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RESEACRH ARTICLE

A histological evaluation of cellular response on bone regeneration scaffold

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ABSTRACT

Biocompatibility testing for a new implant material is necessary before clinical use. The materials for bone scaffolding must also go through biocompatibility tests both *in vitro* and *in vivo*. The purpose of this study was to compare different staining methods in histological preparations to assess the biocompatibility of the material to tissues. Twenty Wistar rats (n= 20), aged 4 months, males, weighing about 250-350 g were divided into 2 research groups, namely sub-cutaneous and bone implantation for 3, 14, and 30 days. The sample was a biodegradable material with gypsum and calcium carbonate as the basic ingredients. Histological stains were prepared in several ways using Hematoxylin Eosin (HE), Mallory, Toluidine Blue, Tartrate-resistant acid phosphatase polyclonal antibody (TRAP), and Chromogen 3,3 diaminobenzidine (DAB) after pre-performing a decalcification process with ethylenedinitril-o-tetra-acetic acid (EDTA). The observation was done using magnification ranging from 10X10 to 100X10 with a light microscope (Nikon eclipse E600) and OptiLab viewer. The results were displayed in the form of descriptions of images generated from different staining methods. Staining by the HE method is most often chosen because it is cheap and easy but the other staining methods such as Toluidine Blue, Mallory, and TRAP show better contrast. Tissue staining techniques with immunohistochemistry (DAB, VEGF) are used to evaluate cell and tissue responses by identifying specific proteins present.

Keywords: DAB; HE; mallory; scaffolding; toluidine blue; TRAP

INTRODUCTION

Bone damage are one of the challenges in the clinical field. Some alloplastic materials have been widely developed as bone scaffolds to facilitate new bone regeneration. Modification of bone substitution is expected to create conditions as similar to extracellular in the implantation area as possible.[1] After an implantation procedure, in the tissues there will be an interaction between the host's immune system and the implant material with cellular reactions and inflammatory responses.[2] Biocompatibility tests of the material must be carried out to ensure its level of safety in the body.

Testing with experimental animals can be used to estimate the biocompatibility of new bone scaffolding materials prior to clinical use in humans.[3] The soft tissues surrounding the implantation location, as well as the hard tissues, must be opened during the implantation process. The soft tissue response to the implant typically indicates more severe inflammation than the bone tissue response.[4] Some observations of the number of inflammatory cells, giant cells, the thickness of the capsule surrounding the bone scaffolding material, the increase in the number of blood vessels, the formation of osteoblasts and osteoclasts are often used as parameters of the degree of biocompatibility of bone scaffolding materials.

Some histological staining methods commonly used are to use HE (Hematoxylin Eosin), Mallory, Toluidine Blue, TRAP (Tartrate-resistant acid phosphatase polyclonal antibody), and immunohistochemistry like DAB (Chromogen 3,3 diaminobenzidine).[5,6,7] In hard tissues, histological staining can be preceded by softening bones or decalcification process using the EDTA method[5] and the Plank Rychlo's solution.[8] Another non-softening method is grown in methyl methacrylate (MMA).[7] This bone softening action

before processing is intended to facilitate slicing the bones into thin slices, thus making staining easier and perfecting the result.[6]

A histological evaluation not only confirms that a bone scaffolding material is biocompatible, but also ensures that the material does not cause adverse effects. Histological observations can show the severity response of the tissues around the bone implant, including: 1) hypersensitive cells (mast), 2) inflammatory cells (neutrophils, macrophages, lymphocytes), 3) response to foreign bodies (multinucleated giant cells), 4) bone formation (osteoblasts), 5) bone remodeling (osteoclasts).[2,9] The staining method should be selected properly. The purpose of this study was to compare different staining methods in the preparation of tissue specimens commonly used for *in vivo* biocompatibility testing.

MATERIALS AND METHODS

The animal experimental procedure was approved by the Ethics Committee of Faculty of Dentistry, Universitas Gadjah Mada, number of 001465/ KKEP/FKG-UGM/2018. Twenty Wistar rats, aged 4 months, males, weighing about 250-350 g were kept in an animal care facility for 7 days to adjust to the daily diet, water, and drums under the 12-hour/12-hour light/dark cycle. The national guidelines for the care and use of laboratory animals were applied during the study. The animals were housed in cages located at the Integrated Research and Testing Laboratory (LPPT), Universitas Gadjah Mada. Each animal was randomized to one of the 2 groups, namely the subcutaneous or bone implantation group. The sample used was bone scaffolding based on a mixture of gypsum, calcium carbonate, and gelatin.[6] The implantation procedure in the experimental animals was performed under general anesthesia by intramuscular injection using ketamine (11-22 mg/kg body weight) combined with xylazine (0.55-1.1 mg/kg body weight). In the area of operation, the fur was previously shaved off and smeared with betadine.[6,7] All the implants were sterilized by EOG (ethylene oxide gas) before use in the animal study. Subcutaneous implants were prepared with four cylindrical implants per animal placed in subcutaneous pockets via 1 cm dorsal midline incisions on either side of the spine**.** The skin was closed with an absorbable suture material of vicryl 2.0 (Ethicon, Johnson and Johnson Indonesia, Jakarta, Indonesia). The bone implantation was prepared by exposing the lateral aspects of the left and right femoral condyles with a 1 cm longitudinal incision. Then, a 2-mm wide and 3-mm deep hole was drilled, which was further widened to reach the final defect diameter of 2.5 mm. The defect was created with a slow drill speed, followed by continuous internal cooling with saline. A cylindrical implant (2.5-mm wide and 3-mm long) was placed in the bone defects as created in each rat. After the implant installation, the muscles and skin were closed in separate layers with the Vicryl 2.0 (Ethicon, Johnson and Johnson Indonesia, Jakarta, Indonesia) suture material. To reduce the risk of perioperative infection, each rat received an Interflox-100 antibiotic (Interchemix, Horsterweg 26, Maastricht, The Netherlands), 10 ml/ 20-40 kg intramuscularly for 3 days after surgery. All the animals were fed with mouse pellets, and water was given ad libitum. The postoperative condition (the surgical wound, food intake, activity, clinical signs of infection) were monitored daily. Implantation times were 3, 14, and 30 days. The rats were sacrificed by an overdose of ketamine and xylazine.

Afterwards, each implant and its surrounding tissue were immediately resected for further histological processing. The condyles were cut into smaller samples with a diamond bur and fixed in 10% buffered formalin solution for 1 day. Subsequently, the specimens were decalcified by 10% ethylenedinitril-o-tetra-acetic acid (Titriplex III, Merck, Darmstad, Germany). All the sections (subcutaneous and bone) were removed and immersed in 10% phosphate-buffered formalin for 24 hours. The bone preparations were softened using EDTA for 2-4 weeks in advance. Processing of tissue included dehydration in graded ethanol from 50% to 100%, infiltration with paraffin, and routine histological processing and staining with

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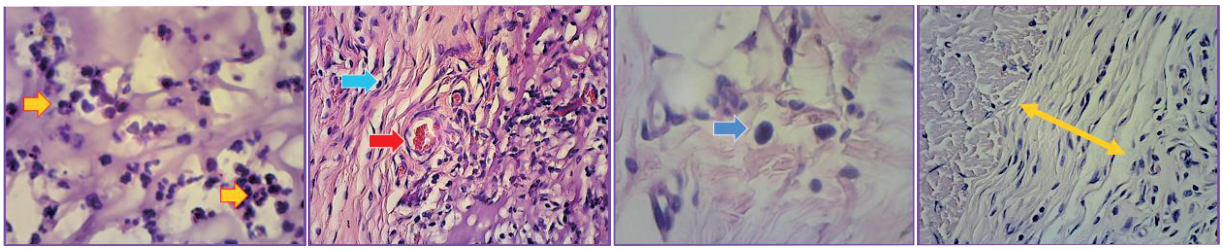
HE, Mallory, Toluidine Blue, TRAP, dan DAB.[6,10] At least three parts of the tissue around the implant (soft and hard tissue) were examined with a light microscope (Nikon eclipse E600) with an Optilabviewer® to draw images at magnification from 10X10 to 100X10. Description analysis was performed on the response of soft tissues and hard tissues to implants.

RESULTS AND DISCUSSION

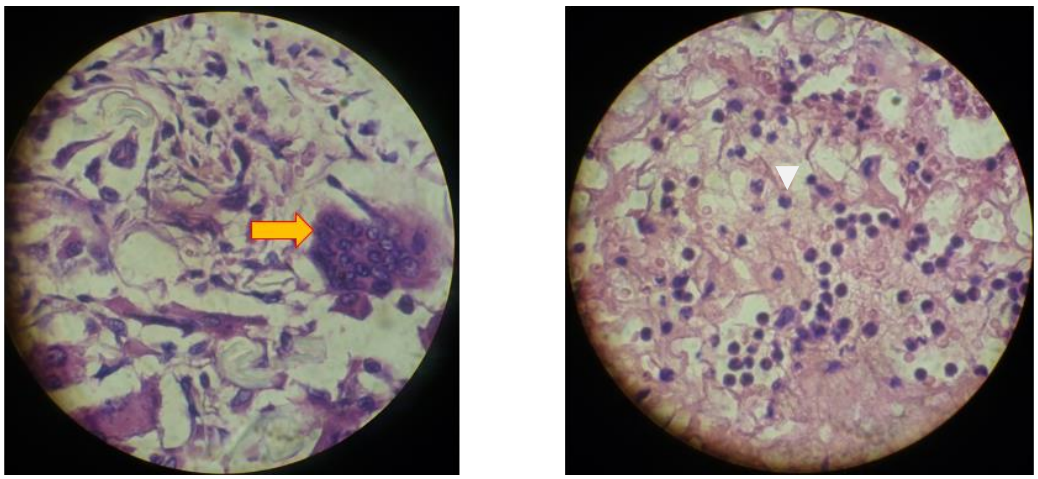
Biocompatibility tests of new implant materials are required to determine the degree of safety and tolerance of tissues to the material before used in human. Some of the histological staining methods used in this study were HE, Mallory, TRAP, Toluidine Blue, and DAB. The staining methods were selected according to individual needs.

Hematoxylin and Eosin are staining methods widely used in in the staining of histological tissues, usually necessary to support medical diagnosis and research. The principle of HE staining is that chromatin in the nucleus binds to an alkaline stain (hematoxylin), cytoplasmic proteins bind to an acidic stain (eosin), and cells become pink with purple-blue nuclei. Histological preparations with HE staining can be used for observation of inflammatory cells such as neutrophils, macrophages, lymphocytes, giant



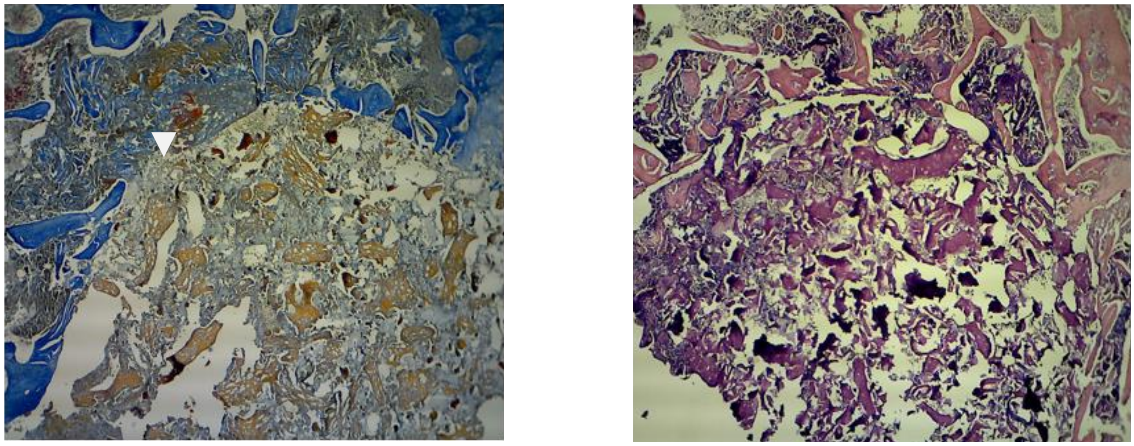
(A) (B) (C) (D)

Figure 1. A 3-days sub-cutaneous implantation, 100X magnification, HE staining (A) and a 14-days sub-cutaneous implantation, 40X magnification, HE staining (B). The yellow arrow is a neutrophil cell (A), the blue arrow is a collagen fiber with fibroblast cell around it (B), and the red arrow indicates a new blood vessel in the implantation area (B). Figure 1B showed that the remaining implant material was still detected on day 14, in the form of thin sheets of purple, smooth and porous. A 30 days sub-cutaneous implantation, 100X magnification, HE staining with a lymphocyte (blue arrow) (C). A 14 days sub-cutaneous implantation, 40X magnification, HE staining (D). Fibroblast capsules pulled outside the implantation area indicated by a yellow arrow.



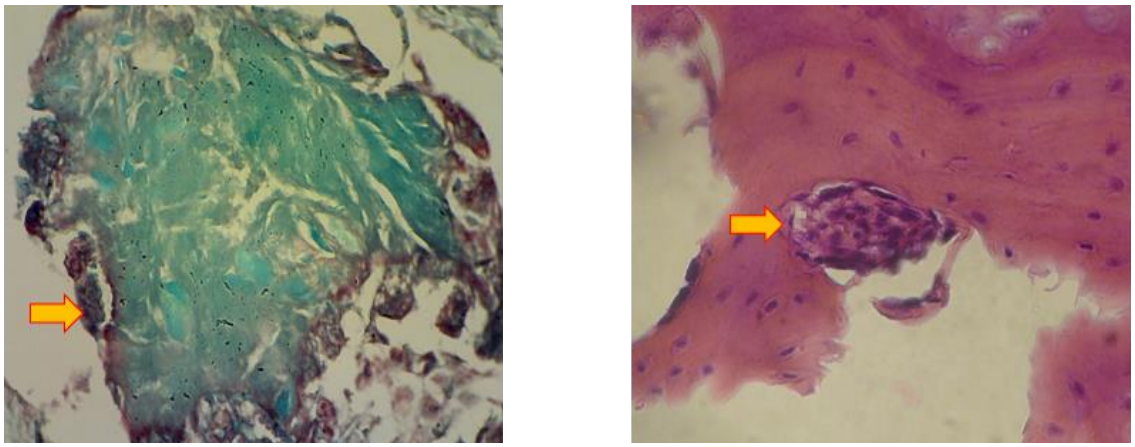
(A) (B)

Figure 2. (A) A 30-day bone implant, 100x magnification, HE staining of giant cells (yellow arrows). (B) A 30-day bone implant, x40 magnification, HE staining with inflammatory cells surround the remaining implant of degradable material.



(A) (B)

Figure 3. It show a comparison of (A) a 3 days bone Implant, staining with mallory, 10X magnification and (B) with HE staining. The remaining material appears in the area of artificial defects with pmn cells scattered around the implant material. Compared to red in HE staining, the bones around the defect look more dark blue in Mallory. The rest of the implant material is more visible contrast in Mallory's coloring than HE. PMN cells are easier to observe with HE staining.



(A) (B)

Figure 4. (A) A 30-days bone implantation, TRAP method staining, 40X magnification. The yellow arrow indicates a large osteoclast cell that has many nuclei, located within the recesses of the bone. (B) Image of osteoclasts (yellow arrows), appearing in pink, coloring using the HE Staining method.

cells, and show the presence of new blood vessels and collagen fibers.[10]

The hematoxylin-eosin staining of the subcutaneous implants clearly showed the presence of inflammatory cells with individually specific characteristics. Three days after the transplantation, the predominant cells were pmn leukocytes, mainly neutrophils and macrophages (1A). Meanwhile, 14 days after the implantation, new blood vessels began to appear around the collagen fibers and thicken among the fibroblasts (Figure 1B).

Mallory's staining is usually used to see the thickness of collagen fibers formed in the area of the defect. The thickness of collagen fibers can be used to indicate the occurrence of a new tissue regeneration process after implantation. In addition to seeing collagen fibers, Mallory's staining method can also observe bone maturity and residual implant material.[6] Collagen fibers are dark blue; mucus, amyloids, bluish-colored hyaline material; nuclei, cytoplasm, fibrin, neuroglia, elastic fibers, muscle fibers are red; erythrocytes and myelin fibers are

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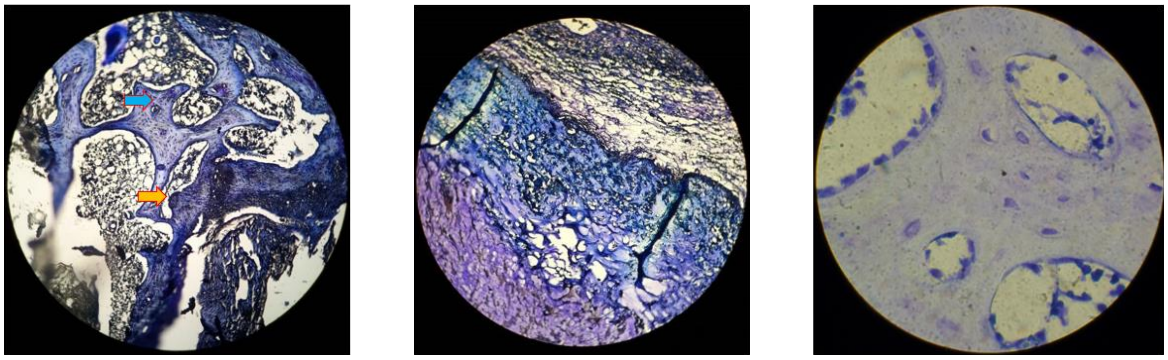

(A) (B) (C)

Figure 5. (A) Bone picture at 3-day implantation, Toluidin Blue staining, 10X magnification. The bones are dyed dark blue, with a degree of bone maturity can be seen from the thickness of the blue color. The yellow arrow indicates osteocytes are clearly visible trapped in bone islands and the red arrows indicate osteoclasts in the old bone niche. (B) 14-day subcutaneous implantation, Toluidin Blue staining, 40X magnification. Collagen fibers (dark blue color) appear to delimit the implantation area (light blue color). Figure 5C shows that bone preparate with Toluidin Blue staining, 40X magnification. Osteoblasts appear to be arranged in a row on top of old bone cells.

yellow to orange. The slices will be stained by a solution of acid fuchsin, aniline blue, orange G, and phosphotungstic acid (Figure 3A).[6]

Assessment of the thickness of collagen fibers with Mallory's staining is usually done by scoring. Collagen density is measured in histology for both soft and hard tissues. Score 0 (no collagen fibers yet), score 1 (thin and irregular collagen fibers), score 2 (thin and/ or thick collagen fibers that appear to be arranged in parallel), score 3 (collagen fibers become thickened to form matrix extracellular) (modification from Anne et al).[6]

TRAP staining is usually used to see osteoclasts. This staining is only for bone tissue and can be used to see the ratio of osteoblasts to osteoclasts that can describe the process of bone remodeling.[5,11]

Staining with the TRAP method shows osteoclasts with a dark red color around the bone with a green color. These cells are located in depressions known as resorption bays (Howship's lacunae) on the surfaces of the bone undergoing resorption. Osteoclasts can also be seen by HE staining method but their color does not contrast too much with the surrounding bones as shown by the TRAP staining. A special feature of osteoclasts is a cell of a considerably big size and attached to the recesses of the bone.

Toluidine blue is a basic thiazine metachromatic dye with high affinity for acidic tissue components and stains tissues rich in DNA and RNA.[12] Toluidine blue staining is mainly used to identify mast cells. Since the blue tint of Toluidine blue gradien indicates mature new bones, bone scaffolding material can be used in histology tests to detect the formation of new bones.

Tissue staining techniques with immunohistochemistry can be used in evaluating cell and tissue responses on bone regeneration scaffolding by identifying specific proteins that are present in cells or tissues during the tissue regeneration process. The presence of specific cell types, inflammatory mediators, growth factors, transcription factor proteins, enzymes, and other proteins is identified with specific antibodies that are further visualized in brown, red, or other colors depending on the chromogen used in the IHC staining. With chromogen 3,3'-diaminobenzidine (DAB), the target protein will be visualized in brown, while with chromogen Aminoethyl carbazole (AEC) it will be visualized in red. The process of angiogenesis can be detected by the presence of Vascular Endothelial Growth Factor (VEGF) proteins. The ongoing inflammatory process can be detected by the presence of inflammatory mediators such as IL-8 and IL-1beta and also the presence of inflammatory cells such as lymphocytes (with CD8 specific antibody),

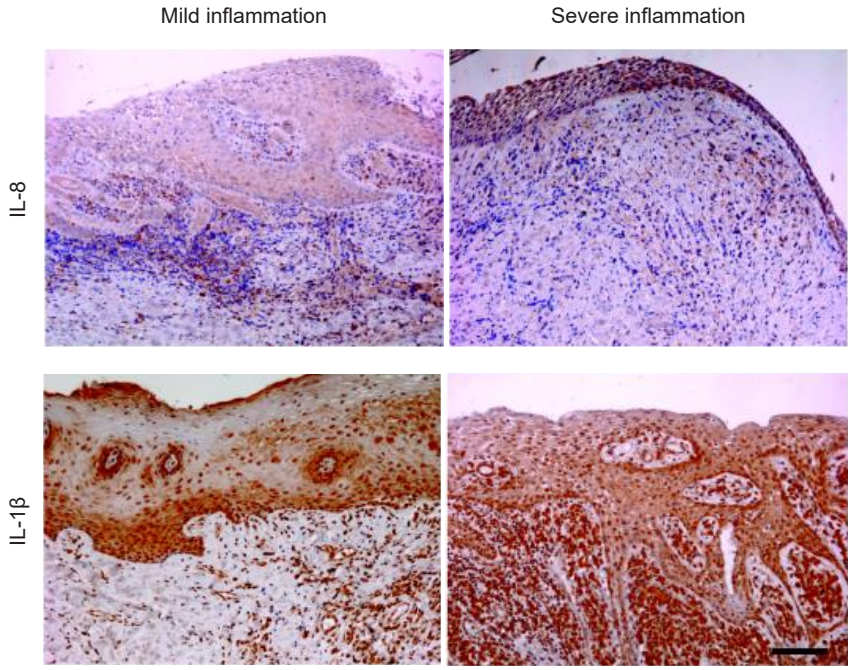

Figure 6. Tissue images involving mild and severe inflammation of tissues with IHC staining with IL-8 and IL-1β-specific antibodies, using DAB chromogens. Brown color indicates the expression of IL-8 and IL-1β in tissues. The intensity of brown color in tissues that experience severe inflammation appears stronger than in tissues that experience mild inflammation. Chromogen DAB. Scale 100µm. Modified from Listyarifah et al (2017).

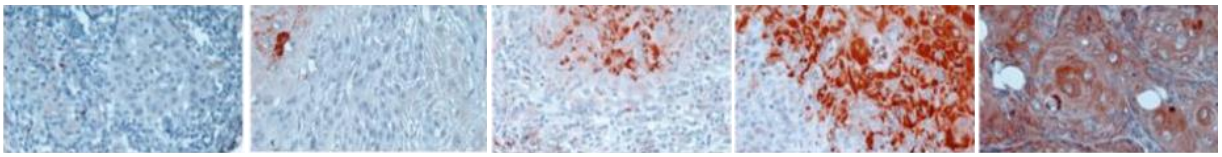

Figure 7. The scoring of immmunohistochemical staining based on percentage of expression was (A) score 0 negative, (B) score 1 < 10%, (C) score 2 11%-50%, (D) score 3 51-90%, and (E) score 4 more than 90%. Chromogen AEC.

mast cells (with specific mast cell tryptase antibody), macrophages (with CD68-specific antibody), or other inflammatory cells.[13] (Figure 6).

The results of this IHC staining can be interpreted and analyzed qualitatively, semi quantitatively, or quantitatively. Qualitative interpretation is a descriptive report regarding the presence or absence of specific protein expression in stained tissue slices. When there is a brownish (with DAB chromogen)[13] or red (with AEC chromogen) tissue[14,15] then it means that the protein is expressed in the tissue. On the other hand, if the tissue appears neither brown nor red, this means that the protein is not expressed in the tissue.

Semiqualitative interplay is carried out by providing a score / scoring of visible coloring. The scoring performed can be a percentage of the extent of protein expression in the tissue or the intensity of the color that appears. Classification of scores based on broad percentages are 0 (negative results), 1 (up to 10%), 2 (11%-50%), 3 (51%-90%), and 4 (more than 90%). Classifying scores based on the color intensity that appears is in the form of scores of 0 (negative results), 1 (weak), 2 (moderate), and 3 (strong)[14] (Figure 7).

Quantitative interpretation can be done manually or automatically with the help of software such as ImageJ® or Optilabviewer®. Qualitative

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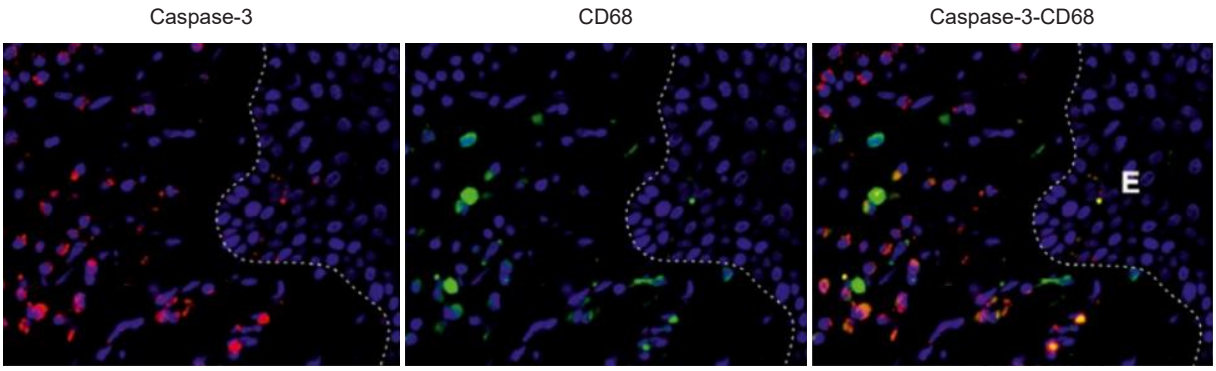

Figure 8. Double immunofluorescence to detect caspase 3 (apoptosis marker) with red fluorochrome and to detect CD68 (macrophage marker) with green flurochrome. The nucleae are colored with a visualized DAPI with a blue color. Macrophage cells that express caspase 3 (arrow) appear orange which is a superimpose between red and green. E indicates the epithel and the white dotted line is made to signify the basal lamina. Modified from Listyarifah et al (2017).

interpretation includes calculating the number of cells expressing the target protein (calculated either manually or automatically), calculating the percentage of area, or calculating the intensity of staining.

The technique and usefulness of tissue staining with immunofluorescence have similarities with IHC in evaluating cell and tissue responses on bone regeneration scaffolding. The difference is mainly in the chromogen and observations. Chromogen for immunofluorescence is fluorophore which has a different color depending on the type of fluorophore used. The fluorophore luminescence cannot be observed with an ordinary light microscope but with a fluorescence microscope. The advantage is that, with the immunofluorescence technique, it will be easier to identify the colocalization of different proteins in 1 tissue. To identify the number of lymphocytes (or other specific cells) that express a particular protein such as caspase 3 or TLR, a staining process is required using 2 antibodies, namely CD68 (as a macrophage marker protein) and capase-3 or TLR or other proteins. Each antibody is visualized in a different colored fluorophore. If there is a superimpose (meaning there is colocalization) it will be visualized in different colors (Figure 8).

CONCLUSION

Biocompatibility testing of implant materials both in vitro and in vivo is required before clinical use. Histologically testing using experimental animals is very often done. The selection of the staining method used will affect the result. The selection of the staining method largely depends on what will be analyzed from the histological preparations. HE staining is most often chosen because it is cheap and easy. On the other hand, other staining methods such as Toluidine Blue, Mallory, and TRAP show better contrast results than HE staining. Meanwhile, tissue staining techniques with immunohistochemistry (DAB, VEGF) are used to evaluate cell and tissue responses by identifying the presence of specific proteins. The difference with the technique and staining of tissues using the immunofluorescence method is mainly in its chromogen and observations.

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CONFLICT OF INTEREST

The author states that there is no conflict of interest between the author and funding agency.

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