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Anticancer effect of flaxseed and Cisplatin in oral squamous cell carcinoma: An in-vitro study

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ABSTRACT

Aims: oral squamous cell carcinoma (OSCC) is the most common form of oral cancer and is currently largely treated by surgery and/or radiation. Significant complications can occur because of these treatments. Adjunct therapies that are safer and less toxic are being explored. This study aims to investigate the potential anti-cancerous effect of the flaxseed on oral squamous carcinoma cells in vitro and determine the possible modulation of the activity of cisplatin by flaxseed using different durations of applications. Material and Methods: OSCC cell line cells were cultured and the MTT assay was used to assess cell metabolic activity. Results: The antiproliferative effect of flaxseed oil at 24 and 48 hours as measured by the IC50 was 71.48±4.26 ug/ml and 18.11±1.36 ug/ml respectively. The mean percentage of viable cells at 24 and 48 hours when treated with flaxseed oil was 66.2+/-14.6 and 56.3+/-12.7, p-value <0.001. At 24 hours, the antiproliferative effect of cisplatin alone vs. when combined with flaxseed oil as measured by the IC50 was 9.59 +/- 0.32 ug/ml vs. 12.91+/- 0.94 ug/ml. The mean percentage of viable cells when treated with cisplatin alone vs. flaxseed oil combined with Cisplatin was 52.8+/- 14.4% vs. 55.1+/-15.5%, p-value=0.02. At 48 hours, the antiproliferative effect of cisplatin alone vs. when combined with flaxseed oil as measured by the IC50 was 2.70±0.11 ug/ml vs. 1.55±0.04 ug/ml. The mean percentage of viable cells when treated with cisplatin alone vs. flaxseed oil combined with Cisplatin was 45.4+/- 12.1 vs. 42.1+/-12.5, p-value=0.004. Conclusion: Further studies to explore potential mechanisms of the anticancer effect of flaxseed oil are needed.

التأثير المضاد للسرطان لبذور الكتان و السيسبلاتين علي مرض سرطان الخلايا الحرشفية الفموية: دراسة معملية

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الكلمات المفتاحية:
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الملخص

الأهداف: يعتبر سرطان الخلايا الحرشفية الفموية اكثر سرطانات الفم شيوعا و حاليا يعالج بنسبة كبيره عن طريق الجراحة و/ او العلاج الاشعاعي. قد تسبب هذه العلاجات بآثار جانبية هامة. يتم حاليا اكتشاف علاجات بديلة آمنة و اقل سمية. هذه الدراسة تهدف الي استكشاف التأثير المضاد للسرطان المحتمل لبذور الكتان علي الخلايا الحرشفية الفموية داخل المعمل و تحديد إمكانية تحوير نشاط السيسلابين بواسطة بذور الكتان باستخدام فترات زمنية مختلفة. المواد و الطرق: تم عمل مزرعة للخلايا الحرشفية الفموية و اجري اختبار الام تي تي لتحديد مستوي النشاط الايضي للخلايا. النتائج: تم قياس تأثير المضاد للسرطان لبذور الكتان عند 24 ساعة و عند 48 ساعة و كان المعدل 71.48±4.26 و 18.11±1.36 علي التوالي. متوسط نسبة الخلايا الحية المعالجة بزيت بذور الكتان عند 24 ساعة و عند 48 ساعة كانت 66.2+/-14.6 و 56.3+/-12.7 p-value <0.001. عند 24 ساعة، التأثير المضاد للسرطان للسيسبلاتين بمفرده و عند اتحاده مع زيت بذر الكتان كان 9.59 +/- 0.32 و 12.91+/- 0.94. متوسط نسبة الخلايا الحية المعالجة بالسيسبلاتين بمفرده مقارنة

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باتحاده مع زيت بذر الكتان كان 52.8+/- 14.4% و 55.1+/-15.5 p-value=0.02. % عند 48ساعة، التأثير المضاد للسرطان للسيسبلاتين بمفرده مقارنة باتحاده مع زيت بذر الكتان كان 2.70±0.11 و 1.55±0.04. متوسط نسبة الخلايا الحية المعالجة بالسيسبلاتين بمفرده مقارنة باتحاده مع زيت بذر الكتان كان 45.4+/- 12.1 و 42.1+/-12.5، p-value=0.004.

Introduction

Oral cancer is one of the most common cancers worldwide, the vast majority are oral squamous cell carcinomas (OSCC)[1] . The five-year survival rate for OSCC is below 55%, this is mainly due to the delayed diagnosis and most patients diagnosed at advanced cancer stages [2, 3]. Radiation therapy and surgery are the treatment of choice for early and advanced OSCC respectively. A combination therapy of radiation therapy and surgery and chemotherapy is the treatment used for recurrent OSCC cases and those with metastasis. Despite improving the survival of OSCC patients, combination therapy has a negative impact on the quality of life of these patients because of disfigurement, and disfunction due to surgery, as well as complications and toxicity of radiation and chemotherapy [4]. Therefore, it is important to develop a safer natural therapeutic and preventive alternative with less toxicity. Cisplatin is considered one of the most common and effective chemotherapy drugs used for treating oral cancer. The anticancer effect of Cisplatin is via different mechanisms, such as generating DNA lesions and cell apoptosis [5, 6]. Despite the effectiveness in treatment of oral cancer, this drug has several adverse effects. Some are late effects which occur months to years post-chemotherapy because of the damage to healthy cells while killing cancer cells. Common side effects of cancer therapy include reduction in blood immune cells, diarrhoea, fatigue, pain, hair loss, and psychological distress. These side effects interfere significantly with the efficacy of cancer treatment [4]. To overcome this problem, it is important to develop new therapeutic strategies such as combinatorial chemotherapeutic regimens that can be used as alternative or adjunct drugs, which would allow for using lower doses of cisplatin, reducing adverse effects and providing better treatment outcomes

To overcome the above-mentioned issues with conventional chemotherapy, plant compounds and their analogues are being studied. It has been found that natural products derived from a variety of sources can stimulate and inhibit numerous physiological pathways, which can be helpful in altering the course of many diseases, such as cancer. Therefore, more than half of medications have been developed from natural compounds, including most anticancer drugs[7]. Promising results have been shown for the use natural products for cisplatin-induced toxicity. There is evidence for health benefits of plant polyphenols, specifically the flaxseed polyphenols have gained increasing attention. These compounds were found to help with the chemo-sensitization of cisplatin-resistant cells by either up or downregulating specific signalling pathways that trigger apoptosis [8] . Therefore, our objective was to study the effects of flaxseed in combination with cisplatin on the proliferation, apoptosis, autophagy, oxidative stress, and DNA damage of oral cancer cells.

Flaxseed, also known as linseed, is derived from the flax plant, a part of the Linaceae family [9]. flax belongs to the species Linum *usitatissumum* L.. It contains numerous nutrient and non-nutrient chemical constituents, like α-linolenic acid, fiber, and lignans, which can provide health benefits [10, 11]. More recently, polyphenols of flaxseed—the lignans—have shown to have antioxidative, anti-inflammatory, anti-atherosclerogenic, and antiestrogenic potential, thus potentially reducing the risk and protect against cancer [12-14].

During intake of flax, the most abundant compound, SDG, is ingested, then metabolized and converted into two mammalian lignans, Enterodiol (END) and Enterolactone (ENL) by the gut microbiota [15]. In vivo, the lignans are acted upon in the upper part of the bowel, by the gastrointestinal microflora . Further hydroxylation and demethylation by the microflora lead to the production of the mammalian lignan END, which is then oxidized to produce ENL [9]. Once produced, END and ENL can either be efficiently absorbed into the bloodstream or conjugated by enterocytes into inactive compounds excreted in the gut [16].

This study aims to investigate the potential anti-cancerous effect of the flaxseed on oral squamous carcinoma cells in vitro and determine the possible modulation of the activity of cisplatin by flaxseed using different durations of applications.

Material and methods:

In this in vitro study, we used cell line cells obtained from American Type Culture Collection, cells were cultured using DMEM (Invitrogen/Life Technologies) supplemented with 10% FBS (Hyclone,), 10 ug/ml of insulin (Sigma), and 1% penicillin-streptomycin. All of the remaining chemicals and reagents were from Sigma, or Invitrogen.

Plate cells (cells density 1.2 – 1.8 × 10,000 cells/well) in a volume of 100µl complete growth medium + 100 ul of the tested compound per well in a 96-well plate for 24 hours before the MTT assay .

Cell culture protocol

1. Remove culture medium to a centrifuge tube.
2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin 0.53 mM EDTA solution to remove all traces of serum which contains Trypsin inhibitor.
3. Add 2.0 to 3.0 ml of Trypsin EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes).

Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal.

4. Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting.
5. Transfer the cell suspension to the centrifuge tube with the medium and cells from step 1, and centrifuge at approximately 125 xg for 5 to 10 minutes. Discard the supernatant.
6. Resuspend the cell pellet in fresh growth medium. Add appropriate aliquots of the cell suspension to new culture vessels.
7. Incubate cultures at 37°C for 24 hrs.

8-After treatment of cells with the serial concentrations of the compound to be tested incubation is carried out for 48 h at 37ºC ,then the plates are to be examined under the inverted microscope and proceed for the MTT assay

MTT – Cytotoxicity assay protocol

The MTT method of monitoring in vitro cytotoxicity is well suited for use with multi well plates. For best results, cells in the log phase of growth should be employed and final cell number should not exceed 106 cells/cm2. Each test should include a blank containing complete medium without cells.

1. Remove cultures from incubator into laminar flow hood or other sterile work area.
2. Reconstitute each vial of MTT [M-5655] to be used with 3 ml of medium or balanced salt solution without phenol red and serum. Add reconstituted MTT in an amount equal to 10% of the culture medium volume.
3. Return cultures to incubator for 2-4 hours depending on cell type and maximum cell density. (An incubation period of 2 hours is generally adequate but may be lengthened for low cell densities or cells with lower metabolic activity.) Incubation times should be consistent when making comparisons.
4. After the incubation period, remove cultures from incubator and dissolve the resulting formazan crystals by adding an amount of MTT Solubilization Solution [M-8910] equal to the original culture medium volume.
5. Gentle mixing in a gyratory shaker will enhance dissolution.

Occasionally, especially in dense cultures, pipetting up and down [trituration] may be required to completely dissolve the MTT formazan crystals.

6. Spectrophotometrically measure absorbance

at a wavelength of 570 nm. Measure the background absorbance of multiwell plates at 690 nm and subtract from the 450 nm measurement. Tests performed in multi well plates can be read using the appropriate type of plate reader or measured using for spectrophotometry.

Statistical Analyses:

The statistical analysis was performed using Jamovi software version 2.3.28, paired t-test was used, and p-value of <0.5 was considered significant between the IC50 values when using flaxseed, cisplatin, and a mixture of both flaxseed and cisplatin.

Results:

i. The potential anticancer effect of flaxseed oil at 24 and 48 hours

The anti-proliferative effect of flaxseed oil at 24 and 48 hours as measured by the IC50 was 71.48±4.26 ug/ml and 18.11±1.36 ug/ml respectively. The mean percentage of viable cells at 24 and 48 hours when treated with flaxseed oil was 66.2+/-14.6 and 56.3+/-12.7, p-value <0.001.

ii. The anticancer effect of Cisplatin alone vs. when combined with Flaxseed oil after 24 hours.

At 24 hours, the antiproliferative effect of cisplatin alone vs. when combined with flaxseed oil as measured by the IC50 was 9.59 +/- 0.32 ug/ml vs. 12.91+/- 0.94 ug/ml. The mean percentage of viable cells when treated with cisplatin alone vs. flaxseed oil combined with Cisplatin was 52.8+/- 14.4% vs. 55.1+/-15.5%, p-value=0.02.

iii. The anticancer effect of Cisplatin alone vs. when combined with Flaxseed oil after 48 hours.

At 48 hours, the antiproliferative effect of cisplatin alone vs. when combined with flaxseed oil as measured by the IC50 was 2.70±0.11 ug/ml vs. 1.55±0.04 ug/ml. The mean percentage of viable cells when treated with cisplatin alone vs. flaxseed oil combined with Cisplatin was 45.4+/-12.1 vs. 42.1+/-12.5, p-value=0.004. Table 1, and figure 1. Show the different IC50 values of our results.

Table 1. shows the IC50 values when using flaxseed and cisplatin separately, and when used mix together at 24 and 48 hours.

Sample	Cytotoxicity IC50 ug/ml SCC25	
	24h	48h
Flax Oil/SCC25	71.48±4.26	18.11±1.36
Cisplatin/SCC25	9.59±0.32	2.70±0.11
Mix/SCC25	12.91±0.94	1.55±0.04

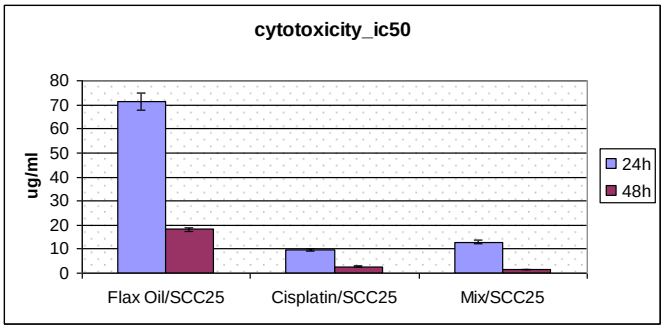

Figure 1. A bar chart shows the IC50 values when using flaxseed and cisplatin separately, and when used mix together at 24 and 48 hours.

Discussion:

Cisplatin is considered the oldest and most used chemotherapeutic agent 17. The anticancer effect of Cisplatin is through different mechanisms, such as generating DNA lesions and cell apoptosis [5]. Due to cisplatin's major side effects, it is important to develop new therapeutic strategies such as combinatorial chemotherapeutic regimens that can be used as alternative or adjunct drugs, which would allow for using lower doses of cisplatin, reducing adverse effects and providing better treatment outcomes.

Promising results have been shown for the use natural products for cisplatin-induced toxicity. Flaxseed has shown promising therapeutic anti-cancerous effect 18. These compounds were found to help with help with the chemo-sensitization of cisplatin-resistant cells by either up or downregulating specific signalling pathways that trigger apoptosis [11,12]. Therefore, our objective was to study the effects of flaxseed in combination with cisplatin on the proliferation of oral cancer cells.

While several studies have explored the anticancer effect of flaxseed on different cancer cells [8], up to our knowledge, this is the first study to assess the effect of flaxseed oil on oral cancer cells in vitro. In our study showed an increase in the antiproliferative effect of cisplatin when used in combination with flaxseed oil for 48 hours. However, interestingly, there was a transient reduction in the antiproliferative effect of cisplatin when used in combination with flaxseed for 24 hours. It is unclear why the addition of flaxseed for 24 hours reduced the anticancer effect of cisplatin.

Conclusion: our study showed that flaxseed oil potentially modulates the effect the cisplatin on oral squamous cell carcinoma cells in vitro. Further studies are needed to understand the underlying mechanisms of the anticancer effect of flaxseed oil. Furthermore, more reliable data via in vivo studies is needed.

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