Assessment of Anti-cancerous Effect of Green, Roasted and Decaffeinated Coffee on Oral Squamous Cell Carcinoma Cell Line

(In Vitro Study)

Presented by

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Evidence based committee (Reviewers)

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Research plan committee

Name	Signature	Date
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I. Administrative information:

1. Title:

Assessment of Anti-cancerous Effect of Green, Roasted and Decaffeinated Coffee on Oral Squamous Cell Carcinoma Cell Line (In Vitro Study)

2. Protocol registration

Cairo University, June 2018

3. Protocol version:

July 2018, Version (1)

4. Funding:

Self-funding.

5. Roles and responsibilities:

1- The Principle Supervisor:

- **Professor Dr. Mohsen Kazem**, Professor of Oral and Maxillofacial pathology, Faculty of Dentistry Cairo University.

2-The Assistant Supervisor :

- **Dr. Manar Abdulwaniss Mohammed**, Lecturer of Oral and Maxillofacial Pathology, Faculty of Dentistry Cairo University.

3-The Investigators:

- **Dr.Esam Rashwan** , Assistant Professor and Head of Confirmatory Diagnostic unit VACSERA – Egypt
- **Asmaa Emad El-Din Mohammed**(The Principle Investigator)

II. Introduction:

6.a.Scientific background

Oral cancer is the sixth most common cancer worldwide (Barrios et al, 2015). Over 90% of all identified oral cavity cancers are invasive oral squamous cell carcinomas (OSCCs) (Johnson et al, 2011). Primary treatments of OSCC are surgery, radiation therapy, and chemotherapy (da Silva et al, 2012). However, anticancer therapies (drugs, irradiation) have undesirable side effects as they may induce mutations or irreversible DNA damage killing healthy cells (Ochwang'I et al, 2014).

One of the most frequently used alternative therapies is herbal medicine that act as anti-ROS agents preventing DNA damage has been used alongside conventional treatment regimens (Li et al, 2016). One of the agents that receives particular strong interest is coffee. Coffee is considered as a major source of dietary antioxidants; some are present in the green bean, whereas others are generated during roasting. Coffee roasting, the process of the heating of green coffee beans transforming them into black coffee beans, transforms the chemical and biological properties of coffee beans (Wei et al. 2012 and Jeong et al, 2013).

Regarding oral cancer, some studies reported an association of high coffee consumption to an augmented risk of oral cancer (Bundgaard et al, 1995 and Franco et al, 1989) while others showed a clear inverse association with the risk of oral cancer (Naganuma et al, 2008 and Hildebrand et al, 2013). Recently, there have been reports of a protective effect of coffee consumption on oral cancer from two recent meta-analysis (Zhang et al, 2015 and Li et al, 2016). To our knowledge, only two studies were done to assess the effect of coffee ingredients (cafestol and kahweol) on oral squamous cell carcinoma cell lines (Chae et al, 2014 and Kotowski et al, 2015).

Due to these controversial findings concerning the effect of roasted coffee and absence of data on unprocessed (green coffee), our study aims to investigate the effect of different coffee beverage as regard apoptosis and proliferation carried out in OSCC cell lines.

Rationale for carrying out this study:

The main objective of our study was to evaluate anti proliferative and apoptotic activity of green coffee, in comparison to roasted coffee and decaffeinated coffee on cultured OSCC cell line.

PICO

Patient / Population: Human Oral squamous cell carcinoma cell line

Intervention1: Green coffee

Intervention2: Roasted coffee

Intervention3: Decaffeinated coffee

Control: no application

Outcome:

Primary outcome 1: assessment of proliferative activity OSCC cell line treated with coffee using **ELISA.**

Primary outcome 2: Assessment of apoptotic cell death of OSCC cell line treated with coffee using **RT-PCR.**

	Outcome measure	Measuring device	Measuring unit
1- **Primary**1	Proliferative activity	**ELISA**	Comparative (light absorbed) numerical value
1- **Primary**2	Apoptotic cell death	**RT-PCR**	Comparative (fold changes) numerical value

Research question

Do different types of coffee affect proliferation and/or apoptosis of human oral squamous cell carcinoma cell line?

III. Methods

A) Samples, intervention and outcomes

1) Material:

1.   Cell Lines:

Oral squamous cell carcinoma cell line (SCC15), purchased form the confirmatory diagnostic unit, R&D sector, Vacsera, Cairo, Egypt.

2.   Coffee extract:

Green coffee, roasted coffee and decaffeinated coffee from local Egyptian market, Cairo, Egypt. The peels and seeds were manually separated.

2) Methods:

Intervention:

Oral squamous cell carcinoma cell line samples will be cultured with and without the addition of green coffee, roasted coffee or decaffeinated coffee extract.

Collecting Samples:

Oral squamous cell carcinoma cell lines will be purchased from (veterinary serum & vaccine research institute, VACSERA).

☐ Inclusion Criteria:

Only human cell lines.

☐ Exclusion Criteria:

Animal cell lines.

Mixed tumors.

Sample preparation:

Cells will be sub-cultured in a 96-well plate.

☐ Monolayer cells will be incubated for 48 hours at 37ºC in the 5% CO2 incubator.

☐ After 48 hours, cells will be fixed in wells for 1 hour in trichloro-acetic acid (50%-5 micro liter) which was added to the wells by a multichannel pipette.

☐ Cells will be then washed with tap water and left to dry for 1 hour.

☐ Sulphorhodamine B stain will be added (50 μl) and left for half an hour.

☐ Excess stain will be washed with 5% glacial acetic acid and then attached stain was recovered with EDTA buffer (100 μl).

Measurements:

a) MTT assay for cellular viability:

1. One ml of cells (50,000-100,000 cells/ml) will be plated into each well of 96-well culture plate for 24 hours before the MTT assay.

2. The cells will be incubated for 24 h in CO2 incubator.

3. After treatment of cells with antibiotics and antifungal for 24-72 h, experimental media will be removed, and cells will be washed with PBS.

4. Coffee extracts will be added for cell lines with serial dilutions (100μg/ml, 10μg/ml, 1μg/ml, 0.1 μg/ml, and 0.01μg/ml), incubated for 48 hours and then will be washed by PBS twice.

5. The cells will be incubated with medium containing 0.5 mg/ml MTT in CO2 incubator at 37°C for 4 h.

6. The medium will be aspirated, and the formazan product will be solubilized with dimethyl sulfoxide (DMSO).

7. Absorbance at 570 nm will be measured for each well using a microplate reader (BioTek,Flx 800).

The percentages of cytotoxicity and cell viability are calculated using the following equations:

% cytotoxicity = 1 - [means absorbance of treated cells/mean absorbance of negative control], % viability = 100 - % cytotoxicity.

Measurement of proliferative activity:

Enzyme assay (ELISA) will be used to estimate the inhibitory effect green coffee, roasted coffee, decaffeinated coffee on **Epidermal growth factor Receptor (EGFR)** protein.

Sample preparation for ELISA:

Cell lysis for cell culture samples will be carried by spinning down cells for 15 minutes at 1200 RPM. Cell pellets are washed once in cold PBS. Cells are re-suspended in Lysis Buffer to a concentration of 1.5x106 cells/mL, are incubated for 1 hour at room temperature with gentle shaking. Cells are centrifuged at 200 x g for 15 minutes. The supernatant will be diluted at least 50-fold in 1X Assay Buffer (5 μL supernatant + 245 μL 1X Assay Buffer) for the assay.

1. Prepared biotin antibody is added to samples in different wells.

2.   Then prepared Streptavidin horseradish peroxidase (HRP) solution is added after washing.

3.   Tetramethylbenzidine (TMB) Solution is added to each well and incubated at room temperature. Stop Solution is added to each well. Readings will be done at 450 nm immediately

Apoptosis activity assay:

   Following treatment with green coffee or roasted coffee or decaffeinated coffee for 24 h, the cells are harvested, lysed and tested with RT-PCR using specific primers to estimate the fold change of the gene expression of Caspase-3, bax, bcl-xl and p53.

7. Calculated sample size

No sample size estimation could be done, as the primary outcome is not a measured outcome.

8. Description of study sample

No sample size could be applied on cell line experiments as we generate millions of naturally randomized cells.

9. Intervention for each group

Green coffee extract will be introduced to the 1st group, while roasted coffee will be added to the 2nd group. The 3rd group will be treated with decaffeinated coffee.

10. Outcomes

 Apoptosis (Gene expression of Caspase – 3, EGFR).

Prioritization of Outcome	Outcome	Method of measurement	Measuring unit
Primary1	EGFR expression using ELISA	**Robonek P2000 ELISA reader**	IC 50 µg / ml
Primary2	Caspase-3, bax, bcl-xl and p53 gene expression using RT – PCR	**Rotorgene PCR system**	Fold change FLD=e-ΔΔCT

B)Assignment to intervention

This study is non-randomized.

11. Sequence generation

Sequence generation is not applied on cell line experiments.

12. Allocation concealment

Allocation concealment is not applied on cell line experiments.

13. Implementation

Implementation is not applied on cell line experiments.

C) Blinding

14. Blinding

Blinding is not applied on cell line experiments.

D) Statistical methods

   Data will be analyzed using IBM SPSS advanced statistics (Statistical Package for Social Sciences), version 21 (SPSS Inc., Chicago, IL). Numerical data will be described as mean and standard deviation or median and range. Categorical data will be described as numbers and percentages.

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