The Pattern of Collagen, Col1A, BSP and MMP-8 in Alveolar Bone Socket Post Tooth Extraction of Rattus Norvegicus Strain Wistar After Induced with Hydroxyapatite Bovine Tooth Graft

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Abstract

Alveolar bone defects can occur as a result of tooth extraction and also endodontic surgical procedures such as hemisection. Regenerative dentistry by using bone graft is promising in clinical settings. To explore the increase of collagen density, type I collagen (Col1A), bone sialoptrotein (BSP) and matrix metalloproteinase-8 (MMP-8) in alveolar bone socket post tooth extraction of Rattus Norvegicus strain Wistar after inducing with hydroxyapatite bovine tooth graft (HAp-BTG). Twenty-eight Wistar rats were randomly divided into 2 groups, treatment and control and 2 sub-groups, day 14th and 28th. Extraction to the lower left incisor of Wistar rat was performed. The post extraction socket was filled with PEG as the control group and PEG+HAp-BTG as the treatment group. On 14th and 28th day, mandible the Wistar rats was taken. By using Masson's Trichrome staining to examine immunology through histopathological examination, the collagen density was observed using a microscope with 400x of magnification. The Col1A, BSP and MMP-8 were examined by immunohistochemistry, and observed by a light microscope 1000x magnification. There is not significant different in collagen density between the treatment and control groups, whereas Col1A, BSP and MMP-8 were significantly different between treatment and control groups. It showed that HAp-BTG can induce the increasing of Col1A that has a pivotal role in strengthening the connective tissue in dental socket, rather than collagen in general. HAp-BTG has a potential to speed-up the recovery of dental socket post extraction and increase the strength of connective tissue by inducing Col1A.

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Introduction

Recovery of bone defects in the oral cavity is one of the main challenges for dental services. Bone defects can occur in alveolar bone as a result of various factors, such as tooth extraction, periodonal disease, trauma, cysts, tumors and infections.[1] Bone defects can also occur in endodontic surgical procedures such as hemisection and apex resection which can cause damage and alveolar bone loss.[2,3] The physiological response to post-surgical damage is socket healing that leads to alveolar ridge resorption and re-building.[4,5]

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The healing process of alveolar bone socket consists of 4 phases, namely hemostasis phase, inflammatory phase, proliferation phase, and bone remodeling phase.[6] Accelerating the healing process is needed to restore normal function of the affected body part, relieve discomfort, and minimize the possibility of further complications.[7] By giving bone graft in alveolar socket after surgical procedur can accelerate the process of bone formation or osteogenesis which in turn can also improve the prognosis of the tooth.[8–11]

Bone grafts can be constructed from a variety of natural materials such as autografts, allografts, xenografts and artificial materials such as ceramics and polymers, and synthetic materials such as growth factors and alloplastics.[11] Several types of animal teeth have been used as substrates for alternative biomaterials such as teeth from primates, bovine, pigs, horses, and shark. Bovine teeth have

become the most widely used substitute for human teeth because they have similar structure or composition with human teeth and easily obtained in large quantities with good condition. Bovine teeth also have a relatively large flat surface of enamel. Compare to the human teeth, bovine teeth are stronger because they are not easily porous and not easily formed lesions.[12] The study of Moharamzadeh et al (2008) using bovine tooth graft showed that it had good biocompatibility properties, also showed a potential as a osteoconductive bone substitute. Processing of bovine teeth into bone graft is very promising because of the abundant availability of materials, can help reduce waste and reduce dependence on imported bone graft materials.[13]

Osteoconductive materials can serve as the medium for stem cells to attach, live and develop or differentiate into osteoblasts. In bone defects, osteoblasts will produce collagen, especially collagen type 1 which serve to support the mineralization of bone matrix and organize the bone matrix to induce the osteogenesis process can occur properly. Collagen density measurement is an important aspect in bone tissue engineering because collagen is one of the markers of osteogenesis activity.[14,15] Collagen is an important factor of the alveolar bone healing process because it provides integrity and strength to connective tissue, especially during the proliferation and remodelling phases.[16] Matrix Metalloproteinases-8 (MMP-8) and type 1 collagen were strongly expressed in infected dentin of primary teeth at baseline and after cavity sealing with glass ionomer cement.[17,18,19]

During the osteogenesis process, the role of bone sialoprotein (BSP) is absolutely needed for functioning the osteoclast, especially for integrity binding of bone cells and bone minerals[20]. The study of Boudiffa et al (2020) showed that among mice with knock-out of BSP gene, impacted on very low bone formation activity than the normal mice. It was also showed the reduce of osteoclast surface and the number. The full expression of osteopontin (OPN) or bone sialoprotein (BSP) is absolutely required, and OPN may compensate the lack of BSP for osteogenesis.[21,22]

An animal experiments was done to observe the expression of type 1 collagen, bone sialoproteinase (BSP) and Matrix Metalloproteinases-8 (MMP-8) in alveolar bone socket of Rattus Norvegicus strain Wistar after induced with Hydroxyapatite Bovine Tooth Graft (*HAp*-BTG) on 14th and 28 th day.

Materials and methods

This study was conducted as an animal experimental laboratory in Wistar rat with a randomized post-test only control group design.

Research Samples

Subjects in this study were male *Rattus norvegicus strain* Wistar aged 3-3,5 months and weighed 250-300 grams. Those animals were healthy and active, had normal appetite, and suffered no tooth decay and no injuries all over the body. Materials used were hydroxyapatite bovine tooth graft (*HAp*-BTG) powder (size approx. 3,5 micron) that was sterilized with gamma-ray in BATAN (Badan Tenaga Atom Nasional = National Atomic Energy Centre) Jakarta, Indonesia. The *HAp*-BTG powder (0,5 gram) was mixed with combination 19,6 grams PEG 400 (liquid) and 4,9 grams of PEG 4000 (powder) as a carrier. PEG is non toxic additional material that usable in tissue was used as a carrier substance to make bovine tooth graft easier to be applied into the socket.[22]

Research Methods

Twenty-eight (28) Wistar rats were randomly divided into two groups (treatment and control with 14 rats each group), and every group was divided into 2 sub-groups of 14 days and 28 days evaluation. During the termination date, the rats were intra-muscular anaesthetized in dextra posterior femoral region with combination of xylazine and ketamin with the dose ratio was 1:1, then extraction of the lower left incisor of Wistar rat was performed using Insisor extraction forceps. The post-extraction socket were inserted with PEG as the control group and PEG+ *HAp*-BTG as the treatment group as much as ± 0,1 cc using *syringe*. After the treatments, those animals were put back into the cage and treated until 14 and 28 days.

On 14th and 28th day, 7 Wistar rats from each group were terminated, decapitated, necropsy, retracted and cut off the left mandible then immersed in 10% formalin solution for tissue fixation. The histopathological (HPA) preparation consist of decalcification of the mandibular bone tissue using EDTA 10% for approximately 3 months, and then paraffin block preparations were made. The paraffin blocks were sliced with a rotary microtome in longitudinal direction with a

thickness of about 4 microns, and placed on a glass object. Deparaffinization was conducted by dissolving in xylol for 2 x 5 minutes then hydrated with absolute alcohol 99%, 95%, 90%, 80%, and 70% for 2 minutes each step. The residual alcohol was washed with running water for 15 minutes. Next, the HPA slides were stained with Masson Trichrome (MT) to identify the collagen fibre and immunohistochemistry stain (IHC) for identification of type 1 collagen (Col1A), BSP and MMP-8.

Collagen density were observed using a light microscope. The area being assessed was around the edge of the socket or socket healing centre (HC) (Salim et al., 2015). Collagen that were observed microscopically will appear in the form of blue coloured fibres. The histopathological scoring for collagen density was carried out based on the observation of one field of view region with 400x magnification.

Score	Operational definition of the density of collagen fibre per microscopic field
0	No collagen fibres were found in the microscopic view
1	Low density of collagen fibres in the wound area (less than 10% per field of microscopic view)
2	Density of collagen fibres in wound area is moderate (10 to 50% per field of microscopic view)
3	Density of collagen fibres in wound area is high (>50 to 90% per field of microscopic view)
4	The density of collagen fibres in the wound area is very tight (>90 to 100% per field of microscopic view)

Table 1. Grading of collagen density in microscopic examination of histology slide preparation.[23,24]

Immunohistochemistry staining was performed in Laboratory of Biochemistry, Brawijaya University, Malang. The laboratory work was carried out as the previous study.[25] Color development was performed with solution of 3.3'-diaminobenzidine (DAB) staining kit (cat noD7304-1SET, Sigma Aldrich, US). Antibody monoclonal for type I collagen using mouse monoclonal anti Col-1A (novus biotech cat#: Nb600-450); MMP8 rabbit polyclonal anti MMP-8 (Bioss cat#BS-1913R), and BSP Mouse monoclonal anti BSPII (LFMb-25, Santa Cruz Biotechnology™, US cat#SC7360).

The Col1A, BSP and MMP-8 were examined in light microscopic with 1000x magnification and oil immersion to identify the cell with brown colour that showed the expression of Col1A, BSP or MMP-8. The number of expressed cell were counted for 20 microscopic field, then the number of expressed cell per field were calculated for statistical analysis using SPSS program version 25.

Results

The scoring of the density of collagen in microscopic visualization, magnification of 400x, is shown in Figure 1. The enumeration of collagen fibres per microscopic field was used as evaluation score of the collagen fibres.

The result showed that the mean collagen density in treatment and control group, in day 14 and 28 were 1.428 and 1.285, the density of collagen in treatment and control group were 4 and 2.2857. There were not significant difference between treatment and control group in both of day-14 and day-28 ($p>0.05$) (shown by Table 2 and Figure 1).

	Group		
Day of evaluation	Treatment group	Control group	p
Day-14	1.428	1.285	0.710
Day-28	4	2.857	0.073
p	0.001	0.017	

Table 2. Mean of collagen density (score) of histopathological examination among dental socket post extraction after inducing the hydroxyapatite bovine tooh graft (*HAp*-BTG) (treatment group), compare to the control group. Note: p=significancy at alpha 5%; significant if ($p<0.05$)

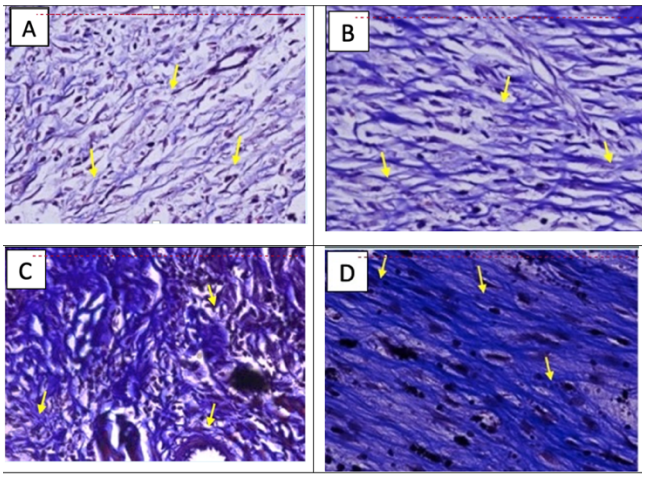

Figure 1. The density of collagen scoring in microscopic view of histological slide stained with Masson Trichrome (MT).

Note: The histologically score the density of collagen were scored 1=A; 2=B, 3=C, 4=D.

The collagen density was significantly increase by the time (day 28 vs day-14), in both of treatment group and control group (Mann Whitney test, $p<0.05$).
The observation of Col1A, BSP and MMP-8 per microscopic field in treatment vs control group on day-14 were 13.71 vs 5.44, 11.63 vs 6.24 and 8.75 vs 11.61, whereas on day-28 were 15.81 vs 8.64, 14.78 vs 9.09 and 6.14 vs 15.66 respectively. There were significantly different of Col1A, BSP and MMP-8 between treatment and control group ($p<0.05$).

	Day-14			Day-28		
	Treatment (n=7)	Control (n=7)	p	Treatment (n=7)	Control (n=7)	p
col1A	13.71	5.44	0.000	15.81	8.64	0.00
bsp	11.63	6.24	0.001	14.78	9.09	0.01
mmp-8	8.75	11.61	0.023	6.14	15.66	0.00

Table 3. The density of expressed cell (Col1A, BSP, MMP-8) per microscopic field of immunohistolochemistry (IHC) staining of the slide tissue preparation.
Note: control=applied with PEG; treatment= applied with PEG+*HAp*-BTG (Hydroxy Appatite Bovine Tooth Graft).

Discussion

Collagen is an important factor for alveolar bone healing process because it provides integrity and strength to connective tissue. The collagen density of alveolar bone socket healing after tooth extraction, were significantly increase on day-28 compare to the day-14 ($p<0.05$), but there was not significantly different of collagen density between treatment group against control group. The other way, the type I collagen expressed cell (Col1A) and BSP were significant different ($p<0.05$) between treatment and control group, 13.71 vs 5.44 and 11.63 vs 6.24 respectively.

The study about bone healing, collagen produced by fibroblast was reached peak on 7th to 14th day and continue increase accumulation until 21st day [27] and other study showed mature collagen in alveolar rat, increased substantially for up to 28 days after tooth extraction.[28]

There are up to 20 types of collagen, that spread among tissues and its role as formation of fibrillar/microfibrillar network of extracellular matrix and basement membrane. Fibril forming collagen are including of type I, II, III, V and XI collagen.[29] The integrity of the tissue is absolutely determined by type 1 collagen, rather than all other mixed collagens. The study about osteogenesis imperfecta (OI) that impact in connective tissue related disorder and showed manifestation of bone fragility, was identified by the mutation of Col1A1 gene.[30] It showed that the type I collagen has the pivotal role in the integrity of the tissue itself, rather than the collagen at general. It was also identified the polymorphisms of Col1A1 associated gene in women with cervix insufficiency that has a defect the cervix connective tissue.[31] Thus the higher type I collagen expressed due to the application of *HAp*-BTG has a positive impact in dental socket recovery and maturation after dental extraction. Type I collagen forms more than 90% of the organic mass of bone and the major collagen of skin, tendon and ligaments.[29]

The expression of bone sialoprotein (BSP) also has a similar effect with type I collagen in bone regeneration. The BSP expressed cell in treatment group was significantly higher than control group ($p<0.05$) in both of day-14 and day-28, 11.63 versus 6.24 and 14.78 versus 9.09 respectively. Application of BPS in site specific in vivo bone calcification, can stimulate osteoblast differentiation in calvarial defects, but not in the sub-cutaneous tissue.[22] The BSP in accordance with osteopontin (OPN) are belong to small integrin binding ligand N-linked glycoprotein (SIBLING) whose interact with bone cells and bone mineral. The study of Boudiffa et al (2010) showed that deficiency of BSP will impairs osteogenesis and mineral resorption.[20] The application of BSP coated scaffold in bone defect was also tendency towards bone ingrowth in rat after 4 weeks inplantation.[32] Chibinski et al (2014) showed that after sealing infected caries dentin in primary teeth, the BSP exhibited strong expression for both of the matrix and around dentin tubules.[19] It also showed the high expression of type I collagen. The central role that BSP plays in bone formation through specific binding of type 1 collagen makes BSP-collagen scaffolds an excellent choice for bone regeneration.[33]

The socket recovery after tooth extraction, is followed by the process of bone formation, collagen metabolisms and inflammation. Matrix

metalloproteinase 8 (MMP-8 = collagenase-2) is an enzyme capable of degrading of almost all extracellular matrix and basement membrane protein components both in physiologic repair and pathologic destruction of tissues, such as a breakdown of extracellular matrix in embryonic development, wound healing, and tissue remodeling,[34] is produced in bone marrow and store in polymorphonuclear leukocyte and odontoblast. The study of Korpi et al (2009) according the healing process of sockets post dental extraction in MMP-8 deficient mice showed that there were decreasing of neutrophil and significantly increase of procollagen III synthesis. It means that MMP-8 is needed for inflammation and collagen metabolisms.[35] The role MMP-8 is mostly responsible for the collagenolytic activity in dentin.[36] Our present study showed that on day-14 and day-28, MMP-8 significantly higher in control group compare to the treatment group mice ($p<0.5$), 11.61 vs 8.75 and 15.66 vs 6.14 respectively. It means that after 14 days and 28 days, the maturation of osteogenesis reached a good progress that was indicated by decreasing of MMP-8 and increasing of type I collagen formation. This study also showed that *HAp*-BTG has a positive impact in accelerating the healing process matrix recovery of dental socket after tooth extraction. The review article conducted by Araujo et al (2019) revealed that the immediate installation of implant was conducive osseointegration and survival rate of bone sockets, but it was still fail to prevent the dimensional reduction of alveolar ridge.[37]

Conclusions

HAp-BTG can induce the increasing of Col1A that has a pivotal role in strengthening the connective tissue in dental socket, rather than collagen in general. HAp-BTG has a potential to speed-up the recovery of dental socket post extraction and increase the strength of connective tissue by inducing Col1A.

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Declaration of Interest

None declared.

Ethical policy and institutional review board statement

This research was approved by the Ethical Committee of Faculty of Dental Medicine, Universitas Airlangga (No. 297/HRECC.FODM/VI/2020). The animal experiment was conducted in Laboratory of Biochemistry Faculty of Medicine, Universitas Airlangga.

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