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Myoinositol causes myometrial contractions in isolated non-pregnant rat myometrium

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ABSTRACT

We aimed to investigate myoinositol effect on contractility of rat uterus in vitro and its mechanism of action. In a standard organ bath spontaneous contractions were recorded. After the cessation of spontaneous contractions, 5µM, 50 µM and 500 µM of myoinositol and 20µM folic acid were applied to each 7 strips and contractions were again enrolled. Same procedures were also repeated in calcium free solution. Real Time Polymerase Chain Reaction (RT-PCR) analysis of ion channels were done only for 50 µM myoinositol which corresponds to the treatment dose. It is demonstrated that in all doses increase in frequency and amplitude of contractions were statistically significant. Any contraction was not observed in calcium free solution which demonstrated us that myoinositol uses extracellular calcium for contraction. It is also demonstrated that folic acid does not cause contraction in non pregnant rat uterine smooth muscle cells. Expression of Transient receptor potential cation channel, subfamily M, member 2 (TRPM2) and Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) channels significantly decreased after application of myoinositol ($p=0.01$, for both) whereas expression of Transient receptor potential cation channel, subfamily M, member 7 (TRPM7) and Calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B) channels did not change significantly when compared to control (for both). We demonstrated for the first time that myoinositol causes contraction in myometrium of nonpregnant rat uterus and it uses the extracellular calcium for contraction.

Key Words: myoinositol, rat uterus, contraction

Introduction

Polycystic ovary syndrome (PCOS) influences up to 20% of women in child bearing age (1). It represents a common cause of female infertility. In recent years, effect of inositol in PCOS is demonstrated by many studies (2).

Inositol was discovered in 1850 (3). Myoinositol is a form of inositol and is naturally present in many kinds of plants especially fruit and beans (4). Myoinositol can also be synthesized in the body from glucose-6-phosphate. It is a component of cell membrane and it is needed for the development and life of human cells in the culture (5).

It has been demonstrated that inositol, by itself or its derivatives, is important in cytoskeleton reaarrangement, morphogenesis, regulation of cell proliferation, control of intracellular calcium and maintainance of cell membrane potential (6,7). It is present in high concentrations in reproductive organs of males and females (8,9).

Contractility of uterus smooth muscle is mostly related to influx of calcium from the extracellular space however release of calcium from endoplasmic reticulum can also increase calcium levels and cause contraction. The endoplasmic reticulum calcium stores can be accessed upon stimulation by inositol. Phospholipase C can produce inositol -1,4,5 triphosphate that causes discharge of sequestrated calcium out of the endolasmic reticulum. Oxytocin is an important hormone and it also uses this phospholipase C pathway. Because of this effect, use of inositol during pregnancy is not recommended (10).

Increase in intracellular calcium occurs by the use of different calcium channels among which the superfamily of transient receptor potential (TRP) proteins are the newly discovered ones. TRP ion channels are classified into seven related families. They are widely distributed in many cell types and they work in many physiological processes. Majority of TRP channels are non-selective and they work together with other ion channels (11).

We herein aimed to determine myoinositol effect on non pregnant rat uterus muscle contraction, its mechanism of action and possible roles of four different channels on contraction. These are

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Transient receptor potential cation channel, subfamily M, member 2 (TRPM2) and Transient receptor potential cation channel, subfamily V, member 1 (TRPV1) channels which are located in intracellular compartments and responsible from the release of calcium and Transient receptor potential cation channel, subfamily M, member 7 (TRPM7) and Calcium channel, voltage-dependent, N type, alpha 1B subunit (CACNA1B) are present on cell membrane and responsible from influx of extracellular calcium (12-14).

To our knowledge despite its common use in infertile patients and its known effect on calcium metabolism, this is the first study demonstrating the effect of myoinositol on uterine contractility and investigating its mechanism of action.

Materials and methods

Animals - Drugs and Solutions: Permission for the study was given by local ethics committee and the experiments were carried out in the Laboratory for Animal Experimentation, Firat University, Elazig, Turkey. 28 female non-pregnant Spraque Dawley rats weighting between 200-250 g, aged 10-12 weeks were used in the study. Vaginal smears were done and animals showing regular cycles were selected on the day of diestrus. After decapitation, we took out the uterus and put in Krebs solution.

Agent used in the study was myoinositol (Inofolic Sachet, Italfarmaco, Milano, Italy). Since folic acid was also present in the preperation used in the experiment, we also examined its effect by using a seperate folic acid preperation (Folbiol, I.E.Ulagay, Turkey). They were dissolved in required amounts before application into the organ bath. The ingredients of Krebs-Henseit solution were obtained from Sigma (Disenhofen, Germany).

Measurement of Contractility: For the analysis of contractility, one longitudinal smooth muscle strip measuring 12x2x1 mm were prepared from each rat and 7 strips were used for each drug dose. One of the tips was attached to a metal hook and the other to an isometric force-displacement transducer. Myometrial strips were ($p<0.05$,for all) suspended in a standard organ bath containing Krebs-Henseit solution. Experiments were performed at 37^{o}C and solution was aerated with a mixture of 95% 0_2 and 5% CO_2.

Experiment medium was washed in every 30 minutes. After an equilibration period under a tension of 1 g, regular spontaneous contractions were recorded for 10 minutes. Physiologic power converter (FDT05, Commat Ltd, Ankara, Turkey) was used and results were enrolled by MP150 WS Windows programme. Contractions were followed and after the complete cessation of contractions 5 μM myoinositol was added into the organ bath and contractions were again recorded for 10 minutes. This procedure was repeated for 50 μM and 500 μM doses. The washing of the bath after each procedure prevented the formation of cumulative dose. To determine the mechanism of action, the effect of the drug is also studied in calcium free solution. At the end of the process, tissue fragments to which 50 μM drug was applied, were taken for genetic analysis.

Contractile activities are quantified by calculating the amplitude, frequency and area under curve (AUC). Results of frequency are reported as number of contractions per 10 minute and data of amplitude are given as miligrams (mg).

Quantitative Real time PCR assays: RT-PCR analysis of ion channels were done only for 50 μM dose of myoinositol which corresponds to the treatment dose.

A piece of myometrium measuring approximately 100 mg were taken from the seven rat uteri, to whom 50 μM dose of drug was planned to apply, for RT-PCR analysis just after removal from the body and put into RNA latter solution. Muscle tissues that were treated with 50 μM dose of myoinositol were also taken into RNA latter solution after the procedure. Total RNA of uteri were isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Random primedcDNAs were generated by reverse transcription of total RNA samples with High Capacity RNA to cDNA Synthesis kit (P/N:4387406, Applied Biosystem, USA). The 20 μl reactions were incubated in an Applied Biosystems 7500 Fast Thermo cycler in a 96-well platefor 30 min. at 16°C, 30 min. at 42°C, 5 min. at 85°C and then held at 4°C. Tag Man Assays ID numbers are shown in Table 1. RNA samples were normalized based on the TaqMan® Gene Expression Assays formouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) endogenous controls. RT-PCR was performed using a Standard TaqMan® PCR kit (P/N:4370074, AppliedBiosystem, USA) protocol on an Applied Biosystems 7500 Fast thermal Cycler. It was incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s. and 60°C for 1 min. All reactions were run in triplicate. The samples were quantified for all genes using the comparative Ct (ΔΔCt) method, as described in the Assays-on-Demand Users Manual

(AppliedBiosystems). In Table 1 gene names and assay IDs are given:

Statistical Anaysis: The data are given as means±SD. All statistics were done by statistical programme SPSS for Windows (version 21.0.1, SPSS, Inc. Chicago, Illinois). Since the datas showed normal distribution, paired t test was performed. p <0.05 was recognized as statistically significant.

Results

We obtained spontaneous contractions in strips.

Results of isolated organ bath: In uterine strips from non pregnant rats, myoinositol caused significant increase in the contractile activity in all doses. Application of 5μM, 50 μM and 500μM of myoinositol increased the frequency of contraction 128.9%, 50,8% and 112.7% respectively (Figure 1). Increase in amplitude of contractions were as 13.8%, 10.3% and 23.4% respectively (Figure 2). All the results were statistically significant (p<0.05).

When the area under curve is calculated, it is observed that increase is statistically significant in all doses. Increase is 202.9% (p<0.05) in 5 μM dose; 186.1% (p<0.05) in the 50 μM dose and 222.8% (p<0.05) in the 500 μM dose (Figure 3).

Same procedures were also repeated in a calcium free solution and any contraction was not observed at any dose in this solution. These results showed that myoinositol most probably causes conractions by using the extracellular calcium.

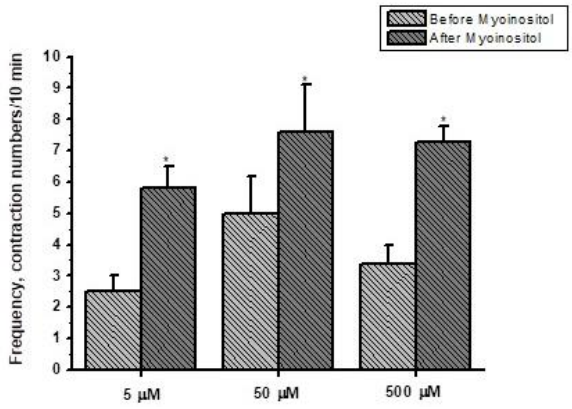

Fig. 1. Effect of different doses of myoinositol on frequency of uterus contraction. (n:7). Results are given as mean±S.D. Asteriks (*) means significant difference from its own control (p<0.05).

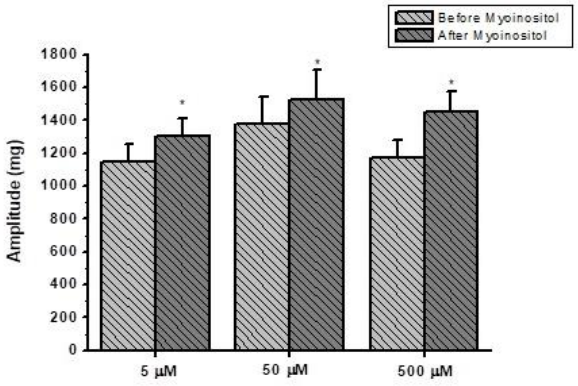

Fig. 2. Effects of different doses of myoinositol on amplitude of uterus contraction. (n:7). Results are given as mean±S.D. Asteriks (*) means significant difference from its own control (p<0.05).

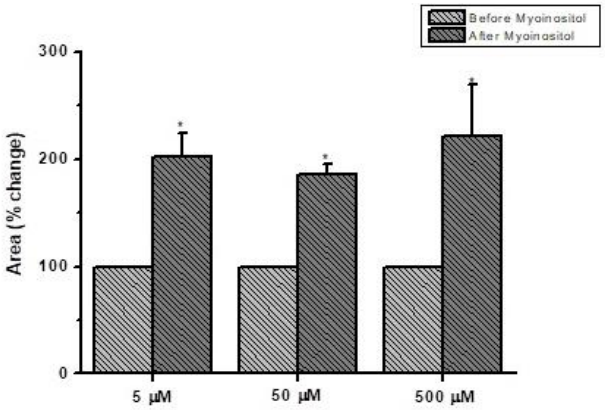

Fig. 3. Effects of different doses of myoinositol on area under curve (AUC) of uterus contraction. (n:7). Results are given as mean±S.D. Asteriks (*) means significant difference from its own control (p<0.05).

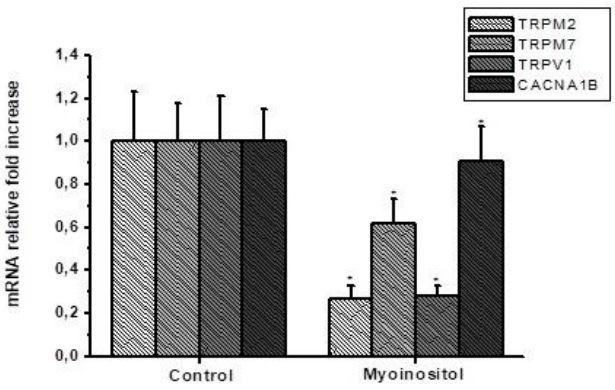

Fig. 4. Quantitative real time PCR analysis of TRPM2, TRPM7, TRPV1 and CACNA1B in uterus tissue of nonpregnant rat before and after the application of 50 μM myoinositol. (n:7). The symbol asteriks (*) indicates significant difference (p<0.05).

Table 1. Assay identifiers (IDs) used for qRT-PCR.

Gene Symbol	Gane Name	AssaysIDs
TRPM2	Transiente receptor potential cationchannel, subfamily M, member 2	Rn01429410_ml
TRPM7	Transiente receptor potential cation channel, subfamily M, member 2	Rn01328216_ml
TRPV1	Transiente receptor potential cationchannel, subfamily V, member 1	Rn00583117_ml
CACNA1B	Calcium channel, voltage-dependent, N type, alpha 1B subunit	Rn01643813_ml
Gapdh	Glycer aldehyde-3-phosphate dehydrogenase	Rn01775763_gl

Folic acid effect is also evaluated. 20 μM folic acid, which corresponds to the dose used in the preperate, was applied and no contraction was observed. After the observation of this effect, oxitocin was also applied to the medium to show the viability of these strips. Contractions were observed in all strips after the application of oxytocin which confirmed that all the tissues were viable and folic acid does not cause contraction in uterine muscle.

Results of Quantitative Real time PCR Assays: TRPM2 and TRPV1 expression significantly decreased after application of myoinositol ($p=0.01$ and $p=0.01$, respectively) whereas TRPM7 and CACNA1B expression did not change significantly when compared to control (for both) (Figure 4). It decreased TRPM2 expression to 0,27 times, TRPV1 to 0,28 times, TRPM7 to 0,62 times and CACNA1B to 0,91 times.

Discussion

We analysed the effect of myoinositol on non pregnant rat uterus muscle contraction for the first time and its results indicated that myoinositol causes uterine contractions possibly by permitting the influx of of extracellular calcium to the intracellular area.

Studies investigating the contractility patterns of human uterus was started in 1899 by Heinricius and by the development of intrauterine devices these studies become more abundant (15). After that days, many studies have been done and it is discovered that uterus is not a quiescent organ.

Uterine peristaltic activity changes throughout the menstruel cycle. Contractility increases with the progression of follicular phase (16). After ovulation, contractility of the uterus decreases due to the progesteron which is known to be important for successful implantation (17).

After the observation of these physiological changes, many studies were done and showed that excessive uterine contractions decrease implantation and pregnancy rates and these results proved the importance of uterine quiescence in implantation period (18-20). After the observation of impact of uterine contraction to implantation, the effects of many hormones have been studied.

A study evaluated the differences in uterus contraction which occurs after hCG application and found that contractions nearly stops at the seventh day of hCG administration. They came to the conclusion that success of transfer of day 5 embryo may be due to this effect (21). Celik et al. (22) showed the utero-relaxing effect of estrogen, progesteron and hCG in the myometrium of non pregnant ooferectomized rats.

Also the effects of some commonly used drugs on uterus contraction are studied. It is observed that glibenclamide and metformin, drugs used in diabetes and PCOS, does not change the amplitude and frequency of spontaneous uterine contractions (23). It was found in another rat study that T4 treatment changes myometrial activity (24).

After the observation of increased frequency of uterine contraction during ovarian stimulation cycles compared with the corresponding phase of natural menstruel cycles in a number of studies, a new pharmaterapeutic target arised and investigation of drugs reducing the contractility of uterus increased and conflicting results are found (25,26). Moon and colleagues reported that piroxicam, a nonsteroidal anti-inflammatory drug (NSAID), increased the implantation and pregnancy rate in women undergoing assisted-reproductive technology (ART) (27). In contrast, indomethacin which is a different type of NSAID did not improve the implantation rates (28). It is reported that hyoscine is more effective than indomethacin in ART cycles (29). On the other hand, ritodrine did not increase implantation (30).

In the light of above studies it can be concluded that management of silency of uterus in the implantation phase is critically important in

patients desiring pregnancy. So we planned to study the effect of myoinositol which is a drug commonly used in infertility clinics. While accumulating evidence suggests that myo-inositol improves the quantity and quality of oocytes in women with PCOS undergoing ART, data on its effects on live birth rates is much more limited.

It is known that smooth muscle contraction is mostly dependent on influx of calcium but release of sarcoplasmic reticulum calcium can also cause contraction. It is known that phosphoinositides play a role in muscle calcium signaling in differentiated muscle by the release of calcium from the sarcoplasmic reticulum. Phaneuf et al. (31) reported that oxytocin causes contraction by the activation of phospholipase C to produce inositol-1, 4, 5-triphosphate which releases calcium from intracellular stores and stimulates uterine contractions. Lan et al. (32) demonstrated that atosiban significantly decreased the mean frequency of uterine contractions and improved pregnancy rates.

We demonstrated that myoinositol causes contractions in rat uterus muscle. By the use of calcium free solution, it is also demonstrated that myoinositol shows its effect by causing influx of calcium from the extracellular area. It does not cause release of calcium from the sarcoplasmic reticulum as predicted.

To support this finding, TRPV1, TRPM2,TRPM7 and CACNA1B channels are studied. Consistent with our results obtained from the isolated organ bath study, it is observed that expression of mRNA levels of channels, TRPV1 and TRP decreased in statistically significant amounts and mRNA levels of channels located on plasma membrane did not change in statistically significant amount. In our study, we only studied four channels that are permeable to calcium. The study of more calcium channels can show other channels responsible from the influx of calcium. In this sense, our study offers a preliminary data.

Our study is important in many aspects. It is the first study showing the effect of myoinositol on uterus contraction. It is also demonstrated that ion channels on cell membrane are used for the calcium influx. Different channels including TRPs are investigated and it is shown that their levels changed differently after the application of drug.

This study has some limitations. It is an in vitro study done on rat myometrium. And only a few channels are studied.

In conclusion although it is demonstrated in our study that myoinositol caused contraction in uterus of an experimental rat model, further in vivo studies should be designed to show the direct effect of myoinositol on uterus contraction and its possible role in fertilization, implantation and continuation of pregnancy. Since importance of TRP channels in calcium homeostasis of smooth muscle has been started to be understood, they can be targets of drugs regulating uterus contraction in obstetrics and reproductive medicine. We hope that our study might contribute researchers in this aspect.

Conflict of interest: None of the authors have conflicts of interest and financial support is not taken.

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