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Phenytoin inhibits both the first ovulation and uterine development in gonadotropin-primed immature rats

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Abstract

This study was planned to determine the effects and possible mechanism of action of phenytoin on development of the reproductive tract and first ovulation in immature rats. Rats were injected s.c. with 5 IU of equine chorionic gonadotrophin (equine CG) on day 26 to induce ovarian and uterine development. Treatment with phenytoin (140 mg/kg) at 1200 h on day 28, which induces serum levels approximately twice those reached with the clinical dose as anticorvulsant drug for humans, was effective for inhibiting the first ovulation and normal secretion of serum follicle – stimulating hormone and luteinizing hormone (LH) on day 29 as well as the preovulatory gonadotrophin surge on day 28. The block of ovulation was overcome by administration of human chorionic gonadotrophin or LH–releasing hormone on day 28. Simultaneous treatment with equine CG and phenytoin at 0800 h on day 26 did not affect either ovarian weight or ovarian hormones secretion, whereas phenytoin clearly inhibited the normal increase in uterine weight on day 27. Furthermore, phenytoin suppressed uterine growth after 17β-oestradiol injection. These results indicate that phenytoin inhibits the first ovulation by inhibiting the gonadotrophin surge and further, that the drug impairs the stimulatory effects of oestrogen on uterine proliferation in the gonadotropin-induced ovulation model.

Keywords: Phenytoin; Ovulation; Oestrogen; Uterus

1. Introduction

It has been reported that female reproductive functions are disturbed in some persons with epilepsy and seizures, and that the disorders may be related to hormonal changes during the menstrual cycles (Mattson and Cramer, 1985; Morrell, 1998; Stoffel-Wagner et al., 1998). For example, reductions of fertility, diminution of sexual responsiveness, and a high incidence of menstrual abnormalities (Dansky et al., 1980; Webber et al., 1986) have been described. Anovulation is thought to be an important cause of infertility in women with epilepsy. Reproductive disorders seen in patients with epilepsy may have two major reasons. One is temporal lobe epilepsy itself (Herzog et al., 1986; Cummings et al., 1995) and another is unexpected adverse actions of antiepileptic drugs. Phenytoin has been widely and effectively used for treatment for epilepsy and arrhythmia, but recent reports have demonstrated that phenytoin has various effects on the reproductive organ. In men taking phenytoin, metabolism of sex hormones was enhanced (Dana-Haeri et al., 1984) and the free androgen index was decreased as a consequence of increased serum sex hormone-binding globulin (SHBG) concentrations (Macphee et al., 1988; Murialdo et al., 1994; Isojarvi et al., 1995) and/or enzyme induction in the liver (Ghosal et al., 1996). Phenytoin enhanced the prolactin response to thyrotropin-releasing hormone (Murialdo et al., 1994) and decreased dehydroepiandrosterone sulphate levels (Macphee et al., 1988). There is a report showing that phenytoin increased the serum levels of oestradiol in patients with epilepsy (Herzog et al., 1991), whereas carbamazepine decreased the serum oestradiol levels and oestradiol/SHBG ratio (Isojarvi et al., 1990). Further, Dana-Haeri et al. (1984) have reported that pituitary responsiveness to gonadotropin-releasing hormone (GnRH) changed in epileptic patients receiving phenytoin treatment. However, little is known regarding the action of phenytoin on oestrogen-induced development of uterus.

In experimental animals, phenytoin (100–200 mg/kg) blocked spontaneous ovulation in mature female rats and

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the inhibition of ovulation was almost overcome by luteinizing hormone (LH) injection or electrolytic stimulation of the medial preoptic area (Quinn, 1965). Additionally, it has been reported that phenytoin inhibits testosterone production in rat Leydig cells in vitro, implying direct inhibitory effects of phenytoin on steroidogenesis (Kuhn-Velten et al., 1990). With these phenytoin studies as background, there are few reports which have systematically examined the effects of antiepileptic drugs on reproductive functions in experimental animals and humans, and also the effects of clinical serum levels of phenytoin on reproductive function in rats. Induction of the first ovulation in immature rats by treatment with equine chorionic gonadotrophin (equine CG) is well characterized and useful for evaluating the influence of various drugs on ovarian development and ovulation without effects on luteal function (Tamura et al., 1998a,b). In the present study, therefore, we examined whether a pharmacological dose of phenytoin influences gonadotrophin-induced ovarian and uterine development, and the first ovulation in immature female rats.

2. Materials and methods

2.1. Animals and induction of ovulation

Immature female rats (at 26 days of age) of the Wistar strain which were supplied by the Imamichi Institute for Animal Reproduction (Ibaraki-ken, Japan) were used. The animals were maintained in temperature (23 ± 1), humidity ($55 \pm 5\%$), and light (12 h light/day) controlled quarters, and were provided with food and water ad libitum. All procedures using rats were performed in accordance with the institutional guidelines for experimental animal care in our University and the study was approved by the steering committee. To induce gonadal development and the first ovulation with a normal number of oocytes, the animals were given a single s.c. injection of 5 IU equine CG (Serotoropin; Teikoku Hormone, Tokyo) in 0.2 ml saline at 0800 h when they were 26 days old. Equine CG, which was originally called pregnant mare serum gonadotropin (PMSG), possesses both follicle-stimulating hormone (FSH)-like and weak LH-like activities to promote the maturation of ovarian follicles and the secretion of ovarian hormone. Ovulation is induced by the preovulatory gonadotrophin surge on the afternoon of day 28, which is triggered by the positive-feedback action of oestrogen on the same day. Immature rats primed with equine CG show a well-characterized response with defined ovulation occurring on the third morning after equine CG injection (Nuti et al., 1975). These animals ovulated an average of nine oocytes on the morning of day 29. The occurrence of ovulation was ascertained by examining whether occytes were present in the ampulla of oviducts, using a dissecting microscope.

2.2. Drug treatment and experimental schedule

Each dose (70, 140, or 210 mg/kg) of phenytoin (Fujinaga Pharmaceutical, Tokyo) was administered i.p. at 1200 h to equine CG-primed rats on day 28. A total of 10 IU of human CG (Gonatropin, Teikoku Hormone, Tokyo) was given at 1700 h on day 28. A 1 μg of luteinizing hormone–releasing hormone (LHRH) (National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK], National Hormone and Pituitary Program) was given at 1600 h on the same day. In the experiments on the effects of phenytoin on equine CG-induced early folliculogenesis, equine CG and phenytoin (140 mg/kg) were given simultaneously at 0800 h on day 26 and the rats were killed after 24 h. Blood was collected via the abdominal aorta under ether anaesthesia, allowed to clot at 4°C, and serum was separated by centrifugation and stored at −80°C until assay for each hormone.

2.3. Radioimmunoassay (RIA) of FSH, LH, 17β-oestradiol, and inhibin

Serum levels of FSH and LH were measured with NIDDK RIA kits for rat FSH and LH. Iodinated preparations were made from rat FSH-I-8 and LH-I-9. Anti-rat FSH-S-11 and anti-rat LH-S-10 were used as antisera (Watanabe et al., 1990). The results were expressed as NIDDK rat FSH-RP-2 and LH-RP-2. The intra- and inter-assay coefficients of variation were 5.7% and 20.4% for FSH and 8.6% and 9.8% for LH, respectively. The serum concentrations of inhibin and oestradiol were measured using antiserum against each hormone, as described before (Tamura et al., 1998a, Watanabe et al., 1990). The intra- and inter-assay coefficients of variation were 5.2% and 11.0% for inhibin and 5.8% and 18.1% for oestradiol, respectively.

2.4. Measurement of serum phenytoin levels in a high-performance liquid chromatographic (HPLC) system

The serum levels of phenytoin were determined as described before (Kouno et al., 1993). Briefly, the HPLC system consisted of a continuous flow delivery system (BIP-I, Jasco, Tokyo), a UV detector (Uvidec-100V, Jasco), a syringe-loading sample injector with a loop (Model 7125, Rheodyne, Cotati, CA, USA) and a single-pen recorder (RC-150, Jasco), was used (Detector wavelength; 240 nm at 0.01 absorbance unit flow unit). A conventional analytical column was packed with silica gel. A total of 5 ml of serum specimens was introduced onto the surface of the support material with a microsyringe. The Extrashot was then attached to the injector of HPLC system. Dichloromethane (130 ml) was introduced gently into the injector through the Extrashot, and HPLC was conducted in the usual manner with the mobile phase solvent, *n*-hexane containing 0.2% acetic acid, 2% ethanol, and 15% dichloromethane, at a flow rate of 1.5 ml/min.

Table 1
Effects of phenytoin on the first ovulation and the weight of reproductive tracts and effect of human CG or LHRH on phenytoin-induced block of ovulation in equine CG-primed immature rats
Immature female rats were given a single injection of equine CG (CG; 5 IU) at 0800 h at 26 days of age. Phenytoin (PHT; 70, 140, or 210 mg/kg) was injected 52 h after equine CG administration (1200 h on day 28). The number of ovulation was determined by counting oocytes in the oviduct. Human CG (10 IU) and LHRH (1 μg) were injected 4 (1600 h on day 28) and 5 h (1700 h on day 28) after phenytoin (140 mg/kg) treatment, respectively, and animals were killed at 0800 h on day 29. Each value is the means ± S.E.M. for 6–13 rats. Parentheses show the dose (mg/kg) of phenytoin.

Groups	Ovulating rats/ rats examined	No. of oocytes in ovulating rats	Ovarian weight (mg)	Uterine weight (mg)
Control	0/11	–	18.1 ± 0.94	51.5 ± 3.16
CG	13/13	9.3 ± 0.40	26.0 ± 0.98	146.2 ± 2.46
CG + PHT (70)	8/8	9.5 ± 0.82	28.6 ± 0.71	138.9 ± 5.38
CG + PHT (140)	0/8	–	20.4 ± 0.49[a]	171.6 ± 3.80[b]
CG + PHT (210)	0/8	–	20.1 ± 0.78[a]	180.0 ± 6.25[b]
CG + PHT (140) + human CG	9/9	8.7 ± 0.91	35.2 ± 4.53	129.6 ± 8.71
CG + PHT (140) + LHRH	6/6	8.3 ± 0.99	36.3 ± 4.75	136.8 ± 8.94

[a] $P < 0.01$.
[b] $P < 0.001$; vs. CG.

2.5. Statistical analysis

Results are expressed as means ± S.E.M. The significance of differences was tested with an unpaired Student's *t*-test, Welch *t*-test or Tukey multiple test. Differences with $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of phenytoin on the equine CG-induced first ovulation in immature rats

Phenytoin was injected to 28-day-old animals (first pro-oestrus) and the occurrence of ovulation was checked on day 29 (72 h after equine CG treatment) (Table 1). Normal ovulation (9 ± 0.4) was observed in equine CG-primed rats. When phenytoin was injected at 1200 h on day 28, which is just prior to 'the critical period' in pro-oestrus, the first ovulation was completely inhibited in phenytoin (140 and 210 mg/kg)-treated animals, although phenytoin (70 mg/kg) did not affect the ovulation. To confirm the mechanisms of the inhibition of ovulation induced by phenytoin, the effects of human CG or LHRH on the ovulation were examined in phenytoin-treated animals. The phenytoin-induced block of ovulation was completely reversed by human CG treatment at 1700 h on day 28, and the number of oocytes ovulated was almost the same as the control (the equine CG group). Treatment with LHRH also completely restored the rates of ovulating animals in the phenytoin-treated group. Fig. 1 shows the

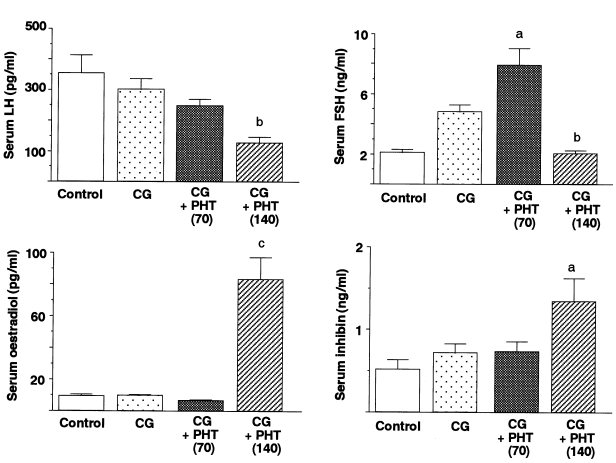

Fig. 1. Effects of phenytoin on the serum levels of LH, FSH, 17β-oestradiol, and inhibin at 72 h after equine CG treatment in immature female rats. Each treatment with equine CG (CG) and/or phenytoin (PHT) was carried out as described in Table 1. Blood was collected at 0800 h on day 29 (72 h after CG administration). Each value is the means ± S.E.M. for 8–13 rats. [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$; vs. CG.

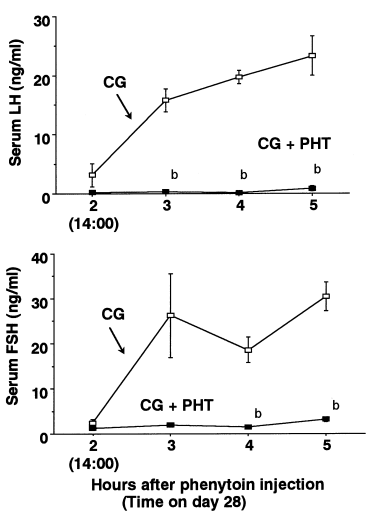

Fig. 2. Changes in the serum levels of LH, FSH, oestradiol, and inhibin after phenytoin treatment until the expected time of LH surge in equine CG-primed immature rats. Each treatment with equine CG and/or phenytoin (PHT: 140 mg/kg) was carried out as described in Table 1, and blood was collected from 1 h after PHT administration (1300 h) once every hour until 1700 h. Each value is the means ± S.E.M. for 8–13 rats. [b] $P < 0.01$; vs. CG.

Table 2
Effects of simultaneous administration of equine CG and phenytoin on the weights of the reproductive tracts in immature rats
Immature female rats were injected with 5 IU of equine CG (CG) and 140 mg/kg of phenytoin (PHT) at 0800 h at 26 days of age. Animals were killed 24 h after equine CG administration (0800 h on day 27). Each value is the means ± S.E.M. for 6 rats.

Groups	Ovarian weights (mg)	Uterine weights (mg)
CG	29.6 ± 1.78	125.9 ± 9.67
CG + PHT	27.4 ± 1.45	96.6 ± 3.87[a]

[a] $P < 0.05$; vs. CG.

serum levels of gonadotrophins and ovarian steroids in phenytoin-treated animals on day 29. Phenytoin (70 mg/kg) did not influence the serum levels of LH, oestradiol or inhibin except for a significant elevation of FSH levels compared to the equine CG group. On treatment with phenytoin (140 mg/kg), however, both FSH and LH levels were markedly suppressed and ovarian hormones (oestradiol and inhibin) were greatly increased. Fig. 2 shows the effects of phenytoin on the serum levels of gonadotrophins on the afternoon of day 28. The serum levels of LH and FSH in the equine CG + phenytoin group were apparently lower than those in the equine CG group between 3 h (1500 h) and 5 h (1700 h) after phenytoin treatment, but estradiol and inhibin levels were not greatly influenced (data not shown). These data support the results in Table 1. When we examined the hourly time course of the serum levels of phenytoin, these reached their maximal level at 1 h (1300 h) and this was maintained for at least 4 h (1700 h) (41 ± 4.0 μg/ml).

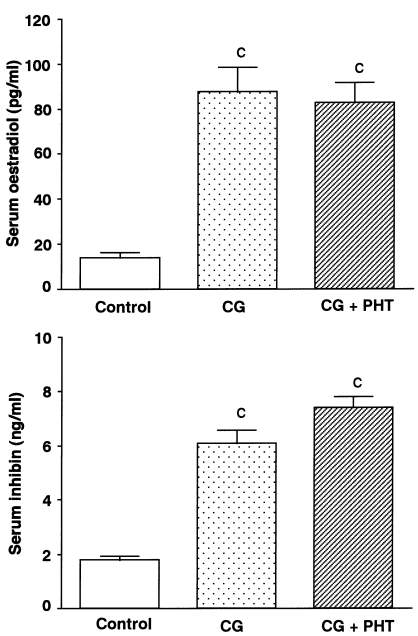

Fig. 3. Effects of simultaneous administration of equine CG and phenytoin on the serum levels of 17β-oestradiol and inhibin in immature female rats. Each treatment with equine CG (CG) and phenytoin (PHT) was carried out as described in Table 2. Blood was collected at 0800 h.

Table 3
Effects of phenytoin on the uterine weight increased by 17β-oestradiol in immature rats
Immature female rats were injected with 500 ng of 17β-oestradiol (Oestradiol) and 140 mg/kg of phenytoin (PHT) at 0800 h at 26 days of age. Animals were killed at 0800 h on day 27 (24 h after oestradiol treatment). Each value shows means ± S.E.M. for 8 or 10 rats.

Groups	Uterine weight (mg)
Control	69.6 ± 2.89
Oestradiol	134.5 ± 10.41[a]
PHT	66.9 ± 6.43
PHT + Oestradiol	109.2 ± 5.20[b]

[a] $P < 0.001$; vs. Control.
[b] $P < 0.05$; vs. Oestradiol.

3.2. Effects of phenytoin on equine CG-induced ovarian and uterine development in immature rats

To examine the influence of phenytoin on equine CG-induced folliculogenesis and uterine growth, phenytoin was administered together with equine CG on day 26 and then reproductive tracts were removed on day 27. Phenytoin treatment clearly decreased uterine weight, although there was no significant change in ovarian weight (Table 2). The basal levels of serum gonadotrophins did not change at 24 h after the combination of equine CG and phenytoin treatment, as compared to the equine CG group, and the levels were not influenced by phenytoin alone, as compared to the control (data not shown). Although the serum levels of oestradiol and inhibin were significantly higher in the equine CG or equine CG + phenytoin group than in the control (Fig. 3), there was no effect of phenytoin on oestradiol and inhibin levels on day 27. To determine whether phenytoin inhibits the uterine development induced by oestrogen, the effects of phenytoin on stimulatory actions of exogenous oestradiol were examined (Table 3). Uterine weight in the phenytoin only group was not changed as compared to that in the control, whereas that in the combined phenytoin and oestradiol group was significantly lower than in the oestradiol only group.

4. Discussion

Our findings in the present study were that phenytoin has an inhibitory effect on the first ovulation, but not on the development of follicles, in equine CG-primed immature rats and that the drug directly suppresses oestrogenic actions on uterine weight. We showed, on the afternoon of first pro-oestrus (day 28), serum levels of approximately 40 μg/ml of phenytoin, which is close to those (10–20 μg/ml) induced by administration of pharmacological doses as antiepileptic or antiarrhythmic drug, and is effective for blocking the first ovulation. The phenytoin-induced block of ovulation appears to be mainly due to suppression of the preovulatory gonadotrophin surge, be-

cause additional treatment with human CG or LHRH completely restored the occurrence of ovulation. The inhibitory action of phenytoin on ovulation was consistent with the results of a study in adult rats (Quinn, 1965). In contrast to our data, in mature animals (8-week-old rats), the same dose of phenytoin did not completely inhibit ovulation and resulted in 40% of the animals ovulating (data not shown). But the author had shown that no all mature animals ovulated when more than 175 mg/kg phenytoin was administered between 1100 and 1200 h in pro-oestrus. These results indicate that immature rats are more sensitive to the drug for the block of ovulation than are mature rats. The serum levels of LH and FSH in the equine CG and phenytoin (140 mg/kg)-treated group were significantly lower than those in the equine OG group at 0800 h on day 29, whereas the levels of oestradiol and inhibin were higher than those in the equine CG group. These results imply that distinct inhibitory effects of phenytoin on pituitary hormone release still remain on the morning of day 29 and the normal decreases in oestradiol and inhibin levels after ovulation were inhibited. The significant inhibition of LH levels on day 29 may have been due to continuous effects of the drug on LH secretion in phenytointreated animals, compared with the control and equine CG groups because phenytoin did not change the basal LH levels in immature rats on day 26. Interestingly, phenytoin has a biphasic effect on FSH levels, inhibiting FSH at the dose of 140 mg/kg and increasing FSH at doses of 70 mg/kg which do not block ovulation, although the reason is not clear. Wise et al. (1979) had suggested that there may be different thresholds of responsiveness to LHRH for LH and FSH secretion. Their results demonstrated that LHRH, when administered under certain restricted conditions, caused the selective release of pituitary FSH with minor effects on LH release. More importantly, it was shown that the frequency of the pulsatile LHRH signal can differentially regulate both LH and FSH subunit messenger RNA expression (Dalkin et al. 1989). Accordingly, a low dose of phenytoin (70 mg/kg) might selectively influence the mechanisms involved in FSH release in the hypothalamus–pituitary axis by affecting the frequency of LHRH stimulation. The suppression of the LH surge in the phenytoin-treated animals is probably generated by the inhibition of LHRH secretion in the hypothalamus and/or a decrease in pituitary responsiveness to LHRH for LH and FSH release.

In the present study, we detected inhibitory effects of phenytoin on oestrogenic action in the uterus. The oestrogen-stimulated elevation of uterine weight was inhibited by phenytoin, even though the serum levels of estradiol did not change. Phenytoin treatment resulted in an overnight loss of 20% of uterine weight. Because of its rapid effect, phenytoin might impair water imbibition, which is one of the early uterine responses to oestrogen stimulation. Although the precise mechanisms of phenytoin action on the uterus need to be addressed, the demonstration that phenytoin affects uterine growth is evidence that phenytoin is active in the peripheral reproductive tracts. Kuhn-Velten et al. (1990) found that phenytoin affects steroid metabolism directly in the testis. However, we could not detect such effects on ovarian steroidogenesis. The mode of phenytoin actions on the block of ovulation as described above seems to be similar to that of phenobarbital. It is well known that phenobarbital inhibits the preovulatory LH surge (Schwartz and Lawton, 1968) by blocking LHRH secretion (Rance and Barraclough, 1981). The phenobarbital-induced inhibition of ovulation was not overcome by oestrogen treatment in pro-oestrus (Brown-Grant, 1969). The hypothalamus–pituitary responsiveness to oestrogen for induction of the preovulatory LH surge which is triggered by the oestrogen surge in pro-oestrus, was hampered by phenobarbital because ovulation was blocked, regardless of the lack of effects of the drugs on oestrogen secretion. If phenytoin has an inhibitory effect similar to that of phenobarbital, the anti-oestrogenic action of phenytoin might account for its ability to block the LH surge, namely the anti-oestrogenic action of phenytoin during the critical period might impair the actions of oestrogen to stimulate hypothalamic neurons, which activate LHRH secretion. The inhibitory mechanisms of anti-epileptic drugs, including phenytoin and phenobarbital, on LH release are not known in detail. One of major mechanisms of the effect of these drugs against epilepsy and seizures is thought to involve the enhancement of γ-aminobutyric acid (GABA)-mediated synaptic inhibition, which leads to reduction of neuronal excitability (McNamara., 1997). Recent studies have suggested that $GABA_A$ receptor subunits are localized in LHRH neurons (Jung et al., 1998) and that activation of $GABA_A$ receptors reduces pituitary LH release and GnRH gene expression in the preoptic area (Leonhardt et al., 1995). Together, the aforementioned indicate that phenytoin may inhibit LH release at least with partial involvement of $GABA_A$ receptors, and that the anti-oestrogenic action of phenytoin might interfere with an important mechanism for $GABA_A$ receptor regulation of LH release.

In conclusion, our studies showed that a phenytoin treatment which induced almost the same concentration in the serum as used for epilepsy therapy causes the block of LH and FSH release, leading to inhibition of the first ovulation. Phenytoin exerted no effect on ovarian follicular development after equine CG treatment, whereas it caused inhibition of uterine development, possibly by blocking oestrogen-stimulated processes. The information obtained from the present study may help elucidate possible adverse actions of phenytoin in patients with epilepsy and arrhythmia.

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