ABSTRACT

Background: Xylazole (Xyl) is a veterinary anesthetic that is structurally and functionally similar to xylazine. However, the effects of Xyl in vitro remain unknown.

Objectives: This study aimed to investigate the anesthetic mechanism of Xyl using fetal rat nerve cells treated with Xyl.

Methods: Fetal rat nerve cells cultured for seven days were treated with 10, 20, 30, and 40 μg/mL Xyl for 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min. Variations of amino acid neurotransmitters (AANTs), Nitric oxide-Cyclic GMP (NO-cGMP) signaling pathway, and ATPase were evaluated.

Results: Xyl decreased the levels of cGMP and NO in nerve cells. Furthermore, Xyl affected the AANT content and Na+-K+-ATPase and Ca2+-Mg2+-ATPase activity in nerve cells. These findings suggested that Xyl inhibited the NO-cGMP signaling pathway in nerve cells in vitro.

Conclusions: This study provided new evidence that the anesthetic and analgesic effects of Xyl are related to the inhibition of the NO-cGMP signaling pathway.

Keywords: Xylazole; anesthesia; cyclic GMP; gamma-aminobutyric acid; glutamic acid

INTRODUCTION

Xylazole [N-(2,6-dimethylphenyl)-1,3-thiazol-2-amine hydrochloride] (Xyl) is an analog of Xylazine that was synthesized by the Chinese Academy of Agricultural Sciences and is widely used in China. Previous studies have shown that Xyl, including Xylazine, is an α2-adrenergic receptor agonist [1,2]. It has obvious sedative, analgesic, and muscle-relaxing effects in a variety of animal models and can be used to produce anesthesia at high doses. Yohimbine and tolazoline, both central α2-adrenergic receptor antagonists, can reverse the effects of Xyl on the central nervous system (CNS) [3-5]. Xyl is a promising α2-adrenergic receptor agonist that can be used as a chemical anesthetic. Gradual elucidation of its mechanism of action will provide useful theoretical guidance for its clinical application, appropriate compatibility, and reduction or avoidance of side effects [2,6].

The Nitric oxide-Cyclic GMP (NO-cGMP) signaling pathway is involved in a variety of physiological activities in the human body, such as diabetes and erectile dysfunction [7].
It is also widely present in the CNS, especially in nerve cells. NO is a recently discovered intracellular messenger that may play a role in information transmission in the CNS [8-11]. It is produced by nitric oxide synthase (NOS), which is catalyzed by L-arginine, and can act as an endogenous activator of soluble guanylyl cyclase (sGC), which then activates sGC to catalyze the generation of cGMP by GTP. As a key component of the NO-cGMP pathway, cGMP can act on cGMP ligand-gated ion channels and exert biological effects by regulating cGMP-dependent protein kinase (PKG) production and phosphodiesterase (PDE) activity, both of which are dependent on the Ca/CaM signal. Moreover, cGMP directly controls the opening of channels via the cyclic nucleotide-gated ion channel [12-14]. Notably, the NO-cGMP signaling pathway plays an important role in a variety of anesthetics [15,16].

According to previous studies, amino acid neurotransmitters (AANTs), which are an important component of central neurotransmitters (NTs), can either activate or inhibit cGMP formation [17-19]. AANTs include excitatory AANTs, such as glutamate (Glu) and aspartate (Asp), as well as inhibitory AANTs, such as γ-aminobutyric acid (GABA), all of which are strongly associated with the mechanism of action of anesthetics [20,21]. Furthermore, AANTs have been linked to brain injury, neurodegeneration, analgesia, learning, and memory. Abnormalities in AANTs can cause excitotoxic reactions, Parkinson’s disease, chorea, epilepsy, and other neurological diseases [22,23].

The available studies on the effects of Xyl evaluated in vivo are relatively concentrated [3,4,6], while few studies reported on the drug’s anesthetic and analgesic mechanisms in vitro. In order to investigate the molecular mechanism of Xyl anesthesia, we cultured nerve cells in vitro to simulate the living environment of nerve cells in vivo and examined the effect of Xyl on the NO-cGMP signaling pathway.

**MATERIALS AND METHODS**

**Animals**

Wistar rats were purchased from the Experimental Animal Center of the Second Affiliated Hospital of Harbin Medical University. All rats were placed in a room with a comfortable temperature (22±3°C) and routinely fed standard rodent chow and water ad libitum. One female and one male Wistar rat of childbearing age were kept together, and the female rats were observed every 12 h. After the appearance of the vaginal plug, the female rats were raised separately, and the time was calculated and used for the experiment on the 17th day.

**Experimental drugs and chemicals**

Xyl was purchased from the Lanzhou Institute of Husbhandry and Pharmaceutical Sciences of Caas, China. DMEM was purchased from Gibco/BRL (USA). FBS was purchased from Invitrogen (USA). Cell Counting Kit-8 (CCK-8) was purchased from APEXBio (USA). The NO, cGMP, Na+-K+-ATPase, and Ca2+-Mg2+-ATPase kits were purchased from Nanjing Jiancheng Bioengineering Institute (China). The Glu and Asp kits were purchased from Sigma (USA).

**Experiment procedure**

Rats were euthanized with CO2, disinfected as part of routine operation, and dissected. Fetal rats were removed from the uterus, followed by the separation of their heads and bodies. The fetal rat brain was extracted as previously described [24]. The brain was removed from the skull, then the cerebral cortex was carefully separated from the hippocampus. The separated
cerebral cortex was collected in order to culture nerve cells. The culture of primary nerve cells was performed according to a previously described method [25]. The cerebral cortex tissue was cut, digested, pipetted, and filtered. After cell counting, $10^{16}$ cells/ml were seeded into Poly-L-lysine solution coated 6-well cell culture plates and 96-well cell culture plates and incubated in a cell culture incubator at 37°C and with 5% CO$_2$. After 48 h, cytarabine was added to the cultures. Half of the medium was replaced twice a week. All animals were kept in conditions that prevented them from experiencing unnecessary pain and discomfort in accordance with the guidelines approved by The Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University (#NEAU-2019-04-0256-12). The number of animals used was minimized, with animal welfare and alleviation of suffering taken into account.

The cells were cultured for 7 days, then immunohistochemistry was performed. After the medium was removed, cells were fixed in 1 mL of 4% paraformaldehyde/sugar mixture for 10 min and placed in 1 mL of 0.1% Triton X-100 for 10 min at room temperature. After being washed twice with PBS, cells were blocked in 10% serum for 1 h. Following that, cells were incubated with mouse anti-MAP-2 antibody (1:300) overnight at 4°C, followed by incubation with the corresponding fluorescence II for 1 h. Glycerol was then mounted and the sample was observed under an inverted microscope.

On day 7 of culture, Xyl was added at concentrations of 10, 20, 30, and 40 μg/mL, with four parallel experiments conducted for each group. Xylazine at a concentration of 40 μg/mL was added as a positive control. Untreated cells were used as a control, with four parallel experiments similarly conducted.

**CCK8 assay**
After Xyl treatment for 120 min, CCK8 reagents (10 μL) were added into 96-well cell culture plates for 2 h. The absorbance at 450 nm was measured using a microplate reader.

**Detection of the NO-cGMP signaling pathway**

For NO: Cells were lysed after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing, and the lysed cell fluid was collected for detection. One EP tube contained 0.5 mL distilled water, another contained 0.4 mL distilled water and 0.1 mL standard, and the third contained 0.4 mL distilled water and 0.1 mL sample. The added reagents and subsequent testing were performed according to the manufacturer’s instructions. After centrifugation at 3,500 rpm, the supernatant from each tube was taken out for a chromogenic reaction and the absorbance at 550 nm was measured.

For cGMP: cGMP was detected using a two-antibody sandwich enzyme-linked immunosorbent assay for biotin. Cells were lysed after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing, and the lysed cell fluid was collected for detection. The sample and standard were added in a blank micropore. The added reagents and subsequent testing were performed according to the manufacturer's instructions. After a chromogenic reaction at 37°C without light for 10 min, termination fluid was added to each micropore and the absorbance at 450 nm was measured.

**Detection of GABA**
The cell supernatant was collected for detection after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing. After filtering through a 0.22 μm filter membrane, 5 μL of the supernatant was taken for sample analysis.
The samples were injected into an Xbridge BEHAmideXP column (2.1×100 mm, 1.7 μm) purchased from Waters (USA) with a temperature of 30°C and a flow rate of 0.25 mL/min. The mobile phase consisted of A [acetonitrile: 5 mmol/L ammonium formate (5:95, v: v)] and B [acetonitrile: 5 mmol/L ammonium formate (15:85, v: v)], with gradient elution. The concentration of mobile phase A decreased from 100% at the beginning to 70% after 3.5 min, then returned to 100% at 5 min. The concentration of mobile phase B increased from 0% at the beginning to 30% after 3.6 min, then returned to 0% at 5 min. After the sample was injected, the external standard method was used to determine the concentration of each substance.

Detection of excitatory Glu and Asp
For Glu: The cell supernatant was collected for detection after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing. The sample and standard were added in a blank micropore. The added reagents and subsequent testing were performed according to the manufacturer's instructions. After a chromogenic reaction at 37°C without light for 10 min, termination fluid was added to each micropore and the absorbance at 450 nm was measured.

For Asp: The cell supernatant was collected for detection after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing. The steps were the same as for the detection of Glu.

Detection of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase
For Na⁺-K⁺-ATPase: Cells were lysed after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing, and the lysed cell fluid was collected for detection. One EP tube contained 0.16 mL distilled water and 0.1 mL standard, and the other contained 0.12 mL distilled water, 0.1 mL sample, and 0.04 mL reagent ten. The added reagents and subsequent testing were performed according to the manufacturer's instructions. After being allowed to react at room temperature for 5 min, the absorbance at 636 nm was measured.

For Ca²⁺-Mg²⁺-ATPase: Cells were lysed after 0, 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min of dosing, and the lysed cell fluid was collected for detection. One EP tube contained 0.16 mL distilled water and 0.1 mL standard, and the other contained 0.1 mL sample, 0.08 mL reagent eight, and 0.08 mL reagent nine. The other steps were the same as for the detection of Na⁺-K⁺-ATPase.

Statistical analysis
All experimental data were presented as the mean ± SEM and were statistically analyzed using GraphPad Prism 5.1 Software (USA). One-way ANOVA and Tukey's post hoc pairwise comparison were used for statistical analyses. p < 0.05 was considered statistically significant.

RESULTS

Nerve cell culture and identification
After three hours of inoculation and culture, most of the nerve cells had adhered to the plate, appearing round and transparent, small, and evenly distributed with some protrusions (Fig. 1A). The number of cells and protrusions increased after three days. The nerve cell bodies took on a pyramidal, oval, or spindle shape, and the protrusions became slender and branched. At this point, most of the cytoplasm had a high refractive index, the background cells were flat, small pieces were distributed at the bottom of the dish, and the protrusions had formed a network (Fig. 1B). After seven days, the primary culture was enriched with
neurons that had clear protrusions, good morphology, and large and clear nuclei (Fig. 1C). A comparison of fluorescent staining in the same field of view is shown in Fig. 1D.

Effects of different Xyl concentrations on fetal rat nerve cells viability
As shown in Fig. 2, Xyl treatment at 10, 20, 30, and 40 μg/mL for 120 min did not have any significant effects on nerve cell viability ($p > 0.05$).

Effects of different Xyl concentrations on the NO-cGMP signaling pathway
The NO and cGMP detection results suggested that Xyl inhibited the NO-cGMP signaling pathway (Fig. 3). The changes in NO content are shown in Fig. 3A-E. In nerve cells treated with 10 and 30 μg/mL Xyl, the content of NO at 5 min was significantly different compared to the control group ($p < 0.05$). The NO content in the 20 μg/mL Xyl group began to significantly decrease at 15 min ($p < 0.01$), while there was a significant decrease in the 40 μg/mL Xyl group after 30 min ($p < 0.01$). After reaching the lowest value, the NO content in each group began to rise rapidly and this upward trend continued for 120 min. The change in NO content in each group was similar to the trend in the xylosine group.
Fig. 3. Effects of Xyl on the NO-cGMP signaling pathway. (A-E) The NO content; (F-J) The cGMP content. Values were expressed as mean ± SEM.

Xyl, xylazole.

* $p < 0.05$, ** $p < 0.01$ vs. control group; * $p < 0.05$, ** $p < 0.01$ vs. xylazine group.
The effect of different Xyl concentrations on cGMP in nerve cells is presented in Fig. 3F-J. The cGMP content in each group began to significantly decrease at 5 min ($p < 0.01$). After reaching the lowest value, the cGMP content in each group began to rise slowly, and this increase continued for 120 min. The general trend of cGMP content was similar between each group and the xylazine group.

**Effects of different Xyl concentrations on AANTs**

Next, we evaluated the changes in AANT content to validate whether the inhibition of the NO-cGMP signaling pathway was responsible for the changes (Fig. 4). The GABA content showed an overall trend of increasing and subsequently decreasing (Fig. 4A-E). Furthermore, the GABA content in the 30 and 40 $\mu$g/mL Xyl groups increased significantly at 5 min ($p < 0.05$), while the 10 and 20 $\mu$g/mL Xyl groups increased significantly at 20 min ($p < 0.05$). The GABA content in each group began to steadily decline after reaching their respective peaks and stabilized after 90 min. The LC-MS/MS analysis chromatogram of GABA was shown in Supplementary Fig. 1.

There was a clear downward trend in Glu content (Fig. 4F-J). At 25 min, the Glu content in the 20 $\mu$g/mL Xyl group was at its lowest ($p < 0.01$). The other three groups also exhibited a slight decline ($p > 0.05$).

As shown in Fig. 4K-O, the Asp content first decreased, then increased. The Asp content in each group gradually decreased for 30 min. At 25 min, the contents in the 30 and 40 $\mu$g/mL Xyl groups were significantly lower than the control group ($p < 0.01$). After reaching the lowest point, the Asp content in each group began to gradually rise at 45 min and stabilized after 90 min.

**Effects of different Xyl concentrations on Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase**

The activities of Na⁺-K⁺-ATPase and Ca²⁺-Mg²⁺-ATPase were investigated to determine whether they were targets for Xyl for the production of anesthesia and analgesia (Fig. 5). The activity of Na⁺-K⁺-ATPase decreased first before increasing (Fig. 5A-E). At 5 min, the Na⁺-K⁺-ATPase activity of each group began to decrease, and treatment with 30 $\mu$g/mL Xyl significantly decreased it ($p < 0.05$). At 10 min, the Na⁺-K⁺-ATPase activity in the other three groups began to decrease rapidly ($p < 0.05$). After reaching the lowest value, the Na⁺-K⁺-ATPase activity in each group began to gradually rise, and the rate was flattened after 60 min.

As shown in Fig. 5F-J, the Ca²⁺-Mg²⁺-ATPase activity showed a significantly decreasing trend. At 5 min, the activity of Ca²⁺-Mg²⁺-ATPase in nerve cells treated with 40 $\mu$g/mL Xyl was significantly different compared to the control group ($p < 0.05$). The 10, 20, and 30 $\mu$g/mL Xyl groups showed a significant decrease after 15 min ($p < 0.01$). After 60 min, the rate in all groups tended to flatten.

**DISCUSSION**

Xyl not only plays an important role in anesthesia and analgesia, but it also decreases Ca²⁺ flow and the production of NO and cGMP. In this study, the content of cGMP reached its lowest level within 25 min after treatment with different concentrations of Xyl, whereas NO content was significantly lower than the control group within 30 min. At 120 min, although the contents of NO and cGMP increased in nerve cells, they were still lower than the control
group. This suggested that anesthesia was more likely to inhibit NO and cGMP after the anesthetic has been removed for 120 min.

Previous studies have shown that the simultaneous inhibition of NO synthesis and cGMP production can inhibit cGMP-mediated neuromodulation [26]. As an intracellular second messenger, NO can activate sGC and increase the content of cGMP in cells [27]. cGMP is a key component of the NO-cGMP signaling pathway, which exerts biological functions by

Fig. 4. Effects of Xyl on the AANTs. (A-E) The GABA content; (F-J) The Glu content; (K-O) The Asp content. Values were expressed as mean ± SEM. Xyl, xylazole; GABA, γ-aminobutyric acid; AANT, amino acid neurotransmitter.
* \( p < 0.05 \), ** \( p < 0.01 \) vs. control group.

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Fig. 5. Effects of Xyl on the ATPase. (A-E) The Na⁺-K⁺-ATPase activity; (F-J) The Ca²⁺-Mg²⁺-ATPase activity. Values were expressed as mean ± SEM.
Xyl, xylazole.
*p < 0.05, **p < 0.01 vs. control group.
regulating the activity of PDE and the production of PKG [12-14]. We previously reported that the effect of xylazine anesthesia may be related to the inhibition of the NO/cGMP signal transduction pathway in each encephalic region of goats [16]. In this study, although the inhibitory effect of Xyl on NO was not as strong as xylazine, it exerted a better inhibitory effect on cGMP compared to xylazine. The animal body is complex, and the anesthetic effect involves multiple pathways. Further in vivo experiments are required to compare the anesthetic effects of Xyl and xylazine.

In this study, nerve cells treated with different concentrations of Xyl showed a significant decrease in NO content and cGMP concentration. General anesthetics can either activate or inhibit the NO-cGMP signaling pathway by activating N-methyl-D-aspartic acid (NMDA) receptors, acetylcholine-activated muscarinic (M) receptor-mediated excitatory pathways, α2-adrenergic receptors, and the GABA receptor-mediated inhibitory pathway, thereby changing the cGMP content in nerve cells [28]. Therefore, inhibiting excitatory pathways or enhancing inhibitory pathways in the CNS can decrease the cGMP content in nerve cells through the NO-cGMP system. As an α2-adrenergic receptors agonist, Xyl can activate the α2-adrenergic receptor to inhibit the NO-cGMP signaling pathway, thereby changing the content of cGMP [2,28]. Hsu and colleagues showed that yohimbine and tolazoline, both α2-adrenergic receptor antagonists, exerted an antagonistic effect on Xyl-induced hypertension [4], and the occurrence of hypertension has been linked to an increase in cGMP content [29]. This could be due to the decreased binding of Xyl and α2-adrenergic receptors, resulting in a weakened inhibitory effect on the NO-cGMP signaling pathway. However, further molecular mechanisms are required to validate it. In this study, the activation of α2-adrenergic receptors by Xyl could be a reason for the reduction in cGMP content, but this has yet to be verified. Sagi and colleagues previously discovered that the activation of GABA receptors inhibited the NO-cGMP signaling pathway [19]. In order to understand the potential relationship between GABA receptors and NO-cGMP in nerve cells treated with Xyl, we examined the changes in inhibitory AANT and GABA content in nerve cells. Similarly, the administration of Xyl increased the content of GABA to activate GABA receptors, resulting in a decrease in the cGMP content in nerve cells.

In contrast to the inhibitory effect of α2-adrenergic receptors and GABA receptors on NO-cGMP signaling, activation of NMDA receptors promotes NO-cGMP signaling. Previous research has shown that anesthetics can inhibit the combination of Glu and NMDA receptors while decreasing the presynaptic release of Glu. This leads to an increase in its uptake and a decrease in Glu concentration in the synaptic cleft, producing anesthetic effects [30-33].

In this study, treatment with different concentrations of Xyl caused a significant decrease in Glu content. This was potentially due to Xyl inhibiting the combination of Glu and NMDA receptors, thereby inhibiting the NO-cGMP signaling pathway. In addition, further findings revealed a positive correlation between the changes in Glu and Asp content, as well as the synthesis of Asp from Glu through transamination. Therefore, we hypothesize that the anesthetic effect of Xyl may also be related to the decrease in Asp synthesis and release, as well as the decrease in neuronal excitability.

The NMDA receptor is an excitatory amino acid receptor abundant in the CNS that can change the Na+ and K+ levels inside and outside the membrane, enabling the synapse to produce a slow excitatory potential. After activation, the influx of Ca2+ can interact with CaM and bind to the binding site on NOS, activating NOS to generate NO. Meanwhile, the Ca2+-CaM complex can activate ATPase, pump free Ca2+ out of the cell, and promote the transportation of free Ca2+ into the endoplasmic reticulum and mitochondria for storage,
thereby maintaining low intracellular Ca\textsuperscript{2+} homeostasis [34]. General anesthetics have been shown to effectively inhibit the activity of Na\textsuperscript{+}-K\textsuperscript{-}ATPase and Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase [35,36]. In this study, different concentrations of Xyl significantly decreased the activity of Na\textsuperscript{+}-K\textsuperscript{-}ATPase and Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase. We hypothesize that Na\textsuperscript{+}-K\textsuperscript{-}ATPase and Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase are also targets of Xyl in the production of anesthesia and analgesia, but further studies are required to verify this.

These results suggested that the anesthetic and analgesic effects of Xyl may be produced by activating \(\alpha\)-adrenergic receptors and GABA receptors, while inhibiting NMDA receptors, leading to the inhibition of the NO-cGMP signaling pathway. In addition, Hammond [17] and Pin and Duvoisin [18] reported that excitatory AANTs activated metabotropic Glu receptors and modulated neurotransmission through the regulation of second messenger systems, including inhibiting adenylyl cyclase and potentiating cAMP formation and cGMP formation. However, many factors can influence the anesthetic effect of Xyl. In this study, only the effect of Xyl on the NO-cGMP signaling pathway was investigated. Further studies are required to determine which specific subtypes of \(\alpha\)-adrenergic receptors, GABA receptors, and NMDA receptors interact with Xyl to inhibit the NO-cGMP signaling pathway in nerve cells.

In conclusion, Xyl can inhibit the NO-cGMP signaling pathway in nerve cells in vitro. Xyl decreased the production of excitatory NTs Asp and Glu while increasing the production of inhibitory NT GABA in nerve cells. Furthermore, Xyl can inhibit the activity of Na\textsuperscript{+}-K\textsuperscript{-}ATPase and Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase in nerve cells.

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**SUPPLEMENTARY MATERIAL**

Supplementary Fig. 1

LC-MS/MS analysis chromatogram of GABA. (A) Standard; (B) Sample.

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