JOURNAL OF DENTISTRY 42 (2014) 329–335

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The inhibitory effects of silver diamine fluorides on cysteine cathepsins

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ARTICLE INFO

Article history:
Received 2 October 2013
Received in revised form
22 November 2013
Accepted 26 November 2013

Keywords:
Cysteine cathepsins
Matrix metalloproteinases
Arrest caries
Dentine
Silver diamine fluoride

ABSTRACT

Aim: The expression of cysteine cathepsins in human carious dentine suggests that this enzyme contributes to the collagen degradation in caries progress. This study investigated whether silver diamine fluoride (SDF) inhibited the activity of cysteine cathepsins.
Methods: Three commercial SDF solutions with concentrations at 38%, 30% and 12% were studied. Two fluoride solutions with the same fluoride ion (F^-) concentrations as the 38% and 12% SDF solutions, and 2 silver solutions with the same silver ion (Ag^+) concentrations as the 38% and 12% SDF solutions were prepared. Five samples of each experimental solution were used to study their inhibitory effect on two cathepsins (B and K) using cathepsin assay kits. Positive control contained assay buffer and cathepsins dilution was used to calculate the percentage inhibition (difference between the mean readings of the test solution and control solution divided by that of the control group).
Results: The percentage inhibition of 38%, 30% and 12% SDF on cathepsin B were 92.0%, 91.5% and 90.3%, respectively ($p < 0.001$); on cathepsin K were 80.6%, 78.5% and 77.9%, respectively ($p < 0.001$). Ag^+ exhibited the inhibitory effect against both cathepsin B and K with or without the presence of F^- ($p < 0.01$). The solutions containing Ag^+ have significantly higher inhibitory effect than the solutions containing F^- only ($p < 0.01$).
Conclusion: According to this study, SDF solution at all 3 tested concentrations significantly inhibited the activity of cathepsin B and K.

1. Introduction

A recent review concluded that silver diamine fluoride (SDF) is a safe, effective, efficient, and equitable caries-preventive agent that appears to meet the criteria of the WHO Millennium Goals and the US Institute.[1] Clinical trials showed that SDF prevented and arrested coronal caries in primary teeth[2,3] and root caries in permanent teeth.[4] Numerous laboratory studies have found that SDF is effective in increasing microhardness[5–7] and the mineral content of dentine caries.[6,8,9] Apart from the demineralisation of hydroxyapatite, caries progression also involves significant degradation of dentine collagen.[10] Mammalian collagenolytic enzymes such as matrix metalloproteinases (MMPs) were found to play a crucial role in collagen degradation.[11] In addition, cysteine cathepsins (or cathepsins)

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0300-5712
http://dx.doi.org/10.1016/j.jdent.2013.11.018

were identified in the degradation of extracellular matrix components.[12,13] Tersariol et al.,[12] reported that cathepsin activities were associated with MMPs activities in dentine. Scaffa et al.,[14] suggested that cathepsins may also be responsible for the collagen degradation in caries lesions. Hence, the inhibition of MMPs and cathepsins may contribute to caries arrest.

Cathepsins are proteolytic enzymes that can be identified in dentine caries and human pulp. They are members of the C1 family of papain-like enzymes, which are the largest and the best-characterised family of cysteine peptidases. Cathepsins can extracellularly degrade type I collagen and proteoglycans, which are the main components of the dentine organic structure. Cathepsins are active and stable in acidic environments and mostly unstable at neutral acidity.[15] The autocatalytic activation of cathepsins is substantially accelerated in the presence of anionic polysaccharides.[16]

Cathepsins are differentiated by their structure, catalytic mechanism, and the proteins they cleave. Cathepsin B can be found in human carious dentine and saliva. Its activity varies in relation to the depth and age of carious lesions, while remaining stable in saliva.[13] Cathepsin B cleaves in the non-helical telopeptide extensions of collagens.[17] Cathepsin K can catabolize collagen and break down dentine. It cleaves the collagen at the triple helical region.[15] These properties suggest that cathepsins may play crucial roles in collagen breakdown in dentine caries lesions.

We found that SDF strongly inhibited the proteolytic activity of MMP-2, MMP-8, and MMP-9, which are three of the main MMPs that take part in collagen degradation,[18] our resent study also demonstrated that SDF prevented dentine collagen degradation from bacterial collagenase challenge.[19] To serve as an effective caries arresting agent, SDF needs to function as a broad-spectrum inhibitor that restrains other dentinal proteolytic enzymes. Unlike the MMPs first discovered in dentine pathologies over 15 years ago, cathepsins were recently detected in dentine.[12,13,15] However, no studies have examined the effectiveness of SDF on cathepsins. Thus, the aim of this study was to investigate the inhibitory effects of 3 common, commercially available SDF solutions at different concentrations on cysteine cathepsins. Freshly prepared silver nitrate ($AgNO_3$) and sodium fluoride (NaF) solutions were used for comparison. Two null hypotheses are tested: firstly, there is no difference in inhibitory effect on cysteine cathepsins by SDF, $AgNO_3$ and NaF solutions; and secondly, there is no difference in inhibitory effect on cysteine cathepsins by 38%, 30%, and 12% SDF solutions.

2. Methods and materials

2.1. Reagents

Commercially available SDF solutions at concentrations of 12% (Cariostop, Biodinamica, Brazil), 30% (Cariostop, Biodinamica, Brazil), and 38% (Saforide, Toyo Seiyaku Kasei, Japan) were selected for this in vitro study. Freshly silver and fluoride solutions were prepared, which contained equivalent concentrations of silver ions (Ag^+) and fluoride ions (F^-) as the 38% and 12% solutions of SDF, respectively. The 3 commercially available SDF solutions had high pH values (pH = 12–13), which could have affected the cysteine cathepsin activity. Therefore, 3 control groups of SDF at 38%, 30%, and 12% buffered with 10% nitric acid (HNO_3) to lower the acidity (pH = 9) were prepared. Five samples of each experimental solution were used in this study. The 10 experimental solutions assessed were numbered groups 1–10, as shown in Table 1.

2.2. Inhibition of cathepsin enzymatic activity by the experimental solutions

We used human recombinant cathepsins and Sensolyte cathepsin assay kits (cathepsins B and K) from AnaSpec Inc. (San Jose, CA, USA). The Sensolyte cathepsin assay kit is a homogeneous assay that can be used to detect enzyme activity and for screening cathepsin inhibitors. The cathepsin B enzyme was diluted 1000-fold in a dithiothreitol (DTT)-containing assay buffer, while the cathepsin K enzyme was pro-incubated with an equivalent amount of activation buffer for 40 min at room temperature before being diluted 300-fold in a DTT-containing assay buffer. The enzyme diluents were prepared immediately before use according to the protocol suggested by the manufacturer. Then, 40 μl diluted cathepsin enzyme and 10 μl experimental solution were added to each well of a black fluorometric 96-well microtiter plate (Fisher Scientific, Gainesville, FL, USA). The wells of the microtiter plate also received 50 μl of diluted cathepsin substrate solution (dilution factor: 1:100) to give a total volume in each well of 100 μl. Thus, the concentration of the experimental solution was diluted 10 times during the reaction. Namely, the

Table 1 – Fluoride and silver content and acidity (pH) of the 10 experimental solutions.

Group	Product	Chemicals	F^- (ppm)	Ag^+ (ppm)	pH
1	Saforide 38%	$Ag(NH_3)_2F$	44,800	255,000	13
2	Buffered saforide	$Ag(NH_3)_2F + HNO_3$	44,800	255,000	9
3	Cariestop 30%	$Ag(NH_3)_2F$	35,400	200,000	12
4	Buffered cariestop 30%	$Ag(NH_3)_2F + HNO_3$	35,400	200,000	9
5	Cariestop 12%	$Ag(NH_3)_2F$	14,150	80,000	12
6	Buffered cariestop 12%	$Ag(NH_3)_2F + HNO_3$	14,150	80,000	9
7	Silver solution A	$AgNO_3$	–	255,000	10
8	Silver solution B	$AgNO_3$	–	80,000	10
9	Fluoride solution A	NaF + KF[a]	44,800	–	9
10	Fluoride solution B	NaF	14,150	–	9

[a] Potassium fluoride was added to 4% NaF to form a solution with 44,800 ppm fluoride ions.

result was the inhibitory effect of diluted experimental solution. A positive control containing recombinant cathepsins (with no potential anti-cathepsin agent) was used as a reference to calculate the percentage of inhibition. A substrate control containing assay buffer only was used to check the background fluorescence of the substrate. Another test control containing experimental solutions, without recombinant cathepsins, was used to measure the auto fluorescence of the experimental solutions. The assay buffer and cathepsin substrate were added to the control wells to obtain the 100 μl total volume.[20]

2.3. *Fluorescence reference standard*

Fluorescence resonance energy transfer (FRET) solutions at serial concentrations were prepared as suggested by the manufacturer and used as the fluorescence reference standard. Serial dilutions of 1:2 were performed to obtain FRET solutions at concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0 μM. Fifty μl of each diluted HiLteFluor™ 488 fluorescence reference solution was added to each well. The cathepsin substrates were then added to the wells to form 100 μl solutions for evaluation. This reference standard is used as an indicator of the amount of final product from the enzymatic reaction.

2.4. *End-point reading*

The reagents were mixed gently in the dark for 10 s. The fluorescence intensity of the assay was measured at excitation/emission (490 nm/520 nm) using http://www.perkinelmer.com/Catalog/Family/ID/VICTOR X Multilabel Plate Reader a multitask plate reader (1420 Victor PerkinElmer, Boston, USA) with an associated computer program (Wallac 1420 manager PerkinElmer, Boston, USA) at room temperature. The data (end-point readings of cathepsin activity) were obtained after 1 h and expressed as relative fluorescence units (RFU). The RFUs were subsequently converted to μM (μg/μl) based on standard curves ($R^2 > 0.99$) generated with the HiLte Fluor™ 488 fluorescence standard. A low concentration value reflected a high inhibitory effect on cathepsins. The percentage inhibition was calculated from the difference between the mean values of the test group and the reference (positive control) group, divided by that of the reference group.[18]

2.5. *Statistical analysis*

All data were assessed for normal distribution using the Shapiro–Wilk test for normality. One-way ANOVA with LSD multiple comparison tests were used to detect differences between the cathepsin concentration values (μg/μl) among all the experimental groups. Two-way ANOVA with simple main effect was used to compare the inhibitory effects of different concentrations of Ag^+ and F^- (as two independent variables) on cathepsins B and K. Analyses were performed with the computer software SPSS Statistics – V20.0 (IBM Corporation, Armonk, USA). The level of statistical significance for all tests was set at 0.05.

3. Results

The mean end-point values of the 10 experimental groups and the positive control are shown in Table 2. No significant difference in end-point values was detected among all the groups containing Ag^+ (including all the SDF groups and 2 silver solution groups, Groups 1–8) for cathepsin B, while the values in Group 1–8 were lower than positive control (Group 11) and two fluoride solution groups (Groups 9 and 10); no significant difference in end-point values was detected among all the SDF groups (Groups 1–6) for cathepsin K, while the values in Group 1–6 were lower than positive control (Group

Table 2 – Mean end-point values (cysteine cathepsins activity) of the experimental solutions.

Group (n = 5 each)	End-point values (cathepsin activity) in μg/μl mean (SD)	
	Cathepsin-B	Cathepsin-K
1. 38% SDF (pH 13)	0.053 (0.002)[a]	0.19 (0.005)[a]
2. 38% SDF (pH 9)	0.054 (0.002)[a]	0.20 (0.010)[a]
3. 30% SDF (pH 12)	0.056 (0.001)[a]	0.21 (0.007)[a]
4. 30% SDF (pH 9)	0.055 (0.001)[a]	0.21 (0.011)[a]
5. 12% SDF (pH 12)	0.064 (0.002)[a]	0.21 (0.008)[a]
6. 12% SDF (pH 9)	0.062 (0.002)[a]	0.23 (0.020)[a]
7. Silver solution A	0.054 (0.002)[a]	0.30 (0.012)[b]
8. Silver solution B	0.064 (0.003)[a]	0.33 (0.013)[b]
9. Fluoride solution A	0.45 (0.012)[b]	0.69 (0.046)[c]
10. Fluoride solution B	0.50 (0.025)[c]	0.80 (0.117)[d]
11. Positive control	0.66 (0.017)[d]	0.96 (0.047)[e]
p value	<0.001	<0.001
LSD comparison	a < b < c < d	a < b < c < d < e
Comparing the effect of the pH of SDF solutions at different concentrations		
38%—Group 1 vs. 2	$p = 0.36$	$p = 0.08$
30%—Group 3 vs. 4	$p = 0.22$	$p = 0.21$
12%—Group 5 vs. 6	$p = 0.28$	$p = 0.18$

Different letters indicate a statistically significant difference between the groups ($p < 0.05$).

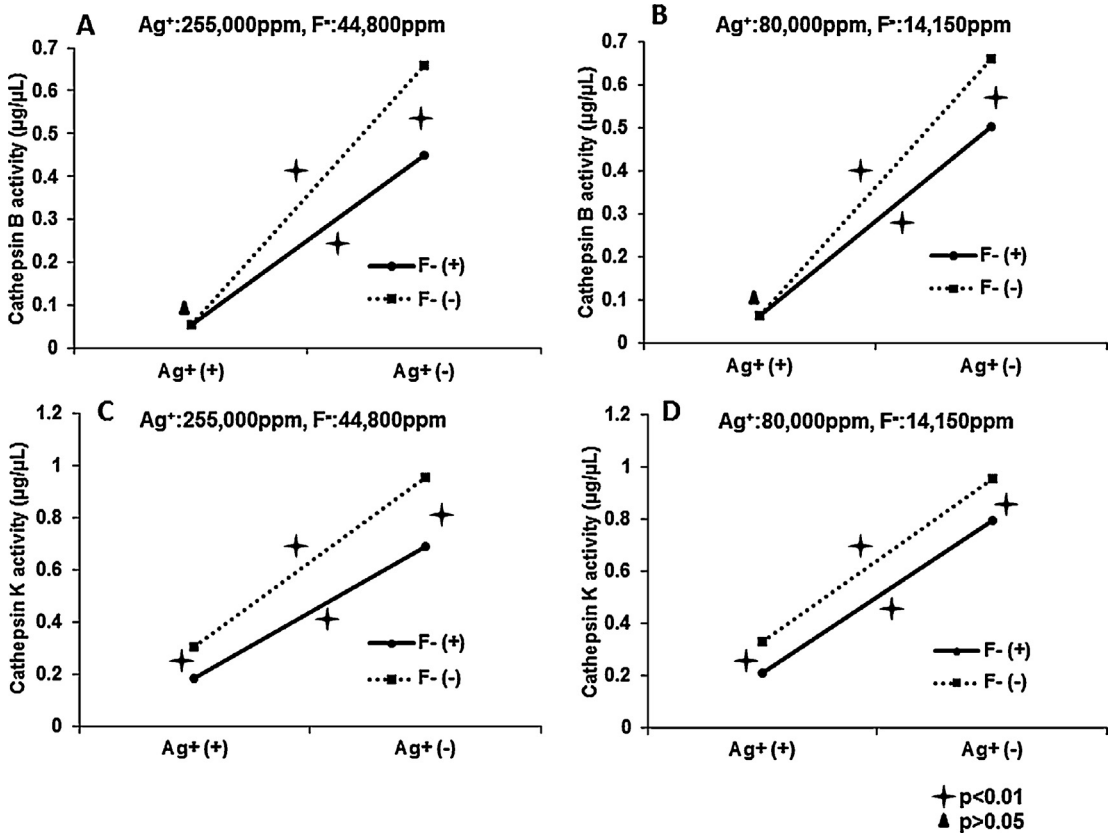

Fig. 1 – Two-way ANOVA with simple main effect analysis. (A) The simple main effect of high-concentration Ag^+ (255,000 ppm) and F^- (44,800 ppm) on the cathepsin B inhibitory effect. Stars in the middle of the lines indicate that Ag^+ has a significant inhibitory effect no matter if F^- is present (solid line) or not (round dot line). Star in the middle of two right points indicates that F^- has a significant inhibitory effect in the absence of Ag^+. Triangle in the left side indicates that F^- does not have a significant inhibitory effect in the presence of Ag^+. (B) The simple main effect of low-concentration Ag^+ (80,000 ppm) and F^- (14,150 ppm) on the cathepsin B inhibitory effect. Similar patterns are observed in Fig. 1A. (C) The simple main effect of high-concentration Ag^+ (255,000 ppm) and F^- (44,800 ppm) on the cathepsin K inhibitory effect. Stars in the middle of the lines indicate that Ag^+ has a significant inhibitory effect no matter if F^- is present (solid line) or not (round dot line). Stars in the middle of two points indicate that F^- has a significant inhibitory effect no matter if Ag^+ is present (left points) or not (right points). (D) The simple main effect of low-concentration Ag^+ (80,000 ppm) and F^- (14,150 ppm) on the cathepsin K inhibitory effect. Similar patterns are observed in Fig. 1C.

11), two fluoride solution groups (Groups 9 and 10) and 2 silver solution groups (Group 7 and 8). No significant differences in end-point values were detected between the commercial SDF products (pH value at 12–13) and the corresponding buffered groups at pH 9 at 38%, 30%, and 12%.

The inhibitory effects of 12% and 38% SDF solutions against cathepsin B and K were summarised in Fig. 1. SDF and Ag^+ solution had similar inhibition against cathepsin B activity, suggesting cathepsin B activity could be inhibited by Ag^+. F^- solution also inhibited cathepsin B activity, but its inhibition effect is less than SDF and Ag^+ solution. In addition, SDF, Ag^+ solution and F^- solution inhibited cathepsin K activity; and SDF had a higher inhibition effect on cathepsin K activity than Ag^+ solution and F^- solution.

The percentage inhibitions of the experimental solutions of the two cathepsins are shown in Fig. 2. The percentage inhibitions of 38% SDF for cathepsins B and K were 92.0% and 80.6%, respectively. The percentage inhibitions of 30% SDF and 12% SDF for cathepsins B and K were also higher than 70%, and no statistically significant difference from percentage inhibitions of 38% SDF. Although F^- also had statistically lower end-point values than the positive control, the percentage inhibitions were only around 30% in high concentration (F^- Solution A) and 20% in low concentration (F^- Solution B). The inhibitory effect of F^- on cathepsins B and K, although statistically significant, is weak (20–30%).

4. Discussion

Clinical trials have demonstrated that SDF can arrest caries.[2,3,21] However, the mechanism of action is not yet fully known. Previous studies on the mechanisms of SDF have mostly focused on antimicrobial effects and mineral changes in tooth structure.[5–8] Caries destruction in dentine is different from that in enamel because dentine contains about 30% by

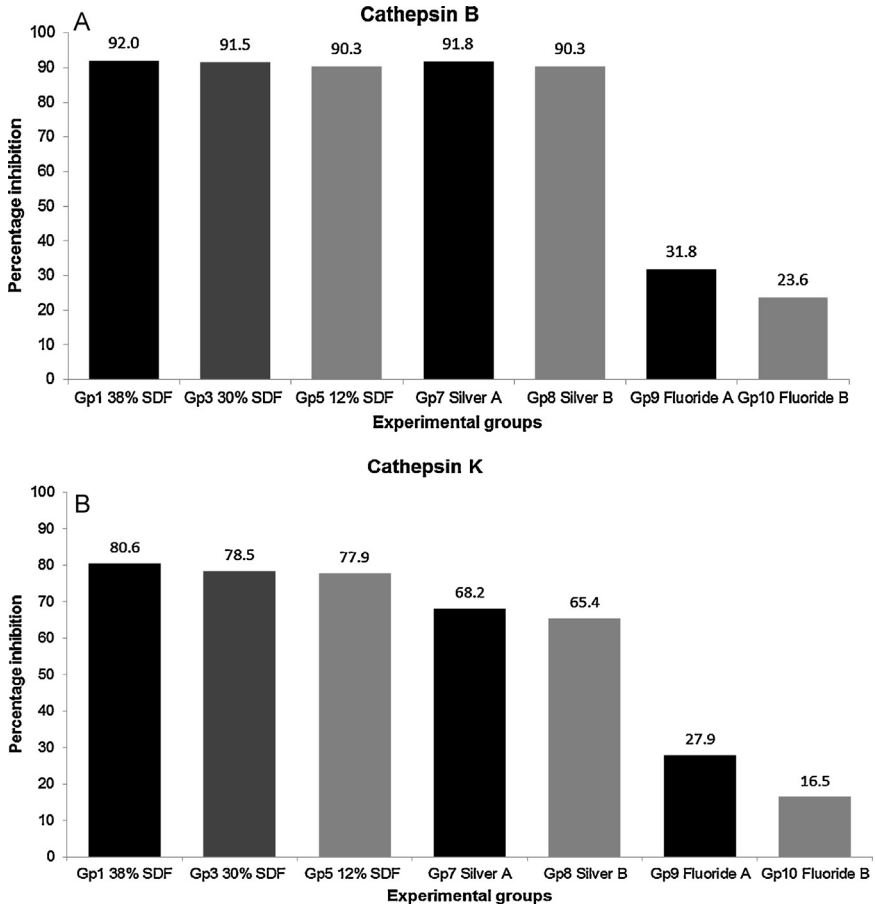

Fig. 2 – Percentage inhibition of (A) cathepsin B and (B) cathepsin K of the experimental groups.

volume of organic matrix. In addition, host MMPs present in the dentine matrix or in saliva play a significant role in dentine collagen degradation.[22] Recent studies have discovered that cathepsins are another crucial enzyme that may activate MMPs and also be directly involved in collagen degradation.[12,13] Therefore, following our previous study on the inhibitory effect of SDF on the activity of MMPs,[18] this study investigated the inhibitory effect of SDF on cathepsins.

According to the results of this study, the first null hypothesis was rejected and the second null hypothesis was accepted. The SDF solutions had high percentage of inhibition on both cathepsin B and K. No statistically significant difference in the percentage inhibition between the three concentrations of SDF solutions.

In this study, a QXL™ 520/HiLyte Fluor™ 488 FRET peptide was used as the substrate for the detection of cathepsin activities. Enzyme substrates based on FRET are sensitive tools for determining protease activity. The fluorescence of HiLyte Fluor™ 488 is quenched by QXL™ 520 until cathepsin B or K cleaves the peptide into two separate fragments. Upon cleavage, the fluorescence of HiLyte Fluor™ 488 is recovered, and can be continuously monitored at Ex/Em = 488/520 nm. Therefore, inhibited cathepsin B or K enzymatic activity will result in a decrease of RFU. It is important to note that proteolytic degradation of the dentinal proteins mediated by the host cathepsins and MMPs in carious lesions is an intricate process. This study used recombinant cathepsins to represent the cathepsins in dentine to act on the FRET substrate, which is a commercial peptide. Acid and enzymatic attacks continue over time during lesion development. In this study, the assay system tested measured a defined end-point effect, which is not the long lasting effect of F^- and Ag^+. Nevertheless, the fluorescent cathepsins assay kit was easy to use and the cathepsins activity was able to be quantified to compare the inhibitory effects of different experimental solutions. Another advantage of this method is that the 96-well plate allowed large numbers of samples to be evaluated simultaneously.[18]

Cysteine cathepsins were recently found to have expression in pulp tissue, and sound and carious dentine.[12,13] Cathepsin B was reported to cleave the non-helical telopeptide extensions of collagens. Moreover, cathepsin B contributes to the potential cathepsin-MMP interplay. Pro-cathepsin B can be activated by active MMPs, and conversely, cathepsin B has been shown to be responsible for the activation of MMPs in fibroblast cultures.[23] Cathepsin K is a unique protease that is both a telopeptidase and a collagenase.[24] It is also the only cysteine cathepsin capable of cleaving collagen at the triple helical region. Cathepsin K is involved in mineralised tissue resorption under normal and pathological conditions. The cathepsin activity in carious dentine significantly increases with increasing depth, indicating that odontoblast- or pulp-derived cathepsins may be important in actively progressing

carious lesions, particularly in young patients.[13] These findings suggest that cathepsins play a significant role in dentinal caries development. The substantial inhibitory effect of SDF on cathepsins B and K could be a reason for the success of SDF in arresting caries in young children.[3]

Cathepsins are active and stable in slightly acidic pH environments and mostly unstable at neutral pH.[15] According to the information provided by the manufacturer, the pH of the assay buffer of the kits used in this study is approximately 4. This provides an acidic condition for the activation of the cathepsin enzymes. To examine whether highly alkaline (high pH) SDF solutions affect cathepsins activity, reference groups of the corresponding SDF concentrations were buffered with 10% nitric acid to investigate whether the acidity influenced the inhibitory effect of SDF. The results did not show any significant difference between the original and the buffered SDF solutions. It is noteworthy that in this experiment, each well of the 100 μl reaction solution contained 90 μl solution prepared from assay buffer and 10 μl experimental solution. Therefore, the experimental solution was diluted 10 times by the assay buffer. Furthermore, the assay itself has a buffer system for each cathepsin to ensure that the pH stays relatively constant during the reaction. In this study, the final pH of the reaction solution ranged between 4.1 and 4.2. Therefore, the difference in the alkalinity of the SDF solution should not affect the acidic environment for cathepsin enzymatic activity. As cathepsins are stable in acidic conditions, the inhibitory effect of SDF on cathepsins should not be due to the high pH of the SDF solutions.

In this study, the solutions with equivalent Ag^+ showed a high rate of inhibition on both cathepsin B and K. However, the solutions with equivalent F^- only showed a slight degree of inhibition. The equivalent concentration of sodium chloride, inert as an ionisable substance, might be a possible comparison to discuss on the mechanism from a view point of effect of the ion strength. Therefore, Ag^+ contributed more to the inhibitory effect of SDF than F^-. Ag^+ nano-particles were shown to have great inhibitory effect on cariogenic bacteria growth, such as *Streptococci mutans* and *Streptococci sanguis*.[25] Nano-particles can break through the permeability of the outer membrane of bacteria cell and inactivate respiratory chain dehydrogenase.[26] The large ionic radius and low oxidation state of Ag^+ have great affinity with protein[27] and this may contribute to its inhibitory effect on the elastase and cathepsin proteinases. The metal ion probably interacts with a reactive side chain of the enzymes to inactivate their catalytic functions. All 3 concentrations of SDF were found to have more profound inhibitory effects on cathepsins than on MMPs.[18] In this study, the 12% SDF solution showed a strong inhibitory effect on cathepsins. This suggests that Ag^+ is a strong inhibitor of cathepsins B and K. In oral cavity, Ag^+ in SDF can be washed away by saliva, the amount of Ag^+ is reduced in carious lesion. There are two possible mechanisms by which the degradation of collagen is inhibited. One is blocking the active site of the collagen against the enzymatic attack. The second is inactivation of the enzymes-mediated collagen degradation.[28] Ag^+ could form less soluble deposits onto dentine with phosphate, chloride, oxide and proteins. These less soluble deposits will release Ag^+, to ambient fluids. The released Ag^+, probably at a minute concentration might exert the inhibitory effect on the presence of enzymes in the fluid. These two mechanisms will be expecting to provide long lasting effectiveness due to the strong reaction of Ag^+ to the collagen and due to a small amount of Ag^+ release.

Acknowledgements

This study was supported by the NSFC/RGC Joint Research Scheme (Grant No. N_HKU 776/10 and NSFC No.81061160511).

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