Influence of Caffeine Consumption on Carcinomatous and Normal Mammary Gland Development in Mice

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ABSTRACT

The influence of caffeine consumption on the development of 7,12-dimethylbenz(a)anthracene-induced mammary carcinomas in BD2F1 female mice and spontaneous mammary carcinomas in nulliparous C3H mice was examined. Caffeine (250 and 500 mg/liter of drinking water) was administered to BD2F1 mice commencing 1 week after a series of 6 weekly 7,12-dimethylbenz(a)anthracene intubations, until experiment termination. Caffeine was administered to C3H mice (via drinking water) commencing at 8 weeks of age to experiment termination. In BD2F1 mice receiving 250 and 500 mg of caffeine, mammary carcinoma multiplicity (number of mammary carcinomas/mouse) was increased by 20 and 40%, respectively. In C3H mice receiving 250 and 500 mg caffeine, mammary carcinoma multiplicity was increased by 13 and 117%, respectively. In both BD2F1 and C3H mice, the higher dose level of caffeine resulted in a significant (P < 0.05) increase in mammary carcinoma multiplicity. Caffeine consumption did not significantly effect the percentage of mice bearing mammary carcinomas or the mean latency period of mammary tumor appearance. In a second series of studies, the influence of caffeine consumption on mammary gland development in female BALB/c mice was assessed in vivo and in vitro (organ culture). In mice consuming caffeine (500 mg/liter of drinking water), mammary gland development was significantly (P < 0.05) increased compared to control mice; this difference in mammary development was more conspicuous in mice treated with mammotrophic hormones. In the organ culture studies, mammary glands derived from caffeine (500 mg/liter of drinking water) consuming BALB/c mice were more responsive in vitro to a mammotrophic hormonal developmental growth stimulus than were mammary glands derived from control mice (P < 0.05). These results provide evidence that caffeine consumption can enhance mammary tumorigenesis in C3H and carcinogen-treated BD2F1 female mice and, in addition, enhance developmental growth of the normal female mouse (BALB/c) mammary gland.

INTRODUCTION

In the preceding reports, we provide compelling evidence that chronic consumption of caffeine can significantly modulate the development of mammary carcinomas in female rats treated with a chemical carcinogen (1, 2). When caffeine was administered (via drinking water) prior to and during carcinogen treatment (initiation phase), mammary tumorigenesis was significantly suppressed. In contrast, when caffeine was administered commencing 3 days after carcinogen treatment (promotion phase), the final yield of mammary carcinomas was not significantly different than that observed in control animals, although an early temperate stimulatory effect on mammary carcinoma development was often observed.

In this report we further examine the relationship between caffeine consumption and the development of mammary tumors in two additional experimental animal models for human breast cancer, i.e., the C3H spontaneous mammary tumor mouse model (exogenous MTV positive, carcinomas of alveolar origin) and the carcinogen-treated BD2F1 mouse mammary tumor model (exogenous MTV negative, carcinomas of ductal origin). The influence of chronic caffeine consumption on the genesis of mammary tumors in these well-known and often-studied mouse models has heretofore not been examined. In addition, we examine whether or not caffeine consumption can influence developmental growth of the normal mouse (BALB/c) mammary gland.

MATERIALS AND METHODS

All animals used in these studies were nulliparous female BD2F1 mice (C57BL x DBA/2F1) (The Jackson Laboratories, Bar Harbor, ME), nulliparous female C3H mice (Cancer Research Laboratory, University of California, Berkeley, CA), and nulliparous female BALB/c mice (Charles River Breeding Laboratories, Inc., Wilmington, MA). They were housed in a temperature-controlled (24°C) and light-controlled (14 h/day) room and were given a standard commercial laboratory mouse/rat chow (Teklad; Harlan Sprague-Dawley, Inc., Winfield, IA) ad libitum throughout the study.

Caffeine Consumption and Chemical Carcinogenesis of the BD2F1 Mouse Mammary Gland

One hundred sixty-four BD2F1 mice were treated with DMBA (Eastman Kodak Co., Rochester, NY) once weekly for 6 weeks beginning at 8 weeks of age. DMBA was dissolved in sesame oil and administered i.g., 1 mg mouse/week. One week after the last carcinogen intubation, the mice were divided into three groups (54–55 mice/group). Animals of Group 1 served as controls. Animals of Groups 2 and 3 received caffeine (ICN Pharmaceuticals, Inc., Cleveland, OH) in their drinking water at a concentration of 250 and 500 mg/liter, respectively. Caffeine solutions (drinking water) were prepared fresh three times per week (MWF). All mice were treated daily as described above until termination of study (20 weeks after the last dose of carcinogen). Beginning 1 week after the last dose of carcinogen, all mice were palpated at 2-week intervals for the presence of mammary tumors. At termination of study, all mammary tumors (palpable and nonpalpable) were excised, fixed in Bouin's fluid, stained with hematoxylin and eosin, and examined histologically.

Caffeine Consumption and Spontaneous Mammary Gland Tumorigenesis in C3H Mice

One hundred twenty-two C3H mice were divided into three groups (37–43 mice/group) at 8 weeks of age. Animals of Group 1 served as controls. Animals of Groups 2 and 3 received caffeine in their drinking water at a concentration of 250 and 500 mg/liter, respectively. This study was terminated when the mice reached 51 weeks of age. Assessment of mammary tumor development was performed as described above. Additionally, at termination of study, the number 4 (inguinal) mammary glands were excised from randomly selected mice, coded, and examined for development by whole-mount evaluation, as described previously (3), on a scale of 1–6 as follows: 1 = few ducts, few or no end buds; 2 = moderate duct growth, moderate number of end buds; 3 = numerous ducts and branches, many end buds; 4 = numerous ducts and branches, minimum lobulo-alveolar development; 5 = numerous ducts and branches, moderate lobulo-alveolar development; 6 = numerous ducts and branches, dense lobulo-alveolar development as in late pregnancy.
Caffeine Consumption and the Developmental Growth of the BALB/c Mouse Mammary Gland

Assessment of Mammary Gland Development in Vivo. Eighty BALB/c mice were divided into four groups (20 mice/group) at 60 days of age. Animals of Groups 1 and 3 served as controls; animals of Groups 2 and 4 received caffeine in their drinking water at a concentration of 500 mg/liter. Caffeine treatments were for 30 days. Ten days prior to the last day of caffeine treatment, mice of Groups 3 and 4 received daily s.c. injections of 17β-estradiol (1 µg) and progesterone (1 mg) (for 10 days); mice of Groups 1 and 2 received daily (for 10 days) injections of saline (0.9% NaCl solution). The steroid hormones were obtained from Sigma Chemical Co., St. Louis, MO. The mice were sacrificed, the number 4 (inguinal) mammary glands were excised from each mouse, coded, and examined for development by whole-amount evaluation on a scale of 1–6 as described above.

Assessment of Mammary Gland Development in Vitro. Twenty BALB/c mice were divided into two groups (10 mice/group) at 60 days of age. Animals of Group 1 served as controls; animals of Group 2 received caffeine via their drinking water, 500 mg/liter. Caffeine treatments were for 30 days. Ten days prior to the last day of caffeine treatment, all mice were treated daily (for 10 days) with 17β-estradiol (1 µg) and progesterone (1 mg). The mice were sacrificed, the number 2 (thoracic) mammary glands were excised and prepared for organ culture analysis.

Detailed procedures for organ culture of the whole mammary gland of the mouse have been thoroughly described by Banerjee et al. (4). The number 2 thoracic mammary glands were placed on sterile dacron rafts in 35-mm Petri dishes, and the glands, resting on the rafts, were then incubated in Waymouth’s medium (MB 752/1; Gibco, Grand Island, NY). Incubations were carried out in a 95% O2:5% CO2 atmosphere in a humidified chamber at 37°C. All incubations were for 6 days. Growth-promoting medium contained the following hormones (and concentrations): bovine insulin (5 µg/ml; Sigma Chemical Co.); bovine prolactin (5 µg/ml; NIH); bovine growth hormone (5 µg/ml; NIH); aldosterone (1 µg/ml; Sigma); 17β-estradiol (0.001 µg/ml; Sigma); and progesterone (1 µg/ml; Sigma).

Each mouse in each group served as its own control; i.e., 1 mammary gland (of each number 2 gland pair) was placed in the basal tissue culture medium, and the contralateral gland was placed in the basal tissue culture medium containing mammotropic hormones (growth-promoting medium). The differential developmental growth response (growth in basal medium versus growth in basal plus mammotropic hormone medium) of each paired number 2 mammary gland for each mouse in each group was determined.

After 6 days of culture, the mammary glands were coded, fixed in glacial acetic acid:100% ethanol (1:3, v/v), stained with alum carmine, and examined for development by whole-mount evaluation on a scale of 1 to 6 as described above. After whole-mount evaluation, the glands were embedded horizontally in a paraffin preparation (Tissue-Prep; Fischer Scientific, Fairlawn, NJ), sectioned at 30 µm, mounted on glass slides, and stained with hematoxylin. Each 30-µm section, throughout the entire gland, was assessed for epithelial area by a computer-assisted image analysis system (R & M Biometrics Corp., Nashville, TN) (5). A television camera attached to a low-power microscope generates an image of the tissue section consisting of light (fat pad) and dark (epithelium) points. This image is relayed to a computer (Apple Iie), which counts the number of dark points. These data are then converted to area (mm²) measurements. The total epithelial area of these sections for each gland was determined. BALB/c mouse mammary glands are ideal for such analysis as the epithelium (ducts and alveoli) of the mammae stain dark (hematoxylin), contrasting exceptionally well with the nonstaining surrounding adipose tissue (fat pad).

Statistics

Percentage of mice bearing mammary tumors was analyzed by chi-square analyses. Mean number of mammary tumors per mouse (mammary tumor multiplicity), mean latency period of mammary tumor appearance, and mean body weights were analyzed by analysis of variance. Mean differences between mammary gland development were evaluated by the nonparametric Wilcoxon rank procedure test. In vitro (organ culture) data were analyzed by split plot analysis of variance.

RESULTS

Effect of Caffeine Consumption on Mammary Tumorigenesis in Female BD2F1 Mice Treated with DMBA. When caffeine at 250 and 500 mg (per liter of drinking water) was administered to BD2F1 mice commencing 7 days after the last dose of carcinogen, a 20 and 40%, respectively, increase in mammary carcinoma multiplicity (number of mammary carcinomas/mouse) was observed (Table 1). Because of the limited number of mammary carcinomas that was observed in these animals, this increase in tumor number just reached the 5% level of statistical probability in the mice receiving the higher dose level of caffeine. Of the mammary carcinomas that occurred in the BD2F1 mice, 49% were adenocarcinomas and 51% were adenocanthomas; no correlation between treatment and tumor type was noted. The percentage of mice with mammary carcinomas, mean latency period of mammary tumor appearance, gain in body weights, and nontumor-related mortality (negligible) were not significantly affected by treatments.

Effect of Caffeine Consumption on Spontaneous Mammary Tumorigenesis in Female C3H Mice. When caffeine at 250 and 500 mg (per liter of drinking water) was administered to C3H mice commencing at 8 weeks of age, a 13 and 117%, increase, respectively, in mammary carcinoma multiplicity was observed (Table 1). The increase in mammary carcinoma multiplicity in mice consuming the higher level of caffeine was significant (P < 0.05). All of the mammary tumors were adenocarcinomas. The percentage of mice bearing mammary carcinomas, mean latency period of mammary tumor appearance, gain in body weights, and nontumor-related mortality (negligible) were not significantly affected by caffeine treatment.

Assessment of mammary gland development by whole-mount evaluation in the C3H mice at termination of the study indicated a slight increase in development scores in caffeine treated mice (Table 2). This difference did not reach the 5% level of statistical probability. Although there appeared to be slightly more mammae lobulo-alveolar development in caffeine-treated mice, compared with controls, this difference was difficult to quantitate due to the presence of large numbers of hyperplastic alveolar nodules in the mammae of both control and caffeine-treated mice.

Effect of Caffeine Consumption on Developmental Growth of the Female BALB/c Mouse Mammary Gland. Mean mammary gland development score in 90-day-old control BALB/c mice was 1.2; in mice receiving caffeine (500 mg/liter of drinking water), mean mammary gland development score was 1.9 (Table 2). The difference between the mean development scores in the two groups of mice was 0.7 (P < 0.05). In estrogen/progesterone-treated control mice, the mean mammary gland development score was 3.0; in mice receiving these hormones and caffeine, the mean mammary gland development score was 4.1. The difference between these development scores was 1.1 (P < 0.05). In general, caffeine consumption increased duct branching in nonhormone-treated mice and increased duct branching and lobulo-alveolar development (differentiation) in hormone (estrogen and progesterone)-treated mice; a greater differential between controls and caffeine consuming mice was observed in hormone-treated mice than in mice not treated with hormones.

The influence of caffeine consumption on hormone responsiveness of the BALB/c mouse mammary gland assessed in...
Table 1 Effect of caffeine consumption on development of DMBA-induced mammary tumors in BD2F1 mice and spontaneous mammary tumors in C3H mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Percentage of mice with mammary carcinomas</th>
<th>Total number of mammary carcinomas</th>
<th>Number of mammary carcinomas per mouse</th>
<th>Mean latency period of mammary tumor appearance (weeks)</th>
<th>Mean body weights (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BD2F1 mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>55</td>
<td>33</td>
<td>28°</td>
<td>0.5 (100)°</td>
<td>11.3</td>
<td>22.8</td>
</tr>
<tr>
<td>Caffeine (250 mg)</td>
<td>54</td>
<td>41</td>
<td>35°</td>
<td>0.6 (120)°</td>
<td>13.8</td>
<td>22.8</td>
</tr>
<tr>
<td>Caffeine (500 mg)</td>
<td>55</td>
<td>44</td>
<td>38°</td>
<td>0.7 (140)°</td>
<td>11.5</td>
<td>22.9</td>
</tr>
<tr>
<td>C3H mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>42</td>
<td>81</td>
<td>100°</td>
<td>2.4 (100)°</td>
<td>34.1</td>
<td>18.6</td>
</tr>
<tr>
<td>Caffeine (250 mg)</td>
<td>43</td>
<td>79</td>
<td>118°</td>
<td>2.7 (113)°</td>
<td>34.1</td>
<td>18.9</td>
</tr>
<tr>
<td>Caffeine (500 mg)</td>
<td>37</td>
<td>86</td>
<td>191°</td>
<td>5.2 (217)°</td>
<td>34.2</td>
<td>18.9</td>
</tr>
</tbody>
</table>

a DMBA was administered (i.g.) once weekly for 6 weeks beginning at 8 weeks of age. Caffeine (mg/liter of drinking water) was provided commencing 1 week after the last dose of carcinogen until termination of study (20 weeks after the last carcinogen treatment).

b 49% and 51% of the mammary tumors were mammary adenocarcinomas and mammary adenoacanthomas, respectively; no correlation between treatment and histological tumor type was noted. The total number of mammary carcinomas is the sum total of adenocarcinomas and adenoacanthomas.

c P < 0.05.

d Caffeine (mg/liter of drinking water) was provided commencing at 8 weeks of age and until termination of study (51 weeks of age).

e 100% of the mammary tumors were mammary adenocarcinomas.

Table 2 Effect of caffeine consumption on mammary gland development in vivo in female C3H and BALB/c mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Mean mammary gland development scores</th>
<th>Mean mammary gland epithelial area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3H mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline-treated mice</td>
<td>20</td>
<td>1.2 ± 0.1°</td>
<td>8.8 ± 1.4°</td>
</tr>
<tr>
<td>Estrogen/progesterone-treated mice</td>
<td>20</td>
<td>1.9 ± 0.2°</td>
<td>21.6 ± 1.8°</td>
</tr>
<tr>
<td>BALB/c mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline-treated mice</td>
<td>20</td>
<td>3.0 ± 0.1°</td>
<td></td>
</tr>
<tr>
<td>Estrogen/progesterone-treated mice</td>
<td>20</td>
<td>4.1 ± 0.2°</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05.

Table 3 Effect of caffeine consumption on mammary gland hormone responsiveness in vitro in female BALB/c mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Mean mammary gland development scores</th>
<th>Mean mammary gland epithelial area (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls (10 mice)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal media</td>
<td>10</td>
<td>2.1 ± 0.1°</td>
<td>8.8 ± 1.4°</td>
</tr>
<tr>
<td>Basal media + hormones</td>
<td>10</td>
<td>4.3 ± 0.2°</td>
<td>21.6 ± 1.8°</td>
</tr>
<tr>
<td>Caffeine-treated mice (10 mice)</td>
<td>10</td>
<td>2.4 ± 0.1°</td>
<td>10.1 ± 2.1°</td>
</tr>
<tr>
<td>Basal media</td>
<td>10</td>
<td>5.1 ± 0.2°</td>
<td>33.1 ± 3.0°</td>
</tr>
<tr>
<td>Basal media + hormones</td>
<td>10</td>
<td>7.2 ± 0.2°</td>
<td>33.1 ± 3.0°</td>
</tr>
</tbody>
</table>

*p < 0.05.

Discussion

In this study, we examined the effect of caffeine consumption on mammary tumorigenesis in two different rodent mammary tumor models, i.e., the carcinogen-treated BD2F1 mouse model (exogenous MTV negative, ductal carcinomas) and the spontaneous C3H mouse model (exogenous MTV positive, alveolar carcinomas). In both models, caffeine treatment increased mammary carcinoma multiplicity; the effect of caffeine was more apparent in the C3H mouse, less apparent in the BD2F1 mouse. These results are not in total accord with the results described in the preceding communications (1, 2) in which chronic and prolonged caffeine consumption during promotion in rats treated with DMBA did not significantly increase the final yield of mammary carcinomas. Instead, these results coincide more closely to the temperate and transient stimulatory effect of caffeine during the early developmental promotional stages of rat mammary gland carcinogenesis. Although the total numbers of mammary tumors that were observed in the carcinogen-treated BD2F1 mice were disappointing low, thus yielding a statistical value that just reached the 5% level of probability, the increase in the number of mammary carcinomas in the caffeine-treated groups was quantitatively similar to that observed in the DMBA-treated rats which consumed caffeine during the early promotional stages of mammary carcinoma development (1, 2). In the C3H mouse model, initiation and/or promotion phases are not readily or easily examined; we cannot conclude, therefore, which phase of tumorigenesis in this animal model was affected (enhanced) by caffeine consumption.

In BALB/c mice chronically treated with caffeine, mammary gland development was significantly increased above that ob-
served in control mice. This was observed in both hormone-treated and nonhormone-treated mice. Enhancement of mammary development by caffeine was more evident in the hormone-treated mice than in mice not treated with hormones. In C3H mice, chronic caffeine treatment appeared to slightly enhance mammary gland development although this effect did not reach the 5% level of statistical probability; these glands were difficult to assess for development because of the presence of large numbers of hyperplastic alveolar nodules. Our results are in accord with a recent report by Nagasawa et al. (6) who observed an increase in mammary development and numbers of hyperplastic alveolar nodules in female SLN mice chronically treated with moderate dose levels of caffeine. In our study, hyperplastic alveolar nodules number and size, as a function of caffeine consumption, was not determined.

When mammary glands were excised from BALB/c mice and plated in 6-day organ culture, the enhancement of mammary development by mammotropic hormones in vitro was more evident in mammas excised from caffeine-treated mice than in control mice. This was apparent when mammas development was assessed by whole-mount evaluation (development scores) and epithelial area. The results of this study would seemingly support the supposition that caffeine enhances mammotropic hormone developmental growth responsiveness of the mouse mammary gland. Although we have shown in both our in vitro and in vivo studies that the administration of mammotropic hormones did increase mammas development more noticeably in mammas derived from (or in) caffeine treated mice, when compared with controls, we cannot conclude that caffeine acts by enhancing hormone-induced developmental growth responsiveness of the mouse mammary gland. Because caffeine enhances mammary gland development in vivo, the mouse mammas (in both the in vivo and in vitro studies) do not doubt already had heightened developmental growth prior to hormone administration. What we can conclude, without hesitation, is that caffeine administration in vivo does significantly increase development of the BALB/c mouse mammary gland; the caffeine-induced increase in mammary development is more conspicuous after administering (in vivo or in vitro) a mammotropic hormonal developmental growth stimulus.

The mechanism by which caffeine consumption modulates mammary tumorigenesis in experimental animals is entirely speculative. It is conceivable that caffeine consumption may modulate normal and neoplastic mammary gland development in rodents by altering neuroendocrine activity. In rodents, caffeine can affect brain monoamine (e.g., catecholamines, serotonin) activity and perhaps the activity of other neurotransmitters (e.g., acetylcholine γ-aminobutyric acid) as well (7). Adenosine-mediated neurotransmission of the brain also appears to be influenced by caffeine (8). Altered activity of the central nervous system by caffeine could consequently influence anterior pituitary hormone secretion. In rodents, relatively high dose levels of caffeine have been reported to decrease serum levels of thyroid-stimulating hormone and increase serum corticosterone levels as well as inhibiting the pulsatile secretion of growth hormone (9, 10). Secretion of prolactin, in contrast, does not appear to be influenced by caffeine treatment (9, 11, 12). Altered secretory rates of these hormones, i.e., thyroid-stimulating hormone, growth hormone, and adrenal corticosterone can clearly influence the development of both the normal and neoplastic rodent mammary gland (13, 14). In the preceding reports (1, 2), we report that moderate dose levels of caffeine consumption consistently inhibit the initiation phase, of DMBA-induced rat mammary gland tumorigenesis while temperately and transiently stimulating the early developmental promotion stages of this tumorigenic process. Although this relationship may appear paradoxical upon first glance, it is important to point out that this relationship has been observed many times previously. For example, endocrine alterations which enhance mammary gland development and differentiation (i.e., the physiological state of pregnancy, the administration of mammotropic hormones), when provided during the initiation phase, inhibits DMBA-induced rat mammary gland tumorigenesis; these same treatments when provided during the promotion phase stimulate this carcinogenic process (14–18). Our results are consistent, therefore, with the proposition that caffeine modulates rodent mammary tumorigenesis by altering systemically the secretion of a hormonal factor or factors affecting the developmental growth of the normal and neoplastic mammary epithelium. Each of the rodent mammary tumor models of human breast cancer used in these studies, i.e., the DMBA-induced rat (Sprague-Dawley) and mouse (BD2F1) mammary carcinoma and the spontaneous C3H mouse mammary carcinoma are eminently developmental growth sensitive to mammotropic hormones (3, 14, 19).

Other mechanisms are also of potential interest. Caffeine has been reported to be mutagenic (20), an activity that could directly influence initiation processes in tumor development via, e.g., its direct binding to DNA or by altering the DNA repair processes. The reported mutagenic activities of caffeine could conceivably influence, indirectly, promotion events as well (21). Caffeine is a 3',5'-phosphodiesterase inhibitor (22); cyclic nucleotides have been reported to be a number of laboratories to influence tumorigenesis in a variety of organ sites (23) including the mammary gland (24). Cyclic nucleotide levels could also be altered by certain brain neurotransmitters (25) or pituitary hormones (26). Caffeine has been reported to increase serum-free fatty acid levels (27); the lipolytic effect of caffeine, presumably due to the ability of the alkaloid to mobilize body fat stores via adrenergic mechanisms, may be important in tumorigenic processes in a variety of organs including the mammary gland (28). Caffeine has also been reported to raise Ca2+ levels in mammalian tissues (29) and intracellular Ca2+ levels have been reported to be elevated in tumor cells (30). Although the results of our studies do not shed light on which of the above cited mechanisms are important in caffeine-induced modulation of rodent mammary gland tumorigenesis, our results provide compelling evidence that caffeine can significantly modify this tumorigenic process, a phenomenon that is clearly dependent upon the time-span of caffeine treatment, the dose of caffeine used, and the animal model examined.

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