



RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

CYTOTOXIC EFFECT OF GREEN TEA INFUSION ON HUMAN HELA CERVICS ADENOCARCINOMA CELL LINE

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ABSTRACT

Tea is a beverage consumed about two thirds of the world population. Several laboratories and epidemiological studies have shown that green tea has anticarcinogenic, antimutagenic and chemopreventive effects related to the polyphenolic compounds present in leaves. Major polyphenolic substances of tea leaves are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). In this study, inhibitory effect of 2% infusion of a commercial green tea product, marketed in Turkey, on HeLa cervics adenocarcinoma cell line has been investigated using direct cytotoxicity test and TNF test. The quantitative analysis of the polyphenolic content of infusion has been performed by HPLC. Results have shown that 2% green tea infusion has cytotoxic effect on HeLa cells, but TNF stimulation may not be the responsible mechanism for this effect.

Anahtar Kelimeler : Green tea, Cytotoxicity, Cancer, TNF- α

YEŞİL ÇAY İNFÜZYONUNUN İNSAN HeLa SERVİKS ADENOKARSİNOMA HÜCRELERİ ÜZERİNE SİTOTOKSİK ETKİSİ

ÖZ

Çay, dünya nüfusunun yaklaşık üçte ikisinin tükettiği bir içecektir. Yapılan birçok laboratuvar ve epidemiyolojik çalışmalar yeşil çayın, yapraklarında bulunan polifenolik bileşenlere bağlı olarak antikarsinojenik, antimutajenik ve kimyasal koruyucu etkilerinin olduğunu göstermiştir. Epikateşin (EK), epigallokateşin (EGK), epikateşin gallat (EKG) ve epigallokateşin gallat (EGKG) çay yapraklarında bulunan ana polifenolik yapıları bileşenlerdir. Bu çalışmada Türkiye’de satılan bir yeşil çay ürününden hazırlanan %2’lik infüzyonun HeLa hücreleri üzerindeki sitotoksitesite direkt sitotoksitesite testi ve TNF testi ile incelenmiştir. Infüzyonun polifenolik bileşenlerinin kantitatif analizi YBSK ile gerçekleştirilmiştir. Sonuçlar %2 yeşil çay infüzyonunun HeLa hücreleri üzerinde sitotoksik etkili olduğunu, ancak bu etkiden sorumlu mekanizmanın TNF stimülasyonu olamayacağını göstermiştir.

İngilizce abstract bölümü

Keywords: Yeşil çay, Sitotoksitesite, Kanser, TNF- α

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1. INTRODUCTION

The history of the tea as a beverage is traced to about 2700 B.C. (Weisburger, 1997). The plant has been growing at Rize area since end of 1800's. Today there are more than 40 plants producing tea products in Turkey (Baytop, 1999). It is believed that tea consumption is second only to water, with a per capita human consumption of approximately 120 mL/day (Ahmad and Mukhtar, 1999).

Three types of tea products are available as green tea, oolong tea and black tea. Whereas green tea and oolong tea are commonly consumed India, Japan, China and some of other eastern countries, black tea is consumed predominantly in western countries (Bruneton, 1999).

Tea leaves have a very rich chemical composition (Table 1). Polyphenols are present to the extent of 30-35% in dry tea leaf and determine the quality of beverage (Kuroda and Hara, 1999). The primary polyphenols present in green tea are epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). The amount of these polyphenolic compounds varies depending on the variety, leaf age and environmental factors such as climate, light, temperature, rainfall and nutrient availability (Ahmad and Mukhtar, 1999; Kuroda and Hara, 1999).

Several laboratories and epidemiological studies have demonstrated that green tea polyphenols have anticarcinogenic and cancer chemopreventive effects (Ahmad et al., 2000; Fujiki, 1999; Inoue et al., 2001; Islam et al., 2000; Kuroda and Hara, 1999; Morre et al., 2000). It is believed that cancer chemopreventive effects of green tea are mainly mediated by EGCG (Ahmad and Mukhtar, 1999).

According to results of several studies, green tea may exhibits its anticarcinogenic activity via inhibition of TNF release (Ahmad et al. 2000, Fujiki 1999, Fujiki et al. 1998, Fujiki et al. 2000). In this study, we aimed to investigate the cytotoxic effect of 2% green tea infusion with TNF test which is a procedure based on the TNF release from the macrophages and direct cytotoxicity test comparatively on human HeLa cervix adenocarcinoma cells.

2.0 MATERIAL AND METHOD

2.1 Material

EC, EGC and ECG were purchased from Sigma Co. EGCG was obtained from Phytoplant Co. (Germany). All the other chemicals used in the experimental procedures were purchased from Sigma Co. (USA). Cytotoxicity tests were performed in a Holten sterile cabinet and Heraeus incubator. Biofuge 17RS centrifuge, Olympus CK2 microscope and Biotech ELX808 ELISA were used in the cytotoxicity

experimental procedures. Quantitative analyses were taken by an HPLC system consisted of an LC10A liquid chromatograph (Shimadzu), a Macherey-Negel C8 column (150 x 4.6 mm, particle size 5µm), a SPD-M10A diode array detector, a LC10AT gradient pump (Shimadzu), a CBM10A connector module (Shimadzu) and a Rheodyne injection port (Cotati) with a 20µL loop.

2.1.1 Cell Culture

Human cervix adenocarcinoma cells (HeLa) were obtained from Ankara University, Faculty of Veterinary. Macrophages were harvested from Swiss albino mice by peritoneal lavage with 5 mL of phosphate buffer solution 4 days after intraperitoneal injection of 4 mL 3% thioglycollate solution.

Table 1. Chemical components of unprocessed tea

| <i>Substance</i> | <i>%</i> |
|--|----------|
| Carbohydrate | 30 |
| Sugars | 4 |
| Starch | 2 |
| Pectins | 11 |
| Pentosans | |
| Crude fibre | 13 |
| Proteins (4% free aminoacids) | 20 |
| Lipids | 2 |
| Polyphenols | 3 |
| Caffeine | 5 |
| Minerals (ash) | 7 |
| Enzymes, flavour chemicals, vitamins, chlorophyll and other pigments (cellulose, lignin) | |

2.2 Method

2.2.1 Preparation of Infusion

2 g of green tea leaves were boiled in 100 mL of boiling water for 5 minutes. Liquid phase was filtered from 0.22 µm filter. This procedure is similar to that of taken out by human to prepare beverage using green tea leaves

2.2.2 Direct Cytotoxicity Test

HeLa cells were suspended in RPMI1640 medium supplemented with 10 mL Hepes buffer solution (1

M)(10x), 5 mL Mem Vitamin (100x), 5 mL NEAA (100x), 5 mL Penicillin-Streptomycine, 10 mL L-Glutamine (200 mM) and and 50 mL FCS. In each well of the plate, 2.104 HeLa cells/100 μ L medium were cultured and incubated at 37°C, 5%CO₂ and 95% humidity. 50, 25, 12.5, 3.25, 3.125, 1.625, 0.812 and 0.406 μ L of green tea infusion doses were applied to the cells directly using RPMI as control and incubated for 24 hours after incubation period, cell viability was assessed by neutral red uptake method (Xie et al., 1999).

2.2.3 TNF Test

The TNF test procedure established by Ruff and Gifford (1981) was performed. Macrophages were suspended in medium prepared in a same manner used in direct cytotoxicity test. 1.104 macrophages/100 μ L medium were cultured in each well. RPMI and LPS, a TNF inducer, were used as control. Same volumes of green tea infusion applied in direct cytotoxicity test were taken on the macrophages and incubated for 18 hours. After this incubation period, supernatants were extracted to another plate containing 2.104 HeLa cells / well and incubated. After 24 hours of incubation, viability of the HeLa cells were assessed using Neutral red uptake method (Xie et al., 1999).

2.2.4 HPLC Method

Quantitative analysis of the polyphenolic compounds in 2% infusion of green tea were made by an HPLC method which has been performed in one of our previous study (Ergun et al., 2004). Briefly, chromatographic separation was performed by delivering the mobile phase, A: acetonitrile:TFA:water (10:0.3:89.7 v/v/v) and B: acetonitrile: water (30:70 v/v) at a flow rate of 1 mL/min. Detection was carried out at 278 nm. Four basic polyphenolic compounds were separated in 17 minutes.

2.2.5 Statistical Analysis

Statistical analyses were performed using Student's t-test and ANOVA varians analysis $p < 0.05$ has accepted as statistically significant. Results have given as mean \pm standard error of mean.

3. RESULTS AND DISCUSSION

Cytotoxic effect of 2% green tea infusion on HeLa cells was investigated with TNF test and direct cytotoxicity test comparatively. In TNF test, cytotoxic effects of LPS which is a substance stimulates TNF release from the macrophages (Luettig et al., 1989) and green tea infusion were evaluated using RPMI as control. According to the results of ANOVA varians analysis, while there was no significant difference between control and LPS applied groups, statistically significant results were obtained from infusion treated

group against LPS and RPMI administered groups ($p < 0.05$) (Fig 1).

In the direct cytotoxicity test, green tea infusion dosages were directly applied to the HeLa cells and RPMI was also used as control. Student's t test was performed for statistical analysis and significant difference was found between control and infusion applied groups (Fig 2).

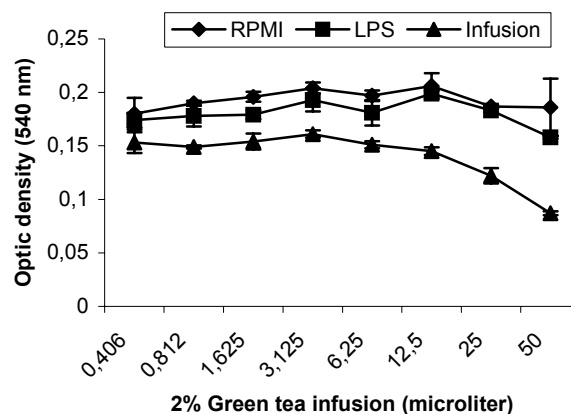


Figure 1. Results of TNF test

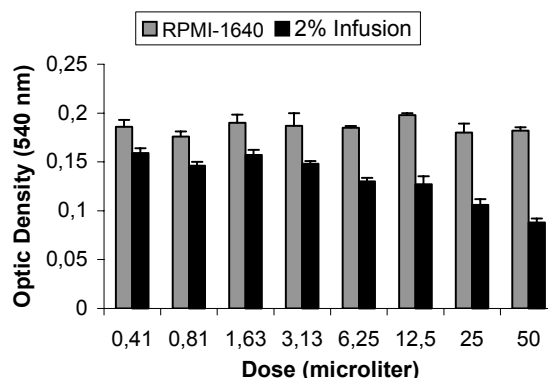


Figure 2. Mean optic densities obtained from direct cytotoxicity test.

The cytotoxic effect of 2% green tea infusion was evaluated accounting the death percentages of the HeLa cells 24 hours of incubation period following infusion administration. As seen in Figure 3, the IC₅₀ values obtained in TNF test and direct cytotoxicity test were quite similar. This similarity has showed that green tea has no TNF stimulating activity on macrophages. So, the cytotoxic effect seen in both tests must have been TNF independent. On the other hand, the cytotoxic effect of green tea is dose dependent when administered directly. However the cytotoxic effect found in TNF test is not linear and dose-dependent.

HPLC results have revealed that 2% green tea infusion used in the experiments contains 92.58 μ g/mL EGC, 186.66 μ g/mL EC, 44.70 μ g/mL EGCG and 24.67 μ g/mL ECG. Since the approximate 50% inhibition on cell viability has occurred in 50 μ L dose of infusion, using the HPLC results, the IC₅₀ values for

EGCG, EC, ECG and EGC were calculated as 2,235 $\mu\text{g/mL}$, 9.433 $\mu\text{g/mL}$, 1.233 $\mu\text{g/mL}$ and 4.629 $\mu\text{g/mL}$ respectively.

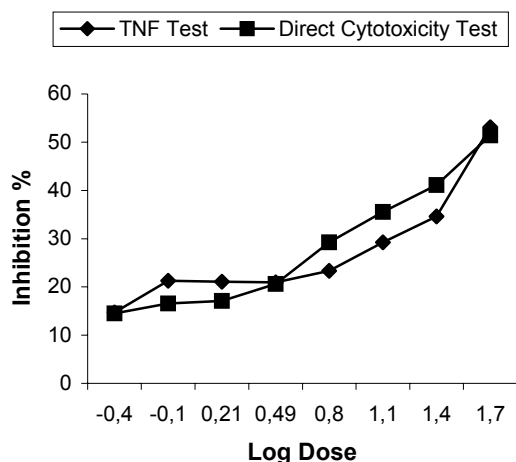


Figure 3. Inhibition percentages obtained in TNF test and direct cytotoxicity test.

In this study, two different test procedures were performed to evaluate the cytotoxic effect of green tea. TNF test was carried out to assess the possible TNF stimulator activity of green tea infusion on macrophages and the possible proliferative or cytotoxic effects of TNF. Direct cytotoxicity test was performed to evaluate the direct toxic effect of the infusion on HeLa cells. Close IC_{50} values obtained in both test procedures have indicated that green tea polyphenols have no TNF release stimulatory activity on macrophages. However, in TNF test, the cytotoxic activity of the supernatants collected from green tea administered macrophage cultured wells were higher than that the effect of the supernatants collected from LPS administered wells (Fig 1). This higher cytotoxic effect may be due either to the direct cytotoxic effect of the remained tea polyphenols in the supernatant or to release of the other cytotoxic cytokines from macrophages under the stimulatory effects of tea polyphenols. But, because the inhibitory activity found in TNF test is not linear and also dose-dependent, we suppose that the inhibitory effect observed in TNF test is related to the direct effects of the polyphenols remained in the supernatant. Fujiki et al. (2000) have reported the suppressive effect of EGCG on TNF- α release at a IC_{50} value of 20 μM . The observation of Fujiki et al. is supported by the fact that we observed cytotoxic activities in infusion applied cells, while normal proliferation was going on in LPS treated cells. If infusion would have TNF stimulatory effect like LPS, normal proliferation would go on in the infusion administered wells.

Direct cytotoxicity test results have revealed the dose dependent toxic effect of the infusion on HeLa cells. This is a good property for the substances which are thought to have a potential to be used in cancer prevention and/or therapy. Also, having a growth inhibitory effect on cancerous cells but not on their normal counterparts (Chen et al., 1998) makes the

green tea polyphenols advantageous choice for chemoprevention and cancer therapeutic.

In conclusion, we suggest that, green tea infusion dose dependently inhibits human HeLa cervix adenocarcinoma cells when administered directly, but TNF release can not be the underlying mechanism of this effect.

REFERENCES

- Ahmad, N. and Mukhtar, H. (1999). Green tea polyphenols and cancer: Biologic mechanisms and practical implications. *Nutrition Reviews* 57(3), 78 – 83.
- Ahmad, N., Gupta, S. and Mukhtar, H. (2000). Green tea polyphenol epigallocatechin-3-gallate differentially modulates nuclear factor KB in cancer cells versus normal cells. *Archives of Biochemistry and Biophysics* 376(2), 338-346.
- Baytop, T. (1999). Türkiye’de Bitkiler ile Tedavi: Geçmişte ve Bugün, 2. Baskı, Nobel Tıp Kitabevi, 180 – 181.
- Bruneton, J. (1999). Pharmacognosy: phytochemistry, medicinal plants, 2nd ed. Hampshire Intercept, 1075 – 1079.
- Ergun, B., Saraçoğlu, A., Şener E. and Altıokka G. (2004). Determination of basic polyphenolic compounds in some Turkish tea samples by HPLC method. *Anadolu University Journal of Science and Technology* 5(2), 285-290.
- Fujiki, H. (1999). Two stages of cancer prevention with green tea. *Journal of Cancer Research and Clinical Oncology* 125, 589-597.
- Fujiki, H., Suganuma and M., Okabe, S. (1998). Cancer inhibition by green tea. *Mutation Research* 402, 307-310.
- Fujiki, H., Suganuma, M. and Okabe, S. (2000). A new concept of tumor promotion by tumor necrosis factor- α , and cancer preventive agents (-)-Epigallocatechin gallate and green tea - A Review. *Cancer Detection and Prevention* 24(1), 91-99.
- Inoue, M., Tajima, K. and Mizutani, M. (2001). Regular consumption of green tea and the risk of breast cancer recurrence: follow-up study from the hospital-based epidemiologic research program at Aichi cancer center (HERPACC). *Japan Cancer Letters* 167, 175-182.
- Islam, S., Islam, N. and Kermode, T. (2000). Involvement of caspase-3 in epigallocatechin-3-gallate mediated apoptosis of human chondrosarcoma cells. *Biochemical and Biophysical Research Communications* 270, 793-797.

Kuroda, Y. and Hara, Y. (1999). Antimutagenic and anticarcinogenic activity of teapolyphenols. *Mutation Research* 436, 69-97.

Luettig, B., Steinmuller, C. and Gifford, G.E. (1989). Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. *Journal of the National Cancer Insitutet*. 81(9), 669 – 675.

Morre, D.J., Bridge, A. and Wu, L.Y. (2000). Preferential inhibition by (-)Epigallocatechin-3-gallate of the cell surface NADH oxidase and growth of transformed cells in culture. *Biochemical Pharmacology* 60, 937-946.

Ruff, M.R. and Gifford, G.E. (1981). Rabbit tumor necrosis factor: Mechanism of action. *Infection and Immunity* 380-385.

Weisburger, J.H. (1997). Tea and health: A historical perspective. *Cancer Letters* 114, 315-317.

Xie, S.P., Pirianov, G., Colston, K.W. (1999). Vitamin D analogues suppress IGF-1 signaling and promote apoptosis in breast cancer cells. *European Journal of Cancer* 35,1717-1723.

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