

Proceeding Book

8th International Dental Scientific Meeting *Dentistry Faculty, Hasanuddin University*

June 20th – 22nd Grand Clarion Hotel & Convention – Makassar, Indonesia

“Comprehensive Dentistry
in the International Community



Editor:
Dr. drg. Susilowati, SU
drg. Maria Tanumihardja, MDSc

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-Makassar : 2014

viii + 272; 15,5 x 23 cm

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Daerah Sulawesi Selatan

First Edition, 2014

ISBN: 978-602-98336-3-8


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The Working Mechanism of Pomegranate (*Punica Granatum Linn*) Fruit Extract Towards Mucus Cell Degradation of Mice Oral Cavity which Experience Transformation through VEGF Expression

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Abstract

Cell transformations are cells that undergo behavioral changes as a result of transcription of an oncogene. Squamous cell carcinoma of the oral cavity is a continuation of cell transformation in the oral cavity squamous cell. The progresses in the fields of surgery, radiation and chemotherapy have not yet provide maximum results. Long-living patients with oral cavity squamous cell carcinoma are less than 50 %. One cause of cancer development is caused by new blood vessels formation or angiogenesis. Whole pomegranate fruit extract (PGL) is an alternative treatment to inhibit angiogenesis formation in cancer. PGL is one of the medicinal plants that contain in vitro anticancer activity. This fact has prompted researchers to study standardized whole pomegranate fruit extract (PGL) with the aim to eliminate the cells undergoing transformation into carcinoma cells through decreased VEGF expression. PGL is compared with ellagic acid (EA, single compound of PGL), and extracts of mahkota dewa (an anti-cancer herb that has been published). The research method used was laboratorial experiment; 30 male mice, aged 5 months which were randomly divided into 5 groups: 2 control groups (K0, normal mice) and (K1, exposed to benzopirene), and 3 treatment groups (P1, ellagic acid), (P2, PGL) and (P3, mahkota dewa). Benzopirene exposure with

a dose of 0.04 mg benzopirene /0.04 ml olium olivarum is for 4 weeks with 3 times a week. Provision of ellagic acid, PGL, and mahkota dewa are with a dose of 75 mg/Kg/weight of mice every day for 4 weeks. The examination applied the immunohistochemistry technique. The results based on the immunohistochemistry technique showed that administration of standardized whole pomegranate fruit extract (PGL) to decrease the VEGF expression is most potent than the ellagic acid and mahkota dewa. The research conclusion is that whole pomegranate fruit extract (PGL) inhibit cells that undergo transformation towards carcinoma through decreasing VEGF expression which result in the inhabitation of angiogenesis formation, then cancer cells do not grow and finally die.

Keywords: *pomegranate (PGL), Cell Transformation, VEGF*

INTRODUCTION

Cell transformations are cells that undergo behavioral changes as a result of an oncogene. The protein-coding genes in oncogene will develop into carcinomas (Sudiana, 2008). More than 90% of oral cavity carcinomas derive from squamous cell; squamous cell carcinoma of the oral cavity becomes a major health problem in developing countries and is a major cause of death. The survival index continues to decline (50%) compared with the advances in cancer diagnosis and treatment (Mehrota and Yadav, 2006). The ability of cancer to induce new blood vessel formation (angiogenesis) is very influential in cancer growth and metastasis. Cancer growth takes place when receiving enough blood supply through vascularization for metabolism and proliferation purposes. To meet its needs, cancer increases the ability of neovascularization (Kresno, 2011). VEGF is an endothelial growth factor that supports new blood vessels formation. VEGF is mitogenesis of vasculogenesis and angiogenesis, most cancer types express high VEGF levels (Kresno, 2011). Various cancer disease management efforts still face many obstacles that result in the lack of success in preventing and treating cancer. Treatments that have been made include surgery, radiation, radiotherapy and the use of chemotherapeutic drugs; surgery to remove cancer has not yet fully ensures healing and there

is a tendency for the occurrence of cancer cells to re-multiply.

The use of radiotherapy poses the risk of other damage to surrounding tissues affected by cancer. Provisions of anti-cancer chemotherapy have less selective pharmacological effects, adverse side effects and has reported the presence of several types of cancer resistance (Rizali and Aurerkari, 2003; Sismindari, 2006). Nowadays, various attempts have been made to study the discovery of anti-cancer drug. One of them is an attempt to explore the potential medicinal plants in Indonesia, namely; pomegranate or *Punica granatum* Linn (PGL).

Pomegranate (PGL) fruit is part of the pomegranate plant that has the most complete components compared to other parts of the pomegranate plant. Pomegranate fruit contains polyphenols compounds, consisting of flavonoids, hydrolyzable tannins (ellagitanins and gallotanins) and condensed tannins. The main active ingredient contained in pomegranates is punicalagin and ellagic acid. Good standardized pomegranate extract generally contains 40% or more ellagic acid. Pomegranate extract with a standard 40% ellagic acid can inhibit the growth of cancer cells, anti-proliferation; induce apoptosis, and in vitro antioxidants (Seeram et al, 2006; Jurenka, 2008). If the effect of whole pomegranate fruit extract (PGL) in Swiss Webster mice strains (Balb/c) is revealed, then the whole pomegranate fruit extract (PGL) can be used as an alternative treatment for squamous cell carcinoma of the oral cavity.

METHODS AND MATERIALS

Method

The research type is a true laboratorial experiment. The study design uses a complete randomized design. Samples and treatments are cultivated under controlled conditions and measurements, so that the treatment effect is more believable or acceptable. The mice used were Swiss Webster Strains (Balb/c), weight range of 30-50 grams, male gender, aged 5-months, and obtained from the animal testing unit of the Gadjah Mada University, Yogyakarta. The experimental unit is the squamous epithelium of the buccal mucosa on bottom right of the mice oral cavity. Mice were divided

into 5 groups; KO (normal mice), K1 (benzopirene), P1 (benzopirene + ellagic acid), P2 (benzopirene + pomegranate (PGL), P3 (benzopirene + *mahkota dewa*), with each group consisting of 6 mice. Mice are exposed to 0.04 mg benzopirene /0.04 ml olium olivarum orally 3 times a week for 4 weeks on the right of the buccal mucosa of the mice oral cavity. At the end of the 9th week, the mice oral mucosal tissues were biopsied and then the mice were terminated. Pomegranate (PGL), ellagic acid, and *mahkota dewa* were given orally every day for 4 weeks, and later on the mice were terminated. The dose of pomegranate, ellagic acid, and *mahkota dewa* is 75 mg/kg/weight/day which were dissolved in 0.3% CMC-Na. Laboratory tests used for VEGF expression applied the immunohistochemistry technique. Pomegranate fruit extract (PGL) that is standardized to 40% ellagic acid is obtained from Xi AN BIOF BIO-TECHNOLOGY CO., LTD in China in the form of ready-to-use extracts that is dissolved in 0.3% CMC-Na.

Materials

The materials are 3% H₂O₂, 0.025% trypsin, PBS, aquadestilata, substart buffer, xylol, absolute ethanol, methanol, anti-VEG antibodies, enzymes, poly L-lysine slide, buffers, formalin, polyclonal antibodies, labeled antiglobulin, secondary antibodies, and streptavidin. Inspection procedures of VEGF expression by immunohistochemistry techniques include reagent preparation (fixation stage, DAB working solution, staining, washing, and labeling).

RESULT

VEGF Expression

The preparation examination results with immunohistochemistry techniques of VEGF expression are seen in table I.

Table 1. The standard mean and deviation of Cells Expressing VEGF on Animal Experiments

Group (n=6)	Number of cells expressing VEGF (mean \pm SD) (%)
Control (-) / K0	0.133 \pm 0.103 ^a
Control (+) / K1 (benzopirene + CMC)	0.350 \pm 0.104 ^c
P1 / (benzopirene + EA)	0.267 \pm 0.103 ^{bc}
P2 / (benzopirene + PGL)	0.183 \pm 0.098 ^{ab}
P3 / (benzopirene + MD)	0.150 \pm 0.104 ^a

*) Different superscripts in the same column differ significantly ($p < 0,05$)

The preparation examination results with immunohistochemistry techniques showed that administration of standardized pomegranate extract (P2/ benzopirene + PGL) can decrease the VEGF expression in the mucosa cell of the buccal oral that undergoes a transformation into squamous cell carcinoma in mice as the experimental animal. Decreased VEGF expression in group P2 (benzopirene + PGL) (0.183 \pm 0.098) did not differ significantly to K0/K- (without benzopirene + CMC) (0.133 \pm 0.103), P3 (benzopirene + MD) (0.150 \pm 0.104) and P1 (benzopirene + EA) (0.267 \pm 0.103), but significantly different when compared to group K1 (benzopirene + CMC) (0.350 \pm 0.104). The mean number of cells expressing VEGF in the group K0, K1, P1, P2 and P3 are shown in Table 1.

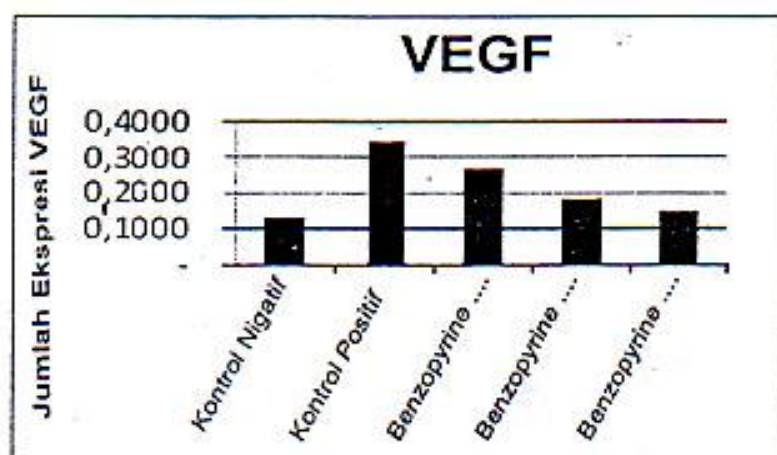


Figure 1. Standard Mean and Deviation Number of Cells Expressing VEGF on Experimental Animal.

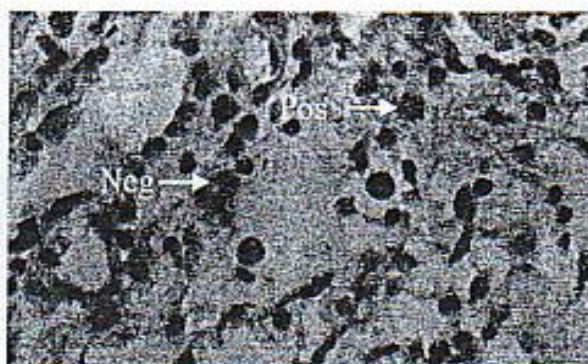
The examination preparation results of VEGF expression at a benzopirene dose of 0.04 mg/ 0.04 ml olium olivarum shows a microscope picture as below:

Group K0



Group K0/negative control (normal mice) → (Positive) Staining immunohistochemistry with polyclonal antibodies against VEGF expression on 400x magnification.

Group K1



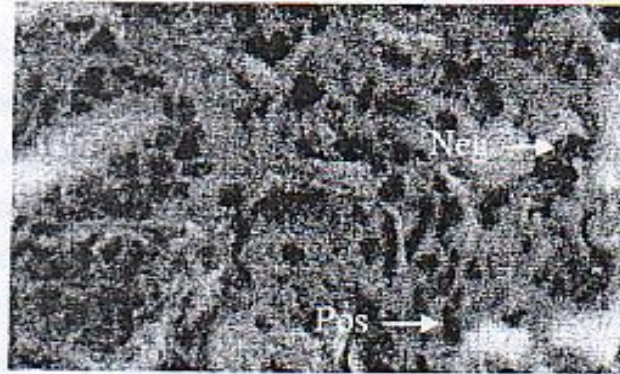
Group K1/positive control (Benzopirene) → (Positive) Staining immunohistochemistry with polyclonal antibodies against VEGF expression on 400x magnification.

Group P1



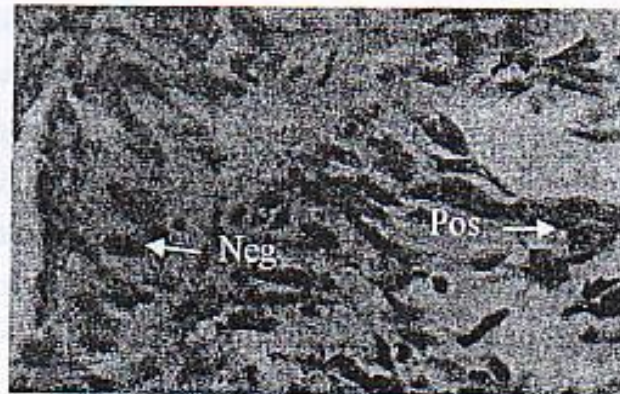
Group P1 (Benzopirene + PGL) → (Positive) Staining immunohistochemistry with polyclonal antibodies against VEGF expression on 400x magnification.

Group P2



Group P2 (Benzopirene + PGL) → (Positive) Staining immunohistochemistry with polyclonal antibodies against VEGF expression on 400x magnification.

Group P3



Group P3 (Benzopirene + MD) → (Positive) Staining immunohistochemistry with polyclonal antibodies against VEGF expression on 400x magnification.

DISCUSSION

Based on Table 1, the results showed that the whole pomegranate (PGL) fruit extract of group P2 (benzopirene + PGL) (0.183 ± 0.098) can decrease the VEGF expression in oral mucosal cells of mice caused by exposure, and the decrease of VEGF expression in group P2 did not

differ significantly to group K0/K- (without benzopirene + CMC) (0.133 ± 0.103), P3 (benzopirene + MD) (0.150 ± 0.104), P1 (benzopirene + EA) (0.267 ± 0.103), but it is significantly different from group K1 / K + (benzopirene + CMC) (0.350 ± 0.104).

VEGF is an angiogenic inducer that can be encountered in various cancer types. VEGF showed specificity to target cells, which are specific for endothelial cells. VEGF, in addition to increasing the proliferation and migration of endothelial cells, also serves to increase the permeability of blood vessels, cause coagulation factor out of the blood vessels and coat perivascular tissues, especially fibrinogen, thus resulting in the formation of fibrin pericancer which facilitates migration of endothelial cells and fibroblasts that excrete proteoglycans to form mature stroma for cancer. Angiogenesis or neovascularization is an important process for the growth of cancer survival (Hasina et al, 2001; Kresno, 2011). Proliferation and migration of endothelial cells are activated by several angiogenesis factors, such as Tyrosine Kinase Receptor which include Vascular Endothelial Growth Factor (VEGF), angiogenin, and angiopoitin. VEGF is used as a predictive indicator of the cancer cell development (Reuben et al, 2011).

VEGF is a major stimulus for cancer growth and neovascularization. The process development of cancer angiogenesis is by triggering the growth factor then stimulating the migration and proliferation of endothelial cells. The blood vessels in cancer showed abnormal morphology, while normal blood vessels are organized by the easily distinguished arterioles, capillaries, and venules (Dvorak, 2005). Research by immunohistochemistry technique showed VEGF is localized in the cancer and endothelial cells (Roskoski, 2007).

Excessive cell proliferation in cancer cells will trigger the arachidonic acid pathway via cyclooxygenase (COX-2), and will excrete prostaglandin that will trigger angiopoitin to excrete VEGF and trigger angiogenesis formation (Cho, 2007). Angiogenesis is the new blood vessels growth from existing blood vessels, which is a complex phenomenon that is absolutely necessary for the growth and survival of cancer cells. Without new blood vessels that supply nutrients to cancer, the cancer will not grow and

develop larger (Kresno, 2011). The results showed that the standardized whole pomegranate fruit extract (PGL) has the ability to reduce the VEGF expression; so with the inhabitation of VEGF, the angiogenesis formation is obstructed, thus nutrient supply to cancer cells will be cut off, then the cancer cells will not grow, and finally die.

Whole pomegranate fruit extract (PGL) can inhibit angiogenesis through regulation of vascular endothelial growth factor (VEGF). This is because the production of angiogenic factors is regulated by NF- κ B (Seeram et al, 2006). Whole pomegranate fruit extract also inhibits cyclooxygenase activity (COX-2), which is an enzyme that is induced by mitogenic agents, inflammation, and thus decrease the excretion of prostaglandins, prostaglandin does not trigger angiopoitin to excrete VEGF, VEGF secretion is inhibited, so that angiogenesis is not formed (Seeram et al, 2006). Caffeic acid, which is one of the active ingredients of whole pomegranate fruit extract (PGL), can inhibit STAT3 activation (signal transducer and activator of transcription 3). STAT 3 is a transcription activator of VEGF gene in various human cancers. STAT3 activity plays a major role in the VEGF overproduction (Jung et al, 2007).

In this study, the activity contained by standardized whole pomegranate fruit extract (PGL) towards the decrease VEGF expression is stronger than ellagic acid. This is because the standardized whole pomegranate fruit extract (PGL) is a complex mixture of different compounds, where some studies have shown that the compounds contained in the standardized whole pomegranate fruit extract (PGL) has the ability to mutually enhance the biological effects of each compounds, such as ellagic acid and quercetin (both are also found in whole pomegranate fruit extract (PGL), which when administered together showed a stronger barrier against the growth of cancer cells than when administered individually (Seeram et al, 2005).

The existence of polyphenols in whole pomegranate fruit extract (PGL) can increase the solubility and absorption of ellagic acid in the digestive tract, and moreover the polyphenols contained in the whole pomegranate fruit extract (PGL) also has the ability to inhibit the ellagic acid metabolism by intestinal microflora into urothilin A and B through

the possessed antibacterial activity of whole pomegranate fruit extract (Seeram et al, 2006).

The research results of group P3 (benzopirene + *mahkota dewa*) herbal medicine that is already available on the market showed decreased VEGF expression that is more powerful than pomegranate fruit extract (PGL) and ellagic acid, this might be due to the content of *mahkota dewa* (*Phaleria macrocarpa*) which are: alkaloids, terpenoids, saponins, polyphenols, and flavonoids. Flavonoids, alkaloids, and saponins can trigger apoptosis. Increasing dose of *mahkota dewa* extract is followed by an increase of apoptotic index (Widyasari et al, 2008). These facts indicate that the result of decreased VEGF expression of *mahkota dewa* is better than pomegranate extract (PGL) and ellagic acid.

Mahkota dewa is a medicinal plant that is toxic, its skin and flesh while in fresh state has a bitter taste, as well as its seeds may contain a compound that works similar to oxytocin and syntocinon, where both of these compounds are known to interfere embryonic development, because it can stimulate muscle contractions and thus making it very dangerous for pregnant women. *Mahkota dewa* is also allegedly containing compounds that are toxic and teratogenic, which can be dangerous if taken in a long term (Suatma et al, 2008).

Repeated high doses of intact pomegranate extract, ellagitannin or punicalagin, as a general dose performed in traditional medicine system is not toxic (Cerde et al., 2003; Vidal et al., 2003).

The study results of group K0 (without benzopirene and without treatment) expressed VEGF; this is because some normal cells, such as fibroblasts, endothelial and keratinocyte produce small amounts of VEGF, the increase VEGF levels occur when it needs angiogenesis (Kresno, 2011).

CONCLUSION

The conclusion of this study is the standardized whole pomegranate fruit extract (PGL) can inhibit the growth and progression of malignant oral mucosal cells of mice that undergo a transformation into squamous cell carcinoma of the oral cavity through a reduction in VEGF expression,

which result the inhabitation of angiogenesis formation, the nutrient supply to the cancer cells will be disconnected, then cancer cells do not grow, and finally die.

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