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RESEARCH ARTICLE

17β-Estradiol Inhibites Nitric Oxide-cGMP-Dependent Pathway but may Activate Independent Pathway in Small Intestinum of Ovariectomized Rat [1] [2]

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Summary

This study was designed to investigate the effect of 17β-estradiol on small intestinal motility of ovariectomized rats and the possible role of nitric oxide (NO) in this activity. A total of 24, 3 to 6 month-old female Sprague Dawley rats (270±20 g) were ovariectomized and divided into four groups as one control and three experimental groups. The control group received 0.2 mL, sesame oil once daily for three days, whereas the experimental groups were treated with 25, 50 and 100 µg/rat intramuscular 17β-estradiol, respectively. The rats were sacrificed by cervical dislocation under anaesthesia 18 h after the termination of last treatment. Immediately, duodenum, jejunum and ileum were isolated for organ bath contractility experiments to evaluate isometric smooth muscle motility *in vitro*. It was observed that application of 100 µg/rat 17β-estradiol showed a decreasing tension in duodenum, whereas none of the different doses of 17β-estradiol showed any significant difference in jejunum. The application of 50 and 100 µg/rat 17β-estradiol decreased the spontaneous contractile tension of ileum. However, L-arginine (10^{-5}M), 8-Br-cGMP (10^{-6}) and SNP (10^{-3}M) decreased the spontaneous contractions of smooth muscle of duodenum, jejunum and ileum. Moreover, it was demonstrated that 17β-estradiol decreased the relaxing activity of L-arginine and 8-Br-cGMP but increased the activity of SNP in dose dependent manner. In conclusion, it is suggested that 17β-estradiol has a relaxative effect in duodenum and ileum and particulary inhibits the activity of cGMP-PK. However, endogenous NO-NOS pathway mediated by 17β-estradiol may play a key role in secretory and/or ciliary activity of intestinum.

Keywords: *17β-estradiol, Small intestine, cGMP, Nitric oxide, Rat*

17β Östradiol Overioktomize Sıçanların İnce Bağırsağında Nitrik Oksit-cGMP'den Bağımsız Yolu Etkinleştiriyor Olabilirken Bağımlı Yolu Engelliyor

Özet

Bu çalışmada ovaryumları çıkarılmış sıçanlarda 17β-östradiolün ince bağırsak kasılımları üzerine etkisi ile bu etkinin ortaya çıkmasında nitrik oksitin rolü incelenmiştir. Bu amaçla çalışmada 3-6 aylık ve ortalama 270±20 g ağırlığında, 24 Sprague Dawley dişi sıçan her grupta 6 sıçan bulunacak şekilde, kontrol (Ov) ve 3 deneme grubu olmak üzere 4 gruba ayrılmıştır. Çalışmada kontrol grubuna günlük olarak kas içi susam yağı enjeksiyonu yapılmış (0,2 ml), deneme gruplarına sırasıyla 25, 50 ve 100 µg/sıçan 17β-östradiol, üç gün uygulanmıştır. Son uygulamadan 18 saat sonra genel anestezisi altında ötenazi yapılmıştır. Takibinde ince bağırsak, duodenum, jejenum ve ileum bölgelerine ayrılmış ve *in vitro* izometrik düz kas hareketleri kaydedilmiştir. 17β-östradiolün 50 ve 100 µg/sıçan dozları spontan ileum kasılımlarının gerimini azaltırken duodenumda aynı etkiyi 100 µg/sıçan dozunun oluşturdu gözlemlendi. Jejenumda ise gruplar arasında farklılık oluşmadığı görüldü. Buna karşın L-arjinine (10^{-5}M), 8-Br-cGMP (10^{-6}M) ve SNP (10^{-3}M) uygulamaları duodenum, jejenum ve ileumda spontan düz kas kasılımlarını azalttı. Ayrıca 17β-östradiol uygulamasının L-arjinin ve 8-Br-cGMP'nin gevşetici etkisini azalttığı tersine SNP'nin etkinliğini ise doza bağımlı olarak arttırdığı gösterildi. Sonuç olarak 17β-östradiolün duodenum ve ileumda özellikle cGMP-PK etkinliğini engellemesine rağmen gevşetici etkiye sahip olduğu buna karşın 17β-östradiol aracılığıyla endojen NO-NOS yolunun bağırsağın sekresyon ve/veya siliyar etkinliklerinde rol oynayabileceği önerilmektedir.

Anahtar sözcükler: *17β-östradiol, İnce bağırsak, cGMP, Nitrik oksit, Sıçan*

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INTRODUCTION

Estrogens are basic female sex hormones and exist in forms of estron, estrol and 17β-estradiol in the body. Estrogens increase the concentration of receptors for oxytocin [1] and adrenergic agents [2], which modulate membrane calcium channels. Estrogens are capable to increase the contraction of uterine smooth muscle [3] however, they produce relaxation in smooth muscles of bile duct [4], trachea [5] and blood vessels [6]. Intestinal motility in human [7] and animals [8] are closely associated with estrous cycle, pregnancy and menopause. It has been demonstrated that changes in concentration of estrogen and progesteron as well as local hormones of digestive tract affects electromechanical behaviour of smooth muscles of gastro-intestinal (GI) tract [9]. It has been reported that estrogens may hyperpolarize the cell membrane by stimulating extracellular discharge of potassium (K^+) in smooth muscle cells, thus the inhibition of spontaneous contraction is occurred [10]. However, the blocking of entrance of calcium (Ca^{+2}) into cells by estrogens inhibits voltage-dependent Ca^{+2} and K^+ channels and this further produces relaxation in smooth muscle [11]. It has been indicated that 17β-estradiol significantly inhibites the carbachol-induced contractions of ileum [12]. Moreover, in a previous study, it has been clearly demonstrated that estrogens increase plasma concentration of cholecystokinin and activates cholecystokinin receptors thus; the inhibitor effect of estradiol on motility of gastrointestinal system occurs [9]. Some molecules mediating 17β-estradiol activity, such as nitric oxide (NO), is also responsible for control of motility and passage speed in GI system. Nitric oxide released from neuronal nitric oxide synthase (nNOS) located in nonadrenergic and noncholinergic (NANC) nerves are very important factor for the control of GI system motility and emptying time [13]. Nonadrenergic and noncholinergic nerves that are dependent on NO release play a key role for regulating the secretion, motility and amplitude of smooth muscles in GI tract [13,14].

As a chemical mediator, NO enters into the cell swiftly, activates cGc in sitozol and stimulates the formation of cGMP, which is an inner cell secondary messanger in simple muscle cells [19]. GMP paves the way for relaxation in a simple muscle via two mechanisms. Firstly, NO_2 reduces levels of Ca^{++} within a cell and increases the permeability of K^+ channels. Thus, it hyperpolarizes the plasma membrane [15,16]. Secondly, cGMP blocks miyozin/aktin interaction by activating cGMP dependent protein kinaz (PKG) which leads to the dephosphorization of light miyozin chains and which is reported to play a key role in NO/cGMP signals [15].

Previous reports have been clearly demonstrated that the effect of estrogens on the GI system motility displayed various responses regarding to different anatomical region of intestinum [12,17]. To the authors' knowledge, the role of NO on 17β-estradiol mediated contractility of small intestine was still unclear. Therefore, this study was designed to evaluate the effect of different doses of 17β-estradiol, a femal sex steroid, on small intestine motility and the possible role of NO in ovariectomized rats.

MATERIAL and METHODS

Animals

A total of 24, 3 to 6 month-old female Sprague-Dawley rats (270±20 g) were used in the study. Rats were fed *ad libitum* with a commercial rat diet. (This research has been approved by Institutional Etchic Committee of Afyon Kocatepe University. Date and number is; 2008/203).

Experimental Groups and Study Design

The ovariectomy procedure was performed under general anesthesia by an intra-peritoneal ketamine (21.2 mg/kg) and xylazine (4.2 mg/kg) combination. Small bilateral incisions were made on the dorsum to expose the ovaries retroperitoneally. The ovarian vessels were then clamped and the ovaries were removed. Afterwards, the uterine tubes were ligated and the muscles and skin were sutured. Two weeks after operation, the ovariectomized rats were randomly assigned to four groups of 6 rats each. Rats in the control group received sesame oil once daily for three days, whereas rats in the experimental groups were treated with 25, 50 and 100 µg/rat 17β-estradiol, intramuscularly.

After 18 h of the last hormone treatment, the rats were killed by cervical dislocation under sodium penthotal (25 mg/kg, intraperitoenal) anaesthesia. Immediately after death, the abdominal cavity of the rats was opened and the mid-duodenum, mid-jejunum and mid-ileum were carefully removed avoiding physical damage. Each individual isolated tissues was placed in Krebs' solution (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1mM $MgSO_4$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose). Subsequently, the surrounding mesentery and fat tissues were carefully removed from the tissues, and a strip-shaped tissue samples with dimensions of 0.1x0.3x1.0 cm were obtained. One edge of each intestine tissue preparation was fixed to platinum ring electrodes. The opposite edge of the tissue was connected to a force-displacement transducer (model 10-A; MAY, Commat, Ankara, Turkey). Isolated intestine tissues were placed in organ baths filled with 20 mL Krebs' solution, which were continuously ventilated with a gas mixture (95% O_2 - 5% CO_2) at 37ºC. The isometric smooth muscle activity of the intestine samples were monitored and recorded by computer via the force transducer and an acquisition system (model MP30 WSW with Biopac Student Lab PRO Software, Biopac Systems). An electrical field stimulation (EFS) device (model ISO 150-

C, MAY, Commat, Ankara, Turkey) was used for maximal contractility.

Recording of Isometric Intestine Contractility

Samples in organ baths were kept in Krebs' solution for at least 1 h prior to the recordings to enable the tissues to adapt to the environment, and the solution was refreshed in 15-min intervals. The appropriate resting tension for the strips was determined in initial experiments. Optimal tension relationships were achieved with resting tensions of 2 g for the intestinum strips. Therefore, a resting tension of 2 g was applied to the tissues. Electrical field stimulation was then used to define submaximal contraction, carried out at various frequencies (2, 4, 8, 16, 32 and 64 Hz) and voltage (10, 20, 30 and 40 voltage). In order to determine endogenous NO activity, L-arginine (10^{-5} M) (Sigma, Cat # A5131) was added to the Krebs' solution. 8-Bromo guanosine 3',5'-cyclic monophosphate sodium salt (8-Br-cGMP, a membrane-permeable analog of cyclic guanosine monophosphate) (10^{-6} M) (Sigma, Cat # B13815131) was added in order to determine the activity of cyclic guanosine monophosphate-protein kinase (cGMP-PK) pathway and sodium nitroprusside (SNP) (10^{-3} M) (Sigma, Cat # S0501) was added in order to determine the exogeneous NO activity. All applications were performed on the same tissue. The mean tension of spontaneous contractions for each strip calculated for a 10-min period before administration of examined substances was set as 100% [18,19]. Thereafter, changes in small intestinal contractions caused by the examined substances were recorded and compared to the control period.

Statistical Analysis

All values are presented as mean ± S.D. For statistical evaluation of the data, one-way ANOVA was used. To compare the individual means of treatment groups, the Tukey test was performed (SPSS 16.0). Group differences were declared significant at P<0.05.

RESULTS

Amplititude of spontaneous contractions obtained in duodenum, jejunum and ileum are given in *Table 1*. It was observed that application of 25 and 50 µg/rat 17β-estradiol did not alter the spontaneous contractile tension of duodenum, whereas application of 100 µg/rat 17β-estradiol showed a decreasing tension as compared to control group (P<0.01). Moreover, none of the different doses of 17β-estradiol showed any significant difference in jejunum. Similarly to duodenum, there was no significant difference between control and 25 µg/rat 17β-estradiol groups of ileum. However, the application of 50 and 100 µg/rat 17β-estradiol decreased the spontaneous contractile tension of ileum (P<0.001).

It was clearly observed that the treatment of L-arginine (10^{-5} M) decreased the percentage inhibiton of spontaneous contractions of smooth muscle of duodenum, jejunum and ileum in 50 and 100 µg/rat 17β-estradiol groups as compared to control (P<0.001) *(Table 2)*. Moreover, 25 µg/rat 17β-estradiol showed a relaxant effect as same as 50 and 100 µg/rat 17β-estradiol groups in smooth muscle of jejunum. The treatment of L-arginine (10^{-5} M) applied after EFS did not show any significant difference between groups of jejunum but a decreasing percentage inhibiton of contractile activity was observed in 50 and 100 µg/rat 17β-estradiol groups of duodenum (P<0.001) and all 17β-estradiol groups of ileum (P<0.05).

It was seen that 8-Br-cGMP (10^{-6}) showed relaxant effect in all 17β-estradiol groups of smooth muscle of duodenum, jejunum and ileum *(Table 2)*. The percentage inhibiton was decreased in 17β-estradiol groups of smooth muscle of duodenum and ileum in a dose-dependent manner (P<0.001), while no significant difference obtained between all 17β-estradiol groups of jejunum. The treatment of 8-Br-cGMP (10^{-6}) applied after EFS caused a decreasing percentage inhibiton of contractile tension in all 17β-estradiol groups of smooth muscle of duodenum (P<0.001), jejunum (P<0.001) and ileum (P<0.01) as compared to spontaneous contractile tension.

It was clearly observed that the treatment of SNP (10^{-3} M) increased the percentage inhibiton of spontaneous contractions of smooth muscle of duodenum (P<0.001) and ileum (P<0.05) in 50 and 100 µg/rat 17β-estradiol groups, whereas the percentage inhibition was increased in 100 µg/rat 17β-estradiol group of jejunum, as compared to control group (P<0.05). The treatment of SNP (10^{-3} M) applied after EFS did not show any significant difference between groups of duodenum, jejunum and ileum *(Table 2)*.

Table 1. *Amplitude values of spontaneous contractile tension of duodenum, jejunum and ileum in groups (g)*
Tablo 1. *Gruplarda duodenum, jejunum ve ileumda spontan kasılım gerimlerinin amplitüt değeri (g)*

Amplititude of Contraction (g)	Control	25 µg 17β- estradiol	50 µg 17β-estradiol	100 µg 17β- estradiol	P<
Duodenum	0.66±0.04[a]	0.64±0.02[a]	0.58±0.03[ab]	0.49±0.01[b]	0.01
Jejunum	0.58±0.02	0.57±0.04	0.55±0.04	0.50±0.02	NS
Ileum	1.05±0.06[a]	1.09±0.06[a]	0.87±0.06[b]	0.78±0.04[b]	0.001

[a, b] Different letters in the same line are statistically significant, ***NS:*** *Not significant*

Table 2. *Percentage inhibition of contractility of smooth muscle of intestinum (%)*
Tablo 2. *Bağırsak düz kas kasılımlarının inhibisyon yüzdesi (%)*

Applications	Control	25 µg 17β- estradiol	50 µg 17β-Estradiol	100 µg 17β-estradiol	P<
Duodenum					
Arg (10^{-5})	25.8±4.11^{a}	20.8±1.72^{a}	9.66±2.41^{b}	7.46±1.65^{b}	0.001
8-Br-cGMP (10^{-6})	15.8±2.55^{a}	9.41±2.98^{b}	3.53±1.44^{bc}	1.61±0.39^{c}	0.001
SNP (10^{-3})	36.6±2.16^{b}	37.7±4.14^{b}	67.5±3.78^{a}	74.12±2.31^{a}	0.001
EFS + Arg (10^{-5})	3.07±0.22^{a}	2.72±0.20^{a}	2.07±0.06^{b}	1.97±0.12^{b}	0.001
EFS + 8-Br-cGMP (10^{-6})	13.2±1.84^{a}	1.72±0.20^{b}	1.07±0.06^{b}	0.97±0.12^{b}	0.001
EFS + SNP (10^{-3})	12.8±0.27	13.6±1.01	16.4±2.47	13.1±1.0	NS
Jejenum					
Arg (10^{-5})	15.8±1.41^{a}	7.30±1.31^{b}	5.94±1.21^{b}	4.47±1.45^{b}	0.001
8-Br-cGMP (10^{-6})	15.1±2.13^{a}	95.0±2.38^{b}	0.90±1.93^{b}	3.82±1.02^{b}	0.01
SNP (10^{-3})	23.8±3.24^{a}	32.9±2.23^{ab},	32.9±1.88^{ab}	38.6±5.39^{b}	0.05
EFS + Arg (10^{-5})	1.03±0.25	1.33±0.33	0.83±0.30	1.01±0.36	NS
EFS + 8-Br-cGMP (10^{-6})	7.33±0.95^{a}	1.40±0.36^{b}	0.70±0.22^{b}	0.80±0.20^{b}	0.001
EFS + SNP (10^{-3})	14.9±1.26	14.9±1.76	20.5±1.92	15.1±2.34	NS
Ileum					
Arg (10^{-5})	27.3±4.77^{a}	19.1±1.64^{a}	7.19±1.68^{b}	4.50±1.11^{b}	0.001
8-Br-cGMP (10^{-6})	9.66±1.28^{a}	5.80±1.41^{b}	2.36±1.20^{c}	0.75±0.40^{c}	0.001
SNP (10^{-3})	25.6±3.85^{b}	34.1±3.04^{ab}	39.3±2.12^{a}	43.3±4.33^{a}	0.01
EFS + Arg (10^{-5})	6.33±1.38^{a}	2.00±0.81^{b}	1.16±0.65^{b}	1.50±0.71^{b}	0.05
EFS + 8-Br-cGMP (10^{-6})	4.33±0.66^{a}	1.83±0.74^{b}	1.16±0.66^{b}	0.83±0.47^{b}	0.01
EFS + SNP (10^{-3})	11.8±1.30	10.3±1.56	9.83±1.07	7.16±2.10	NS

^{a,b,c} *Different letters in the same line are statistically significant,* ***NS:*** *Not significant*

DISCUSSION

It has been reported that 17β-estradiol can be injected at doses of 2-100 µg in rats and it peaks 18 h after administration [20,21]. Therefore, we used 25, 50 ve 100 µg/rat doses of 17β-estradiol and evaluated L-arginine/nitric oxide synthase (NOS)/cGMP pathway on isolated strips of duodenum, jejunum and ileum 18 h after different doses of 17β-estradiol administration.

In the present study, we clearly demonstrated that 17β-estradiol decreased the spontaneous contractile activity of duodenum and ileum in a dose-dependent manner *(Table 1)*. Importantly, 17β-estradiol did not alter the amplitude of spontaneous contractile tension of jejunum. Moreover, it has been reported in many studies that NO pathway directly affecting smooth muscles causes significant decrease in gastro intestinal motility [22,23]. In our study, it is possible to indicate that decreasing spontaneous contractility might be associated the interaction of estrogen with NO. Nitric oxide may directly or indirectly effect smooth muscle contractility of small intestinum. Therefore, the present study focused on the possible linkage between estrogen and NO that might be important in regulation of small intestine contractions.

It has been reported that NO is endogenously synthesized from L-arginine via NOS enzymes [24]. Physiologically, the concentration of L-arginine in mice sera is approximately 0.2-3.0 mM [25]. Moreover, L-arginine at around 0.01-1 mM concentrations totally removes the contractile activity of smooth muscles *in vitro* [26]. It has been clearly showed that the most effective dose of L-arginine is 10^{-5} M [18,27], therefore we preferred to use L-arginine at this dose range. It has been reported that estrogens increase NOS expression in intestine [28]. Therefore, if an endogenous NO system is involved in relaxation of intestinum via increasing NOS expression, smooth muscle relaxation would be expected after L-arginine treatment. In contrast, we observed that the relaxant effect of L-arginine (10^{-5} M) decreased, when the dose of 17β-estradiol increased in duodenum, jejunum and ileum *(Table 2)*. These results suggest that inhibition of relaxant effect of L-arginine by 17β-estradiol may be associated with the inhibition of cyclic guanylate cyclase (GC), cGMP and/or cGMP-dependent protein kinase (PKG 1) pathway rather than NO-NOS pathway. Moreover, we found that the treatment of L-arginine (10^{-5} M) after EFS similarly decreased the contractile tension in 17β-estradiol groups of duodenum and ileum but except jejunum. The deficiency of L-arginine on 17β-estradiol groups of jejunum may be explained by

a prevailing endogenous saturation of the L-arginine for NO synthesis as observed in myometrium [29] or low NOS activity in jejunum as suggested in oviduct [19].

Nitric oxide produced by either endogen or exogen NO donors via NOS enzymes increase the cGMP level by activating soluble guanylate cyclase. Thereafter, cGMP initiates the relaxation by phosphorilisation of cGMP-PK in smooth muscles. 8-Br-cGMP decreases the spontaneous mechanical activity [30]. Moreover, it has been reported that 8-Br cGMP has a relaxant activity in smooth muscles of uterus [18] blood vessels [31] and intestine [32]. Cyclic GMP-PK is an enzyme group and has two different forms which includes cGMP-PKI (cGMP-PK Ia/b) and cGMP-PKII [33-35]. It has been reported that cGMP-PKI produces relaxation in smooth muscles [35] and cGMP-PKII, the major form in gastrointestinal system, is responsible for ion transportation [33,34]. In the present study, we observed that the relaxant effect of 8-Br-cGMP was inhibited in all 17β-estradiol groups of smooth muscle of duodenum, jejunum and ileum. It has been reported that NO may be effective by stimulation of guaniline cyclase in various parts of intestine [30]. In human jejunal longitudinal smooth muscle, NO possibly shows depressor activity throughout guaniline cyclase mechanism rather than other mechanisms [36]. Furthermore, it has been reported that nNOS sourced NO gives rise to hyperpolarization by opening Ca^{+2} dependent K^{+} channels and that it leads to relaxation indipendent from cGMP [37]. This finding supports our previous suggestion that endogenously produced NO shows its relaxative effect mediated by estrogens via NO/NOS independent pathway. Furthermore, it suggests that estrogen inhibites the cGMP-dependent pathway.

Sodium nitroprusside, an exogen NO donor, causes a dose-dependent decrease in spontaneous ileal contraction. 1H-[1,2,4] oxadiazolol [4,3,a] quinoxalin–1-one (QDO), a cGC inhibitor, reduces the effects that are initiated by SNP [38]. The relaxing effect of SNP on smooth muscle can be seen at the highest dose of 10^{-3} M, however the doses higher than this concentration is advocated to be toxic [39]. Motility studies in digestive system indicates that this dose produces relaxation [3,19,32]. In our study, similar to other observations, SNP showed relaxing effect on all examined isolated tissues of intestine that this activity was increased by administration of estrogen. This finding supports our previous suggestion that exogenously produced NO shows its relaxative effect mediated by estrogens and the NO/NOS independent pathway may be associated with this effect. Moreover, SNP generates cGMP-dependent and independent relaxation in smooth muscles [18,27]. In our study, the relaxant activity of L-arginine and 8-Br-cGMP was decreased increasing dose-dependent manner of 17β-estradiol, whereas SNP increased the percentage inhibition of smooth muscle contractility *(Table 2)*. Furthermore, 17β-estradiol inhibited the cGMP-dependent pathway but activated the independent pathway.

In conclusion, above-mentioned results suggest that 17β-estradiol particulary inhibits the activity of cGMP-PK in duodenum and ileum. However, endogenous NO-NOS pathway mediated by 17β-estradiol may play a key role in secretory and/or ciliary activity of intestinum.

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