STUDY TITLE:

CYTOTOXICITY STUDY USING THE ISO ELUTION METHOD

(1X MEM Extract)

TEST ARTICLE:

DLC Coating on Vitox alumina. Zyranox zirconia, Co/Cr, Ti

TEST FACILITY:

NAMSA
6750 Wales Road
Northwood, OH 43619

SPONSOR:

Mark Duchnak
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SUMMARY

An *in vitro* biocompatibility study, based on the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity: *in vitro* Methods guidelines, was conducted on the test article, DLC Coating on Vitox alumina, Zyranox zirconia, Co/Cr, Ti, Not supplied, to determine the potential for cytotoxicity. A single extract of the test article was prepared using single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM). This test extract was placed onto three separate confluent monolayers of L-929 mouse fibroblast cells propagated in 5% CO_2. Three separate monolayers were prepared for the reagent control, negative control and for the positive control. All monolayers were incubated at 37°C in the presence of 5% CO_2 for 48 hours. The monolayer in the test, reagent control, negative control and positive control wells was examined microscopically at 48 hours to determine any change in cell morphology.

Under the conditions of this study, the 1X MEM test extract showed no evidence of causing cell lysis or toxicity. The 1X MEM test extract met the requirements of the test since the grade was less than a grade 2 (mild reactivity). The reagent control, negative control and the positive control performed as anticipated.

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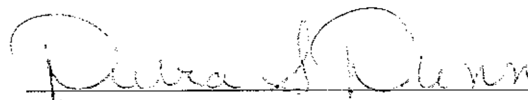

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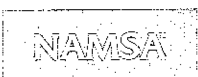

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INTRODUCTION

The test article identified below was extracted, and the extract was subjected to an *in vitro* cytotoxicity study for biocompatibility based on the requirements of the International Organization for Standardization 10993: Biological Evaluation of Medical Devices, Part 5: Tests for Cytotoxicity: *in vitro* Methods. The test was performed to determine whether leachables extracted from the material would cause cytotoxicity. The test article was received on February 7, 2005. The cells were first exposed to the extract on February 15, 2005, and the observations were concluded on February 17, 2005.

MATERIALS

The sample provided by the sponsor was identified and handled as follows:

Test Article:	DLC Coating on Vitox alumina, Zyranox zirconia, Co/Cr, Ti
Identification No.:	Not supplied
Storage Conditions:	Room Temperature
Extraction Vehicle:	Single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM)
Test Article Preparation:	All pieces of the supplied samples were included in the preparation. Based on the USP ratio of 4 g:20 ml, a 203.5 g portion of the test article was covered with 1018 ml of 1X MEM. A single preparation was extracted and agitated at 37°C for 24 hours.
Negative Control Preparation:	High density polyethylene was used as the negative control. Based on the USP ratio of 60 cm^2:20 ml, a single 30.8 cm^2 portion of the control material was covered with 10 ml of 1X MEM. The preparation was subjected to the extraction conditions previously described for the test article.
Reagent Control Preparation:	A single aliquot of 1X MEM without test material was subjected to the same extraction conditions as described for the test article.
Positive Control Preparation:	The current NAMSA positive control, tin stabilized polyvinylchloride, was used to determine a cytotoxic end-point. Based on the USP ratio of 60 cm^2:20 ml, a single 60.8 cm^2 portion of the control material was covered with 20 ml of 1X MEM and extracted and agitated at 37°C for 24 hours. Serial dilutions were prepared (1:2, 1:4, 1:8, 1:16, 1:32) for an end-point titration.
Condition of Extracts:	Test: clear Reagent Control: clear Negative Control: clear Positive Control (undiluted): clear
Sample Disposition:	Any used sample was returned to the sponsor.

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METHODS

Test System Management:

L-929, mouse fibroblast cells, (ATCC CCL 1, NCTC Clone 929, of strain L, or equivalent source) were propagated and maintained in open wells containing single strength Minimum Essential Medium supplemented with 5% serum and 2% antibiotics (1X MEM) in a gaseous environment of 5% carbon dioxide (CO_2). For this study, 10 cm^2 wells were seeded, labeled with passage number and date, and incubated at 37°C in 5% CO_2 to obtain confluent monolayers of cells prior to use. Aseptic procedures were used in the handling of the cell cultures following approved NAMSA Standard Operating Procedures.

Experimental Procedure:

Triplicate culture wells were selected which contained a confluent cell monolayer. The growth medium contained in triplicate cultures was replaced with 2 ml of the test extract. Similarly, triplicate cultures were replaced with 2 ml of the reagent control, negative control and the undiluted and each titer of the positive control. Each well was labeled with the corresponding lab number, replicate number, dilution (as applicable) and the dosing date. The wells were incubated at 37°C in 5% CO_2 for 48 hours.

Following incubation, the cultures were examined microscopically (100X) to evaluate cellular characteristics and percent lysis.

Evaluation Criteria:

The confluency of the monolayer was recorded as (+) if present and (-) if absent. In addition, the color of the test medium was observed and compared to the negative control medium. A color shift toward yellow was associated with an acidic pH range and a color shift toward magenta to purple was associated with an alkaline pH range. Each culture well was evaluated for percent lysis and cellular characteristics using the following USP based criteria:

Grade	Reactivity	Observations	
0	None	Discrete intracytoplasmic granules	No lysis
1	Slight	Not more than 20% of the cells are round, loosely attached, and without intracytoplasmic granules	Not more than 20% lysis
2	Mild	Not more than 50% of the cells are round and devoid of intracytoplasmic granules	Not more than 50% lysis
3	Moderate	Not more than 70% of the cell monolayer contains rounded cells	Not more than 70% lysis
4	Severe	Nearly complete destruction of the cell monolayer	Greater than 70% lysis

For the test to be valid, the reagent control and the negative control must have had a reactivity of none (grade 0) and the positive control must have been a grade 3 or 4. The test sample met the requirements of the test if the biological response was less than or equal to grade 2 (mild). The test would have been repeated if the controls did not perform as anticipated and/or if all three test wells did not yield the same conclusion.

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RESULTS

See Table I for results.

pH Observation: The test medium was similar to the negative control medium at 48 hours.

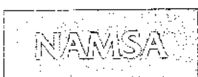
Results and conclusions apply only to the test article tested. No further evaluation of these results is made by NAMSA. Any extrapolation of these data to other samples is the responsibility of the sponsor. All procedures were conducted in conformance with good laboratory practice and ISO 17025.

CONCLUSION

Under the conditions of this study, the 1X MEM test extract showed no evidence of causing cell lysis or toxicity. The 1X MEM test extract met the requirements of the test since the grade was less than a grade 2 (mild reactivity). The reagent control, negative control and the positive control performed as anticipated.

RECORD STORAGE

All raw data pertaining to this study and a copy of the final report are to be retained in designated NAMSA archive files for a period of 5 years.



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