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Research Article

In Vitro Antibacterial Properties of Copper Nanoparticles as Endodontic Medicament against *Enterococcus faecalis*

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Abstract

The aim of this study was to assess fundamental antibacterial properties of Copper Nanoparticles (CuNPs) as intracanal medication against *Enterococcus faecalis (E. faecalis) in vitro*, by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). Single-rooted human teeth were incubated with *E. faecalis* and medicated with CuNPs and calcium hydroxide for 1 and 7 days. A non-medicated group was used as a control. For Colony-Formit Units count, samples from medicated root canals were collected and cultured for 24 hours to detect viable bacteria. Morphology and chemical composition of the biofilms was analyzed by scanning electron microscopy and energy-dispersive X-ray spectroscopy respectively. MIC and MBC values of CuNPs were 150 µg/mL and 225 µg/mL, respectively. CuNPs exhibited an antibacterial effect equivalent to that of calcium hydroxide with no significant differences (p>.05), exhibiting promissory antibacterial properties as intracanal medication to eliminate *E. faecalis* from infected root canal.

Keywords: Intracanal medication; *Enterococcus faecalis*; Copper nanoparticles

Introduction

Periapical disease originates from bacterial infection that invades the root canal system, inducing an immunoinflammatory host response that destroys apical tissues [1,2]. Endodontic therapy seeks to control the intracanal infection and prevent reinfection to achieve periapical reparation. Cleaning and shaping reduce bacterial contamination; however, it is not enough to completely remove it [3]. In addition, more resistant bacteria to endodontic procedures, like *E. faecalis*, are generally present and decrease success rate of the treatment. This Gram-positive facultative anaerobic bacterium has been identified in primary infections or localized in one third of the teeth with persistent periapical disease [4]. Thus, eradication of *E. faecalis* from root canal system is fundamental to improve the prognosis of the endodontically treated teeth.

Intracanal medication is a complementary procedure that involves an antimicrobial agent left inside the root canal between sessions in order to reduce the resistant bacteria to previous therapeutic actions [5]. Calcium hydroxide is the most used intracanal medication; which is a strong alkali that releases calcium and hydroxyl ions, causing protein denaturation and DNA damage [6]. Although, calcium hydroxide has good antimicrobial activity against endodontic pathogens, is less effective against *E. faecalis* and *Candida albicans* [7]. This is attributed to the buffer capacity of dentin, which decreases the pH of medium below the antibacterial value (pH=10.3) [8]. In addition, this material can be difficult to remove from the root canal system through profuse irrigation and instrumentation, remaining more than 45% into the canal surface [9]. Calcium hydroxide residues may interfere with the sealing of the endodontic sealer yielding to micro leakage and possibly interfering with treatment outcome [10].

Exploring alternative antibacterial agents with better performance against resistant bacteria and capable to act effectively within the canals is a new challenge. Due to advances in nanotechnology, metallic nanoparticles, such as silver, zinc or copper appear as a new generation of antimicrobials for biomedical applications [11,12]. Nanoparticles have dimensions smaller than 100 nm and high surface/volume ratio, which allow them to have greater interaction with bacterial membranes [13]. Furthermore, antimicrobial ability of metallic nanoparticles is generally attributed to positively charged ions, which are attracted to the negatively charged bacterial cell membranes, causing changes in their structures, in DNA replication and essential proteins, resulting in the death of the organism [14]. Silver and zinc appear among the most studied nanoparticles [14,15]. In endodontic context, a gel based on silver nanoparticles has been studied against *E. faecalis* producing a disruption of the bacterial biofilm [16]. Silver nanoparticles have been incorporated into calcium hydroxide paste, improving the capacity of the commercial product to kill and remove the *E. faecalis* biofilm from human dentin [17]. On the other, CuNPs exhibit a wide spectrum of antimicrobial activity against different species of microorganisms, including fungi and Gram-positive and Gram-negative bacteria [18]. Copper is also cheaper than silver, mixes easily with polymers and present relatively stable chemical and physical properties [19]. However, the studies about the activity of CuNPs against dental pathogens are scant. Antibacterial effect of CuNPs has been demonstrated against *Escherichia coli* and *Staphylococcus aureus* resistant to methicillin [12], and oral pathogens such as *Aggregatibacter actinomycetemcomitans* [20] and *Candida albicans* [21]. However, until now the antibacterial properties of CuNPs against resistant endodontic bacteria have been not reported.

The aim of this study was to assess fundamental antibacterial properties of CuNPs as intracanal medication against *E. faecalis in vitro*.

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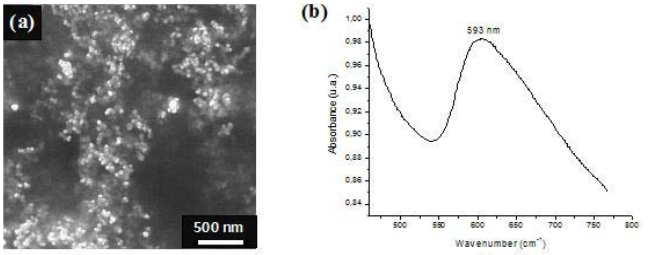

Figure 1: (a) SEM image and (b) resonance surface plasmon of CuNPs confirming nanometric size of obtained particles used in this study.

Materials and Methods

The study protocol was approved by the Ethics and Research Committee of Faculty of Dentistry, University of Chile. Verbal and written consent was obtained from all of the study participants prior to extraction of teeth.

Synthesis and characterization of CuNPs

CuNP suspensions were prepared by mixing 96.84 mL of 10% ascorbic acid, 0.242 g starch (particle stabilizer) and 3.14 mL of a 0.2 M copper acetate solution. Then, the mixture was heated in microwave for 1 minute in two series of 30 seconds to obtain a 1000 µg/mL CuNPs suspension. After that, the suspension was frozen and lyophilized to obtain CuNPs powder. CuNPs were examined by Scanning Electron Microscopy (SEM) (JSM-IT300LV, JEOL, Tokyo, Japan). Specimens were prepared by transferring a small drop of synthesized suspension to carbon tape placed on the SEM specimen holder.

Determination of minimum inhibitory and minimum bactericidal concentration

Macrodilution method was used to determine the MIC. Standard test tubes with 1 mL of Brain-Heart Infusion (BHI) and 1 mL of CuNPs in different concentrations were prepared from a stock solution of 1000 µg/mL; then 100 µL of *E. faecalis* inoculum with a turbidity equivalent to a 0.5 McFarland (1.5 × 108 Colony Formit Units (UFC)) was added. A tube containing only broth inoculated was used as a positive control and a tube containing CuNPs with BHI with no inoculum was used as a negative control. The inoculated tubes were incubated at 37°C for 48 hours. After this period, last test tube in the dilution sequence where there is no microbial growth was identified visually, determining MIC. Then, test tubes with no bacterial growth were culture in BHI Agar and incubated at 37°C for 48 hours. After this period, CFUs were counted under stereomicroscope and visually to determine MBC.

In vitro intracanal medication model

Sixty extracted teeth with orthodontic and/or periodontal indication were collected from Surgery Clinic of Dental School, University of Chile, with signed informed consent. Inclusion terms were single-rooted teeth without apical disease or resorptive pathology and closed apical foramen. Extracted teeth were maintained in alcohol 70% with glycerin until their manipulation to avoid dehydration. Ørstavik & Haapasalo´s modified protocol will be used to infected root canal [22]. Crowns and apices were removed with high velocity diamond burs to produce mid-root sections specimens

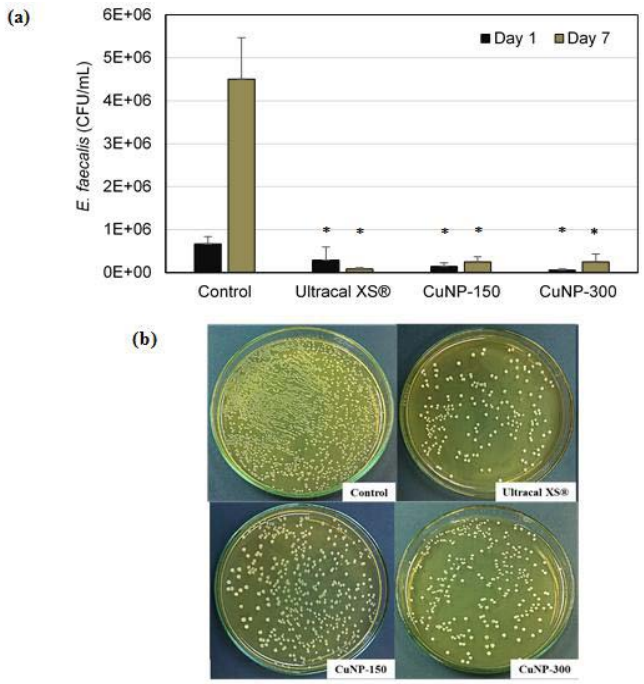

Figure 2: (a) CFU values (mean ± SE of unmedicated control specimen and of those medicated for 1 and 7 days with $Ca(OH)_2$ and CuNPs. (*) samples differ significantly of control ($p < 0.01$). (b) Photographs of CFU grown (dilution 1/100) from control and medicated specimens.

10 mm long. Periodontal tissue was removed with curettes. Volume of root canal was standardized with Gates Glidden #3 burs. Smear layer was removed in ultrasonic bath with sodium hypochlorite 5.25% during 10 minutes, rinsed with sterile saline and finally with EDTA 17% during 30 seconds. Root canals were dried with paper points and sterilized. 24 hours colonies development of *E. faecalis* (ATCC 29212) grown in BHI Agar were suspended in 3mL of BHI, adjusted spectrophotometrically (HALO RB – 10 UV – VIS RATIO BEAM) to obtain 1.5 × 108 UFC/mL of turbidity, equivalent to Mc Farland 0.5. Tubes with specimens were contaminated with 2 mL of bacterial suspension, closed and kept at 37°C during 21 days, replacing 1 mL of saturated suspension for 1 mL of freshly suspension, every 48 hours. Samples were cultivated to confirm the viability and purity of the cultures. The root specimens were rinsed with sterile saline, dried and fixed in plates with wax. Then, samples were randomly divided into 2 experimental groups (n=25) and one control group (n=10). Under aseptic conditions, canals were medicated with calcium hydroxide (Ultracal XS®, Ultradent Products Inc.) and CuNPs/distilled water suspensions of 150 and 300 µg/mL. Control group was not medicated. The medicaments were carried into the root canal using Monoject TM syringes until to observe its complete filling. Specimens were incubated during 1 and 7 days in triplicate at 37°C. After each time, specimens were washed with saline solution and immersed in 1% tween-80 surfactant solution during 10 minutes to obtain viable bacteria from samples. Direct and diluted aliquots (1/10, 1/100, 1/1000) were cultured in BHI agar; plates were kept at 37°C during 24 hours. Aliquots were cultured in BHI agar; plates were kept at 37°C during 24 hours. Colony-Formit Units (CFU) were counted from BHI agar.

Scanning electron microscopy analysis

Random areas from some samples of each group were observed to detect the presence of biofilm of *E. faecalis* and possible action of medicaments tested. For this purpose, infected canals were medicated with Ultracal XS® and CuNPs suspension of 150 µg/mL for 7 days. Specimens without medication were used as control. Adherent bacteria were fixed in 2.5% glutaraldehyde, then progressively dehydrated in ethanol, dried in supercritical CO_2, and finally coated with gold for observation by SEM coupled equipped with Energy-Dispersive X-Ray spectroscopy (EDX) for analysis of chemical composition.

Statistical analysis of the mean values of CFUs counting of each group was performed by one-way analysis of variance [23] followed by multiple comparison Bonferroni's test with the software Graph Pad Prism 5®. Statistical significance was set at $p<.05$.

Results

Characterization of copper nanoparticles

SEM observation indicates that estimated particle size of the CuNPs obtained is about 80 nm (Figure 1a). CuNP suspension exhibits an absorption maximum at 593 nm (Figure 2b) corresponding to its characteristic surface plasmon resonance that confirm the nanometric nature of the particles.

Determination of MIC and MBC

MIC of CuNPs suspension against *E. feacalis* that inhibited the visible growth of the microorganism after incubation period was 150 µg/mL. MBC value that prevented the growth of the bacterium after to be subcultured in agar plates was 225 µg/mL.

In vitro intracanal medication model

Ultracal XS®, CuNP-150 and CuNP-300 notably reduced the number of CFUs after 1 and 7 days of intracanal medication (Figure 2a), compared with the control unmedicated group ($p<.05$). There were qualitatively differences between the CFUs formed from control and those grown from the medicated root canal surfaces (Figure 2b).

SEM images reveals that the biofilm grown on the dentin surface is notably decreased after the different medication treatments (Figure 3b-e), particularly when the CuNP-300 suspension was utilized. In addition, the presence of cocci-like spherical imprints can be observed on the biofilm surfaces treated with CuNPs.

The chemical composition of the surface medicated with CuNP-150 was simultaneously analyzed by EDX. Copper nanoparticles relatively homogeneously distributed were detected on the dentin surface after the medication treatment (Figure 3f). EDX quantitative elemental analysis confirmed that C, O, Ca, P, and Cu are the major element components of CuNP-medicated dentin surface (Figure 3g). EDX mapping on an individual bacterium revealed a high copper concentration localized on the microorganism (Figure 3h).

Discussion

The main etiology of pulp and periapical disease is bacterial infection, which is set in the root canal system forming a biofilm, including areas of difficult instrumental domain. Biofilm characteristics allow microorganisms being more resistant to endodontic therapy, which added to the presence of resistant bacteria, such as *E. faecalis*, lead to explore new intracanal medication agents.

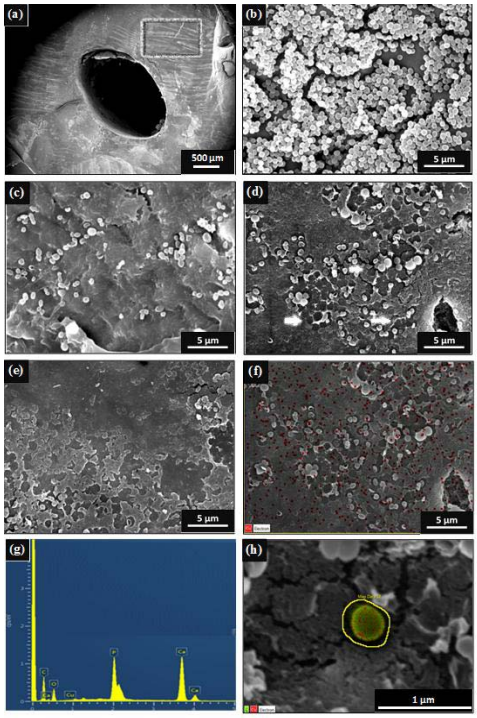

Figure 3: (a) Cross-sectional SEM image of the root intracanal system showing the zone chosen for higher magnification analysis of the medicated biofilm. (b) Representative image of *E. faecalis* biofilm grown on dentin surface, and after medication by using (c) $Ca(OH)_2$, (d) CuNP-150 (white arrows showing cocci-like spherical imprints on the biofilm surface), and (e) CuNP-300. EDX elemental microanalysis of *E. faecalis* biofilm medicated with CuNP-150: (f) EDX mapping showing copper distribution on the biofilm/dentin surface, (g) EDX spectrum with quantitative elemental analysis, (h) Copper distribution on the area occupied by an individual bacterium.

Antibacterial activity of CuNPs against *E. faecalis* was preliminarily assessed by determining the MIC (150 µg/mL) and MBC (225 µg/mL) values. MBC of pure CuNPs against *E. faecalis* had not been previously reported. This value is consistent with the MBC value reported for CuNPs supported on clay (255 µg/mL) [24]. MBC of CuNPs against Gram-positive bacteria (e.g. *Bacillus subtilis*) has been generally reported to be lower than that observed for Gram-negative bacteria (e.g. *E. coli*) [25]. It is believed that copper has great affinity towards amines and carboxylic groups of cell wall, which lead to a stronger damage of cell bacterium structure [26]. Copper particles with nanometric dimensions may also penetrate into the cell or to be oxidized to Cu^+, which form reactive oxygen species that induce DNA damage.

The results of the current study demonstrate that the CuNPs exhibit an equivalent antibacterial effect than that presented by the commercial product Ultracal XS® during an *in vitro* intracanal medication model. Antibacterial mechanism of calcium hydroxide is based on an increasing of the alkalinity of the medium, which is affected by buffer capacity of the dentin, thus reducing its bactericidal properties [8]. In contrast, CuNPs have an antibacterial mechanism independent of pH, penetrating into the bacteria, altering the osmotic balance and inducing DNA damage and cell killing [27]. The multiple

antibacterial mechanisms of CuNPs could be favorable for keeping a long-term antibacterial effect after intracanal medication, which is not generally achieved by using the traditional irrigants and medications, which undergo well-known inactivation processes [8]. Silver nanoparticles mixed with calcium hydroxide have bee also reported to have antibacterial activity against *E. faecalis* [17], providing additional evidences about the potential of metallic nanoparticles as new antimicrobial agents against endodontic pathogens.

In the medication model, CFU counting demonstrated that CuNPs reduced the number of viable bacteria by effectively killing them. In addition, SEM observations revealed the presence of cocci-like spherical imprints on the biofilm surface, which suggests that adherent bacteria are apparently removed from the biofilm. This effect could be attributed to the well-known antifouling property of copper related with its capacity to detach the microorganisms from a surface [28]. This biocide capacity includes eradicating the microorganisms that are in proximity to the surface or degrade the foulants and metabolites that have already settled on it. In addition, EDX analysis showed that copper remains adhered on the dentinal surface even after the medication has been removed. This aspect would be beneficial for endodontic therapy because could prevent the future bacteria recolonization, and thus reducing the possibilities of endodontic failures. Due to that, the *in vitro* endodontic model does not consider the presence of isthmuses and apical deltas, *in vivo* studies could provide further information about the antibacterial behavior of CuNPs during endodontic medication.

Conclusion

CuNPs, exhibit a strong bactericidal effect on *E. faecalis*, adhere on the dentine surface and could detach the bacteria from biofilm, which could have favorable consequences in preventing root canal reinfections. In this study has been demonstrated, for the first time, the antibacterial properties of nanosized copper against *E. Faecalis* and its efficacy as medication in an *in vitro* model. Future studies are required to optimize the cytocompatibility aspects or finding appropriate vehicles in order to achieve a controlled release of the nanoparticles maintaining therapeutic concentrations.

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References

1. Kakehashi S, Stanley HR, Fitzgerald RJ. The Effects of Surgical Exposures of Dental Pulps in Germ-Free and Conventional Laboratory Rats. Oral Surg Oral Med Oral Pathol. 1965; 20: 340-349.
2. Nair PN. Pathogenesis of apical periodontitis and the causes of endodontic failures. Crit Rev Oral Biol Med. 2004; 15: 348-381.
3. Bystrom A, Sundqvist G. Bacteriologic evaluation of the efficacy of mechanical root canal instrumentation in endodontic therapy. Scand J Dent Res. 1981; 89: 321-328.
4. Hancock HH 3rd, Sigurdsson A, Trope M, Moiseiwitsch J. Bacteria isolated after unsuccessful endodontic treatment in a North American population. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001; 91: 579-586.
5. Chong BS, Pitt Ford TR. The role of intracanal medication in root canal treatment. Int Endod J. 1992; 25: 97-106.
6. Siqueira JF Jr, Lopes HP. Mechanisms of antimicrobial activity of calcium hydroxide: a critical review. Int Endod J. 1999; 32: 361-369.
7. Brandle N, Zehnder M, Weiger R, Waltimo T. Impact of growth conditions on susceptibility of five microbial species to alkaline stress. J Endod. 2008; 34: 579-582.
8. Haapasalo HK, Siren EK, Waltimo TM, Orstavik D, Haapasalo MP. Inactivation of local root canal medicaments by dentine: an *in vitro* study. Int Endod J. 2000; 33: 126-131.
9. Lambrianidis T, Margelos J, Beltes P. Removal efficiency of calcium hydroxide dressing from the root canal. J Endod. 1999; 25: 85-88.
10. Margelos J, Eliades G, Verdelis C, Palaghias G. Interaction of calcium hydroxide with zinc oxide-eugenol type sealers: a potential clinical problem. J Endod. 1997; 23: 43-48.
11. Allaker RP. The use of nanoparticles to control oral biofilm formation. J Dent Res. 2010; 89: 1175-1186.
12. Ren G, Hu D, Cheng EW, Vargas-Reus MA, Reip P, Allaker RP. Characterisation of copper oxide nanoparticles for antimicrobial applications. Int J Antimicrob Agents. 2009; 33: 587-590.
13. Cushing BL, Kolesnichenko VL, O'Connor CJ. Recent advances in the liquid-phase syntheses of inorganic nanoparticles. Chem Rev. 2004; 104: 3893-3946.
14. Cioffi N, Ditaranto N, Torsi L, Picca RA, Sabbatini L, Valentini A, et al. Analytical characterization of bioactive fluoropolymer ultra-thin coatings modified by copper nanoparticles. Anal Bioanal Chem. 2005; 381: 607-616.
15. Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J Colloid Interface Sci. 2004; 275: 177-182.
16. Wu D, Fan W, Kishen A, Gutmann JL, Fan B. Evaluation of the antibacterial efficacy of silver nanoparticles against *Enterococcus faecalis* biofilm. J Endod. 2014; 40: 285-290.
17. Afkhami F, Pourhashemi SJ, Sadegh M, Salehi Y, Fard MJ. Antibiofilm efficacy of silver nanoparticles as a vehicle for calcium hydroxide medicament against *Enterococcus faecalis*. J Dent. 2015; 43: 1573-1579.
18. Ramyadevi JKJ, Arumugam Marikani, Govindasamy Rajakumar AA. Synthesis and antimicrobial activity of copper nanoparticles. Materials Letters. 2012; 71: 114-116.
19. Cava RJ. Structural chemistry and the local charge picture of copper oxide superconductors. Science. 1990; 247: 656-662.
20. Massa MA, Covarrubias C, Bittner M, Fuentevilla IA, Capetillo P, Von Marttens A, et al. Synthesis of new antibacterial composite coating for titanium based on highly ordered nanoporous silica and silver nanoparticles. Mater Sci Eng C Mater Biol Appl. 2014; 45: 146-153.
21. Usman MS, El Zowalaty ME, Shameli K, Zainuddin N, Salama M, Ibrahim NA. Synthesis, characterization, and antimicrobial properties of copper nanoparticles. Int J Nanomedicine. 2013; 8: 4467-4479.
22. Haapasalo M, Orstavik D. *In vitro* infection and disinfection of dentinal tubules. J Dent Res. 1987; 66: 1375-1379.
23. Yazlovitskaya EM, Voziyan PA, Manavalan T, Yarbrough WG, Ivanova AV. Cellular oxidative stress response mediates radiosensitivity in Fus1-deficient mice. Cell Death Dis. 2015; 6: e1652.
24. Bagchi B, Kar S, Dey SK, Bhandary S, Roy D, Mukhopadhyay TK, et al. In situ synthesis and antibacterial activity of copper nanoparticle loaded natural montmorillonite clay based on contact inhibition and ion release. Colloids Surf B Biointerfaces. 2013; 108: 358-365.
25. Yoon KY, Hoon Byeon J, Park JH, Hwang J. Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles. Sci Total Environ. 2007; 373: 572-575.
26. Ruparelia JP, Chatterjee AK, Duttagupta SP, Mukherji S. Strain specificity

in antimicrobial activity of silver and copper nanoparticles. Acta Biomater. 2008; 4: 707-716.

27. Pramanik A, Laha D, Bhattacharya D, Pramanik P, Karmakar P. A novel study of antibacterial activity of copper iodide nanoparticle mediated by DNA and membrane damage. Colloids Surf B Biointerfaces. 2012; 96: 50-55.

28. Banerjee I, Pangule RC, Kane RS. Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. Adv Mater. 2011; 23: 690-718.

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