Journal of Inflammation Research Dovepress

Open Access Full Text Article ORIGINAL RESEARCH

The Combination of Super-Active Platelet Lysate and Acellular Amniotic Membrane Enhances Endometrial Receptivity, While Simultaneously Facilitating Endometrial Repair in Rats

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Purpose: To investigate the combined effects of super-active platelet lysate (sPL) and acellular amniotic membrane (AAM) in promoting endometrial repair and enhancing endometrial receptivity in rats.

Methods: The characteristics of sPL-AAM were examined through scanning electron microscopy, contact angle tester, and release experiments. We aimed to establish a rat model for endometrial injury. We divided sixty-four rats into four groups: the Injury group (Control group), the AAM group, the sPL group, and the sPL-AAM group. Our study compared the endometrial thickness, gland count, and fibrotic area recovery in rats at 6 days and 18 days post-treatment. Immunohistochemistry was utilized to assess the expressions of CD34 and ANG. Additionally, we used ELISA to detect the levels of IL-6 and TNF-α, while Western Blot was employed to compare the expressions of CK19, Integrin β3, and TGF-β1. One month after the treatment, we evaluated and compared the pregnancy recovery among the groups.

Results: Compared to the Injury group, the sPL-AAM group demonstrated enhanced endometrial regeneration in rats at both 6 days and 18 days post-treatment, resulting in a favorable pregnancy outcome. This was achieved by promoting angiogenesis, suppressing the inflammatory response, and reducing fibrosis. The observed effects were superior to those of the sPL group alone. While sPL, when administered alone, showed some degree of endometrial restoration at 6 days post-treatment, its efficacy was diminished at 18 days post-treatment. The impact of AAM alone appeared inconspicuous compared to the injury group. This suggests that sPL serves as the primary agent in facilitating endometrial repair, while AAM functions as a carrier to extend the duration of sPL's effectiveness.

Conclusion: sPL-AAM can release effective cytokines, repair endometrial damage in rats, enhance endometrial receptivity, and ultimately improve pregnancy outcomes.

Keywords: sPL, AAM, endometrial damage, endometrial regeneration

Introduction

Endometrial receptivity pertains to the endometrium's capacity to embrace embryos, specifically, its condition that enables embryo implantation.[1,2] When the endometrium is receptive to embryo implantation, this brief yet pivotal phase is known as the implantation window. Ensuring the implantation of embryos with robust developmental prospects within this window holds the essence of a successful pregnancy. However, procedures involving the uterine cavity, alongside inflammation and infection, emerge as prevalent risk factors for endometrial damage. Such damage can diminish endometrial receptivity, ultimately resulting in endometrial thinning, intrauterine adhesions, infertility, and abortions.[3,4]

Promoting endometrial regeneration, enhancing receptivity, and increasing pregnancy rates have been at the forefront of clinical research. Clinically proven methods include hormone replacement therapy to stimulate endometrial growth.[5,6] Additionally, intrauterine perfusion of granulocyte colony-stimulating factor has effectively restored the endometrium.[7]

Received: 15 August 2024
Accepted: 28 November 2024
Published: 17 December 2024

Other strategies include using intrauterine devices, balloons, and biological barriers to prevent adhesions following intrauterine procedures.[8] Recent studies have demonstrated promising results with stem cells from various sources in mitigating endometrial fibrosis, repairing damaged endometrium, and enhancing uterine receptivity.[9–12] These stem cells benefit primarily through their differentiation capabilities, paracrine activity, and immune modulation.[13] However, clinical application still faces limitations, such as immunogenicity and tumorigenicity concerns.[14]

The application of biomedical materials in endometrial repair and regeneration can expedite cell migration, gland formation, and vascular regeneration. Integrating the material's attributes with stem cells and drugs creates a scaffold structure, ensuring the repair's durability. The material must possess characteristics such as biodegradability, excellent biocompatibility, robust mechanical properties, and an absence of immunogenicity. Common scaffold materials utilized in endometrial repair encompass hydrogels and films.[15,16] Currently, a growing number of studies are dedicated to exploring the use of in-utero scaffold structures. By integrating stem cells, drugs, and cell factors with these scaffolds, they can undergo natural degradation or absorption, ensuring that the therapeutic cells remain within the uterine cavity for an extended period, thus facilitating effective treatment.[17,18] When choosing appropriate scaffold materials, it is crucial to evaluate their interactions with stem cells, drugs, or cell factors, taking into account factors such as biocompatibility, pore size, and shape.

Platelet rich plasma (PRP) is a concentrate derived from whole blood via centrifugation, rich in platelets and growth factors. The literature has reported on the role of PRP in promoting endometrial regeneration.[19,20] Super-active platelet lysate (sPL), relies on PRP to stimulate platelets and produce activated platelet-rich plasma. The patented technology involves repeated freezing and thawing, enabling swift platelet induction and activation, and releasing elevated levels of biological cytokines. sPL's superior cytokine content makes it biologically more potent than PRP in fostering tissue repair and cell regeneration.[21] The human amniotic membrane (AM), sourced from the inner lining of the placenta, boasts several favorable properties: excellent biocompatibility, degradability, non-toxicity, and a specific degree of tensile strength.[22,23] This membrane can be tailored to match the dimensions of the uterine cavity, ensuring a more precise fit. Furthermore, the process of amniotic membrane decellularization diminishes its immunogenicity, facilitating the colonization and migration of active factors. Our approach has integrated sPL with acellular amniotic membrane (AAM) to create a composite. The AAM serves as a three-dimensional scaffold for sPL, enabling a higher concentration of the latter to accumulate in the affected area. This ensures an even distribution across the endometrium, allowing sPL to adhere firmly without being dislodged from the uterine cavity. To date, no prior research has explored the combination of sPL and AAM. Therefore, our study aims to investigate the combined effects and mechanisms of sPL and AAM in enhancing endometrial receptivity and facilitating repair, ultimately paving the way for advancing new methods to treat endometrial injury.

Materials and Methods

Ethic

The experimental subjects were exclusively female Sprague Dawley (SD)rats, weighing between 240–260g and aged 8–10 weeks (SPF grade, Department of Experiment Zoology at Harbin Medical University, China). The rats were housed in an environment with controlled lighting conditions, alternating between 12 hours of light and 12 hours of darkness, and had unrestricted access to water and food. All animal experimentation procedures were conducted with the explicit approval of the Ethics Committee for Laboratory Animal Management and Welfare at the First Affiliated Hospital of Harbin Medical University. Furthermore, every researcher involved in these animal experiments had completed the training and assessment administered by the Heilongjiang Society of Laboratory Animals. Additionally, the use of amniotic membranes in the experiments was also sanctioned by the Ethics Committee of the First Affiliated Hospital of Harbin Medical University. The patients provided informed consent, in accordance with the Declaration of Helsinki.

Preparation of AAM

The amniotic membranes were sourced from three mothers who underwent cesarean section deliveries. These mothers had no prior history of human immunodeficiency virus, syphilis, hepatitis B virus, or hepatitis C. Additionally, any

complications during pregnancy related to endometritis, or premature rupture of membranes were ruled out. Under sterile conditions, the amniotic membrane was carefully separated from the placenta and placed in normal saline. The membrane was spread onto a 10cm Petri dish inside a biological safety cabinet, and the villus tissue was meticulously excised. Following this, the membrane underwent repeated rinsing with normal saline. Next, the amniotic membrane was precisely cut into 5cm x 5cm squares and submerged in 0.25% trypsin EDTA (GIBCO). To ensure the complete removal of surface adhesions and pancreatic enzymes, the membrane underwent three thorough washes with normal saline. Subsequently, 1% Triton X-100 (Solarbio) was added to immerse the membrane. This was followed by three additional rinses with normal saline. The now acellular amniotic membrane was then cut into 1.5cm x 0.5cm pieces, rinsed once or twice with 75% ethanol, and washed three times with normal saline. Then it was treated with a solution containing 100 u/mL penicillin and 100 μg/mL streptomycin for 10 minutes. Afterward, the acellular amniotic membranes were dried in a lyophilizer (alpha 1–4 LD plus, Christ, Germany) for 8 hours, sealed, and stored at −20°C for future use.

Preparation of sPL

sPL was supplied by Tian Qing Stem Cell Co., Ltd (Harbin, China). The preparation of sPL followed the methodology outlined in a previous study.[24] A total volume of 100mL of blood was gathered from 10 healthy SD rats via cardiac puncture while under anesthesia. PRP was acquired after two centrifugation processes. To stimulate the platelets and facilitate the release of cytokines, an ultra-low temperature freezing technique was employed. Following centrifugation at 2000 rpm and 4°C, the supernatant underwent filtration through a 0.22 μM filter, yielding the sPL. A total of 20mL of sPL was then prepared and preserved at −80°C after being sub-packaged.

Preparation and Detection of sPL-AAM

The sPL 200μL was securely affixed to a 1.5cm x 0.5cm AAM, and subsequent detections of VEGF and PDGF releases were conducted on days D1, D3, D5, D7, D9, D12, D15, and D18. AAM and sPL-AAM were lyophilized and sprayed with gold, and their surface structure and pore size were examined under a scanning electron microscope (SEM). A contact angle tester (jy-82c, Dingsheng in Chengde, China) was utilized to assess the hydrophilic nature of the materials by measuring the contact angle between fresh acellular amniotic membrane (FAAM), freeze-dried acellular amniotic membrane (FDAAM), sPL-AAM composite, and water. A 1x1cm thin-film sample was meticulously prepared, and the contact angle measurements were conducted using the sessile drop method, wherein 16 microliters of water were gently dispensed onto the center of the sample. Each sample underwent three rounds of measurement to ensure accuracy. The process facilitated real-time, rapid image acquisition, and subsequent determination of the contact angle.

Animal Experiment Design

Sprague Dawley rats underwent surgery under strict sterile conditions. Anesthesia was induced using 1% sodium pentobarbital (30mg/kg), and the abdominal cavity was subsequently accessed. To establish the experimental model, a mechanical infection method was employed. Specifically, an incision was made 0.5cm above the uterine bifurcation, and the uterine cavity was gently scratched using a micro curette until congestion and edema were observed. Then, 1mg/kg of lipopolysaccharide (LPS, Solarbio) was administered. A total of 64 rats in estrus were randomly allocated to four different groups, with 16 rats in each group. These groups were as follows: Injury group, which received no further treatment post-operation; AAM group, a 1.5cm x 0.5cm piece of AAM was implanted in one uterine horn, while the contralateral horn underwent the same procedure; sPL group, 200ul of sPL was injected into one uterine horn, while the contralateral horn underwent the same procedure; and sPL-AAM group, a 1.5cm x 0.5cm sPL AAM complex was positioned in one uterine horn, while the contralateral horn underwent the same procedure. At 6 days and 18 days post-treatment, six rats from each group were sacrificed for histological, immunohistochemical, Western blot, and ELISA analyses. One month after treatment, four rats from each group cohabitated with male rats at a 1:1 ratio for mating purposes. This process was facilitated by nightly mating sessions, and vaginal smears were collected at 9 a.m. the following morning. The conception date was determined based on the weight variation and the presence of sperm under microscope examination. Laparotomy was then performed between 17–19 days of gestation to assess pregnancy outcomes. The number of pregnant rats, the embryo count, and the conception date were all documented.

ELISA

The sPL OD (optical density detected at 450nm by microplate reader) value was ascertained in compliance with the VEGF ELISA Kit (elabscience) and PDGF ELISA Kit (elabscience) prerequisites, and the concentration was delineated in pg/mL as per the designated formula. Uterine lysates were methodically prepared by the kit's directives, and the aggregate protein concentration was subsequently ascertained. The samples underwent rigorous analysis utilizing the TNF-a ELISA Kit (cusabio) and IL-6 ELISA Kit (cusabio), with the concentration being normalized to pg/mg.

Histology and Immunohistochemistry

(1) H&E staining: Rat uterine tissues underwent fixation with 4% paraformaldehyde and were subsequently embedded in paraffin. Serial sections of 5 micrometers were prepared and stained with hematoxylin and eosin. Examination of the stained sections was carried out under a light microscope, where four high-power fields were randomly chosen for the enumeration of glands. The endometrial thickness was measured four times in each image using Image J software (National Institute of Health, USA).

(2) Masson's trichrome staining: The Masson Trichrome Kit (Solarbio) was utilized to stain sections of rat uterine tissue, which were then examined under a light microscope. The fibrotic area was subsequently analyzed using Image J software.

(3) Immunohistochemistry: The sections underwent incubation with the primary antibody (Anti-Angiogenin (ANG), Abcam; Anti-CD34, Abcam) at 4°C overnight. Subsequently, they were treated with a rabbit two-step kit (ZSGB-BIO), biotin-labeled Goat anti-rabbit IgG polymer, and horseradish enzyme-labeled streptavidin working solution for 30 minutes at room temperature. Diaminobenzidine (DAB) was then used for color observation. The expression levels of ANG and CD34 were analyzed using Image J software.

Western Blot

Proteins from rat uterine tissues were efficiently separated using RIPA buffer (Beyotime). These proteins underwent SDS-PAGE separation on polyacrylamide gradient gels and were then transferred onto polyvinylidene difluoride (PVDF) membranes. Following this, the membranes were blocked using sealing fluid. Subsequently, the PVDF membranes were incubated with specific primary antibodies, namely anti-TGF-β1 (Abcam,1:1000), anti-Integrin β3 (Abcam,1:1000), and anti-CK19 (Abcam, 1μg/mL), and allowed to react overnight at 4°C. This was followed by a 1-hour incubation period with the appropriate secondary antibodies (1:10000) at room temperature. After this incubation, the membranes were washed thrice with 1×TBST and then scanned using the Odyssey infrared imaging system. Finally, the scanned images were analyzed using ImageJ software to obtain the desired results.

Statistical Analysis

All data have been represented as mean (M)± standard deviation (SD). To compare the mean values of each group, we employed a one-way analysis of variance (ANOVA) along with Tukey's multiple comparison test. For analysis and graphical representation, we utilized GraphPad Prism 8 (GraphPad Software, USA). A P-value of less than 0.05 was considered statistically significant.

Results

AAM and sPL-AAM Features

The scanning electron microscope revealed a significant quantity of collagen fibers on the surface of AAM, intricately woven in a reticular pattern, accompanied by visible pores of varying dimensions (Figure 1a). Upon sPL binding, it firmly adheres to these pores, resulting in the AAM surface being coated with active factors (Figure 1b). The contact angle serves as an indicator for assessing the hydrophilicity and histocompatibility of scaffold materials. After freeze-drying, the hydrophilicity of the acellular amniotic membrane decreases, and the contact angle increases (Figure 1c, d and f). However, upon combining it with sPL, a notable decrease in the contact angle was observed, along with rapid absorption of water droplets (Figure 1e and f). This suggests that sPL-AAM exhibits excellent tissue compatibility.

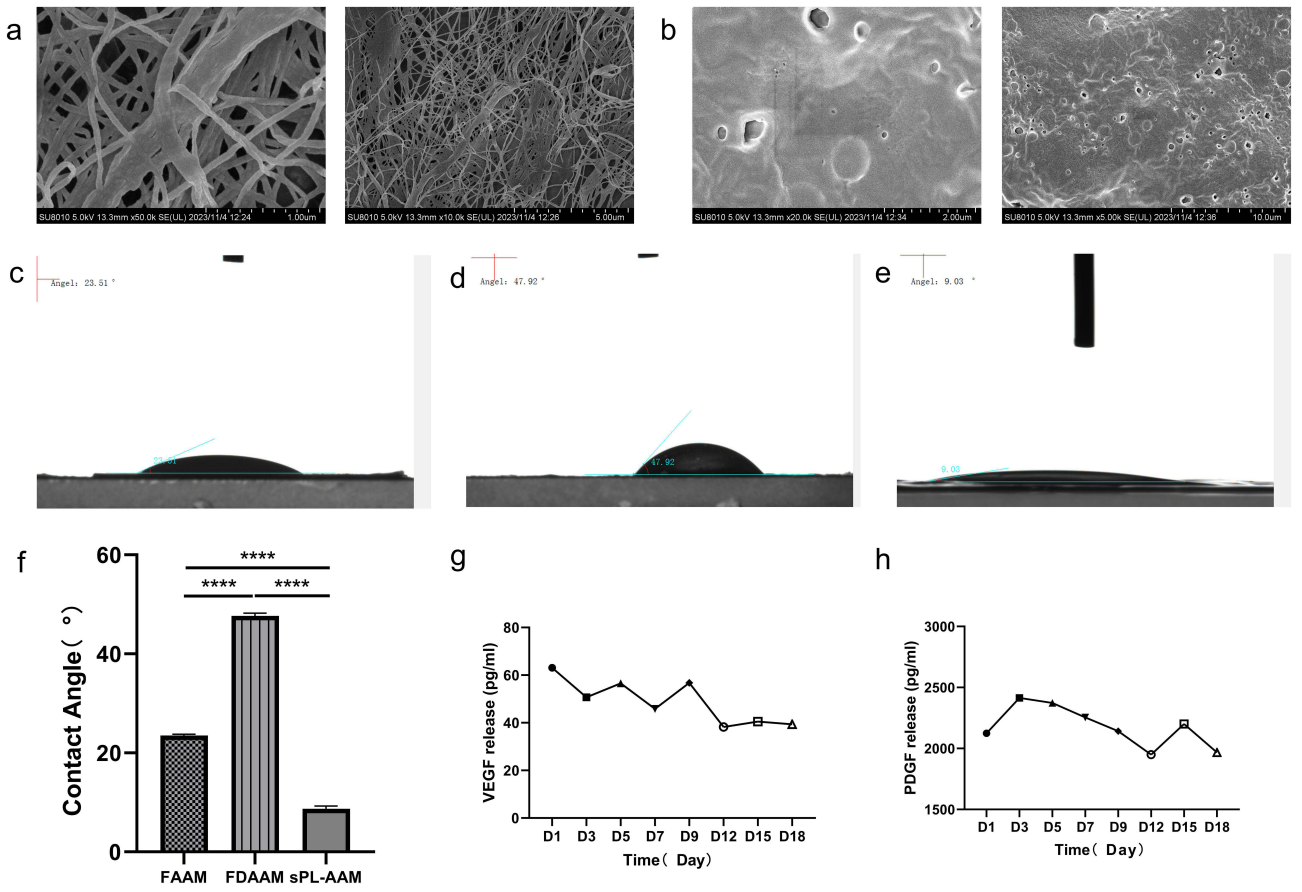

Figure 1 (**a**) AAM under SEM. (**b**) sPL-AAM under SEM. (**c**) Contact angle with water of FAAM. (**d**) Contact angle with water of FDAAM. (**e**) Contact angle with water of sPL-AAM. (**f**) Comparison of contact angles between different samples (n=3). ****P<0.0001. (**g**) Release of VEGF from the sPL-AAM (n=3). (**h**) Release of PDGF from the sPL-AAM (n=3).

Meanwhile, the sPL-AAM complex exhibits a consistent release of VEGF and PDGF active factors over 18 days (Figure 1g and h).

Differential Gene Expression

RNA sequencing was performed on three samples from both the Injury group and the sPL- AAM group. Differential gene expression analysis revealed a noteworthy disparity in gene expression between the two groups post-treatment. Specifically, 2419 mRNAs exhibited differential expression, comprising 1162 genes that were markedly upregulated and 1257 genes that were notably downregulated (Figure 2a). Furthermore, Gene Ontology (GO) analysis indicated that the differentially expressed genes in both groups were predominantly associated with biological processes, including cell integrity, cell development (particularly epithelial tissue proliferation), cell migration, inflammatory response, and stimulus-response (Figure 2b).

sPL-AAM Effectively Enhances Endometrial Repair and Epithelial Regeneration in Rats

After an endometrial injury, the original endometrial structure is compromised. Specifically, the endometrium becomes thinner, the gland count diminishes, and fibrosis proliferates. When comparing the AAM group to the Injury group 6 days post-treatment, it becomes evident that the thickness and gland count in the AAM group have not recovered substantially. While fibrosis (42.44±2.58% vs 51.95±7.47%) has slightly subsided, the distinction is statistically noteworthy (Figure 3a–e). Using sPL as a standalone treatment effectively restores endometrial thickness (532.40±35.41μm vs 412.40±24.56μm), mitigates Fibrosis (37.01±5.61%vs 51.95±7.47%), and rejuvenates the overall uterine morphology (Figure 3a–c and e). However, the gland count remains largely unchanged (Figure 3a and d). In contrast, the sPL-AAM

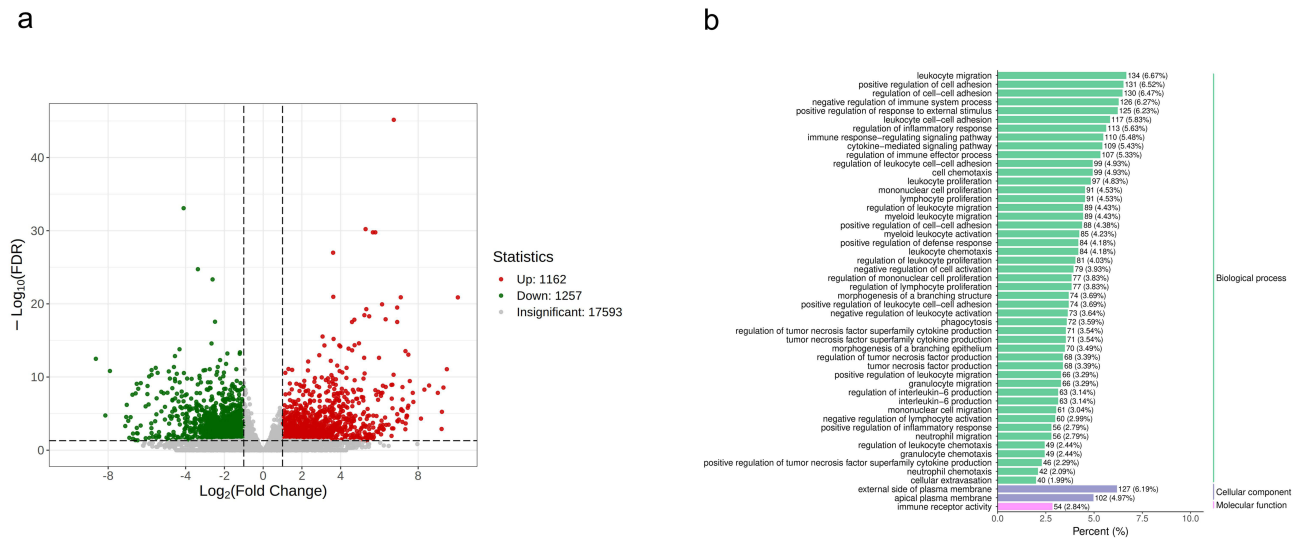

Figure 2 (**a**) Differential gene expression in two groups. (**b**) Go analysis of differential gene expression enrichment regions.

composite group experiences a remarkable endometrium recovery, accompanied by partial epithelial rejuvenation. Both the thickness (634.42±45.06μm vs 412.40±24.56μm) and gland count (6.88±1.23 vs 3.00±0.76) surpass those of the Injury group, exhibiting superior outcomes to both the AAM-only and sPL only groups (Figure 3a, c and d). Furthermore, the fibrotic area (34.22±3.96% vs 51.95±7.47%) is reduced compared to the Injury group (Figure 3b and e). 18 days post-treatment, the endometrium recovery in both the AAM and sPL groups remains unimpressive when juxtaposed with the Injury group (Figure 3f). Nevertheless, the sPL-AAM group demonstrates a notable increase in thickness (734.33±31.84μm vs 416.81±25.84μm) and gland count (7.54±2.76 vs 3.13±1.31), along with a decrease in the fibrotic area (35.96±4.55% vs 50.56±8.37%). These differences are statistically significant (Figure 3f–j). In conclusion, sPL-AAM treatment is a catalytic agent in promoting endometrial repair and regeneration in rats. Simultaneously, we noticed that AAM persisted partially in the uterine cavity for 6 days post-treatment and was fully absorbed after 18 days. The findings revealed that AAM exhibited excellent degradability, making it an apt carrier for sPL.

sPL-AAM Restores Fertility in Rats

One month post-treatment, the pregnancy experiment commenced, involving the cohabitation of female and male rats for a month. Subsequently, the pregnant female rats underwent laparotomy between the 17th and 19th days of gestation. Notably, no pregnancies were observed in the Injury and AAM groups. However, in the sPL group, 2 rats were pregnant with an average embryo count of 6, while in the sPL- AAM group, 3 rats were pregnant with a higher average embryo count of 11.67 (Figure 4a–c and Table 1). These findings suggest that the sPL-AAM group significantly enhanced the rats' fertility, favoring embryo implantation and development.

sPL-AAM Promotes Angiogenesis

The sPL-AAM complex facilitates the healing of damaged intima by enhancing the development of blood vessels. CD34 and ANG are intricately linked to angiogenesis, the process by which blood vessels supply oxygen and vital nutrients to the endometrium,[25] fostering its growth. After 6 days of treatment, the expression of CD34 and ANG in the sPL group and sPL-AAM group were significantly increased compared with the Injury group (Figure 5a and b). This suggests that sPL can be instrumental in boosting blood vessel formation during the initial treatment phase. However, at 18 days of treatment, the expression of CD34 and ANG in the sPL-AAM group was significantly higher than in the sPL group, and the difference was statistically significant (Figure 5c and d). This indicates that while sPL alone may lose its efficacy over time, the sPL-AAM complex can prolong sPL's duration of action, leading to more sustained results.

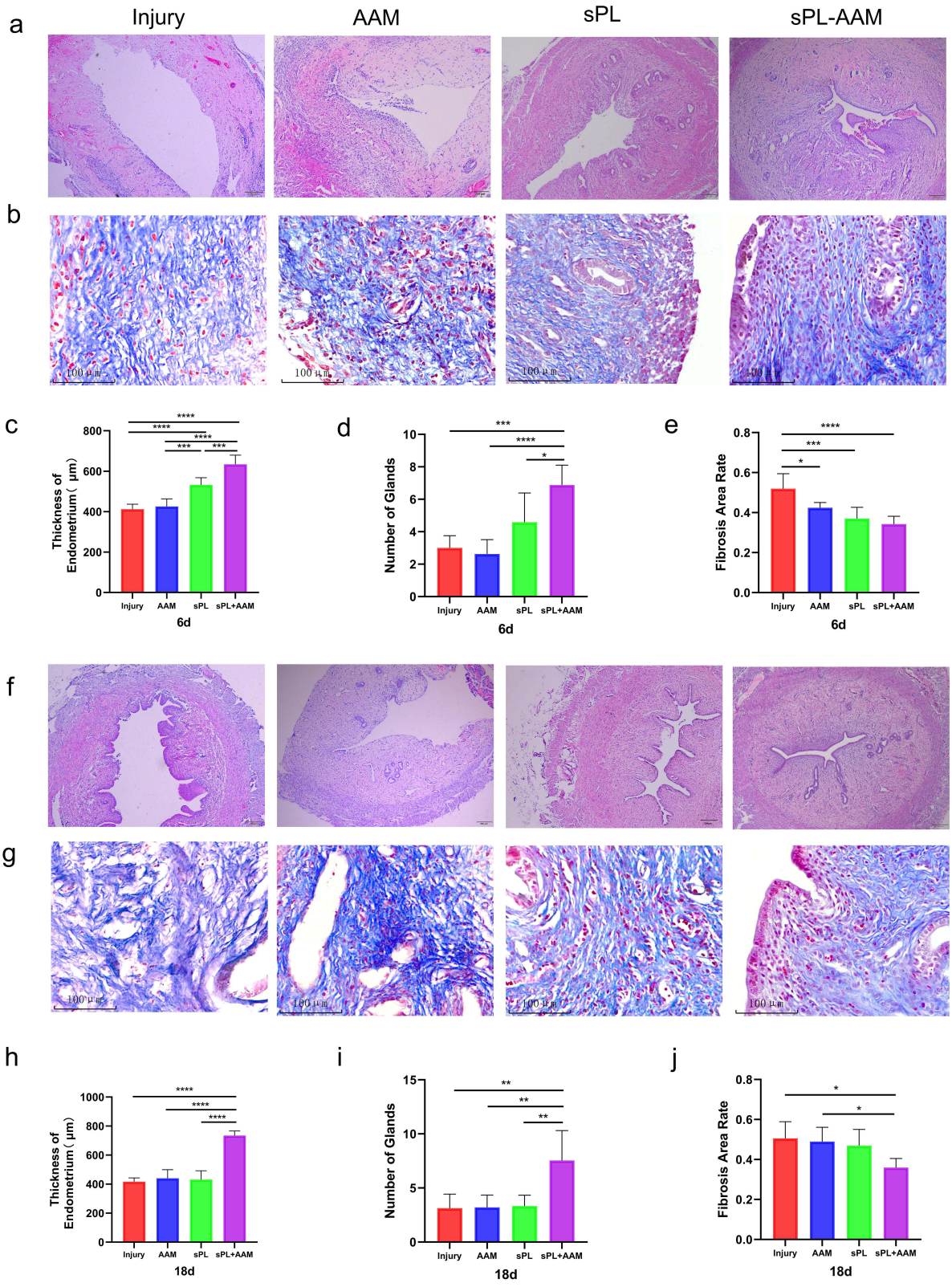

Figure 3 (**a**) Images of uteri treated for 6 days, examined using H&E staining (40X). (**b**) Images of uteri treated for 6 days, examined using Masson's trichrome staining (200X). (**c**) Quantification of endometrial thickness at 6 days after treatment (n=6). (**d**) Quantification of endometrial glands at 6 days after treatment (n=6). (**e**) Quantification of endometrial fibrosis area at 6 days after treatment (n=6). (**f**) Images of uteri treated for 18 days, examined using H&E staining (40X). (**g**) Images of uteri treated for 18 days, examined using Masson's trichrome staining (200X). (**h**) Quantification of endometrial thickness at 18 days after treatment (n=6). (**i**) Quantification of endometrial glands at 18 days after treatment (n=6). (**j**) Quantification of endometrial fibrosis area at 18 days after treatment (n=6). *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

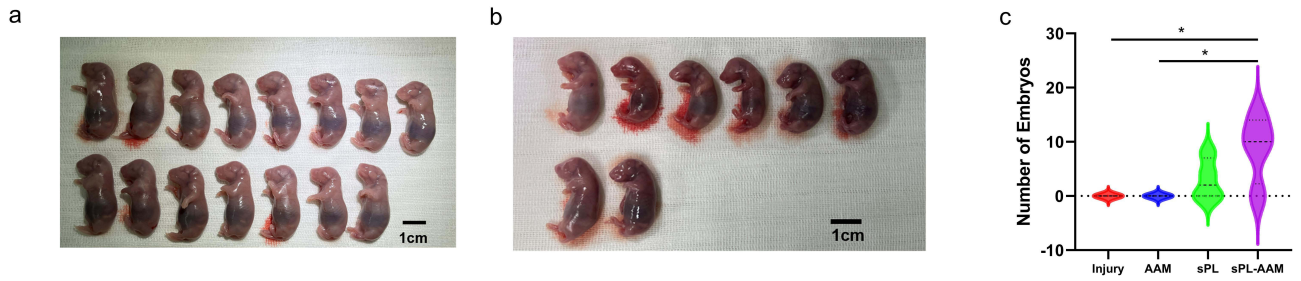

Figure 4 (**a**) Rat embryos from the sPL group. (**b**) Rat embryos from the sPL-AAM group. (**c**) Violin plots showing the number of embryos per uterus following different groups. *P< 0.05.

sPL-AAM Promotes Endometrial Repair by Inhibiting Inflammatory Response

We employed ELISA to assess the inhibitory effects of inflammatory factors in distinct groups post-treatment, revealing that the sPL-AAM complex exerts an immunomodulatory function by suppressing the inflammatory response. After 6 days of treatment, the expression of IL-6 and TNF-α significantly decreased in the sPL group and sPL-AAM group compared to the Injury group (Figure 6a and b). 18 days after treatment, compared with the Injury group, the sPL group showed a decrease in IL-6 expression (Figure 6c); the sPL-AAM group showed a decrease in the expression of IL-6 and TNF-α (Figure 6c and d). This finding underscores the stable efficacy of the sPL-AAM complex.

sPL-AAM Effectively Hinders Fibrosis and Enhances Endometrial Receptivity

sPL-AAM has the potential to stimulate endometrial proliferation and restoration, suppress fibrosis, and improve endometrial receptivity. After 6 days of treatment, the expression of CK19 and Integrin β3 in the sPL and sPL-AAM groups were significantly increased compared with the Injury group, while TGF-β1 decreased (Figure 7a and b). Furthermore, when sPL-AAM was compared to sPL alone, CK19 and Integrin β3 expression levels were notably higher (Figure 7b). 18 days after treatment, the expression of CK19 and Integrin β3 in the sPL-AAM group was significantly increased when compared to both the sPL and Injury groups, while TGF-β1 decreased (Figure 7c and d). The therapeutic effect observed in the sPL-AAM group was superior to that in the sPL alone group.

Discussion

As a result of the recent surge in intrauterine procedures, including uterine cavity operations and abortions, there has been a rise in the number of infertile patients stemming from endometrial damage.[26] Consequently, addressing the repair of the injured endometrium has emerged as a pressing clinical concern. Endometrial repair aims to foster endometrial regeneration, mitigate fibrosis, enhance endometrial receptivity, and optimize pregnancy outcomes.[23]

The amnion does not comprise blood vessels, nerves, or lymphatic vessels. It consists of five distinct layers: the epithelial layer, which adheres to the basement membrane, the dense layer, the fibroblast layer, and the sponge layer.[27] Owing to its excellent biocompatibility, the amnion finds extensive application in tissue engineering.[28] Decellularization effectively removes cells and cellular debris from the amnion while preserving the extracellular collagen structure, thus minimizing the immunogenic response and the potential for infection.[29]

Table 1 Reproductive Outcomes Following Different Groups

Therapy	Number of Pregnancy	Pregnancy Time (day)	Pregnancy Rate
Injury	0		0
AAM	0		0
sPL	2	8	50%
sPL-AAM	3	4	75%

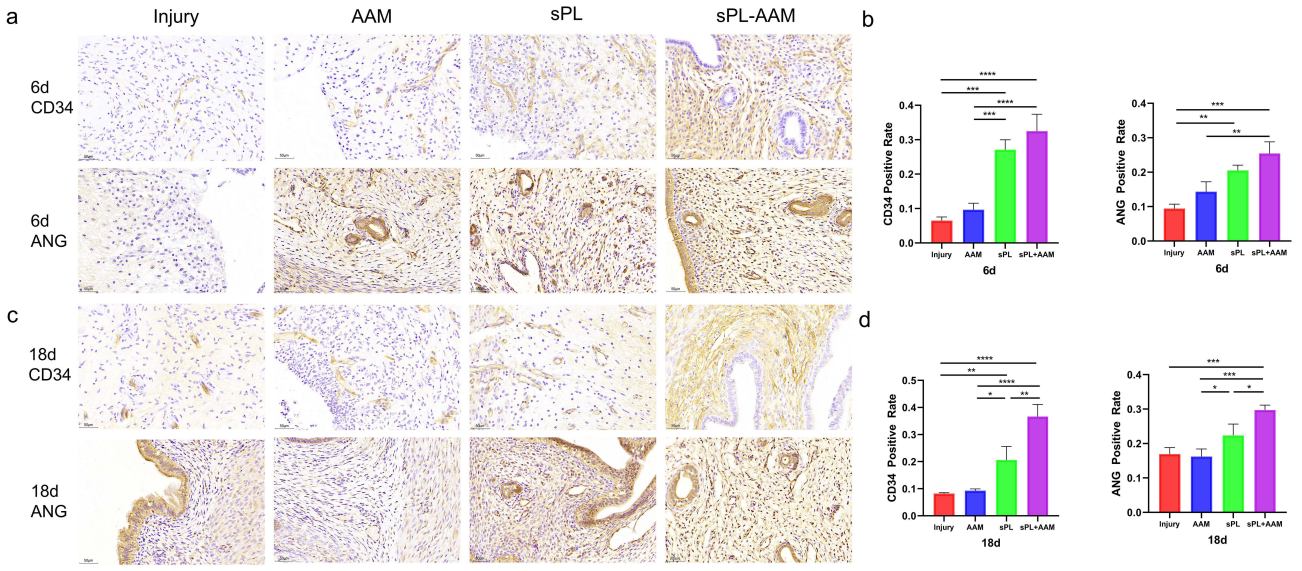

Figure 5 (**a**) CD34 and ANG immunohistochemistry 6 days after treatment of uteri (400X). (**b**) Statistical analysis of CD34 and ANG positive rates after 6 days of treatment (n=3). (**c**) CD34 and ANG immunohistochemistry 18 days after treatment of uteri (400X). (**d**) Statistical analysis of CD34 and ANG positive rates after 18 days of treatment (n=3). *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

sPL, derived from PRP, boasts uniform particles and high concentrations of bioactive factors that actively foster tissue regeneration and cell proliferation.[30] Current research substantiates sPL's therapeutic potential in orthopedic and oral-related maladies.[31,32] When combined with AAM, a loosely porous structure, sPL benefits from a three-dimensional environment that facilitates the stable release of its active factors, thereby extending its therapeutic duration. Notably, the contact angle plays a pivotal role in determining biocompatibility and cell adhesion.[33] Hydrophilic materials enhance droplet spreading on surfaces, exhibiting strong biocompatibility due to their improved interaction with bodily fluids,[34] thereby minimizing the risk of rejection. Upon implantation, the sPL-AAM composite interfaces directly with tissue, leveraging its hydrophilicity to foster adhesion with tissue cells and promote tissue regeneration.

Our study demonstrates that sPL, in isolation, can stimulate intimal proliferation and repair while inhibiting fibrosis during the early phases of injury. However, over an extended period, the continuous release of beneficial factors from sPL becomes restricted, thus diminishing its reparative effects in the later stages of injury. AAM offers a scaffold structure for sPL, prolonging its therapeutic duration, fostering endometrial regeneration, and enhancing endometrial receptivity. Despite this, we noted that rats in both the Injury and AAM groups failed to achieve pregnancy one month post-treatment. Although the pregnancy was not achieved, we opted to perform a laparotomy to investigate the underlying reasons. Upon dissection, it became evident that a majority of the rats in the Injury and AAM groups exhibited upper uterine cavity effusion or ovarian effusion and swelling. This could potentially be attributed to the activation of the inflammatory response triggered by post-modeling curettage and lipopolysaccharide, leading to ovarian damage, endometrial repair dysregulation, and ultimately, the absence of pregnancy.

ANG and CD34 are instrumental in the modulation of angiogenesis. They foster the growth of endothelial cells, thus bolstering angiogenesis. This, in turn, nourishes the damaged site and stimulates endometrial repair.[35,36] When endometrial harm occurs, the blood vessels seal off. However, treatment with sPL-AAM leads to heightened expressions of both ANG and CD34. This is a testament to sPL-AAM's efficacy in spurring endometrial recuperation through enhanced vascular proliferation. Moreover, endometrial harm triggers an acute inflammatory reaction, causing an upsurge in inflammatory markers and participating in immune modulation.[37] Here, inflammatory and fibrotic factors coalesce, jointly propelling fibrosis.[38] The activation of TGF-β1 also hastens the fibrotic process, upping the risks of intrauterine adhesions, and even inducing the Epithelial Mesenchymal Transition (EMT).[39] sPL-AAM mitigates the inflammatory response by downregulating IL-6 and TNF-α expressions. By suppressing TGF-β1 expression, it curtails endometrial fibrosis, thereby facilitating endometrial restoration. CK19, a structural protein maintaining endometrial cell integrity,[40]

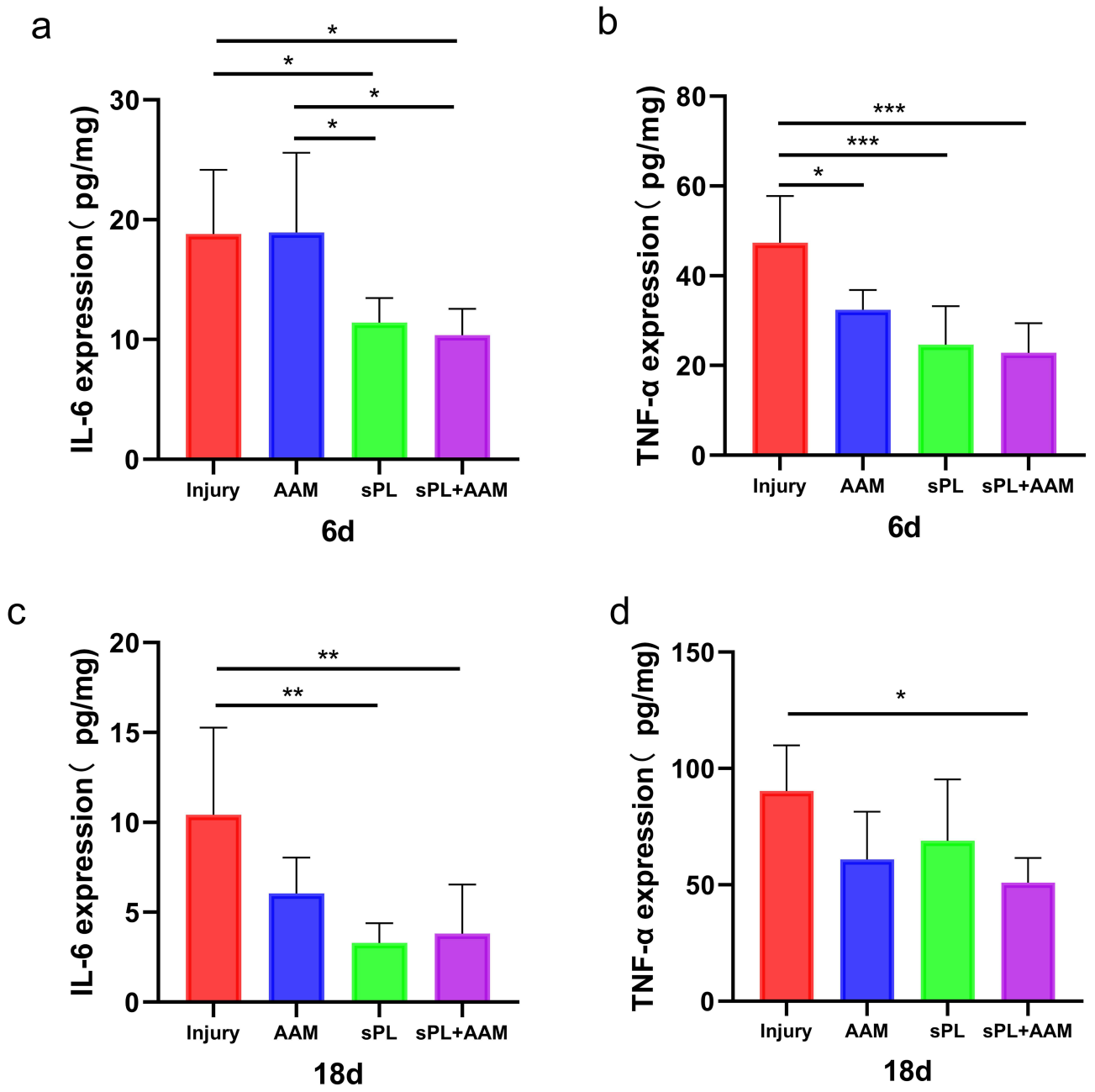

Figure 6 (**a**) ELISA for IL-6 after 6 days of treatment in uteri (n=6). (**b**) ELISA for TNF-α after 6 days of treatment in uteri (n=6). (**c**) ELISA for IL-6 after 18 days of treatment in uteri (n=6). (**d**) ELISA for TNF-α after 18 days of treatment in uteri (n=6). *P< 0.05, **P < 0.01, ***P < 0.001.

is intimately linked with the multiplication and differentiation of endometrial and glandular epithelial cells. Its expression diminishes post-injury.[41] However, CK19 expression was elevated in the sPL-AAM group, indicating its role in advancing endometrial epithelialization during injury. Endometrial receptivity is the capacity of the endometrium to harbor embryos and is related to successful pregnancy outcomes. Key factors evaluating endometrial receptivity are endometrial thickness and uterine artery blood flow.[2,42] Integrin β3 serves as a distinctive marker for endometrial receptivity.[43,44] According to a meta-analysis and systematic review by Wu et al,[45] endometrial thickness and receptivity directly influence pregnancy outcomes. sPL-AAM therapy not only augmented endometrial thickness but also triggered angiogenesis, accompanied by an increase in integrin β3 expression. These observations collectively suggest that sPL-AAM enhances endometrial receptivity and could potentially optimize pregnancy rates.

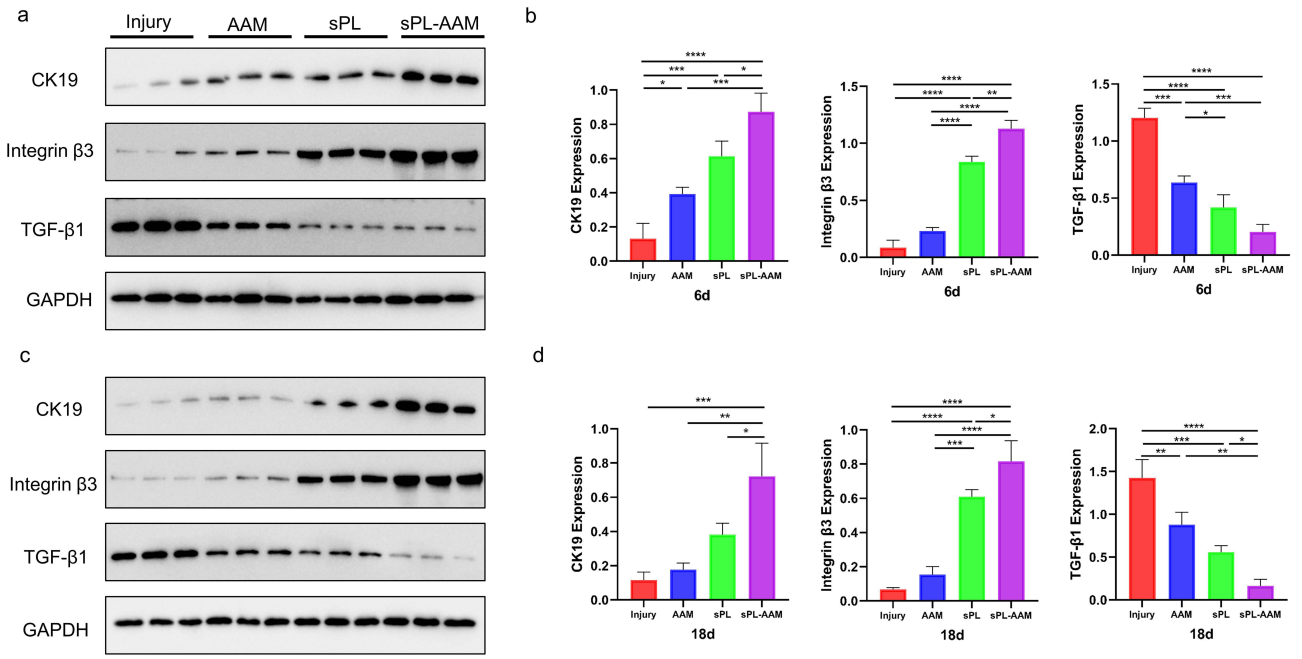

Figure 7 (**a**) CK19, Integrin β3, and TGF-β1 Western Blot 6 days after treatment of uteri. (**b**) Statistical analysis of CK19, Integrin β3, and TGF-β1 expression after 6 days of treatment (n=3). (**c**) CK19, Integrin β3, and TGF-β1 Western Blot 18 days after treatment of uteri. (**d**) Statistical analysis of CK19, Integrin β3, and TGF-β1 expression after 18 days of treatment (n=3). *P< 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

This study did not establish a control group of healthy rats, which is crucial for comparing the degree of endometrial recovery between the treatment group and healthy rats. In the next experimental phase, we will rectify this oversight and make necessary improvements. Simultaneously, we aim to conduct a more comprehensive investigation of the characteristics of sPL-AAM to achieve precise control over the release time and dosage of sPL. We hope to develop sPL-AAM in the future as a novel sustained-release scaffold, offering an innovative treatment approach for patients suffering from endometrial injury.

Conclusions

The sPL-AAM can efficiently release cytokines, repair endometrial damage in rats, stimulate endometrial regeneration, enhance endometrial receptivity, and ultimately achieve favorable pregnancy outcomes. The mechanism of sPL-AAM may encompass the promotion of angiogenesis, inhibition of inflammatory response, and reduction of fibrosis. Our study has demonstrated the potential of the sPL-AAM scaffold structure, paving the way for it to become a promising therapeutic approach for endometrial regeneration in the future.

Funding

The authors thank the funding support by the Postgraduate Research & Practice Innovation Program of Harbin Medical University (YJSCX2023-52HYD).

Disclosure

Ms Chunxiang Liu and Mr Lingqi Meng report a patent A preparation method and application of sPL gel for endometrial injury repair pending to Tian Qing Stem Cell Co., Ltd. The authors state that there is no conflict of interest regarding the publication of this paper.

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