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Biocompatibility of *Portunus pelagicus* Hydroxyapatite Graft on Human Gingival Fibroblast Cell Culture

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Abstract

Introduction: Crab shell (*Portunus pelagicus*) has the potential to be a source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast. Objective: This study aimed to determine the biocompatibility of *Portunus pelagicus* hydroxyapatite graft on human gingival fibroblast cell culture. Methods: Human gingival fibroblast cell cultures were divided into control group and treatment group with the addition of hydroxyapatite graft powder from *Portunus pelagicus* at a concentration of 100 ppm, 50 ppm, and 25 ppm. The synthesis process of hydroxyapatite was conducted by heating at 1000°C then characterizing the compound with SEM-EDX. All samples were incubated in α-MEM medium, then were given MTT material. The cultures on the plate were examined using ELISA reader. The results were analyzed using a Oneway Anova. Results: The percentage of living cells throughout all treatment group shown results that exceeded the LD50 parameter. The highest percentage of living cells was at 25 ppm concentration group. Conclusion: The hydroxyapatite graft powder from crab shells is biocompatible with human gingival fibroblast cell culture.

Keywords

Hydroxyapatite, Graft, Biomaterials, Fibroblasts.

1. Introduction

The development of science and technology is not guaranteed the tooth resistance against disease or trauma completely, so there are still many cases of tooth loss. When tooth loss occurs, bone volume will decrease. Bone resorption due to tooth extraction may cause a problem in prosthodontics because it may lead to poor results of long-term treatment (1). Within 6 months, bone resorption occurs as much as 1.5-2 mm in vertical direction and 40%-50% in horizontal direction, and most cases occur in the first three months. If the treatment is not taken, the bone resorption can reach 40-60% of bone ridge volume in the first three years (2).

At present-day, there are various types of materials and techniques used for bone resorption treatment, including bone graft material, usage of guided tissue regeneration, application of growth factors to stimulate bone regeneration (3). Bone graft is used as scaffolds, matrix attachment and proliferation osteoblast (4). Bone graft material must have a biocompatibility property with living tissue, profitable for osteoconduction, osteoinduction and has ability to support new bone formation. Graft biocompatibility is very crucial in order to prevent rejection by the host and not toxic to the body (5, 6).

The most ideal bone graft to use is an autograft that derived from the patient's own body. However, sometimes the process is not able to support, so that autograft is developed to allograft material. Yet, allografts often transmit infectious diseases, especially HIV and for that reason allograft is developed to xenograft material. The most commonly used xenograft is from bovine, but transmission of bovine spongiform encephalitis (BSE) is commonly occur. Various type of synthetic bone grafts was developed to minimize the risk of transmitting disease. Ideal conditions that need to be fulfilled by synthetic bone grafts are biocompatible, profitable for osteoconduction, osteoinduction and osteogenesis. Those three mechanisms are the most important for resorbable biomaterials that support tissue growth (6). However, alloplast particles cannot be counted on the site at the location of the embedded graft so that bone formation cannot be determined (7).

One type of bone graft, namely hydroxyapatite graft which can be produced from coral reef skeletons, bovine bones, chicken claws, shellfish shells and human cancellous bones (8). In Indonesia, natural raw materials, especially those derived from marine biota are easily found, relatively low-cost and the production process is simple. Among these raw materials are crab shells (*Portunus pelagicus*) which are uncommonly used, even turned into waste (9).

A crab shell contains large amounts of calcium carbonate, about 40% - 70% (9). Calcium carbonate is source of calcium that can be used as synthesis material of hydroxyapatite. Apatite crystals contain many carbon groups in carbonate shapes. Hydroxyapatite is a stable apatite crystal and implanted as bone replacement or filler. Hydroxyapatite biomaterials consist of bioactive components that are compatible with bones and teeth. This supports the use of crab shells as bone replacement alternative biomaterial based on the chemical composition. In addition, the development of crab shells as hydroxyapatite graft has not been carried out further and still requires biocompatibility test to be used on a wide scale (10).

In performing in vitro test of a new materials, fibroblasts are used. Fibroblasts are connective tissue cells that function as defense because they are able to differentiate as odontoblast and osteoblast cells and produce collagen fibers in healing process. The ability to grow fast in tissues and be able to live solitary is the reason why fibroblast cells are easily cultured into cell subjects (11). The biocompatibility test of hydroxyapatite graft produced from crab shells on human gingival fibroblast (HGF) cell cultures is required to determine whether the graft is ideal to be applied to the bone.

2. Materials and Methods

This is an experimental laboratory research with post-test only control group design. The treatment was carried out by giving hydroxyapatite graft powder from crab shell (Portunus pelagicus) on HGF cell culture. This study used 5 samples for each treatment group, those include the addition of hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm as well as cell control group and media control of 5 samples respectively. The sampling technique used is simple random sampling.

Processing of crab shells (Portunus pelagicus) for the synthesis of hydroxyapatite graft conducted at the Center for Biomaterials of Dr. Soetomo General National Hospital Surabaya. It was began by the washing process of crab shells using distilled water, chlorine and H_2O_2 then continued with the heating process of crab shells using furnace at 1000°C for 2 hours. Powder sifting process was performed with sifting machine until the particle size was less than 155μm and continued with the characterization of hydroxyapatite compounds using SEM-EDX.

The in vitro study was conducted at Stem Cell Research Development Center of Universitas Airlangga Surabaya. The isolation process of Human Gingival Fibroblast (HGF) cells was conducted from healthy gingival samples patients aged less than 30 years old. HGF cells are processed by splitting process which aim to multiply cells and replace α-MEM media. Before the treatment phase, cell preparations are processed by doing washing for several times using trypsin enzymes and α-MEM. Cells were divided into 96-well microplate as much as 100 µl with density 3-5x10^3 and incubated for 24 hours at 37°C.

Hydroxyapatite graft powder was diluted with α-MEM media according to the concentration dose, at 100 ppm, 50 ppm and 25 ppm. Each of 5 samples were dripped by hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm. Incubation was carried out for 24 hours then microscopic observation was performed to observe whether cytotoxic effects had occurred. 25µl of MTT was dripped, incubated, then dripped with 10µl of DMSO. The results were analyzed with ELISA reader with wavelength of 620 nm and viability of fibroblast living cells were calculated. The data obtained were tested by Oneway Anova and *Post-hoc Tukey HSD*.

3. Results

The results of SEM micrograph was shown on Figure 1. It showed fineness and homogenity of hydroxyapatite structure. The analysis of the element characerization of the hidroxyapatite graft, the EDX test was conducted and the result shown in Figure 2. The analysis then described in table 1 and showed that the dominant atom in hydroxiapatite graft derived from *Portunus pelagicus* was O followed by Ca and P.

Optical density (OD) value of formazan hydroxyapatite graft powder from Portunus pelagicus was measured using spectrophotometer. The mean value of formazan optical density in 5 samples for each treatment group and percentage of living cells can be seen in Table 2. From the result of MTT assay, it indicated that the treatment at concentrations of 100 ppm and 50 ppm is capable to reduce the number of living cells to a certain extent. The results will be analyzed using the LD_{50} parameter to assess the biocompatibility of hydroxyapatite graft powder from crab shells from the entire concentration group.

Kolmogorov-Smirnov test results showed that all groups have a normality probability value greater than 0.05 ($p > 0.05$) which means that the data was normally distributed. Data analysis was continued by testing the sample variance homogeneity using Levene's test. The homogeneity test results indicated the significance value of 0.987 which means the data was homogeneous ($p > 0.05$). Data was normally distributed and homogeneous so the data needs to be tested using the Oneway Anova test with Post-Hoc Tukey HSD. Oneway Anova test was conducted to find out the overall difference in the sample group. From the test results shown the value of sig. = 0.752 which means there is no significant difference in the whole group because it had not met the sig requirements ($p < 0.05$).

4. Discussion

Bone graft is a material for bone resorption treatment. One type of bone graft is hydroxyapatite graft, which can be produced from marine biota (8). Crab shell has potential to be source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast (HGF) cell cultures which grown on the media. HGF cells can grow easily and have high sensitivity compared to other fibroblast cells. HGF cell cultures was selected because it derived from humans therefore the results are expected to represent material toxicity to human cells (12).

Previous studies by Raya et al. (2015) suggest that CaCO3 content inside shell material is on average 40-70% and varies depending on the species. From these data, it can be proved that the crab shell (*Portunus pelagicus*) is a good source for hydroxyapatite because of high Ca content more than 50%. According to Kartono et al. (2014), apatite crystals contain many carbon groups in carbonate shape (13).

Generally, hydroxyapatite is synthesized in powder shape, thus when observed visually the sample shape is appeared composed of fine granules. With observations using SEM (Scanning electron microscopy), it can be observed the sample morphology and the granules appeared more prominent in shape. The powder particles of sample elements are not completely rounded, but the particles shape varies and resembles the shape crystal. In addition, observations showed that the hydroxyapatite structure had a smooth surface and low porosity (9). The composition and distance of particle as element of the powder sample are quite regular. Moreover, hydroxyapatite size tends to be small. This was obtained from sifting process so that the powder is less than 155 μm.

Furthermore, the results of EDX analysis obtained hydroxyapatite element mapping which showed the atom distribution of three main elements. The composition and mapping confirm the composition of hydroxyapatite element so that it can be concluded that the synthesis results are achieved correspond to the target.

The percentage of living cells above 50% means that hydroxyapatite graft powder has optimal biocompatibility to living tissue. In this case, the material had passed in vitro test before going through the next test, in vivo test and even clinical application tests in humans.The high number of fibroblast cells caused by optimal conditions that inducing work rather than several factors that affect fibroblast cell proliferation, namely Platelet Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Transforming Growth Factor (TGF-β) is associated and influences each other (14). These factors will induce fibroblast cells to migrate, proliferate, and differentiate.

The high number of fibroblast cells can be caused by the high ability of epidermal growth receptor (EGFR) and expression of α5β1 integrin in fibroblast cells, especially human periodontal fibroblasts. Hydroxyapatite plays a role in stimulating fibroblast cell proliferation because it is associated with activation of the epidermal growth factor receptor (EGFR). The active receptor will be phosphorylated in tyrosine 1173 so that an increase in Akt pathway is a signal transduction pathway. Akt pathway activation promotes cell growth and proliferation in response to extracellular signals (20). Increased expression of α5β1 integrin also occured in fibroblast cells associated with EGFR. Increased expression of α5β1 integrin caused increasing attachment of fibroblast cells to hydroxyapatite which activates EGFR and increases cell proliferation (15).

From the results of this study it can be concluded that hydroxyapatite graft powder from *Portunus pelagicus* has biocompatible properties on HGF cell culture and at the lowest concentration of 25 ppm has optimal biocompatibility compared to the other two concentrations.

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Authors contribution: all author was substantial contribution to concept, design, acquisition, analysis and interpretation data and drafting the article.

Conflict of interest not declared.

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ABSTRACT

Introduction: Crab shell (*Portunus pelagicus*) has the potential to be a source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast. **Aim**: This study aimed to determine the biocompatibility of *Portunus pelagicus* hydroxyapatite graft on human gingival fibroblast cell culture. **Material and Methods**: Human gingival fibroblast cell cultures were divided into control group and treatment group with the addition of hydroxyapatite graft powder from *Portunus pelagicus* at a concentration of 100 ppm, 50 ppm, and 25 ppm. The synthesis process of hydroxyapatite was conducted by heating at 1000°C then characterizing the compound with SEM-EDX. All samples were incubated in α-MEM medium, then were given MTT material. The cultures on the plate were examined using ELISA reader. The results were analyzed using a Oneway Anova. **Results**: The percentage of living cells throughout all treatment group shown results that exceeded the LD50 parameter. The highest percentage of living cells was at 25 ppm concentration group. **Conclusion**: The hydroxyapatite graft powder from crab shells is biocompatible with human gingival fibroblast cell culture.

Keywords: Hydroxyapatites, Graft, Fibroblasts.

1. INTRODUCTION

The development of science and technology is not guaranteed the tooth resistance against disease or trauma completely, so there are still many cases of tooth loss. When tooth loss occurs, bone volume will decrease. Bone resorption due to tooth extraction may cause a problem in prosthodontics because it may lead to poor results of long-term treatment (1). Within 6 months, bone resorption occurs as much as 1.5-2 mm in vertical direction and 40%-50% in horizontal direction, and most cases occur in the first three months. If the treatment is not taken, the bone resorption can reach 40-60% of bone ridge volume in the first three years (2).

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The most ideal bone graft to use is an autograft that derived from the patient's own body. However, sometimes the process is not able to support, so that autograft is developed to allograft material. Yet, allografts often transmit infectious diseases, especially HIV and for that reason allograft is developed to xenograft material. The most commonly used xenograft is from bovine, but transmission of bovine spongiform encephalitis (BSE) is commonly occur. Various type of synthetic bone grafts was developed to minimize the risk of transmitting disease. Ideal conditions that need to be fulfilled by synthetic bone grafts are biocompatible, profitable for osteoconduction, osteoinduction and osteogenesis. Those three mechanisms are the most important for resorbable biomaterials that support tissue growth (6). However, alloplast particles cannot be counted on the site at the location of the embedded graft so that bone formation cannot be determined (7).

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2. AIM

In the present study, we determine the biocompatibility of Portunus pelagicus hydroxyapatite graft on human gingival fibroblast cell culture.

3. MATERIAL AND METHODS

This is an experimental laboratory research with post-test only control group design. The treatment was carried out by giving hydroxyapatite graft powder from crab shell (Portunus pelagicus) on HGF cell culture. This study used 5 samples for each treatment group, those include the addition of hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm as well as cell control group and media control of 5 samples respectively. The sampling technique used is simple random sampling.

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4. RESULTS

The results of SEM micrograph was shown on Figure 1. It showed fineness and homogenity of hydroxyapatite structure. The analysis of the element characerization of the hidroxyapatite graft, the EDX test was conducted and the result shown in Figure 2. The analysis then described in Table 1 and showed that the dominant atom in hydroxiapatite graft derived from *Portunus pelagicus* was O followed by Ca and P.

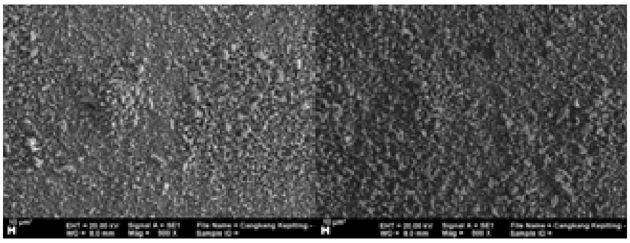

Figure 1. SEM micrograph of hydroxyapatite crab shell samples at 500x magnification from two different fields of view.

Optical density (OD) value of formazan hydroxyapatite graft powder from Portunus pelagicus was measured using spectrophotometer. The mean value of formazan optical density in 5 samples for each treatment group and percentage of living cells can be seen in Table 2. From the result of MTT assay, it indicated that the treatment at concentrations of 100 ppm and 50 ppm is capable to reduce the number of living cells to a certain extent. The results will be analyzed using the LD_{50} parameter to assess the biocompatibility of hydroxyapatite graft powder from crab shells from the entire concentration group.

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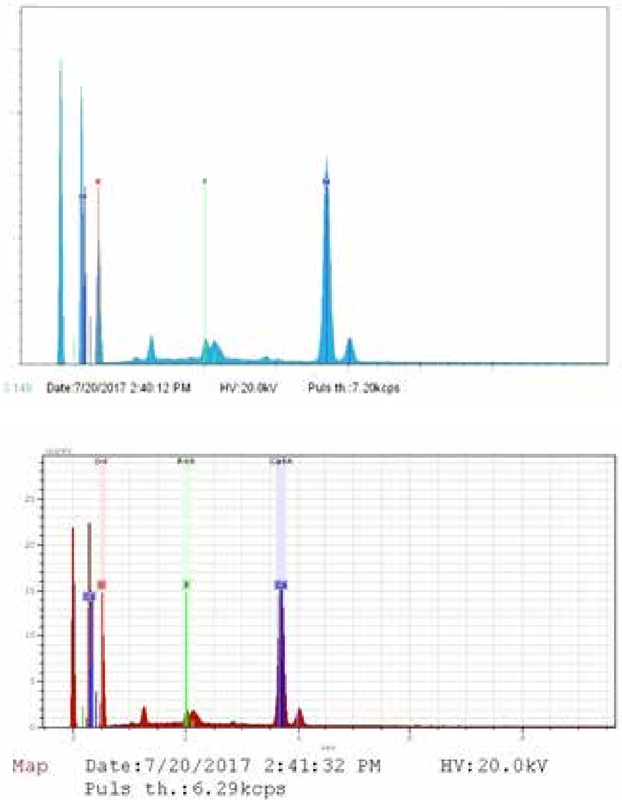

Figure 2. EDX spectrum of hydroxyapatite samples from Portunus pelagicus with three main elements, O, Ca, and P.

Figure 3. Microscopic images of fibroblast cells after hydroxyapatite graft powder exposure at concentration; A) 100 ppm, B) 50 ppm, and C) 25 ppm with 20x magnification.

sample variance homogeneity using Levene's test. The homogeneity test results indicated the significance value of 0.987 which means the data was homogeneous ($p > 0.05$). Data was normally distributed and homogeneous so the data needs to be tested using the Oneway Anova test with Post-Hoc Tukey HSD. Oneway Anova test was conducted to find out the overall difference in the sample group. From the test results shown the value of sig. = 0.752 which means there is no significant difference in the whole group because it had not met the sig requirements ($p < 0.05$).

5. DISCUSSION

Bone graft is a material for bone resorption treatment. One type of bone graft is hydroxyapatite graft, which can be produced from marine biota (8). Crab shell has potential to be source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast (HGF) cell cultures which grown on the media. HGF cells can grow easily and have high sensitivity compared to other fibroblast cells. HGF cell

Element	Normalized Weight Calculation (wt.%)	Atom Calculation (at.%)	Standard deviation
O	67,59	83,54	6,9
Ca	29,16	14,39	0,7
P	3,25	2,08	0,1

Table 1. Calculation of normalized weight and calculation of atoms in Hydroxyapatite Graft derived from Portunus pelagicus.

Treatment Group	N	Optical Density Mean	Standard Deviation	Percentage of Living Cells
Hydroxyapatite graft 100ppm	5	0,0684	0,017785	89,8%
Hydroxyapatite graft 50ppm	5	0,0724	0,018366	93,27%
Hydroxyapatite graft 25ppm	5	0,0816	0,018366	101,2%
Cell Control	5	0,0802	0,014704	100%
Media Control	5	0,0358	0,000837	0%

Table 2. The mean value of optical density and the percentage of living cells for each treatment.

cultures was selected because it derived from humans therefore the results are expected to represent material toxicity to human cells (12).

Previous studies by Raya et al. (2015) suggest that $CaCO_3$ content inside shell material is on average 40-70% and varies depending on the species. From these data, it can be proved that the crab shell (*Portunus pelagicus*) is a good source for hydroxyapatite because of high Ca content more than 50%. According to Kartono et al. (2014), apatite crystals contain many carbon groups in carbonate shape (13).

Generally, hydroxyapatite is synthesized in powder shape, thus when observed visually the sample shape is appeared composed of fine granules. With observations using SEM (Scanning electron microscopy), it can be observed the sample morphology and the granules appeared more prominent in shape. The powder particles of sample elements are not completely rounded, but the particles shape varies and resembles the shape crystal. In addition, observations showed that the hydroxyapatite structure had a smooth surface and low porosity (9). The composition and distance of particle as element of the powder sample are quite regular. Moreover, hydroxyapatite size tends to be small. This was obtained from sifting process so that the powder is less than 155 µm.

Furthermore, the results of EDX analysis obtained hydroxyapatite element mapping which showed the atom distribution of three main elements. The composition and mapping confirm the composition of hydroxyapatite element so that it can be concluded that the synthesis results are achieved correspond to the target.

The percentage of living cells above 50% means that hydroxyapatite graft powder has optimal biocompatibility to living tissue. In this case, the material had passed in vitro test before going through the next test, in vivo test and even clinical application tests in humans.The high number of fibroblast cells caused by optimal conditions that inducing work rather than several factors that affect fibroblast cell proliferation, namely Platelet Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Transforming Growth Factor (TGF-β) is associated and influences each other (14). These factors will induce fibroblast cells to migrate, proliferate, and differentiate.

The high number of fibroblast cells can be caused by the high ability of epidermal growth receptor (EGFR) and expression of α5β1 integrin in fibroblast cells, especially human periodontal fibroblasts. Hydroxyapatite plays a role in stimulating fibroblast cell proliferation because it is associated with activation of the epidermal growth factor receptor (EGFR). The active receptor will be phosphorylated in tyrosine 1173 so that an increase in Akt pathway is a signal transduction pathway. Akt pathway activation promotes cell growth and proliferation in response to extracellular signals (20). Increased expression of α5β1 integrin also occured in fibroblast cells associated with EGFR. Increased expression of α5β1 integrin caused increasing attachment of fibroblast cells to hydroxyapatite which activates EGFR and increases cell proliferation (15).

6. CONCLUSION

From the results of this study it can be concluded that hydroxyapatite graft powder from *Portunus pelagicus* has biocompatible properties on HGF cell culture and at the lowest concentration of 25 ppm has optimal biocompatibility compared to the other two concentrations.

- **Declaration of patient consent**: The authors certify that they have obtained all appropriate patient consent forms
- **Authors contribution**: Each author gave substantial contribution to the conception or design of the work and in the acquisition, analysis and interpretation of data for the work. Each author had role in drafting the work and revising it critically for important intellectual content. Each author gave final approval of the version to be published and they agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.
- **Conflict of interest**: There are no conflicts of interest.
- **Financial support and sponsorship**: Nil

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Author's contribution: FA, PT, MT gave a substantial contribution to the conception and design of the work. FA, NG gave a substantial contribution of data. FA, MT, FT, PT gave a substantial contribution to the acquisition, analysis, or interpretation of data for the work. FA, VA, SZ, FT had a part in article preparing for drafting or revising it critically for important intellectual content. All authors gave final approval of the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Biocompatibility of Portunus Pelagicus Hydroxyapatite Graft on Human Gingival Fibroblast Cell Culture

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ABSTRACT

Introduction: Crab shell (Portunus pelagicus) has the potential to be a source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast. **Aim:** This study aimed to determine the biocompatibility of Portunus pelagicus hydroxyapatite graft on human gingival fibroblast cell culture. **Material and Methods:** Human gingival fibroblast cell cultures were divided into control group and treatment group with the addition of hydroxyapatite graft powder from Portunus pelagicus at a concentration of 100 ppm, 50 ppm, and 25 ppm. The synthesis process of hydroxyapatite was conducted by heating at 1000°C then characterizing the compound with SEM-EDX. All samples were incubated in α-MEM medium, then were given MTT material. The cultures on the plate were examined using ELISA reader. The results were analyzed using a Oneway Anova. **Results:** The percentage of living cells throughout all treatment group shown results that exceeded the LD50 parameter. The highest percentage of living cells was at 25 ppm concentration group. **Conclusion:** The hydroxyapatite graft powder from crab shells is biocompatible with human gingival fibroblast cell culture.

Keywords: Hydroxyapatites, Graft, Fibroblasts.

1. INTRODUCTION

The development of science and technology is not guaranteed the tooth resistance against disease or trauma completely, so there are still many cases of tooth loss. When tooth loss occurs, bone volume will decrease. Bone resorption due to tooth extraction may cause a problem in prosthodontics because it may lead to poor results of long-term treatment (1). Within 6 months, bone resorption occurs as much as 1.5-2 mm in vertical direction and 40%-50% in horizontal direction, and most cases occur in the first three months. If the treatment is not taken, the bone resorption can reach 40-60% of bone ridge volume in the first three years (2).

At present-day, there are various types of materials and techniques used for bone resorption treatment, including bone graft material, usage of guided tissue regeneration, application of growth factors to stimulate bone regeneration (3). Bone graft is used as scaffolds, matrix attachment and proliferation osteoblast (4). Bone graft material must have a biocompatibility property with living tissue, profitable for osteoconduction, osteoinduction and has ability to support new bone formation. Graft biocompatibility is very crucial in order to prevent rejection by the host and not toxic to the body (5, 6).

The most ideal bone graft to use is an autograft that derived from the patient's own body. However, sometimes the process is not able to support, so that autograft is developed to allograft material. Yet, allografts often transmit infectious diseases, especially HIV and for that reason allograft is developed to xenograft material. The most commonly used xenograft is from bovine, but transmission of bovine spongiform encephalitis (BSE) is commonly occur. Various type of synthetic bone grafts was developed to minimize the risk of transmitting disease. Ideal conditions that need to be fulfilled by synthetic bone grafts are biocompatible, profitable for osteoconduction, osteoinduction and osteogenesis. Those three mechanisms are the most important for resorbable biomaterials that support tissue growth (6). However, alloplast particles cannot be counted on the site at the location of the embedded graft so that bone formation cannot be determined (7).

One type of bone graft, namely hydroxyapatite graft which can be produced from coral reef skeletons, bovine bones, chicken claws, shellfish shells and human cancellous bones (8). In Indonesia, natural raw materials, especially those derived from marine biota are easily found, relatively low-cost and the production process is simple. Among these raw materials are crab shells (Portunus pelagicus) which are uncommonly used, even turned into waste (9).

A crab shell contains large amounts of calcium carbonate, about 40% - 70% (9). Calcium carbonate is source of calcium that can be used as synthesis material of hydroxyapatite. Apatite crystals contain many carbon groups in carbonate shapes. Hydroxyapatite is a stable apatite crystal and implanted as bone replacement or filler. Hydroxyapatite biomaterials consist of bioactive components that are compatible with bones and teeth. This supports the use of crab shells as bone replacement alternative biomaterial based on the chemical composition. In addition, the development of crab shells as hydroxyapatite graft has not been carried out further and still requires biocompatibility test to be used on a wide scale (10).

In performing in vitro test of a new materials, fibroblasts are used. Fibroblasts are connective tissue cells that function as defense because they are able to differentiate as odontoblast and osteoblast cells and produce collagen fibers in healing process. The ability to grow fast in tissues and be able to live solitary is the reason why fibroblast cells are easily cultured into cell subjects (11). The biocompatibility test of hydroxyapatite graft produced from crab shells on human gingival fibroblast (HGF) cell cultures is required to determine whether the graft is ideal to be applied to the bone.

2. AIM

In the present study, we determine the biocompatibility of Portunus pelagicus hydroxyapatite graft on human gingival fibroblast cell culture.

3. MATERIAL AND METHOD

This is an experimental laboratory research with post-test only control group design. The treatment was carried out by giving hydroxyapatite graft powder from crab shell (Portunus pelagicus) on HGF cell culture. This study used 5 samples for each treatment group, those include the addition of hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm as well as cell control group and media control of 5 samples respectively. The sampling technique used is simple random sampling.

Processing of crab shells (Portunus pelagicus) for the synthesis of hydroxyapatite graft conducted at the Center for Biomaterials of Dr. Soetomo General National Hospital Surabaya. It was began by the washing process of crab shells using distilled water, chlorine and H2O2 then continued with the heating process of crab shells using furnace at 1000°C for 2 hours. Powder sifting process was performed with sifting machine until the particle size was less than 155μm and continued with the

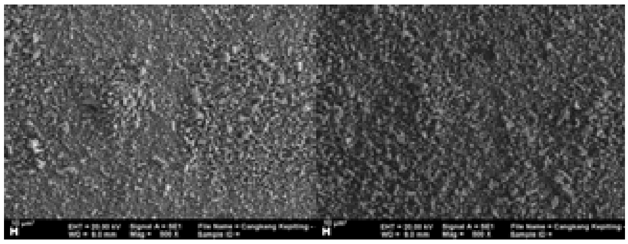

Figure 1. SEM micrograph of hydroxyapatite crab shell samples at 500x magnification from two different fields of view

characterization of hydroxyapatite compounds using SEM-EDX.

The in vitro study was conducted at Stem Cell Research Development Center of Universitas Airlangga Surabaya. The isolation process of Human Gingival Fibroblast (HGF) cells was conducted from healthy gingival samples patients aged less than 30 years old. HGF cells are processed by splitting process which aim to multiply cells and replace α-MEM media. Before the treatment phase, cell preparations are processed by doing washing for several times using trypsin enzymes and α-MEM. Cells were divided into 96-well microplate as much as 100 μl with density 3-5x103 and incubated for 24 hours at 37°C.

Hydroxyapatite graft powder was diluted with α-MEM media according to the concentration dose, at 100 ppm, 50 ppm and 25 ppm. Each of 5 samples were dripped by hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm. Incubation was carried out for 24 hours then microscopic observation was performed to observe whether cytotoxic effects had occurred. 25μl of MTT was dripped, incubated, then dripped with 10μl of DMSO. The results were analyzed with ELISA reader with wavelength of 620 nm and viability of fibroblast living cells were calculated. The data obtained were tested by Oneway Anova and Post-hoc Tukey HSD.

4. RESULTS

The results of SEM micrograph was shown on Figure 1. It showed fineness and homogenity of hydroxyapatite structure. The analysis of the element characerization of the hidroxyapatite graft, the EDX test was conducted and the result shown in Figure 2. The analysis then described in table 1 and showed that the dominant atom in hydroxiapatite graft derived from Portunus pelagicus was O followed by Ca and P.

Optical density (OD) value of formazan hydroxyapatite graft powder from Portunus pelagicus was measured using spectrophotometer. The mean value of formazan optical density in 5 samples for each treatment group and percentage of living cells can be seen in Table 2. From the result of MTT assay, it indicated that the treatment at concentrations of 100 ppm and 50 ppm is capable to reduce the number of living cells to a certain extent. The results will be analyzed using the LD50 parameter to assess the biocompatibility of hydroxyapatite graft powder from crab shells from the entire concentration group.

Kolmogorov-Smirnov test results showed that all groups have a normality probability value greater than 0.05 (p> 0.05) which means that the data was normally distributed. Data analysis was continued by testing the

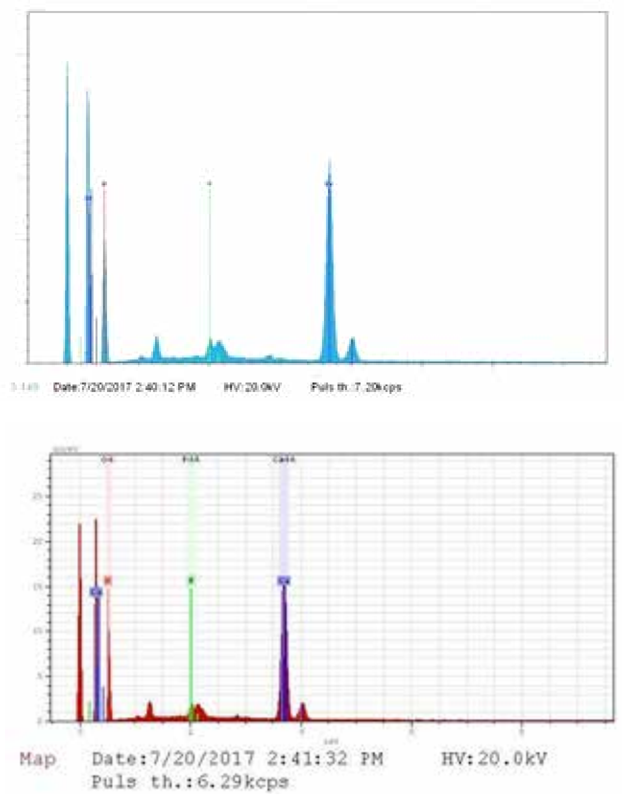

Figure 2. EDX spectrum of hydroxyapatite samples from Portunus pelagicus with three main elements, O, Ca, and P.

Element	Normalized Weight Calculation (wt.%)	Atom Calculation (at.%)	Standard deviation
O	67,59	83,54	6,9
Ca	29,16	14,39	0,7
P	3,25	2,08	0,1

Table 1. Calculation of normalized weight and calculation of atoms in Hydroxyapatite Graft derived from Portunus pelagicus.

Treatment Group	N	Optical Density Mean	Standard Deviation	Percentage of Living Cells
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sample variance homogeneity using Levene's test. The homogeneity test results indicated the significance value of 0.987 which means the data was homogeneous (p > 0.05). Data was normally distributed and homogeneous so the data needs to be tested using the Oneway Anova test with Post-Hoc Tukey HSD. Oneway Anova test was conducted to find out the overall difference in the sample group. From the test results shown the value of sig. = 0.752 which means there is no significant difference in the whole group because it had not met the sig requirements (p <0.05).

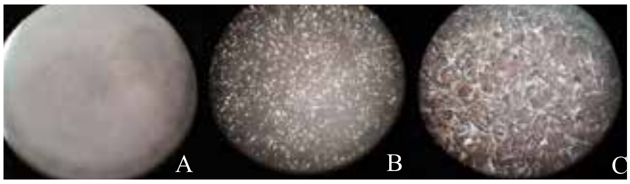

Figure 3. Microscopic images of fibroblast cells after hydroxyapatite graft powder exposure at concentration; A) 100 ppm, B) 50 ppm, and C) 25 ppm with 20x magnification.

5. DISCUSSION

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6. CONCLUSION

From the results of this study it can be concluded that hydroxyapatite graft powder from Portunus pelagicus has biocompatible properties on HGF cell culture and at the lowest concentration of 25 ppm has optimal biocompatibility compared to the other two concentrations.

- **Declaration of patient consent**: The authors certify that they have obtained all appropriate patient consent forms.
- **Authors contribution**: Each author gave substantial contribution to the conception or design of the work and in the acquisition, analysis and interpretation of data for the work. Each author had role in

drafting the work and revising it critically for important intellectual content. Each author gave final approval of the version to be published and they agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Biocompatibility of *Portunus pelagicus* Hydroxyapatite Graft on Human Gingival Fibroblast Cell Culture

Running Title: Hydroxyapatite Graft Biocompatibility Test

ABSTRACT

Introduction: Crab shell (Portunus pelagicus) has the potential to be a source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast. **Objective**: This study aimed to determine the biocompatibility of Portunus pelagicus hydroxyapatite graft on human gingival fibroblast cell culture. **Methods**: Human gingival fibroblast cell cultures were divided into control group and treatment group with the addition of hydroxyapatite graft powder from Portunus pelagicus at a concentration of 100 ppm, 50 ppm, and 25 ppm. The synthesis process of hydroxyapatite was conducted by heating at 1000°C then characterizing the compound with SEM-EDX. All samples were incubated in α-MEM medium, then were given MTT material. The cultures on the plate were examined using ELISA reader. The results were analyzed using a Oneway Anova. **Results**: The percentage of living cells throughout all treatment group shown results that exceeded the LD50 parameter. The highest percentage of living cells was at 25 ppm concentration group. **Conclusion**: The hydroxyapatite graft powder from crab shells is biocompatible with human gingival fibroblast cell culture.

Keywords: Hydroxyapatite, Graft, Biomaterials, Fibroblasts.

BACKGROUND

The development of science and technology is not guaranteed the tooth resistance against disease or trauma completely, so there are still many cases of tooth loss. When tooth loss occurs, bone volume will decrease. Bone resorption due to tooth extraction may cause a problem in prosthodontics because it may lead to poor results of long-term treatment (1). Within 6 months, bone resorption occurs as much as 1.5-2 mm in vertical direction and 40%-50% in horizontal direction, and most cases occur in the first three months. If the treatment is not taken, the bone resorption can reach 40-60% of bone ridge volume in the first three years (2).

At present-day, there are various types of materials and techniques used for bone resorption treatment, including bone graft material, usage of guided tissue regeneration, application of growth factors to stimulate bone regeneration (3). Bone graft is used as scaffolds, matrix attachment and proliferation osteoblast (4). Bone graft material must have a biocompatibility properties with living tissue, profitable for osteoconduction, osteoinduction and has ability to support new bone formation. Graft biocompatibility is very crucial in order to prevent rejection by the host and not toxic to the body (5, 6).

The most ideal bone graft to use is an autograft that derived from the patient's own body. However, sometimes the process is not able to support, so that autograft is developed to allograft material. Yet, allografts often transmit infectious diseases, especially HIV and for that reason allograft is developed to xenograft material. The most commonly used xenograft is from bovine, but transmission of bovine spongiform encephalitis (BSE) is commonly occur. Various type of synthetic bone grafts were developed to minimize the risk of transmitting disease. Ideal conditions that need to be fulfilled by synthetic bone grafts are biocompatible, profitable for osteoconduction, osteoinduction and osteogenesis. Those three mechanisms are the most important for resorbable biomaterials that support tissue growth (6). However, alloplast particles cannot be counted on the site at the location of the embedded graft so that bone formation cannot be determined (7).

One type of bone graft, namely hydroxyapatite graft which can be produced from coral reef skeletons, bovine bones, chicken claws, shellfish shells and human cancellous bones (8). In Indonesia, natural raw materials, especially those derived from marine biota are easily found, relatively low-cost and the production process is simple. Among these raw materials are crab shells (*Portunus pelagicus*) which are uncommonly used, even turned into waste (9).

A crab shell contains large amounts of calcium carbonate, about 40% - 70% (9). Calcium carbonate is source of calcium that can be used as synthesis material of hydroxyapatite. Apatite crystals contain many carbon groups in carbonate shapes. Hydroxyapatite is a stable apatite crystal and implanted as bone replacement or filler. Hydroxyapatite biomaterials consist of bioactive components that are compatible with bones and teeth. This supports the use of crab shells as bone replacement alternative biomaterial based on the chemical composition. In addition, the development of crab shells as hydroxyapatite graft has not been carried out further and still requires biocompatibility test to be used on a wide scale (10).

In performing in vitro test of a new materials, fibroblasts are used. Fibroblasts are connective tissue cells that function as defense because they are able to differentiate as odontoblast and osteoblast cells and produce collagen fibers in healing process. The ability to grow fast in tissues and be able to live solitary is the reason why fibroblast cells are easily cultured into cell subjects (11). The biocompatibility test of hydroxyapatite graft produced from crab shells on human gingival fibroblast (HGF) cell cultures is required to determine whether the graft is ideal to be applied to the bone.

MATERIALS AND METHODS

This is an experimental laboratory research with post-test only control group design. The treatment was carried out by giving hydroxyapatite graft powder from crab shell (Portunus pelagicus) on HGF cell culture. This study used 5 samples for each treatment group, those include the addition of hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm as well as cell control group and media control of 5 samples respectively. The sampling technique used is simple random sampling.

Processing of crab shells (Portunus pelagicus) for the synthesis of hydroxyapatite graft conducted at the Center for Biomaterials of Dr. Soetomo General National Hospital Surabaya. It was began by the washing process of crab shells using distilled water, chlorine and H_2O_2 then continued with the heating process of crab shells using furnace at 1000°C for 2 hours. Powder sifting process was performed with sifting machine until the particle size was less than 155µm and continued with the characterization of hydroxyapatite compounds using SEM-EDX.

The in vitro study was conducted at Stem Cell Research Development Center of Universitas Airlangga Surabaya. The isolation process of Human Gingival Fibroblast (HGF) cells was conducted from healthy gingival samples patients aged less than 30 years old. HGF cells are processed by splitting process which aim to multiply cells and replace α-MEM media. Before the treatment phase, cell preparations are processed by doing washing for several times using trypsin enzymes and α-MEM. Cells were divided into 96-well microplate as much as 100 µl with density 3-5x10^3 and incubated for 24 hours at 37°C.

Hydroxyapatite graft powder was diluted with α-MEM media according to the concentration dose, at 100 ppm, 50 ppm and 25 ppm. Each of 5 samples were dripped by hydroxyapatite graft crab shell at concentration of 100 ppm, 50 ppm and 25 ppm. Incubation was carried out for 24 hours then microscopic observation was performed to observe whether cytotoxic effects had occurred. 25µl of MTT was dripped, incubated, then dripped with 10µl of DMSO. The results were analyzed with ELISA reader with wavelength of 620 nm and viability of fibroblast living cells were calculated. The data obtained were tested by Oneway Anova and *Post-hoc Tukey HSD*.

RESULTS

The results of SEM micrograph was shown on Figure 1. It showed fineness and homogenity of hydroxyapatite structure. The analysis of the element characerization of the hidroxyapatite graft, the EDX test was conducted and the result shown in Figure 2. The analysis then described in table 1 and showed that the dominant atom in hydroxiapatite graft derived from *Portunus pelagicus* was O followed by Ca and P.

Optical density (OD) value of formazan hydroxyapatite graft powder from Portunus pelagicus was measured using spectrophotometer. The mean value of formazan optical density in 5 samples for each treatment group and percentage of living cells can be seen in Table 2. From the result of MTT assay, it indicated that the treatment at concentrations of 100 ppm and 50 ppm is capable to reduce the number of living cells to a certain extent. The results will be analyzed using the LD_{50} parameter to assess the biocompatibility of hydroxyapatite graft powder from crab shells from the entire concentration group.

Kolmogorov-Smirnov test results showed that all groups have a normality probability value greater than 0.05 ($p > 0.05$) which means that the data was normally distributed. Data analysis was continued by testing the sample variance homogeneity using Levene's test. The homogeneity test results indicated the significance value of 0.987 which means the data was homogeneous ($p > 0.05$). Data was normally distributed and homogeneous so the data needs to be tested using the Oneway Anova test with Post-Hoc Tukey HSD. Oneway Anova test was conducted to find out the overall difference in the sample group. From the test results shown the value of sig. = 0.752 which means there is no significant difference in the whole group because it had not met the sig requirements ($p < 0.05$).

DISCUSSION

Bone graft is a material for bone resorption treatment. One type of bone graft is hydroxyapatite graft, which can be produced from marine biota (8). Crab shell has potential to be source of hydroxyapatite biomaterials that used as bone grafts. Before clinical application, crab shell graft should be tested for its biocompatibility in vitro on human gingival fibroblast (HGF) cell cultures which grown on the media. HGF cells can grow easily and have high sensitivity compared to other fibroblast cells. HGF cell cultures was selected because it derived from humans therefore the results are expected to represent material toxicity to human cells (12).

Previous studies by Raya et al. (2015) suggest that CaCO3 content inside shell material is on average 40-70% and varies depending on the species. From these data, it can be proved that the crab shell (Portunus pelagicus) is a good source for hydroxyapatite because of high Ca content more than 50%. According to Kartono et al. (2014), apatite crystals contain many carbon groups in carbonate shape (13).

Generally, hydroxyapatite is synthesized in powder shape, thus when observed visually the sample shape is appeared composed of fine granules. With observations using SEM (Scanning electron microscopy), it can be observed the sample morphology and the granules appeared more prominent in shape. The powder particles of sample elements are not completely rounded, but the particles shape varies and resembles the shape crystal. In addition, observations showed that the hydroxyapatite structure had a smooth surface and low porosity (9). The composition and distance of particle as element of the powder sample are quite regular. Moreover, hydroxyapatite size tends to be small. This was obtained from sifting process so that the powder is less than 155 μm.

Furthermore, the results of EDX analysis obtained hydroxyapatite element mapping which showed the atom distribution of three main elements. The composition and mapping confirm the composition of hydroxyapatite element so that it can be concluded that the synthesis results are achieved correspond to the target.

The percentage of living cells above 50% means that hydroxyapatite graft powder has optimal biocompatibility to living tissue. In this case, the material had passed in vitro test before going through the next test, in vivo test and even clinical application tests in humans.The high number of fibroblast cells caused by optimal conditions that inducing work rather than several factors that affect fibroblast cell proliferation, namely Platelet Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF), and Transforming Growth Factor (TGF-β) is associated and influences each other (14). These factors will induce fibroblast cells to migrate, proliferate, and differentiate.

The high number of fibroblast cells can be caused by the high ability of epidermal growth receptor (EGFR) and expression of α5β1 integrin in fibroblast cells, especially human periodontal fibroblasts. Hydroxyapatite plays a role in stimulating fibroblast cell proliferation because it is associated with activation of the epidermal growth factor receptor (EGFR). The active receptor will be phosphorylated in tyrosine 1173 so that an increase in Akt pathway is a signal transduction pathway. Akt pathway activation promotes cell growth and proliferation in response to extracellular signals (20). Increased expression of α5β1 integrin also occured in fibroblast cells associated with EGFR. Increased expression of α5β1 integrin caused increasing attachment of fibroblast cells to hydroxyapatite which activates EGFR and increases cell proliferation (15).

From the results of this study it can be concluded that hydroxyapatite graft powder from *Portunus pelagicus* has biocompatible properties on HGF cell culture and at the lowest concentration of 25 ppm has optimal biocompatibility compared to the other two concentrations.

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Table and Figure Legend

Table 1. Calculation of normalized weight and calculation of atoms in Hydroxyapatite Graft derived from *Portunus pelagicus*.

Table 2. The mean value of optical density and the percentage of living cells for each group.

Figure 1. Scanning Electron Microscope view of Hydroxyapatite Graft derived from *Portunus pelagicus* at 500x magnification from two different fields of view.

Figure 2. Energy-dispersive X-ray (EDX) spectrum of Hydroxyapatite Graft derived from *Portunus pelagicus*.

Figure 3. Microscopic images of fibroblast cells after Hydroxyapatite Graft derived from *Portunus pelagicus* powder exposure at concentration; A) 100 ppm, B) 50 ppm, and C) 25 ppm with 20x magnification.

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		%	100.0	18.2	31.8	17.1	23.2
	46-55 yrs.	N	0	0	2	8	10
		%	0.0	0.0	9.1	22.9	14.5
	56-65 yrs.	N	0	0	6	14	20
		%	0.0	0.0	27.3	40.0	29.0
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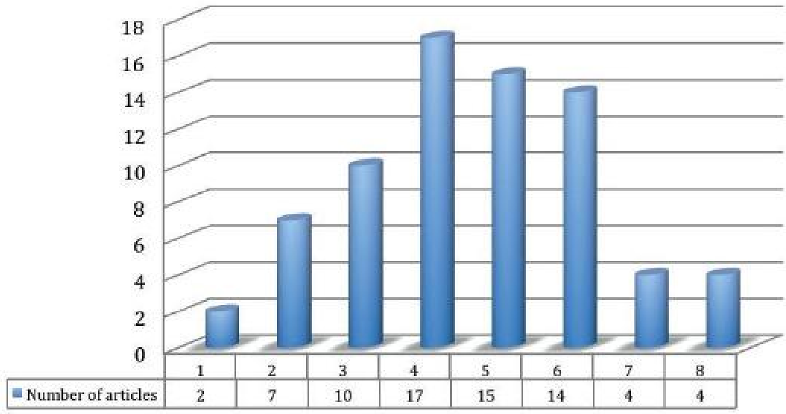

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