ABSTRACT

Purpose: The present study was aimed to investigate the comparative dose response effect of Agaricus bisporus (AB) and Agaricus bisporus loaded chitosan nanoparticles (ABCNPs) against 7, 12-dimethylbenz[a]anthracene (DMBA) induced mammary carcinosgenesis in female Sprague-Dawley rats.

Materials and Method: A single subcutaneous injection of DMBA (25 mg/kg bw) in the mammary gland was induced to develop mammary carcinoma on experimental animals. Rats were treated with different doses of AB (100, 200 & 400 mg/kg bw) and ABCNPs (15, 30 & 60 mg/kg bw) which effectively reduced the oxidative damage on DMBA induced tumor bearing animals, which resulted in the extent of lipid peroxidation with concomitant increase in the activities of enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)) and non-enzymatic (reduced glutathione (GSH), Vitamin C and Vitamin E) levels on experimental animals.

Results: From our results we concluded that among different doses of treatment, 200 mg/kg bw of AB and 30 mg/kg bw of ABCNPs were found to be the optimum dose which effectively altered the DMBA induced oxidative stress on mammary carcinogenesis.

Conclusion: The oral administration of AB and ABCNPs has dose dependently inhibited the DMBA induced oxidative stress on mammary carcinogenesis in experimental animals.

Keywords: Agaricus bisporus, DMBA, Chitosan nanoparticles, Sprague Dawley rats, Lipid peroxidation.

INTRODUCTION

Breast cancer is a complex multi-stage disease, involving the deregulation of a number of different signaling cascades. It is a most common type of cancer next to the lung cancer. The American Cancer Society estimated that approximately 226,870 new patients would be diagnosed with breast cancer and 39,510 women would die from this disease in the year 2012. The incidence rate of breast cancer is rapidly increasing in East Asian countries where the historical rate of occurrence had been relatively low changes in lifestyles, such as alterations in dietary habits, have been suggested as possible causes of this sharp rise in breast cancer incidences. Chemotherapy is a significant anti-cancer therapy which can reduce tumor cell proliferation but also tumor growth. Agents, as they suppress aromatase activity and estrogen biosynthesis as possible causes of this sharp rise in breast cancer incidences.

Chemotherapy has been shown to be beneficial in elderly patients as an alternative therapeutic activities such as anti-carcinogenic, anti-inflammatory, immunesuppressive, cardiovascular, anticancer, antiviral, antibacterial, antiparasitic, hepatoprotective and glycemic. AB contains phytochemicals (flavonoids or lignans) with anti-aromatase containing chitin, which is indigestible. To break down chitin, and allow digestion, a heated extraction process is required. The fatty acids involved in the onset of many diseases like cancer, rheumatoid arthritis, and atherosclerosis. The uncontrolled production of free radicals is involved in the damage of the treatment of various diseases including cancers. Several nanotechnological approaches have been used to improve delivery of chemotherapeutic agents. Nanocarriers of chitosan possess high drug loading where they can avoid clearance by the reticuloendothelial system. However degradable polymers have been used as potential carriers in various drug delivery systems. Among other polymeric carriers chitosan, the second most abundant natural biopolymer in nature plays way for an effective oral delivery system.

Chitosan is produced commercially by deacetylation of chitin, which is the structural element in the exoskeleton of crustaceans (such as crabs and shrimp) and cell walls of fungi. Mushrooms cell walls contain chitin, which is indigestible. To break down chitin, and allow digestion, a heated extraction process is required. It has been extensively used in pharmaceutical and medical fields, due to its favourable biological properties such as biodegradability, bio-compatibility, low toxicity, hemostatic, bacteriostatic, fungistatic, anti-cancerogenic and anti-cholesteremic. Because of its chemical structure, chitosan and its derivative have been investigated in the development of controlled release drug delivery systems, since chitosan mucoadhesive property can enhance drug transmucosal absorption and promote sustained release of drug.

The different antioxidant systems have developed by human body to protect against free radical attacks. High correlation between polyphenolics and scavenging potential of different reactive oxygen species indicates that polyphenolics are considered as main antioxidants. The uncontrolled production of free radicals is involved in the onset of many diseases like cancer, rheumatoid arthritis, and atherosclerosis. The excessive production of reactive oxygen species (ROS) are known to induce lipid peroxidation (LPO), causing the deterioration of foods to induce oxidation of lipids and DNA, resulting in membrane damage, to decrease membrane fluidity, and to cause changes that lead to cancer via DNA mutation. The appropriate balance between LPO and antioxidants should be maintained in the cell because of their potential importance in the pathogenesis of various pathologic diseases including cancer. The compassion of the cell to ROS is attenuated by an array of enzymic and non-enzymic antioxidants. Non-enzymic antioxidants such as GSH, Vitamins C and E play an excellent role in protecting the cells...
from oxidative stress\(^1\). Superoxide dismutase and catalase are enzymatic antioxidants that catalyze the detoxification of superoxide anion and hydrogen peroxide (H\(_2\)O\(_2\)), respectively, protects the cell against ROS induced damage. Reduced glutathione (GSH) in combination with glutathione peroxidase plays a vital role in the defense against free radicals, peroxides, and a wide range of xenobiotics and carcinogens\(^2\). Hence the present study was aimed to investigate the chemotherapeutic effect of \(A.bisporus\) (AB) extracts and its encapsulated chitosan nanoparticles (ABCNPs) on DMBA induced mammary cancer in a dose dependent manner.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Chitosan, Sodium tripolyphosphate, 7,12-Dimethylbenz(a)anthracene, were purchased from Sigma Chemical Company, USA. All the other chemicals used were of analytical grade. MilliQ water was used to synthesize nanoparticles.

**Animals**

All animal studies were conducted in central animal house after approval from the Institutional Animal Ethics Committee endorsed by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (No. 930; dated:29.05.2012). Government of India guidelines. 6-week-old female Sprague Dawley rats were obtained from National Institute of Nutrition, Hyderabad, India and maintained in the Central Animal House, Rajah Muthiah Medical College and Hospital, Annamalai University. The animals were housed in polypropylene cages at room temperature (27 ± 2°C) with relative humidity 55 ± 5%, in an experimental room under a 12h light/12 h dark cycle. They were fed a commercial pellet diet and water freely throughout the study.

**Edible fungal material**

*Agaricus bisporus* (AB) were commercially purchased from Cuddalore in vegetable markets, Tamil Nadu. A voucher specimen (no. 217) was deposited in Department of Botany, Annamalai University.

**Preparation of Ethanol extracts**

Powder of AB (50g) were extracted by stirring with 500 ml of ethanol (30°C) at 150 rpm for 24 h and filtered through Whatman No. 4 filter paper. The residues of ethanol extract was then rotary evaporated at 40°C to dryness, re-dissolved in ethanol to a concentration of 10 mg/ml and stored at 4°C for further use.

**Preparation of AB loaded chitosan nanoparticles**

AB loaded chitosan nanoparticles were synthesized by ionic gelation method using tripolyphosphate as a gelating agent. A known amount of chitosan was dissolved in 1% (v/v) acetic acid and allowed to stir for 1 hour. 3mg/ml AB ethanol extract was then added to the freshly prepared chitosan dispersion. The pH of the medium was maintained at 5.0 using 1M NaOH and then further stirred for 1 hr. Finally, 1mg/ml of TPP was added to the chitosan-AB ethanol extract under mild magnetic stirring. The resulting mixture was allowed to stir for 2 hr to form AB encapsulated chitosan nanoparticles. The AB loaded chitosan nanoparticles were collected after the centrifugation of 10,000 rpm for 45 minutes at 4°C. The powdered samples were collected with the help of lyophilizer and stored at 4°C for further use.

**Mammary carcinogenesis Induction**

Sprague-Dawley rats were induced with a single subcutaneous injection of DMBA (25 mg/kg bw) in 1 ml emulsion of sunflower oil (0.5 ml) and physiological saline (0.5 ml)\(^3\).  

**Experimental design**

Totally 60 rats were divided into ten groups, comprising of six rats in each groups as follows: Group 1: control rats, Group 2-8 rats induced with 25 mg/kg bw of DMBA in 0.75 ml of sunflower oil and 0.25 ml of physiological saline to induce breast cancer. Group 2 received no other treatment. After 90 days of tumor induction, Group 3-5 breast cancer bearing rats treated with ethanol extracts of AB at a dose of 100, 200 & 400 mg/kg bw and group 6-8 breast cancer bearing rats treated with encapsulated ABCNPs at a dose of 15, 30 & 60 mg/kg bw for once a week for 4 weeks. Group 9 and 10, rats were treated with ethanol extracts of AB at a dose of 400 mg/kg bw and ABCNPs at a dose of 60 mg/kg bw throughout the experimental periods. After the experimental period, the animals were fasted for overnight and then sacrificed by cervical decapitation. Breast tissues were used for various biochemical estimations.

**Biochemical analysis**

Animal tissue samples were washed with ice cold saline and dried between folds of filter paper, weighed and homogenized using appropriate buffer and used for various biochemical estimations.

Lipid peroxidation was estimated by measurement of thiobarbituric acid reactive substances (TBARS) using the method of Yagi (1987)\(^4\). The antioxidant enzymes superoxide dismutase (SOD) was analyzed by kakkar et al (1984)\(^5\). Catalase (CAT) was assayed by the method of Sinha (1972)\(^6\). GPx activity was assayed by the method of Rotruck et al. (1973) with modifications\(^7\). The activity of reduced glutathione (GSH) was analyzed by the method of Ellman (1959)\(^8\). Vitamin C was measured by the method of Omaye et al. (1979)\(^9\). Vitamin E was measured by the method of Desai (1984)\(^10\). Tumor weight was estimated according to the method of Geren et al. (1972)\(^11\).

**Histopathological procedure**

For the histopathological study, The tissues were sliced and embedded in paraffin wax; 3-5 µm thick sections were cut using a microtome, dehydrated in graded alcohol, and stained with hematoxylin and eosin. The specimens were evaluated with a light microscope. All histopathological changes were examined by the pathologist.

**Statistical analysis**

Statistical analysis was performed using SPSS 11.5 (SPSS Inc., Chicago) statistical package. Data were expressed as mean ± Standard Deviation (SD). One way analysis of variance (ANOVA) followed by Duncan multiple comparison method was used to correlate the difference between the variables. Data were considered statistically significant if \(P\) value was less than 0.05.

**RESULTS**

Figure 1, illustrate the histopathological examination of DMBA induced control and experimental groups. The tumors were histopathologically established as reasonably and feebly differentiated adenocarcinoma. Oral administration of AB to DMBA treated rats with different doses 100 mg, 200 mg and 400 mg have reduced the tumor incidence in a dose dependent manner, where 200 mg/kg bw was found to be effective which showed 67% reduced tumor incidence on experimental animals. While treatment with ABCNPs (30 mg/kg bw) extremely reduced the tumor incidence in 84% of the animals. However, we noticed dactyl hyperplasia in their mammmary tissues (Fig 1D and G). Tumors noticed in remaining animals were histopathologically confirmed as well-differentiated adenocarcinoma with reasonable to severe dysplasia (Fig. 1C, E, F, and H). In control animals and animals treated with AB and ABCNPs alone, the epithelium normally contained multiple layers of cells (Fig.1A, 1land J). Rats treated with DMBA alone exhibited higher level of epithelial cell proliferation as evidenced by multi-layered epithelium with very high density and cellular atypia (Fig. 1B).

Table 1, explained the tumor incidence, volume and burden of various experimental animals. There appears to be a significant increase in the tumor size of animals which is treated by DMBA alone (Group 2) than those of untreated control (Group 1). The levels of tumor incidence, volume and burden were significantly suppressed by oral administration of AB and ABCNPs treated groups (Group 3-8). AB extracts with effective dose of 200 mg/kg bw and ABCNPs at a dose of 30 mg/kg bw effectively increased the body weight and decreased the tumor incidence, volume and burden (Group 4 & 7). Rats treated AB and ABCNPs alone showed no significant differences as compared to control rats (Group 9-10).
Fig. 1: The histopathological changes observed in control and experimental animals in each group

(A) Group 1 – Control, Microphotograph showing normal glandular structure in control rat. (B) Group 2 – DMBA, Microphotograph showing adenocarcinoma in DMBA treated rats. (C) Group 3 – DMBA+AB (100mg) showing dysplasia, (D) Group 4 – DMBA+AB (200mg) showing ductal hyperplasia, (E) Group 5 – DMBA+AB (400mg) showing dysplasia, (F) Group 6 – DMBA+ABCNPs (15mg) showing dysplasia, (G) Group 7 – DMBA+ABCNPs (30mg) showing ductal hyperplasia, (H) Group 8 – DMBA+AB (60mg) showing dysplasia, (I) Group 9 – AB (400mg) showing normal glandular structure, (J) Group 10 – ABCNPs (60mg) showing normal glandular structure.

Table 1: The tumor incidence, tumor volume and tumor burden of various experimental animals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Tumor incidence</th>
<th>Total number of tumors</th>
<th>Tumor volume (mm³)/animal</th>
<th>Tumor burden (mm³)/animal</th>
</tr>
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<tbody>
<tr>
<td>1 Control</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>2 DMBA</td>
<td>100%</td>
<td>(6)/6</td>
<td>9639.7±832.14</td>
<td>9639.7±832.14</td>
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<tr>
<td>3 DMBA + AB (100 mg/kg/bt.)</td>
<td>66%</td>
<td>(4)/6</td>
<td>7426.1±621.12</td>
<td>7426.1±621.12</td>
</tr>
<tr>
<td>4 DMBA + AB (200 mg/kg/bt.)</td>
<td>33%</td>
<td>(2)/6</td>
<td>1424.5±356.45</td>
<td>1424.5±356.45</td>
</tr>
<tr>
<td>5 DMBA + AB (400 mg/kg/bt.)</td>
<td>50%</td>
<td>(3)/6</td>
<td>2247.0±113.01</td>
<td>2247.0±113.01</td>
</tr>
<tr>
<td>6 DMBA + ABCNPs (15 mg/kg/bt.)</td>
<td>66%</td>
<td>(4)/6</td>
<td>664.23±54.12</td>
<td>664.23±54.12</td>
</tr>
<tr>
<td>7 DMBA + ABCNPs (30 mg/kg/bt.)</td>
<td>16%</td>
<td>(1)/6</td>
<td>330.15±21.01</td>
<td>330.15±21.01</td>
</tr>
<tr>
<td>8 DMBA + ABCNPs (60 mg/kg/bt.)</td>
<td>50%</td>
<td>(3)/6</td>
<td>782.20±42.20</td>
<td>782.20±42.20</td>
</tr>
<tr>
<td>9 AB alone (400 mg/kg/bt.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ABCNPs alone (60 mg/kg/bt.)</td>
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</table>

Values are expressed as mean±S.D. for 6 rats in each group. Tumor volume was measured using the formula $V = \frac{4}{3}\pi \left(\frac{D_1}{2}\right) \left(\frac{D_2}{2}\right) \left(\frac{D_3}{2}\right)$, where $D_1, D_2, and D_3$ are the three diameters (in mm) of the tumor. ( ) indicates total number of animals bearing tumors.

Fig. 2: Effect of AB and ABCNPs on TBARS in the breast tissue of control and experimental animals

Figure 2, illustrates the levels of TBARS in mammary tissues of various experimental groups. AB and ABCNPs treated rats showed a significant alteration in the status of TBARS when compared with the untreated (Group 1) and DMBA treated animals (Group 3-8). In which AB at a dose of 200 mg/kg bw and ABCNPs at a dose of 30 mg/kg bw were effectively improved the status of TBARS (Group 4 & 7). Rats treated with AB and ABCNPs alone showed no significant difference as compared to control rats (Group 9 & 10).

Figure 3A, 3B & 3C showed the activities of enzymic antioxidants like SOD, CAT, GPx in control and experimental animals. A significant reduction of SOD, CAT & GPx was observed in the animals treated with DMBA (Group 2) than those of untreated control (Group 1). The rats treated with AB and ABCNPs showed a significant increased of the SOD, CAT, GPx status when compared with the untreated and DMBA treated animals (Group 4-8). In which AB at a dose of 200 mg/kg bw and ABCNPs at a dose of 30
mg/kg bw were effectively improved the status of enzymic antioxidant status (Group 4 & 7). Rats treated with AB and ABCNPs alone showed no significant difference as compared to control rats (Group 9 & 10).

Fig. 3A: Effect of AB and ABCNPs on the activities of SOD in breast tissue of control and experimental animals

Fig. 3B: Effect of AB and ABCNPs on the activities of CAT in breast tissue of control and experimental animals

Fig. 3C: Effect of AB and ABCNPs on the activities of GPx on breast tissue of control and experimental animals

Values are expressed as mean ± SD. Units for SOD was expressed as the amount of enzyme required to inhibit 50% of NBT reduction. Units for CAT were expressed micromoles of H2O2 utilized/second. Units for GPx were expressed as micromoles of glutathione utilized/minute. P<0.05 NS – non-significant, a P<0.05 of DMBA positive control compared with control group, b* b** b*** P<0.05 of AB groups compared with DMBA untreated group, c* c** c*** P<0.05 of ABCNPs groups compared with DMBA untreated group.

Fig. 4A: Effect of AB and ABCNPs on GSH in the breast tissue of control and experimental animals
anticancer activity and inhibit tumor growth through the inhibition of cancer cell proliferation. In our study, we have also investigated the antioxidation status in DMBA induced mammary carcinogenic animals. Antioxidants are the chemical substances that reduce or prevent oxidation and have a leading role in the early phases of tumor growth. Thioharbituric acid reactive substances (TBARS) is a product of LPO which is the oxidation of unsaturated fatty acids in membranes induced by free radicals, is an indicator of oxidative damage. Many studies have observed the possibility of a connection between LPO and cancer. In the present study, an increase in the levels of TBARS was found in breast cancer bearing animals and these were significantly reduced after treatment with ethanol extracts of AB and ABCNPs. Our data suggest that the significant increase in the level of LPO may be due to its poor antioxidant defense or the inactivation of antioxidant enzymes in cancerous conditions. Recent reports suggest that oxidative stress can cause up regulation of antioxidant enzymes that render cells more resistant to subsequent oxidative insult. The finding of the present study concludes that AB and ABCNPs exert a therapeutic effect by modulating the production of free reactive oxygen radicals.

In our study, we have also investigated the antioxidation status in DMBA induced mammary carcinogenic animals. Antioxidants are the chemical substances that reduce or prevent oxidation and have the ability to neutralize the damaging effects of free radicals in tissues and thus are believed to protect against cancer, arteriosclerosis, heart disease and other diseases. Over expression of antioxidants has been documented in a wide variety of malignant tumors, including breast cancer. The available reports were documented that the supplementation of the diet induced mammary carcinogens showed that the tumor is very necrotic in the AB and ABCNPs treated rats which might be due to induction of apoptotic and necrotic cell death. The polyaromatic hydrocarbon of DMBA acts as a potent carcinogen by generating various reactive metabolic intermediates leading to oxidative stress. DMBA-induced mammary tumors in rat are essentially similar in morphology, pathogenesis, and ER status to human breast cancer and show estrogen dependent growth.

FIG. 4B: Effect of AB and ABCNPs on Vitamin C in the breast tissue of control and experimental animals

Values are expressed as mean±S.D. for six animals in each group. Units—μg/mg protein. P<0.05 NS – non-significant, a P<0.05 of DMBA positive control compared with control group, b* b** b*** P<0.05 of AB groups compared with DMBA untreated group, c* c** c*** P<0.05 of ABCNPs groups compared with DMBA untreated group.

FIG. 4C: Effect of AB and ABCNPs on Vitamin E in the breast tissue of control and experimental animals

DISCUSSION
White button mushrooms are a potential breast cancer chemopreventive agent, as they suppress aromatase activity and estrogen biosynthesis. It is a widely consumed edible mushroom shows beneficial effect towards various cancers. Its potent activity is mainly due to the presence of its active compound lectin. Extensive research has been focused on Agaricus bisporous lectin for its antitumor activity. However, several studies showed certain reversible changes in the antiproliferative action of AB and its active compound lectin on various cancers. Polysaccharides found in white button mushrooms may stimulate the immune system and inhibit tumorigenesis. The phytochemicals in mushroom extract prevent tumor growth through the inhibition of cancer cell proliferation. In order to overcome the low bioavailability of AB, a suitable drug delivery system in nanotechnology is needed to deliver its full potential. Nowadays, researchers are showing considerable interest in developing nanocarriers for effective drug delivery. Therefore to circumvent this issue, nanotechnology has led to the emergence of entirely new research strategies in the field of drug delivery. To circumvent this issue, nanotechnology has led to the emergence of entirely new research strategies in the field of drug delivery. In the present study, this nanotechnology has led to the emergence of entirely new research strategies in the field of drug delivery. Therefore chitosan a suitable nanocarrier for oral drug delivery system has been selected for our study. Chitosan decreased the rate of drug release. Histopathological examination of DMBA treated tumor showed significant difference as compared to control rats (Group 9 & 10).

Histopathological examination of DMBA treated tumor showed significant difference as compared to control rats (Group 9 & 10).
with antioxidants could conceivably protect the human body from the deleterious effects of free radicals and ROS and therein retard the progress of many chronic diseases\textsuperscript{41}.

We have observed a declined superoxide dismutase (SOD) activities, which may be due to the increase in circulating lipid peroxides. This can result in accumulation of superoxide anion, a highly diffusible and potent oxidizing radical capable of traversing membranes causing deleterious effects at sites far from the tumor\textsuperscript{42}. Catalase (CAT) and Glutathione peroxidase (GPX) detoxify significant amount of H$_2$O$_2$ produced during electron transport chain and protect mitochondrial membranes from lipid peroxidative damage\textsuperscript{43}. The decreased activities of catalase found in the cancerous condition may be due to exhaustion of these enzymes in catalyzing the overproduction of hydrogen peroxide by the cancerous cells.\textsuperscript{44} GPX is an important defence enzyme against oxidative damage and this in turn requires glutathione as a cofactor. Several studies have been reported the decreased activities of GPX in various cancerous conditions\textsuperscript{45}. There was a decline in the activities of GPX in the present study, which may be due to the altered antioxidant defense system caused by enormous production of free radicals in DMBA-induced carcinogenesis.

Apart from the enzymic antioxidants, non-enzymic antioxidants such as reduced Glutathione (GSH), Vitamin C and E plays an excellent role in protecting the cells from oxidative stress. The non-enzymic antioxidant systems are the second line of defense against free radical damage\textsuperscript{46}. The observed decrease in the level of Vitamin C and E in mammary carcinoma bearing rats may be due to the excessive utilization of these antioxidants for quenching enormous free radicals generated in cancer condition\textsuperscript{47}. Oral administration of AB and ABCNPs significantly restored the Vitamin C and E levels to near normal. The present study adds to the current knowledge by showing that the baseline levels of vitamin C significantly decreased in drug treated animals.

Glutathione, as a reductant, is very important in maintaining the stability of erythrocyte membranes. It is implicated in the cellular defense against xenobiotics and deleterious compounds, such as free radicals and hydroperoxides\textsuperscript{48}. The AB and ABCNPs were significantly improved the glutathione status which could be brought about by the constituents of the mushroom extract. However, administration of AB at the dose of 200 mg and ABCNPs at the dose of 30 mg has effectively reversed the alterations of DMBA induced oxidative stress to near normal when compared to other doses.

**CONCLUSIONS**

Lower intake of white button mushroom frequently as a routine food constituent would be effective in preventing the initiation of breast tumors in an average woman. But in nanotechnology, pharmacologic dosage of nano drug delivery with sustained release in vivo could inhibit the growth of tumor cells. Oral administration of AB and ABCNPs has dose dependently inhibited the DMBA induced mammary carcinogenesis in experimental animals. Thus our results provide evidence that AB and ABCNPs proves to have a potent antioxidant and also might act as a potential intermittent therapy against DMBA induced breast carcinogenesis. Hence, this preliminary dose fixation study will facilitate us to undergo a detailed investigation on therapeutic effect of AB and ABCNPs against mammary carcinogenesis.

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