Clinical Nutrition Open Science 35 (2021) 77–85

Contents lists available at ScienceDirect

Clinical Nutrition Open Science

journal homepage: www.clinicalnutritionopenscience.com

Original Article

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

Hariadi Yuseran [a, b, *], Edi Hartoyo [c], Tatit Nurseta [d], Handono Kalim [e]

[a] Doctoral Program in Medicine, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia
[b] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Lambung Mangkurat/Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia
[c] Department of Pediatric, Faculty of Medicine, Universitas Lambung Mangkurat/Ulin General Hospital, Banjarmasin, South Kalimantan, Indonesia
[d] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Brawijaya/Dr. Saiful Anwar General Hospital, Malang, East Java, Indonesia
[e] Department of Internal Medicine, Faculty of Medicine, Universitas Brawijaya/Dr. Saiful Anwar General Hospital, Malang, East Java, Indonesia

ARTICLE INFO

Article history:
Received 12 November 2019
Accepted 14 October 2020
Available online 21 October 2020

Keywords:
Cell cycle
Estrogen receptor
Genistein
Malignancy
Trophoblast

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 ($p < 0.05$).

* Corresponding author. Doctoral Program in Medicine, Faculty of Medicine, Universitas Brawijaya, Jalan Veteran, Malang, East Java, Indonesia.
E-mail addresses: hariadiyuseran@yahoo.com (H. Yuseran), edihartoyo@yahoo.com (E. Hartoyo), tatitnurseta@yahoo.co.id (T. Nurseta), hkalim333@gmail.com (H. Kalim).

https://doi.org/10.1016/j.yclnex.2020.10.001

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.

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 ($p < 0.05$).

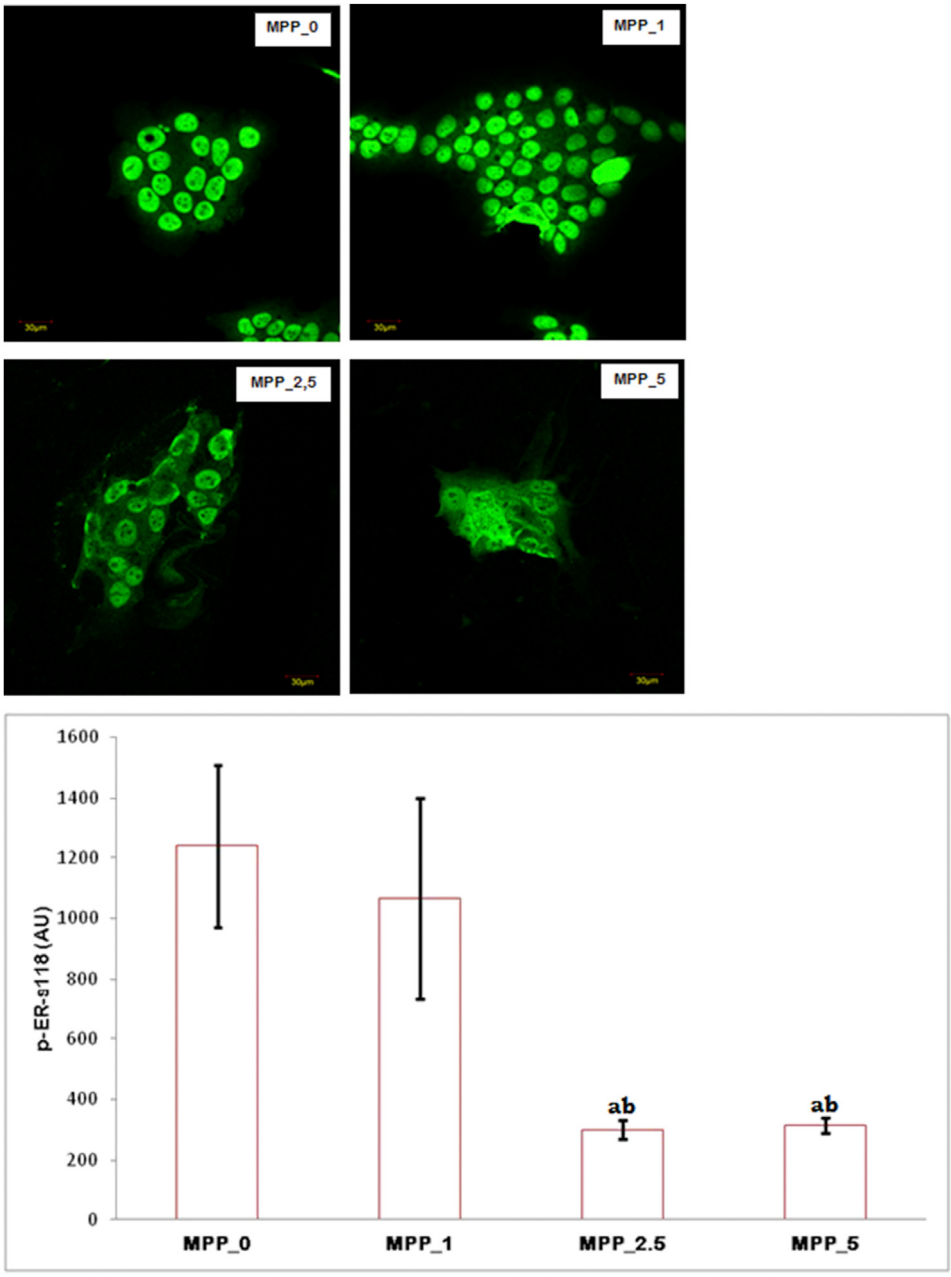

Fig. 1. Confocal micrographs of phosphorylated-ER-α at S118 (p-ER-S118) in JEG-3 choriocarcinoma cells (Magnification x400; confocal laser scanning microscopy; upper panel). The lower panel shows the levels of p-ER-s118 in JEG-3 cells. Note: Data was represented as mean ± standard deviation. a: $p <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

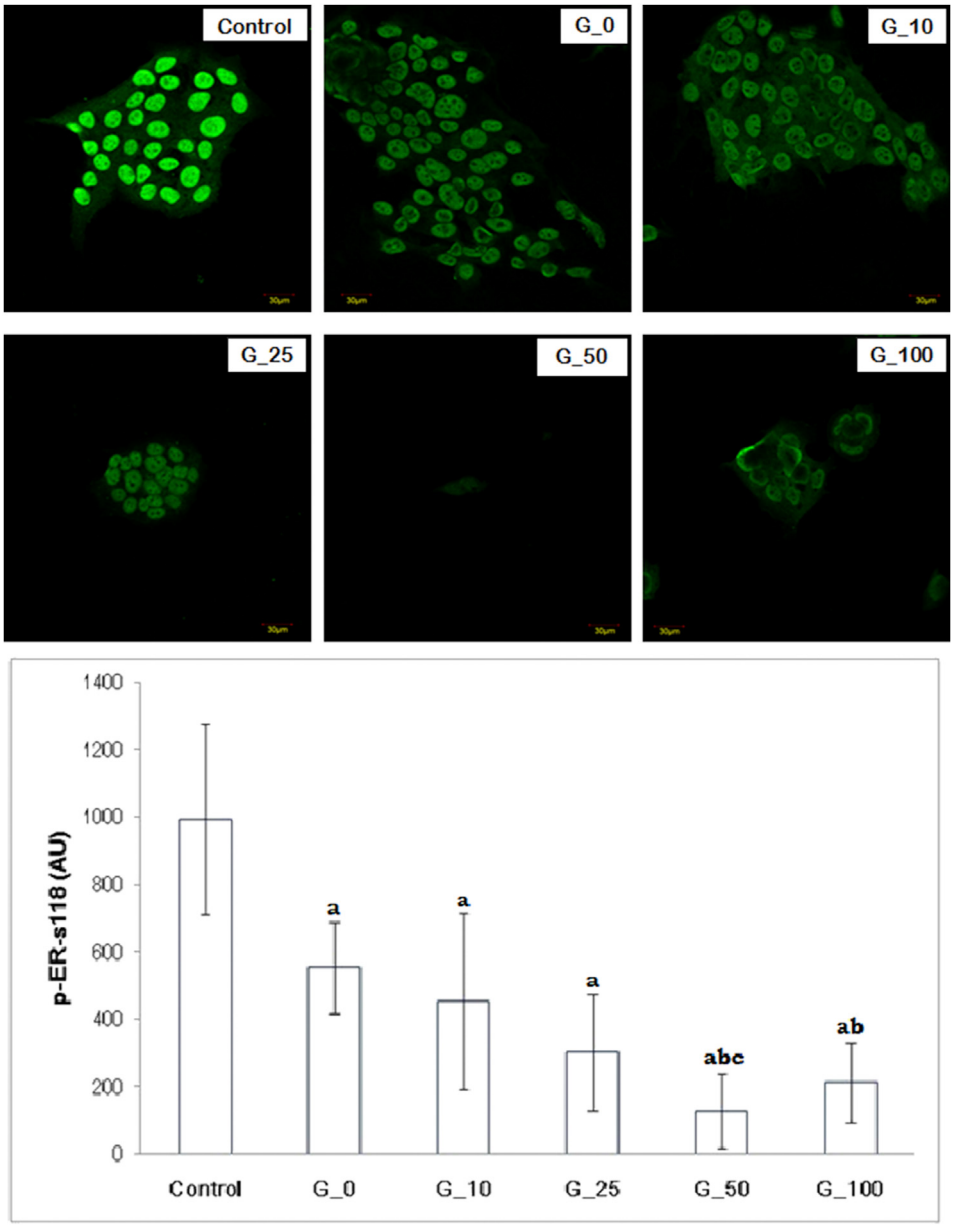

Fig. 2. Confocal micrographs of p-ER-S118 JEG-3 choriocarcinoma cells (Magnification x400; confocal laser scanning microscopy; FITC staining; upper panel). The lower panel shows the levels of p-ER-s118 in genistein-treated JEG-3 cells. Note: Data was represented as mean ± standard deviation. a: $p < 0.05$ 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.

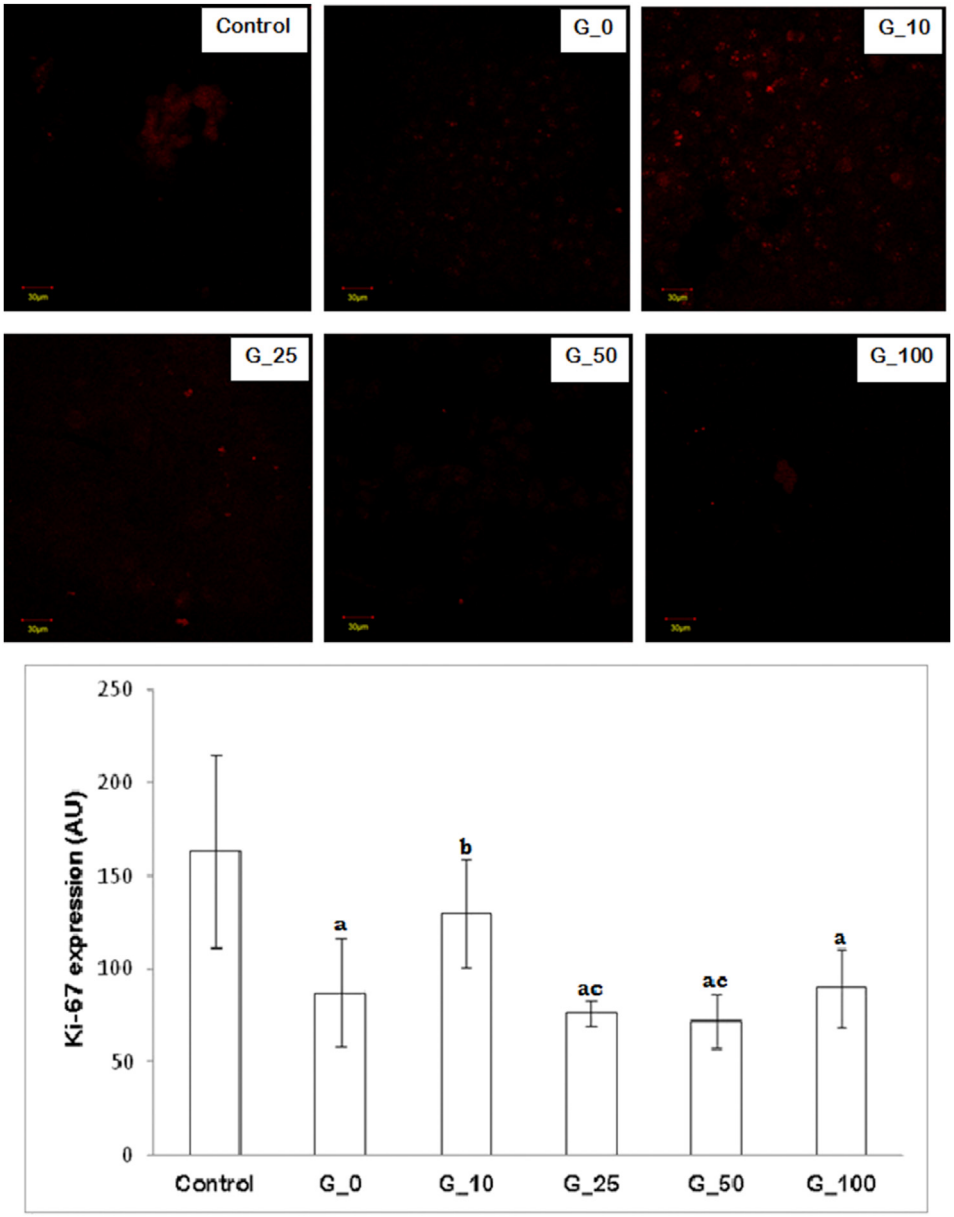

Fig. 3. Confocal micrographs for the expression of Ki-67 expression in JEG-3 choriocarcinoma cell line (Magnification x400; confocal laser scanning microscopy; Rhodamine staining; upper panel). The lower panel shows the signal from Ki-67 in JEG-3 cells. Note: Data was represented as mean ± standard deviation. 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.

Funding source

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.

Acknowledgment

We acknowledged to Mrs Choirunil Chotimah and Mrs Helly Nurul Karima for valuable technical assistance.

References

[1] Strohl AE, Lurain JR. Clinical epidemiology of gestational trophoblastic disease. New York: Curr Obstet Gynecol Rep. Springer Science and Business Media; 2013.
[2] Cheung AN, Zhang HJ, Xue WC, Siu MK. Pathogenesis of choriocarcinoma: clinical, genetic and stem cell perspectives Future. Oncol 2009;5:217–31.
[3] Mello JB, Ramos Cirilo PD, Michelin OC, Custódio Domingues MA, Cunha Rudge MV, Rogatto SR, et al. Genomic profile in gestational and non-gestational choriocarcinomas. Placenta 2017;50:8–15.
[4] Lurain JR. Gestational trophoblastic disease II: classification and management of gestational trophoblastic neoplasia. Am J Obstet Gynecol 2011;204(1):11–8.
[5] Lurain JR. Gestational trophoblastic disease I: epidemiology, pathology, clinical presentation and diagnosis of gestational trophoblastic disease, and management of hydatidiform mole. Am J Obstet Gynecol 2010:531–9.
[6] Xu Q, Tan Y, Zhang K, Li Y. Crosstalk between p38 and Smad3 through TGF-beta1 in JEG-3 choriocarcinoma cells. Int J Oncol 2013;43:1187–93.
[7] Kawamura K, Kawamura N, Okamoto N, Manabe M. Suppression of choriocarcinoma invasion and metastasis following blockade of BDNF/TrkB signaling. Canc Med 2013;2:849–61.
[8] Hankinson SE, Colditz GA, Willett WC. Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones. Breast Cancer Res 2004;6:213–8.
[9] Lannigan DA. Estrogen receptor phosphorylation. Steroids 2003;68:1–9.
[10] Murphy LC, Seekallu SV, Watson PH. Clinical significance of estrogen receptor phosphorylation. Endocr Relat Canc 2011;18:R1–14.
[11] Ward RD, Weigel NL. Steroid receptor phosphorylation: assigning function to site-specific phosphorylation. Biofactors 2009;35:528–36.
[12] Al-Dhaheri MH, Rowan BG. Protein kinase A exhibits selective modulation of estradiol-dependent transcription in breast cancer cells that is associated with decreased ligand binding, altered estrogen receptor alpha promoter interaction, and changes in receptor phosphorylation. Mol Endocrinol 2007;21:439–56.
[13] Duplessis TT, Williams CC, Hill SM, Rowan BG. Phosphorylation of estrogen receptor alpha at serine 118 directs recruitment of promoter complexes and gene-specific transcription. Endocrinology 2011;152:2517–26.
[14] Huderson BP, Duplessis TT, Williams CC, Seger HC, Marsden CG, Pouey KJ, et al. Stable inhibition of specific estrogen receptor alpha (ERalpha) phosphorylation confers increased growth, migration/invasion, and disruption of estradiol signaling in MCF-7 breast cancer cells. Endocrinology 2012;153:4144–59.
[15] Shah YM, Rowan BG. The Src kinase pathway promotes tamoxifen agonist action in Ishikawa endometrial cells through phosphorylation-dependent stabilization of estrogen receptor (alpha) promoter interaction and elevated steroid receptor co-activator 1 activity. Mol Endocrinol 2005;19:732–48.
[16] Morice L, Benaîtreau D, Dieudonné MN, Morvan C, Serazin V, Mazancourt P, et al. Antiproliferative and proapoptotic effects of bisphenol A on human trophoblastic JEG-3 cells. Reprod Toxicol 2011;32:69–76.
[17] Yamashita H, Nishio M, Kobayashi S, Ando Y, Sugiura H, Zhang Z, et al. Phosphorylation of estrogen receptor α serine 167 is predictive of response to endocrine therapy and increases postrelapse survival in metastatic breast cancer. Breast Cancer Res 2005;7:R753.
[18] Yamashita H, Nishio M, Toyama T, Sugiura H, Kondo N, Kobayashi S, et al. Low phosphorylation of estrogen receptor α (ERα) serine 118 and high phosphorylation of ER α serine 167 improve survival in ER-positive breast cancer. Endocr Relat Canc 2008;15:755–63.
[19] Wang M, McLaren S, Jeyathevan R, Allanson BM, Ireland A, Kang A, et al. Laboratory validation studies in Ki-67 digital image analysis of breast carcinoma: a pathway to routine quality assurance. Pathology 2019;51(3):246–52.
[20] Xu J, Liu P, Da J, Hao J, Peng W, Sun G. Prognostic value of Ki-67 in stage I non-small-cell lung cancer: a meta-analysis involving 1931 patients. Pathol Res Pract 2019;215:855–60.
[21] Park J, Lee Y. Hypoxia induced phosphorylation of estrogen receptor at serine 118 in the absence of ligand. J Steroid Biochem Mol Biol 2017;174:146–52.
[22] Fukutake M, Takahashi M, Ishida K, Kawamura H, Sugimura T, Wakabayashi K. Quantification of genistein and genistin in soybeans and soybean products. Food Chem Toxicol 1996;34(5):457e461.
[23] Jiang J, Yan HL, Du AY, Guo YL, Song LH. Genistein enhances radiosensitivityof hepatoma Bel-7404 cells and its possible mechanisms. Chin J Canc Bio-ther 2017;24:395–9.
[24] Banerjee S, Li Y, Wang Z, Sarkar FH. Multi-targeted therapy of cancer by genistein. Canc Lett 2008;269:226–42.
[25] Spagnuolo C, Russo GL, Orhan IE, Habtemariam S, Daglia M, Sureda A, et al. Genistein and cancer:current status, challenges, and future directions. Adv Nutr 2015;6:408–19.
[26] Choi E, Kim G. Effect of Artemisia species on cellular proliferation and apoptosis in human breast cancer cells via estrogenreceptor-related pathway. J Tradit Chin Med 2013;33(5):658–63.
[27] Wang Q, Zhao H, Xiang Q, Ju H, Han S-M, Wang L-Y, et al. Effect of Yikun Neiyi Wan on the expression of aromatase P450, COX-2, and ER Related receptor in endometrial cells in vitro from patients with endometriosis. J Tradit Chin Med 2009;29(4):296–300.
[28] Kurzer MS, Xu X. Dietary phytoestrogens. Annu Rev Nutr 1997;17:353–81.
[29] Liu X, Li X, Yin L, Ding J, Jin H, Feng Y. Genistein inhibits placental choriocarcinoma cell line JAR invasion through ERβ/MTA3/Snail/E-cadherin pathway. Oncol Lett 2011;2:891–7.

[30] Richter DU, Mylonas I, Toth B, Scholz C, Briese V, Friese K, et al. Effects of phytoestrogens genistein and daidzein on progesterone and estrogen (estradiol) production of human term trophoblast cells *in vitro*. Gynecol Endocrinol 2009;25:32–8.
[33] Yuseran H, Hartoyo E, Nurseta T, Kalim H. Molecular docking of genistein on estrogen receptors, promoter region of BCLX, caspase-3, Ki-67, cyclin D1, and telomere activity. J Taibah Univ Med Sci 2019;14(1):79–87.
[34] Zhou HB, Carlson KE, Stossi F, Katzenellenbogen BS, Katzenellenbogen JA. Analogs of methyl-piperidino pyrazole (MPP): antiestrogens with estrogen receptor a selective activity. Bioorg Med Chem Lett 2009;19:108–10.
[35] Murphy LC, Skliris GP, Rowan BG, Al-Dhaheri M, Williams C, Penner C, et al. The relevance of phosphorylated forms of estrogen receptor in human breast cancer in vivo. J Steroid Biochem Mol Biol 2017;174:146–52.
[36] Waldschläger J, Bergemann C, Ruth W, Effmert U, Jeschke U, Richter DU, et al. Flax-seed extracts with phyto estrogenic effects on a hormone receptor-positive tumour cell line. Anticancer Res 2005;25:1817–22.
[37] Notch EG, Mayer GD. Efficacy of pharmacological estrogen receptor antagonists in blocking activation of zebrafish estrogen receptors. Gen Compar Endocrinol 2011;173:183–9.
[38] Acconcia F, Fiocchetti M, Marin M. Xenoestrogen regulation of ERα/ERβ balance in hormone-associated cancers. Mol Cell Endocrinol 2017;45:3–12.
[39] La Rosa P, Pellegrini M, Totta P, Acconcia F, Marino M. Xenoestrogens alter estrogen receptor (ER)a intracellular levels. PloS One 2014;9(2):e88961.
[40] Yan GR, Yin XF, Xiao CL, Tan ZL, Xu SH, He QY. Identification of novel signaling components in genistein-regulated signaling pathways by quantitative phosphoproteomics. J Proteomics 2011;75:695–707.
[41] Sokolov DI, Furaeva KN, Stepanova OI, Ovchinnikova OM, Viazmina LP, Kozonov GR, et al. Changes in functional activity of JEG-3 trophoblast cell line in the presence of factors secreted by placenta. Arch Med Res 2015;46:245–56.
[42] Zhao M, Hou Y, Fu X, Li D, Sun J, Fu X, et al. Selenocystine inhibits JEG-3 cell growth *in vitro* and *in vivo* by triggering oxidative damage-mediated S-phase arrest and apoptosis. J Canc Res Therapeut 2018;14:1540–8.
[43] Barnes S. Effect of genistein on *in vitro* and *in vivo* models of cancer. J Nutr 1995;125. 777S–783S.
[44] Davis JN, Kucuk O, Sarkar FH. Genistein inhibits NF-κβ activation in prostate cancer cells. Nutr Canc 1999;35:167–74.
[45] Li Y, Sarkar FH. Inhibition of nuclear factor kappaB activation in PC3 cells by genistein is mediated via akt signaling pathway. Clin Canc Res 2002;8:2369–77.
[46] Li Y, Upadhyay S, Bhuiyan M, Sarkar FH. Induction of apoptosis in breast cancer cells MDA-MB-231 by genistein. Oncogene 1999;18:3166–72.