Original Study

GROWTH INHIBITION AND CYTOTOXICITY EFFECT OF GREEN TEA EXTRACT ON SQUAMOUS CELL CARCINOMA CELL LINE: AN IN VITRO STUDY

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ABSTRACT

Aim: Cancer and malignancies are the leading cause of death in developed countries and the second leading cause of death in developing countries. Recent studies on natural ingredients, including herbs with targeted anti-malignancy properties and the potential for fewer side effects, have taken place. The aim of the present study is to evaluate the growth inhibition and cytotoxicity potential selective effects of green tea extract on malignant cells compared to normal cells.

Materials & Method: To assess the cytotoxicity effects of green tea extract, a photometry method using MTT (Microculture Tetrazolium Test) was used. All tests on normal and malignant cell lines were repeated three times. In order to analyze the effects of the extract concentration and cell type effects together on cell vitality, a two-way ANOVA analysis was carried out. In order to analyze the effects of the extract concentration on cell vitality, a two-way and one-way ANOVA analysis was carried out.

Results: According to the test results, the cells vitality amount was significantly associated with different concentrations of green tea extract (p-value < 0.001). The cell vitality was also significantly associated with the cell type in concentrations above 20% (p-value =0.023), but not significantly associated with the cell type at concentrations below 20% (p-value=0.123).

Conclusion: The results of this study showed that the cytotoxicity effects of green tea extract on malignant cell lines (SCC, Squamous Cell Carcinoma) are higher than on normal cell lines at concentrations above 20%, while they are similar in concentrations below 20%. According to the study, green tea extract could be used as a natural substance with selected anti-cancer properties and less side effects against SCC.

Key words: Antitumor, Drug Screening Assays, Herb-Drug Interactions, Squamous Cell Carcinoma, Tea.

Introduction

Malignancies, particularly squamous cell carcinoma, are among the main causes of death in the world. Since 1915, an increase in the incidence of head and neck squamous cell carcinoma shows that environmental factors must play an important role in the development of oral cancers.[1] The most important oral carcinogenic environmental factors are: tobacco use,[2] alcohol consumption, diet, body mass index (BMI),[3] oral hygiene and viral infections.[1] The development in chemotherapy and radiotherapy methods, with the goal of improving survival rates, have resulted in an increase in the incidence of adverse effects of these treatments.[4]

Tea is the most popular worldwide beverage after water. Previous studies have shown the anti-tumor effect of green tea and its components on different malignancies, including melanoma skin,[5] lung cancer,[6] colon cancer and pancreatic cancer[7] and malignant mouth tumor[8] and head and neck carcinoma.[9] Chung and colleagues in a 2003 study[10] showed that green tea and its components, including EGCG, exhibit anti-tumor effects through a combination of anti-oxidative effect, anti-proliferative effect and induction of apoptosis.in another study by Lambert JD *et al* in 2003 showed that green tea can inhibit the proliferation of tumor cells and the molecular pathway towards it.[11]

Catechins can affect the cellular signals associated with cell death.[9] One of the most important polyphenolic compounds in green tea are catechins. The therapeutic effect of catechin contained in green tea is related to the EG (epicatechin) – ECG (Epicatechin – 3 - gallate) - EGC (epigallocatechin) and EGCG (Epigallocatechin-3-gallate).[12] According to other studies, EGCG works by inhibiting a molecule such as vascular endothelial growth factor (VEGF) [10,13,14]

According to Kohet *et al* in 2011,[15] the green tea EGCG inhibits the HGF hepatocyte growth factor in oral squamous cell carcinoma and thereby, prevents the growth and invasion of cancer. Ramirez-Mares MV showed that EGCG increased cell apoptosis and caused an anti-cancerous effect on malignant cells.[13]

The failures of routine oral SCC treatment, the failure to improve the prognosis of this disease during the past four decades, as well as the serious side effects of oral SCC treatments, indicate the necessity of finding new treatments. Due to anti-cancerous effect of green tea because of its polyphenolic compounds like catechins. In this study we decided to evaluate the inhibitory and cytotoxicity effects of Lahijan green tea extract for the first time on malignant cell lines compared to normal cells.

Materials and Method

In this experimental *in vitro* study. This study lasted for three weeks and all the procedures were carried out at Isfahan University of Medical Science.

To assess the cytotoxicity effects of green tea extract, a photometry method using MTT (Microculture Tetrazolium Test) was used.[16]

Preparation of plant extracts

To prepare the green tea extract, the plant was dried for three days at room temperature (25°C). The dried plant (300 grams) was ground to a powder in a mortar. The powder was then combined with n-hexane (500 ml/m^3) (CH_3 (CH_2) $4CH_3$) (Sigma, Germany). The combined powder extract was distillate with rotary device (Vargha Tajhiz, Iran) in a vacuum. The operation lasted for approximately 24 hours. A solvent removing operation was performed three times using Rota Vapor (R300, BUCHI, Switzerland). During this operation, the solvent was evaporated and the extract was concentrated. The remaining powder from the previous step was dissolved in chloroform ($CHCl_3$) (> 99 wt %) (Sigma, Germany).

Cell Culture

Cell KB (KERATIN-forming based on Isoenzyme Pattern-squamous cell carcinoma) and L929 (mouse fibroblast cells) was obtained from the Pasteur Institute of Iran Cell Bank. The cells were cultured in the DMEM medium (Dulbecco's Modified Eagle'smedium), (Merck, Germany) containing 10% fetal bovine serum and L-glutamine. The cells were incubated in 5% CO_2 ($HF_2$12UV, Heal Force, China) and the medium was refilled every three days (temp= 37°C, 5% CO_2, 95% RH).

Cytotoxicity Evaluation using Microculture Tetrazolium Test (MTT assay)[17]

To study the cytotoxic effect of the plant extracts, a photometry method using MTT (Sigma, Germany) was used. This method is based on living cells mitochondrial succinate dehydrogenase enzyme activity that converted yellow soluble MTT to purple formazan crystals. [Figure 1]

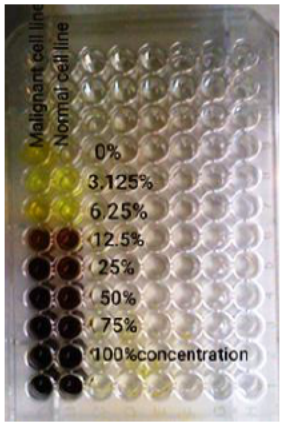

Figure 1: MTT Process (concentrations of herbal extract on malignant and normal cell line)

In order to perform this test, cells of 96-well plates (Gene fanavaran, Iran) were poured with 180 ml cell suspension. Then, 20 microliters of extract from different concentrations were added to the wells. [Figure 2] The final volume per well was 200 microliters. A well containing medium and 5% DMSO (Dimethyl sulfoxide) (Sigma, Germany), without extract, was considered as a negative control, and a well containing medium alone was considered as blank. Plates were incubated for 48 hours in 5% CO_2 at 37°C. Then, 20 microliters of MTT were added to each well and incubated for 2 hours. After this, 100 ml DMSO was added to dissolve the formazan grains in each well. Finally, absorption of 560 nm wavelength of each well was measured by an Elisa reader (Biotech, USA). Cell viability in the control wells was considered as 100% vitality and cell viability in the wells tested were calculated by the following formula.[18] The concentration of the herbal extract that reduced cell vitality was considered as IC50 (IC50 is the drug concentration causing 50% inhibition of the desired activity).[19]

Cell survival rate = (blank cell absorption – treated cell absorption) / (blank cell absorption – negative control absorption) x 100.

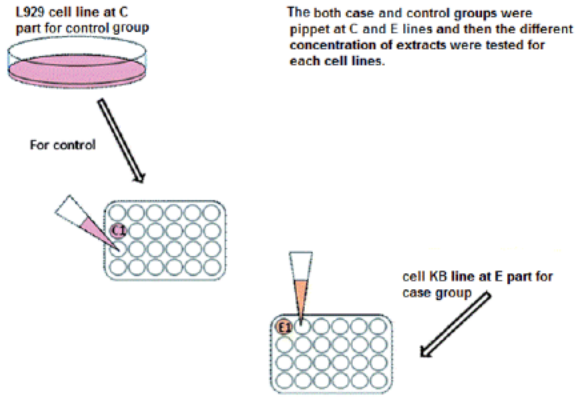

Figure 2: MTT process for L929 cell and cell KB line.

The entire process was repeated three times on normal and malignant cell lines,due to make sure that our actions have been consistent with our values.

Statistical analysis

The results were entered into the software SPSS version 12. The effects of the green tea extract concentration and type of cell (normal cells/malignant cells) on cell viability were analyzed using a two-way analysis of variance (ANOVA). The effects of the plant extracts on the percentage of living cells remaining in each cell line to separate one-way analysis of variance (One-way ANOVA) and two-way ANOVA at a significance level of (α= 0/50) was carried out.

Results

The results showed that the percentage of vital cells was significantly associated with different concentrations of the extract (p-value<0.001) and with cell type in concentrations above 20% (p-value =0.023). However, it was not significant in concentrations below 20% (p-value =0.123). [Table 1]

According to the results, there is an inverse relationship (linear function) between living cells percentage and various concentrations of green tea extract. The linear equation for normal cells is y = -3.85x + 382.5. For malignant cells, the linear equation is y=-2.84x + 284.2,

where y equals the percentage of vital cells and x is equal to the concentration of extract. Vital cells percentages calculated after exposure to different concentrations of the extract by using these equations.

Mean ± SD of normal vital cells after exposure (%)	Mean ± SD of the vital malignant cells (%)	Concentrations of Green Tea (%)
76.95 ± 1.25	71.05 ± 1.48	100
76.94 ± 2.89	81.24 ± 1.57	75
84.69 ± 0.86	89.16 ± 2.18	50
93.59 ± 0,93	94.16 ± 2.59	25
92.25 ± 5.26	96.26 ± 1.17	12.5
94.09 ± 3.82	79.9 ± 0.44	6.25
98.46 ± 1.5	98.95 ± 0.61	3.125
100 ± 0	100 ± 0	0

Table 1: The mean and standard deviation and P-value of normal vital cells and vital malignant cells after exposure to different concentrations of green tea extract.

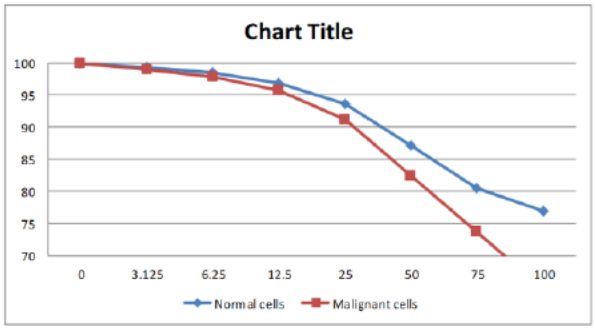

Chart 1: The survival rate after exposure to different concentrations of green tea extract in normal and malignant cell lines.The percentage of viable cells after exposure to different concentrations of green tea extract in normal and malignant cell lines

Discussion

The result showed that green tea inhibits the growth of malignant (SCC) and normal cells.The cytotoxicity effect for concentrations higher than 20% on malignant cells is higher than a normal cell line (mouse fibroblasts). Since comparative studies of the effects of green tea extract on normal and SCC malignant cells have not been done previously, it is not clear whether these pharmacological effects of green tea extract are specific for SCC cells. Our studies showed for the first time that green tea extract at an appropriate dosage inhibited the growth of transformed cell KB, but not their normal cells (L929). There is a liner relationship between cytotoxicity and the growth inhibition effect and green tea extracts concentration.

The therapeutic effects of tea are associated with its polyphenols compounds. Polyphenol compounds in green tea make up more than 30% of its dry weight. There is a higher amount of polyphenol compounds in green tea compared to black tea.[20] This could be because of the method of preparation and processing of green tea.

The results of current study, as with studies by Hsu *et al*. in 2002,[21] Kohet *et al*. in 2011[15] and Liu *et al*. in 2011,[22] indicate that green tea inhibits the growth of oral squamous cell carcinoma. Unlike the mentioned studies, green tea extract used in this study (Lahijan) in concentrations of more than 20% had a higher cytotoxicity effect on cancer cells compared to normal cells. However, in concentrations of less than 20%, the cytotoxicity of extracts on malignant cells and normal cells was the same. Unlike the current study, in the results of Hsu *et al*.,[21] Koh *et al*[15]and Liu *et al*.,[21] all the concentrations of green tea had a higher cytotoxicity effect on malignant cells compared to normal cells.

The difference in results of these studies may be due to the different combinations of green tea. Ramirez-Mares MV Studies by Chow *et al*.,[24] Lee *et al*.[25] and Pister *et al*.[26] showed that the bioavailability of green tea compounds, including polyphenols, for oral use is low, at less than 1%. According to the current study,green tea extracts in low concentrations have the same cytotoxicity effects on normal and malignant cells. Because of the low bioavailability of green tea compounds after oral consumption, oral administration of green tea may not lead to selective anti-tumor effects in oral squamous cell carcinoma. (Selective anti-tumor effects means the applied substance has cytotoxicity and inhibition effects on malignant cells, while it has the minimum effects on normal cells.)

One of the limitations of the present study is that the MTT method is the first method used for cell cytotoxicity, but it could not evaluate the molecular and mitocondrial and cytoplasmic factors that affect the study results. It does not distinguish between cell apoptosis and necrosis. Because of that, and in order to solve this limitation, we decided to use the microarray plus the MTT method to distinguish between cell apoptosis and necrosis.[27]

Conclusion

The results of this study showed that the cytotoxicity effects of green tea extract on a malignant cell line (SCC, Squamous Cell Carcinoma) is higher than on normal cell lines at concentrations above 20%, being similar in concentrations below 20%. According to the study, green tea extract could be used as a natural substance with selected anti-cancer properties and will have less side effects when used against SCC.

References

1. Castillo M, Scheen A, Lefebvre P, Luyckx AS. Insulin-stimulated glucose disposal is not increased in anorexia nervosa. J Clin Endocrinol Metab 1985;60(2):311-4.
2. Schmidt BL, Dierks EJ, Homer L, Potter B. Tobacco smoking history and presentation of oral squamous cell carcinoma. J Oral Maxillofac Surg 2004;62(9):1055-8.
3. Bagnardi V, Rota M, Botteri E, Tramacere I, Islami F, Fedirko V *et al*. Alcohol consumption and site-

specific cancer risk: a comprehensive dose–response meta-analysis. Br J Cancer 2015;112(3):580-93.
4. Saadeh CE. Chemotherapy- and radiotherapy-induced oral mucositis: review of preventive strategies and treatment. Pharmacotherapy 2005;25(4):540-54.
5. Wu Y, Lin Y, Liu H, Li J. Inhibition of invasion and up-regulation of E-cadherin expression in human malignant melanoma cell line A375 by (-)-epigallocatechin-3-gallate. J Huazhong Univ Sci Technolog Med Sci 2008;28(3):356-9.
6. Hazgui S, Bonnomet A, Nawrocki-Raby B, Milliot M, Terryn C, Cutrona J *et al*. Epigallocatechin-3-gallate (EGCG) inhibits the migratory behavior of tumor bronchial epithelial cells. Respiratory Research 2008;9(1):1.
7. Shankar S, Ganapathy S, Hingorani SR, Srivastava RK. EGCG inhibits growth, invasion, angiogenesis and metastasis of pancreatic cancer. Front Biosci 2008;13:440-52.
8. Li N, Chen X, Liao J, Yang G, Wang S, Josephson Y *et al*. Inhibition of 7,12-dimethylbenz [a] anthracene (DMBA)-induced oral carcinogenesis in hamsters by tea and curcumin. Carcinogenesis 2002;23(8):1307-13.
9. Gouni-Berthold I, Sachinidis A. Molecular mechanisms explaining the preventive effects of catechins on the development of proliferative diseases. Curr Pharm Des 2004;10(11):1261-71.
10. Kojima-Yuasa A, Hua JJ, Kennedy DO, Matsui-Yuasa I. Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. Life Sci 2003;73(10):1299-313.
11. Lambert JD, Yang CS. Mechanisms of cancer prevention by tea constituents. J Nutr 2003;133(10):3262S-7S.
12. Zaveri NT. Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications. Life Sci 2006;78(18):2073-80.
13. Ramirez-Mares MV, Kobayashi H, de Mejia EG. Inhibitory effect of Camellia sinensis, Ilex paraguariensis and Ardisia compressa tea extracts on the proliferation of human head and neck squamous carcinoma cells. Toxicology Reports. 2016;3:269-78.
14. Yuan CH, Horng CT, Lee CF, Chiang NN, Tsai FJ, Lu CC *et al*. Epigallocatechin gallate sensitizes cisplatin-resistant oral cancer CAR cell apoptosis and autophagy through stimulating AKT/STAT3 pathway and suppressing multidrug resistance 1 signaling. Environ Toxicol 2016;32(3):845-855.
15. Koh YW, Choi EC, Kang SU, Hwang HS, Lee MH, Pyun J *et al*. Green tea (−)-epigallocatechin-3-gallate inhibits HGF-induced progression in oral cavity cancer through suppression of HGF/c-Met.J Nutritional Biochem 2011;22(11):1074-83.
16. Wang P, Henning SM, Heber D. Limitations of MTT and MTS-based assays for measurement of antiproliferative activity of green tea polyphenols. PloS One 2010;5(4):e10202.
17. Ferrari M, Fornasiero MC, Isetta AM. MTT colorimetric assay for testing macrophage cytotoxic activity in vitro. J Immunol Methods 1990;131(2):165-72.
18. Gavanji S, Sayedipour SS, Larki B, Bakhtari A. Antiviral activity of some plant oils against herpes simplex virus type 1 in Vero cell culture. J Acute Med 2015;5(3):62-8.
19. Constantin M, Bromont C, Fickat R, Massingham R. Studies on the activity of bepridil as a scavenger of free radicals. Biochem Pharmacol 1990;40(7):1615-22.
20. Del Rio D, Stewart AJ, Mullen W, Burns J, Lean ME, Brighenti F *et al*. HPLC-MSn analysis of phenolic compounds and purine alkaloids in green and black tea. J Agric Food Chem 2004;52(10):2807-15.
21. Hsu SD, Singh BB, Lewis JB, Borke JL, Dickinson DP, Drake L *et al*. Chemoprevention of oral cancer by green tea. Gen Dent 2002;50(2):140-6.
22. Liu X, Zhang DY, Zhang W, Zhao X, Yuan C, Ye F. The effect of green tea extract and EGCG on the signaling network in squamous cell carcinoma. Nutr Cancer 2011;63(3):466-75.
23. Masuda M, Suzui M, Lim JT, Deguchi A, Soh JW, Weinstein IB. Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction. J Exp Ther Oncol 2002;2(6):350-9.
24. Chow HS, Cai Y, Hakim IA, Crowell JA, Shahi F, Brooks CA *et al*. Pharmacokinetics and safety of green tea polyphenols after multiple-dose administration of epigallocatechin gallate and polyphenon E in healthy individuals. Clin Cancer Res 2003;9(9):3312-9.
25. Lee MJ, Maliakal P, Chen L, Meng X, Bondoc FY, Prabhu S *et al*. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans formation of different metabolites and individual variability. Cancer Epidemiol Biomarkers Prev 2002;11(10):1025-32.
26. Pisters KM, Newman RA, Coldman B, Shin DM, Khuri FR, Hong WK *et al*. Phase I trial of oral green tea extract in adult patients with solid tumors. J Clin Oncol 2001;19(6):1830-8.
27. Ball CA, Sherlock G, Parkinson H, Rocca-Sera P, Brooksbank C, Causton HC *et al*. Standards for microarray data. Science 2002;298(5593):539.

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