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ORIGINAL ARTICLE

Genotoxicity assessment of locally produced dental nanocomposite using Comet assay

Siti Robayah Mohd Zakri [a], Thirumulu Ponnuraj Kannan [a,b]*, Nora Aziz [a], Siti Fadilah Abdullah [a], Dasmawati Mohamad [a], Ismail Ab Rahman [a], Abdul Rashid Ismail [c]

[a] School of Dental Sciences, [b] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, Health Campus, 16150 Kubang Kerian, Kelantan, Malaysia. [c] Faculty of Dentistry, Melaka Manipal Medical College, Jalan Batu Hampar, Bukit Baru, 75150 Melaka, Malaysia.

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Abstract The aim of this study was to determine the genotoxicity of a locally produced nanocomposite by Universiti Sains Malaysia, Malaysia using Comet assay. Stem cells from human exfoliated deciduous teeth (SHED) were treated with the nanocomposite at five different concentrations (0.006, 0.0125, 0.025, 0.05, and 0.1 mg/ml) along with concurrent negative (medium alone) and positive control (zinc sulfate heptahydrate) and incubated at 37°C for 24 hours in an incubator at 5% CO_2. The tail moment was used to assess the extent of DNA damage. The tail moment for the group of SHED treated with nanocomposite (for all the five different concentrations) was not statistically significant as compared to the negative control, suggesting that the locally produced dental nanocomposite did not induce any DNA damage. Hence, it can be concluded that the locally produced nanocomposite is non-genotoxic on stem cells from human exfoliated deciduous teeth.

Introduction

Biocompatibility is the ability of a material to perform with an appropriate host response in a specific application (Williams, 1999). Recognition of an implant material as biocompatible nowadays depends on a large number of factors such as absence of cytotoxicity, mutagenicity, carcinogenicity, exclusion of allergenic properties, physical, chemical and biological "inertia" and stability in its biological environment. Therefore, before new materials are approved for medical use, mutagenesis system to exclude cytotoxic, mutagenic or carcinogenic properties is applied worldwide. Many of the tests are based on the principle of genotoxicity or mutagenicity, which serves as an indicator for the carcinogenic potential of a substance (Williams, 1980). Biocompatibility is measured with 3 types of biologic tests: in vitro tests, animal tests and usage tests (Wataha, 2001).

The International Standard Organization (ISO) ISO10993-3 (1992) maintains that certain genotoxicity tests be performed in the biological evaluation of medical devices which should preferably cover the three levels of genotoxic effects: DNA effects, gene mutations and chromosomal aberrations. ISO10993-1 (1997) states that any external communicating device that is in contact with tissue/bone/dentin or any implant device that is in contact with bone/tissue for more than a day should be tested for genotoxicity. To assess the DNA damage, Ostling and Johanson (1984) developed a method based on electrophoresis of cells embedded and lysed in agarose on a microscope slide. When the cells were gamma irradiated, the DNA, which is stained with a fluorescent dye, resembles a comet with a head and a tail. Singh *et al*. (1988) modified the technique by using alkaline electrophoresis. The DNA is

* **Corresponding author:** Assoc. Prof. Dr. T.P. Kannan, Tel.: +609-7675847, e-mail: tpkannan@kb.usm.my

organized in the nucleus (or nucleoid) as loops, which retain the supercoils that are contained in the nucleosomes. Cook *et al*. (1976) observed that the supercoiling was relaxed and loops spilled out into a 'halo' around the nucleoid core, when DNA was broken by irradiation. The comet tail is made up of relaxed loops and the number of loops in the tail or the relative tail intensity indicates the number of DNA breaks. The tail length is presumably determined, primarily by the length of the loops (Collins *et al*., 1997). Damaged cells due to the presence of tails, appear as comets, whereas, undamaged cells have no tails because of the intact nucleus.

The general class of nanocomposite organic/inorganic materials is a fast growing area of research. They are characterized by filler-particle sizes of $\leq$100 nm, which can offer esthetic and strength advantages over conventional microfilled and hybrid resin-based composite systems, primarily in terms of smoothness, polishability and precision of shade characterization, plus flexural strength and microhardness (Saunders, 2009). The aim of the present study was to evaluate if the locally produced nanocomposite had any genotoxic effects based on Comet assay on Stem cells from exfoliated deciduous teeth (SHED). SHED have been identified as a novel population of stem cells that have the capacity of self-renewal and multi-lineage differentiation (Gronthos *et al*., 2000; Miura *et al*., 2003). SHED were obtained from the deciduous teeth from patients who attended the Dental Clinic, Hospital Universiti Sains Malaysia (USM). SHED was employed in this study because SHED may come into close contact with the nanocomposite as this locally produced nanocomposite is aimed for use in dental fillings. Moreover, using SHED as test cell in comet assay also mimic the testing of nanocomposite on human subject's tooth as SHED consists of remnant pulp of human deciduous teeth.

Materials and methods

The test material

The locally produced dental nanocomposite used in this study was developed by a group of researchers in the School of Dental Sciences, Universiti Sains Malaysia (USM), Malaysia (Rahim *et al.,* 2011). The components of the locally produced nanocomposite are tetraethylorthosilicate (TEOS, 99%, Fluka, Switzerland), absolute ethanol (C_2H_5OH, 99.8%, Merck, Germany), ammonia (NH_3, 25%, Merck, Germany), γ-methacryloxypropyltrimethoxysilane (γ-MPS, Sigma Aldrich, Germany), acetic acid (CH_3COOH, 25%, Merck, Germany), Bisphenol-A-glycidyl dimethacrylate (BisGMA, Esstech Inc., USA), urethane dimethacrylate (UDMA, Germany), triethylene glycol dimethacrylate (TEGDMA, Fluka, Switzerland), camphorquinone (CQ, Aldrich, Germany), 2-dimethylaminoethyl methacrylate (DMAEMA, Merck, Germany) and distilled water. All the chemicals were used without any further purification. Nanosilica was synthesized using TEOS, ammonia, ethanol, γ-MPS, acetic acid and distilled water. The component which makes this locally produced nanocomposite different from other commercial ones is the nanosilica. It is synthesized via a sol-gel process, particles ranging in size from 10-20 nm, obtained as monodispersed, spherical silica nanoparticles.

Chemicals, reagents and medium

TRIS (CAS No. 77-86-1) was purchased from Bio-Rad Laboratories (USA), sodium hydroxide (CAS No. 1310-73-2) and trypsin (T9935) from Sigma (USA) and Dulbecco's phosphate buffered saline (Gibco) (14190) from UK. Normal melting agarose (CAS No. 9012-36-6), low melting agarose (CAS No. 9012-36-6), ethidium bromide (CAS No. 1239-45-8) and ethylene diamine tetraacetic acid (CAS No. 60-00-4) were purchased from Sigma (USA). Alpha Modified Eagle's Medium (α-MEM) (12-169F) was purchased from Bio Whittaker® (USA), penicillin-streptomycin (15070-063), L-glutamine 200 mM (25030-081) and Fetal Bovine Serum (FBS) (10270) from Gibco, UK. Zinc sulfate heptahydrate (CAS No. 7446-20-0) obtained from AnalaR® BDH Laboratory Supplies (England) served as the positive control.

Ethical approval

Ethical approval was accorded by the Research Ethics Committee (Human), Universiti Sains Malaysia vide reference USMKK/PPP/JEPeM [224.4.(3.29)] dated 10th May 2010.

Cell culture

The culture procedure was followed as per the protocol established by previous researchers in the laboratory (Fazliah *et al.,* 2010; Lutfi *et al.,* 2010). The pulp tissue which contained the stem cells were cultured with complete growth media containing α-MEM supplemented with 20%

FBS, 100 µM L- ascorbic acid, 2 mM L-glutamine and 1% penicillin streptomycin. The cells were incubated in CO_2 incubator with 5% CO_2 at 37°C. Once the cells reached confluence in about 7 days, they were detached using 0.25% trypsin for 2 min. Cells were re-suspended in 5 ml of α-MEM and mixed with 20 µl of the cell suspension and 20 µl of trypan blue and later counted using a Neubauer chamber (Hirschmann Laborgeräte, Eberstadt, Germany), following which they were seeded in 6-well plate (NUNC, Denmark) at a density of 1 x 10^5 cells per well. The stem cells used in this study were from 4^{th} to 6^{th} passages.

Treatment of cells

The locally produced nanocomposite was suspended in complete growth media and incubated at 37°C for 24 h (ISO 10993-12, 1996) at five different concentrations (0.006, 0.0125, 0.025, 0.05, and 0.1 mg/ml). These concentrations were based on Jin *et al.* (2007), who found out that a concentration of 0.1 mg/ml of nanoparticles caused a reduction in the cell survival percentage of human lung epithelial cells. The suspension was filtered using a 0.45 µm syringe filter. The test material and the negative control (only growth media) were treated with the cells for 24 h. In order to ensure the reproducibility and sensitivity of the assay, an independent positive control test was performed with zinc sulfate heptahydrate at a concentration of 240 µg/ml for 4 hours as reported by Noushad *et al.,* (2009). Zinc sulphate causes a significant effect on DNA damage (Banu *et al.,* 2001; Sliwinski *et al.,* 2009; Sharif *et al.,* 2011).

Comet assay

The protocol for the Comet assay was followed as per the guidelines proposed by Tice *et al.* (2000). For each treatment, the slides were prepared in triplicates. The cells were washed with 0.5 ml PBS and centrifuged at 2500 rpm for 5 min. The supernatant was discarded and the pellet was suspended in 70 µl of 0.6% low-melting point agarose and layered on to the slides pre-coated with 0.6% regular agarose and covered with coverslips. The slides were placed on ice to facilitate solidification of agarose. The coverslips were removed and the slides were carefully immersed in a coplin jar containing lysis solution (Trevigen, USA) for about 1 hour. Slides were placed in alkaline buffer (NaOH, EDTA) for 20 min for alkali unwinding prior to electrophoresis and later the slides were electrophoresed for 20 min at 25 V and 300 mA. Then, the slides were neutralized by rinsing the slides twice for 5 min each with Tris–HCl (pH 7.5). The slides were then stained with 50µl ethidium bromide. All the above steps were done in reduced illumination to prevent extraneous DNA damage.

Comet capture

Fifty cells were captured randomly from each slide and examined blindly under a fluorescence microscope (Olympus, Japan) at 40x magnification and analyzed using an image analysis system (Comet Assay IV, Perceptive Instruments Ltd., UK).

Statistical analysis

The parameters from the Comet assay (tail moment) was analyzed as per Wiklund and Agurell, (2003). The migration data was compared by one-way ANOVA followed by a post-hoc analysis using the SPSS statistical software package, version 17.0 for Windows (SPSS Inc., USA). Differences were considered statistically significant when the p value was <0.05.

Results

To evaluate the genotoxic effect using the Comet assay in this study, DNA strand breaks were represented by the mean tail moment for 150 comets/sample (Table 1). Locally produced dental nanocomposite exposed to SHED at five different concentrations in the current study did not induce DNA damage as measured in the Comet assay (Fig. 1). Moreover, there was no dose-dependent relationship. Dose-dependent relationship refers to situation where the effects of a treatment changes, when the dose of the treatment is changed. The absence of these changes makes the effect not dose-dependent. As in the current study, even with different doses of concentration of locally produced nanocomposite tested, no significant difference in tail moments were noticed as compared to the negative control. However, zinc sulfate heptahydrate, which was used as a positive control induced DNA damage, which was statistically significant.

Table 1 DNA damage (tail moment) in SHED exposed to locally produced nanocomposite, positive control and negative controls

Material	Tail moment (Mean ± SE)
Negative control[a]	0.48 ± 0.57
0.006 mg/ml[b]	2.30 ± 0.15
0.0125 mg/ml[b]	0.67 ± 0.88
0.025 mg/ml[b]	0.94 ± 0.11
0.05 mg/ml[b]	0.68 ± 0.11
0.1 mg/ml[b]	1.33 ± 0.14
Positive control[c]	5.87 ± 5.46 [d]

[a] α-MEM, [b] Locally produced nanocomposite, [c] Zinc sulfate heptahydrate, [d] $p < 0.05$

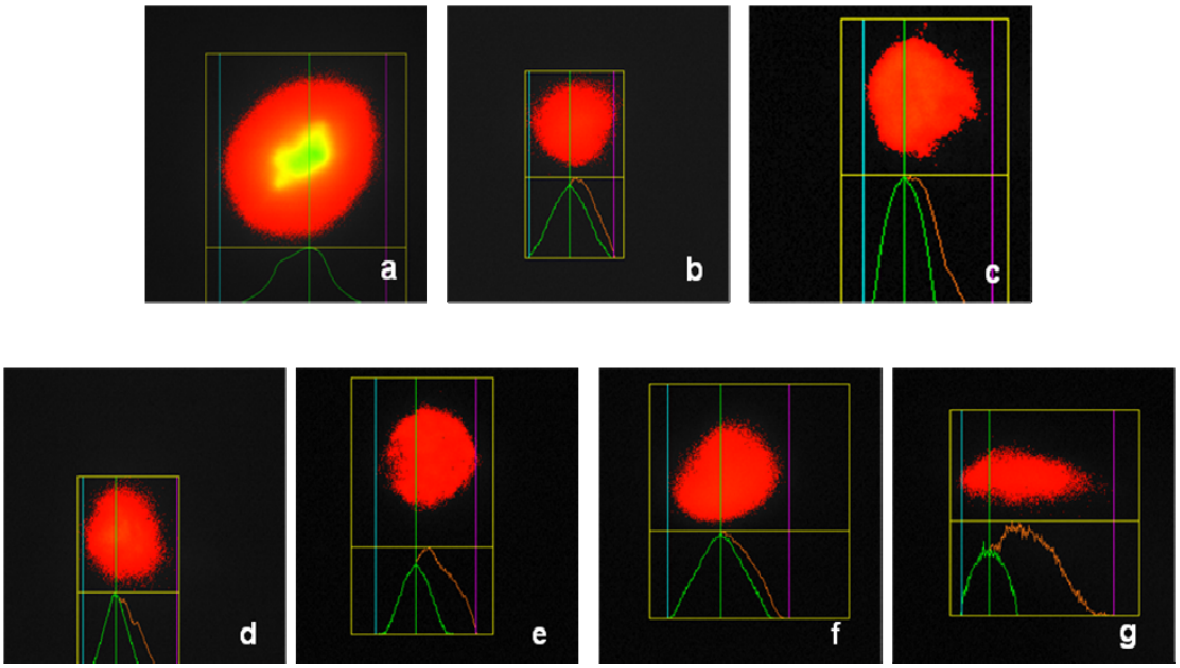

Figure 1 Representative comet images from: (a) α-MEM (negative control), (b) Locally produced nanocomposite 0.006 mg/ml, (c) Locally produced nanocomposite 0.0125 mg/ml, (d) Locally produced nanocomposite 0.025 mg/ml, (e) Locally produced nanocomposite 0.05 mg/ml (f) Locally produced nanocomposite 0.1 mg/ml and (g) Zinc sulfate heptahydrate 240 µg/ml (positive control).

Discussion

The Comet assay is a quick, simple, sensitive, reliable and fairly inexpensive genotoxicity test (Collins *et al.,* 1997). It is widely used to evaluate the genotoxic potential of chemical and physical substances and environmental contaminants and for environmental monitoring purposes. The advantages of the comet assay include the collection of data at the individual cell level, allowing for more robust types of statistical analysis, the need for small number of cells per sample (<10,000), its sensitivity for detecting DNA damage and that virtually any eukaryote cell population is amenable to analysis (Tice *et al.,* 2000).

Composite resin technology has continuously evolved since its introduction by Bowen (1963) as a reinforced Bis-GMA system. A major breakthrough in composite technology was the development of photo-curable resin. A continued development resulted in materials with reduced particle size and increased filler loading that significantly improved the universal applicability of light-cured composite resins. In composite resin technology, particle size and concentration within the matrix is responsible for the polishability, wear and fracture resistance (Soh *et al.,* 2006).

Nanotechnology, also known as nanoscience or molecular engineering, is defined as the creation of functional materials and structures with a characteristic dimension in the range of 0.1-100 nanometers by different physical or chemical techniques (Kirk *et al.,* 1991). This new technology that has become an important discipline in science and technology over the past ten years has shown promise in potential applications areas such as aerospace, computers, telecommunications, microelectronics, biomedical, dental adhesives and dental composites (Soh *et al.,* 2006). This technology has also allowed for tougher, lighter, uncontaminated and more precise materials to be developed. These great advances in nanotechnology have also resulted in the development of several dental nanocomposites with enhanced properties (Soh *et al.,* 2006). Dental nanocomposites used for restoring carious tooth structure have gained considerable attention due to their good biocompatibility and aesthetics (Terry, 2004).

The aim of this study was to assess DNA damage caused by locally produced nanocomposite on SHED *in vitro* using Comet assay. To the best of our knowledge, this is the first study where Comet assay has been carried out using SHED. SHED was employed in this study due to its highly proliferative nature and clonogenicity. A study provides evidence that SHED represent a population of postnatal stem cells capable of extensive proliferation and multipotential differentiation (Miura *et al.,* 2003).

There have been some studies on the genotoxicity or carcinogenicity of some of the components of nanocomposite which are similar to those present in our locally produced nanocomposite. Jin *et al.* (2007) studied on luminescent nanoparticle toxicity on living cells and found that there were no significant toxic effects at the molecular and cellular levels below a concentration of 0.1 mg/ml. A genotoxicity (S.O.S. Chromotest) assay by Couture *et al.* (1989) failed to show any DNA-related effects linked to zirconia. Our results are in agreement with these previous studies as no genotoxic effect was observed with the locally produced nanocomposite. There are a few major comonomers of filling materials, e.g. TEGDMA and HEMA, and monomers, such as Bis-GMA and UDMA. A study by Kleinsasser *et al.* (2004) stated that at higher concentration levels, the methacrylates HEMA, TEGDMA, Bis-GMA and UDMA induced significant but mild enhancement of DNA migration in the Comet assay as a possible sign for limited genotoxic effects. Similarly, genotoxic studies on silica have also been reported. In one Comet assay study, Zhong *et al.* (1997) indicated that silica can induce DNA damage in mammalian cells and that crystalline silica has a higher DNA-damaging activity than amorphous silica. However, both studies vary from the results noticed in the current study as the locally produced nanocomposite failed to induce genotoxicity, even though genotoxic test was not carried out individually for the various components present in the nanocomposite, which forms the limitation of the study.

Not only did the dental nanocomposite cause any DNA damage at all the five different concentrations but also did not exhibit dose-dependent relationship. The tail moment for the group of SHED treated with nanocomposite (for all the five different concentrations) was not statistically significant as compared to the negative control, suggesting that the locally produced dental nanocomposite did not induce any DNA damage. In the case of the positive control using zinc sulfate heptahydrate, only two comets were noticed, which were not statistically significant. From the results of the current study, it can be inferred that the locally produced dental nanocomposite is non-genotoxic under the present test conditions.

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