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Order of magnitude differences between methods for maintaining physiological 17β-estradiol concentrations in ovariectomized rats

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Running head: 17β-estradiol administration to ovx rats

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ABSTRACT

The use of animal, especially rat, models, is crucial for elucidating the biological effects and mechanisms of the widely used hormone 17β-estradiol. Unfortunately there is a lack of consensus on optimal means of obtaining and maintaining physiological 17β-estradiol concentrations in plasma. This may be the reason for varying results in several studies including the disagreement on whether 17β-estradiol is neuroprotective or not. Very few studies have been devoted to investigating the characteristics and biological relevance of different methods of 17β-estradiol administration. We therefore ovariectomized 75 Sprague-Dawley rats and, following a 2 weeks wash-out period, administered 17β-estradiol using three different methods; daily injections (10 μg 17β-estradiol/kg), slow-release pellets (0.25 mg 60 day-release pellets, 0.10 mg 90 day-release pellets) and silastic capsules (with/without wash-out periods) (silastic laboratory tubing, inner/outer diameter: 1.575/3.175 mm, filled with 20 mm columns of 180 μg 17β-estradiol/mL sesame oil). Further 45 animals were used as ovariectomized and native controls, studied in different parts of the estrous cycle. Silastic capsules produced concentrations of 17β-estradiol within the physiological range for 4-5 weeks independent of whether a prior wash-out period was included or not. The slow-release pellets, irrespective of dose or release period, resulted in initial concentrations which were an order of magnitude above physiological concentrations during the first two weeks followed by a substantial decrease. Daily injections resulted in increasing 17β-estradiol concentrations, however within physiological levels. Silastic capsules are conveniently manufactured and used, and are superior to pellets and injections in reliably producing long-term 17β-estradiol concentrations within the physiological range.

Keywords: Estrogens, Injections, Pharmacokinetics, Silastic capsules, Slow-release pellets, Wash-out

INTRODUCTION

Estrogens constitute a family of endogenously synthesized steroid hormones which are widely used for contraception and amelioration of post-menopausal symptoms (Hormone Replacement Therapy, HRT). The extensive use and wide spectrum of biological effects have made estrogens the subject of substantial research efforts and public debate during recent years. Notable is the controversy on whether estrogens decrease [1] or increase [2] ischemic brain damage, a question which has been investigated in a number of laboratory models in rats. Much of the inquiry into the possible favorable effects of estrogens consumption was put on hold when the huge Women Health Initiative (WHI) epidemiologic study was presented, where it was found that estrogen consumption, inter alia, increases the risk of coronary heart disease, breast cancer and stroke but has no effect on the all-cause mortality [3]. In the wake of this report almost half of Swedish HRT treated women ceased their estrogen intake [4], despite the fact that the women included in the WHI study were on the average 10 years older than the typical menopausal women and had elevated body mass index.

The strong evidence of the WHI study makes it practically impossible to perform further studies on the biological effects on estrogens in women past the menopause. This currently leaves us with the use of animal models as practically the only key to many of the questions concerning estrogens and the use of estrogens as drugs. Therefore the importance of well characterized and controlled animal models has increased, particularly in rats. The fundament of conducting experimental studies is a thorough knowledge of the methods and models used. The aforementioned dispute on the neuroprotective or neurodamaging effects of estrogens could partly be a result of the lack of consensus on how to work with rat models with estrogens as a variable.

In rat model experiments designed to investigate the effects or mechanisms of estrogens it is common practice to first ovariectomize (ovx) the female rat, and then to administer 17β-estradiol, the

most biologically active member of the estrogen family. The purpose of this is to achieve physiological levels of 17β-estradiol without having to consider the hormone's natural cyclicity. The difference of opinion on how to do this is most obvious in the choice of methods for administering 17β-estradiol. Three commonly used modes of administration are 1) subcutaneous injections [5-9], 2) inserting a commercially produced hormone pellet subcutaneously [8,10-13] or 3) implanting a silastic capsule filled with 17β-estradiol subcutaneously in the animal [1,12,14-19]. Another question concerns the choice of wash-out period between ovx and 17β-estradiol administration. The examples ranges from no wash-out at all [1,8,13] to several weeks [9,10,16].

Despite the substantial number of studies in which estrogens have been administered to rats, very few experiments have been devoted to investigating the characteristics and biological relevance of the methods themselves. Therefore we considered it of interest to compare the effects on the serum concentrations of 17β-estradiol of the commonly used methods of administering 17β-estradiol to ovx female rats for 6 weeks. A secondary aim of the study was to investigate the possible effects of the wash-out time on the 17β-estradiol concentrations.

MATERIAL AND METHODS

Animals

One hundred and twenty Sprague-Dawley female rats were obtained from B&K Universal (Sollentuna, Sweden). The rats were kept at constant room temperature (21°C) with 12-h light/dark and sound (soft radio music) cycles for at least 1 week prior to the experiment. At the start of the study the rats were 12 weeks of age and weighed 291±11g (mean± SEM) . The animals were housed 5 in each cage during the first 2 weeks, and subsequently 2 in each cage for the remainder of the experiment, with food (Lactamin, Vadstena, Sweden) and water provided ad libitum. All procedures were conducted in accordance with the National Committee for Animal Research in Sweden and Principles of Laboratory Animal Care (NIH publication no. 86-23, revised 1985). The study was

approved by the Local Ethics Committee for Animal Care and Use at Linköping University. The animals used in this experiment were also included in another study of immunochemical measurement methods of 17β-estradiol (separate manuscript simultaneously submitted for publication).

Grouping

Seventy-five animals were randomly allocated to 5 treatment groups (n=15) differing in methods of 17β-estradiol administration. The remaining 45 animals were divided into a native control group (n=30), studied in different parts of the estrous cycle (proestrus, diestrus and estrus), and an ovx control group (Gr.Ovx, n=15).

Ovariectomy and hormone replacement

Fourteen days before day 0 of the experiment, the rats in the first four treatment groups and Gr.Ovx were ovx via the dorsal route. Day 0 of the experiment the rats in the fifth treatment group were ovx.

The next step was administration of 17β-estradiol. The first treatment group (Gr.Inj) received daily subcutaneous injections of 10 µg 17β-estradiol/kg bodyweight (Sigma-Aldrich Sweden AB, CAS Nr [50-28-2], Stockholm, Sweden) solved in 30 µL of sesame oil (Sigma-Aldrich Sweden AB, CAS Nr [8008-74-0], Stockholm, Sweden). This is a frequently used injection dosage to achieve physiological levels of 17β-estradiol [5,6]. For injections a Hamilton® syringe, (1700 series, 100 µL, Sigma-Aldrich Sweden AB, Stockholm, Sweden) was used. Before injection the solution was stirred with a magnetic stirrer for at least 20 hours. Injections were made at 10-12 am every day.

The second (Gr.P0.25) and third (Gr.P0.10) treatment groups received subcutaneously implanted pellets containing 0.25 mg and 0.1 mg 17β-estradiol, claimed by the manufacturer to give an even release of 4.2 and 1.1 µg 17β-estradiol/day respectively (60-day release, SE-121, 0.25mg/pellet and 90-day release, NE-121, 0.1mg/pellet, Innovative Research of America (IRA), Sarasota, FL, USA, http://www.innovrsrch.com/faq.asp#R1). These two dosages were chosen on the basis that they were

in the proximity of the daily dose injected in Gr.Inj. The reason for including two groups receiving 17β-estradiol using the same administration technique, but with different release rates, was to be able to evaluate the impact of different dosages on serum concentrations of 17β-estradiol.

The two last treatment groups (Gr.Sil.1 and Gr.Sil.2) both received identical silastic capsules. These were assembled according to the method described by Dubal and Wise [1]. Thirty mm segments of silastic laboratory tubing (Inner/outer diameter: 1.575/3.175 mm, Dow Corning, VWR International, Buffalo Grove, IL, USA) were filled with a solution of 180 μg 17β-estradiol/mL sesame oil. Five mm pieces of wooden applicator sticks (Birch, length: 15 cm, diameter: 2 mm, SelefaTrade AB, Spånga, Sweden) were cut using a fine tooth saw and used to seal the silastic tubing, resulting in an oil-17β-estradiol-filled column 20 mm in length. The capsules were stored overnight in a vial containing sesame oil with the same concentration of 17β-estradiol as inside the capsules. Before implantation the capsules were carefully wiped. A 0.5 cm incision was made in the loose skin of the rat's neck, and a pocket was bluntly dissected caudally, in which the silastic capsule was gently installed using forceps. The incision was subsequently closed by a suture. Two important aspects of the silastic capsule handling were not described by Dubal and Wise and thus left for the current authors to decide: how to make 5 mm pieces from the wooden applicator sticks (a fine tooth saw, and not e.g. a nipper, was chosen to avoid making cracks in the wood) and where in the rat sub cutis to implant the capsules (the neck was chosen because of its abundance of loose skin, and to minimize the risk of mechanical stress on the capsule). (Fig. 1).

The rats in Gr.Inj received amounts of administered estradiol adjusted according the individual rats' body weight, while the rats in Gr.P0.25, Gr.P0.10, Gr.Sil.1 and Gr.Sil.2 received the same amounts.

The hormone treatments were started on day 0 of the experiment in all treatment groups, hence with a 14 day wash-out, except for Gr.Sil.2 which was not allowed a wash-out period, in accordance with

Dubal & Wise [1]. Thus, since Gr.Sil.1 and Gr.Sil.2 had identical capsules, they differed only in wash-out period.

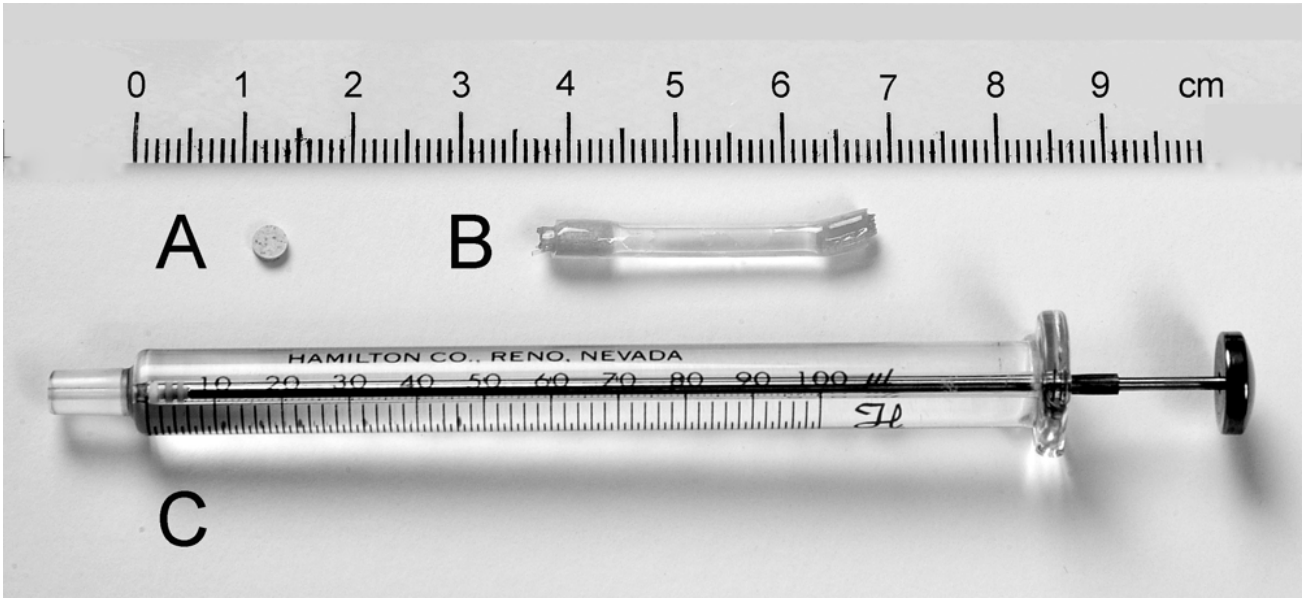

Fig. 1.: *Equipment for administration of 17β-estradiol: slow-release pellet (A), silastic capsule (B) and syringe for daily injections (C). Pellets containing 0.25 mg (60-day release) and 0.10 mg (90-day release) 17β-estradiol claimed to result in an even release of 4.2 and 1.1 µg 17β-estradiol/day respectively were implanted subcutaneously. Silastic laboratory tubing with inner/outer diameter: 1.575/3.175 mm was filled with 20 mm columns of 180 µg 17β-estradiol/mL in sesame oil and implanted subcutaneously in the neck of the animal. 10 µg 17β-estradiol/kg bodyweight dissolved in 30 µL of sesame oil was daily injected subcutaneously.*

Blood sampling

Treatment groups:

Blood samples of 1 mL were collected by venipuncture of the hind limb into serum tubes (Vacuette® Serum Tubes, Hettich Labinstrument AB, Sollentuna, Sweden) on days 2, 7, 14, 21, 28 and 35 of the experiment. Day 42 the animals were sacrificed and trunk blood collected. The blood sampling was done between 8 and 12 am (always prior to the daily injections in Gr.Inj.)

Native controls:

The 30 native control animals' estrous cycles were monitored by vaginal smears [20]. Ten rats were sacrificed in estrus, diestrus and proestrus respectively and concurrently trunk blood was collected from each animal.

Ovariectomized controls:

The 15 animals in Gr.Ovx were sacrificed on day 0 of the experiment, fourteen days after ovx, and trunk blood was collected.

After sampling the blood was let to clot and then centrifuged at 2500 rpm for 10 minutes. Then the serum was aspired, transferred to another vial and frozen until analysis.

Anesthesia and analgetics

During ovx, blood sampling and administration of pellets and silastic capsules the animals were anesthetized with 1.4% (initially 4.2%) isoflurane (Forene® inhal-v 250 ml, Abbott Laboratories. Abbott Park, IL, USA) in an oxygen / nitrous oxide mixture (30% / 70%). The animals were also injected with 0.1 mL Rimadyl®/kg body weight (50 mg/mL Pfizer, Dundee, Scotland) dissolved in 1 mL of saline subcutaneously in the neck during ovx. Eye gel (Lubrithal 15 g Leo laboratories Ltd., Dublin, Ireland) was utilized for protection of the rats' eyes during anesthesia.

Hormone assays

The serum samples were analyzed with an I^{125} radioimmunoassay kit (17β-estradiol double antibody, KE2D, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA), and measured for 300 seconds in a gamma counter (Gamma Master 1277; Wallac-Pharmacia, Turku, Finland). This method has, according to the manufacturer, a lowest detectable concentration of 1.4 pg/mL and intra- and inter-assay coefficients of variation of 4-13% and 3.5-5.5%, respectively, depending on the concentration. All kits used were of the same LOT-number. Before the analysis, the reagents from the different kits were pooled to avoid unnecessary intra-assay variation. The entire analysis was performed in a single

séance. Three standard curves were analyzed and all samples were read against their closest standard curve. A volume of 100 µL of serum was used for each tube, both for standard curve and samples. Standards and samples were analyzed in duplicate (this part is thoroughly described in a separate manuscript simultaneously submitted for publication).

Excluded animals

One animal was excluded from the native control group because of an anatomical anomaly that made vaginal smear impossible. Day 16 of the experiment one of the animals in Gr.P0.25 and 4 of the animals in Gr.P0.10, were found to have large crusts under the fur on their bellies. Day 36 this was also found in one animal in Gr.Inj. These were excluded from the study from the day of discovering the crusts, and sacrificed. One animal from Gr.Sil.1 died during blood sampling anesthesia on day 28 of the experiment. In all, 8/120 animals were excluded at some time during the experiment.

Excluded test results

The results of some samples were excluded due to unacceptable differences between duplicates. This was the case for one sample analyzed with the DPC kit, three samples analyzed with the DSL kit and for three samples analyzed with the MPB kit.

Four samples (one sample day 7 and one day 21 in Gr.Inj, one sample day 42 in Gr.Sil.1 and one sample day 14 in Gr.Sil.2) were considered as extreme outliers and were therefore excluded from the significance analysis. Their outlier status was confirmed ($p<0.005$) by Dixon's test [21].

Statistical analysis

17β-estradiol concentrations of treatment groups and sampling occasions were analyzed for differences using ANOVA (Systat version 11, Systat Software, Inc. San Jose, California). P-values <0.05 were considered significant. 17β-estradiol concentrations were logarithmically transformed before ANOVA due to non-Gaussian distribution of the non-transformed data. In the diagrams, significant differences are visualized by stars in connection to lines linking the values in question.

One star corresponds to $p<0.05$, two stars to $p<0.01$ and three stars to $p<0.001$. The vertical bars from the depicted mean values represent standard error of the mean. A translation of the 17β-estradiol concentrations from the pg/mL-notation to the SI-unit nmol/L is depicted to the right in the diagrams.

RESULTS

17β-estradiol concentrations in the hormone treated groups

Group receiving daily injections (Gr.Inj)

During the time of the study the mean concentrations of 17β-estradiol in Gr.Inj increased from a level of 6.9±2.3 (mean±SEM) pg/mL day 2 and 10.4±4.4 day 7 to 109.9±60.7 pg/mL day 42 ($p<0.001$). The concentrations were not significantly above or below the physiologic range (5.9-63.1 pg/mL) at any time. The mean concentration on day 42 was 74% higher compared to the native proestrus control group mean concentration, however, not statistically significant (Fig. 2). The coefficients of variation for the concentrations in Gr.Inj were high (59.4-218.4%) in comparison to the other experimental groups (Table 2) which were similar to or less than of those observed during normal ovarial cycle (Table 1).

Group receiving 60-day release 0.25 mg pellet (Gr.P0.25)

The mean 17β-estradiol concentrations in Gr.P0.25 showed a plateau day 2 to day 14 with mean concentrations ranging from 304.9±34.6 pg/mL to 327.5±22.2 pg/mL. On day 28 the level had decreased to157.3±7.3 pg/mL which was significantly lower than the plateau ($p<0.001$). On day 42 the minimum mean concentration of 93.2±6.5 pg/mL was reached in this group, which was significantly lower than on day 28 ($p<0.001$). The mean concentrations in Gr.P0.25 were significantly higher than the native control groups until day 21 and not within the physiological

range during the entire duration of the experiment (Fig. 3). Coefficients of variation are presented in Table 2.

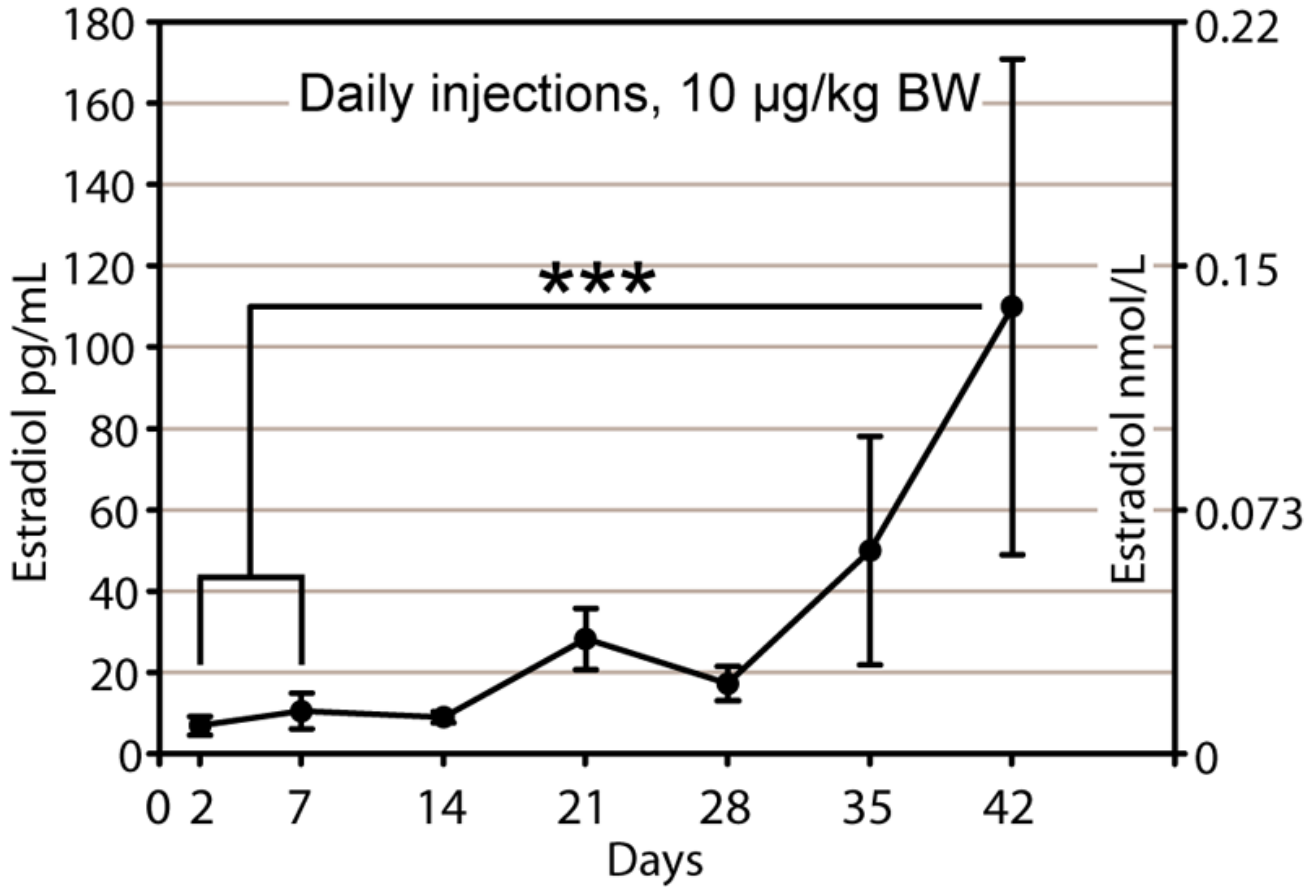

Fig. 2.: *Serum concentrations of 17β-estradiol (mean±SEM) in the group (n=15) receiving daily injections of 17β-estradiol in sesame oil (10 µg/kg body weight/day). (***=p<0.001)*

Table 1: *Relative variation in the concentrations of estradiol during natural ovarial cycle expressed as coefficient of variation in percent (CV%).*

Proestrus	Estrus	Diestrus	Ovariectomized
38.6	73.4	62.0	78.8

Table 2: *Relative variation in the concentrations of estradiol in ovariectomized rats receiving 17β-estradiol by different modes, expressed as coefficient of variation in percent (CV%).*

Mode	**2 days**	**7 days**	**14 days**	**21 days**	**28 days**	**35 days**	**42 days**
Pellet 0.25 mg, 60 days	26.3	43.9	39.9	24.2	17.4	24.8	26.1
Pellet 0.10 mg, 90 days	37.8	61.4	62.8	40.9	49.7	73.1	59.4
Silastic capsule w. washout	37.5	24.7	37.2	37.8	50.1	139.7	52.6
Silastic capsule no washout	41.1	27.0	40.6	43.7	62.6	183.8	67.2
Daily injections	130.1	159.3	59.4	99.7	94.8	218.4	207.5

Group receiving 90-day release 0.10 mg pellet (Gr.P0.10)

The mean 17β-estradiol concentration in Gr.P0.10 was 528.0±51.5 pg/mL day 2, but had decreased to 287.9±45.6 pg/mL day 7 ($p<0.05$). Both these values were significantly above the physiological range ($p<0.01$). On 14 day the concentration had decreased even further to 37.9±6.2 pg/mL ($p<0.001$). During the remainder of the experiment the 17β-estradiol concentration in this group decreased more slowly to reach a minimum concentration of 4.2±0.7 pg/mL on day 42. The mean concentrations were within the physiological range day 14 to 35, but not significantly higher than the 17β-estradiol levels in Gr.Ovx day 35 to 42.

The animals in Gr.P0.10 had significantly lower serum concentrations of 17β-estradiol than those in Gr.P0.10 day 14 to 42 ($p<0.001$) (Fig. 3). Coefficients of variation are presented in Table 2.

Groups receiving silastic capsules, with (Gr.Sil.1) and without (Gr.Sil.2) wash-out period

There was no statistically significant difference (with nor without logarithmic transformation) in the concentration of 17β-estradiol at any single occasion between the two groups receiving silastic capsules. The groups had their maximum concentrations of 38.7±3.7 pg/mL (Gr.Sil.1, with wash-out) and 37.8±4.2 pg/mL (Gr.Sil.2, without wash-out) respectively on day 2. The levels decreased steadily throughout the course of the experiment in both groups to reach minimum levels of 7.0±1.0

pg/mL (Gr.Sil.1) and 4.9±0.9 pg/mL (Gr.Sil.2) on day 42. Day 21 the 17β-estradiol concentration was significantly lower than day 2 and 7 in both groups ($p<0.05$). During the entire experiment, except for the last sampling in Gr.Sil.2, the serum concentrations of 17β-estradiol in these groups were within the physiological range, and also significantly higher than in Gr.Ovx day 2 to 28 ($p<0.01$) (Fig. 3). Coefficients of variation are presented in Table 2.

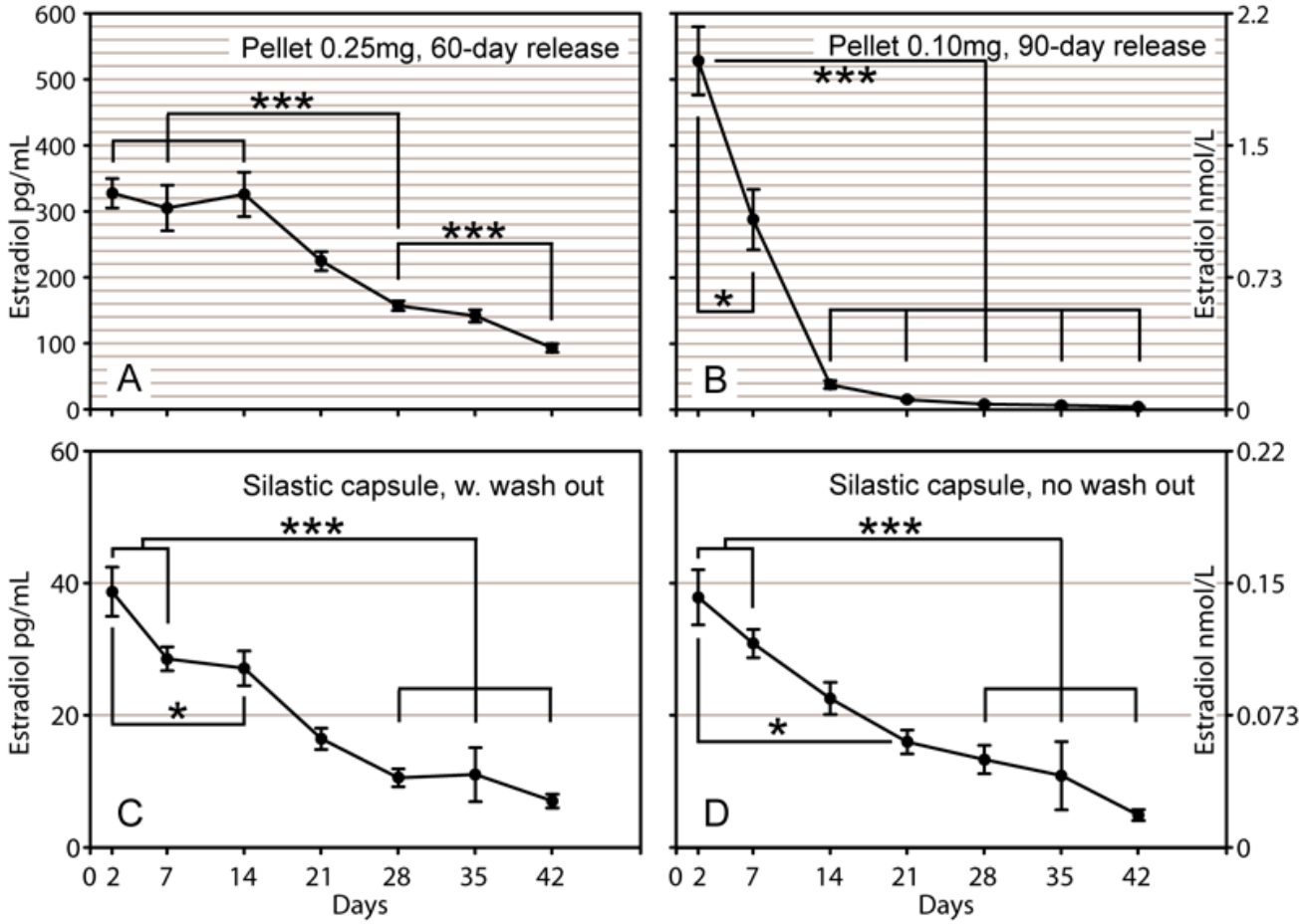

Fig. 3.: *Serum concentrations of 17β-estradiol (mean±SEM) in the hormone treated groups receiving 60-day release 0.25 pellet (Gr.P0.25) (A), 90-day release 0.10 mg pellet (Gr.P0.10) (B), silastic capsules after a 14 day wash-out period (Gr.Sil.1) (C) and silastic capsules without a wash-out period (Gr.Sil.2) (D) (n=15 in each group). The difference in scale on the Y-axis should be noted. The concentration difference between the light horizontal lines is 20 pmol/L in all figures. (*=p<0.05, ***=p<0.001)*

17β-estradiol concentrations in the native and ovx control groups

The serum concentrations of 17β-estradiol in the proestrus group (63.1±7.7 pg/mL) were 961% higher than in the estrus group (5.9±1.4 pg/mL) ($p<0.001$), 373% higher than in the diestrus group (13.3±2.8 pg/mL) ($p<0.001$) and 1553% higher than in Gr.Ovx (3.9±0.8 pg/mL) ($p<0.001$). There was also a significant difference between the diestrus group and Gr.Ovx ($p<0.001$) (Fig. 4). Coefficients of variation are presented in Table1.

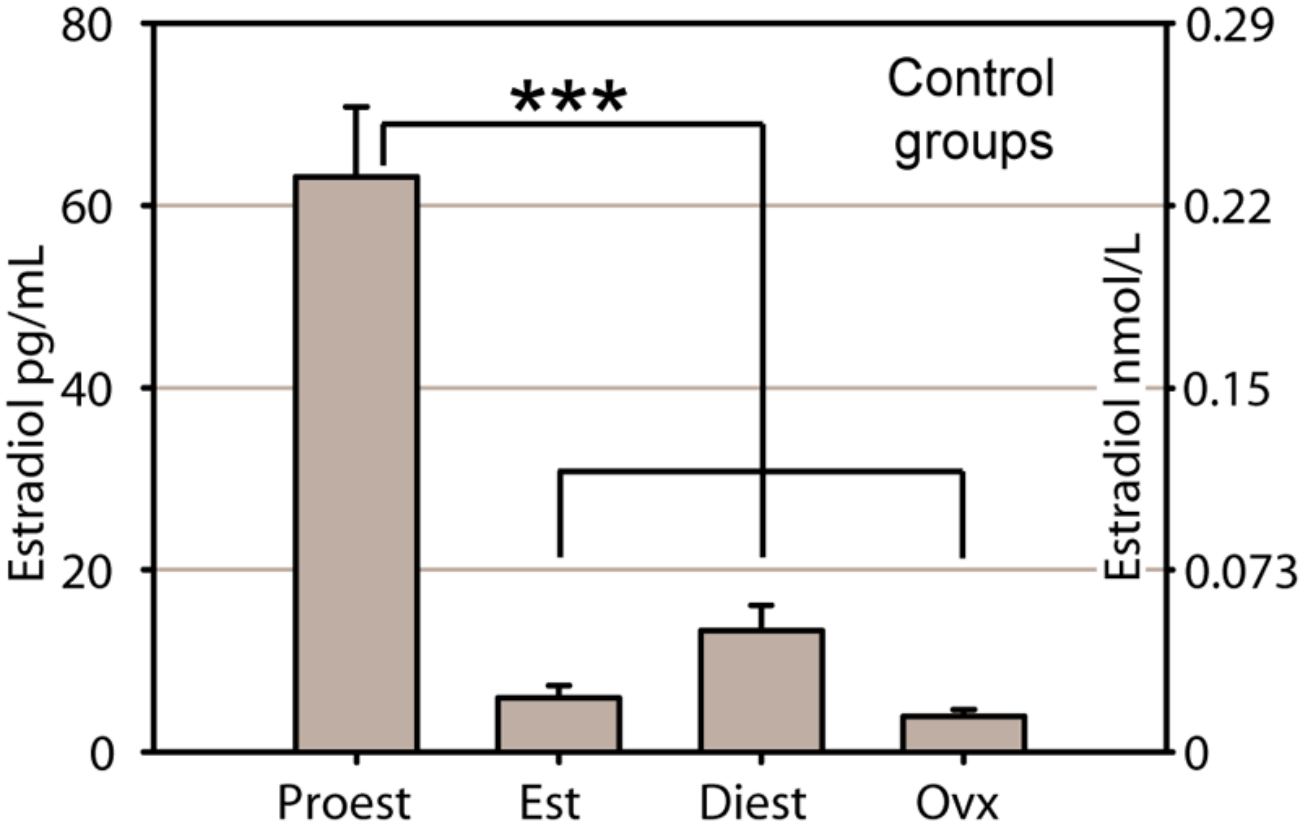

Fig. 4.: *Serum concentrations of 17β-estradiol (mean±SEM) in the native (n=30) and ovariectomized (n=15) control groups. The animals in the native control group were studied in different parts of the estrous cycle; proestrus, diestrus and estrus. (***=p<0.001)*

DISCUSSION

The main finding of the current study is that the silastic capsules are superior to pellets and injections in reliably inducing serum concentrations of 17β-estradiol that are, although steadily sinking, within the physiological range for several weeks. A wash-out period of the endogenous 17β-estradiol does

not affect the 17β-estradiol levels produced by this administration method. The slow release pellets, irrespective to dose or release period, resulted in initial 17β-estradiol concentrations which were an order of magnitude above physiological concentrations during the first two weeks which subsequently substantially decreased.

17β-estradiol is mainly produced in the ovaries, but the adrenal cortex and other organs also participate. While the production in the adrenal cortex is fairly constant, the production of estradiol in the ovaries shows substantial variations during the female rat's 3-6 day estrous cycle [22]. The cycle is commonly divided into three phases, which differ in levels of 17β-estradiol and other hormones: proestrus, diestrus and estrus. Earlier studies have shown that the 17β-estradiol concentrations range from 4.6-21 pg/mL during estrus to 57-88 pg/mL during proestrus [15,19,23-25], observations consistent with the results from the current study.

17β-estradiol circulates in the blood approximately 98% bound to plasma proteins, mainly to the low affinity and high capacity binder albumin (60%) and to the high affinity binder glycoprotein sex hormone binding globulin (SHBG) (38%). The free fraction (remaining 2-3%) is the physiologically active [26]. 17β-estradiol is mainly metabolized in the liver, primarily by conversion to the urinary metabolite estriol via estrone, but also by other oxidation reactions catalyzed by members of the cytochrome P450 family, and by sulphate and glucuronide conjugation. The biotransformation of 17β-estradiol is rapid and its plasma half-life is measured in minutes [27].

It is difficult to assess and interpret studies that measure levels of 17β-estradiol in animals after hormone replacement treatment because of the multitude of factors that can differ, including the method used for 17β-estradiol analysis, choice of wash-out time between ovx and estrogen administration, administered concentrations, administration methods and when blood was sampled in relation to time of administration, only to mention a few. The use of silastic capsules contributes to even more inter-study differences, since the possibilities of variation regarding the capsules

measures, hormone concentration, seal and incubation etc. are infinite. In addition, most studies including measurements of 17β-estradiol after 17β-estradiol administration suffer from the disadvantage of only a single blood sampling occasion. Such a measurement of 17β-estradiol is only relevant if the level of 17β-estradiol is assumed to be perfectly stable throughout the experiment.

The low concentrations initially produced by the daily injections in the current study are probably due to the fact that all blood samplings were performed 24 h after the last injection, in combination with 17β-estradiol's short half life. It is likely that the injection regimen results in an early peak and then in a rapid decrease in the hormone concentration [9]. In a previous study we showed a 70 % decrease in 17β-estradiol concentrations measured 1 respectively 4 hours after administration of a single dose of 40 μg/kg 17β-estradiol to ovx rats [28]. This means that daily injections of 17β-estradiol result in pulsatile concentrations of 17β-estradiol in plasma in contrast to the stable concentrations obtained with silastic tube. These hormone level variations in combination with the substantial intra-group variability (Table 2) produced by the injection regimen makes it an unpredictable mode of 17β-estradiol administration. However, we thought it of interest to compare the method of single daily injection to the other methods, because of its widespread use [5,6]. The rise in the concentration of 17β-estradiol seen in Gr.Inj towards the end of our experiment could be the effect of accumulation of the sesame oil containing the hormone in the animal adipose tissue. Another possibility is that the formation of scar tissue, because of the daily syringe trauma in the rat subcutis, eventually could prolong the release from the injection site and thus increase the measurable serum 17β-estradiol concentration 24 h later. The formation of scar tissue could also offer an explanation to the big intra-group variations seen day 35 and 42. There is also the explanation that the hormone treatment could alter the circulating levels of carrier molecules, which in turn could influence on the measurable 17β-estradiol concentrations. In an earlier study we investigated the pharmacokinetics of 17β-estradiol administered by injections (15 μg/day). In this study the levels of 17β-estradiol decreased from supraphysiological levels during four weeks to reach

a steady state, contrary to the current study. The explanation of this discrepancy possibly represents the difference in dosages used [8].

A crucial finding in the current study is that the IRA pellets do not result in stable serum concentrations. The 0.25 mg pellet kept the rat serum concentrations on a relatively steady, yet supraphysiological, level for 3 weeks (224.8±14.5 to 327.5±22.2 pg/mL), but not the 60 days claimed. This difference was even more striking in the case of the 0.10 mg pellet, claimed to give a steady release for 90 days. This pellet produced a very high 17β-estradiol peak on day 2 (528.0±51.5 pg/mL) to then substantially decrease to physiological levels day 14 (37.9±6.2pg/mL) and sub-physiological levels day 42 (4.2±0.8 pg/mL). IRA pellets have also been tested in several previous studies. In a previous study we didn't manage to show a stable level of 17β-estradiol in the rat until after six weeks of treatment with a 1.5 mg 90-day release pellet [8]. This lack of an early steady state was corroborated by Liu and co-workers who reported substantial differences between levels of 17β-estradiol after one and two weeks of treatment with 0.5 mg 21-day release IRA pellets [12]. In both aforementioned pellet studies, the 17β-estradiol levels were clearly above what is considered to be the physiological range [15,19,23-25]. In 2003 Degano tested two IRA pellets with different amounts of 17β-estradiol (0.05 and 0.5 mg, 21-day release) on large groups of rats with one single blood sampling occasion without seeing a linear dose-serum relation. The 0.05 mg pellet resulted in high physiological concentrations of 55±5 pg/mL while the 0.5 mg pellet gave supraphysiological concentrations of 238±7 pg/mL after 17-19 days of treatment [13]. The possibility of a biphasic dose-response curve -"hormesis"- for 17β-estradiol, that could make peaks and valleys in the hormone concentration exert qualitatively different effects [29], further underscores the crucial importance of a thorough knowledge of the method of administration used.

Despite the humble appearance of the home made silastic tubes, the administration of 17β-estradiol by them was the only method that yielded serum 17β-estradiol concentrations that were, although

steadily decreasing, within the physiological range during the entire length of the experiment (with the aforementioned exception of the last sampling in Gr.Sil.2). Day 35 and 42 of the current experiment the 17β-estradiol concentrations in the silastic capsule groups did not differ significantly from ovx levels, why any experiments to elucidate the effects of 17β-estradiol when using this method should be performed within four weeks after administration. Dubal and Wise tested in 2001 silastic capsules assembled exactly as in the current study by measuring the serum concentrations of 17β-estradiol after 7 days. The mean concentration was found to be around 20 pg/mL, which is slightly lower than in the present study (28.5±1.8 and 30.9±2.1 pg/mL). It is notable that this – to our knowledge – is the only published experiment conducted by Dubal and Wise to validate the use of these exact capsules, and that the samplings were performed on one occasion in a relatively small (n=7-11) group of animals [1]. In 1981 Wise et al. conducted an experiment with slightly different silastic capsules (inner/outer diameter: 1.57 mm/3.8 mm) containing 20 mm lengths of three different concentrations (37.5, 75 and 150 µg/mL) of 17β-estradiol. Higher concentration in the capsules yielded higher hormone levels in the animals, but without showing a linear relation. Rats receiving 150 µg/mL capsules were sacrificed at seven occasions during a two day period. A stabilization of the 17β-estradiol concentrations of 15±1.2 pg/mL was seen in this group after 2 days and 7 hours [30]. Mannino et al. measured levels of 17β-estradiol after administration via silastic capsules (inner/outer diameter: 1.47 mm/1.96 mm) at eight occasions during a 24-day period, and observed a steady-state with an average concentration of 92±8 pg/mL between days 7 to 24 after implantation. Each rat received two capsules 10 mm of length, capped with silastic medical adhesive, containing 10% of 17β-estradiol dissolved in cholesterol. In a dose area- serum level trial in the same experiment a linear relation between dose area and serum concentration was seen on day 10 [18]. However, a concern with this study regarding the serum concentrations of 17β-estradiol, is that progesterone was also administered to the rats, which has been shown to lower the serum concentrations of 17β-estradiol [12].

Serum concentrations of 17β-estradiol have been measured in ovx rats in several studies. Wise showed that rats, seven days after ovx, had 17β-estradiol levels of 5.1±0.5 pg/mL [30], while Bottner and Wuttke reported that young ovx rats had 17β-estradiol levels at 4.3±0.5 pg/mL [31]. Corresponding 17β-estradiol concentrations in the current study were 3.9±0.8 pg/mL.

There was no significant difference between the 17β-estradiol concentrations in the two groups receiving silastic capsules, indicating that having an initial wash-out period is not of importance for the measured 17β-estradiol concentrations in this experimental setup. It is known that some of the main enzymes metabolizing 17β-estradiol are induced by increasing levels of the hormone [32-35]. This could hypothetically lead to higher levels of 17β-estradiol after a period of wash-out because of a down regulation of the metabolizing enzymes. However, this effect was not observed in the present study. A possible explanation may be that the induction of the enzymes could be fast enough to eliminate the effect of the wash-out down regulation at the time of the first sampling occasion, two days after administration. We have found no previous studies comparing the effects of 17β-estradiol administration with and without a wash-out period.

The current study has a bearing on the divergent findings on the possible neuroprotective effects of 17β-estradiol administered to rats. It is reasonable to assume that the administration method previously used in our laboratory (even stronger IRA pellets than tested in the current study; 90-day release, NHH-115, 1.5 mg) [2] have resulted in supraphysiological serum 17β-estradiol concentrations in the rats, while the method adopted by Dubal and Wise [1] have probably yielded physiological, and therefore more biologically relevant, levels.

CONCLUSION

The use of silastic capsules composed as described in this study represents a reliable method for producing physiological levels of 17β-estradiol in ovx rats for 2-4 weeks after administration. It should however be realized that the hormone levels produced by this method decrease by almost

40% during an experiment lasting 2 weeks. Commercially manufactured IRA pellets result in uneven and unpredictable 17β-estradiol concentrations in ovx rats. The use of daily injections is time consuming, and result in spiking 17β-estradiol concentrations and great intra-group variability. Thus - as in the case of the pellets - the induced concentrations produced from the injection regimen are hard to predict. A wash-out period seems to be unnecessary since it does not significantly influence the levels of 17β-estradiol, and may therefore be omitted from experiments of this kind.

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