Effect of genistein on DMBA-induced oral carcinogenesis in hamster

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Genistein, an isoflavone present in soy at high concentrations and being considered the primary antitumor constituent in soy, has been known to be a natural anticancer agent in in vitro studies and experimental animal models. The present study was aimed to investigate the putative chemopreventive effect of genistein on oral carcinogenesis in hamster cheek pouch at the post-initiation stage, as well as its effect on angiogenesis during this process. DMBA solution (0.5% in mineral oil) was applied topically to the left cheek pouch of male Syrian golden hamsters three times a week for 6 weeks. Two days after the last treatment of DMBA, genistein suspended in distilled water (10 mg/kg body Wt/day) or same volume of distilled water was administered into the animals by gavage daily for 12 weeks. The genistein treatment decreased the visible oral tumor incidence to 40.7 (11/27) from 53.6% (15/28) of the positive control, but the difference was not statistically significant (P = 0.34). And no significant difference in the average number of tumors/tumor-bearing hamster, the average tumor volume, or latency was observed between the control and the genistein-treated group. Vascular density in OSCC of the genistein-treated group and that of the control group was similar and there was no significant difference. Importantly, three animals in the genistein-treated group produced poorly-differentiated fibrosarcomas in the DMBA-painted cheek pouches. The exact mechanism behind this needs further investigation. In conclusion, no inhibitory effect of genistein on chemically-induced post-initiation stage of oral carcinogenesis was observed in the present study. On the contrary, genistein appeared to promote oral submucosa stroma tumorigenesis in concert with DMBA. So caution should be warranted for people with predisposition to oral cancer.

Introduction

Oral squamous cell carcinoma (OSCC) is the most common of the head and neck cancers and has a poor prognosis. Multiple primary tumors are a known phenomenon in head and neck cancer (1). Furthermore, local–regional recurrence is a common and challenging oncological problem in patients affected by this disease (2). It has been proposed that carcinomas would arise from multifocal areas of precancerous change involved in the process of field cancerization (3–6). Thus the possibility of addressing the issue of precancerous tissue behavior would be contributory. Preventing or treating oral premalignant lesions with natural or synthetic chemical agents is now the most promising approach to prevent oral cancer.

The low incidence of breast cancer in Asians has been attributed, in part, to the high intake of soy products (7–9). Experiments in animal models suggest that soy consumption decreases tumor number, incidence, latency, multiplicity and metastasis (10,11). Soy contains several potential anticancer agents, including protease inhibitors, phytosteroids, saponins, phytates and isoflavones in a high level (12,13). Genistein, one of the most studied isoflavones, is considered as the principle compound responsible for soy’s beneficial effects (14). As a weak antiestrogen, genistein has been shown to have multiple biological activities such as inhibiting topoisomerase II activity (7,15) and tyrosine kinase activity (8,16), inducing cancer cell death, stimulating apoptosis (15,17), and suppressing angiogenesis (18). However, clinical and preclinical laboratory animal and in vitro studies, aimed at evaluating the association between soy intake and reduction of breast cancer, are conflicting. Petракіs et al. (19) demonstrated that consumption of soy protein isolate had stimulatory effects on the breast tissue of premenopausal women. Clinton et al. (20) reported that soy protein isolates containing increasing concentrations of genistein stimulate the growth of estrogen-dependent breast cancer cells in vivo in a dose-dependent manner. They also reported that the glycoside genistin, like the aglycone genistein, can stimulate estrogen-dependent breast cancer cell growth in vivo. Removal of genistin or genistein from the diet caused tumors to regress (21).

Most reports about the anticancer effect of genistein were focused on estrogen-dependent cancers, such as breast cancer, prostate cancer, uterine cancer and so on. There are relatively few reports associating this compound with OSCC. The published in vitro and in vivo studies of the anticancer effect of soy or genistein on oral cancer are, too, inconsistent. Ye et al. (22) demonstrated that genistein inhibited squamous cell carcinoma-25 (SCC-25) cell growth (a human oral squamous carcinoma line). But results from in vivo studies have appeared to be disappointing. Myoung et al. (23) reported that, in an OSCC-bearing nude mice model, the tumor growth and metastatic behavior in the genistein-treated group and the control group were similar and there were no significant differences, although a significantly lower vascular density was found. Moreover, people who have been diagnosed with oral leukoplakia or other oral premalignant lesions often inquire whether soy products, including genistein, will protect from malignant transformation of their diseases. But right now there are even less reports about the preventive effect of genistein on oral precancerous lesions.

Abbreviations: DMBA, 7,12-dimethylbenz[a]anthracene; MVD, microvessels density; OSCC, oral squamous cell carcinoma; SCC, Squamous cell carcinoma.

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The development of oral cancer is a multi-step process requiring initiation, promotion and progression. Application of 7,12-dimethylbenz[a]anthracene (DMBA) to the cheek pouch of the Syrian golden hamster produces SCC and premalignant lesions that are histologically similar to the lesions in humans (24,25). After 6 weeks of DMBA treatment, mucosa lesions of the hamster cheek pouches are just at the premalignant stage, which are similar with human cases with oral leukoplakia or with heavy smokers. This post-initiation stage could be a good time to test the primary preventive effect of chemopreventive agents.

The purpose of the present study was to address two objectives. First, to investigate whether genistein, at a dosage relevant to the real-life consumption pattern, has inhibitory effect on the malignant conversion of oral precancerous lesions. Second, to test whether genistein has antiangiogenic effect during this process of transformation.

Materials and methods

Treatment of animals

All the experiments were conducted at Shanghai Ninth People’s Hospital, affiliated to Shanghai Second Medical University, under Protocol no. 2002-0041. A total of 80 male Syrian golden hamsters (6 weeks old) weighing 60–80 g were purchased from Slac (Shanghai, China). The animals were housed, four per cage, in a room with controlled temperature and humidity with 12 h light–dark cycles. All animals were given sterilized soy-free diet (Slac, Shanghai, China) and tap water ad libitum. After 1 week of acclimatization, the animals were randomly divided into five groups. Left pouches of hamsters in Group A (n = 30) and Group B (n = 30) were topically treated with 100 μl of 0.5% DMBA (Sigma Chemical, St Louis, MO) with a paintbrush three times/week for 6 weeks whilst each animal from Group C (n = 10) was similarly treated with only mineral oil. Two days after the last DMBA treatment, the hamsters in Group B received genistein (Sigma Chemical) suspended in distilled water (10 mg/kg body wt/day) daily by gavage, and animals in Groups A and C received the same volume of distilled water for 12 weeks. Another 10 animals (Group D) received no treatment and served as blank control. Beginning from week 7, each animal was examined once a week in order to record the presence, size and date of detection of all tumors. At the end of week 18, the animals were killed and tissue samples were collected for histopathological and immunohistochemical examination.

Pathological and histopathological examinations

At the completion of the study, animals were killed by cervical dislocation. The number of visible tumors in the oral cavity was counted; the length, width and height of each tumor were measured with a caliper. The tumor volume was calculated by the formula

\[
\text{Volume} = \frac{4}{3} \pi r^3
\]

(where \( r \) is the average radius of the three diameter measurements in mm). Tumors and cheek pouch tissue samples were harvested. Samples from each animal were fixed in 10% formalin and embedded. Six sections (5 μm) of each sample were cut and the slides were H&E stained for histopathological analysis. Basal cell hyperplasia, dysplasia, SCC and papillomas were diagnosed with established criteria (26). The dysplasia of oral epithelium was indicated by increased number of basal cells. The dysplasia was characterized by irregular epithelial stratification, increased number of mitotic figures, increased nuclear-to-cytoplasmic ratio and loss of polarity of basal cells. Papilloma was diagnosed by stratified squamous epithelium over branching fibrovascular cores, including papilloma-hyperplasia and papilloma-dysplasia. Carcinoma was diagnosed by the invasion of underlying tissues, including those originating from papilloma and those from apparently normal epithelium.

Angiogenesis

To assess the angiogenesis of the squamous epithelium of the oral mucosa, tissue sections were immunostained with a rabbit anti-factor VIII antibody (1:500; Dako, Carpinteria, CA). Briefly, sections were pretreated with 0.25% trypsin for 10 min at 37°C. After washing, the samples were incubated in 10% normal swine serum for 20 min, followed by rabbit antihuman von Willebrand factor (1:500), biotinylated swine antirabbit antibody (1:400) for 30 min, and streptavidin biotin complex/horseradish peroxidase for 30 min. All of the sera and avidin biotin reagents were obtained from Dako. In non-lesioned area and preinvasive lesions including hyperplasia, dysplasia and benign papillomas the blood vessels in submucosa were counted. In carcinoma, peritumoral vessels were counted. Highly vascularized areas were identified by scanning sections at low power (40× and 100×). After six areas of highest neovascularization were identified, the number of vessel count was performed on a 200× field (20× objective and 10× ocular), and the average counts of the six fields were determined. Vessel lumens were not necessary for a structure to be defined as a vessel.

Statistical analysis

The tumor incidence of different groups was compared using the χ²-test and Fisher’s exact test. The log-rank test was used to analyze tumor latency. One way ANOVA test was used to compare the number of various oral lesions and micro vessel density (MVD) between positive control group and genestein-treated group. The tumor volume and tumor multiplicity were analyzed with signed rank test, using the computer software SPSS 10.0. Differences with calculated \( P \)-values <0.05 were regarded as significant.

Results

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At week 6, all DMBA-treated animals had a visibly roughened granular surface on the mucosa with varying degrees of erythema and occasional white plaque-like lesion. Cheek pouches of animals from the mineral oil-treated and untreated groups revealed no obvious changes.

In hamsters treated with DMBA followed by genistein, tumors took a little bit longer period of time to develop compared with animals in Group A, the mean appearance time for the first tumor was 12.50 ± 2.22 and 13.30 ± 1.49 (mean ± SD) weeks, repectively, not approaching statistical difference (\( P = 0.30 \)).

At week 18, the genistein treatment (10 mg/kg body wt/day) (Group B) decreased the visible oral tumor incidence to 40.7 (11/27) from 53.6% (15/28) of the positive control (Group A), but the difference was not statistically significant (\( P = 0.34 \)). The average number of tumors/tumor-bearing hamster was 1.46 ± 1.04 and 1.16 ± 0.69 (mean ± SD) for positive control and genistein group, respectively. The average tumor volume was 81.71 ± 67.90 and 77.51 ± 76.39 for positive control and genistein group, respectively. For both parameters, the differences did not reach any statistical significance (Table I).

Histologically, in accordance with previous reports, the left buccal pouches of DMBA-treated animals presented areas of leukoplakia with hyperplasia or dysplasia (Figure 1B) as well as papillomas (papilloma-hyperplasia and papilloma-dysplasia, Figure 1C) and SCC (Figure 1D and E). Histopathological analysis revealed that in the positive control group the dysplasia content was 76.39% (mean ± SD) of the tumors. The peritumoral area had 8.99% (mean ± SD) of the tumors, indicating that the process of transformation was not completed. In the genestein group, the dysplasia content was 57.90% (mean ± SD) of the tumors. The peritumoral area had 8.76% (mean ± SD) of the tumors, indicating that the process of transformation was not completed.

Table I. Effect of genistein on tumor incidence, multiplicity and volume

<table>
<thead>
<tr>
<th>Group</th>
<th>Tumor-bearing hamster</th>
<th>Percentage mean volume (mm³)</th>
<th>Mean multiplicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>15/28</td>
<td>53.6</td>
<td>81.71 ± 67.90</td>
</tr>
<tr>
<td>Genistein</td>
<td>11/27</td>
<td>40.7</td>
<td>77.51 ± 76.39</td>
</tr>
</tbody>
</table>

The animals were treated with genistein (10 mg/kg body wt/day) (Group B) or saline (Group A) for 12 weeks after topical application of 0.5% DMBA to the left oral pouch of hamsters three times/week for 6 weeks. Two animals in Group A and three animals in Group B died during the study period. Multiplicity and volume were compared using ANOVA followed by Dunnett’s test where Group B is compared with Group A. There were no significant differences for any of the two parameters. Tumor volume (mm³) was calculated by the formula:

\[
\text{volume} = \frac{4}{3} \pi r^3
\]

(where \( r \) represents the average radius of three diameter measurements in mm).

Mean (SD) volume = total tumor volume/no. tumor-bearing animals.

Mean (SD) multiplicity = no. tumors/no. tumor-bearing animals.
74% OSCC appeared to have developed from papilloma (Figure 1D). In the genistein-treated group, 8 out of the 11 palpable masses were OSCCs, and the remaining 3 masses were fibrosarcomas. The squamous epithelium appeared intact, but abnormal and frequent mitotic figures as well as extensive cellular atypia were seen in the submucosa connective tissue (Figure 1F). Although the genistein treatment decreased the oral tumor incidence to 40.7 (11/27, Group B) from 53.6% (15/28, Group A), the difference was not statistically significant. When considering the degree of tumor differentiation, an increase of poorly differentiated tumors in the genistein-treated group was observed; this difference reached statistical significance ($P < 0.01$) (Table II).

The number of dysplastic lesions and papilloma per animal was not different between the positive group and the genistein-treated group (Table III). No oral lesions were found in animals from the mineral oil-treated and untreated groups.

### Table II. Degree of differentiation of DMBA-induced oral tumors in hamsters

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of tumor masses</th>
<th>Degree of tumor differentiation (% of masses)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>15</td>
<td>Well 86.7, Moderately 13.3, Poorly —</td>
</tr>
<tr>
<td>Genistein</td>
<td>11</td>
<td>Well 63.6*, Moderately 9.1, Poorly 27.3**</td>
</tr>
</tbody>
</table>

* $P < 0.05$.

** $P < 0.01$ versus control, Fisher’s exact test.

Angiogenesis

In DMBA-induced oral lesions, the neovasculatures primarily concentrated in the stromal areas and spread along stromal ridges on the periphery of epithelial lesions (Figure 2A–C). The MVD of DMBA-induced various oral lesions were significantly higher than that of the non-lesioned area ($P < 0.01$), with increasing small-caliber ($<15 \mu m$ diameter) vessels. Examination of tumor tissue sections revealed that tumor vessels varied greatly in size, with predominating small-caliber ($<15 \mu m$ diameter) vessels. And the MVD in the areas of SCC (Figure 2D) was significantly higher than those of leukoplakia (Figure 2B) and papilloma (Figure 2C) (Table IV). Compared with Group A, genistein treatment did not lead to any significant decrease of vessel density in OSCC (Figure 2E).

In the fibrosarcoma tissue sections of the genistein-treated group, neovasculatures scattered in the tumor areas, with vascular vessels lacking complete lumen and regular formation. The vascular density is much lower than that in the OSCC tissues (Figure 2F).

### Table III. Effect of genistein on DMBA-induced oral carcinogenesis in hamsters

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of hyperplasia</th>
<th>No. of dysplasia</th>
<th>No. of papilloma</th>
<th>No. of carcinoma</th>
<th>No. of fibrosarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive control</td>
<td>2</td>
<td>8</td>
<td>3</td>
<td>15</td>
<td>—</td>
</tr>
<tr>
<td>Genistein</td>
<td>5</td>
<td>7</td>
<td>4</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

The number of dysplastic lesion and papilloma per animal was not different between the positive group and the genistein-treated group (by one way ANOVA test). The incidence of the total tumor was not significantly different between these two groups, although the incidences of carcinoma and fibrosarcoma were different (by the $\chi^2$-test).

Discussion

The present study was aimed at investigating whether genistein, administered since post-initiation stage and at a dosage relevant to the real-life consumption pattern, can influence chemically-induced oral carcinogenesis in male hamsters as well as its possible effect on angiogenesis during this process. In order to find out whether the eastern eating habit of soy foods has any protective effect against oral cancer, we tried to make the dosage we used relate to the real-life consumption...
patterns. However, we did not see any statistical differences in tumor incidence, tumor volume, tumor multiplicity and tumor latency, although genistein treatment decreased the tumor-bearing animals to 11/27 from 15/28. Despite the anticancer effect of genistein on OSCC was shown in some in vitro studies (22,27), it is noted that the concentration required to obtain this effect was higher than the plasma level of genistein in people consuming large amounts of soyfoods. Estimate of average daily genistein consumption by soy consumers range from 0.3 to 1 mg/kg (28–31). On a body weight basis, extrapolating from hamster to human, based on an average weight of a Syrian golden hamster of 150 g and of a man 70 kg, hamsters should be exposed from 5 to 10 times the genistein intake of people eating a diet high in soy in order to achieve a similar pharmacological effect. Hamsters receiving 10 mg/kg body Wt/daygenistein were exposed to 10 times more genistein than the average Asian adult. Hence, the genistein dosage used in the present study is relevant to soy consumption patterns in Asian countries. So according to the results of the present study, genistein, administered since post-initiation stage and at a dosage relevant to the real-life consumption pattern, may not be helpful in inhibiting the malignant transformation of oral premalignant lesions.

But thinking of the possible dose-dependent response of genistein, it is probable that varied concentrations of genistein may exert different effects on oral carcinogenesis. In addition, with view to the timing of exposure determining the metabolism, bioavailability and biological action of genistein, dosing genistein after exposure to DMBA may not be able to maximize the chemopreventive effect, since it takes a few days to weeks for genistein to reach a pharmacologically steady state in vivo. So we are carrying out a series of studies presently in our laboratory, attempting to find out the effects of genistein, in varied concentrations, on oral carcinogenesis when dosed with before exposure to carcinogens or at the same time with carcinogens exposure. We hope to find out the dose–response relationship and the timing of the genistein treatment with oral carcinogenesis.

With regard to the in vivo studies, Myoung et al. (23) did not find any inhibitory effect of genistein on the transplanted human OSCC all through the study period, although a decrease of MVD was observed. In the present study, genistein treatment did not lead to statistically significant decrease of vessel density in OSCC or lesions with dysplasia. In Myoung’s study, the rapidly growing transplantable mouse tumor model was employed, which is usually grown as a solid, localized tumor.

Table IV. Effect of genistein on angiogenesis during the DMBA-induced oral carcinogenesis

<table>
<thead>
<tr>
<th>Group</th>
<th>Normal</th>
<th>Inflammation</th>
<th>Hyperplasia</th>
<th>Dysplasia</th>
<th>Papilloma</th>
<th>OSCC</th>
<th>Fibrosarcoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>—</td>
<td>—</td>
<td>7.67 ± 1.05</td>
<td>10.80 ± 1.10</td>
<td>15.80 ± 0.84</td>
<td>19.60 ± 0.64</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>—</td>
<td>—</td>
<td>7.51 ± 0.85</td>
<td>10.19 ± 1.00</td>
<td>15.60 ± 0.99</td>
<td>18.87 ± 1.23</td>
<td>15.14 ± 0.99</td>
</tr>
<tr>
<td>C</td>
<td>5.50 ± 0.59</td>
<td>6.21 ± 0.79</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>D</td>
<td>5.14 ± 0.34</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

MVD = vessel count/ high powered field, magnification is 200×. The number of vWF-positive blood vessels was counted as described in Materials and methods. The MVD in fibrosarcomas of the genistein-treated group was significantly lowered than that in OSCC both of the genistein-treated group (Group A) and the positive control group (Group B) (P < 0.01). But no decrease of MVD was observed between the genistein-treated group (Group A) and the positive control (Group B) (by one way ANOVA test).

Fig. 2. Angiogenesis in DMBA-induced various oral lesions (factor VIII immunostaining). (A) Normal epithelium; (B) Leukoplakia; (C) Papilloma; (D) OSCC from the positive control group OSCC developed from papillomas; (E) papillary SCC from the genistein-treated group, no decrease in MVD was observed compared with (D); (F) Fibrosarcoma from the genistein-treated group, the MVD is much lower than that in the OSCC, the vessels were irregular, lacking of visible lumens.
in the subcutaneous space. This kind of tumor models will contain an extremely high proportion of newly formed immature vessels, and it is such vessels that appear to be especially vulnerable to the effects of most antiangiogenic drugs (32). Consequently, this approach almost certainly exaggerates the antiangiogenesis responses. Secondly, the response of a tumor mass growing ectopically may be abnormal compared with the same tumor growing in a physiologically relevant site (33). Thirdly we investigated the effects of genistein through oral intake instead of subcutaneous injection as described in Myoung’s study; the former is just the same way humans take genistein. Thus we believe that the results of the present study would provide a more faithful picture of what will occur later in the clinic, although there are still much difference between animal tumors and human tumors.

We also showed in the present study that genistein treatment caused occurrence of poorly-differentiated fibrosarcomas in three of the DMBA-treated hamsters. To the best of our knowledge, this was the first time that fibrosarcoma was induced in the hamster cheek pouches by DMBA treatment conjuncted with genistein. The Syrian hamster buccal pouch is thought of as an excellent model for studying oral carcinogenesis which, under the induction of DMBA, consistently produces SCCs as well as the precancerous leukoplakias that precede them. However, in the present study, we found that after 6 weeks of DMBA induction and subsequently 12 weeks of genistein intervention, the cheek pouches of 3 out of 27 hamsters produced fibrosarcoma, another 8 hamsters produced OSCC. It was conceivable that these fibrosarcomas resulted, at least partly, from the effect of genistein. The precise reasons for this are unclear. We speculated that it may be a result of estrogenic activity of genistein (34). Many studies have shown that estrogen has a biological role in oral mucosa, with estrogen receptor-beta the predominant estrogen receptor subtype (35,36). Another study also showed that the gingival stroma of rats is a target organ of estrogen. In this study, the author noticed that estradiol induces alterations of gingival lamina propria, such as increase in activity of fibroblasts, number of eosinophils and intercellular substance, and the promotion effect of estrogen on the growth of stoma was strongly suggested (37). Therefore, it is possible that the hormonal activity of genistein acted in concert with the carcinogen DMBA to give rise to the carcinogenic effects.

Besides its estrogen-like activity, genistein was also reported to be genotoxic. Metzler et al. (38) reported that genistein was clastogenic in cultured mammalian cells and leads to gene mutation; it was proved to be a strong inducer of DNA strand breaks and micronuclei containing acentric fragments (39). On the other hand, it should be noted that, for most people, soy is a healthy food. As one of our previous studies (unpublished data) showed, genistein treatment only induced no tumor masses or leukoplakia-like lesions on hamster cheek pouches. Histopathological examination also showed that all cheek pouches of these hamsters were normal. In this study, no carcinogenic activity was observed with genistein per se. Different experimental protocol and marked differences in concentration and qualitative–quantitative composition of the soy products used may lead to such contrary results. Further investigations are needed to clarify this confusion. After all, isoflavones are more than phytoestrogens, and they also possess non-hormonal properties; for example, they exert antioxidant effects under some experimental conditions (40,41) and influence the activity of enzymes involved in the metabolism of estrogen and that of regulate cell growth and differentiation (42,43).

It has been suggested that women with estrogen-dependent breast cancer or a predisposition to it may want to reduce their consumption of soy products with high isoflavone content (44). According to the results of our study, not only for women with breast cancer but also for people with oral leukoplakia or with prior exposure to carcinogens, such as heavy smokers, there is a need for additional consideration into the possibility of enhancing or promoting tumor growth by consumption of isoflavone containing products.

In conclusion, our research did not find genistein, administered since post-initiation stage and at a dosage relevant to the real-life consumption pattern, to have any preventive effect on DMBA-induced oral carcinogenesis in hamster cheek pouch. Moreover, it showed that genistein might modify the histomorphological characteristic of the occurring tumors which expressed a rather poor differentiation. Under the joint action of genistein, not only the squamous epithelium but also the submucosa stroma may undergo malignant transformation during the DMBA-induced carcinogenesis. Though this novel finding needs validation and the mechanism behind this needs further investigation, caution is necessary for people with oral leukoplakia or former and current smokers consuming dietary genistein.

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Conflict of Interest Statement: None declared.

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