EDITORS' CHOICE

Cannabinoids and the human uterus during pregnancy

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Objective: The aim of this study was to investigate the expression of cannabinoid receptors in human uterine smooth muscle during pregnancy and to evaluate the effects of endogenous and exogenous cannabinoids on myometrial contractility in vitro.
Study design: Human myometrial biopsy specimens were obtained at elective cesarean delivery and snap frozen or mounted for isometric recording under physiologic conditions. Cumulative doses of the endogenous cannabinoid anandamide or the exogenous cannabinoid Δ^9 (indicates a double bond between carbons 9 and 10) tetrahydrocannabinol were added in the range 1 nmol/L to 100 μmol/L. Selectivity of the cannabinoid receptor agonists was investigated with specific antagonists for the CB_1 and the CB_2 receptors. Reverse transcription–polymerase chain reaction with primers for the CB_1 and CB_2 receptors was performed on messenger RNA that was isolated from human pregnant myometrium.
Results: Both anandamide and Δ^9-tetrahydrocannabinol exerted a direct relaxant effect on human pregnant myometrium in vitro, which was of equal potency for both compounds. This relaxant effect was antagonized by the specific CB_1 receptor antagonist, SR 141716, but not by the specific CB_2 receptor antagonist, SR 144528 (n = 6 specimens, $P < .01$). Both the CB_1 and CB_2 receptors are expressed in human myometrium.
Conclusions: Both endogenous and exogenous cannabinoids exert a potent and direct relaxant effect on human pregnant myometrium, which is mediated through the CB_1 receptor. This highlights a possible role for endogenous cannabinoids during human parturition and pregnancy. These results also support the view that the use of exogenous cannabinoids during pregnancy is not linked independently with preterm labor.
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There has been a marked increase in marijuana (cannabis) use during pregnancy in the last 20 years.[1-3] This increase has raised questions about its potential adverse effects for the mother and developing fetus.[3-7] Specific problems (such as low fetal birth weight, preterm delivery, miscarriage, stillbirth, congenital malformation, and neonatal complications) have been associated with cannabis use during pregnancy,[3-8] with evidence that frequent or regular use is more hazardous than occasional use.[2]

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The principal biologically active component of marijuana, Δ^9-tetrahydrocannabinol (Δ^9-THC), is a member of the cannabinoid family, which is known to exert the effects by way of the cannabinoid receptor subtypes, CB_1 and CB_2.[8,9] In 1992, after the discovery of the cannabinoid receptors, it was reported that arachidonoyl ethanolamine, also known as anandamide, is an endogenous ligand for these receptors (ie, an endogenous cannabinoid or endocannabinoid).[10] Since then much research has focused on the role and effects of cannabinoids in different tissue systems.[11-13] Little is known about the direct effects of cannabinoids on human reproductive tissues. In rats, it has been shown that the administration of anandamide or Δ^9-THC prolonged the duration of pregnancy and increased the frequency of stillbirths.[14,15] It was suggested that this prolongation of pregnancy in rats may be linked to a reduction in uterine prostaglandin synthesis. However, CB_1 and CB_2 receptors are coupled through $G_{i/o}$ proteins to other intracellular signaling pathways (such as adenylate cyclase, mitogen-activated protein kinase, and ion channels)[16-18] and therefore may have the ability to modulate uterine contractility directly. The aims of this study were to investigate for expression of the CB_1 and CB_2 receptors in human uterine smooth muscle (myometrium) that was obtained during pregnancy, to evaluate the direct effects of the cannabinoids anandamide and Δ^9-THC on human uterine contractility during pregnancy, and to determine whether the uterine effects of these cannabinoids were mediated by the stimulation of the CB_1 or CB_2 receptors.

Methods

Tissue collection

Biopsy specimens of human myometrial tissue during pregnancy were obtained at elective cesarean delivery (ie, women not in labor) performed at term (37-42 weeks of gestation, n = 11 women). The biopsy specimens were excised from the upper midline portion of the incision in the lower uterine segment. Ethical committee approval was obtained from the Research Ethics Committee at University College Hospital, Galway, and recruitment was by written informed consent. Immediately on collection, the tissue for organ bath studies was placed in Krebs-Henseleit physiologic salt solution of the following composition: potassium chloride 4.7 mmol/L, sodium chloride 118 mmol/L, magnesium sulfate 1.2 mmol/L, calcium chloride 1.2 mmol/L, potassium phosphate 1.2 mmol/L, sodium bicarbonate 25 mmol/L, and glucose 11 mmol/L (Sigma-Aldrich, Dublin, Ireland). Tissue for the organ bath studies was stored at 4°C and used within 12 hours of collection. Immediately on collection tissue for RNA extraction was rinsed in normal saline solution, snap frozen in liquid nitrogen, and stored at −70°C.

Myometrial tissue bath experiments

Longitudinal myometrial strips were dissected, measuring approximately $2 \times 2 \times 10$ mm, and mounted under $2g$ of tension in organ tissue baths for isometric recording as previously described.[19-21] Each tissue bath contained 20 mL of Krebs-Henseleit physiologic salt solution, which was maintained at 37°C, pH 7.4, and gassed continuously with a mixture of 95% oxygen/ 5% carbon dioxide. The strips were allowed to equilibrate for at least 1 hour before the addition of oxytocin as a uterotonic agent. The Krebs-Henseleit physiologic salt solution in the tissue baths was changed every 15 minutes during the equilibration period. After equilibration, contractions were stimulated by bath exposure of the strips to oxytocin (0.5 nmol/L) for a period of 30 minutes, until regular phasic contractions were achieved. Bath addition of either the selective CB_1 antagonist SR 141716[22] or the selective CB_2 antagonist SR 144528[23] or of the respective vehicle for each antagonist was then performed for a further 30-minute period. This was followed by the addition of the endocannabinoid anandamide or the exogenous cannabinoid Δ^9-THC, in a cumulative manner, at bath concentrations in the range 1 nmol/L to 100 μmol/L (ie, 10^{-9}-10^{-4} mol/L) at 20-minute intervals. The cumulative increases in bath concentration of anandamide or Δ^9-THC were achieved in a pattern of 1 log molar increase every 20 minutes (ie, 1 nmol, 10 nmol, 100 nmol). Control experiments that tested for tachyphylaxis, with a single dose for a similar time period, revealed no difference between isolated exposure and cumulative exposure during this time.

The mechanical response of tissues was measured by the calculation of the integral of selected areas for 20-minute periods with the PowerLab hardware unit (AD Instruments, Oxfordshire, UK) and Chart software (version 3.6; AD Instruments). The integrated tension for the 20-minute period before the addition of cannabinoid, either anandamide or Δ^9-THC, was calculated, and this value served as a control because no significant spontaneous reduction in myometrial contractility was observed over the duration of the experiments in the control strips. The effects of anandamide and Δ^9-THC, in the presence or absence of antagonist, were assessed by the integral that was calculated during the 20-minute period after the addition of each drug concentration and given as a percentage of the integral that was obtained in the 20-minute period before any drug addition (ie, percentage contractility).

RNA extraction and reverse transcription

Total RNA was isolated with TRIzol reagent (Life Technologies, Grand Island, NY).[24] RNA was then reverse transcribed into complementary DNA (cDNA) for use as a template for polymerase chain reaction (PCR). One microgram of RNA was DNAase I treated

(Ambion Inc, Austin, Tex). The RNA samples were then denatured at 65°C for 10 minutes. Reverse transcription was performed at 42°C for 60 minutes in a reaction volume of 20 μL that contained oligo dT primer (500 ng), Maloney murine leukemia virus, reverse transcription buffer (50 mmol/L TRIS−hydrochloric acid, pH 8.3, 75 mmol/L potassium chloride, 3 mmol/L magnesium chloride, 10 mmol/L dithiothreitol; Promega, Madison, Wis), diethylpyrocarbonate-treated water (BDH, Dorset, UK), deoxyribonucleotide triphosphates (0.1 μmol/L; Promega), and 200 μ Maloney murine leukemia virus reverse transcriptase (Promega). Reverse transcriptase activity was stopped when the samples were heated at 65°C for 10 minutes. Control RNA samples, in which no reverse transcription enzyme was added, were included to confirm that no genomic DNA contamination was present.

PCR

The 5 μL of each 20-μL reverse transcriptase reaction was then used in subsequent PCR reactions. PCR was performed in a final volume of 50 μL that contained 1.5mmol/L magnesium chloride, 20 mmol/L TRIS−hydrochloric acid, 50 mmol/L potassium chloride, pH 8.3 (Life Technologies), 1.25 μ Taq DNA polymerase (Life Technologies), 40 μmol/L deoxyribonucleotide triphosphates (Promega), and 0.2 pmol of each sense and antisense primer. cDNA amplification was carried out by an initial denaturation step of 5 minutes at 95°C followed by 40 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 45 seconds, and elongation at 72°C for 20 seconds. The 5 μL of each PCR product was then separated by gel electrophoresis on a 1.2% agarose gel. Products were run alongside a 100-bp DNA mass ladder (Sigma-Aldrich, Dublin, Ireland) for sizing. Primers that were used were designed according to published DNA and messenger RNA (mRNA) sequences from GenBank (Los Alamos, NM; CB_1 receptor accession code U73304, CB_2 receptor accession code X74328).

Drugs and solutions

A stock solution of oxytocin (1 mmol/L, Sigma-Aldrich) was made in ethanol. Anandamide and Δ^9-THC were obtained from Sigma-Aldrich (St Louis, Mo). A stock solution (10^{-2} mol/L) of anandamide or Δ^9-THC was prepared with ethanol. The series of dilutions were made with deionized water on the day of the experimentation and were maintained at room temperature for the duration of the experiment. Fresh Krebs-Henseleit physiologic salt solution was made daily. SR 141716 was donated by B. R Martin, PhD, Virginia Commonwealth University, and also by Sanofi Recherche, Montpellier France. The SR 144528 was donated by Sanofi Recherche. Stock solutions (10^{-3} mol/L) of all three compounds were made with deionized water. A license was

obtained for the possession and importation of Δ^9-THC under the "Misuse of Drugs Acts, 1977 and 1984" and the "Misuse of Drugs Regulations, 1988" as set down by the government of the Republic of Ireland.

Statistical analysis

With the calculated integrals of contractile activity at each bath, the concentration dose response curves were analyzed by fitting the logistic equation: $Y = Y \max \times D^{n_H}/EC_{50} + D^{n_H}$, where Y is the response (percentage contractility), Y max is the maximal relaxation achieved, D is the dose of agonist (anandamide or Δ^9-THC), n_H is the slope function, and EC_{50} is the agonist dose giving one-half the maximal response. Curve fitting was performed with the software package Prism (Graphpad Software, San Diego, Calif). For myometrial tissue, the concentration of agonist (ie, anandamide or Δ^9-THC) that resulted in one half the maximal inhibitory effect (ie, the EC_{50}) was measured and represented in pharmacologic terms as its appropriate $-\log_{10}$ value (ie, $-\log_{10} EC_{50}$), which is also known as the pD_2. For example, if the EC_{50} value were 1 μmol/L (ie, 10^{-6} mol/L), the pD_2 would be 6.0. Comparisons of pD_2 ($-\log EC_{50}$) and mean maximal inhibitory (MMI) response values were carried out with a two-way analysis of variance. Post hoc comparisons were made with the Newman-Keuls test. A probability value of $<.05$ was accepted as statistically significant. The statistical package GBSTAT (version 6.5; Dynamic Microsystems Inc, Silver Spring, Md) was used for statistical calculations.

Results

Myometrial biopsy specimens were obtained from 11 women who underwent cesarean delivery between 38 and 40 weeks of gestation (median gestation, 39 weeks). All cesarean deliveries were carried out electively for the following reasons: breech presentation, previous cesarean delivery, previous myomectomy, previous shoulder dystocia, placenta previa, and fetal intrauterine growth restriction. The mean maternal age at delivery was 34.7 years (range, 21-37 years). The median parity value of the women at the time of delivery was 1 (range, 0-3). All women had regional anesthesia for cesarean delivery.

Both anandamide and Δ^9-THC exerted a significant concentration-dependent relaxant effect on human myometrial contractility in vitro. Recordings of oxytocin-induced myometrial contractions and the effects of cumulatively increasing concentrations of anandamide and Δ^9 THC are shown in Figures 1, A, and 2, A, respectively. For anandamide, the pD_2 value was 5.58 ± 0.42 (n = 6 specimens), the MMI response value was
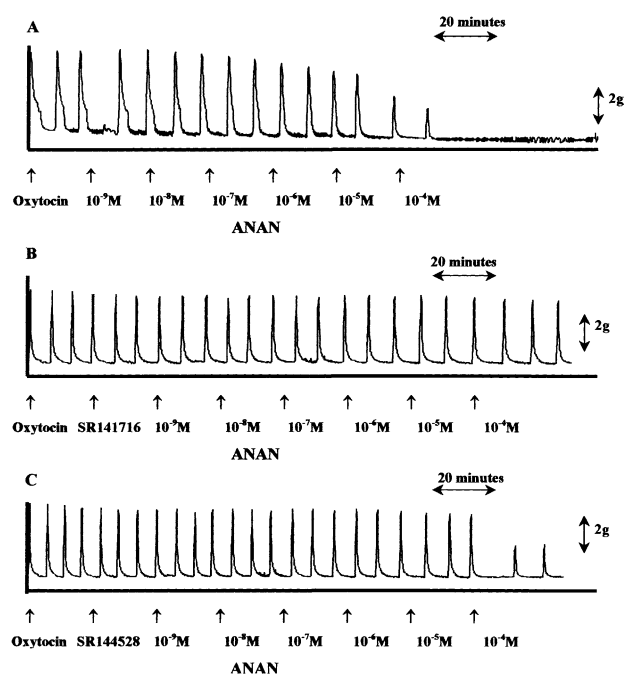
Figure 1 Representative recordings of oxytocin-induced contractions in human myometrial strips and the effects of anandamide are shown. **A,** The relaxant effect of anandamide *(ANAN)* in myometrium is not preincubated with antagonist. **B,** Antagonism of the effects of anandamide in the presence of the CB_1 antagonist SR 141716 is shown. **C,** No antagonism was observed with the CB_2 antagonist SR 144528.
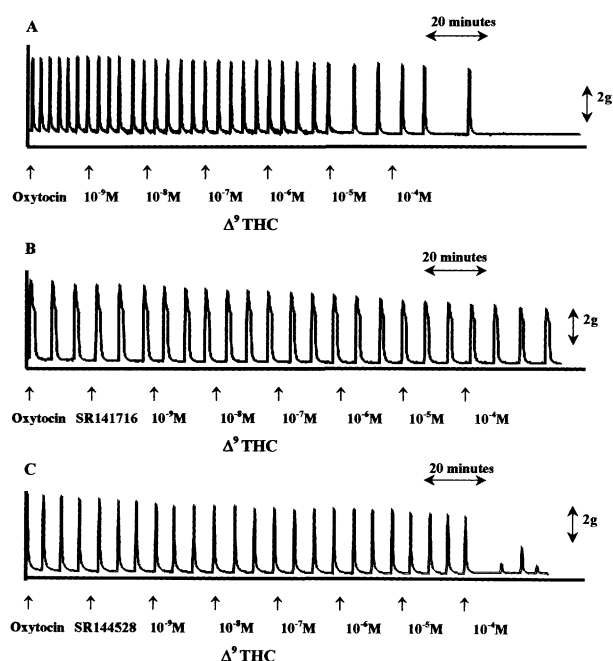

Figure 2 Representative recordings of oxytocin-induced contractions in human myometrial strips and the effects of Δ^9-THC are shown. **A,** The relaxant effect of Δ^9-THC in myometrium is not preincubated with antagonist. **B,** Antagonism of the effects of Δ^9-THC in the presence of the CB_1 antagonist SR 141716 is shown. **C,** No antagonism was observed with the CB_2 antagonist SR 144528.

Table I Uterorelaxant effect of the cannabinoid agents in the absence and presence of antagonist

Agonist	No antagonist		+SR 141716 (CB$_1$ anatgonist)		+SR 144528 (CB$_2$ antagonist)	
	pD$_2$	MMI	pD$_2$	MMI	pD$_2$	MMI
Δ^9-THC	5.19 ± 0.91	75.05 ± 1.15	2.57 ± 0.18*	34.44 ± 2.28*	5.73 ± 0.30	74.07 ± 3.72
ANAN	5.58 ± 0.42	75.75 ± 2.20	2.95 ± 0.14*	38.39 ± 2.08*	6.21 ± 0.29	75.43 ± 1.95

Data expressed as means ± SEM.
* $P < .01$ versus antagonist-treated counterparts.

75.74% ± 2.20% (n = 6 specimens), and the corresponding values for Δ^9-THC were 5.20 ± 0.94 (n = 6 specimens) and 75.04% ± 1.15% (n = 6 specimens, Table I). There was no significant difference between the potencies of both compounds.

The selective CB_1 antagonist SR 141716 produced a parallel rightward shift of the dose response curve for both anandamide (Figure 3, *A*) and Δ^9-THC (Figure 3, *B*), which indicated antagonism of their effects. Examples of raw data recordings of myometrial contractions in vitro that demonstrated antagonism by SR 141716 are shown in Figures 1, *B,* and 2, *B,* for anandamide and Δ^9-THC, respectively. There was a significant difference between the pD$_2$ values for both anandamide and Δ^9-THC in the absence and presence of SR141716

(n = 6 specimens, $P < .01$, Table I). There was also a significant difference between the MMI response values for both anandamide and Δ^9-THC in the absence and presence of SR141716 (n = 6 specimens, $P < .01$, Table I). On the contrary, the CB_2 antagonist SR 144528 did not result in any significant alteration in the dose response curve for either anandamide or Δ^9-THC, as demonstrated in Figures 1, *C,* and 2, *C,* respectively, and also in Figure 3, *A* and *B*, respectively. There was no significant difference between the pD$_2$ values or the MMI response values for either anandamide or Δ^9-THC in the presence or absence of SR 144528 (Table I).

Reverse transcriptase reaction products were subjected to PCR amplification with oligonucleotide primer sets that corresponded to both cannabinoid receptor
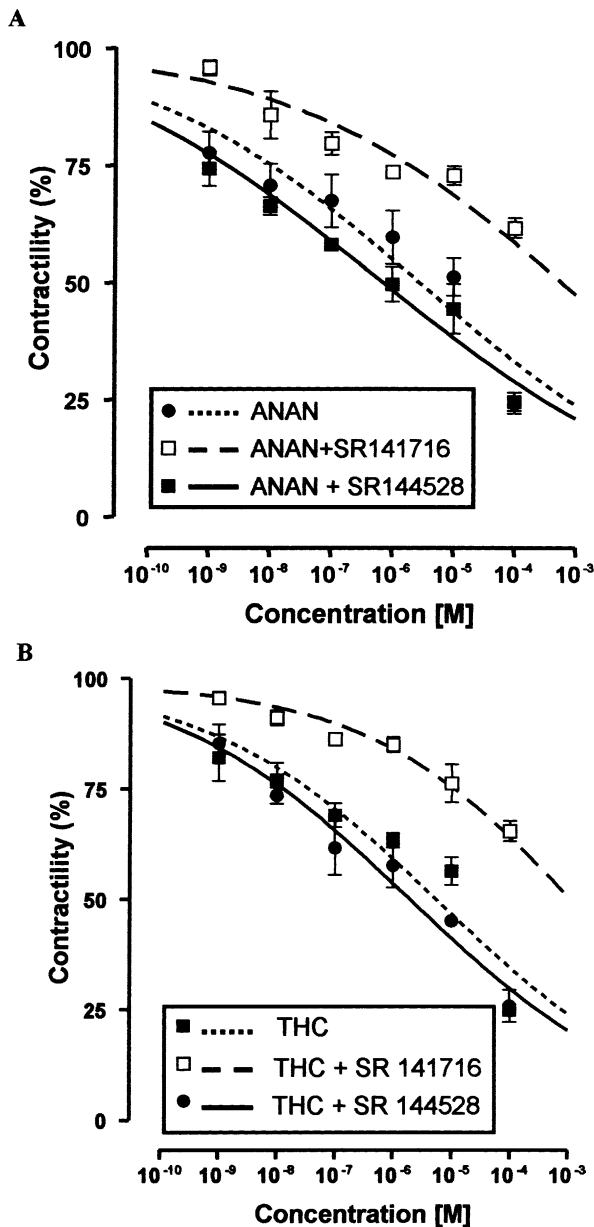
A

B

Figure 3 **A,** The dose response curves demonstrates the uterorelaxant effect of the endogenous cannabinoid, anandamide alone *(closed circles)* and with bath pre-exposure to the CB_1 receptor antagonist SR 141716 *(open squares)* or the CB_2 receptor antagonist SR 144528 *(closed squares)*. **B,** The dose-response curves demonstrate the uterorelaxant effect of the exogenous cannabinoid, Δ^9-THC alone *(closed squares)* and with bath pre-exposure to SR 141716 *(open squares)* or SR 144528 *(closed circles)*. Percentage contractility is shown on the *y-axis,* and the concentration of agonist is shown on the *x-axis.* The *points* represent the means, and the *error bars* represent the SEM.

subtypes. Both primer sets yielded PCR products of the expected sizes (n = 2 sets, Figure 4 and Table II). Amplification with the CB_1 primer set resulted in a 345-bp PCR product. This product was sequenced (MWG-Bio-

tech Ltd., Milton Keynes, UK), and the results verified that it was a part of the CB_1 receptor gene. Amplification of myometrial cDNA with CB_2 primers yielded a 439-bp product for which sequence analysis confirmed it to be part of the CB_2 receptor gene sequence. PCR of the reverse transcriptase negative controls showed no amplification that confirmed no genomic DNA contamination. Similarly, the PCR negative control (no cDNA template) showed no amplification product.

Comment

This is the first demonstration of the presence of cannabinoid receptors in human uterine smooth muscle and the first report that outlines that the endogenous cannabinoid anandamide and the exogenous cannabinoid Δ^9-THC exert a potent relaxant effect on oxytocin-induced human myometrial contractility. Endogenous cannabinoids have been the subject of much research interest in vascular[11] and nonvascular[12] smooth muscle systems in recent years. Little is known about the role of endogenous cannabinoids in human reproduction. In the mouse, it has been suggested that the aberrant synthesis of anandamide or the aberrant expression of cannabinoid receptors in the uterus or embryo may account for early pregnancy failure or female infertility.[25] Similarly, low fatty acid amide hydrolase activity, the anandamide degrading enzyme, has been implicated as a cause of implantation failure and pregnancy loss in humans.[26] In rats it has been demonstrated that the administration of endogenous (anandamide) or exogenous (Δ^9-THC) cannabinoids results in an increase in the duration of pregnancy and in the frequency of stillbirths.[15] It was suggested that this may be due to the central effects of the cannabinoids. Our findings demonstrate a clear direct effect of endogenous and exogenous cannabinoids on myometrial function and raise questions about a possible role of endogenous cannabinoids in the maintenance of uterine quiescence during normal pregnancy.

We have shown that the relaxant effect of the endogenous and exogenous cannabinoids in human uterine smooth muscle appears to be mediated predominantly through the CB_1 receptor. Previously CB_1 mRNA has been detected in the central nervous system and in peripheral tissues that include heart, lung, bladder, and adrenal gland.[16,27] In vascular tissues, the vasodilatation and hypotension that is elicited by endocannabinoids appear to be also mediated through the CB_1 receptor.[11,28] In the gastrointestinal system, the activation of CB_1 receptors inhibits gastrointestinal motility, intestinal secretion, and gastric acid secretion.[27] The proposed signal transduction pathways that are linked to cannabinoid receptor agonists include the inhibition of N-type and P/Q-type voltage sensitive calcium
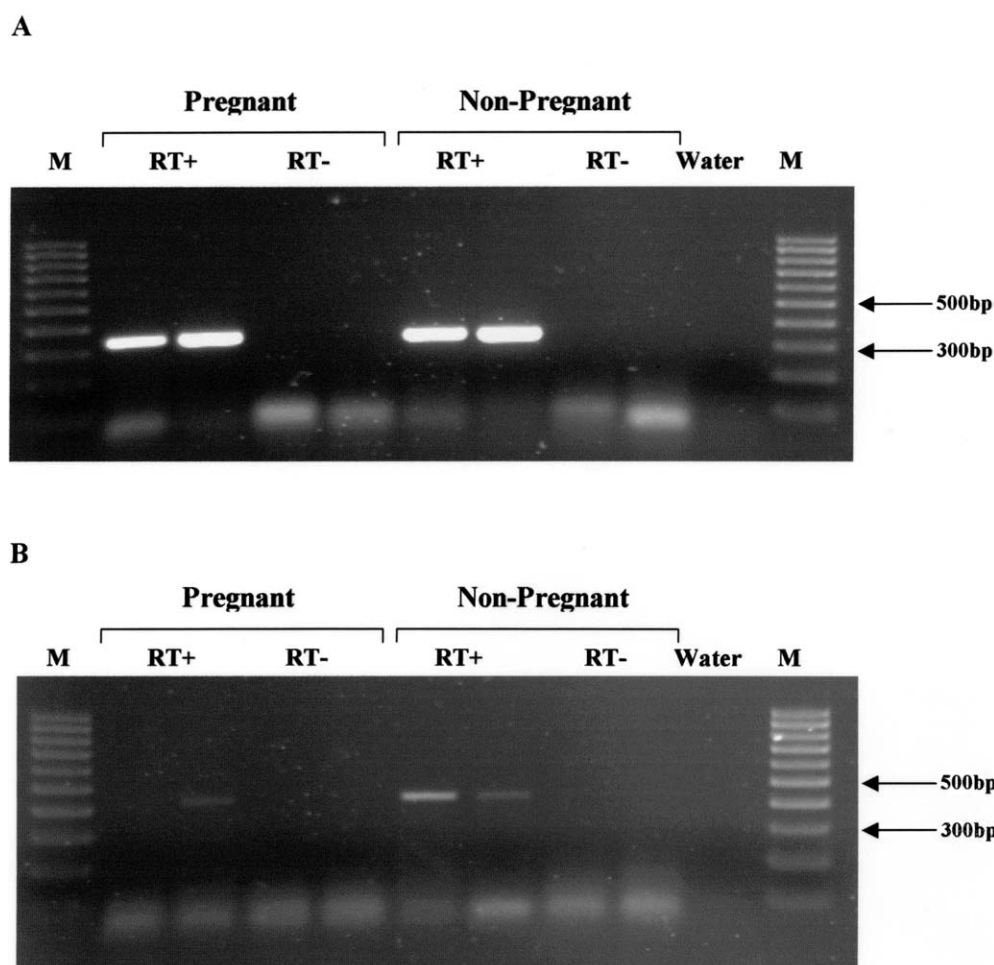
A

B

Figure 4 Representative agarose gel that was stained with ethidium bromide demonstrates the expression of CB_1 mRNA **(A)** and CB_2 mRNA **(B)** in human pregnant (n = 2 specimens) and nonpregnant (n = 2 specimens) myometrium. Reverse transcriptase—negative (*RT*−) controls for each candidate gene are shown alongside reverse transcriptase—positive (*RT*+) PCR products. A water control is also included (no cDNA template). *M,* 100-bp marker.

channels and the activation of inwardly rectifying potassium channels, which would lead to membrane hyperpolarization and the inhibition of contractile activity.[27] The mechanisms that are operative in human uterine tissue, which result in the degree and potency of relaxation observed in this study, remain a topic for future research. Little is known about the physiologic role of the CB_2 receptors, but CB_2 mRNA is present mainly in immune cells with particularly high levels in B cells and natural killer cells,[29] which suggests an immunomodulatory role. Although we have demonstrated CB_2 mRNA in human myometrium during pregnancy, the CB_2 receptor did not appear to have a major role in the relaxant effect that is elicited by cannabinoids.

Approximately 5% of women admit to using cannabis during pregnancy,[2,30] but it is believed that this figure underestimates the true prevalence of cannabis smoking

Table II Primers used for reverse transcription—PCR

Gene	Sense 5′-3′	Antisense 5′-3′
CB_1 receptor	GGTCCTCTCAGCACT-AGTCTCCT	TCCACTCCGTAGAGCATA-GATGA
CB_2 receptor	CAGACATTTTCCCACACA-TTGAT	CAAACACCGTCTTAATGA-GCTTG

at this time. According to previously measured serum concentrations of Δ^9-THC in the serum of patients after the intake of marijuana, we have estimated that the serum levels would be in the micromolar range, which lies in the middle of our dose response curve.[31,32] Our findings therefore suggest that smoking cannabis during pregnancy would exert a significant uterorelaxant effect. The link between cannabis use during pregnancy and the risk of

preterm delivery has been the focus of much research, with conflicting reports from different studies.[2,33,34] From a physiologic point of view, our results support the theory that cannabis use in itself, after standardization for other socioeconomic variables, should not be an independent risk factor for preterm delivery. The possibility that regular use after term might delay the onset of labor and therefore increase stillbirth rates, as shown in animal studies,[14] is raised however by our findings.

The studies that are mentioned here can only be performed in vitro in human uterine tissue for obvious ethical reasons. There are some limitations in extrapolation from in vitro studies to the in vivo situation. Our findings do not account for a possible central effect of endogenous and exogenous cannabinoids that, it appears, may have further relaxant effects on peripheral smooth muscle tissues.[35] The possibility that cannabinoid effects on human uterine tissue may be altered during pregnancy, in comparison with nonpregnant myometrium, requires further evaluation. Finally, our study has focused on the effects on uterine smooth muscle and has not addressed the effects of cannabinoids on the fetus or the fetoplacental circulation.

In conclusion, endogenous and exogenous cannabinoids exert a potent relaxant effect in vitro on human uterine smooth muscle during pregnancy. This direct effect is mediated through the CB_1 receptor. This highlights the possibility of a physiologic role for endogenous cannabinoids during human pregnancy and parturition. Our results support the view that exogenous cannabis use during pregnancy is not linked independently to preterm delivery.

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