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New evidence for the selective, long-lasting central effects of the brain-targeted estradiol, Estredox

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Abstract

The present study examined the dose- and time-dependent central effects of an estradiol chemical delivery system cyclodextrin complex (E_2-CDS–CD) on the reestablishment of copulatory behavior of castrated male and ovariectomized female rats with concomitant determination of the blood luteinizing hormone (LH) and E_2 levels. In orchidectomized males, Estredox, after single doses of 0.3 and 3.0 mg/kg iv, reestablished the mounting and intromission up to 4 weeks. The LH suppressive effect lasted to Day 7 and 28, respectively. After repeated administration for 10 days at a dose of 0.01mg/kg iv, significant effect was obtained by Day 14.

Ovariectomized females were treated iv daily for 5 days either with E_2-CDS–CD, estradiol benzoate (EB) or vehicle, and the lordosis quotient was determined. At a dose of 0.03 mg/kg the duration of EB's effect was 10 days shorter and only one-third of that of E_2-CDS–CD. The LH suppression lasted to Day 18. On the other hand, after EB treatment there was no significant decrease in LH levels. The low plasma E_2 levels indicated fast rate of peripheral elimination in both males and females.

The brain-targeting E_2 indicates better efficacy and increased safety in replacement therapies because of the reduced peripheral side effects.

Keywords: Estradiol; Brain targeting; Castration; Copulatory behavior; Lordosis behavior; Central vs. peripheral effect

1. Introduction

Estrogens are among the most ubiquitous and important hormones in the female organism (Ruggiero and Likis, 2002). More than 400 different cellular actions of estrogens have been described (Sarrel et al., 1994). They affect female reproductive function and anatomy by interacting with multiple organ systems. On one hand, steroids cause changes in biological activity via binding to estrogenic receptors in the cell cytoplasm followed by gene transcription; on the other hand, they are mediators of nongenomic events, such as action on excitability of neuronal and pituitary cells and modulation of G-protein coupling (McEwen, 2001). Estradiol (E_2) is the most potent natural human estrogen, as it has the highest affinity for estrogen receptors (Ruggiero and Likis, 2002). Many of E_2's pharmacological effects are mediated through the CNS. Brain estrogen deprivation causes syndromes such as male or female sexual dysfunction, menopausal vasomotor symptoms (''hot flushes'') (Yen, 1977) and decline of cognitive function in postmenopausal women (Hogervorst et al., 2002).

Estrogens can protect from amyloid beta-induced apoptotic cell death (Hosoda et al., 2001), stroke-like ischemic injury (Simpkins et al., 1997; Wise et al., 2001), and estrogenic modulation may play a role in schizophrenia and Parkinson's disease (Cyr et al., 2002). The stress response can also be influenced by E_2 (Luine, 2002).

In males it may be potentially useful in the treatment of prostate cancer (Gerber et al., 2000). Thus, a number of potential therapeutic applications can be envisaged for a CNS-targeted system where a brain-specific sustained hormone level could be maintained. Many existing pharma-

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ceuticals are rendered ineffective in the treatment of cerebral diseases by our inability to effectively deliver them and sustain the appropriate level within the brain.

Brain-targeted chemical delivery system (CDS) represents a rational drug design approach that exploits sequential metabolism not only to deliver, but also to target drugs at their site of action (Bodor and Brewster, 1991; Bodor and Buchwald, 1999). In case of E_2-CDS the method of delivery is based upon a dihydropyridine↔pyridinium-type salt redox system. The lipophilic 17-dihydrotrigonelline ester (E_2-CDS) of E_2 is enzymatically converted to the hydrophilic trigonellinate ester (E_2-Q^+), which is specifically retained in the brain due to the BBB. The slow and sustained hydrolysis to E_2 of the hydrophilic and so "locked-in" (E_2-Q^+) takes place in the brain, by esterases. Similar (E_2-CDS) → (E_2-Q^+) conversion in the rest of the body accelerates peripheral elimination and improves targeting. By localizing drugs at their desired site of action many side effects including cancer, hypertension and altered metabolism can be avoided. (Kaplan, 1978; Lobo, 1995; Yager and Liehr, 1996) Investigation of the sexual behavior of rats represents a good in vivo model of testing the central effect of E_2.

Castration causes the termination of sexual behavior in rats (Anderson et al., 1987), but the sexual activity of castrated male and female rats can be reestablished by administration of E_2 (Beyer et al., 1976; Clark and Roy, 1983).

In males testosterone is converted to E_2 in the brain and so influences the copulatory activity (Jones et al., 1986; Krey et al., 1980).

In female rats E_2 acts in the hypothalamus and preoptic area to regulate the expression of lordosis, an important component of female reproductive behavior (Etgen et al., 1992, 1999). Lordosis is a characteristic posture of the females for a sexually active male to allow copulation (Clark and Roy, 1983; Zipse et al., 2000). E_2 acts on multiple molecular targets that may converge on common biochemical pathways to ensure integration of sensory and neurochemical cues that regulate lordosis expression.

Beyond confirming earlier results obtained in orchidectomized males (Anderson et al., 1987; Rasia-Filho et al., 1991), the main interest of the present investigation focused on the influence of E_2 on the sexual activity of females, using equimolar doses of estradiol-17-benzoate (EB) as reference compound. Aqueous solution of E_2-CDS as a 2-hydroxypropyl-β-cyclodextrin complex (E_2-CDS–CD) represents an optimal solution for the administration of the drug (Szejtli et al., 1982; Pitha et al., 1986). 2-Hydroxypropyl-β-cyclodextrin (HPCD) (Brewster et al., 1988) was selected for solubilizaton.

The circulating luteinizing hormone (LH) is a biomarker reflecting the CNS effects of E_2. Estrogen diminishes the secretion of luteinizing hormone-releasing hormone (LHRH), hence, reduces the secretion of LH. Consequently, we have also investigated LH and E_2 levels as the measure of central and peripheral effects of E_2-CDS–CD, respectively.

2. Methods

2.1. Animals

Adult male (300–400g) and female (220–250g) Sprague–Dawley rats (Charles River, Godollo, Hungary) were used. Animals were kept in community cages (four animals/cage) in a climate-controlled room (23 ± 2 °C, 50–60% humidity) with a 14-h light, 10-h dark cycle of artificial lighting, using reversed light–dark cycle. Food and water were available ad libitum.

2.2. Surgery

Under ether anesthesia, animals were bilaterally orchidectomized or ovariectomized, then left to recover for 4 weeks before measuring the sexual activity.

All animals were treated in accordance with the guidelines of the European Communities Council Directive (86/609/EEC) and studies were permitted by the Institutional Animal Care Commission.

2.3. Drugs

Estradiol benzoate and progesterone were obtained from Sigma Chemical Budapest, Hungary.

2-Hydroxy-propyl-β-cyclodextrin was purchased from Cerestar, Hammond, USA.

EB was dissolved in 40% (wt/vol) HPCD solution and diluted with 27% (wt/vol) HPCD (0.29 mg/kg is equimolar to 0.3 mg/kg E_2-CDS–CD).

Estradiol-17-*N*-methyl-1,4-dihydronicotinate ester (E_2-CDS) as a 3% complex with HPβCD (E_2-CDS–CD, Estredox) was dissolved in distilled water and diluted with 27% HPCD solution. E_2-CDS–CD was synthesized by Alchem Laboratories, Alachua, USA.

2.4. Sexual behavioral tests

2.4.1. Experiments in orchidectomized male rats

To establish baseline behavior each male ($n = 60$) was tested every 5 days until four successive and consistent behavioral patterns were achieved. The selected rats were bilaterally castrated via a single midventral incision.

After repeated testing at the 28th days after castration (Day 0) rats were randomly divided among four experimental groups (8–12 animals/ group). Only those animals that showed an ejaculation latency greater than 15 min were used. Groups were treated with different doses (0.03, 0.3 and 3 mg/kg) of E_2-CDS–CD via a single tail vein injection in a volume of 0.1 ml/100 g body weight. The control group

received 27% HPCD solution intravenously in the same volume. Examinations of male sexual behavior were conducted on Days 3, 7, 14, 28 and 35 after drug administration or until the effect disappeared. Three weeks after the experiment had been completed the sexually inactive animals were newly randomized and the test was repeated with daily administration of E_2-CDS–CD at a dose of 0.01 mg/kg for 10 days.

Female rats weighing 200–250 g were brought to receptivity by subcutaneous injection of EB (50 μg/animal) 48 h before testing and progesterone, dissolved in sunflower oil (0.5 mg/animal), 4 h prior to the experiments. Mating tests were done during the dark cycle placing the animals into a large Plexiglas observation cage where only a dim light was on. The male was always introduced into the test area 5 min prior to the female. Behavioral patterns and related times were recorded by skilled observers.

The following parameters of male copulatory behavior were measured: mount latency (ML), the time from the introduction of the female to the initial mount or intromission; mount frequency (MF), number of mounts during the observation period; intromission latency (IL), the time from introduction of the female to the first intromission; intromission frequency (IF), number of intromissions during the observation period; ejaculation latency (EL), time from first intromission to ejaculation. Tests were considered negative if IL exceeded 15 min.

2.4.2. Experiments in ovariectomized female rats

After recovery from the surgery, ovariectomized female rats were divided into four groups and treated once a day for 5 days with the following drug doses of E_2-CDS–CD: 0.003, 0.01 and 0.03 mg/kg via a bolus injection through a tail vein. The control group received the vehicle in the same volume. Testing started 2 days after the first treatment with E_2-CDS–CD.

The investigation of EB was performed in the same newly randomized sexually inactive females after 3 weeks of washout period. The animals were treated similarly to the treatment schedule applied for E_2-CDS–CD at a dose equimolar to that of E_2-CDS–CD.

The experienced and active male rats were placed individually into the Plexiglas arena 5 min prior to the female. Females were observed for maximum 15 min or for 10

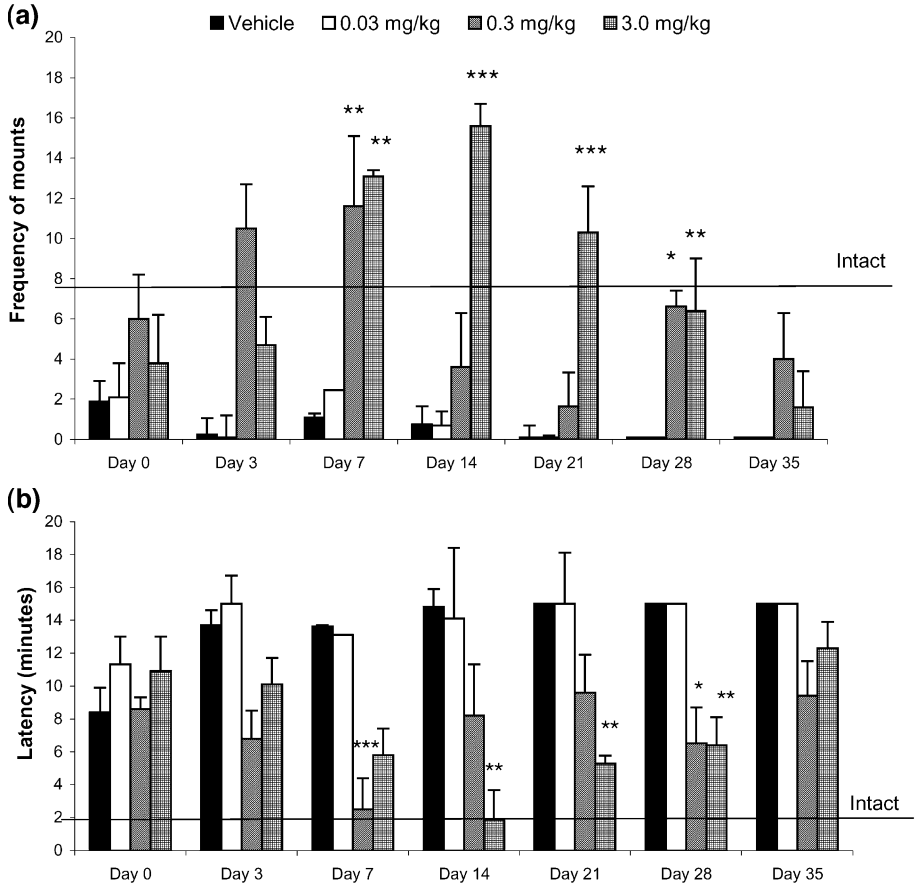

Fig. 1. Effect of E_2-CDS–CD on the mounting frequency of castrated male rats from Day 0 to Day 35 after a single iv injection (a). Effect of E_2-CDS–CD on the mounting latency of castrated male rats from Day 0 to day to Day 35 after a single iv injection (b). Each point represents the group mean ± S.E.M. * $P<.05$, ** $P<.01$, *** $P<.001$ vs. vehicle-treated control group, analyzed by Mann–Whitney U test ($n=8–12$). The intact levels were determined before castration (mean value of four groups, $n=44$).

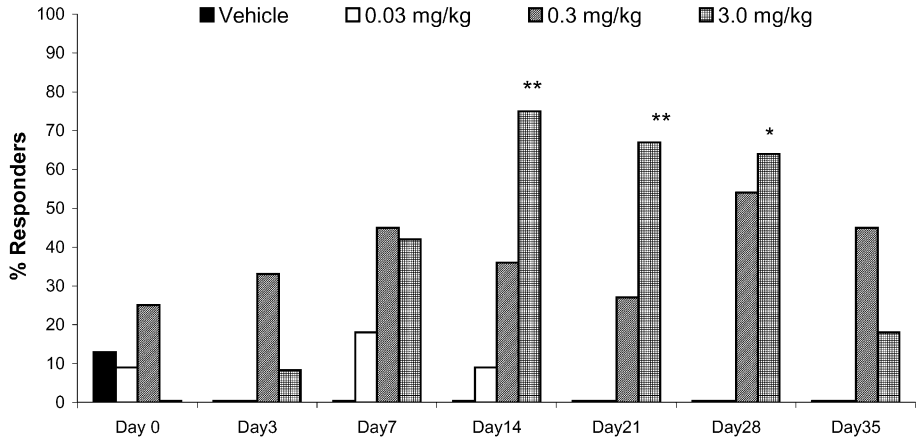

Fig. 2. Effect of E_2-CDS–CD on the intromission performance (percent responders) of castrated male rats from Day 0 to Day 35 after a single iv injection. The intromission performance in all the four groups was 100% before castration. Groups were analyzed by the Fisher exact test. $*P<.05$, $**P<.01$ vs. vehicle-treated control group ($n=8–12$).

successful mounts per test session and the number of lordosis responses was recorded. The lordosis quotient (LQ) reflecting the estrogen effect on sexual receptivity was calculated as follows: LQ = 100 × number of lordoses/10 mounts.

Investigations on female sexual behavior were performed every day for 10 (EB) and 22 (E_2-CDS–CD) days, respectively.

2.5. Radioimmunoassay of LH and E_2

Citrated blood samples were taken by the retro-orbital sinus puncture under light ether anesthesia to determine plasma LH and E_2 levels. The samples were stored at 4 °C for 1 h then centrifuged at 1000 × *g* for 10 min. Plasma was separated and stored at − 80 °C until assayed. Plasma LH concentrations from individual samples were measured by double antibody radioimmunoassay kits obtained from Amersham Pharmacia Biotech, Rome, Italy. Plasma E_2 levels were determined by double antibody ^{125}I isotope RIA kits obtained from BioChem Immuno System. The limit of detection was 15 pg/ml.

2.6. Statistics

Behavioral changes were analyzed by using Mann–Whitney *U* test (Siegal, 1956). Fisher exact test was used for percentage comparisons (Zar, 1974). The serum LH data

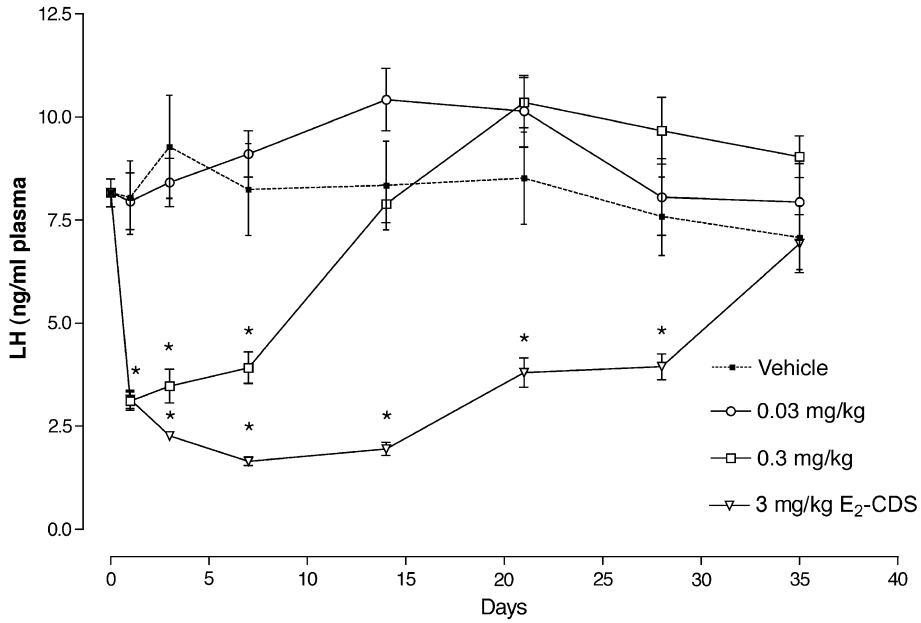

Fig. 3. Plasma LH levels of male rats after bilateral castration and E2-CDS–CD treatment. $*P<.05$ vs. vehicle-treated group. Each point represents the group mean ± S.E.M. Analyzed by ANOVA followed by Bonferroni post hoc test ($n=8–12$).

were analyzed for each time and treatment group by analysis of variance (ANOVA) followed by Bonferroni post hoc test. Plasma LH and E_2 concentrations were evaluated by computerized standard curve program of Prism software (Version 3.0, Graph Pad, San Diego, CA, USA).

3. Results

3.1. Experiments in orchidectomized male rats

Mounting frequency was significantly increased by Day 28 in doses of 0.3 and 3.0 mg/kg (Fig. 1a) and, in parallel, mounting latency was sharply reduced from Day 7 to Day 28 in the two higher doses (Fig. 1b). The intromission performance was also significantly improved from Day 14 to Day 28 at the dose of 3.0 mg/kg (Fig. 2) Again, a significant increase of intromission frequency and decrease of intromission latency was observed from Day 14 to Day 28 at the dose of 3.0 mg/kg (data not shown). A single dose of 0.03 mg/kg did not show significant effect at any day investigated.

When E_2-CDS–CD was administered at a daily intravenous (iv) dose of 0.01 mg/kg for 10 days, this dosing schedule had significant effect both on mounting and intromission performance by Day 14: it restored mounting performance by 67% and intromission performance by 50% (data not shown).

Mean plasma LH concentration of intact (i.e., not yet orchidectomized) male rats was 1.10 ± 0.15 ng/ml. Four weeks after bilateral orchidectomy LH levels increased roughly to eightfold of the normal level (8.13 ± 0.33 ng/ml, Fig. 3). At the low dose of E_2-CDS–CD (0.03 mg/kg) plasma LH levels were not reduced. At the dose of 0.3 mg/

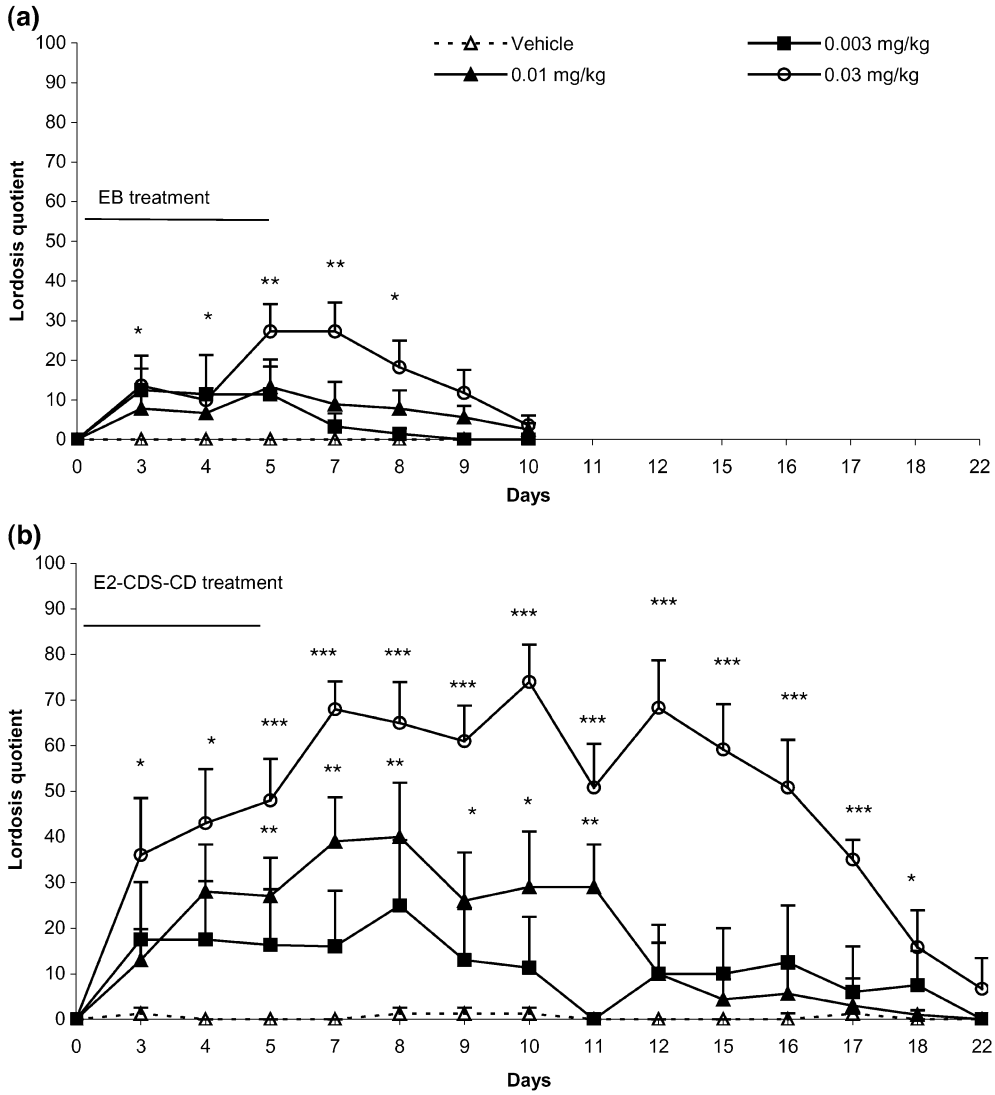

Fig. 4. Effect of different doses of EB on lordosis behavior of castrated female rats (a). Effect of different doses of E2-CDS–CD on lordosis behavior of castrated female rats (b). Animals were treated intravenously once a day for 5 consecutive days. Each point represents the group mean ± S.E.M. * $P < .05$, ** $P < .01$ vs. vehicle-treated control group analyzed by Mann–Whitney U test ($n = 7–12$).

kg, significantly reduced LH levels were found on Days 1, 3 and 7. At the high dose of E_2-CDS–CD (3.0 mg/kg) the LH levels were suppressed significantly throughout 28 days. When E_2-CDS–CD was administered daily for 10 consecutive days plasma LH level was significantly reduced from Day 3 to Day 14 (data not shown).

Plasma E_2 levels were detectable only at the dose of 3 mg/kg. On the first day after treatment the E_2 level was 258 ± 9 ng/ml, which decreased to 61 ± 8 ng/ml by Day 7. From Day 7 E_2 level was below the limit of detection.

3.2. Experiments in ovariectomized female rats

After recovering from ovariectomy (3 weeks after surgery) female rats were randomized and treated with different doses of E_2-CDS–CD or vehicle. The minimum number of animals per group was seven. The investigation of the positive reference compound EB was performed in the same newly randomized females after 3 weeks of washout.

At the equimolar single dose of 0.03 mg/kg both compounds significantly enhanced the LQ value. In case of E_2-CDS–CD this effect lasted from Day 3 to Day 18 (Fig. 4). The potency of EB was less pronounced, lasted from Day 3 only to Day 8 and the LQ value was about three times lower compared to E_2-CDS–CD: the maximal values of LQ after E_2-CDS–CD and EB treatment were 73 and 27.3, respectively. The 0.01 mg/kg dose of E_2-CDS–CD significantly enhanced LQ from Day 5 to day 11. On the contrary, the equimolar low and mid doses of EB had no significant effect (Fig. 4).

At the dose of 0.03 mg/kg of E_2-CDS–CD the plasma LH level of ovariectomized female rats was decreased from 9.5 to 5.1 on Day 10 and to 5.6 on Day 18. Following equimolar EB treatment no significant changes in plasma LH levels were observed. The plasma E_2 levels were not detectable at any doses investigated.

4. Discussion

In the present study the reestablishment of the sexual activity in orchidectomized and ovariectomized rats was investigated as an easy in vivo measure of the central effects of a brain-targeted E_2.

E_2-CDS–CD dose dependently improved the sexual activity both in castrated female and male rats. This effect was long lasting. By contrast, results obtained in females showed that equimolar doses of EB were considerably less effective and its effect was much shorter.

The central effect of E_2-CDS–CD was confirmed by plasma LH suppression. The elevation of plasma E_2 level was transiently observed only at the highest dose (3 mg/kg); however, the improvement of sexual behavior lasted much longer. These changes indicate the prolonged central effect of E_2-CDS as a result of the sustained release of the E_2 from the locked-in E_2-Q^+ (Sarkar et al., 1989).

Anderson et al. (1987) demonstrated that E_2-CDS dissolved in DMSO reestablished male rat's copulatory behavior for 28–35 days at a single iv dose of 3 mg/kg. Rasia-Filho et al. (1991) found that E_2 implanted into the corticomedial amygdala normalizes impaired sexual behavior of castrated male rats on the sixth and ninth day after drug implantation. We have confirmed these results and shed more light for the potential advantage of E_2-CDS. In our study, this compound effectively reestablished the estrogen-dependent components of sexual behavior in experienced castrated male rats. When it was administered at a daily iv dose of 0.01 mg/kg for 10 consecutive days it also had a significant effect on mount frequency and on mount and intromission latency, indicating the central effect and the lock in of the E_2-Q^+ in the brain. The LH suppression lasted three times longer than the duration of the treatment.

In ovariectomized female rats the influence of E_2-CDS–CD on the sexual behavior was not investigated before; only the changes in blood LH level were studied upon administration of the brain-targeted compound. Estes et al. (1987) found that a single iv dose of 0.5 mg/kg E_2-CDS–CD caused 3- to 6-week-long LH suppression. In a further investigation, using a dose of 0.1 mg/kg, the LH suppressive effect of E_2-CDS–CD lasted for 18 days (Brewster et al., 1988). In the present study, we have not only confirmed the former results but also found that the plasma LH level was suppressed even at as low an iv dose of E_2-CDS–CD as 0.03 mg/kg. Additionally, in our present experiment we demonstrated that Estredox treatment has a long-lasting (18 days postadministration) effect on the lordosis behavior of female rats at the single dose of 0.03 mg/kg.

The observed concomitant LH suppression confirmed that this E_2 chemical delivery system has a long half-life in the brain, thus sustaining elevated E_2 level in the brain. However, the slowly released E_2 that crossed the blood–brain barrier is quickly cleared from the peripheral tissues; this idea is supported by normal blood E_2 levels. Clark and Roy (1983) have demonstrated that the greatly enhanced behavioral response to repeated pulses of E_2 is consistent with the hypothesis that an initial action of E_2 may sensitize target tissues to further stimulation by the hormone in ovariectomized female rats. In according with this finding we observed that repeated low doses of Estredox (0.01 mg/kg once daily for 10 days) have been more effective than a single low dose of the same compound.

As the CNS is the target site for estrogen treatment of postmenopausal syndrome, brain-targeted delivery system of the hormone represents a safe and effective therapeutic possibility in most cases. Constant elevated peripheral exposure to estrogens may lead to a number of pathological conditions including breast cancer, coronary heart disease, and pulmonary embolism (Beral et al., 2002). Contrary to earlier expectations, hormone replacement therapy (HRT)

does not benefit the incidence of coronary heart disease (Low et al., 2002). The estrogen plus progestin combination of the Women's Health Initiative trial, in postmenopausal women, was stopped prematurely due to an unacceptably increased risk for invasive breast cancer, stroke and heart attack (Rossouw et al., 2002). Therefore, there is less justification for a *systemic* estrogen replacement with the current preparations, but there is a need for a treatment based on differential distribution of E_2.

Our results suggest better efficacy and increased safety obtained with the brain-targeted form of E_2 in replacement therapies. First clinical studies with buccal administration of E_2-CDS–CD confirm the results of the animal experiments (Estes et al., 1994). Phase I/II clinical evaluations in postmenopausal volunteers have shown E_2-CDS–CD to be safe and produce significantly longer lasting central estrogenic effects than the parent E_2 while also maintaining sustained effective but safe peripheral levels of the active drug.

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