UNIVERSITY OF SZEGED

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCES

NON-GENOMIC ACTIONS OF STEROID HORMONES IN PREGNANT AND NON-PREGNANT UTERINE TISSUES: IN VIVO AND VITRO STUDIES

PH.D. THESIS

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List of publications

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List of the abbreviations

ALD	Aldosterone
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CS	Corticosteroids
DEX	Dexamethasone
E2	17-β estradiol
FLD	Fludrocortisone
FLU	Flutamide
FUL	Fulvestrant
GC	Glucocorticoids
GR	Glucocorticoid Receptor
IV	Intravenous
MC	Mineralocorticoids
MIF	Mifepristone
MR	Mineralocorticoid Receptor
PsD_{max}	Maximum of power spectrum density
P4	Progesterone
SEM	Standard error
SPR	Spironolactone
SPRD	Sprague-Dawley rats
T	Testosterone
TCA	Trichloroacetic acid

1. Introduction:

Steroids are specific configuration of 4 ringed organic compounds which have important within specific endocrine glands [1].

The reproductive steroids group also known as sex steroid hormones are collectively responsible for the primary and secondary features of these hormones through human body. Sex hormones can influence pregnant uterine contractility; their ratio may be an important key in the parturition process. Progesterone is well-known as a pro-gestational hormone reducing uterine contractility and maintaining pregnancy [2]. On the other hand, estrogens increase the contraction of the pregnant uterus and contribute to the parturition process [3]. However, the effect of testosterone (T) on pregnancy has not exactly been clarified yet, it is presumed to increase the rate of miscarriage [4].

The corticosteroid hormones are another group of steroid hormones secreted from the adrenal cortex and exert various ranges of actions through regulating functional responses on different organs and tissues targets such as the brain, kidney, liver, fat tissue, and muscles. The result of their action can affect immune system function, muscle contractions, or relaxations, as well as a disturbance in fat distribution and secretory gland function.

The classical signaling pathway of steroids is the “genomic pathway”. Steroids first pass the membrane, bind to specific steroid receptors and make a ligand-receptor complex, which goes into the nucleus; then, by binding to the hormone response element or functional proteins like nuclear factor kappa B, they alter gene transcription and protein synthesis [5]. This action has a significant gap time between the drug administration or the secretion of hormones and the desired effect.

However, there is prompt action for all types of steroids which occurs immediately called “non-genomic pathway”. One of the first studies about the non-genomic action of sex steroids demonstrated that immediately after the administration of 17-β estradiol (E2) to ovariectomized rat, the level of uterine cyclic adenosine monophosphate (cAMP) was doubled [6]. Several other studies showed that sex hormones exert a variety of prompt functional effects on different tissues, such as cancer cells in breast [7], pituitary glands [8], sperms [9], nerve cells [10] and many other targets.

The fast action of glucocorticoids (GCs) was already described more than 60 years ago proving that administration of GC led to fast inhibition of stimulus-induced

adrenocorticotropic hormone secretion [11]. Since then, fast GC effects especially on central nervous and cardiovascular systems, smooth and skeletal muscles were investigated. For example, corticosterone inhibits hypothalamic–pituitary–adrenocortical axis in a fast manner, and stimulates adaptive reflex to the situation like cognitive aspects [12]. Furthermore, it can also temporarily elevate the levels of excitatory amino acids aspartate and glutamate [13]. Corticosterone quickly elevates blood sugar during stress by inhibiting glycogenesis in high concentration at early stages of stress response [14], therefore, the GC fast action also increases carbohydrate ingestion [11] and decreases insulin secretion [15]. Apart from that, beneficial fast effect of GC have been reported on airway smooth muscles in asthmatic condition [16]. In addition, GCs mediate T-cell immunosuppressive response by non-genomic action [17]. Uterine contraction of non-pregnant rat can be inhibited by cortisol [18,19] and dexamethasone (DEX) [20]. Endometrial GSs deficiency increases the inflammation and the angiogenesis therefore leads to heavier menstrual bleeding by the impaired subsequent vasoconstriction [21].

The fast response of aldosterone (ALD) on the cardiovascular system was reported after 5 minutes of its administration, when peripheral vascular resistance and cardiac output were increased and decreased, respectively. Due to the short timeframe of response, the hypothesis of a new non-genomic pathway was made [22]. ALD has been reported to change the cell volume of human mononuclear leukocytes by modifying the Na^+/H^+ antiporter and inducing alkalization. This action was not blocked by mineralocorticoid receptor (MR) blocker spironolactone (SPR) within15 minutes [23]. In addition, ALD has a rapid positive inotropic effect on rat cardiac muscle [24], and its fast effect has been reported on $Na^+/K^+/2Cl^-$ cotransporter and Na^+/K^+ pump activity in rabbit cardio-myocytes [25]. In the collecting ducts, ALD induces Na^+/H^+ exchange and promotes intercellular Ca^{2+} flux, but in the medullary thick ascending limb, it decreases Na^+/H^+ exchange and bicarbonate absorption in a non-genomic way [26]. ALD can also moderately inhibit the uterine contraction in Wistar rats [18].

It is known that the results of the non-genomic action can be the same as or even different from the effects mediated through the genomic pathway. For example, in the cardiovascular system and diabetes mellitus, the outcome of both signaling pathways is the same [27–30], but in breast cancer cell lines, their actions can be the opposite [26].

Since the prompt actions of steroids on uterine contractility are not fully explored yet, we aimed to investigate the fast, non-genomic action of sex steroids (E2, progesterone (P4), T) and corticosteroids (MC fludrocortisone (FLD) and GC (DEX)) on uterine

contractions and signaling pathways both late (22-day) pregnant and non-pregnant rats in vitro and in vivo as well.

2. Materials and methods:

2.1 Animals:

Sprague-Dawley rats (SPRD) (180-200 g, Animalab Hungary Ltd, Vác, Hungary) were kept under controlled temperature, humidity, and light (20-23 °C and 40–60 % and 12 h light/dark regime, respectively). Diet (Altromin 1324, Charles-River Laboratories, Sulzfeld, Germany), and tap water were available ad libitum. The animals were treated in accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (Article 32 of Act XXVIII). All experiments involving animal subjects were carried out with the approval of the National Scientific Ethical Committee on Animal Experimentation (registration number: IV./3071/2016.).

For the experiment, we used non-pregnant rats in the estrus phase and 22-day pregnant rats. The estrus phase was detected by vaginal impedance with Estrus Cycle Monitor (IM-01, MSB-MET Ltd., Balatonfüred, Hungary). For mating, rats in the estrus cycle were chosen and placed separately in an automated breeding cage with male rats (240-260g). The sexual intercourse was evaluated by native vaginal smear or copulation plugs. The confirmed pregnant animals were kept in new cages. The positive cases were considered as first-day pregnant animals.

2.2 Isolated organ bath contractility studies:

The experimental protocol is shown in Figure 1. The animals were terminated in a carbon dioxide chamber and the uterus samples were cut from both sides of the uterine horns. After cleaning from connective and adipose tissue, 3-4-mm dissected uterine tissues were tied with silk thread and mounted vertically in an isolated organ bath filled with 10 ml de Jongh buffer consisting of 137 millimolar (mM) NaCl, 3 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 4 mM NaH_2PO_4, 6 mM glucose, the pH was adjusted between 7.35-7.40 with constant temperature (37 °C) and with carbogen (95% O_2 + 5% CO_2) support. Tissues were attached to a gauge transducer (SG-02; MSB-MET Ltd., Balatonfüred, Hungary), with initial resting tension of 1.5 g, the contractions were measured, recorded, and analyzed with a SPEL Advanced ISOSYS Data Acquisition

System (MSB-MET Ltd., Balatonfüred, Hungary). The tissues were washed periodically every 15 minutes during the 1-hour equilibrium incubation period.

To achieve a satisfactory rhythmic contraction response, KCl (25 mM) was added to each chamber for 7 minutes. Each steroid was added in a cumulative manner (for T, E2, P4, and FLD 10^{-8}-10^{-3} M, and DEX 10^{-6}-10^{-8}M) every 5 minutes. Concentration-response curves were plotted against the KCl-stimulated contraction response and the effects of steroids were expressed in percentage change.

In another set of experiments, pregnant uterine tissues were pretreated with cycloheximide (10^{-6} M), a protein synthesis inhibitor, and actinomycin D (10^{-6} M) [31], a transcriptional inhibitor separately for 30 minutes. Tissues were pretreated with the following steroid hormone receptor antagonists for 10 minutes before KCl stimulation: fulvestrant (FUL) (10^{-6} M) for E2, flutamide (FLU) (10^{-6} M) for T, spironolactone (10^{-6}M) for FLD [25], mifepristone (MIF) 10^{-8} and 10^{-6} M for DEX [32] and all types of steroids respectively.

Finally, the endometrium of the uterine tissues was removed by scraping and the experiments were repeated to observe the effect of the steroids on the myometrium. The experimental protocol of the isolated organ bath study is shown in Figure 1.

The samples for each experiment were collected from both sides of the uterine horns of 2 animals (8 rings/experiment) and repeated at least 3 times for each individual set of experiments (n=132).

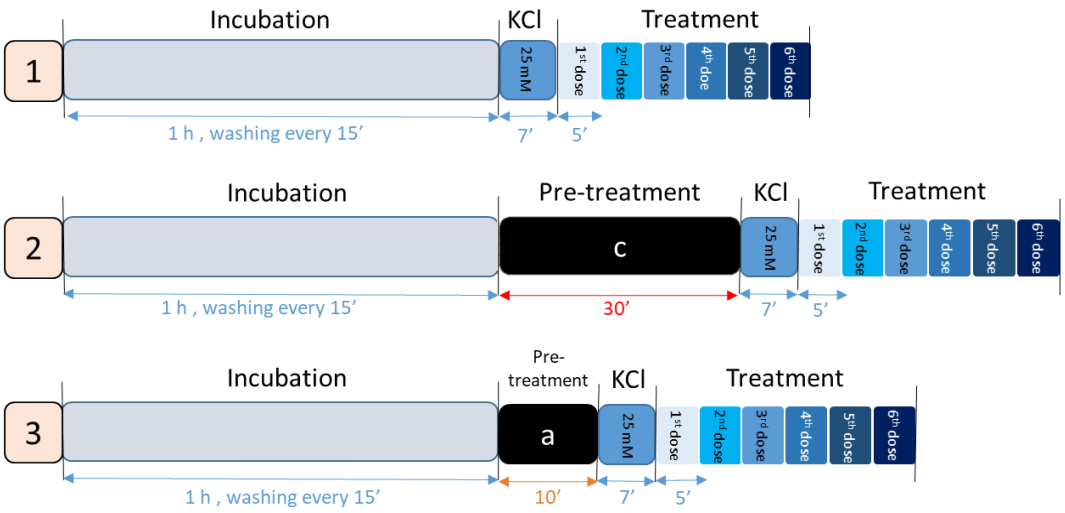

Figure 1. The isolated organ baths experimental protocol, the 1-hour incubation period, 7-min KCl stimulation, and cumulative dose treatment in 5-min interval time were the same for all experiments; (1) intact or endometrium removed pregnant and non-pregnant uterus samples treated with T, E2, P4, and DEX; (2) pregnant uterine samples with pre-treatment with actinomycin D and cycloheximide (c) for 30 min then treated with T and E2 treatment; (3) 10-min pre-treatment with steroid receptor antagonist (a): fulvestrant 10^{-6} M for E2, flutamide 10^{-6} M for T, spironolactone for FLD and MIF 10^{-8} and 10^{-6} M for DEX and all steroid treatments respectively, then treatment with T, E2, P4, F, and DEX.

2.3 In-vivo studies:

Non-pregnant (Groups 1, 2, and 3), and 22-day pregnant (Groups 4, 5, and 6) rats were anesthetized with isoflurane inhalation and the jugular vein was cannulated for later intravenous (IV) drug administration.

In the case of the non-pregnant experiment after laparotomy, an implantable strain gauge was sutured onto the surface of the left uterine horn, while for the pregnant study a bipolar disk electrode pair was fixed subcutaneously 1 cm right from the midline above the uterus. To cover the incision, the surfaces of the abdominal wall were closed with surgical staples. Both the mechanical (strain gauges) and myoelectric signals (disk electrode) were recorded for 30-min time intervals both before and after the administration of the investigated drugs (Figure 2).

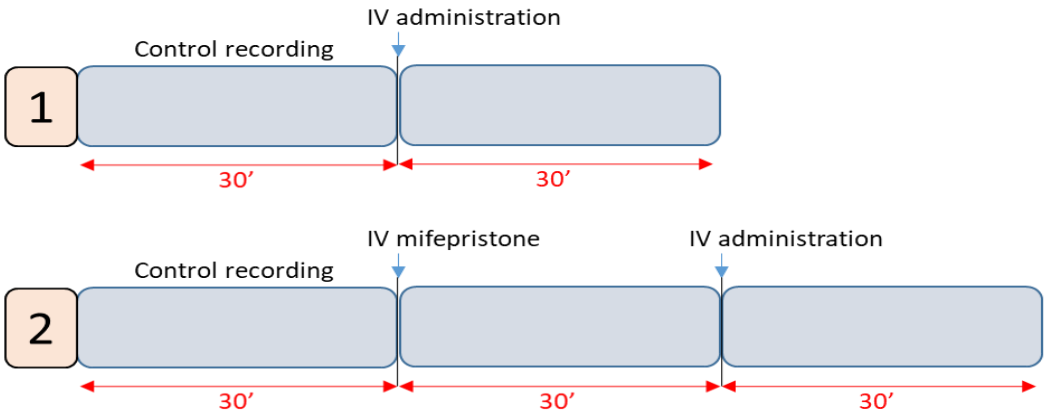

Figure 2. Protocol for the in vivo contractility study. The animals were first anesthetized, and strain gauges or electrodes were inserted on the abdomen of non-pregnant or pregnant animals, respectively. The 30-minute recording of spontaneous contractions (control period) was the same for all experiments; (1) dexamethasone (DEX) (4 mg/kg) or fludrocortisone (FLD) (25 mg/kg) was administered IV after the control period. (2) Following the control period, mifepristone (MIF) in 10 mg/kg was injected IV, then DEX or FLD was administered IV 30 min. after MIF administration.

Rats in Group 1 (n=6) and Group 4 (n=7) were treated with DEX (4 mg/kg IV) [33] only, while animals in Group 2 (n=6) and Group 5 (n=7) received MIF (10 mg/kg IV) [34] and then DEX (4 mg/kg IV) 30 min. apart. Group 3 (n=4) and Group 6 (n=6) were treated with FLD (25 mg/kg IV) [35]. In non-pregnant rats, the uterine contractions were evaluated by the AUCs (area under the curve) of the recorded contraction. In pregnant rats, the electromyographic responses were evaluated by fast Fourier transformation and the maximum of power spectrum density (PsD_{max}) values were compared in the frequency range of 1-3 cpm, which is characteristic for late pregnant uteri (Figure 3) [36].

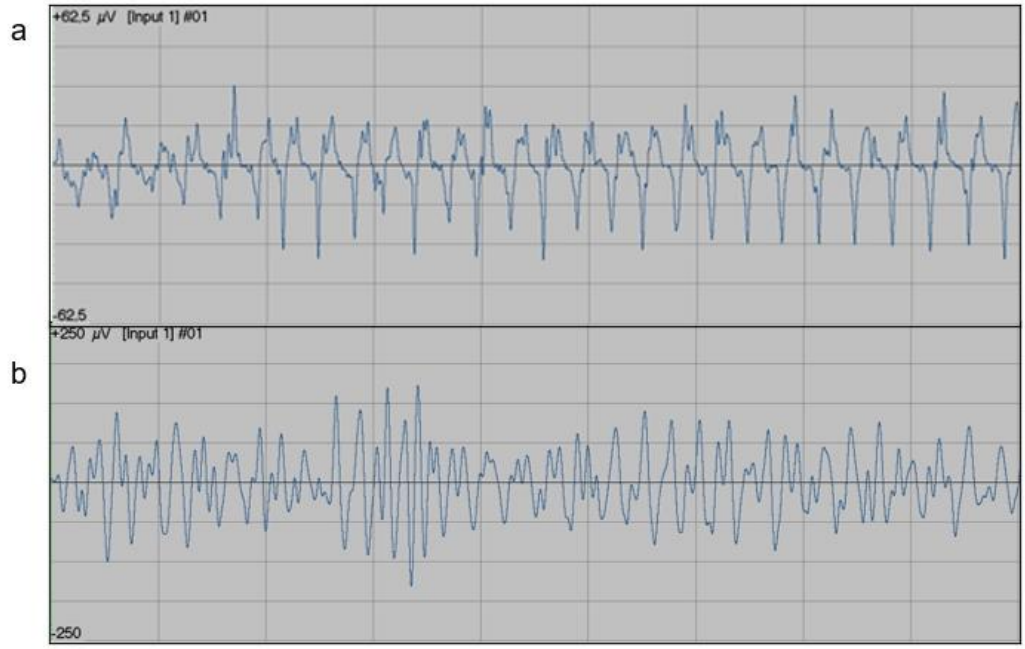

Figure 3. Representative primary signals in rat uterus within 30 minutes of experiment. a: mechanical signals of the non-pregnant tissue. The mechanical contractions were measured with strain gauges sutured onto the surface of the uterus. AUC analysis was then performed on the recorded mechanical signals. b: myoelectric signals of the 22-day pregnant tissue. The myoelectric signals of the myometrium were detected with silver disk electrodes positioned on the abdomen. The recorded myoelectric signals were then analyzed by fast Fourier transformation [36].

2.4 [^{35}S]GTPγS studies:

To investigate the efficacy of steroids on G-protein-coupled receptors (GPCRs), especially $G_{\alpha i/o}$ in a non-genomic manner, the changes of GDP to GTP were measured with radiolabeled, non-hydrolysable GTP. The experiment of [^{35}S]GTPγS was performed as described in our previous study [37]. Uterine tissue samples of both pregnant and non-pregnant animals (n=10) were grounded and homogenized with Ultra-Turrax® (IKA-Werke GmbH & Co. KG, Staufen in Breisgau, Germany) in an ice bath for 2 × 30 s with 20 volumes (W/V) of ice-cold Tris-EDTA buffer (composed of 10 mm Tris–HCl, 1 mM EDTA, 0.6 mM $MgCl_2$, and 0.25 M sucrose, pH 7.4), and afterwards suspended by a 4-layer gauze filter. Then the pellets were suspended by centrifugation at 40000 g for 20 min at 4 °C. Finally, the protein content of the pellets was measured with a Nanodrop™ 2000

spectrophotometer (Thermo Fisher Scientific, Wilmington, US) and the pellets were diluted to 10 mg/ml sample.

The uterine tissue fractions (in a final concentration of 10 µg/ml) and Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HC, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 mbq/0.05 cm^3 [^{35}S]GTPγS (0.05 nm) were pre-incubated in 24 polystyrene test tubes (Starstedt Co.) with or without MIF (10^{-6} M) at 30 °C. After 15 minutes, different doses of E2, P, T, FLD (10^{-8}-10^{-4}) and DEX (10^{-6}-10^{-4}) were added separately for 20 min. Total binding or basal activity and non-specific binding were evaluated by measuring the buffer without protein sample as basal activity and 10µm unlabeled GTPγS and subtraction from total binding for non-specific binding. The reaction in the incubation period was terminated by fast vacuum filtration (through Whatman GF/B filters with Brandel M24R Cell harvester). To separate the bound and free [^{35}S]GTPγS completely, the filters were washed with ice-cold buffer (pH 7.4) 3 times. Then the filters were dried, and their radioactivity was detected in Ultima GoldTM MV aqueous scintillation cocktail with Packard TriCarb 2300TR liquid scintillation counter. Each experiment was designed in triplicate, repeated 2 times.

2.5 Cyclic AMP studies:

The changes in cyclic AMP (cAMP) levels of the sample tissue by non-genomic action of steroids were investigated using the commercial cAMP Enzyme Immunoassay Kit (Cayman Chemical, USA). The experiment was followed by an isolated organ bath study. Uterine tissues of both pregnant and non-pregnant SPRD rats were collected and incubated in an organ bath filled with 10 ml of de Jongh buffer. After incubation with MIF (10^{-6} M) (half of the samples - the other half without MIF) for 10 minutes and KCl 25 mM for 7 minutes, 2 doses of T, E2, P, FLD or control (10^{-4}-10^{-6}) and DEX (10^{-4}-10^{-5} M) were added for 5 minutes. Finally, forskolin (10^{-5} M) was added to all chambers for another 10 minutes. Then, by using liquid nitrogen, the samples were immediately frozen and kept at -70 °C. Liquid samples were prepared from frozen tissues. the frozen tissues were first pulverized, weighted, homogenized, mixed with 10 volumes of an ice-cold aqueous solution of 5% trichloroacetic acid (TCA), and centrifuged at 1500 g for 15 minutes. The supernatants were separated from TCA with water-saturated ether. The separation was repeated 3 times. Ether residue was evaporated by heating the liquids and the final liquid samples were stored at -70 °C to be used in the cAMP assay. The cAMP level of the samples was expressed in nmol/mg tissue.

2.6 Drugs and chemicals:

1,3,5-Estratriene-3,17β-diol (E2), 4-pregnene-3,20-dion (P4), 17β-Hydroxy-3-oxo-4-androstene (T), 9-Fluorocortisol acetate (FLD), Spironolactone (SPR), cycloheximide, actinomycin D, mifepristone (MIF), and flutamide (FLU) were all purchased from Sigma-Aldrich, Budapest, Hungary. Dexamethasone sodium phosphate (DEX) was purchased from Ratiopharm, Budapest, Hungary. Fulvestrant (Falsoldex-FUL) 250 mg/ml injection was purchased from AstraZeneca Pharmaceutical, Budapest, Hungary. Forskolin was purchased from Tocris, Norderstedt, Germany.

DEX was dissolved in distilled water for both in vivo and in vitro experiments. For the in vitro study, T, E2, P4 and FLD was dissolved in water; ethanol; Macrogol 400 = 90; 0.2; 0.8 in the highest concentration, further dilutions were made with water. In the in vivo experiments, FLD was dissolved in water; dimethyl sulfoxide (DMSO); Macrogol 400 = 12.5; 12.5; 75.

2.7 Statistical analysis:

The response curves for the in vitro experiments were plotted by analysis of the AUC of contraction response against concentration. Based on the evaluated AUCs, the E_{max} and EC_{50} values were determined and presented as the mean ± SEM (standard error).

The recorded mechanical and myoelectric signals of the in vivo experiments were analyzed by AUC and fast Fourier transformation, respectively. The AUC and PsD_{max} values were determined and compared statistically [36,38].

Data analysis and statistical assays were done by using the Prism 8.0 (GraphPad Software Inc. San Diego, CA, USA) computer program by applying the ANOVA Dunnett's test.

3. Results:

3.1 Studies with testosterone:

3.1.1 **Isolated organ bath study:**

T elicited a relaxing effect especially at high concentrations (10^{-5}- 10^{-3} M) and reached 50% relaxation of the non-pregnant and 70% in pregnant uteri (Figure 4.a and d, Figure 5.a and b, and Table 1.). The presence of cycloheximide and actinomycin D (Figure 5.c, and Table 1.), the removal of the endometrium (Figure 5.a and b, and Table 1.) or even

FLU (Figure 4.b and e, Figure 5. a and b, and Table 1.) did not modify the relaxing effect of T in both pregnant and non-pregnant uterine tissues. However, MIF shifted the T concentration-response curve to the right and reduced its maximal inhibitory effect (Figure 4. c and f, Figure 5.a and b, and Table 1.) In the subsequent experiments investigating the signaling pathway, we measured the T action alone or in the presence of MIF. The interventions (endometrium removal) and drug treatments (actinomycin D, cycloheximide, FLU) with non-significant modifications were omitted from further studies.

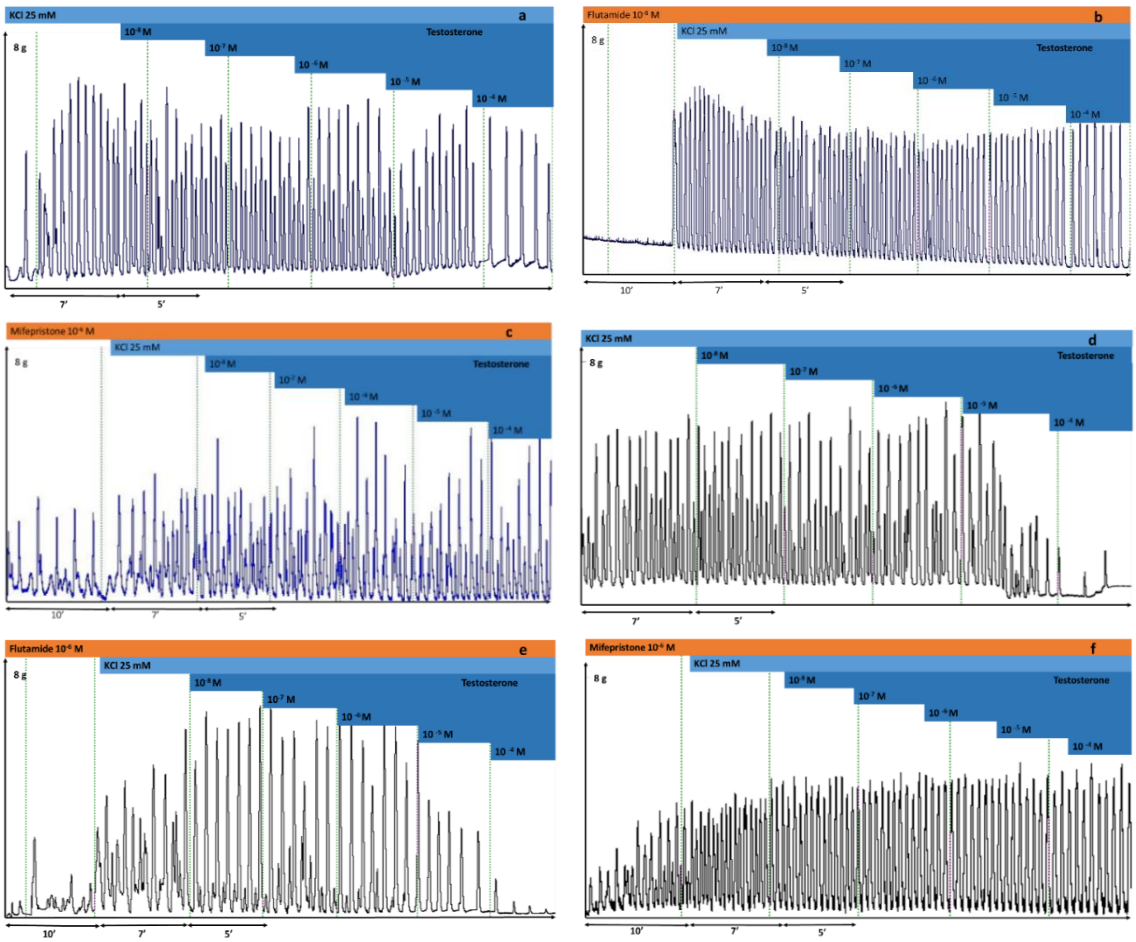

Figure 4. Effects of Testosterone on KCl-induced (25 mM) non-pregnant (a) and pregnant (d) uterine contractions at concentrations of 10^{-8} -10^{-4} M in a cumulative manner. The effects of Testosterone were also investigated in the presence of 10^{-6} M Flutamide or 10^{-6} M MIF both in non-pregnant (b, c) and pregnant uteri (e, f). Each figure is a representative record.

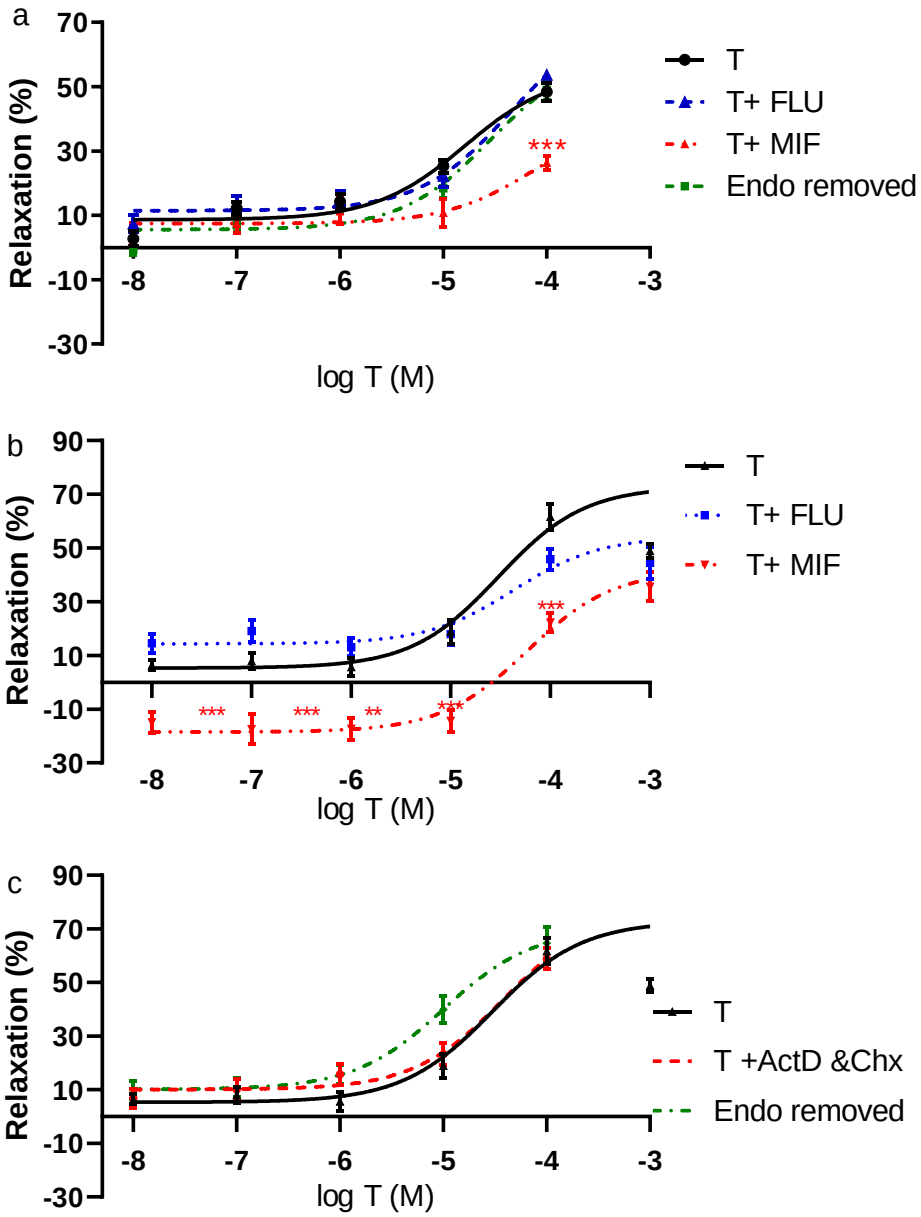

Figure 5. Effects of T on uterine contractions stimulated with KCl (25 mM), intact or with endometrium removal (a, c), and with pre-treatment with flutamide or mifepristone (a, b) for pregnant and non-pregnant respectively. Also, with pre-treated of pregnant tissues with actinomycin D and cycloheximide(c). data presented by percent of relaxation. **: $p<0.01$; ***: $p<0.001$; ActD, actinomycin D; Chx, cycloheximide; Endo, endometrium; FLU, flutamide; MIF, mifepristone; T, testosterone.

a	T	T+ FLU	T+ MIF	Endo removal
E_{max} (%±S.E.M)	54±4.1	57±2.5	26±2.1	57±1.7
EC_{50} (M)	1.6e-005	3.2e-005	7.8e-005(***)	2.8e-005

b	T	T+ ActD+ Chx	T+ FLU	T+ MIF	Endo removal
E_{max} (%±S.E.M)	73±7.8	77±2.4	62±8	42±7.2	70±2.7
EC_{50} (M)	3.0e-005	3.8e-005	3.3e-005	5.8e-005(***)	1.0e-005

Table 1. Changes in the E_{max} and EC_{50} values of the non-pregnant(a) and pregnant(b) uterine relaxing effect of T alone, with pre-treatment with actinomycin D and cycloheximide and mifepristone and also after the removal of the endometrium in the 22-day-pregnant rat. ***: $p<0.001$; ActD, actinomycin D; Chx, cycloheximide; Endo, endometrium; FLU, flutamide; MIF, mifepristone; T, testosterone.

3.1.2 **[^{35}S]GTPγs binding assay studies:**

T increased the [^{35}S]GTPγS binding in a concentration dependent manner on both pregnant and non-pregnant tissues. The pre-treatment with MIF reduced specific binding and shifted the curve to the right, indicating less activation of G-proteins. Although this inhibition was more significant in pregnant tissues (Figure 6.a and b, and Table 2**.**).

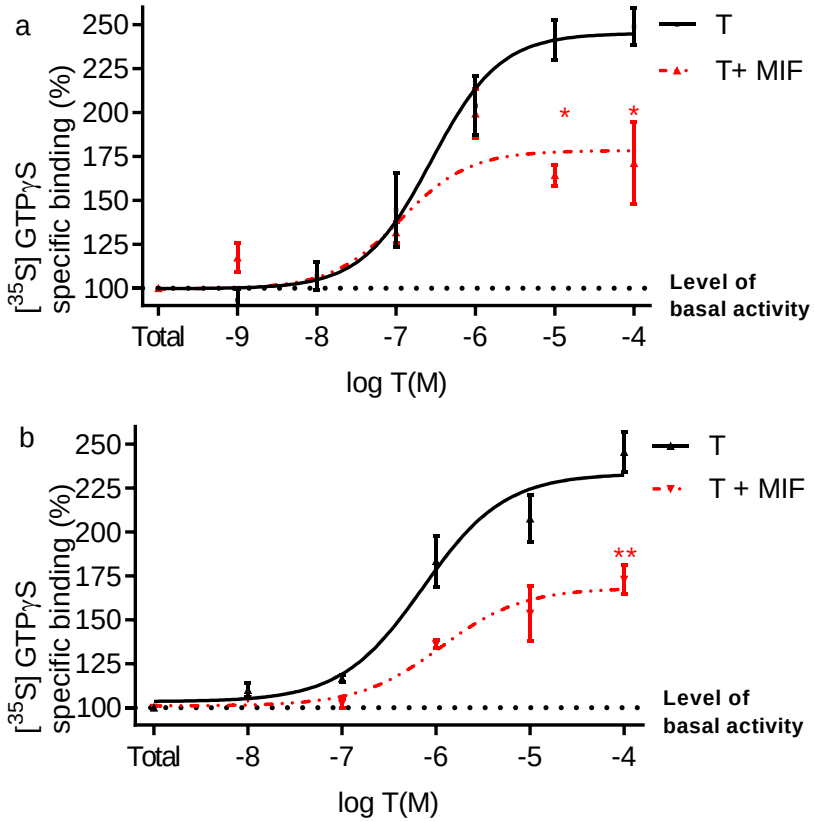

Figure 6. Effect of T (10^{-8}-10^{-4} M) on [^{35}S]GTPγS binding of non-pregnant(a) and pregnant (b) tissues with or without pre-treatment with mifepristone. Mifepristone reduced the T-induced increase in [^{35}S]GTPγS binding. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without any substances. *: p <0.05; **: p<0.01; MIF, mifepristone; T, testosterone.

a	**T**	**T+ MIF**
E_{max} (%±S.E.M)	230±8.5	178±95(**)
EC_{50} (M)	2.7e-007	1.3e-007
b	**T**	**T+ MIF**
E_{max} (%±S.E.M)	230±9.3	166±7.2(**)
EC_{50} (M)	7.1e-007	1.2e-006

Table 2. Changes in the [^{35}S]GTPγS binding induced by T alone and with pre-incubation with mifepristone in the 22-day-pregnant rat uteri. **: p<0.01; MIF, mifepristone; T, testosterone.

3.1.3 **cAMP study:**

The pregnant and non-pregnant cAMP level was raised by T compared to the control at both high and low concentrations (10^{-4}, 10^{-6} M), But at lower dose (10^{-6}M) this elevation of cAMP was less significant. Moreover, the pre-treatment with MIF significantly reduced the uterine cAMP levels by T specially in pregnant tissues (Figure 7.a and b).

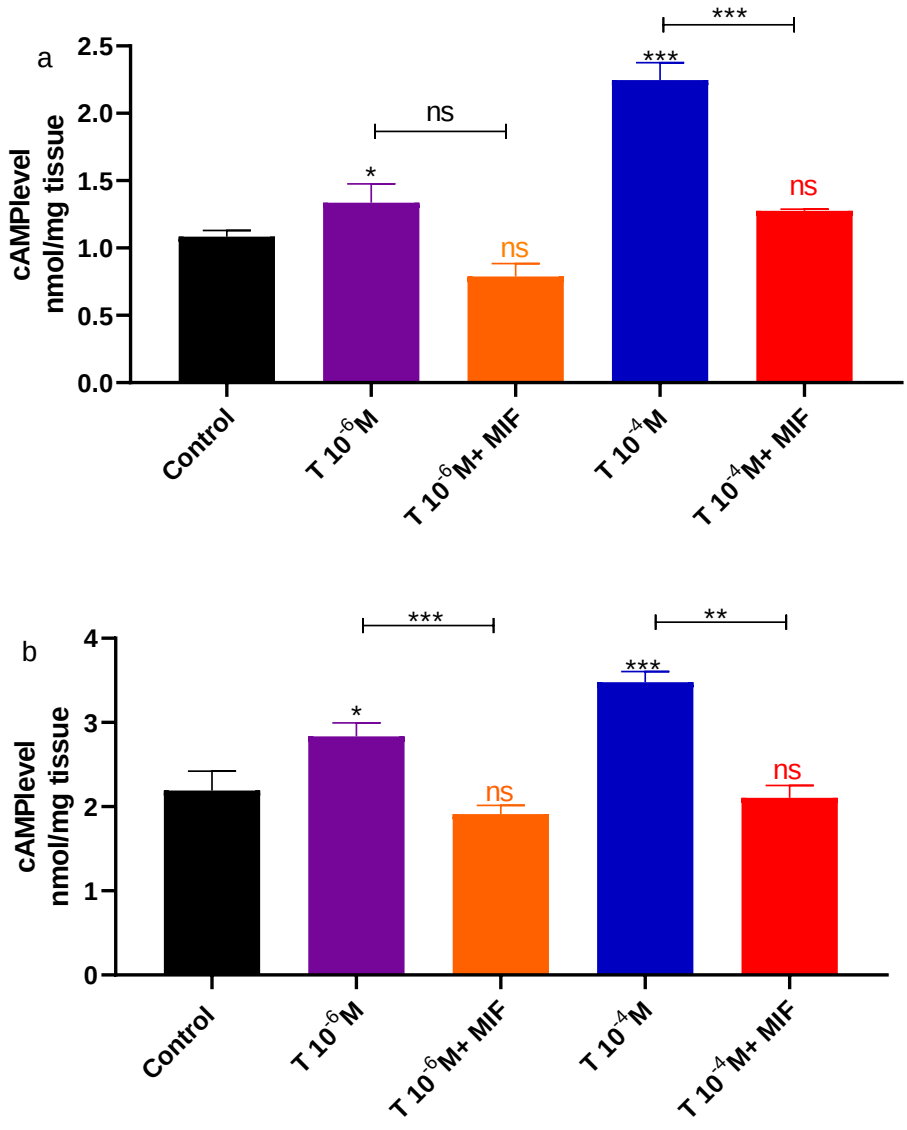

Figure 7. Change in the level of uterine cAMP in the presence of T alone and after pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues. The uterine cAMP level was expressed in nmol/mg tissue. ns: non-significance; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; MIF, mifepristone; T, testosterone.

3.2 Studies with 17-β estradiol:

3.2.1 **Isolated organ bath study:**

E2 relaxed non-pregnant and pregnant uterine contractions specially at the highest concentration (10^{-4}M), the inhibition was 50 and 70% respectively (Figure 8.a and d , Figure 9.a and b, and Table 3.). Actinomycin D and cycloheximide (Figure 9.b, Table 3.), the removal of the endometrium (Figure 9.a and b, Table 3.) or even pre-treatment with FUL, (Figure 8.b and e, Figure 9a and b) did not influence the effect of E2 in both tissues. In contrast, pre-treatment of both tissues with MIF reduced the relaxing effect of E2, which was more significant in all doses in pregnant tissues (Figure 8.f, Figure 9.b), while in non-pregnant the effect was only in high doses was significant (Figure 8.c, Figure 9.a, and Table 3.). In the subsequent experiments investigating the signaling pathway, we measured the E2 action alone or in the presence of MIF. The interventions (endometrium removal) and drug treatments (actinomycin D, cycloheximide, FLU) with non-significant modifications were omitted from further studies.

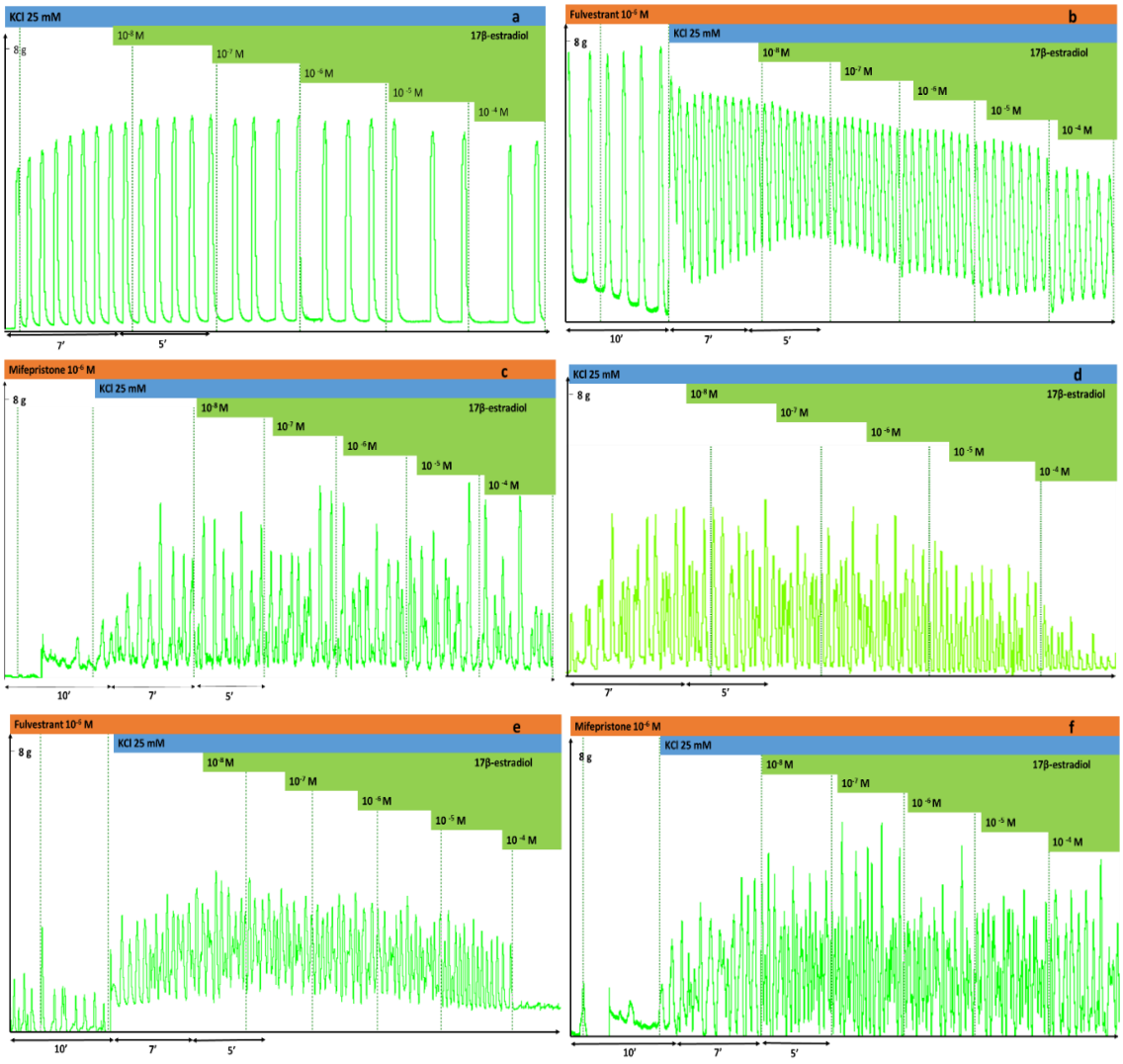

Figure 8. Effects of E2 on KCl-induced (25 mM) intact or after endometrium removal on pregnant (a, d) and non-pregnant (f, i) uterine contractions at concentrations of 10^{-8} -10^{-4} M in a cumulative manner. The effects of E2 were also investigated in the presence of 10^{-6} M Fulvestrant or 10^{-6} M mifepristone both in non-pregnant (g, h) and pregnant uteri (d, e). and also, actinomycin and cycloheximide pre-treatment (c). Each figure is a representative record.

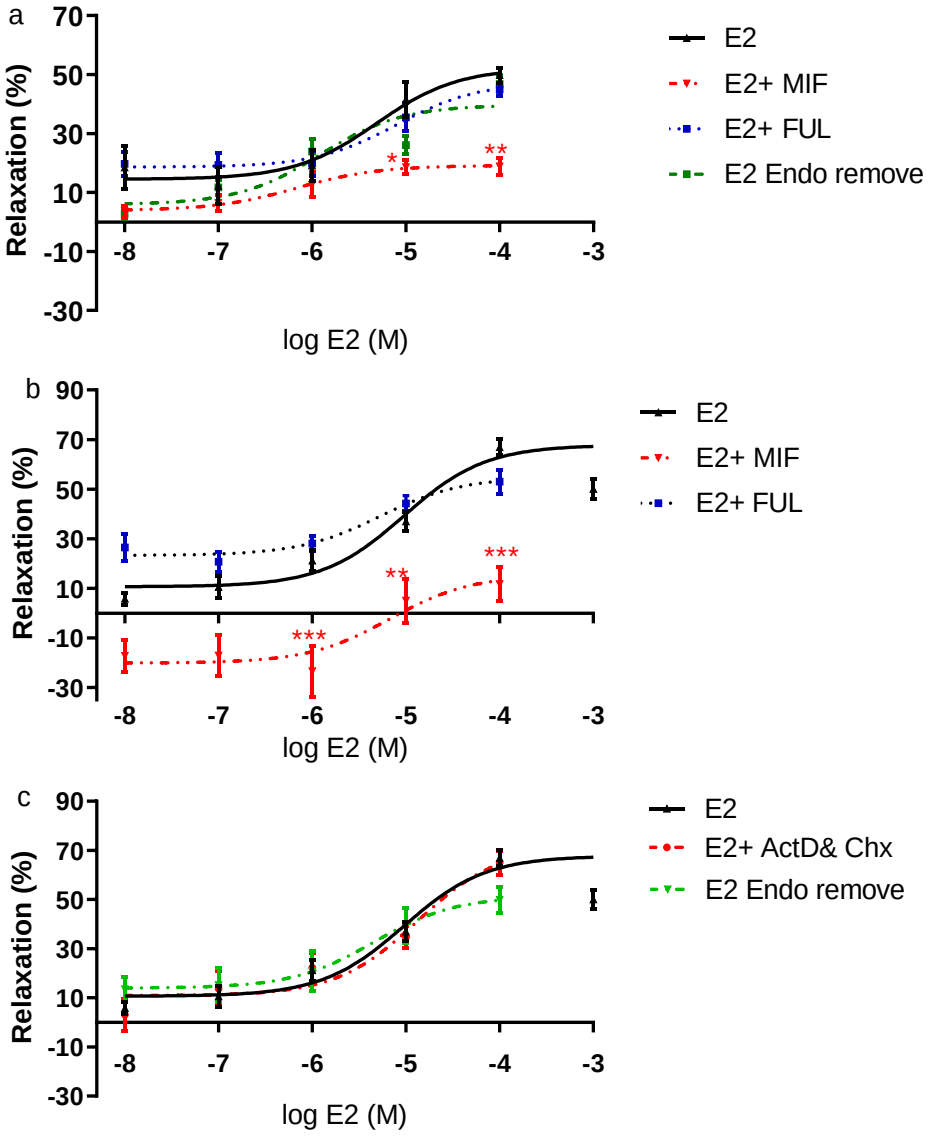

Figure 9. Effect of E2 on pregnant uterus tissue stimulated with KCl (25 mM) in the presence of actinomycin D and cycloheximide (a), and with pre-treatment with fulvestrant or mifepristone, and after endometrium removal (b) presented by percent of relaxation. *:p <0.05; **: p<0.01; ***: p<0.001; ActD, actinomycin D; Chx, cycloheximide; E2, 17-β estradiol; MIF, mifepristone; FUL, fulvestrant.

	E2	E2 + FUL	E2+ MIF	Endo removal
E_{max} (%±S.E.M)	52.2±9.6	52.8±4.1	19.2±2.4(**)	40.7±3
EC_{50} (M)	4.8e-006	2.7e-006	6.8e-007	1.2e-006

	E2	E2+ ActD +Chx	E2 + FUL	E2+ MIF	Endo removal
E_{max} (%±S.E.M)	67.8±2.8	71.7±3.8	54.8±2.9	15.4±5.5(***)	51.3±4.6
EC_{50} (M)	9.5e-006	1.3e-005	5.3e-006	6.7e-006	4.6e-006

Table 3. Changes in the E_{max} and EC_{50} values of the non-pregnant (a) and pregnant (b)uterine relaxing effect of E2 alone, with pre-treatment with actinomycin D and cycloheximide, fulvestrant and mifepristone and after removing the endometrium. **: $p<0.01$; ***: $p<0.001$; ActD, actinomycin D; Chx, cycloheximide; E2, 17-β estradiol; FUL, fulvestrant; MIF, mifepristone.

3.2.2 **[^{35}S]GTPγS binding assay studies:**

The [^{35}S]GTPγS binding on pregnant and non-pregnant samples was increased by E2 in a concentration dependent manner, which was reduced by MIF specially in high dose and pregnant tissue (Figure 10.a and b, Table 4.).

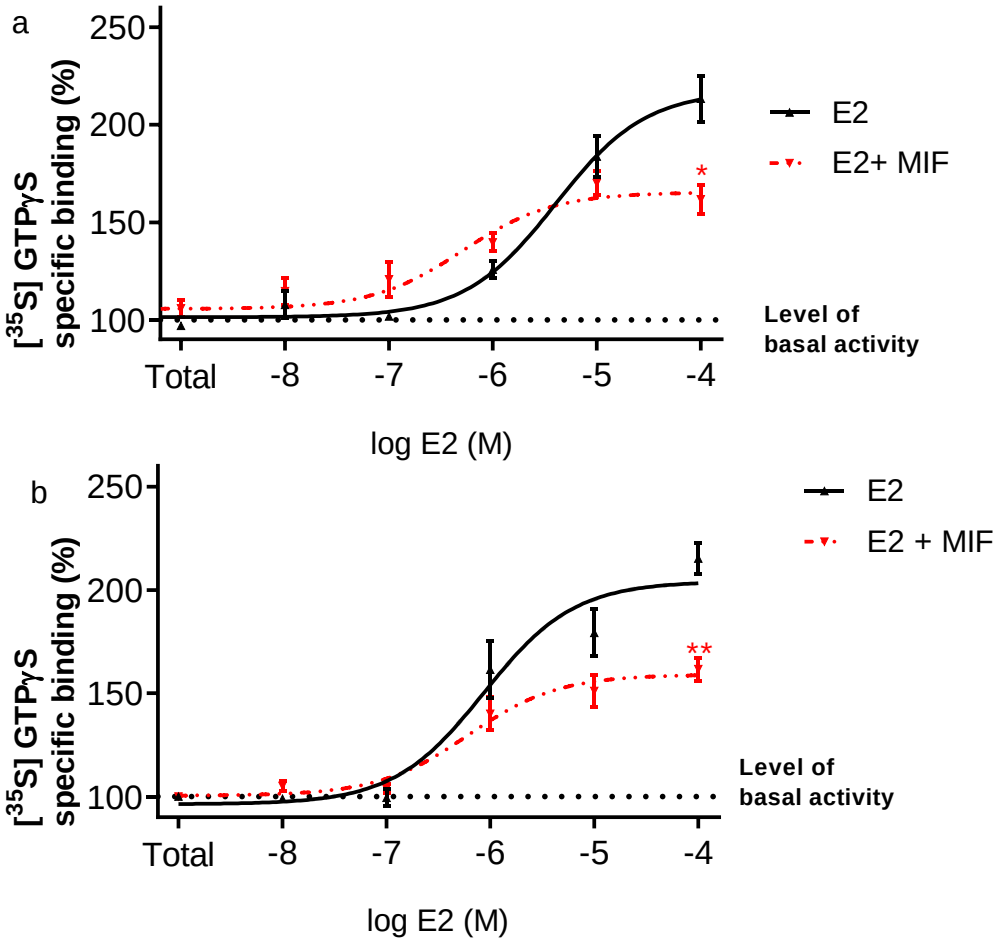

Figure 10. The effect of E2 (10^{-8}-10^{-4} M) on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues. Mifepristone reduced the E2-induced increase in [^{35}S]GTPγS binding. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without substance. *:p <0.05; **: p<0.01; E2, 17-β estradiol; MIF, mifepristone.

a	E2	E2+ MIF
E_{max} (%±S.E.M)	217±8.2	106±11.8(**)
EC_{50} (M)	4.1e-006	1.4e-006

b	E2	E2+ MIF
E_{max} (%±S.E.M)	196 ±7.7	155±4.5(**)
EC_{50} (M)	7.1e-007	8.5e-007

Table 4. Changes in the [^{35}S]GTPγS binding effect of E2 alone and with pre-incubation with mifepristone in the non-pregnant (a) and 22-day-pregnant (b) rat. **: $p<0.01$; E2, 17-β estradiol; MIF, mifepristone.

3.2.3 **cAMP study:**

E2 increased the level of cAMP of the non- pregnant and pregnant tissue compared to the control in high concentrations the elevation was more in non-pregnant, while it had no effect in a low dose in both tissues. The pre-treatment with MIF reduced the high E2 concentration-induced cAMP increase ($p<0.01$), but MIF had no action in the case of low E2 concentration. (Figure 11.a and b).

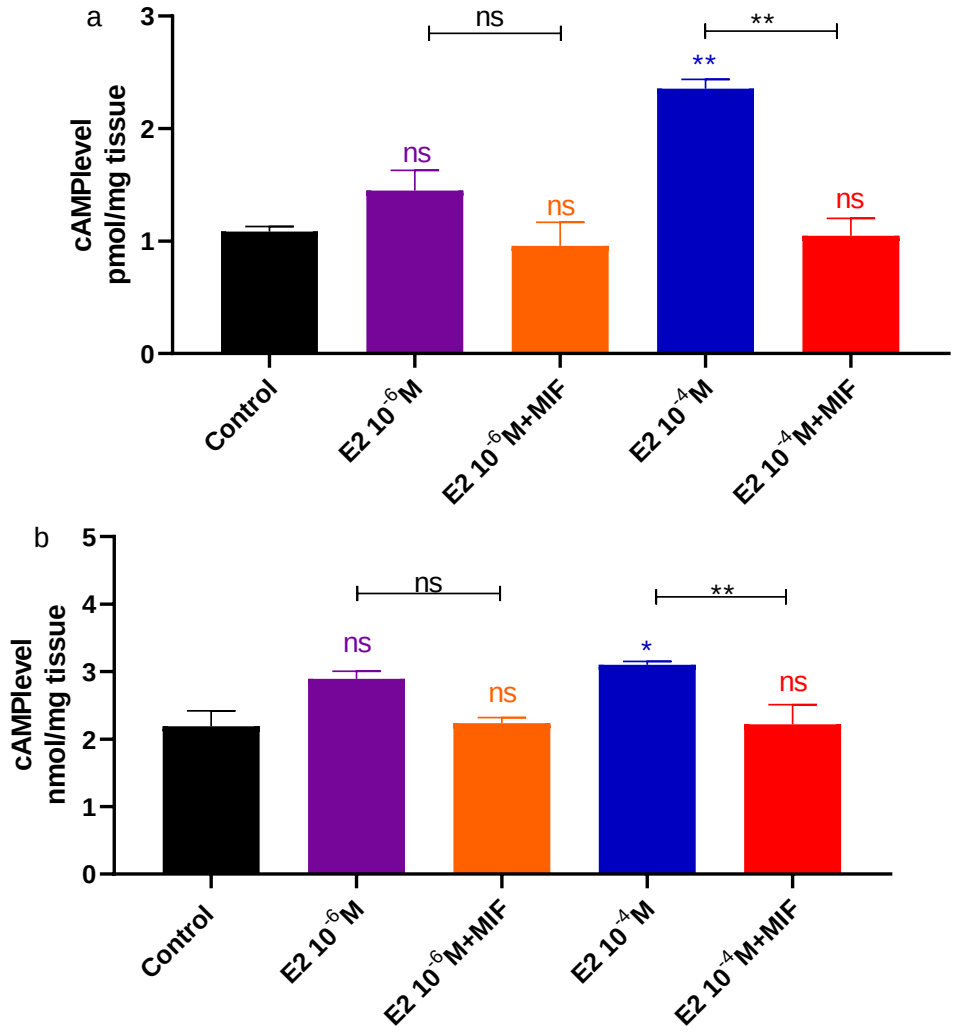

Figure 11. Changes in the level of cAMP in the presence of E2 alone (10^{-6} and 10^{-4} M) and with pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues expressed in nmol/mg tissue. ns: non-significance; *: $p < 0.05$ **: $p<0.01$; E2, 17-β estradiol; MIF, mifepristone.

3.3 Studies with progesterone:

P4 had a negligible effect on KCl-stimulated non-pregnant and pregnant uterine contractions (Figure 12.a and c, Figure 13.a and b, and Table 5.). The presence of MIF did not modify its action (Figure 12.b and d., Figure 13.a and b, and Table 5.). Since the relaxing effect of P4 was missing, we did not investigate it further.

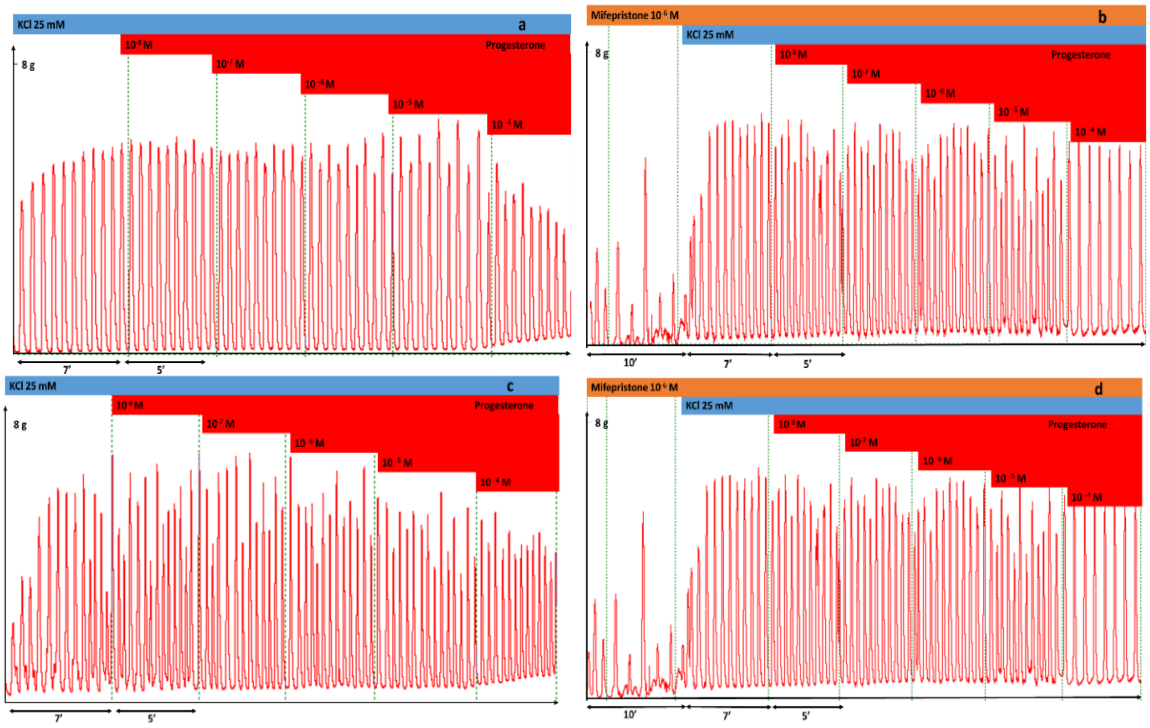

Figure 12. Effects of Progesterone on KCl-induced (25 mM) non-pregnant (a) and pregnant (c) uterine contractions at concentrations of 10^{-8} -10^{-4} M in a cumulative manner. The effects of progesterone were also investigated in the presence of 10^{-6} M mifepristone in non-pregnant (b) and pregnant uteri (d). Each figure is a representative record.

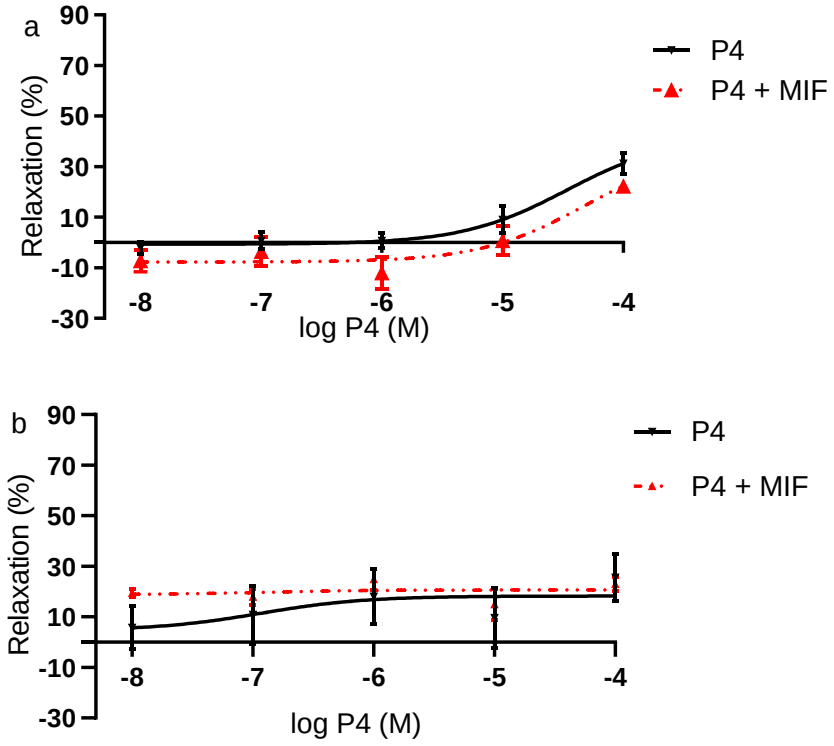

Figure 13. Effect of P4 on non-pregnant (a) and pregnant (b) uterus smooth muscle contractions stimulated by KCl, alone and in the presence of mifepristone. MIF, mifepristone; P4, progesterone.

a	P4	P4+MIF
E_{max} (%±S.E.M)	42±11.1	36±23
EC_{50} (M)	3.8e-005	4.7e-005
b	**P4**	**P4+MIF**
E_{max} (%±S.E.M)	16±6.9	20±2.6
EC_{50} (M)	1.2e007	1.2e-007

Table 5. Changes in the uterine-contracting effect of P4 alone and in the presence of mifepristone in pregnant(a) and non-pregnant (b) uterine tissues. P4; progesterone. MIF, mifepristone.

3.4 Studies with dexamethasone:

3.4.1 **Isolated organ bath studies:**

DEX elicited a moderate relaxing effect at high concentration (10^{-4} M) in non-pregnant uteri (Figure 14.a, Figure 15.a, and Table 6.), but in pregnant uteri this relaxing effect was slightly less (Figure 14.d, Figure 15.b, and Table 6) (37% and 33%, respectively). Only the relaxing effect of DEX at the dose of 10^{-4} M was inhibited by the higher dose of MIF (10^{-6} M), while neither dose of MIF changed significantly the response at the lower and moderate doses of DEX in non-pregnant uteri (Figure 14.b and c, Figure 15.a), whereas in the pregnant uterus, the effect was shifted to the right and even changed to a slight contraction only in the presence of a higher dose of MIF (10^{-6} M) (Figure 14.e and f, Figure 15.b, and Table 6.). Actinomycin D pre-treatment did not modify the effect in either non-pregnant or pregnant tissues (Figure 15.a and b, and Table 6.). Endometrium removal did not change the action either (data not shown). In the subsequent experiments for the investigation of the signaling pathway, the action of DEX alone and in the presence of MIF was measured. Since there were no significant changes with the intervention (endothelium removal) and the lower dose of MIF (10^{-8} M), we omitted them from further steps of our study.

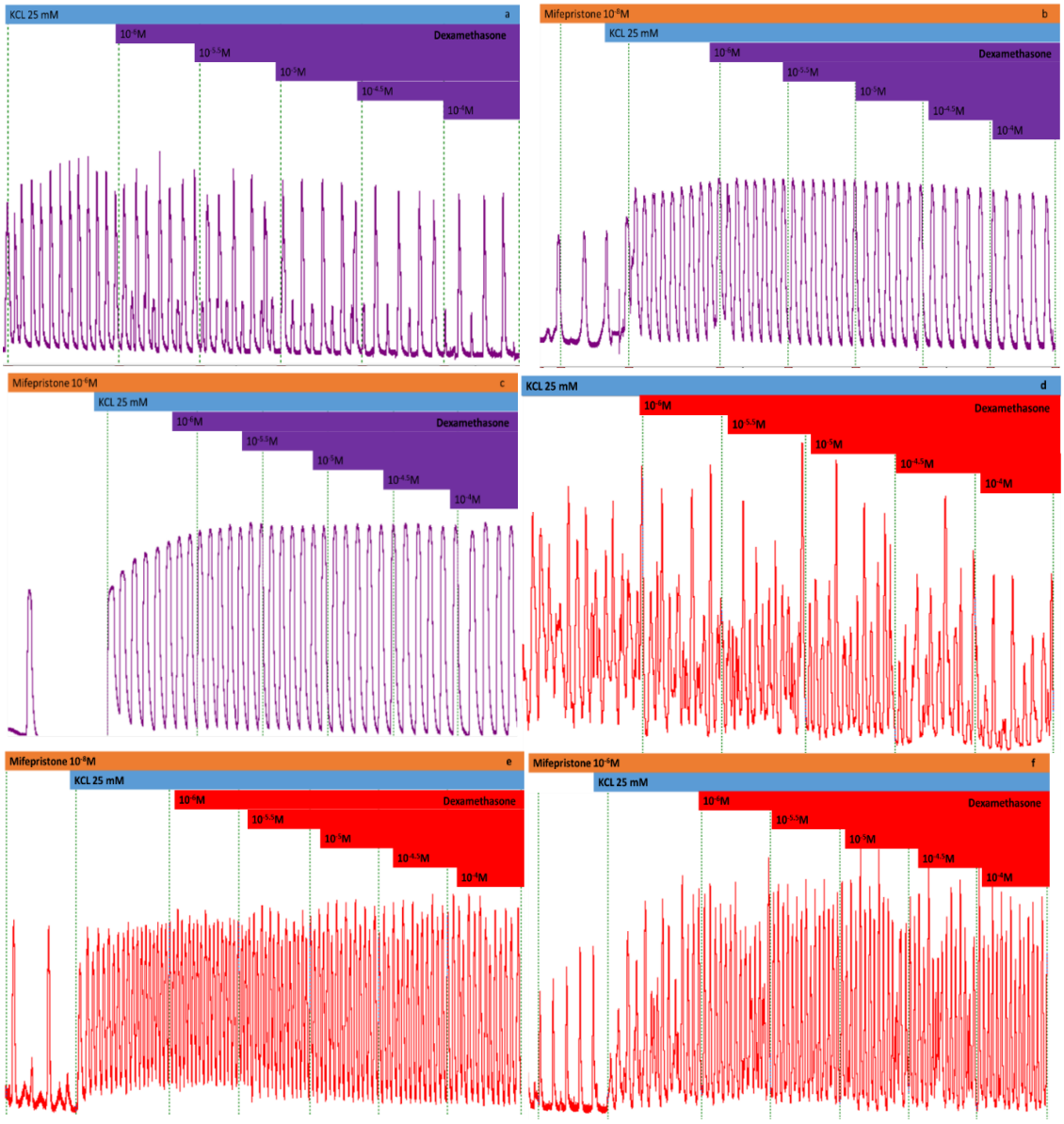

Figure 14. Effects of dexamethasone on KCl-induced (25 mM) non-pregnant (a) and pregnant (d) uterine contractions at concentrations of 10^{-6} -10^{-4} M in a cumulative manner. The effects of dexamethasone were also investigated in the presence of 10^{-8} and 10^{-6} M mifepristone both in non-pregnant (b, c) and pregnant uteri (e, f). Each figure is a representative record.

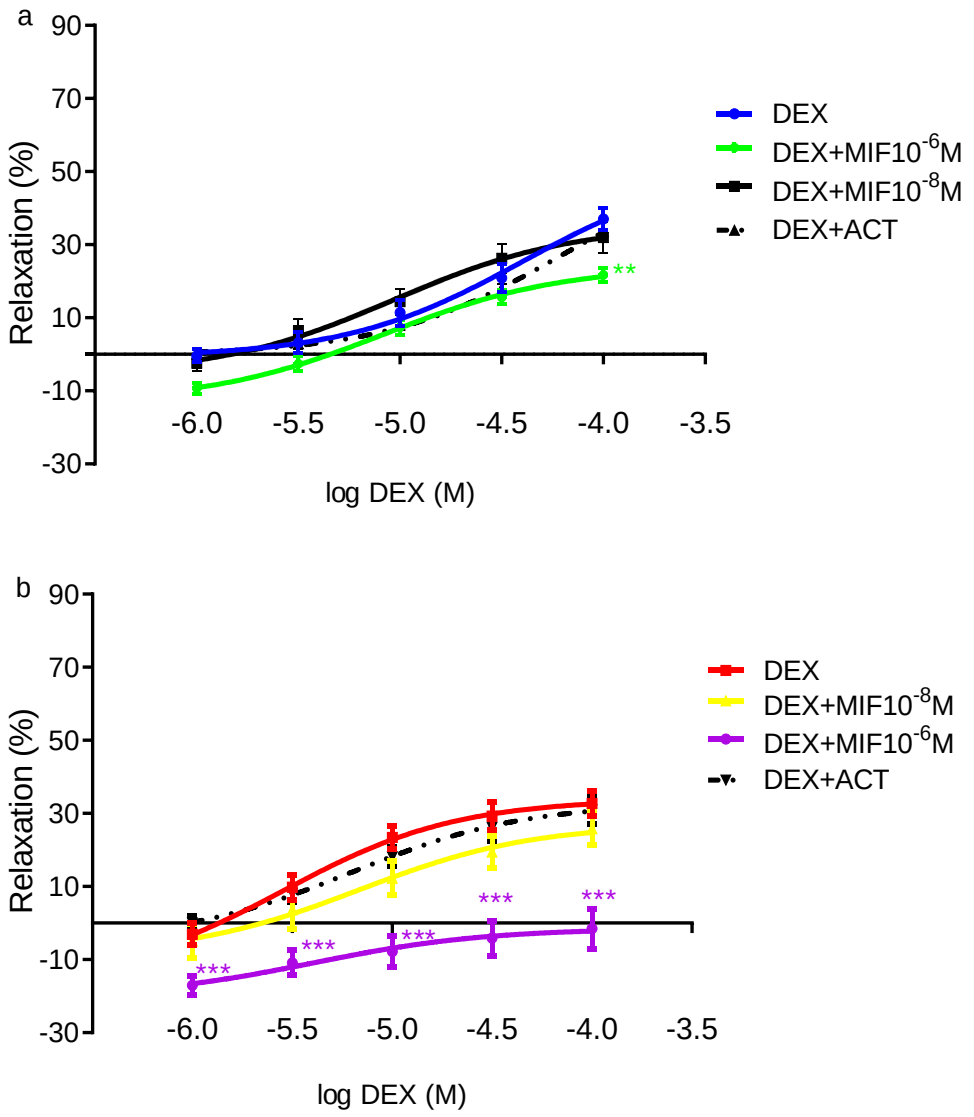

Figure 15. Effects of dexamethasone on non-pregnant (a) and pregnant (b) uterine contractions in vitro stimulated with KCl (25 mM) and pre-treated with mifepristone in 2 doses or Actinomycin D, presented as a percentage of relaxation. **: $p<0.01$; ***: $p<0.001$; ACT, Actinomycin D; DEX, dexamethasone; MIF, mifepristone.

a	DEX	DEX+ MIF8	DEX+ MIF6	DEX+ ACT
E_{max} (%±S.E.M)	51±2.8	35.3±4.9	24.14±2.8(**)	54.9±1
EC_{50} (M)	4e-005	9.2e-006	8.3e-006	6.1e-005
b	**DEX**	**DEX+ MIF8**	**DEX+ MIF6**	**DEX+ ACT**
E_{max} (%±S.E.M)	34±10.1	27.3±7.6	-1.3±7(**)	32.9±4.9
EC_{50} (M)	2.9e-006	6.8e-006	4.2e-006	6.3e-006

Table 6. Changes in the E_{max} and EC_{50} values of the non-pregnant (a) and pregnant (b)uterine relaxing effect of DEX alone, with pre-treatment with actinomycin D and cycloheximide or two doses of mifepristone and after removing the endometrium. **: $p<0.01$; ***: $p<0.001$; ActD, actinomycin D; Chx, cycloheximide; DEX, dexamethasone; MIF, mifepristone.

3.4.2 **[^{35}S]GTPγS binding assay studies.**

The elevation of [^{35}S]GTPγS binding in a concentration-dependent manner was observed both in pregnant (Figure 16.b, and Table 7.) and non-pregnant tissues (Figure 16.a, and Table 7.). MIF pre-treatment shifted to the right by inhibiting this elevation, which means less activation of G-proteins.

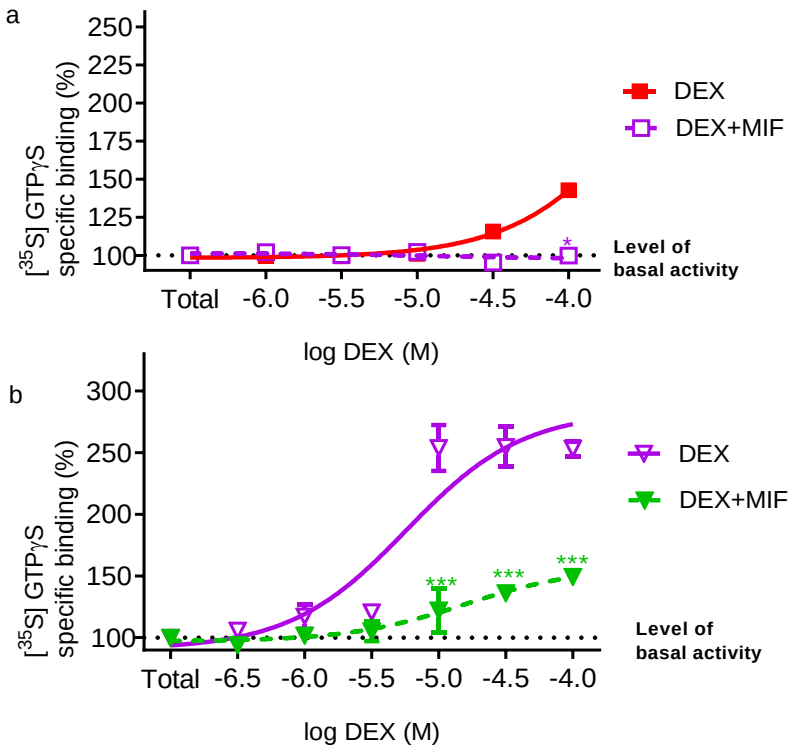

Figure 16. Effect of dexamethasone (10^{-6}-10^{-4} M) on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone. Mifepristone reduced dexamethasone-induced increase in [^{35}S]GTPγS binding in non-pregnant (a) and pregnant (b) uterine tissues. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without any substances. *: p<0.05; ***: p<0.001; MIF, mifepristone; DEX, dexamethasone.

a	DEX	DEX+ MIF
E_{max} (%±S.E.M)	185±35.8	101±2
EC_{50} (M)	4.3e-005	1.3e-005
b	**DEX**	**DEX+ MIF**
E_{max} (%±S.E.M)	28±20.3	157±11.6(***)
EC_{50} (M)	5.7e-006	1.5e-005

Table 7. Changes in the [^{35}S]GTPγS binding effect of DEX alone and with pre-incubation with mifepristone in the non-pregnant (a) and 22-day-pregnant (b) rat. ***: p<0.001; DEX, dexamethsone; MIF, mifepristone.

3.4.3 **cAMP study.**

Uterine cAMP levels both in pregnant and non-pregnant uteri rose in the presence of a high dose of DEX (10^{-4} M), moreover, this rise was inhibited significantly by pre-treatment with MIF. The moderate dose of DEX (10^{-5} M) raised cAMP just in non-pregnant tissues (Figure 17.a and b)

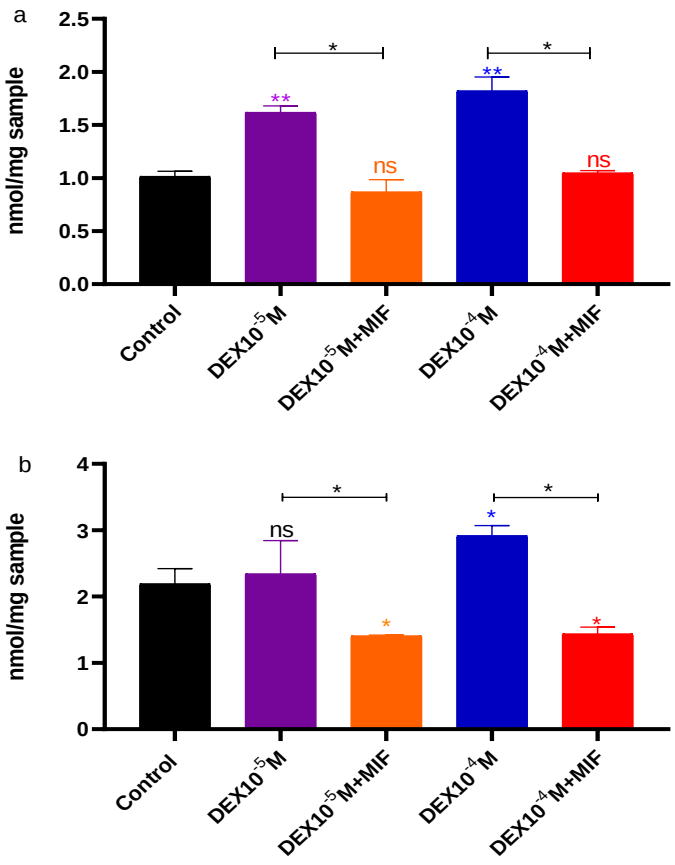

Figure 17. Changes in the cAMP level of uterine non-pregnant (a) and pregnant (b) tissues in the presence of dexamethasone alone and after pre-treatment with mifepristone. The uterine cAMP level was expressed in nmol/mg tissue. ns: non-significance; *: $p < 0.05$ **: $p<0.01$; DEX, dexamethasone; MIF, mifepristone.

3.4.4 **In vivo study.**

The injection of one high dose of DEX led to the inhibition of contraction for both pregnant and non-pregnant animals, the action was stronger in pregnant ones. In addition, we observed that half an hour of pre-treatment with MIF blocked the effect of DEX specially in pregnant animals which was significant, furthermore, MIF alone did not affect the uterus (Figure 18.).

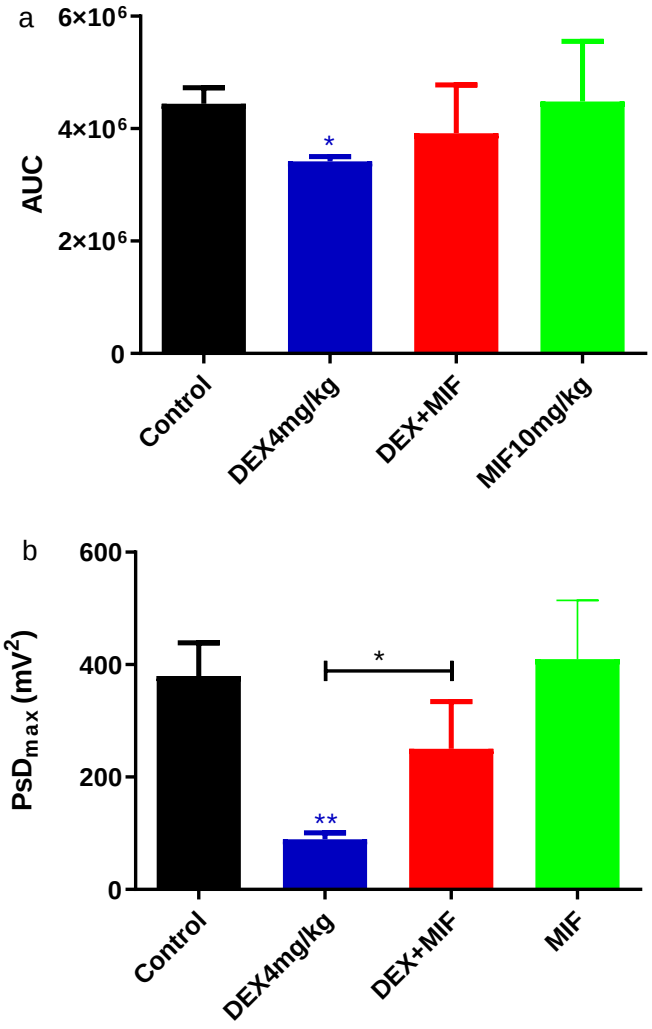

Figure 18. Effect of dexamethasone and mifepristone on non-pregnant (a) and pregnant (b) animals, alone and with pre-treatment with MIF, in vivo, *: $p<0.05$; **: $p<0.01$; MIF, mifepristone; DEX, dexamethasone.

3.5 Studies with fludrocortisone:

3.5.1 **Isolated organ bath studies.**

FLD showed a very slight relaxing action only at the highest dose (10^{-4}M) both in pregnant (Figure 19.d, Figure 20.b) and non-pregnant groups (Figure 19.a, Figure 20.) (31 and 34%, respectively). Spironolactone and MIF did not modify the action in either tissue (Figure 19.b and e, Figure 20., Table 8.), except for MIF in the highest dose in non-pregnant uteri, which inhibited the relaxing action moderately. Similarly, Actinomycin D did not

alter the action (Figure 19.c and f, Figure 20.), and endothelium removal had no effect on these actions either (data not shown).

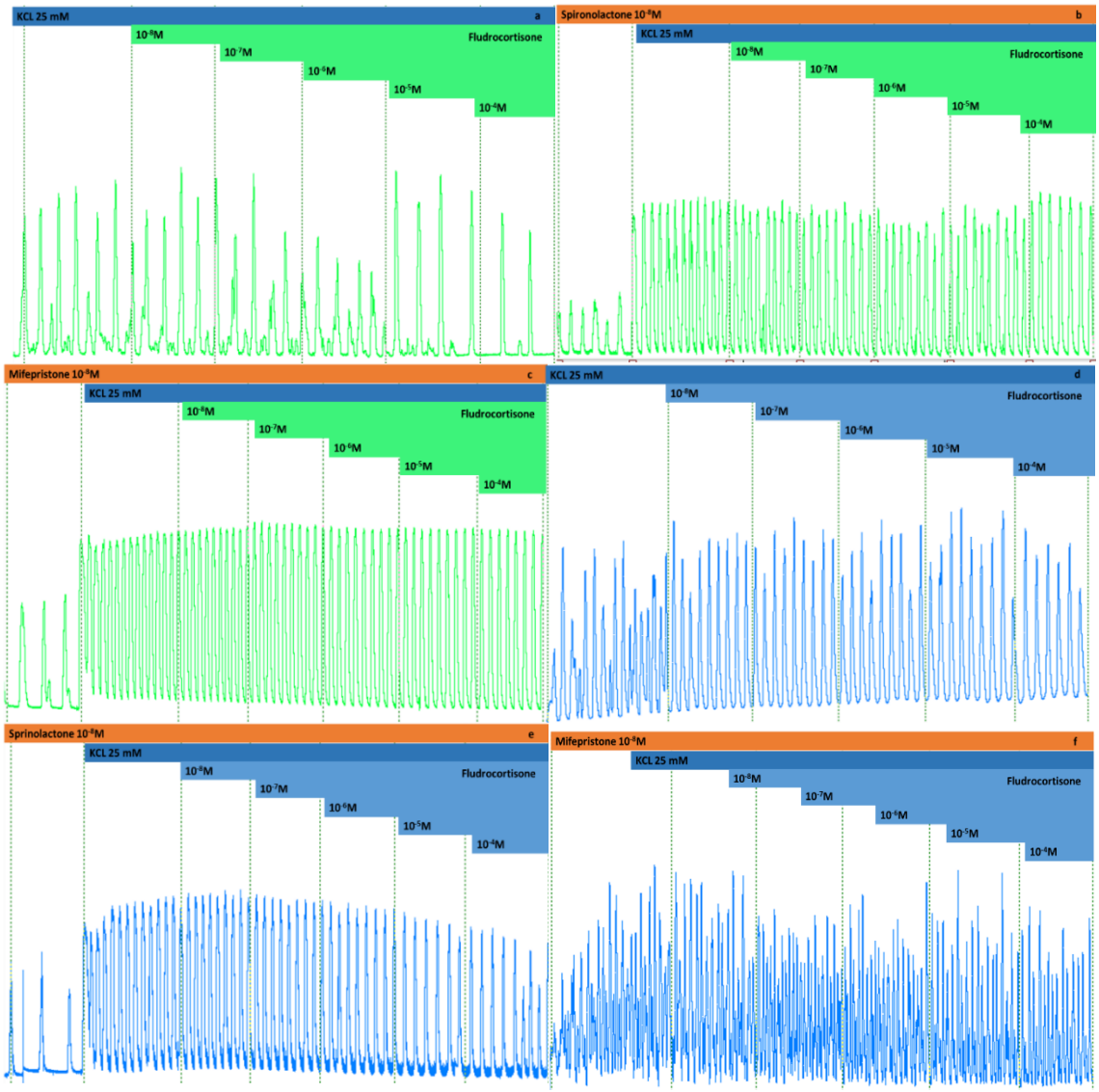

Figure 19. Effects of fludrocortisone on KCl-induced (25 mM) non-pregnant (a) and pregnant (d) uterine contractions at concentrations of 10^{-6} -10^{-4} M in a cumulative manner. The effects of fludrocortisone were also investigated in the presence of 10^{-6} M spironolactone or 10^{-6} M mifepristone both in non-pregnant (b, c) and pregnant uteri (e, f). Each figure is a representative record.

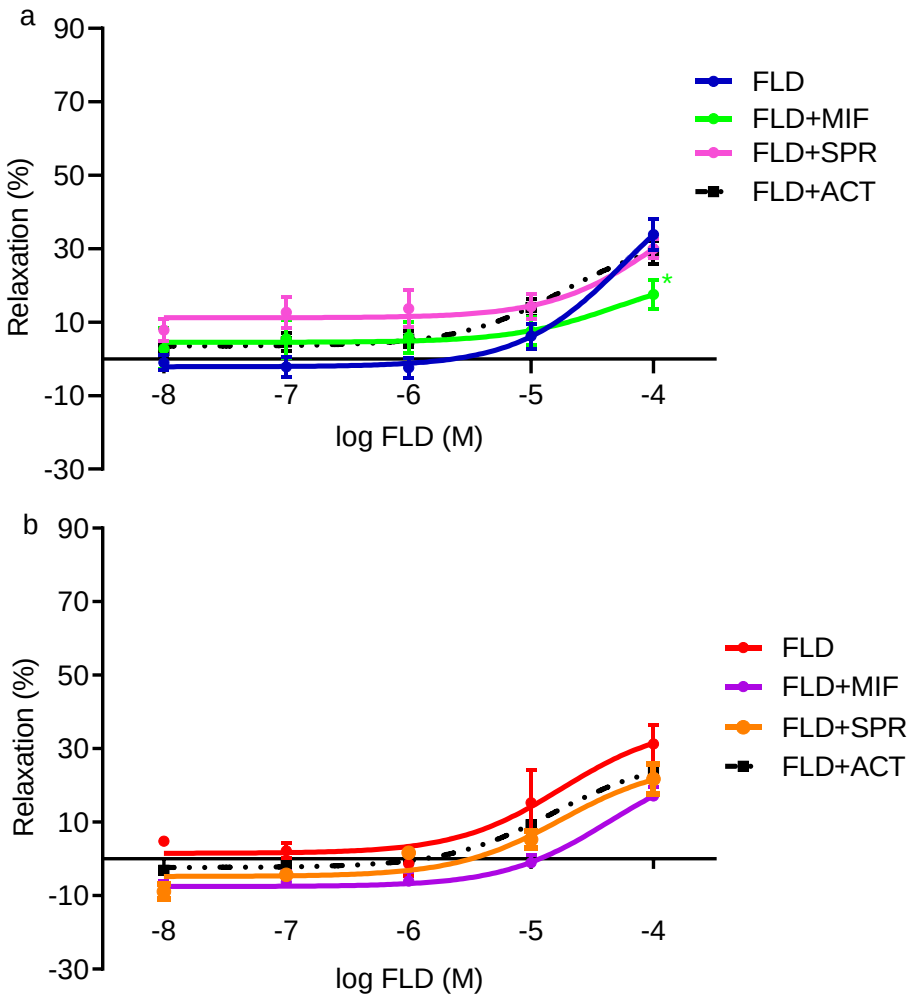

Figure 20. Effects of fludrocortisone on non-pregnant (a) and pregnant (b) uterine contractions stimulated in vitro with KCl (25 mM) and pre-treated with spironolactone and mifepristone (10^{-6} M), presented as a percentage of relaxation. *: $p<0.05$; ACT, actinomycin D; MIF, mifepristone; FLD, fludrocortisone.

a	**FLD**	**FLD+ SPR**	**FLD+ MIF**	**FLD+ ACT**
E_{max} (%±S.E.M)	55±1.8	51±2.3	24±2.8(*)	34±1.5
EC_{50} (M)	6.5e-005	1.1e-005	5.2-005	1.8e-005
b	FLD	FLD+ SPR	FLD+ MIF	FLD+ ACT
E_{max} (%±S.E.M)	37±3.1	37±3.1	28±1.6	27±1.8
EC_{50} (M)	1.7e-005	1.7e-005	4.3e-00-5	1.5e-005

Table 8. Changes in the E_{max} and EC_{50} values of the non-pregnant (a) and pregnant (b)uterine relaxing effect of FLD alone, with pre-treatment with actinomycin D and cycloheximide, spironolactone and mifepristone and after removing the endometrium. *: $p<0.05$; ActD, actinomycin D; Chx, cycloheximide; DEX, dexamethasone; MIF, mifepristone; SPR, spironolactone.

3.5.2 **[^{35}S]GTPγS binding assay studies:**

The [^{35}S]GTPγS binding was very faintly elevated only by higher doses of FLD in non-pregnant tissues. However, MIF did not change this small elevation (Figure 21.a and b, and Table 9.).

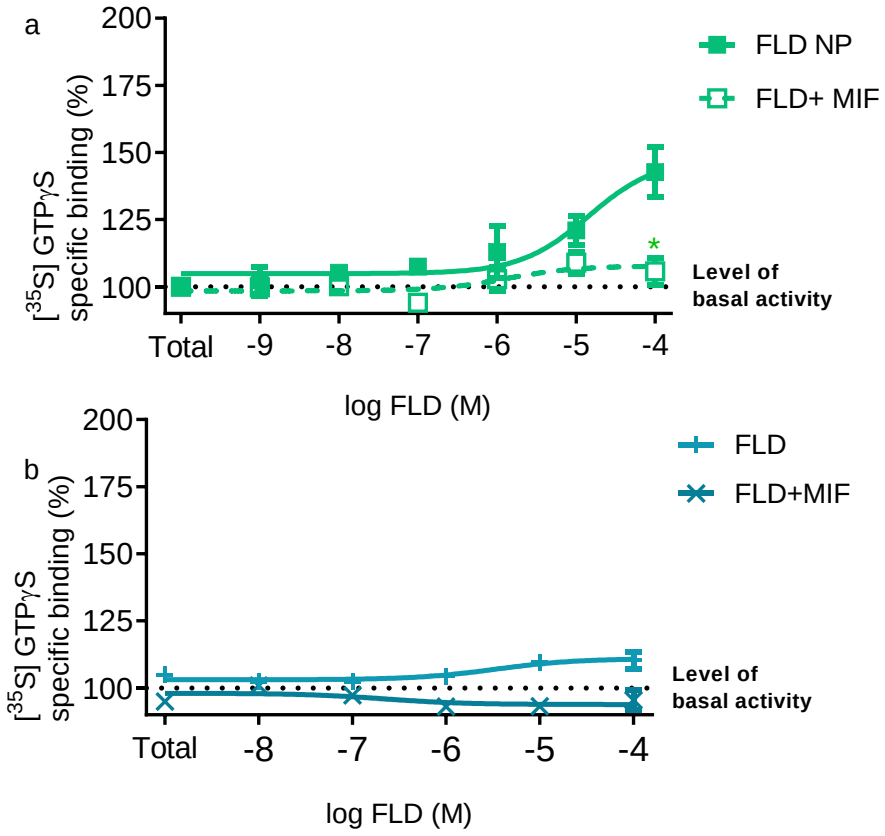

Figure 21. Effect of fludrocortisone (10^{-8}-10^{-4} M) on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone. Mifepristone reduced FLD-induced increase in [^{35}S]GTPγS binding in non-pregnant (a) and pregnant (b) uterine tissues. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without any substances. *: $p<0.05$; MIF, mifepristone; FLD, fludrocortisone, SPR; spironolactone.

a	**FLD**	**FLD+ MIF**
E_{max} (%±S.E.M)	148±9.1	107±3
EC_{50} (M)	1.4e-005	1.4e-006
b	**DEX**	**DEX+ MIF**
E_{max} (%±S.E.M)	111±2.3	93±1.6
EC_{50} (M)	3.4e-006	2.3e-007

Table 9. Changes in the [^{35}S]GTPγS binding effect of DEX alone and with pre-incubation with mifepristone in the non-pregnant (a) and 22-day-pregnant (b) rat. **: $p<0.01$; DEX, dexamethasone; MIF, mifepristone.

3.5.3 **cAMP study.**

FLD moderately increased cAMP level in non-pregnant tissues, while MIF pre-treatment inhibited it. In pregnant tissues, there was basically no significant change in cAMP level compared to the control (Figure 22.).

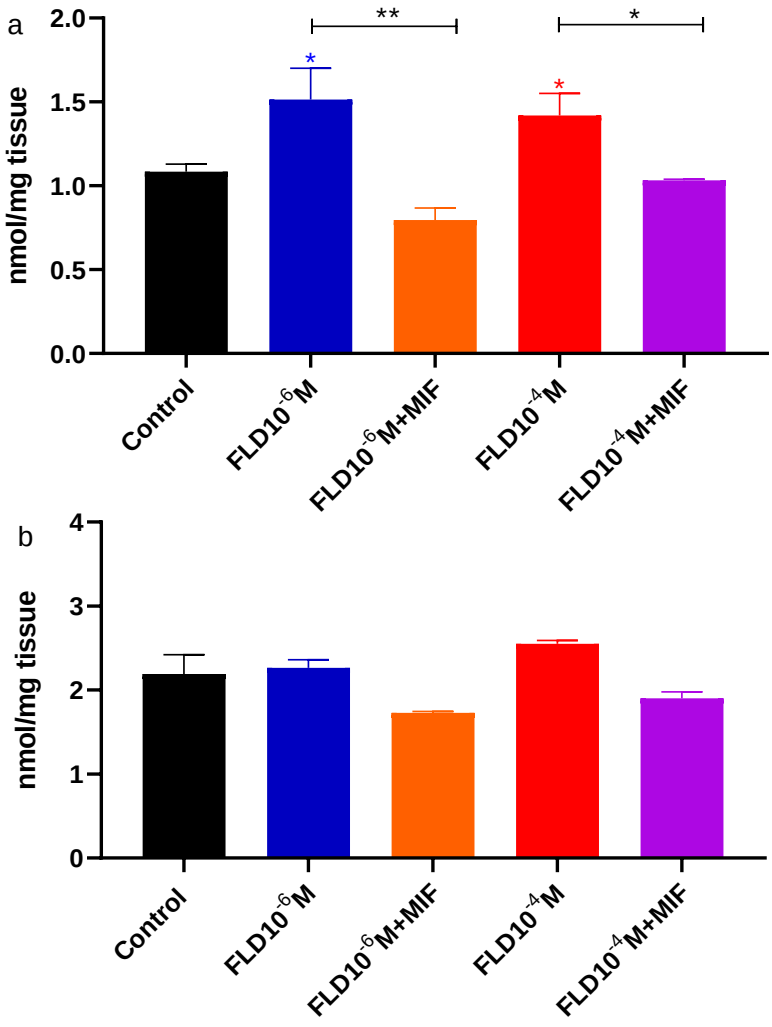

Figure 22. Changes in the cAMP level of uterine non-pregnant (a) and pregnant (b) tissues in the presence of fludrocortisone alone and after pre-treatment with mifepristone. The uterine cAMP level was expressed in nmol/mg tissue. *: $p<0.05$; **: $p<0.01$; MIF, mifepristone; FLD, fludrocortisone.

3.5.4 **In vivo study:**

FLD had no significant action on either animal group (Figure 23.).

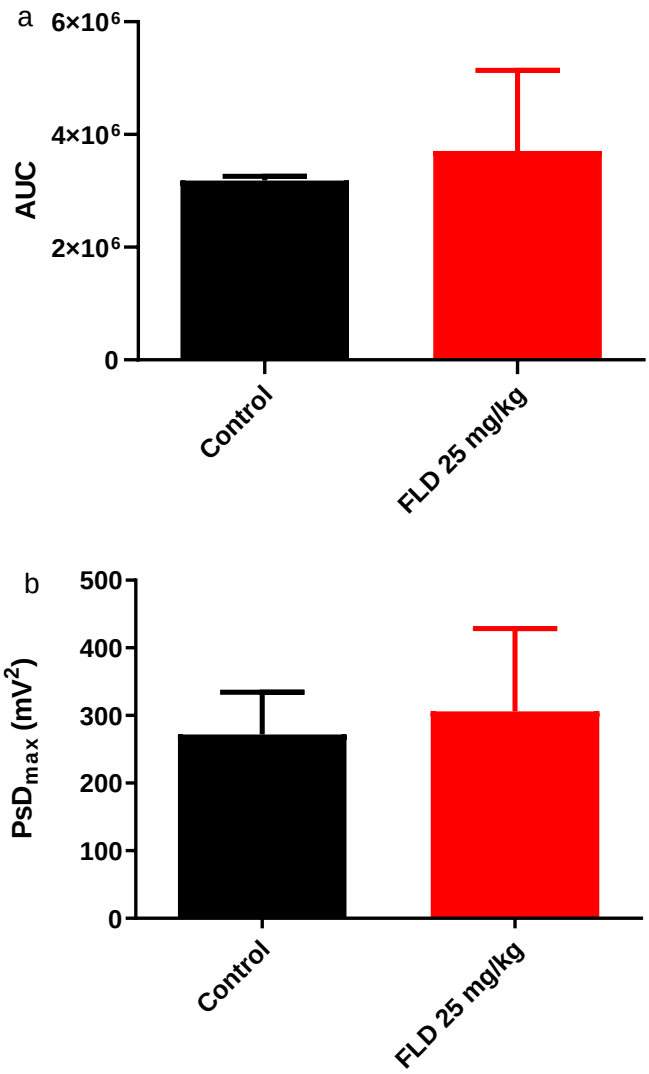

Figure 23. Effect of fludrocortisone (FLD) on non-pregnant (a) and pregnant (b) animals, in vivo. FLD did not modify either non-pregnant or pregnant contractions.

4. Discussion:

The non-genomic action of steroid hormones, especially on smooth muscles from different organs, has been investigated in several studies. It was proved that E2 and P4 had a vasorelaxant action through a non-genomic pathway. Studies on rat arterial beds showed that E2 induced vascular relaxation [39]. The same results were found on the arterial tissues in human [40,41] lamb [42], monkey [43] and mice [29]. On the contrary, the non-genomic action of E2 induced hyperreactivity and contraction on tracheal smooth muscles [44]. Additionally, E2 elicited non-genomic vasoconstriction in mice, which led to the reduction of skin cooling action [45]. The non-genomic smooth muscle relaxing effect of T was proved in human coronary arteries [46], umbilical arteries [47,48], peripheral vasculature of rats [49] and even in the trachea of guinea pigs [50,51]. However, a comparative investigation of the non-genomic effect of steroid hormones on the uterine contractions has not been carried out yet clearly.

Therefore, we aimed to investigate the effects of the 3 basic sex hormones (E2, P4 and T) and also 2 group of corticoid hormones (DEX and FLD) on non-pregnant and late stage pregnant uterine contractions in rats in vitro and in vivo.

The in vitro non-genomic action of T, E2, DEX and FLD in uterine tissues inhibit induced contraction. Both E2 and T had a remarkable relaxing effect (approximately 70% and 50% inhibition in pregnant and non-pregnant respectively). Although such an action of T was described earlier on human and pregnant rat uteri [52,53], such a result about E2 has not been published yet. In contrast, E2, T and P4 were reported as ineffective on both pregnant rat and human myometrial contractions induced by oxytocin in vitro [54], but in that study the sex steroids were used in lower concentrations (below 10^{-6} M), while we applied them in 10^{-4} or 10^{-3} M as the highest concentrations. Thus, the high concentrations can explain why we could detect quite a strong relaxing effect with T and E2. The other surprise was the ineffectiveness of P4 on pregnant contractions considering its clinical use against premature contractions in threatening preterm birth [55], although in that case it is applied as a preventive agent. Our result suggests that there is no prompt relaxing action of P4 on pregnant uterine contractions. Similarly, earlier studies did not find any non-genomic relaxing effect for P4 either [56], although a synthetic P4 derivative, dydrogesterone was found to inhibit the pregnant myometrial contractions by the inhibition of voltage dependent Ca-channels [54]. Some other experiments found that P4 had an ability to relax human non-pregnant or pregnant uterine tissues in high dose [57–59] which findings are

virtually in conflict of our results. However, the findings may be a result of genomic feature of P4 since the P4 incubation period in both reported studies were more than 1 hour.

CSs and particularly GCs inhibit induced contraction. The in vitro study of DEX showed a concentration-dependent relaxing effect, while FLD did not show such a relationship and had a slight moderate relaxing action only in a very high dose. In vivo experiments with DEX revealed a relaxing action which was more significant in pregnant animals. The results are similar to previous research about prompt GC action in the rat myocyte cell [60] as well as in the airway smooth muscles of guinea pigs [61] and mice [62]. In contrast, FLD had no action at all in vivo, which may be explained by the weak water solubility of the compound and the subsequent limitation to administer high intravenous doses to non-pregnant rats. In the case of pregnant rats, we did not anticipate any in vivo effect after the lack of in vitro effectivity, which suggests that FLU cannot act through the non-genomic pathway in late pregnant uteri. Because of same solubility difficulty of sex steroid hormones, we could not administer them in vivo.

Since the exposure time of the uterine tissues to the steroid hormones lasted a maximum of 30 minutes, which is considered to be too short to initiate the genomic response [63], the genomic action was ruled out. We also proved this, since the blockade of the genomic pathway by the RNA transcription inhibitor actinomycin D and the protein synthesis inhibitor cycloheximide did not modify the effects of E2 or T similarly to earlier studies [18,64]. Subsequently, the removal of the endometrium did not modify the steroid hormone effects either, so we also proved that the relaxation effects of E2, T, DEX and FLD are linked to myometrial steroid receptors. This finding is in contrast with earlier studies of airway smooth muscles, where the prompt relaxing action of CSs is endothelium-dependent [65]. This suggests the different functions of the endometrium and the endothelium in the uterus and lungs, respectively.

The prompt effect can be mediated by a non-specific interaction between the ligand and the cell membrane or by a specific interaction with the cytosolic receptor. The specific receptor antagonists of sex hormones (FLU for T and FUL for E2) did not reduce their actions, which is further evidence that the genomic pathway as well as ER and androgenic receptor is not involved in the relaxing effects of T and E2. Since SPR (the specific MC receptor antagonist) did not change the action of FLD, the role of MR at both membrane and cytosolic sites was ruled out, similarly to previous results [66]. Furthermore, MIF in a low concentration (10^{-8} M, acting as a GC receptor antagonist) [32] did not inhibit the prompt action of DEX, suggesting that GR has no non-genomic action in the uterus. Others have

also shown that the non-genomic action of GCs is insensitive to GR because receptor blockade did not modify the action [66]. However, besides its genomic action, the GR receptor may act through a non-genomic action as well in the airway smooth muscle [61]. Surprisingly, the actions of T and E2 were MIF sensitive, their maximum effects were reduced significantly by the compound.

Surprisingly, higher dose of MIF (10^{-6} M, progesterone receptor antagonist) significantly inhibited the prompt relaxing action of T, E2 and DEX both in pregnant and non-pregnant tissues, and that of FLD in non-pregnant uteri. The same concentration has also been reported to inhibit the relaxing effect of budesonide in the skeletal muscle of mice [31]. This suggests that MIF generally inhibits the non-genomic target of steroids for uterus relaxation, which is possibly independent of its progesterone and glucocorticoid receptor (GR) inhibitory action. We also found that MIF can inhibit the prompt action of steroids by reducing cAMP levels. Hence, we hypothesized that the acute effect of GC may be either GR-dependent in certain tissues such as airway smooth muscles [16] or GR-independent in the uterine tissue, blocking other possible pathways.

These effects might be mediated by GPCRs, especially the G-protein estrogen receptor (GPER), which is coupled to Gs protein and enhances the intracellular cAMP level, has already been identified as a target of sex steroids in several tissues [67,68] as well as in human myometrium [69,70]. A study reported that the relaxing action of ALD in vascular endothelial cells is mediated through GPER [71]. In our [^{35}S]GTPγS binding and cAMP measurements we proved a significant increase in G-protein and cAMP levels after stimulation by steroids in both pregnant and non-pregnant tissues, and their effects could be inhibited by MIF. The elevation of GTP and later cAMP requires $G_{\square}$ stimulation, therefore the relaxing action may suggest this [64], and probably MIF is a blocker of this non-genomic pathway. The previously reported signaling pathway for putative sex steroid membrane receptors involves phospholipase, kinase [72], calcium [73] and other second messengers such as IP3 or cAMP [74]. It is also possible that the activation of a G protein receptor by rising cAMP regulates the voltage-gated ion channels (e.g. BK_{Ca} and K_V) [48] and the intracellular calcium regulation [28,44]. Consequently, GPER can also inhibit uterine smooth muscle contraction as a non-genomic action. Therefore, our results suggest that T, E2 and DEX possibly activate GPR30 and MIF might be a competitive antagonist on this receptor. Besides this, the non-genomic pathway could be mediated through the G_i or $G_{q/11}$ pathway, which was reported for corticosterone in PC12 cells [75] or hippocampal neurons,

respectively [16]. Since the GTPγS study reveals activated GTP only, Gi or $G_{q/11}$ can also be involved in the uterine pathway.

5. Conclusion:

T, E2, DEX can significantly inhibit KCl-stimulated contractions in the non-pregnant and also late pregnant uterus in high concentrations and in a non-genomic manner while FLD is effective just in non-pregnant uteri. Their actions are mediated by a G-protein coupled receptor (possibly GPR30) that can be blocked by MIF. However, P4 seems to be inefficient as a non-genomic relaxant of uterin tissue. Based on our results, a single and high dose of efficacous steroids might prevent premature delivery and extend the gestational period, while MCs and P4 are practically not useful for this purpose. Considering the fact that GCs are widely used in threatening premature birth to enhance surfactant secretion preventing respiratory distress syndrome [76], they may have a further benefit in delaying the time of delivery and reducing the risk of prematurity compare with sex steroids. Despite of the possible side effects of GCs related to this indication [77] the possible steroid tocolytic therapy would be safe if we excluded the genomic effect and only trigger the non-genomic (rapid) pathways. To solve this problem, further basic experiments are needed to search for suitable steroid analogues or specific antagonists.

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8. Appendix