Article

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Daphnetin Modulates Immune Balance and Enhances Pregnancy Viability in a Mouse Model of Unexplained Recurrent Abortion

Sheng-gen Long[1], Zhi-qin Zhang[2], Jun Tan[2,*]

[1]Department of Gynecological Oncology, Jiangxi Maternity and Child Healthcare Hospital, 330006 Nanchang, Jiangxi, China
[2]Department of Reproductive Center, Jiangxi Maternity and Child Healthcare Hospital, 330006 Nanchang, Jiangxi, China
*Correspondence: tanjun561127@163.com (Jun Tan)
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Background: Our previous research revealed that daphnetin (7,8-dihydroxycou-marin) positively influences the balance between forked transcription factor P3 (*Foxp3*+) regulatory T cells (Treg) and T helper 17 (Th17) cells in the peripheral blood mononuclear cells of individuals with unexplained recurrent pregnancy loss. However, the specific mechanism remains unclear. This research aims to further examine how daphnetin regulates the Th17 cell/*Foxp3*+ Treg cell imbalance in a mouse model with unexplained recurrent spontaneous abortion (URSA).

Methods: Mice (n = 40) were allocated into the following groups: daphnetin high dose (4 mg/kg·day), daphnetin low dose (1 mg/kg·day), URSA model, and normal pregnancy (control). We used flow cytometry for assessing the Th17/Treg cell ratio in peripheral blood mononuclear cells, quantitative real-time polymerase chain reaction for measuring cytokine expression levels, and transmission electron microscopy for observing ultrastructural changes in decidual tissues and calculating the embryo absorption rate.

Results: Compared to the URSA model group, daphnetin significantly reduced the T17cell/*Foxp3*+ Treg cell ratio in peripheral blood mononuclear cells. Daphnetin also decreased the expression of Th17 cell-related cytokines, including orphan nuclear receptor γt (*RORγt*) and signal transduction and transcriptional activator 3 (STAT3), as well as increase the expression of *Foxp3*+ Treg cells-related cytokines, including STAT5 and *Foxp3*+. Furthermore, daphnetin reduced the embryo absorption rate and improved the decidual tissue ultrastructure of URSA model mice.

Conclusion: Daphnetin improves the Th17 cell/*Foxp3*+ Treg cell imbalance in URSA model mice, thereby contributing to the repair of decidual tissue damage and reducing the embryo absorption rate. These findings suggest that daphnetin may offer a new method for treating URSA.

Keywords: daphnetin; unexplained recurrent spontaneous abortion; T cells; immune regulation; cytokine

Introduction

Recurrent spontaneous abortion (RSA), which affects approximately 5% of fertile women worldwide, is defined as having two or more spontaneous miscarriages before 20 weeks of gestation [1,2]. More than 60% of RSA cases are unexplained, known as unexplained recurrent spontaneous abortion (URSA) [3,4]. Current treatments, including heparin and aspirin, have various side effects such as osteoporosis, ulcers, bleeding and allergic reactions. Therefore, it is important to develop new drugs that are safe, effective, and low in toxicity for URSA patients.

Recent studies have identified an important relationship between URSA and abnormal immune tolerance [5–8]. Specifically, cellular immune responses, such as T cell differentiation and T cell-related cytokine production, can disrupt the immune tolerance necessary for a successful pregnancy [9]. It has been proven that $CD4^+$T lymphocyte differentiation into various T lymphocyte subtypes occurs under distinct conditions, enabling them to perform diverse biological functions [10]. Among the T cell subsets, regulatory T cells (Treg) and T helper 17 (Th17) cells, two recently discovered T cell subsets that are crucial in maintaining maternal-fetal immune balance, are risk factors for URSA [11]. Our earlier study revealed a significantly higher Th17/Treg ratio in the peripheral blood of individuals with URSA compared to those with normal pregnancies [11]. This imbalance is a risk factor for URSA because an elevated Th17/Treg ratio indicates a shift towards a pro-inflammatory state and reduced immune tolerance, increasing the likelihood of pregnancy complications and spontaneous abortion [11]. Therefore, understanding and modulating the Th17/Treg balance could be key in developing effective treatments for URSA.

Currently, there is no cure for URSA. Daphnetin (7,8-dihydroxycou-marin), derived from *Daphne odora* var. marginata (D. marginata), is rich in coumarin compounds praised for their anti-inflammatory, anti-bacterial, and anti-

Table 1. Primer sequences.

Gene	Species	Primer sequence
Foxp3	mice	Forward: 5′-AGACCCCTGTGCTCCAAGTG-3′ Reverse: 5′-CAGACTCCATTTGCCAGCAG-3′
RORγt	mice	Forward: 5′-CCTTCCCTCCACTCTATAAGGA-3′ Reverse: 5′-GTCAGAGGGCTGAAGGAAATAG-3′
STAT3	mice	Forward: 5′-AATATAGCCGATTCCTGCAAGAG-3′ Reverse: 5′-TGGCTTCTCAAGATACCTGCTC-3′
STAT5	mice	Forward: 5′-CAGCCGTGGGATGCTATTGA-3′ Reverse: 5′-GGGACAGCGGTCATACGTG-3′
β-actin	mice	Forward: 5′-TGACAGGATGCAGAAGGAGA-3′ Reverse: 5′-TAGAGCCACCAATCCACACA-3′

Foxp3, forked transcription factor P3; *RORγt*, orphan nuclear receptor γt; *STAT3*, signal transduction and transcriptional activator 3.

tumor properties [12–15]. For instance, an earlier study reported that daphnetin can regulate the balance between Tregs and Th17 cells [16]. We previously described the positive impact of daphnetin on URSA through an improvement of the Th17/Treg cell balance [17]. However, the exact molecular mechanism remains unclear. Therefore, this research sought to investigate how daphnetin regulates the Th17/Treg cell imbalance in an animal model of URSA, potentially providing a novel approach for treating the condition in the future.

Materials and Methods

Establishment of URSA Model Mice

Female CBA/J mice aged 8 weeks and weighing 18–22 g were purchased from Beijing Huafukang Biotechnology Co. (Beijing, China). Male BALB/c mice aged 6 weeks and weighing 18–20 g were purchased from Cloud-Clone Corp (No.11401300086323, Wuhan, China). Male DBA/2 mice aged 8 weeks and weighing 18–22 g were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mating of female CBA/J and male BALB/c mice at a 2:1 ratio was used to establish a normal pregnancy mouse model, and mating of female CBA/J mice and male DBA/2 mice, also at a 2:1 ratio, was used to generate the URSA mouse model. Vaginal thrombus was considered day 0 of pregnancy. This experiment was conducted by Cloud-Clone Corp (Wuhan, China). All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and the protocols approved by Laboratory Animals of CLOUD-CLONE CORP. WUHAN Ethics Committee (IACU18-0155).

Experimental Grouping and Administration Treatment

Forty experimental animals were grouped as follows: URSA model (n = 10), normal pregnancy (control) model (n = 10), daphnetin high dose (4 mg/kg·day) (n = 10), and daphnetin low dose (1 mg/kg·day) (n = 10). Intragastric administration commenced on day 0 of pregnancy for all groups. The doses of daphnetin (HY-N028, MedChemExpress, Monmouth Junction, NJ, USA) were selected based on our earlier study [13]. The intragastric volume of daphnetin administered was 0.4 mL per 20 g of body weight. For a span of 14 days, control, and URSA model mice were provided with equivalent amounts of distilled water. One hour after the final administration, the whole blood of the mice was collected and used for density gradient centrifugation with Ficoll-Paque. After centrifugation, peripheral blood mononuclear cells (PBMCs) were collected, washed with phosphate buffered saline (PBS) to remove any cell debris, and then resuspended for further analyses. The mice were euthanized with 2% pentobarbital sodium (150 mg/kg), and the abdominal cavity was opened to expose the uterus. Next, we observed the growth of uterus and recorded the number of implanted and absorbed embryos.

Flow Cytometry

After isolation of peripheral blood mononuclear cells (PBMCs), phorbol ester was added according to the working concentration of 50 ng/mL and incubated with ionomycin (1 μg/mL) and monensin (2 μM) (eBioscience, San Diego, CA, USA) at 37 °C for 4 h to stimulate the cells and inhibit protein transport, ensuring the accumulation of cytokines within the cells for accurate detection by flow cytometry. Homotypic control and detection tubes were also set up. To block the Fc receptor, the cells were first incubated with an Fc receptor blocking solution to prevent nonspecific binding. Following this, the CD4 surface antibody (cat.#14-9766-82, eBioscience, San Diego, CA, USA) was introduced for staining and then thoroughly mixed to ensure proper labeling of the CD4 molecules on the cell surface. After a 15-min incubation at ambient temperature in darkness, each tube was added to the lyzed blood cells, followed by fixation. After rinsing the cells, the experimental tube was furnished with two sets of color-matched antibodies specific for Th17 and Treg cell markers (eBioscience, San Diego, CA, USA), whereas the control tube was en-

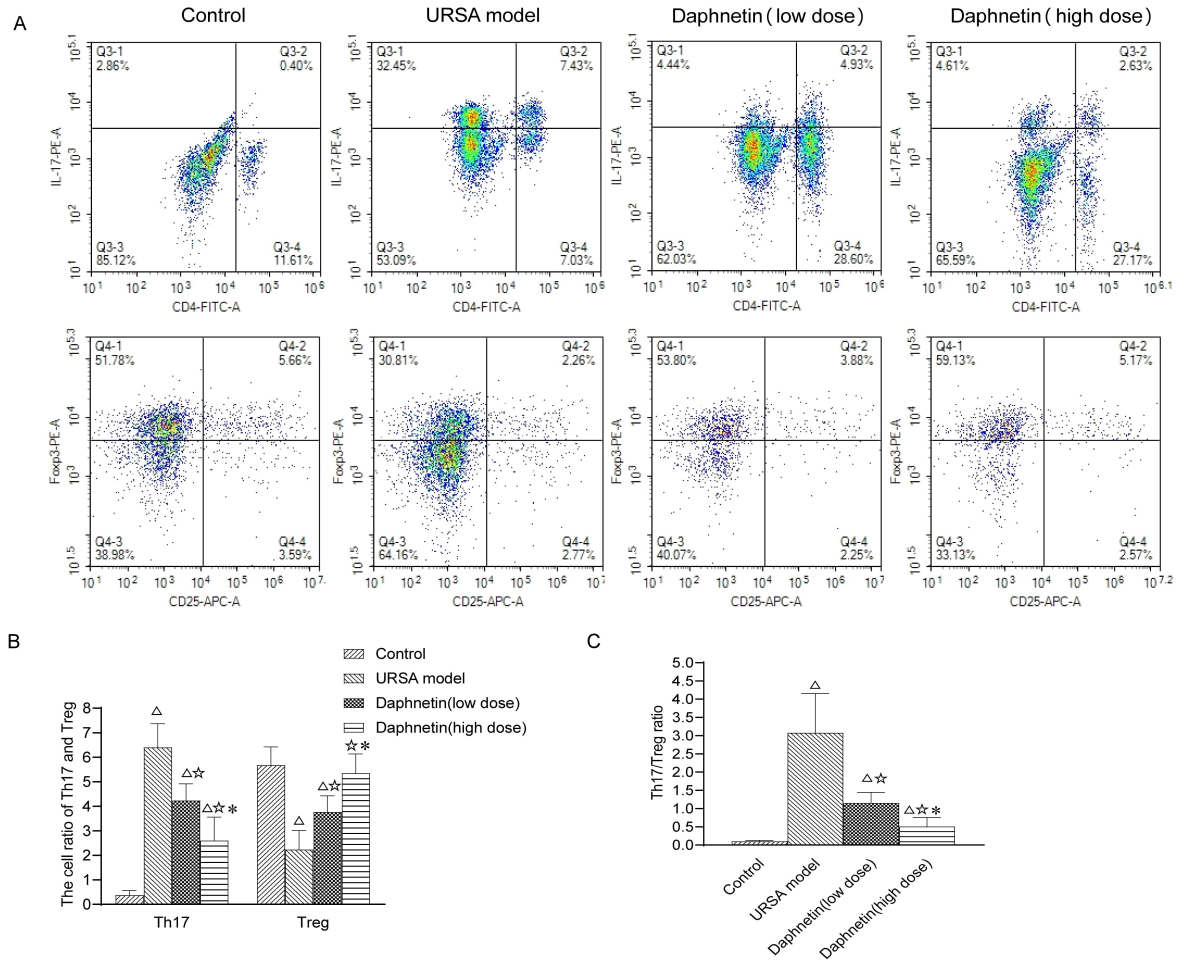

Fig. 1. Daphnetin affects the T helper 17 (Th17)/regulatory T cells (Treg) ratio in mice's peripheral blood mononuclear cells. (A,B) Flow cytometry analysis of Th17 and Treg cell ratios in peripheral blood mononuclear cells (PBMCs) of mice in each group. (C) Th17/Treg ratio in PBMCs of mouse in each group. n = 10 per group, $\triangle p < 0.05$ vs. control, $\star p < 0.05$ vs. unexplained recurrent spontaneous abortion (URSA) model, $*p < 0.05$ vs. daphnetin low dose. Daphnetin, 7,8-dihydroxycou-marin; IL-17, interleukin-17.

dowed with isotype control antibodies to ensure specificity (eBioscience, San Diego, CA, USA). After incubation in darkness to prevent photobleaching of the fluorescent antibodies, the cells were washed and the supernatant was centrifuged with a detergent to remove unbound antibodies. The cells were then resuspended and analyzed within 24 hours to ensure optimal viability and staining integrity. The analysis was completed through the CD4 histogram, and the Th17 and Treg cells were expressed via the scatter plot. The data were obtained and analyzed using Cellsquest software (BD Biosciences, San Jose, CA, USA, staining scheme, Th17: interleukin-17 (IL-17)-PE, CD4-FITC; Treg: CD25-APC, CD4-FITC, forked transcription factor P3 (Foxp3)-PE).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from PBMCs using TRIzol reagent (10296028CN, Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, followed by reverse transcription to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (4368814, Applied Biosystems, Foster City, CA, USA). cDNA was amplified using SYBR Green PCR Master Mix (4309155, Applied Biosystems, Foster, CA, USA) in a 7500 Fast Real-Time PCR System (4351104, Applied Biosystems, Foster, CA, USA). The reaction was composed of initial denaturation at 95 °C for 10 minutes, followed by 40 cycles of 95 °C for annealing and extension. The fluorescence emitted during the PCR reactions was measured in real-time, and the cycle threshold (Ct) values were recorded. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method with *β-actin* serving as an internal control. The primer sequences are provided in Table 1.

Transmission Electron Microscopy

Uterine tissue ($1 \times 1 \times 1$ mm^3) was collected and fixed in 2.5% glutaraldehyde at 4 °C for 2–4 h. After rinsing three times with PBS (pH 7.4), the tissue was fixed at ambient temperature for 2 h in 1% osmic acid in PBS (pH

7.4), followed by rinsing with PBS (pH 7.4) 3 times for 15 min each time. The tissue was dehydrated and embedded overnight at 37 °C, followed by oven polymerization at 60 °C for 48 h. Next, the tissue was sectioned to generate ultra-thin slices (thickness, 60–80 nm), which were subject to uranium-lead double staining (2% saturated uranium acetate alcohol solution, lead citrate) for 15 min and dried overnight at ambient temperature. Sections were viewed under an HT7700 transmission electron microscope (HITACHI (China) Co., Ltd., Shanghai, China).

Statistical Analysis

The measurement data were displayed as the mean ± standard deviation. Pairwise comparisons were made using SPSS software (version 22.0, IBM, Armonk, NY, USA), Tukey test, and one-way analysis of variance. $p < 0.05$ was considered a statistically significant difference.

Results

Impact of Daphnetin on the Th17/Treg Proportion in Peripheral Blood Mononuclear Cells of Mice

Flow cytometry was used to assess the impact of daphnetin on the Th17/Treg ratio in mouse PBMCs. The URSA group exhibited a notably higher Th17 cell ratio and a lower Treg cell ratio in PBMCs compared to the control group ($p < 0.05$). Treatment with daphnetin decreased the Th17 cell ratio and increased the Treg cell ratio ($p < 0.05$), with more pronounced effects at higher doses. Thus, treatment with daphnetin significantly reduces the Th17/Treg cell ratio ($p < 0.05$) (Fig. 1).

Effects of Daphnetin on the Foxp3, RORγt, STAT3, and STAT5 Expression in Decidual Tissues of Mice

As shown in Fig. 2, compared to the URSA group, the daphnetin groups showed lower expression of the Th17-related cytokines orphan nuclear receptor γt (*RORγt*) and signal transduction and transcriptional activator 3 (*STAT3*) ($p < 0.05$), while the expression of the Treg-related cytokines *Foxp3* and *STAT5* were higher ($p < 0.05$) (Fig. 2). These findings suggest that daphnetin modulates key immunoregulatory genes involved in URSA.

Observation of Alterations in the Ultrastructure of Decidual Tissue via Transmission Electron Microscopy

We investigated the ultrastructural changes of decidual tissue in mice from each group by transmission electron microscopy. In the control group, the decidual cells of mice were large and compact, arranged neatly, with the cytoplasm rich in organelles and the nucleus large, round, rich in euchromatin. The vascular walls of the decidual tissue in the control group were intact, surrounded by single or multiple vascular endothelial cells, with cell membranes protruding into the cavity surface (Fig. 3A). Conversely, the decidual cells in the URSA model group were smaller, the intercellular space was significantly enlarged, and the cytoplasm was dense. The endoplasmic reticulum was rough, mitochondria showed vacuolar degeneration, and the curvature of the nucleus increased, with increased heterochromatin near the nuclear membrane. The vascular walls were distinctly incomplete, and the vascular endothelial cells showed a fusiform nucleus (Fig. 3B). After treatment with daphnetin, decidual cells increased in quantity and the intercellular space enlarged (Fig. 3C), and these effects were more pronounced at higher doses of daphnetin (Fig. 3D).

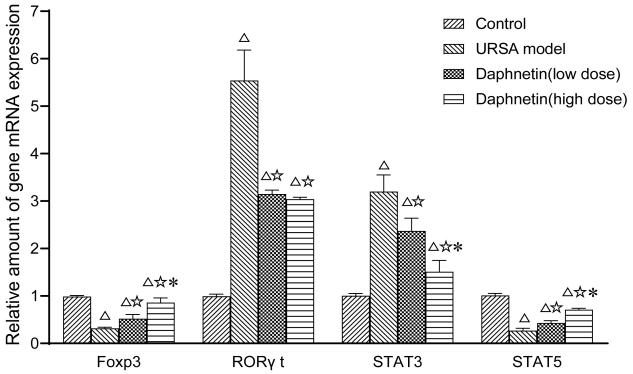

Fig. 2. Effects of daphnetin on *Foxp3*, *RORγt*, *STAT3*, and *STAT5* mRNA expression in peripheral blood of mice. The *Foxp3*, *RORγt*, *STAT3*, and *STAT5* mRNA levels in PBMCs of mouse in each group were measured by quantitative real-time polymerase chain reaction (qRT-PCR). n = 6 per group, △$p <$ 0.05 vs. Control, ☆$p < 0.05$ vs. recurrent spontaneous abortion (RSA) model, *$p < 0.05$ vs. daphnetin low dose.

Uterine Morphology and Embryo Absorption Rate

After daphnetin treatment, the embryo absorption rate was significantly decreased ($p < 0.05$), and there were no significant differences between control and high dose groups ($p > 0.05$) (Table 2). The control group exhibited a healthy and consistent uterine appearance, indicative of normal pregnancy. Conversely, the URSA model demonstrated reduced size, with embryo atrophy or disappearance, consistent with the pathology of URSA. Low dose daphnetin treatment appeared to partially restore uterine morphology, with some improvement in uniformity and size. High dose daphnetin treatment showed further morphological improvement, with uterine features more closely resembling the control group, suggesting a dose-dependent therapeutic effect (Fig. 4).

Discussion

This study illustrates the therapeutic potential of daphnetin in balancing Th17/Treg levels in URSA model mice, a key factor of the condition. Daphnetin effectively reduced

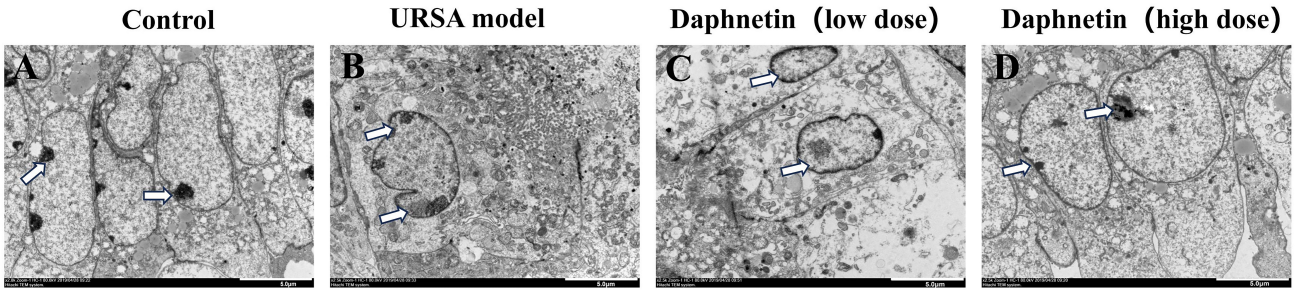

Fig. 3. Ultrastructural changes of decidual tissue of mice observed by transmission electron microscopy. Transmission electron microscope images of the control (A), URSA model (B), daphnetin low dose (C) and daphnetin high dose (D) groups. Scale bar = 5 µm. (A) White arrows indicate large and compact decidual cells of mice, neatly arranged cells, large and round nucleus, and cytoplasm rich in organelles. (B) White arrows highlight that the vascular wall of the decidual tissue appeared notably incomplete, with vascular endothelial cells exhibiting fusiform nuclei. (C) White arrows show an increase in decidual cell number and a corresponding enlargement of the intercellular space. (D) White arrows indicate that the quantity of decidual cells was up-regulated and the intercellular space was larger.

Table 2. Embryo absorption rates' comparison in each group.

Groups	Number of mice	Number of absorbed embryos	Number of viable embryos	Embryo absorption rate
Control	10	12	90	11.76%
URSA model	10	43	42	50.59% △
Daphnetin (low dose)	10	20	72	21.74% ☆
Daphnetin (high dose)	10	15	83	15.31% ☆*

Note: $\triangle p < 0.05$ vs. control, $\star p < 0.05$ vs. URSA model, $^{*}p < 0.05$ vs. daphnetin low dose. Embryo absorption rate = number of absorbed embryos/(number of absorbed embryos + number of viable embryos) × 100%.

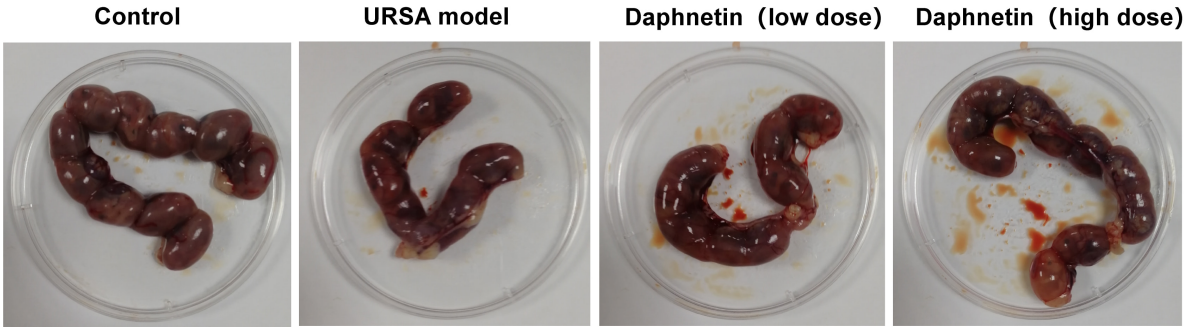

Fig. 4. Embryo morphology of each group. Embryo morphology in control, URSA model, and daphnetin-treated mice were observed. Control uteri appeared normal, while the URSA model uteri showed abnormalities. Low-dose daphnetin treatment resulted in some improvement, and high-dose treatment led to further normalization.

the Th17/Treg ratio, thereby mitigating inflammatory responses and enhancing immune tolerance at the maternal–fetal interface. These outcomes suggest daphnetin could improve pregnancy outcomes and offer a promising therapeutic alternative with potentially fewer side effects compared to traditional treatments such as heparin and aspirin. Therefore, this study's insights into daphnetin's modulatory effects pave the way for novel interventions aimed at correcting immune dysregulation associated with URSA.

Despite the unknown cause of URSA, multiple studies have established a strong correlation between the imbalance of Th17/Treg cells and URSA [18–20]. Maintaining a proper maternal–fetal immune balance is crucial for ensuring a successful pregnancy; however, disturbances in this balance can lead to various pregnancy-correlated complications, including spontaneous abortion. Research has shown that Treg and Th17 cells play crucial roles in the establishment of immune tolerance and immune defense mechanisms [21,22]. Treg cells can regulate Th17 cells to protect embryos from maternal rejection and maintain pregnancy [23]. Evidence demonstrates that the restrictive regulation between Th17 and Treg cells is pivotal for maintaining immune balance, thus contributing to pregnancy maintenance. When the ratio of Th17/Treg cells is elevated, the incidence of URSA increases significantly, illustrating that disrupted immune tolerance potentially leads

to fetal rejection [24,25]. Our investigation found that the Th17/Treg ratio was markedly elevated in the URSA group compared to the control group, consistent with previous results [26,27]. This finding underscores its association with increased pregnancy complications, including fetal rejection and spontaneous abortion. Targeted interventions that effectively modulate this balance are crucial to improve pregnancy outcomes.

The mechanism underlying the Th17/Treg balance involves the reverse differentiation patterns of Th17 and Treg cells [28]. $CD4^+$T lymphocytes can differentiate into Th17/Treg intermediate cells that express both *RORγt*, a key transcription factor of Th17 cells, and *Foxp3*, a specific transcription factor of Treg cells [21]. Significantly, the STAT family of proteins, particularly STAT3 and STAT5, play crucial roles in this process [29]. STAT3 promotes Th17 cell differentiation, while STAT5 supports Treg cell development. An imbalance in STAT5 expression is particularly detrimental, leading to a reduction of *Foxp3*+ Tregs cells and an inhibition of the induction of Tregs *in vitro* [30]. Our previous studies showed that, compared with the URSA group, there was a reduction in the quantity of Th17 cells along with an elevation in the population of Treg cells in PBMCs of URSA patients treated with daphnetin, indicating that daphnetin can regulate the Th17/Treg cell balance. In this study, daphnetin treatment of URSA model mice resulted in decreased mRNA expression of the Th17-related cytokines *RORγt* and *STAT3* and increased mRNA expression of the Treg-related cytokines *Foxp3* and *STAT5*. The observed decrease in Th17 cytokine expression along with an increase in Treg cytokine expression in treated URSA model mice underpins daphnetin's potential as a therapeutic agent to stabilize the maternal immune environment and to improve pregnancy outcomes.

The therapeutic potential of daphnetin was evident through marked improvements in embryonic viability and decidual tissue integrity in treated URSA model mice. Daphnetin treatment led to a significant decrease in the embryonic absorption rate, a common complication of URSA, and a significant improvement in the ultrastructure of decidual tissue. These outcomes not only underscore the compound's efficacy in reducing embryonic absorption rates but also in ameliorating the structural anomalies observed in decidual tissues of affected models. The decrease in Th17/Treg ratio after treatment further corroborates the immune-modulatory capability of daphnetin, aligning with observed clinical benefits. Taken together, these findings indicate that daphnetin has a certain therapeutic efficacy on URSA model mice, which may offer a new approach for URSA treatment in the future.

Despite these advancements, our study has limitations that warrant consideration. First, the use of an animal model, while invaluable for understanding complex biological processes in a controlled environment, may not fully replicate the human physiological responses to daphnetin. The extrapolation of these results to clinical settings should, therefore, be approached with caution. Additionally, our investigation was limited to short-term observations. The long-term effects of daphnetin, especially those concerning sustained pregnancy and postnatal outcomes, remain unknown and should be the focus of future studies. Moreover, the molecular pathways by which daphnetin exerts its effects, particularly the interactions between STAT3 and STAT5 signaling, have not been exhaustively delineated. Further research is needed to clarify these mechanisms and to explore potential synergistic effects of daphnetin with other therapeutic agents.

Conclusion

In summary, daphnetin exerts a therapeutic effect on URSA model mice by modulating the imbalance between Th17 and Treg cells and repairing damaged tissue. The findings highlight daphnetin's potential as an innovative treatment for URSA, offering therapeutic benefits with minimal side effects compared to conventional treatments such as heparin and aspirin. By restoring immune homeostasis at the maternal–fetal interface, daphnetin significantly improves pregnancy outcomes for women with URSA. Future research should prioritize clinical trials to validate these effects in humans and further explore the underlying molecular mechanisms, potentially establishing daphnetin as a cornerstone of therapeutic strategies against URSA.

Availability of Data and Materials

The data underlying this article will be shared on reasonable request to the corresponding author.

Author Contributions

SL, ZZ, and JT contributed to the study conception and design. Material preparation, data collection and analysis were performed by SL and ZZ. The first draft of the manuscript was written by SL and ZZ. JT was responsible for the rigorous revision of important intellectual content. All authors contributed significantly to editorial changes of important content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and the protocols approved by Laboratory Animals of CLOUD-CLONE CORP. WUHAN Ethics Committee (IACU18-0155).

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Conflict of Interest

The authors declare no conflict of interest.

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