VOLUME 14 No. 1, 22 December 2024 Pages 18-28

VIABILITY OF BHK-21 FIBROBLAST CELLS TOWARD ACRYLIC DENTURE BASES AFTER REINFORCED BY NATURAL FIBERS

VIABILITAS SEL FIBROBLAST BHK-21 AKIBAT PERLAKUAN BASIS GIGI TIRUAN AKRILIK SETELAH DIPERKUAT SERAT ALAMI

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Submitted: 2023-10-31; Revised: 2024-05-16; Accepted:2024-05-16

ABSTRAK

Basis gigi tiruan akrilik harus memenuhi kriteria biokompatibilitas untuk penggunaan di dalam mulut. Penelitian ini menguji viabilitas sel fibroblas BHK-21 setelah perlakuan dengan basis gigi resin akrilik yang dimodifikasi menggunakan serat alami. Serat rami dan pelepah batang pisang digunakan sebagai alternatif serat sintetik yang lebih terjangkau. Sebanyak 42 spesimen resin akrilik berdiameter 10 mm dan tebal 2 mm yang tebagi dalam kelompok antara lain resin tanpa serat, serat rami 0,5%, 1,5%, 2,5% dan serat pelepah batang pisang 0,5%, 1,5%, 2,5%, serta media kultur sel tanpa spesimen. Resin diinkubasi dengan media kultur sel di dalam inkubator suhu 37°C selama 7 hari. Uji sitotoksisitas dari rendaman pada sel BHK-21 dilakukan menggunakan metode MTT dan viabilitas sel dihitung menggunakan rumus Freshney. Analisis statistik dilakukan menggunakn One-Way ANOVA, Post-hoc LSD ($p<0.05$). Hasil uji sitotoksisitas menunjukkan bahwa semua kelompok perlakuan memiliki viabilitas sel lebih dari 70%, sesuai dengan standar ISO 10993-5. Semua kelompok perlakuan tidak menyebabkan perbedaan viabilitas sel yang signifikan terhadap kontrol media tanpa spesimen. Selain itu, penambahan rami 0,5%, 1.5%, dan 2,5% tidak mempengaruhi viabilitas sel BHK-21 dibandingkan kontrol resin tanpa serat, sedangkan penambahan serat pisang 0,5%, 1.5%, dan 2.5% meningkatkan viabilitas sel dibandingkan kontrol resin ($P = 0,035$; $P = 0,021$; dan $P = 0,011$). Kesimpulannya, peningkatan konsentrasi serat alami pada basis gigi akrilik tidak berdampak negatif pada pertumbuhan sel fibroblas.

Keywords: *viabilitas sel; sel fibroblas BHK-21; basis gigi tiruan akrilik; serat alami.*

ABSTRACT

The use of acrylic denture bases in the oral cavity requires biocompatibility. This study investigated the viability of BHK-21 fibroblast cells after treatment with an acrylic denture base modified using natural fibers. Ramie and banana stem fibers were used as cost-effective alternatives to synthetic fibers. The study involved 42 acrylic resin specimens (10 mm diameter, 2 mm thickness) divided into groups: resin without fibers, 0.5%, 1.5%, and 2.5% ramie fibers, and 0.5%, 1.5%, and 2.5% banana stem fibers. The resin was incubated with cell culture media at 37°C for 7 days. Cytotoxicity testing using the MTT method revealed that all treatment groups had cell viability exceeding 70%, meeting ISO 10993-5 standards. No significant differences in cell viability were observed between

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the treatment groups and the control (media without specimens). Additionally, adding 0.5%, 1.5%, and 2.5% ramie fibers did not affect BHK-21 cell viability compared to the resin-only control, while adding banana stem fibers increased cell viability compared to the control (P = 0.035; P = 0.021; and P = 0.011). In conclusion, increasing the concentration of natural fibers in acrylic denture bases did not negatively impact fibroblast cell growth.

Keywords: *cell viability; BHK-21 fibroblast cell; acrylic denture base; natural fiber.*

INTRODUCTION

The most widely used denture base is heat-cured acrylic resin (HCAR). It is a dental material containing polymethyl methacrylate (PMMA). Despite many other superior materials and technological developments, acrylic denture use is still a choice because HCAR has many advantages, namely pleasing aesthetics, easy to clean, and relatively more cost effective prices (Alqutaibi et al., 2023; Hassan et al., 2019). However, these acrylic dentures shatter readily after being worn for a long time due to their low mechanical qualities, which is a problem (Kanie et al., 2000).

The mechanical qualities of acrylic denture bases can be enhanced in many ways; one is by modification with fiber materials, including synthetic and natural fibers. Natural fibers are more cost-effective than synthetic fibers and may provide alternatives to enhance HCAR quality. Natural fibers are promising biomaterials because of their higher tensile strength and Young's modulus than denture base materials, which are more biocompatible (Xu et al., 2013). However, using natural fibers in dentistry still needs to be investigated (Golbidi, 2007; Kunarto & Ernawan, 2018).

Ramie fiber *(Boehmeria Nivea L. Gaud)* is made from natural, lustrous, and hard bark fiber. This plant fiber satisfies the aesthetic requirements of a denture base due to its small diameter (10–60 μm) and white tint (Xu et al., 2013). Meanwhile, the fiber found in banana stems, originating from the banana plant, is abundant in Indonesia. Banana tree trunk fiber is infrequently used waste (Kunarto & Ernawan, 2018; Supraptiningsih, 2012).

This acrylic denture base must be biocompatible, meaning that it must be acceptable to the host, non-toxic, non-irritant, non-carcinogenic, and allergenic when used in the mouth (Alqutaibi et al., 2023; Anusavice et al., 2013; Geurtsen, 2002). A cytotoxicity test must be carried out before one material is applied as a denture base in the mouth. The cytotoxicity of a material indicates the possibility of toxic effects if the material is applied clinically. This test determines a material's toxic effect directly on tissue or cell cultures (Anggraeni, 2022).

The cytotoxicity test often used to test dental material is an in vitro cell culture-based assay using BHK-21 fibroblast cells. Researchers often use BHK-21 cells because fibroblasts are most commonly found in connective tissue such as the gingiva (Anusavice et al., 2013; Jang & Lee, 2015; Schmalz, 1998; Yuliati, 2005).

Fibroblast cells function as defense cells because of their ability to differentiate into odontoblasts and osteoblasts during the healing process. The power of fibroblast cells to proliferate in wound tissue and their ability to live on their means that these cells are fundamental and have become the most popular cell subject for biological research (Cevanti et al., 2023; Kurniawati et al., 2015).

The toxicity of materials used in dentistry is related to cell viability, in which a commonly used cytotoxicity test is the MTT {3-(4,5-dimethylthiaziol-2yl)-2,5-di- phenyltetrazolium bromide} assay for quantitative measurement of the living cells based on the enzymatic activity (Djustiana et al., 2021; Ismiyati et al., 2017; Rose et al., 2000; Yuliati, 2005). Djustiana et al. (2021) mentioned that the cytotoxicity test for the acrylic was also carried out based on the cytotoxicity test of PMMA microfiber on L-929 primary cell culture as a reinforcement for direct bridge dentures. Likewise, research on the cytotoxicity test of a mixture of acrylic resin and chitosan as an anti-fungal denture material has been carried out by Ismiyati et al. (2017).

This study aimed to evaluate the effect of HCAR base plates on the cell viability of BHK-21 fibroblast cells when different concentrations of ramie or banana stem fibers were added as reinforcement. The results of this research will provide information about the cell viability of acrylic resin reinforced with natural fibers with different concentrations so that it can be chosen which fiber to add, and which concentration is safe and produces a non-toxic modified acrylic resin denture base.

METHOD

This research was a laboratory experiment with a post-test only with a control group design. The specimen is a disc plate with a diameter of 10 mm and a thickness of 2 mm. The specimen was made from heat-cured acrylic resin. The total number of specimens was 42 discs and then divided into seven groups, namely six samples of each: control acrylic resin (fiber-free), resin added with 0.5%, 1.5%, or 2.5% of ramie fiber, and resin added with 0.5%, 1.5%, 2.5% of banana stem fiber.

Preparation of Reinforced Fibers

This research uses banana stems and ramie as natural fibers. The bark of ramie *(Boehmeria nivea L.Gaud),* which is extremely hard and bright white, makes ramie fiber. Meanwhile, banana stem fiber is taken from the midrib of the *Kepok* banana *(Musa paradisiaca L)*, removed from the interior, and dried until the water content is lowered, and the banana stem's physical characteristics are set, allowing the fiber to be seen. Drying was carried out for 12 days to ensure that the samples did not become moldy because this would reduce the quality of the fiber.

Fiber collection was carried out in 2 stages, using the decorticator method and a wire brush tool to produce clean fibers (Widiyantoro et al., 2021). It used to be necessary to alkalize natural fibers using a 5% NaOH solution to improve the surface layer roughness, promote adhesion between the fiber and the polymer matrix, and decrease the fiber's water absorption (Ku, H. et al., 2011). According to the following formula, the fiber volume in this study was 0.5%, 1.5%, and 2.5% of the acrylic plate's weight: fiber volume (%) = fiber weight (g)/sample weight (g) x 100% (Hadianto et al., 2013).

Preparation of Fiber Modified HCAR

The specimens were made from HCAR material (BasiQ20, Vertex, Netherlands), and the fibers were cut to match the diameter of the disc plate-shaped specimen, weighed until qualifying the weight requirements, and then dipped in monomer until everything was wetted. The polymer (powder) and monomer (liquid) were stirred in a mixing container in proportions according to the manufacturer's instructions (the ratio of 10 ml liquid to 24 g powder). Next, before the dough reached the kneading stage, it was inserted into the mold space one-third of the way up; the fiber was then impregnated and positioned in the middle one-third of the mold.

Plastic was first layered on top of the dough, and the dental flask was pressed using a hand press, following the addition of two-thirds of the dough and the cuvette's closure. After opening the dental flask and cleaning the leftover acrylic, it was closed again and pressed with a table press at a pressure of 2200 psi (50 kg/cm2). The next step was curing, which involved (by the manufacturer's instructions) submerging the dental flask in boiling water for 20 minutes (100°C), then taking it out and cooling it. The specimen was then taken out of the dental flask, and the surface was polished using abrasive sheets with numbers 360, 600, and 1000. Then, the specimen was cleaned with air spray and an ultrasonic cleaner for 5 minutes to clean the remaining polymer or monomer. The grouped specimens are ready to be tested (Figure 1).

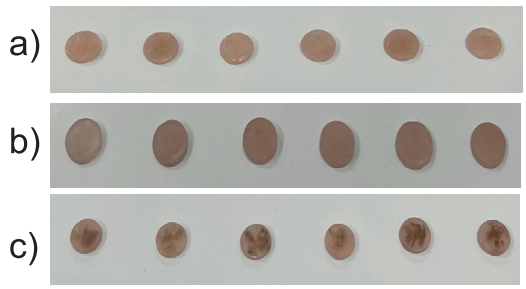


Figure 1.
Heat-cured acrylic resin (HCAR) disk specimens: a). Control resin without fiber (fiber free); b). Resin reinforced with ramie fiber, and c). banana stem fiber.
Source: Author (2023)

BHK-21 Cell Culture

BHK-21 cells (Kerafast EH1011, USA) were cultured. They propagated in DMEM (Sigma, St. Louis, USA) growth medium containing 5% FBS (Biosera, France) and 1% Penicillin-Streptomycin antibiotic (Thermo-Fisher Sci., USA) in a humid incubator with 5% CO_2 at 37°C.

Soaking of HCAR Disc in PBS or Culture Medium for Cytotoxicity Test

Samples of HCAR disc plates with free and added fiber were sterilized by soaking in 70% ethanol for 15 minutes in a biosafety cabinet. The samples were then washed with 2 mL of PBS twice and with 2 mL of sterile aquabidest once. The sample was transferred into a 24-well plate, added with 2 mL of PBS or DMEM culture medium (5%FBS), and stored in an incubator at 37°C for seven days. Soaking PBS and medium were transferred into a 1.5 mL tube and then used for the MTT assay.

MTT Cytotoxicity Test

For the cytotoxic test, confluent cells were harvested using trypsin-EDTA (Thermo Fisher Sci., USA), counted, and seeded into 96-well plates with a cell number of 6,000 cells/well. After overnight incubation, the cell medium was discarded, and 100 µl of samples (soaking PBS or medium) were added to each well and incubated inside the incubator for 24 hours. Then, the samples were removed, and the cells were washed with PBS and treated with MTT 0.5 mg/ml in a culture medium for about 2-4 hours. After the formazan crystals formed, the medium was discarded, and formazan was solubilized with 100 µl DMSO in each well and shaken for 10 minutes.

A microplate reader is used for absorbance measurement with a wavelength of 570 nm. Cytotoxicity test was measured based on relative cell viability to the control cells (fiber-free resin group for soaking PBS experiment and control medium without specimen for soaking medium experiment) using the Freshney formula (Freshney, 2008; Meiyanto et al., 2006) (Equation 1).

$$cell\ viability = \frac{(\text{Abs treatment-Abs blank})}{(\text{Abs control cell-Abs blank})} \text{x}100\% \text{..... (1)}$$

Statistical Analysis

The data obtained in this study was tested for normality using Shapiro-Wilk. Based on the significance value ($p < 0.05$), the data distribution results were expected because all groups had a p-value> 0.05; then, continued with the Lavene homogeneity test, the p-value was obtained > 0.05, so the data in the two groups is considered homogeneous. Then, the significant difference of each treatment was analyzed using One-Way ANOVA and post-hoc LSD ($p<0.05$).

RESULTS AND DISCUSSION

The cytotoxicity test in this study used PBS and culture medium as the soaking material. This cell culture method is often used to test the initial toxicity effects of materials used in dentistry (Anusavice et al., 2013; Schmalz, 1998; Yuliati, 2005). In this study, we used BHK-21 fibroblasts, the most widely used by researchers for material toxicity tests in dentistry (Apriasari et al., 2014; Freshney, 2008). Cell viability is the possibility of cells

being able to live after being exposed to the culture medium of the material sample (Anggraeni, 2022).

The BHK-21 cell morphology after treatment with soaking PBS (25% in culture medium) can be seen in Figure 2A. There was no significant difference in cell morphology between treated and control groups. In addition, the cytotoxicity test of the soaking PBS for the control resin, resin reinforced with ramie 0.5%, 1.5%, and 2.5%, and banana stem 0.5%, 1.5%, and 2.5% exhibited cell viability values of 100%, 95.04%, 91.68%, 94.83%, 97.77%, 99.34%, and 100.16%, respectively. No significant difference in cell viability was observed after natural fiber reinforcement toward the HCAR discs (Figure 2B).

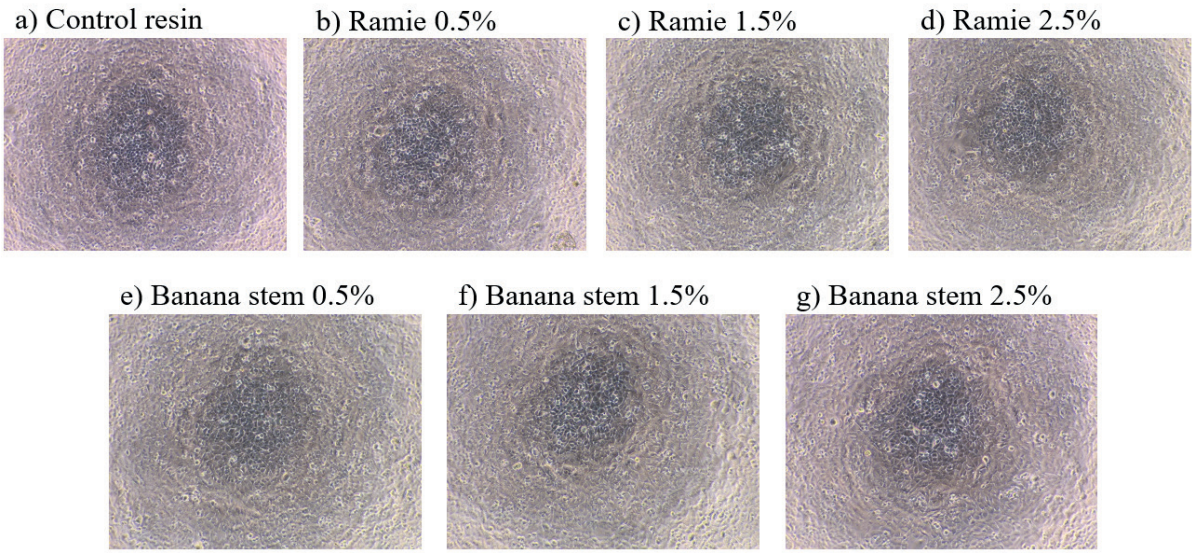
BHK-cell morphology after treatment with soaking medium also did not exhibit remarkable change (Figure 3A). The cytotoxicity test of soaking medium for control resin, resin reinforced with ramie 0.5%, 1.5%, and 2.5%, banana stem 0.5%, 1.5%, and 2.5%, also medium without specimen exhibited cell viability values: 94.60%, 89.37%, 91.02%, 106.17%, 120.24%, 123.57%, 126.23%, and 100%, respectively. The group adding 2.5% banana stem fiber had the most significant cell viability, followed by the 1.5% banana stem fiber group.

In contrast, the smallest cell viability was found in the ramie fiber group. All treatment groups were not statistically different from the control medium with no specimen. Moreover, the addition of ramie 0.5%, 1.5%, and 2.5% did not affect BHK-21 cell viability compared to the control resin (fiber-free HCAR), whereas the addition of banana stem fiber 0.5%, 1.5%, and 2.5% increased cell viability compared to fiber-free HCAR ($P = 0.035$; $P = 0.021$; and $P= 0.011$) (Figure 3B).

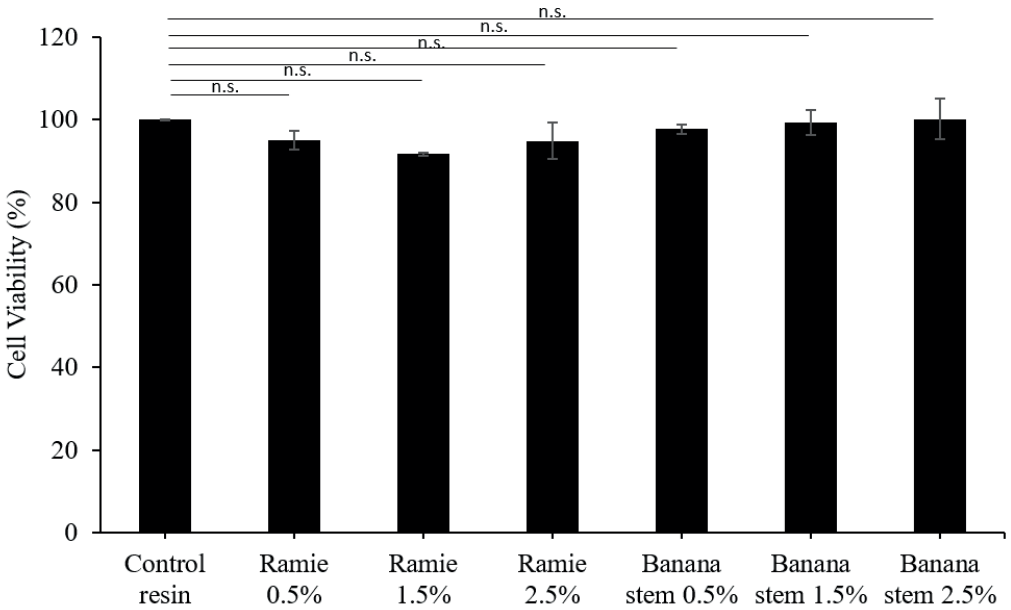
The Cytotoxicity test is the initial stage of biocompatibility testing of dental materials. This test is necessary because the material placed in the mouth is required to be non-toxic, non-irritant, non-carcinogenic, and non-allergenic so that it does not harm the user (Anusavice et al., 2013; Yuliati 2005).

The results of this research showed that the cell viability values in all groups, both groups fiber-free HCAR and HCAR with the addition of ramie fibers and banana stem fibers, showed cell viability values of more than 70%, meaning that according to ISO 10993-5, the specimen is clinically acceptable and can be used in the oral cavity (Kazak et al., 2020).

The cell viability after treatment with the HCAR group with the addition of 0.5%, 1.5%, and 2.5% banana stem fibers seem higher than the fiber-free HCAR group, HCAR with the addition of 0.5%, 1.5%, 2.5% ramie fibers as well as the control medium group. From the microscopic images, cells did not appear to be pycnotic (no damage to the cell nucleus), did not swell, the cell membrane boundaries were regular, and did not lose their attachment to the microtiter plate (Figure 2A and 3A).

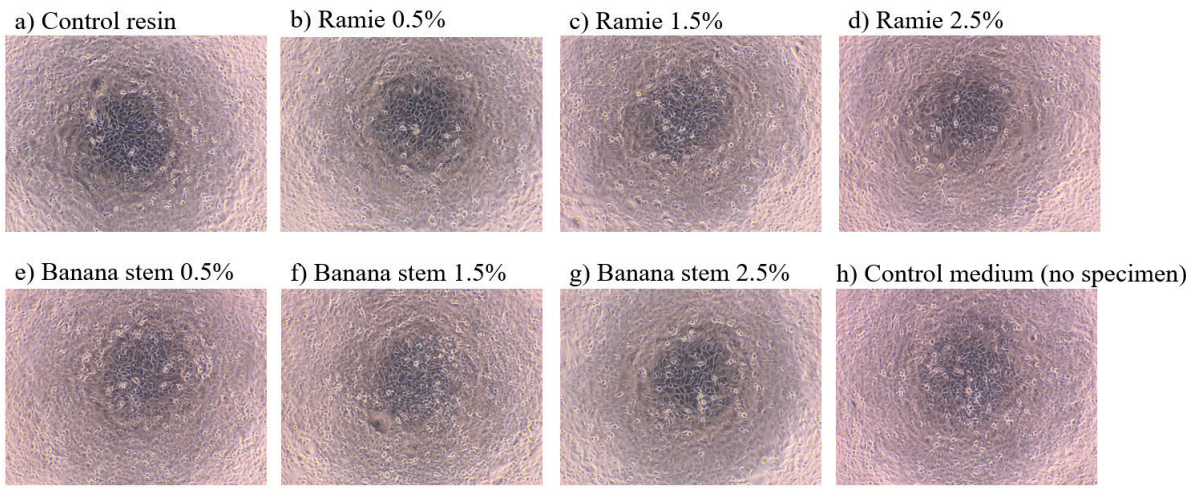


(A)

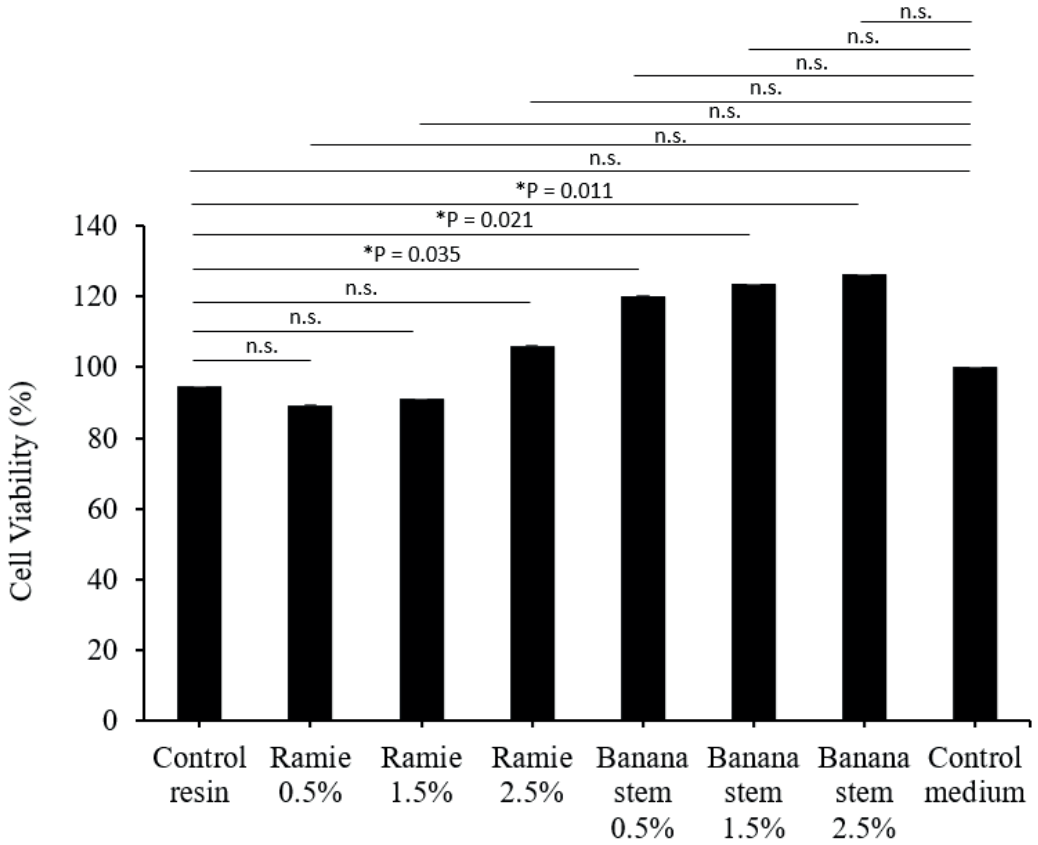


(B)

Figure 2.
Effect of HCAR disc soaking PBS on BHK-21 fibroblast cell viability. A. Microscopic images of BHK-21 cells after treatment with the HCAR disc soaking PBS. B. Graph represents cell viability of BHK-21 cells after treatment with 25% of soaking PBS. Data represented as a mean of cell viability ± SEM (n = 3). n.s. = not significant.
Source: Author's analysis (2023)



(A)



(B)

Figure 3.
Effect of HCAR disc soaking medium on BHK-21 fibroblast cell viability. A. Microscopic images of BHK-21 cells after treatment with the HCAR disc soaking complete DMEM medium. B. Graph represents cell viability of BHK-21 cells after treatment with 25% of soaking medium. Data represented as a mean of cell viability ± SEM (n = 6). n.s. = not significant. *P <0.05.
Source: Author's analysis (2023)

The greater the fiber concentration, the higher the cell viability value, both in the HCAR group added with ramie fibers and banana stem fibers. This shows that increasing the fiber concentration in the heat-cured acrylic plate or different fiber concentrations does not have a negative effect on the growth of BHK 21 fibroblast cells. All groups (fiber-free or with added fibers) are categorized as non-toxic because the cell viability value is more than 90%.

The soluble residual monomers could cause a decrease in cell viability in the soaking medium, which results from the degradation process of the polymerized polymer residue matrix. This soluble monomer is challenging to react to again and thus may affect the soft tissues of the oral cavity (Cevanti et al., 2023; Kamalak et al., 2018).

In samples with banana stem fibers, BHK-21 cell viability was higher than the control medium, even though it was not statistically significant. This is in line with the research (Aspriyanto et al., 2018) regarding the effect of giving Mauli banana stem extract on the quality of wound healing in mice by assessing the number of fibroblast cells, where an increase in the number of fibroblast cells was obtained during the healing process of incision wounds induced with Mauli banana stem extract. Mauli banana stem extract (*Musa acuminata*) contains the same substances as other bananas, namely terpenoid saponins. This research used *kepok* banana stem fiber (*Musa paradisiaca L*), which also contains bioactive triterpenoid saponin, which has immunostimulatory properties and can increase the activity and number of fibroblast cells. Meanwhile, (Promdontree et al. 2023) in the research using ramie fiber cellulose as a composite hydrogel material for medical materials, Promdontree et al. (2023) found that the toxicity value was low against fibroblast cells. The natural ramie and banana stem fibers have relatively high cellulose content. Banana stem fiber has 63-64% cellulose, while ramie fiber has 80-85% cellulose.

Natural fibers have a low interface between the fiber and the polymer matrix because cellulose contains polar hydroxyl groups that make the fiber hydrophilic. Water interacts with cellulose fibers, like ramie and banana stems, throughout the fiber, as opposed to glass fiber, which only absorbs water on the surface (Supraptiningsih, 2012; Xu et al., 2013). Many hydroxyl groups in the cellulose structure can form hydrogen bonds with water, and cellulose may be very insoluble in water and insoluble in other solvents. The crystalline level of cellulose fibers is relatively high due to the high strength and forces between chains and hydrogen bonds between hydroxyl groups of adjacent chains (Cevanti et al., 2023).

The fibers used in this research contain relatively high levels of cellulose and hemicellulose, and by the alkalization process (treatment with NaOH) on the fiber, the hemicellulose content will be converted into cellulose. Treatment using base (NaOH) of natural fibers is one of the chemical treatments known to increase cellulose content through the removal or conversion of hemicellulose and lignin, in addition to reducing surface tension and increasing adhesion between natural fibers and polymer matrices (Bachtiar et al., 2008; Kim & Netravali, 2010).

This study showed no significant difference in cell viability between the control fiber-free HCAR group and the fiber-added HCAR groups compared with the control medium group (soaking medium without specimen). This indicates that acrylic plates with or without added fiber do not affect the viability value of fibroblast cells, and acrylic plates with added fibers can be used safely as reinforcement for acrylic denture bases without causing cytotoxicity in the oral cavity.

CONCLUSION

In this study, among the soaking medium-treated cells, the highest cell viability was shown after cell treatment with the 2.5% banana stem fiber group (126.23% ± 0.82), and the lowest cell viability was shown after cell treatment with 0.5% ramie fiber group (89.37% ± 1.18). According to ISO 10993-5, all sample groups are clinically acceptable based

on more than 70% cell viability values. The higher the fiber concentration, the higher the cell viability. Increasing the concentration of these fibers up to 2.5% in the acrylic plate did not harm fibroblast cells' growth, representing no toxic materials released into the soaking medium.

ACKNOWLEDGMENTS

The author thanks the Director of the Polytechnic of Health, Ministry of Health and Research Center for Genetic Engineering, National Research and Innovation Agency, Indonesia, which supported the implementation of research activities.

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