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An Evaluation of Cytotoxicity of Iron Oxide Nanoparticles with Hydrogen Peroxide Endodontic Irrigant

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Abstract

Aims: The aim of this research was to determine the cytotoxic effect of different concentrations of iron oxide nanoparticles (IONPs) in combination with hydrogen peroxide (H_2O_2) in vitro as a potential endodontic irrigating solution**. Materials and Methods:** Cytotoxicity of IONPs at concentrations (8, 4, 2, 1, 0.5, and 0.25 mg/ml) with 3% H_2O_2 were evaluated on cultured Human Dermal Fibroblast (HDFn) cell line. Cytotoxicity was assessed after 10 minutes of exposure using the Mossman's Tetrazolium Toxicity (MTT) assay. **Results**: Tukey's test indicated that no significant difference in cell viability was found at lower concentrations (0.2, 0.5, 1, and 2 mg/ml). While at a concentration of 4 and 8 mg/ml, the cell viability was not significantly different from each other whereas, significantly different from previous concentrations. **Conclusion**: From the gained data, it can be deduced that IONPs+H_2O_2 had almost no cytotoxic effect on HDFn cell viability at concentrations 0.2, 0.5, 1, and 2 mg/ml, while a moderate decrease of survival has been observed at concentrations 4 and 8 mg/ml.

الخلاصة

الأهداف: تهدف الدراسة الى تحديد التأثير السام لتركيزات مختلفة من جزيئات أكسيد الحديد النانوية مع بيروكسيد الهيدروجين فى المختبر كمحلول محتمل لرى لب الأسنان. **مواد وطرائق البحث**: تم تقييم السمية الخلوية لجزيئات أكسيد الحديد النانوية بتركيزات (8 ، 4 ، 2 ، 1 ، 0.5 ، و 0.25 مجم / مل) مع 3 ٪ بيروكسيد الهيدروجين على خط الخلايا الليفية الجلدية البشرية المستزرعة، تم تقييم السمية الخلوية بعد 10 دقائق من التعرض (MTT assay) **النتائج**: اشارت النتائج الى عدم وجود فرق كبير فى حيوية الخلية بتركيزات أقل (0.2 ، 0.5 ، 1 ، 2 مجم / مل)، بينما تراكيز 4 و 8 مجم / مل ، لم تكن حيوية الخلية مختلفة بشكل كبير عن بعضها البعض بينما كانت مختلفة بشكل كبير عن التركيزات السابقة. **الاستنتاجات**: من البيانات المكتسبة ، يمكن استنتاج أن جزيئات أكسيد الحديد النانوية مع بيروكسيد الهيدروجين لم يكن لها تقريبًا أى تأثير سام على خط الخلايا الليفية الجلدية البشرية المستزرعة بتركيزات 0.2 و 0.5 و 1 و 2 مجم / مل ، بينما لوحظ انخفاض معتدل فى البقاء على قيد الحياة بتركيزات 4 و 8 مجم / مل.

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INTRODUCTION

The use of biocompatible dental material is an important factor in endodontics. Biocompatibility is defined by Williams (2008) as "the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effect in the recipient, but generating the most appropriate beneficial cellular or tissue response in that specific situation, and optimizing the clinically relevant performance of that therapy"[1]. Biocompatibility is a collective term that includes mutagenicity, genotoxicity, carcinogenicity, histocompatibility, cytotoxicity, and microbial effect[2]. One of the requirements of an ideal intracanal irrigating solution is its ability to produce an antimicrobial effect while remaining biologically compatible with the periapical tissue and not provoke irritation[3].

Several chemical substances have been proposed and used as irrigants during the chemomechanical preparation of a root canal[4]. Sodium hypochlorite in concentration 0.5-5.25%[5], 2%. Chlorhexidine digluconate[6], Ethylenediaminetetraacetic Acid[7] and hydrogen peroxide[8]. Although sodium hypochlorite is a common irrigant due to its potent antibacterial and organic debris dissolving actions[9], its shortcomings include the unpleasant taste, toxicity, incapability to remove the smear layer completely, and the possible deterioration of the flexural strength of dentin[10].

A wide range of nanoparticles with antimicrobial activity has been developed including inorganic (especially silver) and chitosan-based nanoparticles. Whereas these nanoparticles are potentially useful endodontic disinfection technologies, the prolonged contact time needed to successfully kill bacteria and the toxicity problems of silver nanoparticles present significant drawbacks[11,12].

Iron oxide nanoparticles (IONPs) are a unique type of metal nanoparticles with magnetic properties and good biocompatibility[13]. IONPs show an unusual biomimetic behavior by exhibiting enzyme-like activity and were thus called nanocatalyst[14]. In 2007, Gao *et al* showed that IONPs have an intrinsic peroxidase-like activity that enables them to catalyze H_2O_2 breakdown and rapid production of bioactive free radicals[15]. H_2O_2 is a widely used disinfectant which exhibits antimicrobial effect by free radical generation, but the cycle is slow when used alone with minimal antibiofilm effects[16].

IONPs+H_2O_2 showed promising antimicrobial activity against *Enterococcus. Faecalis*[8], However, with the increasing concern about the cytotoxic effect of using the nanoparticles in human tissue, cytotoxicity testing is essential to validate the use of IONPs+H_2O_2 as an intracanal irrigating solution. The aim of this study was to evaluate the cytotoxic effect of different concentrations of IONPs+ H_2O_2 in *vitro* as a potential endodontic irrigating solution.

MATERIALS AND METHODS

Preparation of Nanoparticle Suspension

IONPs (purity more than 99.7%) with a size 25-50 nm were purchased from US NANO (US Research Nanomaterials Inc, Houston, Texas, USA). To prepare a stock solution with 8 mg/ml IONPs, 160 mg of the nanoparticles were turned into the suspension in 20 ml of deionized water.

For a proper dispensing of the particles, the vortex system was used for 15 minutes.

Cell Culture Procedure

Cytotoxicity of the suspension has been evaluated on cultured human dermal fibroblast cell line (HDFn). Fibroblast cells were obtained from the Centre for Natural Products Research and Drug Discovery, University of Malaya.

HDFn has been cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin. The culture was incubated at 37ºC in a humidified atmosphere, 95% air, and 5% Co_2. The medium has been changed every other day. When the cells reached confluence, they were removed using 0.2% (w/v) trypsin and transferred to new culture flasks[17].

After sufficient growth for experimentation, the cells were trypsinized and plated in 96-cluster well culture plates at a concentration of 1×10^4 cells/well. Each well had 100µl of cell suspension. After 24 hours of incubation at 37ºC under 5% CO_2, the cells formed a confluent monolayer on the base plate of the culture well. The cells' adherence was checked by examining under a phase-contrast microscope. Only those wells containing a cell layer that was evenly spread across the base of the well were included in the study.

Stock IONPs suspension was serially diluted with concentrated DMEM culture medium (10% FBS and 1% penicillin/streptomycin supplements) into 6 dilutions (8, 4, 2, 1, 0.5, 0.25 mg/ml) and were used in combination with 3% H_2O_2.

Cell cultures were divided into six groups with eight wells per group (n=8). For each group, a fixed volume of tested irrigant (200µL) was added into each culture well. One group of eight wells with normal culture medium served as control**.**

Mosmann's Tetrazolium Toxicity (MTT) assay

After 10 minutes of treating HDFn cell with the tested solution, the plate was washed to remove excess solution and fresh medium was added to culture the cells for another 24 hours. The percentage of viable cells in each well was estimated by MTT assay[18]. A solution of 0.1 ml MTT was applied to the 96-well plate and incubated for 4 hours. The medium was then removed and 200µL of formazan dimethyl sulfoxide (DMSO) was added. In a spectrophotometer (EMC LAB, Germany), the optical density of DMSO was measured at 590 nm. Percentage of cell viability was calculated as the ratio of the average absorbance of triplicate measurements to the mean absorbance of control cells without nanotherapy [19]:

Cell viability = $(I_{sample}/I_{control}) \times 100$.

Statistical analysis:

The statistical analysis was carried out using Social Sciences Statistics System version 25 (SPSS). The normality of data was tested by Kolmogorov-Smirnov test and Shapiro-Wilk test. One-Way ANOVA test was used to determine if there are significant differences between groups. After it, a post hoc Tukey's test was applied to

determine the differences between them. A p-value of ≤ 0.05 was considered statistically significant.

RESULTS

Descriptive statistics, including mean, standard deviation, minimum, and maximum values for the concentration of IONPs+H_2O_2 used in MTT assay are listed in the Table (1). The obtained mean indicated that the toxicity of different concentrations of nanoparticles on HDFn cell lines follows a concentration dependent manner.

Table (1): Mean, standard deviation, minimum, and maximum values of the different concentrations of IONPs+H_2O_2

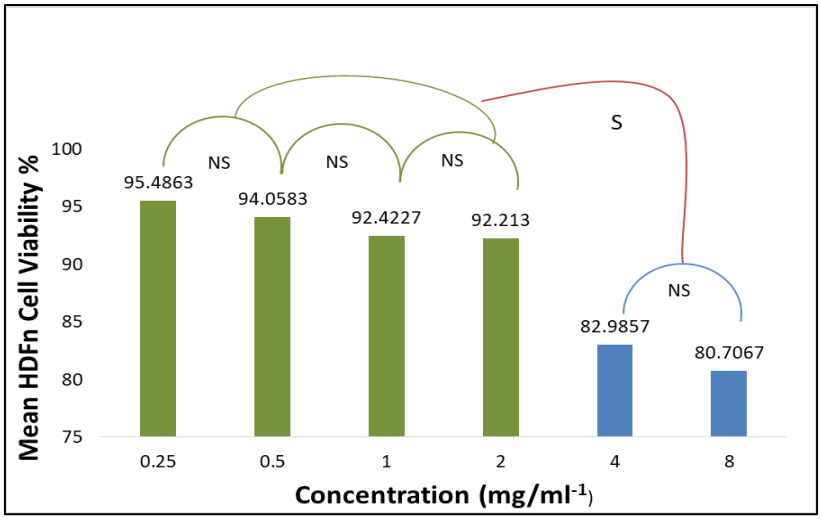
Concentration (mg/ml)	Mean	SD	Minimum	Maximum
0.25	95.4863	1.44564	93.87	96.64
0.5	94.0583	1.20472	92.71	95.02
1	92.4227	1.01112	91.51	93.51
2	92.2130	.84775	91.26	92.88
4	82.9857	1.42222	81.94	84.61
8	80.7067	1.81698	79.45	82.79

The result of One-Way ANOVA for MTT cytotoxicity test (Table 2) showed there is a significant difference in cell viability with different concentrations (P< 0.05).

Table (2): Analysis of One-Way ANOVA for the six conc. (8, 4, 2, 1, 0.5, 0.25 mg/ml) used in the study in combination with H_2O_2 with analysis of variance in between groups and within groups.

Sum of squares		Df	Mean square	F	p-value
Between Groups	576.448	5	115.290	65.219	.000
Within Groups	21.213	12	1.768		
Total	597.661	17			

Post hoc Tukey's test indicated that there is no significant difference in cell viability at lower concentrations (0.2, 0.5, 1, and 2 mg/ml). On the other hand, at concentration of 4 and 8 mg/ml, the cell viability of IONPs+H_2O_2 was not significantly different from each other, whereas they significantly different from previous lower concentrations. The result is shown in Figure (1).



NS: non significant
S: significant

Figure (1): Histogram for comparison among different concentration of IONPs+H_2O_2

DISCUSSION

The toxicity of materials used in endodontic therapy is a special concern because damage or irritation could cause degeneration of the periapical tissue and delayed wound healing. The ideal endodontic irrigating solution should be selectively toxic and act as an antimicrobial agent but with low periradicular tissue toxicity [20].

In our study, MTT, an enzyme-based procedure was used to assess the cell viability with a colorimetric method. The MTT assay is a sensitive and accurate predictor of cellular metabolic act ivity and is favored over the other methods measuring this end-point such as the ATP[21], and H-thymidine incorporation assay due to its ease in usage, safety, and high reproducibility[22].

This assay is based on the reduction of MTT, a yellow water-soluble tetrazolium dye, mainly by the mitochondrial dehydrogenases, to purple formazan crystals. The formazan product has been analyzed spectrophotometrically (590 nm) after dissolution in DMSO, the spectra of nanoparticle-treated and untreated cells providing an estimation of the degree of cytotoxi city[23].

The concentration of nanoparticles has been considered a crucial factor in cell cytotoxicity[24]. As a result , cells exposed to lower concentrations of nanoparticles can clear their load of particles via the usual phagocytosis process, but at higher concentrations, the clearing process is disrupted and gradually health effects appear[25].

Our results are in a line with Amin *et al*,[26] who researched the toxicity of IONPs on human skin Fibroblasts cell lines in sizes ranging from approximately 54 nm and concentrations ranging from 10 ug/ml to 500 ug/ml for up to 24 and 48 hours. Standard Human fibroblasts display either no decrease or minimal decrease in cell survival after 24 and 48 hours of IONP exposure. Also, Our results agrees with previous

studies which show that the viability of cells was unaffected by any of the low concentrations tested, which can be due to the role of iron as key component of the body-to-cell life cycle[27], suggesting the biocompatibility of these nanoparticles[28,29]. The findings of numerous experiments have demonstrated the essential role of iron oxide in the regular functioning of cellular organelles, but at higher concentrations (4 and 8 mg/ml), this mechanism could be reversed and serve as an initiator of the Fenton reaction. Exposure to higher concentrations of nanoparticles resulted in increased generation of reactive oxygen species through H_2O_2 oxidation, causing some abnormalities in cell life and growth[30,31].

CONCLUSIONS

From the gained data, it can be deduced that IONPs+H_2O_2 had almost no cytotoxic effect on HDFn cell viability at concentrations 0.2, 0.5, 1, and 2 mg/ml, while a moderate decrease of survival has been observed at concentrations 4 and 8 mg/ml. However, according to Mahmoudi *et al*.,(2009)[32] IONPs reducing cell viability for less than 20% can be considered as being biocompatible.

Conflict of Interest

Authors declare no conflict of interest.

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