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CNAS L13034

In Vitro Cytotoxicity Test

MTT Method

Final Report

Article Name: Single-use medical face mask (non-sterile)

Report Number: CSTBB20030143

Method Standard: ISO 10993-5：2009

Sponsor

Henan Siyuan Medical Equipment Co., Ltd

Industrial cluster ,Huanglou Road, Shaolin Avenue , Dengfeng City

Test Facility

CCIC Huatongwei international inspection (Suzhou) Co., Ltd

Room 101, Building G, Ruoshui Road 388, Suzhou, Jiangsu, China

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Notices

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Abstract

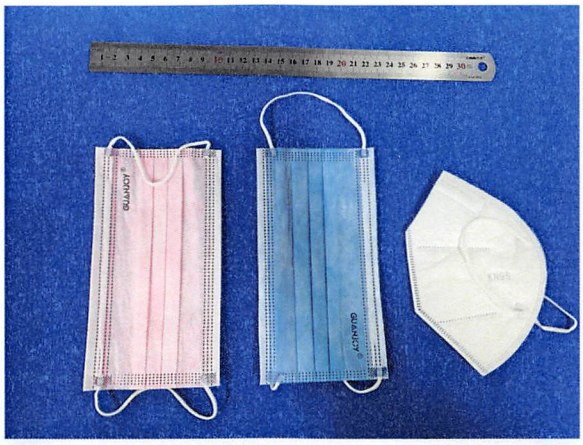
In this study, mammalian L-929 cells were cultured in vitro according to ISO 10993-5:2009 to test the potential cytotoxicity of the test article.

The test articles and the control material were separately placed in MEM medium containing 10% fetal bovine serum, and extracted in a 37 ℃ incubator for 24 hours. After the end of the extraction, the cell culture medium in the 96-well plate (10^4 cells/well) cultured for 24 hours was removed and replaced with the corresponding extract, cultured in 37 ℃, 5% CO_2, >90% humidity for 24 hours. After the culture, the morphology and cell lysis of the cells were observed under the microscope, and the cytotoxicity of the test samples was determined by MTT assay.

The results showed that the cells in the blank control group and the negative control group (high density polyethylene) were well-formed throughout the experiment and showed no cytotoxic reaction. A severe cytotoxic response was shown in the positive control group (ZDEC). The 100% concentration of the test extract retained a normal appearance after 24 hours of incubation, and the cell viability was 82.3%. The data of each group met the acceptance criteria, and the results of this test were valid.

Based on the above results, it can be concluded that under the experimental conditions, the test article Single-use medical face mask (non-sterile) have no potential toxicity to L-929 in the MTT method.

Study Verification and Signature



Protocol Number	SST2003009101BB
Protocol Effective Date	2020-03-17
Technical Initiation Date	2020-03-18
Technical Completion Date	2020-03-20
Final Report Completion Date	2020-03-20

Personnel _____ 2020-03-20
Date Completed

Approved
Study Director _____ 2020-03-20
Date Completed

Supervisory
Test Facility Manager _____ 2020-3-20
Date Completed

Huatongwei international inspection (Suzhou) Co., Ltd.

Quality Assurance Statement and GLP Statement

Quality Assurance Statement

The Quality Assurance Unit conducted inspections on the following dates. The findings were reported to the Study Director and to the HTW's Management.

The final report was reviewed to assure that the report accurately describes the methods and standard operating procedures. The reported results accurately reflect the raw data of the nonclinical study conducted per the protocol.

Phase Inspected	Date	Study Director	Management
Experiment	2020-03-18	2020-03-18	2020-03-18
Raw Data	2020-03-20	2020-03-20	2020-03-20
Final Report	2020-03-20	2020-03-20	2020-03-20

The findings of these inspections have been reported to Management and the Study Director.

Hui Jiang
Quality Assurance

2020-03-20
Date

GLP Statement

This study was conducted in compliance with current U.S. Food and Drug Administration regulations set forth in 21 CFR, Part 58.

The sections of the regulations not performed by or under the direction of HTW, exempt from this Good Laboratory Practice Statement, included characterization and stability of the test article and its mixture with carriers, 21 CFR, Part 58.105 and 58.113.

Xiaoyu Wen
Study Director

2020-03-20
Date

1.0 Purpose

The purpose of the test is to determine the potential cytotoxicity toxicity of a mammalian cell culture (mouse fibroblast L-929 cells) in response to the test article.

2.0 Reference

Biological evaluation of medical devices-Part 5: Tests for In Vitro Cytotoxicity (ISO 10993-5：2009)

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

3.0 Test and control articles

Groups	Test article	Negative Control Article	Positive Control Article	Blank Control
Name	Single-use medical face mask (non-sterile)	High Density Polyethylene Film	ZDEC	MEM medium, with addition 10% FBS
Manufacture	Henan Siyuan Medical Equipment Co., Ltd	Hatano Research Institute. FDSC	Sigma-Aldrich.	Hyclone
Size	Not provided	3 cm×10 cm (5 sheets)	25 g	500 ml
Model	Flat Type(red) Flat Type(blue) Folded Type	/	/	/
Lot Batch#	20200218	C-161	BCBQ6847V	AD23420275
Test Article Material	Not provided	/	/	/
Physical State	Solid	Solid	Solid	Liquid
Color	White, blue, red	White	White	Pink
Packaging Material	Not provided	/	/	/
Sterilized or Not	Not provided	No	No	Yes
Concentration	/	/	0.1%	/
Total Surface	Not provided	/	/	/
Storage Condition	Room Tep.	Room Tep.	Room Tep.	4℃
Note：The information about the test article was supplied by the sponsor wherever applicable.				

4.0 Identification and justification of test system

L-929 mouse fibroblast cells obtained from American Type Culture Collection (ATCC).

L-929 cells have been used for cytotoxicity studies because they demonstrate sensitivity to extractable cytotoxic articles. Also, the test article is extracted and administered in vitro to mouse fibroblast L929 cells through a solvent compatible with the test system, which is the optimal route of administration available in this test system as recommended in ISO 10993-5.

5.0 Equipment and reagents

5.1 Instruments

Vertical pressure steam sterilizer (SHB026), CO_2 Incubator (SHB002), Steel Straight Scale (SHB076), Electronic Balance (SHB016), Clean bench (SHB014), Multiskan Spectrum Microplate Spectrophotometer (SHB003), Bench type low speed centrifuge (SHB022), Inverted microscope (SHB005)

5.2 Reagents

MEM (Hyclone, AD23420275), FBS (Clark, JC65113), Penicillin-Streptomycin (Gibco, 15140122), Try psin (Gibco, 25200056), PBS (Hyclone, AD21380277), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyletrazo lium bromide) (Sigma, MKBG20386), Isopropyl alcoho (Macklin, C10394867)

6.0 Experiment design and dose

6.1 Sample preparation

According to the table below, aseptic extraction of the test article sealed and incubated in MEM medium (10% FBS) at 37 ℃, 5% CO_2 and 60 rpm for 24 hours.

Groups	Sampling		Sterilization	Aseptic Extraction In Inert Container				Final Extract
	Sampling Manner	Actually sampling	Method	Ratio	Extracts	Condition	pH	Clear or Not
Test article	Whole	1518.8 cm^2	UV	6 cm^2: 1 ml	253.1 ml	37 ℃ 24 h	7.4	Clear
Negative Control	Random	60 cm^2	UV	3 cm^2: 1 ml	20.0 ml	37 ℃ 24 h	7.4	Clear
Positive Control	Random	0.02 g	Filter	0.1 g: 100 ml	20.0 ml	37 ℃ 24 h	7.4	Clear
Blank Control	/	/	/	/	10.0 ml	37 ℃ 24 h	7.4	Clear

The changes of the leaching solution was observed after extraction. No particulates or color changes were observed in pre- and post-extraction, and immediately be used in the follow-up experiment. The color and pH of the extraction solution did not change before and after use, and the pH value was 7.4 after leaching.

6.2 Test method

Aseptic procedures were used for handling cell cultures. L-929 cells were cultured in MEM medium (10% FBS, 1% Penicillin-Streptomycin solution) at 37 ℃ in a humidified atmosphere of 5% CO_2, then digested by 0.25% trypsin containing EDTA to get single cell suspension. 1×10^5 cells/ml suspension were obtained by centrifuging (1000 rpm, 5 min) and re-dispersing in MEM medium.

The suspended cells were dispensed at 100 µl per well in 96-well plate, and cultured in cell incubator (5% CO_2, 37 ℃, >90% humidity). Cell morphology was evaluated to verify that the monolayer was satisfactory.

After the cells grew to about 70% and form a monolayer, original culture medium was discarded. The 96-well plates were then treated with 100 µl of extract of test article (100%、75%、50%、25%), control article, negative article and positive article respectively. The 96-well plate was incubated at 37 ℃ in cell incubator of 5% CO_2 for 24 h. Six replicates of each test were tested.

After incubation, observe the cell morphology first and then discard the culture medium. Add 50 µl MTT (1mg/ml) to each well and then incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 for 2 hours. The liquid in

each well was tipped out and 100 µl Isopropyl alcoho was added to each well to suspend the cell layer.

Evaluate the suspension above with a dual-wavelength spectrophotometer with the measurement wavelength at 570 nm.

7.0 Statistical method

Mean±standard deviation ($\bar{x}$±s)

The cell cytotoxicity ratio = OD_{570} of test (or positive or negative) article group/ OD_{570} of blank control group×100%.

8.0 Evaluation criteria

8.1 The 50% extract of the test article should have at least the same or a higher viability than the 100% extract. Otherwise the test should be repeated.

8.2 The lower the Viab.% value, the higher the cytotoxic potential of the test article is.

8.3 If viability is reduced to < 70% of the blank, it has a cytotoxic potential.

8.4 The Viab.% of the 100% extract of the test article is the final result.

9.0 Results of the test

9.1 Results of the cell morphology

Table 1 Observation of the cell morphology

Group	Before inoculation	Before treated with extract	24 h after treatment
Blank control	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
Negative control			Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
Positive control			Nearly complete or complete destruction of the cell layers.
100% Test article extract			The cells showed a round shape and a change in cell morphology occasionally, and there were particles in the cytoplasm, occasionally cell lysis and slight growth inhibition.
75% Test article extract			The cells showed a round shape and a change in cell morphology occasionally, and there were particles in the cytoplasm, occasionally cell lysis and slight growth inhibition.
50% Test article extract			Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.
25% Test article extract			Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth.

9.2 Results of the cell vitality

Table2 Results of the cell vitality

Group	OD value								Viab. (%)
	1	2	3	4	5	6	$\bar{x}$	s	
Blank control	0.894	0.859	0.897	0.885	0.893	0.854	0.880	0.019	100.0
Negative control	0.911	0.855	0.862	0.817	0.898	0.873	0.869	0.033	98.8
Positive control	0.057	0.067	0.052	0.049	0.069	0.074	0.061	0.010	7.0
100% test article extract	0.712	0.712	0.760	0.724	0.727	0.709	0.724	0.019	82.3
75% test article extract	0.754	0.742	0.756	0.749	0.761	0.757	0.753	0.007	85.6
50% test article extract	0.801	0.812	0.806	0.811	0.798	0.815	0.807	0.007	91.7
25% test article extract	0.805	0.818	0.856	0.813	0.829	0.860	0.830	0.023	94.3

10.0 Conclusion

Under the conditions of this study, the test article have no potential toxicity to L-929 cells.

11.0 Compliance

US FDA Good Laboratory Practice Regulations 21 CFR 58, effective June 20, 1979, as amended 52 FR 33780, Sept. 4, 1987, and subsequent amendments

Standard operating procedure of CCIC Huatongwei international inspection (Suzhou) Co., Ltd.

12.0 Record

All raw data pertaining to this study and a copy of the final report are to be stored in the designated archive files at Huatongwei.

13.0 Confidentiality Agreement

Statements of confidentiality were as agreed upon prior to study initiation.