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Modulation effect of tea polyphenol toward *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine-induced precancerous gastric lesion in rats

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Abstract

The chemopreventive effect of tea polyphenol (TP) on precancerous gastric lesion was examined. A rat model was established by gavage of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), and different concentrations of TP were given to Wistar rats in drinking water during the 16 weeks of the experiment. The histopathological data showed an effect of TP to lighten the lesions induced by MNNG. By flow cytometry, we demonstrated that TP treatment decreased the proliferation and apoptosis index (AI) induced by MNNG. The arrest in the G_0-G_1 phase of the cell cycle was also obtained. The results suggested that TP had preventive effect against gastric carcinogenesis at the preinitiation stage and such prevention may be related to the modulation of the balance of cell death and cell proliferation.
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Keywords: MNNG; Tea polyphenol; Precancerous lesion; Apoptosis; Proliferation

1. Introduction

Gastric cancer is a global health problem of major proportions [1]. Asian countries have high stomach cancer incidence and mortality rates. Based on a currently accepted model of human gastric carcinogenesis [2–4], gastric cancer usually develops from precancerous lesion to neoplasia. Atrophic gastritis, intestinal metaplasia and atypical proliferation are the most common precancerous lesion of gastric cancer. In recent years, research on gastric cancer has concentrated primarily on identifying environmental and genetic risk factors. Studies have shown that high intake of smoked, salted and nitrated foods, high intake of carbohydrates and low intake of fruits, vegetables and milk significantly increase the risk for stomach cancer [5]. Current studies suggests that the dietary consumption plays a key role in the development of gastric cancer, indicating that natural products in the traditional diet that have been reported to have anticancer activities may exert their protective effect against gastric cancer.

Consumption of green tea has been reported to afford protection against carcinogenesis of human esophagus, fore stomach, duodenum, colon, liver and lung [6–8]. The chemopreventive effect of green tea against cancer has also been demonstrated in mouse and rat models [9–11]. The main responsible component of green tea is tea polyphenol (TP). It has been assumed in several researches that these compounds' chemopreventive activities include antioxidant and free radical scavenging activity, and stimulation of detoxification systems through selective induction or modification of phase I and phase II metabolic enzymes. In addition, green tea may inhibit biochemical markers of tumor initiation and promotion, including the rate of cell replication, and thus inhibits the growth and development of neoplasms and prevents mutagenicity and genotoxicity [12,13]. Current studies, which indicate an inverse association between green tea consumption and cancer risk, support a possible chemopreventive effect of green tea [14,15]. However, studies on tea components are still limited, especially at a mechanism level.

Hence, the purpose of the present study was to investigate if TP plays an active chemopreventive role in chemical carcinogenesis. *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was used in this work as a potent mutagen,

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which causes methylation of nucleic acid and protein and is known to induce adenocarcinomas in rats, mice, hamsters, ferrets, rabbits and dogs [16,17]. We also aimed to understand chemopreventive mechanisms of TP by measuring cell proliferation and apoptosis.

2. Materials and methods

2.1. Materials

Tea polyphenol was purchased from Sigma, USA, MNNG was purchased from Pfaltz and Bauer, USA.

2.2. Treatment of animals

Male Wistar rats (4 weeks old) weighing 80–100 g were purchased from the animal center of the Chinese Academy of Science, Shanghai, China. The rats were housed five per cage in a room with controlled temperature and humidity. After 1 week of acclimatization, rats were randomly divided into six groups (10 rats per group). Except the blank control group, the others were treated with gavage of 400 mg/kg body weight MNNG (in dimethyl sulfoxide) one time/day for 10 days. After the last MNNG treatment, the rats were randomly divided into five groups and received no treatment (negative control group), 0.5% TP, 1.0% TP, 1.5% TP or 40 mg/kg body weight retinoic acid (positive control group) as drinking fluid for 16 weeks. The body weights were monitored once every week and drinking water was quantified every 3 days. All rats were killed on day 120. The blood samples were collected for the assay of Ca^{2+} and lactate dehydrogenase (LDH), and stomachs were separated for histopathological studies and flow cytometric analysis.

2.3. Lactate dehydrogenase release

Cell injury was monitored by measuring the LDH released in the blood. Lactate dehydrogenase was assayed using an LDH reagent kit (Long March-Trace Chiron Medical Science, Shanghai) through measuring the increase in NADH absorbance at 340 nm by a spectrophotometer (Antai, Shanghai) during the oxidation of lactate to pyruvate.

Table 1
Water consumption in various experimental groups

Group	Drinking water (ml)
1	21.9±2.2
2	18.0±2.9
3	16.3±1.5
4	18.5±2.7
5	16.2±2.8
6	19.3±1.9

Values are means±S.D. 1: blank control group; 2: negative control group; 3: 0.5% TP treatment group; 4: 1.0% TP treatment group; 5: 1.5% TP treatment group; 6: positive control group.

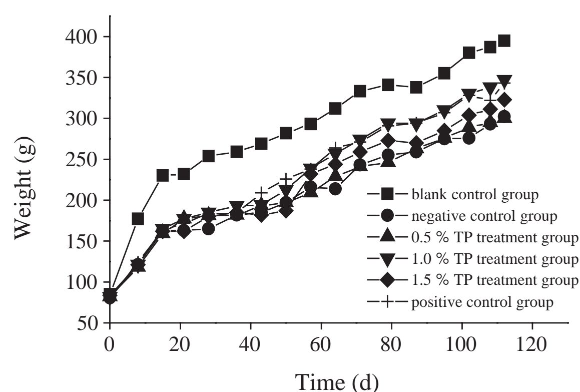

Fig. 1. Effect of TP on rat body weight.

2.4. Ca2+ assay

Ca^{2+} in the blood was assayed using arsenazo III (Long March-Trace Chiron Medical Science) method through measuring the absorbance of Ca^{2+}-arsenazo III chelate at 660 nm by a spectrophotometer (Antai).

2.5. Histopathological studies

Gastric antrums from the rats were preserved in 10% neutral formalin. Gastric antrum tissues were processed, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin (H&E), and evaluated histopathologically.

2.6. Flow cytometric analysis of cell-cycle status and apoptosis

Tissues of rat stomachs were cut into pieces and filtrated through nylon net of mesh 200 to collect cells. Cells diluted to the density of 1×10^6/ml were trypsinized, washed twice with cold phosphate-buffered saline (PBS), centrifuged and fixed in 90% cold methanol in PBS for 1 h at 4°C. After that, the cells were centrifuged at 1100 × g for 5 min, washed twice with cold PBS and incubated with RNase for 10 min. Then the cells were chilled over ice for 10 min,

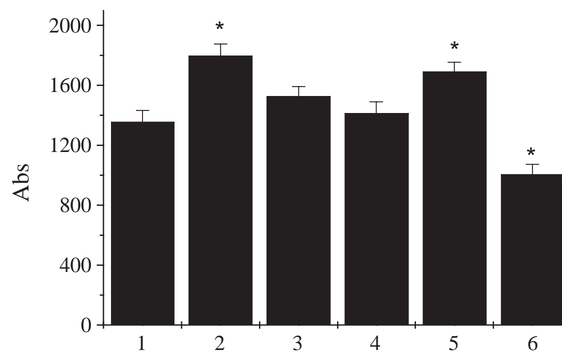

Fig. 2. Effect of TP on the LDH release in blood sample. Each value was expressed as the mean±S.D. of four separate experiments. Significant differences from untreated control are indicated by $*P<.05$. 1: blank control group; 2: negative control group; 3: 0.5% TP treatment group; 4: 1.0% TP treatment group; 5: 1.5% TP treatment group; 6: positive control group.

Table 2
Effect of TP on the Ca^{2+} concentration in blood sample

Group	Concentration of Ca^{2+}
1	2.56±0.04
2	2.56±0.03
3	2.66±0.05
4	2.48±0.02
5	2.48±0.07
6	2.70±0.07

1: blank control group; 2: negative control group; 3: 0.5% TP treatment group; 4: 1.0% TP treatment group; 5: 1.5% TP treatment group; 6: positive control group.

stained with propidium iodide for 15 min and analyzed by flow cytometry (FACSalibur, Becton Dickinson, USA). The apoptosis index (AI)=the apoptosis cells/the total cells. The proliferation index (PI)=(cells of S and G_2M)/(cells of G_0/G_1, S and G_2M).

2.7. Statistical analysis

Quantitative data were analyzed using Student's *t* test and statistical significance level was set at $P<.05$ or $P<.01$.

3. Result

3.1. General observations

The water consumption among different groups during the whole experimental period showed no significant difference (Table 1). Considering the fact that drugs were dissolved in water, we could conclude that there was not any significant difference of rats in drugs intake. We also observed that the body weight increased more slowly in rats treated with drugs compared with that of the blank control group. The body weight growth in the groups treated with drugs during the experimental period showed no significant difference. However, the body weight of rats in negative control group is the lowest (Fig. 1).

3.2. Assay for LDH and Ca2+ in blood samples

The effects of drug treatment on LDH release are shown in Fig. 2. Compared with the blank control group, the LDH in blood sample was increased in negative control group. Different concentrations of TP treatment reduced the increasing LDH level, and such effect was most notable in the 1.0% TP-treated group. However, there were no significant differences in the concentration of Ca^{2+} of blood sample among different groups (Table 2).

3.3. Histopathological studies in the stomach

In contrast to the sections from the blank control, where gastromucosal epithelium was integrated, lamina propria had many ramifications and tubular glands ranged orderly (Fig. 3A), atrophic gastromucosal lamina propria glands decreased distinctly, glands ranged irregularly and atypical glandular epithelium and lymphocytic infiltration were observed in the negative control group (Fig. 3B). The result

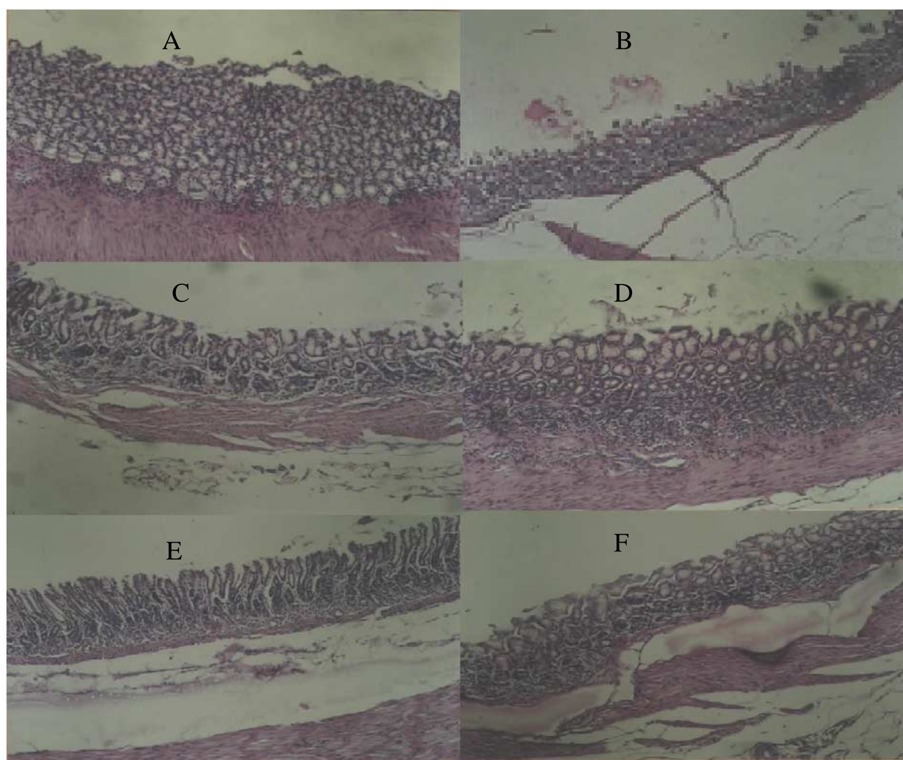

Fig. 3. Effect of TP on the gastric lesion induced by MNNG (H&E staining). A: blank control group; B: negative control group; C: 0.5% TP treatment group; D: 1.0% TP treatment group; E: 1.5% TP treatment group; F: positive control group. Magnification: 200×.

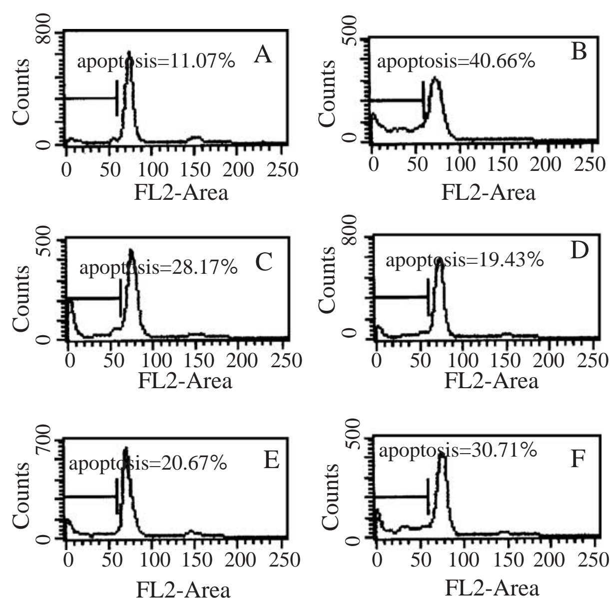

Fig. 4. Effect of TP on apoptosis by flow cytometry. A: blank control group; B: negative control group; C: 0.5% TP treatment group; D: 1.0% TP treatment group; E: 1.5% TP treatment group; F: positive control group.

indicates that using MNNG to establish the rat model of gastric precancerous lesion is feasible. In the positive control group (Fig. 3F), the situations of atrophic gastromucosal and atypical glandular epithelium were lightened than that in the negative control group, especially in the TP treatment groups. Therefore, the rat model to evaluate the preventive effect of TP on gastric precancerous lesion is credible. Among the groups of TP treatment with different concentrations, the preventive effects on lesion of 1.0% TP treatment (Fig. 3D) were the best compared with the 0.5% TP treatment (Fig. 3C) and 1.5% TP treatment (Fig. 3E), which suggested the chemopreventive capacities of TP on the gastric cancer.

3.4. Assay for apoptosis, cell proliferation and cell cycle

The extent of apoptosis was quantified by flow cytometric analysis of the cells labeled with propidium iodide. In the negative group (Fig. 4B), the AI was significantly higher than that of the blank control (Fig. 4A). The AI of 0.5%, 1.0% and 1.5% TP-treated groups were 28.17%, 19.43% and 20.67%, respectively (Fig. 4C, D and E). When compared with that in the negative control, the AI induced by MNNG decreased significantly in TP-treated groups, where the preventive effects on apoptosis were better than that in the positive control (Fig. 4F). Among the TP treatments, the 1.0% TP treatment was the best one.

The cell cycle perturbations were also examined in Table 3. Compared with the blank control in which the arrest of cells in G_0-G_1 phase is 92.48%, the arrest of cells of negative control group in G_0-G_1 phase was 87.67%. The TP treatments resulted in the increase of cells arrested in G_0-G_1 phase.

Since the cell proliferation was correlated with histological severity, the result of cell proliferation was shown in Table 4. In the negative control, the PI was higher than that of blank control. The data suggested that MNNG enhanced cell proliferation and such effect was inhibited by the TP treatments.

Table 3
Effect of TP on cell cycle by flow cytometry

Group	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
1	92.48±0.98	5.39±0.35	2.13±0.41
2	87.68±0.96*	3.96±0.45	8.36±0.52**
3	90.59±0.96	3.14±0.38	6.27±0.53**
4	92.06±0.97	4.30±0.34	3.64±0.39
5	91.04±0.95	3.86±0.35	5.11±0.49**
6	90.38±0.95	4.62±0.42	5.00±0.46**

Each value was expressed as the mean±S.D. of four separate experiments. Significant differences from untreated control are indicated by $*P<.05$ or $**P<.01$. 1: blank control group; 2: negative control group; 3: 0.5% TP treatment group; 4: 1.0% TP treatment group; 5: 1.5% TP treatment group; 6: positive control group.

Table 4
Effect of TP on index of apoptosis and proliferation by flow cytometry

Group	AI	PI	AI/PI
1	11.07±1.96	7.52±0.86	1.47
2	40.66±3.77**	12.32±1.10*	3.30
3	28.17±2.56**	9.41±1.03	2.99
4	19.43±1.85	7.94±0.56	2.45
5	20.67±2.65*	8.96±0.77	2.31
6	30.71±2.64**	9.62±0.83	3.19

The AI=the apoptosis cells/the total cells. The PI=(cells of S and G_2M)/(cells of G_0/G_1, S and G_2). Each value was expressed as the mean±S.D. of four separate experiments. Significant differences from untreated control are indicated by $*P<.05$ or $**P<.01$. 1: blank control group; 2: negative control group; 3: 0.5% TP treatment group; 4: 1.0% TP treatment group; 5: 1.5% TP treatment group; 6: positive control group.

4. Discussion

In this study, the rat model was established by the application of MNNG to investigate the chemopreventive effect of TP toward the carcinogenesis potential of gastric cancer. Moreover, attempts were made to elucidate the mechanisms of protection.

The results indicated that TP given after the MNNG treatment inhibited the histological severity of gastric mucosa. At the concentrations used, the 1.0% TP treatment appeared to have the stronger preventive effect. The result of the present study are in agreement with most previous studies concerning the inhibitory activity of green tea against tumorigenesis in the skin, colon, esophagus, lung and other organs [7,18,19]. To our knowledge, this is the first report showing an inhibitory effect of tea on gastric precancerous lesion.

It has been well known that the free radical production and subsequent oxidative stress play a role in tumor

initiation, promotion and progression [20–23]. On one hand, numerous free radical generators have been demonstrated to act as tumor promoters [24]. On the other hand, antioxidant agents are believed to protect against cancer by scavenging reactive radical species, resulting in a reduced level of radical-mediated DNA damage [25]. One proposed mechanism of action for antioxidant is modulation of cell death and proliferation.

Apoptosis in recent years has become an important issue in biomedical research. The life span of both normal and cancer cells within a living system is regarded to be substantially affected by the rate of apoptosis. In addition, apoptosis is a discrete way of cell death different from necrotic cell death and regarded to be an ideal way of cell elimination. Thus, the chemopreventive agents, which can modulate apoptosis, may be able to affect the steady-state cell populations that are often useful targets in the management and therapy for cancer.

The development of gastric cancer is a multistep and multifactor process. Studies have shown that the balance between cell apoptosis and cell proliferation is of great importance for maintaining gastric mucosal integrity. Gastric cancer is a collection of diseases based on disruption of a delicate cell number balance controlled by cellular proliferation and cell death. In this investigation, compared with the blank control, the AI and PI of negative control group were both increased, indicating that the balance of the cell apoptosis and proliferation was interrupted by the application of MNNG. We therefore hypothesized that in the preinitial stage of gastric cancer, the instability of gastromucosal epithelium cells is enhanced, represented by the fact that the proliferation of active cells and the apoptosis of mass cells exist at the same time, resulting in the high update rate of gastromucosal epithelium cells. With the lesion progression, the dominant apoptosis cells would be eliminated continually through the apoptosis mechanism, and the dominant proliferous cells would be reserved through continual proliferation, until in the end, cancer comes into being. In the experiment of this investigation, with the TP treatment, the AI/PI was decreased and tended to the level of normal state, suggesting that TP could protect gastromucosal epithelium cells from developing into cancer through modulating the balance of cell apoptosis and proliferation.

Our results have demonstrated that TP inhibited carcinogenesis at the precancerous lesion stage. This agent may be explored as chemopreventive drug for human at high risk of gastric cancer. It has also been demonstrated that cancer initial stage is correlated with the relation of cell apoptosis and proliferation. Proliferation and apoptosis may be used as surrogate biomarkers for chemoprevention studies. Furthermore, the underlying mechanism of how antioxidant agents exert their chemopreventive effects through the modulation of apoptosis and proliferation is going to be clarified in the future.

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