Evaluation of Heavy Metals Content, Mutagenicity, and Sterility of Indonesian Coral Goniopora sp. as Bone Graft Candidate

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Abstract. It has been reported that *Goniopora sp.* coral originating from sea water can induce osteogenesis and regeneration of bone. However, biocompatibility and safety aspects of this material have not been reported yet. We have evaluated the mutagenicity of the raw material and the sterility of processed material of Indonesian coral *Goniopora sp.* as a potential candidate for osteogenic bone graft. In addition, heavy metals were also identified and determined. A mutagenicity test was conducted using the Ames test, while a sterility test was carried out based on the direct inoculation method. The heavy metals tested – including arsenic, lead, cadmium, chrome, cobalt, silver, and mercury – were analyzed by neutron activation analysis or atomic absorption spectrophotometry. The results showed that the Indonesian coral did not display mutagenic properties and proved sterile after irradiation. Arsenic, lead, cadmium, mercury, chrome, cobalt, and silver were identified at a concentration level of ≤ 2.65, ≤3.60, 25.23, 1.72, 34.67, 0.51, and 44.01 ppm, respectively. Taking into account arsenic, lead, cadmium and mercury contents and their provisional tolerable daily intake (PTDI) values, the maximum daily safe exposure level of Indonesian coral *Goniopora sp.* was predicted to be 1 g/person. It was concluded that the coral can be developed as a potential osteogenic bone graft.

Keywords: *biocompatibility; bone graft; coral; Goniopora sp., heavy metals; mutagenicity; sterility.*

1 Introduction

In the field of oral surgery, patients with bone defects in oral and maxillofacial regions due to tumors, traumas, cysts and infectious diseases are common cases. Up to now, bone graft is one of the materials of choice to fix up those defects. As bone substitute, bone graft should be compatible with endogenous tissue, non toxic, non allergenic and not induce inflammation.

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The application of autogenous bone graft to fix bone defects is still the method of choice due to its less allergenic properties. However, on the one hand, this method can increase patient morbidity due to second surgery that should be carried out to take the bone; on the other hand, it is sometime difficult to obtain enough bone due to limitation of donor availability. Non-autogenous bone graft such as allograft, xenograft and aloplastic materials have been developed as alternatives. Alograft is bone graft from cadavers but has no osteogenic properties and consequently only slowly induces bone formation. Furthermore, there is a risk of virus contamination, for example HIV because the preparation process cannot inactivate the HIV virus. The application of aloplastic material has advantages, i.e. it does not increase patient morbidity and there is no risk of virus contamination. However this material is expensive and still difficult to obtain.

The Indonesian coral *Goniopora sp.* has been reported to show osteoconductive properties and is suitable to be applied as a scaffold in the regeneration process of bone. In an in vitro and in vivo test, the coral that had been sieved through a 200 mesh sieve and showed an average particle size of 1000 nm, was able to increase the osteogenic process, induce regeneration of bone, increase formation of collagen tissue and induce bone trabekula [1].

However, biocompatibility and safety aspects of this material still have not been reported. In this research we have evaluated the mutagenicity of the raw material and the sterility of processed material of Indonesian coral *Goniopora sp*. as a potential candidate for osteogenic bone graft. In addition, heavy metals that may be contained in this coral were identified and determined.

The mutagenicity test was performed by applying the Ames test [2], while the sterility test performed by applying the compendial method [3]. Heavy metals tested included arsenic (As), lead (Pb), cadmium (Cd), chrome (Cr), cobalt (Co), silver (Ag) and mercury (Hg). The analysis of As, Pb and Cd was performed by means of atomic absorption analysis (AAS) [4] while Cr, Co, Ag and Hg were analyzed applying neutron activation analysis (NAA) [5-7].

2 Material and Method

2.1 Mutagenecity Test

2.1.1 Microorganisms and Other Materials

Salmonella typhimurium TA 1535 was used in this experiment. Coral *Goniopora sp*. samples were used in several concentrations. Growth medium for Salmonella was prepared in 2 parts: *top agar* and *bottom agar*. *Bottom agar*

contains glucose, while *top agar* contains histidin or histidin-biotin with the proportion of top agar : histidin-biotin being 10:1.

Other materials used were solution of histidin and biotin (0.5 mM), ampicillin and tetracyline, LB agar plate, nutrient agar, *Nutrient Broth Oxoid* No. 2, aquadest, *agar media*, NaCl, Vogel Bohrer solution, crystal violet and sodium azide.

Apparatus and instruments used were: 37 °C incubator, petri dishes, 10 mL vial, erlenmeyer flasks, water bath, *homogenizer,* autoclave, microscope, colony counter, UV light (15 watt), refrigerator.

2.1.2 Master Plate Culture

The bacterial culture was inoculated in *Nutrient Broth Oxoid* No. 1 medium, with a density of 1-2 x 10^9 cells per mL and incubated at 37 °C. A genotype strain test and a spontaneous reversion test were carried out before the Ames test was performed. The bacteria of the stock culture were inoculated in 10 mL of *Nutrient Broth Oxoid* No. 2 and then incubated at 37 °C overnight. The bacteria were then transferred into 20 mL of medium (master plate 1) containing histidin. The culture was then incubated at 37 °C for 48 hrs. The 2nd master plate was prepared by transferring the bacteria from master plate 1 to the medium containing histidin, after which the plate was incubated at 37°C overnight.

2.1.3 Genotype Test

The genotype test was carried out to guarantee that the bacteria were in good condition and valid for the testing procedure. The genotype test consisted of several tests, i.e. requirement of histidin test, *rfa* mutation test, R-factor, PAQ1 plasmid for tetracycline resistancy test, *uvrB* deletion test for sensitivity against UV light [8].

2.1.4 Spontaeous Revertion

Spontaeous revertion was also checked to see the strain dependence to histidine and counting the number of mutants that appeared spontaneously on the agar plates. 20 mL of *bottom agar* and 3 mL *top agar* was prepared and 0.15 mL of *Salmonella typhimurium* TA 1535 culture was added to the agar. The culture was incubated at 37 °C for 48 hrs. The colonies that appeared were counted.

2.1.5 Mutagenicity Test

In the Ames assay, a test tube containing a suspension of *Salmonella typhimurium* TA 1535, with and without S9 mix, were incubated for 20 min at 37 ºC with the sample material (coral powder). Control cultures, with the same ingredients except for the coral, were also incubated. In addition, positive control cultures were prepared, which contained the bacteria and a known potent mutagen (sodium azide). Then agar was added to the cultures and the contents of the tubes were thoroughly mixed and poured onto the surface of petri dishes containing standard bacterial culture medium.

The plates were incubated and bacterial colonies that do not require an excess of supplemental histidine appeared and grew. These colonies comprised of bacteria that have undergone reverse mutation to restore the function of the histidine-manufacturing gene. The number of colonies was counted after 48 hrs. The formula of inhibition rate (%) = (A-B)/A x 100, where A is revertants in the positive control and B is revertants in the infusion samples, after substracting the spontaneous revertants [9].

2.2 Sterility Test

For conducting the sterility test of the coral after irradiation, several tests were carried out, including sterility and fertility tests of the media and bactericidal and fungicidal activity tests of the coral. These tests were performed to guarantee the validity of the sterility test.

2.2.1 Sterility Test of Media

FTM and TSB media were used in the sterility test. The media were incubated at 37 °C and 25 °C respectively after sterilization. After 14 days the media were observed.

2.2.2 Fertility Test of Media

FTM and TSB fertility was tested to prove that the media were able to support microorganism growth. 20 mL of each medium was inoculated by 10^6 CFU of *B. subtilis* and 10^3 CFU of *C. albicans*, respectively. The media were then incubated at 37°C for FTM and 25°C for TSB, for 14 days. The turbidity of the media was observed.

2.2.3 Bactericidal and Fungicidal Activity Test of Coral

The coral was tested for its antibacterial and antifungal activity by inoculating 10^6 CFU of *B. subtilis* and 10^3 CFU of *C. albicans* into 20 mL of medium containing 25 mg of coral. The medium was incubated at 37°C for FTM and

25°C for TSB for 14 days. If the media remained clear, this proved the antibactericidal and antifungicidal activity of the coral.

2.2.4 Sterility Test of the Coral

The coral after irradiation was tested for its sterility by transferring 25 mg of coral into 20 mL of FTM and TSB media. The medium was incubated at 37°C for FTM and 25°C for TSB for 14 days.

2.3 Determination of As, Pb and Cd in Coral Sample:

2.3.1 Sample Preparation

0.1 g of coral sample (accurately weighed) was transferred into a PTFE vessel. 2.5 ml of demineralized water and 7.5 ml concentrated nitric acid were added into the vessel. The sample was dissolved and digested using a microwave digester for 20 minutes at 200°C and then transferred into a beaker glass. The sample solution was then evaporated at a temperature of less than 60°C. The residue was then re-dissolved with demineralized water and quantitatively transferred into a 10.0 ml measuring flask.

2.3.2 Standard Solutions Preparation

A series of standard solutions of each Pb and Cd with a concentration of 0.2, 0.4, 0.8 and 1.2 ppm respectively were prepared by stepwise dilution of Pb- and Cd-tritisol (each 4000 ppm) standard stock solutions using 1 N nitric acid solution. A series of standard solutions of As with a concentration of 5.0, 10.0, 20.0, 30.0 and 40.0 ppm respectively were prepared in the same manner from As-tritisol standard stock solution (4000 ppm), but the final standard solutions (in a 100.0 ml measuring flask) contained 10 ml of 12 N hydrochloric acid (HCl), 2 drops of concentrated formic acid and a small amount of potassium iodide (KI).

2.3.3 Measurement of As

To sample solutions, concentrated HCl and potassium iodide (KI) solution were added so that the final concentration of HCl and KI was 2M and 200 ppm, respectively. The solution was left for one hour and was then heated to 80°C and kept heated for 10 minutes. Then the sample was cooled to room temperature and diluted with demineralized water to 50.0 ml. Concentrated HCl and sodiumborohydride (NaBH4) were prepared as generating reagents according to the instruction manual of the AAS instrument. The sample and standard solutions were aspired. As was measured at 193.7 nm.

2.3.4 Measurement and Calculation of Pb and Cd

The sample and standard solutions of Pb and Cd were aspired. Pb was measured at 283.3 nm, while Cd was measured at 228.8 nm. The calculation of Pb and Cd concentrations in the coral sample was carried out using a common calibration curve method.

2.4 Determination of Cr, Co, Ag and Hg in Coral Sample:

2.4.1 Sample Preparation

0.02 g of sample (accurately weighed) was transferred into a polyethylene vial.

2.4.2 Standard Solution Preparation

A standard solution of Cr, Co, and Ag, each of 2 ppm and of Hg (1 ppm), was prepared in a polyethylene vial by mixing and stepwise dilution of standard stock solution of Cr, Co, and Ag, and Hg (each 4000 ppm). The mixed standard solution was placed on a rack and dried in a drying oven.

2.4.3 Irradiation of Sample and Standard

The sample and standard solution were placed in an aluminium container and irradiated in a Rabbit System Reactor with a power of 15 MW and neutron flux of 10^{-13} $n.cm^{-2}.s^{-1}$, for 1 h. After irradiation, the sample and standard solution were put in a post-irradiation box for 2 to 3 weeks.

2.4.4 Measurement of Cr, Co, Ag and Hg

Gamma radiation of the sample and standard solution were counted using gamma detector HPGe for 10000 seconds at 320.08, 1332.5, 657.76, and 279.19 keV each for Cr, Co, Ag and Hg, respectively. The mass of each element in the sample and standard solution was calculated using a one-point method based on the ratio of the radionuclide activity of the sample to the standard of each element multiplied by the known concentration of the standard.

3 Result and Discussion

3.1 Mutagenicity Test

The results after incubation are shown in Table 1.

According to Maron and Ames [2], to calculate the mutagenicity potency, the numbers of revertants of the samples was compared to the negative and positive control. The substances can be categorized as mutagen if the value of the

comparison is higher than 2.0. If it is higher than 1.7 but lower than 2.0 it means that the substance has the potential to be mutagenic, and if it is lower than 1.6 means there are no potential mutagenic properties.

Table 1 Ames test results for coral.

Samples	Numbers of revertants	
	Without S9	**With S9**
Negative control (spontaneous reversion)	259	332
Positive control (Sodium Azide 1 ppm)	857	949
Coral	170	243

It was shown in Table 1 that the coral sample tested without eukaryotic metabolic enzyme (S9) gave values of 170/259 (0.65) and 243/332 (0.73) with S9, whereas the sodium azide gave values of 857/259 (3.3) and 949/332 (2.8) for the test without S9 and with S9 respectively. The sodium azide showed a higher number of revertants and indicated that sodium azide has a high potency for mutagenicity as its value is higher than 2.0, whereas the coral showed a value lower than 1.6, both without S9 and with S9.

3.2 Sterility Test

The results of the sterility test taken after 14 days showed that the media solutions (FTM and TSB) were valid to be used; they had remained clear, which means that the media were sterile. For the fertility test, the media displayed turbidity, which means that the media were perfectly fertile and could be used to grow the microorganisms, since the *Bacillus subtilis* and *Candida albicans* used in the test are representative of bacteria and fungi that grow well in the media. For the bactericidal and fungicidal test, the media remained turbid, which means that the samples had no bactericidal or fungicidal activity and we could proceed with the sterility test of the sample. In the sterility test of the samples the media remained clear, which means that the coral was sterile because there had been no growth of microorganisms after sterilization by irradiation.

3.3 Heavy Metals Content

As, Pb, Cd and Hg were identified in the coral samples and have been quantitatively determined. Table 2 shows the concentration level of each element in the ten coral samples. From this table it is clear that the average concentration of Cd (25.23 mg/kg) was especially much higher in comparison to that of As, Pb and Hg.

Table 2 Concentration of some elements in coral samples.

Number of Samples	Average concentration(mg/kg)						
	As*	Pb*	Cd	Hg	Cr	Co	Ag
10	2.65	3.60	25.23	1.72	34.67	0.51	44.01
Deviation Std.	0.00	0.00	6.83	1.04	13.27	0.20	17.96

*) Concentration of As and Pb in the coral sample was lower than the method detection limit (MDL) and hence could only be determined semi-quantitatively. MDL for As = 2.65 mg / kg, for Pb = 3.60 mg / kg. For the calculation of As and Pb exposure, the MDL values were applied.

As representatives of toxic heavy metals, As, Pb, Cd, and Hg were chosen for calculation of the maximum safe amount of the coral as bone graft. Taking into account the concentrations as well as the provisional tolerable daily intake (PTDI) values of these metals [10-16] and assuming the average body weight of population to be 60 kg, the exposure level of these metals could be predicted. PTDI values of As, Pb, Cd and Hg, stated in µg/kg body weight/day, are as follows: 2.14, 3.57, 1.00 and 0.47, respectively. These were calculated by taking the provisional tolerable weekly intake (PTWI) reported in the literature [10-16] and dividing the PTWI values by seven (PTDI = PTWI / 7). The predicted exposure levels, stated as percentages of PTDI, were calculated as follows:

% PTDI = [(concentration of metal in coral (µg/kg) x weight of applied coral (kg)) / (PTDI (µg/kg body weight/day) x 60 kg)] x 100%.

Table 3 shows the predicted exposure levels of each metal at different weights of application.

Table 3 Predicted Exposure Level of As, Pb, Cd and Hg.

Metal	Concentration (µg/kg)	Predicted exposure level at different weights of application, stated as percentage of PTDI (%)			
		0.5 g	**1.0 g**	**1.5 g**	**2.0 g**
As	≤ 2650	1.03	2.06	3.09	4.12
Pb	≤ 3600	0.84	1.68	2.52	3.36
Cd	**25230**	**21.03**	**42.05**	**63.08**	**84.10**
Hg	1720	3.04	6.08	9.12	12.16
Predicted accumulated daily exposure (%)		**25.94**	**51.87**	**77.81**	**103.74**

Due to Cd being the metal with the highest concentration in the coral, it is also the main contributor to the exposure level of toxic metals, which limits the amount of coral that can be applied as bone graft. From Table 2, it is clear that the application of the coral up to 2 g daily is already equivalent to more than 100% of the PTDI. Taking into account possible intake of these toxic metals

from other sources, e.g. foods, it is suggested that the amount of the coral to be applied as bone graft should not exceed 1 g for one application, which is equivalent to nearly 52% of the PTDI.

4 Conclusion

It can be concluded that *Goniopora sp.* coral has no mutagenic activity and proved sterile after irradiation. The coral contained As, Pb, Cd and Hgat in a concentration of 2.65, 25.23, 3.0 and 1.72 mg/kg, respectively. Due to these toxic metal contents the safe amount of this coral to be applied as bone graft is around 1 g for one application. Based on these results, it can be concluded that the Indonesian coral *Goniopora sp.* can be further developed as an osteogenic bone graft candidate.

References

[1] Julia, V., Latief, B.S. & Winiati, E., *The Influence of Coral Addition (Goniopora sp) on Osteoblast Activity* (Research Report), University of Indonesia, Jakarta, 2006 (Text in Indonesian).

[2] Maron, D. & Ames, B., *Resived Methods for the Salmonella Mutagenicity Test, Mutation Research*, **113**(3-4), pp. 173-215, 1983.

[3] Directorate General of Food and Drug Control, *Indonesian Pharmacopeia,* 4th edition, Department of Health of Republic of Indonesia, pp. 855-863, Jakarta, 1995.

[4] Cantle, J.E., *Techniques and Instrumentation in Analytical Chemistry. Atomic Absorption Spectrometry*, Elsevier Scientific Publishing Company, pp. 139, New York, 1982.

[5] Friedlander, G., Kennedy, J.W., Macias, E.S. & Miller, J.M. *Nuclear and Radiochemistry,* 3rd ed., John Willey & Sons, pp. 424-427, New York, 1981.

[6] International Atomic Energy Agency (IAEA), *Training Course Series No.4: Sampling and Analytical Methodologies for Instrumental Neutron Activation Analysis of Airborne Particulate Matter*, IAEA, December, pp. 28-37, Vienna, Austria, 1992.

[7] William, D.E. & Diane, E.V., *Radiochemistry and Nuclear Methods of Analysis*, John Wiley and Sons Inc., pp. 253, New York, USA, 1991.

[8] Mortelsman, K. & Zeiger, E., *The Ames Salmonella/Microsome Mutagenicity Assay*, Mutation Research, **455**(1-2), pp. 29-60, 2000.

[9] Horn, R.C. & Vera, M.V., *Antimutagenic activity of Extracts of Natural Substances in the Salmonella/Microsome Assay*, Mutagenesis, **18**(2), pp. 113-118, 2003.

[10] Joint FAO/WHO Expert Committee on Food Additives, *Toxicological Evaluation of Certain Food Additives and Contaminants*. WHO Food

Additives Series, No. 24, Cambridge University Press, pp. 155-162, Cambridge, 1989.

[11] Joint FAO/WHO Expert Committee on Food Additives, *Toxicological Evaluation of Certain Food Additives and Contaminants.* WHO Food Additives Series, No. 24, Cambridge University Press, pp. 163-219, Cambridge, 1989.

[12] Joint FAO/WHO Expert Committee on Food Additives, *Evaluation of Certain Food Additives and Contaminants: Forty-First Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series, No. 837, World Health Organization, pp. 28-32, Geneva, 1993.

[13] Joint FAO/WHO Expert Committee on Food Additives, *Toxicological Evaluation of Certain Food Additives and Contaminants, WHO Food Additives Series*, No. 21, Cambridge University Press, pp. 223-255, Cambridge, 1987.

[14] Joint FAO/WHO Expert Committee on Food Additives, *Evaluation of Certain Food Additives and Contaminants: Forty-First Report of The Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series, No. 837, World Health Organization, pp. 32-35, Geneva, 1993.

[15] Joint FAO/WHO Expert Committee on Food Additives, *Evaluation of Certain Food Additives and the Contaminants Mercury, Lead, and Cadmium: Sixteenth Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series, No. 505, World Health Organization, pp. 11-24, Geneva, 1972.

[16] Joint FAO/WHO Expert Committee on Food Additives. *Evaluation of Certain Food Additives and Contaminants: Twenty-Second Report of the Joint FAO/WHO Expert Committee on Food Additives*, WHO Technical Report Series, No. 631, World Health Organization, pp. 25-28, Geneva, 1978.