Temporal Exposure of Dermal Fibroblasts to Silver Diamine Fluoride and Fluorine NMR Quantitation

Cannon ML*/Duchaj S**/ Patel R***/Kabat B****

Objective: Silver diamine fluoride has been advocated as a caries arresting material for Early Childhood Caries (ECC) and has received considerable public attention as the "silver bullet". However, cytotoxicity tests on the current concentrations of Silver Diamine Fluoride (SDF) to soft tissue have not been thoroughly assessed and analyzed at selected time intervals. The level of fluoride that is present within human cells has yet to be quantified. Preliminary SDF toxicity studies in our lab determined exposures of Dermal Fibroblasts to 0.03% SDF for 18 hours resulted in 100% cytotoxicity and complete monolayer loss. Endpoint titration of SDF determined that morphologic cytotoxic effects were ameliorated at input SDF levels lower than 0.002%. Because of the small culture sample volumes, we were unable to effectively assay fluoride concentrations using commercially available assays. In this study we attempted to assess fluoride levels in culture supernatants in a temporal fashion using quantitative Nuclear Magnetic Resonance (NMR). ***Study design:*** *Dermal Fibroblast (DF) cells were grown in 24 well cluster plates fitted with 0.4 micron Transwell™ inserts to confluency in 0.9mL of DF culture media. Then the DF cells were challenged with 0.1 mL of SDF in sterile water in the Transwell chamber to achieve a final concentration of 0.03% SDF. Cultures were reincubated for 30 minutes, 1, 2, 4 and 8 hours. At the selected time points Transwell inserts were removed. SDF culture media was removed and replaced with fresh media and allowed to re-incubate up to 8 hours. Harvested SDF culture media was centrifuged at 15,000 x g to remove any resulting SDF precipitates and supernatants were harvested and stored at -70°C for fluoride assay. After 8 hours, media was aspirated from all wells and DF cells were fixed and then stained with methylene blue and assessed for cytotoxicity. Harvested supernatants were assessed for fluoride content. While SDF is soluble in pure water, it precipitates instantly in the presence of other media constituents and 0.85% saline. Transwells inserts capture the precipitate but allow soluble SDF and constituents pass through to the cell monolayer. NMR was used to assess SDF (fluoride) prepared in water, in DF media or in normal saline at the same concentrations used in the DF cell studies. The ^{19}F NMR spectra were acquired at 25 °C on an Agilent DD2 500 MHz spectrometer equipped with a 5mm HFX z gradient probe operating at 470.3 MHz for fluorine. For quantitative measurements, all spectra were collected with 64 scans and a delay of 5 seconds. The spectrum width is 220 ppm with offset at resonance of -110 ppm. The processing and analyzing were done by MNOVA. The dataset consists 45371 complex points and is zero-filled to the size of 128k points after applying 5Hz exponential line broadening. The ^{19}F chemical shift was referenced indirectly based on proton chemical shift, which was referenced with respect to the water proton signal of 4.75 ppm at 25°C.* ***Results:*** *Visible DF cell morphology changes begin to appear as early as 1 hour exposure to 0.03% SDF in Transwells and continue with degradation of cell morphology through 8 hours exposure at which point 100% of the cell monolayer is lost. The 8 hour image shows complete cell loss which is consistent with earlier studies using 24 hour exposures at 0.03% concentration. Note that the actual concentration of SDF affecting cell viability is shown in this study to be far lower than the 0.03% input because of the aggregate precipitation captured with the Transwell inserts. In this study, our NMR fluoride assessments showed that only 6-12 % of the input SDF fluoride reaches the lower cell chamber.* ***Conclusions:*** *Considering that the SDF reagent is applied orally at ~40%, these results warrant more refined testing to identify true lower limit of toxicity end points of SDF. SDF should be utilized only by trained professionals and never contact soft tissue. NMR may be utilized to determine relative amounts of fluoride both in cell culture media and within fluoride exposed cells.*

Keywords: SDF, cytotoxicity, dermal fibroblasts cells, Transwells

*ML Cannon, Ann and Robert Lurie Children's Hospital of Chicago, Northwestern University Feinberg School of Medicine
** S Duchaj, Ann and Robert Lurie Children's Hospital of Chicago
***R Patel, Ann and Robert Lurie Children's Hospital of Chicago
****B Kabat, Ann and Robert Lurie Children's Hospital of Chicago

Corresponding author:
Cannon M. L.,
Northwestern University and Ann and Robert Lurie Children's Hospital, Chicago, IL. USA
Phone: +(847)8996720
E-mail: drmarkcannon@outlook.com, markcannon@northwestern.edu

INTRODUCTION

Silver diamine fluoride (SDF) has been advocated as a caries arresting material for Early Childhood Caries. [1] Clinical application of SDF has been reported to arrest the caries process for a period of up to 6 months thereby requiring subsequent re-applications. [2] This may pose a problem with non-compliant families that delay or ignore follow up visits. In addition, a systematic review of the clinical trials reveals that many trials did not include positive controls, often comparing SDF to either no treatment or just a placebo. [3] Normally, clinical trials would include both negative and positive controls. Interestingly, a one-time application of SDF did not significantly prevent dental caries. [4] Therefore, due to the requirement for multiple treatments, there is a need for SDF cytotoxicity studies over time.

Cytotoxicity tests on current concentrations of SDF to soft tissue should be thoroughly assessed and analyzed at selected time intervals. Also, the potential long-term effect of SDF on mitochondria should be investigated. A study by Lou et al on the influence of chronic fluorosis on mitochondrial dynamics morphology and distribution in cortical neurons of the rat brain indicated that chronic fluorosis induces abnormal mitochondrial dynamics, which might in turn result in a high level of oxidative stress. [5] In addition, the effects of fluoride ion in dental medicaments on cell vitality, specifically the mitochondria should be investigated. NMR analysis of fluoride ion levels may be a technique useful in determining if fluoride ions are anti-mitochondrial and should be further investigated as mitochondrial health is linked to aging and cancer. Recently published studies on the possible effects of chronic over exposure to fluoride have created increased interest in the dental products using fluoride. Twenty-one of twenty-three epidemiology studies have reported a possible negative effect on cognitive abilities or intelligence due to chronic fluoride exposure. [6] Animal studies have presented plausible biochemical mechanisms for the neurologic effects. [7] The effect of fluoride on the brain microglia has also been established. [8] Oddly enough, the anti-microbial nature of the fluoride ion that is partially responsible for dental benefits, may be the reason for the anti-mitochondrial effect. For example, anti-bacterial antibiotics are also anti-mitochondrial. [9] The use of non-dental personnel in an un-controlled environment with lack of proper suctioning and lighting may contribute to un-necessary exposure to any cytotoxic effects. In addition, the risk benefit ratio should always be considered.

Fluoride varnish was once touted as an effective preventative for young children. [10] Unfortunately, a systematic review of Fluoride varnish clinical trials revealed a discrepancy between the much-heralded preventative predicted effectiveness and it's actual performance.[11] Indeed, of nine clinical trials, only 3 showed any positive benefit in little children, although fluoride varnish may be recommended for older patients. Other interventions in these three studies may have been responsible for the positive result. Additionally, the release of fluoride into the saliva by fluoride varnishes does NOT correlate with the absorption of fluoride into the enamel.[12] Possibly, the release of fluoride into the saliva only gives a systemic dosing to the recipient and may actually be associated with more potential side effects and little enamel protection. Perhaps the "Old Standard" of Acidulated Phosphate Fluoride foam/gel treatment for younger children under the age of 6 should still be utilized as a dental preventative [13]

Fluoride induces cell stress, including endoplasmic reticulum stress and oxidative stress, which leads to impairment of ameloblasts responsible for dental enamel formation. Recently it was reported that fluoride activates SIRT1 and autophagy as an adaptive response to protect cells from stress. [14] Interestingly, fluoride dentifrice was the main source of fluoride intake by the children evaluated. However, the fluoride concentration in food items also significantly contributed to the daily ingestion by 2-6-year-old children. [15] Most of children in the study were exposed to a daily fluoride intake above the suggested threshold for dental fluorosis. The dentifrice alone is responsible for an average of 81.5% of the daily fluoride intake, while among the constituents of the diet, water and milk are the most important contributors. [16] Children ingested around 60% of the dentifrice loaded onto the brush, but no significant differences were seen among the groups ($p > 0.05$). Mean daily fluoride intake from dentifrice for the three groups (G-A, G-B and G-C) was 0.022(a), 0.032(a) and 0.061(b) mg F/kg body weight, respectively ($p < 0.01$). There was a strong positive correlation ($r = 0.86$, $p < 0.0001$) between the amount of dentifrice used and the amount of fluoride ingested during toothbrushing. The results indicate the need for instructing children's parents and care givers to use a small amount of dentifrice (< 0.3 g) to avoid excessive ingestion of fluoride. [17]

Children with low height-for-age were more likely to have dental fluorosis in the TFI (Thylstrup-Fejerskov Index) categories that affect the entire tooth surface. The results suggest that subpopulations with chronic undernutrition are more susceptible to dental fluorosis. [18] If future studies are done, this preliminary information should help in designing SDF assays going forward. For now, it is fair to assume that the actual SDF cytotoxic endpoints are lower than what has been obtained from our DF culture studies. Among human populations, fluoride-related health issues span from mild effects on teeth and bones to severe kidney problems, neurotoxicity, and even cancer. [19-23] Nowadays, fluoride toxicity is gaining much attention in the fluoride endemic areas due to its adverse impact on children's cognitive function, since children are more susceptible to fluoride toxicity as compared to adults. The primary mechanism behind the neurotoxicity of fluoride is still not clearly understood. However, fluoride reportedly disrupts the biochemical mechanism and thus alters the normal functioning of the brain. Several human studies are suggestive of lowered IQs in children exposed to high fluoride during their childhood. [24] The conclusive findings of previous published research suggest that the fluoride concentration in drinking water tends to grossly affect the children's IQ level. [25] However, proof is limited, because most of the studies are concentrated in a few countries and have limitations and data gaps. [26] The grave potential implications of fluoride toxicity demand extensive and meticulously designed research to bridge the data gap in developing countries. Furthermore, spanning research work across all the fluoride endemic areas of the world could generate a larger database, helping resolve the issue on a large scale. [27]

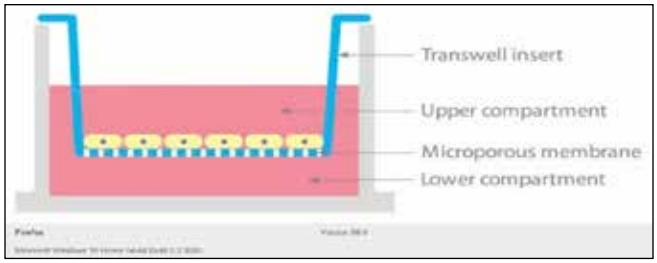
Unfortunately, the mothers' report estimating the amount of toothpaste on the toothbrush using demonstrative figures was not a reliable method to judge the inadvertent ingestion of fluoride from dentifrices. [28] Fluoride ingestion is probably much greater than estimated, which may cause one to speculate as to the greater over-all effect. Some parents may be less concerned because they utilize

a "children's" toothpaste. However, the findings suggest that the ingested dose should be calculated based on TSF (Total Systemic Fluoride). The dose of TSF ingested by children is similar whether adult or children's toothpaste is used. [29] The majority of dentifrices used in that study contained F (96%) and in 84% of them, Total Fluoride concentration was representative of that declared by the manufacturers. In the F-toothpastes, 78% showed TSF concentration ≥ 1000 ppm, varying from 422.3 to 1432.3 ppm F (mean ± SD of 1017.6 ± 239.4). [30] So most children in that study were exposed to a high fluoride concentration. [29] However, a high concentration is necessary to prevent decay according to published results if only fluoride is considered. "There are benefits of using fluoride toothpaste at certain strengths to prevent tooth decay when compared with non-fluoride toothpaste. The stronger the fluoride concentration, the more decay is prevented. For many of the comparisons of different strengths of toothpaste, the findings are uncertain and could be challenged by further research. The choice of fluoride toothpaste for young children should be balanced against the risk of fluorosis," reported a Cochrane database article.[31] This guideline does not consider potential side effects or the reported neurologic toxicity. Frequent ingestion of low but excessive quantities of fluoride during the period of tooth formation can certainly lead to dental fluorosis. That risk is well understood. But particular concern is warranted for the ingestion of fluoride-containing toothpastes by young children and the inappropriate use of dietary fluoride supplements in communities with sufficient fluoride already present in drinking water. [32]

MATERIALS AND METHOD

Dermal Fibroblast (DF) cells were grown in 24 well cluster plates to confluency. For experimental procedures, test wells were fitted with 0.4 micron Transwell™ inserts (depicted in Figure 1.). Each well of DF cells contained 0.9mL of DF culture medium. DF cells were challenged with 0.1 mL of SDF in sterile water in the Transwell insert chamber to achieve a final concentration of 0.03% SDF in 1 ml total volume. Cultures were reincubated for 30 minutes, 1, 2, 4 and 8 hours. SDF at levels above 0.008% precipitate in the presence of culture media forming an insoluble precipitate as determined in preliminary studies (Data not shown). Trans-wells were employed to capture precipitate but permit the soluble SDF portion to pass through the membrane. At the selected time points Transwell inserts were removed. SDF culture media was removed and saved for fluoride assay. Fresh culture media (1.0mL) was added to the harvested wells and allowed to re-incubate up to 8 hours. Harvested SDF culture media was centrifuged @ 15,000 x g for 10 minutes, harvested and stored at -70°C for fluoride assay. After 8 hours, all remaining Transwell inserts were removed, media was aspirated and saved while DF cells were fixed with 95% Ethanol for 10 minutes and then stained with methylene blue. All DF cell images were taken at 600X. For additional NMR studies SDF was prepared diluted in water, in DF media at a 10-fold concentration and in normal saline at the same concentrations used in the DF studies. All samples were subjected to centrifugation at 15,000 x g to remove any resulting precipitates and supernatants were harvested for the NMR (nuclear magnetic resonance) studies.

Figure 1: Diagram of a Transwell Iinsert – courtesy of Vandateb. com (Transwells- Corning Life Sciences One Riverfront Plaza, Corning, NY 1483 The lower compartment contains 0.9mL of DF culture media . 0.1mL of SDF at 0.3% is added to the upper chamber.



RESULTS

The temporal cell changes to SDF exposure are seen in Figures 2 through 7 below. Morphological changes (Initial changes from striated cells to rhomboid appearance followed by cell loss) are clearly seen within 1 hour exposure with complete loss of all cells by 8 hours.

Figure 2. Untreated Dermal Fibroblasts (DF).

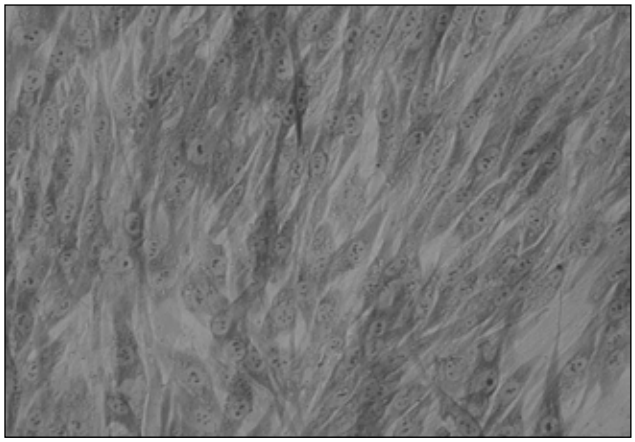


Figure 3. Dermal Fibroblasts after 30 min. exposure to 0.03% SDF (600X magnification exposure to SDF (600X magnification)

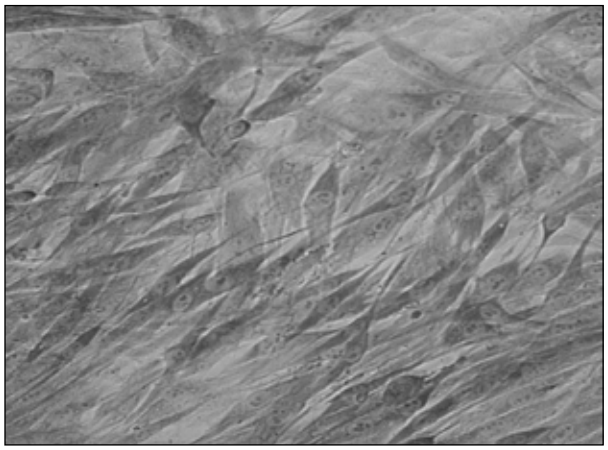


Figure 4: Dermal Fibroblasts after 1 hour exposure

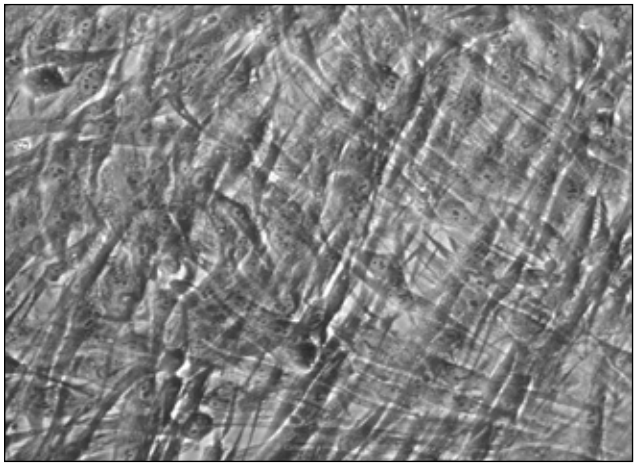


Figure 5: Dermal Fibroblasts after 2 hour exposure to 0.03% SDF (600x magnification) to 0.03% SDF (600X magnification)

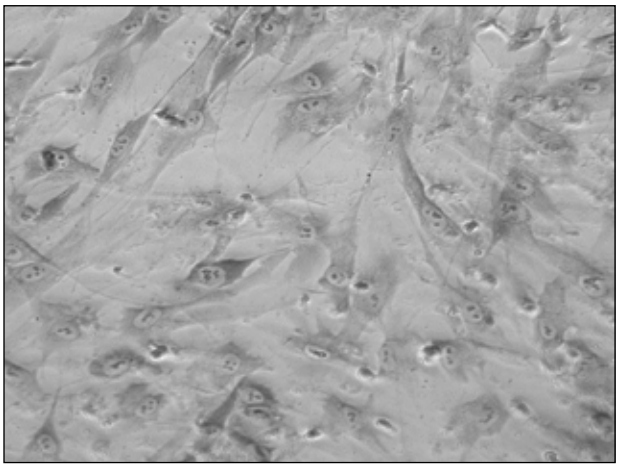
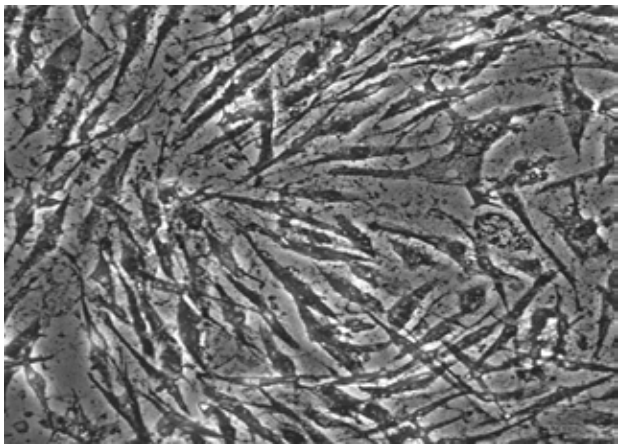


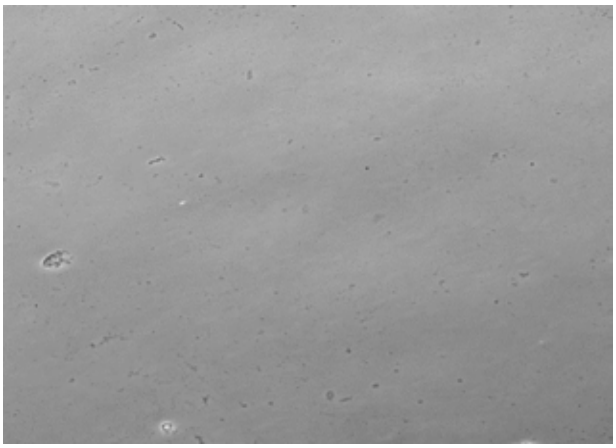
Figure 6: Dermal Fibroblasts after 4 hour exposure



NMR Fluorine quantitation in Dermal Fibroblast (DF) Culture system:

Silver Diamine Fluoride (SDF) used for dental applications is supplied as a 38% (2.36M) unbuffered aqueous preparation. Cytotoxicity studies in the SID lab have shown that SDF is toxic to dermal fibroblasts in unfiltered concentrations as low as 0.002% (0.116mM) after the 24 hour exposure to DF cells. When SDF is

Figure 7: Dermal Fibroblasts after 8 hour exposure to 0.03% SDF (600X magnification) to 0.03% SDF (600X magnification)



applied to the cells separated by a 0.4 micron Transwell™ membrane the toxic endpoint is slightly higher (0.004%). The reason for using Transwell systems is that SDF precipitates instantly when exposed to proteinaceous complete culture media, normal saline (0.85%) and a host of other common chemical entities. The characteristic precipitation of SDF confounds our ability to measure the actual concentrations of SDF or its ionic fluoride component that target the DF cells in a cytotoxic manner. Typical environmental Fluoride assays require large volumes of liquid preparations to quantitate the ion and are impractical for small volume biological assay systems. For that reason, we turned to a more sensitive NMR based assay which was recommended by the Northwestern University Chemistry department and conducted in their IMSERC NMR facility. Our sample sizes available were sufficient to estimate the amount of fluoride generated post SDF precipitation that might be expected to affect DF cells.

Preliminary NMR Study: Determination of detectability of SDF/F- in DF Culture media.

The preliminary results suggest that fluoride can be detected at relatively low concentrations but the presence of DF media components may interfere to some extent on the quantitative detection of fluoride ion when binding occurs in minor DF media components. We re-examined a higher concentration of SDF in DF medium with an internal Trifluoroacetic acid (TFA) standard. Figure 11 shows the NMR results of 0.3% SDF on DF media post centrifugation. Triflouroacetic acid (TFA) is used a quantitation standard as described in the methods section. TFA standard peak is seen at -75.4 ppm as a single peak. Three SDF related fluoride peaks are seen at ppm positions -130.06, -143.33, and -157.85 in the presence of DF media. The multiple peaks are not unexpected for SDF because of the complex culture media matrix that potentially bind fluorine. The ppm frequency shifts are also due to the pH changes caused by TFA. The values for each peak relative to the ~10mM TFA peak are shown in the plot table. All of the peaks are in the µM range. The total fluorine recovered is the sum of the three peaks.

As stated, normal (0.85%) saline precipitates SDF in the same way as DF media without the additional binding entities. That said, we replicated our DF studies using normal saline as a precipitant using Transwells to capture precipitated SDF and measured Fluorine as described. We also looked at SDF in water at the 0.03% SDF level.

Figure 8. Generic Diagram of NMR Spectrometer (From Barron, P. M. V. R. & A. R. (2020, June 9). NMR Spectroscopy– https://chem.libretexts.org/@go/page/55887)

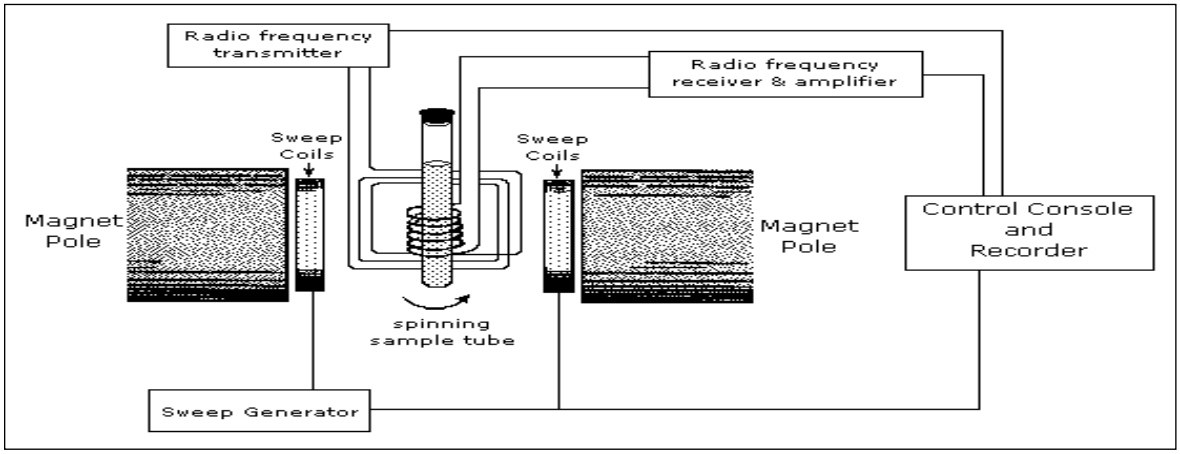


Figure 9 Two standard qualitative NMR plots are shown to assess background fluoride in DF medium. The upper plot is 0.06% (3.72mM) SDF in DF media. A single small fluoride peak can be seen at -120ppm position (see arrow).The lower black line is a plot of DF media without any SDF showing no fluorine peak at –120 ppm or elsewhere in this expanded plot.

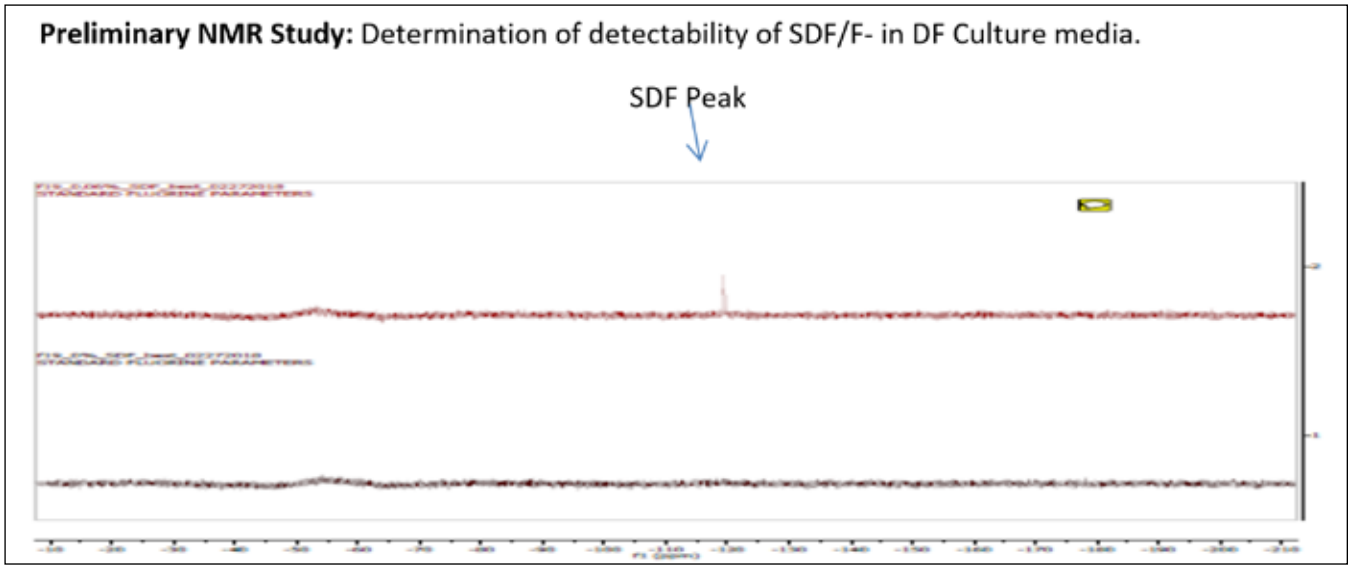


Figure 10. Expanded 0.06% peak. The broad peak is due to fluorine binding to media components which is further evaluated below.

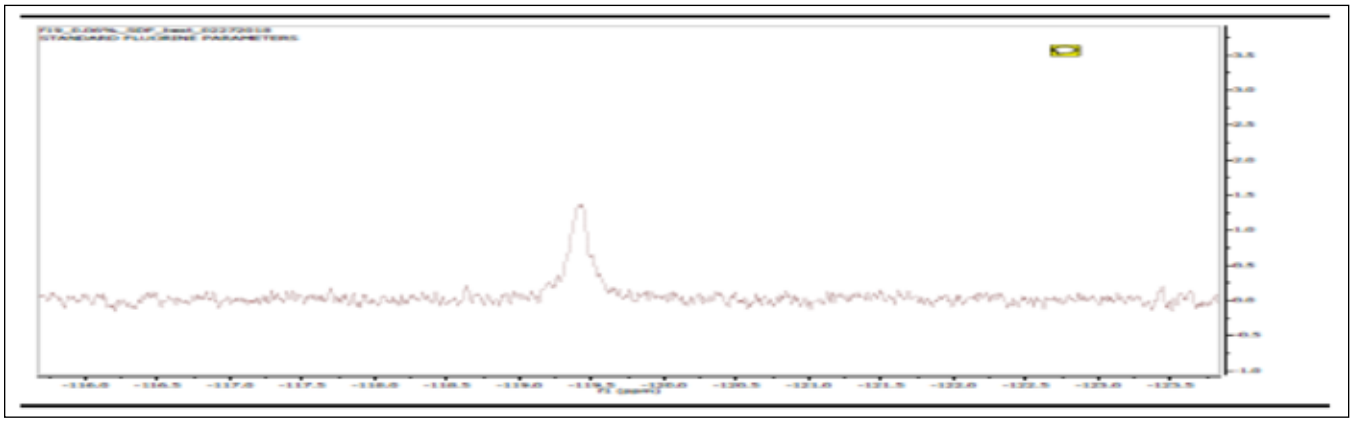


Figure 11. TFA SDF Peaks

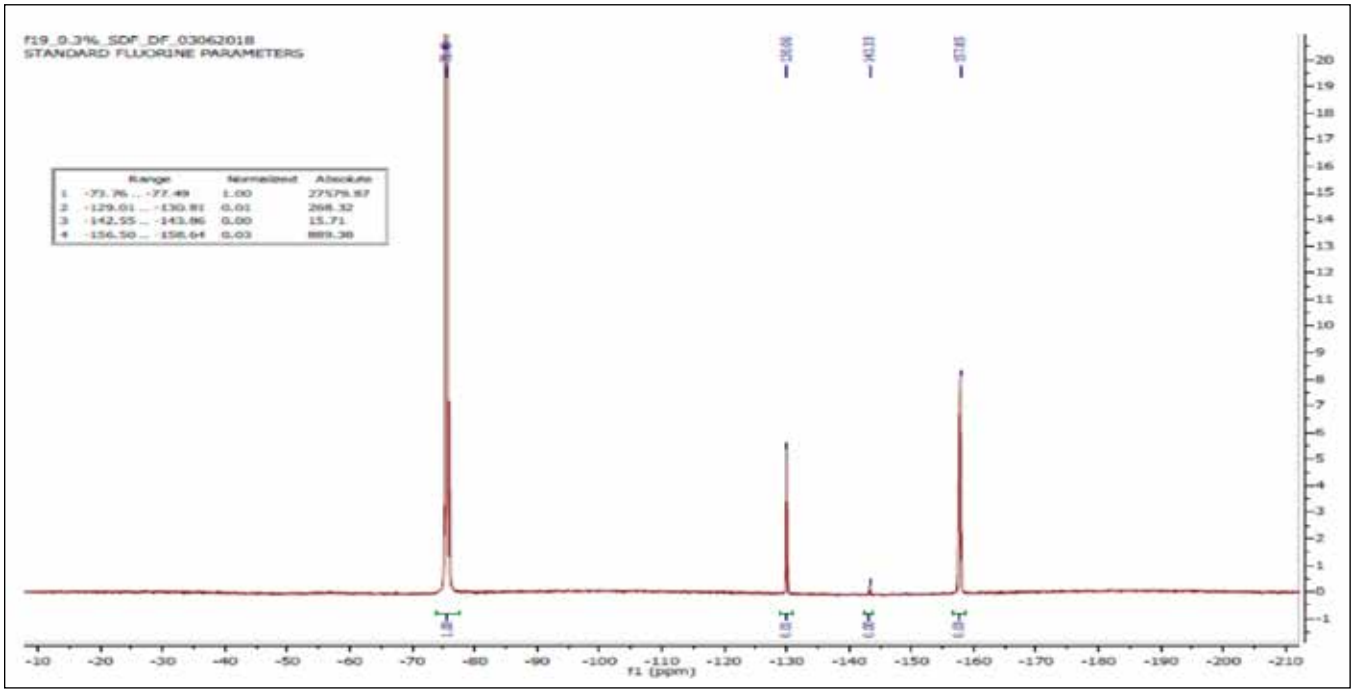
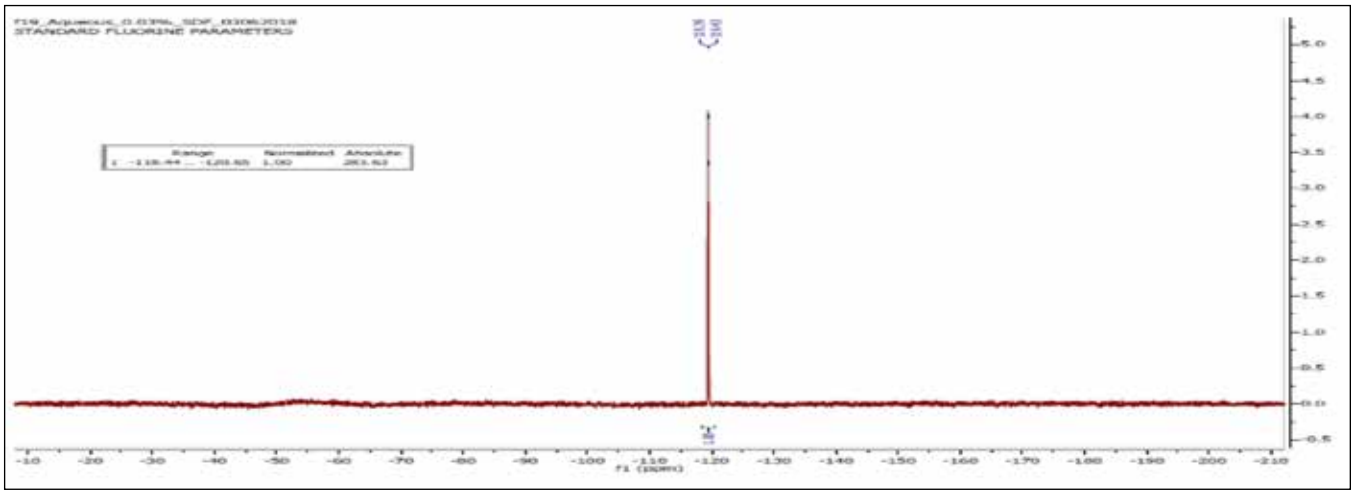


Figure 12: 0.03% SDF in H_20



To determine the NMR position of SDF in an aqueous environment, we looked at a 0.03% (1.86mM) preparation of SDF in molecular grade water. As can be seen, a very well defined single peak at -119.4 is generated that is very narrow in the absence of other molecular entities. There was no visible precipitate post-centrifugation with this aqueous preparation. Estimated recovery ~0.2836mM (15.2%) relative to absolute NMR value.

As seen in DF media, the TFA changes the matrix pH and the frequency shifts the SDF peak position to -161.41. In this case some SDF precipitates in the presence of normal saline and some signal (~ one third) is lost compared to that in H_2O.

Figure 13 (below) is a composite of 6 samples normalized to TFA standards used to estimate SDF (F-) concentrations and to demonstrate the SDF fluorine ppm shifts that are related to the use of TFA as an internal standard compared to samples assayed without TFA Ultimately, the peak shifts do not significantly affect quantitation of fluorine which is supported by the assay linearity check in Figure 14 below. The spectra below are (from top to bottom) 0.004% SDF in saline, 0.008% SDF in saline, 0.015% SDF in saline, 0.03% SDF in saline with TFA, 0.03% SDF in H2O, and 0.3%sdf in DF media with TFA.

Table 1 summarizes the SDF data and Figure 14 below plots the absolute quantitative NMR peak value vs. the nominal SDF input value. As can be seen, relative quantities of fluoride can be assessed with NMR. It is clear that the fluorine levels from SDF can be measured in the in-vitro system used in this study. The levels of SDF are small but clearly toxic to DF cells. But only represent a small fraction of fluorine from the nominal input SDF. We can also see that we can measure differences in fluorine in a dose dependent manner in a linear manner.

Figure 13: NMR of 0.03% SDF in Normal saline with ~10mM TFA internal standard.

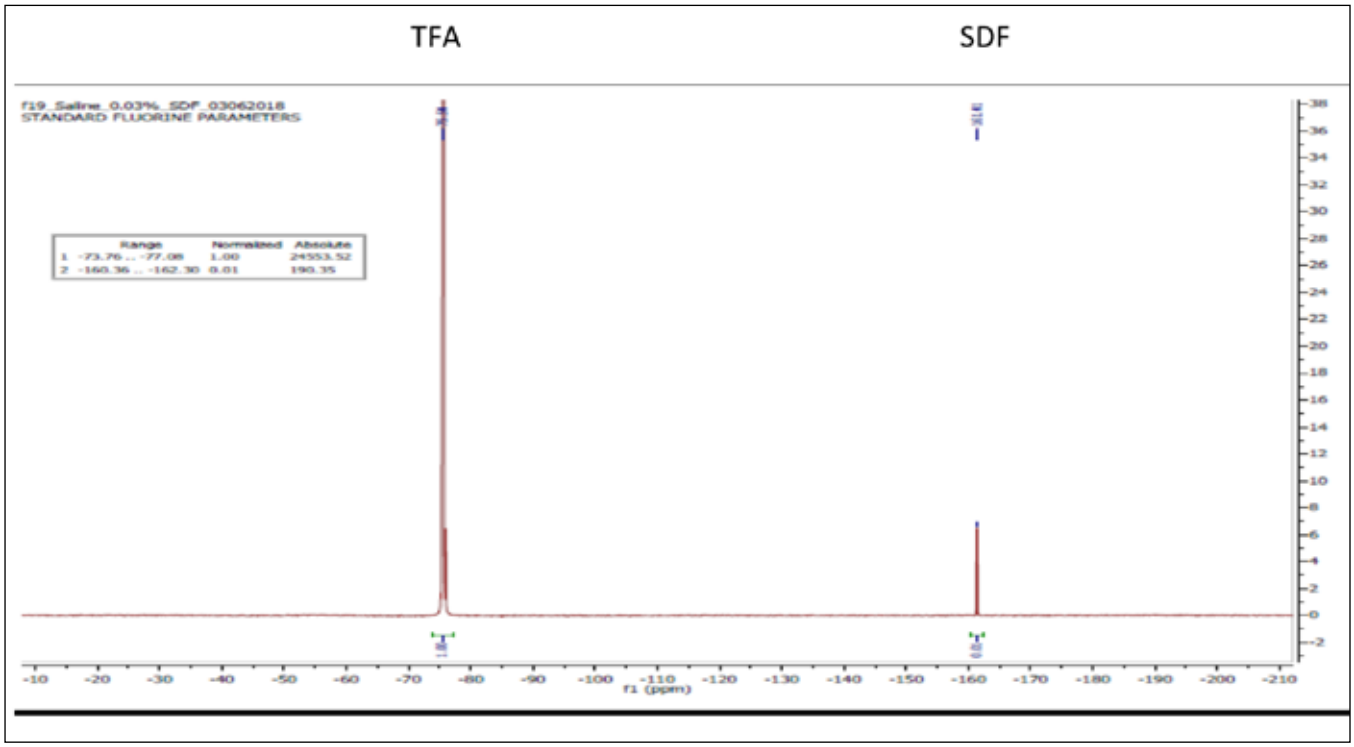


Table 1. NMR Data Summary

Nominal SDF input concentrations	Absolute NMR SDF values	Fluorine recovery (% SDF)	{SDF} normalized to TFA Standard	Fluorine Peak values (ppm)
	µM values		µM values	
0.3% SDF in DF media	1173.4	6.3%	425.49	Peak 1: (-130.6) Peak 2(-143.33), Peak 3: (-157.85)
0.03% SDF in H2O	283.63	15.2 %	N/A	(-119.4) to (-120.65)
%SDF in 0.85% NaCl				
0.03% (1860 µM)	190.35	10.2 %	77.5	((-160) to (-162.3) w/TFA
0.015% (930 µM)	64.7	6.96 %	N/A	(-119.4) to (-120.65)
0.008% (465 µM)	51.09	11.0 %	N/A	(-119.4) to (-120.65)
0.004% (232 µM)	26.04	11.2 %	N/A	(-119.4) to (-120.65)

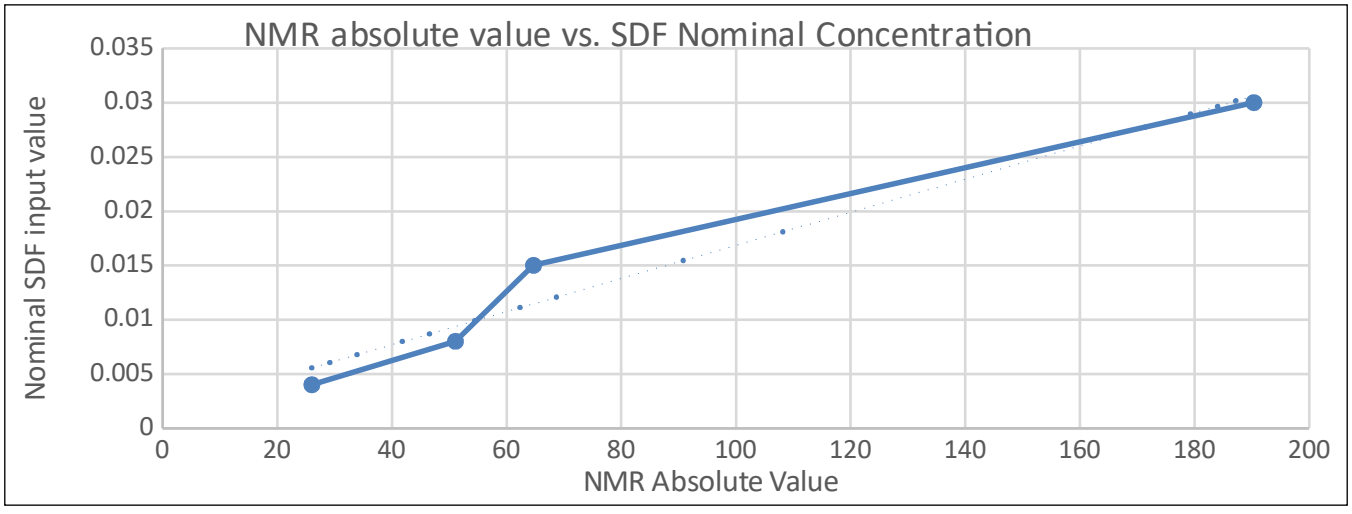
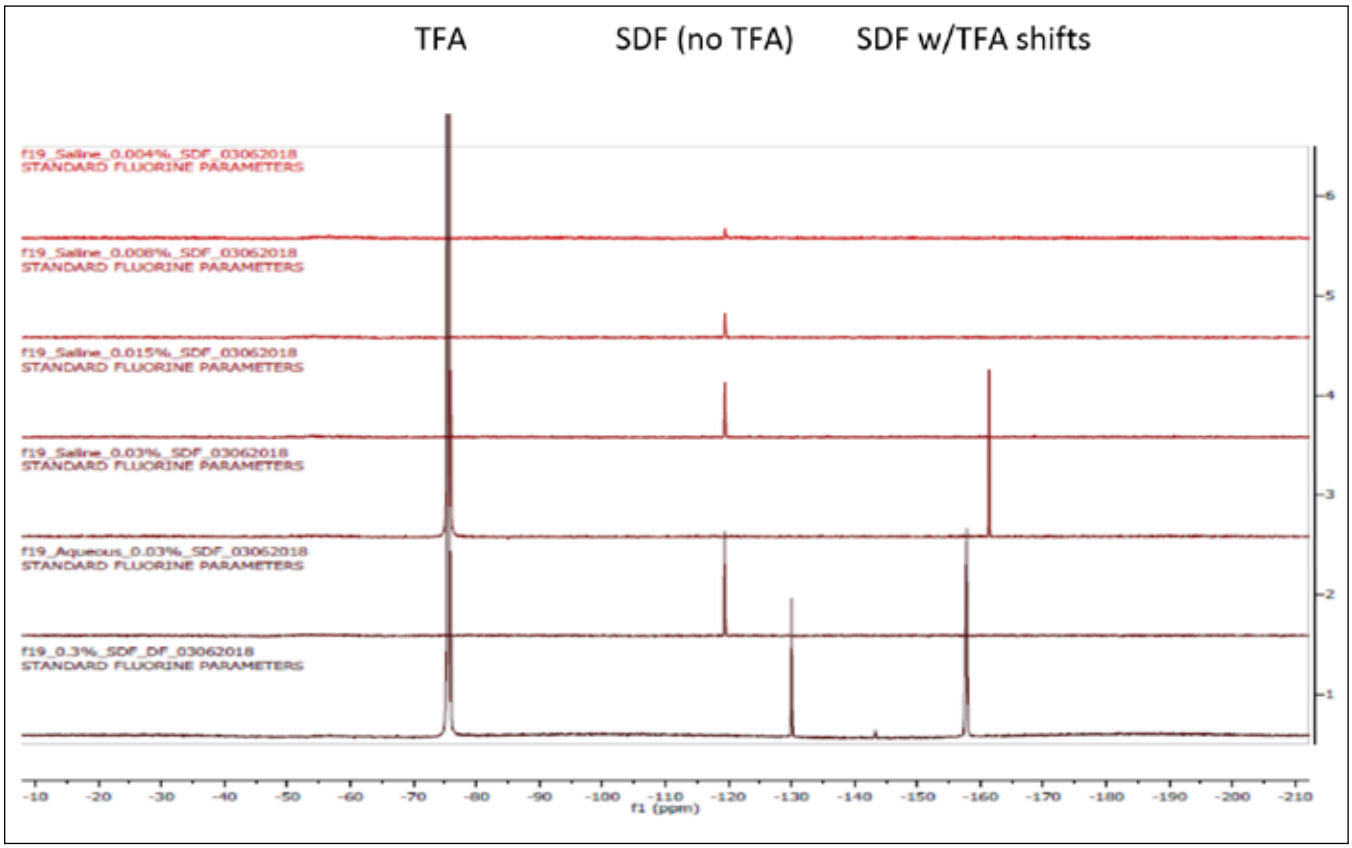


Figure 14. Linearity of Fluorine NMR Assay y for SDF in Saline:



DISCUSSION

Determining cytotoxicity of materials often utilizes dermal fibroblasts in media containing the test material and has been used to study silver ions. Silver nano particles have been shown in rat studies to accumulate in various organs from highest to lowest: lungs, spleen, liver, kidney, thymus, and heart. [33] Some observed effects include tissue irritation, chromosome breakage with potential genotoxicity and carcinogenic effects. [34] The use of silver nano particles could be further examined due to the concern that the public has previously expressed over the effect of long-term exposure to silver dental amalgam restorations. [35] Although the use of mercury in silver dental amalgam restorations was always stated as the chief concern, the anecdotal symptoms reported by some individuals may have been related to the release of nano silver particles. [36 and 37] Silver, either in colloidal form, or ionic, or as nano particles has been associated with marked cytotoxicity. [38] Dermal fibroblasts exposed to silver have hindered proliferation and that has been associated with the delayed healing seen with anti-microbial products using silver particles. [39-40] The very strong inhibitory effect of SDF on the dermal fibroblasts may have been the result of the combined cytotoxicity of both fluoride and silver. Unfortunately, we did not analyze the silver ion concentrations within the dermal cells, and most likely the precipitation that occurred in the media contained the silver ions. Plus, the concentration of the fluoride within the cells mostly matched the levels of SDF solution which correlated with the cytotoxicity observed. The international standard ISO 7405, which addresses the biocompatibility of dental materials and devices, states that biocompatibility tests should be performed on materials in an "as-used state". [41] The amounts of SDF used by practitioners, especially non dental trained clinicians, may well exceed the amount expected with proper following of the manufacturer's instructions (Six sites and air dry. For use in adults over the age of 21). [42] The cytotoxicity studies used adults and not little children who may not cooperate with the SDF placement, allowing for soft tissue contact or even ingestion of the product. In addition, some practitioners place a Fluoride varnish on top of the SDF without knowledge of the potential toxicity of the combined treatment and with no evidence for increased effectiveness in decay prevention. [43]

The Cytotoxicity of SDF on DF cells, HeLa cells and bacteria was clear in a previously completed study by the authors. This study was designed to try to quantify the actual cytotoxic endpoint of SDF. The results of this study further illustrate the scope of the problem in that the uncertainty persists. First, SDF when placed in a complex matrix like DF culture media, generates different fluoride containing components probably by associating with media proteins and other media components even after significant loss of SDF due to precipitation. Secondly, while NMR generates very concise values in soluble water matrix, the same is not true even in a simple saline or buffered matrix because of the loss of some material due to precipitation in these matrices even though the degree of precipitation is less. Finally, the use of internal fluorine containing standards alters the NMR signals (somewhat expected) so that one must plan future studies so that the internal standards are used as a template but not incorporated in the SDF containing samples to be quantified. This will still result in estimated values but is better when the other confounding variables are limited.

Considering that the SDF reagent is applied orally at a roughly 38% concentration (24.4-28.8% (w/v) Ag, 5.0-5.9% fluoride, at pH 10 and 8-10% ammonia) in aqueous solution, these results warrant more refined testing to identify true lower limit of toxicity end points of SDF. SDF should be utilized only by trained professionals and never contact soft tissue due to toxicity concerns. The TF should be considered from all sources; the public water, to dietary sources, from ingested toothpaste, professionally applied fluorides, both varnishes and SDF. The concern should not just be fluorosis, the risk we all understand, but rather neurological status, as in average IQ. The problem is that standardized weighted mean difference in IQ score between exposed and reference populations was -0.45 (95% confidence interval: -0.56, -0.35) using a random-effects model. [44] Thus, this study concluded that children in high-fluoride areas had significantly lower IQ scores than those who lived in low-fluoride areas. The potential for neurologic damage far out-weighs dental concerns. [45] Of interest is that, at concentrations higher than 0.7 parts per million, a systematic review found a low to moderate level-of-evidence that suggests adverse effects on learning and memory in animal models exposed to fluoride. The evidence is strongest (moderate level-of-evidence) in animals exposed as adults and weaker (low level-of-evidence) in animals exposed during development. [46] So what is the effect on humans? 21 of 23 recent epidemiological studies report an association between high fluoride exposure and reduced intelligence. This discrepancy between experimental and epidemiological evidence may be the result of deficiencies inherent in most of these epidemiological studies on a potential association between fluoride and intelligence, especially with respect to other confounding factors, e.g., breast feeding, socioeconomic status, maternal intelligence, residence, low birth weight, and exposure to other possible neurotoxic chemicals. In conclusion, based on the "totality of currently available scientific evidence, the present review does not support the presumption that fluoride should be assessed as a human developmental neurotoxicant at the current exposure levels in Europe". [6] This research review article may not have considered the full effects of swallowed fluoride dental preventatives. This would present as a serious and obvious error. Indeed, the "most common sources of acute overexposures today are dental products–particularly dentifrices because of their relatively high fluoride concentrations, pleasant flavors, and their presence in non-secure locations in most homes. For example, ingestion of only 1.8 ounces of a standard fluoridated dentifrice (900-1,100 mg/kg) by a 10-kg child delivers enough fluoride to reach the 'probably toxic dose' (5 mg/kg body weight)." [47]

It has been reported that with a fluoride concentration of 44,800 parts per million, SDF is nearly twice the strength of commercially available 5% sodium fluoride varnishes used in primary care. Application of both agents on the same day is to be avoided as the fluoride dose would be additive, and its safety profile in children is still unknown. [21] However, some clinicians are apparently applying both at the same visit. Notwithstanding, Silver Diamine Fluoride (SDF) 38% has been receiving a great deal of attention by U.S. dental professionals since it was cleared for use by the Food and Drug Administration in August 2014 under the provisions of the Federal Food, Drug and Cosmetics Act. The Cleared Indications for Use are for the "Treatment of dentinal hypersensitivity. For use in adults over the age of 21." Even though originally approved for only over the age of 21, this product is widely used on little children and even endorsed by major dental organizations as an off-label use. This is somewhat unexpected due to the reported cytotoxicity of the product. [48] One should assume a 'cautionary approach" due to the vulnerability of young children. Even though, at "concentrations of 30% and 38%, SDF shows potential as an alternative treatment for caries arrest in the primary dentition and permanent first molars." [49] Dental caries is, of course, a major concern, but so should be neurologic health and the lack of negative side-effects. There is an anti-microbial effect, "fluoride-treated tooth surfaces inhibit bacterial acid production at the bacteria/tooth interface. The SDF-coated RD had the strongest inhibitory effect." [50] This may also imply an anti-mitochondrial effect, especially with males. Interestingly, a recent publication states "research confirms that SDF has long lasting antimicrobial effects at very low concentrations although it does raise concerns regarding cytotoxicity." [51]

Dental prevention does not rely only on fluoride, but also on diet modification and the reduction in refined carbohydrates creates improved systemic health. [52] Processed sugar has been linked to diabetes, obesity, increased blood pressure, and even cancer. [53-54] Obviously, dental professionals should discuss nutrition with all their patients, first and foremost. In addition, sugar overwhelms the protective effect of fluoride supplementation. [55] Hence leaving only the potential cytotoxicity and none of the dental preventive benefit for the patient. Other options are also available, such as, probiotic supplementation, which arguably is superior in preventive benefit to the patient. [56-57] Of course, fluoride amounts could safely be reduced as long as the toothpaste, mouthwash or varnish had xylitol added to increase the effectiveness of the preventative. [58-62]

In summary, SDF has an important role to play in the managing of dental caries and should be utilized by dental professionals as directed in the manufacturer's instructions for use. The overall concern should be the TSF consumed by the young child. More of anything is not always better when treating dental patients, especially children. Dental professionals should also utilize SDF carefully preventing any ingestions or soft tissue contact and be aware of the TSF amounts the child patient is exposed to.

CONCLUSION

SDF has the potential for cytotoxicity and should be applied only by a trained dental professional according to the manufacturer's indications. SDF application should be an important part of Preventive Dentistry but not the only dental preventive utilized. The practitioner should also consider the total systemic fluoride exposure for each patient, especially young children, and be cognizant of potential cytotoxicity.

Acknowledgement:

We are grateful to Dr. Yongbo Zhang PhD at Northwestern University's IMSERC group for performing the NMR assessments and interpretations of the data included in this study.

REFERENCES

1. Crystal YO, Marghalani AA, Ureles SD, et al. Use of silver diamine fluoride for dental caries management in children and adolescents, including those with special health care needs. Pediatr Dent 2017; 39(5):E135-E145.
2. Menardia, V., Ramos, T., & Morato, M. (2005). Efficacy of silver diamine fluoride for caries reduction in primary teeth and first permanent molars of schoolchildren: 36-month clinical trial. Journal of dental research, 84(8), 721–724. https://doi.org/10.1177/154405910508400807
3. Oliveira, B. H., Rajendra, A., Veitz-Keenan, A., & Niederman, R. (2019). The Effect of Silver Diamine Fluoride in Preventing Caries in the Primary Dentition: A Systematic Review and Meta-Analysis. Caries research, 53(1), 24–32. https://doi.org/10.1159/000488686
4. Monse, B., Heinrich-Weltzien, R., Mulder, J. et al. Caries preventive efficacy of silver diamine fluoride (SDF) and ART sealants in a school-based daily fluoride toothbrushing program in the Philippines. BMC Oral Health 12, 52 (2012).
5. Lou, D. D., Guan, Z. Z., Liu, Y. J., Liu, Y. F., Zhang, K. L., Pan, J. G., & Pei, J. J. (2013). The influence of chronic fluorosis on mitochondrial dynamics morphology and distribution in cortical neurons of the rat brain. Archives of toxicology, 87(3), 449–457. https://doi.org/10.1007/s00204-012-0942-z
6. Guth, S., Hüser, S., Roth, A., Degen, G., Diel, P., Edlund, K., Eisenbrand, G., Engel, K. H., Epe, B., Grune, T., Heinz, V., Henle, T., Humpf, H. U., Jäger, H., Joost, H. G., Kulling, S. E., Lampen, A., Mally, A., Marchan, R., Marko, D., … Hengstler, J. G. (2020). Toxicity of fluoride: critical evaluation of evidence for human developmental neurotoxicity in epidemiological studies, animal experiments and in vitro analyses. Archives of toxicology, 94(5), 1375–1415. https://doi.org/10.1007/s00204-020-02725-2
7. Chen J, Chen X, Yang K, Xia T, Xie H. [Studies on DNA damage and apoptosis in rat brain induced by fluoride]. Zhonghua yu Fang yi xue za zhi [Chinese Journal of Preventive Medicine]. 2002 Jul;36(4):222-224.
8. Yan, N., Liu, Y., Liu, S. et al. Fluoride-Induced Neuron Apoptosis and Expressions of Inflammatory Factors by Activating Microglia in Rat Brain. Mol Neurobiol 53, 4449–4460 (2016). https://doi.org/10.1007/s12035-015-9380-2
9. Kalghatgi, S., Spina, C. S., Costello, J. C., Liesa, M., Morones-Ramirez, J. R., Slomovic, S., Molina, A., Shirihai, O. S., & Collins, J. J. (2013). Bactericidal antibiotics induce mitochondrial dysfunction and oxidative damage in Mammalian cells. Science translational medicine, 5(192), 192ra85. https://doi.org/10.1126/scitranslmed.3006055
10. Weyant, R. J., Tracy, S. L., Anselmo, T. T., Beltrán-Aguilar, E. D., Donly, K. J., Frese, W. A., Hujoel, P. P., Iafolla, T., Kohn, W., Kumar, J., Levy, S. M., Tinanoff, N., Wright, J. T., Zero, D., Aravamudhan, K., Frantsve-Hawley, J., Meyer, D. M., & American Dental Association Council on Scientific Affairs Expert Panel on Topical Fluoride Caries Preventive Agents (2013). Topical fluoride for caries prevention: executive summary of the updated clinical recommendations and supporting systematic review. Journal of the American Dental Association (1939), 144(11), 1279–1291. https://doi.org/10.14219/jada.archive.2013.0057
11. Horst, J. A., Tanzer, J. M., & Milgrom, P. M. (2018). Fluorides and Other Preventive Strategies for Tooth Decay. Dental clinics of North America, 62(2), 207–234. https://doi.org/10.1016/j.cden.2017.11.003
12. Bolis, C., Härtli, G. P., & Lendenmann, U. (2015). Fluoride Varnishes—Is There a Correlation Between Fluoride Release and Deposition on Enamel? Oral health & preventive dentistry, 13(6), 545–556. https://doi.org/10.3290/j.ohpd.a34373
13. Evans, D. APF foam does reduce caries in primary teeth. Evid Based Dent 8, 7 (2007). https://doi.org/10.1038/sj.ebd.6400461
14. Suzuki, M., Bandoski, C., & Bartlett, J. D. (2015). Fluoride induces oxidative damage and SIRT1/autophagy through ROS-mediated JNK signaling. Free radical biology & medicine, 89, 369–378. https://doi.org/10.1016/j.freeradbiomed.2015.08.015
15. Miziara, A. P., Philippi, S. T., Levy, F. M., & Buzalaf, M. A. (2009). Fluoride ingestion from food items and dentifrice in 2-6-year-old Brazilian children living in a fluoridated area using a semiquantitative food frequency questionnaire. Community dentistry and oral epidemiology, 37(4), 305–315. https://doi.org/10.1111/j.1600-0528.2009.00477.x
16. de Almeida, B. S., da Silva Cardoso, V. E., & Buzalaf, M. A. (2007). Fluoride ingestion from toothpaste and diet in 1- to 3-year-old Brazilian children. Community dentistry and oral epidemiology, 35(1), 53–63. https://doi.org/10.1111/j.1600-0528.2007.00328.x
17. Moraes, S. M., Pessan, J. P., Ramires, I., & Buzalaf, M. A. (2007). Fluoride intake from regular and low fluoride dentifrices by 2-3-year-old children: influence of the dentifrice flavor. Brazilian oral research, 21(3), 234–240. https://doi.org/10.1590/s1806-83242007000300008
18. Irigoyen-Camacho, M. E., García Pérez, A., Mejía González, A., & Huizar Alvarez, R. (2016). Nutritional status and dental fluorosis among school-children in communities with different drinking water fluoride concentrations in a central region in Mexico. The Science of the total environment, 541, 512–519. https://doi.org/10.1016/j.scitotenv.2015.09.085
19. Song, C., Fu, B., Zhang, J., Zhao, J., Yuan, M., Peng, W., Zhang, Y., & Wu, H. (2017). Sodium fluoride induces nephrotoxicity via oxidative stress-regulated mitochondrial SIRT3 signaling pathway. Scientific reports, 7(1), 672. https://doi.org/10.1038/s41598-017-00796-3
20. Shashi A (2003) Histopathological investigation of fluoride-induced neurotoxicity in rabbits. Fluoride 36:95–105
21. Grandjean P, Olsen JH. Extended follow-up of cancer incidence in fluoride-exposed workers. Journal of the National Cancer Institute. 2004;96(10):802–803. [PubMed] [Google Scholar]
22. Tohyama E. Relationship between fluoride concentration in drinking water and mortality rate from uterine cancer in Okinawa prefecture, Japan. Journal of Epidemiology. 1996;6(4):184–191. [PubMed] [Google Scholar]
23. Bassin EB, Wypij D, Davis RB, Mittleman MA. Age-specific fluoride exposure in drinking water and osteosarcoma (United States) Cancer Causes and Control. 2006;17(4):421–428. [PubMed] [Google Scholar]
24. Trivedi MH, Verma RJ, Chinoy NJ, Patel RS, Sathawara NG (2007) Effect of high fluoride water on intelligence of school children in India. Fluoride 40:178–183
25. Xiang Q, Liang Y, Chen L, Wang C, Chen B, Chen X, Zhou M (2003) Effect of fluoride in drinking water on children's intelligence. Fluoride 36:84–94
26. Tang Q, Du J, Ma H, Jian S, Zhou X (2008) Fluoride and children's intelligence, a meta-analysis. Biol Trace Elem Res 126(1–3):115–120
27. Saeed, M., Malik, R. N., & Kamal, A. (2020). Fluorosis and cognitive development among children (6-14 years of age) in the endemic areas of the world: a review and critical analysis. Environmental science and pollution research international, 27(3), 2566–2579. https://doi.org/10.1007/s11356-019-06938-6
28. de Siqueira, M., de Sousa, E. T., Alves, V. F., Sampaio, F. C., & Diniz, M. B. (2019). Maternal report underestimates the amount of dentifrice used by children during toothbrushing: a cross-sectional study. European archives of paediatric dentistry: official journal of the European Academy of Paediatric Dentistry, 20(6), 603–607. https://doi.org/10.1007/s40368-019-00443-7
29. Oliveira, M. J., Martins, C. C., Paiva, S. M., Tenuta, L. M., & Cury, J. A. (2013). Estimated fluoride doses from toothpastes should be based on total soluble fluoride. International journal of environmental research and public health, 10(11), 5726–5736. https://doi.org/10.3390/ijerph10115726
30. Cury, J. A., Oliveira, M. J., Martins, C. C., Tenuta, L. M., & Paiva, S. M. (2010). Available fluoride in toothpastes used by Brazilian children. Brazilian dental journal, 21(5), 396–400. https://doi.org/10.1590/s0103-64402010000500003
31. Walsh, T., Worthington, H. V., Glenny, A. M., Marinho, V. C., & Jeroncic, A. (2019). Fluoride toothpastes of different concentrations for preventing dental caries. The Cochrane database of systematic reviews, 3(3), CD007868. https://doi.org/10.1002/14651858.CD007868.pub3
32. Heifetz, S. B., & Horowitz, H. S. (1986). Amounts of fluoride in self-administered dental products: safety considerations for children. Pediatrics, 77(6), 876–882.
33. Wen, Hairuo, et al. "Acute Toxicity and Genotoxicity of Silver Nanoparticle in Rats." Plos One, vol. 12, no. 9, 27 Sept. 2017. US National Library of Medicine National Institutes of Health, doi:10.1371/journal.pone.0185554.

34. Wiemann, Martin, et al. "Silver Nanoparticles in the Lung: Toxic Effects and Focal Accumulation of Silver in Remote Organs." Nanomaterials, vol. 7, no. 12, 2017, p. 441., doi:10.3390/nano7120441
35. Jirau-Colón, H., González-Parrilla, L., Martinez-Jiménez, J., Adam, W., & Jiménez-Velez, B. (2019). Rethinking the Dental Amalgam Dilemma: An Integrated Toxicological Approach. International journal of environmental research and public health, 16(6), 1036. https://doi.org/10.3390/ijerph16061036
36. Butler, K. S., Peeler, D. J., Casey, B. J., Dair, B. J., & Elespuru, R. K. (2015). Silver nanoparticles: correlating nanoparticle size and cellular uptake with genotoxicity. Mutagenesis, 30(4), 577–591. https://doi.org/10.1093/mutage/gev020
37. Zhang, T., Wang, L., Chen, Q., & Chen, C. (2014). Cytotoxic potential of silver nanoparticles. Yonsei medical journal, 55(2), 283–291. https://doi.org/10.3349/ymj.2014.55.2.283
38. Lansdown A. B. (2010). A pharmacological and toxicological profile of silver as an antimicrobial agent in medical devices. Advances in pharmacological sciences, 2010, 910686. https://doi.org/10.1155/2010/910686
39. Hidalgo, E., & Domínguez, C. (1998). Study of cytotoxicity mechanisms of silver nitrate in human dermal fibroblasts. Toxicology letters, 98(3), 169–179. https://doi.org/10.1016/s0378-4274(98)00114-3
40. Huang, Y., Lü, X., & Ma, J. (2014). Toxicity of silver nanoparticles to human dermal fibroblasts on microRNA level. Journal of biomedical nanotechnology, 10(11), 3304–3317. https://doi.org/10.1166/jbn.2014.1974
41. ISO 7405:2018 Dentistry — Evaluation of biocompatibility of medical devices used in dentistry
42. Elevate Oral Care, LLC.West Palm Beach, FL 33411877-866-9113© 2018 Elevate Oral Care ELE489-0318
43. Sarvas E., Karp J. M., Silver diamine fluoride arrests untreated dental caries but has drawbacks. AAP News. https://www.aappublications.org/news/2016/08/05/SilverDiamine080516.
44. Choi, A. L., Sun, G., Zhang, Y., & Grandjean, P. (2012). Developmental fluoride neurotoxicity: a systematic review and meta-analysis. Environmental health perspectives, 120(10), 1362–1368. https://doi.org/10.1289/ehp.1104912
45. Choi, A. L., Zhang, Y., Sun, G., Bellinger, D. C., Wang, K., Yang, X. J., Li, J. S., Zheng, Q., Fu, Y., & Grandjean, P. (2015). Association of lifetime exposure to fluoride and cognitive functions in Chinese children: a pilot study. Neurotoxicology and teratology, 47, 96–101. https://doi.org/10.1016/j.ntt.2014.11.001
46. National Toxicology Program. (2016). NTP Research Report on Systematic Literature Review on the Effects of Fluoride on Learning and Memory in Animal Studies: Research Report 1. National Toxicology Program.
47. Whitford G. M. (2011). Acute toxicity of ingested fluoride. Monographs in oral science, 22, 66–80. https://doi.org/10.1159/000325146
48. García-Contreras R, Scougall-Vilchis RJ, Contreras-Bulnes R, et al. Cytotoxic impact of silver and silver diamine fluoride in six oral cells culture. Rev ADM. 2013;70(3):134-139.Elise Sarvas, D.D.S., M.S.D., M.P.H. and Jeffrey M. Karp, D.M.D., M.S.
49. Contreras, V., Toro, M. J., Elías-Boneta, A. R., & Encarnación-Burgos, A. (2017). Effectiveness of silver diamine fluoride in caries prevention and arrest: a systematic literature review. General dentistry, 65(3), 22–29.
50. Ishiguro, T., Mayanagi, G., Azumi, M., Otani, H., Fukushima, A., Sasaki, K., & Takahashi, N. (2019). Sodium fluoride and silver diamine fluoride-coated tooth surfaces inhibit bacterial acid production at the bacteria/tooth interface. Journal of dentistry, 84, 30–35. https://doi.org/10.1016/j.jdent.2018.12.017
51. Fancher, M. E., Fournier, S., Townsend, J., & Lallier, T. E. (2019). Cytotoxic effects of silver diamine fluoride. American journal of dentistry, 32(3), 152–156.
52. Vos, M. B., Kaar, J. L., Welsh, J. A., Van Horn, L. V., Feig, D. I., Anderson, C., Patel, M. J., Cruz Munos, J., Krebs, N. F., Xanthakos, S. A., Johnson, R. K., & American Heart Association Nutrition Committee of the Council on Lifestyle and Cardiometabolic Health; Council on Clinical Cardiology; Council on Cardiovascular Disease in the Young; Council on Cardiovascular and Stroke Nursing; Council on Epidemiology and Prevention; Council on Functional Genomics and Translational Biology; and Council on Hypertension (2017). Added Sugars and Cardiovascular Disease Risk in Children: A Scientific Statement From the American Heart Association. Circulation, 135(19), e1017–e1034. https://doi.org/10.1161/CIR.0000000000000439
53. Patnode, C. D., Evans, C. V., Senger, C. A., Redmond, N., & Lin, J. S. (2017). Behavioral Counseling to Promote a Healthful Diet and Physical Activity for Cardiovascular Disease Prevention in Adults Without Known Cardiovascular Disease Risk Factors: Updated Systematic Review for the U.S. Preventive Services Task Force. Agency for Healthcare Research and Quality (US).
54. Yoshida, Y., & Simoes, E. J. (2018). Sugar-Sweetened Beverage, Obesity, and Type 2 Diabetes in Children and Adolescents: Policies, Taxation, and Programs. Current diabetes reports, 18(6), 31. https://doi.org/10.1007/s11892-018-1004-6
55. Moynihan P. (2016). Sugars and Dental Caries: Evidence for Setting a Recommended Threshold for Intake. Advances in nutrition (Bethesda, Md.), 7(1), 149–156. https://doi.org/10.3945/an.115.009365
56. Cannon M. A Review of Probiotic Therapy in Preventive Dental Practice, Probiotics and Anti-microbial Proteins, Vol 3, Num 2, July 2011.
57. Cannon M. Vorachek A., Le C., White K. Retrospective Review of Oral Probiotic Therapy. Journal of Clinical Pediatric Dentistry. Volume 43, Number 6/2019
58. Sintes, J. L., Escalante, C., Stewart, B., McCool, J. J., Garcia, L., Volpe, A. R., & Triol, C. (1995). Enhanced anticaries efficacy of a 0.243% sodium fluoride/10% xylitol/silica dentifrice: 3-year clinical results. American journal of dentistry, 8(5), 231–235.
59. Sintes, J. L., Elías-Boneta, A., Stewart, B., Volpe, A. R., & Lovett, J. (2002). Anticaries efficacy of a sodium monofluorophosphate dentifrice containing xylitol in a dicalcium phosphate dihydrate base. A 30-month caries clinical study in Costa Rica. American journal of dentistry, 15(4), 215–219.
60. Amaechi, B.T., Higham, S.M., Edgar, W.M., 1998. The influence of xylitol and fluoride on dental erosion in vitro. Arch. Oral Biol. 43, 157–161. https://doi.org/10.1016/S0003-9969(97)00109-X.
61. Chunmuang, S., Jitpukdeebodintra, S., Chuenarrom, C., Benjakul, P., 2007. Effect of xylitol and fluoride on enamel erosion in vitro. J. Oral Sci. 49, 293¬–297. https://doi.org/10.2334/josnusd.49.293.
62. Souza, J.G., Rochel, I.D., Pereira, A.F., Silva, T.C., Rios, D., Machado, M.A., Buzalaf, M.A., Magalhães, A.C., 2010. Effects of experimental xylitol varnishes and solutions on bovine enamel erosion in vitro. J. Oral Sci. 52, 553–559. https://doi.org/10.2334/josnusd.52.553.