PERPUSTAKAAN KAMPUS KESIHATAN
UNIVERSITI SAINS MALAYSIA

RUJUKAN

Genotoxicity Study of Sea Coral Used as Bone Replacement Material

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USM Short Term Grant
304/PPSG/6131244

1558X

BAHAGIAN PENYELIDIKAN & PEMBANGUNAN
CANSELORI
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Laporan Akhir Projek Penyelidikan Jangka Pendek

1) Nama Penyelidik

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2) Pusat Pengajian/Pusat/Unit :

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KUBANG KERIAN, KELANTAN

3) Tajuk Projek :

GENOTOXICITY STUDY OF SEA CORAL USED AS BONE REPLACEMENT MATERIAL

4) (a) Penemuan Projek/Abstrak

(Perlu disediakan makluman diantara 100 - 200 perkataan didalam Bahasa Malaysia dan Bahasa Inggeris ini kemudiannya akan dimuatkan ke dalam Laporan Tahunan Bahagian Penyelidikan & Pembangunan sebagai satu cara untuk menyampaikan dapatan projek tuan/puan kepada Pihak Universiti).

Kebanyakan kaedah untuk mengesan karsinogen dan mutagen masa kini adalah berdasarkan kenyataan bahawa genotoksisiti/mutagenisiti boleh dijadikan penunjuk potensi karsinogenik.

Ujian *in vitro* ini ialah untuk menganalisa sifat genotoksisiti batu karang dari spesis *Porites* yang dihasilkan oleh Bank Tisu Negara, Universiti Sains Malaysia (USM), menggunakan ujian Asai Mutan Bakteria Berbalik ("Bacterial Reverse Mutation Assay") atau ujian Ames.

Asai Mutan Bakteria Berbalik batu karang dijalankan ke atas *Salmonella typhimurium* TA1535, TA1537, TA98, TA100 and *E. coli* WP2 *uvrA* menggunakan kaedah pengeraman awal dengan kehadiran dan tanpa kehadiran system pengaktifan metabolik eksogenus.

Keputusan menunjukkan bilangan koloni revertan untuk kesemua jenis bacteria yang dirawat dengan bahan ujian adalah kurang dari dua kali ganda untuk setiap kawalan negatif bersama atau tanpa Campuran S9.

Kesimpulannya, keputusan ujian ini menunjukkan bahan batu karang tersebut boleh dianggap tidak mempunyai potensi mutagenik berbalik.

Most of the methods available today to detect carcinogens and mutagens are based upon the principle that genotoxicity/mutagenicity may serve as an indicator of carcinogenic potential.

This *in vitro* study was made to evaluate the genotoxicity characteristic of coral of *Porites sp.* produced by the National Tissue Bank, Universiti Sains Malaysia (USM), by using the Bacterial Reverse Mutation Assay test or Ames test.

The Bacterial Reverse Mutation Assay of the coral material was performed on *Salmonella typhimurium* strains TA98, TA100, TA1535, TA1537 and *Escherichia coli* strain WP2 *uvr*A using the preincubation method in the presence and absence of an exogenous metabolic activation system.

The results showed the numbers of revertant colonies of all strains which were treated with the test substance were less than twice that of each negative control with and without S9 Mix.

In conclusion the results of the tests showed that the coral material was considered to have no reverse mutagenic potential under the present test condition.

(b) Senaraikan Kata Kunci yang digunakan di dalam abstrak :

Bahasa Malaysia	Bahasa Inggeris
Batu Karang	Coral
Genotoksisiti	Genotoxicity
Mutagenisiti	Mutagenicity
Ujian Ames	Ames test

5) Output Dan Faedah Projek

(a) Penerbitan (termasuk laporan /Seminar)
(Sila nyatakan jenis, tajuk, pengarang, tahun terbitan dan dimana telah diterbit/ dibentangkan)

1. Poster presentation
Genotoxocity Study of the Osseous Substitutes: Ames Test.
A. Azlina, O. Shamsuria A. H. Suzina, and A. R. Samsudin,
8th National Conference on Medical Sciences 08 –09/ 05/ 2003, Universiti Sains Malaysia, Kelantan.

2. Abstract publication
Genotoxocity Study of the Osseous Substitutes: Ames Test.
A. Azlina, O. Shamsuria A. H. Suzina, and A. R. Samsudin,
The Malaysian Journal of Medical Sciences, Vol 10 No 2, 2003. 145

3. Submitted for oral presentation
Mutagenicity of CORAGRAF and REKAGRAF in the Ames Test.
A. H. Suzina, A. Azlina, O. Shamsuria and A. R. Samsudin,
International Conference on Biomaterials and Tissue Engineering 2004, 24 – 26/ 05/ 2004, Kuala Lumpur.

(b) Faedah-faedah lain Seperti Perkembangan Produk, Prospek Komersialisasi Dan Pendaftaran Paten
(jika ada dan perlu, sila gunakan kertas berasingan)

Tiada

(c) Latihan Gunatenaga Manusia

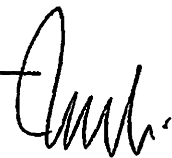
i)	Pelajar Siswazah :	Tiada
ii)	Pelajar Prasiswazah:	Tiada
iii)	Lain-lain:	Tiada

6) Peralatan Yang Telah Dibeli: Tiada

UNTUK KEGUNAAN JAWATANKUASA PENYELIDIKAN UNIVERSITI

1) Final report Ok

2) Paper for publication.



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TITLE

Genotoxicity Study of Sea Coral Used as Bone Replacement Material

INTRODUCTION

Autograft is the gold standard for bone repairing procedures as it contains the triggering factors necessary for bone formation in cases of bone defect. However, the availability of autograft bone is limited and the harvesting of autograft bone causes morbidity at the donor site. Allografts have become alternatives to autografts. However allografts have disadvantages such as the possibility of disease transmission. Therefore, alternative bone substitute materials have been developed.

An ideal bone graft substitute is a material that is a biologically inert, readily available, easily adaptable to the site in terms of size and shape, and replaceable by the host bone (Bajpai, 1983). Most importantly the bone graft substitute must be biocompatible, osteoconductive and resorbable after new bone has bridged across the defect.

Bone is composed of calcium salts, primarily calcium phosphate and the major constituent is in the form of poorly crystalline calcium phosphate compound known as hydroxyapatite. Substantially lower amount of calcium carbonate is also present. Both hydroxyapatite and calcium carbonate are bioactive and osteoconductive. Using these naturally occurring calcium salts as the scaffold component of bone graft substitute has been appealing (White and Shors, 1986).

The coral skeletons are composed primarily of calcium carbonate (99%) in the form of aragonite. The remaining 1% is composed of simple amino acids (Ouhayan, *et al.*, 1992). The porosity and the three dimensional structure of the coral implants promote bony ingrowth, thus it is classified as an osteoconductive material (Guillemin, *et al.*, 1987; Ouhayan, *et al.*, 1992; Guillemin, *et al.*, 1989). In addition resorption of the implants has been observed simultaneously with apposition of new bone, initially onto the coral implants and later totally replacing the implant (Guillemin, *et al.*, 1989; Sautier, *et al.*, 1990). Many studies had shown that coral implants are well tolerated by tissues when implanted into animals (Shahgaldi, 1998; Catherine, et al., 1995). Similarly, experience in human implantations has been encouraging (Marchac and Sandor, 1994).

History has shown that some implants used in human proved to be mutagenic and caused cancer after many years of implantation. Thus, before humans can be exposed to chemicals or other materials, it must be confirmed that this exposure will not endanger human health. This study looked at genocompatibility of a locally produced osseous substitutes, natural coral of *Porites sp.* to ensure its safety as bioimplants in human, using specific genotoxic test. Genotoxicology (mutagenicity) tests evaluate the ability of a material to cause mutation or chromosomal damage. One of the tests is Ames test or also known as bacterial reverse mutation assay (OECD, 1997; Mortelmans and Zeiger, 2000; Maron and Ames, 1983). This assay uses bacteria, *S. typhimurium* and *E. coli*, which have the ability to reverse some of the mutations with the gene regaining their original genocompatible functions.

PURPOSE OF STUDY

This study was performed in order to retrieve information and to detect mutation on the bacterial reverse mutation assay caused by coral of *Porites sp.* produced by the National Tissue Bank, USM, in the presence and absence of an exogenous metabolic activation system as its in vitro biocompatibility.

The results obtained from this study are perhaps helping to develop a non-genotoxic biomaterial similar to the standard biomaterial that is already being used in clinical practice.

The purpose of this study was to determine the mutagenic potential of the coral material using *Salmonella typhimurium* and *Escherichia coli.*

MATERIALS AND METHODS

Test substance and positive control

1. **Test substance**

Produced by : National Tissue Bank, USM

Name: Coral of *Porites sp.*

Form : fine granules

Coral processing

In our study we had identified a species of dead sea coral of *Porites sp.* which we harvested from Malaysian costal region (A licence was provided by the Department of Fisheries Malaysia to harvest dead coral for this purpose). The coral materials were processed using innovative techniques at National Tissue Bank, USM. The material were cleaned from debris and washed with distilled water. Then, samples were soaked in sodium hypochloride for 36-40 hours and treated with ultrasound bath for 20-40 minutes at 40-60 °C. Then again was rinsed with deionized water until the samples were completely eliminated of the organic matter and organic impurities and was freeze dried. Those treated coral was sorted and crushed to powder form. The samples were triple packed and radiosterilized at Malaysian Institute for Nuclear Technology Research (MINT) using gamma irradiation.

3. Bacterial strains

a. Strains selected

Salmonella typhimurium strains TA 98, TA 100, TA 1535 and TA 1537, and *Escherichia coli* strain WP2 *uvr*A were obtained from Dr. T. Nohmi, National Institute of Health Science, Tokyo, Japan. *S. typhimurium* strains TA 100, TA 1535 and *E. coli* strain WP2 *uvr*A were used for detection of base-pair substitution mutations, while *S. typhimurium* strains TA 98 and TA 1537 were used for the detection of frameshift mutations.

b. Storage

Test strains were stored as frozen stock cultures (0.0035 ml of dimethyl sulfoxide (DMSO)/0.4 ml of broth culture) at –80 °C in ultra-deep freezer.

c. Characterization of the strains

Strains	Mutation on synthesis of acid amino	Mutation on excision repair	Membrane Mutation (LPS)	R-factor (pKM101)
Salmonella typhimurium				
TA98	hisD3052	Δ uvrB	rfa	+
TA100	hisG46	Δ uvrB	rfa	+
TA1535	hisG46	Δ uvrB	rfa	-
TA1537	hisC3076	Δ uvrB	rfa	-
Escherichia coli				
WP2 uvrA	trp	Δ uvrA	+	-

The amino acid requirement for growth was demonstrated by using histidine for *S. typhimurium* strains and tryptophan for *E. coli* strain. The presence of R-factor,

b. Storage

They were stored in ultra-deep freezer at -80 °C.

c. Composition of S9 Mix

One ml of S9 Mix contains protein (25.19 mg/ml), cytochrome p-450 (0.97 nmol/mg protein), DMN demethylase activity (4.97 nmol HCHO formed/mg protein/min), aniline hydroxylase activity (24.02 nmol p-aminophenol formed/mg protein/hr) and B[a] P hydroxylase activity (16.85 higher than non induced S9).

From the stock cultures, 36 µl of bacterial suspension was inoculated in a L-tube containing 18ml nutrient broth No. 2 (Oxoid Ltd.), the bacterial culture was incubated at 37± 0.5 °C for 6 to 9 hours with shaking at 57 times/min in a Monod shaker. The viable cells counts were calculated from the values, which were determined at 660 nanometer by spectrophotometry at the end of incubation.

		TA100	TA1535	WP2 uvrA	TA98	TA1537
No. of viable cells (x 10 ^{9}/ml)	Dose finding Test	1.00	1.12	1.08	1.06	1.02
	Main test	1.07	1.00	1.40	1.09	1.03

PREPARATION OF TEST SUBSTANCE AND POSITIVE CONTROLS

Test substance

a. Preparation

The test substance was prepared in sterile pure water and ultrasonicated for 30 minutes in a sonicator bath to make 5% w/v concentration and diluted with the same solvent to give lower concentrations.

b. Stability of test solution

No denaturation of test solution was observed in respect to colour change and exothermic reaction up to 2 hours after preparation.

c. Preparation time

Test solution was used within 2 hours after preparation at room temperature.

Positive controls

a. Preparation

NaN3 was dissolved in pure water, AF-2, ICR-191 and 2AA were dissolved in DMSO.

b. Storage condition

Stored in ultra-deep freezer at -80 °C.

METHODS

The test was carried out for *S. typhimurium* TA98, TA100, TA1535, TA1537 and *E. Coli* strain WP2 uvrA using the pre-incubation method both with and without metabolic activation system. Plating was done in triplicate for the negative control and in duplicate for the substance and positive controls.

Procedures

After 0.1 ml of the test substance solution, 0.5ml of 0.1 M Sodium phosphate buffer (pH 7.4) or S9 Mix and 0.1ml of the bacterial culture were added to a tube, the mixture were incubated for 20 minutes at 37±0.5 °C. 2 ml of soft agar was then added to each tube and poured onto a minimal glucose agar plate. After incubation for 48 hours at 37±0.5 °C, the number of revertant colonies was counted.

As the sterility test, 0.1ml of test substance solution, S9 Mix and 0.1 M sodium phosphate buffer (pH 7.4) were poured onto a minimal agar plate and incubated at 37±0.5 °C for 48 hours to check for the bacterial contamination. Pure water was used as negative control and the following positive control were used for each bacterial strain.

Positive Controls of Bacterial Strains

	TA100	TA1535	WP2 uvrA	TA98	TA1537
S9 Mix (-)	AF-2	NaN_3	AF-2	AF-2	ICR-191
	0.1 μg/plate	0.5 μg/plate	0.05 μg/plate	0.01 μg/plate	1 μg/plate
S9 Mix (+)	2AA	2AA	2AA	2AA	2AA
	0.5 μg/plate	2 μg/plate	10 μg/plate	1 μg/plate	2 μg/plate

Dose selection

a. Dose finding test

The test was carried out at the highest dose of 5000 μg/plate and 4 doses of 2500, 1250, 625 and 313 μg/plate. Growth inhibition was not observed at any of these doses for all the bacterial strains tested both in the presence or absence of S9 Mix. Test plates of TA100, TA1535, TA98, TA 1537 and WP2 *uvr*A with and without S9 Mix showed no increase of revertant colonies for all the doses tested.

b. Main test

Based on the results of the dose finding test, a maximum dose was decided at 5000 μg/plate. The lower 4 doses were obtained by dilution with a geometric progression of 2, i.e. replicating exactly the procedure carried out in the dose finding test.

EXAMINATION AND COLONY COUNTING

a. Microscopic observation

The state of the revertant colonies (size or number of colonies) and growth inhibition was examined with a stereoscopic.

b. Colony counting

The number of colony was counted with a manual counter or a colony analyzer (ProtoCol). Each plate was counted three times and the average of the three counts was adopted as the number of revertant colonies on the plate. The average number of revertant colonies for each dose was calculated as the average plate counts for a set of duplicate. Decimals of the average figures were rounded off.

c. Interpretation of the results

Signs of toxicity or growth inhibition for all the bacterial strains under the test condition were described in this report. The test substance was judged to be negative, when the number of revertant colonies was less then twice that the negative control.

RESULTS

a. Results of the plate counts of range finding tests

The highest dose of 5000 μg/plate was used to detect cytotoxicity in the preliminary experiment. Result shows no growth inhibition and test plate showed no increase of revertant colonies.

The test substance was tested at different concentrations of 313 μg/plate, 625 μg/plate, 1250 μg/plate, 2500 μg/plate and 5000μg/plate. For the test substance, negative control and positive control tests were performed on *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 *uvr*A using the preincubation method in the presence and absence of exogenous metabolic activation system.

The test substance was judged to be negative, when the number of revertant colonies was less than twice that of the negative control. No dose-response relationship and reproducibility was obtained. No statistical procedure was used.

b. Results of plate count of main tests

Based of the results of the range finding tests, a maximum dose was decided at 5000 μg/plate. The test substance was tested at different concentration of 313 μg/plate, 625 μg/plate, 1250 μg/plate, 2500 μg/plate and 5000 μg/plate. For the test substance, negative control and positive control tests were performed on *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537 and *Escherichia coli* strain WP2 *uvr*A using the

preincubation method in the presence and absence of exogenous metabolic activation system.

The test substance was judged to be negative, when the number of revertant colonies was less than twice that of the negative control as shown in Tables 1.1 and 1.2. No dose-response relationship and reproducibility was obtained. No statistical procedure was used.

Table 2.1: Result of Main Test Without S9 Mix for Coral

	Number of revertants [(number of colonies/plate) ± SD]				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA 98	TA 1537
Control (Water)	181 ± 15	14 ± 2	21 ± 5	39 ± 7	13 ± 7
Test substance concentration(μg/plate)					
313	203 ± 30	19 ± 4	13 ± 6	44 ± 4	13 ± 1
625	193 ± 8	13 ± 4	17 ± 2	51 ± 6	18 ± 5
1250	218 ± 12	16 ± 3	29 ± 6	34 ± 7	17 ± 2
2500	216 ± 21	21 ± 3	29 ± 8	48 ± 2	21 ± 10
5000	231 ± 54	20 ± 2	36 ± 1	45 ± 0	26 ± 11
Positive control	AF-2	NaN3	AF-2	AF-2	ICR-191
Concentration	0.01	0.5	0.05	0.1	1
Number of revertant	417 ± 4	383 ± 9	1785 ± 23	352 ± 0	2305 ± 3

AF-2: 2-(2-Furyl)-3-(5-nitro-2-furyl) acrylamide
NaN3: Sodium azide
ICR-191: 2-Methoxy-6-chloro-9[3-(2-chloroethyl)-aminopropylamino]acridine-2HCl

Table 2.2: Result of Main Test With S9 Mix for Coral

	Number of revertants [(number of colonies/plate) ± SD]				
	Base-pair substitution type			Frameshift type	
	TA 100	TA1535	WP2uvrA	TA 98	TA 1537
Control (Water)	186 ± 47	17 ± 1	25 ± 7	42 ± 10	19 ± 3
Test substance concentration (μg/plate)					
313	189 ± 3	15 ± 1	20 ± 4	28 ± 5	16 ± 1
625	170 ± 35	16 ± 5	26 ± 7	40 ± 6	19 ± 2
1250	180 ± 26	17 ± 1	17 ± 3	45 ± 8	27 ± 1
2500	192 ± 2	9 ± 3	22 ± 14	42 ± 5	32 ± 2
5000	221 ± 36	19 ± 11	31 ± 8	40 ± 3	36 ± 1
Positive control	2AA	2AA	2AA	2AA	2AA
Concentration	1	2	10	0.5	2
Number of revertant	825 ± 35	103 ± 22	115 ± 22	267 ± 6	173 ± 21

2AA: 2-Aminoantharacene

DISCUSSION

Research innovation in forming a variety of porous biomaterial such as of coral origin was conceived and developed all over the world but thus far not in Malaysia. Malaysian biodiversity has abundance of sea coral that can be studied and utilized for health restoration.

Today, a large variety of bone substitutes are used for bone replacement in humans. The biocompatibility of these so-called osseous substitutes must be tested by current multiple-stage evaluation before implantations in patients can be carried out. Animal experiments must be preceded by in vitro testing for cytotoxicity, mutagenicity and/or carcinogenicity.

More than 100 methods are available today to identify carcinogens and mutagens. Many of them are based upon the principle that genotoxicity, i.e. mutagenicity may serve as an indicator of carcinogenic potential.

To detect the genotoxic potentials of tissue replacement material we employed the reverse mutation test using bacteria (Ames test). In this study, all the bacterial tester strains treated with and without S9 Mix showed no increase of revertant colonies with increase in concentration of test substance for both the range finding test and the main test. The number of revertant colonies was less than twice that of the solvent control for all the five bacterial strains and this was reproducible for both the dose finding test and the main test.

Positive results from the bacterial reverse mutation test indicate the substance induces point mutations by base substitutions or frameshifts in the genome of either *Salmonella typhimurium* and/or *Escherichia coli*. Negative results indicate that under the test conditions, the test substance is not mutagenic in the tested species and clearly justify further animal experiments.

CONCLUSION

In conclusion the coral material was considered to have no reverse mutagenic potential and non-genotoxic biomaterial under the present test condition. The data suggest that the coral bone graft substitute produced by the National Tissue Bank, USM, does not pose a human cancer risk.

ACKNOWLEDGEMENTS

We acknowledge with gratitude the assistance and guidance from Dr. Chen Sau Soon and Ms. Hasnah Mohd Zin from Environmental & Energy Technology Centre, SIRIM Berhad. We wish to thank Ms Azlina Ahmad for her contribution in the development of Genotoxicity Lab in Universiti Sains Malaysia Health Campus.

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