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Inhibitory effect of a new steroidal saponin, OSW-1, on ovarian functions in rats

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1 This study was undertaken to determine the effects of OSW-1 (3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-*O*-(2-*O*-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-(2-*O*-acetyl-α-L-arabinopyranoside)) on the pituitary-ovarian system and the functions of aortic smooth muscle.

2 A single s.c. injection of OSW-1 (9 μg kg^{-1}) on the morning of pro-oestrus inhibited the occurrence of the expected next pro-oestrus, whereas administration of OSW-1 at a dose of 4.5 μg kg^{-1} did not affect the oestrous cycle. OSW-1 treatment on the day of dioestrus-l did not affect the oestrous cycle.

3 At doses of 4.5 and 9 μg kg^{-1} OSW-1, the serum oestradiol (E_2) levels at the expected next pro-oestrus were significantly lower than in control (pro-oestrus). The serum luteinizing hormone (LH) levels 4 days after 9 μg kg^{-1} OSW-1 treatment were also markedly lower than those of control. OSW-1 (4.5 μg kg^{-1}) did not affect the levels of inhibin, progesterone and gonadotrophins on the same day.

4 OSW-1 did not inhibit the preovulatory LH surge which occurs on the afternoon of pro-oestrus day.

5 The expression of mRNA coding for the cholesterol side chain cleavage cytochrome P-450 (p450scc), an ovarian steroidal limiting enzyme, was suppressed at 24 and 96 h after OSW-1 treatment.

6 Administration of OSW-1 (9 μg kg^{-1}) tended to reduce the relaxation of isolated thoracic aorta ring preparations induced by acetylcholine, while there was no difference in the relaxation induced by sodium nitroprusside.

7 Our results show that OSW-1 inhibits ovarian E_2 secretion and that the decrease in E_2 secretion may contribute to its effects on the oestrous cycle and the sensitivity of the thoracic aorta to relaxation. The decrease in the levels of ovarian steroids induced by OSW-1 may be due to its direct inhibitory action on the gene expression of the steroidal enzyme and on the proliferation of granulosa cells in the ovary.

Keywords: Steroidal saponin; OSW-1; oestradiol; aorta relaxation; oestrous cycle; nitric oxide (NO)

Introduction

A novel cholestane glycoside called OSW-1 (3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-*O*-(2-*O*-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-(2-*O*-acetyl-α-L-arabinopyranoside)) was recently isolated from *Ornithogalum saundersiae* (Kubo *et al*., 1992) and has been found to have a potent growth inhibitory activity on a human tumour cell line. There is increasing evidence to suggest that OSW-1 has potential for clinical use as a new anti-cancer drug (Rouhi, 1995; Mimaki *et al*., 1997). Many Schilloideae plants (*Liliaceae*) including *Ornithogalum* spp. commonly produce cardiac glycosides which have a common structure, a steroid nucleus containing various residues. OSW-1 also has a steroid structure with a unique sugar moiety having *p*-methoxybenzoyl residue at the C-16 position. It has been suggested that cardiac glycosides may exert oestrogenic activity in certain cases because of their chemical similarity to the sex hormone (Hoffman & Bigger, 1990). Steroidal saponin sometimes causes gynecomastia in man (Dall, 1965). This compound may induce endocrine changes in part by affecting steroid metabolism or receptor-mediated signal transduction via its agonistic/antagonistic activity.

Further, it has been found that cardiac glycosides, such as ouabain and saponin, inhibit the physiological functions of arterial smooth muscle and endothelial cells (Rubanyi *et al*., 1985; Samata *et al*., 1986; Rodriguez-Manas *et al*., 1992). The action of ouabain is mediated by the blockade of nitric oxide (NO) release from endothelial cells or by the impairment of endothelium-dependent hyperpolarization in smooth muscle cells. In contrast to such common characteristics of cardiac glycosides, OSW-1 did not affect the function of the rat isolated aortic ring preparation, even when more than 100 times the concentration of the drug which exerts anti-tumour activity was used *in vitro* (Honda *et al*, 1997b).

Several studies have suggested that hormonal changes in pregnancy produce various effects on the cardiovascular system (Griending *et al*., 1985; Paller, 1987; Weiner *et al*., 1992). Oestrogen may modulate the contractility of vascular smooth muscle cells in part by increasing NO in endothelial cells (Weiner *et al*., 1994; Honda *et al*., 1996). Hidaka *et al*. (1991) showed that the contractile response of the iliac arteries in pregnancy was suppressed by progesterone treatment. Similar to these observations, we have recently shown that aortic ring preparations derived from rats on the day of oestrus are more sensitive to the relaxant effect of acetylcholine (Honda *et al*., 1997a). Endocrine changes induced by OSW-1, therefore, may influence the functions of arterial smooth muscle.

On the basis of these findings, we have examined whether OSW-1 affects oestrous cycle and the secretion of ovarian and pituitary hormones, and whether the changes in hormonal levels induced by OSW-1 treatment affect the function of aortic smooth muscle.

Methods

Animals and drug treatment

All procedures were carried out in accordance with institutional guideline for animal research in our university. Eight week-old mature female rats weighing 170–200 g of the Wistar-Imamichi strain which were supplied by Imamichi Institute for animal reproduction (Ibaragi-ken, Japan) were

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used. Animals were maintained in an air-conditioned room under controlled lighting (12 h-light/day schedule: 7 h 00 min – 19 h 00 min) and provided with food and water *ad libitum*. Vaginal smears were checked daily and rats with at least two consecutive 4 day oestrous cycles were used. OSW-1 (4.5 μg kg^{-1} or 9 μg kg^{-1}) was dissolved in a small amount of ethanol (final concentration, 0.2%) followed by addition of sesame oil. Animals were given a single s.c. injection in a volume of 0.54 ml kg^{-1} at 9 h 00 min in pro-oestrus or dioestrus-I. The oestrous cycle was checked daily after OSW-1 treatment. In the group injected with 9 μg kg^{-1} OSW-1 in pro-oestrus, the next pro-oestrus did not appear. Blood was collected via the abdominal aorta under ether anaesthesia on the day of expected pro-oestrus, 4 days (96 h) after drug treatment, for the assay of hormone levels. Blood was allowed to clot at 4°C. Serum samples were separated by centrifugation and stored frozen at −80°C until the measurement of hormones. For Northern analysis for detecting ovarian p450scc mRNA, ovaries at 24 and 96 h after drug treatment were excised, weighed and immediately frozen in liquid nitrogen. To determine the sites where OSW-1 causes an abnormal cycle, effects of saponin injected just before 'the critical period' for the gonadotrophin surge and ovulation were examined. OSW-1 (9 μg kg^{-1}) was administered s.c. at 9 h 00 min or 12 h 00 min in pro-oestrus and blood samples were collected, for radioimmunoassay (RIA) of various hormones, via the tail vein under ether anaesthesia at 17 h 00 min, 5 h after drug treatment, which is the expected time of gonadotrophins surge, as previously described (Watanabe *et al*., 1990; Kogo *et al*., 1992). Blood samples in control were also collected in the same way. At 9 h 00 min of the next day (oestrus), all animals were autopsied and blood was collected via the abdominal aorta under ether anaesthesia. The occurrence of ovulation was determined by examining under the microscope whether oocytes were present in the ampulla of oviducts. For the measurement of isometric tension, rats were anaesthetized by ether and killed by bleeding on the day of expected oestrus 5 days after drug treatment. The thoracic aorta was carefully dissected to remove connective tissue and placed in modified Krebs-Henseleit solution.

Radioimmunoassay of inhibin, ovarian steroids and gonadotrophins

The concentration of inhibin in serum was measured by use of a rabbit antiserum against bovine inhibin and ^{125}I-labelled 32 kDa bovine inhibin, as described previously (Hamada *et al*., 1989). The intra- and inter-assay coefficients of variation were 4.6% and 4.9%, respectively. Oestradiol-17β (E_2) and progesterone (P_4) levels in the serum were determined by double-antibody RIA, as in inhibin RIA, with antiserum against E_2 (GDN244; Korenman *et al*., 1974) and P_4 (GDN337; Gibori *et al*., 1977). The intra- and inter-assay coefficients of variation were 5.3 and 17.9% for E_2 and 5.5 and 21.7% for P_4, respectively. Serum levels of follicle stimulating hormone (FSH) and luteinizing hormone (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-11 were used as antisera. Results are expressed as NIDDK rat FSH-RP-2 and LH-RP-3. The intra- and inter-assay coefficients of variation were 5.5 and 7.1% for FSH and 5.1 and 8.9% for LH, respectively.

RNA preparation and Northern analysis

Ovaries were homogenized in 500 μl 4 M guanidine isothiocyanate (GTC) solution. Total RNA was extracted according to the method of Chomczynski & Sacchi (1987). Denatured RNA (10 μg) was separated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane (Zeta-Probe, BioRad). The 28 S and 18 S ribosomal RNAs in gel were stained with ethidium bromide to demonstrate the integrity of applied RNA and to verify that the same amounts of RNA were applied to each lane. Before hybridization with the cholesterol side chain cleavage cytochrome P-450 scc (p450scc) cDNA probe, the membrane was incubated at 42°C for 4 h in a solution containing 50% (v/v) formamide, 50 mM NaH_2PO_4 (pH 6.5), 5 × SSC (1 × SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt's solution, 0.1% sodium dodecyl sulphate (SDS) and 300 μg ml^{-1} denatured herring sperm DNA. The rat p450scc cDNA cloned by Dr K. Morohashi (Morohashi *et al*., 1984) was used. The filter was then hybridized at 42°C for 16 h in the above buffer, which contained ^{32}P-labelled p450scc. The specific activity of the cDNA probe was 3.4×10^4c.p.m. μg^{-1}. After hybridization, the filter was washed twice at room temperature in a solution with 5 × SSC and 0.1% SDS for 10 min each. The filter was dried briefly and exposed to X-ray film (Reflection NEF, Dupont).

Measurement of isometric tension

The thoracic aorta was cut into rings about 5 mm long and set in an organ bath containing modified Krebs-Henseleit solution, as described previously (Honda *et al*., 1996). Two hooks were inserted through opposite sides of the aorta wall with the smooth muscle between them. One hook was attached to a fixed point in the organ bath and the other was connected to an isometric transducer (SB-1T Nihonkoden, Japan), which was joined to a polygraph (LECT-horiz-8K NEC San-ei, Japan). The resting tension was 0.5 g. Acetylcholine (ACh) or sodium nitroprusside (SNP) was added to the bath to study relaxation of the aorta after the submaximal tone had been induced by 3×10^{-7} M noradrenaline.

Drugs

Cholestane glycoside, OSW-1 (3β, 16β, 17α-trihydroxycholest-5-en-22-one 16-*O*-(2-*O*-4-methoxybenzoyl-β-D-xylopyranosyl)-(1→3)-(2-*O*-acetyl-α-L-arabinopyranoside)) was isolated and purified from a methanol extract of bulbs of *Ornithogalum saundersiae* as described previously (Kubo *et al*., 1992).

Statistical analysis

Data are presented as the mean ± s.e. Statistical analysis of data was performed with multiple Tukey's test and Student's *t* test. Differences between the control and drug-treated groups were evaluated with $P < 0.05$ as the level of significance.

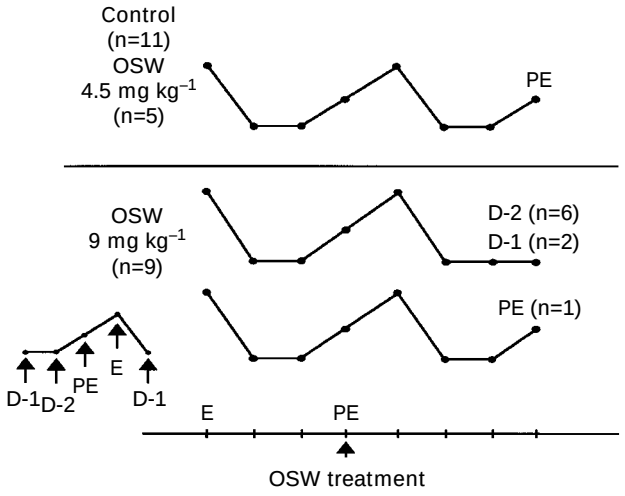


Figure 1 Changes in oestrous cycle after OSW-1 treatment. The figure at left bottom shows the four stages (D-1: dioestrus-I, D-2: dioestrus-II, PE: pro-oestrus, E: oestrus) of the oestrous cycle.

Results

Effects of OSW-1 on oestrous cycle, the weight of reproductive tract and the serum levels of ovarian hormones (steroids and inhibin) and gonadotropin (FSH and LH)

As shown in Figure 1, about 90% of animals treated with 9 μg kg^{-1} OSW-1 did not show pro-oestrus 4 days after the drug was injected at 9 h 00 min on the day of pro-oestrus of the oestrous cycle. Six rats were dioestrus-II and 2 rats were at dioestrus-I. Administration of OSW-1 at a dose of 4.5 μg kg^{-1} showed no abnormal oestrous cycle. In addition, we examined the effects of OSW-1 on oestrous cycle when the drug was injected at 9 h 00 min on the day of dioestrus-I, instead of pro-oestrus. Administration of OSW-1 on the day of dioestrus-I, had no effect on the regular oestrous cycle (data not shown). We also observed that the prolonged dioestrus induced by

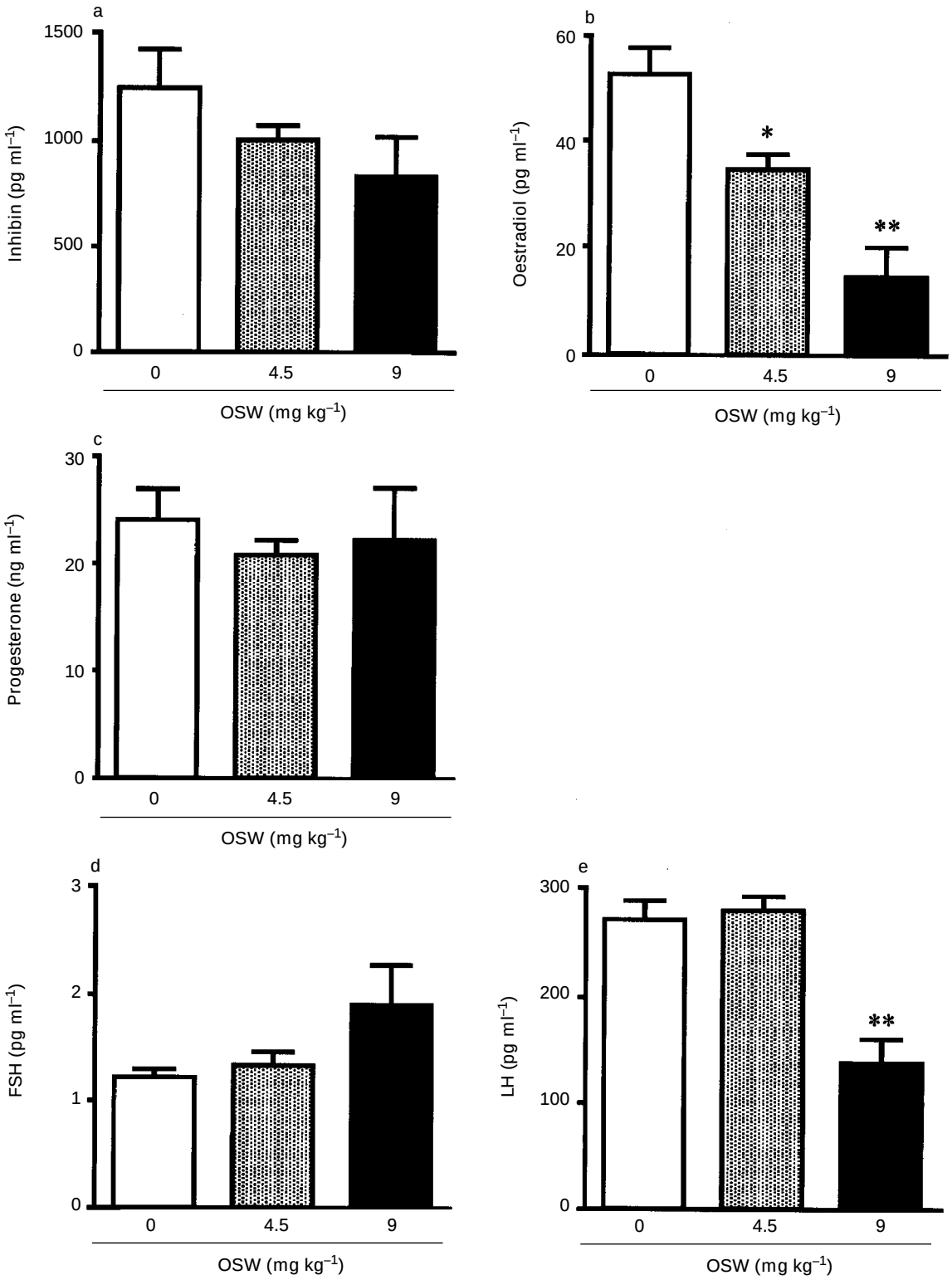


Figure 2 Effects of OSW-1 on the serum levels of inhibin (a) ovarian steroids ((b) oestradiol, (c) progesterone), and gonadotrophins ((d) FSH and (e) LH) on the next expected day of pro-oestrus. After 4 days of vehicle or OSW-1 treatment in pro-oestrus, blood was collected via the abdominal aorta under ether anaesthesia. Each column shows the mean$\pm$s.e. of 5 animals. *$P<0.05$, **$P<0.001$, significantly different from control.

9 μg kg^{-1} OSW-1 had recovered within 2 or 3 days (data not shown). Thus, the inhibitory effect of OSW-1 on the oestrous cycle appeared only in the next oestrous cycle after drug treatment. We also examined the effects of two OSW-1 analogues (OSW-17 and OSW-33), which have no p-methoxy benzoyl residue, on the oestrous cycle. Neither OSW-17 nor OSW-33 had any effect on the oestrous cycle even when 20 times the concentration of OSW-1 used was injected, indicating that the residue is essential for the inhibitory activity on the oestrous cycle. Ovarian weight was not changed by OSW-1 treatment (73.5$\pm$3.09 mg), although uterine weight in the 9 μg kg^{-1} OSW-1 treated group was lower than in the control and 4.5 μg kg^{-1} OSW-1-treated groups (222$\pm$24.4 mg vs 345$\pm$11.0 mg and 358$\pm$13.4 mg, respectively).

We measured the serum levels of hormones to determine whether OSW-1 affects ovarian steroidogenesis and inhibin synthesis at 4 days after drug treatment, which is the day of the expected pro-oestrus of oestrous cycle (Figure 2) and when the irregular effect of OSW-1 on oestrous cycle was observed. Serum E_2 levels were significantly decreased both in rats injected with 4.5 μg kg^{-1} OSW-1, whose oestrous cycle did not change, and in rats treated with 9 μg kg^{-1} OSW-1, which

Table 1 Effects of OSW-1 on ovulation and ovarian weight in rats

Group	*Ovulating rats/ rats examined*	*No. of oocytes in ovulating rats*	*Ovarian weight* (mg)
Control	5/5	12$\pm$1.2	78.3$\pm$4.68
OSW-1 (9 h)	5/5	10$\pm$1.2	84.1$\pm$8.02
OSW-1 (12 h)	6/6	12$\pm$1.7	78.3$\pm$5.60

OSW-1 (9 μg kg^{-1}) was administered s.c. at 9 h 00 min or 12 h 00 min in pro-oestrus. Animals were killed at 9 h 00 min on the next day after drug treatment. Values are the mean$\pm$s.e.

Table 2 Effects of OSW-1 on the serum levels of the preovulatory gonadotrophins surge and ovarian steroids

Hormones	*Control*	*OSW-1*
LH (ng ml^{-1})	29.7$\pm$4.54	25.3$\pm$1.95
FSH (ng ml^{-1})	9.9$\pm$3.19	11.4$\pm$3.08
Progesterone (ng ml^{-1})	38.5$\pm$8.43	43.4$\pm$3.26
Oestradiol (pg ml^{-1})	64.3$\pm$12.1	60.8$\pm$10.6

OSW-1 (9 μg kg^{-1}) was administered s.c. at 12 h 00 min in pro-oestrus. Blood samples were collected via the tail vein under ether anaethesia 5 h (17 h 00 min) after drug treatment. Each column shows the mean$\pm$s.e.

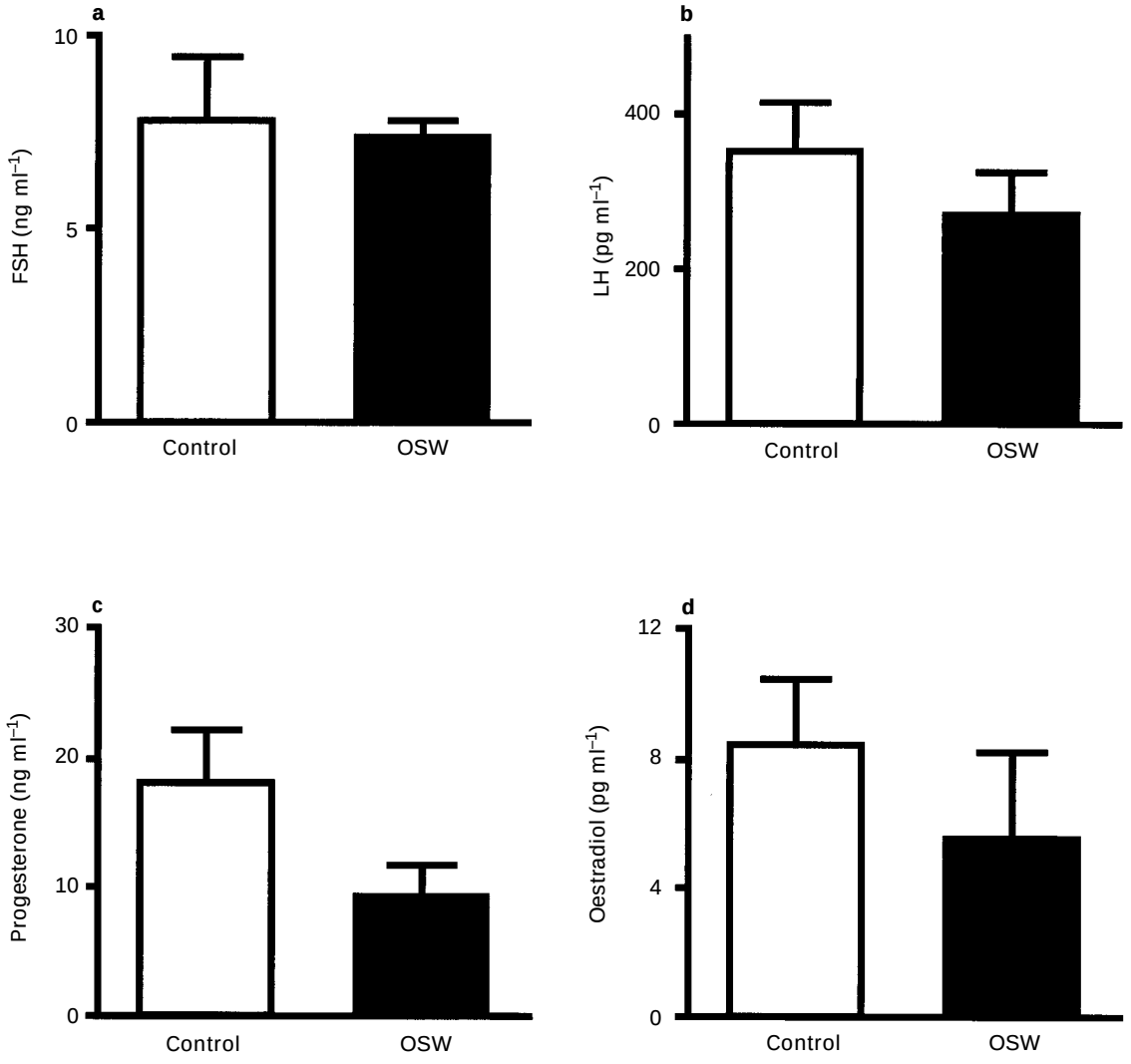


Figure 3 Effects of OSW-1 on the serum levels of gonadotrophins (a and b) and ovarian steroids (c and d) on the next day (oestrus) after drug treatment. OSW-1 (9 μg kg^{-1}) was administered s.c. at 12 h 00 min in pro-oestrus. Blood samples were collected at 9 h 00 min on the next day after OSW-1 treatment. Each column shows the mean$\pm$s.e. of 5–6 animals.

showed a disorder in the oestrous cycle compared to control. The levels in rats treated with 4.5 and 9 μg kg^{-1} OSW-1 were 66% and 28% of that of control rats, respectively. Serum inhibin levels showed a tendency to decrease, but serum P_4 levels were not changed compared to the levels of control (pro-oestrus) rats. To confirm the effects of OSW-1 on the secretion of gonadotrophins, the serum levels of FSH and LH were measured at 4 days after OSW-1 treatment. LH levels in the 9 μg kg^{-1} OSW-1-treated group significantly decreased compared to control and the 4.5 μg kg^{-1} OSW-1-treated group, although FSH levels in the 9 μg kg^{-1} OSW-1-treated group had a tendency to increase. The FSH levels seemed to be correlated to the levels of inhibin decreased by OSW-1 treatment.

Effects of OSW-1 on the surge of gonadotrophins (pro-oestrus 17 h 00 min) and ovulation (oestrus), and on the serum levels of gonadotrophins and ovarian steroids on the next day (oestrus) after drug treatment

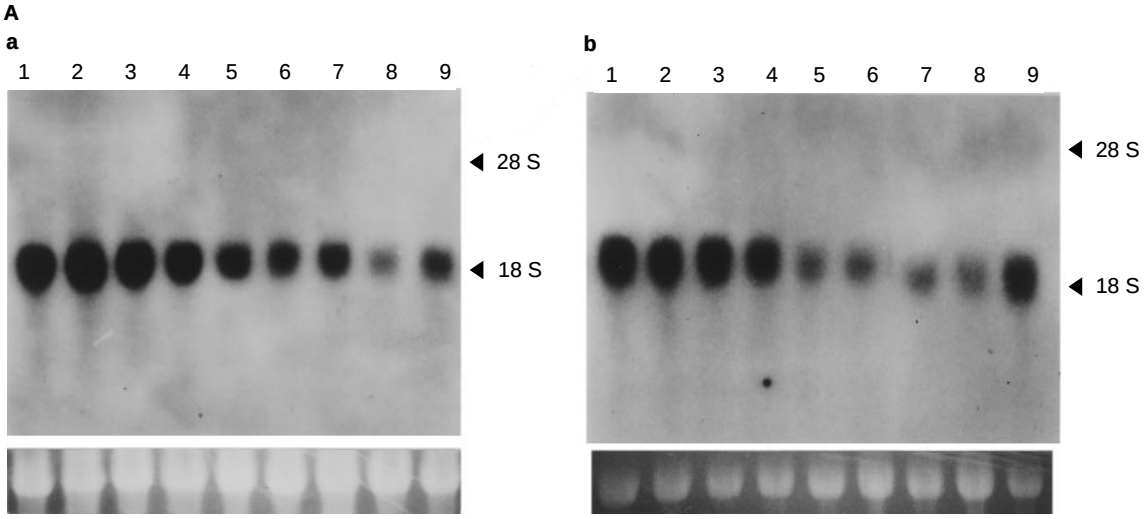
To determine whether OSW-1 blocks ovulation on the next day after drug treatment, OSW-1 (9 μg kg^{-1}) was administered at 9 h 00 min or 12 h 00 min on the day of pro-oestrus and animals were autopsied and blood collected at 9 h 00 min on the next day. As shown in Table 1, OSW-1 did not significantly influence the occurrence of ovulation and the number of oocytes in ovulating rats was normal in both groups (drug treatment at 9 h 00 min or at 12 h 00 min). We further determined the serum levels of FSH, LH, P_4 and E_2 in both pro-oestrus (17 h 00 min, Table 2) and oestrus (9 h 00 min, Figure 3) after OSW-1 treatment at 12 h 00 min in pro-oestrus. We have not presented the data from animals treated with OSW-1 at 9 h 00 min because similar results were obtained with those treated at 12 h 00 min. Blood collection at 17 h 00 min at the expected time of the LH surge in pro-oestrus was performed via the tail vein to examine the ovulation in the same animals on the next morning, as shown in Table 1. As shown in Table 2, gonadotrophin levels surged whereas ovarian steroids levels at 5 h after drug treatment in OSW-1-treated animals were not changed compared to controls. At 9 h 00 min on oestrus day, 21 h after OSW-1 treatment, both serum P_4 and E_2 levels in OSW-1-treated animals showed a tendency to decrease (46% and 65% of control, respectively), whereas FSH and LH were not changed (Figure 5).

Effects of OSW-1 on ovarian p450scc gene expression

To clarify whether the inhibitory effects of OSW-1 on serum levels of ovarian steroids are mediated by the inhibition of p450scc mRNA expression, we examined the effect of OSW-1 treatment on the gene expression. OSW-1 was administered s.c. at 9 h 00 min in pro-oestrus and animals were killed on the next day, at 24 h after drug treatment (Figure 4a), and on the expected day of pro-oestrus, 96 h later (Figure 4b). OSW-1 (4.5 and 9 μg kg^{-1}) significantly decreased the levels of ovarian p450scc mRNA 24 and 96 h after treatment.

Effects of OSW-1 on isometric relaxation in rat aorta

We examined the relaxation of isolated thoracic aorta to determine whether OSW-1-induced hormonal changes influence the cardiovascular system, because ACh-induced relaxation is dependent on the serum levels of E_2 (Honda *et al*., 1996; 1997a). Cumulative addition of ACh (10^{-9} to 3×10^{-6} M) in-



B

Group	a	b
Control	1.00 ± 0.014	1.00 ± 0.060
OSW-1 (4.5 μg kg^{-1})	–	0.82 ± 0.062*
OSW-1 (9.0 μg kg^{-1})	0.83 ± 0.044*	0.78 ± 0.69*

Figure 4 Representative Northern blot analysis of ovarian p450scc mRNA in OSW-1-treated animals. OSW-1 (9 μg kg^{-1}) was administered s.c. at 9 h 00 min in pro-oestrus. At 24 h (a) or 96 h (b) after vehicle or OSW-1 treatment, total ovarian RNA was extracted and 10 μg RNA was subjected to the analysis. (A) Bottom picture in each panel shows ethidium bromide-stained 18 S rRNA on each sample. (a) lane 1–4: control, lanes 5–9: OSW-1 (9 μg kg^{-1}); (b) lanes 1–3: control, lanes 4–6: OSW-1 (4.5 μg kg^{-1}), lanes 7–9: OSW-1 (9 μg kg^{-1}). (B) Densitometric analysis of the levels of p450scc mRNA detected by Northern blot analysis. The mean value of mRNA in control is represented as 1.00. Each value shows the mean ± s.e. of 3–5 animals. *$P<0.05$, significantly different from control.

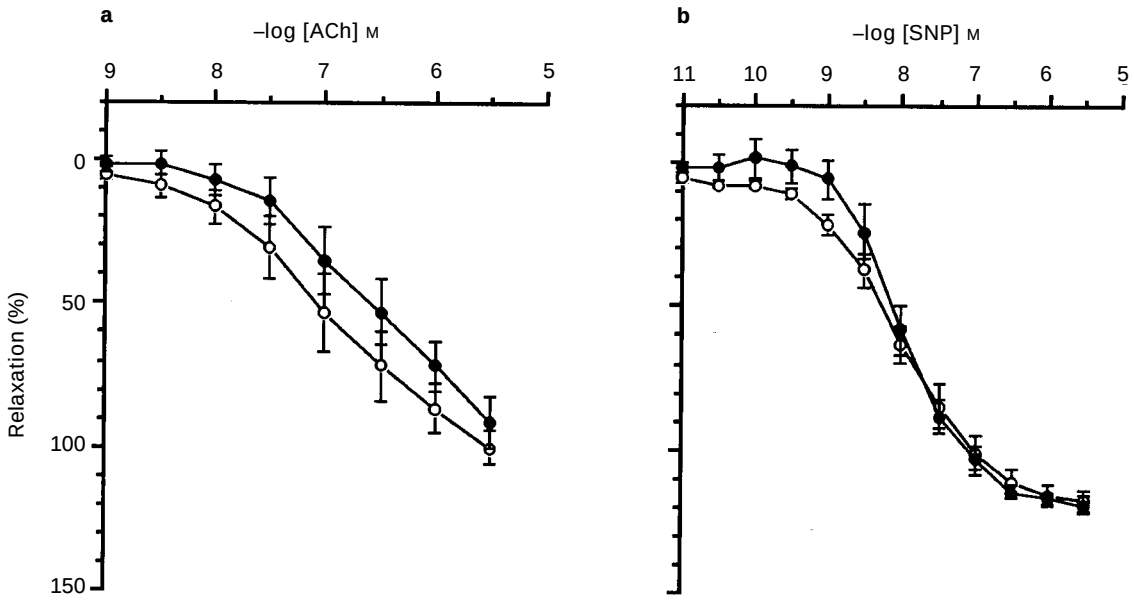


Figure 5 Concentration-relaxation response curves for (a) ACh and (b) SNP in the aortic ring preparations isolated from control and OSW-1-treated rats. After 5 days of vehicle or OSW-1 treatment in pro-oestrus, the thoracic aorta was isolated. Each point represents the mean of 7 experiments; vertical lines show s.e.mean. The figures show responses of the preparations isolated from animals on the day of oestrus (○) and from OSW-1-treated animals (●).

duced concentration-dependent relaxation in the aortic ring preparations isolated from OSW-1 treated and untreated (oestrus) animals (Figure 5a). OSW-1 decreased ACh-induced relaxation and shifted the concentration-dependent relaxation curve for ACh to the right. ED_{50} values for the relaxation in control and OSW-1 treated groups were 2.1 ± 0.80 and $8.5 \pm 4.73 \times 10^{-7}$ M, respectively. However, the SNP-induced relaxation was not affected by treatment with OSW-1 (Figure 5b). The ED_{50} values in the control and treated groups were 7.9 ± 2.37 and $8.6 \pm 2.60 \times 10^{-9}$ M, respectively.

Discussion

The present study demonstrated that OSW-1, which belongs to a steroidal saponin like ouabain, can inhibit ovarian E_2 production. This compound has strong cytostatic activities against various human malignant tumour cells. It has recently been shown that its suppressive effect on the growth of human pronyelocytic leukaemia HL-60 cells is about 10–100 times more potent than that of other anti-cancer agents, such as vincristine and methotrexate, which are in clinical use at present (Mimaki *et al.*, 1997). OSW-1 (10 μg kg^{-1}) is effective against mouse P388 leukaemia, with a single injection increasing their life span by about 60% compared to control. Thus, the potentiality of the drug as a new anti-cancer drug is being focused. However, until now there has been no study with regard to its effects on the reproductive system.

OSW-1, (9 μg kg^{-1}), which is almost the same dose as that effective against murine leukaemia, suppressed the occurrence of next pro-oestrus (Figure 1) and the serum levels of E_2 (Figures 2 and 3). In addition, half of the dose (4.5 μg kg^{-1}) also decreased serum E_2 levels by 34% compared to control, although it had no effect on the oestrous cycle. OSW-1 (9 μg kg^{-1}) delayed the occurrence of the next pro-oestrus by prolonging the duration of dioestrus, as shown in Figure 1. The inhibition of the normal increment in ovarian E_2 production induced by OSW-1 from the day of dioestrus to pro-oestrus is thought to result in the prolongation of the period of dioestrus. However, this inhibitory effect on the oestrous cycle was not severe, because the regular cycle was restored within about 2 days in the next cycle. On the other hand, in the case of the low dose of OSW-1, it was thought that E_2 levels decreased by OSW-1 were within the limit of the levels to cause pro-oestrus. The levels of serum LH in the 9 μg kg^{-1} OSW-1-treated animals were also significantly lower than in control. The reason for this is probably because most animals treated with OSW-1 showed dioestrus on the day of expected pro-oestrus. In fact, the serum LH levels were almost the same as those on the day of normal dioestrus, although the E_2 levels (16 ± 7.0 pg ml^{-1}) in OSW-1-treated animals were slightly higher than normal on the days of dioestrus (8 ± 0.4 pg ml^{-1}). The difference in LH levels between the 4.5 μg kg^{-1} and 9 μg kg^{-1} OSW-1-treated groups probably depends upon the difference in oestrous cycle (pro-oestrus vs dioestrus). Our data do not indicate that OSW-1 prolongs the duration of dioestrus by blocking ovulation via a suppressive effect on the central nervous system, because administration of OSW-1 at 12 h 00 min in pro-oestrus just before the 'critical period' for gonadotrophins surge did not block ovulation and the preovulatory LH surge (Tables 1 and 2). Although ovulation occurred in OSW-1 treated animals, ovarian steroid levels in serum on the day of oestrus tended to be inhibited by OSW-1 (Figure 3). These results (Figures 2 and 3) suggest that OSW-1 directly suppresses the production of ovarian steroids, not by inhibition of gonadotrophin release. In order to examine the possible effects of OSW-1 on ovarian cell functions and steroidogenesis, we analysed ovarian total RNA by measuring p450scc mRNA which plays an important role in steroidogenesis in the ovary, catalysing the initial rate-limiting reaction in the steroid synthesis. At both 1 day and 4 days after OSW-1 treatment, the expression of mRNA coding p450scc was inhibited (Figure 4). This long-acting effect may contribute to the decrease in serum E_2 levels. In contrast to E_2 levels, P_4 levels were not changed at 4 days after OSW-1 treatment. These data, therefore, suggest that OSW-1 acts at granulosa cell to inhibit the conversion of androstendione to E_2 in the cell. OSW-1 might block the biosynthesis pathway of E_2 possibly via inhibition of the activity and/or production of aromatase in ovarian granulosa cells.

The relaxation of aorta induced by ACh tended to be decreased by treatment with OSW-1, as shown in Figure 5. As mentioned in the Introduction, we recently found that the sensitivity of rat thoracic aorta to ACh changes during the 4 days of oestrous cycle, and a maximal increment in the relaxation was obtained on preparations isolated from animals at oestrus, compared to those of dioestrus-II. Thus, as a

functional parameter of E_2 activity, uterine weight and sensitivity of aortic smooth muscle towards ACh are reduced in line with the lower levels of E_2 secretion. We have already shown that OSW-1 does not directly influence the relaxation of blood vessel *in vitro* (Honda *et al*., 1997b). Therefore, the inhibitory actions of OSW-1 on the relaxation of aorta are partially mediated by the inhibition of E_2 secretion. The relaxant effect of smooth muscle by SNP, a donor of NO, is thought to be mediated by an elevation of guanosine 3′, 5′-cyclic monophosphate (cyclic GMP). Weiner *et al*. (1994) recently showed that the mRNA for NO synthases (NOS) isozymes (eNOS and nNOS) and NOS activity were induced by both pregnancy and E_2. The relaxation of the preparations supplied with exogenous NO by SNP was not affected by OSW-1 treatment. Our data suggest that the sensitivity of smooth muscle towards exogenous NO is not affected by OSW-1 treatment, and that the decrease in the ACh-induced relaxation caused by OSW-1 may be associated with the amount of endogenous NO produced by E_2 in the aorta.

Although we do not have enough data to explain why OSW-1 inhibits E_2 production and prolongs the period of dioestrus only when the drug is injected to animals on the day of pro-oestrus, OSW-1 might directly suppress the proliferation of developing granulosa cells in mature follicles. It is well known that granulosa cells in ovarian follicles developed by FSH proliferate rapidly and produce a large amount of E_2, inducing the gonadotrophins surge for ovulation. OSW-1 seems to act on the growth of granulosa cells in terms of its specificity shown against proliferating tumour cells. If adverse or side effects of OSW-1 can be controlled, the drug might be used for drug therapy on neoplastic diseases, especially oestrogen-dependent breast cancer and endometriosis.

In conclusion, the present study shows that OSW-1 inhibits ovarian E_2 secretion and induces an irregular oestrous cycle, which is recovered within a few days, depending on the day of its administration. The decrease in E_2 secretion induced by OSW-1 probably contributes to the decreased thoracic aorta relaxation. Further, the inhibitory actions of OSW-1 on the gene expression of steroidal enzyme and on the growth of ovarian follicles might at least in part mediate a blockage of E_2 secretion, independent of the central hypothalamus-pituitary system.

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