Synergistic effect of Elephantopus scaber L and Sauropus androgynus L merr extracts in modulating prolactin hormone and erythropoiesis in pregnant typhoid mice

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Abstract

Purpose: To investigate the efficacy of Elephantopus scaber L. and Sauropus androgynus (L.) merr formulations in prolactin and erythrocyte cell production in pregnant typhoid mice.

Methods: In the experiment, 21 pregnant BALB/c mice, divided into seven treatment groups (n = 3) were used. All groups, except control group (T1), were orally infected with Salmonella typhi (10^7 CFU/mL). Individual initial doses of 200 mg/kg E. scaber and 150 mg/kg S. androgynus were given to the mice. Extracts of E. scaber and S. androgynus were then administered orally in the following formulation ratios: 100:0 (T3), 75:25 (T4), 50:50 (T5), 25:75 (T6) and 0:100 % (T7). Blood was isolated from the orbital veins of pregnant mice at 4, 8 and 12 days post-infection, and centrifuged at 2500 rpm and 10 ºC for 5 min. The supernatant was separated from the pellets to obtain the serum. Hematopoietic cells were isolated from bone marrow at 12 days post-infection. Prolactin hormone level was determined by enzyme-linked immunosorbent assay (ELISA), and erythrocytes were measured by fluorescence-activated cell sorting (FACS).

Result: Salmonella typhi infection in pregnant mice reduces levels of prolactin and TER119+ cells. T7 formulation increased (p < 0.05) prolactin levels on days 8 (3.83 ± 0.93 ng/mL) and 12 (3.45 ± 0.39 ng/mL) post-infection. Furthermore, the T3, T5, and T7 formulation may have elevated the number of TER119+ cells compared to the control group. Mice given the T3 formulation showed increased numbers of TER119+VLA4 (68.73 %), those given T5 showed comparable numbers of TER119+VLA4 (45.81 %) and TER119’VLA4+ (54.19 %), while those given T7 showed increased the numbers of TER119’VLA4+ (97.45 %).

Conclusion: E. scaber and S. androgynus leaves extracts significantly increased (p < 0.05) the levels of prolactin and erythrocytes to support the pregnancy of BALB/c mice with typhoid model.

Keywords: Elephantopus scaber, Erythropoiesis, Pregnancy, Prolactin, Salmonella typhi, Sauropus androgynus

INTRODUCTION

Pregnant women have a higher risk of infectious diseases because they have different immunological conditions compared to non-pregnant women [1]. The concentrations of hormones in pregnant women change when they are infected by pathogens such as Salmonella typhi. A previous study has shown that prolactin is one hormone that changes in response to...
**Salmonella typhi** infection [2]. During the first and second trimesters of pregnancy, prolactin levels were significantly reduced by infection with the parasite compared to a control group; however, during the third trimester, this hormone increased but remained lower than in the control. The reduction of prolactin levels affects the production of progesterone by the corpus luteum during pregnancy and decreases production of breast milk after birth [2].

A recent study demonstrates reduced erythrocyte levels in pregnant mice infected with *Salmonella typhi* [3]. The lipopolysaccharide produced by *Salmonella typhi* reduces erythropoietin (EPO) mRNA expression in the kidney and disturbs erythropoiesis [4,5]. Prolactin has been found to stimulate erythropoiesis and increase red blood cell mass [6]. Therefore, a reduction in prolactin will decrease erythrocyte numbers and induce iron deficiency in pregnant women. Suplemental iron is important during pregnancy, especially for placental growth and in iron fulfillment for fetus.

**Experimental**

**Experimental animals**

The experiment used 21 6-week-old female BALB/c mice, which were kept in pathogen-free condition and were 5 days pregnant at the beginning of the experiments. The mice were collected from LPPT Gadjahmada University and were housed in a pathogen-free chamber under a 12/12 h light/dark cycle (beginning at 06.00) at room the temperature (25 °C). The standard mouse pellets and water were provided *ad libitum*. The Brawijaya University Ethics Committee approved all protocols used in this study (approval ref no. 523-KEP-UB). All animal experiments were performed according to the Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) [10].

**Preparation of *E. scaber* and *S. androgynus* leaf ethanol extract**

Fresh leaves of *E. scaber* and *S. androgynus* were collected in September 2015 from Balai Materia Medica Batu, Malang, Indonesia. The plants were identified by Dr. Seraphina Indriyani, M.Sc, Departement of Biology, Brawijaya University, Malang, Indonesia, and specimens voucher (0194/Tako.Identifikasi/03/216; 0195/Tako.Identifikasi/03/216) were prepared and deposited in the herbarium of the Biology Departement. The leaves were air-dried at room temperature and pulverized into a powder for a day. The pulverized powder was soaked in 70 % ethanol for 24 h at room temperature. The ethanol extract of each plant was concentrated to dryness under reduced pressure at 50 °C in a vacuum pump evaporator.

**Salmonella typhi**

The *Salmonella typhi* isolate was collected from the Microbiology Laboratory in the Faculty of Medicine at Brawijaya University, Malang, Indonesia. Experimental animals (except controls) were administered orally up to 10^7* CFU in 0.5 mL solvent.

**Treatments**

All animals were treated for 16 days and were provided food and water *ad libitum*. *E. scaber* and *S. androgynus* were administered orally during the first 4 days of pregnancy. On day 5 of pregnancy, *Salmonella typhi* was administered orally. Administration of *E. scaber* and *S. androgynus* continued until the last day of treatment, according to the treatment group. The pregnant mice were divided randomly into seven treatment groups, with three mice each group. Group T1 was normal mice without any extract administration and *Salmonella typhi* infection. Group T2 was given *Salmonella typhi* infection and without extract. Group T3 – T7 were infected with *Salmonella typhi* and various ratio doses of *E. scaber* and *S. androgynus* extract: 100:0 (T3), 100:75 (T4), 100:50 (T5), 100:25 (T6), 100:0 (T7).
Dissection of animals

Dissection for analysis of prolactin levels was performed at 4, 8 and 12 days post-infection, while analysis of TER119⁺VLA4⁺ cells (representing erythrocyte precursors), and TER119⁺VLA4⁻ cells (representing mature erythrocytes) were performed at 12 days post-infection.

Serum and bone marrow cell isolation

Blood isolation from the orbital veins for prolactin measurements was performed using a 1-mL hematocrit pipette capillary. The blood was collected in microtubes, incubated at 37 °C for 2 h, and then centrifuged at 4000 rpm and 37 °C for 5 min. The supernatant (serum) was moved to another microtube. Bone marrow (BM) was isolated from femoral bone separated from muscles and tissues. The bone was washed twice with phosphate-buffered saline (PBS), and then both epiphyses were trimmed with scissors. The marrow shaft was flushed using a 26-gauge needle attached to a 20-mL syringe containing PBS. Cell homogenates were centrifuged at 2500 rpm, at 10 °C, for 5 min, and the pellet was resuspended in 1 mL PBS.

ELISA assay

The levels of prolactin were measured using an Elabscience ELISA kit.

FACS analysis

The BM cell suspension was stained with FITC-conjugated rat anti-mouse VLA4 and PE-conjugated rat anti-mouse TER119. All samples were incubated for 20 min at 10 °C, after which 500 µL PBS was added. Each sample was transferred to a flow cytometry cuvette and analyzed by a flow cytometer.

Statistical analysis

Data from the FACS analysis were analyzed using BD Cell Quest PROTM software (BD Biosciences, San Jose, CA, USA). Both ELISA and FACS data were analyzed using parametric one-way ANOVA with a significance level of p < 0.05, followed by Tukey’s test. The statistical analysis software used was SPSS version 16.0 (PASW Statistics for Windows, SPSS Inc., Chicago, IL, USA) and Excel 2007 (Microsoft).

RESULTS

Influence of *S. androgyrus* on the level of prolactin in a mouse model of typhoid infection during pregnancy

Figure 1 shows that *Salmonella typhi* infection (T2) at day 8 post infection significantly (p < 0.05) reduced the level of prolactin in pregnant mice. The effects of different formulations of ethanol extracts from *E. scaber* and *S. androgyrus* leaves on prolactin levels at (A) day 4, (B) day 8, and (C) day 12 post-infection. T1, control; T2, *Salmonella typhi* infection without treatment; T3, *Salmonella typhi* infection with 100% *E. scaber*; T4, *Salmonella typhi* infection with 75% *E. scaber* and 25% *S. androgyrus*; T5, *Salmonella typhi* infection with 50% *E. scaber* and 50% *S. androgyrus*; T6, *Salmonella typhi* infection with 25% *E. scaber* and 75% *S. androgyrus*; T7, *Salmonella typhi* infection with 100% *S. androgyrus*. *p < 0.05 compared to T2*
mice compared to uninfected pregnant mice (T1), from 1.676 ± 0.097 ng/mL (T1) to 1.144 ± 0.045 ng/mL (T2). We have observed fluctuations in the levels of prolactin from 8 to 12 days post-infection. At 8 days post-infection, T3 increased the level of prolactin (1.952 ± 0.535 ng/mL), but not significantly (p < 0.05) compared to T2 (1.144 ± 0.045 ng/mL). T7 significantly (p < 0.05) (3.832 ± 0.934 ng/mL) increased the level of prolactin compared to T2 (1.144 ± 0.045 ng/mL) and T1 (1.676 ± 0.097 ng/mL). At 12 days post-infection, T7 significantly (p < 0.05) increased the level of prolactin (3.446 ± 0.386 ng/mL) compared to T2 (1.992 ± 0.685 ng/mL). As mentioned previously, the T3, T5, and T7 formulations of E. scaber and S. androgynus elevated the levels of TER119+ cells. The control group's (T1) numbers of the two types of cells were 46.56 % TER119+VLA4- and 53.44 % TER119+VLA4+ (Figure 3). Figure 3 also shows that infection with Salmonella typhi reduced TER119+ cell by decreasing the number of TER119+VLA4- erythrocyte precursors.

As shown in Figure 2, infection with Salmonella typhi (T2) reduced the number of TER119+ cells significantly (p < 0.05) compared to uninfected pregnant mice (T1), from 21.36 to 3.13 %. All combinations of E. scaber and S. androgynus leaf extracts (T3-T7) elevated the number of TER119+ cells compared to those in T2 mice. However, the numbers of TER119+ cells did not significantly different (p < 0.05) when the T3, T5, and T7 groups were compared to the control group (T1).

**E. scaber** and **S. androgynus** modulated erythrocytes levels

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Figure 3: Effect of different formulations of ethanol extracts from *E. scaber* and *S. androgynus* leaves on the numbers of erythrocyte precursors (TER119^*VLA4*^+) and mature erythrocytes (TER119^*VLA4*^−). T1, control; T2, *Salmonella typhi* infection; T3, *Salmonella typhi* infection with 100% *E. scaber*; T4, *Salmonella typhi* infection with 75% *E. scaber* and 25% *S. androgynus*; T5, *Salmonella typhi* infection with 50% *E. scaber* and 50% *S. androgynus*; T6, *Salmonella typhi* infection with 25% *E. scaber* and 75% *S. androgynus*; T7, *Salmonella typhi* infection with 100% *S. androgynus*. Values are mean ± SD (n = 3, p < 0.05) indicating that *Salmonella typhi* infection disrupts erythropoiesis. T3 increased the number of TER119^*VLA4*^+ cells, T5 resulted in equal numbers of TER119^*VLA4*^ and TER119^*VLA4*^+ cells, while T7 increased the levels of TER119^*VLA4*^+ cells. However, among these three treatments, the T5 group produced numbers comparable to those of the T1 control group: 45.81% TER119^*VLA4*^ and 54.19% TER119^*VLA4*^+ cells.

**DISCUSSION**

This study demonstrated that infection with *Salmonella typhi* reduces prolactin levels and erythrocyte numbers. Prolactin is a hormone that plays an important role in immunoregulation in hosts as a natural means of protection against infection [13]. Therefore, prolactin levels increase during pregnancy. Parasitic infection can influence prolactin levels [2]. *Salmonella* spp. bacteria produce lipopolysaccharide, typical of gram-negative bacteria, to inhibit the synthesis and release of decidual prolactin [14]. Infection with *Salmonella typhi* affects erythrocyte levels by influencing both the prolactin and EPO hormones. The prolactin receptor is a type 1 cytokine receptor with a similar structure to those of receptors such as IL-1–7, granulocyte-macrophage colony stimulating factor,

Inflammation caused by bacteria can reduce EPO production and iron availability [15]. Reducion of EPO levels leads to reduced maturation of erythrocyte precursors. The reduced iron availability results in hepcidin activation during inflammation. Hepcidin is a major regulator of iron that binds to the iron exporter ferroportin, causing internalization of iron and inhibiting its release [15]. Reduced EPO production and iron availability lead to anemia, and during pregnancy this can result in pre-term labor, pre-eclampsia, and sepsis [16]. Thus, disruption of erythrocyte precursor maturation is harmful to pregnant women.

The T7 mice (100 % of S. androgynus) exhibited increased levels of prolactin and erythrocytes, particularly mature erythrocytes (TER119/VLA4), compared to the control group. The administration of S. androgynus leaf extract has been shown to significantly increase prolactin gene expression in mice [17]. S. androgynus acts as a secondary messenger in cell signal transduction of hormones and growth factors. Increasing prolactin indirectly increases the number of erythrocytes [15]. Prolonged administration of prolactin increases the red blood cell mass in normal mice [6].

Production of erythroid is influenced by JAK2, which is a kinase associated with, and activated by, the prolactin receptor. In normal erythroid cell production, the body produces EPO, which stimulates MGF-STAT5 DNA binding activity, while MGF-STAT5 is a substrate of kinase JAK2 [18,19]. The presence of prolactin also replaces EPO to activate STAT5 in hematopoietic cells. STAT5 activates JAK2 and causes erythroid cell proliferation [19]. S. androgynus has a vitamin C content of 22–244 mg/100 g fresh weight [20]. Vitamin C is capable of inducing Fe release from ferritin and mobilizing Fe from the reticuloendothelial system to transferrin. The resulting increase in Fe levels induces production of erythroid cells [21].

The 100 % E. scaber treatment (T3) increased the number of erythrocytes, especially erythrocyte precursors (TER119/VLA4). Our previous study demonstrated that E. scaber leaf extract influenced the number of TER119° cells in a mouse model of typhoid infection during pregnancy [3]. E. scaber contains 45.4 % Fe, which is an important nutrient involved in the production of new red blood cells. Iron in plasma is transported by transferrin to the bone marrow for hemoglobin synthesis and integration in erythrocytes [21]. Furthermore, the 50 % E. scaber/50 % S. androgynus treatment (T5) increased the number of erythrocytes, especially in balancing the numbers of mature (TER119/VLA4) and precursor erythrocytes (TER119/VLA4°), compared to the control pregnant mice (T1). Based on the mechanisms explained previously, E. scaber and S. androgynus extract combinations act synergistically to increase the levels of prolactin and erythrocytes.

CONCLUSION

This study demonstrates that E. scaber and S. androgynus modulate erythropoiesis and prolactin levels in pregnant typhoid mice. Administration of E. scaber extract increases the number of erythrocyte precursors, whereas administration of S. androgynus extract increases the number of mature erythrocytes and prolactin levels. Administration of the combination of 50 % E. scaber/50 % S. androgynus results in balanced numbers of precursor and mature erythrocytes.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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