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# Comparison of Two Methods of Estradiol Replacement: their Physiological and Behavioral Outcomes

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## Abstract

Fluctuating sex steroids during the estrous or menstrual cycle of mammalian females make it difficult to determine their role on behaviors and physiology. To avoid this, many investigators ovariectomize their animals and administer progesterone, estradiol or a combination of both. Several different strategies are used to administer estradiol, which confounds interpretation of results. This study compared two methods of estradiol replacement implants: Silastic tubes filled with crystalline estradiol benzoate (E2) and commercially available estradiol benzoate pellets. Implants were placed subcutaneously in adult ovariectomized (OVX) rats and blood samples obtained weekly. Control OVX rats received empty Silastic tubes or placebo pellets. Our data shows that E2 plasma levels from rats with Silastic implants peaked after one week and decreased slowly thereafter. In contrast, plasma E2 from commercial pellets peaked after two weeks, increasing and decreasing over time. To validate hormone release, body weight was monitored. All E2 treated animals maintained a similar body weight over the four weeks period whereas an increase in body weight over time was observed in the OVX group that received empty implants, confirming E2 release and supporting the role of E2 in the regulation of body weight. Furthermore, the effects of E2 on basal locomotor activity were assessed using animal activity cages. Results showed no difference between E2 and control group in several locomotor activities. These results indicate that Silastic implants achieve more stable plasma estradiol levels than pellets and thus are a better alternative for studies of estradiol on brain function and behavior.

**Keywords:** Estradiol; Silastic implants; Body weight; Estradiol pellets; Locomotor activity

## Abbreviations

E2: Estradiol; OVX: Ovariectomized

## Introduction

Estrogens are a family of steroid hormones derived from cholesterol that include estrone, estriol, and estradiol. Estradiol is the most abundant sex steroid in pre-menopausal woman and has the highest estrogenic activity. Estradiol is produced mainly in the ovaries and to a lesser extent, by the adrenal cortex. Several tissues, such as the brain, produce estradiol for local use, mainly by aromatization of androgens to estradiol. Estradiol's effects are exerted mainly by binding to intracellular receptors. The estrogen-receptor (ER) binds to another ER, and this dimer is translocated to the nucleus. The hormone-receptor complex binds to specific sites on the DNA (estrogen response elements) and activates or represses transcription of target genes. Estradiol may also bind, or interact, with membrane receptors, and modulate aperture of ionic channels or promote activation of second messenger systems, resulting in a faster response [1]. Indeed, the membrane receptor, GPER (also known as GPR30), a 7 transmembrane G-protein coupled receptor that binds estradiol, was recently described to mediate many of estradiol's rapid effects [1].

In addition to its well-known role in the regulation of reproductive function, particularly in the female, estradiol exerts multiple effects on non-reproductive tissues, such as adipose, bone and neural tissue. The brain, particularly areas involved in reproductive function and in learning and memory, are rich in estrogen receptors [2]. Estradiol also plays a regulatory role in several aspects of neural plasticity, such as cell growth, axonal elongation, synapse formation, and neuronal differentiation [3,4]. Studies in vitro and in vivo have clearly shown that estradiol is also a potent neuroprotective agent [3], protecting and minimizing the deleterious effects of several neurodegenerative disorders, such as Parkinson's and Alzheimer's disease [5] as well as and in brain and spinal cord injury [6-8].

Estradiol also plays an important role in modulating sexual and social behavior in many species [9]. In studies of gender differences, estradiol is found to influence mood, aggressiveness, locomotor activity, and a large range of affective disorders. For example, changes in circulating levels of this steroid are associated with anxiety and depressive disorders such as postpartum depression, perimenopausal depression, and premenstrual dysphoric symptoms [10]. Estradiol also regulates adiposity, energy balance and the expression of several of the peptides involved in hypothalamic regulation of appetite. Indeed, gonadectomy usually results in an increase in body weight, although the exact mechanism has not been clearly established [11,12].

Our laboratories study the effects that estradiol exerts on neural substrates that regulate the response to drugs of abuse and recovery after spinal cord injury. Females in our studies are usually ovariectomized and receive estradiol replacement by Silastic implants. The method of estradiol replacement is the subject of much debate among neuroendocrinologists. In some studies estradiol is administered by daily subcutaneous injections. This method has the disadvantage of the stress associated with the injection and daily declining estradiol plasma levels as the estradiol bolus is metabolized [13,14]. To avoid daily injections, which can cause distress to the

animal, and in some cases ulceration, estradiol replacement therapy can also be accomplished by implanting a device (Silastic tube, commercial pellet, osmotic pump) that releases estradiol [15].

The use of a subcutaneous Silastic tube filled with estradiol benzoate has several advantages. It provides constant hormone release and it can be prepared in the laboratory at a relatively low cost. The disadvantage is that it is time consuming and the consistency in the amount of estradiol released will depend on the amount of estradiol packed into the Silastic tube, as well as the length, diameter and permeability of the Silastic tube. Other commonly employed methods for estradiol administration include subcutaneous insertion of commercial pellets (Innovative Research (Sarasota, FL) or osmotic mini-pumps (for example Alzet™). Commercial pellets and Alzet mini-pumps are good alternatives to the Silastic tubes, but are much more expensive.

In our fields of study, the literature contains many reports of contradictory effects of estradiol. We surmised that these discrepancies may be attributed in part to differences in the method of estradiol replacement and in the methodology used to measure plasma estradiol, particularly when purchasing commercially available RIA kits. Several studies have demonstrated the great variability that exists in the values of plasma estradiol obtained depending on the method (RIA, ELISA), type of kit (coated tubes versus secondary antibodies) and company used [16,17].

This study was designed to evaluate plasma levels of estradiol attained by two commonly used methods of estradiol replacement: commercial pellets and Silastic tube implants. For each method we used different doses of estradiol pellets and of Silastic tubes and measured plasma estradiol and body weight every week for 4 weeks. In addition, we assessed the effect of constant exposure to estradiol on locomotor activity and anxiety behaviors.

## Materials and Methods

### Animals and housing

Adult female Sprague-Dawley rats of approximately 200 g were purchased from Hilltop Lab Animals (Scottdale, PA). Rats were maintained in a 12:12-h light-dark cycle (lights off at 6 pm), in a temperature (25°C) and humidity controlled room at the University of Puerto Rico, Medical Sciences Campus (UPR MSC) AAALAC accredited animal facility. Animals were housed two per cage with tap water and food (TEK 22/5 rodent diet 8640) provided ad libitum. A period of 1 week was given for acclimation to the animal facilities before any animal manipulation. All animal experimental procedures followed the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) from the UPR MSC.

### Estradiol implants

Silastic tubing implants were prepared according to Legan et al. and modified according to Febo et al. [18,19]. Briefly, 5 mm Silastic tubes (1.47 mm internal diameter, 1.97 mm outside diameter; Dow Corning, distributed by Fisher Scientific, Cayey, Puerto Rico) were filled with 3, 4 and 5 mg of 17-β-estradiol 3-benzoate (Sigma-Aldrich, St. Louis, MO, USA) or left empty. These tubes were sealed at each end with sterile silicone adhesive sealant. Silastic implants were placed in a 0.9% sterile saline solution 3 hrs prior to use to confirm the integrity of the tubes. Those that did not float at the end of this period were discarded. Commercial pellets of 17-β-estradiol (3 mg and 4 mg) were purchased from Innovative Research (Sarasota, FL) to compare its delivery efficiency with that of the estradiol-filled Silastic tubing. Placebo pellets composed of cholesterol and other "inert" ingredients (personal communication) were purchased to use as controls. All implants were inserted in the midscapular region immediately after ovariectomy to reduce alterations in the physiology of the animal, such as the increase in appetite, weight and anxiety among others.

### Surgical procedures

To eliminate the main endogenous source of plasma estradiol and thus control for estradiol cyclical variability, animals were bilaterally ovariectomized. Rats were anesthetized with ketamine (87.5 mg/kg) and xylazine (4.2 mg/kg) diluted in 0.9% sterile saline and administered intraperitoneally. The procedure was performed under aseptic and sterile conditions. Briefly, the anesthetized rat was placed on its ventral surface and the ovaries were removed via a 1 cm incision on the dorsum of the animal caudal to the posterolateral border of the ribs. The fascia was separated from the skin and a 7 mm incision was made in the muscle to gain access to the peritoneal cavity. The oviducts were clamped, ligated and the ovaries removed. The muscle was then sutured and the skin incision closed with staples. Immediately after removal of the ovaries, the empty or estradiol filled Silastic tubes, as well as the placebo or estradiol pellets, were placed subcutaneously in the midscapular region and the skin sealed with a clamp.

### Animal groups and treatment

Female rats were divided into 9 groups. Our first group, gonadally intact rats, served as a control, they represented a group of gonadally intact female rats with plasma estradiol fluctuating throughout their estrous cycle. The second control group, ovariectomized, was comprised of rats that had both their ovaries removed; these represent rats devoid of ovarian plasma estradiol. The third control group, ovariectomized with an empty Silastic implant, was a control for ovariectomized rats that received a Silastic implants with 3, 4 or 5 mg of estradiol (Groups 5-7). The fourth group, ovariectomized rats with a cholesterol pellet, served as a control for ovariectomized rats that received a commercial pellet of 3 or 4 mg of estradiol dissolved in cholesterol. Groups 5 to 9 were experimental groups, designed to evaluate two methods of estradiol replacement and determine which provided the most consistent method of estradiol delivery. These were ovariectomized rats that received a Silastic implant of 3, 4 or 5 mg of estradiol benzoate (Groups 5-7) and ovariectomized rats that received an estradiol pellet with 3 or 4 mg of estradiol (Groups 8 and 9). Below is a list of these groups:

Group 1: Gonadally intact rats.

Group 2: Ovariectomized.

Groups 3: Ovariectomized that received an empty Silastic implant.

Group 4: Ovariectomized that received a pellet containing cholesterol.

Group 5: Ovariectomized that received 3 mg of estradiol in a Silastic implant.

Group 6: Ovariectomized that received 4 mg of estradiol in a Silastic implant.

Group 7: Ovariectomized that received 5 mg of estradiol in a Silastic implant.

Group 8: Ovariectomized that received a 3 mg estradiol pellet.

Group 9: Ovariectomized that received a 4 mg estradiol pellet.

### Animal weight determination

To obtain a physiological measurement of the effectiveness of ovariectomy and of estradiol replacement, we measured body weight, since body weight can be used as a physiological parameter of estradiol [12,20]. Animal body weight was determined weekly for 4 weeks after ovariectomy and implant insertion, and evaluated in relation to the dose of estradiol given.

### Behavioral tests

An automated animal activity cage system (Versamax TM system), purchased from AccuScan Instrument (Columbus, Ohio, USA) was used to determine the effect of plasma estradiol on locomotion, exploratory and anxiety related behaviors. The cages were located in an isolated room, at 25°C with low illumination. The system consisted of 10 acrylic cages (42cm × 42cm × 30cm) with 16 infrared beams equally spaced across the length and width of the cages at a height of 2 cm from the cage floor (to monitor horizontal movement). Another set of infrared beams was located at a height of 10 cm from the cage floor to monitor vertical movement [21]. Beam data was displayed through Versadat$^{R}$, a windows-based program. The program differentiates between stereotyped and horizontal locomotor activity based on repeated interruption of the same beam or sequential breaking of different beams, respectively. Every week during the afternoon for a period of 4 weeks, animals were placed individually in the cages for 60 minutes. Horizontal and vertical activity, total distance, and time spent in the center of the cage were assessed.

### Radioimmunoassays

A weekly blood sample (0.8 ml) was obtained from the tail, and placed in a 1.5 ml microtube. Samples were centrifuged at 5,000 rpm for 5 minutes (4°C), to separate the plasma from the hematocrit. Plasma was stored at -80°C until the day of the assay. Total plasma estradiol level was determined using the Double Antibody RIA kit (MP biomedical Costa Mesa, California, USA) or the Coat-A-Count RIA kit (TKE22 Diagnostic Product Corporation, Los Angeles, California, USA). The size of the sample required for the Double Antibody RIA kit was 50 µl, and for the Coat-A-Count RIA kit was 100 µl. The assays were conducted according to the instructions supplied by the manufacturer. After the procedure was concluded, the samples were counted for 1 min in a gamma counter (Beckman Gamma 5500B) to determine the counts per minute. Determination of hormonal levels was interpolated from a standard curve prepared in triplicate using standard calibrators and quality control serum specific for each RIA kit. The calculation of the data reduction was performed by linear regression and logit-log representation with the assistance of computer software. All samples, and calibration standards, were assayed in duplicate. Standardization of the results was done by calculating the difference in estradiol concentration measured by both RIA kits in the same plasma sample.

### Statistical analysis

Plasma estradiol levels obtained from each hormone delivery system were compared using One-way ANOVA, followed by the FISHER multiple comparison test or Student-Newman-Keuls multiple comparisons test. The effect of estradiol on body weight was determined with One-way ANOVA, followed by Tukey-Kramer multiple comparisons test. Behavioral assays were analyzed using a Repeated Measures ANOVA with Newman-Keuls used for post-hoc analysis. Factorial analysis was used to analyze the behavioral data represented in the bar graphs data that show total behavior. Graphs were plotted using Sigma Plot 10.0 and statistical analysis performed using the STATISTICA™ software package by StatSoft.

## Results

Plasma estradiol levels in gonadally intact, and ovariectomized rats that received no implant, an empty Silastic tube and a placebo pellet As expected, ovariectomy decreased plasma 17-β-estradiol levels (Table 1 and Figure 1). Ovariectomy caused a 46% decrease in plasma estradiol levels by day 7 (FISHER LSD test $p<0.001$). Estradiol continued to decline slightly up to day 28, where a 63.7% decrease from original levels was detected (Table 1). OVX animals that received an empty Silastic implant were no different from OVX rats with no implant. However, estradiol levels of rats implanted with placebo pellets did not decline to the same extent as those that received an empty Silastic implant or no implant whatsoever, and in fact were not significantly different than levels observed in intact females at day 0 (Figure 1 and Table 1). Indeed, an increase in plasma estradiol levels was observed on days 14 and 28 in animals that received a placebo implant. Curiously, significant weekly fluctuations in plasma estradiol levels were observed in this group of animals compared to the other groups at 7, 14, 21 and 28 days (ANOVA, $p<0.0001$).

| Days after OVX | Weight (g) | Change in Body Weight relative to day 7 | Levels of Estradiol (E2) (pg/ml) | Ratio (Weight/E2) |
|---|---|---|---|---|
| Silastic Tubes Empty | | | | |
| 7 | 227 ± 3 | - | 13.30 ± 0.74 | 17.10 |
| 14 | 274 ± 4 | 47 ± 5 | 11.41 ± 0.60 | 24.03 |
| 21 | 298 ± 5 | 71 ± 6 | 10.37 ± 0.41 | 28.78 |
| 28 | 320 ± 7 | 92 ± 8 | 8.96 ± 0.41 | 35.67 |
| Pellets of Cholesterol | | | | |
| 7 | 203 ± 4 | - | 16.6 ± 3.39 | 12.24 |

| | | | | |
|---|---|---|---|---|
| 14 | 237 ± 6 | 34 ± 3 | 35.59 ± 9.16 | 6.66 |
| 21 | 264 ± 5 | 61 ± 3 | 14.18 ± 1.00 | 18.65 |
| 28 | 281 ± 4 | 78 ± 3 | 29.03 ± 4.37 | 9.69 |

**Table 1:** Comparison between weight of the rats and estradiol levels in ovariectomized animals with Empty Silastic tubes or Pellets with cholesterol.

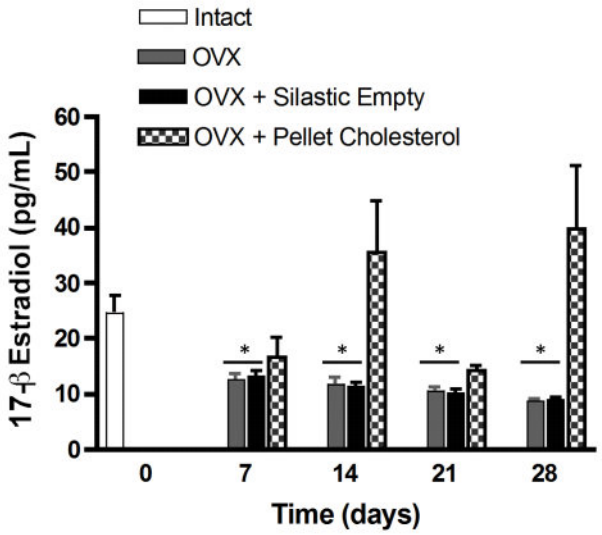


Figure 1: Changes in 17-β-estradiol plasma levels after ovariectomy. Plasma estradiol before ovariectomy (Day 0) and 7, 4,21 and 28 days after ovariectomy. Plasma estradiol decreased over time in ovariectomized rats that received an empty Silastic implant (OVX +Silastic Empty) and in those that did not receive an implant (OVX); (ANOVA $p<0.0001$ $F_{(94, 29)}=25.86$ followed by FISHER-LSD multiple comparison test]. No difference in estradiol levels was detected between these groups throughout the 4 weeks post-surgery. Rats that received the placebo cholesterol pellet (OVX +Placebo Pellet) did not show a significant decrease in basal estradiol levels after ovariectomy ($p>0.05$). The estradiol value of the intact animals (white bar) is the average of the all the animals, before they were ovariectomized and assigned to the different groups. At time point 0, n=29; All OVX n=7; OVX+ empty Silastic n=14; OVX+Cholesterol pellet n=8. Data are expressed as mean ± standard error of the mean (S.E.M.).

## Ovariectomized rats with silastic implants containing estradiol

The estradiol released by the Silastic tubing implants was highest at day 7 and decreased gradually with time (Figure 2). The highest values were observed in those animals that received the 5 mg implant (compare 183 ± 19.9, 176 ± 27.8, and 257 ± 62.9 pg/ml for the 3, 4, or 5 mg Silastic tubing implants, respectively). By day 28, plasma estradiol had decreased significantly in all groups 36.2%, 49.3%, and 39.4% compared to their values on day 7 (implants of 3, 4 and 5 mg of estradiol, respectively) (Fischer LSD test $p=0.0255$, $p=0.001084$, $p=0.010639$). We did not observe significant differences in plasma estradiol levels attained by the 3 mg and 4 mg Silastic implants within the time points studied. However, significant differences were observed between Silastic implant of 4 and 5 mg of estradiol at 7, 14, and 28 days (FISCHER LSD test $p=0.021$, $p=0.015$, $p=0.041$, respectively).

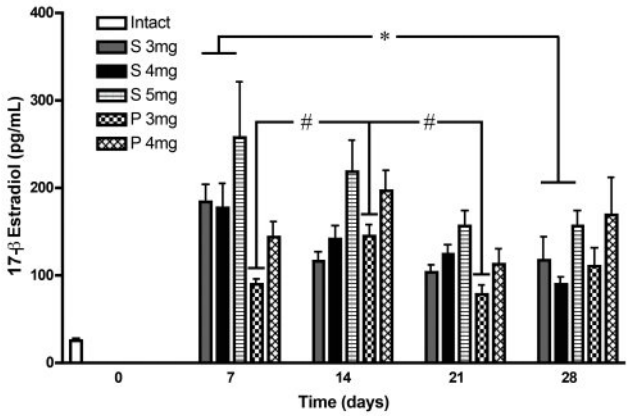


**Figure 2:** Comparison of estradiol levels produced by two steroid replacement systems. Bars show weekly estradiol (E) levels achieved by Silastic implants (S) and commercial pellets (P). Plasma estradiol of rats implanted with an estradiol-filled Silastic tubing decreased gradually with time, being the highest values at 7 days versus the concentrations observed at 28 days (at least $^{*}p<0.02$), according to FISHER multiple comparison test. Plasma estradiol of rats implanted with commercially available pellets showed a wavelike pattern with estradiol levels fluctuating between the time-points studied. Statistical analysis demonstrated that the levels of estradiol were significantly higher at 14 days relative to day 7 (at least $\#p<0.05$), but returned to a similar level by day 21. All values are significantly different from that of intact rats at day 0 (with ovaries: white bar). Intact n=29; OVX+S3 n=10; OVX+S4 n=11; OVX ± S5 n=5; OVX+P3 n=8; OVX+P4 n=7. Data are expressed as mean ± S.E.M.

## Ovariectomized rats with commercial pellets containing estradiol

The estradiol-pellet delivery system (Hormone Pellet Press) produced estradiol levels that fluctuated between time points. Plasma estradiol showed a wavelike pattern with estradiol levels fluctuating significantly ($p<0.0061$) between sampling days (Figure 2), similar to what was observed with the placebo pellets. Estradiol levels were higher at days 14 and 28, and lower at days 7 and 21. The highest concentration was reached at 14 days, 145 ± 12.4 pg/ml for the 3 mg

estradiol pellet and 195.7 ± 23.05 pg/ml for the 4 mg estradiol pellet. One-way ANOVA, followed by Student-Newman-Keuls multiple comparison test demonstrated that estradiol levels in rats treated with 3 mg estradiol pellets were significantly different ($p<0.05$) between samples at 7 days (89.8 ± 5.1 pg/ml) versus 14 days (145 ± 12.4 pg/ml). In addition, the statistical analysis revealed that estradiol levels were significantly different ($p<0.01$) between the samples obtained at 14 days versus the 21 days (78 ± 10.2 pg/ml). However, no differences were observed between the samples obtained at 7 days versus the hormone levels at 21 days or between estradiol levels at 14 versus 28 days, confirming the wavelike pattern fluctuations with the use of pellets.

## Estradiol levels obtained by two different estradiol RIA kits

Total plasma estradiol levels of weekly blood samples were measured using a Double Antibody RIA kit (MP biomedical; Costa Mesa, California, USA) and a Coat-Count RIA kit (TKE22 Diagnostic Product Corporation, Los Angeles, California, USA). Results obtained using the MP biomedical kit was 10.4 times higher than those detected using the Coat-A-Count kit. For example, estradiol levels in animals with empty Silastic implants at 7, 14, 21 and 28 days were: 146+27, 115+12, 105+22 and 97+21 pg/ml, respectively. Rats with placebo pellets presented estradiol levels of 173+35 at 7 days, 370+95 at 14 days, 147+10 at 21 days and 302+46 pg/ml at 28 days. In addition, animals with Silastic tubes containing estradiol (3-5 mg) or estradiol pellets (3-4 mg) gave values of estradiol in the plasma between 934+53 and 2,859+539 pg/ml using the double antibody RIA kit (MP Biomedical) within the first three weeks of estradiol administration. Thus the values we present are those obtained with the Coat-A-Count kit and those of MP Biomedical with a conversion factor of 10.4 lower, values that are similar to those reported previously by our laboratory and of others [14,19].

## Body weight

Body weight increased following ovariectomy, estradiol treatment attenuated the effect of ovariectomy (Figures 3 and 4). Body weight increased significantly ($p<0.0001$) in rats without estradiol (Silastic implants empty) compared to animals treated with Silastic implants containing 3, 4 or 5 mg of the hormone, according to one-way ANOVA analysis. Rats that were ovariectomized, regardless of whether they received no implant or an empty Silastic implant (Figure 3), or placebo pellet (Figure 4), showed an increase in body weight (Table 1).

This increase was evident beginning at day 7 and the body weight change continued throughout days 14, 21 and 28. The weight of these rats was significantly different compared to their weight prior to ovariectomy (data not shown). This increase in body weight was not observed in ovariectomized rats that received estrogen replacement, regardless of the amount and/or method of replacement (Figures 3 and 4). Interestingly, despite the significant differences in plasma estradiol levels between the two methods of estradiol replacement, body weights were not altered.

Rats that received an empty Silastic implant weighed more, and overall had lower plasma estradiol, than rats that received a placebo pellet (Table 1). However, the ratio between body weight and plasma estradiol is higher in rats that received the empty Silastic implant (Table 1).

## Behavior: locomotor activity

No major differences in locomotor activity were observed between OVX and OVX-EB rats that received a 3 or 4 mg Silastic implant [(data combined) Figure 5). We did find that rats treated with estradiol showed greater rearing during the first 10 minutes in the activity chamber (Figure 5 bottom panel].

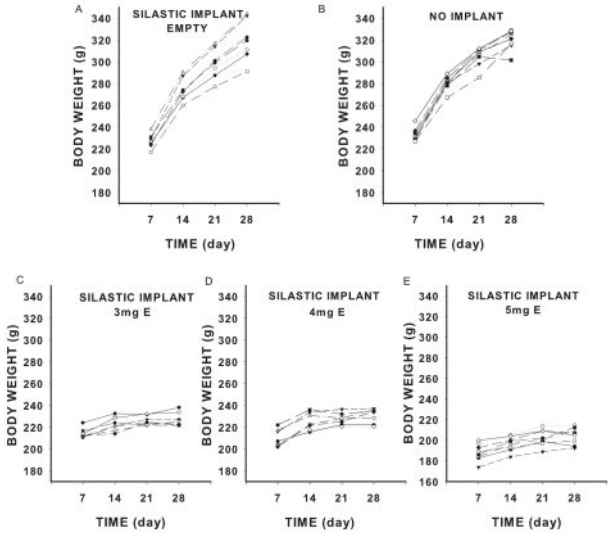


**Figure 3: Body weight of ovariectomized rats that received a Silastic tubing implant with and without estradiol.** Rats were ovariectomized and received (A) an empty Silastic tubing implant, (B) no implant, (C) Silastic tubing with 3mg estradiol, (D) Silastic tubing with 4mg estradiol, and (E) Silastic tubing with 5mg estradiol. Removal of endogenous estradiol results in a significant ($p<0.0001$) change of body weight over time (n=7-8 for each group), according to one-way ANOVA, followed by a Tukey-Kramer multiple comparisons test. Rats treated with estradiol maintained a relative constant body weight through the 4 weeks of the study. OVX+SE n=7; OVX+S3 n=8; OVX+S4 n=8; OVX+S5 n=8. Each plot represents one animal.

We also recorded the time spent in the center of the activity chamber, as a measure of estradiols reported anxiolytic effect (Figure 6). A trend to spend more time in the center of the activity chamber was observed in rats that received a 3 or 4 mg Silastic implant. This trend became significantly different by day 21 and disappeared by day 28 (Figure 6).

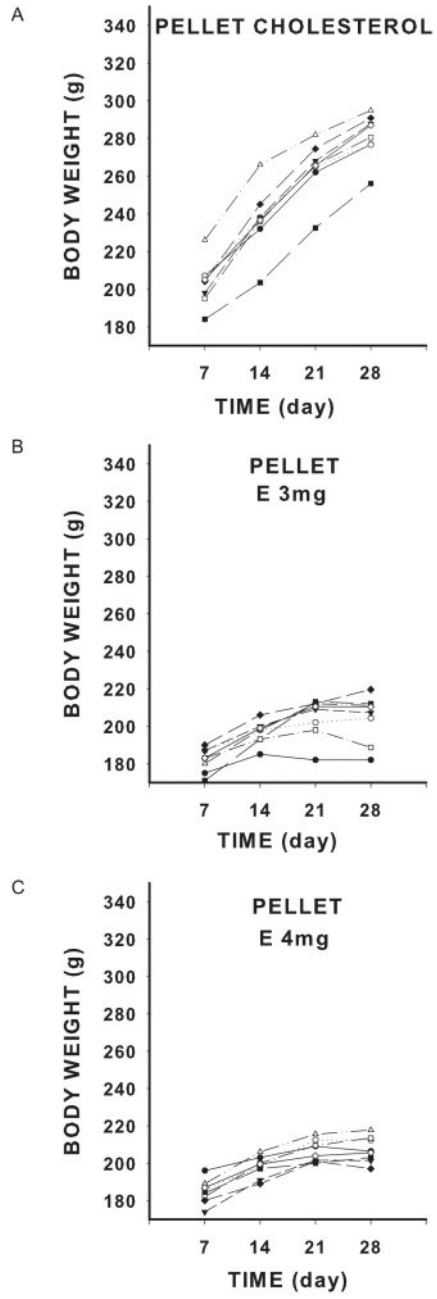


**Figure 4: Body weight of ovariectomized rats that received a pellet implant with or without estradiol.** Ovariectomized rats that received a pellet implant that contained (A) cholesterol (placebo), (B) 3 mg estradiol/cholesterol and (C) 4 mg estradiol/cholesterol. The bilateral removal of ovaries increased the change of body weight significantly ($p<0.0001$) in comparison to animals treated with estradiol, according to one-way ANOVA followed by a Tukey-Kramer multiple comparisons test. Estradiol replacement by pellets was effective in preventing the increase in body weight observed in the placebo group. Changes in body weight were similar between the groups that received 3 or 4 mg pellet of estradiol (n=8 for each group). Each plot represents one animal.

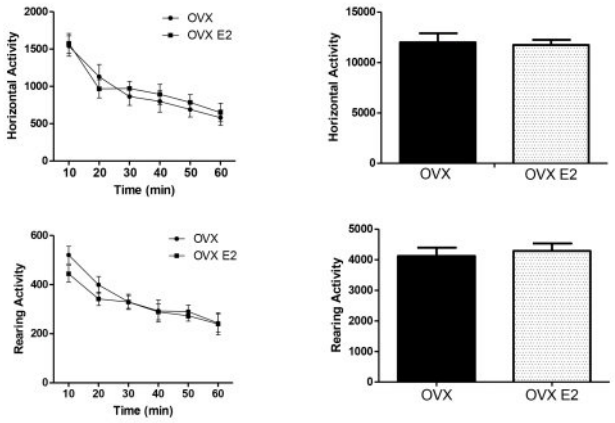


**Figure 5: Horizontal and rearing activity of ovariectomized rats that received a Silastic implant with or without estradiol.** Horizontal (Top Panel) and Rearing (Bottom Panel) activity on day 28 of ovariectomized rats that received a Silastic tube without or with estradiol (data combined of 3 and 4 mg estradiol). No significant difference was observed between ovariectomized (OVX) and ovariectomized plus estradiol (OVX E2) rats (n=7-8 per group). Data are expressed as mean ± S.E.M.

## Discussion

Our results show that Silastic implants provide a more stable delivery system of estradiol than the commercially available pellets, i.e., delivery by pellets fluctuated significantly from week to week. Although both hormone replacement methods were effective, they differ in their release pattern. Estradiol levels achieved by Silastic implants peaked at our first sampling period (day 7) and decreased slightly thereafter, following a similar pharmacokinetic profile as previously reported [22]. In contrast, hormone release by pellets increased and decreased from week to week, similar to the pattern observed with their control "placebo" pellets. Our recommendation coincides with that of Strom et al. and Ingberg et al. that Silastic implants are a more reliable and effective method for administering estradiol than pellets [14,23]. Our study also indicates that both methods, and all doses tested, are effective in deterring the increase in body weight observed after ovariectomy. This effect of estradiol on body weight has been extensively documented [12,24,25]. This study also found that after 28 days, the Silastic tube and pellet implants continue to release estradiol. Surprisingly, we found that plasma estradiol in animals that received the placebo pellet (vehicle) were similar to levels observed in gonadally intact females. Gonadally intact female rats go through a 4-5 day estrous cycle, during which plasma estradiol fluctuates almost 100 pg/ml. Thus, the average value of plasma estradiol in gonadally intact rats depends on the number of rats in each stage of the cycle.

Our study also found that rats that received a 3 or 4 mg Silastic implant show a trend toward an increase in the time spent in the center of the activity chamber at day 14 which becomes significant at day 21 and is lost by day 28. Estradiol plasma levels of OVX animals that received an empty Silastic implant were no different from OVX rats that didn't receive an implant, indicating that the Silastic implant does

not contain an estrogenic compound that can significantly contribute to detectable estradiol in plasma.

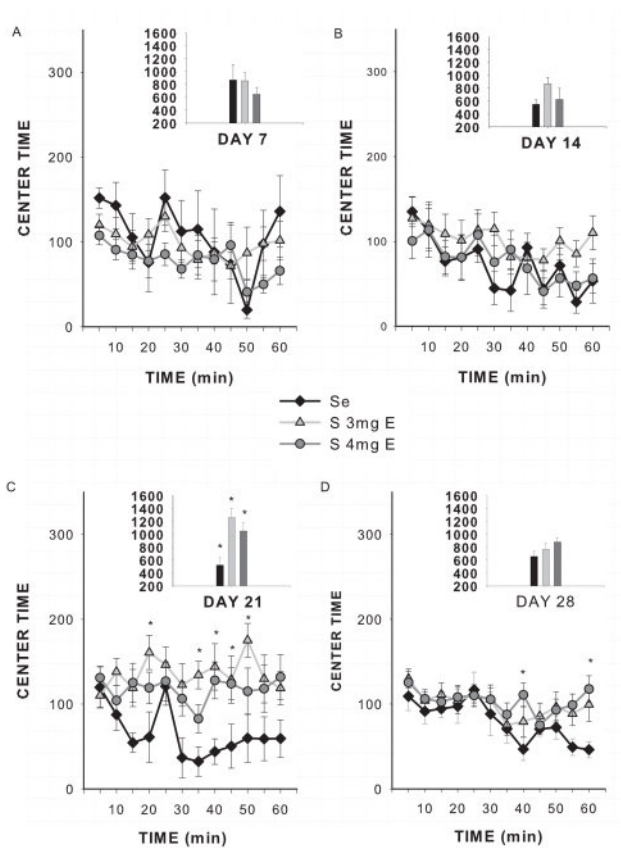


**Figure 6:** Time spent in the center of an open field by ovariectomized rats that received a Silastic tube with and without estradiol. Ovariectomized rats received a Silastic tube implant that was empty (Se) or filled with 3 mg (S 3 mg E) or 4 mg (S 4 mg E) estradiol benzoate and tested in an open field to assess the time spent in the Center box. Time spent in the Center box after (A) 7 days, (B) 14 days, (C) 21 days or (D) 28 days of estradiol treatment. Estradiol treated rats spent more time at the center at 21 days than non-treated rats (Se, Silastic empty) ($p=0.009$ $F(2,25)=5.7550$, repeated measures ANOVA followed by Newman-Keuls multiple comparison test). (n=7-8 per group). Data are expressed as mean ± S.E.M.

Differences in plasma estradiol could account for the variability and sometimes contradictory findings between studies [26,27]. We found that estradiol release peaked at 7 days in rats that received Silastic implants and produced relatively stable estradiol levels that slowly declined until our last time point of 28 days. However, in rats receiving the pellets, estradiol levels peaked at 14 days, decreased at 21 days, and went up again at day 28. These differences in the release pattern of estradiol by the pellets may reflect manufacturing conundrums. It is possible that when the pellet is produced and implanted subcutaneously, some layers are metabolized more easily than others and this may be contributing to differences in the rate of estradiol delivery. This information needs to be taken into consideration in the experimental design of studies that require estradiol replacement. Indeed, recent studies of ischemic brain damage show that the neuroprotective properties of estradiol can vary depending on the method of administration [28].

At first, estradiol plasma levels were measured using the MP Biomedical estradiol double antibody RIA kit. However, we became concerned when the values we obtained were approximately 10 fold higher than those reported in the literature. We ordered the Coat-A-Count RIA total estradiol kit by Diagnostic Products Corporation and ran the same samples. We observed that the values were 10.4 times lower, a difference of an order of magnitude. We used this as a conversion factor to standardize all the values obtained with the MP Biomedical kit to those of the Coat-A-Count kit.

Although Legan et al. and several others showed that Silastic tubing of 5 mm produced approximately 75-100 pg/ml [18,29,30] of circulating estradiol, others have found widespread variability. For example, in previous experiments we reported total plasma estradiol concentrations of 141.4 ± 17.0 pg/ml (range, 94–192 pg/ml), 15 days after initial subcutaneous placement [19]. In this study we prepared the Silastic tubing implants as described by Legan et al. [18]. In addition, implants were weighed after filling them with the appropriate dose of estradiol, making sure all implants contained the same amount of steroid. After 14 days, the plasma levels produced by the Silastic implant containing 3, 4 and 5 mg of estradiol, were 116.2 ± 9.9, 140.7 ±14.9 and 218.0 pg/ml respectively.

Variations in estradiol concentration reported in the literature may be attributed to differences in the amount of estradiol placed inside the tubing. To minimize variability, we recommend weighing the amount of estradiol to be placed inside the Silastic tube. Differences in the methodology for measuring estradiol (RIA vs ELISA), manufacturing differences in the production of RIA and ELISA kits that varies with between companies, in addition to individual differences in metabolism and adipose tissue content may also contribute to these differences. Indeed, variability of the RIA kit may be due to differences in antibody recognition of epitopes or poor separation of free vs. bound hormone.

Plastics are known to contain estrogen-like molecules such as bisphenol A. In this study, we did not observe any significant contribution of the empty Silastic tube to estradiol in blood. In both groups, removal of the ovaries decreased plasma estradiol levels. Although the largest decline was seen by day 7, levels continue to decrease slightly. As shown by many investigators, estradiol levels decline gradually and do not tend to reach 0 because fat sources and aromatization from precursor molecules are still available [31-33]. Thus, we also recommend the use of empty Silastic tubes as controls, as they do not provide estradiol.

Caution must be taken if using commercial pellets to replace estradiol. Rats implanted with a 3 and 4 mg estradiol pellet, as well those implanted with the placebo pellet, had fluctuating estradiol plasma levels, increasing and decreasing between the 4 weekly samplings. This fluctuation was not observed in ovariectomized rats that received Silastic tubes that were empty or filled with estradiol benzoate. Furthermore, rats that received placebo-cholesterol pellets had estradiol plasma values similar to those observed in intact rats. Cholesterol serves as the precursor in the synthesis of gonadal and adrenal steroids. Reduced levels of circulating estradiol due to ovariectomy are known to increase FSH secretion. FSH in turn activates the cholesterol side chain cleavage enzyme (also known as desmolase and CYP 11A1) that converts cholesterol to pregnenolone.

Conversion of cholesterol to pregnenolone is the rate-limiting step in the synthesis of these steroid hormones. Thus it is not surprising that rats that received the placebo pellet had higher plasma estradiol values.

Estradiol blood level during proestrous, the stage of the estrous cycle where estradiol is highest, has been shown to fluctuate significantly. Values of estradiol during proestrous have been reported from 16-88 pg/ml range [34-36]. During pregnancy values may increase upto 80 pg/ml [29]. Thus, the plasma estradiol levels reported here are in the high physiological range.

Body weight was monitored to provide a reliable bioassay of estradiol. All ovariectomized rats showed an increase in body weight by 7 days after ovariectomy, whereas those that received estradiol by implants or pellets did not, confirming previous studies that show an inverse relationship between estradiol and body weight [11,24,37-39]. Interestingly, despite the significant differences in plasma estradiol levels between the two methods of estradiol replacement, body weights were not significantly different between the groups that received estradiol. Apparently, the dose of estrogen was sufficient to maintain body weight at levels similar to those prior to ovariectomy. It would be of great clinical value to determine the minimum dose of estradiol that has an anti-obesity effect to offer perimenopausal women a safer hormone replacement alternative that may counteract the gain in weight that accompanies menopause [37,40].

The recognized effects of estradiol on the brain lead us to assess anxiety related behaviors. All behavioral assays were conducted with ovariectomized rats that received the Silastic implants. We did not use rats that received the estradiol pellet replacement, since the data presented previously showed variability in the release and in the time that peak estradiol levels were achieved. Our data shows that animals with estradiol replacement spent more time in the center of the activity chamber than in the periphery. These results are in accordance with those in the literature that support an anxiolytic role of estradiol [41,42]. Estradiol is a pleiotropic hormone, thus it is important to provide a consistent estradiol delivery system in animal models. Therefore, our results show that Silastic implants provide a quick, steady and cost effective strategy in estrogen replacement studies.

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## Author Disclosure Summary

None of the authors have any potential conflicts of interest associated with this research.

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