Section A-Research paper
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Evaluation of the Effect of Hyaluronic Acid on Proliferation of Periodontal Ligament Fibroblast - An In-Vitro Study

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ABSTRACT

Background: In the connective tissue, synovial fluid, and other tissues, hyaluronic acid (HA), plays a crucial role in the processes of inflammation, granulation tissue development, epithelial remodelling and the healing of chronic wounds in both mineralized and non-mineralized periodontal tissues.

Therefore, this study aimed to investigate the effect of HA on PDL cell line proliferation in vitro at baseline, 3rd day, 5th day as well as to compare the rate of proliferation between the test and control group, baseline and day 3 as well as baseline and day 5 in the test group.

Methods: A total of 30 samples of PDL fibroblast cell line were taken, which were divided into two groups consisting of 15 samples each. Group 1 includes the "control group" where the PDL fibroblast cell was unstimulated, while group 2 includes the "test group" consisting of stimulated PDL fibroblast cells by HA. Cell proliferation of the two groups was checked at baseline, 3rd and 5th day and the proliferation activity was determined by Alamar blue assay indicator.

Results: Hyaluronic acid showed high PDL cell viability. It significantly increased cell proliferation compared to the controlled samples, and a significant increase in proliferation was seen between the 3rd and 5th day in the test group.

Conclusion: HA sustained high PDL cell viability and promoted the growth of the cultured PDL fibroblast cell line. To better understand how HA affects periodontal growth, additional in vitro and animal study is required.

KeyWord: Hyaluronic Acid (HA), Periodontal fibroblast cell line, Proliferation, Alamar Blue.

INTRODUCTION

Hyaluronic acid (HA), often referred to as hyaluronate or hyaluronan, is a nonsulphated, high molecular weight polysaccharide that belongs to the glycosaminoglycan family and is found in the extracellular matrix of numerous tissues, including skin, synovial joints, and periodontal tissues.[1,2] The promotion of cell migration and differentiation during tissue development and repair is one such capability of HA.

Due to its capacity to maintain the integrity of cartilage tissue HA has been used extensively for individuals with knee osteoarthritis.[3,4] Moreover, HA injections have been utilized to treat temporomandibular joint symptoms in the oral and maxillofacial region[5,6] In more recent times, HA has also been used for aesthetic benefits, especially to lessen or eradicate face wrinkles, interdental papilla loss, and many other abnormalities.[7,8,9]

HA has been speculated to have an impact on periodontal regeneration since it is being used more frequently in dentistry.[10] In both mineralized and non-mineralized periodontal tissues, it plays a key role in the processes of inflammation, granulation tissue development, and epithelial remodelling during the healing of chronic wounds.[11] In the early phases of inflammation, hyaluronic acid plays several roles. It acts as a structural framework by interacting with a fibrin plug, which controls the infiltration of inflammatory cells into the host's extracellular matrix. It stimulates the production of several polypeptide molecules from fibroblasts, keratinocytes, cementoblasts, and osteoblasts.[12] These molecules support the inflammatory response and subsequently encourage the synthesis of hyaluronic acid by the endothelial cells of the blood vessel.[13] In the event of colonization and growth of anaerobic pathogenic bacteria in the gingival sulci and surrounding periodontal tissue, HA continues to be involved in the activation of inflammatory cells, including polymorphonuclear leukocytes and macrophage function, including migration and adherence at the site of injury, phagocytosis, and destruction of microbial pathogens.[14,15] Hyaluronic acid also indirectly influences the development of inflammation and the maintenance of granulation tissue by limiting the release of proteases from inflamed cells that break down extracellular matrix proteins as healing progresses.[16] A rise in PDL cell proliferation was seen as a result of hyaluronic acid's actions. As part of the granulation phase, HA has been demonstrated to be a crucial protein that is abundantly expressed in a variety of tissues and is in charge of encouraging cell migration, proliferation, and granulation tissue organization. HA is momentarily raised during the development of tissue regeneration in non-mineralized tissues and aids in the re-establishment of the epithelium.

Thus, the present study aimed to investigate the effect of hyaluronic acid on periodontal fibroblast cell line proliferation at baseline, 3rd day and 5th day.

MATERIALS AND METHOD

The clinical study for the evaluation of the effect of HA on the proliferation of PDL fibroblast an in vitro study was conducted. The primary human PDL cells were obtained from the tissue culture lab and HA used was obtained from a commercially injectable syringe which contains a formulation of 8.0 mg/mL of sodium hyaluronate. A total of 30 samples were taken and were divided into two groups the control and the test group.
The control group consisted of unstimulated PDL fibroblast cell line, while the test group consisted of stimulated PDL fibroblast cell line with hyaluronic acid.

The two groups were further subdivided into 3 subgroups, each containing 5 samples. In the 1st subgroup, the proliferation activity of the stimulated and unstimulated periodontal ligament fibroblast cell line was observed after 24 hours by adding Alamar Blue to the first 10 culture plates. In the 2nd subgroup, the proliferation activity of both

the groups was observed after 72 hours, i.e., on 3rd day post seeding by adding Alamar Blue to the next 10 culture plates. In the 3rd subgroup, the proliferation activity of both the groups was observed after 120 hours, i.e., on 5th day post seeding by adding Alamar Blue to the next 10 culture plates.

Hyaluronic acid was directly pre-coated in all 15 culture plates pospost-seeding the test group. (Figure 1, 2)

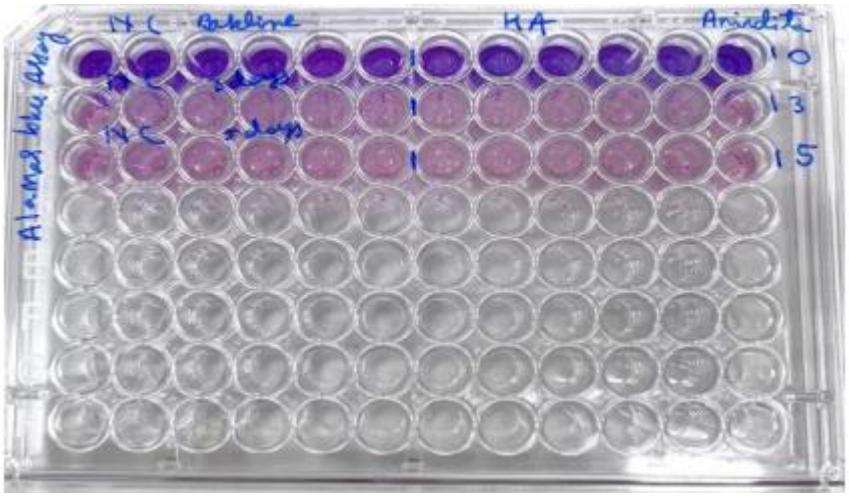

Figure 1: Control Group (Left) and Test Group (Right)

Figure 2: HyaluronicAcid

Primary human PDL cells were seeded with hyaluronic acid. At day 1 post seeding, cells were evaluated using Alamar blue assay. Moreover, to investigate the effects of HA, the cells were stimulated for 5 days with HA. Samples were then investigated for cell proliferation. (Figure 3,4)

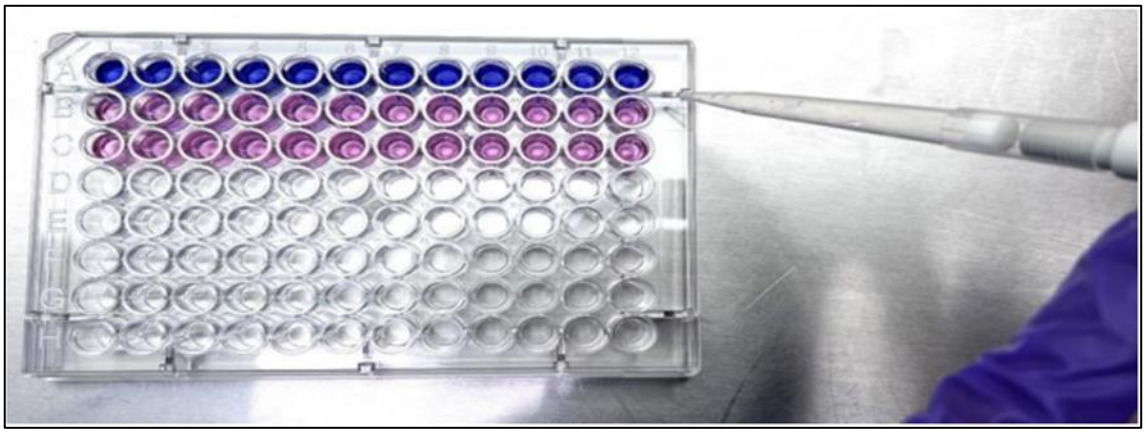

Figure 3: Alamar blue added to the culture media

Figure 4: Alamar Blue

A total of 30 samples of PDL fibroblast cell line were taken and were divided into 2 groups: Group 1 the "Control Group" and Group 2 the "Test Group". In group 1, the PDL fibroblast was seeded without adding HA to it, while in group 2, the PDL fibroblast cell line was seeded with HA.

The two groups were further divided into 3 subgroups containing 5 samples each. The cells in the test group were seeded into the culture plate with HA. The culture media used for the invitro study consisted of Dulbecco's modified eagle medium, Fetal bovine serum, Antibiotic solution which consisted of 100µ/ml Penicillin and 100µg/ml Streptomycin and 100µg/ml Gentamycin.

24 hours after seeding, Alamar blue was added to the 1st 5 samples in both group 1 and group 2 to check for the proliferation rate. After the addition of Alamar blue in both the

groups, the culture plate was inserted into the incubator at 37°C in a 5% CO2 atmosphere and was checked for the rate of proliferation of the PDL fibroblast after 3 hours under the microscope. (Figure 5,6).

Figure 5: Culture plate inside an incubator

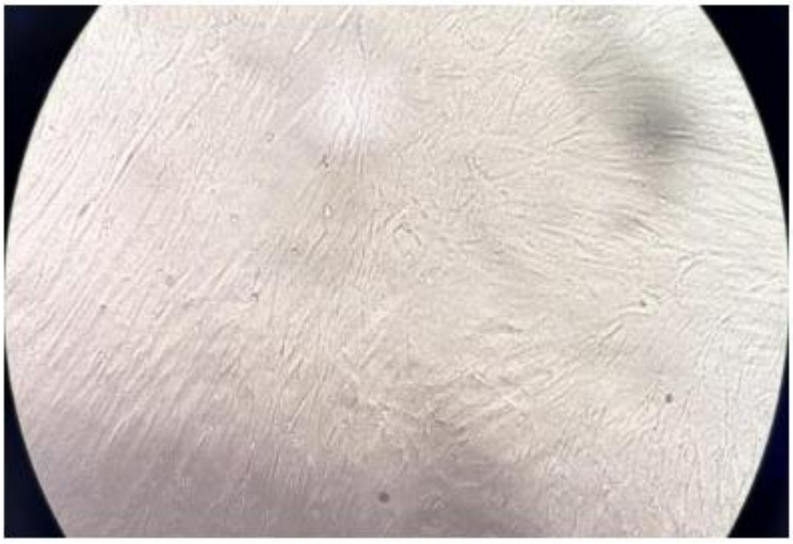

Figure 6: Microscopic view at baseline

The culture plate was then taken and viewed under SpectraMax 190, a multi-mode microplate reader that measures the optical density under two wavelengths 570nm and 600nm respectively. (Figure 7).

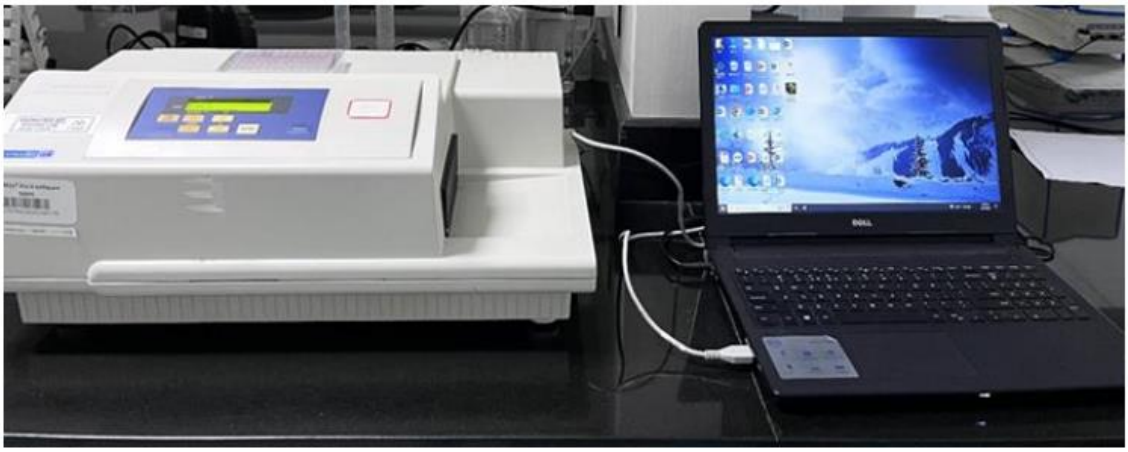

Figure 7: SpectraMax 190

The same procedure was used to check for the proliferation rate of the PDL cells on the 3rd day and the 5th day. The cell viability for each group was checked using the following formula.

CELL VIABILITY (PROLIFERATION) (% Alamar Blue Reduction) =

$$\left\{ \frac{(\text{Ɛox\_600nm X A570nm\_tx}) - (\text{Ɛox\_570nm X A600nm\_tx})}{(\text{Ɛred\_570nm X A600nm\_t0}) - (\text{Ɛred\_600nm X A570nm\_t0})} \right\} \text{ X } 100$$

In the formula,

Ɛ ox_600nm and Ɛ ox_570nm = Molar Extinction coefficient for oxidized state of Resazurin at 600nm
and 570nm respectively

Ɛ red_600nm and Ɛ red_570nm = Molar Extinction coefficient for reduced state of Resazurin at 600nm
and 570nm respectively

A570nm_tx and A600nm_tx = Absorbance measured at 570nm and 600nm at given time tx respectively

A570nm_t0 and A600nm_t0 = Absorbance measured at 570nm and 600nm at time t0 (Baseline)

RESULTS

To investigate the biocompatibility of HA towards human PDL cells, the Alamar blue assay was used. It is a simple non-radioactive assay used to monitor and determine the proliferation of various cell lines. (Ahmed et al 1994, Gloeckner et al 2001).

It was shown that HA maintained a significant amount of cell viability, at 1, 3, and 5 days post seeding. SpectraMax 190, a multi-mode microplate reader which measures the optical density under two wavelengths 570nm and 600nm respectively was used to determine the proliferation rate of the PDL cell upon adding HA. (Table 1,2 and 3)

Table 1: Spectrophotometric readings (od value) at baseline

Negative Control				**TEST**			
570nm	**MEAN**	**600nm**	**MEAN**	**570nm**	**MEAN**	**600nm**	**MEAN**
1.561	1.559	0.839	0.833	1.694	1.689	0.813	0.822
1.562		0.839		1.685		0.821	
1.558		0.830		1.688		0.825	
1.570		0.835		1.692		0.821	
1.552		0.828		1.689		0.826	
1.553		0.825		1.688		0.828	

Table 2: Spectrophotometric readings (od value) at day 3

Negative Control				**TEST**			
570nm	**MEAN**	**600nm**	**MEAN**	**570nm**	**MEAN**	**600nm**	**MEAN**
2.118	2.115	1.600	1.598	1.825	1.830	0.856	0.858
2.112		1.597		1.828		0.859	
2.110		1.599		1.829		0.860	
2.108		1.598		1.831		0.854	
2.118		1.595		1.832		0.858	
2.122		1.599		1.836		0.861	

Table 3: Spectrophotometric readings (od value) at day 5

Negative Control				**TEST**			
570nm	**MEAN**	**600nm**	**MEAN**	**570nm**	**MEAN**	**600nm**	**MEAN**
2.375	2.370	0.402	0.410	2.885	2.884	0.410	0.410
2.371		0.413		2.881		0.412	
2.380		0.403		2.880		0.409	
2.369		0.415		2.886		0.410	
2.365		0.417		2.885		0.409	
2.361		0.407		2.888		0.412	

Cell viability proliferation % was determined by Alamar blue assay under two wavelengths. (Table 4)

Table 4: Cell viability (proliferation%) by Alamar blue assay under two wavelengths

WAVELENGTH	**REDUCED RESAZURIN (ε_{red})**	**OXIDIZED RESAZURIN (ε_{ox})**
570nm	155677	80586
600nm	14652	117216

Proliferation % at baseline, day 3, and day 5 in both control and test group respectively. (Table 5, 6 and 7)

Table 5: Cell viability, proliferation % at baseline

DAYS	**BASELINE**			
	Negative control		**Test**	
WAVELENGTH	570nm	600nm	570nm	600nm

MEAN OD VALUES	1.559	0.833	1.689	0.822
CELL VIABILITY (PROLIFERATION) (%)	108		127	

Table 6: Cell viability (proliferation) (%) at day 3

DAYS	**3 DAYS**			
	Negative control		**Test**	
WAVELENGTH	570nm	600nm	570nm	600nm
MEAN OD VALUES	2.115	1.598	1.830	0.858
CELL VIABILITY (PROLIFERATION) (%)	111		141	

Table 7: Cell viability (proliferation) (%) at day 5

DAYS	**5 DAYS**			
	Negative control		**Test**	
WAVELENGTH	570nm	600nm	570nm	600nm
MEAN OD VALUES	2.370	0.410	2.844	0.410
CELL VIABILITY (PROLIFERATION) (%)	229		291	

Overall comparison of cell proliferation in the control group was determined at baseline, day 3 and day 5. (Table 8, Figure 8)

Cell proliferation (%)	Control group	P value (Friedman test)
Baseline	108.00 ± 2.83	0.001*
Day 3	111.00 ± 3.06	
Day 5	229.00 ± 4.98	

*p≤ 0.05 is statistically significant

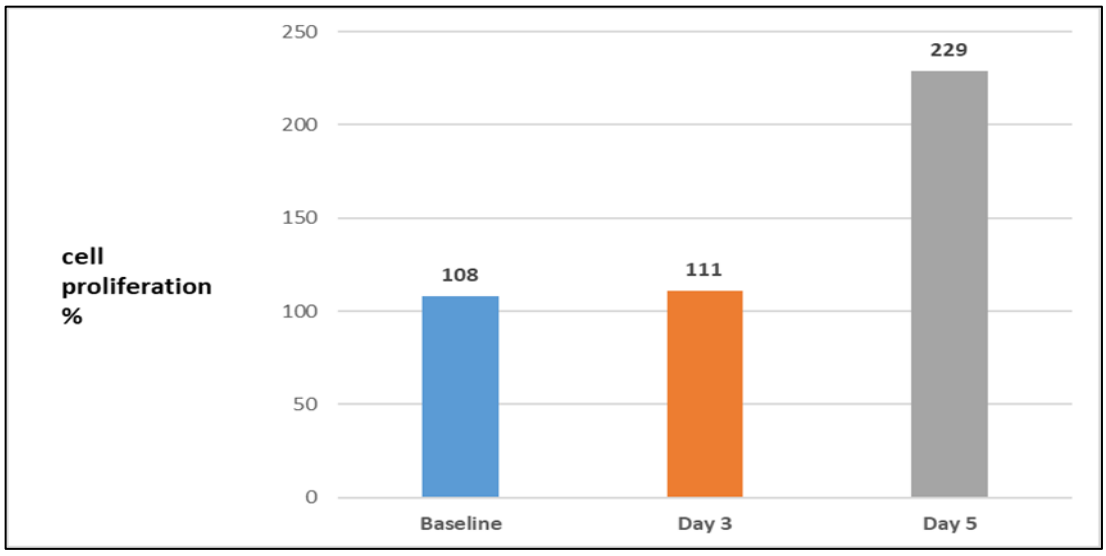

Figure 8: Overall comparison of cell proliferation in control group at baseline, day 3 and day 5.

Overall comparison of cell proliferation due to hyaluronic acid was investigated at baseline, day 3 and day 5. (Table 9, Figure 9)

Cell proliferation (%)	Test Group	P value (Friedman test)
Baseline	127 ± 2.83	0.002*
Day 3	141 ± 4.00	
Day 5	291 ± 6.26	

*p≤ 0.05 statistically significant

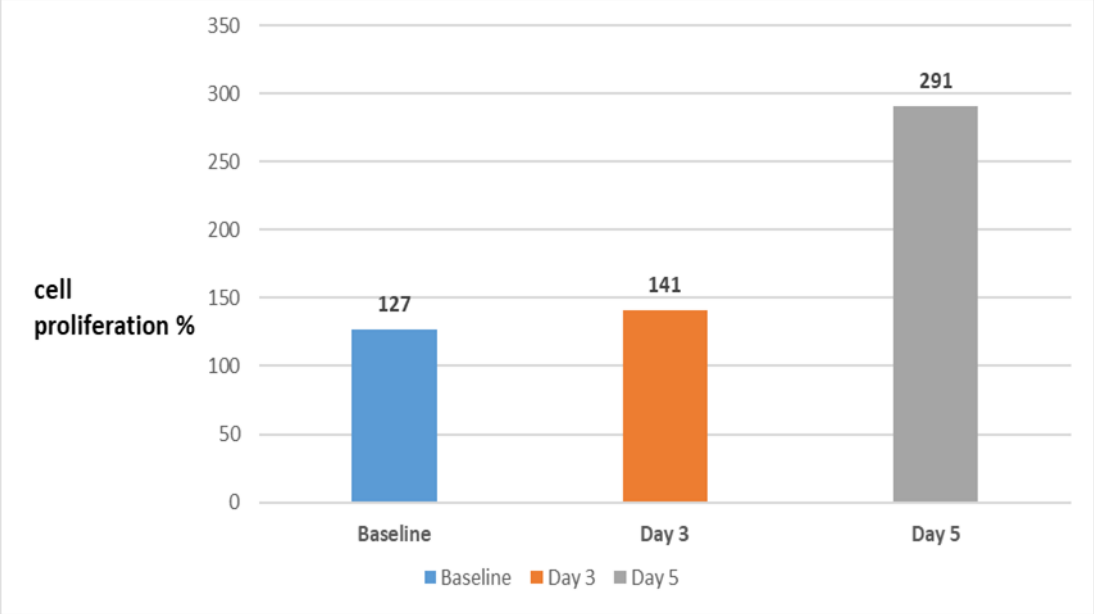

Figure 9: Overall comparison of cell proliferation due to hyaluronic acid at baseline, day 3 and day 5.

Comparison of cell proliferation was investigated between test group and control group at different time periods. (Table 10, Figure 10)

Cell proliferation (%)	Negative control	Test group	P value (Mann Whitney U test)
Baseline	108 ± 2.83	127 ± 2.83	0.002*
Day 3	111 ± 3.06	141 ± 4.00	0.002*
Day 5	229 ± 4.97	291 ± 6.26	0.002*

*p≤ 0.05 is statistically significant

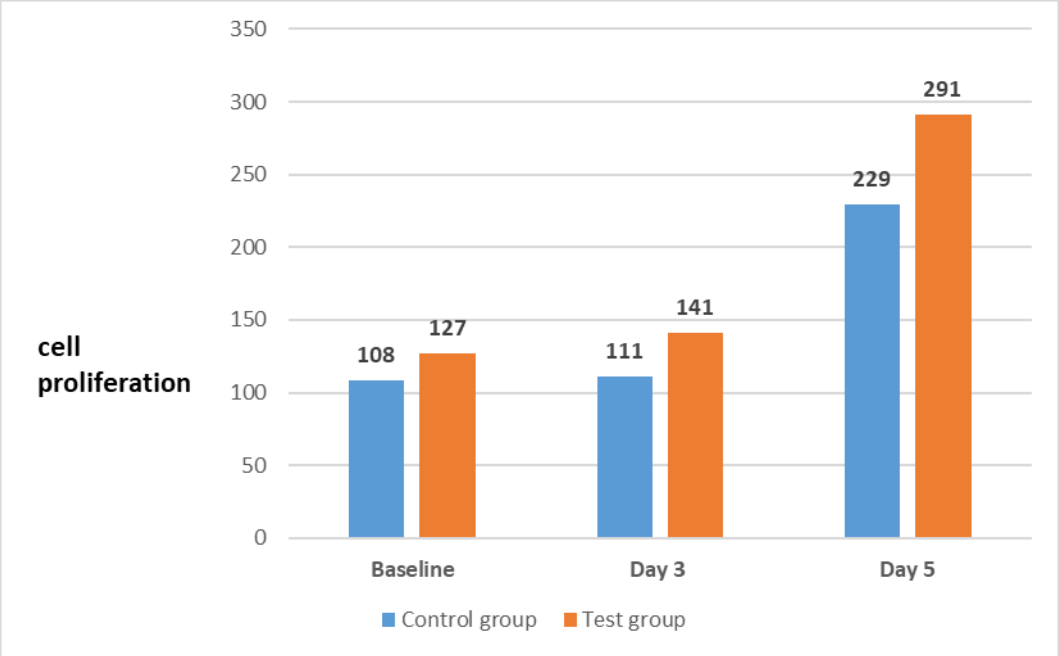

Figure 10: Comparison of cell proliferation between test group and control group at different time periods.

Comparison of cell proliferation was investigated in group 2 at baseline and day 3. (Table 11, Figure 11)

Cell proliferation (%)	Test Group	P value (Wilcoxon Signed Rank test)
Baseline	127 ± 2.83	0.027*
Day 3	141 ± 4.00	

*p≤ 0.05 is statistically significant

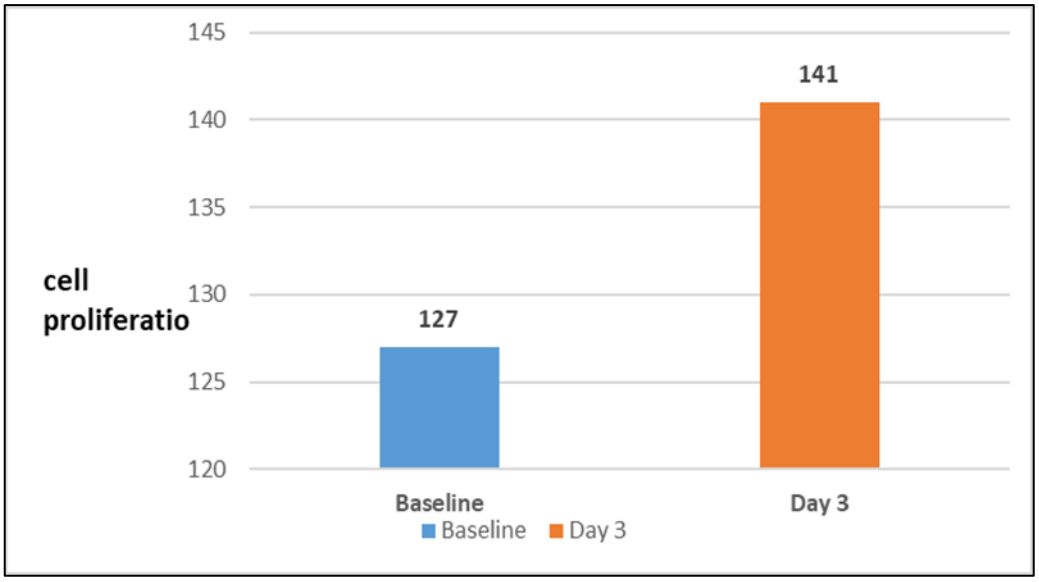

Figure 11: Comparison of cell proliferation in group 2 at baseline and day 3.

Comparison of cell proliferation was investigated in group 2 at baseline and day 5. (Table 12, Figure 12)

Cell proliferation (%)	Test Group	P value (Wilcoxon Signed Rank test)
Baseline	127 ± 2.83	0.027*
Day 5	291 ± 6.26	

*p≤ 0.05 is statistically significant

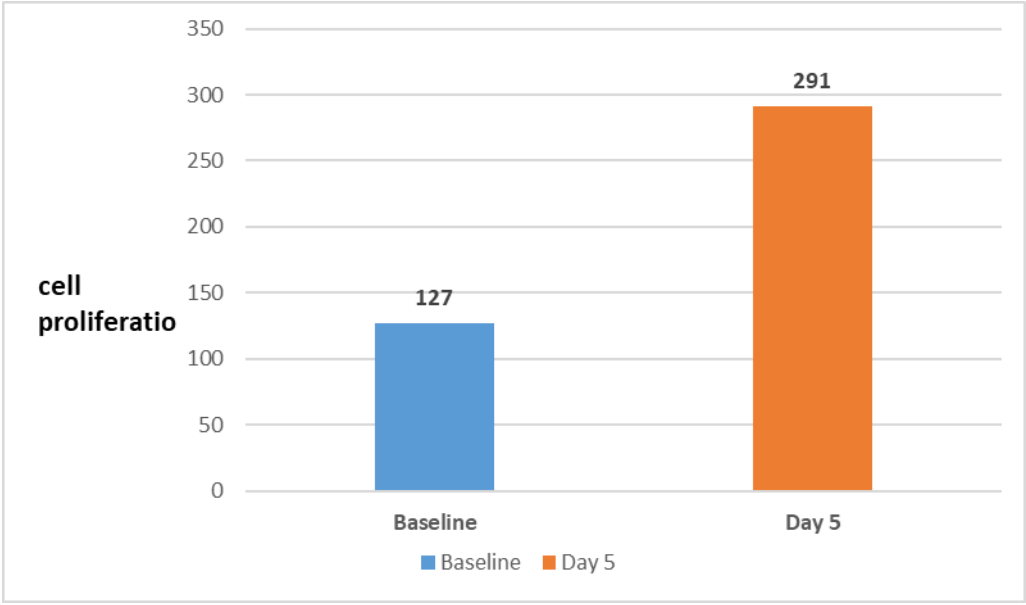

Figure 12: Comparison of cell proliferation in group 2 at baseline and day 5.

DISCUSSION

In the present study, the effect of hyaluronic acid on periodontal fibroblast cell line proliferation was evaluated in vitro. The rate of proliferation was checked between the test and control group at baseline, 3rd day, and 5th day respectively.

Hyaluronan plays a variety of roles in the mediation of the tissue healing process, from the early inflammatory activation process through to granulation tissue development and to the reepithelization process, similar to other biological processes including morphogenesis and metastasis.[17–20] It stands out among biological molecules in its physicochemical characteristics and particular interactions with cells and extracellular matrix.

It has not been demonstrated that increased hyaluronan has direct mitogenic activity, even though cell proliferation is a crucial component of tissue regeneration and is necessary for fibroblast dissociation from the matrix.[21,22] Hyaluronan, however, may have a significant, indirect, role in cell proliferation as well by aiding cell mitosis in response to mitogenic stimuli.

On the other hand, hyaluronidase therapy prevented the proliferation of periodontal ligament cells. It is widely acknowledged that during chain elongation, hyaluronic acid is maintained on the membrane-bound synthase complex, generating the hyaluronic acid matrix that surrounds cells and is cleavable by hyaluronidases.[23] Thus, it is hypothesized that matrix formation and hyaluronic acid synthesis may be essential for cell proliferation. In addition, it was proposed that exogenous hyaluronidase therapy results in the breakdown of hyaluronic acid CD44 bindings, a clustering of CD44 on the cell surface, and the elimination of the hyaluronic acid matrix.[24] It is thought that disrupting the hyaluronic acid CD44 association and preventing the proliferation of periodontal cells occurs when hyaluronidase breaks down the hyaluronic acid around the cells or when anti-CD44 blocks the interaction between the hyaluronic acid and CD44. Thus, it is proposed that the interaction between CD44 and hyaluronic acid is essential for the growth of periodontal ligament cells.

Hyaluronan gel is effective in controlling inflammation and gingival bleeding. HA containing preparations applied topically, offer a potentially beneficial adjuvant in the treatment of gingivitis, although their usage does not eliminate the necessity for plaque reduction as the main therapeutic strategy.[25] These findings were at odds with a study that assessed the potential advantages of a topical subgingival application of hyaluronic acid gel as a supplement to scaling and root planing. In comparison to scaling and root planing alone, the adjuvant use of hyaluronic acid gel did not show any clinical or microbiological benefit.[11] These contradicting results can be the result of varying inclusion criteria or hyaluronic acid in a different form.

With the application of hyaluronic acid gel, studies have shown that gingival pocket depth is reduced, and lymphocyte and epithelial cell proliferation are both significantly reduced.[26] Gel that contains 0.2% hyaluronan is effective in treating plaque-induced gingivitis. After 7, 14, and 21 days, there was a noticeable decline in the peroxidase and lysozyme activities at every study site.[27] Additionally HA gel is useful in reducing gingival bleeding and inflammation. Treatment of deep periodontal problems with esterified hyaluronic acid also showed reduction in pocket depth and increase in attachment gain after one year of treatment.[28]

Despite its apparent paradoxical function, hyaluronic acid can control the inflammatory response by removing reactive oxygen species[29] that are generated by inflammatory cells, which may help to stabilize the matrix of granulation tissue. Hyaluronic acid may also indirectly influence the onset of inflammation and the maintenance of granulation tissue by limiting the release of proteases from inflamed cells that break down extracellular matrix proteins as healing progresses.[16]

Granulation tissue gradually gives way to calcified callus in mineralized periodontal tissues like alveolar bone.[30] Hyaluronic acid engages in a variety of biological processes at this time, including cell proliferation, migration of extracellular matrix-derived cells into the injury matrix, and granulation tissue organization.

These changes enable the basal layer of the gingival epithelium to reattach to the basal lamina and the mineralized tissues to fully mature. As a result, the union of the tooth surface is restored. Hyaluronic acid synthesis slows in the later stages of granulation,

and the already-existing hyaluronic acid is depolymerized by host enzymes called hyaluronidase. This alters the composition of the granulation tissue and produces low molecular compounds. This showed that, although the precise mechanism of action is still unknown,[31] low molecular hyaluronic fragments produced by subsequent hyaluronidase activity enhance the development of blood vessels (angiogenesis) in the lesion.

To better identify the ideal applications and delivery mechanisms of HA for enhanced clinical use, additional in vitro models, especially those in inflammatory situations, as well as animal research are required.

CONCLUSION

In the domains of tissue engineering, drug delivery, and disease therapy, hyaluronic acid is appealing due to its wide availability in body tissues and inherent biocompatibility.
In several medical specialties, including ophthalmology, dermatology, and rheumatology, the use of exogenous hyaluronan and hyaluronan-based biomaterials has been helpful in modifying and speeding the wound healing process. It has also been demonstrated to be helpful in the treatment of gingivitis and periodontitis due to its bacteriostatic and anti-inflammatory properties.

In subsequent studies, it was discovered that HA demonstrated anti-inflammatory, anti-edematous, anti-bacterial, and pro-angiogenic properties. Some authors also have spoken about its significant antioxidant capacity, which was attained by scavenging reactive oxidative species, or ROS.

Topical HA can be helpful as an adjunctive treatment for gingivitis, chronic periodontitis, as well as during the postoperative phase for implant and sinus lift treatments to promote faster healing and lessen patient discomfort.

Studies further suggest that hyaluronan and hyaluronan-based biomaterials may be appropriate transporters of cells from periodontal tissues or known tissue-regenerating extracellular matrix components in the augmentation of both mineralized and non-mineralized periodontal tissues.

To fully appreciate the therapeutic benefits of hyaluronan administration in periodontal healing and surgery, laboratory research and extensive randomized, controlled clinical studies into the application of hyaluronan and hyaluronan-based biomaterial to periodontal sites are needed.

Ethics approval and consent to participate.
Not applicable. The primary human PDL cells within the present manuscript did not require ethical approval as the tissues were to be discarded.

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