Journal of Steroid Biochemistry & Molecular Biology 114 (2009) 152–160

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Antiovulatory effect of a single injection of pure antiestrogen ZK 191703 at early stage of rat estrus cycle

Tsuyuki Nishino[a], Hiroshi Yamanouchi[b,1], Kanako Ishibashi[c], Christian Hirtreiter[d,*], Yukishige Nishino[e,2]

[a] Experimental Toxicology and Ecology, Reproduction Toxicology, BASF SE, Carl-Bosch-Straße 38, 67056 Ludwigshafen, Germany
[b] Research and Development, Nihon Schering K.K., Nishimiyahara 2-6-64, Yodogawa-ku, Osaka 532-0004, Japan
[c] Japan Clinical Research, Lilly Research Laboratories Japan, Ely Lilly Japan K.K., Sannomiya Plaza Building 7-1-5, Isogamidori, Chuo-ku, Kobe 651-0086, Japan
[d] Institute of Organic Chemistry, University of Regensburg, Universitätsstraße 31, 93053 Regensburg, Germany
[e] Research and Development, Nihon Schering K.K., Lanzendorfer Weg 14, 14089 Berlin, Germany

ARTICLE INFO

Article history:
Received 9 October 2008
Received in revised form 23 January 2009
Accepted 28 January 2009

Keywords:
Antiestrogen
Tamoxifen
Hormone levels
Rat
Antiovulatory effect

ABSTRACT

The effects of ZK 191703 (ZK), a pure antiestrogen, on ovulation, follicle development and peripheral hormone levels were investigated in rats with 4-day estrus cycle and gonadotropin-primed immature rats in comparison to tamoxifen (TAM)-treatment. In adult rats, a single s.c. injection of ZK (5 mg/kg) or TAM (5 mg/kg) at an early stage of the estrus cycle (diestrus 9:00) inhibited ovulation, and was associated with suppression of the surge of preovulatory LH, FSH and progesterone. In rats treated with ZK or TAM at a late stage of the estrus cycle (proestrus 9:00), no inhibitory effects on ovulation, the gonadotropin and progesterone surge were detected. ZK treatment at diestrus 9:00, in contrast to TAM, increased the baseline LH level. When immature rats were treated with antiestrogens in the earlier stage of follicular development, 6 and 30 h but not 48 h or later after injection of gonadotropin (PMSG), ovulation was attenuated, associated with a lowered progesterone level. Unruptured preovulatory follicles were found in most of the ovaries from anovulatory animals treated with ZK or TAM. Antiestrogens, ZK and TAM administered at an early phase of the estrus cycle delay the follicular development functionally and inhibit ovulation in rats and suppression of the preovulatory progesterone surge.

1. Introduction

Estrogens exert multiple effects in the process of follicular maturation and ovulation [1–4], indicating the existence of estrogen receptors (ERs) in the ovaries of several species [5–9]. Recent studies demonstrated a reproductive deficit of the consistent absence of ovulatory follicles in ERα-knockout mice [10], and a partial arrest of follicular development with anovulatory cycles in ERβ-knockout mice [11]. Moreover, findings in early studies indicated that the synthetic estrogen, diethylstilbestrol alone stimulates the growth of small preantral follicles in the absence of FSH in rats, suggesting the participation of estrogens in the early stage of the estrus cycle in the rat [12]. On the other hand, Shirely et al. reported that estrogen-deficiency induced by either ovariectomy or treatment with the antiestrogen, MER-25 (partial agonist) at a certain critical stage of estrus cycle, led to an interruption in the estrogen-dependent processes in the reproductive organ systems and functions (changes in genital tracts, ovulation and mating behavior). These findings suggested the participation of estrogens in the process of follicular maturation and ovulation in rats.

Recently, Donath and Nishino have reported that repeated administration of the pure antiestrogen, ZM 182780 and the partial agonist, tamoxifen (TAM) to rats results in the inhibition of ovulation, relating to the suppression of the preovulatory progesterone surge [13].

However, it was not yet clarified at what stage of follicular maturation and ovulatory process antiestrogens modulate the biological effects of estrogens. Therefore, the study using the pure antiestrogen ZK 191703 [23] and the partial agonist, TAM was undertaken to investigate the physiological role of estrogens in the process of ovulation by examining the exact timing of administration of antiestrogens for their antiovulatory effect, hormonal changes in peripheral blood and histological changes in the ovaries of adult rats with regular estrus cycles and gonadotropin-primed immature rats.

* Corresponding author at: Research and Development, Nihon Schering K.K., Nishimiyahara 2-6-64, Yodogawa-ku, Osaka 532-0004, Japan.
Tel.: +49 941 943 4290; fax: +49 941 943 1515.
E-mail addresses: tsuyuki.nishino@basf.com (T. Nishino), hyamanouchi@schering.co.jp (H. Yamanouchi), ishibashi_kanako@lilly.com (K. Ishibashi), christian.hirtreiter@chemie.uni-regensburg.de (C. Hirtreiter), yukishige.nishino@freenet.de (Y. Nishino).
[1] Tel.: +81 6 6396 2551; fax: +81 6 6398 2462.
[2] Tel.: +49 30 365 3663; fax: +49 30 365 3663.

0960-0760/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jsbmb.2009.01.021

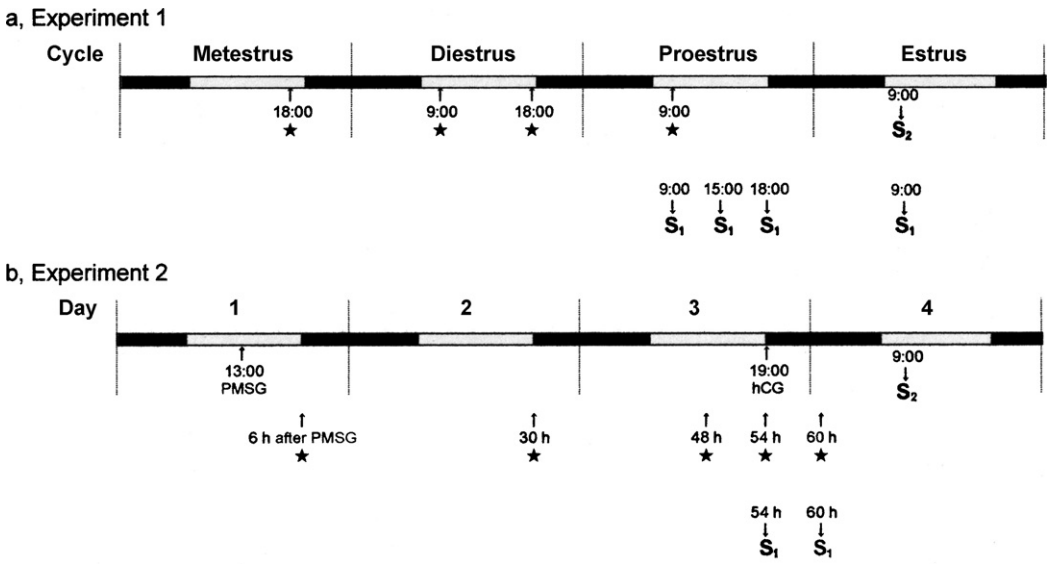

Fig. 1. Experimental procedures for experiment 1 (a) and experiment 2 (b). *A single administration (s.c.) of TAM 5 mg/kg or ZK 5 mg/kg. "S_1" indicates sampling time of blood for RIA of GTH and steroids. "S_2" indicates sampling time for counting ovulation rate and histological analyses.

2. Material and methods

2.1. Animals

Adult female rats (9–11 weeks old) weighing about 200 g (experiment 1) and 24-day-old female rats weighing about 50 g (experiment 2) (Wistar-strain, Clea Japan, Inc., Tokyo, Japan) were used. All animals were kept at 23 °C under controlled illumination (12 h light, 12 h dark). They were fed on a standard diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and water *ad libitum*.

2.2. Compounds

The antiestrogen ZK 191703 (ZK) (11-β-Fluoro-7α-(14,14,15,15-pentafluoro-6-methyl-10-thia-6-azapentadecyl)estra-1,3,5(10)-triene-3,17β-diol) was synthesized in the laboratory of Schering AG (Berlin, Germany). Tamoxifen (trans-1-(4-β-dimethylamino-ethoxyphenyl)-1,2-diphenyl-but-1-ene) was purchased from Sigma Corp. (MO, USA). Both compounds were dissolved in arachis oil containing 10% ethanol. Pregnant mare serum gonadotropin (PMSG) (Teikoku Hormone, Co. Ltd., Tokyo, Japan) and human chorionic gonadotropin (hCG) (Mochida Pharmacological Corp., Tokyo, Japan) were dissolved in saline.

2.3. Experimental procedure (experiment 1)

Prior to treatment animals were sorted by vaginal smear test. In order to determine the optimum time to inhibit ovulation with the compounds, animals were treated with compounds at varying times during the estrus cycle. ZK (5 mg/kg) or TAM (5 mg/kg) were subcutaneously administered to adult animals showing regular 4-day cycles in the previous 8 days, once at 18:00 of metestrus, 9:00, 18:00 of diestrus or 9:00 of proestrus (Fig. 1a). Following estrus manifestation, the fallopian tube and the ovary on the left side were isolated and ova in the tube counted after flushing with saline. The following day the animals were killed by decapitation and the contralateral tubes examined for ova to assess possible delayed ovulation in the drug-treated animals.

Trunk blood was collected from the decapitated animals for the determination of hormone levels. The isolated ovaries were excised for histological analyses.

2.4. Experimental procedure (experiment 2)

PMSG at a dose of 20 I.U. was subcutaneously injected to 24 days old immature female rats to stimulate follicular development. Ovulation was induced 54 h later by i.p. injection of 10 I.U. hCG (Fig. 1b).

Table 1
The effects of ZK 191703 (ZK) and tamoxifen (TAM) on organ weights of the adult rat (experiment 1) and hCG/PMSG primed immature rat (experiment 2).

	Injection	Uterus weight (mg/100 g b.w.)			Ovary weight (mg/100 g b.w.)		
		Control	TAM	ZK	Control	TAM	ZK
Experiment 1	Control	219.4 ± 18.0 (5)			23.2 ± 2.5 (11)		
	Metestrus 18:00		160.6 ± 11.0 (5)[a]	129.4 ± 3.8 (6)[a]		23.4 ± 2.3 (10)	23.7 ± 3.5 (10)
	Diestrus 9:00		150.6 ± 11.0 (5)[a]	123.1 ± 7.1 (5)[a]		21.4 ± 2.1 (10)	22.3 ± 2.2 (10)
	Diestrus 18:00		169.5 ± 10.9 (3)[a]	138.2 ± 6.5 (3)[a]		22.9 ± 2.4 (8)	24.8 ± 2.3 (8)
	Proestrus 9:00		236.6 ± 14.2 (5)	203.2 ± 15.9 (5)		24.2 ± 2.2 (10)	23.4 ± 1.8 (10)
Experiment 2	Control	239.9 ± 25.6 (19)			50.7 ± 9.0 (19)		
	6 h after PMSG		201.6 ± 21.9 (10)[a]	186.1 ± 16.8 (10)[a]		56.7 ± 8.7 (10)	51.1 ± 9.7 (10)
	30 h after PMSG		242.3 ± 29.8 (5)	178.9 ± 17.3 (5)[a]		49.6 ± 6.8 (5)	46.4 ± 10.0 (5)
	48 h after PMSG		230.9 ± 46.5 (5)	209.7 ± 11.0 (5)		53.0 ± 13.0 (5)	50.4 ± 6.4 (5)
	54 h after PMSG		259.2 ± 10.4 (5)	239.5 ± 25.5 (5)		48.1 ± 5.6 (5)	47.5 ± 6.1 (5)
	60 h after PMSG		200.3 ± 17.1 (5)[a]	232.2 ± 16.6 (5)		45.8 ± 3.9 (5)	47.7 ± 4.4 (5)

Mean ± S.D. Figures in parentheses indicate the number of animals.
[a] $P < 0.01$ compared with control value.

Animals were treated with ZK (5 mg/kg) or TAM (5 mg/kg) s.c. once at 6, 30, 48, 54 or 60 h after PMSG injection. Animals were killed by decapitation at 14 h after hCG injection which followed 68 h after the PMSG injection. The fallopian tubes and ovaries were processed as mentioned above in experiment 1. To determine the serum level of the hormones, the animals were killed for the collection of trunk blood at 54 or 60 h after PMSG injection.

2.5. Determination of hormones in serum

The blood was allowed to clot and centrifuged to obtain serum. Sera were frozen at −20 °C until assayed for steroids and gonadotropin. Serum levels of estradiol, progesterone and testosterone were measured by radioimmunoassay using commercially available kits (for estradiol and progesterone: IMMUNOTECH, Inc., Marseille, France; for testosterone: Diagnostics Products Corporation, CA, USA): the intraassay and interassay coefficients of variation were 5.6% and 8.5% for estradiol RIA, 7.5% and 10.0% for progesterone RIA, 6.5% and 8.6% for testosterone RIA respectively. Serum LH and FSH were determined by double-antibody radioimmunoassay with reagents kindly provided by the National Hormone and Pituitary Program (MD, USA). The reference preparations NIDDK-rat LH-RP-3 and rat FSH-RP-2 were used as the standards for assays with the first antiserum NIDDK-anti-rLH-S-10 and anti-rFSH-S-11. NIDDK-rLH-I-9 and rFSH-I-8 were used as the radioligands and iodinated by a lactperoxidase method [14]. The second antiserum, HAC-RBA2-05GTP91, was provided by the Institute for Molecular and Cellular Regulation, Gunma University, Japan. The intraassay and interassay coefficients of variation were 3.4% and 6.0% for LH RIA, 9.4% and 14.2% for FSH RIA respectively.

2.6. Histological analysis

Ovaries were fixed in 10% formalin, dehydrated and embedded in paraffin. Three μm sections were made, and stained with hematoxylin and eosin for microscopic examination.

2.7. Statistical analyses

Data are expressed as mean ± S.D. Statistical analysis of the data was performed according to one-way analysis of variance or Kruskal–Wallis test, followed by Dunnett's multiple comparison test. The statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Experiment 1 (adult animals with estrus cycle)

3.1.1. Organ weight

Animals treated with ZK at metestrus 18:00, diestrus 9:00 or diestrus 18:00 showed a decrease in uterine weight, compared to the control animals, while no decrease was seen in those at proestrus 9:00 (Table 1). Likewise, TAM showed changes in the uterine weight. By contrast, no significant differences were observed in the weight of ovaries between treated animals and control.

3.1.2. Ovulation

Ovulation was completely inhibited by treatment with a single dose of either antiestrogen at 18:00 of metestrus (Fig. 2). When antiestrogens were given at 9:00 of diestrus, none and two animals showed ovulation out of ten examined for TAM and ZK, respectively. By a single injection of ZK or TAM at 18:00 of diestrus, the number of ova released into the fallopian tubes was decreased to 34% and 15% respectively, compared to that of the control animals. However, no significant inhibition of ovulation was observed in the animals treated at 9:00 of proestrus.

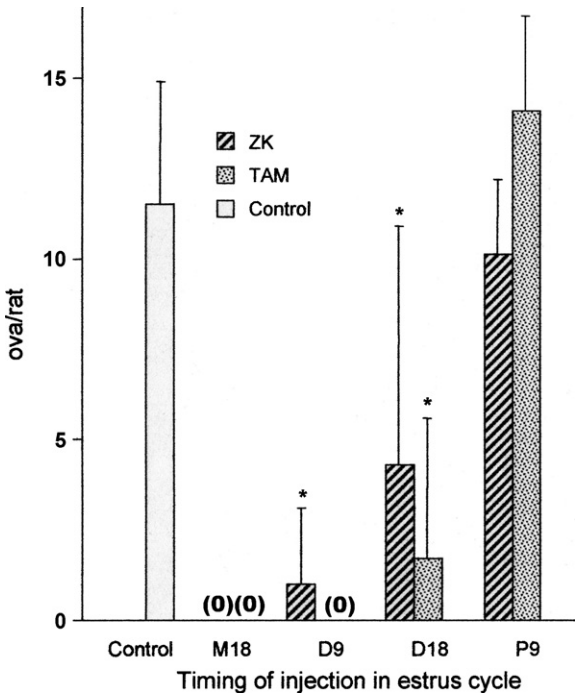

Fig. 2. Inhibitory effects of ZK 191703 (ZK) and tamoxifen (TAM) on spontaneous ovulation in adult rats (experiment 1). Treatment with ZK or TAM was performed at different time during estrus cycle as indicated below *x*-axis; M18: 18:00 at metestrus; D9: 9:00 at diestrus; D18: 18:00 at diestrus; P9: 9:00 at proestrus. Expressed as mean ± S.D. ($n = 10–12$). Significantly different from the control, $^{**}P < 0.01$.

3.1.3. Hormone concentrations in serum

In the control animals, LH in serum was low in a range of 0.3–4.2 ng/ml during a phase of metestrus 18:00 to proestrus 15:00 (Fig. 3a). A marked increase in LH was observed at 18:00 of proestrus, declining to the baseline level of 0.3 ng/ml at 9:00 of estrus. Thus, the preovulatory surge manifested at 18:00 of proestrus. Pretreatment with TAM or ZK at diestrus 9:00 suppressed the preovulatory LH surge significantly. Baseline LH levels at proestrus 9:00 and estrus 9:00 did not alter with TAM, but were maintained higher than those for control modestly with ZK. When looking at animals in which the antiovulatory effect was not clearly induced by antiestrogens, i.e. in a group treated at 9:00 of proestrus, no significant suppression of the preovulatory LH surge was observed. An increase in basal LH were observed at proestrus 15:00 in ZK treated rats, and at estrus 9:00 in ZK or TAM-treated rats (Fig. 3b).

The preovulatory FSH surge, which appeared at proestrus 18:00 and estrus 9:00 in control animals, was significantly suppressed by an injection of ZK or TAM at diestrus 9:00 (Fig. 4a). When antiestrogens were given at 9:00 of proestrus, ZK or TAM did not suppress the FSH surge at proestrus 18:00, whereas modest changes were seen, i.e. an increase and a decrease in the FSH surge at 15:00 of proestrus and 9:00 of estrus, respectively, by ZK, and an increase at 18:00 of proestrus and 9:00 of estrus by TAM (Fig. 4b).

Progesterone surges at proestrus 15:00 and 18:00 were suppressed by ZK or TAM given at diestrus 9:00 (Fig. 5a). TAM at proestrus 9:00, the treatment of which did not show an antiovulatory effect, reduced the progesterone surge at 15:00 and 18:00 (Fig. 5b).

Testosterone levels in serum for both the control group and TAM-treated group during the estrus cycle were lower than the sensitivity limit of the assay. ZK elevated the level of testosterone by treatment at both diestrus 9:00 and proestrus 9:00. However, the

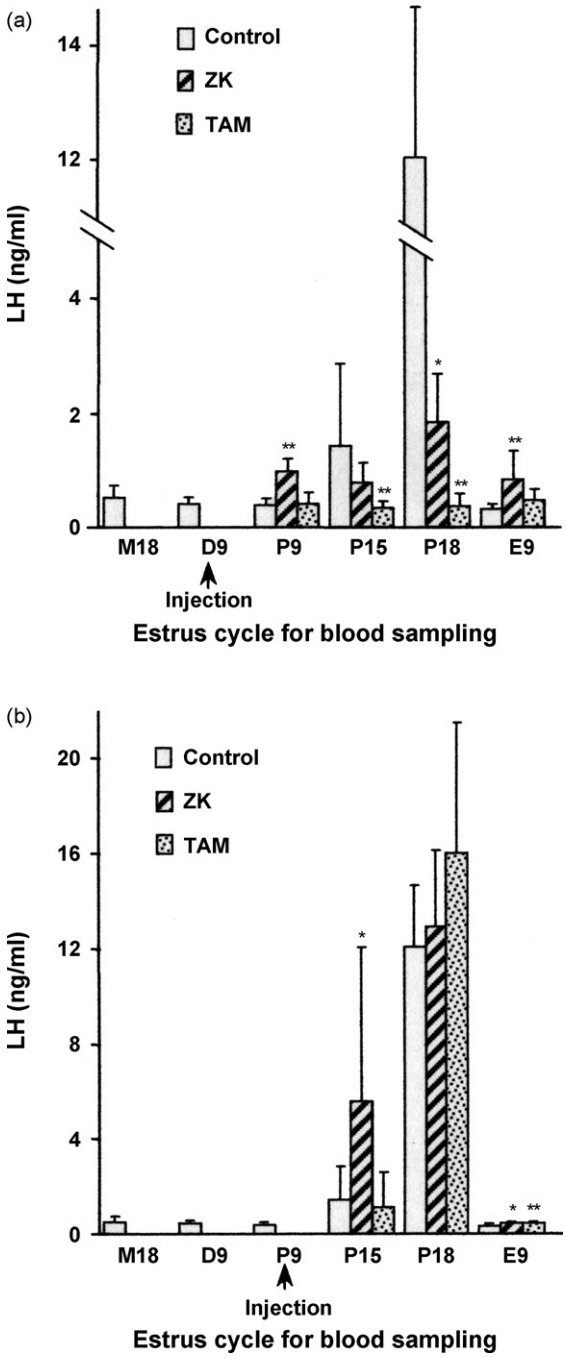

Fig. 3. Serum concentration of LH measured at different times during estrus cycle after treatment with ZK or TAM at 9:00 of diestrus (a) or 9:00 of proestrus (b) in adult rats (experiment 1). Expressed as mean ± S.D. (n = 9–10). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

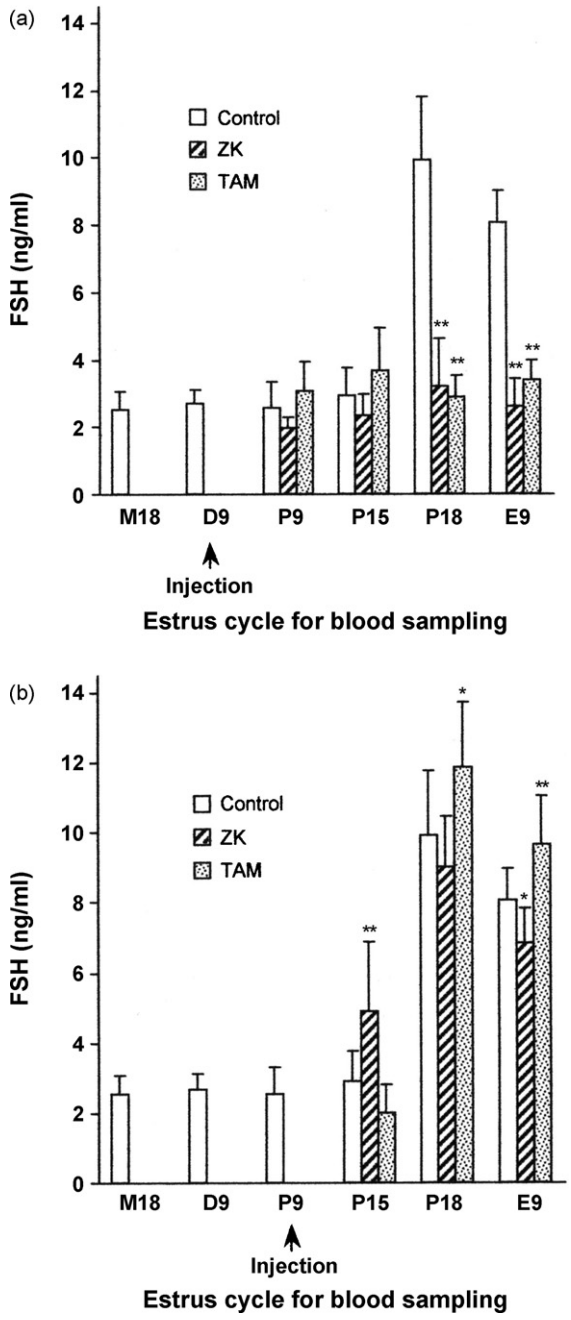

Fig. 4. Serum concentration of FSH measured at different times during estrus cycle after treatment with ZK or TAM at 9:00 of diestrus (a) or 9:00 of proestrus (b) in adult rats (experiment 1). Expressed as mean ± S.D. (n = 7–10). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

treatment at diestrus 9:00 was more effective than the treatment at proestrus 9:00 (Fig. 6a and b).

3.1.4. Histological analyses

As shown in Fig. 7, rats treated with ZK or TAM at diestrus 9:00 showed healthy late tertiary follicles, indicating that ovulation did not take place. These histological findings are comparable to those mentioned above regarding the inhibitory effects of antiestrogens on ovulation. By the treatment at 9:00 of proestrus with ZK or TAM, corpora lutea was newly formed in ovaries, as usually seen in control rats.

3.2. Experiment 2 (immature rats)

3.2.1. Organ weight

When ZK or TAM were given to immature rats at 6 h after PMSG-priming, the weight of uterus was decreased (Table 1). Later administration of antiestrogens to rats did not change the weight, except ZK treatment at 30 h. No significant differences were observed in the weight of ovaries between antiestrogens-treated and control groups.

3.2.2. Ovulation

When immature rats were treated with ZK or TAM at 6 h after PMSG-priming, ovulation was suppressed significantly (Fig. 8). The

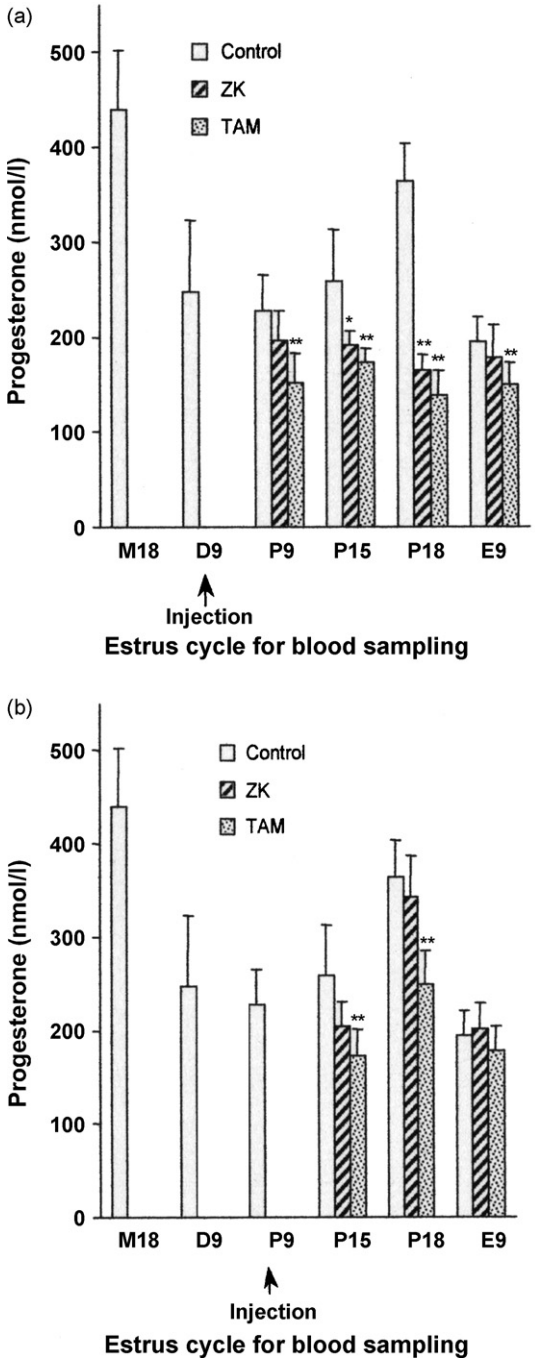

Fig. 5. Serum concentration of progesterone measured at different times during estrus cycle after treatment with ZK or TAM at 9:00 of diestrus (a) or 9:00 of proestrus (b) in adult rats (experiment 1). Expressed as mean ± S.D. ($n = 10$). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the control.

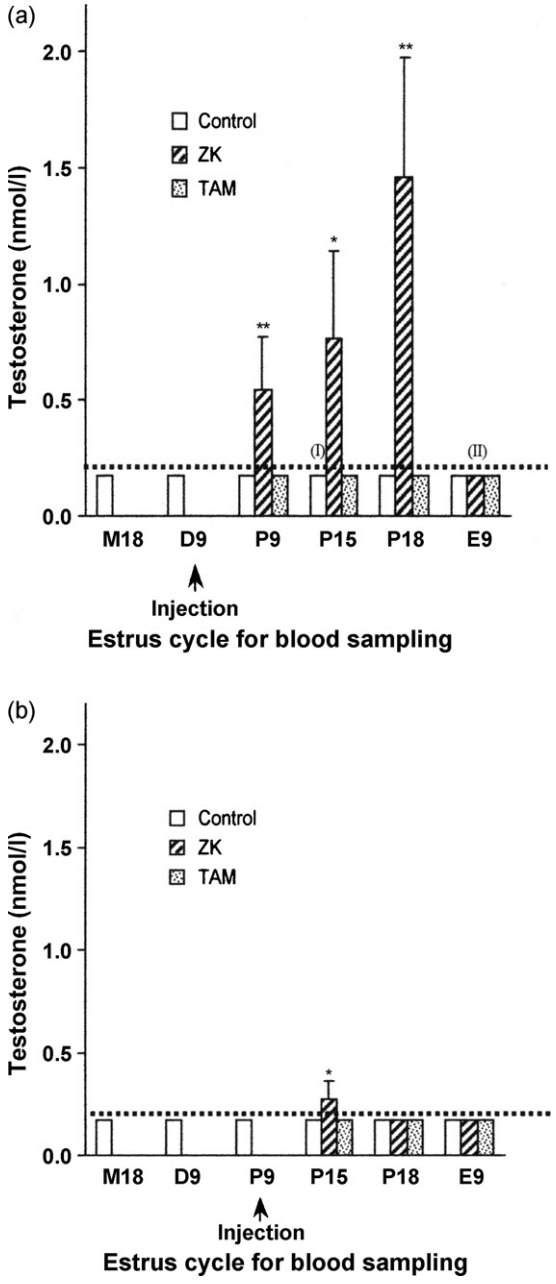

Fig. 6. Serum concentration of testosterone measured at different times during estrus cycle after treatment with ZK or TAM at 9:00 of diestrus (a) or 9:00 of proestrus (b) in adult rats (experiment 1). Expressed as mean ± S.D. ($n = 5$–10). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the sensitivity limit of the assay (0.17 nmol/l: indicated by dotted line). Column under dotted line indicate the value <0.17 nmol/l except for (I) and (II). (I) one animal in five rats in this group showed 0.21 nmol/l, the other values were under 0.17 nmol/l. (II) 4 animals in 10 rats showed 0.37 ± 0.33 nmol/l and the others showed the value under 0.17 nmol/l.

animals treated with ZK at 30 h after PMSG also showed significant decrease in ovulation. TAM at 30 h after PMSG tended to suppress ovulation with no statistically significant difference since 1 animal out of 10 tested showed an unexpectedly high rate of superovulation (62 ova per rat). No ovulatory suppression was induced by 48 h or later treatment with antiestrogens.

3.2.3. Hormone concentrations in serum

No significant difference in estradiol between TAM-treated and control animals was observed with blood sampling at 54 and 60 h, when TAM was injected at 30 h after PMSG (data not shown). Effects of ZK on estrogen level were not shown because of possible cross-reaction.

Progesterone levels, which increased at 54 h following PMSG-priming, were markedly suppressed by ZK or TAM, when given at 30 h (Fig. 9). This decreased levels of progesterone were restored at 60 h after PMSG.

3.2.4. Histological analyses

Histological comparison of ovaries was made between animals treated with ZK or TAM at 30 h after PMSG injection (ovulation was suppressed) and those at 48 h after PMSG (ovulation was

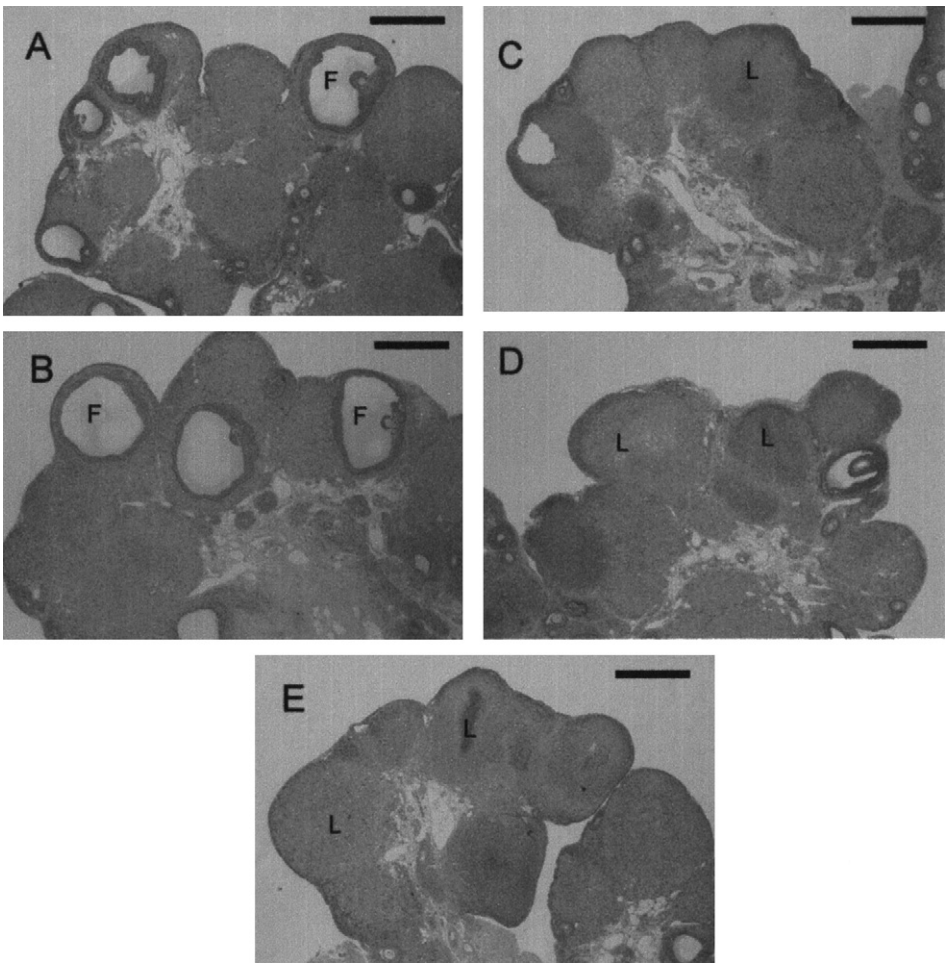

Fig. 7. Histological changes of ovaries from adult rats (experiment 1) treated with ZK or TAM at 9:00 of diestrus (A or B) or at 9:00 of proestrus (C or D). Ovary from control animal was also showed (E). Ovaries were isolated from respective animals at 9:00 of estrus. F: late tertiary follicle; L: newly formed corpora lutea; bar = 500 μm.

not inhibited). Late tertiary follicles were clearly found in ovaries isolated from animals treated at 30 h after PMSG injection (Fig. 10). By contrast, there was a sizeable area of newly formed corpora lutea found in ovaries from animals treated with ZK or TAM at 48 h after PMSG, and also in ovaries from control animals.

4. Discussion

In the present study, the pure antiestrogen ZK and the partial agonist TAM in a single dose administered at the early phase of the estrus cycle (metestrus or diestrus) in rats caused a significant decrease in the preovulatory surge of gonadotropins and inhibited ovulation. Neither the preovulatory LH/FSH surge nor ovulation was, however, inhibited by antiestrogens administered at a late phase of the estrus cycle (proestrus). Therefore, antiestrogens may disturb sequential processes of hormonal change and effect the trigger of ovulatory processes when administered at an early stage. The development of ovulatory follicles is dependent on the delicately balanced interactions of FSH, LH and ovarian steroids [15]. Any changes in the levels of the gonadotropins or of steroids, caused by administration of antiestrogens, at critical stages of follicular development may lead to failure of ovulation.

The present findings indicate a significantly high level of basal LH/FSH and testosterone following treatment with a single dose of ZK at the early phase of estrus cycle. The elevation of basal LH and inhibition of preovulatory LH/FSH surge implies that both negative and positive feedback processes may be modulated by ZK. A recent study in rats showed that the pure antiestrogen, ZM 182780, administered repeatedly throughout the estrus cycle, causes an increase in the basal level of LH which stimulates the androgen production in theca cells, suggesting that an excess of these aromatizable androgens may suppress the preovulatory surge of LH/FSH and induce atresia [13]. The ovaries obtained from this study [13] have been histologically investigated by Rumpel et al. [24] who showed also a stimulation of the theca cells and a high incidence of atretic follicles to coincidence with an increase in the basal LH level after the repeated treatment of female rats with ZM 182780. In the present study, however we found neither any remarkable stimulation of the theca layer or an increase in atretic cells after the single injection of ZK in rats. These different observations in above mentioned three studies may be due to different frequency of the treatment with pure antiestrogens.

In contrast, TAM, like estrogens, has been known to exert a negative feedback effect on pituitary-ovarian axis, when administered repeatedly [13]. In the present study, a single dose of TAM also caused a decrease in the basal and preovulatory level of LH/FSH. TAM may act as an agonist on the pituitary ERα which regulates negative feedback [16]. These findings suggest that ZK and TAM differ in affecting hormonal milieu in rats: whereas TAM totally blocks pituitary-ovarian axis, ZK increases in the basal LH level. However, these antiestrogens exerted a similar inhibitory effect against ovulation when administered at the early stage of the estrus cycle. Therefore, changes in hormonal milieu caused by these antiestrogens may not be directly related to the antiovulatory effect of antiestrogens.

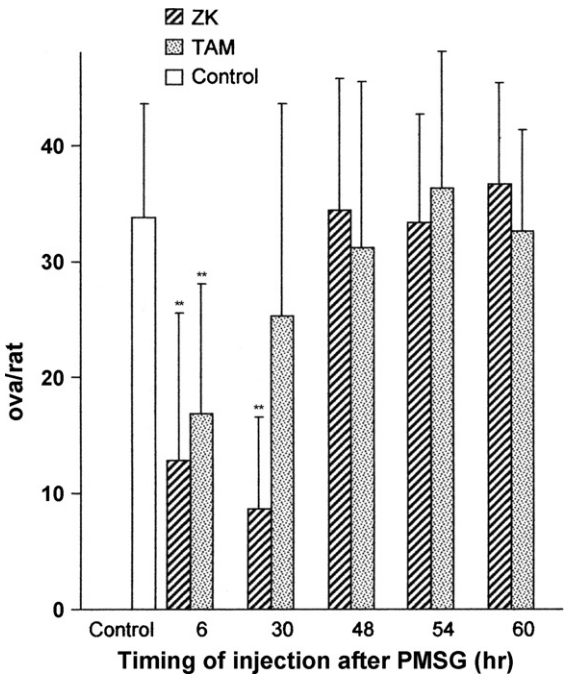

Fig. 8. Inhibitory effects of ZK 191703 (ZK) and tamoxifen (TAM) on PMSG/hCG (experiment 2). The immature rats received PMSG at time 0 and hCG 54 h later. ZK or TAM was administered once at 6–60 h after PMSG as indicated below *x*-axis. Expressed as mean ± S.D. (n = 10–19). Significantly difference from the control, $^{**}P < 0.01$.

Additionally, the inhibition of the preovulatory surge of gonadotropins may not be essential in the antiovulatory effect of antiestrogens, since *superovulation* induced by exogenous gonadotropins (PMSG/hCG) in immature rats was also inhibited by both antiestrogens. The progesterone surge is thought to be more important than the gonadotropin surge in rats, as has been mentioned in our previous papers (Donath and Nishino): the correlation between a suppression of the preovulatory progesterone surge and

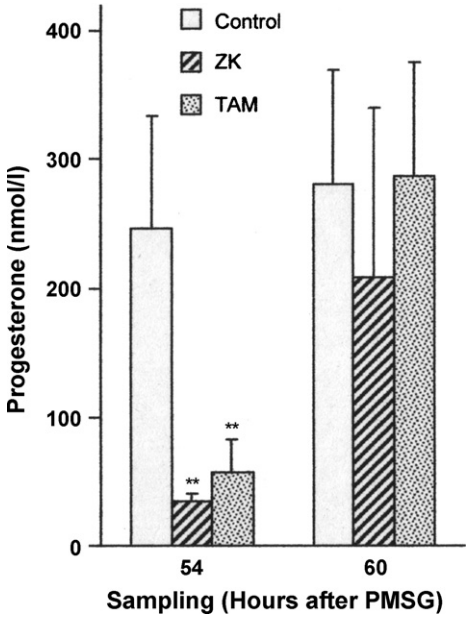

Fig. 9. Serum concentration of progesterone at different times in immature rats treated with PMSG/hCG (experiment 2). The animals received PMSG at time 0 and hCG 54 h later. Blood sampling was made just before administration of hCG. ZK or TAM was administered at 30 h after PMSG. Expressed as mean ± S.D. (n = 9–10). Significantly difference from the control, $^{**}P < 0.01$.

inhibition of ovulation by daily administration of antiestrogens [13] is shown. The present observation in PMSG/hCG-primed immature rats clearly indicates that treatment with both antiestrogens leads to an inhibition of the preovulatory progesterone surge. This antiovulatory effect was more pronounced, when antiestrogens were administered at an earlier time prior to the induction of ovulation by hCG. In adult rats, the inhibitory effect of both antiestrogens on the preovulatory progesterone surge and spontaneous ovulation was also more pronounced in animals treated at an earlier stage of the estrus cycle (metestrus or diestrus) than at the late stage (proestrus). Thus, it is conceivable that the anovulation of animals induced by ZK and TAM may be due to a decrease in the preovulatory progesterone surge, which may be regarded as a result of a disturbance to estrogen-dependent functions at an early phase of the estrus cycle.

Interestingly the effects of antiestrogens on ovulation and hormonal milieu are very similar to those of antiprogestins in rats with estrus cycle and in immature rats treated with PMSG/hCG [17,25,26].

Progesterone and the progesterone receptors (PRs) are known to be a necessary component in the induction of the gonadotropin surge [18] and may be considered more important for the induction of ovulation than for the gonadotropin surge: Schubert et al. showed that the antiovulatory activity of a progesterone antagonist could be better correlated to the inhibition of the preovulatory progesterone production than to the suppression of the LH surge [17]. Donath et al. reported that the inhibition of ovulation induced by the antiprogestin, onapristone, could be related to decreased intraovarian progesterone production and down-regulation of progesterone receptor in the ovary [19]. Supportive for the importance of progesterone and PR is the fact that the preovulatory gonadotropin surge is lacking in PR-knockout mice.

Moreover, Lydon et al. have reported that PR-knockout mice primed with PMSG/hCG are anovulatory, and the ovaries from these animals have unruptured preovulatory follicles [20]. Interestingly, the histology of ovaries from superovulated ERβ-knockout mice also indicates the presence of numerous preovulatory but unruptured follicles [11]. These histological findings in PR- and ERβ-knockout mice are very similar to the present observation that the histological pictures of ovaries revealed an increased number of unruptured preovulatory follicles in rats treated with ZK or TAM. Since preovulatory PR expression in ovaries is not regulated by ER [21], the disturbance of early follicular development mediated by ERβ may indirectly result in a down-regulation of intraovarian PR and finally in an inhibition of ovulation.

According to Couse and Korach, ovaries of ERα-knockout mice possess primary and secondary follicles, suggesting that ERβ is essential to the early stage of folliculogenesis [22]. In rat ovaries, ERβ is also expressed preferentially in granulosa cells of small and growing follicles, but is down-regulated in those in preovulatory follicles [5]. It therefore appears that both ZK and TAM as pure estrogen antagonists similarly inhibit ovulation in rats by interfering with the follicular maturation at an early stage in the ERβ-mediated process and causing a delay in follicular development. Consequently, there is a possibility that functional disturbance of follicular development, which cannot be shown in the present morphological analyses, is involved in the mechanism of the antiovulatory effect of these antiestrogens. The present finding that the partial agonist, TAM exhibits the same effect on follicular development as does the pure antagonist may be not surprising so far as it is known that TAM behaves as a pure antagonist of ERβ-function [8].

In summary, these results suggest that antiestrogens, ZK and TAM administered at an early phase of the estrus cycle delay the follicular development functionally in rats, probably via inhibition of ERβ-mediated functions in the ovary, associated with an imbalance

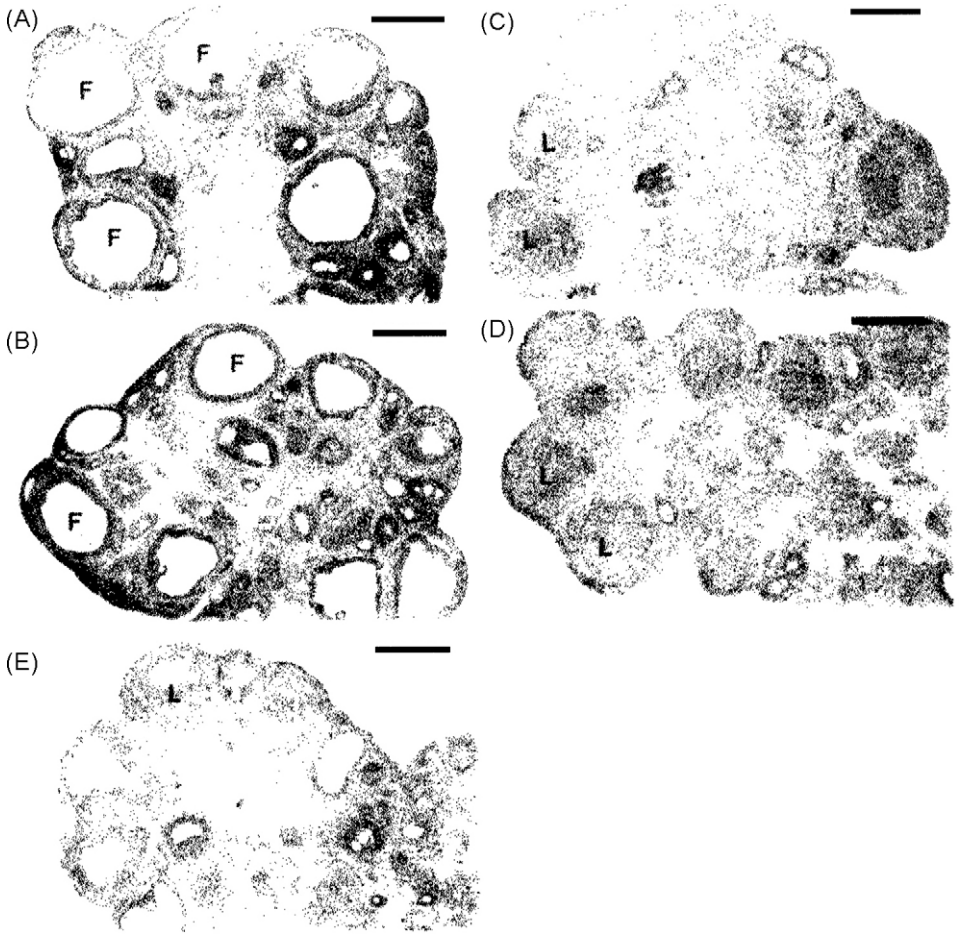

Fig. 10. Histological changes of ovaries from PMSG/hCG-primed immature rats (experiment 2) treated with ZK or TAM at 30 h (A or B) or 48 h (C or D) after PMSG. Ovary from control animal was also showed (E). Ovaries were isolated from respective animals 68 h after PMSG. F: late tertiary follicle, L: newly formed corpora lutea; bar = 500 μm.

of hormonal milieu, suppressed surge of preovulatory progesterone and gonadotropins, and finally anovulation. Thus estrogen plays an important role during early follicular development in sequential processes.

Long-term effects of ZK on reproduction and carcinogenicity should be examined in future studies.

Acknowledgements

We wish to express our gratitude to the National Hormone and Pituitary Program and the Institute for Molecular and Cellular Regulation of Gunma University for supplying rat LH, rat FSH, RIA kits and the second antiserum for RIA.

References

[1] S.G. Hillier, A.J. Zeleznik, R.A. Knazek, G.T. Ross, Hormonal regulation of preovulatory follicle maturation in the rat, J. Reprod. Fertil. 60 (1980) 219–229.
[2] J.A. Jonassen, K. Bose, J.S. Richards, Enhancement and desensitization of hormone-responsive adenylate cyclase in granulosa cells of preantral and antral ovarian follicles: effects of estradiol and follicle-stimulating hormone, Endocrinology 111 (1982) 74–79.
[3] G.F. Erickson, An analysis of follicle development and ovum maturation, Sem. Reprod. Endocrinol. 4 (1986) 233–254.
[4] J.E. Levine, New concepts of the neuroendocrine regulation of gonadotropin surges in rats, Biol. Reprod. 56 (1997) 293–302.
[5] M. Byers, G.G. Kuiper, J.A. Gustafsson, O.K. Park-Sarge, Estrogen receptor-beta mRNA expression in rat ovary: down-regulation by gonadotropins, Mol. Endocrinol. 11 (1997) 172–182.
[6] C.L. Chaffin, R.L. Stouffer, D.M. Duffy, Gonadotropin and steroid regulation of steroid receptor and aryl hydrocarbon receptor messenger ribonucleic acid in macaque granulosa cells during the periovulatory interval, Endocrinology 140 (1999) 4753–4760.
[7] M. Tomanek, C. Pisselet, P. Monget, T. Madigou, M.L. Thieulant, D. Monniaux, Estrogen receptor protein and mRNA expression in the ovary of sheep, Mol. Reprod. Dev. 48 (1997) 53–62.
[8] G.B. Tremblay, A. Tremblay, N.G. Copeland, D.J. Gilbert, N.A. Jenkins, F. Labrie, V. Giguere, Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta, Mol. Endocrinol. 11 (1997) 353–365.
[9] N. Walther, C. Lioutas, G. Tillmann, R. Ivell, Cloning of bovine estrogen receptor beta (ERbeta): expression of novel deleted isoforms in reproductive tissues, Mol. Cell. Endocrinol. 152 (1999) 37–45.
[10] D.B. Lubahn, J.S. Moyer, T.S. Golding, J.F. Couse, K.S. Korach, O. Smithies, Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 11162–11166.
[11] J.H. Krege, J.B. Hodgin, J.F. Couse, E. Enmark, M. Warner, J.F. Mahler, M. Sar, K.S. Korach, J.A. Gustafsson, O. Smithies, Generation and reproductive phenotypes of mice lacking estrogen receptor beta, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 15677–15682.
[12] R.L. Goldenberg, J.L. Vaitukaitis, G.T. Ross, Estrogen and follicle stimulation hormone interactions on follicle growth in rats, Endocrinology 90 (1972) 1492–1498.
[13] J. Donath, Y. Nishino, Effects of partial versus pure antiestrogens on ovulation and the pituitary-ovarian axis in the rat, J. Steroid Biochem. Mol. Biol. 66 (1998) 247–254.
[14] Y. Miyachi, J.L. Vaitukaitis, E. Nieschlag, M.B. Lipsett, Enzymatic radioiodination of gonadotropins, J. Clin. Endocrinol. Metab. 34 (1972) 23–28.
[15] A. Tsafriri, R.H. Braw, Experimental approaches to atresia in mammals, Oxf. Rev. Reprod. Biol. 6 (1984) 226–265.
[16] E.F. Rissman, S.R. Wersinger, J.A. Taylor, D.B. Lubahn, Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects, Horm. Behav. 31 (1997) 232–243.
[17] C. Schubert, J. Donath, H. Michna, Y. Nishino, The antiovulatory activity of progesterone antagonists is not correlated to their antiprogestational potency in the rat, J. Steroid Biochem. Mol. Biol. 59 (1996) 75–82.
[18] P.E. Chappell, J.P. Lydon, O.M. Conneely, B.W. O'Malley, J.E. Levine, Endocrine defects in mice carrying a null mutation fort he progesterone receptor gene, Endocrinology 138 (1997) 4147–4152.

[19] J. Donath, H. Michna, Y. Nishino, The antiovulatory effect of the antiprogestin onapristone could be related to down-regulation of intraovarian progesterone (receptors), J. Steroid Biochem. Mol. Biol. 62 (1997) 107–118.
[20] J.P. Lydon, F.J. DeMayo, C.R. Funk, S.K. Mani, A.R. Hughes, C.A. Montgomery Jr., G. Shyamala, O.M. Conneely, B.W. OïMalley, Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities, Genes Dev. 9 (1995) 2266–2278.
[21] O.K. Park, K.E. Mayo, Transient expression of progesterone receptor messenger RNA in ovarian granulosa cells after the preovulatory luteinizing hormone surge, Mol. Endocrinol. 5 (1991) 967–978.
[22] J.F. Couse, K.S. Korach, Exploring the role of sex steroids through studies of receptor deficient mice [see comments], J. Mol. Med. 76 (1998) 497–511.
[23] J. Hoffmann, R. Bohlmann, N. Heinrich, H. Hofmeister, J. Kroll, H. Künzer, R.B. Lichtner, Y. Nishino, K. Parczyk, G. Sauer, H. Gieschen, H.F. Ulbrich, M.R. Schneider, Characterization of new estrogen receptor destabilizing compounds: effects on estrogen-sensitive and tamoxifen-resistant breast cancer, J. Natl. Cancer Inst. 96 (February (3)) (2004) 210–218.
[24] E. Rumpel, C. Schubert, Y. Nishino, J. Donath, Effects of suppression of estrogen action on the hypophyseal-ovarian axis in rats, in: Annual Meeting of the Anatomische Gesellschaft, Greifswald, 1998, Conference Book, vol. 93, Greifswald, 1998.
[25] C. Schubert, Y. Nishino, H. Michna, Disturbance of follicular development and endocrine reactions induced by the antiovulatory effective progesterone antagonist Onapristone, Ann. Anat. 177 (March (2)) (1995) 139–146.
[26] J. Donath, Y. Nishino, T. Schulz, H. Michna, The antiovulatory potential of progesterone antagonists correlates with a down-regulation of progesterone receptors in the hypothalamus, pituitary and ovaries, Ann. Anat. 182 (March (2)) (2000) 143–150.