The Effect of *Acidithiobacillus ferrooxidans* on Different Types of Endodontic Files

Acidithiobacillus ferrooxidans'ın Farklı Endodontik Eğeler Üzerine Olan Etkisi

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ABSTRACT Objective: Endodontic instruments cannot be removed in an orthograd way when they are fractured during endodontic therapy. Many mechanical techniques have been identified for removing broken instruments in the root canal, but bioleaching has not yet been used for this purpose. The aim of this study was to investigate the effects of acidophil species on different types of endodontic files. **Material and Methods:** Ninety-one samples of different endodontic files were chosen to investigate the effects of acidophil species on the files. Seventy-eight of these instruments were cut off at 2 mm from the tip of the file and divided into three groups: Group SS for ISO #20K stainless-steel (Dentsply-Maillefer, Ballaigues, Switzerland); Group Ni-Ti for ISO #20K Ni-Ti files (Dentsply-Maillefer); and Grup ProFile of ProFile™ #20 4% tapered (Dentsply-Maillefer). Thirteen samples served as control. All the samples were weighted with a precision balance three times. Seventy-eight of the samples were placed in sterile test tubes with Thiobacillus medium and $1x10^{11}$ CFU/mL of Acidophile species was added to the test tubes. Groups were divided into two subgroups and samples were re-weighted on the 15^{th} and 30^{th} days. Three samples were randomly selected from all groups for SEM investigation. After variance homogeneity testing, variance analysis (ANOVA) was applied to the data. The statistical differences between groups were analysed using Duncan's Multiple Range test ($p<0.01$). **Results:** Time and material in weight change were found to be statistically effective ($p<0.000$). Group SS was most affected by the time factor and provided the highest change at the end of the 30^{th} day, ($p<0.000$). In addition, there was a significant difference among all groups at both time intervals ($p<0.000$). **Conclusion:** Acidophil species has potential for solving fractured endodontic files, especially for stainless steel files.

Key Words: *Acidithiobacillus ferrooxidans*; dental instruments; root canal therapy; retreatment

ÖZET Amaç: Endodontik eğeler, kök kanal tedavisi sırasında kırıldıklarında çoğu zaman ortograd yolla uzaklaştırılamamaktadır. Kırık kanal aletini, birçok mekanik yöntemle kök kanalından uzaklaştırılması tanımlanmış olsa da bioleach bu amaçla daha önce hiç kullanılmamıştır. Bu çalışmanın amacı asidofil türlerinin farklı tipte endodontik eğeler üzerine olan etkisini incelemektir. **Gereç ve Yöntemler:** Asidofil türlerinin endodontik eğeler üzerine olan etkinliğini incelemek için üç farklı tip toplamda doksan bir adet endodontik eğe kullanıldı. Yetmiş sekiz adet eğe 2mm kesilerek; ISO #20K paslanmaz çelik (Dentsply-Maillefer, Ballaigues, İsviçre) (Grup SS), ISO #20K Ni-Ti files (Dentsply-Maillefer) (Grup Ni-Ti), ProFile #20 %4 taper açılı döner alet (Dentsply-Maillefer) (Grup ProFile) olmak üzere üç gruba ayrıldı. On üç adet örnek kontrol grubu olarak ayrıldı. Bütün örnekler hassas terazi ile üçer kez tartılarak ağırlıkları kaydedildi. Örnekler Thiobacillus özel besiyeri içeren test tüplerine yerleştirilerek, her bir tüpe $1x10^{11}$ CFU/mL Asidofil bakteri ekimi yapıldı. Gruplar, on beş gün ve otuz gün sonrası incelenme için iki alt gruba ayrılarak, aynı ağırlık ölçümleri bu dönemlerde de tekrarlandı. Her gruptan SEM incelemesi için rastgele üçer adet örnek seçildi. Varyans homojenite testi ve ANOVA uygulandı. Ağırlık ortalamaları (mg) arasındaki farklılık değerleri Duncan'ın çoklu dağılım testi ile değerlendirildi. **Bulgular:** Ağırlıkların zamana ve materyale göre değişiminin istatistiksel olarak etkili olduğu bulundu ($p<0,000$). Otuz gün sonrasında paslanmaz çelik eğelerin zamandan en fazla etkilenen eğeler olduğu bulundu. Bununla birlikte bütün eğe grupları arasında her inceleme döneminde aralarında farklılık olduğu saptandı ($p<0,000$). **Sonuç:** Asidofil türlerinin paslanmaz çelik eğelerde daha fazla olmak üzere, endodontik eğeler üzerinde potansiyel çözücü nitelikte olduğu sonucuna ulaşıldı.

Anahtar Kelimeler: *Acidithiobacillus ferrooxidans*; dental araçlar; kök kanali tedavisi; yeniden tedavi

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Bioleaching is the solubilization of metals from metallic ores and concentrates of compounds by using catalysts of bacteria under normal pressure and in the temperature range of 5-90°C. Bioprocesses-using microorganisms have become well-established technology over the years and have found applications for the extraction of copper and uranium from metal-bearing ores and scrap materials.[1-6] Likewise, bio-oxidative pretreatment of refractory gold ores and concentrates is now commercially practiced as an alternative to traditional processes.[4,7-9] In recent years, bioleaching technology has been extended to extracting other metals, including zinc, nickel, cobalt and manganese from sulfide ores and concentrates.[7,10-14] Mesophilic iron-and/or sulfur-oxidizing bacteria, notably *Acidithiobacillus ferrooxidans (Af)* is the most extensively used microorganism within the mining and metallurgical industries for the oxidation of sulfide minerals. It obtains its carbon by fixing atmospheric carbon dioxide and is obligatory autotrophic.[15] Considerable interest has been shown in *Af* because of its use in industrial mineral processing, agriculture and for refining waste water.[16-19]

In endodontic treatment fractured rotary nickel-titanium (Ni-Ti) rotary files are a common procedural problem. This constitutes one of the most troublesome procedural considerations, especially given that the frequency of use of Ni-Ti in endodontic practice is increasing.[20] The most common causes for file separation are limitations in physical properties, inadequate access, root canal anatomy, and possible manufacturing defects.[21] A number of studies have concluded that attempts at removing fractured instruments may lead to unwanted effects such as excessive dentine removal and weakening of the tooth, ledge formation, root perforation and apical extrusion of the fragment into the periradicular tissues.[22-26] Until now, the procedure involved attempting to remove the fractured instrument from the root canal system as a whole.

In this study, a bioleaching method for removing the separated part of the instrument in the root canal system is discussed.

The aim of this study was to investigate the effects of *Af* species on different types of fractured instruments.

MATERIAL AND METHODS

Ninety-one endodontic files were used in this study. These files were divided into three groups: Group SS of stainless-steel hand ISO #20 K-files (Dentsply-Maillefer, Ballaigues, Switzerland); Group Ni-Ti of nickel-titanium hand ISO #20 K-files (Dentsply-Maillefer); and Group ProFile™: of nickel-titanium rotary #20 4% tapered files (Dentsply-Maillefer) (n=91). Thirteen samples served as a control. Pieces 2 mm in-length were cut off from the tip of files and placed in sterile 10 ml screw-capped test tubes. Seventy-eight test tubes were numbered randomly and noted for file type. The entire pieces of a file in the test tubes from the three groups were weighed, noted and then autoclaved at 134 °C for 10 minutes (Hiclave HV-8.5; Hirayama Manufacturing Co.; Japan).

CULTURE AND MICROBIOLOGICAL PREPARATIONS OF AF

For the microbiological process, *Af* (ATCC 19859) was obtained for the bacterial culture of the strains. *Thiobacillus* medium with thiosulfate was used. This medium was prepared according to the description of DSMZ (Deuthsche Sammlung von Mikroorganismen und Zellkulturen GmbH.; Braunschweig, Germany). *Thiobacillus* medium [KH_2PO_4 (3.0 g), $MgSO_4$, $7H_20$ (0.5 g), $(NH_4)_2SO_4$ (3.0 g), $CaCl_2x2H_2O$ (0.25 g), $Na_2S_2O_3x5H_2O$ (5.0 g)] with thiosulfate in 1000 mL distilled water was prepared in a sealed vessel under conditions specified by the DSMZ guidelines. The medium was prepared and autoclaved at 121°C for 15 min. *Thiosulfate* and $FeSO_4x7\ H_2O$ (10.0 mg/L) were sterilized separately by filtration and then added to the autoclaved medium in the vessel and the final pH of the medium was adjusted to 4.4-4.7. The bacterial concentration was adjusted to $1x10^{11}$ CFU/mL. A 1 mL amount of bacterial suspension was dispensed to each of 78 sterilized screw-capped test tubes; completed with the medium, the final amount in each test tube was 8 mL. Then the file tips were placed in the screw-capped test tubes

containing the medium and bacteria and incubated at 37°C under standard atmospheric conditions for 15 days. After 15 days of the incubation period, half of the tubes from each of three groups were taken off (n=13). A specimen from each group was taken for SEM (Scanning Electron Microscopy; JSM-6400-JOEL, 1995; Tokyo, Japan) investigation, fixed with 10% glutaraldehyde and coated with a 15 μm layer of gold (n=3). The rest of the specimens were vibrated for 2 minutes with sterile deionized water and re-weighed (n=12).

For each of the three groups, the remaining test tubes were incubated for a period until 30th day. After the time exposure, another 3 test tubes containing the file tips from each of the three groups were also prepared for SEM screening in the manner previously described (n=3). The rest of the specimens in the tubes were removed and weighed after having been vibrated for 2 minutes with sterilized deionized water (n=12). The differences in the weight of the files between the first and the second weighing were obtained, and were calculated as a percentage.

Statistical analyses were carried out using the SPSS 11.00 software. After variance homogeneity testing, variance analysis (ANOVA) was applied to the data and the differences between the averages of the groups were determined by Duncan's Multiple Range test ($p<0.01$).

RESULTS

Only 4 of the 13 control samples showed a weight difference and the median of the control group was smaller than 0.0001 g. Therefore, the control group was discounted from the statistical analysis. Interactions between materials and time were significant. Time and material were found to be statistically effective ($p<0.000$) in weight changes.

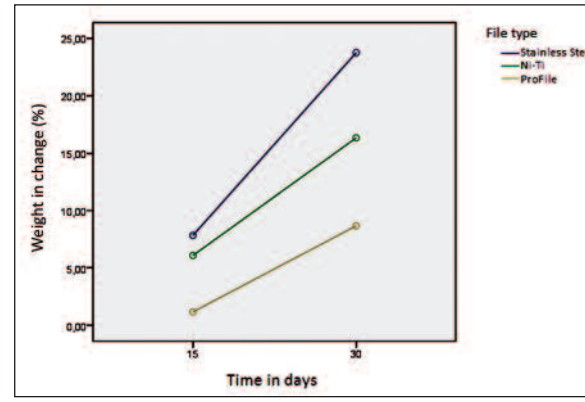

FIGURE 1: Correlation between percentage of changing weight and time. (See for colored form http://dishekimligi.turkiyeklinikleri.com/)

Weight loss by time of each material differed in groups. At the end of the 30th day, the stainless steel group was most affected by the time factor, while the Ni-Ti and ProFile™ groups showed a similar rate of change in weight (Figure 1). Also, statistical analysis of the data revealed a significant difference in weight of the experimental groups at both the 15th day ($p<0.000$) and the 30th day ($p<0.000$). At the 15th day, the ProFile™ group showed the least change in weight, whereas at the 30th day, the stainless steel group showed the most change. The mean and standard deviations of percentage in weight change, for all of the experimental groups are listed in Table 1. In addition, there were significant differences among all groups at both time intervals ($p<0.000$).

The SEM investigation supports the effect of acidophil species on the endodontic files (Figure 2). The bacillus created a large quantity of various types of metal debris scattered in type, small pieces, and large pieces of metal (Figure 3). SEM images showed that the bacillus produced a biofilm layer near the edges of the fractured part (Figure 4). The most interesting result of the SEM investigation

TABLE 1: The mean and standard deviations of percentage in weight change (mg) and time.

	Group SS		Group NiTi		Group ProFileTM	
	15th day	30th day	15th day	30th day	15th day	30th day
Average, SD (%)	7.83, 0.93	23.75, 0.94	6.08, 0.90	16.33, 1.47	1.17, 0.40	8.67, 0.70

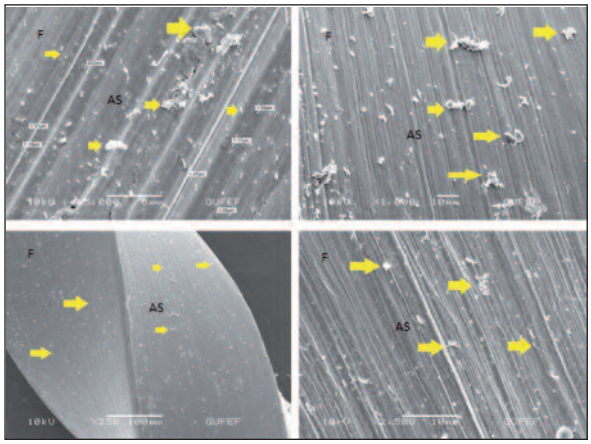

FIGURE 2: The SEM image showed the effect of acidophil species (AS-yellow arrows) on the endodontic files (SEM image x5000 magnification, samples covered with gold.
F: File.
(See for colored form http://dishekimligi.turkiyeklinikleri.com/)

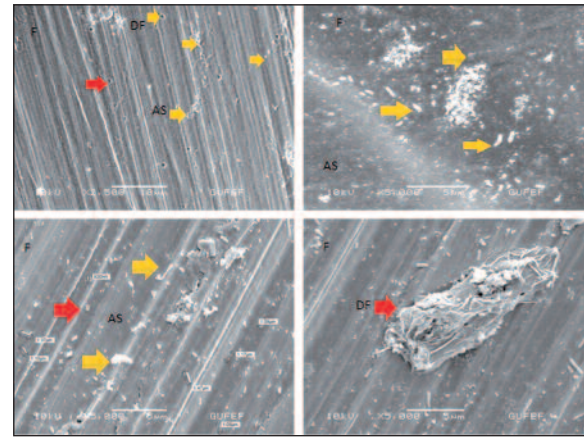

FIGURE 3: The SEM image showed that the bacillus created a large quantity of various types of metal debris scattered type, small pieces, and big pieces of metal (SEM image x5000 magnification, samples covered with gold).
F: File; DF: Defect area (red arrows); AS: Acidophil species (yellow arrows).
(See for colored form http://dishekimligi.turkiyeklinikleri.com/)

was that the bacillus made wide colonies, while the density of the bacillus increased close to the fractured edge of the endodontic files (Figure 5). The defects on the body surfaces of the files were rare when compared with the defects that occurred on the tips of the instruments (Figure 6).

DISCUSSION

Af is a chemolithotrophic, gram-negative, rod-shaped microorganism with a cell size in the range of 0.3-0.8 μm in diameter and 0.9-2 μm in length.[27,28] The optimum growth temperature of these microorganisms lies in the range of 20-40°C, depending to some extent, upon the particular strain and other growth conditions such as acidity of environment.[28,29] The bacteria can grow in acidic environments ranging from pH 1 to pH 5 with optimum growth at pH 1.8-2.5.[11,16] *Af* derives its energy required for growth and other metabolic functions from the oxidation of ferrous iron and a variety of inorganic sulfur compounds including elemental sulfur, thiosulfate, sulfite and polythionates.[27] It secures all of its cellular carbon by fixing atmospheric carbon dioxide. Although *Af* is generally considered to be strictly aerobic, it has been demonstrated that some strains, of the species are capable of anaerobic growth on elemental sulfur in extremely acidic environments causing dissimilatory reduction of ferric iron by using it as a terminal electron acceptor.[30]

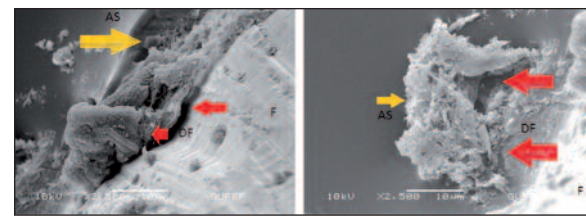

FIGURE 4: The SEM image showed that the bacillus used to produce a biofilm layer near the edges of the fractured part (SEM image x2500 magnification, samples covered with gold).
F: File; DF: Defect area (red arrows); AS: Acidophil species (yellow arrows).
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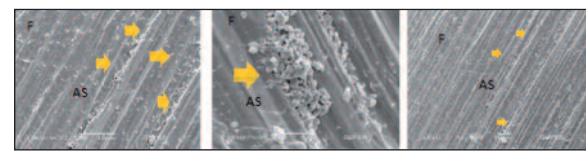

FIGURE 5: The SEM image showed that the bacillus made wide colonies, while the density of the bacillus increased close to the fractured edge of the endodontic files (SEM image x5000 magnification, samples covered with gold). F: File; F: File; AS: Acidophil species (yellow arrows).
(See for colored form http://dishekimligi.turkiyeklinikleri.com/)

In addition to the oxidation of sulfur and sulfur compounds *Af* is able to oxidize ferrous to ferric iron and so derive its energy from this exergonic reaction. In this reaction hydrogen ions

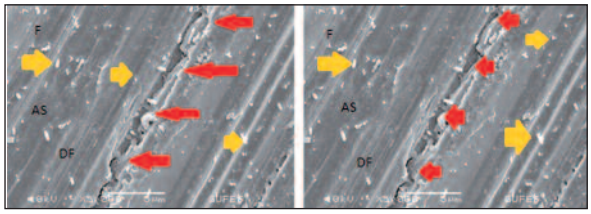

FIGURE 6: The SEM image showed that the defects on the body surfaces of the files were rare when compared with the defects that occurred on the tips of the instruments (SEM image x5000 magnification, samples covered with gold).
F: File; DF: Defect area (red arrows); AS: Acidophil species (yellow arrows).
(See for colored form http://dishekimligi.turkiyeklinikleri.com/)

are consumed and so the pH of the medium should rise. But at pH values higher than 2 the ferric iron precipitates as ferric hydroxide, jarosites or similar compounds, and this results in the formation of hydrogen ions, so that the pH of the medium is lowered as is the case with oxidation of sulfur compounds:

$$2Fe^{+2}+2H^{+}+\tfrac{1}{2}O_2 \xrightarrow{\text{bacteria}} 2Fe^{+3}+H_2O \quad (1)$$

$$2Fe^{+3}+6H_2O \longrightarrow 2Fe(OH)_3+6H^{+} \quad (2)$$

$$2Fe^{+2}+5H_2O+\tfrac{1}{2}O_2 \longrightarrow 2Fe(OH)_3+4H^{+} \quad (3)$$

The actual role of acidophil species in the oxidation process has not been completely resolved and until recently direct and indirect mechanisms have been extensively discussed in the literature.[31-36] Theoretically, the bioleaching process is primarily achieved by two mechanisms and two mechanisms of bacterial metal leaching have been proposed. In the first, referred to as the direct (bacterial) mechanism, bacteria directly interact with minerals and enhance the rate of mineral dissolution above the rate of chemical leaching driven by Fe^{3+} (e.g. Eq. 1).[19,37]

$$MeS_2+H_2O+3.5O_2 \xrightarrow{\text{bacteria}} Me^{2+}+2SO_4^{-2}+2H^{+} \quad (1)$$

Here, MeS is an insoluble metal sulfide and Me^{+2} is a free metal ion. In Eq. 1, bacteria adhere to the surface of sulfide mineral and solubilize the metal directly.

The second mechanism involves bacterial oxidation of Fe^{2+} to Fe^{3+} (e.g. Eq. 2) and subsequent chemical leaching of metal-bearing minerals by Fe^{3+} (e.g. Eq. 3). During this indirect (chemical) mechanism, bacterially-produced Fe^{3+} plays a critical role in metal solubilization.[20,38]

$$14Fe^{+2}+3.5O_2+14H \xrightarrow{\text{bacteria}} 14Fe^{3+}+7H_2O \quad (2)$$

$$MeS_2+8H_2O+14Fe^{+3} \longrightarrow Me^{+2}+14Fe^{+2}+2SO_4^{-2}+16H^{+} \quad (3)$$

The reaction represented by Eq. 2 requires the participation of bacteria, whereas Eq. 3 is a purely chemical process. Since Fe^{+2} produced in the reaction of Eq. 3 is recycled to Eq. 2, high amounts of heavy metals can be continuously leached from solid substrates.[39] In a closed system, microorganisms have a typical growth pattern, which for convenience, is divided into stages, starting with the lag phase, following through to the exponential phase (the stage of logarithmic growth, in which the number of cells doubles during each unit time period), the stationary phase, and ending with the death phase. *Af*, as with other microorganisms, usually has a period of adaptation, which leads to a delay before the onset of rapid growth. This period, in which demonstrable growth is slow, but in which new enzymes are being produced, is called the lag phase. The logarithmic phases were from 10 to 32 h for *Af* cultivated with Fe^{+2} and from 4 to 12 days for *Af* cultivated with elemental sulfur.[40]

Maximum weight loss was found in the stainless steel files. Ni-Ti files were composed of 55% nitinol alloy (by weight) and 45% titanium (by weight).[41] Stainless steel endodontic files were composed of 68.58% Fe, 20% Cr, 8.95% Ni, 1.42% Mn and 1.05% Si.[42] Because of the high Fe content of the stainless steel files, acidophil species had a greater affect on the stainless steel files than the Ni-Ti files.

Various matrices have been used for immobilization of *Af* by adhesion; for example to surface glass beads, activated carbon, ion exchange resin, or by entrapment within calcium alginate, agar, K-carrageenan and gerlite.[43-45] Other supports, such as polyurhane foam, combine the advantages of adhesion with those of entrapment.[46] Because of the special feature of bacillus, it can be applied easily to any surface. In this study, SEM images showed that bacillus produced a biofilm layer near

the edge of the fractured part. The most interesting result of the SEM investigation was that the bacillus made wide colonies, while the density of the bacillus increased close to the fractured edges of the endodontic files. The defects on the body surfaces of the instruments were rare when compared with the defects that occurred on the tips of the instruments. In addition, it is necessary to determine whether there is a sufficient amount of oxygen in the root canal system or to ensure sufficient oxygen is supplied when investigating such applications.

CONCLUSION

Acidophil species has a potential for destroying endodontic files, and it was effective on stainless steel files. These results suggest that bioleaching may be an alternative or adjunct to conventional techniques for removal of fractured instruments in the root canal system.

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