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Original Research Article

Effect of platelet-rich plasma on limbal and amniotic stem cell viability and proliferation: An in vitro study for ocular surface regeneration

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Abstract

Background: Limbal and amniotic stem cells are crucial for corneal regeneration, positioning them as essential components for ocular surface repair. Platelet-rich plasma (PRP) holds significant potential for promoting regeneration; however, its effects on the viability and proliferation of stem cells in vitro remain largely unexamined.
Objective: This study seeks to assess the ability of PRP to improve the viability and proliferation of limbal and amniotic stem cells, which has important implications for corneal tissue engineering.
Materials and Methods: The research studied PRP supplementation's effect on stem cell cultures, focusing on cell viability and proliferation. A quantitative analysis compared PRP-treated and control groups over five passages.
Results: After 24 hours, limbal stem cells exposed to PRP exhibited a significantly higher viability rate of 90.60% compared to 83.40% in the control group ($p = 0.001$). Similarly, PRP-treated amniotic cells demonstrated 86.40% viability, compared to 81.00% in controls ($p = 0.002$). After 48 hours, viability in PRP-treated limbal cells rose to 93.80%, compared to 86.60% in controls ($p < 0.001$), while amniotic cell viability increased to 90.40% versus 85.80% in controls ($p = 0.015$). Proliferation rates also increased significantly in both cell types with PRP treatment. Limbal cells showed a proliferation rate of 202.55%, compared to 195.61% in controls ($p < 0.001$), and amniotic cells showed 173.43% versus 168.32% in controls ($p < 0.001$).
Conclusion: PRP significantly enhances both the viability and proliferation of rabbit limbal and amniotic stem cells, highlighting its potential application in tissue engineering and corneal regeneration.

Keywords: Platelet-rich plasma, Limbal stem cells, Amniotic stem cells, Cell viability, Cell proliferation, Tissue engineering, Corneal regeneration.

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1. Introduction

Limbal stem cells, found in the limbal niche, are essential for regenerating and maintaining the corneal epithelium, which is critical for maintaining corneal clarity and structural stability.[1,2] A more thorough understanding of the mechanisms governing these cells could lead to innovative therapies for addressing limbal stem cell deficiencies, thereby enhancing vision and overall quality of life.[3-6] Likewise, amniotic stem cells exhibit considerable potential for tissue regeneration, especially concerning eye disorders, thereby positioning them as promising candidates for therapeutic interventions in tissue repair.[7,8]

PRP has garnered significant attention in regenerative medicine due to its ability to promote tissue repair and healing.[9,10] This influence stems from its delivery of elevated levels of growth factors, cytokines, and bioactive compounds that significantly affect cellular activities.[11,12] Specifically, PRP has demonstrated the ability to enhance the proliferation

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and differentiation of stem cells, paving the way for new approaches to treating corneal diseases.[13,14]

This study builds upon earlier research involving corneal regeneration in a rabbit model, where we examined the efficacy of stem cells in treating chemical burns.[15] Our current investigation aims to refine the in vitro cultivation techniques for limbal and amniotic stem cells for future clinical use. In particular, we are concentrating on the effects of PRP on boosting cell viability and proliferation, with the overarching objective of improving strategies for ocular surface repair in experimental models.

2. Materials and Methods

2.1. Study rationale and experimental design

This research is part of the Med-Biotech regenerative medicine project, based at the Faculty of Medicine and Pharmacy, Mohammed V University in Rabat. The project focuses on corneal restoration in rabbits and has produced several recent publications.

2.2. Sampling

We sourced the stem cells from samples previously cryogenically stored in Med-Biotech's cryobank and collected the blood needed for PRP from five healthy rabbits.

2.3. PRP preparation

To prepare PRP, we used 0.32 M trisodium citrate as an anticoagulant, maintaining a 9:1 blood-to-anticoagulant ratio. Blood samples underwent centrifugation at 200g for 10 minutes, followed by a second spin at 350g for 15 minutes, yielding approximately 1.5 to 2 ml of plasma per tube. The plasma was centrifuged at 800g for 10 minutes to concentrate the platelets into a pellet, which was resuspended in Gibco PBS to reach a final volume of 10 ml. For riching total activation, we added 0.2 ml of 10% calcium gluconate (PROAMP) for chemical activation and subjected the mixture to five freeze-thaw cycles in liquid nitrogen for physical activation. PRP was filtered using a 0.2-micrometer filter.[16-18]

2.4. Media preparation

The cell culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM, Gibco, supplemented with 10% fetal bovine serum, 0.1M non-essential amino acids, 2mM GlutaMAX, 1% penicillin-streptomycin, and 1% nystatin (all from Gibco).[19]

2.5. Cell culture processing

Limbal and amniotic cell vials were thawed, washed with 3 ml of Gibco PBS, and centrifuged at 200g for 10 minutes to concentrate the cells. After counting cell pellets, we seeded 5000 cells/cm² into 25 cm² flasks containing 7 ml of culture medium (two flasks per group). We incubated cells until they reached 70% confluence, then proceeded to trypsinization and reseeding at the same density. Cells were also plated in 96-well plates (5000 cells per well) for viability and growth assessments, performed 24 and 48 hours post-inoculation using Trypan Blue assays.

2.6. Statistical analysis

Five sample sections were evaluated microscopically for morphology and confluence, while viability and proliferation rates were quantified using microplate data. The data were analyzed using SPSS software. The Shapiro-Wilk test was applied to confirm the normality of data distributions, and the independent samples t-tests were involved for parametric comparisons between PRP and control groups.

3. Results

3.1. Qualitative assessment

3.1.1. Cell morphology

Cell morphology analysis revealed marked differences between cells cultured with PRP and those under standard conditions (**Table 1**). Limbal cells treated with PRP exhibited an elongated, well-organized structure with minimal stress signs, whereas the control group's limbal cells displayed irregularities and early signs of fragmentation. Similarly, PRP-treated amniotic cells demonstrated a coherent, rounded morphology with uniform shape and structural integrity, while the control group showed more irregular and fragmented cells.

3.1.2. Cell culture confluence

The assessment of confluence demonstrated noticeable growth differences between PRP-treated and control groups. Limbal cells supplemented with PRP reached confluence more swiftly, forming a dense and uniform cell layer, while the control group achieved slower confluence with visible gaps between cell clusters. PRP-treated amniotic cells also exhibited rapid confluence, covering most of the culture vessel's surface, in contrast to the control group, which showed slower confluence and less cohesive coverage (**Table 1**).

3.2. Quantitative assessment

3.2.1. Cell viability

Table 2 shows the viability of limbal and amniotic stem cells over five passages in the PRP and control groups. The Shapiro-Wilk test confirmed normal data distribution (p-values > 0.05), validating parametric statistical methods.

Table 1: Morphology, health, and confluence of limbal and amniotic stem cells in PRP vs. control groups

Cell Type	Condition	Cell Morphology	Overall Health	Confluence
LSC	PRP-Group	Uniform, elongated, well-structured	Healthy, minimal stress observed	High confluence; most of the flask surface is covered; dense and uniform cell layer.
	Control Group	Slightly irregular, some fragmentation	Moderate stress, early signs of deterioration	Lower confluence; smaller portion of flask surface covered; gaps between cell clusters.
ASC	PRP-Group	Rounded, consistent, well-structured	Healthy, active proliferation	High confluence; most of the flask surface covered; continuous cell layers with few gaps.
	Control Group	Irregular, some fragmentation	Noticeable stress, signs of degeneration	Lower confluence; more pronounced spaces between cells; less uniform coverage.

LSC: Limbal stem cells; **ASC:** Amniotic stem cells

Table 2: Limbal and amniotic cell viability (PRP vs. control group) at 24 and 48 hours

Cell Type	Condition	Time (h)	N	Mean Viability (%)	Standard Deviation	95% Confidence Interval for Mean	Shapiro-Wilk p-value
LSC	PRP Group	24	5	90. 60	2.70	87.25 - 93.95	0.980
		48	5	93.80	1.92	91. 41 - 96.19	0.928
	Control Group	24	5	83.40	1.95	80.98 – 85.82	0.758
		48	5	86.60	1.14	85.18 – 88.02	0.814
ASC	PRP Group	24	5	86.40	2.07	83.83 – 88.97	0.754
		48	5	90.40	2.70	87.05 – 93.75	0.980
	Control Group	24	5	81.00	1.58	79.04 – 82.96	0.967
		48	5	85.80	1.92	83.41 – 88.19	0.928

LSC: Limbal stem cells; ASC: Amniotic stem cells

For limbal cells, the mean viability rate in the PRP group at 24 hours was 90.60% (SD = 2.70, p = 0.980), increasing to 93.80% (SD = 1.92, p = 0.928) at 48 hours. In contrast, the control group's mean viability rate at 24 hours was 83.40% (SD = 1.95, p = 0.758), rising to 86.60% (SD = 1.14, p = 0.814) at 48 hours.

For amniotic cells, the PRP group's viability at 24 hours was 86.40% (SD = 2.07, p = 0.754), increasing to 90.40% (SD = 2.70, p = 0.980) at 48 hours. The control group showed lower viability at 81.00% (SD = 1.58, p = 0.967) at 24 hours, rising to 85.80% (SD = 1.92, p = 0.928) at 48 hours.

Figure 1(A) displays histograms illustrating the viability distributions for both groups at different time points, while **Figure 1**(C) shows boxplots comparing viability rates across groups at 24 and 48 hours.

Independent samples t-tests (**Table 4**) indicated that, after 24 hours, PRP-treated limbal cells had a significantly higher viability (90.60%, SD = 2.70) compared to controls (83.40%, SD = 1.95), with a mean difference of 7.20% (p = 0.001). Similarly, amniotic cells in the PRP group had higher viability (86.40%, SD = 2.07) compared to the control group (81.00%, SD = 1.58), with a mean difference of 5.40% (p = 0.002). After 48 hours, PRP-treated limbal cells maintained significantly higher viability (93.80%, SD = 1.92) compared to controls (86.60%, SD = 1.14), with a mean difference of 7.20% (p < 0.001). Likewise, amniotic cells in the PRP group showed increased viability (90.40%, SD = 2.70) compared to controls (85.80%, SD = 1.92), with a mean difference of 4.60% (p = 0.015).

3.2.2. Cell proliferation

Table 3 shows the proliferation rates of limbal and amniotic cells over five passages. The Shapiro-Wilk test confirmed normal data distribution (p-values > 0.05), supporting parametric statistical methods.

In the PRP group, the average proliferation rate of limbal cells was 202.55% (SD = 0.38, p = 0.484), while the control group had a lower mean rate of 195.61% (SD = 1.09, p = 0.286). Similarly, for amniotic cells, the PRP group exhibited an average proliferation rate of 173.43% (SD = 0.23, p = 0.474) compared to 168.32% (SD = 0.19, p = 0.501) in the control group.

Figure 1(B) shows histograms of proliferation values, while **Figure 1**(D) illustrates the differences between PRP and control groups using boxplots.

Independent samples t-tests (**Table 4**) revealed notably higher proliferation rates for both limbal cells (202.55%, SD = 0.38) and amniotic cells (173.43%, SD = 0.23) in the PRP group compared to the control group, with p-values of < 0.001 for both.

Table 3: Limbal and amniotic cells proliferation (PRP vs. control group) at 24 and 48 hours

Cell Type	Condition	N	Mean Proliferation Rate (%)	Standard Deviation	95% Confidence Interval for Mean	Shapiro-Wilk p-value
LSC	PRP Group	5	202.55	0.38	202.08 – 203.03	0.484
	Control Group	5	195.61	1.09	194.26 – 196.95	0.286
ASC	PRP Group	5	173.43	0.23	173.14 – 173.72	0.474
	Control Group	5	168.32	0.19	168.08 – 168.56	0.501

LSC: Limbal stem cells; **ASC:** Amniotic stem cells

Table 4: Summary of viability and proliferation rates for limbal and amniotic cells in PRP and control groups

Condition	Group	N	Mean (%)	Std. Deviation	Std. Error Mean	t	df	p-value	Mean Difference	Std. Error Difference	95% Confidence Interval
LCVR (24h)	PRP Group	5	90.60	2.70	1.21	4.832	8	0.001	7.200	1.490	3.764 – 10.636
	Control Group	5	83.40	1.95	0.87						
ACVR (24h)	PRP Group	5	86.40	2.07	0.93	4.630	8	0.002	5.400	1.166	2.711 – 8.089
	Control Group	5	81.00	1.58	0.71						
LCVR (48h)	PRP Group	5	93.80	1.92	0.86	7.200	8	0.000	7.200	1.000	4.894 – 9.506
	Control Group	5	86.60	1.14	0.51						
ACVR (48h)	PRP Group	5	90.40	2.70	1.21	3.101	8	0.015	4.600	1.483	1.180 – 8.020
	Control Group	5	85.80	1.92	0.86						
LCPR	PRP Group	5	202.55	0.38	0.17	13.49	8	0.000	6.95	0.52	5.76 – 8.14
	Control Group	5	195.61	1.09	0.49						
ACPR	PRP Group	5	173.43	0.23	0.10	37.93	8	0.000	5.11	0.13	4.80 – 5.42
	Control Group	5	168.32	0.19	0.09						

LCVR: Limbal cells viability rate; **ACVR:** Amniotic cells viability rate; **LCPR:** Limbal cells proliferation rate; **ACPR:** Amniotic cells proliferation rate

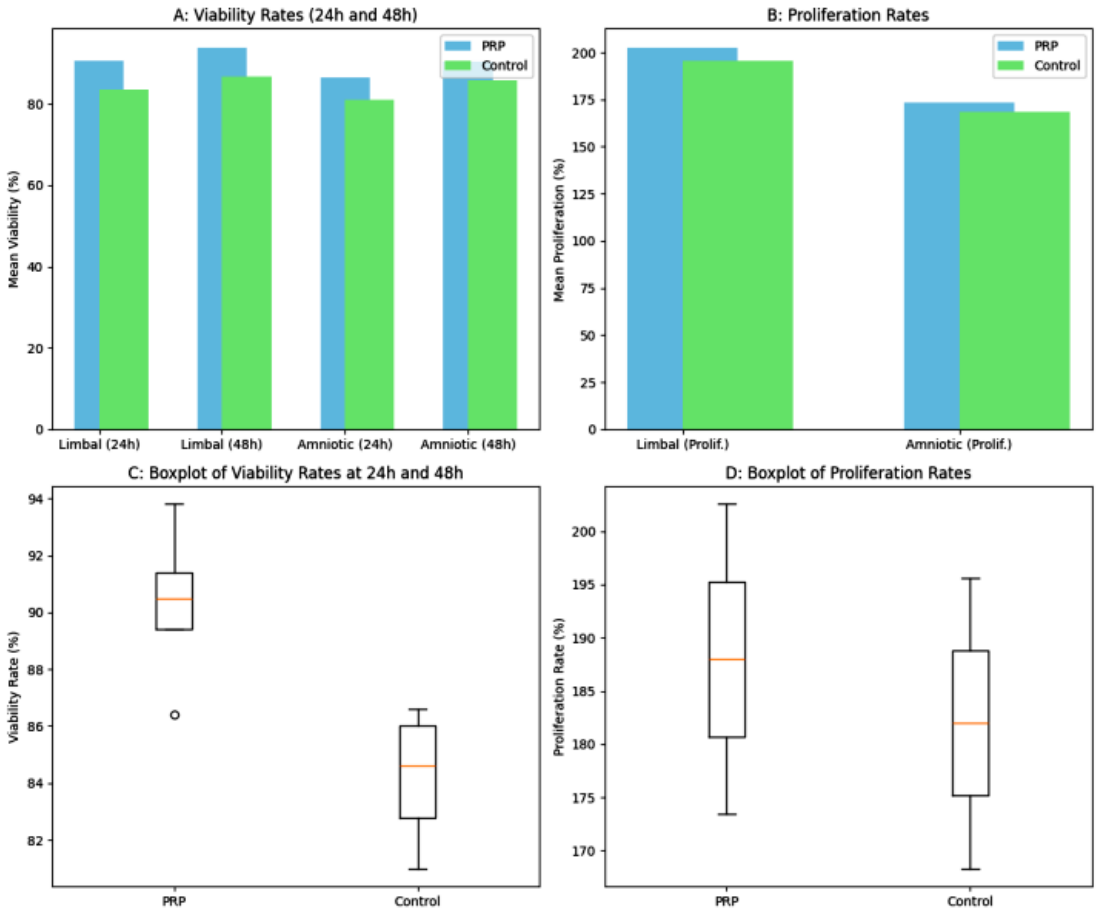

Figure 1: Comparison of viability and proliferation rates of limbal and amniotic cells (PRP vs. Control). **A:** Viability rates (24h and 48h); **B:** Proliferation rates; **C:** Boxplots of viability rates at 24h and 48h; **D:** Boxplots of proliferation rates

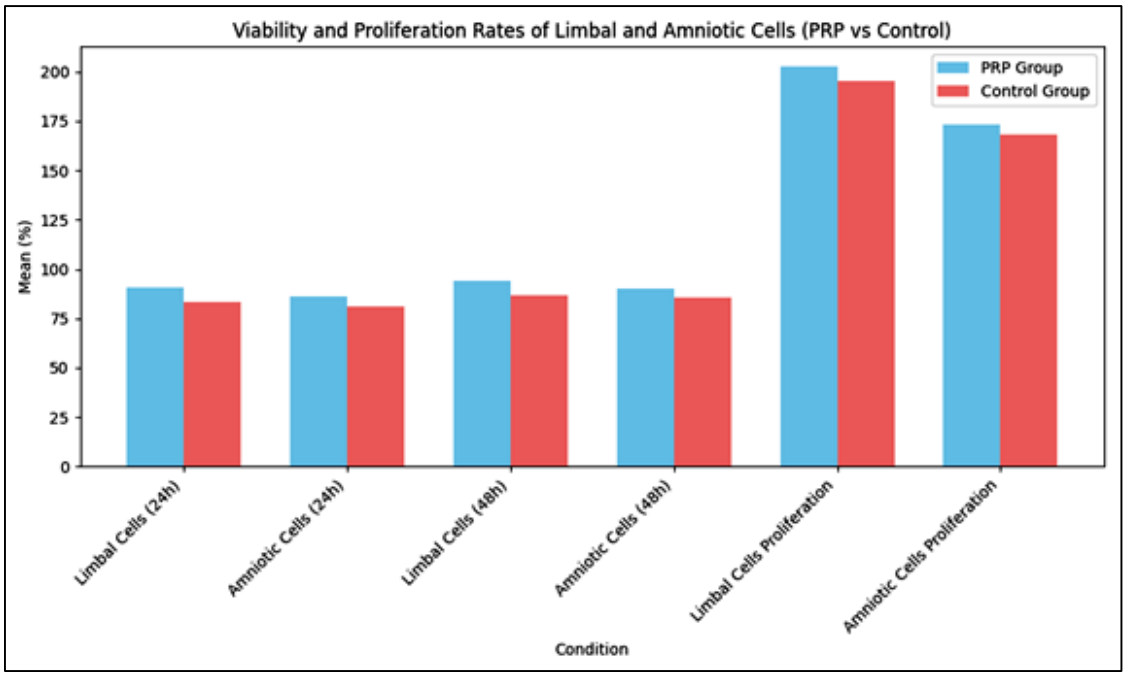

Figure 2: Histogram comparing viability and proliferation rates of limbal and amniotic cells (PRP vs. Control)

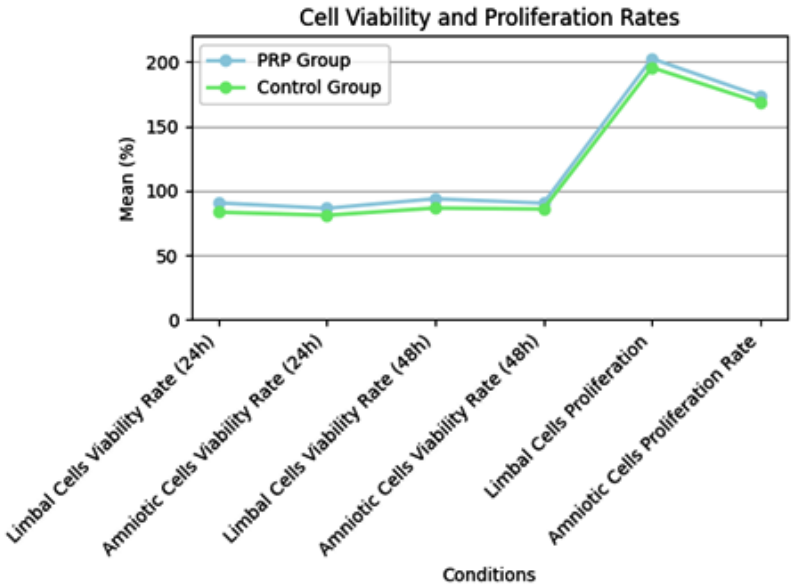

Figure 3: Curves comparing limbal and amniotic cell viability and proliferation rates (PRP vs. Control)

4. Discussion

4.1. How PRP works

PRP (Platelet-Rich Plasma) contains a variety of biologically active substances that influence cellular growth and signaling pathways, positioning it as a potential stimulator for stem cell development and tissue regeneration. Growth factors such as Platelet-Derived Growth Factor (PDGF), Transforming Growth Factor-beta (TGF-β), and Vascular Endothelial Growth Factor (VEGF) play pivotal roles in promoting cell proliferation, differentiation, and angiogenesis. These elements likely contribute to the enhanced viability and proliferation observed in limbal and amniotic cells treated with PRP.[20-23]

In addition to growth factors, PRP contains bioactive molecules like ATP, calcium, and zinc, as well as cytokines such as Interleukins (ILs) and Tumor Necrosis Factor (TNF). These components stimulate natural growth factor production, enhance cell proliferation, and reduce oxidative stress. PRP also contains antioxidants and mediators that promote a favorable cellular environment, encouraging tissue repair and regeneration.[24-27] The improved cellular structure and increased proliferation observed in the study's limbal and amniotic cells suggest that PRP decreases cell death and oxidative damage, further underscoring its regenerative potential.

4.2. Descriptive analysis

The data, confirmed to have a normal distribution via the Shapiro-Wilk test ($p > 0.05$), allowed for parametric testing. Independent samples t-tests revealed significant improvements in cell viability and proliferation in limbal and amniotic stem cells treated with PRP compared to controls ($p < 0.005$). Qualitative and quantitative results consistently demonstrated the positive impact of PRP on cellular morphology, confluence, viability, and proliferation. Visual data further substantiated these effects, with PRP supplementation significantly enhancing the therapeutic potential of limbal and amniotic cells. These results have promising implications for improving cell culture techniques that promote tissue engineering and cell therapy.

4.3. Comparative analysis

Both qualitative and quantitative assessments indicate that PRP supplementation positively influences cell morphology, confluence, viability, and proliferation. Statistical analysis, particularly the independent samples t-tests, confirmed these benefits, showing meaningful enhancements in viability and proliferation rates ($p < 0.05$). Figures such as histogram (**Figure 2**) and curve plot (**Figure 3**) visually emphasize these differences, highlighting the superiority of PRP over control treatments in fostering stem cell growth and viability. This study presents compelling evidence that PRP is a valuable tool for cultivating and maintaining limbal and amniotic stem cells, with substantial applications in regenerative medicine.

4.4. Comparison with existing studies

Previous studies have investigated the effects of PRP on various stem cell types in different laboratory models, consistently demonstrating PRP's capacity to promote cell growth. Our research builds on previous findings and specifically examines the effects of PRP on limbal and amniotic stem cells in rabbits. While the positive influence of PRP on stem cell proliferation is well established, this study aims to deepen the understanding of its potential for ocular surface regeneration.[28-31]

4.5. Limitations and clinical relevance

The findings from this research are encouraging, but it is important to recognize several limitations. Firstly, the small

sample size (N=5 per group) restricts the applicability of the results. While the Shapiro-Wilk test confirmed that the data followed a normal distribution, a larger sample size would significantly improve the statistical robustness and accuracy. Additionally, the brief duration of the study (limited to 48 hours) limited the ability to evaluate the long-term effects of PRP on cell viability and growth. Future studies should focus on understanding the lasting impact of PRP, especially in clinical environments where extended cell survival is crucial. Despite these constraints, PRP shows considerable promise for ocular surface reconstruction and wound healing, primarily due to its capacity to enhance cell viability and proliferation. Nonetheless, the differing responses among various cell types highlight the necessity for customized PRP treatments tailored to specific therapeutic goals.

5. Conclusion

This research underscores the effectiveness of PRP in enhancing the survival and growth of limbal and amniotic stem cells derived from rabbits in an in vitro setting. The significant response observed in limbal cells following PRP treatment showcases its potential to improve cellular therapy techniques. This research contributes to ongoing studies on corneal regeneration and reinforces prior investigations into stem cell treatments for limbal deficiencies in rabbit models affected by chemical burns. By promoting the proliferation of stem cells through PRP supplementation, this study establishes a groundwork for future innovations in cellular therapies targeting ocular surface conditions, particularly limbal stem cell deficiency. Further research is essential to explore the differing responses of limbal and amniotic cells to PRP treatment, focusing on optimizing protocols to enhance therapeutic results. Such advancements could pave the way for revolutionary clinical applications, offering new strategies for corneal repair and restoring vision for patients suffering from severe ocular surface damage.

6. Source of Funding

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7. Conflicts of Interest

The study authors state that they have no personal or financial interests that could influence the outcomes or interpretation of their work.

8. Ethical Committee Approval

Ethical approval was not required, as the use of frozen animal cells at our facility is not subject to oversight by the ethics committee.

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