The contribution of nitric oxide on the relaxation effects of diethylstilbestrol

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ABSTRACT

الأهداف: تقييم مساهمة أكسيد النتريك (NO) على آثار الاسترخاء لعقار ديثيلستيلبيسترول على رحم أنثى الجرذ.

الطريقة: أجريت الدراسة بقسم مختبر الصيدلة بكلية الطب بجامعة ديار بكير – تركيا. تم تعليق حلقات الرحم من أنثى 8 جرذان غير حامل نوع وستار ألبينو، يتراوح وزنها مابين (300-350g) **جرام**، في مرحلة تعزيز الودق في حمام للأعضاء والتحفيز بالمجال الكهربائي، تم تطبيق ذلك من أجل تسجيل التوتر القياسي المتناظر. تم التحقق من أثر أكسيد النتريك (NO) على الاستجابات المتقلصة لحلقات الرحم لدى الجرذان. تمت دراسة آثار طليعة أكسيد النتريك (NO) إل–آرجينيـن (10^{-7}-10^{-4}M) ومثبط سينثيس أكسيد النتريك (NO) إل– نتيرو– آرجينين– ميثيل إستر (10^{-7}-10^{-4}M) واتحاد تركيبهما على الاستجابات المتقلصة في وجود وغياب ديثيلستيلبيسترول ($2x10^{-4}$M).

النتائج: تم فحص إجمالي عدد 30 عينة (عدد= 6 لكل مجموعة، خمس مجموعات). كانت الاستجابات المتقلصة المثبطة لديثيلستيلبيسترول 64.2±4.5% (عدد=6 – $p<0.05$). انخفضت الاستجابات المتقلصة في وجود إل– نتيرو– آرجينين– ميثيل إستر (عدد=6 – $p<0.05$). تم تكمين التثبيط على الاستجابات المتقلصة الى ديثيلستيلبيسترول في وجودة إل– آرجينين تحت تحفيز الأحوال المشابهة (عدد=6 – $p<0.05$). لم تتأثر الاستجابات المتقلصة لتحفيز المجال الكهربائي في وجود ديثيلستيلبيسترول بواسطة إل–نتيرو– آرجينين– ميثيل إستر (عدد=6 – $p>0.05$).

خاتمة: بينت هذه الدراسة أن أكسيد النتريك قد يدعم الآثار المثبطة لعقار ديثيلستيلبيسترول بآليات عمل مختلفة على الانقباضات المُحفزة بالمجال الكهربائي لرحم أنثى الجرذ الغير كامل.

Objectives: To evaluate the contribution of nitric oxide (NO) on the relaxation effects of diethylstilbestrol on rat uterus.

Methods: Uterine rings from 8 nonpregnant Wistar Albino rats (300-350g) in the pro-estrous phase were suspended in an organ bath and electrical field stimulation applied for recording isometric tension. The influence of NO on contractile responses of rat uterine rings was investigated. The effects of NO precursor L-arginine (10^{-7}-10^{-4}M) concentration and NO synthase inhibitor L-nitro-arginine-methyl ester (10^{-7}-10^{-4}M) concentration and a combination of them on contractile responses were studied in the presence and absence of diethylstilbestrol ($2x10^{-4}$M) concentration. The study was carried out at the Department of Pharmacology Laboratory, Faculty of Medicine, Dicle University, Diyarbakir, Turkey.

Results: Totally, 30 samples were investigated (n=6 for each group, 5 groups). Diethylstilbestrol inhibited contractile responses 64.2±4.5% (n=6, $p<0.05$). Contractile responses decreased in the presence of L-arginine (n=6, $p<0.05$) and this inhibition was abolished in the presence of L-nitro-arginine-methyl ester (n=6, $p<0.05$). The inhibition on contractile responses to diethylstilbestrol was potentiated in the presence of L-arginine under similar conditions (n=6, $p<0.05$). The contractile responses to electrical field stimulation in the presence of diethylstilbestrol were not affected by L-nitro-arginine-methyl ester (n=6, $p>0.05$).

Conclusions: These data provide evidence that NO may potentiate the inhibitory effects of diethylstilbestrol by different mechanisms on the electrically induced contractions of the non-pregnant rat uterus.

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Unlike other smooth muscles, the uterus is largely under hormonal control. Estrogen is an important mediator in reproductive events, such as uterine growth, control quiescence during gestation, and the set up of the uterus for labor and delivery. In addition to steroid hormones, there are a number of factors that modulate myometrial contractility, such as oxytocin, prostaglandins, endothelin, platelet activating factors and relaxation, including corticotropin releasing hormone, prostacyclin, and nitric oxide (NO).[1] Nitric oxide is a potent relaxant of smooth muscle and possibly plays a role in the maintenance of uterine quiescence.[2] Some studies have shown increased variation in NO production and nitric oxide synthase (NOS) expression during the estrous cycle.[3] Estradiol, whose concentration peaks before implantation, might regulate NOS activity in the uterus. Intraluminal injections of L-nitro-arginine-methyl ester (L-NAME), a competitive inhibitor of NOS, reduced the number of implanted embryos by 50%, this suggests that the NO system plays a relaxant role during implantation.[4] Furthermore, clinical trials have shown a positive effect of the NO-precursor L-arginine (L-Arg) on the pregnancy rate of poor responder patients.[5] In contrast, there are also theories that estradiol administration causes inhibition in total NO production, suppression of both mRNA, and protein levels of NOS enzyme.[6] Hence, the factors that regulate NO production in the uterus remain unclear. The steroid hormone 17ß-estradiol (E2) exhibits dramatic effects in the uterus with respect to hyperemia, gene expression, and proliferation of the endometrium. There is a wide array of changes after E2 exposure in the expression of multiple genes and biochemical processes within the uterus.[7] However, as a whole, it is unclear how these responses mediate the contractile responses of the uterine tissue. Evidence demonstrates that there is an inverse correlation between the frequency of uterine contractions and the pregnancy rate.[8] However, the elevated NO generation that occurs during pregnancy decreases at term.[9-11] Uterine relaxation linked to NO has been described previously.[12] Data suggests that estradiol might be a modulator of NOS activity during nidation and that NO production is necessary to achieve a successful embryo implantation and to prevent preterm labor.[4] Differences between conditions and experimental methods have provided contrary results on the effects of estrogen on NO dependent uterus relaxation. Therefore, in the present study we aimed to investigate the possible role of NO on the inhibitory effects of diethylstilbestrol (DES) in the rat uterus.

Methods. The study was carried out in the Department of Pharmacology Laboratory, Faculty of Medicine, Dicle University, Diyarbakir, Turkey between May 2004 and July 2004. Uterine rings were obtained from our initial organ bath study.[13] The study was approved by the Dicle University Ethical Committee. Animals had no pretreatment with any drugs or special conditions. Female Wistar Albino rats used in the pro-estrous phase were obtained from the Dicle University Health Sciences Practice and Research Center. Experiments conformed to the provisions of the Declaration of Helsinki in 1995 (as revised in Edinburgh 2000).

Measurements. Ether anesthetized 8 female Wistar Albino rats in proestrous phase (300-350 g) were sacrificed by cervical dislocation. Uterus tissue was rapidly removed. Both horns of the uterus were sliced out, and the uterus rings obtained and prepared for isolated tissue experiments. Four uterus rings were obtained from every rat. Thirty samples were used throughout the study from 8 rats. Each ring was used for each treatment. The rings were 40-60 mm in length, and were kept in Kreb's solution until used. The Kreb's solution had the following composition: sodium chloride (NaCl) (118.0 mmol/l), potassium chloride (KCl) (4.7 mmol/l), calcium chloride ($CaCl_2$) (2.5 mmol/l), magnesium sulphate ($MgSO_4$) (1.0 mmol/l), monopotassium phosphate (KH_2PO_4) (1.0 mmol/l), glucose (11.0 mmol/l), and sodium bicarbonate ($NaHCO_3$) (25.0 mmol/l). The rings were mounted in a 10 ml organ bath filled with Krebs solution maintained at 37°C. After mounting, each ring was allowed to equilibrate with a basal tension of 1 g for 45 minutes. During this time, the solution was replaced every 15 minutes with fresh solution. The solution was gassed with 95% oxygen (O_2) and 5% carbon dioxide (CO_2) during the study, and the temperature was maintained at 37°C. Each ring was connected to a force transducer (FDT 10-A, May IOBS 99, COMMAT Iletisim Co., Ankara, Turkey) for the measurement of isometric force, which was continuously displaced and recorded on an online computer via 4-channel transducer data acquisition system (MP30B-CE, Biopac Systems Inc., Santa Barbara, CA) using software (BSL PRO Manuel Professional v 3.6.6, Biopac Systems Inc.). Drugs were added directly to the organ bath. In all series, uterine rings were first stimulated with electrical field stimulation (EFS) generated by a MAY ST95 Point Stimulator and MAY ISO 150. A Stimulus Isolated Power Supply was used to apply the electrical field using circular 0.5 cm diameter platinum ended electrodes. Neurogenic contractions were elicited by EFS in uterine rings. The parameters of EFS were the following: duration 30 ms, frequency range of nerve stimulation 20 Hz, supra-maximal voltage 40 V. The square pulse duration was set at 30 msec. Each 30-second stimulation was followed by a 2-minute recovery period. In the first series of experiments, the tissues were stimulated by EFS

followed by addition of a single concentration of DES ($2x10^{-4}$M) to the organ bath. Contractile responses were elicited by EFS in the presence or absence of drugs. In the following experiments, L-Arg and L-Name, respectively, were added in cumulatively graded concentrations to the organ bath and EFS induced contractile responses were elicited in the absence and presence of them in different series. The same procedures were repeated in the presence of DES ($2x10^{-4}$□) in L-Arg and L-NAME groups. A cumulative concentration-response curve was constructed in a stepwise manner after the response to the previous concentration had reached a plateau.

Groups. Group I: EFS+DES, Group II: EFS+L-arginine (10^{-7}-10^{-4}□), Group III: EFS + DES ($2x10^{-4}$□)+L-arginine (10^{-7}-10^{-4}□), Group IV: EFS+L-NAME (10^{-6}-10^{-4}□), Group V: EFS + DES ($2x10^{-4}$□) + L-NAME (10^{-6}-10^{-4}□).

Chemicals. Drugs and their sources were: Diethylstilbestrol, L-arginine, Nw-Nitro-L-arginine, from Sigma Chemicals (St. Louis, USA). Stock solutions were prepared in distilled water. All drugs were stored at -20°C and freshly dissolved in distilled water to the appropriate molar concentration for the organ bath.

Statistical analysis. Contractile responses were expressed as a percentage of the contraction induced by EFS. Continuous variables were expressed as mean ± SD. The contractile effects of the drugs were expressed as a percentage of the baseline contractile force resulting from EFS. Paired Student's t test for the means of 2 groups was used. An analysis of variance (ANOVA) with a post-hoc Tukey's-HSD (Honestly Significantly Different) test was performed for more experiments with more than 2 groups. Two-sided P values were considered statistically significant at $p<0.05$. Statistical analyses were carried out by using the statistical packages for SPSS 15.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results. The DES ($2x10^{-4}$M) caused inhibition of the contractile responses resulting from the electrical stimulation 64.2 ± 4.5% of control responses (n=6) ($p<0.05$) (**Figure 1**). Contractile responses to the electrical field stimulation were also inhibited in the presence of L-Arg in a dose dependent manner (n=6) ($p<0.05$) (**Table 1**) (**Figure 1**). Uterine rings showed spontaneous activity. The inhibition on the contractile responses given to DES was potentiated by cumulatively added L-Arg (10^{-7}-10^{-4}□) (n=6) ($p<0.05$) (**Table 1**) (**Figure 2**). The L-NAME (10^{-7}-10^{-4}□) abolished the inhibition of the contractile responses (n=6) ($p<0.05$) (**Table 2**) (**Figure 3**), however, there were no significant differences when combined with DES (10^{-7}-10^{-4}□), (n=6) ($p>0.05$) (**Table 2**) (**Figure 3**). The L-Arg combination with L-NAME in the presence of DES inhibition significantly abolished when compared with L-Arg + DES group, (n=6) ($p>0.05$) (**Table 2**) (**Figure 4**).

Discussion. Previous studies have investigated the levels of the various factors within this pathway in the uterus, in hopes of understanding how they participate in the above-mentioned physiological and pathological states. Several studies have implicated a variety of regulatory factors of uterine activity, including steroid hormones,[14] and substances that suppress or stimulate myometrial contractility.[15] In our study, DES caused inhibition of the contractile responses to EFS. Reports

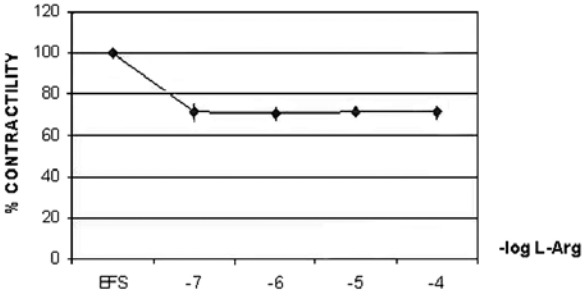

Figure 1 - The effects of DES (10^{-7}□ -10^{-4}□) on EFS induced contractile responses. Values are means ± SD for 6 rats. There were significant differences on contractile responses with respect to control responses given to the EFS only ($p<0.05$). EFS - electrical field stimulation, DES - diethylstilbestrol

Table 1 - Dose-response values (%) ± SD of L-Arg (10^{-7}-10^{-4}□) (-log mM) and DES ($2x10^{-4}$M)+L-Arg (10^{-7}-10^{-4}□) (-log mM) on EFS induced contractile responses. There were significant differences between groups (n=6).

Sequence of application	L-Arg	DES+L-Arg	*P*-value
Control EFS	100	100	
-8	84.0 ± 2.1	56.8 ± 3.8	<0.05
-7	64.7 ± 4.1	49.2 ±2.7	<0.05
-6	62.6 ± 3.5	43.2 ± 5.4	<0.05
-5	62.2 ± 3.1	38.9 ±4.6	<0.05

$p<0.05$ considered statistically significant. -8 to -5 are expressed as -log of molar concentrations
L-Arg - L-arginine, DES - diethylstilbestrol, EFS - electrical field stimulation

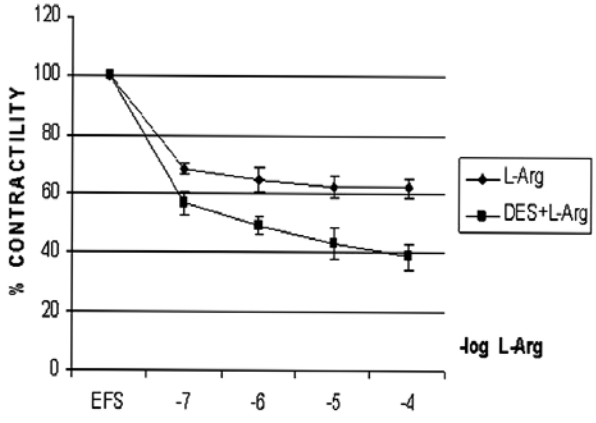

Figure 2 - The effects of L-Arg (10^{-7}-10^{-4}M) and DES ($2x10^{-4}$M) + L-Arg (10^{-7}-10^{-4}M) on EFS induced contractile responses. Dose-response curves for L-Arg. Values are means ± SD for 6 rats. There were significant differences on contractile responses between groups (p<0.05). L-Arg - L-arginine, DES - diethylstilbestrol, EFS - electrical field stimulation

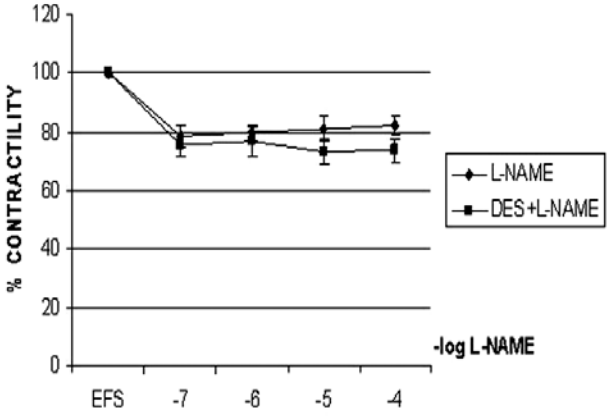

Figure 3 - The effects of L-NAME (10^{-7}-10^{-4}M) and DES ($2x10^{-4}$M) + L-NAME (10^{-7}-10^{-4}M) on EFS induced contractile responses. Dose-response curves for L-Arg. Values are means ± SD for 6 rats. There were no significant differences on contractile responses between groups (p>0.05). L-NAME - L-nitro-arginine-methyl ester, DES - diethylstilbestrol, EFS - electrical field stimulation

Table 2 - Dose-response values (%) ±SD of L-NAME (10^{-7}-10^{-4}□) (-log mM), DES ($2x10^{-4}$M)+L-NAME(10^{-7}-10^{-4}□) (-log mM) and DES ($2x10^{-4}$M)+L-NAME(10^{-7}-10^{-4}□) + L-Arg (10^{-7}-10^{-4}□) (-log mM) on EFS induced contractile responses. There were no significant differences between groups (n=6). L-NAME - L-nitro-arginine-methyl ester, L-Arg - L-arginine, DES - diethylstilbestrol, EFS - electrical field stimulation

Sequence of application	L-NAME	DES+L-NAME	DES+L-NAME + L-Arg	*P*-value
Control EFS	100	100	100	
-8	78.5 ± 3.6	75.7 ± 4.2	74.3 ± 6.1	>0.05
-7	79.8 ± 2.7	76.6 ± 5.2	71.2 ± 2.5	>0.05
-6	81.2 ± 4.3	73.1 ± 4.1	68.1 ± 4.2	>0.05
-5	82.1 ± 3.1	73.6 ± 3.7	69.3 ± 5.4	>0.05

p<0.05 considered statistically significant. -8 to -5 are expressed as -log of molar concentrations
L-NAME - L-nitro-arginine-methyl ester, L-Arg - L-arginine, DES - diethylstilbestrol, EFS - electrical field stimulation

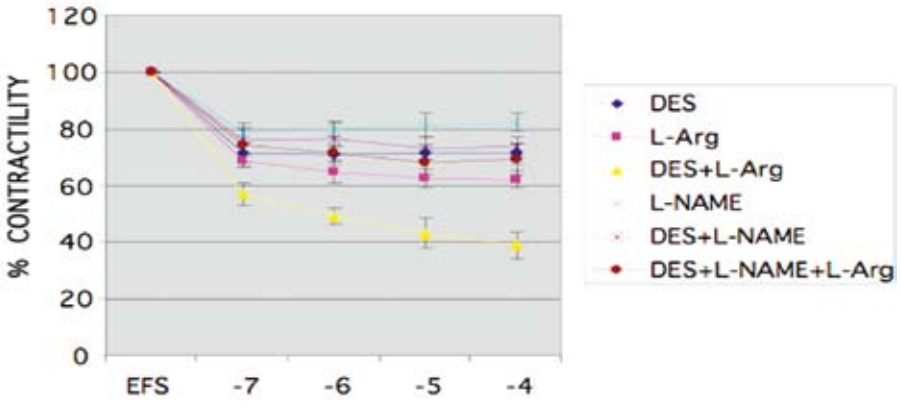

Figure 4 - The effects of L-NAME (10^{-7}-10^{-4}M) and DES ($2x10^{-4}$M) + L-NAME (10^{-7}-10^{-4}M) on EFS induced contractile responses. Dose-response curves for DES, L-Arg and DES+L-Arg. Values are means ± SD for 6 rats. There were no significant differences on contractile responses between groups (p>0.05). L-NAME - L-nitro-arginine-methyl ester, L-Arg - L-arginine, DES - diethylstilbestrol, EFS - electrical field stimulation

indicate that estrogens regulate the NO/soluble guanylyl cyclase (sGC)/ 3',5'-monophosphate (cGMP) cell signaling pathway and the levels of NO and the second messenger cGMP in many tissues with subsequent relaxation of smooth muscle cells, including the uterus.[16,17] In this study, we demonstrated the effects of NO that potentiate the inhibitor effect of DES in the uterus and also that it modulates contractility. The observation that exogenous L-arginine had the capability of relaxing the spontaneous contractions of myometrial rings from non-pregnant uterus in vitro suggests the presence of functional NOS,[18] and that the effects of L-arginine are reversed by inhibitors of NO synthase L-NAME. Compelling evidence for the presence of NOS has been provided by immunocytochemistry and in-situ hybridization of NOS messenger ribonucleic acid (mRNA) in uteri from pre- and post- menopausal women,[19] or by Western immunoblot analysis,[20] as well as by biochemical analysis of NOS activity in both pregnant and non-pregnant women.[21] Han et al[22] claims that estradiol stimulates the expression of NOS isoforms in endometrial derived primary and human embryonic stem (HES) cells through a genomic mechanism.[22] These studies indicate that the NO system is present in the uterus and may contribute to the maintenance of uterine quiescence during pregnancy, but not during delivery, depending on the circulating estrogen levels that potentiate its effects. In guinea pig myometrium, the NO donor S-nitroso-L-cysteine (CysNO) had no effect on spontaneous contractile activity, either in pregnant or non-pregnant uterine tissue rings, but had a relaxing activity on agonist-evoked contractions.[23] The various effects of NO donors may depend on the species studied and the experimental conditions. Nitric oxide generation is elevated in non-pregnant rat uterus during pro-estrous and in response to estradiol treatment. This increase in uterine NO generation by estradiol is associated with increased NOS III expression.[10] Yallampali et al,[10] also indicated that estradiol inhibits NOS II and total NO generation and stimulates NOS III expression. It was previously reported that anti-estrogens increased NOS II expression in the pregnant rat uterus.[24] In previously published data on the uterine NOS, there appears to be considerable species differences with respect to both characteristics and subcellular localization.[25] The NOS was generally found to be higher in the particulate fraction than cytosolic fraction.[26] Reports indicate that decidual NO synthase activity, which has the characteristics of the inducible isoform of the enzyme, is significantly lower on the last day of gestation. This suggests a role for NO in the control of uterine contractility during pregnancy.[11] A decrease in estrogen levels after birth and increased contractile activity of the uterus may also indicate the effect of estrogen on NO. We proposed that changes in contractility responses and compliance may depend on the endocrine status and the levels of circulating androgens and estrogens. The fact that both the protein synthesis inhibitor cycloheximide and the transcriptional inhibitor actinomycin D inhibited the smooth muscle relaxing effect of estradiol in the isolated rat uterus suggest that genomic mechanisms may also contribute to the response.[27] In a previous study, it was demonstrated that NO generated from sodium nitroprusside (SNP) stimulated the synthesis of platelet-activating factor (PAF) and evoked contractility in uterine horns from mice treated with estrogen. This result suggests the possibility that these tissue conditions might be favorable for the generation of peroxynitrites, possible mediators of both effects. It is also shown that the contractility evoked by the addition of SNP was not due to production of PAF, because its antagonist, thieno-triazolodiazepine (WEB 2086) (10-30 mumol/L, a concentration that blocked contractions evoked by PAF 1 nmol/L), had no effect on the SNP-evoked contractions.[28] However, there are also contrary theories since, the NO precursor L-arginine inhibited spontaneous contractions of non-pregnant human myometrium in vitro.[29] There are many systems and pathways at work here. We examined the effect of L-Arg and L-NAME on the electrically stimulated contractile responses of rings from non-pregnant rat uteri in the presence of DES. The observation that L-Arg increased the inhibition of the contractile responses on non pregnant rat uteri in the presence of DES, and these effects were abolished in the presence of L-NAME suggest that NO has a mediator role in this phenomenon, but possibly with different mechanisms. However, data on animal and human pregnant uterine contractions are still controversial. Since the NOS substrate L-arginine induced a dose-dependent inhibition of the uterine contractile activity, our results support the presence of a functional NOS in non-pregnant rat uterus.

In conclusion, the present study provides evidence that in rat non-pregnant uterus, L-Arg may contribute to the inhibition of the electrically stimulated contractile responses of DES, but possibly with different mechanisms. However, the exact mechanism is still unclear. With regard to the variability of the effect of L-arginine and NO donors based on the species, the experimental conditions and the hormonal status, these data highlight the need for more experiments on human uterine preparations. Further experiments are required to better understand the modulation of uterine contractility. This would spur further investigation regarding the possibility for using an exogenous NO donor and estrogen supplementation. These data could

open the field for the intra-vaginal administration of L-arginine and estrogen in the very early stages of embryo transfer in order to improve implantation rates.

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