OPG and RANKL Expression in Osteoblast Culture after Application of Osphronemus Gourami Fish Scale Collagen Peptide

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Abstract

Periodontal disease can be treated with surgical procedures using bone graft. This research investigated the potency of Osphronemus gourami fish scales as bone graft material. Scaffold derived from collagen is employed in tissue engineering because of its biocompatibility, high porosity, low antigenicity, and ability to be resorbed by tissue. The study aims to analyse the potential effect of gourami fish-scale collagen peptide on expression of Osteoprotegerin (OPG) and Receptor Activator of Nuclear Factor-κB Ligand (RANKL) in osteoblast cell culture. This research constituted a study of rat calvaria osteoblasts by post-test only controls group design. Fish scale collagen derived from gourami was hydrolysed into collagen peptides, before being applied to osteoblasts culture. Application of fish scale collagen at concentrations of 0.0125 mg/ml, 0.05 mg/ml, 0.2 mg/ml, and 0.8 mg/ml in osteoblast culture was observed at 24 hours, 48 hours and 72 hours. Statistical analysis used a One Way Anova test with a significance level of 5%. Our study showed there was a significant difference in the expression of OPG at time observation intervals of 24 hours (p=0.002), 48 hours (p=0.001), and 72 hours (p=0.019) between groups. There is no significant difference in the expression of RANKL at an observation time. Gourami fish-scale collagen peptide administration on osteoblast cell culture enhance the expression of OPG and RANKL.

Experimental article (J Int Dent Med Res 2021; 14(2): 618-622)

Keywords: Collagen, Osphronemus gourami fish scales, OPG, infectious Disease, RANKL.

Received date: 10 February 2021 **Accept date:** 22 April 2021

Introduction

Collagen is the most promising material for tissue engineering due to its biocompatibility and biodegradability. Collagen scaffold must meet several basic requirements, such as high porosity, appropriate pore size, adequate cell-adhesion properties, differentiation, and proliferation with desirable mechanical integrity to maintain the predesigned tissue structure, non-cytotoxicity and osteoconductivity.[1,2] One body part of a fish containing collagen is the scales. Research conducted by Nagai *et al.*[3] states that fish scales are a potential alternative source of collagen. Biocompatibility, in vitro cytotoxicity and viability tests indicated that collagen derived from fish scales did not induce cytotoxic effects and possessed good cell viability.

Osteogenesis need bone forming cells for healing process.[4,5] Osteogenesis is induced by osteoblasts which promote cell attachment and proliferation followed by the expression of osteoblast phenotype.[6] Collagen contains specific cell adhesion domains, including the arginine-glycine-aspartic acid (RGD) motif. After the integrin receptor on the cell surface binds to the RGD motif on the collagen molecule, cell adhesion is actively induced. This interaction contributes to the promotion of cell growth and differentiation and the regulation of various cell functions, included osteoblasts.[7,8] Osteoblasts produce collagen type 1, proteoglycan and cytokines that effect the bone remodeling process. Cytokines produced by osteoblasts include receptor activators of nuclear factor-κβ ligand (RANKL) and osteoprotegerin (OPG). Interaction between OPG and RANKL can activate osteoblasts and induce osteogenesis.

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Currently, fish collagen has been widely researched and used in the fields of food, cosmetics and medicine. However, the use of fish collagen, especially from fish scales, for research in the field of biomaterials, especially bone regeneration, is still not widely carried out. Previous study found that gourami fish-scale collagen peptide was save for baby hamster kidney fibroblasts-21 culture[9], and osteoblast cell culture[10]. gourami fish-scale collagen peptide also induced vascular endothelial growth factor and bone morphogenetic protein expression on osteoblast cell culture.[11] The aim of this study is to analyze the ability of osteogenic differentiation of osteoblast cell culture after administration different concentration of gourami fish-scale collagen peptide by OPG and RANKL expression.

Materials and methods

Ethical clearance.

This study incorporated an experimental laboratory with post-test only control group design. Ethical clearance for the research was obtained from the Health Research Ethical Clearance Commission (No.278/HRECC.FODM/X/ 2018).

Extraction of Osphronemus gourami fish scale collagen.

Fish scale collagen is extracted using a combination of chemical and enzymatic methods.[12,13] Osphronemus gourami fish scales were washed with water until clean and dried under the sun. Osphronemus gourami fish scales were soaked in 1 M NaOH solution at 4° C for 24 hours to remove non-collagen protein. Every eight hours, the NaOH solution is replaced with stirring occasionally being carried out before it was washed with distilled water to neutralize pH and soaked in isobutane to remove any fat present. A chelating agent (EDTA) 1N was then added to induce decalcification followed by the addition of 0.5M of acetic acid (acid solubility collagen) and 0.1 gr of the enzyme pepsin (pepsin solubility collagen). The solution was then stirred with an ultrasonic device at 4° C before screening and the addition of 0.5 M NaCl. Centrifugation in small tubes at 4000 rpm and subsequent washing with distilled water and salting out was completed. A lyophilization process was completed which involved using a freeze dryer with a condenser temperature of -76° C and ambient temperature of 23.6° C for 12 hours to remove water content. Finally, the results of collagen extract of carp scales were sterilized.

Hydrolysis of Osphronemus gourami fish scales collagen.

A sterile tube containing 250mg of fish scales collagen, 20mg of enzyme papain and sodium phosphate buffer liquid (0.1 M, pH 7.5) and 250mg /5ml of concentrated fish scale collagen) was placed in an orbita shaker at 55^0 C and 50 rpm for 120 hours. Denaturation process and inactivation by enzyme in water bath (75^0C, 10 minutes). The result of the hydrolysis of Osphronemus gourami fish scales collagen is peptide collagen.[14]

Calvaria rat osteoblast cell culture.

Preparation of primary osteoblast culture from calvaria in two day- old rats involved washing with phosphate buffer saline (PBS) and cutting into small pieces with scissors before crushing five times with collagenase for ten minutes at 37^0C. Supernatant was taken from each cut and transferred to fetal bovine serum (FBS). The collected 3.4 and 5 supernatants were centrifuged at 1500 rpm for five minutes and subsequently resuspended in basic medium culture (α-modified Eagle's medium (α-MEM) with 15% FBS, penicillin G (50U / mL) and streptomycin (50µg / mL). The cells were maintained in basic medium culture and passaged three times before being used for experimentation in the supplemented medium.

Examination of OPG and RANKL Expressions by Immunocytochemical Method.

A cover slip was placed on a 24-well plate. Approximately 25,000 osteoblasts were planted into the well plate causing confluence of around 60-70% the following day. The cell culture media was discarded and the cells rinsed twice with PBS. 1.0 ml of methanol at -20oC was added to the well plate and stored at -20°C for 5-10 minutes. Methanol was removed and carefully added to 1.0 ml of PBS on a well plate before being agitated slowly and thoroughly for ten minutes. 0.5ml of 0.05% saponin was added to the well plate for 15 minutes at room temperature. The cells were washed with PBS three times for three minutes on each occasion and subsequently blocked with buffers (serum from the same species as secondary antibodies) for 30-60 minutes. Primary antibodies were diluted (1µl antibodies to 200µl serum-PBS) and the

resulting solutions added to each well plate and incubated at room temperature for one hour. Cells were washed with PBS for three minutes on each occasion. Diluted secondary antibodies (1µl antibodies to 200µl serum-PBS) were added and then incubated at room temperature for 30-60 minutes. Cells were washed with PBS three times, for three minutes on each occasion prior to immunocytochemical staining.

Statistical analysis of data.

The data obtained was analyzed statistically with a Statistical Package for the Social Sciences Software 21.0 edition (IBM™, Illinois, Chicago, USA) by the one-way analysis of variance (ANOVA) ($P < 0.05$) based on a Shapiro–Wilk normality test and a Levene's variance homogeneity test ($P > 0.05$). Tukey HSD test was done for post hoc test.

Results

The positive expression of OPG in osteoblast showed brown cells (Figure 1). The highest expression of OPG was found in the 0.2 mg/ml concentration group (E3) after 72 hours observation. In contrast, during observation at 24 hours the highest average OPG was found in the 0.8 mg/ml concentration group. The data obtained was normal and homogeneous distributed ($P > 0.05$). There was a significant increase in the expression of OPG in E3 group comparing to control on 72 hours observation (table 1).

Group	OPG expression					
	Control	0.0125 mg/ml (E1)	0.05 mg/ml (E2)	0,2 mg/ml (E3)	0.8 mg/ml (E4)	One-way ANOVA (*P*)
24 hours	7,42 ± 0,76	9,08 ± 1,62 †	13,83 ± 2,77 †	15,17 ± 2,99 †a	15,67 ± 1,53 †a	0.02*
48 hours	12,33 ± 2,02	23,17 ± 4,16 †	28,67 ± 1,38 †	29,33 ± 3,55 †	29,67 ± 1,53 †	0.00*
72 hours	13,67 ± 3,78	27,08 ± 7,15	30,75 ± 6,08 †	31,58 ± 3,33 †	34,33 ± 7,21†	0.01*

Table 1. Mean ± standard deviation, one-way analysis of variance test and post hoc analysis result of OPG expression between groups.
*Significant at $p<0.05$, †Significant at $p<0.05$ comparing to control group on same observation time, [a]Significant at $p<0.05$ comparing to E1 group on same observation time, ANOVA: analysis of variance, OPG: Osteoprotegerin.

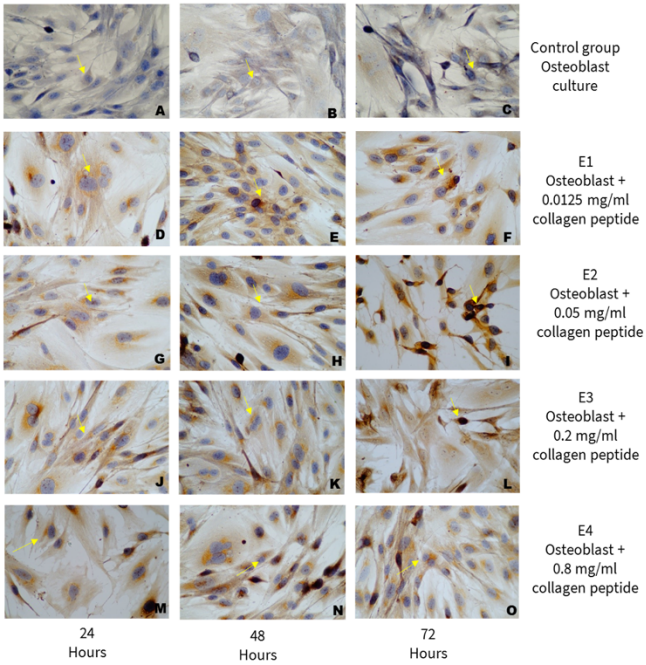

Figure 1. OPG expression in osteoblast cell culture after administration of gourami fish scales with various concentration and time of observation. Positive OPG expression marked in brown (yellow arrow) at 400x magnification.

The positive expression of RANKL in osteoblast showed brown cells (Figure 2). The highest expression of RANKL after 72 hours observation was found the 0.8 mg/ml concentration group (E4), but there is no significantly difference of RANKL expression between group on the same observation time (table 2).

Group	RANKL expression					
	Control	0.0125 mg/ml (E1)	0.05 mg/ml (E2)	0,2 mg/ml (E3)	0.8 mg/ml (E4)	One-way ANOVA (*P*)
24 hours	7,42 ± 1,94	10,41 ± 1,37	12,50 ± 2,82	13,92 ± 3,18	14,33 ± 3,21	0.05
48 hours	11,42 ± 2,38	12,25 ± 2,65	14,33 ± 3,36	14,75 ± 3,03	16,00 ± 3,61	0.31
72 hours	11,28 ± 3,78	13,67 ± 7,15	15,00 ± 2,41	15,48 ± 1,08	17,00 ± 2,00	0.18

Table 2. Mean ± standard deviation, one-way analysis of variance test and post hoc analysis result of OPG expression between groups.
ANOVA: analysis of variance, RANKL: Receptor Activator of Nuclear Factor Kβ-Ligand.

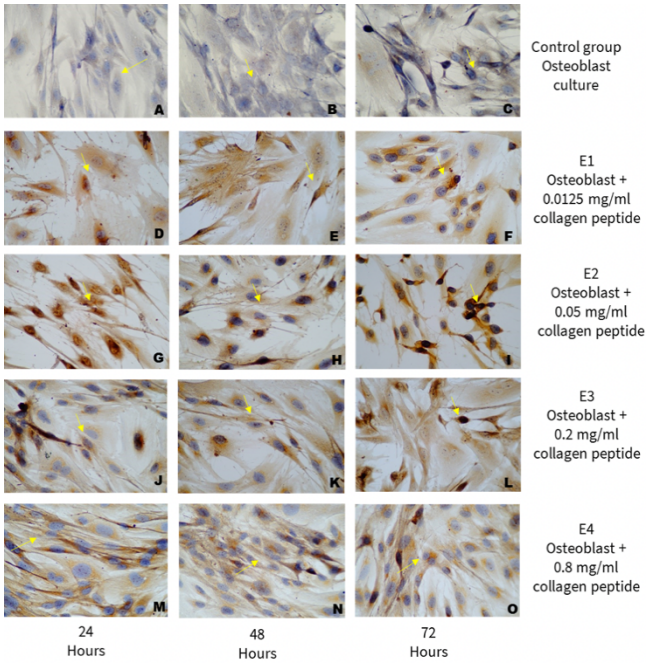

Figure 2. RANKL expression in osteoblast cell culture after administration of gourami scales with various concentration and time of observation. Positive OPG expression marked in brown (yellow arrow) at 400x magnification.

Discussion

This in vitro study shown that the OPG and RANKL expression increased in proportion to the increase of the collagen concentration and also observation time during initial phase of osteogenesis. However, OPG expression was greater than RANKL expression in the application of gouramy scales collagen to osteoblast cell culture. This is consistent with the statements of Boeyce et al[15] and Belibasakis[16] that the OPG and RANKL expressions will always be inversely related.

This in vitro research employed primary osteoblasts from rat calvaria. Osteoblasts are mononucleated cells, derived from mesenchymal stem cells and responsible for the synthesis and mineralization of bone during initial bone formation and later bone remodeling. Osteoblasts also play a role in the regulation of osteoclast activity through RANKL and OPG.[17] In bone homeostasis, OPG as decoy receptor binds RANKL thereby inhibiting RANKL interaction with RANK. Interaction between OPG and RANKL can activate osteoblasts and stimulate osteogenesis.[18]

A significant difference existed between the OPG expression observed at 24 hours and that observed at 48 and 72 hours. However, there was no significant difference in expression of RANKL observed at 48 hours and 72 hours. These findings show that the concentration of fish scale collagen in osteoblast culture enhance osteoblast growth, while differentiation is directly proportional with OPG enhancement and the possibility of increased RANKL production 24 hours after application of fish scale collagen. Observation at 72 hours indicated that OPG and RANKL expression was still increasing, although not significantly. This was due to the aging of the osteoblasts which can affect the capacity of the proliferation and differentiation of osteoblasts to produce OPG and RANKL.[7]

Collagen contains specific cell adhesion domains, including the arginine-glycine-aspartic acid (RGD) motif. After the integrin receptor (α1β1) on the cell surface binds to the RGD motif on the collagen molecule, cell adhesion is actively induced. This interaction contributes to the promotion of cell growth and the differentiation and regulation of osteoblasts. Mature osteoblasts can produce OPG and RANKL.[7,8]

In the process of differentiation of monocyte cell derivatives into mature osteoclasts, there is an interaction between osteoblasts and osteoclasts. This process requires RANKL which plays an important role in the development and activation of osteoclasts and plays a role in regulating bone remodelling. In implication, RANKL stimulates the fusion of mononuclear precursor cells to multinuclear, which stimulates differentiation into mature osteoclasts, adhesions to the bone surface and induces bone resorption activity, and is able to maintain osteoclast life by slowing down the occurrence of apoptosis.[16] RANKL is expressed mostly by osteoblasts and mesenchymal layer cells. RANKL is also expressed by periosteal cells, chondrocytes, endothelial cells and active T cells. Next, RANKL will interact with RANK on the surface of osteoclast progenitor cells to stimulate cell differentiation. In addition, OPG can act as a decoy-receptor to inhibit osteoclast formation by binding to RANKL, thereby preventing the interaction between RANKL and RANK on osteoclast progenitors and inhibiting the process of osteoclastogenesis.[8,15]

The comparison of OPG and RANKL expression is determined by the average fish

scale concentration at the same observation time. The average OPG expression is greater than RANKL expression, whereas over-expression of OPG reduces osteoclast formation with the result that the potential interaction of OPG and RANKL will increase and activate osteoblasts. Osteoblast activation can enhance osteogenesis.[15–17] Fish scale collagen can stimulate the differentiation of osteogenic in mesenchymal stem cell (MSC) and inhibit MSC modulation of RANKL by enhancing OPG expression.[19,20] The expression of OPG and RANKL is always inversely proportional.

Conclusions

The expression of OPG and RANKL is enhanced after the application of Osphronemus gourami fish scale collagen in osteoblast culture.

Acknowledgements

The authors would like to thank the Faculty of Dental Medicine Universitas Airlangga for its support of this research. Financial disclosure; The research was funded by Universitas Airlangga.

Declaration of Interest

The authors declared that there is no conflict of interest

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