogen. For other experimental groups: goats were sacrificed at each time point. The tissues in different encephalic regions of the brain were removed from the liquid nitrogen tank and stored at -80°C for further investigation.

Determination of NOS activity: The activity of NOS was determined with a NOS assay kit (Nanjing Bioengineering Institute Batch). A tube was filled with 100 μl distilled water and 100 μl sample were added to the tube. 200 μl substrate buffer, 10 μl accelerator, and 100 μl chromogenic agent were added to both tubes. Then, the samples were mixed at 37°C in a water bath reaction for 15 min. After wards, 100 μl transparent agent and 2000 μl termination fluid were added to both tubes. After full mixing, 1 cm light path correction was done at 530 nm with distilled water zero adjustment, and measured via colorimetry.

Determination of NO content: The content of NO was determined with a NO kit (Nanjing Jiancheng Bioengineering Institute). A tube was filled with 0.5 ml of distilled water and 0.4 ml of distilled water and 0.1 ml sample were added to the tube. We added the 0.2 ml each of reagent one and reagent two to three branches, and mixed the contents in a water bath reaction for 60 min. We then added 0.2 ml of reagent three and 0.1 ml of reagent four. Full vortex mixing for 30 s, was followed by letting the mixture settle for 10 min at room temperature, and subsequent centrifugation for 10 min (3,500-4,000 g). The resulting clear supernatant extract was used in a chromogenic reaction.

Determination of cGMP concentration: cGMP in the sample was determined via double antibody sandwich enzyme standard immunoassay using the cGMP kit (US R & D systems company). 50 μl of sample were added at a 1:1 dilution to be tested with blank micropore. To the standard micropore and sample micropore, 50 μl biotin labeled antibody solution was added and incubated at 36±2°C for 60 min. The mixture was washed five times, each time letting it settle for 10-20 s. Then, 60 μl affinity streptavidin-HRP was added to all micropores and mixed well before incubation at 36±2°C for 30 min. The mixture was again washed five times, letting it settle for 10 s. Each pore received an addition of 50 μl A solution and 50 μl B solution, incubating it at 36±2°C without light for 15 min. Then, 50 μl termination solution was added to each micropore to terminate.

Data analysis: Mean data±standard deviation was used for data analysis by utilizing the SPSS 13 for Windows

statistical analysis software, for one-dimensional variance analysis. Two variables simple correlation analysis was conducted using linear correlation regression analysis, with P<0.05 suggesting a significant difference, P<0.01 suggesting a very significant difference. The experimental data were plotted with GraphPad Prism 5.

RESULTS

Results of NOS activity: The activity of NOS in the cerebrum, hippocampus, thalamus, cerebellum, and brainstem was inhibited to some extent. Compared to the C group, the I group decreased by 46.1 and 36.8% in the cerebrum and thalamus, respectively (P<0.01, Fig. 1A and D); while the hippocampus and brainstem were reduced by 28.1 and 16.4% (P<0.05, Fig. 1C and E), respectively. The NOS activity of A group compared to C group decreased in cerebrum, hippocampus, thalamus, cerebellum and brainstem by 51, 39.7, 41.1, 39.1 and 25.4%, respectively (P<0.01, Fig. 1). In group R1, the activity of NOS in the above encephalic region was significantly increased, was and a significant difference was found compared to group A (P<0.01, Fig. 1). However, the activity of NOS in the cerebrum was still significantly inhibited compared to the C group (P<0.01, Fig. 1A). The activity of NOS in the encephalic region recovered to a normal level in the R2 group (Fig. 1A).

Results of NO content: The content of NO in each brain area decreased significantly following injection. I group compared to C group contents of NO in thalamus and cerebellum decreased by 13.8% and 17.9% (P<0.01, Fig. 2B and D), respectively. Compared to C group, A group had content of NO within the cerebrum, hippocampus, thalamus, cerebellum, and brainstem of 39, 17.1, 21.7, 31.1 and 27.4%, respectively (P<0.01, Fig. 2). Compared to I group, NO levels differed in the cerebrum, hippocampus, and brainstem in A group (P<0.01, Fig. 2A, C, and E) as well as the thalamus and cerebellum (P<0.05, Fig. 2B and D). R1 group had significantly increased NO content in the encephalic region compared to A group (P<0.01, Fig. 2). The content of NO returned to normal levels in the encephalic region of the R2 group compared to A group (P<0.01, Fig. 2).
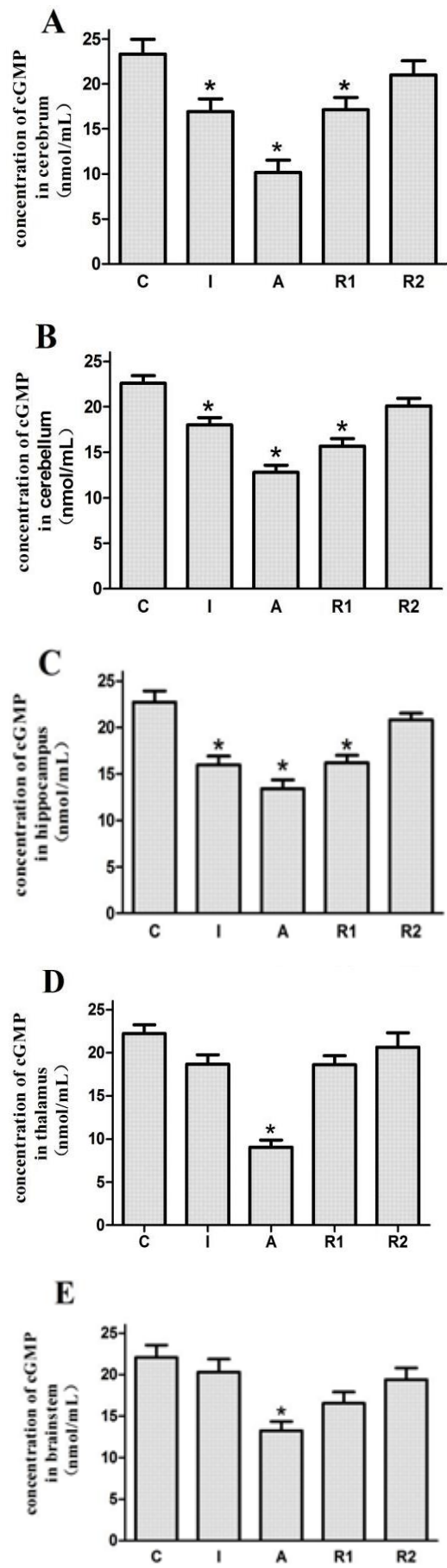
Results of cGMP concentration: The concentration of cGMP in the five encephalic regions decreased significantly after anesthesia. I group had a reduced concentration within the cerebrum, the hippocampus, and the cerebellum compared to C group; reduction was 27.5 and 29.7%, respectively (P<0.01, Fig. 3A, C) and 20.2% (P<0.05, Fig. 3B). For the cerebrum, the hippocampus, thalamus, cerebellum, and the brainstem, A group had a reduced concentration of cGMP compared to C group; levels were respectively reduced by 56.4, 40.9, 59.2, 43.4 and 40.0% (P<0.01, Fig. 3), within the cerebrum, thalamus, and brainstem (P<0.01, Fig 3A, D, E) as well as the cerebellum (P<0.05, Fig 3B); In group R1, the concentration of cGMP increased significantly compared to I group within the cerebrum and the thalamus compared to A group (P<0.01, Fig. 3A, D). The concentration of cGMP of the encephalic region of R2 group returned to normal levels.
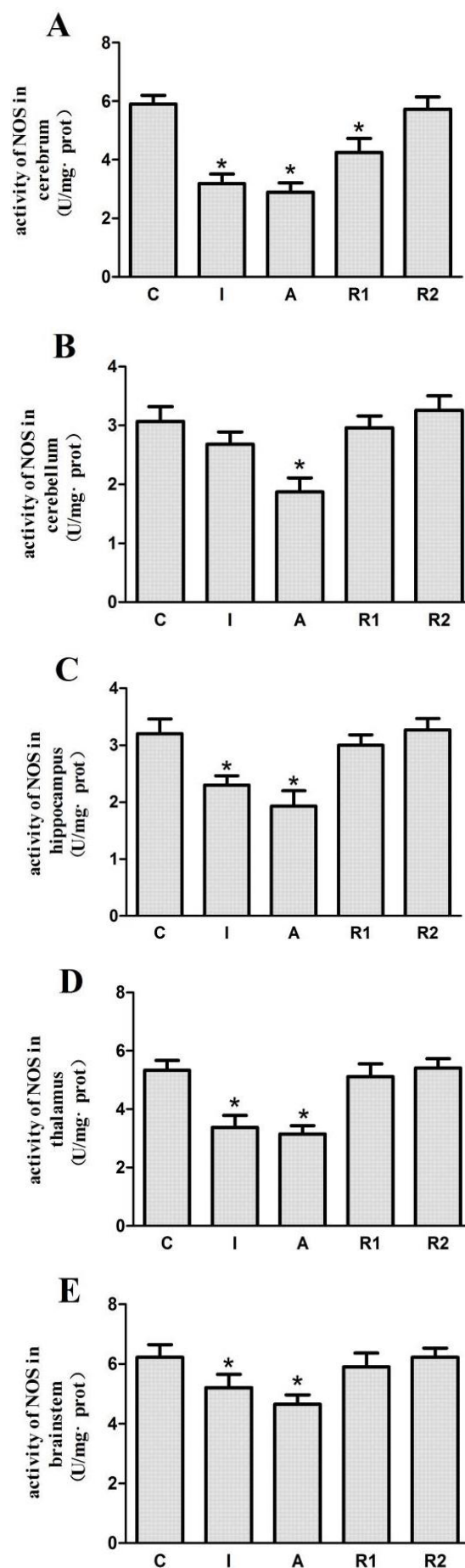
Fig. 1: NOS activity in five encephalic regions at different periods. *Significant differences (P<0.05) are in comparison to the control group.
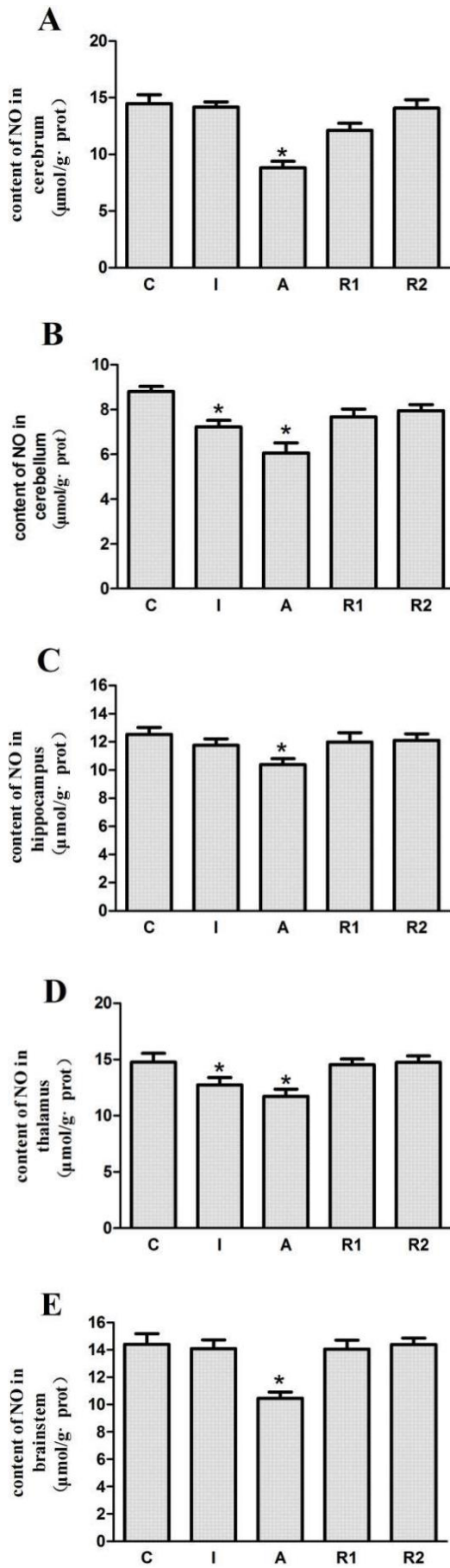
Fig. 2: NO content in five encephalic regions at different periods. *Significant differences (P<0.05) are in comparison to the control group.

Fig. 3: cGMP concentration in five encephalic regions at different periods. *Significant differences (P<0.05) are in comparison to the control group.

DISCUSSION

According to our results, the mechanism of xylazine action as a goat anesthesia is associated with the NO/cGMP pathway. NOS is the rate-limiting enzyme of NO synthesis, and NOS exerts its biological effects through the formation of NO (Buchwalow et al., 2017). As a type of biological information transmitter, NO is a highly reactive free radical, which exists widely in goats. NO is produced by NOS, catalyzing the reaction of L-Arg with molecular oxygen (Grasemann et al., 2017). A previous study has confirmed that NMDA and M cholinergic receptors produce cGMP by activating NOS in the excitatory pathways. Numerous intravenous anesthetics have been suggested to be involved in the NO/cGMP signaling pathway (Romero et al., 2011). The link can change with cGMP depending on the activity of phosphodiesterase, changing the NO half-life, and preventing the release of NO or the direct effect of NOS, thus activating the NOS after NMDA receptor activation (Ostadhadi et al., 2016). Therefore, the mechanism of anesthesia may be related to the NMDA receptor and the M cholinergic receptor excitatory pathway. These results furthermore suggest that α2-adrenergic receptor agonists may be involved in the regulation of α2-adrenergic receptor mediated sedation, analgesia, and muscle relaxation. This effect had been reported to be related to the NO/cGMP signal transduction system (Tonner et al., 1999). During R1 group treatment, the activity of NOS in each of the encephalic regions was significantly increased, resulting in a significant difference compared to A group (P<0.01, Fig. 1). However, the activity of NOS in the cerebrum was still significantly inhibited compared to the C group (P<0.01, Fig. 1A). The activity of NOS in the encephalic region recovered to normal levels in group R2 (Fig. 1). This suggests that anesthesia is more likely to inhibit NOS activity in the cerebrum than in other encephalic regions. This may be because anesthesia affects the excitatory pathways of NMDA receptors and the M cholinergic receptors in the cerebrum.

NO plays an important role in both the formation and maintenance of neuropathic pain (Wong et al., 2017). However, if anesthesia blocks the formation of NO, this leads to a reduction in excitatory neurotransmission and consequently, in inhibitory conduction enhancement. Then, the sedation state is presented, which is consistent with the overall enhancement of the anesthetic state (Srebro et al., 2016). The study of NO target molecules also showed that downstream signal molecules of NO play different roles in the processing of a pain signal. Therefore, reducing the content of NO can relieve the pain, which is one of the critical indicators for successful anesthesia (Fan et al., 2016). NO affects the processing of pain signals at multiple levels in the nervous system, and can enhance the excitability of neurons, thus abolishing the descending inhibition of the spinal cord (Musicki et al., 2017). In group I, the NO content in each brain area was clearly decreased, and in the thalamus region, it was 13.8% lower than in group C (P<0.01, Fig. 2D). The NO content in each brain region increased slightly in group R1; however, compared to the C group in cerebrum, the difference was still significant. These effects were associated with a simultaneous low NOS activity. This

also suggests that the NOS activity was greatly inhibited, which led to NO synthesis.

A prior study reported that the NO/cGMP signal transduction system is closely related to the α2-adrenergic receptor pathway (Thorin et al., 1998). Inhibited NO production can lead to a reduced cGMP content. In the NO/cGMP pathway, NO is an important information substance and plays an important role in the transmission and maintenance of nociceptive transmission within the central nervous system (Banuls et al., 2014). NO activates sGC and promotes cGMP synthesis and cGMP is the central link of the entire system (Spinelli et al., 2016). The downstream target molecules of cGMP include protein kinase G (Zhang et al., 2016), nucleotide gated cation channels, cGMP regulated phosphodiesterase, and the AMPA receptor. NO is mainly involved in a variety of regulatory mechanisms linked via cGMP and it also plays an important role in regulating peripheral cells (Kim et al., 2016). Clonidine and dexmedetomidine can reduce the content of cGMP in the cerebellum and enrich the cerebrum, hippocampus, and the caudate nucleus with α2-adrenergic receptors. Furthermore, the content of cGMP decreased (Vulliemoz et al., 1996), which could be reversed by the selective receptor yohimbin and the dose dependent atipamezole antagonist imidazole (Murahata et al., 2014). During anesthesia, the concentration of cGMP was significantly lower in each brain area than for the control group. A significant difference was detected in cGMP concentration compared to the control group in the cerebrum and hippocampus during induction. During the same period, we suggest that the concentration of cGMP was consistent with the trend of NOS concentration and no content in brain activity. In the hippocampus, anesthesia may affect other substances the contributed to the change of cGMP, such as the content of α2-adrenergic receptor. Although the effects of xylazine on the activity of NOS, the content of NO, and the concentration of cGMP were different during anesthesia, the trend followed a similar changing tendency, which was similar to that of goats. This shows that the regulation of the NO/cGMP signal transduction system in various encephalic regions of goats may be involved in the molecular mechanism of xylazine anesthesia. Furthermore, the effect of anesthetics may be related to the inhibition of the NO/cGMP signal transduction system in these regions.

A previous study has shown that the NO/cGMP signal transduction system is closely related to the GABA pathway (Fedele et al., 1997). In the central nervous system, GABAA receptors are always present in the same region as NOS, NO, and cGMP and have the function of regulating GABA release and the GABAA receptor. NO and cGMP can inhibit the activity of the GABAA receptor, and the activation of GABAA receptor in turn inhibits the NO/cGMP signal transduction system (Yu et al., 2003). Therefore, as soon as the NO/cGMP signaling pathways is verified to be involved in the xylazine anesthesia mechanism in goats, the GABA pathway is a likely candidate to be involved as well, playing a role in the process of goat anesthesia. In a future study of this anesthesia mechanism, other substances should be investigated that will affect the NO/cGMP, thus enabling to systematically summarize the effect of the anesthesia

mechanism. This will have a positive impact on the development of novel narcotic drugs.

Authors contribution: YW and BJ designed the research experiment. RL, LL, YL and DW fed animals and collected samples. YW, YZ, and CG were involved in the data analysis. LG provided technical assistance for conducting the study. YW wrote and edited the manuscript. All authors have participated in the study and concur with submission and subsequent revisions submitted the corresponding author. The authors declare that they have no competing interests.

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