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Short Communication

Type 1 Collagen from Scales of *Osphronemus gouramy* Enhances Osteocalcin Expression: An *In Vivo* Study

Noer Ulfah[1,2]*, Chiquita Prahasanti[2], Theresia I. Budhy[3], Apriani W. Nelly[4], Esi Y. Fitrina[4], Zalfaa F. Yuda[5]

[1]*Doctoral Student of Dental Medicine Science, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*
[2]*Department Periodontology, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*
[3]*Department Pathology, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*
[4]*Periodontology Specialist Study Program, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*
[5]*Dental Medicine Education Study Program, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia*

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ABSTRACT

Type 1 collagen is the predominant component of the extracellular matrix and serves as a scaffold in tissue engineering. Type 1 collagen fibrils constitute the primary organic component of scales, mimicking their function in bone. Osteocalcin expression provides a definitive marker for the terminal phase of the formation of bone. This study aimed to evaluate the effect of the application of gourami fish scales on osteocalcin expression. Twenty-seven experimental animals were allocated into the control group, the CHA group, and the group receiving collagen derived from gourami fish scales. Osteocalcin expression was detected 14 days later using immunohistochemical examination. The mean and standard deviation of osteoclastin expression after 14 days of using gourami fish scales were 16.56 and 3.909, respectively. The study of the One-Way ANOVA test yielded a significant value of 0.000 ($p < 0.05$), and the Tukey HSD test results with Multiple Comparisons reveal significant differences among the three groups. The group collagen in gourami fish scales exhibited the highest expression of osteocalcin. The application of collagen to gouramy fish scales (*Osphronemus gouramy*) may enhance osteocalcin expression.

Keywords: Gourami Fish Scale, Type 1 Collagen, Osteocalcin, Bone Tissue Engineering .

Introduction

Tissue engineering is an interdisciplinary arena that merges engineering ideas with science to construct biological materials intended at restoring, preserving, or improving tissue functionality.[1] Scaffold matrix, a component of bone tissue engineering, serves as a template for tissue formation and is usually drawn to cells and growth agents.[2,3] The most suitable scaffold for bone tissue engineering must be biocompatible, osteoinductive, and osteoconductive. As a result, it can reproduce the features of bone tissue.[3] Type 1 collagen is the most prominent element of the extracellular matrix (ECM) and serves as a scaffold, promoting the migration of cells, tissue regeneration, and wound healing. Analogous to bone extracellular matrix, which is abundant in type 1 collagen, collagen is a crucial component in bone tissue engineering, as collagen-based scaffolds deliver the necessary biological signals for cell proliferation, adhesion, orientation, and eliciting chemotactic responses.[4] Collagen possesses significant potential as a biomaterial for bone tissue engineering due to its abundance availablity, high porosity, biocompatibility, capacity to integrate with other substances, hydrophilicity, ease of processing, bioabsorbability, and low antigenicity.[3,4]

*Corresponding author. Email: noer-u@fkg.unair.ac.id
Tel: +62 8385993001

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Freshwater fish, particularly the gourami fish (*Osphronemus gouramy*), are distinguished by their flat and large bodies, yellowish-silvery ventral side, and reddish-brown dorsal surface.[5] Fish scales contain osteoblasts and osteoclasts, identical to those found in higher vertebrates, yet the control of cellular activity in their tissue is still unclear.[6] Gourami fish scales are comparable to teeth and bones in that they are composed mainly of three elements: water, hydroxyapatite (a mineral), and collagen (an organic component).[7] Fish scales are rich in protein, and keratin and collagen, particularly type 1 collagen fibrils, are commonly found in gourami fish scales.[8] Similar to how type 1 collagen fibrils function in bones, they are the main organic component of fish scales.[9]

Bone formation markers correspond to osteoblast activity and are generated by active osteoblasts during the developmental phase. The activity of bone-specific alkaline phosphatase isoform, type 1 amino procollagen concentration, and osteocalcin concentration can be considered indicators of bone development.[10] Osteocalcin, also known as bone GLA protein (BGP), is a protein specific to bone that acts as a sensitive and specific marker of osteoblast activity in various metabolic bone disorders. The synthesis of osteocalcin depends on vitamin D and vitamin K for converting the carboxylation products of three glutamate residues into gamma-carboxyglutamate (Gla).[11] Osteocalcin is primarily produced by osteoblasts during bone formation, with most of the secreted osteocalcin retained in the extracellular matrix (ECM).[12] Meanwhile, odontoblasts also produce osteocalcin in limited amounts.[13] Similar to other secreted proteins, osteocalcin experiences signal sequence removal in the rough endoplasmic reticulum, forming pro-osteocalcin. Before osteoblast secretion, specific glutamic acid residues are carboxylated via a vitamin K-dependent enzymatic mechanism, forming Gla.[12] After propeptide cleavage and secretion, most native osteocalcin is incorporated into the mineralized matrix, aided by calcium binding from Gla residues. Conversely, approximately 10% to 30% of the osteocalcin synthesized by osteoblasts is released into the

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bloodstream. This research evaluated the increase in osteocalcin expression resulting from the application of collagen obtained from gourami fish scales (*Osphronemus gouramy*).[13]

Materials and Methods

Animals

Male albino rats (*Rattus norvegicus*) of the Wistar strain, 2 - 3 months old, and weighing between 200 - 250 grams were used in this post-test-only *in vivo* study. The rats were obtained from the Biochemistry Experimental Animal Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. They were kept in well-ventilated cages, and fed with Hi Pro Vite brand pelleted diet (PT Charoen Co., Ltd. Pokwang No. 591) and had free access to drinking water.

Ethical approval

Ethical Clearance with reference number 756/HRECC.FODM/X/2022 was obtained from the Ethics Commission for Health Research, Faculty of Dental Medicine, Universitas Airlangga, Indonesia.

Extraction of collagen

Gourami fish were obtained from local supplier in Surabaya, East Java, Indonesia. They fish (1 kg) were submerged in 6% acetic acid 9100 mL) for seven days to acquire their scales. After the scales have expanded, they were rinsed under flowing water until the pH indicator paper indicates a neutral pH has been attained. The resulting collagen was dehydrated by freeze-drying, and the dehydrated collagen was stabilized by exposure to γ-ray irradiation.[14,15]

Study design

The Lemeshow technique was used to analyze the sample size for this study, which yielded a sample size of nine for each group after which the samples were randomly collected, and divided into three groups: Control group, carbonated hydroxyapatite (CHA) group, and gourami fish scales group.[15]

The mandibular incisor extraction socket in the control group was filled with blood. CHA composite was filled into the mandibular incisor extraction socket of the CHA group. Gourami fish scale collagen was administered to the extraction socket of the mandible incisor of the third group. After 14 days, osteocalcin expression was detected using by immunohistochemical examination.

Determination of osteocalcin expression

On day 14, euthanasia was performed using chloroform. Both mandibular central incisors and the surrounding tissue were harvested and fixed in 10% buffered formalin solution for at least 24 hours. The tissue was sectioned to a thickness of 2-4 mm and further fixed in 10% neutral buffered formalin (NBF) for 3 days to preserve the integrity of the tissue. The tissue was then dehydrated by immersion in 70% alcohol for approximately 15 minutes, followed by 80% alcohol for 1 hour, 95% alcohol for 2 hours, and repeated twice with 95% alcohol for 1 hour each, then placed in ethanol for 1 hour. The tissue was then infiltrated twice with paraffin at 56-58°C for 2 hours, followed by treatment with xylene for 1 hour, and repeated once with xylene for 2 hours. Paraffin blocks were embedded and sectioned at a thickness of 4 µm using a microtome. The sections containing alveolar tissue were then placed on a hot plate at 30°C to 35°C for at least 12 hours for proper tissue processing. Deparaffinization was performed using xylene, followed by hydration with graded alcohol and washing with Tris-buffered saline (TBS) for 10 minutes, followed by rinsing with water. The preparations were then mounted and observed under a Nikon H600L light microscope. The expression of osteocalcin was assessed by counting the number of epithelial cells in the gingival tissue that expressed osteocalcin, using immunohistochemical (IHC) staining at ×400 magnification.

Statistical analysis

Data were analyzed by One-Way analysis of variance (ANOVA) using the statistical package for the social sciences (SPSS IBM 29 Software, 2021). The Tukey HSD test was used to assess the differences between the intervention and control groups. In each analysis, P-value less than 0.05 was considered statistically significant.[16,17]

Results and Discussion

Osteocalcin expression

The osteocalcin expression values for each group on day 14 are shown in Table 1 and Figure 1. The Shapiro-Wilk test results for all groups showed a p-value > 0.05, demonstrating the normal distribution of the data. The homogeneity test applied the Levene test at a significance level of $p > 0.05$, showing that all data were the same. The Oneway ANOVA test indicated a significant difference ($p < 0.05$) between the groups. Multiple comparisons using Tukey HSD test also revealed that there were significant difference between the gourami fish scales groups, CHA, and control group. This study sought to determine osteocalcin expression after administration of collagen derived from gourami fish scales. Mature osteoblasts demonstrated considerable expression of osteocalcin, a marker specific to bone tissue and cells within the osteoblastic lineage. Osteocalcin expression is regulated during specific embryonic stages of osteoblast phenotypic differentiation. Osteocalcin is a late marker for mature osteoblasts; however, it is absent during the initial stages of osteoblastic development.[15] Osteocalcin expression is stimulated *in vitro* at the onset of mineralization, producing additional osteoblast markers, including type I collagen and alkaline phosphatase. The expression of osteocalcin is a characteristic hallmark of the final phase of bone formation.[12] Collagen, a naturally occurring biomaterial, possesses osteoinductive and osteoconductive properties, making it an ideal scaffold for facilitating cell penetration and new bone formation.

Table 1: Osteocalcin expression levels after 14 days

Variable	Value		
	Control group	**CHA**	**Gourami fish scales**
Osteocalcin	3.89 ± 1.364	9.22 ± 1.856	16.56 ± 3.909

Values are mean ± standard deviation.

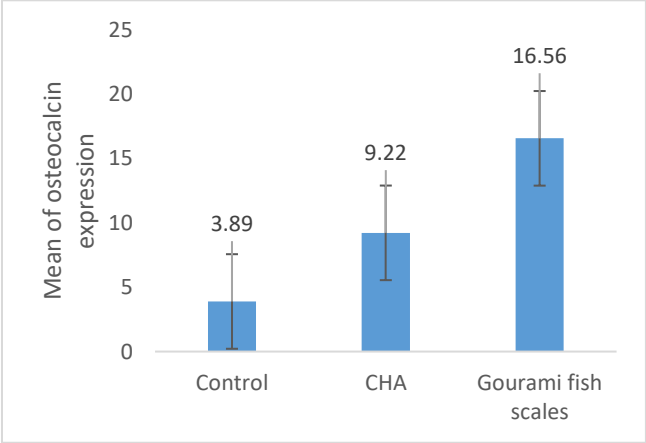

Figure 1: Osteocalcin expression chart

Scanning electron microscopy

Scanning electron microscopy (SEM) examination indicated that the collagen extract from gourami fish scales exhibited a structure resembling a meshwork of interwoven fibres with intervening pores. The three-dimensional structure of the collagen scaffold is designed to closely match the geometry of bone components, thereby promoting vascularization growth and creating an optimal environment for bone formation.[14,18] Porosity, which is geometrically fully connected within a highly porous structure to facilitate cell growth and distribution, and the capacity to support neovascularization from the surrounding tissue, is essential for scaffolds.

Porosity size is a critical factor, as tiny pore scaffolds may result in pore closure by cells, thereby hindering cellular penetration, extracellular matrix formation, and neovascularization within the scaffold. The optimal range of pore diameters for bone tissue engineering is 200-900 µm. The SEM results demonstrated that the collagen extract from gourami fish scales exhibited pore sizes ranging from 191.6 to 385.3 µm, consistent with the specifications for scaffolding in tissue engineering.[19,20]

This study indicated a phase of bone development marked by osteocalcin expression on day 14. Compared to the control and CHA groups, the average osteocalcin expression in the collagen group derived from gourami fish scales significantly increased ($p < 0.05$). The scales of gourami fish are composed of type 1 collagen, exhibiting osteoinductive and osteoconductive properties. This collagen is a structural scaffold for connective tissue and guides the maturation of new bone from osteoprogenitor cells.[21] Type 1 collagen promotes chondrocyte adhesion, proliferation, and differentiation, as well as the activity of osteoblasts and osteoclasts, indicating its osteoinductive properties. Type 1 collagen interacts with other extracellular matrix proteins, including cell surface integrins.[22] Collagen protein plays a role in the phenotypic differentiation of osteoblasts, as well as in cell adhesion and proliferation. Type 1 collagen protein is considered an early indicator of osteoblastic differentiation and is essential for the osteoblast phenotype.[19,23] Thus, it has demonstrated promise as a scaffold in bone tissue engineering.

A previous study found that the collagen isolated from gourami fish scales totally broke down in approximately seven days. Optimizing this degradation rate is necessary in order to employ the most suitable scaffold. Since the body is supposed to absorb the scaffold, regulated resorption is essential to make room for the development of new bone tissue.[22,24] This study supports previous research showing that the collagen from gourami fish scales increases the bone-formation biomarker osteocalcin.[25]

Conclusion

The findings from this study has shown that the expression of osteocalcin after the administration of collagen derived from gourami fish scales is higher compared to the control and CHA groups. Thus, it can be concluded that collagen derived from gourami fish scales enhances osteocalcin expression, a marker for bone formation. Further research using different markers should be taken to strengthen the potency of gourami fish scales in enhancing bone formation.

Conflict of Interest

Authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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