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Mutagenicity Testing and DNA Damage Analysis of Malaysian White Portland Cement Using Ames Test and Comet Assay

Abstract—The aim of the current study was to determine the mutagenic effect and DNA damage of Malaysian white Portland cement (MWPC) and White mineral trioxide aggregate (WMTA) using Ames test and Comet assay. The cytotoxicity of WMTA and MWPC was evaluated using MTT assay, mutagenicity using Ames test in the absence and presence of metabolic activation system (S9 mix) on *Salmonella* strains (TA98, TA102, TA1535, TA1537 and TA1538) and DNA damage using Comet assay on human periodontal ligament fibroblast (HPLF) cell line. Concurrent negative and positive controls were included. Based on the MTT assay, the IC50 for WMTA and MWPC was 18.71 and 19.91 mg/ml, IC25 was 3.33 and 3.55 mg/ml and IC10 was 0.59 and 0.63 mg/ml respectively. These values were further employed in Comet assay. Ames test revealed that WMTA and MWPC did not cause any mutagenic effect as the number of revertant colonies was less than that of the negative control. In the Comet assay, no significant comet formation was found in HPLFs treated with WMTA, MWPC and negative control except the positive control. Hence, it can be concluded that MWPC is non-mutagenic and does not cause any DNA damage under the present test conditions.

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1 INTRODUCTION

A number of materials have been used as root-end filling materials comprising composite resins, cavit, glass ionomer cements, gutta-percha, amalgam, zinc oxide-eugenol cements, and gold foil [1]. Due to their shortcomings in sensitivity to the presence of moisture, microleakage, and varying degrees of toxicity, Mineral Trioxide Aggregate (MTA) was created and presented as a root end filling material [2]. Additionally, it has been utilized as a feasible option for different clinical applications, such as capping of pulp tissue, root end closure and for repairing furcal perforations [3]. Initially, a grey formulation of MTA was produced called grey MTA (GMTA), but due to aesthetic concerns, white formulation was then manufactured (ProRoot MTA, Tulsa Dental, Tulsa, OK, USA). Many *in vitro* and *in vivo* studies of White Mineral Trioxide Aggregate (WMTA) have demonstrated a good sealing ability, favourable biological profile, and induction of formation of hard tissue similar to that of GMTA [4-8]. Despite these positive values, MTA has some shortcomings which includes long setting time (~3h), poor handling properties, and high cost [2,9-10].

In general, MTA consists of 75% Portland cement, 20% bismuth oxide, and 5% calcium sulphate as a setting modifier [11]. Based on X-ray diffraction, MTA and Portland Cement (PC) were almost identical macroscopically and microscopically [12]. Yet, another study affirms that PC contains similar chemical elements and physical properties as MTA [13]. Asgary and colleagues [14] reported that there was no significant difference between the dominant compounds in both WMTA and White PC (WPC) except for the presence of bismuth oxide in WMTA

that is necessary as a radio pacifier. Various studies and reported cases regarding good sealing ability and favourable biological profile continue to support WPC as a potential alternative material to WMTA [7,14-15]. Hence, WPC has acquired extraordinary interest as a viable substitute due to its lower cost and wide availability compared to WMTA [13,16].

Based on *in vitro* studies on mouse lymphoma cells, human endothelial cells, Chinese hamster ovary cells, human peripheral lymphocytes cells and human osteosarcoma cells, it has been reported that WPC has good biocompatibility [17-20]. Malaysian WPC (MWPC) (Aalborg, Malaysia) is widely available in Malaysia. The main purpose of this research was to investigate the potential use of MWPC as an alternative substitute material to WMTA for use in furcation perforation repair, pulp capping, pulpotomy, apexification and root end filling material in Malaysia. The information available on the biocompatibility for the potential use of Malaysian WPC (MWPC) in clinical dentistry is scant [21] which warrants further investigations. In ensuring the compatibility and toxic effect of biomaterials, International Organization for Standardization guidelines has listed several tests for biological evaluation of medical devices for both *in vitro* and *in vivo* to assess the biocompatibility of the material. Thus, the aim of the present study was to evaluate the mutagenicity effects of MWPC using Ames test and DNA damage using Comet assay on human periodontal ligament fibroblast (HPLF) cell line.

2 MATERIALS AND METHODS

Preparation and extraction of test materials

WMTA (Dentsply, USA) was prepared according to the manufacturer's instructions and MWPC (Aalborg, Malaysia) based on a previous study [21-22]. After mixing powder and liquid, both materials were applied into Perspex moulds (diameter 10 mm x 2 mm thickness) which were then left to set. After 24 hours, both materials were weighed and sterilized using ultraviolet radiation for 30 min (15 min on each side). They were then incubated in culture media or sterile distilled water according to the respective experiment at 37°C for 7 days [17]. Subsequently, both materials were filtered to obtain the extracts.

Cytotoxicity testing

Cell culture

Human periodontal ligament fibroblasts, HPLFs (LONZA, USA) were cultured in alpha minimum essential medium (α-MEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin solution incubated 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 solution before centrifuging at 1200 rpm for 5 min. The cells were counted by re-suspending in 1 ml of medium and later 10 µl of trypan blue was added and mixed with 10 µl of cell suspension. The mixtures were then pipetted onto the counting chamber of the haemocytometer using capillary action. The cells were counted using the formula, cell count = Av x 2n x 10^4 cells/ml, where Av is the average number of cells and n is the dilution factor.

MTT assay

MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was followed according to guidelines proposed by Mosmann [23]. Confluent HPLFs were trypsinised and seeded as mentioned earlier. 1 x 10^4 HPLFs were counted and seeded in 96 well plates. For treatment groups, various concentrations of extracts of WMTA and MWPC (200, 100, 50, 25, 12.5, 6.25, 3.125, 1.52 mg/ml) were prepared by serial dilution before adding it to the cells. These concentrations were selected based on ISO 10993-12 [24], which recommends the use of a maximum dose of 0.2 g/ml followed by serial dilution. Control consisted of only the cell suspension and complete medium. The complete medium comprised α-MEM, 10% FBS and 1% penicillin-streptomycin solution. The plate was incubated for 72 hours in humidified incubator at 37°C with 5% CO_2. Then, the MTT (Calbiochem, Germany) solution (with a final concentration of 0.5 mg/ml) was added into the wells before incubating the plate for further 3-4 hours.

Following the incubation period, formazan crystals formed by the viable cells was dissolved by addition of 100 µl of DMSO (dimethyl sulfoxide, Merck, Germany) to each well and the plate was shaken gently to make sure that the DMSO dissolved completely. The absorbance was read at 570 nm using ELISA plate reader (Tecan, Switzerland). The relative viability of the cells treated with MWPC, and MTA extracts were compared to control cells. The cell viability was calculated using the formula below:

% Cell viability = [A_{570} of treated cells] / [A_{570} of control cells] x 100%

The viability of treated HPLFs was calculated with regard to the untreated cell control which was set at 100% viability. The values of each well were plotted on a graph to derive the inhibitory concentration (IC) [25]. For determination of the inhibitory concentration IC10, IC25, and IC50, dose response graph was constructed from series of different concentrations of MWPC and MTA using GraphPad prism 6 software. These three inhibitory concentrations of both materials' extracts were applied in the Comet assay.

Mutagenicity assay

Tester strains

Five strains of *Salmonella typhimurium* bacteria were used, TA98, TA102, TA1535, TA1537 and TA1538. The tester strains were obtained from MOLTOX (USA). All the tester strains were checked for their genetic integrity for histidine dependence, biotin dependence, histidine/biotin dependence, rfa marker (crystal violet) and the presence of plasmid pKM101 (ampicillin resistance) and plasmid pAQ1 (tetracycline resistance) for strain TA102 before the experiments were carried out [26].

Ames test

The *Salmonella* mutagenicity assay was carried out according to the method described by Mortelmans and Zeiger [26]. The test was carried out at the highest dose of 5 mg/plate, and four lower doses of 2.5, 1.25, 0.625, and 0.3125 mg/plate which was obtained by a dilution with a progression of 2. Various aliquots (5, 2.5, 1.25, 0.625, 0.3125 mg/plate) were tested for mutagenicity in the pre-incubation assay.

The pre-incubation assay

An overnight culture of 0.1 ml of *Salmonella* strain was pipetted into the sterile tube along with 0.05 ml of test materials and 0.5 ml of metabolic activation, S9 (liver microsomal enzyme, S9 homogenate, Sigma, USA), or sodium phosphate buffer (pH 7.4). This step was repeated later by replacing the test materials with positive and negative control (sterile distilled water). All the solutions above were then mixed and incubated for 20 min at 37°C. Two ml of molten top agar maintained at temperature of 43-48°C was then added into each tube. The mixtures were mixed and poured onto the surface of glucose minimal agar plates. When the top agar had hardened, the plate was inverted and incubated for 48 hours at 37°C in the incubator. The colonies were counted by a colony counter (aCOLyte, SYMBIOSIS, UK) and the result was expressed as the number of revertant colonies per plate (mean value from triplicates obtained in one experiment). In this study, the two-fold rule was selected to interpret the results. A compound is considered non-mutagenic if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments. If the number of revertant colonies is more than 2 folds over the solvent control, then, it is considered mutagenic [26].

Preparation and treatment of HPLFs for Comet assay

HPLFs were cultured until it reached confluence. Then, the cells were trypsinized as mentioned previously. 1 x 10^5 of HPLFs were counted and seeded into 6-well plate together with 5 ml of complete medium. The cells were incubated overnight to allow for cell attachment. The extraction of WMTA and MWPC was prepared as mentioned previously. After incubation, the medium was discarded, and the cells were washed using phosphate buffered saline (PBS). Subsequently, the cells were exposed to the

WMTA and MWPC extract for 24 hours at the concentrations of IC10, IC25, and IC50 values obtained from the MTT assay. For negative control, the cells were treated with complete medium and for positive control, the cells were treated with 100 µM of hydrogen peroxide (H_2O_2) which was prepared beforehand and incubated with cells for 15 min at 4°C.

Comet assay

The Comet assay kit from Trevigen, (USA) was employed in the current study and the protocol was followed according to guidelines proposed by Tice and his colleagues under alkaline conditions [27]. The complete medium from 6-well plate was discarded and the cells were washed twice with PBS. Then, the cells were trypsinised with 0.5 ml of trypsin before adding 1 ml of complete medium. 0.5 ml of the cell solution was then transferred into 1.5 ml of sterile centrifuge tube. The tubes were kept 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. Finally, all the tubes were incubated at 4°C for 5 min before centrifuging again at 2500 rpm for 5 min.

Then, low melting agarose (LMA) was added into each tube and carefully resuspended with the cell pellet and deposited on pre-coated slides. LMA was used as it melts at low temperature which will not denature proteins and nucleic acids in comparison to other normal melting agarose (NMA) that can cause DNA damage leading to false positives. The slides were placed on ice for 5 min or left at 4°C for 10 min before submerging in lysis buffer at 4°C for 1 hour. Slides were then placed in pre-chilled alkaline solution (pH>13) for 20 min for DNA unwinding. Electrophoresis was conducted at 25 V and 300 mA for 20 min using an electrophoresis chamber (Aurogene, USA). This was followed by washing of the slides thrice with cold neutralization buffer for 5 min each, fixed in 99% ethanol for 5 min and dried at room temperature overnight. Ethidium bromide (Sigma, USA) was used for DNA staining and comet visualization. The slides were prepared in triplicates for each treatment. Slides were examined at 40x magnification using a fluorescence microscope (Olympus BX41, Japan). Comet assay IV software from Perceptive Instruments (UK) was used for evaluation of the head and tail regions of the comets. The tail moment was chosen as the parameter and a total of 50 cells were scored randomly from each slide. Results are presented as the mean tail moment ± SEM.

Statistical analyses

The results of Ames test are expressed as mean revertant colonies ± SE. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by post-hoc (Tukey's method) using IBM SPSS Statistics Version 22. The difference was considered significant when the *p*-value was <0.05.

3 RESULTS

Cytotoxicity assessment using MTT assay

Both tested materials showed similar pattern on viability of cells (Figures 1 and 2). Both materials exhibited 60 to 90% cell viability at all concentrations except for 100 and 200 mg/ml. Both the tested materials displayed a higher value which was more than 90% cell viability at the concentration of 100 mg/ml. This indicates that both the tested materials were non-toxic to HPLFs at this concentration, whereas, at 200 mg/ml concentration, WMTA showed 65.43% of cell viability compared to MWPC which exhibited 46.05% cell viability indicating that WMTA exhibited mild cytotoxicity while, MWPC showed moderate cytotoxicity towards HPLFs at this particular concentration. From both the graphs, the IC was determined using GraphPad prism (version 6.07) software. The software demonstrated that the IC50 was 18.71 mg/ml for WMTA and 19.91 mg/ml for MWPC. On the other hand, the concentration for IC25 for WMTA and MWPC were 3.33 mg/ml and 3.55 mg/ml respectively. As for IC10, they were 0.59 mg/ml and 0.63 mg/ml for WMTA and MWPC respectively. These concentrations were then applied in Comet assay.

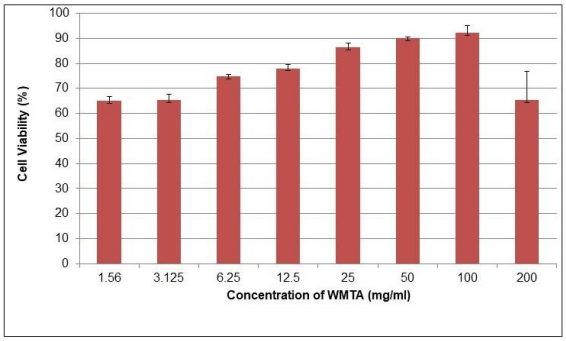

Figure 1. Cell viability of White Mineral Trioxide Aggregate on human periodontal ligament fibroblast cell line after 72 hours.

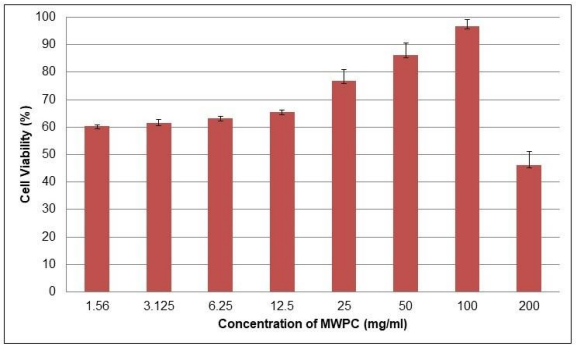

Figure 2. Cell viability of Malaysian White Portland Cement on human periodontal ligament fibroblast cell line after 72 hours.

Mutagenicity assessment using Ames test

The number of revertant colonies for TA98, TA1535, TA1537 and TA1538 for both the tested materials and conditions displayed low reversion rates (Tables 1 and 2). In contrast, TA102 strain displayed high number of revertant colonies as shown in Tables 1 and 2 because of the phenotype of the strain which is a wild type. There was no dose-dependent relationship between the concentrations and the number of revertant colonies for both the tested materials in the absence or presence of S9 mix. Concomitantly, the number of revertant colonies of all the positive controls showed high reversion rates both in absence and presence of S9 mix.

Table 1: Mutagenicity of *Salmonella typhimurium* strains in the absence of S9

Treatment	mg/ml	Number of revertant colonies (Mean±SE)				
		TA98[a]	TA102[b]	TA1535[c]	TA1537[d]	TA1538[a]
WMTA	5	19.00±1.73	175.00±1.73	27.33±3.18	36.67±0.33	26.00±0.58
	2.5	17.67±1.45	182.33±1.45	24.00±1.73	37.67±0.88	25.33±1.76
	1.25	17.67±1.33	165.00±1.73	25.33±3.38	32.67±1.76	23.67±1.86
	0.625	17.33±1.76	174.00±2.31	23.67±3.18	36.67±1.20	23.00±0.58
	0.3125	17.00±0.58	183.33±2.03	24.33±2.33	34.67±2.40	25.00±1.73
MWPC	5	25.33±1.45	174.00±1.53	25.33±0.88	26.33±1.33	24.00±0.58
	2.5	26.33±0.88	176.00±1.15	27.33±0.67	23.00±1.73	27.00±0.58
	1.25	15.33±1.45	179.00±2.08	23.00±0.58	26.67±0.88	25.67±0.33
	0.625	19.67±2.19	145.00±2.65	26.00±1.00	23.67±2.18	24.00±1.16
	0.3125	23.67±1.86	168.00±1.53	21.67±1.20	21.67±0.88	22.67±1.20
Negative control	0	25.33±1.45	273.00±2.52	33.00±1.53	41.67±0.56	36.33±1.3
Positive control	0.0005-0.05	144.33±2.33	449.33±2.96	219.00±0.58	215.00±1.53	243.00±1.73

Positive control: [a]4-nitro-o-phenylenediamine (Sigma, USA), [b]Mitomycin C (Calbiochem, Germany), [c]Sodium azide (Wako Pure, Japan), [d]9-aminoacridine (Acros Organic, USA)

Table 2: Mutagenicity of *Salmonella typhimurium* strains in the presence of S9

Treatment	mg/ml	Number of revertant colonies (Mean±SE)				
		TA98	TA102	TA1535	TA1537	TA1538
MTA	5	24.33±2.19	256.33±1.20	36.67±1.76	50.33±2.03	28.33±2.60
	2.5	25.00±2.08	242.67±1.45	49.00±0.58	51.67±2.60	31.33±0.88
	1.25	23.00±0.58	263.67±2.03	34.67±1.45	46.00±1.15	27.00±1.53
	0.625	25.67±2.40	236.33±1.20	31.00±2.08	54.67±1.76	37.00±1.53
	0.3125	27.67±0.88	226.33±1.20	31.00±1.53	53.33±2.91	23.67±1.86
MWPC	5	31.33±1.76	252.00±0.58	38.00±0.58	35.67±0.67	25.00±0.58
	2.5	33.00±1.53	273.00±1.15	45.00±1.73	33.67±0.67	31.00±1.53
	1.25	36.00±1.73	259.67±2.91	40.00±1.15	45.67±2.85	27.00±0.58
	0.625	33.67±1.45	237.67±1.45	39.00±0.58	40.00±1.16	36.67±1.45
	0.3125	36.33±1.20	251.33±0.88	40.33±0.88	45.33±0.88	21.67±0.67
Negative control	0	36.67±0.88	274.67±1.76	43.33±1.76	55.00±0.58	52.00±0.58
Positive control[a]	0.0005-0.05	170.33±0.33	482.33±2.33	265.33±2.91	223.33±2.40	365.00±2.65

[a]2-aminoanthracene (Acros Organic, USA).

DNA damage assessment using Comet assay

The mean tail moment for all tested groups of WMTA, MWPC and negative control group showed low values, in contrast to positive control which displayed extremely higher values for both parameters (Table 3). Besides, no comet formation was observed in HPLFs when treated with all the tested groups of WMTA and MWPC, respectively based on the Figures 3 and 4. Similar findings were also found with the negative control group (Fig. 5A). In this instance, undamaged cells appeared round in shape and no formation of tail was observed. In contrast, Figure 5B showed an obvious formation of comets when treated with positive control. The formation of comets indicates that the DNA was damaged and migrated towards the anode during electrophoresis.

A parametric statistical test was chosen for analysis of tail moments. The tail moments of all the tested groups were analysed using one-way analysis of variance (ANOVA) followed by post-hoc (Tukey's test). The post-hoc comparison (Tukey's test) was performed to compare the differences in tail moments between two independent tested groups. The results are summarised in Table 4. Based on the results, no significant difference was observed in tail moment between all the groups of WMTA and MWPC (p>0.05). Tail moments between all the tested groups of WMTA, MWPC and negative control group were also not significantly different (p>0.05). However, a significant difference was observed in tail moments between WMTA, MWPC, negative and the positive control groups (p<0.05).

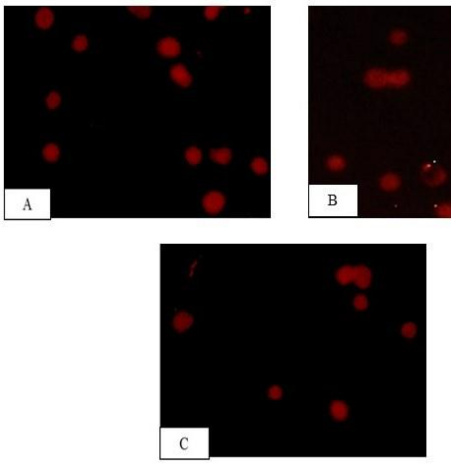

Figure 3. Images of human periodontal ligament fibroblasts treated with WMTA A: 0.59 mg/ml of WMTA; B: 3.33 mg/ml of WMTA; C: 18.71 mg/ml of WMTA.

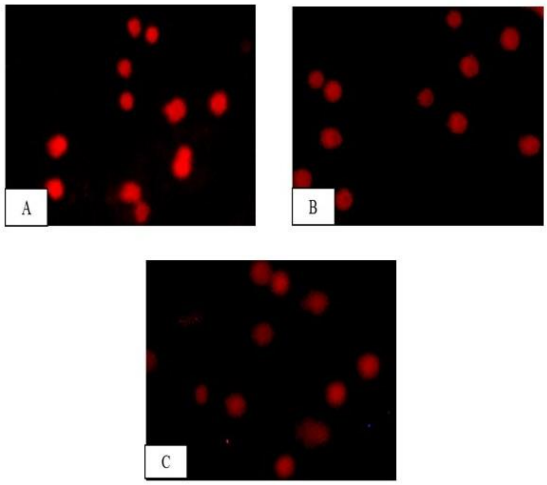

Figure 4. Images of human periodontal ligament fibroblasts treated with MWPC A: 0.63 mg/ml of MWPC; B: 3.55 mg/ml of MWPC; C: 19.91 mg/ml of MWPC.

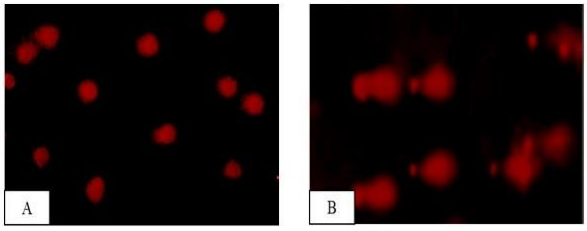

Figure 5. Images of human periodontal ligament fibroblasts treated with negative and positive controls A: Negative control (α-MEM); B: Positive control (100 µM H_2O_2).

Table 3: Mean tail moments of all the tested groups

Group	Mean tail moment[a] ± SEM	*p*-value
Negative control (α-MEM)	0.0214 ± 0.0008	
0.59 mg/ml of WMTA	0.0346 ± 0.0015	
3.33 mg/ml of WMTA	0.0328 ± 0.0023	
18.71 mg/ml of WMTA	0.0332 ± 0.0024	0.001
0.63 mg/ml of MWPC	0.0344 ± 0.0024	
3.55 mg/ml of MWPC	0.0470 ± 0.0106	
19.91 mg/ml of MWPC	0.0372 ± 0.0006	
Positive control (100 µM H_2O_2)	45.4969 ± 0.2745	

[a]Mean of three independent tests

Table 4: Post-hoc comparisons of tail moments of human periodontal ligament fibroblasts treated with WMTA and MWPC extract (Tukey's test). The level of significance was set at (p<0.05)*

Group-wise comparison	*p*-value
Negative control versus 0.59 mg/ml of WMTA	1.000
Negative control versus 3.33 mg/ml of WMTA	1.000
Negative control versus 18.71 mg/ml of WMTA	1.000
Negative control versus 0.63 mg/ml of MWPC	0.205
Negative control versus 3.55 mg/ml of MWPC	0.999
Negative control versus 19.91 mg/ml of MWPC	0.944
0.59 mg/ml of WMTA versus 0.63 mg/ml of MWPC	0.282
3.33 mg/ml of WMTA versus 3.55 mg/ml of MWPC	1.000
18.71 mg/ml of WMTA versus 19.91 mg/ml of MWPC	0.970
0.59 mg/ml of WMTA versus 3.33 mg/ml of WMTA	1.000
0.59 mg/ml of WMTA versus 18.71 mg/ml of WMTA	1.000
3.33 mg/ml of WMTA versus 18.71 mg/ml of WMTA	1.000
0.63 mg/ml of MWPC versus 3.55 mg/ml of MWPC	0.444
0.63 mg/ml of MWPC versus 19.91 mg/ml of MWPC	0.774
3.55 mg/ml of MWPC versus 19.91 mg/ml of MWPC	0.999
Positive control versus negative control	0.001*
Positive control versus 0.59 mg/ml of WMTA	0.001*
Positive control versus 3.33 mg/ml of WMTA	0.001*
Positive control versus 18.71 mg/ml of WMTA	0.001*
Positive control versus 0.63 mg/ml of MWPC	0.001*
Positive control versus 3.55 mg/ml of MWPC	0.001*
Positive control versus 19.91 mg/ml of MWPC	0.001*

4 DISCUSSION

MWPC consists of several compositions that comprise calcium (Ca), oxygen (O), carbon (Ca), silica (Si), aluminium (Al) and magnesium (Mg) which are also present in WMTA. Small percentages of sulphur (S) and potassium (K) were merely detected in MWPC while bismuth oxide was detected in WMTA only. Meanwhile, the phase analyses of MWPC and WMTA consists of calcium cement formula (calcium silicate, tricalcium silicate, tricalcium silicon penta-oxide, and calcite). Only calcium sulphite and potassium carbonate were detected in MWPC compared to WMTA and Egyptian white Portland cement (EWPC) as reported [21]. These components may react differently with regard to their toxicity and genotoxicity.

MTT assay was conducted to study the cytotoxicity effect of WMTA and MWPC extract on HPLFs and to determine the dose for Comet assay. The cell viability for both materials showed favourable biological response towards HPLFs. The results of this study were in agreement with studies conducted on human osteosarcoma [17], on mouse lymphoma cell [28]. on Chinese hamster ovary [20], and on dental pulp stem cells [22], which demonstrated favourable biological responses. This indicates that MWPC was comparable to WMTA and WPC that originated

from other countries like Spain, Brazil, and South Korea [17,19,28-30]. Three different concentrations were selected from each material to assess the DNA damage of WMTA and MWPC at that particular level of cytotoxicity. The three different concentrations for each material represented the concentrations that produced 50%, 25%, and 10% reduction of cell population that were employed in Comet assay. Based on the MTT assay, the concentrations of WMTA and MWPC extract demonstrated favourable biological profile towards HPLFs. In the current study, genotoxicity was assessed using Ames test and Comet assay based on ISO 10993-3 guidelines [31]. Gene mutations are reliably evaluated using bacteria or prokaryotic cells when they cause any changes in the growth requirements of the cells [32]. Ames test was first introduced by Ames and colleagues in 1973 who described an improved bacterial test system in detecting and classifying carcinogens and mutagens with *Salmonella* strains [33]. Nowadays, Ames test is commonly used worldwide as an initial screening of mutagenic potential for new biomaterials [26,34]. The mutagenic potential of WMTA and MWPC was evaluated using Ames test using five strains of *Salmonella typhimurium* (TA98, TA102, TA1535, TA1537 and TA1538). These tester strains were used to detect any potential mutagenic materials which lead to two classes of gene mutations which are base pair and frameshift mutations [33]. Performing this test may indicate as the starting point in assessing the mutagenicity potential of both materials. A minimum fold increase, 2-fold has been used in this study to interpret between mutagenic and non-mutagenic response as compared to negative control [35]. In the present study, low reversion rate was displayed by both the materials either in the absence or presence of S9 mix and the reversion rate was less than of the negative control. This demonstrated that WMTA and MWPC did not cause any potential mutations as the number of revertant colonies was less than the number of revertant colonies of negative control. In contrast, higher reversion rate was seen in positive control as expected. Positive controls that were used in this study were mutagenic to the tested strains. These positive controls results coincide with previous studies [36-37]. They found that the positive controls gave higher reversion rate colonies when compared to the number of revertant colonies of negative control. In addition, according to Mortelmans and Zeiger [26], the study is only reliable and valid when the number of revertant colonies of positive control is twice than the number of revertant colonies of negative control. This demonstrated that all the positive controls exhibited mutagenic responses towards all the tested *Salmonella* strains whereby it indicates the validity and reliability of the experiments conducted.

In this case, no mutagenicity was detected from all five *Salmonella* strains either in the absence or presence of S9 mix when treated with WMTA and MWPC. This may probably be due to the chemical compositions in WMTA and MWPC which were not mutagenic. Ca, O, C, Si, Al, and Mg were the major chemical compositions that exist in both WMTA and MWPC as reported through Energy-dispersive X-ray micro-analysis (EDX) [22]. Higher percentage of Al was detected in MWPC which was more than 2-3 times in WMTA. Although Al is known as a common toxic element in PC based cement, the results showed that MWPC did not exhibit any potential mutagenic activity [38]. Thus, it can be assumed that Al in MWPC did not induce mutagenicity towards all the *Salmonella* strains tested. Besides, no dose-dependent relationship was observed based on the increasing concentrations for both materials in the tested conditions. A previous study examined WMTA and other commonly used root end filling materials using Ames test [39]. They concluded that WMTA did not induce any mutagenic potential towards TA98 and TA1535 *Salmonella* strains which corresponds to the results obtained in the present study. The results showed low number of revertant colonies for both materials when S9 mix was not added to the culture medium. In contrast, when S9 mix was added to the culture, the number of revertant colonies was higher for TA102. This may be due to the characteristics of strain TA102 which is very sensitive and responsive towards the mutagenic substances compared to other strains [40]. S9 mix was used due to inability of the bacteria to metabolize chemicals via cytochrome

P450 as in mammals. Thus, S9 is necessary to be added to mimic the mammalian metabolic condition so that the mutagenic potential of the materials can be assessed [41]. Nevertheless, as mentioned previously, only WMTA had been investigated so far [39], and there are no previous reports on the evaluation of mutagenic potential of WPC.

The *in vitro* alkaline Comet assay was used in the present study due to its sensitivity in assessing the DNA damage that are induced by genotoxic materials in cells. Comet assay is varied depending on pH of buffers that are used in lysis step. Singh and colleagues introduced and developed the *in vitro* alkaline Comet assay which utilized pH>13 [42]. Since then, it has gained a wide range of applications and become popular among the researchers compared to other genotoxicity tests [27]. The alkaline Comet assay is capable of detecting various forms of DNA damage such as double strand breaks (DSBs), single strand breaks (SSBs), alkaline labile sites (ALSs) that are expressed as SSB which is associated with incomplete excision repair [42]. To the best of our knowledge, this is the first study employing alkaline Comet assay to assess the DNA damage caused by MWPC on HPLFs. The results showed that WMTA and MWPC extracts did not affect the HPLFs on all selected concentrations including the negative control. This was displayed in the present study (Figures 3, 4 and 5A), whereby, the unaffected HPLFs appeared round in shape with no formation of tail. WMTA and MWPC also showed similar results. The unaffected cells were indicated by the mean tail moment and % DNA in tail for all the tested groups of WMTA, MWPC and negative control group which demonstrated lower values of the parameters. Ahmed et al. reported that there were some variations in the compositions of MWPC [22]. The variations were calcium sulphite and potassium carbonate which were absent in WMTA. This result is consistent with the findings of Braz et al. [19] which showed that alkaline Comet assay failed to detect the presence of DNA damage on human peripheral lymphocytes although the WPC originated from Brazil and may have some variations in its compositions. By contrast, the formation of tail in the comets was observed when treated with positive control, H_2O_2 as presented in Figure 5B. H_2O_2 is a typical DNA damage-inducing agent and has been used frequently as a positive control in the Comet assay studies [43,44]. H_2O_2 is easily permeable into the cell membrane and converted to hydroxyl radicals by a nonenzymatic process which induces DNA damage to the cells [45]. This indicates that H_2O_2 caused DNA damage on HPLFs which is in agreement by Musa et al. [46], where H_2O_2 was used as the positive control in assessing locally produced hydroxyapatite-silica on human lung fibroblast cell line using alkaline Comet assay. They used the same concentration of H_2O_2 (100 µM) as in the present study. In addition, it has been reported that higher the concentration of H_2O_2 used, greater is the DNA damage in the cells [43].

5 CONCLUSION

The cytotoxicity profile of WMTA and MWPC on human periodontal ligament fibroblast cells was comparable with each other. Both WMTA and MWPC did not show any significant difference in the mutagenic activity on *Salmonella* strains, TA98, TA102, TA1535, TA1537 and TA1538 based on Ames test and no DNA damages were present based on Comet assay. Thus, it can be concluded that MWPC is non-mutagenic and does not cause DNA damage under the present test conditions which provides additional evidence on the potential use of MWPC in clinical dentistry application.

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