EASTDERM HYDROGEL WOUND DRESSING

<Lot No.: 2016.01.18.001>

In Vitro Cytotoxicity Test

-MTT Assay

FINAL REPORT

Sponsor: **Easting Biotech Co Ltd**

Testing Institution: **SGS Taiwan Ltd.**
Ultra Trace &Industrial Safety Hygiene

Report No.: **UB/2016/30966**

Note:
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STUDY SCHEDULE

In Vitro Cytotoxicity Test- MTT Assay
EASTDERM HYDROGEL WOUND DRESSING

Report No.:	UB/2016/30966
Test Article Received Date	2016.03.16
Experimental Starting Date:	2016.03.30
Experimental Completion Date:	2016.04.21
Study Completion Date:	See Study Director's signature date in the report
Name of Study Personnel:	Allison Lai & Tanya Tan (only for mycoplasma test)

ADDRESS INFORMATION

Testing Facility/Test Site

Name: SGS TAIWAN LTD. Ultra Trace &Industrial Safety Hygiene

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Study Director

Name: Benson Liu

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Sponsor

Name: Easting Biotech Co Ltd

Address: 3F., No.49, Xiwei St., Sanchong Dist., New Taipei City 24155, Taiwan (R.O.C.)

INFORMATION FOR TEST ARTICLE

台灣檢驗科技股份有限公司
SGS Taiwan Ltd.

INFORMATION FOR ☒TEST ARTICLE/☐CONTROL ARTICLE

Sponsor Company Name	Easting Biotech Co Ltd
Sponsor Address	3F., No.49, Xiwei St., Sanchong Dist., New Taipei City 24155, Taiwan (R.O.C.)
Name of Test Article/ Control Article	EASTDERM HYDROGEL WOUND DRESSING
Amount (Note 2)	A、Quantity/Unit: 15/pcs (e.g.10ml / bottle * 6 bottles) B、☒One Test (No Retention)☐Two Test (For Retention) C、Packing Condition: ☐In Bulk ☒Intact Packing
Sterilization	Has been Sterilized ☐NO ☒YES, If Yes, Please Select the Following Method, ☐EO Sterilization☒Gamma Sterilization☐Steam Sterilization☐Other_____
Expiry Date (Note 3)	☒Expiry Date: 2019 . 01 . 18 (YYYY.MM.DD) ☐Not Provided.
Batch/Lot Number	☒Specific Number：2016.01.18.001 ☐Not Provided.
Sample Description	A、Major Components: Hydrogel, Polyurethane Film B、Purity: N.A. C、Concentration: N.A. D、Stability：N.A E、Color : Translucent F、External Features: ☐Liquid ☐Irregular ☐Powder ☐Granule ☒Flat ☐Other:_____ G、Solvent and Solubility ：N.A
Attachment(Note 4)	☐Certificate of Analysis ☐Material Safety Data Sheet ☐Stability Test Result ☐Others：_____ ☒No Attachment (Note4)
Storage Condition	☒Room Temperature☐2~8℃☐-10~-25℃☐Others_____
Others	N.A

Test Article No.
UB/2016/30966
It will be labeled by SGS sample receiving personnel.

Note1. Above all information including the blanks which sponsor cannot provide are disclosure by the sponsor.
Note2. If the sponsor doesn't provide the retention of test article/control article, the retention of a reserved test article/control article from each batch of test article /control article is the responsibility of the Sponsor.
Note3. Should prepare extra amount of the same lot test/control article for the retention. For retention, if the effective period is less than 5 years, the test article/control article will be retained till the expiry date. If the expiry date is longer than 5 years, the test article/control article will be retained for 5 years only. If the expiry date remained incomplete, it mentions that sponsor will agree that test facility will determine the earliest date, e.g. Exp. Date: 2015(YYYY), sponsor didn't identify the MM/DD, the expiration date should be 2015.01.01.
Note4. Sponsor should determinate, document and confirm the identity, strength, purity, stability, composition, method of synthesis, fabrication, derivation or other characteristics of the test article/control article before study. If the sponsor cannot provide the information, determination and documentation of the test article/control article are the responsibility of the Sponsor.
Note5. Note 'N/A' or 'N.A'if not applicable. Do not leave blank.
Note6. Test article and control article should be filled individually in "INFORMATION FOR TEST ARTICLE/CONTROL ARTICLE".
Note7. GLP test：(1) Corrections and additions to a final report should be in the form of amendments. Amendments should clearly specify the reason for the corrections or additions. Lot number, test article photos and raw data cannot be amended by sponsor's requirement. (2)One protocol only can generate one report except for translation version. If the report with more than two languages version, we only issue English version protocol. We only issue amendment or additional language GLP report within three years and non-GLP report within one year. (3)The GLP compliance statement will state that we follow TFDA GLP and TAF OECD GLP norm unless sponsor's requirement. SGS Taiwan Ltd. UTIS have acquired Good Laboratory Practice Statement of Compliance from TFDA and TAF.
Note8. Please write down it carefully and in detail. "INFORMATION FOR TEST ARTICLE/CONTROL ARTICLE" will be placed in the protocol and report along with a copy of this official document. If the information is not clear, we will exclude them from GLP statement.

Sponsor Signature/ Date：王以仁 2016/03/16

版次：3.5 試驗物質/對照物質資料表 Information For Test Article/Control Article
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SIGNATURE OF PERSONNEL

In Vitro Cytotoxicity Test- MTT Assay
EASTDERM HYDROGEL WOUND DRESSING

Study Director:

Benson Liu 2016.05.04

Benson Liu / SGS Taiwan Ltd. Date

Facility Manager:

2016.05.04

Yuanmin Wen / SGS Taiwan Ltd. Date

ABSTRACT

In vitro cytotoxicity test was performed in this study to evaluate the biological compatibility of "EASTDERM HYDROGEL WOUND DRESSING", which was provided by Easting Biotech Co Ltd. Extraction of test article and treatment of mouse lung fibroblast cells (L929 cells) with test article extracts were performed according to ISO10993-5. Cell viability determined by MTT assay showed that the test article extract had in average <30% inhibitory effects to the viability of cells. The 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, the test article was subjected to *in vitro* cytotoxicity test to evaluate toxicity of substances that could be extracted or released from the medical device according to the ISO 10993-5 Biological evaluation of medical device – Part 5: Tests for *in vitro* cytotoxicity guidance. Therefore, the test system was mouse lung fibroblast cells (L929 cells). The original source was from Food Industry Research and Development Institute, Strain No. BCRC RM60091. The test article preparation was carried out according to ISO10993-12 guidance. Based on recommendations described in ISO10993-5, quantitative determination of cell viability by MTT assay was carried out. This result 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 RM60091. Subculture passage number: P13. Bank No.:20160307-5D-25-25
B. Morphology: Fibroblast-like
C. Culture properties: Adherent
D. Incubation condition: Incubate in αMEM medium with 10% horse serum at 37±1°C in the presence of 5±1% CO_2.

2. Reagents

A. 100X L-Glutamine solution (contained 200 mM L-Glutamine) (Hyclone, Cat No. SH30034.01, Lot No.:ABA211011)
B. 100X Penicillin-Streptomycin solution (contained 10,000 units/mL Penicillin and 10,000 µg/mL Streptomycin) (contain 10,000 units/mL Penicillin and 10,000 µg/mL Streptomycin) (HyClone, Cat No. SV30010, Lot No.: J150023)
C. 10X Phosphate buffer solution, PBS (UniRegion Bio-Tech, Product No. UR-PBS001, Lot No.: PBS001-5C)
D. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT (Sigma, Cat No. M5655, Lot No.: MKBV3098V)
E. Dimethyl sulfoxide, DMSO (Sigma, Cat No. 34943, Lot No.: SZBE0170V)
F. Horse serum (Gibco, Cat No. 16050-122, Lot No.:1671319)
G. MEM Alpha Modification (1X), αMEM (Hyclone, Cat No.: SH30568.01, Lot No.: AAL209402)
H. 0.25% Trypsin solution (HyClone, Cat No.SH30042.01, Lot No.: J150021)

3. Equipments

A. Orbital Shaker Incubator (PRECISION , CI-60, Equipment No.: INB-08)
B. Balance (DENVER, TB-214, Equipment No.: BAL-13)
C. Biological safety cabinet (LABCONCO, 3450801, Equipment No.: BSC-01)
D. CO_2 Incubator (ASTEC, SCA-165DS, Equipment No.: INB-01)
E. Microscope (OLYMPUS, CKX41, Equipment No.: MIS-02)
F. Centrifuge (Eppendorf, 5804R, Equipment No.: CEN-07)
G. Water bath (Kansin, WB212-B2, Equipment No.: WAB-02)
H. Microplate Spectrophotometer (BioTek™, Eon, Equipment No.: MPS-02)
I. Ruler (Equipment No.: RUL-01)

4. Preparation of Test Article and Controls

A. Test Article

The test article was handled under sterile environment and operated with aseptic technique during preparation. αMEM complete medium contain 10% horse serum was used as extraction buffer. The test article was extracted with a ratio of 6 cm^2 ±10% /1mL in extraction buffer for 24±2 hour at 37±1°C with constant agitation at 100 rpm per criteria described in ISO10993-12. In this study, 204 cm^2 of test article was extracted in 34.000mL extraction buffer. The pH adjustment; filtration and centrifugation are not conducted. The extract was used within 24 hours after preparation.

B. Controls

a. Blank control: αMEM complete medium contain 10% horse serum as blank control.
b. Positive control: Polyurethane film – ZDEC (Polyurethane film containing Zinc Di-Ethyldithio-Carbamate, RM-A, Hatano Research Institute, Japan) extracted with 0.1 g ± 10% /1mL αMEM complete medium was as positive control.
c. Negative control: HDPE film (High Density Poly-Ethylene film, RM-C, Hatano Research Institute, Japan) extracted with 0.1 g ± 10% /1mL αMEM complete medium was as negative control.
d. Extraction Condition: Extractions were performed at 37±1°C for 24±2 hours with constant agitation at 100 rpm. The pH adjustment, filtration and centrifugation did not conduct. The extract was used within 24 hours after preparation.

5. *In vitro* cytotoxicity test-MTT assay

A. Cell incubation

a. Preparation of αMEM complete cell culture medium
For 500mL complete cell culture medium as example, complete cell culture medium was prepared by mixing 440 mL of αMEM , 5 mL of 100X Penicillin- Streptomycin solution, 5 mL of 100X L-Glutamine solution and 50 mL of horse serum. The completed medium was stored at 4±2°C.

b. Cell culture
Mouse lung fibroblast cells were used here for cytotoxicity test. The L929 cells were grown on a 10-cm dish containing 10 mL of αMEM complete medium and incubated at 37±1°C in the presence of 5±1% CO_2. 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°C. Enzymatic activity of trypsin was terminated by adding αMEM complete medium and then transferred to new 10-cm dish for subculture.

B. MTT assay

a. 100 µL of L929 cell suspension (1×10^5cells/mL) was transferred into each well of a 96-well cell culture plate. The cells were then incubated at 37±1°C 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 the cells was observed under microscope.
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 °C.
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.) If the mean of cytotoxicity was less than 30%, the result showed "<30%".
g. The average of inhibition of cell viability was used to give final interpretation of cytotoxicity.
h. All the experiment procedure was referring to SGS SOP: TESP-UB-1016.

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 – ZDEC film; Negative reference material was HDPE film.
(3) The inhibition of cell viability of positive control was greater than 30%(>30%) and negative control was less than 30% (≦30%)

B. Blank

(1) Measure the absolute value of optical density, OD_{570}. The acceptance criterion 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 (contained 12 replicates).
(3) The difference between the OD average of left blanks and the right blanks were less than 15% compares to the total average mean.

DATA MANAGEMENT

The quantitative data was showed as mean and standard deviation (S.D.). The achievement of a reduction of cell viability by more than 30% (>30%) is considered as cytotoxic effect.

RESULTS

1. **Appearance**
   The extracts of the test article was not different than the blank control.

2. **Inhibition of cell viability**
   The acquired readings of OD_{570} absorbance of blank control were averaged and set as 0% inhibition of cell viability. In proportion to blank control, we determined inhibition of cell viability of negative control, positive control, test article extract extraction as <30%, 100.18±0.06% and <30% respectively. The relative values of inhibition of cell viability were shown in Table 2.

CONCLUSION

The inhibition of cell viability was averaged and listed in Table 2. Based on the averaged result which concluded that the" EASTDERM HYDROGEL WOUND DRESSING" extract did not induce cytotoxic to L929 cells.

REFERENCES

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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.
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9. SGS SOP: EOMP-USL-0027 Operating procedures of the biosafety cabinet and laminar flow, UV-lamp verification and aerobic plate counts. Version 2.4
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11. SGS SOP: EOMP-USL-0030 Maintenance and operating procedures of the microscope. Version 1.1
12. SGS SOP: EOMP-USL-0035 CO_2 incubator Operating procedures. Version 1.0
13. SGS SOP: EOMP-USL-0036 Operating Procedures of MilliQ. Version 1.0
14. SGS SOP: EOMP-USL-0037 Manual of freezer-refrigerators. Version 1.0
15. SGS SOP: EOMP-USL-0038 Microorganism incubator operating Procedures Version 1.0
16. SGS SOP: TESP-UB-0217 Operating Procedures of the cells' activation and verification. Version 2.1
17. SGS SOP: TESP-UB-1016 Medical Device *In Vitro* Cytotoxicity Test- MTT Assay. Version 1.9

TABLES

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 cm^2 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- Cytotoxic effect of test article extract in inhibition of L929 cell viability (%)

Groups	Exp 1	Exp 2	Exp 3	Mean	SD
Blank control	<30%	<30%	<30%	<30%	0.60%
Positive control	100.21%	100.21%	100.12%	100.18%	0.06%
Negative control	<30%	<30%	<30%	<30%	1.24%
UB/2016/30966	<30%	<30%	<30%	<30%	2.83%

TEST ARTICLE PHOTO

UB/2016/30966