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Regular paper

Rhein inhibits endometriosis by targeting microRNA-135

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Endometriosis is a serious human gynecological disorder of women of reproductive age. The present study was designed to explore the therapeutic implications of rhein in the management of endometriosis. The results showed that rhein significantly ($p<0.05$) inhibited the proliferation of endometrial stroma cells in a dose-dependent fashion. Besides, the rhein treated endometrial stroma cells showed significantly ($p<0.05$) lower migration and invasion, *in vitro*. Transwell and wound healing assays showed that rhein also suppressed the migration and invasion of the endometrial stroma cells. Rhein was shown to target miR-135 at the molecular level to exert its anti-proliferative effects against the human endometrial stroma cells. Conversely, overexpression of miR-135 could nullify the anti-proliferative effects of rhein. Taken together, the findings of the present study highlight the therapeutic utility of rhein against human endometriosis. However, more *in vivo* studies are required.

Keywords: endometriosis, rhein, rhubarb, proliferation, micro-RNA, miR-135, invasion

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Abbreviations: DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; EdU, 5-ethynyl-2′-deoxyuridine; FBS, Fetal bovine serum; HESCs, human endometrial stromal cells; miRs, Micro-RNAs; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-trtrazolium bromide; ROS, reactive oxygen species

INTRODUCTION

Endometriosis is characterized as the clinical condition in which endometrial glands and stroma grows ectopic to the uterine cavity at various locations and in particular within the ovaries (Foti *et al*., 2018). Endometriosis affects about 6–10% of women of reproductive age and is generally associated with secondary clinical manifestations, such as dysmenorrhea, pain in pelvic cavity, and infertility with severe impact on the over-all health of the patient (Dekker *et al*., 2021; Renner *et al*., 2017). Although, the endometriosis is considered to be benign, its increased invasiveness is equivalent to tumorous growth (Lal *et al*., 2020; McCluggage, 2020). Therefore, it is crucial to explore potential preventive and curative measures against this disorder to mitigate the non-desirous clinical conditions and more importantly to preserve the fertility.

Rhein, a 4,5-dihydroxyanthraquinone-2-carboxylic acid, is one of the main anthraquinone derivatives present in the rhubarb root (*Rheum palmatum*) (Liu *et al*., 2017). The molecule is known for its anti-proliferative potential and has been in use for more than a thousand years in Chinese traditional medicine to treat inflammatory disorders like diabetic nephropathy and osteoarthritis (Henamayee *et al*., 2020; Hu *et al*., 2019). There is growing support that rhein has pro-apoptotic property through which it restricts the proliferation of malignant human cells like breast, lung, and colon cancer cells (Tang *et al*., 2017). There are also reports that rhein alleviates the levels of reactive oxygen species (ROS) (Zhou *et al*., 2017). In addition, rhein has been shown to inhibit the adenomyosis by targeting the NF-ϰB and β-Catenin signaling pathways (Feng *et al*., 2017). Considering this, rhein administration was hypothesized for its possible effect on the proliferation of endometrial stromal cells.

Micro-RNAs (miRs) are an important class of short length non-coding RNAs which regulate the expression of their target genes at post-transcriptional levels by binding to the untranslated regions of their mRNA transcripts (Selvarajan *et al*, 2019). MiRs regulate many essential cellular and biological processes including proliferation, apoptosis, and invasion. It has been reported that the progression of endometriosis is linked with the aberrant expression of several micro-RNAs (miRs) (Liu *et al*., 2018). The expression of microRNA-135 (miR-135) has been shown to be up-regulated in endometriosis and has been deduced to promote the proliferation and invasion of endometrial stroma cells (Petracco *et al*., 2019; Mirabutalebi *et al*., 2017). Therefore, the present work aimed at exploration of the effects of rhein on endometrial stroma cell proliferation with the focus on examination of the mechanism of its action at molecular level, primarily *via* miR-135.

MATERIALS AND METHODS

Cell culture and treatment

The viable human endometrial stromal cells (HESCs) were procured from the Cell Bank of Type Culture Collection of the Chinese Academy of Science. For their propagation and *in vitro* maintenance, the cells were cultured using 10% FBS (Gibco Inc.) supplemented DMEM (Thermo Fisher Scientific, Inc.) at 37°C with 5% CO_2.

Rhein was purchased from the M/s Sigma-Aldrich Inc. and its stock was made by dissolving it at a concentration of 100 mM in DMSO, which was then used to make final working treatment concentrations (in μM).

MTT proliferation assay

Cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-trtrazolium bromide (MTT) assay. In brief, the HESCs (2.5×10^3 cells/well) were placed into the wells of a 96-well plate, treated with different treatment concentrations and incubated at 37°C for specific time durations of 0 h, 24 h, 48 or 72 h. At this

point, each well was added with 15 μL of MTT solution (0.5%) and cells were again incubated for 4 h at 37°C, after which the culture medium was replaced with 150 μL DMSO. OD (560) nm was then measured for each cell sample to determine the cell proliferation.

Colony formation assay

For colony formation assay, the cells were seeded at 600 cells/well into the wells of a 6-well plate. The cells were then added with 0 μM or 10 μM rhein and incubated at 37°C for 15 days till the colonies became distinctly visible. Colonies were then ethanol fixed, stained with 0.1% crystal violet and photographed.

EdU incorporation assay

The 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay was performed to analyze the proliferative viability of HESCs with the help of a Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. DAPI (Sigma-Aldrich) was used as a counter-stain.

Quantitative RT-PCR

TRIzol (Thermo Fisher Scientific) was used to perform the extraction of total RNA from HESCs as per the manufacturer's protocol. RNA isolated was reverse transcribed to cDNA using RevertAid First strand cDNA synthesis kit (Thermo Fisher Scientific). MiR and mRNA expression analysis was performed with the help of a SYBR Green PCR Kit (Takara, Japan) on QuantStudio 5.0 RT-PCR System. β-actin was used as a reference control for normalization of Ki67, PCNA, and MMPs. The snRNA U6 was used as a reference for the normalization of miR-135 expression. The expression levels were quantified with 2^{-ddCt} method. The RT primers used were: Ki-67, 5'-TCA GAC TCC ATG TGC CTG-3' (Forward) and 5'-GAC ACA CAC ATT GTC CTC-3' (Reverse); PCNA, 5'-CTT AGC ACT AGT ATT CGA-3' (Forward) and 5'-CAC CCG ACG GCA TCT TTA-3' (Reverse); miR-135, 5'-GCC CGC TAT GGC TTT CAT TCC T-3' (Forward) and 5'-CAG TGC AGG GTC CGA GGT-3' (Reverse); β-actin, 5'-CAC CAT TGG CAA TGA GCG GTT C-3' (Forward), 5'-AGG TCT TTG CGG ATG TCC ACG T-3' (Reverse) and U6 5'- ACGAATTTG CGT GTCATC CT -3' (Forward) and 5'- AAG CCTGCCGGTGACTAA C -3' (Reverse).

Scratch-heal assays

The migratory potential of HESCs treated with 0 μM or 10 μM rhein was analyzed with the scratch-heal assay. Briefly, the cells were added into a six-well plate at a density of 4×10^5 cells per well. The cells were cultured at 37°C till a confluence of 80–90% was reached. At this point, medium was removed, and a uniform scratch was carved using a sterile pipette tip and photographed under light microscope. After 24 h incubation at 37°C, the scratch was observed under microscope and photographed again.

Transwell chamber assay

For the analysis of their invasion, the HESCs were assessed using the transwell chamber invasion assay. Herein, 2×10^5 cells were added in the upper chamber of each transwell insert (Corning, Cambridge, USA) with Matrigel coated membrane. Only serum free 10% FBS supplemented culture medium was added into the lower chambers. Afterwards, the cells were incubated at 37°C for 24 h and then the cells from the upper surface of the membrane were removed with cotton, while the cells from its lower surface were methanol fixed and stained with 0.1% crystal violet for 25 min. Finally, the cells were imagined under a light microscope.

Transfection

Pre-synthesized miR-135 mimics and its negative control miR-NC were ordered from GenePharma (Shanghai, China). The cell transfection was carried out with the help of Lipofectamine 3000 (Thermo Fisher Scientific) following the manufacturer's instructions. RT-PCR was used to assess the transfection efficiency.

Statistical analysis

Each experiment was performed in triplicate and the values were expressed as mean ± standard deviation (S.D.). The statistical analysis was performed with SPSS 20.0 (SPSS/IBM, Armonk, New York, USA) software. The significance of the data was analyzed through Student's *t*-test or one-way analysis of variance (ANOVA with a significance level of *p*-value less than 0.05).

RESULTS

Rhein inhibits the proliferation of HESCs

To visualize the effects of rhein on the proliferation of HESCs, the latter were administered with increasing concentrations of rhein (0–80 μM) for 24 h. The MTT assay was then performed to examine the proliferation of differentially treated HESCs. It was seen that proliferation of HESCs decreased with increasing concentrations of rhein treatment and the IC_{50} of rhein against the HESCs was found to be 10 μM (Fig. 1A). Further, the 10 μM rhein treated HESCs showed significantly lower proliferation at 24, 48, and 72 h treatment durations with respect to control untreated cells (Fig. 1B). The anti-proliferative effects of rhein against HESCs cells were also evident in terms of significant decline in their clonogenicity (Fig. 1C). Again, the HESCs cells treated with 10 μM rhein showed markedly lower EdU incorporation (Fig. 1D). Moreover, the administration of rhein significantly repressed the expression of proliferation markers, Ki-67 and PCNA (Fig. 1E and 1F). The results are thus reflective of anti-proliferative action of rhein against HESCs.

Rhein restricts *in vitro* HESC migration and invasion

The effects of rhein administration were also analyzed on the migration and invasion of HESCs using scratch-heal and transwell chamber assays, respectively. HESCs cells treated with 10 μM rhein were shown to exhibit significantly lower migration, *in vitro*, in comparison to the untreated cells (Fig. 2A). The relative HESC migration decreased by more than 50 percent under rhein treatment. Similarly, the HESCs exhibited significantly lower invasion when administered with rhein, *in vitro* (Fig. 2B). Inhibitory potential of rhein against the migration and invasion of HESCs was besides evident from the significant down-regulation of MMP2 and MMP9 protein levels in rhein treated HESCs (Fig. 2E).

Rhein represses miR-135 to inhibit HESC growth, *in vitro*

The expression analysis of miR-135 from control and 10 μM rhein treated HESCs showed that rhein targets

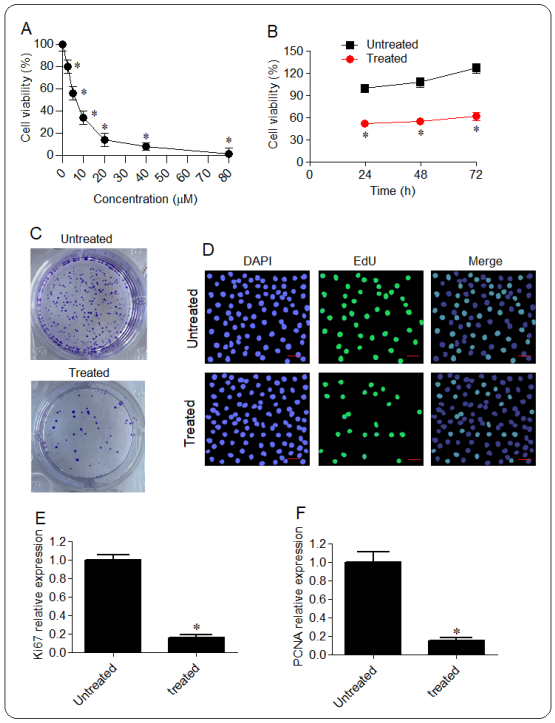

Figure 1. Rhein inhibits human endometrial stromal cell viability.
(**A**) MTT assay of HESCs treated with 0 to 80 μM rhein for 24 h (**B**) MTT assay of HESCs treated with 0 or 10 μM rhein for 24, 48 or 72 h (**C**) colony formation assay of HESCs treated with 0 or 10 μM rhein (**D**) EdU incorporation assay of HESCs treated with 0 or 10 μM rhein for 24 h (**E**) qRT-PCR of Ki-67 from HESCs treated with 0 or 10 μM rhein for 24 h (**F**) qRT-PCR of PCNA from HESCs treated with 0 or 10 μM rhein for 24 h. Experiments were performed in triplicates and statistical significance was assessed at *p*-value <0.05.

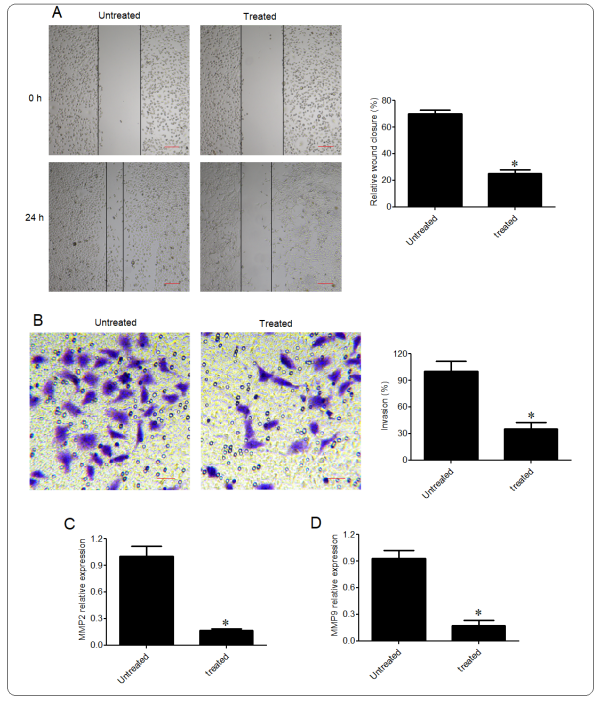

Figure 2. Rhein inhibits migration and invasion of human endometrial stromal cells.
(**A**) Scratch-heal migration assay of HESCs treated with 0 μM or 10 μM rhein for 24 h (**B**) transwell chamber invasion assay of HESCs treated with 0 or 10 μM rhein for 24 h (**C**) qRT-PCR of MMP2 and (**D**) MMP9 from HESCs treated with 0 or 10 μM rhein for 24 h. Experiments were performed in triplicates and statistical significance was assessed at $p<0.05$.

and represses miR-135 in HESCs (Fig. 3A). To assess whether rhein inhibited proliferation of HESCs *via* miR-135 down-regulation, miR-135 was transiently over-expressed in HESCs by transfecting the latter with miR-135 mimics and the same was confirmed with respect to miR-NC control transfected cells using qRT-PCR (Fig. 3B). MTT assay showed that the proliferation of miR-135 over-expressing HESCs was minimally affected by rhein (Fig. 4A). Similarly, the over-expression of miR-135 was shown to attenuate the effects of rhein administration on clonogenicity and proliferative viability of HESCs (Fig. 4B and 4C). The results indicate that rhein targets miR-135 in HESCs to inhibit their growth, *in vitro*.

DISCUSSION

Clinically manifested as growth of endometrial glands and stroma ectopic to uterine cavity, endometriosis is often linked with the devastating conditions of pelvic pain and infertility among human females of reproductive age (Foti *et al.*, 2018). Although endometriosis is considered a benign pathological condition, endometrial stroma cells here exhibit profound invading capacity, which mimics the malignant behavior of human cancer cells (Mutter *et al.*, 2007). Moreover, endometriosis and endometrial cancer are believed to posses' numerous possible links (Painter *et al.*, 2018; Kajiyama *et al.*, 2019). Additionally, endometriosis has been shown to be associated with mutation in cancer causing genes (Koppolu *et al.*, 2021). Therefore, it becomes necessary to look for the possible measures to restrict the growth, migration, and invasion of endometrial cells.

Rhein has huge medicinal importance and exhibits nephroprotective, hepatoprotective, ROS-scavenging, anti-inflammatory, and anticancer properties (Zhou *et al.*, 2015). Rhein is known for its anti-proliferative activity against several human cancer cells, which include cervical cancer, lung cancer, breast cancer, colon cancer and so on (Wu *et al.*, 2017; Lin *et al.*, 2009). Feng *et al.* in 2017 deduced that rhein could alleviate adenomyosis via the inhibition of NF-κB and β-catenin signaling pathways (Feng *et al.*, 2017). Adenomyosis resembles endometriosis to a great extent and thus it suggests that rhein might exhibit similar inhibitory action against endometriosis. Exploring the same, the rhein administration was shown

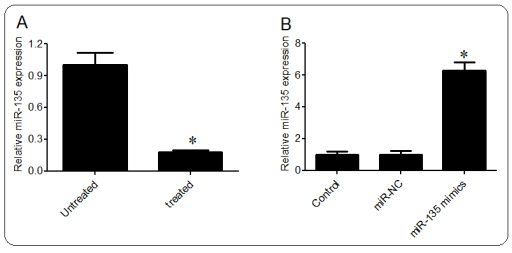

Figure 3. Rhein represses miR-135 in human endometrial stromal cells.
(**A**) qRT-PCR of miR-135 from HESCs treated with 0 or 10 μM (**B**) qRT-PCR of miR-135 from non-transfected HESCs and ones transfected with miR-NC or miR-135 mimics. Experiments were performed in triplicates and statistical significance was assessed at $p<0.05$.

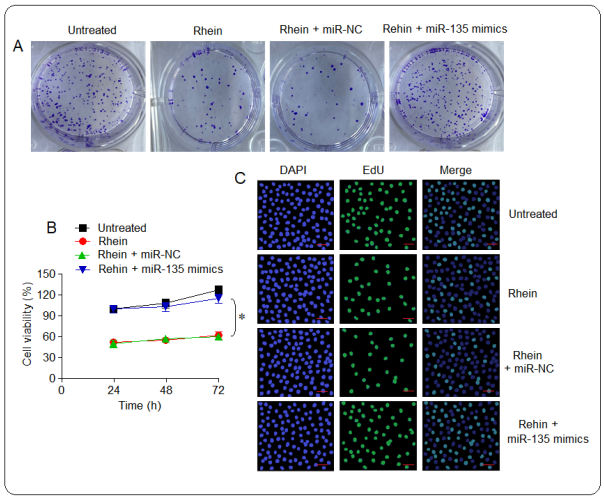

Figure 4. Rhein targets miR-135 to inhibit human endometrial stromal cell proliferation.
(**A**) MTT assay of non-transfected HESCs treated with 0 μM and miR-NC or miR-135 mimics transfected HESCs administered with 10 μM rhein for 24, 48 or 72 h (**B**) colony formation assay of non-transfected HESCs treated with 0 μM and miR-NC or miR-135 mimics transfected HESCs administered with 10 μM rhein for 24 h (**C**) EdU incorporation assay of non-transfected HESCs treated with 0 μM and miR-NC or miR-135 mimics transfected HESCs administered with 10 μM rhein for 24 h. Experiments were performed in triplicates and statistical significance was assessed at p-value<0.05.

to limit the proliferation of HESCs considerably in the present study. The anti-proliferative effects of rhein were also evident as significant decline in the colony formation and EdU incorporation of HESCs. The decline in cell proliferation by rhein can be attributed to its pro-apoptotic property and mitotic arrest (Chang *et al*., 2012; Han *et al*., 2018). The MMP2 and MMP9 proteins have been shown to be responsible for regulating the migration and invasion of different cells (Lin *et al*., 2010). Several cancers have been shown to be associated with the upregulation of MMP2 and MMP9 (Lu *et al*., 2018). Inhibition of these genes causes inhibition of cell migration and invasion (Chien *et al*., 2018). The HESCs also exhibited significant decline in migration and invasion, *in vitro*, once administered with rhein, which was evident as the fall in the expression of MMP2 and MMP9 proteins. Rhein has been shown to variously mediate its role by targeting specific micro-RNAs or long non-coding RNAs (Zhang *et al*., 2020). For instance, it was shown to inhibit the renal inflammatory injury of uric acid nephropathy through lncRNA-Cox2/miR-150-5p/STAT1 axis (Hu *et al*., 2020). Similarly, it has been reported that lncRNA ANRIL regulates the nephroprotective property of rhein in uric acid nephropathy rats (Hu *et al*., 2019). Endometriosis has been previously shown to exhibit up-regulation of microRNA-135 (miR-135) (Petracco *et al*., 2019; Mirabutalebi *et al*., 2018). In the present study, rhein administration was found to repress the expression of miR-135. Further experimentation revealed that rhein inhibits the proliferation of HESCs *via* the targeting of miR-135. Summing up, the results of the current study are indicative of therapeutic potential of rhein against the human endometriosis.

CONCLUSION

Rhein inhibits the growth, migration, and invasion of human endometrial stroma cells. At molecular level, rhein targets miR-135, which is over-expressed in endometrial stroma cells, to exert its anti-proliferative effects. The results are thus indicative of therapeutic potential of rhein against human endometriosis, which can be enhanced through semi-synthetic chemistry approaches. Additionally, more studies, especially *in vivo,* are required to confirm the therapeutic potential of rhein.

Conflict of interest

Authors declare that there are no conflicts of interest.

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