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Cytotoxicity assessment of zirconia-reinforced experimental nanohybrid dental composite using MTT assay

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ABSTRACT

The physical properties of experimental nanohybrid dental composite (NHDC) have been successfully enhanced by the use of nano-sized zirconia as a reinforcement filler. The study aims to assess the cytotoxicity of this newly developed zirconia-reinforced experimental NHDC on the L929 mouse fibroblasts cell line using MTT assay. The zirconia-reinforced (0, 3, 5 and 10 wt.%) experimental NHDCs were fabricated into disc-shaped and ground to powder form. The powder was diluted in complete Dulbecco's Modified Eagle Medium and serially diluted five times. Then the dilution assays were incubated for 24, 48 and 72 hours with the 80% confluence fibroblasts cells in 96-well cell culture plates, followed by MTT assay. The experiment was carried out in six replicates and repeated three times. The cytotoxicity effect of NHDC was assessed based on the IC50 value. The nonparametric Kruskal-Wallis test was used for comparing more than two groups of variables. Significant level was set at $p = 0.05$. The zirconia-reinforced experimental NHDCs were found to be non-cytotoxic with the cell viability of all sample concentrations and incubation periods were above 50% when assessed on the L929 mouse fibroblasts cell line using the MTT assay.

Keywords
Zirconium, MTT formazan, Composite Resins, IC50, Cell Culture Techniques.

Introduction

The resin-based dental composite was introduced in the early 1960s and is now becoming a commonly used restorative material for carious teeth, replacing the once popular dental amalgam. The change was due to the restoration's aesthetic demands and the concern about the amalgam's mercury safety (Patki, 2013). Additionally, the adhesive properties of a resin-based dental composite make it a better restorative material. It offers good enamel and dentin bonding, as well as helps strengthen the tooth (Abdelaziz & Saleh, 2018; Arola *et al.*, 2001). Owing to its tooth-coloured properties, resin-based dental composites have been preferred for anterior and posterior restorations (Ferracane, 2013; Roulet, 1997).

In Malaysia, the use of resin-based dental composites relies on imported products, which are costly. The high cost of dental material would inevitably lead to a rise in dental care cost which would be burdensome for the patient, particularly for the lower-income community. In an attempt to reduce the treatment cost of resin-based dental composite restoration, a group of researchers from the Universiti Sains Malaysia have taken the initiative to develop locally produced nanohybrid dental composite (NHDC) using silica extracted from rice husk as a filler (Noushad *et al.*, 2016; Noushad *et al.*, 2012). Although this experimental NHDC used only nanohybrid silica as a filler with a resin-to-filler ratio of 50/50, the dental composite was considered a success

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with a flexural strength ranging from 82 to 107 Mpa (Noushad et al., 2016), which surpassed the minimum requirement for the posterior dental composite (International Organization for Standardization [ISO], 2009). However, the flexural strengths of the experimental NHDC were significantly inferior compared to the commercial dental composite used in the study, Filtek™ Z250 (3M ESPE, St. Paul, MN, USA).

We have studied the effects of zirconia reinforcement filler in improving the physical and mechanical properties of this experimental NHDC. Apparently, our zirconia-reinforced (3 and 5 percent) experimental NHDCs were found to significantly increase the dental composite's physical and mechanical properties as analyzed for Vickers hardness, flexural strength, and compressive strength (Ismail *et al.*, 2020). As part of a larger project, this study aims to evaluate the cytotoxicity effect of zirconia-reinforced experimental NHDC on the L929 mouse fibroblasts cell line using MTT assay, testing the hypothesis that the zirconia-reinforced experimental NHDC has no cytotoxic effect on the L929 mouse fibroblast cell line.

Methods

Sample preparation

The study was approved by the Human Research Ethics Committee of Universiti Sains Malaysia (USM/JEPeM/17020137). In this study, zirconia-reinforced experimental NHDC was fabricated using resin matrixes and fillers at a 50/50 ratio (Table 1). Silica extracted from the rice husk was used as a primary filler, and zirconia (3 – 10 wt.%) was used as a reinforcement filler. Four groups of NHDCs were fabricated in the study based on zirconia reinforcement amounts: Group 1: 0 wt.% zirconia reinforcement; Group 2: 3 wt.% zirconia reinforcement; Group 3: 5 wt.% zirconia reinforcement; and Group 4: 10 wt.% zirconia reinforcement.

Table 1. Zirconia-reinforced experimental NHDC composition.

Zirconia reinforcement amount	Filler (50 wt.%)		Resin Matrix (50 wt.%)	
	Silica (wt.%)	Zirconia (wt.%)	Bis-GMA (wt.%)	TEDGMA (wt.%)
Group 1: 0 wt.%	100	0	60	40
Group 2: 3 wt.%	97	3		
Group 3: 5 wt.%	95	5		
Group 4: 10 wt.%	90	10		

A total of 0.5 wt.% of camphorquinone and dimethylaminoethyl methacrylate, which were a photoinitiator and co-initiator, respectively, were also incorporate into the NHDC. NHDC =

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Nanohybrid Dental Composite; Bis-GMA = Bisphenol A-glycidyl Methacrylate; TEDGMA = Triethylene Glycol Dimethacrylate.

Disc-shaped dental composite samples with a diameter of 15 mm and a thickness of 1 mm were prepared using a stainless steel mould. Each sample was light-cured for 40 seconds using a light-curing unit, Bludent Led Smart (BG Light Ltd, Plovdiv, Bulgaria) with a standard mode and wavelength range of 800 - 1200 nm. The cured dental composite sample was then crushed and ground into a fine powder using pestle and mortar.

A dilution assay of 100mg/ml NHDCs and 0.46mg/ml Zinc Sulfate Heptahydrate (positive control) (Sigma-Aldrich, St. Louis, MO, UK) was then prepared. A 100mg and 3.68mg of NHDCs powder and Zinc Sulfate Heptahydrate powder, respectively, were diluted with 8ml of complete Dulbecco's Modified Eagle Medium (Thermo Fisher Scientific Inc, Waltham, MA USA) in an autoclaved bijou bottle.

Samples were then incubated in the shaker at 37°C for 72 hours before filtering using the 0.45µM pore membrane filter, MF-Millipore™ (Merck KGaA, Darmstadt, Germany). The dilution assays of NHDC and Zinc Sulfate Heptahydrate were serially diluted five times. The concentrations for NHDCs dilution were 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml, and the concentrations for Zinc Sulfate Heptahydrate dilution were 0.48, 0.24, 0.12, 0.06, 0.03 and 0.015 mg/ml. The dilutions were kept in the SI-300 incubator (GMI, Ramsey, MN, USA) with a continuous shake at 37°C until further use.

Cell culture procedures

The L929 mouse fibroblast cell line (ATCC, Manassas, VA, USA) was used in this study. Cells were cultured in a high glucose DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. All reagents were purchased from Thermo Fisher Scientific Inc (Waltham, MA USA). Cells were allowed to grow in the T-25 tissue culture flask (Greiner Bio-One, Maybachstrabe, Frickenhausen, Germany) in a humidified atmosphere with 5% CO2 at 37ºC. The growth media was replaced every two or three days until the cells reached a confluence of 80%. For subculture, the confluent cell was washed using 1 ml of Dulbecco's Phosphate Buffered Saline (Thermo Fisher Scientific Inc, Waltham, MA USA) and then trypsinized with 0.01% of Tryple™ Express trypsin (Thermo Fisher Scientific Inc, Waltham, MA USA) for cell detachment.

The cells in the 6^{th} to 9^{th} passages were used in this study. Trypan blue, 0.4 % (w / v) (Sigma-Aldrich, St. Louis, MO, USA) was used to stain and discriminate dead cells from viable cells, and the number of cells was determined using an inverted light microscope, Axiovert 40 C (Carl Zeiss, Göttingen, Germany). Briefly, 100 µl media containing 1 x 104 cells were seeded in a 96-well cell culture plate (Jet Bio-filtration Co Ltd, Guangzhou, China). The plate was incubated overnight in a humidified atmosphere with 5% CO_2 at 37ºC. After overnight incubation, cells were treated with sample-dilution assay prepared in complete DMEM at concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml. For comparison, the cells were treated with the Zinc Sulfate Heptahydrate dilution assay (positive control) at the concentration of 0.48, 0.24, 0.12, 0.06, 0.03

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and 0.015 mg/ml, and the complete DMEM was used as a negative control. The cells were then incubated for 24, 48 and 72 hours, and the MTT assay was carried out at the end of each incubation period. The experiment was performed in six replicates and was repeated three times.

MTT assay

After each incubation period, 10 µl of the 5mg/ml MTT (Sigma-Aldrich, St Louis, MO, USA) was added to each culture plate well and incubated for 2 hours at 37 ºC and 5% CO_2. The MTT dilution was then removed, and 100 ul of 100% DMSO was added to the adherent cells to dissolve the violet formazan crystals. Samples were agitated for 15 minutes, and the absorbance was read at 570 nm using a Sunrise^{TM} 96-well microplate reader (Tecan Group Ltd., Männedorf, Zurich, Switzerland). The relative cell viability (%) was calculated using the following formula: Relative cell viability (%) = [A]test / [A]control X 100; where [A]test is the average absorbances of treated cells and [A]control is the average absorbances of control cells.

Statistical Analysis

Descriptively, the cytotoxicity effect of experimental NHDCs on cell viability was assessed based on the IC50 value. Experimental NHDCs with cell viability greater than 50% are considered non-cytotoxic (ISO, 2009). Statistical analyses were carried out using SPSS (Version 24.0). The Shapiro-Wilk test was performed to evaluate data normality. Since the data were not normally distributed, the Kruskal-Wallis tests were used to compare more than two numerical variables, followed by a Bonferonni pairwise comparison test. The significance level was set at $p = 0.05$.

Results

Figure 1 shows the cytotoxicity effect of zirconia-reinforced (0, 3, 5 and 10 wt.%) experimental NHDC on the L929 mouse fibroblast cell line using the MTT assay. In all sample groups, for each incubation period of 24, 48 and 72 hours, the mean percentage of cell viability was greater than 50%, which was considered as non-cytotoxic. There was no statistically significant difference in cell viability between all concentrations of 0, 3, 5 and 10 wt.% of NHDC in the same group for all incubation periods.

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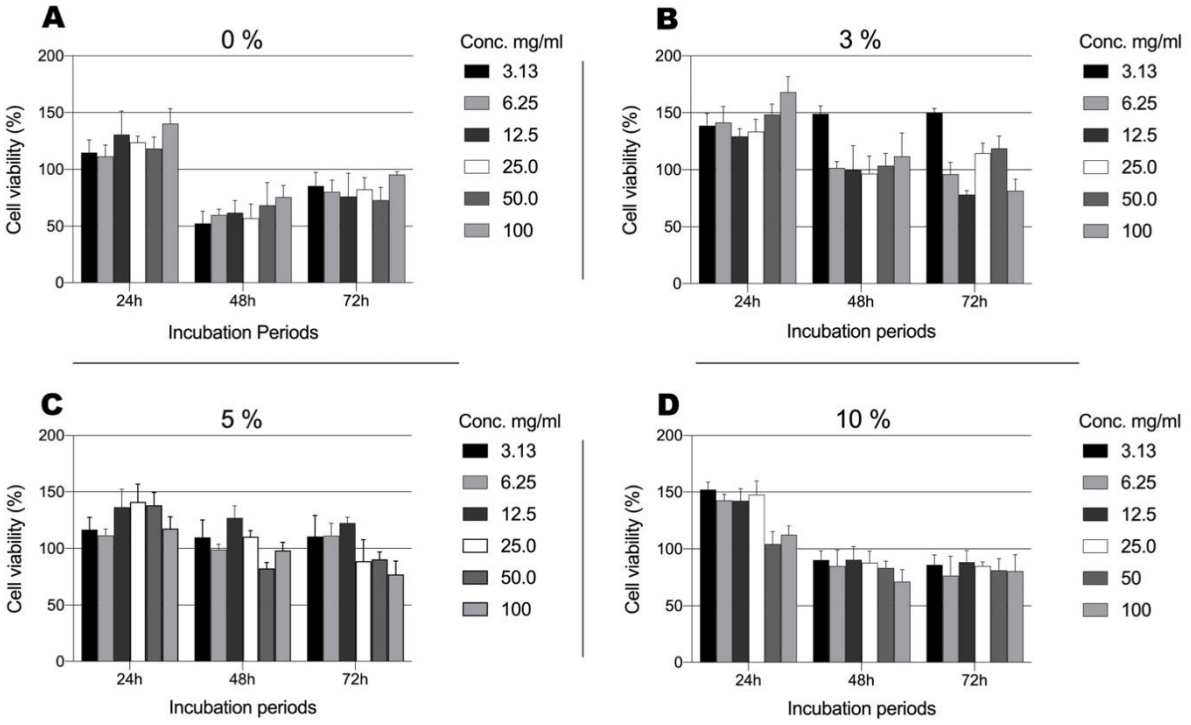

Figure 1. Effect of zirconia-reinforced (0, 3, 5 and 10 wt.%) experimental NHDC on the viability of the L929 mouse fibroblast cell line evaluated using MTT assay. Error bar indicates the standard error of the mean (SEM)

Discussions

In this study, we evaluated the cytotoxicity effect of the zirconia-reinforced experimental NHDC. The cytotoxicity evaluation is an essential requirement for a newly developed dental restorative material that has direct contact with the cells and tissues of the body (Kunzmann *et al.*, 2011; Reddy *et al.*, 2012). Dental restorative materials require not only excellent physical and mechanical properties but also biocompatibility (Reddy *et al.*, 2012). These materials have the potential to cause harm to oral tissue due to the possibility of a leachable toxic substance that could enter the circulatory system and cause systemic toxic reactions (Karaoğlanoğlu *et al.*, 2010).

Biocompatibility is generally described as a material that is in harmony with living tissues. In dentistry, biocompatibility tests have been conducted to evaluate a tissue reaction to a dental device or material that has direct or indirect contact with the patient's cells, tissues or body fluids. Several approaches are available to assess material biocompatibility, including cytotoxicity, sensitization, systemic toxicity, genotoxicity, implantation and haemocompatibility (ISO, 2018). Cytotoxicity testing is where the cell culture technique is used to assess cell death, cell growth inhibition, colony formation and other effects on cells induced by medical devices/ materials. At least nine colourimetric assays are available for the cytotoxicity test (Aslantürk, 2017). While

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they vary in nature, they have similar cellular processes and outcomes (Istifli *et al.*, 2019). Among them, MTT, lactate dehydrogenase (LDH), and neutral red uptake (NRU) assays are well established because they are better in sensitivity and reliability (Tobólska *et al.*, 2018). The MTT assay has been reported to be capable of detecting the most substantial cell count variety and sensitivity compared to the NSU assay (Van Tonder *et al.*, 2015). As for the LDH assay, the downside is its high cost (Kaja *et al.*, 2015). In the present study, the MTT rapid colourimetric assay was used due to its convenient nature. In addition, MTT is highly reproducible and low experimental cost (Mangis *et al.*, 2019; Mosmann, 1983), and the MTT assay has also been reported to provide a better overview of nanoparticle toxicity (Sahu *et al.*, 2016).

In this study, zirconia nanoparticles were incorporated into the experimental NHDC to improve the composite properties. Zirconia nanoparticles could be dislodged from a dental composite and may pose a potential threat to the surrounding tissue. Our study shows that zirconia-reinforced experimental NHDCs are non-cytotoxic when evaluated using an MTT assay. All groups, at all concentration and incubation period, show a mean percentage of viable cells above the half-maximal inhibitory concentration (IC50), where the majority of the group shows a percentage of viable cells above 70%. IC50 was used as a non-cytotoxicity indicator, where the concentration of zirconia-reinforced experimental NHDCs required to inhibit 50% of cell biological processes was estimated *in vitro* (ISO, 2009). This finding was expected as in vitro studies showed that zirconia alone was biocompatible and had minimal cytotoxicity. Shin *et al.* (2016) showed that zirconia, either alone or embedded in dental cement, had a limited cytotoxic effect when tested using an MTT assay. Zirconia is one of the biosphere trace metals distributed in soil, water, vegetation and animals. Human exposure to zirconia occurs through the consumption of water, vegetable and animal products, and daily intakes could be as high as 125 mg (Ghosh *et al.*, 1992). Zirconia has been identified to be biocompatible both in vitro and in vivo. Zirconia powder dilutions of 100, 50, 10 and 1% were shown to be non-cytotoxic when evaluated on murine 3T3 fibroblasts and human umbilical endothelial cells (HUVEC) using an MTT assay (Dion *et al.*, 1994). In vivo, cylindrical yttria-stabilised zirconia polycrystal (Y-TZP), a surface-treatment zirconia implant inserted into the maxillary teeth socket of rats, has been shown to enhance soft and hard tissue response when evaluated histologically (Iinuma *et al.*, 2020). However, an animal study showed that intraperitoneal injection of high-dose (110 ppm) zirconia oxide (ZrO_2) nanoparticles was capable of inducing cellular toxicity and also affect the normal function of the liver and kidney of the rat (Arefian *et al.*, 2015).

NHDC also uses silica as a filler, which is also known to be non-cytotoxic. A study reported that dental composites containing silanised mesoporous silica fillers offered good cytocompatibility to human gingival fibroblasts (HGFs) when evaluated using time-lapse confocal laser scanning microscopy (Attik *et al*., 2017). In addition, silica-based dental composite reinforced with glass fibre was also found to be non-cytotoxic when evaluated using an MTT assay with cell viability of more than 90% (Meric *et al.*, 2008). Furthermore, rats treated with intravenous injections of nano- and micro-particle silica did not show any immunogenic and toxicity issues based on the assessment of LDH levels in the spleen and liver, and the assessment of white blood cells infiltrating major organs (Jaganathan & Godin, 2012).

Our experimental NHDC contains common dental composite resin ingredients such as Bis-GMA, TEGDMA, a silane coupling agent (γ-methacryloxypropyltrimethoxy silane) and activator (Camphorquinone), which are known to be non-cytotoxic (Aranha *et al.*, 2010; Shin *et al.*, 2016).

Although uncured dental composite resin was shown to have a certain degree of cytotoxicity, this effect was not found in cured dental composites (Rajic *et al.*, 2018). The cytotoxic effect found in the uncured dental composite is most likely caused by a volatile unpolymerised monomer. In fact, better biocompatibility was found in the Bis-GMA/TEGDMA based composite resin compared to the 2-hydroxy-ethyl-methacrylate (HEMA) containing resin-modified glass ionomer. Pluripotent mesenchymal precursor C2C12 cells were also shown to be more prominent in cell adhesion and proliferation when treated with a solvent extract of Bis-GMA/TEGDMA based composite resin compared to glass ionomer, as evaluated using the MTT assay (Imazato *et al.*, 2010).

Conclusion

The main limitation of the study was the cell selection in the MTT assay. This study used the L929 mouse fibroblast cell line, which is less clinically relevant compared to human primary cells. Due to the high cost and additional expertise involved in handling and maintaining the cells, we did not use human primary cells. Within the limitation of the study, we can conclude that zirconia-reinforced experimental NHDCs are non-cytotoxic when evaluated on the L929 mouse fibroblast cells line using MTT assay.

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