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GENETICS

An *in vitro* Study of Genotoxicity of Silver Amalgam

Akram Hassan, Muhammad Hidayat Muhamad Daud, Zaihan Ariffin, Mohammad Khursheed Alam

ABSTRACT

Background: **Silver amalgam/Silverfil Argentum® is a 'Malaysian made amalgam' has already been approved to be free from cytotoxicity, however its genotoxic effect has not been explored yet as biocompatible material. The objective of this study was to identify the genotoxic characteristic of silver amalgam by using Bacterial Reverse Mutation Assay (Ames test).**

Materials and Methods: **This was a descriptive experimental study involving two strains of mutated *Salmonella*. The test material was evaluated in two mutated strains of *Salmonella typhimurium* TA98 and TA100 with and without an external metabolic activation system (S9 Mix). The bacteria were incubated for 48 hours at 37 ± 0.5℃ before the colony growth or revertant colonies were counted. Data obtained were analyzed by using non-statistical method.**

Results: **The investigation of the genotoxic reaction on the test material revealed that the numbers of revertant colonies in both strains with and without S9 Mix were less than twice of the negative control even in the presence of high silver amalgam concentrations (5.0 µg/ml).**

Conclusion: **This study demonstrated that the test material did not exhibit any mutagenic activity under the chosen conditions. Thus, silver amalgam could be considered to have no genotoxicity effect.**

KEY WORDS

silver amalgam, genotoxicity, Ames test

INTRODUCTION

Amalgam was recognized to be the most ancient and long lasting restorative material ever been made until now. In spite of good hardness properties, level of toxicity of mercury content is keep declining over the years. Many companies of amalgam product passionately running in research and put full effort in order to notify their product to be free from mutagenesis. Silverfil Argentum® is a Malaysian made amalgam (Mohamad *et al*. 2013) developed since 10 years ago has successfully received FDA 510 (K) clearance from the United States Department of Health and Services in America.

An international standard (ISO) lays down specific requirements for biocompatibility, including the tests based on the nature of the contact and the duration of implantation of the biomaterial. The standard stipulates that all materials that will be in contact with mucous membrane, bone or dentinal tissue where the contact exceeds 30 days, as well as all implantable devices where the contact exceeds 24 hours, must undergo genotoxicity testing (Chauvel-Lebret *et al*., 2001). According to International Organization for Standardization (ISO) (1992), when the genetic toxicity of a medical device has to be experimentally assessed, a series of *in vitro* tests shall be used, this series shall include at least three assays and at least two of these should preferably use mammalian cells as a target.

Kirkpatrick *et al*. (1997) and Katzer (2002) suggested to use an *in vitro* study after allowing for cost intensive and time consuming. Moltelmans and Zeiger (2000) strongly agreed that *Salmonella typhymurium* / microsome assay to be widely accepted as a short-term bacterial assay in order to identify the substance that can produce genetic damage that leads to gene mutations. To reduce the number of biomechanical studies, evaluation of new biomaterials should nowadays begin with *in vitro* cytotoxicity and mutagenicity tests. This applies for the development of both temporary and permanent implants and prostheses and for permanent implant particularly under the aspect that carcinogenic potential is often the consequence of chronic exposure to minute concentrations (Katzer *et al*., 2002). Although Silverfil Argentum® had successfully explored in cytotoxic effect studies, the genotoxic characteristics have to be carried out before it can be known as biocompatible material.

Therefore, the aim of this study was to identify the effect of genotoxicity in this product.

MATERIALS AND METHODS

Test Material

Silverfil Argentum® which obtained from Dunia Perwira Manufacturing Sdn. Bhd. in capsule form was triturated by using amalgamator for 5 seconds. The bolus was let to be dried and hardened before it can be powderized. The powder was weighted into 5 unit of scale of 5000 µg/2500 µg/1250 µg/625 µg/313 µg per plate respectively by using micro weight machine (mg scale). Each unit scale of powder was then diluted into 1 ml of Dymethylsulfoxide (DMSO) solution and kept inside incubator for 24 hours at 37 ± 0.5℃ rolling prior to the procedure. Apart of it, 60% reactive silver and 40% silver mercury makes Silverfil Argentum® less expose into cytotoxic effect. It saved inside autoclave machine with 121℃ heated as the boiling point reaching 357℃.

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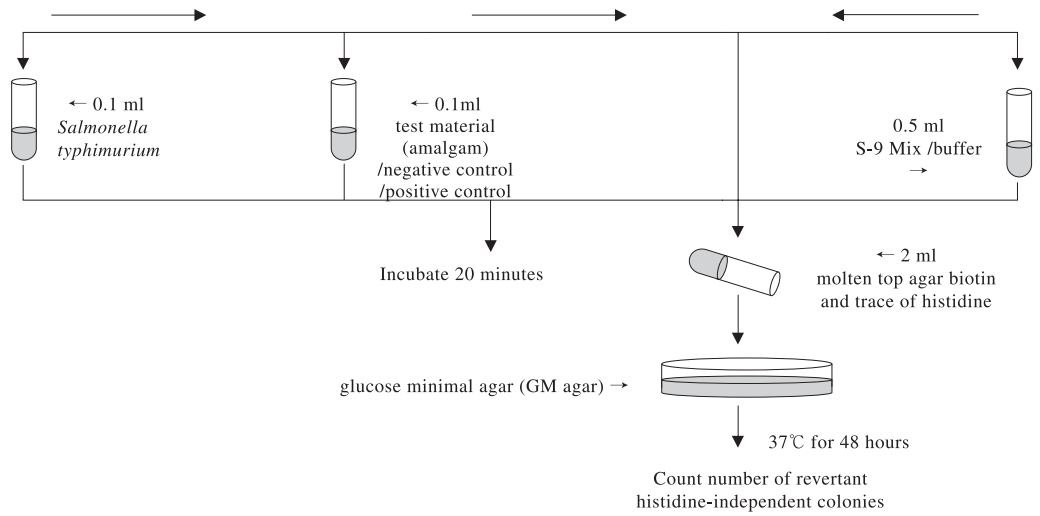

Figure 1. Overview of bacterial reverse mutation test (Ames Test) process

Table 1. Positive Controls

Strain	Positive control chemical (-S9)	Molar concentration (mg/plate)[a]	Positive control chemical (+S9)	Molar concentration (mg/plate)[a]
TA98	4-Nitro-o-phenylenediamine	2.5	2-Animoantracene	2.5
TA100	Sodium Azide	5.0	2-Animoantracene	2.5

a - Concentration based on 100 x 15 mm petri plate containing 20 to 25 ml of GM agar (Mortelmans and Zeiger, 2000).

Ames Test

Ames test is a method introduced by people named B.Ames, J.Mc Cann and E.Yamasaki in 1975 using special genotype variants of the bacterium *Salmonella typhymurium* (Katzer, 2001). Basically, the bacteria was mutated and histidine dependent (lack of histidine genes) which unable to grow onto histidine-free agar. Once being exposed to the mutagens, it will revert and become histidine independent that make it able to grow and form a colony on the same plate. Here, TA98 and TA100 were selected strains used under samples 471 approved by International Organization for Standardization (1992). Genetically, TA98 is going to be for detection of frameshift mutation while TA100 is responsible on base-pair substitution mutation (Chang, 2002).

Therefore, reviving these two strains before use are very important particularly for genetic analysis. Those strains were obtained from Dr. T. Nohmi from National Institute of Health Science, Tokyo, Japan and were stored as frozen stock cultures (0.035 ml of DMSO/0.4 ml of broth culture) at -80 ± 5℃ in ultra-deep freezer (MDF 392AT, SANYO).

In this study, metabolic activation system was practically used to activate the Silverfil Argentum® to be more mutagenic, thus increase the number of colony growth on the agar. It contained cytochrome-based P450 mimicked endogenous human liver. An exogenous mammalian organ activation system that consists of a 9000xg supernatant fraction of a rat liver homogenate (S9) with presence of cofactors for NADPH-supported oxidation was used (Mortelmans and Zeiger, 2000).

Sterile distilled water was used as negative control (Chang, 2002). Positive control chemicals were specifically designed as highly mutagenic to each strain. 4-Nitro-o-phenylenediamine (4-NoPD) was definitely for strain TA98 and Sodium azide (NAN3) was specifically for strain TA100. Both were applied without S9 (replaced with Sodium phosphate buffer). In another hand, 2- Aminoantracene (2AA) was used in S9 inclusion (Mortelman and Zeiger, 2000).

S9 mix and buffer were obtained from Kikkoman Co. Ltd. and stored inside ultra-deep freezer under -80 ± 5℃ (MDF 392AT, SANYO).

Reviving (Frozen Working Cultures)

1 ml of *Salmonella typhymurium* was inoculated into 5 ml of nutrient broth and nutrient agar. This procedure was aseptically done in a 'Lamina Flow'. These two mediums were then incubated for overnight in the incubator (SANYO) at 37 ± 0.5℃. The result was shown cloudy look in nutrient broth and colonies were formed on the nutrient agar. One isolated healthy colony was transferred onto Glucose Minimal (GM) agar and incubated for 48 h at 37 ± 0.5℃. This would later produced colony and subculture was repeated for another 48 hours. The last colony formed on the GM agar so-called master plate was being run for genetic analysis by streaking the bacteria onto histidine, biotin and histidine-biotin filled agar for 15 hours (Mortelmans and Zeiger 2000, Chang 2002). There were no growths seen in histidine and biotin agar but presence on histine-biotin agar, rfa marker and presence of plasmid pKM101 with ampicillin resistance had shown growth inhibition which proved that TA98 and TA100 were mutated on specific genotype.

Main Test

After preparing frozen working cultures which underwent genetic analysis, TA98 and TA100 were ready to be used. Silverfil Argentum® was incubated at 37 ± 0.5℃ for 24 hours prior to the procedure. Amount of S9 fraction was measured within the range 10-30% (v/v) in the S9 mix by using the formula calculated below:-

$$10\% = \frac{\text{0.04 ml S9 to 0.1 ml S9}}{\text{1 ml cofactor per plate}}$$

So, 10% (v/v) was used for this test and was ready inside sterile glass tube containing ice cube and maintained cold along the procedure.

For the 13 x 100 mm sterile glass tube, the following orders were

Table 2. Result for *Salmonella typhymurium* TA98

	Substance	Number of revertant colonies		
	Negative control	455		
	Positive control	932		
	Test substance(μg/plate)	Plate 1	Plate 2	Mean
without S9 Mix	313	483	880	682
	625	683	1001	842
	1250	432	584	508
	2500	296	1017	657
	5000	669	584	627
	Negative control	125		
	Positive control	298		
	Test substance(μg/plate)	Plate 1	Plate 2	Mean
with S9 Mix	313	232	139	186
	625	156	155	156
	1250	125	168	147
	2500	203	104	154
	50001	137	143	140

Table 3. Result for *Salmonella typhymurium* TA100

	Substance	Number of revertant colonies		
	Negative control	321		
	Positive control	791		
	Test substance(μg/plate)	Plate 1	Plate 2	Mean
without S9 Mix	313	69	109	89
	625	593	277	435
	1250	541	130	336
	2500	101	85	93
	5000	135	142	139
	Negative control	410		
	Positive control	858		
	Test substance(μg/plate)	Plate 1	Plate 2	Mean
with S9 Mix	313	997	275	636
	625	804	61	433
	1250	96	99	98
	2500	658	230	444
	5000	264	460	362

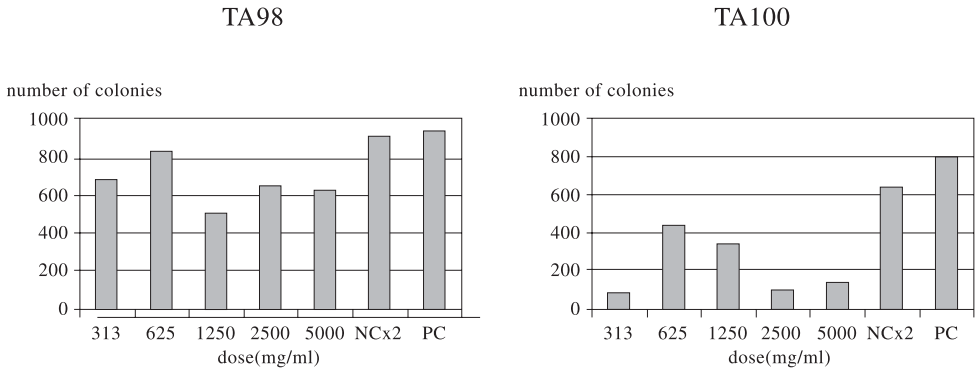

Figure 2. Tests in two strains without metabolic activation (-S9 Mix)

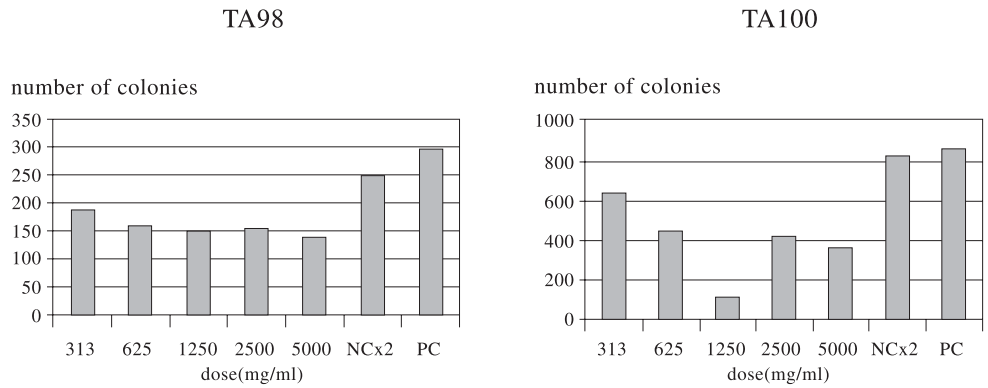

Figure 3. Tests in two strains with metabolic activation (+S9 Mix)

added with mild mixed:

* 0.1 ml Silverfil Argentum® extracts
* 0.1 ml *Salmonella* strain
* 0.5 ml sodium phosphate buffer, 0.1 M pH 7.4

The mixture was incubated for 20 minutes at 37 ± 0.5℃. Then, 2 ml of molten top agar was added, gently mixed and quickly poured onto the surface of GM plate agar. In order to make sure of evenly distributed, this procedure was done within 2 minutes and manually stirred. After the top agar was hardened, the plate was incubated in inverted position at human temperature for 48 hours. Final result was obtained by counting the colony growth using Colony Analyzer (ProtoCol.). The procedure was standardized for S9 mix, solvent and positive controls.

Amount of positive control chemicals were calculated by using a formula gained below:-

M1V1 = M2V2 (M = molar, V = volume)
M1 = molar concentration (x) M2 = molar concentration of chemical
V2 = 0.7 ml solution V1 = 0.1 ml solution

No ethical clearance needed for this test seems it was not involved human and animal product.

Statistical Consideration

This approach also has its weaknesses. It has been shown that the two-fold rule may be too insensitive for *Salmonella* strains with relatively high reversion frequencies, such as TA100, TA97, and TA102, and too sensitive for chemicals with low reversion frequencies, such as TA1535 and TA1537. Therefore, non-statistical approach was applied here. Mortelmans and Zeiger (2000) implemented following criteria to interpret results.

- *Positive:* A compound is considered a mutagen if it produces a reproducible, dose-related increase in the number of revertant colonies in one or more strains.
- *Negative:* A compound is considered a nonmutagen if no dose-related increase in the number of revertant colonies is observed in at least two independent experiments.
- *Inconclusive:* If a compound cannot be identified clearly as a mutagen or a nonmutagen, the results are classified as inconclusive.

RESULTS

The test substance is considered to be mutagenic when the number of counted colonies exceeds the number of colonies in the negative controls by at least double and a relationship between dose and response can be observed. The results in the *Salmonella* tester strains are presented in Table 2 and 3 and shown in Figure 2 and 3.

The results showed the numbers of revertant colonies which were treated with the test substance were less than twice of negative control with and without S9 Mix at all concentrations. No dose-response relationship was obtained and no statistical procedure was used.

The results showed the numbers of revertant colonies which were treated with the test substance were less than twice of negative control with and without S9 Mix at all concentrations. No dose-response relationship was obtained and no statistical procedure was used.

Figure 2 showed that the test for mutagenesis in two strains without metabolic activation (-S9 Mix) with different concentrations of Silverfil Argentum®, gave no evidence for mutagenic effects.

Figure 3 showed that the test for mutagenesis in two strains with metabolic activation (+S9 Mix) with different concentrations of Silverfil Argentum®, gave no evidence for mutagenic effects.

DISCUSSION

Based on International Standard, ISO 10993-2 (1992), approximately eight different *in vitro* assays are listed down for researchers to choose. However, the best two tests would be Organization for Economic Cooperation and Development (OECD) tests which were *Salmonella typhimurium* reverse mutation assay (471) and mammalian cytogenetic test (473). In this study, the assay used was *Salmonella typhimurium* reverse mutation alone and it showed very significant findings. Margolin et al. (1985) mentioned of using TA98 and TA100 as a two out of 6 recommended strains of bacteria with and without metabolic activation (S9) system in this test was sufficient to identify approximately 90% of the mutagens in a population containing about 35% mutagenic chemicals.

Although all known assays can yield false-positive and false-negative results, experience shows that the combination of two different test methods is a reliable parameter for determining carcinogens which are a risk to human health (Katzer *et al*., 2002).

A minimum of two assays is needed as a battery that is capable of detecting most potential mutagens and genotoxic carcinogens. And this study is only a part of an *in vitro* evaluation of genotoxicity of Silverfil Argentum. Concentrations of Silverfil Argentum® with a range from 313 µg to 5000 ug were chosen as standard values given by Mortelmans and Zeiger (2000) because this material has previously approved to be out of cytotoxic effect and yet the 5000ug was the highest indicator value to respond to any genotoxic activity.

To ensure the comparability of results, the extraction temperature should preferably be 37 ± 0.5℃ that mimicked the human body temperature and the time must be at least 24 hours. Silverfil Argentum® was dissolved into distilled water and not DMSO simply because DMSO solution is known to be high cytotoxic. This study involves the mixture of S9 as metabolic activation system because *Salmonella typhymurium* is only a prokaryote (basic cell living structure) and cannot represent the human being as a perfect model; therefore the addition of the exogenous liver enzyme could enhance the quality of the result.

After 48 hours incubated, the visible colonies were only *Salmonella typhymurium* and not other organisms. It was due to highly mutated characteristic of *Salmonella typhymurium* both at the level of gene transcription and gene translation that had been proven through genetic analysis. This would make the bacteria to be strong enough to kill other organisms and able to grow in the absence of histidine plate agar. The revertants were counted by using Colony Analyzer (ProtoCol.). The specificity and sensitivity was fixed to 96% with 8cm diameter of countered area. This measurement homogenously has been done to all plates.

From the data, we found that Standard Deviation (SD) value generally was high and it was due to small samples and replications. However, if we deal with experimental environment, it is enough to only duplicate or triplicate the test if the first trial gives a set of data within expected result. Nevertheless, in the future *in vitro* genotoxicity test which will preferably use more strains and mammalian cells as target should be carried out. *In vivo* genotoxicity test can be undertaken if scientifically indicated or *in vitro* test results indicate potential genotoxicity. Technique sensitive and low sterility were the main problems here and need to be overcome in order to get better results. A nanotechnology measurement technique hopefully can be applied to cut the tester material into more identical size according to reference doses.

CONCLUSION

These data were demonstrating as negative result and showing the test material Silverfil Argentum did not exhibit mutagenic activity and no dose-related increase in the number of revertant colonies under the present test conditions. Thus, the material was considered to have no genotoxic effect.

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