Experimental Research

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The optimum oxygen level in hypoxic culture conditions of ligament derived stem cells: experimental research

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Background: The hypoxic condition is a physiological norm for various stem cells. The natural microenvironment contains lower oxygen pressures. Recent studies reported significant increases in the cultured cells' proliferation in the presence of a low oxygen pressure.
Objective: This study aimed to investigate the optimum oxygen level for rabbit cruciate ligament fibroblast cells culture and Ligament Derived Conditioned Medium/LD-CM (Secretome) preparation in vitro.
Materials and methods: Fibroblasts were isolated from the cruciate ligament of the rabbit's knee. Cultured of rabbit cruciate ligament Fibroblast Cells (fifth passage) were assigned to the slight (5% O_2), middle (3% O_2), and severe hypoxia (1% O_2) groups and the normoxia (21% O_2) group. Measurement of growth factors: TGF-β1, PDGF, FGF, and VEGF in LD-CM (Secretome) used an enzyme-linked immunosorbent assay.
Results: The highest number of cultured cells were in the 5% O_2 group compared to the normoxia, 1 and 3% groups. The hypoxia 5% group also had increased productions of PDGF, FGF, and VEGF proteins in LD-CM (secretome) compared to the 1, 3%, and normoxia groups. TGF-β1 production was slightly higher in the 3 group than the 5% group.
Conclusion: The hypoxic precondition of 5% oxygen was the optimum condition for ligament culture and ligament derived conditioned medium (secretome) preparation in vitro.

Keywords: hypoxia, ligament derived conditioned medium, normoxia, proliferation, secretome

Introduction

In orthopedic medicine, injuries to the knee cruciate ligament are common, especially the anterior cruciate ligament[1,2]. Without treatment, many knee cruciate ligament injuries will not heal properly and result in instability, restricted knee motion, and prolonged knee pain[3]. With the recent developments in functional tissue engineering, there are new approaches to the treatment of these challenging knee injuries.

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HIGHLIGHTS

- Numerous anterior cruciate ligament injuries located in the knee will not heal properly and may result in instability, restricted knee motion, and prolonged knee pain.
- As an alternative to traditional autograft reconstruction in repairing damaged anterior cruciate ligament, tissue engineering using stem cells, growth factors, and scaffolds has gained attention as a viable method.
- In tissue engineering, amplification of the proliferation of cells cultured in the presence of a low oxygen pressure (hypoxia) has been found successful in some research. However, previous review study found no of consensus concerning the best oxygen concentration/hypoxic condition for ligament culture.
- This study aimed to investigate the optimum oxygen level for rabbit cruciate ligament fibroblast cells culture and LD-CM (secretome) preparation in vitro by comparing O_2 concentrations under hypoxia 1, 3, 5%, and normoxia (21%).
- The hypoxic precondition of 5% oxygen was the optimum condition for ligament culture and ligament derived conditioned medium (secretome) preparation in vitro toward the increased cell proliferation of several important growth factors that are vitally needed for ligament healing and the cellular regeneration process.

As an alternative to traditional autograft reconstruction in repairing damaged anterior cruciate ligament, tissue engineering using stem cells, growth factors, and scaffolds has gained

attention as a viable method. However, there are still few publications that describe using ligament derived stem cells and mesenchymal stem cells in Ligament Derived Conditioned Medium (LD-CM) (secretome)[4].

Numerous studies showed that transforming growth factor (TGF) β1, pPlatelet derived growth factor (PDGF), fibroblast growth factor (FGF), and vascular endothelial growth factor (VEGF) have important roles in ligament regeneration. In a rabbit model, endogenous growth factors including PDGF and TGF-β1 were found in high concentrations during the acute phase of ligament injury, but returned to normal within one month after injury[5]. In ligament reconstruction FGF was found to aid in improving cell proliferation and cellular matrix repair[5].

The hypoxic condition is a physiological norm for a variety of stem cells. Previously, most cell cultures were maintained in vitro at about 20% oxygen level. The natural cellular environment contains lower oxygen pressures[6]. In arterial blood, the average oxygen concentration is ~12–3% in the tissue, with considerable variations observed based on the location. Some studies have used hypoxia in tissue culture techniques. Amplification of the proliferation of cells cultured in the presence of a low oxygen pressure has been found successful in some research[6].

An increasing number of studies have reported the effects from various types of hypoxic conditions. The use of regenerative medicine with ligament cell culture has begun to be developed and applied both in vitro and in vivo, but optimal oxygen levels used in ligament culture have never been studied or published so far, so in our opinion, it is necessary to start research on determining optimal oxygen levels in ligament culture[7]. A previous review study found no of consensus concerning the best oxygen concentration/hypoxic condition for ligament culture[6]. However, all studies in that review used less than or equal to 5 O_2

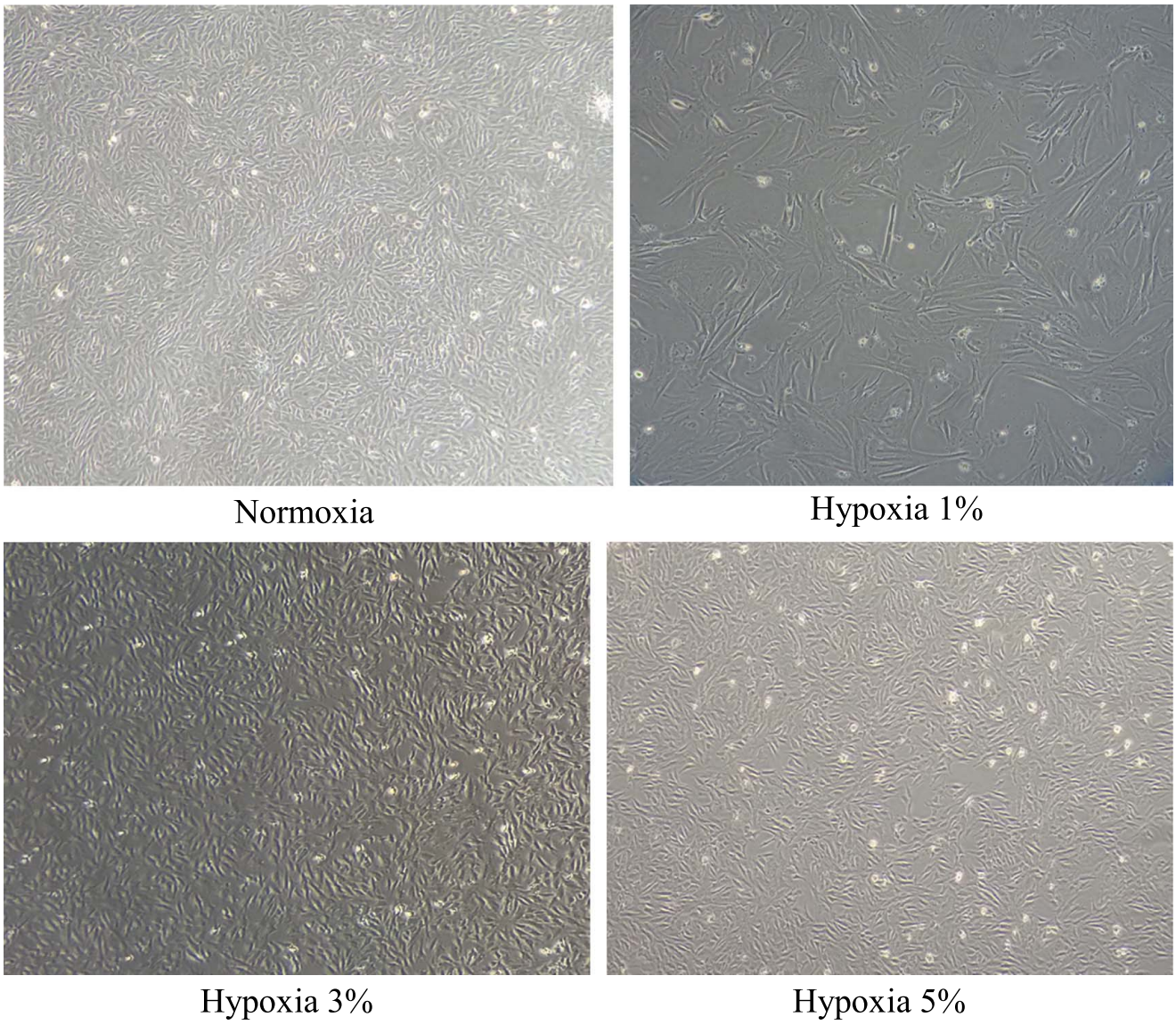

Figure 1. Morphology of rabbit Cruciate Ligament Fibroblast Cells (rCLFCs); Inverted microscopy showed flat fusiform or dendroid shapes that were arranged in a monolayer with normal cell spacing; original magnification: ×100.

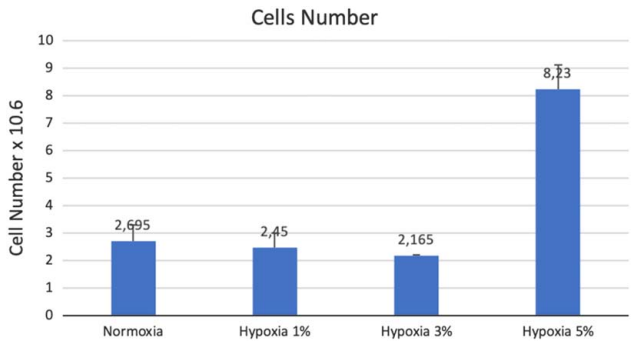

Figure 2. Cell number per group; data presented as mean ± SD.

concentration[6]. Accordingly, this study aimed to investigate the optimum oxygen level for rabbit cruciate ligament Fibroblast Cells (rCLFCs) culture and LD-CM (secretome) preparation in vitro by comparing O_2 concentrations of 1, 3, 5%, and normoxia (21%).

Materials and methods

The experiments were carried out in compliance with the principles of medical ethics and research standards for animal care and use, and the study was reported according to the ARRIVE Criteria, after being approved by the Animal Care and Use Committee, No.2.KE.027.04.2020[8].

Isolation and expansion of rabbit cruciate ligament Fibroblast Cells (rCLFCs)

Using the methods described by Banavar *et al.*[9], rCLFCs from rabbit cruciate ligaments were prepared in culture media and placed in a humidified atmosphere of 37°C and 5% CO_2 for 2 weeks. The cultured cells were examined under an inverted microscope with a magnification of × 400 (Olympus CKX53) and the medium was changed every 3 days. After 2 weeks, cells had reached 80% of confluency, and were passaged. Modifications in this experiment included using either passage (P) 4 or 5 cells.

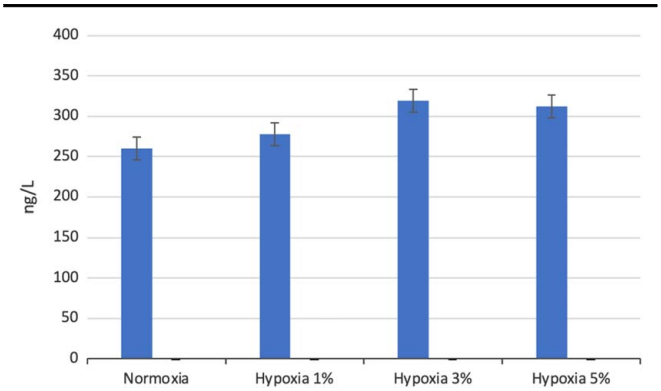

Figure 3. TGF β1 measurement results.

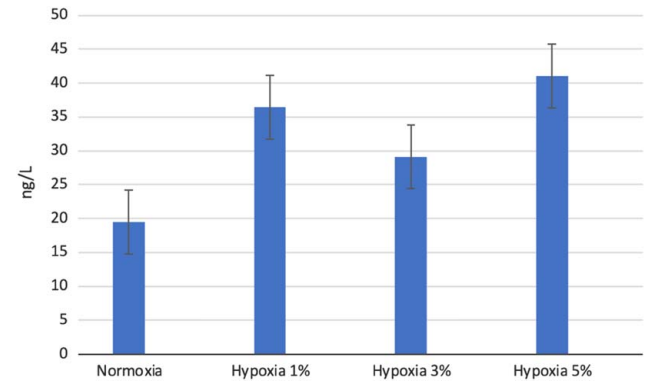

Figure 4. PDGF measurement results.

Hypoxia treatment

The hypoxia group was simulated in a water-saturated gas mixture of 1, 3, or 5% O_2, 5% CO_2, and 94% N_2 at 37°C, then after 24 h the medium was collected.

Cell counting

After 24 h of treatment every petri dish of normoxia (21%) and 1, 3, 5% hypoxia, were counted using an automatic cell counter TC20 (Bio-Rad) to determine total live cells and cell viability.

TGF-β1, PDGF, FGF, and VEGF measurements

Measurements of growth factor levels in LD-CM (secretome) in each group were done using an ELISA assay according to the laboratory protocol (Bioassay Technology Laboratory, E0052Rb (PDGF); E0133Rb (TGF-β1); E0026Rb (VEGF); E0227Rb (FGF)). The collection of LD-CM followed the instructions provided by the manufacturer. Within 10 min after adding the stop solution to each well (Bioassay Technology Laboratory), the optical density value was immediately measured using a microplate reader set to 450 nm.

Statistical analysis

The data were presented as mean and SD. All analyses were done with the SPSS 26.0 statistical package (IBM Corp.), and $P < 0.05$ considered as statistically significant and 95% CI.

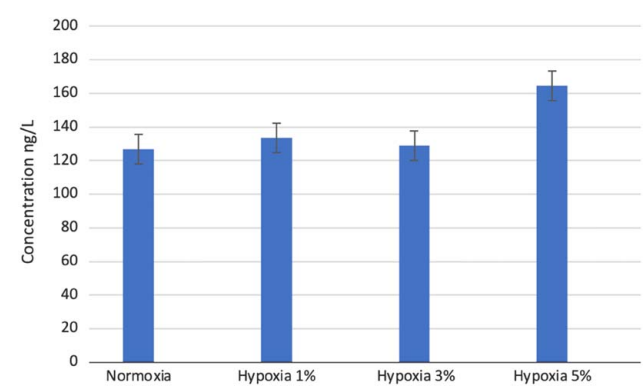

Figure 5. FGF measurement results.

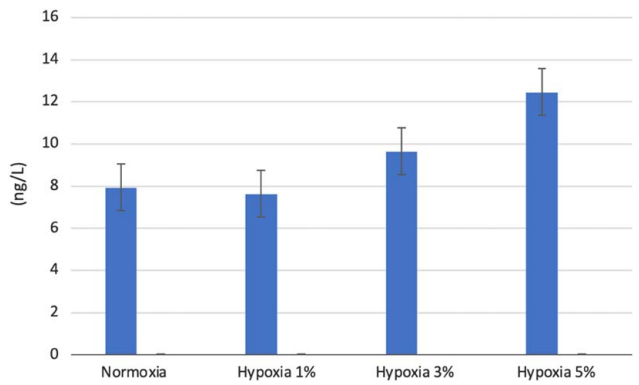

Figure 6. VEGF measurement results.

Results

Proliferation rate of rabbit cruciate ligament fibroblast cells (rCLFCs)

The cell proliferation rates of the normoxia and Hypoxia 1, 3, and 5% groups were calculated. The Hypoxia 5% group showed the highest proliferation rate compared with the other groups (Figs. 1 and 2, Table 1).

Transforming growth factor (TGF) β1 measurement

Concentration the hypoxia 3% group had the highest concentration level compared to the other groups (Fig. 3, Table 2).

The results showed that the TGF β1 level in the 3% Hypoxia group had higher levels and a significant difference compared to the 1% normoxia and 1% hypoxia groups. However, even though the TGF β1 level in the 3% hypoxic group was higher than the 5% hypoxic group, there was no significant difference.

Platelet-derived growth factor (PDGF) measurement

The mean concentration levels of the hypoxia 5% group had the highest concentration level compared to the other groups (Fig. 4, Table 3).

All data is normally distributed, then the homogeneity test is used. Levene obtained a *P*-value based on a mean of 0.025 (<0.05), that the data was not homogeneous. The comparative test used the Brown–Forsythe test and Games–Howell Post-hoc.

The results show that the 5% hypoxia group had the highest concentration and was significantly different when compared to the normoxia, 3% hypoxia and 1% groups.

Table 1

Cell number counting: data analyzed using nonparametric Kruskal–Wallis followed by Mann–Whitney post-hoc test

Group	Mean (.10^6)	SD	Median (.10^6)	Min (.10^6)	Max (.10^6)	*P*
Normoxia	2.32[a]	0.37	2.09	2.04	2.87	
Hypoxia 1%	2.61[a]	0.41	2.83	2.06	2.94	0.002*
Hypoxia 3%	2.44[a]	0.37	2.24	2.15	2.98	
Hypoxia 5%	6.82[b]	2.57	8.01	3.1	9.05	

Nonparametric Kruskal–Wallis.
*significancy <0.05.
[a,b]Different superscripts show significant group differences based on the post-hoc Mann–Whitney test.

Table 2

The mean and SD of TGF β1 levels of each group

Group	Mean (ng/l)	SD	Median (ng/l)	Min (ng/l)	Max (ng/l)	*P*
Normoxia	260.05	0.03	260.05[a]	260	260.09	
Hypoxia 1%	277.41	0.10	277.46[a,c]	277.27	277.49	0.000*
Hypoxia 3%	319.26	0.01	319.27[b]	319.25	319.28	
Hypoxia 5%	312.46	0.18	312.47[b,c]	312.44	312.49	

Nonparametric Kruskal–Wallis.
*Significancy <0.05.
[a,b,c,d]Different superscripts show significant group differences based on the post-hoc Mann–Whitney test.

Fibroblast growth factor (FGF) measurement

The mean concentration levels of FGF of the hypoxia 5% group had the highest concentration level compared to the other groups (Fig. 5 and Table 4).

All data is normally distributed, then the homogeneity test is used. Levene obtained a *P*-value based on the mean of 0.859 (>0.05), that the data is homogeneous. The comparative test used a one-way ANOVA parametric test.

The 5% Hypoxia group had the highest concentration of FGF and was significantly different when compared to the other groups.

Vascular endothelial growth factor (VEGF) measurement

Concentration levels of VEGF of the hypoxia 5% group had the highest concentration level compared to the other groups (Fig. 6 and Table 5).

VEGF levels in all groups were normally distributed ($P > \alpha$). Then the homogeneity test used by Levene obtained a *P*-value based on the mean of 0.789 (>0.05), the data was homogeneous. The comparative test used a one-way Anova parametric test

The 5% Hypoxia group had the highest VEGF levels and was significantly different when compared to the other groups.

Discussion

The novelty of this observation study was determining the optimum of oxygen level for precondition for ligament culture and LD-CM (secretome) preparation in vitro. While previous review studies had shown that severe hypoxic levels enhanced proliferation of fibroblast cells(6), this study's findings are the first to document the optimum oxygen level for ligament derived stem cells cultures and LD-CM (secretome) preparation for further study.

While most tissue cultures are maintained at oxygen levels of ~20% in vitro, previous study conducted by Zhang *et al.*[10]

Table 3

The mean and SD of PDGF levels of each group

Group	Mean (ng/l)	SD	Min (ng/l)	Max (ng/l)	*P*
Normoxia	19.51[a]	0.03	19.47	19.55	
Hypoxia 1%	36.42[b]	0.18	36.4	36.45	0.000*
Hypoxia 3%	29.14[c]	0.01	29.13	29.16	
Hypoxia 5%	41.06[d]	0.01	41.05	41.08	

The Brown–Forsythe test statistic.
*significancy <0.05.
[a,b,c,d]Different superscripts show significant group differences based on the post-hoc Games–Howell.

Table 4

The mean and SD of FGF levels of each group

Group	Mean (ng/l)	SD	Min (ng/l)	Max (ng/l)	*P*
Normoxia	126.9[a]	0.013	126.88	126.91	
Hypoxia 1%	133.66[b]	0.016	133.63	133.68	0.000*
Hypoxia 3%	128.75[c]	0.013	128.74	128.78	
Hypoxia 5%	164.37[d]	0.017	164.35	164.40	

Parametric one-way ANOVA test.
*significancy <0.05.
[a,b,c,d]Different superscripts show significant group differences based on the post-hoc Tukey.

reported varying results in their analysis of the effects of hypoxia on stem cell proliferation and differentiation.

Various studies have reported about ligament culture in hypoxic precondition. Matsuda *et al*.[11] studied ligament cultures using human periodontal ligament exposed to 5% oxygen. There has been no consensus between the studies regarding the best oxygen concentration for ligament or tendon culture. In this study, we demonstrated that the optimum concentration was found in the hypoxia 5% group, which had significantly increased cell proliferation compared to the other groups. Previous study[12] showed that culture in oxygen 3% enhanced the in vitro proliferation potential of satellite cells from old skeletal muscles. Zhang *et al*.[10] used O_2 less than or equal to 5% and showed that human periodontal ligament fibroblast growth was accelerated with a substantial increase in cell proliferation.

In the ligament regeneration process, TGFβ, TGFβ, VEGF, PDGF, and bFGF are up-regulated[13]. In the initial stages of healing, these growth factors, especially PDGF and bFGF, have important roles in fibroblast migration and proliferation as well as stimulating extracellular matrix synthesis. Additionally, TGFβ and VEGF are vital in the remodeling phases, and aid in the regulation of angiogenesis in the damaged areas[13].

Our study showed that LD-CM resulting from ligament culture with oxygen precondition 5% had the highest level of PDGF, VEGF, and FGF compared to the other groups, except that the concentration of TGF-β1 was slightly higher in the O_2 3% group than the 5% group. Previous study that investigated hypoxic conditioned media from mesenchymal stem cells used oxygen level 0.5% showed that hypoxic condition expressed lymphangiogenic factors including epidermal growth factor, FGF, hepatocyte growth factor, Insulin-like growth factor (IGF-1), and VEGF-A more than the normoxic group[14]. Another study of hypoxia in adipose MSC-hypoxic conditioned media using oxygen level 2% found significantly increased secretions of insulin-like growth factor binding protein (IGFBP)-1, IGFBP-2, macrophage colony-stimulating factor (M-CSF), platelet-derived growth factor receptor-β, and VEGF[14]. In previous studies about ligament culture under hypoxic condition, all studies showed hypoxia could significantly increase the levels of VEGF[6], but using various oxygen level such as 0.1[14], 1[15,16], 2[17,18] 5%[19,20].

Table 5

The mean and SD of VEGF levels of each group

Group	Mean (ng/l)	SD	Min (ng/l)	Max (ng/l)	*P*
Normoxia	7.93[a]	0.01	7.9	7.95	
Hypoxia 1%	7.63[b]	0.01	7.6	7.66	0.000*
Hypoxia 3%	9.63[c]	0.13	9.62	9.66	
Hypoxia 5%	12.45[d]	0.20	12.3	12.49	

Parametric one-way ANOVA test.
*significancy <0.05.
a,b,c,d: Different superscripts show significant group differences based on the post-hoc Tukey.

This study showed that hypoxia 5% was the optimum level of oxygen precondition for ligament culture and LD-CM preparation in vitro. The limitation of this study is that this study is still in the in vitro stage, so further in vivo studies and longer observations are needed to determine optimal oxygen levels in ligament culture. Further study is needed to confirm our findings and evaluate the hypoxia effect in vivo.

Conclusions

The hypoxic precondition of 5% oxygen was the optimum condition for ligament culture and LD-CM (secretome) preparation in vitro. This condition could increase cell proliferation and enhance several important growth factors that are vitally needed for ligament healing and the cellular regeneration process.

Ethics approval

This study was approved by Animal Care and Use Committee, Universitas Airlangga (No.2.KE.027.04.2020).

Consent for publication

Not applicable.

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Authors' contributions

S.R., D.N.U., C.R.S.P., H.S., D.T., H.B.N., N.A., F.A.R., and F. M.: concepted and designed this study; S.R., D.N.U., C.R.S.P., H.S.,F.A.R., and F.M.: analyzed and interpretated of the data; S.R., D.T., H.B.N., N.A., F.A.R., and F.M.: drafted of the paper; S.R., D.N.U., C.R.S.P., H.S., and D.T.: revised it critically for intellectual content; S.R., D.N.U., C.R.S.P., H.S., D.T., H.B.N., N.A., F.A.R., and F.M.: finalized approval of the version to be published. All authors agree to be accountable for all aspects of the work.

Declaration of conflict of interest

The authors declare no conflict of interest.

Availability of data and material

Not applicable.

Provenance and peer review

Not commissioned, externally peer reviewed.

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