Differential Modulatory Effect of Epigallocatechin-3-gallate in Suppression of Tumor Proliferation

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Despite the remarkable progress in selecting the chemotherapeutic drugs, most are expensive and associated with many adverse effects targeting both cancer and normal cells. The using of polyphenols as natural materials for chemoprevention is considered a promising approach in reducing the tumor proliferation. This study aims to investigate whether a difference between the use of green tea and its component Epigallocatechin-gallate (EGCG) in treatment and protection against tumor. Sixty female Swiss albino mice weighted 20–22 g divided into 6 groups (n=10). The tumor suppression of green tea and EGCG was mirrored by evaluating their antioxidant and the anti-inflammatory effect on tumor markers and DNA integrity. Our results showed that the administration of EGCG showed a significant elevation of both antioxidants and anti-inflammatory markers in serum of EAC-bearing animals and revealed its high curative power to protect than treat tumor growth. Moreover, genomic DNA fragmentations assay present EGCG as a modulatory agent in keeping genome integrity. The administration of green tea and its major constituent EGCG showed a significant a potent protective role in suppressing tumor proliferation than its use in treatment due to its antioxidant and anti-inflammatory effect and maintaining the integrity of underlying genomic DNA that make it a strong barrier which arrest the process of oncogenesis.

Keywords: EGCG, tumor, oxidative stress, cell proliferation, DNA fragmentation.

Cancer is still the much–feared disease that threat human health. Its victims are still frightened and depressed. It considered as an end-product of multiple physiological and genomic instability which disturb the machineries regulating cells proliferation. In addition, the elevation of oxidative stress could stimulate pro-inflammatory cytokines and inhibit cellular antioxidant capacity and this damage continues attach biological molecules including DNA. Most of chemotherapeutic drugs still have adverse effects. The naturally existing compounds from plants known as phytochemicals are not only serve as interest resources for novel drugs but also revealed the importance of the diet and its impact, not only on body health but also in prevention.
of diseases development such as cancer. So, the cellular and its underlying genomic stabilities could be restored by nutritional treatment or protection with herbs which has no side effects.

The first barrier of defense against oxidative stress involves two main enzymes superoxide dismutase (SOD) and glutathione peroxidase (GSH), which are antioxidants usually measured in the realization of the natural compounds antioxidant activity. SOD enzyme modifies the highly reactive superoxide radicals in hydrogen peroxide (H$_2$O$_2$) and molecular oxygen and makes the first antioxidant defense in an oxidative stress condition. Similarly, GSH preserves the polyunsaturated cell membranes.

Several studies were reported that the antioxidant properties of fruits and vegetables can modulate various biological processes including growth and cell proliferation. The use of phytochemical in cancer therapy could understood via regulating cell proliferation, increasing antioxidant status, regulation of the immune system, inactivation of carcinogens, induction of cell cycle arrest and apoptosis. Green tea obtained from the Camellia sinensis plant, the major constituents of green tea are polyphenols comprising phenolic acids and catechins those belong to the free iron scavengers, flavonoids. Many botanical flavonoids possess strong antioxidant activities.

Evidences presented green tea possessing chemotherapeutic potential, but still a question, whether the administration of green tea and its component Epigallocatechin-gallate (EGCG) are more effective in treatment of existing tumor or rather prevention against tumor formation. Thus, the main objective of this study was to investigate differential activity of green tea and its active constituent EGCG as a natural therapeutic and preventive agent for tumor proliferation.

**MATERIAL AND METHODS**

**Animals**

60 female Swiss albino mice (of age about 8 weeks, 20–22 g body weight) were divided into 6 groups ($n=10$). The animals were acclimatized for one week before each experiment or one week.

**Ethical consideration**

This experimental protocol was reviewed and approved by The Institutional Animal Care and Use Committee (ZU-IACUC Committee), Zagazig University and the approval number is ZU-IACUC/1/F/108/2020.

**Ehrlich Ascites Carcinoma (EAC)**

Ehrlich’s ascites carcinoma (EAC) is an undifferentiated carcinoma, has a high transplantable capability, no-regression, rapid proliferation, short life span, obtained from National cancer institute (Cairo, Egypt). EAC has been used as an experimental model to evaluate the effect of natural plant on tumor cell proliferation.

The tumor was maintained in Swiss mice in the ascitic form, collected by aspiration with a Pasteur pipette, centrifuged for 10 min at 200 xg, and washed twice with phosphate-buffered saline (PBS, pH 7.2). Cell viability was evaluated by trypan blue (0.2%) exclusion test and only cell suspensions with more than 95% viability were used. In all experimental protocols, mice were injected intraperitoneally by $2.5 \times 10^6$ cells/mouse.

**Drugs**

Green tea (Camellia sinensis): Green tea was prepared by steeping 2.5 g in 250 mL of boiling water for 5 minutes. After cooling, the mice were administered orally at a daily dose of (250 mg/kg. b.wt.) as 1 % of solution. Tea bags (Authentic Green Tea) from Rabea company (Egypt).

EGCG: (-)-EpigallocatechinGallate (EGCG) with purity 99% was purchased from National bio-lab company for Trade, Egypt Cat. No.4524. It was freshly prepared by dissolving in water, and then administered orally at a daily dose of 50mg/kg. b. wt.

Sorafenib: Sorafenib (purity 99% it was purchased from National bio-Lab. Company for Trade, Egypt cat no 17307032. It was freshly prepared by dissolved in distal water, then administered orally and daily at a dose of 50 mg/kg. b. wt.

**Experimental design**

Control group: Animals were received only the normal laboratory diet and tap water.

Ehrlich group: Animals were intraperitoneally injected by 0.2 ml Ehrlich Ascites Carcinoma ($2.5 \times 10^6$ Ehrlich cells).

Ehrlich+ Sorafenib group: Animals were intraperitoneally injected 0.2 ml Ehrlich Ascites Carcinoma ($2.5 \times 10^6$ Ehrlich cells). Animal then left for one week and then treated with sorafenib orally (50mg/kg/day) for 5 weeks.
Ehrlich + Green tea group: Animals were intraperitoneally injected by 0.2 ml Ehrlich Ascites Carcinoma (2.5 ×10⁶ Ehrlich cells), and left for one week and then treated with green tea orally (250 mg/kg/day) for 5 weeks.

Ehrlich + EGCG group: Animals were intraperitoneally injected by 0.2 ml Ehrlich Ascites Carcinoma (2.5 ×10⁶ Ehrlich cells). Mice were left for one week and then treated with EGCG orally (50 mg/kg/day) for 5 weeks.

EGCG group: Animals were treated with EGCG orally (50 mg/kg/day) for 10 days and then intraperitoneally injected once 0.2 ml Ehrlich Ascites Carcinoma (2.5 ×10⁶ Ehrlich cells).

Collection of samples

Blood
At the end of the experiments, animals were fasted overnight, sacrificed and blood samples were collected into plasma tubes and the samples were harvested for determining the activity of GSH, SOD, AFP, IL-8 and IL-8 levels.

The ascetic fluid
After treatment, the ascetic fluid was collected from the peritoneal cavity in graduated falcon tubes. Phosphate buffered saline (PBS) was added to each tube for Ehrlich Ascites Count.

Biochemical analysis
All the biochemical parameters in this study were estimated at National cancer institute (Cairo, Egypt).

Evaluation of antioxidant effect
The status of oxidative stress was mirrored in serum by measuring levels. Superoxide dismutase activity¹⁶ and reduced glutathione¹⁷ by using Kits obtained from Sigma-Aldrich; Cat. No.; 19160 and CS0260, respectively and the developed colors were measured at wavelength given on kits.

Evaluation of tumor proliferation markers
To evaluate the tumor proliferation, animals were dissected and the ascitic fluid was collected from the peritoneal cavity, the volume was measured by a graduated centrifuge tube¹⁸. Tumor Necrosis Factor Alpha (TNF-α) was determined according to the method of Swardfager¹⁹ by using ELISA Kit obtained from Sigma-Aldrich, Cat. No.; RAB0479.

Evaluation of anti-inflammatory effect
Serum α-fetoprotein (AFP) was determined according to the method of Maiolini and Masseyeff²⁰ by using ELISA Kit obtained from Mybiosource, Cat. No.; MBS90043. Detection is based on using Purified AFP antibody to coat Micro Elisa Strip plate wells to make solid-phase antibody, then add AFP and AFP-antibody labeled with HRP, the color change was measured at wavelength 450 nm. Serum interferon-gamma was determined according to the method of Fischer²¹ by using ELISA Kit obtained from ThermoFisher scientific Co. Cat. No.; ERIFNG. While interleukin-6²² and interleukin-8²³ by using ELISA Kit obtained from Mybiosource Cat. No.; MBS535410 and MBS9141543, respectively. The kits based on enzyme-linked immune-sorbent assay.

DNA fragmentation by agarose gel electrophoresis
Genomic DNA extraction of Ehrlich Ascites Carcinomas (EAC) cells was extracted according to the protocol of genomic DNA extraction Cat. No; 51304 from animal tissues²⁴ according to the manufacturer’s instructions of the QIAamp DNA Mini Kit. The tissue was homogenized in 1 ml lysis buffer [20 mM Tris-Cl (pH 7.5), 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, and 25 mM disodium pyrophosphate] at 37°C for 1 h. Then, 0.4 ml of saturated NaCl was added to each set of cell lysates and incubated on ice for 5 min and centrifuged at 3,000 ×g for 30 min. The DNA was precipitated using chilled ethanol, which was separated by centrifugation. Separated DNA was re-suspended in TAE buffer (40 mM Tris-acetate and 1 mM EDTA), and the purity of the extracted DNA was checked by 2% agarose gel of 0.5 ug/ml ethidium bromide in TAE buffer according to Raj²⁵. The DNA bands were observed and photographed under a UV trans-illuminator.

Statistical analysis
The obtained data were subjected to one-way analysis by software package SPSS versions 2015 used for interactive or patched, statistical analysis, produced by SPSS Inc., it was acquired by IBM in 2009; data were expressed as mean ± S.E.

RESULTS
Antioxidants effect
Our results in table (1) and fig. (1) revealed that administration of green tea (250 mg/kg/day)
Fig. 1. The chemotherapeutic and chemopreventive effect of green tea (250 mg/kg. b.wt.) and its active ingredient EGCG (50mg/kg/day), sorafenib (50mg/kg/day) on SOD (U/ml) and GSH (ng/dl) levels in blood plasma of female mice (20±22 gm b.w.), Data are presented as (mean ± S.E.). S.E = Standard error.
Fig. 2. The effect of Green tea (250 mg/kg/day), its active ingredient EGCG (50 mg/kg/day) and sorafenib (50mg/kg/day) on levels of proliferation markers in blood plasma of female mice (20±22 gmb.w.) a) Ehrlich carcinomas cell count (x 10^6), b) Tumor necrosis factor-alpha (TNF-alpha) (pg/ml), c) Leutinizing hormone LH (µl/ml) and d) Growth hormone (ng/ml)
or EGCG (50mg/kg/day) in EAC- bearing mice, in group 4 and 6, respectively, were significantly, increased the antioxidant potential as mirrored by measuring the levels of SOD and GSH in blood when compared with control animals (group 1) and EAC-bearing untreated animals (group 2). This antioxidant property against tumor progression revealed to be more effective when administered prior EAC transplantation (group 6).

**Tumor suppressor and anti-inflammatory effect**

To investigate whether the antioxidant properties of green tea and EGCG would affect tumor proliferation, the count per volume of EAC, levels of tumor markers including tumor necrosis factor-α, luteinizing hormone (LH) and growth hormone (GH) were estimated in blood fig. (2). Our measurements shown that administration of green tea (250 mg/kg/day) or EGCG (50mg/kg/day) in EAC- bearing mice, in group 4 and 6, respectively, were significantly decreased the count of EAC and levels of TNF-α, LH and GH in blood when compared with control animals (group 1) and EAC-bearing untreated animals (group 2). And that tumor formation was suppressed more significantly when green tea or EGCG administered prior EAC transplantation (group 6). On the other hand Results of this study clarified that the anti-tumor activity of green tea and EGCG not only based on their antioxidant properties but also dependent on their anti-inflammatory properties which were evaluated by estimation of a number of inflammatory markers including; α-fetoprotein (AFP), interferon-α (IF-α), interleukin-6 (IL-6) and interleukin-8 (IL-8) as in fig. (3). Results revealed that administration of green tea (250 mg/kg/day) or EGCG (50mg/kg/day) in EAC- bearing mice, in group 4 and 6, respectively, were significantly decreased the levels of (AFP), (IF-α), (IL-6) (IL-8) in blood when compared with control animals (group 1) and EAC-bearing untreated animals (group 2). And that we found their anti-inflammatory effect was increased significantly as noticed by significant decrease in these inflammatory markers when green tea or EGCG administered in (group 6) prior EAC transplantation.

**DNA Fragmentation Assay**

Isolated DNA from EAC-bearing mice (group 2) showed complete degradation into oligonucleotide fragments forming a clear ladder of apoptosis when separated by agarose gel electrophoresis as shown in fig. (4). While, DNA extracted from treated animals in groups 3,4 and 5 with sorafenib, green tea and EGCG, respectively showed a pattern significantly fragmented as
compared to control DNA. The genomic DNA of animals, treated with EGCG prior to EAC transplantation, was highly intact and similar to the normal pattern (fig. 4).

**DISCUSSION**

The processes of tumorgenesis, and even the chemotherapy are associated with release of a number of elevated reactive oxygen species (ROS) and (RNS) in an affected area which activates signal transduction cascades and changes transcription factors and mediates the biological reactions of cell stress. The generated metabolites connected to oxidative stress cause damage to biological molecules including protein, lipids and nucleic acids. The degradation of genomic DNA into nucleosomal fragments was considered the hallmark of mutated unstable cells and by time it becomes tumor. The use of anticancer drugs is no longer the best solution to overcome cancer since multiple side effects cannot be ignored as well as its slow-term effect could also lead to its return.

Here, the present study designed to investigate whether a preference exist between the use of EGCG as chemotherapeutic and protective agent in suppressing the tumor. Our choice to use Ehrlich Ascites Carcinomas EAC since it proliferates and stimulates the oxidative stress in mice and hens elevates its proliferation.

Our results obviously confirmed two main results; firstly, the treatment with green tea or EGCG offered chemotherapeutic effect against cell proliferation and that when EGCG administered in EAC-bearing mice, it significantly reduced
the volume of tumor and tumor markers in blood such as TNF-α, GH and LH and diminished the inflammatory markers including α-fetoprotein, IL-6 and IL-8 which are considered as tumor marker for hepatic cell carcinomas\(^3\). This effect has been clearly attributed to its antioxidant activities\(^34\),\(^35\) which confirmed its ability to scavenge a wide range of free radicals. Secondly, Supplementation of EGCG prior EAC transplantation, the anti-inflammatory, antioxidant effects were apparently improved and kept the DNA integrity (as shown by DNA fragmentation assay) and this helped in suppressing the tumor proliferation. These results revealed that EGCG can influence tumor suppressors and inhibit cellular proliferation, interfering in this way with the steps of carcinogenesis. The anticancer and anti-inflammatory properties of EGCG may be due to the antioxidant effect of hydroxyl groups which are the main functional constituent of catechins, the family to which epigallo-catechin-gallate EGCG belongs, which can neutralize the reactive oxygen and nitrogen reactive species\(^36\). The integrity of genomic DNA maintained in orally administrated EGCG (50 mg/kg/b.wt.) is proposed to be due to its interfere in initiation, development and progression carcinogenesis by modulating the critical processes of cellular proliferation, differentiation, apoptosis, angiogenesis and metastasis\(^7\). The use of green tea or its component EGCG as a traditional treatment is recommended to increase the benefits as a natural therapeutic and preventive agent for tumor proliferation.

**CONCLUSION**

The administration of green tea and its major constituent EGCG showed a significant potent protective role in suppressing tumor proliferation than its use in treatment due to its antioxidant and anti-inflammatory effect and maintaining the integrity of underlying genomic DNA that make it a strong barrier which arrest the process of oncogenesis.

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**Competing interests**

No conflict of interest.

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**Ethical approval**

The study was approved by the Institutional Animal Care and Use Committee of Zagazig University [ZU-IACUC/3/F/75/2019].

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