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Original Article

Genotoxic evaluation of synthetic hydroxyapatite using mammalian bone marrow chromosome aberration test

Thirumulu Ponnuraj Kannan[a,b,*], Nik Ahmad Shah Nik Lah[c], Azlina Ahmad[a], Siti Fatimah Ramli[d], Narazah Mohd Yusoff[d], Ab Rani Samsudin[e]

[a] School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[b] Human Genome Centre, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[c] Occupational Safety and Health Unit, Health Campus, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan, Malaysia.
[d] Advanced Medical and Dental Institute, Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Pulau Pinang, Malaysia.
[e] College of Dentistry, University of Sharjah, P.O. Box 27272, Sharjah, United Arab Emirates.

* Corresponding author: kannan@usm.my

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Abstract Some of the beneficial biocompatible properties of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]; the major component and an essential ingredient of normal bone and teeth, are that it is rapidly integrated into the human body and will bond to bone forming indistinguishable unions. But, before new materials are approved for medical use, mutagenesis systems to exclude cytotoxic, mutagenic or carcinogenic properties are applied worldwide. This study aimed to detect any chromosomal aberrations induced by the synthetic hydroxyapatite granules [Manufactured by Universiti Sains Malaysia, (USM) Penang, Malaysia] in the bone marrow cells of mice. The mitotic indices of the groups treated with synthetic hydroxyapatite granules did not show any significant difference as compared to the negative control group treated with distilled water. Also the groups of mice treated with synthetic hydroxyapatite granules and distilled water did not induce significant change in chromosome aberrations as compared to the positive control group treated with Mitomycin C. The mitotic indices and chromosomal analyses indicate that under the present test conditions, synthetic hydroxyapatite granules (manufactured by USM) are non cytotoxic and do not induce chromosome aberrations in the bone marrow cells of mice.

Keywords: Chromosome aberration, cytotoxicity, genotoxicity, hydroxyapatite, mice.

Introduction

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] is the major component and an essential ingredient, of normal bone and teeth. It is hydroxyapatite that makes up bone mineral and the matrix of teeth. There is a necessity for replacing bone substance, which has been lost due to traumatic or non-traumatic events. The lost bone can be replaced by endogenous or exogenous bone tissues. The use of endogenous bone (autogenous bone) has remained the golden standard in restoring bone defects but it involves additional surgery (Wojciech and Masahiro, 1998). Moreover, the endogenous bone is available only in limited quantities. The major disadvantage of exogenous bone (allograft bone) is the risk of viral or bacterial transmission and the human body may reject them. When implanted into the body, the synthetic hydroxyapatite is accepted by the body and because of its porous nature, allows normal tissue integration to take place. Hydroxyapatite has excellent biocompatibility and when placed in contact with viable bone result in osseoconduction and osseointegration. Hydroxyapatite does not cause inflammatory response, toxic reactions or foreign body giant cell reactions (Costantino *et al*., 1991). Due to these reasons, there is a growing need for fabrication of artificial hard tissue replacement implants (Wojciech and

Masahiro, 1998). Biomaterials may provide a solution for these problems. An ideal bone substitute should be biocompatible, which means acceptance of the implant to the tissue surface (Costantino *et al*., 1991). Solid hydroxyapatite can be carved and trimmed to the requirements of the correction. Because bone and soft tissue growth into the pores of the implant occurs quickly after implantation, the implant is securely held in place. Over time the implant is partially resorbed and replaced by natural bone. Moreover, when a bone substitute with some mechanical strength is needed, hydroxyapatite seems most appropriate as it represents the natural mineral in human bone and approximates the structure of cancellous bone (Cooke, 1992). However, synthetic hydroxyapatite is relatively a new biomaterial and before new materials is approved for medical use, mutagenesis systems to exclude cytotoxic, mutagenic or carcinogenic properties are applied worldwide (Katzer *et al*., 2002). This study aims to find whether synthetic hydroxyapatite granules (Manufactured by USM, Penang) produce any chromosome aberrations in the bone marrow cells of mice.

Materials and methods

Ethical approval

Approval to carry out this research was obtained from the Animal Ethics Committee of Universiti Sains Malaysia, Health Campus as per the reference PPSG/07 (A)/044 dated 28th May 2003. The experimental procedures including handling of the animals were strictly adhered to as per the guidelines.

Experimental animals

Adult healthy Swiss albino strain of mice aged 10 to 12 weeks was chosen as our experimental animal. Each group of animals comprised of 5 females and 5 males. The animals were reared in cages and commercial pellet diet (Gold Coin Feedmills Sdn. Bhd., Malaysia) and distilled water were given *ad libitum.*

Experimental groups

Negative control: The animals in this group were injected with 0.5 ml of distilled water intra peritoneally and were sacrificed after 24 hours.

Positive control: The animals in the positive group were injected with a positive control chemical, Mitomycin C at the dose rate of 1.5 mg/kg body weight. They were sacrificed 24 hours after treatment.

Treatment group: The mice in the treatment group were divided into 3 and were injected with synthetic hydroxyapatite granules (Manufactured by USM, Penang) dispersed in 0.5 ml of distilled water at the dose level of 2000 mg / kg body weight and the mice were sacrificed at different intervals. The experimental protocol is presented in Table 1.

Table 1 Experimental protocol

Group	Number of animals		Time of harvesting bone marrow following treatment
	Males	Females	
Distilled water (Negative control)	5	5	24 hours
Mitomycin C (Positive control)	5	5	24 hours
Treatment (Synthetic hydroxyapatite granules)	5	5	6 hours
	5	5	24 hours
	5	5	48 hours

Biomaterial

The biomaterial used in this study is a porous form of synthetic hydroxyapatite granules, manufactured by the School of Materials and Mineral Resources Engineering, Universiti Sains Malaysia, Penang, Malaysia. The granules ranged from 100 to 200 microns in size.

Experiment

All the animal experiments were conducted in the Animal Research and Service Centre and the culture work in the Human Genome Centre of Universiti Sains Malaysia, Health Campus. The animals were subjected to the treatment protocol as per their respective group shown in Table 1. After appropriate time of treatment, the animals were injected intra peritoneally with colchicine (4 mg/kg) 90 minutes prior to sacrifice. The animals were then sacrificed by cervical dislocation. The bone marrow cells were collected from both the femurs by flushing in warm (37°C)

potassium chloride solution in centrifuge tubes and the volume was adjusted to 15 ml following which they were incubated at 37°C for 20 minutes. This was followed by fixation using 3:1 Methanol:Acetic acid. The slides were prepared as per the procedure described elsewhere (Henegariu *et al*., 2001). The slides were stained with 10 per cent Leishman's stain in phosphate buffer saline (pH 6.8) for 5 minutes, rinsed in distilled water and air dried.

Mitotic Index and screening for chromosome aberrations

The mitotic index was calculated to determine the cytotoxicity as follows:

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

A total of 1000 cells were counted per culture to determine the mitotic index values whereas, a total of 100 cells per animal were scored for the detection of chromosomal aberrations, both numerical and structural. The slides were screened under oil objective (100x) of Leica CTR MIC Image Analyzer and pictures were taken using Leica Chantal software (Leica Microsystems, Germany). The statistical analyses was carried out using SPSS 12.0.1 (Release: July 2004) to calculate the mean and standard error and Duncan's multiple range test was carried out to detect significance of differences, if any, among the different experimental groups.

Results

The biomaterial used in the present study to evaluate the mutagenicity was porous form of synthetic hydroxyapatite granules manufactured by School of Materials and Mineral Resources Engineering, USM, Penang, Malaysia. The colchicine injected at a concentration of 4 mg/kg body weight 90 minutes prior to harvesting, hypotonic treatment using 0.075 M KCl (0.56 per cent) at 37°C for 20 minutes and fixation using 3:1 Methanol:Acetic acid all yielded consistent results with regard to chromosome preparation and good number of analyzable cells at metaphase. More evenly controlled chromosome spreading was obtained by following the protocol as described (Henegariu *et al*., 2001). The mean mitotic index values for the different treatment groups are shown in Table 2.

Chromosomal analyses

No statistically significant chromosome aberrations were observed, either numerical or structural in the chromosomes of mice treated with distilled water and those treated with synthetic hydroxyapatite granules (Fig. 1a). However, mice injected with Mitomycin C showed several fold increase in chromosome aberration levels, validating the experimental conditions used (Fig. 1b). The aberrations included breaks, deletions and gaps. The details are presented in Table 3.

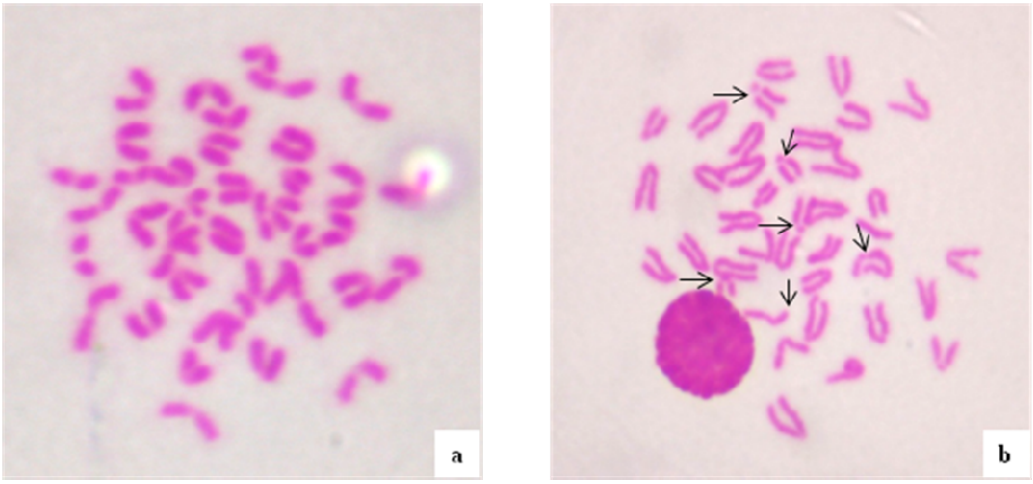

Fig. 1 Metaphase spread of mice **a:** treated with hydroxyapatite (normal chromosomes without any numerical and structural aberrations) **b:** treated with positive control (Mitomycin C); arrows indicate the chromosome aberrations.

Table 2 Mean mitotic indices observed in bone marrow cells of mice exposed to different treatments

Treatment	Dose	Time of harvesting bone marrow cells of mice (in hours)	Mitotic Index (%)	
			Mean ± SE	SEV
Mitomycin C (Positive control)	1.5 mg / Kg body weight	24	1.34 ± 0.11	a
Distilled water (Negative control)	0.5 ml	24	3.29 ± 0.06	b
Synthetic hydroxyapatite granules	2 g / kg body weight	6	3.07 ± 0.05	b
Synthetic hydroxyapatite granules	2 g / kg body weight	24	3.16 ± 0.10	b
Synthetic hydroxyapatite granules	2 g / kg body weight	48	3.05 ± 0.08	b

1000 cells were scored per each sample. Each value is the mean of 10 samples. SE is the standard error of the mean; SEV is the statistical evaluation obtained following ANOVA test and subsequent comparing with Duncan's new multiple range test. Values in vertical column (Mean ± SE) with the same alphabet (b,b) are not significantly different at 5% level whereas values with different alphabets (a,b) are significant at 5% level.

Table 3 Chromosome aberrations observed in bone marrow cells of mice exposed to different treatments

Treatment	Dose	Time of harvesting bone marrow cells of mice (in hours)	Chromosome aberrations	Chromosome aberrations per cell	
				Mean ± SE	SEV
Mitomycin C (Positive control)	1.5 mg/ kg body weight	24	84	0.084 ± 0.011	b
Distilled water (Negative control)	0.5 ml	24	5	0.005 ± 0.002	a
Synthetic hydroxyapatite granules	2 g / kg body weight	6	7	0.007 ± 0.004	a
Synthetic hydroxyapatite granules	2 g / kg body weight	24	3	0.003 ± 0.002	a
Synthetic hydroxyapatite granules	2 g / kg body weight	48	5	0.005 ± 0.002	a

100 metaphase spreads were scored per each sample. Each value is the mean of 10 samples. SE is the standard error of the mean; SEV is the statistical evaluation obtained following ANOVA test and subsequent comparing with Duncan's new multiple range test. Values in vertical column (Mean ± SE) with the same alphabet (a,a) are not significantly different at 5% level whereas values with different alphabets (a,b) are significant at 5% level.

Discussion

The chromosome aberration test using cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and or carcinogens (Ishidate *et al*., 1998). The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in the bone marrow cells of animals, usually rodents (Adler, 1984; Preston *et al*., 1987; Richold *et al*., 1990; Hayashi *et al*., 1994). Bone marrow is a highly vascularized tissue and it contains a population of rapidly cycling cells that can be readily isolated and processed (Environmental Protection Agency, 1998). This chromosome aberration test is especially relevant to assessing mutagenic hazard in that it allows consideration of factors of *in vivo* metabolism, pharmacokinetics, and DNA-repair processes although these may vary among species and among tissues. One of the factors that that contribute to the inter-individual differences in drug-metabolizing enzymes (DMEs) expression and drug metabolism include the male and female subjects (Cotreau *et al.*, 2005; Kennedy, 2008). Studies carried out in rats and mice have established that sex-based differences in liver function also characterize many phase II DMEs, including sulfotransferases (Klaassen *et al.,* 1998; Clodfelter *et al.,* 2007; Kocarek *et al.,* 2008), glutathione transferases (Srivastava and Waxman, 1993; Clodfelter *et al.,* 2007; Knight *et al.,* 2007), and UDP-glucuronosyltransferases (Takeuchi *et al.,* 2004; Clodfelter *et al.,* 2007; Buckley and Klaassen, 2007). Moreover, the Health Effects Test Guidelines (EPA, 1998) recommends the use of at least five female and five male animals per experimental and control group for this purpose.

Colchicine

Colchicine or its synthetic analog; colcemid, is a spindle fiber poison. It disrupts the microtubules or spindle fibers and prevents the cells in metaphase of mitosis from proceeding to anaphase. The animals were injected with colchicine at a dose of 4 mg per kg body weight 1½ hours prior to sacrifice, which is in accordance with the reports of the earlier workers (Das *et al*., 1996; Choudhury *et al*., 1997; Poddar *et al*., 2000; Giri *et al*., 2002). Then, the mice were sampled at an appropriate interval thereafter. The injection of colchicine 1½ hours prior to sacrifice yielded quite a number of metaphase spreads for analyses.

Harvesting of bone marrow

Two things are very important in hypotonic treatment: the right strength of solution and the length of time in solution. This hypotonic treatment causes the cells and nuclei to swell so that the chromosomes will be separated more widely. Hypotonic treatment using 0.075 M KCl (0.56 per cent) at 37°C for 20 minutes yielded good number of swollen cells in the present study as per the protocol of Henegariu *et al*, (2001). More evenly controlled chromosome spreading was obtained by following the method of Octavian Henegariu *et al.* (2001), who opined that just 1 to 1.5 ml of fixative is sufficient for fixation of the cells followed by micro centrifugation at 6000 to 7000 rpm for 1-2 minutes. In the present study, however, the conventional method was followed.

Positive control chemical

Positive controls should employ a known clastogen at exposure levels expected to give a reproducible and detectable increase over background, which demonstrate the sensitivity of the test system. Positive control concentrations should be chosen so that the effects are clear (EPA, 1998). Mitomycin C that was used as the positive control chemical in the present study produced chromosomal aberrations in the bone marrow cells of mice.

Mitotic Index

The values of mitotic index are an indication of the degree of cytotoxicity. A reduction greater than 50 per cent in the mitotic index value when compared to the control indicates the cytotoxic nature of the test substance. In the present study there was no significant difference ($p < 0.05$) in the mitotic index values between the treatment group with synthetic

hydroxyapatite granules (3.07 ± 0.05 for 6 hours; 3.16 ± 0.10 for 24 hours and 3.05 ± 0.08 for 48 hours) and the negative control group treated with distilled water (3.29 ± 0.06 for 24 hours). However, the positive control group treated with Mitomycin C, showed significant difference (1.34 ± 0.11 for 24 hours) as compared to the negative control. This indicates that the biomaterial in the present study, synthetic hydroxyapatite granules are non cytotoxic to the bone marrow cells of mice. Only in the case of positive control group treated with Mitomycin C, significant difference was observed in the mitotic indices when compared to the negative control group. This could be attributed to the cytotoxic nature of the positive control chemical.

Chromosomal analysis

Three different treatment timings were followed in this study, namely, 6, 24 and 48 hours. In the case of rodents, the central sampling time is 24 hours (EPA, 1998). Since the cell cycle kinetics can be influenced by the test substance, Health Effects Test Guidelines (EPA, 1998) recommends one earlier and one later sampling interval adequately spaced within the range of 6 to 48 hours. Except the positive control, which was treated with Mitomycin C, all the other groups did not show any significant numerical or structural chromosomal aberrations. The groups of mice treated with synthetic hydroxyapatite granules and distilled water did not induce significant change ($p < 0.05$) in chromosome aberrations as compared to the group treated with Mitomycin C. Their respective values of chromosome aberration per cell are 0.007 ± 0.004 for 6 hours; 0.003 ± 0.002 for 24 hours and 0.005 ± 0.022 for 48 hours for hydroxyapatite treated groups, 0.005 ± 0.002 for distilled water treated group and 0.084 ± 0.011 for Mitomycin C treated group. The production of chromosome aberration is a complex cellular process and the mechanism of chromosome breakage and rejoining is not yet completely understood (Palitti, 1998; Savage *et al*., 1988). All the cells that were scored, both in the negative control and treatment groups (with hydroxyapatite) had a normal complement of 2n=40 without any significant chromosomal aberrations. An increase in polyploidy may indicate that the test substance has the potential to induce numerical chromosome aberrations. An increase in endoreduplication may indicate that the test substance has the potential to inhibit cell-cycle progression (Lücke-Huhle, 1983; Huang *et al*., 1983). This indicates that the result is negative and the test substance, synthetic hydroxyapatite granules (porous form) does not induce any chromosome aberrations in cultured mammalian cells (EPA, 1998).

Conclusions

The value of the mitotic indices and the absence of chromosome aberrations indicate that the biomaterial, synthetic hydroxyapatite granules (Manufactured by School of Materials and Mineral Resources Engineering, USM) is non cytotoxic and does not induce both numerical and structural chromosomal aberrations under the present test conditions in the bone marrow cells of mice.

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