Evaluation of the re-mineralization capacity of a gold nanoparticle-based dental varnish: An in vitro study

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Abstract
Background: Dental caries is an infectious microbial disease caused by acidogenic bacteria. It leads to the dissolution of enamel, dentin, and cementum. Enamel demineralization is often appreciated as ‘White Chalky lesions or Chalky enamel’. Standard procedures for protection of these teeth are fissure sealing and topical fluoride application. A varnish is generally a material in which a resin such as copal is dissolved within an organic solvent such as ethanol. Gold is one of the most biocompatible dental materials. Gold nanoparticles were biosynthesised using aspartic acid in previous studies.

Aim and Objectives: To prepare a gold nanoparticle based dental varnish and to evaluate its re-mineralizing capacity.

Materials and Methods: Gold nanoparticle dental varnish was prepared using all the necessary constituents. This newly prepared dental varnish was compared with G.C Fuji/SnF2 dental varnish. Demineralizing capacity of the dental varnishes were analysed. The tooth specimens were prepared according to methodology and mounted on resin blocks. They were subjected to demineralization remineralization cycles. ICP-OES and Knoop Hardness tests were performed.

Results: AuNP dental varnish had a satisfactory remineralization effect on demineralised enamel. For calcium analysis, the AuNP group showed significantly more total calcium loss when compared to the SnF2 group (P < 0.05) and was statistically significant. For phosphorus analysis, AuNP group showed significantly greater net phosphorus loss compared to the SnF2 group (P < 0.05) and was statistically significant. It was also observed that the KHN increased more significantly in Group A (SnF2) as compared to Group B (AuNP) and was also statistically significant (P < 0.05).

Conclusion: AuNP dental varnish showed considerable re-mineralizing property; however, it was not superior to dental varnishes like SnF2 dental varnish. Further research needs to be done in vitro to better modify the AuNP dental varnish before proceeding to in vivo studies.

Keywords: Caries, Enamel Caries, Dental Varnish, Gold nanoparticle, Incipient lesion, Initial Caries, Remineralization

INTRODUCTION

Dental caries is an infectious microbial disease caused by acidogenic bacteria. It leads to the dissolution of enamel, dentin, and cementum. It is the most common oral disease.

Sixty to ninety percent of the child and adult population are said to be affected by dental caries. Dental caries often results in the dissolution of the hydroxyapatite crystal structures of the tooth and subsequent loss of calcium, phosphate, and other ions in the tooth. This leads to the demineralization of the tooth substrate. Demineralization is often appreciated as “White Chalky lesions or Chalky enamel. Clinically, these white spot lesions are the earliest macroscopic evidence of enamel caries. Typically, the enamel surface layer stays intact during subsurface demineralization, but in the absence of an intervention, it will eventually collapse into cavitation.
Standard procedures for the protection of these teeth are fissure sealing and topical fluoride application.\textsuperscript{[3]} So far, none of these procedures are completely efficient. Therefore, attempts have been made to find an effective anticariogenic and re-mineralizing agent to have ions directly delivered to when and where they are needed most. A varnish is generally a material in which a resin such as copal is dissolved within an organic solvent such as ethanol. These are dispensed as sticky liquids in small quantities in amber-colored bottles.\textsuperscript{[10]} They are generally applied to the surface of the tooth at the site of demineralization and also in caries prone areas by the dentist in office using an applicator tip and then allowed to air dry or light-cured based on the mechanism of setting.

Often patients with high caries index scores and high susceptibility to dental caries, pediatric patients, terminally ill patients and patients with special needs who lack hand dexterity and ability to brush regularly and maintain good oral hygiene are ideal candidates for varnish application.\textsuperscript{[7]} The varnishes usually contain active agents that are derivatives of fluorides. These fluoride substances are known to have sustained fluoride ion releasing capacity\textsuperscript{[8]} and help in continuously providing fluoride to the demineralized site to help in the process of aided re-mineralization. It is believed that when hydroxyapatite crystals form ionic and covalent bonds with the fluoride ion, fluorapatite ions are produced. Fluorapatite ions are said to make the enamel re-mineralize beside, making it harder and more resistant to future decalcification.

Gold is said to be one of the oldest materials used in dentistry.\textsuperscript{[9]} It is extremely biocompatible and is also known to have recognizable antimicrobial properties. When gold is turned into nanoparticles, the properties of gold further improve.\textsuperscript{[10]} This is because gold nanoparticles (AuNP) are said to have a high surface plasmon resonance and surface ratio, hence can aid in the movement of any biomolecules that it comes in contact with. AuNP while being synthesized can be synthesized by biological eco-friendly methods.\textsuperscript{[11]} This, in turn, combines the property of the gold and the biomolecule to make it highly superior in its physical and chemical properties.

Aspartic acid is an amino acid containing one amino group and one carboxylic group. The carboxylic group is the free end of the amino acid and can form bonds with other atoms or elements. There are several previous studies where AuNP have been synthesized using aspartic acid and have been used to cause aided bone regeneration or repair of the long bone or tibia in fracture healing.\textsuperscript{[12]} The open carboxylic group and the increased plasmon resonance of the AuNP are said to attract phosphate and calcium ions to the site of injury and cause aided repair of the bone. With evidence from the above studies and several others, the current study was undertaken. The aim of the present study was to prepare a AuNP based dental varnish and to evaluate its re-mineralizing capacity.

**MATERIALS AND METHODS**

**Gold nanoparticle synthesis and characterization**

Gold Chloride solution (0.266 M) was slowly added to 250 ml of aspartic acid with stirring at 45°C, maintained in a thermostat regulated water bath (Serological Water Bath-META LAB; Mumbai). The mixture of the solutions was kept in a long-necked borosilicate flask and continuously stirred on a magnetic stirrer. The formation of AuNP was confirmed by the change of the colorless solution to a reddish hue. The solution was stirred for approximately 9 h. The synthesized AuNP were then purified by centrifugation (10,000 rpm: 30 min, 14-inch radius of centrifugation) at 4°C (Refrigerated Universal Centrifuge-HOVER LABS; India). The nanoparticles collected were thoroughly washed with deionized water and re-dispersed in Millipore water. Nano particles were formed in approximately 9 h with peak absorbance at 24 h at 525 nm. The synthesized nanoparticles were spherical in shape, with an average size of 20 nm. The synthesized nanoparticles showed excellent surface plasmon resonance and optical properties.

**Specimen preparation**

Forty sound human incisor teeth that were extracted for periodontal or orthodontic reasons were included in this study. Teeth with caries, fractures, surface cracks, and enamel hypoplasia were excluded from the study. Samples were thoroughly cleaned of organic debris and sterilized by autoclaving and were stored in 10% formalin. The calculation of sample size was done using G Power software with the confidence level set at 95%. All the specimens were randomly divided into two groups (n = 20). They were sectioned at the cervical level to produce enamel slabs of 6 mm × 6 mm × 2 mm dimensions. The enamel slabs were embedded in acrylic resins and polished with silicon carbide discs to obtain a smooth and flat surface. Representative samples were subjected to analysis by Knoop hardness testing and induced coupled plasma-optical emission spectrometry (ICP-OES) to determine the baseline data.

**Preparation of artificial saliva**

Artificial saliva containing buffering capacity was prepared by mixing 0.213 g/L of CaCl\textsubscript{2}, 2H\textsubscript{2}O; 0.738 g/L of KH\textsubscript{2}PO\textsubscript{4}; 1.114 g/L of KCl; 0.381 g/L of NaCl; and 12 g/L of tris buffer for remineralization. All the enamel specimens were immersed in the salivary pool for 2 h, at 37°C under gentle agitation to allow the formation of the acquired pellicle.\textsuperscript{[13]}
Preparation of test and control varnish samples
In this study, stannous fluoride dental varnish was readily procured. G. C Fuji Varnish, G. C. America was used as control group (Group A), as its re-mineralizing properties are well established. The AuNP dental varnish was used as a test group (Group B).

Preparation of gold nanoparticles dental varnish
A total of 0.05 g of 0.266 M AuNPs were added to 5 ml copal resin gum. This sticky mixture was further dissolved in 5 ml of 70% Ethanol. The active agent added was 5% sodium fluoride. In addition, a cosmetic flavoring agent of natural origin, Peppermint, was added. The prepared AuNP dental varnish was carefully stored in an airtight amber colored glass bottle and maintained at room temperature. All chemicals were procured from Sisco Chemicals, Mumbai, and were of analytical grade.

Demineralization-remineralization cycle
For the demineralization challenge, 0.01 M HCl was used to simulate the dissolution of surface enamel and cause white spot lesion formation. One demineralization-remineralization cycle consisted of an initial 10 s immersion in 20 ml of HCl, followed by 60 s immersion in 20 ml of artificial saliva and then a 30 s exposure to 20 ml of the commercial varnish in Group A or in Group B. Following this, both samples were subjected to 60-min immersion in 20 ml of artificial saliva. This cycle was repeated thrice during the same day. This cycle was designed to simulate conditions within the oral cavity.

Microhardness hardness test
Tooth specimens were then subjected to Knoop hardness testing to measure the change in surface hardness. A diamond pyramid indenter with 100 g force for 10 s was used for the Knoop hardness test. The Knoop hardness test was initially done on a representative sample before subjecting it to the demineralization cycle, to establish a baseline value. Following the completion of the remineralization demineralization cycle the samples were tested again to access the change in Knoop hardness value.

Mineral loss analysis
ICP atomic emission spectrometry (ICP-OES) (Perkin Elmer 2000) was used to evaluate for mineral loss. After the 3rd demineralization-remineralization cycle, the acidic solution of each sample of the respective groups was individually collected in plastic-coated glass containers and was labeled. The net loss of calcium and phosphorus was determined for each group using ICP-OES that allows semi-quantitative analysis of the elemental composition of the specimens. It works on the principle that calcium and phosphorus emit photons of specific wavelength when excited; this wavelength is converted into electrical signals using the photodetector and processed by a computer to give data as numerical values.

Both the Knoop Hardness test and ICP-OES were conducted at the Central Leather Research Institute, Chennai, Tamil Nadu.

RESULTS

Mineral loss analysis
The elemental analysis or mineral loss analysis was done by ICP-OES analysis. (Perkin Elmer, 2000). For calcium analysis, the AuNP group showed significantly more total calcium loss when compared to the SnF2 group (P < 0.05) and was statistically significant. For phosphorus analysis, AuNP group showed significantly greater net phosphorus loss.

| Table 1: Mean calcium and phosphorus loss in the tooth specimen when subjected to demineralization-remineralization cycle using the gold nanoparticle varnish group and SnF2 varnish group, respectively |
|---------------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
| Calcium                        | Phosphorous         |
| Mean                            | SD                  | z^2                 | P (P < 0.05)        | Mean                            | SD                  | z^2                 | P (P < 0.05)        |
| Group A (SnF2)                 | 14.9987             | 0.9546              | 1.232               | 0.005               | 28.934              | 2.234               | 1.033               | 0.002               |
| Group B (AuNP)                 | 16.3541             | 0.7749              | 1.027               | 0.001               | 31.334              | 3.799               | 1.172               | 0.000               |

The SnF2 dental varnish was superior to the AuNP dental varnish at re-mineralizing demineralised enamel lesions. SD: Standard deviation, AuNP: Gold nanoparticle.

| Table 2: Baseline and experimental knoop hardness values |
|----------------------------------------------------------|
| Group          | Decalcified | Recalcified |
| Mean (HK)      | SD          | P (P < 0.05) | Mean (HK)      | SD          | P (P < 0.05) |
| Group A (SnF2) | 184.37      | 0.372        | 0.041           | 234.55      | 0.322        | 0.005           |
| Group B (AuNP) | 184.62      | 0.226        | 0.002           | 212.88      | 0.292        | 0.004           |

SnF2 dental varnish was superior to AuNP dental varnish in re-mineralizing demineralised enamel lesions. SD: Standard deviation, AuNP: Gold nanoparticle.
loss compared to the SnF2 group ($P < 0.05$) and was statistically significant [Table 1]. This showed that despite having satisfactory re-mineralizing properties, the AuNP dental varnish was a less potent re-mineralizing than the gold standard SnF2 dental varnish.

**Microhardness hardness test**

For the microhardness analysis, the Knoop microhardness test was done. The tooth specimens were subjected to load under a Diamond Pyramid indenter with 100 g force for 10 s. A preliminary test of the specimen was done before the demineralization remineralization cycle to establish a baseline value.

The Knoop hardness number, which was initially lesser, was seen to increase after subjecting the demineralized specimen to both Group A (SnF2) and Group B (AuNP) dental varnishes, respectively. The data obtained was seen to be statistically significant. However, it was also observed that the KHN increased more significantly in Group A (SnF2) as compared to Group B (AuNP) and was also statistically significant ($P < 0.05$; Chi-square test [Table 2]). This inferred that AuNP dental varnish had a satisfactory re-mineralizing property, however, it was not superior to the gold standard SnF2 dental varnish.

**DISCUSSION**

The timely and prompt diagnosis of initial signs of dental caries plays a vital role in maintaining good oral hygiene in an individual. The oral cavity is said to be the gateway to the digestive system and, therefore, the body. Hence, good oral hygiene is especially important in maintaining the overall well-being of an individual. Often it is seen that the pH in the oral cavity drops beneath the critical level, i.e. a pH range of 4.5–5. There could be various reasons for this such as intermittent snacking, failure to rinse mouth, increased consumption of stick foods and sugars, etc. In such a scenario, when the pH falls beneath the critical level, demineralization of the tooth surface begins by the action of the organic acids formed due to the fermentation of various sugars within the oral cavity. If this level of acidic pH remains in the oral cavity in a sustained manner, it can cause the first or initial signs of caries, known as incipient lesions. These often present with chalky white lesions and can easily go unnoticed by the individual. The absence to treat or arrest such a lesion can cause further progression of the carious process leading to cavitated lesions.

To avoid this various protocols of treatment are being followed at both the primary and primordial prevention levels. This could include the application of dental varnishes in susceptible areas such as deep cusp and fissures, placement of sealants or fluorides, etc. Dental varnishes are basically inorganic molecules dispersed within an organic solvent. This could most often be a resin such as copal or natural gum within ethanol or acetone like organic solvent. Conventionally, the application of a dental varnish is an in-office treatment procedure and done as per judgment of the clinician. Several commercial brands of dental varnishes are present. Most often, they have an active agent or a therapeutic agent such as fluorides. These fluorides may be in the form of stannous fluoride, sodium fluoride, etc., a fluoride varnish, when applied to the tooth surface, is said to substantially release fluoride ions and has a good protective mechanism on the tooth surface or the tooth enamel. When a tooth undergoes demineralization, it loses its structural core elements such as calcium and phosphorus. This element loss causes the clinical breakdown of the tooth structure.

Over the years, several metallic ions and compounds have been tested in various dental materials for various properties. Gold is also one such noble metal and has been known to be used in dentistry for several years. It is known to have high biocompatibility and also antimicrobial activity. However, its properties as a re-mineralizing agent on tooth enamel have not been explored until date. Further, when gold is converted into biologically synthesized nanoparticles, it exhibits an added array of properties, namely surface plasmon resonance and increased surface ratio. This means, when the property of surface plasmon resonance helps in the precise synthesis of the AuNP, the large surface ratio can be used for the attachment and delivery of other biomolecules from one site to another. In the AuNP used in the present study, amino acid, aspartic acid was used as the biological agent during synthesis. The aspartic acid has a free amino end terminal and can be used to further increase the surface area of the AuNP. Documented evidence in studies involving AuNP aided bone repair of long bone/tibia has shown that these nanoparticles with free carboxyl terminals have the potential to attract phosphorus and Calcium ions from the bloodstream to the site of healing. In our work, we have hypothesized that a similar mechanism causes the free phosphorus and calcium in the saliva to move to the zone of initial caries that is being treated with our AuNP dental varnish and can help promote remineralization. It would act as an added mechanism to the already known action of the fluorides on the site of demineralization.

The current study is a sequel to our previous preliminary studies studies wherein the AuNP were synthesized from Aspartic acid by a method of Biosynthesis. In this study, *in vitro* tests were done on tooth specimens to assess the remineralization capacity of the newly prepared AuNP dental varnish. Results of ICP-OES and Knoop hardness test showed that AuNP dental varnish had a satisfactory remineralization effect on demineralized enamel. However, it was not superior to the gold standard SnF2 dental varnish that was commercially available. Further studies
can be carried out in a clinical setup (in vivo), to ascertain and assess if similar encouraging results can be obtained even clinically. If so, it could prove to be an eco-friendly, biocompatible, and potential alternative to existing re-mineralizing dental varnishes.

**CONCLUSION**

AuNP dental varnish showed considerable re-mineralizing property; however, it was not superior to dental varnishes like SnF2 dental varnish. Further research needs to be done in vitro to better modify the AuNP dental varnish before proceeding to in vivo studies.

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**Conflicts of interest**

There are no conflicts of interest.

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