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Estrogen Depletion Differentially Affects Blood Pressure Depending on Age in Long–Evans Rats

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Normotensive female rats exhibit age-related decreases in estrous cyclicity and increases in blood pressure. In spontaneously hypertensive rats, estrogens, including dietary phytoestrogens, prevent or attenuate the increased blood pressure associated with estrogen depletion. The present studies examine the effects of ovariectomy (OVX) at either 3 or 10 mo of age. Although blood pressure increases from 3 to 9 mo, OVX at 3 mo of age has no added effect—despite the fact that OVX (compared to ovary-intact) rats weighed significantly more. In contrast, aging from 10 to 16 mo is associated with a further increase in blood pressure, which is potentiated by estrogen depletion. Removal of dietary phytoestrogens exacerbated the hypertensive effects of OVX in these middle-aged rats. As in younger rats, estrogen depletion at 10 mo of age was associated with greater weight gain. Whereas estrogen depletion at 3 mo of age was without effect on fluid intake over the next 6 mo, OVX at 10 mo of age was associated with decreased fluid intake. In a final study, rats were OVX at 3 mo of age with estradiol (E2) treatment initiated at 10 mo of age. Long-term OVX (>10 mo) was associated with increased blood pressure and mortality at 14–16 mo of age. Circulating levels of E2 were decreased by OVX. Plasma aldosterone was increased by OVX, an effect which was prevented by either E2 or phytoestrogens. Neither E2 nor aldosterone was affected by age. These data indicate that (a) the physiological effects of estrogen depletion vary with age; (b) phytoestrogens in the diet exert some protective effects; and (c) long-term OVX in the absence of hormone replacement is associated with premature mortality. We suggest that chronic increases in aldosterone and sympathetic tone underlie the hypertensive effects of estrogen depletion.

Key Words: Aging; ovariectomy; blood pressure; body weight; aldosterone; estradiol; phytoestrogens; Long–Evans rats.

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Introduction

Hypertension is a common medical syndrome in developed countries that contributes significantly to premature death (1). With increasing age, humans experience an increased incidence of hypertension *(2,3)*. Young women typically have lower arterial blood pressure than age-matched men. This advantage is lost in the decades following menopause *(2,3)*. Female sex steroids modify autonomic function, and the age-related decline in the circulating levels is correlated with several age-related diseases, such as hypertension in both humans and animals *(5–8)*. Our recent report shows that the incidence of regular estrous cycles decreases in aging Long–Evans (a normotensive strain) rats *(4)*. Although female rats do not undergo a menopause *per se*, they do transition from regular cycles to irregular cycles, to periods of extended and persistent estrus. Young female rats exhibit estrous cycle–related increases in plasma estradiol, whereas middle-aged rats in persistent estrus do not. Middle-aged females in persistent estrus have plasma levels of estradiol similar to those in young cycling rats on diestrous. Young female rats have lower blood pressure than males, but at 14 mo of age there are no differences between the sexes. Interestingly, young male rats exhibit a marked circadian periodicity for plasma testosterone, but with aging both circadian periodicity and circulating concentrations decrease. Thus, our previous data clearly indicate that "normotensive" rats exhibit age-related increases in blood pressure in association with decreases in reproductive function and circulating sex steroid levels *(4)*. The effects of aging appear analogous to those observed in humans.

Until recently, it was accepted that estrogen provided some protection against adverse cardiovascular and cerebrovascular events in humans and rats. However, publication of the results of the Women's Health Initiative *(5–8)* indicated that the beneficial health effects of estrogen replacement therapy were outweighed by the negative effects, thus reopening heated debate on the benefits of hormone replacement therapy. In rats, the effects of ovariectomy and estrogen replacement on blood pressure remain somewhat equivocal, but estrogen treatment is almost universally associated with some degree of neuroprotection in experimental models of stroke *(9–12)*. Many laboratories use young rats to

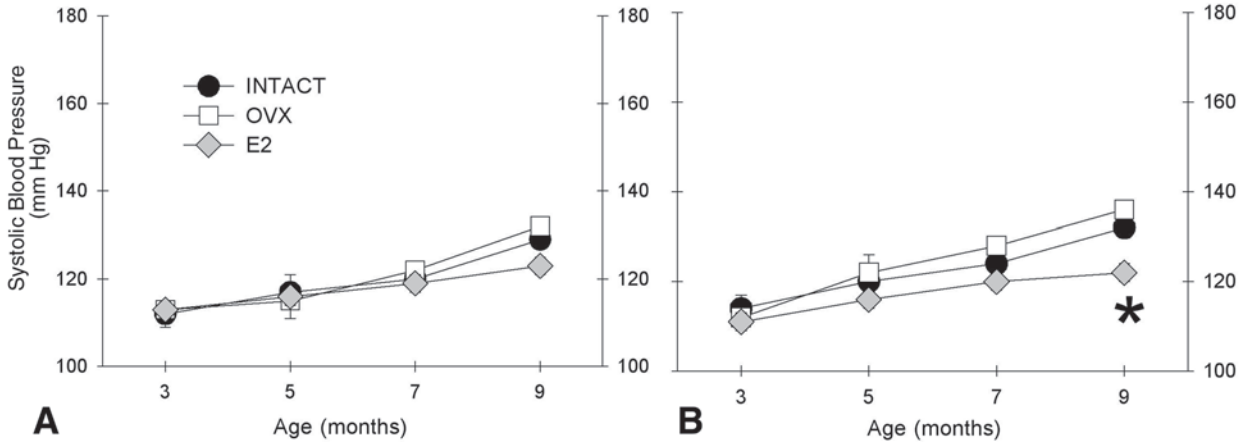

Fig. 1. Effects of ovariectomy (OVX) at 3 mo of age, ± estradiol (E2), and substitution of a phytoestrogen-free diet at 3 mo of age, on systolic blood pressure. Data are presented as mean ± SEM, with 12–14 rats per group. (**A**) Rats were maintained on a normal diet (Teklad 8640). From 3 to 6 mo of age all rats exhibited a modest increase in systolic blood pressure; (**B**) immediately after OVX, rats were placed on a phytoestrogen-free diet (Teklad AIN-76A). From 3 to 6 mo of age there was a modest increase in systolic blood pressure in all groups. At 9 mo of age, 6 mo after OVX and substitution of the phytoestrogen-free diet, the OVX + E2 rats had lower blood pressure than that seen in the intact or OVX rats on the phytoestrogen-free diet (*$p < 0.01$).

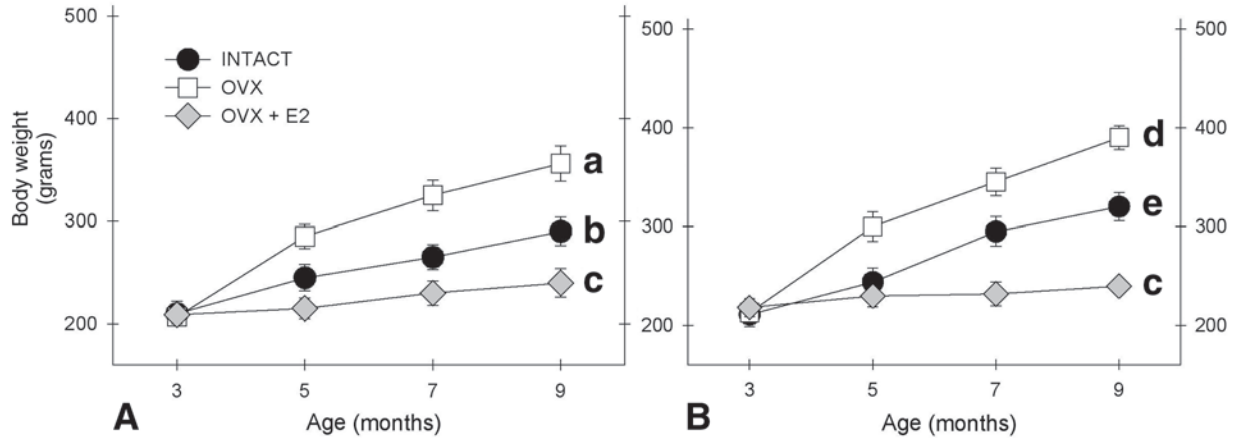

Fig. 2. Effects of OVX at 3 mo of age, with or without E2 and substitution of a phytoestrogen-free diet at 3 mo of age, on body weight. Data are presented as mean ± SEM, with 12–14 rats per group: (**A**) a normal diet; (**B**) phytoestrogen-free diet. All groups demonstrated age-related increases in body weight. Different lettered superscripts indicate differences between groups. In rats with or without phytoestrogens, at all time points OVX > intact > OVX + E2. At 7 and 9 mo of age phytoestrogen-depleted intact and OVX rats weighed more than phytoestrogen replete rats. (ANOVA and pairwise comparisons $p < 0.01$.)

assess the effects of ovariectomy and estrogen treatment, with variable results. We suggest that several factors may contribute to these discrepancies, most notably the age of the rats at ovariectomy, the health status of the rats, and the presence of phytoestrogens in the diet. Therefore, in the present studies we assessed the effects of ovariectomy at 3 or 10 mo of age, with or without substitution of a phytoestrogen-free diet and/or estradiol replacement at the time of ovariectomy. In the Women's Health Initiative there was an additional complicating factor—hormone therapy was initiated after a variable period of hormone deprivation, averaging about 10 yr *(5–8)*. The issue of time from menopause to hormone treatment was examined by Brownley and colleagues *(13)* who observed that, compared to placebo-treated and women receiving conjugated estrogens and medroxyprogesterone acetate that were >5 yr post-menopause, women that initiated replacement therapy <5 yr after menopause demonstrated decreases in blood pressure. To address the effects of a prolonged period of reduced hormone levels, a final experiment assessed the effects of delayed (7 mo delay between ovariectomy and estradiol treatment) on hypertension in ovariectomized rats.

Results

Study 1: Effects of Ovariectomy at 3 mo of Age (Figs. 1–3)

Aging from 3 to 9 mo is associated with a modest increase in systolic blood pressure (10–20 mmHg). Ovariectomy (OVX) at 3 mo of age, or the removal of dietary phytoestrogens, does not affect this increase in blood pressure. In contrast, estradiol (E2) treatment from 3 to 9 mo of age to

Table 1
Estradiol (E2; pg/mL) Levels in Female Rats That Were Ovariectomized (OVX) or Sham Ovariectomized (Intact), Treated with Estradiol or Vehicle, and Maintained on a Normal or a Phytoestrogen-free Diet Starting at 3 mo of Age

		Age at surgery (months after surgery)			
Gonadal status	Diet	3 mo (0)	4 mo (1)	6 mo (3)	8 mo (5)
Intact	Normal	31 ± 9^a	29 ± 9^a	31 ± 9^a	31 ± 9^a
OVX	Normal	29 ± 8^a	7^b	7^b	7^b
OVX + E2	Normal	32 ± 8^a	73 ± 9^c	79 ± 8^c	76 ± 8^c
Intact	Phytoestrogen-free	31 ± 7^a	30 ± 10^a	29 ± 11^a	28 ± 7^a
OVX	Phytoestrogen-free	31 ± 8^a	7^b	7^b	7^b
OVX + E2	Phytoestrogen-free	32 ± 9^a	75 ± 12^c	71 ± 10^c	73 ± 9^c

Data are mean ± SEM. (Intact = sham surgery; OVX = ovariectomized; OVX + E2 = ovariectomized and implanted with Silastic capsules containing estradiol; $n \geq 12$ per group; ^{a,b,c}groups sharing lettered superscripts are similar, groups with differing lettered superscripts are different from each other, $p < 0.01$.)

rats OVX at 3 mo of age (OVX + E2) and placed on a phytoestrogen-free diet attenuated the age-related increase in systolic blood pressure (Fig. 1). Aging from 3 to 9 mo is also associated with an increase in body weight. The increase in body weight is potentiated by OVX and, further, by substitution of a phytoestrogen-free diet. The potentiating effect of estrogen depletion on body weight gain is reversed by E2 (Fig. 2). It is important to note that there is no increase in blood pressure despite a profound increase in body weight.

Circulating E2 levels were consistently highest in OVX + E2 rats, intermediate in intact (sham-ovariectomized) females (on diestrous), and lowest in OVX rats, at 8 mo of age, E2 levels were approx 30 pg/mL in intact, undetectable (<7 pg/mL) in OVX, and approx 75 pg/mL in OVX + E2 females. Plasma E2 levels were unaffected by substitution of the phytoestrogen-free diet (Table 1). No age-related decline in plasma E2 was observed (rats were either sampled on diestrous or in persistent vaginal estrus). Thus, similar E2 levels were observed at 4, 6, and 8 mo of age (Table 1), when sampled at the low point of the estrous cycle. Lordosis quotients (LQ) 6 mo after OVX were very low, and were not affected by the substitution of the phytoestrogen-free diet. In contrast, OVX + E2 rats exhibited high levels of receptivity (LQ 78 ± 20), which was reduced in females on the phytoestrogen-free diet (LQ 45 ± 20; $p < 0.05$).

Aldosterone levels at 8 mo of age in females that were intact, OVX, or OVX + E2 at 3 mo of age were modified by gonadal status and by diet (Fig. 3). Intact and OVX + E2 rats had the lowest aldosterone levels, irrespective of diet. OVX rats had higher levels of aldosterone, and dietary phytoestroegens partially ameliorated this effect. Fluid intake (mL/rat/d) from 3 to 9 mo of age was not affected by OVX or substitution of a phytoestrogen-free diet (Table 2).

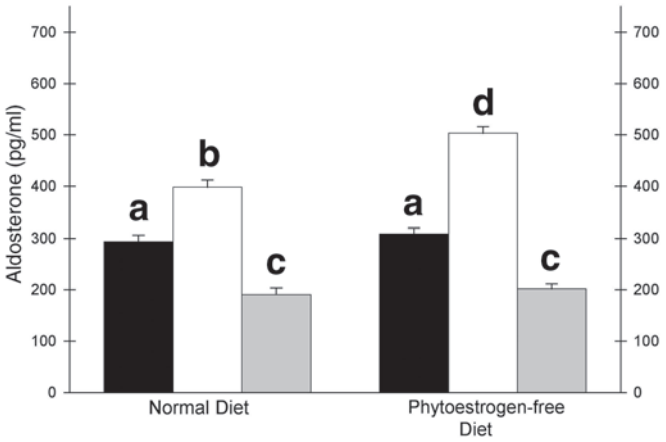

Fig. 3. Plasma aldosterone levels 5 mo after OVX at 3 mo of age and maintained on a normal or a phytoestrogen-free diet from 3 mo of age. Circulating levels of aldosterone were increased in the OVX rats, and to a greater extent in the OVX rats deprived of phytoestrogens. Data are mean ± SEM for six rats per group. Black bars are intact, white bars are OVX, and gray bars are OVX + E2. Different superscripts indicate differences between the groups ($p < 0.01$).

Study 2: Effects of Ovariectomy at 10 mo of Age (Figs. 4–6)

Aging from 10 to 16 mo was associated with a further, albeit modest, increase in systolic blood pressure (approx 15–20 mmHg). This age-related increase was not affected by OVX or E2 replacement in rats on a phytoestrogen-replete diet. However, in rats deprived of dietary phytoestrogens as well as endogenous estrogens, a slowly developing hypertension was observed. The elevated blood pressure, relative to intact and OVX + E2 rats, seen in the OVX rats on the phytoestrogen-free diet developed between 14 and 16 mo of age (Fig. 4). Aging from 10 to 16 mo was associated

Table 2
Daily Fluid Intake (mL/rat/d) in Rats That Were Intact, OVX, or OVX + E2 at 3 mo of Age[a]

		Age at surgery (months after surgery)			
Gonadal status	Diet	3 mo (0)	4 mo (1)	6 mo (3)	8 mo (5)
Intact	Normal	35 ± 1.8	32 ± 1.5	37 ± 1.3	34 ± 0.9
OVX	Normal	34 ± 1.7	31 ± 1.4	34 ± 1.4	29 ± 2.1
OVX + E2	Normal	36 ± 1.3	33 ± 1.2	38 ± 1.5	36 ± 1.7
Intact	Phytoestrogen-free	34 ± 0.9	30 ± 1.9	32 ± 1.3	30 ± 0.9
OVX	Phytoestrogen-free	35 ± 1.2	29 ± 1.8	26 ± 1.9	28 ± 1.9
OVX + E2	Phytoestrogen-free	37 ± 1.4	33 ± 0.9	30 ± 1.7	35 ± 1.2

[a]Data are mean ± SEM. (Intact = sham surgery; OVX = ovariectomized; OVX + E2 = ovariectomized and implanted with Silastic capsules containing estradiol; $n \geq 12$ per group; there were no differences.)

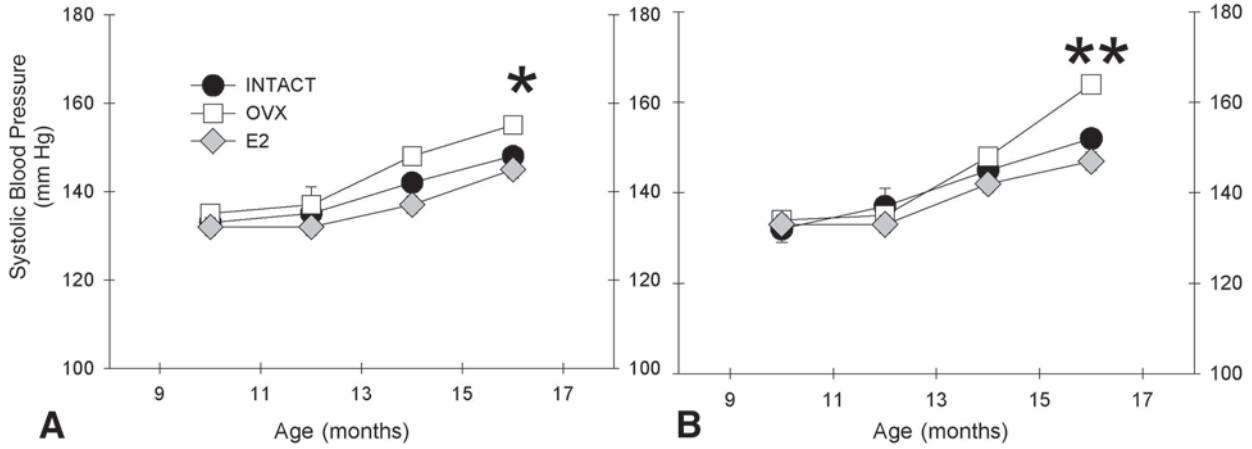

Fig. 4. Effects of OVX at 10 mo of age, with or without estradiol replacement and substitution of a phytoestrogen-free diet at 10 mo of age, on systolic blood pressure: (**A**) normal diet; (**B**) phytoestrogen-free diet. Data are presented as mean ± SEM, with 12–14 rats per group. From 10 to 16 mo of age all rats exhibited a modest increase in systolic blood pressure. There were no differences between any of the groups at 12 mo of age (2 mo post-OVX), but at 16 mo of age the OVX > intact = OVX + E2 on either diet (*$p < 0.01$) roups ($p < 0.01$), blood pressures of the OVX rats on normal chow were lower than those on the phytoestrogen-free diet (**$p < 0.01$).

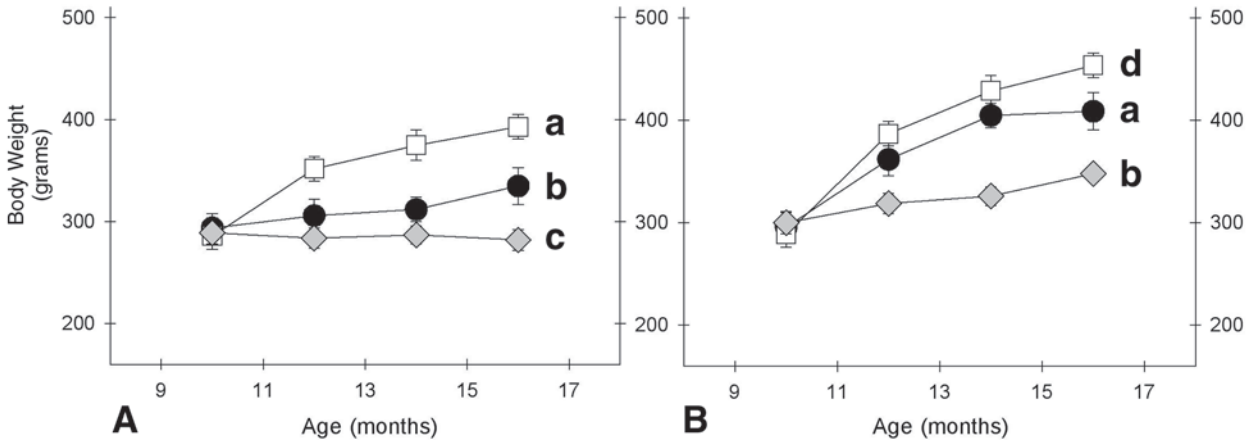

Fig. 5. Effects of OVX at 10 mo of age, with or without E2 and substitution of a phytoestrogen-free diet at 10 mo of age, on body weight: (**A**) normal diet; (**B**) phytoestrogen-free diet. Data are presented as mean ± SEM, with 12–14 rats per group. All groups displayed age-related increases in body weight except the OVX + E2 rats on the phytoestrogen replete diet, who maintained their body weight. Differences in superscripts denote differences between the groups ($p < 0.01$ for all comparisons).

Table 3
Circulating E2 Levels (pg/mL) in Female Rats That Were OVX or Intact, Treated with E2 or Vehicle, and on a Normal or a Phytoestrogen-free Diet Starting at 10 mo of Age

		Age at surgery (months after surgery)			
Gonadal status	Diet	10 mo (0)	11 mo (1)	13 mo (3)	15 mo (5)
Intact	Normal	29 ± 8^a	29 ± 9^a	31 ± 9^a	31 ± 9^a
OVX	Normal	33 ± 9^a	7^b	7^b	7^b
OVX + E2	Normal	31 ± 10^a	75 ± 10^c	77 ± 8^c	80 ± 10^c
Intact	Phytoestrogen-free	30 ± 7^a	32 ± 9^a	29 ± 8^a	27 ± 9^a
OVX	Phytoestrogen-free	28 ± 8^a	7^b	7^b	7^b
OVX + E2	Phytoestrogen-free	27 ± 9^a	80 ± 8^c	78 ± 9^c	82 ± 9^c

Data are mean ± SEM. (Intact = sham surgery; OVX = ovariectomized; OVX + E2 = ovariectomized and implanted with Silastic capsules containing estradiol; $n \geq 12$ per group; ^{a,b,c}groups sharing lettered superscripts are similar, groups with differing lettered superscripts are different from each other, $p < 0.01$.)

Table 4
Daily Fluid Intake (mL/rat/d) in Female Rats That Were OVX, Intact, or OVX + E2, With or Without Dietary Phytoestrogens at 10 mo of Age

		Age at surgery (months after surgery)			
Gonadal status	Diet	9 mo (0)	11 mo (1)	13 mo (3)	15 mo (5)
Intact	Normal	33 ± 0.9^a	32 ± 1.0^a	29 ± 1.1^a	24 ± 1.0^b
OVX	Normal	34 ± 1.1^a	29 ± 0.8^a	23 ± 1.2^b	16 ± 0.9^c
OVX + E2	Normal	32 ± 0.9^a	30 ± 2.0^a	29 ± 0.9^a	27 ± 0.8^a
Intact	Phytoestrogen-free	31 ± 0.9^a	24 ± 1.8^b	20 ± 2.2^b	16 ± 0.2^c
OVX	Phytoestrogen-free	34 ± 1.1^a	20 ± 1.1^b	16 ± 0.9^c	13 ± 0.7^d
OVX + E2	Phytoestrogen-free	32 ± 1.3^a	27 ± 1.9^a	24 ± 1.1^b	20 ± 0.8^b

Data are mean ± SEM. ($n \geq 12$ per group; ^{a,b,c}groups sharing lettered superscripts are similar, groups with differing lettered superscripts are different from each other; $p < 0.01$.)

with a continued increase in body weight, which was accentuated by OVX and attenuated by E2. Dietary phytoestrogen deprivation at 10 mo of age was associated with a further increase in body weight (Fig. 5).

Plasma E2 levels for intact rats in diestrus, or in persistent vaginal estrus, at 10–15 mo of age were similar to those seen in study 1 for rats between 3 and 8 mo of age. OVX rats had circulating levels of E2 that were below the sensitivity of the assay, and OVX + E2 treated rats had elevated levels of estradiol (Table 3). In tests 6 mo after ovariectomy, essentially no receptive behavior was exhibited by the OVX rats, whereas OVX + E2 rats exhibited good lordosis irrespective of dietary phytoestrogens (LQ 96 ± 3 for normal diet and LQ 91 ± 4 for phytoestrogen-free diet).

Fluid intakes were equivalent across groups at 9 mo of age, prior to the study. Fluid intake decreased from 10 to 16 mo of age, an effect that was exacerbated by OVX and the phytoestrogen-free diet, but reversed by E2 treatment (Table 4). Aldosterone levels at 15 mo of age in females that were OVX and hormone treated at 10 mo of age were different by gonadal status and by diet (Fig. 6). Intact and OVX + E2

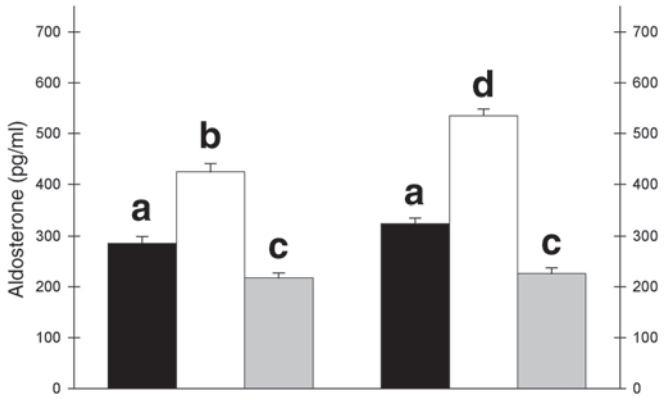

Fig. 6. Plasma aldosterone levels 5 mo after OVX at 10 mo of age maintained on a normal or a phytoestrogen-free diet from 10 mo of age. Circulating levels of aldosterone were increased in the OVX rats, and to a greater extent in the OVX rats deprived of phytoestrogens. Data are mean ± SEM for six rats per group. Black bars are intact, white bars are OVX, and gray bars are OVX + E2. For either diet, OVX > intact > OVX + E2; phytoestrogen deprivation was associated with increased aldosterone in OVX and intact rats. Different superscripts indicate differences between the groups ($p < 0.01$).

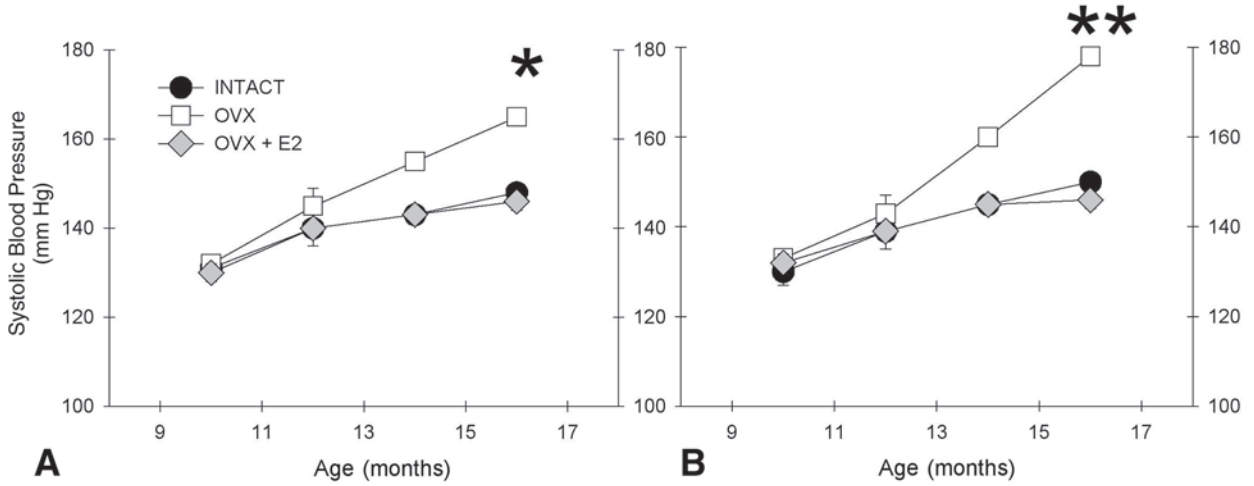

Fig. 7. Effects of OVX at 3 mo of age, with or without E2 and a diet with or without phytoestrogens at 10 mo of age, on systolic blood pressure. Data are presented as mean ± SEM > 12 rats per group for all points ≤ 14 mo of age: (**A**) normal diet; (**B**) phytoestrogen-free diet. From 10 to 14 mo of age the intact and OVX + E2 rats exhibited modest increases in blood pressure. In contrast, larger increases were observed in the OVX rats (*$p < 0.01$ vs intact an OVX + E2 groups). OVX rats on the phytoestrogen-free diet had higher blood pressure at 14 and 16 mo of age than phytoestrogen replete OVX rats (**$p < 0.01$).

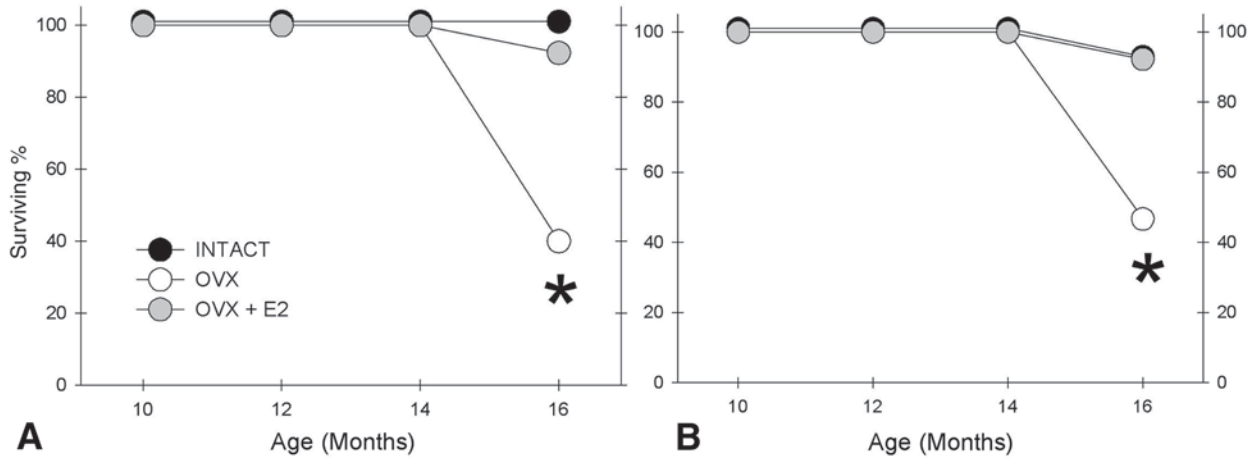

Fig. 8. OVX at 3 mo of age without hormone replacement is associated with premature death on either (**A**) normal or (**B**) phytoestrogen-free diet. Data are percentage surviving. All groups started with 13–14 rats. (*$p < 0.01$).

rats had the lowest aldosterone levels. OVX rats had higher levels of aldosterone. Dietary phytoestrogens were associated with lower aldosterone levels in OVX rats, but higher aldosterone levels in intact rats, and had no effect in OVX + E2 rats.

Study 3: Effects of Ovariectomy at 3 mo of Age with Estradiol Replacement at 10 mo of Age (Figs. 7–9)

OVX at 3 mo of age was associated with a greater weight gain (149 ± 5 g in OVX) over the next 6 mo than that seen in intact females (70 ± 5 g; $p < 0.001$). Despite the difference in weight gain, there was no difference in systolic blood pressure at 9 mo of age (124 ± 3 mmHg for rats OVX, and 126 ± 3 mmHg for intact females). Systolic blood pressure in intact females exhibited modest age-related increases from 10 to 16 mo of age, similar to those seen in study 2. In contrast, females OVX at 3 mo of age demonstrated a greater increase in blood pressure during this period. This further increase was not seen in those rats receiving E2 (OVX at 3 mo + E2 at 10 mo). Removal of dietary phytoestrogens at 10 mo of age did not alter blood pressure in intact or OVX + E2 females, but potentiated the increased blood pressure seen in OVX rats (Fig. 7). Between 14 and 16 mo of age most of the rats OVX at 3 mo of age died (6/15 and 7/15 survived until 16 mo of age for the OVX on the normal and phytoestrogen-free diets, respectively), whereas essentially all of the intact (13/13 on normal and 12/13 on phytoestrogen-free diet) and OVX + E2 treated (12/13 on either normal or phytoestrogen-free diets) rats survived (Fig. 8). In the OVX rats, death was preceded by precipitous weight loss and signs of stroke. During the period from 10 to 14 mo

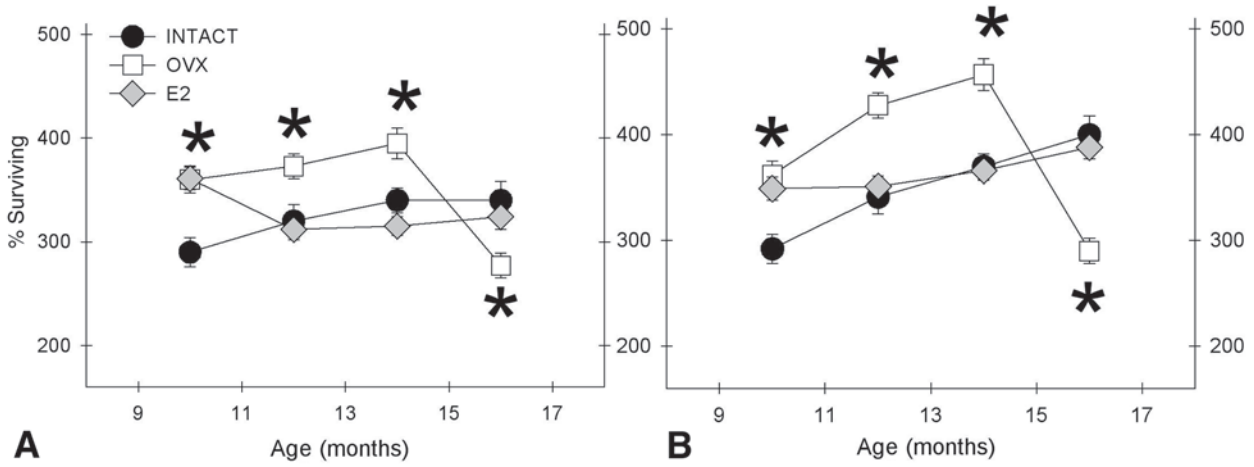

Fig. 9. Effects of OVX at 3 mo of age, with or without E2 and dietary phytoestrogens at 10 mo of age, on body weight. Data are presented as mean ± SEM: (**A**) normal or (**B**) phytoestrogen-free diet. At 10 mo of age, intact rats weighed much less that rats OVX at 3 mo of age ($p < 0.01$). From 10 to 14 mo of age there was a parallel increase in body weight in the intact and OVX, but the OVX + E maintained their 10 mo body weight if they were phytoestrogen replete. From 14 to 16 mo of age, OVX rats lost weight and exhibited signs of stroke and increased mortality. (*$p < 0.01$ vs intact)

Table 5
Plasma E2 Levels (pg/mL) in Rats That Were OVX at 3 mo of Age

		Age at surgery (months after surgery)			
Gonadal status	Diet	10 mo (7)	11 mo (8)	13 mo (10)	15 mo (12)
Intact	Normal	31 ± 9^a	29 ± 9^a	31 ± 9^a	31 ± 9^a
OVX	Normal	7^b	7^b	7^b	7^b
OVX + E2	Normal	7^b	83 ± 8^c	81 ± 9^c	79 ± 12^c
Intact	Phytoestrogen-free	31 ± 7^a	30 ± 10^a	29 ± 11^a	28 ± 7^a
OVX	Phytoestrogen-free	7^b	7^b	7^b	7^b
OVX + E2	Phytoestrogen-free	7^b	79 ± 12^c	78 ± 10^c	79 ± 9^c

E2 and/or substitution of a phytoestrogen-free diet started at 10 mo of age (after 7 mo of hormone deficiency for OVX). Data are mean ± SEM. (Intact = sham surgery; OVX = ovariectomized; OVX + E2 = ovariectomized and implanted with Silastic capsules containing estradiol; $n \geq 12$ per group; ^{a,b,c}groups sharing lettered superscripts are similar, groups with differing lettered superscripts are different from each other, $p < 0.01$.)

of age OVX and phytoestrogen deprivation modified weight gain similarly to study 1 and study 2, but decreased from 14 to 16 mo of age, in conjunction with increased mortality (Fig. 9).

At 10 mo of age, intact rats in diestrus, or in persistent vaginal estrus, had circulating levels of E2 similar to those seen in studies 1 and 2, whereas rats that were OVX 7 mo earlier had undetectable levels of E2. Plasma E2 levels for intact females (approx 30 pg/mL) and OVX rats (≤7 pg/mL) were maintained through 15 mo of age (Table 5). In OVX + E2 rats E2 levels from 11 to 15 mo of age were similar to those reported for study 2, and OVX + E2 rats displayed good receptive behavior at 16 mo of age (LQ > 90, irrespective of diet), whereas the surviving OVX rats did not.

As in Study 2, aging during the period from 10 to 16 mo of age was associated with decrements in fluid intake that were accentuated by ovariectomy and the phytoestrogen-free diet and reversed by estradiol treatment (Table 6). Aldosterone levels at 15 mo of age in females that were ovariectomized and hormones treated at 10 mo of age were different by gonadal status and by diet (Fig. 9). Intact and OVX + E2 rats had the lowest aldosterone levels. OVX rats had higher levels of aldosterone. Dietary phytoestrogens were associated with lower aldosterone levels in ovariectomized rats, but higher aldosterone levels in intact rats, and had no effect in the OVX + E2 rats.

Discussion

The present studies demonstrate that age at estrogen depletion can markedly influence many of the physiological effects. Furthermore, we demonstrate that phytoestrogens

Table 6
Daily Fluid Intake in Female Rats That Were OVX or Left Intact at 3 mo of Age

		Age at surgery (months after surgery)			
Gonadal status	Diet	10 mo (0)	11 mo (1)	13 mo (3)	15 mo (5)
Intact	Normal	35 ± 1.9^{a}	30 ± 1.8^{a}	28 ± 1.6^{a}	23 ± 1.2^{b}
OVX	Normal	33 ± 1.7^{a}	28 ± 1.6^{a}	24 ± 1.7^{b}	17 ± 1.6^{d}
OVX + E2	Normal	31 ± 1.8^{a}	33 ± 1.8^{a}	29 ± 2.1^{a}	25 ± 1.9^{b}
Intact	Phytoestrogen-free	34 ± 1.5^{a}	26 ± 1.9^{b}	21 ± 1.9^{c}	17 ± 1.3^{d}
OVX	Phytoestrogen-free	32 ± 1.9^{a}	22 ± 2.1^{c}	15 ± 1.6^{d}	12 ± 1.0^{e}
OVX + E2	Phytoestrogen-free	30 ± 2.0^{a}	28 ± 1.7^{a}	25 ± 1.9^{b}	21 ± 1.8^{c}

[a,b,c]Treatment with E2 or vehicle, with or without phytoestrogens in the diet at 10 mo of age. Data are mean ± SEM. ($n \geq 12$ per group; [d,e]groups sharing lettered superscripts are similar, groups with differing lettered superscripts are different from each other; $p < 0.01$.)

can contribute to estrogenic modulation of physiological function.

While most studies of the mechanisms of hypertension have focused on men, it is clear that women also experience significant morbidity and mortality from the effects of hypertension. Prior to menopause, women experience significantly less cardiovascular disease than age-matched men. However, following menopause the incidence of cardiovascular disease increases dramatically in women and eventually approaches the incidence in men *(2,14)*.

On average, arterial pressure is significantly higher in males compared to females, but by age 60 the average arterial pressure is higher in females than males *(2,3)*. Even after adjusting for age and body mass index, postmenopausal women are 2.2 times more likely to develop hypertension than premenopausal women *(15)*, and hypertension nearly triples a woman's risk for cardiovascular diseases, including stroke and coronary disease *(16)*.

Despite numerous observational reports, the beneficial effects of estrogen on blood pressure remain controversial. Previous studies had reported that postmenopausal women taking hormone replacement therapy displayed smaller increases in blood pressure over time compared to age-matched women who were not on hormone replacement therapy, and the benefit appeared to be greater at older ages (for example, see ref. *17*). Several other groups have reported that hormone replacement therapy does not lower arterial pressure in similar women. There are several possible explanations for disparate results. One possibility is that resting blood pressure is not altered, but that responses to challenges are increased in the placebo compared to treated women. Hunt et al. *(18)*, for example, reported that estrogen therapy did not change systolic blood pressure or cardiac–vagal baroreceptor gain, but did increase vascular sympathetic baroreceptor gain. These investigators concluded that long-term estrogen replacement has effects on cardiovascular regulation that may not be reflected in resting (i.e., non-stressed) arterial pressure. Similarly, Matthews and colleagues *(19)* reported that women who did not use hormone replacement therapy had higher systolic blood pressure and pulse pressure responses to stressful tasks than did users of hormone replacement therapy. However, a short course (6–8 wk) of transdermal estradiol treatment failed to modify cardiovascular responses to stress *(19)*.

Sympathoinhibitory effects of estrogen in response to sympathoexcitation (apnea, cold pressor test) have been reported in the absence of differences in blood pressure or heart rate *(20)*. Overall, the data on cardioprotective effects of estrogen are equivocal, but, until quite recently the beneficial effects were well accepted. The controversy regarding the beneficial effects of estrogen has been rekindled by the recent reports from the Women's Health Initiative (WHI), and leading to the early cessation of both treatment arms of the study, one arm examined the effects of treatment with PremPro [i.e., conjugated equine estrogens (Premarin) plus medroxyprogesterone acetate (Provera) *(5,6)*], while the other examined the effects of treatment with Premarin only *(7)*. The results of the WHI demonstrated that hormone replacement therapy with PremPro or Premarin provided few of the benefits promised by hormone replacement therapy and had an adverse effect on breast cancer and stroke *(5–8)*. While these data have led many to abandon HRT (e.g., ref. *21*), except for short-term treatment of select groups of women, the true interpretation of the data are clouded by several issues. First, medroxyprogesterone acetate appears to impair arterial endothelial function and may thus counteract some of the beneficial actions of estrogen *(22)*. Second, Premarin is derived from the urine of pregnant horses and contains many estrogenic metabolites that are not normally present in women. Thus, the effectiveness of hormone replacement therapy for the general prevention of disease remains ambiguous. Other important issues are the effects of the duration of estrogen depletion and, a related issue, the age at which hormone replacement starts.

To counter the interpretation put forth by the WHI investigators, several recent studies provide support for a protective effect of estrogen on arterial pressure. In postmenopausal women, estrogen replacement lowers systolic blood pressure (approx 10 mmHg) and pulse pressure (approx 6 mmHg), and reduces age-associated increases in arterial stiffness. Furthermore, the beneficial effects of estrogen appear to be attenuated by the addition of progesterone *(17)*. These effects of estrogen were dependent on the age and status of the individual receiving therapy. Others *(23)* have reported that continued estrogen replacement therapy is associated with improved forearm resistance artery endothelial function, and that this effect is greater in hypertensive women. Alecrin and colleagues *(24)* conducted a prospective double-blind trial and reported that hypertensive postmenopausal women receiving E2 over 12 wk showed significant improvement in left ventricular diastolic function compared to placebo. Thus, the present studies address the important questions of if and how estrogen depletion affects blood pressure in a rat model of aging.

Dietary phytoestrogens have been considered as an alternative to estrogen replacement therapy. Phytoestrogens can directly activate estrogen receptors *(25)*. Compared to E2, phytoestrogens have a relatively low binding affinity for estrogen receptor (ER) α, but binding to ERβ is only about threefold lower than E2 *(25,26)*. ERβ are present in various organs and high concentrations are found in several brain areas in male and female rats *(26)*. In both rats and humans consuming a soy-based diet the circulating plasma concentrations of genistein (one of the more commonly found phytoestrogens) are 100 to 1000 fold greater than those of E2 *(27)*. We have suggested that estrogen and phytoestrogens may act though a common mechanism, via interacting with ERβ *(28,29)*. The present studies demonstrate that dietary phytoestrogens can participate in the regulation of physiological function.

During their lifespan, female rats transition from normal 3–5 d cycles to variable cycles and constant vaginal estrus to chronic anestrus. The initial shift to irregular cycles and constant estrus begins prior to 10 mo of age *(30,31)*. We have observed similar changes in Long–Evans females *(4)*. A previous study from our laboratories indicated that endogenous and dietary estrogens can protect female SHR from salt-sensitive hypertension *(32)*. Our earlier studies, and most studies evaluating the effects of ovariectomy, had used "standard" commercial diets that contain about 0.06% phytoestrogen. Other studies have suggested that this concentration of phytoestrogens could decrease cardiovascular and neuronal damage in animal models *(33–35)*. We, therefore, tested the hypothesis that removal of the phytoestrogens from the diet would exacerbate salt-sensitive hypertension in the ovariectomized SHR. Female SHR were OVX at 3 wk of age and placed on one of four diets: 0.6% or 8.0% NaCl with (0.06 ± 0.02%) or without dietary phytoestrogens *(29)*. In the SHR on the phytoestrogen-free diet, the 8% (vs 0.6%) NaCl caused a 68 ± 8 mmHg increase in mean arterial pressure. In contrast, in the phytoestrogen-replete diet, the 8% NaCl caused only a 23 ± 4 mmHg increase in mean arterial pressure. Furthermore, in the OVX SHR on the basal NaCl diet, elimination of phytoestrogen from the diet slightly increased arterial pressure. Histological examination following 14 wk on the diets demonstrated significant damage (frequent protein casts) in the kidneys of the high salt, phytoestrogen-free diet group but no appreciable damage in any other group. Together, these data indicated that the elimination of all dietary phytoestrogens dramatically exacerbates the hypertensive effect of a high salt diet in OVX SHR *(36)*. Thus, to ensure that estrogen depletion is as complete as possible, OVX rats should be placed on a phytoestrogen-free diet.

In a subsequent study, SHR were OVX at 10 mo of age and placed on a phytoestrogen-free diet, containing either basal or high NaCl. Each rat was then implanted with a Silastic tube containing 17β estradiol or vehicle (E2 treated rats had circulating levels of approx 70 pg/mL). Three months later arterial pressure and anterior hypothalamic norepinephrine metabolite levels [3-methoxy-4-hydroxyphenyl (ethylene) glycol (MOPEG)] were measured. On the basal salt diet, estrogen depleted rats had increased arterial pressure (12 mmHg) and decreased anterior hypothalamic nucleus MOPEG *(36)*. The anterior hypothalamic nucleus is sympatoinhibitory and lesions cause hypertension *(37)*. The increased blood pressure and decreased MOPEG release caused by estrogen deprivation were reversed by E2 *(36)*. In all groups, the high salt diet increased arterial pressure by over 35 mmHg and reduced anterior hypothalamic nucleus MOPEG by >60%. In the rats on the high salt diet, chronic estrogen depletion (compared to intact controls) modestly increased the NaCl-sensitive hypertension and decreased anterior hypothalamic nucleus norepinephrine release. Estradiol replacement prevented these changes. Across all groups, there was a significant inverse correlation between arterial pressure and anterior hypothalamic nucleus MOPEG. These data suggest that both dietary salt excess and estrogen depletion raise arterial pressure in middle-aged female SHR by decreasing anterior hypothalamic norepinephrine *(36)*.

SHR are morbidly hypertensive. The present studies examined the ability of estrogen depletion to raise arterial pressure in young and middle-aged, "normotensive" (Long–Evans) rats. Ovary intact female Long–Evans rats exhibit an age-related increase in blood pressure similar to those in our previous report *(4)*, and still well below that seen in age-matched female SHR *(36)*. In young normotensive females, estrogen deprivation (partial–OVX; or complete–OVX with a phytoestrogen-free diet) or E2 replacement did not significantly alter blood pressure, although in the groups on the phytoestrogen-free diet blood pressures were significantly lower in the OVX + E2 (Fig. 1). In contrast, middle-aged,

intact Long–Evans female rats on a diet that contained phytoestrogens, displayed small increases in arterial pressure (15 mmHg) between 10 to 16 mo of age, and removal of phytoestrogens from their diet at 10 mo of age did not accelerate the age-related increase in blood pressure. In the group fed the normal (with phytoestrogens) diet, OVX at 10 mo of age augmented the arterial blood pressure rise. Combined removal of phytoestrogens from the diet and OVX further elevated arterial pressure. Estrogen treatment of OVX Long–Evans rats prevented the arterial pressure differences (Fig. 4). In normotensive rats OVX at 3 mo of age, removal of phytoestrogens from the diet at 10 mo of age was associated with a greater increase in blood pressure (Fig. 7). Long-term OVX was associated with premature death irrespective of dietary phytoestrogens (Fig. 8).

There are important differences in OVX females depending on whether phytoetrogens were present in the diet. These data are corroborated by ongoing studies in our laboratories, and others. Genistein or estrogen therapy was reported to correct endothelial dysfunction *(38)*, and to reduce contractile responses and to lower blood pressure in OVX rats *(39)*. Another group reported that dietary soy exerted an antihypertensive effect in OVX SHR, which was dependent on sympathetic activity *(40)*. There are also reports of beneficial effects of phytoestrogens in humans (e.g., ref. *41–43*).

The effectiveness of E2 replacement with the Silastic capsules was verified with (a) periodic hormone measurements (Tables 1, 3, and 5), (b) monitoring the decreased weight gain in intact and estradiol treated rats (Figs. 2, 5, and 9), an effect which does not appear to be age-dependent, and (c) by the display of lordosis behavior in long-term estradiol-treated rats.

Interestingly, substitution of a phytoestrogen-free diet for 6 mo was associated with reduced receptive behavior (lordosis) in response to male mounting in OVX + E2 at 3 mo of age, but not in those that were OVX + E2 at 10 mo of age. These data support the suggestion that the effects of phytoestrogens may include a positive modulation of sexual function in young, but not middle-aged, rats. We have hypothesized that estrogens may exert their beneficial effects through ERβ in the anterior hypothalamic nucleus *(29,38)*. The anterior hypothalamic–preoptic area is thought to be inhibitory to the expression of female sexual behavior *(44)*. In contrast to our observation that phytoestrogen depletion may reduce sexual receptivity, Patisaul et al. have reported that soy isoflavones inhibit steroid-induced lordosis in short-term OVX [several weeks post-OVX *(45,46)*], whereas genistein had no effect *(47)*. This group has reported that soy isoflavones or genistein preferentially affect ERβ, and not ERα regulated gene expression in brain, and exert agonist as well as antagonistic effects on ERβ *(45–47)*. The disagreement between our studies could be related to the amount of phytoestrogen, length of depletion or exposure, or to the post-OVX interval.

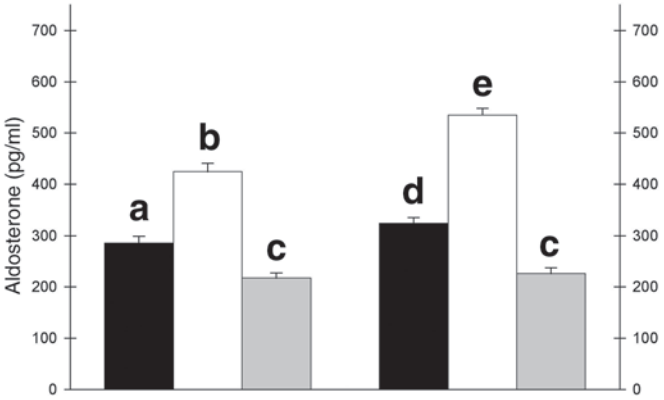

Fig. 10. Plasma aldosterone levels 12 mo after OVX at 3 mo of age and maintained on a normal or a phytoestrogen-free diet from 10 mo of age. Circulating levels of aldosterone were increased in the OVX rats, and to a greater extent in the OVX rats deprived of phytoestrogens. Data are mean ± SEM for six rats per group. Black bars are intact, white bars are OVX, and gray bars are OVX + E2. For either diet, OVX > intact > OVX + E2; phytoestrogen deprivation was associated with increased aldosterone in OVX and intact rats. Different superscripts indicate differences between the groups ($p < 0.01$).

We also observed an age-dependent effect of estrogen depletion on fluid intake (Tables 2, 4, and 6). There was no effect of estrogen depletion in young rats, but fluid intake was reduced in rats that were estrogen depleted at 10 mo of age. Fluid intake and urinary output are important homeostatic mechanisms to regulate extracellular fluid volume and electrolytes, and, consequently, blood pressure. Estrogenic stimulation (or maintenance) of fluid intake may be one mechanism for estrogen-mediated decreased blood pressure because we observed that increased fluid intake was associated with lower blood pressures. Furthermore, the effects of estrogen depletion on blood pressure and fluid intake are dependent on the age at which depletion occurs.

A number of reports (e.g., refs. *48–51*) support the suggestion that estrogen has multiple effects on components of the renin–angiotensin–aldosterone system. Aldosterone is an important regulator of extracellular fluid sodium, and sodium is a major regulator of extracellular fluid volume. Paradoxically, aldosterone is increased in states of fluid expansion, such as long-standing congestive heart failure. We hypothesized that estrogen depletion would be associated with increased circulating levels of aldosterone, and that long-term increases in aldosterone are associated with increased blood pressure. Our data indicate that there is no age-related increase in aldosterone in intact or OVX + E2 rats, but that OVX is associated with increased aldosterone levels, which are tempered by dietary phytoestrogens (Figs. 3, 6, and 10). Tang *(52)* reported that females have higher aldosterone levels than age-matched males, and the absence of age-related decrements in circulating aldosterone has been reported *(52,53)*. We suggest that there is an inverse inter-

action between estradiol and aldosterone in the regulation of blood pressure. This interaction, most likely, involves peripheral (kidney and vasculature) and central (autonomic) sites.

Gonadal status also influences body weight gain in Long–Evans females. In rats OVX at 3 mo of age there was a dramatic increase in weight gain from 3 to 9 mo of age, which was prevented by estradiol treatment, and blunted by dietary phytoestrogens (Fig. 2). Rats that were OVX at 10 mo of age showed a greater increase in weight from 10 to 16 mo of age, which was reversed by estradiol treatment and blunted by dietary phytoestrogens (Fig. 5). It should be noted that the early increase in body weight is not associated with increased blood pressure, but that long-term OVX and the associated increase in body weight, coupled to decreased fluid intake and increased aldosterone, are associated with greater middle-aged increases in blood pressure (Fig. 7) and premature mortality (Fig. 8). The experimental literature associating excess weight with reduced longevity is sparse, but overweight is a well-established risk factor for human cardiovascular disease and stroke *(54–56)*. Conversely, a voluminous literature supports the suggestion that chronic reductions in caloric intake are associated with increased lifespan in animals and humans *(57–60)*.

In "normal" Long–Evans females, systolic pressure increases from 115 mmHg at 3 mo of age, to 135 mmHg at 10 mo of age, further increasing to 150 mmHg by 16 mo of age *(4)*. These data parallel those obtained in humans and support the use of the middle-aged rat as a valid model for age-associated human disease. It is important to note that most studies examining the effects of estrogen depletion on blood pressure and stroke damage have utilized young females. Many physiological functions change with age, and, thus, an estrogen-depleted young female is not equivalent to an estrogen-depleted older female. Similarly, recent epidemiological data indicated that optimal prevention of late-life stroke will likely require control of mid-life increases in blood pressure *(61)*.

In summary, our present data indicate that (a) in general, estrogens, including dietary phytoestrogens, prevent or attenuate OVX-induced increases in blood pressure; (b) the effects of estrogen depletion are dependent on age—OVX at 3 mo age had no effect on systolic blood pressure over the next 6 mo, whereas OVX at 10 mo of age was associated with higher systolic blood pressure at 16 mo of age, and this effect was exacerbated in rats consuming a phytoestrogen-free diet; (c) OVX at 3 mo and initiation of estrogen therapy at 10 mo revealed that long-term ovariectomy was associated with increased systolic blood pressure and mortality at 14–16 mo of age; (d) circulating levels of estradiol do not change with age (3–16 mo), but estrogen depletion increases plasma aldosterone. We conclude that the effects of estrogen depletion vary with age (at least in rats); that phytoestrogens exert some protective effects; and that long-term ovariectomy is associated with premature mortality. We suggest that the deleterious effects of estrogen depletion involve the renin–angiotensin–aldosterone system, as well as the sympathetic nervous system.

Materials and Methods

Animals

Female Long–Evans rats (Simonsen Laboratories, Gilroy, CA) arrived in the laboratory at 6–7 wk of age and were housed two per cage. They were provided food and water *ad libitum* and maintained in a temperature and light (lights off, 12:00 h; lights on, 22:00 h) controlled environment within an AALAC accredited facility. The 14 h of light and 10 h of darkness was used for all studies. All experimental procedures were conducted in accordance with National Institutes of Health guidelines and were approved by the Institutional Animal and Use Committee at Meharry Medical College.

In our laboratory we have repeatedly observed that manipulations performed in the late light phase (3–4 h prior to lights off) do not affect changes in activity and ingestive behavior that typically occur with the light:dark transition. These manipulations have included vaginal smears, tail-cuff blood pressure measurements, erectile reflex tests, and blood sampling *(4)*. Furthermore, this period is a period of stable blood pressure *(62,63)*, and previous reports indicate that diurnal–nocturnal differences in blood pressure are reduced with aging (e.g., ref. *63*; Wyss and Clark, unpublished observations). Thus, in the present studies all manipulations were done during the late part of the light phase, being completed at least 2 h prior to the light:dark transition. Reproductive status (diestrous, proestrous, estrous, or persistent vaginal estrous) in intact females was monitored by examination of vaginal smears, as previously described by us *(4)*.

Circulating Hormone Levels

Estradiol

In previous studies estradiol was quantified using radioimmunoassay methods (e.g., refs. *4,36*). In the present studies, plasma estradiol was quantified using a competitive binding enzyme immunoassay kit (Active Estradiol EIA DSL-10-4300; Diagnostic Systems Laboratory, Inc., Webster, TX) using a Bio-Rad Model 680 Microplate Reader. Data were analyzed using the microplate manager software (v 5.2, Bio-Rad Laboratories, Hercules, CA). The sensitivity of the assay was 7 pg/mL. Where levels were below the sensitivity of the assay, a value of 7 pg/mL was used. Assays were performed at the conclusion of the studies, and all samples from any individual animal were measured in the same assay. Intra- and interassay coefficients of variation were 7.5% and 10.2%, respectively, for pooled sampled averaging 50 pg/mL.

Aldosterone

In previous studies (e.g., ref. *64*), we measured circulating aldosoterone levels using radioimmunoassay methods. In the current studies, aldosterone was measured in the final samples from six rats for each group in each study, using an EIA kit from Cayman Chemical, with incubation for 18 h at 4°C prior to development. The antibody does not cross react with other relevant corticosteroids, androgens, or estrogens (<0.01%). Sensitivity of the assay was 8 pg/mL. All samples for a study were done in the same assay. Intra- and interassay coefficients of variation were 9.2% and 13.6%, respectively, for pooled samples averaging 100 pg/mL.

Measuring Systolic Blood Pressure

Systolic blood pressures were obtained using tail-cuff plethysmography, as previously described by us *(64–66)*. Briefly, indirect arterial pressures were monitored using a tail-cuff plethysmography system *(67,68)*. Systolic arterial blood pressure was measured in conscious animals with an automated system (Model 179; IITC Life Science Instruments, Woodland Hills, CA) that utilizes tail cuffs and photoelectric sensors to detect tail pulses *(64–66)*. Rats are restrained in holders appropriate for their body weight in a test chamber maintained at 29°C. Blood pressure was evaluated in three sessions during a 5 d period, and values will represent the mean of those obtained in the last two sessions. Each session involved at least five blood pressure determinations.

Study 1: Effects of Ovareictomy at 3 Months of Age

After a 2 wk acclimation period, systolic blood pressure was measured noninvasively using tail-cuff plethysmography. After three to five blood pressure measurements were obtained, the animals were weighed. The female rats were then assigned to one of six treatments groups such that there were no differences in blood pressure or body weight prior to surgery. The six groups were (a) intact (Sham ovariectomy) with normal rat chow (Teklad 8640; $n = 12$); (b) OVX with normal rat chow ($n = 14$); (c) OVX + E2 and normal rat chow ($n = 13$); (d) intact with a phytoestrogen-free diet (Teklad AIN-76A; $n = 12$); (e) OVX with a phytoestrogen-free diet ($n = 14$); (f) OVX + E2 and a phytoestrogen-free diet ($n = 13$). Ovariectomies were done using a ventral approach under isoflurane anesthesia (e.g., ref. *4*). After the ventral incision was closed, the rat was placed on its stomach and the midscapular region was shaved. Estradiol treatment used a Silastic capsule 10 mm in length, filled with crystalline estradiol benzoate, and sealed at both ends with Silastic Medical Adhesive that had been preincubated in phosphate-buffered saline, which was implanted subcutaneously in the midscapular region in those rats receiving estradiol. Previous studies from our laboratories indicate that these pellets release estradiol at the rate of approx 20 mg/kg/d *(13)*. The remaining groups received empty implants. All animals received 500 µg gentamycin, im, after surgery. Silastic capsules were replaced after the blood pressure measurements taken 3 mo after surgery were completed (in this case, at 6 mo of age). Immediately preceding surgery, and periodically (at 4, 6, and 8 mo of age—that is, 1, 3, and 5 mo after surgery), intact females exhibiting diestrous vaginal smears *(4)*, as well as OVX and OVX + E2 females, were anesthetized with isoflurane and a blood sample (1.5 mL) was obtained by jugular venisection, 3–4 h prior to lights off *(4)*. Blood was collected in heparinized syringes, and right and left sides were alternated for jugular sampling. Approx 3 min elapsed between the initial disturbance of the animal to completion of sampling. Animals received 500 µg gentamycin, im, after each venisection. Plasma was separated and stored at –20°C for subsequent determinations of estradiol and aldosterone.

Systolic blood pressure was measured immediately prior to surgery, and bimonthly thereafter. In intact rats, blood pressure was determined while the rats were exhibiting vaginal smears indicative of diestrus. Body weight was also recorded bimonthly. During the week preceding the blood pressure measurements, fluid intake was determined. For this, the rats were given a premeasured amount of water and the amount of fluid consumed was recorded daily. An average daily fluid intake was determined for the 7 d period and is expressed as mL/rat/d for each animal. As an additional index of estrogen action, or lack thereof, the ovariectomized rats (with or without estradiol treatment) were tested for the display of receptive behavior (lordosis) following 6 mo after surgery. This was done by placing the female in a cage with a sexually vigorous male and observing for lordosis in response to mounting by the male.

Study 2: Effects of Ovariectomy at 10 mo of Age

After a 2 wk acclimation period, systolic blood pressure was measured noninvasively. After three to five blood pressure measurements were obtained, the animals were weighed. An additional series of blood pressure measurements and body weights were obtained at 10 mo of age (presurgery values). The female rats were then assigned to one of six treatments groups, such that there were no differences in blood pressure or body weight prior to surgery. The six groups were identical to those for study 1: (a) intact with normal rat chow (Teklad 8640; $n = 12$); (b) OVX with normal rat chow ($n = 14$); (c) OVX + E2 and normal rat chow ($n = 13$); (d) intact with a phytoestrogen-free diet (Teklad AIN-76A; $n = 12$); (e) OVX with a phytoestrogen-free diet ($n = 14$); (f) OVX + E2 and a phytoestrogen-free diet ($n = 13$). As in study 1, ovariectomies were done using a ventral approach under isoflurane anesthesia. A Silastic capsule filled with crystalline estradiol benzoate was implanted subcutaneously in those rats receiving estradiol. All animals received 500 µg gentamycin, im, after each surgery. Silastic capsules were replaced after the blood pressure measurements taken 3 mo (in this case, at 12 mo of age) after sur-

gery were completed. Immediately preceding surgery, and periodically thereafter (at 10, 12, and 14 mo of age—that is, 1, 3, and 5 mo after surgery) intact females exhibiting vaginal smears indicative of diestrus or persistent vaginal estrus, as well as OVX and OVX + E2 rats were anesthetized with isoflurane and a blood sample (1.5 mL) obtained by jugular venisection, 3–4 h prior to lights off. Blood was collected in heparinized syringes, and right and left sides were alternated for jugular sampling. Approximately 3 min elapsed between the initial disturbance of the animal to completion of sampling. Animals received 500 μg gentamycin, im, after each venisection. Plasma was separated and stored at –20°C for subsequent determinations of estradiol and aldosterone.

Systolic blood pressure was measured immediately prior to surgery, and bimonthly thereafter. In intact rats, blood pressure was determined while the rats were exhibiting vaginal smears indicative of diestrus, or persistent vaginal estrus. Body weight was also recorded bimonthly. During the week preceding the blood pressure measurements, fluid intake was determined. For this, the rats were given a premeasured amount of water and the amount of fluid consumed was recorded daily. An average daily fluid intake was determined for the 7 d period and is expressed as mL/rat/d for each animal. As an additional index of estrogen action, or lack thereof, the OVX and OVX + E2 rats were tested for the display of receptive behavior (lordosis) following 6 mo after surgery. This was done by placing the female in a cage with a sexually vigorous male and observing for lordosis in response to mounting by the male.

Study 3: Effects of Ovariectomy at 3 mo of Age with Estradiol Replacement at 10 mo of Age

After a 2 wk acclimation period, systolic blood pressure was measured noninvasively. After three to five blood pressure measurements were obtained, the animals were weighed (presurgery values). Animals were assigned to undergo either ovariectomy ($n = 60$) or sham surgery ($n = 26$). Ovariectomies were performed using the ventral approach under isoflurane anesthesia with 500 μg gentamycin injected im after each surgery. An additional series of blood pressure measurements and body weights were obtained at 10 mo of age (pretreatment values). The intact (sham-ovariectomized) rats were then assigned to one of two groups, such that there were no differences in blood pressure or body weight prior to assignment. One-half of the rats remained on the normal rat chow, while the other half was switched to a phytoestrogen-free diet at 10 mo of age. Two OVX rats died while aging from 3 to 9 mo of age. The rats that had been OVX at 3 mo of age were assigned to one of four treatment groups; (a) OVX at 3 mo of age with normal rat chow and empty capsule at 10 mo of age ($n = 15$); (b) OVX at 3 mo of age with a phytoestrogen-free diet and an empty capsule at 10 mo of age ($n = 15$); (c) OVX at 3 mo of age with a normal rat chow diet and an estradiol-containing capsule implanted at 10 mo of age (OVX + E2; $n = 14$); and (d) OVX at 3 mo of age with a phytoestrogen-free diet and an estradiol-containing capsule at 10 mo of age (OVX + E2; $n = 14$). A Silastic capsule filled with crystalline estradiol was implanted sc in those rats receiving estradiol under isoflurane anesthesia. Silastic capsules were replaced after the blood pressure measurements taken 3 mo (in this case, at 12 mo of age) after surgery was completed. Immediately preceding surgery, and periodically thereafter (at 10, 12, and 14 mo of age—that is, 1, 3, and 5 mo after estradiol or dietary change), intact females exhibiting vaginal smears indicative of diestrus or persistent vaginal estrus, as well as OVX and OVX + E2 rats were anesthetized with isoflurane and a blood sample (1.5 mL) obtained by jugular venisection, 3–4 h prior to lights off. Blood was collected in heparinized syringes, and right and left sides were alternated for jugular sampling. Approximately 3 min elapsed between the initial disturbance of the animal to completion of sampling. Animals received 500 μg gentamycin, im, after each venisection. Plasma was separated and stored at –20°C for subsequent determinations of estradiol and aldosterone.

Systolic blood pressure was measured immediately prior to surgery, and bimonthly thereafter. In intact rats, blood pressure was determined while the rats were exhibiting vaginal smears indicative of diestrus, or peristent vaginal estrus. Body weight was also recorded bimonthly. During the week preceding the blood pressure measurements, fluid intake was determined. For this, the rats were given a premeasured amount of water and the amount of fluid consumed was recorded daily. An average daily fluid intake was determined for the 7 d period and is expressed as mL/rat/d for each animal. As an additional index of estrogen action, or lack thereof, the ovariectomized rats (with or without estradiol treatment) were tested for the display of receptive behavior (lordosis) following 6 mo after surgery. This was done by placing the female in a cage with a sexually vigorous male and observing for lordosis in response to mounting by the male.

Statistics

Data are presented as mean ± SEM. Within each study, data were evaluated using a two-factor ANOVA (factors were gonadal status, three categories; and diet, two categories) for each data collection point, with repeated measures for estradiol, body weight, fluid intake, and systolic blood pressure. Age interactions were assessed using a three-factor ANOVA. Post-hoc tests where utilized as appropriate. Where data failed normalcy tests, nonparametric statistical analyses were used. Values where $p \leq 0.05$ were considered significant.

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