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Cytotoxicity and Antibacterial Activity of Toothpastes and Mouthwashes Available in the Iranian Market

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A B S T R A C T

Objectives: Toothpastes and mouthwashes contain chemicals that may be harmful to oral tissues. This study assessed the cytotoxicity and antibacterial activity of toothpastes and compare the Iranian and foreign toothpastes and mouthwashes available in the Iranian market in this respect.

Materials and Methods: Twenty samples (13 toothpastes and 4 mouthwashes) were selected. The cytotoxicity of 1, 10, and 50 mg/mL of toothpastes and 0.05, 2 and 10 µL of mouthwashes was measured after 1, 15 and 30 min of exposure to human gingival fibroblasts, each in triplicate. The methyl thiazolyl tetrazolium (MTT) assay was used for cytotoxicity testing. The serial dilution method was utilized to determine the minimum inhibitory concentration (MIC) of each sample against *Lactobacillus acidophilus* (*L. acidophilus*) and *Streptococcus mutans* (*S. mutans*). Two-way ANOVA and Tukey's test were used for data analysis.

Results: A significant difference in cytotoxicity was noted among different products (P=0.00). The difference in cytotoxicity of each sample was not significant at 1, 15 and 30 min (P=0.08). The obtained MIC for all toothpastes and mouthwashes was between 0.0039 mg/mL and 0.0156 mg/mL, except for Sensodyne toothpaste and Oral B mouthwash.

Conclusion: Some brands of toothpastes have higher cytotoxicity due to their composition, and their cytotoxicity should not be overlooked. The antibacterial activity of the samples was almost equal when they were in contact with *L. acidophilus* and *S. mutans* except for the Irsha mouthwash, Sehat, Darugar and Bath toothpastes. The antibacterial effect of toothpastes and mouthwashes increased with an increase in exposure time.

Keywords: Mouthwashes; Toothpastes; Cytotoxicity Tests, Immunologic; *Streptococcus mutans*; *Lactobacillus acidophilus*

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INTRODUCTION

Toothpastes are among the most commonly used oral hygiene products. However, they contain chemicals such as fluoride, abrasives, and detergents that may be harmful to oral tissues. Toothbrushing is a widely practiced

oral hygiene measure. However, some patients require chemical control (by use of mouthwashes) in addition to mechanical plaque removal. Mouthwashes also contain chemicals such as fluoride that may be toxic to oral tissues [1,2].

Fluoride plays an important role in oral health promotion. It induces enamel remineralization and prevents demineralization. It also has antibacterial properties. The cariostatic effects of fluoride are mainly exerted when applied topically [3]. The most important toxic effect of fluoride on cells is its interaction with enzymes. In most cases, fluoride serves as an enzyme inhibitor. However, fluoride ions may occasionally stimulate the enzymatic activity. In micromolar concentrations, fluoride can serve as an effective element in anabolism because it increases cell proliferation while it inhibits several enzymes such as phosphatase [4]. In the recent years, many studies have shown that fluoride can induce oxidative stress and balance the intracellular oxidation-reduction homeostasis, lipid peroxidation and the carbonyl protein content. It can also alter the expression of genes and cause apoptosis [5-8].

Sodium lauryl sulfate, which is added as detergent to the toothpastes' formula, has been studied in-vitro and has shown significant toxic effects on the oral mucosa and gingiva [9-11]. Allergic reactions have been reported following the use of some brands of toothpastes [12]. Cocamidopropyl betaine, a foaming agent found in toothpastes, is responsible for such reactions. Preservatives such as paraben and sodium benzoate, also have adverse effects. Paraben is a carcinogen, and sodium benzoate can cause anaphylactic shock [12].

The toxic effects of chemical ingredients of toothpastes and mouthwashes can affect many cell types. However, time- and concentration-dependent responses vary in different cell types (e.g. necrosis, as the primary mechanism of cell death, is seen following the use of relatively high concentrations of fluoride) [4].

Although mechanical plaque removal is the best strategy to maintain oral hygiene, evidence shows that plaque remains on some oral surfaces after tooth brushing [13]. Therefore, antibacterial ingredients of toothpastes and mouthwashes can help preserving oral health [14,15]. Information about the cytotoxicity of toothpastes is limited in Iran. This study aimed to assess the cytotoxicity and antibacterial activity of toothpastes and mouthwashes and compare the Iranian and foreign toothpastes and mouthwashes available in the Iranian market in this respect. The null hypothesis was that there would be no difference in the cytotoxicity and antibacterial effects of different toothpastes and mouthwashes.

MATERIALS AND METHODS

Study materials:

This study was conducted in the fall of 2017. Thirteen Iranian and foreign toothpastes available in the Iranian market were selected, including Sehat, Oral B PRO-EXPERT, Crend3, Pooneh, Paradontax Daily Fluoride, Colgate Sensitive Multi Protection, Darugar, Close-Up Deep Action, Nasim, Sensodyne Daily Care Gum Protection, Bath, Signal Complete 8, and Crest 7 (Complete).

Listerine Total Care Zero Alcohol, Irsha Antiseptic, Oral B Pro-Expert, and Vi-one General Total Care mouthwashes were also evaluated in this study.

Cytotoxicity test:

Human gingival fibroblasts were obtained from the cell bank of the Iranian Biological Resource Center. The cells were detached using trypsin, and 100 µL of the cell suspension containing 10,000 cells was added to each well of a 96-well plate. The methyl thiazolyl tetrazolium (MTT) assay was used for cytotoxicity testing, which is based on the conversion of MTT salt to formazan crystals by the viable cells. This test is used for in vitro assessment of the toxic effects of medications on the cells. The toxicity of 1, 10 and 50 mg/mL of toothpastes and 0.05, 2 and 10 µL of mouthwashes was measured after 1, 15 and 30 min of exposure, each in triplicate. In order to obtain the concentrations to be tested, first, the cytotoxicity of two products in 10 concentrations was experimentally tested; out of which, 3 concentrations with 0%, 50% and

100% cytotoxicity were selected for testing of other products. In order to obtain different concentrations of toothpastes, 1, 10 and 50 mg of toothpastes were weighed and dissolved in 1 mL of culture medium. Two well-plates were selected and each concentration of products was added to 3 wells (for 3 repetitions). The concentration of 1 mg/mL was used as positive control while 50 mg/mL served as negative control. After 1 min, 10 µL of the MTT solution was added to each well to reach a final concentration of 5 mg/mL. The same protocol was repeated for assessments at 15 and 30 min, in which the MTT was added to the wells 15 and 30 min after exposure of the cells to the tested materials. In order to calculate the mass of toothpaste that is used by patients at each time of tooth brushing (pea size), three individuals were requested to apply a pea-size amount of the toothpaste on a digital scale for 10 times. The mean weight of the mass applied by the three individuals was found to be 0.7, 0.6 and 0.5 g. The mean of the three values i.e. 0.6 g was used for analysis of the results.

Antibacterial activity:

In this study, *Lactobacillus acidophilus* (*L. acidophilus*; ATCC 4356) and *Streptococcus mutans* (*S. mutans*; ATCC 35668) were investigated. Lyophilized ampoules of bacterial species were obtained from the Iranian Research Organization for Science and Technology and were activated according to the provided instructions.

In order to achieve the appropriate value of minimum inhibitory concentration (MIC), firstly one toothpaste, Parodontax, and two mouthwashes, Oral B and Listerine, were used, which caused a decrease in the number of the studied samples. The serial dilution method with concentrations of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, were utilized. Afterwards, 0.5 McFarland concentration of the solution was prepared from each of the bacteria using the European standard, EN 1040:2005 CSN EN [16]. Then, the dilutions and suspensions were placed in contact with each other for 90 s, and 100 µL of the produced solutions was inoculated into MRS agar, separately. The plates were incubated at 37°C in a CO2 incubator for 24 h. The dilution with no bacterial colony was considered as the MIC in the control test. Three dilutions with a concentration more than the MIC in the control test as well as three dilutions with a concentration less than that were also provided. Then, these dilutions were placed in contact with *S. mutans* and *L. acidophilus* for 1 min and 30 min, respectively. In order to calibrate the experiments, the harvesting process was repeated three times.

Statistical analysis:

Two-way ANOVA was applied to analyze the difference in cytotoxicity of different toothpastes at different time points. The Tukey's HSD test was used for pairwise comparisons if the difference among the groups was found to be significant by two-way ANOVA. Although the antibacterial tests were repeated three times, the achieved results were similar and as a result, the variance of the MIC in each studied group was zero. Therefore, a statistical test was not performed.

RESULTS

Cytotoxicity:

Figures 1 and 2 show the mean values of optical density of the samples at each time point. Cell viability was calculated by subtracting the optical density values from 100.

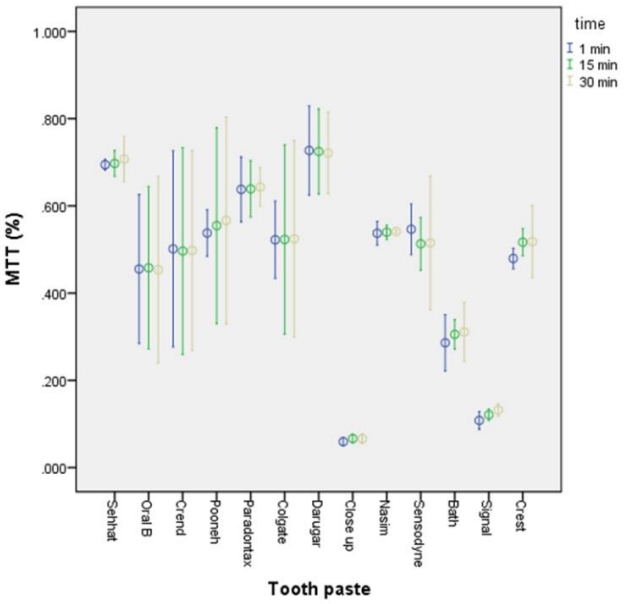

Fig. 1. Mean and 95% confidence interval of the optical density of the studied toothpastes at 10 mg/mL concentration

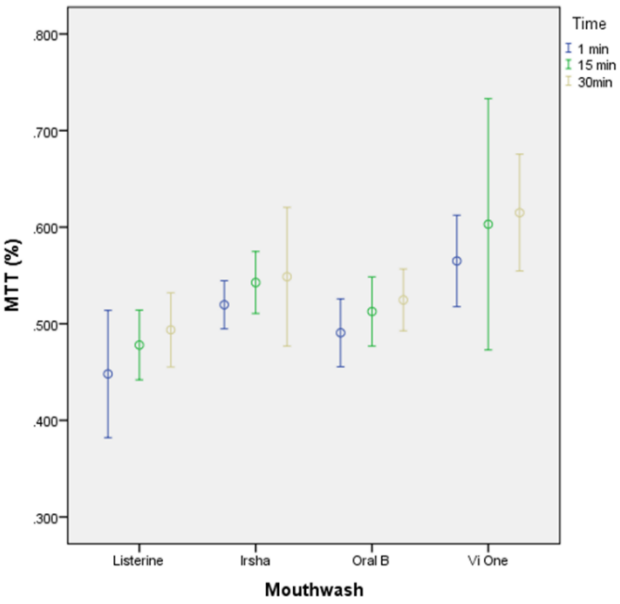

Fig. 2. Mean and 95% confidence interval of the optical density of the studied mouthwashes at 10 mg/mL concentration

Two-way ANOVA showed a significant difference in cytotoxicity among different products (P=0.00). However, the difference in cytotoxicity of each sample was not significant at 1, 15 and 30 min (P=0.08). The interaction effect of material and time was not significant (P=1.00). In this study, 1, 10 and 50 mg/mL concentrations of toothpastes and 1, 2 and 10 µL volumes of mouthwashes were evaluated. In 50 mg/µL concentration and 10 µL volume, the cytotoxicity of the samples was over 90% while in 1 mg/mL concentration and 1 µL volume, the cytotoxicity was <10%.

The current results showed that the Close-Up toothpaste had maximum cytotoxicity, which was significantly different from that of other toothpastes (P<0.05), except Signal (P=0.319). The cytotoxicity of Bath toothpaste was significantly lower than that of Close-Up and Signal and higher than that of other toothpastes (P<0.05). The cytotoxicity of Oral B had no significant difference with the cytotoxicity of Crend, Crest, Colgate, and Sensodyne (P=0.8, P=0.579, P=0.079, and P=0.64, respectively) but it had significant differences with other toothpastes (P<0.05). The cytotoxicity of this toothpaste was found to be moderate.

The cytotoxicity of Crend and Nasim were found to be average. The cytotoxicity of Crend had significant differences with the other toothpastes (P<0.05), except for Oral B, Crest, Colgate, Sensodyne, Nasim, and Pooneh (P=0.8, P=1, P=0.999, P=0.850, and P=0.373, respectively); while, the cytotoxicity of Nasim had no significant difference with that of Crend, Pooneh, Colgate, Sensodyne, and Crest (P=0.850, P=1, P=1, P=1 and P=0.962, respectively) but it had significant differences with other toothpastes (P<0.05).

The cytotoxicity of Paradontax did not have significant differences with that of Sehhat (P=0.221) but its difference with other toothpastes was significant (P<0.05). The cytotoxicity of Sehhat toothpaste did not have a significant difference with that of Darugar and Paradontax toothpastes (P=0.999 and P=0.221, respectively) but it had significant differences with other toothpastes (P<0.05). The cytotoxicity of Darugar had significant differences with other toothpastes (P<0.05), except that of Sehhat (P=0.999). The cytotoxicity of these toothpastes was the lowest among all.

The cytotoxicity of Listerine mouthwash had no significant difference with that of Irsha and Oral B (P=0.134 and P=0.946, respectively) but it had a significant difference with Vi One (P<0.05). The cytotoxicity of Irsha was moderate and had no significant difference with other mouthwashes (P=0.134 for Listerine, P=0.997 for Oral B, and P=0.28 for Vi One). The cytotoxicity of Oral B had a significant difference with Vi One (P<0.05).

Antibacterial activity:

All toothpastes and mouthwashes were examined against *S. mutans* and *L. acidophilus* and showed antibacterial activity. Tables 1 and 2 show the MIC of the products. The obtained MIC for all toothpastes and mouthwashes was between 0.0039 mg/mL and 0.0156 mg/mL, except for the corresponding values for Sensodyne toothpaste and Oral B mouthwash, which were 0.5 mg/mL and 0.0312 mg/mL when they were in contact with *S. mutans* and *L. acidophilus* for 1 min and 30 min, respectively.

Table 1. Maximum inhibitory concentrations of the studied mouthwashes against S. *mutans* (SM) and *L. acidophilus* (LA)

Mouthwash	SM		LA	
	1 min	30 min	1 min	30 min
Listerine	1:64	1:256	1:64	1:256
Irsha	1:16	1:32	1:16	1:32
Oral B	1:2	1:32	1:2	1:32
Vi-One	1:32	1:256	1:32	1:256

Table 2. Maximum inhibitory concentrations of the studied toothpastes against *S. mutans* (SM) and *L. acidophilus* (LA)

Toothpastes	SM		LA	
	1 min	30 min	1 min	30 min
Sehat	1:128	1:128	1:128	1:128
Oral B	1:64	1:256	1:64	1:256
Crend3	1:128	1:256	1:128	1:256
Pooneh	1:128	1:256	1:128	1:256
Paradontax	1:64	1:256	1:64	1:256
Colgate	1:64	1:256	1:64	1:128
Darugar	1:64	1:64	1:64	1:64
Close-Up	1:128	1:256	1:256	1:256
Nasim	1:64	1:256	1:64	1:128
Sensodyne	1:2	1:32	1:2	1:32
Bath	1:64	1:64	1:64	1:64
Signal	1:64	1:256	1:128	1:256
Crest	1:64	1:256	1:64	1:128

DISCUSSION

All chemical agents used in the oral cavity should be evaluated in terms of cytotoxicity. A number of experiments are performed by the American Dental Association and Food and Drug Administration for comprehensive assessment of dental materials. Cell culture is among the primary tests performed to determine the toxic effects of dental products. Toothpastes are used on a daily basis for oral hygiene. Since they are in direct contact with the oral mucosa, all their negative effects should be investigated. However, their effects on oral mucosa have not been thoroughly investigated [2].

The main objective of the present study was to assess the cytotoxicity and antibacterial activity of toothpastes and mouthwashes from several Iranian and foreign manufacturers. The results of the MTT assay showed that all products had some degrees of inhibition on cell viability, which increased by an increase in concentration. However, the cytotoxicity of products was not significantly different following different exposure times. It was found that the antibacterial activity of the studied toothpastes and mouthwashes was almost equal when they were in contact with *L. acidophilus* and *S. mutans*.

The MTT assay is a fast and reliable method for assessing cytotoxicity based on the activity of enzymes present in viable cells. The optical density of the samples at each time point shows the number of viable cells, as well as the amount of metabolic activity. The higher the optical density, the more viable cells are present in the sample, showing lower cytotoxicity of the tested material. A material that shows more than 50% cell viability is considered non-toxic [11,17].

Toothpastes have many constituents. Plaque removal and caries prevention are the main goals behind the use of toothpastes. Abrasive and insoluble particles such as silica, aluminum hydroxide, and calcium carbonate are used for plaque removal. Fluoride compounds such as sodium fluoride and sodium monofluorophosphate are used for caries prevention. Toothpastes also contain a detergent such as sodium lauryl sulfate and cocamidopropyl betaine. These are the main constituents of toothpastes. Sodium lauryl sulfate is known as one of the most cytotoxic components in the composition of toothpastes [2,11]. Other ingredients added for other purposes such as desensitization, anti-plaque, anti-inflammatory and anti-odor properties, preservatives, dyes and flavors may also have toxic effects [11]. Menthol and limonene are both flavors added to toothpastes; however, menthol has the potential to cause allergy, and the cariogenic property of limonene has been confirmed [18]. Benzoic acid and its derivatives, parabens and sorbitol derived from the ascorbic acid, are antimicrobial agents. Evidence shows that these substances have low cytotoxicity and the Food and Drug Administration has categorized them in "generally recognized as safe" category given that they are used in safe amounts [19].

Ghapanchi et al. [2] evaluated the cytotoxicity of 16 commercial toothpastes and stated that the cytotoxicity of samples increased from 1 to 2 and 5 min. Some of the toothpastes evaluated in their study were also evaluated in our study. In their study, the Bath toothpaste was among the most toxic toothpastes, which was in line with our study. However, they reported that Darugar and Sehat toothpastes were also among the most toxic toothpastes while in the present study, these two toothpastes had the lowest cytotoxicity. Moreover, in the present study, Close-Up and Signal had the highest cytotoxicity while their study showed these two toothpastes had low cytotoxicity. They did not mention the constituents of toothpastes, their fluoride dosage, and the concentrations tested. Although their methodology was similar to ours, difference in the findings of the two studies can be due to the difference in the concentration of tested toothpastes and the type of cells evaluated since they assessed epithelial and HELA cells.

Torrado et al. [20] evaluated the cytotoxicity of Crest Extra Whitening toothpaste and a new experimental toothpaste named NMTD against mouse fibroblasts using the MTT assay. They showed that none of the tested toothpastes had any significant effect on cell viability. No significant increase in cytotoxicity was noted by increasing the incubation time. The first part of their results was different from our findings since in our study, all samples showed some degrees of cytotoxicity. This difference is probably due to the difference in type of cells evaluated. However, the second part of their results was in agreement with our findings because no significant change in cytotoxicity of the samples was noted by an increase in exposure time in our study.

Toothpastes are usually supplied in 100 g tubes and contain 1450 ppm fluoride. Approximately 0.6 g of toothpaste, which contains about 9 ppm fluoride, is used at each time of tooth brushing. Among mouthwashes, Vi One mouthwash, for instance, is supplied in 330 mL bottles and contains 500 ppm fluoride. The manufacturer recommends using 15 mL of the mouthwash for each time of consumption, which contains about 22 ppm fluoride. Jeng et al, [21] in their study on the cytotoxicity of sodium fluoride against human oral fibroblasts showed that sodium fluoride in 4 mM/L (80 ppm) and higher concentrations is toxic for oral mucosal fibroblasts. In concentrations lower than 2 mM/L (40 ppm), no significant negative effect was noted. According to this study, toothpastes and mouthwashes, at each time of use, did not increase the level of fluoride in oral mucosal cells to the toxic level, which is 40 ppm. This study revealed that rinsing the oral cavity with 0.2% sodium fluoride can increase the fluoride level of oral mucosal cells to 40 ppm. Evidence shows that protein synthesis is the first and most sensitive parameter affected by the toxic effects of fluoride on cells, and fluoride exerts its effect by inhibition of protein synthesis. These effects were clearly noticeable in use of 2 mM/L and higher concentrations of fluoride in this study. Due to the frequent long-term use of fluoride, more detailed studies are required regarding its toxic effects in vitro and in vivo.

Detergents may also be responsible for the cytotoxicity of toothpastes. Cvikl et al. [11] evaluated the effect of constituents of toothpastes on cell viability. They assessed the cytotoxicity of 9 toothpastes with different detergents in their composition, and demonstrated that toothpastes containing sodium lauryl sulfate and amine fluoride strongly affected the cell viability while toothpastes containing cocamidopropyl betaine had lower effects on cell viability. In our study, sodium lauryl sulfate was the detergent in the composition of all toothpastes except for Paradontax and Sensodyne that contained cocamidopropyl betaine and were both found to be less cytotoxic.

Evidence shows that the cytotoxicity of sodium monofluorophosphate is lower than that of sodium fluoride [22]. In the present study, all toothpastes that had low cytotoxicity contained sodium monofluorophosphate, except for Paradontax and Sensodyne that contained sodium fluoride. However, the detergent in their composition was

cocamidopropyl betaine instead of sodium lauryl sulfate. Song et al. [23] used the MTT assay to assess the cytotoxicity of ammonium hexafluorosilicate against gingival fibroblasts. They tested 0.01% and 1% concentrations of the product, which were similar to 0.05% and 2% concentrations used in our study. They revealed that the cytotoxicity of ammonium hexafluorosilicate was dose- and time-dependent and under in vitro conditions, it had low or no cytotoxicity in up to 0.01% concentrations and less than 5 min of exposure time. Similarly, in our study, 0.05% concentration showed a mean cytotoxicity of <15%. However, the cytotoxicity of our tested samples was not time-dependent.

Antimicrobial mechanisms of toothpastes containing fluoride are via the inhibition of glucose transport, carbohydrate storage, extracellular polysaccharide formation, and acid formation by oral streptococci [14]. It appears that there was no change in antibacterial activity by changing the concentration of fluoride compounds to minor amounts in over-the-counter toothpastes and mouthwashes. Systematic reviews have shown that formulations containing triclosan/copolymer significantly improve plaque control and periodontal health, and are more effective than regular fluoride toothpastes [24,25]. In the present study, despite the presence of triclosan in Bath toothpaste, it did not show greater antibacterial activity, which may be related to the concentration of this substance or other compounds in this toothpaste.

In this study, after 1 min of exposure, the maximum antibacterial effect was found in Pooneh, Crend, Sehat and Close-Up toothpastes. After 30 min of exposure, all toothpastes showed increased antibacterial effect, except for Bath, Sehat and Darugar toothpastes. It appears that the antibacterial effect of toothpastes and mouthwashes increased as the result of longer contact with the oral environment. It was found that the amount of MIC of the studied toothpastes was almost equal when they were in contact with *L. acidophilus* and *S. mutans*. However, higher efficacy was observed, as an exception, for the Signal and Close-Up toothpastes when they were in contact with *L. acidophilus* for 1 min, and for the Crest, Nasim and Colgate toothpastes when they were in contact with *L. acidophilus* for 30 min. It was found that the amount of MIC of the Listerine and Vi One mouthwashes was the highest while that of Irsha and Oral B was the lowest, when they were in contact with *L. acidophilus* and *S. mutans*.

CONCLUSION

Within the limitations of this in vitro study, the following results were obtained:

1. Close-Up, Signal and Bath toothpastes had maximum cytotoxicity while Darugar, Sehat and Paradontax had minimum cytotoxicity.
2. Listerine and Oral B mouthwashes had the highest and Vi One and Irsha mouthwashes had the lowest cytotoxicity.
3. The antibacterial activity of the toothpastes and mouthwashes was almost equal when they were in contact with *L. acidophilus* and *S. mutans*.
4. In general, except for the Irsha mouthwash, and Sehat, Darugar and Bath toothpastes, the antibacterial effect of toothpastes and mouthwashes increased when the contact time increased.

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CONFLICT OF INTEREST STATEMENT

None declared.

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