Published in final edited form as:

Life Sci. 2011 January 17; 88(3-4): 178–186. doi:10.1016/j.lfs.2010.11.008.

Dose-dependent toxic effects of high-dose estrogen on renal and cardiac injury in surgically postmenopausal mice

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Abstract

Aims—We previously found that in mice with experimental myocardial infarction (MI), 17 β -estradiol (E2) increased mortality and worsened cardiac remodeling and these deleterious effects were associated with renal enlargement and hydronephrosis in a dose-dependent manner. In the present study we questioned whether E2-induced renal damage predisposes to rather than results from its adverse effects on the heart.

Main methods—Ovariectomized (ovx) mice received either placebo (P) or E2 at 0.02 (E2-L, low dose), 0.42 (E2-M, moderate dose) or 4.2 μ g/d (E2-H, high dose) for 8 weeks.

Key findings—E2-L partially restored uterine weight and plasma estrogen levels without affecting heart, lung and liver weight, hemodynamic parameters, or heart and kidney morphology and function. E2-M restored normal uterine weight, but this was accompanied by a significant increase in kidney weight, albuminuria, glomerular matrix formation and markers for oxidative stress. E2-H increased uterine weight 4.5-fold and resulted in higher plasma creatinine levels, severe albuminuria, renal tubular dilatation, tubulointerstitial injury, hydronephrosis, glomerulosclerosis and oxidative stress. E2-H also caused ascites, hepatomegaly and fluid retention in the uterine horns but had no significant effect on blood pressure or heart function.

Significance—Our data demonstrated that an excessive dose of E2 that raises uterine weight beyond physiological levels adversely affects the kidney even before it damages the heart. We believe estrogen dosage should be taken into account when considering hormonal replacement therapy, since inappropriate doses of E2 may damage not only the heart but also the kidney.

Keywords

estrogen dosage; heart; kidney; hydronephrosis and albuminuria

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Disclosures: None.

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INTRODUCTION

Women have a lower incidence and prevalence of cardiovascular and renal disease and slower progression from chronic renal disease to end-stage renal failure compared to age-matched men (Adams, Jr. et al. 1999; Isles et al. 1992; Brett and Madans 1995; Silbiger and Neugarten 2008; Neugarten et al. 2000; Eriksen and Ingebretsen 2006). This gender advantage for females becomes far less or disappears with increased age and reduced estrogen levels after menopause, suggesting that ovarian hormones, most likely estrogen, protect women against cardiovascular and renal disease (Lerner and Kannel 1986; Eaker et al. 1993). Observational studies have demonstrated that postmenopausal women who receive hormone replacement therapy (HRT) have a lower rate of cardiovascular disease and cardiac death than those who do not (Stampfer et al. 1991; Grodstein et al. 2000; Blum et al. 2000). However, two recent randomized prospective primary or secondary prevention trials, the Women's Health Initiative (WHI) (Rossouw et al. 2002) and the Heart and Estrogen/Progestin Replacement Study (HERS II) (Hulley et al. 2002), showed that HRT can have an unfavorable outcome on the risk and events of CVD. Although these data are convincing, it is not clear whether such unfavorable outcomes are at least partly related to the age of the patients and the time when HRT is begun (Hodis 2008; Grodstein et al. 2003; Schnatz 2006), nor whether HRT also exerts an adverse effect on the kidney.

Estrogen dosage may contribute to the variable outcome of HRT. In most observational and clinical trials, 0.625 mg/day oral conjugated equine estrogen (CEE) plus 2.5mg/day medroxyprogesterone acetate (MPA) was used. Few studies have addressed the possible impact of a single-dose regimen on the outcome of HRT. Grodstein et al reported that a low dose of CEE (0.3 mg/day) lessened major coronary events, whereas at 0.625 mg or higher and combined with progestin it significantly increased the risk of stroke (Grodstein et al. 2000). Sanada et al (Sanada et al. 2003) confirmed that low-dose CEE (0.3 mg/day for 3 months) improved endothelial function and decreased LDL cholesterol nearly as well as the standard dose of 0.625 mg/day. More recently, Villa et al (Villa et al. 2010) reported that low-dose estrogen (1 mg) in combination with drospirenone (a new progestin derived from 17 α -spironolactone) had a favorable effect on lipid profile and endothelial function. In animal studies, wide range of estrogen dosages (from 0.17 to 83.3 μ g/day) has been used. However, nearly all studies employed only a single dose, and its effects on the heart were reported to be beneficial, ineffective or detrimental (Beer et al. 2007; H $\ddot{u}$ gel et al. 1999; van Eickels et al. 2003). Thus it is difficult to be sure whether these divergent findings were dose-related or due to differences in species or strain. We previously tested the dose-effect of 17 β -estradiol (E2) in mice myocardial infarction (MI) and found that at 0.42 μ g/day it tended to be cardioprotective; however, at increased doses (4.2 and 18.8 μ g/day) it not only increased mortality but also worsened cardiac hypertrophy, LV chamber dilatation and heart dysfunction. Moreover, when we treated mice with high doses of E2 we found dose-dependent enlargement of the liver and kidney (Zhan et al. 2008). These findings were provocative; however, there are questions remained to be answered, namely, 1) whether the low dose was low enough to provide cardioprotection; and 2) whether the renal damage was directly due to the cardiac damage or an entirely separate effect of the hormonal treatment.

In the present experiment, we added a much lower dose of E2 (0.02 μ g/day) to two doses from our previous study (0.42 and 4.2 μ g/day), focusing on the effect of E2 on renal morphology and function in ovariectomized mice without MI in an attempt to clarify 1) whether E2 causes structural and functional injuries in the kidney in a dose-dependent manner; and 2) whether E2-induced renal damage predisposes rather than results from its adverse effects on the heart.

MATERIALS AND METHODS

Animals

Female C57BL/6J mice 4 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). They were housed in an air-conditioned room with a 12-hour dark/light cycle and given standard chow with free access to tap water. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Henry Ford Health System in accord with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Ovariectomy and estrogen replacement

Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and bilateral ovariectomy (ovx) was performed *via* a pair of flank incisions (Cavasin et al. 2004; Cavasin et al. 2003). 60-day-release pellets containing placebo or 17 β -estradiol (E2 0.02, 0.42, or 4.2 μ g/day; Innovative Research of America, Sarasota, FL) were implanted subcutaneously at the back of the neck immediately after ovx (Zhan et al. 2008). Sham ovx groups had the same procedure except that the ovaries were left intact.

Experimental protocol

Mice were divided into five groups: 1) sham-ovx; 2) ovx + placebo; 3) ovx + low dose (E2-L, 0.02 μ g/d); 4) ovx + moderate dose (E2-M, 0.42 μ g/d); and 5) ovx + high dose (E2-H, 4.2 μ g/d). Treatment was continued for 8 weeks.

Systolic blood pressures (SBP), cardiac function and organ harvest

At the end of the experiment, SBP was determined by tail cuff (Visitech BP-2000, Apex, NC) following a 7-day training period (Xu et al. 2007). Transthoracic echocardiography was performed in conscious mice using a Doppler echocardiographic system equipped with a 15-MHz linear transducer (Acuson C512, Mountain View, CA) as described previously (Yang et al. 1999; Xu et al. 2007). Mice were then anesthetized with pentobarbital (50 mg/kg, i.p.) and a Millar micromanometer pressure catheter (Millar Instruments, Houston, TX) was inserted into the left ventricle (LV) *via* the right carotid artery to measure LV pressure and $dp/dt_{max}/iP$ (maximum rate of the rise in LV pressure divided by pressure at maximum dp/dt), an indicator of isovolumic contraction (Cingolani et al. 2003; Sun et al. 2005). After blood was collected, the heart was stopped at diastole by injecting 15% KCl. The heart, lung, kidney, liver and uterus were removed and weighed. The LV was sectioned transversely into 3 slices, and the middle section was rapidly frozen in isopentane and stored at -70°C for determination of interstitial collagen fraction (ICF) and cardiomyocyte cross-sectional area (MCSA) as described previously (Xu et al. 2007). Kidneys were fixed in 10% formalin, processed and embedded in paraffin for histological analysis.

Blood collection and plasma estrogen and creatinine

After measuring cardiac hemodynamics and function, 500 μ l of blood was withdrawn from the vena cava and plasma was kept at -70°C until assay. Plasma estrogen and creatinine were determined by enzyme immunoassay using a commercially available kit (Cayman Chemicals, Ann Arbor, MI).

Urine volume, albumin, creatinine clearance and 8-isoprostane

During the last week of the experiment, mice were placed in metabolic cages. After 5 days of acclimatization, 24-hour urine was collected, centrifuged, filtered and stored at -20°C until assay. Urinary albumin was measured with a commercially available ELISA kit (Alpha Diagnostics, San Antonio, TX) and expressed as μ g/24 hr. Creatinine clearance (CrCl) was

calculated using the formula: (urinary creatinine x urine volume)/(plasma creatinine x 24 hr) and expressed as $\mu\text{l}/\text{min}/10\text{ mg kidney weight}$. 8-isoprostane was measured with an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI) (Schnackenberg and Wilcox 1999) and expressed as $\text{ng}/24\text{ hr}$.

Western blot for NADPH oxidase subunit gp91^{phox} (Nox2) protein expression

Kidney tissue was homogenized in lysis buffer, the supernatant collected and protein content detected with Coomassie protein assay reagent (Thermo Scientific, Waltham, MA). Protein extracts (60 $\mu\text{g}/\text{lane}$) were separated out in 10% SDS-PAGE under reducing conditions and electrotransferred to a nitrocellulose membrane. Membranes were blocked in buffer (TBS/0.1% Tween 20 containing 5% non-fat milk) and incubated overnight at 4°C with a monoclonal antibody against Nox2 (1:500, BD Transduction, Lexington KY). They were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies (1:2000, Amersham Pharmacia Biotech, Piscataway, NJ) for 1 hr at room temperature. Immunoreactive bands were detected with a chemiluminescent reaction kit (Amersham) and semi-quantified by densitometry. Nox2 protein was normalized by GAPDH.

Histology of the kidney

i) Tubulointerstitial injury—Renal sections were deparaffinized in xylene, dehydrated by ethanol gradient and stained with hematoxylin and eosin (H&E). Renal interstitial collagen was assessed by picrosirius red staining and calcium deposition assessed by von Kassa staining; dark-colored calcium crystals were visualized microscopically using a dark field with polarized light illumination. Tubulointerstitial injury was evaluated using a modified semi-quantitative scoring system (Hayakawa and Raij 1998). Severity of injury was scored from 0 to 3 according to four criteria: 1) *distance between two neighboring tubules*: 0 = no change, 1 = slightly increased, 2 = moderately increased, 3 = greatly increased associated with interstitial fibrosis; 2) *number of dilated tubules*: 0 = none, 1 = a few, 2 = a moderate number, 3 = most of them; 3) *pathology of tubules*: 0 = no change, 1 = a few flattened epithelial cells combined with loss of the brush border, 2 = dead cells or cell fragments in the lumen, 3 = casts in the lumen, luminal obstruction and tubular necrosis; 4) *Calcium phosphate deposition*: 0 = No calcium crystal identified; 1 = a few dark-colored crystals observed in the cortex at 40x magnification; 2 = calcium crystals appeared in the cortex or medulla at 10x or 20x magnification and easily identified under 40x magnification; 3 = calcium crystals easily seen in many areas of the renal tissue at 10x and 20x magnification and extensively distributed in the tubules and interstitial space of the cortex and medulla. Each criterion was evaluated in at least 10 microscope fields and averaged. Scores from all four criteria were added together. Injury was scored independently by three investigators in a blind fashion.

ii) Glomerular matrix—Sections were deparaffinized, dehydrated and fixed in 4% formaldehyde for 20 min, then stained with Schiff's reagent (PAS; Sigma, St. Louis, MO) for 15 min as described previously (Liao et al. 2008). Dark purple areas of the glomerulus were considered to present the extracellular matrix. Twenty-five to 30 glomeruli in each section were imaged at 40x magnification. Data were analyzed by computerized imaging software (Microsuite Biological, Olympus, Center Valley, PA). The glomerular matrix was expressed as a percentage of the glomerular area.

Data analysis

All data were summarized by mean $\pm$ SE. Student's two-sample *t*-test was used to compare differences and calculate *p*-value between two groups. Since multiple comparisons were involved, Hochberg's step-up procedure was used to adjust *p*-values so that the family-wise

type I error rate (at the 0.05 level) would be controlled. Differences were considered significant if the adjusted *p*-value was less than 0.05.

RESULTS

Effect of E2 replacement on organ weight

Efficacy of 8 weeks of E2 replacement was determined based on uterine weight and plasma estrogen levels (Figure 1). Ovariectomy (ovx) significantly reduced uterine weight. E2 at low doses (E2-L) partially but significantly restored uterine weight. E2-M restored uterine weight to the same level as sham-ovx but E2-H caused an excessive increase (about 5-fold higher than sham-ovx). All mice in the E2-H group exhibited fluid retention in the uterine horns and stiffening of the uterus as well as moderate to severe urine retention in the bladder, hydronephrosis and ascites, although the ureter was not obstructed. OvX with or without E2 replacement had no effect on heart and lung weight (Table 1).

Effect of E2 on systolic blood pressure, heart rate, cardiac remodeling and function

Systolic blood pressure (SBP) and heart rate (HR) were similar among groups, indicating that neither one was affected by ovx or E2 replacement (Table 2). OvX and ovx + E2 at all doses had no significant effect on LV pressure, $dP/dt_{max}/iP$ (an indicator of isovolumic contraction), dP/dt_{min} or ejection fraction. However, at moderate and high doses E2 increased LV chamber dimension, area and mass (Table 2). Histological evaluation showed that ovx increased both interstitial collagen fraction (ICF) and myocyte cross-sectional area (MCSA) significantly compared to sham-ovx, which was reversed by E2-L but not E2-M or E2-H (Table 2).

Dose-Effect of Estrogen on Renal Function and Histology

Kidney weight and renal function: We found that ovx alone or combined with E2-L had no effect on kidney weight, whereas E2-M and E2-H significantly increased the ratio of kidney to body weight (Figure 2A). Urine volume was not affected by ovx *per se* or ovx plus E2-L or E2-M but was significantly increased by E2-H compared to sham ovx controls (Figure 2B); however, 24-hr urinary albumin excretion was increased significantly in both E2-M and E2-H groups (Figure 2C). Plasma creatinine remained unchanged with ovx alone or ovx + E2-L or E2-M but increased significantly with E2-H (Figure 3A). CrCl tended to decrease with E2-M compared to s-ovx, although the difference was not statistically significant. E2-H significantly reduced CrCl (Figure 3B).

Tubular dilatation and glomerular matrix formation: OvX alone or combined with E2-L had no effect on the renal tubules. However, 1 out of 8 mice given E2-M developed tubular dilatation, while 7 out of 8 mice in the E2-H group had severe dilatation and many tubules contained waxy casts (Figure 4). On PAS staining, ovx alone or combined with E2-L had no effect on the glomerular matrix; however, both E2-M and E2-H increased matrix formation significantly and similarly (Figure 5).

Tubulointerstitial injury: Representative images of tubulointerstitial injury caused by E2-H and semi-quantitative injury scores are shown in Figure 6. OvX *per se* increased tubulointerstitial injury, influenced mainly by increased calcium deposition scores. This injury was partly reversed by E2-L but unaffected by E2-M. In contrast, E2-H worsened renal injury, as evidenced by a greater distance between neighboring tubules, an increased number of dilated tubules, flattened epithelial cells with loss of the brush border, dead cells, cell fragments and/or casts in the lumen along with interstitial fibrosis and calcium phosphate deposition.

Nox2 protein expression and urinary 8-isoprostane excretion: OvX increased Nox2 protein expression, which was not affected by E2-L but was enhanced by E2-M and E2-H in a dose-dependent manner (Figure 7A). Urinary 8-isoprostane excretion was significantly increased by ovx, but E2-L reversed it to sham-ovx levels. Both E2-M and E2-H groups exhibited significantly higher levels of 8-isoprostane than sham-ovx controls (Figure 7B).

DISCUSSION

Using a mouse model of surgically induced menopause (ovx), we examined the dose-dependent effects of estrogen replacement on the heart and kidney. We found that in mice with an intact heart E2 did not affect systolic blood pressure or contractile function at any of the doses we tested, although LV chamber dimension and wall thickness were slightly but significantly increased by moderate (E2-M) and high doses (E2-H). However, in the kidney dose-dependent detrimental effects of E2 (especially E2-H) on morphology and function became apparent, including: 1) increased kidney weight and hydronephrosis; 2) increased plasma creatinine and albuminuria; 3) increased glomerular matrix formation and interstitial fibrosis; 4) tubular dilatation and tubulointerstitial injury as estimated by semi-quantitative scoring; and 5) increased oxidative stress as indicated by Nox2 protein expression and urinary 8-isoprostane excretion. We believe this is the first reported evidence that E2 causes dose-dependent renal damage in ovariectomized mice with an intact heart, which may contribute to its adverse cardiac effect.

Women have a lower incidence and prevalence of renal disease and slower progression from chronic renal disease to end-stage renal failure (Silbiger and Neugarten 2008; Neugarten et al. 2000; Eriksen and Ingebretsen 2006). This gender advantage for women becomes less or disappears with increased age and reduced estrogen levels after menopause. Animal studies also showed that females are protected from hypertension and renal injury and this protection diminishes or disappears when they are ovariectomized to surgically induce menopause (Reckelhoff and Maric 2010; Sartori-Valinotti et al. 2007; Zhang et al. 2007; Sullivan et al. 2010). These results suggest that ovarian hormones, most likely estrogen, protect females against cardio-renal disease. However, the outcome of hormonal replacement therapy (HRT) on the kidney remains controversial. Agarwal et al (Agarwal et al. 2005) and Karl et al (Karl et al. 2006) found that HRT was associated with reduced urinary albumin excretion and glomerulosclerosis in postmenopausal women; yet Manning et al reported that HRT did not improve microalbuminuria in postmenopausal diabetic women (Manning et al. 2003), whereas others showed that both HRT and contraceptives increased the risk of microalbuminuria (Monster et al. 2001; Ribstein et al. 1999). In animal studies, E2 was reported to either improve renal function and reduce tubulointerstitial fibrosis (Mankhey et al. 2005; Mankhey et al. 2007) or worsen glomerulosclerosis and accelerate renal injury in obese Zucker rats (Gades et al. 1998; Stevenson et al. 2000). The reasons for these conflicting results remain unclear. Since all of these studies involved a single-dose regimen, one cannot be sure whether dosage affects the outcome of E2 treatment. Our present data provide evidence that whether E2 is protective or harmful to the kidney largely depends on the dosage.

Using a mouse model of surgically induced menopause along with MI, we previously found that moderate and high doses of E2 not only increased mortality and deteriorated cardiac remodeling and function but also caused kidney and liver damage as evidenced by severe hydronephrosis, liver enlargement and ascites (Zhan et al. 2008). Since that study involved mice with MI, we could not be sure whether such renal damage was due to heart failure or a direct adverse effect of E2 on the kidney that in turn contributed to deterioration of cardiac dysfunction and remodeling post-MI. To clarify this, we used ovariectomized mice without MI to see if the toxic effects of E2 on the kidney occur prior to heart dysfunction. We found

that ovx *per se* caused a slight but significant increase in interstitial collagen deposition and myocyte size and that these effects were reversed by a low dose of E2 but augmented by moderate and high doses. Despite those morphological changes, no dosage of E2 had any significant impact on cardiac hemodynamics and function. However, in the kidney moderate or high doses of E2 not only caused structural damage but also reduced renal function as evidenced by hydronephrosis, tubular dilatation and increased glomerular matrix deposition as well as increased plasma creatinine and albuminuria, whereas these effects were not seen with a low dose. Since E2-induced renal damage developed before any noticeable adverse effects on cardiac function, our data suggest that the renal damage was not secondary to cardiac dysfunction; rather, these renal toxic effects may have contributed to and even enhanced cardiac damage.

The precise mechanisms responsible for these detrimental renal effects of E2 are not known. In diabetic animals, E2 reportedly increases plasma creatinine and triglyceride-rich lipoproteins, and it has been suggested that their binding to glomerular cells initiates or accelerates glomerulosclerosis (Gades et al. 1998; Stevenson et al. 2000). In the present study, we found that a high dose of E2 increased plasma creatinine and this was accompanied by heightened albuminuria and glomerular matrix formation as well as reduced creatinine clearance. Unfortunately, we did not measure lipoproteins and do not know whether E2-induced renal damage was at least partially due to an increase in triglyceride-rich lipoproteins. This warrants further investigation in the future.

In addition to glomerular injury, E2 also caused urine retention, tubular dilatation, hydronephrosis, and hydroureter, mostly at high doses. E2-induced urine retention, cystitis and hydronephrosis have been reported by others (Pearse et al. 2009; Gakhar et al. 2009; Levin-Allerhand et al. 2003). The severe tubular dilatation and hydronephrosis could be due to increased pressure and backward flow resulting from urine retention. E2 has been shown to enhance β -adrenergic receptor-mediated relaxation of the detrusor muscle in the bladder (Yono et al. 2000). Chronic E2 treatment also reportedly inhibits micturition (Buhl et al. 1985) and produces thickening of the urethral epithelium, even to the point of hermetically sealing the urethra (Blakeman et al. 2000). All of these conditions can cause urine retention and increase bladder pressure, contributing to the development of hydronephrosis observed with E2 treatment. Clinically, E2 has been used to treat urinary incontinence (UI) in elderly women (Fantl et al. 1994; Fantl 1994), but the recent clinical trials HERS (Steinauer et al. 2005) and WHI (Hendrix et al. 2005) failed to show that HRT has a beneficial effect on UI. Since E2-induced urine retention and hydronephrosis appear to be dose-dependent as evidenced by the present study and others (Pearse et al. 2009; Gakhar et al. 2009; Ba and Friedman 2004), it is reasonable to assume that urine retention and increased pressure in the bladder may contribute to the disappointing results of E2 or HRT used to treat UI in postmenopausal women, which could be dose-related.

Generation of reactive oxygen species (ROS) that leads to increased oxidative stress plays an important pathophysiological role in cardio-renal disease (Sullivan et al. 2010; Sartori-Valinotti et al. 2007; Ji et al. 2007). ROS can serve as both intercellular and intracellular second messengers, altering the signaling molecules involved in cell growth/proliferation, apoptosis and release of pro-inflammatory mediators and leading to tissue and end-organ damage (Takimoto and Kass 2007; Touyz 2004). Studies have shown that E2 inhibits NADPH oxidase (Nox2, a major source of ROS generation), reduces ROS production and increases NO availability, thereby slowing or reversing progression of renal injury (Javeshghani et al. 2009; Ji et al. 2007; Blush et al. 2004; Zhang et al. 2007). In the present study we showed that ovx by itself increased renal Nox2 protein expression and urinary 8-isoprostane excretion while the increase in 8-isoprostane was reversed by a low dose of E2, supporting the concept that E2 reduces oxidative stress. However, Nox2 and 8-isoprostane

excretion were heightened by E2-M and -H, which correlated with renal damage and as well as cardiac structural and morphological changes such as increased myocyte size and cardiac mass induced by those doses of E2, although these adverse cardio-renal effects were independent of blood pressure. While we do not know precisely how E2 at high doses enhances oxidative stress dose-dependently, we believe these data may have significant implications for increased cardiovascular events in women on HRT. The potential toxic effects of high doses of E2 on the kidney could very well contribute to the unfavorable outcome of HRT on the heart, which warrants further investigation.

In summary, our data suggest that E2 replacement that increases plasma estrogen uterine weight far beyond physiological levels is harmful to the heart but can even more severely damage the kidney. At lower doses that partially restored uterine weight, E2 did not adversely affect the kidney but in fact tended to offer cardio-renal protection in ovariectomized mice as indicated by decreased myocardial interstitial collagen deposition and myocyte hypertrophy as well as reduced renal injury and oxidative stress. Thus before we can be sure whether E2 replacement is detrimental or beneficial, we first need to establish a safe and effective dose.

Acknowledgments

This work was supported by National Institutes of Health Grants HL -078951 (XPY) and Henry Ford Health System institutional funds (XPY).

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Figure 1.

Dose-effect of 17 β -estradiol (E2) replacement on plasma estrogen levels (A) and uterine weight corrected by body weight (UW/BW) (B) in ovariectomized (ovx) mice. s-ovx: sham ovariectomy; pl: placebo; 0.02 μ g/day: E2-low dose; 0.42 μ g/day: E2-moderate dose; 4.2 μ g/day: E2-high dose(all doses given for 8 weeks).

Figure 2.

Dose-effect of 17β-estradiol (E2) replacement on kidney weight corrected by body weight (KW/BW) (A), 24-hr urine volume (B), and 24-hr urinary albumin excretion (C) in ovariectomized (ovx) mice. s-ovx: sham ovariectomy; pl: placebo; 0.02 µg/day: E2-low dose; 0.42 µg/day: E2-moderate dose; 4.2 µg/day: E2-high dose.

Figure 3. Dose-effect of 17β-estradiol (E2) replacement on plasma creatinine (A) and creatinine clearance (CrCl) (B) in ovariectomized (ovx) mice. s-ovx: sham ovariectomy; pl: placebo; 0.02 μg/day: E2-low dose; 0.42 μg/day: E2-moderate dose; 4.2 μg/day: E2-high dose.

Figure 4.

Dose-effect of 17 β -estradiol (E2) replacement on renal tubule dilatation in ovariectomized (ovx) mice. Left panels: representative images showing E2-induced tubular dilatation (seen mostly with high-dose E2) (E). Right panel: number of mice exhibiting tubular dilatation in each treatment group. s-ovx: sham ovariectomy; pl: placebo; 0.02 μ g/day: E2-low dose; 0.42 μ g/day: E2-moderate dose; 4.2 μ g/day: E2-high dose.

Figure 5. Dose-effect of 17β-estradiol (E2) replacement on glomerulosclerosis in ovariectomized (ovx) mice. Left panels: representative images showing E2-induced increase in glomerular matrix (dark purple-stained area seen with periodic acid-Schiff staining). Right panel: quantitative analysis of E2-induced glomerulosclerosis. s-ovx: sham ovariectomy; pl: placebo; 0.02 μg/day: E2-low dose; 0.42 μg/day: E2-moderate dose; 4.2 μg/day: E2-high dose.

Figure 6.

Dose-effect of 17β -estradiol (E2) replacement on tubulointerstitial injury score. Left panels: representative images of high-dose E2-induced tubulointerstitial injury; A: dilated tubules combined with flattened epithelial cells, loss of the brush border, dead cells and/or cell fragments and casts in the lumen as well as increased distance between two neighboring tubules; B: interstitial fibrosis (picrosirius red staining); and C: renal calcium deposition (von Kossa staining, showing dark calcium crystals using a dark field and polarized light). s-ovx: sham ovariectomy; pl: placebo; 0.02 µg/day: E2-low dose; 0.42 µg/day: E2-moderate dose; 4.2 µg/day: E2-high dose.

Figure 7. Dose-effect of 17 β -estradiol (E2) replacement on Nox2 protein expression (A) and urinary 8-isoprostane excretion (B) in ovariectomized (ovx) mice. s-ovx: sham ovariectomy; pl: placebo; 0.02 μ g/day: E2-low dose; 0.42 μ g/day: E2-moderate dose; 4.2 μ g/day: E2-high dose.

Table 1Dose-Effect of 17 β Estradiol (E2) on Organ Weight.

Parameters	Sham-ovx <i>n</i> = 7		ovx + placebo <i>n</i> = 8		ovx + L <i>n</i> = 9		ovx + M <i>n</i> = 8		ovx + H <i>n</i> = 8	
	Groups		ovx + E2		ovx + E2		ovx + E2		ovx + E2	
BW (g)	19.3±0.6		20.5±0.3		21.5±0.5		19.9±0.4		20.0±0.4	
Uterine weight/BW (mg/10 g)	41±6		7±1 [*]		14±2 ^{*†}		50±12 ^{†§}		170±30 ^{*†§#}	
Kidney weight/BW (mg/10 g)	116±4		105±4		109±5		127±3 ^{*†§}		125±2 ^{*†§}	
Heart weight/BW (mg/10g)	39.6±0.8		39.9±1.7		36.7±2.5		41.3±1.1		39.6±1.4	
Lung weight/BW (mg/10 g)	54±2.6		53±1.1		51±1.6		51±1.3		54±2.8	
Liver weight/BW (mg/10 g)	468±22		430±36		443±42		481±17		516±16 ^{*†}	

ovx: ovariectomy; E2: 17 β -estradiol; L: low dose (0.02 μ g/day); M: moderate dose (0.42 μ g/d); H: high dose (4.2 μ g/d). BW: body weight.^{*}
^{*} $p < 0.05$ vs sham-ovx;[†]
[†] $p < 0.05$ vs ovx + placebo;[§]
[§] $p < 0.05$ vs ovx + L;[#]
[#] $p < 0.05$ vs ovx + M.

Table 2

Dose-Effect of Estrogen on Left Ventricular Function, Morphology and Histology.

Groups		ovx + E2			
Parameters	Sham-ovx	ovx + placebo	ovx + L	ovx + M	ovx + H
SBP (mmHg)	105±5	104±2	106±4	106±3	101±3
HR (beats/min)	659±19	649±14	659±10	658±13	629±15
dP/dt _{max} /IP (sec ⁻¹)	98±3	91±7	107±13	122±7	94±1
dP/dt _{min} (mmHg/sec)	-3022±107	-2837±270	-3293±431	-3539±347	-2957±245
LVdDs (mm)	1.06±0.06	1.11±0.03	1.08±0.02	1.16±0.04	1.18±0.04*
LVdD (mm)	2.07±0.09	2.23±0.06	2.19±0.04	2.35±0.04*	2.27±0.06*
PWTd (mm)	0.77±0.02	0.80±0.02	0.78±0.01	0.84±0.02	0.87±0.01*§
LVAs (mm ²)	0.71±0.10	0.75±0.01	0.79±0.02	0.88±0.04*†	1.0±0.09*†§
LVAd(mm ²)	3.62±0.03	3.95±0.16	4.07±0.13	4.35±0.18*	4.41±0.2*†
LV mass (mg/10 g)	21.2±4.1	22.7±0.5	20.9±0.8	27.4±1.5*†§	27.5±1.4*†§
EF (%)	80.5±1.3	80.9±0.5	80.7±0.5	79.7±0.7	77.5±1.1
ICF (%)	4.7±0.3	5.9±0.4*	4.7±0.4†	5.7±0.5*	6.1±0.2*§
MCSA (mm ²)	141±3	151±2*	142±3†	150±2*	148±5*§

ovx: ovariectomy; E2: 17β-estradiol; L: low dose (0.02 µg/day); M: moderate dose (0.42 µg/d); H: high dose (4.2 µg/d). SBP: systolic blood pressure; HR: heart rate; IP: left ventricular instant pressure; LVdDs and LVdD: LV systolic and diastolic dimensions; PWTd: diastolic posterior wall thickness; LVAs and LVAd: LV systolic and diastolic area; EF: ejection fraction; ICF: interstitial collagen fraction; MCSA: myocyte cross-sectional area.

* $p < 0.05$ vs sham -ovx;

† $p < 0.05$ vs ovx +placebo;

§ $p < 0.05$ vs ovx +L.