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Original Article

Expression of T-type calcium channels $ca_v3.1$ and $ca_v3.2$ in different segments and different gestational ages of mouse uterus

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Abstract: Objective: To explore the expression of T-type calcium channels $Ca_v3.1$ and $Ca_v3.2$ in different segments of mouse uterus as well as different gestational ages during pregnancy. Study design: Uterus samples were collected from C57BL/6 mice at 0, 7, 14, 19 days of pregnancy and one day postpartum. The mRNA expression levels of T-type calcium channel were detected by quantitative RT-PCR. Protei n expression levels of $Ca_v3.1$ and $Ca_v3.2$ at different gestational ages and different segments [upper segment (US) and lower segment (LS)] were determined by immunohistochemistry and western blot, respectively. Results: We only observed the expression of $Ca_v3.1$ and $Ca_v3.2$ in mouse uterus but not $Ca_v3.3$. The expression levels of $Ca_v3.1$ and $Ca_v3.2$ increased during pregnancy and declined rapidly after labor. Besides, $Ca_v3.1$ and $Ca_v3.2$ were significantly higher expressed in US compared to LS during late pregnancy ($P < 0.05$). Conclusion: Our results provide a comprehensive analysis of expression of T-type calcium channel subunit in US and LS of non-pregnant, pregnant and postpartum mouse myometrial cells.

Keywords: T-type calcium channels, pregnancy, uterus

Introduction

As smooth muscles of uterus, the myometrium experiences major transition from relaxed during pregnancy to an extremely contractile muscle layer at term. The regulation of myometrial contraction is crucial for pregnancy and parturition. In this process, calcium dynamics in myometrial cells are modulated to facilitate myometrial and uterine contractility [1, 2]. Calcium entry from the extracellular milieu is important in elevating the concentration of Ca^{2+} in smooth muscle cells and involves various calcium channels including T-type calcium channels [3].

The subfamily of T-type calcium channel is classified based on α1 subunit composition termed α1G ($Ca_v3.1$), α1H ($Ca_v3.2$) and α1I ($Ca_v3.3$) [4, 5]. $Ca_v3.1$ and $Ca_v3.2$ have similar activation and could be differentiated by sensitivity to nickel [6]. In mice, when $Ca_v3.1$ is lacked, $Ca_v3.2$ plays a main role in wild-type spermatogenic cells [7]. In comparison, $Ca_v3.3$ is easily distinguishable by its voltage dependence of activation and inactivation kinetics which occurs at slightly more positive potentials [8, 9]. T-type calcium channel has been found to be expressed in many mouse organs, including rat brain [10] and sinoatrial node of heart [11]. In addition, recent research in rats has revealed that both $Ca_v3.1$ and $Ca_v3.2$ are expressed in the circular and longitudinal layers of myometrium, and the expression levels differ between longitudinal and circular muscle cells [12]. However, it did not elaborate its expression levels at different uterine segments [upper segment (US) and lower segment (LS)].

In this study, in order to determine the different expression levels of mouse uterus during pregnancy and after labor, we detected the mRNA and protein expression levels of T-type calcium channels α1 subunit in mouse uterus at different gestational ages and different uterine segments (US and LS).

Materials and methods

All studies have been approved by Shengjing Hospital of China Medical University Ethics Committee and performed in accordance with the ethical standards.

Table 1. PCR primer sequences for the amplification of T-type calcium channels

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
$Ca_v3.1$	GGCATCGAGTACCACGGAGCA	CAGCTTCAGCAGCATCTCCAA
$Ca_v3.2$	TTCTTCGTATTCTTCATCTT	CTCCTCTGTCTGGTAGTAT
$Ca_v3.3$	CAAAGCCATCAACCGTGTTCC	ACGCCGATGATGCCAAAGA
β-actin	CCTCGCCTTTGCCGATCC	GGATCTTCATGAGGTAGTAGTC

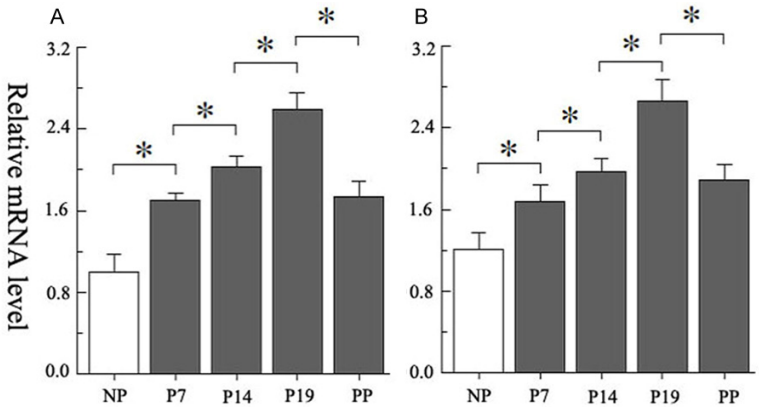

Figure 1. QRT-PCR for relative mRNA levels of $Ca_v3.1$ (A) and $Ca_v3.2$ (B) in mouse uterus during different gestational ages. The expression values were expressed as the ratio of $Ca_v3.1$ (A) and $Ca_v3.2$ (B) compared to GAPDH. $^*P < 0.05$.

Animals and treatments

Total 80 adult C57BL/6 mice (40 female and 40 male, 8-10 weeks old, weighing 18-20 g) were purchased from the Animal Department of Peking Union Medical College (Beijing, China). The animals were allowed free access to food and water at all times and were maintained at cycles of 12 hours light: 12 hours dark. Day 0 of pregnancy (non-pregnant) was determined by the presence of a copulatory plug. Mice were euthanized by CO_2 inhalation on specific days relative to pregnancy duration [non-pregnant (NP, n=7), days 7 (P7, n=7), 14 (P14, n=9), and 19 (P19, n=8) of non-laboring gestation and one day postpartum (PP, n=9)]. All animals were treated in accordance with the Guide for Care and Use of Laboratory Animals and the study was approved by the Animal Care and Use Committee of China Medical University (Liaoning, China).

Tissue preparation

All 40 female mice achieved pregnancy and were euthanized. The uterus was rapidly excised and washed in cold sterile saline (0.9% NaCl). Some uterus samples were placed in TRIzol reagent for RNA extraction and others were stored at -80°C for western blot and immunohistochemical analysis. In addition, the mouse US and LS tissue were also collected for PCR and western blot analysis, respectively. For immunohistochemical analysis, biopsies were fixed in 4% paraformaldehyde and embed in ice-cold phosphate-buffered saline.

RNA isolation and quantitative RT-PCR (qRT-PCR)

RNAiso Plus (TakaRa, Japan) was used to obtain RNA from mouse uterine tissue. The cDNA was obtained by reverse transcriptase PCR (RT-PCR) using the PrimeScript RT reagent Kit and amplified in triplicate with SYBR Premix Ex Taq (Roche Diagnostics td., Burgess Hill, West Sussex, RH159RY) by specific primers (**Table 1**) for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal standard to normalize gene expression. QRT-PCR was performed in the presence of SYBR Green by running 45 cycles (95°C for 20 s, 60°C for 20 s and 72°C for 20 s) and a final extension at 72°C for 15 s with GAPDH as the reference gene. The cycle threshold (CT) values of the samples were detected at a level where the exponential increase in amplicon abundance was approximately parallel between all samples.

Western blot

The tissues were homogenized in lysis buffer at 4°C for 5 minutes. After centrifugation, proteins (30 μg) were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane. After being blocked with skim milk, the membrane was incubated with mouse anti-$Ca_v3.1$ or mouse anti-$Ca_v3.2$ primary antibodies (Alomone Labs Ltd. Jerusalem, Israel; 1:500) at 4°C overnight and then with HRP-linked sheep anti-mouse secondary antibody (GE Healthcare; 1:1000) for 1 h at room

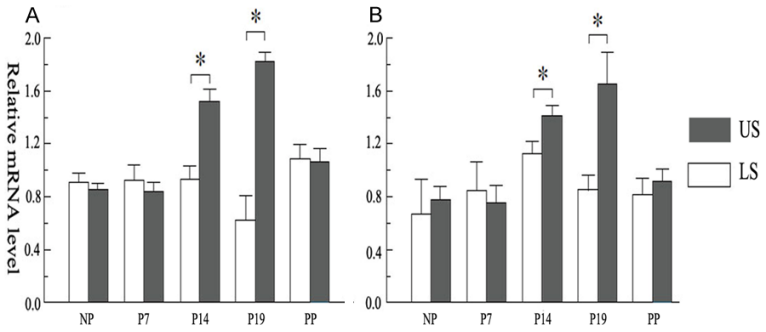

Figure 2. QRT-PCR for the mRNA expression levels of $Ca_v3.1$ (A) and $Ca_v3.2$ (B) in upper segment of mouse uterine (US) and the lower segment of uterus (LS) at different gestational ages. $^*P < 0.05$.

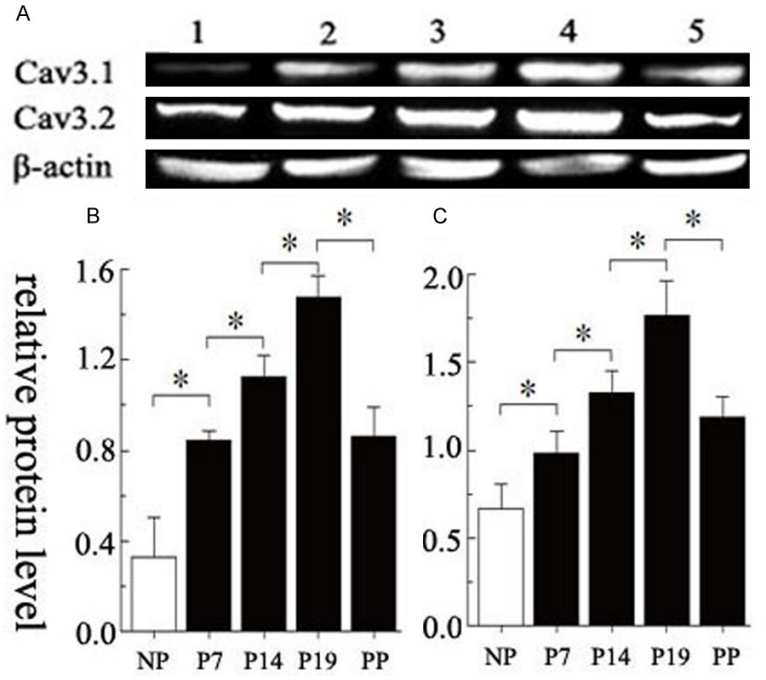

Figure 3. Western blot analysis for protein expression of $Ca_v3.1$ and $Ca_v3.2$ in mouse uterus at different gestational ages. (A) The molecular weights of $Ca_v3.1$ and $Ca_v3.2$ are 262 kDa. (B, C) The protein expression values of $Ca_v3.1$ (B) and $Ca_v3.2$ (C) in mouse uterus at different gestational ages. $^*P < 0.05$. β-actin served as a loading control.

temperature. The resulting band intensities were quantified through the image scanning densitometer (Furi Technology, Shanghai, China) and calculated according to the β-actin reference bands. β-actin served as a loading control.

Immunohistochemistry analysi

Immunohistochemistry analysis of $Ca_v3.1$ and $Ca_v3.2$ at different gestational ages was performed by the UltraSensitive-SP-kit (MaiXin Biotechnology, Fuzhou, China) using a biotinylated second antibody. The specific $Ca_v3.1$ and $Ca_v3.2$ antibodies (anti-$Ca_v3.1$ and anti-$Ca_v3.2$ antibody) were purchased from Alomone Labs (Alomone Labs Ltd. Jerusalem, Israel) using a 1:200 dilution. Negative control was performed by substituting the primary antibody with normal serum in the same dilution. In order to search antigens, 4 μm-paraffin sections were cut, rehydrated and microwaved in citric acid buffer. Then unspecific antigen-bindings were blocked with goat serum for 30 min and the samples were incubated overnight at 4°C with diluted primary antibodies. Finally, the bound antibodies were detected using biotin-streptavidin-peroxidase system with diaminobenzidine (Sigma, St Louis, MO, USA) as chromogen, followed by counterstaining with hemalum.

Statistical analysis

All data values are expressed as the mean ± SEM. Population sample numbers (n) indicate the number of animals used. All data for homogeneity of variance were tested by Bartlett's test. Individual comparisons for paired data were analyzed by Student's t test. Multiple comparisons were tested using one-way ANOVA with Student-New-man-Keuls. All statistical analyses were performed with SPSS 16.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Expression of mRNA for T-type calcium channels

Expression of T-type calcium channels was identified in mouse uterine tissue from non-

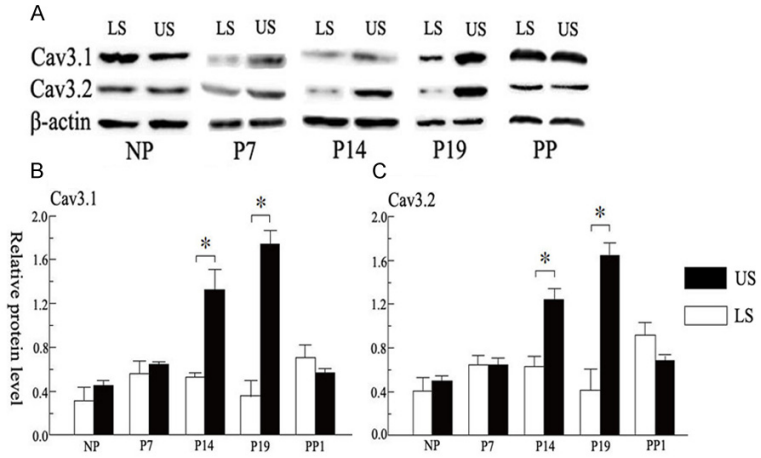

Figure 4. Western blot analysis for protein expression of $Ca_v3.1$ and $Ca_v3.2$ in US and LS at different gestational ages. (A) The molecular weights of $Ca_v3.1$ and $Ca_v3.2$ are 262 kDa. (B, C) The expression values of $Ca_v3.1$ (B) and $Ca_v3.2$ (C) in mouse US and LS at different gestational ages. $^*P < 0.05$. β-actin served as a loading control.

pregnant, pregnant and postpartum mouse. QRT-PCR revealed that both $Ca_v3.1$ and $Ca_v3.2$ were expressed in uterine tissue from all detected gestational ages while $Ca_v3.3$ was not observed as below the limit of detection.

There were significant differences in mRNA expression levels of $Ca_v3.1$ at each two detected gestational ages (**Figure 1A**, $P < 0.05$), the same as $Ca_v3.2$ (**Figure 1B**). Expression of mRNA levels of both $Ca_v3.1$ and $Ca_v3.2$ increased with the increase of gestational age, and declined rapidly after labor. Moreover, mRNA expression levels of $Ca_v3.1$ in mouse US and LS tissue revealed that the levels in US were significantly higher compared to that in LS during both P14 and P19 stages of gestation (**Figure 2A**, $P < 0.05$). The same results were also found for $Ca_v3.2$ (**Figure 2B**, $P < 0.05$).

Protein expression of $Ca_v3.1$ and $Ca_v3.2$

Western blot analysis, using $Ca_v3.1$ and $Ca_v3.2$ specific antibodies, detected bands of 262 kDa (**Figure 3A**). Expression of $Ca_v3.1$ and $Ca_v3.2$ was significantly increased in the term of pregnancy with increase of gestational ages and reached the highest at P19, and significantly declined after labor (**Figure 3B** and **3C**, $P < 0.05$), which was the same as the mRNA expression levels. Additionally, a band of 262 kDa about $Ca_v3.1$ or $Ca_v3.2$ was also observed in the US and LS of mouse at different (**Figure 4A**) gestational ages. Besides, the protein levels of $Ca_v3.1$ (**Figure 4B**) and $Ca_v3.2$ (**Figure 4C**) in US were significantly higher compared to LS ($P < 0.05$) at the pregnancy of P14 and P19.

Positive immunoreactivity for $Ca_v3.1$ and $Ca_v3.2$ was identified in both US and LS from non-pregnant, pregnant and postpartum mouse uterine myometrium. Immunohistological analysis demonstrated that $Ca_v3.1$ (**Figure 5**) and $Ca_v3.2$ (**Figure 6**) were highly expressed in US and LS myometrial smooth muscle cells.

Discussion

An increase in intracellular calcium concentration in smooth muscle cells was essential for contraction of the myometrium and parturition and T-type calcium channel might play an important role in this process. In our study, we found that both the mRNA and protein of $Ca_v3.1$ and $Ca_v3.2$ were expressed in non-pregnant, pregnant and postpartum mouse uterine myometrium in US and LS, while $Ca_v3.3$ was not expressed or expressed at undetectable levels. In addition, the expression levels of $Ca_v3.1$ and $Ca_v3.2$ increased with gestational age growth, and declined rapidly after labor.

T-type calcium channel has been reported to be involved in regulation of initiation of action and spontaneous phasic contraction of smooth muscle such as the urinary smooth muscle of human [13] and uterine smooth muscle in pregnant rats [14]. Previous studies have been reported the expression of T-type α subunits (α 1G and α 1H) by RT-PCR and western-blot and found that both the mRNA and protein of T-type α subunits are expressed in rats myometrium [12] and the expression levels are different during gestation [14], which are consistent with our study.

In addition, we found that the protein levels of T-type Ca^{2+} channel $Ca_v3.1$ and $Ca_v3.2$ increased with gestational age growth, and declined rapidly after labor. In the nervous system, estrogen has been shown to up-regulate T-type cal-

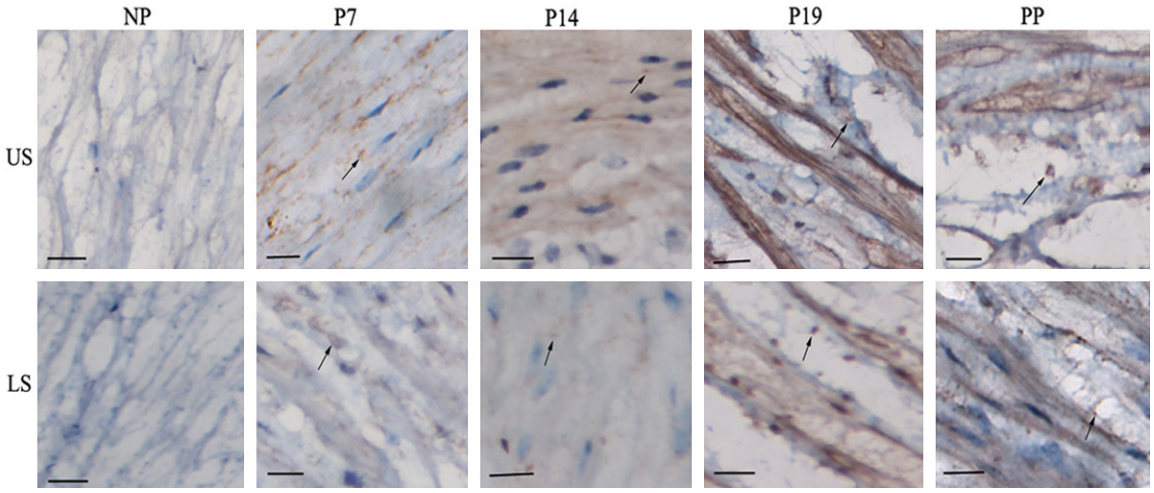

Figure 5. Immunohistochemistry of $Ca_v3.1$ in mouse US and LS at different gestational ages (SP × 400).

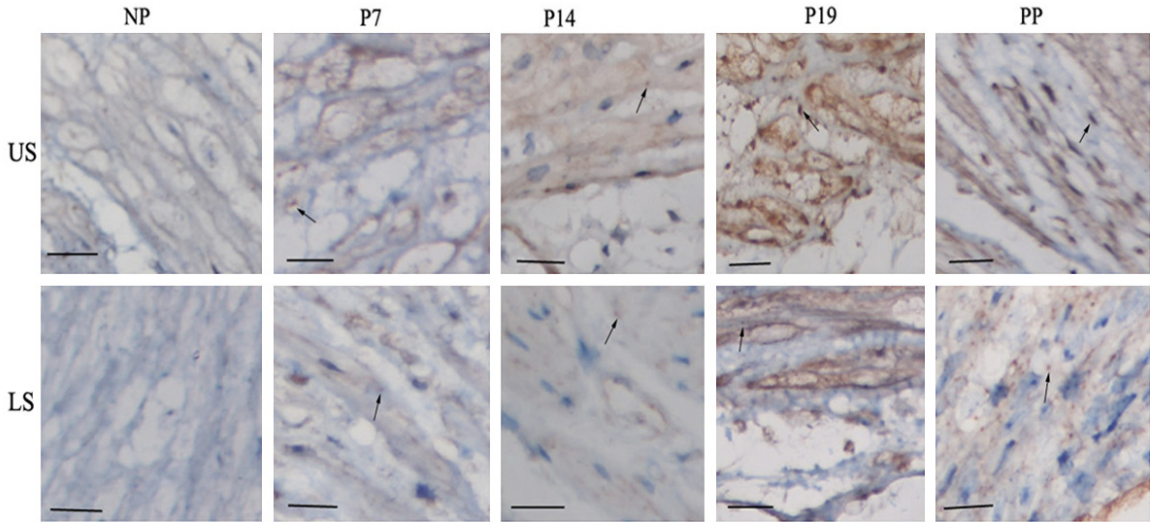

Figure 6. Immunohistochemistry of $Ca_v3.2$ in mouse US and LS at different gestational ages (SP × 400).

cium channel expression [15-17]. The levels of estrogen have close relationship with gestational age, and estrogen increases with gestational age growth and reaches a peak a few weeks before labor [18]. Thus, we hypothesize that, in mouse uterine tissue, estrogen may regulate calcium channel expression and the increased expression may facilitate uterine contractility required for labor. However, more studies are needed to verify our hypothesis.

It has been confirmed that T-type Ca^{2+} channels contribute to a wide variety of physiological functions, most predominantly in the nervous, cardiovascular and endocrine systems [19]. But the role of T-type Ca^{2+} channels in uterine tissue still remains unclear, and changes in the uterine expression of these isoforms may influence physiological functions during pregnancy. The increased expression of $Ca_v3.1$ and $Ca_v3.2$ during pregnancy that may facilitate uterine contractility required for labor. It has been reported that as one of the intracellular signals, Ca^{2+} entry mediated by $Ca_v3.2$ channels might act in a complex network which is involved in embryonic stem cell self-renewal [20]. *In vitro,* mibefradil, a T-type Ca^{2+} channel blocker, could inhibit spontaneous rhythmic contractions and oxytocin-induced contraction in the pregnant goat myometrial strips [21]. In vitro experi-

ments on human myometrial tissue strips, mibefradil can not only inhibit uterine contract, but also can block bioelectrical signal. Mibefradil plays a essential role in inhibiting the bioelectrical signal and uterine contractile forces, suggesting the importance of T-type Ca^{2+} channels in the initiation of contraction [22]. A lot of studies indicate differential expression of a variety of proteins including BK_{Ca} protein and prostaglandin receptors between US and LS myometrium with labour [23-25], which support the idea that increased contractility of the fundus compared to the lower segment during labour. Our results indicate that the similar expression pattern of T-type Ca^{2+} channels in US and LS was occurred during late pregnancy. Uterine contractions originate in the fundus of uterus, and then spread down the higher expression of T-type calcium channels in US of uterine may explain this phenomenon.

The current study revealed the different expression levels of $Ca_v3.1$ and $Ca_v3.2$ in mouse US and LS at different gestational age. However, there is a lack of information regarding specific regulatory mechanism of $Ca_v3.1$ and $Ca_v3.2$ in mouse uterus, and the role they play in functional regionalization of the uterus during pregnancy and labor.

There are still some limitations in the current study, For example, our study did not involve the uterine longitudinal and circular muscle cells. Besides, the number of animals and the gestational ages we chose were limited. A deeper study of the expression of $Ca_v3.1$ and $Ca_v3.2$ in different myometrial cells remain an area for further research.

In conclusion, our results indicate that T-type calcium channel subunits Cav3.1 and Cav3.2 are expressed in UC and EU of non-pregnant, pregnant and postpartum mouse myometrial cells and the expression levels increased during pregnancy and declined rapidly after labor. Upregulation of the T-type calcium channels may be necessary for parturition. The level expression of T-type calcium channels higher in UC of pregnant uterus suggest T-type calcium channels may involve in starting uterine contraction. But the mechanism responsible for the upregulation of T-type calcium channels expression needs further research.

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Disclosure of conflict of interest

None.

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