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In vitro inhibition of uterine contractions using electrospun nanofibers loaded with nifedipine and ML7

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Abstract. – OBJECTIVE: To formulate a nanofiber-based controlled drug delivery system that could be effective in preventing uterine contractions and can be used for the treatment of preterm labor.

PATIENTS AND METHODS: We utilized uterine tissue samples obtained from ten pregnant women who underwent cesarean section at term to investigate the effect of nanofibers on spontaneous and induced myometrial contractions. We prepared nifedipine and ML7-loaded nanofibers using the electrospinning method with Poly(D,L-lactide-co-glycolide) (PLGA) polymer, resulted in seven groups of nanofibers, including a control group. Group I served as the control, Group II was non-drug loaded nanofiber, Group III was nifedipine (10^{-5} M) loaded nanofiber, Group IV was ML7 ($3x10^{-5}$ M) loaded nanofiber, Group V was ML7 ($3x10^{-5}$ M) and nifedipine (10^{-5} M) nanofiber, Group VI was ML7 ($3x10^{-5}$ M) and nifedipine ($3x10^{-5}$ M) nanofiber, and Group VII was ML7 ($3x10^{-5}$ M) and nifedipine (10^{-4} M) nanofiber. To evaluate the contractile response, the nanofibers loaded with different doses of ML7 and nifedipine were applied onto the tissue strips, and *in vitro* organ bath experiments were performed. Full-thickness uterine samples were cleared of the serosa and surrounding tissues, and eight strips (3x10 mm) were prepared from each sample. The seven different nanofiber formulations were gently placed and sutured onto the strips, with one strip always kept as the time control. We recorded spontaneous, KCl-induced, and stimulated cumulative oxytocin-induced contractions from all samples in all groups. After completing all experiments, the viability of the strips was checked, and weight measurement was recorded.

RESULTS: The administration of drug-loaded polymers resulted in a significant decrease in both the frequency and intensity of spontaneous and induced contractions in all groups ($p<0.01$). No significant difference was observed between the control group and the non-drug-loaded nanofiber group in post hoc analysis ($p=0.704$). In terms of amplitude and frequency of contractions, the most significant decrease was observed in group VII at cumulative oxytocin doses compared to the control and non-drug-loaded nanofiber groups ($p<0.05$). Moreover, group VI also showed a significant decrease in contraction intensity and frequency compared to the control and non-drug-loaded nanofiber groups ($p<0.05$). While the use of nifedipine and/or ML7-loaded nanofibers decreased both intensity and frequency of contraction, this attenuation was not significant compared to the control and empty polymer groups. However, a more significant inhibition was observed when ML7 was used with nifedipine at doses of $3x10^{-5}$ M and 10^{-4} M.

CONCLUSIONS: The results indicate that human uterine contractions can be inhibited using calcium channel blocker (nifedipine) and myosin light chain kinase inhibitor (ML7) loaded nanofibers in uterine tissue strips. These results strongly suggested the potential for the development of locally effective and safe controlled drug release systems to prevent premature birth.

Key Words:
Preterm Birth, Ca^{+2} channel blocker, Myosin light chain kinase inhibitor, Polymeric nanofiber formulation.

Introduction

World Health Organization (WHO) defines preterm birth (PTB) as delivery between $24^{0/7}$ to

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$36^{6/7}$ weeks of gestation[1]. Its worldwide incidence is relatively high; 15 million babies are documented to be born preterm, and the global PTB rate is 11% (5 to 18)[2]. As about one million babies die from prematurity, and of those who survive are disabled, PTB is considered one of the most important causes of neonatal mortality and morbidity[3-5]. Mortality increases with increasing immaturity, and the risk is very high in newborns born under 1,500 g.

Despite the considerably high incidence, the etiology of preterm labor is still under investigation. Premature activation of fetal hypothalamic-pituitary-adrenal axis, inflammation-infections, decidual hemorrhages, and pathological uterine distension are among the known causes of preterm birth[6-8]. The complex pathophysiology makes it difficult to take preventive measures.

Considering its very important consequences, it is crucial to intervene in the PTB process. The treatment strategy should aim to prolong the duration of the pregnancy as much as possible so that the chance of survival for the baby increases and the risks for complications and long-term disabilities are minimized. The primary goal for PTB treatment is to delay delivery for at least 48 hours; in this way, adequate time for fetal lung maturation with glucocorticoid administration and transfer of the patient to an advanced center can be gained. This strategy is proven[9] to increase the survival rate in newborns before 34 weeks of gestation.

Tocolytic drugs are the common choice of treatment to inhibit myometrial contractions, stop preterm labor, and postpone delivery. The choice among different tocolytic agents used in obstetric practice, cyclooxygenase (COX) inhibitors, calcium channel blockers, beta-agonists, and magnesium sulfate, is made by considering the efficacy, risks for both mother and baby and the side effects. The common complications vary from relatively tolerable flushing, nausea, vomiting, etc., to very serious side effects such as cardiac arrhythmias and even cardiac arrest[10-12]. Ca^{+2} channel blockers, specifically nifedipine, are the first choice if there are no contraindications, with a more favorable profile than other tocolytics. However, they may cause hypotension-related symptoms, nausea, flushing, lower extremity edema, headache, decreased uterine blood flow, and fetal oxygen saturation in extreme cases[13]. In extreme cases, hypotension may cause myocardial dysfunction[14,15]. Ca^{+2} channel blockers may also cause decreased uterine blood flow and fetal oxygen saturation[14,16].

Inhibition of preterm labor depends on the effective inhibition of myometrial contractions. Myometrial contractions involve a series of mechanisms initiated by increased intracellular calcium and the interaction of actin and myosin, specifically *via* myosin light-chain phosphorylation with myosin light-chain kinase (MLCK)[14,17]. The measures that induce tocolysis, i.e., effectively prevent the initiation and propagation of myometrial contractions, should decrease intracytoplasmic Ca^{+2} concentration and/or regulate the MLCK activity.

In recent years, there has been a tremendous evolution in controlled drug delivery systems, from macroscale to nanoscale to smart, targeted drug delivery. Novel drug delivery platforms help achieve maximum efficacy and safety in the treatment of diseases, as the drugs can be administered to the target area at a controlled rate and as precisely as possible. Among those different nanosystems designed and studied for various active pharmaceutical ingredients (API) and for different diseases, nanofibers have been widely investigated[18] not only as drug delivery systems but also as healthcare materials for use in tissue engineering and wound dressing. Nanofibers are well suited as carriers for drug delivery due to their unique properties to encapsulate other substances with a high surface-to-volume ratio, low density, tunable porosity, and excellent mechanical properties. They are widely used for drug release applications owing to their high drug loading capability, ease of handling, and cost effectiveness[19,20]. New advances in this area will help facilitate the targeted delivery of drugs while also helping to alleviate their side effects.

Considering the side effects limiting the systemic use of drugs for PTB, direct application of a drug delivery system to the uterus can reduce the side effects and effectively control uterine contractions. After the 24^{th} week of pregnancy, the uterus takes up a lot of space in the abdomen and becomes an easily accessible organ. Therefore, nanofibers loaded with API that can inhibit uterine contractions during PTB and applied directly to the surface of the uterus can effectively control PTB. For this purpose, nanofiber-based-controlled drug delivery formulations releasing Ca^{+2} channel blocker nifedipine and/or myosin light chain kinase inhibitor ML7 were prepared, and their effectiveness in reducing myometrium contractility was tested with *in vitro* uterine strips obtained from pregnant women at term.

Patients and Methods

Materials

This study was conducted between October 1, 2020, and October 1, 2021, at Hacettepe University Faculty of Medicine, Department of Physiology, Faculty of Pharmacy, Department of Pharmaceutical Technology and VM Medical Park Ankara Hospital, Gynecology and Obstetrics Clinic.

The study protocol was approved by the Hacettepe University Non-Interventional Clinical Research Ethics Board (Approval date: 17.09.2019 and number: 2019/23-10). We obtained uterine tissue samples from 10 pregnant women who had given their written informed consent and delivered by cesarean section at term. The participants had no history of drug use during pregnancy or chronic diseases, nor any complication related to the pregnancy. They did not have a history of COVID-19 infection, and they had not been vaccinated for COVID-19, as the vaccination program had not been initiated in Turkey at the time of the experiments. The uterine samples were then used to test the effect of the produced nanofibers on spontaneous and induced myometrial contractions.

The efficacy of nanofiber mats loaded with varying doses of Ca^{+2} channel blocker (nifedipine) and MLCK inhibitor (ML7) was tested on myometrial contraction. The selected doses were based on previous data[16,21] and their effectiveness was assessed in preliminary *in vitro* experiments prior to testing on uterine tissue samples.

The chemicals and API used were nifedipine (1,4-Dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinedicarboxylic acid dimethyl ester (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), MLCK inhibitor (ML7 hydrochloride, Biovision, Waltham, MA, USA) Resomer® RG 504 H; Poly(D,L-lactide-co-glycolide) (PLGA) 50:50, molecular weight MW=38.000-54.000, (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) and Tetrahydrofuran (99.8%, Lab Scan, Rong Pathum Wan, Bangkok, Thailand) and N,N-Dimethylmethanamide (99.9%, Carlo Erba, Val-de-Reuil, France). The ingredients of the Krebs-Henseleit were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany).

Preparation of Poly(lactic-co-glycolic acid) Nanofibers

Nifedipine and ML7-loaded nanofiber formulations were prepared using PLGA polymers and the electrospinning method. The API was completely dissolved in the organic solvent mixture system containing different solvents such as tetrahydrofuran, N, N-dimethyl formamide (DMF), and ethanol in certain proportions in a magnetic stirrer at room temperature and protected from light. The ratios of these solvents were determined by the characterization of nanofibers obtained during the formulation development studies and by evaluating the nanofiber structure formations.

After this process, PLGA was dissolved in the solution prepared by DMF: Tetrahydrofuran (THF) in 35:65 volume/volume (v/v) ratio. The final PLGA solution was in 25% weight/volume (w/v) ratio, i.e. 25 g of PLGA in 100 ml of DMF/THF (35:65 v/v) solution. Then, nifedipine or ML7, or a combination of them, were added to the solution separately. The loaded doses of nifedipine and ML7 were given to all groups, as shown in Table I.

The solutions were mixed and stirred for 2 h. Afterward, the polymer solution was transferred to a 10 mL syringe and connected to the electrospinning device by attaching it horizontally to the injector pump (NE-1000 Programmable single syringe pump, New EraPump Systems, Farmingdale, NY, USA). The electrospinning method parameters used in the production were adjusted as follows: In the Inovenso Ne300 Electrospinning device (Inovenso Technology, Cambridge, MA, USA), the flow rate was 4 mL/min, the applied voltage was 19 kV, and the distance between the

Table I. The experimental groups and the doses of nifedipine and ML7 loaded to the nanofibers.

Group	Name	Concentration
I	Control (No nanofiber)	N/A
II	Non-drug loaded empty nanofiber	No drug
III	Nifedipine loaded nanofiber	10^{-5} M
IV	ML7 loaded nanofiber	$3x10^{-5}$ M
V	ML7 and Nifedipine loaded nanofiber	$3x10^{-5}$ M/10^{-5} M
VI	ML7 and Nifedipine loaded nanofiber	$3x10^{-5}$ M/$3x10^{-5}$ M
VII	ML7 and Nifedipine loaded nanofiber	$3x10^{-5}$ M/10^{-4}M

injector tip and the collector was 14 cm. Randomly oriented nanofibers were removed from the surface of the collector in the form of a rotating cylinder (rotation speed 200 rpm) covered with aluminum foil, and formulations were obtained.

Characterization of Nanofibers

The surface morphology of the electrospun nanofibers was observed with Scanning Electron Microscopy- (SEM, Quanta 400F, FEI Company, Hillsboro, OR, USA) at a voltage of 30 kV. Prior to analysis, the samples were mounted on aluminum stubs and sputter-coated with gold-palladium (AuPd) under an argon atmosphere. The average diameter of the resulting nanofibers was measured using ImageJ software (National Institute of Health, Bethesda, Maryland, USA). A minimum of 100 nanofibers were randomly selected from scanning electron microscopy images and analyzed.

The nanofibers loaded by the different doses of MLCK inhibitor, ML7, and Ca^{+2} channel blocker, nifedipine applied on the tissue strips, and *in vitro* organ bath experiments were performed to evaluate the contractile response.

Preparation of Tissue Strips and Evaluation of Myometrial Contraction

A full-thickness tissue sample of approximately 10x10 mm was taken from the upper edge of the uterine incision during the cesarean section. The tissue samples were transferred to the laboratory in cold oxygenated Krebs-Hanseleit solution (118.4 mM NaCl, 1.2 mM KH_2PO_4, 4.7 mM KCl, 25.0 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 12.2 mM glucose; pH 7.35- 7.40) in 30-45 min. The full-thickness uterine samples were cleared of serosa, and all the surrounding tissues and eight strips were prepared (3x10 mm) from each sample. The seven different nanofiber formulations (Table I) were gently placed and sutured on the strips. One of the strips was always spared as time control (TC), and neither any nanofiber was attached to nor stimulated with oxytocin.

The myometrium strips were mounted in double-jacketed organ baths filled with Krebs-Hanseleit solution bubbled with 95% O_2 and 5% CO_2 mixture at 37°C under 0.5-1 g tension. The strips were connected to the isometric force displacement transducers (MAY FDT05, Commat, Ankara, Turkey), and the force of contraction was recorded in real-time by a data acquisition/analysis system (BIOPAC MP36, Biopac Systems Inc., Goleta, CA, USA). The bath fluid was renewed with washouts every 15 minutes for an hour until spontaneous contraction appeared, and the strips stabilized.

The experimental protocol is described below.

Spontaneous contractions

After equilibration, spontaneous contractions of the strips were recorded for 30 minutes, and the frequency of the contractions (Hz) and the maximum contraction amplitude (gr/mg tissue) were determined and presented as the percentage of the response evoked by KCl stimulation.

Stimulated contractile response

The strips in all four baths were tested with 120 mM KCl to ensure the viability of the tissue and determine the maximum contractile response and provide a standard value for all the other measurements. The contractile responses of the strips were recorded for 10 minutes after KCl application, followed by three washouts and equilibration for at least 60 minutes before the next protocol was initiated.

The KCl-induced contraction response was determined as the maximum contraction amplitude (100%), and the spontaneous and induced responses of the strips were standardized accordingly.

In the second stimulation protocol, cumulative dose-contraction response to oxytocin was recorded (10^{-11}-10^{-5} M). The consecutive higher dose was applied every 10 minutes. The contraction frequency (Hz) and the maximum force of contraction (gr/gr tissue) were determined, and the values as the percentage of the response evoked by KCl were calculated. After all the protocols were completed, the viability of the strips was checked by applying 120 mM KCl to all strips. The strips were detached from the nanofibers and weighed.

After the protocols for the first 4 strips were completed, the other strips were attached to the respective nanofibers, and the same experimental protocol was applied. The order of the strips was determined randomly for each patient, and the tissue was kept in a cold oxygenated buffer the whole time until they were placed in the organ baths.

Statistical Analysis

The data was processed by BSLpro software (Biopac Systems Inc., Goleta, CA, USA), the contraction responses, both amplitude and AUC were normalized for strip weight and presented as the percent of KCl-induced responses. The obtained data were analyzed using SPSS 22.0 (IBM Corp.,

Table II. Demographic and clinical findings of the patients.

	n=10
Age (Years)	29.5 (25-34)
Gravida (number)	2 (2-3)
Para (number)	1 (1-2)
BMI (kg/m^2)	28.3 (26.3-33.1)

Armonk, NY, USA) data analysis program. After the distribution of the obtained data was determined with the Shapiro-Wilks test, the normally distributed variables were evaluated with repeated measures of ANOVA and post-hoc Tukey test. Emax (Maximum) and pEC_{50} values (–log EC_{50}, drug concentration evoking half-maximal response) were calculated using GraphPadPrism (v.5.01; GraphPad Software, San Diego, CA, USA). $p<0.05$ was considered statistically significant.

Results

The myometrium samples were obtained from 10 women who underwent cesarean section at term and gave consent to participate in this study. The demographic and obstetric data of the patients are given in Table II.

Morphology of Nanofibers

All nanofiber formulations were produced successfully by the electrospinning process. Figure 1 shows SEM images and average diameters of the nanofibers (produced from PLGA solution by electrospinning). The fiber diameter, although different between groups, had no statistical significance ($p>0.05$).

Results of the In-Vitro Functional Studies

The study evaluated the effects of drug-loaded nanofibers on spontaneous, KCl-induced, and oxytocin-induced myometrial contractions. The force and frequency of spontaneous contractions varied among the different groups. The spontaneous activity of the strips exposed to drug-loaded nanofibers was attenuated both for frequency and force of contraction. The lowest frequency was observed in strips treated with both nifedipine and ML7, and the force of contraction was reduced by both nifedipine, ML7, and their combination. The nanofibers carrying both agents were particularly effective in reducing the force of contraction when compared to strips with no API or single agent application (Table III). The KCl is a depolarizing agent used to obtain reference contraction response (Table III). The maximum response to KCl stimulation was different between groups. All the API significantly reduced the maximum force of the KCl-induced contraction; the lowest values were obtained from Group IV, ML7 alone, and Group VII (10^{-4} M nifedipine + $3x10^{-5}$ M ML7). The results were comparable between all the drugs and their combinations.

The cumulative oxytocin dose response of the strips treated with nanofibers was evaluated for the maximum force of contraction, AUC for 10 minutes, and the frequency of myometrial contractions. The myometrium response to cumulative oxytocin stimulation in different experimental groups is exemplified in Figure 2. The cumulative oxytocin response exhibited an increase in the amplitude and frequency of the contractions with increasing doses. The findings suggest a significant decrease in both the force of contraction and frequency in the combined drug-loaded nanofiber groups ($p<0.001$), which is also supported by the AUC values. While all the API-loaded fibers led to significant attenuation in response to oxytocin, the most effective inhibition of myometrial contractions was observed in Group VII (10^{-4} M nifedipine+$3x10^{-5}$ M ML7) (Figure 3). Oxytocin was not applied to the TC strips; instead, an equivalent volume of Krebs solution was added at the time points of oxytocin application.

Post hoc analysis between groups showed no statistical difference between the control group and the non-drug-loaded nanofiber group ($p=0.704$). On the contrary, drug-loaded nanofibers responded less to oxytocin ($p<0.05$) (Figure 3). Group VII exhibited the most significant inhibition even at the highest oxytocin doses compared to the control and non-drug-loaded nanofiber groups (p VII *vs.* I=0.005 and p VII *vs.* II=0.045).

The attenuation by nifedipine-only was not as effective as ML7-only or the combination groups. The pairwise comparisons of drug-loaded nanofiber groups did not show any significant differences ($p>0.05$) except the difference between Group VII and Group III ($p<0.05$), which is more prominent at higher oxytocin doses (Figure 3).

The AUC represents the total force applied at unit time and is crucial for partition. The AUC values exhibited a similar pattern to the maximum force of contraction so that the most significant inhibition was recorded in Group VII, significantly lower than the control and non-drug-loaded nanofiber groups (p VII *vs.* I and VII *vs.*

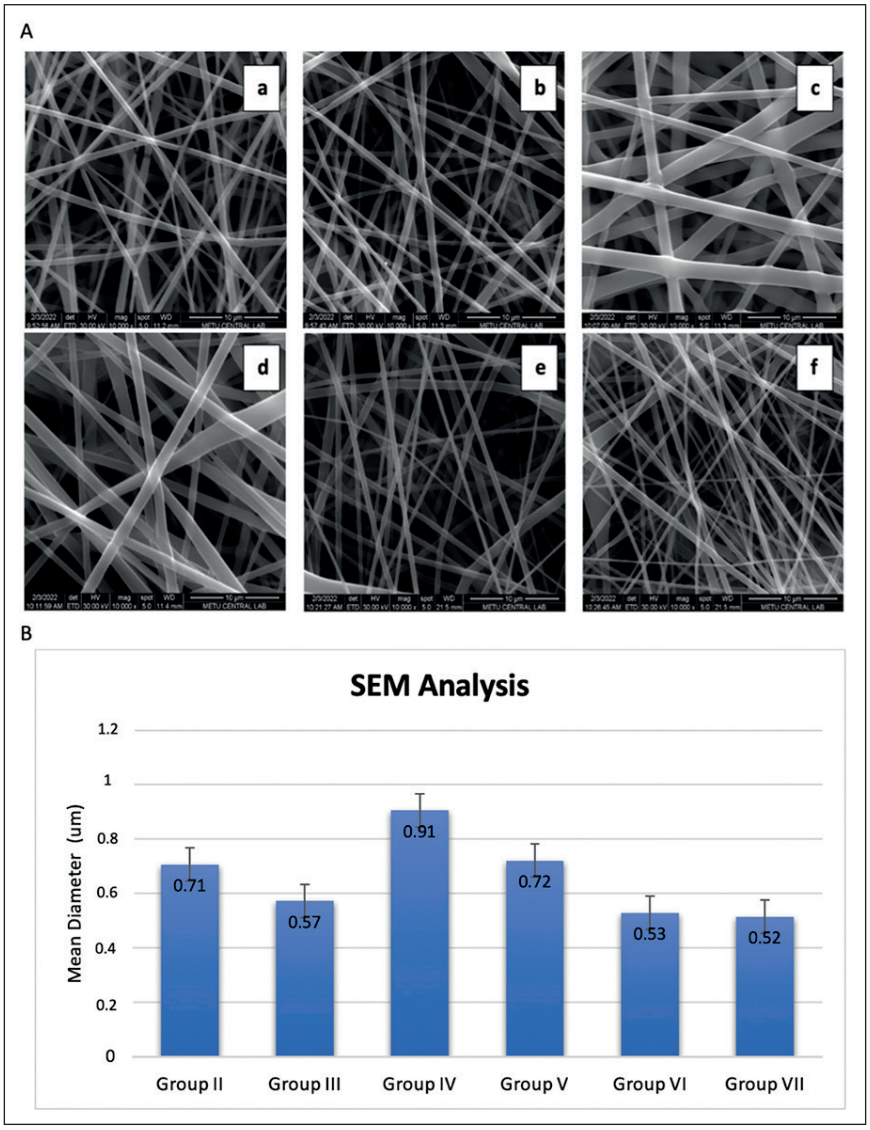

Figure 1. **A**, Scanning electron microscopic appearance of the nanofibers (magnification ratio: X 10.000; voltage 30 kv), (**a**) Group II; (**b**) Group III; (**c**) Group IV; (**d**) Group V; (**e**) Group VI; (**f**) Group VII and (**B**), fiber diameters (Mean±SEM) of the nanofibers. Group II: Non-drug loaded nanofiber; Group III: Nifedipine (10^{-5} M) loaded nanofiber; Group IV: ML7 ($3x10^{-5}$ M) loaded nanofiber; Group V: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-5} M) nanofiber; Group VI: ML7 ($3x10^{-5}$ M) and Nifedipine ($3x10^{-5}$ M) nanofiber; Group VII: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-4} M) nanofiber.

II<0.001). Moreover, Group VII was more effective in the inhibition of oxytocin-induced myometrial contractions than any other formulation used (p VI *vs.* I=0.022) and non-drug loaded nanofiber groups (p VII *vs.* VI, V, IV and III <0.005).

There was a statistically significant difference in the frequency of contractions between the groups (p<0.001) (Table IV). Pairwise comparisons revealed that all drug-loaded polymers significantly decreased contraction frequencies at oxytocin doses lower than 10^{-8} M (p<0.05). At higher oxytocin doses, the combined polymers (nifedipine and ML7) remained effective, while the ML7-only polymer lost its effectiveness (p<0.05).

The Emax values revealed a significantly lower response in drug-exposed strips, indicating the effectiveness of nanofiber polymers. Likewise, lower pEC_{50} values supported the effective inhibition of contractions. The most significant effects were observed in Group VII (Table V).

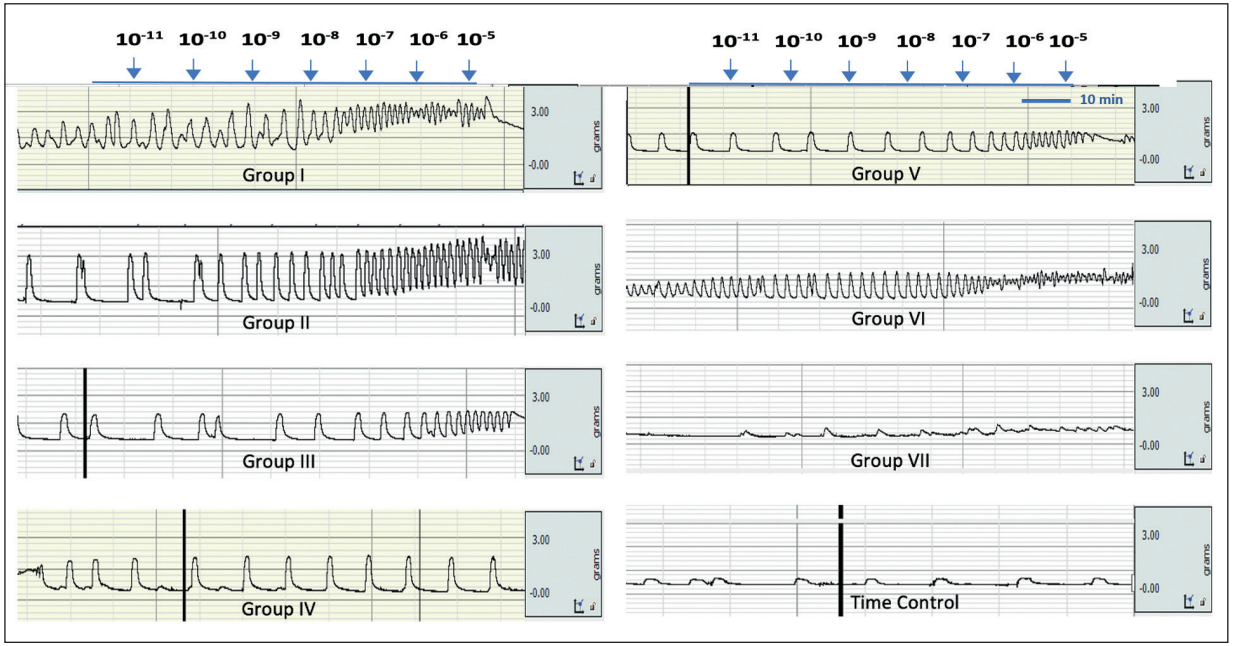

Figure 2. The real-time recording of oxytocin dose-contraction responses of the myometrial strips in experimental groups. Group I: Control; Group II: Non-drug loaded; Group III: Nifedipine (10^{-5} M) loaded; Group IV: ML7 ($3x10^{-5}$ M) loaded; Group V: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-5} M) loaded; Group VI: ML7 ($3x10^{-5}$ M) and Nifedipine ($3x10^{-5}$ M) loaded; Group VII: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-4} M) loaded nanofibers.

Table III. The frequency and maximum force of contraction of spontaneous activity and KCl-induced response of myometrial strips in experimental groups.

		Time control	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Spontaneous contraction	Frequency (contraction number/5 min)	1.23±0.02	1.31±0.01	1.25±0.01	1.10±0.02	1.03±0.02*	1.0±0.03*	0.92±0.03*	0.88±0.02*
	Force of contraction (% of KCl-induced contraction)	21.33±1.89	20.86±3.01	22.16±2.96	16.90±2.38	17.04±2.45	13.18±0.99*	11.36±1.69*,**	9.08±1.35*,**
KCl-induced	Maximum Force of Contraction (% of KCl-induced contraction)	100.0	103.28±4.80	85.85±1.92	68.45±1.68*	54.90±6.03*,***	64.01±4.85*,	56.83±2.95*,***	52.49±4.20*,***

*p<0.05 *vs.* TC, Group I and II **p<0.05 *vs.* Group III and IV, ***p<0.005 *vs.* Group III.

Table IV. Frequency (contraction number/5 min).

		Time control	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Oxytocin concentration (M)	10^{-11}	1.2±0.01	1.4±0.01	1.4±0.02	1.1±0.02	1.1±0.02	1.1±0.03	1.1±0.02	0.9±0.02*
	10^{-10}	1.2±0.02	1.7±0.2	1.5±0.04	1.1±0.02	1.1±0.03	1.1±0.03	1.0±0.03	0.9±0.02*
	10^{-9}	1.2±0.02	2.0±0.1	1.9±0.1	1.1±0.02	1.2±0.03	1.2±0.03	1.1±0.03	1.0±0.02*
	10^{-8}	1.2±0.01	2.5±0.2	2.4±0.2	1.3±0.03*	1.3±0.03*	1.3±0.03*	1.1±0.03*	1.0±0.02*
	10^{-7}	1.2±0.02	3.3±0.2	3.2±0.2	1.4±0.03*	1.4±0.03*	1.4±0.03*	1.2±0.04*	1.0±0.02*
	10^{-6}	1.2±0.01	4.1±0.2	3.8±0.2	1.6±0.05*	1.4±0.03*	1.4±0.03*	1.3±0.05*	1.1±0.03*
	10^{-5}	1.2±0.02	4.7±0.2	4.5±0.2	1.8±0.06*	1.6±0.05*	1.6±0.05*	1.4±0.05*	1.1±0.04**

*p<0.05 *vs*. TC, Group I and II **p<0.05 *vs*. Group III.

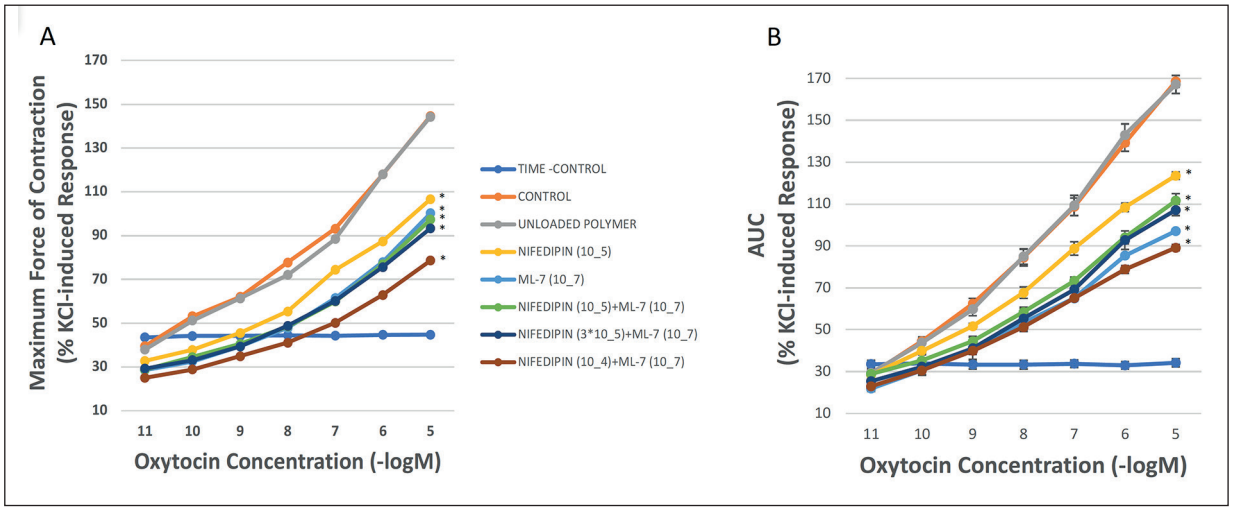

Figure 3. Oxytocin dose-response curves of the myometrial strips. **A**, The maximum force of contraction at each dose is used. **B**, Area under the curve (AUC) recorded between the application of two consecutive doses. The values are given as percent of the KCl-induced contractions. *p<0.05 *vs*. Group I and II. Group I: Control; Group II: Non-drug loaded; Group III: Nifedipine (10^{-5} M) loaded; Group IV: ML7 ($3x10^{-5}$ M) loaded; Group V: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-5} M) loaded; Group VI: ML7 ($3x10^{-5}$ M) and Nifedipine ($3x10^{-5}$ M) loaded; Group VII: ML7 ($3x10^{-5}$ M) and Nifedipine (10^{-4} M) loaded nanofibers.

Discussion

In this study, we successfully prepared drug-loaded nanofiber mats and applied them to myometrial strips. The functional experiments revealed that the utilization of nifedipine + ML7-loaded nanofibers resulted in a reduction of both the amplitude and frequency of uterine contractions. The effectiveness of nanofibers in reducing uterine contractions was found to be higher when the dose of nifedipine exceeded 10^{-5} M.

Premature birth remains a multifaceted issue resulting from numerous factors and continues to be the leading cause of both mortality and morbidity among newborns, with rates ranging from 5% to 18% in high- and low-income countries[22-24]. Although extensive research is ongoing, the molecular mechanisms responsible for the onset of PB

Table V. E_{max} and pEC_{50} values for the inhibitor effects of the drug-loaded nanofibers on oxytocin-stimulated myometrium strips.

Group	E_{max}	pEC_{50}
I	144.63±1.47	7.38±0.15
II	144.29±1.94	7.11±0.14
III	106.64± 1.60*	5.23±0.17*
IV	100.18±1.68*	4.99±0.17*
V	97.32±2.04*	4.9±0.19*
VI	93.33±1.74*,**	3.78±0.13*
VII	78.59±1.89*,**	3.15±0.10*,**

*p<0.005 *vs*. Group I and Group II **p<0.05 *vs*. Group III. E_{max}: Maximum contraction. pEC_{50}: -log EC_{50}, oxytocin concentration evoking a half-maximal response.

remain unclear. The prevailing hypothesis suggests that PB may be caused by mechanisms that prematurely stimulate or bypass a "parturition complex cascade"[24,25]. Due to the unclear cause, preventive measures for PB are limited, making treatment even more critical. Effective treatment can provide the necessary time to improve fetal maturation and increase the newborn's chance of survival. Typically, treatment is not required after the 34th week of gestation due to a lower risk of perinatal morbidity and mortality. However, PRB occurring before the 34th week of gestation must be treated. Unfortunately, the available treatment options, such as cyclooxygenase inhibitors, calcium channel blockers, beta mimetics, and magnesium sulfate, are not very effective, and complications are common, limiting their use[26]. A Cochrane systematic analysis[10] on the effectiveness of tocolytic agents revealed that all the tocolytic drugs, including beta mimetics, calcium channel blockers, magnesium sulfate, oxytocin receptor antagonists, and nitric oxide donors, as well as their combinations, were found to be potentially useful in postponing preterm birth by 48 hours to 7 days. However, the analysis[10] also noted that these tocolytic treatments may have adverse side effects that could result in treatment discontinuation.

Our objective was to create a treatment method for PTB that would have lower systemic side effects and be effective over the long term. Our motivation for developing a controlled drug release system to be applied directly to the uterus stemmed from several factors. First, we believed that direct application would allow for more effective control or inhibition of myometrial contractions. Additionally, administering drug-loaded formulations directly to the uterus could help prevent systemic side effects, reduce the required tocolytic dose, and extend the duration of uterine contraction's inhibition, potentially leading to improved fetal outcomes.

The smooth muscles in different organs exhibit variations in their response to stimulating agonists, receptors, and dominant signaling pathways, in addition to their common contraction mechanisms[27,28]. Therefore, interventions aimed at inducing contraction or relaxation should target specific receptors or key steps in the process. Figure 4 illustrates a simplified diagram of the myometrial contraction and relaxation cycle. A crucial requirement for contraction in smooth muscle, including myometrial tissue, is the increase in cytoplasmic calcium concentration and myosin light chain phosphorylation *via* MLCK. Regardless of the stimulus that triggers contraction, calcium enters the myocytes through voltage-gated L-type calcium channels from the extracellular fluid and is released from intracellular stores in the sarcoplasmic reticulum (SR)[29,30].

Elevated levels of intracellular calcium bind with calmodulin, thereby augmenting the activity of myosin light chain kinase, resulting in the phosphorylation of MLC and ultimately leading to muscle contraction[30]. It may seem simple to prevent contractions by intervening in these steps, but it is a complex task. Smooth muscles are present in various hollow organs like the stomach, intestines, bladder, blood and lymphatic vessels, and airways, making it difficult to implement a systemic treatment to target these steps in the contraction-relaxation cascade without causing severe systemic side effects. Tissue-specific approaches and local drug delivery systems may offer a new perspective on treating medical conditions efficiently without causing systemic side effects. This approach could be particularly useful for treating hollow organs and short-term conditions, such as preventing uterine contractions in preterm birth. To date, there has been no prior research on using a nanofiber formulation with controlled drug release for direct administration to the uterus to halt uterine contractions. Previous studies[1,10] on preterm birth treatment have focused on systemic tocolytic drug use and cervical pessary applications. Therefore, we believe that our research is important and has the potential to be very effective. In this study, nanofiber polymers were developed by incorporating two drugs: a calcium channel blocker nifedipine, and myosin light chain kinase inhibitor ML7. Calcium channel blockers are commonly prescribed as a first-line treatment option for PTB because they can effectively relax smooth muscles[13]. Ca^{+2} channel blockers impede both the influx of calcium ions across the cell membrane and their release from the sarcoplasmic reticulum, leading to a reduction in the amount of calcium ions within the cytoplasm. This decline in cytoplasmic calcium concentrations obstructs the phosphorylation of myosin light chain kinase that depends on calcium, eventually resulting in the relaxation of myometrial smooth muscle[17]. Most trials[10,13] evaluating the use of calcium channel blockers to prevent acute preterm labor have utilized nifedipine. According to a meta-analysis[13] of randomized trials comparing placebo or no treatment with calcium channel blockers for PTB, the administration of these blockers lowered the likelihood of delivery within 48 hours (RR 0.30, 95% CI 0.21-0.43: two studies, n=173 participants).

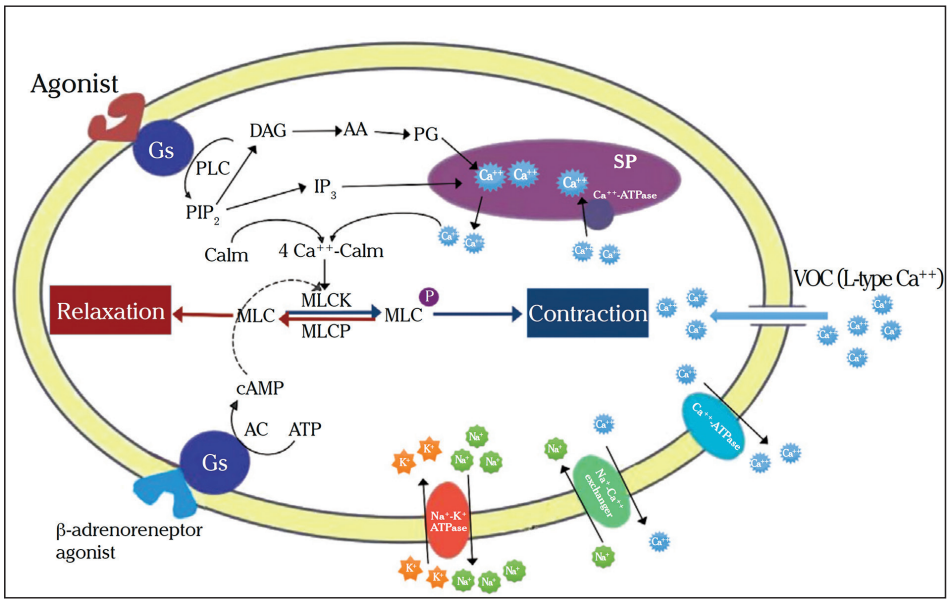

Figure 4. Schema of contraction and relaxation cycle in smooth muscle cell (Figure is adopted with permission from Pehlivanoğlu et al[17]).

Nifedipine is associated with systemic side effects, the most important and limiting of which is hypotension. Moreover, due to its rapid elimination and relatively short half-life of around two hours, it requires repeated daily administration to achieve and maintain effective plasma levels[10,32]. Additionally, it has a low and unregulated bioavailability of roughly 50% after oral administration due to a high first-pass effect. Given these characteristics, sustained-release formulations could be a suitable option for nifedipine and other drugs with biological half-lives in the range of 2-8 hours[33]. Nifedipine is primarily utilized for the management of hypertension and angina, but research[34,35] has been conducted to create controlled drug delivery systems using water-soluble polymers or nanoparticles. These systems are intended to enhance the bioavailability of nifedipine and ensure its regulated release. However, these studies are not for obstetric practice.

ML7 (Hexahydro-1-[(5-Iodo-1-naphthaleny) sulfonyl]-1H-1,4-diazepine hydrochloride) is a highly effective and specific inhibitor of smooth muscle myosin light chain kinase, which plays a crucial role in regulating smooth muscle contraction through myosin phosphorylation activated by Ca^{2+}-calmodulin. Suppressing MLCK activity results in attenuated smooth muscle contraction. While no research has been conducted on uterine smooth muscle, reports[36] have shown that inhibiting MLCK can lead to long-term relaxation in vascular smooth muscle. ML7 has also been demonstrated[37] to inhibit the contraction of the trabecular meshwork in the eye. During our research, ML7 was administered either alone or in conjunction with nifedipine. Results showed that the administration of ML7 alone was effective in reducing both the amplitude and frequency of contractions. When used in combination with nifedipine, a more substantial inhibition of myometrial activity was observed.

Polymeric nanofibers have been increasingly employed in pharmaceutical applications in recent years due to their distinctive features, including the ease of production, the ability to process various materials into fibers, a vast and adaptable surface area, and a multifaceted pore structure[38]. Nanofibers that exhibit effective drug-loading capability, slow release, and excellent stability are garnering significant attention due to their potential to control local drug release. In addition, nanofibers offer several advantages in topical drug applications. The fibrous surface structure facilitates strong adhesion to mucous layers, while their nanoporous structure instantly absorbs moisture from the mucous layers through small particles[39]. This superior adhesion to biological surfaces makes nanofibers an excellent choice for topical drug delivery devices[40]. In our research, we observed that the electrospun nanofibers exhibited relatively good adhesion when applied to uterine tissue. However, during experiments in the organ bath, the fibers were found to slide off the tis-

sue strips, indicating a need for fixation. To address this limitation, we sutured the nanofibers onto the strips using 5.0 vicryl. It is important to note that this study is the first of its kind, and there are no comparable studies in the literature. To improve adhesion, future research may explore producing the nanofibers in a microneedle structure or adding a mucoadhesive layer to the drug delivery system.

Our study had a limitation in that it was not possible to conduct release pharmacokinetic experiments on drug-loaded polymers due to the low drug doses used in the experiments. However, we are currently planning new studies to assess the drugs' release times and stability. In the next phase, toxicology tests will also be conducted to determine the safety of these polymeric formulations.

The application of a controlled drug delivery system to the uterus in real life is a topic of debate due to the invasive procedure it requires. Nevertheless, given the large size of the pregnant uterus within the abdomen and its accessibility beneath the anterior abdominal wall, a minimally invasive laparoscopic technique could be used to place a drug-loaded polymer on one or more points of the uterus. By placing nanofiber mats that provide controlled release on various areas of the uterus, it is believed that the onset and spread of myometrial contractions could be prevented.

The discussion should also include the identification of the specific patient population for whom this invasive procedure will be suitable. The primary focus is on patients who go into labor before completing 34 weeks of gestation, as they stand to benefit the most. This procedure has the potential to delay delivery by several days, thanks to its prolonged tocolytic effect, leading to a more significant reduction in neonatal morbidity and mortality. Furthermore, by avoiding systemic side effects, the tocolytic activity can be sustained for an extended period of days and even weeks. In the future, the development of nanofibers that contain varying drug doses and have a more extended-release time may offer patients the opportunity to deliver closer to term. In addition, as we experienced COVID-19 pandemic and ZIKA virus infections in pregnant women and their devastating consequences, future perspectives should include the history of these and similar diseases, since there are various reports[41] regarding the fetal and maternal outcome after infections.

Undoubtedly, childbirth is a very complex process, with several mechanisms involved, including inflammation, stress hormones, and other hormonal factors. Nanofibers have the potential to be utilized as carriers of substances that can locally modulate inflammatory and hormonal processes, thereby preventing uterine contractions. Given these factors, we believe that there is a substantial opportunity for advancement in this field not only for preterm delivery but also for other gestational pathologies such as preeclempsia[42].

Conclusions

Our study reveals that the drug-loaded nanofibers, particularly those containing both nifedipine and ML7, effectively attenuated myometrial contractions and frequency in response to oxytocin. These results could potentially have important implications for the treatment of conditions such as preterm labor. However, further research is needed to confirm these findings and explore the potential clinical applications.

Conflict of Interest

The authors declare no conflict of interest.

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Ethics Approval

The study protocol was approved by the Hacettepe University Non-Interventional Clinical Research Ethics Board (issued by 2019/23-10).

Informed Consent

The protocol was performed in accordance with the Declaration of Helsinki, and the participants provided written informed consent.

Authors' Contribution

YK, BP designed, analyzed, and wrote the article. YK, BP, MDE, DÖ performed in-vitro electrophysiological experiments. BT, HE prepared nanofibers. All authors contributed to and approved the final version of the paper.

Data Availability

All data associated with this paper are available from the corresponding author upon reasonable request.

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