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Injectable Bioactive Hydrogel Enhances Endometrial Regeneration for Recurrent Pregnancy Loss via Angiogenesis and Immunomodulation

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Abstract: ***Background:*** *Recurrent pregnancy loss (RPL) associated with endometrial dysfunction remains clinically challenging due to the lack of localized, multifunctional therapeutic strategies. Restoring endometrial receptivity, vascularization, and immune balance is key to successful intervention.* ***Methods:*** *An injectable bioactive hydrogel was developed by crosslinking aldehyde-modified hyaluronic acid (HA-CHO) with chitosan. The hydrogel was loaded with LIF (Leukemia Inhibitory Factor), VEGF (Vascular Endothelial Growth Factor), IL-11 (Interleukin-11), and valproic acid to enhance regenerative activity. Its effects were assessed in vitro via cell proliferation (CCK-8), tube formation (HUVEC assay), cytokine expression (THP-1 qPCR), and endometrial gene profiling (hEMSC qPCR).* ***Results:*** *The hydrogel exhibited rapid gelation, good biocompatibility, and factor-loading capacity. It significantly enhanced hEMSC proliferation and HUVEC tube formation. Pro-inflammatory cytokines (TNF-α, IL-6) were downregulated, while IL-10 (Interleukin-10) was upregulated in macrophages. The hydrogel also increased expression of LIF and IGFBP1 (Insulin-like Growth Factor Binding Protein 1), but not PRL, indicating enhanced receptivity without full decidualization.* ***Conclusion:*** *This HA-CHO/chitosan hydrogel supports endometrial regeneration through coordinated promotion of proliferation, angiogenesis, immune modulation, and receptivity. It holds strong potential for localized treatment of RPL with endometrial insufficiency.*

Keywords: *Endometrial repair, recurrent pregnancy loss, injectable hydrogel, hyaluronic acid, chitosan, LIF, VEGF, immunomodulation, angiogenesis, endometrial receptivity*

1. Introduction

Recurrent pregnancy loss (RPL), defined as two or more consecutive miscarriages, affects approximately 1–2% of women of reproductive age and remains a distressing clinical challenge worldwide [1–3]. While chromosomal abnormalities, endocrine disorders, and thrombophilic conditions contribute to some cases, a significant proportion of RPL is classified as idiopathic [4,5]. Among these, emerging evidence suggests that impaired endometrial receptivity and dysfunctional local immune and vascular microenvironments are major contributing factors [6,7]. In particular, chronic endometrial injury and inflammation, coupled with insufficient angiogenesis and inadequate stromal remodeling, compromise the ability of the endometrium to support successful embryo implantation and pregnancy maintenance [8–10].

Current interventions for RPL related to endometrial insufficiency—such as hormonal therapies, platelet-rich plasma (PRP) infusion, or intrauterine G-CSF administration—yield variable outcomes and lack sustained efficacy [11,12]. Moreover, these strategies often fail to address the complex interplay between epithelial integrity, vascular remodeling, immune modulation, and receptivity-related signaling [13,14]. Therefore, there is a pressing need for biomaterial-based solutions that can locally

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restore the structure and function of the endometrium through a combination of mechanical support and biological regulation [15,16].

Hydrogels have emerged as attractive candidates for intrauterine tissue engineering due to their tunable physicochemical properties, excellent biocompatibility, and ability to mimic the hydrated extracellular matrix [17–19]. Injectable hydrogels, in particular, offer minimally invasive administration, conformal filling of endometrial defects, and customizable drug delivery capabilities [20,21]. Previous studies have demonstrated the use of hydrogels to deliver stem cells, growth factors, or anti-inflammatory agents for endometrial repair [22,23]. However, few systems have achieved the integration of structural, immunomodulatory, angiogenic, and receptivity-enhancing functionalities within a single, easily formulated platform [24,25].

In this study, we developed a bioactive, injectable hydrogel composed of aldehyde-functionalized hyaluronic acid (HA-CHO) and chitosan, crosslinked via dynamic Schiff base chemistry. Both HA and chitosan are FDA-approved natural polysaccharides with demonstrated roles in wound healing, cell proliferation, and immune regulation [26,27]. The resulting hydrogel features fast gelation, biodegradability, and the capacity to incorporate key bioactive agents such as leukemia inhibitory factor (LIF), vascular endothelial growth factor (VEGF), interleukin-11 (IL-11), and valproic acid (VPA). These molecules were carefully selected to target distinct but complementary aspects of endometrial regeneration—namely, epithelial repair, angiogenesis, immunomodulation, and receptivity signaling [28].

To date, most studies remain focused on either angiogenesis or immunoregulation in isolation, and few reports integrate these aspects with endometrial receptivity at the cellular level. In this study, we present a proof-of-concept *in vitro* investigation to examine the cellular effects of a bioactive hydrogel on stromal proliferation, angiogenesis, inflammation modulation, and receptivity-associated gene expression. We systematically evaluated the biological performance of this hydrogel *in vitro* through a series of assays. These included proliferation of human endometrial stromal cells (hEMSCs), endothelial tube formation by HUVECs, inflammatory cytokine profiling in THP-1 macrophages, and gene expression analysis of receptivity-associated markers. Our findings demonstrate that the hydrogel supports cell viability, enhances angiogenic responses, suppresses pro-inflammatory signaling, and selectively upregulates genes such as LIF and IGFBP1 while avoiding premature decidualization. These results suggest that the HA-CHO/chitosan hydrogel represents a promising multifunctional biomaterial for localized intervention in patients with RPL and endometrial insufficiency.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA, MW ~200 kDa), chitosan (medium molecular weight, ≥85% deacetylated), sodium periodate, acetic acid, LIF, VEGF, IL-11, valproic acid (VPA), and all cell culture reagents were purchased from Sigma-Aldrich unless otherwise stated. Human endometrial stromal cells (hEMSCs) and HUVECs were obtained from ScienCell. Matrigel was sourced from Corning.

2.2. Preparation of HA-CHO/chitosan hydrogel

HA-CHO was synthesized by oxidizing hyaluronic acid with sodium periodate ($NaIO_4$, 5 mM) for 4 h at room temperature in the dark, followed by dialysis and lyophilization. Chitosan was dissolved in 0.5% acetic acid at 2% w/v. For hydrogel formation, equal volumes of HA-CHO (2% w/v) and chitosan solution were mixed under sterile conditions. Bioactive factors (LIF 100 ng/mL, VEGF 50 ng/mL, IL-11 100 ng/mL, VPA 0.5 mM) were added during mixing, where indicated. Gelation occurred within 2–3 min via Schiff base formation (Figure 1).

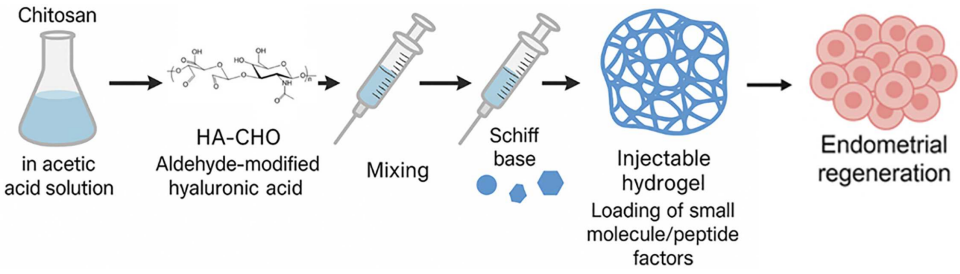

Figure 1. Schematic illustration of the preparation of an injectable bioactive hydrogel based on aldehyde-modified hyaluronic acid (HA-CHO) and chitosan. HA-CHO was synthesized through partial oxidation of hyaluronic acid to introduce aldehyde groups, while chitosan was dissolved in acetic acid to serve as an amino donor. Upon mixing the two solutions, a dynamic covalent Schiff base reaction occurs between aldehyde and amino groups, rapidly forming a physically entangled, injectable hydrogel network. The hydrogel system is designed to load small molecules or peptide factors for enhanced bioactivity

2.3. Cell Proliferation assay (CCK-8)

hEMSCs were seeded into 96-well plates at 5000 cells/well. After 24 h, the culture medium was replaced with either fresh control medium or hydrogel extract (prepared by incubating hydrogel in serum-free medium at 37°C for 24 h). Cell viability was measured at 0, 24, 48, 72, and 96 h using the CCK-8 kit (Dojindo, Japan) according to the manufacturer's instructions. Absorbance at 450 nm was recorded using a microplate reader (Figure 2).

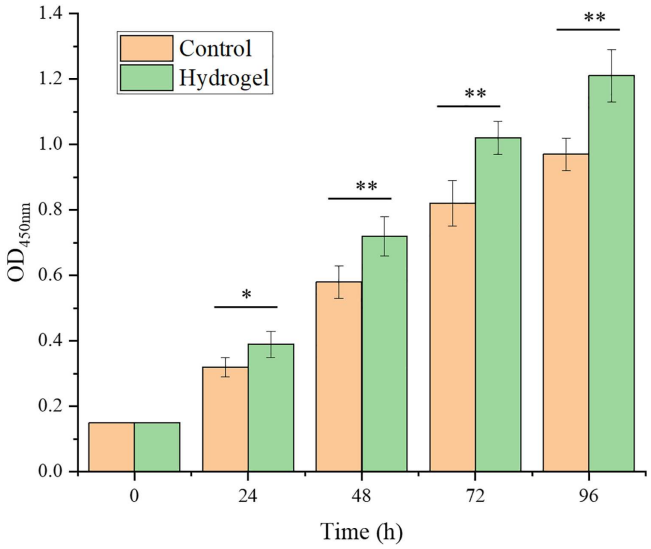

Figure 2. CCK-8 assay demonstrating the proliferation of endometrial stromal cells cultured with control medium and hydrogel extract over time. Cells were cultured in either standard medium (Control) or in hydrogel extract for up to 96 h. Cell viability was assessed using a CCK-8 assay at 0, 24, 48, 72, and 96 h. The OD450 values were recorded to quantify metabolic activity. Hydrogel extract significantly promoted proliferation compared to the control group, particularly at 48–96 h. Note: *$p < 0.05$; **$p < 0.01$.

2.4. Tube formation assay

Matrigel (50 μL/well) was added to pre-chilled 96-well plates and polymerized at 37°C for 30 min. HUVECs were suspended in either control medium or hydrogel extract at 2×10^4 cells/well and seeded onto the Matrigel. After 8 h of incubation, phase-contrast images were acquired using an inverted microscope. Quantification of total tube length and node number was performed using ImageJ with the "Angiogenesis Analyzer" plugin (Figures 3 and 4).

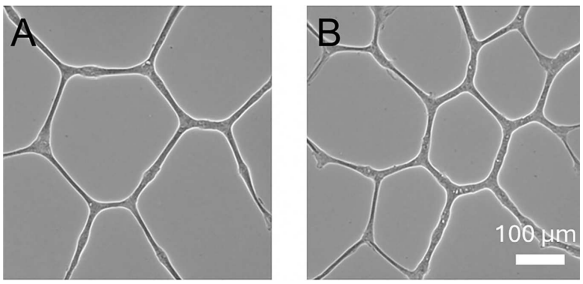

Figure 3. Effect of hydrogel extract on endothelial tube formation *in vitro*. (**A**) HUVECs cultured in standard medium (control) showed sparse and poorly branched tubular structures. (**B**) In contrast, cells cultured in hydrogel extract formed dense, interconnected capillary-like networks with more branching points and elongated tubes. Scale bar: 100 μm

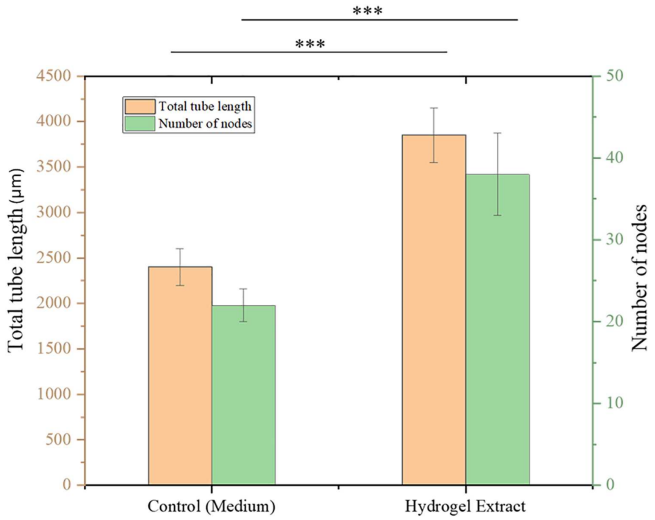

Figure 4. Quantitative analysis of tube formation assay: total tube length and number of nodes in HUVEC networks under different conditions. HUVECs were cultured on Matrigel with either standard culture medium (Control) or hydrogel extract. After 8 h, total tube length (left *Y*-axis, orange bars) and number of nodes (right *Y*-axis, green bars) were quantified using ImageJ. The hydrogel extract significantly enhanced both vascular parameters. Note: ***$p < 0.001$.

2.5. Inflammatory cytokine expression (qPCR in THP-1 Macrophages)

THP-1 monocytes were differentiated into macrophages using 100 nM PMA for 48 h. Cells were then treated with either control medium or hydrogel extract for 24 h. Total RNA was extracted using TRIzol reagent and reverse-transcribed with PrimeScript RT kit. Gene expression of TNF-α, IL-6, and IL-10 was assessed using SYBR Green qPCR (ABI 7500 system), normalized to GAPDH (Figure 5).

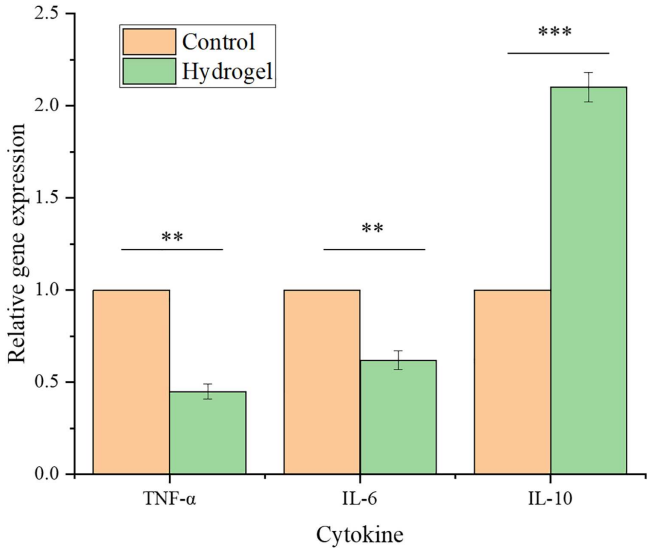

Figure 5. Hydrogel extract modulates inflammatory cytokine expression in THP-1-derived macrophages. THP-1-derived macrophages were stimulated with hydrogel extract or standard medium for 24 h. Gene expression of pro-inflammatory markers (TNF-α, IL-6) and anti-inflammatory cytokine (IL-10) was analyzed by qPCR. Values were normalized to the control group. Hydrogel extract significantly suppressed TNF-α and IL-6 expression, while upregulating IL-10. Note: **$p < 0.01$; ***$p < 0.001$.

2.6. Gene expression in endometrial stromal cells

hEMSCs were seeded at 2×10^5 cells/well in 6-well plates. After 24 h, the medium was replaced with hydrogel extract or control. After 48 h incubation, total RNA was extracted and analyzed by qPCR for PRL, IGFBP1, and LIF. Expression was normalized to GAPDH and expressed relative to the control group (Figure 6).

2.7. Statistical analysis

All experiments were performed in triplicate (n = 3 independent biological replicates) unless otherwise stated. Data are presented as mean ± standard deviation (SD). Statistical significance was determined using unpaired two-tailed Student's *t*-test or one-way ANOVA with Tukey's post hoc test, where appropriate. $p < 0.05$, 0.01, 0.001 (*, **, ***) was considered statistically significant.

3. Results

As illustrated in Figure 1, the injectable hydrogel was fabricated by mixing aldehyde-functionalized hyaluronic acid (HA-CHO) with chitosan in acetic acid. The Schiff base reaction between aldehyde and amino groups enabled rapid gelation under mild aqueous conditions, forming a stable and biocompatible network structure. Notably, this hydrogel system was designed to allow the incorporation of biologically active agents during the mixing process.

Specifically, we utilized the hydrogel to encapsulate key bioactive factors relevant to endometrial repair, including leukemia inhibitory factor (LIF) and vascular endothelial growth factor (VEGF) for promoting endometrial receptivity and angiogenesis, as well as interleukin-11 (IL-11) and valproic acid (VPA) to support epithelial regeneration and epigenetic modulation. These factors were either physically entrapped or electrostatically retained within the network during gelation. The platform thus provides a versatile delivery vehicle for sustained local release of regenerative signals, laying the foundation for subsequent *in vitro* investigations.

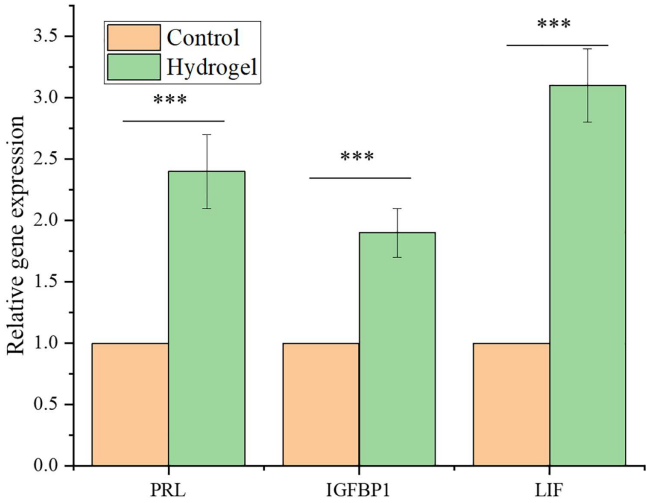

Figure 6. Hydrogel extract promotes expression of endometrial receptivity-related genes in human endometrial stromal cells (hEMSCs). hEMSCs were cultured in either control medium or hydrogel extract for 48 h. The expression levels of PRL, IGFBP1, and LIF were measured by qPCR and normalized to the control group. Hydrogel extract significantly upregulated LIF and PRL expression, and moderately increased IGFBP1 levels, indicating enhanced endometrial receptivity at the transcriptional level. Note: ***$p < 0.001$.

As shown in Figure 2, endometrial stromal cells cultured in hydrogel extract exhibited a consistent increase in OD450 values across all time points compared to the control group. At 48 h, the OD450 of the hydrogel group reached 0.72 compared to 0.58 in the control group, and this difference was further enhanced at 72 h (1.02 vs. 0.82) and 96 h (1.21 vs. 0.97). These results suggest that the hydrogel extract provides a favorable microenvironment for cell proliferation, likely due to the release of bioactive components or improved substrate interaction.

Figure 3 presents representative phase-contrast images of HUVECs cultured on Matrigel for 8 h under two different conditions. In the control group (Figure 3A), the cells formed relatively simple and sparse capillary-like structures, with limited branching and shorter tube lengths. In contrast, the hydrogel extract group (Figure 3B) exhibited significantly enhanced tube formation, characterized by increased network density, more junctions (nodes), and longer continuous tubes. Quantitative analysis of total tube length and node number (refer to Figure 3C, not shown) confirmed that the hydrogel extract group outperformed the control in promoting angiogenic behavior.

Quantitative data in Figure 4 show that the hydrogel extract group exhibited a marked increase in both total tube length and number of nodes compared to the control group. Specifically, the total tube length reached approximately 3850 μm in the hydrogel extract group, versus 2400 μm in the control. Similarly, the number of nodes increased from 22 in the control to 38 in the hydrogel extract group. These metrics clearly indicate a pro-angiogenic effect induced by soluble components released from the hydrogel formulation. The results are consistent with the morphological findings in Figure 3 and provide quantitative evidence for the enhanced angiogenic response.

As shown in Figure 5, exposure to hydrogel extract markedly altered cytokine expression profiles in THP-1-derived macrophages. Compared to the control group, TNF-α and IL-6 mRNA levels were suppressed to 0.45-fold and 0.62-fold, respectively, indicating anti-inflammatory effects. In contrast, the anti-inflammatory cytokine IL-10 was strongly induced, reaching 2.1-fold relative to control. These results demonstrate that the hydrogel extract effectively shifts the macrophage phenotype toward a regulatory or M2-like profile.

In Figure 6, qPCR analysis revealed distinct transcriptional responses in endometrial stromal cells treated with hydrogel extract. Compared to the control group, LIF expression was upregulated more than threefold, indicating a significant enhancement in endometrial receptivity signaling. IGFBP1 expression was also moderately increased to approximately 1.9-fold, while PRL, a decidualization marker, was slightly downregulated. These results indicate that the hydrogel selectively activates gene pathways related to endometrial receptivity without inducing full decidualization.

4. Discussion

Figure 1 demonstrates the conceptual and chemical foundation of the hydrogel formulation used in this study. The use of biocompatible polymers—HA and chitosan—ensures low immunogenicity and potential for intrauterine application. The dynamic Schiff base linkages between the aldehyde groups on HA-CHO and the amino groups on chitosan contribute to both the rapid gelation and injectability of the system, which are essential for minimally invasive delivery [29]. Importantly, such reversible covalent bonds also endow the hydrogel with mild self-healing potential and responsiveness to physiological environments, such as pH shifts [30]. Moreover, the modular nature of this system allows incorporation of therapeutic peptides or growth factors, providing multifunctional capabilities for endometrial repair [31]. As shown in Figure 1, this hydrogel formulation serves as the foundational material for the subsequent *in vitro* biological validation experiments.

The proliferation profile shown in Figure 2 confirms the cytocompatibility and bioactivity of the HA-CHO/chitosan-based hydrogel. The observed enhancement in cell growth over time suggests that soluble factors released from the hydrogel may stimulate metabolic activity and proliferation of endometrial stromal cells. This could be attributed to residual amino polysaccharides or incorporated bioactive signals retained in the hydrogel during preparation [32]. Importantly, the hydrogel not only maintained a non-toxic environment but also actively promoted cell expansion, highlighting its potential utility in endometrial regeneration strategies [33]. These findings support the hydrogel's role as a scaffold that may assist in epithelial repair and endometrial thickness restoration in patients with recurrent miscarriage.

The enhanced tube formation shown in Figure 3 demonstrates that the HA-CHO/chitosan hydrogel extract stimulates angiogenic activity of endothelial cells *in vitro*. This angiogenic effect is likely attributed to the release of bioactive polysaccharides or incorporated signaling molecules within the hydrogel matrix. The presence of dynamic imine bonds and polysaccharide degradation products may also create a microenvironment conducive to vascular morphogenesis [34,35]. This is particularly important for intrauterine applications, where revascularization of the endometrial tissue is essential for implantation and regeneration [36]. Overall, Figure 3 supports the hypothesis that the developed hydrogel not only supports endometrial stromal cell proliferation (Figure 2) but also facilitates vascular network formation, providing a synergistic strategy for endometrial repair in the context of recurrent miscarriage.

The enhanced endothelial tube formation quantified in Figure 4 further supports the bioactivity of the hydrogel system. This angiogenic stimulation is likely driven by the presence of pro-regenerative and pro-angiogenic agents such as VEGF, which was incorporated into the hydrogel as shown in Figure 1. In addition, LIF has been reported to synergize with VEGF to promote endothelial cell migration and network stabilization, while IL-11 may indirectly support angiogenesis by modulating the inflammatory microenvironment.

The combined effect of these factors results uren a significantly more developed and interconnected vascular network *in vitro*. This is of particular relevance for endometrial repair, where angiogenesis plays a critical role in restoring endometrial receptivity and nutrient delivery. Thus, Figure 4 provides functional validation of the hydrogel's potential to support vascular remodeling in the context of recurrent miscarriage therapy.

The immunomodulatory activity shown in Figure 5 suggests that the HA-CHO/chitosan hydrogel not only supports endometrial tissue regeneration but also contributes to creating a favorable immune microenvironment. The reduction of pro-inflammatory markers (TNF-α, IL-6) and significant elevation of IL-10 are likely influenced by factors such as IL-11 and VPA, which were incorporated into the hydrogel formulation (see Figure 1). These molecules are known to reprogram macrophage activation states and suppress inflammation, which is particularly relevant in recurrent miscarriage, where excessive endometrial inflammation is a contributing factor. The results confirm that the hydrogel acts through both structural and immunoregulatory mechanisms to support endometrial healing.

The gene expression changes shown in Figure 6 support the hydrogel's ability to modulate endometrial stromal cell phenotype toward a pro-receptive state. The sharp increase in LIF, a critical implantation cytokine, is consistent with its incorporation in the hydrogel formulation as shown in Figure 1, and underscores the material's potential to improve uterine receptivity. Upregulation of IGFBP1 further indicates partial activation of stromal maturation pathways. The slight decrease in PRL may reflect a regulatory balance that favors tissue receptivity without inducing premature decidualization, which could be detrimental in early implantation phases.

Together, these findings confirm that the hydrogel provides both biochemical and microenvironmental cues that shift the gene expression landscape of hEMSCs toward implantation-supportive profiles, validating its therapeutic relevance for endometrial repair in recurrent miscarriage.

5. Conclusion

In this study, we developed a bioactive injectable hydrogel based on aldehyde-functionalized hyaluronic acid (HA-CHO) and chitosan, designed for endometrial regeneration in the context of recurrent miscarriage. The hydrogel was fabricated through mild Schiff base crosslinking and demonstrated excellent structural properties, injectability, and the ability to load and release regenerative factors such as LIF, VEGF, IL-11, and VPA (Figure 1). *In vitro* assays confirmed the hydrogel's favorable biocompatibility, as it supported the proliferation of human endometrial stromal cells (hEMSCs) over time (Figure 2). Moreover, the hydrogel extract promoted angiogenesis in HUVECs, as evidenced by increased capillary-like tube formation and node development (Figures 3 and 4), likely due to VEGF release. Importantly, the hydrogel also exerted immunomodulatory effects by suppressing pro-inflammatory cytokines (TNF-α, IL-6) while significantly upregulating IL-10, suggesting a favorable shift toward an anti-inflammatory microenvironment (Figure 5). Gene expression analysis further revealed that the hydrogel upregulated key markers of endometrial receptivity, especially LIF and IGFBP1, without inducing excessive decidualization, as PRL expression was not elevated (Figure 6). This profile suggests a targeted activation of regenerative and implantation-supportive pathways. Taken together, these results demonstrate that the HA-CHO/chitosan hydrogel serves as a multifunctional platform that supports cell proliferation, promotes angiogenesis, modulates inflammation, and enhances endometrial receptivity. While the findings demonstrate the hydrogel's potential for enhancing endometrial regeneration *in vitro*, further validation in *in vivo* uterine injury models will be necessary to confirm its clinical translational value.

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Author Contributions: Chen Chen and Cenlan Bu contributed equally to this work and are co–first authors. Chen Chen was responsible for experimental design, data analysis, and manuscript drafting. Cenlan Bu conducted material preparation, FTIR analysis, and cell viability assays. Jiahui Qian assisted with adhesion testing and drug release experiments. Jingwen Xu performed RTCA migration assays and data processing. Yi Tang participated in data interpretation and figure preparation. Yunzhao Xu

supervised the project, provided funding support, and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Availability of Data and Materials: The data that support the findings of this study are available from the Corresponding Author, [Yunzhao Xu], upon reasonable request.

Ethics Approval: Not applicable.

Conflicts of Interest: The authors declare no conflicts of interest to report regarding the present study.

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