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Fungal Derived Biosynthesised Silver Nanoparticles: a New Approach for Root Canal Disinfection- a Review

Rahul Halkai [1], Kiran. R. Halkai [2,*], Swapna Munaga [3], RajKiran Chitumalla [3], Shishir Ram Shetty [4], Jyothi C. [5]

1 Specialist Endodontist, Ajman, United Arab Emirates; drrahulendo@yahoo.co.in (R.H.);
2 College of Dentistry, Ajman University, Ajman, United Arab Emirates; drkiranhalkai@gmail.com (K.R.H.);
3 Faculty of Dentistry, Department of Restorative and Prosthetic Dental Sciences, College of Dentistry, King Saud Abdulaziz University of Health Sciences, Riyadh, Kingdom Of Saudi Arabia; swapnamunagadr@gmail.com (S.M.); rajkchitu@yahoo.com (R.C.);
4 Department of Oral Medicine & Radiology, College of Dental medicine, University of Sharjah, Sharjah, United Arab Emirates; drshishirshettyomr@yahoo.com (S.R.S.);
5 Department of Periodontics, Oxford Dental College& Hospital, Bengaluru, India; drcjyothi@gmail.com (J.C.);
* Correspondence: drkiranhalkai@gmail.com;

Scopus Author ID 55782762100

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Abstract: Complex root canal morphology, biofilm mediated endodontic infection, and growing microbial resistance to conventional root canal disinfectants lead to endodontic failures. Hence there is an ongoing search for new antimicrobial agents for root canal disinfection. Several nanoparticles were used for root canal disinfection; however, in recent days, biosynthesized silver nanoparticles (AgNPs) have gained immense interest due to their unique properties. Hence, biosynthesized AgNPs provide a new horizon for root canal disinfection due to effective antimicrobial activity and biocompatibility. This review article focuses on the biosynthesis of AgNPs using fungi and their application as antimicrobial agents in root canal disinfection.

Keywords: Biosynthesis; fungi; biosynthesized silver nanoparticles; antimicrobial agents; root canal disinfection.

1. Introduction

The main causative factors for root canal infection are the microorganisms [1]. Even after rapid development in endodontic practice, we encounter failures either due to persistent infection or reinfection; therefore, the goal of the root canal treatment is to achieve a complete microbial free environment [2]. It is a known fact that along with mechanical preparation, root canal irrigation and intracanal medicaments are essential for endodontic disinfection [3,4]. However, complex root canal morphology, adverse effects, and limitations of conventional root canal disinfectants and growing bacterial resistance to available antimicrobial agents pose a challenge for thorough root canal disinfectant [5].

Antimicrobial agents are commonly employed for root canal disinfection as irrigants and intracanal medicaments (ICMs); hence, their role is critical in endodontic treatment outcome [2]. Root canal irrigants play a key role as they penetrate the root canal irregularities, dentinal tubules, ramifications, fins, isthmus, etc., where instruments cannot reach [6]. The most commonly used endodontic irrigants are sodium hypochlorite (NaOCl), ethylene-

diamine-tetra-acetic acid (EDTA) solution, and a chlorhexidine (CHX) [7,8]. The disadvantages with NaOCl include limited penetrability in the complex root canal system, toxicity, risk of emphysema, allergy, offensive smell, taste, and also its inability to remove the smear layer[9]. Chlorhexidine (CHX) has been used effectively as an alternative to NaOCl for root canal irrigation due to its broad-spectrum antimicrobial activity and substantivity effect on root dentine. However, the disadvantages include discoloration of the tooth, can cause dryness and burning sensation in oral mucosa, and forms brown precipitate upon interaction with NaOCl [10]. Chelating agents such as 17% ethylenediaminetetraacetic acid (EDTA), 10% citric acid, tannin, and maleic acid are used for smear layer removal [11,12]. However, irrigation with EDTA could demineralize the dentine and produce erosions in coronal as well as the middle part of the root canal system, and it does not remove the organic debris [13]. Therefore, antimicrobial agents with effective antimicrobial activity without drug resistance and improved properties have to be developed [14].

Nanoparticles(NPs) have emerged as newer antimicrobial agents for root canal disinfection due to their unique properties such as smaller size in the range of 1-100 nm, high surface area; also, they can penetrate deeper into tissues at much smaller doses and biocompatibility [15]. Drug resistance has not been reported against NPs till now, as it needs several genetic mutations to occur, which need a long time for the microorganisms to overcome this [16]. Synthetic nanoparticles such as chitosan, zinc oxide nanoparticles, and AgNPs, etc., have been studied as root canal irrigants and ICMs with promising antimicrobial efficacy [17-19].

Recently, nanotechnology has shifted to biological approaches due to the concern regarding various chemicals used during processing and causing health hazards to human health and the environment. The synthetic process for producing nanoparticles is very expensive, technical difficulty, and tedious process. On the other hand, biological approaches for the production of NPs, using natural entities like fungi, bacteria, viruses, leaf extract, bark, etc., are economical, and no toxic chemicals are used in the process, much effective and biocompatible [20]. Several nanoparticles, such as silver, gold, copper, zinc, etc., are produced by biological approaches. Silver nanoparticles (AgNPs) exhibit potential antimicrobial activity against various microorganisms and are biocompatible [2]. Fungi have more advantages compared to other microorganisms for AgNPs synthesis such as: can be easily grown with simple media on a large scale, economic, fast, secrete a large amount of bioactive substances, enzymes such as nitrate reductase which act as reducing agents, possess high wall binding capabilities acting as stabilizing and capping agents hence, considered as naturally occurring nano factories [2, 21]. The present study aims to review the biosynthesis of AgNPs using fungi and their role in root canal disinfection.

2. Methodology for search

2.1. Search strategy.

Studies were identified through a search using the following electronic databases: PubMed, Web of Science, Scopus, Google Scholar, and Cochrane database of systematic reviews. The following terms were used as keywords: biosynthesized / green synthesized silver nanoparticles, fungal derived, silver nanoparticles, root canal failure, root canal disinfectants, endodontic pathogens, and antimicrobial activity and combination of these keywords. The literature was reviewed from January 1990- June 2020.

2.2 Study selection.

According to selection criteria, studies were required to (a) be published in peer-reviewed journals in the English language only (b) provide original data, and (c) explicitly report on the antimicrobial efficacy of biosynthesized silver nanoparticles against endodontic pathogens or their role in root canal disinfection. Articles published as abstracts only, thesis work, and other languages were excluded. The search results are summarized, as mentioned below.

3. Bio-based methods for AgNP synthesis

According to most of the studies, NPs produced by synthetic processes using chemical approaches cause hazards to human health and the environment. Thus, there is a growing need to develop eco-friendly processes that are economical and do not use toxic chemicals in the process [20]. This led to the search of natural entities such as microorganisms, which include bacteria, viruses, fungi, and herbal extracts, etc., which can be used to produce NP's due to their biological properties. The potential microorganisms capable of producing NPs range from simple prokaryotic bacterial cells to eukaryotic fungi and plants. The biological entities can reduce the bulk metallic compounds and produce NPs [15].

Highly stable NPs are produced if certain critical aspects have been considered, such as types of microorganisms, their genetic properties, optimal conditions for cell growth, and enzyme activity. The size and shape of the NPs can be controlled by altering substrate concentration, pH, light, temperature, buffer strength, electron donor, biomass and substrate concentration, mixing speed, and exposure time [22].

4. Fungal derived silver nanoparticles

4.1. Biosynthesis of AgNPs using fungi.

The process of biosynthesis of AgNPs using fungi is same as any other natural entities, such as isolation of microorganisms from different sources such as leaves of some plants, soil samples, bark, and roots, etc., and need to be cultured and grown depending upon the growth requirements [2, 20]. Once grown, the fungi are isolated and sub-cultured. The fungal isolate is then added to any suitable liquid growth media and cultured until lavish fungal biomass is obtained, usually within 3-4 days. The biomass is washed several times and placed in distilled water for another 24-48 hrs and filtered; the obtained filtrate without biomass is used further [2]. Usually, 1 mmol concentration of aqueous silver nitrate ($AgNO_3$) solution is added to the filtrate. If other NPs are to be produced, any other suitable ion solution such as silver sulfate, etc., are used instead of $AgNO_3$ and monitored for NPs formation. Another technique includes the direct addition of the fungal hyphae to the ion solution instead of fungal filtrate [2]. The biological reaction includes the reduction of ions in solution by fungal derived enzymes and AgNPs, thus formed were stabilized by capping peptides [23]. The reaction can be observed by visual observation of color change of the solution, which turns to dark brown color or the hyphae become dark in color, indicating the formation of AgNPs [2]. The color change after addition of $AgNO_3$ to fungal filtrate is due to excitation of surface plasmon vibrations essentially the vibrations of the group conduction electrons in the AgNPs and increase in color intensity of culture filtrate occurs due to increased number of nanoparticles produced as a result of reduction of silver ions [24, 25]. Several studies were reported using various fungi for the

biosynthesis of AgNPs. Stable AgNPs were synthesized extracellularly using *Fusarium oxysporum.* The long-term stability of the nanoparticle solution might be due to the stabilization of the AgNPs by fungal derived proteins [26]. AgNPs have been reported to interact strongly with proteins, including cytochrome *c* (Cc) [27]. In *Fusarium oxysporum*, the bioreduction of silver ions occurs due to an enzymatic process involving NADH-dependent reductase [28]. The secreted enzyme nitrate reductase was found to be dependent on NADH cofactor, and it is responsible for the formation of AgNPs in solution [29]. The high stability of NPs in solution was due to the release of capping proteins by *F. oxysporum*, which act as capping agents. It is shown that the NPs in solution remained stable at higher pH values (>12), and they aggregated at lower pH values (<2) as the protein was denatured; hence the stability of the capping protein was found to be pH-dependent [22].

Kumar et al., demonstrated *in vitro* biosynthesis of AgNPs using α-NADPH-dependent nitrate reductase enzyme purified from *F. oxysporum* and *Phytochelatin.* The advantages of this protocol have led to the development of a new approach for the biosynthesis of NPs over a range of chemical compositions and shapes without possible aggregation and also eliminates the downstream processing required for the use of these NPs in homogeneous catalysis and other applications such as non-linear optics [29]. Mukherjee et al. proposed a novel biological method using *Vericillum* sp. for AgNps synthesis using a two-step mechanism. The first step includes the trapping of the silver ions at the surface of the fungal cells. In the second step, the enzymes present in the fungal cell reduce silver ions, therefore forming AgNPs [30].

AgNPs were green synthesized using *Aspergillus flavus,* and the particles were found to be stable more than 3 months with no significant aggregation because of surface binding of stabilizing substances secreted by the fungus [31]. Extracellular biosynthesis of AgNPs *was reported* using *Aspergillus fumigatus* and *Cladosporium cladosporioides* biomass [32,33]. It was suggested that proteins, organic acids, and polysaccharides released by *C. cladosporioides* were responsible for the formation of spherical crystalline silver NPs [33]. Endophytic fungi such as *Fusarium semitectum* was found to be effective in producing stable AgNPs [34]. Endophytic fungi live inside the internal tissues of plants and do not cause any side effects. The potential advantages are they are rich sources of bioactive secondary metabolites with unique structures for the production of AgNPs [35,36].

4.2. Characterization.

Characterization of AgNPs is essential for better understanding the morphology, size, shape, presence of biomolecules, characteristics, and for confirmation of the formation of AgNPs [2, 21]. The basic characterization techniques include visual observation showing color change which can be attributed to the enzymatic reduction of silver ions when exposed to the fungi and this leads to change in the color of the solution, however, sometimes the solution remains clear, and only the fungal hyphae changes /darkens the color in solution [2]. UV Spectrum shows the absorbance peak in the range of 380-450nm due to the excitation of surface Plasmon resonance of AgNPs [20]. Scanning electron microscope (SEM) and high-resolution Transmission electron microscope (TEM) allows us to ascertain the size, shape, morphology, and aggregation level of AgNPs. The Selected Area Electron Diffraction (SAED) pattern of TEM allows confirming the crystalline nature of AgNPs [2,20]. Surface tomography, xeroradiography, and Fourier Transform Infrared Spectroscopy (FTIR) are some of the other commonly employed characterization techniques [20,21]. Extracellular biosynthesis of AgNPs employing the fungus *Cladosporium cladosporioides* was confirmed by UV–vis

spectrophotometer, and the particles were found to be 10–100 nm in dimensions as measured by TEM images. The structure of AgNPs was analyzed using XRD technique and protein–AgNP interaction assessed by FTIR spectroscopy [34]. The presence of the bands in the FTIR spectra of AgNPs indicates that the secondary structure of the proteins is not affected during the formation of AgNPs or by the binding of the proteins with the AgNPs [35]. The presence of functional groups in the FTIR spectrum indicates the fungal derived proteins act as capping agents and stabilization of AgNPs [20]. In a study, AgNps were synthesized by both *C. tropicum* and *F. oxysporum*. The study concluded the size of AgNps increased as the pH of the ion solution decreased, and at a lower temperature, the nanoparticles were smaller in size while higher temperatures produced larger nanoparticles [37].

5. Antimicrobial efficacy

5.1. Antimicrobial efficacy of fungal derived AgNPs against various pathogens.

AgNPs were employed as antimicrobial agents against several pathogens like Gram-positive, Gram-negative, MDR resistant strains and found to be effective even against tumor cells [35,36,38]. AgNPs were produced using fungus, *Penicillium Sp* isolated from healthy leaves of *C. Longa* (turmeric), characterized by visual observation, UV visible spectroscopy, Transmission Electron Microscopy (TEM) and Fourier Transform Infrared Spectroscopy (FTIR) and evaluated antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Salmonella typhimurium,* and *Enterobacter aerogenes.* The results showed that AgNPs had efficient antibacterial activity against *P. aeruginosa, K. pneumoniae,* with a maximum zone of inhibition of 21 and 15 mm. The study concluded that endophytic fungi are rich sources of secondary metabolites, which can reduce metals producing AgNPs, and exhibit considerable antibacterial activity [35].

5.2. Antimicrobial efficacy of fungal derived AgNPs against endodontic pathogens.

Relatively very few studies show the antimicrobial efficacy of AgNPs against oral pathogens [20, 21]. It is reported that biosynthesized AgNPs exhibit efficient antimicrobial activity against resistant bacteria, including *E. faecalis* with 12–16 mm zones of inhibition[39]. Antibiofilm efficacy of AgNPs was evaluated as a vehicle for calcium hydroxide medicament and shown to exhibited effective antibacterial activity against *E. faecalis* [40]. Similarly, Halkai *et al*. showed effective antimicrobial efficacy against *P.gingivitis, E.faecalis,* and *B.pumilus* [20]. AgNPs were effective even against the biofilms of several resistant strains, such as *E. faecalis* [41]. Endo-perio lesions present a great clinical challenge due to the involvement of both the tissues, hence the use of AgNPs during treatment of these lesions will be beneficial in a successful outcome. It is shown that AgNPs exhibited effective antimicrobial activity against endo-perio pathogens both in planktonic and biofilm form *in vitro* models [42].

6. Mechanism of antimicrobial activity

The antimicrobial property of silver is defined by the release rate of silver ion. Silver in bulk status is considered an inert material. However, when it gets ionized by moisture, it will result in a highly reactive state. At this stage, silver can interact with bacterial cell walls leading to structural changes due to its binding to tissue protein [43]. Studies suggest that the antibacterial action of AgNPs includes the reaction between the positively charged AgNP

molecules with the bacterial cell wall of negative charge leading to the damage of the bacteria cell wall [44]. In addition to being able to release silver ions, AgNPs may induce pits in the bacterial cell membrane and then get accumulated in the pits leading to fragmentation of the cell [45,46]. In addition to this, AgNPs can also penetrate bacterial cell walls and subsequently change the structure of the cell membrane which leads to the release of Ag ions, disruption of the cell wall and cellular contents, realize of reactive oxygen species (ROS), DNA damage and ultimately the cell death [47,48].

AgNPs have a higher affinity for Gram-negative strains, which is due to the lower peptidoglycan content and narrower cell walls in Gram-negative bacteria. The thicker cell walls of Gram-positive bacteria inhibit the penetration of AgNPs into the cells [49]. The difference in affinity for bacteria is due to the ability of AgNPs for binding to the enzymes containing disulfide or sulfhydryl (SH) groups of enzymes that lead to disruption of metabolic processes, which in turn causes the cell death [50]. And it suggests that uptake of AgNPs is important to impart the antimicrobial effect on bacteria [51]. Other factors, such as the concentration, size, and shape of NPs, also affect the efficacy against the microorganisms [2]. Further, a decrease in the size of NPs increases antimicrobial efficacy due to increased surface area and biocompatibility; however, using AgNps in low concentrations can cause bacterial resistance [52].

Endodontic infection is biofilm mediated infection that poses the greatest difficulty for endodontic disinfection. It is shown that complete eradication of biofilm was not achieved with AgNPs, however, the same concentration has eradicated all of the planktonic bacteria [53]. The complicated structure and composition of the biofilm, diffusion rate, size, and shape, physicochemical characteristics of the AgNPs may determine its effect on biofilm [54]. The possible mechanism for biofilm inhibition to AgNPs may be attributed due to (1)Transport of AgNPs through biofilm can be greatly obstructed for particles larger than 50 nm (2) the chemical composition of nanoparticles can arouse adsorption and accumulation of silver nanoparticles in the biofilm, thereby reducing their diffusion (3) the electrostatic interaction between bacteria and AgNPs can influence charged nanoparticles penetration through the biofilm [55].

7. Applications of AgNPs in endodontics

AgNPs can be alternatively used to conventional irrigants for intracanal irrigation during endodontic treatment. Gutta-percha coated with AgNPs has been developed as an antimicrobial obturator for root canal obturation [56]. AgNPs are also incorporated as antibacterial material into mineral trioxide aggregate to enhance the success of pulp-capping, apexification, and sealing perforations in teeth [57]. Due to the unique properties and effective antimicrobial activity of biosynthesized AgNPs against the endodontic pathogens including the most resistant microbe *E. faecalis*, it has been recommended to incorporate AgNPs for root canal disinfection; they can be effectively used as root canal irritants, ICMs and can be combined with other ICMs for synergistic effects [2, 41].

However, several concerns need to be addressed for effective clinical use, such as discoloration of root dentin or the tooth, if it is used for root canal disinfection, any irritation to periapical tissues if it leaches, whether they can dissolve with contact to periapical tissues. It has been suggested that nanosilver particles slowly dissolve into a more toxic form, usually slowly with time[58]. However, studies show that fungal derived biomolecules act as stabilizing agents and prevent the dissolution of silver [35, 36].

Recently, the cytotoxicity of fungal derived biosynthesized AgNPs was evaluated on normal Human gingival fibroblast (HGF) cell line and found to be least cytotoxic, and a dose range below 256 micrograms was effective without causing any cytotoxicity [59]. However, literature is scarce in terms of *in vivo* studies. Therefore, further studies need to be conducted for the effective clinical use of these particles.

8. Conclusion

It is well anticipated that the application of nanoparticles has revolutionized clinical endodontic practice. The utilization of biosynthesized AgNPs for root canal disinfection is of considerable importance due to their eco-friendly and biological properties and pave a new horizon for endodontic practice as effective antimicrobial agents. This review showed an insight into the biosynthesis of AgNPs using fungi, properties, and mechanisms of antimicrobial activity and the effective use of these novel nanoparticulate systems to combat root canal infections. However, it is crucial to understand their shortcomings and their probable cellular effects. Therefore, further *in vitro, in vivo* studies and clinical trials are required to evaluate the efficacy of biosynthesized silver nanoparticles in reducing bacteria from the root canal system and effective use of these particles with the highest safety for patients.

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Conflicts of Interest

The authors declare no conflict of interest.

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