https://doi.org/10.36377/1726-7242-2022-20-3-251-254

Results of PCR diagnostics after gingiva soft tissue augmentation

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Abstract:

Aim. To demonstrate the results of polymerase chain reaction in real time.

Materials and methods. The gum fragment was placed in 1 ml of RNA-leiter ("QIAGEN", Germany), incubated for a day at +4C, samples were stored at -70 °C.

To obtain cDNA from the RNA matrix, a ready-made set of reagents MMLV RT Kit ("Eurogen", Russia) was used, the reaction was carried out according to the attached instructions. 2 µl of random decanucleotide primer (Random dN) and 1 µl of sterile RNase-free water were added to 6 µl of RNA, heated at +70C in a “Termit” thermostat (“DNA-Technology”, Russia) for 2 min to melt secondary RNA structures, then stored on ice (+4C). 4 ml of 5X buffer was added to the reaction mixture for the synthesis of the first chain (280 mM Tris-HCl, 375 mM KCl, 30 mM MgCl2, pH 8,7), 2 ml of dNTP mixture, 2 ml of DTT and 2 ml of sterile water free of RNase. Immediately before the reaction, 1 ml of MMLV revertase (reverse transcriptase of mouse leukemia virus) was added to the mixture and added to the RNA. The test tubes were heated in the Gnome thermostat (“DNA- Technology”, Russia) at +39C for 60 minutes, then at +70C for 10 minutes in the “Termit” thermostat (“DNA-Technology”, Russia).

Results. The expression of IL-4, IL-10, IL-6, IL-12b, IL-1ß, MMP9 mRNA was not detected in the studied samples. The results of the polymerase chain reaction in real time showed that the expression levels of the proinflammatory cytokine TNFa and TIMP1 genes did not differ in both the autograft group and the collagen membrane group. The expression of MMP 2 and TIMP 2 was higher in the group using a collagen membrane, which is probably associated with tissue regeneration processes.

Conclusions. Fibro-guide collagen matrix shows no less effective clinical results in comparison with autografts

Keywords: need for orthodontic care; orthodontics; IOTN.

Received: 15.05.2022; **revised:** 20.07.2022; **accepted:** 29.07.2022.

Conflict of interests: The authors declare no conflict of interests.

Acknowledgments: there are no funding and individual acknowledgments to declare.

For citation: Anzhela B. Adzhieva, Zurab S. Khabadze, Hakob M. Nalchajyan, Sergey S. Ivanov, Elizaveta A. Vasyuta. Results of PCR diagnostics after gingiva soft tissue augmentation. Endodontics today. 2022; 20(3):251-254. DOI: 10.36377/1726-7242-2022-20-3-251-254.

INTRODUCTION

Remodeling and resorption processes are started after tooth extraction, mainly including the part of the alveolar bone that is directly adjacent to the periodontal space and into which the fibers of the periodontal ligaments in the buccal region are woven. These processes mainly occur during the first weeks after removal, which leads to a significant loss of volume, especially in the buccal part of the alveolar ridge [1-3]. Biological processes lead to a decrease in the volume of both soft and hard tissues [3]. The health and stability of the tissues around the implant are considered key factors of aesthetic treatment. Various methods and materials for soft tissue augmentation are described in the literature [6-8].

The use of subepithelial connective tissue grafts is considered the gold standard [4], but since there is an additional second surgical field in the oral cavity during this procedure, the healing process can proceed with complications [5]. Obtaining autogenic transplants is not always possible due to tissue deficiency, but alternative methods have been developed to increase soft tissues with a collagen matrix [6,9,10].

To confirm the effectiveness of the Fibro-guide collagen matrix for use in soft tissue augmentation in the oral cavity, we conducted clinical, histological, and morphometric studies [11]. And in this paper we will focus in more detail on the methods of polymerase chain reaction and demonstrate the results of polymerase chain reaction in real time.

MATERIALS AND METHODS

Real-time polymerase chain reaction.

Isolation of RNA.

The gum fragment was placed in 1 ml of RNA-leiter ("QIAGEN", Germany), incubated for a day at +4C, samples were stored at -70 °C.

To isolate total RNA, the RNeasy Plus Mini Kit ("QIAGEN", Germany) was used. The tissue (about 30 mg) was homogenized in the attached buffer “Buffer RLT Plus” with mercaptoethanol on a TissueLyser LT homogenizer ("QIAGEN", Germany) for 4 min at 40 Hz, the homogenate was centrifuged for 3 min at 9500g in an Eppendorf Mini Spin Plus centrifuge ("Eppendorf", Germany), the supernatant was transferred to a gDNA Eliminator for removal of genomic DNA. Centrifuged for 30 seconds at 8000g, an equal volume of 70% ethyl alcohol was added to the precipitate, thoroughly mixed. The sample was transferred to the RNeasy Spin Column, centrifuged for 30 sec at 8000g, Buffer RW1 was added, centrifuged for 30 sec at 8000g, Buffer RPE was added, centrifuged for 30 sec at 8000g. Buffer RPE was added, centrifuged for 2 min at 8000g, then at 9500g for drying, 40 µl of sterile water free of RNase was added, and centrifuged for 1 min at 8000g. The resulting RNA was stored at -70C.

Reverse transcription

To obtain cDNA from the RNA matrix, a ready-made set of reagents MMLV RT Kit ("Eurogen", Russia) was used, the reaction was carried out according to the attached instructions. 2 µl of random decanucleotide primer (Random dN) and 1 µl of sterile RNase-free water were added to 6 µl of RNA, heated at +70C in a "Termit" thermostat ("DNA-Technology", Russia) for 2 min to melt secondary RNA structures, then stored on ice (+4C). 4 ml of 5X buffer was added to the reaction mixture for the synthesis of the first chain (280 mM Tris-HCl, 375 mM KCl, 30 mM MgCl2, pH 8,7), 2 ml of dNTP mixture, 2 ml of DTT and 2 ml of sterile water free of RNase. Immediately before the reaction, 1 ml of MMLV revertase (reverse transcriptase of mouse leukemia virus) was added to the mixture and added to the RNA. The test tubes were heated in the Gnome thermostat ("DNA-Technology", Russia) at +39C for 60 minutes, then at +70C for 10 minutes in the "Termit" thermostat ("DNA-Technology", Russia). The synthesized cDNA was diluted with sterile water free of RNase. The resulting cDNA libraries were frozen and stored at -70C.

Real-time polymerase chain reaction

The expression levels of MMP2, MMP9, TIMP1, TIMP2, TNFa, IL-1β, IL-12b, IL-4, IL-10 and IL-10 mRNA were determined using a mixture for PCR qPCRmix-HS SYBR (Eurogen, Russia) containing the fluorescent intercalating dye Sybr Green I, according to the attached instructions.6 regarding the level of GAPDH mRNA expression on the DTprime Real-Time amplifier ("DNA-Technology", Russia). 1 ml of cDNA solution and 1 ml of primer were added to the sample. Primers for PCR were selected using the on-line Primer-BLAST program in accordance with generally accepted requirements. Primers synthesized by Eurogen (Russia) were used. The volume of the mixture was adjusted to 25 µl. To analyze gene expression, the method of determining the threshold cycle (Ct) and calculating the relative gene expression according to M.W. Pfaffl (2001) was used. The relative mRNA concentration of these genes was calculated by direct comparison of the data according to the formula: [A]0/[B]0 = EDC(T), where [A]0 is the initial concentration of the gene mRNA in the PCR mixture, [B]0 is the initial concentration of GAPDH mRNA in the PCR mixture, E is the reaction efficiency (assumed to be equal to 1.98), DS(T) is the difference between the threshold cycles of GAPDH and the desired gene.

List of primers used

Gene	Primer	5' к 3'
GAPDH	Forward	GCACCGTCAAGGCTGAGAAC
	Reverse	TGGTGAAGACGCCAGTGGA
MMP2	Forward	AGGTTCGACGTGAAGGCG
	Reverse	GCCGTCCTGTACTGAAGGAG
MMP9	Forward	AGGTTCGACGTGAAGGCG
	Reverse	GCCGTCCTGTACTGAAGGAG
TIMP1	Forward	CCTTCCAGGTGTTTCCCTGTT
	Reverse	TCCGGAAGAAAGATGGGAGTG
TIMP2	Forward	GACCCACAAGGAGATTGGGG
	Reverse	CGGAGACGACTGGTCTATGC
TNFα	Forward	CAGGCAGTCAGATCATCTTC
	Reverse	CTGGGAGTAGATGAGGTACA
IL-1β	Forward	CAGGGACAGGATATGGAGCA
	Reverse	GGCAGACTCAAATTCCAGCT
IL-12b	Forward	TAAGATGCGAGGCCAAGAATTA
	Reverse	TACTCATACTCCTTGTTGTCCC
IL-4	Forward	CTGAGAAGGAAACCTTCTGC
	Reverse	CACAGGACAGGAATTCAAGC
IL-10	Forward	CCTTCAGCAGAGTGAAGACT
	Reverse	CACTCATGGCTTTGTAGATGC
IL-6	Forward	GGTATACCTAGAGTACCTCCA
		CCCATGCTACATTTGCCGAA

Table 1. **The level of gene expression in the human gum. The data are presented in the form of Me (25%; 75%). p – statistical significance of differences, Mann-Whitney criterion.**

Conventional unit	*TNFα*	*MMP2*	*TIMP1*	*TIMP2*
Group A	0,003 (0,002-0,005)	0,0005 (0,0004-0,001)	0,26 (0,08-0,44)	0,35 (0,09-0,74)
Group F	0,014 (0,013-0,015)	0,0017 (0,0015-0,0018)	0,40 (0,31-0,56)	1,13 (0,6-1,15)
p – statistically significant differences between groups A and F	0,034	0,008		0,024

Statistical methods

The obtained data were subjected to statistical processing. The nature of the distribution of indicators was established using the Kolmogorov-Smirnov test. Since the data were distributed abnormally according to the test results, the nonparametric Mann-Whitney U-test (Statistica 8.0) was used to establish the reliability of the differences. The differences were considered statistically significant at $p<0.05$. The indicators were expressed as median and interquartile range Me (25%; 75%). The data were presented graphically using span diagrams depicting median, interquartile span, lower (25%) and upper (75%) extremes in the programs "Statistica 8.0" and Microsoft Office Excel 2016.

RESULTS

The expression of IL-4, IL-10, IL-6, IL-12b, IL-1ß, MMP9 mRNA was not detected in the studied samples.

Differences were revealed between groups A and F in the expression of TNFa, MMP2, TIMP2 (Table 1).

It was shown that the expression of MMP2 was statistically significantly higher in group F (Fig. 1). The expression level of TIMP1 in the compared groups did not differ (Fig. 2).

Differences between groups A and F in TIMP2 expression were revealed (Fig. 3). It was shown that TIMP2 expression was statistically significantly higher in Group F.

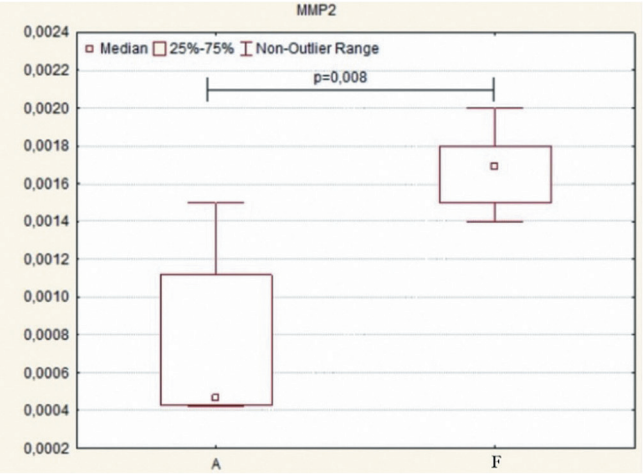

Fig. 1. **Differences in MMP2 expression between groups A and F. The data are presented in the form of Me (25%; 75%). p – statistical significance of differences, Mann-Whitney criterion.**

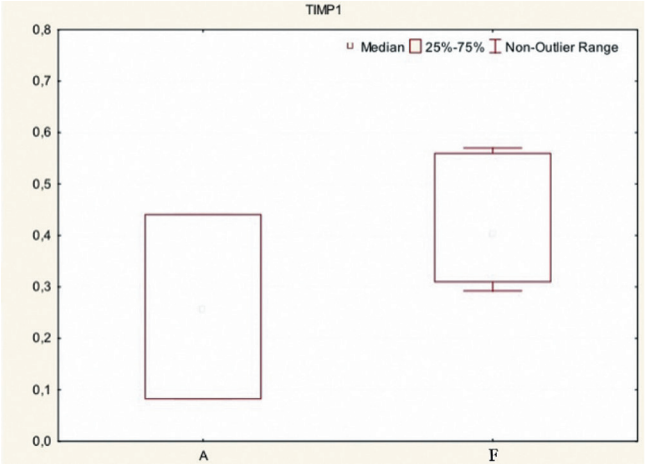

Fig. 2. **TIMP1 expression in groups A and F. The data are presented in the form of Me (25%; 75%).**

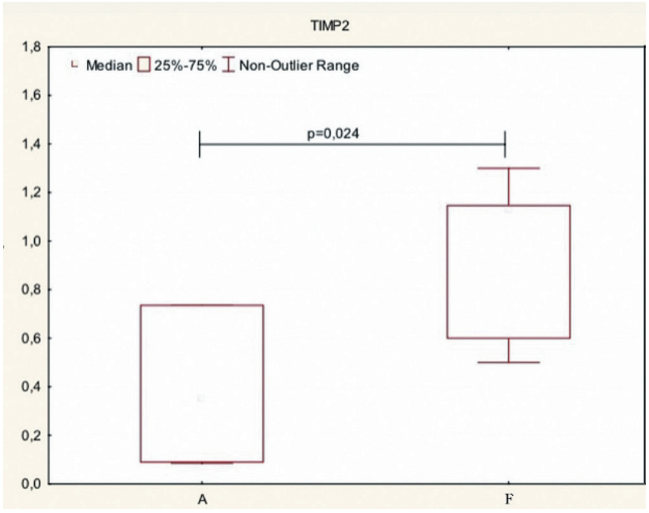

Fig. 3. **Differences in TIMP2 expression between groups A and F. The data are presented in the form of Me (25%; 5%). p – statistical significance of differences, Mann-Whitney criterion.**

Differences were revealed between groups A and F in TNFa expression (Fig. 4). It was shown that TNFa expression was statistically significantly higher in Group F.

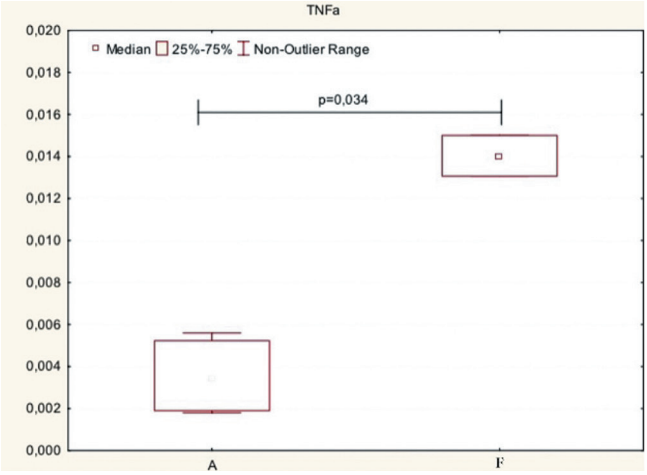

Fig. 4. **Differences in TNFa expression between groups A and F. The data are presented in the form of Me (25%; 75%). p – statistical significance of differences, Mann-Whitney criterion.**

Fig. 4. Differences in TNFa expression between groups A and F. The data are presented in the form of Me (25%; 75%). p – statistical significance of differences, Mann-Whitney criterion

DISCUSSION

The results of the polymerase chain reaction in real time showed that the expression levels of the proinflammatory cytokine TNFa and TIMP1 genes did not differ in both the autograft group and the collagen membrane group. The expression of MMP 2 and TIMP 2 was higher in the group using a collagen membrane, which is probably associated with tissue regeneration processes.

Consequently the use of the Fibro-guide collagen matrix is a good alternative to autografts, since it does not cause the development of pronounced inflammation and an increase in the expression of the proinflammatory cytokine TNFa, and also contributes to an increase in the expression of genes regulating regeneration processes.

CONCLUSIONS

Fibro-guide collagen matrix shows no less effective clinical results in comparison with autografts

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AUTHOR'S CONTRIBUTION:

Anzhela B. Adzhieva – drafted the article or revised it critically for important intellectual content.

Zurab S. Khabadze – approved the version to be published.

Hakob M. Nalchajyan – has made a substantial contribution to the concept or design of the article.

Sergey S. Ivanov – the acquisition, analysis, or interpretation of data for the article.

Elizaveta A. Vasyuta – the acquisition, analysis, or interpretation of data for the article.

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