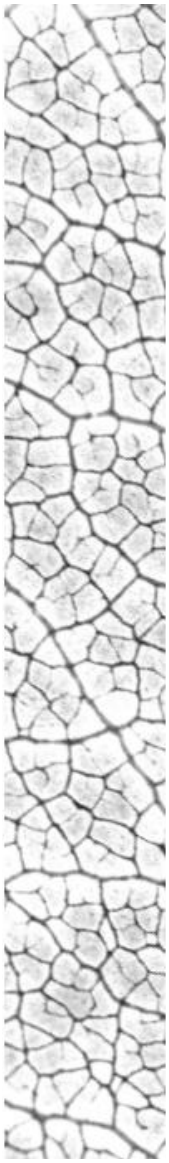

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Collagen fiber increase due to hydroxyapatite from crab shells (*Portunus pelagicus*) application in post tooth extraction in Wistar rats

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Abstract
Tooth extraction will be followed by alveolar bone resorption process. Loss of alveolar bone support will affect retention, stability and comfort in the use of dentures. This can be solved by doing bone graft with hydroxyapatite (HA) material. This study used HA-derived crab shell (Portunus pelagicus) because it contains protein, calcium carbonate, and chitin. A good synthetic bone graft is the structural and composition similar to natural bone. Bone consists of collagen and HA as the main component and a few percent of the other components. The type of collagen that has an important role in bone formation is type I collagen, because this collagen can undergo the process of mineralization. Determine the enhancement of collagen fibers in the post-extraction after being given hydroxyapatite from crab shells on the 14th and 28th days. 36 Wistar rats were divided into 4 groups consisting of control and the treatment group on day 14 and day 28. HA from crab shell was applied to the rats' sockets after extraction of lower left incisor. After the 14th and 28th days, the mandible was cut and HPA microscope slide set were made by staining Masson's Trcihome (MT). The collagen fibers was seen using a binocular light microscope at 400x magnification. The research data were analyzed using Kruskall Wallis and Mann-Whitney test. The collagen fibers increased significantly between the control and treatment groups. Giving hydroxyapatite from crab shells in post extraction Wistar tooth sockets can increase the amount of collagen.

Keywords: hydroxyapatite, crab shells, collagen, Wistar rats

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INTRODUCTION

Tooth extraction is the process of extracting teeth from the socket and alveolar bone. This action is usually carried out as a last option in dental and oral care for various reasons, including for the purpose of orthodontic treatment, wisdom teeth or molar 3 (M3) that do not erupt perfectly, dental caries that cannot be treated, trauma, periodontal disease, supernumerary tooth, root fracture, and ankylotic deciduous teeth (Wray, & Clark, 2003; Hong-juan, et al, 2017).

The act of tooth extraction will be followed by the process of alveolar bone resorption because this process is unavoidable and is a physiological process. The loss of alveolar bone support will affect retention, stability and comfort in the use of dentures thus it can interfere with the installation of dentures and implants, therefore it is very important to maintain the alveolar ridge at the time of extraction. Socket preservation is a procedure with aim to reduce bone loss after tooth extraction. Proper treatment for bone damage are essential because bone acts as a support for bodily functions, thus the proper material is a factor in the success of bone implantation. Bone graft is a choice that widely used to repair bone damage. There are three types of bone grafts, namely; autograft, a bone substitution from other parts of the bone that belong to the same patient, Allograft, a graft from other individual, Xenograft, implantation of body parts from different species. Each bone grafts has their own weaknesses, therefore synthesis bone graft are made. The terms that must be met by synthetic bone grafts are acceptable to the body or biocompatible. In addition, the terms that must be fulfilled by synthetic bone grafts are beneficial to the osteoconduction process, osteoinduction process, and osteogenesis process. Osteocondutive and osteoinductive are the crucial factor for restorable biomaterial to direct and encourage the formation of tissue growth (Wahl, & Czernuszka, 2006). Osteoconductivity and osteointegration from bone graft related to the level of porosity and pore size (Develioğlu, et al. 2005).

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An ideal synthetic bone graft is a bone graft that is structurally similar to natural bone. Bone consisted of collagen and hydroxyapatite as the main component and a few percent of other components (Vaccaro, 2002). Collagen is a non-living organic polymer and arrange bone connective tissue structures. There are 13 types of collagen inside human body. The type of collagen that play pivotal role in bone formation is collagen type I, because this collagen can undergo the process of mineralization (Lieberman, & Friedlaender, 2005).

Hydroxyapatite (HA) with chemical formulas $Ca_{10}(PO_4)$ (Saphiro, 2008), $(OH)_2$ is one ceramic that has good biocompatibility properties, in chemically and physically the mineral content is the same as bone and teeth in humans. Hydroxyapatite is bioactive ceramics which has been widely used in medical applications, among the damaged bone repair, metal coating prostheses (implants) to improve biological and mechanical properties, and also as a drug delivery medium. Thermodynamically hydroxyapatite is very stable at pH, temperature and composition of fluid physiology (Peroos, & de Leeuw, 2006).

Hydroxyapatite is a calcium phosphate ceramic that is truly biocompatible and non-toxic and is an integral part of the existing bone and tooth tissue. So, it is important that these materials to be produced independently. Raw materials for the production of hydroxyapatite biomaterials very easily available and abundant in Indonesia. The production process is easy and relatively cheap in cost if done on a large scale. Among the abundant raw materials are crab shells, which are one of Indonesia's main export commodities.

Crab in Indonesia is obtained from the capture of natural stocks in coastal waters, especially in the mangrove, estuary area and from aquaculture in aquatic ponds (Wijaya, & Yulianda, 2010). In present there are around six tons of crabs per day for consumption in Jakarta. The use of crabs is generally limited to food needs, and usually only crab meat is taken while the shell is removed. Removed crab shell makes it a waste that does not have added value to the community. But actually, crab shells contain 15.60-23.90% protein, 53.70-78.40% calcium carbonate, and 18.70-32.20% chitin, which also depends on the type of crab and living place (Alexandru, 2011). Chitin in the body plays a role in accelerating the wound healing in the oral cavity, controlling bleeding, and increasing bone tissue formation (Puspawati, Simpen, 2010).

This study used Wistar rats (Rattus Norvegicus). Wistar rats are laboratory rat bred for scientific research. For years, rats have been used in many experimental studies, among them are genetics, disease, drug effects, and other topics in the field (Alexandru, 2011).

This study aims to determine the enhancement of collagen fibers in the post-extraction after being given hydroxyapatite from crab shells on the 14th and 28th days.

MATERIAL AND METHODS

This research is an experimental laboratory research, and have received an ethical clearance from the Ethic Commite of Faculty of Dental Medicine, Universitas Airlangga No. 057/HRECC.FODM/V/2018. The research is divided into five steps. Step one, Taro leaf extracting (Peroos, Du, & de Leeuw, 2006)., taro leaves were taken and washed out, then cut into parts and dried for 6 hours. The leaves were inserted into extractor machine, then added solvent ethanol 96% twice the weight of the taro leaves and shaken for 24 hours. Following after, the leaves were filtered so that clear filtrate is obtained. The extract was evaporated with the evaporator machine at 50-60° for 3-5 hours and it will be obtained a thick greenish taro leaves extract which has been free of solvents of ethanol which is considered 100%.

Step two is the dilution of Taro leaves (Peroos, Du, & de Leeuw, 2006) to obtain extract with a concentration 40%, 60% and 80% dilution by using solvent aquades. To obtain concentration 40% dilute 40 ml of taro leaves extract with 60 ml aquades, for 60% concentration dilute 60 ml of taro leaves extract with 40 ml aquades, and for 80% concentration, dilute 80 ml of taro leaves extract with 20 ml aquades.

Step three is the sample treatment (Wijaya, & Yulianda, 2010). Acrylic plate was sterilized in 121º of autoclave machine during 18 minutes, and then soaked the acrylic plate into sterile saliva for 1 hour and rinsed with PBS as much as 2 times. After that, the acrylic plate was soaked into *Candida albicans* for 24 hours. In Tube 1, the acrylic is soaked in 5ml of aquades for 15 minutes as control; in tube 2, the acrylic plate is soaked in 5ml of 40% taro leaves extract in 15 minutes; ; in tube 2, the acrylic plate is soaked in 5ml of 60% taro leaves extract in 15 minutes; ; in tube 2, the acrylic plate is soaked in 5ml of 80% taro leaves extract in 15 minutes. After that, PBS is used to rinse the plate twice to removed the taro leaves extract from the surface of the plate.

Step four is the cultivation of *Candida* albicans (Wijaya, & Yulianda, 2010). This is done by putting the acrylic plates that have been rinsed in 5ml of SDB (Sabouraud Dextrose Broth) and soaked into vibrator machine to remove the *Candida albicans* from the surface of the plate. After that, put the liquid into media of SDA (Sabaroud Dextrose Agar) then spride the liquid and incubate for 48 hours with 37ºC.

And the last step is the calculation of *Candida albicans* colonies (Puspawati, Simpen, 2010). After incubation for 48 hours, the calculation of colony is done using a Quebec Colony Counter colony count. The results then were analyzed by using the Kolmogrov Smirnov, Levene test, one way ANOVA, and Tukey HSD test.

Table 1. Table collagen fiber

Groups	N	Mean
Control Group (14th day)	9	1.5556
Control Group (28th day)	9	2.3333
Treatment Group (14th day)	9	3.4444
Treatment Group (28th day)	9	3.7778

Table 2. Mann-Whitney Analysis Result

	Control Group Day 14	Control Group Day 28	Treatment Group Day 14	Treatment Group Day 28
Control Group Day 14				
Control Group Day 28	0.024*			
Treatment Group Day 14	0.000*	0.004*		
Treatment Group Day 28	0.000*	0.001*	0.159	

RESULTS

The study of increasing collagen fibers in administration of hydroxyapatite from crab shells *(Portunus pelagicus*) after tooth extraction was done on 36 rats by extraction of the lower left incisors. Before the hydroxyapatite from crab shells was applied to Wistar rats, the content of the crab shells and the hydroxyapatite gel of crab shells.

The data in each sample is quantitative data, where the mean collagen density is calculated by dividing the area of collagen connective tissue in the microscopic slides into four visual fields. All of these examinations use an electronic microscope equipped with a calibrated micrometer. Making the tooth socket after extraction after the 14th day and 28th day microscopic slides with MT stained Observation was done by looking at collagen fiber scoring in preparations using a 400x magnification electronic microscope. The results of the average calculation of collagen fibers in preparations can be seen in **Table 1**.

Kruskall Wallis test was conducted to find out whether all treatment groups had a mean difference in groups obtained p value of 0.000 ($p < 0.05$) and it can be concluded that there was a significant difference in the increase of collagen fiber density between study groups (control day 14, control 28th day, 14th day treatment, and 28th day treatment).

To discover the different groups followed by the Mann-Whitney test which can be seen in **Table 2**.

Based on **Table 2** showed there was a significant difference in the collagen fibers density in all groups except between treatment on the 14th day and treatment on the 28th day. This is due to the high content of calcium carbonate in hydroxyapatite from crab shells (*Portunus pelagicus*).

DISCUSSION

This study is an experimental study to determine the effect of hydroxyapatite from crab shells (Portunus pelagicus) application to the dental socket post tooth-extraction to the collagen fibers density. This study used a hydroxyapatite gel from crab shell (Portunus pelagicus) to observe the description of collagen fiber density on the 14th day and 28th day after tooth extraction. Rats are used as experimental animals because of the same wound healing mechanism as humans, but the rat wound healing in is shorter than in humans.

The act of tooth extraction will be followed by the process of alveolar bone resorption. This process is unavoidable and is a physiological process. This will cause significant changes and could interfere with the installation of dentures and implants. Bone graft is a choice that is widely used to repair bone damage, one of the bone graft materials is hydroxyapatite which functions as a bone graft scaffold that can become a medium for stem cells and osteoblasts to attach, live and develop well in the defected bone.

The study was conducted on the 14th and 28th day after tooth extraction because according to a research by Kuboki et al. (1988) who examined "*time changes in cross-linked collagen in post tooth-extraction to rabbits*", saying that collagen decreased after the fifth day after extraction (5% of total protein) and increased steadily on days 7, 10 and 14th day to 45%. This increase indicates the development of granulation tissue and gradual replacement into lammelar bone as histologically observed. According to Andreasen JO13, he said that pre-collagen fibers were found after 4 days, while mature collagen fibers found in new cementum formation were seen after 14 days. The amount of collagen fiber increased substantially up to 28 days after extraction.

The results showed a significant difference in collagen between controls on the 14th day and 28th day. On the 14th day the mean number of collagen fibers was found to be 1,556 and on the 28th day there was an increase in mean number of collagen fibers up to 2,333 because the repair phase occurred on the 28th day. In this phase osteoblast activity increase. Osteoblast will help the soft callus mineralization process by secreting the matrix (collagen type l) which will later become hard callus or woven bone. This type l collagen has a space called the "hole zone" and creates a condition that will stimulate the deposition of calcium hydroxyapatite crystals between collagen fibers. Then the chondrocyte cells and osteoblasts will release the "prepacked" calcium phosphate complex into the matrix by releasing matrix vesicle buds from the cell membrane. The vesicles will carry neutral protease consisting of endopeptidase, alanyl-beta napthylamidase, and aminipeptidase and alkaline phosphatase enzymes

which will degrade proteoglycans-rich matrix and hydrolyze ATP and energy-rich phosphate esters to provide phosphate ions that are useful for deposition calcium. During the mineralization process, the ends of the bone fragments are gradually enveloped by a fusiform period which contains woven bone which continues to increase. The more minerals deposited, the harder the callus is (Saphiro, 2008; Ardhiyanto, 2015).

Based on the collagen fiber density observation in control group of Wistar rats with treatment on the 14th and 28th day by hydroxyapatite gel from the crab shell (Portunus pelagicus) application showed a significant increase in density of collagen fibers, this was in accordance with the theory that Hydroxyapatite made from crab shells (Portunus pelagicus) has a high calcium carbonate (CaCO3) content of around 40-70% (Raya, et al. 2015). Calcium carbonate has properties that can be absorbed by tissue (bioresorbable), easily biodegradable, and osteoconductive. The ideal bone grafting material must be replaced by host bones and therefore need to have bioresorbable, osteoconductive and biodegradable properties. The presence of osteoconductive properties of crab shells hydroxyapatite, it is able to induce and stimulate stem cells and osteoblasts to proliferate and differentiate a new bone formation or bone regeneration processes. Osteoblasts as metabolically active secretory cells produce a number of superfamily bone morphogenetic protein (BMP), including BMP-2, BMP-7, and β factor changes, with the addition of Insulin-Like Growth Factor, (IGF-I and IGF-II), Platelet-Derived Growth Factor (PDGF), Fibroblastic Growth Factors (FGF), TGF-β, interleukin I and PDGF (Platelet-Derived Growth Factor) and osteoid which consist of type-I collagen for bone matrix mineralization by secreting osteocytes and bone matrix (Lieberman, & Friedlaender, 2005). TGF-β1 extend the expression of matrix genes specifically by inhibiting the production and activity of collagenase so that collagen deposition stimulation occurs (Schwartz, Shires, & Spencer, 2000)., this can lead to the formation of strong and mature collagen fibers in bone given hydroxyapatite from crab shells (Portunus pelagicus).

Between treatment groups on the 14th day and 28th day there were no significant differences, this was suspected on the 14th and 28th day after extraction, fibrous matrix formation in the post extraction socket consisting of collagen fibers which is called fibrous callus is enough, after 14 days after extraction is the phase of tissue maturation. At the stage of tissue maturation, collagen collagen fibers are formed more organized. This phase will continue to the next healing phase as time goes by, soft callus and hard callus will be formed (Ardhiyanto, 2007).

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