ABSTRACT

Aim: To screen the possible antimicrobial activity of a range of clinically used, silver-based compounds on cariogenic organisms: silver diamine fluoride (SDF), silver fluoride, and silver nitrate.

Materials and methods: Preliminary screening disk-diffusion susceptibility tests were conducted on Mueller–Hinton agar plates inoculated with Streptococcus mutans, Lactobacillus acidophilus, and Actinomyces naeslundii, organisms known to be cariogenic. In order to identify which component of the silver compounds was responsible for any antibacterial (AB) effect, and to provide controls, the following were also investigated at high and low concentrations: sodium fluoride, ammonium fluoride, ammonium chloride, sodium fluoride, sodium chloride, and sodium nitrate, as well as deionized water as control. A volume of 10 µL of a test solution was dispensed onto a paper disk resting on the inoculated agar surface, and the plate incubated anaerobically at 37°C for 48 hours. The zones of inhibition were then measured.

Results: Silver diamine fluoride, silver fluoride, silver nitrate, and ammonium fluoride had significant AB effect (p < 0.05) on all three test organisms, although ammonium fluoride had no effect at low concentration; the remaining other compounds had no effect.

Conclusion: Silver ions appear to be the principal AB agent at both high and low concentration; fluoride ions only have an AB effect at high concentration, while ammonium, nitrate, chloride and sodium ions have none. The anticaries effect of topical silver solutions appears restricted to that of the silver ions.

Clinical significance: Silver compounds, such as SDF, silver fluoride, and silver nitrate have AB effect against cariogenic organisms and these may have clinical impact in arresting or preventing dental decay. Sodium fluoride did not have AB effect under the conditions tested.

Keywords: Agar diffusion test, Ammonium fluoride, Antibacterial, Caries, Fluoride, Silver diamine fluoride, Silver nitrate.

INTRODUCTION

The effect of silver as an AB agent for public health care, such as for water or milk storage and water purification, has been known for centuries. This beneficial effect has been used in many other contexts, including soft tissue wound care, bone prostheses, reconstructive orthopedic surgery, cardiac devices, catheters and surgical appliances, as well in the form of ionizable silver in fabrics for clinical use (to reduce the risk of nosocomial infections) and personal hygiene products. New commercial contexts continue to be found and promoted.

The AB effect of silver has been suggested to have three possible mechanisms:

1. Prevention of cellular respiration: Silver ions are thought to bind nonspecifically to bacterial cell surfaces, causing disruption of membrane transport functions; this disruption then allows silver ions to penetrate the microbe.

2. Inhibition of cell division (reproduction): Silver ions react with the base pairs of deoxyribonucleic acid, thus preventing replication.
3. Disruption of cell metabolism: Silver ions are highly reactive and readily bind to thiol groups (SH) which are present in enzymes, denaturing them. The energy system of the organism is thereby incapacitated, osmotic pressure cannot be maintained, and this leads to vital substrate leakage, causing death.\(^1\),\(^12\),\(^13\)

These are clearly not mutually exclusive. Indeed, other more general effects are anticipated because Ag\(^+\) is a strong oxidizing agent, reacting with many organic compounds and materials.

Lansdown and Williams\(^3\) have given a comprehensive account of the use of silver in health-related contexts, but offer no indication of when it was introduced to dentistry as such. It has been claimed that the value of silver nitrate was recognized in 1846,\(^14\) but no source was given for this. The first clear report of clinical observations following the treatment of cavities with silver nitrate appears to be from 1891.\(^15\) Miller investigated the action of silver nitrate on “ivory” and claimed that it may be protective by forming a barrier in the surface of the dentin.\(^16\) The addition of ammonium hydroxide to a silver nitrate solution\(^17\) converts the acidic, irritating solution (salt of weak base and strong acid) to an alkaline one said to give little or no irritation to the pulp.\(^14\) While numerous investigations have reported on its disinfectant effect,\(^18\)\(^\text{22}\) its use was questioned for its potentially injurious action on the contents of vital dentinal tubules and odontoblasts as well as the pulp.\(^23\) It is said to have become unpopular in medical health care due to the irremovable black stain that it causes, and the emergence of antibiotics appeared to make it redundant.\(^24\)

It has been claimed that silver and fluoride ions together have a better anticaries effect than either silver nitrate or sodium fluoride alone.\(^25\) It was then suggested that SDF\(^26\) might react with the tooth mineral hydroxyapatite to form calcium fluoride and silver phosphate, which was thought to be responsible for the prevention of dental caries and the hardening of existing lesions.\(^27\)

A simplified chemical reaction scheme was suggested:

\[
\text{Ca}_{10}(PO_4)_{6}(OH)_2 + Ag(NH_3)_2F \rightarrow \text{CaF}_2 + Ag_3PO_4 + NH_4OH
\]

X-ray diffraction was used to support the conclusion that calcium fluoride (CaF\(_2\)) and silver phosphate (Ag\(_3\)PO\(_4\)) are the major products of reaction with SDF.\(^28\),\(^29\)

However, the relative roles of silver and fluoride were not determined, and it is not clear whether the fluoride as such improves the anticaries effect in a direct AB. For example, Klein et al\(^30\) studied a bacterial model system to compare four agents in respect of their abilities to inhibit carious lesion progression in enamel. They found that both silver fluoride (AgF) + stannous fluoride (SnF\(_2\)) and silver nitrate (AgNO\(_3\)) alone significantly decreased caries progression better than did either SDF or chlorhexidine.

Other clinical investigations have shown the beneficial use of SDF in arresting enamel and dentin caries in children \textit{in vivo}.\(^31\)\(^\text{33}\) However, laboratory studies have all been on sound or demineralized permanent dentition enamel, while the clinical studies have been on the primary dentition. Thus, although no distinction is expected, the absence of a direct demonstration of equivalence leaves some doubt about comparability. Subsequently, using the agar-diffusion method and serial dilution, both 12 and 30% SDF were found to have AB activity in buccal swab cultures from both high and low caries risk pediatric patients.\(^34\)

The reaction of SDF and AgNO\(_3\) tooth tissue components has been studied further.\(^35\) SDF produced globular particles of CaF\(_2\) on the surface of hydroxyapatite, but these disappeared on washing. With AgNO\(_3\), Ag\(_3\)PO\(_4\) crystals were formed which were not dissolved on washing, but which darkened, converting gradually to metallic silver, on exposure to light. On gelatin, both SDF and AgNO\(_3\) produced particles of silver which were resistant to washing.

 Despite the evident importance, very little specific work relevant to the subject has been reported in the last 50 years. Thus, given the lack of explicit mechanism for the effect of SDF, the aim of the present study was to determine the relative AB effects on cariogenic organisms of silver and fluoride ions in silver- and fluoride-based agents used in dentistry by means of diffusion-gradient sensitivity testing, i.e., using the disk-diffusion susceptibility test, also known as the agar diffusion test (ADT),\(^36\) which is simple, fast, and reliable.\(^37\) This was to ascertain whether more elaborate testing would be appropriate.

**MATERIALS AND METHODS**

**Test Principle**

Since organisms must vary in their sensitivity to agents, testing by means of serial dilution in culture media, while feasible, is of low resolution and inefficient. Utilizing the continuity of a diffusion gradient from a high concentration source, the location of the inhibition concentration is automatically identified and the distance from the source can be used as a proxy for sensitivity, narrowing the range required for any subsequent work.

**Test Agents**

Stock solutions were prepared of each agent and of several control compounds with ions in common in a combinatorial scheme (Table 1). Thus, Ag(NH\(_3\))\(_2\)F (SDF) (J. Morita Corporation, Osaka, Japan), silver fluoride (AgF), silver nitrate (AgNO\(_3\)), ammonium chloride (NH\(_4\)Cl), ammonium fluoride (NH\(_4\)F), sodium chloride (NaCl), sodium fluoride (NaF), and sodium nitrate (NaNO\(_2\)) (all Sigma Chemical, St.
Louis, Missouri, USA) were dissolved in deionized water (Milli-Q Plus, Millipore, Billerica, Massachusetts, USA) at two concentrations. To avoid adventitious organisms which might interfere with the analysis, all solutions were filtered using a nonpyrogenic, sterile, single-use syringe filter (0.2 µm Super Membrane Acrodisc, Millex-GS, Millipore, France). Deionized water was used as a negative control. Approximate pH values were estimated using pH paper (Macherey-Nagel, Germany), pH electrodes being compromised by some of the test solutions.

Due to the very large AB effect subsequently found for 400 mg/mL NH₄F solution, additional concentrations of 100 and 200 mg/mL were also tested in the same way for this agent.

**Bacteria**

Inocula of *S. mutans* [American Type Culture Collection (ATCC) 35668], *L. acidophilus* (ATCC 9224), and *A. naeslundii* (ATCC 12104), some of the main organisms known to be involved in the caries process, were prepared from a 24-hour anaerobic incubation on blood-agar. Organisms were harvested to produce suspensions in sterile brain-heart infusion media (Oxoid, Unipath, Basingstoke, England) to a MacFarland optical density of 0.5 at 660 nm.

**Preparation, Incubation, and Reading of Agar Diffusion Plates**

For each suspension, 20 µL was dispersed onto a Mueller–Hinton agar plate (pH 7, Oxoid) using the lawn-deposition mode of a spiral plater programmed to give a uniform bacterial coverage (Autoplate 4000, Spiral Biotech, Norwood, Massachusetts USA). All plates were prepared on the same day. Ten minutes after plating, 10 µL portions of the test solutions were applied to filter paper disks (6 mm diameter, Macherey–Nagel) which had been placed on the agar. After 48-hour anaerobic incubation of the plates, inverted, at 37°C, the diameter of the zone of inhibition around each disk was measured with a Vernier caliper to the nearest half-millimeter. The area of inhibition was then calculated since the diffused concentration is expected to fall with the square of the radius and thus balance the weighting of results for larger diameters. The area of the filter paper disk (28 mm²) was ignored, treating a diameter of 6 mm as zero. All trials were conducted with ten replicates. In the event of a filter paper disk separating from the agar, the data were dropped. To minimize variation, all plates were prepared in one session at a controlled temperature (25°C), with constant agar volume.

**Table 1**: Test solutions. For NH₄F, 100 and 200 mg mL⁻¹ were also tested

<table>
<thead>
<tr>
<th>Agent</th>
<th>High concentration</th>
<th>Low concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg mL⁻¹</td>
<td>mol L⁻¹</td>
</tr>
<tr>
<td>SDF</td>
<td>380</td>
<td>2.36</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>380</td>
<td>2.24</td>
</tr>
<tr>
<td>AgF</td>
<td>380</td>
<td>3.00</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>330*</td>
<td>–</td>
</tr>
<tr>
<td>NH₄F</td>
<td>400</td>
<td>–</td>
</tr>
<tr>
<td>NaF</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaCl</td>
<td>400</td>
<td>–</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>220*</td>
<td>–</td>
</tr>
</tbody>
</table>

*Due to limited solubility, high concentration was not possible for NaF, and only limited values for NH₄Cl and NaNO₃

**Statistical Analysis**

Statistical tests were conducted in software (Statistical Package for the Social Sciences for Windows, version 15, SPSS, Chicago, Illinois, USA). Three-way analysis of variance (agent × concentration × species) (AoV) was followed by lower level tests as indicated by the results, applying Bonferroni protection as appropriate in post hoc multiple comparisons. The significance cut-off was set at α = 0.05.

**RESULTS**

The three-way AoV indicated highly significant effects for all three main factors as well as all interactions (p < 0.001). Accordingly, the design was broken down to separate high- and low-concentration results.

**High Concentration**

Two-way AoV (agent × species) showed that *A. naeslundii* was significantly more sensitive than *S. mutans* (p < 0.001), itself significantly more sensitive than *L. acidophilus* (p < 0.001). However, the interaction was significant (p < 0.001).

The inhibition zone areas are shown in Graph 1, and the numerical comparisons in Table 2. For all silver compounds, a circular black zone of reduced silver was formed around the paper disk; this was always of a smaller diameter than the inhibition zone. AgF, AgNO₃, SDF, and NH₄F had significant bacterial inhibition (p < 0.001), while NH₄Cl, NaCl, and NaNO₃ did not show any such effect. The AB effect of NH₄F (at high concentration) was significantly greater than for all other agents for *S. mutans* and *A. naeslundii*, while for *L. acidophilus*, NH₄F
and SDF were significantly more effective than other agents, but with no significant difference between them. The edge of the inhibition zone for NH₄F to S. mutans, A. naeslundii, and L. acidophilus was obscure whilst that for silver compounds was very sharp.

### Low Concentration

The two-way AoV as above showed no significant sensitivity difference between A. naeslundii and S. mutans (p > 0.05), although both were significantly more sensitive than L. acidophilus (p < 0.001). Again, there was a significant interaction (p < 0.001).

Again, for all silver compounds, a circular black zone of reduced silver was formed around the paper disk, again, smaller than the inhibition zone. The numerical comparisons are in Table 3. AgF, AgNO₃, and SDF showed significant bacterial inhibition compared with the other test agents (p < 0.001). Although NH₄F showed a high AB effect at high concentration, none was shown at low concentration. This was investigated further.

### Ammonium Fluoride

There was no effect for any species at a concentration ≤ 100 mg/mL. The results for 200 mg/mL are shown in Table 2.

### DISCUSSION

In the mouth, bacteria grow in complex biofilms. Hence, the use of an oral biofilm model might be considered a more appropriate means of simulating the oral environment for assessing AB agents. However, the creation of such a model or chemostat is complex, expensive, and time consuming. A preliminary in vitro assessment is therefore, valuable to determine whether further investigation is warranted. The ADT is the generally accepted procedure for determining in vitro sensitivity under routine laboratory conditions. An ADT is simple to perform, relatively reproducible, direct, well-controlled, and allows bacteria to grow in a simple biofilm on the agar surface. Results can be obtained in a short period of time. Nevertheless, it is recognized that the ADT does not simulate the clinical environment, and further work would follow for a detailed understanding.

It can be noted that the concentrations used in the paper disk are necessarily high, and may well be far higher than would be feasible in a treatment context, simply to provide a large enough gradient and an inhibition zone that can be measured with sufficient resolution to be discriminatory. It is also true that both pH and osmotic effects could be involved. However, these effects will be present anyway should such agents be used in practice, and still have the relevant gradients in the test medium. To disentangle these factors would require much more complex experimentation, but in the absence of information to the effect that adjustment to either is appropriate for better efficacy (with otherwise benign species, but noting that osmolarity cannot be reduced independently), all that can be done is to test each agent as-is, as used clinically. "Adjuvants" would have to be explored separately.

In view of the possibility of confounding of results by both synergies and interferences between the cations and anions, the potentially active moieties were tested separately with (reasonably assumed) benign counterions, Na⁺, Cl⁻, so that such behavior could be disentangled. The known reaction products of SDF, i.e., calcium fluoride and silver phosphate, logically should be tested on this basis, but, of course, they have very low solubility.

### Table 2: Inhibition zone area (mean ± SD, mm²) of S. mutans, A. naeslundii, and L. acidophilus for high concentration tests (Table 1)

<table>
<thead>
<tr>
<th></th>
<th>S. mutans</th>
<th>A. naeslundii</th>
<th>L. acidophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF</td>
<td>395.8 ± 15.1²</td>
<td>386.1 ± 44.1²</td>
<td>362.6 ± 23.7²</td>
</tr>
<tr>
<td>AgF</td>
<td>423.6 ± 82.4³</td>
<td>528.9 ± 50.3³</td>
<td>208.7 ± 13.9³</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>177.8 ± 14.9⁵</td>
<td>225.4 ± 47.4⁵</td>
<td>171.2 ± 20.8⁵</td>
</tr>
<tr>
<td>NH₄F</td>
<td>1666 ± 174⁶</td>
<td>1437 ± 270⁶</td>
<td>437 ± 121⁶</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0d</td>
<td>0d</td>
<td>0c</td>
</tr>
<tr>
<td>NaF</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>NaCl</td>
<td>0d</td>
<td>0d</td>
<td>0c</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0d</td>
<td>0d</td>
<td>0c</td>
</tr>
<tr>
<td>Deionized water</td>
<td>0d</td>
<td>0d</td>
<td>0c</td>
</tr>
</tbody>
</table>

Within species, values with the same letter are not significantly different from each other (p > 0.05). Others are significantly different at p < 0.005; SD: Standard deviation

### Table 3: Inhibition zone area (mean ± SD, mm²) of S. mutans, A. naeslundii, and L. acidophilus for low concentration tests (Table 1)

<table>
<thead>
<tr>
<th></th>
<th>S. mutans</th>
<th>A. naeslundii</th>
<th>L. acidophilus</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF</td>
<td>229.9 ± 24.0⁴</td>
<td>199.1 ± 6.1⁴</td>
<td>142.2 ± 40.3⁴</td>
</tr>
<tr>
<td>AgF</td>
<td>213.7 ± 18.7⁴</td>
<td>213.7 ± 18.7⁴</td>
<td>81.2 ± 4.7⁷</td>
</tr>
<tr>
<td>AgNO₃</td>
<td>213.2 ± 22.7⁴</td>
<td>199.8 ± 18.4⁵</td>
<td>155.4 ± 23.5⁵</td>
</tr>
<tr>
<td>NH₄F</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
<tr>
<td>NaF</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
<tr>
<td>NaCl</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
<tr>
<td>Deionized water</td>
<td>0⁰</td>
<td>0⁰</td>
<td>0⁰</td>
</tr>
</tbody>
</table>

Within species, values with the same letter are not significantly different from each other (p > 0.05). Others are significantly different at p < 0.005; SD: Standard deviation

Graphs 1A and B as for medium concentration. Two-way AoV (species × concentration) showed a clear effect for both factors (p < 0.001), as well as significant interaction (p < 0.001). Again, there was no significant difference between S. mutans and A. naeslundii (p > 0.05); both showed a clear dose effect (multiple comparison test, p < 0.001). Lactobacillus acidophilus was less sensitive than the other two (p < 0.001), there being no AB effect at 200 mg/mL.
and so are not capable of a suitable solution. In any case, the only relevant ions are Ag⁺ and F⁻, since calcium and phosphate are not expected to be inhibitory, and these are dealt with separately.

Amongst the large number of species of bacteria normally present in the oral flora, Streptococcus, Lactobacillus, and Actinomyces species have been reported to be associated with both dentin and root caries⁴¹-⁴³ and to have been isolated from carious dentin using anaerobic techniques.⁴⁴-⁴⁶

Since NH₄Cl had no AB effect at either concentration, it is clear that neither NH₄⁺ nor Cl⁻ is AB as such, which was to be expected. Similar arguments show that none of Na⁺, Cl⁻, and NO₃⁻ is AB, again as expected (gross osmotic effects excepted, although incidentally these results show that they are not significant here). NaF ([F⁻] = 17,000 ppm) was not AB; however, NH₄F was only effective at high concentration ([F⁻] = 190,000 ppm). Taken together, this implies that F⁻ is AB at high concentration. This was confirmed by the dilution series test of NH₄F: cut-off points were 200 (L. acidophilus) and 100 mg/mL (Graph 1C). For the ADT of NH₄F at 400 mg/mL, after 5 days of incubation all bacteria grew back into the former inhibition zone. This supports the concentration effect deduction as ion diffusion is continuous (in the absence of removal by binding or precipitation), while the indistinct edge of the inhibition zone indicates that F⁻ is indeed a bacterial inhibitor rather than bactericidal under the present circumstances, as indicated elsewhere.⁴⁷

NH₄F has not been widely used, presumably due to its toxicity and the pungency of the released NH₃, and it has been little studied. Only Maltz and Emilson⁴⁸ have investigated the susceptibility of oral bacteria to NH₄F, along with NaF, SnF₂, and CuF₂, concluding that fluoride has an AB effect only at high concentration. They also remarked that, for SnF₂ and CuF₂, the metal ions seem to play a major role, with both of which observations the present results are consistent.

Comparing the low-concentration results for NaNO₃ and NaF, which showed no AB effect, with those for AgNO₃ and AgF, which did, it is concluded that it is only the silver ions that are AB.

The weaker AB effect of SDF at high concentration compared with NH₄F may be due to chloride in the agar precipitating silver ions.⁹ In addition, since there was no significant difference between the AB effects of AgF and

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**Graphs 1A to C:** Inhibition zone areas for the three test bacteria for the various test agents. Error bars: ±1 standard deviation. “High” and “low” concentrations as in Table 1, “medium” = 200 mg mL⁻¹ (A) S. mutans, (B) A. naeslundii, (C) L. acidophilus
In summary, at low concentration, the AB effect of SDF is due only to silver ions. Fluoride inhibits bacteria at very high concentration ([F⁻] ≥ 97,000 ppm), but no effect was found at [F⁻] < 49,000 ppm, and especially, there is no clear cooperative or synergistic action with silver ions. NH₄⁺, Na⁺, Cl⁻, and NO₃⁻ had no detectable AB effect. The effect of Ag⁺ in broth may be constrained by the chloride present.

These results must be put into the context of the biofilm of the oral environment. It is understood that agar-diffusion is only a screening test and that it cannot be used to determine the efficacy of a process in vitro. This arises simply because the biofilm itself has different properties, chemically and physically, while the community of organisms behaves differently to isolated species. However, in the present work, we are showing an absence of effect for fluoride (and other certain other chemical species) in a system that would be expected to show the greatest possible sensitivity. But, it is understood that the sensitivity of the organisms in a monospecific culture cannot be less than that in a biofilm. It follows then that the screening test can be no worse, and is probably rather more sensitive, for detecting the possibility of an effect. Obviously, were there to be one, then a biofilm test would be required to confirm and assess sensitivity. Thus, we can conclude that, simplistic though it may be, the ADT suggests now that an AB effect for clinically relevant concentrations of fluoride in the context of a biofilm is unlikely. Equally, in the case of silver (whose efficacy has, as far as we know, never been challenged) in the context of a biofilm, some lesser effect might be encountered and that such a system would need to be studied. A distinction also needs to be drawn between the general exposure of biofilms to fluoride through mouth rinses, APF and the like, and the precisely targeted treatment of caries with silver solutions. Nevertheless, the lethality of Ag⁺ should it contact the organism is confirmed, and this provides a good positive control for any more elaborate screening system.

CONCLUSION

Consistent with the bulk of related observations and the expectations of the chemistry, it was concluded that it is silver ions that exert the principal AB effect in the compounds studied, so that if ‘optimization’ of SDF or the like as an anticaries agent is to be pursued, it should be with regard to the AB effect of silver ions, the role of fluoride being complementary with respect to de- or remineralization, but not AB at the relevant concentrations. However, these are in vitro results and this needs to be confirmed
in vivo or on more suitable tooth models. Further outcomes should examine how to conduct more elaborate trials involving dilution methods and biofilms over different time periods ultimately leading to clinical trials.

**CLINICAL SIGNIFICANCE**

Silver compounds, such as SDF, silver fluoride, and silver nitrate have AB effect against cariogenic organisms and these may have clinical impact in arresting or preventing dental decay. Sodium fluoride did not have AB effect under the conditions tested.

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