

The in vitro Effects of a Tomato Extract on Neoangiogenesis-Controlling Molecules in Colon Carcinoma Cells

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Abstract

The aim of this study was to reveal the antiproliferative effect of two tomato extracts on colorectal cancer cells in vitro, and to show their influence on the secretion of growth factors involved in tumor neoangiogenesis: VEGF and Endothelin-1, in order to reveal some of the inhibitory mechanisms exerted by the lycopene-containing tomato extracts. Human colon cancer cell lines DLD1 and HT29 were treated with two different tomato extracts, obtained from fresh and frozen tomato fruits. The cytotoxicity of the compounds was measured using the MTT test, while the expression of VEGF and Endothelin-1 was assessed by ELISA. Results showed that the extract from frozen tomatoes was more cytotoxic as the analogue from fresh tomatoes on both cell types, and both extracts were more toxic against DLD-1 cell line. VEGF and Endothelin-1 expression was also reduced by the tomato extracts, especially in DLD1. In vitro tests demonstrated that the lycopene-containing tomato extracts reduce the growth of malignant cells by tumor neovascularization hindrance, opening new perspectives to their employment in human therapeutic purposes.

Keywords: tomato extract, lycopene, colon carcinoma, neoangiogenesis.

1. Introduction

The role of nutrition gains more and more importance in cancer prevention, and a very frequent and invasive tumor, the colorectal carcinoma was proven to be particularly related to the diet [1]. Some natural compounds display not only cancer-preventing properties, but also therapeutic potential in tumor diseases; one of them is lycopene [2], which is already in clinical

trials [3]. Lycopene is a carotenoid with antioxidant properties, contained in tomatoes extracts and abundant in European diet. Previous studies revealed that lycopene is able to diminish the tumor cells proliferation, counteract the angiogenesis and cellular adhesion through vascular endothelial growth factor (VEGF) inhibition in various experimental models: human normal endothelial cells [4], human fibroblasts [5], benign hyperplasia [6] or hepatic cancer cells [7].

In vitro [8] and in vivo [9] experimental models proved that lycopene is able to modulate the vascular endothelial function in normal endothelial cells, through the inhibitory effect

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exerted on endothelin-1 (ET-1) release; but this phenomenon was not studied in tumors until now. The colorectal carcinoma cells constitutively express VEGF [10] and ET-1 [11], two growth factors of major importance which regulate the vascular system [12]. Human DLD-1 K-ras mutant colon cancer cells [13] and the HT-29 K-ras wild type cells [14] are able to secrete these two proteins; therefore the two cell populations are a good model to study the implication of these molecules in lycopene antitumor action.

The aim of this study was to reveal the antiproliferative effect of two tomato extracts on colorectal cancer cells *in vitro*, and to show their influence on the secretion of growth factors involved in tumor neoangiogenesis: VEGF and Endothelin-1, in order to reveal some of the inhibitory mechanisms exerted by the lycopene-containing tomato extracts.

2. Materials and methods

The human colon cancer cell lines DLD1 and HT29 were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK). The cells were grown in RPMI (DLD1) and McCoy's 5 (HT29) medium, supplemented with 10% Fetal bovine serum, 1% Glutamine and 1% Penicillin-Streptomycin solution (all media and supplements from Sigma Aldrich, St Louis, MO, USA) at 37°C in an incubator with humidified atmosphere and 5% CO₂ content.

For cytotoxicity test the cells were plated on 96-well microplates (Nunc delta surface plates from Thermo Fischer Scientific, Waltham, MA, USA) at a concentration of 15×10^3 cells/well in 200 μ l media. For Elisa testing, the cells were plated in 6-well plates, 5×10^5 cells in 3 ml medium.

Two lycopene-containing tomato extracts were obtained by a previously described method [15] and solubilized in tetrahydrofuran (THF, Sigma Aldrich) to obtain stock solutions: 19.53 mg/ml fresh tomatoes extract (1), and 14.08 mg/ml frozen tomatoes (2). The cell cultures were treated with the fresh tomatoes extract, the frozen tomatoes extract and with a reference compound- lycopene (from Fluorochem Ltd, Hadfield, UK). Serial dilutions were prepared in phosphate buffered saline solution (PBS, Sigma Aldrich) to obtain a series of concentrations between 0.1- 15 mg/ml

for extract 1, and between 0.1-14 mg/ml for extract 2. The reference lycopene concentration was 10.73 mg/ml, and the range of working solutions was 0.1-10 mg/ml. Considering that the proportion of compounds: cell culture media was 1:20, the final concentration of the extracts in cell culture media was from 50 μ g/ml to 950 μ g/ml for extract 1, and 50 to 750 μ g/ml for extract 2.

The cytotoxicity of the compounds was measured in triplicate, as described before [16], using the MTT dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, from Sigma Aldrich), which is transformed into its insoluble formazan form by the mitochondrial oxidoreductase enzymes, only in viable cells. The formazan crystals were solubilized in dimethylsulphoxyde (from Titolchimica, Italy), and the 96-well plates were measured in colorimetry using a Synergy 2.0 microplate reader (from BioTek Company, Winooski, USA) at 570nm wavelength. The absorbance of each well reflected the number of viable cells present. As reference we used untreated cells, and as positive control the aqueous lycopene solution.

For Elisa testing, after 24 hours of treatment the supernates were collected from the 6-well plates and analyzed. The protein concentration in supernates was measured as described before [17], using the ELISA immunoenzymatic method. The VEGF Elisa kit was provided by BlueGene Biotech (Shanghai, China) and Endothelin-1 kit from Cusabio Life Science (Wuhan, China). The pure proteins concentrations for standard curve were in the range of 0 to 1000 picogram/ml for VEGF and 0 to 200 picogram/ml for Endothelin-1. The methods were specific for human probes and the sensitivity was below 0.7 pg. For both tests, we used the antibody-coated plates provided by the manufacturers; the standards and samples were pipetted into wells in duplicate. The VEGF/ ET-1 present in the sample were bound by the immobilized antibody from the plate surface. After removing any unbound substances by 3 or 4 wash procedures, a biotin-conjugated antibody specific for VEGF or ET-1 was added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Following a wash to remove any unbound enzymes, a substrate solution was added to the wells which develop a color reaction, in direct proportion to the amount of the target protein. The color development was stopped with a sulphuric

acid solution and the plate was measured with a Tecan Sunrise Elisa reader (Tecan Group, Männedorf, Switzerland), at 450nm, and the results were generated using the Magellan software. Results were normalized: cells were counted in each well, and the protein production corresponding to 5×10^5 cells was calculated. The measurements data were processed using Graph Pad Prism 5 biostatistics software (from GraphPad Software, La Jolla, USA).

3. Results and discussion

The tomato extracts exerted cytotoxicity against both DLD-1 and HT-29 colon cancer cell lines in vitro (Table 1, Figure 1); the cell populations

subjected to the 24-hours treatment with the extracts showed poorer viability and lower proliferation rate when compared with the untreated cells. To quantify the magnitude of the extracts antiproliferative effect, we established the IC₅₀ value for each one (the concentration which reduces 50% of the living cells within a population). Being natural extracts, IC₅₀ values were moderate (range of 26.66 to 257.40 micrograms per milliliter), as expected. The extract from frozen tomatoes was more cytotoxic as the analogue from fresh tomatoes on both cell types, and the extracts were more toxic against the aggressive, K-ras mutant DLD-1 cell line (one-way Anova test, Bonferroni multiple comparison post-test, $p < 0.001$, very significant differences).

Table 1. IC₅₀ values

Cell line	DLD-1		HT-29	
	Fresh tomato extract	Frozen tomato extract	Fresh tomato extract	Frozen tomato extract
IC ₅₀ values (µg/ml)	46.91	26.66	257.40	141.72
Log IC ₅₀	1.671	1.426	2.411	2.151
Log IC ₅₀ Standard error	0.08815	0.07117	0.1083	0.07828

Sigmoidal dose-response curves

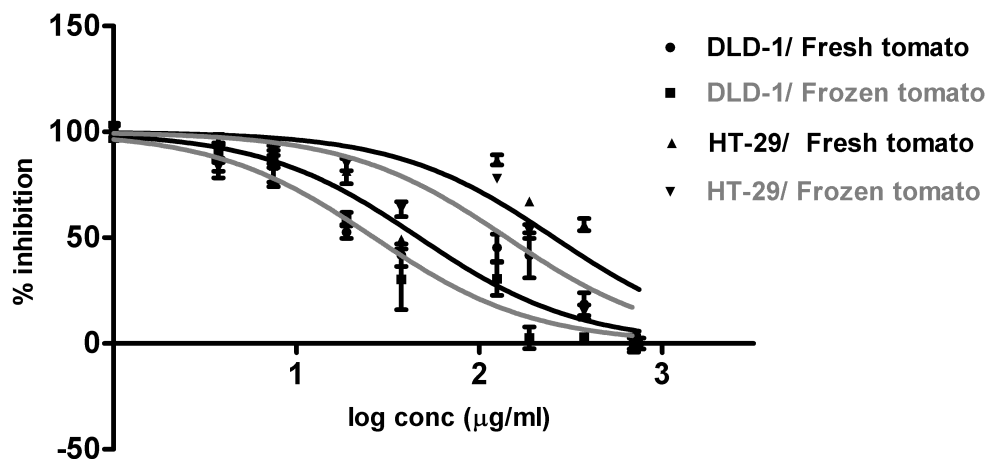


Figure 1. Relationship between the concentration of tomato extracts (black line: extract from fresh tomatoes, gray line: extract from frozen tomatoes) and the proliferation of the tumor cell lines DLD-1 and HT-29 in vitro (representation generated by Graph Pad Prism software)

The concentration of vascular endothelial factor in treated DLD-1 cells (Figure 2) was diminished by both extracts, especially by the fresh tomato extract (One-way Anova test, Bonferroni post-test, in the 95% confidence interval, $p < 0.5$). In HT-29 cells, the extracts reduced VEGF in a very significant manner ($p < 0.01$), and between them was no substantial difference. The reference

lycopene values show no significant differences versus the extracts ($p > 0.5$) which proves the compounds high efficacy. Despite the superior cytotoxicity, the frozen tomato extract VEGF-suppressing capacity is lesser as the fresh tomato extract in DLD-1, and the two extracts effect is comparable in HT-29.

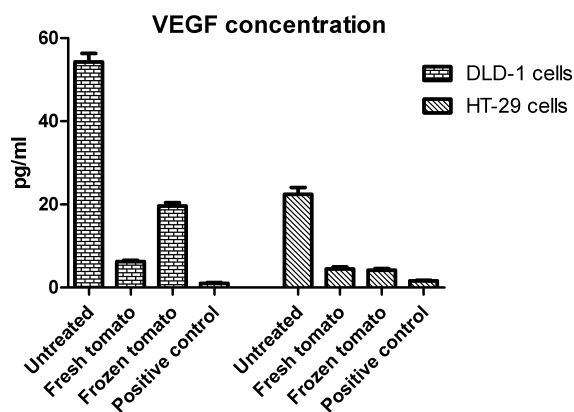


Figure 2. The tumor cells VEGF production in vitro within 24 hours of treatment with tomato extracts was quantified using ELISA measurements (concentrations expressed in picogram/ml).

The extracts inhibited the ET-1 secretion in treated DLD-1 cells (Figure 3), the reduction being significant (One-way Anova test, Bonferroni post-test, in the 95% confidence interval, $p < 0.1$) in fresh and frozen tomato extracts versus untreated control. In HT-29 cells, only the fresh tomato extract reduced to some extent the ET-1 level secreted by the cells, but the differences were not significant ($p > 0.05$). The action of frozen tomatoes extract on HT-29 ET-1 level displayed rising tendency, and the positive control values were nearly zero, out of the detection range. The fresh tomato extract does not inhibit the cell viability to the same extent as its frozen counterpart, but its ET-1 inhibitory effect is constant in both DLD-1 and HT-29 cells, while the extract from frozen tomatoes acted divergent between the two cell lines.

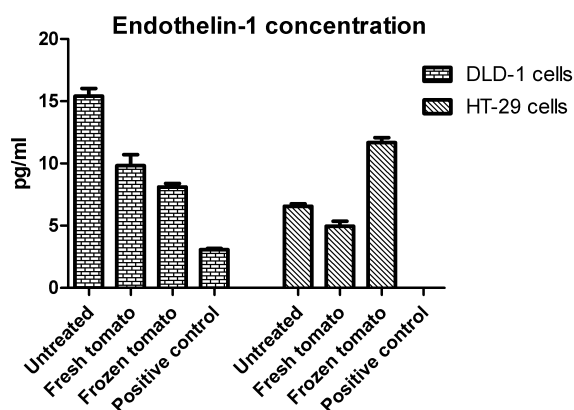


Figure 3. Concentration of ET-1 (picograms/ml) secreted by the malignant colon cells treated with tomato extracts in vitro for 24 hours

The bioactive compounds such as lycopene have promising chemopreventive effects in colorectal carcinoma, without serious side effects [18] by interferences with the phosphoinositide 3-kinase (PI3K) and Wnt cellular signaling, and they are able to inhibit the human colorectal tumors by suppressing the Akt signaling pathways [19]. The tomato extracts used in this study, having lycopene content, act similarly against HT-29 K-ras wild type/p53-mutant cells [19], and additionally, we demonstrated that they have even better effect on K-ras mutant DLD-1 cells in vitro, therefore both extracts show a good potential to suppress the tumor growth related signaling in K-ras mutant colorectal tumors, where the Wnt/beta-catenin pathway plays a critical role in tumor growth, as demonstrated in vitro [20].

Since neoangiogenesis, the formation of blood vessels in tumor mass is a critical step in tumor progression and metastasis, several therapeutic efforts are directed to development of drugs which counteract this phenomenon. The expensive monoclonal targeted therapy in colorectal cancer has a confirmed efficiency only in K-ras wild-type tumors, without being a real benefit in cancer cells which display K-ras mutations [21]. The anti-angiogenesis targeted therapy in K-ras wild-type colorectal cancer modulates the vascular endothelial growth factor (VEGF) expression, which acts as a biomarker for tumor early response to therapy [22]. The extracts studied in the present manuscript had a markedly reducing effect on VEGF production in vitro, in both K-ras mutant and wild-type cells, showing a good therapeutic potential, an inexpensive choice as compared with the monoclonal drugs.

ET-1 stimulates cell proliferation [14] by increased DNA replication in colorectal cancer cells, and has a mitogenic action mediated by PI3K [23], therefore the inhibitory effect of lycopene on ET-1 secretion is of major importance in colon cancer cell growth arrest. Since the extract from fresh tomatoes inhibited VEGF and ET-1 in both cell lines, it is expected that phosphoinositide 3-kinase will be influenced by this bioactive compound.

4. Conclusions

In vitro tests demonstrated that the lycopene-containing tomato extracts act against colorectal

tumor cells in multiple ways; the capacity of this extract to reduce the growth of malignant cells is enhanced by tumor neovascularization hindrance, opening new perspectives to their employment in human therapeutic purposes.

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