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Research Article
Effect of Nano Herbal Andaliman (*Zanthoxylum acanthopodium*) Fruits in NOTCH1 and Hes1 Expressions to Human Placental Trophoblasts

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Abstract

Background and Objective: Andaliman fruit (*Zanthoxylum acanthopodium*) is a well-known spice antioxidant in Northern Sumatera (Indonesia). The cellular activity requires antioxidants in counteracting free radicals. The cellular proteins that play a role in development, proliferation, differentiation and embryonic processes in the human placenta are NOTCH1 and Hes1. The aim of this research was to analyze the expression of NOTCH1 and Hes1 genes after administering nano herbal andaliman to the trophoblast cells of the human placenta. **Materials and Methods:** HTR8 trophoblast cells were divided into two groups, namely, control and treatment (nano herbal andaliman). RNA isolation, reverse transcription and RT-PCR (real-time polymerase chain reaction) were performed to analyze the NOTCH1 and Notch target gene (Hes1) expressions. The NOTCH1 and Hes1 gene expressions were quantified using the CT method (2-ΔΔCT) and normalized with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expressions. **Results:** Nanoherbal andaliman reduced the expression of NOTCH1 genes in the human placental trophoblast. However, it increased the expression of Hes1 when the incubation time was 16 hrs. **Conclusion:** Nanoherbal andaliman decreases the expression of genes that are crucial in hypoxia and free radicals in the placenta, namely, NOTCH1 and Hes1 increased after incubation for 16 hrs. Therefore, this herb needs to be evaluated further.

Key words: Andaliman, Hes1, HTR-8 cells, NOTCH1, nano herbal, trophoblast, *Zanthoxylum acanthopodium*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesia has various herbs and spices that are unique to food and beneficial to health. One of the popular spices with antioxidants is andaliman (*Zanthoxylum acanthopodium*). Andaliman is a wild plant in Northern Sumatera in Indonesia[1]. This herb has been used by the Batak tribe for centuries for flavoring, health and anti-inflammation purposes[1,2]. Ethyl acetate, methanol and andaliman fruit extracts show antioxidant activity[3]. Nano-sized andaliman can also reduce MDA levels, increase HSP-70 and improve liver in pre-eclampsia (PE)[4-7].

A Pregnant problem can occur because of problems with the placental trophoblast. The Notch gene functions in the processes of cell death, proliferation, organogenesis and placental development[8]. Trophoblast stem cells originate from placental precursors and express NOTCH1[8,9]. NOTCH1 is a transmembrane receptor protein that functions in many tissues, but this protein is activated by differentiated ligands[10]. In particular, placental trophoblast cells appear to be tumor-like sometimes because they can invade the uterus and change the blood vessels of a pregnant mother[11,12]. Pregnancy can decrease the NOTCH1 protein in cytotrophoblasts and syncytiotrophoblasts from the first to the third trimester[13]. Notch involvement is also a regulator of vascular formation in the placenta[14,15]. Releasing the NOTCH1 gene in human placental trophoblasts can produce variable defects in arterial development[15]. Notch-induced Hes1 expression can be maintained at a relatively high level[16]. Hes1 is the key gene in maintaining stem cells or cancer stem cells in a non-dividing state[17]. The expression of Hes1 induced by NOTCH1 can be tagged or detected in muscle stem cells in the bone, embryonic nerve stem cells and progenitor cells[17,18]. The Hes1 gene in the NOTCH1 pathway gene facilitates cell differentiation during development and decides on cell fate. The purpose of this study was to analyze and the role of cellular genes in the human placenta, such as Notch-1, Hes1 after being administered with nano herbal andaliman.

MATERIALS AND METHODS

Study area: The study was carried out at Department of Biology, Physiology Laboratorium, Universitas Sumatera Utara, Indonesia from June, 2019-September, 2020. RT-PCR analysis at Department of Biomedicine and the prevent, University of Rome Tor Vergata, Italy from December, 2019-May, 2020.

Nano herbal andaliman: Andaliman was obtained from Berastagi Market, Northern Sumatra Province. Nano herbal andaliman was manufactured via high-energy milling (HEM)[4-7] in the Indonesian Research Institute in Jakarta. Ten miligram of nano herbal andaliman was dissolved in 1% DMSO. Researchers received permission from the ethical clearance of animal handling at the Faculty of Mathematics and Natural Sciences, University of Sumatera Utara (No. 010/KEPH-FMIPA/2020).

HTR-8 cell culture and treatments: The HTR-8 trophoblast cell was kindly provided by Dr. Luisa Campagnolo University of Rome 'Tor Vergara' Rome, Italy. The cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium contains 5% foetal serum, 2 mM L-glutamine and 50 mg mL^{-1} of streptomycin (product code: L0498-500, Biowest, USA) at 37°C in an atmosphere of 5% CO2/95% air. The cells were seeded in 6-multiwell before the experiments to RPMI 1640. Cells were seeded into 6-well plates, 100 mm dishes for RT–PCR. The HTR-8 cell trophoblast cell consisted of two treatment groups. Namely, control and treated (nano herbal andaliman) with four-time periods of 30 min, 1, 3 and 16 hrs (overnight). So, the total treatment is 48 wells (8 wellsx6 replication).

RNA extractions and real-time reverse transcription-polymerase chain reaction (RT-PCR): Total RNA (30 min, 1, 3 and 16 hrs) was isolated from HTR-8 cells at term using triazole reagent (Invitrogen) as the manufacturer's suggested procedure. Isolated RNA samples were quantitated by NanoDrop 3300 spectrophotometer (Thermo Scientific, NanoDrop Products, Wilmington, DE, USA). A 2 µg of the isolated RNA was used to prepare the cDNA using random hexamers, dNTP mixture, RT buffer and Superscript III reverse transcriptase following the manufacturer's protocol (Superscript III RT PCR System; Invitrogen, Carlsbad, CA, USA). Real Master Mix SYBR ROX (Eppendorf, Hamburg, Germany) was used to calculate gene expressions. About 2 µg of total RNA was reverse-transcribed into cDNA according to the kit's protocol (Applied Biosystems 7300 real-time PCR system, Foster City, USA). The NOTCH1 and Hes1 gene expressions were quantified using the CT method ($2^{-\Delta\Delta CT}$) and normalized with Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expressions. The GAPDH primer sequences were as follows: Forward primer, 5-TCCCTGAGCTGAAACGGGAAG 3; reverse primer, 5-GGAGGAGTGGGTGTCGCTGT 3. The NOTCH1 primer sequences were as follows: Forward primer, 5 GTC CCA

CCC ATG ACC ACT ACC CAG TTC 3; reverse primer, 5 GGG TGT TGT CCA CAG GTG A 3. The Hes1 primer sequences were as follows: Forward primer: 5-AGG CGG ACA TTC TGG AAA TG 3; reverse primer, 5-TCG TTC ATG CAC TCG CTG A 3.

Statistical analysis: The research data were analyzed using the one-way analysis of variance (ANOVA) test in Sigmaplot software. Asterisks indicate of the data the data level in each treatments of statistical significance (*p<0.05; **p<0.001).

RESULTS

The HTR8 cell image obtained under a light microscope shows that no difference was noted amongst the control, vehicle and nano herbal andaliman treatment groups at 30 min (Fig. 1a-c1). The same observation was obtained for the control, vehicle and nano herbal andaliman treatment groups at 1 (Fig. 1a-c2), 3 (Fig. 1a-c3) and 24 hrs (Fig. 1a-c4). However, the HTR8 cell image from a light microscope showed

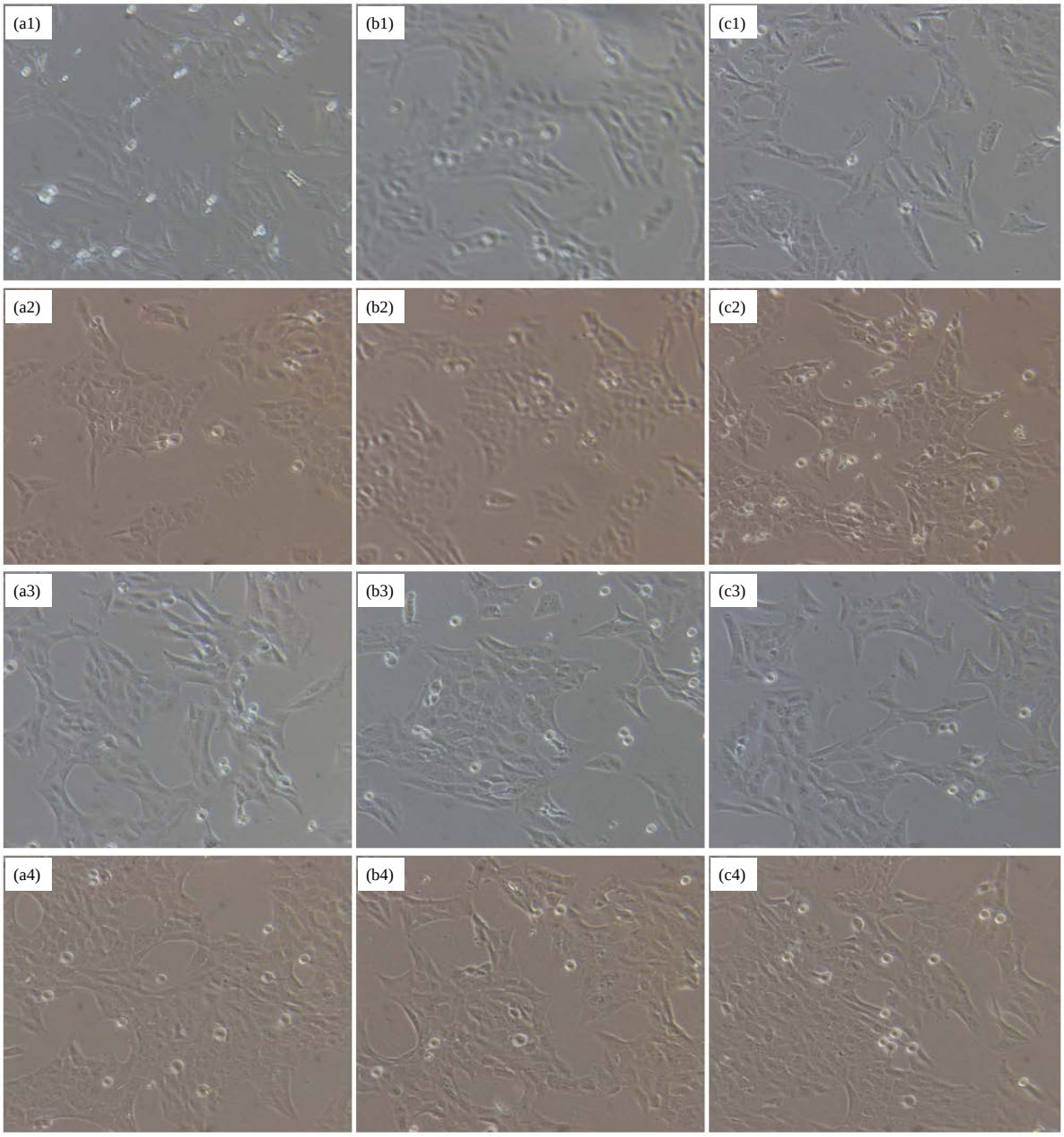

Fig. 1(a-c): Development of HTR8
(a) Control (untreated), (b) Vehicle (DMSO 1%) and (c) Treatments (nano herbal andaliman), 1: 30, 2: 1 h, 3: 3 hrs and 4:16 hrs

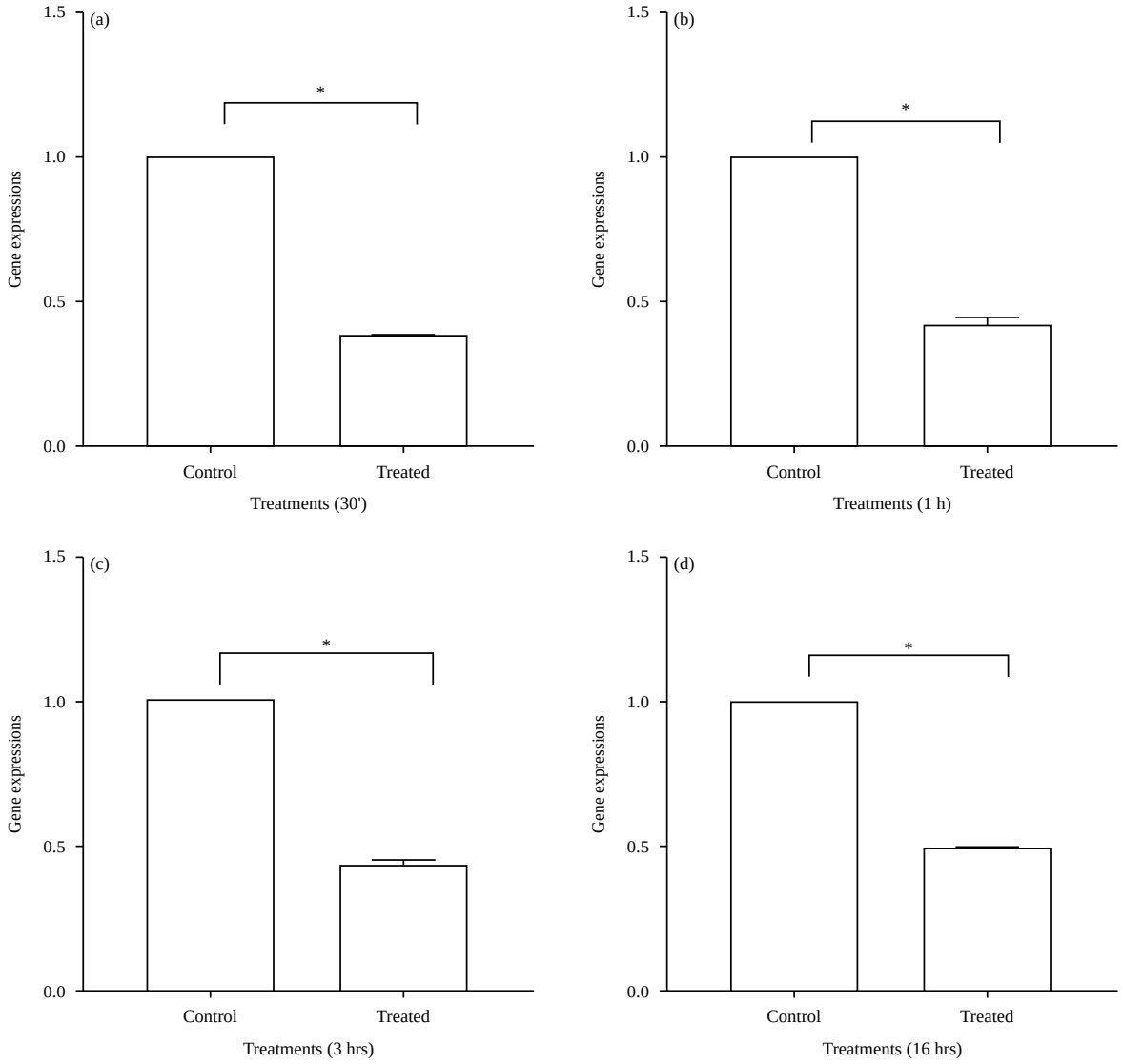

Fig. 2(a-d): Expression of the NOTCH1 gene on HTR8 cells in control and nanoherbal andaliman
(a) 30, (b) 1 h, (c) 3 hrs and (d) 16 hrs (*p<0.05)

that HTR8 proliferated and the amount of cell death and morphology were not different. The histology revealed that the nano herbal andaliman treatment group had the same morphology and almost the same development as the control or DMSO only groups.

Figure 2a showed that a significant difference in NOTCH1 gene expression with a value of F = 0.019 (p<0.05) was observed in the vehicle and nano herbal andaliman treatment groups at 30 min. The same was observed for 1 h of incubation, in which a significant difference of F = 0.039 (p<0.05) was noted for control and nano herbal andaliman treatment groups (Fig. 2b). After 3 hrs of incubation, a significant difference of 0.018 (p<0.05) was determined between the two groups (Fig. 2c). The treatment in the 16 hrs group also had a value of F = 0.027 (p<0.05) (Fig. 2d). These data indicate that nano herbal andaliman reduced the expression of the NOTCH1 gene in the human placental trophoblast.

Figure 3a showed that a significant difference with a value of F = 0.012 (p<0.05) existed between the control and nano herbal andaliman groups in terms of the Notch target gene (Hes1) in Htr8 cells under30 min of incubation. At 1 h of incubation, a significant difference of 0.028 (p<0.05) was observed between the control and nano herbal andaliman groups (Fig. 3b). A significant difference of F = 0.019 (p<0.05) was likewise noted between the two groups in the incubation time of 3 hrs (Fig. 3c). However, in the treatment of the 16 hrs group, no significant difference was observed with the value

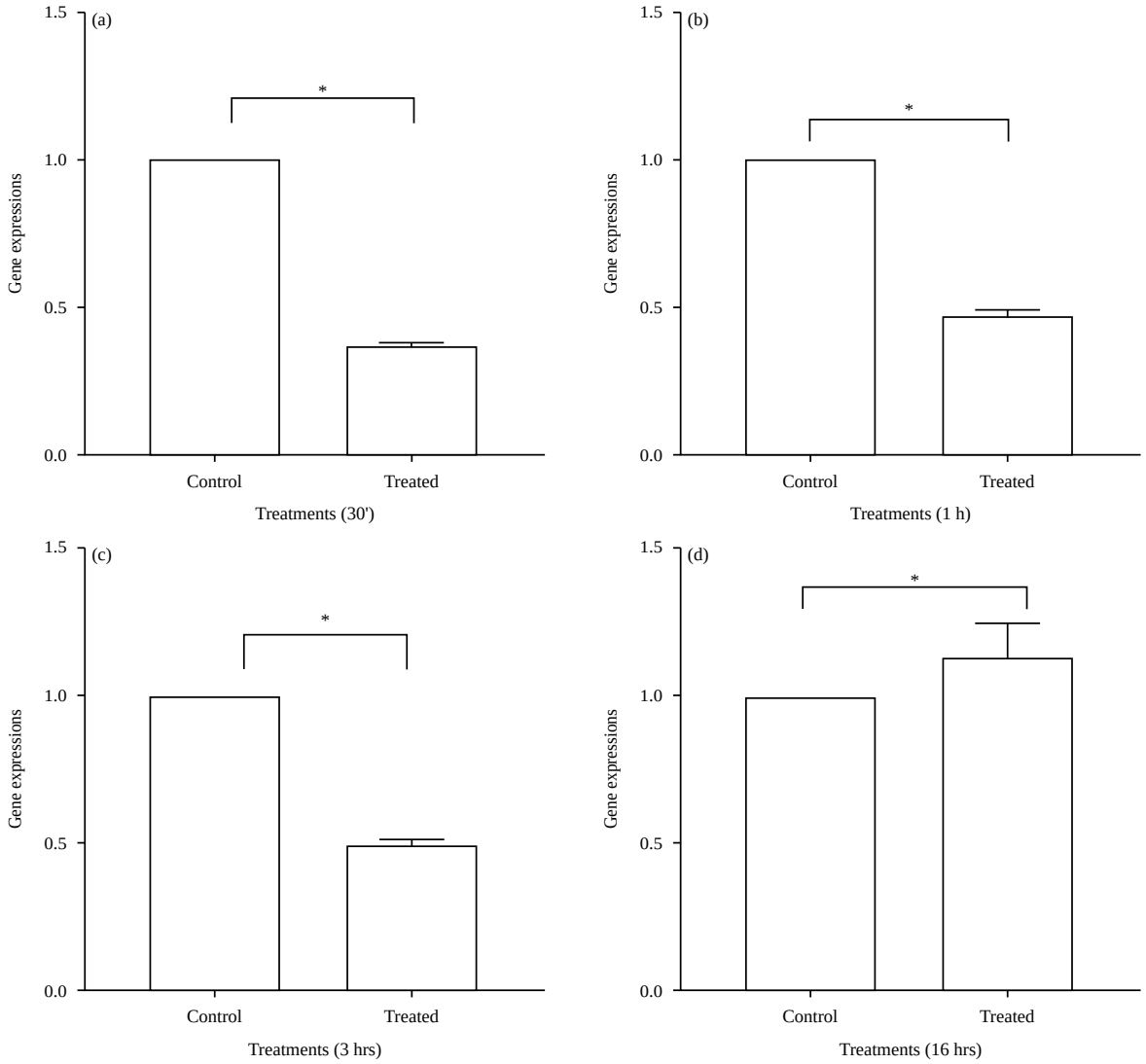

Fig. 3(a-d): Expression of notch target gene (Hes1) on HTR8 cells in the control and nanoherbal andaliman
(a) 30, (b) 1 hr, (c) 3 hrs and (d) 16 hrs (* $p<0.05$)

of $F = 0.127$ ($p>0.05$) (Fig. 3d). These data indicate that nano herbal andaliman reduced the expression of the Notch target gene (Hes1) in less than 16 hrs.

DISCUSSION

HTR8 cells showed good proliferation and the number of deaths and morphology were not different. The histological results showed that the andaliman nano herbal treatment had the same morphology and development as the control group or DMSO alone. Nanoherbal Andaliman is not toxic to HTR8 cells. Proliferation is the phase of the cell when it experiences a repetition of the cell cycle or uninhibited growth. The provision of certain herbs with antioxidants can affect cell growth activities. Antioxidant properties (vitamins C and E) are crucial for the growth of trophoblast cells and in preventing the appearance of signs of PE. Antioxidants are substances that delay oxidative damage to the cells of organisms or act as inhibitors of free radicals (hydroperoxide or peroxide)[19]. Andaliman fruit has antioxidant activity and in nano size, it can reduce MDA levels and increase HSP-70[4,5].

In the current study, a decrease occurred in the NOTCH1 expression. Notch signaling is a conserved evolutionary pathway involved in inter-cell signaling and important for the appropriate choice of cell fate during development[8,9]. Notch signaling plays a role in cell differentiation, proliferation and apoptosis. The process is regulated in the human placenta to improve fetal growth. Notch signaling also plays a role in the

development of rat pre-implantation where inter-cell signaling is essential, especially between deep cell mass and blastocyst trophectoderm[9]. The majority of patients with breast cancer carry a NOTCH1 mutation because cellular Notch signaling is correlated with cancer, such as in T-cell lymphoblastic leukaemia[20,21]. Notch signaling is required in maintaining old trophoblasts and inhibiting differences in the growth of excess and premature trophoblasts along the invasive trophoblast lineage.

In the current study, Notch activity and expression were the reasons why one of the main target genes (i.e., Hes1) increased after incubation for 16 hrs and decreased under 16 hrs of incubation (Fig. 3). In intestinal stem cells, Hes1 inhibits the release of the cell cycle and differentiation by suppressing the c21 inhibitors of p21KIP1 and p57KIP2 inhibitors, which is concurrent with the decline in the regulation of Hes1[22]. The mutant embryo of Hes1has proneural genes Mash1 and Math3, which are regulated together with the differentiation of premature neurons[23,24]. The expression of neuron markers and the prevention of cortical progenitors from migrating out of the ventricular zone are an over-expression of Hes1[23]. The increased activity of the Notch signaling pathway that contributes to tumor growth is an upregulation of the target gene Notch, Hes1[25,26].

The implication of the research is the examination of the NOTCH1 and Hes1 gene in the human trophoblast placenta very required in the discovery of pregnancy drug. Nano herbal andaliman needs to be further evaluated with other notch target gene expression. Because the notch signaling gene is very important in the process of cell death, proliferation, organogenesis and placental development.

CONCLUSION

Nano herbal andaliman showed that almost the same morphology in each treatment. However, it decreased the expression of genes that are crucial in hypoxia and free radicals in the placenta, namely, NOTCH1. The Hes1 gene in the NOTCH1 pathway gene facilitates cell differentiation during development and decides on cell fate and this gene increased after incubation for 16 hrs. So, further analysis is needed for the reliable role of nano herbals in NOTCH1 and notch signaling of other target genes for drug development in pregnancy placental health.

SIGNIFICANCE STATEMENT

This study discovers that nano herbal andaliman in the Hes1 and NOTCH1 pathway gene facilitates cell differentiation and increased after 16 hrs incubation. This study will help the researcher to uncover for the reliable role of nano herbal andaliman in Notch signaling of other target genes for drug development in pregnancy placental health. Because the notch gene functions in the processes of cell death, proliferation, organogenesis and placental development. Thus, a new theory on the role of nano herbal in the notch target gene signaling in pregnancy may be arrived at.

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