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FACULTY OF PHARMACY

DOCTORAL SCHOOL OF PHARMACEUTICAL SCIENCE

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

SUMMARY OF PH.D. THESIS

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List of Contents

1. Introduction:

Steroids are specific configuration of 4 ringed organic compounds which have important roles and mediate different functions in live system. They have 2 basic physiological roles: as structural in cell membrane and as signaling molecules such as endocrine hormones.

The 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. Progesterone is well-known as a pro-gestational hormone reducing uterine contractility and maintaining pregnancy. On the other hand, estrogens increase the contraction of the pregnant uterus and contribute to the parturition process. However, the effect of testosterone (T) on pregnancy has not exactly been clarified yet.

The corticosteroid hormones are another group of steroid hormones secreted from the adrenal cortex and exert various ranges of actions through regulating functional responses. 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, alter gene transcription and protein synthesis. 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”. For example, beneficial fast effect of GC have been reported on airway smooth muscles in asthmatic condition . In addition, uterine contraction of non-pregnant rat can be inhibited by cortisol and dexamethasone (DEX).

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, but in breast cancer cell lines, their actions can be the opposite.

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.

Materials and methods:

1.1 Animals:

Sprague-Dawley rats (SPRD) (180-200 g, Animalab Hungary Ltd, Vác, Hungary) were kept under controlled temperature, humidity, and light. Diet, 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.

1.2 Isolated organ bath contractility studies:

The experimental protocol is shown in Figure 1. The dissected uterine tissues were tied with silk thread and mounted vertically in an isolated organ bath filled with 10 ml de Jongh buffer with constant temperature and with carbogen 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.

KCl (25 mM) was added to each chamber for 7 minutes. Each steroid was added in a cumulative manner every 5 minutes. Concentration-response curves were plotted against the KCl-stimulated contraction response.

In another set of experiments, pregnant uterine tissues were pretreated with cycloheximide, and actinomycin D, genomic pathway blockers separately for 30 minutes. Tissues were pretreated with the following steroid hormone receptor antagonists for 10 minutes before KCl stimulation. Finally, the endometrium of the uterine tissues was removed by scraping and the experiments were repeated.

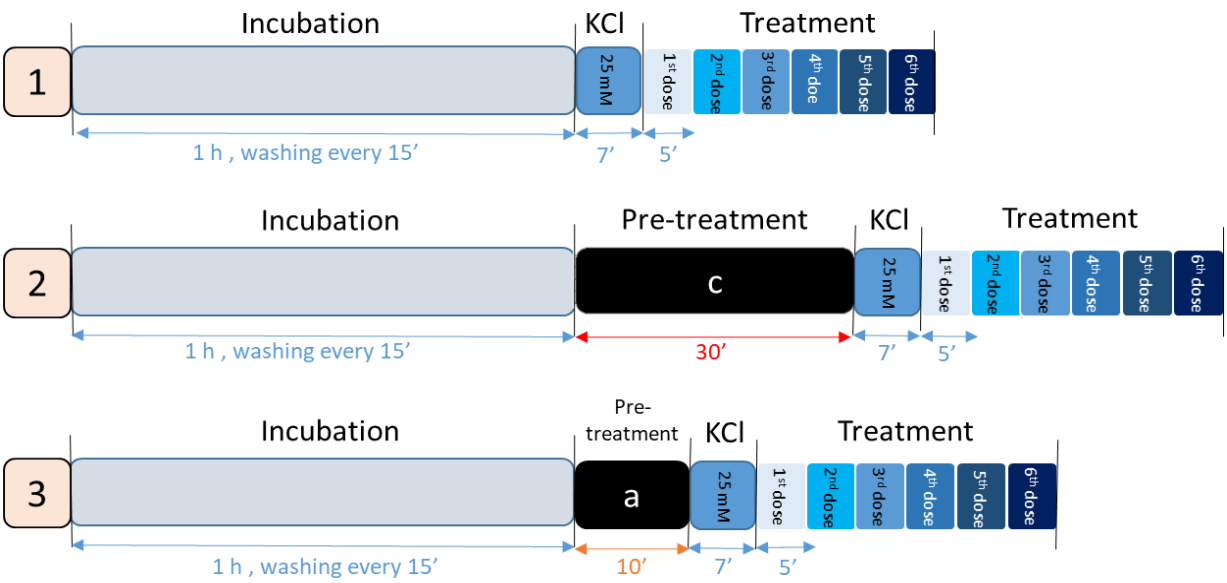

Figure 1. The isolated organ baths experimental protocol, the 1-hour incubation period, 7-min KCl stimulation, and cumulative dose treatment in 5-min interval time were the same for all experiments; (1) intact or endometrium removed pregnant and non-pregnant uterus samples treated with T, E2, P4, and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

1.3 In-vivo studies:

Non-pregnant, and 22-day pregnant 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 uterine horn, while for the pregnant study a bipolar disk electrode pair was fixed subcutaneously 1 cm right from the midline above the uterus. 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).

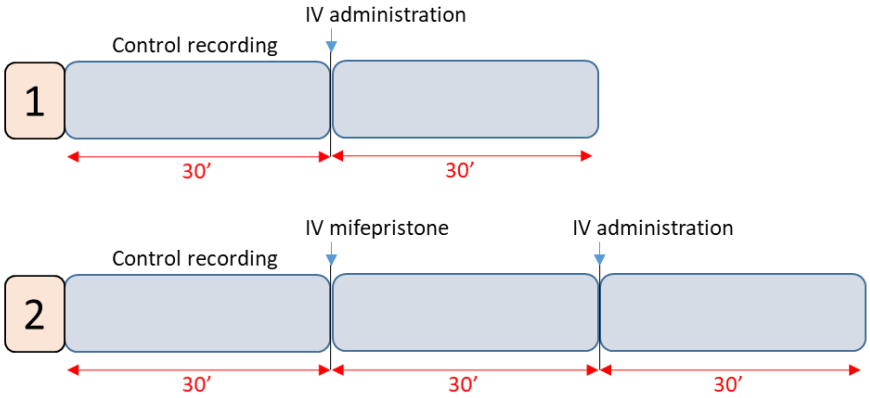

Figure 2. Protocol for the in vivo contractility study. The animals were first anesthetized, and strain gauges or electrodes were inserted on the abdomen of non-pregnant or pregnant animals, respectively. The 30-minute recording of spontaneous contractions (control period) was the same for all experiments;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

1.4 [^{35}S]GTPγS studies:

To investigate the efficacy of steroids on G-protein-coupled receptors (GPCRs), especially $G_{\alpha i/o}$, the changes of GDP to GTP were measured with radiolabeled, non-hydrolysable GTP.

1.5 Cyclic AMP studies:

The changes in cyclic AMP (cAMP) levels of the sample tissue of steroids were investigated using the commercial cAMP Enzyme Immunoassay Kit (Cayman Chemical, USA).

1.6 Statistical analysis:

The response curves for the in vitro experiments were plotted by analysis of the area under the curve (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.

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.

2. Results:

2.1 Studies with testosterone:

2.1.1 Isolated organ bath study:

T elicited a relaxing effect and reached 50% relaxation of the non-pregnant and 70% in pregnant uteri at high concentrations. The presence of cycloheximide and actinomycin D, removal of the endometrium or FLU did not modify the relaxing effect of T in both pregnant and non-pregnant uterine tissues. MIF shifted the T response curve to the right and reduced its maximal inhibitory effect (Figure 3.).

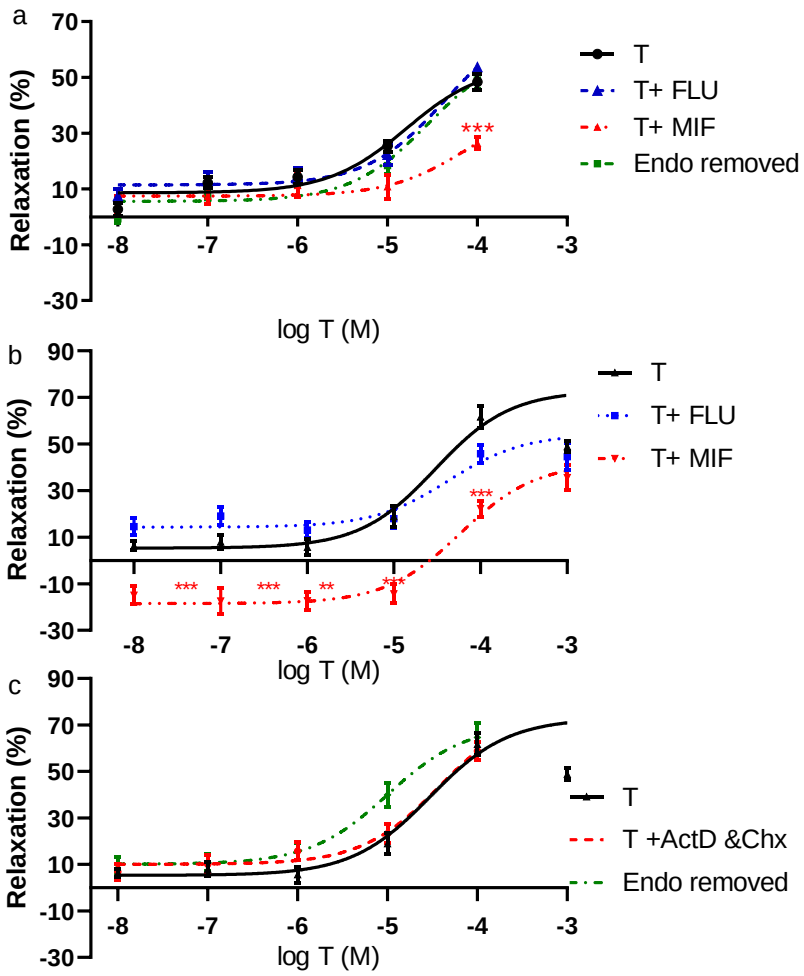

Figure 3. Effects of T on uterine contractions stimulated with KCl (25 mM), intact or with endometrium removal (a, c), and with pre-treatment with flutamide or mifepristone (a, b) for pregnant and non-pregnant respectively. Also with pre-treated of pregnant tissues with actinomycin D and cycloheximide(c). 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.

2.1.2 **[^{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. (Figure 4.).

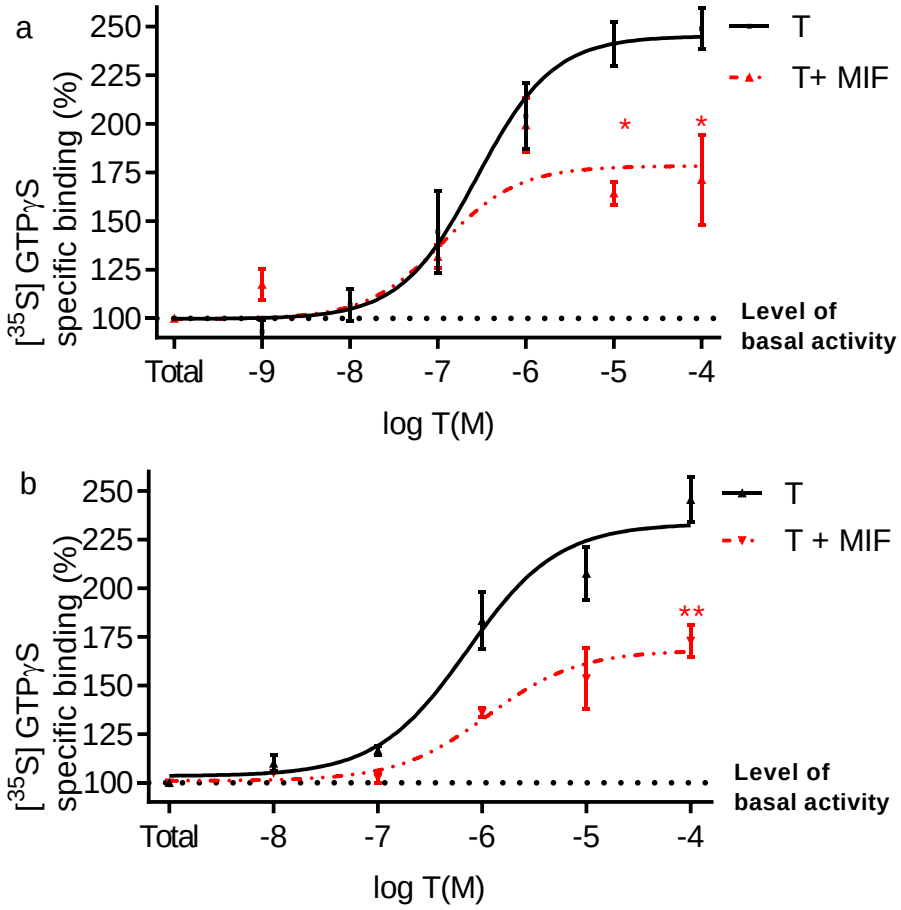

Figure 4. Effect of T (10^{-8}-10^{-4} M) on [^{35}S]GTPγS binding of non-pregnant(a) and pregnant (b) tissues with or without pre-treatment with mifepristone. Mifepristone reduced the T-induced increase in [^{35}S]GTPγS binding. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without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

2.1.3 **cAMP study:**

The pregnant and non-pregnant cAMP level was raised by T compared to the control at both high and low concentrations. But at lower dose 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 5.).

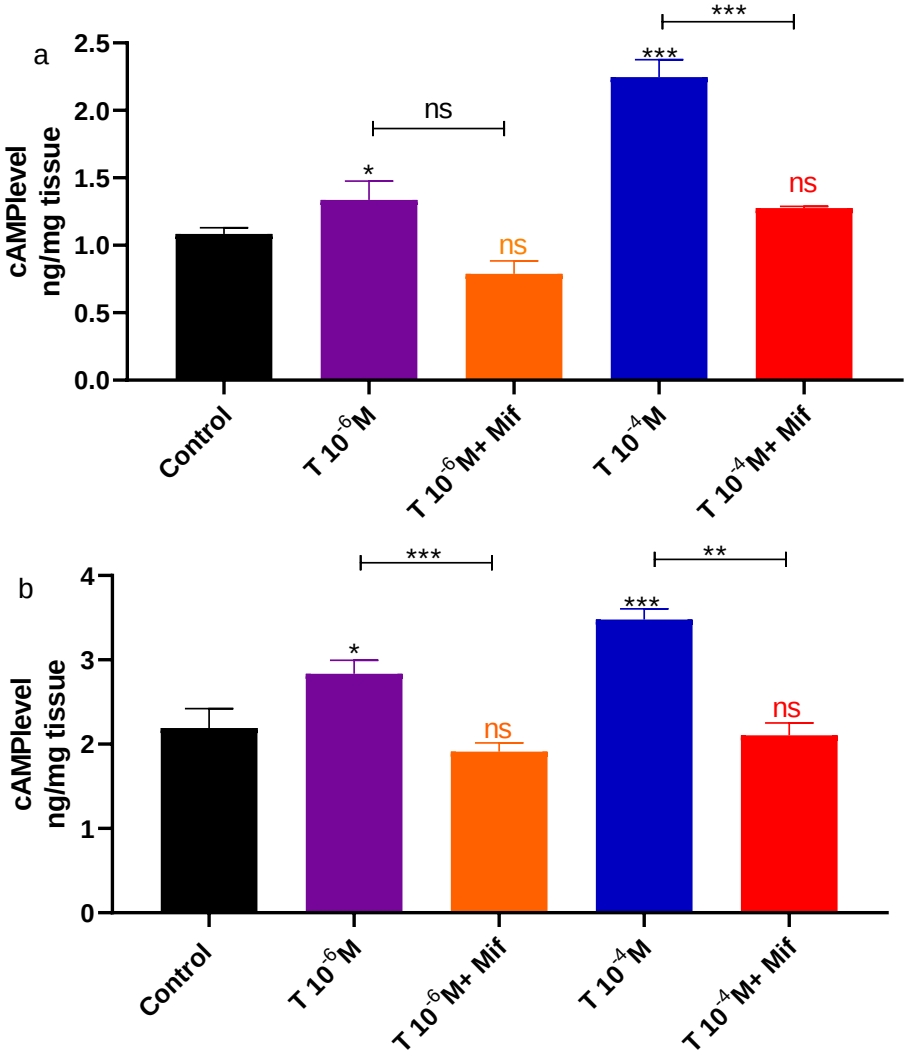

Figure 5. Change in the level of uterine cAMP in the presence of T alone and after pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues. The uterine cAMP level was expressed in ng/mg tissue. ns: non-significance; *: p <0.05; **: p<0.01; ***: p<0.001; MIF, mifepristone; T, testosterone.

2.2 Studies with 17-β estradiol:

2.2.1 **Isolated organ bath study:**

E2 relaxed non-pregnant and pregnant uterine contractions specially at the highest concentration. Actinomycin D and cycloheximide, the removal of the endometrium or pre-treatment with fulvestrant, did not influence the effect of E2. Pre-treatment of both tissues with MIF reduced the relaxing effect of E2 (Figure 6.).

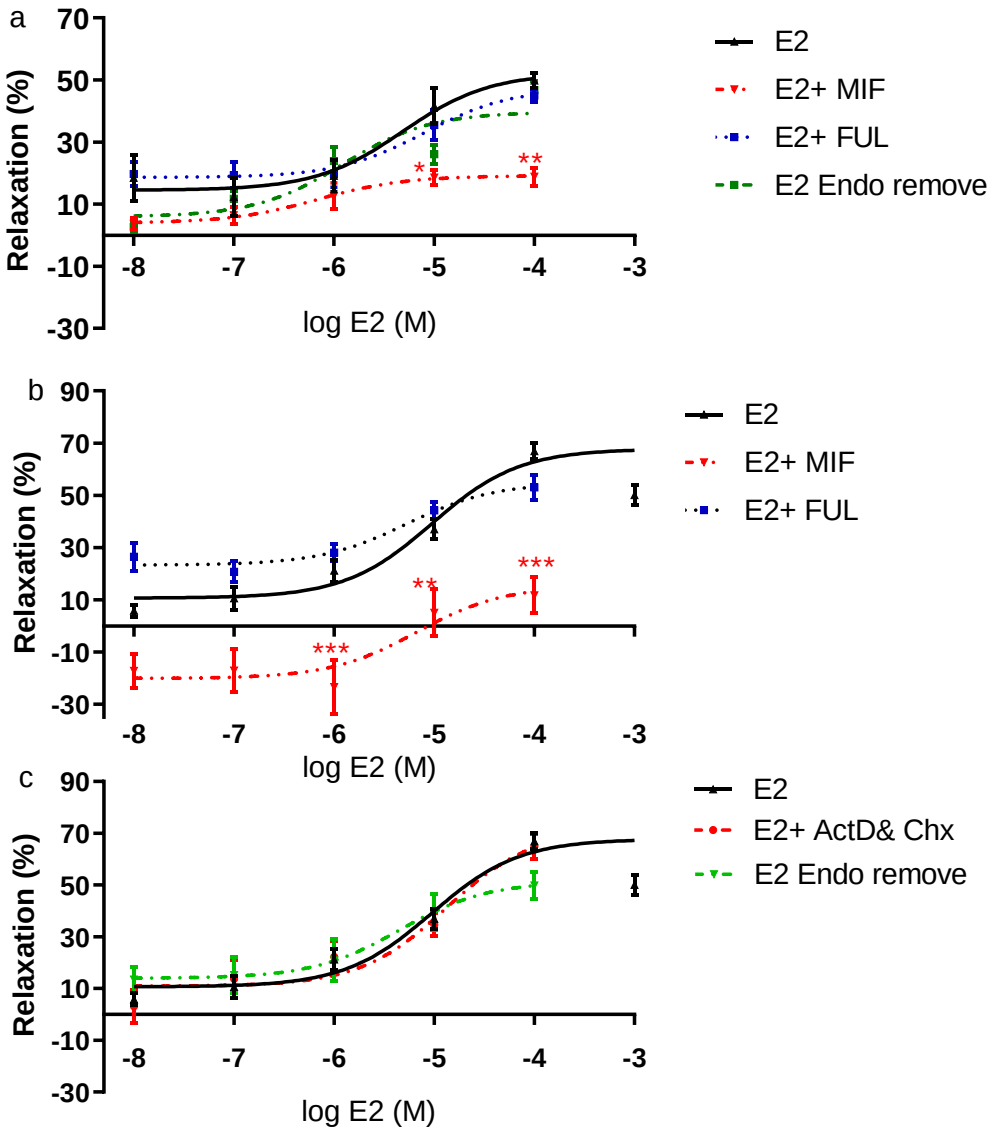

Figure 6. Effect of E2 on pregnant uterus tissue stimulated with KCl (25 mM) in the presence of actinomycin D and cycloheximide (a), and with pre-treatment with fulvestrant or mifepristone, and after endometrium removal (b) presented by percent of relaxation. *:p <0.05; **: p<0.01; ***: p<0.001; ActD, actinomycin D; Chx, cycloheximide; E2, 17-β estradiol; MIF, mifepristone; FUL, fulvestrant.

2.2.2 **[^{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 mifepristone specially in high dose and pregnant tissue (Figure 7.).

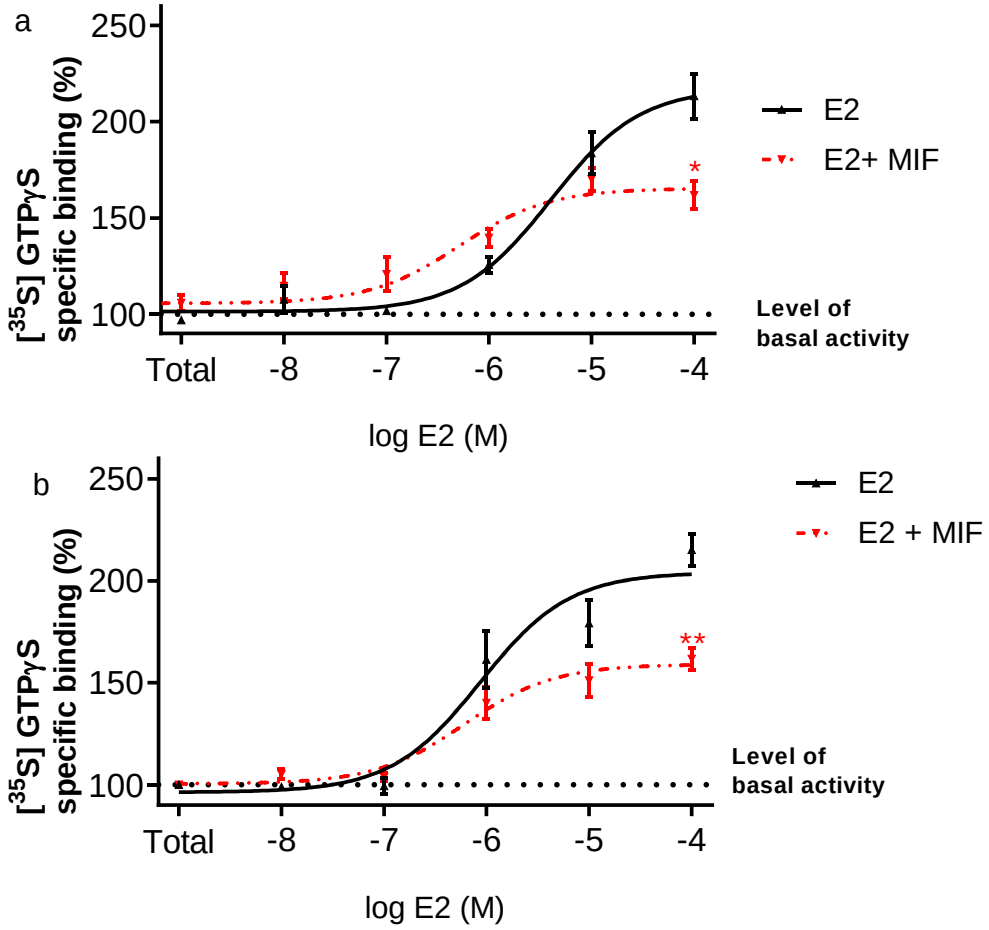

Figure 7. The effect of E2 on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without substance. *:p <0.05; **: p<0.01; E2, 17-β estradiol; MIF, mifepristone.

2.2.3 **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 (Figure 8.).

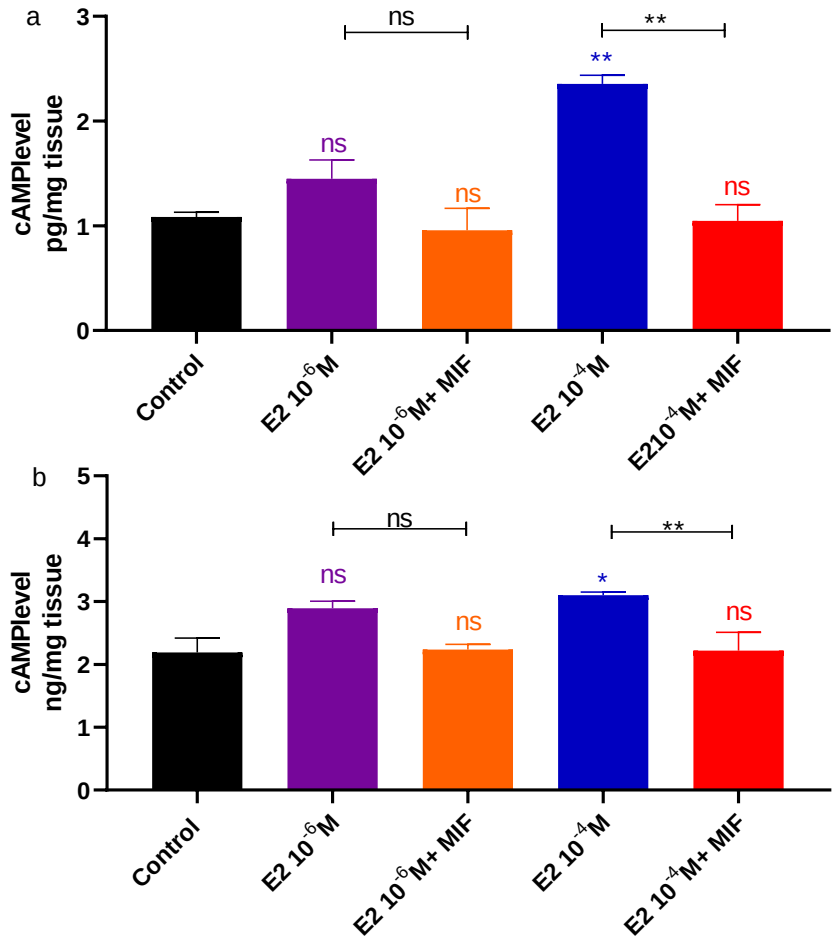

Figure 8. Changes in the level of cAMP in the presence of E2 alone (10^{-6} and 10^{-4} M) and with pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues expressed in ng/mg tissue. *: p <0.05 **: p<0.01; E2, 17-β estradiol; MIF, mifepristone.

2.3 Studies with progesterone:

P4 had a negligible effect on KCl-stimulated non-pregnant and pregnant uterine contractions (Figure 9.). The presence of mifepristone did not modify its action (Figure 9.). Since the relaxing effect of P4 was missing, we did not investigate it further.

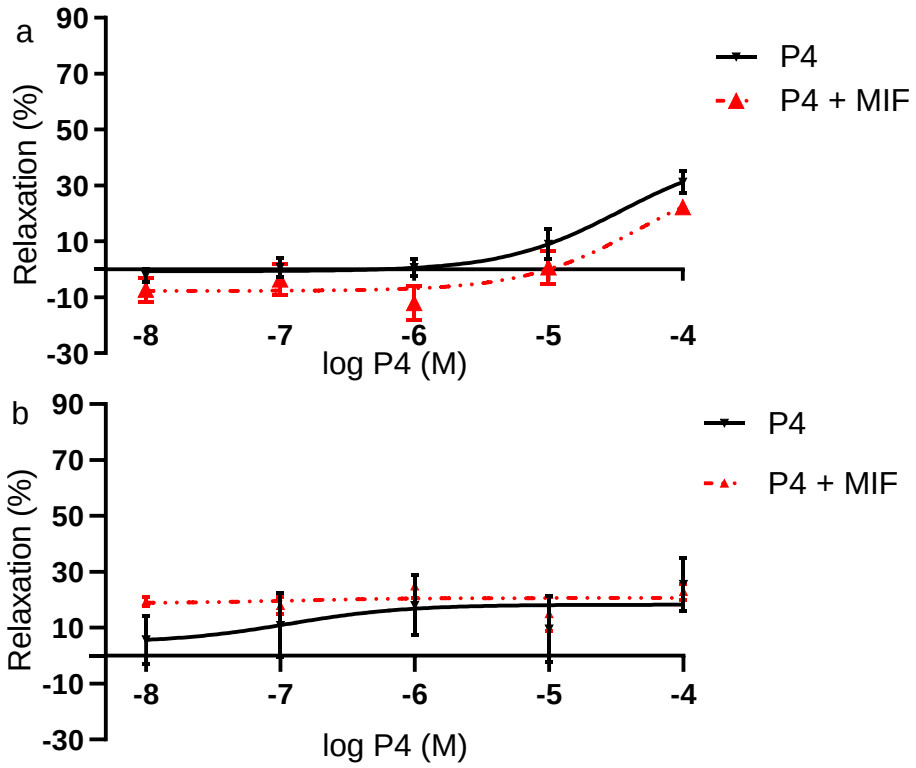

Figure 9. Effect of P4 on non-pregnant (a) and pregnant (b) uterus smooth muscle contractions stimulated by KCl, alone and in the presence of mifepristone. MIF, mifepristone; P4, progesterone.

2.4 Studies with dexamethasone:

2.4.1 **Isolated organ bath studies:**

DEX elicited a moderate relaxing effect at high concentration in non-pregnant uteri, in pregnant uteri relaxing effect was slightly less. The relaxing effect of DEX was inhibited by the higher dose of MIF in the pregnant uterus. Actinomycin D pre-treatment and endometrium removal did not modify (Figure 10.).

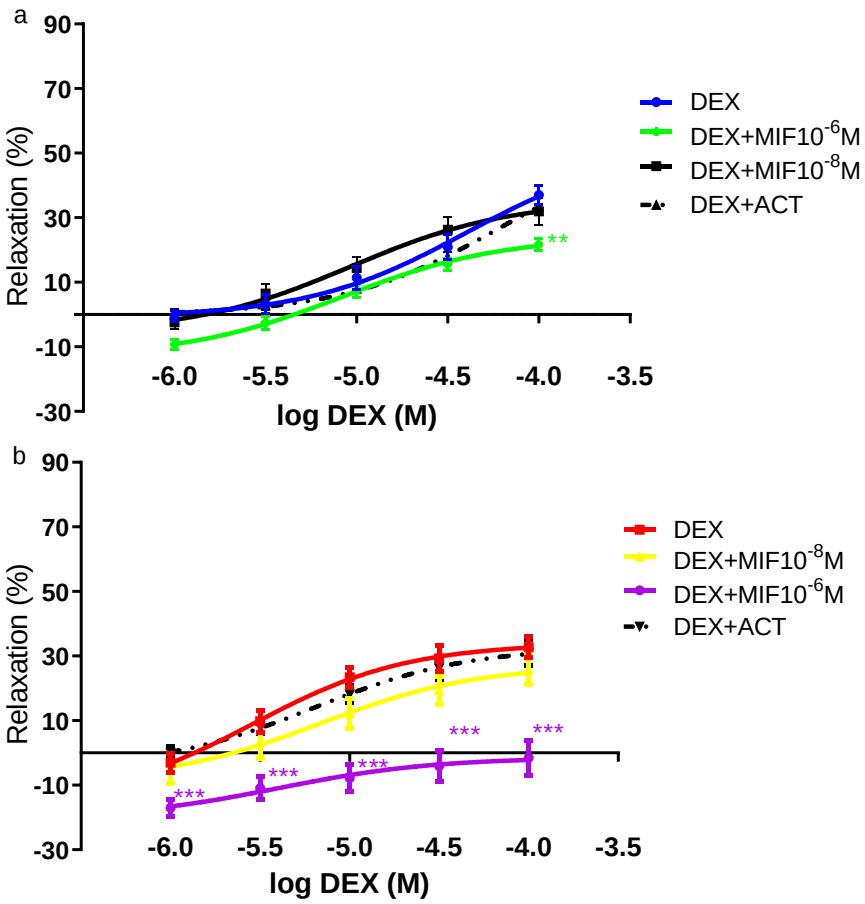

Figure 10. Effects of dexamethasone on non-pregnant (a) and pregnant (b) uterine contractions in vitro stimulated with KCl (25 mM) and pre-treated with mifepristone in 2 doses or Actinomycin D, presented as a percentage of relaxation. **: p<0.01; ***: p<0.001; ACT, Actinomycin D; DEX, dexamethasone; MIF, mifepristone.

2.4.2 **[^{35}S]GTPγS binding assay studies.**

The elevation of [^{35}S]GTPγS binding in a concentration-dependent manner was observed both in pregnant (Figure 11.b) and non-pregnant tissues (Figure 11.a). MIF pre-treatment shifted to the right by inhibiting this elevation, which means less activation of G-proteins.

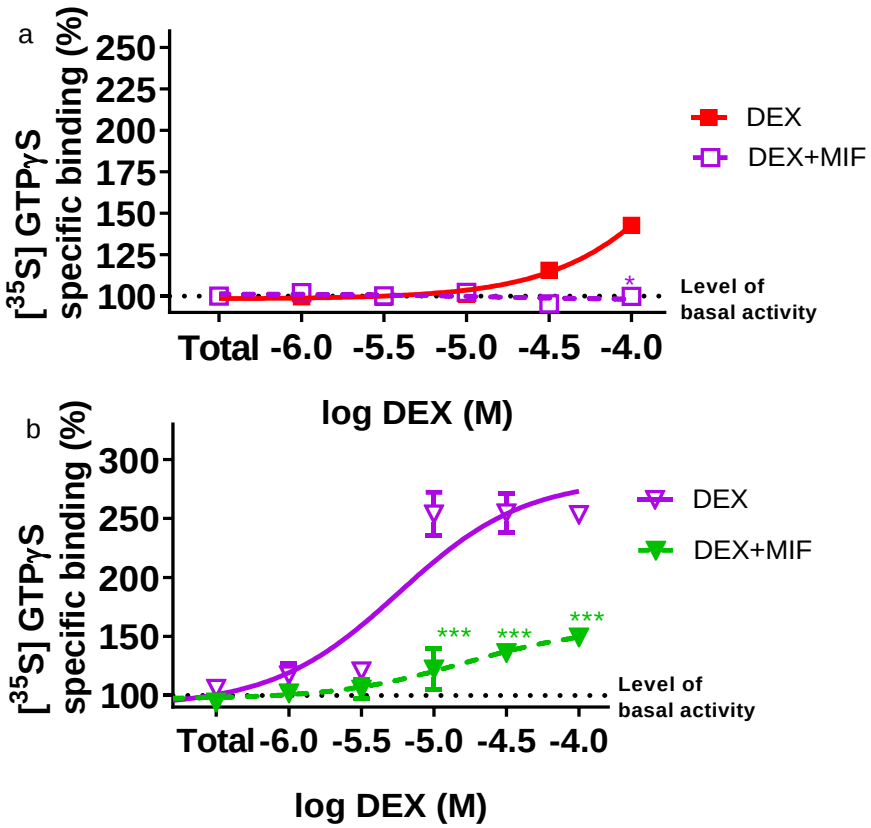

Figure 11. Effect of dexamethasone (10^{-6}-10^{-4} M) on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone. Mifepristone reduced dexamethasone-induced increase in [^{35}S]GTPγS binding in non-pregnant (a) and pregnant (b) uterine tissues. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without any substances. *: $p<0.05$; MIF, mifepristone; DEX, dexamethasone.

2.4.3

2.4.4 **cAMP study.**

Uterine cAMP levels both in pregnant and non-pregnant uteri rose in the presence of a high dose of DEX, moreover, this rise was inhibited significantly by pre-treatment with MIF. The moderate dose of DEX raised cAMP just in non-pregnant tissues (Figure 12.a and b)

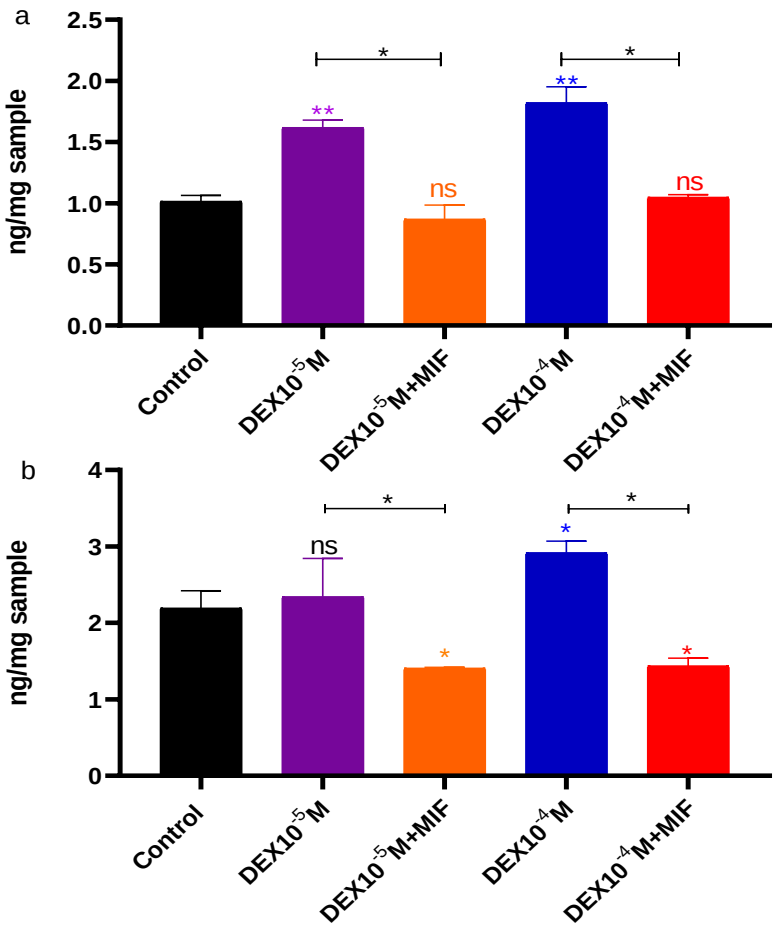

Figure 12. Changes in the cAMP level of uterine non-pregnant (a) and pregnant (b) tissues in the presence of dexamethasone alone and after pre-treatment with mifepristone. The uterine cAMP level was expressed in ng/mg tissue. *: p <0.05 **: p<0.01; MIF, mifepristone; DEX, dexamethasone.

2.4.5 **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 in both groups of animals, furthermore, MIF alone did not affect the uterus (Figure 13.).

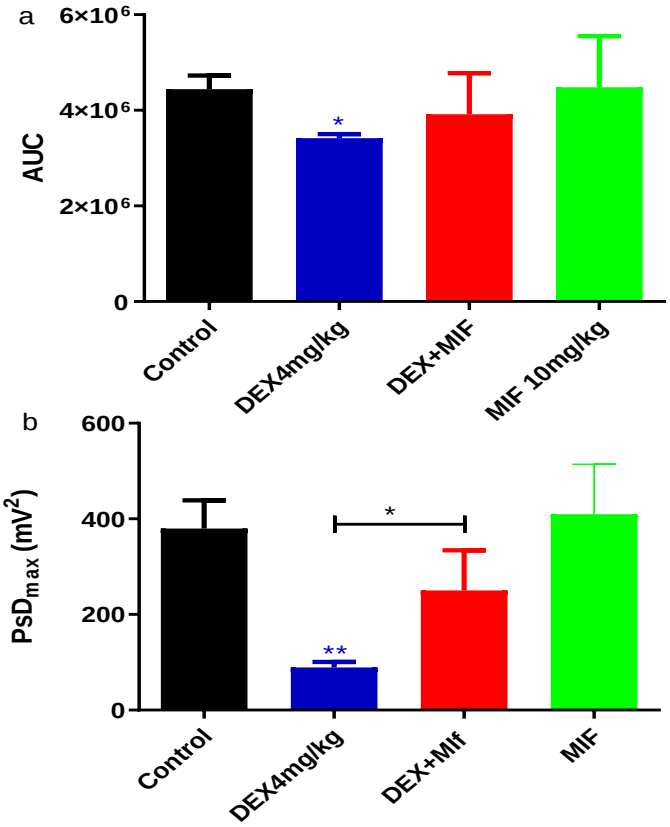

Figure 13. Effect of dexamethasone and mifepristone on non-pregnant (a) and pregnant (b) animals, alone and with pre-treatment with MIF, in vivo, *: $p<0.05$; **: $p<0.01$; MIF, mifepristone; DEX, dexamethasone.

2.5 Studies with fludrocortisone:

2.5.1 **Isolated organ bath studies.**

FLD showed a very slight relaxing action only at the highest dose both in pregnant and non-pregnant. Spironolactone and MIF did not modify the action in either tissue, except for MIF in the highest dose in non-pregnant uteri. Actinomycin D and endothelium removal did not alter the action (Figure 14).

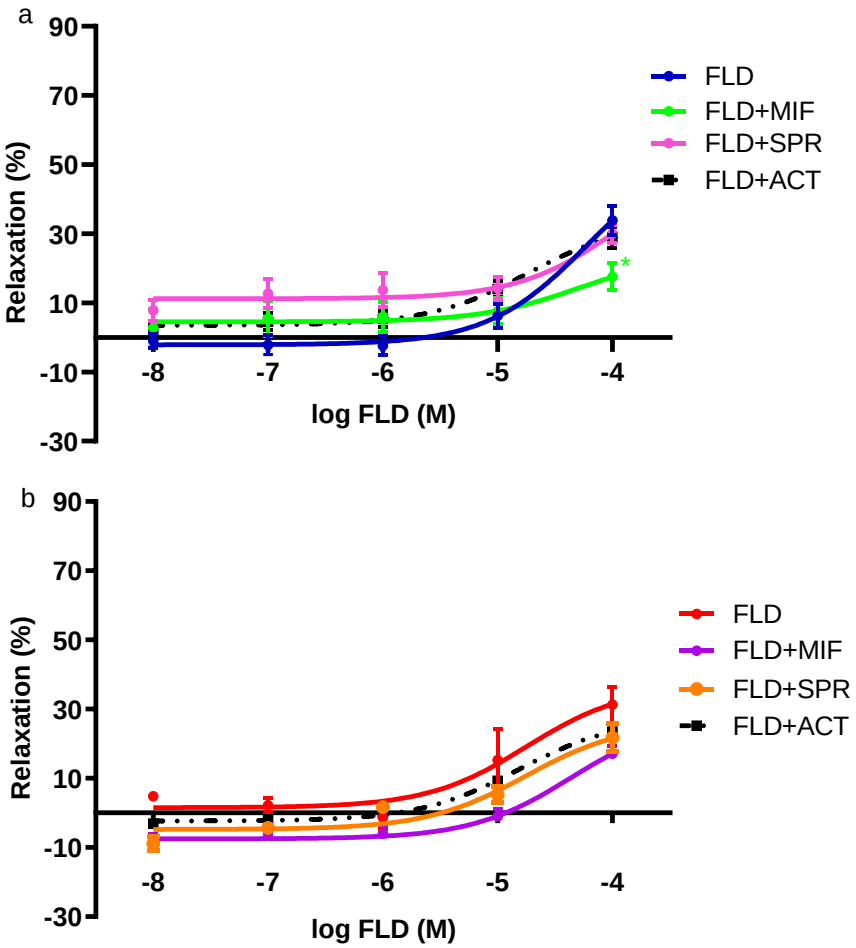

Figure 14. Effects of fludrocortisone on non-pregnant (a) and pregnant (b) uterine contractions stimulated in vitro with KCl (25 mM) and pre-treated with spironolactone and mifepristone (10^{-6} M), presented as a percentage of relaxation. *: $p<0.05$; ACT, actinomycin D; MIF, mifepristone; FLD, fludrocortisone.

2.5.2 **[^{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 15.a and b).

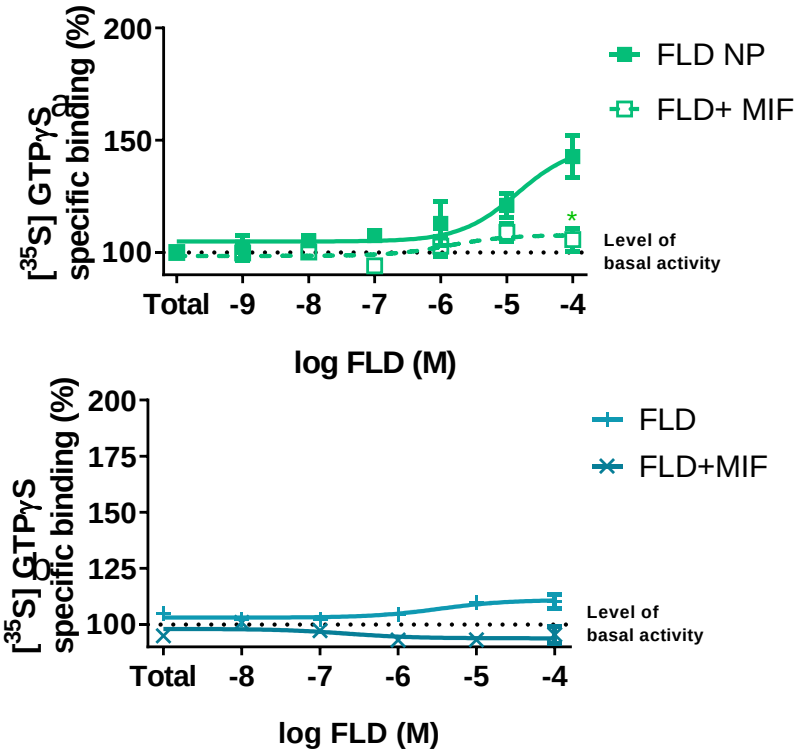

Figure 15**.** Effect of fludrocortisone (10^{-8}-10^{-4} M) on [^{35}S]GTPγS binding with or without pre-treatment with mifepristone in non-pregnant (a) and pregnant (b) uterine tissues. Basal activity (100%) refers to the level of [^{35}S]GTPγS binding without any substances. *: $p<0.05$; MIF, mifepristone; FLD, fludrocortisone, SPR; spironolactone.

2.5.3 **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 16.).

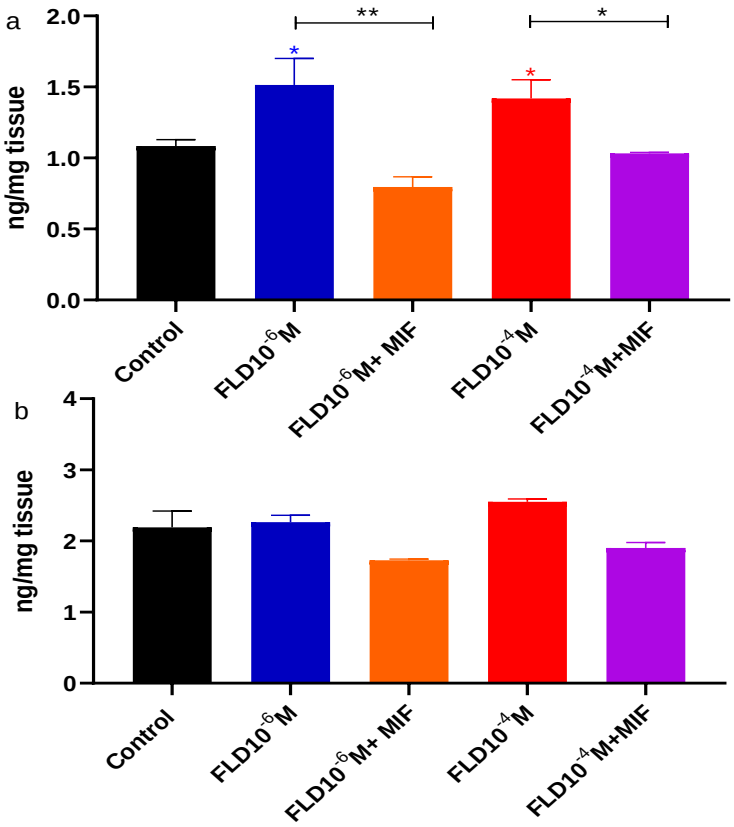

Figure 16. Changes in the cAMP level of uterine non-pregnant (a) and pregnant (b) tissues in the presence of fludrocortisone alone and after pre-treatment with mifepristone. The uterine cAMP level was expressed in ng/mg tissue. MIF, mifepristone; FLD, fludrocortisone.

2.5.4 **In vivo study:** FLD had no significant action on either animal group (Figure 17.).

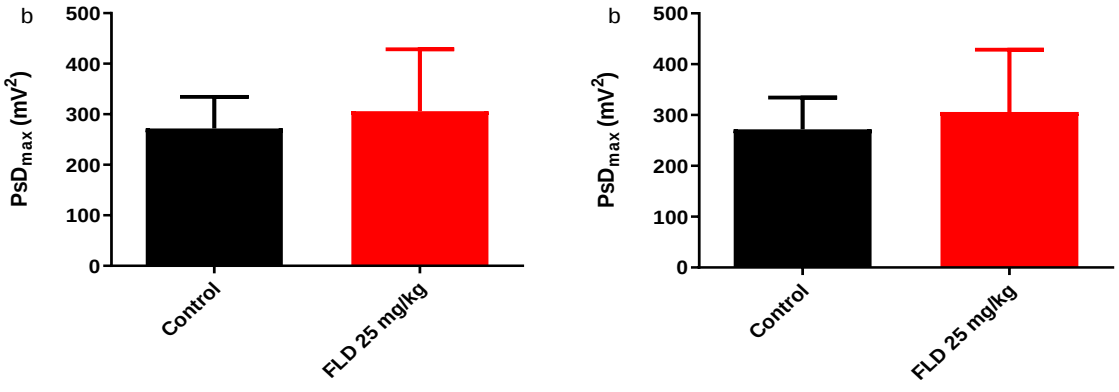

Figure 17. Effect of fludrocortisone (FLD) on non-pregnant (a) and pregnant (b) animals, in vivo. FLD did not modify either non-pregnant or pregnant contractions.

3. Discussion:

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. Although such an action of T was described earlier on human and pregnant rat uteri, such a result about E2 has not been published yet. 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.

CSs and particularly GCs inhibit induced contraction. 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 airway smooth muscles of guinea pigs and mice. 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.

Since the exposure time lasted a maximum of 30 minutes, which is considered to be too short to initiate the genomic response, the genomic action was ruled out. The blockade of the genomic pathway did not modify the effects of E2 or T similarly to earlier studies. Also, 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.

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 did not reduce their actions, which proved that the genomic pathway as well as ER, androgenic receptor and MR is not involved in the relaxing effects of E2, T and ALD.

Surprisingly, higher dose of MIF (PR 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. This suggests that mifepristone 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 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. In [^{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_{α} stimulation, 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**,** calcium and other second messengers such as IP3 or cAMP. 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.

4. 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. 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, they may have a further benefit in delaying the time of delivery and reducing the risk of prematurity compare with sex steroids. Further preclinical and clinical investigations are necessary to determine the required dose and duration, along with the potential risks, of GCs in the application of premature birth therapy.

List of publications

This thesis is based on the following publications:

I. M. Mirdamadi, A. Kothencz, E. Szűcs, S. Benyhe, M. Szécsi, R. Gáspár, **Non-genomic actions of sex hormones on pregnant uterine contractility in rats: An in vitro study at term**, Life Sci. 263 (2020) 118584. https://doi.org/10.1016/j.lfs.2020.118584.

II. Mirdamadi, M., Schaffer, A., Barna, T., Samavati, R., Szűcs, K.F., Szűcs, E., Benyhe, S., Szécsi, M., Gáspár, R., 2022. **Non-genomic uterorelaxant actions of corticosteroid hormones in rats: An in vitro and in vivo study.** Eur. J. Pharmacol. 175346. https://doi.org/10.1016/j.ejphar.2022.175346

Other publication unrelated to this thesis:

I. D.L. Vidács, Z. Veréb, R. Bozó, L.B. Flink, H. Polyánka, I.B. Németh, S. Póliska, B.T. Papp, M. Manczinger, R. Gáspár, S. Mirdamadi, L. Kemény, Z. Bata-Csörgő, **Phenotypic plasticity of melanocytes derived from human adult skin**, Pigment Cell Melanoma Res. (2021). https://doi.org/10.1111/pcmr.13012.

II. Schaffer, E. Ducza, N. Bódi, M. Bagyánszki, Z. Szalai, M. Mirdamadi, T. Barna, K.F. Szűcs, R. Gáspár, **The ontogenies of endometrial and myometrial leptin and adiponectin receptors in pregnant rats: Their putative impact on uterine contractility**, Life Sci. 297 (2022) 120465. https://doi.org/10.1016/j.lfs.2022.120465.

III. R. Gáspár, J. Hajagos-Tóth, A. Schaffer, A. Kothencz, L. Siska-Szabó, E. Ducza, A. Csányi, T. Tábi, F. Bagaméry, É. Szökő, O. Kovács, T. Barna, R. Samavati, M. Mirdamadi, A. Sztojkov-Ivanov, K.F. Szűcs, S.G. Vari, **High Fat High Sucrose Diet Modifies Uterine Contractility and Cervical Resistance in Pregnant Rats: The Roles of Sex Hormones, Adipokines and Cytokines**, Life. 12 (2022) 794. https://doi.org/10.3390/life12060794.