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**TOXICOLOGICAL SCREENING OF ELLAGIC ACID IN POMEGRANATE
FRUIT AND HYDROXYAPATITE COMBINATION AS BONE GRAFT
MATERIAL ON BHK-21 FIBROBLAST CELL**

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ABSTRACT

Background: Bone graft is an alternative therapy for periodontitis and other bone destructive lesions. Several studies had revealed Ellagic Acid (EA) ability in increasing osteogenesis process. EA contains polyphenols, such as Ellagitannin, Gallotannin, and Anthocyanin, which demonstrate anti-inflammatory and antibacterial activity as well as growth factor stimulating effect. EA combination with bone graft material (hydroxyapatite) is anticipated to enhance bone osteogenesis yet no investigation was performed to identify its toxicity towards fibroblast cell. **Objective:** To analyze EA toxicity on fibroblast cell in vitro. **Methods:** This was a true experimental study using post-test only with control group design. Fibroblast cell was exposed with EA in eight different concentrations: 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4% and 5%. Control group comprised of cell control and media group. All groups were exposed to MTT Assay test and measured using Elisa Reader. **Result:** The calculation of cell viability value in EA groups at 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4% and 5% concentration were 88.2%, 92.3%, 97.5%, 89.5%, 84.2%, 90.7%, 88.9% and 89.4% respectively. **Conclusion:** All EA and hydroxyapatite combinations are not toxic towards BHK-21 fibroblast cells.

Keywords: Bone graft, ellagic acid, fibroblast cells, hydroxyapatite, MTT assay

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INTRODUCTION

Bone destruction and periodontal problems are the highest disease prevalence in Indonesia with annual increase in the current decade. Based on Basic Health Research conducted by Ministry of Health Indonesia, this disease prevalence reached 23.4% in 2007 and increased to 57.6% in 2018.¹⁻³ Newman *et al* (2012) mentioned that 50% of adult population in the United States suffered from bone and periodontal tissue destruction, while National Household Health Survey Ministry of Health Indonesia in 2011 reported that bone and periodontal problems constituted 60% of oral and dental diseases within the country.

Conventional therapy, in extensive bone destruction specifically, is by utilizing graft or bone transplant from various types such as autograft, allograft, xenograft or synthetic graft.⁴

Autograft is bone transplant method originated from individual's body. This type of transplant demonstrates excellent osteogenic and osteoconductive activity. Notwithstanding, autograft results in several drawbacks such as painful sensation at donor site, blood loss, infection, donor tissues death, and limited number of organ available.⁵ So does allograft obtained from cadavers or donor from similar species or xenograft (from animal or different

species) that are presented with countable disadvantages like less osteogenic activity as well as risk of cross infection and immunological rejection.⁶⁻⁸ As the gold standard of therapeutic material for bone destruction, the three types of graft exhibit limitation in material quantity. This leads to the disproportion between material availability and higher demand of graft material therefore synthetic substances are being developed consequently. Synthetic graft is considered as the solution for limited supply of adequate material because it may be produced without being dependent on donors like other types while providing good osteoinductive activity.^{5,9,10}

Main component of bone graft material is hydroxyapatite. It is an inorganic compound identified in the structure of human bone and teeth.¹¹ Hydroxyapatite in the form of graft or scaffold is used as a therapy for bone defect by presenting good biocompatibility, bio-affinity, bioactivity, osteoinductive, osteoconductive and osteointegrative properties.^{6,12} The occurrence of bone destruction will stimulate bone healing process initiated with inflammatory process. Inflammatory process induces the increase of osteoclast activity for bone resorption. By diminishing inflammatory reaction, the activity of osteoclast will decrease thus accelerating new bone formation.¹³ Hydroxyapatite material has no activity as anti-inflammation so that anti-inflammatory effect in bone graft material may be furnished by the addition of ellagic acid (EA). EA is a compound mostly found in pomegranate together with other polyphenols such as gallotannin, anthocyanin and others.¹⁴ A number of researchers revealed that phenolic compound is the major bioactive substance required for bone health. Ellagic acid has been proven to possess anti-inflammatory, antioxidant, free radical scavenging, antiapoptotic, antimutagenic, and antiviral effect in addition to antifibrotic activity. Further to previous study, EA is also confirmed to accelerate bone healing process in rats after tooth extraction.^{15,13,16,17}

EA combination with hydroxyapatite is anticipated to enhance anti-inflammatory activity which may reduce bone resorption and accelerate new bone remodeling. Combination of this material should be identified to be safe

with no disadvantageous effect for tissues in human body.^{18,19} To corroborate this aspect, cytotoxic screening should be performed using BHK-21 fibroblast cell culture originated from hamster kidney. BHK-21 fibroblast cell is used as it is the common cell lines deployed for investigating drug and material toxicity in dental field.²⁰ Toxicological screening in this study exerts *methylthiazol tetrazolium* (MTT) assay method. This method is reported as a valid screening with rapid process. The result of MTT assay will display purplish color in cell culture well after given the reagent. This result is measured by observing the absorbance using spectrophotometer to obtain OD (optical density) value. The value of OD is equivalent to the number of living cell in cell culture well.^{23,24} Toxicological screening of hydroxyapatite combination with EA on BHK-21 fibroblast cell has yet been conducted. Henceforth, a study about the toxicity of hydroxyapatite combination with EA upon fibroblast cell is needed.

MATERIALS AND METHODS

This research had been approved by Ethics Committee Faculty of Dentistry Universitas Lambung Mangkurat No. 190/KEPKH-FKGULM/EC/V/2019. This was a true experimental study with post-test only and control group design. Samples were selected randomly and divided into 11 groups comprised of 8 treatment groups and 2 control groups. Eight treatment groups comprised of hydroxyapatite combination with EA at 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4% and 5% concentration while two control groups comprised of positive control using hydroxyapatite, cell control and media control. Replication was performed five times per group based on Federer formula. Population in this study was BHK-21 fibroblast cell obtained from LPPT Gajah Mada University.

Bone graft material applied in this study was synthetic hydroxyapatite from *Batan*. Ellagic Acid (EA) used was a pure EA (96%) from pomegranate fruit of *Xi'an Biof Bio-Technology Co., Ltd. China*. EA and hydroxyapatite (HA) were combined based on weight ratio of each material using digital

analytic balance. Combination of material was executed until eight compositions were obtained: (i) 99.9% HA + 0.1%, (ii) 99.8% HA + 0.2% EA, (iii) 99.5% HA + 0.5% EA, (iv) 99% HA + 1% EA, (v) 98% HA + 2% EA, (vi) 97% HA + 3% EA, (vii) 96% HA + 4% EA and (viii) 95% HA + 5% EA.

Fibroblast culture cell in the form of cell line was planted in a bottle. After confluent, culture was collected using *trypsin versene*. The result of collection was taken a little and replanted in eagle media containing 10% bovine serum albumin and incubated for 24 hours. Soon the cell was transferred to a small bottle at 2×10^5 cell/ml solidity; cell was prepared for sample testing. 96-well cell culture plates were deployed for cell viability testing on acrylic resin. The testing was performed based on standardized protocol for MTT assay. In each well, 200 μ l media containing cell at 2×10^5 cell/ml solidity was inserted. Prior to the testing, cell was sterilized using ultraviolet for 15 minutes and subsequently inserted in well plate. Well plate was later incubated for 20 hours at 37°C. Following the incubation, each well was inserted with 20 μ l MTT solved in PBS to be incubated further for 5 hours at 37°C temperature. The sample was collected from each well and each well was added with 200 μ l DMSO using vertical up-down pipetting motion to dissolve formulated crystalline. Well plates were incubated for 5 minutes at 37°C. Further, well plate was measured using spectrophotometer at 620 nm wavelength. The result was obtained in the form of optical density (absorbent). The value of absorbent from each well represents the number of cell viability in media culture.²⁵ Measurement of cell viability was performed using Optical Density formula as follows:^{23,24}

$$\text{Cell viability (\%)} = \frac{\text{OD treatment} - \text{OD media control}}{\text{OD cell control} - \text{OD media control}} \times 100\%$$

Notes:

Cell viability (%): Percentage of living cell after treatment

OD treatment : Optical Density of fibroblast cell in each sample

OD media control : Optical Density of fibroblast cell in media control

OD cell control : Optical Density of cell control without any additional treatment

A material is categorized as non-toxic when the number of living cell or cell viability results greater than 60%.

RESULTS

The result of MTT assay for the toxicological screening of hydroxyapatite combination with EA demonstrates the presence of purplish color in cell culture. Deeper colour demonstrates higher viability of fibroblast cell in the well. Dark purplish colour was observed in positive control group treated with hydroxyapatite only while well-distributed purplish color was observed in all treatment groups.

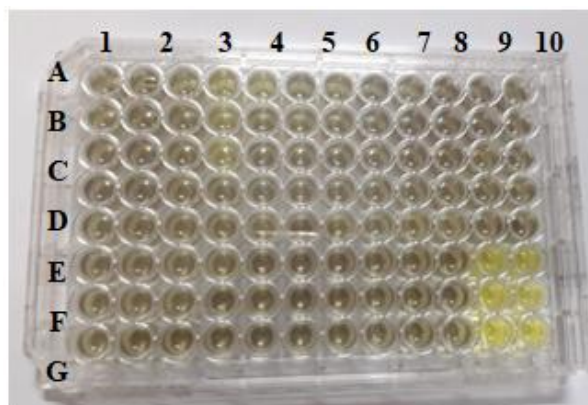


Figure 1. a. Material with 0.1% EA concentration (61, 6B, 6C, 6D, 6E); b. Material with 0.2% EA concentration (6F, 6G, 6H, 7A, 7B); c. Material with 0.5% EA concentration (7C, 7D, 7E, 7F, 7G); d. Material with 1% EA concentration (7H, 8A, 8B, 8C, 8D); e. Material with 2% EA concentration (8E, 8F, 8G, 8H, 9A); f. Material with 3% EA concentration (9B, 9C, 9D, 9E, 9F); g. Material with 4% EA concentration (9G, 9H, 10A, 10B, 10C); h. Material with 5% EA concentration (10D, 10E, 10F, 10G, 10H); i. Hydroxyapatite as positive control (11A, 11B, 11C, 11D, 11E); j.

Fibroblast as cell control (12A, 12B, 12C, 12D, 12E); k. Media control (11F, 11G, 11H, 12F, 12G, 12H)

Table 1. Viability of BHK-21 fibroblast cell after treatment (%)

| Concentration | Fibroblast cell viability (%) |
|-------------------|-------------------------------|
| Ellagic Acid 0,1% | 88,21 |
| Ellagic Acid 0,2% | 92,33 |
| Ellagic Acid 0,5% | 97,58 |
| Ellagic Acid 1% | 89,56 |
| Ellagic Acid 2% | 84,24 |
| Ellagic Acid 3% | 90,77 |
| Ellagic Acid 4% | 88,92 |
| Ellagic Acid 5% | 89,42 |

Cell viability measurement in table 1 reveals that OD value in all treatment groups were greater than 60%. This may be clarified that all treatment groups exhibit no toxicity.

DISCUSSION

Toxicological screening for bone graft material combination of hydroxyapatite and ellagic acid from pomegranate fruit resulted in non-toxic effect upon fibroblast cell culture after treated with ellagic acid at 0.1%, 0.2%, 0.3%, 0.5%, 1%, 2%, 3%, 4% and 5% concentration. It is based on cell viability value which was scored more than 60% in all groups. The highest viability was observed in 0.5% ellagic acid concentration group with 97.59% OD value while the lowest viability was prevailed in 2% ellagic acid concentration group with 84.24% OD value. This result is in accordance with previous study mentioning that 40% standardized pomegranate fruit extract possessed ideal concentration between 2.5% to 7.5% while topical application of pure ellagic acid (98-100%) on wound healing of rats showed the best result at 1% concentration. Ellagic acid at 1-3% concentration in several studies also demonstrates antitumor, anti-inflammatory and anticancer activity on lung, prostate and brain cancer cells.^{16,26-28}

Previous study claimed that EA exposure on fibroblast and osteoblast cell at viability testing may increase the proliferation of osteoblast and fibroblast cell *in vitro*.²⁹ Other studies also revealed non-toxic property of EA towards RAW264.7 cells which is a progenitor for bone and fibroblast formation.^{30,31} In this study, cell viability was less than 100% showing the presence of cell death that yet to be classified as toxic. This occurs as the result of cytotoxic effect from EA material as it initiates changes in the permeability of fibroblast cell membrane.¹⁹ Damage on cell membrane may cause the cell to be viable and develop into cell death. In non-viable cell, membrane cell shall be penetrated by *trypsine blue* that is hardly occurs in viable cell. The higher the number of non-viable cell, the higher the percentage of cell death. Percent cell death is demonstrated by formazan optical density value where higher formazan optical density value indicates higher number of living cell. This means that BHK-21 fibroblast cell is able to maintain membrane integrity so that cell death may not occur.²³ Percent of cell death in this study is classified in low category inferring that EA is non-toxic towards fibroblast cell.

Low percentage of cell death in this study is generated by EA content which may stimulate the proliferation of fibroblast cell. Ellagic acid contains polyphenols that are largely composed by punicalagin. Punicalagin phenolic compound is reported to improve non-specific immunity through cell activity that regulates immune response. This phenolic compound is also proven to stimulate pulp cell proliferation and accelerate wound healing process.^{19,32} Punicalagin also act as pro-oxidant that increase oxidative stress activity thus initiating apoptosis in carcinoma cell whilst excluding normal cell.^{26,27} Ellagitanin compound at particular level may operate as antioxidant, antibacterial and anti-inflammatory agent. This compound is evinced to be safe for normal cell in limited amount of dosage and intensify wound healing process.^{14,33} From this study, it may be concluded that no toxicity was observed from ellagic acid in pomegranate fruit at 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4% and 5% concentration

with hydroxyapatite combination towards BHK-21 fibroblast cells.

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