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Evaluation of the Apoptotic Effect of Punicalagin on Tongue Carcinoma Cell (In-vitro study)

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Background: Squamous cell carcinoma most frequently develops in the tongue in the oral cavity (SCC), (SCC). Due to increased local invasion, a high likelihood of recurrence, and metastasis to the cervical lymph node, tongue cancer has a poor prognosis. It's been demonstrated that the pomegranate compound punicalagin (PUN) causes amazing biological reactions.
Aim: The aim of this study was to assess the cytotoxic effect of punicalagin.
Materials and Methods: This was in-vitro study that was done on Human tongue squamous cell carcinoma cell line which supplied from the Cell Culture Department – Nawah Scientific Research Center, Cairo, Egypt at Central Lab of Stem Cells, and Biomaterial Applied Research (CLSBAR) in Faculty of Dentistry, Ain Shams University. we assess cytotoxicity of punicalagin supplied from "Sigma Aldrich, Germany through MMT assay, evaluation of apoptosis by annexin-v/Propidium iodide using flowcytometry method and test the underlying apoptotic effect of punicalagin on tongue cancer cells as one of the major anti-tumor effects.
Results: The half maximal cytotoxic outcome of Punicalagin was (185 µmol) concentration. In test group treated with (185µmol/ml) of punicalagin the percentage of apoptotic cell, necrotic cells and live cells was (51.3% ,2.3%, 46.7%) respectively.
Conclusion: Punicalagin has a cytotoxic effect on Human tongue squamous cell carcinoma cell line and considered as valued anticancer agent.

Keywords: apoptosis, punicalagin,Tongue cancer

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Introduction

The tongue is the place where squamous cell carcinoma (SCC) occurs most frequently, followed by the floor of the mouth, the retromolar region, the alveolar ridge, the hard palate, and the buccal mucosa. It continues to spread more widely. This shows that other unknown etiological elements, such as the molecular biology component, are crucial in its development in addition to the most frequent risk factors (1).

Due to increased local invasion, a high probability of recurrence, and metastasis to the cervical lymph node, oral cancer patients have a poor prognosis. Due to the tongue's distinctive histological characteristics, such as its high lymphatic supply and extremely rich muscle construction, which make it difficult for it to easily defend itself against invasion and metastasis, tongue cancer is regarded as a significant community healthcare problem with one of the worst prognoses (2).

Cell apoptosis is programmed cell death that occur under physiological and pathological conditions. The eradication of malignant cells was all linked to apoptosis. it assists in thoughtful the etiology of diseases generated by interrupted apoptosis (3).

The most important polyphenol found in the pomegranate (Punica granatum) husk is punicalagin (PUN). PUN, a bioactive component, accounts for more than half of the pomegranate juice's overall antioxidant capacity (4). It has been demonstrated that it has a variety of beneficial health effects, including neuroprotective cardiovascular protecting, anticancer anti, atherosclerotic and anti-obesity qualities. Punicalagin has been shown to have a wide range of exceptional biological effects, including genotoxic, anti-inflammatory, and hepatoprotective actionsn (5). Punicalagin is thought to be converted by the gut microbiota into several urolithins, each of which has distinct anti-cancer properties. Punicalagin's bioavailability and absorption profile are unknown, which is one of the difficulties in taking Punicalagin orally (6). Punicalagin has been discovered to be toxic to cancer cells but not to healthy cells, leading to the discovery that it can trigger apoptosis in several cancer cell lines (7).

Material and methods

This in vitro study was based on services in Central Lab of Stem Cells and Biomaterial Applied Research (CLSBAR) in Faculty of Dentistry, Ain Shams University.

The cell line used in this study Human tongue squamous cell carcinoma cell line (HNO-97), was supplied from the Cell Culture Department – Nawah Scientific Research Center, Cairo, Egypt. Punicalagin was provided from Sigma Aldrich, Germany. 18 samples were divided into Group 1 negative control group incubated for 24 hours. And Group 2 test group treated with IC-50 punicalagin incubated for 24 hours. A stock solution of punicalagin 500 mM was set in molecular grade Methanol and aliquoted and stored at −20°C until use. For all experiments, many concentrations (250µmol, 200 µmol, 150 µmol, 100 µmol, 50 µmol) of punicalagin were set by diluting the stock with medium. The carrier solvent (Methanol) was additional to the control cells. Assessment of cell viability by cell proliferation assay (MTT) then Calculation of half maximal inhibitory concentration (IC50) using the Graph pad prism software 9. The tongue cancer cells were treated with dose of IC50(185 µmol/mL) concentration of Punicalagin, and cells were collected at 48 hours or Assessment of Apoptosis using Annexin-V (Ann-v) /Propidium iodide (PI) staining by using Flowcytometry.

Results

1-Determination of the half maximal cytotoxic effect (IC50) of Pomegranate (punicalagin) on HNO97 Tongue carcinoma cells.

The half maximal toxic result of Punicalagin was found at concentration (185 µmol), with 95

% confidence intervals ranging from 165.4 to 216.5 µmol (figure 1).

2-Assessment of cytotoxic effect in cultured cells:

A) Optical density of MTT assay at 570nm

ANOVA test discovered that the difference between dissimilar concentrations of Punicalagin was statically significant (p=0.00). Post hoc test revealed a significant difference between each 2 concentrations, as well as between each concentration and negative control (p=0.000). The percentage of cell viability was 99.64% in negative control (Dulbecco's Modified Eagle Medium). Cell viability diminished to 91.28% using 50 µmol/mL Punicalagin. Cell viability gradually reduced with increasing Punicalagin to reach (38.74%) using 250 µmol/mL Punicalagin, denoting an inverse correlation between the punicalagin concentration and cell viability

b) Detection of apoptosis using Annexin/propidium iodide staining. (Figure 2)

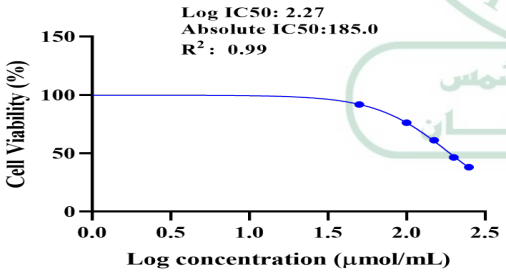

Figure (1) Linear regression curve illustrating the log dose of Punicalagin versus the normalized response in HNO97 cells. IC50: half maximum cytotoxic effect, CI: confidence interval.

Discussion

One of the most common malignant tumors, tongue squamous cell carcinoma (TSCC), carries a high risk of metastasis and recurrence. Despite significant recent breakthroughs in clinical diagnostic and treatment techniques, the aggressive invasion and metastasis of TSCC remain difficult to reverse and the overall 5-year survival rate is still low. (8) Therefore, it is imperative that we look for new therapeutic modalities.

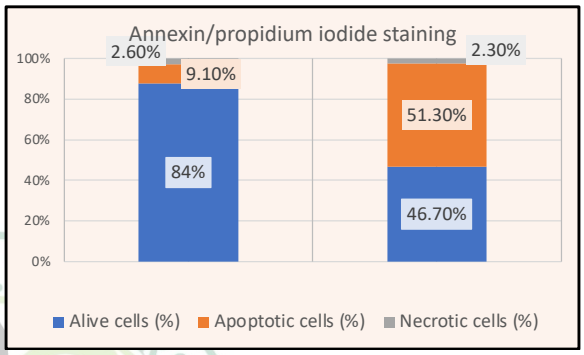

Figure (2): Bar chart illustrating percentage of alive, necrotic and apoptotic cells in Untreated HNO-97 cells and HNO-97 cells treated with 185 µmol/mL Punicalagin

Many cancer cells evade the usual apoptotic mechanisms, which leads to uncontrolled proliferation. Furthermore, the absence of apoptotic regulation allows cancer cells to multiply and accumulate several mutations, increasing their invasiveness during the tumor's development. As a result, apoptosis has received more attention in the study of the life sciences, especially in the field of tumor research (9).

The components of pomegranates are currently the subject of interesting research into good nutrition and medicine. The credit for these advantages is given to polyphenols, which mostly consist of hydrolyzed tannins (10). More than 50% of the antioxidant properties of pomegranate juice are attributed to punicalagin (PUN), a bioactive substance. Numerous in vitro and in vivo studies have confirmed the anti-cancer properties of punicalagin. Punicalagin (PUN) is thought to be the bioactive component responsible for more than fifty percent of the antioxidant benefits of pomegranate juice. Punicalagin anti-cancer properties have been supported by several in vitro and in vivo investigations (11).

The present study the cytotoxic effect of punicalagin on Human Tongue Squamous cell carcinoma cell line (HNO-97) through the MTT

assay assessment the apoptotic effect of punicalagin by detection of apoptosis that might occur in test group with IC-50 (185µmol/ml) in comparison to control group through Ann-v /PI staining using flowcytometry technique.

In test group we use a serial increased gradually concentrations (50 ,100 ,150 ,200 ,250 µmol/ml) which revealed that Punicalagin had a clear cytotoxic effect on test group when compared to negative control group in dose dependent manner, the viability percentage means of test group decreased (91.28 %, 76.03%, 60.44%, 45.95 %38) respectively after 48 hours post treatment. These findings indicated that increasing the concentration of punicalagin is highly toxic to the viable cancer cells.

This observation was in accordance with many studies on different cancer cell lines as on leukemic (NB4 and MOLT-4) and colorectal (HCT 116) carcinoma cell line cells using various Punicalagin concentration show that decrease cell viability percentage in cells dose dependent manner (12 -13)

On other hand several studies done on different cell lines although using lower PUN concentrations , show higher percentage of decrease viability of cancer cells as lung cancer cell line(A549) and hepatoma (HepG2) cell line from (11,14) IN present study we use different higher concentrations of PUN because previous studies on treatment of several types of cancer has proved that OSCC respond poorly to chemotherapy treatment (15.16) , tongue cancer especially was resistance to chemotherapies (17) ,and PUN is believed to be natural chemotherapeutic drug.

Since the MTT assay demonstrated only whether the cells were viable or not, the need for other assay to clarify whether the recorded loss of viability was resulting from which type of death (apoptosis or necrosis) become critical So An assessment of occurrence of apoptosis and / or necrosis using annexin-v/ PI staining by flowcytometry technique used to detect of percentage of apoptotic cells, dead necrotic cell, and live cells.

In the present study the test group treated with (185µmol/ml) of punicalagin stained with Ann-V and PI after 48 hours post treatment revealed that there is a statistically significance difference between test and control and the percentage of apoptotic cells was elevated from control to test group this suggest that Punicalagin induced cell apoptosis in TSCC and explained by Reactive oxygen species(ROS) generation by Punicalagin that prevent the proliferation of cancer cells and encourage cell apoptosis(11)

This result was also in accordance with a study had proved a significant difference between Human hepatoma cells (HepG2) treated with PUN and normal Human hepatic(L-02) cells as control group using the same technique. this study revealed that. early apoptosis shows slight changes, and the percentage of late apoptosis were elevated in contrast to control group suggested that PUN induced cell apoptosis in a dose-dependent manner (11). But our result was proved that increase percentage of apoptotic cells due to using a higher concentration of PUN.

On the other hand an additional study on HCT 116 (colorectal carcinoma) in contrast to normal colon epithelium (CCD 841) cells by using the same technique showing There was no significant differences between treated with IC50 (87 ± 3.825 µg/mL) of PUN and untreated group as it utilize a low PUN concentration the result of percentage of apoptotic cells is low (13).

Conclusion

Punicalagin has a cytotoxic effect on the human tongue squamous cell carcinoma cell line, according to the findings of the current study, and it can be regarded as a potent anticancer drug that induces apoptosis.

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