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In Vitro Cytotoxicity Test

MTT Method

Final Report

Verification

Report Number: CSTBB21031166

Article Name: Medical Clean Paper Wiper

Method Standard: ISO 10993-5：2009

Sponsor

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Abstract

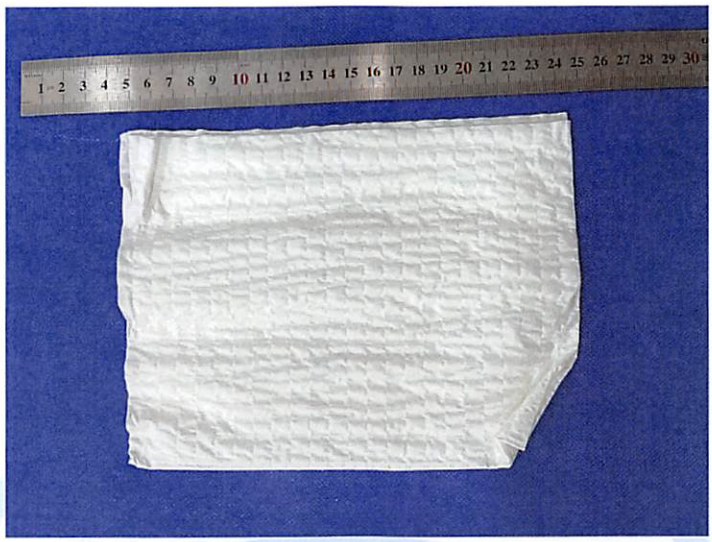
In this study, mammalian L-929 cells were cultured in vitro according to ISO 10993-5:2009 to test the potential cytotoxicity of the test article.

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 85.3%. 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 has no potential toxicity to L-929 in the MTT method.

Study Verification and Signature



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CCIC Huatongwei International Inspection (Suzhou) Co., Ltd.

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 10993-12：2021)

3.0 Test and control articles

Groups	Test article	Negative Control Article	Positive Control Article	Blank Control
Name	Medical Clean Paper Wiper	High Density Polyethylene Film	ZDEC	MEM medium, with addition 10% FBS
Manufacturer	suzhou Virgil medical Technology Co.,Ltd	Hatano Research Institute. FDSC	Sigma-Aldrich.	Hyclone
Size	30*40cm	3 cm×10 cm (5 sheets)	25 g	500 ml
Model	/	/	/	/
Lot Batch#	20210302	C-161	BCBQ6847V	AF29628620
Test Article Material	Paper	/	/	/
Physical State	Solid	Solid	Solid	Liquid
Color	White	White	White	Pink
Packaging Material	carton	/	/	/
Sterilized or Not	No	No	No	Yes
Concentration	/	/	0.1%	/
Total Surface or weight	Not provided	/	/	/
Storage Condition	Room Temp.	Room Temp.	Room Temp.	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, AF29628620), FBS (Clark, JC65927), Penicillin-Streptomycin (Gibco, 2175429), Tryps in (Gibco, 2085461), PBS (Gibco, 8120015), MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bro mide) (Solarbio, 715S051), Isopropyl alcohol (Rhawn, RH246947)

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		Aseptic Extraction In Inert Container				Final Extract
	Sampling Manner	Actually sampling	Ratio	Extracts	Condition	pH	Clear or Not
Test article	Random	2.0 g	0.1 g : 1 ml	20.0 ml	37 ℃ 24 h	7.4	Clear
Negative Control	Random	60.0 cm^2	3 cm^2: 1 ml	20.0 ml	37 ℃ 24 h	7.4	Clear
Positive Control	Random	0.02 g	0.1 g: 100 ml	20.0 ml	37 ℃ 24 h	7.4	Clear
Blank Control	/	/	/	20.0 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, the color and pH of the extraction solution did not change before and after use, and the pH value was 7.4, the status of the extract was shown in the figure below. The extraction solution and the pH value did not been adjusted, filtered, centrifuged, diluted and other processes before used.The extraction of the test article could be stored at 4℃ for no more than 24 h, but in our test, the test article extract were immediately be used after leaching.

Vehicle	Time Observed	Extracts	Condition of Final Extracts		
			Color	Clear or Not	Particulates
MEM medium (10% FBS)	Before Extraction	Test article	Pink	Clear	None
		Negative Control	Pink	Clear	None
		Positive Control	Pink	Clear	None
		Blank Control	Pink	Clear	None
	After Extraction	Test article	Pink	Clear	None
		Negative Control	Pink	Clear	None
		Positive Control	Pink	Clear	None
		Blank Control	Pink	Clear	None

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 24 h incubation which made 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 alcohol 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 ($\bar{x} \pm s$)

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

Table 1 Qualitative morphological grading of cytotoxicity of extracts

Grade	Conditions of all cultures
0	Discrete intracytoplasmatic granules, no cell lysis, no reduction of cell growth
1	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; only slight growth inhibition observable.
2	Not more than 50 % of the cells are round, devoid of intracytoplasmatic granules, no extensive cell lysis; not more than 50 % growth inhibition observable.
3	Not more than 70 % of the cell layers contain rounded cells or are lysed; cell layers not completly destroyed, but more than 50 % growth inhibition observable.
4	Nearly complete or complete destruction of the cell layers.

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 2 Observation of the cell morphology

Group	Before inoculation	Before treated with extract	24 h after treatment
Blank control	0	0	0
Negative control	0	0	0
Positive control	0	0	4
100% Test article extract	0	0	0
75% Test article extract	0	0	0
50% Test article extract	0	0	0
25% Test article extract	0	0	0

9.2 Results of the cell vitality

Table3 Results of the cell vitality

Group	OD value								Viab. (%)
	1	2	3	4	5	6	$\bar{x}$	s	
Blank control	0.631	0.620	0.615	0.613	0.615	0.613	0.618	0.007	100.0
Negative control	0.609	0.604	0.605	0.633	0.628	0.611	0.615	0.012	99.6
Positive control	0.060	0.054	0.051	0.055	0.052	0.053	0.054	0.003	8.7
100% test article extract	0.529	0.527	0.532	0.520	0.520	0.534	0.527	0.006	85.3
75% test article extract	0.542	0.538	0.538	0.548	0.537	0.541	0.541	0.004	87.5
50% test article extract	0.563	0.557	0.569	0.583	0.578	0.582	0.572	0.010	92.6
25% test article extract	0.606	0.583	0.592	0.592	0.604	0.574	0.592	0.012	95.8

10.0 Conclusion

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

11.0 Protocol amendment/deviations

There were no amendments or deviations that occurred during the course of this study.

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.