Determination of Cytotoxic Effects of Silver Diamine Fluoride/ Potassium Iodide agent on human peripheral lymphocytes

Running Title: Cytotoxic Effects of Silver Diamine Fluoride/ Potassium Iodide Agent

ABSTRACT:

Aim: The present study aimed to investigate cytotoxicity effect of different concentrations of Silver Diamine Fluoride/Potassium Iodide agent on human peripheral blood lymphocytes.

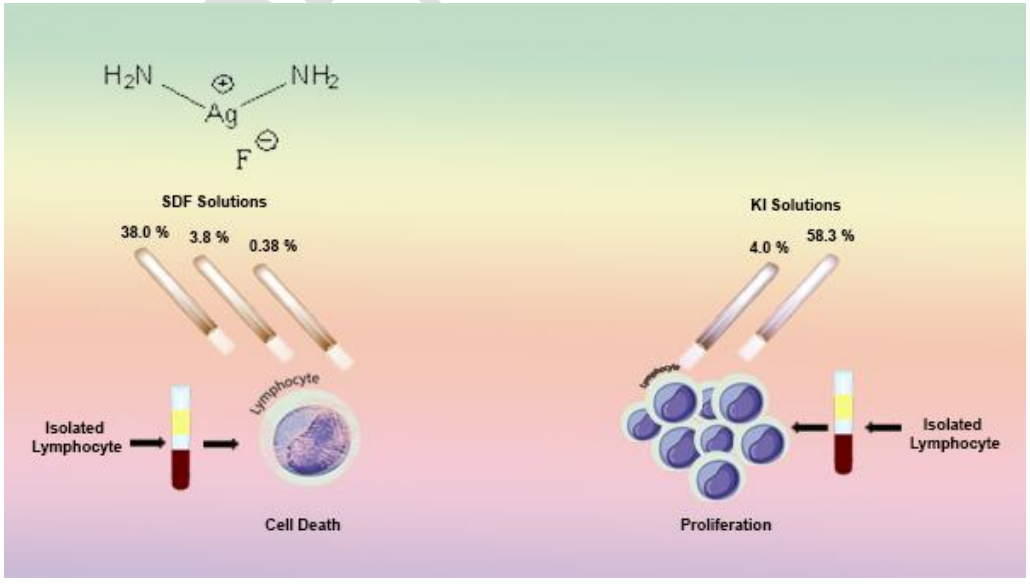
Materials and Methods: The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the effects of SDF (0.38%, 3.8% and 38%) and KI (4%, 58.3%) on cell viability on human lymphocytes after 24 h, 48 h and 72 h of incubation.

Results: MTT test results demonstrated that cell viability decreased as concentrations of SDF increased. 0.38 % SDF was observed to induce the highest cell death after 72 h of contact. At 3.8 % and 38 %, SDF were found to be cytotoxic on human lymphocytes at all application periods. KI solutions at 4 %, 58.3 % concentrations did not show cytotoxicity effect at 24 h exposure, conversely, which resulted in cell proliferation, while moderate cytotoxicity was observed at incubation periods for 48 h and 72 h.

Conclusion: SDF was found to be cytotoxic to human peripheral lymphocytes at all three concentrations and at all exposure times. KI solutions showed cell proliferation in the first 24 hours, which is important in determining the protocol for the use of this solution in clinical procedures, especially in dentistry.

Clinical relevance: Given the cytotoxicity of the SDF and KI solutions, the risk / benefit factors for their clinical applications should be seriously evaluated.

Keywords: cell viability, silver diamine fluoride, potassium iodide, cytotoxicity



Graphical Abstract

Main Text

Introduction

Silver compounds have a long history of use in both medicine and dentistry. Silver Diamine Fluoride (SDF) is described as a solution which involves silver, ammonia and fluoride ($Ag(NH_3)_2F$). It is a mixed heavy-metal halide coordination complex. Silver has exhibited good antibacterial properties and has historically been utilized for disinfection and sterilization in a wide range of fields in dentistry. Silver's antibacterial properties as well as the ability of fluoride's promoting remineralization act together with the aim of prevention of the progression and management of caries. Ammonia ensures that the solution is maintained at a constant concentration during a period of time [1-3].

SDF is the sole agent among chemotherapeutic agents in the field of dentistry. SDF can potentially arrest caries, prevent the formation of new carious lesions and manage dentinal hypersensitivity, particularly in young, socially, vulnerable children who don't cooperate, adults with fears, children and adults with special needs, patients having salivary dysfunction and as well as controlling root caries in the elderly [4-7]. It is supported by many laboratory [7-11] and clinical [12-15] studies that SDF can be used as a successful treatment in order to manage caries and the most widely used concentration in these studies was 38 %, but the rates of 35 %, 30 % and 12 % were utilized as well.

Moreover, SDF is recommended to be used as an antimicrobial root canal irrigant or endodontic medicament for dressings between appointments. Its potential antimicrobial activity has been investigated in several studies at various concentrations (3.8 %,0.38 % and 0.038 %) and has been revealed that % 3.8 SDF is more effective than lower concentrations [16-18].

The main known adverse effect is that applying SDF potentially leads to browning or blackening of enamel or dentin as well as paving the way for esthetic concerns in terms of parents and children. In recent times, it has been reported that potassium iodide (KI) can diminish dark staining after SDF application by means of reacting with free silver ions with the aim of forming a yellow silver iodide precipitate. In the studies conducted, good results have been obtained at concentrations of 10% and 58.3% of the KI concentration [19-21]. In addition, iodine potassium iodide (IKI) solution used as an endodontic irrigant contains iodine (2%) and potassium iodide (4%) [22-24].

An SDF / KI solution is available in a two-stage commercial product consisting of 38 % SDF (first step) and 58.3 % KI (second step) (Riva Star, SDI, Bayswater, Australia). However, the mechanism of SDF action is not yet fully known. Previous studies on the mechanisms of SDF have shown that when applied to active carious lesions, it stops the carious process by killing bacteria present in the lesion, altering the mineral structure of the tooth and occluding the dentinal tubules affected by Ca^{+2} and PO_4^{-3} crystal deposits. Recent studies have found out that it acts on MMPs and cathepsins, which play an important role in dentin collagen degradation [1,10,25].

In order to increase the use of SDF in clinical dentistry, their safety is required to be better understood [26]. Cytotoxicity tests are one of the essential tests determining the safety of a material as it evaluates the hemolytic potential of the material when subjected to blood. The toxicity and cytotoxicity of chemical agents to be used in clinical application must be determined in bacterial or human cells because of containing heavy ion metals [27,28].

The bactericidal properties of SDF and KI solutions have been confirmed by many studies in the literature [2,3,6,7,17,18]. But, the cytotoxic properties of SDF and KI solutions

have been less studied [29-34]. In this respect, the present study aimed to evaluate the cytotoxicity of SDF and KI solutions at various concentrations on human lymphocyte cells.

MATERIALS AND METHODS

Participants provided written informed consent to participate in this study. The Ethical Committee of the Kafkas University of Medical Sciences approved the use of human cells (Reg. No. 2020-14).

Materials

Chemicals and Instruments

The materials used in the study were supplied commercially. SDF/KI agent (Riva Star, SDI, Bayswater, Australia) consisting of 38% SDF and 58.3 % KI were used. Phosphate Buffered Saline (PBS), Fetal Bovine Serum (FBS), Penicillin-Streptomycin-Amphotericin B Solution, Histopaque-1077, Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and MTT (Acros) were purchased commercially. In this study, Nüve BM 101 Water bath, J.P. Selecta Digiheat drying and sterilization oven, ISOLAB vortex mixer, HETTICH EBA 200 centrifuge device, Panasonic MCO 170AICUVH-PE CO_2 Incubator, Thermoscientific-Countess II cell counter and Thermoscientific-Multiskan Sky Microplate Spectrophotometer were used.

Methods

MTT Analysis

In this study, MTT experiments used to determine cell viability were applied with slight modifications to the Mosmann method. For the preparation of the culture medium, 89 % DMEM, 10 % FBS and 1 % penicillin- streptomycin-amphotericin were added in a sterile tube and kept at 37 °C. Lymphocytes were isolated from a human peripheral blood sample [27] and cell counting was performed using a cell counter. Then, 100 µL culture medium and 100 µL cell suspension ($1x10^5$ cells/well) were added to the 96-well plates, respectively. The cells were kept for 24 h in 5% CO_2 incubator at 37 °C to proliferate and adherence to the surface. After incubation, 100 µL aliquots of the materials (0.38 %, 3.8 %, 38 % SDF and 4 %, 58.3 % KI) were added to the wells. The microplates were incubated at 37 °C for 24 h. When the incubation period was complete, 10 µL of MTT solution (5 mg/mL of MTT in PBS buffer) was added to each well. The plates were gently mixed on the shaker and kept in the incubator for 4 h. After incubation, formazan crystals were seen in the bottom of the wells. After 4 h of incubation in a CO_2 incubator, the medium of each well was removed and 200 µL DMSO was added to dissolve the formazan crystals formed as a result of live cell activity. The absorbance values at 570 nm were recorded by spectrophotometer [28]. The absorbance values were used to determinate the cell viability. The cell viability percentages were calculated according to the formula (1). The statistical analysis was conducted with IBM SPSS statistics for Windows package program (v.18.0, IBM Corp., Armonk, New York, USA). Two-way ANOVA (Tukey) was used to evaluate whether any treatment significantly differed from the control or each other's. Statistically significance level was accepted at % 95 ($p<0.05$).

$$\text{Cell Viability (\%)} = \frac{A_1}{A_0} x100 \quad (1)$$

(A_1= Mean of test concentration's absorbance and A_0= Mean of cell controls' absorbance)

RESULTS

The cell viability values were evaluated by comparing both test groups among themselves and with cell control groups (Fig. I). In addition, comparisons of cell viability and cell death percentages were given in Table I.

Cell viability percentages were determined as 83.70 % (24 h), 82.96 % (48 h) and 66.09 % (72 h) when SDF at 0.38 % concentration was used. In addition, it was observed that the highest cell death occurred at 0.38 % concentration of SDF and 72 h exposure time. SDF was found to be severely cytotoxic at these concentrations as cell viability was not observed at 3.8 % and 38 % concentrations and all application dose. KI did not cause any cytotoxic effect at 4 % and 58.3 % concentrations for 24 h exposure, on the contrary, cell proliferation was determined to occur. It was also determined that 67.2 % and 82.99 % cell proliferation occurred at 4% and 58% concentrations, respectively. KI caused moderate cytotoxicity on lymphocyte cells due to the increasing concentration for 48 h and 72 h incubation times. The results showed that the SDF material applied in the first step of this kit, which was applied in two steps, was cytotoxic at the concentration required for treatment (38 %) and at a concentration 10 times diluted (3.8 %) than this concentration. In addition, it was determined that the material applied in the second step of the treatment did not reduce cell viability below 50 % both the treatment concentration (58.3 %) and even at 14.5 times lower concentration (4 %).

Discussion

The biocompatibility assessment is crucial for the clinical validation of dental materials and, to date, only limited number of studies have investigated the cytotoxicity of SDF. The cell culture assays provide controllable and repeatable method of assessment and are ethically more acceptable in comparison to in vivo animal studies, what is more important is that the results may lead to significant clinical conclusions in biomaterial research [28,30,31]. There are different cell types that may be used *in vitro* studies. Previous studies regarding the toxicity of SDF solutions were conducted on cells derived from pulp or soft tissues of the oral cavity [29-31]. Acra MA et al. [29] and García-Contreras R et al. [31] tested the effects of SDF on normal oral cells (gingival fibroblast (HGF), pulp cells (HPC), periodontal ligament fibroblast (HPLF)) for 24 h and 48 h and found SDF to be cytotoxic on the cells. Likewise, SDF incubated in hydroxyapatite discs was shown to be cytotoxic to human gingival fibroblasts at 0.01 % and it further retained its cytotoxic effects even after 9 weeks of rinsing with artificial saliva [30]. Similar to our study, only one study was performed on blood cells and showed that 38 % SDF on human erythrocytes had hemolytic influence in a great number of cases when it was performed at full strength and possessed a high level of toxicity at a 50 % dilution which confirmed the previous observations regarding toxicity in studies on different oral cells [32]. To the best of our knowledge, the current study was the first to investigate the cytotoxic effect of SDF solutions on human lymphocytes. Unlike previous studies, our study was carried out in three different incubation times (24 h, 48 h and 72 h). The present study found out that cell viability was not observed at 3.8 % and 38 % concentrations at all incubation times. At 0.38 % SDF concentration, the highest cell death (33.91 %) was realized to take place during 72 h exposure compared to 24 h or 48 h. The results, in this regard, are of great significance since a better understanding of the immune system response to these agents is important for the future advances in dentistry.

The findings of the current study revealed that KI did not cause any cytotoxic effect at 4 % and 58.3 % concentrations for 24 h exposure; on the contrary, cell proliferation was observed to occur. 2% IKI solution containing 4% concentration of KI was used as an

endodontic irrigant due to its high antimicrobial properties. Previous studies show that IKI possesses low cytotoxicity and is well tolerated by human gingival fibroblasts [23,33]. From this point of view, the cell proliferation observed in our study confirms the results of studies claiming that the IKI solution provides healing in tissues. According to the results of this study, KI caused moderate cytotoxicity in lymphocyte cells at longer exposure times compared to increasing concentration, in contrast to cell proliferation after 24 h of exposure. These results of the study are important in determining the protocol for the use of this solution in root canal therapy and other clinical procedures.

Taking the results and limitation of the present study into consideration, the biocompatibility of the solutions should be further investigated in vivo studies so as to confirm the conclusions of the current study.

Conclusion

The findings presented suggest that SDF have cytotoxicity effect on human peripheral blood lymphocytes dependent on increase in concentrations. This is important because cytotoxicity is a factor that adversely affects biocompatibility, thus attention should be paid to the risk/benefit factor during the clinical application of the compounds analyzed.

Furthermore, KI did not cause any cytotoxic effect on both concentrations. As a result of 24 h exposure; in contrast, cell proliferation was observed to occur. As a result of conducting the present research, we propose that it should be used carefully at longer exposures on cells due to the fact that increasing concentrations and exposure times might result in moderate cytotoxicity. It would be fruitful to pursue further research about cytotoxicity of SDF and KI solutions by considering the findings obtained through the study.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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Materials		Cell Viability (%)	Cell Death (%)
Cell Control		100.00	-
SDF % 0,38	24 h	83.70	16.30

	48 h	82.96	17.04
	72 h	66.09	33.91
SDF % 3,8	24 h	0.00	100.00
	48 h	0.00	100.00
	72 h	0.00	100.00
SDF % 38	24 h	0.00	100.00
	48 h	0.00	100.00
	72 h	0.00	100.00
KI % 4	24 h	167.20	-
	48 h	80.32	19.68
	72 h	86.29	13.71
KI % 58,3	24 h	182.99	-
	48 h	61.67	38.33
	72 h	64.93	35.07

Table I. Cell viability and cell death percentages (%)

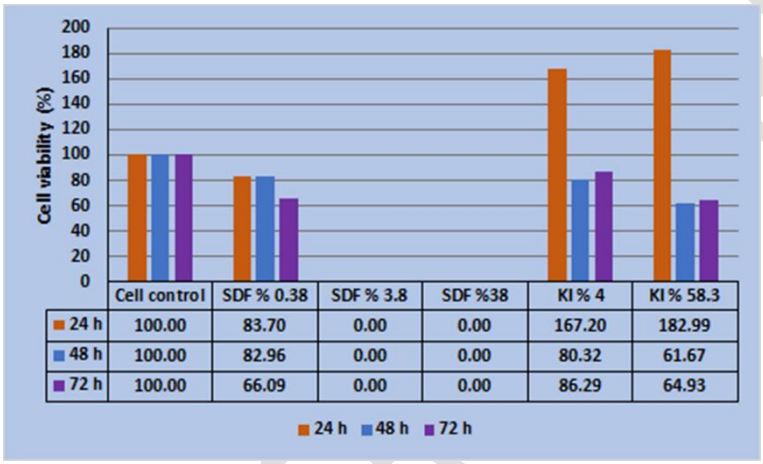

Figure I. Cell viability (%) of SDF and KI at different concentrations.