Tuesday, September 22, 2011

Independent Laboratory Cytotoxic Effects on Marine Mammals Testing:

FreshAWL contracted with TOXIKON (Bedford, MA) in August, 2009 for the purpose of testing FreshAWL® FUEL-WASH™.

The purpose of the study was to determine the biological reactivity of a mammalian monolayer cell culture (L929) in response to the test article. The study design was suitable for liquid test articles in a variety of shapes (e.g., elastomeric closures, etc.), liquid test articles or extracts. The agar layer protects the cells from mechanical damage while allowing the diffusion of leachable chemicals from the test article.

The study was conducted based upon the following references:

2.1 ISO 10993-5, 2009, Biological Evaluation of Medical Devices – Part 5: Tests for *In Vitro* Cytotoxicity.
2.2 ISO 10993-12, 2007, Biological Evaluation of Medical Devices – Part 12: Sample Preparation and Reference Materials.
2.3 ISO/IEC 17025, 2005, General Requirements for the Competence of Testing and Calibration Laboratories.

Mild biological reactivity (Grade 2) was observed in the L929 mammalian cells at 48 hours post exposure to the test article. The observed cellular response obtained from the positive control article (Grade 3) and negative control article (Grade 0) confirmed the suitability of the test system. Based on the criteria of the protocol, the test article, FUEL-WASH is considered non-cytotoxic and meets the requirements of the Agar Diffusion Test defined in ISO 10993-5 guidelines.

FreshAWL, LLC • 1950 Olivera RD., Suite D • Concord, CA 94520
p (888) 400-FAWL (3295) e info@freshawl.com

Leaders in Life Science and Technology

TOXIKON FINAL GLP REPORT: 09–3274–G1

AGAR DIFFUSION TEST – ISO

Test Article
FreshAWL® FUEL-WASH™

Author
Franck Grall, Pharm.D., Ph.D.

Final Report Date
August 14, 2009

COMPLIANCE
21 CFR, Part 58
Good Laboratory Practice for Non–Clinical Laboratory Studies

MANAGEMENT OF THE STUDY

Performing Laboratory
Toxikon Corporation
15 Wiggins Avenue
Bedford, MA 01730

Sponsor
FreshAWL, LLC
1950 Olivera Rd., Suite D
Concord, CA 94520

STUDY SUMMARY

Mild biological reactivity (Grade 2) was observed in the L929 mammalian cells at 48 hours post exposure to the test article. The observed cellular response obtained from the positive control article (Grade 3) and negative control article (Grade 0) confirmed the suitability of the test system. Based on the criteria of the protocol, the test article, **FUEL-WASH™,** is considered non-cytotoxic and meets the requirements of the Agar Diffusion Test defined in ISO 10993-5 guidelines.

FreshAWL, LLC • 1950 Olivera RD., Suite D • Concord, CA 94520
p (888) 400-FAWL (3295) e info@freshawl.com

7.2 Pre–Dose Procedure:

Cell Culture Preparation:
Cultures of L929 cells were initiated in complete MEM and incubated at 37 ± 1 °C in a humidified atmosphere containing 5 ± 1% carbon dioxide. Cultures that had grown to approximate confluence at the end of the log phase of the growth curve were used in the assay. The liquid medium was replaced with a serum–supplemented medium/agar mixture that was stained with a vital dye, neutral red, prior to testing (final concentration of agar = 1%). The culture was protected from light for the duration of the assay to prevent cell damage elicited by photo–activation of the stain.

7.3 Dose Administration:

7.3.1 The test article (100 µL volume) was placed on a sterile filter disc with a surface area ≥ 100 mm² at 100% concentration. In addition, complete MEM was placed on a sterile filter disc with surface area ≥ 100 mm² at 100 µL volume (Disc Control).

7.3.2 The negative and positive control articles were placed directly on the surface of the agar.

7.3.3 The test was performed in triplicate.

7.4 Post–Dose Procedure:

7.4.1 Incubation:
All plates were incubated for 48 ± 2 hours at 37 ± 1 °C, in a humidified atmosphere containing 5 ± 1% carbon dioxide.

7.4.2 The extent of decolorization of the cells stained with neutral red was evaluated at 0, 24, and 48 hours.

8.0 EVALUATION CRITERIA

8.1 The response of the cell monolayer is evaluated under a microscope for cytotoxicity. The zone of biological reactivity (cellular degeneration and malformation) under and around the test and control articles is measured and rated on a scale of 0 to 4. The test system is considered suitable if no signs of cellular reactivity (Grade 0) are noted for the negative control article and the positive control article shows greater than a Mild reactivity (Grade 2). The test article meets the requirements of the test if none of the cultures treated with the test article shows greater than a Mild reactivity (Grade 2). If the suitability of the system had not been confirmed, the test would have been repeated.

FreshAWL, LLC • 1950 Olivera RD., Suite D • Concord, CA 94520
p (888) 400-FAWL (3295) e info@freshawl.com

Grade	Reactivity	Description of Reactivity Zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerated cells under specimen
2	Mild	Zone limited to the area under the specimen
3	Moderate	Zone extends up to 1.0 cm beyond specimen
4	Severe	Zone extends greater than 1.0 cm beyond the specimen

8.2 The study and its design employ methodology to minimize uncertainty of measurement and control of bias for data collection and analysis.

9.0 RESULTS

REACTIVITY GRADES

Date	Time	Dish	Test Article		Controls					
					Positive		Negative		Disc Control	
			Zone Size (cm)	Grade	Zone Size (cm)	Grade	Zone Size (cm)	Grade	Zone Size (cm)	Grade
08/05/09	0 Hours*	A	0.0	0	0.0	0	0.0	0	0.0	0
		B	0.0	0	0.0	0	0.0	0	0.0	0
		C	0.0	0	0.0	0	0.0	0	0.0	0
08/06/09	24 Hours	A	0.0	1	0.5	3	0.0	0	0.0	0
		B	0.0	1	0.5	3	0.0	0	0.0	0
		C	0.0	1	0.5	3	0.0	0	0.0	0
08/07/09	48 Hours	A	0.0	2	0.7	3	0.0	0	0.0	0
		B	0.0	2	0.7	3	0.0	0	0.0	0
		C	0.0	2	0.7	3	0.0	0	0.0	0

* 0 Hours = Pre–dose

10.0 CONCLUSION

Mild biological reactivity (Grade 2) was observed in the L929 mammalian cells at 48 hours post exposure to the test article. The observed cellular response obtained from the positive control article (Grade 3) and negative control article (Grade 0) confirmed the suitability of the test system. Based on the criteria of the protocol, the test article, FUEL-WASH™ , is considered non–cytotoxic and meets the requirements of the Agar Diffusion Test defined in ISO 10993–5 guidelines.

11.0 RECORDS

11.1 Original raw data are archived at Toxikon Corporation.

11.2 A copy of the final report and any report amendments is archived at Toxikon Corporation.

FreshAWL, LLC • 1950 Olivera RD., Suite D • Concord, CA 94520
p (888) 400-FAWL (3295) e info@freshawl.com