# Genotoxicity evaluation of dental restoration nanocomposite using comet assay and chromosome aberration test

**Article** *in* Nanotechnology · December 2012

DOI: 10.1088/0957-4484/24/1/015105 · Source: PubMed

CITATIONS
7

READS
137

**4 authors:**

Marahaini Musa
University of Oxford
**5** PUBLICATIONS **31** CITATIONS
SEE PROFILE

Kannan Thirumulu Ponnuraj
Universiti Sains Malaysia
**75** PUBLICATIONS **338** CITATIONS
SEE PROFILE

Dasmawati Mohamad
Universiti Sains Malaysia
**77** PUBLICATIONS **765** CITATIONS
SEE PROFILE

Ismail Ab Rahman
Universiti Sains Malaysia
**102** PUBLICATIONS **1,854** CITATIONS
SEE PROFILE

# Genotoxicity evaluation of dental restoration nanocomposite using comet assay and chromosome aberration test



2013 Nanotechnology 24 015105

(http://iopscience.iop.org/0957-4484/24/1/015105)

Nanotechnology **24** (2013) 015105 (13pp) doi:10.1088/0957-4484/24/1/015105

# Genotoxicity evaluation of dental restoration nanocomposite using comet assay and chromosome aberration test

**Marahaini Musa**[1], **Kannan Thirumulu Ponnuraj**[1,2,3], **Dasmawati Mohamad**[1] **and Ismail Ab Rahman**[1]

[1] School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia
[2] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia

E-mail: tpkannan@kb.usm.my

Received 24 September 2012, in final form 12 November 2012
Published 7 December 2012
Online at stacks.iop.org/Nano/24/015105

**Abstract**

Nanocomposite is used as a dental filling to restore the affected tooth, especially in dental caries. The dental nanocomposite (KelFil) for tooth restoration used in this study was produced by the School of Dental Sciences, Universiti Sains Malaysia, Malaysia and is incorporated with monodispersed, spherical nanosilica fillers. The aim of the study was to determine the genotoxic effect of KelFil using *in vitro* genotoxicity tests. The cytotoxicity and genotoxicity of KelFil was evaluated using MTT assay, comet assay and chromosome aberration tests with or without the addition of a metabolic activation system (S9 mix), using the human lung fibroblast cell line (MRC-5). Concurrent negative and positive controls were included. In the comet assay, no comet formation was found in the KelFil groups. There was a significant difference in tail moment between KelFil groups and positive control ($p < 0.05$). Similarly, no significant aberrations in chromosomes were noticed in KelFil groups. The mitotic indices of treatment groups and negative control were significantly different from positive controls. Hence, it can be concluded that the locally produced dental restoration nanocomposite (KelFil) is non-genotoxic under the present test conditions.

(Some figures may appear in colour only in the online journal)

## 1. Introduction

Dental caries (tooth decay) is a multifactorial disease. It is considered a public health problem as it is a widespread disease that is costly to treat and affects the quality of life of children of all ages [1]. Restoration of an affected tooth or teeth using biomaterials such as amalgam, resin, porcelain and gold can be performed [2]. Nanocomposite also could be used as a dental filling for restorative purposes.

An example of the classes of composites is the polymer matrix composite. Among the common methacrylates (monomer resin) in the matrix of restorative materials which make polymer composite are bisphenol glycidyl methacrylate (BisGMA), urethane dimethacrylate (UDMA), and triethylene glycol dimethacrylate (TEGDMA) [3]. To further improve the properties and reduce costs, polymers are usually reinforced with inorganic fillers such as silica [4]. Nanofilled composite resins which are characterized by filler particle sizes of $\leq$100 nm, offer better aesthetic and strength properties compared to conventional microfilled and hybrid resin-based composite (RBC) systems [5]. The most important criterion of a biomaterial is biocompatibility. Assessment of biocompatibility of implantable devices involves complex experiments, both *in vitro* and *in vivo*, on cell culture, tissue sections, and the whole body in order to study the effects of the particular material on the local and systemic effects [6]. The International Organization for

[3] Address for correspondence: School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.

 

Standardization (ISO) (10993-Part 1) (2009) has listed several tests for biological evaluation of medical devices, which includes cytotoxicity and genotoxicity testing [7].

Cytotoxicity test is performed to determine the cytotoxic effect of a chemical or drug. This test also can be used to estimate the starting dosage for a toxicity test [8]. An example of cytotoxicity tests using the colorimetric principle is MTT assay [9]. MTT assay is a quantitative and reliable colorimetric assay that determines cell survival by using different end points. The principle of this assay is based on the reduction of yellow water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) by viable cells after they are treated with a test chemical or device. Mitochondrial dehydrogenase at the cytochrome b and cytochrome c sites of living cells convert formazan salt to an insoluble purple formazan metabolite. Solubilization of the formazan by alcohol or dimethyl sulfoxide (DMSO) can be measured quantitatively, where the results are proportional to viable cells [10].

Since nanocomposite used in dental restoration as a biomaterial will be in contact with the oral tissues for a long time, the need for genotoxicity testing is essential. Genotoxicity testing is usually indicated for identification of hazards caused by new chemical entities (NCE) with respect of damage to DNA [11]. Alteration and damage to DNA can result in mutation of genes and also numerical and structural chromosomal aberrations. These phenomena were responsible for heritable effects on germ cells which affected future generations [12].

The genotoxicity tests to be performed on KelFil have been selected according to ISO guidelines and focus has been made on the DNA effects and changes in chromosomes on a mammalian cell line induced by this material. The tests are the comet assay and chromosome aberration test.

Comet assay, also known as single cell gel electrophoresis assay, is a widely used genotoxic test, as it is quick, simple, sensitive, reliable, fairly inexpensive and produces special images. The alkaline comet assay detects single (and double) DNA breaks which are indicated in the number of relaxed loops in the comet tail. An increasing amount of damage is shown by the increased intensity of the tail, where, with increasing doses, more material moves out from the head [13]. DNA damage plays an essential role in mutagenesis, carcinogenesis and aging. Hydrolysis, exposure to reactive oxygen species (ROS) and other reactive metabolites are the chemical events that lead to DNA damage. Factors that trigger these reactions include exposure to exogenous chemicals or from metabolic, endogenous processes [14].

Another genotoxicity test, the chromosome aberration (CA) test, is conducted to study the CAs that are induced by agents that cause damage to chromosomal DNA [15–17]. The majority of chemical mutagens induce aberrations of the chromatid type, but chromosome type aberrations also occur [18]. CA can be divided into intra-chromosomal (lesions in the same chromosome) and inter-chromosomal (lesions in the arms of different homologous or non-homologous chromosomes) aberrations. The first class involves aberrations within a single chromosome (e.g. terminal and interstitial deletions and inversions) and the second class consists of rearrangements between at least two chromosomes (e.g. translocation and dicentrics) [19].

## 2. Experimental methods

### *2.1. Nanocomposite*

The material tested in this study was a locally produced dental restoration nanocomposite (KelFil). This nanocomposite was produced by the School of Dental Sciences (PPSG), Universiti Sains Malaysia (USM), Kelantan, Malaysia. The nanosilica fillers, synthesized locally by a sol–gel process, were in the size range of 10–20 nm. The experimental nanocomposite (ENC2) contained 35 wt% of nanosilica fillers. This material (ENC2) will be called KelFil (brand name) hereafter. The nanosilica fillers obtained were relatively monodispersed, spherical with low agglomeration. The chemicals used to synthesize nanosilica were tetraethylorthosilicate (TEOS, 99%, Fluka, Switzerland), absolute ethanol ($C_2H_5OH$, 99.8%, Systerm, Shah Alam, Malaysia), ammonia ($NH_3$, 25% Merck, Germany), $\gamma$-methacryloxypropyltrimethoxysilane ($\gamma$-MPS, Sigma Aldrich, Germany) and acetic acid ($CH_3COOH$, 25%, Merck, Germany). Zinc oxide fillers and zirconia fillers, which were synthesized in-house at the School of Dental Sciences and School of Materials and Mineral Resources, USM, were added as antimicrobial agents.

These nanosilica fillers were subsequently mixed with monomer resins and a photoinitiator system to prepare a dental nanocomposite. The monomer resins used in synthesis of KelFil include bisphenol A glycidyl methacrylate (Bis-GMA, Esstech, USA), diurethane dimethacrylate (UDMA, Aldrich, Germany) and triethylene glycol dimethacrylate (TEGDMA, Fluka, Switzerland), while the photoinitiator system consists of camphorquinone (CQ, Aldrich, Germany) and *N*, *N*-dimethylaminoethyl methacrylate (DMAEMA, Merck, Germany) [20]. KelFil used in this research was a light-cured sample, where the test material was cured by an Elipar$^{TM}$ Freelight LED with a light intensity of 1200 mW cm$^{-2}$ and a wavelength in the range 430–480 nm. Light curing is a procedure used in dentistry to cure, set or polymerize various types of resin-based dental materials. The curing process turns the soft and putty-like texture of the material into a hard and rigid structure [21].

### *2.2. Extraction of test material*

For the extraction, light-cured KelFil was pounded into irregular, smaller pieces using a pestle and mortar. Then, 0.2 g of the sample was weighed and put into a microcentrifuge tube. The material was sterilized by gamma radiation at dosage of 25 kGy. Preparation of extract was performed according to the International Organization for Standardization (ISO) (10 993: Part 12) (2007) guidelines [22]. The KelFil sample was transferred to a 15 ml centrifuge tube and 1 ml of culture medium was added into the tube, making a final concentration of extract of 0.2 g ml$^{-1}$. The tube was incubated for 24 h at 37 °C on

a roller. Then the extraction was filtered using a 0.45 $\mu$m filter. Nanocomposite extract was freshly prepared before each experiment.

### *2.3. Cell culture*

Human lung fibroblast cells, MRC-5 (CCL-171, ATCC, USA) were cultured in Alpha Minimal Essential Medium ($\alpha$-MEM) (GIBCO, New York) supplemented with 10% fetal bovine serum (FBS) (GIBCO, New Zealand), 1% of antibiotics—penicillin (5000 IU $ml^{-1}$) and streptomycin (5000 $\mu g$ $ml^{-1}$) (GIBCO, New Zealand), in a humidified incubator supplied with a 95% air, 5% $CO_2$ atmosphere (NuAire, USA) at 37 °C for 4 days until confluence. The cells were trypsinized using 0.25% trypsin-EDTA solution (GIBCO, New Zealand) before centrifugation at 1200 rpm for 5 min. The cell pellet was re-suspended in 1 ml of medium. The counting of cells was done using a hemocytometer (Hirschmann Laborgeräte, Eberstadt, Germany). 10 $\mu$l of 0.4% trypan blue solution (Invitrogen, USA) was added to 10 $\mu$l of cell suspension (ratio 1:2). The mixture of cells and trypan blue solution was then pipetted into the counting chamber of the hemocytometer using capillary action. Then the cells were counted using the formula below:

$$\text{cell count} = \text{Av} \times 2^n \times 10^4 \text{ cells ml}^{-1}$$

where Av is the average number of cells and $n$ the dilution factor.

### *2.4. Cytotoxicity test (MTT assay)*

MTT assay was performed according to Mosmann [23]. Confluent MRC-5 cells were trypsinized and seeded as mentioned previously. $1 \times 10^4$ MRC-5 cells were counted and seeded in a 96-well plate. The cells were incubated for 24 h prior to the treatment with KelFil extract. For treatment groups, various concentrations of KelFil (2, 1, 0.5, 0.25, 0.125, 0.063, 0.0313, 0.016 and 0.008 g $ml^{-1}$) were prepared by serial dilution before adding to the cells. In the control well, only the cell suspension and culture medium were used. The plate was then incubated for 72 h, in a humidified incubator at 37 °C with 5% $CO_2$. After that, 0.5 mg $ml^{-1}$ of MTT (Calbiochem, Darmstadt, Germany) solution diluted with PBS was added to all the wells before incubating the plate for a further 4 h. Following the incubation period, the formazan crystals that were formed by the viable cells were dissolved by adding 100 $\mu$l of DMSO (Merck, Germany) to each well, with gentle shaking of the plate to aid in the process. The absorbance ($A$) of each well was read at 570 nm using an ELISA plate reader (Tecan, Switzerland). The relative viability of the cells treated with KelFil compared to control cells was calculated using the formula below:

$$\%\text{ cell viability} = [A_{570} \text{ of treated cell}] \times 100\%/[A_{570} \text{ of control cells}].$$

The viability of treated cells was calculated with regard to the untreated cell control, which was set to 100% viability. The test material was considered cytotoxic when the cell viability decreased to less than 50%. For determination of inhibitory concentrations (ICs) of $IC_{10}$, $IC_{25}$ and $IC_{50}$, dose–response graphs were constructed from a series of different concentrations of KelFil using GraphPad Prism 5 software. These three concentrations of KelFil extract were applied in both the comet assay and chromosome aberration test.

### *2.5. The comet assay*

Detection of DNA damage induced by KelFil was studied using the alkaline version of comet assay [24]. In this test, $5 \times 10^4$ MRC-5 cells were seeded in a 60 mm Petri dish along with 5 ml of culture medium. Cells were incubated overnight to allow cell attachment. The extraction of KelFil was prepared as mentioned earlier. Following the incubation period, medium was discarded and cells were washed with PBS. Then, the cells were exposed to the KelFil extract at concentrations which gave $IC_{10}$, $IC_{25}$ and $IC_{50}$ values in the MTT assay (0.08, 0.79 and 8.00 mg $ml^{-1}$ KelFil respectively) for 24 h. For negative control, cells were treated with culture medium only and for positive control, 50 $\mu$M of hydrogen peroxide ($H_2O_2$) was prepared by diluting 30% $H_2O_2$ (Merck, Germany) in PBS and incubated with cells for 5 min at 4 °C. Triplicate cultures were set up for each concentration.

After the incubation period, medium from the Petri dish was discarded and cells were washed twice with PBS. Cells were then trypsinized before medium was added. The cell solution was transferred into a sterile 1.5 ml microcentrifuge tube. The tubes were left at 4 °C for 20 min and then centrifuged at 2500 rpm for 5 min. After that, the supernatant was discarded and 1 ml of PBS was added. All tubes were incubated at 4 °C for 20 min before centrifuging again at 2500 rpm for 5 min. 80 $\mu$l of 0.6% low melting point agar (LMA) (Fermentas, Lithuania) in PBS was added to each tube and re-suspended gently with the cell pellet. An 80 $\mu$l aliquot of the mixture was transferred with a pipette onto a slide that was previously coated with 0.6% normal melting agar (NMA) (Invitrogen, UK) in PBS. The slides were covered with coverslips and the gel left to solidify at 4 °C for 3–5 min.

The slides were then placed in fresh working lysis buffer consisting of 1% Triton-X 100 (Sigma Aldrich, Germany), 100 mM disodium EDTA (Bioshop, Canada), 2.5 M NaCl (Fisher Scientific, UK), 10 mM tris-HCl (Bio-Rad Laboratories, USA) and sterile distilled water, and left at 4 °C for 1 h. Following lysis treatment, the slides were immersed in alkaline (pH > 13) electrophoresis buffer (300 mM NaOH and 1 mM EDTA in sterile distilled water) for 20 min to allow DNA unwinding before electrophoresis at 25 V and 300 mA for 20 min using an electrophoresis chamber (Aurogene, USA). The electrophoresis was carried out in the dark. The slides were then rinsed thrice with cold neutralization buffer (0.4 M tris-HCl, pH 7.5) for a duration of 5 min each. After neutralization, the slides were dehydrated in 99% ethanol (Merck, Germany) for 5 min and dried at room temperature overnight. The slides were coded and stained with 20 $\mu$l of

0.5 $\mu$g ml$^{-1}$ ethidium bromide (Sigma Aldrich, Germany) solution for more than 15 min.

Comet images were examined under a fluorescence microscope (Olympus BX41, Japan) at 40× magnification. All slides, including those of positive and negative controls, were independently coded before microscopic analysis and scored without the knowledge of the code. Comet assay IV software from Perceptive Instruments (UK) was used for quantification of the head and tail regions of the comets. The tail moment (the length from the center of the head to the center of the tail × the intensity of the head (%)) was chosen as the measurement parameter. Results are represented as the mean tail moment ± SEM. A total of 50 cells were scored per culture.

### *2.6. Chromosome aberration (CA) test*

CA tests were performed not only to study the cytotoxicity of the material on cells but also to determine the aberrations induced by the particular material on chromosomes of the human lung fibroblast cell line. The MRC-5 cell line was exposed to different concentrations of KelFil (0.08, 0.79 and 8.00 mg ml$^{-1}$ KelFil) for 6, 24 and 48 h. The experiment for *in vitro* CA test was performed in two conditions; either in the presence or absence of metabolic activation system (S9 mix). S9 mix used in the test was purchased from Sigma Aldrich (Germany) and dissolved in 1 ml of cofactor. For 100 ml of cofactor solution, the chemicals used were 0.16 g D-glucose-6-phosphate (Sigma Aldrich, Germany), 0.35 g nictotinamide adenine dinucleotide phosphate (NADP) (Sigma Aldrich, Germany), 0.18 g $MgCl_2$ (Merck, Germany), 0.27 g KCl (Sigma Aldrich, Germany), 1.28 g sodium phosphate dibasic dihydrate ($Na_2HPO_4{\cdot}2H_2O$) (Sigma Aldrich, Germany), 0.28 g sodium phosphate monobasic dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and distilled water. The S9 mix was aliquoted in a 15 ml centrifuge tube and stored at −20 °C until use.

Before the treatment, cells were seeded at a density of $2.0 \times 10^4$ cells/60 mm plate in 5 ml of culture medium and kept at 37 °C in a humidified incubator supplied with 5% $CO_2$ for 72 h. After the incubation period, the culture medium in each plate was discarded. KelFil extract, according to the $IC_{10}$, $IC_{25}$ and $IC_{50}$ concentrations from MTT assay (0.08, 0.79 and 8.00 mg ml$^{-1}$ KelFil respectively), were added to cells at a volume of 5 ml. Negative and positive controls were also tested concurrently along with the test groups. All groups were treated for 6, 24 and 48 h, either with the test material, culture medium or positive control agents. For the treatment with metabolic activation enzyme, S9 mix was added in a culture medium at 5% in volume (0.5 ml). Culture medium was added to the negative control group while positive control groups were treated with either mitomycin C (MMC) (Sigma Aldrich, Germany) or cyclophosphamide monohydrate (CP) (Calbiochem, Darmstadt, Germany), with S9 and without S9 mix respectively. The concentration of positive control used in this experiment was different, depending on the incubation time. For 6 h incubation, 0.1 $\mu$g ml$^{-1}$ MMC and 10 $\mu$g ml$^{-1}$ CP were used. For 24 h incubation, 0.05 $\mu$g ml$^{-1}$ MMC and 6 $\mu$g ml$^{-1}$ CP were used. For 48 h incubation, 0.02 $\mu$g ml$^{-1}$ MMC and 6 $\mu$g ml$^{-1}$ CP were used. Duplicate cultures were set up for each group in this experiment.

For arresting cells in the metaphase, colcemid (Invitrogen, USA) at a concentration of 1 $\mu$g ml$^{-1}$ was added 4 h before harvesting of cells and incubated at 37 °C and 5% $CO_2$. The medium was then withdrawn and discarded. Cells were washed with PBS twice and trypsinized. The cells were then collected in a 15 ml centrifuge tube and centrifuged at 1000 rpm for 10 min.

After centrifugation, the supernatant was discarded, leaving about 1 ml of supernatant along with the cell pellet. The cell pellet was then mixed gently. Eight ml of pre-warmed 0.075 M hypotonic potassium chloride (KCl) (Invitrogen, USA) solution was added slowly into the centrifuge tube and gently mixed. The tube was placed in an incubator at 37 °C for 50 min before centrifugation at 1000 rpm for 10 min. The supernatant was removed, leaving 1 ml of hypotonic solution along with the cell button. The cells were then mixed with the solution.

Next, the cells were fixed in 6 ml of chilled and freshly prepared 3:1 methanol:acetic acid fixative solution. The solution was mixed gently with cells before centrifuging at 1000 rpm for 10 min. The fixation step was repeated and the cells were incubated with the fixative at 4 °C overnight. The fixative wash was repeated once the next day. Cells were centrifuged again at 1000 rpm for 10 min. Most of the fixative was removed and the cells were suspended in a small amount of fixative solution. Slides were prepared by pipetting 50 $\mu$l of cell suspension onto the surface of slide. Slides were then air dried before being labeled. Slides were stained with freshly prepared working Giemsa stain for 30 min. After that, the slides were rinsed with distilled water and air dried.

A total of 1000 cells were counted per culture to determine the Mitotic index (MI). MI was calculated for determination of cytotoxicity. The formula is stated as below:

$$\text{Mitotic index} = \frac{\text{Number of cells in metaphase}}{\text{Total number of cells counted}} \times 100\%.$$

The slides were screened under a light microscope (Nikon Eclipse E600, Japan) for the detection of chromosome aberrations. Any numerical and structural aberrations were observed and noted. Good metaphase spreads were selected and photographed under an oil immersion objective (100×) using Dino Eye software (Taiwan). 100 metaphase spreads were examined for each culture. Hence a total of 200 cells were evaluated per group, which consisted of duplicate cultures.

### *2.7. Statistical analyses*

For comet assay results, a non-parametric Kruskal–Wallis statistical test followed by pairwise comparison using Mann–Whitney *U* test was employed. Statistical analysis of the CA test was performed using an independent-*t* test. All statistical analyses were performed using IBM SPSS Statistics 19 software (SPSS Inc., USA). The difference was considered significant when the *p*-value was $<0.05$.

**Table 1.** Mean tail moments of treatment groups.

| Group | Mean tail moment[a] ± SEM | Median (IQR)[b] |
|---|---|---|
| Negative control ($\alpha$-MEM) | 0.0976 ± 0.0058 | 0.1008 (0.0861–0.1090) |
| 0.08 mg ml$^{-1}$ of KelFil | 0.1000 ± 0.0059 | 0.1041 (0.0883–0.1116) |
| 0.79 mg ml$^{-1}$ of KelFil | 0.0968 ± 0.0050 | 0.1007 (0.0869–0.1066) |
| 8.00 mg ml$^{-1}$ of KelFil | 0.1020 ± 0.0060 | 0.1031 (0.0902–0.1139) |
| Positive control ($H_2O_2$—50 $\mu$M) | 36.0842 ± 1.1618 | 36.2240 (33.7881–38.3804) |

[a] Mean of three independent tests.

[b] Interquartile range.

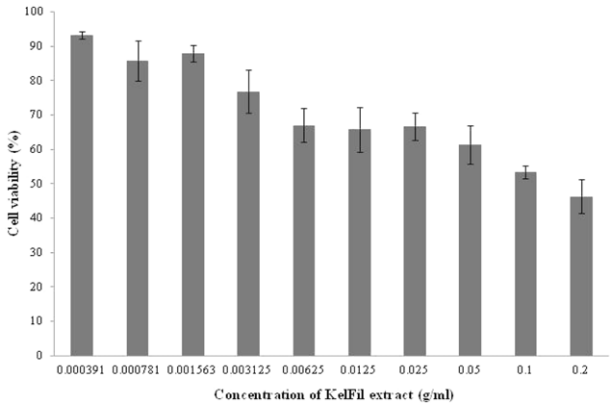


**Figure 1.** Dose–inhibition graph of KelFil on MRC-5 cells.

## 3. Results

Two types of testing were performed in the present study. The first test, MTT assay as a cytotoxicity test, was conducted to evaluate the cytocompatibility of KelFil, while comet assay and the CA test were used to assess the genotoxicity of KelFil. To ensure that any observed damage to genetic material was not due to cytotoxicity-related mechanisms, only those concentrations leading to more than 50% cell viability were analyzed and used in genotoxicity tests.

### *3.1. MTT assay*

Based on the dose–inhibition graph (figure 1) constructed, it was found that the viability of the cells was not proportional to the concentration of KelFil, where greater cell inhibition was seen at a higher concentration of KelFil extract. A lower concentration of KelFil extract produced greater cell viability. From GrapPad Prism software, the $IC_{50}$ value was determined as 8.00 mg ml$^{-1}$. The concentrations for $IC_{25}$ and $IC_{10}$ were 0.79 mg ml$^{-1}$ and 0.08 mg ml$^{-1}$ respectively.

### *3.2. Comet assay*

The mean tail moment ± standard error mean (SEM) for each group is shown in table 1. No comet formation was observed in MRC-5 cells treated with three different concentrations of KelFil extract, the same as in the negative control. Undamaged cells appeared round in shape, with no tail formation. This was different from the positive control, where there was formation of comets indicating the migration of damaged DNA during electrophoresis (figure 2). No dose-dependent relationship was observed.

**Table 2.** Pairwise comparisons of tail moments of MRC-5 cells treated with KelFil extract. (Note: Mann–Whitney *U* test (Bonferroni correction); statistically different ($p < 0.05$) are underlined. Positive control: 50 $\mu$M $H_2O_2$, negative control: $\alpha$-MEM.)

| Group-wise comparison | Mann–Whitney *U* test | *p*-value |
|---|---|---|
| Negative control versus 0.08 mg ml$^{-1}$ of KelFil | 10 698 | 0.460 |
| Negative control versus 0.79 mg ml$^{-1}$ of KelFil | 11 245 | 0.995 |
| Negative control versus 8.00 mg ml$^{-1}$ of KelFil | 10 711 | 0.471 |
| 0.08 mg ml$^{-1}$ KelFil versus 0.79 mg ml$^{-1}$ of KelFil | 10 469.5 | 0.298 |
| 0.08 mg ml$^{-1}$ KelFil versus 8.00 mg ml$^{-1}$ of KelFil | 11 181.5 | 0.927 |
| 0.79 mg ml$^{-1}$ KelFil versus 8.00 mg ml$^{-1}$ of KelFil | 10 588 | 0.377 |
| Positive control versus negative control | 0 | <u>≤0.001</u> |
| Positive control versus 0.08 mg ml$^{-1}$ of KelFil | 0 | <u>≤0.001</u> |
| Positive control versus 0.79 mg ml$^{-1}$ of KelFil | 0 | <u>≤0.001</u> |
| Positive control versus 8.00 mg ml$^{-1}$ of KelFil | 0 | <u>≤0.001</u> |

A non-parametric statistical test was chosen for the analysis of tail moments. Pairwise comparison (Mann–Whitney *U* test) was performed to compare the differences in tail moment between two independent treatment groups. The results are summarized in table 2. There was no significant difference in tail moments between KelFil groups ($p > 0.05$). The tail moment between KelFil groups and the negative control was also not significantly different ($p > 0.05$). However, there was a significant difference in the tail moment between KelFil groups and the positive control ($p < 0.05$). This indicated that 50 $\mu$M $H_2O_2$ induced significant DNA damage.

Besides tail moment as the measurement parameter, the level of DNA damage occurring in the treatment group also could be divided into several classifications (Class 0: undamaged, Class 1, Class 2, Class 3, Class 4: maximum damage and apoptotic cell) [25]. DNA damage in the positive control group was of Class 3 and Class 4.

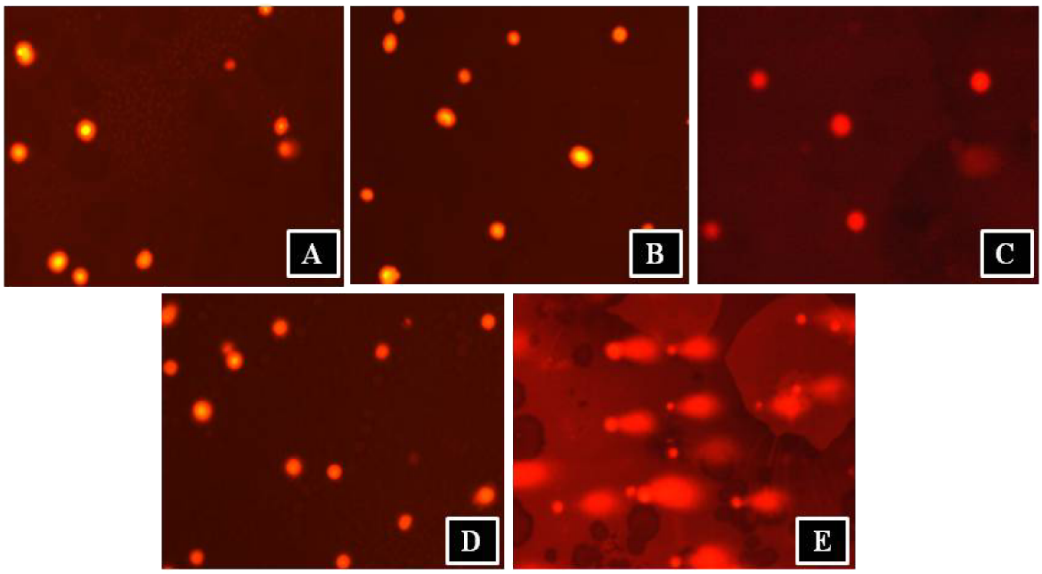


**Figure 2.** Representative images from comet assay. (A) 8.00 mg $ml^{-1}$ of KelFil; (B) 0.79 mg $ml^{-1}$ of KelFil; (C) 0.08 mg $ml^{-1}$ of KelFil; (D) negative control ($\alpha$-MEM); (E) positive control (50 $\mu$M $H_2O_2$).

**Table 3.** Mean mitotic indices of KelFil groups, negative and positive controls. (Note: MMC: mitomycin C; CP: cyclophosphamide monohydrate (positive controls).)

| | | Mean mitotic index (%) | |
|---|---|---|---|
| Group | Hours | Without S9 | With S9 |
| 0.08 mg $ml^{-1}$ of KelFil | 6 | 3.50 | 4.80 |
| | 24 | 3.50 | 4.90 |
| | 48 | 3.20 | 3.95[a] |
| 0.79 mg $ml^{-1}$ of KelFil | 6 | 3.55 | 5.10[b] |
| | 24 | 4.05 | 5.05 |
| | 48 | 3.30 | 3.95[a] |
| 8.00 mg $ml^{-1}$ of KelFil | 6 | 3.20 | 4.75 |
| | 24 | 3.55[b] | 5.20 |
| | 48 | 3.50 | 3.95 |
| Negative control ($\alpha$-MEM) | 6 | 3.60 | 4.55 |
| | 24 | 4.15 | 4.60 |
| | 48 | 3.90 | 4.05 |
| 0.1 $\mu$g $ml^{-1}$ (MMC) | 6 | 1.80[b] | — |
| 0.05 $\mu$g $ml^{-1}$ (MMC) | 24 | 1.60[b] | |
| 0.02 $\mu$g $ml^{-1}$ (MMC) | 48 | 1.40[b] | |
| 10 $\mu$g $ml^{-1}$ (CP) | 6 | — | 3.35 |
| 6 $\mu$g $ml^{-1}$ (CP) | 24 | | 3.20 |
| 6 $\mu$g $ml^{-1}$ (CP) | 48 | | 2.85 |

[a] $p > 0.05$ compared to CP.

[b] $p < 0.05$ compared to negative control.

### *3.3. Chromosome aberration (CA) test*

The effect of KelFil in inducing CA was studied at 6, 24 and 48 h. Three different incubation periods were selected in order to investigate the genotoxicity effect of KelFil at different stages of the cell cycle. The analysis of the cytotoxicity effect of KelFil also was done by calculating the MI value of each group. Table 3 shows the mean mitotic indices of the treatment groups.

Determination of the cytotoxicity level of KelFil was performed by comparing the MI of KelFil groups with that of positive and negative controls. When compared with the negative control, MMC produced a significant cytotoxic effect to cells ($p < 0.05$), unlike CP ($p > 0.05$). This finding indicated that treatment with CP was not cytotoxic to MRC-5 cells. For the treatment in the absence of S9 mix, there were significant differences ($p < 0.05$) in MI between KelFil groups and MMC. It can be concluded that KelFil groups (presence of S9 mix) did not produce significant cytotoxicity.

In the presence of S9 mix, MI at 0.08 and 0.79 mg $ml^{-1}$ of KelFil at 48 h of incubation were insignificantly different from CP ($p > 0.05$). As mentioned previously, CP did not cause a significant cytotoxic effect. Thus, these concentrations of KelFil (0.08 and 0.79 mg $ml^{-1}$ KelFil at 48 h of incubation) also were non-cytotoxic. Other KelFil groups produced mitotic indices that were significantly different from CP. However, the differences in mitotic indices of these groups were not significant compared to the negative control ($p < 0.05$), which indicated the lack of cytotoxic effect.

As for comparison of MI between KelFil groups and the negative control, no significant differences ($p > 0.05$) were found except for 8.00 mg $ml^{-1}$ KelFil at 24 h incubation (absence of S9 mix) and 0.79 mg $ml^{-1}$ KelFil at 6 h incubation (presence of S9 mix). These two KelFil groups produced significant cytotoxic effect compared to the negative control. However, both these groups also produced significant difference in cytotoxicity in comparison with positive controls. As these values (8.00 and 0.79 mg $ml^{-1}$ KelFil) were pre-determined from MTT assay, which resulted in viability of cells of 50% and 25% respectively, it can be concluded that these two concentrations are non-cytotoxic to the cells.

The results of the *in vitro* chromosome aberration test at 6, 24 and 48 h incubation times are shown in tables 4, 5 and 6 respectively. KelFil did not induce significant structural and numerical aberrations of the chromosomes,

**Table 4.** Induction of chromosome aberrations by dental restoration nanocomposite (KelFil) in MRC-5 cells in the absence and presence of S9 mix after 6 h of incubation period. (Note: Ctg: chromatid gap; Csg: chromosome gap; Ctb: chromatid break; Csb: chromosome break; Exch: triradial, quadriradial and other chromatid exchanges; Dic + Oth: dicentric chromosomes and other aberrations (e.g. rings); >10: multiaberrant cells, Pol: polyploids except endoreduplications; End: endoreduplications; negative control: $\alpha$-MEM; MMC: mitomycin C; CP: cyclophosphamide monohydrate.)

| | | Structural aberrations[a] | | | | | | | | | | Numerical aberrations[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Test material | S9 mix | Ctg | Csg | Ctb | Csb | Exch | Dic+Oth | >10 | Loss of centromere | Total | Total aberrant cells[b] (%, in brackets) | Pol | End | Total (% in bracket) |
| 0.08 mg ml$^{-1}$ of KelFil | − | 1 | 4 | 0 | 0 | 0 | 1 | 0 | 5 | 10 | 3 (1.5) | 0 | 2 | 2 (1.0) |
| 0.79 mg ml$^{-1}$ of KelFil | − | 0 | 6 | 1 | 0 | 0 | 0 | 0 | 6 | 13 | 7 (3.5) | 0 | 3 | 3 (1.5) |
| 8.00 mg ml$^{-1}$ of KelFil | − | 0 | 5 | 1 | 1 | 0 | 0 | 0 | 5 | 12 | 6 (3.0) | 0 | 4 | 4 (2.0) |
| Negative control | − | 0 | 2 | 1 | 0 | 0 | 0 | 0 | 5 | 8 | 3 (1.5) | 0 | 3 | 3 (1.5) |
| 0.08 mg ml$^{-1}$ of KelFil | + | 0 | 3 | 1 | 0 | 0 | 0 | 0 | 5 | 8 | 4 (2.0) | 0 | 2 | 2 (1.0) |
| 0.79 mg ml$^{-1}$ of KelFil | + | 0 | 9 | 2 | 0 | 0 | 0 | 0 | 9 | 20 | 9 (4.5) | 0 | 4 | 4 (2.0) |
| 8.00 mg ml$^{-1}$ of KelFil | + | 0 | 10 | 1 | 0 | 0 | 1 | 0 | 7 | 18 | 6 (3.0) | 0 | 3 | 3 (1.5) |
| Negative control | + | 0 | 5 | 0 | 1 | 0 | 1 | 0 | 8 | 15 | 6 (3.0) | 0 | 2 | 2 (1.0) |
| MMC (0.1 $\mu$g ml$^{-1}$) | − | 10 | 34 | 97 | 5 | 5 | 18 | 1 | 49 | 219 | 144 (72.0) | 0 | 11 | 11 (5.5) |
| CP (10 $\mu$g ml$^{-1}$) | + | 0 | 12 | 27 | 0 | 2 | 6 | 0 | 35 | 82 | 57 (28.5) | 0 | 1 | 1 (0.5) |

[a] 200 cells were analyzed in each group.

[b] Excluding gaps.

**Table 5.** Induction of chromosome aberrations by dental restoration nanocomposite (KelFil) in MRC-5 cells in the absence and presence of S9 mix after 24 h of incubation period. (Note: Ctg: chromatid gap; Csg: chromosome gap; Ctb: chromatid break; Csb: chromosome break; Exch: triradial, quadriradial and other chromatid exchanges; Dic + Oth: dicentric chromosomes and other aberrations (e.g. rings); >10: multiaberrant cells, Pol: polyploids except endoreduplications; End: endoreduplications; negative control: $\alpha$-MEM; MMC: mitomycin C; CP: cyclophosphamide monohydrate.)

| | | Structural aberrations[a] | | | | | | | | | | Numerical aberrations[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Test material | S9 mix | Ctg | Csg | Ctb | Csb | Exch | Dic+Oth | >10 | Loss of centromere | Total | Total aberrant cells[b] (%, in brackets) | Pol | End | Total (% in bracket) |
| 0.08 mg ml$^{-1}$ of KelFil | − | 0 | 5 | 0 | 0 | 0 | 1 | 0 | 5 | 11 | 5 (2.5) | 0 | 3 | 3 (1.5) |
| 0.79 mg ml$^{-1}$ of KelFil | − | 0 | 4 | 4 | 0 | 0 | 1 | 0 | 7 | 16 | 8 (4.0) | 0 | 1 | 1 (0.5) |
| 8.00 mg ml$^{-1}$ of KelFil | − | 0 | 6 | 2 | 0 | 0 | 1 | 0 | 5 | 13 | 7 (3.5) | 0 | 4 | 4 (2.0) |
| Negative control | − | 0 | 3 | 2 | 0 | 0 | 0 | 0 | 6 | 11 | 5 (2.5) | 0 | 4 | 4 (2.0) |
| 0.08 mg ml$^{-1}$ of KelFil | + | 1 | 3 | 0 | 0 | 0 | 0 | 0 | 6 | 10 | 5 (2.5) | 0 | 3 | 3 (1.5) |
| 0.79 mg ml$^{-1}$ of KelFil | + | 0 | 6 | 1 | 0 | 0 | 2 | 0 | 10 | 19 | 11 (5.5) | 0 | 3 | 3 (1.5) |
| 8.00 mg ml$^{-1}$ of KelFil | + | 0 | 8 | 2 | 0 | 0 | 0 | 0 | 8 | 18 | 8 (4.0) | 0 | 3 | 3 (1.5) |
| Negative control | + | 1 | 7 | 0 | 0 | 0 | 0 | 0 | 11 | 19 | 9 (4.5) | 0 | 2 | 2 (1.0) |
| MMC (0.05 $\mu$g ml$^{-1}$) | − | 3 | 32 | 62 | 3 | 1 | 19 | 0 | 23 | 143 | 86 (65.6)[c] | 0 | 7 | 7 (5.34) |
| CP (6 $\mu$g ml$^{-1}$) | + | 2 | 22 | 23 | 2 | 2 | 4 | 1 | 29 | 85 | 69 (34.5) | 0 | 6 | 6 (3.0) |

[a] 200 cells were analyzed in each group.

[b] Excluding gaps.

[c] 131 cells were analyzed in the MMC group.

regardless of treatment conditions (absence and presence of S9 mix) and incubation period when compared to the negative control ($p > 0.05$). Also no dose-relationship was observed. However, there was slight difference in the total aberrant cells between two treatment conditions, whereby the number of structural aberrations in treatment in the presence

**Table 6.** Induction of chromosome aberrations by dental restoration nanocomposite (KelFil) in MRC-5 cells in the absence and presence of S9 mix after 48 h of incubation period. (Note: Ctg: chromatid gap; Csg: chromosome gap; Ctb: chromatid break; Csb: chromosome break; Exch: triradial, quadriradial and other chromatid exchanges; Dic + Oth: dicentric chromosomes and other aberrations (e.g. rings); >10: multiaberrant cells, Pol: polyploids except endoreduplications; End: endoreduplications; negative control: $\alpha$-MEM; MMC: mitomycin C; CP: cyclophosphamide monohydrate.)

| | | Structural aberrations[a] | | | | | | | | | | Numerical aberrations[a] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Test material | S9 mix | Ctg | Csg | Ctb | Csb | Exch | Dic + Oth | >10 | Loss of centromere | Total | Total aberrant cells[b] (%, in brackets) | Pol | End | Total (% in bracket) |
| 0.08 mg ml$^{-1}$ of KelFil | − | 0 | 5 | 1 | 0 | 0 | 1 | 0 | 6 | 13 | 6 (3.0) | 0 | 2 | 2 (1.0) |
| 0.79 mg ml$^{-1}$ of KelFil | − | 0 | 5 | 2 | 0 | 0 | 0 | 0 | 9 | 16 | 5 (2.5) | 0 | 2 | 2 (1.0) |
| 8.00 mg ml$^{-1}$ of KelFil | − | 1 | 5 | 3 | 0 | 0 | 1 | 0 | 7 | 17 | 9 (4.5) | 0 | 4 | 4 (2.0) |
| Negative control | − | 1 | 6 | 0 | 0 | 0 | 0 | 0 | 5 | 12 | 3 (1.5) | 0 | 2 | 2 (1.0) |
| 0.08 mg ml$^{-1}$ of KelFil | + | 0 | 2 | 2 | 0 | 0 | 1 | 0 | 3 | 8 | 3 (1.5) | 0 | 3 | 3 (1.5) |
| 0.79 mg ml$^{-1}$ of KelFil | + | 0 | 7 | 4 | 0 | 0 | 0 | 0 | 10 | 21 | 11 (5.5) | 0 | 2 | 2 (1.0) |
| 8.00 mg ml$^{-1}$ of KelFil | + | 0 | 8 | 2 | 0 | 0 | 2 | 0 | 10 | 22 | 9 (4.5) | 0 | 4 | 4 (2.0) |
| Negative control | + | 0 | 9 | 0 | 0 | 0 | 0 | 0 | 13 | 22 | 9 (4.5) | 0 | 3 | 3 (1.5) |
| MMC (0.02 $\mu$g ml$^{-1}$) | − | 7 | 23 | 38 | 8 | 6 | 14 | 1 | 17 | 113 | 62 (73.8)[c] | 0 | 8 | 8 (9.5) |
| CP (6 $\mu$g ml$^{-1}$) | + | 12 | 17 | 48 | 0 | 6 | 17 | 2 | 37 | 139 | 76 (38.0) | 0 | 9 | 9 (4.5) |

[a] 200 cells were analyzed in each group.

[b] Excluding gaps.

[c] 84 cells were analyzed in the MMC group.

of S9 mix was higher compared to treatment without S9 mix. Even though KelFil produced endoreduplication, there was no significant difference when compared to the negative control. No polyploidy was found in any of the groups.

Positive controls (MMC and CP) induced strong chromosomal aberrations and the frequency of the aberrations increased from 6 to 48 h. In the case of MMC, the total number of cells counted for the 24 and 48 h incubation periods was 131 cells and 84 cells respectively. A total of 200 cells could not be counted as there was insufficient metaphase spreads available for analysis. The aberrations of chromosomes which were observed in the present study include chromatid and chromosome gaps, chromatid and chromosome breaks, chromosomal exchange, dicentric, polyploidy and endoreduplications.

Representative images of the metaphase spread of KelFil, negative and positive control groups are shown in figure 3. Positive controls (both MMC and CP) produced chromosomal aberrations. When compared to positive controls, KelFil treated groups did not produce any significant aberrations to the chromosomes.

## 4. Discussion

Appropriate testing needs to be performed to evaluate the effect of the material on the genetic material of cells. Even if the cells initially appear cytocompatible, genotoxic effects will eventually result in abnormal and reduced cell growth [26]. According to the International Organization for Standardization (ISO), (10993-Part 3) (2003) guidelines, genotoxicity testing is indicated where there is review of possibilities of composition in the medical device, such as dental material that interacts with genetic material, or when the medical device's chemical composition is not known [27]. As the public awareness of the safety of products has increased nowadays, biocompatibility testing of products to be commercialized is a must. A factor which determines the biocompatibility of a biomaterial is the genotoxicity effect. Among biomaterials in dentistry which have been found to be non-genotoxic are locally produced dental porcelain [28] and locally produced hydroxyapatite–silica nanocomposite [29].

Prior to the genotoxicity testing, MTT assay was conducted. This cytotoxicity test was conducted not only to study the cytotoxic effect of KelFil but also to determine the starting dosage for the experiments. The term 'cytotoxicity' is defined as the cascade of molecular events that interrupt macromolecular synthesis, resulting in unequivocal cellular and functional and structural damage [30]. Three different concentrations, which represented concentrations that produced 50%, 25% and 10% reduction of cell population, were selected for the genotoxicity test in order to study the genotoxicity of the test material at that particular level of cytotoxicity. From MTT assay, the concentration of KelFil was indirectly proportional to the viability of cells. This shows that the higher the concentration of KelFil extract,

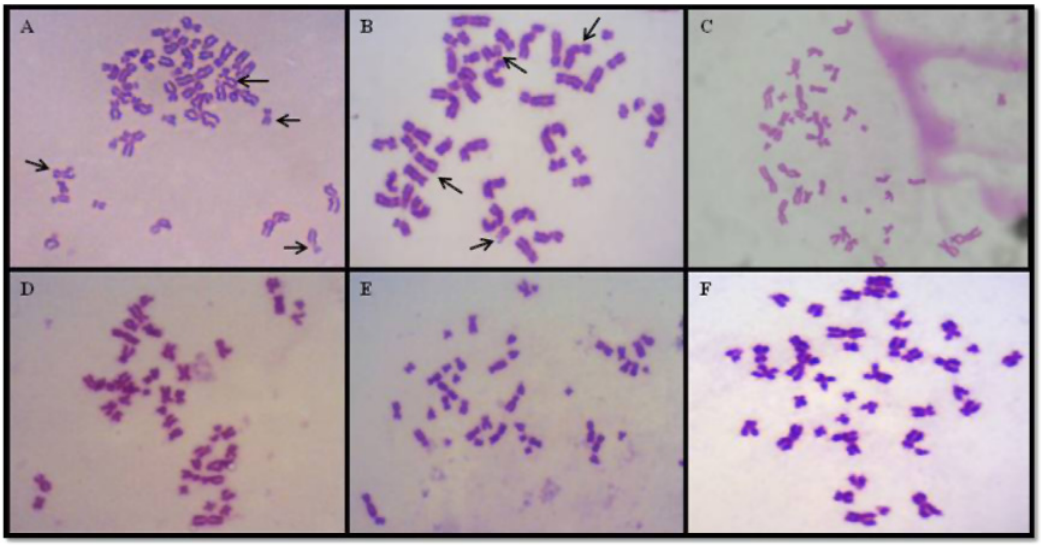


**Figure 3.** Representative images of KelFil groups, positive and negative controls. Aberrations in the chromosome are denoted by arrows. Among aberrations found in the positive control were chromosome and chromatid breaks, chromosome and chromatid gaps, deletions, loss of centromere and exchanges. (A) MMC; (B) CP; (C) negative control ($\alpha$-MEM); (D) 0.08 mg ml$^{-1}$ of KelFil; (E) 0.79 mg ml$^{-1}$ of KelFil; (F) 8.00 mg ml$^{-1}$ of KelFil.

the higher the inhibition of growth and proliferation of cells compared to more diluted KelFil extract.

DNA damage plays an essential role in mutagenesis, carcinogenesis and aging. Exposure to exogenous and endogenous agents which induce damage to DNA constantly put the eukaryotic genome under mutational stress. Genetic alteration induced by these agents could result in cell death. An alteration to genetic material is DNA double strand breaks (DSBs), which present a threat to viability of cells and stability of the genome if left unrepaired or when incorrectly repaired [31]. Research has shown that DSBs are the principal lesions in the formation process of CA [32–34]. DSB may lead to breakage of chromosomes when left unrepaired. DSB also could lead to mutations, chromosome rearrangements and oncogenic transformation when improperly repaired [35].

Duesberg *et al* suggested that cancer is a disease of chromosomal disorganization rather than a genetic disease [36]. Approximately 350 genes from 25 000 genes in the human genome have been linked with cancer development [37, 38]. Aberration in the function of these genes (also called cancer genes) may result from DNA polymorphism which occur naturally, changes in genome copy number (from amplification, deletion, chromosome loss or duplication), alteration in the gene and structure of chromosome (through chromosomal translocation, inversion or other rearrangement which cause chimeric transcripts or deregulated gene expression) and point mutations, which includes base substitutions, deletions, or insertions in coding regions and splice sites [39].

As there is a strong correlation between DNA damage, CA and the onset of cancer, it is important that the effect on genetic material induced by KelFil should be analyzed. KelFil showed negative results in both genotoxicity tests (comet assay and CA test) selected and was inferred to be non-genotoxic. The negative results in CA test showed that the test material did not cause any changes to chromosomes of the selected cell line *in vitro*.

In comet assay, the presence of comets indicated the DNA breakage in cells. Absence of any comet formation in KelFil treated groups represented the non-genotoxic property of the test material. $H_2O_2$ as a positive control induced DNA damage to the cells. $H_2O_2$ has been known as a damaging agent to DNA in various cell types [40]. It caused a spectrum of lesions in DNA, including single and DSBs. Damage to DNA due to $H_2O_2$ resulted from production of the hydroxyl radical [41]. Hydroxyl radical is an example of ROS [42]. Endogenous ROS resulted from normal oxidative metabolism in the mitochondria [43, 44]. However, when there is imbalance between the activity of antioxidant enzymes and the level of ROS, this will lead to build-up of superoxide and oxidative damage [45]. The presence of damage to DNA could be observed from the length of the comet tail. The damaged DNA migrated from the cathode to anode and is visualized as a tail. A greater extent of DNA damage resulted in an increased intensity of the tail, thus producing a higher percentage of tail moment. Damage caused by $H_2O_2$ was lethal to MRC-5 cells, as was observed in this present work.

There were also questions on the association between nanoparticles and cell membrane and whether the cells internalize some of the particles. Hirsch *et al* demonstrated the presence of visible internalized, insoluble nanomaterial agglomerates which interfere with the scoring of comet assay [46]. Our research found no visible sign of these internalized nanomaterials in MRC-5 cells through comet assay image analysis, thus eliminating the possibility of error/interruption in data analysis.

In CA test, the cytotoxicity effect of KelFil was studied by comparing the MI between the treatment and control groups (positive and negative control). This was essential considering that chromosomal aberrations are caused by

secondary mechanisms associated with cytotoxicity [47]. From the comparison of MI, it was found that MMC was highly cytotoxic to the cells, different from CP. Kelfil groups proved to cytocompatible based on the assessment of MI. From the analysis of metaphase spreads and chromosomal aberrations these damaging agents, MMC and CP proved to be genotoxic. Even though CP did not produce significant a cytotoxic effect, it was genotoxic to the cells, causing aberrations to the chromosomes. From CA analysis, MMC was found to be both cytotoxic and genotoxic. KelFil did not produce any significant aberrations to the chromosomes, unlike the positive controls.

Three incubation periods (6, 24 and 48 h) were employed in the CA test to investigate the genotoxicity effect of KelFil at different phases of the cell cycle. Cellular division of a typical eukaryotic cell cycle is illustrated by human cells in culture, which is approximately every 24 h. The $G_1$ phase might occur for about 11 h, the S phase about 8 h, the $G_2$ phase about 4 h, and the M phase about 1 h [48]. In the current study, the 6 h incubation period enables the study of the genotoxic potential of KelFil at the beginning of DNA synthesis ($G_1$ phase). Meanwhile the 24 and 48 h incubation periods are used to examine the effect of the test material on the chromosomes after one and two cell cycles respectively.

A composite consists of a combination of two or more materials. As dental filling material, KelFil would be implanted in the patient's oral cavity for a long time. The duration of implantation, the environment/condition in the oral cavity and the integrity of dental filling material may affect the toxicity differently. More than 30 various compounds have been extracted from polymerized dental composites. Among the detected components in the extract are major monomers, co-monomers, various additives, and reaction products [49]. The toxicity of dental resin composites was attributed to the release of residual monomers due to process of degradation or incomplete polymerization of the material [50]. These components could be brought into contact with mucosal tissues and may be extracted into dentine where they could diffuse towards the pulp. The process where leachable materials were released was affected by the degree of cure of the polymer network (e.g. the number of unreacted monomers), chemistry of the solvent, size and chemical composition of the elutable species [51]. An *in vitro* study suggested that TEGDMA (monomer resin) caused mutations, probably by binding covalently with DNA [52]. Schweikl *et al* determined the mutagenic effect of an aqueous extract of composite resins, whereby components of materials like TEGDMA might induce undesirable genotoxic effects to the chromosomes [53]. Exposure to an aqueous extract of the dental composite resins caused homologous mitotic recombination, point and chromosomal mutation effects [54]. Another agent used as in resin composites, camphorquinone (photoinitiator), also has been found to generate free radicals, including reactive oxygen species [55]. However, it has also been reported that the mutagenic effect of TEGDMA was completely absent in treatment with S9 mix [56]. Furthermore, Tanaka *et al* concluded that the polymerized resin composite containing BisGMA and TEGDMA does not leach a high quantity of monomers in aqueous extracts and most of the unbound monomers remained in the polymerized material [57]. This factor might have contributed to the non-genotoxic property of KelFil, as low or insignificant levels of leachable compounds released by the dental composite would have been incapable of producing a strong genotoxic effect.

As a nanocomposite, KelFil also contains nanosilica fillers among other components. Due to its smaller size (nano-size), greater surface area for reaction, and its ability to penetrate cells, nanosilica presents greater risks in imposing undesirable effect on biological organisms. Studies have demonstrated the role of the oxidative membrane and DNA damage as the main mechanism involved in the toxicity of micron-sized crystalline silica [58].

*In vitro* studies have been conducted to investigate the toxicity of nanosilica. Previous research by Gerloff *et al* showed that amorphous nanosilica (14 nm in size) caused oxidative DNA damage [59]. This was supported by Yang *et al*, who reported increased ROS generation after exposure to silica nanoparticles (SNPs) [60]. The toxicity of SNPs was dose- time- and size-dependent, where smaller particles were more toxic and capable of inducing generation of ROS [61]. Ye *et al* has compared the toxicity effect of three different sizes of SNPs (21, 48 and 86 nm sized), with the smallest size SNP (21 nm) proving capable of inducing generation of ROS [62]. Furthermore, Sohaebuddin *et al* also added that toxicity of SNP depended on the type of cell line used. Silica fillers used in KelFil are 10–20 nm in size [63]. Even though many researchers reported on the toxicity of nanosilica in this size range, no significant genotoxic effect was identified on DNA or on the chromosomes produced by KelFil. This is supported by another study which has proved the non-genotoxic property of amorphous silica (size range from 20 to <40 nm) through comet assay [64]. As only one type of cell line was used in the present study, which was MRC-5 cells, it is impossible to make a comparison of the toxicity effect of KelFil with other type of cells.

There was also a study which reported that non-cytotoxic SNPs are capable of inducing slight chromosome breakage and at higher concentrations might lead to mitotic arrest [65]. At the $IC_{10}$, $IC_{25}$ and $IC_{50}$ values of KelFil extracts which were used in the present study, no significant mutagenic effect on treated MRC-5 cells was detected at these non-cytotoxic concentrations.

There are also reports on the different effect of SNP on DNA and chromosomes, where nano-sized silica powder (size of 10 nm) was identified to elevate the level of DNA damage but did not cause damage to chromosomes [66]. We found negative results in both tests (comet assay and CA test), thus eliminating the different effects of KelFil on DNA and chromosomes. The method in which nanosilica was synthesized also plays a role in non-genotoxic property of KelFil. Napierska *et al* concluded that the exposure to SNPs is probably minimal for sols and gels as the nanoparticles are trapped/immobilized within their matrix [58]. Nanosilica used in KelFil was produced using a sol–gel technique. As the nanoparticle remains in the matrix and is not released into the

medium, this will reduce or might eliminate the chances for nanosilica to exhibit a genotoxic effect.

There are other nanocomposites containing nanosilica commercially available in the market. One of the popular commercial nanocomposites which contains nanosilica used in dentistry is Filtek$^{TM}$ Z500. The filler of FilTek$^{TM}$ Z500 consists of a combination of 20 nm nanosilica filler and agglomerated zirconia/silica particles in the size range of 0.6 to 1.4 $\mu$m. Other nanocomposites derived from the same brand as Filtek$^{TM}$ Z500 are Filtek$^{TM}$ Z350 (containing non-agglomerated/non-aggregated, 20 nm silica nanofiller and 0.6–1.4 nm agglomerated silica nanocluster) and Filtek$^{TM}$ Supreme XT (containing non-agglomerated/non-aggregated, 20 nm silica nanofiller and 0.6–1.4 nm agglomerated silica nanocluster) (3M ESPE Dental Products, St Paul, MN, USA) [67]. Tadin *et al* reported that Filtek$^{TM}$ Supreme XT was not genotoxic, based on *in vitro* comet assay on human leukocytes [68]. However, the genotoxic potential of Filtek$^{TM}$ Z350 and Filtek$^{TM}$ Z500 has not been found in the literature. Only cytotoxicity assay was performed for Filtek$^{TM}$ Z350 [69]. Another brand of nanocomposite containing nano-sized silica is Esthet X (containing nanofiller silica particle size 0.04 $\mu$m, 60% by volume) (Dentsply/Caulk, Milford, DE, USA) [70]. The elution of Esthet X was found to induce oxidative DNA damage when incubated with gingival fibroblasts [71], which is an indication of genotoxicity.

## 5. Conclusion

The MTT assay (cytotoxicity test) conducted in this study revealed that $IC_{10}$, $IC_{25}$ and $IC_{50}$ values for dental restoration nanocomposite (KelFil) were 0.08, 0.79 and 8.00 mg ml$^{-1}$ respectively. These three concentrations of KelFil extract were applied in genotoxicity tests. Both comet assay and CA test results show the non-genotoxic effect of KelFil in the current study. However, further *in vivo* tests are necessary to validate the safety of KelFil before commercialization.

## Acknowledgments

The authors would like to acknowledge the Craniofacial Science Laboratory staff, School of Dental Sciences, USM, Malaysia and USM Fellowship for the financial support. This work was supported by USM short term grant (304/PPSG/61311024). There is no competing interest for this study.

## References

[1] Ismail A 2004 Diagnostic levels in dental public health planning *Caries Res.* **38** 199–203
[2] Chan K H S, Mai Y, Kim H, Tong K C T, Ng D and Hsiao J C M 2010 Review: resin composite filling *Materials* **3** 1228–43
[3] Peutzfeldt A 1997 Resin composites in dentistry: the monomer systems *Eur. J. Oral. Sci.* **105** 97–116
[4] Mittal V 2009 Polymer layered silicate nanocomposites: a review *Materials* **2** 992–1057
[5] Saunders S A 2009 Current practicality of nanotechnology in dentistry. Part 1: focus on nanocomposite restoratives and biomimetics *Clin. Cosm. Inv. Dent.* **1** 47–61
[6] Onuki Y, Bhardwaj U, Papadimitrakopoulos F and Burgess D J 2008 A review of the biocompatibility of implantable devices: current challenges to overcome foreign body response *J. Diabetes. Sci. Technol.* **2** 1003–15
[7] ISO International Organization of Standardization 2009 Biological evaluation of medical devices. Part 1: evaluation and testing within a risk management process, ISO 10993-1
[8] OECD Organisation for Economic Co-operation and Development 2010 Environment directorate joint meeting of the chemicals committee and the working party on chemicals, pesticides and biotechnology: series on testing and assessment: No. 129. OECD Guidance document on using cytotoxicity tests to estimate starting doses for acute systemic toxicity tests Available from: http://iccvam.niehs.nih.gov/SuppDocs/FedDocs/OECD/OECD-GD129.pdf
[9] Vega-Avila E and Pugsley M K 2011 An overview of colorimetric assay methods used to assess survival or proliferation of mammalian cells *Proc. West. Pharmacol. Soc.* **54** 10–4
[10] Bean T A, Zhuang W C, Tong P Y, Eick J D, Chappelow C C and Yourtee D M 1995 Comparison of tetrazolium colorimetric and 51Cr release assays for cytotoxicity determination of dental biomaterials *Dent. Mater.* **11** 327–31
[11] Madle S, Korte A and Ball R 1987 Experience with mutagenicity testing of new drugs: view point of a regulatory agency *Mutat. Res.* **182** 187–92
[12] Wassom J 1992 Origins of genetic toxicology and the environmental Mutagen Society *Environ. Mol. Mut.* **14** 1–6
[13] Collins A R, Dobson V L, Dusinska M, Kennedy G and Stetina R 1997 The comet assay: what can it really tell us? *Mutat. Res.* **375** 183–93
[14] De Bont R and van Larabeke N 2004 Endogenous DNA damage in human: a review of quantitative data *Mutagenesis* **19** 169–85
[15] Natarajan A T 1976 Molecular aspects of the origin of chromosome structural changes *Biol. Zbl.* **95** 139–56
[16] Roberts J J 1978 The repair of DNA modified by cytotoxic, mutagenic, and carcinogenic chemicals *Advances in Radiation Biology* ed J T Lett and H Adler (New York: Academic) pp 211–435
[17] Singer B and Grunberger D 1983 *Molecular Biology of Mutagens and Carcinogens* (New York: Plenum) (Mutagens and Carcinogens—A Molecular Approach) pp 1–347
[18] OECD Organisation for Economic Co-operation Development 1997 OECD guideline for the testing of chemicals. *In vitro* mammalian chromosome aberration test—473
[19] Pfeiffer P, Goedecke W and Obe G 2000 Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations *Mutagenesis* **15** 289–302
[20] Rahim T N A T, Mohamad D, Ismail A R and Akil H M 2011 Synthesis of nanosilica fillers for experimental dental nanocomposites and their characterisations *J. Phys. Sci.* **22** 93–105
[21] Ritter A V 2002 Talking with patients: light-curing *J. Esthet. Restor. Dent.* **14** 128
[22] ISO International Organization of Standardization 2007 Biological evaluation of medical devices. Part 12: sample preparation and reference materials, ISO 10993-12
[23] Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays *J. Immunol. Methods* **65** 55–63
[24] Singh N P, McCoy M T, Tice R R and Schneider E L 1988 A simple technique for quantitation of low levels of DNA damage in individual cells *Exp. Cell. Res.* **175** 184–91

[25] Silva J, de Freitas T R O, Marinho J R, Speit G and Erdtmann B 2000 An alkaline single-cell gel electrophoresis (comet) assay for environmental biomonitoring with native rodents *Genet. Mol. Biol.* **23** 241–5
[26] Keong L C and Halim A S 2009 *In vitro* models in biocompatibility assessment for biomedical-grade chitosan derivatives in wound management *Int. J. Mol. Sci.* **10** 1300–13
[27] ISO International Organization of Standardization 2003 Biological evaluation of medical devices. Part 3: tests for genotoxicity, carcinogenicity and reproductive toxicity, ISO 10993-3
[28] Noushad M, Kannan T P, Husein A, Abdullah H and Ismail A R 2009 Genotoxicity evaluation of locally produced dental porcelain—an *in vitro* study using the Ames and comet assays *Toxicol. In Vitro* **23** 1145–50
[29] Musa M, Kannan T P, Masudi S M and Ismail A R 2012 Assessment of DNA damage caused by locally produced hydroxyapatite–silica nanocomposite using comet assay on human lung fibroblast cell line *Mol. Cell. Toxicol.* **8** 53–60
[30] Aldridge W N 1993 The biochemical principles of toxicology *Experimental Toxicology: The Basic Issues* 2nd edn, ed D Anderson and D M Conning (Cambridge: Royal Society of Chemistry) pp 56–78
[31] Summers K C, Shen F, Sierra Potchanant E A, Phipps E A, Hickey R J and Malkas L H 2011 Phosphorylation: the molecular switch of double-strand break repair *Int. J. Proteom.* **2011** 373816
[32] Natarajan A T and Obe G 1978 Molecular mechanisms involved in the production of chromosomal aberrations. I. Utilization of neurospora endonuclease for the study of aberration production in G2 stage of the cell cycle *Mutat. Res.* **52** 137–49
[33] Obe G, Johannes C and Schulte-Frohlinde D 1992 DNA doublestrand breaks induced by sparsely ionizing radiation and endonucleases as critical lesions for cell death, chromosomal aberrations, mutations and oncogenic transformation *Mutagenesis* **7** 3–12
[34] Bryant P 1998 The signal model: a possible explanation for the conversion of DNA double-strand breaks into chromatid breaks *Int. J. Radiat. Biol.* **73** 243–51
[35] Obe G, Pfeiffer P, Savage J R, Johannes C, Goedecke W, Jeppesen P, Natarajan A T, Martínez-López W, Folle G A and Drets M E 2002 Chromosomal aberrations: formation, identification and distribution *Mutat. Res.* **504** 17–36
[36] Duesberg P, Li R, Fabarius A and Hehlmann R 2005 The chromosomal basis of cancer *Cell. Oncol.* **27** 293–318
[37] Futreal P A, Coin L, Marshall M, Hubbard T, Wooster R, Rahman N and Stratton M R 2004 A census of human cancer genes *Nature Rev. Cancer.* **4** 177–83
[38] The Wellcome Trust Sanger Institute Cancer Genome Project 2005 Available from: www.sanger.ac.uk/genetics/CGP
[39] Willman C L and Hromas R A 2006 Genomic alterations and chromosomal aberrations in human cancer *Cancer Medicine* vol 7, ed D W Kufe, R C Bast, W N Hait, W K Hong, R E Pollock, R R Weichselbaum, J F Holland and E Frei (London: BC Decker inc) pp 104–34
[40] Kim S Y, Kim R H, Huh T L and Park J 2000 a-phenyl-Nt-butylnitrone protects oxidative damage to HepG2 cells *J. Biochem. Mol. Biol.* **34** 43–6
[41] Collins A R 1999 Oxidative DNA damage, antioxidants, and cancer *Bioessays* **21** 238–46
[42] Scandalios J G 2002 Oxidative stress response—what have genome-scale studies taught us? *Genome. Biol.* **3** 1019.1–6
[43] Hermes-Lima M 2004 Oxidative stress and medical sciences *Functional Metabolism: Regulation, and Adaptation* ed K B Storey (New York: Wiley) pp 269–82
[44] Traber M G 2006 Relationship of vitamin E metabolism and oxidation in exercising human subjects *Br. J. Nutr.* **96** S34–7
[45] Jornot L, Petersen H and Junod A F 1998 Hydrogen peroxide-induced DNA damage is independent of nuclear calcium but dependent on redox-active ions *Biochem. J.* **335** 85–94
[46] Hirsch C, Kaiser J, Wessling F, Fischer K, Roeslein M, Wick P and Krug H F 2011 A novel comprehensive evaluation platform to assess nanoparticle toxicity *in vitro* *J. Phys.: Conf. Ser.* **304** 012053
[47] Hilliard C A, Armstrong M J, Bradt C I, Hill R B, Greenwood S K and Galloway S M 1998 Chromosome aberrations *in vitro* related to cytotoxicity of nonmutagenic chemicals and metabolic poisons *Environ. Mol. Mut.* **31** 316–26
[48] Cooper G M 2000 *The Cell: A Molecular Approach* 2nd edn (Sunderland, MA: Sinauer Associates)
[49] Spahl W, Budzikiewicz H and Geurtsen W 1998 Determination of leachable components from four commercial dental composites by gas and liquid chromatography/mass spectrometry *J. Dent.* **26** 137–45
[50] Wataha J C, Rueggeberg F A, Lapp C A, Lewis J P, Lockwood P E, Ergle J W and Mettenburg D J 1999 *In vitro* cytotoxicity or resin-containing restorative materials after aging in artificial saliva *Clin. Oral. Invest.* **3** 144–9
[51] Ferracane J L 1994 Elution of leachable components from composites *J. Oral. Rehabil.* **21** 441–52
[52] Schweikl H and Schmalz G 1999 Triethylene glycol dimethacrylate induces large deletions in the hprt gene of V79 cells *Mutat. Res.* **438** 71–8
[53] Schweikl H, Hiller K, Bolay C, Kreissl M, Kreismann W, Nusser A, Steinhauser S, Wieczorek J, Vasold R and Schmalz G 2005 Cytotoxic and mutagenic effects of dental composite materials *Biomaterials* **26** 1713–9
[54] Arossi G A, Dihl R R, Lehmann M, Reguly M L and de Andrade H H 2010 Genetic toxicology of dental composite resin extracts in somatic cells *in vivo* basic *Clin. Pharmacol. Toxicol.* **107** 625–9
[55] Volk J, Geurtsen W, Leyhausen G, Beckedorf A, Rahmer H and Zieman C 2008 Genotoxicity of camphorquinone in HGF and mouse lymphoma cells *Int. Asssociation for Dental Research, Division Pan European Federation (PEF, Mtg) (London)*
[56] Schweikl H, Schmalz G and Spruss T 2001 The induction of micronuclei *in vitro* by unpolymerized resin monomers *J. Dent. Res.* **80** 1615–20
[57] Tanaka K, Taira M, Shintani H, Wakasa K and Yamaki M 1991 Residual monomers (TEDGMA and Bis-GMA) of a set visible-light-cured dental composite resin when immersed in water *J. Oral. Rehabil.* **18** 353–62
[58] Napierska D, Thomassen L C, Lison D, Martens J A and Hoet P H 2010 The nanosilica hazard: another variable entity *Part. Fibre Toxicol.* **7** 39
[59] Gerloff K, Albrecht C, Boots A W, Forster I and Schins R P F 2009 Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells *Nanotoxicology* **3** 355–64
[60] Yang H, Wu Q, Tang M, Kong L and Lu Z 2009 Cell membrane injury induced by silica nanoparticles in mouse macrophage *J. Biomed. Nanotechnol.* **5** 528–35
[61] Napierska D, Thomassen L C, Rabolli V, Lison D, Gonzalez L, Kirsch-Volders M, Martens J A and Hoet P H 2009 Size-dependent cytotoxicity of monodisperse silica nanoparticles in human endothelial cells *Small* **5** 846–53
[62] Ye Y, Liu J, Xu J, Sun L, Chen M and Lan M 2010 Nano-$SiO_2$ induces apoptosis via activation of p53 and Bax mediated by oxidative stress in human hepatic cell line *Toxicol. In Vitro* **24** 751–8

[63] Sohaebuddin S K, Thevenot P T, Baker D, Eaton J W and Tang L 2010 Nanomaterial cytotoxicity is composition, size, and cell type dependent *Part. Fibre Toxicol.* **7** 22
[64] Barnes C A *et al* 2008 Reproducible comet assay of amorphous silica nanoparticles detects no genotoxicity *Nano Lett.* **8** 3069–74
[65] Gonzalez L *et al* 2010 Exploring the aneugenic and clastogenic potential in the nanosize range: A549 human lung carcinoma cells and amorphous monodisperse silica nanoparticles as models *Nanotoxicology* **4** 382–95
[66] Choi H S, Kim Y J, Song M, Song M K and Ryu J C 2011 Genotoxicity of nano-silica in mammalian cell lines *Toxicol. Environ. Health Sci.* **3** 7–13
[67] 3M ESPE Dental Products *Filtek™ Dental Restorative* Available at http://solutions.3m.com/wps/portal/3M/en_US/3M-ESPE-NA/dental-professionals/products/brand/filtek/
[68] Tadin A, Marovic D, Galic N, Milevoj A, Medvedec Mikic I and Zeljezic D 2012 Genotoxic biomonitoring of flowable and non-flowable composite resins in peripheral blood leukocytes *Acta Odontol. Scand.* at press
[69] Huang X, Lei L, Zheng B, Zhang M, Zhong S and Lin Y 2007 *In vitro* cytotoxicity test of three dental restorative material *The Preliminary Program for 8th Annual Scientific Mtg of IADR Chinese Division*
[70] Dentsply/Caulk *Esthet X HD* Available at www.esthetxhd.com/about-esthetx-hd
[71] Sykes B, Tipton D, Garcia-Godoy F and Babu J 2011 Inflammatory and toxicity effects of dental composites on gingival fibroblasts *The Preliminary Program for IADR/AADR/CADR 89th General Session and Exhibition (March, 2011)*