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Polyherbal formulation containing *Saoropus androgynous, Trigonella foenum-graceum,* and *Moringa oleifera* increased the expression of mRNA *smooth muscle* α-*actin* (ACTA2) and *cytokeratin* 14 (CK14) in lactating rats

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ABSTRACT

Polyherbal formulation (PHF) containing extracts of *Sauropus androgynous, Trigonella foenum-graceum* and *Moringa oleifera* has been proven can induce milk production in animal model. However, its molecular of action has not been elucidated, yet. This study aimed to investigate the effect of the PHF on the mRNA expressions of α-actin smooth muscle (ACTA2) and cytokeratin 14 (CK14) on the myoepithelial cells of the lactating rats mammary glands. Thirty female Wistar rats were divided into five groups with six of each. Group I was orally administered aquadest. Group II, III, and IV rats were orally administered the PHF at dose level of 26.25, 52.5, and 105 mg/kg once a day, for 15 days, respectively. Group V was orally administered 2.7 mg domperidone. On 16th day, rats were sacrificed. Mammary glands were isolated and processed for mRNA expression analysis using real-time polymerase chain reaction (qRT-PCR). The results demonstrated that the mRNA expression of ACTA2 and CK14 increased in dose-dependent manner in the groups of PHF. Significantly different between the Group III, IV, and V compared to Group I was observed ($p < 0.05$). However, there was no significantly different between Group IV and Group V ($p>0.05$). In conclusion, the PHF increases the mRNA expression of ACTA2 and CK14 on myoepithelial cells of the mammary glands on lactating rats.

Keywords:
polyherbal formulation
myoepithelial cell
α-actin smooth muscle
cytokeratin 14
lactation

ABSTRAK

Sediaan poliherbal (SPH) yang mengandung ekstrak *Sauropus androgynous, Trigonella foenum-graceum* dan *Moringa oleifera* terbukti dapat meningkatkan air susu pada hewan coba. Namun mekanis aksi molekulernya belum pernah dikaji. Penelitian ini bertujuan untuk mengkaji efek PHF pada ekspresi mRNA α-*actin smooth muscle* (ACTA2) dan *cytokeratin* 14 (CK14) pada sel mioepitelial kelenjar mamae tikus menyusui. Tiga puluh ekor tikus Wistar betina dibagi menjadi lima kelompok dengan enam ekor setiap kelompok. Kelompok I diberi akuades. Kelompok II-IV diberi SPH oral dengan dosis berturut-turut 26,25; 52,25 dan 105 mg/kg sekali sehari selama 15 hari. Kelompok V diberi domperidone oral 2,7 mg. Pada hari ke 16, tikus dikorbankn. Kelenjar mamae diambil dan dianalisis ekspresi mRNAnya dengan qRT-PCR. Hasil penelitian menunjukkan bahwa ekspresi mRNA ACTA2 dan CK14 meningkat sesuai dengan dosis pemberian pada kelompok SPH. Terdapat perbedaan bermakna antara Kelompok III, IV dan V dibandingkan Kelompok I ($p<0,05$). Namundemikian, tidak terdapat perbedaan bermakna antara Kelompok IV dan Kelompok V ($p>0,05$). Dapat disimpulkan, pemberian SPH dapat meningkatkan ekspresi mRNA ACTA2 dan CK14 sel mioepitelial kelenjar mamae tikus menyusui.

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INTRODUCTION

Breastfeeding is essential for a child's health, development, and nutrition. However, many mothers inadequately breastfeed their infants after giving birth due to emotional stress, anxiety, and illness.[1] Therefore, the percentage of exclusive breastfeeding in most countries across the world, including Indonesia, remains below the target set by the World Health Assembly (at least 50 percent).[2,3] Maternal perception of low supply breastmilk is one of the reasons for breastfeeding cessation.[4,5]

Several kinds of efforts to increase milk supply are being made, such as improving infant sucking techniques, initiating early breastfeeding, and pharmacological interventions. Domperidone, metoclopramide, and oxytocin have been used to increase the milk supply. However, their use are restricted due to safety aspects,[6,7] Medicinal plants have been used traditionally by the community as alternative medicine to increase the milk production in nursing mothers with lack of breast milk in various countries including in Indonesia. Some these Indonesian medicinal plants are *Sauropus androgynous* (local name *katuk*), *Trigonella foenum-graceum* (*klabet*), and *Moringa oleifera* (*kelor*). In addition, these medicinal plants have been proven to have galactagogue effect.[8-13] In our previous study we prepared a polyherbal formulation containing these three plants and proved its galactagogue effect in lactating rats (data unpublished).

Milk ejection is necessary for delivering the secreted milk stored in the alveoli from mother to infant. This mechanism is affected by the myoepithelial cell, one of the mammary gland's main cells, which, in response to oxytocin, produces contractile activity.[14,15] Myoepithelial cells express α-actin smooth muscle (ACTA2), a smooth muscle actin isoform that provides an essential function in myoepithelial contraction and maintains the lactation. Previous *in vivo* studies revealed that the disruption of lactation occurred on rats with ACTA2 *null* mammary glands.[15,16] In addition, filament-forming proteins in myoepithelial cells called cytokeratins provide scaffold structures within cells to maintain tissue structure and function.[17,18] Cytokeratin 14 (CK14), one of the types of cytokeratins expressed in myoepithelial cells, was thought to play an important role in providing resistance to mechanical stress and maintaining cell proliferation. CK14 knockdown cell lines demonstrated cell proliferation reduction and delayed in cell cycle progression.[19] Therefore, where the conditions differ in ACTA2 and CK14 expressions will show disruption in myoepithelial cells and reduce breast milk supply.[15,19,20]

In this study we reported the effect of the polyherbal formulation containing the extracts of *S. androgynous folium*, *T. foenum-graceum* seed, and *M. oleifera folium* on ACTA2 and CK14 mRNA expressions on myoepithelial cells of lactating rat mammary glands.

MATERIALS AND METHODS

Study products

The polyherbal formulations capsules were manufactured by a traditional medicine industry in Banten, Indonesia. Each capsule (500 mg) contains three kinds of herbal extracts of *S. androgynous folium* 300 mg, *T. foenum-graceum* seed 150 mg, and *M. oleifera folium* 50 mg.

Animal

Thirty pregnant female Wistar rats, procured from the Department of Pharmacology and Therapy, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia were maintained under

standard laboratory conditions (room temperature of 25 ± 2°C and relative humidity of 60 ± 5%) with free access to food and water *ad libitum*. Rats were divided into five groups of six animals each. Group I as negative control was orally administered aquadest. Group II, III, and IV rats were orally administered the PHF at dose level of 26.25, 52.5, and 105 mg/kg once a day at 6.00 PM, for 15 days, respectively. Group V as positive control was orally administered 2.7 mg domperidone, from 3rd day to 15th day of parturition. On 16th day, rats were sacrificed, and mammary glands were isolated and processed for mRNA expression analysis.

Quantitative real time PCR (qRT-PCR)

All the qRT- PCR assays were purchased from Gene-All Hybrid and used according to the manufacturer's instructions. qPCRs were performed using One-Step qRT-PCR with KAPA SYBR FAST Universal. Reactions of the PCRs were conducted using the forward primer GADPH (5'-GCA TCC TGG GCT ACA CTG AG-3'), ACTA 2 (5'-TGT GCT GGA CTC TGG AGA TG-3'), CK14 (5'-CCT CTG GCT CTC AAT CAT CC-3'), and the reverse primer GAPDH (5'TCC ACC CTG TTG CTG TA-3'), ACTA2 (5'-TAG AGG TCC TTC CTG ATG TC-3'), and CK14 (5'-ATG ACC TTG GTG CGG ATC T-3'). An individual reaction was carried out using the DT-Lite Real-Time PCR System (DNA Technology) with a reverse transcription at 42°C for 5 minutes, followed by enzymatic activation at 95°C for 3 minutes, denaturation for 1-3 seconds at 95°C, and elongation for up to 20 seconds at 60°C.

Gene expression levels were calculated based on the cycle threshold (Ct) value using the following formula: Fold change = $2^{(-\Delta\Delta CT)}$, with a ΔCT = CT ACTA2 or CT CK14 − CT GAPDH (internal control). ΔΔCT = Δ CT (treatment)−Δ CT (control).

Statistical analysis

Data was analyzed using XLSTAT Version 2019.1.2 for Macintosh OS (Addinsoft, Boston, USA). Data was expressed as the mean ± standard deviation (SD). Differences in gene expression among multiple group comparisons were first assessed by a one-way analysis of variance (Anova) followed by a Tukey post-hoc comparison test with control animals. Significant differences were defined as those with a p-value smaller than 0.05. All the calculations were implemented using XLSTAT for Mac version 16.0.

Ethical considerations

The protocol was approved by the Medical and Health Research Ethics Committee (MHREC), the Faculty of Medicine, Public Health and Nursing at Universitas Gadjah Mada, and Dr. Sardjito General Hospital, Yogyakarta (Ref: KE/FK/0904/EC/2017).

RESULTS

The mRNA expression of ACTA2 and CK-14 in the mammary glands of the lactating rat were performed using qPCR. These gene expressions were compared between rat groups treated with three different doses of PHF (Group II-IV) and their respective control group (Group I and V). The PHF treated groups showed a dose-dependent increase of mRNA expression of ACTA2 and CK14 during a 14-day lactation period.

As shown in FIGURE 1, the mRNA expression of ACTA2 in mammary glands indicated upregulation in all groups (Group I-V). The negative control group treated with aquadest (Group I) showed the lowest expression of mRNA among other groups, whereas the highest was the positive control group (Group V). The mRNA expression of the ACTA2 in mammary glands of lactating rats treated with 52.5 and 105 mg/kg PHF (Group II

and III) significantly increased by 4.07 and 4.50 fold, respectively, compared to negative control group ($p<0.05$). However, no significantly different between the mRNA expression of ACTA2 after treated with 105 mg/kg PHF (Group IV) and 2.7 mg/kg domperidone as positive control (Group V) was observed.

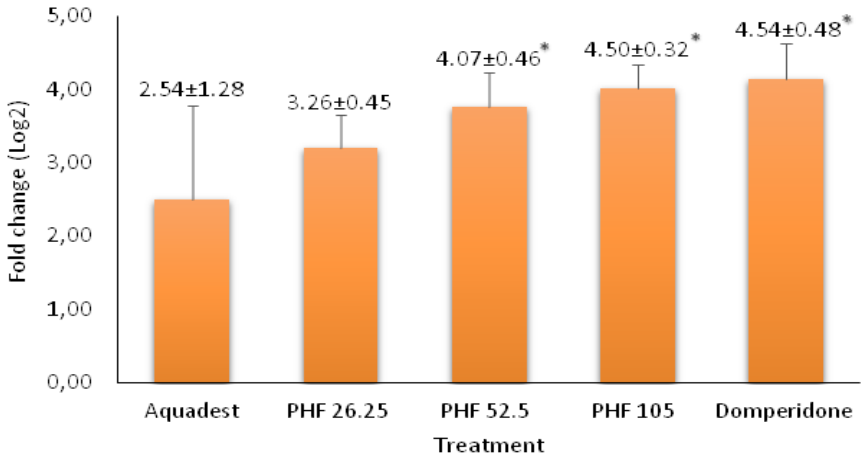

FIGURE 1. The mRNA expressions of ACTA2 in mammary glands of all group. Lactating rats treated with the two highest doses of PHFs were significantly higher than negative control group (aquadest). (*p <0.05).

The mRNA expression of CK14 in mammary glands also indicated upregulation in all groups (Group I-V) as shown in FIGURE 2. The mRNA expression of the CK14 in mammary glands of lactating rats treated with 52.5 and 105 mg/kg PHF (Group II and III) significantly increased by 3.77 and 4.02 fold, respectively, compared to negative control group ($p<0.05$). However, no significantly different between the mRNA expression of CK14 after treated with 105 mg/kg PHF (Group IV) and 2.7 mg/kg domperidone as positive control (Group V) was also observed.

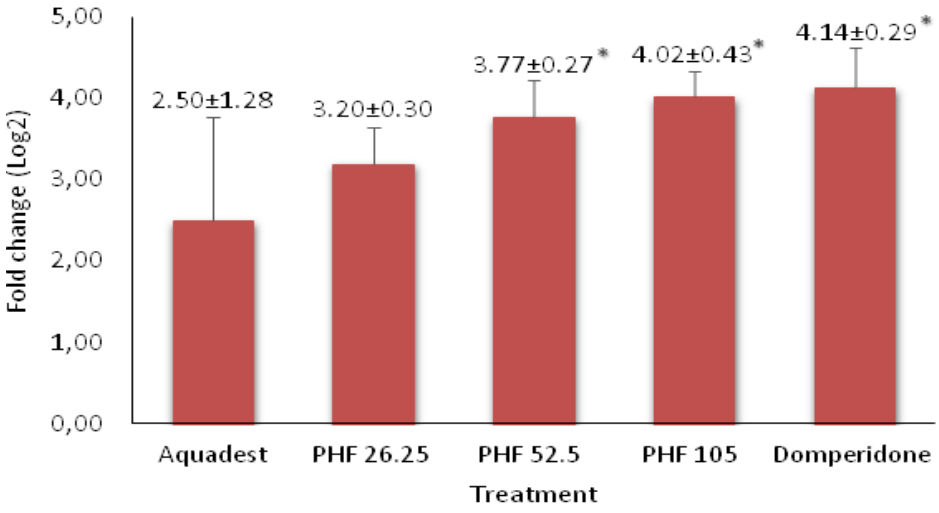

FIGURE 2. The mRNA expressions of CK14 in mammary glands of all group. Lactating rats treated with the two highest doses of PHFs were significantly higher than negative control group (aquadest). (*p <0.05).

DISCUSSION

The qRT-PCR measurements showed PHF containing extracts of *S. androgynous*, *T. foenum-graceum*, and *M. oleifera* significantly increased the mRNA expressions of both ACTA2 and CK14 in the mammary glands of lactating rats. The both genes are expressed in myoepithelial cells. The mammary epithelium consists of two cell layers, the luminal and basal myoepithelial. The luminal cells are involved in the production and secretion of milk, while basal myoepithelial cells contract to eject milk during lactation.[21] The myoepithelial cells are contractile and the manner, as well as some architectural characteristics (caveolae, microfilaments, and dense body), are considered somewhat similar to smooth muscle.[22] Myoepithelial cells contain large quantities of microfilaments, express smooth-muscle specific cytoskeleton, and contractile proteins. ACTA2 and cytokeratins 5, 14, and 17 are included as the markers of myoepithelial cells.[21,22] ACTA2, a smooth muscle actin isoform, provides an essential function in myoepithelial contraction, and maintains the lactation. Previous *in vivo* studies revealed that the disruption of lactation occurred in rats with ACTA2 null mammary glands, so that they were unable to productively nurse their offspring, as the mammary myoepithelial contraction and milk ejection was impaired.[15,16] Moreover, filament-forming proteins in myoepithelial cells called cytokeratins provide scaffold structures within cells to maintain the tissue structure and function.[17,18] Cytokeratin 14, one of the types of cytokeratin expressed in myoepithelial cells, was thought to have a function in providing resistance to mechanical stress and maintaining cell proliferation and integrity. Cytokeratin 14 knockdown cell lines demonstrated cell proliferation reduction and was delayed in cell cycle progression.[19]

During lactation, myoepithelial cells generate the contracting force required to eject milk in response to oxytocin.[20,23] Oxytocin induces the contraction by the pulsatile release from the pituitary gland and binds G protein-coupled receptors on the surface of the myoepithelial cell.[24] The contraction of myoepithelial cells is regulated by myosin light chains (MLC). Phosphorylation on the MLC induces myosin ATPase activity, the binding of myosin to actin, and contraction.[25] In addition, oxytocin also stimulates the production of prolactin that is able to vitalize lactation and mammary gland development.[26]

The increase of the mRNA expressions of ACTA2 and CK14 in the rat groups treated with PHF might be the result of the synergistic mechanism of upregulated hormones in the lactation period and the effect of PHF contents. These both genes are considered highly expressed in myoepithelial cells as the result of oxytocin stimulated by pups sucking and the PHF contents. Papaverine in *S. androgynous* leaves might influence the blood vessels dilatation that provides the oxytocin hormone circulation become more smoothly through the bloodstream.[12] Moreover, *S. androgynus* leaves contain sterol that plays a role in the hormone precursor and increases estrogen production, which was also found in *M. oleifera* leaves like stigmasterol, sitosterol and kampesterol.[12] An estrogen-like effect or phytoestrogen and diosgenin (a type of steroidal sapogenin) in fenugreek or *T. foenum-graceum* are considered the cause of the galactagogue effect in this plant. Estrogen hormone has a regulatory function for oxytocin by influencing its transcription, which, in turn, stimulates the secretion of oxytocin and eventually a contraction of myoepithelial cells.[4,27]

We used domperidone as the reference drug, as this drug is widely used as a galactagogue. It has a potent antidopaminergic effect on D2 receptors

of the lactotrophic cells of the anterior pituitary gland. As domperidone also showed the ability to increase the mRNA expression of ACTA2 and CK14, it might be explained that the content in PHF has a similar mechanism of action with the drug. Papaverine content in PHF might also lead to the blockage of the dopamine receptor. Papaverine has a function in inhibiting PDE10A, an enzyme that predominantly regulates dopamine and cAMP-regulated phosphoprotein, Mr 32 kDA (DARPP-32), which thus inhibits protein phosphatase-1 (PP-1) and the effects of dopaminergic signaling. The inhibition of the signaling can stimulate the prolactin release[12,28] and consequently influence an oxytocin level increase. Parker *et al.*[29] suggested that prolactin had a stimulatory action on the release of oxytocin. This mechanism can explain why PHF and domperidone affect the contraction of myoepithelial cells by showing a high mRNA expression of ACTA2 and CK14 in mammary glands in response to an increase in oxytocin.

CONCLUSION

In conclusion, the PHF containing the extracts of *S. androgynous*, *T. foenum-graceum*, and *M. oleifera* induces the milk production on lactating rats by increase the mRNA expression of ACTA2 and KC14 on the myoepithelial cells of the mammary glands. Further study will be conducted to evaluate acute and subchronic toxicity in animal before clinical study in human conducted.

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