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Niosomal Doxycycline and Triamcinolone: A Novel Approach to Minimize Cytotoxicity in Endodontic Medicaments

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Introduction: Mechanical root canal preparations and irrigation solutions are essential for reducing microbial counts in the root canal system. However, these methods do not completely eliminate microorganisms. Intracanal medicaments are used to further decrease microbial counts. This study aims to assess the cytotoxicity of various intracanal medicaments. **Materials and methods:** In this in vitro study, murine fibroblast cell lines (L929) were cultured in a controlled environment. The MTT assay was employed to evaluate the cytotoxicity of different medicament combinations, including calcium hydroxide and triamcinolone (D1), niosomal doxycycline and triamcinolone (D2), calcium hydroxide (D3), and a combination of doxycycline and triamcinolone (D4). Statistical analysis was performed using ANOVA and Dunnett's test. **Results:** The results indicated that D1 and D2 had lower cytotoxicity, while D4 exhibited the highest cytotoxicity. D1 was found to be non-cytotoxic up to a concentration of 500 µg/mL over a period of 72 hours. D2 and D3 showed similar effects up to concentrations of 250 µg/mL and 100 µg/mL, respectively, for 72 hours. In contrast, D4 exhibited cytotoxicity at concentrations above 75 µg/mL at 72 hours. **Conclusion:** This study suggests that encapsulating doxycycline in niosomal structures (D2) reduces cytotoxicity in murine fibroblast cell lines (L929) for at least 24 and 48 hours. These findings offer promising implications for the development of endodontic medicaments with improved biocompatibility.

Keywords: Calcium Hydroxide; Corticosteroid; Cytotoxicity; Niosomal Doxycycline

Introduction

One of the important aims of root canal treatment is to eliminate microorganisms from the root canal system. Most root canal preparation and debridement techniques do not lead to the complete elimination of tissues and microorganisms from the root canal system, leaving untouched areas in the root canals. In addition, most root canal irrigation methods cannot eliminate 100% of microorganisms from the root canal system [1]. In addition, Endodontic treatments are mostly performed in one appointment, and when more appointments are needed, such as in acute apical abscesses, cases with suppuration from the root canal, emergency treatments, and treatments with a time limitation, intracanal medicaments are used between appointments [2]. If no intracanal medicaments are used between treatment sessions and the root canal is left empty, the microbial counts will return even to the level before treatment [3].

Calcium hydroxide has been the first choice among all the intracanal medicaments until now [4-7]. Calcium hydroxide paste creates an alkaline environment due to its high pH, in which most microorganisms cannot survive [4]. This antimicrobial property of calcium hydroxide is achieved by mixing it with water, leading to the release of hydroxyl ions into the environment, which penetrate the dentinal tubules. Notably, this function is time-

dependent for the penetration into the dentinal tubules and the persistence of the proper pH of the environment [8, 9]. Calcium hydroxide requires a long time for its antimicrobial effect and does not inhibit all the different types of microorganisms [10]. In addition, the complete removal of calcium hydroxide from the root canal system is difficult [11, 12].

Corticosteroids are another group of medications that effectively decrease inflammation [13], and their local use in the root canal decreases postoperative pain [13-15]. Corticosteroids prevent inflammation by inhibiting the synthesis of icosanoids [16]. The effect of systemic prescription of corticosteroids usually appears several hours or even several days after their use, depending on the corticosteroid type, because their function requires time to change the expression of genes and synthesis of proteins [14]. The most common commercial product containing antibiotics and corticosteroids is Ledermix, an intracanal medicament. This medicament with a corticosteroid base was introduced by Schroeder and Triadan [17, 18]. The Ledermix paste is a combination of triamcinolone and demeclocycline, with 1 and 3.21% concentrations of triamcinolone and demeclocycline, respectively. This paste reaches a maximum concentration through radicular dentin in two hours. Then, the release rate of demeclocycline decreases, reacting 0.1-fold of the original rate one week after treatment [19, 20]. The application of this medicament has been recommended from two weeks to two months. It is still unclear whether routine root canal irrigation procedures can completely remove this medication from the root canal system [21, 22].

Nanoparticle technology has shown great potential in endodontics, and several studies have investigated its implications in this field [23-27]. Nanoparticles from natural and synthetic materials have been used in various applications in endodontics, including incorporation in sealers, obturating materials, irrigation, and intracanal medicament [28]. The various nanoparticles that have been studied include graphene, silver nanoparticles, chitosan, hydroxyapatite nanoparticles, iron compound, zirconia, poly (lactic) co-glycolic acid, bioactive glass, mesoporous calcium silicate, titanium dioxide nanoparticles, magnesium, calcium oxide, and copper oxide [28]. These nanoparticles have shown promising results in improving antimicrobial efficacy, mechanical integrity, and drug elutions in endodontic treatment [29]. The cytotoxicity of nanoparticles can vary depending on the concentration and duration of exposure.

The mixture of doxycycline and triamcinolone has been investigated as an intracanal medication and is being used commercially. But Nano form of doxycycline and triamcinolone as intracanal medication have not yet been investigated and further research is needed to evaluate the cytotoxicity and potential benefits of it as intracanal medicaments. The present study was aimed to evaluate the cytotoxicity of mixture of niosomal doxycycline and triamcinolone, mixture of doxycycline and triamcinolone, mixture of calcium hydroxide and triamcinolone, calcium hydroxide.

Materials and Methods

Murine fibroblasts (L929) cells commonly have been used to evaluate the cytotoxicity of endodontic materials [30, 31]. A cell line derived from L929 was obturated from the cell bank of the Iran Pasteur Institute. These cells are in the form of a suspension and a multilayer mass. The cells were cultured in the 1640RPMI medium enriched with 10% bovine fetal serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin at 37°C and incubated at a relative humidity of 85–90% in the presence of 5% CO_2. First, the culture suspension was centrifuged, and after eliminating the supernatant, the cellular precipitate was dissolved in 1 mL of the culture medium. Then, its 1:10 dilution was prepared; 10 µL of this liquid was mixed with 90 µL of the culture medium, and 10 µL of this solution was placed under a microscope using a Neobar plate to determine the cell count. The cell count on the Neobar plate was multiplied by 100,000 to obtain the cell count in 1 mL of the solution volume. The cytotoxicity was determined using the MTT assay (3-{4,5-dimethylthiazol-2-yl}-2,5- diphenyl tetrazolium bromide colorimetric assay, *aka* Mosmann's Tetrazolium Toxicity assay).

To evaluate cell viability percentage using the viability test, one drop of the cellular suspension (1×10^6 cells/mL) was placed on a conventional microscope plate with an equal volume of trypan blue, and at least 100 cells and at most 200 cells were counted. In non-viable cells, the dye penetrated the cells in 3 min, staining the cell, especially its nucleus, blue. However, the dye did not penetrate the viable cells. The percentage of viable cells was calculated using the following formula:

$$\text{Vitability \%} = \frac{\textit{the number of viable cells (in 100 cells)}}{\textit{the number of viable cells} + \textit{non} - \textit{viable cells (a total of 100 cells)}} \times 100$$

A triplicate MTT assay was used to determine cytotoxicity. First, a cellular suspension was prepared; 200 µL of this cellular suspension was added to each well of a 96-well plate. Trypan blue test and cell counting were carried out to ensure 5×10^3 cells in each well. The plate was incubated for 24 h at 5% CO_2 under 100% relative humidity to ensure the cells' attachment to the bed, and the medication was added at the intended dilutions.

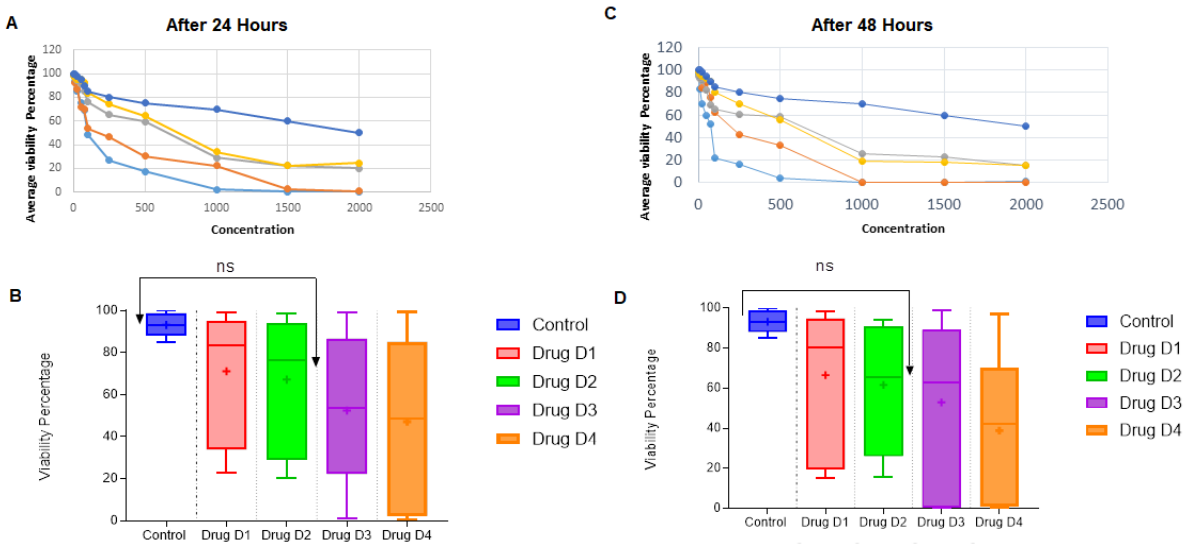

Figure 1. *A, C)* The cytotoxicity of different concentrations; *B, D)* Comparison of medications at maximum nontoxic concentration with the control group

The plates were incubated for 24, 48, and 72 h and one week under CO_2. After 24 h, the supernatant was removed by rotating the first plate, and 200 µL of the new culture medium was added to each well. In addition, 20 µL of MTT stain was added to each well, and the plate was wrapped in aluminum foil and incubated for 4 h. Then the cell culture media were evaluated by turning the plates, and 100 µL of DMSO and 10 µL of glycine buffer were added to each well. On the other hand, the cells were properly mixed with a medium sampler to remove any possible precipitating granules. Finally, an ELISA reader was used to determine light absorption in each well at a wavelength of 570 nm. Overall, observing L929 cells up to 7 days after exposure to endodontic materials allows for a sufficient period of time to evaluate potential cytotoxic effects and determine the effect of the material on the cells [30, 31]. All the procedures were repeated for other plates after 48 and 72 h and one week, and the results were recorded to compare cellular growth quantitatively. All the procedures were carried out three times. After determining the concentration with the lowest cytotoxicity for each mixture, they were compared with the control group with a sample size of 10.

The samples were randomly assigned to 5 groups:

- **Group D1:** calcium hydroxide
- **Group D2:** the paste consisting of a mixture of the niosomal form of doxycycline and triamcinolone
- **Group D3:** the paste consisting of a mixture of calcium hydroxide and triamcinolone
- **Group D4:** the paste consisting of a mixture of doxycycline and triamcinolone
- **Group D5 (control):** distilled water

All the samples were incubated twice for seven days at 37°C.

After determining the cell viability percentages at each dilution of medications, ANOVA was used to compare the groups with the control group, followed by the post hoc Dunnett's test. All the data was analyzed by SPSS software (SPSS version 21.0; IBM, Chicago, USA).

Results

Effect of different concentrations of medications on the cells in 24 h

Figure 1A shows the effects of different concentrations of medications 1 to 4 (D1 to D4) in the first 24 h. D1 had protective effects up to a maximum concentration of 500 µg/mL; D2 and D3 had protective effects up to a maximum concentration of 250 mg/mL; and D4 had protective effects up to a maximum concentration of 75 µg/mL. The medications were cytotoxic at concentrations higher than those mentioned above. According to the multiple comparisons made using Dunnett's tests, the survival rates of D1 and D2 were not significantly different from the control group in the first 24 h (P=0.15 and P=0.27, respectively); however, D3 and D4 were significantly different from the control group (P=0.003 and P=0.001, respectively) and caused increased cytotoxicity (Figure 1B).

Effects of different concentrations of the medications on the cells after 48 hours

In the first 48 h, D1, D2, D3, and D4 had protective effects up to maximum concentrations of 500, 250, 100, and 75 µg/mL, with cytotoxicity at concentrations higher than those mentioned above (Figure 1C).

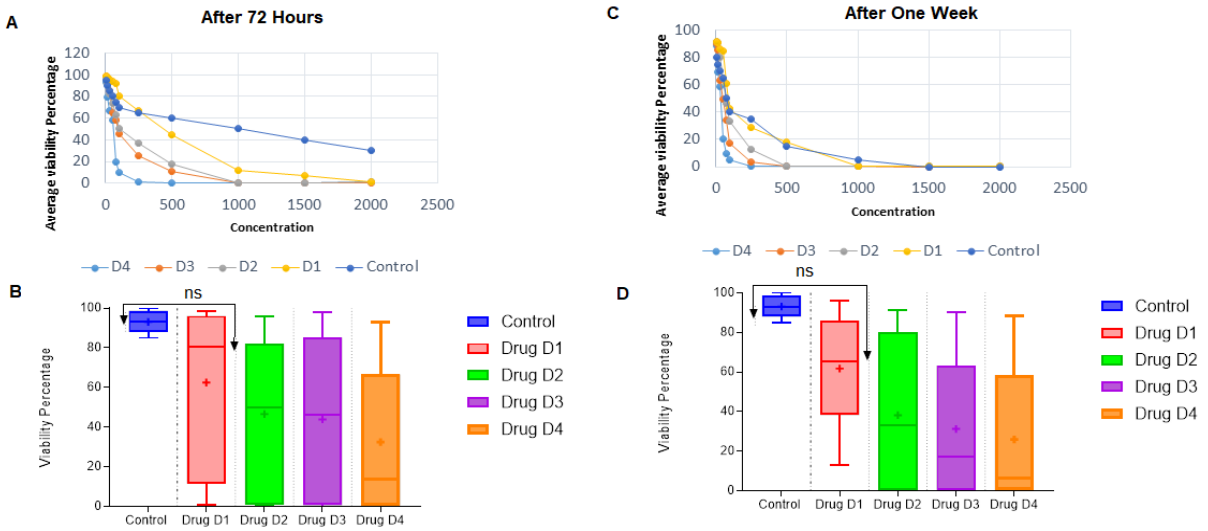

Figure 2. *A, C*) The cytotoxicity of different concentrations; *B, D*) Comparison of medications at maximum nontoxic concentration with the control group

According to Dunnett test results, in the first 48 h, D1 and D2 were not significantly different from the control group (P=0.16 and P=0.07, respectively); however, D3 and D4 were significantly different from the control group (P=0.01 and P=0.001, respectively) and exhibited increased cytotoxicity (Figure 1D).

Effects of different concentrations of the medications on the cell after 48 h

According to Figure 2A, after 72 h, D1, D2, D3, and D4 had protective effects up to maximum concentrations of 250, 75, 75, and 50 µg/mL, with cytotoxicity at concentrations higher than those mentioned above.

According to Figure 2B and Dunnett's test results, D1 was not significantly different from the control group (P=0.13); however, D2 and D3 were in the mean range and had similar effects after 72 h, significantly different from the control group (P=0.009 and P=0.005). In addition, the cell viability under the effect of D4 was significantly different from the control group (P=0.001), with increased cytotoxicity at a concentration of >50 µg/mL after 72 h.

Effects of different concentrations of the medications on the cells after one week

According to Figure 2C, D1, D3, and D4 had protective effects up to maximum concentrations of 75, 50, 25, and 25 µg/mL, respectively, with cytotoxicity at concentrations higher than those mentioned above.

According to Figure 2D, all the mediations exhibited significant changes. D1 was different at all the intervals from the control group; however, the mean difference was less than that of the other medications. The results of Dunnett's test were not significant (P=0.06). Therefore, at a 95% confidence interval, D1 was still active after a week and had no cytotoxicity. On the other hand, D2, D3, and D4 exhibited effects higher than the mean and resulted in cell death at 50, 25, and 25 µg/mL after a week (P=0.0004, P<0.0001, and P<0.0001, respectively).

Discussion

According to the present study, confining doxycycline within the niosomal structure (formulation D2) decreased cytotoxicity to the murine fibroblast cell line (L929) for at least 24 and 48 h, which might be attributed to the slow release of doxycycline after confirming it within the lipid layers of niosomes.

Mechanical preparation and debridement, followed by irrigation with root canal irrigation solutions, significantly decrease microbial counts in the root canal system without completely removing them [10, 32]. Therefore, intracanal medicaments should be used to further decrease microbial counts and inflammation [33]. Intracanal medicaments have good biocompatibility properties and are used for various purposes, including decreasing intracanal microbial counts, preventing their re-entry and growth into the coronal area, and destroying microorganisms in inaccessible areas and root canal irregularities that are not affected during mechanical preparation [5, 11, 34].

In recent years, different agents, including calcium hydroxide, corticosteroids, and antibiotics, have been used as intracanal medicaments that have undergone many changes in their chemical structure and composition [4, 11, 35-37]. Biologically, one of the most important factors for applying a material clinically is its cytotoxicity [38].

Different methods are available for evaluating cytotoxicity, including Neutral Red Uptake(NRU), antibody (AB) test, and the enzyme-linked immunosorbent assay (ELISA) [18, 19]. The ELISA technique is very important due to its high sensitivity and specificity, rapid and easy nature for a large number of samples, no neutralizing risk, and isotope stability [39]. Fibroblasts are the main cells of the connective tissue and can produce and support the connective tissue matrix. During the inflammatory stage, fibroblasts absorb toxic inflammatory products and bacterial products. Fibroblast cell cultures are used to determine the cytotoxicity of dental materials. These cells are procured easily and cultured with no difference from the basic cells [20].

The cytotoxicity of materials is first tested using *in vitro* tests, followed by *in vivo* evaluations. *In vivo* tests have some limitations and principally evaluate acute reactions after applying these materials in the oral cavity or their long-term effects. One of the most common methods to evaluate cytotoxicity is the MTT assay [21].

This method is used to measure proliferative lymphokines, mitogens, complement-mediated lysis, and cell growth factors and evaluate the cytotoxicity of different materials and medications.

MTT is the tetrazolium salt dissolved in water. When prepared in a saline environment without phenol red, this compound produces a yellow-colored solution. This material is converted to an insoluble compound called formazan by the dehydrogenase enzyme in the mitochondria of living cells, which is dissolved by solvents such as acidic isopropanol or DMSO, creating a violet-purple color. The absorption of this color is measured at a wavelength of 370 nm using an ELISA plate reader (MR 600 Microplate Reader; DYNA Tech Laboratories, Alexandria, VA, USA) [32]. A decrease in formazan concentration and determining the turbidity help evaluate the cell viability and cytotoxicity of the materials used.

New techniques are available to evaluate drug delivery in dentistry. One of these methods is introducing low concentrations of medications into injectable methylcellulose hydrogel in association with molecular weight control [12, 13]. In this line, McIntyre *et al.* evaluated the cytotoxicity and antimicrobial properties of methylcellulose hydrogels loaded with low concentrations of a double antibiotic paste using 1-, 5-, and 10-mg/mL concentrations of the double antibiotic paste [14]. The results of the present study using the MTT assay showed that 5- and 10- mg/mL concentrations of the hydrogel had toxic effects. Applying nanotechnology in dental treatments has attracted considerable attention in recent years, and one of its applications is drug delivery [15]. Neshaat *et al.* [16] evaluated the cytotoxicity of nano triple antibiotic paste and the anti-inflammatory effects of nano-drugs after their application as intracanal medicaments, concluding that the cytotoxicity of nano triple antibiotic paste was lower than that of calcium hydroxide and higher than that of triple antibiotic paste. In addition, although nano triple antibiotic paste exhibited maximum apoptotic values compared to calcium hydroxide and triple antibiotic paste, the difference was not significant statistically.

Another method is to use calcium-containing zinc oxide crystals associated with glycol propylene or polyethylene glycol and propylene glycol. A study by de Soouza *et al.* [17] showed that medications containing calcium-containing zinc oxide nanocrystals exhibited lower biocompatibility and pH than calcium hydroxide. To carry out the method used by de Souza *et al.* [17] the industrialization of this method should be promoted.

Another method for the controlled release of medications is to use liposomal and niosomal mechanisms. These mechanisms release drugs and carry out controlled release operations in a controlled manner. Niosomes are a combination of non-ionic surfactant and cholesterol with a layered structure, which trap the medications between their layers and release them slowly, gradually, and continuously, increasing the medication's substantivity in the location. In addition, due to the surfactant in their structure, niosomes increase penetration; therefore, they have a dual role. Since this method has not been used concerning intracanal medications, it was used in the present study; in the D2 group, the niosomal form of doxycycline was used.

Athanassiadis *et al.* [9] evaluated the effects of calcium hydroxide on the corticosteroid component of Ledermix, reporting that incorporating calcium hydroxide into Ledermix destroys its triamcinolone component in 72 h. The findings of the above study do not coincide with the present study because, in the present study, calcium hydroxide was mixed with Ledermix, which itself contains triamcinolone corticosteroid and the antibiotic demeclocycline. However, in the present study, in group D1, calcium hydroxide was mixed only with triamcinolone. Since a combination of corticosteroids and antibiotics is theoretically more effective, some products that are a mixture of both have been marketed. Ledermix is a mixture of antibiotics and corticosteroids and is used as an intracanal medicament between appointments. This mixture is not available as a commercial product on the Iranian market. Therefore, its formulation and evaluation of its effects during clinical use to introduce an Iranian product will be useful. In the present study, the medications in group D4 (*i.e.*, a mixture of doxycycline and triamcinolone) were similar to the commercial form of Ledermix and were evaluated.

The results of the present study showed that different concentrations of D1 in the MTT assay exhibited a minimum lethal effect on cells at 500-, 500-, 250-, and 75-µg/mL doses at 24-, 48-, and 72-h and one-week intervals, respectively, after treatment with these medications. At these time intervals, the groups treated with

the 250-, 25-, 75-, and 50-µg/mL doses of D2, groups treated with 250-, 100-, 75-, and 25- µg/mL doses of D3, and the groups treated with 75-, 75-, 50-, and 25-µg/mL doses of D4, respectively, exhibited the least fatal effects on cells in the same period.

Muincharem *et al.* [7] evaluated the effects of a glucocorticoid, fluocinolone, on the pulp cells of a freshly extracted third molar tooth and used the MTT assay to evaluate cytotoxicity and cell proliferation. According to the results, fluocinolone has no cytotoxicity and increases the proliferation of pulpal cells. In addition, this mixture induces the production of fibronectin and type I collagen.

Zhang *et al.* [40] evaluated the effects of ropivacaine, bupivacaine, and triamcinolone on several cell lines, including fibroblasts, concluding that triamcinolone did not cause cellular necrosis and apoptosis. In the present study, triamcinolone from the glucocorticoid family was also evaluated. On the other, Zhan *et al.* [41] evaluated the biocompatibility of calcium hydroxide and composite resin on human fibroblasts and murine fibroblasts by the cell counting method, reporting that the inability of murine fibroblasts under the effect of calcium hydroxide was three folds lower. In addition, Gheorghiu *et al.* [42] reported the lowest toxicity under the effect of Ledermix, with the highest toxicity related to Calxyl (calcium hydroxide) and Cresophene.

In the present study, D1 and D2 medications exhibited less toxicity, and D4 was the most toxic. D4 (a mixture of doxycycline and triamcinolone) exhibited the highest cytotoxicity in the present study, which appears to be attributable to the antibiotic component [40]. Since D4 was similar to the commercial form of Ledermix, the difference in the results of the present study and those of the study by Gheorghiu *et al.* [42] might be attributed to different compositions and doses of the medications used.

One of the limitations of the present study was that although *in vitro* studies are necessary for the evaluation of dental materials, extending the results to clinical applications is a significant challenge since the laboratory environment and the confounding factors can be more properly controlled than the oral environment. In addition, considering the complexities of the root canal system, the inability to completely debride and eliminate microorganisms might affect the outcomes. Therefore, more clinical studies are required to further evaluate these intracanal medications.

Conclusion

In the present study, confining doxycycline in the niosomal structure resulted in decreased cytotoxicity to murine fibroblast cell line (L929) at least at 24- and 48-hour intervals, which might be attributed to the slow release of doxycycline after confining it in the lipid layers of niosomes.

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Conflict of interest

None.

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Authors' contributions

Conceptualization: A. Shahravan Data Curation: A. Shahravan, R. Fereidooni, H. Manochehrifar. A. Pardakhty, HR. Mollaie. Formal Analysis: A. Shahravan, AH. Nekouei. Methodology: A. Shahravan. A. Pardakhty, HR. Mollaei. Project Adminstration: A. Shahravan, H. Manochehrifar. Validation: A. Shahravan, H. Manochehrifar. Writing- Original Draft: All authors. Writing- Review & Editing: All authors.

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