CONTENTS

ABSTRAK

UJIAN KEROSAKAN KROMOSOM *IN VIVO* BAGI HIDROKSIAPATIT DI DALAM MENCIT

Pengenalan: Dalam bidang pergigian, pencarian untuk mendapatkan material yang ideal sebagai penganti kepada tulang yang rosak telah lama dijalankan. Material hidroksiapatit telah digunakan secara meluas sebagai pengganti kepada tulang rosak. Ini kerana hidroksiapatit adalah komponen mineral yang asas kepada tulang. Tambahan pula kdanungan hidroksiapatit adalah bioserasi yang cepat berpadu dengan badan manusia, tulang terutamanya dan menghasilkan pembentukan tulang baru. Bagaimanapun, sebelum material yang baru dihasilkan dibenarkan penggunaannya dalam bidang perubatan, satu analisa mutagenesis yang dapat mengesan sitotoksisiti, mutagenisiti dan bahan karsinogenik diwajibkan di seluruh dunia. Tujuan kajian in vitro menggunakan mamalia ini adalah untuk mengesan sebarang perubahan/ kerosakan pada kromosom akibat pendedahan kepada bahan ujian iaitu granul sintetik hidroksiapatit (dihasilkan oleh USM, Pulau Pinang) dalam sel sum-sum tulang mencit.

Objektif:

1. Untuk menstdanardkan teknik penghasilan kromosom daripada sum-sum tulang mencit.
2. Untuk mengkaji indeks mitotik haiwan berkenaan selepas didedahkan kepada granul sintetik hidroksiapatit
3. Untuk mendapatkan kadar perubahan/ kerosakan pada kromosom sel sum-sum tulang mencit yang disebabkan oleh granul sintetik hidroksiapatit

Bahan dan kaedah: Mencit (*Mus musculus* – strain Swiss Albino) telah digunakan sebagai model haiwan dalam kajian ini. Mencit itu dibahagikan kepada 3 kumpulan iaitu kawalan positif, negatif dan kumpulan yang menerima rawatan. Kumpulan yang menerima rawatan terdiri daripada tiga sub-kumpulan berdasarkan kepada masa sum-sum tulang tersebut diambil. Setiap kumpulan terdiri daripada 5 mencit jantan dan 5 mencit betina. Kumpulan kawalan negatif akan disuntik dengan air suling, kawalan positif dengan Mitomycin C dan kumpulan rawatan disuntik dengan granul sintetik hidroksiapatit secara intra-peritoneal. Pembahagian sel dihentikan pada peringkat

metafasa dengan suntikan colchicines secara intra-peritoneal pada 1 ½ jam sebelum mencit berkenaan dimatikan. Selepas itu, sum-sum tulang berkenaan diambil daripada bahagian femur mencit berkenaan. Sel-sel tersebut kemudiannya diberi rawatan hipotonik dan *fixative*, kromosom disediakan dan dianalisis untuk melihat kesan sitotoksisiti dan perubahan/ kerosakan pada kromosom.

Hasil: Peratusan indeks mitotik bagi kumpulan yang menerima rawatan granul sintetik hidroksiapatit (3.07 ± 0.05 untuk 6 jam; 3.16 ± 0.10 untuk 24 jam dan 3.05 ± 0.08 untuk 48 jam) tidak menunjukkan perbezaan yang signifikan ($p < 0.05$) jika dibdaningkan dengan kumpulan kumpulan kawalan negatif yang diberi suntikan air suling (3.29 ± 0.06 untuk 24 jam). Bagaimanapun, jika dibdaningkan dengan kumpulan kawalan positif, yang diberi suntikan Mitomycin C, terdapat perbezaan yang signifikan (1.34 ± 0.11 untuk 24 jam). Kumpulan yang dirawat dengan granul sintetik hidroksiapatit dan kawalan negatif juga tidak menunjukkan perbezaan yang signifikan ($p < 0.05$) dalam perubahan/ kerosakan pada kromosom jika dibandingkan dengan kawalan positif yang diberi Mitomycin C. Jumlah kerosakan kromosom per sel adalah 0.007 ± 0.004 untuk 6 jam; 0.003 ± 0.002 untuk 24 jam dan 0.005 ± 0.022 untuk 48 jam bagi kumpulan granul sintetik hidroksiapatit, 0.005 ± 0.002 untuk kawalan negatif yang diberi suntikan air suling dan 0.084 ± 0.011 untuk kawalan positif, yang diberi suntikan Mitomycin C.

Kesimpulan: Hasil negatif yang diperolehi dalam kajian ini menunjukkan yang dalam ujian ini, granul sintetik hidroksiapatit(dihasilkan oleh USM) adalah tidak sitotoksik dan tidak menyebabkan kerosakan kromosom dalam sum-sum tulang mencit.

Kata kunci: *sitotoksisiti, kerosakan kromosom, hidroksiapatit, mencit*

ABSTRACT

IN VIVO CHROMOSOME ABERRATION TEST FOR HYDROXYAPATITE IN MICE

Introduction: Dentistry has searched for the ideal material to place in osseous defects for many years and hydroxyapatite has been extensively used for such purposes. Hydroxyapatite is the primary mineral component of bone. The beneficial biocompatible properties of hydroxyapatite 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. The purpose of this mammalian *in vivo* test is to detect any chromosomal aberrations induced by the test compound, synthetic hydroxyapatite granules (Manufactured in USM, Penang) in the bone marrow cells of mice.

Objectives:

1. To standardize the technique of production of chromosomes from bone marrow cells of mice
2. To study the mitotic index after exposure of animals to synthetic hydroxyapatite granules
3. To determine the extent of chromosomal aberrations induced by synthetic hydroxyapatite granules in the bone marrow cells of mice

Materials and methods: Mice (*Mus musculus* - Swiss Albino strain) were used as the animal models in this study. The mice were divided into three groups, namely, positive control, negative control and treatment groups. The treatment group comprised of three sub-groups based on the timing of harvesting bone marrow. Each group consisted of five males and five females respectively. The negative control group animals were injected with distilled water, positive control with Mitomycin C and the treatment groups with synthetic hydroxyapatite granules intra-peritoneally. The cell division was arrested at metaphase by injecting colchicine intra peritoneally 1½ hour prior to sacrificing the animals and later the bone marrow samples were collected from the femur. The cells were then subjected to hypotonic and fixative treatments; chromosomes were prepared and analyzed for indication of cytotoxicity and for chromosome aberrations.

Results: The mitotic indices in percentage of the groups treated 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) did not show any significant difference ($p < 0.05$) as compared to the negative control group treated with distilled water (3.29 ± 0.06 for 24 hours) unlike the positive control group treated with Mitomycin C, which showed significant difference (1.34 ± 0.11 for 24 hours). Also 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 treated group.

Conclusions: A negative result obtained in the present study indicates that under the test conditions, synthetic hydroxyapatite granules (manufactured by USM) are non cytotoxic and do not induce chromosome aberrations in the bone marrow cells of mice.

Key words: *cytotoxicity, chromosome aberration, hydroxyapatite, mice*

INTRODUCTION

Dentistry has searched for the ideal material to place in osseous defects for many years. 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. 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.

For these reasons, there is a growing need for fabrication of artificial hard tissue replacement implants. 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 (Wojciech Suchanek and Masahiro Yoshimura, 1998). The ultimate objective, of course, is to come up with a synthetic biomaterial that will produce the same results as a normal bone.

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. When implanted into the body, the synthetic implant 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 (Constantino *et al.*, 1991). For these reasons, it has become widely popular among craniofacial and

neurosurgeons and is often quoted as being a near-ideal bone substitute material (Jarcho, 1981).

Facial augmentation with hydroxyapatite has been used for correcting cheek, chin, jaw, nose and brow bone. It has been used extensively in restoring alveolar ridges, particularly mandibular ridges, where severe atrophy is so often seen, as well as act as a bone graft substitute in orthognathic surgery. 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. However, synthetic hydroxyapatite is relatively a new biomaterial and before new materials are 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.

2. REVIEW OF LITERATURE

2.1 Biomaterials

A biomaterial is defined as "a material intended with biological system to evaluate, treat, augment, or replace any organ or function of the body (Williams *et al*, 1992). It can be classified as biological biomaterial (allografts, xenografts and autografts) and synthetic biomaterial (metals, polymer and ceramics).

2.1.1 Biological biomaterial

Biological biomaterials are materials that are naturally produced and they can be subdivided into

a. **Autografts:** Autogenous bone graft is the bone graft from the same organism. Until today, it is still the "gold standard" in bone grafting. It has several advantages compared to allografts or xenografts. It has greater osteogenic capacity and it is biocompatible. As the autografts resorbs, revascularization recruits mesenchymal-type cells, which differentiate into osteogenic, chondrogenic or other cell lines. However, autografts has limitation e.g. secondary operation, limited availability of bone and operation morbidity (Habal and Reddi, 1994).

b. **Allografts:** Allograft is the bone graft from the same species. It demonstrates a lower osteogenic capacity, higher resorption rate and larger immunogenic response and also less revascularization of the grafts than autografts (Friedlaender *et al*, 1994). Despite these drawbacks, allograft bone offers a useful adjunct to the range of bone grafting materials. Bone can also be mixed with autogenous grafts in hip prosthesis operations but to maintain the availability of allograft bone, a well-organized bone bank is needed (Tomford *et al*, 1987).

c. **Xenografts:** The xenogenic bone graft is a graft made with bone from other species. It has similar problem to the allograft. It elicits an acute antigenic response with high failure rate. Xenografts are indeed rarely used.

2.1.2 Synthetic Biomaterials

The synthetic biomaterials can be subdivided into

a. **Metals:** The main metals used clinically are titanium, vitallium, aluminium and stainless steel. All of them are inert and biocompatible (Mofid *et al*, 1997).

b. **Polymers:** Polymers comprise of a large group of materials of heterogenous origin. For example, methyl methacrylate is widely used in traumatic skull defects. Other polymers used include polyhydroxyethylmethacrylate, polycolide, polylactide and porous high density polyethylenes.

c. **Ceramics:** Ceramics consists of crystalline metallic oxides, carbides, nitrides and borides fused by sintering. They are brittle, have low electrical and heat conductivity and elicit very little tissue reaction. Ceramics comprise materials derived from calcium. Such ceramics are composed of calcium sulphate, phosphate and carbonate derivatives and their mixture in dense, porous and granular forms.

2. 2 Hydroxyapatite

Hydroxyapatite, [Ca10 (PO4)6 (OH)2] is a synthetic biomaterial produced from calcium nitrate, phosphoric acid, ammonia and distilled water (Braye *et al*,1996). When a bone substitute with some mechanical strength is needed, hydroxyapatite appears most appropriate, as it represents the natural mineral in human bone and approximates the natural structure of cancellous bone (Cooke, 1992). It has been used clinically in dental, craniofacial and orthopedic surgery, mainly in granular form. As a biomaterial advance

over their hydroxyapatite predecessors, which were preformed granules and blocks, they offer an off-the-shelf powder and liquid mixture that can be easily molded into almost any shape and intimately adapted to the irregularities of any cranial defect while converting into hydroxyapatite in the early postoperative period. Furthermore, this biomaterial maintains its intra-operative position and shape postoperatively and is very biocompatible with bone due to its inorganic mineral content. Hydroxyapatite has many of the qualities of ideal bone substitute like osteoconductive, osteoinductive, biocompatible and easy to shape but it resorbs very slowly and is brittle. The use of hydroxyapatite cements (HACs), which became available for widespread clinical use in the United States in 1995, was a welcome alternative to the traditional use of polymethylmethacrylate (acrylic) for cranioplastic applications. While being misnamed as cements, they really are a bone substitute material for non–load-bearing applications in the superior craniofacial skeleton. For these reasons, it has become widely popular among craniofacial and neurosurgeons and is often quoted as being a near-ideal bone substitute material (Jarcho, 1981). Hydroxyapatite seems to be the most appropriate ceramic material for artificial teeth or bones due to excellent biocompatibility and bioactivity (Wojciech Suchanek and Masahiro Yoshimura, 1998).

2.3 Biocompatibility testing

Recognition of an implant material as biocompatible depends on

a. absence of cytotoxicity, mutagenenicity, carcinogenicity

b. exclusion of allergenic properties

c. physical-chemical and biological 'inertia' and

d. stability in its biological environment (Katzer *et al.*, 2002)

The following three tests are generally recommended for biocompatibility testing with regard to mutagenicity and cytotoxicity.

I. A test for gene mutation in bacteria

II. An *in vitro* test for chromosomal effects in mammalian cells

III. An *in vivo* test for chromosomal effects using rodent haemopoeitic cells

2.3.1 *In vivo* chromosomal aberration test

The mammalian *in vivo* chromosome aberration test is used for the detection of structural chromosome aberrations induced by test compounds in bone marrow cells of animals, usually rodents cells of animals, usually rodents (Adler, 1984; Preston *et al.*, 1987; Richold *et al,* 1990 and Tice *et al.*, 1994). Structural chromosome aberrations may be of two types, chromosome or chromatid. An increase in polyploidy may indicate that a chemical has the potential to induce numerical aberrations. With the majority of chemical mutagens, induced aberrations are of the chromatid-type, but chromosome-type aberrations also occur. Chromosome mutations and related events are the cause of many human-genetic diseases and there is substantial evidence that chromosome mutations and related events causing alterations in oncogenes and tumour-suppressor genes are involved in cancer in humans and experimental systems (Health Effects Test Guidelines – OPPTS 870.5385, 1998). Similar *in vivo* chromosome aberration protocols have been carried out by other scientists to test compounds like garlic (Das *et al.*, 1996); arsenic (Arati *et al.*, 1997); turmeric and curcumin (Mukhopadhyay *et al.*, 1998) iron (Poddar *et al.*, 2000); fenvalerate (Giri *et al.*, 2002); hydroxyapatite granules (Hasib *et al.*, 2004).

2.4 Selection of animal species

Rats, mice and Chinese hamsters are commonly used, although any appropriate mammalian species may be used. Commonly used laboratory strains of young healthy adult animals are employed and at the commencement of the study, the weight variation of animals should be minimal (OECD, 1997; Health Effects Test Guidelines – OPPTS 870.5385, 1998).

2.5 Spindle inhibitor

To arrest cells in metaphase, the cell division has to be blocked. Colchicine, a plant alkaloid or its synthetic analog, colcemid is used for the purpose. Dividing cells thus stop at metaphase because they are unable to proceed to anaphase. The length of application of colchicine is limited because in addition to stopping the cells in metaphase, if applied too strong a concentration or for too long time, it causes further condensation and distortion of the chromosomes (Jean H priest, 1969).

2.6 Harvesting of bone marrow

2.6.1 Hypotonic treatment

Hypotonic solution swells the cells though osmosis. As a result, the chromosomes are widely spread apart and available for critical studies (Hsu and Pomerat, 1953). For this purpose, a solution of 0.075 M potassium chloride was used (Chan *et al.*, 1977). Most of the researchers used 0.075 M Potassium chloride whereas sodium citrate was used by Stranzinger *et al.*, (1974).

2.6.2 Fixation

The commonly used fixative is the methanol and acetic acid in the ratio of 3:1. The fixative penetrates the cells rapidly and preserves the chromosome structure (Yunis

and Sanchez, 1975). Several authors have pointed out that the methanol and acetic acid fixatives extract a substantial part of histones from the metaphase chromosomes (Sumner *et al*, 1973; Retief and Ruchel, 1977). In a study conducted on the chromosomes of the domestic rabbit, *Oryctolagus cuniculus* by Issa *et al.* in 1968, they used six parts of ethanol, three parts of chloroform and one part of glacial acetic acid as the fixative of choice.

2.6.3 Staining

Giemsa stain is the most commonly used staining material though certain other stains have also been extensively by many researchers. Issa *et al.* (1968) used 2% lactic-acetic-orcein to stain the chromosomes.

2.7 Modal Chromosome number of mice

The mice (*Mus musculus*) have a normal diploid number of 40 chromosomes with all the chromosomes being acrocentric.

2.8 Mitotic Index

Mitotic index is the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population (Health Effects Test Guidelines – OPPTS 870.5385, 1998).

2.9 Chromosomal aberrations

There are 2 types of chromosomal aberrations

i) Numerical Aberrations: Change in the number of the chromosomes from the normal number characteristic of the cells utilized

ii) Structural Aberrations: Change in the chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, intrachanges, and interchanges etc.,

2.10 Mutagenicity

Mutagenicity refers to the induction of permanent transmissible changes in the amount or structure of genetic material of cells or organisms. These changes "mutations" may involve a single gene or gene segment, a block of genes or whole chromosomes (ETAD, 1998). A test substance that does not produce any chromosomal aberrations is considered non-mutagenic in this system (Health Effects Test Guidelines – OPPTS 870.5385, 1998).

3. Materials and method

3.1 Animal Ethics Committee 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 under the Project Titled (008): "*In vivo* Chromosome Aberration Test for Hydroxyapatite in Mice". The experimental procedures including handling of the animals were strictly adhered to as per the guidelines.

3.2 Experimental animal

Mice (*Mus musculus* - Swiss Albino strain - Fig 1) were chosen as our experimental animal due to the reasons of ready availability, easy handling, cheap and its unpretentious nature. The source of the animals was from the Animal House Unit of the USM Health Campus. Adult healthy mice were chosen for the present study. The animals ranged from 10 to 12 weeks of age and their overall mean body weights were 29.51 ± 0.22 and 36.28 ± 0.37 grams for the females and males respectively. 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.*

3.3 Experimental groups

The groups of mice were divided into positive control, negative control and treatment groups, each group consisting of five males and five females.

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

Positive control: The animals of 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 also 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 following treatment as shown in Table 1.

Table 1

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

3.4 Biomaterial

The biomaterial used in this study is a porous form of synthetic hydroxyapatite granules (Fig 2), which is 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.

3.5 Aseptic precautions and procedures

A universal problem encountered in tissue culture is contamination, which has probably caused more failures than any other reason. To avoid contamination, all steps were carried out under sterile and aseptic conditions. All the culture work was performed in the Human Genome Centre of USM Health Campus.

3.6 Animal experiment

All the animal experiments were conducted in the Animal House Unit of Universiti Sains Malaysia, Health campus. The animals were subjected to the treatment as per the protocol of their respective group as presented in table 1.

3.6.1 Colchicine treatment

After appropriate time of treatment, the animals were injected intra peritoneally with colchicine (4 mg/kg) 90 minutes prior to sacrifice.

3.6.2 Sacrificing the animals

The animals were euthanised by giving an overdose of pentobarbitone sodium (500 mg/kg body weight) and later death was ensured by cervical dislocation.

3.6.3 Harvesting of bone marrow

The bone marrow cells were collected from both the femurs by flushing in warm (37°C) potassium chloride solution (KCl - Sigma - P 9327 - hypotonic treatment) and the

volume was adjusted to 15 ml. The centrifuge tubes were incubated at 37 °C for 20 minutes.

3.6.4 Fixative treatment

The tubes were centrifuged at 1000 rpm for 10 minutes, the supernatant was discarded and 6ml of freshly prepared 3:1 Methanol:Acetic acid (Methanol - BDH 10158BG and Acetic acid - Merck K 32585963-344) was added. Centrifugation and fixation was repeated twice with an interval of 30 minutes.

3.6.5 Preparation of slides

With an automatic pipette, 50 µL of cell suspension was evenly distributed on several locations on the slide and the liquid was spread by gently moving the pipette tip parallel to the surface. Then, the slide was placed face down into the steam of the hot water bath (80°C) for 3 seconds and then dried by placing the slide on the metal plate of the water bath. After the slide surface became grainy, four drops of glacial acetic acid were placed on the slide and allowed to cover the surface. Immediately, the slide was exposed for 5 seconds to the water vapors and then quickly dried on the hot metal plate of the water bath (Octavian Henegariu *et al.*, 2001).

3.6.6 Staining

The slides were stained with 10 per cent Leishman's stain (Sigma L 6254) in phosphate buffer saline (pH 6.8) for 5 minutes, rinsed in distilled water and air dried. Leishman's staining solution was prepared by dissolving 2.4 g of Leishman's powder in one litre of methanol.

3.7 Mitotic Index

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.

3.8 Screening for chromosome aberrations

Slides were screened to detect if there were both the numerical and structural aberrations in all the samples collected prior to and after the implantation of the biomaterial. A total of 100 cells per animal were scored for the chromosomal aberrations. The slides were screened under oil objective (100x) of Leica CTR MIC Image Analyzer and pictures were taken using Leica Chantal software.

3.9 Statistical analysis

The statistical analyses was carried out using SPSS software version 12.0.1 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 experimental groups.

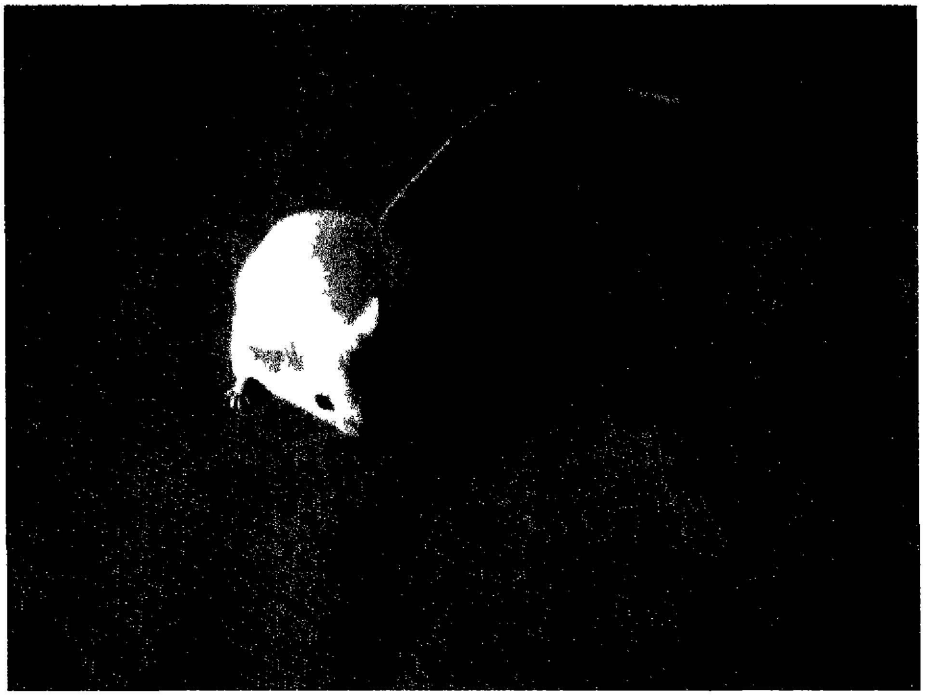

Fig 1: Swiss Albino mice (*Mus musculus*)

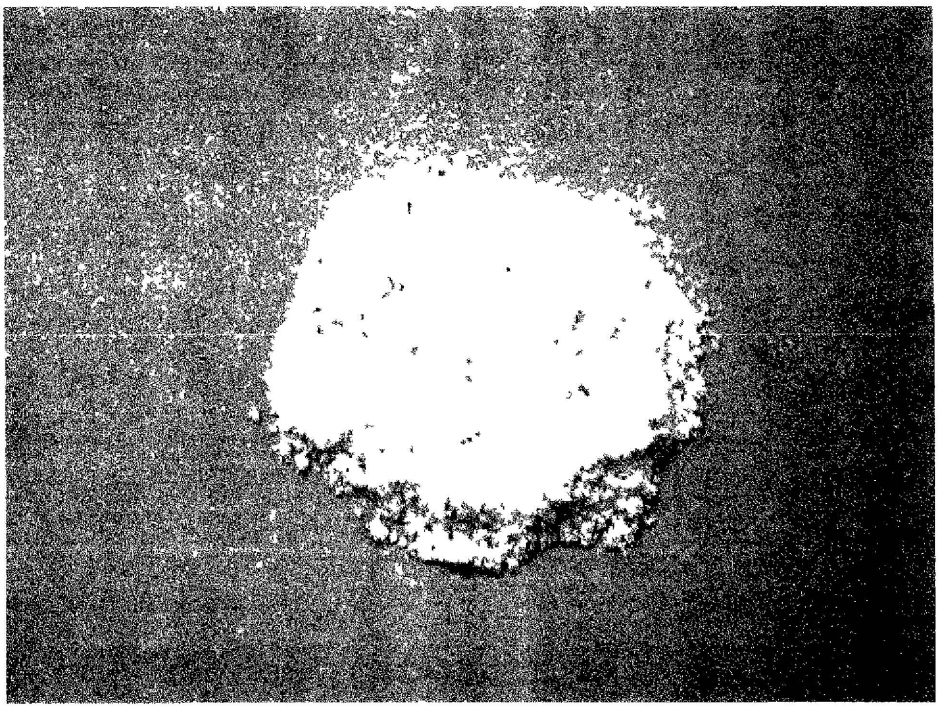

Fig 2: Synthetic hydroxyapatite granules (Manufactured by USM, Penang)

4. 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, Universiti Sains Malaysia, Penang.

4.1 Colchicine

Colchicine injected at a concentration of 4 mg /kg body weight 90 minutes prior to harvesting yielded quite a number of analyzable metaphase spreads in the present study.

4.2 Harvesting of bone marrow

4.2.1 Hypotonic treatment

Hypotonic treatment using 0.075 M KCl (0.56 per cent) at 37°C for 20 minutes yielded good number of swollen cells.

4.2.2 Fixation

3:1 Methanol:Acetic acid was found to be a very good fixative for the fixation of cells in the present study. Chilled fixative yielded consistent results with regard to fixation and preparation of chromosomes.

4.2.3 Chromosome preparation

More evenly controlled chromosome spreading was obtained when the protocol of Octavian Henegariu *et al.*, (2001) was followed in comparison to the conventional method.

4.3 Mitotic Index

The mitotic index was calculated as the ratio of number of cell in metaphase to the total number of cells counted in percentage. A total of 1000 cells were counted per

animal to determine the mitotic index values. The mean mitotic index values for the different treatment groups are presented in Table 2.

Table 2

MEAN MITOTIC INDICES 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) followed by the same letter (b,b) are not significantly different at 5% level whereas values with different letters (a,b) are significant at 5% level.

4.4 Chromosomal Analysis

A total of 100 metaphases were analyzed to calculate the extent of chromosomal aberrations per animal. No statistically significant chromosome aberrations were observed, either numerical or structural in the chromosomes of mice treated with distilled water (Fig 3) and those treated with synthetic hydroxyapatite granules (Fig 4). However, mice injected with Mitomycin C showed several fold increase in chromosome aberration levels, validating the experimental conditions used (Fig 5). The details are presented in table 3.

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) followed by the same letter (a,a) are not significantly different at 5% level whereas values with different letters (a,b) are significant at 5% level.

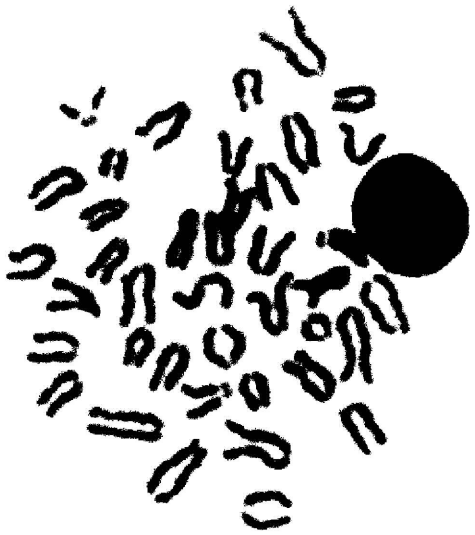

Fig 3: Metaphase spread – Mice (Negative control – Distilled water)

Fig 4. Metaphase spread – Mice (Treatment with Hydroxyapatite)

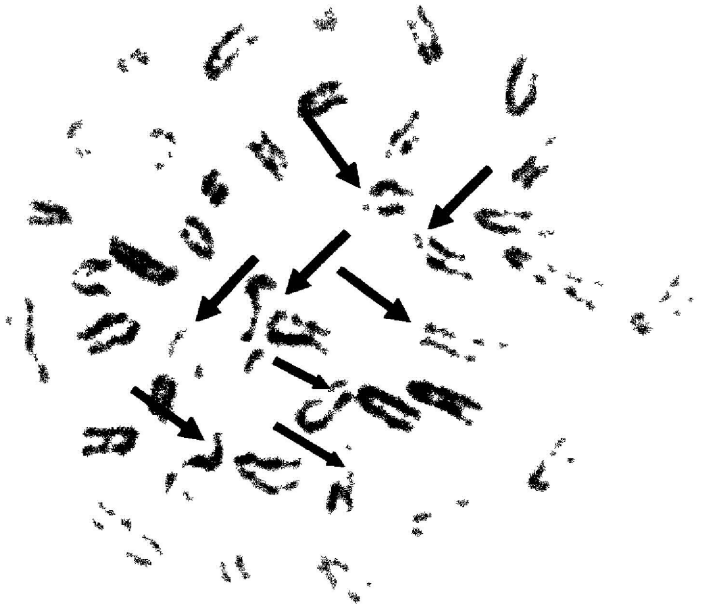

Fig 5. Metaphase spread – Mice (Positive control - Mitomycin C)

5. DISCUSSION

5.1 The mammalian *in vivo* chromosome aberration test

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 and Tice *et al.*, 1994). It is obligatory that sterile procedures are required for any cell culture and chromosomal analysis procedure. Bone marrow is a highly vascularized tissue and it contains a population of rapidly cycling cells that can be readily isolated and processed (Health Effects Test Guidelines, OPPTS 870.5385, 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. An *in vivo* test is also useful for further investigation of a mutagenic effect detected by an *in vitro* test.

5.2 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. 1½ hours prior to sacrifice, the animals were injected with colchicine at a dose of 4mg per kg body weight. Then the mice were sampled at an appropriate interval thereafter. The injection of colchicines 1½ hours prior to sacrifice yielded quite a number of metaphase spreads for analyses.

5.3 Harvesting of Bone Marrow

5.3.1 Hypotonic treatment

Two things are very important in this procedure: 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 Octavian Henegariu *et al.*,(2001). More evenly controlled chromosome spreading was obtained by following this method.

5.3.2 Fixation

Octavian Henegariu *et al.* (2001) 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.

5.4 Metabolic Activation

The metabolic activation system (S9) usually obtained from rat liver is designed to simulate mammalian liver enzyme systems. The purpose of adding S9 in *in vitro* cultures is to increase the level of metabolizing enzymes to active forms. Substances, which are reactive, and form DNA adducts directly, either with DNA in test tube or with DNA in a living cell are known as directly-acting agents whereas substances which require metabolic activation system before they are genetically active are known as indirectly-acting agents (Kristien and Errol, 2000). However this *in vivo* study takes into consideration the metabolism, pharmacokinetics, and DNA-repair processes (Health Effects Test Guidelines, OPPTS 870.5385, 1998).

5.5 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 (Health Effects Test Guidelines, OPPTS 870.5385, 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.

5.6 Modal chromosome number

The modal chromosome number of mice (*Mus musculus*) in the present study was found to be 2n=40. All the chromosomes were found to be acrocentric.

5.7 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 is 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.

5.8 Chromosomal Analysis

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 treated group.

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 (Locke-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 (Health Effects Test Guidelines – OPPTS 870.5385, 1998).

5.9 Mutagenicity

The mitotic index values and chromosomal analyses in this study indicate that the test substance, synthetic hydroxyapatite granules is non-mutagenic in the bone marrow cells of mice under the present test conditions.

Conclusions

The value of the mitotic indices and the absence of chromosome aberrations indicate that the biomaterial, synthetic hydroxyapatite granules (Manufactured by USM, Penang) is non cytotoxic and non mutagenic under the present test conditions in the bone marrow cells of mice.

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