Viability of BHK-21 Fibroblast Cells Resulting from Synthesis of Collagen Powder (*Thunnus albacares*) with Variation of NaCl Concentration

Kartika Theresia Zefanya, Fitria Rahmitasari**, Widaningsih***, Widyasri Prananingrum***

** Student of Faculty of Dentistry, Universitas Hang Tuah, Surabaya, Indonesia*
*** Department of Dental Materials, Faculty of Dentistry, Universitas Hang Tuah, Surabaya, Indonesia*
**** Department of Prosthodontics, Faculty of Dentistry, Universitas Hang Tuah, Surabaya, Indonesia*

Correspondence: ***fitria.rahmitasari@hangtuah.ac.id***

Received 19 June 2024; 1st revision 1 July 2024; 2nd revision 18 July 2024; Accepted 19 July 2024; Published online 31 July 2024

Keywords:

Collagen, Thunnus Albacares, Papain Soluble, Nacl, Viability

ABSTRACT

Background: *Collagen plays a crucial role in post-extraction wound healing. Thunnus albacares skin, a collagen source synthesized through the papain soluble collagen (PaSC) method with varying NaCl concentrations, is assessed for non-toxicity using the MTT assay on BHK-21 fibroblast cell cultures. This research is to determine cell viability resulting from synthesizing collagen powder from the skin of yellowfin tuna (Thunnus albacares) with varying NaCl concentrations using the papain soluble collagen (PaSC) method.*

Method: *Collagen powder was synthesized by cleaning Thunnus albacares skin measuring 1x1 cm, soaking it in 0.1 M NaOH, hydrolyzing it with CH_3COOH, extracting it with papain enzyme, and then dividing the samples into four groups: without NaCl, NaCl 0.9 M, NaCl 1.3 M, and NaCl 1.7 M. Samples were centrifuged (6000 rpm) and followed by freeze-drying. Cell viability was obtained by conducting a cytotoxicity test using the MTT Assay method on BHK-21 fibroblast cells.*

Result: *The percentage of cell viability in groups K, P_1, P_2, P_3, and P_4 were 100%, 10.708%, 113.750%, 107.833%, and 105.958%, respectively. The Kruskall-Wallis test yielded a significance value 0.000, indicating a significant difference ($p<0,005$). The Mann-Whitney test confirmed significant differences between groups.*

Conclusion: *Collagen powder from Thunnus albacares skin with NaCl concentrations of 0.9 M, 1.3 M, and 1.7 M showed no toxic effects, while the group without NaCl showed toxic effects. Collagen powder with a NaCl concentration of 1.7 M yielded ideal results and showed no toxic effects.*

Copyright ©2024 National Research and Innovation Agency. This is an open access article under the CC BY-SA license (https://creativecommons.org/licenses/by-sa/4.0/).

doi: http://dx.doi.org/10.30659/odj.11.1.94-104
2460-4119 / 2354-5992 ©2024 National Research and Innovation Agency
This is an open access article under the CC BY-SA license (https://creativecommons.org/licenses/by-sa/4.0/)
Odonto : Dental Journal accredited as Sinta 2 Journal (https://sinta.kemdikbud.go.id/journals/profile/3200)
How to Cite: *Zefanya et al*. Viability of BHK-21 Fibroblast Cells Resulting from Synthesis of Collagen Powder (Thunnus albacares) with Variation of NaCl Concentration. Odonto: Dental Journal, v.11, n.1, p.94-104, July 2024.

INTRODUCTION

Collagen is one of several important components in the body in the form of structural proteins that are in the extracellular matrix and can be found in large quantities in the body's connective tissues such as skin, ligaments, bones, tendons, and cartilages[1]. Collagen is employed in tissue engineering endeavors due to its favorable biocompatibility, biodegradability, and minimal antigenic properties[2]. Collagen has a role in the field of dentistry in healing post-extraction wounds that create tooth socket formation. Collagen also participates in forming new tissue and alveolar bone healing that encompasses various cellular and molecular activities, such as the breakdown and reconstruction of both soft and hard tissues in the form of scaffold, reducing postoperative pain and swelling[3,4]. Collagen can reduce bleeding while accelerating the wound healing process which can be obtained in the form of plug, sponge, scaffold, drug delivery carrier, microfibrillar, and matrix[5]. Collagen can be made into scaffold that is useful for bone regeneration bone regeneration because it can be absorbed by the body and has good attachment to cells[6].

In general, collagen can be obtained from cattle, chicken, mammals, pigs, and birds[7]. Marine biota can be used as an option as a source of collagen with good prospects because its use is not limited to all religions and does not cause infectious diseases[8]. Fisheries waste is currently generated quite a lot around the world so that the utilization of these wastes is currently increasing to increase economic value, utilization in the field of biotechnology, and the desire to minimize the amount of waste in society[8].

This study uses the skin of yellowfin tuna (*Thunnus albacares*) which will be utilized as a source of collagen because it contains glycine, glutamic acid, proline, arginine, hydroxyproline, alanine, serine, and aspartic acid in large quantities so that it has the potential to be a halal source of collagen[9,10]. The protein content of yellowfin tuna skin (*Thunnus albacares*) is higher than other body parts at 37.32%, while the meat is 28.34%, the egg is 22.83%, and the swim bubble is 17.52%[11].

Yellowfin tuna (*Thunnus albacares*) has the highest catch than other tuna species with 69% of the 1,297,062 tons of tuna catch from 2004 to 2011 in Indonesia[12]. Therefore, this study used the skin of yellowfin tuna (*Thunnus albacares*) as the main source of collagen powder.

The collagen extraction method used in this study is an enzymatic extraction method, namely papain soluble collagen (PaSC) because the amount of collagen obtained during fish skin extraction is more than extraction by the acid method[13]. Papain enzyme comes from papaya plants that can be found in many places and already has a halal certificate because the source taken comes from plants and is easy to obtain[14].

The requirement for a material to be implemented in the oral cavity is to have biocompatibility and not disturb the surrounding tissue so that it is necessary to conduct a cytotoxicity test as an evaluation of dental materials to obtain cell viability[15]. The cytotoxicity test performed in this study was the MTT assay method by calculating the total reduction of MTT salt with a wavelength of 595 nm which has a yellow color to formazan which has a purple color by the respiratory chain in mitochondria, namely tetrazolium succinate reductase[16].

The media used for the cytotoxicity test in this study were BHK-21 (Baby Hamster Kidney-21) fibroblast cells because BHK-21 fibroblast cells originated from embryos so they have advantages such

as good stability, not easy to mutate, easy to culture and grow[17,18]. A material is said to be non-toxic if after calculating the percentage of cell viability > 50% and is said to be toxic if the percentage of cell viability < 50%[19].

The addition of NaCl is done for precipitation process before centrifugation to obtain a precipitate[20]. Utilization of salt with high concentration can precipitate proteins that act as wet collagen precipitates by attracting water that binds protein molecules[21]. Giving NaCl with various concentrations aims to get the best collagen extract results so it is necessary to determine the appropriate NaCl concentration in the collagen precipitation process[22]. The addition of NaCl with a high concentration can damage and become toxic due to changes in osmotic power due to high Na ions[23].

RESEARCH METHOD

The research used was true experimental laboratories research with posttest-only control group design. The number of groups in this research were 5 consisting of the control group, without NaCl, NaCl 0.9 M, NaCl 1.3 M, NaCl 1.7 M and each group consisted of 6 samples so that the total number of samples was 30. This research was conducted in April 2023-January 2024 at Mas Junet Fresh Fish Shop located in Mergan Market Malang, Widya Mandala Catholic University Surabaya Pharmacy Laboratory, Hang Tuah University Surabaya Chemistry Laboratory, and Surabaya Pharmaceutical Veterinary Center Laboratory (PUSVETMA).

The tools used in this study are cutting board, knife, analytical balance, micropipette, beaker, glass jar, sieve, basin, glass stirring rod, erlenmeyer, measuring cup, 6000 rpm centrifugator, 10 ml conical tube, 5 L jerry can, freeze dryer, specimen container, 4 ºC refrigerator, magnetic stirrer, conical tube, 96 plates mold, laminar flow, UV stelirizer, ELISA reader, and syringe.

The materials needed in this study are yellowfin tuna skin (*Thunnus albacares*), papain enzyme with activity of 30,000 USP/mg (EC. 3.4.22. 2), NaOH, NaCl, CH_3COOH, aquabidest, whatman filter paper, 14 kDa dialysis bag, flannel cloth, water, pH indicator paper, fibroblast cell line Baby Hamster Kidney-21 (BHK-21) from PUSVETMA Surabaya, Eagle's Minimum Essential Medium (MEM), phosphate buffer saline (PBS), dimethylsulfoxide (DMSO), kanamycin, penicillin streptomycin, foetal bovine serum (FBS), fungizone.

Sample preparation

This study begins with sample preparation by collecting the skin of yellowfin tuna (*Thunnus albacares*) and making sure there is no meat attached to the fish skin. Then the skin was cleaned with running water until clean and cut into small square parts with a size of 1x1 cm.

Pre-treatment

Next, the sample pre-treatment stage was carried out by soaking the 1x1 cm yellowfin tuna (*Thunnus albacares*) skin in a 0.1 M NaOH solution in a ratio of 1:10 (b/v) for 24 hours and replacing the NaOH solution every 8 hours at a temperature of 10 ºC. Rinse the skin of yellowfin tuna (*Thunnus albacares*) with aquabidest until it reaches neutral pH (pH 7-7.5).

Hydrolysis and extraction

Then the hydrolysis and extraction stage was carried out by mixing papain enzyme (EC 3.4.22.2) with a concentration of 10,000 U/g followed by mixing 0.5 M CH_3COOH solution. Soaking was carried out for 72 hours (3 days). The filtrate was filtered with Whatman filter paper and flannel cloth and divided into 4 groups with equal amount of solution and precipitation process with NaCl in groups 2 to 4 with concentrations of 0.9 M, 1.3 M, and 1.7 M for 24 hours. Each group was put into a 10 ml conical tube for centrifugation with a 6000 rpm centrifugator for 1 hour and collect the pellet. The dialysis process was carried out with aquabidest with a 14 kDa dialysis bag for 24 hours to dismiss the other chemical materials and then collected in the specimen container.

Freeze dry

Wet collagen was stored in a 4℃ refrigerator for 24 hours and then freeze dried with a freeze dryer (Labfreeze FD-12-MR) for 72 hours to obtain dry collagen powder.

Viability test

Cytotoxicity test was performed by sterilizing collagen powder using UV sterilizer for 30 minutes. Then the first column well was filled with BHK-21 fibroblast cells in Eagle's minimum essential medium (MEM) culture medium, kanamycin, penicilinn streptomycin 1%, foetal bovine serum (FBS) 10%, and fungizone 100 units/ml as much as 100 µl in each well as cell control. Wells in the twelfth column were filled with Eagle's minimum essential medium (MEM), kanamycin, penicilinn streptomycin 1%, foetal bovine serum (FBS) 10%, and fungizone 100 units/ml in 100 µl in each well as media control.

Wells in the 2nd-5th columns were filled with BHK-21 cells at a density of $3x10^3$ in Eagle's minimum essential medium (MEM) culture medium, kanamycin, penicilinn streptomycin 1%, foetal bovine serum (FBS) 10%, and fungizone 100 units/ml at 100 µl in each well. Yellowfin tuna skin (*Thunnus albacares*) collagen powder was added into each well as much as 50 µl in each of the 2nd, 3rd, 4th, and 5th columns. Perform the incubation process with 5% CO_2 for 20 hours at 37ºC.

Then remove the microplate from the incubator and cell culture media. Remove the culture media and collagen powder in the well with the help of a syringe so that the BHK-21 cells remain in the well. Each well is refilled with 100 µl of cell culture media. Perform MTT and Phosphate Buffer Saline (PBS) filtration with 0.20 µl millipore in each well and incubate for 3 hours in the hope that MTT can be metabolically active. Total incubation time in 37ºC incubator is 24 hours. Take MTT and culture media with syringe. Then add DMSO 50 µl to each well to dissolve the formazan crystals and then shaker the microplate for 5 minutes.

Read the optical density (OD) value of formazan using an ELISA reader with a wavelength of 620 nm. Calculate the percentage of total BHK-21 fibroblast cell viability using the cell viability formula:

$$\%\ \text{Viability of cells} = \frac{\text{OD treatment} - \text{OD media control}}{\text{OD cell control} - \text{OD media control}} \times 100\%$$

Data analysis

The data obtained will be statistically analyzed using SPSS 27 with a significant value used is (α=0.05). The research data were analyzed using the Shapiro Wilk normality test and the Levene test

for homogeneity test. In this study, the data were not normally distributed and not homogeneous so that non-parametric tests were carried out with the Kruskall-Wallis test and the Mann-Whitney test was carried out.

RESULTS

The results obtained the viability value of collagen powder from yellowfin tuna skin (*Thunnus albacares*) with various NaCl concentrations using the papain soluble collagen (PaSC) method against BHK-21 fibroblast cells, by looking at the absorbance value using the MTT Assay method.

Table 1. Mean absorbance value, standard deviation, and percentage of live cells of yellowfin tuna skin collagen powder (*Thunnus albacares*) using MTT Assay method.

Group	Replication	Mean ± Standard Deviation
K	6	100.000% ± 0.000
P_1	6	10.708% ± 6.901
P_2	6	113.750% ± 7.682
P_3	6	107.833% ± 4.203
P_4	6	105.958% ± 5.816

Description:
K = Control group containing BHK-21 fibroblast cells.
P1 = Treatment 1 (BHK-21 fibroblast cells treated with *Thunnus albacares* skin collagen powder without NaCl administration).
P2 = Treatment 2 (BHK-21 fibroblast cells treated with *Thunnus albacares* skin collagen powder with 0.9 M NaCl concentration).
P3 = Treatment 3 (BHK-21 fibroblast cells treated with *Thunnus albacares* skin collagen powder with 1.3 M NaCl concentration).
P4 = Treatment 4 (BHK-21 fibroblast cells treated with *Thunnus albacares* skin collagen powder with 1.7 M NaCl concentration).
Mean (%) = The average value of the number of living cells in each group after the MTT test in percentage form

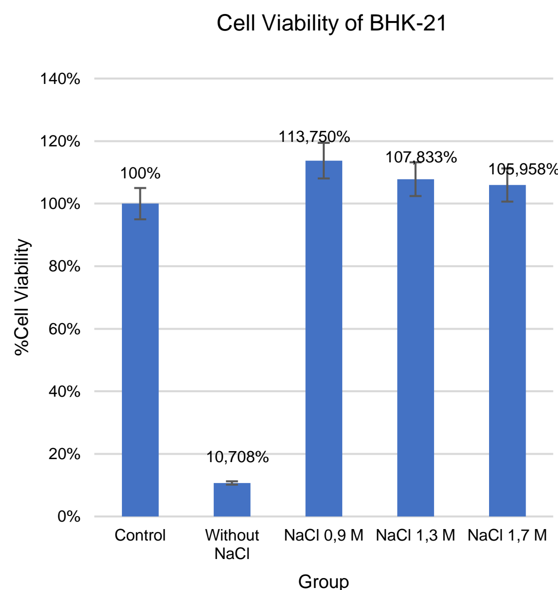


Figure 1. Mean cell viability values of BHK-21 fibroblasts

Based on the results of the study, it is known that the group that has the highest fibroblast cell viability value (%) is the NaCl 0.9 M group (113,750%) while the group that has the lowest fibroblast cell viability value (%) is the group without NaCl (10,708%).

The Saphiro-Wilk test was used as a normality test because the number of samples used in this study was less than 50 samples. The normality test results showed that group K had a significant value of <0.05 while in groups P1, P2, P3 and P4 had a significant value of p>0.05. This indicates that the cell viability test results of yellowfin tuna skin collagen powder (*Thunnus albacares*) against BHK-21 fibroblast cells are not normally distributed so that it can be continued with non-parametric tests.

Based on the results of the homogeneity test using the Levene test, the significance value is 0.006 (p>0.05), which means that the results of the cell viability test of yellowfin tuna skin collagen powder (*Thunnus albacares*) against BHK-21 cells are not homogeneous so that it can be continued with a non-parametric test, namely the Kruskall-Wallis test and the Mann-Whitney test.

Kruskall-Wallis test was conducted to determine the difference in cell viability between all groups of yellowfin tuna skin collagen powder (*Thunnus albacares*) with varying NaCl concentration using papain soluble collagen (PaSC) method and cell control group.

Table 2. Kruskall-Wallis test results

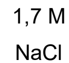
Kruskall-Wallis Test	***Df***	***Asymp.Sig.***
21.709	4	0.000

Table 2 shows the results of the Kruskall-Wallis test which shows p=0.000 (p<0.05) which can be concluded that there are significant differences between each treatment group. Then the Mann-Whitney test can be done to determine the significant difference between the viability of BHK-21 fibroblast cells with groups K, P1, P2, P3, and P4.

Significant differences in each group can be done by performing the Mann-Whitney test with a significance value of p<0.05.

Table 3. Mann-Whitney test results

	Control	Without NaCl	0,9 M NaCl	1,3 M NaCl	1,7 M NaCl
Control		0.002*	0.007*	0.002*	0.040*
Without NaCl			0.004*	0.004*	0.0-4*
0,9 M NaCl				0.109	0.055
1,3 M NaCl					0.810



Based on **Table 3**, it is known that there is a significant difference ($p<0.05$) between the control group and groups P1, P2, P3, and P4, group P1 and groups K, P2, P3, and P4, group P2 and groups K and P1, group P3 and group K and P1, group K and P1. There is no significant difference between groups P2 with P3 and P4. There is no significant difference between group P3 and P4.

DISCUSSION

This study generally aims to determine the differences in cell viability of the synthesized *Thunnus albacares* skin collagen powder with variations in NaCl concentration using the papain soluble collagen (PaSC) method. There is a difference in the average percentage of living cells in group K (100%) when compared to groups P1 (10.708%), P2 (113.750%), P3 (107.833%), P4 (105.958%) in the form of the number of living cells due to the treatment of collagen powder so that there is a reaction on the number of fibroblast cells that affect cell toxicity.

Based on the results of the study, it shows that there is a significant difference between group K (100%) and P1 (10.708%) which can be caused by group K is a control group of cells not given treatment and only contains BHK-21 fibroblast cells so that no changes occur when reading absorbance values and calculating cell viability. Group P1 (without NaCl) has a lower percentage of cell viability than the control group because in group P1 no NaCl was given during the synthesis process. This causes the collagen powder of yellowfin tuna skin (*Thunnus albacares*) to remain in acidic condition due to the provision of acetic acid at the hydrolysis stage. NaCl has a function in purifying collagen which is soluble in acid. The absence of NaCl causes the collagen to have an acidic pH (pH <7) because there is no purification process of collagen by NaCl. The hydrolysis process with acetic acid causes collagen to degrade and the solvent becomes toxic because the effect of acidic pH in the MTT Assay test causes an increase in toxicity to fibroblast cells because the cells do not proliferate[24]. Acidic pH levels in collagen cause cells to undergo a protein denaturation process so that damage occurs between intramolecular disulfide covalent bonds with hydrophobic bonds, ionic bonds, and hydrogen bonds so that low cell viability is obtained[25].

Based on the results of the study, it shows that there is a significant difference between group K (100%) with P2 (113.750%), P3 (107.833%), and P4 (105.958%) which can be caused by group K which is a cell control group that only contains BHK-21 fibroblast cells so that cell viability is 100%. Collagen powder in groups P2 (113.750%), P3 (107.833%), and P4 (105.958%) had more cell viability than group K 100% which means the ability of fibroblast cells to proliferate is high because the dose of collagen given to cells provides a good therapeutic effect. Research has shown that collagen, as a biomaterial, can promote the attachment and proliferation of cells and tissues[2]. Another influence that makes the cells proliferate highly is that the collagen powder is given NaCl during the collagen extraction process so that there is a precipitation process in the form of wet collagen precipitation which gives an influence on cell viability. Giving NaCl with various concentrations causes collagen to undergo a

purification process by NaCl so that collagen precipitates with a pH close to neutral and has a higher amino acid content.

The results showed a significant difference between group P1 (10.708%) and group P2 (113.750%) because in group P1 the sample was not given NaCl during the collagen extraction process, so that the collagen powder of yellowfin tuna skin (*Thunnus albacares*) remained in acidic conditions due to the provision of acetic acid at the hydrolysis stage. Acetic acid that is still contained in the sample causes collagen to degrade and the solvent becomes toxic because the effect of acidic pH on collagen causes cells to undergo a protein denaturation process so that damage occurs between intramolecular disulfide covalent bonds with hydrophobic bonds, ionic bonds, and hydrogen bonds so that low cell viability is obtained[25]. Group P2 (NaCl 0.9 M) has a high cell proliferation ability because the dose of collagen given to the cells provides a good therapeutic effect. Giving a concentration of 0.9 M NaCl is a concentration of salt administration that is not too high so that changes in osmotic power due to the amount of Na ions are low and do not make collagen toxic but rather produce higher cell viability and make cells proliferate high.

The results showed that there was a significant difference between group P1 (10.708%) and P3 (107.833%). In group P1, the sample was not given NaCl during the collagen extraction process, so the collagen powder of yellowfin tuna skin (*Thunnus albacares*) remained in acidic condition due to the provision of acetic acid at the hydrolysis stage. Acetic acid that is still contained in the sample causes collagen to degrade and the solvent becomes toxic because the effect of acidic pH on collagen causes cells to undergo a protein denaturation process so that damage occurs between intramolecular covalent disulfide bonds with hydrophobic bonds, ionic bonds, and hydrogen bonds so that low cell viability is obtained[25]. Group P3 (NaCl 1.3 M) has a high cell proliferation ability because the dose of collagen given to the cells provides a good therapeutic effect. Another influence that makes the cells proliferate highly is that the collagen powder is given NaCl during the collagen extraction process so that there is a precipitation process in the form of wet collagen precipitation which gives an influence on cell viability. Giving 1.3 M NaCl concentration is a concentration of salt that is not too high so that changes in osmotic power due to the amount of Na ions are not too high and do not make collagen toxic but rather produce higher cell viability and make cells proliferate high.

The results showed a significant difference between group P1 (10.708%) and group P4 (105.958%). The significant difference can be caused because in group P1 the sample was not given NaCl during the collagen extraction process, so that the collagen powder of yellowfin tuna skin (*Thunnus albacares*) remained in acidic condition due to the provision of acetic acid at the hydrolysis stage. The absence of NaCl caused the collagen to have an acidic pH (pH <7) because there was no purification process of collagen by NaCl. Group P4 (NaCl 1.7 M) produced collagen powder that could dissolve well and homogeneous during the MTT Assay test which was conducted by administering NaCl 1.7 M. Group P4 (NaCl 1.7 M) has a high cell proliferation ability because the dose of collagen given to the cells gives a good therapeutic effect. Another influence that makes the cells proliferate highly is that the collagen powder is given NaCl during the collagen extraction process so that there is a precipitation process in the form of wet collagen precipitation which gives an influence on cell viability. Giving NaCl with various concentrations causes collagen to undergo a purification process by NaCl so that collagen

precipitate is obtained with a pH close to neutral and has a higher amino acid content. Giving a concentration of 1.7 M NaCl is a concentration of salt that is high enough but still safe so that changes in osmotic power due to the number of Na ions are higher than the P1 and P2 groups but does not make collagen toxic but results in higher cell viability and makes cells proliferate high.

In this study, it was found that the P2 (NaCl 0.9 M), P3 (NaCl 1.3 M), and P4 (1.7 M) groups had differences in the average percentage of cell viability but there were no significant differences and had high cell viability of >50%, which means that the three groups were not toxic[26]. This is due to the provision of NaCl concentrations that are not too high but the higher the concentration of NaCl can cause cell viability to decrease. Giving too high a concentration of NaCl can reduce cell viability, damage, and become toxic due to high Na ions[23,27]. Na ions cannot be maintained in cells at high concentrations because they can cause toxicity in metabolism. Meanwhile, the content of amino acids such as proline as an osmotic agent that can control and as an osmotic agent in collagen makes the proline act as a negative charge against the Na ions[28].

Another influencing factor is that the higher the concentration of NaCl given, the larger the particle size produced, while the group that was not given NaCl produced a small particle size. The smaller the particle size it can reduce cell viability and increase the number of dead cells because it makes it easier for these particles to penetrate the intercellular space and cause rupture of the cell wall[29]. The resulting particle size can affect cell viability in the MTT Assay test conducted on yellowfin tuna skin collagen powder (*Thunnus albacares*). The smaller the particle size, the lower the cell viability and the larger the particle size, the higher the cell viability.

Other factors that can affect the results of the percentage of cell viability besides particle size are the concentration of electrolyte solution (NaCl), type of solvent (CH_3COOH), temperature, pH, and the presence of external intruders that can survive with various temperatures and water pressure such as bacterial and fungal microorganisms and other factors that occur during the process of making collagen powder[30]. Collagen powder is said to have good quality if the color produced has a white or near white base color.

The results of this study showed that although there were significant differences between groups K, P1, P2, P3, and P4, but in the P2, P3, and P4 treatment groups showed the results of a high percentage of BHK-21 fibroblast cell viability and more than 50%, namely 113.750%, 107.833%, and 105.958% but in the P1 treatment group showed the results of a low percentage of BHK-21 fibroblast cell viability and less than 50%, namely 10.708%. This proves that the P1 group is toxic and unsafe to use while the P2, P3, and P4 groups are not toxic and indicate that the P2, P3, and P4 groups of yellowfin tuna skin collagen powder (*Thunnus albacares*) with variations in NaCl concentration using the papain soluble collagen (PaSC) method do not have toxic effects and are safe to use and qualify according to SNI standards.

CONCLUSION

Collagen powder synthesized from yellowfin tuna skin (*Thunnus albacares*) treated with variations in NaCl concentration of 1.7 M using the papain soluble collagen (PaSC) method is the best group with an average percentage of BHK-21 fibroblast cells viability of 105.958% than other group.

ACKNOWLEDGEMENT

In the end, the researchers would like to express gratitude to those who have helped in completing this article, they are Faculty of Dentistry, Hang Tuah University and the teaching staffs.

REFERENCES

1. Jafari H, Lista A, Siekapen MM, et al. Fish collagen: Extraction, characterization, and applications for biomaterials engineering. *Polymers (Basel)*. 2020;12(10):1-37.
2. Rahmitasari F, Rahayu RP, Munadziroh E. The Chitosan-chicken Shank Collagen Used as Scaffold Through Lymphocyte Cell Proliferation in Bone Regeneration Process. *Acta Med Philipp*. 2022;56(8):43-48.
3. Santosa AH, Kintawati S, Sugiaman VK. Effect of Resorbable Collagen Plug (RCP) on Extraction Wound Healing. *e-GiGi*. 2022;10(1):81.
4. Sularsih S, Mulawarmanti D, Rahmitasari F, Siswodihardjo S. In Silico Analysis of Glycosaminoglycan-Acemannan as a Scaffold Material on Alveolar Bone Healing. *Eur J Dent*. 2022;16(3):643-647.
5. Binlateh T, Thammanichanon P, Rittipakorn P, Thinsathid N, Jitprasertwong P. Collagen-Based Biomaterials in Periodontal Regeneration: Current Applications and Future Perspectives of Plant-Based Collagen. *Biomimetics*. 2022;7(2):1-16.
6. Rahmitasari F. Scaffold 3D kitosan dan kolagen sebagai graft pada kasus kerusakan tulang. *Jurnal Material Kedokteran Gigi*. 2016;5(2):1-7.
7. Sembiring TES, Reo AR, Onibala H, et al. Ekstraksi Kolagen Tulang Ikan Tuna (Thunnus sp) DENGAN ASAM KLORIDA. *Media Teknologi Hasil Perikanan*. 2020;8(3):107-110.
8. Coppola D, Oliviero M, Vitale GA, et al. Marine collagen from alternative and sustainable sources: Extraction, processing and applications. *Mar Drugs*. 2020;18(214):1-23.
9. Handaratri A, Hudha MI. Ekstraksi Kolagen dari Ikan Tuna Sirip Kuning dengan Bantuan Microwave (Microwave-Assisted Collagen Extraction of Yellowfin Tuna). *Reka Buana : Jurnal Ilmiah Teknik Sipil dan Teknik Kimia*. 2021;6(2):104-111.
10. Kusa SR, Naiu AS, Yusuf N. Karakteristik Kolagen Kulit Tuna Sirip Kuning (*Thunnus albacares*) pada Waktu Hidro-Ekstraksi Berbeda dan Potensinya dalam Bentuk Sediaan Nanokolagen. *Media Teknologi Hasil Perikanan*. 2022;10(2):107-116.
11. Hadinoto S, Idrus S. Proporsi dan Kadar Proksimat Bagian Tubuh Ikan Tuna Ekor Kuning (*Thunnus Albacares*) dari Perairan Maluku. *Majalah BIAM*. 2018;14(02):51-57.
12. Agustina M, Setyadji B, Prawira D, Tampubolon ARP. Perikanan Tuna Sirip Kuning (Thunnus albacares Bonnaterre, 1788) pada Armada Tonda di Samudera Hindia Selatan Jawa. *Bawal Widya Riset Perikanan Tangkap (BAWAL)*. 2019;11(3):161-173.
13. Cahyono H, Trilaksani W, Uju. Karakteristik Karakteristik Fisikokimia Papain Soluble Collagen dari Gelembung Renang Ikan Tuna (Thunnus sp.). *J Pengolah Has Perikan Indones*. 2022;25(1):1-17.
14. Nurjanah, Baharuddin TI, Nurhayati T. Ekstraksi Kolagen Kulit Ikan Tuna Sirip Kuning (Thunnus albacares) menggunakan Enzim Pepsin dan Papain. *J Pengolah Has Perikan Indones*. 2021;24(2):174-187.
15. Aini NQ, Agustantina TH, Rianti D. Uji sitotoksisitas seng oksida ekstrak Allium sativum Linn terhadap sel fibroblas gingiva manusia. *Jurnal Material Kedokteran Gigi*. 2018;7(2):37-44.
16. Zulfa E, Susilowati S, Budiarti A. Uji Sitotoksisitas Ekstrak Metanol Umbi Bit (*Beta vulgaris L. var. rubra L.*) Terhadap Cell Line T47D. *Jurnal Ilmu Farmasi dan Farmasi Klinik*. 2015;12(1):20-25.
17. Fitriani F, Subiwahjudi A, Soetojo A, Yuanita T. *Sitotoksisitas Ekstrak Kulit Kakao (Theobroma Cacao) Terhadap Kultur Sel Fibroblas BHK-21*. Vol 9.
18. Emilda Y, Budipramana E, Kuntari DS. Uji toksisitas ekstrak bawang putih (*Allium Sativum*) terhadap kultur sel fibroblast. *Dental Journal Majalah Kedokteran Gigi*. 2014;47(4):215-219.
19. Cevanti TA, Aprilia. Sitotoksisitas Ekstrak Kulit Batang Spesies Mangrove Rhizopora Mucronata Terhadap Sel Ginggival Mesenchymal Stem Cells Sebagai Bahan Medikamen Saluran Akar Secara Invitro. 2017:1-8.
20. Hukmi NMM, Sarbon NM. Isolation and characterization of acid soluble collagen (ASC) and pepsin soluble collagen (PSC) extracted from silver catfish (Pangasius sp.) skin. *Int Food Res J*. 2018;25(6):2601-2607.
21. Ata ST, Yulianty R, Sami FJ, Ramli N. Isolasi Kolagen Dari Kulit Dan Tulang Ikan Cakalang (*Katsuwonus pelamis*). *Journal of Pharmaceutical and Medicinal Sciences*. 2016;1(1):27-30.
22. Nursyam H. Ekstraksi Kolagen dari Limbah Kulit Ikan Tuna (*Thunnus sp*) dengan Berbagai Konsentrasi NaCl. *Jurnal Penelitian Perikanan*. 2010;13(1):107-113.

23. Fatikhasari Z, Lailaty IQ, Sartika D, Ubaidi MA. Viabilitas dan Vigor Benih Kacang Tanah (*Arachis hypogaea L.*), Kacang Hijau (*Vigna radiata* (*L.*) *R. Wilczek*), dan Jagung (*Zea mays L.*) pada Temperatur dan Tekanan Osmotik Berbeda. *Jurnal Ilmu Pertanian Indonesia*. 2022;27(1):7-17.
24. Lu WC, Chiu CS, Chan YJ, Mulio AT, Li PH. Characterization and biological properties of marine by-product collagen through ultrasound-assisted extraction. *Aquac Rep*. 2023;29:1-18.
25. Ramadhani PN. Efek Variasi Komposisi Kolagen - Kitosan - Gliserol Karakterisasi Scaffold Untuk Gingival Therapy. 2018.
26. Prananingrum W, Setiawan GC, Rizal MB, Prabowo PB, Pratama AF, Resaldi MF, Annisa NY, Fadhilah Y, Sari RP. Cytotoxicity and mechanical properties of biphasic calcium phosphate scaffold from Tegilarca granosa due to its composition. Odonto: Dental Journal. 2023 Dec;10(2):247-56.
27. Sun J, Aoki K, Zheng JX, Su BZ, Ouyang XH, Misumi J. Effect of NaCl and Helicobacter pylori Vacuolating Cytotoxin on Cytokine Expression and Viability. *World J Gastroenterol*. 2006;12(14):2174-2180.
28. Naiola BP. Akumulasi Solut Dan Regulasi Osmotik Dalam Sel Tumbuhan Pada Kondisistres Air [Solute Accumulation and Osmotic Adjustment in Stressed Plant Cells During Drought]. *Ber Biol*. 2005;7(6):333-340.
29. Putri KKZP, Prahasti AE. Pengaruh Metode Maserasi dan Ultrasonik terhadap Ukuran Partikel Ekstrak Kulit Buah Kakao (*Theobroma cacao*). *Jurnal Kedokteran Gigi Terpadu (JKGT)*. 2022;4(1):1-6.
30. Prahasanti C, Rizqi Romadhona W, Kurnia S, Dinaryanti A. *Viability Test of Collagen Extract from Gouramy Scale (Oshpronemusgouramy) on Bone Marrow Mesenchymal Stem Cell Culture*. Vol 20.