Anticancer effects of punicalagin and 5-fluorouracil on laryngeal squamous cell carcinoma: an in vitro study

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ABSTRACT

The purpose of this study was to assess the apoptotic effects of punicalagin alone and in combination with 5-fluorouracil (5-FU) on laryngeal squamous cell carcinoma (Hep-2) cell line. Hep-2 cells were cultured and divided into four groups: Group 1 received no therapy and served as control, Group 2 received 5-FU only, Group 3 received punicalagin only, and Group 4 received a combination of 5-FU and punicalagin. After 48 hours of incubation, cellular changes were examined under an inverted microscope. The methyl thiazolyl tetrazolium assay, caspase-3 gene level, and vascular endothelial growth factor (VEGF) level were assessed. The control group showed the highest mean value of cancer cell proliferation rate (1.595±0.58), followed by the punicalagin group (1.263±0.447), then the 5-FU group (0.827±0.256), while the combination group showed the lowest proliferation rate (0.253±0.111). The combination group showed the highest mean value of caspase-3 concentration (3.177±0.736), followed by the 5-FU group (1.830±0.646), and punicalagin group (0.741±0.302), while the control group showed the lowest mean value (0.359±0.117). Regarding VEGF levels, the control group had a statistically significant higher mean value, followed by the punicalagin and 5-FU groups, and finally, the combination group which showed the lowest value. Punicalagin exerts an anticancer effect through anti-proliferative action and induction of apoptosis on Hep-2 cell line. Combining punicalagin with 5-FU potentiates its anti-proliferative, apoptotic, and anti-angiogenic actions. It, further, helps in mitigating the putative side effects of 5-FU by reducing the dose required for its therapeutic effects.

Introduction

Head and neck cancers are a heterogeneous group of cancers. Most of them derive from the epithelium of the mucosal lining of the mouth, pharynx, and larynx.1 Ranking the sixth among the most frequently diagnosed carcinomas globally,2 a high annual incidence rate of 30% of head and neck squamous cell carcinoma (HNSCC) is reported, with approxi-
Materials and Methods

This study has received ethical approval from the Human Research Ethics Committee of Al Azhar University for Girls, Faculty of Dental Medicine, code # P-PD-23-07.
shaken on a shaker with foil covering them to completely dissolve the MTT formazan. Within an hour, the absorbance at optical density (OD) = 590 nm was measured. By dividing the total OD of the column wells by the number of wells, the mean OD of each column on the 96-well plate was determined. To calculate the percentage of viability and cytotoxicity for each concentration treatment, the mean OD of each column with a particular concentration treatment was divided by the mean of the untreated control cells.

Quantitative real-time polymerase chain reaction

For evaluating the caspase-3 gene and the amounts of nucleic acids (DNA, cDNA, and RNA) quantitative real-time polymerase chain reaction (qRT-PCR) was used. This is achieved by quantification and detection of fluorescence emitted from a reporter molecule in real-time. With every cycle of amplification, the PCR product accumulates and is detected. An applied biosystem with software version 3.1 (Step OneCFM, USA) was used for the qRT-PCR amplification and analysis. This assay was performed as explained by Overbergh et al. using the same reagents and kits.19

Enzyme-linked immunosorbent assay technique for assessment of vascular endothelial growth factor

The human VEGF ELISA kit was used following the manufacturer's instructions. The human VEGF level can be measured quantitatively using in vitro quantitative sandwich enzyme-linked immunosorbent assay. The protocol for the human VEGF ELISA kit was done in the following steps: i) cells were grown as mentioned in experimental groups for 48 hours; ii) supernatants were collected from each group and placed in 96 well plates for ELISA assays. Supernatants were added to appropriate wells; iii) after washing the plates, a prepared biotin antibody was added to every well; iv) after washing the plates, the ready streptavidin horseradish peroxidase enzyme (HRP) conjugate solution was added; v) each well received an addition of tetra-methyl benzidine one-step development solution; vi) each well received a dose of stop solution. Plates were immediately read at 450 nm.

Statistical analysis

The statistical package for social science V23.0 (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The ranges and mean ± standard deviation were calculated for the quantitative data. Frequencies and percentages were also used to represent qualitative variables. A one-way analysis of variance (ANOVA) and the Post Hoc test were used to compare group means. Multiple comparisons between various variables were conducted using Tukey’s test.

Results

Morphological assessment

The cells were examined under the inverted microscope phase to assess their viability and possible morphological changes that occurred after exposure to punicalagin and 5-FU in the four groups. Expected morphological changes during apoptotic cell death include nuclear shrinkage and fragmentation, chromatin condensation, and irregular nuclear outline. In group 1 (control group), Hep-2 cells showed a monolayer of densely packed cohesive slightly elongated fusiform and angular malignant cells. In group 2, there was a reduced number of viable malignant cells with obvious necrotic areas and a significant number of cells showing morphological changes and appearing rounded with decreased size (shrunken) which indicated apoptotic changes. No obvious changes were detected in group 3 when compared to the control group with small areas of necrosis and a slight increase in apoptotic cell numbers. The number of apoptotic cells increased in group 4 with large areas of necrosis (Figures 1-4).

Cell proliferation evaluation by methyl thiazolyl tetrazolium assay

After 48h of incubation, the ANOVA test revealed a statistically significant difference between groups (P<0.001) (Table 1). The control group showed the highest mean value of Hep-2 cell proliferation, followed by punicalagin, then 5-FU, with the least mean value recorded in punicalagin-5-FU combination.

Caspase-3 concentration

The concentration of caspase-3 was measured in different groups to assess the apoptotic effect. There was a highly statistically significant difference (P<0.001) between groups, with the combination group showing the highest mean value, followed by punicalagin alone, and the least mean value identified in the control group. The efficacy of the punicalagin-5-FU group was 173.6% more than 5-FU alone (Table 1).

Table 1. Values of cell viability, vascular endothelial growth factor and caspase-3, and statistical significance of difference among experimental groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell viability (μg/ml) Mean±SD</th>
<th>VEGF Mean±SD</th>
<th>Caspase-3 (μg/ml) Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>1.595±0.582 A</td>
<td>52.110±16.423 A</td>
<td>0.359±0.117 D</td>
</tr>
<tr>
<td>5-FU</td>
<td>0.827±0.256 C</td>
<td>28.459±4.091 C</td>
<td>1.830±0.646 B</td>
</tr>
<tr>
<td>Punicalagin</td>
<td>1.263±0.447 B</td>
<td>34.348±7.060 B</td>
<td>0.741±0.302 C</td>
</tr>
<tr>
<td>Punicalagin + 5-FU</td>
<td>0.253±0.111 D</td>
<td>13.940±2.486 D</td>
<td>3.177±0.736 A</td>
</tr>
<tr>
<td>ANOVA</td>
<td>F-test</td>
<td>P-value</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.851</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
</tbody>
</table>

SD, standard deviation; VEGF, vascular endothelial growth factor; 5-FU, 5-fluorouracil.
IV-vascular endothelial growth factor level

A highly statistically significant difference is noted between groups for the VEGF levels with the highest VEGF mean value in the control group, followed by punicalagin group, 5-FU group, then punicalagin+ 5-FU, which showed the lowest value (P<0.001) (Table 1).

Discussion

This study explored the potential antitumor efficacy of punicalagin and 5-FU when used on the Hep-2 cell line. Previous research highlighted the antitumor roles of punicalagin in a few types of cancer. Within the context of laryngeal cancer, one study concluded that cytotoxicity of punicalagin is only achieved at higher concentrations; however, there were no conclusive results. The laryngeal squamous cell carcinoma (Hep-2) cell line was used as a model similar to other studies because it is widely used to represent head and neck malignancies as squamous cell carcinoma of the larynx that arises from the intra-oral site and has similar genetic phenotypes.

This study explored the antitumor effects by morphological assessment, and evaluating caspase 3 and VEGF levels. We used punicalagin with constant concentration (20 μg/ml ≈ 18.5 μM). Tang et al. used punicalagin with increasing concentrations of 0, 12.5, 25, 50, 100, and 200 μM, for 24, 36, and 48h in the treatment of HeLa cells to evaluate cell viability. They found that after 48h inhibition of the viability of HeLa cells in vitro became statistically significant at 12.5 μM. Similarly, Seeram et al. found that punicalagin, ellagic acid, a total pomegranate tannin extracts reduced cell proliferation in human oral, prostate, and colon cell lines in a dose-dependent manner ranging from 12.5 to 100 g/ml.

In order to clarify the punicalagin anti-carcinogenic effect in comparison to 5-FU, morphological changes of Hep-2 cells were assessed under the inverted phase microscope. According to Sangour et al., cell viability was assessed at 48 hours to enable adequate time for the substance to inhibit or kill the cells.

Regarding morphological changes of cancerous cells, signs of apoptosis in treated groups were identified such as cell body shrinkage, reduction in cell volume, and the detection of a circular shape, as well as nuclear shrinkage, frag-
mented irregular outline, and chromatin condensation and margination. These signs were markedly noticed in the punicalagin+5-FU treated group followed by 5-FU and punicalagin monotherapy groups, respectively. These changes were not detected in the control group. Similarly, Li et al. found the same morphological changes after treating thyroid cancer cell lines with pomegranate (punica granatum) extract.22 In the current study, the highest mean value of cell proliferation assay was identified in the control group, followed by punicalagin, and finally, 5-FU while the least mean value was recorded in the combination group. This indicates that punicalagin amplifies the anti-proliferative effect of 5-FU. Punicalagin with a high 5-FU concentration induced the highest cytotoxic effect with the lowest percentage detected for viable and proliferating cells. This could be attributed to the antioxidant effect of punicalagin, which eventually undergoes reactive oxygen species modulation in cancer cells.23 Furthermore, Mirzaei et al. reported that Punicalagin was capable of reducing oxidative phosphorylation and glycolysis leading to autophagy of cancer cells.24 5-FU is a widely used chemotherapeutic medication that has remained an important treatment for a variety of solid tumors, including LSCC. However, the systemic use of 5-FU as chemotherapy is associated with considerable toxic side effects and the development of drug resistance. The main mechanisms of action of 5-FU include the inhibition of thymidylate synthase activity, DNA synthesis, and DNA repair via the incorporation of its metabolites into the DNA/RNA of cancer cells.25 All of these activities could ultimately lead to apoptosis. Punicalagin was previously used as an adjuvant chemo-preventive agent in combination with 5-FU, to reduce the 5-FU dose, thereby, decreasing its toxic side effects and improving treatment outcomes.26

In the current investigation, the antiangiogenic efficacy was assessed by evaluating VEGF levels which is used to evaluate the angiogenic potential of cancer cells. The greatest mean value for VEGF was recorded in the control group, followed by punicalagin, then 5-FU, with the lowest mean value recorded in the Punicalagin+5-FU group, indicating that punicalagin enhances the anti-angiogenic activity of 5-FU. Punicalagin has also shown promise as an adjuvant treatment with 5-fluorouracil (5-FU) to decrease its negative effects. In rats and human beings, Souchon et al. (1975) observed that intravenous hyperalimentation, a method of delivering nutrients straight into the bloodstream, reduced the gastrointestinal toxicity of 5-FU.27 This suggested that punicalagin, which protects against diabetes and brain impairment could also help to minimize the adverse effects of 5-FU.28 Several investigations have demonstrated that the combination of punicalagin with 5-fluorouracil has promising antitumor effects. Anitha concluded that 5-FU and curcumin, a chemical related to punicalagin, have improved anticancer effects in colon cancer.29 Ghiringhelli also emphasized 5-FU’s ability to improve immune responses and remove immunosuppressive cells, indicating a possible synergy with punicalagin.30 Both Huang et al. and Suruli et al. showed that punicalagin can decrease cancer cell growth and invasion, in addition to lowering inflammatory cytokines which support its potential as a supplemental treatment to 5-FU.31,32 In both Liu et al. and Tang et al. studies, punicalagin caused apoptosis and limited proliferation in human cancer cells, including HepG2 hepatoma cells and HeLa cervical cancer cells.33,34 These effects were linked to mitochondrial targeting and suppression of the catenin pathway, respectively.15 Yan et al. and Zhang et al. demonstrated punicalagin’s ability to protect against lipotoxicity, a condition linked with nonalcoholic fatty liver disease, by activating the Keap1-Nrf2 antioxidant defense system and the SIRT1/autophagy pathway.28,33

**Conclusions**

These findings emphasize that punicalagin has a broad therapeutic potential in cancer management. Punicalagin exerts an anticancer effect through anti-proliferative action and induction of apoptosis on the Hep-2 cell line. The addition of punicalagin to 5-FU potentiates its anti-proliferative, apoptotic, and anti-angiogenic actions. Punicalagin could be used as adjunctive treatment with 5-FU to diminish its side effects.

**References**


