THE RODENT UTEROTROPHIC ASSAY : CRITICAL PROTOCOL FEATURES, STUDIES WITH NONYLPHENOLS, COMPARISON WITH A YEAST ESTROGENICITY ASSAY

by

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SUMMARY

The major protocol features of the immature rat uterotrophic assay have been evaluated using a range of reference chemicals. The protocol variables considered include the selection of the test species and route of chemical administration, the age of the test animals, the maintenance diet used and the specificity of the assay for estrogens. It is concluded that three daily oral administrations of older rats, followed by determination of absolute uterus weights on the fourth day, provides a sensitive and toxicologically relevant *in vivo* estrogenicity assay. Rats are favoured over mice for reasons of toxicological practice, but the choice of test species is probably not a critical protocol variable, as evidenced by the similar sensitivity of rats and mice to the uterotrophic activity of methoxychlor. Vaginal opening is shown to be a useful, but non-definitive, ad chemicals to reduce or abolish the uterotrophic response of estradiol is suggested to provide a useful extension of the uterotrophic assay for the purpose of detecting

The results of a series of studies on the environmental estrogen nonylphenol (NP), and its linear isomer n-nonyl of the aliphatic side chain is important that b for activity. 17-Desoxyestradiol similar activity to estradiol in the uterotrophic assay, and is suggested to represent the 'parent' estrogen of NP. Benzoylation of NP and 17-desoxyestr uterotrophic activity, in contrast to the enhancing effect of benzoylation on estradiol.

Selected chemicals shown to be active in the immature rat uterotrophic assay were also evaluated in an *in vitro* yeast human estrogen receptor transactivation assay. Most of the chemicals gave similar qualitative responses to those seen in the uterotrophic assay, and the detection of the estrogen methoxychlor by the yeast assay evidenced a degree of intrinsic metabolic competence. However, the assay had a reduced ability (compared to rodents) to hydrolyse the benzoate ester of estradiol, and the estrogenic benzoate derivative of NP was not active in the yeast assay. These last results indicate that current metabolic deficiencies of *in vitro* estrogenicity assays will limit the value of negative data for the immediate future.

The results described illustrate the intrinsic complexity of evaluating chemicals for estrogenic activities and confirm the need for rigorous attention to experimental design and criteria for assessing estrogenic activity.

INTRODUCTION

There is concern that some natural and synthetic chemicals have the potential to disturb normal sexual differentiation/development in animals and humans (Colborn and Clement, 1992; McLachlan, 1993; McLachlan and Korach, 1995). The ability of some chemicals to elicit an estrogenic response in intact organisms forms one of the primary mechanisms by which such adverse effects could be produced (McLachlan and Korach, 1995). One of the most extensively used assays for estrogenic activity is the rodent uterotrophic assay in which the ability of chemicals to stimulate uterine growth is monitored (Reel *et al*., 1996; O'Connor *et al.*, 1996). There are a variety of protocols described for this assay, including the use of rats or mice; immature, hypophysectomized or ovariectomized animals, and the use of oral or subcutaneous routes of chemical administration. Given that most toxicity studies are currently conducted in rats, using the oral route of administration, we decided to evaluate the utility of the immature rat uterotrophic assay, using the oral route of chemical administration, as an *in vivo* assay for estrogenic activity. An associated objective was to discern the critical features of the assay protocol.

Having confirmed the practicality of the immature rat uterotrophic assay it was used to study a range of reference hormonally active chemicals and to elucidate the chemical basis for the estrogenic activity reported for branched chain p-nonylphenol. Finally, relationship between estrogenic activities observed *in vitro,* and those observed *in vivo,* some of the chemicals found positive in the uterotrophic assay were evaluated in the yeast human estrogen receptor (hER) transactivation assay described by Routledge and Sumpter (1996).

MATERIALS AND METHODS

Chemicals

Estradiol (E_2; for chemical structures, see Scheme 1), ethinyl estradiol (EE), cyproterone acetate (CPA), methoxychlor (MC; 98%), corn oil and arachis oil were obtained from Sigma (Poole, Dorset, UK). Estradiol benzoate (E_2B) was obtained from Intervet (Cambridge, UK) as a solution in arachis oil (peanut oil). Coumestrol was obtained from Apin Chemicals (Abingdon, Oxfordshire, UK) and ICI 182,780 was a gift from Dr A Wakeling, Zeneca Pharmaceuticals (Alderley Park, Macclesfield, UK). p-Nonylphenol was obtained from two sources: Fluka Chemika-BioChemika (Gillingham, Dorset, UK; 85% para isomers: [NP(F)]) and Schenectady International (... Texas, USA; 94.2%: [NP(S)]). NMR analysis of these two samples indicated them to be a mixture ... (nNP; 98%) was obtained from Lancaster synthesis (Morecambe, Lancashire, UK). The benzoate ester of NP(F), (NPB), was prepared by standard benzoylation of the parent phenol (NP; Fluka). The structural integrity of the oily product was confirmed by NMR spectroscopy and elemental analysis. The benzoate ester of nNP (nNPB) was prepared by benzoylation of the parent phenol (nNP; source as above). The product was a soft wh... °. NMR and mass spectroscopy confirmed its structure and elemental analysis indicated that it contained 1/6 molecule of water. 17-Desoxyestradiol [estra-1,3,5(10)-... was synthesized from estrone by the method described by Francois and Levisalles (1968). After recrystallisation from cyclohexane it was ... mp 138-140°. NMR and mass spectroscopy confirmed its structure and elemental analysis indicated that it contained 1/10 molecule of water. The corresponding benzoate derivative, 17-desoxyestradiol benzoate [estra-1,3,5-(10)-...] (dE_2B), was prepared by a standard benzoylation reaction. The product was recrystallized from methylated spirits (74 OP) giving colourless ... °. NMR, mass

spectroscopy and elemental analysis confirmed the structure and purity of the product. The diphenol derivative bis(*p*-methoxyphenyl)[1,2-diphenol; 99%] was a gift from MD Shelby, originally supplied by Cedra Corp., Austin, Texas, USA.

Animals

Immature female Alpk, except where indicated otherwise, with body weights 38-48g were ordered from Zeneca, Alderley Park. Immature Alderley Park Swiss Albino mice were obtained from the same source (2 weights 12-17g). Animals were housed in wi was controlled and a 12h/12h light dark cycle was maintained. Animals were weaned on Harlan Teklad TRM diet (Harlan UK, Bicester, Oxfordshire, UK) at 1 maintained on pelleted PCD diet (Special Diet Services Ltd, Witham, Essex, UK) from 21 days onwards. Diet and water were available *ad libitum.* A range of additional diets were used in the rat dietary study, as described in the legend to Figure 9. Rat and mouse No.3 Breeding diet (R&M No.3), rat and mouse No.1 maintenance diet (R&M No.1) and casein diet (Super Casein batch RHM 92/117) were also obtained from Special Diet Services Ltd. All animals were acclimatized for 24h before being dosed.

Uterotrophic assays

The protocol for the uterotrophic assays was based on that described by Wakeling *et al.*, (1991). Animals were dosed by either oral gavage or subcutaneous (sc) injection. Test agents were dissolved or homogeneously suspended in arachis oil (except where indicated otherwise). The dosing volume for both routes of exposure was 5ml/kg body weight, except for the assays of coumestrol where a dosing volume of 10ml/kg was used. Animals received 3 daily doses of the test compound and were killed by an overdose of halothane 24h after the final dose. The dose levels shown in the Figures are the daily dose levels. Vaginal opening (or otherwise) was recorded at the time of death. Uteri were excised, trimmed free of fat, pierced and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The uterus was then weighed (wet weight). Uterine dry weight was also determined by drying the uteri at 70° for 24h before *et al.*, 1988). Dose levels were usually selected based on previous findings for estradiol, and in the case of those chemicals giving positive uterotrophic responses, the maximum tolerated dose (MTD) was probably not achieved. The top dose level of NP evaluated (285 mg/kg) was the MTD (data not shown). The analogs of NP were evaluated to this same top dose level. The top dose level of cyproterone acetate evaluated (2.5 mg/kg) was ~5-100 times the h

E_2, or E_2B, was used as a positive control agent in all of the studies, and each study was accompanied by a vehicle (negative) control group. Uterus wet weights are shown in the Figures, but uterus dry weights and uterus weight/body weight data were derived routinely and are discussed in the text. Generally, five animals per exposure group were employed, but this number was increased in some cases (see Figures).

Yeast assays

The yeast assay for estrogenic activity of chemicals, as described by Routledge and Sumpter (1996), has the DNA sequence of hER incorporated into the yeast genome. The molecular construct was designed to allow the activated receptor to bind to e sequences contained within a strong promoter region of an expression plasmid. When an active ligand binds to the estrogen receptor a *lac*Z reporter gene expresses the enzyme -galactosidase, which itself is quantitated using a chromogenic substrate. The methods used

were exactly as described earlier (Routledge and Sumpter, 1996). Briefly, the test chemicals were dissolved in ethanol and added to 96 well plastic plates at the concentrations shown in Figure 15. The ethanol was allowed to evaporate before the addition of medium containing the yeast . The plates were incubated at 32 for 4 days and quantitated in terms of the absorbance of wells at 540 nm. Vehicle and estradiol controls accompanied each experiment, and representative data are shown.

RESULTS AND DISCUSSION

The results and discussion are presented in relation to the three topics evaluated in this study. Chemical structures are shown in Scheme 1.

Critical protocol features of the uterotrophic assay

Estradiol benzoate (E_2B) gave an approximately 3-fold increase in uterus weights in animals aged between 22-25 days, whereas animals aged 27 days at the start of the experiment had developed uteri and failed to respond further to the estrogenic challenge (Figure 1). Vaginal opening showed the same trend. The uterus weights of animals aged 25 days at the end of the experiment were identical (Figure 1) despite a 20% difference in body weight at termination (data not shown). These observations indicate that uterotrophic assays should commence at about 22 days of age, and that absolute uterus weight is probably a better index of uterine growth than the uterus/body weight index (see later for extension of the latter point).

Comparisons of the oral and sc routes of administration of the three established estrogens estradiol (E_2), estradiol benzoate (E_2B) and ethinyl estradiol (EE). E_2 is more active in the uterotrophic assay when injected subcutaneously, but a similar maximal effect can be achieved following oral administration of higher 2). Based on these combined results, oral administration of 400 g/kg of the endogenous estrogen E_2 was adopted as the standard positive control agent for the uterotrophic assay. The relationship between uterus wet weights, uterus wet weights relative to body weight, and uterus dry weights was essentially constant for all of the animals in the present study, and representative data for animals exposed to E_2 are shown in Table 1. Uterus wet weights expressed relative to body weights are subject to chemically-induced cha (Ashby *et al.*, 1997c), as illustrated by the slightly larger uterus/body weight ratio for animals exposed to the highest s.c. dose of E_2, an effect dependant upon a small reduction in body weight for these animals (Table 1). We decided to regard absolute uterus wet weights as the primary assay parameter, with recourse to the determination of uterus dry weights in cases where it was considered necessary to confirm that a weak uterotrophic assay response was not due totally to increased water imbibation (c.f. such a suggestion; Evans *et al.*, 1996).

The enhanced sensitivity of the uterotrophic assay to E_2 when using sc injection, as opposed to oral administration, was also observed for both E_2B (Figure 3) and EE (cutaneously administered EE was the most potent estrogen encountered in the present study, showing trophic activity at 0.5 g/kg. The choice of route of administration for chemical studies will be influenced by the purpose for which the data are to be used. Oral administration is generally employed in chemical toxicity studies to be employed for human risk assessments, and it seems reasonable to stay with this route for routine toxicity studies. In some cases, such as when the shape of the dose response relationship is the critical issue, it may be appropriate to employ parenteral administration, but it would be inappropriate to use such routes routinely, especially if the only justification for that is that these routes have been

used historically. The specificity of the uterotrophic assay for estrogens was indicated by the negative response observed at the dose level (Neumann, 1977; Figure 5).

The observation that premature vaginal opening appears to be a less sensitive marker of estrogenic activity than does the stimulation of uterine growth (Reel *et al.*, 1996) has been confirmed by the present studies. In particular, premature vaginal opening (Figures 2-4) does not always parallel increases in uterine weight. For example, at doses giving approximately equivalent uterotrophic effects, orally administered E_2 and EE produced premature vaginal opening while their subcutaneous counterparts did not. To date, none of our control rats administered has had an open vagina at the time of the uterotrophic assay (post-puberty); nonetheless, some chemicals produce a clear uterotrophic effect in the absence of vaginal opening (see coumestrol, later herein). This indicates that while premature vaginal opening can strengthen the classification of an agent as an estrogen, it cannot be taken as a definitive requirement.

The uterotrophic response produced by E_2 was ablated, in a dose-related manner, by the estrogen receptor antagonist ICI 182,780 (Wakeling and Valcaccia, 1983) when the latter was administered concomitantly with each dose of E_2 (Figure 6). This indicates that estrogens can be detected using the uterotrophic assay by the simple expedient of challenging the assay positive control agent with the test chemical. Animals exposed to ICI 182,780, with or without E_2, gave uterus weights that were decreased by ~50% when compared to concurrent control values. This led us to evaluate whether either the test vehicle, or the test diet, contained estrogenic contaminants. First, we evaluated whether the arachis oil vehicle was itself weakly estrogenic, some vegetable oils being known to contain phytoestrogens (Gunstone *et al.*, 1994). The data shown in Figure 7 confirm that there was no difference in the uterus weights of untreated, arachis oil treated, or distilled water treated rats. In each case, ICI 182,780 gave an approximately 50% reduction in uterus weights compared to concurrent controls. This indicated either that low levels of endogenous estrogens, or dietary phytoestrogens, were responsible for the elevated control uterus weights. The uterotrophic activity of the phytoestrogen coumestrol (Markarevich *et al.*, 1995) (Figure 8) confirmed that estrogenic dietary contaminants have the potential to influence control uterus weights in uterotrophic assays. The data shown in Figure 9 confirmed that dietary estrogens were the probable reason why our control uterus weights could be reduced by administration of ICI 182,780. Based on these data, the R&M no.3 / R&M no.1 diet combination was adopted for routine use with the assay. However, the experiments discussed in this paper were conducted before that change of diet occurred, thus the relatively high control uterus weights observed herein. Overall, the data shown in Figure 9 underline that diet should be considered as an important variable in any endocrine disruption assay, and that care should be taken to specify its source and constitution.

The rat was selected as the primary test species for the present studies. However, the mouse has been used equally in the past, and the general sensitivity and utility of the mouse uterotrophic assay was recently confirmed by Shelby *et al.* (1996). One of the xenobiotic estrogens evaluated by Shelby *et al.* , methoxychlor (MC), was found to be inactive in the two *in vitro* estrogenicity assays employed, probably because of the inability of these systems to remove the methyl groups of MC to yield the active diphenolic estrogenic species (Bulger *et al.* 1978). In fact, Shelby *et al.* (1996) demonstrated the latter diphenol to be estrogenic *in vitro*. In contrast, these authors reported MC to be uterotrophic to the mouse (subcutaneous). These data are shown in Figure 10 reveal that MC shows similar uterotrophic activity in the rat and the mouse, using either oral

administration. Shelby *et al.* (1996) expressed their uterotrophic assay data corrected for body weight, and when the mouse data shown in Figure 10 are similarly corrected, MC is found to have given a positive response of similar magnitude in the two lab increase in uterus weights over concurrent control levels, at 500 mg/kg MC). These limited data for MC suggest that the choice of test species and route of chemical administration may only lead to quantitative, rather than qualitative, differences in uterotrophic assay outcomes. However, qualitatively different test results will inevitably emerge for chemicals that are differentially absorbed and/or differentially metabolized in different test species. The choice of test species and route of administration for the uterotrophic assay should be kept under review as new data accumulate.

Structural basis of the estrogenicity of nonylphenol

Commercially available nonylphenol (NP) cont isomers. The commercial material is known to possess estrogenic properties (Soto *et al.*, 1995; Routledge and Sumpter, 1997), and these have been suggested to arise due to the structural similarity to E_2 of one or more of the isomers of NP. Warhurst (1994) has suggested one particular isomer of NP as being the most likely to bind to and activate the estrogen receptor (Scheme 1). This isomer happens to be similar in structure to octylphenol, a related compound that contains only one isomer (as shown in Scheme 1) and which is active as an estrogen *in vitro* (Routledge and Sumpter, 1997) and *in vivo* (Bicknell *et al.*, 1995). Recently, Sanborn (1996) erroneously associated the estrogenic properties of commercial NP with the linear aliphatic sidechain isomer nNP, a compound which is not estrogenic (see below). Sanborn (1996) also suggested that hydroxylation of the aliphatic sidechain of nonylphenols (shown for *nNP* in Scheme 1) was required to complete their structural alignment with E_2. However, that suggestion is not supported by the observation that NP, *per se*, competes with E_2 in bindings to isolated cytosolic estrogen receptors, i.e., without the need for prior metabolic transformation (Soto *et al.*, 1995; White *et al.*, 1994).

As recently reported by Lee and Lee (1996), NP gave a dose-related posi immature rat uterotrophic assay (Figure 11). This response was abolished by co-administration antagonist ICI 182,780, thereby confirming that the effect was mediated *via* the estrogen receptor (Figure 11). Similar levels of uterotrophic activity were observed for the two different samples of NP tested [NP (Figure 12). The inactivity of the straight chain analog of NP (nNP; Figure 12) was supported by its inactivity in the yeast assay (see below). The inactivity of nNP favors the suggestion that the in NP is important to the interaction of the side-c chemical with the estrogen receptor (Routledge and Sumpter, 1997; Warhurst, 1994). It is interesting to note the weak assay responses for NP shown in Figure 12. The reproducibility of these weak effects, and the observation of cases of vaginal opening among the test animals, establish uterotrophic activity in these cases; nonetheless, these data indicate the urgent need to agree criteria for discerning activity in the uterotrophic assay (Ashby *et al.*, 1997c).

It was noted earlier that E_2B is a more potent estrogen in the uterotrophic assay than is E_2 itself (Figures 2 and 3). This enhancement of activity is probably caused by a combination of factors, such as reduced conjugative excretion of the free phenol (E_2) by protection of its acidic phenol substituent, and the enhanced lipophilicity of the benzoate (E_2B) in comparison to the parent phenol (E_2). It therefore became of interest to evaluate if the benzoate derivative of NP (NPB) would be more potent than NP itself, and in particular, if the benzoate derivative (nNPB) of the inactive straight-chain analog (nNP) in the uterotrophic assay. The data shown in Figure 13 demonstrate that these two benzoates

were of similar activity to the parent phenols; equally as positive, and negative, respectively. These rather surprising findings indicate that the activity-enhancing effe estradiol cannot be generalized, and that nNP seems to be devoid of intrinsic estrogenic activity.

The suggestion that the estrogenic activity of NP is due to the ability of some of its constituent isomers to mimic estradiol in binding to the estrogen receptor (Routledge and Sumpter, 1997; Warhurst, 1994) indicates that the 17-desoxy derivative (desoxy-E_2) should represent the progenitor 'estrogenic alkylated phenol' reported to be uterotrophic to hypophysectomised rats (Huggins and Jensen, 1955) and to be able to compete with estradiol in binding to isolated estrogen receptors (Fanchenko *et al.*, 1979). The latter activity suggests that desoxy-E_2 was uterotrophic to immature rats (Figure 14), showing a slightly higher minimum detection level than E_2 and a more flattened dose response relationship (Figures 3 and 14). As observed for NP and nNP, the desoxy-E_2 showed similar activity in the uterotrophic assay to that of the parent compound (Figure 14). It therefore appears that the suggestion that one or more of the branched chain isomers of NP is able to mimic estradiol in binding to the estrogen receptor is a valid and useful observation, and that 17-desoxy-E_2 is the optimised form of However, the suggestion that the aliphatic sidechain of NP requires to be hydroxylated before NP acquires estrogenic activity (Sanborn, 1996) seems to be unlikely.

Relative sensitivity of the yeast estrogenicity assay and the uterotrophic assay

In vitro assays will be of particular value in cases where a lead chemical showing estrogenic activity is shown to be positive in the *in vitro* assay, and where the activity of structural analogs of the lead chemical are the subject of study. The yeast hER assay described by Routledge and Sumpter (1996), has shown sensitivity to a wide range of chemical classes of estrogens, and the assay has intrinsic metabolic competence, as evidenced by its ability to detect as positive the estrogen MC (Figure 15; Ashby *et al.*, 1997b), despite the inability of some other *in vitro* assays to detect it (Shelby *et al.*, 1996). This attribute of the yeast assay is probably due to the enhanced metabolic competence of growing yeast cells and their presumed ability to demethylate MC to the active estrogenic diphenol derivative (MC-diphenol; Bulger *et al.*, 1978). As expected, the yeast assay was more sensitive to the ultimate estrogenic species derived from MC (MC-dipl to MC itself (Figure 15).

In the yeast assay, both desoxy-E_2 (Figure 15). This reduced activity is consistent with the relative activity in the uterotrophic assay (Figures 14 and 3). The *in vivo* has implications for the derivation of structure activity relationships among xenobiotic estrogens (Katzenellenbogen, 1995; Waller *et al.*, 1996) where the prese hydroxy and the 17-hydroxy groups of considered to be necessary for estrogenic activity.

The yeast assay is less sensitive to E_2B than it is to E_2 (Figure 15). This is in contrast to the marginally enhanced activity of E_2B, compared to E_2, in the uterotrophic assay (Figures 2 and 3). This indicates that the yeast assay is probably able to hydrolys group present in E_2B, but that this is not completed under the conditions of the test. As observed with the uterotrophic assay, the yeast assay found the two different samples of NP to be of similar estrogenic activity, and nNP to be inactive (Figure 15). In surprising contrast to in the uterotrophic assay, the benzoate derivative of NP (NPB) was inactive in the yeast

assay (Figure 13). This suggests that the yeast cells possess only selective esterase activity, being able to hydrolyse partially the benzoate ester of E_2B, while being unable to hydrolyse the benzoate ester of NPB.

In summary, the yeast estrogenicity assay has demonstrated high general sensitivity, and a level of metabolic competence, in the present studies. A potential weakness of this (and probably of all other *in vitro* assays) is highlighted by the failure of the yeast assay to detect as positive the *in vivo* estrogen NPB. That failure is perhaps reflected quantitatively by the reduced sensitivity of the assay to E_2B in comparison to E_2. Although the problem posed by the inadequate metabolic competence of the current *in vitro* estrogenicity assays must be solved before they can achieve their true potential (Ashby *et al.*, 1997a; Ashby, 1997), the partial metabolic competence of the present yeast assay confirms it as a valuable screening test.

CONCLUSIONS

The results described here illustrate the intrinsic complexity of evaluating chemicals for estrogenic activities. Suitable detection systems exist, but the manner in which they are deployed can influence the toxicological value of the data derived. It is recommended that when designing experiments in this area of toxicology it is important consider the purposes for which the test data will be used. This will influence practical decisions, such as choice of *in vitro* or *in vivo* assays, and the choice of dose levels and route of chemical administration in rodent studies. At the practical level, there is an urgent need to agree criteria for assessing the activity of chemicals in the available estrogenicity assays, and to enhance the metabolic competence of the currently available *in vitro* assays.

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Table 1. The effect of 17 - e s t r a d i o l o n d i f f e r e n t p a r a m e t e r
administered by either oral gavage or subcutaneous (sc) injection. Group sizes are as
described in Figure 2.

Estradiol g/kg	Mean body weight (g; SD)	Uterus wet weight (mg)		Uterus wet weight (mg)/ body weight (g)		Uterus dry weight (mg)	
		mean SD	% of control values	mean SD	% of control values	mean SD	% of control values
Oral gavage							
0	63.5 4.6	33.7 8.1	100	0.531 0.13	100	6.69 1.06	100
10	61.7 1.8	37.5 7.5	112	0.609 0.12	115	6.30 0.85	94
20	62.8 4.0	39.0 7.4	116	0.624 0.13	117	6.99 1.16	104
40	60.9 3.6	38.3 4.3	114	0.630 0.07	119	7.66 0.84	114
100	60.8 3.4	68.1 17.5	202	1.120 0.29	211	12.15 3.20	182
200	60.7 3.2	70.9 14.9	210	1.165 0.22	219	13.05 3.70	195
400	63.2 5.4	101.7 16.7	302	1.614 0.26	304	19.37 2.36	289
sc injection							
0	63.8 4.8	38.2 7.3	100	0.597 0.10	100	7.09 1.13	100
0.5	66.0 3.2	40.5 3.2	106	0.616 0.06	103	7.83 0.65	111
1	63.4 3.7	42.3 7.0	111	0.666 0.08	112	8.10 1.29	114
2	65.3 4.8	43.9 5.8	115	0.670 0.04	112	8.34 1.01	118
5	63.1 5.2	86.4 9.8	226	1.368 0.11	229	15.00 2.21	212
10	62.7 3.1	95.4 17.0	250	1.519 0.25	254	16.92 2.31	239
20	61.8 3.6	86.4 13.4	226	1.482 0.12	248	15.73 2.32	222
40	63.1 2.9	84.9 6.0	222	1.348 0.09	226	15.61 1.28	220
200	60.6 3.6	89.0 11.8	235	1.486 0.20	249	16.45 1.65	232
400	61.9 4.5	101.2 14.5	265	1.628 0.16	273	17.98 2.31	254

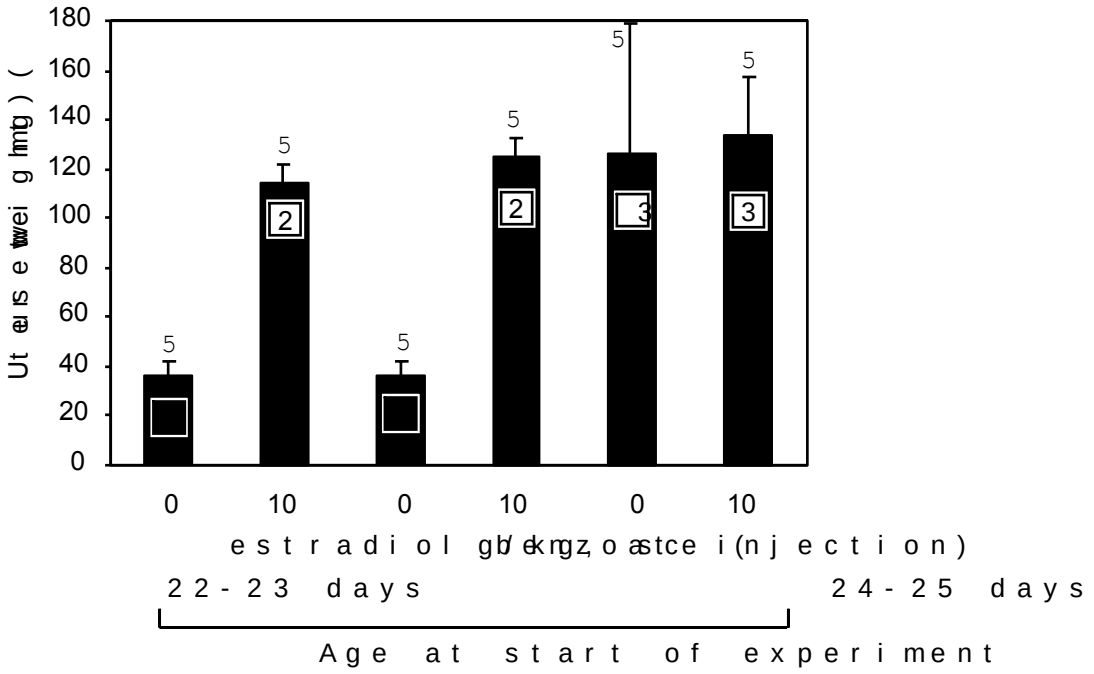


FIGURE 1. The effects of age and weight on uterine grow in response to estradiol benzoate. Controls received No animal with open vagina, one animal with vagina open (number open shown in the box). Data represent group above the bar represents the group size.

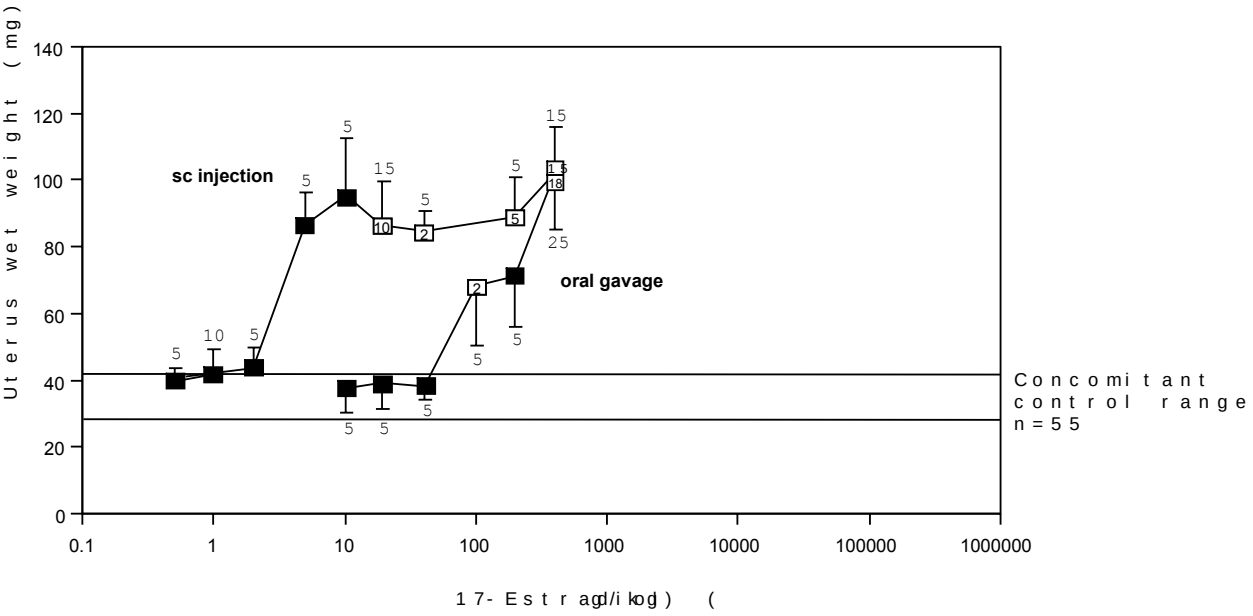


FIGURE 2. The effects of 17 on uterine wet weight in immature rats. Controls received the vehicle (sesame oil, 0.1 ml/kg). This paper allows comparison of data between experiments. Closed symbols denote no open symbols denote at least one animal with open vagina (the number of within the datapoint box). Data represent group means ÑSD. Values were independent studies, each of which the control group. The number above or the bar represents the combined group size.

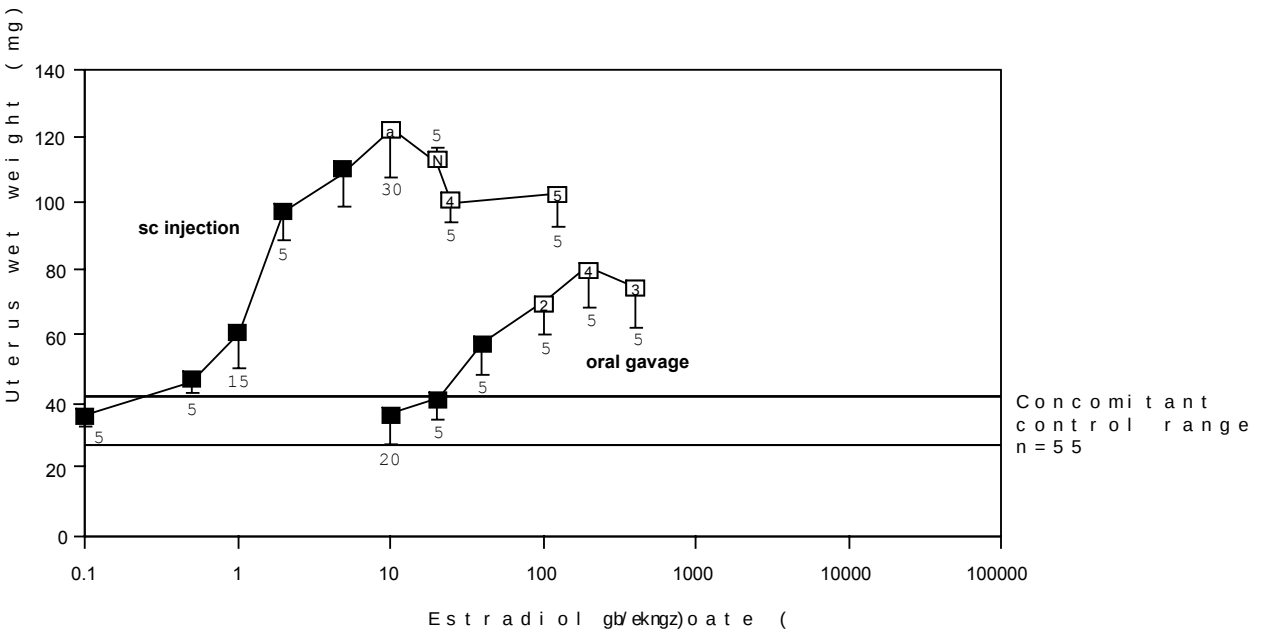


FIGURE 3. The effects of 17β-estradiol benzoate on uterus wet weight and vaginal opening in immature rats. Controls received arachis oil only. Closed symbols denote no animal with open vagina (the number of open vaginas is shown within the symbol); open symbols denote at least one animal with open vagina (the number of open vaginas is shown within the symbol, N = vaginal opening not determined). Data represent group means ± SD. Values were combined from 9 independent studies, each of which contained a vehicle control group. The number below the bar represents the combined group size.

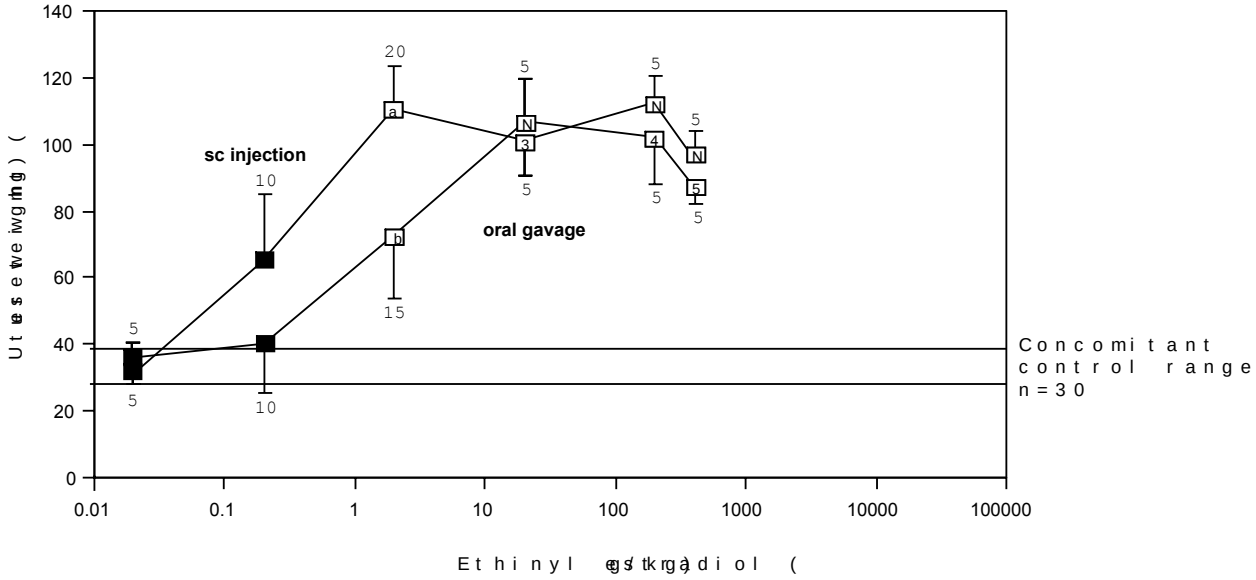


FIGURE 4. The effects of ethinyl estradiol administered via oral gavage on uterine wet weight in immature rats. Controls received arachis oil only. Closed symbols denote no animal with open vagina (the number of open vaginas is shown within the symbol); open symbols denote at least one animal with open vagina (the number of open vaginas is shown within the symbol, N = vaginal opening not determined). Data represent group means ± SD. Values above or below the bar represents the combined group size. Values were combined from 5 independent studies, each of which contained a vehicle control group.

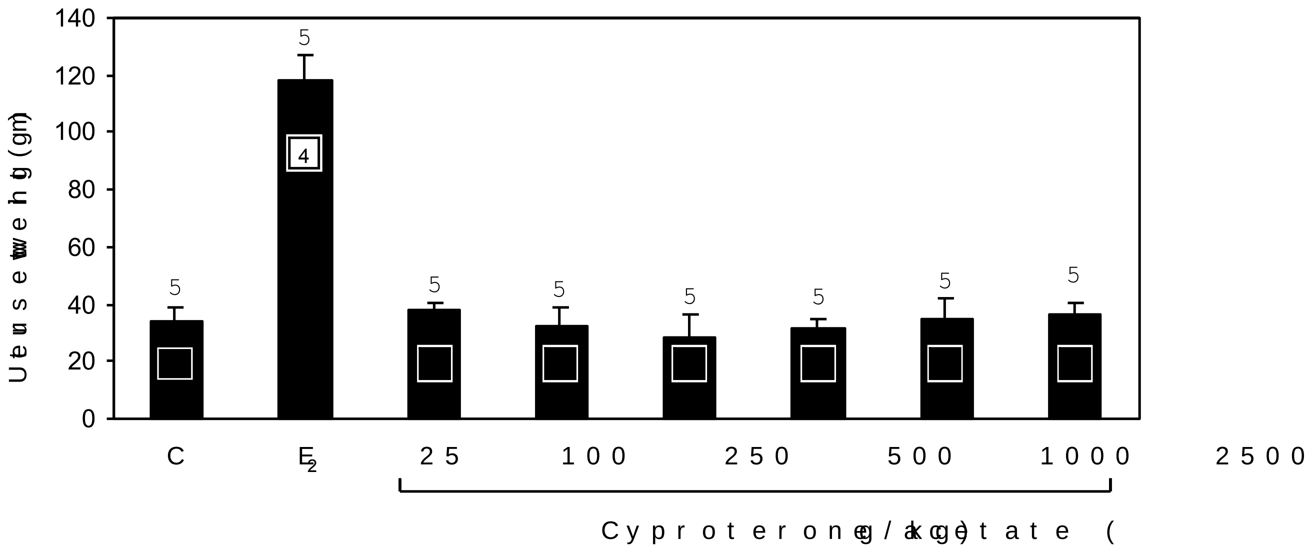


FIGURE 5. The effects of oral administration of the anti
uterus wet weight and vaginal opening in immature rats (400 g/kg, oral gavage) E2 used
positive control. Controls received arachis oil only.
one animal with open vagina (number of open vaginas sh
group means Ñ SD. The number above the bar represents t

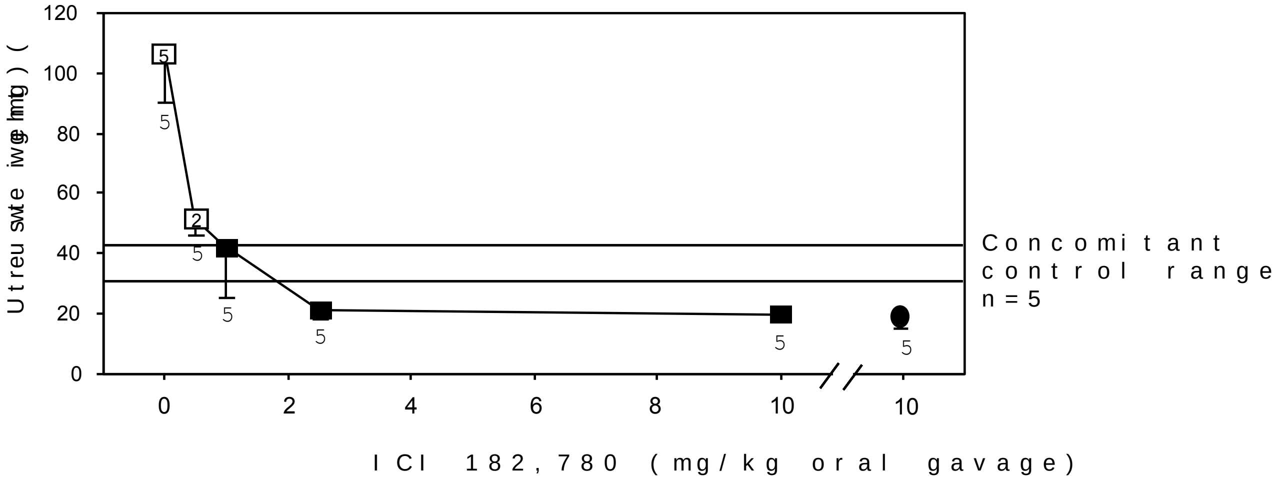
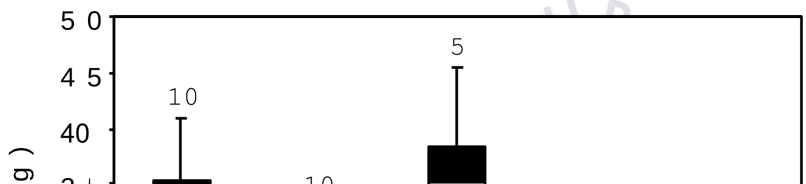


FIGURE 6. The effects of the estrogen receptor antagonist I
uterotrophic activity of estradiol (400 g/kg oral gavage) in immature rats. Con
animals received arachis oil only. The effects of ICI 18
are shown on the right of the figure (●). Closed sy
open vagina, open symbols denote at least one animal wi
number of open vaginas is shown within the datapoint box
means Ñ SD. The number below the bar represents the grou



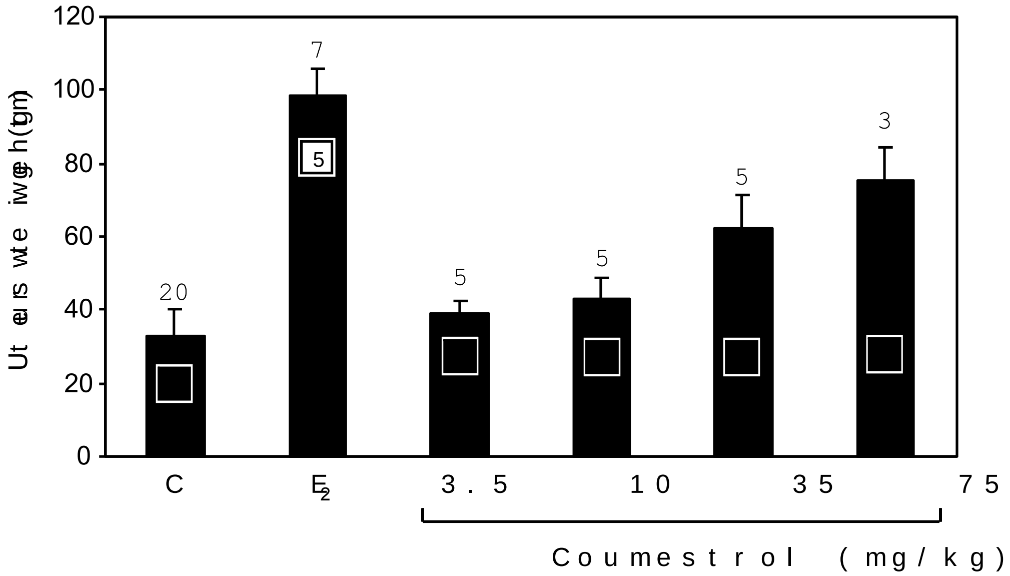
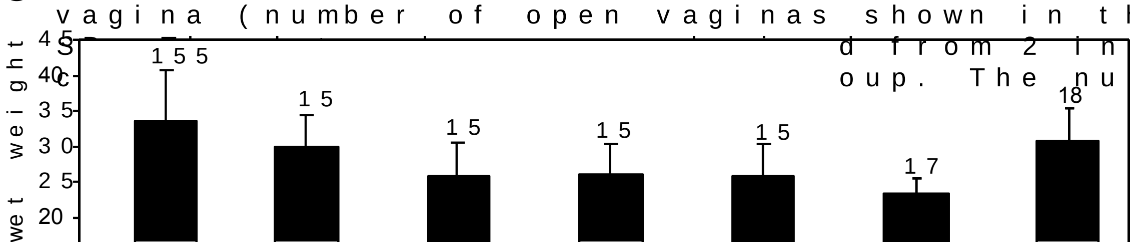


FIGURE 8. The effects of oral administration of coume
vaginal opening. Estradiol (E$_2$) (4 µg/kg, oral gavage) was used a posit
received arachis oil only. ■ □ No animal with op
vagina (number of open vaginas shown in the open box
S d from 2 independent e
C oup. The number above



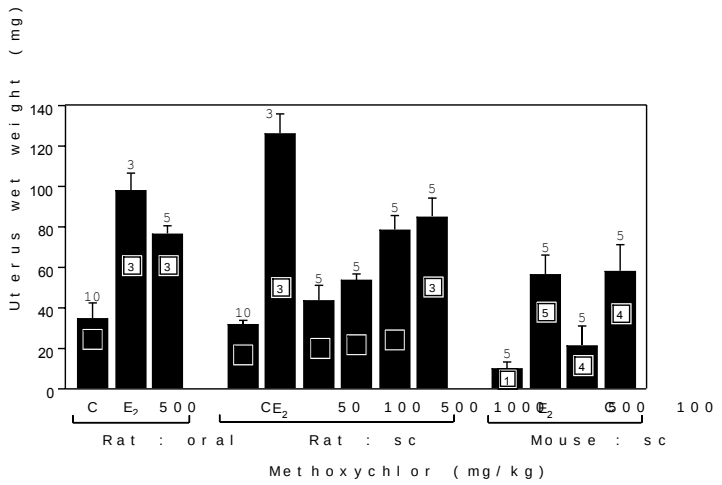
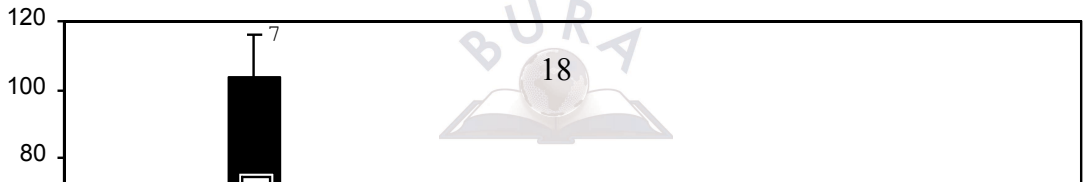


FIGURE 10. The effects of oral and sc administration of methoxychlor on uter immature rats and mwiacse .usEesdt raa dpi oostgi/tki5v, e ocroanlt rgodalvg(g4eGo ras50appropriate). Control rats received arachis oil ly and contro mice receivedcorn oil one animal with open vagina (number of open vaginas shown in theopen box) above the bar represents the group size.



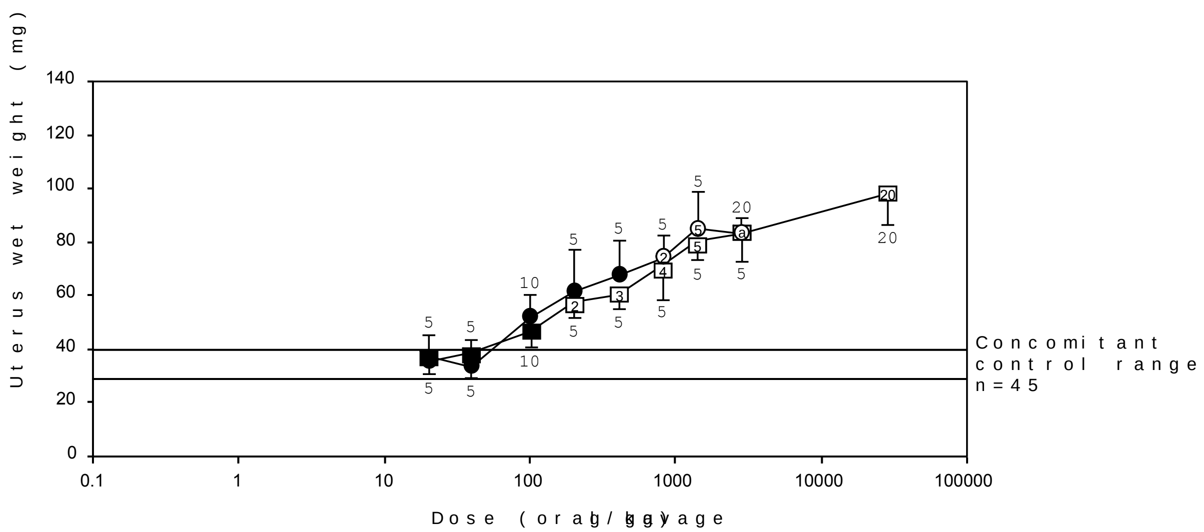
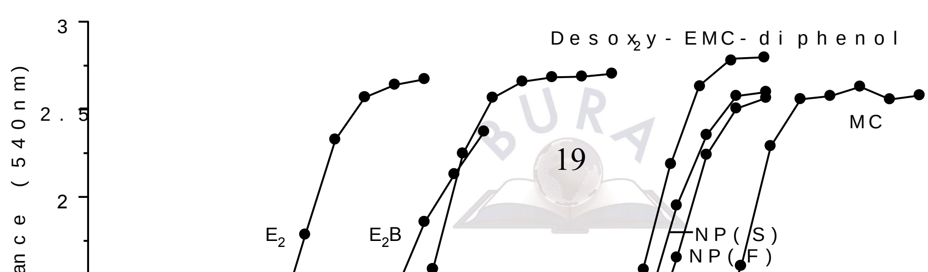


FIGURE 14. The effect of desoxy-■ radiol (●) and its benzoate derivative ( )on ut
immature rats. Controls received arachis oil only. The scale allows comparison w
denote animal with open vagina, open symbols denote at least one animal with open
within the data point: a 5/5 for both, 1/5 for E_2B with open vagina. Data represent group means Ñ
data shown represent the results of 8 studies and E_2B with desoxy-E was accompanied by
positive and negative control groups (positive control data not shown). The number
group size.



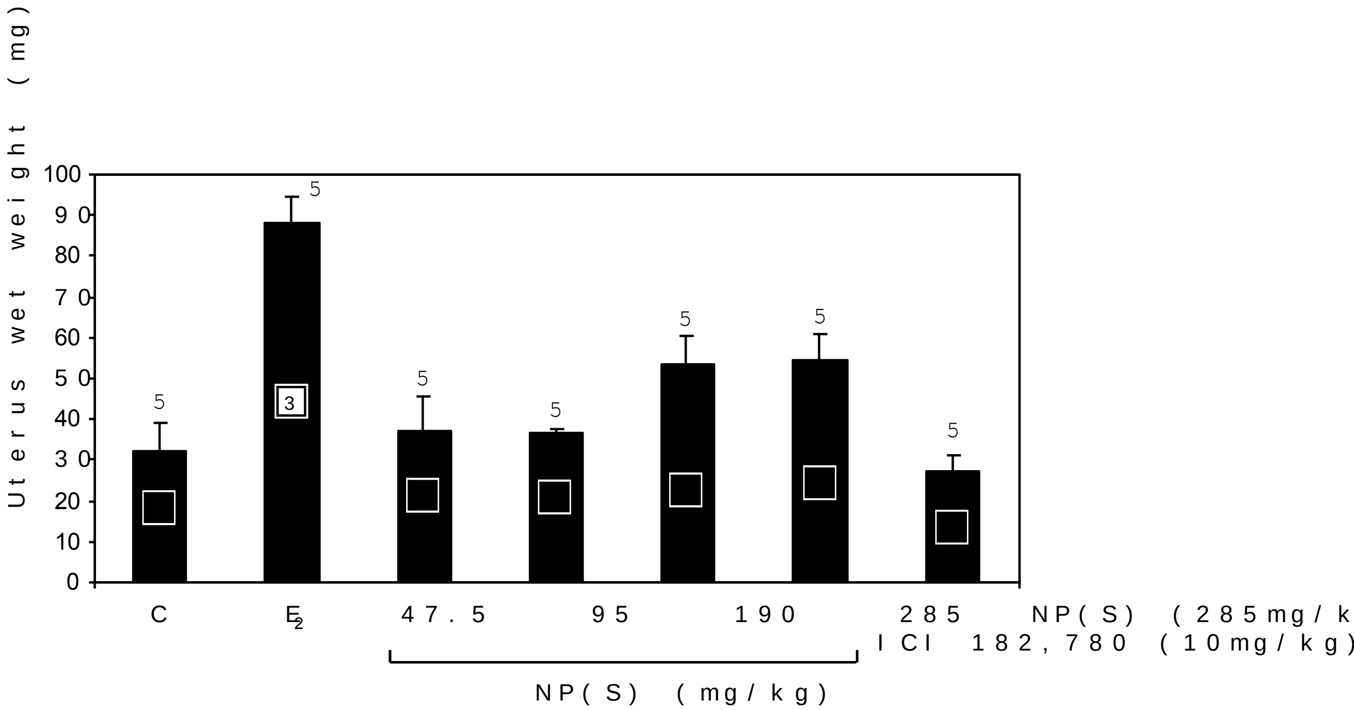


FIGURE 11. The effects of oral administration of branched sample; NP(S)] on uterus wet weight and vaginal opening E2 (400 µg/kg, oral gavage) was used a positive control. Contro hand side bar represents data for the addition of ICI 18 285mg/kg of NP(S) (the maximum tolerated dose level). least one animal with open vagina (number of open vagina represent group means ±SD. The number above the bar repr

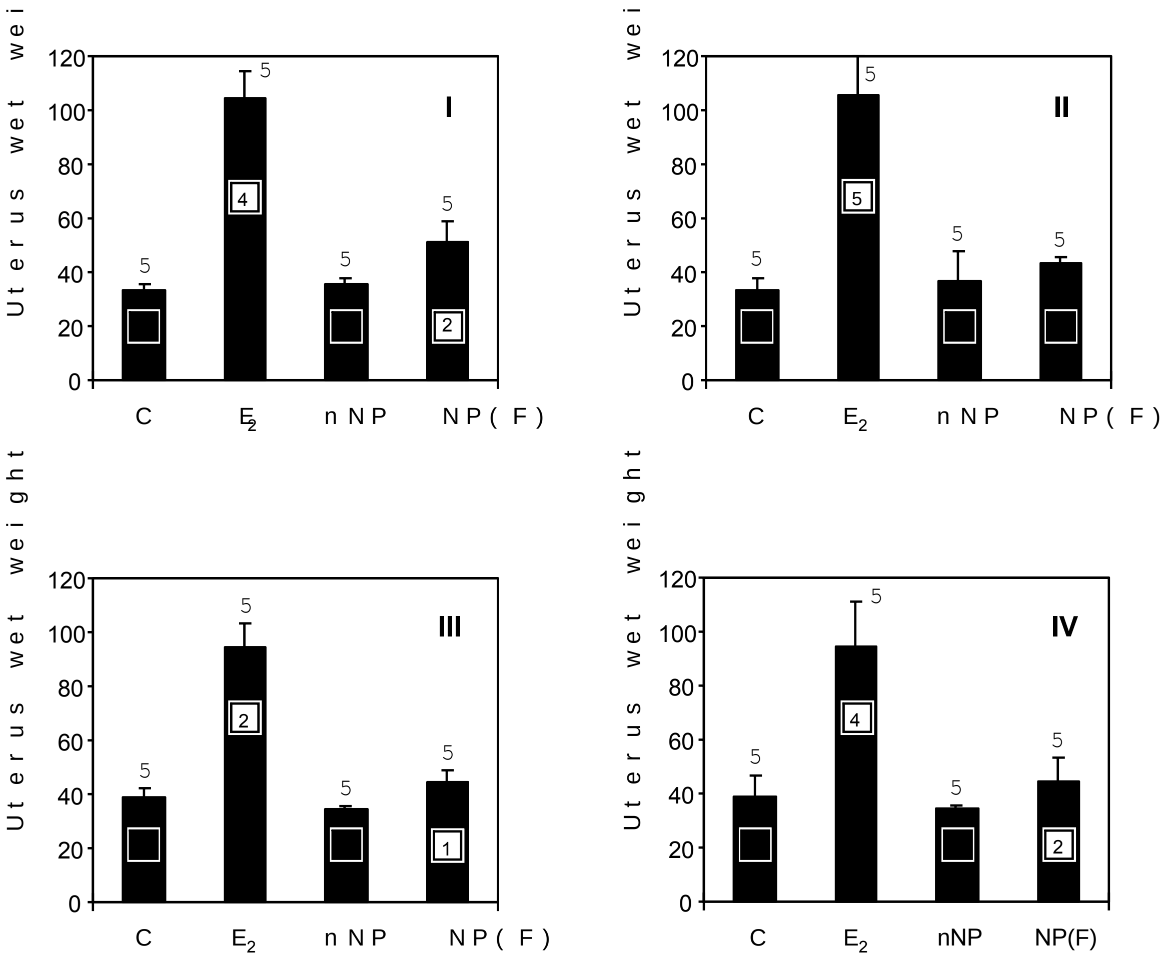


FIGURE 12. The effects of the Fluka sample of branched nonyl (nNP) on uterus wet weight and vaginal opening in 4 indep samples were administered by oral gavage at the maximum Estradiol (E2) (400 µg, oral gavage) was used as a positive contro only. No animal with open vagina, at least o vaginas shown in the box). Data represent group means ±SD. The number above t represents the group size.