REPORT

TEST FACILITY

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CONFIDENTIAL

STUDY TITLE

Cytotoxicity Study Using the ISO Agarose Overlay Method

TEST ARTICLE NAME

WaterShield

TEST ARTICLE IDENTIFICATION

Blue Lantern 200/300 Coating

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Summary

The test article, WaterShield, was evaluated to determine the potential for cytotoxicity. This study was conducted based on the requirements of ISO 10993-5, Biological evaluation of medical devices - Part 5: Tests for *in vitro* cytotoxicity. Triplicate wells were dosed with a 1 cm x 1 cm portion of the test article. Triplicate wells were dosed with a 1 cm length portion of high density polyethylene as a negative control. Triplicate wells were dosed with a 1 cm x 1 cm portion of latex as a positive control. Each was placed on an agarose surface directly overlaying a subconfluent monolayer of L-929 mouse fibroblast cells. After incubating at 37°C in the presence of 5% CO_2 for 24 hours, the cultures were examined macroscopically and microscopically for any abnormal cell morphology and cell lysis.

The test article showed no evidence of causing any cell lysis or toxicity. The test article met the requirements of the test since the grade was less than or equal to a grade 2 (mild reactivity).

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2-25-15
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1. Introduction

1.1 Purpose

The purpose of this study was to determine the potential of a test article to cause cytotoxicity.

1.2 Testing Guidelines

This study was based on the requirements of the International Organization for Standardization 10993-5, Biological evaluation of medical devices - Part 5: Tests for *in vitro* cytotoxicity.

1.3 Dates

Test Article Received:	January 30, 2015
Cells Dosed:	February 19, 2015
Observations Concluded:	February 20, 2015

2. Identification of Test and Control Articles

The test article provided by the sponsor was identified and handled as described below:

Table 1: Test Article

Name:	WaterShield
Identification:	Blue Lantern 200/300 Coating
Physical Description of the Test Article:	Hear Aid BioChip
Storage Conditions:	Room Temperature

Table 2: Negative Control Article

Name:	High density polyethylene (HDPE)
Purity, Composition, and Other Characteristics:	Purity: Meets USP <661> Polyethylene Containers, Multiple Internal Reflectance, Thermal Analysis, Heavy Metals, and Non-Volatile Residue; Composition: polyethylene

Table 3: Positive Control Article

Name:	Latex
Purity, Composition, and Other Characteristics:	Composition: natural rubber latex, zinc carbamate accelerators, zinc oxide, and titanium dioxide

Table 4: Ancillary Materials

Growth Media:	Single strength Minimum Essential Medium supplemented with 5% fetal bovine serum, 2% antibiotics (100 units/mL penicillin, 100 µg/mL streptomycin, and 2.5 µg/mL amphotericin B) and 1% (2 mM) L-glutamine (1X MEM) Double strength Minimum Essential Medium supplemented with 10% fetal bovine serum, 4% antibiotics (200 units/mL penicillin, 200 µg/mL streptomycin and 5.0 µg/mL amphotericin B) and 2% (4 mM) L-glutamine (2X MEM)

3. Test System

3.1 Test System and Justification of Test System

Mammalian cell culture monolayer consisting of L-929 mouse fibroblast cells (ECACC Cat #85103115, or equivalent source) was used. *In vitro* mammalian cell culture studies have been used historically to evaluate cytotoxicity of biomaterials and medical devices.

3.2 Test System Management

L-929 mouse fibroblast cells were propagated and maintained in flasks containing 1X MEM at 37°C with 5% carbon dioxide (CO_2). For this study, cells were seeded in 10 cm^2 cell culture wells, labeled with passage number and date, and incubated at 37°C in the presence of 5% CO_2 to obtain subconfluent monolayers of cells prior to use. Aseptic procedures were used in the handling of the cell cultures following approved NAMSA Standard Operating Procedures.

3.3 Preparation of Agarose Overlay

The culture wells were selected which contained a subconfluent cell monolayer. The agarose mixture was prepared with equal amounts of 2% agarose and 2X MEM supplemented with neutral red. The growth medium in each well was replaced with 2.0 mL of the agarose mixture. The agarose mixture was allowed to solidify over the cells to form the agarose overlay.

4. Method

4.1 Test Article Preparation

Triplicate wells were dosed with a 1 cm x 1 cm portion of the test article.

4.2 Control Article Preparation

Negative Control: Triplicate wells were dosed with a 1 cm length portion of HDPE.

Positive Control: Triplicate wells were dosed with a 1 cm x 1 cm portion of latex.

4.3 Test Procedure

The prepared test article was placed on the solidified agarose surface in each of three cell culture wells. Similarly, the negative control and the positive control were each placed on the solidified agarose surface in three cell culture wells. The wells were labeled with the corresponding lab number and dosing date, and incubated at 37°C in the presence of 5% CO_2 for 24 –26 hours.

Following incubation, the cells were examined macroscopically for cell decolorization around the test article and controls to determine the zone of cell lysis (if any). After macroscopic examination, the cell monolayers were examined microscopically (100X) to verify any decolorized zones and to determine cell morphology in proximity to the article. Scoring for cytotoxicity was based on the following criteria:

Table 5: Test Scoring

Grade	Reactivity	Condition of Cultures
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 1.0 cm
4	Severe	Zone extending farther than 1.0 cm beyond specimen

For the suitability of the system to be confirmed, the negative control must have been a grade of 0 (reactivity none) and the positive control must have been a grade equal to or greater than a grade of 3 (reactivity moderate to severe). The test article passed the test if all three monolayers were less than or equal to a grade of 2 (reactivity mild).

All times and temperatures reported herein are approximate and are within ranges established by the external standards described in the References section of this report and/or NAMSA standard operating procedures.

5. Results

The scores obtained were as follows:

Table 6: Individual Scores

Articles		Zone of Lysis (mm)	Grade	Reactivity
Test Article:	(1)	0	0	None
	(2)	0	0	None
	(3)	0	0	None
Negative Control:	(1)	0	0	None
	(2)	0	0	None
	(3)	0	0	None
Positive Control:	(1)	8	3	Moderate
	(2)	8	3	Moderate
	(3)	8	3	Moderate

Note: 1, 2, and 3 denote replicates.

6. Conclusion

The test article showed no evidence of causing any cell lysis or toxicity. The test article met the requirements of the test since the grade was less than or equal to a grade 2 (mild reactivity).

Results and conclusions apply only to the test article tested. Any extrapolation of these data to other articles is the sponsor's responsibility.

7. Records

All raw data pertaining to this study and a copy of the final report are retained in designated NAMSA archive files in accordance with NAMSA SOPs.

8. ISO Compliance

All procedures were certified to ISO 13485 and accredited to ISO 17025.

9. References

International Organization for Standardization (ISO) 10993-1, Biological evaluation of medical devices - Part 1: Evaluation and testing within a risk management process (2009/Technical Corrigendum 1 2010).

International Organization for Standardization (ISO) 10993-5, Biological evaluation of medical devices - Part 5: Tests for *in vitro* cytotoxicity (2009).

International Organization for Standardization (ISO) 10993-12, Biological evaluation of medical devices - Part 12: Sample preparation and reference materials (2012).

International Organization for Standardization (ISO) 13485, Medical devices - Quality management systems - Requirements for regulatory purposes (2003/Technical Corrigendum 1 2009).

International Organization for Standardization/International Electrotechnical Commission (ISO/IEC) 17025, General requirements for the competence of testing and calibration laboratories (2005/Technical Corrigendum 1 2006).

United States Pharmacopeia 37, National Formulary 32 (USP), General Chapter <87>, Biological Reactivity Tests, In Vitro (2014).