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Investigation of Murine Fertility by Kampo Formula Containing *Astragalus* Root Enhanced Endometrial Wnt/β-catenin Signaling Factors

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Abstract

Astragalus root (Root of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus membranaceus var. mongholicus* (Bunge) P.K.Hsiao) is a prescription drug constituting Kampo formulae with the aim of cardiotonic, hidroschesis, diuresis, and hypotensive effect in Japan. In this study, we report that hot water extract of Astragalus root (AsR) increased the number of neutrophils and the secretion of mucus from the endocervical gland in ovariectomized mice treated with estradiol. These secretions block bacterial access through the uterine cavity. Moreover, AsR and its main chemical component, AstragalosideIV, increased the levels of uterine Wnt4 and Wnt7a involved in endometrial proliferation and the maintenance of the stroma-smooth muscle boundaries of the uterus, respectively. To explore the possibility of fertility improvement, we determine the litter size of maternal mice administrated with hot-water extract of *Astragalus* root or *Kampo* formula containing *Astragalus* root. AsR-treated maternal mice exhibited increased litter size by natural mating, whereas Keishikaogito which is a Kampo formula containing *Astragalus* root showed a trend towards decreased the litter size. These results suggest that Astragalus root mediates endometrial proliferation, leading to the improved fertility; a crude drug-component of Keishikaogito, except *Astragalus* root, might interfere with embryo implantation. The discovery of Kampo formulae showing increase of litter size is needed for treatment of fertility in medical care.

Keywords: *Astragalus* Root; Endometrium; ERα; Fertility; Wnt/β-catenin

Introduction

Young female athletes or dieter often exhibit dysmenorrhea (including amenorrhea and oligomenorrhea), which is induced by an estrogen deficiency caused by thickness or stress, leading to infertility. *Astragalus* root (Root of *Astragalus membranaceus* (Fisch.) Bunge or *Astragalus membranaceus var. mongholicus* (Bunge) P.K.Hsiao) is a prescription drug constituting Kampo formulae with the aim of cardiotonic, hidroschesis, diuresis, and hypotensive effect in Japan. Pharmacological or biological activities of the root have been reported, such as anti-inflammatory [1], anti-tumor [2], hepatoprotective [3], renal-protective [4], and immune-enhancement affects [5]. In our previous report, a hot water extract of *Astragalus* root (AsR) increased blood estradiol and uterine ERα levels in female ICR mice. Furthermore, mitochondrial PPARα-mRNA and β-oxidation enzyme levels in the ovary were enhanced [6]; we concluded

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that AsR increased estrogen secretion through energy generation by ovarian PPARα activation, promoting the endometrial proliferation. Peroxisome proliferator-activated receptor (PPAR) α, which is a ligand-induced transcription factor, is highly expressed in hepatocytes, cardiomyocytes, enterocytes, and renal proximal tubule cells. Additionally, it is expressed, moderately in uterine glands, cervix, and ovarian follicular cells. Fenofibrate (PPARα agonist) increased ERα and β-catenin expression levels in the luminal and glandular epithelium of the endometrium, whereas the uterine proliferation was attenuated [7].

Estrogen is synthesized in ovarian granulosa cells, and is secreted from follicular lutein cells. Estrogen receptor α (ERα), which belongs to the family of the nuclear receptor, binds to estrogen receptor elements (ERE) to regulate the expression of target genes involved in the proliferation of the functional endometrial layer. Endometrial ERα stimulation induces an increase in the Wnt/β-catenin signaling factor [8]. Wnt combines the frizzled receptor to inhibit the degradation of β-catenin, which in a complex with cadherin, contributes to cellular adhesion essential for embryonic patterning and cell growth, migration, and differentiation. Wnt4, Wnt5a, and Wnt7a are highly expressed in the mouse uterus, and the expression pattern is responsive to change in circulating sex hormones levels. Wnt4, which primarily expresses through stroma subjacent to the luminal epithelium of mouse uterus [9], increases when there are high circulating levels of estradiol. Furthermore, Wnt4 mediates progesterone-induced stromal decidualization of mouse endometrium [10]. Wnt7a, which is expressed in the luminal epithelium of the endometrium, is released to smooth muscle and maintains the organization of the smooth muscle in the adult mouse uterus.

The chemical components of hot-water extract of *Astragalus* root have been well defined, as have its absorption and metabolism. Eleven flavonoids and 12 triterpenoid saponins were detected in mouse plasma after the oral administration of the extract, and "flavonoids and saponins were found to have a relatively high response value and relatively faster absorption, indicating that flavonoids and saponins might play an important role in the therapeutic effect" [11]. Accordingly, we determined uterine ERα, PPARα, Wnt and β-catenin levels of ovariectomized mice treated with hot water extract of *Astragalus* root or its main flavonoid and saponin compounds, formononetin and astragalosideⅣ, respectively.

Astragalus root is commonly used as a prescription drug for Kampo formulae in clinical environment. Keishikaogito (KO) is one of the *Astragalus* root-containing Kampo formula, whose primary usage is the recovery from fatigue and night sweets. To explore the possibility of fertility improvement, we determine the litter size of maternal mice administrated with hot-water extract of *Astragalus* root or Keishikaogito.

Materials and Methods

Hot-Water Extract of Astragalus Root and Keishikaogito

Medical-grade *Astragalus* root, cinnamon bark, ginger, glycyrrhiza, jujube, peony root were purchased from Tochimoto Tenkaidou Co., Ltd. (Osaka, Japan). The *Astragalus* root (3.0 g) or crude-drug components of Keishikaogito (*Astragalus* root, 3.0 g; Cinnamon bark, 3.0 g; Ginger, 1.0 g; Glycyrrhiza, 2.0 g; Jujube, 4.0 g; Peony root, 3.0 g) were boiled in 600 ml distilled water, until the final volume was reduced by half. The solution was concentrated under reduced pressure at 40°C and then freeze-dried.

Animals

Male and female SPF/ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan), and housed and maintained under standardized temperatures (25 ± 1 °C) and humidity (55 ± 5 %) in a light cycle room (light from 07:00 a.m. to 07:00 p.m.; dark from 07:00 p.m. to 07:00 a.m.). The mice were allowed to acclimatize for a week. The Animal Experimental Committee of Tohoku Medical and Pharmaceutical University approved all experiments, and experimental procedures were conducted following the ethical guidelines of the University.

Free Feeding of *Astragalus* Root to Ovariectomized Mice

Female 11-week-old mice were divided randomly into four groups, with six mice in each group. The mice groups were fed a standard chow (CE-2; CLEA Japan, Inc., Tokyo, Japan). They were ovariectomized under isoflurane anesthesia controlled by Inhalation Anesthesia Systems for Rodents (NARCOBIT-E). Two weeks later, each group was started on a standard chow containing 0.2 % fenofibrate, 5 % hot-water extract of *Astragalus* root (AsR), or 10 % AsR, and all groups were injected subcutaneously with estradiol (0.05 mg/kg/day) once daily for 48 days. The mice were weighed on random days. We collected vaginal smears from days 0 to 9, once-daily, from the control and 5 % AsR -treated mice. The vaginal smears were modified by Giemsa stain and observed by microscopy at 40 x magnification. Liver, uterine, mammary gland and visceral fat were collected and weighed after euthanasia by an excess of isoflurane anesthesia at 49th day. The uterus was kept at -80 °C until use.

Subcutaneous Injections of Formononetin or AstragalosideⅣ to Ovariectomized Mice

Fenofibrate (Wako Pure Chemical Industries Ltd., Osaka,

Japan), formononetin (Tokyo Chemical Industry Co., Ltd., Tokyo Japan), astragalosideIV (Carbosynth Ltd., Berkshire, UK), β-estradiol (Wako Pure Chemical Industries Ltd.) were used for subcutaneous injections.

Female 11-week-old mice were divided randomly into five groups, with five mice in each group. The mice were fed a standard chow (CE-2). The mice in four groups were ovariectomized under isoflurane anesthesia controlled by Inhalation Anesthesia Systems for Rodents (NARCOBIT-E). Two weeks later, ovariectomized mice were subcutaneously injected saline (as control group) or fenofibrate (0.0156 mmol/kg) or formononetin (0.0156 mmol/kg) or astragalosideIV (0.0156 mmol/kg) together with estradiol (0.05mg/kg/day) once daily. Uterine was collected and weighed after euthanasia by an excess of isoflurane anesthesia on the 26th day. The uterus was kept at -80 °C until use.

ERα, PPARα, β-catenin, Wnt4 and Wnt7a Levels Assay

The ERα level was determined by enzyme-linked immunosorbent assay (ELISA) using 96-well plastic plates. The uterus (30 mg) was homogenized in 1000 µl of 20 mM Tris-HCl buffer (pH 7.5) containing 1 % Triton-100, 150 mM NaCl, 2 mM EDTA, 250 mM sucrose, 2 mM EGTA and protease inhibitor cocktail (Sigma-Aldrich, Co. LLC., MO, USA). The homogenates were centrifuged at 3300 rpm for 10 min at 4°C to separate supernatant. The total amount of protein in the supernatant was measured using a protein assay kit (TaKaRa BCA Protein Assay kit; Takara Bio, Inc., Shiga, Japan). The supernatant containing 0.5 µg protein was immobilized in each well. 3 % skim milk was used as the blocking reagent. Rabbit polyclonal ERα antibody (Signal way Antibody LLC, MD, USA) as the primary antibody and Goat anti-rabbit polyclonal IgG conjugated alkaline phosphatase (Bio-Rad Laboratories Inc., CA, USA) as the secondary antibody was immobilized. The *p*-Nitrophosphate Tablet (Sigma-Aldrich Co. LLC, MO, USA) was used as a color reagent. Absorbance was measured at 405 nm, and the ERα level was represented as a relative ratio of the absorbance against that of the control group.

PPARα, Wnt4, Wnt7a, and β-catenin expression levels were measured using the same method as described above, except for the primary antibody. PPARα antibody (GeneTex Inc., CA, USA), Wnt4 polyclonal antibody (Bioss Antibodies Inc., MA, USA), Wnt-7a antibody (Novus Biologicals LLC, CO, USA), and β-catenin (Ab-33) antibody (EnoGene Biotech Co, Ltd., NY, USA) were used as the primary antibodies.

Natural Mating of Mice

Female mice were assessed the fertility by vaginal smear stained with Giemsa's Azur eosin methylene blue solution (Merck KGaA, Darmstadt, Germany). Twenty-four 6-week-old female ICR mice were divided randomly into four groups, with six mice in each group. The 2 % *Astragalus* root (AsR), 2.5 % Keishikaogito (KO), 5 % KO groups were started on a standard chow (CE-2) containing 2 % of hot-water extract of AsR, 2.5 % or 5 % of hot-water extract of KO to female mice for 24 days. AsR- or KO- treated female mice withdrew the samples just before natural mating. The male mouse was interbred with 2 female mice in one cage for 12 days. Vaginal plugging was monitored at 09:00~09:30 each day. After mating, female mice were single-housed, and the birthdates of each litter, litter size and weight were recorded. Each pups' weight and length at weaning day (day 16) were measured and recorded for the litter that consisted of 14 pups.

Statistical Analysis

All data are expressed as the mean ± SD. ANOVA (Dunnett's test) was performed using SigmaStat version 2.03 (Systat Software Inc.), and a $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Bodyweight Changes and Organ Weights

As depicted in Figure 1, 0.2 % fenofibrate-treated mice had significantly lower body weights compared to controls at the 20, 30, 40, and 49th days. Mice fed 5% and 10% AsR -treated chow showed a trend towards decreased body weights.

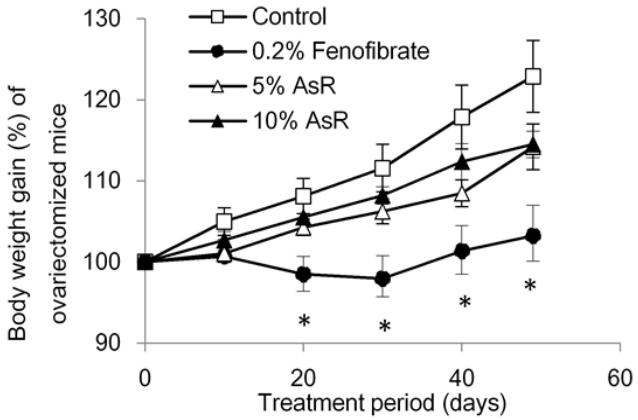

Figure 1: Monitoring body weight gain of AsR-treated mice. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test. AsR, hot-water extract of Astragalus root.

In Figure 2, 0.2 % fenofibrate-treated mice showed significantly increased liver weight (Figure 2A) and decreased

uterus, mammary gland, and visceral fat weights (Figure 2B, 2C, 2D). Ten percent of AsR-treated mice showed decreased mammary gland weight (Figure 2C) and significantly decreased visceral fat weight (Figure 2D).

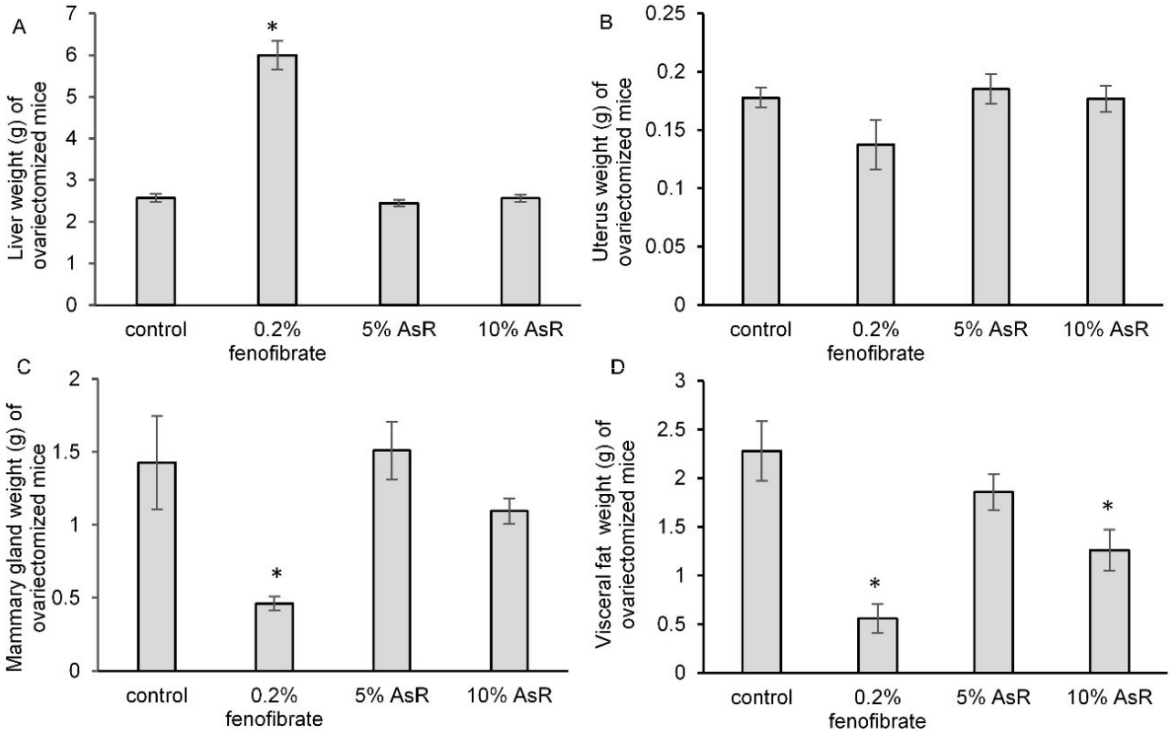

Figure 2: Wet organ weight of AsR-treated mice. (A) liver (B) uterus (C) mammary gland (D) visceral fat. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test. AsR, a hot-water extract of Astragalus root.

Nuclear Receptors PPARα and ERα Levels in the Uterus

The levels of uterine PPARα (Figure 3A) and ERα (Figure 3B) were significantly higher in the 5 % and 10 % AsR-treated mice.

Uterine PPARα level of AstragalosideⅣ-injected mice was significantly higher, as well as the level of formononetin-injected mice (Figure 4A). AstragalosideⅣ-injected mice showed significantly higher uterine ERα levels (Figure 4B).

Secretory Proteins Wnt4 and Wnt7a and their Signaling Factor β-catenin Levels in the Uterus

The levels of uterine Wnt4 (Figure 5A), Wnt7a (Figure 5B) and β-catenin (Figure 5C) were significantly higher in the 5 % and 10 % AsR-treated mice.

The astragalosideⅣ-injected mice had significantly higher levels of uterine Wnt4 (Figure 6A), Wnt7a (Figure 6B), and β-catenin (Figure 6C). The formononetin-treated mice had a significantly higher uterine Wnt7a levels (Figure 6B).

Vaginal Neutrophils and Mucus of *Astragalus* Root - Treated Mice

Vaginal smears were collected from control and 5% AsR -treated mice from days 0 through 9. We used a modified Giemsa stain to compare the 5% AsR-treated and control mice. In 5 % AsR-treated mice on the 7th day, the round and non-ruptured neutrophils abundantly emerged with mucus in the vaginal smears (Figure 7A). In control mice, the neutrophils mostly ruptured, and mucus was not clearly visible (Figure 7B) from days 0 through 9.

Comparison of Litter Size and Survival Pups

Although there were no significant differences in the average of litter size between AsR-treated mice and control mice, AsR-treated mice tended to produce increased average litter sizes with a narrower SD for size (Figure 8).

All pups of AsR-treated maternal mice survived and satisfactorily grew, while several control mice pups exhibited

slow growth and died before the weaning day (at 16th day). This was especially true for the pups from litters of 16 or more. In KO-treated maternal mice, one pup born from a 2.5% KO-treated maternal mouse died before weaning day, two maternal mice fed 5% KO had no pregnancies, despite the fact that the maternal mice were observed with a virginal plug and mounting a male mouse.

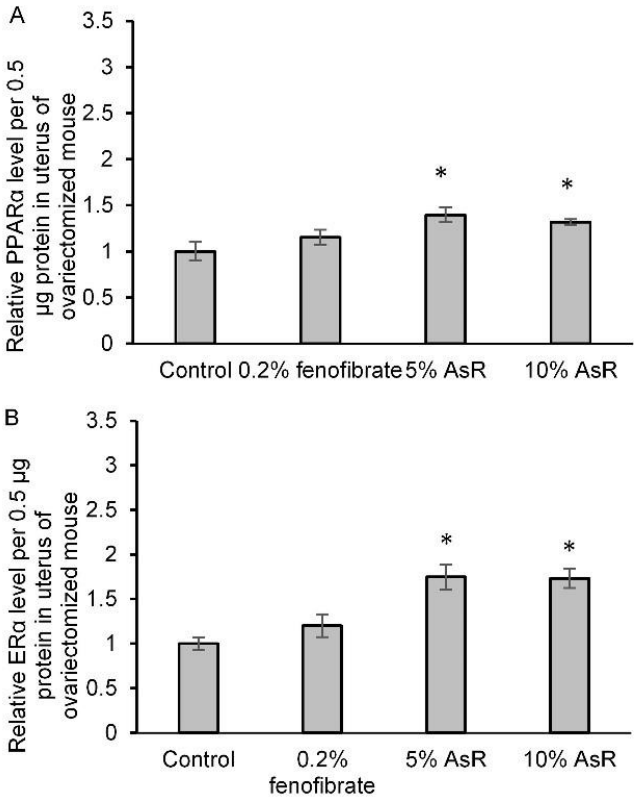

Figure 3: Expression levels of nuclear receptors PPARα and ERα expressed in the uterus of AsR-treated mice. (A) Uterine PPARα level; (B) uterine ERα level. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test. AsR, a hot-water extract of Astragalus root.

Comparison of Pup's Weight and Length among Identical Litter Size

A litter of 14 pups were compared for body weight and length. The pups born from 5% KO-treated maternal mice showed a trend towards increased body weight (Figure 9A) and significantly increased body length at the weaning day (Figure 9B).

Relationship between the Pup's Growth and Litter Size

We evaluated the relationship between the pup's growth and the litter size using scatterplots (Figure 10). The pup's average weight decreased in proportion to increasing litter size. When the pup's weight (g) and the litter size were regarded as x and y, respectively, $y = -1.2391x + 22.737$ ($R^2 = 0.8949$) was obtained as the regression equation.

Discussion

Astragalus root and its Component AstragalosideⅣ Influence to Endometrial ERα, Wnt and β-Catenin

Wnt4 is strongly expressed in the endometrial stroma and luminal epithelium of the mouse uterus, with the epithelium essentially doubled in thickness secondary to the effects of estradiol. Wnt4 increases in the presence of high circulating levels of estradiol, and mediate progesterone-induced decidualization in the mouse uterus. In this study, the levels of ERα, Wnt4, and β-catenin were significantly increased in the mouse uterus treated with *Astragalus* root (Figures 3B, 5A & 5C) or AstragalosideⅣ (Figures 4B, 6A & 6C). Therefore, AsR and its component AstragalosideⅣ are thought to contribute to endometrial proliferation and to mediate endometrial decidualization.

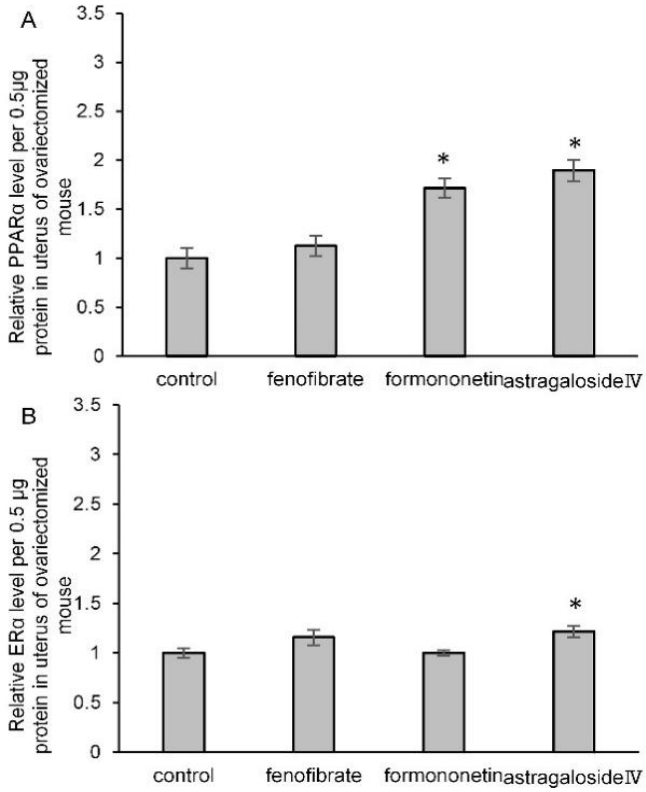

Figure 4: Expression levels of nuclear receptor PPARα and ERα expressed in the uterus of formononetin or astragalosideⅣ -treated mice. (A) Uterine PPARα level; (B) uterine ERα level. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test.

Wnt7a, which is restricted to the oviduct and uterine luminal epithelium in the adult mouse, acts to maintain the formation of the stroma-smooth muscle boundaries of the adult uterus and plays a vital role as a cell death suppressor [9]. In this study, the Wnt7a level was significantly increased in the AsR, formononetin, and AstragalosideIV-treated mice (Figures 5B & 6B). *Astragalus* root and its components formononetin and AstragalosideIV are believed to help maintain the organization of the uterus and prevent to aberrant cell death within the uterus.

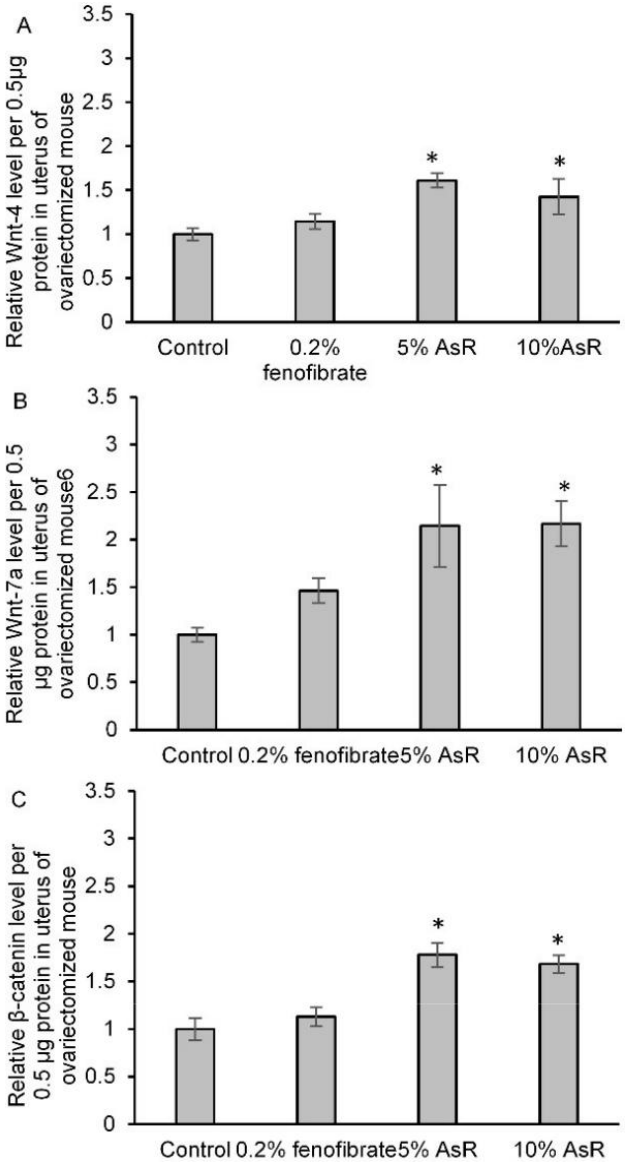

Figure 5: Expression levels of Wnt/β-catenin signaling factor expressed in the uterus of AsR-treated mice. (A) Wnt4; (B) Wnt7a; (C) β-catenin levels. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test. AsR, hot-water extract of Astragalus root.

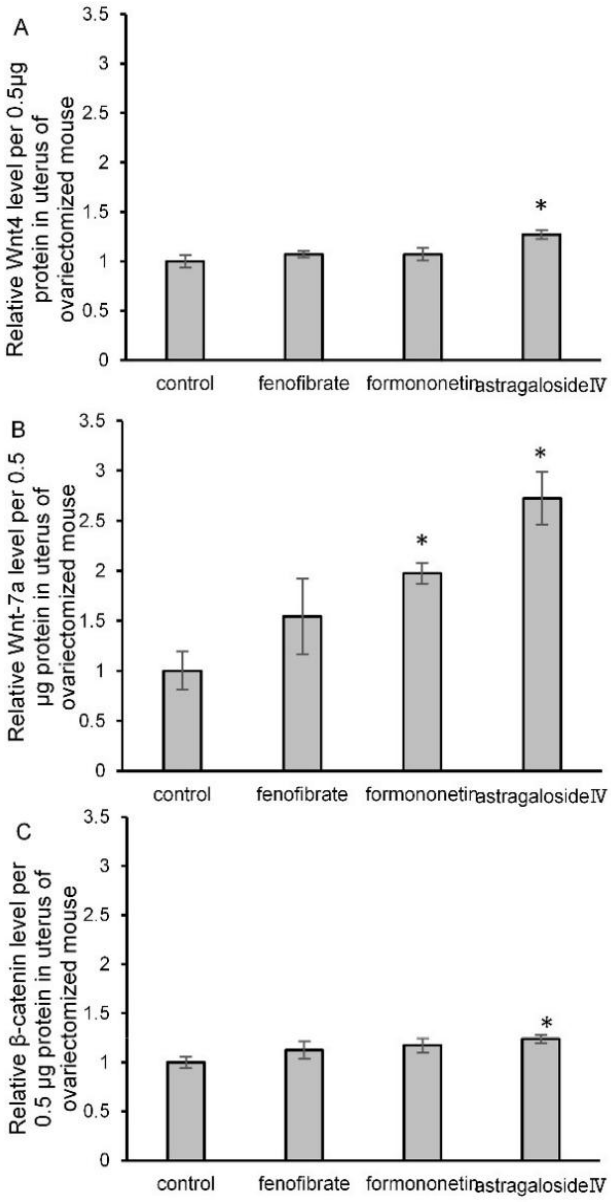

Figure 6: Expression levels of Wnt/β-catenin signaling factor expressed in the uterus of formononetin or astragalosideIV -treated mice. (A) Wnt4; (B) Wnt7a; (C) β-catenin levels. Each value represents the mean ± SD (n=6). *; p<0.05, one-way ANOVA performed after Dunnett's test.

Influence to Vaginal Neutrophils and Mucus by *Astragalus* root

A large number of neutrophils emerge in the vagina for phagocytosis of bacteria. Neutrophils are generally ruptured or condensed during the collection or preparation of vaginal smears. In Figure 5B, neutrophils of the control group, were

ruptured; AsR-treated mice, however, showed non-ruptured, non-condensed, and crowding neutrophils (Figure 7A). AstragalosideIV, the main component of *Astragalus* root, promotes the expression of CXCR2 (Chemokine C-X-C motif receptor 2) on neutrophils [12], thereby increasing the filtration of neutrophils to the vagina in Figure 7A.

Furthermore, there was a remarkable amount of mucus secreted from the endocervical gland (pointing arrows in Figure 7A). It is known that the secretion from the endocervical epithelium is maximal at ovulation to block bacterial access through the uterine cavity. Therefore, *Astragalus* root helps prevent bacterial invasion by increasing neutrophils phagocytosis and mucus secretion in the luminal epithelium of the vagina.

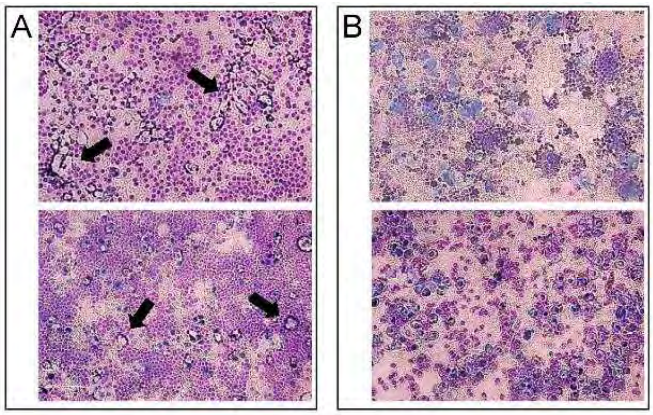

Figure 7: Vaginal smears of AsR-treated mice. (A) A vaginal smear of 5% AsR- treated mice; (B) Control. Vaginal neutrophils were stained with Giemsa to reddish-violet. The black arrows indicate mucus.

Astragalus root Influences to Litter Size

In the control group, litters of 16 pups or more grew insufficiently, and a few of the pups died before weaning day. All pups of the 2% AsR-treated maternal mice, however, survived until weaning day. In 2% AsR-treated maternal mice, there was a trend towards increased average the litter size, with a narrower SD for litter size, compared to that of control (Figure 8).

The main component of *Astragalus* root, polysaccharides, and saponins promote wound healing by improving immune function [13]. Subsequently to ovulation from the follicle, the secretion of progesterone from granulosa lutein cells induces the decidualization of the endometrium. Decidual $CD4^+$ $CD25^+$ regulatory T cells (Treg) are increased during maternal immunotolerance for the fetus, while cytotoxic $CD8^+$ T cells are decreased. $CD4^+$ Treg mediates maternal immunotolerance for the fetus [14]. Gattinoni L, et al. [15] indicates that 'Activation of Wnt/β-catenin signaling inhibits cytotoxic $CD8^+$ T cell differentiation', and 'Wnt/β-catenin signaling enhances survival of $CD4^+$ Treg'. The polysaccharides of *Astragalus* root promote the expression of β-TGF [13] secreted from $CD4^+$ Treg to prevent cytotoxic T cell activation. Moreover, astragalosideIV enhances $CD4^+CD25^+$Foxp3 Treg [16]. Taken together with observations from these reports, we propose that *Astragalus* root improves decidual immunotolerance for embryo implantation and fetus through activation of Wnt/β-catenin signaling, leading to increased litter size.

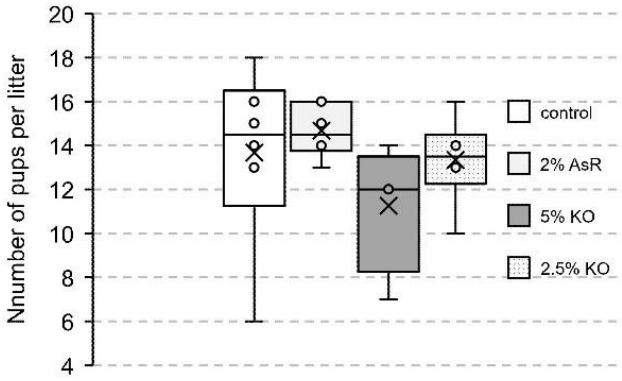

Figure 8: Box-whisker plot applied to litter size. Litter size of each maternal mouse was presented by "○" and the ends of the whiskers (n=6 in each group) the lower and upper edge of the box signal the 25th percentile and 75th percentile, respectively. Across the line parallel to the edge of the box indicates the medium of the distribution. The means of litter sizes were represented by "×". There are no outliers in the plot. AsR, a hot-water extract of Astragalus root; KO, a hot-water extract of Keishikaogito.

Keishikaogito Promotes Fetal Growth

Astragalus root is a prescription drug constituting Keishikaogito whose primary usage is the recovery from fatigue and night sweats. In 5 % KO-treated mice, two females failed to get pregnant, even though the mice were observed with virginal plugs and mounting male mice. Meanwhile, all of the maternal mice in the control, 2% AsR and 2.5% KO groups became pregnant. As shown by Figure 10, the bigger the litter size, the less the pups grow. To exclude the influence of litter size from the comparison of pup's growth, litter with equal numbers of pups were chosen from each group. When we compared litters with 14 pups, the pups from 5% KO-treated maternal mice showed a trend towards increased body weight (Figure 9A), and significantly increased body

length (Figure 9B).

Astragalus Root is also used as a component of Hochuekkito, where it is used for fatigue, weakness after illness, anorexia, and night sweats. Hochuekkito augments the reduction of immunotolerance by B cells, the activation of INF-γ (cytotoxic T cells activating factor) [17]. These factors are believed to reduce the immunotolerance for the fetus. These findings suggested that a crude drug-component of Keishikaogito, except *Astragalus* Root, might interfere with embryo implantation while promoting fetus growth.

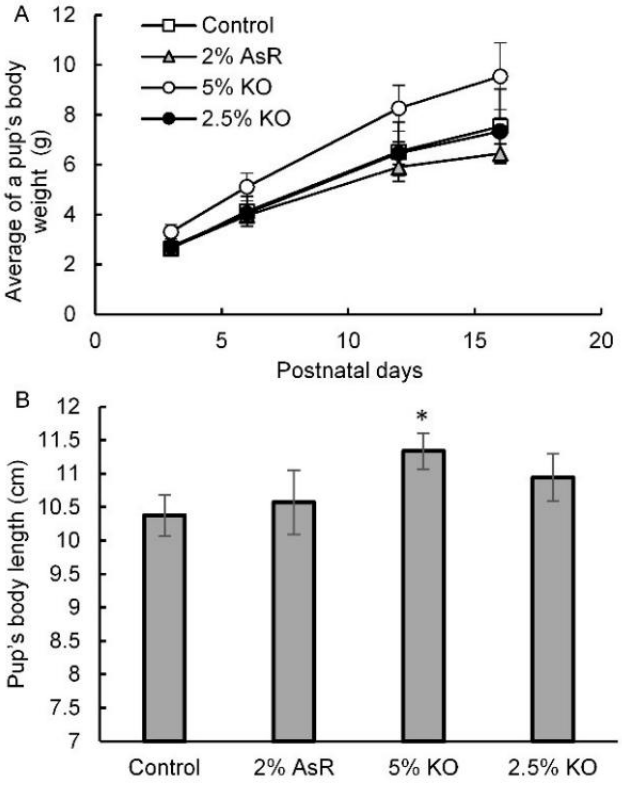

Figure 9: Body weight and length of the litter size constituting of 14 pups from each group. (A) monitoring of body weight of pups; (B) body length of pups at weaning day. *; p<0.05, one-way ANOVA performed after Dunnett's test. AsR, a hot-water extract of Astragalus root; KO, a hot-water extract of Keishikaogito.

Conclusion

Astragalus root thought to improve embryo implantation through activating endometrial Wnt/β-catenin signaling factors. However, *Astragalus* root was not to increase litter size when used as a component of Keishikaogito. The discovery of *Astragalus* root-containing Kampo formulae showing increase of litter size is needed for treatment of fertility in medical care.

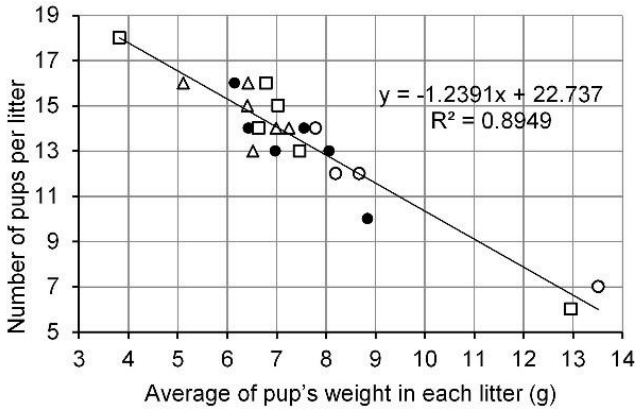

Figure 10: Relationships between litter size and pup's growth. □ control; △, 2% AsR; ○, 5% KO, ●, 2.5% KO -treated mice. When the number of pups per litter and the average of pup's weight in each litter were regarded as y and x, respectively, liner regression equation, y = -1.239x + 22.737; coefficient of determination, R2 = 0.895. AsR, a hot-water extract of Astragalus root; KO, a hot-water extract of Keishikaogito.

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Conflict of Interests

The authors declare no conflicts of interest associated with this manuscript.

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