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EGFL7 and *HIF-1α* Expression on Human Trophoblast Placentalby *Rhodomyrtus tomentosa* and *Zanthoxylum acanthopodium*

INTRODUCTION

Hypoxia-induced Factor 1 alpha (*HIF-1a*) is a gene found in the body and placenta. This gene in the placenta plays an important role in the regulation of trophoblast differentiation and molecular pathways[1]. Over expression of this protein can lead to inflammatory disease, preeclampsia and high blood pressure[1]. Hypoxia induces nuclear translocation to form HIF and then binds to hypoxia response elements of related genes[2]. The target genes involved in hypoxia are glycolysis, red blood cell production and angiogenesis[1,2]. Low oxygen in trophoblast cells is an extrinsic factor for cell migration, invasion and proliferation[2]. The role of autophagy in hypoxic trophoblast has a role in placentation[3]. Autophagy can lead to poor placentation in some cases of placental problems such as hypertension or preeclampsia[3]. Epidermal Growth Factor- like domain 7 (*EGFL7*) is an endothelial-restricted gene in embryonic vascular development[4]. *EGFL7* in the placenta is expressed on maternal and fetal vascular endothelium throughout placental development[4]. *EGFL7* can regulate cell migration and trophoblast cell invasion by activating the MAPK, PI3K and NOTCH signalling pathways[5]. *EGFL7* is also referred to as a soluble, extracellular matrix-bound gene in the developing embryo[5]. However, this gene is also found in embryonic stem cells, pre-and peri-implantation embryos and primordial germ cells[5,6]. *EGFL7* is largely derived during late embryogenesis and in the endothelium and is upregulated during pathological and physiological angiogenesis, such as in utero[6]. *EGFL7* is associated with HIF because it is regulated in response to hypoxia[6].

Indonesia has a wealth of herbs because it is located in a tropical climate and is traversed by the equator. Some of the plants from Indonesia that have high antioxidants are andaliman (*Zanthoxylum acanthopodium*) and haramonting (*Rhodomyrtus tomentosa*). Both of these plants are often used by the public for traditional health medicine and anti-inflammatory properties[7,8]. Haramonting fruit extracts showed antioxidant activity[9-11]. In studies using rats placenta, andaliman and nano-sized haramonting can reduce MDA levels, increase HSP-70 and improve liver and placenta[12-14]. Molecularly, andaliman can inhibit apoptosis through cytochrome c and FasL in the placenta[15] and affect the activity of Hes1 and notch1 genes in human trophoblasts[16].

This study aimed to analyze the role of *EGFL7* and *HIF-1a* genes after being given andaliman and haramonting in human trophoblasts. Thus providing information to us whether this herb is beneficial for the *EGFL7* and *HIF-1a* genes that affect the embryo and placenta in pregnant women.

MATERIALS AND METHODS

Study area: The research project was conducted from April, 2019-2020. The research was carried out in the Physiology Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Sumatera Utara, Medan, Indonesia and the Department of Biomedicine and the prevention. Faculty of Medicines, University of Rome Tor Vergata, Rome, Italy.

Preparation of nanoherbal *Zanthoxylum acanthopodium* (NZA) and *Rhodomyrtus tomentose* (NRT): Andaliman (*Zanthoxylum acanthopodium*) and haramonting (*Rhodomyrtus tomentosa*) originate from a plantation in Berastagi, Kabanjahe, Sumatera Utara, Indonesia. Andaliman fruit and haramonting leaves are washed and then air-dried. Andaliman takes 3 weeks to dry while haramonting leaves only 7 days (1 week). The dried samples were sent to the Indonesian Institute of Sciences (LIPI, Jakarta) to be made into nanoherbs using High Energy Milling (HEM).

Study design: The cells used were HTR8 trophoblast cells as many as 300,000 cells/well. Because it uses 48 wells, the total cells needed are 144.10^6. This study used 4 incubation times, namely 30 min, 1, 3 and 16 hrs (overnight) with a total of 48 wells and then observed the cells. 24 wells for Nanoherbal *Zanthoxylum acanthopodium* (NZA) and another 24 wells for Nanoherbal *Rhodomyrtus tomentosa* (NRT). About 10 mg of nanoherbals andaliman and haramonting were dissolved in 1% DMSO then filtered using a 0.45 μm filter membrane and then stored at room temperature and can be used for treatment using HTR8 cells.

Cell culture: HTR8/SVneo EVT cells in the Department of Biomedicine and the prevent University of Rome Tor Vergata, Rome, Italy). The cell line used was HTR8-ATG4BC74A. HTR8/SVneo cells were cultured in RPMI1640 supplemented with 10% FBS, 100 U mLG^1 penicillin and 100 g mLG^1 streptomycin under a 5% CO_2 atmosphere at 37EC16.

RNA isolation: RNA was derived from cell cultures that had been prepared using TRIZOL Reagent (Roche Diagnostics GmbH). HTR8 in 4 time zones namely 30 min, 1, 3 and 16 hrs (overnight)16. DNA contamination was removed by DNAse treatment. mRNA was reverse transcribed using random primers and the superscript first-strand synthesis system (Invitrogen). Gene expression was measured using Real Master Mix SYBR ROX (Eppendorf, Hamburg, Germany). Differences between gene expression were quantified using the Ct method with normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

RT-PCR: RNA was isolated using Trizol (Invitrogen) and reverse-transcribed using qScript cDNA Supermix (Quanta Biosciences). Gene expression was measured quantitatively using SYBR green (applied biosciences) and a specific primerset for-*HIF-1a* and *EGFL7*.

Differences between target expressions were quantified using the CT method with normalization to GADPH with the following primers:

C *EGFL7* : 5'-CCACAAAAAAGAAGAAGGCTACCC-3'

C 5'-TCCAAGAAGGACCCTGCTCACTC-3'
C GAPDH: 5'-TCGGAGTCAACGGATTTGGT-3'
C 5'-GAATTTGCCATGGGTGGAAT-3
C 5'-GAATTTGCCATGGGTGGAAT-3
C *HIF-1a* : 5' GCTCATCAGTTGCCACTTCC3'
C 5' CGCTGRGTGTTTWGTTCTT3'

Data analysis: All gene analysis results were performed 3 times. Data were expressed as Mean±Standard error and analyzed using a one-way analysis of variance (ANOVA) test with sigmaplot application, (*$p<0.05$ and **$p<0.001$).

RESULTS

Administration of NZA and NRT on the morphology of human trophoblast cells: The administration of Nanoherbal andaliman (NZA) and haramonting (NRT) did not affect the life of human trophoblast cells. The number of viable, differentiated, active and proliferating cells in the control group (Fig.1a) was almost the same as that of human trophoblast cells when the nanoherbal andaliman (Fig.1b) and haramonting (Fig.1c) were administered. Based on the microscopic picture, it is known that these 2 herbs are nottoxic and safe on human trophoblast cells.

***EGFL7* expression on human trophoblasts by nanoherbal *Zanthoxylum acanthopodium* (NZA):** Administration of Nanoherbal *Zanthoxylum acanthopodium* (NZA) at an incubation time of 30 min (Fig. 2a) to the *EGFL7* gene showed a significant difference ($p<0.05$) in human trophoblast cells compared to the control group. When the incubation time became 1 hr (Fig. 2b), it was found that the expression of *EGFL7* decreased significantly ($p<0.05$). The decrease in *EGFL7* expression continued to occur at 3 hrs of incubation (Fig. 2c) and overnight (Fig. 2d). Based on this analysis, it is known that the longer the incubation time of human trophoblast cells with Nanoherbal *Zanthoxylum acanthopodium* (NZA) treatment, the lower the expression ofthe *EGFL7* gene.

HIF-1a* expression on human trophoblasts by nanoherbal *Zanthoxylum acanthopodium* (NZA):** Administration of Nanoherbal *Zanthoxylum acanthopodium* (NZA) at 30 min incubation time (Fig. 3a) to the *HIF-1a* gene showed an increase ($p<0.05$) in human trophoblast cells compared to the untreated group. In the 1 hr incubation group (Fig. 3b), *HIF-1a* expression remained significantly increased ($p<0.05$).However, there was a decrease in *HIF-1a* expression at 3 hrsof incubation (Fig. 3c) although not significant ($p>0.05$) and a significant decrease in *HIF-1a* expression overnight (Fig. 3d)EGFL7* expression on human trophoblasts by nanoherbal *Rhodomyrtus tomentose* (NRT):** The administration ofNanoherbal *Rhodomyrtus tomentose* (NRT) at an incubation time of 30 min (Fig. 4a) to the *EGFL7* gene showed significant differences ($p<0.05$) in human trophoblast cells compared to the control group. In the 1 hr incubation group there was adecrease but not significantly (Fig. 4b), when the incubation time was increased to 3 hrs (Fig. 4c) there was a significantincrease in *EGFL7* expression ($p<0.05$). The increase in *EGFL7*expression continued to occur at 16 hrs incubation time (Fig. 4d) with

$p<0.01$). Based on this analysis, it is known that the longer the incubation time of human trophoblast cells on NRT administration, the higher the expression of the *EGFL7* gene.

***HIF-1a* expression on human trophoblasts by nanoherbal Rhodomyrtus tomentose (NRT):** Administration of Nanoherbal *Rhodomyrtus tomentosa* (NRT) at 30 min incubation time (Fig. 5a) to the *HIF-1a* gene showed non-significant differences ($p>0.05$) in human trophoblast cells compared to the untreated group. In the group with an incubation time of 1 hr (Fig. 5b), *HIF-1a* expression decreased significantly ($p<0.05$). The decrease in *HIF-1a* expression continued to occur at the incubation time of 3 hrs (Fig. 5c)significantly ($p<0.05$). The longest incubation (overnight) showed a significant difference (Fig. 5d) with $p<0.05$). Based on this analysis, it is known that the longer the incubation time of human trophoblast cells on NRT administration, the more the *HIF-1a* gene expression decreases.

DISCUSSION

Based on the microscopic, it is known that these 2 herbsare not toxic and may affect genes expression in pregnancy. The administration of Nanoherbal andaliman (NZA) and Nanoherbal haramonting (NRT) are safe for human trophoblast cells. The trophoblast is very important for placental perfusion in maintaining fetal growth[16,17]. Failure ofinterstitial and endovascular trophoblast invasion may result in inadequate spiral artery transformation, resulting in preeclampsia or fetal growth restriction[17]. So both of these herbs are still relatively safe in pregnancy.

In the administration of Nanoherbal *Zanthoxylum acanthopodium* (NZA) to the *EGFL7* gene, the longer the incubation time of human trophoblast cells, the less

expression of the *EGFL7* gene. On the other hand, the longerthe incubation time of human trophoblast cells in the administration of Nanoherbal *Rhodomyrtus tomentose* (NRT),the higher the expression of the *EGFL7* gene. *Rhodomyrtus tomentose* gave a more significant effect than *Zanthoxylum acanthopodium*. Flavonoids, steroids, glycosides, saponins and tannins are among the ingredients found in NRT. Furthermore, NRT has emulsion properties that meet drug requirements as well as strong antioxidant activity. The LC_{50} and LD_{50} values in the toxicity test were 2961.535 ppm and 10.4 0.135 mg kgG^{1} b.wt., respectively. The *Zanthoxylum* family has quite a lot of steroid compounds. The danger of excessive steroids on cells can cause fat accumulation and hypertension[18].

In the *HIF-1a* gene, only incubation time >16 hrs of human trophoblast cells treated with Nanoherbal *Zanthoxylum acanthopodium* (NZA) can reduce *HIF-1a* gene expression. However, the longer the incubation time ofhuman trophoblast cells on the administration of Nanoherbal *Rhodomyrtus tomentose* (NRT), the more the *HIF-1a* geneexpression decreased. Incubation time also affects gene expression because cells also need time and adapt to the environment or medium. *HIF-1a* is sensitive to oxygen because it acts as a regulator of cellular transcription at low oxygen levels. Increased levels and activity of these genesare associated with cell state and inhibition of trophoblast differentiation[19]. The loss of the *HIF-1a* gene is also detrimental in placental development, so it is

known that inappropriate *HIF-1a* stabilization can result in prolonged *HIF-1a* activity that can adversely affect trophoblast differentiation and ultimately can lead to placental abnormalities[20]. Rhodomyrtus tomentose can significantly reduce *HIF-1a* because this plant has higher antioxidants than *Zanthoxylum acanthopodium*[10,11]. The *Zanthoxylum* family has quite a lot of steroid compounds. The danger ofexcessive steroids on cells can cause fat accumulation andhypertension[18]. Based on this analysis, it was found that NRTwas better in the expression of *HIF-1a* and *EGL7* genes inhuman trophoblasts than NZA.

The implications of this study include the examination of the *EGFL7* and *HIF-1a* genes in the human placental trophoblast, which is required in drug discovery pregnancy. The nano herbs andaliman and haramonting should be studied further to the expression of other target genes. Placental markers undergo hypoxia, proliferation, organogenesis and placental development because signallinggenes *EGFL7* and *HIF-1a* are important in the process oflinking maternal and fetal cells.

CONCLUSION

Rhodomyrtus tomentose gave a more significant effect than *Zanthoxylum acanthopodium* because *Rhodomyrtus tomentose* has emulsion characteristics that meet drug requirements and strong antioxidant activity than *Zanthoxylum acanthopodium*. This was evidenced by asignificant difference ($p<0.01$) in the expression of HIF and *EGFL7* genes on human trophoblast cells.

SIGNIFICANCE STATEMENT

This study discovers that *Rhodomyrtus tomentosa* (haramonting) fruits can as herbal for placental problem therapy molecularly. This study will help the researcher to uncover the role of *Rhodomyrtus tomentosa* in molecular signalling of other target genes for drug development in hypertension. Thus, a new theory on the role of andaliman and haramonting in the HIF and *EGFL7* pathways expression in human trophoblast may be arrivedat.

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