Original Research Report

CHONDROREGENERATIVE POTENTIAL OF PLATELET-RICH FIBRIN (PRF)-IMPREGNATED DECELLULARIZED BOVINE CARTILAGE SCAFFOLD IMPLANTED SUBCUTANEOUSLY

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ABSTRACT

The invention of alternative implants with regenerative potential comparable to autologous cartilage continues to be encouraged due to high morbidity of the donor site related to autologous harvesting process. This research attempted an invention of alternative implant using tissue engineering techniques in the form of endogenous regeneration by combining decellularized bovine cartilage scaffold with platelet-rich fibrin (BCPRF) that was implanted subcutaneously. The study aimed to compare the chondroregenerative potential between BCPRF and autologous cartilage in terms of the formation of newly-regenerated chondrocyte, the thickness of type II collagen produced, and the rate of cartilage resorption following the subcutaneous implantation. This study was conducted in a pretest-posttest control group design using New Zealand white rabbits. Forty-eight experimental samples were divided into two groups, then treated with subcutaneous implantation of BCPRF and autologous cartilage respectively. The results were evaluated after six weeks of implantation. Thirty-nine samples were evaluated. There was a significant difference found from both groups in terms of the formation of newly-regenerated chondrocyte, the thickness of type II collagen (p=0.000), and the implant resorption rate (p=0.000). The microscopic images demonstrated a superior chondroregenerative potential in the group receiving implantation of autologous cartilage compared to the group receiving BCPRF. The chondroregenerative potential for autologous cartilage and BCPRF differed significantly in terms of the formation of newly-regenerated chondrocyte, the deposition of type II collagen matrix, as well as the resorption rate.

***Keywords**: Autologous cartilage; platelet-rich fibrin; bovine scaffold; chondrocyte regeneration; medicine*

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Highlights:

1. This research compared the hondroregenerative potential betw een bovine cartilage scaffold w ith platelet-rich fibrin (BCPRF) and autologous cartilage.
2. The formation of new ly-regenerated chondrocyte, the thickness of type II collagen, and the rate of cartilage resorption follow ing the subcutaneous implantation w ere assessed.
3. BCPRF is highly biocompatible and can be developed as an alternative to alloplastic porous polyethylene (Medpor) implant material.

INTRODUCTION

The usage of cartilage grafts in corrective surgery for congenital craniofacial abnormalities, trauma, aesthetics, and tumor resection may be considered relatively high. Reconstruction using autologous cartilage is an oftenly used modality. However, autologous cartilage application slowly begins to be restricted due to some drawbacks in harvesting, such as donor-site morbidity, likelihood of dislocation, donor-site scarring, and risk of pneumothorax (Araco et al. 2006,Mischkow ski et al. 2008,G unter et al. 2008, Revell & Athanasiou 2009,Moon et al. 2012,Wee et al. 2015)

Autogenous cartilage graft is a biocompatible alternative available in the same surgical field (Soria-Gondek et al. 2022). Autologous cartilage is always the tissue of choice for transplantation purposes. Preserved or fresh homogenous cartilage graft is a valuable second choice. Heterogeneous cartilage from stingray or shark should only be considered if homogeneous

cartilage is not available There was good evidence that young cartilage grafts, with or without perichondrium, do not grow or increase in size after implantation in humans (Peer 1954). This encourages the discovery of other alternative modalities that can provide the same efficacy as autologous cartilage with minimal complications.

The new paradigm in tissue engineering nowadays has provided an opportunity to develop xenograft for tissue regeneration, especially cartilage. Cartilage tissue engineering has proven its effectiveness for cartilage regeneration. Xenografts have different properties, depending on their origin, nature, and processing. Bovine cartilage is a xenograft material that is often used as a scaffold due to its availability in nature. It is typically derived from cattles and pigs because of its osteoinductive and osteoconductive properties, low cost, and availability. However, it has shortcomings in immune response and carries the risk of transmitting animal diseases. Research have not yet reported significant differences in the use of animal-derived or synthetic biomaterials. Several lines of evidence suggest that synthetic materials have a lower risk of disease transmission (Barakat et al. 2008, Rhatomy et al. 2021). Deproteinization is an essential process to eliminate the antigenicity of xenograft bone. Effective strategies to eliminate the antigenicity of foreign bone are important in the development of xenogenic bone substitutes (Barakat et al. 2008).

Platelet-rich fibrin (PRF) is one of the sources of growth factors that has been widely studied lately. It is a fibrin matrix containing cytokines, growth factors, and cells that are progressively launched into wounds over time. Platelets are very vital for tissue healing (He et al. 2009). General traits of PRF consist of the amendment of centrifugation speed and time in addition to the dearth of anticoagulants/polymerization agents, differentiating it extensively from first-generation platelet concentrates or platelet-rich plasma (PRP). PRF differs from PRP because of its ability in providing the biochemical structure of a fibrin clot with platelets, cells, and circulating cytokines, and growth factors that have high affinity (Choukroun et al. 2006a, 2006b, Dohan Ehrenfest et al. 2009, Ghanaati et al. 2014). In addition, the discharge of growth factors is controlled and sustained owing to the fibrin scaffold, which can benefit the regeneration process (He et al. 2009, Dohan Ehrenfest et al. 2010, Pradeep et al. 2012, Kobayashi et al. 2016).

This research was conducted based on the tissue engineering-endogenous regeneration concept. The aim of this research was to examine whether the implant made from a combination of bovine cartilage and PRF (hereafter referred to as decellularized bovine cartilage scaffold-PRF implant (BCPRF)) has a potential comparable to autologous cartilage in regenerating cartilage tissue. In the future, the BCPRF can be developed as an alternative implant capable of replacing the use of autologous cartilage.

Rhatomy et al. (2021) stated that decellularized biomaterial scaffold limits the use of growth factor, resulting in better cost and resource efficiency. Organic materials are preferred because they have better biocompatibility and biodegradability than synthetic materials (Park & Cho 2010). Sponge bovine cartilage scaffold is a byproduct, which has no economic value and is usually discarded. This biomaterial is cheap and easy to acquire. Bovine cartilage scaffold will not damage the stem cell (Utomo & Rantam 2017, Mahyudin et al. 2018).

Tissue engineering technique requires three main components to regenerate a network, the cells, scaffolds, and growth factors (Vinatier et al. 2009). In terms of implant manufacturing technology, many studies combine these three components exogenously, with the aim of regenerating tissue in vivo (Utomo & Rantam 2017). In the endogenous regeneration concept, the potential of scaffold and growth factors combination is optimized exogenously, so it can endogenously stimulate the recruitment and differentiation of cells (e.g. endogenous mesenchymal stem cells/MSC). The result expected is in vivo regeneration of the tissue (Gulati & Poluri 2015). The advantage of this concept is that it does not require complicated procedure or large cost for exogenous stem cell preparation. In this study, the decellularized bovine cartilage scaffold proved to contain bone morphogenetic protein 4 (BMP4), which plays an important role in the differentiation of MSC towards chondrogenic lineage.

MATERIALS AND METHODS

Fabrication of BCPRF implant

Bovine articular cartilage obtained from the surface of cow joints was decellularized using 5% sodium dodecylsulfate (SDS) solution for 72 hours. To produce PRF, 10 ml of rabbit blood was centrifuged at 2700 rpm for 12 minutes. The gel-shaped middle layer was then extracted as PRF. The decellularized bovine cartilage was combined with the PRF through a mechanical mixing process with ratio of 5 g decellularized bovine cartilage and 1 mL PRF. Afterwards, the result went through the lyophilization process (freeze drying) for 2x24 hours. The outcome was a porous compact implant that was divided into 2x1x0.2 cm size (Figure 1).

Figure 1. Macroscopic appearance of BCPRF implants showing porous compact texture

Harvesting autologous cartilage

Autologous cartilage was obtained from rabbit auricle (2x1 cm in size, without its perichondrium layer), as seen in Figure 2. The dimension (length, width, and thickness) was then measured using a micrometer. The donor site was closed by primary intention using nylon.

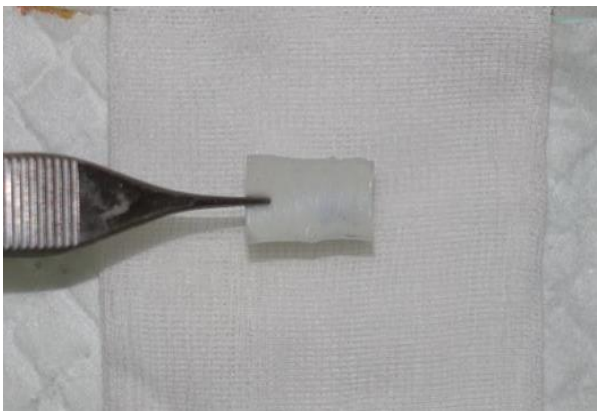

Figure 2. Autologous cartilage harvested from auricular region

Subcutaneous implantation

Two experimental groups, each consisted of 12 New Zealand male white rabbit (*Oryctolagus cuniculus)* weighed 3-3.5 kg, were prepared to undergo subcutaneous implantation using the BCPRF implant and autologous cartilage respectively. The number of samples for each group was 24 samples from 12 rabbits.

In the first group, BCPRF were implanted subcutaneously in the back area of the rabbits by creating a pocket. The implant sites were treated by primary intention using nylon. In the second group, autologous cartilages were implanted using the same method (Figure 3). Those implants were maintained for six weeks. Injection of 200 mg cefazoline was given intramuscularly once a day for three days after the implantation. The drop-out criteria included the emersion of signs of infection in the implant sites, implant extrusion, implant loss, and death of the rabbit.

Harvesting

After implantation, the implant and peri-implant tissue were then harvested from both experimental groups. The specimen volume was measured using VisiTrak for the length and width, while the thickness was measured histopathologically. The specimens were then preserved in 10% neutral-buffered formalin (NBF) solution.

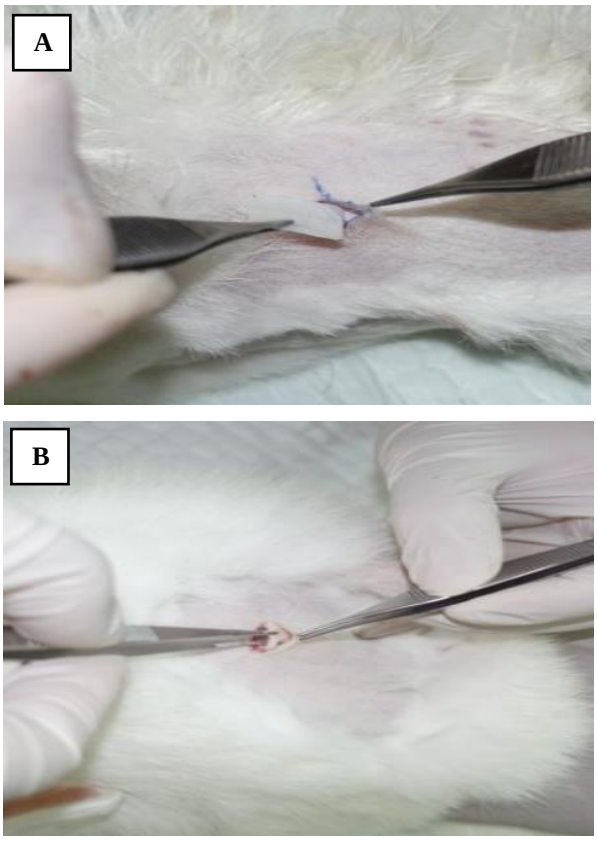

Figure 3. (A) subcutaneous implantation of autologous auricular cartilage; (B) similar process involving BCPRF implant

Evaluation and statistics

There are three parameters of chondroregenerative potential, i.e. the amount of chondrocyte formation, the thickness of type II collagen, and implant resorption rate, were assessed in a histopathological examination. The chondrocyte formation was counted after a hematoxylin-eosin staining from three fields of view

with 400x magnification. The thickness of type II collagen was measured by immunohistochemical staining with 100x magnification. The implant resorption rate was known by calculating the reduction/change in implant volume pre- and post-implantation. The results were then tested statistically using the Independent t-Test and Mann-Whitney U Test with 95% confidence interval (CI).

RESULTS

During 6 weeks of observation, the results yielded 2 dead rabbits, 2 implant exposures, and 2 infected implant sites in the autologous cartilage group, so the total evaluated samples was 16. The BCPRF group lost 1 implant, so the total evaluated samples became 23.

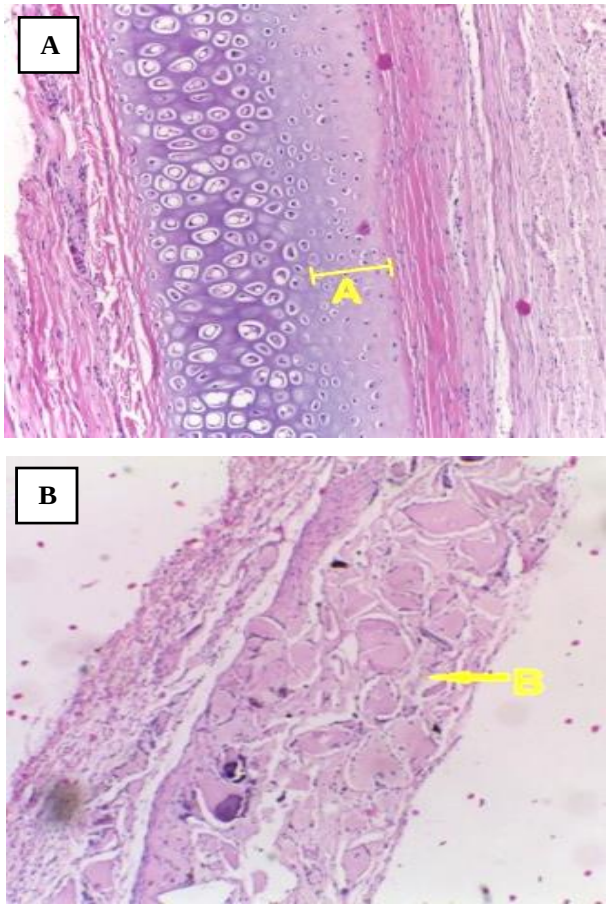

Figure 4. Hematoxyllin-eosin staining showing new chondrocyte formation in implanted autologous cartilage (A), compared to those none observed in BCPRF implantation (B)

The median of new chondrocytes from the peri-implant site was calculated from three fields of view with 400x magnification. The growth of new chondrocytes in the autologous cartilage group was 16.84±4.47 cells, meanwhile there was no growth of chondrocyte cells in the BCPRF group (Figure 4).

The thickness of type II collagen formed on the matrix between chondrocyte cells or peri-implant tissues in both groups was assessed using immunohistochemical staining with 100x magnification. In the autologous cartilage group, type II collagen thickness reached 23.05±7.59 μm. Whereas, in the BCPRF group, the thickness was 7.63±3.21 μm. Significant differences were found in the two groups (p=0.0000).

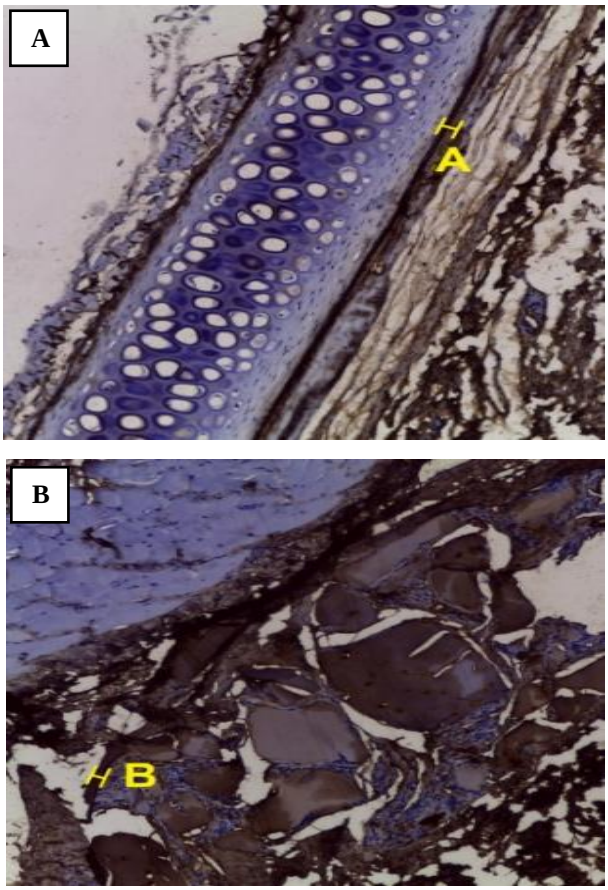

Figure 5. Immunohistochemical staining of type II collagen observed on peri-implant tissue of autologous cartilage (A) and BCPRF implant (B)

The implant resorption rate was measured by the percentage change in implant volume pre- and post-implantation. The volume was known by calculating the length, width, and thickness of the implant. In the autologous cartilage group, the percentage of resorption reached -6.94±12.86%. Whereas, in the BCPRF group, a reduction of -76.25±17.31% was obtained (Figure 6). Both were significantly different (p=0.0000).

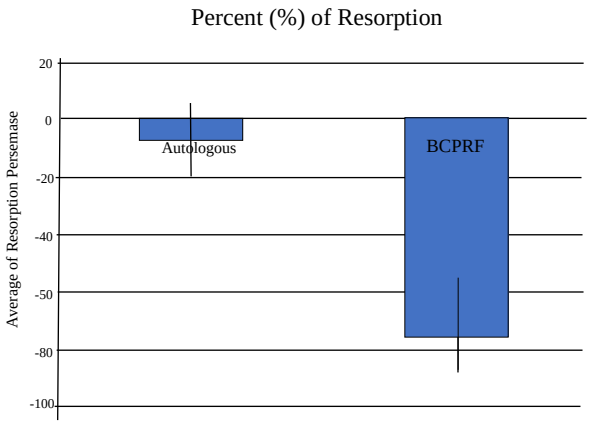

Figure 6. Rate of implant resorption comparing autologous cartilage implant (left bar) and BCPRF implant (right bar)

DISCUSSION

The results concluded that there was a significant difference in chondroregenerative capacity between autologous cartilage implant and BCPRF implant. The autologous cartilage implants proved to be superior in regenerating chondrocytes, producing chondroid matrix that was predominantly comprised of type II collagen, in addition to having lower implant resorption. In this study, autologous cartilage and BCPRF were implanted in a distant region that did not share equal characteristics with their native environments (Bimoseno et al. 2022). Subcutaneous tissue found on the back of rabbits is generally constituted of fibrocollagenous connective tissue and is devoid of cartilaginous tissue.

The ability of autologous cartilage implants to regenerate even outside of its native environment, as demonstrated in this study, may be attributed to the presence of active chondrocytes within the implants. The survival of autologous cartilage as graft stems from the process of plasma imbibition, which promotes chondrocytes to undergo a regenerative process termed as appositional growth. The recruitment of MSC to the wound of autologous cartilage implantation induces the differentiation of MSC towards the chondrocytic lineage due to an adequate differentiation signal. This signal is composed of active bone morphogenetic protein (BMP) and cartilage-derived morphogenetic protein (CDMP) molecules that are produced by active chondrocytes. The chondroid matrix found in autologous cartilage implants is optimal to direct the differentiation of MSC to form new cartilaginous structures. Adequate differentiation of MSC sustains the survival of autologous cartilage as a graft.

Complications found in this study, such as exposed (extruded) implants and infected autologous cartilage implant sites, might be caused by the fact that the skin pockets created were around the same size as the autologous cartilage implant, thus made them unable to accommodate the migration of the rigid autologous cartilage implant. This may have caused excessive surface tension of the skin suture, resulting in suture dehiscence. Surface tension may also generate inflammation and infection even though several studies claim that the use of autologous cartilage graft is relatively safer, with lower rates of infection and extrusion attributed to its minimal immune reaction (Wee et al. 2015).

The results of this study exhibited that autologous cartilage had greater regenerative potential than BCPRF implants when measured by the growth of chondrocytes, the production of type II collagen matrix, and the rate of resorption. The BCPRF implants demonstrated a relatively high rate of resorption or implant degradation, with an average volume post-implantation of 76%. In a study using polylactide (PLA) material, Odelius et al. (2011) suggested that pore size and porosity of implants are directly correlated with the degradation or resorption of implants. A highly porous implant is more likely to increase hydrolysis and degradation. Big pore size, porosity, and large network provide a good media for cell regeneration and the flow of nutrients into the scaffold (Loh & Choong 2013, Gariboldi & Best 2015).

The final process of BCPRF implant manufacturing is freeze-drying or lyophilization. This process produces microarchitecture that is dense and porous and contains relatively large pores. The high porosity of the microarchitecture allows for a greater surface area of the implant to undergo hydrolysis and degradation. This explains the high resorption and degradation rate observed in the BCPRF implants. The degradation process that occurred was hydrolytic, instead of immunologic or enzymatic. Therefore, the rates of inflammation, infection, and extrusion were lower in the BCPRF implants than the autologous cartilage. Optimal decellularization process allows the immunologic component of an implant to be omitted, thereby minimizing the degradation process that involves immune reactions.

The concept of tissue engineering triad combines the role of cells, scaffold, and morphogenic factor in the regeneration of tissue (Vinatier et al. 2009). According to the endogenous regeneration paradigm, BCPRF implants are designed to regenerate cartilaginous tissue by optimizing the recruitment of endogenous MSC into the injured/implanted area (Gulati & Poluri 2015). The endogenous MSC will differentiate to chondrogenic lineage via active morphogenetic proteins (CDMP1,

CDMP2, BMP2, BMP4, BMP6, and BMP9) that are found within the scaffold in the form of decellularized bovine cartilage matrix (Utomo & Rantam 2017). This differentiation signal is strengthened by supportive growth factors and BMP2 found in PRF. Thus, it is expected that a porous and chondroinductive scaffold media, due to its composition of morphogenic proteins and adequate growth factor signals from PRF, will influence the recruitment of MSC to the injured area and will differentiate it into mature chondrocytes.

The results of this study revealed that the regenerative capacity in the BCPRF implants, in terms of chondrocyte growth, was not proportionate to those found in the autologous cartilage implants. The lack of chondrocyte growth post-implantation of BCPRF might be attributed inadequate signals for morphogenic factors that direct the differentiation of MSC towards chondrogenesis. A study by Nakayama et al. (2003) explained that BMP4, a morphogenetic protein used during chondrogenesis, works in a dose-dependent manner. The study claimed that a greater dose of BMP4 (50 ng/mL) will stimulate greater production of cartilage. Conversely, a minimal dosage of BMP4 (5 ng/mL) is not sufficient to stimulate the formation of cartilage. This suggests that the inability of the BCPRF implants to stimulate chondrocyte production occured because of an inadequate number of morphogenetic proteins, thus resulting in a suboptimal MSC differentiation signal. This phenomenon might explain the inability of the implants to direct the differentiation of MSC. Unfortunately, this study did not evaluate the concentration of morphogenetic proteins contained within the BCPRF implants, in particular the CDMP1, CDMP2, BMP2, BMP4, BMP6, BMP9, and transforming growth factor beta (TGF-β).

The conditions explained above would influence the MSC to differentiate according to the more dominant differentiation signal, which would stimulate the formation of fibrocollagenous tissue. According to the histological examinations performed in this study, the fibrocollagenous tissue grew and invaded the porous structure of the BCPRF implants between the chondroid matrixes. The fibrocollagenous tissue was comprised of fibroblasts and collagen fibers that, upon immunohistochemical staining, also expressed type II collagen fibers. Nonetheless, the thickness of type II collagen of the fibrocollagenous tissue that grew in the BCPRF implants was not compared to the thickness of collagen produced by the autologous cartilage implants. The type II collagen matrixes that developed in the BCPRF implants were produced by fibrocollagenous tissue instead of mature chondrocytes. It may be concluded that the regeneration of tissue requires a combination of cells, scaffold, and an adequate signal of morphogenetic proteins. The role of morphogenetic proteins has often been discussed in many studies regarding tissue engineering that involves implants, be it in vivo or in vitro (Li et al. 2015, Crecente-Campo et al. 2017, Lin et al. 2019).

Nevertheless, positive results were found in this study. The growth of fibrocollagenous tissue may be favorable with optimal integration of the BCPRF implant to its surrounding tissue. Though it lacks regenerative ability, the BCPRF may be developed as an implant material that relies on fibrocollagenous tissue formation (Utomo & Sari 2019, Utomo & Yusbida 2019). BCPRF has high biocompatibility because it is made from decellularized bovine cartilage matrix. This material may be developed as an alternative to alloplastic implant material, made of porous polyethylene (Medpor), that is widely used in reconstructive and aesthetic plastic surgery. The integration of alloplastic implants relies on their ability to facilitate fibrocollagenous tissue growth within the porous internal structure. The difference lies in the mechanical stability and resorption of the implants. Redesigning the manufacturing process of BCPRF implant can produce a strong and mechanically stable implant with low resorption rate. BCPRF implant may be developed into biomaterial that can compete with alloplastic implant material. The composition of its natural material may allow BCPRF implant to be superior to its alloplastic counterpart.

Strength and limitations

This study proposed that one of the parameters used to measure chondroregenerative capacity should be the production of type II collagen matrix produced by chondrocytes. On the other hand, the implantation of BCPRF implants did not result in the growth of chondrocytes.

CONCLUSION

The chondroregenerative potentials of autologous cartilage and BCPRF differ greatly with respect to the newly regenerated chondrocyte formation, type II collagen matrix deposition, and resorption rate. However, the BCPRF is made from decellularized bovine cartilage matrix, which makes it highly biocompatible. It can be developed as an alternative to alloplastic porous polyethylene (Medpor) implant material.

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Conflict of interest

There was no conflict of interest in this researchs.

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Author contribution

Putu Ardhy Parama Widyatmika conceptualized the study, wrote and prepared the original draft, and reviewed and edited the manuscript. Muhammad Sjaifuddin Noer developed the methodology and collected the resources. Magda Rosalina Hutagalung gave the validation of the study. All authors have read and agreed to the published version of the manuscript.

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