Enhancing Effects of β-Estradiol 3-Benzate but Not Methoxychlor on the Promotion/Progression Stage of Chemically-induced Mammary Carcinogenesis in Ovariectomized Rats

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Modifying effects of β-estradiol 3-benzoate (EB) and methoxychlor (MXC), a pesticide which possesses weak estrogenic activity, on 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary carcinogenesis were investigated in ovariectomized or intact female Sprague-Dawley rats. Twenty-eight weeks after a single DMBA (100 mg/kg body weight) initiation, when the incidence of mammary tumor-bearing rats had reached 75%, a number of the animals were subjected to ovariectomy in order to obtain 3 groups: i) tumor-bearing, ovariectomized group; ii) tumor-bearing, intact group; iii) no-tumor, ovariectomized group. Subsequently animals of each group were subjected to subcutaneous implantation of 0.5 mg EB or given diet containing 1000 ppm MXC for 13 weeks. Although the incidences, multiplicities and volumes of the palpable tumors gradually decreased after ovariectomy, EB treatment stimulated tumor growth in the tumor-bearing, ovariectomized group thereafter. A similar effect of EB treatment was also observed in the no-tumor, ovariectomized group. However, MXC did not show any effect in the tumor-bearing, or no-tumor ovariectomized groups, except that the multiplicity of tumors was significantly decreased by MXC treatment in the tumor-bearing, intact group. The results of our study suggest that MXC has no promotion/progression effect, but rather possesses a weak inhibitory effect, whereas the strongly estrogenic substance EB clearly enhanced DMBA-induced mammary tumorigenesis.

Key words: Mammary carcinogenesis — β-Estradiol 3-benzoate — Methoxychlor — Rat

Endocrine-disrupting effects of environmental xenoestrogens in humans and animals have become an important issue. In the mammary glands, xenoestrogens have been reported to alter glandular differentiation and epithelial proliferation. Since natural and synthetic estrogens have been shown to be potent modifiers of breast cancer in humans, related risk assessment of endocrine disrupting chemicals is clearly a high priority. Methoxychlor (MXC) is a pesticide derived as the methoxylated isomer of dichlorodiphenyltrichloroethane (DDT), and is thought to be less toxic. MXC is approved for use on agricultural crops and livestock, and humans could be exposed to it. MXC is approved for use on agricultural crops and livestock, and humans could be exposed to it.6) Estrogenic effects of MXC have been shown in numerous in vivo rodent studies, impacting on the development of the female reproductive organs in rats; and increasing prolactin release in male rats; uterine epithelial height in ovariectomized mice; uterine weight in immature mice and number of epidermal growth factor receptors in the uteri of immature female rats. MXC itself has weak affinity for estrogen receptor (ER) α and β, and its metabolite 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is considered to be the principal active metabolite, because it shows agonistic activity to ERα with an affinity about 100-fold higher than that of MXC, and 15-fold lower than that of 17β-estradiol. Therefore, in vivo estrogenic activity of MXC is generally considered to be derived from HPTE. Stresser and Kupfer showed the presence in human hepatic microsomes of enzymes that convert MXC to estrogenic metabolites, and they suggested the compound to have estrogenic potency in humans as it does in animals. With regard to its carcinogenic potential, MXC has been classified as a Group 3 agent by the International Agency for Research on Cancer, while DDT could be associated with a breast cancer risk in humans. There is some evidence that MXC may induce mammary tumors in female rats, but further studies are needed to clarify whether MXC is indeed carcinogenic for the mammary glands in rodents and human beings.

Ovariectomy (OVX) removes the promoting effects of endogenous estrogens on mammary gland carcinogenesis. For accessing the impact of estrogenicity of endocrine-disrupting chemicals, ovariectomized female rats are necessary, since they are more sensitive to estrogenic effects than their intact counterparts. It has been shown

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that β-estradiol 3-benzoate (EB) is a potent synthetic estrogen which enhances the growth of breast tumors induced by chemicals or γ-rays in rodents.21, 22)

In the present study, we examined the modifying effects of EB and MXC on development of 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in the promotion or progression stage using both intact and ovariectomized female rats.

MATERIALS AND METHODS

Animals and housing One hundred and sixty female Sprague Dawley rats were purchased at 5 weeks of age from Charles River Japan Co., Ltd. (Yokohama). Through the acclimatization and experimental periods, animals were housed at a maximum of 5 to a plastic cage with absorbent hardwood bedding (White Flakes, Charles River, Inc., Tokyo) in an air-conditioned animal room (room temperature, 24±2°C; relative humidity, 60±10%; lighting cycle, 12-h light/12-h dark). All animals were transferred to clean cages with fresh bedding twice a week. The rats were quarantined for 2 weeks and only those without any abnormal findings at the end of this acclimatization period were selected for the experiment. Powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo) and tap water via automatic stainless steel nozzles were freely available throughout the study. This study was carried out in accordance with the Guide for Animal Experimentation, National Institute of Health Sciences, Japan.

Chemicals DMBA (purity: ≥95.0%) and MXC (purity: ≥95.0%) were purchased from Sigma Chemical Co. (St. Louis, MO), and EB (purity: 97.0–103%) and cholesterol from Wako Pure Chemical Industries, Ltd. (Osaka). EB-containing pellets were prepared by mixing 1000 mg of cholesterol and 1.0 ml of olive oil with 33 mg of EB and introducing the mixture into medical-grade silicone tubes which were then cut into segments. The EB content in each pellet was approximately 0.5 mg.

Experimental design The experimental design is outlined in Fig. 1. All female rats received an intragastric intubation of 100 mg/kg body weight of DMBA (10 mg/ml sesame oil) at the age of 7 weeks. Twenty eight weeks thereafter, they were divided into 3 groups: tumor-bearing ovariectomized; tumor-bearing intact; and no tumor ovariectomized. Each group consisted of 3 subgroups; receiving no (basal diet + vehicle pellet), EB (basal diet + EB pellet), or MXC treatment (1000 ppm MXC in diet + vehicle pellet) for 13 weeks. DMBA (100 mg/kg, i.g.), ovariectomy, basal diet, basal diet + vehicle pellet, basal diet + EB (0.5 mg) pellet, MXC (1000 ppm) + vehicle pellet.

Table I. Survival Rate, Body Weight and Number of Regressing Palpable Tumors in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

| Tumors at the start of dosing | Ovariectomy | Treatment | Survival rate (%) | Body weight | No. of regressed tumors (%) |
|-----------------------------|------------|-----------|-------------------|-------------|---------------------------|
| Bearing Subjected           | Control    | 8/10 (80) | 395.8±57.1        | 15/24 (63)  |
|                             | EB         | 17/20 (85)| 322.0±55.6**     | 21/48 (44)  |
|                             | MXC        | 15/20 (75)| 288.6±17.8**     | 24/40 (60)  |
| Bearing Intact              | Control    | 5/8 (63)  | 348.1±35.1        | 0/16 (0)    |
|                             | EB         | 4/9 (44)  | 323.6±33.9        | 4/19 (21)   |
|                             | MXC        | 5/8 (63)  | 295.8±41.8        | 8/16 (50)** |
| None Subjected              | Control    | 12/13 (92)| 422.4±73.0        | —           |
|                             | EB         | 15/15 (100)| 314.8±29.3**   | —           |
|                             | MXC        | 13/14 (93)| 291.0±21.7**     | —           |

* P<0.05, ** P<0.01.
the age of 7 weeks. Animals were checked every day and individual body weights were determined every week. When the incidence of palpable tumor-bearing animals reached 75% (28 weeks after the administration of DMBA), 50 tumor-bearing and 42 no-tumor rats were ovariectomized under ether anesthesia (32 rats died of mammary tumors before OVX). The tumor-bearing, ovariectomized rats [Tumor(+)-OVX group] were divided into 3 subgroups of 10, 20 and 20 animals for no, EB and MXC treatment, respectively. No-tumor, ovariectomized animals [Tumor(−)-OVX group] were also divided into 3 subgroups of 13, 15 and 14 animals, respectively. The remaining 26 tumor-bearing rats [Tumor(+)-intact group] were similarly divided into subgroups of 8, 9 and 9 animals. EB and MXC were given for 13 weeks. The control and EB subgroups were allowed free access to powdered

Fig. 2. Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for the tumor-bearing ovariectomized rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

Fig. 3. Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for the tumor-bearing intact rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

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basal diet throughout the administration period. EB pellets were implanted in the subcutis of the interscapular area under light anesthesia with ether and replaced every 4 weeks. For the control and MXC subgroups, pellets containing only cholesterol and olive oil were implanted. MXC was mixed into powdered basal diet at a concentration of 1000 ppm for ad libitum consumption. The location, number and size (long and short diameters) of subcutaneous thoracic and abdominal tumors of all animals were examined by palpation and recorded weekly throughout the EB or MXC-treated period. The tumor volumes were calculated as follows:

\[
\text{Tumor volume} = \frac{(\text{long diameter})^2 \times (\text{short diameter})}{2}
\]

After the end of the 13-week administration period, the surviving animals were killed by exsanguination from the posterior vena cava under ether anesthesia and subjected to autopsy.

**Histopathology** Mammary tumors, other grossly abnormal lesions and major organs including the uterus, vagina,

**Fig. 4.** Sequential changes in body weight (A), incidence of palpable mammary tumor-bearing rats (B), mean number of tumors per rat (C), and mean tumor volume (D) for no-tumor ovariectomized rats. ● control, ■ β-estradiol 3-benzoate, ▲ methoxychlor.

**Table II.** Incidence, Volume, Multiplicity and Histological Classification of Mammary Tumors in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

| Tumors at the start of dosing | Ovariectomy | Treatment | Incidence of tumor-bearing rats (%) | Multiplicity (Number of tumors/rat) | Tumor volume (cm³/tumor) | Tumor type (%) |
|-------------------------------|-------------|-----------|-----------------------------------|------------------------------------|-------------------------|---------------|
| Bearing                       | Subjected   | Control   | 4/8 (50)                           | 1.00±0.05                          | 12.28±13.52             | 2/8 (25)       |
|                               |             | EB        | 17/17 (100)**                      | 4.77±8.28                          | 14.26±31.76             | 7/90 (8)       |
|                               |             | MXC       | 13/15 (87)                         | 1.80±1.65                          | 6.80±16.26*             | 13/27 (48)     |
|                               | Intact      | Control   | 5/5 (100)                          | 5.80±4.90                          | 6.70±9.22               | 2/28 (7)       |
|                               |             | EB        | 4/4 (100)                          | 6.50±8.43                          | 13.18±15.41             | 3/26 (11)      |
|                               |             | MXC       | 5/5 (100)                          | 2.00±1.22                          | 13.70±15.77             | 1/10 (10)      |
| None                          | Subjected   | Control   | 1/12 (8)                           | 0.17±0.39                          | 1.38±1.24               | 0/2 (0)        |
|                               |             | EB        | 14/15 (93)**                       | 3.80±2.40                          | 5.61±13.97              | 10/57 (17)     |
|                               |             | MXC       | 5/13 (39)                          | 0.38±0.51                          | 3.00±5.32               | 2/5 (40)       |

* P<0.05, ** P<0.01.

a) Adenoma, fibroadenoma, fibroma.
pituitary gland and ovaries (except for ovariectomized animals) were fixed in 10% neutral buffered formalin. The mammary tumors and these organs were processed routinely, embedded in paraffin, sectioned at 4–5 µm and stained with hematoxylin and eosin (H-E) for microscopic examination.

**Statistical analysis** The survival rates, number of regressing tumors after OVX, and incidences of mammary tumors and other histological lesions were analyzed for inter-group differences by using Fisher’s exact test. Body weights were analyzed using Student’s t test. Multiplicity and volume of mammary tumors were compared with the Mann-Whitney U test.

**RESULTS**

**Mortality and body weight changes** One MXC-treated animal in the Tumor(+)-intact group which died of a mammary tumor after 3 days of OVX was excluded from the evaluation. The survival rates at the termination were 75–85%, 44–63% and 92–100% in the Tumor(+)-OVX, Tumor(+)-intact and Tumor(−)-OVX groups, respectively, and there were no significant differences among control, EB and MXC subgroups within each group (Table I). The cause of death in almost all cases of mortality was mammary tumor-related. Pituitary tumors were also observed in 4 of 5 dead animals in the Tumor(+)-intact-EB group. In the Tumor(+)-OVX and Tumor(−)-OVX groups, body weights of EB or MXC-treated rats were significantly (P<0.01) lower than those of the controls (Figs. 2A, 4A, Table I). Also in the Tumor(+) intact group, body weights showed a tendency for decrease in the MXC subgroups, but this did not reach statistical significance (Fig. 3A, Table I).

**Sequential changes of palpable tumors** Incidences, multiplicities and volumes of palpable tumors gradually regressed after OVX in the Tumor(+)-OVX groups in the initial few weeks (Fig. 2, B, C and D). EB treatment seemed to inhibit these OVX effects on incidence and mean volume. However, examination of individual tumors indicated that EB treatment did not significantly affect the disappearance of tumors (Table I). On the other hand, remaining tumors obviously grew under EB treatment, resulting in a continuous increase in mean volume (Fig. 2D). In the last half of the dosing period, the mean number of tumors also gradually increased in the EB treatment subgroup. In the Tumor(−)-OVX group, the incidence, multiplicity and volume of palpable tumors gradually increased in the EB treatment subgroup from the middle of the treatment period (Fig. 4, B, C and D). In the Tumor(−)-intact group, EB treatment caused no clear change (Fig. 3, B, C and D). In the MXC treatment subgroups, no clear changes were observed in the Tumor(+) OVX group (Fig. 2, B, C and D, Table I). In the Tumor(+) intact group, no tumors disappeared throughout the experimental period in the control subgroup, whereas MXC treatment induced statistically significant (P<0.01) regression (Fig. 3B, Table I). MXC treatment also inhibited the development of palpable tumors (Fig. 3C). In the Tumor(−)-OVX group, MXC treatment increased the vol-

![Graph](image-url)
ume of palpable tumors in the Tumor(−)-OVX group (Fig. 4D), which was considered not to be significant, since the small number of larger tumors affected the data. **Histopathological findings for mammary tumors** At the terminal kill, histologically defined mammary tumors were classified as benign lesions including adenomas, fibroadenomas and fibromas and malignant adenocarcinomas (Table II, Fig. 5). The incidence and multiplicity of mammary tumors were significantly elevated and apparent volume increase in several adenocarcinomas was observed in the EB treatment subgroup of the Tumor(+)−OVX group. EB treatment also increased the incidence, volume and multiplicity of mammary tumors in the Tumor(−)-OVX group. In the MXC treatment subgroups, such enhancing effects were not observed. MXC treatment significantly decreased the number of mammary tumors compared to the corresponding control in the Tumor(+)−intact group. A similar change was observed in the Tumor(+)−OVX group, though the multiplicity was increased compared to the control subgroup, and this volume change was

| Tumors at the start of dosing | Ovariectomy Treatment | Ovary | Uterus | Vagina | Epithelial thickening/mucinification (%) |
|------------------------------|------------------------|-------|--------|--------|----------------------------------------|
|                              |                        | Atrophy (%) | Atrophy (%) | Hypertrophy (%) | Atrophy (%) |                                    |
| Bearing Subjected            | Control                | —     | 8/8 (100) | 0/8 (0) | 8/8 (100) | 0/8 (0)                                |
|                              | EB                     | 0/16 (0)** | 16/16 (100)** | 0/16 (0)** | 1/16 (6) |                                        |
|                              | MXC                    | 0/15 (0)** | 15/15 (100)** | 0/15 (0)** | 13/15 (87)** |                                    |
| Bearing Intact               | Control                | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0)                                |
|                              | EB                     | 4/4 (100)** | 4/4 (100)** | 0/4 (0) | 4/4 (100)** |                                        |
|                              | MXC                    | 5/5 (100)** | 5/5 (100)** | 0/5 (0) | 1/5 (20) |                                    |
| None Subjected               | Control                | 12/12 (100) | 0/12 (0) | 12/12 (100) | 0/12 (0) |                                     |
|                              | EB                     | 0/13 (0)** | 13/13 (100)** | 0/13 (0)** | 2/13 (15) |                                        |
|                              | MXC                    | 0/13 (0)** | 13/13 (100)** | 0/13 (0)** | 7/13 (54)** |                                    |

| — not examined.             |                        |       |        |        |                                        |

*P < 0.05, **P < 0.01.

### Table III. Histopathological Findings for Reproductive Organs in DMBA-initiated Female Rats after 13-Week Dosing with β-Estradiol 3-Benzoate (EB) or Methoxychlor (MXC)

| Tumors at the start of dosing | Ovariectomy Treatment | Ovary | Uterus | Vagina | Pituitary Castration cells (%) | Adenoma (%) | Adrenal Necrosis (%) |
|------------------------------|------------------------|-------|--------|--------|--------------------------------|--------------|---------------------|
|                              |                        |       |        |        | + | ++ |                   |               |                    |
| Bearing Subjected            | Control                | 0/6 (0) | 6/6 (100) | 0/6 (0) | 7/8 (87) |
|                              | EB                     | 0/17 (0) | 0/17 (0)** | 12/16 (75)** | 15/16 (94) |
|                              | MXC                    | 12/15 (80)** | 0/15 (0)** | 14/15 (93) |                         |
| Bearing Intact               | Control                | 0/5 (0) | 0/5 (0) | 0/5 (0) | 5/5 (100) |
|                              | EB                     | 0/4 (0) | 0/4 (0) | 4/4 (100)** | 3/3 (100) |
|                              | MXC                    | 0/4 (0) | 0/4 (0) | 0/4 (0) | 5/5 (100) |
| None Subjected               | Control                | 0/13 (0) | 12/13 (92) | 0/13 (0) | 10/12 (83) |
|                              | EB                     | 0/15 (0) | 0/15 (0)** | 14/15 (93)** | 15/15 (100) |
|                              | MXC                    | 10/12 (83)** | 0/12 (0)** | 0/12 (0) | 12/12 (100) |

+ slight, ++ moderate.  
*P < 0.05, **P < 0.01.
Histopathological findings for other organs As shown in Table III, hypertrophy of the uterine epithelium and thickening/mucinification of the vaginal epithelium were observed in the EB and MXC subgroups of each group. Furthermore, both treatments induced ovarian atrophic changes such as decrease or absence of corpora lutea in intact animals. In the pituitary, ‘castration cells,’ which are generally observed in the pituitary of castrated animals, were frequently observed in ovariec tomized animals, and the frequency and severity were relatively low in MXC-treated subgroups compared to the corresponding controls. In the EB-treated subgroups, pituitary adenomas were frequently observed in ovariectomized animals, and the breast while acting as agonists in bone and the uterus in the case of tamoxifen. Pre viously MXC was found to have antiestrogenic effects on the ovary, but an estrogenic influence on the uterus and oviduct in mice. HPTE, the principal active metabolite of MXC, exhibits agonistic activity against ERα in contrast to antagonism of ERβ in estrogen-responsive promoter-transfected HepG2 and HeLa cells. The available reports suggest that MXC could be a SERM and it may be antagonistic in mammary tissue, like tamoxifen or raloxifen. Furthermore, MXC can increase the concentrations of mRNAs for two estrogen-responsive proteins, lactoferrin and glucose-6-phosphate dehydrogenase, in the uterus of mice by an unknown mechanism that is not mediated through ERα or β. Further investigations, including determination of the levels of such estrogen-responsive mRNAs in mammary tumors, are needed to elucidate the mechanisms of MXC-induced tumor suppression.

In conclusion, the present study indicated that subcutaneous application of 0.5 mg EB pellet results in promoting or progressing effects on DMBA-induced mammary tumors, under ovariec tomized conditions. In contrast, 1000 ppm MXC in the feed was without influence after ovariec tom y, while showing suppressive effects when ovarian hormones were present.

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