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Original Article

The effect of childbirth on carcinogenesis of DMBA-induced breast cancer in female SD rats

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Abstract

Many epidemiologic and clinical studies have indicated that the frequency of breast cancer was lower in parous women than in nulliparous women. Moreover, the incidence of breast cancer has been reported to be lower in women with early childbirth than in women with late childbirth. To verify the effect of childbirth and the age at first childbirth on carcinogenesis and progression of breast cancer, we induced breast cancer by 7,12-dimethylbenanthracene (DMBA) in 120 female Sprague-Dawley (SD) rats, and divided them into control or experimental (DMBA-treated) nulliparous, early childbirth, and late childbirth groups to observe the incidence, latency, and size of breast cancer. Argyrophilic nucleolar organizer regions (AgNOR) count and the expression of C-erbB-2, proliferating cell nuclear antigen (PCNA), Ki-67, and minichromosome maintenance protein 2 (MCM2) in breast cancer tissues were detected by immunohistochemistry. The breast cancer incidences were 95.0%, 16.7%, and 58.8% in the experimental nulliparous, early childbirth, and late childbirth groups, respectively (all $P < 0.05$). Between any two of these groups, the latency was significantly different, but tumor size was similar. AgNOR count and the expression of C-erbB-2, PCNA, Ki-67, and MCM2 were significantly higher in the experimental nulliparous group than in the experimental early or late childbirth groups ($P < 0.05$), but no significant differences were observed between the latter two groups. Taken together, the results suggest that childbirth, especially early childbirth, can reduce the incidence and postpone the onset of DMBA-induced breast cancer.

Key words 7,12-Dimethylbenanthracene (DMBA), childbirth, nulliparity, breast cancer, rat

In recent years, the incidence of breast cancer has continued to rise across the world. Despite increasing attention to prevention, early diagnosis, and early treatment, the average 10-year disease-free survival rate for breast cancer patients after treatment remains at 40% to 50%. Although improving therapy may increase the survival rate, understanding the cause of breast cancer, and thereby providing insight into measures to reduce incidence, is critical and remains a key goal in this field.

For economic reasons, more and more women postponed childbearing, resulting in higher age at parity. Juxtaposed to this trend is a rapidly rising incidence of breast cancer among women. Many researchers believe that breast cancer has a variety of etiologies, one of which is childbirth. Several epidemiologic studies indicate that breast cancer incidence is higher among nulliparous women than among parous women[1]. In addition, studies further suggest that age of first childbirth affects risk of breast cancer[2].

Yang *et al.*[3] showed that childbearing and breast-feeding rats had longer time to disease onset and less mammary tumors than did nulliparous rats under the same carcinogenic conditions. Moon[4] believed that pregnancy protects the mammary gland against

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carcinogenic reagents even at 100 to 130 days after delivery, suggesting a long-lasting change in the sensitivity of mammary gland to carcinogenic reagent. Russo *et al*. [5] found that parous rats and human chorionic gonadotropin (HCG)–treated nulliparous rats had lower breast cancer incidence and smaller tumor size than did HCG-untreated nulliparous rats after 7,12-dimethylbenanthracene (DMBA) treatment, and found no difference between the first two groups. They proposed that the difference between parous and HCG-untreated nulliparous rats was due to pregnancy-based mammary gland differentiation, which led to decreased cell proliferation, reduced aggregation of carcinogenic reagents, and increased DNA repair capacity in breast epithelial tissue[6,7], and that the similarity in cancer incidence and tumor size between parous rats and HCG-treated rats was due to HCG-caused mammary gland differentiation[8,9], similar to pregnancy-based mammary gland differentiation. Chie *et al*.[10] conducted a large scale retrospective study on women with first full-term pregnancy at different ages and found that women with a younger age of first childbirth had a lower risk of breast cancer than did those with an older age of first childbirth, which was due to early protection from pregnancy-based mammary gland differentiation and hormonal changes. After the first full-term pregnancy, mammary epithelial cells become fully differentiated and cease proliferation, making them insensitive to carcinogenic reagents. Similarly, changes in hormone levels, including reduced prolactin and estrogen and increased sex hormone-binding globulin, may provide additional protection against breast cancer[11,12]. Furthermore, full-term pregnancy also reduces the aggregation of estrogen and other potential breast carcinogenic agents[13].

Although many researchers have shown that childbirth, especially early childbirth, can inhibit tumor cell growth by retrospective studies on women, this concept is still controversial due to different sample sizes and methodologies used in these studies. In this study, we divided Sprague-Dawley (SD) rats into nulliparous, early childbirth, and late childbirth groups and induced breast cancer by DMBA to explore the effect of childbirth on breast carcinogenesis.

Materials and Methods

Animals and reagents

A total of 130 healthy SD rats (80 8-week-old females, 40 36-week-old females, and 10 12-week-old males used for mating) were provided by the Animal Center of Hebei Medical University (Animal Certificate of Conformity: 902147). DMBA was provided by Sigma-Aldrich Corp. (St. Louis, MO, USA). A staining kit for argyrophilic nucleolar organizer region (AgNOR) was purchased from Shanghai Chemical Reagent Company. C-erbB-2 (HER-2/neu), proliferating cell nuclear antigen (PCNA), Ki-67, and minichromosome maintenance protein 2 (MCM2) antibodies were purchased from Zymed Company (Carlsbad, CA, USA). The SP immunohistochemistry kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd.

Methods

Establishment of rat breast cancer model Forty 8-week-old pregnant rats, 40 36-week-old pregnant rats, and 40 8-week-old unpregnant rats were randomly divided into control or experimental (DMBA-treated) early childbirth, late childbirth, and nulliparous groups, with 20 rats in each group. One week after childbearing (without breast feeding), the rats from the experimental groups were lavaged twice with DMBA (10 mg/100 g body weight) at a dose interval of 2 weeks; the rats from control groups were fed by normal diet without any treatment.

Morphology observation and pathology assessment After 24 weeks of feeding, the rats were killed when at diestrum as confirmed by vaginal smear examination. Hairs were removed to count tumors, observe tumor morphology, and measure the maximum diameter of tumors. Maximum diameter was measured directly for single tumors or determined from the biggest tumor if multiple tumors occurred. Breast tissues were resected, fixed routinely, and embedded in paraffin to prepare 4-μm sections for HE staining, argyrophilic staining and AgNOR counting, and two-step immunohistochemical staining using the SP method to detect the expressions of C-erbB-2, PCNA, Ki-67, and MCM2.

The criteria of pathologic and immunohistochemical assessment AgNOR staining was assessed according to the diagnostic criteria for Dunn rat atypical breast hyperplasia and breast cancer. At least 100 cells in each section were counted under the optical microscopy. A stained mass with a diameter of $\geqslant$2.5 μm and $\leqslant$5 μm was counted as 5 AgNOR particles, a diameter of >5 μm and $\leqslant$10 μm counted as 10 particles, and a diameter of >10 μm counted as 15 particles. Finally, the number of AgNOR particles per nucleus was calculated.

C-erbB-2 expression was shown as brown particles in the cell membrane. C-erbB-2 staining was scored according to the Hercep Test recommended by the Food Drug Administration: (−) was defined as $\leqslant$10% of cells with light yellow staining on the membrane; (+) was defined as >10% of cells with scattered light yellow or brown fine particles on the membrane; (++) was defined

as >10% of cells with brown fine and coarse particles around the membrane; and (+++) was defined as >10% of cells with dark brown or brown particles around the membrane. Grades 0 and (+) were defined as negative, and grades (++) and (+++) were defined as positive.

PCNA was expressed in the nuclei, with light yellow staining as weak expression, brown staining as moderate expression, and dark brown staining as strong expression. A total of 500 tumor cells were counted for each section under high magnification view (×400), and PCNA staining intensity index (SII) was calculated as follows: SII = (percentage of weakly positive cells × 1) + (percentage of moderately positive cells × 2) + (percentage of strongly positive cells × 3). Based on PCNA SII scores, samples were graded as (−), with SII scores of 0% to 25%; (+), 26% to 50%; (++), 51% to 75%; and (+++), 76% to 100%. Grades (−) and (+) were defined as negative, and grades (++) and (+++) were defined as positive.

Ki-67 expression was shown as brown particles in the nuclei and graded according to the percentage of positive tumor cells, with (−) defined as <5% of positive cells, (+) defined as 5% to 25% of positive cells, (++) defined as 26% to 50% of positive cells, and (+++) defined as >50% of positive cells. Grades (−) and (+) were defined as negative, and grades (++) and (+++) were defined as positive.

MCM2 was expressed in the nuclei in brown or dark brown. The most intensely stained positive regions were selected by microscopy under low magnification, after which 100 tumor cells were counted under high magnification (×400). The percentage of positive cells was calculated in each section, and the average value of five regions was taken as the labeling index. MCM2 expression was graded according to the labeling index: (−) represented a labeling index of 0 to 25%; (+), 26% to 50%; (++), 51% to 75%; (+++), 76% to 100%. Grades (−) and (+) were defined as negative, and grades (++) and (+++) were defined as positive.

Statistical analysis

Data were analyzed by the Fisher's exact test and rank sum test using SPSS13.0 statistical software. A P value <0.05 was considered statistically significant.

Results

Breast tumor formation

After 24 weeks of feeding, 5 of the 60 experimental rats died: 2 in the DMBA-treated early childbirth group and 3 in the DMBA-treated late childbirth group; 2 died from accidental lavage of DMBA into the trachea, and another 3 died of body failure due to jaundice. None of the 60 control rats died. In the experimental groups, 32 rats had breast cancer (19 in the DMBA-treated nulliparous group, 3 in the DMBA-treated early childbirth group, and 10 in the DMBA-treated late childbirth group), resulting in a total of 54 tumors. No tumors were observed in the control groups. Fisher's exact test showed that the occurrence rate of breast cancer was significantly lower in the DMBA-treated early and late childbirth groups than in the nulliparous group (P <0.001; P = 0.014), and was also significantly lower in the DMBA-treated early childbirth group than in the DMBA-treated late childbirth group (P = 0.015) (Table 1). The peak onset of breast cancer was 16 weeks after DMBA treatment. The first tumor was observed after 8 weeks in the DMBA-treated nulliparous group. The latency between the initial observation of breast cancer and the first DMBA administration was significantly longer in the DMBA-treated early and late childbirth groups than in the nulliparous group (P = 0.001; P <0.001), and was also significantly longer in the DMBA-treated early childbirth group than in the DMBA-treated late childbirth group (P = 0.014) (Table 1).

Tumor size

The maximum tumor diameter was measured in all rats with breast cancers. The mean maximum tumor diameter was similar in three DMBA-treated groups (Table 1).

Table 1. Occurrence rate, latency, and tumor diameter of breast cancer in DMBA-treated nulliparous, early childbirth, and late childbirth groups

Group	Occurrence rate	Latency (weeks)	Tumor size (cm)
Nulliparous	95.0%	10.25 ± 0.83	2.86 ± 0.54
Early childbirth	16.7%[a]	17.25 ± 3.20[a]	2.65 ± 1.67
Late childbirth	58.8%[b,c]	14.50 ± 0.96[a,c]	2.41 ± 0.50

DMBA, 7,12-dimethylbenanthracene. [a]P < 0.001, [b]P < 0.05, vs. the nulliparous group; [c]P < 0.05, vs. the early childbirth group.

Morphologic examination of breast tumors

Of the 54 breast tumors, 5 were ductal carcinoma *in situ*, 8 were invasive lobular carcinoma, and 41 were invasive ductal carcinoma. Most tumors located at the inferior outside of the breast, and were hard with an uneven surface. A few tumors had ulcerated skin adhesions, which appeared dark purple at sites of necrosis. Hyperplastic thick blood vessels, which contained thrombosis and were usually incompletely coated, were observed around tumors after skin incision. The tumors felt sandy during anatomical dissection and were yellow-white at the incision sites. Epithelial hyperplasia or atypical hyperplasia was observed in the remaining breast without visible tumors.

Pathologic examination of breast cancer with HE staining

The tumors were solid without glandular-like structure. Cancer cells were small, surrounded by interstitial fibrosis, and displayed frequent mitosis (Figure 1).

Immunohistochemical examinations of protein expression in breast cancer

Argyrophilic staining resulted in light yellow and dark brown coloring of argyrophilic granules in the nuclei (Figure 2A). C-erbB-2 expression was observed as brown granules on the membrane of breast cancer cells (Figure 2B); PCNA was expressed in light yellow in the nuclei and cytoplasm of a few cells (Figure 2C); Ki-67 expression was visualized as brown granules in the nuclei (Figure 2D). MCM2 expression was visualized as brown or dark brown granules in the nuclei (Figure 2E). The AgNOR count per nucleus as well as percentages of positive tumors for C-erbB-2, PCNA, Ki-67, and MCM2 were significantly lower in the DMBA-treated early and late childbirth groups than in the DMBA-treated nulliparous group (all $P < 0.01$), but showed no significant differences between the early and late childbirth groups (Table 2).

Discussion

In recent years, the incidence of breast cancer has continued to rise across the world. Childbirth and age of childbirth are critical factors for breast cancer. Epidemiologic and clinical studies indicate that breast cancer incidence is significantly lower in parous women than in nulliparous women, as well as lower in women with early childbirth than in women with late childbirth[1].

Epidemiologic and clinical studies indicate that

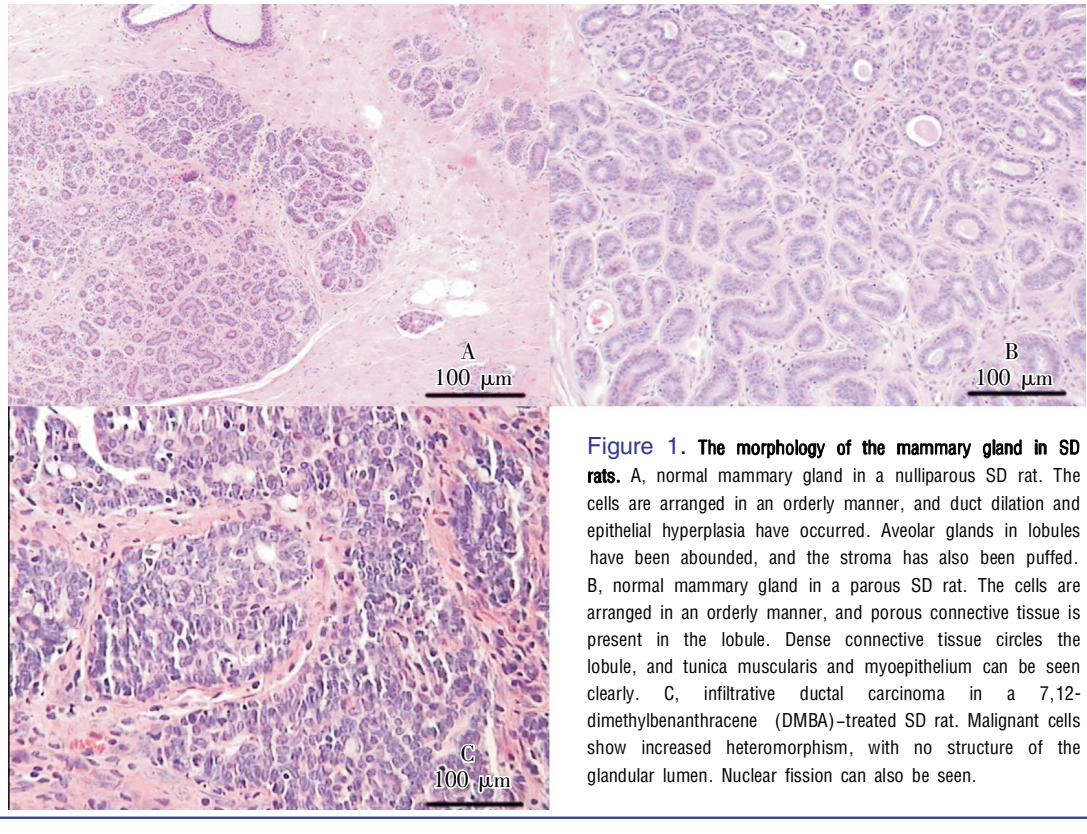

Figure 1. **The morphology of the mammary gland in SD rats.** A, normal mammary gland in a nulliparous SD rat. The cells are arranged in an orderly manner, and duct dilation and epithelial hyperplasia have occurred. Aveolar glands in lobules have been abounded, and the stroma has also been puffed. B, normal mammary gland in a parous SD rat. The cells are arranged in an orderly manner, and porous connective tissue is present in the lobule. Dense connective tissue circles the lobule, and tunica muscularis and myoepithelium can be seen clearly. C, infiltrative ductal carcinoma in a 7,12-dimethylbenanthracene (DMBA)–treated SD rat. Malignant cells show increased heteromorphism, with no structure of the glandular lumen. Nuclear fission can also be seen.

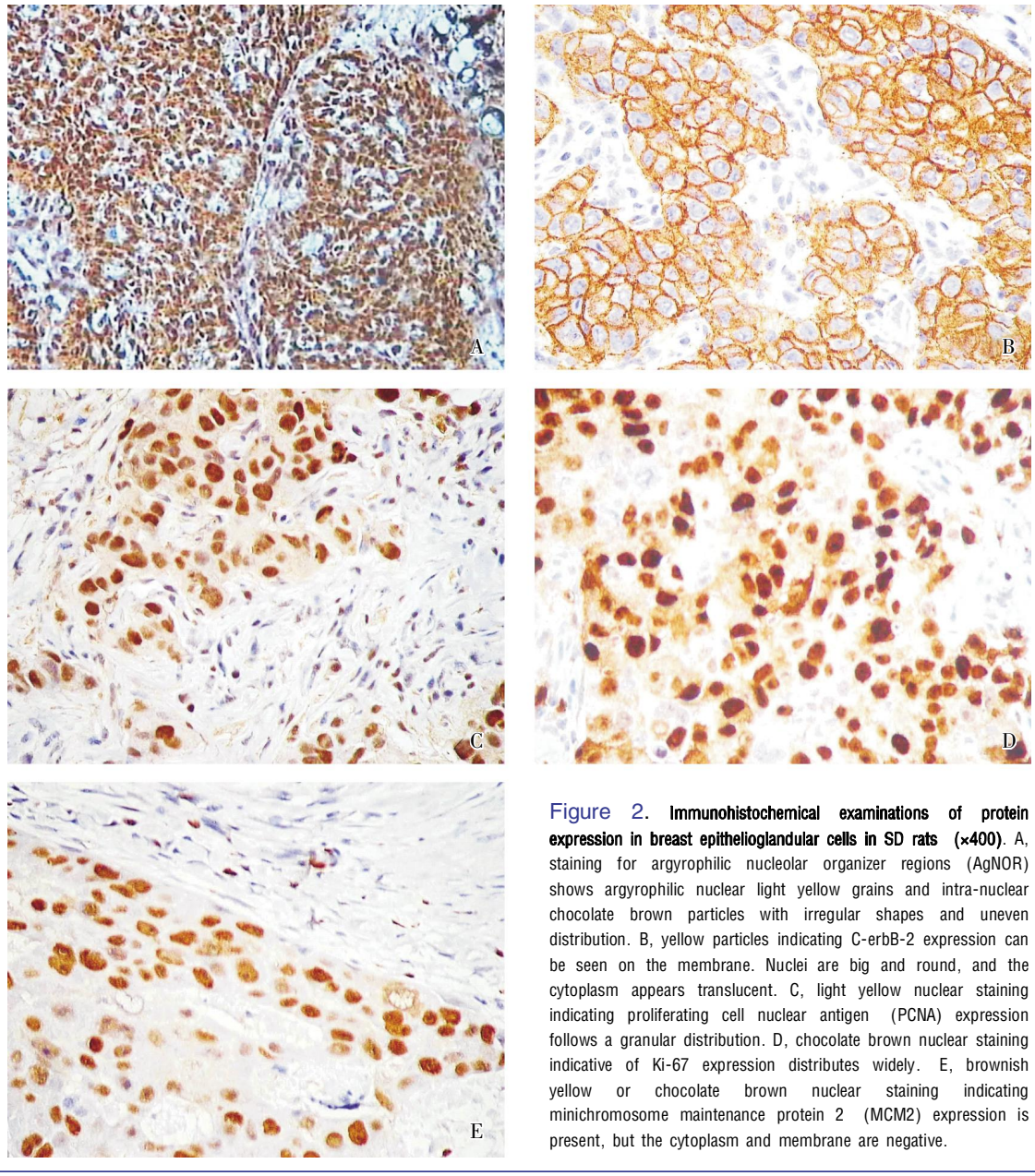

Figure 2. **Immunohistochemical examinations of protein expression in breast epithelioglandular cells in SD rats (×400)**. A, staining for argyrophilic nucleolar organizer regions (AgNOR) shows argyrophilic nuclear light yellow grains and intra-nuclear chocolate brown particles with irregular shapes and uneven distribution. B, yellow particles indicating C-erbB-2 expression can be seen on the membrane. Nuclei are big and round, and the cytoplasm appears translucent. C, light yellow nuclear staining indicating proliferating cell nuclear antigen (PCNA) expression follows a granular distribution. D, chocolate brown nuclear staining indicative of Ki-67 expression distributes widely. E, brownish yellow or chocolate brown nuclear staining indicating minichromosome maintenance protein 2 (MCM2) expression is present, but the cytoplasm and membrane are negative.

Table 2. Expression of AgNOR, C-erbB-2, PCNA, Ki-67, and MCM2 in DMBA-treated nulliparous, early childbirth, and late childbirth groups

Group	AgNOR count per nucleus	Percentage of protein-positive tumors (%)			
		C-erbB-2	PCNA	Ki-67	MCM2
Nulliparous	7.52±1.24	80.0	70.0	85.0	80.0
Early childbirth	3.52±3.00[a]	16.7[b]	16.7[a]	16.7[b]	16.7[b]
Late childbirth	4.76±1.20[a]	29.4[a]	23.5[a]	35.3[a]	35.3[a]

AgNOR, argyrophilic nucleolar organizer regions; PCNA, proliferating cell nuclear antigen; MCM2, minichromosome maintenance protein 2. [a]$P < 0.01$, [b]$P < 0.001$, vs. the nulliparous group.

childbirth history affects the risk of breast cancer [2]. Jensen *et al*. [14] analyzed 50 000 Danish nulliparous women and found that the carcinogenesis of breast cancer was significantly higher in nulliparous women than in parous women. Similarly, other studies show that childbirth, especially early childbirth, is important for decreasing the risk of breast cancer[15,16]. After determining the carcinogen-sensitive factors of breast cancer in animal models and the pathogenesis of human breast cancer, Russo *et al*.[17] concluded that the protective role of childbirth against breast cancer is due to the mammary gland differentiation induced during pregnancy. Yang *et al*.[3] treated nulliparous and parous rats with DMBA and found that exposure to carcinogenic agents after full-term pregnancy significantly reduced breast cancer incidence in rats. This pregnancy-induced protective effect resulted from structural and functional changes in breast and mesenchymal cells during pregnancy and pregnancy-promoted breast cell differentiation, both of which made breast epithelial cells less sensitive to carcinogenic agents[18].

Animal models can be used to simulate carcinogenesis and development of human breast cancer and to study its molecular mechanism and intervention. DMBA, a polycyclic aromatic hydrocarbon compound belonging to anthracene class, has a strong carcinogenic effect, especially in SD rats. DMBA is relatively safe and most commonly used in animal experiments to induce breast cancer. The histology of DMBA-induced breast cancer and precancerosis in SD rats has been widely accepted to be very similar to human breast cancer, making this model ideal for breast cancer studies. Enhanced expression of AgNOR, PCNA, Ki-67, and MCM2 indicate abnormal proliferation of breast tissue. Similarly, expression of C-erbB-2 is closely related to carcinogenesis and development of breast cancer, and its abnormal expression suggests active proliferation and malignancy of tumor tissue. Our results showed that DMBA treatment led to increased expression of AgNOR, C-erbB-2, PCNA, Ki-67, and MCM2 in breast epithelial cells in SD rats and that breast epithelial hyperplasia or atypical hyperplasia and carcinogenesis occurred at the same time, suggesting that DMBA significantly induces the development of breast cancer.

In this study, we divided SD rats into nulliparous, early and late childbirth groups. These rats were lavaged with DMBA to induce breast cancer. The incidence, latency, and tumor diameters of breast cancer were recorded for each group to determine the protective role of childbirth against breast cancer in rats. Our results showed that pregnancy and childbirth decreased sensitivity of rats to DMBA, resulting in decreased breast cancer incidence and extended latency of breast carcinogenesis. We propose that the mammary gland differentiation that occurs after the first full-term pregnancy reduces the risk of breast cancer. After the first full-term pregnancy, breast epithelial cells fully differentiate and cease to proliferate in normal circumstances, thereby becoming insensitive to carcinogenic reagents[19]. The pregnancy-induced protective effect begins upon the first full-term pregnancy and is maintained through life [20]. A large number of undifferentiated type 1 lobules exist in the breast tissue of nulliparous women, mainly in pre-menarche immature female breast; in contrast, the breast tissue of parous women consists of type 3 lobules, which are not present in nulliparous women [17]. Because of pregnancy and childbirth, undifferentiated type 1 lobules become well differentiated type 3 lobules[17]. Therefore, as the degree of mammary gland differentiation determines the level of sensitivity to carcinogenic reagents, the incidence of breast cancer in parous women is lower than that in nulliparous women.

We also found that breast cancer incidence was lower and latency was shorter in the early childbirth group than in the late childbirth group. Early childbirth results in early differentiation of mammary epithelial cells and reduced exposure to carcinogenic reagents. In a large-scale study in Denmark, Jensen *et al*.[14] found that the incidence of breast cancer increased as the age of first childbirth was postponed by every 2.5 to 4 years. Premenopausal women who had first childbirth at the ages of 30 to 34 did not have an increased risk of breast cancer. However, breast cancer incidence in women who had first childbirth at the age of 35 or later increased by 80% [14]. This is in contrast to the findings of MacMahon *et al*.[18], who proposed that the risk of breast cancer in parous women who had first childbirth at the ages of 30 to 34 was equivalent to the risk in nulliparous women, whereas that the risk was reduced in women who had first childbirth at the ages of 30 or younger.

In summary, pregnancy and childbirth, especially early childbirth, decrease the carcinogenetic effect of DMBA and postpone the onset of breast cancer in SD rats.

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