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Suppressive effect of neonatal treatment with a phytoestrogen, coumestrol, on lordosis and estrous cycle in female rats

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Abstract

The neural control systems for the ovulatory cycle and lordosis behavior are sexually differentiated by estrogen during the perinatal period in rats. In the present study, the effects of a single neonatal injection with the phytoestrogen, coumestrol, on female reproductive functions were investigated. Female rats were injected subcutaneously with 1 or 3 mg coumestrol (CM1, CM3), 1 mg genistein (GS1), 1 mg estradiol (E_2), or oil at day 5 after birth (birth day = day 1) and an estrous cycle check and lordosis behavior test were performed. As a result, vaginal opening was advanced in CM1-, CM3- or E_2-treated females. A vaginal smear check indicated that oil- or GS1-treated females showed a constant 4- or 5-day estrous cycle, whereas CM1-, CM3- or E_2-treated rats showed a persistent or prolonged estrus. Ovariectomy was performed in all females at 60 days of age. The ovary weights in the CM1-, CM3- or E_2-treated groups were lower than those in the oil- and GS1-treated groups and no corpora lutea were found in any rats of these three groups, except for two E_2-treated rats. Behavioral tests were carried out after implantation of E_2-tubes. All rats in the CM1-, GS1-treated groups showed a high lordosis quotient (LQ), being comparable to that in the oil-treated females. On the other hand, LQs in the CM3, E_2 or male groups were lower than that in the control female group. These results suggest that a single neonatal injection of 3 mg coumestrol was effective in suppressing the functions of ovulation-inducing mechanisms and the induction of lordosis, but 1 mg coumestrol was effective in only the estrous cycle of female rats.

Keywords: Phytoestrogen; Coumestrol; Sex differentiation; Brain; Lordosis; Estrous cycle; Rat

1. Introduction

Sexual differentiation of the brain function is known to occur during the perinatal period in the rat. Conspicuous sexually dimorphic features are estrus-cyclicity and the sexual behavioral pattern in mammals. These functions are controlled by the hypothalamus: by the prepoptic area (POA) for cyclicity [7,25], and by the ventromedial hypothalamic nucleus (VMN) and the septum for female sexual behavior [20,32]. During the perinatal period, the presence of androgen or estrogen causes these functions to become the male type, consisting of acyclic estrus and low lordosis [1,8,27]. In perinatal rats, testosterone in the neurons is converted by aromatase in many brain areas that are concerned with estrous cyclicity and lordosis. In the critical period for sexual differentiation of the brain, substances that act on the receptor for estrogen have the potency to cause the male brain to lack cyclicity and have a low female sexual behavioral pattern.

Many estrogenic substances, such as bisphenol A or nonylphenol, are known to exist in industrial products [13]. Plants also contain many kind of estrogenic compounds, such as phytoestrogen [10,13], one of which is isoflavone [21,22]. A considerable amount of evidence suggests that isoflavone acts as an estrogenic agent on reproductive functions in experimental animals [31]. One of the potent isoflavones is genistein (GS) in the soybean. Although, GS has the capacity to bind to estrogen receptors (ER) [16], this substance is used as an antiestrogen, because the estrogenic activity of this phytoestrogen is thought to be weaker than that of estradiol and prevents estrogen from binding to the receptor [10,11]. In contrast, there are reports that GS acts as an agonist of the

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estrogen receptor in the neonatal period in rats, because a daily injection of 1 mg GS for 5 days from birth induced an acyclic condition and low lordotic activity in female rats [15]. Furthermore, injections of 1 mg GS for 10 days in the neonatal period caused the male type of sexually dimorphic nucleus to appear in the POA and a low response of LH secretion of the pituitary to GnRH [5,6]. GS has been reported to have a 20-fold higher affinity to ERβ than to ERα [16]. In addition to isoflavones, coumestrol (CM), which belongs to the coumestans, is also known to be a potent phytoestrogen, which is obtained from clover and alfalfa sprouts [2]. Similar to GS, CM has been reported to have a higher affinity to ERβ than to ERα and its binding affinity to ERβ is comparable or superior to that of estradiol [16]. Neonatal feeding with CM influences development of the reproductive functions [29,30]. However there is no clear evidence for the effect of CM on sexual differentiation of the brain.

In this experiment, to clarify the estrogenic potency of coumestrol on sexual differentiation of the regulatory systems for estrous cyclicity and female sexual behavior, female rats were injected neonatally with CM and their female reproductive functions were analyzed in adulthood.

2. Materials and methods

2.1. General procedures

Pregnant Wistar rats were purchased from Takasugi Animal Farm (Saitama, Japan). The animals were kept in a light-controlled (14L:10D, lights on at 05:00 h) and temperature-controlled (23–25 °C) room with free access to water and food. All experiments were carried out according to the Guidelines for the Care and Use of Laboratory Animals in the School of Human Science of Waseda University.

Litter size was adjusted to 10 pups on the birth day. Female pups were injected with CM or GS at day 5 (birth day = day 1). Vaginal opening and vaginal smears were checked. At day 60, all animals were ovariectomized. The ovaries were weighed and fixed to check for the existence of the corpus luteum (CL). One to two weeks after the ovariectomy, silicon tubes containing estradiol (E_2) were implanted subcutaneously and three behavioral tests were performed.

2.2. Injection of phytoestrogens

Female pups were subcutaneously injected with 1 or 3 mg of coumestrol (CM1, CM3; LKT Laboratories, Inc., St. Paul, MN; suspended in 0.05 ml of sesame oil; $n = 10$, 11, respectively), or 1 mg genistein (GS1; Nakahara Co., Ltd., Gifu, Japan; suspended in 0.05 ml of oil; $n = 6$) at day 5. In 11 female pups, 1 mg 17β-estradiol (E_2; Sigma, St. Louis, MO; suspended in 0.05 ml of oil) was injected at day 5. Control female pups ($n = 11$) received 0.05 ml sesame oil at day 5. All female pups within a litter received the same treatment. One to three litters were used in one treatment group.

2.3. Vaginal opening and vaginal smear check

In all female pups, the day of vaginal opening was recorded. The differences in the day of vaginal opening among the groups were detected by the Mann–Whitney *U*-test.

Vaginal smears were checked from the vaginal opening until day 60 when ovariectomy was performed. A female rat that showed a constant 4- or 5-day vaginal estrous cycle was regarded as an animal with a regular estrous cycle. When a vaginal smear contained cornified cells through the examination term, it was judged to be persistent estrus, even if a few leukocytes were seen occasionally. If the diestrous condition appeared in some intervals in persistent estrus, the condition was regarded as a prolonged estrus. Incidence of regular or irregular cyclicity was analyzed by the χ^2-test.

2.4. Gonadectomy and histological examination

On day 60, all female rats were ovariectomized under ether anaesthesia. Ten males from six litters were orchidectomized to use as male control in lordosis behavioral test. The ovaries were weighed and fixed in Bouin's fluid. To confirm the existence of CL histologically, 10 μm-thick paraffin sections of the ovaries were made and stained with hematoxylin and eosin.

2.5. Behavioral tests

One to two weeks after the gonadectomy, the animals received subcutaneous implants of two silicon tubes (inner diameter, 1.57 mm; outer diameter, 3.18 mm; length, 30 mm: Kaneka Medics, Osaka, Japan) filled with E_2 (Sigma, St. Louis, Missouri). A behavioral test was performed 2, 4 and 6 days after E_2 tube implantation. Under a red light, each experimental animal was placed in a transparent-plastic observation cage (width, 60 cm; length, 50 cm; depth, 40 cm) with two vigorous male rats, and the lordosis quotient (LQ, number of lordosis reflexes/10 mounts by male × 100) was recorded in each rat. Differences in the mean LQs among the groups were analyzed by two-way ANOVA for repeated measures followed by contrast analysis between the groups.

3. Results

3.1. Vaginal opening

The vaginal orifice opened at day 34 (median) in the oil-treated females (Table 1). The median day of vaginal opening was 34.5 in the GS1 group. In the E_2 group, the median day was advanced to day 26 ($P < 0.01$ versus oil, GS1, and CM3 groups, respectively). As in the E_2 group, the median day of vaginal opening was day 25 in the CM1 group ($P < 0.01$ versus oil and GS1 groups, respectively), and day 24 in the CM3 group ($P < 0.01$ versus oil, E_2, and GS1 groups, respectively).

Table 1
Effects of neonatal treatment with coumestrol (CM), genistein (GS), or estradiol (E_2) on vaginal opening and estrous cycle in female rats

Treatment	Number of rats	Vaginal open (median date)	BW (g) at 30-day age	Vaginal estrous cycle		
				Regular	Irregular	
					Prolonged	Persistent
Oil	11	34 (31–38)	113 ± 5	11	0	0
1 mg E_2	11	26[c] (23–29)[a]	123 ± 3	0[f]	9	2
1 mg CM	10	25[d] (22–28)[b]	114 ± 2	0[f]	3	7
3 mg CM	11	24[e] (20–24)	95 ± 8	0[f]	8	3
1 mg GS	6	34.5 (29–37)	112 ± 4	6	0	0

Parentheses indicate the range of value.
[a] $n = 6$.
[b] $n = 5$.
[c] $P < 0.01$ vs. oil, 3 mg CM and 1 mg GS.
[d] $P < 0.01$ vs. oil and 1 mg GS.
[e] $P < 0.01$ vs. oil, E_2, and 1 mg GS.
[f] $P < 0.01$ vs. oil and 1 mg GS.

At weaning (day 30), there are no statistical difference in the body weights between all of E2- or CM-treated groups and oil-treated group.

3.2. Vaginal cycle

A regular 4-day estrous cycle was observed in all oil-treated control animals (Table 1). In contrast, none of the E_2-rats showed a regular estrous cycle ($P < 0.01$ versus oil and GS1 groups, respectively). A Prolonged estrus was seen in 9 out of 11 E_2-rats, and 2 rats showed a persistent estrus. In the CM1 group, 7 out of 10 females showed a persistent estrus, and another 3 rats showed a prolonged estrus ($P < 0.01$ versus oil and GS1 groups, respectively). In the CM3 group, 8 out of 11 females showed a prolonged estrus, and another 3 showed a persistent estrus ($P < 0.01$ versus oil and GS1 groups, respectively). As in the oil group, all rats in the GS1 group showed a 4-day estrous cycle.

3.3. Ovarian condition

Mean ovarian weight was 90.3 mg in the control group (Table 2). In the GS1 group, ovarian weight was almost the same as that in the control group. On the contrary, in the E_2 group, mean ovarian weight was almost one-half the value of the control group ($P < 0.01$ versus oil and GS1 groups, respectively). As in the E_2 group, mean ovarian weights in both CM1 and CM3 groups were also lower ($P < 0.01$ versus oil and GS1 groups, respectively).

Table 2
Effects of neonatal treatment with coumestrol (CM), genistein (GS), or estradiol (E_2) on ovarian condition at 60-day age in female rats

Treatment	Number of rats	Ovarian weight (mg)	Corpus luteum	Body weight (g)
Oil	11	90.3 ± 3.3	11/11	297 ± 8
1 mg E_2	11	50.1 ± 4.9[a]	2/11[b]	358 ± 14[c]
1 mg CM	10	35.3 ± 2.6[a]	0/10[b]	326 ± 9
3 mg CM	11	38.4 ± 3.8[a]	0/11[b]	306 ± 16
1 mg GS	6	106.5 ± 5.1	6/6	331 ± 11

[a] $P < 0.01$ vs. oil and 1 mg GS.
[b] $P < 0.01$ vs. oil and 1 mg GS.
[c] $P < 0.05$ vs. oil.

Histological examination showed that all rats in the control group had CL in their ovaries (Table 2, Fig. 1). As in the control group, all GS1-rats had ovaries with CL. Nine of eleven E_2-rats had no CL, but two E_2-rats, which showed a prolonged estrus, had ovaries with CL ($P < 0.01$ versus oil and GS1 groups, respectively). CL was not seen in all CM1- and CM3-rats ($P < 0.01$ versus oil and GS1 groups, respectively).

3.4. Lordosis behavior

Through three behavioral tests, repeated measures of ANOVA showed statistically significant differences in the results of the groups ($P < 0.0001$) (Fig. 2). Mean LQ in the control group increased from 15.5 ± 7.7 in the first test to 70.9 ± 9.2 in the third test. Both CM1- and GS1-treated females also showed a similar increase in LQ to those seen in the control rats. In contrast, 7 of 10 male rats showed lordosis, and the mean LQs were lower than those in control females in the 2nd and 3rd tests ($P < 0.01$). As in males, mean LQs were low in the E_2-treated group, although lordosis was observed in 7 of 11 rats. In CM3-treated females, lordosis was observed in 8 of 11 rats, and the mean LQ at the final test was lower than that in the control female rats ($P < 0.01$) and comparable to the values in males and the E_2 groups.

4. Discussion

In this experiment, all female rats treated with E_2 or CM on day 5 after birth developed an anovulatory condition, because of persistent or prolonged estrus with no CL in these animals. Conversely, a single injection of GS was not effective, although daily injections for the first 5 days after birth have been shown to suppress the ovulatory cycle [15]. These results suggest that 1 mg CM has an estrogenic potency

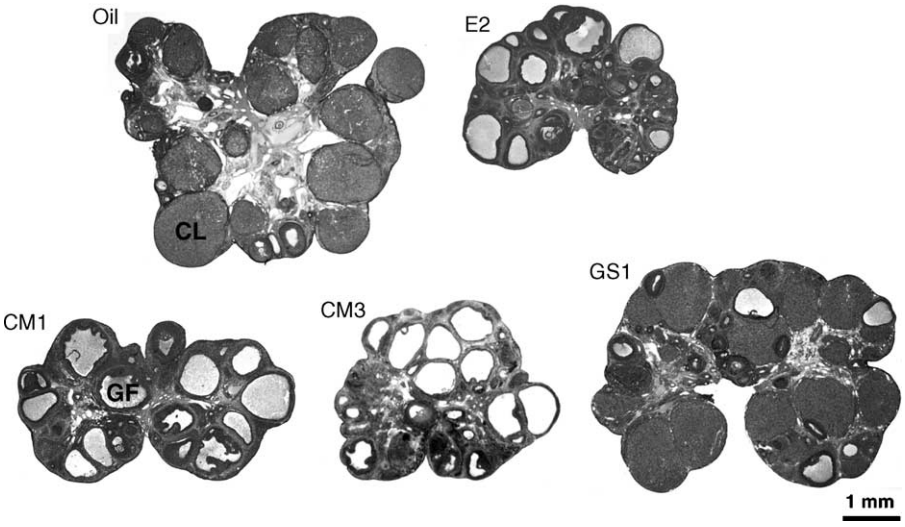

Fig. 1. Representative microphotographs of ovaries of rats neonatally treated with sesame oil (Oil), 1 mg estradiol (E_2), 1 mg coumestrol (CM1), 3 mg coumestrol (CM3), or 1 mg genistein (GS1) at day 5. Corpola lutea are absent from the ovaries in the E_2 and CM groups. CL, corpus luteum; GF, Graafian follicle.

that is comparable to the same dose of E_2 and is higher than that of GS in masculinization of the neonatal rat brain.

Ovarian weight is influenced by the size and number of Graafian follicles and CL, which are in turn influenced by the amounts of circulating LH and FSH. The absence of CL indicates the lack of LH surge. Although, no hormonal assay was conducted in this study, differences in ovarian weight among the groups suggested that neonatal E_2- or CM-treatment suppressed the surge of gonadotropin.

The male secretory pattern of gonadotropin is formed by estrogen intraneuronal converted from testosterone in rats [18]. The preoptic area is responsible for cyclic regulation of gonadotropin [25]. In the present experiment, the function of the center for cyclic ovulation in the preoptic area was

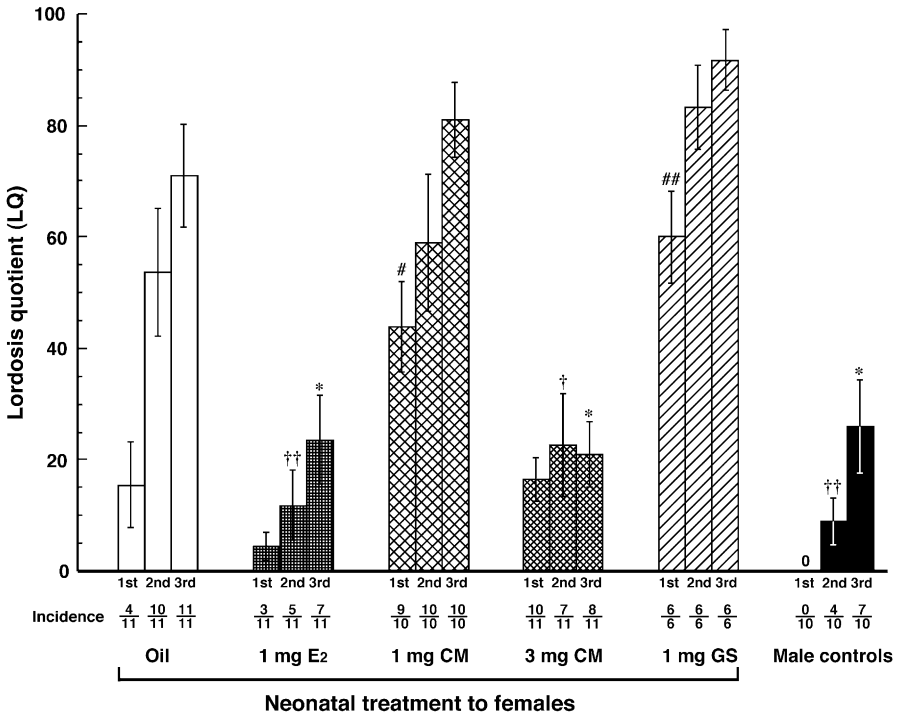

Fig. 2. Mean lordosis quotient in each group in three tests. Female rats were treated with sesame oil (Oil), 1 mg estradiol (E_2), 1 mg-, 3 mg-coumestrol (CM1, CM3, respectively), or 1 mg genistein (GS1) at day 5. Behavioral tests were performed 2, 4, and 6 days after the implantation of E_2 tubes. Bar on the top of each column is S.E. $^{*}P < 0.01$ vs. Oil, CM1, and GS1: $^{\dagger}P < 0.05$ vs. Oil and CM1; $P < 0.01$ vs. GS1: $^{\dagger\dagger}P < 0.01$ vs. Oil, CM1 and GS1: $^{\#}P < 0.05$ vs. Oil; $P < 0.01$ vs. E_2 and Male: $^{\#\#}P < 0.01$ vs. Oil, E_2, and CM3. Bar on the top of each column is S.E.

probably disrupted by coumestrol in the neonatal period in female rats, despite the lack of direct evidence that coumestrol can pass through the blood–brain barrier. It has been reported that the isoflavone, genistein, has the potential to cross the barrier [4].

Similar to E_2, a single injection of 1 mg CM in the neonatal period advanced the timing of vaginal opening in the present experiment. This result was consistent with previous results showing that neonatal estrogen [17] and phytoestrogen [15] accelerated canalization of the vagina. However, the present result suggested that a single injection of 1 mg GS was not sufficient to advance the day of vaginal opening, although the injections of 1 mg GS for 5 days after birth, was effective [15]. CM has been reported to advance vaginal opening even when the administration was started from day 20 after birth [28]. Thus, CM is as effective as estrogen for the development of peripheral tissue as well as the brain.

On the other hand, a single injection of 1 mg E_2 on postnatal day 5, but not 1 mg CM suppressed lordosis behavior in the present result. When the dose of CM was increased to 3 mg, a lordosis-suppressing effect appeared. This result suggests that the defeminizing effect on the lordosis-regulating system of CM is not strong when compared to E_2, and defeminization of the lordosis-regulating system requires a higher level of estrogen than masculinization of the estrous-cycle system. The difference in lordotic activity in adulthood is dependent on the dose of injected estrogen during the neonatal period, because a high dose of estrogen causes severe loss of lordosis, compared to a low dose [3,26]. Neonatal estrogen is thought to act on the lordosis-inhibiting mechanism in the lateral septum [32] and strengthen the inhibition [12]. It can be said that 3 mg CM, but not 1 mg CM was sufficient to strengthen the inhibition and suppress the induction of lordosis in female rats.

As well as adult animals, the neonatal brain contains both ERα [33] and ERβ [9]. In the hypothalamus, mRNA expression of both ERs is influenced by neonatal estrogen [24]. The binding affinities of CM and E_2 to both ERα and ERβ is stronger than that of GS [16]. CM has been reported to have greater potency for increasing ER binding to the estrogen response element than GS [14]. If this phenomenon can be generalized to the neonatal brain, the order of binding affinities to ER is consistent with the present functional results. Although the binding affinities of E_2 to ERα and ERβ were similar, those of CM and GS to ERβ were greater than to ERα [16]. Furthermore, the affinity of CM to ERβ was higher than that of E_2 [16]. In the present functional results, the masculinizing effect on estrous cyclicity was seen in female rats treated with E_2 and CM, but the defeminizing effect on the lordosis-inducing mechanism induced by CM was weak, when compared to that induced by E_2. From the aspect of ER-binding affinity together with the present functional facts, it can be predicted that ERα rather than ERβ is closely involved in the defeminizing system, although the possibility of correlation of ERβ to defeminization was suggested in our previous report [15]. However, it has been reported that a region-specific difference exists in the influence of CM on ERβ in the brain [19]. Furthermore, ERβ has been found to be involved in the regulation of sexually dimorphic neural responses to E_2 [23]. Further experiments are needed to clarify these points.

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