COMPARISON OF RANKL/OPG RATIO BETWEEN COMMON CARP OPERCULUM GRAFT AND BOVINE BONE GRAFT FOR THE TREATMENT OF ORBITAL DEFECTS IN NEW ZEALAND WHITE RABBITS

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ABSTRACT

Background: Large traumatic orbital defects can cause vision defects such as enophthalmos and diplopia so that reconstruction using graft is often necessary. Autografts are the gold standard for the treatment of large orbital defects, but the availability of materials and donor morbidity often limit the usefulness of autografts. Alternative grafts, such as xenografts are relatively easy to manufacture and are metabolically inert. The hydroxyapatite content of xenografts also has osteoconductive properties. The RANKL/OPG ratio act as an indicator that determines the density of bone and can be used as a marker of bone healing in graft reconstruction.

Aims: This study aims to determine the ratio of RANKL/OPG in orbital base defects in rabbits that underwent common carp operculum and bovine bone grafting.

Method: This study is a cross-sectional study using a dataset from RT-PCR results from a previous study. The subjects were two groups of rabbits undergoing common carp operculum and bovine group grafting. The RT-PCR was performed on periosteum tissue after 30 days of graft implantation on the two groups of rabbits. Thirty days after grant implantation, enucleation was performed on the rabbits and the periosteum was sampled for RT-PCR.

Results: The ratio of RANKL/OPG in the bovine bone graft group was 4.54±6.224, and 3.75±5.326 in the carp operculum graft group. There was no statistically significant difference between the average RANKL/OPG expression in the two groups with a *p-value* of 0.841 ($p > 0.05$). The average RANKL expression in the carp operculum group was 6.03±5.668, and the bovine bone group was 9.69±8.003. There was no significant difference between the RANKL expression in the two groups, with a *p-value* of 0.428 ($p > 0.05$). The average OPG expression in the carp operculum group was 5.97±6.356, and the bovine bone group was 7.28±6.274. There was no significant difference between the RANKL expression in the two groups, with a *p-value* of 0. 752 ($p > 0.05$).

Conclusion: The RANKL/OPG ratio during the recovery of orbital defects with carp operculum graft is comparable to that of bovine bone graft.

Keywords: orbital defects, RANKL, OPG, xenograft, carp operculum graft, bovine bone graft

INTRODUCTION

Orbital defects are a complex problem that can be caused by trauma, tumor resection, congenital malformations, and degenerative processes. Orbital bone defects caused by fractures with extensive bone loss cannot heal on their own and requires surgical management. The gold standard for the management of large orbital defects that exceed critical-size defects is graft reconstruction to fill in the bony defect. In general, orbital reconstruction consists of defect exposure, the liberation of tissue around the site of defect, and the implantation of graft in the defect areas. The choice of graft material influences the success of orbital reconstruction. The gold standard for grafts is autograft bone grafts originating from the patient, but the use of autografts is often constrained by several factors, including the availability of materials, morbidity of the donor site, and immunological reactions. Therefore, the development of biomaterials for the reconstruction of orbital has gained significant attention during recent years.[1-3]

Xenografts are often considered as the alternative for autografts, due to the availability of materials. Graft materials that are widely used include grafts from bovine and pig bones. A study by Purnomo et al. (2012) showed that bovine bone bones are osteoconductive, which can facilitate cell growth and unite bone fragments in cases of bone loss due to fractures.[4,5] Bovine bone is relatively expensive in terms of cost, and if not properly processed can lead to disease transmission. Research by Rodriguez et al. (2018) points to the possibility of long-term complications due to undegraded bovine bone graft tissue.[6]

In addition to bovine bone, carp operculum can also be used as orbital graft material due to its calcium and phosphate content that are similar to humans, namely the content of hydroxyapatite (HAP), which serves as a bridge during bone regeneration with low immunogenicity. HAPs from natural materials such as carp operculum are relatively inexpensive, easy to manufacture, and have good metabolic activity and respond dynamically to human tissue. A study by Yamamoto (2015) reported a low risk of disease transmission from fish to humans.[7] Research by Kartiwa in 2018 showed a higher calcium and phosphorus content in carp operculum bones of 0.555% and 0.171%, respectively, compared to bovine bones of 0.080% calcium and 0.028% phosphorus.[8] Research by Mustafa et al. (2015) showed a stable fishbone Ca / P ratio which is almost identical to the human bone.[9] In Indonesia, the cost of carp operculum graft manufactured by the National Nuclear Energy Agency (BATAN) tissue bank is also much lower compared to bovine bone grafts.

Cox et al said that biochemical markers of bone turn over have long been used to see the quantitative changes in skeletar turn-over. These bone turnover markers are commonly subdivided into three categories: 1) Bone resorption amrkers, 2) osteoclast regulatory proteins, and 3) bone

formation markers. Bone healing consists of acute. RANKL/OPG balance is an important indicator of osteoclast activity, the healing process of the graft, and will determine bone density. Taking into account the available literature and the potential of common carp operculum as an alternative graft material in orbital bone defect reconstruction, the researcher intends to compare the RANKL/OPG ratio between common carp operculum and bovine bone grafts.

METHODS

This study is a cross-sectional study with a retrospectively obtained data from a 2018 study, approved under the number 01/IACUC-BF/IX/17 issued by the Laboratory Animal Welfare and Utilization Commission of PT. BIOFARMA (PERSERO). Data was obtained from RT-PCR results of the previous study, which uses rabbits's periosteum after 30 days of common carp operculum and bovine grafting. The minimal sample size of this study was 10, divided into two groups using the meads resource equation. The first group was common carp group and the second group was the bovine group. The inclusion criteria of this study is RT-PCR data from rabbit's periosteum after 30 days implantation of common carp and bovine grafting. Exclusion criteria was the RNA data tht cannot be processed and uncomplete data.

RANKL and OPG expression were measured using RT-PCR using OPG and RANKL primer, with statistical analysis using independent samples t-test for RANKL and OPG expression separately, and Mann-Whitney test for RANKL/OPG ratio.

RESULTS

The normality of data distribution was tested using the Shapiro-Wilk test as a preliminary statistical analysis. The results of the normality test of RANKL and OPG distribution in the bovine bone graft group and common carp operculum are described in Table 3.1.

Table 3.1 Normality Test Results of RANKL and OPG Expression Data in Bovine Bone Graft Group and Common Carp Operculum Graft Group

Variable	Group	*p*	Data Distribution
OPG Normality	Bovine bone	0.477	Normal
RANKL Normality	Bovine bone	0.802	Normal
OPG Normality	Carp operculum	0.293	Normal
RANKL Normality	Carp operculum	0.405	Normal

The results of the normality test showed that data from all groups were normally distributed, and are suitable for analysis using independent t-test for RANKL and OPG expression separately. The results of the independent t-test for RANKL expression are described in Table 3.2.

Table 3.2 Comparison of RANKL Expression in Bovine Bone Graft Group and Common Carp Operculum Graft Group using Independent t-test

Variable	Group		p
	Bovine Bone (n = 5)	Common Carp Operculum (n = 5)	
RANKL			
Mean±Std	9.69±8.003	6.03±5.668	
Median	8.64	5.12	0.428
Range (min-max)	0.95-20.56	0.65-15.26	

Table 3.2 showed that the average expression of RANKL in the bovine bone graft group was 9.69±8.003, and the average expression of RANKL in the common carp operculum graft was 6.03±5.668. The independent t-test showed no statistically different results between the two groups with a p-value of 0.428 (> 0.05).

Table 3.3 Comparison of OPG Expression in Bovine Bone Graft Group and Common Carp Operculum Graft Group using Independent t-test

Variable	Group		p
	Bovine bone (n = 5)	Common Carp Operculum (n = 5)	
OPG			
Mean±Std	7.28±6.274	5.97±6.356	
Median	9.47	3.18	0.434
Range (min-max)	0.99-14.86	0.49-15.33	

Table 3.2 showed that the average expression of OPG in the bovine bone graft group was 8.95±5.026, and the average expression of RANKL in the common carp operculum graft was 5.97±6.356. The independent t-test showed no statistically different results between the two groups with a p-value of 0.434 (> 0.05). The RANKL/OPG ratio comparison between the two groups was tested using the Mann-Whitney test, with results as described in Table 3.4.

Table 3.4 RANKL/OPG Ratio Comparison Between Bovine Bone Graft Group and Common Carp Operculum Group using Mann-Whitney Test

Variable	Group		p
	Bovine Bone (n = 5)	Common Carp Operculum (n = 5)	
RANKL/OPG Ratio			
Mean±Std	4.54±6.22	3.75±5.32	
Median	1.96	0.70	0.841
Range (min-max)	0.06-15.4	0.20-12.61	

Note: For numerical data, the p-value was tested with independent t-test for normally distributed data, and otherwise using Mann-Whitney test. Significance levels were based on p <0.05. Asterisk (*) sign shows p < 0.05 or statistically significant results.

The results described in Table 3.4 showed a RANKL/OPG ratio in the bovine bone graft group of 4.54±6.224 and a RANKL/OPG ratio of 3.75±5.326 in the common carp operculum group. Mann-Whitney test was used because the data for RANKL/OPG ratio was not normally distributed. The results of the Mann-Whitney test showed no statistically significant difference between the two groups with a p-value of 0.841 (> 0.05).

DISCUSSION

Large orbital defects due to fractures that exceed critical size cannot heal spontaneously. Orbital defects can cause diplopia and eyeball position problems, which can interfere with the quality of life. The management of large orbital defects that exceed critical size is reconstruction using grafts to fill the missing bone. The gold standard for graft reconstruction is autogenous bone grafts, but this approach has several disadvantages, including difficulty in obtaining materials, morbidity on the donor site, and immunological reactions.[1,3, 10] Xenograft from bovine ribs and common carp operculum bone with calcium and phosphate in the form of hydroxyapatite can be used as alternative materials for bone grafts. Xenograft materials are available in large quantities to meet the needs of graft products. Bovine bone xenograft with a Ca/P ratio of 4.8/2.8 is widely used in bone reconstruction, especially in the field of oral surgery, because of low biodegradation and good osteoconductive ability. Another xenograft that can be used is common carp operculum bone which has a Ca/P ratio of 1.67.[4, 5, 11]

A previous study by Kartiwa et al. (2017) is an initial study examining the morphology of common carp operculum using Scanning Electron Microscopy (SEM). Xenograft examination of bovine ribs was used as a comparison. The study showed that the carp operculum bone could be used as an alternative graft for the reconstruction of orbital bone defects. Carp operculum bones have large pores of >200 microns and small pores of <10 microns which are good characteristics for scaffolding. The calcium and phosphate content in the bones of carp

operculum is higher than that of bovine bones and has a more stable ratio of around 1.67. The study also showed that the expression of RANKL as a marker of bone resorption in the carp operculum group was lower than that of bovine bone.[8] This could indicate a lower level of biodegradation in bovine bones compared to common carp operculum, according to research by Rodriguez et al.[6, 11, 12] Previous studies were done by making broad orbital defects that exceeded the critical size in rabbit models. Rabbit model was used because of the fast bone turnover and mineral content similar to humans. Examination of RANKL and OPG gene expression was performed after 30 days of implantation on the orbital defect using RT-PCR. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is a sensitive examination and can detect minute changes in gene expression. RT-PCR is easy to perform and can produce fast and accurate genetic measurements.[13, 14]

This study uses normalized data from previous research samples. The results of this study indicate that the expression of RANKL and OPG in the common carp operculum bone group is lower than that of bovine bone group, but there is no statistically significant difference, so it can be concluded that the expression of RANKL and OPG in orbital bone defects after the implantation of common carp operculum graft is equivalent to that of bovine bone graft, with p values of 0.428 and 0.434. Bone remodeling occurs continuously in both physiological and pathological conditions. The healing process of bones after grafting occurs through a remodeling process consisting of osteoclastogenesis, where the graft is degraded by osteoclasts and followed by osteoblastogenesis, namely the deposition of new bone by osteoblast cells. Histological studies have shown that the use of biomaterials in bone defects will induce foreign body reactions and activate osteoclasts. Osteoclast activity lasts for 3-9 months after implantation, which will replace the bone with new bone. Bone resorption by osteoclasts is influenced by signals from the RANKL-RANK-OPG system. In recent years, RANKL and OPG are considered important signals for osteoclast differentiation which plays an active role in the process of bone remodeling and growth. RANKL is a type 2 homotrimeric protein produced by osteoblasts. The results of this study show that RANKL expression is higher in bovine bones, implying a higher resorption rate of bovine bones caused by higher mineral content in bovine bones.

OPG expression in common carp operculum is lower than that of bovine bone, but this may be due to the response to compensate for RANKL which serves to balance bone resorption and formation. Balance between RANKL/OPG will determine bone density, formation rate, and resorption rate. If the ratio >1 indicates bone resorption and indicates formation if the ratio < 1. The desired ratio is balance beetween RANKL and OPG. In this study, the RANKL / OPG ratio in the common carp operculum group

was lower than that of bovine bones in the fourth week (mean ± std, 3.75 ± 5,326; 4.54 ± 6.224). This difference can be attributed to the higher calcium and phosphate content in common carp operculum with a more stable Ca/P ratio so that in the fourth week the resorption process and bone formation in the common carp operculum group are closer to balance compared to the bovine bone group.[13, 15-18] Research by Kartiwa compared the expression of matrix metalloproteinase-2 (MMP-2) in bovine bone graft and common carp operculum graft. MMP-2 is an enzyme for the degradation of the extracellular matrix. The results of the study showed that the mean expression of MMP-2 was not statistically different in both groups, which meant that the implantation of common carp operculum graft was comparable to that of bovine bone graft. The expression of RANKL as a marker of osteoclast activity in the two study groups is in accordance with the results of the study by Kartiwa, which means that the resorption process in the two groups is comparable.[19]

This study shows that common carp operculum and bovine bone xenografts have potential as scaffolds for large orbital defect reconstruction. The limitations of this study include the low quantity of samples and the one-time examination of RANKL and OPG, which limits the results of RANKL/OPG ratio only to one point in time. The formation of new bone on the defect area, degraded xenograft particles, and rejection reaction against the xenograft are not evaluated in this study.

CONCLUSIONS

The RANKL/OPG ratio in orbital bone defects after the implantation of common carp operculum graft and bovine bone graft is equivalent.

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