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Prevention of mammary carcinogenesis by short-term estrogen and progestin treatments

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Abstract

Introduction Women who have undergone a full-term pregnancy before the age of 20 have one-half the risk of developing breast cancer compared with women who have never gone through a full-term pregnancy. This protective effect is observed universally among women of all ethnic groups. Parity in rats and mice also protects them against chemically induced mammary carcinogenesis.

Methods Seven-week-old virgin Lewis rats were given *N*-methyl-*N*-nitrosourea. Two weeks later the rats were treated with natural or synthetic estrogens and progestins for 7–21 days by subcutaneous implantation of silastic capsules.

Results In our current experiment, we demonstrate that short-term sustained exposure to natural or synthetic estrogens along with progestins is effective in preventing mammary carcinogenesis in rats. Treatment with 30 mg estriol plus 30 mg progesterone for 3 weeks significantly reduced the incidence of mammary cancer. Short-term exposure to ethynyl estradiol plus megesterol acetate or norethindrone was effective in decreasing the incidence of mammary cancers. Tamoxifen plus progesterone treatment for 3 weeks was able to confer only a transient protection from mammary carcinogenesis, while 2-methoxy estradiol plus progesterone was effective in conferring protection against mammary cancers.

Conclusions The data obtained in the present study demonstrate that, in nulliparous rats, long-term protection against mammary carcinogenesis can be achieved by short-term treatments with natural or synthetic estrogen and progesterone combinations.

Keywords: cancer, estrogen, mammary, prevention, progestin

Introduction

Breast cancer is one of the most prevalent types of cancer in women especially in the United States and other Western countries. The risk of developing breast cancer is reduced by 50% in women who have undergone a full-term pregnancy by the age of 20, as compared with nulliparous women [1–3]. This phenomenon of parity protection against breast cancer is observed universally among women from all ethnic groups. The protective effect of parity is not only observed in humans, but is also found in rats and mice [4–9].

The mechanism involved in parity protection against breast cancer is still not defined. Understanding the mechanism would help in developing strategies for the prevention of breast cancer. Several studies have supported the hypothesis that the mammary glands in parous rats have decreased proliferation, have higher capacity to repair DNA, have lower binding of carcinogen, and are more differentiated as compared with the mammary gland of age-matched virgin rats [10–13]. On the contrary, other studies have found no difference in the structure, in the proliferative activity, or in carcinogen binding to DNA of

mammary cells between parous rats and age-matched virgin rats [5,6].

During pregnancy, several hormones in a cohort cause proliferation and differentiation of the mammary gland. There is a dramatic increase in the levels of circulating estrogens, progesterone, prolactin, growth hormone and placental lactogens. As a consequence of these hormonal exposures, the mammary gland proliferates and differentiates in preparation for lactation [14]. At the end of pregnancy, the mammary gland under the influence of lactogenic hormones becomes fully lactational. After weaning of the offspring, the highly differentiated lobuloalveolar structures subsequently involute as a result of the decrease in lactogenic hormones [14].

Pregnancy before or soon after exposure to a chemical carcinogen is protective against mammary cancers in rodents. Russo and colleagues [10–13] have suggested that the protective effect is due to the differentiation of target structures during pregnancy. Others have reported that there are persistent alterations in the levels of circulating hormones in parous women and rats compared with their respective age-matched controls. The blood level of prolactin is reduced in parous women [15,16]. Thordarson and colleagues [17] have reported a decrease in the circulating concentration of growth hormone in parous rats. They have also demonstrated that mammary glands of parous rats have decreased levels of estrogen receptors and epidermal growth factor receptors compared with age-matched virgin rats. These persistent alterations might be involved in refractoriness to mammary carcinogenesis in parous rats.

Administration of high doses of estradiol and progesterone in combination [18–23] or of human chorionic gonadotropin [24] before or after carcinogen treatment was protective against mammary carcinogenesis in rats. The widely accepted explanation for hormone-induced refractoriness to mammary carcinogenesis is that either the target cells for cancer in the mammary gland are altered to a nonsusceptible state by early hormone treatment [23], or that the initiated cells are differentiated following carcinogen treatment [20,22] or are killed following hormone treatment [24]. We have recently demonstrated that short-term treatment with high pregnancy levels of estradiol with or without progesterone is highly effective in decreasing the mammary cancer incidence [25,26]. There are also reports implicating alteration in gene expressions as an explanation for the protective effect of pregnancy or the protective hormone treatments against mammary cancer [27–30].

The objective of the present study was to examine whether different kinds of natural and/or synthetic estrogens and progestins at different doses and durations would modify the incidence of mammary carcinogenesis following exposure to *N*-methyl-*N*-nitrosourea (MNU). We tested estriol alone or in combination with progesterone because it is known that estriol levels increase during pregnancy [31]. We also used the synthetic compounds (ethynyl estradiol in combination with megesterol acetate or norethindrone). These compounds are used in combination oral contraceptives, and the risk of uterine and ovarian cancers are reduced by almost 50% in women using contraceptives containing these compounds [32,33]. Moreover, oral contraceptives do not confer an increased breast cancer risk [34].

We also wanted to test the protective effect, if any, of short-term treatment with tamoxifen combined with progesterone. Long-term treatment with tamoxifen is used as a hormonal therapy for breast cancers [35]. The estrogen metabolites, 2-hydroxy estradiol and 2-methoxy estradiol, which have been reported to inhibit angiogenesis and tumor cell growth [36], were administered alone or in combination with progesterone. Since it is known that estradiol metabolites bind with low affinity to estradiol receptors [37–39] and they are less potent estrogens [40,41] than the parent compound, we attempted to determine whether a weak estrogen alone or in combination with progesterone would be able to mimic the protective effect of pregnancy protection against mammary cancers. We hypothesized that short-term treatment with different estrogens alone or in combination with progestins would be able to mimic the protective effect of pregnancy and to reduce the incidence of mammary carcinogenesis in these treated rats in comparison with untreated controls following exposure to MNU.

Materials and methods
Animals
Virgin Lewis rats were purchased from Harlan Sprague Dawley (Indianapolis, IN, USA and San Diego, CA, USA). The rats were housed in a temperature-controlled room with a 12-hour light and 12-hour dark schedule. They were given food (Teklad, Madison, WI, USA) and water *ad libitum*.

Carcinogen treatment
A single intraperitoneal injection of MNU (Ashe Stevens, Detroit, MI, USA) at a dose of 50 mg/kg body weight was given to all rats at 7 weeks of age. MNU was dissolved in physiological saline that had been adjusted to pH 5.0 [42].

Hormone treatment
All the hormone treatments in the following experiments were given 2 weeks after the administration of the carcinogen. The hormones, natural or synthetic, were packed in individual silastic capsules (0.078 inch ID × 0.125 inch OD, 2 cm long; Dow Corning, Midland, MI, USA). Rats that received a combination hormone treatment were implanted with individual capsules containing the respec-

tive hormone. The hormones were packed in the silastic capsules in a cellulose matrix, except for the 30 mg dose that was packed with the hormone alone. The control group received empty silastic capsules. All silastic capsules were implanted subcutaneously dorsally. There were 10–13 rats in the different hormone treatment groups.

Effects of natural and synthetic estrogens and progestins on the prevention of mammary carcinogenesis after exposure to MNU

Effect of estriol plus or minus progesterone on MNU-induced mammary carcinogenesis
When the rats were 9 weeks old they were divided into five groups, receiving the following treatments: (1) control, (2) 30 mg estriol, (3) 200 μg estriol (Sigma, St Louis, MO, USA), (4) 30 mg estriol plus 30 mg progesterone (Sigma), and (5) 200 μg estriol plus 30 mg progesterone in silastic capsules. Each treatment was continued for 1 or 3 weeks.

Effect of ethynyl estradiol plus megesterol acetate or norethindrone on mammary carcinogenesis
Rats were implanted with silastic capsules containing 100 μg ethynyl estradiol (Sigma) and 30 mg megesterol acetate (Sigma) or norethindrone (Sigma). Treatment with 100 μg ethynyl estradiol plus 30 mg megesterol acetate was given for both 1 and 3 weeks. Treatment with 100 μg ethynyl estradiol plus norethindrone was given for 1 week.

Effect of tamoxifen plus progesterone on MNU-induced carcinogenesis
Rats were implanted with silastic capsules containing 30 mg tamoxifen (Sigma) and 30 mg progesterone. The silastic capsules were removed after 3 weeks of treatment.

Effect of 2-hydroxy estradiol or 2-methoxy estradiol with or without progesterone on mammary carcinogenesis induced by MNU
The rats were divided into five groups and were treated as follows: (1) control, (2) 2 mg 2-hydroxy estradiol (Steraloids, Newport, RI, USA), (3) 2 mg 2-hydroxy estradiol plus 30 mg progesterone, (4) 2 mg 2-methoxy estradiol (Steraloids), and (5) 2 mg 2-methoxy estradiol plus 30 mg progesterone.

Mammary carcinogenesis
Rats were palpated once every week after carcinogen exposure for 9 months to monitor for mammary cancer development. Histopathological examination was performed to confirm the carcinomatous nature of the palpable tumors.

Statistics
The effects of the different hormonal treatments were analyzed using the chi-square test for 2×2 contingency tables and the Student t test. $P < 0.05$ was considered significant.

Results
Effect of estriol alone or with progesterone on mammary cancer incidence and multiplicity
In the present study to determine whether estriol alone or in combination with progesterone could be protective against MNU-induced carcinogenesis, rats treated with 30 mg estriol plus 30 mg progesterone for 3 weeks had a significantly reduced incidence of mammary cancers (25%). The treated rats had an average of 0.4 cancers per rat 9 months after MNU injection, compared with control rats that had a high mammary cancer incidence of 80% and a cancer multiplicity of 1.7 cancers per rat. Treatments with estriol alone for 1 week at a dose of 30 mg (83%) or 200 μg (100%), or with 200 μg estriol plus 30 mg progesterone (75%) did not prevent MNU-induced mammary carcinogenesis. Even when the same doses were given for 3 weeks, the mammary cancer incidence was 64%, 75% and 82%, respectively. Mammary cancer multiplicity in these treatments was not significantly different from that of the controls (Table 1).

Effect of ethynyl estradiol plus megesterol acetate or norethindrone on mammary cancer incidence and multiplicity
The mammary cancer incidence (23%) and the average number of cancers per rat (0.2 cancers) were significantly lower in rats treated with ethynyl estradiol and megesterol acetate for 3 weeks compared with controls that had a mammary cancer incidence of 75% and had 1.5 cancers per rat. The same combination of hormones given for 1 week was effective in preventing mammary carcinogenesis for the first 6 months, when the mammary cancer incidence was only 8%, but by 9 months the incidence had increased to 38%. However, the average number of cancers per rat in the 1-week treatment group (0.4 cancers) was significantly lower than the controls. Treatment with ethynyl estradiol and norethindrone for 1 week was effective in drastically decreasing the mammary cancer incidence (25%) and the average number of cancers per rat (0.2 cancers) compared with the controls (Table 2).

Effect of tamoxifen plus progesterone on mammary cancer incidence and multiplicity
Treatment with tamoxifen and progesterone for 3 weeks was effective in preventing mammary carcinogenesis for the first 6 months after MNU injection, when the incidence reached 30%. Nine months after MNU injection the mammary cancer incidence was 58% and the multiplicity was 0.9, which was not significantly lower than the controls in cancer incidence or multiplicity (Table 2).

Effect of 2-hydroxy estradiol and 2-methoxy estradiol with or without progesterone on mammary cancer incidence and multiplicity
Administrations of estrogen metabolites alone or in combination with progesterone were ineffective in preventing

Table 1

Effect of estriol on mammary carcinogenesis

Treatment	Duration	Mammary cancer incidence	Rats with mammary cancer (%)	Average number of cancers per rat
Control	3 weeks	8/10	80	1.7 ± 0.4
30 mg estriol	1 week	10/12	83	1.6 ± 0.3
200 µg estriol	1 week	11/11	100	1.7 ± 0.3
30 mg estriol + 30 mg progesterone	1 week	7/12	58	1.2 ± 0.3
200 µg estriol + 30 mg progesterone	1 week	9/12	75	1.6 ± 0.3
30 mg estriol	3 weeks	7/11	64	1.0 ± 0.3
200 µg estriol	3 weeks	9/12	75	1.6 ± 0.3
30 mg estriol + 30 mg progesterone	3 weeks	3/12*	25	0.4 ± 0.2*
200 µg estriol + 30 mg progesterone	3 weeks	9/11	82	2.0 ± 0.4

Rats were treated with *N*-methyl-*N*-nitrosourea at 7 weeks of age. At 9 weeks of age, the rats were treated with hormones for 1 or 3 weeks. Control rats received empty silastic capsules. *$P < 0.05$ compared with controls.

Table 2

Effect of synthetic estrogens and progestins on mammary carcinogenesis

Treatment	Duration	Mammary cancer incidence	Rats with mammary cancer (%)	Average number of cancers per rat
Control	3 weeks	9/12	75	1.5 ± 0.3
100 µg eE + 30 mg megesterol	3 weeks	3/13*	23	0.3 ± 0.2*
30 mg tamoxifen + 30 mg progesterone	3 weeks	7/12	58	0.9 ± 0.3
100 µg eE + 30 mg megesterol	1 week	5/13	38	0.4 ± 0.1*
100 µg eE + 30 mg norethindrone	1 week	3/12*	25	0.2 ± 0.1*

Rats were treated with *N*-methyl-*N*-nitrosourea at 7 weeks of age. At 9 weeks of age, the rats were treated with hormones for 1 or 3 weeks. Control rats received empty silastic capsules. eE, 17α-ethynyl estradiol. *$P < 0.05$ compared with controls.

MNU-induced mammary carcinogenesis, except for the combination of 2-methoxy estradiol plus progesterone. Two milligrams of 2-hydroxy estradiol (80%) alone or in combination with 30 mg progesterone (60%) did not lower the mammary cancer incidence compared with the controls (75%). Treatment with 2 mg 2-methoxy estradiol alone for 3 weeks did not decrease the mammary cancer incidence (60%) compared with the controls. However, when 2 mg 2-methoxy estradiol was given along with 30 mg progesterone the mammary cancer incidence was lowered (30%) in comparison with the controls (Table 3). This treatment also lowered the average number of cancers per rat (0.4 cancers) compared with the controls (1.3). Even though 2-methoxy estradiol alone was not effective in decreasing the cancer incidence, it was effective in decreasing the average number of cancers per rat by almost 50% (0.7 cancers). Treatments with 2-hydroxy estradiol alone or in combination with progesterone did not affect cancer multiplicity compared with the controls (Table 3).

Discussion
The results of the present study show that short-term treatments with natural or synthetic ovarian steroids are effective in long-term protection against MNU-induced mammary carcinogenesis in female Lewis rats. It is well established that full-term pregnancy is very effective in preventing mammary carcinogenesis in humans, rats and mice [1–9]. We have previously demonstrated that 1 and 3 weeks of treatment with estradiol 17β with or without progesterone is highly effective in reducing the mammary cancer incidence and cancer multiplicity [25,26].

Lemon [43] demonstrated that administration of estriol inhibited 7,12-dimethylbenzanthracene-induced mammary carcinogenesis in rats. The data from our studies show that a high dose of estriol plus progesterone administered for 3 weeks provides long-term protection against mammary carcinogenesis. A similar combination given for 1 week was not effective in reducing mammary carcinogenesis. Estriol alone or at a lower dose in combination

Table 3

Effect of estrogen metabolites on mammary carcinogenesis

Treatment	Duration	Mammary cancer incidence	Rats with mammary cancer (%)	Average number of cancers per rat
Control	3 weeks	9/12	75	1.3±0.5
2 mg 2-hydroxy estradiol	3 weeks	8/10	80	1.4±0.3
2 mg 2-hydroxy estradiol + 30 mg progesterone	3 weeks	6/10	60	1.5±0.5
2 mg 2-methoxy estradiol	3 weeks	6/10	60	0.7±0.2
2 mg 2-methoxy estradiol + 30 mg progesterone	3 weeks	3/10*	30	0.4±0.2

Rats were treated with *N*-methyl-*N*-nitrosourea at 7 weeks of age. At 9 weeks of age, the rats were treated with hormones for 3 weeks. Control rats received empty silastic capsules. *$P < 0.05$ compared with controls.

with progesterone did not have a protective effect. The data suggest that high levels of estriol in combination with progesterone administered for 3 weeks is effective while a lower dose and a lower duration of treatment were ineffective in preventing mammary carcinogenesis, indicating that prevention of mammary cancer depends on the dose and duration of hormones used.

We demonstrated earlier that the pregnancy level of estradiol with or without progesterone is efficient in conferring protection against mammary cancers. Huggins and colleagues [18] and Grubbs and colleagues [20] previously reported that high levels of estradiol and progesterone given daily for 40 days, beginning 2 weeks after the administration of carcinogen, inhibited the appearance of mammary cancers in rats treated with 7,12-dimethylbenzanthracene or MNU. Huggins and colleagues suggested that the potential cancer cells were killed due to differential sensitivity to high levels of ovarian steroids, while Grubbs and colleagues suggested that the protection offered by ovarian steroids was due to differentiation of preneoplastic cells.

Russo and Russo [11,13] have shown that human chorionic gonadotropin (hCG) treatment also provided protection against mammary carcinogenesis. It has also been suggested that protective hormone treatments result in persistent changes in the intracellular pathways, which mediate proliferation responses to carcinogens [23]. Thordarson and colleagues [17] demonstrated that parous rats given carcinogen and followed by continuous treatment with estradiol and progesterone had a high cancer incidence. These data suggest that parous mammary epithelial cells do undergo transformation, but they have a reduced promotional environment. Short-term hCG treatment resulted in activation of several apoptotic genes like TRMP2, IL-1β converting enzyme, p53, c-*myc* and bcl-XS [27]. You and colleagues [44] suggested that hCG-conferred protection against mammary cancer was due to an increase in connexin 26, a tumor suppressor gene.

D'Cruz and colleagues have reported persistent downregulation of many growth-promoting factors (amphiregulin, pleiotrophin and insulin-like growth factor 1) and upregulation of genes involved in differentiation (whey acidic protein, caesins, lactoferrin) and in immune response (immunoglobulins, macrophage metalloelastase, macrophage expressed gene 1), and upregulation of transforming growth factor β3 and several of its transcriptional targets (clusterin, Eta, Id2) [28]. Short-term estradiol plus progesterone treatment persistently upregulated RbAp46 and G.B7, genes implicated in chromatin remodeling [29]. Medina and colleagues [30] attributed an increment in p53 gene expression as one of the mechanisms for hormone-induced protection.

Our studies with synthetic estrogenic and progestogenic compounds show that short-duration treatments are sufficient to prevent mammary carcinogenesis. Ethynyl estradiol and norethindrone administered for 1 week are highly effective in preventing the occurrence of mammary cancers. The latent period for the appearance of mammary cancers was very long as no mammary cancer appeared for the first 7 months after the administration of the carcinogen. A combination of 100 μg ethynyl estradiol and 30 mg megesterol acetate was able to prevent MNU-induced mammary carcinogenesis when given for 3 weeks. The same combination was less effective when administered for only 1 week. This indicates that the preventive effect of these compounds depends on the dose, the duration and the nature of treatments. Medroxyprogesterone acetate did not increase the risk of carcinoma development when administered to virgin rats at the clinical dose used for contraception. However, a 10-fold dose increase resulted in a higher tumorigenic response, while a similar dose of the mostly estrogenic northynodrel-mestranol was protective [45,46].

These synthetic hormones are used in oral contraceptives and have been found to decrease the incidence of uterine and ovarian cancers [33]. Previous studies have reported a slight increase or no increase in breast cancer risk in

women taking oral contraceptives [47,48]. It has recently been reported that oral contraceptives do not increase the risk of breast cancers [34]. In our study, we found that these synthetic hormones given for a short duration are capable of preventing mammary carcinogenesis. When we used tamoxifen plus progesterone treatment, there was a significant difference in mammary cancer incidence for the first 6 months. By the end of 9 months, however, there were no differences between the treated group and the control group of rats. This combination had some preventive effect on mammary cancers but it was a short-term effect. In previous studies, tamoxifen treatment given continuously prevents mammary carcinogenesis [35] and the short-term treatment, which we administered here, was not sufficient to prevent mammary cancers. Jordan and colleagues [49] also reported that short-term treatment with various doses of tamoxifen was not able to confer protection against chemical carcinogen-induced mammary carcinogenesis. Jordan [50] later suggested that tamoxifen appears to act as an inhibitor of the tumor cell cycle rather than as a tumoricidal agent in the rat model.

It has been reported that certain estrogen metabolites have a preventive effect in the process of mammary carcinogenesis [36]. It has been reported that 2-hydroxy estradiol is a potent inhibitor of tumor cell proliferation [51–53] and angiogenesis [52,53]. In addition, 2-methoxy estradiol also inhibits the growth of many cancer cell lines [51–53]. In the current study, we investigated the effects of two estrogen metabolites on mammary carcinogenesis (namely, 2-hydroxy estradiol and 2-methoxy estradiol) at a dose of 2 mg alone or in combination with 30 mg progesterone. Neither metabolite alone nor 2-hydroxy estradiol in combination with progesterone was effective in preventing mammary cancers. A combination of 2-methoxy estradiol and progesterone, however, was able to decrease the mammary cancer incidence. Both these estrogen metabolites bind to the estrogen receptor, but with a markedly reduced binding affinity as compared with estradiol [37–39]. Also, both these metabolites possess much weaker estrogenic potency than estradiol [40,41]. The reason for the lack of protective effect may be that the reduced binding affinity and weaker potency or the dose of the metabolites we used was not sufficient to produce a strong estrogenic effect.

Lamartiniere and colleagues [54] have shown that phytoestrogen genistein protects against mammary cancer. They also showed that the effect was dose dependent. Since genistein is a weak estrogen, higher doses were needed to achieve protection. It is clear from our data and from data of previous studies that a mid- to late-pregnancy level of estrogen alone or in combination with progesterone is necessary for the protective effect against chemically induced mammary cancers. The protective effect of estrogen plus progestin combinations, whether natural or synthetic, could be similar to parity-induced protection against mammary cancers. It has been shown that there is permanent decrease in the mammogenic hormones, growth hormone and prolactin in parous rats [18,55]. Parous women also have reduced blood levels of prolactin compared with age-matched nulliparous women [15,16]. The reduced levels of mammogenic hormones like prolactin and growth hormone resulting in a decrease in promotional environment and the altered sensitivity of the mammary glands to estradiol may also be factors involved in the protective effect against mammary carcinogenesis. Unlike many of the earlier studies where protection was achieved against mammary cancer by administration of high doses of estradiol plus progesterone for a longer duration, we have been able to demonstrate that administration of estrogens and progestins (natural or synthetic) for a short duration is as effective as high-dose combinations and long-duration treatments in preventing mammary cancers.

Conclusions
The overall data from the present study suggest that a variety of estrogenic compounds are able to protect against chemical-induced mammary carcinogenesis. Short-term treatment with low doses of strong estrogenic compounds in combination with progestins are highly effective in preventing mammary carcinogenesis. Our treatments for 1 week with synthetic estrogens and progestins indicate that treatments shorter than the length of gestation in rats will also be able to induce a long-term protection against mammary carcinogenesis. This would help in developing new strategies for prevention of mammary cancers using natural or synthetic hormones for a short duration.

Competing interests
None declared.

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