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Original research article

Stability of bioactive compounds and wound healing activities of porcine placental extract

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ABSTRACT

Porcine placental extract (PPE) is a biologically active substance enriched with proteins and regenerative mediators, offering therapeutic potential in wound healing applications. However, the long-term stability of PPE under various storage conditions remains unclear. This study investigated the biochemical stability and functional bioactivity of PPE stored at 4 °C and −20 °C for 1, 3, 6, and 12 months. Total protein content was quantified using the Bradford assay, while levels of vascular endothelial growth factor-A (VEGF-A) and platelet-derived growth factor-BB (PDGF-BB) were measured using enzyme-linked immunosorbent assay (ELISA). Functional assays included endothelial cell proliferation using the MTT assay and migration assessed by scratch wound healing assay on EA.hy926 cells. Results showed that total protein content declined significantly after as early as one month, with more than 50% reduction by 12 months at both temperatures. VEGF-A levels remained stable for one month before decreasing, whereas PDGF-BB levels dropped significantly from the first month. Despite these biochemical losses, PPE maintained stable bioactivity in promoting endothelial cell proliferation and migration throughout the 12-month period, regardless of storage temperature. These findings demonstrate that PPE retains functional efficacy for at least 12 months under refrigerated or

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frozen storage and support its potential development as a stable, bioactive wound-healing agent. Improved preservation techniques may further enhance its long-term biochemical integrity.

Keywords: porcine placental extract, growth factors, VEGF-A, wound healing, endothelial cells

1. Introduction

Wound healing is a highly regulated and dynamic biological process that involves the coordination of various cellular and molecular events, broadly categorized into four overlapping phases: hemostasis, inflammation, proliferation, and remodeling.[1] Among these, the proliferative phase plays a crucial role in restoring tissue integrity through processes such as angiogenesis, fibroblast activation, re-epithelialization, and extracellular matrix (ECM) synthesis.[2] These events are orchestrated by a network of cytokines and growth factors that regulate cellular proliferation, migration, and differentiation.[3]

Vascular endothelial growth factor-A (VEGF-A) and platelet-derived growth factor-BB (PDGF-BB) are two of the most studied pro-angiogenic growth factors. VEGF-A serves as the principal driver of endothelial proliferation, migration and neovascularization,[2,3] while PDGF-BB stabilizes new vessels by recruiting pericytes and smooth muscle cells, thereby supporting vascular remodeling.[4] Both factors are abundantly detected in placental extracts and are widely recognized as representative markers of angiogenic activity.[5,6] The availability of these factors in the wound environment is vital for timely healing and tissue regeneration.

Placental tissues - both human and animal-derived - are rich natural sources of these biomolecules and have been used historically in various traditional medicine systems to accelerate healing.[7] Modern studies have corroborated the therapeutic value of placental extracts by demonstrating their efficacy in promoting angiogenesis, reducing inflammation, and enhancing wound closure in both in vitro and in vivo models.[6,8-9] The composition of placental extracts includes not only VEGF-A and PDGF-BB but also a spectrum of bioactive peptides, amino acids, nucleotides, cytokines, enzymes, and extracellular vesicles,[10–12] contributing to their multifunctional healing potential.

Porcine placental extract (PPE) in particular has emerged as a promising alternative to human placental products due to its compositional similarity, abundant availability, and fewer ethical constraints.[13] It has been reported to stimulate fibroblast proliferation, collagen synthesis, and endothelial cell migration, all of which are key processes in skin regeneration and vascular remodeling.[14–16] Previously, our group demonstrated that PPE significantly enhances angiogenesis in vitro by upregulating VEGF-A expression and promoting capillary-like tube formation in endothelial cells,[17] thereby reinforcing its potential utility in regenerative medicine.

However, a major challenge in translating PPE into clinical or commercial applications lies in its biochemical and functional stability during storage. Biological extracts are inherently prone to degradation through a variety of physicochemical mechanisms such as proteolytic activity, oxidation, deamidation, microbial contamination, and structural denaturation.[18–19] These degradative changes can impair the structure and function of proteins and peptides, leading to reduced efficacy or loss of biological activity over time.[20]

Despite these advances, data on the long-term storage stability of porcine placental extract - particularly under commonly used storage conditions such as refrigeration (4°C) or standard freezing (-20°C) - remain limited. It is essential to understand how bioactive constituents such as total proteins, VEGF-A, and PDGF-BB behave under these conditions, and whether PPE retains its regenerative functions over time.

Therefore, the present study aimed to investigate the storage stability of PPE over a 12-month period by monitoring total protein concentration, VEGF-A and PDGF-BB levels, and functional bioactivities relevant to wound healing, including endothelial cell proliferation and migration. The findings are expected to support the development of shelf-stable PPE formulations for therapeutic use in wound care and regenerative medicine.

2. Materials and Methods

2.1 Porcine placental extract (PPE)

PPE was prepared and provided by the Faculty of Science, Mahidol University, Bangkok, Thailand. In brief, approximately 500 g of fresh porcine placenta was washed with phosphate-buffered saline (PBS) pH 7.4. The tissue was then homogenized and subjected to ultrasonic disruption on ice. The homogenate was centrifuged at 4°C for 1 hour, and the resulting supernatant was sterilized by filtration through a 0.2 µm membrane filter. No protease inhibitors were added in this preparation. The sterile extract was aliquoted and stored at 4°C or -20°C for further experiments.

2.2 Cell culture

The human endothelial cell line EA.hy926 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Thermo Fisher Scientific, USA) enriched with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher Scientific, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in a humidified incubator at 37°C with 5% CO_2 and 95% air.

2.3 Bradford assay

The total protein content of PPE was quantified using the Bradford assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Briefly, 10 µL of sample was mixed with 200 µL of Bradford reagent in a 96-well microplate and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Bovine serum albumin (BSA) was used to generate a standard curve ranging from 0 to 1000 µg/mL. All measurements were performed in triplicate, and protein concentrations were expressed as mean ± standard deviation (SD).

2.4 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of angiogenic growth factors, including PDGF-BB and VEGF-A, in PPE were quantified using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions.

Specifically, the Porcine PDGF-BB ELISA Kit (RayBiotech, Norcross, GA, USA) and the Porcine VEGF-A ELISA Kit (Thermo Fisher Scientific, Waltham, MA, USA) were employed to detect PDGF-BB and VEGF-A levels, respectively. Briefly, 100 µL of each PPE sample or standard was added to the pre-coated 96-well plates and incubated at room temperature for the recommended duration. After washing, biotinylated detection antibodies and horseradish peroxidase (HRP)-conjugated streptavidin were sequentially added. Following incubation with TMB substrate, the reaction was stopped using stop solution, and absorbance was measured at 450 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA).

Standard curves were constructed using serial dilutions of known standards provided in each kit. Each sample was analyzed in triplicate. The concentrations of

VEGF-A and PDGF-BB were initially calculated in pg/mL and subsequently normalized to the total protein content of each sample, which had been determined by Bradford assay. Final values were expressed as pg of growth factor per mg of total protein (pg/mg protein) and reported as mean ± SD.

2.5 MTT assay

The proliferation of endothelial cells in response to PPE was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. EA.hy926 cells were seeded in 96-well plates at a density of 8,000 cells/well and incubated at 37°C with 5% CO_2 for 24 hours to allow cell attachment. After incubation, the culture medium was removed and replaced with fresh medium containing 20 µg/mL PPE. This working concentration was selected based on preliminary experiments (data not shown), which showed that 20 µg/mL consistently stimulated endothelial cell proliferation without cytotoxic effects. Cells were then incubated for an additional 48 hours, a duration chosen to ensure sufficient time for measurable growth responses. Following treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours at 37°C. The resulting formazan crystals were dissolved by adding 100 µL of DMSO per well. Absorbance was measured at 540 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Cell proliferation was calculated as a percentage of absorbance relative to the untreated control group. The proliferation data were reported as the percentage increase in cell proliferation compared to untreated cells (% increase in cell proliferation).

2.6 Wound healing

The wound healing assay was performed to evaluate the migratory capacity of endothelial cells in response to PPE. EA.hy926 cells were seeded in 12-well plates and cultured in complete medium under standard conditions (37°C, 5% CO_2) for 24 hours, until a confluent monolayer was formed. A linear scratch wound was created in the center of each well using a sterile 200 µL pipette tip. The wells were gently washed with PBS to remove detached cells. The cells were bathed with fresh medium containing 20 µg/mL PPE and incubated for 24 hours. This incubation time was selected to capture early wound closure events while avoiding potential confounding effects from cell overgrowth. Cell migration into the wound area was observed under an inverted phase-contrast microscope at 0 and 24 hours post-scratch. Images were captured at designated time points, and the wound area was quantitatively analyzed using ImageJ software (NIH, USA). The wound closure area was calculated by comparing the remaining wound gap at 24 hours to the initial gap at 0 hours. The results were expressed as the percentage of wound closure, indicating the cell migration capacity.

2.7 Statistical analysis

All experiments were conducted in triplicate, and the data were expressed as mean ± SD. Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. A p-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software (version 9.0, GraphPad Software, San Diego, CA, USA).

3. Results

Storage stability of PPE was evaluated under two temperature conditions (4°C and -20°C) over storage durations of 1, 3, 6 and 12 months. Key biochemical components and relevant bioactivities were assessed as follows:

3.1 Total protein

The total protein content of PPE was determined using the Bradford assay, and the results are summarized in Table 1. A significant decline in protein concentration was observed after just one month of storage at both 4°C and -20°C, with reductions of

31.4% and 29.5%, respectively, compared to the initial levels. These findings suggest that proteins in porcine placental extract are highly susceptible to degradation within the first month of storage, regardless of temperature. Over the course of 12 months, protein content continued to decline progressively. At the 12-month time point, the remaining protein levels were 41.3% and 33.29% of the baseline values for samples stored at 4°C and -20°C, respectively. Despite the ongoing degradation, the rate of protein loss was comparable between the two storage conditions, as illustrated in Fig. 1.

Table 1. Total protein content of porcine placental extract (PPE) stored at 4°C and -20°C for 1, 3, 6, and 12 months, expressed as a percentage relative to the initial concentration. Data are presented as mean ± SD.

Month	Total Protein (µg/mL)	
	4°C	-20°C
0	3106±151.6 (100%)	3106±151.6 (100%)
1	2137±124.2 (68.6±4 %)	2189±59.0 (70.5±1.9%)
3	1474±46.6 (47.5±1.5%)	1669±48.8 (53.7±1.6%)
6	1567±56.0 (50.5±1.8%)	1673±5.4 (53.9±0.17%)
12	1282±32 (41.3±1.8%)	1034±23 (33.3±0.23%)

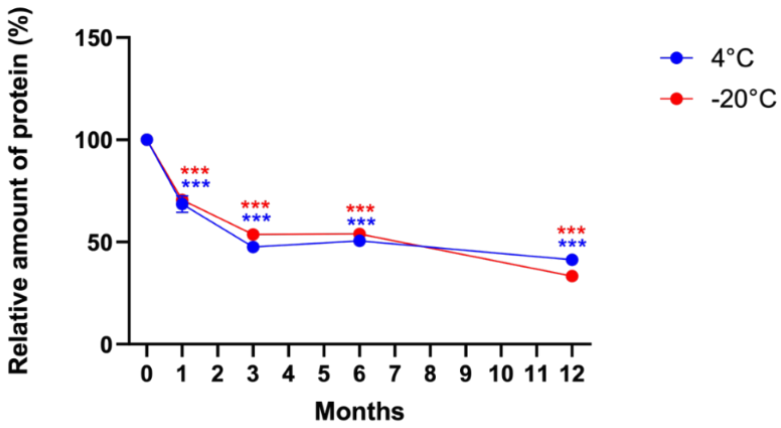

Fig 1. Protein content of porcine placental extract (PPE) after storage at 4°C and -20°C for various time periods, expressed as a percentage relative to the initial concentration. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 compared to baseline.

3.2 Growth factors

The stability of two key growth factors, PDGF-BB and VEGF-A, in PPE was analyzed after storage at 4°C and -20°C for 0, 1, 3, 6, and 12 months. At the initial time point, the concentrations of PDGF-BB and VEGF-A were approximately 600 and 200 pg/mg protein, respectively. These results demonstrate that both growth factors were readily detectable in PPE, with PDGF-BB present at relatively higher levels than VEGF-A. Such detectable concentrations are consistent with previous reports of placental extracts containing VEGF-A and PDGF-BB within similar ranges.[5,6]

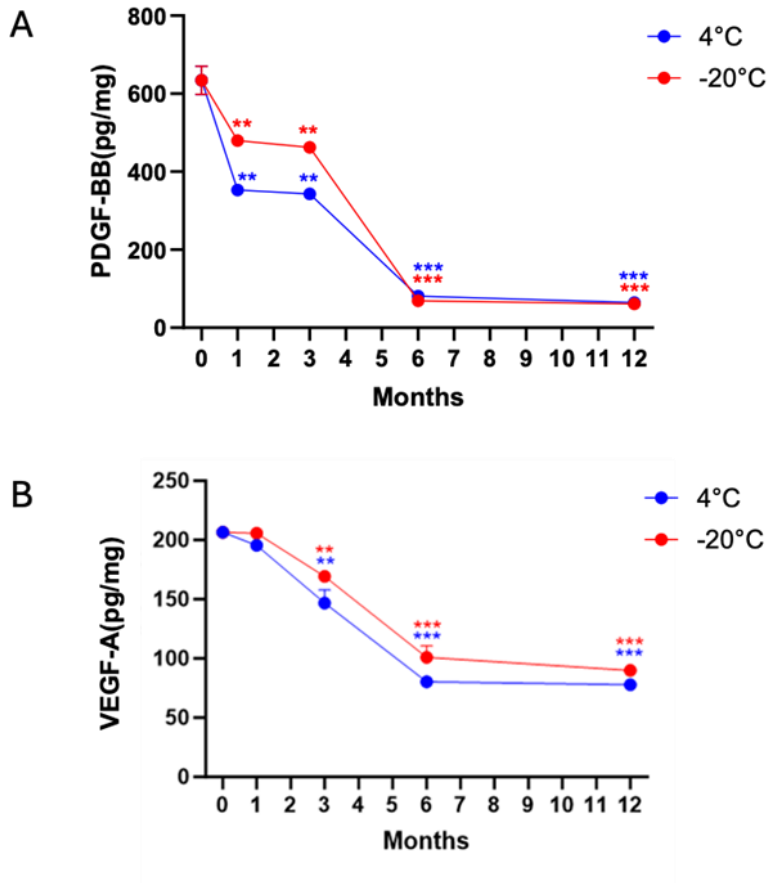

Fig 2. Growth factor levels of porcine placental extract (PPE) stored at 4°C and -20°C for 1, 3, 6, and 12 months compared to the initial time point: (A) PDGF-BB and (B) VEGF-A. Data are presented as mean ± SD. *p < 0.05, **p < 0.01, ***p < 0.001 versus baseline.

After 1 month of storage, PDGF-BB levels significantly declined at both 4°C and -20°C ($p < 0.01$). In contrast, VEGF-A levels remained stable under both temperature conditions (Fig. 2B), suggesting its lower stability (Fig 2A). By month 3, both VEGF-A and PDGF-BB concentrations had decreased significantly compared to baseline levels under both storage conditions ($p < 0.01$). At 6 months, this downward trend continued. PDGF-BB content declined by more than 70%, from approximately 600 pg/mg protein to less than 200 pg/mg protein, while VEGF-A levels decreased by over 50% relative to baseline. After 12 months of storage, the concentrations of both growth factors remained comparable to those observed at

the 6-month time point under both temperature conditions.

These findings suggest differential stability between the two growth factors. VEGF-A remained stable for up to 1 month at both storage temperatures, whereas PDGF-BB exhibited significant instability from the first month onward under both 4°C and -20°C conditions.

3.3 Cell proliferation

The PPE stored at 4°C and -20°C for 1, 3, 6, and 12 months were evaluated using the EA.hy926 human endothelial cell line. At the initial time point, PPE at a protein concentration of 20 µg/mL significantly enhanced endothelial cell proliferation by more than 20% compared to the untreated control. PPE samples stored at both temperatures continued to exhibit consistent pro-proliferative effects across all tested time points, with no significant reduction in activity compared to the freshly prepared extract (Fig. 3). These findings indicate that, when normalized by protein content, PPE retains its biological function in promoting endothelial cell proliferation over extended storage periods under both temperature conditions.

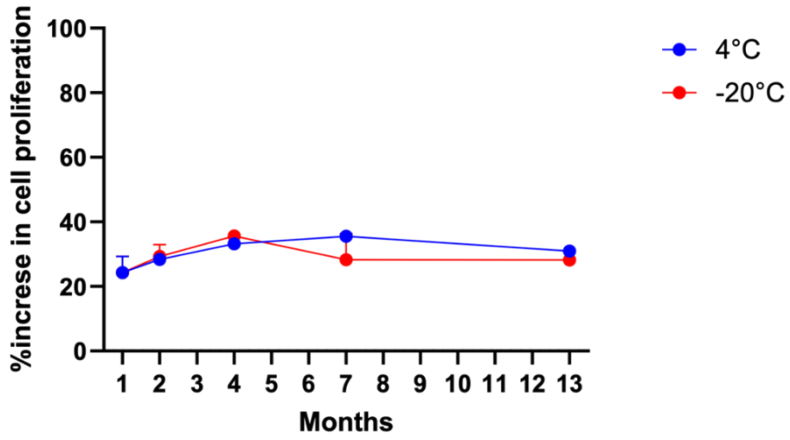

Fig 3. Proliferative activity of human endothelial cells (EA.hy926) treated with porcine placental extract (PPE) stored at 4°C and -20°C for 1, 3, 6, and 12 months, compared to freshly prepared extract. Data are presented as mean ± SD.

3.4 Cell migration

The effect of PPE on endothelial cell migration was assessed following storage at 4°C and -20°C for 1, 3, 6, and 12 months using the EA.hy926 cell line. At the initial time point, PPE at the tested concentration enhanced endothelial cell migration by 30% compared to the untreated control (Fig. 4A, B). Throughout the entire storage period, PPE samples stored at both temperatures maintained a consistent ability to promote cell migration, with no significant decline observed at any time point. These results indicate that, when normalized to protein content, PPE retained its pro-migratory activity under both storage conditions for up to 12 months.

4. Discussion

This study demonstrated that PPE maintains key biological activities associated with wound healing - including the promotion of endothelial cell proliferation and migration - even after long-term storage at 4°C and -20°C for up to 12 months. Although there was a progressive reduction in total protein content and levels of pro-angiogenic growth factors such as VEGF-A PDGF-BB, the

retained bioactivity indicates that PPE remains functionally viable under these conditions. The early and significant decline in total protein content, detectable within the first month of storage, underscores the biochemical vulnerability of placental proteins to degradation. Biological extracts, particularly those rich in peptides and growth factors, are highly susceptible to various physicochemical processes including proteolytic degradation, oxidation, deamidation, and structural denaturation.[18–20] These reactions can lead to protein unfolding, aggregation, fragmentation, or chemical modifications that result in the loss of therapeutic efficacy.[21–22]

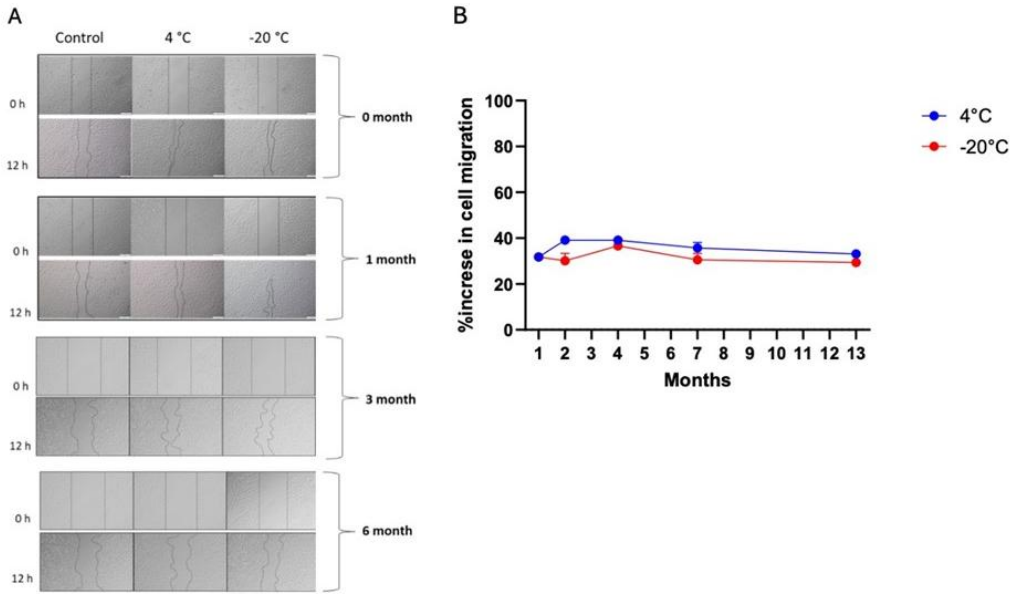

Fig 4. Endothelial cell migration in response to porcine placental extract (PPE) stored at 4°C and -20°C for different durations. (A) Representative images of scratch wound healing assay at 0 and 12 h after treatment with PPE stored for 0, 1, 3, and 6 months. (B) Quantitative analysis of the percentage increase in cell migration relative to the control group after storage up to 12 months. Data are presented as mean ± SD.

Placental tissue is known to contain endogenous proteases that remain active in aqueous environments. When not properly inhibited or stabilized, these enzymes contribute to autolysis and further degradation of proteins, especially under non-frozen or fluctuating temperature conditions.[23] For example, aqueous PPE stored at 4°C without the addition of stabilizing excipients has been shown to undergo substantial protein loss within weeks.[24] In this context, our results are consistent with the literature and emphasize the importance of storage condition optimization.

Both VEGF-A and PDGF-BB were detectable throughout the 12-month storage period, although VEGF-A showed relative short-term stability while PDGF-BB declined significantly from the first month. This indicates differential vulnerability between the two growth factors. Importantly, despite these reductions, PPE consistently promoted endothelial proliferation and migration, suggesting that even reduced concentrations of VEGF-A and PDGF-BB, together with other bioactive constituents, were sufficient to maintain biological activity.

Normalization of growth factor levels to total protein was performed to control for variability in protein yield across samples and to provide relative values. However, this approach has inherent limitations, as the marked decline in total protein observed from the first month may mask the true extent of VEGF-A and PDGF-BB reduction. This limitation should be considered when interpreting our results. To improve transparency, both normalized and non-normalized values are provided in the Supplementary Information (Supplementary Table S1). These findings emphasize the importance of considering synergistic effects among multiple components in placental extracts rather than focusing solely on individual factors.

Endothelial cell migration is a fundamental step in angiogenesis, enabling endothelial cells to move into wound sites, form capillary sprouts, and establish new microvessels. This process is essential for restoring blood supply and supporting tissue regeneration. The finding that PPE retained its ability to promote endothelial migration even after prolonged storage suggests a meaningful impact on neovascularization, which may accelerate wound closure and improve healing outcomes.

A limitation of the present study is that functional assays were performed using only one endothelial cell line (EA.hy926). While this model provides useful insight into angiogenic activity, additional studies employing other relevant cell types (e.g., primary endothelial cells, fibroblasts, or keratinocytes) and in vivo validation will be necessary to fully establish the wound-healing potential of PPE.

Previous proteomic and functional analyses of placental extracts have identified additional wound-healing mediators such as epidermal growth factor (EGF), hepatocyte growth factor (HGF), insulin-like growth factors (IGFs), and transforming growth factor-β (TGF-β), which may remain stable longer or function additively.[25] These factors could compensate for the loss of VEGF and PDGF, ensuring sustained bioactivity over time. In addition to these growth factors, placental extracts are also known to contain cytokines, bioactive peptides, and extracellular vesicles that may exert paracrine effects on cell migration, angiogenesis, and tissue remodeling. The presence of such multifactorial constituents likely explains why PPE maintained endothelial proliferation and migration activity despite reduced levels of VEGF-A and PDGF-BB, highlighting the synergistic nature of the extract's biological activity.

From a translational perspective, the findings support the feasibility of using cold storage - particularly at 4°C - as a practical strategy for PPE preservation, especially in settings where deep freezing (-80 °C) is not feasible. This could facilitate the development of PPE-based formulations such as topical gels, hydrocolloid dressings, or injectable products for wound care without the burden of cold-chain logistics.[26] From a translational perspective, cold storage at 4°C or -20°C may represent practical short- to mid-term strategies for PPE preservation. Advanced stabilization techniques such as lyophilization or encapsulation could further enhance long-term stability,[27-29] however, additional experiments beyond the present study would be required to validate their applicability.

Additionally, future work should focus on in vivo validation and real-time stability testing in actual wound care settings. Analytical methods such as peptide mapping, HPLC, or mass spectrometry can also be employed to track structural integrity of key biomolecules in stored PPE.[30] Understanding the molecular degradation kinetics and identifying optimal preservation strategies are essential steps toward the successful commercialization of PPE as a biotherapeutic product.

5. Conclusion

This study demonstrates that porcine placental extract (PPE) maintains key wound

healing-related bioactivities - namely, the stimulation of endothelial cell proliferation and migration - for up to 12 months when stored at either 4°C or -20°C, despite progressive reductions in total protein content and angiogenic growth factor levels. These findings underscore the functional stability of PPE under common storage conditions and highlight its potential as a shelf-stable bioactive material for regenerative medicine. Furthermore, the use of PPE represents a promising strategy for the valorization of livestock-derived biological materials, aligning with principles of sustainability and circular biomedicine. Future research should explore advanced preservation techniques and in vivo validation to support its clinical translation and formulation development.

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Conflicts of Interest

The authors declare no conflict of interest.

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Supplementary Table S1

Supplementary Table S1. Absolute concentrations of PDGF-BB and VEGF-A in porcine placental extract (PPE) stored at 4 °C and -20 °C for 0, 1, 3, 6, and 12 months.
Data are expressed as pg/mL (mean values).

Month	PDGF (pg/mL)		VEGF (pg/mL)	
	4°C	-20°C	4°C	-20°C
0	204.20±8.18	204.20±8.18	66.12±1.14	67.81±1.17
1	165.32±1.09	219.13±1.04	91.22±1.69	91.59±1.55
3	232.59±7.46	277.05±3.13	105.72±2.56	105.01±2.50
6	51.66±1.79	41.44±0.37	60.93±2.19	66.25±2.04
12	50.27±0.58	59.31±0.60	70.35±2.57	97.32±3.52

Supplementary Table S1 presents the absolute (non-normalized) concentrations of VEGF-A and PDGF-BB measured by ELISA in porcine placental extract (PPE) stored under two temperature conditions (4 °C and −20 °C) over 12 months. These data complement the normalized values shown in the main text, providing a clearer view of the actual decline in growth factor levels due to protein degradation during storage.