Extracts from *Paeonia lactiflora Pallas, Rehmannia Glutinosa var. Purpurea Makino, Perilla Frutescens var. Acuta Kudo* may increase the endometrial receptivity through expression of leukemia inhibitory factor and adhesion molecules

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**Abstract**

**OBJECTIVE:** To find out the combination of the extracts from *Paeonia lactiflora Pallas* (PL), *Rehmannia Glutinosa var. Purpurea Makino* (RG), *Perilla Frutescens var. Acuta Kudo* (PF) to increase endometrial receptivity.

**METHODS:** Herbal medicines were extracted with boiling water and polysaccharides were removed. We examined the effect of PL, RG, and PF (PRP), a most effective herbal formula deduced from constitutive ingredient herbs of Antai Yin which is composed of PRP, on the leukemia inhibitory factor (LIF) expression and endometrial receptivity.

**RESULTS:** The combination of the extracts from PRP induced the LIF expression in Ishikawa cells and increased the adhesion between Ishikawa and JAr cells. In addition, PRP-induced attachment of JAr cells onto Ishikawa cells and expression of adhesion molecules, ITGA, ITGB5, CD44s, and L-selectin, are significantly reduced by knock-down of LIF expression.

**CONCLUSION:** Induced by the combination of the PRP extracts, the adhesion between trophoblast and endometrial cells are mediated by expression of LIF and adhesion molecules. Thus, we suggest the combination of the PRP extracts may be a novel therapy for enhancing embryo implantation rate.

**Keywords:** Paeonia; Rehmannia; Perilla; Embryo implantation; Endometrium; Leukemia inhibitory factor; Cell adhesion molecules

**INTRODUCTION**

The main causes of female infertility included the problems of ovulation, embryo development, embryo
Table 1 Composition of a Medicinal herbal formula, Antai Yin

<table>
<thead>
<tr>
<th>Voucher specimen number</th>
<th>Scientific name</th>
<th>Dose (g, t.i.d.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KMRC-AT01</td>
<td>Roots of Atractylodes japonica Koidze.</td>
<td>2.50</td>
</tr>
<tr>
<td>KMRC-AT02</td>
<td>Roots of Scutellaria Baiicalensis Georgi.</td>
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</tr>
<tr>
<td>DC-H03</td>
<td>Roots of Angelica Gigas Nakai</td>
<td>1.25</td>
</tr>
<tr>
<td>DC-H21</td>
<td>Roots of Peonia Lactiflora Pallas</td>
<td>1.25</td>
</tr>
<tr>
<td>DC-H16</td>
<td>Processed roots of Rehmannia glutinosa var. Purpurea Makino</td>
<td>1.25</td>
</tr>
<tr>
<td>DC-H27</td>
<td>Leaves and stems of Perilla Frutescens var. Acuta Kudo</td>
<td>1.00</td>
</tr>
<tr>
<td>DC-H23</td>
<td>Peels of Citrus reticulata Markovitch</td>
<td>1.25</td>
</tr>
<tr>
<td>DC-H24</td>
<td>Roots of Cnidium Officinal Makino</td>
<td>1.00</td>
</tr>
<tr>
<td>KMRC-AT03</td>
<td>Fruits of Amomum Xanthioides Wallich.</td>
<td>1.25</td>
</tr>
<tr>
<td>KMRC-AT04</td>
<td>Roots of Glycyrrhiza uralensis Fischer</td>
<td>0.50</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>13.13</strong></td>
</tr>
</tbody>
</table>

transport, and embryo implantation. Despite advances in the assisted reproductive technologies (ART) solved the problems of ovulation, embryo development and transport, failure of embryo implantation remains an unmet need in the treatment of infertility. 

Embryo implantation is a complex biological process, requiring the communication between appropriately developed trophoblast and the receptive endometrium. This process is established and maintained by diverse molecules, such as cytokines, growth factors, and receptors. Among these factors, leukemia inhibitory factor (LIF), a member of interleukine-6 family cytokine, plays a key role in regulating endometrial receptivity.

Traditional herbal medicine has been used for long history to treat infertility in East Asian countries and the efficacy was generally accepted by clinical studies. Several previous studies reported the effectiveness of herbal medicines, such as Bangdeyun, Zhuyun recipe, and Bushen Antai recipe. In addition, previous studies reported that LIF was reported as an important molecular target of herbal remedies as well as acupuncture. Among these herbal formulas, Bushen Antai recipe, a transform of Shoutai Wan, is composed of Cuscuta chinensis Lamark, Dipssacus asper Wall., Salvia miltiorrhiza Bunge, Astragalus mongholicus Bunge, and Angelica sinensis (Oliv.) Diels, and used for prevention and treatment of threatened abortion. Because abortion is a failure of pregnancy maintenance, herbal drugs used for treating threatened abortion may be effective to infertility.

Antai Yin, is an herbal formula composed of *Atractylodes japonica Koidze.* (AJ, *Atractylodes* Rhizoma), *Scutellaria Baiicalensis Georgi.* (SB, *Scutellariae Radix*), *Angelica Gigas Nakai* (AG, *Angelicae Radix*), *Peonia Lactiflora Pallasi* (PL, *Peoniae Radix Alba*), *Rehmannia glutinosa var. Purpurea Makino* (RG, *Rehmanniae Radix Preparata*), *Perilla Frutescens var. Acuta Kudo* (PF, *Perillae Herba*), *Citrus reticulata Markovitch* (CR, *Auranti Pericarpium*), *Cnidium Officinal Makino* (CO, *Cnidii Rhizomatis*), *Amomum Xanthioides Wallich.* (AX, *Amomi Semen*), and *Glycyrrhizaeuralenis Fischer* (GU, *Glycyrrhizae Radix*). This prescription have been used for preventing threatened abortion, originally established by Gong. In addition, several previous studies showed that the formula has no mutagenic or hepatocytotoxic effect and showed anti-inflammatory and analgesic effects. However, there is no previous report mentioning the effect of Antai Yin or its ingredient herbs on endometrial receptivity.

In this study, we examined the effect of the combined extracts from PF, RG, and PL (PRP), a most potent from the constitutive ingredient herbs of Antai Yin on the expression of LIF and receptivity of endometrium.

**METHODS**

**Chemicals and reagents**

Antibodies against LIF and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals and reagents, including 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyterazolium bromide (MTT), 5-hydroxymethyl-2-furaldehyde (5-HMF), ferulic acid, protocatechuic acid, rosmarinic acid, and dimethyl sulfoxide (DMSO), were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

**Plant materials and sample preparation**

Antai Yin, an herbal formula registered to Korean Medical Insurance, was generously gifted by Hankookshinyak Pharmaceutical Co. (Nonsan, Korea). Ten ingredient medicinal herbs contained in Antai Yin were purchased from Omniherb Co. (Daegu, Korea), identified by herbal expert of supplier, and presented in Table 1. Voucher specimens was deposited at the Korean Medical Research Center for Healthy Aging.
Pusan National University. The Antai Yin and its ingredient herbs were extracted according to our previous report. Briefly, each medicinal herb (100 g) was extracted with distilled water (1 L) for 2 h at 100 °C, and then centrifuged at 4000 rpm for 10 min. The supernatant was extracted with 70% ethanol for polysaccharide precipitation at 4 °C. After centrifugation at 4000 rpm for 10 min, the supernatant was evaporated and lyophilized by freeze-drier to give a powder. The powders were freshly dissolved in dimethyl sulfoxide (DMSO) before experiments. The combinations of herbal medicines, including PRP, are composed by same amount of each ingredient herbs from Antai Yin.

**High performance liquid chromatography (HPLC) analysis of herbal materials**

Phytochemical characteristic of RG and PF was identified by HPLC analysis by using an Agilent 1200 series system (Agilent Technologies, Santa Clara, CA, USA), which composed by a solvent delivery unit, an on-line degasser, a column oven, an autosampler, and a multi-wavelength detector. The data was analyzed by LC solution software (Agilent Technologies, Santa Clara, CA, USA, version 1.24). The column used for analysis was a KR100-5C18 column (AlzinoNobel, Arhen, Netherlands; 4.6 mm × 250 mm; pore size, 5 μm). For analysis of RG, the mobile phases were solvent A (water) and solvent B (acetonitrile). The gradient flow was as follows: (A)/(B) = 95/5 (0 min) → (A)/(B) = 30/70 (60 min). The column temperature was maintained at 25 °C. The analysis was carried out at a flow rate of 0.2 mL/min with detection at 280 nm. In addition, for analyzing PF, the mobile phases were solvent A [0.1% formic acid aqueous (v/v)] and solvent B (methanol). The gradient flow was as follows: (A)/(B) = 100/0 (5 min) → (A)/(B) = 50/50 (25 min) → (A)/(B) = 0/100 (50 min). The column temperature was maintained at 33 °C. The analysis was carried out at a flow rate of 1 mL/min with detection at 254 nm. The column injection volume was 20 μL. A standard solution, containing 5-HMF for analyzing RG and ferulic acid, protocatechuic acid, and rosmarinic acid for analysis of PF was prepared by dissolving in distilled water.

**Cell culture**

The human endometrial Ishikawa cell line was generously provided by Dr. Jacques Simard (CHUL Research Center, Quebec, Canada). The cells were cultured as monolayers at 37 °C in an atmosphere containing 5% CO2/air and Roswell Park Memorial Institute 1640 (RPMI1640; Welgene) containing 10% heat-inactivated FBS and 1% penicillin/streptomycin.

**Cell viability assay**

Ishikawa cells were cultured in 24-well plates with indicated concentrations of Antai Yin and PRP in serum free medium for 24 h. After then, the medium was replaced with MTT solution (2 mg/mL) and incubated in 37 °C in a cell culture incubator for 3 h. The formazan crystals formed by MTT solution were fused with DMSO and EtOH solution and measured at 540 nm with microplate reader.

**Western blot analysis**

Total proteins were isolated from Ishikawa cells treated with each herbal medicine or not. The proteins were separated by SDS-PAGE and electro-transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked by 5% skim milk in Tris-buffered solution (TBS) and then incubated with each antibody specific for target proteins, including LIF and GAPDH, during overnight. After reaction, the membranes were washed and incubated with appropriate secondary antibodies conjugated with the horseradish peroxidase. The signals were visualized by using the ECL chemiluminescence system (GE Healthcare, Uppsala, Sweden).

**Reverse-transcription polymerase chain reaction (RT-PCR)**

Total RNA was isolated from PRP-treated Ishikawa cells with GeneJET RNA Purification Kit (Thermo Fischer Scientific, Waltham, MA, USA). Equal amount of total RNA of each sample was used for reverse transcription reaction by using M-MLV reverse transcriptase (Enzymonics, Daejeon, Korea) with oligo-dT primers. Synthesized cDNA was subjected for amplification by PCR using DiaStartM Taq DNA Polymerase (Solgent Co., Daejeon, Korea). The primers and PCR conditions used for amplifying LIF, ITGAV, ITGB1, ITGB3, ITGB4, ITGB5, CD44s, ICAM-1, L-selectin, and β-actin were shown in Table 2. The mixtures were denatured at 95 °C for 5 min, annealed at each annealing temperature for 30 s, extended at 72 °C for 30 s. The reactions from annealing to extension were repeated for indicated in Table 2. After final extension 72 °C for 10 min, the amplified DNA were electrophoresized in 1% agarose gels containing ethidium bromide and visualized under UV light. The images were taken with GelDoc-It TS Imaging System (UVP, Upland, CA, USA).

**Adhesion assay**

Briefly, Ishikawa cells (1.5 × 10⁵ cells) were seeded in 6 well plates during overnight. Media were replaced and incubated in serum free-DMEM with herbal drugs for

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48 h. The JAr cells were labeled with 5-chloromethylfluoresceindiacetate (CMFDA) fluorescence dye (CellTracker Green; Invitrogen, Carlsbad, CA, USA) for 10 min at 37 °C. Then, the labeled JAr cells were washed in 1 × phosphate-buffered saline (PBS) and incubated with Ishikawa cells shaking for 30 min gently in room temperature. The cells were vigorously washed to remove non-attached JAr cells. The attached JAr cells were visualized by using a fluorescent microscope and the cell numbers were calculated.

**Knockdown of LIF by shRNA**

For knockdown of hLIF expression, five lentiviral shRNA constructs were purchased from Open Biosystems (Thermo Scientific, Waltham, MA, USA). The lentiviral vector encoding shRNA against hLIF (3 μg) was transfected in 293T packaging cells by using Lipofectamine 2000 (Invitrogen). After 48 h, supernatant having lentiviruses was collected and subsequently infected into Ishikawa cells. The transfected cells were selected with puromycin (3 μg/mL) for 1 week. The efficiency of genetic knockdown against LIF expression was verified by Western blot analysis and most effectively knockdown clone was used for experiment.

**Densitometry and statistical analysis**

The intensities of the bands acquired from Western blot analysis and the number of attached cells from adhesion assay were measured by using ImageJ software (NIH, Bethesda, MA, USA). The results from cell viability and adhesion assay were indicated as the value of percentage. The band intensities from Western blot analysis were expressed as the fold to control. The data from Western blot analysis and the number of attached cells from adhesion assay were measured by using ImageJ software (NIH, Bethesda, MA, USA). The results from cell viability and adhesion assay were indicated as the value of percentage. The band intensities from Western blot analysis were expressed as the fold to control. The data was expressed as the mean ± standard deviation (x ± s) and the differences between the two groups were determined by standard t-tests using GraphPad Prism (GraphPad Software, San Diego, CA, USA). The minimum significance level was set at a P value of 0.05. All experiments were independently performed at least three times.

**RESULTS**

**Six ingredient herbs of Antai Yin increase the expression of LIF**

The result presented that Antai Yin has no cytotoxicity up to concentration of 100 μg/mL (Figure 1A). Next, we examined whether the expression of LIF is increased by treatment of Antai Yin. To disappoint to us, Western blot analysis showed that Antai Yin did not increase LIF expression up to concentration of 100 μg/mL (Figure 1B).

Next, as shown in Figure 2, among 10 ingredient herbs, PL, RG, PF, CR, CO, and GU significantly increased the expression of LIF by more than 1.5 fold to that of control. In addition, SB slightly increased the expression of LIF, however there is no statistical significance.

**Determine optimal combination of 6 ingredient herbs inducing LIF expression**

We examined the additional or synergic effect produced by combination of each ingredient herb. As PF most potently increased the expression of LIF, we combined the other 5 ingredients with PF. The result indicated that PF and RG most highly increased the expression of LIF by 3.4 fold than that of control, among these combinations (Figure 3A). Secondly, we co-treated the other 4 ingredient herb in presence of the combination of PF and RG (PR). The data from Western blot demonstrated that the combination of

| Table 2 Primers used for PCR of LIF, integrins, and β-actin |
|-----------------|-----------------|-----------------|
| Gene            | Primer sequence | PCR condition  |
| LIF             | Forward: 5'-GGCCCGGGGACACCCCAT AGACG-3' | 60 °C 35 cycles |
|                 | Reverse: 5'-CCACGGCGCCATCCAGGTA AA-3' | 35 cycles |
| ITGV            | Forward: 5'-ATGCCTCCATGTAGATCAACAGAT-3' | 60 °C 35 cycles |
|                 | Reverse: 5'-TTCCAAAAGTCCCTTGCTGCT-3' | 35 cycles |
| ITGB1           | Forward: 5'-CTGCTGTGTTGAGTGATCAAACT-3' | 60 °C 30 cycles |
|                 | Reverse: 5'-GCTGTTGTTAATTGTGCAGCA-3' | 35 cycles |
| ITGB3           | Forward: 5'-CTGCCGTGACGAGAT TGAGT-3' | 62 °C 35 cycles |
|                 | Reverse: 5'-TGC CCC GGT AGC TGA TAT TG-3' | 35 cycles |
| ITGB5           | Forward: 5'-ACCTGGAACACGGTGAGA-3' | 60 °C 35 cycles |
|                 | Reverse: 5'-AAAAAGTAGCGGTGTCGGCAAA-3' | 127 |
| CD44s           | Forward: 5'-AGGGATCCCTCCAGCTCCTT-3' | 62 °C 30 cycles |
|                 | Reverse: 5'-AAAGGATGTCGGCGTTGTGCAGT-3' | 35 cycles |
| ICAM-1          | Forward: 5'-CAGTGACCACCTCTACAGCTTTCGG-3' | 62 °C 30 cycles |
|                 | Reverse: 5'-GCTGCTCACACAGTGATGAGCAAA-3' | 209 |
| L-selectin      | Forward: 5'-AAACCCATGAACCTGCGAAA AG-3' | 62 °C 30 cycles |
|                 | Reverse: 5'-GGGAGTTACCGTCCTGACCT-3' | 336 |
| β-actin         | Forward: 5'-CAAGAGATGCGCGCGCTGCTG-3' | 60 °C 30 cycles |
|                 | Reverse: 5'-TCTCCGCTGACCTGCGGC-3' | 275 |

Notes: PCR: polymerase chain reaction; LIF: leukemia inhibitory factor.
PRP is most potent to induce LIF expression. The combination increased LIF expression by 10.5 fold than that of control (Figure 3B).

Next, we further combined the other 3 herbs to PRP and treated to Ishikawa cells, to find more effective combination composed of 4 ingredient herbs. However, the additional combination of other 3 herbs to PRP did not show significant increase in LIF expression. On the contrary, the combination of CO to PRP decreased the PRP-induced LIF expression by about 0.6 fold (Figure 3C). As shown in Figure 2, the arithmetic sum of induction rate of LIF induced by PF, RG, and PL is about 6.1 fold. In addition, the increase rates of LIF expression induced by RG and PL were not prominent than that by CR or CO.

**PRP increase the adhesion between endometrial and trophoblast through LIF-dependent manner**

The phytochemical characteristics of three component herbs of PRP were confirmed by HPLC analysis by using standard ingredient chemicals. The results from recent our study\(^1\) and shown in Figure 4, clearly showed that these herbs are identical with previous study.\(^{19,20,22}\) To determine the effect of PRP on the trophoblast adhesion, we performed adhesion assay using human trophoblastic JAr cells and human endothelial Ishikawa cells. The results clearly showed that treatment of PF to Ishikawa cells increased the receptivity to trophoblastic JAr cells. The treatment of

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**Figure 1** Effect of Antai Yin on cell viability and LIF expression in human endometrial Ishikawa cells

A: Ishikawa cells were treated with indicated concentration of Antai Yin. Cell viability was estimated by using an MTT assay. \(P < 0.01\) and \(P < 0.001\), compared to vehicle treated control (mean ± standard deviation). B: Ishikawa cells were treated with indicated concentration of Antai Yin for 24 h. The expression levels of LIF protein were measured by Western blot analysis. The expression of GAPDH was used for internal control. MTT: 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; LIF: leukemia inhibitory factor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

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**Figure 2** Screening of effective ingredient herbs from Antai Yin on the LIF expression

A: Ishikawa cells were treated with each ingredient herb of Antai Yin (50 μg/mL) for 24 h. After then, the expression of LIF was examined by Western blot analysis. A1: AJ: Atractylodes Japonica Koidz.; SB: Scutellaria Baicalensis Georgi.; AG: Angelica Gigas Nakai.; PL: Paeonia Lactiflora Pallas; RG: Rehmannia Glutinosa var. Purpurea Makino; A2: PF: Perilla Frutescens var. Acuta Kudo; CR: Citrus Reticulata Markovich; CO: Cnidium Officinale Makino; AX: Amomum Xanthioides Wallich.; GU: Glycyrrhizauralensis Fischer. B: the intensities from Western blot analysis were estimated by densitometric analysis and calculated by fold of GAPDH. LIF: Leukemia inhibitory factor; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.
PR, and PRP combinations gradually increased the attached of JAr cells onto Ishikawa cells (Figure 3D). Next, we confirmed the effect of PRP on the cytotoxicity and expression of LIF. The results showed that PRP effectively increased the expression of LIF at non-toxic dose of PRP in a dose-dependent manner (Figure 5). In addition, to evaluate that the positive effect of PRP on the trophoblast adhesion was due to LIF expression, we treated JAr cells with 50 μg/mL of PF and CO, CR, GU, PL, RG, and LIF knock-down-Ishikawa cells. Viral vector harboring shRNA against LIF was introduced into Ishikawa cells (shLIF), and the knockdown efficiency was verified by Western blot analysis using antibody against LIF (data not shown). Control cells transfected with pLKO.1 mock vector (pLKO.1) and shLIF cells were incubated with PRP or not for 48 h, and adhesion assay was performed with fluoresce-labeled JAr cells. The result demonstrated that the PRP-induced attachments of JAr cells onto Ishikawa cells were significantly reduced by shLIF (Figure 6).

**Figure 3 Analysis of additional effect of ingredient herb combination on the LIF expression and adhesion between JAr and Ishikawa cells.**

A: Ishikawa cells were co-treated with 50 μg/mL of PF and CO, CR, GU, PL, RG (50 μg/mL, respectively) for 24 h. B: Ishikawa cells were co-treated with PF + RG (50 μg/mL, respectively) and CO, CR, GU, PL (50 μg/mL, respectively) for 24 h. C: Ishikawa cells were co-treated with PF + RG + PL (50 μg/mL, respectively) and CO, CR, GU (50 μg/mL, respectively) for 24 h. The expression of GAPDH was used for internal control. The intensities from Western blot analysis were estimated by densitometric analysis and calculated by fold of GAPDH. The relative expression of LIF compared with control was shown. D: Ishikawa cells were cultivated in 6-well plates and treated PF, PF + RG, and PF + RG + PL (50 μg/mL, respectively) in serum free culture medium for 48 h, respectively. JAr cell labeled with CellTracker were added onto Ishikawa cell monolayer and gently shaken for 30 min. After a brief wash, the cells were fixed by using formaldehyde and attached JAr cells were counted. The data was calculated by percentage of control and shown as mean ± standard deviation of three independent experiments. *P < 0.001 in comparison with CON. #P < 0.001 in comparison with PF and $P < 0.001$ in comparison with PR. E: The pictures were taken by fluorescent microscopy. E1: CON; E2: PF; E3: PR; E4: PRP; LIF: leukemia inhibitory factor; PF: Perilla Frutescens var. Acuta Kudo; CO: Cnidium Officinale Makino; CR: Citrus Reticulata Markovich; GU: Glycyrrhiza-ralensis Fischer; PL: Paeonia Lactiflora Palllas; RG: Rehmannia Glutinosa var. Purpurea Makino; CON: control; PR: PF and RG; PRP: PL, RG and PF; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

**PRP increase the adhesion molecules, including ITGAV, ITGB5, CD44s, and L-selectin, through LIF-dependent manner**

To figure out which adhesion molecules are regulated by PRP treatment, we analyzed the expression of adhesion molecules, such as ITGAV, ITGB1, ITGB3, ITGB5, CD44s, ICAM-1, and L-selectin. The mRNA expression levels of ITGV, ITGB3, CD44s, and L-selectin were increased by PRP treatment. However, the expressions of ITGB1, ITGB3 were not affected by PRP, and the expression of ICAM-1 was reduced by PRP treatment (Figure 7A). In addition, to evaluate the effect of PRP on the expressions of these adhesion molecules are involved with LIF expression, we confirmed the expressions of these molecules using the
The results showed that the expressions of ITGAV, ITGB5, CD44s, and L-selectin were increased by PRP treatment in pLKO.1 cells. However, in the shLIF cells, PRP treatment did not affect the expression of these adhesion molecules (Figure 7B). These results suggest that PRP-stimulated expression of adhesion molecules is directly mediated by LIF expression.

**DISCUSSION**

Traditional herbal medicines have been used for improving pregnancy rate in clinic and clinical efficacy of these herbal medicines were supported by several clinical studies. 8-10 Previous studies showed that some formulas including Diaojing Zhongyu Tang,11 Kaiyu Zhongyu Tang,12 Jiawei Diaojing San,13 Fuyi Dihuang Wan,14 Wentu Yulin Tang,15 and Yulin Zhu16,17 are effective for increasing pregnancy rate through regulating menstruation cycle and promoting ovulation. However, the studies focusing on embryo implantation are very few. Several previous studies showed that traditional Chinese medicinal herbal formulas used for preventing miscarriage have positive effect on the expression of LIF, a cytokine plays a key role in embryo implantation.12,13 LIF is a cytokine playing key roles in mammalian reproduction through regulating endometrial receptivity, decidualization, trophoblast differentiation, and placenta formation.18-21 In human uterus, the expression of LIF is relatively low in the proliferative phase of menstrual cycle, rise after ovulation, and reached peak level during mid and late secretory phases of the cycle.22 The LIF is secreted from glandular endometrium, interacts with its receptor, LIF receptor (LIFR), expressed on the luminal endothelium and subsequently enhances the expression of adhesion
Figure 6 Involvement of LIF on the PRP induced adhesion between JAR and Ishikawa cells.

Ishikawa cells transfected with pLKO.1 or shLIF were treated with PRP (150 μg/mL) for 48 h in 6-well plates. A: cellTracker fluorescence-labeled JAR cells were added onto Ishikawa cells for 30 min and attached cells were counted. Data were calculated by percentage of control and expressed as mean ± standard deviation of three independent experiments. *P < 0.001 and **P < 0.001, in comparison with CON transfected with pLKO.1. †P < 0.001 compared with PRP transfected with shLIF. B: the pictures were taken by fluorescent microscopy. B1: Ishikawa cells transfected with pLKO.1; B2: Ishikawa cells transfected with pLKO.1 treated with PRP; B3: Ishikawa cells transfected with shLIF; B4: Ishikawa cells transfected with shLIF treated with PRP. LIF: leukemia inhibitory factor; PRP: Paeonia Lactiflora Pallas, Rehmannia Glutinosa var. Purpurea Makino and Perilla Frutescens var. Acuta Kudo; pLKO.1: pLKO.1 mock vector; CON: control.

Figure 7 Effect of PRP on the LIF-dependent expression of adhesion molecules, such as ITGAV, ITGB5, CD44s, L-selectin

A: Ishikawa cells were treated with PRP (150 μg/mL) for 24 h. The expression of adhesion molecules, including ITGAV, ITGB1, ITGB3, ITGB5, CD44s, ICAM-1, and L-selectin, were estimated by RT-PCR. B: Ishikawa cells transfected with pLKO.1 or shLIF were treated with PRP (150 μg/mL) for 24 h. The expression of adhesion molecules, such as ITGAV, ITGB5, CD44s, and L-selectin, were determined by RT-PCR. The expression of β-actin was used as internal control. C: the schematic presentation of this study was shown. PRP increased the expression of adhesion molecules through inducing LIF expression. Increased adhesion molecules, such as ITGAV, ITGB5, CD44s, and L-selectin, which are known as mediator of adhesion between trophoblast and endometrium, may be involved in PRP-stimulated endometrial receptivity toward trophoblast. PRP: Paeonia Lactiflora Pallas, Rehmannia Glutinosa var. Purpurea Makino and Perilla Frutescens var. Acuta Kudo; LIF: Leukemia inhibitory factor; RT-PCR: reverse-transcription polymerase chain reaction; pLKO.1: pLKO.1 mock vector; shLIF: viral vector harboring shRNA against LIF; LIFR: LIF receptor.
molecules, such as integrin β3, intercellular adhesion molecule 1 (ICAM-1), podoplanin and junctional adhesion molecule 2. The critical role of LIF was evidenced by several clinical studies as well as genetic or antagonizing abolishment in mice model. Polysaccharides were contained in herbal decoction were broken down or excreted in digestive systems. In addition, polysaccharides from herbal medicines can also cause the abnormal cellular immune response and complex structures of polysaccharides can interfere with the role of adhesion molecules in the cell-cell interaction. The results showed that LIF expression was not increased by the whole formula of Antai Yin at non-toxic concentration. The results demonstrated that six herbal ingredients, such as PL, RG, PF, CR, CO, and GU, significantly increased the LIF expression. The results showed that only some ingredient herbs were effective in the increment of LIF expression, whereas the whole formula was not. The phenomenon may be due to the negative interaction, i.e., some ingredient herb interferes the effect of the other herbs. As suggested by Wagner and Ulrich-Merzenich, although the concept of synergy effect is prevalent in traditional medicine, proving the synergy effects in herbal medicine is very difficult. On the contrary, the result from Figures 1 and 2 demonstrated the possibility of negative interaction between the ingredients of Antai Yin. Therefore, we examined the optimal combination from six ingredient herbs having effect on LIF expression through checking the additional or synergic effect produced by combination of each herb. The data presented that the combination of PRP extracts is most potent to induce LIF expression. The additional combination of other 3 herbs, such as CO, CR, and GU, to PRP did not show significant increase in LIF expression. In addition, the combinational effect of PRP (about 10 fold) on induction of LIF expression is much more than the arithmetic sum of effects induced by each herb (about 6 fold). Moreover, the increase rates of LIF expression induced by RG and PL are not prominent than that by CR or CO. Thus, we postulated that the combined effect of PF, RG, and PL can be explained by synergy effect by positive interaction between three ingredient herbs.

Therefore, we suggest PRP as an optimal combination that increase the expression of LIF among the 10 ingredient herbs composing Antai Yin.

The expression and function of LIF plays crucial role in embryo implantation. However, implantation rate can be down-regulated by another factor, such as reduction of LIF receptor. Thus, to determine the effect of PRP on the trophoblast adhesion, adhesion assay was performed by using human trophoblastic JAr cells and human endothelial Ishikawa cells. These cells are commonly used to examine adhesion between the endometrial and trophoblast cells. The results clearly showed that the treatment of PF and PR, PRP combinations to Ishikawa cells gradually increased the attachment of JAr cells onto Ishikawa cells. In addition, PRP increased the expression of LIF in a dose dependent manner and PRP-induced attachment of JAr cells of Ishikawa cells are significantly reduced by shLIF. These results clearly indicate that the effect of PRP on the adhesion between trophoblast and endometrial cells are mediated by LIF expression.

To elucidate the exact molecular mechanism underlying PRP-stimulated endometrial receptivity, the understanding on LIF-dependency of the adhesion molecules expression is crucial. Endometrial receptivity toward trophoblast is mainly regulated by the expression of adhesion molecules including integrins. Among several integrins, integrin αv is a main component of receptor for Arg-Gly-Asp motif that mediates cell-cell interaction, especially in embryo implantation. In addition, we previously presented that the expression of ITGAV, ITGB, and ITGB5 is regulated by LIF. The other adhesion molecules, such as CD44s, ICAM-1, and L-selectins, also were reported as regulators of the interaction between trophoblast and endometrial cells. The results in this study clearly demonstrated that among these molecules, the expression of ITGAV, ITGB5, CD44s, and L-selectin are up-regulated by PRP treatment in LIF-dependent manners. These results suggest that LIF-dependent induction of endometrial receptivity by PRP treatment may be mediated by these adhesion molecules. To develop of a novel herbal formula having efficacy and safety on human embryo implantation, further extensive in vivo experiments and clinical researches are needed.

In conclusion, the combination of the PRP extracts is potent to induce LIF expression. In addition, it increased the endometrial receptivity toward trophoblast cells through the expression of LIF and adhesion molecules. Because there is no effective treatment for enhancing endometrial receptivity until now, to develop alternative treatments such as traditional herbal remedies are focused recently. Therefore, the combination of PRP extracts may be a promising therapy for enhancing endometrial receptivity.

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