



## RESEARCH ARTICLE

### Ketamine Anesthesia on Neurocyte in Fetal Rat via cAMP and NO/cGMP Pathway

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#### ARTICLE HISTORY (18-167)

Received: May 13, 2018  
Revised: October 08, 2018  
Accepted: October 14, 2018  
Published online: December 21, 2018

#### Key words:

cAMP  
Fetal Rat  
Ketamine  
Neurocyte  
NO-cGMP

#### ABSTRACT

Ketamine anesthesia during infancy has an impact on development of nervous system. This study is to demonstrate effects of ketamine on neuronal cells via cAMP and NO-cGMP pathway. The cortex neurocyte of Wistar fetal rat were divided into four groups. Every group was given different doses, low dose group was 0.7 µg/mL, anesthesia groups were 1 µg/mL, 3 µg/mL, 5 µg/mL at 0, 5, 10, 15, 20, 25, 30, 45, 60, 90 and 120 min, respectively. The content of cGMP and cAMP were detected by ELISA, and the activity of NOS and content of NO were detected by spectrophotometric method. After different concentrations of ketamine were applied to isolated neurons, cAMP and cGMP levels were lowered first and then increased, the content reached the bottom at 25 min and then increased. In the 30 min, the content of NO and NOS decreased to the lowest value and then went up, respectively. In the 120 min, their values were both lower than 0 min. Ketamine probably expressed its anesthesia effect with the increasement of cAMP and inhibition of NO-cGMP pathway.

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**To Cite This Article:** Chen Y, Liu W, Liu M, Zhao J, Li X, Chang T, Wang Q, Zhang X, Chang D and Gao L, 2018. Ketamine anesthesia on neurocyte in fetal rat via camp and NO/cGMP pathway. Pak Vet J. <http://dx.doi.org/10.29261/pakvetj/2018.113>

#### INTRODUCTION

Ketamine, a non-competitive NMDA antagonist that regulates the neurotransmission of the postsynaptic receptors, blocking the NMDA receptors and causing anesthesia (Kawamata et al., 2000). Research has shown that ketamine increased functional coupling of adenylyl cyclase to increase intracellular cyclic adenosine monophosphate (cAMP) (Wray et al., 2017). In the nervous system, cAMP, acting as an intracellular second messenger, which plays a role in multiple signaling pathways, especially the hyperpolarization-activated CGN pathway (HCN channels, hyperpolarization-activated CGN channels), thereby participating in the body's learning, memory, and pain functions. cAMP can be produced by ATP decomposed by adenylyl cyclase (AC). Several studies have shown that a variety of anesthesia affect the change of cAMP content (Carceles et al., 2004; Cao et al., 2016). The NO-cGMP signaling pathway can be widely involved in the physiological activities of the body, such as the development of the cardiovascular system and the nervous system (Gao et al., 2017; Wobst et al., 2018). Over the years, researchers have found that

Nitric oxide (NO) is a novel biosignal molecule with a variety of physiological functions such as second messengers, neurotransmitters, and effector molecules. It is an important carrier for the exchange of information between cells and plays an important physiological role in living organisms. NO is produced by NOS, catalyzing the reaction of L-Arg with molecular oxygen (Grasemann et al., 2017). NO activated soluble guanosine (SGC), which increased the content of the intracellular cyclic guanosine (cGMP). cGMP, the second messenger in the cell, plays a role in information transfer by regulating ion channels, regulating the activity of phosphatase (PDE), activating cGMP-dependent protein kinase (GPK) and interacting with camp-dependent protein kinase (PKA) (Korkmaz et al., 2004; Francis et al., 2010). The previous study demonstrated that ketamine stimulates the L-arginine/NO/cyclic GMP pathway via neuronal NO synthase to induce peripheral antinociceptive effects (Romero et al., 2011). Each site in the NO pathway may be a potential target site for general anesthetics, and there are different views on how the drug interacts with pathways and specific sites of action. Meanwhile, previous study has indicated that anesthetics can affect

NO/cGMP pathway significantly (Wang *et al.*, 2008; Yin *et al.*, 2016; Nagasaka *et al.*, 2017).

The aim of this study was to investigate the effects of cAMP and NO-cGMP signaling pathways in vivo neurons of fetal rats during different periods of ketamine anesthesia.

## MATERIALS AND METHODS

**Animals:** Five pregnant Wistar rats weighing 280-300 g were offered by laboratory animal center of Second Affiliated Hospital of Harbin Medical University (Harbin, China). Rats were placed in a room with comfortable temperature ( $22\pm 3^\circ\text{C}$ ).

**Experiment procedure:** 17-day pregnant rats were sacrificed and fetal rats were removed from uterus and killed. The process of collection and culture of hippocampal neurons in fetal rats was according to Kaech's article (Kaech and Banker, 2006). All animal experiments were based on the University's "Guide to Animal Experiments".

**Identification of isolated neurons:** The cells were seeded into 6-well plates precoated with polylysine and cultured for 8 days for immunohistochemistry. Before fixing with 4% paraformaldehyde fixative for 10 min, they were washed two times with PBS. After washing with PBS, cells were added 0.1% Triton X-100 1 mL and placed 10 min. Blocked in 10% serum for 1 h, they were incubated with mouse anti-MAP-2 polyclonal antibody (1:300) overnight at  $4^\circ\text{C}$ . Next day, the corresponding fluorescence II was added and incubated for 1 h at room temperature without light. The glycerol was mounted and observed under microscope.

**Determination of NO content:** The content of NO in neuronal cells with different concentration of ketamine was determined by NO kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). A tube was contained with 0.5 mL distilled water, 0.4 mL distilled water and 0.1 mL sample. We added the 0.2 mL each of reagent one and reagent two to three branches, and mixed the contents in a water bath for 1 h. Then 0.2 mL of reagent three and 0.1 mL reagent four were added. The tube is blended with vortex 30 sec, then centrifuged at  $3000 \times g$  for 10 min. The supernatant was used in a chromogenic reaction and measured at 530 nm according to standard curve and cytokines were expressed as  $\mu\text{mol/L}$  or U/mL.

**Determination of NOS activity:** The NOS activity was detected by NOS assay kit (Nanjing Bioengineering Institute). A tube was filled with 100  $\mu\text{l}$  distilled water and 100  $\mu\text{l}$  sample. 200  $\mu\text{l}$  substrate buffer, 10  $\mu\text{l}$  accelerator and 100  $\mu\text{l}$  chromogenic agent were added to both tubes. Then, the samples were mixed at  $37^\circ\text{C}$  in a water bath for 15 min. After wards, 100  $\mu\text{l}$  transparent agent and 2 mL termination fluid were added to both tubes. Fully mixing, 1 cm light path correction was done at 530 nm with distilled water zero adjustment and measured by colorimetry.

**Determination of cAMP and cGMP:** cAMP and cGMP were detected by two-antibody sandwich enzyme-linked immunosorbent assay for biotin (ELISA) via the cAMP and cGMP kit (Nanjing Bioengineering Institute), respectively. The sample (50 $\mu\text{l}$ ) was added at a 1:1 dilution to be tested with blank micropore. For the standard and sample micropore, 50 $\mu\text{l}$  biotin labeled antibody solution was added and incubated at  $36\pm 2^\circ\text{C}$  for 1 h. Then, affinity streptavidin-HRP (60 $\mu\text{l}$ ) was added to all micropores and mixed before incubation at  $36\pm 2^\circ\text{C}$  for 0.5 h. Each pore received an addition of solution A (50 $\mu\text{l}$ ) and solution B (50 $\mu\text{l}$ ), which was incubated at  $34\text{--}38^\circ\text{C}$  without light for 15 min. Then, the termination fluid of 50 $\mu\text{l}$  was added to each micropore and the absorbance was measured at 450 nm.

**Statistical analysis:** Data were expressed as the mean  $\pm$  standard deviation (SD). Two-way analysis of variance (ANOVA) was performed using Statistical Product and Service Solutions (SPSS) 18.0 (Shanghai, China) The simple correlation analysis between two variables was linear regression analysis.  $P < 0.05$  was considered as significant difference,  $P < 0.01$  means extremely significant. The GraphPad prism 7 (California, USA) were used to draw figures.

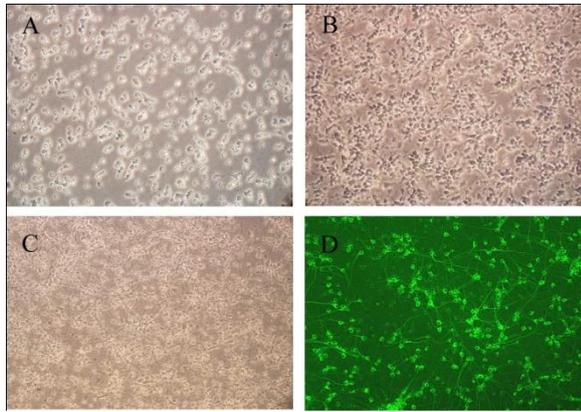
## RESULTS

**Nerve cell culture and identification:** The morphological changes during nerve cell culture were observed under a phase contrast microscope for 8 days. The neurons just obtained were round, small, translucent, scattered, and the cells began to adhere to the wall 3 hours after planting (Fig. 1a). After 4 days of culture, the cells were significantly enlarged, and many projections emerged from the cell body, showing a vertebral or star shape. At this time, the neurons showed obvious morphological features, and the neurons were clear and bright, with prominent protrusions, and they could form contact with adjacent cell protrusions (Fig. 1b). After 8 days, the cell body of the neuron increased, the nucleus was obvious, and the processes increased further and gradually became network. The cells migrated closer to each other, some neurons clustered together, and dense neuronal cell networks were seen. No obvious glial cells were observed (Fig. 1c).

The cerebral cortical neurons were cultured for 8 days and identified by immunofluorescence staining. The cultured neurons were stained by MAP2 under a laser confocal microscope. The primary cultures were rich in neurons, the protrusions were clear, the morphology was good, and the nucleus was large (Fig. 1d).

**Different concentrations of ketamine on cAMP:** The effect of different concentrations of ketamine in nerve cell on cAMP is represented in Fig. 2. Under the action of different concentrations of ketamine, the content of cAMP showed a tendency to decrease first and then increase later. 0.7 $\mu\text{g/mL}$  ketamine was applied to nerve cells, the cAMP content was reduced by 51, 63, 75, 74, 70, 61, and 33% from 15 minutes to 90 min compared with 0 min ( $P < 0.01$ ). After 1  $\mu\text{g/mL}$  ketamine was applied to nerve cells, the cAMP content at 10 min was reduced by 22% ( $P < 0.05$ ); the cAMP content at 5 min to 60 min was

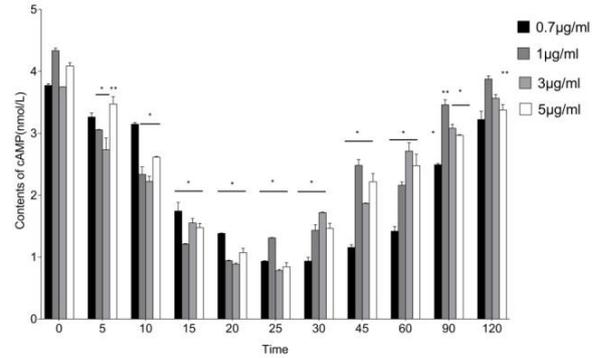
reduced by 28%, 43%, 71%, 78% and 69%, 68%, 43%, 50% respectively. The difference was extremely significant compared with 0 min ( $P<0.01$ ); cAMP content decreased 17%, in 90 min ( $P<0.05$ ). 3  $\mu\text{g}/\text{mL}$  ketamine was applied to nerve cells, the cAMP content was reduced by 31%, 42%, 60%, 77%, 80%, 54%, 50%, 30% and 19% from 5 min to 90 min, with extremely significant differences ( $P<0.01$ ). The cAMP content at 90 min was reduced by 21% and the difference was significant ( $P<0.05$ ). The cAMP content of 5  $\mu\text{g}/\text{mL}$  ketamine decreased by 16% and 18% for 5 min and 120 min, respectively ( $P<0.05$ ). The cAMP content decreased by 36%, 65%, 75%, 80%, 65%, 48%, 42% and 27% from 10 min to 90 min, respectively ( $P<0.01$ ).



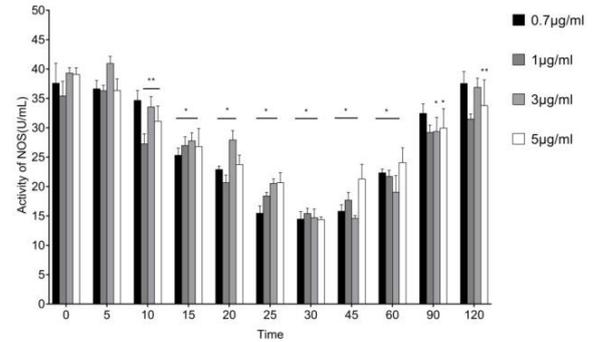
**Fig. 1:** A: Observation of fetal rat nerve cells cultured in vitro for 3h B: Observation of fetal rat nerve cells cultured in vitro for 4 days, C: Observation of fetal rat nerve cells cultured under 8 days, D: Observation of fetal rat nerve cells under 8 days by immunofluorescence staining.

**Different concentrations of ketamine on activity of NOS:** The different concentrations of ketamine on NOS of every point in time were represented in Fig. 3. Compared to 0 min, there was a significant ( $P<0.01$ ) decrease and increase in the NOS activity on the time point of 15 to 60 min for 0.7  $\mu\text{g}/\text{mL}$  ketamine. However, there was no significant variation in the NOS activity for other point in time. Meanwhile, the activity of NOSase from 20 min to 60 min varied significantly compared to 0 min with 1  $\mu\text{g}/\text{mL}$  ketamine on nerve cells ( $P<0.01$ ). Nevertheless, in 10 and 15 min, the activity of NOSase decreased significantly compared to 0 min ( $P<0.01$ ). There was no significant difference in 5, 90 and 120 min compared to 0 min. In 10 min, there was a significant difference in 3  $\mu\text{g}/\text{mL}$  ( $P<0.01$ ) and 5  $\mu\text{g}/\text{mL}$  ( $P<0.01$ ), respectively. And in the point of 15 to 60 min, the difference of 3 and 5  $\mu\text{g}/\text{mL}$  ketamine was significant ( $P<0.05$ ). Besides, the content of NOSase changed significantly after adding 5  $\mu\text{g}/\text{mL}$  ketamine ( $P<0.01$ ).

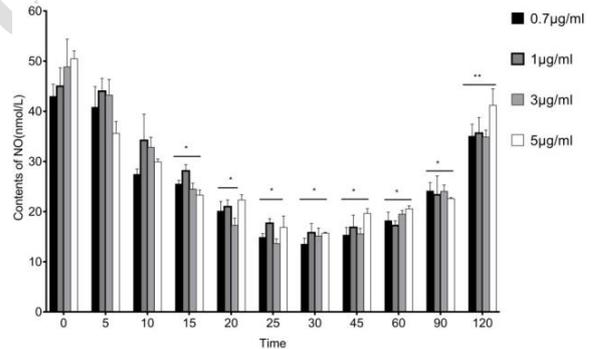
**Different concentrations of ketamine on NO:** The different concentrations of ketamine on NO of every point in time were represented in Fig. 4. Compared to 0 min, there was a significant ( $P<0.01$ ) variation in NO activity on 10 to 90 min for 0.7, 1 and 3  $\mu\text{g}/\text{mL}$ , respectively. Similarly, there was incredibly significant at 120 min in NO activity for all concentrations of ketamine ( $P<0.01$ ). In particular, the time range of 5  $\mu\text{g}/\text{mL}$  in the nerve cells is 5-90 min, the level of NO in the neuron cell changed greatly ( $P<0.05$ ).



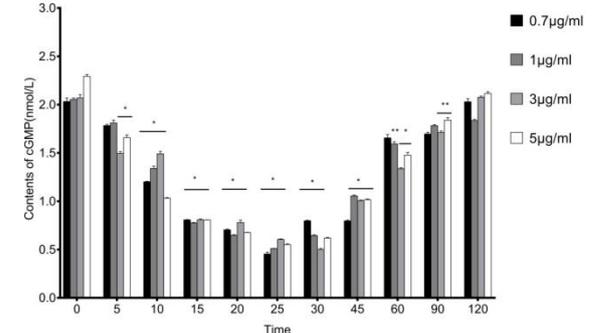
**Fig. 2:** Content of cAMP (nmol/L) in neurons treated with different concentrations of ketamine. Data were expressed as mean  $\pm$  SD ( $n=3$ ). One-way ANOVA followed by Turkey's *post-hoc* test was performed. \* $P<0.05$ ; \*\* $P<0.01$  \*\* vs. 0 min.



**Fig. 3:** Activity of NOS (U/mL) in neurons treated with different concentrations of ketamine. Data were expressed as mean  $\pm$  SD ( $n=3$ ). One-way ANOVA followed by Turkey's *post-hoc* test was performed. \* $P<0.05$ ; \*\* $P<0.01$  vs. 0 min.



**Fig. 4:** Activity of NO ( $\mu\text{g}/\text{mL}$ ) in neurons treated with different concentrations of ketamine. Data were expressed as mean  $\pm$  SD ( $n=3$ ). One-way ANOVA followed by Turkey's *post-hoc* test was performed. \* $P<0.05$ ; \*\* $P<0.01$  vs. 0 min.



**Fig. 5:** Activity of cGMP (nmol/L) in neurons treated with different concentrations of ketamine. Data were expressed as mean  $\pm$  SD ( $n=3$ ). One-way ANOVA followed by Turkey's *post-hoc* test was performed. \* $P<0.05$ ; \*\* $P<0.01$  \*\* vs. 0 min.

**Different concentrations of ketamine on cGMP:** The different concentrations of ketamine on cGMP of every point in time were represented in Fig. 5. Compared to 0 min, there was a significant ( $P < 0.01$ ) variation in cGMP on 10 to 45 min for 0.7 and 1  $\mu\text{g}/\text{mL}$  ketamine ( $P < 0.01$ ). Simultaneously, in 60 min, there was exceedingly significant variation in cGMP for 1  $\mu\text{g}/\text{mL}$  ( $P < 0.01$ ). From 5 to 60 min, there was a significant change in 3 and 5  $\mu\text{g}/\text{mL}$  ( $P < 0.01$ ), respectively. In addition, in 90 min, there was a tremendous difference in compared to 3 and 5  $\mu\text{g}/\text{mL}$  compared to 0 min ( $P < 0.01$ ), respectively. However, there was no significant variation in the other detection time points in four concentrations compared to 0 min.

## DISCUSSION

The primary culture of neural cells can truly simulate the environmental state of the central nervous system. Therefore, we chose primary cells extracted and cultured from the fetal rat hippocampus and cortex as research objects. The mechanism of ketamine anesthesia in fetal rat is related to a variety of signaling pathways, but it is unclear whether cAMP and NO/cGMP pathways are involved. The results of this study show that after the action of neurons by ketamine, various detection indicators have changed.

Ketamine, a NMDA receptor antagonist that may inhibit the growth of the phosphatase activity by blocking the NMDA receptor pathway, preventing the passage of calcium ( $\text{Ca}^{2+}$ ) and sodium ( $\text{Na}^{+}$ ) and inhibiting the increase in the activities of the phosphate esterase, thereby inhibiting the cyclic adenosine monophosphate (cAMP) degradation caused by phosphatase (Oliver *et al.*, 1990). The results of the animal experiment showed that cAMP content increased with anesthesia, and cAMP increased significantly in the cortex under ketamine anesthesia, and the cAMP content increased after the inverted reflex was restored (Li-Na *et al.*, 2001). cAMP, a typical second messenger of nerve impulse conduction, is transformed from adenosine triphosphate (ATP) under the action of adenylyl cyclase (AC). In our experiment, we observed a significant decrease in cAMP with ketamine anesthesia from 0 to 25 min on the isolated neurons, but the cAMP content increased more obviously in 30 to 120 min. Among them, the effect of ketamine at 5  $\mu\text{g}/\text{mL}$  was significantly different from the other three groups. This was most likely due to the anesthetic drugs can activate AC and inhibit the phosphoric acid esterase to increase cAMP in neurons and hyperpolarize cell membrane potential (Carceles *et al.*, 2004).

Ketamine not only plays an important role in anesthesia and analgesia, but also reduces  $\text{Ca}^{2+}$  flow and the production of NO and cGMP and the activity of NOS enzyme. In our study, after treated by different concentrations of ketamine, the activity of NOS and content of NO in 30 min was significantly lower than the 0 min, meanwhile, the content of cGMP reached to the bottom in 25 min. At 120 min, NOS, NO and cGMP increased in neurons but still remained below normal levels compared to 0 min. This suggests that anesthesia is more likely to inhibit NOS activity, NO and cGMP after removing anaesthetic for 120 min. Based on our previous work, it was indicated that effect of xylazine anesthesia

may be related to the inhibition of the NO/cGMP signal transduction pathway in each encephalic region of goats (Wang *et al.*, 2017).

NO is an intracellular messenger discovered in recent years, which may play a role in information transmission in the central nervous system (Wong *et al.*, 2017). NO can be produced by L-arginine catalyzed by nitric oxide synthase (NOS) (Grasemann *et al.*, 2017). NO activates soluble guanylate cyclase (sGC) and increases the content of cGMP in cells. As an intracellular second messenger, a series of cells are triggered by modulating ion channels to regulate phosphodiesterase (PDE) activity, activation of cGMP-dependent protein kinase (GPK), and cAMP-dependent protein kinase (PKA) interactions. The cascade reaction exerts information transmission (Andoh *et al.*, 2002; Korkmaz *et al.*, 2010).

From the results of this experiment, under the effect of different concentrations of ketamine, NO content, NOS activity and cGMP concentration showed a significant decline. Previous study indicated that general anesthetics can activate or inhibit NO-cGMP signaling by activating NMDA receptors, acetylcholine-activated muscarinic (M) receptor-mediated excitatory pathways, GABA receptors and  $\alpha_2$  adrenergic receptor-mediated inhibitory pathway, thereby changing the cGMP content in nerve cells (Vulliemoz *et al.*, 1996). In the central nervous system (CNS), NMDA receptors, as a high content of excitatory amino acid receptors, which can change  $\text{Na}^{+}$ ,  $\text{K}^{+}$  levels inside and outside the membrane, so that the synapse produces a slow excitatory potential. At the same time, after being activated, it will also lead to the influx of  $\text{Ca}^{2+}$  and the influx of  $\text{Ca}^{2+}$  can interact with CaM and bind to the binding site on NOS, which activates NOS to generate NO. Romero *et al.* found that when NMDA receptors are activated, ketamine binds to the phencyclidine (PCP) site on the gated pathway, blocking the transmission of excitatory substances and reducing the production of cGMP (Romero *et al.*, 2011). These results suggest that ketamine's inhibitory effect on cGMP may be related to NMDA receptor and AMPA receptors, but not ketamine's inhibition of NOS (Yagami *et al.*, 2010). Simultaneous inhibition of NOS activity, NO synthesis, and cGMP production inhibited cGMP-mediated neuromodulation (Dächsel *et al.*, 2010). In addition, Orser *et al.* believe that ketamine may affect NMDA receptors through two different mechanisms: the combination of ketamine and open channels leads to a shortened mean channel opening time; ketamine reduces the frequency of channel opening through a biochemical allosteric mechanism (Orser *et al.*, 1997).

All above, this study found that anesthetics can block the activation of NO-cGMP signaling pathway by blocking pathway mediated by NMDA receptors, thereby inhibiting the excitation and conduction of the nervous system, and ultimately producing anesthetic effects. However, Galley *et al.* found during the study of rat thoracic aorta that ketamine was able to resist the effect of acetylcholine-induced elevation of cGMP, but acetylcholine could not activate NOS through acting on NMDA receptor, suggesting that ketamine inhibits cGMP. The content is not completely mediated through NMDA receptors and NOS (Zhu *et al.*, 2017). The specific reasons for this need further study to give corresponding

explanations. At present, there are few reports about the relationship between M receptor-mediated pathway and NO-cGMP signaling pathway.

Studies have shown that there is a closely relationship between  $\alpha_2$  adrenergic receptors and NO/cGMP signaling pathways. Activated  $\alpha_2$  adrenergic receptors can inhibit the activation of NO/cGMP signaling pathway by inhibiting GC activity or NO production, inhibiting neurotransmitters that can activate NOS activity, or directly inhibiting NOS activity. The previous study indicated that the NO-cGMP signaling pathway in specificity anesthetic for deer play an important role in anesthesia (Yin *et al.*, 2016). Based on our previous study, Wang *et al* found that the general anesthesia effect of xylazine is achieved by inhibiting the activation of NO-cGMP signaling pathways in goat brain regions (Wang *et al.*, 2017). In summary, the anesthetic mechanism of ketamine anesthetic may be achieved by inhibiting the activation of  $\alpha_2$ -adrenoceptor-mediated pathways and thereby inhibiting the function of NO-cGMP signaling pathway.

**Conclusions:** A simple and feasible method of nerve cell culture was established. At the same time, the anesthetic concentration of ketamine may be mainly by reducing the content of the relevant messenger molecule and the activity of the corresponding enzymes to play its role in ketamine anesthesia.

**Acknowledgements:** This research was supported by the National Natural Science Foundation of China (31572580 and 31372491).

**Authors contribution:** YC and ML made contributions to conception and design and draft the manuscript. WL revised critically for important content. TC, QW, XZ and DC did animal husbandry and acquisition of data. JZ and XL WHL did well in analysis and interpretation of data. LG provided technical assistance. YC wrote and modified this manuscript. All authors have participated in study and all declare no conflict of interest.

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