PROLIFERATION AND APOPTOSIS IN RAT MODELS OF ORAL SQUAMOUS CELL CARCINOMA INDUCED BY CAPSAICIN EXTRACT NANOPARTICLE GEL

PROLIFERASI DAN APOPTOSIS ORAL SQUAMOUS CELL CARCINOMA PADA TIKUS DENGAN INDIKSI GEL NANOPARTIKEL CAPSAICIN

Pratiwi Nur Widyaningsih*, Fitri Aniowati1, Cantika Nadrotan Naim1, Nova Dwi Anggraeni2

1 Department of Dentistry, Faculty of Medicine, Universitas Jenderal Soedirman, Indonesia
2 Department of Pharmacy, Faculty of Health Science, Universitas Jenderal Soedirman, Indonesia

ABSTRACT

Background: Oral Squamous Cell Carcinoma (OSCC) is highly invasive neoplasm of oral epithelial tissue that is moderately differentiated and tends to metastasize rapidly. Capsaicin, a pungent phytochemical in red peppers, exhibits both anti-proliferative and potential pro-cancer effects on human cancer cell lines. Purpose: Determine the proliferation and apoptosis between five groups of rats induced OSCC. Method: 5 groups of treatment; C- (untreated rats), C+ (induced by DMBA), E1 (exposed to DMBA and given cisplatin), E2 (induced by DMBA and Capsaicin extract nanoparticle gel with 1% concentration), and E3 (induced by DMBA and Capsaicin extract nanoparticle gel with a concentration of 3.3%). Maceration method was used to obtain Capsaicin extract from green cayenne pepper, then made into nanoparticle gel. Tissue samples were taken after the treatment was completed, then they were pathologically observed histopathologically using IHC to assess apoptic activity via the TUNEL method and proliferative activity using PCNA. Result: The proliferative activity in the E1 group had a significant difference compared to the E2 and E3 groups. E2 and E3 were not significantly different (p-value ≥0.05). Apoptotic activity in Group E1 indicated a significant difference from the E2 group and was not significantly different from the E3 group, whereas the E2 and E3 groups were significantly different from each other (p-value ≤0.05). Conclusion: The research showed that Capsaicin nanoparticle gel increased apoptotic activity while decreasing proliferative activity in different treatment groups of the OSCC rat model.

ARTICLE INFO

Received 14 December 2022
Revised 04 January 2023
Accepted 13 September 2023
Online 11 November 2023

Correspondence: Pratiwi Nur Widyaningsih
E-mail: drgpratiwinw@unsoed.ac.id

Keywords: OSCC, Proliferation, Apoptosis, Capsaicin

ABSTRAK

Latar belakang: Karsinoma sel skuamosa rongga mulut (OSCC) merupakan neoplasma invasif jaringan epitel rongga mulut yang berdiferensiasi sedang serta cenderung bemetastasis dengan cepat. Capsaicin adalah fitokimia dalam berbagai genus cabai yang dapat memberikan efek anti-proliferatif pada berbagai lini sel kanker manusia. Namun, Capsaicin juga dianggap mampu mendorong pertumbuhan sel kanker. Tujuan: Mengetahui proliferasi dan apoptosis tikus model OSCC pada lima kelompok. Metode: Terdapat 5 kelompok yaitu C- (tikus tidak diberi perlakuan), C+ (tikus diinduksi dengan DMBA), E1 (tikus diinduksi DMBA dan diberi cisplatin), E2 (tikus yang diinduksi DMBA dan gel nanopartikel ekstrak Capsaicin dengan konsentrasi 1%), dan E3 (tikus yang diinduksi DMBA dan gel nanopartikel ekstrak kapsaisin dengan konsentrasi 3,3%). Ekstrak Capsaicin dari cabai rawit hijau dibuat dibuat dengan metode magerasi. Sampel jaringan diambil setelah perawatan selesai. Jaringan diamati secara histopatologi dengan IHC, kemudian diamati aktivitas apoptosis menggunakan metode TUNEL dan diamati aktivitas proliferatif melalui PCNA. Hasil: Aktivitas proliferatif pada kelompok E1 memiliki perbedaan signifikan dibandingkan dengan kelompok E2 dan E3. Kelompok perlakuan, E2 dan E3 tidak terdapat perbedaan signifikan (p-value ≥0,05). Aktivitas apoptosis pada kelompok E1 memiliki perbedaan signifikan dibandingkan kelompok E2 dan E3 yang tidak memiliki perbedaan yang signifikan dengan kelompok E3. Kelompok perlakuan, E2 dan E3 memiliki perbedaan yang signifikan (p-value ≤0,05). Kesimpulan: Hasil penelitian menunjukkan gel nanopartikel Capsaicin berpengaruh terhadap peningkatan aktivitas apoptosis dan penurunan aktivitas proliferasi antar kelompok perlakuan pada tikus model OSCC.

Kata kunci: OSCC, Proliferasi, Apoptosis, Capsaicin
INTRODUCTION

Oral Squamous Cell Carcinoma (OSCC) is an invasive neoplasm of the epithelial tissue of the oral cavity with varying degrees of differentiation (Riskayanti et al., 2021). OSCC can occur on the lower lip, floor of the mouth, ventral and lateral to the tongue, retromolar area, tonsils and lateral soft palate. Most of the OSCC is a squamous cell carcinoma moderately differentiated and tends to metastasize rapidly (Bugshan and Farooq, 2020). It could potentially lead to direct tumor invasion into bone, muscle, and neurovascular tissue or regional and distant node metastasis (Lin et al., 2021).

The progression of OSCC is influenced by two interconnected pathways in particular called proliferation and apoptosis (He et al., 2022). In advanced cancer cells, progressive Epithelial-Mesenchymal Transition (EMT) can occur due to dysregulation of additional transcription factors. EMT is a process that involves loss of epithelial cells lose polarity and adhesion, leading to resistance to apoptosis, expression of matrix-degrading enzymes such as metalloproteinases, and increased motility and invasiveness (Choi et al., 2019). One of the main characteristics of cancer cells is the ability of tumor cells to maintain their proliferative signaling due to dysregulation control, making apoptosis induction a potential mechanism for eliminating them (Kim et al., 2018). Cancer cells can stimulate the growth of normal cells in the tumor microenvironment to release growth factor ligands which is referred to as control dysregulation via endocrine or autocrine pathways (Hanahan, 2022).

Capsaicin is a phytochemical derived from homovanillic acid which causes the spicy taste of chili peppers. Scientifically Capsaicin demonstrates anticancer effects on various types of human cancer cells by inducing apoptosis (Friedman et al., 2018). Conversely, Capsaicin is believed to promote cancer cell growth (Lin et al., 2018). The anticancer effects induced by Capsaicin on osteoma cells involve several MAPK signaling pathways (increasing tumor cell proliferation and anti-apoptosis). Overall Capsaicin has anti-cancer potential by targeting cell cycle regulators to inhibit the cancer growth and division (Huang et al., 2021). The aim of this study was to determine the proliferation and apoptosis in five OSCC-induced rat groups.

MATERIAL AND METHOD

This study was conducted for four months at Jenderal Soedirman University and Brawijaya University based on an experimental laboratory using the randomized post-test control group design method. The Capsaicin extract was taken from green cayenne pepper (Capsicum frutescens L.) using maceration method in Kemutug Kidul Village, Baturaden District, Banyumas and analyzed at the Environmental Laboratory of the Faculty of Biology, Jenderal Soedirman University. The study employed 20 Wistar rats divided into 5 test groups (Table 1), with ethical approval obtained from Jenderal Soedirman University Faculty of Medicine under ethical number 125/KEPK/VII/2021. The examination of cell proliferation and apoptosis was carried out at Brawijaya University Faculty of Dentistry.

The green cayenne pepper extraction involves two methods: simplicia and maceration. Green cayenne pepper simplicia is made by drying the chilies in an oven at 80°C for 6 hours (Fadhilatunnur et al., 2022). Dried chilies are pounded to get fine powder. On the other hand, maceration was carried out by dissolving chili simplicia in 96% ethanol solvent at 50°C, stirring at 200 rpm for 4 hours in multiple stages to obtain a thick extract (Sapitri et al., 2021).

Capsaicin extract nanoparticle gel was prepared in 100 g gel preparations, with variations of 3.3% and 1% Capsaicin extract. The gel preparation was done by weighing all the ingredients needed for carbopol 0.5 g; triethanolamine 4 gr; methylparaben 0.3 gr; propylene glycol 15 gr; sodium metabisulphite 1 gr; Capsaicin extract each 1 gr and 3.3 gr; and distilled water 78.2 gr and 75.9 gr. The gel was prepared by mixing the gelling agent, namely carbopol, with heated water, then allowing the gel mass to form and swell. Methylparaben was dissolved in propylene glycol and sodium metabisulfite was added and stirred until a homogeneous solution was formed. The solution is added to the carbopol base and homogenized, followed by mixing the extract into the base and homogenizing again. The remaining water was added to the base and homogenized again (Megawati et al., 2019). Nanoparticles were prepared by stirring on a magnetic stirrer for 4 hours until achieving the desired nanoparticle gen consistency.

The active compound, Capsaicin, was isolated separately to determine its presence in green cayenne chili samples. The Capsaicin compound isolation test was carried out by dissolving green cayenne pepper powder in a single solvent n-hexane and methanol. As much as 5 grams of chili powder was dissolved in 100 ml of methanol, then 10 ml was taken for isolation. A solution of chili powder and methanol was added to 10 ml of distilled water and 10 ml of n-hexane to form an n-hexane fraction containing a purer Capsaicin compound. Furthermore, the solution was shaken, and the n-hexane fractions formed were separated and placed in different vials. The fraction was placed in a desiccator for 24 hours and cooled in the refrigerator until crystals were formed, indicating the presence of pure Capsaicin compounds (Ghozaly and Elfahmi, 2020).

Before the test animals were given treatment, male wistar rats (Rattus norvegicus) aged 2 months with body weight of 150 - 200 grams were given markers to differentiate each test group, and the acclimation process was carried out for one week to trigger the growth of cancer cells, carcinogen induction was required using 7 - 12 Dimethylbenzeno(a)anthracene (DMBA) as much as 96 mg which is dissolved in 24
mL of corn oil and homogenized using a vortex for ± 15 minutes. The dose of DMBA was 20 mg/kg BW rats by injection of the right buccal mucosa for two weeks with a frequency of twice a week. Ketamine was used as an anesthetic agent intramuscularly at a dose of 0.2 mL/200 gr BW rats. Rats were diagnosed with cancer by palpation method four times a week. Interpretation of the diagnosis of cancer occurs when there is a palpable nodule on the buccal mucosa. Cancer treatment was started once a day for seven days after the rats were diagnosed with cancer.

Table 1. Type of experiment group and rats treatment

<table>
<thead>
<tr>
<th>Experiment group</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control – (C-)</td>
<td>Rat without treatment</td>
</tr>
<tr>
<td>Control + (C+)</td>
<td>Rat induced to DMBA</td>
</tr>
<tr>
<td>Experimental control 1 (E1)</td>
<td>Rat induced to DMBA and cisplatin</td>
</tr>
<tr>
<td>Experimental control 2 (E2)</td>
<td>Rat induced to DMBA and Capsaicin extract nanoparticle gel with a concentration of 1%</td>
</tr>
<tr>
<td>Experimental control 3 (E3)</td>
<td>Rat induced to DMBA and Capsaicin extract nanoparticle gel with a concentration of 3.3%</td>
</tr>
</tbody>
</table>

Based on Table 1, the treatment was carried out after observation with a frequency of once a day for 1 week. In the experimental control 1 (E1) group, cisplatin was used intravenously at a dose of 5 mg/kg BW. In the experimental control 2 (E2) group used 1% Capsaicin nanoparticle gel at a dose of 1 mg/kg BW. In the experimental control 3 (E3) group used 3.3% Capsaicin nanoparticle gel at a dose of 3 mg/kg BW, applied to the right buccal mucosa. Samples were drawn using a random sampling, then they were divided into five treatments which were carried out once a day for seven days. Preparation for a surgical procedure using the cervical dislocation method was carried out after it was declared that the sample had cancer (a nodule was palpable in the buccal) and had been given treatment. The heads of the rats were cut to observe the tumor tissue, cleaned using saline, and stored in a sample pot containing 10% Neutral Buffered Formalin (NBF). Immunohistochemical preparations were made.

The rats were observed for proliferation and apoptosis in oral squamous cell carcinoma induced by Capsaicin gel extract nanoparticles through tissue histological examination. Increased proliferation indicates an increase in tumor malignancy, while a decrease in apoptosis indicates a local failure to deal with cancer growth and indicates an increase in metastasis (Gartner and Hiatt, 2012). The method chosen to detect cell apoptosis was Terminal deoxynucleotidyl Transferase-mediated dUTP Nick End Labeling (TUNEL) while cell proliferation was observed using the Proliferating Cell Nuclear Antigen (PCNA) method. Apoptotic staining will appear brownish.

Figure 1 shows the mean of cells in the tissue. Group E1 (administration of cisplatin in the OSCC model) had the highest proliferative and apoptotic activity, as evidenced by the lowest number of cells detected in the preparation. Group C+ (negative control) showed the highest number of cells among all groups. Groups E2 and E3 (administration of 1% and 3.3% Capsaicin nanoparticle gel) showed lower cell numbers than the C+ group (negative control).

Figures 2(a) and 2(b) show the proliferative and apoptotic activity in the histological picture. The low number of cells were detected in the tissue exhibiting high proliferative and apoptotic activity. These cells could be characterized by non-spherical and amorphous shapes, showing proliferative activity, and the reddish colored cells indicating apoptotic activity. The results of the observations were analyzed using a Post-Hoc test and the differences between groups were found as follows. Table 2 shows the results of the post hoc LSD proliferative activity. Group C- (healthy control) had a significant difference (p-value ≤0.05) compared to groups E2 and E3 (administered Capsaicin nanoparticle gel 1% and 3.3%). The E1 group (with cisplatin) had a significant difference compared to the E2 and E3 groups. Between treatment groups, E2 and E3 were not significantly different (p-value ≥0.05).

Table 3 shows the results of the Post-Hoc Mann-Whitney test for apoptotic activity. Group C- (healthy control) had a significant difference compared to groups E2 and E3 (given Capsaicin nanoparticles gel 1% and 3.3%). The E1 group (with cisplatin) had a significant difference compared to the E2 group and was not significantly different from the E3 group. Between treatment groups, E2 and E3 were significantly different (p-value ≤0.05).

RESULT

Proliferative and apoptotic activity in OSCC cells was observed histologically. On average, it can be seen in Figure 1 that there is a difference in the number between groups.
Figure 2. Cells undergoing proliferation and apoptosis can be seen, stained brownish (a) The proliferative in the histological picture; (b) Apoptotic activity in the histological picture.

Table 2. Results of the Post-Hoc of proliferative activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>C-</th>
<th>C+</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-</td>
<td>6.8</td>
<td>1.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C+</td>
<td>14.2</td>
<td>2.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>7.2</td>
<td>1.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>12.0</td>
<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>10.2</td>
<td>2.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Results of the Post-Hoc of apoptotic activity

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>C-</th>
<th>C+</th>
<th>E1</th>
<th>E2</th>
<th>E3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-</td>
<td>4.0</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C+</td>
<td>14.6</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E1</td>
<td>6.4</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2</td>
<td>12.0</td>
<td>1.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E3</td>
<td>8.0</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

The principle of chemotherapy is generally to induce cell apoptosis to eliminate cancer cells. Capsaicin induced significant apoptosis in numerous cancer cell lines. Furthermore, the high concentration of Capsaicin can trigger apoptotic cell death for most cancer cells in the range between 200 and 400 µM (Zhang et al., 2002). Research has demonstrated that Capsaicin can induce apoptosis in oral cancer cells through the intrinsic pathway at a concentration of 200 µM (Kamaruddin et al., 2019).

The main hallmark of cancer is uncontrolled cell development (proliferation) due to dysfunctional apoptosis. Hence, in cancer patients, tissue immortality, invasion, and metastasis to other organs can occur. Apoptosis is a process of programmed cell death that occurs regularly to eliminate unnecessary cells without an inflammatory response. It occurs normally during development and aging. This mechanism is a homeostatic mechanism to maintain cell populations. It also functions as a defense mechanism during an immune response or when cells are damaged by disease (Huang et al., 2021). It is characterized by DNA fragmentation, chromatin condensation, the cytoplasm shrinkage and non-lytic cell death preserving the surrounding cells or tissues. It is the process of cell death to maintain the integrity of the entire body. One of the most important roles of apoptosis is to maintain homeostasis of cell proliferation and to restrict the excessive proliferation in cancer cells. Multifunctional targets and activation of multiple specific apoptotic signaling pathways are important in cancer therapy to prevent the development of drug resistance in cancer treatment (Sun and Zhang, 2021). Cancer treatment drugs for OSCC include platinum drugs (cisplatin and carboplatin), taxanes, anthracyclines, and antimetabolites. Platinum drugs generally work by triggering a molecular cascade that leads to cell cycle arrest or cell death in cancerous tumors through the use of a mechanism known as DNA Damage Response (DDR), resulting in DNA repair and recovery, cell cycle arrest, or apoptosis in damaged cells (Law et al., 2021).

The anticancer effects of Capsaicin are primarily dependent on the induction of apoptosis (Zhang et al., 2002). Two major signaling systems that can lead to apoptosis are the intrinsic mitochondrial death pathway and the extrinsic death receptor pathway. The intrinsic pathway is involved in the complete execution of apoptosis and can be an important target for new treatments (Arul and Kothai, 2020). Capsaicin can induce apoptosis in the intrinsic pathway by activating CD95 as a mediated apoptotic of both intrinsic and extrinsic pathways. Capsaicin also suppresses the expression of antiapoptotic proteins, leading to caspase-9 and caspase-3 activation, loss of mitochondrial membrane potential, and subsequent release of cytochrome c (Kamaruddin et al., 2019). Nevertheless, the induction of apoptosis by Capsaicin is not considered a major pathway, especially in cases of defective apoptosis, which is an important feature of cancer cells. Furthermore, the apoptotic effect induced by Capsaicin usually is observed at high concentrations. Therefore, Capsaicin may work through other pathways to exert its effects. The anticancer effect on cancer cells is regulated through the cell cycle, which is divided into G0/G1 phase, S phase, and G2/M phase. DNA checkpoints throughout the cell cycle ensure the integrity of DNA replication, and growing evidence suggested that Capsaicin caused DNA damage, affecting cell cycle regulation. This type of cell cycle termination depends on the phenotype and genotype diversity of different cancer cells (Zhang et al., 2002).

The diagnosis of OSCC can be made through various methods, but the primary method for determining the diagnosis and assessing the severity of the disease is through the biopsy (Mosqueda-Solis et al., 2021). Collecting tissue at a biopsy enables clinicians to examine tissue conditions, including epithelial proliferation to the basal layer, stratified epithelial dysplasia, and abnormal microvascularization (Abati et al., 2020). Apoptosis is one of the responses of cancer cells when they are unable to replicate and divide after an invasion, whether it is endogenous signals from within the cell or exogenous administration, such as radiotherapy, chemotherapy or other cytotoxic chemicals. Observing apoptosis can be a determinant of cancer cell survival within a tissue (Carneiro and El-Deiry, 2020). On the other hand, Proliferating-Cell Nuclear Antigen (PCNA) is one of the activities associated with cancer cell proliferation and division. PCNA serves as an indicator of cancer severity-progression. The number of PCNAs in this study proved the success of DMB induction in the OSCC rat model (Sadikin, 2019).

Capsaicin is one of the compounds contained in cayenne pepper, and it exerts antitumor effects through several mechanisms. Specifically, Capsaicin triggers apoptosis in cancer cells by activating the TRPV1 membrane receptor, leading to mitochondrial damage caused by alterations in cytoplasmic cell arrangement in the form of a decrease in Ca²⁺ levels. Additionally, Capsaicin’s mechanism of action may involve the generation of Reactive Oxygen Species (ROS), thereby promoting DNA and mitochondrial damage, ultimately leading to apoptosis (Chen et al., 2021).

The results of observations, based on histological observations of the right buccal cell mucosa, using the PCNA revealed that actively proliferating cells exhibited a dark brown color (Pakaya et al., 2020). Among the treatments, the administration of 3% Capsaicin nanoparticle gel (E3) with DMB induction showed the most significant proliferation, evident as a broad dark brown color in tissue histology. Notably, the histological structure of treatment (E3), induced by DMBA and administered with a 3% nanoparticle gel, resembled the proliferation results observed in the positive control treatment (C+) where DMBA and cisplatin induced were used as inducers.
The TUNEL Assay method is used to detect apoptosis using a fluorescence microscope. Cells undergoing apoptosis will be indicated by green fluorescence, while those not undergoing apoptosis will be indicated by red fluorescence (Fan et al., 2021). The results of the histological examination revealed that the majority of cells not undergoing apoptosis were observed in (E1) induced by DMBA and cisplatin. Examination of (E2), with the administration of 1% Capsaicin nanoparticle gel, showed that the extent of red fluorescence results was greater compared to the administration of 3% Capsaicin nanoparticle gel.

CONCLUSION

The research showed that Capsaicin nanoparticle gel had an effect on increasing apoptotic activity and decreasing proliferative activity in the OSCC rat model. Further research is needed to investigate the effects of Capsaicin on apoptotic and proliferative activity in human cancer cells.

ACKNOWLEDGMENTS

The author is grateful to Unsoed Pharmacy Laboratory and Brawijaya University Laboratory for technical support for this research and to the lecturers for input and revisions to the article. There were no conflicts of interest while conducting the research and write up.

REFERENCE


