Evaluation of antibacterial effect of concentrated growth factor on \textit{Aggregatibacter actinomycetemcomitans} and \textit{Porphyromonas gingivalis}

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\textbf{ABSTRACT}

\textbf{Introduction:} \textit{Aggregatibacter actinomycetemcomitans} and \textit{Porphyromonas gingivalis} are the two main bacteria that cause periodontitis which is an inflammatory disease of periodontal tissues. Numerous antibacterial agents have been introduced to fight against these periodontal pathogens, but the antibacterial efficacy of concentrated growth factor has not been studied yet. Therefore, this study is aimed to investigate the antibacterial impact of concentrated growth factor on the growth of \textit{Aggregatibacter actinomycetemcomitans} and \textit{Porphyromonas gingivalis}. \textbf{Methods:} In this study, concentrated growth factor was obtained from blood samples of healthy people with no systemic disease and no antibiotics used for at least 3 months before the experiments. The concentrated growth factor from each blood sample was divided into two parts, i.e., three samples in \textit{P. gingivalis} group and three samples in \textit{A. actinomycetemcomitans} group, and a positive control group to verify the accuracy of the procedure and a negative group to rule out any contamination. The minimum inhibitory concentration and minimum bactericidal concentration of CGF against \textit{A. actinomycetemcomitans} and \textit{P. gingivalis} were determined by the broth microdilution method. Statistical analysis was performed using SPSS 22 software, and \textit{P} value significance was set to 0.05. \textbf{Results:} The antibacterial property of CGF on the studied bacteria in concentrations of MIC and \(\frac{1}{4}\) MIC is the same for both bacteria, but in \(\frac{1}{2}\) MIC, its impact on \textit{A. actinomycetemcomitans} is significantly higher than \textit{P. gingivalis}. Examining the MBC showed that no MBC dose was obtained. Results of the disk diffusion test indicated the lack of the zones of bacterial growth inhibition. \textbf{Conclusions:} Considering the limitations of our study, it can be concluded that CGF has antibacterial effectiveness against \textit{A. actinomycetemcomitans} and \textit{P. gingivalis} with no significant difference between two bacteria.

\textbf{Keywords:} \textit{Actinomycetemcomitans}, antibacterial, \textit{Porphyromonas gingivalis}

\section*{Introduction}

Periodontitis is a complicated, multifactorial, and inflammatory disease of periodontal supporting tissues which can cause tooth loss. Although bacterial plaque consists of over 700 types of bacteria, just few of them are linked with the disease. These pathogenic bacteria, such as \textit{Porphyromonas gingivalis} and \textit{Aggregatibacter actinomycetemcomitans}, have a large battery of virulence factors, one of which is the ability to adhere to
intraoral surfaces. That leads to survival and therefore more destructive activities.[1]

P. gingivalis is a keystone pathogen which can cause dysbiosis of the oral microbiota in periodontal disease which reinforces and exacerbates the inflammation by causing a shift in microbial community.[2] However, A. actinomyctetemcomitans is highly associated with a rapid progression and early onset disease. A. actinomyctetemcomitans can escape from stressful challenges to a more protected subgingival domain and crush the host response to stimulate the growth of other pathogens.[3]

Nowadays, the autologous platelet concentrates (APCs) used in different medical fields including dentistry, oral surgery, orthopedics, skin, eye, and cosmetic surgery, and plastic surgery have become highly popular. Because during the activation, the platelets release the growth factors and also other molecules which heal the wounds, they also generate the positive responses both in hard and in soft tissues on regeneration.[4,5] Furthermore, the anti-inflammatory properties of the APC result in the considerable reduction in pain and postsurgical swelling.[6]

Concentrated growth factor (CGF) is an autologous biological substance with leukocyte-rich and platelet-rich fibrin; it is called the new generation of platelet concentrates.[7] CGF has a high adhesion strength, tensile strength, and higher viscosity than other platelets.[8] CGF is an organic matrix full of fibrin containing the growth factors, platelets, leukocytes, and CD34+ stem cells which help the regeneration process.[7] It also contains the immunological cells which are effective in regulating the inflammation and reducing the risk of infection.[9] Due to the high concentration of leukocytes, CGF has the antimicrobial properties. This serves as an anti-antigen factor on the unhealed chronic wounds.[9]

Unlike platelet-rich fibrin (PRF), CGF uses the centrifugation speeds of 2400–2700 rpm for decomposing the venous blood cells. This results in the fibrin-rich blocks which are much larger, denser, and richer than PRF in terms of growth; this, in turn, leads to the better regeneration and more adaptation in using the fibrin-rich block.[10]

A main difference between CGF and platelet-rich plasma (PRP) is that CGF generation does not require the addition of other reagents, i.e., it does not use the anticoagulant during the blood sampling or heterozoic thrombin and calcium chloride for activating the platelets and fibrin polymerization. Instead of using such reagents, CGF is polymerized slowly during the centrifugation in the same way as the natural in vivo polymerization. Such polymerization is necessary for the appropriate organization of a fibrin network.[11] The fibrin structure derived from CGF is desired for entrapping cytokines and cellular migration (because of the 3D structure of fibrin network) and releases slowly the growth factors of platelet for at least 7–10 days.[11,12]

In recent studies, CGF is identified as a natural scaffold with a reservoir of growth factors and cytokines in healing, proliferation, and regeneration of tissues.[9,13] No study has been conducted on the antibacterial property of CGF to this date, while there are studies on the antimicrobial characteristics of other APCs.[14–16] This study, hence, was conducted aiming at investigating the antibacterial impact of CGF on the growth of A. actinomyctetemcomitans and P. gingivalis.

**Materials and Methods**

In the present study, blood of healthy people was used to prepare CGF. All subjects voluntarily participated and were informed about the goal of the study. The ethical approval code (IR.TBZMED.REC.1399.824) was obtained from the Tabriz University of Medical Sciences. The persons had no systemic disease and infection symptoms and used no antibiotics for at least 3 months before the experiments. CGF prepared from each patient was divided into two parts, i.e., three samples in P. gingivalis group and three samples in A. actinomyctetemcomitans group. To ensure the accuracy of stages, positive control group was utilized with chlorhexidine 0.2% and negative control group was used to rule out any contamination, and the antimicrobial activity of CGF was investigated against the P. gingivalis and A. actinomyctetemcomitans.

**Preparation of CGF**

Each blood sample was collected in a sterilized test tube with no anticoagulant. Blood sample tubes were centrifuged in the special CGF centrifuge (Silfradent, Italy). CGF clots were taken from the tube and removed from red blood cells by a microscopic scissor. Clots were blended and set on a sterile gauze to discard excess serum, and then CGF clots were minced, homogenized, and carried out at -80°C for 1 h, after that centrifuged at 3000 rpm for 10 min at room temperature. The superior liquid (CGFs) was sifted using a 0.22-μm sterile syringe filter (Memberan, CA) and put away at -80°C till use.

**Lyophilization of CGFs**

According to the Wang et al.[17] study, CGFs were pre-frozen at ~80°C for 12 h and then lyophilized using a freeze dryer (Lyotrap/Plus, UK) for 24 h. Freeze-dried CGFs were stored at -4°C before use.

**Bacterial strains and culture conditions**

A. actinomyctetemcomitans ATCC 33384 and P. gingivalis ATCC 33277 were a kind gift of Prof. Abbas Bahador (Tehran University of Medical Sciences). The brain heart infusion (BHI) agar (Merck, Germany) enriched with 5% defibrinated sheep blood, menadione 1 mg/L, vancomycin 1 mg/L, and hemin 5 mg/L was applied for A. actinomyctetemcomitans, and the Brucella agar (Merek, Germany) medium enriched with 5% defibrinated sheep blood, menadione 1 mg/L, yeast extract 5 g/L, and hemin 5 mg/L was used for P. gingivalis.

**Determination of the minimum inhibitory concentration (MIC) of CGF**

The MIC of CGF against A. actinomyctetemcomitans and P. gingivalis was determined by the broth microdilution method.
as recommended by the Clinical and Laboratory Standards Institute (CLSI).\textsuperscript{18} 100 μL of CGF at the final concentration of $20 \times 10^4$ g/L was diluted as twofold serial dilutions with 100 μL of BHI broth (Merck, Germany) in the wells of a 96-well microplate. 100 μL of each bacterial suspension with a concentration of $1.5 \times 10^8$ CFU/mL was then added separately to the wells. The microplate was incubated for 24 hours at 37°C in anaerobic condition. The MIC was determined as the lowest concentration of CGF in which visible bacterial growth was inhibited. Also, the quantitative evaluation of microbial growth was determined based on the previous study.\textsuperscript{19,20} [Figure 1]

**Determination of the minimum bactericidal concentration (MBC) of CGF**

The MBC was considered the lowest concentration of CGF which prevented the growth and reduced the inoculum by $>99.9\%$ within 24 hours, irrespective of counts of survivors at higher CGF concentrations.\textsuperscript{18} For this purpose, 2 μL of contents of the well determined as the MIC and also the wells with a concentration of $2 \times _4 \times$ MIC were cultured in drops in the BHI agar enriched with 5% defibrinated sheep blood, menadione 1 mg/L, vancomycin 1 mg/L, and hemin 5 mg/L and were then incubated for 24 hours in anaerobic condition.

**Determination of the antimicrobial impact by disk diffusion test**

The blank disks were impregnated with supernatant obtained from the centrifuge of CGF samples in aseptic conditions. The suspension of half a McFarland from each of the microorganisms was then prepared in BHI broth and spread on enriched blood agar medium using a sterilized swab. The disks were placed at 2-cm intervals, and the plates were incubated at 37°C for 24 hours under anaerobic condition. After incubation, the zones of bacterial growth inhibition were measured.

**Statistical analysis**

Data were analyzed by SPSS version 22, and the significance chosen level was $P < 0.05$. Kolmogorov–Smirnov test was used as a normality test. To compare antibacterial efficacy against *A. actinomycetemcomitans* and *P. gingivalis*, one-way analysis of variance (ANOVA) was applied.

**Results**

Results of broth microdilution method showed that the MIC value of CGF for *A. actinomycetemcomitans* and *P. gingivalis* was $20 \times 10^4$ g/L. The antibacterial property of CGF on the studied bacteria in concentrations of MIC ($20 \times 10^4$ g/L) and $\frac{1}{2}$ MIC ($5 \times 10^4$ g/L) is the same for both bacteria, but in $\frac{1}{4}$ MIC ($10 \times 10^4$ g/L), its impact on *A. actinomycetemcomitans* is significantly higher than *P. gingivalis* [Table 1].

**Examining the results of minimum bactericidal concentration:** Examination of the MBC shows that no MBC dose was obtained against the studied one. So, MBC must be more than $20 \times 10^4$ g/L.

**Determining the antimicrobial impact of CGF samples by disk diffusion test:** Results of the disk diffusion test indicated the lack of the zones of bacterial growth inhibition around the disks showing the non-diffusibility of CGF samples from the disk in plate surface [Figure 2].

**Discussion**

To our recognition, this is the first study to investigate the antimicrobial

![Figure 1: Determination of the antimicrobial impact of CGF samples against *A. actinomycetemcomitans* and *P. gingivalis*](image)

![Figure 2: Lack of zones of bacterial growth inhibition around the disks in the enriched blood agar medium impregnated with bacteria in agar disk diffusion, showing the non-diffusibility of CGF samples. 1: CGF sample 1, 2: CGF sample 2, 3: CGF sample 3, B: blank (as a control)](image)

| Table 1: The antibacterial property of CGF on bacteria *A. actinomycetemcomitans* and *P. gingivalis* |
|-------------------------------------------------|
| Control ($n=3$) CFU/mL $\times 10^5$ | MIC CFU/mL $\times 10^5$ | $\frac{1}{2}$ MIC CFU/mL $\times 10^5$ | $\frac{1}{4}$ MIC CFU/mL $\times 10^5$ |
|-------------------------------------------------|
| *A. actinomycetemcomitans* | 42.96±4.16 | 33.60±3.43 | 36.85±3.00 | 40.27±3.12 |
| *P. gingivalis* | 45.06±3.28 | 31.38±1.65 | 40.86±2.16 | 43.10±2.08 |
| P* | 0.12 | 0.38 | 0.005 | 0.085 |

P: Mann-Whitney U compared to the control group; P*: Mann-Whitney U compared to both bacteria
effect of CGF, and it proved some content of antimicrobial activity which can be so helpful in the struggle versus periodontal pathogens, reducing bacterial contamination in surgical procedures. Results of this study showed that the antibacterial property of CGF on A. actinomycetemcomitans and P. gingivalis in direct contact method was significantly higher than that of the control group in terms of MIC, but it was the same as the control in ½ MIC and ¼ MIC. This study also revealed that the antibacterial property of CGF on the studied bacteria in MIC and ½ MIC is the same for both bacteria, but in ¼ MIC, its impact on A. actinomycetemcomitans is significantly higher than that on P. gingivalis.

The antibacterial property based on the non-growth halo diameter in disk diffusion test was zero for both bacteria of A. actinomycetemcomitans and P. gingivalis. The MBC dose against the studied bacteria was obtained by none of the CGF samples (1 MIC and ½ MIC).

In the present study, the antibacterial property was different based on the disk diffusion method and direct contact assay (with conditions more similar to the physiological environment).

Numerous studies indicate the impact of antibacterial properties of various forms of platelets concentrates on the bacteria including A. actinomycetemcomitans and P. gingivalis. In this line, the study by Castro et al., (2019) showed that leukocytes and platelet-rich fibrin (L-PRF) had severe inhibition against P. gingivalis in disk diffusion, but it had no effect on other bacterial strains including A. actinomycetemcomitans. In this study, the diameter of non-growth halo in disk diffusion technique was zero for both A. actinomycetemcomitans and P. gingivalis.

Kour et al., (2018) showed the antibacterial activity of three platelet concentrates of PRP, PRF, and injectable PRF (I-PRF) on the standard strains of A. actinomycetemcomitans and P. gingivalis by the diffusion disk method. Although PRP and I-PRF were more active than PRF, these scholars suggested that due to being autologous and easy preparation, I-PRF can be an extra contribution to surgical treatment in reducing the number of bacteria which help to heal and regenerate the wound.

In direct contact assay, CGF had the same impact on both bacteria and the antibacterial property of CGF was higher than that of the control group (chlorhexidine 0.2%).

The study by Chandra Gnr et al., (2017) indicated the antibacterial impact of PRP on P. gingivalis and A. actinomycetemcomitans in 2 and 7 days, respectively. But no area of inhibition by PRF was observed. In the study by Badade et al., (2016) A. actinomycetemcomitans and P. gingivalis were also inhibited by PRP, not by PRF.

The study by Yang et al., (2015) presented different results. They tested the antibacterial properties of three periodontal bacteria based on the MIC and showed that all plasma preparation methods can prevent the growth of bacteria where the PRP shows superior activity.

Results of the aforementioned studies confirm the antibacterial property of PRP in connection with the strains of A. actinomycetemcomitans and P. gingivalis, but different results were obtained concerning other platelet concentrates. In other words, studies had introduced the PRPs as a substance against the colonization and accumulation of bacteria, and generally, as a powerful substance against the postsurgical infections. Meanwhile, since the difference of APCs lies in the settings of centrifuge device speed, one can expect that the antibacterial properties of CGF are similar to other platelet concentrates.

While comparing PRP, plasma rich in growth factors (PRGF), advanced platelet-rich fibrin (A-PRF), and CGF, Masuki et al., (2016) showed that the levels of growth factor are higher than the PRP. CGF is used not only as a scaffold, but also as a source for growth factors.

CGF is an organic fibrin-rich matrix which contains growth factors, platelets, leukocytes, and CD34+ stem cells helping to the regeneration process. It also contains the immunological cells which are effective in regulating the inflammation and reducing the risk of infection.

Since no study has been conducted on the antibacterial property of CGF, its antibacterial impact mechanism is unknown. What is clear is that CGF contains many platelets and high concentration of leukocytes of which the concentration is 2–4 times higher than the whole blood. This issue can result in the antibacterial properties.

Platelets play a critical role in the host antimicrobial defense. In the host defense against the bacteria and fungi, leukocytes and neutrophils are involving effectively; this occurs through the activity of existing myeloperoxidases in neutrophils and lymphocytes produced by the immune cells in immunological defense. The leukocytes diluted in these materials, therefore, play an important role in the host defense against the bacterial infections.

One of the limitations of the current study was the in vitro design, which restricts its generalization to clinical situations. In periodontal pockets, the bacteria arrange as biofilm which is more organized and resist versus antibacterial agents. Also, considering the small literature available, more studies are needed to investigate the antimicrobial effect of APCs, especially CGF.

**Conclusions**

Considering the limitations of our study, it can be concluded that CGF has antibacterial effectiveness against A. actinomycetemcomitans and P. gingivalis with no significant difference between two bacteria. CGF can be utilized as an efficient antibacterial agent in periodontal treatments.
Acknowledgements

The authors want to thank the Department of Periodontics at Tabriz University of Medical Sciences for supporting this work and also express their gratitude to Ms. Afshaneh Ghalami, the person in charge of nursery part of periodontics department in Tabriz Faculty of Dentistry because of her aids during the blood sampling stages.

Ethics approval

All the participants signed a consent form before blood sampling.

Financial support and sponsorship

The study was funded by the dental branch of Tabriz University of Medical Sciences.

Conflicts of interest

There are no conflicts of interest.

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