CANNABINOID INDUCED CHANGES IN RAT UTERINE CONTRACTILITY

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ABSTRACT

Background: Endocannabinoids have been demonstrated in many mammalian tissues. It was reported that exposure to exocannabinoids (e.g. marijuana) is associated with adverse pregnancy outcomes.

Objective: The present study was designed to demonstrate the effect of anandamide on spontaneous contraction of pregnant and non pregnant rat uterus and the possible involvement of cannabinoid CB1 receptors, Nitric Oxide(NO), and small conductance Ca^{+2} activated K^+ channels in anandamide induced effect.

Design: The present study was carried out on a total number of 30 adult albino rats (24 females and 6 males). The animals were obtained from the laboratory animals' farm unit Faculty of Veterinary Medicine, Zagazig University, with an average weight, 180-200 grams. The male rats were used for induction of pregnancy. The first day of pregnancy was determined by the presence spermatozoa in the vaginal smear examined microscopically. The female rats were divided into four equal groups (6 for each). Three groups (non pregnant, day 10 and day 19 of gestation) were used to study the effects of anandamide (10^{-6}, 10^{-5} and 10^{-4} M/ml organ bath fluid) on spontaneous contractile activity of isolated uterine strips. The fourth group (day 10 of gestation) was used to study the possible mechanisms of action of anandamide using CB (1) receptor antagonist (AM251, 10^{-6} M/L), N^G-nitro-L-arginine methyl ester (L- NAME, 3×10^{-5} M/L), and small conductance Ca^{+2} activated K^+ channels blocker (Apamin, 10^{-8} M/L).

Results: The present study showed that anandamide exerted a significant dose dependant reduction in frequency and amplitude of spontaneous contraction of uterine strips isolated from both pregnant and non pregnant rats. The present study also revealed that the utero-relaxant effect of anandamide was significantly more potent in uterine strips isolated from pregnant rats on day 10 of gestation than that in both non pregnant rats and pregnant rats on day 19 of gestation. After incubation of uterine strips isolated from pregnant rats on day 10 of gestation with the specific CB_1 receptor antagonist AM251, the utero-relaxant effect of anandamide was almost completely abolished. This finding indicates that CB1 receptors are present in the rat uterus and may be the main receptor subtype involved in endocannabinoid-induced uterine relaxation. It was also found that pretreatment of uterine strips with NO synthase inhibitor (L-NAME) and small conductance-Ca^{+2} activated K^+ channel blocker (Apamin) significantly decreased the anandamide induced utero-relaxant effect.

Conclusion: In conclusion, anandamide exerts a potent relaxant effect in vitro on uterine strips isolated from pregnant and non pregnant rat uterus. This relaxant effect is higher in mid-gestation and this may help uterine quiescence during pregnancy, and then diminishes in late pregnancy which may allow effective uterine contraction during labor. In addition, the direct relaxant effect of anandamide is mediated through binding with CB1 receptors. Moreover, activation of nitric oxide generation and opening of small conductance Ca^{++} activated K^+ channels play a role in this anadamide induced utero-relaxant effect. This study highlights the possibility of a physiologic role for endogenous cannabinoids during human pregnancy and parturition and supports the view that exogenous cannabis use during pregnancy may have adverse effects on pregnancy and delivery.

Key word: Endocannabinoids, uterine contractility and pregnancy.

INTRODUCTION

A number of endogenous compounds that act as ligands for the cannabinoid receptors have been discovered. These compounds have been called endocannabinoids [1]. The most important are arachidonoyl ethanolamide (anandamide), 2-arachidonoyl glycerol (2-AG), and 2-archidonyl glyceryl ether [2]. Endocannabinoids are not stored in intracellular compartments, but are synthesized, through multiple biosynthetic pathways, and released 'on demand' by neurons and peripheral cells [3].

Endocannabinoids have been demonstrated in many mammalian tissues and are widely distributed in the CNS, peripheral nerves, leukocytes, spleen and testicles [1]. The uterus contains the highest levels of anandamide; the first discovered Endocannabinoid, suggesting a particular role of anandamide in female reproductive system [4].

The plasma levels of anandamide fluctuate through the menstrual cycle being higher in the follicular than luteal phase [5] and are higher in reproductive age women compared with postmenopausal age women. Anandamide level was reported to affect fertility, down-regulation is associated with uterine receptivity, while up-regulation has been shown to impair pregnancy and embryo development in mice and is correlated with uterine refractoriness to embryo implantation [6]. These observations have kindled new researches investigating the clinical potential of anandamide as a predictor of fertility and pregnancy outcomes [1,7]. Furthermore, anandamide plasma levels fluctuate during pregnancy, falling in the late first and early second trimester, and increasing 4-fold before labor [1]. In addition, some investigators reported that cannabinoid receptors (CB) are expressed in the oviduct, uterus [8], and placental membranes

[9]. Moreover, **Das et al.**[10] demonstrated that CB_1 mRNA is present in mouse uterus and it showed a higher accumulation on days 4 and 7 of pregnancy than that on day 1. Moreover, **Fonesca et al.**[11] found a significant difference in expression of cannabinoid receptors during pregnancy being upregulated during mid-pregnancy, with decreasing density as gestation advances. These data indicate that enocannabinoids may play an important role during pregnancy and labor.

As Expression of cannabinoid receptors was found to be high during embryogenesis, with CB1 and CB2 expression (at least in rodents) observed from the early four- to eight-cell embryo stage through to the preimplantation blastocyst, many researchers suggested that endocannabinoids may play a modulatory role in human reproduction [8,12].

Besides pregnant uterus and preimplantation embryo, oviduct is also a target for endocannabinoid action. In fact, in the mouse CB1 deficiency causes early pregnancy loss due to retention of embryos in the oviduct [13].

Numerous reports indicate that cannabinoids affect uterine motility. Cannabis has been used for more than a century to treat dysmenorrhea and menorrhagia [14]. On the other hand it was reported that exposure to Δ^9-tetrahydrocannabinol, the major active component of marijuana which bind to cananbinoid receptors, CB1 [15] and CB2 [16], is associated with adverse pregnancy outcomes [17], including spontaneous and preterm labor [18], fetal growth restriction and miscarriage [19]. In rats, it has been shown that administration of anandamide or Δ^9- tetrahydrocannabinol (THC) prolonged the duration of pregnancy and increased the frequency of stillbirths [20].

There are contradictory reports about the effect of endocannabinoides on uterine contractility. **Dennedy et al.** [21] found a relaxant effect of the endocannbiniod, anandamide on uterine contractility and demonstrated that this relaxant effect may be linked to a reduction in uterine prostaglandin synthesis. In contrast, **Dmitrieva and Berkley** [22] found an increase in the force of spontaneous uterine contraction under the effect of cannabinoid receptor agonists which was attributed, at least in part, to cannabinoid-induced production of PGE2 and PGF2α which decreases the intracellular concentration of cAMP [23].

As regards the mechanisms of action of endocannabinoids, many contradictory reports have been encountered. While some investigators reported that endocannabinoids directly regulate uterine contraction via binding with CB1 and CB2 receptors that preferentially couple to inhibitory $G\alpha_{i/o}$ proteins to inhibit adenylate cyclase activity, and hence reduce intracellular cAMP levels [24], others observed increased cAMP levels following CB1 activation [25], implying possible coupling to $G\alpha_s$ proteins. Similar observations, however, were not reported for CB2 receptors [26].

Numerous other signaling events, including increased activity of mitogen activated protein kinases (MAPKs) [27], inhibition of voltage-gated Ca^{+2} channels, activation of K^+ channels, and nitric oxide (NO) generation, have also been reported to follow CB receptor subtypes activation under different conditions [28,29].

Because of these contradictory reports, the present study was designed to demonstrate the effect of anandamide on spontaneous contraction of uterine strips isolated from both pregnant and non pregnant rats and the possible involvement of cannabinoid CB1 receptors, Nitric Oxide (NO), and small conductance Ca^{+2} activated K^+ channels in anandamide induced effect.

MATERIAL AND METHODS
Materials:
1-Animals: Thirty healthy adult albino rats (24 female rats and 6 male rats) were obtained from the laboratory animals' farm unit Faculty of Veterinary Medicine, Zagazig University, with an average weight, 180-200 grams. The animals were kept in steel wire cages (6/cage) under hygienic conditions and kept on the diet which consisted of mixed commercial rat laboratory chow and supplied in separate clean containers. Animals had free access to water and kept at room temperature. All animals were bred in the animal house. The rats were accommodated to laboratory conditions for two weeks before the experiments going on. The male rats were used for induction of pregnancy.

Groups: The animals were divided into four equal groups (6 for each):

Group (1): adult non pregnant female rats.

Group (2): pregnant female rats on day 10 of gestation.

Group (3): pregnant female rats on day 19 of gestation.

In these three groups, the effects of anandamide on spontaneous contractile activity of isolated uterine strips were studied.

Group (4): pregnant female rats on day 10 of gestation to study the effects of anandamide on spontaneous contractile activity of isolated uterine strips in the presence of:

1. Selective cannabinoid CB (1) receptor antagonist, AM251.

2. Nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME).

3. Small conductance Ca^{++} activated K^+ channels blocker, Apamin.

2-Drugs and chemicals:

*   Anandamide (Arachidonylethanolamide), non selective cannabinoid receptor agonist.
*   AM251, selective cannabinoid CB (1) receptor antagonist.
* N^G-nitro-L-arginine methyl ester (L- NAME), nitric oxide synthase inhibitor.
*   Apamin, blocker of small conductance Ca^{++} activated K^+ channels.

NB: Anandamide was dissolved in ethanol, AM251 was dissolved in dimethylsulfoxide (DMSO) and N^G-nitro-L-arginine methyl ester (L-NAME) and Apamin were dissolved in distilled water**.**

All the previous agents and their solvents were purchased from Sigma Chemicals CO. (Aldrich, St. Louis, Mo).

* De Jalone solution gm/2 L (NaCl, 18; KCl, 0.84**;** Glucose,2**;** Na HCO_3,2**;** $CaCl_2$,0.4)**.** The pH of this solution was 7.4 and it was bubbled with Carbogen (95%O2 and 5% CO2) to be used as a bath fluid for isolated uterine strips [30]. All the chemicals used for preparing De-Jalone solution were purchased from El Nasr Pharmaceutical Chemicals CO. Abu Zaabal, Egypt.

Methods:

1- Preparation of the non pregnant group

The non pregnant female rats were prepared with subcutaneous injection of estrogen (1 ml in sesame oil) for three successive days before the experiments for sensitization of the uterine smooth muscle.

2- Timed- pregnant group:

Determination of the first day of pregnancy:

Vaginal smears taken from the female rats were examined daily by using light microscope to ensure that they were in regular estrus cycle. The estrus phase of the estrus cycle was detected by the presence of cornified epithelial cells which increase in number and eventually predominate as the estrus progresses [31]**.**

The female proved to be in estrus phase was paired with a mature male rat in a separate cage. After mating, females were subsequently isolated until the time of analysis to ensure accurate gestation timing, and in the next morning a vaginal smear taken. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The presence of sperms indicated the first day of gestation [32]**.** Parturition usually occurs in the evening of day 21 or the morning of day 22 as the duration of pregnancy in rats is about 21 days [33]**.**

3- Isolated uterine tissue protocol:

Rats were sacrificed in the estrus phase in the non pregnant group (group 1), on day 10 of gestation (group 2 and 4), and on day 19 of gestation (group 3) by decapitation. The abdomen was opened, the uterine horns were dissected, and transferred immediately to a dish containing De-Jalone solution, then the extraneous tissues were removed e.g. pregnant uteri were cleaned from fat, placenta, fetus, fetal membrane and then rinsed thoroughly.

Afterwards each horn was opened longitudinally along its mesenteric border and divided by a long cut into two equal length segments to produce strips of about 0.4 cm in width x 1.3 cm in length [34]**.** A thread was then attached to the end of each strip, and the preparation was mounted in De Jalone solution of pH 7.4 at temperature of 37°C, aerated with a mixture of 95% O_2 and 5% CO_2 in the organ bath (50 ml volume). One end of the strip was attached to a fixed pin in the aerator of the bath and the other to an ink writing lever. The load on the lever was 2-3 gm. The preparation required approximately 1 hour to equilibrate after dissection.

The strips were bathed with De-Jalone solution. After spontaneous activity became regular various agents were added. After recording the effect of each dose, the uterine strips were washed 2 to 3 times with 5 minutes interval and left for about half an hour to return to their inherited conditions.

The drugs were added as follow:

* ***Anandamide*** was added in three separate doses: 10^{-6}, 10^{-5} and 10^{-4} M/ml organ bath fluid [35] to organ baths containing uterine strips isolated from;

    * 6 non pregnant adult female rats.
    * 6 rats on day 10 of gestation.
    * 6 rats on day 19 of gestation,

In additional experiments, the contractile activity of the uterine strips isolated from 6 rats on day 10 of gestation was recorded in response to addition of the third dose of ***anandamide*** (10^{-4} M/ml) in the presence of:

* AM251 (10^{-6} mol/ L) [35].
* N^G-nitro-L-arginine methyl ester (L-NAME) (3 x 10^{-5} mol/L) [36].
* Apamin (10^{-8} mol/L) [37].

The isolated uterine strips were incubated for 15 min with each of the previously mentioned chemicals followed by a period of 2-5 min incubation with anandamide (10^{-4} M / ml).

The amplitude (mm) and frequency (cycle/20min) of contractions developed by the strips after the addition of each dose of

anandamide alone or anandamide in the presence of different types of chemicals , were quantitated and expressed as the percentage of the amplitude or the frequency generated during the spontaneous contractile activity before the addition of these agents (the control).

Statistical analysis:

Data were presented as mean ± SEM. Statistical significance was determined by paired "t" test for differences within the same group. Differences between groups were determined by a one-way ANOVA and correlation coefficient (r). $P<0.05$ was considered statistically significant. SPSS version 10.0 program for Windows (SPSS Inc. Chicago, IL, USA) was used.

RESULTS

Tables (1a, b; 2a, b; 3a, b) and tracing I (a, b, c), II (a, b, c) ,III (a, b, c) demonstrate the effect of different doses of anandamide (10^{-6}, 10^{-5} and 10^{-4} M/ml organ bath fluid) on spontaneous contractility of uterine strips isolated from non pregnant, pregnant rats on day 10 and day 19 of gestation. It was found that anandamide had a significant utero-relaxant effect as it produced a significant decrease in the amplitude and frequency of spontaneous uterine contraction. This relaxant effect was found to be dose dependant because there was a significant positive correlation between the relaxant effect and the doses used (r = 0.804 for amplitude and 0.809 for frequency in non pregnant, r = 0.749 for amplitude and 0.750 for frequency in pregnant rats on day 10 of gestation and r = 0.899 for amplitude and 0.889 for frequency in pregnant rats on day 19 of gestation).

Tables (4 a, b) and figure (1a, b) show a comparison between the percentages of reduction ($\overline{X}$±SE) of amplitude and frequency of spontaneous contraction of uterine strips isolated from non pregnant rats, pregnant rats on day 10 and pregnant rats on day 19 of gestation in the presence of different doses of anandamide. It was observed that, the utero-relaxant effect of anandamide was significantly higher in uterine stripe isolated from the group of pregnant rats on day 10 of gestation when compared with both non pregnant group and group of pregnant rats on day 19 of gestation. However, a non significant difference in the utero-relaxant effect of anandamide was observed when comparing the non pregnant group with the pregnant group on day 19 of gestation.

Tables (5a,b; 6a,b), figure (2a, b) and tracing VI (a,b,c) show the effect of anandamide (10^{-4} M/ml organ bath fluid) on spontaneous contractility of uterine strips isolated from pregnant rats on day 10 of gestation in the presence of CB1 receptor antagonist (AM251, 10^{-6} M/L), nitric oxide synthase inhibitor (L-NAME, 3×10^{-5} M/L) and small conductance Ca^{+2} activated K^{+} channel blocker (Apamin, 10^{-8} M/L).

It was found that, while the utero-relaxant effect of anandamide was significantly and almost completely abolished in the presence of CB1 receptor antagonist (AM251), it was partially but significantly blocked in the presence of either L-NAME or Apamin.

Table (1a): Effect of different doses of anandamide (10^{-6}, 10^{-5}, 10^{-4}M/ml organ bath fluid) on the amplitude (mm) of spontaneous contraction of uterine strips isolated from adult non pregnant rats.

N= 6	Amplitude (mm)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	28.67	27.67	2.23	42	22.33	46.05	38.0	9.33	74.73
±SE	3.62	3.61	1.29	2.94	1.83	4.68	2.25	2.36	5.87
"t"	2.24 (P >0.05)			7.55 (P<0.001)			8.06 (P <0.001)		
r	+ 0.804 (P <0.001)								

Table (1b): Effect of different doses of anandamide (10^{-6}, 10^{-5}, 10^{-4}M/ml organ bath fluid) on the frequency of spontaneous contraction of uterine strips isolated from adult non pregnant rats.

| N=6	frequency (cycle/20 min)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	8.33	7.50	9.08	9.00	5.17	42.8	7.67	2.33	70.7
±SE	0.72	0.56	4.19	0.58	0.48	2.82	0.67	0.56	6.60
"t"	2.08 (P >0.05)			12.47 (P <0.001)			10.79 (P <0.001)		
r	+ 0.809 (P <0.001)								
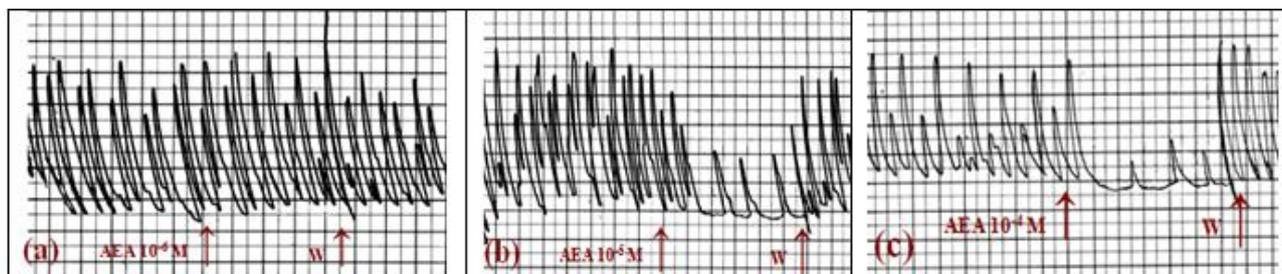
Tracing I (a, b, c): representative recordings of the effect of different doses of anandamide (AEA, 10^{-6}, 10^{-5}, 10^{-4} M/ml) on spontaneous contractility of uterine strips isolated from non pregnant rats.

Table (2a): Effect of different doses of anandamide (10^{-6}, 10^{-5}, 10^{-4}M/ml organ bath fluid) on the amplitude (mm) of spontaneous contraction of uterine strips isolated from adult pregnant rats at day 10 of gestation.

N=6	Amplitude (mm)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	63.6	43.5	31.45	67.16	19.83	71.5	59.50	5.50	91.73
±SE	4.4	3.2	2.71	5.17	4.42	5.39	3.27	3.48	4.98
"t"	8.55 (P <0.001)			13.45 (P <0.001)			21.92 (P <0.001)		
r	+ 0.749 (P <0.001)								

Table (2b): Effect of different doses of anandamide (10^{-6}, 10^{-5}, 10^{-4}M/ml organ bath fluid) on the frequency of spontaneous contraction of uterine strips isolated from adult pregnant rats at day 10 of gestation.

N=6	frequency (cycle/20 min)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	8.6	6.1	28.41	11.33	3.6	67.3	8.00	1.17	86.96
±SE	0.88	0.60	3.52	0.49	0.33	3.19	0.73	0.54	5.89
"t"	5.84(P <0.01)			12.47(P <0.001)			14.32(P <0.001)		
r	+ 0.75 (P <0.001)								
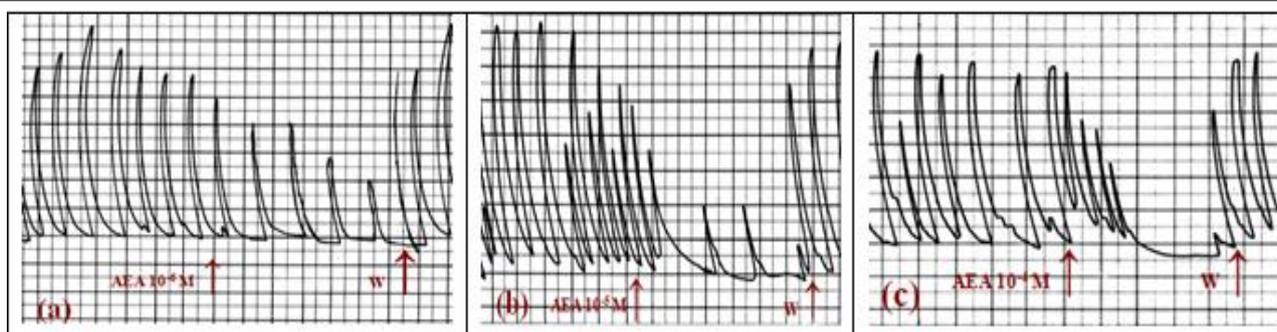
Tracing II (a, b, c): representative recordings of the effect of different doses of anandamide (AEA, 10^{-6}, 10^{-5}, 10^{-4} M/ml) on spontaneous contractility of uterine strips isolated from pregnant rats on day10 of gestation.
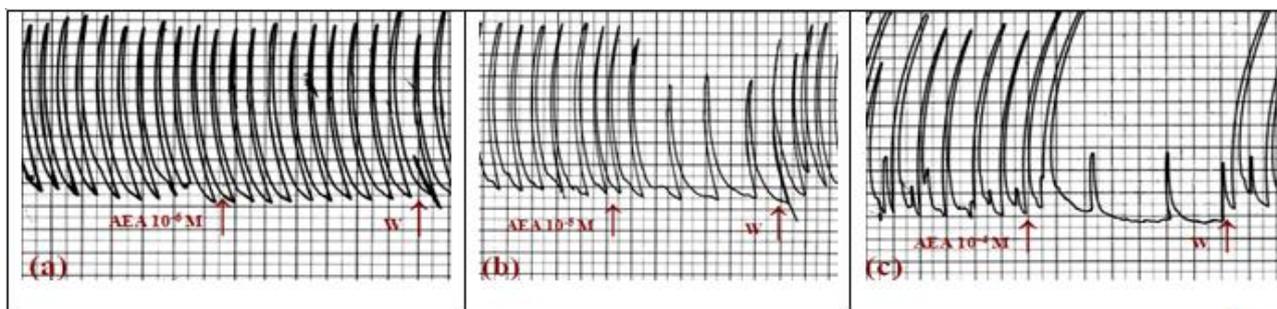
Table (3a): Effect of different doses of anandamide (10^{-6},10^{-5},10^{-4}M/ml organ bath fluid) on the amplitude (mm) of spontaneous contraction of uterine strips isolated from adult pregnant rats at day 19 of gestation.

N=6	Amplitude (mm)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	77.50	72.33	6.71	86.83	58.83	32	85.17	30.00	65.22
±SE	3.49	4.18	2.86	7.06	5.41	4.02	4.81	3.36	1.82
"t"	2.29 (P >0.05)			6.16(P <0.01)			33.20(P <0.001)		
r	+ 0.899 (P <0.001)								

Table (3b): Effect of different doses of anandamide (10^{-6},10^{-5},10^{-4}M/ml organ bath fluid) on the frequency of spontaneous contraction of uterine strips isolated from adult pregnant rats at day 19of gestation.

N=6	frequency (cycle/20 min)								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	9.83	9.50	3.50	9.83	6.33	35.80	9.00	2.83	68.48
±SE	0.60	0.67	2.21	0.60	0.49	2.02	0.58	0.31	2.72
"t"	1.58 (P >0.05)			15.65 (P <0.001)			12.92 (P <0.001)		
r	+ 0.889 (P <0.001)								

NB: r = correlation between dose of anandamide (M/ml organ bath fluid) and percentage of reduction ($\overline{X}$ ± SE) of amplitude and frequency of contraction of uterine strips.


Tracing III (a, b, c): Representative recordings of the effect of different doses of anandamide (AEA, 10^{-6}, 10^{-5}, 10^{-4} M/ml) on spontaneous contractility of uterine strips isolated from pregnant rats on day 19 of gestation.

Table (4a): Comparison between the percentages of reduction ($\overline{X}$ ±SE) of amplitude of spontaneous contraction of uterine strips isolated from non pregnant rats, rats on day 10 and day 19 of gestation in the presence of different doses of anandamide.

N=6	Percentage of reduction of amplitude								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Non pregnant	Day 10	Day 19	Non pregnant	Day 10	Day 19	Non pregnant	Day 10	Day 19
$\overline{X}$	2.23	31.45	6.71	46.05	71.5	32	74.73	91.73	65.22
±SE	1.29	2.71	2.86	4.68	5.39	4.02	5.87	4.98	1.82
F	42.99 (P<0.001)			17.92 (P<0.001)			8.62 (P<0.01)		
P of LSD		<0.001	N.S		<0.01	NS		<0.05	N.S
			<0.001			<0.001			<0.01

Table (4b): Comparison between the percentages of reduction ($\overline{X}$ ±SE) of frequency of spontaneous contraction of uterine strips isolated from non pregnant rats, rats on day 10 and day 19 of gestation in the presence of different doses of anandamide.

N=6	Percentage of reduction of frequency								
	Anandamide10^{-6} M/ml			Anandamide10^{-5} M/ml			Anandamide10^{-4} M/ml		
	Non pregnant	Day 10	Day 19	Non pregnant	Day 10	Day 19	Non pregnant	Day 10	Day 19
$\overline{X}$	9.08	28.41	3.5	42.8	67.3	35.8	70.7	86.96	68.48
±SE	4.19	3.52	2.21	2.82	3.19	2.02	6.6	5.89	2.72
F	14.68 (P<0.001)			36.82 (P<0.001)			3.56 (P>0.05)		
P of		<0.01	N.S		<0.001	N.S		<0.05	N.S
LSD			<0.001			<0.001			<0.05
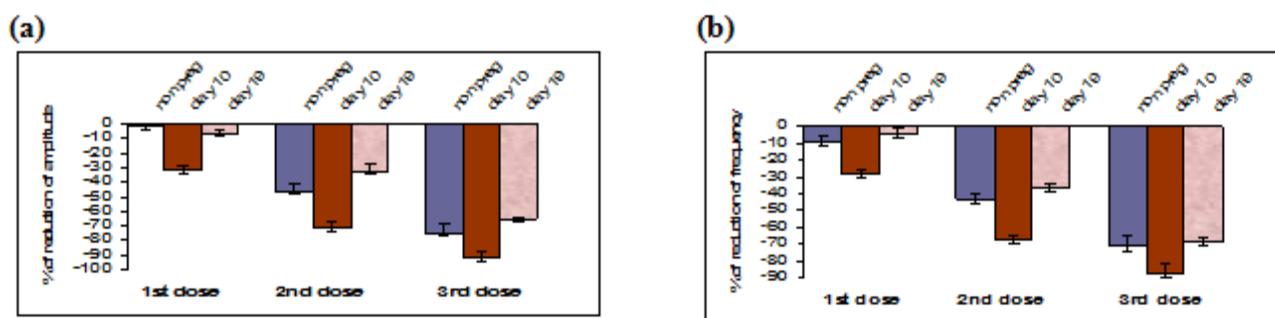
Fig (1a,b): The percentages of reduction ($\overline{X}$ ±SE) of amplitude (a) and frequency (b) of spontaneous contraction of uterine strips isolated from non pregnant rats, rats on day 10 and day 19 of gestation in the presence of different doses of anandamide (1st dose 10^{-6}, 2nd dose 10^{-5} and 3rd dose 10^{-4} M/ml organ bath fluid).

Table (5a): Effect of anandamide (10^{-4}M/ml organ bath fluid) on the amplitude (mm) of spontaneous contraction of uterine strips isolated from pregnant rats (day 10) before and after incubation with AM251 (10^{-6}M/L), L-NAME (3×10^{-5} M/L) and Apamin (10^{-8} M/L).
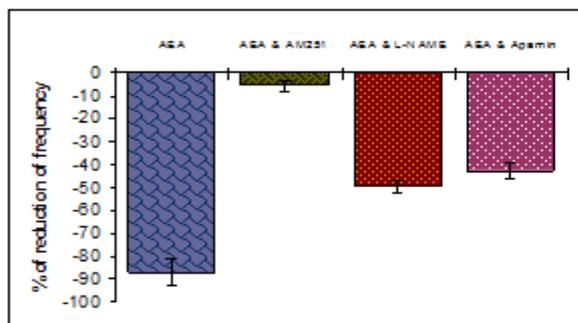
N=6	Amplitude (mm)								
	Anandamide &AM251			Anandamide &L-NAME			Anandamide &Apamin		
	Before	After	% of reduction	Before	After	% of reduction	Before	After	% of reduction
$\overline{X}$	73	65.66	9.22	60.83	28.33	53.53	63.5	43.5	30.88
±SE	4.44	4.31	5.31	5.25	3.14	3.49	6.08	4.85	4.15
"t"	1.67(P >0.05)			10.18(P <0.001)			5.91(P <0.01)		

Table (5b): Effect of anandamide (10^{-4}M/ml organ bath fluid) on the frequency of spontaneous contraction of uterine strips isolated from pregnant rats (day 10) before and after incubation with AM251 (10^{-6}M/L), L-NAME (3×10^{-5} M/L) and Apamin (10^{-8} M/L).
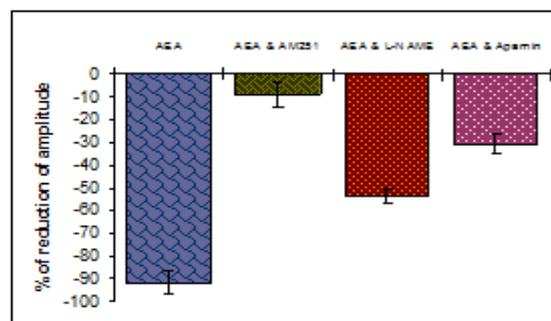
N=6	Frequency (cycle/20 min)								
	Anandamide&AM251			Anandamide-NAME			Anandamide Apamin		
	Before	After	% of reduction	After	After	% of reduction	Before	After	% of reduction
$\overline{X}$	9.83	9.33	5.6	9.66	4.83	49.85	9.66	5.5	42.85
±SE	0.6	0.76	2.52	0.49	0.31	2.6	0.49	0.43	3.94
"t"	2.24(P >0.05)			12.04 (P <0.001)			8.73(P <0.001)		

Table (6a): Comparison between the percentages of reduction ($\overline{X}\pm SE$) of amplitude of contraction produced by addition of anandamide (10^{-4} M/ml organ bath fluid) to uterine strips isolated from pregnant rats (day 10) before and after incubation with AM251 (10^{-6} M/L), L-NAME (3×10^{-5} M/L), and Apamin (10^{-8} M/L).

N=6	Percentage of reduction of amplitude			
	Anandamide (10^{-4} M/ml)	AM251 & Anandamide (10^{-4} M/ml)	L-NAME & Anandamide (10^{-4} M/ml)	Apamin & Anandamide (10^{-4} M/ml)
$\overline{X}$	91.73	9.22	53.53	30.88
±SE	4.98	5.31	3.49	4.15
F	60.18 (P<0.001)			
P of LSD		<0.001	<0.001	<0.001
			<0.001	<0.01
				<0.01

Table (6b): Comparison between the percentages of reduction ($\overline{X}\pm SE$) of frequency of contraction produced by addition of anandamide (10^{-4} M/ml organ bath fluid) to uterine strips isolated from pregnant rats (day 10) before and after incubation with AM251 (10^{-6} M/L), L-NAME (3×10^{-5} M/L), and Apamin (10^{-8} M/L).

| N=6	Percentage of reduction of frequency			
	Anandamide (10^{-4} M/ml)	AM251 & Anandamide (10^{-4} M/ml)	L-NAME & Anandamide (10^{-4} M/ml)	Apamin & Anandamide (10^{-4} M/ml)
$\overline{X}$	86.96	5.60	49.85	42.85
±SE	5.89	2.25	2.599	3.94
F	70.123 (P<0.001)			
P of LSD		<0.001	<0.001	<0.001
			<0.001	<0.01
				>0.05

(a)　　　　　(b)

Fig (2): The percentages of reduction ($\overline{X}\pm SE$) of amplitude (a) and frequency (b) of contraction produced by addition of anandamide (10^{-4} M/ml organ bath fluid) to uterine strips isolated from pregnant rats (day 10) before and after incubation with AM251 (10^{-6} M/L), L-NAME (3×10^{-5} M/L), and Apamin (10^{-8} M/L).
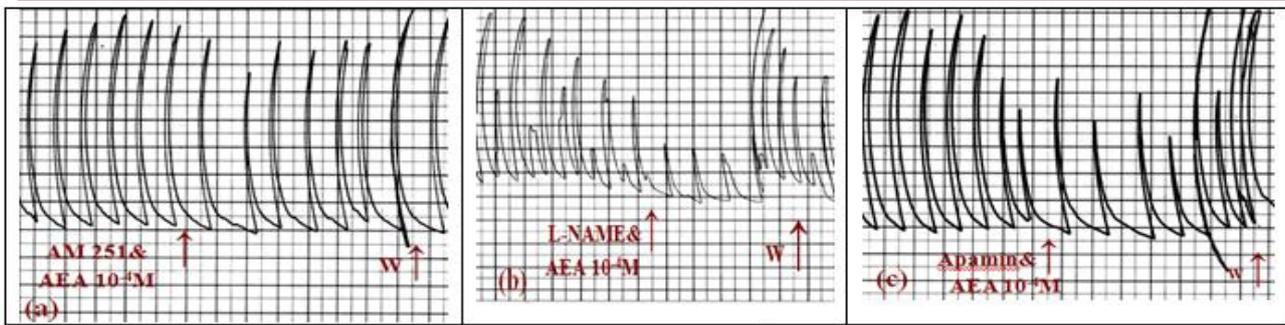
Tracing (Va, b, c) show the effect of anandamide (10^{-4} M/ml organ bath fluid) on spontaneous contractility of uterine strips isolated from pregnant rats on day 10 of gestation in the presence of AM251, 10^{-6} M/L, L-NAME, 3×10^{-5} M/L and Apamin, 10^{-8} M/L.

DISCUSSION

The results of the present study showed that anandamide exerted a significant dose dependant reduction in frequency and amplitude of spontaneous contraction of uterine strips isolated from both pregnant and non pregnant rats. This utero-relaxant effect of anandamide was almost completely abolished when anandamide was added after incubation of uterine strips isolated from pregnant rats on day 10 of gestation with the specific CB_1 receptor antagonist, AM251. This finding indicates that CB1 receptors are present in the rat uterus and may be the main receptor subtype involved in endocannabinoid-induced uterine relaxation.

These results are in accordance with those reported by **Dennedy et al.** [21] who demonstrated that the endogenous cannabinoid, anandamide and the exogenous cannabinoid, Δ^9-THC exerted a potent relaxant effect on human myometrial contractility. This relaxant effect was found to be prevented by CB1 antagonist SR141716 but not by CB2 antagonist SR144528.Therefore, they suggested that the relaxation component is under control of CB1 receptor only.

In support to the relaxant effect of anandamide on uterine smooth muscles that was observed in the present study, anandamide was found to have a CB1 mediated relaxant effect on vascular smooth muscles, leading to vasodilatation and hypotension [38] and gastrointestinal smooth muscles, causing inhibition of gastrointestinal motility and gastric acid secretion [39].

Our results are at variance with those of **Dmitrieva and Berkley** [22] who found an increase in the force of spontaneous uterine contraction under the effect of cannabinoid receptor agonists.

In humans, while **Dennedy et al.** [21] reported that the human endometrium expresses both CB1 and CB2 receptor subtypes, **Brighton et al.** [12] found that CB_1 mRNA is expressed in the human myometrial smooth muscle cells and that CB_2 mRNA appears to be very low if present at all. In pregnant rats, **Fonesca et al.** [11] described CB1 and CB2 receptor mRNA in the outer longitudinal and inner circular layer of the myometrium. In contrast, other investigators demonstrated that in mice, both CB1 and CB2 receptor subtypes are expressed in preimplantation embryos, whereas only CB1 is expressed in the oviduct and uterus [13].

The first possible mechanism which accounts for the utero-relaxant effect of anandamide is that, under certain conditions, increased cAMP levels following CB1 activation have been observed [25], implying possible coupling to $G\alpha_s$. Similar observations, however, were not reported for CB2 [26]. Elevation of cAMP levels leads to smooth muscle relaxation by activation of the cAMP-dependent protein kinase (PKA), which in turn phosphorylates the myosin light chain kinase and renders it inactive. This causes the myosin light chain to remain unphosphorylated and thus induces a relaxant response [40]. In contrast, others demonstrated that AEA signaling inhibits adenylate cyclase through binding with $G\alpha_i$ protein, thereby reducing cAMP levels [12]. However, an AEA-mediated reduction in cAMP levels does not result, as one may expect, in myometrial contraction [21], implying that alternative mechanisms control AEA-stimulated myometrial relaxation [21]. Furthermore, some investigators reported that smooth muscle relaxation could be mediated via either increase cAMP and this is probably due to increased intracellular binding of Ca^{+2} ion, or decrease cAMP which is associated with increased Ca^{+2} ion efflux from the muscle cells [41].

Contraction of the uterus is primarily dependent on the activity of L-type Ca^{2+} channels, particularly at term [42]. Thus, the second possible mechanism explaining the utro-relaxant effect of anandamide is the reduction of intracellular Ca^{+2} concentrations. CB1 receptor signaling is known to inhibit L-type Ca^{2+} channels and inhibit intracellular Ca^{2+} store release in muscle cells leading to relaxation [43].

It has been reported that only CB1 and not CB2 regulates ionic currents (inhibition of voltage-gated L, N and P/Q-type Ca^{2+} channels, activation of K^+ channels) [29]. There is mounting evidence accumulated in the last few years, showing that lipid rafts (LRs) are involved in the trafficking and functioning of CB_1 receptors but not CB_2 [44]. LRs are specialized membrane microdomains biochemically defined by the insolubility of their components in cold non-ionic detergents [45].

Interestingly, a role for LRs in reproduction has been recently documented. For instance, LRs have been recognized as a critical factor in the pathways involved in Ca^{2+} signalling in the uterus, with an impact on the prevention of preterm and difficult labours [46]. In fact, the increases in cytosolic Ca^{2+} and contractility that occur with lipid raft disruption are due, at least in part, to effects on large conductance Ca^{2+}-activated K^+ channels, localized within LRs [47].

K^+ channels critically regulate smooth muscle contractility by opposing membrane potential depolarization and hence Ca^{2+} elevation. Certain K^+ channels, including ATP-sensitive K^+ (K_{ATP}) channels [48] large conductance Ca^{2+}-activated K^+ (BK) channels [49] and small conductance Ca^{2+} activated K^+ (SK) channel [45], have been implicated in the control of uterine excitability in both pregnant and non-pregnant states.

The present study revealed that, the relaxant effect of anandamide on spontaneous uterine contraction was partially prevented by incubation of uterine strips with Apamin which is a small conductance-Ca^{+2} activated K^+ channel blocker. Therefore the third possible mechanism which explains the utero-relaxant effect of anandamide is the activation of K^+ channels.

These results are in agreement with those of many investigators who demonstrated that activation of K^+ channels is one of the signal-transduction pathways regulated by CB1 receptor [29,50]

Our results also showed that the relaxant effect induced by anadamide was partially blocked by incubation of uterine strips isolated on day 10 of gestation with NO synthase inhibitor, L-NAME. This finding indicates that, the fourth possible explanation of the utero-relaxant effect of anandamide is the generation of NO.

Our results are supported by the findings of **Maccarrone *et al.*** [51] who reported that activation of CB1 cannabinoid receptors by AEA causes stimulation of the inducible NO synthase activity. Also it was found that methanandamide, the stable synthetic analogue of anandamide, induces iNOS protein expression and NO production in uterine explant tissue [52].

Moreover, throughout gestation myometrial NO production is up regulated to reach high levels in midgestation despite low circulating level of anandamide at this time of pregnancy. This up regulation of NO production could be attributed to the up regulation of cannabinod receptors in midgestation and thus may contribute to pregnancy maintainance by inhibiting uterine smooth muscle contraction and then, close to term NO production decreases in the myometrium thus promoting effective contractions that result in labor [53]. In contrast to the myometrium, NO production in the cervix is low during gestation and becomes up-regulated once pregnancy advances to term thus helping cervical dilatation during labour [47].

In addition, it was reported that CB1 activates, whereas CB2 inhibits nitric oxide synthase [29]. The opposite effect of CB1 versus CB2 on NO release might be relevant for the in vivo control of reproduction. Since human endometrium expresses both CB1 and CB2 [21], it is believed that these two receptor subtypes are engaged at different time points to modulate in opposite ways NO content and thus NO-dependent effects [47].

Many studies showed that, in majority of smooth muscles including uterine smooth muscles, the relaxing effect of NO is related to opening of large conductance Ca^{2+} activated and voltage dependent K^+ channels by hyperpolarization of cell membrane [54]. Other studies indicated that NO-induced relaxation of uterine smooth muscle was counteracted by the small conductance K^+ channel blocker, Apamin and this indicates that small conductance K^+ channel are also present in the rat myometrium [55].

The in vitro utero-relaxant effect encountered in the present study could lead to the hypothesis that anandamide may cause a delay in the onset of labor and prolongation of the gestation period. This hypothesis was proved by **Wenger et al.** [56] who showed that daily intra-peritoneal injection of anandamide over the third week of rat pregnancy caused a significant increase in the duration of pregnancy and the frequency of stillbirths and attributed this prolongation to a reduction in prostaglandin (PG F 1α and PG F 2α) synthesis. In contrast, **Habayeb et al.** [57] reported that the rise of anandamide level during labor is associated with an increased local production of prostaglandins. This high level of anandamide could be to provide a large reservoir for arachidonic acid, the precursor for prostaglandin production.

Another possible mechanism of action is that anandamide may be related to alteration in myometrial gene expression. **Brighton et al.** [12] demonstrated that AEA oactivates extra cellular regulated kinase 1/2 (ERK1/2) in human mymetrial

cells. ERK1/2 proteins are members of the mitogen activated protein kinase (MAPK) family, which can provide a link between extracellular stimuli and transcription factors to regulate gene expression. These effects were mediated directly through CB receptor-$G\alpha_{i/o}$ coupling. Indeed, longer term AEA exposure suppresses calponin and smoothlin expression in human myometrial smooth muscle cell line (ULTR). Taken together these data suggest that AEA may further confer a relaxatory phenotype on the myometrial cells [7].

One of the outstanding observations in the present study is that the utero-relaxant effect of anandamide was more potent in uterine strips isolated from pregnant rats on day 10 of gestation than that in both non pregnant rats and pregnant rats on day 19 of gestation.

This can be explained by the results of **Fonesca et al.** [11] who found a significant difference in the protein levels of cannabinoid receptors between days of gestation in rat uterus. CB1 protein levels on day 10 and 12 were significantly higher than those on days 16 and 19 of pregnancy. They also detected immunoreactivity for CB1 receptors in the circular muscle layer that was upregulated during midpregnancy, with decreasing intensity as gestation advances. In addition, it was found that CB_1 mRNA is present in mouse uterus and it shows a higher accumulation on days 4 and 7 of pregnancy than that on day 1[10].

Anandamide has also been shown to have non CB1 and non CB2-dependent effects suggesting the existence of a CB3 receptor [58] and evidence exists that anandamide can also bind to other receptors that are not exclusively associated with cannabinoids [59]. It has been shown that anandamide binds to and activates the transient receptor potential vanilloid 1 receptor (TRPV1 or VR1), which is characterized as a ligand-gated non selective cationic channel [60]. The concentrations of AEA required to fully activate TRPV1 as assessed by measuring intracellular Ca^{+2} are 1- to 10-fold higher than those required to evoke CB1-mediated functional responses [61].

A strong reactivity for the vanilloid receptor in the longitudinal muscle layer of rat uterus was detected throughout gestation [11]. In humans, a dramatic increase in plasma anandamide levels during term labor compared with non-laboring women has been described suggesting a role for anandamide in labor [57]. Thus, it was hypothesized that TRPV1 activation mediated by the high levels of anandamide might contribute to the ability of the outer myometrial layer to generate optimal contractile activity during labor [11].

In light of these reports, the reduction of the utero-relaxant effect of anandamide recorded in the present study from uterine strips isolated from pregnant rats on day 19 could be accounted for by the down- regulation in CB1 receptors which is accompanied by the activation of TRPV1. This down-regulation of CB1 receptors may be estrogen mediated [62]. In addition, the results of the present study together with other studies could reflect the beneficial effects of the changes in the circulating levels of anandamide that occur during gestation. **Habayeb et al.** [57] demonstrated the remarkable changes in the levels of AEA during pregnancy and labor. They found that Plasma AEA levels fell from the first to the second and third trimesters with no change between the second and third trimesters. The levels rose at term before the onset of clinically apparent labor and rose further during labor to represent a 6-fold increase from third-trimester levels.

The levels detected in the first trimester, which were similar to those reported in successful in vitro fertilization (IVF) preg-nancies [63], were similar to those in the luteal phase of the menstrual cycle. This implies that the low levels of AEA proposed to be required to support early pregnancy are already established in the luteal phase, enabling successful implantation, and that successful pregnancy represents a successful maintenance of these suppressed levels [57].

The low levels in postmenopausal women and the high levels in the follicular phase suggest that steroid hormones primarily regulate AEA levels, with estradiol increasing the levels and progesterone suppressing them [57]. The effect of progesterone could result from regulation of the degradation of peripheral AEA by peripheral blood mononuclear cells given that the levels of fatty acid amide hydrolase (FAAH), the principal enzyme involved in AEA degradation, in these cells are regulated by progesterone [64]. The induction of high AEA levels by estradiol could be mediated by its effect upon endothelial cells given that it has been reported that estradiol increases the release of AEA from these cells into the circulation [63].

Moreover, labor is a painful process and AEA has a well-documented role in pain transmission [65], the rise in plasma AEA may be a byproduct of the labor process [12]. Recent data have confirmed that elevated plasma AEA concentrations occur when a women converts to the active labor state, suggesting that AEA may play an important role in labor [66].

There are some limitations in the extrapolation from in vitro studies to the in vivo situation and from animal to human studies. The in vitro studies do not account for a possible central effect of endogenous cannabinoids which may have further

relaxant effects on peripheral smooth muscle tissues [67].

Further studies are required to evaluate the cannabinoid effects on human uterine tissue during pregnancy, in comparison with nonpregnant myometrium and to examine the possible mechanisms of this effect. Further studies are also needed to investigate the effects of cannabinoids on the fetus or the feto-placental circulation.

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التغيرات المحدثة بالكانابينود فى انقباضية رحم الفئران

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أكتشفت مركبات الإندوكنابينويد فى كثير من الخلايا و تنتشر بشكل واسع فى الجهاز العصبي المركزي، الأعصاب الطرفية ، كرات الدم البيضاء ، الطحال و الخصية.

وقد سجل العلماء ان التعرض للكنابينويد الخارجية كالماريجوانا يصاحبه تأثيرات سيئة علي الحمل مثل الولادة المبكرة، تأخر نمو الجنين و فشل الحمل. كما وجد أيضا أن مستقبلات الكنابينويد موجودة في قناة المبيض، الرحم و أغشية المشيمة. وكذلك يحتوي الرحم علي أعلي نسبة من مركب الأناندامید وهو أول إندوكنابينويد تم أكتشافه مما يرجح بأن الأناندامید يلعب دورا فى عملية الإنجاب.

و بسبب وجود تقارير متضاربة حول تاثير مركبات الكنابينويد علي انقباضية الرحم و تقارير متضاربة عن كيفية هذا التاثير لذلك صممت هذه الدراسة لبحث تأثير الأناندامید علي الانقباضية التلقائية لرحم الجرذان الحوامل و غير الحوامل. ولإيضاح الكيفية التي يؤثر بها الأناندامید علي الرحم و مدي ارتباطها بمستقبلات الكنابينويد (١)، اكسيد النيتريك و ممرات البوتاسيوم التي تنشط بالكالسيوم.

أجريت هذه الدراسة علي عدد كلي ٣٠من الجرذان البيضاء البالغة (٢٤إناث و ٦ ذكور) تم احضارها من وحدة مزرعة حيوانات التجارب بكلية الطب البيطري جامعة الزقازيق و تراوحت أوزانها بين 180 الي 200 جرام و استخدمت الذكور لاحداث الحمل. و تم تحديد اليوم الأول من الحمل بوجود حيوانات منوية في الفحص الميكرسكوبي لمسحة من الافرازات المهبلية. و قد قسمت الاناث إلي أربع مجموعات متساوية تحتوي كل منها علي ٦ فئران.

* ثلاث من هذه المجموعات (غير الحوامل ، حوامل في اليوم العاشرمن الحمل، و حوامل في اليوم التاسع عشرمن الحمل) استخدمت لدراسة تأثيرالأناندامید علي الانقباضية التلقائية لشرائح رحم الجرذان المعزولة.

* والمجموعة الرابعة (حوامل في اليوم العاشرمن الحمل) استخدمت لدراسة الكيفية التي يؤثر بها الأناندامید علي الرحم وذلك باستخدام مضاد مستقبلات الكنابينويد (AM251) ، مثبط انزيمات تصنيع اكسيد النيتريك (L-NAME) ، و غالق ممرات البوتاسيوم التي تنشط بالكالسيوم (Apamin).

و قد أظهرت الدراسة أن الأناندامید له تأثير إنبساطي ذو دلالة احصائية علي شرائح الرحم المعزولة من الجرذان الحوامل وغير الحوامل و يزيد هذا التأثيربزيادة تركيز الأناندامید. و أن هذا التأثيرالانبساطي للاناندامید يحدث غاليا عن طريق التاثير المباشر علي مستقبلات الكنابينويد (١) لأن مضادات هذه المستقبلات ألغت هذا التأثير بشكل شبه كامل مما يوحي بان مستقبلات الكنابينويد (١) موجودة في رحم الجرذان و انها ربما تكون النوع الرئيسي من مستقبلات الكنابينويد المسؤل عن هذا التاثير الانبساطي للاناندامید. و قد أظهرت الدراسة ايضا أن كل من مثبط انزیمات تصنيع اكسيد النيتريك، و غالق ممرات البوتاسيوم التي تنشط بالكالسيوم تقلل بشكل ذو دلالة احصائية التأثير الإنبساطي للأناندامید.

كما أظهرت الدراسة أن التأثيرالإنبساطي للأناندامید كان اقوي بدرجة ذات دلالة احصائية علي شرائح الرحم المعزولة من الجرذان الحوامل في اليوم العاشرمن الحمل مقارنة بكل من مجموعة الحوامل في اليوم التاسع عشرمن الحمل وكذلك غير الحوامل.

و مما سبق يمكن ان نستنتج أن:

● الأناندامید له تأثير إنبساطي علي الانقباضية التلقائية لرحم الجرذان الحوامل و غير الحوامل و أن هذا التأثير اقوي في منتصف الحمل مما قد يساعد علي هدوء الرحم خلال فترة الحمل ثم ينخفض هذا التأثير الإنبساطي في نهاية الحمل مما قد يساعد علي انقباض فعال للرحم اثناء الولادة.

● الأناندامید يحدث هذا التأثير الإنبساطي المباشر غاليا عن طريق ارتباطه بمستقبلات الكنابينويد (١). كما أن كل من زيادة انتاج اكسيد النيتريك و فتح ممرات البوتاسيوم التي تنشط بالكالسيوم يلعب دورا هاما في هذا التأثير الإنبساطي.

● تلقي هذه الدراسة الضوء علي احتمالية وجود دورا فسيولوجيا تلعبه مركبات الاندوكنابينويد اثناء فترة الحمل و الولادة. كما تدعم الراي القائل بان استخدام مركبات الكنابينويد الخارجية له تاثير سلبي علي الحمل و الولادة.