Yeosin-san Increases Female Fertility through Inducing Uterine Receptivity and Ovarian Function

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Despite the development of assisted reproduction technologies (ART) including *in vitro* fertilization (IVF), the poor ovarian response and endometrial receptivity remains clinically a major unmet need. Although these problems are difficulties to solve in infertility treatment, there are no good therapeutic option yet. Traditional herbal remedies and acupuncture, therefore are being proposed as alternative treatment. Our group found that traditional herbal medicines such as *Paeonia lactiflora* L.(PL, 芍藥), *Cyperus rotundus* L.(CR, 香附子), and *Perilla frutescens* (PF, 紫蘇葉) could improve endometrial receptivity. In this study, we found out Yeosin-san (如神散) as an optimal herbal formula via combination of the previously established herbal medicines. Yeosin-san is a traditional Korean medical formula which was established by Ziming Jin (陳自明) and recorded in Furendaiquanliangfang (婦人大全良方) at first. The formula traditionally used for treating abnormal uterine bleeding and leukorrhea. It showed a highest effect on leukemia inhibitory factor (LIF) expression and on the adhesion between trophoblastic cells and endometrial cells. In addition, it has been shown that the Yeosin-san not only increases the endometrial receptivity to improve the embryo implantation but also enhances the ovary function by expressing the angiogenesis-related genes. Here we suggest that Yeosin-san could be a novel and effective candidate for treating female infertility.

keywords : Yeosin-san, Fertility, Embryo implantation, Cell adhesion, Leukemia inhibitory factor (LIF)

Introduction

About 10% of women experience infertility and half of these undergo assisted ART, such as intra-uterine insemination (IUI) and IVF[1)]. Although breakthrough of ART in recent decades has improved outcomes for struggling couples, poor ovarian response (POR) and repetitive implantation failure (RIF) are major unmet needs in female infertility[2,3)]. However, there are very limited knowledge on the molecular mechanisms regulating ovarian function and embryo implantation[4,5)]. In addition, there are very limited options for increasing pregnancy rates through improving ovarian function and endometrial receptivity[6)]. Thus, numerous studies are struggling to develop a novel therapeutic option for improving endometrial receptivity.

Traditional medicines, such as acupuncture, moxibustion, and herbal medicines, have been used for many centuries to treat female infertility in Korea, Japan, and China[7,8)]. Many studies focused on the remedies for ovarian failure and RIF[9,10)]. Although the excise mechanism underlying the pro-pregnant function of herbal medicines are not fully elucidated, LIF, a cytokine of interleukin (IL)-6 family, is focused as a key mediator enhancing female fertility rate by herbal medicines[11-13)]. LIF is a well-known regulator of reproductive microenvironment, especially in ovarian and uterus function[14,15)]. Thus, we previously reported that several herbal medicine and its ingredient compounds enhanced the expression of LIF[16-19)].

In this study, we found out Yeosin-san as an optimal herbal formula via combination of the previously established

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·Received : 2019/03/07 ·Revised : 2019/04/05 ·Accepted : 2019/04/22

pISSN 1738-7698 eISSN 2288-2529 http://dx.doi.org/10.15188/kjopp.2019.04.33.2.141
Available online at https://kmpath.jams.or.kr
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herbal medicines. Yeosin-san is composed of PL and CR, formerly used for treating abnormal uterine bleeding and leukorrhea[20]. It showed the enhancive effect on endometrial receptivity and ovarian function by means of in vitro and in vivo experiments. Therefore, here we suggest that Yeosin-san might be a novel candidate of approved herbal medicine preparation for treating female infertility.

Materials and Methods

1. Materials

Antibody against LIF and GAPDH was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), mifepristone (antagonist of the progesterone receptor: RU486), and human chorionic gonadotropin (hCG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gonadotropin from pregnant mare serum (PMSG) was LEE Biosolutions, Inc (Maryland, MO, USA).

2. Preparation of herbal medicines and formulae

Herbal formulae were made by mixing the herbal medicine. Briefly, herbal formula was boiled with distilled water. The extractions were concentrated and gave a powder using a spray-drying process. After the powders were dissolved in distilled water, it filtered and stocked for experiments.

3. High-performance liquid chromatography (HPLC) analysis of Yeosin-san

The phytochemical profiling of Yeosin-san was examined by HPLC analysis using paeoniflorin as a standard compound, according to previous protocols with some modifications[21]. The HPLC analysis was performed by using an Agilent 1200 series system (Agilent Technologies, CA, USA). Separation was performed on ZORBAX eclipse XDB C18 column (5μm particle size, 4.6 x 250 mm, i.d.) from Agilent Technologies. The mobile phase was composed of 0.1% TFA in Water (mobile phase A) and Acetonitrile (mobile phase B) = 85 : 15. The flow late was 1mL/min, operating temperature was maintained at 35℃ and the detection was performed at the wave length of 231nm. All the analytes were dissolved in HPLC grade water (Honeywell Burdick and Jackson™). The samples were filtered with a 0.45 μm PTFE filters before analysis. The column injection volume was 10 μL. The gradient flow was as follows : (A)/(B) = 85-85/15-15 (0-3 min) → (A)/(B) = 85-60/15-40 (3-33 min) → (A)/(B) = 60-60/40-40 (33-36 min) → (A)/(B) = 60-0/40-100 (36-39 min) → (A)/(B) = 0-0/100-100 (39-44 min) → (A)/(B) = 0-85/100-15 (44-49 min) → (A)/(B) = 85-85/15-15 (49-52 min).

4. Cell culture

The human endometrial Ishikawa cells were provided by Dr. Jacques Simard (CHUL Research center, Québec, Canada). The human trophoblast JAr cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Respectively, the cells were maintained at 37℃ in a 5% CO_2/ 95% air atmosphere in Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) (Welgene, Daegu, Korea) containing 10% heat-inactivated fetal bovine serum (FBS Thermo Fisher Scientific, MA, USA)/1% penicillin streptomycin (Pen Strep; Thermo Fisher Scientific).

5. Cell viability assay

The cytotoxicity of herbal formulae was examined using MTT assay. Ishikawa cells were cultured in 24-well plates with the indicated concentrations of herbal formulae for 24h. After washing, MTT (0.5mg/mL) was added and incubated for 4h at 37℃ in a CO_2 incubator. Then, Formazan crystals formed in viable cells were dissolved in dimethyl sulfoxide/ethanol (v/v, 1:1) solution. The absorbance was measured at 540 nm with microplate reader (SpectraMax M2; Molecular Devices, CA, USA). The percentage of living cells was calculated against untreated cells.

6. Adhesion assay

Ishikawa cells were cultured in 6-well plates with herbal formulae treatment for 48 h. The JAr cells were incubated with 5-chloromethylfluorescein diacetate (CMFDA) fluorescence dye (CellTracker; Life Technologies, Carlsbad, CA, USA) for 15 min at 37℃. The labeled JAr cells were washed in three times and added onto herbal formulae-treated Ishikawa cells. After gently shaking for 30 min, the cells were washed to remove non-binding JAr cells, the attached JAr cells were visualized using a fluorescence microscope (Axio Imager M1, Zeiss, Oberkochen, Germany) and calculated.

7. Western blot analysis

Total protein (20 μg) from herbal formulae-treated Ishikawa cells were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose membranes. The membranes were blocked in 5% blocking solution and then incubated with anti-LIF and GAPDH antibodies. After the reaction with horseradish peroxidase-linked secondary

antibodies, the signals were visualized using ECL chemiluminescence system (GE Healthcare, Little Chalfont, UK).

8. Animals

Male and female C57BL/6 mice, inbred in a specific pathogen-free facility (SPF), (7-8 weeks old, weight 20-22 g) were obtained from Orient Bio, Co. (Seongnam, Korea). They were bred separately and had free access to water and a standard diet with a 12 h light/12 h dark cycle. All experimental procedures were approved by the Animal Research Ethics Committee at the Pusan University of Korea (no. PNU-2018-1943).

9. Establishment of implantation failure model

Forty female mice were randomly divided into five groups: control (Con), RU, 0.25X, 0.5X, and 1X Yeosin-san (PL+CR). Each mouse was orally administered 0.25X, 0.5X, and 1X Yeosin-san (7.98 mg/kg/day, 15.96 mg/kg/day, 31.92 mg/kg/day) in 100 μL of normal saline solution by zonde for 15 days. After superovulation by intraperitoneal injection with 100 μL of 5 IU PMSG followed by injection of 5 IU of hCG, all female mice were mated with males (ratio 1:1). On day 4 from pregnancy estimated by vaginal plugs, the female mice were injected with RU486 (4 mg/kg/day) for 4 days. After separating from male, all mice were sacrificed and both uterine horns were excised to determine the number of implantation sites. The number of implanted embryos on each uterine horn was recorded.

10. Establishment of natural aging mouse model

Female C57BL/6 mice of 12 months (weight 30-35 g) were purchased from the Koatech Inc. (Gyunggi-do, Korea). They were bred under a 12 h light/dark cycle with free access to water and food in an animal facility of SPF class with a temperature of 21 ± 2ºC and the relative humidity of 55 ± 10%.

11. Histological hematoxylin and eosin (H&E) staining and ovary follicle counting

After 2-weeks of Yeosin-san treatment, both ovaries were isolated and fixed in 4% paraformaldehyde at 4℃ overnight, and dehydrated using ethanol series, cleared in xylene, embedded in paraffin, and sectioned for H&E staining. After mounting, sections were analyzed histologically under a light microscope. Follicles were counted in all sections from each ovary, and results were corrected for double counting. Follicles were classified into primordial follicle (an oocyte surrounded by one layer of flattened granulosa cells), primary follicle (an oocyte surrounded by one layer of cuboidal granulosa cells), secondary follicle (two or three layers of cuboidal granulosa cells with no antral space), and antral follicle (more than four layers of granulosa cells with one or more independent antral spaces, or with a cumulus granulosa cell layer). Follicles containing degenerated oocytes were deemed atresia based on the presence of apoptotic bodies in the granulosa cell layer, disorganized granulosa cells, a degenerating oocyte, or fragmentation of the oocyte nucleus.

12. Superovulation, zygotes collection, and embryo culture

After another female mice were administered with Yeosin-san in the same way, they were superovulated by intraperitoneal injection with 0.1 μL of 5 IU PMSG (LEE Biosolutions, Inc) followed by injection of 5 IU of hCG (Sigma-Aldrich) approximately 48 h later. Then the mice were immediately paired with an 8~12-week-old individual male. The following morning the mice were inspected, and those with a confirmed vaginal plug were considered fertilized. Eighteen hours after hCG injection, female mice with a confirmed vaginal plug were killed by cervical dislocation, and cumulus-enclosed one-cell embryos (zygotes) were retrieved from the oviductal ampulae and denuded by incubation for 1 min with 0.1% hyaluronidase (Sigma-Aldrich) in PBS (Giboc BRL, Grand Island, NY, USA). Zygotes were pooled and washed three times in G-IVF-plus medium (Vitrolife, V. Frolunda, Sweden) with 10% serum substitute supplement (SSS; Irvine, Inc. Santana, USA). Healthy zygotes only were cultured in 20 μL drops of Gl-plus medium (Vitrolife) with 10% SSS for the first 2 days, and then G2-plus medium (Vitrolife) with 10% SSS for the latter 2 days under paraffin-oil at 37℃ in a 5% CO_2 incubator, and the media were changed daily.

13. Quantitative real-time PCR

Just after the retrieval of the zygotes, both ovaries of each mouse were collected and mRNA expressions of genes associated with angiogenesis, including vascular endothelial growth factor (VEGF), visfatin, and fibroblast growth factor (FGF)-2, were measured by quantitative real-time PCR. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Complementary DNA was synthesized from 1μg of total RNA using AMV Reverse Transcriptase (Promega, Madison, WI, USA) and a random hexamer (Takara Bio, Inc., Otsu, Japan) at 42℃ for 1 h. Real-time PCR was performed using TOPreal™ qPCR 2X PreMIX SYBR

(Enzynomics, Daejeon, Korea). Reaction mixtures were prepared using TOPreal™ qPCR 2X PreMIX, 0.5 pmol/μl of each primer, 100 ng of cDNA, and sterile water (RNase free). The reaction conditions consisted of denaturation at 95℃ for 10 min, followed by 30 cycles of 95℃ for 10 sec, 60℃ for 30 sec. Each cDNA was subjected to polymerase chain reaction (PCR) amplification using gene-specific primers(Table 1).

Table 1. Primers sequences used for real-time PCR amplification

Gene	Sequence (5'→3')	
	Forward	Reverse
VEGF	AGGCTGCTGTAACGATGAAG	GTCTGCATTCACATCTGCTG
Visfatin	CTTGTTCAGTCCTGGTATCC	GCGAAGAGACTCCTCTGTAA
FGF2	GAGTTGTGTCTATCAAGGGAGTG	CAGCTCTTAGCAGACATTGG
GAPDH	TCAACGGCACAGTCAAGGC	CTCCACGACATACTCAGCAC

14. Estradiol measurement using ELISA assay

Immediately after sacrifice of mice, blood was collected by cardiac puncture with a syringe. Serum was separated by centrifugation at 1,000g for 15 min and stored at -80℃ until use for assays. Serum levels of estradiol (E2) were measured using specific ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer's instructions. Intra- and inter-assay coefficient of variation (CV) was less than 15 % for E2. The absorbance was read at 450 nm within 10 min, against a blanking well in an ELISA Reader (BioTek, winooski, USA). All samples were run in duplicate. A standard curve was created by professional computer software "Curve Expert" capable of generating four parameters logistic curve-fit.

15. Statistical analysis

Statistical analysis of results was performed by one-way analysis of variance with Tukey's post-hoc test or a Student's t-test using GraphPad Prism (GraphPad Software, CA, USA) and a SPSS program (ver. 12.0). Values are expressed as the mean ± SD. The minimum significance level was set at a P value of 0.05. The number of zygotes retrieved and blastocyst formation rate were analyzed by one-way analysis of variance. Comparison of mRNA expression level of angiogenic factors and numbers of follicles at each developmental stage was analyzed by Student t-test.

Results

1. Effect of various herbal medicine combinations on cell viabilities.

Previously, we reported that several medicinal herbs including PL, CR, and PF have effect of increasing embryo implantation rate via inducing LIF-mediated endometrial receptivity[17-19]. In this study, we experimented for founding out a novel formula composed by these herbal medicines to develop an effective herbal medicinal preparation. Firstly, we evaluated the phytochemical properties of Yeosin-san by HPLC analysis using Paeoniflorin as a standard compound (Fig. 1). Next, the cytotoxic effects of herbal medicine combinations were determined. Four different combination of herbal medicines had low cytotoxicity on human endometrial Ishikawa cells (Fig. 2). The concentration of inhibitory 50 percent (IC_{50}) of these combinations were over than 1000 μg/ml. In addition, at the concentrations of 250 μg/ml, these four combinations have no significant cytotoxic effect on Ishikawa cells. Thus, we used 250 μg/ml of these combinations for further experiments.

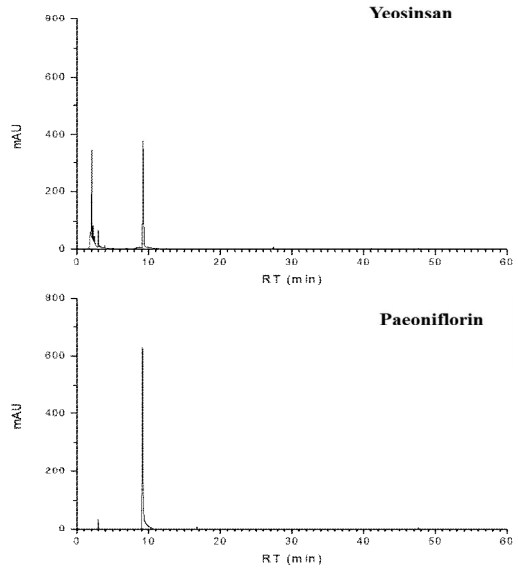

Fig. 1. The extraction of herbal formulae and HPLC analysis of Yeosin-san. Phytochemical profiling of Yeosin-san was performed by HPLC analysis using paeoniflorin as a standard compound.

2. Effect of various herbal medicine combinations on trophoblast adhesion and LIF expression.

The adhesion of trophoblastic JAr cells to endometrial Ishikawa cells were determined by adhesion assay after fluorescent staining of JAr cells. The results showed that combination of PL and CR had highest effect on the adhesion between JAr cell and Ishikawa cells (Fig. 3A and B). Interestingly, the combination of three herbal medicines including PL, CR, and PF had relatively lower effect on trophoblast adhesion compared with combination of PL and CR. In addition, the combination of PL and CR showed highest expression of LIF, among the four combinations

(Fig. 3C). From the results from in vitro experiments, Yeosin-san, a PL and CR combination 20, might be a good drug candidate for increasing endometrial receptivity.

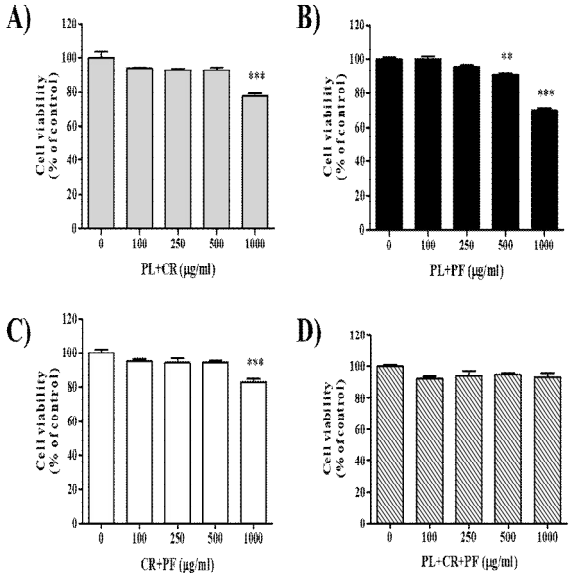

Fig. 2. Cytotoxicity of herbal formulae on the endometrial Ishikawa cells. (A-D) The herbal formulae were treated with indicated concentration of the herbal formulae for 24 h. The viabilities of the cells were measured by MTT assay. The absorbance was calculated to percentage of control group and presented as mean ± SD. **p<0.01 and ***p<0.001 compared to control group.

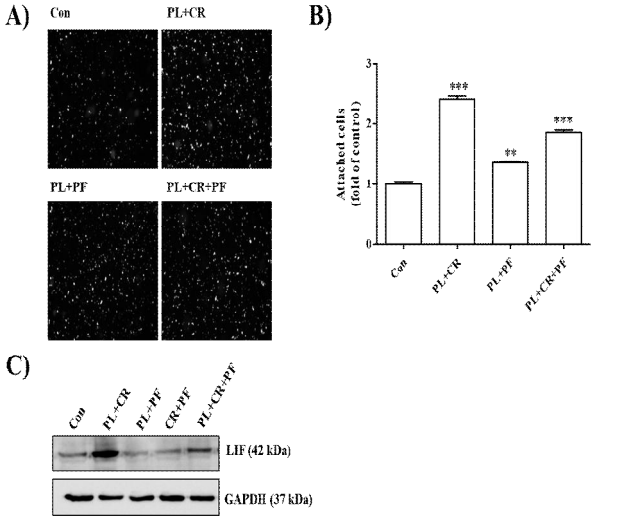

Fig. 3. The effect of herbal formulae on the adhesion between Ishikawa cells and JAr cells and on the expression of LIF. (A) The Ishikawa cells were treated with indicated 250 μg/ml of herbal formulae for 48 h. The fluorescent-labeled JAr cells were incubated onto the Ishikawa cells for 30 min with gentle shaking and unattached cells were wash out. The represented pictures of attached JAr cells were captured by fluorescent microscopy (Magnification x50). (B) The attached JAr cells were counted, calculated to fold of control, and presented as mean ± SD. **p<0.01 and ***p<0.001 compared to control group. (C) The Ishikawa cells were treated with indicated 250 μg/ml of herbal formulae for 24 h. The expression of LIF was estimated by Western blot analysis. The expression of GAPDH was used for loading control.

3. *In vivo* effect of Yeosin-san on embryo implantation.

To examine in vivo efficacy of Yeosin-san had on endometrial receptivity, we adopted a previously established implantation failure mouse model by RU486 treatment 19. The result presented that depleted embryo implantation by RU486 treatment was significantly reversed by administration of Yeosin-san from dose of 0.25X (Fig. 4). The dose of 0.5X showed highest effect on endometrial receptivity. From these results, Yeosin-san had an effect increasing embryo implantation in both model of in vitro and *in vivo*.

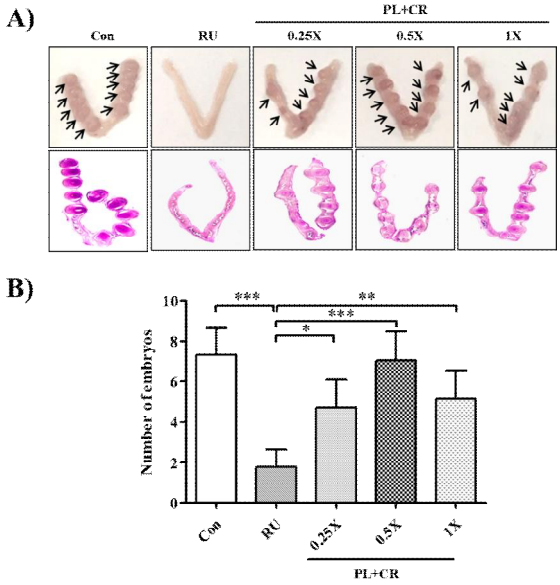

Fig. 4. The effect of Yeosin-san on the in vivo embryo implantation. (A) The female C57BL/6 mice were orally administrated with indicated concentration (1X = 31.92 mg/kg/day) of Yeosin-san (PL+CR) for 17 days. After 7 days from starting Yeosin-san treatment, the mice were mate with male. On day 4 from pregnancy estimated by vaginal plug, the female mice were subcutaneously injected with RU486 (4 mg/kg/day) for 4 days. After 7 days from RU486 injection, all mice were sacrificed and the uteri were pick out. The representative pictures of uteri implanted with embryo were presented. The tissue section of uteri was stained with H&E and the slides were scan. (B) The numbers of embryo implanted sites were counted and demonstrated as mean ± SD. *p<0.05, **p<0.01, and ***p<0.001 comparing each other.

4. Yeosin-san improved ovarian function in natural ovarian aging mice

In the first experiment, after Yeosin-san was administered to mice aged 12 months for 2 weeks, H&E were carried out ovarian tissues to assess the effect of Yeosin-san treatment on follicle development, follicle loss and numbers of follicles at each stage (primodial, primary, secondary, antral, atretic follicles, and corpora lutea). The histological characteristics of follicles at each stage after 2 weeks of Yeosin-san treatment were shown in Fig. 5A. The H&E stained ovarian tissues showed that only 1X of

Yeosin-san increased the total number of follicles (109±0.3) compared with the control group (72±0.2) with an increase rate of 51.4% at 1X Yeosin-san. Of these, numbers of surviving follicles (including primordial, primary, secondary and antral follicles) in the Yeosin-san treatment group were 93±0.3 at 1X Yeosin-san dose, and 72±0.2 at 0.5X Yeosin-san dose, which was more than that of the control group (63±0.3). Surviving follicle numbers were significantly increased by 47.6% at 1X Yeosin-san, and 14.3% at 0.5X Yeosin-san, respectively (Fig. 5B). Numbers of atretic follicles in the 1X Yeosin-san treatment group were 16±0.5, which was increased than that of the control group (9±1.0). The mean number and percentage of follicles at each stage were counted and calculated. The number of primordial follicles in the Yeosin-san treatment group was 27 ± 0.5 at 1X Yeosin-san and 32 ±1.2 at 0.5X Yeosin-san, which was more than that of the control group (24±0.6). The number of primary follicles in the Yeosin-san treatment group was 28 ± 1.0 at 1X Yeosin-san and 19 ±0.8 at 0.5X Yeosin-san, which was more than that of the control group (17±1.0). Numbers of secondary and antral follicles were significantly increased just at 1X Yeosin-san (23 ± 1.2 and 15±0.5, respectively) compared to the control group (13±0.8 and 9±1.4, respectively). Especially, the number of antral follicles increased to almost double in the 1X Yeosin-san treatment. The number of corpora lutea had no significant difference was similar in control group and the 1X Yeosin-san treatment groups (Fig. 5C). The mean body, ovary and uterus weights are shown in Fig. 6. There were no significant differences in the body, ovary, or uterus weight between the control group and the Yeosin-san-treated groups.

In order to examine whether Yeosin-san improves ovarian response and oocyte quality, the mice were treated with Yeosin-san for 2-weeks, superovulated followed mated with males, and then the zygotes were retrieved and cultured for 4 days. The mean number of zygotes retrieved was 110, 87, and 93 at 0.25X, 0.5X, and 1X Yeosin-san, respectively, which were significantly increased by about twice compared with 47 of control group ($P < 0.05$). The embryo development rate to blastocyst was 3.8% at 0.25X Yeosin-san, 5.7% at 0.5X Yeosin-san, and 17.2% at 1X Yeosin-san which were significantly higher than 0% of control group (Table 2). However, no significant increases in serum estradiol levels were observed in all Yeosin-san-treated group and control group (Fig. 7).

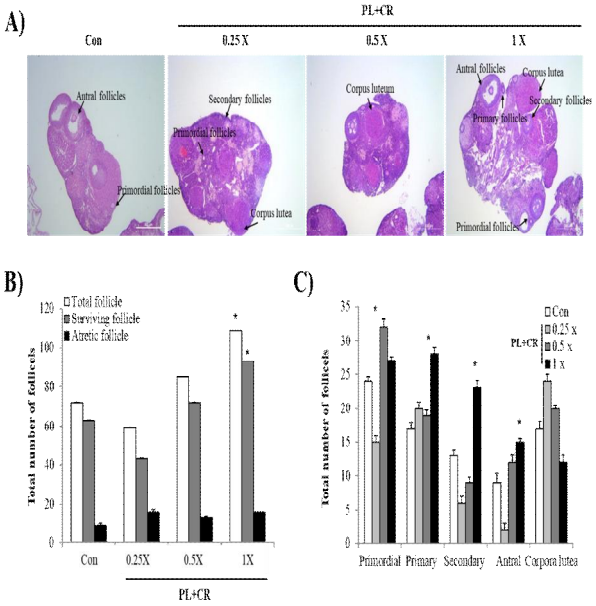

Fig. 5. Effect of Yeosin-san on follicular development. (A) Representative hematoxylin and eosin stained histological images of mouse ovary after Yeosin-san treatment. (B) Comparison of the total number of follicles, surviving follicles, and atretic follicles. (C) The distribution of follicles at different stages. *P < 0.05 (versus control group).

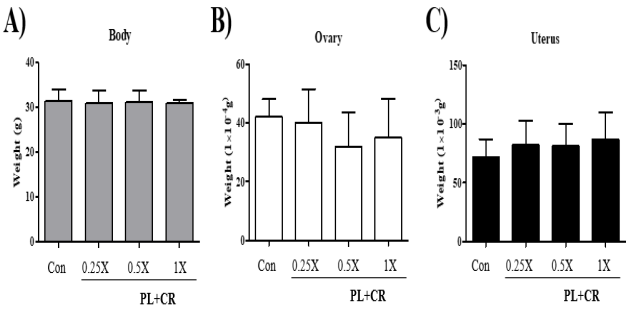

Fig. 6. Effect of Yeosin-san on the weight of the body, uterus, and ovaries. Values are presented as mean ± SD of at least 3 independent experiments.

Table 2. Effect of Yeosin-san on number of zygotes retrieved and embryo development

Yeosin-san concentration	No. of mice provided	No. of zygotes retrieved	Mean No. of Zygotes flushed/mouse	No. of zygotes fragmented (%)	No. of zygotes cultured	No. of 2-cell embryos (%)	No. of blastocysts (%)*
0	6	47	7.8±3.6	17 (36.1)	30	8 (26.7)	0 (0.0%)
0.25X	8	110	13.7±5.2[a]	30 (27.7)	80	13 (16.3)	3 (3.8%)
0.5X	6	87	14.5±3.8[a]	30 (34.5)	57	11 (19.3)	3 (5.7%)
1.0X	7	93	13.3±3.2[a]	35 (37.6)	58	13 (22.4)	10 (17.2)[a]

The number of Zygotes per mouse were presented as mean ± SD. *%= No. of blastocysts/ No. of zygotes cultured. [a]P < 0.05 (vs controls).

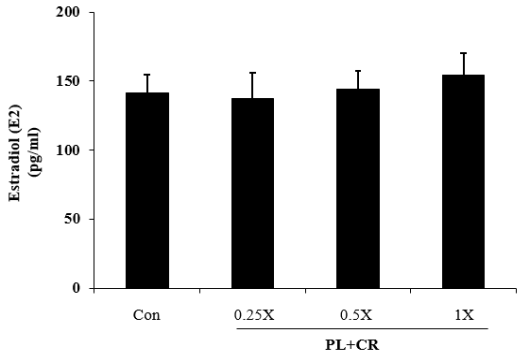

Fig. 7. Measurement of serum hormone levels. Values presented as mean ± SD of at least 3 independent experiments.

5. Yeosin-san increased ovarian expression of genes associated with angiogenesis

To investigate the effect of Yeosin-san on the expression of angiogenesis-related genes in the ovarian tissues, we examined mRNA expression of VEGF, visfatin, FGF-2 in ovaries collected just after the retrieval of the zygotes. Expressions of these two genes (visfatin and FGF-2) were increased after Yeosin-san treatment compared to the control group ($P < 0.05$). In particular, expressions of FGF-2 were significantly increased at all dose of Yeosin-san than control group ($P < 0.05$). Expressions of visfatin were also higher at 0.25X and 1X of Yeosin-san than control group ($P < 0.05$), but there was no significant difference at 0.5X of Yeosin-san compared to the control group (Fig. 8).

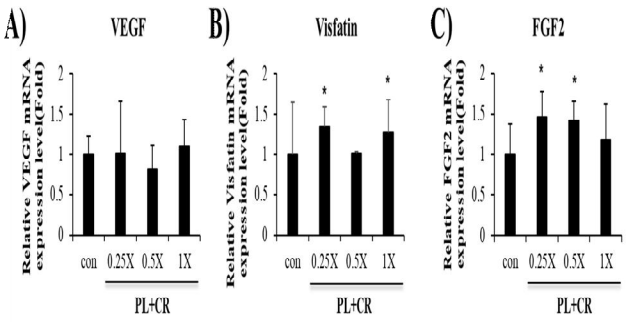

Fig. 8. Expression of angiogenesis-related genes (VEGF, visfatin, FGF-2) determined by quantitative real-time PCR. Whole ovaries were collected just after the retrieval of the zygotes. PCR was performed in duplicate on each sample. Relative gene expression levels were calculated versus GAPDH. Data are presented as mean ± SD. *P<0.05 (vs control).

Discussion

Infertility is a disease defined as the failure to establish a clinical pregnancy after 12 month of regular and unprotected sexual intercourse[22]. Although various factors are regarded as cause of infertility[22,23], ART could have been a solution for most of patients suffering from infertility[1]. However, the problems of poor responding ovary and insufficient uterine receptivity are remained as unsolved clinical needs[2-4]. To overcome these problems, our group have been screened potent candidates of improving endometrial receptivity and ovarian function from Korean medicinal herbs[16-19]. In this study, we found out the optimal herbal formula via combination of previously established herbal medicines, such as PL, CR, and PF. The formula composed by PL and CR, formerly named as Yeosin-san[20], showed a highest effect on the adhesion between trophoblastic cells and endometrial cells (Fig. 3). In addition, we confirmed the effect of Yeosin-san on endometrial receptivity and ovarian function by means of in vivo experiments(Fig. 4, 5 and Table 2).

Yeosin-san is a traditional Korean medical formula which was established by Ziming Jin and recorded in Furendaiquanliangfang at first[24]. The formula traditionally used for treating abnormal uterine bleeding and leukorrhea[20,24]. The ingredient herb, PL has been used for treating diverse female disease, such as amenorrhea, dysmenorrhea, cramp in pelvic abdomen, uterine bleeding, and female infertility, via tonyfing and activating blood (補血活血)[25]. Another component, CR also has been used for treating pain in trunk, stomachache, leukorrhea, and uterine bleeding through smoothing the liver and regulating qi (疏肝理氣)[25]. Previously, these two medicinal herbs was confirmed as candidates for increasing female fertility rates [17,19]. Thus, we supposed that Yeosin-san might be a potent herbal formula for treating female infertility which was induced by depression of liver qi and blood deficiency (肝鬱血虛).

LIF is well-known as a key regulator in mammalian implantation process[15]. Genetic depletion, pharmacological downregulation, neutralizing by monoclonal antibody, and antagonizing its receptor abolished the embryo implantation in various animal models[26-32]. In women patients suffering from infertility, the mutation of LIF gene was related with decreased activity of LIF in the uterus and cause implantation failure[33]. In addition, LIF expression is detected in human ovarian stroma, granulosa cells, and in follicular fluid where its levels correlated with estrogen production and embryo quality[34]. In mice, LIF enhanced the growth of cultured preantral follicles, but not their maturation, via acting as a proliferative factor for granulosa and theca cells[35]. In this study, the LIF expression was highest in the endometrial cells which were treated with Yeosin-san compared to other combined herbal formulae (Fig. 3C).

Angiogenesis is an important process for proper functioning of the female reproduction system including ovary and uterus[36]. Especially, angiogenesis is critical for the function of follicles, including steroidogenesis and maturation of the oocyte[37]. It is locally regulated by multiple angiogenic factors including VEGF and visfatin[38,39]. FGFs is also the representative growth factor that can contribute to vascularization. FGFs stimulate vessel cell proliferation and differentiation and are regulators of endothelial cell migration, proliferation, and survival[40]. Previously, our group reported that administration of the angiogenic factors could improve ovarian function and oocyte quality[41]. Considering the sexual maturity (around 6 weeks) and lifespan (1~2 years) in the laboratory for mice, 12 months of mouse corresponds to late 50 years of humans. The mouse ovary of this age is extremely aged as before and after menopause of human, and their ovarian function and fertility are abruptly decreased[42]. In this study, we confirmed that these factors were related with effect of Yeosin-san on ovarian function in natural aging mouse model(Fig. 8). However, Yeosin-san does not affect to estrogen production and weight of body, uterus, and ovaries (Fig. 6 and 7).

In endometrial receptivity experiment, 0.5X dose of corresponding to human uptake showed a highest effect on embryo implantation (Fig. 4). The dose of 0.25X and 1X also have significant effects. However, in the experiments of follicular development and embryo development, 1X dose of Yeosin-san demonstrated most potent effects (Fig. 5 and Table 2). From these results collectively, we assume that 1X dose of Yeosin-san is an optimal amount for designing further clinical study. In addition, Yeosin-san does not affect to weight of total body and reproductive organs (Fig. 6) and to serum estradiol levels (Fig. 7). These results indicate that dosage of Yeosin-san used in this study is generally acceptable for pharmacological use. To examine the safety of Yeosin-san, further extensive single dose or multiple dose toxicity test by good laboratory practice level should be performed.

Conclusion

In this study, we found out Yeosin-san as an effective herbal formula which was composed of PL and CR. Yeosin-san showed a highest effect on LIF expression and on the adhesion between trophoblastic cells and endometrial cells. In addition, we confirmed the effect of Yeosin-san on endometrial receptivity and ovarian function by in vivo experiments. The optimal dose of Yeosin-san is 1X deduced from traditional human administration and experimental evidences. Here we suggest that Yeosin-san could be a novel and effective candidate for treating female infertility.

Acknowledgement

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI17C0935).

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