

Original Research Article

Development of a hyaluronic acid – bovine pericardium composite membrane for biocompatible biomedical use

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Abstract

Purpose: To evaluate the effect of different molecular weights and concentrations of bovine pericardium membranes (BPM) - hyaluronic acid (HA) complex on Baby Hamster Kidney-21 (BHK-21) cells' viability and cytotoxicity.

Methods: Bovine pericardium membranes-HA complex were divided into seven groups, namely Group A (1300 kDa HA, 0.5 %), Group B (1300 kDa HA, 1.0 %), Group C (1300 kDa HA, 2.0 %), Group D (120 kDa HA, 0.5 %), Group E (120 kDa HA, 1.0 %), Group F (120 kDa HA, 2.0 %), and Group G (unmodified BPM). Baby hamster kidney fibroblast cells (BHK-21 cells) were exposed for 24 h. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay), and cytotoxicity was observed morphologically. Data were analyzed using one-way ANOVA with a significance level set at $p < 0.05$.

Results: The 1.0 % 1300 kDa HA group achieved the highest cell viability (96.70 %), which was significantly higher compared to other treatment groups ($p < 0.000$). Both molecular weights peaked at 1.0 % with slight decreases at 2.0 %, indicating a threshold effect. The unmodified bovine pericardium membrane had the lowest viability (82.84 %), with significant differences ($p = 0.000$).

Conclusion: Incorporating 1.0 % high-molecular-weight BPM-HA enhances the biocompatibility of bovine pericardium membranes, supporting application in regenerative biomaterials.

Keywords: BHK-21 cells, Bovine pericardium membrane, Hyaluronic acid, Toxicity, Viability test

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INTRODUCTION

The collagen content and structure of bovine pericardium membranes obtained from the

National Nuclear Energy Agency (BATAN), Indonesia, are comparable to those of human tissues. They are among the biomaterials frequently employed in medical applications,

including tissue engineering, vascular grafts, and other prostheses. The bovine pericardial membrane is an excellent option for creating biomaterials because of its mechanical properties, biocompatibility, and capacity to promote cellular regeneration [1,2]. However, the use of chemicals such as hyaluronic acid is growing in popularity to enhance the biocompatibility and functionality of bovine pericardium membranes. Hyaluronic acid, being a polysaccharide that occurs naturally in the human body, plays a crucial part in tissue repair and wound healing [3].

Hyaluronic acid has several molecular sizes, with two main molecular sizes often used in biomaterial applications, namely hyaluronic acid with high (1300 kDa) and low molecular weights (120 kDa). These two types of hyaluronic acid have different properties and benefits in terms of viscosity, tissue penetration, and influence on cell growth [4]. Hyaluronic acid with a large molecular size tends to form denser tissues and has better hydration ability, while hyaluronic acid with a small molecular size is more easily absorbed by tissues and can accelerate wound healing [5].

When assessing a biomaterial's biocompatibility, cell viability and toxicity are crucial factors. While toxicity quantifies a material's capacity to harm cells or tissues, cell viability shows a cell's capacity to survive, thrive, and function following exposure to biomaterials [6]. To investigate the effects of bovine pericardium membrane and the combination of bovine pericardium membrane and hyaluronic acid on cell survival and toxicity, BHK-21 (Baby Hamster Kidney-21) cells were chosen as the cell type for this investigation. Because BHK-21 cells grow well in cell cultures and are sensitive to injury, they are frequently utilized in cytotoxicity experiments [7].

The purpose of this study is to assess how the bovine pericardium membrane's hyaluronic acid concentrations, which have molecular weights of 1300 and 120 kDa, affect the toxicity and viability of BHK-21 cells, a continuous fibroblast-like cell line derived from the Syrian golden hamster (*Mesocricetus auratus*) commonly used as an in vitro model for cytotoxicity and biomaterial biocompatibility studies [8]. Additionally, compared to the bovine pericardium membrane alone, the study sought to determine whether larger quantities of hyaluronic acid had a greater effect on cell viability and decreased cell toxicity. It is believed that by understanding this influence, the study's findings may contribute to the creation of biovine-based biomaterials for the membrane of the bovine pericardium that are

safer and more biocompatible for use in medical applications [9].

EXPERIMENTAL

Source of Bovine pericardium membrane

Bovine pericardium membrane samples were obtained from the pericardial tissues of adult cattle sourced from the National Nuclear Energy Agency (BATAN), Indonesia. Tissues were collected under aseptic conditions and subjected to sterilization using ultraviolet (UV) radiation for 30 minutes, followed by decellularization through enzymatic and chemical treatments to remove undesirable remnants, including immunogenic proteins. After processing, the membrane was dried and rinsed with a sterile saline solution. Prior to use, small segments of the bovine pericardium membrane (e.g., 5 mm × 5 mm) were re-sterilized using ultraviolet (UV) radiation for 30 minutes [10]. These membranes were used as the unmodified (non-HA-treated) bovine pericardium membrane group in the subsequent cell viability and cytotoxicity experiments.

Preparation of hyaluronic acid modified membranes

Hyaluronic acid (HA) with molecular weights of 1300 and 120 kDa was obtained from ECOORI Pte Ltd, Singapore. These molecular weights were selected based on previous studies demonstrating that low- and high-molecular-weight HA exhibit distinct biological activities influencing cell proliferation, inflammation, and biomaterial biocompatibility [11]. Hyaluronic acid (1300 kDa and 120 kDa) was dissolved separately in 50 mL of sterile saline solution to prepare final concentrations of 0.5 % (0.25 g), 1.0 % (0.5 g), and 2.0 % (1.0 g) for each molecular weight, and subsequently filtered using a 0.22 µm filter to ensure sterility before application in experiments [12].

The combination of hyaluronic acid and bovine pericardium membrane was achieved by immersing the membrane in sterile HA solutions of varying concentrations (0.5, 1.0, and 2.0 %) for 24 hours at room temperature under aseptic conditions. After soaking, the membranes were gently dried with sterile filter paper to remove any residual HA solution. These HA-modified bovine pericardium membranes were then prepared for use in BHK-21 cell viability and toxicity assays [6].

Protocol

Baby Hamster Kidney-21 (BHK-21) cells, a fibroblast-like continuous cell line commonly used in cytotoxicity and biomaterial biocompatibility studies, were selected as an in vitro model to evaluate cell viability and cytotoxic responses to the tested membranes. The cells were obtained from FKG Research Center, Faculty of Dental Medicine, Universitas Airlangga, Surabaya, Indonesia, and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), 1 % penicillin-streptomycin, and 1 % L-glutamine. Cells were maintained in culture dishes within an incubator at 37°C, with 5 % CO₂ and 95 % relative humidity [10].

For treatment, BHK-21 cells were seeded at a density of approximately 10,000 cells per well in 96-well plates and allowed to adhere for 24 hours. The medium was then replaced with fresh medium containing the unmodified bovine pericardium membrane, as well as bovine pericardium membrane with 1300 kDa HA at concentrations of 0.5, 1.0, and 2.0 %, and bovine pericardium membrane with 120 kDa HA at concentrations of 0.5, 1.0, and 2.0 %. The experimental groups were defined as follows: Group A (1300 kDa HA, 0.5 %), Group B (1300 kDa HA, 1.0 %), Group C (1300 kDa HA, 2.0 %), Group D (120 kDa HA, 0.5 %), Group E (120 kDa HA, 1.0 %), Group F (120 kDa HA, 2.0 %), and Group G (bovine pericardium membrane without HA; membrane-only group). Treatments were maintained for 24 hours to evaluate the effect of exposure on cell viability and toxicity [13].

Cell viability was assessed using the MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). After incubation, the culture medium in each well was replaced with MTT solution (5 mg/mL), followed by incubation at 37 °C for four hours. Afterward, the MTT solution was discarded, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. Cell viability was expressed as a percentage relative to the untreated control group [14]. To evaluate cell toxicity, morphological observations were conducted using an inverted microscope, focusing on cell shape, density, and membrane integrity. Toxicological responses were indicated by morphological changes such as swelling, membrane rupture, or significant alterations in shape. The experimental groups consisted of bovine pericardium membrane treated with 1300 kDa HA at 0.5, 1.0, and 2.0 %, bovine

pericardium membrane treated with 120 kDa HA at 0.5, 1.0, and 2.0 %, and bovine pericardium membrane without HA. The control consisted of BHK-21 cells cultured in medium without any membrane or HA treatment, representing baseline morphology and viability. Each experimental group was compared to the control to determine the degree of toxicity associated with each HA-bovine pericardium combination [3].

Statistical analysis

Data obtained from the MTT assay and morphological evaluations were analyzed using statistical software such as SPSS. Results for each condition were expressed as mean $\pm$ standard deviation (SD) from three replicates. Statistical significance between groups was determined using one-way analysis of variance (ANOVA), with $p < 0.05$ considered statistically significant [15].

RESULTS

The fibroblast viability assay demonstrated that the incorporation of hyaluronic acid into the bovine pericardium membrane significantly influenced BHK-21 fibroblast viability (Table 1), as confirmed by one-way ANOVA analysis ($p < 0.001$; Table 4). Among all treatment groups, the combination of 1300 kDa hyaluronic acid at a concentration of 1.0 % (group B) showed the highest average viability of 96.70 ± 1.11 %, surpassing both lower (0.5 %; Group A: 84.27 ± 3.16 %) and higher (2.0 %; Group C: 85.60 ± 2.38 %) concentrations within the same molecular weight group. Meanwhile, the lowest fibroblast viability was observed in Group G, which consisted of bovine pericardium membrane without hyaluronic acid supplementation (membrane-only group), with a mean viability of 82.84 ± 3.81 % (Table 1). In contrast, Groups A - F included membranes supplemented with hyaluronic acid at molecular weights of 120 and 1300 kDa and concentrations of 0.5, 1.0, and 2.0 %, which generally demonstrated improved viability. The selection of these molecular weights was based on their distinct biological activities and previously reported effects on cell behavior and biomaterial biocompatibility [16,17].

Within the 120 kDa hyaluronic acid treatment group, the 1.0 % concentration (group E) achieved the highest cell viability at 86.159 %, followed closely by 2.0 % (group F) at 85.018 and 0.5 % (group D) at 84.915 %. Despite the relatively small difference, the trend suggests that 1.0 % concentration remains optimal for cell

survival, regardless of molecular weight. Cell viability trends further revealed that increasing the concentration of hyaluronic acid from 0.5 to

1.0 % significantly improved cell survival, while a further increase to 2.0 % resulted in a slight decline.

Table 1: BHK-21 cell viability (%) of bovine pericardium membrane on hyaluronic acid modification (Groups A–G)

Replication	Cell Control	A	B	C	D	E	F	G
1	100	88.42	97.61	84.53	88.12	84.83	83.53	76.90
2	100	84.31	97.58	83.31	76.13	85.98	82.47	88.47
3	100	85.79	96.60	88.93	86.57	89.89	83.44	85.05
4	100	81.34	96.89	83.60	89.07	81.06	88.03	82.80
5	100	81.48	94.84	87.62	84.68	89.05	87.62	80.96
Mean±SD	100±0.00	84.27±3.16 ^a	96.70±1.11 ^b	85.60±2.38 ^a	84.92±5.25 ^a	86.16±3.84 ^a	85.02±2.40 ^a	82.84±3.81 ^a

Note: Abbreviations and Treatment Groups; A = 1300 kDa 0.5% + Bovine Pericardium Membrane; B = 1300 kDa 1.0% + Bovine Pericardium Membrane; C = 1300 kDa 2.0% + Bovine Pericardium Membrane; D = 120 kDa 0.5% + Bovine Pericardium Membrane; E = 120 kDa 1.0% + Bovine Pericardium Membrane; F = 120 kDa 2.0% + Bovine Pericardium Membrane; G = Bovine Pericardium Membrane (Batan). n = 5. Different superscript letters indicate statistically significant differences among groups ($p < 0.05$). Groups sharing the same superscript letter are not significantly different. Group B (^b) demonstrated significantly higher cell viability compared to the other groups (^a)

Table 2: Normality test

Parameter	Material	Kolmogorov-Smirnova			Shapiro-Wilk		
		Statistics	Df	P-value	Statistics	Df	P-value
Viability	1300 Kda 0.5%+BPM	0.224	5	0.200*	0.920	5	0.527
	1300 Kda 1.0%+BPM	0.200	5	0.200*	0.893	5	0.371
	1300 Kda 2.0%+BPM	0.242	5	0.200*	0.873	5	0.281
	120 Kda 0.5%+BPM	0.282	5	0.200*	0.827	5	0.132
	120 Kda 1.0%+BPM	0.193	5	0.200*	0.947	5	0.717
	120 Kda 2.0%+BPM	0.316	5	0.115	0.818	5	0.114
	BPM	0.167	5	0.200*	0.990	5	0.980

Note: where $P > 0.05$, then the distributed data is normal; where $P < 0.05$, means the distributed data is abnormal; BPM = Bovine Pericardium membrane

Table 3: Homogeneity of variances

Parameter	Treatment	Levene statistic	df1	DF2	P-value
Viability	Based on Mean	1.078	6	28	0.399
	Based on Median	0.630	6	28	0.705
	Based on Median and with adjusted df	0.630	6	15.635	0.705
	Based on the trimmed mean	1.043	6	28	0.419

Note: where $P > 0.05$, then the data of the tested group is homogeneous; where $P < 0.05$, then the data of the tested group is not homogeneous

Statistical normality analysis (Table 2) using both the Kolmogorov-Smirnov and Shapiro-Wilk tests confirmed that most data were normally distributed [18], with $p > 0.05$. For instance, 1300 kDa 0.5 % + BMP ($P = 0.527$) and 120 kDa 1.0 % + BMP ($P = 0.717$) displayed clear normal distribution. However, certain treatments, such as 120 kDa 2.0% + BMP, showed a borderline abnormal distribution in the Shapiro-Wilk test ($P = 0.114$), suggesting the need for careful interpretation in further inferential statistical analysis. The results of the homogeneity of variance test using Levene's method are presented in Table 3. The test employed various statistical approaches, including trimmed mean, median, median adjusted to the degree of freedom, and mean. According to the test

findings, all approaches showed P values greater than 0.05, with the mean approach yielding $P = 0.399$ and the median approach yielding $P = 0.705$. These results indicate that the data groupings exhibit homogeneous variances.

In Table 4, the mean difference between the tested groups was statistically significant ($p < 0.05$). This indicates that the group under comparison experienced distinct impacts from the treatment.

Table 4: ANOVA test

Viability	Df	P-value
Between Groups	6	0.000
Within Groups	28	
Total	34	

Table 5: Tukey-HSD multiple comparisons

Viability	Tukey HSD			
	Material	P-value	95% Confidence Interval	
			Lower Bound	Upper Bound
1300 Kda 0.5%+BPM	1300 Kda 1.0%+BPM	0.000	-193.162	-53.374
	1300 Kda 2.0%+BPM	0.998	-82.118	57.670
	120 Kda 0.5%+BPM	1.000	-76.376	63.412
	120 Kda 1.0%+BPM	0.976	-88.818	50.970
	120 Kda 2.0%+BPM	1.000	-77.406	62.382
	BPM	0.984	-52.524	87.264
1300 Kda 1.0%+BPM	1300 Kda 0.5%+BPM	0.000	53.374	193.162
	1300 Kda 2.0%+BPM	0.000	41.150	180.938
	120 Kda 0.5%+BPM	0.000	46.892	186.680
	120 Kda 1.0%+BPM	0.001	34.450	174.238
	120 Kda 2.0%+BPM	0.000	45.862	185.650
	BPM	0.000	70.744	210.532
1300 Kda 2.0%+BPM	1300 Kda 0.5%+BPM	0.998	-57.670	82.118
	1300 Kda 1.0%+BPM	0.000	-180.938	-41.150
	120 Kda 0.5%+BPM	1.000	-64.152	75.636
	120 Kda 1.0%+BPM	1.000	-76.594	63.194
	120 Kda 2.0%+BPM	1.000	-65.182	74.606
	BPM	0.826	-40.300	99.488
120 Kda 0.5%+BPM	1300 Kda 0.5%+BPM	1.000	-63.412	76.376
	1300 Kda 1.0%+BPM	0.000	-186.680	-46.892
	1300 Kda 2.0%+BPM	1.000	-75.636	64.152
	120 Kda 1.0%+BPM	0.997	-82.336	57.452
	120 Kda 2.0%+BPM	1.000	-70.924	68.864
	BPM	0.928	-46.042	93.746
120 Kda 1.0%+BPM	1300 Kda 0.5%+BPM	0.976	-50.970	88.818
	1300 Kda 1.0%+BPM	0.001	-174.238	-34.450
	1300 Kda 2.0%+BPM	1.000	-63.194	76.594
	120 Kda 0.5%+BPM	0.997	-57.452	82.336
	120 Kda 2.0%+BPM	0.998	-58.482	81.306
	BPM	0.654	-33.600	106.188
120 Kda 2.0%+BPM	1300 Kda 0.5%+BPM	1.000	-62.382	77.406
	1300 Kda 1.0%+BPM	0.000	-185.650	-45.862
	1300 Kda 2.0%+BPM	1.000	-74.606	65.182
	120 Kda 0.5%+BPM	1.000	-68.864	70.924
	120 Kda 1.0%+BPM	0.998	-81.306	58.482
	BPM	0.914	-45.012	94.776
BPM	1300 Kda 0.5%+BPM	0.984	-87.264	52.524
	1300 Kda 1.0%+BPM	0.000	-210.532	-70.744
	1300 Kda 2.0%+BPM	0.826	-99.488	40.300
	120 Kda 0.5%+BPM	0.928	-93.746	46.042
	120 Kda 1.0%+BPM	0.654	-106.188	33.600
	120 Kda 2.0%+BPM	0.914	-94.776	45.012

Note: where $P > 0.05$, the average is equal; where $P < 0.05$, the mean differs significantly

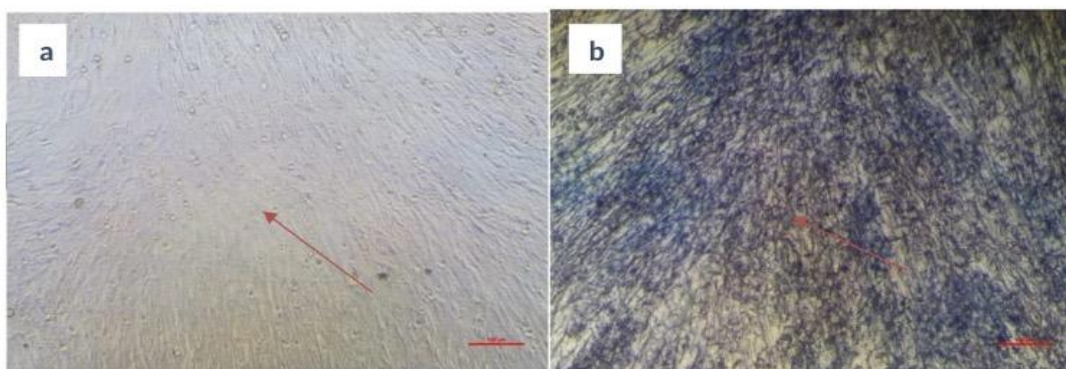


Figure 1: Histology of MTT cells after assay. (a) non-viable cells (1300 kDa 1.0%) (b) viable cells exhibiting strong purple coloration due to formation of formazan crystals (1300 kDa)

Results of ANOVA comparing cell viability across different molecular weights and concentrations of hyaluronic acid combined with bovine pericardium membrane revealed that the group that differed significantly from the other test groups, specifically the 1300 kDa 1.0 % + bovine pericardium membrane, had good and noteworthy cell viability. As shown in Figure 1 a, cells that are inactive or non-viable show no visible staining, indicating the absence of metabolic activity. In contrast, Figure 1 b presents the MTT assay result, where viable cells exhibit a strong purple coloration due to the formation of formazan crystals. The intensity of the purple color reflects high cellular metabolic activity, confirming that treatment with 1300 kDa HA at 1.0% concentration supports cell viability.

DISCUSSION

The combination of hyaluronic acid with bovine pericardium membrane significantly increased cell viability compared to the use of bovine pericardium membrane alone. The 1.0 % hyaluronic acid concentration yielded the highest viability at both 1300 kDa (96.702 %) and 120 kDa (86.159 %), indicating that this concentration was optimal for supporting cell survival. In contrast, lower concentrations (0.5 %) resulted in lower viability, while higher concentrations (2.0 %) indicated a decrease in viability that may be due to the effects of saturation or mild toxicity at high concentrations [19].

This pattern was consistently observed in both molecular weight groups, suggesting the presence of a concentration threshold beyond which hyaluronic acid may exert saturation effects or mild cytotoxicity. Therefore, the 1.0 % concentration appears to represent an optimal balance between enhancing cell viability and minimizing potential adverse effects [20].

Compared across molecular weights, the 1300 kDa variant consistently yielded better results than the 120 kDa at equivalent concentrations, emphasizing the positive role of higher molecular weight in supporting cell viability [21]. For example, at a concentration of 1.0 %, 1300 kDa hyaluronic acid achieved a cell viability of 96.702 %, while 120 kDa hyaluronic acid was 86.159 %. This suggests that high molecular weight hyaluronic acid has better hydration capabilities and supports a more conducive environment for cell growth [22].

The addition of hyaluronic acid to the bovine pericardium membrane provides a significant synergistic effect, increasing cell viability compared to the use of bovine pericardium

membrane alone (G), which had the lowest cell viability of 82.84 %. Statistical analysis supported these results, with mostly normally distributed data and homogeneous variance between groups. The Tukey HSD post-hoc test revealed that the 1300 kDa 1.0 % hyaluronic acid group showed significant differences in comparison with nearly all other groups, including the bovine pericardium membrane without hyaluronic acid, while the ANOVA results indicated significant differences between the groups ($p = 0.000$).

Taken together, these findings support the conclusion that the optimal formulation for maintaining BHK-21 cell viability is a combination of bovine pericardium membrane with 1.0 % high molecular weight (1300 kDa) hyaluronic acid [23]. This combination suggests enhanced biocompatibility and potential applicability in regenerative biomaterial development. The enhanced viability profiles highlight the synergistic effect of hyaluronic acid, particularly at optimal concentrations, in improving the biological performance of bovine-derived pericardial membranes [24].

These results have significant ramifications for the creation of biomaterials based on the use of bovine pericardium membrane in tissue engineering and wound healing, among other medical applications. However, the decrease in cell viability at high concentrations (2.0 %) demonstrates the importance of determining the optimal concentration to ensure the safety and effectiveness of the biomaterial. Although this study provides significant insights, some limitations, such as the use of one cell type (BHK-21) and short-term observations, need to be noted. Further research is needed to explore clinical applications and evaluate the long-term effects of this combination on more complex tissue or animal models. This combination provides an important foundation for the development of biomaterials that are more biocompatible, safe, and effective for various medical needs [25].

CONCLUSION

This study confirms that the integration of hyaluronic acid (HA) into bovine pericardium membrane significantly enhances cell viability, with the most favorable result observed at a concentration of 1.0 % HA with a high molecular weight of 1300 kDa. This specific combination yielded the highest cell viability (96.702 %) and demonstrated superior biocompatibility compared to both lower and higher concentrations and to the membrane alone. The results highlight the synergistic role of high-molecular-weight HA in

promoting a cellular environment conducive to survival and regeneration. Therefore, the formulation of bovine pericardium membrane with 1.0 % 1300 kDa HA shows strong potential as a safe and effective biomaterial for application in tissue engineering and wound healing. Further *in vivo* and long-term studies are warranted to validate these findings and expand their clinical relevance.

DECLARATIONS

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Ethics approval and consent to participate

The study received ethical approval from the Health Research Ethical Clearance Commission, Faculty of Dental Medicine, Universitas Airlangga, under certificate number 1186/HRECC.FODM/XII/2024.

Use of Artificial intelligence/Large language models

The authors confirm that AI-based tools were used solely for language editing and manuscript polishing purposes (e.g., grammar, phrasing, and clarity). No AI tools were used to generate content, interpret data, or influence study design, results, or conclusions. All intellectual contributions, study analyses, and interpretations reported in the manuscript are the original work of the authors.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article, and all liabilities pertaining to claims relating to the content of this

article will be borne by the authors. Dwi Wahyu Indrawati was responsible for the conceptualization and methodology, along with Ernie Maduratna Setiawatie, as well as formal analysis, together with Retno Pudji Rahayu. The investigation was conducted by Rini Devijanti Ridwan and Hendrik Setia Budi, while data curation was carried out by Coen Pramono, Ida Bagus Narmada, and Anita Yulianti. Dwi Wahyu Indrawati prepared the original draft of the manuscript, and the review and editing were performed by Devi Rianti, Dwi Wahyu Indrawati, and Rizky Brilliant Syah Manurung. Visualization was handled by Rizky Brilliant Syah Manurung, and overall supervision was provided by Hari Basuki Notobroto. All authors have read and agreed to the published version of the manuscript.

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