瑞德生物科技有限公司
MASTER LABORATORY CO.,LTD.

In Vitro Cytotoxicity Test

Master Laboratory Co., Ltd. Genetic Toxicology Testing Laboratory

Address: Rm. A209, 2F., No.2, Sec. 2, Shengyi Rd., Zhubei City, Hsinchu County 302, Taiwan (R.O.C.) Tel: (886-2) 25176117

Ear-loop
In Vitro Cytotoxicity Test
STUDY REPORT

Sponsor: CHIN HSIUNG FIBER CO., LTD

Testing Institution: Master Laboratory Co., Ltd.

November 2020

Report No.: MSC-202011-003-CE-R01

Experimental starting date: 10.27.2020

Test article registration date: 10.22.2020

Test article extraction date: 11.05.2020

Experimental completion date: 11.06.2020

Study Announcement

1. The study report is valid for the test article used only.
2. The study report could not be recopied or extracted only if the permission from Master Laboratory Co., Ltd.
3. The study report is invalid without the endorsement of Master Laboratory Co., Ltd.

SIGNATURE OF STUDY PERSONNEL

Study Director

Jyun Cheng Liu 11. 12. 2020

Jyun Cheng Liu **Date**

Facility Management

Alan Hsieh 11.12.2020

Alan Hsieh **Date**

GLP COMPLIANCE STATEMENT

The study met with the technical requirements of the protocol, and all applicable guidance and regulations, which included the Good Laboratory Practice for Non-clinical Laboratory Studies (FDA, 21 CFR, Part 58, 2019) and Good Laboratory Practice for Non-clinical Laboratory Studies (Food and Drug Administration, R.O.C., 2019). There were no deviations from the approved study plan and no adverse problem that would affect the integrity of this study or the interpretation of the study result. Because the test article is a proprietary product of the sponsor, all the contents related with test article in 21 CFR Part 58 (US FDA) are not applicable to this study (21 CFR §58.105, §58.113, FDA).

Study Director

Jyun Cheng Liu

Jyun Cheng Liu

11. 12. 2020

Date

QUALITY ASSURANCE STATEMENT

The Quality Assurance personnel had inspected the conduct of different phases of the study according to a predetermined study schedule. To the best of our knowledge, there were no deviations from the study plan and standard operating procedures that would affect the integrity of this study. This report has been audited by the QA personnel in accordance with the appropriate standard operating procedures of Master Laboratory Co., Ltd. described the methods and procedures used in the study, and the reported results accurately reflect the raw data generated during this study.

Listed below are the phases in this study that were audited by the QA personnel and the dates the audits were performed and findings reported to facility management (FM).

Inspection record:

Date of inspection	Phase	Date Reported to SD	Date Reported to FM
10.27.2020	Study protocol, Personnel quality and test article. (Study base)	10.27.2020	10.27.2020
11.05.2020	Date of dosing. (Process base)	11.05.2020	11.05.2020
11.06.2020	Environment, equipment, and SOP. (Facility base)	11.06.2020	11.06.2020
11.10.2020	Study system, study report, raw data, and final report. (Study base)	11.10.2020	11.10.2020

Quality Assurance unit in charge

Ying Chun Chen

Ying Chun Chen

11. 12. 2020

Date

Contract Research Organization and Sponsor Information

1. CRO:
   a. Title: Master Laboratory Co., Ltd.-Genetic Toxicology Testing Laboratory
   b. Address: Rm. A209, 2F., No. 2, Sec. 2, Shengyi Rd., Zhubei City, Hsinchu County 302, Taiwan (R.O.C.)

2. Sponsor:
   a. Title: CHIN HSIUNG FIBER CO., LTD
   b. Address: NO.37-12, Ta Hsin RD., Pu Yen Hsiang, Chang Hua Hsien, Taiwan

CONTENTS

SUMMARY

The present study was to evaluate the short-term (24h) *in vitro* cytotoxicity of extract from the test article "Ear-loop". The testing was performed in compliance with ISO 10993-5:2009. The cell morphology of mouse fibroblast cell line (L929) and the confluency of monolayer were scored under the microscope for qualitative determination. The MTT assay was performed in order to check the cytotoxicity of the test article extract quantitatively. Both qualitative and quantitative results showed that no cytotoxicity of the test article extract was observed in this study.

INTRODUCTION

Evaluation of the *in vitro* cytotoxicity of a medical device was the initial step of a biocompatibility study. It was usually performed using immortalized cell lines, for example, L929. The aim of this study was to investigate the cytotoxic effect of the test article extract. The cytotoxicity was determined qualitatively and quantitatively.

TEST ARTICLE

A. Test article identification (Based on information provided by sponsor)

Test article: Ear-loop

Test article Lot No. Not provided

Sterilization condition: Not Sterilized

Expiry day: Not provided

B. Characteristics of Test article

Physical description	Solid
Composition	Spandex Yarn+ Nylon Yarn
Test article as received or treated	The test article was extracted as received without pretreatment.
Quantity received	2 unit

C. Extraction condition of test article

According to ISO 10993-12:2012, the extraction ratio of test article surface/culture medium volume was approximately 3 cm^2/mL.

MATERIALS AND METHODS

A. Chemicals and Materials

1. Cell system

   L929 cells were obtained from Food Industry Research and Development Institute (BCRC RM60091), referred to as L929 mouse fibroblasts.

2. Medium and reagents

   a. MEM/Alpha modification (Hyclone, SH30568.01, AF29526954)
   b. Donor Equine Serum (Hyclone, SH30074.04, AC10233340)
   c. Penicillin-Streptomycin solution (Hyclone, SV30010, J190003)
   d. L-glutamine (GIBCO, 25030-081, 2185851)
   e. MEM-non essential amino acid (Hyclone, SH30238.01, AF29526917)
   f. DPBS (Hyclone, SH30256, AE29431651)
   g. MTT cell proliferation/viability assay kit (R&D systems, 4890-25-K, Reagent LOT: 40353H17, Detergent LOT: 40341H17)
   h. Neutral red (SIGMA-ALDRICH, N2889, RNBH5611)
   i. Trypsin 0.25% (1X) solution (Hyclone, SH30042.01, J200011)
   j. Trypan Blue solution (SIGMA-ALDRICH, T8154, RNBF6220)

3. Controls

   a. Blank(B): culture medium (MEM/ alpha modification inclusive of 10% (v/v) horse serum, 1% (v/v) penicillin-streptomycin solution, 1% (v/v) L-glutamine and 1% (v/v) non-essential amino acid)
   b. Negative control (NC): high density polyethylene (HDPE)
   c. Positive control (PC): culture medium inclusive of 10% (v/v) DMSO

B. Methods

1. Cell culture

The cryopreserved L929 cells were thawed, and cultured in MEM/alpha modification medium at 37±1℃ in 5% CO_2 atmosphere. Up to 80% cell growth was observed by microscope, subculture until obtaining passage number 2 prior to use. The culture medium should be replaced twice a week.

2. Sample preparation

2.1. Test Sample(S):

According to ISO 10993-12:2012, the extraction ratio of test article surface /culture medium volume shall be 3 cm^2/mL, extract at a rotation speed of 100 rpm under 37±1℃ for 24±2h. After extraction, the extract solution was used immediately; the appearance of test article extract was clear and colorless without particulates present.

2.2. Positive control (PC):

Culture medium with 10% (v/v) DMSO, and incubated at a rotation speed of 100 rpm under 37±1℃ for 24±2h.

2.3. Negative control (NC):

Based on the ISO 10993-12:2012, the extraction ratio was 0.2 g/mL, took 1.0g HDPE, immersed it in 5.0 mL culture medium, and extracted at a rotation speed of 100 rpm under 37±1℃ for 24±2h.

2.4. Blank (B):

Took 5.0 mL of culture medium and then incubated at a rotation speed of 100 rpm under 37±1℃ for 24±2h.

3. Qualitative and quantitative (MTT assay) determination

a. Qualitative analysis

5×10^4 of L929 mouse fibroblast cells were seeded in 24-well culture plates and then incubated at 37±1℃ in 5% CO_2 atmosphere for 24±2h in order to obtain confluent monolayers of cells. After cell attachment, the original culture medium was removed and replenished 0.5mL in each of culture well (B, NC, PC, S). Then the test plates were subsequently incubated at 37±1℃ in 5% CO_2 atmosphere for 24±2h.The plate was incubated for 24h. After 24h, the cells of each well were stained with the neutral red solution and scored by the change of cell morphology and viability under inverted microscope in accordance with criteria of appendix 1.

b. Quantitative analysis (MTT assay)

L929 mouse fibroblast cells were seeded in 96-well culture plates with the number of 1×10^4 cells, and then incubated at 37±1℃ in 5% CO_2 atmosphere for 24±2h. After 24h, the original culture medium was removed and replenished 0.1mL in each of culture well (B, NC, PC, S). Then the test plates were subsequently incubated at 37±1℃ in 5% CO_2 atmosphere for 24±2h. At the end of the incubation period, 10μL MTT reagent (kit component, 4890-25-01) was added into each well. The reaction was performed at 37℃ in 5% CO_2 atmosphere for 2-3h and kept from light. 0.1mL of detergent reagent (kit component, 4890-25-02) was added into each well and incubated in dark for 2-3h before measuring the absorbance. The absorbance of test samples was measured at 570 nm (reference wavelength: 630 nm) by a microplate reader (ELx800, BioTek). The absorbance of test samples was represented as mean ± standard deviation (SD).

Compared to the blank (as 100% viability), the percentage of viability and

mortality were calculated by the following formula:

$$\text{Viability (\%)} = \frac{OD_{570e}}{OD_{570b}} \times 100\%$$

Mortality (%) = 100 – Viability (%)

Where

OD_{570e} is the mean value of the measured absorbance of the test samples;

OD_{570b} is the mean value of the measured absorbance of the blank (B).

If viability is reduced to < 70% of the blank , indicating the test article has a cytotoxic potential.

RESULTS

To evaluate the cytotoxicity of test article "Ear-loop" extract, test article extract was tested towards the cell growth, morphology and viability. After cell exposure to the extract for 24 h, the following items were evaluated:

A. Qualitative determination (Appendix 1, Figure 1)

The morphology of B, NC, PC, and S treated L929 cells stained with NR were observed after 3h under inverted microscope (100X). The morphology of B and NC cells showed long spindle shape with obvious lamellipodia and filopodia instead of lysed, rounded shape, and inhibited growth. However, PC showed nearly complete rounded cells lysed morphology; cell layers almost completely destroyed, and growth inhibition was observed. The result of test sample showed the same long spindle shape as B and NC. According to the results of microscopic assay, the percentages of rounded or lysed cells of B, NC, PC, and S were evaluated at 0%, 0%, 100%, and 0% respectively. Therefore, the cytotoxicity of B, NC, PC, and S was graded at 0, 0, 4, and 0 (Table 1).

B. Quantitative determination (Table 2)

The cell viability was evaluated after L929 cells treated with B, NC, PC, and S for 24 h through MTT cell proliferation/viability assay. The absorbance of B, NC, PC, and S at 570 nm were 0.553±0.003, 0.524±0.008, 0.061±0.001 and 0.409±0.017 respectively; the cell viability represented 100%, 95%, 11%, and 74%; the mortality showed 0%, 5%, 89%, and 26%.

CONCLUSION

According to the results of qualitative and quantitative assays, the results (Table 1 and Table 2) showed "Zero" reactivity. Therefore, none *in vitro* cytotoxicity could be considered in the extract solution of the test article "Ear-loop".

REFERENCES

1. Good Laboratory Practice for Nonclinical Laboratory Studies (2019) Food and Drug Administration, R.O.C.
2. Good Laboratory Practice for Nonclinical Laboratory Studies. Title 21 of the U.S. Code of Federal Regulations, Part 58 (2019) United States Food and Drug Administration.
3. ISO 10993-5:2009, Biological evaluation of medical devices-Part 5: Tests for *in vitro* cytotoxicity.
4. Biological evaluation of medical devices-Part 12; Sample preparation and reference materials ISO 10993 (2012).
5. Dijkhuizen-Radersma R, Hesseling SC, Kaim PE, Groot K, Bezemer JM (2002) Biocompatibility and degradation of poly (ether-ester) microspheres: *in vitro* and *in vivo* evaluation. Biomaterials. 23, 4719-4729.
6. 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.

Table 1. Cytotoxicity evaluated using neutral red stain

Test item	Cell lysis (%)	Grade
Blank (B)	0	0
Negative control (NC)	0	0
Positive control (PC)	100	4
Test sample extract (S)	0	0

Table 2. The results of MTT assay for evaluation of cell viability

Test Item	Absorbance ($OD_{570\ nm}$)	Viability (%)	Mortality (%)
Blank (B)	0.553±0.003	100	0
Negative control (NC)	0.524±0.008	95	5
Positive control (PC)	0.061±0.001	11	89
Test sample extract (S)	0.409±0.017	74	26
50% test sample extract	0.431±0.017	78	22

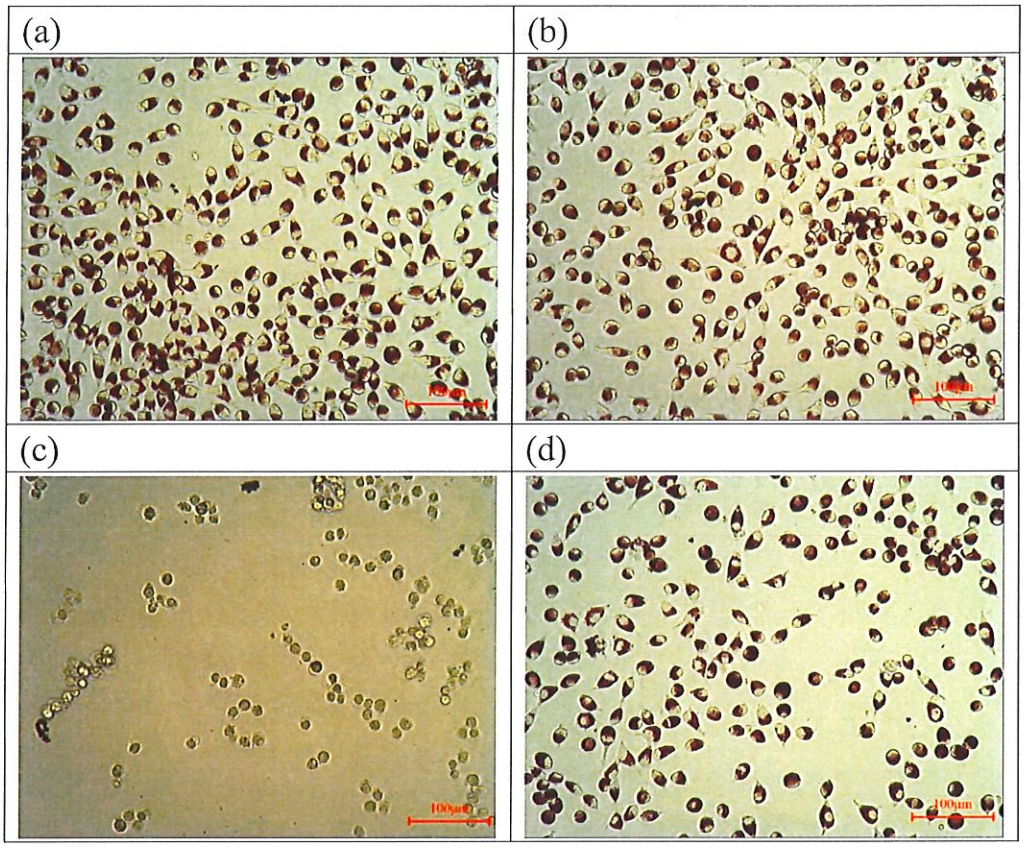

Figure 1. The observed cell morphology of L929 cells after being treated for 24 h under 100X inverted microscope.
a. Blank (B): culture medium
b. Negative control (NC): HDPE
c. Positive control (PC): 10% DMSO
d. Test sample (S): Ear-loop

Appendix 1. Qualitative morphological grading of cytotoxicity

Grade	Reactivity	Conditions of all cultures
0	None	Discrete intracytoplasmic granules, no cell lysis, no reduction of cell growth
1	Slight	Not more than 20% of the cells are round, loosely attached and without intracytoplasmic granules, or show changes in morphology; occasional lysed cells are present; only slight growth inhibition is observed.
2	Mild	Not more than 50% of the cells are round, devoid of intracytoplasmic granules; not more than 50% growth inhibition is observed.
3	Moderate	Not more than 70% of the cell layers contain rounded cells or are lysed; cell layers not completely destroyed, but more than 50% growth inhibition is observed.
4	Severe	Nearly complete or complete destruction of the cell layers.

Appendix 2. Test Article Information Sheet

Master Laboratory Co., Ltd.

Information for ☑Test Article /□Control Article

Sponsor Company	CHIN HSIUNG FIBER CO., LTD
Sponsor Address	NO.37-12, Ta Hsin RD., Pu Yen Hsiang, Chang Hua Hsien, Taiwan
Contract study item	☑ Base on the contract ☐ Others:
Name of test article	Ear-loop
Major components	Spandex Yarn+ Nylon Yarn
Sample status	☐Sterilized (☐Gamma ☐EO ☐Steam) ☑ Not Sterilized
Storage condition	☑ Room temperature (10°C~30°C) ☐ 4°C ☐ Dry ☑ Away from light ☐ Others:
Expiry day	
Specific requirement	
Batch/ Lot number	☑ Base on the specific number on the package:_____ ☐ Base on the date on the package ☐ Base on the arrived date ☐ Others:
Extract by	☐ Weight (0.2g/ml) Total weight of each test article: ☑ Surface (Sample thickness: ☑>1.0mm ☐0.5-1.0mm ☐<0.5mm) Total area surface of each test article:
Absorption	☑ Non absorption ☐ Water absorption rate: / Oil absorption rate:
Sponsor Signature	Hank Shih

Appendix 3. Test Article Photo