S-adenosyl methionine regulates calcium channels and inhibits uterine smooth muscle contraction in rats with infectious premature delivery through the transient receptor protein 3/protein kinase Cβ/C-kinase-activated protein phosphatase-1 inhibitor of 17 kDa signaling pathway

JING GE[1*], TAO HAN[2*], XIAOQIU LI[3*], LILI SHAN[1], JINHUAN ZHANG[1], YAN HONG[1], YANQIU XIA[1], JUN WANG[1] and MINGXIAO HOU[4]

Departments of [1]Maternity, [2]Oncology, [3]Neurology and [4]Cardiothoracic Surgery, General Hospital of Shenyang Military Area Command, Shenyang, Liaoning 110016, P.R. China

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Abstract. The aim of the present study was to investigate the effects of S-adenosyl methionine (SAMe) on infectious premature inflammatory factors and uterine contraction, and to further explore its mechanism of action via the transient receptor protein 3 (TRPC3)/protein kinase Cβ (PKCβ)/C-kinase-activated protein phosphatase-1 inhibitor of 17 kDa (CPI-17) signaling pathway, following intervention by a TRPC3 inhibitor. A rat model of premature delivery induced by lipopolysaccharide (LPS) was established. Following treatment with SAMe and inhibiting TRPC3 expression, rat serum and uterus were isolated. Hematoxylin and eosin staining was used to observe the histopathological changes in the uterus. Uterine muscle strips *in vitro* were selected to measure the changes in muscle tension. ELISA was utilized to measure the changes in serum inflammatory factor and oxidative stress indexes. Immunohistochemistry, western blot assay and reverse transcription-quantitative polymerase chain reaction were applied to detect calcium channel protein expression in the uterus. Western blot analysis was employed to measure the expression of TRPC3/PKCβ/CPI-17 signaling pathway-related proteins. TRPC3 was highly expressed in the uterus of rat models of premature delivery induced by LPS. Following treatment with SAMe, inflammatory cell infiltration markedly reduced in the uterus and the tension of *in vitro* uterine muscle strips significantly decreased. SAMe treatment suppressed inflammatory reaction and oxidative stress, and diminished L-type and T-type calcium channel protein expression. TRPC3/PKCβ/CPI-17 signaling pathway-related protein expression was also reduced. When TRPC3 expression was suppressed, the effects of SAMe against inflammation and oxidative stress were diminished. TRPC3/PKCβ/CPI-17 signaling pathway-related protein expression significantly increased. SAMe was able to reduce inflammatory reaction and oxidative stress in the uterus of rat model of infectious premature delivery induced by LPS, prolong delivery time, reduce the mortality rate of offspring rats, and serve a therapeutic role. This effect is likely achieved via the regulation of uterine contractions and childbirth through the TRPC3/PKCβ/CPI-17 signaling pathway.

Correspondence to: Dr Jun Wang, Department of Maternity, General Hospital of Shenyang Military Area Command, 83 Wenhua Road, Shenyang, Liaoning 110016, P.R. China
E-mail: wj202fck@163.com

Dr Mingxiao Hou, Department of Cardiothoracic Surgery, General Hospital of Shenyang Military Area Command, 83 Wenhua Road, Shenyang, Liaoning 110016, P.R. China
E-mail: houmingxiao188@163.com

*Contributed equally

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Introduction

Premature delivery is a common complication of pregnancy. The World Health Organization reported in 2013 that ~15 million premature babies are born every year worldwide (1). Each year, >1 million premature babies succumb to premature complications, and many surviving children also suffer from defects in the nervous system, respiratory tract, and digestive tract (2). It has previously been demonstrated that infection is the most prevalent cause of premature delivery, accounting for ~40% (3). The placenta is the intermediary between the mother and the fetus, and has an important barrier effect on the mother-fetus interface. Once bacterial endotoxin lipopolysaccharide (LPS) enters the organism, it can cause placental infection, and a number of pathological changes occur in the placenta (4). Under normal conditions, extracellular Ca^{2+} primarily flows through calcium channels in the cell membrane, triggering the release of Ca^{2+} via

calcium release channels in the sarcoplasmic reticulum (5-7). Thus, increased intracellular Ca^{2+} concentrations may result in uterine contraction and premature delivery. Taken together, calcium channels serve an important role in regulating smooth muscle activity. Pyr3 is a transient receptor protein 3 (TRPC3) channel blocker, which potentiates dexamethasone sensitivity and apoptosis in acute lymphoblastic leukemia cells by disturbing $Ca2^{+}$ signaling, mitochondrial membrane potential changes and reactive oxygen species production (8). TRPC is a non-selective cation channel composed of four monomers with six transmembrane helices, participates in many pathophysiological processes, and can transport sodium, calcium and magnesium ions (9,10). When cellular calcium stores are depleted, the TRPC channel on the cell membrane is opened, resulting in the influx of extracellular Ca^{2+} and calcium stores are restored. This Ca^{2+} influx is known as store-operated calcium entry (11). Protein kinase Cβ (PKCβ)/C-kinase-activated protein phosphatase-1 inhibitor of 17 kDa (CPI-17) is a Ca^{2+}-activated phospholipid-dependent protein kinase that regulates different cell functions, such as gene expression, cell proliferation, apoptosis and cell migration (12,13). Myosin is a basic constituent of a thick filament and is a hexamer of macromolecular proteins consisting of two myosin heavy chains and four myosin light chains (MLC) (14). Ozaki *et al* (15) previously demonstrated that a PKC agonist could significantly increase the phosphorylation of MLC and the contraction of vascular smooth muscle. However, PKC agonists cannot increase the phosphorylation level of MLC20 and the contractile function of vascular smooth muscle (16). PKCβ/CPI-17 serves a critical role in the calcium sensitization mechanism of smooth muscle (15).

As an organic nutrient, if amino acids can be used as therapeutic agents, it is relatively safe and reliable, has no obvious side effects, and has no adverse effects on fetal development, intelligence and heredity (17). Therefore, the use of amino acids is of great clinical value. It has previously been demonstrated that S-adenosyl methionine (SAMe) is an important metabolic intermediate in methionine, a methyl donor for the catalytic reaction of >100 different methyltransferases *in vivo*, and serves an important role in the metabolism of living cells (18). At present, SAMe is used in clinic settings for intrahepatic cholestasis and fatty liver in pregnancy, effectively improves the clinical symptoms and outcomes of pregnant women, and reduces the rate of premature delivery (19). Furthermore, SAMe is also a prescription drug for arthritis, has few side effects and has exhibited greater efficacy than conventional clinical drugs, such as indomethacin (20,21). Recent studies also demonstrated that SAMe has antidepressant and anti-tumor effects (22,23).

The present study sought to establish rat models of premature delivery induced by LPS, to investigate the effects of SAMe on infectious premature inflammatory factors and uterine contraction, and to further explore the underlying mechanism of the TRPC3/PKCβ/CPI-17 signaling pathway following intervention with a TRPC3 inhibitor.

Materials and methods

Animals and ethical approval. A total of 45 female and 30 male specific-pathogen-free Sprague-Dawley rats (weight, 220-260 g; age, 7 weeks) were provided by the Experimental Animal Center of the General Hospital of Shenyang Military Area Command [Shenyang, China; license no. SCXK (JUN) 2012-0002]. Female and male rats were placed in the same cage overnight at a ratio of 2:1 (3 rats per cage). Animals were housed at a constant temperature (22±1˚C) with 50% humidity in a 12 h light/dark cycle. The rats had access to food and autoclaved water *ad libitum*. The next morning, the detection of sperm on the vaginal plug or vaginal smear was considered as the day 0 of pregnancy. All animal procedures were approved by the Animal Experiments Ethics Committee of the General Hospital of Shenyang Military Area Command. Pregnant rats were randomly assigned to the control group (n=9), LPS group (model of infectious premature delivery; n=12), SAMe group (SAMe + LPS; n=12), and Pyr3 group (Pyr3 + SAMe + LPS; n=12).

Preparation of rat models of infectious premature delivery and sample collection. LPS salt solution (100 μg/kg; cat. no. L8880; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China; saline solution was used as a solvent) was intraperitoneally administered to pregnant rats in the LPS group at day 15 of pregnancy. In the SAMe group, 30 mg/kg SAMe (physiological saline was used as the solvent; cat. no. S9990; Beijing Solarbio Science & Technology Co., Ltd.) was intraperitoneally injected at 0.5 h following LPS injection. In the Pyr3 group, 30 mg/kg Pyr (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was intraperitoneally administered at 30 min following SAMe injection. In the control group, an equal volume of physiological saline was intraperitoneally administered concurrently with LPS administration in the other groups. The occurrence of premature delivery (<19 days of pregnancy) was recorded. Following premature delivery, rats were anesthetized with 2% sodium pentobarbital (40 mg/kg; Beijing Huaye Huanyu Chemical Co., Ltd., Beijing, China). Arterial blood was collected and ~5 ml serum was isolated by centrifugation at 1,000 x g for 5 min at 4˚C. The blood plasma was isolated, packed separately and stored at -80˚C for ELISA. Rats were subsequently sacrificed via anesthesia overdose with 2% sodium pentobarbital (120 mg/kg). The route of sodium pentobarbital administration for anesthesia and sacrifice was intraperitoneal injection. The uterus and placenta was harvested and adipose tissue was removed. Blood was washed away with physiological saline. A random section of each sample was fixed in 10% paraformaldehyde at room temperature for 48 h and the other was stored in liquid nitrogen.

Determination of latency and live birth rate in each group. Following the evaluation of premature delivery in each group, latency (the time between LPS administration and the first fetus delivery) and live birth rate in each group were recorded.

Determination of muscle tension of uterine muscle strips of pregnant rats. Uterus tissue samples (~1 cm longitudinal direction) were washed with physiological saline, fixed on the specimen hook of a multi-channel physiological signal acquisition system, and placed in a tank filled with 8 ml Tyrode's solution. The sample was also connected to a tension converter to record uterine contraction.

Hematoxylin and eosin (HE) staining. Rat uterus samples were dehydrated with a graded alcohol series, permeabilized with xylene, immersed and embedded in paraffin, and cut into 5-μm-thick sections. Sections were stained with hematoxylin for 5 min at room temperature, washed with PBS, differentiated with hydrochloric acid ethanol for 3 sec, stained with eosin for 1 min at room temperature and mounted with neutral resin. Changes in rat uterine tissue were observed under light microscopy (magnification, x12.6).

Immunohistochemistry. Paraffin-embedded 5-μm-thick sections were placed in a 67˚C oven for 2 h, dewaxed, hydrated in a descending alcohol series and treated with citrate buffer (pH, 6.0; cat. no. C1010; Beijing Solarbio Science & Technology Co., Ltd.) at 100˚C for 10 min. Antigen retrieval was performed via microwave heating at 100˚C. Each section was treated with ~30 μl 3% hydrogen peroxide aqueous solution (cat. no. 10011208; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) at room temperature for 10 min, and blocked with 5% bovine serum albumin for 45 min at room temperature. After abundant liquid was discarded, sections were incubated with antibodies against TRPC3 (ab51560; rabbit; 97 kDa; 1:500; Abcam, Cambridge, UK); calcium channel, voltage-dependent, L type, α 1C subunit (Cav1.2; ab58552; rabbit; 249 kDa; 1:1,000; Abcam); calcium channel, voltage-dependent, T type, α 1G subunit (Cav3.1; ab95092; rabbit; 262 kDa; 1:1,000; Abcam); and calcium channel, voltage-dependent, T type, α 1H subunit (Cav3.2; ab95092; rabbit; 262 kDa; 1:1,000 Abcam) at 4˚C overnight. Sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-goat immunoglobulin G secondary antibodies (1:1,000; cat. no. ab6721; Abcam) for 2 h at room temperature, washed with PBS, visualized with 3,3'-diaminobenzidine, counterstained with hematoxylin for 2 min at room temperature, dehydrated through a graded alcohol series, permeabilized with xylene, mounted with neutral resin and observed using a light microscope (magnification, x25.2).

ELISA. The following ELISA kits were used to analyze rat serum samples, according to the manufacturers' protocols: Interleukin (IL)-1β (SEA563Ra; Uscn Life Sciences, Inc., Wuhan, China), IL-8 (H008; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), tumor necrosis factor α (TNF-α; SEA133Ra; Uscn Life Sciences, Inc.), IL-10 (SEA056Ra; Uscn Life Sciences, Inc.), malondialdehyde (MDA; A003-2; Nanjing Jiancheng Bioengineering Institute), superoxide dismutase (SOD; A001-3; Nanjing Jiancheng Bioengineering Institute) and nitric oxide (NO; A013-2; Nanjing Jiancheng Bioengineering Institute). Blank wells, standard wells and detected wells were used. Blank wells contained 100 μl PBS; the other wells contained 100 μl standard preparation (provided with the kits) or rat serum samples. All were incubated at 37˚C for 2 h. Following removal of the liquid, the plate was washed three times with the reagent provided by the kits. HRP-conjugated fluid (100 μl) was added to each well at 37˚C for 1 h. Following removal of the liquid, sections were dried, and 100 μl substrate solution was added to each well and incubated at 37˚C in the dark for ~15 min. Stop buffer (50 μl) was subsequently added to each well. Optical density values were measured in each well at 450 nm using a microplate reader.

Western blot analysis. Rat placenta was lysed in radioimmunoprecipitation assay lysate containing protease inhibitor (cat. no. R0010; Beijing Solarbio Science & Technology Co., Ltd.) on ice for 30 min. Following centrifugation at 4,000 x g for 20 min at 4˚C, supernatant was collected. Proteins were quantified using a Bicinchoninc Acid Assay Protein Quantification kit. The protein samples (30 μg/lane) were subjected to 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. The membrane was blocked with 5% skimmed milk for 2 h at room temperature, and incubated with antibodies against Cav1.2 (1:1,000), Cav3.1 (1:1,000), Cav3.2 (1:1,000), PKCβ (ab227490; rabbit; 76 kDa; 1:1,000; Abcam) CPI-17 (ab32213; rabbit; 17 kDa; 1:1,000; Abcam), phosphorylated (p-)CPI-17 (ab52174; rabbit; 17 kDa; 1:1,000; Abcam) and GAPDH (ab181602; rabbit; 36kDa; 1:10,000; Abcam) at 4˚C overnight. Subsequently, the membrane was washed with TBST, and incubated with HRP-conjugated secondary antibody (1:2,000; cat. no. ab6721; Abcam) at room temperature for 2 h. Proteins were visualized using an enhanced chemiluminescence kit and gel imaging system. Absorbance values were analyzed using Image Tools (Image J 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Rat uterus samples were triturated and TRIzol reagent (15596018; Life Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to extract RNA, according to the manufacturer's protocol. RNA was reverse-transcribed into cDNA using a High-Capacity RNA-to-cDNA kit (4387406; Invitrogen; thermo Fisher Scientific, Inc.). qPCR was performed using a QuantiFast SYBR Green PCR kit (204057; Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol. Relative gene expression data was quantified using the $2^{-\Delta\Delta Cq}$ method (24). GAPDH was used as the reference gene. Primers used for qPCR are presented in Table I.

Statistical analysis. All data were statistically analyzed using SPSS 19.0 (IBM Corp., Armonk, NY, USA). Data are presented as the mean + standard deviation. One-way analysis of variance followed by Tukey's post-hoc test was used for comparison among groups. All experiments were repeated in triplicate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Therapeutic effect of SAMe on infectious premature delivery. The effect of SAMe on rats with infectious premature delivery was assessed as latency and live birth rate (Fig. 1A and B). In the control group, latency time was ~70.4 h, or ~19 days of pregnancy, with a live birth rate of 96%. In the LPS group, the fetus was delivered within 24 h, ~15 h, with a live birth rate of 4.3%. These results indicated the model of infectious premature delivery was established successfully. In the SAMe group the latency time was 59.7 h and the live birth rate was 72.5%. However, the therapeutic effect of SAMe was significantly reduced following addition of Pyr3. Compared with the LPS group, latency was prolonged by ~28.9 h, and the live birth rate was 43.5%.

Table I. Gene primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequence (5'-3')
Cav1.2	Forward: CGGATCTGGAAGCTCGGAT
	Reverse: GTGATTGCGGAGCCCGA
Cav3.1	Forward: TTAAGAGCTACCTGATCGAG
	Reverse: TGTATCCGCACCTTCTGCA
Cav3.2	Forward: GCCATTCTCTCCTTCCTGCA
	Reverse: CGCAGCAGCAAATTTATG
GAPDH	Forward: GCATGATGCCGGCAGCTTT
	Reverse: CAGCAACTGAATGAGGCCA

Cav1.2, calcium channel, voltage-dependent, L type, α 1C subunit; Cav3.1, calcium channel, voltage-dependent, T type, α 1G subunit; Cav3.2, calcium channel, voltage-dependent, T type, α 1H subunit.

SAMe suppresses inflammatory factors IL-1β, IL-8, TNF-a, IL-10, MDA, SOD and NO serum content. To verify the effect of SAMe against inflammatory reaction and oxidative stress, rat serum was collected for ELISA (Fig. 2A-D). Results demonstrated that IL-1β, IL-8 and TNF-α expression was significantly increased in the LPS group (P<0.05 vs. control), whereas IL-10 expression was significantly reduced (P<0.05 vs. control). Following treatment with SAMe, IL-1β, IL-8 and TNF-α expression was significantly diminished (P<0.05 vs. LPS), whereas IL-10 expression significantly increased (P<0.05 vs. LPS). Following treatment with Pyr3, the anti-inflammatory effect of SAMe was significantly reduced; IL-1β, IL-8 and TNF-α expression significantly increased (P<0.05 vs. SAMe) and IL-10 expression significantly decreased (P<0.05 vs. SAMe). These data suggest that SAMe treatment significantly reduces the inflammatory reaction induced by LPS.

Further detection of serum oxidative stress-related factors (Fig. 2E-G) revealed that MDA and NO expression was significantly increased in the LPS group (P<0.05 vs. control), and SOD expression was significantly diminished (P<0.05 vs. control). Following SAMe treatment, MDA and NO expression was significantly reduced (P<0.05 vs. LPS), and SOD expression was significantly increased (P<0.05 vs. LPS). Following treatment with Pyr3, MDA and NO expression was significantly increased (P<0.05 vs. SAMe), while SOD expression was significantly reduced (P<0.05 vs. SAMe). These data indicate that SAMe can significantly inhibit LPS-induced inflammatory reaction and oxidative stress. Following inhibiting TRPC3, the effect of SAMe was significantly weakened. These findings suggest that SAMe exerted its effect through the TRPC3 signaling pathway.

SAMe improves muscle tension of the uterus. H&E staining was utilized to observe the pathological changes in rat uterus (Fig. 3A). Results indicated that following LPS-induced premature delivery, in the LPS group, a large number of inflammatory cells infiltrated into the smooth muscle layer of uterus in rats, and cells were irregularly distributed. Following SAMe treatment, this inflammatory cell infiltration was markedly reduced. Following suppressing TRPC3 expression with Pyr3, inflammatory cell infiltration markedly increased in the smooth muscle layer of rat uterus.

Muscle tension of the rat uterus (Fig. 3B) indicated that following LPS-induced premature delivery, muscle tension of *in vitro* uterine muscle strips significantly increased (P<0.05 vs. control). The muscle tension in the SAMe group significantly diminished (P<0.05 vs. LPS). Following suppressing TRPC3, muscle tension significantly increased (P<0.05 vs. SAMe).

SAMe reduces the expression of calcium channel in rats with infectious premature delivery. Western blot analysis (Fig. 4A) was used to measure Cav1.2, Cav3.1 and Cav3.2 expression in the rat uterus. Data suggested that Cav1.2, Cav3.1 and Cav3.2 expression increased in rats with premature delivery induced by LPS (all, P<0.05 vs. control). Following treatment with SAMe, Cav1.2, Cav3.1 and Cav3.2 expression significantly decreased (all, P<0.05 vs. LPS). Following inhibition of TRPC3, Cav1.2, Cav3.1 and Cav3.2 expression significantly increased (all, P<0.05 vs. SAMe). RT-qPCR (Fig. 4B) results were consistent with western blot assay results. Immunohistochemical analysis (Fig. 4C) indicated that compared with the control group, Cav1.2, Cav3.1 and Cav3.2 expression notably increased in rats with premature delivery induced by LPS. Following treatment with SAMe, Cav1.2, Cav3.1 and Cav3.2 expression significantly decreased compared with the LPS group. Following inhibition of TRPC3, Cav1.2, Cav3.1 and Cav3.2 expression significantly increased compared with the SAMe group. Immunostaining was indicated with a blue color. These findings suggest that L-type calcium channels and T-type calcium channels participate in the occurrence of premature delivery. SAMe can effectively weaken the expression of L-type and T-type calcium channel-related proteins and uterine contraction.

SAMe promotes uterine contraction and delivery via the TRPC3/PKCβ/CPI-17 signaling pathway. Western blot analysis was utilized to measure the expression of TRPC3/PKCβ/CPI-17 signaling pathway-related proteins in the rat uterus (Fig. 5). Data suggested that following premature delivery induced by LPS, PKCβ and p-CPI-17 expression significantly increased (P<0.05 vs. control). Following treatment with SAMe, PKCβ and p-CPI-17 expression significantly diminished (P<0.05 vs. LPS). These data indicate that the TRPC3/PKCβ/CPI-17 signaling pathway participated in the occurrence and development of premature delivery. Following the application of Pyr3, inflammatory factor expression increased, and PKCβ and p-CPI-17 expression significantly increased (P<0.05 vs. SAMe). These results indicate that the anti-inflammatory effect of SAMe is exerted via the TRPC3/PKCβ/CPI-17 signaling pathway.

Discussion

Infection has been demonstrated to be associated with premature delivery. Histological evidence of intrauterine infection is exhibited in 19-74% preterm infants (25). At present, it is believed that infectious preterm delivery is associated with Gram-negative bacteria endotoxin LPS that stimulates an increase in local cytokines, and subsequently stimulates

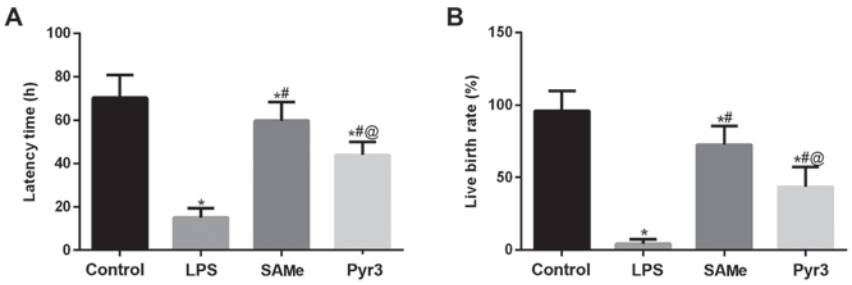

Figure 1. Therapeutic effect of SAMe on infectious premature delivery. Following establishing the infectious premature delivery models in rats, (A) latency and (B) live birth rate were observed. *P<0.05 vs. control group; #P<0.05 vs. LPS group; @P<0.05 vs. SAMe group. SAMe, S-adenosyl methionine; LPS, lipopolysaccharide. Data are provided as the mean ± standard deviation (n=45).

abnormal uterine contraction (26). In the present study, pregnant rat models of premature delivery were successfully established via LPS administration. SAMe treatment revealed that SAMe could reduce the rate of premature delivery, elevate survival rate of fetal rats, improve muscle tension, and increase calcium-specific protein expression, indicating that SAMe has a therapeutic effect on infectious premature delivery. Administration of the TRPC3 protein inhibitor Pyr3 demonstrated that the therapeutic effect of SAMe was suppressed, and that the TRPC3/PKCβ/CPI-17 signaling pathway has an important regulatory effect.

SAMe is a physiologically active substance found in all tissues and fluids, and is converted from methionine under the action of adenosylmethionase (18). As a methyl donor and a physiological sulfur compound, SAMe can participate in important biochemical reactions in the human body and transform thioxo into taurine (18). Previous studies have also verified that SAMe could regulate IL-10 production in monocytes, and increase IL-6 synthesis in monocytes and liver Kupffer cells following LPS stimulation (27,28). Its metabolite, 5-methylthioadenosine, has an important immunomodulatory effect on the inflammatory response of hepatocytes (29). Simultaneously, SAMe is able to reduce serum TNF-α and TGF-β contents (30,31). In the present study, SAMe treatment increased IL-1β, IL-8, TNF-α and IL-10 contents in rats with infectious premature delivery, and had an inhibitory effect on oxidative stress factors. These findings indicated that SAMe could suppress inflammatory reaction and the resulting stress response in rats with infectious premature delivery.

The mechanism of infectious premature delivery is complex and is the result of a number of factors. There are many theories about the causes of premature childbirth, including neurotransmitter theory, mechanical theory, hormone control theory and immunity theory (32-34). These theories share a common final path; the induction of the contraction of uterine smooth muscle (35). The contraction of the uterus is determined by the concentration of free calcium in the muscle cells (36). Prior to uterine contraction, cytoplasmatic free calcium concentration increases significantly. Mark *et al* (37) recently presented a mechanism of intracellular calcium signaling that leads to cyclic uterine contractions. Normally, extracellular calcium ions flow through the calcium channel of the cell membrane, triggering Ca^{2+} release from the sarcoplasmic reticulum calcium release channel. Therefore, elevated intracellular calcium concentration results in uterine contraction and premature delivery. It can be observed that the calcium channel serves an important role in regulating smooth muscle activity. The present study detected the expression of L-type calcium channel Cav1.2 and T-type calcium channel Cav3.1 and Cav3.2 expression in the rat uterus (38,39). Results indicated that each protein expression increased in premature delivery in rats of the LPS group, and confirmed that L-type and T-type calcium channels participated in the occurrence of premature delivery. The addition of SAMe revealed that calcium channel protein expression decreased in each group, and muscle tension test verified this conclusion. It is thus clear that SAMe could effectively weaken L-type and T-type calcium channel-related protein expression, and weaken uterine contraction.

TRPC channel has an extensive physiological role in addition to mediating extracellular calcium influx and inducing uterine contraction, which has been confirmed in previous studies of the nervous system, vascular smooth muscle, myocardium, and skin (40-43). A variety of physiological phenomena depend on the opening of TRPC channels. A previous study verified that TRPC channel was expressed in the pregnant uterus (44). The upregulation of TRPC3 channel, and L-type and T-type calcium channel functions enhances the contractility of uterine spiral arteries during pregnancy, which is associated with labor onset. In the present study, on the basis of SAMe treatment, Pyr3 was administered, so that the inhibitory effect of SAMe on inflammation and regulatory effect on calcium channels were suppressed, indicating that the therapeutic effect of SAMe on infectious premature delivery is associated with TRPC channel. It has previously been demonstrated that TRPC channels are expressed in mammalian uterine muscle tissue (45). Mechanical traction of human uterine smooth muscle *ex vivo* can increase the expression of TRPC 3 channel (46).

The PKCβ/CPI-17 pathway serves an important role in the contraction of vascular smooth muscle and the mechanism of calcium sensitization. Su *et al* (47) previously induced CPI-17 gene silencing in bronchial smooth muscle using RNA interference technology, and observed that calcium sensitization, contraction frequency and contractility of bronchial smooth muscle decreased, which suggested that CPI-17 has an effect on the contraction of smooth muscle. Ozaki *et al* (15) previously demonstrated that PKC agonist can significantly increase the phosphorylation of MLC and the contraction of vascular smooth muscle. In the presence of the specific MLCP activity inhibitor, Rho-associated kinase, exogenous phosphorylated

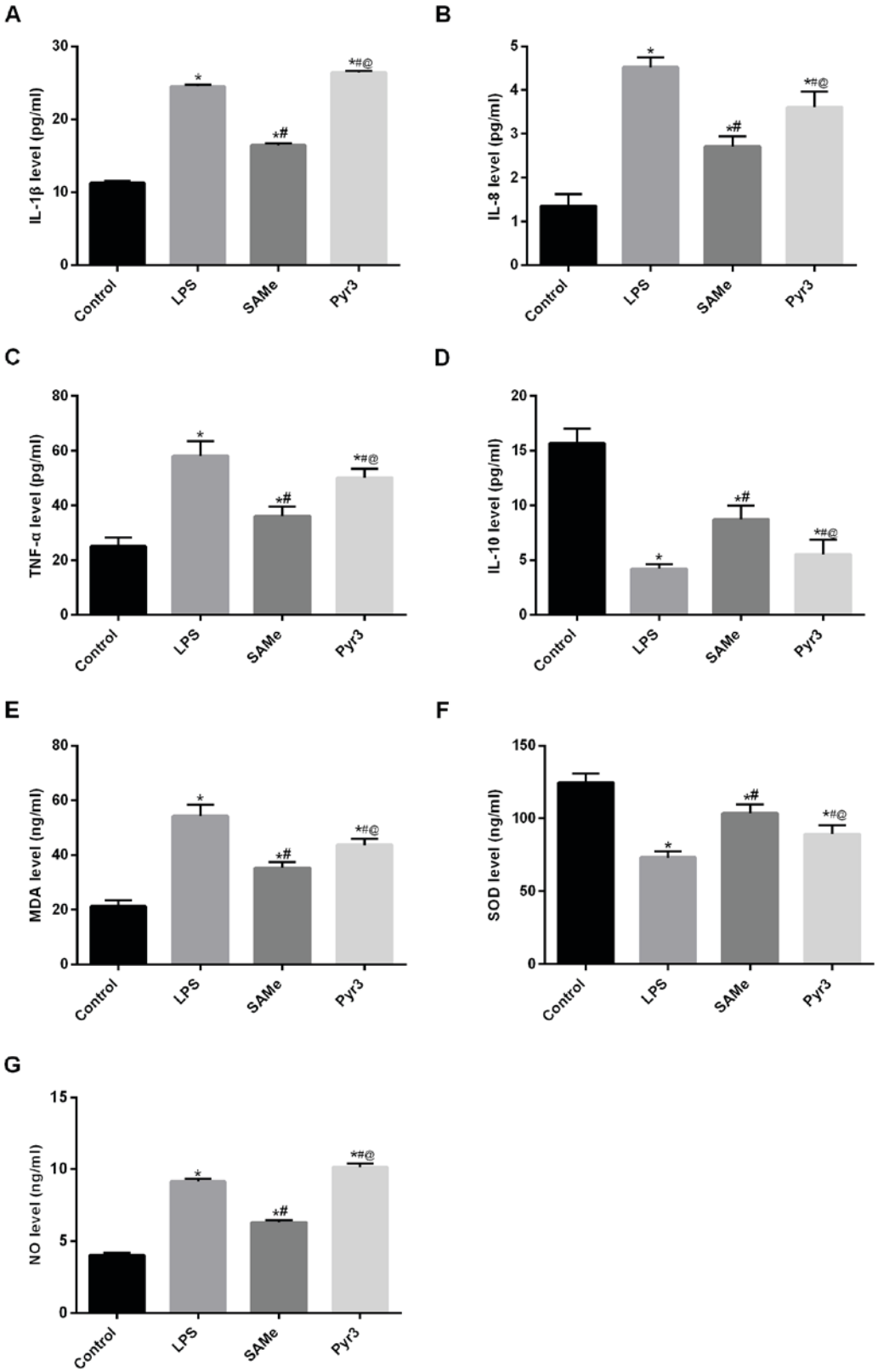

Figure 2. SAMe suppresses inflammatory factors IL-1β, IL-8, TNF-α, IL-10, MDA, SOD and NO. (A) IL-1β, (B) IL-8, (C) TNF-α, (D) IL-10, (E) MDA, (F) SOD and (G) NO serum levels were detected by ELISA. *P<0.05 vs. control group; #P<0.05 vs. LPS group; @P<0.05 vs. SAMe group. SAMe, S-adenosyl methionine; IL, interleukin; TNF-α, tumor necrosis factor α; MDA, malondialdehyde; SOD, superoxide dismutase; NO, nitric oxide; LPS, lipopolysaccharide. Data are provided as the mean ± standard deviation (n=45).

CPI-17 can increase the contraction of vascular smooth muscle in a dose-dependent manner (48). The present study detected proteins in the TRPC3/PKCβ/CPI-17 signaling pathway, and demonstrated that SAMe could downregulate the increased expression of TRPC3, PKCβ and p-CPI-17 induced by infectious premature delivery. When TRPC3 inhibitor was added,

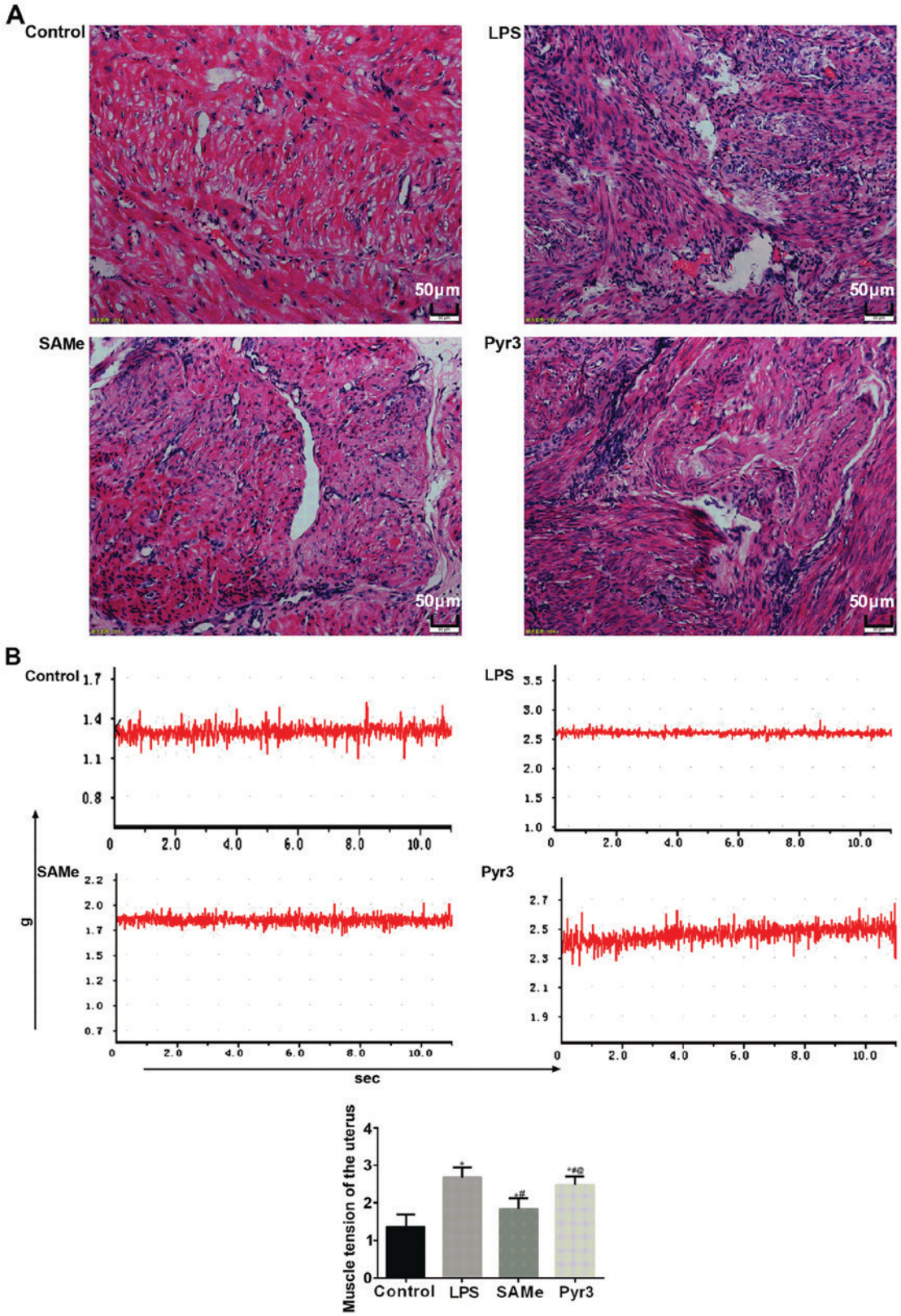

Figure 3. SAMe improves muscle tension of the uterus. (A) Hematoxylin and eosin staining. (B) Muscle tension of the rat uterus. *P<0.05 vs. control group; #P<0.05 vs. LPS group; @P<0.05 vs. SAMe group. SAMe, S-adenosyl methionine; LPS, lipopolysaccharide. Data are provided as the mean ± standard deviation (n=45).

the effect of SAMe was no longer apparent. These data indicate that SAMe improves muscle tension, suppresses the influx of exogenous calcium ions, and inhibits inflammatory reaction through the TRPC3/PKCβ/CPI-17 pathway, thereby ameliorating infectious premature delivery. The present study is a preliminary exploration of the mechanism of action of SAMe, and the underlying regulatory mechanism in improving infectious premature delivery requires further investigations.

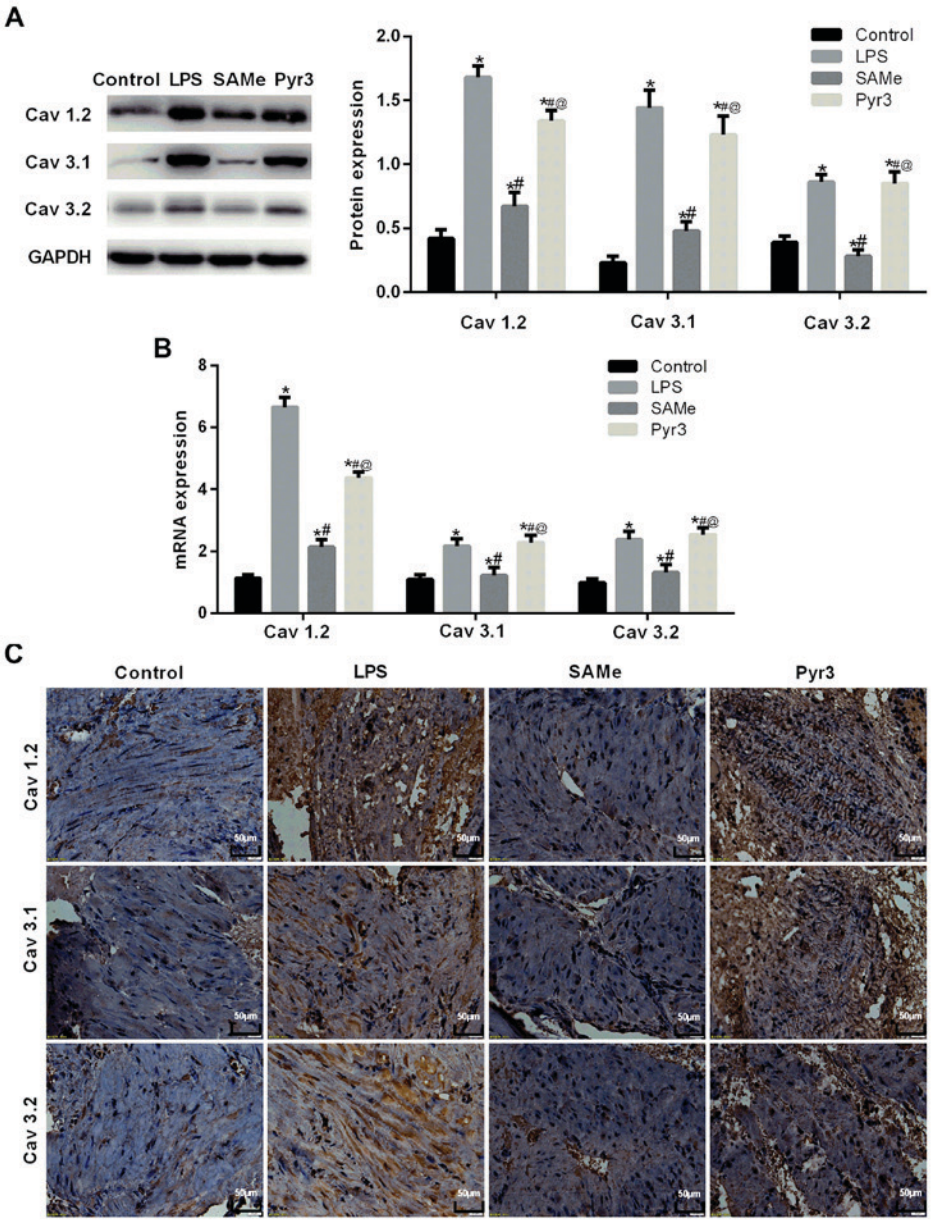

Figure 4. SAMe reduces the expression of calcium channels in rats with infectious premature delivery. Cav1.2, Cav3.1 and Cav3.2 expression were detected by (A) western blot analysis, (B) reverse transcription-quantitative polymerase chain reaction and (C) immunohistochemistry. *P<0.05 vs. control group; #P<0.05 vs. LPS group; @P<0.05 vs. SAMe group. SAMe, S-adenosyl methionine; Cav1.2, calcium channel, voltage-dependent, L type, α 1C subunit; Cav3.1, calcium channel, voltage-dependent, T type, α 1G subunit; Cav3.2, calcium channel, voltage-dependent, T type, α 1H subunit; LPS, lipopolysaccharide. Data are provided as the mean ± standard deviation (n=45).

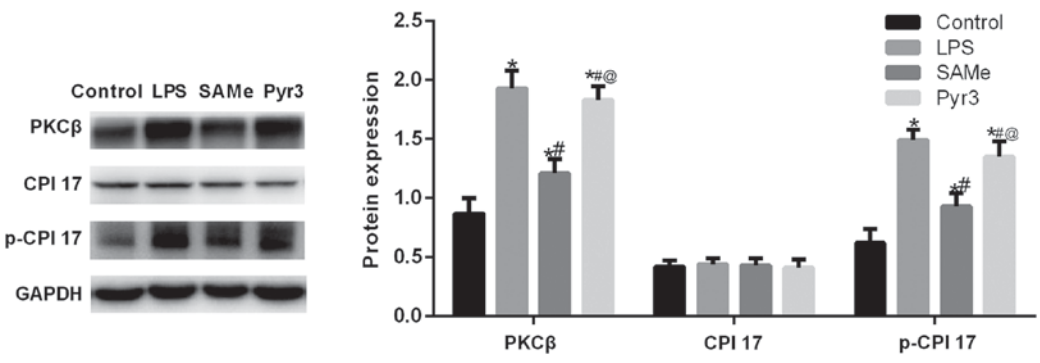

Figure 5. SAMe promotes uterine contraction and delivery through the TRPC3/PKCβ/CPI-17 signaling pathway. TRPC3/PKCβ/CPI-17 signaling pathway-related protein expression levels were detected by western blot analysis. *P<0.05 vs. control group; #P<0.05 vs. LPS group; @P<0.05 vs. SAMe group. SAMe, S-adenosyl methionine; TRPC3, transient receptor protein 3; PKCβ, protein kinase Cβ; CPI-17, C-kinase-activated protein phosphatase-1 inhibitor of 17 kDa; LPS, lipopolysaccharide; p, phosphorylated. Data are provided as the mean ± standard deviation (n=45).

In conclusion, the present study successfully established a rat model of infectious premature delivery. SAMe was able to prolong the delivery time, decrease the mortality rate, inhibit the inflammatory reaction and oxidative stress, and regulate the calcium channel expression in this model. Its regulatory mechanism is possibly associated with the PKC/PLCβ/CPI-17 signaling pathway. These findings provide a basis for the clinical prevention and treatment of infectious premature delivery and targeted therapy of novel drugs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JG, TH, XL, JW and MH conceived and designed the study, acquired the data, interpreted the results and drafted the manuscript. MH contributed to the acquisition of funding and support. JG, TH, XL, LS and JZ performed the experiments. YH and YX analyzed the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Animal Experiments Ethics Committee of the General Hospital of Shenyang Military (Shenyang, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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