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DOSE-DEPENDENT THERAPEUTIC EFFECTS OF 2-METHOXYESTRADIOL ON MONOCROTALINE-INDUCED PULMONARY HYPERTENSION AND VASCULAR REMODELLING

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A b s t r a c t: 2-Methoxyestradiol (2ME) is a major non-oestrogenic metabolite of oestradiol. Our previous studies, performed in several models of cardiac and/or vascular injury, suggest that 2ME strongly inhibits both pressure-dependent and pressure-independent cardiac and vascular remodelling. Furthermore, recently we have shown that in male rats 2ME attenuates the development and retards the progression of monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH); and in female rats 2ME eliminates the exacerbation of PAH and increased mortality due to ovariectomy. In the present study we compared the therapeutic effects of three different doses of 2ME (3, 10 and 30 μg/kg/hour; 2ME-3, 2ME-10 and 2ME-30, respectively) in male rats with MCT-induced PAH. The animals were also monitored for plasma 2ME levels and potential oestrogenic effects. Treatments were initiated 12 days after administration of MCT (60 mg/kg, i.p.). Twenty-eight days post MCT, right ventricular peak systolic pressure (RVPSP) was measured and morphometric analysis was conducted.

All three doses of 2ME produced beneficial therapeutic effects in pulmonary hypertensive animals, i.e. reduced pulmonary artery pressure and right ventricular hypertrophy, attenuated pulmonary vascular remodelling and inflammatory response, and had favourable effects on survival. Notably, none of the three doses had any effect on plasma testosterone levels or on seminal vesicle or testicle weight. Dose-dependent increases in 2ME plasma levels were observed only with 2ME-3 and 2ME-10; 2ME-30 produced 2ME plasma levels similar to those seen with 2ME10. Nonetheless, 2ME-30 was significantly more efficacious than 2ME-3 or 2ME-10 and eliminated the high mortality (34%) induced by MCT. In summary, the present study indicates that 2ME, used in doses that produce plasma levels similar to those seen in the last trimester of

pregnancy (1000–3000pg/ml), is effective and safe (i.e. has no oestrogenic effects) in experimental PAH. These data also suggest that 2ME disposition, rather than plasma concentration, determines the therapeutic effects of 2ME in PAH.

Key words: Pulmonary hypertension, 2-methoxyestradiol, vascular remodelling.

Introduction

2-Methoxyestradiol (2ME), a major metabolite of 17β-oestradiol (oestradiol), is a product of the sequential conversion of oestradiol to 2-hydroxyoestradiol (2HE) and 2ME by the enzymes cytochrome P450s and cathechol-O-methyltransferase, respectively [1]. Notably, although 2HE has mild and 2ME little or no oestrogenic activity, these metabolites have cardiovascular protective effects mediated by oestrogen receptor-independent mechanisms [2, 3].

2ME attenuates blood pressure, cardiac hypertrophy, and/or vascular and renal injury in spontaneously hypertensive rats [4], obese and lean ZSF1 rats [5], apolipoprotein-deficient mice [6], rats with balloon injury [7], and rats with chronic NOS inhibition – or angiotensin II-induced hypertension and cardiovascular and renal disease [8, 9]. Importantly, our initial studies also suggest that 2ME exhibits direct, i.e. pressure-independent, inhibition of cardiac and vascular remodelling: in a constricted-aorta rat model, 2ME inhibits not only cardiac hypertrophy and vascular remodelling (media hypertrophy) of the aorta above the constriction, but also reduces media hypertrophy of the abdominal aorta below the constriction [10]. Furthermore, 2ME and its metabolic precursor 2HE inhibit pressure-independent right ventricular hypertrophy and reduce cardiac fibrosis induced by isoproterenol [11].

Previously, we have shown that, in male rats with monocrotaline (MCT)-induced pulmonary arterial hypertension (PAH), 2ME attenuates the development and retards the progression of disease [12], and that in female rats 2ME eliminates the exacerbation of PAH and increased mortality due to ovariectomy [13]. 2ME also attenuates bleomycin-induced PAH and pulmonary fibrosis in ovariectomized rats [14], as well as PAH induced by exposure to chronic hypoxia or pneumotoxin ANTU [15, 16]. Finally, we have recently demonstrated that in intact and ovariectomized female rats with severe "human-like" occlusive PAH, 2ME, but not estradiol, has preventive and therapeutic effects [17].

In all of our previous studies, the beneficial effects of 2ME were produced by a dose of 10 μg/kg/h of 2ME delivered by osmotic minipump over 10–21 days. We have documented that this dose of 2ME produces plasma concentrations of 1000–3000 pg/ml, i.e., plasma levels seen in the last trimester of pregnancy [1, 8, 18], and therefore should be considered a "high physiologi-

cal" dose. However, it is unknown whether smaller doses of 2ME are still effective in PAH, whether higher doses provide additional benefits, or whether a correlation exists between plasma concentrations and the therapeutic efficacy of 2ME in PAH. 2ME has little or no affinity for oestrogen receptors, but does bind to tubulin [3], and significant intracellular disposition and micromolar intracellular concentrations of 2ME have been reported [19]. It has also been suggested that at high concentration demethylation may occur, converting 2ME back to the mild oestrogenic metabolite 2HE (xx). The purpose of the present study was to compare the efficacy of three different doses of 2ME, to examine whether a correlation exists between 2ME plasma concentration and therapeutic response, and to determine whether increases in 2ME dose eventually produce oestrogenic effects in male rats with monocrotaline-induced PAH.

Material and methods

A total of 65 male, 13-week-old Sprague Dawley rats (323 ± 6g) were used to compare the efficacy of three different doses of 2-methoxyestradiol on the progression of pulmonary hypertension induced by pneumotoxin monocrotaline. Animals were randomly assigned to one of the experimental groups. The control group (n = 10) received intraperitoneally 10 ml/kg of 1 ml 1.0 N HCl neutralized with 1.0 N NaOH and diluted with distilled water. The remaining groups received monocrotaline (MCT, 60 mg/kg, dissolved in 1.0 N HCl at a concentration of 100 mg/ml, neutralized with 1.0 N NaOH and diluted with distilled water to 6 mg/ml). Some of the MCT treated animals were examined for right ventricular function and pulmonary vascular remodelling 12 days (n = 10) and 28 days (n = 15) after MCT administration (MCT-12d and MCT groups, respectively). Twelve days after administration of MCT, treatments with 2ME (3, 10 and 30 μg/kg/h via osmotic minipump, 2ME-3, 2ME-10 and 2ME-30 groups, respectively; n = 10 per group) were initiated. All animals not treated with 2ME were implanted with osmotic minipumps (model 2ML4, Alzet, Palo Alto, CA) delivering vehicle (PEG-400, 2.5 μl/h). Monocrotaline and PEG-400 were purchased from Sigma-Aldrich (St. Louis, MO) and 2ME was purchased from Steraloids (Newport, RI).

Twenty-eight days after administration of MCT the surviving animals were anaesthetized and instrumented for measurement of right ventricular peak systolic pressure (RVPSP), as described earlier [12, 13]. Briefly, a PE-240 polyethylene catheter was inserted into the trachea to facilitate breathing. A PE-50 catheter was inserted into the left carotid artery and connected to a digital blood pressure analyzer (BPA-400, Micro-Med. Inc., Louisville, KY) for continuous measurement of systolic, diastolic and mean arterial blood pressure and heart rate. The PE-240 catheter was connected to a rodent ventilator (Model 683,

Harvard Apparatus, South Natick, MA), the thorax was opened, and the heart was exposed. The right ventricle was punctured with a 23-gauge needle attached by a fluid-filled catheter to a Heart Performance Analyzer (HPA-200 τ, Micro-Med. Inc., Louisville, KY). After a 30-minute stabilization period, RVPSP was recorded over 15 minutes. Animals were euthanized by anaesthetic overdose and heart and lungs were taken for morphometric analyses. Lungs were perfused via the trachea with 10% buffered formalin under constant low pressure (25 mmHg), and immersed in 10% buffered formalin for 72 h before being embedded in paraffin.

To study media hypertrophy of the pulmonary arteries, arterial wall smooth muscle cells were stained using a mouse monoclonal anti-smooth muscle-alpha actin antibody at a dilution of 1/100 (Lab Vision, Fremont, CA). To assess pulmonary vascular remodelling, measurements of media thickness and media and adventitia surface were conducted using an Image Analyzing System (Diagnostic Instruments, Inc., Sterling Heights, MI) that included a SPOT RT Camera mounted on a NIKON Eclipse 50 light microscope and a specialized software package (SPOT Software, Version 4.1). Briefly, after calibrating each objective, measurements were made at 20 × magnification on ten cross-sectioned pulmonary artery branches 50–200 μm in diameter. Measurements of thickening of the vascular wall and media of pulmonary arteries were taken in the peripheral lung fields at approximately equal distances from the pleural lining, and only vessels with an approximately circular cross-sectional profile were studied. For each blood vessel, two rectangular diameters and their four respective media were measured. The media % index was calculated as (2 × media) / (diameter × 100) and is presented as the average of four media measurements per vessel. Since MCT induced significant widening of adventitia, a corrected media index was calculated using the diameter that includes only media+lumen measurement. For each blood vessel, two rectangular diameters and their four respective media were measured, and averages of four individual values of media thickness and media % index were calculated. A polyclonal anti-ED1 antibody (Serotec, Raleigh, NC) was used to assess cell number of interstitial monocytes/macrophages. This antibody specifically stains the cytoplasm of alveolar and interstitial macrophages. Nonspecific staining was assessed by replacing the primary antibody with affinity-purified, nonimmune, rabbit IgG (R&D Systems). Twenty high power fields per section were randomly selected and assessed for ED1 positively stained macrophages.

Blood samples were analysed for plasma testosterone levels using a testosterone immunoassay kit (R&D Systems by Assay Designs, Inc.). An ultrasensitive LC-MS/MS method developed by the Analytical Development Corporation (Colorado Springs, CO) was used to measure plasma 2ME levels (285.2/189.1 m/z). The detection limit was 100 pg/ml plasma and 2-fluoroestradiol (273.1/177.3 m/z) was used as an internal standard.

Statistical analysis

All results are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Number Crunchers Statistical System software and significance was defined as $p < 0.05$. Comparisons among multiple groups were performed by a one-factor analysis of variance (1F-ANOVA). If this analysis indicated a significant difference among the means, multiple comparisons were made with a post-hoc Fisher's LSD test. A Student's T test was used to compare specific pairs of groups determined a priori to be of particular importance.

Results

Single injections of monocrotaline induced pulmonary hypertension within twelve days. On Day 12, MCT treated animals had increased RVPSP (40.7 ± 1.5 vs. 27.6 ± 0.8 mmHg, MCT-12d vs. Cont; $p < 0.001$), established right ventricular hypertrophy (i.e. increased RV/LV+S ratio; Figure 1), significant vascular remodelling of small size pulmonary arteries, and presence of significant numbers of inflammatory cells in the lungs (Table 1, Figures 2, 5B, and 6B). The disease continued to progress and on Day 28 a significantly higher RVPSP, more pronounced RV hypertrophy, pulmonary vascular remodelling and presence of inflammatory cells were detected in the MCT group as compared to the MCT-12d group. By Day 28, 33% of animals in the MCT group had died prematurely (Figure 3, bottom panel).

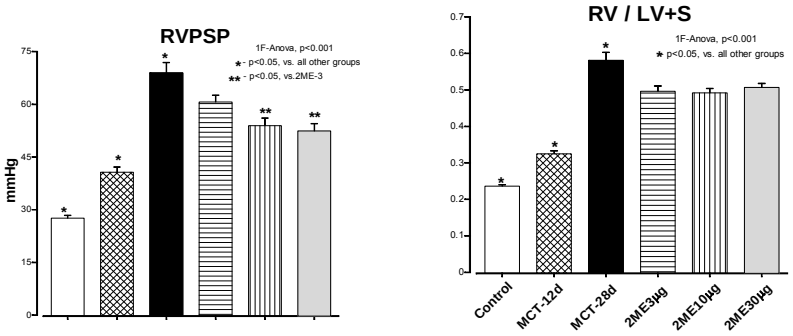

Figure 1 – Right ventricular (RV) peak systolic pressure (RVPSP) and RV to left ventricle + septum (RV/LV+S) ratio in monocrotaline pulmonary hypertensive rats treated with 2-methoxyestradiol (2ME)

Слика 1 – Десно вентрикуларен максимален систолен притисок (RVPSP) и сооднос на десна комора/лева комора + септум (RV/LV+S) кај стаорци третирани со 2-метоксиестрадиол (2ME)

Table 1 – Табела 1

Effects of 2-methoxyestradiol on vascular remodelling in pulmonary arteries of male rats with monocrotaline (MCT)-induced pulmonary hypertension (M ± SE)
Ефекти на 2-метоксиестрадиол врз монокроталински индуцирани морфометриски промени во белодробните артериоли кај машки стаорци

Treatment	Vessels Range (μ)	Diameter (μ)	Media (μ)	Media Surface (μ2)	Adventitia Surface (μ2)	Wall Surface (μ2)	Wall to Lumen Ratio	Media to Lumen Ratio
Cont	67–228	115.73	11.08	3872	779**	4651	91.1	76.0
		4.64	0.49	280	58	320	5.4	5.0
MCT-12d	62–230	133.85	21.33	8154	2424	10578	262.0	200.6
		5.85	1.36	802	205	965	28.4	22.8
MCT	56–259	133.09	24.66	8742	2830	11256	284.3	211.1
		1.68	0.25	984	265	48	9.5	7.3
2ME-3	79–223	134.17	18.85[a]	6308*	2741	9049[a]	198.7*	140.4*
		4.85	0.78	486	218	645	15.3	11.7
2ME-10	63–225	115.28	17.78*	5247* [b]	2145*	7392*	219.6 [a]	145.6*
		4.77	0.94	550	266	720	21.0	9.2
2ME-30	85–192	122.91	16.08*	5174* [b]	1504**	6678 [b,c]	133.6 [b,c,d]	104.3 [c,d]
		4.29	0.69	368	121	452	7.8	7.0
1F ANOVA	p <	0.009	0.001	0.001	0.001	0.001	0.001	0.001

Post hoc Fisher LSD test, $p < 0.05$: * – vs Cont, MCT-12d & MCT; ** – vs. all other groups; a – vs CONT & MCT; b – vs 2ME-3; c – vs MCT-12d & MCT; d – vs 2ME-10

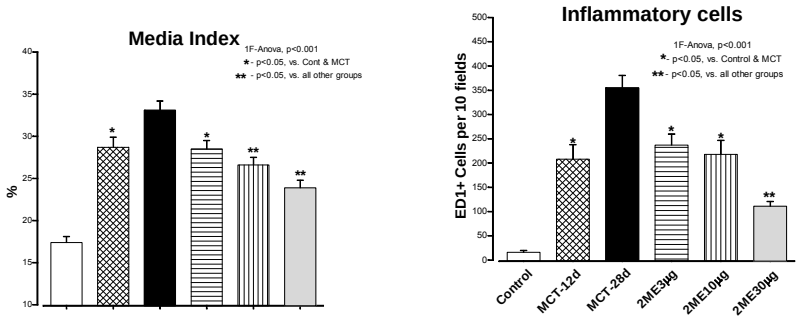

Figure 2 – The effects of 2-methoxyestardiol (2ME) on small size pulmonary arteries media remodelling (media index) in and inflammation (ED1+ positive cells) in lungs from monocrotaline (MCT) pulmonary hypertensive rats
Слика 2 – Дејство на 2-метоксиестрадиол врз хипертрофија на медијата (медија индекс) во пулмонарни артерии со мала големина и воспалителен одговор (ЕД1+ позитивни клетки) во бели дробови од стаорци со МКТ белодробна хипертензија

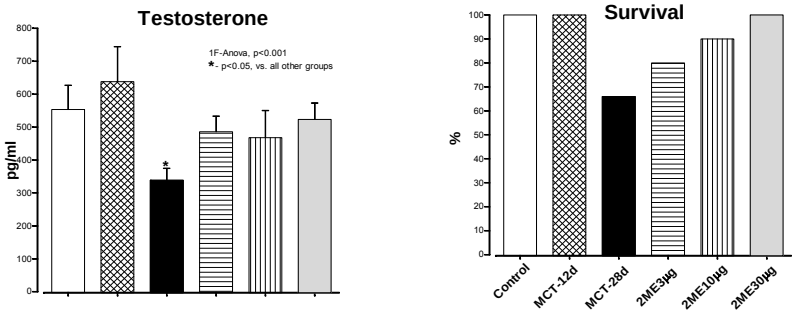

Figure 3 – Plasma testosterone levels and survival in monocrotaline (MCT) pulmonary hypertensive rats treated with 2-methoxyestradiol (2ME)
Слика 3 – Концентрации на тестостерон и преживување на стаорци со монокроталин – индуцирана белодробна хипертензија третирани со 2-метоксиестрадиол (2ME)

All three doses of 2ME reduced RVPSP, right ventricular hypertrophy (Figure 1), and vascular remodelling of small size pulmonary arteries (Figure 2, upper panel and Figure 5), but 2ME-10 and 2ME-30 were more efficacious than 2ME-3 in the reduction of pulmonary hypertension and vascular remodelling. Different 2ME treatments had remarkable effects on the influx of ED1+ positive

Table 2 – Табела 2

Effects of 2-methoxyestradiol on lungs weight and cardiac remodelling and testicle and seminal vesicle weight in male rats with monocrotaline (MCT)-induced pulmonary hypertension (M ± SE)
Ефекти на 2-метоксиестрадиол на тежина на бели дробови и срцева хипертрофија, и тежина на тестиси
и семени везикли кај машки стаорци со монокроталин-индуцирана белодробна хипертензија

	B.W. G	Lungs g	Heart g	RV	LV+S	Sem.Ves. G	Testicles g	L/B.W. mg/g	H/B.W. mg/g	RV/B.W. mg/g	LV+S/B.W. mg/g	SV/B.W. mg/g	T./B.W. mg/g
Cont	444**	1.463**	1.149	0.201	0.851	1.420**	3.088	3.302**	2.585	0.452**	1.915	3.208	6.991**
	12	0.045	0.045	0.008	0.032	0.046	0.083	0.083	0.058	0.013	0041	0.104	0.232
MCT-12d	327	1.925	0.925**	0.211	0.649**	1.083	3.047	5.913	2.817	0.644**	1.977	3.310	9.345
	7	0.049	0.037	0.011	0.027	0.088	0.150	0.203	0.059	0.025	0.049	0.271	0.483
MCT	345	2.613**	1.365	0.441	0.763[e]	1.041	2.931	7.423**	3.947	1.292**	2.221[e]	2.999	8.520
	6	0.105	0.047	0.017	0.022	0.066	0.081	0.327	0.132	0.060	0.077	0.151	0.205
2ME-3	363	2.165*	1.298	0.393*	0.788[e]	0.982	2.934	6.142[a]	3.686*	1.084*	2.271[e]	2.724	8.147[c]
	10	0.063	0.047	0.024	0.020	0.065	0.102	0.203	0.146	0.060	0.171	0.196	0.349
2ME-10	352	2.104[a]	1.275[c]	0.394*	0.800[e]	1.004	2.856	5.769[a]	3.536	1.120*	2.071[e]	2.864	8.126[c]
	6	0.061	0.042	0.018	0.030	0.045	0.081	0.196	0.146	0.047	0.183	0.148	0.239
2ME-30	382*[d]	1.965[a]	1.245[c]	0.385*	0.760[e]	1.196	3.143	5.178*[b]	3.26*[b]	1.005*	1.990	3.113	8.248[c]
	8	0.091	0.029	0.017	0.016	0.084	0.091	0.285	0.045	0.035	0.030	0.193	0.254
1F-ANOVA p<	0.001	0.001	0.001	0.001	0.001	0.002	0.456	0.001	0.001	0.001	0.002	.245	0.001

Post hoc Fisher LSD test, $p < 0.05$: * – vs Cont, MCT-12d & MCT; ** – vs. all other groups; a – vs CONT & MCT; b – vs 2ME-3; c – vs MCT-12d & MCT; d – vs 2ME-10; e – vs MCT-12d; f – vs CONT, MCT-12d, 2ME-30

inflammatory cells into MCT injured lungs; both 2ME-3 and 2ME-10 inhibited further inflammatory response and produced ED1+ cell numbers comparable to those seen in the MCT-12d group, and more importantly, 2ME-30 treatment initiated on Day 12 post MCT even reversed the inflammatory response. On Day 28, the 2ME-30 group had ~50% fewer inflammatory cells than on Day 12 (MCT-12d group; Figure 2 bottom panel, Figures 6B and 6F). This was associated with marked attenuation of MCT-induced increase in lung weight (Table 2).

We detected significant remodelling of small size pulmonary arteries in MCT treated animals. On Day 12 post MCT, significant media hypertrophy was present, and media thickness, media surface, and media index were increased by 92.5%, 210.6% and 65.3%, respectively (Figure 2 upper panel, Figure 6B and Table 1). Early activation of adventitia was detected, and by Day 12 adventitia surface area was increased by 311% compared to control animals, and wall surface area and wall-to-lumen and wall-to-media ratios were increased by 227.4%, 287.7% and 263.9%, respectively. Over the next 16 days, only mild further remodelling of small size pulmonary arteries took place, and by Day 28 an additional 20–30% increase in measured parameters of vascular remodelling was detected (Table 1). Treatment with 2ME-3 had only mild effect and only prevented further vascular remodelling, whereas 2ME-10 reversed media hypertrophy and had no effects on adventitia widening. Notably, 2ME-30 markedly reversed media hypertrophy and adventitial widening, and on Day 28, all measured parameters of vascular remodelling in the 2ME-30 group were significantly reduced compared to those in the MCT-12d group (Figure 2, upper panel, Figures 6B & 6F, Table 1).

The beneficial effects of 2ME treatments on disease progression were associated with reduced mortality (Figure 3, bottom panel). Over the 28 days post MCT, five out of 15 rats in MCT group died prematurely, i.e. only 66% of animals in the MCT group survived compared to 80% and 90% of rats surviving in the 2ME-3 and 2ME-10 groups, respectively. Remarkably, 2ME-30 eliminated the high mortality (34%) caused by pneumotoxin MCT, i.e. all 2ME-30 animals survived versus only 66% of MCT animals.

Plasma levels of 2ME on Day 14 and Day 28 (2 and 16 days into treatments) are presented in Figure 4. As expected, 2ME-10 produced plasma concentrations of 2000–3000pg/ml of 2ME. Furthermore, a 3.3-fold reduction in the dose resulted in proportionally lower plasma 2ME levels (700–900 pg/ml) in the 2ME-3 group but, surprisingly, a 3.3-fold increase in dose did *not* result in a proportional increase in plasma concentrations, and the 2ME-30 group had 2ME plasma levels similar to those measured in the 2ME-10 group.

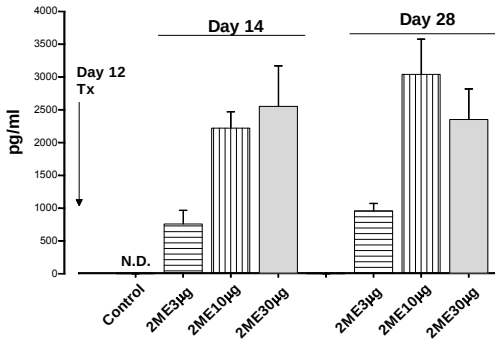

Figure 4 – Concentrations of 2-methoxyestradiol in plasma from monocrotaline pulmonary hypertensive rats, after 2 and 16 days of treatment (14 and 28 days post MCT, respectively)
Слика 4 – Концентрации на 2-метоксиестрадиол во плазма од стаорци со монокроталин – индуцирана белодробна хипертензија по 2 и 16 дена третман (14 и 28 дена по администрација на МКТ)

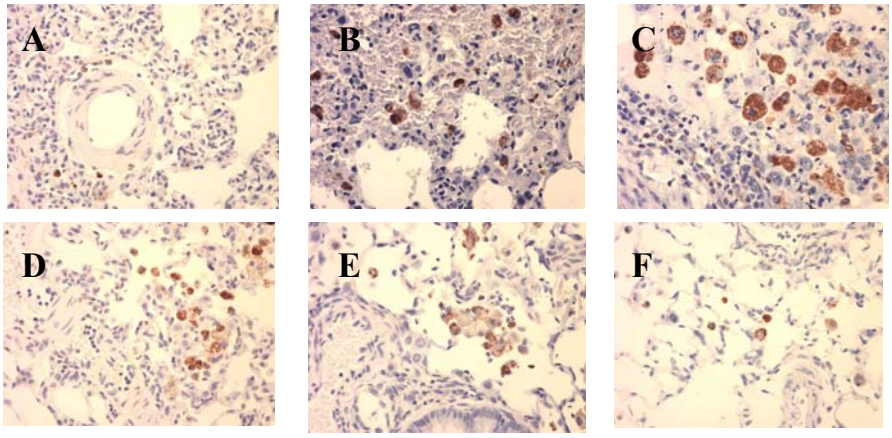

Figure 5 – Inflammatory cells (ED1 staining) in lungs from control rat (A), animals with monocrotaline-induced pulmonary hypertension 12 and 28 days post MCT (B & C, respectively)and MCT animals treated from Day12 to Day 28 with 3, 10 and 30 µg/kg/h of 2-methoxyestradiol (D, E and F, respectively)
Слика 5 – Инфламаторни клетки (ED1 боење) во бели дробови од контролен стаорец (А), животни со монокроталин (МКТ) – индуцирана белодробна хипертензија на ден 12 и 28 (Б и Ц) и кај МКТ животни третирани од ден 12 до ден 28 со 3, 10 и 30 µg/kg/час на 2-метоксиестрадиол (Д, Е и Ф, соодветно)

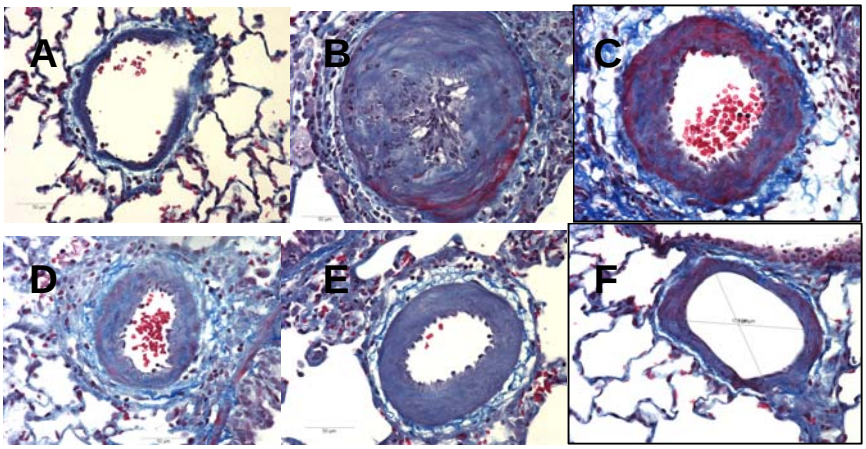

Figure 6 – Media hypertrophy in control rat (A), animals with monocrotaline-induced pulmonary hypertension 12 and 28 days post MCT (B & C, respectively) and MCT animals treated from Day12 to Day 28 with 3, 10 and 30 μg/kg/h of 2-methoxyestradiol (D, E and F, respectively)

Слика 6 – Белодробни артерии(хипертрофија на медијата) во бели дробови од контролен стаорец (А), животни со монокроталин (МКТ) – индуцирана белодробна хипертензија на ден 12 и 28 (Б и Ц) и кај МКТ животни третирани од ден 12 до ден 28 со 3, 10 и 30 μg/kg/час на 2-метоксиестрадиол (Д, Е и Ф, соодветно)

Data related to the potential oestrogenic activity of 2ME are presented in Figure 3 (upper panel) and Table 2. On Day 28, MCT treated animals had significantly reduced plasma testosterone levels, most likely due to MCT-induced inflammation. Treatment with 2ME prevented the MCT-induced reduction in plasma testosterone, and testosterone levels in the 2ME-3, 2ME-10, and 2ME-30 groups were similar to those in the control group and significantly higher than those in the MCT group. None of the three doses had any effect on seminal vesicle or testicle weight.

Discussion

2-Methoxyoestradiol was long held to be biologically inactive, partially because of the weak binding of methoxyoestrogens to oestrogen receptors [21]. Attention has since been focussed on highly interactive catecholoestrogens because of their unique structural resemblance to both oestrogens and catecholamines and their potential carcinogenic effects. The antimitogenic effects of 2ME were first described in 1989 by Spicer and Hammond, who reported that

2ME, in a dose-dependent manner, inhibits growth of porcine granulosa cells [21]. At the same time, Seeger and colleagues reported that both in oestrogen receptor-positive and oestrogen receptor-negative cells, 2ME is a more potent antimitogen than 2HE or E2, suggesting that 2ME has estrogen receptor-independent effects. Furthermore, Seeger and colleagues demonstrated that co-administration of a COMT-inhibitor abolishes the antimitogenic effects of 2HE, but not those of 2ME, and that 2ME is required for inhibition of cell growth to occur [22]. Similar antimitogenic effects were later reproduced by Nishigaki and colleagues [23], who showed that oestradiol and its downstream metabolites of the 2-hydroxylation pathway inhibited, in the order 2ME>2HE>E2, the proliferation of vascular smooth muscle cells from rabbit aorta. These authors suggested that 2ME may be useful to suppress excessive vascular remodelling and the progression of atherosclerosis [23].

During the past twenty years most attention has been focussed on the anti-angiogenic and apoptotic effects of 2ME; 2ME was shown to be an effecttive antiproliferative agent in various cancer cell lines, to inhibit tumour growth in animal models, and to be effective in early clinical trials in patients with prostate and ovarian cancer [24]. However, in the last decade, a growing body of evidence has strongly supported the notion that 2ME (the major non-oestrogenic metabolite of oestradiol) exerts many cardiovascular effects as well, namely, mediating the effects of oestradiol [25]. We were first to report that 2ME exhibits protective effects in animal models of cardiovascular and renal disease [8–10] and to demonstrate that 2ME mediates the protective effects of oestradiol (E2) in PAH [12]. We have also very recently shown that in an experimental model of occlusive "human-like" PAH in female rats, 2ME, but not E2, has both preventive and therapeutic effects [17]. Importantly, a recent study in patients with PAH suggests that the higher prevalence of PAH in females may be due altered to altered E2 metabolism and lower levels of its downstream metabolites [26]. The above discussion underscores the need for further investigation of the physiological and pharmacological properties of 2ME in PAH.

The purpose of the present study was to address three clinically relevant questions: (i) whether higher doses of 2ME provide additional benefits; (ii) whether plasma concentrations of 2ME correlate with its therapeutic effects; and (iii) whether increasing doses of 2ME eventually produce oestrogenic effects in male rats with monocrotaline-induced PAH. We used 10 μg/kg/h 2ME as a standard dose that we have previously shown to provide therapeutic effects in various models of experimental PAH, including MCT-, bleomycin-, chronic hypoxia- and alfa naftiltiourea-induced PAH in male and/or female rats, and in occlusive "human-like" PAH in female rats [12–17]. This should be considered a high physiological dose, because it produces plasma concentrations of 2ME (2000-3000pg/ml) similar to those seen in the last trimester of pregnancy [1, 8].

In the present study, we compared the therapeutic efficacy and safety of three different doses of 2ME, initiating treatments on Day 12 post MCT in animals that already had significant PAH and pulmonary vascular remodelling and inflammation. Increasing the 2ME dose to 30 μg/kg/h provided additional therapeutic benefits, the most remarkable of which being prevention of death and reversal of vascular remodelling and inflammation. The measured parameters of vascular remodelling were, on average, 20–35% lower compared with vascular remodelling at the beginning of the treatment in the MCT-12d group; there was also 50% reduction in inflammation in the 2ME-30 group as compared with the MCT-12d group. Surprisingly, these remarkable additional therapeutic benefits of increased 2ME doses were achieved in the absence of further increase in plasma 2ME concentrations. The cause of this non-linear relationship is unclear. 2-Methoxyoestradiol is extensively metabolized by the ubiquitous enzyme type-2 17β-hydroxysteroid dehydrogenase to 2-methoxyestrone (2ME1), a metabolite that lacks biologic activity but which may be converted back to 2ME by type-1 17β-hydroxysteroid dehydrogenase (17β-HSD). Recently we have demonstrated that 2ME1 attenuates MCT-induced PAH [27] and that combination therapies of 2ME with retinoic acid (type-1 17βHSD, which facilitates the conversion of 2ME1 back to 2ME) have significantly greater effect on the amelioration of MCT-induced PAH than single treatments [28]. It is also worth mentioning that very fast cellular uptake and high (micromolar) intracellular plasma concentrations of 2ME have been reported [19], suggesting that 2ME disposition may play critical roles in the biological and pharmacological effects of 2ME.

Unsurprisingly, pulmonary hypertensive animals (MCT group) had reduced testosterone levels, as chronic inflammation is associated with reduced testicular interstitial fluid and plasma testosterone levels [29]. What *is* surprising is that 2ME treatment prevented reduction in testosterone levels and that the 2ME groups had plasma testosterone levels similar to those in the control group. The similar weight of seminal vesicles and testicles among experimental groups provides further evidence for a lack of any oestrogenic activity by any of 2ME treatments. Notably, in a phase I clinical trial in healthy volunteers, we recently detected no oestrogenic effects from a subcutaneously injected long-acting formulation of 2ME in doses up to 10 mg/kg [30].

In summary, the present study indicates that 2ME is safe and effective in experimental PAH and that cellular disposition rather than plasma concentration plays a critical role in the biological and pharmacological effects of 2ME in PAH.

REFERENCES

1. Ball P., Emons G., Kayser H., Teichmann J. (1983): Metabolic clearance rates of catechol estrogens in rats. *Endocrinology;* 113: 1781–1783.

2. Liu D., Bachmann KA. (1998): An investigation of the relationship between estrogen, estrogen metabolites and blood cholesterol levels in ovariectomized rats. *J Pharmacol Exp Ther;* 286: 561–568.

3. Lakham NJ., Sarkar MA., Venitz J., Figg WD. (2003): 2-Methoxyestardiol, a promising anticancer drug. *Pharmacotherapy;* 23: 165–172.

4. Bonacasa B., Sanchez M L., Rodriguez F., Lopez B, Quesada T., Fenoy F J., Hernández I. (2008): 2-Methoxyestradiol attenuates hypertension and coronary vascular remodeling in spontaneously hypertensive rats. *Maturitas;* 61: 310–316.

5. Zhang X., Jia Y., Jackson EK., Tofovic SP. (2007): 2-Methoxyestradiol and 2-ethoxyestradiol retard the progression of renal disease in aged, obese, diabetic ZSF1 rats. *J Cardiovasc Pharmacol;* 49: 56–63.

6. Borghardt J., Bergstrom G., Krettek A., Sjoberg S., Boren J., Tivesten A. (2007): The endogeous estradiol metabolite 2-methoxyestradiol reduces atherosclerotic lesion formation in female ApoE-deficient mice. *Endocrinology;* 148: 4128–4132.

7. Barchiesi F., Jackson EK, Fingerke J., Gillespie DG., Odermatt B., Dubey RK. (2006): 2-methoxyestradiol, an estradiol metabolite, inhibits neointima formation and smooth muscle cell growth via double blockade of the cell cycle. *Cir Res;* 99: 266–274.

8. Tofovic SP., Salah EM., Dubey R., Melhem MF., Jackson EK. (2005): Estradiol metabolites attenuate renal and cardiovascular injury induced by chronic nitric oxide synthase inhibition. *J Cardiovasc Pharmacol;* 46: 25–35, 2005.

9. Tofovic SP., Mady H., Jackson EK. (2003): Estradiol metabolites attenuate renal and cardiovascular injury induced by chronic angiotensin II administration. *Hypertension;* 42: 416.

10. Tofovic SP., Jackson EK., Mady H. (2003): 2-Methoxyestradiol attenuates hypertension and cardiac and vascular remodelling in constricted aorta rat model. *J Am Soc Nephrol;* 14, 620A.

11. Tofovic SP., Zhang X., Zhu H., Jackson EK., Rafikova O., Petrusevska G. (2008): 2-Ethoxyestradiol is antimitogenic and attenuates monocrotaline-induced pulmonary hypertension and vascular remodelling. *Vasc Pharmacol;* 48: 174–183.

12. Tofovic SP., Eman S., Mady H., Jackson EK., Melhem M. (2005): Estradiol metabolites attenuates monocrotaline-induced pulmonary hypertension in rats. *J Cardiovasc Pharmacol;* 46: 430–437.

13. Tofovic SP., Zhang X., Jackson EK., Dacic S., Petrusevska G. (2006): 2-Methoxyestradiol mediates the protective effects of estradiol in monocrotaline-induced pulmonary hypertension. *Vascular Pharmacol.;* 45: 358–367.

14. Tofovic SP., Zhang X., Jackson EK., Zhu H., Petrusevska G. (2009): 2-Methoxyestradiol Attenuates Bleomycin-Induced Pulmonary Hypertension and Fibrosis in Estrogen-Deficient Rats. *Vascular Pharmacol;* 51: 190–197.

15. Tofovic SP., Jackson EK., Zhang X. (2005): 2-Methoxyestradiol attenuates pulmonary hypertension induced by α-naphthylthiourea. *FASEB J*, 19(5): A1333.

16. Tofovic SP., Jackson EK., Zhang X. (2004): 2-Methoxyestradiol Mediates Protective Effects of Estradiol in Monocrotaline-Induced Pulmonary Hypertension. *Circulation;* 110 (Suppl III): III–18.

17. Tofovic SP., Rafikova O. (2009): Preventive and Therapeutic Effects of 2-Methoxyestradiol, but not Estradiol, in Severe Occlusive Pulmonary Arterial Hypertension in Female Rats. *Am J Res Crit Care Med;* 179: A1802, 2009.

18. Tofovic SP., Jackson EK., Tofoski GJ. (2007): Dysregulated estradiol metabolism in preeclampsia. *Physiologist;* 50: 21, 10–17.

19. Kamath K., Okouneva T., Larson G., Panda D., Wilson L., Jordan MA. (2006): 2-Methoxyestradiol suppresses microtubule dynamics and arrests mitosis without depolymerizing microtubules. *Mol Cancer Ther;* 5: 2225–2233.

20. Merriam GR., MacLusky NJ., Picard MK., Naftolin F. (1980): Comparative properties of the catechol estrogens, I: Methylation by catechol-O-methylstransferase and binding to cytosol estrogen receptors. *Steroids;* 36: 1–11.

21. Spicer LJ., Hammond JM. (1989): catecholestrogens inhibit proliferation and synthesis of porcine granulosa cells in vitro: comparison with estradiol, 5-alpha-dihydrotestosterone, gonadotropins and catecholamines. *Mol Cell Endocrinol;* 64: 119–126.

22. Seegers JC., Aveling ML., Van Aswegen CH., Cross M., Koch F., Joubert WS. (1989): The cytotoxic effects of estradiol-17 beta, catecholestradiols and methoxyestradiols on dividing MCF-7 and HeLa cells. *J Steroid Biochem;* 32: 797–809.

23. Nishigaki I., Sasaguri Y., Yagi K. (1995): Anti-proliferative effect of 2-Methoxyestradiol on cultured smooth muscle cells from rabbit aorta. *Atherosclerosis;* 113: 167–170.

24. Sutrherland TE., Anderson RL., Huges RA., Altmann E., Schuliga M., Ziogas J., Stewart AG. (2007): 2-Methoxyestradiol – a unique blend of activities generating a new class of anti-tumor/anti-inflammatory agents. *Drug Discovery Today;* 12: 577–584.

25. Dubey RK., Tofovic SP., Jackson EK. (2004): Cardiovascular pharmacology of estradiol metabolites. *J Pharm Exp Ther;* 308: 403–409.

26. Austin ED., Cogan JD., West JD., Hedges LK., Hamid R., Dawson EP., Wheeler LA., Parl FF., Loyd JE., Phillips JA. (2009): Alterations in oestrogen metabolism: implications for higher penetrance of familial pulmonary arterial hypertension in females *Eur Respir J;* 34: 1093–1099, 2009.

27. Tofovic SP., Zhu H., Jackson EK., Rafikova O. (2008): 2-Methoxyestrone inhibits vascular remodelling and attenuates monocrotaline-induced pulmonary hypertension. *Eur Res J;* 32: Suppl. 52, 164S.

28. Tofovic SP., Jackson EK., Rafikova O. (2008): Synergistic effects of 2-Methoxyestradiol and retinoic acid on amelioration of monocrotaline-induced pulmonary hypertension. *Am J Res Crit Care Med;* Abstract Issue 177: A592, 2008.

29. O'Bryan MK., Schlatt S., Phillips DJ., de Kretser DM., Hedger MP. (1982): Bacterial lipopolysaccharide-induced inflammation compromises testicular function at multiple levels in vivo. *Endocrinology;* 141: 238–246.

30. Tofovic SP., Jackson EK., Piche C. (2009): Pharmacokinetics and safety of a subcutaneously injected long-acting formulation of 2-methoxyestradiol (2ME) in healthy volunteers. *Basic & Clin Pharmacol & Toxicol;* 105 (Suppl. 1): 107.

Резиме

ДОЗНО-ЗАВИСНИ ЕФЕКТИ НА 2-МЕТОКСИЕСТРАДИОЛ ВРЗ МОНОКРОТАЛИН-ИНДУЦИРАНАТА БЕЛОДРОБНА ХИПЕРТЕНЗИЈА И ВАСКУЛАРНИТЕ ОШТЕТУВАЊА

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Апстракт: 2-метоксиестрадиол (2ME) е главен метаболит на естрадиол без естрогенско дејство. Нашите претходни истражувања, изведени на неколку модели на кардиоваскуларни оштетувања, сугерираат дека 2ME силно ги инхибира притисок-зависните и притисок-независните срцеви и васкуларни оштетувања. Неодамна, ние докажавме дека кај машки стаорци, 2ME го намалува развојот и го успорува понатамошното влошување на белодробната хипертензија (БХ) индуцирана со монокроталин (МКТ), додека кај женски стаорци 2ME го спречува влошувањето на БХ и ја отстранува зголемената смртност кои се резултат на овариектомијата на животните. Во ова испитување, кај машки стаорци, ние ги споредивме тераписките ефекти на три различни дози на 2ME (3, 10 и 30 μг/кг/час; 2ME3, 2ME10, и 2ME30 група) врз МКТ-индуцираната БХ. Животните беа следени за можна појава на естрогенин ефекти. Исто така беа мерени и плазма концентрациите на 2ME. Терапијата беше започната 12 дена по администрацијата на МКТ (60мг/кх/и.п.). Дваесет и осум дена после давањето на МКТ, крвниот систолен притисок на десната комора (DK), (DKVSP) беше мерен, и беше направена морфометриска анализа. Сите три дози имаа тераписки ефект, односно, го намалија притисокот во белодробната артерија, хипертрофијата на десната комора, белодробните васкуларни оштетувања и смртноста. Значајно е да се напомене дека сите три дози немаа никакво дејство врз плазма концентрациите на тестостерон, и на тежината на тестисите и семиналните везикули. Дозно-зависно покачување на плазма концентрациите на 2ME беа забележани само со 2ME3 и 2ME10, додека групите 2ME10 и 2ME30 имаа слични плазма концентрации на 2ME. Сепак, 2ME30 беше поефикасен од 2ME3 и 2ME10 и го елиминира високиот морталитет (34%) индуциран со МКТ. Заклучок: оваа студија укажува дека 2ME, во дози кои постигнуваат

плазма концентрации на 2ME слични на оние во задниот тирместар на бременост (1000–3000 pg/ml) е ефикасен и безбеден (нема естрогени ефекти) во есперименталната БХ. Презентираните податоци исто така укажуваат дека дистрибуцијата во ткивата/органите, а не концентрациите во плазма, го одредуваат терапискиот ефект на 2ME во БХ.

Клучни зборови: белодробна хипертензија, 2-метоксиестрадиол, естрогени хормони, белодробни васкуларни оштетувања.

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