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Effects of Short-term Pregnancy Hormone Treatment against *N*-Methyl-*N*-nitrosourea-induced Mammary Carcinomas in Female Lewis Rats

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Abstract. *Aim: To study the effects of short-term pregnancy hormone treatment, estradiol and progesterone or prolactin, against pre-existing mammary cancer as well as newly developing mammary cancer by using the N-methyl-N-nitrosourea (MNU)-induced rat mammary cancer model. Materials and Methods: Three-week-old female Lewis rats (n=103) received an intraperitoneal injection of 50 mg/kg MNU. Nine weeks after the MNU injection, the rats received 3 weeks of hormone treatments, consisting of a subcutaneously implanted 0.5 mg estradiol- and 32.5 mg progesterone-containing pellet (E/P group, n=35) or four subcutaneous injections per week of 5 mg/kg perphenazine (PPZ group, n=38), a prolactin-releasing agent. The remaining rats did not receive hormones (control group, n=30). At 12 weeks of age (when the hormone treatments were started), 6 rats in each of the three groups had palpable tumors. These rats were sacrificed at 15 weeks of age, and the remaining rats were sacrificed at 19 weeks of age. The entire mammary glands and the palpable mammary tumors were histologically examined, and the development and growth of mammary tumors was compared. Results: Newly developing mammary tumors were suppressed and pre-existing ones regressed in the E/P group, while the development and growth of both newly developing cancers and pre-existing tumors were accelerated in the PPZ group as compared with the control group. Conclusion: Short-term, pregnancy levels of estradiol and progesterone (but not prolactin) may be the most effective treatment against MNU-induced mammary tumors in female Lewis rats.*

Breast cancer is one of the most common malignancies in women. It is estimated that each year there are more than one million new cases of breast cancer worldwide and 410,000 breast cancer-related deaths (1). The etiology of breast cancer is largely unknown. However, genetic susceptibility, environmental factors, and hormonal effects appear to be the major determinants. Many aspects of hormonal effects confer increased risk, and a relationship between female reproductive history and breast cancer risk has consistently been found as early as the 18th century, when Italian physician Bernardino Ramazzini recorded that breast cancer was found more frequently in nuns than in any other group of women (2). In the 1970s, MacMahon and co-workers reported that the inverse relationship between breast cancer risk and early full-term pregnancy was one of the most consistent findings in research regarding the etiology of human breast cancer (3). Subsequent research has continued to show that women who experience a full-term pregnancy at a young age have a substantially decreased risk of breast cancer (3-6). Women who had a full-term pregnancy when they were younger than 20 years have half the risk of developing breast cancer as nulliparous women. Lactation seems to be of marginal importance in breast cancer risk (7). Although early full-term pregnancy provides long-lasting protection against breast cancer, there is a transient increase in breast cancer risk during and soon after pregnancy (8). In short, pregnancy leads to an early increase in breast cancer risk followed by an overall later decrease in breast cancer risk.

Animal models play an important role in understanding the pathological events in and effective treatment of human diseases. Parity protection can be seen in the *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary cancer model (9). Young rats (≤3 months of age) that undergo a full-term pregnancy before, during or shortly after carcinogenic exposure have a significantly reduced overall mammary cancer incidence and multiplicity and have a prolonged latency (reviews: 10, 11). Although the overall incidence of mammary cancer is reduced when compared with nulliparous rats, pregnancy occurring after MNU or 7,12-dimethylbenz[α]anthracene (DMBA) exposure transiently increases the incidence of mammary cancer during the period of pregnancy and lactation (12, 13).

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During pregnancy and lactation, many hormones act in a complex pathway to cause proliferation and differentiation of the mammary gland, and these hormones may alter sensitivity to carcinogenic insult. The major hormones involved in this pathway include estradiol (EST), progesterone (PRG), and prolactin (PRL). Serum EST and PRG levels progressively increase during pregnancy and decrease after parturition, while serum PRL levels remain low during pregnancy, increase sharply on the day of parturition, and remain high during lactation (14). Although long-term EST and PRG treatment increases breast cancer risk (15-17), short-term treatment (equivalent to the gestational period of rats; approximately 21 days) with EST, alone or in combination with PGR, reduces the incidence and multiplicity of rat mammary carcinogenesis and prolongs its latency (10, 11). The short-term EST and PRG treatment protocol established by Huggins *et al*. (18) and followed by many other researchers has been consistently shown to reduce rat mammary carcinogenesis as effectively as pregnancy (11). EST alone at serum levels equivalent to those of pregnancy is effective at reducing mammary cancer risk, and the addition of PRG exerts stronger protective effects, whereas PRG alone is ineffective (19, 20). Neuroleptic drugs (dopamine antagonists) interfere with dopaminergic inhibition of PRL secretion and produce a persistent and marked increase in serum PRL levels, and long-term hyperprolactinemia is associated with mammary/breast carcinogenesis in rats and humans (21). However, in one study, short-term neuroleptic perphenazine (PPZ) exposure did not alter mammary cancer risk (19). In animal experiments related to parity protection, the interval between carcinogenic exposure and hormone treatment is usually short (*ca*. 2 weeks, before the development of palpable tumor) to see the effect of hormone treatment on newly developing mammary tumors and their growth. However, the effects of pregnancy hormones on pre-existing (macroscopically visible) mammary cancer has not been fully evaluated. Therefore, the goals of this study were: (i) to determine whether short-term pregnancy hormone treatment, EST and PRG or PRL, influences the growth of pre-existing tumors as well as newly developing tumors, and (ii) to identify the hormone acting to transiently increase the risk of mammary cancer during or shortly after pregnancy. For this purpose, short-term pregnancy hormone treatment (EST and PRG, or PRL) was performed after several animals had developed macroscopically visible tumors.

Materials and Methods

MNU-induced rat mammary carcinogenesis. One hundred and three female Lewis rats that were 2 weeks of age were purchased from Charles River Japan (Hino, Japan). All pups were housed 5 or 6 per cage with one nursing mother in an environment-controlled animal room (22±2˚C, 60%±10% humidity, and 12-h light/dark cycle). After an acclimatization period of 1 week, all animals received an intraperitoneal injection of 50 mg/kg body weight MNU (Sigma, St. Louis, MO, USA) dissolved in physiologic saline containing 0.05% acetic acid immediately prior to injection (Figure 1). The rats were then weaned and randomly divided into three groups: the hormone-untreated, age-matched control group (n=30); the short-term EST and PRG pellet-treated group (E/P, n=35); and the short-term PPZ-treated group (PPZ, n=38). All rats had *ad libitum* access to a commercial pellet diet (CMF 30 kGy, Oriental Yeast, Chiba, Japan) and fresh water. At 12 weeks of age (9 weeks after MNU injection), a total of 18 rats (6 in each group) had developed palpable mammary tumors (≥1 cm in diameter) that were approximately the same size. The rats in the E/P group received a subcutaneously implanted, 21-day-release pellet that contained 0.5 mg of EST and 32.5 mg PGR (Innovative Research of America, Sarasota, FL, USA) in the middle of their back; the pellet was removed at 15 weeks of age (3 weeks after implantation). The rats in the PPZ group received subcutaneous (*s.c.*) injections of 5 mg/kg PPZ dissolved in 0.03 M HCl (5 mg/ml) for 4 consecutive days followed by 3 days without injections every week for 3 weeks.

Six tumor-bearing rats in each group (E/P, PPZ, and control group) were sacrificed at 15 weeks of age, and the remaining rats were sacrificed at 19 weeks of age. At 15 weeks of age, the size of the pre-existing tumors (mammary tumors palpated at 12 weeks of age) was measured with calipers, and the volume was calculated by using the standard formula (width^2×length×0.5). Non-tumorous mammary tissues as well as palpable mammary tumors (pre-existing and newly growing) were collected immediately after sacrifice for pathological examination. The animals were cared for in accordance with the Guidelines for Animal Experimentation Committee of Kansai Medical University.

Histological analysis. All palpable mammary tumors and bilateral cervical-inguinal mammary fat chains (mammary glands no. 1-6) were excised, fixed with formalin overnight, embedded in paraffin, and cut into 4-μm-thick serial sections. The sections were stained with hematoxylin and eosin (HE) or used for histochemistry.

Histological classification of mammary tumors. Mammary tumors were histologically classified according to previous criteria (22). The mammary tumors were evaluated by incidence [(number of rats with cancer(s)/total number of rats sacrificed) ×100] and multiplicity (number of tumors/total number of rats sacrificed).

Histochemistry. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) was performed with anti-PCNA antibody (clone PC10, Novocastra, New Castle upon Tyne, UK) and a labeled streptavidin-biotin (LSAB) staining kit (Dako, Carpinteria, CA, USA). For quantitative analysis, PCNA-stained sections of tumors were scanned with a high-resolution digital slide scanner (NanoZoomer 2.0 Digital Pathology; Hamamatsu Photonics, Hamamatsu, Japan) to prepare digital images. The ndpi image files were opened in color mode with NDP.view software (Hamamatsu Photonics, Hamamatsu, Japan). These images were converted to TIFF files. PCNA-positive signals (brown color) and total tumor mass were individually selected from TIFF images using Adobe Photoshop CS2 software (Adobe Systems, Tokyo, Japan). Black and white images were prepared for the morphometric analysis, and the total tumor area and PCNA-positive area were individually measured using a computer-based image analysis system (Scion Image, Scion Corporation, Frederick, MD, USA). The percentage of PCNA-positive area was morphometrically determined by two pathologists certified by the Japanese Society of Toxicologic Pathology (K.Y. and A.T.).

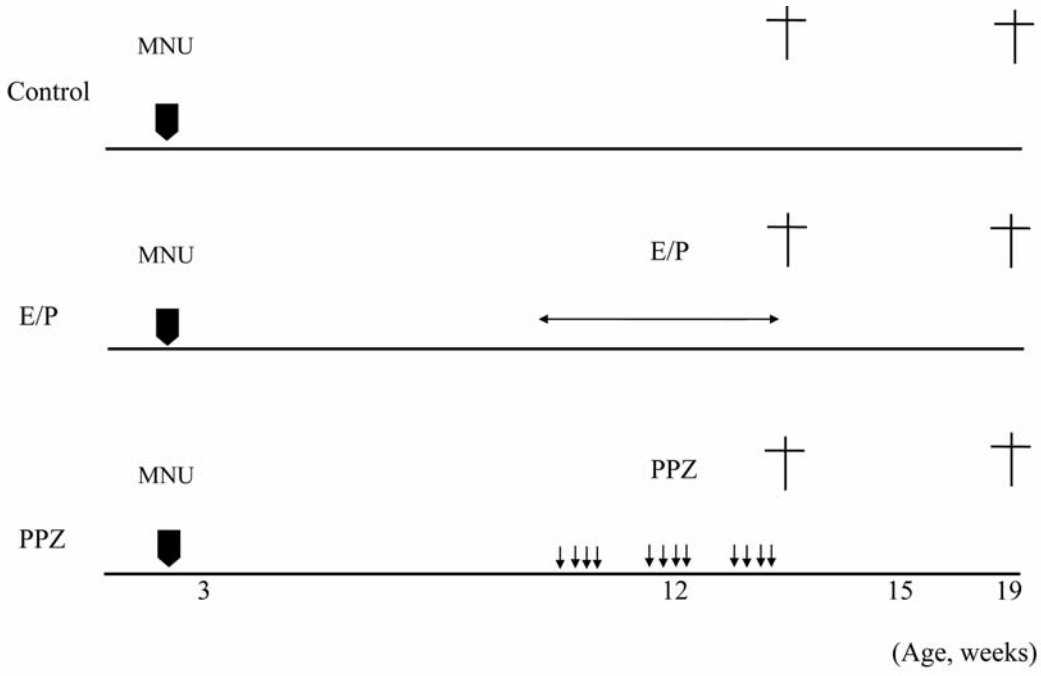

Figure 1. *Schematic representation of the experimental protocol. Control, hormone-untreated; MNU, N-methyl-N-nitrosourea; †, sacrifice; E/P, estradiol and progesterone; PPZ, perphenazine.*

TdT-mediated dUTP nick-end labeling (TUNEL) staining was performed using an apoptosis detection kit (Apop-Tag, Millipore, Bedford, MA, USA). Quantitative analysis of apoptosis was difficult to perform due to karyolysis in tumor cells; thus, only qualitative comparisons were performed.

Statistical analysis. All values were expressed as the mean±standard error (SE). Tumor volume and cancer multiplicity were analyzed by Student's *t*-test, and the incidence of mammary cancer was analyzed using the χ^2 test. A probability value of $p<0.05$ was considered statistically significant.

Results

Pre-existing mammary cancer at 15 weeks of age. Six rats in each of the three groups (control, E/P, and PPZ groups) had developed one or two palpable mammary tumors (≥1 cm) at 12 weeks of age. There were a total of 7 tumors in the control group, 7 tumors in the E/P group, and 6 tumors in the PPZ group. After 3 weeks of treatment (at 15 weeks of age), the pre-existing tumor volume tended to decrease after E/P treatment (mean volume, 383±271 mm^3) and increase after PPZ treatment (mean volume, 6895±2982 mm^3), as compared with the hormone-untreated tumors in the control group (mean volume, 1036±435 mm^3), although these differences were not statistically significant (Figure 2). All tumors in the control and PPZ groups were continuously growing, while the tumors in the E/P group were not (2 non-regressing, 3 partially regressing, and 2 completely regressing, Table I).

Mammary tumors from the control and PPZ groups were typical adenocarcinomas with papillary and/or cribriform features, no secretory activity, and generally viable cancer cells (Figure 3a and c), while mammary tumors from the E/P group were characterized by degenerative features (Figure 3b). As compared with the tumors in the control and PPZ groups (Figure 3d and f), the tumors in the E/P group contained fewer PCNA-positive cells (Figure 3e) and more TUNEL-positive signals (Figure 3h), which was a reflection of tumor degeneration. As compared with the control tumors, there was a quantitative reduction in the percentage of PCNA-positive area in the E/P tumors, while a significant increase was not seen in PPZ tumors (Figure 4a). Thus, as compared to no hormone treatment (the control group), E/P treatment caused regression of the pre-existing tumors due to decreased cell proliferation and increased apoptosis, while PPZ treatment tended to accelerate cancer cell growth.

Newly developing mammary cancer at 15 and 19 weeks of age. At 15 weeks of age, the number of newly developing tumors in the control, E/P and PPZ groups was 12, 4 and 22, and the cancer multiplicity was 2.0, 0.7 and 3.7, respectively (Table I). At 19 weeks of age, the mammary cancer incidence was 75%, 34% and 97%, and the cancer multiplicity was 2.4, 0.5 and 3.3, respectively (Table II). At 19 weeks of age, the newly growing tumors from the three groups showed identical morphology (see Figure 3a and c) and contained a similar

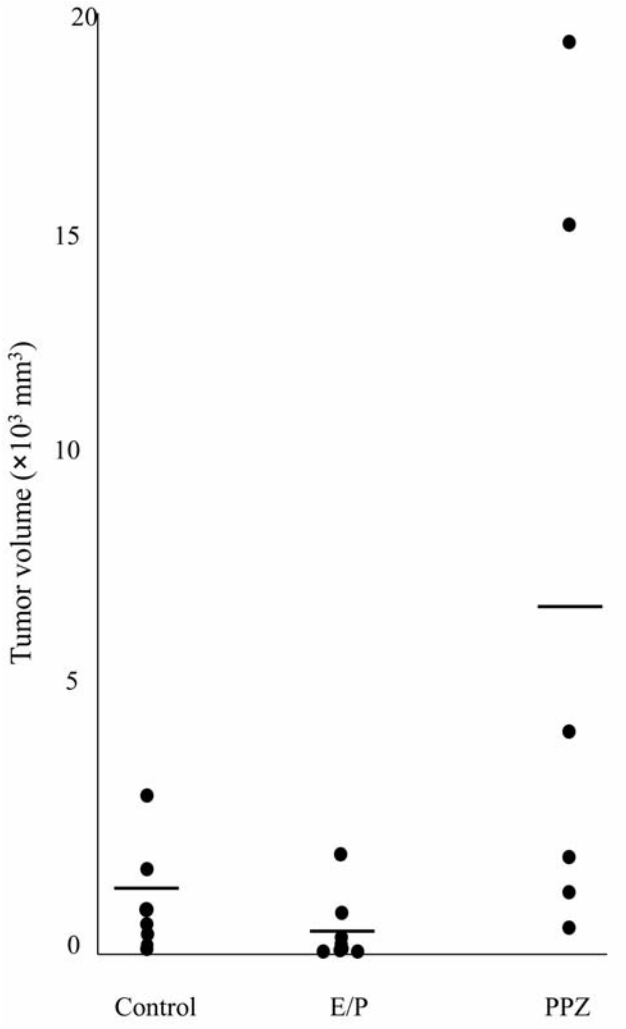

Figure 2. *Tumor volume of pre-existing (palpable mammary tumor at 12 weeks of age) mammary cancer 3 weeks after pregnancy hormone treatment. Control, hormone-untreated, E/P, estradiol and progesterone treatment; PPZ, perphenazine treatment. Tumor volume was calculated by using the standard formula, width²×length×0.5.*

percentage of PCNA-positive areas (Figure 4b). Thus, E/P suppressed and PPZ accelerated the appearance of newly developing mammary tumors; however, tumor growth, as evaluated by cell proliferation (percentage of PCNA-positive area), was not affected by short-term hormone treatment.

Discussion

Huggins *et al*. (18) demonstrated that the development of DMBA-induced mammary cancer in nulliparous Sprague-Dawley rats could be suppressed by the intramuscular injection of EST and PRG dissolved in ethanol and diluted in sesame oil at a dose of 20 μg and 4 mg, respectively, administered 6 times per week for 30 days; the circulating EST and PRG levels were not recorded. However in another report, nulliparous Sprague-Dawley rats that received *s.c*. injections of 20 μg EST and 4 mg PRG 5 times per week for 5 weeks beginning at 40 days of age developed pregnancy levels of serum EST and PRG during the treatment period (23). Although PRG in addition to EST accelerates mammary cancer-suppressing activity, EST alone is effective, while PRG alone is not (19). A silastic capsule containing 100 μg of EST and implanted into nulliparous Lewis rats for 3 weeks elevates serum EST levels to pregnancy levels and is highly effective in preventing mammary cancer, while a silastic capsule containing 20 μg of EST does not increase serum EST levels equivalent to those of pregnancy and does not reduce the incidence of mammary cancer (20). Thus, EST at pregnancy level for a short period is the key hormone for the suppression of mammary cancer development. The doses of EST and PRG administered do not directly reflect the serum hormone levels because different administration methods result in different serum hormone levels (11).

When a 21-day-release 0.5 mg EST and 32.5 mg PRG pellet was *s.c*. implanted into nulliparous Lewis rats for 3 weeks (similar to the present work), the serum EST and PRG levels reached pregnancy levels and effectively reduced the development of MNU-induced rat mammary cancer (16). Thus, a short-term increase in the EST and PRG milieu to pregnancy levels may effectively suppress the development of mammary cancer. When the interval between carcinogen administration and pregnancy is prolonged, the mammary cancer-suppressing effects are weakened (12). The interval between carcinogenic exposure and pregnancy (or pregnancy hormone treatment) in experimental animal models is usually set at 2 weeks (11). When the interval between carcinogenic exposure and EST and PRG treatment was increased to 17 or 20 weeks, the mammary cancer-suppressing effect was lost (24, 25). However, although the interval between carcinogenic exposure and EST and PRG treatment was 9 weeks in the present study, the development of newly developing tumors was significantly suppressed.

Surprisingly, the pregnancy level of EST and PRG for 3 weeks caused the regression of pre-existing mammary tumors (≥1 cm in diameter); complete regression occurred in 2 out of the 7 tumors, partial regression occurred in 3, and 2 remained the same size. In these regressing tumors, the loss of proliferation and induction of apoptosis were characteristic features. Although the circulating EST levels were not recorded, daily injection of 1 mg (but not 10 μg) of EST dissolved in ethanol and sesame oil given for 28 days suppressed the growth of *s.c*. transplanted rat MT6 mammary tumors, as compared with EST-untreated controls (26), and daily *s.c*. injection of 100 μg EST dissolved in ethanol and olive oil for 10 days significantly reduced the size of DMBA-induced mammary tumors 1 cm in diameter (27). A high dose of EST induces the regression of pre-existing (macroscopically

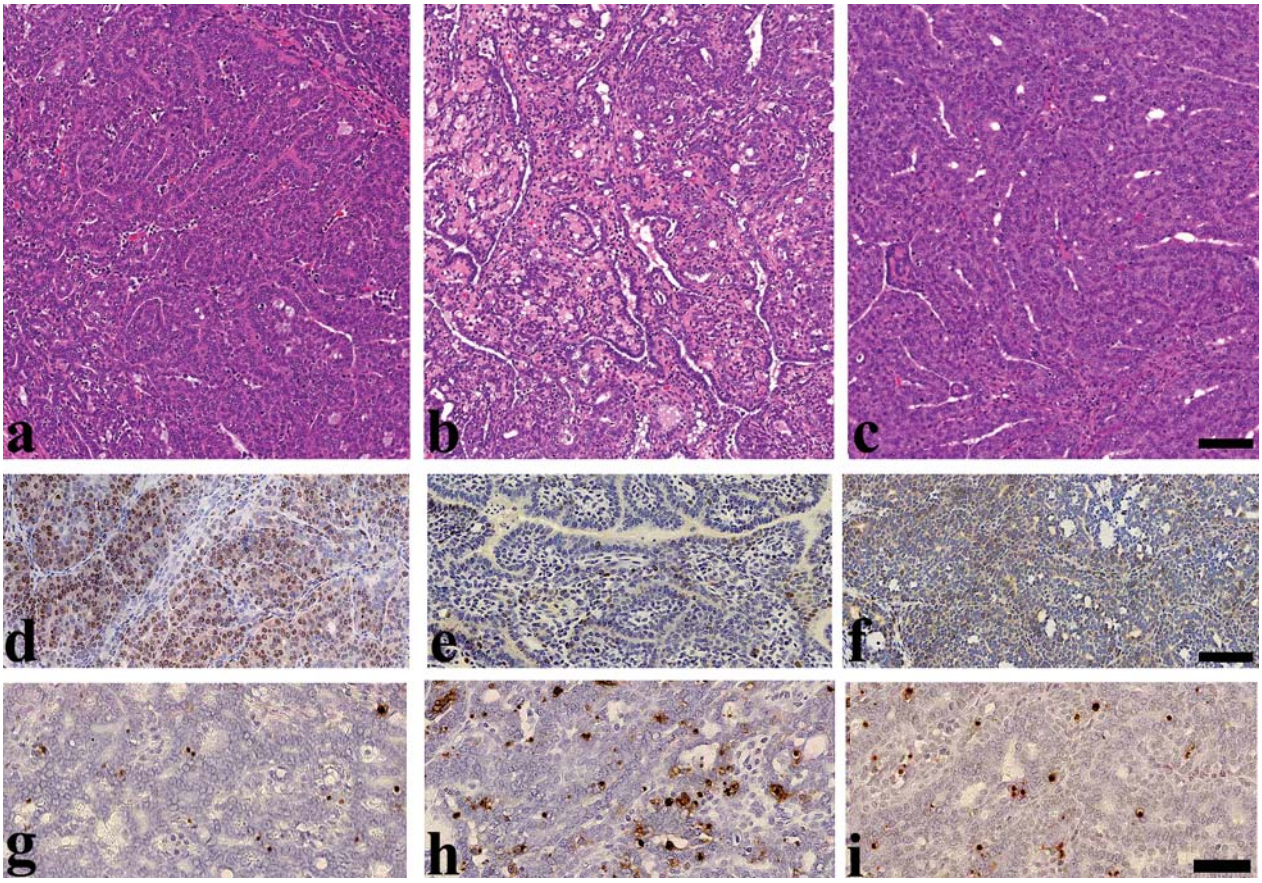

Figure 3. *Effects of short-term estradiol and progesterone or perphenazine treatment on MNU-induced mammary cancer in female Lewis rats. a, d and g: Hormone-untreated (control). b, c and h: Estradiol and progesterone (E/P)-treated. c, f and i: Perphenazine (PPZ)-treated. a, b and c: HE staining (bar=100 μm). d, e and f: PCNA staining (bar=100 μm). g, h and i: TUNEL staining (bar=50 μm).*

visible) mammary tumors, while a low dose is ineffective. Importantly, in the present study, pregnancy levels of EST and PRG reduced the growth of established tumors by an apoptotic mechanism and suppressed the development of newly growing tumors during or shortly after hormone treatment. This evidence suggests that pregnancy hormones EST and PRG may not be involved in the development of so-called pregnancy-associated breast cancer (breast cancer occurring during or shortly after pregnancy).

Neuroleptics elevate serum PRL levels (hyperprolactinemia) and are associated with a small but significant risk of breast cancer in humans (28). In nulliparous adult Wistar rats, the serum PRL level was 50 ng/ml in normal controls, 870 ng/ml in lactating rats, and 670 ng/ml in rats that received *s.c.* injection of 5 mg/kg PPZ once a day for 2 weeks (29). Thus, PPZ injection caused serum PRL levels similar to those of lactating rats. PPZ *s.c.* injected at a dose of 5 mg/kg 5 times a week for 3 weeks increased the serum PRL levels and caused the production of pregnancy levels of PRG by corpora lutea, while levels of EST remained comparable to those in nulliparous rats; when MNU was given 2 weeks prior to the start of the 3-week PPZ treatment at a dose of 5 mg/kg 5 times a week, mammary carcinogenesis was unaffected (19). In contrast, the same dose of PPZ administered 4 times a week for 3 weeks accelerated the development of newly growing mammary tumors and stimulated the growth of pre-existing ones when given 9 weeks after MNU treatment. The different results of these two experiments may be caused by the different intervals between carcinogenic exposure and PPZ treatment; carcinogen-induced initiated cells may have time to gradually acquire preneoplastic potential and be more prone to neoplastic transformation. Breast tissue of young women is more sensitive to radiation-induced carcinogenesis (30); therefore, (pre)neoplastic cells may exist before the first pregnancy.

Long-term treatment with the neuroleptic drug fluphenazine causes hyperprolactinemia, increases the number of DMBA-induced mammary tumors, and shortens the latency (31).

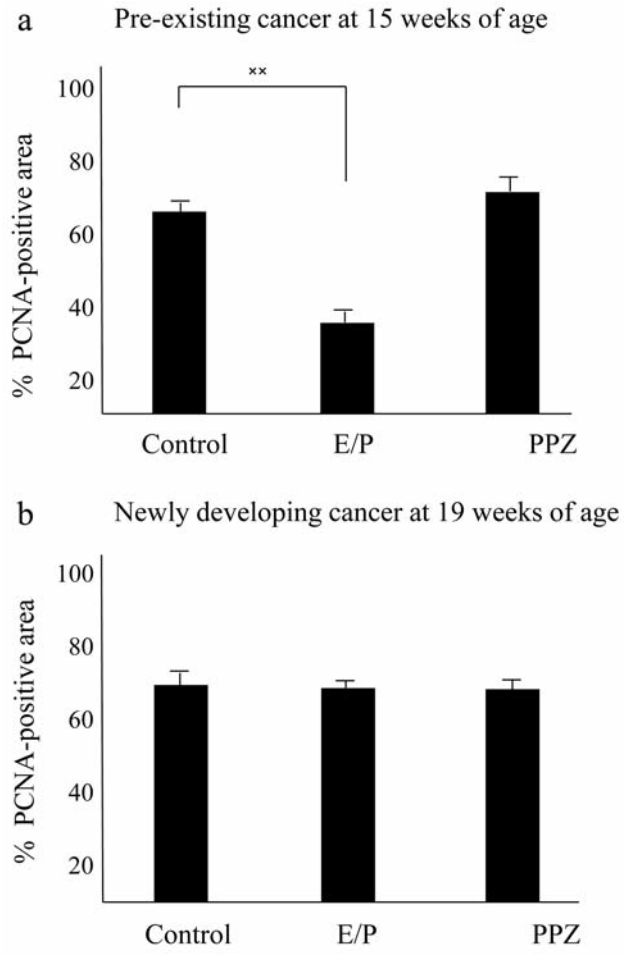

Figure 4. *Comparison of percentage of PCNA-positive area. a: Pre-existing cancer evaluated at 15 weeks of age. b: Newly developing cancer evaluated at 19 weeks of age. Five or six tumors were arbitrarily selected from each group and used for comparison. Control, hormone-untreated; E/P, estradiol and progesterone; PPZ, perphenazine; **p<0.01.*

Thus, as in long-term PRL exposure, short-term PRL exposure at a level comparable to pregnancy may be a breast cancer risk. In humans, a higher frequency of multiplicity (31%, 4/13) and the development of lipid-secreting carcinomas (12%, 2/17), which account for only 1%-2% of all primary breast cancers, were characteristic in neuroleptic users (32). Rat R3230AC and human T-47D breast cancer cells develop lipid droplets after PRL treatment (33, 34). However, although a high frequency of cancer multiplicity was seen after short-term PPZ treatment, no lipid accumulation in mammary cancer cells was detected in the present study. A pituitary isograft under the kidney capsule increases the serum PRL titer to pregnancy levels. Parous BALB/c mice refractory to MNU-induced mammary cancers regain susceptibility to carcinogenic stimuli when given a pituitary isograft for 5 weeks followed by MNU administration (35). Therefore, short-term PRL may negate the cancer-suppressing effects of EST and PRG and may significantly accelerate the growth of newly developing cancer as well as pre-existing cancer. During pregnancy or shortly after pregnancy, although the overall incidence of mammary cancer will be reduced, a transient increase in the incidence of mammary cancer has been observed (36). Although EST and PRG treatment did not cause increased mammary cancer risk, PRL may be partially involved in this risk.

It remains unclear how short-term pregnancy levels of EST and PRG reduce the mammary cancer risk. Systemic alterations of the host hormonal environment and/or local changes in mammary gland architecture have been postulated to be involved. A possible mechanism of parity protection is that parous rats are refractory to carcinogen-induced mammary epithelial cell proliferation (37, 38). Alterations in the n-6/n-3 polyunsaturated fatty acid (PUFA) ratio may also be involved in the mechanism of parity protection. A high-fat diet rich in n-6 PUFA stimulates mammary cancer growth and metastasis, while n-3 PUFA has a cancer-suppressing effect (39). A transient decrease in the serum n-6/n-3 PUFA ratio is seen in short-term E/P pellet-exposed rats as compared with controls (40). The proposed mechanisms *per se* are insufficient to explain the mechanisms of parity protection. However, although pregnancy levels of PRL increase breast cancer risk, EST and PRG may be the most effective treatment against breast cancer without transient increase in risk during or shortly after the exposure to hormones. Further studies are desired because parity protection, especially EST and PRG treatment for a short duration, may be the most physiological way to control breast cancer.

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Table I. *MNU-induced mammary cancer at 15 weeks of age after estradiol and progesterone or perphenazine treatment for 3 weeks.*

Group		Pre-existing cancer					Newly developing cancer	
		At 12 weeks	At 15 weeks				At 15 weeks	
	No. of rats	No. of tumors	CG	NR	PR	CR	No. of tumors	Multiplicity
Control	6	7	7	0	0	0	12	2.0±0.8
E/P	6	7	0	2	3	2	4	0.7±0.3
PPZ	6	6	6	0	0	0	22	3.7±0.9

MNU (50 mg/kg) was intraperitoneally administered at 3 weeks of age. At 12 weeks of age, rats received hormone treatments that consisted of a 0.5 mg estradiol- and 32.5 mg progesterone-containing 21-day release pellet subcutaneously inserted (E/P group) or subcutaneous injections of 5 mg/kg perphenazine 4 times a week for 3 weeks (PPZ group). The control group contained rats that did not receive any hormone treatments. Multiplicity is expressed as the mean±SE. MNU, *N*-Methyl-*N*-nitrosourea; CG, continuously growing; NR, non-regressing; PR, partial regressing; CR, completely regressing; E/P, estradiol and progesterone-treated; PPZ, perphenazine-treated.

Table II. *MNU-induced mammary cancer at 19 weeks of age after estradiol and progesterone or perphenazine treatment for 3 weeks.*

Group	No. of rats	No. of rats with MC (%)	No. of MCs	Multiplicity
Control	24	18 (75)	58	2.4±0.4
E/P	32	11 (34)	16	0.5±0.1
PPZ	29	28 (97)	95	3.3±0.4**

MNU (50 mg/kg) was intraperitoneally administered at 3 weeks of age. At 12 weeks of age, rats received hormone treatments that consisted of a 0.5 mg estradiol- and 32.5 mg progesterone-containing 21-day release pellet subcutaneously inserted (E/P group) or subcutaneous injections of 5 mg/kg perphenazine four times a week for 3 weeks (PPZ group). The control group contained rats that did not receive any hormone treatments. Multiplicity is expressed as the mean ±SE. **p<0.01 vs. E/P group. MNU, *N*-Methyl-*N*-nitrosourea; MC, mammary cancer; E/P, estradiol and progesterone-treated; PPZ, perphenazine-treated.

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