Magnesium Alloy Specimen

In Vitro Cytotoxicity Test

-MTT Assay

FINAL REPORT

Sponsor: Metal Industries Research & Development Centre

Testing Institution: SGS Taiwan Ltd.

Report No.: UP/2014/60049

Date of Writing: 2014.06.30

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STUDY SCHEDULE
In Vitro Cytotoxicity Test-MTT Assay
Magnesium Alloy Specimen

Report No.:	UP/2014/60049
Study Initiation date:	2014.06.20
Experimental starting date:	2014.06.20
Experimental completion date:	2014.06.26
Study completion date:	See Study Director's signature date in the report
Name of study Personnel:	張恩慈

Testing Institution

Name: SGS TAIWAN LTD .

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Sponsor/Client

Name: Metal Industries Research & Development Centre

Address: 3F, No.88, Luke 5th Rd., Luzhu Dist., Kaohsiung City, Taiwan 82151, R.O.C. (Kaohsiung Science Park)

INFORMATION FOR TEST ARTICLE

試驗物質資料表

☒試驗物質/☐對照物質資料表

委託單位名稱	金屬工業研究發展中心
委託單位地址	82151高雄市路竹區路科五路88號3樓
委託試驗項目	☒ 以合約為主 ☐ 其它 ：
試驗物質/對照物質名稱	鎂合金試片
批號	☐依據特定編號：_____ ☐其它：_____ ☐依據包裝上日期：_____ ☒無批號可提供
規格數量	45.6cm2/片*11片 (如10mL/瓶*6瓶)
提供之同批號量 (註2)	☒1次之測試使用 ☐2次以上之測試使用(留樣用)
外觀型態	型態：☐液狀☐粉狀☐錠狀☐膠囊狀 ☒其他：片狀 / 顏色：乳白色
主要成分及純度	成分：鎂 / 純度：90%以上
適合之溶劑及其溶解度	N/A
保存條件	保存條件：☐室溫 ☐4℃☐避光 ☒其他 真空包裝
保存期限(註3)	☐有效期限：西元_____年_____月_____日 或 ☐保存期間：共 _____年_____月_____日或 ☒依SGS UB之留樣保存期限為主
檢附之文件(註4)	☐分析證明☐安全資料表 ☐安定性測試結果 ☒無附件(註4) ☐其他：_____
滅菌	產品是否已滅菌☒是☐否 (如勾選YES請再勾選下方滅菌方法) 滅菌方法是 ☐EO滅菌☒Gamma滅菌 ☐蒸汽滅菌☐其他_____
醫療器材使用之範疇 (非醫療器材者免填)	1.☐與皮膚或黏膜短期接觸(接觸人體累積時間) ☐短期接觸(不超過4 hr) ☐長期接觸(超過4 hr以上) ，最長累積時數為_____小時 2.☒ 植入式的醫療器材
特殊需求(註5)	N/A

客戶簽名/日期：[signature] 2014.6.9

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版次：3.3 試驗-對照物質資料表 Information for test article-control article
發行日期：2013.12.25 page 1

SIGATURE OF STUDY PERSONNEL
In Vitro Cytotoxicity Test-MTT Assay
Magnesium Alloy Specimen

Study Director:

劉錦誠　　2014.07.04

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ABSTRACT

In vitro cytotoxicity test was performed in this study to evaluate the biological compatibility of "Magnesium Alloy Specimen", which was provided by Metal Industries Research & Development Centre. Extraction of test article and treatment of mouse lung fibroblast cells (L929 cells) with test article extracts were performed according to ISO10993-12 and ISO10993-5, respectively. Cell viability determined by MTT assay showed that the test article extract had in average <30% inhibitory effects to the viability of cells. Together with qualitative observations of cell morphology, these results suggested that the test article extract did not induce cytotoxic effect in L929 cells.

PURPOSE

According to the nature and duration of the anticipated contact with human tissues when in use medical device should be carefully tested for biocompatibility to avoid potential physiological damage by toxic substances produced or contaminated during manufacturing. In this study, “Magnesium Alloy Specimen” was subjected to in vitro cytotoxicity test to evaluate toxicity of substances that could be extracted or released from the medical device. Therefore, the test system was mouse lung fibroblast cells (L929 cells). The original source was from BCRC. Based on recommendations described in ISO10993-5, quantitative determination of cell viability by MTT assay and qualitative observation of cell morphology were carried out, followed by concluding level of cytotoxicity according to the scoring criteria listed in the document. These results provided practical information for assessing the *in vitro* cytotoxicity of the medical device.

EXPERIMENTAL DESIGN

1. Test System

A. Cell line: Mouse lung fibroblast L929 cells. The original source was supplied by Food Industry Research and Development Institute, Strain No.: BCRC 60091. Bank No.: 20110506-1E-NA-04

B. Morphology: Fibroblast-like

Culture properties: Adherent

C. Incubation condition: Incubate in Minimum essential medium Eagle with 10% horse serum at 37±1℃ in the presence of 5±1% CO_2

2. Reagent

A. 100X L-Glutamine solution (Gibco, Cat No. 25030-081, Lot No.:1293468; Expiry date: 2015.01.31)

B. 100X Penicillin-Streptomycin solution (Gibco, Cat No. 15140-122, Lot No.: 1491906; Expiry date: 2014.11.30)

C. 100X Sodium pyruvate solution (Gibco, Cat No. 11360-070, Lot No.: 1500935; Expiry date: 2014.11.13)

D. 10X Phosphate buffer solution (UniRegion Bio-Tech, Product No. UR-PBS001, Lot No.: PBS001-5B; Expiry date: 2016.08.30)

E. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Sigma, Cat No. M5655, Lot No.: MKBP4399V; Expiry date: 2017.03.26)

F. Dimethyl sulfoxide, DMSO (Sigma, Cat No. 34943, Lot No.: SZBD1790V; Expiry date: 2017.04.11)

G. Horse serum (Gibco, Cat No. 16050-122, Lot No.: 1312382; Expiry date: 2017.04.16)

H. Minimum Essential Medium, MEM (Gibco, Cat No. 10370-021, Lot No.: 1374742;

Expiry date: 2015.06.27)

I. Trypsin solution (Gibco, Cat No. 25200-056, Lot No.: 1374941; Expiry date: 2015.06.30)

3. Equipment

A. Orbital Shaker Incubator (HILES, E-600, Equipment No.: INB-3)

B. Balance (DENVER, TB-214, Equipment No.: BAL-13)

C. Biological safety cabinet (LABCONCO, 3450801, Equipment No.: BSC-1)

D. CO2 Incubator (ASTEC, SCA-165DS, Equipment No.: INB-1)

E. Microscope (OLYMPUS, CKX41, Equipment No.: MIS-2)

F. Centrifuge (Eppendorf, 5804R, Equipment No.: CEN-7)

G. Water bath (Kansin,WB212-B2, Equipment No.: WAB-2)

H. Microplate Spectrophotometer (BioTekTM, Eon, Equipment No.: MPS-2)

4. Preparation of Test Article and Control Article

A. Test Article

The test article was handled under sterile environment and operated with aseptic technique during preparation. In this study 1.3920 g of test article was extracted in 6.960 mL MEM compete medium (contained 10% horse serum). The test article was extracted with a ratio of 0.2g /1mL in MEM compete medium for 24±2 hour at 37±1℃ with constant agitation at 150 rpm per criteria described in ISO10993-12. The pH adjustment, filtration and centrifugation were not conducted.

B. Control Articles

a. Blank control: MEM complete medium was as blank control.

b. Positive control: Polyurethane film – ZDEC (Polyurethane film containing

Zinc Di-Ethyldithio-Carbamate, RM-A, Hatano Research Institute, Japan) extracted with 0.1g ± 10% /1mL MEM compete medium was as positive control.

c. Negative control: HDPE film (High Density Poly-Ethylene film, RM-C, Hatano Research Institute, Japan) extracted with 0.1g ± 10% /1mL MEM complete medium was as negative control.

d. Extraction Condition: Extractions were performed at 37±1℃ for 24±2 hours (ISO10993-12) with constant agitation at 150rpm.

5. *In vitro* cytotoxicity test-MTT assay

A. Cell incubation

a. Preparation of complete MEM cell culture medium

Complete cell culture medium was prepared by mixing 435 mL of MEM, 5 mL of 100X Penicillin- Streptomycin solution, 5 mL of 100X L-Glutamine solution, 5mL of 100X sodium pyruvate and 50 mL of Horse serum. The completed medium was stored at 4℃.

b. Cell culture

Mouse lung fibroblast cells (L929 cells, Food Industry Research and Development Institute, Strain No. BCRC 60091) were used here for cytotoxicity test. The L929 cells were grown on a 10-cm dish containing 10 mL of complete MEM medium and incubated at 37±1℃ in the presence of 5±1% CO2. Detachment of the cells was performed by washing the cells with 1X PBS followed by treatment with 1.0 mL/dish of trypsin solution for 3 minutes at 37±1℃. Enzymatic activity of trypsin was terminated by adding complete MEM medium and then transferred to new 10-cm dish for subculture.

B. *In vitro* cytotoxicity test

a. 100 μL of L929 cell suspension (1×105cells/mL) was transferred into each

well of a 96-well cell culture plate. The cells were then incubated at 37±1℃ for 24±2 hours in a humidified atmosphere containing 5±1% CO_2.

b. Culture medium was replaced with 100 μL of test article extracts or controls. The cells were then incubated for another 24 hours. Treatments of the cells with the extracts were performed in triplicates.

c. Morphology of cells were observed under microscope and scored in accordance with ISO10993-5. The scoring criteria were summarized in Table 2.

d. Following evaluation of cell conditions, the culture medium was aspirated from the plates. 50 μL of the MTT solution was then added to each well and the plate was further incubated for 2 hours ± 10 mins at 37±1 ℃.

e. MTT solution was replaced with 100 μL of DMSO. The plate was incubated at room temperature for 10 minutes and subsequently subjected to a microplate reader equipped with a 570 nm filter for colorimetric measurement (reference 650 nm).

f. The triplicate results of MTT assay were presented as mean ± standard deviation (S.D.) and were scored in accordance with ISO10993-5 (as in Table 2). If the mean of cytotoxicity was less than 30%, the result showed "<30%".

g. Scores of cell morphology and inhibition of viability were averaged to give final interpretation of cytotoxicity.

6. Quality criteria

a. Positive control and negative control

(1)Positive and negative controls should be included in every cytotoxicity test.

(2)Positive control was Polyurethane film – ZDEC; Negative reference material

was HDPE film.

b. Blank

(1)Measure the absolute value of optical density, OD570, The acceptance criteria of blank was ≧ 0.2.

(2)Blanks were placed both at the left side (row 2) and the right side (row 11) of the 96-well plate.

(3)The left and right mean of the blanks do not differ by more than 15% from the mean of all blanks.

DATA MANAGEMENT

The quantitative data (Table 4) are showed as mean ± standard deviation (S.D.) and the qualitative data (Table 3) are scored using “Criteria for scores in cytotoxicity test” (Table 2). The individual score represents the average of triplicates (Table 5). The achievement of a numerical grade greater than 2 or reduction of cell viability by more than 30 % is considered a cytotoxic effect.

RESULT

1. Appearance

The extracts of the test article was no different than the blank control.

2. Cell Morphology

As shown in Table 3 and Figure 1, the cells exposed to negative control showed no significant change in cell morphology compared to that of reagent control and resulted in a score as 0. Positive control extract caused severe cellular damage and obvious morphological alteration in almost all cells. Therefore, the positive control experiment was scored as 4. The cells treated with test article extract showed discrete intracytoplasmatic granules and no cell lysis. Therefore, the cell morphology was scored as 0.

3. Inhibition of cell viability

The acquired readings of OD_{570} absorbance of reagent control were averaged and set as 0% inhibition of cell viability. In proportion to reagent control, we determined inhibition of cell viability of negative control, positive control, and test article as <30%, 98.22±0.03% and <30% respectively. The relative values of inhibition of cell viability were shown in Table 5.

CONCLUSION

The scores of the morphological evaluation and the relative inhibition of cell viability were averaged and listed in Table 5. Based on the ISO10993-5 guidelines, the "Magnesium Alloy Specimen" extract did not induce cytotoxic to L929 cells.

REFERENCES

1. Abiraman S, Varma HK, Kumari TV, Umashankar PR, John A (2002) Preliminary in vitro and in vivo characterizations of a sol-gel derived bioactive glass-ceramic system. Bull. Mater. Sci. 25(5), 419-429.

2. Mendes SC, Reis RL, Bovell YP, Cunha AM, Blitterswijk CA, Bruijn JD (2001) Biocompatibility testing of novel starch-based materials with potential application in orthopaedic surgery: a preliminary study. Biomaterials. 22, 2057-2064.

3. ISO 10993 (2009) Biological evaluation of medical device—Part 5: Tests for *in vitro* cytotoxicity.

4. ISO 10993 (2012) Biological evaluation of medical devices-Part 12：Sample preparation and reference materials.

5. SGS SOP: EOMP-USL-0003 Operating procedures and analysis of the Balance. Version 1.3

6. SGS SOP: EOMP-USL-0020 Eppendorf High-speed centrifuges Operating procedures. Version 1.1.

7. SGS SOP: EOMP-USL-0027 Operating procedures of the biosafety cabinet and laminar flow, UV-lamp verification and aerobic plate counts. Version 2.3

8. SGS SOP: EOMP-USL-0029 Operating Procedures of Microplate Spectrophotometer-BioTek EON. Version 1.1

9. SGS SOP: EOMP-USL-0030 Maintenance and operating procedures of the microscope. Version 1.1

10. SGS SOP: EOMP-USL-0035 CO2 incubator Operating procedures. Version 1.0

11. SGS SOP: EOMP-USL-0036 Operating Procedures of MilliQ. Version 1.0

12. SGS SOP: EOMP-USL-0037 Manual of freezer-refrigerators. Version 1.0

13. SGS SOP: EOMP-USL-0038 Incubator operating Procedures Version 1.0

14. SGS SOP: TESP-UB-0217 Operating Procedures of the cells' activation and verification. Version 2.0

15. SGS SOP: TESP-UB-0224 The management and validation of medium preparation. Version 1.1

16. SGS SOP: TESP-UB-1016 Medical Device *In Vitro* Cytotoxicity Test- MTT assay. Version 1.7

TABLE

Table 1 - Summary of extraction ratio for medical device

Thickness (mm)	Extraction ratio (surface area or mass/volume) ± 10%	Examples of forms of materials
< 0.5	6 cm^2/mL	Film, sheet, tubing wall
0.5 to 1.0	3 cm^2/mL	Tubing wall, slab, small moulded items
> 1.0	3 cm^2/mL	Larger moulded items
> 1.0	1.25 cm^2/mL	Elastomeric closures
Irregularly shaped solid devices	0.2 g/mL	Powder, pellets, foam, non-absorbent, moulded items
Irregularly shaped porous devices (low-density materials)	0.1 g/mL	Membranes, textiles

NOTE: While there are no standardized methods available at present for testing absorbents and hydrocolloids, a suggested protocol is as follows:
-determine the volume of extraction vehicle that each 0.1 g or 1.0 cm2 of material absorbs;
-then, in performing the material extraction, add this additional volume to each 0.1 g or 1.0 cm^2 in an extraction mixture.

Table 2 –Scoring criteria for cytotoxicity tests

Grade	Reactivity	Cell morphological change
0	None	Discrete intracytoplasmatic granules and no cell lysis.
1	Slight	Not more than 20 % of the cells are round, loosely attached and without intracytoplasmatic granules, or show changes in morphology; occasional lysed cells are present.
2	Mild	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis.
3	Moderate	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed.
4	Severe	Nearly complete or complete destruction of the cell layers.

Table 3 –Scores of cytotoxicity of test article extract in L929 cell morphology

Extracts	Exp 1	Exp 2	Exp 3
Reagent control	0	0	0
Positive control	4	4	4
Negative control	0	0	0
UP/2014/60049	1	1	1

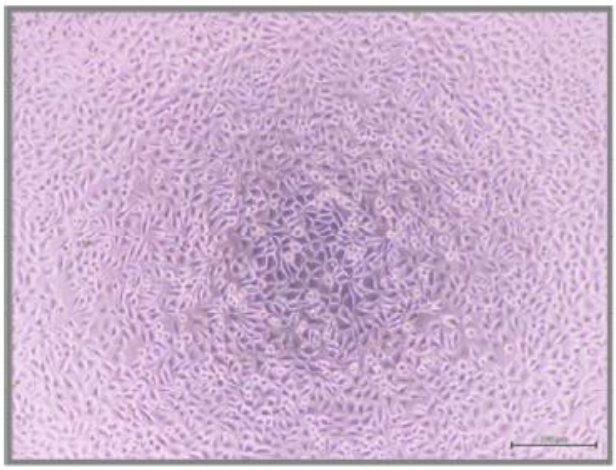
Table 4- Cytotoxic effect of test article extract in inhibition of L929 cell viability (%)

Extracts	Exp 1	Exp 2	Exp 3	Mean+SD
Reagent control	<30%	<30%	<30%	<30%
Positive control	98.26%	98.20%	98.20%	98.22%±0.03%
Negative control	<30%	<30%	<30%	<30%
UP/2014/60049	<30%	<30%	<30%	<30%

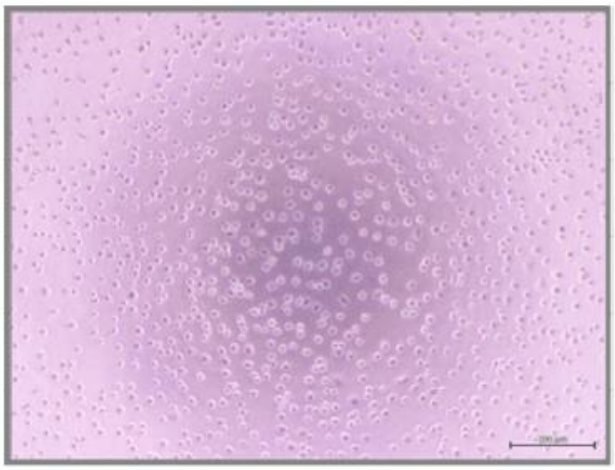
Table 5 - Summary of cytotoxicity test results

Extracts	Cell morphology	Inhibition of viability	Cytotoxicity
Reagent control	0	<30%	None
Positive control	4	98.22%	Cytotoxic
Negative control	0	<30%	None
UP/2014/60049	1	<30%	None

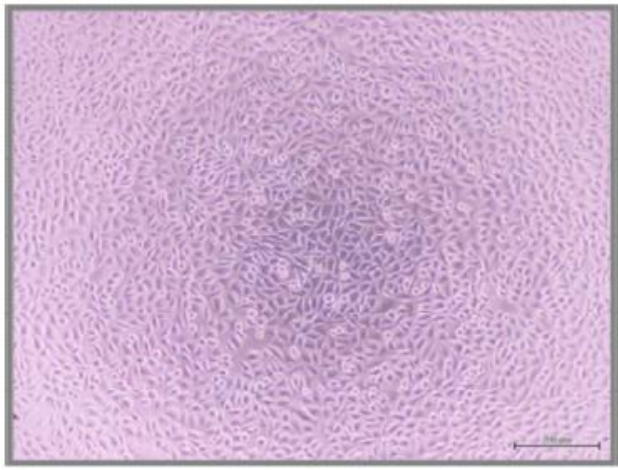
FIGURES



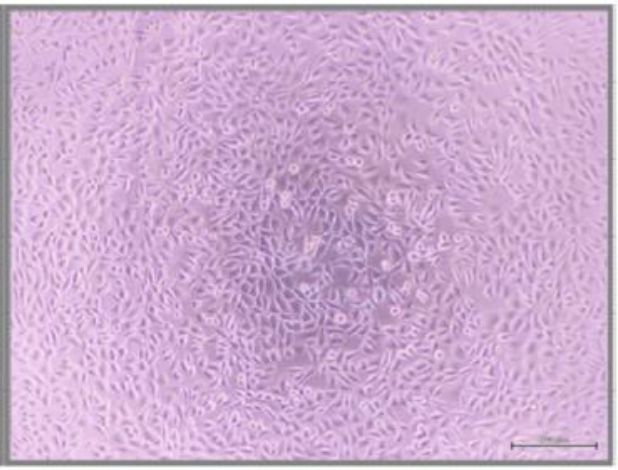
Blank control



Positive control



Nefative control



UP60049-50℃, 72hr

***Morphology and confluency of L929 cells after being exposed to test article or control extracts**

TEST ARTICLE PHOTO