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Original Article

Genistein inhibits the proliferation of human choriocarcinoma cells via the downregulation of estrogen receptor-α phosphorylation at serine 118

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SUMMARY

Background & aims: Choriocarcinoma is a malignant trophoblastic tumor. The phosphorylation of estrogen receptor-α at serine 118 (p-ER-s118) decreases cancer cell proliferation. However, the effect of genistein as a modulator of p-ER-s118 and proliferation of chorioarcinoma cells remains to be understood. This study aims at determining the function of genistein on p-ER-s118 levels and human choriocarcinoma JEG-3 cell proliferation.

Methods: After reaching confluency, cells were divided into six groups, the control group (without methyl-piperidino-pyrazole (MPP) pre-treatment and genistein treatment); and groups with cells treated with genistein at concentrations of 0, 10, 25, 50, and 100 μM (cells were pretreated with MPP). Expression of p-ER-s118 and Ki-67 were analyzed using immunocytochemistry.

Results: Different doses of genistein decreased p-ER-s118 levels compared to those in the control ($p < 0.05$). JEG-3 cell proliferation was inhibited by MPP pre-treatment, concomitant with genistein treatment with a dose of 0 μM, 25 μM, 50 μM, and 100 μM compared to the proliferation of the control cells ($p < 0.05$).

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Conclusion: Taken together, treatment with methyl-piperidino-pyrazole downregulated p-ER-s118. The addition of genistein further decreased the levels of p-ER-s118 and inhibited cell proliferation. Thus, *in vivo* studies nedd to follow this *in vitro* study to elucidate the mechanism(s) employed by genistein as an alternative therapy for choriocarcinoma.

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1. Introduction

Choriocarcinoma is a malignant trophoblastic tumor derived from conceptus trophoblast cells. These tumors contain cytotrophoblast cells, intermediate trophoblast cells, and syncytiotrophoblasts without chorionic villi [1,2]. The incidence of choriocarcinoma in Southeast Asian and Japanese population is higher than that in the European and North American population [3]. Choriocarcinoma is found in 1 out of 40.000–50.000 pregnancies or 1 in 40 M pregnancies [4,5]. Choriocarcinoma is effectively treated during the initial stages. Early-stage patients exhibit high rates of remission. These tumors become aggressive and metastasize rapidly and extensively through the lymphatic and venous systems during the late stage of disease [6]. The diagnosis of choriocarcinoma is characterized by excessive uterine bleeding and high levels of human chorionic gonadotropin in the blood [7].

Estrogen/estradiol ligands activate estrogen receptor-α (ER-α) that is a nuclear receptor transcription factor [8]. As a modular protein, ER-α contains several functional domains. In addition to ligand binding, ER-α undergoes post-translational modifications, like phosphorylation. ER-α phosphorylation targets all domains, most frequently for the N-terminus with a ligand-dependent and independent manner [9,10]. Phosphorylated ER-α functions involved in various biological processes of normal or malignant cells [11–16]. Phosphorylated of estrogen receptor-α at serine 118 (p-ER-s118) positively associated with resistance to endocrine therapy in breast carcinoma [17]. In MCF-7 breast cancer cells, increased levels of p-ER-s118 decrease cell proliferation [14]. Low levels of p-ER-s118 improve survival in ER-positive breast cancer [18]. The balance between cell division and apoptosis determines cell proliferation [19]. Choriocarcinoma is a very proliferative and invasive tumor that induces placental malignancy. Ki-67 is a nuclear antigen that is expressed during all the phases of cell proliferation [20,21]. However, the correlation between the levels of p-ER-s118 and choriocarcinoma cell behavior, especially proliferation remains to be understood.

Genistein is a natural phytoestrogen of the isoflavone group. These active compounds are found in soybeans and have long been used as a source of food protein [22]. Various studies have demonstrated the role of genistein as an anticancer agent by modulating the expression of cell cycle and apoptosis-related genes, inhibition of metastasis, suppression of proliferation, and regulation of estrogen receptors [23–28]. Genistein inhibits the invasion and migration of JAR choriocarcinoma cells by modulating the expression of metastasis-related genes [29]. Other studies have shown that genistein stimulates estrogen production in JEG-3 cells [30]. However, the effect of genistein on the levels of p-ER-s118 and choriocarcinoma cell proliferation is yet to be studied. Therefore, this study aims at determining the role of genistein on p-ER-s118 levels and proliferation of the human choriocarcinoma cell line.

2. Material and methods

2.1. Ethics

This study has been approved by the local ethics committee, Faculty of Medicine, Universitas Lambung Mangkurat, Banjarmasin, South Kalimantan, Indonesia (Number 1087/KEPK-FK UNLAM/EC/II/2018).

2.2. Cell culture

JEG-3 human choriocarcinoma cells were culture as previously described [29]. These cells were purchased from the American Type Collection of Cells (Manassas, VA, USA). JEG-3 cells were grown in Minimum Essential Medium Eagle's salts without L-glutamine (Biowest, France, catalog number L0415-500) supplemented with 10% fetal bovine serum (Biowest, France, catalog number S181H-100), 1 mM sodium pyruvate (Sigma Aldrich, Singapore, catalog number S8636), antibiotic and antimytotic (Sigma Aldrich, Singapore, catalog number 5955). Cells were cultured in a 75 cm2 flask in a 5% CO2 incubator at 370C. Once the cells reached 90% confluency, they were split, using 0.25% trypsin (Biowest, France, Catalog number L0931). Cells were treated after reaching a density of 15.000 cells/cm^2.

2.3. Determination of the dosage of methyl-piperidino-pyrazole (MPP)

We determined the optimum dose of MPP (Santa Cruz, USA, catalog number sc-204098) to the block of p-ER-s118. Cells were incubated 3 h with MPP at concentrations of 0; 1; 2.5; and 5 μM and were used to analyze the levels of p-ER-s118. We used the lowest dose that blocked phosphorylation for our subsequent experiments. Experiments were performed in triplicates.

2.4. Genistein treatment

We determined the optimum dose of genistein used to treat JEG-3 cells to understand its role on the levels of p-ER-s118 and cell proliferation. MPP-treated cells were incubated with 0,10, 25, 50 and 100 μM of genistein for 3 h (Nacalai Tesque Inc. Japan, catalog number 16659-36). Experiments were performed in triplicates.

2.5. Immunocytochemistry

Immunocytochemistry was performed as described previously study [30]. We used primary antibodies targeting recombinant phospho ER-α S118 (GeneTex, Ca USA, catalog number GTX50139) and Ki-67 (Invitrogen, USA, catalog number MA5-14520). Subsequently, the cells were washed and incubated with secondary antibodies. The coverslip containing the cells were mounted on the slides that were subjected to confocal microscopy.

2.5. Statistical analysis

Data have been represented as mean ± standard deviation and analyzed using analysis of variance in SPSS version 16 for Windows. Data that were significant omitted subjected to post-hoc test. $p < 0.05$ was considered statistically significant.

3. Results

Figure 1 shows the levels of p-ER-s118 in the various cell groups. The levels of p-ER-s118 decreased at MPP concentrations of 2.5 μM and 5 μM compared to those in the control groups ($p < 0.05$). p-ER-s118 levels were not significantly different in cells treated with 2.5 mM and 5 mM of MPP ($p > 0.05$).

Figure 2 shows the levels of p-ER-s118 in the control and genistein-treated groups. As observed before, MPP without genistein significantly reduced p-ER-s118 levels compared to those in the control group ($p < 0.05$). p-ER-s118 levels were further lowered upon the addition of genistein than those in the control group ($p < 0.05$).

Figure 3 shows the expression of Ki-67 in different group of cells. Genistein treatment (25, 50, and 100 μM) downregulated Ki-67 compared to the levels of Ki-67 in the control group ($p < 0.05$).

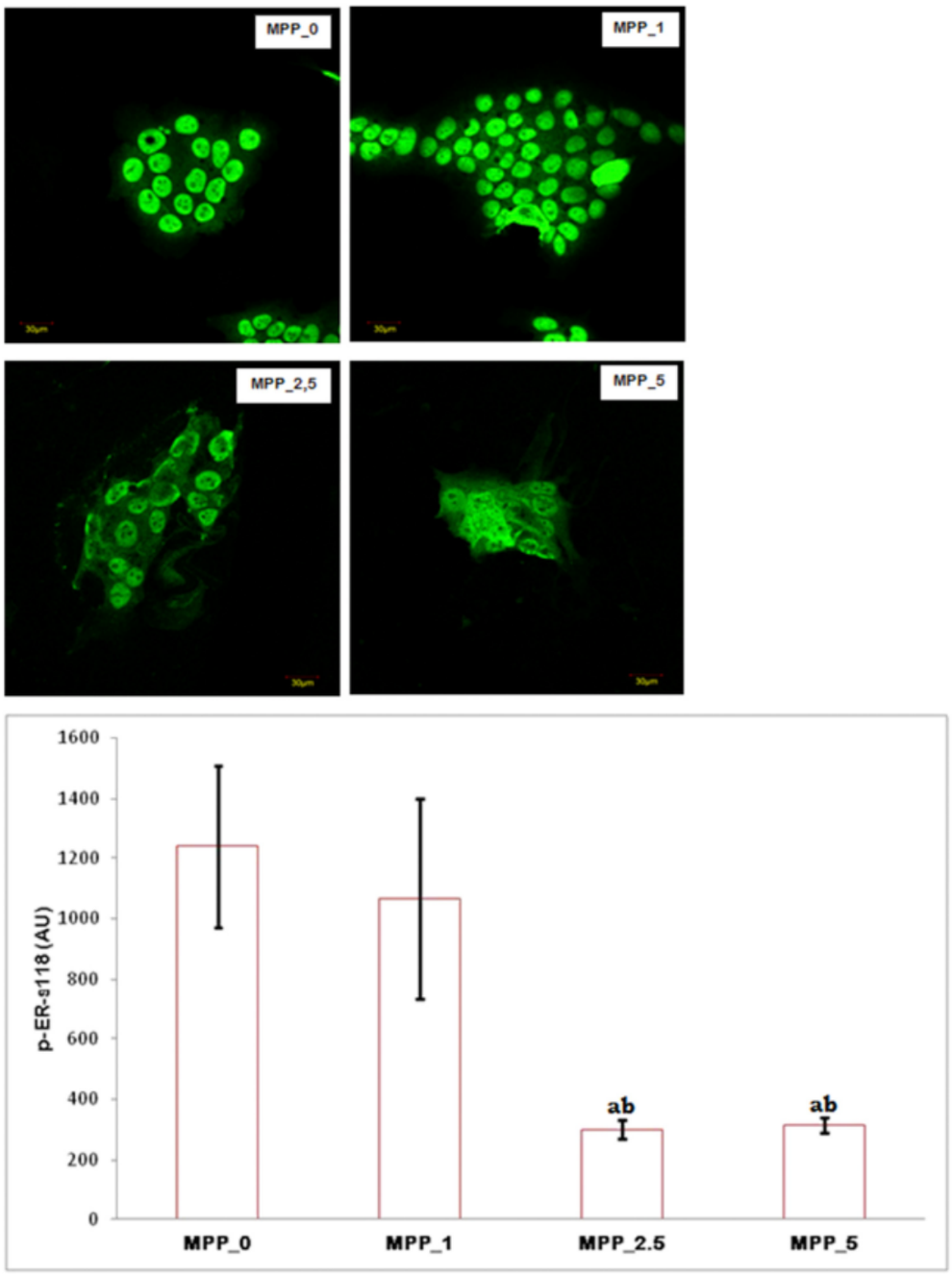

Fig. 1. Confocal micrographs of phosphorylated-ER-α at S118 (p-ER-S118) in JEG-3 choriocarcinoma cells (Magnification x400; confocal laser scanning microscopy; upper panel). The lower panel shows the levels of p-ER-s118 in JEG-3 cells. Note: Data was represented as mean ± standard deviation. a: $p < 0.05$ compared with the control group; b: $p < 0.05$ compared to MPP-treated cells (1 μM).

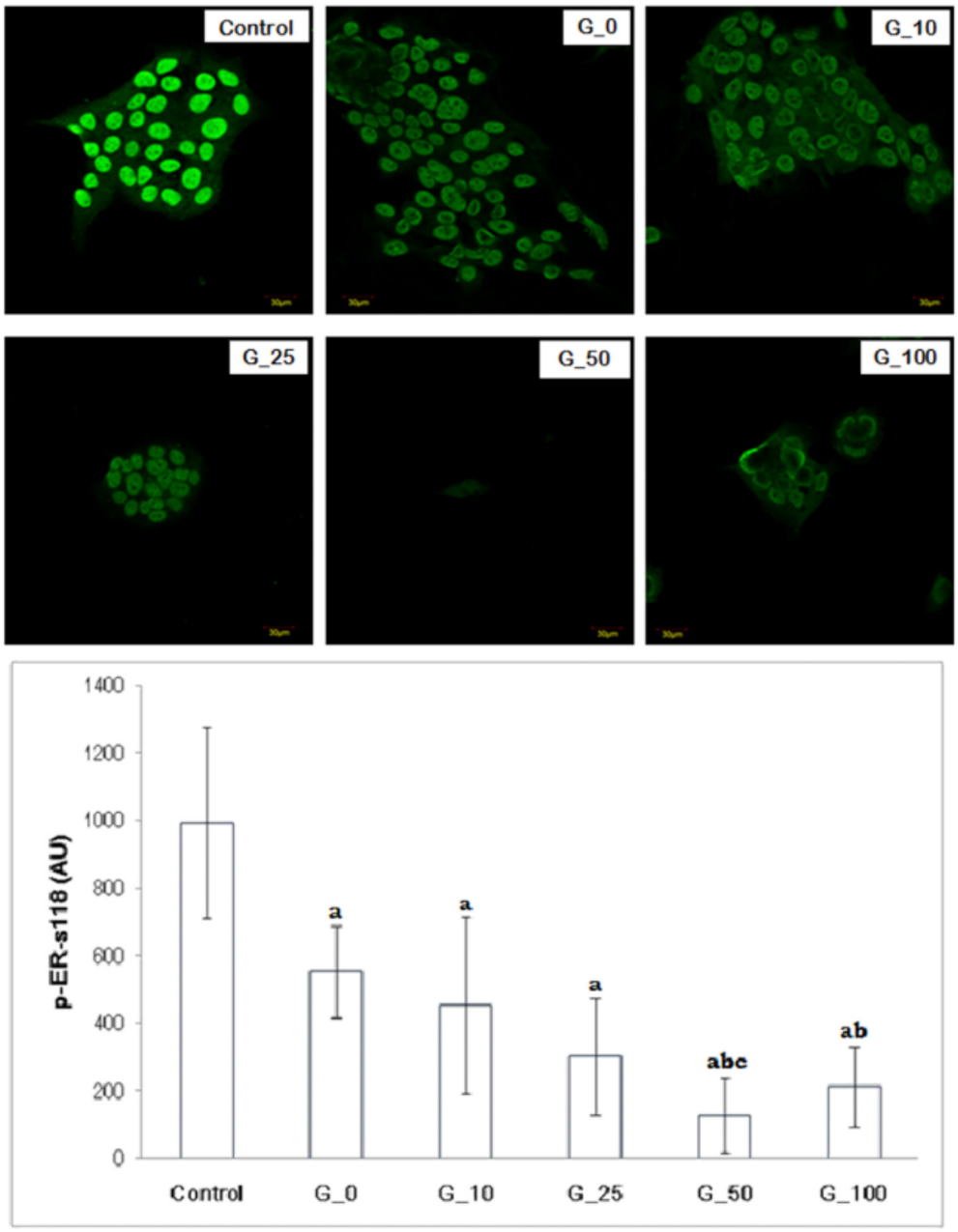

Fig. 2. Confocal micrographs of p-ER-S118 JEG-3 choriocarcinoma cells (Magnification x400; confocal laser scanning microscopy; FITC staining; upper panel). The lower panel shows the levels of p-ER-s118 in genistein-treated JEG-3 cells. Note: Data was represented as mean ± standard deviation. a: $p < 0.05$ compared to the control group; b: $p < 0.05$ compared to genistein treatment at a dose of 0 μM; G_0:, G_10, G_25, G_50, and G_100 represent JEG-3 cells treated with genistein at concentrations of 0, 10, 25, 50, and 100 μM, respectively.

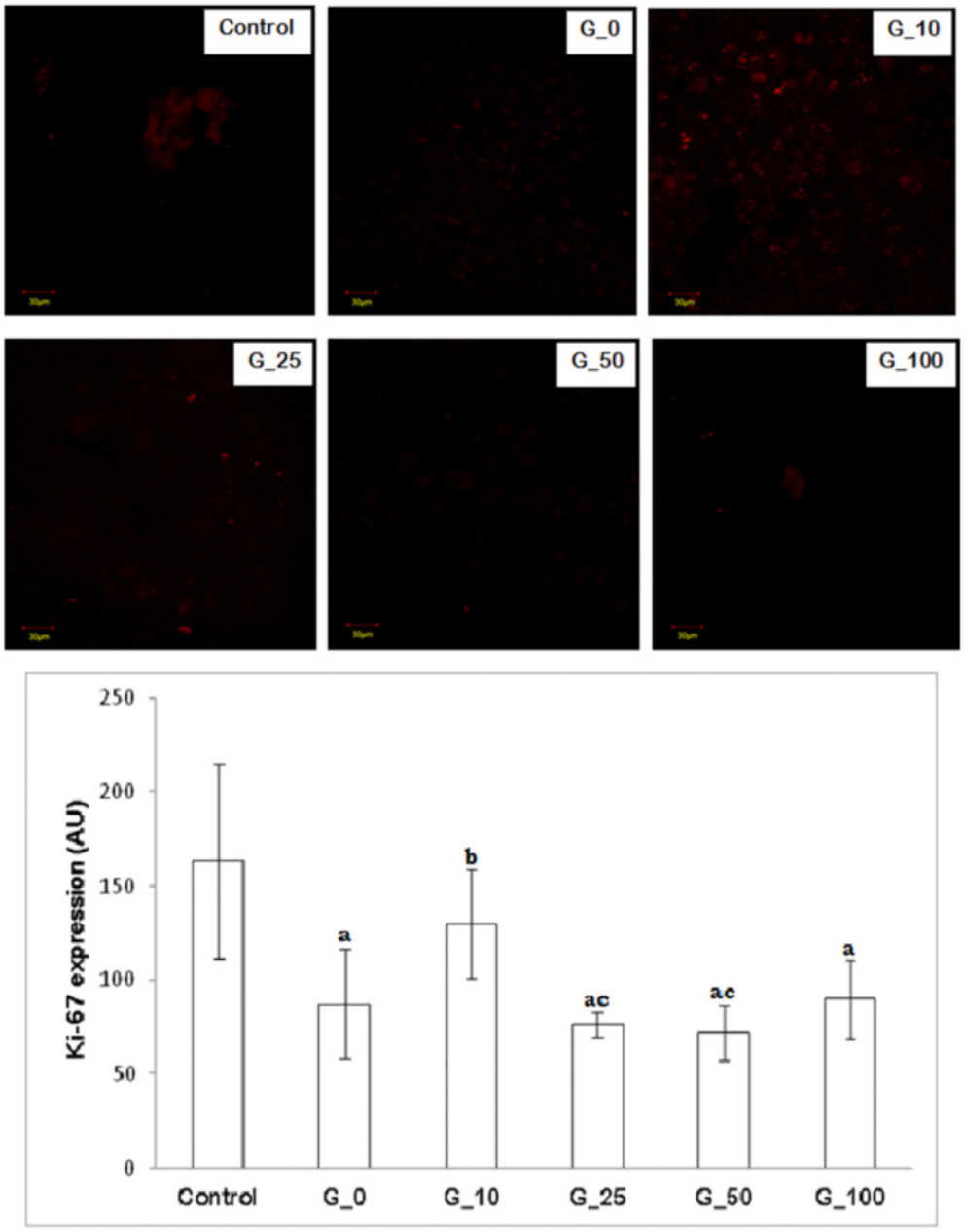

Fig. 3. Confocal micrographs for the expression of Ki-67 expression in JEG-3 choriocarcinoma cell line (Magnification x400; confocal laser scanning microscopy; Rhodamine staining; upper panel). The lower panel shows the signal from Ki-67 in JEG-3 cells. Note: Data was represented as mean ± standard deviation. a: $p < 0.05$ compared to the control group; b: $p < 0.05$ compared to genistein treatment at a dose of 0 μM; c: $p < 0.05$ compared to genistein treatment at a dose of 10 μM; G_0, G_10, G_25, G_50, and G_100 represent JEG-3 cells treated with genistein at concentrations of 0, 10, 25, 50, and 100 μM, respectively.

4. Discussion

Our previous in silico study found that a stronger interaction between genistein and ER-α as compared to that between genistein and ER-β [33]. In this study, we used MPP to block ER-α. MPP, an ER-α-selective antagonist, is active at concentrations that do not have an agonist or antagonistic effect on ER-β [34].

Understanding the phosphorylation profiles of ER-α has helped identify reliable biomarkers for evaluating the efficacy of endocrine or herbal therapies against the development of cancer [35]. In this study, we found basal expression of p-ER-s118 in choriocarcinoma cell line. This indicates that JEG-3 cells as one of the choriocarcinoma cell lines have functional ER-α due to the presence of phosphorylated serine 118. Our result is contrasted with previous findings that JEG-3 cell does not express ER-α [5], but consistent with previous findings that demonstrate the expression of ER-α on JEG-3 cells [36]. We also showed that p-ER-s118 could be suppressed by MPP at doses of 2.5 and 5 μM. These results are consistent with the theory that MPP is a selective antagonist of ER-α. The affinity of MPP is 200 times greater for ER-α than that compared to ER-β [37]. Furthermore, a 2.5 μM dose of MPP was used to evaluate the action role of genistein against p-ER-s118 and proliferation of choriocarcinoma cells.

Treatment with genistein significantly decreased the levels of p-ER-s118. At a dose of 10 mM, the down-regulation of p-ER-s118 was similar to that using MPP alone. The decrease in p-ER-s118 levels were more pronounced at higher doses of genistein. This shows that genistein can modulate the phosphorylation of estrogen receptor-α at serine 118 in JEG-3 choriocarcinoma cells. This down-regulation may occur via proteasome-dependent degradation [38]. This study highlights the activity of xenoestrogens in inducing the phosphorylation of the ER [39]. This study also extends the results from previous quantitative phosphoproteomic studies [40].

Previous studies have shown that Ki-67 is expressed by JEG-3 choriocarcinoma cells [41] and in choriocarcinoma-induced experimental animals [42] as a marker for cell proliferation. In this study, Ki-67 was downregulated significantly in cells treated with genistein at concentrations of 0, 25, 50, and 100 μM compared to Ki-67 levels in the control group. These results indicate that MPP administration alone or in combination a specific dose of genistein suppressed the proliferation of choriocarcinoma cells. This antiproliferative mechanism involved the suppression of p-ER-s118. This study is consistent with the fact that genistein is a protein kinase inhibitor that modulates cell growth [43–46].

It conclusion, treatment of cells with MPP downregulated the p-ER-s118. Genistein increases the potency of MPP in modulating the level of p-ER-s118 and inhibiting cell proliferation. Thus, the results of this *in vitro* study should be validated by *in vivo* tests to study the mechanism(s) employed by genistein to serve as an alternative therapy for choriocarcinoma.

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None.

Authors contribution

All authors have critically reviewed and approved the final version of the manuscript. HY, EH, TN, HK conceived and designed the study. HY conducted research, provided research materials, and collected and organized data, analyzed and interpreted data. HY, EH, TN, HK wrote an initial and final draft of the article.

Conflict of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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