

The Effect of Vitamin D₃ on the Contractile Response of Isolated Rat Uterus

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Abstract

Background: Vitamin D receptors (VDR) are expressed in many reproductive tissues indicating a potential role of vitamin D₃-VDR in the regulation of reproductive functions. Nevertheless, the data about the effect of vitamin D₃ in the uterus are scarce.

Aim of Study: This study was designed to evaluate the potential effects of vitamin D₃ on spontaneous, KCl-induced and oxytocin-induced contractions in the rat uterus in vitro.

Material and Methods: The study was conducted on 10 healthy adult female albino non-pregnant rats. Full-thickness longitudinal muscle strips were dissected from each non-pregnant rat and then myometrial tension was recorded. The strips were mounted vertically in organ baths and exposed to vitamin D₃ and different uterotonic agents to delineate the potential action of vitamin D₃ on the rat myometrial contractility. Spontaneous contractions were recorded using mechanical activity recording system. We evaluated the effects of 3 different doses of vitamin D₃ on spontaneous uterine contractions; then on concentrated KCl-induced uterine contractions and oxytocin (OT)-induced uterine contractions. Furthermore, the effects of vitamin D₃ on spontaneous uterine contractions pretreated with nifedipine; a voltage-gated L-type Ca²⁺ channel antagonist were investigated.

Results: Vitamin D₃ significantly inhibited the spontaneous uterine contractions in a dose dependent manner. Moreover, vitamin D₃ inhibited the uterine contractions whether induced by oxytocin or concentrated KCl. Administration of nifedipine resulted in a significant decline in the force amplitude of spontaneous uterine contractions. In the presence of vitamin D₃, the uterine relaxant effect of nifedipine was significantly augmented.

Conclusions: The inhibitory effects of the in vitro administration of vitamin D₃ on rat uterus may yield novel insights into its therapeutic use. Further, it may be recommended to be used as a novel tocolytic agent for preventing unwanted uterine contractions in early pregnancy and relieving pain related to dysmenorrhea.

Key Words: Vitamin D₃– Oxytocin – Uterus – Contractility.

Introduction

ADEQUATE and precise uterine contractility is necessary for various reproductive processes, including sperm transport, fruitful embryo transport, implantation, pregnancy and labor [1]; hence, the physiological functions of uterus smooth muscle are essential. The abnormalities of uterine contractility may trigger different clinical defects such as implantation failure, spontaneous miscarriages, infertility, ectopic pregnancies and preterm birth [2-5].

Vitamin D₃ is a distinguished steroid and fat soluble compound that regulates bone health predominantly via calcium-phosphorus homeostasis [6]. The biological effects of vitamin D₃ are applied via binding to vitamin D receptors (VDR) which are expressed in many reproductive tissues, therefore, growing evidence indicates that many reproductive functions in human and animals are regulated by vitamin D₃ [7-9]. In female reproductive system, vitamin D₃ may affect various physiological processes in the placenta, ovary and uterus. It is necessary for the normal decidual cells differentiation [10]. Hence, it may contribute to various reproductive pathologies such as endometriosis, uterine fibroids, endometrial carcinoma, preeclampsia and polycystic ovarian syndrome [11-13]. Therefore, little data is available about the exact role of vitamin D₃ and VDR in the uterine tissues and its contribution to the uterine function. There is limited data concerning uterine contractility under the effect of vitamin D₃. We hypothesized that vitamin D₃ may inhibit the rat uterine contraction in vitro

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and investigated the effects of changing vitamin D3 doses on non-pregnant rat uterine contractility *in vitro*. Specifically, we studied the effect of vitamin D3 on spontaneous uterine contractions; uterine contractions induced by oxytocin (OT) and uterine strips depolarized by high concentrations of KCl, in addition to uterine contractility pretreated with nifedipine.

Material and Methods

Animals and uterine tissue preparation:

This research was conducted in the scientific and medical research center (ZSMRC) in Faculty of Medicine, Zagazig University in the period from 1st to 30th of July 2019 and involved 10 healthy non-pregnant adult female albino rats aged 20 weeks, were used in this study. This study protocol was approved by the experimental animal Ethics Committee, Faculty of Medicine, Zagazig University. The experimental procedures were performed in accordance with the guide for the use and care of laboratory animals. The oestrous cycle stages were monitored daily using vaginal smears and the rats were sacrificed only in the metoestrous or dioestrous stages because the uterus produces regular spontaneous contractions in these stages [14]. Additionally, Estradiol was administered 24 h before decapitation to assist in obtaining a spontaneous periodic contraction of myometrium [15]. To get the estrogenized uterus, virgin female rats were subcutaneously injected with 17- β -estradiol benzoate (1mg/kg) and killed 24h later by decapitation. The abdomen was opened longitudinally and the uterus was promptly removed, cleaned of the connective tissue and cut into strips of about 1cm of length, then immediately placed in a buffered physiological salt solution (PSS) of the following composition per liter: 9gm NaCl, 420mg KCl, 240mg CaCl₂, 0.16g KH₂PO₄, 0.5 g NaHCO₃, 0.5g dextrose, pH 7.40 [16]. It was maintained at 37 \pm 0.50 $^{\circ}$ C and continuously bubbled with air. The preparation was allowed to equilibrate for 30min. during which the bathing solution was changed every 10min.

In vitro contractility study:

The dissected uterine strips were transferred and mounted vertically in a 5mL tissue organ bath (Panlab muti-chambre, ADInstruments Australia), continuously perfused with PSS at a rate of 4mL/min and bubbled with 95% O₂/5% CO₂ at 37 $^{\circ}$ C; the pH was maintained at 7.40 throughout the experiment [17]. The uterine strips were connected to an isometric force transducer using surgical silk.

Changes in isometric force were recorded, amplified, and displayed using LabChart software. At first, 1gm of resting tension was applied; the strips were allowed to equilibrate for at least 60min to obtain stable and regular uterine contractions [16].

Drugs and chemicals:

Chemicals for physiologic buffer solution used in this study were purchased from El Gomhouria Co. For Drugs & Medical Supplies (Egypt). Vitamin D3 (cholecalciferol), oxytocin (α -Hypophamine) and nifedipine (BAY 1040) are obtained from (Sigma Aldrich). All stock solutions were made and stored in accordance with the manufacturer's guidelines. Oxytocin was prepared by dissolving it in distilled water and used at a final working concentration of (10-2IU/mL) [17]. Concentrated KCl (60mM/L) solution was made fresh by isotonic replacement of NaCl [18]. Vitamin D3 was dissolved in ethanol (0.1% in the organ bath) before dilution with the PSS. Nifedipine was kept in a darkened container and dissolved in ethanol.

Experimental protocols:

Dose-dependent effect of vitamin D₃ on spontaneous uterine contractions:

Three different concentrations of vitamin D₃ (10⁻³, 10⁻⁶, 10⁻⁹ M) were used to find the concentration that produces a 50% inhibition of 100% of the force amplitude (IC₅₀) of uterine strips. Each dose was applied for a period of 10min, the uterine strips were then washed with physiologic PSS and the recovery of uterine contractions was monitored. The IC₅₀ was determined and then used throughout the study.

Effects of vitamin D₃ on KCl-induced uterine contractions:

To investigate the effect of vitamin D₃ on the depolarized uterine strip, 60mM KCl was applied twice (each for 10min) to the same uterine strip. Applications of KCl were done twice separated by a recovery period in physiologic PSS for 10min. The first KCl application was applied without vitamin D₃ (taken as a 100% control) and the second application was applied in the presence of vitamin D₃.

Effects of vitamin D₃ on oxytocin (OT)-induced uterine contractions:

Once steady and regular spontaneous contractions were obtained, uterine strips were stimulated with (10-2IU/mL) oxytocin for 10min to obtain regular phasic contractions (this period was used as a 100% control). The vitamin D₃ was then added

for 10min in the continued presence of oxytocin. At the end of experiments, the strip was washed with normal PSS.

Investigations on the calcium (Ca²⁺) channels involvement:

To test the involvement of calcium channels in the mechanism of action of vitamin D₃, nifedipine (Dihydropyridines) which is a Ca²⁺ channel antagonist was used. Once steady and regular spontaneous contractions were obtained for 10 minutes (this period was used as a 100% control). Nifedipine was added with (0.03 μ mol) for another 10min (The vitamin-D₃ was then added for 10min in the continued presence of nifedipine. At the end of experiments, the strip was washed with normal PSS.

Data analysis and statistics:

For each experiment, the results are the means of eight different uterine samples. Data were presented as mean \pm SEM and analyzed using version 24 SPSS program (SPSS Inc. Chicago, IL, USA). Student *t*-test and One way analysis of variance (ANOVA) was used followed by student-least significant differences (LSD) test to compare statistical differences between groups. *p*-value less than 0.05 was considered to be significant. We compare the means of the parameters before and after exposure to vitamin D₃ during spontaneous contraction and contraction induced by KCl, oxytocin, in addition to changes accompanying nifedipine. Force amplitude was used as the main parameter to assess the uterine contractile activity, other parameters were measured, such as the frequency (the number of contractions in 10min). The spontaneous contractile activity for the last 10min in control solution was calculated as 100%. The 10-minute vitamin D₃ application was analyzed and expressed as a percentage of the preceding control period.

Results

Vitamin D₃ dose dependence and its effects on spontaneous uterine contractions:

Cumulative applications of vitamin D₃ progressively and significantly inhibited the spontaneous uterine contractions in a dose dependent manner as shown in Table (1), (Fig. 1A, B, C and D).

Application of 10⁻⁶ M of vitamin D₃ significantly decreased the force amplitude to 50.5 \pm 2.3%. Therefore, 10⁻⁶ M of vitamin D₃ was taken as the IC₅₀ and this concentration was used throughout the remainder of the study.

Effects of vitamin D₃ on KCl-induced uterine contraction:

Concentrated KCl solution increased the uterine force amplitude and it was maintained as long as KCl was applied; uterine force returned to normal regular contractions when the strip was washed with normal PSS, however, when vitamin D₃ was added with the concentrated KCl solution, the force amplitude significantly declined to 77.2% \pm 1.9 of control values (*p*<0.05, n=10, Fig. 2A,2B).

Effects of vitamin D₃ on Oxytocin (OT)-induced uterine contraction:

Application of 10-2IU/mL of oxytocin to a spontaneously-contracting uterus caused an increase in the force amplitude and frequency as shown in Fig. (3A). In the presence of vitamin D₃, the force amplitude and frequency significantly decreased to 50% \pm 4.5 and 60% \pm 3.5 respectively of control values (*p*<0.001, n=10) (Fig. 3B).

Effects of vitamin D₃ on uterine contraction pretreated with Ca²⁺ channel antagonist:

Application of 0.03 μ mol of nifedipine to a spontaneously-contracting uterus caused a significant decrease in the force amplitude (51.1% \pm 2.3) of control value (spontaneous contraction), (Fig. 4A,4B). In the presence of vitamin D₃, the force amplitude significantly decreased to 26% \pm 1. 1 of control values (*p*<0.001).

Table (1): The effect of different concentrations of vitamin D₃ on the mean force amplitude of isolated rat uterine preparations (mean \pm SEM).

Vitamin D ₃ (M)	Mean force amplitude (%)	N
0/(Control)	100.0000	10
10 ⁻³ M	87.57 \pm 4*	10
10 ⁻⁶ M	50.5 \pm 2.3**	10
10 ⁻⁹ M	20.1 \pm 3.1***	10

**p*<0.05 compared to the control.

***p*<0.01 compared to the control.

****p*<0.001 compared to the control.

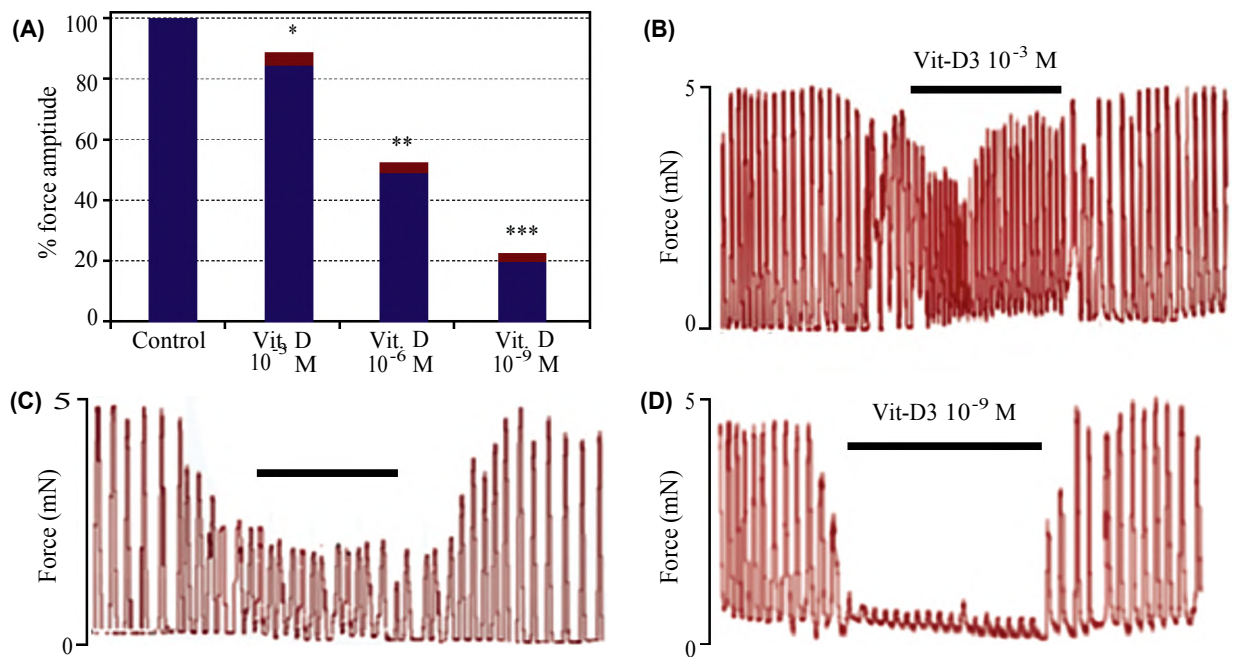


Fig. (1): The effect of different concentrations of vitamin D3 on rat spontaneous uterine contractions. (A) - Bar chart showing dose dependence decrease of % force amplitude after increasing concentrations of vit D₃ (10⁻³, 10⁻⁶, and 10⁻⁹ M), data are the mean ± SEM of 10 independent samples, **p*<0.05, ***p*<0.01, and ****p*<0.001. (B) The effect of 10⁻³ M (C) 10⁻⁶ M, and (D) 10⁻⁹ M of vitamin D3 on spontaneous contractions of isolated non-pregnant estrogenized rat uterine preparations.

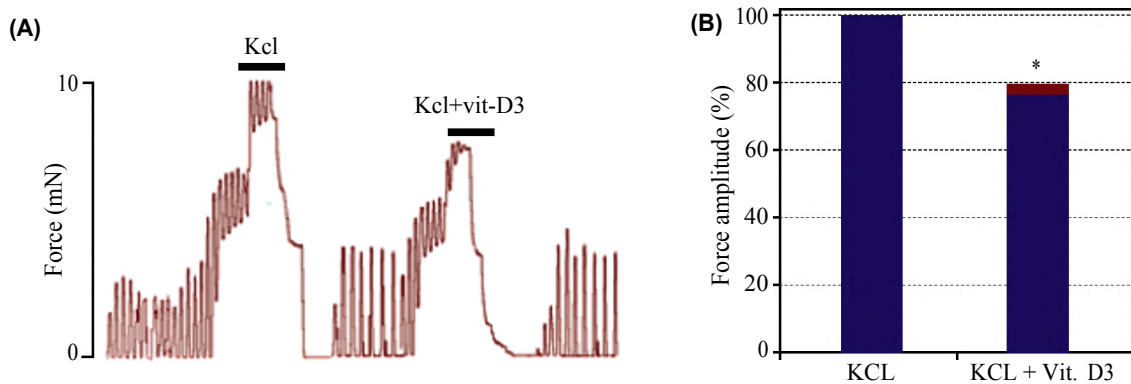


Fig. (2): The effect of vitamin D3 on rat uterine contractions in the presence of KCl solution. (A) Response of uterine strip to 60mM of KCl in the absence and presence of vitamin D3 (10⁻⁶ M). (B) Bar chart showing the significant decrease in % force amplitude after vitamin D3 treatment (mean ± SEM). Control: KCl alone, **p*<0.05.

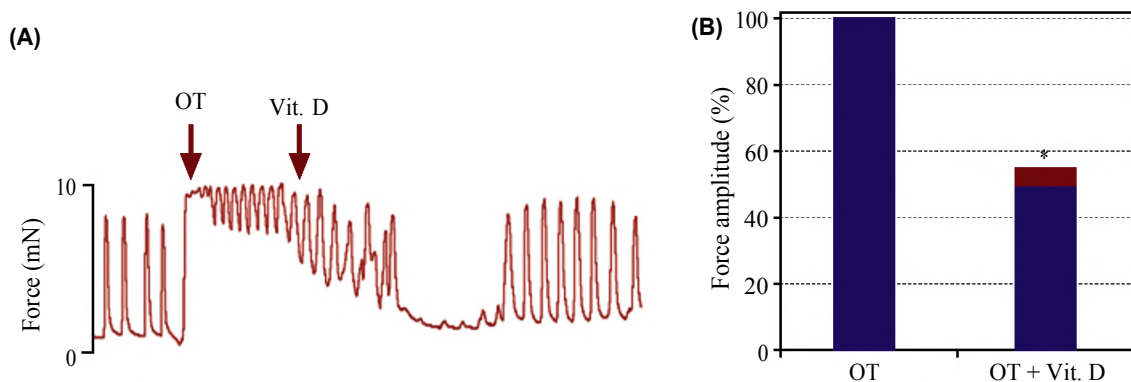


Fig. (3): The effect of vitamin D3 on oxytocin (OT)-induced rat uterine contractions. (A) Recording of uterine contractions induced by 10-2IU/ml OT and after the application of vitamin D3 (10⁻⁶ M). (B) Bar charts showing a significant decrease in % force amplitude after vitamin D3 treatment (mean ± SEM), Control: OT alone, n=10 independent samples *p*<0.001.

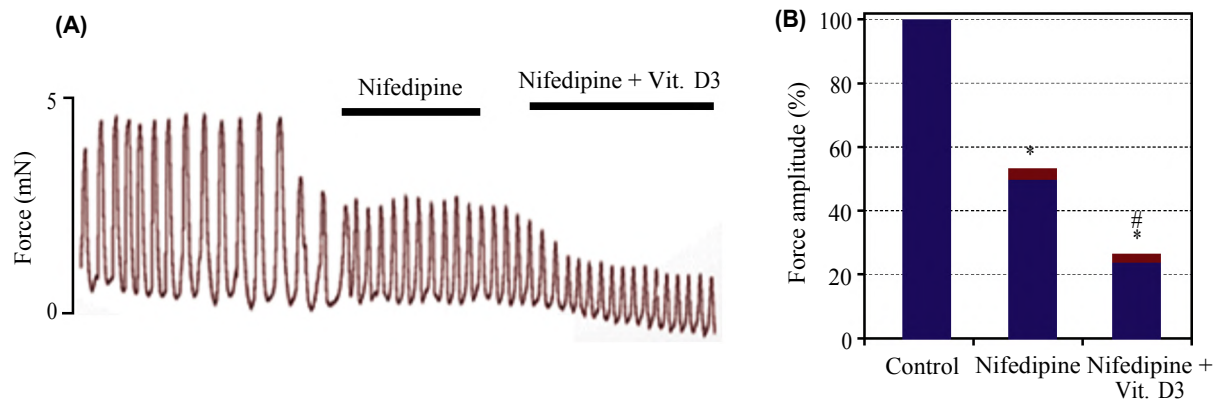


Fig. (4): The effect of vitamin D₃ on rat uterine contractions in the presence of nifedipine. (A) Response of uterine strip to nifedipine in the absence and presence of vitamin D₃ (10⁻⁶ M). (B) Bar chart showing the significant decrease in % force amplitude after vitamin D₃ treatment (mean ± SEM). Control: spontaneous contraction; *Significant to control, #Significant to nifedipine alone.

Discussion

Vitamin D has a crucial role in reproductive function and health in the human and animal. In addition, VDR is expressed in the ovary, endometrium and myometrium [19]. However, the available data on the impact of vitamin D on the uterine function is limited. The role of vitamin D₃-VDR system in the rat uterus is interesting and worth to be investigated. Our obtained findings indicate that the rat uterus is a target tissue for vitamin D₃, which should be considered as a locally acting regulator of uterine physiology. The present study show for the first time the influence of vitamin D₃ on rat myometrial strips in vitro. The main finding is that vitamin D₃ reversibly inhibits spontaneous periodic uterine contraction in a concentration dependent manner. Whether spontaneously initiated or oxytocin and KCl-induced. The current findings are consistent with the previous study performed on smooth muscles of rat aorta [20], where significant decreases in endothelium-dependent contractions were observed after vitamin D₃ application by reducing the increase in cytosolic-free calcium concentration in the endothelial cells helping to explain the link between vitamin D deficiency and hypertension. The relaxing effect of vitamin D₃ is dose-dependent and detected in all types of uterine contractions with or without endometrium, suggesting that it may directly or indirectly act to inhibit calcium channels or reduce intracellular calcium [Ca²⁺], hence inhibiting contractions [21,22]. We also observed that vitamin D₃ inhibited oxytocin-induced periodic uterine contraction and high K⁺-induced tonic contraction. These results suggest that the inhibitory effect of vitamin D₃ could be operated by the prevention of mechanisms for spontaneous, oxytocin-induced periodic uterine

contraction and high K⁺-induced tonic contraction. Physiologically, extracellular Ca²⁺ enters the myocyte via the voltage-gated dihydropyridine channels located at the plasma membrane [23]. Then, it enters down its concentration gradient initiating the release of Ca²⁺ from the intracellular stores [24]. Uterine contractions induced by oxytocin is caused by increasing [Ca²⁺]_i via calcium release from the sarcoplasmic reticulum and/or calcium influx via L-type Ca²⁺ channels [25]. Moreover, oxytocin is the chief pathway linked to the onset of preterm labor among the physiological pathways. Oxytocin binds to its G protein-coupled receptor, phospholipase C (PLC) is activated resulting in increased inositol trisphosphate (IP₃) and diacylglycerol (DAG) levels. At the sarcoplasmic reticulum membrane, IP₃ stimulates the IP₃R receptor leading to Ca²⁺ release from the stores into the cytosol. Elevated cytosolic Ca²⁺ further encouraged extracellular Ca²⁺ influx [26], hence a further rise in the intracellular Ca²⁺. Ca²⁺ binds to calmodulin stimulating the myosin light chain kinase resulting in myosin light chains phosphorylation, promoting contraction [27].

Vitamin D₃ significantly reduced the oxytocin effect on uterine muscle, this suggest the probable relaxant mechanism/s of vitamin D₃. These results are supported by the findings of Wong et al., [20] who showed that that vitamin D₃ reduces the vascular tone of the aorta of hypertensive rat by reducing calcium influx into the endothelial cells and hence decreasing the production of endothelium-derived contracting factors. However, Thota et al., demonstrated that vitamin D treatment decreased inflammation induced cytokines and contractile associated factors in the uterine myometrial smooth muscle cells via NF κB pathway [28].

In addition, vitamin D₃ significantly attenuated the tonic contraction induced by concentrated KCl. Concentrated KCl solution induces depolarization, opening of L-type Ca²⁺ channels, and increasing the intracellular Ca²⁺ resulting in maintained uterine tonic contractions. KCl-induced contraction can be attenuated by Rho A (a small GTPase protein) inhibitors in uterine smooth muscle [29], suggesting that depolarization by KCl could also activate ROCK (Rho associated protein kinase), which is serine kinase that determine the calcium sensitivity in smooth muscle cells and contribute to the regulation of smooth muscle contraction [30]. Therefore, the inhibitory effect of vitamin D₃ on KCl-induced contraction can be partially attributed to the direct blockade of L-type Ca²⁺ channels or through inhibition of ROCK pathways.

Administration of nifedipine, a dihydropyridine L-type Ca²⁺ channel antagonist, is an effective smooth muscle relaxant, resulted in a significant decline in the force amplitude of spontaneous uterine contractions. These findings are in accordance with the results of Lovasz et al., [31]. This contractility study revealed that the relaxant effect of nifedipine on spontaneous uterine contractions was significantly increased by the vitamin D₃ application suggesting that vitamin D₃ depends not only on inhibition of extracellular Ca²⁺ influx in induction of uterine relaxation but another possible mechanism regulating intracellular Ca²⁺. The synergy of nifedipine with vitamin D₃ may be of novel therapeutic significance in the management of unwanted uterine contractions.

Conclusion:

Collectively, these data, for the first time, suggest that the in vitro administration of vitamin D₃ inhibits the spontaneous uterine contractions; KCl-induced uterine contraction and oxytocin-induced uterine contraction. This significant relaxing effect of vitamin D₃ on the uterine contractility may be due to its inhibitory effect on L-type calcium channels in addition to inhibition of calcium release within the uterine smooth muscle. Therefore, vitamin D₃ may be used as a novel approach in the relief of painful uterine contractions during dysmenorrhea or in the avoidance of the unwanted uterine contractions in early pregnancy that that involve the risk of miscarriage or preterm labor.

Using isolated uteri of rats, this in-vitro study offered preliminary proof that might be used to further evaluate the in vivo influence of vitamin D₃ on human and animal uterine contractility. Further studies are needed to investigate the effect

of vitamin D₃ on human uterus, opening a new window for management of some gynecological disorders. It is also of great interest to further investigate the possible molecular mechanisms of vitamin D₃, such as its physiological effect on ROCK activity, prostaglandin biosynthesis, and other calcium or potassium channels.

Declaration of interest statement:

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgment:

The authors are grateful to the members of Zagazig Scientific and Medical Research Center (ZSMRC) in Zagazig University and Physiology Departments, Egypt for its support.

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تأثير فيتامين د₃ على استجابة تقلص الرحم المعزول للفئران

دراسات عديدة أكدت على وجود مستقبلات فيتامين (د₃) في العديد من من الأنسجة التناسلية مما يشير إلى الدور المحتمل لفيتامين د₃ في تنظيم وظائف الإنجاب. ومع ذلك، فإن البيانات حول تأثير فيتامين د₃ على الرحم نادرة لذلك، تم تصميم هذه الدراسة لتقييم الآثار المحتملة لفيتامين د₃ على الانقباضات التلقائية وأيضاً الانقباضات المستحثة باستخدام كلوريد البوتاسيوم المركز والأوكسيتوسين في رحم الفئران المعزول في المختبر.

أجريت الدراسة على ١٠ فئران أنثى بالغين تبلغ ٢٠ أسبوعاً أصحاء غير حوامل. تم تشريح شرائط العضلات الطولية كاملة السماكة من كل فأره ومن ثم تم تسجيل التوتر العضلي بشكل متساوي القياس. تم تركيب الشرائط عمودياً في حمامات الأعضاء وتعرضت للفيتامين د₃ وعوامل توتر الرحم المختلفة لتحديد التأثير المحتمل لفيتامين د₃ على انقباض عضلات الرحم. سجلت الانقباضات التلقائية باستخدام نظام تسجيل النشاط الميكانيكي. قمنا بتقييم آثار ٣ جرعات مختلفة من فيتامين D₃ على تقلصات الرحم التلقائية ثم على تقلصات الرحم التي يسببها كلوريد البوتاسيوم المركز وتقلصات الرحم الناجم عن الأوكسيتوسين وأيضاً تم دراسة آثار فيتامين د₃ على تقلصات الرحم المعالجة مع نيفيديبين وهو نواء مضاد لبوابات الكالسيوم في جدار الرحم.

النتائج: فيتامين د₃ يثبط بشكل كبير انقباضات الرحم التلقائية بدرجات مرتبطة بالجرعة. علاوة على ذلك، فإن فيتامين د₃ يثبط انقباضات الرحم سواء الناجم عن الأوكسيتوسين أو كلوريد البوتاسيوم المركز. كما أدى استخدام نيفيديبين في انخفاض كبير في قوة انقباضات الرحم التلقائية ومع إضافة فيتامين د₃ تم زيادة تأثير إرخاء نيفيديبين للرحم بشكل ملحوظ.

الاستنتاجات: الآثار المثبطة لفيتامين د₃ على رحم الجرذان في المختبر قد تسفر عن رؤى جديدة في استخدامه العلاجي. علاوة على ذلك، قد يوصى باستخدامه لمنع الانقباضات الرحمية غير المرغوب فيها في الحمل المبكر وتخفيف الألم الناتج عن عسر الطمث.