Scanning electron microscopy imaging to assess bone implant contact enhancement after immediate bioactive compound placement

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ABSTRACT
Along with the increasing use of implant-supported dentures, the use of biomaterials to accelerate the process of new bone formation is favorable. Chlorella vulgaris is a natural product that contains elements of calcium, minerals, and vitamin D acting in mineralizing bones and teeth. In addition, the content of flavonoids and phenols in Chlorella vulgaris that are applied topically can inhibit TNF-α therefore inducing bone healing. This study aims to assess the effect of bioactive material on bone implant contact (BIC) by using SEM imaging. Nine Landrace pigs were used in this study and surgical procedures were performed in the mandible. Chlorella vulgaris extract gel was placed in the left socket and without gel in the right socket, afterward followed by titanium implant placement. Both treatments were carried out in the same way, then three pigs were observed per one time period, namely the 30th, 60th, and 90th days by using SEM test. The BIC of the sample showed the greatest at day 90th after application of gel and implant placement. It is concluded that the use of bioactive material, gel extract Chlorella vulgaris 15%, can stimulate the osseointegration better, and improve the BIC percentage.
Keywords: osseointegration, biomaterial, bone implant contact

INTRODUCTION

The healing process in the implant system is similar to bone healing in general. At first, the blood gets between the apparatus and the bone, and then a blood clot form. Blood clots are transformed by phagocytic cells, such as polymorphonuclear leukocytes, lymphoid cells, and macrophages. The level of phagocyte activity peaked during the time between days 1 and 3 after surgery. At this time, the prosthesis is attached to the apparatus and with stimulation, bone remodeling occurs. Calcification of the Haversian bone becomes dense and homogeneous. Occlusal pressure stimulates the surrounding bone. With remodeling, the osseointegrated apparatus can retain masticatory function.[1]

Bone formation begins at the implant surface in response to surface physicochemical properties, referred to as contact osteogenesis. The use of natural materials that are considered to have minimal toxicity is needed to assist the osseointegration process. The use of 15% Chlorella vulgaris extract gel as a bioactive ingredient that is injected into the socket before implant placement can stimulate the growth and development of fibroblasts and has antipreteolytic properties and stimulates tissue formation.[2,3]

The active components in Chlorella vulgaris extract include chlorellin (anti-inflammatory), chlorella growth factor (CGF), an extract consisting of various substances including essential amino acids, peptides, proteins, vitamins, sugars, and nucleic acids. In addition, there are other ingredients of Chlorella vulgaris including carotenoids (antioxidant compounds), chlorophyll, and phycobilin (complex protein pigments found only in phytoplankton).[3–6]

In order to assess the effectiveness of the natural biomaterial compound which refer to Chlorella vulgaris extract, bone to implant contact (BIC) imaging is considered to become one of many ways to evaluate this study. There are several methods which can be used to evaluate the degree of osseointegration, by using invasive or non-invasive methods.[7] However there are limitations for each methods besides of the advantages has given. The most commonly and frequently technique being used is a micro-CT instrumentation, while the information has acquired by using 2D imaging methods which is histologycal or refered as back scattered electron SEM (BSE-SEM) and secondary electron SEM (SE-SEM).[2,7] SEM as a part of micro-CT test instrument has some advantages in assessing local variations within tissue mineraliation and could be scanning rapidly. The resolution of the imaging conducted from SEM has been improved and gives high resolution of the bone and implant interface.[8]

The presence of various active components in the Chlorella vulgaris extract which is considered capable of assisting the process of new bone formation after implant placement which assess by imaging of BIC using SEM test, thus became the purpose of writing this research report.

METHODS

Preparation of Chlorella vulgaris extract gel

Chlorella vulgaris extract filtrate through maceration technique in the form of a powder prepara-

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ration which will then be made into a gel preparation. Mixing 10 g of Chlorella vulgaris extract, propylene glycol, glycerol, methylparaben, NaCMC, and aquades to form a Chlorella vulgaris gel with a concentration of 15%. Furthermore, in vitro tests were carried out to assess the gel formulation so that it was ready to be applied.

Experimental animal treatment

The study was conducted on 9 Landrace pigs that had been adapted before treatment. The surgical procedure was performed after administration of inhalation and induction anesthesia. Placement of implants between the canine region and mandibular left and right premolars was performed on each experimental animal. After the final drilling, the sample is irrigated using saline and the socket is then dried. Next, 2 mL of 15% Chlorella vulgaris extract gel was injected into the mandibular left socket sample and the titanium implant was inserted. In the right mandibular socket, a titanium implant was inserted without Chlorella vulgaris extract gel. Both treatments were carried out in the same way on 9 pigs, then 3 pigs were observed per 1 time period, namely the 30th, 60th, and 90th days.

Preparation of gel

Taking bone tissue segments with an implant in the middle with a size of 2x1 cm. The implant preparation was cleaned with 10% formaldehyde solution for 10 minutes, then dehydrated with 70% ethanol, and then dried or vacuumed. The bone around the implant was cut with a micromotor to 2 mm at the implant margin. Bone and surrounding tissue are prepared for further cutting using a microtome at half the diameter of the implant so that it is divided into 2 parts.

The implant-embedded parts were prepared for analysis using a SEM test kit (Tescam VEGA 3, Germany) for surface morphology assessment. Observation of the preparation was done by looking at the bone-implant to contact or BIC formed. Observations were made with magnification of 100x, 1200x, and 2000x.

RESULT

Descriptive analysis of the sample describes the mean, range, and standard deviation of the amount of BIC formed in landrace male pigs after implantation of either injected extract gel Chlorella vulgaris 15% or without Chlorella vulgaris extract gel. A significant increase (P<0.05) in the BIC value in the treatment group with the BIC value day 30 of 13.79%, day 60 of 16.14%, and day 90 of 18.39% (Fig.1 & 2).

There was a significant difference between the control group not added with Chlorella vulgaris extract gel and the treatment group added with 15% Chlorella vulgaris extract gel on day 90 (P<0.05) (Table 1, Fig.3 & 4).

Table 1 The BIC value of 15% chlorella vulgaris extract gel

Group	30th	60th	90th	P value
Treatment	13.79	16.14	18.39	0.002**
Control	11.97	14.39	16.87	0.000*

* Repeated ANOVA, p value < 0.05; Significant **Friedman test p < 0.05; Significant

From the results of the SEM test on implants based on the day of observation, it was shown that there was an increase in the formation of new bone attachments with the interface distance between the bone and implants getting smaller based on the time period. This can be seen in the calculation of the BIC value with the increasing value along with the length of the observation period using SEM test with magnifications of 40x, 100x, and 1200x.

DISCUSSION

Micrometre scale technique which had been used in this study was SEM method. This method has several advantages such as using 2D imaging,

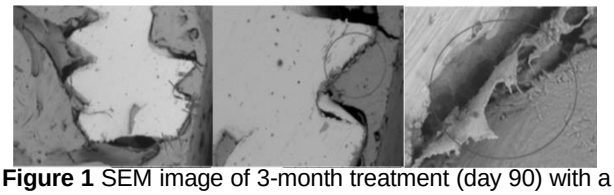

Figure 1 SEM image of 3-month treatment (day 90) with a BIC value of 18.39%

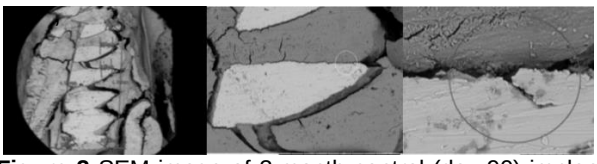

Figure 2 SEM image of 3-month control (day 90) implant with a BIC value of 16.85%

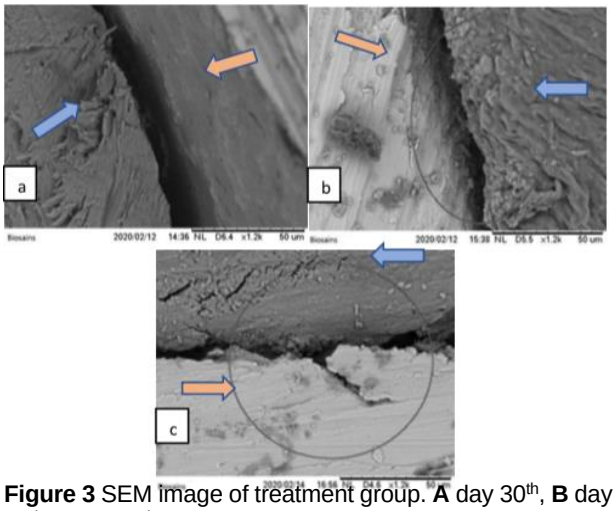

Figure 3 SEM image of treatment group. **A** day 30th, **B** day 60th, **C** day 90th (orange arrow is implant, blue arrow is bone).

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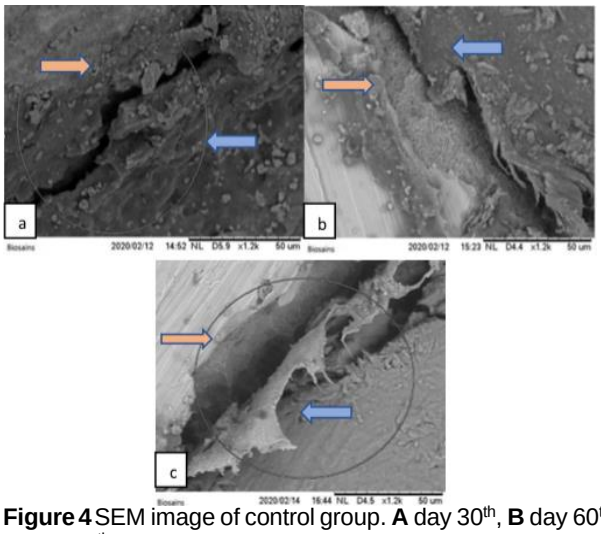

Figure 4 SEM image of control group. **A** day 30th, **B** day 60th, **C** day 90th (orange arrow is implant, blue arrow is bone)

analysis element, can be using different contrast phenomenas, low to very high spatial resolution, and high dept of field.[2] The SEM examine the rough surface of the sample preparation, and evaluate bone formation around and inside solid and porous metal implants and degradable materials, the voxels containing information of the immediate bone-implant interface are obscured by various imaging artefacts.[2,8]

Initial tissue response to implant osseointegration can be seen between titanium dental implants and new bone regeneration. Damage to both hard and soft tissues initiate the wound healing process which ultimately allows the implant to become ankylotic with bone. This condition can be seen and analyzed through BIC conducted in vivo in experimental animals. This study is an assessment of the early stage of osseointegration after implant insertion in experimental animals, by looking at the difference in BIC values between the test and reference surfaces and statistically analyzing to compare the osteogenic potential of the implant surfaces. The highest bond formation in bone and implants was characterized by an increase in trabecular density and an increase in the BIC ratio.[9-13]

After the surgical placement of the implant into the endosteal location, the traumatized bone around the implant begins the wound healing process, which starts from the inflammatory phase, the proliferative phase, to the maturation phase. A few seconds after implant placement, the entire surface of the implant is covered with a thin layer of serum protein which is a growth factor, the surface characteristics of the material have a major influence on the adhesion of the serum protein. Serum protein is associated with the activation of physiological processes of platelets (platelets) and the release of granules. This platelet degranulation releases growth factors and triggers chemotactic signals. When platelets come into contact with synthetic surfaces, they release serotonin and histamine which cause platelet aggregation and further thrombosis.[7,11]

During the proliferative phase, vascular growth occurs from the surrounding vital tissues, a process called neovascularization. In this phase, the effect of Chlorella can reduce the secretion of cytokines so that it is hoped that new tissue is more easily formed and wound healing is faster. The metabolism of local inflammatory cells, fibroblasts, progenitor cells, and other local cells creates an area of relative hypoxia in the wound area that triggers local mesenchymal cells to differentiate into fibroblasts, osteoblasts, and chondroblasts.[6,7]

Chlorella vulgaris has a myelostimulating effect through cytokine induction, interferes with the production of IFN-c, IL-1a and TNF-a, and increases the production of IL-10 and IL-6 suppress the infla-flammatory cytokine TNF-alpha and inflammatory mediators (nitric oxide) also stimulate the production of IFN-c, IL-1a, TNF-a and NK cell activity. The main component of Chlorella is chlorella growth factor (CGF), an extract consisting of a variety of substances including essential amino acids, peptides, proteins, vitamins, sugars, and nucleic acids. Activation of fibroblasts by Chlorella with the accelerated formation of granulation tissue where the granulation tissue consists of connective tissue and fibroblasts, new blood vessels (angiogenesis), and inflammatory cells. The CGF stimulates increased growth and development of fibroblasts. The extracellular matrix is created by these cells and eventually, a fibro-cartilaginous callus is formed which turns into a bone callus. Early immature bones are called woven bones.[3.6]

One of the antibiotics contained in Chlorella is called Chlorellin and acts as a new anti-inflammamatory agent from natural sources with fewer side effects. Meanwhile, fibrin nets have formed that cover the wound, and the infiltration of leukocyte cells into the wound area is carried out first by neutrophils. Its migration is influenced by cytokines such as IL-1 and TNF alpha. In this phase, the effect of Chlorella triggers an increase in the levels of several cytokines that are useful in increasing leukocyte activity. Chlorella's anti-inflammatory effects include inhibiting the production of IL-5 by mast cells, inhibiting GM-CSF cytokines, and inhibiting angiotensin I-converting enzyme (ACE).[3]

Inflammation is an important host defense mechanism and is characterized by complex interac-

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tions between inflammatory mediators and inflammatory cells. When the tissue is inflamed, fibroblasts will immediately migrate towards the wound, proliferate and produce a collagen matrix to repair damaged tissue. Activation of fibroblasts by Chlorella with accelerated formation of granulation tissue where the granulation tissue consists of connective tissue and fibroblasts, new blood vessels (angiogenesis), and inflammatory cells. CGF stimulates increased growth and development of fibroblasts. Chlorella also stimulates the activity of T-cells and macrophages by increasing interferon levels, thereby increasing the immune system's ability to fight bacteria, viruses, chemicals, or foreign proteins.[3,6,14,15]

The remodeling process begins at week 12 and the strength of the interface between implant and bone increases after 0-12 weeks of placement, this implant is related to the amount of bone surrounding the bone. In the treatment group or implants previously injected with 15% Chlorella vulgaris as a bioactive ingredient into the socket, the mean BIC value was higher than the group without extract.

In weeks 2-4, namely the stage of cell proliferation and callus formation, osteoblast cell deposition occurs on both implant and bone surfaces. At this stage the BIC value on days 30-60 increased in the treatment group, indicating that Chlorella vulgaris can trigger local acceleration of bone formation, early expression of growth factors, bone differentiation, and osteogenesis which accelerates the formation of young bone or woven bone. control group. The flavonoids and CGF present in Chlorella vulgaris stimulate the acceleration of angiogenesis and bone formation around the implant. CGF induces bone morphogenetic protein and osteogenetic synergy occurs.[11,12]

It is concluded that application of 15% Chlorella vulgaris extract gel resulted in better adhesion between bone and titanium implant surface which was indicated by a higher BIC value on day 90 and accelerated the osseointegration process observed by SEM test.

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