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Original Research Article

Atractylenolide promotes trophoblast cell proliferation and migration in recurrent spontaneous abortion via ERK pathway

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Abstract

Purpose: *To investigate the effect of atractylenolide on recurrent spontaneous abortion (RSA).*
Methods: *The HTR-8/SVneo was established as an in vitro cell model of RSA. Cell viability and proliferation were determined using CCK8 and BrdU staining, while cell migration and invasion were determined by cell scratch and transwell assays.*
Results: *Atractylenolide significantly increased cell viability, and enhanced the number of BrdU-positive cells of HTR-8/SVneo ($p < 0.01$). Atractylenolide also significantly promoted cell migration and invasion ($p < 0.01$), and increased protein expression of MMP-9, MMP-2, and N-cadherin, but reduced E-cadherin. Atractylenolide also increased the phosphorylation of ERK ($p < 0.01$).*
Conclusion: *Atractylenolide enhances cell proliferation and migration of HTR-8/SVneo through activation of ERK signaling. Further studies using animal models are recommended to determine the protective role of atractylenolide against RSA, in vivo.*

Keywords: *Atractylenolide, Trophoblast cell, Proliferation, Migration, Invasion*

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INTRODUCTION

Recurrent spontaneous abortion (RSA) occurs in 2 - 5 % of women of childbearing age [1]. The main cause of RSA are immune regulatory protein defects, stressful life events, psychological trauma, smoking and alcohol consumption, infection, environmental factors, genetics, and hormonal abnormalities are considered to be the main causes of RSA [2]. Therefore, understanding the pathogenesis of RSA is essential for its treatment. The placenta functions as a maternal-fetal barrier and provides nutrients necessary for the developing fetus [3]. During pregnancy, trophoblasts from trophectoderm are implanted into the blastocyst, and the proliferation and migration of placenta trophoblasts are involved in embryo implantation and pregnancy [3]. For example, extravillous trophoblasts migrate from an attached embryo and enter the spiral arteries and uterine epithelial cells, establishing a maternal-fetal connection [4]. Deficiency in trophoblast cell proliferation and migration has been shown to induce pregnancy-

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related complications, including RSA [5]. Atractylodes macrocephala Koidz (Baizhu in China) is widely used as a traditional Chinese herb for treating Alzheimer's disease, osteoporosis, cancer, and gastrointestinal diseases [6]. Atractylodes macrocephala Koidz is also used in treatment of unexplained RSA [7]. Atractylenolide is a lactone compound of Atractylodes macrocephala Koidz, and is involved in glucose and lipid metabolism. Atractylenolide exerts anti-bacterial, anti-osteoporosis, anti-platelet, anti-inflammatory, and anti-cancer activities [8]. Atractylenolide reduced hydrogen peroxide-induced HTR-8/SVneo cell apoptosis, thus alleviating preeclampsia [9]. However, the role of Atractylenolide in RSA remains unclear. Thus, this study was aimed at investigating the effect of atractylenolide on recurrent spontaneous abortion through trophoblast cell proliferation and migration pathways.

EXPERIMENTAL

Cell viability and proliferation assays

The HTR-8/Svneo (1×10^3 cells/well) was seeded in a 96-well plate and treated with 20, 40 or 60 μM atractylenolide (Sigma-Aldrich, St. Louis, MO, USA) for 24 h. Cells were then incubated with a CCK8 test kit (Solarbio, Beijing, China) 24, 48 or 72 h later. Absorbance at 450 nm was determined using microplate auto-reader. To assess cell proliferation, HTR-8/Svneo post-atractylenolide incubation was treated with 10 μM BrdU (Abcam, Shanghai, China) for 24 h, digested with trypsin, fixed in 70 % ethanol, and then permeabilized. The cells were probed with BrdU-specific antibody (1:100, Abcam). The cells were also incubated with CY5 conjugated fluorescence secondary antibody for 30 min and then measured by FACS flow cytometry (BD Biosciences, Bedford, MA, USA).

Cell migration and invasion assays

The HTR-8/Svneo cells were seeded in a 24-well plate, and treated with different concentrations of atractylenolide for 24 h. A plastic pipette tip was used to scratch the cell monolayer. Wound width was measured using a microscope (Olympus, Tokyo, Japan) 24 h later. To determine cell invasion, HTR-8/Svneo post-atractylenolide incubation was seeded into upper wells of Boyden chamber with matrigel-coated membrane (BD Biosciences). Medium containing 15 % serum was added to the lower chamber wells. After 25 h, cells in the lower wells were measured under a microscope (Olympus, Tokyo, Japan).

Western blot assay

Protein samples of HTR-8/Svneo were obtained after incubation in radioimmunoprecipitation assay (RIPA) buffer, separated using sodium doceyl-sulfate polyacrylamide gel electrophoresis SDS-PAGE, electro-transferred onto polyvinyl-idene diflouride (PVDF) membranes and then blocked with 5 % skim milk. Membranes were probed with primary antibodies: anti-MMP-9 and anti-GAPDH (1:2000), anti-MMP-2 (1:3000), anti-E-cadherin and anti-N-cadherin (1:4000), anti-p-ERK and anti-ERK (1:5000). Membranes were then incubated with a secondary antibody (1:4000), and subjected to enhanced chemiluminescence (Sigma-Aldrich).

Statistical analysis

Data analysis was performed using GraphPad prism software (10.0). Data were expressed as mean ± standard error of mean (SEM), analyzed with student's t test and $p < 0.05$ was considered statistically significant.

RESULTS

Atractylenolide stimulated proliferation of HTR-8/Svneo cells

HTR-8/Svneo cells were induced with 20, 40 or 60 μM atractylenolide (Figure 1 A). Atractylenolide significantly increased cell viability (Figure 1 B), the number of BrdU-positive cells in HTR-8/Svneo (Figure 1 C and D), suggesting its proliferative effect on RSA.

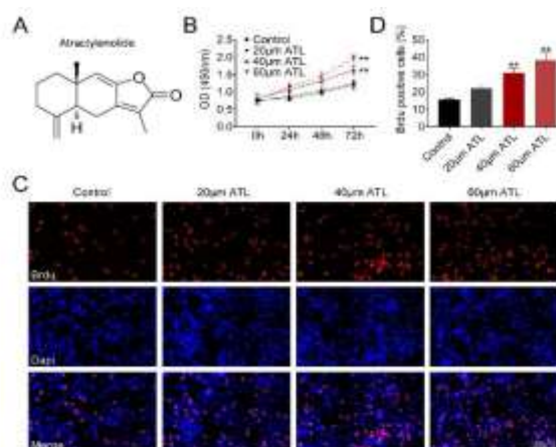

Figure 1: Atractylenolide stimulated proliferation of HTR-8/Svneo. (A) Chemical structure of Atractylenolide. (B) Atractylenolide increased viability of HTR-8/Svneo cells. (C) Atractylenolide increased cell proliferation of HTR-8/Svneo. (D) Atractylenolide enhanced the number of BrdU -positive cells of HTR-8/Svneo. ***P* < 0.01 vs. control

Atractylenolide stimulated migration and invasion of HTR-8/Svneo cells

Atractylenolide enhanced cell migration (Figure 2 A and B). Cell invasion of HTR-8/Svneo was also stimulated by atractylenolide (Figure 2 C and D), revealing its prometastatic effect on RSA.

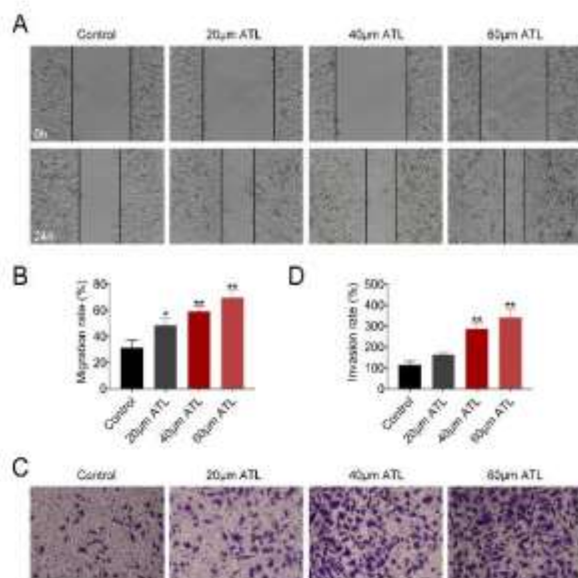

Figure 2: Atractylenolide stimulated migration and invasion of HTR-8/Svneo cells. (A) Atractylenolide promoted migration of HTR-8/Svneo in a dose-dependent way. (B) Relative migration of HTR-8/Svneo. (C) Atractylenolide promoted invasion of HTR-8/Svneo, (D) Relative invasion of HTR-8/Svneo. *$P < 0.05$, **$p < 0.01$ vs. control

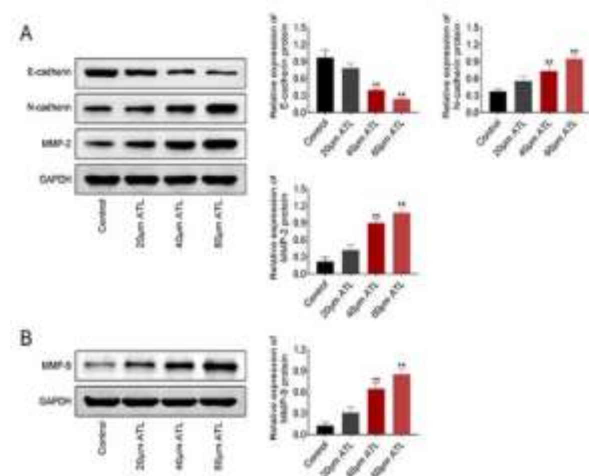

Figure 3: Atractylenolide stimulated epithelial-mesenchymal transition (EMT) of HTR-8/Svneo. (A) Atractylenolide reduced protein expression of E-cadherin, enhanced N-cadherin, MMP-2 of HTR-8/Svneo. (B) Atractylenolide enhanced expression of MMP-9 in HTR-8/Svneo. **$P < 0.01$ vs. control

Atractylenolide stimulated epithelial-mesenchymal transition (EMT) of HTR-8/Svneo cells

Atractylenolide reduced protein expression of E-cadherin (Figure 3 A). However, Atractylenolide enhanced expressions of N-cadherin, MMP-2 (Figure 3 A) and MMP-9 (Figure 3 B) in HTR-8/Svneo cells.

Atractylenolide stimulated phosphorylation of ERK in HTR-8/Svneo

Atractylenolide did not affect expression of ERK but increased p-ERK expression (Figure 4).

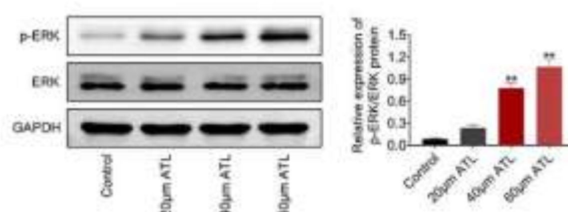

Figure 4: Effect of atractylenolide on phosphorylation of ERK in HTR-8/Svneo cells. Atractylenolide increased p-ERK of HTR-8/Svneo. **$P < 0.01$ vs. control

DISCUSSION

Traditional Chinese medicine is used for recurrent miscarriages [10]. The bioactive natural product of Traditional Chinese Medicine (TCM) exerts various pharmacological activities, which have also shown great therapeutic potential in treatment of RSA [11]. For example, Atractylodes macrocephala Koidz is used for unexplained RSA [7]. This study has found that atractylenolide, the bioactive component of Atractylodes macrocephala Koidz, promoted cell proliferation, migration, and invasion of HTR-8/Svneo, and might also attenuate RSA.

Trophoblast cell migration and proliferation are essential for maternal-fetal connection during pregnancy [12]. Therefore, HTR-8/Svneo is used as an *in vitro* model of RSA [12]. Atractylenolide increased cell viability, proliferation, and migration of HTR-8/Svneo [9]. This indicated that atractylenolide exerts proliferative and prometastatic effects on RSA.

Epithelial-mesenchymal transition (EMT) is characterized by acquisition of mesenchymal phenotype, loss of epithelial phenotype, and is related to trophoblast cell invasion and migration [13]. The EMT is implicated in differentiation of trophoblast cells, and immune microenvironment of maternal-fetal interface [13]. Suppression of

EMT of HTR-8/Svneo has been reported to promote progression of RSA [14]. The TGF-β1-induced invasion, migration, and EMT of small intestine epithelial cells were repressed by atractylenolide III [15]. Atractylenolide decreased protein expression of E-cadherin and increased MMP-9, MMP-9, and N-cadherin, thereby promoting EMT and attenuating RSA.

The ERK signaling is implicated in distinct cellular proliferation, migration, and invasion [16]. Activation of ERK signaling contributes to trophoblast migration, invasion [17], and down-regulation of ERK phosphorylation suppresses EMT of trophoblast cells [18]. Atractylenolide promoted activation of MAPK/ERK in HTR-8/Svneo [9]. Phosphorylation of ERK was repressed by atractylenolide, demonstrating that atractylenolide might ameliorate RSA through the activation of ERK signaling.

CONCLUSION

Atractylenolide promotes proliferation and EMT of trophoblast, as well as stimulates activation of ERK signaling. Therefore, atractylenolide might serve as a therapeutic agent for RSA. However, *in vivo* animal models should be used to investigate the protective role of atractylenolide against RSA.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Beili Lv, Haiyan Wang, Xinrong Li and Minjie Tang designed the study and carried it out; supervised the data collection, analyzed the data, interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript for publication.

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