Placenta 36 (2015) 559–566

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

# Chinese herbal medicine for miscarriage affects decidual micro-environment and fetal growth

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ARTICLE INFO

*Article history:*
Accepted 11 February 2015

*Keywords:*
Chinese herbal medicine
Decidual cells
IUGR
Macrophage
Spontaneous abortion

ABSTRACT

*Introduction:* Intrauterine growth restriction complicates 5–10% of pregnancies. This study aims to test the hypothesis that Chinese herbal formula, JLFC01, affects pregnancy and fetal development by modulating the pro-inflammatory decidual micro-environment.
*Methods:* Human decidua from gestational age-matched elective terminations or incomplete/missed abortion was immunostained using anti-CD68 + anti-CD86 or anti-CD163 antibodies. qRT-PCR and Luminex assay measured the effects of JLFC01 on IL-1β- or TNF-α-induced cytokine expression in first trimester decidual cells and on an established spontaneous abortion/intrauterine growth restriction (SA/IUGR)-prone mouse placentae. The effect of JLFC01 on human endometrial endothelial cell angiogenesis was evaluated by average area, length and numbers of branching points of tube formation. Food intake, litter size, fetal weight, placental weight and resorption rate were recorded in SA/IUGR-prone mouse treated with JLFC01. qRT-PCR, Western blot and immunohistochemistry assessed the expression of mouse placental IGF-I and IGF-IR.
*Results:* In spontaneous abortion, numbers of decidual macrophages expressing CD86 and CD163 are increased and decreased, respectively. JLFC01 reduces IL-1β- or TNF-α-induced GM-CSF, M-CSF, C–C motif ligand 2 (CCL2), interferon-γ-inducible protein-10 (IP-10), CCL5 and IL-8 production in first trimester decidual cells. JLFC01 suppresses the activity of IL-1β- or TNF-α-treated first trimester decidual cells in enhancing macrophage-inhibited angiogenesis. In SA/IUGR-prone mice, JLFC01 increases maternal food intake, litter size, fetal and placental weight, and reduces fetal resorption rate. JLFC01 induces IGF-I and IGF-IR expression and inhibits M-CSF, CCL2, CCL5, CCL11, CCL3 and G-CSF expression in the placentae.
*Discussion:* JLFC01 improves gestation by inhibiting decidual inflammation, enhancing angiogenesis and promoting fetal growth.


## 1. Introduction

Spontaneous abortion (SA) complicates 15% of human pregnancies. Although chromosomal anomalies account for more than 50% of SA, abnormal fetal development, maternal systemic disorders and environmental insults contribute to its incidence [1]. The mechanisms causing SA remain unknown thereby precluding the development of effective prevention and treatment. Various attempts including immunological approaches [2–6] have not

*Abbreviations:* CCL, C–C motif ligand; CHM, Chinese herbal medicine; CM, conditioned media; EVTs, extravillous trophoblasts; FTDCs, first trimester decidual cells; GA, gestational age; GD, gestational day; HEEC, human endometrial endothelial cell; IP-10, interferon-γ-inducible protein-10; IRB, institutional review board; IUGR, intrauterine growth restriction; NK, natural killer; OSU, The Ohio State University; SA, spontaneous abortion.

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http://dx.doi.org/10.1016/j.placenta.2015.02.006

significantly improved the SA-associated live birth rate. Chinese herbal medicine (CHM) has been widely used in Asia for centuries. Although studies suggest that CMH prevents SA [7], evidence is insufficient to assess the effectiveness of CHM in treating SA. First appearing in Chung-Ching Chang's "Synopsis of Golden Chamber" about 1800 years ago, JLFC01 is derived from a traditional Chinese formula that successfully treats blood stagnation.

Intrauterine growth restriction (IUGR) complicates 5–10% of pregnancies [8]. Many studies indicate that preeclampsia and IUGR [9] are initiated in the first trimester as a consequence of insufficient uteroplacental blood flow to the developing fetal-placental unit and suggest a similar origin for SA. Blastocyst-derived semi-allogeneic extravillous trophoblasts (EVTs) traverse the decidua and inner third of the myometrium and interact with resident decidual cells, decidual natural killer (NK) cells and macrophages. Invading EVTs transform uterine spiral arteries into high-capacitance vessels accompanied by expression of angiogenic factors and microvascular angiogenesis [10]. The resulting increased uterine blood flow to the intervillous space is pivotal for fetal-placental development [9,11]. Disturbances of this environment disrupt early fetal development and elicit long-term complications in affected children.

An aberrant pro-inflammatory decidual micro-environment elicits preeclampsia, SA and IUGR. In addition to mediating spiral artery transformation, decidual macrophages are critical modulators of the immune balance at the fetal–maternal interface. The function, differentiated state and responsiveness of macrophages are governed by their micro-environment and proximity to adjacent cells [12,13]. Generally, macrophage polarization is divided into classically (M1) and alternatively (M2) activated groups [14]. Besides bridging both innate and adaptive immunity, which defend against pathogens, decidual macrophages are important mediators of implantation, placental development and cervical ripening [15]. In early pregnancy, M2 macrophages play a key role in inducing immunotolerance of the fetal semi-allograft [15]. Tight regulation of macrophage trafficking/function plays crucial roles during placentation. Under physiological steady state, macrophages isolated from first trimester decidua are polarized toward an immunotolerant M2 phenotype [16]. By contrast, pro-inflammatory M1 polarization of macrophages is linked to adverse pregnancy outcomes [17]. Our studies indicate that potent pro-inflammatory cytokines, IL-1β and TNF-α, stimulate human first trimester decidual cells (FTDCs) to secrete several chemokines that recruit NK cells [18] and monocytes [19–21]. The decidual cells then promote macrophage differentiation toward an M1 subtype via the regulation of colony-stimulating factors [13].

The current study postulates that these decidual M1 macrophages are integral to the onset of SA and proposes to treat and/or prevent SA by counteracting the resulting pro-inflammatory decidual milieu. Initial observations determined that unlike the dominant M2 immunotolerant macrophage population of normal first trimester human decidua, the decidual macrophage population accompanying SAs displays a pro-inflammatory M1 phenotype. Complementing these *in situ* observations, the modulating effects of JLFC01 on: 1) IL-1β- or TNF-α-induced expression of several cytokines by FTDCs; 2) FTDCs in modulating human endometrial endothelial cell (HEEC) angiogenesis-inhibiting activity of macrophages were examined. These *in vitro* observations were extended to include CBA/J x DBA/2J mice, an established SA/IUGR-prone model, in which the effects of JLFC01 ingestion were assessed. An aberrant decidual pro-inflammatory micro-environment [22] can also interfere with normal fetal growth by disrupting fetal programming. The insulin-like growth factors (IGFs) and their receptors are potent regulators of protein turnover, mitogenesis and differentiation [23] and implicated in fetal-placental development [24]. Abnormal IGF expression, malfunctioning IGF receptors or defective downstream signaling pathways are proposed to contribute to the development of IUGR [25]. Thus, the effects of JLFC01 ingestion were compared on placental expression of mRNA and protein levels of IGFs and their receptors in SA/IUGR-prone mice.

## 2. Methods

### 2.1. Immunofluorescent staining of decidua for macrophage markers

Decidua was obtained under Institutional Review Board (IRB) approval at Mackay Memorial Hospital, Taipei, Taiwan. Gestational age (GA)-matched tissue was obtained from elective terminations of normal pregnancies between 6 and 12 weeks of gestation without uterine contraction, vaginal bleeding or evidence of fetal demise. Upon diagnosis of missed/incomplete abortion, decidual basalis was evacuated within 24 h from patients without infection or systemic diseases. Serial sections of OCT-embedded specimens were immunostained with mouse anti-human CD68 (1:25, Dako, Carpinteria, CA) followed by Rhodamine–conjugated donkey anti-mouse antibody (1:50, EMD Millipore, Billerica, MA). Sections were then incubated with rabbit anti-human CD163 (1:250, Sigma–Aldrich, St. Louise, MO) or CD86 (1:200, GeneTex, Irvine, CA) followed by corresponding FITC–conjugated secondary antibody (1:100) and 4′,6′-diamidino-2-phenylindole (1:500,000, Sigma–Aldrich). Morphometric analysis of cell numbers used Axiovision 3.1 software (Carl Zeiss, Oberkochen, Germany). Five randomly selected fields from each section (three sections/tissue) were examined. Cell numbers per field ($3 \times 10^6$ pixel$^2$) were counted and calculated as the mean of 15 fields for each tissue. A total of 15 cases per group were examined.

### 2.2. Cell isolation and culture

FTDCs were isolated and cultured as previously described [20]. Briefly, decidua from elective termination of 6–12 weeks gestation was obtained under IRB approval at The Ohio State University (OSU) and Beth Israel Medical Center, New York, NY. Cells were purified using Ficoll-Hipaque Plus (GE Healthcare, Piscataway, NJ). CD45 staining confirmed the absence of leukocytes. Cultured FTDCs were found to be vimentin-positive and cytokeratin-negative and displayed morphological changes and enhanced prolactin and plasminogen activator inhibitor-1 as well as inhibited interstitial collagenase and stromelysin-1 expression with prolonged tissue factor expression during incubation with a progestin. Confluent FTDCs were primed with estradiol ($10^{-8}$ M) + medroxyprogesterone acetate ($10^{-7}$ M) for 7d and pre-treated with or without 1.25 μg/ml of a Chinese herbal formula, JLFC01, for 24 h then incubated with 1 ng/ml of IL-1β or TNF-α (R&D Systems, Minneapolis, MN) with or without JLFC01. JLFC01 was manufactured from herbs and processed in stainless steel extractors at a low temperature (below 100 °C) in order to preserve the activity of essential ingredients and generate a water decoction. This procedure follows the sequence described in canonical Chinese medicine book since the sequence of the herbs processed within a decoction determines the efficacy of the formula. The extracted liquid is then spray dried to form a powder. This procedure follows good manufacturing practice (GMP) guidelines. In addition, both the presence and levels of heavy metal and microbes are also assessed. Conditioned medium (CM) supernatants were collected. Monocytes were isolated from peripheral blood of healthy reproductive age female donors using Ficoll-Hipaque and purified using anti-CD14-paramagnetic beads according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA).

For HEECs [26], blood vessels in the endometrium obtained from hysterectomy for myomas was dissected, then, minced and digested with type VII collagenase/dispase/DNase I followed by filtration through a 70-μm cell strainer. The cells was labeled with biotinylated UEA-1 (*Ulex europaeus*) lectin, and then, separated from non-labeled cells by panning on activated surface/AIS MicroCELLector flasks coated with streptavidin. Cells were cultured in EBM-2 medium supplemented with 15% fetal calf serum.

### 2.3. Cell proliferation assay

Cell proliferation was examined using a CellTiter 96 One Solution Cell Proliferation Assay (Promega, Madison, WI). Briefly, FTDCs were treated with vehicle, 0.08, 0.16, 0.31, 0.63, 1.25, 2.5, 5 or 10 μg/ml of JLFC01 for 48 h. Absorbance was detected at 490 nm after adding MTS reagent.

### 2.4. Bio-Plex assay

Bio-Plex assays (Bio-Rad, Hercules, CA) measured GM-CSF, M-CSF, C–C motif ligand 2 (CCL2), interferon-γ-inducible protein-10 (IP-10), CCL5 and IL-8 levels in CM from FTDC cultures as well as M-CSF, CCL11, CCL2, CCL3, CCL5 and G-CSF levels in mouse placenta lysates. Data acquisition and analyses were completed with the Bio-Plex 200 system using Bio-Plex Manager Software v6. Bicinchoninic acid protein assay (Thermo scientific, Rockford, IL) measured total cell protein levels.

### 2.5. Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted using total RNA purification plus kit (Norgen Bioteck, ON, Canada). Reverse transcription used SuperScript™III First-Strand Synthesis