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Nongenomic inhibition of coronary constriction by 17 β -estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol

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Abstract

The cardioprotective effects of 17 β -estradiol (E2) in women are hypothesized to be partially mediated by its metabolites, 2-hydroxyestradiol (2-HOE) and 2-methoxyestradiol (2-MeOH). Therefore, the purpose of our study was to determine the acute effects of E2, 2-HOE, and 2-MeOH to inhibit coronary arterial constriction. Right coronary arteries were dissected out of hearts obtained from breeding sows, cut into 4 mm rings, and suspended in organ baths. Incubation of the rings with E2, 2-HOE, and 2-MeOH (10 μ mol/L) for 60 min attenuated a subsequent KCl-induced contraction by ~ 50%; the protein synthesis inhibitor, cycloheximide, and the estrogen receptor antagonists, ICI 182,780 and tamoxifen, did not effect the attenuation. Moreover, E2, 2-HOE, and 2-MeOH antagonized the contraction induced by the vasospasm agonist, endothelin-1 (0.1 μ mol/L) by ~ 36%; when the L-type Ca²⁺ channel blocker, nifedipine, was added at the conclusion of the experiment no additional contractile attenuation was present. Our results suggest that E2, 2-HOE, and 2-MeOH demonstrate a similar nongenomic inhibition of agonist-induced extracellular Ca²⁺-dependent contractions.

Keywords

17 β -estradiol; 2-hydroxyestradiol; 2-methoxyestradiol; endothelin-1; acetylcholine; artery; coronary; calcium

Introduction

Because the development of coronary heart disease dramatically increases after women reach menopause this suggests that 17 β -estradiol (E2) protects women against the development of vascular disease (Orshal and Khalil, 2004). Rosano et al. (1997) even demonstrated that the short-term administration of E2 has an anti-ischemic effect in postmenopausal women with coronary artery disease. As suggested by Dubey et al. (2004), the cardiovascular benefits of E2 may also be mediated by its biologically active metabolites, 2-hydroxyestradiol (2-HOE) and 2-methoxyestradiol (2-MeOH). Thus far no studies have directly compared coronary artery reactivity between E2, 2-HOE, and 2-MeOH. Kitazawa et al. (1997) did measure the ability of E2, 2-HOE, and 2-MeOH to relax pre-constricted (using a high KCl solution) denuded rat femoral arteries and found the potency order to relax a KCl-induced contraction was E2 = 2-MeOH $\gg$ 2-HOE.

Our interest in the ability of the E2 metabolites to regulate coronary arterial diameter is based upon the findings that 2-MeOH does not demonstrate the mitogenic effects associated with E2 (Gui and Zheng, 2006; Nishigaki et al., 1995); therefore, our long-term hypothesis

is that these metabolites may help maintain blood flow through the coronary vasculature without promoting abnormal cell growth and proliferation. E2 does appear to suppress the occurrence of coronary arterial spasms and the resultant reduction in the development of myocardial ischemia (Kawano and Ogawa, 2005). However, there are a lack of studies evaluating the ability of E2, 2-HOE, and 2-MeOH to inhibit a subsequent coronary artery contraction and/or spasm. Sudhir et al. (1997) initially found that the intracoronary infusion of E2 can reduce coronary vasospasms mediated by the atherosclerotic and vasoconstrictive peptide, endothelin-1 (ET-1). Moreover, Dubey et al. (2001) reported that 2-MeOH is able to inhibit the synthesis of ET-1. Based upon these reports by Sudhir et al. (1997) and Dubey et al. (2001) this study tested the hypothesis that a short-term exposure to E2, 2-HOE, and 2-MeOH is able to similarly attenuate coronary arterial vasoconstriction. Because the generation of coronary arterial tone is principally due to Ca^{2+} influx through voltage-gated Ca^{2+} channels (Quignard et al., 1997), we predict that our hypothesized contractile inhibition is due to blockage of extracellular Ca^{2+} influx.

Materials and Methods

Isometric Tension Measurements

Pig hearts from female Yorkshire pigs 3 to 4 years of age ("retired" breeding sows) were freshly obtained from a local packing plant, placed on ice, and brought back to the laboratory. The right coronary artery was immediately dissected from each heart in a cold, modified low Ca^{2+} Krebs's solution containing (in mmol/L) 138 NaCl, 5 KCl, 0.1 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, and a pH of 7.4.

Coronary arteries were sectioned into 4 mm rings (endothelium intact) and suspended in 25 mL organ baths containing an oxygenated (95% O_2 : 5% CO_2) modified Krebs's solution (PSS; composition in mmol/L: 138 NaCl, 5 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose) having a pH of 7.4 at 37°C. The inner diameter of these arterial rings were ~ 250 μm , and the optimal length for each ring was determined as previously described (Hill et al., 2000). Endothelial integrity was confirmed by relaxation to bradykinin (0.1 $\mu\text{mol/L}$). For each experiment we evaluated the effects of 17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), 2-methoxyestradiol (2-MeOH), and the EtOH solvent on different ring segments of the same artery. A total of 27 pigs were used to complete this study.

To determine the effective short-term incubation time to cross-compare E2, 2-HOE, and 2-MeOH we determined the relaxation kinetics for each of these estradiols. The amount of time (in minutes) to achieve a 25%, 50%, 75%, and 100% relaxation of a pre-constricted artery (using a 60 mmol/L KCl solution) is shown in Table 1. Based upon these data all incubation times were 60 minutes. This kinetic analysis is similar to the E2 kinetics reported by Kitazawa et al. (1997) using the rat portal vein. They found that E2 (5 $\mu\text{mol/L}$) induced a decline in a KCl constricted vein by ~80% in 60 minutes, and that a 60 minute pre-incubation before the administration of a high KCl solution reduced the KCl-induced contraction by a similar magnitude (~80% of control).

To evaluate the relaxation ability of E2, 2-HOE, and 2-MeOH, rings were initially pre-constricted with a depolarizing 60 mmol/L KCl solution (60K; composition in mmol/L: 83 NaCl, 60 KCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose; pH of 7.4) and a concentration-response relationship generated to each respective estradiol (0.3 $\mu\text{mol/L}$ to 100 $\mu\text{mol/L}$) and its ethanol (EtOH) vehicle. Each estradiol concentration was allowed to equilibrate with the rings for 60 minutes before adding the next sequential concentration.

The ability of E2, 2-HOE, and 2-MeOH to attenuate vascular tone development was evaluated against known vasoconstrictive agonists, a depolarizing 60K solution,

endothelin-1 (ET-1), and acetylcholine (ACH). The 60K-induced constriction was evaluated before and after incubation with E2, 2-HOE, and 2-MeOH (10 $\mu\text{mol/L}$). This was done in the presence and absence of the protein synthesis inhibitor, cycloheximide (10 $\mu\text{mol/L}$). Moreover, the reversibility of any estradiol-induced attenuation of the 60K contraction was further evaluated with a subsequent washout period (30 minutes) before exposing the rings to 60K for a third time.

To determine the vascular reactivity to ET-1 and ACH in the presence of the estradiols, a repeatable contractile response to 60K was initially obtained before the 60 minute incubation period with the estradiols or EtOH. Incubation with the estradiols or EtOH was done in the presence (PSS, 2 mmol/L Ca^{2+}) and absence (0 mmol/L Ca^{2+}) of extracellular Ca^{2+} . The 0 mmol/L Ca^{2+} solution had the following composition (in mmol/L: 138 NaCl, 5 KCl, 1 MgCl_2 , 5 EGTA, 10 HEPES, 10 glucose) having a pH of 7.4 at 37 °C. Finally, the rings were exposed to endothelin-1 (0.1 $\mu\text{mol/L}$) or acetylcholine (10 $\mu\text{mol/L}$) while still in the 0 mmol/L or 2 mmol/L Ca^{2+} solution. This was followed by the addition of the L-type Ca^{2+} channel antagonist, nifedipine (10 $\mu\text{mol/L}$). The contractions to ET-1 and ACH are expressed as a percentage of the initial 60K response.

The effect of the α and β estrogen receptor modifiers, ICI 182,780 and tamoxifen, were also evaluated on the 60K-induced contraction. The 60K-induced contraction was evaluated before and after incubation with EtOH, E2, 2-HOE, and 2-MeOH (30 $\mu\text{mol/L}$). This was done in the presence and absence of a 60 minute incubation period with ICI 182,780 and tamoxifen (10 $\mu\text{mol/L}$). The isometric tension was amplified by a Transbridge 4M amplifier (World Precision Instruments, Florida, USA) and the data recorded by the Windaq data acquisition system (Dataq Instruments, Ohio, USA).

Chemicals

Acetylcholine HCl, nifedipine, cycloheximide, tamoxifen, 17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), 2-methoxyestradiol (2-MeOH) were purchased from Sigma Aldrich (St. Louis, MO, USA). ICI 182,780 and endothelin-1 were obtained from Tocris Bioscience (Ellisville, MO, USA) and American Peptide Company (Sunnyvale, CA, USA), respectively. The solvent for the estradiols, nifedipine, and ICI 182,780 was ethanol, and stock solutions were made 1000-fold greater than the final bath concentration; thus, the EtOH concentration in the bath was 0.1% (except when generating the concentration-response relationship to the estradiols and EtOH to relax the pre-constricted arterial rings).

Statistical Analysis

Data are expressed as the mean $\pm$ S.E.M. for the number (n) of the animals within each group. The EC_{50} values ($-\log$ of the effective concentration to generate a 50% response) were calculated and analyzed using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). The relaxation kinetics were determined on each arterial ring by conducting a natural log transformation of the data ($r^2 > 0.98$) using Microsoft Excel (Microsoft Corporation, Redmond, WA); this generated an equation that was used to determine the percent relaxation for each arterial segment. Statistical analyses of the data were performed using a one-way or two-way ANOVA followed by Bonferroni's post-hoc analysis (SigmaStat 3.5; SyStat Software, Point Richmond, CA), depending on the number of factors of interest. Significance was defined as $p < 0.05$.

Results

A concentration-response relationship (0.3 $\mu\text{mol/L}$ to 100 $\mu\text{mol/L}$) to 17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH) was generated to determine

the approximate pD_2 value ($-\log EC_{50}$) for each estradiol that would be used in subsequent experiments. As shown in figure 1, all the estradiols similarly relaxed the 60 mmol/L (60K)-induced contraction from 3 $\mu\text{mol/L}$ to 100 $\mu\text{mol/L}$ ($n=6$). The pD_2 values for E2, 2-HOE, and 2-MeOH were 4.91 ± 0.04 , 4.72 ± 0.04 , and 4.89 ± 0.15 , respectively. Likewise, E2, 2-HOE, and 2-MeOH almost achieved complete relaxation to baseline ($87.89 \pm 8.21\%$, $87.15 \pm 6.25\%$, and $87.66 \pm 6.65\%$, respectively).

The approximate relaxation pD_2 values (10 $\mu\text{mol/L}$) for E2, 2-HOE, and 2-MeOH also attenuated the contraction to a depolarizing 60K solution ($n=5$). As shown in figure 2A, the rings were exposed to 60K before and after treatment with E2, 2-HOE, and 2-MeOH. Then after a washout period with a physiological saline solution (PSS), 60K was reapplied to the rings. The percent change from the first (60K1) to the second (60K2) 60K-induced contraction for the ethanol (EtOH) solvent was $119.77 \pm 16.28\%$. In contrast, the 60K2/60K1 ratio for E2, 2-HOE, and 2-MeOH was $52.70 \pm 10.20\%$, $67.76 \pm 6.04\%$, and $64.13 \pm 13.92\%$, respectively (figure 2B). Cycloheximide (10 $\mu\text{mol/L}$) had no effect on 60K-induced contraction in the presence of EtOH, or the E2, 2-HOE, and 2-MeOH attenuation of the 60K-mediated contraction. After the washout period, the 60K-induced contraction (60K3) was similar to the initial (60K1) 60K contraction. The 60K3/60K1 ratio for EtOH, E2, 2-HOE, and 2-MeOH was $111.06 \pm 6.50\%$, $81.32 \pm 5.73\%$, $97.14 \pm 12.08\%$, and $93.09 \pm 11.22\%$, respectively. The attenuation of the 60K-induced contraction by the estradiols was further investigated using the known estrogen receptor antagonists, ICI 182,780 ($n=5$) and tamoxifen ($n=2$). Teoh *et al.* (1999; 2000b) has previously used these antagonist concentrations to effectively confirm if E2 operates through an estrogen receptor dependent mechanism in porcine coronary arteries. As shown in figure 3, ICI 182,780 (10 $\mu\text{mol/L}$) had no effect on the estradiols' antagonism of the contraction by 60K. Tamoxifen (10 $\mu\text{mol/L}$) also had no effect (data not shown).

E2, 2-HOE, and 2-MeOH (1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$) were also evaluated against the potent, vasoconstrictive peptide, endothelin-1 (ET-1, 0.1 $\mu\text{mol/L}$). As shown in figure 4A, incubation in E2, 2-HOE, and 2-MeOH (10 $\mu\text{mol/L}$, $n=7$) similarly attenuated the ET-1-mediated constriction by $\sim 36\%$ compared to the EtOH solvent. While the addition of nifedipine (10 $\mu\text{mol/L}$) relaxed the ET-1-induced contraction to pre-constriction levels in the EtOH group, nifedipine had no further effect on the estradiol-mediated inhibition (data not shown). No attenuation of the ET-1 contraction was present using 1 $\mu\text{mol/L}$ of E2 and its metabolites (data not shown; $n=7$). As demonstrated in figure 4A, removal of extracellular Ca^{2+} (0 mmol/L Ca^{2+}) significantly lowered the ET-1-induced contraction in the presence of EtOH and the estradiols (10 $\mu\text{mol/L}$); however, the estradiols did not effect the contraction to ET-1 in the absence of extracellular Ca^{2+} .

In contrast to ET-1, incubation in E2, 2-HOE, and 2-MeOH (1 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$) did not attenuate the contraction induced by the coronary vasoconstrictive agonist, acetylcholine (10 $\mu\text{mol/L}$, $n=4$). As similarly demonstrated by ET-1, the absence of extracellular Ca^{2+} significantly reduced the acetylcholine-induced contraction (figure 4B); this contraction was not effected by the estradiols.

Discussion

Prior studies have suggested that a short-term exposure (< 60 minutes) to E2 elicits a nongenomic relaxation response in the vasculature. These nongenomic responses include the generation of NOS (Mendelsohn, 2002), activation of cAMP (Teoh and Man, 2000), and the inhibition of Ca^{2+} influx, thus inducing vascular relaxation (Crews and Khalil, 1999; Kitazawa *et al.*, 1997; Salom *et al.*, 2002) and attenuating constrictive agonists (Kitazawa *et al.*, 1997; Teoh *et al.*, 2000a). However, few studies have directly compared the nongenomic

response of E2 with its most efficacious metabolites of the vascular system, 2-HOE and 2-MeOH. The purpose of our study was to determine the short-term response of E2 with 2-HOE and 2-MeOH on coronary vasoconstriction. Our results indicated that E2, 2-HOE, and 2-MeOH similarly inhibited a depolarizing KCl-induced contraction, and also the contraction mediated by the vasospastic peptide, endothelin-1. The estradiols failed to inhibit the vasoconstriction induced by another vasospastic agent, acetylcholine. Our results indicate that the estradiols inhibit the voltage-gated entry of extracellular Ca^{2+} , therefore, the difference in the agonist-induced attenuation by the estradiols appears to be due to the Ca^{2+} influx pathways used to elicit a contraction.

Inhibition of Ca^{2+} influx by the estradiols appears to be nongenomic, estrogen receptor independent, and occur at supraphysiological concentrations. Because of the high E2, 2-HOE, and 2-MeOH concentrations used to attenuate the contractile activity of the coronary arteries, we predict that the estradiols primarily target the arterial smooth muscle in lieu of the endothelium. Although the aim of this study was not to determine if E2, 2-HOE, and 2-MeOH mediate their contractile inhibition through their action on smooth muscle versus the endothelium, our lab has previously found that the mechanical removal of the endothelium does not change the relaxation kinetics of E2, 2-HOE, and 2-MeOH in this study (unpublished observations). A reoccurring concern with many of the studies that have evaluated the short-term (<60 minutes) effects of E2 on vascular tone is that E2 elicits its effects at superphysiological concentrations. However, our lab has found that a longer exposure time (> 24 hrs) to E2 will increase its efficacy. Using coronary arteries we have found that 1 nmol/L E2 attenuates the arterial constriction in response to a high KCl solution due to the downregulation of voltage-gated Ca^{2+} channels (Hill and Rusch, 2008). Future studies will be aimed to investigate whether physiological concentrations of the E2 metabolites can elicit a similar genomic effect as E2 based upon a longer exposure time.

We confirmed that the short exposure time used in this study represented nongenomic E2, 2-HOE, and 2-MeOH effects by demonstrating that washing out the arterial rings with a physiological saline solution could reverse the estradiols' inhibition of the KCl-induced contraction. Also, the presence of the protein synthesis inhibitor, cycloheximide, did not effect the inhibition of the KCl contraction. In further support of this nongenomic effect of E2, 2-HOE, and 2-MeOH, we found that the relaxation response and inhibition of the KCl-induced contraction was not effected by the E2 receptor antagonists, ICI 182,780 and tamoxifen. In agreement, Nadal et al. (2000) and Hayes et al. (2002) previously suggested that the acute effects of estradiols occur independent of the classical α and β estrogen receptors.

The estradiols' inhibition of the depolarizing KCl-induced contraction suggests that that E2, 2-HOE, and 2-MeOH interfere with voltage-gated Ca^{2+} influx. This corroborates a study by Han et al. (1995) who demonstrated E2 inhibition of Ca^{2+} influx in female porcine coronary arteries. Likewise, our data indicate the estradiols also inhibited the extracellular Ca^{2+} -dependent vasoconstriction mediated by the coronary vasospastic peptide, endothelin-1 (ET-1). ET-1 is known to mediate force generation by a sustained influx of extracellular Ca^{2+} into vascular smooth muscle cells primarily by voltage-dependent mechanisms (i.e. L-type Ca^{2+} channels) (Bowles et al., 1995; Hill et al., 2000). In our study we suspect that the estradiols do primarily inhibit the voltage-dependent mechanisms because (1) the attenuation of the KCl and ET-1-induced contractions (in 2 mmol/L extracellular Ca^{2+}) had a similar magnitude, and (2) in the presence of extracellular Ca^{2+} the addition of the L-type Ca^{2+} channel antagonist, nifedipine, had no additional effect on the estradiols'-induced attenuation of the ET-1 contraction.

In contrast to the KCl and ET-1 contractions, E2, 2-HOE, and 2-MeOH had little effect on the acetylcholine-mediated contraction. Because our data suggest that the estradiols inhibit voltage-dependent Ca^{2+} channels we suspect that acetylcholine must mediate its contractile response via a voltage-independent Ca^{2+} influx mechanism. Our results are corroborated by a study by Tanz and Nayler (1991) who used pig coronary arteries to demonstrate that the acetylcholine-induced contraction is mediated by a Ca^{2+} influx mechanism that is not sensitive to the L-type Ca^{2+} channel antagonists, nifedipine and diltiazem. We did not attempt to mechanistically characterize the acetylcholine-induced contraction in these arteries; however, Weirich et al. (2005) has previously identified that acetylcholine may induce Ca^{2+} influx via Ca^{2+} -permeable nonselective cation channels and store-operated Ca^{2+} channels.

Overall, our studies suggest that the metabolites of E2 (2-HOE and 2-MeOH) are equally effective as its parent compound, E2, to inhibit voltage-gated Ca^{2+} influx (i.e. L-type Ca^{2+} channels) and attenuate agonist-induced extracellular Ca^{2+} -dependent contractions. 2-MeOH has already been evaluated in phase I and II clinical trials with mixed results to treat various myelomas (James et al., 2007; Rajkumar et al., 2007). Rajkumar et al. (2007) has suggested that part of the problem with the oral administration of 2-MeOH (> 1,000 mg/day) is its low bioavailability in the plasma (increase of 1-2 ng/mL). However, Yan et al. (2006) demonstrated that the localized administration of 2-MeOH can reduce cerebral vasospasms and inhibit vascular smooth muscle cell proliferation. The clinical use of the E2 metabolites (particularly 2-MeOH) needs to be further analyzed because 2-MeOH may have a protective role against the development of coronary artery disease.

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Figure 1.

17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH) demonstrate a similar ability to induce coronary relaxation. Rings were initially pre-constricted with a 60 mmol/L KCl solution followed by the generation of a cumulative concentration response relationship to each respective estradiol (0.3 μ mol/L to 100 μ mol/L). Each estradiol-induced relaxation is expressed as percentage (%) of the maximum contractile response to 60 mmol/L KCl (n=6 pigs for each group). ^ indicates 2-MeOH $p < 0.05$ from the EtOH solvent. Also, * suggests $p < 0.05$ for all the groups from the EtOH control using a one-way ANOVA.

Figure 2. 17β -estradiol (E2), 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH) exhibit a similar nongenomic inhibitory effect on a depolarizing KCl-induced contraction. (A) Typical tracing illustrating the experimental protocol used to determine the nongenomic inhibition of the 60 mmol/L KCl (60K)-induced contraction by E2, 2-HOE, and 2-MeOH. Separate arterial rings incubated with either 10 μ mol/L of E2, 2-HOE, or 2-MeOH from the same artery ($n=5$) were incubated in the presence or absence of cycloheximide (10 μ mol/L). The horizontal lines indicate the exposure time to each drug. 60K1, 60K2, and 60K3 represent the first, second, and third application of the 60K solution. The composite data are shown in panel (B), which demonstrates that the KCl-induced contraction is attenuated by

E2, 2-HOE, and 2-MeOH. The data shown are in the absence of cycloheximide and are expressed as a percentage of the first 60K-mediated contraction (60K2/60K1). * indicates $p < 0.05$ (one-way ANOVA) from the EtOH solvent.

Figure 3.

Inhibition of the KCl-induced contraction by 17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH) is independent of estrogen receptors. The 60 mmol/L KCl (60K)-induced contraction was evaluated in the absence (60K1) and presence (60K2) of the selective estrogen receptor modifier, ICI 182,780, * indicates $p < 0.05$ from the EtOH solvent ($n = 5$ pigs for each group) using a two-way ANOVA.

Figure 4.

17 β -estradiol (E2), 2-hydroxyestradiol (2-HOE), and 2-methoxyestradiol (2-MeOH) attenuate the endothelin-1-induced (ET-1) constriction, but not the acetylcholine-mediated constriction. Both agonist-induced contractions were evaluated in the presence (2 mmol/L Ca²⁺) and absence (0 mmol/L Ca²⁺) of extracellular Ca²⁺. (A) E2, 2-HOE, and 2-MeOH (10 μ mol/L) demonstrate a similar ability to inhibit the ET-1-induced (0.1 μ mol/L) contraction in 2 mmol/L Ca²⁺. * indicates $p < 0.05$ from the EtOH solvent ($n = 7$ pigs for each group). Also, $p < 0.05$ between EtOH groups (2 mmol/L Ca²⁺ versus 0 mmol/L Ca²⁺). (B) E2, 2-HOE, and 2-MeOH (10 μ mol/L) have no effect on the acetylcholine-induced (10 μ mol/L) contraction ($n = 4$ pigs for each group). All data are represented as the % of an initial 60 mmol/L KCl-induced contraction for each arterial ring. Statistical analysis was performed using a two-way ANOVA.

Table 1

Percent relaxation kinetics (in minutes) for 30 $\mu\text{mol/L}$ 17 β -estradiol, 2-hydroxyestradiol, and 2-methoxyestradiol.

Treatment	n	25%	50%	75%	100%
17 β -Estradiol	7	12.86 $\pm$ 1.20	24.60 $\pm$ 1.37	38.59 $\pm$ 1.50	56.27 $\pm$ 1.32
2-Hydroxyestradiol	7	12.66 $\pm$ 1.75	24.82 $\pm$ 1.65	38.44 $\pm$ 1.46	53.99 $\pm$ 1.16
2-Methoxyestradiol	7	10.49 $\pm$ 1.65	23.63 $\pm$ 1.17	37.57 $\pm$ 1.02	53.59 $\pm$ 1.14