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Research Article
Assessment of Anti-carcinogenic Effect of Pomegranate in Oral Squamous Cell Carcinoma (Pre-clinical Study)

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Abstract

Background and Objective: The survival portion of patients treated from oral cancer isn't in progress. This is why researchers are continuously looking for new anti-cancer drugs either natural product or natural product derivatives. Studies have shown that pomegranate and its constituents might have an anti-tumorigenic effect. So the aim of this study was to assess the anticarcinogenic roles of pomegranate on oral squamous cell carcinoma as an adjuvant of chemotherapy. **Materials and Methods:** The Hep-2 cells were propagated and maintained under basic culture media. Cells were grouped according to culture media and each group was tested for cell proliferation as well as VEGF expression and caspase-3 expressions using ELISA and RT-PCR, respectively. **Results:** Regarding cell proliferation and VEGF expression, a higher mean value was recorded in group 1 in comparison to group 3 with a significant difference ($p = 0.001$) and in group 2 in comparison to group 4, with a significant difference ($p = 0.049$). While concerning caspase-3 expression, a higher mean value was recorded in group 3 in comparison to group 1 with a significant difference ($p = 0.045$) and in group 4 in comparison to group 2, with a significant difference ($p = 0.00$). **Conclusion:** Pomegranate can be an adjuvant, natural product for oral cancer treatment in combination with 5-fluorouracil to reduce its dose and nullify its toxic side effects on normal body organs.

Key words: Oral squamous cell carcinoma, pomegranate, 5-fluorouracil, anti-proliferative, anti-angiogenic, apoptotic activity, chemoprevention

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oral cancer, is one of the most aggressive and invasive malignant tumors with a high metastatic potential. Squamous cell carcinoma (SCC) is an invasive epithelial tumor representing more than 90% of head and neck cancers[1]. It affects oral cavity, oropharynx, hypopharynx, larynx and nasopharynx with a propensity to early and extensive lymph node metastasis. It is considered as a major health problem that affects more than half a million people in the world every year[2].

Oral cancer treatment protocol involves 3 well known steps, surgical intervention, radiotherapy and chemotherapy[1]. Each step has its benefits as well as its drawbacks. Patients who are disallowed for surgery mainly because of advanced local tumor growth, distant metastasis or severe medical co-morbidities are most commonly advised chemotherapy as induction, adjuvant, neoadjuvant or palliative therapy[3].

The concept of chemoprevention was originally detected in the 1920s and was reintroduced with vast hope for cancer research by Sporn *et al.*[4]. Amongst a variety of chemotherapeutic drugs, cisplatin, carboplatin, 5-fluorouracil (5-FU), paclitaxel and docetaxel are most frequently used against OSCC[5]. Fluorouracil is also known as FU or 5FU and is one of the most commonly used drugs to treat cancer. Fluorouracil is part of a group of chemotherapy drugs known as anti metabolites. They discontinue the cells for creating and repairing DNA, so that they cannot cultivate and multiply[6]. Its common side effects are loss of appetite, tiredness, diarrhea, loss of fertility, mouth sores and ulcers, bleeding gums, nail problems, arrhythmia, tiredness and weakness. Fluorouracil can cause slow wound healing[7].

Researchers are continuously seeking new anti-cancer drugs either natural product or natural product derivatives. This is a trial to overcome deleterious effect of synthetic agents[8]. Pomegranate (*Punica granatum* L.), an ancient fruit, is acquired from a deciduous tree belonging to the family Lythraceae. Pomegranate as a fruit has been known for its beneficial effect on health. All parts of pomegranate have been used in medicine, the peel, the leaf, the root and the pulp[8]. This may be attributed to its antioxidant and antiatherosclerotic activities.

Pomegranates enclose a lot of polyphenolic compounds with elevated antioxidant and free-radical-scavenging action, such as flavonoids, condensed tannins and hydrolyzable tannins (ellagitannins [ETs] and gallotannins). The ETs are measured to be the most bioactive polyphenols of pomegranates. The most plentiful ET in pomegranates is punicalagin (PU).

Studies have exposed that pomegranate and its components can capably affect numerous signaling pathways concerned in inflammation, cellular transformation, hyperproliferation, initiation of tumorigenesis, angiogenesis and ultimately suppressing the final steps of tumorigenesis and metastasis[9]. The PU repressed the growth of human lung, breast, colon and cervical cancer cells *in vitro*. It decreased the frequency of chemically induced lung and mammary tumors, reduced the volume and multiplicity of estrogen-induced mammary tumors and produced apoptosis in cancer cells *in vitro*. These data established that pomegranate phytochemicals offers protection against diverse cancer-related processes[9]. But its anticarcinogenic effect on oral cancer hasn't been well demonstrated[5,6].

To date, there are few studies that have investigated the anticarcinogenic roles of pomegranate on oral squamous cell carcinoma. Thus this study will be conducted to focus on understanding the benefit of using pomegranate as an adjuvant in combination with 5-fluorouracil to reduce its dose and nullifies its toxic side effects on normal body organs in treating oral cancer.

MATERIALS AND METHODS

All the steps of the present study have been conducted at the national research centre (NRC) starting from January, 2018- January, 2019.

Punicalagin (Pomegranate polyphenol) and 5-fluorouracil were purchased from Sigma Aldrich (USA). Oral squamous cell carcinoma cell line was maintained *in vitro* under standard culture conditions. The cells were treated with different concentrations of 5-fluorouracil and Pomegranate was used in a concentration of (20 μg mL^{-1}) for 24 h.

Experimental groups: Group 1: 5-fluorouracil in low dose (2 mg mL^{-1}), Group 2: 5-fluorouracil in high dose (8 mg mL^{-1}), Group 3: 5-fluorouracil with low dose and Pomegranate (Punicalagin), Group 4: 5-fluorouracil with high dose and Pomegranate (Punicalagin).

Cytotoxicity assay: The viability of the cells was assessed by measuring the formation of a formazan from MTT spectrophotometrically (MTT cell proliferation kit Cayman Chemical Company®, USA) in all groups after 24 h of treatment. Briefly,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well (25 μL/well) and incubated for 2 h. The blue dye taken up by cells was dissolved in dimethyl sulfoxide (100 μL/well) and the absorbance was measured with a spectrophotometer (Bio-Rad Laboratories, Hercules, CA, USA) at 490 nm. All assays were run in triplicate[5].

VEGF assessment in different groups using ELISA kit: Vascular endothelial growth factor (VEGF) was assessed to evaluate the ability of cancer cells for angiogenesis (pg mL^{-1}). Human VEGF ELISA kit (WUHAN HUAMEI BIOTECH CO., LTD, Donghu, China) was used according to manual instructions. The supernatant of cultured cell was collected in each group to measure VEGF presence using ELISA reader[7].

RNA isolation from PE treated cells: Genomic RNA was isolated from squamous cell carcinoma cell line by standard treatment with SDS and EDTA in the presence of 200 µg mL^{-1} proteinase K (Sigma), followed by NaCl extraction and isopropanol precipitation.

Quantitative real-time PCR to measure quantity of caspase 3: Real-time PCR was assessed to estimate quantity of caspase 3 gene. We used a Step One (Applied Biosystems, Foster City, CA, USA) for quantitative real-time PCR. Primer specific for caspase 3 used for quantitation reactions is listed in Table 1. Exon 8 of caspase 3 gene was amplified separately by incubating on a Step One (Applied Biosystem) for 10 min at 94°C for initial denaturation followed by 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min. The final extension step was 72°C for 7 min. The standard reaction mixture (25 µL) contained 100 mg of genomic RNA, 0.25 µmol L^{-1} of the primer and SYBR green reagent supermix (Bio-Rad laboratories, Hercules, CA). All PCR reactions were performed in sets of four. The means of the specific gene RNA and GAPDH DNA copy numbers were calculated for each specimen separately and relative quantization ratios were generated[5,6].

Gene	Primers sequence from 5'-3'
Caspase 3	Forward: 5′-GCTATTGTGAGGCGGTTGT-3′
	Reverse: 5′-TGTTTCCCTGAGGTTTGC-3′
GAPDH	CCTCTACTGGCGCTGCCAAGGCT
	GTCCACCACTGACACGTTGG

Statistical analysis: Values were presented as mean and standard deviation (SD) values. Data were explored for normality using Kolmogorov-Smirnov test of normality. The results of Kolmogorov-Smirnov test indicated that most of data were normally distributed (parametric data), therefore, one way analysis of variance (ANOVA test) was used for intergroup comparison. This was followed by Tukey's *post hoc* test and independent t-test for pairwise group comparisons.

The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 19.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

RESULTS

Using low dose 5F: A higher mean value was recorded in group 1 in comparison to group 3 with a significant difference ($p = 0.001$). Using high dose 5F: A higher mean value was recorded in group 2 in comparison to group 4, with a significant difference ($p = 0.049$) (Table 1).

Using low dose 5F: A higher mean value was recorded in Group 3 in comparison to group 1 with a significant difference ($p = 0.045$). Using high dose 5F: A higher mean value was recorded in group 4 in comparison to group 2, with a significant difference ($p = 0.00$) (Table 2).

Using low dose 5F: A higher mean value was recorded in group 1 in comparison to group 3, with a significant difference ($p = 0.000$). Using high dose 5F: A higher mean value was recorded in group 2 comparison to group 4 with a significant difference ($p = 0.00$) (Table 3).

Table 1: Comparison of cell proliferation between group 1-4 (t-test)

	Low dose-5F		High dose-5F	
Parameters	Group 1	Group 3	Group 2	Group 4
Mean	0.970	0.303	0.685	0.203
Std deviation	0.308	0.139	0.205	0.083
Difference	0.66727*	0.48159*		
p-value	0.001*	0.049*		

Significance level $p<0.05$, *Significant

Table 2: Comparison of caspase-3 between group 1-4 (t-test)

	Low dose-5F		High dose-5F	
Parameters	Group 1	Group 3	Group 2	Group 4
Mean	1.647	2.411	2.014	3.943
Std deviation	0.682	0.697	0.611	0.775
Difference	0.764	1.929		
p-value	0.045*	0.00*		

Significance level $p<0.05$, *Significant

Table 3: Comparison of VEGF expression between group 1-4 (t-test)

	Low dose-5F		High dose-5F	
Parameters	Group 1	Group 3	Group 2	Group 4
Mean	30.600	20.200	26.319	7.676
Std deviation	3.017	3.223	5.165	1.750
Difference	10.40	18.643		
p-value	0.000*	0.000*		

Significance level $p<0.05$, *Significant

DISCUSSION

The OSCC is the most prevalent malignant neoplasm of the oral cavity representing almost 90% of all oral malignancies[1-3]. Regularly used treatment for highly developed head and neck cancer consisted of radical surgery with adjuvant radiotherapy or radiotherapy alone[4].

Although chemotherapy has enormous role in treating oral cancer, it is recognized that chemotherapeutic agents have quite a few undesirable consequences on various systems in the body. Chemotherapy may cause deleterious effects because it can equally damage healthy cells as well as malignant cancer cells. These side effects can happen at any time during treatment[10].

Amongst several categories of anticancer drugs, 5-fluorouracil (5-FU) has been broadly useful clinically for more than 50 years[10]. Resistance to 5-FU has developed and this represented a chief trouble for successful cancer treatment. Also 5-fluorouracil has many restrictions including its short biological half-life related to its quick metabolism, its incomplete and non-uniform absorption orally, cardiac toxicity, GIT disturbances, nausea, vomiting, hematologic toxicity, thrombophlebitis and skin rash[6,11].

Numerous natural compounds have been widely investigated to assess their chemopreventive potential. Pomegranate has proved a chemopreventive potential for breast cancer, prostate cancer and skin cancer[8]. This cancer-fighting capability of pomegranate lies in its abundance as antioxidants with an anti-inflammatory effect. This leads researchers to declare that pomegranate juice has a superior level of antioxidants than do green tea and red wine, which have also been investigated for their potential cancer prevention effects[12]. In this study, the team assessed the potential of punicalgan, as one of the components of pomegranate extract, to have a synergistic anticancer effect on Hep-2 cell line when used in conjunction with 5-FU. The hypothesis was replacing the use of a high dose of 5-FU with many side effects by a low dose of 5-FU supplemented with punicalgan, a naturally occurring agent.

There are no adequate studies testing pomegranate extract effect on oral cancer. So, this makes the presented research a valuable pioneer in the field of oral cancer treatment using natural products. The reached results are promising to continue further preclinical and *in vivo* studies. Adding punicalgan to either a high or low dose of 5FU showed a synergistic effect in lessening the viability of these malignant cells. Also, these combinations helped in increasing the expression of caspase-3, one of the apoptotic markers and decreasing the VEGF expression, so lowering the angiogenic capability of these cells.

It is well known that cancerous cells in a solid tumor such as OSCC proliferate beyond the apoptotic brakes of the normal tissues and persuade the formation of new blood vessels to help in providing nutrients. If the treatment succeeds in cutting this cycle, malignant cells will be not capable to continue cancer progression.

In accordance, Khan[13] studied the anticancer effect of pomegranate juice versus the use of punicalgan alone on colon cancer cell line. He showed that using the whole juice is more helpful in terms of diminishing proliferation and rising apoptosis of cancer cells. This may be due to synergistic property of various constituents found in the juice.

Kohno *et al.*[14] in a separate study included pomegranate seed oil in the diet and this patently reduced the incidence and multiplicity of colonic carcinoma (measured as no of tumors/rat) induced by azoxymethane . In accordance to the presented results, Larossa *et al.*[15], in a study, proved that Punicalagin induced apoptosis of colon cancer cells.

Toi *et al.*[16] confirmed for the first time an anti-angiogenic potential of pomegranate fractions in estrogen sensitive (MCF-7) or estrogen resistant (MDA-MB-231) human breast cancer cells or immortalized normal human breast epithelial cells (MCF-10A), grown in the attendance or lack of pomegranate seed oil (SESCO) or fermented juice polyphenols .

Albrecht *et al.*[17] showed that a range of concentrations of PE (pomegranate extract) (20-100 μg mL^{-1}) repressed proliferation, invasion of the cells all the way through Matrigel component and produced apoptosis of different cells as LNCaP, PC3 and DU145 cells[18-20]. These results propose an overall significant anti-proliferative and pro-apoptotic action of PE against human prostate cancer.

CONCLUSION

Pomegranate can be an adjuvant, natural product in oral cancer treatment through its anti-proliferative, anti-angiogenic as well as apoptotic activity.

SIGNIFICANCE STATEMENT

This study discovered that punicalgan, one of the components of pomegranate extract, have a synergistic anticancer effect on Hep-2 cell line when used in conjunction with either a high or low dose of 5-fluorouracil (5-FU) in decreasing the viability of these malignant cells, that can be beneficial for substitution of a high dose of 5-FU with many side effects by a low dose of 5-FU supplemented with punicalgan, a naturally occurring agent. This study will help the researcher to cover the area of oral cancer regarding

treatment with chemotherapeutic agents that many researchers were not able to explore. Thus, a new theory on this naturally occurring agent may be arrived at.

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