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**ACUTE DERMAL TOXICITY STUDY OF AIRCRAFT ENGINE
OILS USING NEW ZEALAND WHITE RABBITS
(*ORYCTOLAGUS CUNICULUS*)**

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14. ABSTRACT There is little data available on toxicity levels of used aircraft engine oils relative to their unused (new) versions. This study was conducted to determine if new engine oils and their used versions have the potential to induce irritation. Twelve Male New Zealand White Rabbits (<i>Oryctolagus cuniculus</i> , 19 weeks old) were used to determine the acute dermal toxicity potential of four aircraft engine oils in their unused and used states: two MIL-PRF-7808 oils, a MIL-PRF-23699 HTS oil and an experimental MIL-PRF-23699 type oil. Five fur-free test sites (6 cm ² each) located lateral to the midline of the back were treated with two undiluted (0.5 ml) new engine oils and their used versions. The fifth site received RO water as a control. Each treatment was repeated 3 times (3 rabbits/oil type). Each oil was tested under both semi-occluded and occluded conditions. E-collars were placed on each animal for at least 72 h to prevent ingestion of the test substance and/or gauze plus wrappings and disturbance of site recovery. The 4 hour exposure was followed by gauze plus wrappings removal, and gently cleaning of sites prior to scoring for erythema and edema at 0.5-1, 24, 48 and 72 h post exposure based on Draize (1959). Additional observations were made on days 7, 10 and 14 to determine recovery. Exposure to both used and new oils produced dermal irritation consisting of no more than very slight to well-defined erythema and very slight edema. Calculated Primary Dermal Irritation Index (PDII) indicate that all the oils were slightly irritating. Although the PDII values for new oils and their used versions were not significantly different, they were all statistically higher (p<0.05) than those obtained for the control regardless the type of occlusion binding applied. The used oils under semi-occlusion conditions yielded larger size effects (Cohen's d) relative to their unused versions suggesting an enhancement in irritation when the oil is aging. Grade 4 in the used state yielded the largest size effect which was d = 5.9 versus 2.6 for its unused version. The slight dermal irritation resulting from four hours of exposure to oils raises concerns about the magnitude of impact related to prolonged and/or repeated exposure (in compliance with DODI 3216.01).					
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PREFACE

Support for this research was provided through the Aerospace Toxicology Program in the Air Force Research Laboratory, 711th Human Performance Wing, Airman Systems Directorate, Human-Centered ISR Division, Molecular Mechanisms Branch, 711 HPW/RHXJ (formerly Molecular Bioeffects Branch, 711 HPW/RHDJ) at Wright-Patterson Air Force Base (AFB) OH. This research was conducted under cooperative agreement FA8650-15-2-6608 with the Henry M. Jackson Foundation for the Advancement of Military Medicine (HJF). The program manager for HJF was David R. Mattie, PhD (711 HPW/RHXJ), who was also the Technical Manager for this project.

The acute dermal irritation study was designed to be in compliance with the U.S. Environmental Protection Agency (EPA), Health Effects Test Guidelines, Office of Prevention, Pesticides, and Toxic Substances (OPPTS) 870.2500, Acute Dermal Irritation published in August 1998 and the Organization for Economic Cooperation and Development (OECD) Guideline for Testing of Chemicals, Acute Dermal Irritation/Corrosion, Section 404, adopted on July 28th 2015. This study was performed in a Good Laboratory Practice (GLP) Standards certified laboratory at Charles River Laboratories, Inc. (640 N. Elizabeth Street, Spencerville, OH 45887).

The dermal irritation study protocol, “An Acute Skin Irritation Study of Aircraft Engine Oils by Dermal Administration in Rabbits,” was approved as FWR-2017-0001A by the Air Force Surgeon General’s Office of Research Oversight & Compliance (OROC) and as 20132252 by the Installation Animal Care and Use Committee (IACUC) of Charles River Laboratories, Preclinical Services, Ohio. The study was conducted in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC), International, in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011). The study was performed in compliance with DODI 3216.1.

The views expressed in this report are those of the authors and do not necessarily reflect the official policy or position of the U.S. Air Force, Department of Defense, nor the U. S. Government. The authors are employees, contractors and subcontractors of the U.S. Government. This work was prepared as part of official duties. Title 17, U.S.C., §105 provides that copyright protection under this title is not available for any work of the U.S. Government. Title 17, U.S.C., §101 defines a U.S. Government work as a work prepared by a military service member or employee of the U.S. Government as part of that person’s official duties.

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1. SUMMARY

Depression prevalence in aircraft maintenance workers has been reported due to a suggested link with exposure to organophosphate esters in hydraulic fluids and engine oils. Studies have indicated that people who are chronically exposed to a low level of organophosphate compounds could develop neuropathy and neuropsychiatric problems such as depression. Currently, there is little data available on toxicity levels of used aircraft engine oils relative to their unused (new) versions. Twelve male New Zealand White rabbits (*Oryctolagus cuniculus*, 19 weeks old) were used to determine the acute dermal toxicity potential of two MIL-PRF-7808 oils (Grades 3 and 4), a MIL-PRF-23699 High Thermal Stability (HTS) oil (Grade 5 HTS) and an experimental MIL-PRF-23699 (Experimental Grade 5) oils. All these aircraft engine oils were tested in their unused and used/laboratory stressed (aged) states. Five fur-free test sites (6 cm² each) located lateral to the midline of the back were treated with two undiluted (0.5 ml) new engine oils and their used versions. The fifth site received reverse osmosis deionized (RODI) water as a control. Each treatment was repeated 3 times (3 rabbits/oil type). Each oil was tested under both semi-occluded and occluded conditions. E-collars were placed on each animal for at least 72 h to prevent ingestion of the test substance and/or gauze plus wrappings and disturbance of site recovery. The 4 hour exposure was followed by gauze plus wrappings removal, and gently cleaning of sites prior to scoring for erythema and edema at 0.5-1, 24, 48 and 72 h post exposure based on Draize (1959). Additional observations were made on days 7, 10 and 14 to determine recovery. Exposure to both used and new oils produced dermal irritation consisting of no more than very slight to well-defined erythema and very slight edema. Calculated Primary Dermal Irritation Index (PDII) indicate that all the oils were slightly irritating. Although the PDII values for new oils and their used versions were not significantly different, they were all statistically higher ($p < 0.05$) than those obtained for the control regardless the type of occlusion binding applied. The used oils under semi-occlusion conditions yielded larger size effects (Cohen's d) relative to their unused versions suggesting an enhancement in irritation when the oil is aging. Grade 4 in the used state yielded the largest size effect which was $d = 5.9$ versus 2.6 for its unused version. The slight dermal irritation resulting from four hours of exposure to oils raises concerns about the magnitude of impact related to prolonged and/or repeated exposure (in compliance with DODI 3216.01).

2. INTRODUCTION

Air Force Research Laboratory (AFRL) has unique skill sets and mission focus to address critical Airmen-centric needs. There is limited data available on toxicity inherent to aircraft engine oil exposure. Concern has been raised regarding the possible occupational exposure to aircraft engine oils among individuals who work on aircraft during maintenance operations. The aircraft engine oils contain a mixture of organophosphate compounds and some of them are known to inhibit esterase enzymes (Aldridge, 1954; Barrett and Oehme, 1994; Carletti *et al.*, 2013). As dermal contact is a major route of exposure, it is very important to evaluate the irritation potential of current and proposed new and used aircraft engine oils. Dermal contact with the engine oils poses a health risk because they contain a mixture of organophosphate compounds that can penetrate the skin and some of them have been associated with a range of neurological and neuropsychological effects (Rosenstock *et al.*, 1991; Steenland, 1996; Leon-S *et al.*, 1996).

The skin shields the body from an excessive loss of water, electrolytes and other body constituents and minimizes the entry of toxic substances from external environment (Zhai and Maibach, 2001). However, various factors such as exposure to chemicals can contribute to perturbation of the skin barrier function, resulting in increased entrance of exogenous substances into the body (Denda, *et al.*, 1998). Other factors that can contribute to increased dermal entry of exogenous chemicals include occlusion of the skin. Dermal occlusion can improve the hydration of stratum corneum, the principal barrier, thus, progressively decreasing the efficiency in its barrier function (Bucks *et al.*, 1991; Treffel *et al.*, 1992 and Bucks *et al.*, 1999) and serving as a reservoir of the chemical for body entry (Wester and Maibach, 1983). The compromised skin barrier function leads to impaired transepidermal water loss which aggravates the irritation at the site of the chemical entry (Berardesca and Maibach, 1988; Bucks *et al.*, 1991; Hogan and Maibach, 1991; Klingman *et al.*, 1996; Bucks *et al.*, 1999). We cannot rule out that these events are possible with exposure to the aircraft engine oils when the oil gets trapped under the aircraft maintenance worker's clothes.

The safety data sheet (SDS) of each aircraft engine oil lists ingredients of the oil and the potential toxicity associated with each ingredient. The SDS shows that these toxic ingredients are at very low levels. However, the SDS does not show the toxicity associated with exposure to the mixture. Since the overall toxicity of a particular mixture depends on the proportion and toxicity of each ingredient as well as the synergic interactions between ingredients, an ideal evaluation of the hazardous effects of exposure to the compound mixture requires a toxicity test on the entire mixture, not solely on each component. Thus, our study was designed to assess the toxicity of each engine oil as a mixture of ingredients. Although toxic ingredients in the engine oils are at a very low level, little is currently known about the oil transformations occurring in running engines, due to breakdown of ingredients and/or worn engine components that may end up in oils. This change in composition could potentially change the oil properties, yielding a more toxic oil mixture. This study was also designed to determine the dermal irritation potential of used/laboratory stressed (aged) oils relative to their unused/unstressed versions. Toxicity was assessed through dermal exposure since skin is the primary route of exposure. The study characterized the irritation potential of a MIL-PRF-7808 Grade 4 (Grade 4), a MIL-PRF-7808 Grade 3 (Grade 3), a MIL-PRF-23699 HTS (Grade 5 HTS) and an experimental MIL-PRF-23699 (Experimental Grade 5) aircraft engine oils in their unused and used/laboratory stressed

(aged) states under occlusive and semi-occlusive wrapping conditions. To our best knowledge, this was the first study designed to examine and compare the dermal irritation associated with exposure to unused engine oils and their used versions.

2.1 Objective / Hypothesis

The objective of this first study was to compare the irritant potential of aircraft engine oils in both their new (unused) and used/laboratory stressed (aged) states following a single acute exposure to the skin of albino rabbits. The primary USAF engine oils are MIL-PRF-7808 Grades 3 and 4 used in the majority of USAF aircraft from legacy aircraft to 5th generation fighters. We also performed a comparison of new (unused) and laboratory stressed (simulation of used state) MIL-PRF-23699 engine oils that may be used in USAF aircraft in the future (a Grade 5 HTS and an Experimental Grade 5). The hypothesis of this proposed study was that an exposure to used or laboratory stressed aircraft engine oils is toxic (an irritant) as compared to the new (unused or stressed) oils.

3. MATERIALS AND METHODS

This study was conducted using Good Laboratory Practices (GLP) at Charles River Laboratories, 640 N. Elizabeth Street, Spencerville, Ohio.

3.1 Test and control substances

3.1.1 Test substance 1

Identification:	Grade 4 (Used)
Batch (Lot) No./Source:	Unknown; removed from <u>F-22 aircraft at Langley AFB</u>
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown; removed from aircraft
Physical Description:	Red liquid
Storage Conditions:	Kept in a controlled room temperature area

Identification:	Grade 4 (Unused/New)
Batch (Lot) No./Source:	CT1702120; <u>received from AF Petroleum Office, WPAFB</u>
Receipt Date:	21 Feb2018
Expiration Date:	April 2020
Physical Description:	Red liquid
Storage Conditions:	Kept in a controlled room temperature area

3.1.2 Test substance 2

Identification:	Grade 3 (Used)
Batch (Lot) No./Source:	Unknown; removed from <u>C-17 aircraft @ WPAFB</u>
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown; removed from aircraft
Physical Description:	Red liquid
Storage Conditions:	Kept in a controlled room temperature area

Identification:	Grade 3 (Unused/New)
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Batch (Lot) No./Source:	2017202525; <u>received from AF Petroleum Office, WPAFB</u>
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown; not provided on container
Physical Description:	Colorless liquid
Storage Conditions:	Kept in a controlled room temperature area

3.1.3 Test substance 3

Identification:	Grade 5 HTS (Laboratory stressed/aged)
Batch (Lot) No./Source:	Laboratory Stressed
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown how many years; testing still in progress
Physical Description:	Brown liquid
Storage Conditions:	Kept in a controlled room temperature area

Identification:	Grade 5 HTS (Unstressed/New)
Batch (Lot) No./Source:	Laboratory Sample
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown how many years; testing still in progress
Physical Description:	Brown liquid
Storage Conditions:	Kept in a controlled room temperature area

3.1.4 Test substance 4

Identification:	Experimental Grade 5 (Laboratory stressed/aged)
Batch (Lot) No./Source:	Laboratory Stressed
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown how many years; testing still in progress
Physical Description:	Brown liquid
Storage Conditions:	Kept in a controlled room temperature area

Identification:	Experimental Grade 5 (Unstressed/New)
Batch (Lot) No./Source:	Laboratory Sample
Receipt Date:	21 Feb 2018
Expiration Date:	Unknown how many years; testing still in progress
Physical Description:	Red liquid
Storage Conditions:	Kept in a controlled room temperature area

3.1.5 Control substance

Identification:	RODI Water
Physical Description:	Clear liquid

3.1.6 Induced engine oil aging process

The used versions of the Grade 5 HTS and Experimental Grade 5 oils were not available as these oils are either not widely used in Department of Defense (DoD) systems or they have been proposed for future use. To obtain aged versions that reflect the properties of used oil with respect to viscosity and total acid number (TAN) change, these oils were laboratory stressed

(aged) through the use of SAE ARP5921 “Evaluation of Coking Propensity of Aviation Lubricants in an Air-Oil Mist Environment using the Vapor Phase Coker (VPC)”. The VPC was selected for use in this study due to its ability to moderately age approximately a quart of oil in one testing period. To provide a thermal and oxidative environment for oil aging, 900 g oil were subjected to the following conditions: 204°C sump, dry air 765 mL/minute bubble through oil, oil vapor 371°C, for 18 hours.

3.2 Test system

3.2.1 Animals

On 13 Mar 2018, 14 male New Zealand White rabbits were received at Charles River Laboratories, Inc. (640 N. Elizabeth Street, Spencerville, OH 45887) from Covance Laboratories, Denver, PA. The animals chosen for study were arbitrarily selected from healthy stock animals. These animals were 19 weeks old on the day before dose initiation and weighed between 2.7 kg and 3.1 kg.

3.2.2 Justification for test system and number of animals

The New Zealand White rabbits were chosen as the animal model for this study since this species is accepted as the non-rodent species for preclinical toxicity testing by regulatory agencies. Presently, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

3.2.3 Husbandry

3.2.3.1 Housing

The animals were individually housed throughout the study in suspended stainless steel cages equipped with an automatic watering valve. The animals were acclimated to their designated housing for 7 days before the first day of dosing. Housing and care were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals* from the National Research Council (NRC, 2011).

3.2.3.2 Environmental conditions

Room temperature and relative humidity were maintained in the ranges of 69°F to 71°F (21°C to 22°C) and 49% to 52%, respectively. A 12-hour light/12-hour dark cycle was maintained, except when interrupted for designated procedures. Additionally, ten or greater air changes per hour with 100% fresh air (no air recirculation) were maintained in the animal rooms.

3.2.3.3 Food

PMI Nutrition International Certified Rabbit Chow No. 5322 was provided ad libitum throughout the study, except during designated procedures. To avoid potential gastrointestinal disturbances, food was withheld for approximately 24 to 48 hours after receipt. Food was then gradually increased over a 3-day period. The feed was analyzed by the supplier for nutritional components

and environmental contaminants. Results of the dietary analyses were provided by the supplier for each lot of diet. There were no known contaminants in the feed that would interfere with the objectives of the study.

3.2.3.4 Water

After treatment by reverse osmosis and ultraviolet irradiation, municipal tap water was freely available to each animal via an automatic watering system, except during designated procedures. Periodic analysis of the water was performed. Results of these analyses indicated that there were no known contaminants in the water that could interfere with the outcome of the study.

3.2.3.5 Animal enrichment

For psychological/environmental enrichment, animals were provided with a floor toy, except when interrupted by study procedures/activities. In addition, a timothy cube was provided to each animal 3 times per week. One NutraBlock per animal was offered at least once per week and/or offered up to 2 times per week. Occasional edible enrichment treats were provided.

3.2.3.6 Veterinary care

Veterinary care was available throughout the course of the study. However, no examinations or treatments were required by the veterinary staff.

3.4 Experimental Design and general procedures

3.4.1 Route and rationale of test article administration

The test substances were dermally administered on clipped and intact skin in order to evaluate the dermal irritation potential of both unused aircraft engine oils and their used/laboratory stressed versions. This study was intended to provide information on the health hazards likely to arise from a short-term exposure to engine oils by the dermal route.

3.4.2 Animal grouping

At the start of the study, animals were randomly assigned into 4 groups ([Table 1](#)) of 3 rabbits each. The $n = 3$ per group was considered to be the minimum required by Environmental Protection Agency (EPA) test guidelines for acute dermal irritation (EPA Health Effects Test Guidelines, OPPTS 870.2500, Acute Dermal Irritation published in August 1998) to properly characterize the effects of the test substance. This study was designed to minimize the number of animals to accomplish its objectives.

3.4.3 Animal identification and preparation

Each animal was identified by a subcutaneously implanted electronic identification chip. One day prior to the start of testing, fur was removed from the dorsal area of the trunk using a small animal clipper. Care was taken to avoid abrading the skin during the clipping procedure.

3.4.4 Justification of route and dose levels

The dermal exposure was selected because the skin is a route of human exposure.

3.4.5 Mortality/moribundity checks

Throughout the study, animals were observed for general health/mortality and moribundity twice daily (morning and afternoon).

3.4.6 Detailed clinical and cage observations

Animals were removed from the cage and examined in detail at animal assignment and prior to dosing. Cage side observations were performed at least once daily, beginning pre-test and throughout the dosing and observation periods. Cage side observations were not required on the days of detailed clinical observations during the pre-test and observation periods, or on the day of scheduled euthanasia.

3.4.7 Administration of test materials

On the treatment day, five test sites (6 cm² each) located lateral to the midline of the back of the rabbit ([Fig. 1](#)) were delineated with an indelible marker. Four test sites on each rabbit were treated with two undiluted (0.5 ml) new engine oils and their used/laboratory stressed versions. The first site received RODI water and served as control. A control group of animals was not needed as each animal served as its own control. Each treatment was repeated 3 times (3 rabbits/oil type). The test site was covered with about 1 inch x 1 inch 4-ply gauze patch secured in place with a nonirritating surgical tape. Rabbits were divided into groups based on whether semi-occlusive or occlusive wrappings were applied ([Table 1](#); [Fig. 2](#)). Semi-occlusive wrapping ([Table 1](#), groups 1 and 2) consisted of a stockinette placed over the rabbit trunk and test area while an occlusive wrapping ([Table 1](#), groups 3 and 4) included a plastic wrap placed over the gauze patches prior to stockinette application. E-collars was placed on each animal for at least 72 h to prevent ingestion of the test substance and/or wrappings and disturbance of the site for recovery.

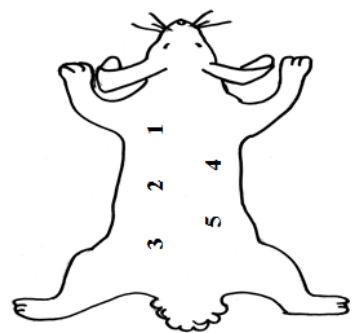


Figure 1. Location and number of test sites on each animal.

Table 1. Test articles, exposure time and method, number of test sites per animal. No used versions of Grade 5 HTS and Experimental Grade 5 oils were available. They were laboratory stressed (aged) to obtain the mimics of their used versions.

Group No.	Test Material	Test Material Status	Dose (mL)	Exposure Time	Exposure Method	Test Site	Number of Animals
1	Water Control	-	0.5	4 hours	Semi-occluded	1	3
	Grade 4	Used	0.5			2	
	Grade 4	Unused/New	0.5			3	
	Grade 3	Used	0.5			4	
	Grade 3	Unused/New	0.5			5	
2	Water Control	-	0.5	4 hours	Semi-occluded	2	3
	Grade 5 HTS	Stressed/Used	0.5			3	
	Grade 5 HTS	Unstressed/New	0.5			4	
	Experimental Grade 5	Stressed/Used	0.5			5	
	Experimental Grade 5	Unstressed/New	0.5			1	
3	Water Control	-	0.5	4 hours	Occluded	3	3
	Grade 4	Used	0.5			4	
	Grade 4	Unused/New	0.5			5	
	Grade 3	Used	0.5			1	
	Grade 3	Unused/New	0.5			2	
4	Water Control	-	0.5	4 hours	Occluded	4	3
	Grade 5 HTS	Stressed/Used	0.5			5	
	Grade 5 HTS	Unstressed/New	0.5			1	
	Experimental Grade 5	Stressed/Used	0.5			2	
	Experimental Grade 5	Unstressed/New	0.5			3	

- = not applicable

3.4.8 Test site cleaning and dermal observations

After four hours of treatment, gauze plus wrappings were removed, the corners of each test site delineated using a marker and the sites gently cleaned. Since RODI water was not sufficient to remove the test substance, the residual test substance was removed using gauze moistened with acetone, followed by dry gauze, then gauze moistened with RODI water, followed by dry gauze. Erythema and edema scoring was performed at 0.5-1 (D0), 24 (D1), 48 (D2) and 72 h (D3) post exposure (**Fig. 2**) based on Draize (1959; **Table 2**). Additional observations and scorings were made on days 7 (D7), 10 (D10) and 14 (D14) to determine recovery.

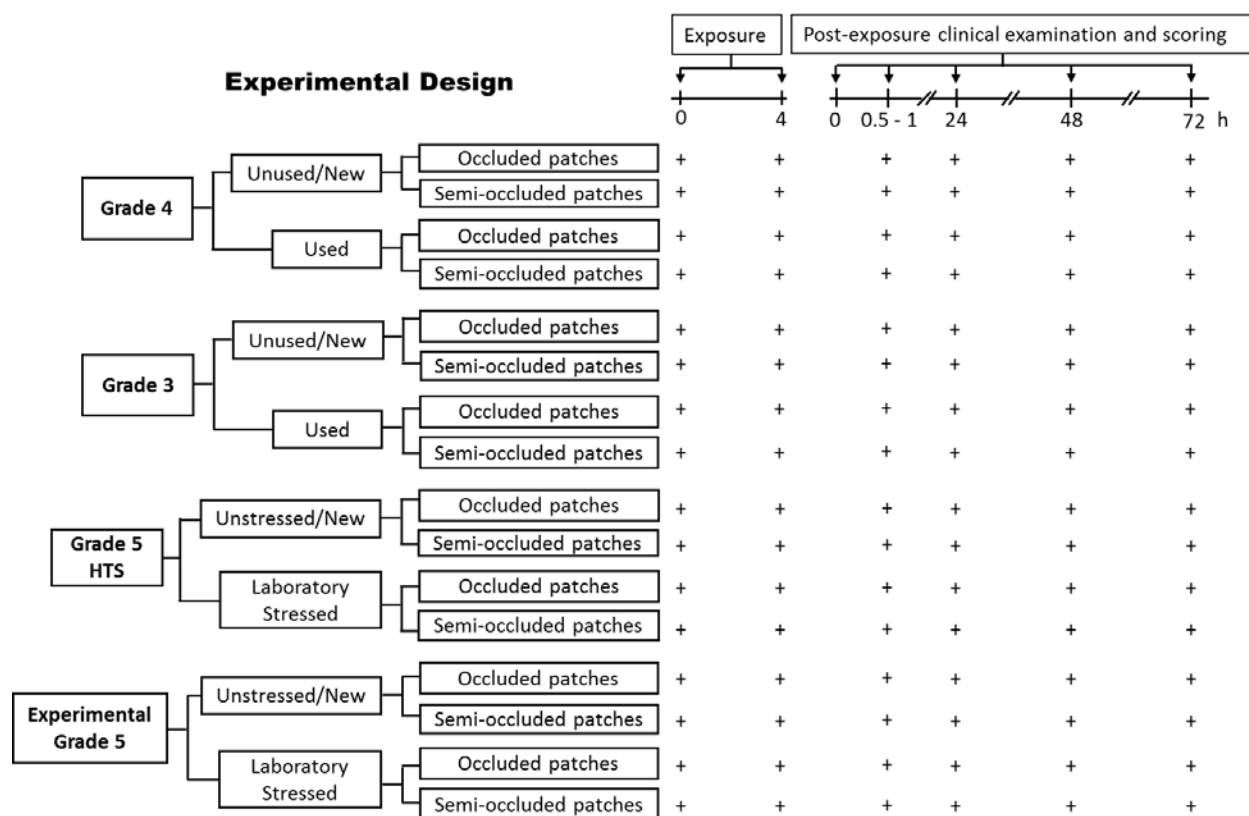


Figure 2. Diagram depicting experimental design.

3.4.9 Body weights

The weight of each rabbit was recorded on the day they were assigned into groups, prior to dosing, and on the day of scheduled euthanasia.

3.4.10 Scheduled euthanasia

All rabbits were euthanized by sodium pentobarbital injection and the bodies were discarded in an appropriate manner.

3.5 Computerized systems

Critical computerized systems used in the study are listed below ([Table 3](#)). All computerized systems used in the conduct of this study have been validated. If a particular system did not satisfy all requirements, appropriate administrative and procedural controls would be implemented to assure the quality and integrity of data. However, there were no discrepancies to report for this study.

Table 2. Scoring criteria for dermal reactions (Draize, 1959)

Erythema and Eschar Formation

Value

- 0 No erythema
- 1 Very slight erythema (barely perceptible, edges of area not well defined)
- 2 Slight erythema (pale red in color and edges definable)
- 3 Moderate to severe erythema (definite red in color and area well defined)
- 4 Severe erythema (beet or crimson) to slight eschar formation (injuries in depth)

- 4 Maximum possible erythema score

Edema Formation

- 0 No edema
- 1 Very slight edema (barely perceptible, edges of area not well defined)
- 2 Slight edema (edges of area well defined by definite raising)
- 3 Moderate edema (raised approximately 1 mm)
- 4 Severe edema (raised more than 1 mm and extending beyond area of exposure)

- 4 Maximum possible edema score
- 8 Maximum total possible primary irritation score

DESCRIPTIVE RATINGS**Mean Primary Dermal Irritation Index****Range of Values****Descriptive Rating**

0	Nonirritating
0.1 – 2.0	Slightly irritating
2.1 – 5.0	Moderately irritating
5.1 – 8.0	Severe irritating

Table 3. Critical computerized systems

System Name	Version No.	Description of Data Collected and/or Analyzed
Provantis	8 and/or 10	In-life and postmortem data
Systems 600 Apogee Insight System	3.13	Temperature and/or humidity (animal rooms, refrigerators, freezers, and compound storage, as applicable)
Instem Life Science Systems, DISPENSE	8 and/or 10	Test material receipt, accountability, and/or formulation activities

3.6 Statistical analysis

Erythema scores were subjected to one-way ANOVA to test the differences in irritation between oil treated sites and control sites. This test was also run to assess if exposure to used/laboratory

stressed versions of engine oils yields enhanced dermal irritation compared to exposure to new oils. Levine's test was used to check the homoscedasticity of the data, and the Welch test was conducted if the data displayed unequal variance (Levine test, $p \leq 0.05$). Results are expressed as mean $\pm$ S.E.M and considered statistically significant at $p \leq 0.05$.

Primary Dermal Irritation Indices (PDII) were calculated from erythema and edema scores recorded at 0.5-1, 24, 48, and 72 hours post treatment (after patch removal). The total scores for erythema and edema were calculated separately, divided by the number of rabbits (3) x time points (4), rounded to the nearest tenth, and added together. Based on these values, the grading system in [Table 2](#) was used to arrive at a primary dermal irritation index for each test article separately for the occluded and semi-occluded methods of exposure. PDII data for oil treated and control sites were used to calculate the effect size (Cohen's d) that indicated the magnitude of difference in irritability of oil *versus* control (water). We also used PDII data obtained from sites treated with unused (new) oils and those exposed to used/laboratory stressed oils to calculate the effect sizes that assessed the magnitude of difference in irritability of new oil *versus* its used/laboratory stressed version. The general equation used for computing effect size is shown below, where $(M)_T$ and $(M)_R$ are the average PDII values in the treatment (used or stressed) and reference (new) groups, respectively, while $(\sigma)_T$ and $(\sigma)_R$ are the standard deviations for PDII values in the treatment and reference groups, respectively.

$$d = \frac{(M)_T - (M)_R}{\sqrt{\frac{(\sigma)_T^2 + (\sigma)_R^2}{2}}} \quad (1)$$

Effect size values were graded as, small ($d = 0.2$), medium ($d = 0.5$) and large ($d = 0.8$) based on Cohen's effect size (d) classification (Cohen, 1988). Cohen's d values were then used to construct graphs shown in the results section.

4. RESULTS

4.1 Mortality and clinical observations

All animals survived until scheduled euthanasia. Clinical observations were limited to red fur staining and scabs. These findings are normal for animals of this age and strain.

4.2 Body weights

There were no apparent treatment-related effects for body weights during the study.

4.3 Laboratory aged aircraft engine oils

Grade 5 HTS and Experimental Grade 5 oils are oils that may be used in United States Air Force (USAF) aircraft in the future. Their used versions were not available at the time of this study. To obtain aged versions that reflect the properties of used oil, these oils were laboratory aged (stressed) as described in the methods section.

The results for kinematic viscosity (ASTM D445) and total acid number (TAN, SAE ARP5088) for these oils in their new states and their laboratory aged (stressed) versions are shown in [Table 4](#).

Table 4. Kinematic viscosity and total acid number (TAN) for Grade 5 HTS and Experimental Grade 5 oils in new states and their laboratory stressed (aged) versions.

Oils	Oil status					
	New oil		Laboratory aged (stressed) oil			
	Viscosity at 40 °C	TAN (mg KOH/g)	Viscosity at 40 °C	% Viscosity change	TAN (mg KOH/g)	TAN change
Grade 5 HTS	26.15	0.26	26.69	2.07	0.43	0.17
Experimental Grade 5	26.82	0.02	27.49	2.50	0.41	0.39

4.4 Dermal irritation scores

4.4.1 Individual erythema and edema scores and the recovery process

4.4.1.1 Control treatment

Individual erythema and edema scores under semi-occlusive and occlusive wrapping conditions for all oils (unused and used/laboratory stressed versions) are shown in [Tables 5 and 6](#), respectively. Under semi-occlusive wrapping conditions, exposure to the control article produced very slight erythema in 4 out of 6 rabbits by 1 hour (D0) post treatment ([Table 5](#)), while under occlusive wrapping conditions, only 2 out of 6 animals showed very slight redness ([Table 6](#)). The dermal irritation was completely resolved in these animals by day D1 post exposure scoring interval except two animals (animal numbers 1004 and 1007) that displayed irritation again at D3. All irritation was resolved completely by D7.

4.4.1.2 Grade 4

All 3 animals exposed to Grade 4 (G4) oil in a new state (unused; G4-N) displayed slight redness by 1 hour post exposure under semi-occlusive wrapping conditions (**Table 5, D0**) while those exposed to the same oil under occlusive wrapping conditions at D0 were not affected (**Table 6**). The skin redness for all animals subjected to semi-occlusive wrappings conditions was resolved by D10. However, under occlusive wrapping conditions, all 3 animals displayed a delayed response to this oil by D2. The slight erythema was resolved completely by D7.

Exposure to the used version (G4-U) of Grade 4 oil produced very slight erythema in all 3 rabbits by 1 hour post treatment under both semi-occlusive and occlusive wrapping conditions (**Tables 5 and 6**). The dermal irritation associated with this version of G4 oil was resolved completely by D7 in all 3 animals subjected to semi-occlusive wrapping conditions (**Table 5**). For those that had test sites occluded, one rabbit had irritation resolved by D1, one by D7 and for the remaining rabbit, skin redness disappeared by D14 (**Table 6**).

Under semi-occlusive wrapping conditions, two out three rabbits exposed to G4-U and G4-N oils were characterized by brown skin staining by the beginning of D7 and the issue was still present on 1 animal on D14. However, under occlusive wrapping conditions, brown skin staining was observed by the beginning of D7 on all 3 animals only treated with G4-N oil and the staining was still visible on 1 animal on D14.

A very slight edema was only observed on 1 animal at D2 post exposure to G4-U oil under occlusive wrapping conditions (**Table 6**).

Table 5. Individual erythema and edema scores under semi-occlusive wrapping conditions. All animals scored zero for edema except the animal shown by asterisk with the score equal to 1 at Day 0 (D0).

Group #	Animal #	Test Material	Material Status	Labels	Test Site	D0	D1	D2	D3	D7	D10	D14
1	1001	Water (Control)	-	Control	1	0	0	0	0	0	0	-
	1001	Grade 4	Used	G4-U	2	1	1	1	1	0	0	-
	1001	Grade 4	Unused/New	G4-N	3	1	1	1	1	0	0	-
	1001	Grade 3	Used	G3-U	4	0	1	1	1	1	0	-
	1001	Grade 3	Unused/New	G3-N	5	1	1	1	1	1	0	-
	1002	Water (Control)	-	Control	1	1	0	0	0	0	0	0
	1002	Grade 4	Used	G4-U	2	1	1	1	1	0	0	0
	1002	Grade 4	Unused/New	G4-N	3	1	1	1	1	1	0	0
	1002	Grade 3	Used	G3-U	4	1	1	1	1	1	0	0
	1002	Grade 3	Unused/New	G3-N	5	1	0	0	1	0	0	0
	1003	Water (Control)	-	Control	1	1	0	0	0	0	0	-
	1003	Grade 4	Used	G4-U	2	1	1	1	1	0	-	-
	1003	Grade 4	Unused/New	G4-N	3	1	0	0	1	0	0	-
	1003	Grade 3	Used	G3-U	4	1	1	1	1	1	0	-
	1003	Grade 3	Unused/New	G3-N	5	1	1	1	2	1	0	-
2	1004	Water (Control)	-	Control	2	1	0	0	1	0	-	-
	1004	Grade 5 HTS	Stressed/Used	G5-U	3	0	1	1	1	0	-	-
	1004	Grade 5 HTS	Unstressed/New	G5-N	4	0	1	1	1	0	-	-
	1004	Experimental Grade 5	Stressed/Used	EG5-U	5	0	1	1	1	0	-	-
	1004	Experimental Grade 5	Unstressed/New	EG5-N	1	0	1	1	2	0	-	-
	1005	Water (Control)	-	Control	2	1	0	0	0	0	0	0
	1005	Grade 5 HTS	Stressed/Used	G5-U	3	1	0	0	1	0	0	0
	1005	Grade 5 HTS	Unstressed/New	G5-N	4	0	1	1	1	1	1	1
	1005*	Experimental Grade 5	Stressed/Used	EG5-U	5	1*	1	1	1	1	1	1
	1005	Experimental Grade 5	Unstressed/New	EG5-N	1	0	0	0	0	0	0	0
	1006	Water (Control)	-	Control	2	0	0	0	0	0	0	-
	1006	Grade 5 HTS	Stressed/Used	G5-U	3	1	1	1	2	0	0	-
	1006	Grade 5 HTS	Unstressed/New	G5-N	4	1	1	1	2	1	0	-
	1006	Experimental Grade 5	Stressed/Used	EG5-U	5	1	1	1	2	1	0	-
	1006	Experimental Grade 5	Unstressed/New	EG5-N	1	0	1	1	1	0	0	-

D: day; Asterisk indicates that the score for edema was also 1; -: Severity not recorded

Table 6. Individual erythema and edema scores under occlusive wrapping conditions. All animals scored zero for edema except the animal shown by asterisk with the score equal to 1 at Day 2 (D2).

Group #	Animal #	Test Material	Material Status	Labels	Test Site	D0	D1	D2	D3	D7	D10	D14
3	1007	Water (Control)	-	Control	3	1	0	0	1	0	0	0
	1007*	Grade 4	Used	G4-U	4	1	1	1*	1	1	1	0
	1007	Grade 4	Unused/New	G4-N	5	0	1	1	1	0	0	0
	1007	Grade 3	Used	G3-U	1	1	1	1	0	0	0	0
	1007	Grade 3	Unused/New	G3-N	2	1	1	1	0	0	0	0
	1008	Water (Control)	-	Control	3	1	0	0	0	0	0	-
	1008	Grade 4	Used	G4-U	4	1	0	1	1	0	0	-
	1008	Grade 4	Unused/New	G4-N	5	0	0	1	1	0	0	-
	1008	Grade 3	Used	G3-U	1	1	1	1	0	1	0	-
	1008	Grade 3	Unused/New	G3-N	2	0	0	0	1	0	0	-
	1009	Water (Control)	-	Control	3	0	0	0	0	0	-	-
	1009	Grade 4	Used	G4-U	4	1	0	0	0	0	-	-
	1009	Grade 4	Unused/New	G4-N	5	0	1	1	1	0	-	-
	1009	Grade 3	Used	G3-U	1	0	1	0	1	0	-	-
	1009	Grade 3	Unused/New	G3-N	2	0	0	0	1	0	-	-
4	1010	Water (Control)	-	Control	4	0	0	0	0	0	-	-
	1010	Grade 5 HTS	Stressed/Used	G5-U	5	0	0	0	1	0	-	-
	1010	Grade 5 HTS	Unstressed/New	G5-N	1	0	0	0	0	0	-	-
	1010	Experimental Grade 5	Stressed/Used	EG5-U	2	0	1	1	1	0	-	-
	1010	Experimental Grade 5	Unstressed/New	EG5-N	3	1	1	1	1	0	-	-
	1011	Water Control	-	Control	4	0	0	0	0	0	0	-
	1011	Grade 5 HTS	Stressed/Used	G5-U	5	1	1	1	1	0	0	-
	1011	Grade 5 HTS	Unstressed/New	G5-N	1	0	1	1	1	1	0	-
	1011	Experimental Grade 5	Stressed/Used	EG5-U	2	0	1	1	1	0	0	-
	1011	Experimental Grade 5	Unstressed/New	EG5-N	3	1	1	1	1	0	0	-
	1012	Water (Control)	-	Control	4	0	0	0	0	0	-	-
	1012	Grade 5 HTS	Stressed/Used	G5-U	5	1	0	0	1	0	-	-
	1012	Grade 5 HTS	Unstressed/New	G5-N	1	0	1	1	1	0	-	-
	1012	Experimental Grade 5	Stressed/Used	EG5-U	2	1	1	1	1	0	-	-
	1012	Experimental Grade 5	Unstressed/New	EG5-N	3	0	1	1	1	0	-	-

D: day; Asterisk indicates that the score for edema was also 1; -: Severity not recorded

4.4.1.3 Grade 3

Exposure to Grade 3 (G3) oil in a new state (G3-N) and under semi-occlusive wrapping conditions produced very slight erythema in all 3 rabbits by 1 hour post treatment ([Table 5](#)). This irritation was resolved by D7 in only 1 rabbit and D10 in the remaining two rabbits. Under occlusive wrapping conditions, this oil induced a very slight skin redness in 1 animal by 1 hour post treatment, but was resolved by D2 ([Table 6](#)). However, there was a delayed erythema that appeared D3 on the two animals that did not initially show irritation at 1 hour post exposure but this issue was resolved by D7.

The used version of this oil (G3-U) produced very slight erythema in 2 out of 3 animals by 1 hour post exposure under both semi-occlusive and occlusive wrapping conditions ([Tables 5 and 6](#)). By D1, all of the 3 animals scored very light erythema under semi-occlusive conditions; this persisted through D7 but was resolved by D10 ([Table 5](#)). Similarly, all 3 animals displayed a very slight skin redness by day 1 under occlusive wrapping conditions but one resolved by D3, one by D7 and one by D10 ([Table 6](#)).

Brown skin staining was also noted in 2 out of 3 animals exposed to G3-U oil under semi-occlusive wrapping conditions, beginning D7 while only 1 animal from those subjected to occlusive wrapping conditions displayed staining on this day. However, brown skin staining was still present in 1 animal on D14 under both wrapping conditions.

No animal displayed a sign of edema after exposure to G3-N and G3-U oils under both semi-occlusive and occlusive wrapping conditions.

4.4.1.4 Grade 5 HTS

Although 1 out of 3 rabbits exposed to a new version (G5-N) of Grade 5 HTS (G5) oil produced very slight erythema by 1 hour post treatment under semi-occlusive wrapping conditions, all the animals displayed skin redness from D1 through D3 (**Table 5**). Erythema for one animal increased from very slight to slight erythema at D3 but was resolved by D10. Erythema was resolved by D7 in one animal but in the third animal irritation persisted till the end of the study (D14). Under occlusive wrapping conditions, no rabbit showed irritation at 1 hour post exposure (**Table 6**). However, by D1, two rabbits produced very slight erythema and this was resolved completely by D7 in one and D10 in the other. One rabbit never showed irritation.

Exposure to the laboratory stressed (aged) version (G5-U) of G5 oil produced very slight erythema in 2 out of 3 rabbits by 1 hour post treatment under both semi-occlusive and occlusive wrapping conditions (**Tables 5 and 6**). However, by D3 all six rabbits showed very slight erythema. In one animal, irritation increased from very slight to slight erythema under semi-occlusive wrapping conditions on D3. Regardless of the pattern of erythema in all six animals, it was resolved completely in all the rabbits by D7 (**Table 5**).

Exposure to G5-N oil under both semi-occlusive and occlusive wrapping conditions was characterized by brown skin staining that was noted in 1 out of 3 animals on D7. However, this issue persisted through D14 in 1 rabbit among those that were subjected to semi-occluded wrappings.

No animal displayed a sign of edema after exposure to G5-N or G5-U oil under both semi-occlusive and occlusive wrapping conditions.

4.4.1.5 Experimental Grade 5

Although exposure to new (not stressed; EG5-N) Experimental Grade 5 (EG5) oil under semi-occlusive wrapping conditions did not produce erythema by 1 hour post treatment, two rabbits displayed skin irritation from D1 through D3 and this was resolved completely by D7 (**Table 5**). Irritation increased from very slight to slight erythema for one of these rabbits on D3. The third rabbit never showed irritation with semi-occlusive wrappings. Under occlusive wrapping conditions, 2 out 3 rabbits produced very slight erythema by 1 hour post exposure (**Table 6**). By day 1, all three rabbits displayed skin redness through D3, but this was resolved completely by D7.

The laboratory stressed version (aged; EG5-U) of EG5 oil induced very slight erythema in 2 out of 3 animals by 1 hour post exposure under semi-occluded wrapping conditions (**Table 5**). By D3, all the animals displayed dermal irritation and this was resolved by D7 in one and D 10 in

another. In one animal, the very slight erythema persisted till the end of the study. Under occluded wrapping conditions, 1 animal produced very slight erythema by 1 hour post exposure (Table 6). On days 1 through 3, all animals displayed skin redness but it was resolved completely by D7.

Brown skin staining was also noted on one rabbit among those exposed to EG5-U oil under semi-occlusive wrapping conditions from D7 and was still visible at the end of the study.

Only one case of very slight edema was noted at 1 hour post treatment for one rabbit exposed to EG5-U oil under semi-occlusive wrapping conditions.

4.4.2 Averaged erythema scores and primary dermal irritation indices

4.4.2.1 Averaged erythema scores

The averaged erythema scores for treatment groups indicates that irritation scores for new oils and their used/laboratory stressed versions were not statistically different under both semi-occlusive and occlusive wrapping conditions (Fig. 3A and 3B). However, erythema scores for all the oils under semi-occlusive wrapping conditions were significantly higher ($p < 0.05$) than those obtained for rabbits treated with control substance (water) except scores for rabbits exposed to EG5-N (Fig. 3A). Under occlusive wrapping conditions, only erythema scores for both new and used/laboratory stressed versions of G4, EG5 and the used version of G3 were significantly higher than the scores obtained for the control group.

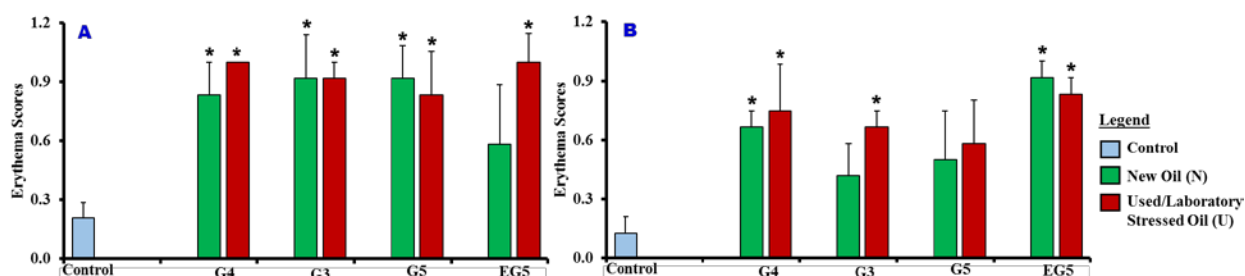


Figure 3. Rabbit erythema scores induced by a 4 hour dermal exposure to new (unused) aircraft engine oils and their used/laboratory stressed versions under (A) semi-occlusive and (B) occlusive wrapping conditions (see methods for details). Asterisk denotes significant differences from control group; $p < 0.05$.

4.4.2.2 Primary Dermal Irritation Indices

Exposure to both used/laboratory stressed and new aircraft engine oils produced dermal irritation consisting of no more than very slight to slight erythema and very slight edema. Calculated Primary Dermal Irritation Index (PDII) indicates that all the oils (new and used/laboratory stressed) were slightly irritating under both semi-occlusive and occlusive wrapping conditions (Table 7; Fig. 4A and 4B). Although the PDII values for new oils and used/laboratory stressed versions were not significantly different, they were all statistically higher ($p < 0.05$) than those obtained for the control under semi-occlusive wrapping conditions, except PDII value for EG5-N (Fig. 4A). Under occlusive wrapping conditions, the PDII values for all the oils were also

significantly higher than the value obtained for the control, except PDII values obtained for G3-N and G5-N (**Fig. 4B**).

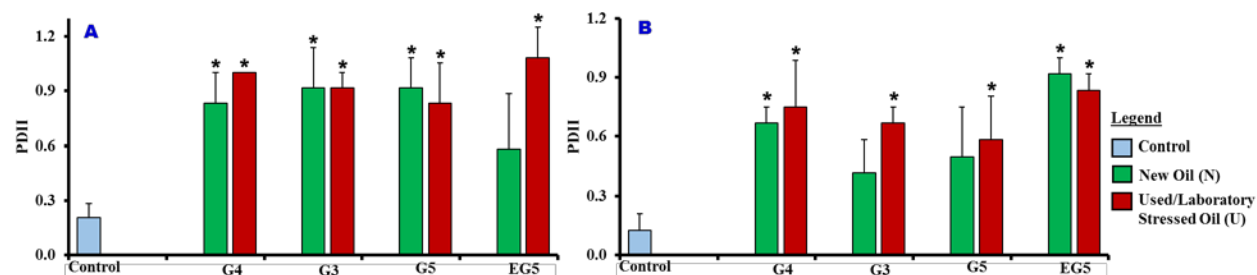


Figure 4. PDII calculated based on erythema and edema scores in rabbits induced by a four hour dermal exposure to new (unused) aircraft engine oils and their used/laboratory stressed versions under (A) semi-occlusive and (B) occlusive wrapping conditions (see methods for details). Asterisk denotes significant differences from control group; $p < 0.05$.

Table 7. Calculated Primary Dermal Irritation Indices (PDII) for test articles.

Group No.	Exposure Method	Test Material	Test Material Status	Labels	PDII values	Irritation Rating
1	Semi-occluded	Water Control	-	Control	0.21	Slight Irritant
		Grade 4	Used	G4-U	1.00	Slight Irritant
		Grade 4	Unused/New	G4-N	0.83	Slight Irritant
		Grade 3	Used	G3-U	0.92	Slight Irritant
		Grade 3	Unused/New	G3-N	0.92	Slight Irritant
2	Semi-occluded	Water Control	-	Control	0.21	Slight Irritant
		Grade 5 HTS	Laboratory stressed	G5-U	0.83	Slight Irritant
		Grade 5 HTS	Unstressed/New	G5-N	0.92	Slight Irritant
		Experimental Grade 5	Laboratory stressed	EG5-U	1.08	Slight Irritant
		Experimental Grade 5	Unstressed/New	EG5-N	0.58	Slight Irritant
3	Occluded	Water Control	-	Control	0.13	Slight Irritant
		Grade 4	Used	G4-U	0.75	Slight Irritant
		Grade 4	Unused/New	G4-N	0.67	Slight Irritant
		Grade 3	Used	G3-U	0.67	Slight Irritant
		Grade 3	Unused/New	G3-N	0.42	Slight Irritant
4	Occluded	Water Control	-	Control	0.13	Slight Irritant
		Grade 5 HTS	Laboratory stressed	G5-U	0.58	Slight Irritant
		Grade 5 HTS	Unstressed/New	G5-N	0.50	Slight Irritant
		Experimental Grade 5	Laboratory stressed	EG5-U	0.83	Slight Irritant
		Experimental Grade 5	Unstressed/New	EG5-N	0.92	Slight Irritant

4.4.3 Magnitude of skin irritation induced by exposure to aircraft engine oils

To more clearly illustrate the magnitude difference in irritability between exposure to engine oils and the control, we calculated the size effect (Cohen's d) by subtracting the averaged PDII value obtained for the control group from that obtained for the engine oil treated group and the difference was assessed relative to the pooled standard deviations of the treated group and its corresponding control group (Equation 2), where $(X)_T$ and $(X)_C$ are the average PDII values in the treatment and control groups, respectively, while $(\sigma)_T$ and $(\sigma)_C$ are the standard deviations for PDII values in the treated group and its corresponding control group, respectively.

$$d = \frac{(X)_T - (X)_C}{\sqrt{\frac{(\sigma)_T^2 + (\sigma)_C^2}{2}}} \quad (2)$$

We then constructed a graph using the Cohen's d values as shown in [Fig. 5](#).

Under both semi-occlusive and occlusive wrapping conditions, all the oils yielded large effect sizes ($d = 0.8$) based on Cohen's classification (Cohen, 1988), suggesting that the effect associated with exposure to these oils is not negligible regardless the state of oil (new or used/laboratory stressed). A comparison of the magnitude of difference between dermal irritation for rabbits exposed to the oils and those treated with the control (RO) indicates that semi-occlusive wrapping conditions produced elevated effect sizes higher than those obtained under occlusive conditions, except for both versions of EG5 oil ([Fig. 5 A and 5B](#)).

Under semi-occlusive conditions, all used/laboratory stressed versions were associated with elevated effect sizes as compared to the performance of the new oils except for G5 (**Fig. 5 A**), suggesting that these oils increase their toxicity as they age. The G4-U produced the highest effect size ($d = 5.9$ versus 2.6 for the new oil) while the smallest effect size was obtained with rabbits treated with the EG5-N ($d = 0.96$) (**Fig. 5 A**).

Under occlusive wrapping conditions, both versions (new and laboratory stressed/aged) of EG5 yielded the highest effect size ($d = 4.4$ and 3.9) relative to those obtained with the rest of oils (**Fig. 5B**). Only effect sizes for G3-U and G5-U were elevated relative to those obtained for their unused/unstressed versions (G3-N and G5-N).

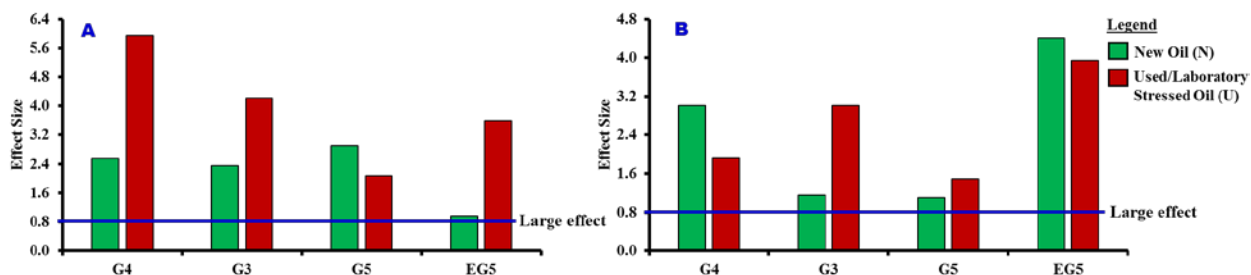


Figure 5. The magnitude difference in dermal irritability (effect size also known as Cohen's d) between rabbits exposed to aircraft engine oils (unused oils shown in green bars and their used/laboratory stressed versions shown in red bars) and controls for 4 hours under (A) semi-occlusive and (B) occlusive wrapping conditions (see methods for details).

To more clearly illustrate the magnitude difference in irritability between exposure to used/laboratory stressed engine oils and their unused versions, we calculated the size effect (Cohen's d) by subtracting the averaged PDII value obtained for the unused engine oil group from that obtained for the group treated with its used version. The difference was assessed relative to the pooled standard deviations for the group exposed to the unused oil and the group treated with its used version (Equation 3), where $(A)_N$ and $(A)_U$ are the average PDII values for the new and used oil treated groups, respectively, while $(\sigma)_N$ and $(\sigma)_U$ are the standard deviations for PDII values obtained for the new and used oil treated groups, respectively.

$$d = \frac{(A)_U - (A)_N}{\sqrt{\frac{(\sigma)_U^2 + (\sigma)_N^2}{2}}} \quad (3)$$

We then constructed a graph using the Cohen's d values as shown in **Fig. 6**.

A comparison of the magnitude of difference between dermal irritation for rabbits exposed to used/laboratory stressed versions of engine oils and those treated with the new versions of these oils under both semi-occlusive and occlusive wrapping conditions indicates that the type of wrapping applied on the test sites had an effect for the strength of skin irritation. Comparing the magnitude of irritation difference between exposure to new oils and their used/laboratory stressed versions indicates that semi-occlusive and occlusive wrapping conditions yielded opposite effects on the strength of skin irritation associated with exposure to these oils (**Fig. 6A and 6B**). The magnitude of difference between irritation for G4-U 4 and its unused version G4-N was elevated ($d = 0.82$) under semi-occlusive wrapping conditions (**Fig. 6A**) while this

difference was decreased ($d = 0.27$) under occlusive wrapping conditions (Fig. 6B). Although G4-U was more irritating than G4-N under both wrapping conditions, semi-occlusive wrapping conditions tended to enhance irritability as the oil aged. Similarly, the magnitude of difference between irritation for EG5-U and EG5-N was elevated ($d = 1.19$) under semi-occlusive wrapping conditions (Fig. 6A) whereas this difference decreased ($d = -0.58$) under occlusive wrapping conditions (Fig. 6B). Applying semi-occlusive wrappings on test sites for EG5-U oil enhanced irritability of this oil as compared to EG5-N. Interestingly, applying occlusive wrappings on the test sites for this oil lessened the irritability of EG5-U as compared to the performance of EG5-N oil. Taken together, these observations suggest that occlusive wrapping conditions lessened the skin irritation potential for EG5-U oil relative to the performance of this oil in its original state (EG5-N). G4-U and EG5-U oils are more irritating than their original versions (G4-N and EG5-N) when the test sites are subjected to semi- occlusive wrapping conditions.

It was also interesting to note that exposure to G3 and G5 yielded opposite effects to those observed with exposure to G4 and EG5 oils. Our data indicate that there was no irritation difference ($d = 0$) between exposure to G3-U and G3-N under semi-occlusive wrapping conditions (Fig. 6A). Conversely, this difference was elevated ($d = 1.10$) when occlusive wrappings were applied to test sites (Fig. 6B), suggesting that occlusive wrappings enhanced irritability of the used version of this oil relative to its unused version. Likewise, G5-U oil was less irritating ($d = -0.25$) as compared to G5-N under semi-occlusive wrapping conditions (Fig. 6A) but became more irritating ($d = 0.20$) under occlusive wrapping conditions (Fig. 6B), suggesting that occlusive wrapping conditions enhanced the irritation potential of G5-U oil. Taken together, these observations suggest that occlusive wrappings of the test sites enhanced the skin irritation of both G3-U and G5-U oils relative to the performance of these oils in their original states (G3-N and G5-N).

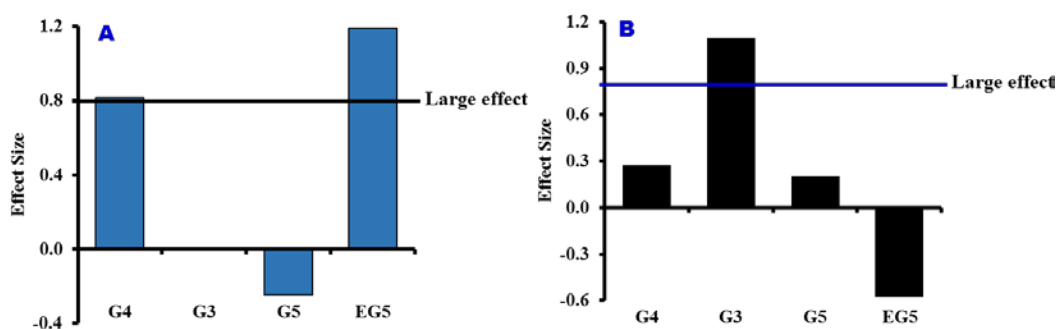


Figure 6. The magnitude difference in dermal irritability (effect size also known as Cohen's d) between rabbits exposed to new (unused) aircraft engine oils and their used/laboratory stressed versions for 4 hours under (A) semi-occlusive and (B) occlusive wrapping conditions (see methods for details).

5. DISCUSSION

The current study was designed to achieve two different goals. First, the safety data sheet (SDS) of each aircraft engine oil lists ingredients of oil and the potential toxicity associated with each ingredient. However, the SDS does not show the toxicity associated with exposure to the mixture. Since the overall toxicity of a particular mixture depends on the proportion and toxicity

of each ingredient as well as the synergistic interactions between ingredients, an ideal evaluation of the hazardous effects of exposure to the compound mixture requires a toxicity test of the entire mixture not solely for each component. Thus, the first goal for this study was to assess the dermal irritation potential for aircraft engine oils, each oil considered as a mixture of ingredients.

Second, there is no available data to indicate that the level of toxicity associated with exposure to aircraft engine oils is not related to their age. In other words, there is no data to indicate that the aging process of oils (due to their usage in running engines) has no effect on their potential toxicity. While engine oils are known to contain toxic ingredients at a very low level, little is currently known about oil transformations (due to breakdown of ingredients and/or worn engine components) occurring during engine operation. Wear products of engine components may end up in oils. This could potentially change the oil properties, yielding a more toxic oil mixture as compared to the new oil. Thus, the second goal for this study was to determine the dermal irritation potential of used/laboratory stressed (aged) oils relative to their unused/unstressed versions. Four aircraft engine oils, a MIL-PRF-7808 Grade 4, a MIL-PRF-7808 Grade 3, a MIL-PRF-23699 HTS and an experimental MIL-PRF-23699 type oil, each regarded as a mixture of ingredients were studied. Testing was conducted through dermal exposure since the skin is a major route of exposure. The treatment sites were covered by semi-occluded or occluded wrappings mimicking what may happen in the real world environment when the oil gets trapped under the aircraft maintenance worker's clothes.

All animals were healthy and survived until scheduled euthanasia. Clinical observations were limited to red fur staining and scabs. The findings were normal for animals considering their age and strain. No apparent treatment-related effects on body weights were observed during the study.

The results reported in this study highlight three main observations: (1) irritation in control test sites for some rabbits exposed to RODI water (control); (2) exposure to same oil yielded different responses under semi-occlusive and occlusive wrapping conditions. In general, semi-occlusive wrapping conditions tended to produce higher erythema scores and PDII values relative to those obtained under occlusive wrapping conditions; and (3) exposure to used/laboratory stressed oils enhanced or decreased skin irritation relative to the performance of their unused/unstressed versions depending on the type of dressing applied to test sites.

Very slight erythema was noted at the early post-exposure observations on control test sites in 4 out of 6 rabbits subjected to semi-occlusive dressing conditions. Applying occlusive dressing was less likely to produce irritation as this was observed for only 2 out of 6 rabbits. The control-induced irritation was rapidly and completely resolved for all affected rabbits. Exposure to both used/laboratory stressed and new oils under either semi-occlusive or occlusive wrapping conditions generally produced very slight dermal irritation. Cases of a well-defined erythema were only observed with three animals at Day 3 post treatment and irritation was resolved or degraded to very slight erythema by D7. Two cases of a very slight edema were observed only with G4-U (at D2 post exposure) and EG5-U oils (at 1 h post treatment). Both cases were resolved completely by the following 24 hours. No edema case was observed with test sites exposed to control test substance. The disparity in scores obtained for control test sites under semi-occluded *versus* occluded dressing conditions suggests that dressings might have also

introduced some variations in data obtained for the test sites exposed to oils. The use of acetone to clean the test sites might have also contributed to data variations.

All oils produced erythema at various observation time points under both semi-occlusive and occlusive wrapping conditions. Averaged erythema scores obtained for the test sites put all oils (unused and used/laboratory stressed) and controls under the same category of very slight irritation based on irritation classification by Draize (1959). Since only two cases of edema were observed with oil exposure, calculated PDII values depended mainly on erythema scores. This resulted in PDII score for each oil being almost similar to averaged erythema score. Comparing the irritation strength under both semi-occlusive and occlusive wrapping conditions, all oils yielded higher PDII values under semi-occlusive wrapping conditions except the EG5-N oil. Our data suggest that applying occlusive wrappings on test sites treated with this oil enhanced skin irritation. Semi-occlusive conditions strengthened toxicity of the rest of oils, regardless of their aging states. Under semi-occlusive wrapping conditions, EG5-N yielded the lowest PDII score (0.58) while its laboratory stressed version (EG5-U) produced the highest score (1.08). These observations suggest that stressing this oil enhanced its dermal irritation potential. Under occlusive dressing conditions, G3-N oil yielded the least PDII value (0.42) while both versions of EG5 (unstressed and stressed) produced the highest PDII values (0.92 and 0.83, respectively). Considering that irritation index for EG5-N was at the lowest level (PDII = 0.58) relative to PDII level (1.08) obtained with EG5-U when semi-occlusive dressings were applied on test sites, we could speculate that occlusive dressing conditions can potentially increase the toxicity of this oil.

Our data clearly demonstrate that exposure to aircraft engine oils can significantly induce dermal irritation regardless of the oil's age status (unused, used and laboratory stressed) as illustrated in **Fig. 3 and 4**. In general, occlusive wrapping of test sites lessened dermal irritation for engine oils as compared to semi-occlusive dressings. Studies have suggested that occlusion disrupts skin barrier function by impairing passive transdermal water loss at the treatment site, thus aggravating effects associated with the applied treatment (Bucks *et al.*, 1991; Kligman, 1996; Berardesca and Maibach, 1988). There are also reports indicating that skin occlusion improves stratum corneum hydration, which can gradually decrease its barrier efficiency (Bucks *et al.*, 1991; Treffel *et al.*, 1992 and Bucks *et al.*, 1999). The widely accepted dogma is that occlusive dressing enhances percutaneous absorption (Berry, 1983; Schaefer *et al.*, 1982) and transdermal penetration for compounds (Bucks *et al.*, 1991; Treffel *et al.*, 1992 and Bucks *et al.*, 1999). This suggests that occlusive dressing conditions are more conducive to irritation than semi-occlusive conditions. However, our results contradict this dogma. As reports show, occlusion does not increase absorption of all compounds (Bucks *et al.*, 1988; Bucks *et al.*, 1991; Treffel *et al.*, 1992 and Bucks *et al.*, 1999) and the occlusion-induced hydration of skin enhances the penetration of non-polar compounds but has a minimal effect on polar molecules (Bucks *et al.*, 1988; Treffel *et al.*, 1992). Other factors such as the compound's physicochemical properties (aqueous solubility, volatility, partition coefficient, etc.), anatomy of the test site may also contribute to occlusion's effect on absorption (Bucks *et al.*, 1988; Bucks *et al.*, 1991; Treffel *et al.*, 1992; Hotchkiss *et al.* 1992; Leow and Maibach 1997). Although we did not assess the physicochemical properties of the oils used in this study, we cannot rule out that these properties may have contributed to differences we observed in irritation potentials of oils under semi-occlusive and occlusive wrapping conditions.

A comparison of the magnitude of difference (effect size) between dermal irritation for rabbits exposed to used/laboratory stressed versions of oils and those treated with the unused versions of these oils under both semi-occlusive and occlusive wrapping conditions indicated that the type of wrapping applied on the test sites has an effect on the strength of skin irritation. G4-U oil tended to be more irritating than G4-N under both wrapping conditions. Similar observations were noted for EG5-U oil subjected to semi-occlusive dressing conditions. Interestingly, applying occlusive wrappings on the test sites exposed to EG5-U oil lessened its irritability potential. Under occlusive dressing conditions, the treatment penetrates the stratum corneum upon skin exposure and after removing the dressing, the stratum corneum dehydrates, absorption of the compound slows resulting in stratum corneum serving as a reservoir for the compound (Wester and Maibach, 1983). This may have been the case for G3-U and G5-U since occlusive wrappings of the test sites that received these versions of oils enhanced irritation in comparison to test sites treated with the unused/unstressed versions. It is interesting to note that irritation of the test sites exposed to these oils in their new states was less pronounced. Bucks *et al.* (1988) have reported that occlusion enhances the absorption of more lipophilic steroids while it does not affect the most water-soluble steroids. The observations that G3-N and G5-N oils and their used/laboratory stressed versions (G3-U and G5-U) have different dermal irritation potentials clearly suggesting that oils go through changes in chemical properties as they age.

In summary, this study shows that a 4 hour dermal exposure to aircraft engine oils results in slight skin irritation. This raises concerns about the magnitude of impact related to prolonged exposure as the shifts for aircraft maintenance workers last more than 4 hours. It is also unknown what could be the magnitude of impact associated with repeated exposure that may be happening in the real world environment. Applying occlusive wrappings on test sites tended to provide conditions that lessen irritation levels as compared to semi-occlusive wrappings. In general, used oils tended to enhance the PDII relative to the performance of their unused versions, suggesting an increase in toxicity as the oil age.

6. CONCLUSION

The slight dermal irritation associated with four hours exposure to aircraft engine oils raises concerns about the magnitude of the impact of prolonged and/or repeated exposure. Our data show that used oils tended to be more irritating as compared to new versions, suggesting that as the oils age, they increase their potential toxicity. While personal protection equipment needs to always be used when handling the oils, more research is also needed to elucidate the health issues associated with repeated dermal exposure to both new and used versions, which reflects what happens in a real world environment where the maintenance workers may be repeatedly exposed to engine oils.

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LIST OF ACRONYMS

AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AFB	Air Force Base
AFRL	Air Force Research Laboratory
EPA	Environmental Protection Agency
EG5	Experimental Grade 5
EG5-N	Experimental Grade 5 New (unstressed/aged)
EG5-U	Experimental Grade 5 Used (laboratory stressed/aged)
G3	Grade 3
G3-N	Grade 3 New (unused)
G3-U	Grade 3 Used
G4	Grade 4
G4-N	Grade 4 New (unused)
G4-U	Grade 4 Used
G5	Grade 5 HTS
G5-N	Grade 5 HTS New (unstressed/aged)
G5-U	Grade 5 HTS Used (laboratory stressed/aged)
GLP	Good Laboratory Practices
HJF	Henry M. Jackson Foundation for the Advancement of Military Medicine
HTS	High Thermal Stability
IACUC	Installation Animal Care and Use Committee
NRC	National Research Council
NTE	Neuropathy Target Esterase
OECD	Organization for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides, and Toxic Substances
OROC	Office of Research Oversight & Compliance
PDII	Primary Dermal Irritation Index
RODI	Reverse Osmosis Deionized Water
SDS	Safety Data Sheet
TAN	Total Acid Number
TCP	Tri-Cresyl Phosphate
TOCP	Tri-Ortho-Cresyl Phosphate
USAF	U.S. Air Force

APPENDIX A: PROTOCOL

An Acute Skin Irritation Study of Aircraft Engine Oils by Dermal Administration in Rabbits

SPONSOR:

HJF
2728 Q Street, Bldg 837
WPAFB, OH 45433-5707
United States

TESTING FACILITY:

Charles River Laboratories, Inc.
640 N. Elizabeth Street
Spencerville, OH 45887
United States

OBJECTIVE(S)

The objective of this study is to assess the irritant effects of Aircraft Engine Oils in both their new (unused) and used states, when given as a single dermal administration to rabbits.

1. GUIDELINES FOR STUDY DESIGN

The design of this study was based on the study objective(s), the overall product development strategy for the test substance, and the following study design guidelines:

- EPA Health Effects Test Guideline OPPTS 870.2500: *Acute Dermal Irritation*.
- U.S. Consumer Products Safety Commission, Federal Hazardous Substances Act Regulations, Subchapter C, 16 CFR Part 1500.41.

2. REGULATORY COMPLIANCE

The study will be performed in accordance with the United States Code of Federal Regulations, Title 40, Parts 160 and 792: Good Laboratory Practice Standards and as accepted by Regulatory Authorities throughout the European Union (OECD Principles of Good Laboratory Practice), Japan (MAFF and METI), and other countries that are signatories to the OECD Mutual Acceptance of Data Agreement.

Exceptions to GLPs include the following study elements:

- Characterization of the test substance were performed by the Sponsor according to established SOPs, controls, and approved test methodologies to ensure integrity and validity of the results generated; these analyses were not conducted in compliance with the GLP or GMP regulations.
- Stability testing of the supplied test substance was performed by the Sponsor at a laboratory that follows FDA GMP regulations.
- Concentration, stability, and homogeneity of the test substance formulations will not be/were not determined in this study.

3. QUALITY ASSURANCE

3.1. TESTING FACILITY

The Testing Facility Quality Assurance Unit (QAU) will monitor the study to assure the facilities, equipment, personnel, methods, practices, records, and controls are in conformance with Good Laboratory Practice regulations. The QAU will review the protocol, conduct inspections at intervals adequate to assure the integrity of the study, and audit the Final Report to assure that it accurately describes the methods and standard operating procedures and that the reported results accurately reflect the raw data of the study.

4. TEST AND CONTROL SUBSTANCES

4.1. TEST SUBSTANCE 1

Identification: Grade 4 (Used)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Red liquid

Storage Conditions: Kept in a controlled room temperature area

Identification: Grade 4 (Unused)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Red liquid

Storage Conditions: Kept in a controlled room temperature area

4.2. TEST SUBSTANCE 2

Identification: Grade 3 (Used)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Red liquid

Storage Conditions: Kept in a controlled room temperature area

Identification: Grade 3 (Unused)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Colorless liquid

Storage Conditions: Kept in a controlled room temperature area

4.3. TEST SUBSTANCE 3

Identification: Grade 5 HTS (Used)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Brown liquid

Storage Conditions: Kept in a controlled room temperature area

Identification: Grade 5 HTS (Unused)

Batch (Lot) Number: To be included in the Final Report

Expiration Date: To be included in the Final Report

Physical Description: Brown liquid

Storage Conditions: Kept in a controlled room temperature area

4.4. TEST SUBSTANCE 4

Identification: Experimental Grade 5 (Used)

Batch (Lot) Number: To be included in the Final Report
Expiration Date: To be included in the Final Report
Physical Description: Brown liquid
Storage Conditions: Kept in a controlled room temperature area
Identification: Experimental Grade 5 (Unused)
Batch (Lot) Number: To be included in the Final Report
Expiration Date: To be included in the Final Report
Physical Description: Red liquid
Storage Conditions: Kept in a controlled room temperature area

4.5. CONTROL SUBSTANCE

Identification: Reverse Osmosis Deionized (RODI) Water
Physical Description: Liquid

4.6. TEST SUBSTANCE CHARACTERIZATION

The Sponsor will provide to the Testing Facility documentation of the identity, strength, purity, composition, and stability for the test substances. A Certificate of Analysis or equivalent documentation will be provided for inclusion in the Final Report. The Sponsor will also provide information concerning the regulatory standard that was followed for these evaluations.

The Sponsor has appropriate documentation on file concerning the method of synthesis, fabrication or derivation of the test substances, and this information is available to the appropriate regulatory agencies should it be requested.

4.7. ANALYSIS OF TEST SUBSTANCE

The stability of the bulk test substance will not be determined during the course of this study. Information to support the stability of each lot of the bulk test substance will be provided by the Sponsor.

4.8. TEST SUBSTANCE INVENTORY AND DISPOSITION

Records of the receipt, distribution, storage, and disposition of test substances (including empty containers) will be maintained. All unused Sponsor-supplied bulk test substances will be returned to the Sponsor (after issue of the Final Reports of all studies using these materials, unless otherwise instructed by the Sponsor). All empty containers will be maintained for the duration of the study.

5. SAFETY

The following safety instructions apply to this study:

Standard laboratory safety procedures will be employed for handling the test and control substance(s). Specifically, laboratory gloves, laboratory coat, and eye protection will be worn. Safety information on the test substance will be provided by the Sponsor in the form of a Material Safety Data Sheet or equivalent, if available.

6. DOSE FORMULATION AND ANALYSIS

6.1. PREPARATION OF CONTROL SUBSTANCE

The control substance, RODI Water, will be dispensed on the day of dosing.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.2. PREPARATION OF TEST SUBSTANCE

The test substances will be administered as received.

Any residual volumes will be discarded unless otherwise requested by the Study Director.

6.3. SAMPLE COLLECTION AND ANALYSIS

The test substances will be used as received from the Sponsor; therefore, samples for dose formulation analysis will not be collected by the Testing Facility.

7. TEST SYSTEM

Species:	Rabbit
Strain:	New Zealand White rabbit
Source:	Covance Laboratories
Number of Males Ordered:	14
Target Age at the Initiation of Dosing:	13 to 24 weeks
Target Weight at the Initiation of Dosing:	3.0 to 4.5 kg

The actual age and weight of animals received will be listed in the Final Report.

7.1. JUSTIFICATION OF TEST SYSTEM AND NUMBER OF ANIMALS

The New Zealand White rabbit was chosen as the animal model for this study as it is an accepted nonrodent species for preclinical toxicity testing by regulatory agencies.

The total number of animals to be used in this study is considered to be the minimum required to properly characterize the effects of the test substance and has been designed such that it does not require an unnecessary number of animals to accomplish its objectives.

At this time, studies in laboratory animals provide the best available basis for extrapolation to humans and are required to support regulatory submissions. Acceptable models which do not use live animals currently do not exist.

7.2. ANIMAL IDENTIFICATION

Each animal will be identified using a subcutaneously implanted electronic identification chip.

7.3. ENVIRONMENTAL ACCLIMATION

The animals will be acclimated to their designated housing for at least 5 days before the first day of dosing.

7.4. SELECTION, ASSIGNMENT, REPLACEMENT, AND DISPOSITION OF ANIMALS

The animals chosen for study will be arbitrarily selected from healthy stock animals. Animals in poor health will not be assigned to groups.

The disposition of all animals will be documented in the study records.

8. HUSBANDRY

8.1. HOUSING

Animals will be single housed in stainless steel cages equipped with an automatic watering valve as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2 and 3) and as described in the *Guide for the Care and Use of Laboratory Animals*.¹ These housing conditions will be maintained unless deemed inappropriate by the Study Director and/or Clinical Veterinarian. The room(s) in which the animals will be kept will be documented in the study records.

Each cage will be clearly labeled with a color-coded cage card indicating study, group, animal number, and sex.

8.2. ENVIRONMENTAL CONDITIONS

The targeted conditions for animal room environment will be as follows:

Temperature:	61°F to 72°F (16°C to 22°C)
Humidity:	30% to 70%
Light Cycle:	12 hours light and 12 hours dark (except during designated procedures)
Ventilation:	10 or more air changes per hour

8.3. FOOD

PMI Nutrition International Certified Rabbit Chow No. 5322 will be provided ad libitum throughout the study, except during designated procedures. To avoid potential gastrointestinal disturbances, food will be withheld for 24 hours after receipt. Food will then be gradually increased over a 3-day period.

Supplemental diet may be provided to the animals as warranted by clinical signs or other changes. Any food supplementation will be approved by the Study Director and Clinical Veterinarian and documented accordingly.

The feed is analyzed by the supplier for nutritional components and environmental contaminants. Results of the analysis are provided by the supplier and are on file at the Testing Facility.

It is considered that there are no known contaminants in the feed that would interfere with the objectives of the study.

8.4. WATER

Municipal tap water after treatment by reverse osmosis and ultraviolet irradiation will be freely available to each animal via an automatic watering system (except during designated procedures). Water bottles can be provided, if required.

Periodic analysis of the water is performed, and results of these analyses are on file at the Testing Facility.

It is considered that there are no known contaminants in the water that could interfere with the outcome of the study.

8.5. ANIMAL ENRICHMENT

For psychological/environmental enrichment, animals will be provided with items, such as a certified toy and/or stainless steel manipulative device, except when interrupted by study procedures/activities. In addition, the animals will receive a certified timothy hay cube at least 3 times per week. One NutraBlock per animal may be offered at least once per week and may be offered up to 2 times per week.

8.6. VETERINARY CARE

Veterinary care will be available throughout the course of the study and animals will be examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments, if any, will be documented in the study records.

In the event that animals show signs of illness or distress, the responsible veterinarian may make initial recommendations about treatment of the animal(s) and/or alteration of study procedures, which must be approved by the Study Director. All such actions will be properly documented in the study records and, when appropriate, by protocol amendment. Treatment of the animal(s) for minor injuries or ailments may be approved without prior consultation with the Sponsor representative when such treatment does not impact fulfillment of the study objectives. If the condition of the animal(s) warrants significant therapeutic intervention or alterations in study procedures, the Sponsor representative will be contacted, when possible, to discuss appropriate action. If the condition of the animal(s) is such that emergency measures must be taken, the Study Director and/or attending veterinarian will attempt to consult with the Sponsor representative prior to responding to the medical crisis, but the Study Director and/or veterinarian has authority to act immediately at his/her discretion to alleviate suffering. The Sponsor representative will be fully informed of any such events.

9. EXPERIMENTAL DESIGN

Table 1. Test materials, dose volume, exposure time and number of patches per animal

Group No.	Test Material	Test Material Status	Dose Volume (mL)	Exposure Time	Exposure Method	Test Site	Number of Animals
							Males
1	Water (Control)	-	0.5	4 hours	Semi-occluded	1	3
	Grade 4	Used	0.5			2	
	Grade 4	Unused	0.5			3	
	Grade 3	Used	0.5			4	
	Grade 3	Unused	0.5			5	
2	Water (Control)	-	0.5	4 hours	Semi-occluded	2	3
	Grade 5 HTS	Used	0.5			3	
	Grade 5 HTS	Unused	0.5			4	
	Experimental Grade 5	Used	0.5			5	
	Experimental Grade 5	Unused	0.5			1	
3	Water (Control)	-	0.5	4 hours	Occluded	3	3
	Grade 4	Used	0.5			4	
	Grade 4	Unused	0.5			5	
	Grade 3	Used	0.5			1	
	Grade 3	Unused	0.5			2	
4	Water (Control)	-	0.5	4 hours	Occluded	4	3
	Grade 5 HTS	Used	0.5			5	
	Grade 5 HTS	Unused	0.5			1	
	Experimental Grade 5	Used	0.5			2	
	Experimental Grade 5	Unused	0.5			3	

9.1. ADMINISTRATION OF TEST SUBSTANCES

On Day -1, the animals chosen for use on study will have the fur removed from the dorsal area of the trunk using a small animal clipper (No. 40 blade). Care will be taken to avoid abrading the skin during the clipping procedure.

On the following day (Day 0), the test substance will be applied to five test sites (6 cm² each) on each animal for a total of 5 test sites per animal (Fig. 1). The test sites will be delineated with an indelible marker. The test sites will remain intact. The test substance will be applied as indicated below:

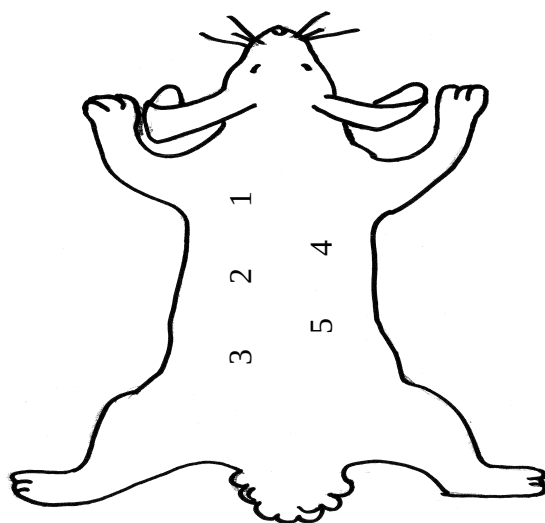


Figure 1. Location and number of test sites on each animal.

A 0.5 mL dose of the material will be administered to each site under an approximate 1 inch x 1 inch square 4-ply gauze patch. The gauze patch(es) will be held in contact with the skin at the cut edges with a nonirritating tape. After dosing, collars will be placed on each animal and will remain in place until removal on Day 3.

For Groups 1 and 2 (Table 1) – removal and ingestion of the test substance will be prevented by placing a stockinette over the trunk and test area (semi-occlusive binding).

For Groups 3 and 4 (Table 1) – removal and ingestion of the test substance will be prevented by placing plastic wrap applied over the gauze. A stockinette will then be placed over the trunk and test area (occlusive binding).

Following dosing, the Study Director will be notified by the technician if severe local reactions occur or if the animals exhibit overt clinical indications of pain/distress immediately postdose. Patch removal will be performed for each exposure period as indicated below:

Following completion of the exposure period, the tape, stockinette, and gauze patch will be removed from each animal and the corners of the test site delineated using a marker. Residual test substance will then be removed using gauze moistened with RO (Reverse Osmosis) water followed by dry gauze. If the RO water does not sufficiently remove the test substance residue, the Study Director/Sponsor may choose to use another appropriate solvent.

9.2. JUSTIFICATION OF ROUTE AND DOSE LEVELS

The dermal route of exposure was selected because this is a possible route of human exposure.

There have been reports about a high mental depression prevalence in aircraft maintenance workers and suggestions have been made on a link between this health issue with exposure to chemicals containing phosphate present in hydraulic fluids and engine oils. The aircraft engine oils contain a mixture of these chemicals and some of them are known to interfere with normal function of nervous systems. While reports suggest that the toxicity of the engine oil ingredients is at a very low level, little is currently known about the oil transformations that may occur while

they are being used in running engines. During the engine operations, the oil may go through transformations due to breakdown of its ingredients. A cocktail of chemicals that could form from worn or broken engine components may end up in the oils and could potentially change their properties. This may yield a more toxic oil mixture as compared to the unused version (new oil). Since the overall toxicity of a particular mixture depends on the proportion and toxicity of each ingredient among other things, an ideal evaluation of the hazardous effects of exposure to the compound mixture requires a toxicity test on the entire mixture not solely on each component. This study intends to characterize the toxicity and compare the dermal irritation of both unused and used versions of engine oils. The objective is to determine the irritation potential of new and used aircraft engine oils (Grade 4, Grade 3, Grade 5 HTS and Experimental Grade 5) following a single exposure to the skin of albino rabbits.

10. IN-LIFE PROCEDURES, OBSERVATIONS, AND MEASUREMENTS

10.1. MORTALITY/MORIBUNDITY CHECKS

Frequency: Twice daily, once in the morning and once in the afternoon, throughout the study.

Procedure: Animals will be observed for general health/mortality and moribundity. Animals will not be removed from cage during observation, unless necessary for identification or confirmation of possible findings.

10.2. CLINICAL OBSERVATIONS

10.2.1. DETAILED CLINICAL OBSERVATIONS

Frequency: At animal assignment and prior to dosing.

Procedure: Animals removed from the cage for examination.

10.2.2. CAGE SIDE OBSERVATIONS

Frequency: At least once daily, beginning pretest and throughout the dosing and observation periods. Cage side observations are not required on the days of detailed clinical observations during the pretest (prior to Day 1) and observation periods, or on the day of scheduled euthanasia.

Procedure: Animals will not be removed from the cage during observation, unless necessary for identification or confirmation of possible findings.

10.3. DERMAL SCORING

Frequency: 1 hour after patch removal, and 24, 48, and 72 hours after patch application.

Procedure: Animals will be examined for signs of erythema and edema and the responses scored according to Draize.² If there is no evidence of dermal irritation at the 72-hour scoring interval, the study will be terminated. If dermal irritation persists at any test site, the observation period may be extended for the affected animals (e.g., scored at 7, 10, and 14 days after patch removal). Animals requiring an extended observation period will remain on test until the irritation has resolved, permanent injury is evident, or the Study Director/Sponsor determines that additional scoring intervals are unnecessary. The dermal test sites may be reclipped as necessary to allow clear visualization of the skin. An alternative light source may be used to aid in dermal scoring.

10.4. BODY WEIGHTS

Frequency: At least at animal assignment, prior to dosing and the day of scheduled euthanasia.

Procedure: Animals will be individually weighed.

11. TERMINAL PROCEDURES

Terminal procedures are summarized in the following table:

Terminal Procedures for Main Animals

Group No.	Number of Males	Scheduled Euthanasia Day	Necropsy Procedures	
			Necropsy	Tissue Collection
1	3	a	-	-
2	3	a	-	-
3	3	a	-	-
4	3	a	-	-
Unscheduled Deaths			X	-
Replaced animals			X	-

X = procedure to be conducted; - = not applicable.

^a If there is no irritation after the 72-hour scoring interval, then animals may be euthanized. If irritation persists on any of the test sites, the observation period may be extended for the affected animals (e.g., scored on Days 7, 10, and 14).

11.1. UNSCHEDULED DEATHS

If a main study animal dies on study, a necropsy will be conducted. If necessary, the animal will be refrigerated to minimize autolysis.

Main study animals may be euthanized for humane reasons as per Testing Facility SOPs. These animals will undergo necropsy. If necessary, the animal will be refrigerated to minimize autolysis.

11.2. SCHEDULED EUTHANASIA

Main study animals surviving until scheduled euthanasia will be euthanized by sodium pentobarbital injection (with a 6 mL syringe) and discarded.

11.3. NECROPSY

Main study animals found dead or euthanized moribund will be subjected to a complete necropsy examination, which will include evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues.

Necropsy procedures will be performed by qualified personnel with appropriate training and experience in animal anatomy and gross pathology.

Images may be generated for illustration of or consultation on gross observations. Generation of such images will be documented. Images and associated documentation will be retained and archived.

12. STATISTICAL ANALYSIS

Corrosion will be considered to have resulted if the substance in contact with rabbit skin has caused destruction or irreversible alteration of the tissue on at least one-third of the rabbits tested. Tissue destruction is considered to have occurred if, at any of the readings, there is ulceration or necrosis. Tissue destruction does not include merely sloughing of the epidermis, or erythema, edema, or fissuring.

In the event that any exposure period is non-corrosive, the data from that exposure period will be classified as indicated below.

Data will be presented as individual values by animal. The individual body weight data tables will also include the calculated means and standard deviations for each group.

12.1. EPA-FIFRA DERMAL IRRITATION DESCRIPTIVE CLASSIFICATION

The 1- (or initial observation), 24-, 48-, and 72-hour erythema and edema scores for all animals will be added and the total divided by the number of test sites x 4 to yield the Primary Irritation Index (P.I.I.). If an animal dies during the first 72 hours of the study, the Primary Irritation Index will be adjusted to include only the days the animal was scored. The calculated Primary Irritation Index (P.I.I.) will be classified according to the Dermal Irritation Descriptive Classification³ presented in Attachment A. If any animal shows evidence of irreversible tissue destruction during the study (as judged by the Study Director) or at study termination (Day 14, 21, or as determined by the Study Director/Sponsor), the P.I.I. will not be calculated and the test material will be classified as Corrosive.

13. COMPUTERIZED SYSTEMS

The following critical computerized systems may be used in the study. The actual critical computerized systems used will be specified in the Final Report.

Data for parameters not required by protocol, which are automatically generated by analytical devices used will be retained on file but not reported. Statistical analysis results that are generated by the program but are not required by protocol and/or are not scientifically relevant will be retained on file but will not be included in the tabulations.

Critical Computerized Systems

System Name	Description of Data Collected and/or Analyzed
Compaq Alpha DS10 Computer using the Toxicology Analysis System Customized, Acute Toxicology Module or Provantis	applicable in-life data
Systems 600 Apogee Insight System	temperature and/or humidity (animal rooms, refrigerators, freezers, and compound storage)
Instem Life Science Systems, DISPENSE	test material receipt, accountability and/or formulation activities

14. AMENDMENTS AND DEVIATIONS

Changes to the approved protocol shall be made in the form of an amendment, which will be signed and dated by the Study Director. Every reasonable effort will be made to discuss any necessary protocol changes in advance with the Sponsor.

All protocol and SOP deviations will be documented in the study records. Deviations from the protocol and/or SOP related to the phase(s) of the study conducted at a Test Site shall be documented, acknowledged by the PI/IS, and reported to the Study Director for authorization/acknowledgement. The Study Director will notify the Sponsor of deviations that may result in a significant impact on the study as soon as possible.

15. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, electronic data, documentation, protocol, retained samples and specimens, and interim (if applicable) and final reports will be archived by no later than the date

of final report issue. All materials generated by Charles River from this study will be transferred to the archives at Charles River Laboratories, Inc., Horsham, PA. At least one year after issue of the draft report, the Sponsor will be contacted.

16. REPORTING

A comprehensive Draft Report will be prepared following completion of the study and will be finalized following consultation with the Sponsor. The report will include all information necessary to provide a complete and accurate description of the experimental methods and results and any circumstances that may have affected the quality or integrity of the study.

The Sponsor will receive an electronic version of the Draft and Final Report provided in Adobe Acrobat PDF format (hyperlinked and searchable) along with a Microsoft Word version of the text. The PDF document will be created from native electronic files to the extent possible, including text and tables generated by the Testing Facility. Report components not available in native electronic files and/or original signature pages will be scanned and converted to PDF image files for incorporation. An original copy of the report with the Testing Facility's handwritten signatures will be retained.

Reports should be finalized within 6 months of submission of the Draft Report. If the Sponsor has not provided comments to the report within 6 months of draft submission, the report will be finalized by the Testing Facility unless other arrangements are made by the Sponsor.

17. ANIMAL WELFARE

This study will comply with all applicable sections of the Final Rules of the Animal Welfare Act regulations (Code of Federal Regulations, Title 9), the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* from the Office of Laboratory Animal Welfare, and the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.^{1,4} The protocol and any amendments or procedures involving the care or use of animals in this study will be reviewed and approved by the Testing Facility Institutional Animal Care and Use Committee before the initiation of such procedures.

If an animal is determined to be in overt pain/distress, or appears moribund and is beyond the point where recovery appears reasonable, the animal will be euthanized for humane reasons in accordance with the *American Veterinary Medical Association (AVMA) Guidelines on Euthanasia* and with the procedures outlined in the protocol.⁵

By approving this protocol, the Sponsor affirms that there are no acceptable non-animal alternatives for this study, that this study is required by a relevant government regulatory agency(ies) and that it does not unnecessarily duplicate any previous experiments.

18. REFERENCES

1. National Research Council. *Guide for the Care and Use of Laboratory Animals*. 8th edition. Washington, DC: National Academy Press. 2011.
2. Draize, JH. *Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics*. The Association of Food and Drug Officials of the United States; 1959:49-51.

3. U.S. Environmental Protection Agency. *Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals-Addendum 3 on Data Reporting*; 1988.
4. Office of Laboratory Animal Welfare. *Public Health Services Policy on Humane Care and Use of Laboratory Animals*. Bethesda, MD: National Institutes of Health. August 2002.
5. American Veterinary Medical Association. *AVMA Guidelines on Euthanasia*. February 2013.

ATTACHMENT A
Dermal Evaluation Criteria

EPA CRITERIA	
Primary Irritation Index (P.I.I.)	Irritation Rating
0.00	Nonirritant
0.01 to 2.00	Slight Irritant
2.01 to 5.00	Moderate Irritant
5.01 to 8.00	Severe Irritant

APPENDIX B: DEVIATIONS

All deviations that occurred during the study have been acknowledged by the Study Director, assessed for impact, and documented in the Study Records. All protocol deviations and those SOP deviations regarded as significant by the Study Director are listed below. None of the deviations were considered to have impacted the overall integrity of the study or the interpretation of the study results and conclusions.

In-life Observations, Measurements, and Evaluations

- On Day 2, the 48-hour dermal grade for Group 2 male Animal No. 1004 was outside of the acceptable ± 30 -minute time range by 11 minutes, the dermal grade for Group 2 male Animal No. 1005 was outside of the acceptable ± 30 -minute time range by 16 minutes, and the dermal grade for Group 2 male Animal No. 1006 was outside of the acceptable ± 30 -minute time range by 21 minutes. This deviation had no impact on the study as the excursions from the expected times of dermal scoring were minimal and the results were able to be interpreted. The dermal grades were not expected to change in the short duration of time that the dermal grades were performed earlier than the intended time.

APPENDIX C: INDIVIDUAL MORTALITY

Individual Mortality Explanation Page

Abbreviations

AM SIRT	:	Mortality/moribundity check in the morning
PM SIRT	:	Mortality/moribundity check in the afternoon
DE	:	Detailed examination
CSO	:	Cage side observation
PreRx	:	Observation predosing
Post Rx	:	Observation post dosing
TE	:	Terminal Euthanasia
TERM	:	Terminal Euthanasia
UE	:	Unscheduled Euthanasia
UNSC	:	Unscheduled Euthanasia
FD	:	Found Dead
REC	:	Recovery Euthanasia
INTM	:	Interim Euthanasia
AD	:	Accidental Death
ACCD	:	Accidental Death
REL	:	Released

Note: This is a comprehensive list of abbreviations. All of the abbreviations listed may not be applicable to this report.

Individual Mortality

20192252

Group	Dose Level	Sex	Animal	Cage	Removal Day	Removal Week	Removal Date	Removal Time	Time Slot	Removal Symptom	Pathology Reason
1		Male	1001	1	14	2	03APR2018	14:05	.	TE	.
			1002	2	14	2	03APR2018	14:06	.	TE	.
			1003	3	14	2	03APR2018	14:06	.	TE	.
2		Male	1004	4	10	1	30MAR2018	9:17	.	TE	.
			1005	5	14	2	03APR2018	14:06	.	TE	.
			1006	6	14	2	03APR2018	14:06	.	TE	.
3		Male	1007	7	14	2	03APR2018	14:06	.	TE	.
			1008	8	14	2	03APR2018	14:06	.	TE	.
			1009	9	10	1	30MAR2018	9:14	.	TE	.
4		Male	1010	10	10	1	30MAR2018	9:21	.	TE	.
			1011	11	14	2	03APR2018	14:06	.	TE	.
			1012	12	10	1	30MAR2018	9:21	.	TE	.

APPENDIX D: INDIVIDUAL CLINICAL OBSERVATIONS

Individual Clinical Observations Explanation Page

Abbreviations/Descriptions

0	:	White
1	:	Slight
2	:	Moderate
3	:	Severe
4	:	Black
5	:	Blue
6	:	Brown
7	:	Clear
8	:	Green
9	:	Red
A	:	Slight group housed
B	:	Moderate group housed
C	:	Severe group housed
M	:	Mass present
N	:	Severity not applicable
X	:	Present
Y	:	Yellow
-	:	Severity not recorded
L	:	Lesion present
S	:	Scab present
G	:	Lesion ended
D	:	Scab ended
CSO	:	Cage side observation
DE	:	Detailed examination
Unsc	:	Unscheduled examination
Post	:	Observation post dosing
AM	:	Observation in the morning
PM	:	Observation in the afternoon
PreRx	:	Observation predosing
Post Rx	:	Observation post dosing
During Rx	:	Observation during dosing
AM SIRT	:	Mortality/moribundity check in the morning
PM SIRT	:	Mortality/moribundity check in the afternoon

Note: This is a comprehensive list of abbreviations. All of the abbreviations listed may not be applicable to this report.

Note: Only animals and/or time points with findings are presented in this appendix.

Individual Clinical Observations

Group: 1 Group 1 Sex: Male	Observation Type: All Types	Day(s) Relative to Start Date					
		-7 CSO	-6 CSO	-4 DE	0 DE		
1001	Fur, Staining, Red, Interscapular	X	X	.	.		
Group: 2 Group 2 Sex: Male	Observation Type: All Types	Day(s) Relative to Start Date					
		-7 CSO	-6 CSO	-4 DE	0 DE		
1005	Skin, Scab, Dorsal Thoracic	.	.	.	X		
	Skin, Scab, Lumbar	.	.	.	X		
1006	Fur, Staining, Red, Interscapular	X	.	.	.		
Group: 3 Group 3 Sex: Male	Observation Type: All Types	Day(s) Relative to Start Date					
		-7 CSO	-6 CSO	-4 DE	0 DE		
1008	Skin, Scab, Interscapular	.	.	X	.		
Group: 4 Group 4 Sex: Male	Observation Type: All Types	Day(s) Relative to Start Date					
		-7 CSO	-6 CSO	-4 DE	0 DE		
1010	Skin, Scab, Lumbar	.	.	.	X		
1012	Fur, Staining, Red, Interscapular	X	.	.	.		

APPENDIX E: INDIVIDUAL DERMAL SCORES

Individual Dermal Scores

ERYTHEMA AND EDEMA OBSERVATIONS		
OBSERVATION	DEFINITION	CODE
Erythema - Grade 0	No erythema	0
Erythema - Grade 1	Very slight erythema (barely perceptible)	1
Erythema - Grade 2	Well-defined erythema	2
Erythema - Grade 3	Moderate to severe erythema	3
Erythema - Grade 4	Severe erythema (beet redness)	4
Maximized Grade 4	Notable dermal lesions (see below)	M - 4 (see below)
Edema - Grade 0	No edema	0
Edema - Grade 1	Very slight edema (barely perceptible)	1
Edema - Grade 2	Slight edema (edges of area well defined by definite raising)	2
Edema - Grade 3	Moderate edema (raised approximately 1 millimeter)	3
Edema - Grade 4	Severe edema (raised more than 1 millimeter and extends beyond the area of exposure)	4

NOTE: Each animal was assigned an erythema and edema score. The most severely affected area within the test site was graded. If eschar, blanching, ulceration and/or necrosis greater than grade 1 were observed, then the "Maximized Grade 4" was assigned to the test site in place of the erythema score and the type of notable dermal lesion(s) (e.g., eschar - grade 2, blanching - grade 3, ulceration - grade 4) was noted. The presence of any other dermal changes (e.g., desquamation, fissuring, and eschar exfoliation) was also recorded.

NOTABLE DERMAL LESIONS		
OBSERVATION	DEFINITION/EXPLANATION	CODE
Eschar	A crust-like formation within or on the test area. Characterized as scab-like (dried blood or lymph) or dead layers of tissue/crust. The area is hardened to the touch and not very pliable. Note: Because erythema cannot be observed through eschar and eschar is considered to be a notable dermal lesion, the erythema score was maximized when eschar was present greater than ES-1. The test site was observed for reversibility in order to determine if the eschar was an in-depth injury. Coded using an area designation (see below).	--
Eschar - Grade 1	Focal and/or pinpoint areas up to 10% of test site	ES-1
Eschar - Grade 2	> 10% < 25% of test site	ES-2
Eschar - Grade 3	> 25% < 50% of test site	ES-3
Eschar - Grade 4	> 50% of test site	ES-4
Blanching	Characterized by areas of white to yellow or tannish discoloration in the test site due to a decreased blood flow to the skin. Note: An erythema score cannot be determined and blanching is considered a notable dermal lesion; therefore, the erythema score was maximized when blanching was present greater than BLA-1. The test site was observed for reversibility in order to determine if the blanching was an in-depth injury. Coded using an area designation (see below).	--
Blanching - Grade 1	Focal and/or pinpoint areas up to 10% of the test site	BLA-1
Blanching - Grade 2	> 10% < 25% of test site	BLA-2
Blanching - Grade 3	> 25% < 50% of test site	BLA-3
Blanching - Grade 4	> 50% of test site	BLA-4
Ulceration	An open lesion in the skin possibly due to the exfoliation of necrotic tissue or eschar formation. Characterized by a crater-like area which is generally inflamed and has a moist exudate. The erythema score was maximized when ulceration was present greater than U-1. Ulceration is considered an in-depth injury. Coded using an area designation (see below).	--
Ulceration - Grade 1	Focal and/or pinpoint areas up to 10% of the test site	U-1
Ulceration - Grade 2	> 10% < 25% of test site	U-2
Ulceration - Grade 3	> 25% < 50% of test site	U-3
Ulceration - Grade 4	> 50% of test site	U-4

NOTABLE DERMAL LESIONS (Continued)		
OBSERVATION	DEFINITION/EXPLANATION	CODE
Necrosis	The apparent death of a portion of tissue which may result in irreversible damage depending on the severity of injury based on the color, area and texture. It is characterized by a dark (ranging from gray to black) and often in-depth discoloration of the tissue. Because this term is considered to be diagnostic, this observation was only made with the approval of the Study Director and accompanied by a full description (the color was noted). The erythema score was maximized when necrosis was present greater than NEC-1. Necrosis is considered a notable dermal lesion and an in-depth injury. Coded using an area designation (see below).	--
Necrosis - Grade 1	Focal and/or pinpoint areas up to 10% of the test site	NEC-1 (color)
Necrosis - Grade 2	> 10% < 25% of test site	NEC-2 (color)
Necrosis - Grade 3	> 25% < 50% of test site	NEC-3 (color)
Necrosis - Grade 4	> 50% of test site	NEC-4 (color)

ADDITIONAL DERMAL OBSERVATIONS		
OBSERVATION	DEFINITION/EXPLANATION	CODE
Desquamation or Skin Flaking	Characterized by scaling or flaking of dermal tissue with or without denuded areas. May consist of a range from dry flaking of the skin to more pronounced flaking with denuded areas (in these cases the affected area may have a slight harder "feel" to it as compared to normal tissue; however, this should not be confused with a notable dermal lesion such as eschar). Areas of eschar were not scored for desquamation/skin flaking. This finding is generally not considered significant if the test site is otherwise clear for erythema, edema, etc.	DES or SFLA
Fissuring	Characterized by cracking of the skin or eschar formation (slough and/or scab) that is associated with moist exudate. Fissuring was checked prior to removing the animal from the cage and manipulating the test site.	FIS
Eschar Exfoliation	The process by which areas of eschar flake off the test site. This observation was noted only with an ES observation. May be graded with the following criteria:	EXF
Eschar Exfoliation – Grade 1	Barely perceptible scales.	EXF-1
Eschar Exfoliation – Grade 2	Distinct scales.	EXF-2
Eschar Exfoliation – Grade 3	Pronounced flaking with denuded sites.	EXF-3
Test Site Staining or Skin Staining	Skin located at the test site appears to be stained/dischored possibly due to test substance (note color of staining).	TSS (color) or SSTA
Erythema Extends Beyond the Test Site or Skin Red	The erythema extends beyond the test site. May be referred to as "Skin Red" with an appropriate location. Note: A Study Director was contacted for erythema extending beyond the test site.	ERB or SRED
Superficial Lightening or Skin Pale	Characterized by pale area(s) (almost a burn-like appearance) in the test site. However, erythema may still be observed through the pale area. Note: This observation may affect the overall erythema score of the test site. This observation may progress to other observations resulting in notable dermal lesions, but by itself was not considered a notable dermal lesion that resulted in a maximized dermal score. May be graded with the following criteria:	SL or SPAL
Superficial Lightening - Grade 1	Focal and/or pinpoint areas up to 10% of the test site	SL-1
Superficial Lightening - Grade 2	> 10% < 25% of test site	SL-2
Superficial Lightening - Grade 3	> 25% < 50% of test site	SL-3
Superficial Lightening - Grade 4	> 50% of test site	SL-4

Individual Dermal Observations Explanation page

Abbreviation/Description

0	:	White
1	:	Slight
2	:	Moderate
3	:	Severe
4	:	Black
5	:	Blue
6	:	Brown
7	:	Clear
8	:	Green
9	:	Red
A	:	Slight group housed
B	:	Moderate group housed
C	:	Severe group housed
M	:	Mass present
N	:	Severity not applicable
X	:	Present
Y	:	Yellow
-	:	Severity not recorded
L	:	Lesion present
S	:	Scab present
G	:	Lesion ended
D	:	Scab ended
CSO	:	Cage side observation
DE	:	Detailed examination

Note: This is a comprehensive list of abbreviations. All of the abbreviations listed may not be applicable to this report.

Individual Dermal Observations

Legend:

0 = Grade0; 1 = Grade 1; 2 = Grade 2; 6 = Brown

Group: 1 Group 1 Sex: Male	Observation Type: Local Irritation Ext 1	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1001	Erythema, Treatment Site No.01	0	0	0	0	0	0	.
	Edema, Treatment Site No.01	0	0	0	0	0	0	.
1002	Erythema, Treatment Site No.01	1	0	0	0	0	0	0
	Edema, Treatment Site No.01	0	0	0	0	0	0	0
1003	Erythema, Treatment Site No.01	1	0	0	0	0	0	.
	Edema, Treatment Site No.01	0	0	0	0	0	0	.
Group: 2 Group 2 Sex: Male	Observation Type: Local Irritation Ext 1	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1004	Erythema, Treatment Site No.01	0	1	1	2	0	.	.
	Edema, Treatment Site No.01	0	0	0	0	0	.	.
1005	Erythema, Treatment Site No.01	0	0	0	0	0	0	0
	Edema, Treatment Site No.01	0	0	0	0	0	0	0
1006	Erythema, Treatment Site No.01	0	1	1	1	0	0	.
	Edema, Treatment Site No.01	0	0	0	0	0	0	.
Group: 3 Group 3 Sex: Male	Observation Type: Local Irritation Ext 1	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1007	Erythema, Treatment Site No.01	1	1	1	0	0	0	0
	Edema, Treatment Site No.01	0	0	0	0	0	0	0
1008	Skin Staining, Treatment Site No.01	.	.	.	.	6	6	.
	Erythema, Treatment Site No.01	1	1	1	0	1	0	.
	Edema, Treatment Site No.01	0	0	0	0	0	0	.
1009	Erythema, Treatment Site No.01	0	1	0	1	0	.	.
	Edema, Treatment Site No.01	0	0	0	0	0	.	.
Group: 4 Group 4 Sex: Male	Observation Type: Local Irritation Ext 1	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1010	Erythema, Treatment Site No.01	0	0	0	0	0	.	.
	Edema, Treatment Site No.01	0	0	0	0	0	.	.
1011	Erythema, Treatment Site No.01	0	1	1	1	1	0	.
	Edema, Treatment Site No.01	0	0	0	0	0	0	.
1012	Skin Staining, Treatment Site No.01	.	.	.	.	6	.	.
	Erythema, Treatment Site No.01	0	1	1	1	0	.	.
	Edema, Treatment Site No.01	0	0	0	0	0	.	.
Group: 1 Group 1 Sex: Male	Observation Type: Local Irritation Ext 2	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1001	Erythema, Treatment Site No.02	1	1	1	1	0	0	.
	Edema, Treatment Site No.02	0	0	0	0	0	0	.
1002	Skin Staining, Treatment Site No.02	.	.	.	.	6	6	6
	Erythema, Treatment Site No.02	1	1	1	1	0	0	0
	Edema, Treatment Site No.02	0	0	0	0	0	0	0
1003	Skin Staining, Treatment Site No.02	.	.	.	.	6	6	.
	Erythema, Treatment Site No.02	1	1	1	1	0	0	.
	Edema, Treatment Site No.02	0	0	0	0	0	0	.
Group: 2 Group 2 Sex: Male	Observation Type: Local Irritation Ext 2	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1004	Erythema, Treatment Site No.02	1	0	0	1	0	.	.
	Edema, Treatment Site No.02	0	0	0	0	0	.	.
1005	Erythema, Treatment Site No.02	1	0	0	0	0	0	0
	Edema, Treatment Site No.02	0	0	0	0	0	0	0
1006	Erythema, Treatment Site No.02	0	0	0	0	0	0	.
	Edema, Treatment Site No.02	0	0	0	0	0	0	.

Group: 3 Group 3 Sex: Male	Observation Type: Local Irritation Ext 2	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1007	Erythema, Treatment Site No.02	1	1	1	0	0	0	0
	Edema, Treatment Site No.02	0	0	0	0	0	0	0
1008	Erythema, Treatment Site No.02	0	0	0	1	0	0	.
	Edema, Treatment Site No.02	0	0	0	0	0	0	.
1009	Erythema, Treatment Site No.02	0	0	0	1	0	.	.
	Edema, Treatment Site No.02	0	0	0	0	0	.	.
Group: 4 Group 4 Sex: Male	Observation Type: Local Irritation Ext 2	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1010	Erythema, Treatment Site No.02	0	1	1	1	0	.	.
	Edema, Treatment Site No.02	0	0	0	0	0	.	.
1011	Erythema, Treatment Site No.02	0	1	1	1	0	0	.
	Edema, Treatment Site No.02	0	0	0	0	0	0	.
1012	Erythema, Treatment Site No.02	1	1	1	1	0	.	.
	Edema, Treatment Site No.02	0	0	0	0	0	.	.
Group: 1 Group 1 Sex: Male	Observation Type: Local Irritation Ext 3	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1001	Erythema, Treatment Site No.03	1	1	1	1	0	0	.
	Edema, Treatment Site No.03	0	0	0	0	0	0	.
1002	Skin Staining, Treatment Site No.03	.	.	.	.	6	6	6
	Erythema, Treatment Site No.03	1	1	1	1	1	0	0
	Edema, Treatment Site No.03	0	0	0	0	0	0	0
1003	Skin Staining, Treatment Site No.03	.	.	.	.	6	6	.
	Erythema, Treatment Site No.03	1	0	0	1	0	0	.
	Edema, Treatment Site No.03	0	0	0	0	0	0	.
Group: 2 Group 2 Sex: Male	Observation Type: Local Irritation Ext 3	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1004	Erythema, Treatment Site No.03	0	1	1	1	0	.	.
	Edema, Treatment Site No.03	0	0	0	0	0	.	.
1005	Erythema, Treatment Site No.03	1	0	0	1	0	0	0
	Edema, Treatment Site No.03	0	0	0	0	0	0	0
1006	Erythema, Treatment Site No.03	1	1	1	2	0	0	.
	Edema, Treatment Site No.03	0	0	0	0	0	0	.
Group: 3 Group 3 Sex: Male	Observation Type: Local Irritation Ext 3	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1007	Erythema, Treatment Site No.03	1	0	0	1	0	0	0
	Edema, Treatment Site No.03	0	0	0	0	0	0	0
1008	Erythema, Treatment Site No.03	1	0	0	0	0	0	.
	Edema, Treatment Site No.03	0	0	0	0	0	0	.
1009	Erythema, Treatment Site No.03	0	0	0	0	0	.	.
	Edema, Treatment Site No.03	0	0	0	0	0	.	.
Group: 4 Group 4 Sex: Male	Observation Type: Local Irritation Ext 3	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1010	Erythema, Treatment Site No.03	1	1	1	1	0	.	.
	Edema, Treatment Site No.03	0	0	0	0	0	.	.
1011	Erythema, Treatment Site No.03	1	1	1	1	0	0	.
	Edema, Treatment Site No.03	0	0	0	0	0	0	.
1012	Erythema, Treatment Site No.03	0	1	1	1	0	.	.
	Edema, Treatment Site No.03	0	0	0	0	0	.	.
Group: 1 Group 1 Sex: Male	Observation Type: Local Irritation Ext 4	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1001	Erythema, Treatment Site No.04	0	1	1	1	1	0	.
	Edema, Treatment Site No.04	0	0	0	0	0	0	.
1002	Skin Staining, Treatment Site No.04	.	.	.	.	6	6	6
	Erythema, Treatment Site No.04	1	1	1	1	1	1	0
	Edema, Treatment Site No.04	0	0	0	0	0	0	0
1003	Skin Staining, Treatment Site No.04	.	.	.	.	6	6	.
	Erythema, Treatment Site No.04	1	1	1	1	1	0	.
	Edema, Treatment Site No.04	0	0	0	0	0	0	.

Group: 2 Group 2 Sex: Male	Observation Type: Local Irritation Ext 4	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1004	Erythema, Treatment Site No.04	0	1	1	1	0	-	-
	Edema, Treatment Site No.04	0	0	0	0	0	-	-
1005	Skin Staining, Treatment Site No.04	-	-	-	-	6	6	6
	Erythema, Treatment Site No.04	0	1	1	1	1	1	1
	Edema, Treatment Site No.04	0	0	0	0	0	0	0
1006	Erythema, Treatment Site No.04	1	1	1	2	1	0	-
	Edema, Treatment Site No.04	0	0	0	0	0	0	-
Group: 3 Group 3 Sex: Male	Observation Type: Local Irritation Ext 4	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1007	Erythema, Treatment Site No.04	1	1	1	1	1	1	0
	Edema, Treatment Site No.04	0	0	1	0	0	0	0
1008	Erythema, Treatment Site No.04	1	0	1	1	0	0	-
	Edema, Treatment Site No.04	0	0	0	0	0	0	-
1009	Erythema, Treatment Site No.04	1	0	0	0	0	-	-
	Edema, Treatment Site No.04	0	0	0	0	0	-	-
Group: 4 Group 4 Sex: Male	Observation Type: Local Irritation Ext 4	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1010	Erythema, Treatment Site No.04	0	0	0	0	0	-	-
	Edema, Treatment Site No.04	0	0	0	0	0	-	-
1011	Erythema, Treatment Site No.04	0	0	0	0	0	0	-
	Edema, Treatment Site No.04	0	0	0	0	0	0	-
1012	Erythema, Treatment Site No.04	0	0	0	0	0	-	-
	Edema, Treatment Site No.04	0	0	0	0	0	-	-
Group: 1 Group 1 Sex: Male	Observation Type: Local Irritation Ext 5	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1001	Erythema, Treatment Site No.05	1	1	1	1	1	0	-
	Edema, Treatment Site No.05	0	0	0	0	0	0	-
1002	Erythema, Treatment Site No.05	1	0	0	1	0	0	0
	Edema, Treatment Site No.05	0	0	0	0	0	0	0
1003	Erythema, Treatment Site No.05	1	1	1	2	1	0	-
	Edema, Treatment Site No.05	0	0	0	0	0	0	-
Group: 2 Group 2 Sex: Male	Observation Type: Local Irritation Ext 5	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1004	Erythema, Treatment Site No.05	0	1	1	1	0	-	-
	Edema, Treatment Site No.05	0	0	0	0	0	-	-
1005	Skin Staining, Treatment Site No.05	-	-	-	-	6	6	6
	Erythema, Treatment Site No.05	1	1	1	1	1	1	1
	Edema, Treatment Site No.05	1	0	0	0	0	0	0
1006	Erythema, Treatment Site No.05	1	1	1	2	1	0	-
	Edema, Treatment Site No.05	0	0	0	0	0	0	-
Group: 3 Group 3 Sex: Male	Observation Type: Local Irritation Ext 5	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1007	Skin Staining, Treatment Site No.05	-	-	-	-	6	6	6
	Erythema, Treatment Site No.05	0	1	1	1	0	0	0
	Edema, Treatment Site No.05	0	0	0	0	0	0	0
1008	Skin Staining, Treatment Site No.05	-	-	-	-	6	6	-
	Erythema, Treatment Site No.05	0	0	1	1	0	0	-
	Edema, Treatment Site No.05	0	0	0	0	0	0	-
1009	Skin Staining, Treatment Site No.05	-	-	-	-	6	-	-
	Erythema, Treatment Site No.05	0	1	1	1	0	-	-
	Edema, Treatment Site No.05	0	0	0	0	0	-	-
Group: 4 Group 4 Sex: Male	Observation Type: Local Irritation Ext 5	Day(s) Relative to Start Date						
		0	1	2	3	7	10	14 CSO
1010	Erythema, Treatment Site No.05	0	0	0	1	0	-	-
	Edema, Treatment Site No.05	0	0	0	0	0	-	-
1011	Erythema, Treatment Site No.05	1	1	1	1	0	0	-
	Edema, Treatment Site No.05	0	0	0	0	0	0	-
1012	Erythema, Treatment Site No.05	1	0	0	1	0	-	-
	Edema, Treatment Site No.05	0	0	0	0	0	-	-

APPENDIX F: INDIVIDUAL BODY WEIGHTS

Individual Body Weights

Sex: Male Bodyweight (kg)

Group: 1	Day(s) Relative to Start Date		
Group 1	-4	0	14
1001	2.9	3.1	3.4
1002	2.6	2.8	3.2
1003	2.7	2.9	3.3
Mean	2.73	2.93	3.30
SD	0.15	0.15	0.10

Sex: Male Bodyweight (kg)

Group: 2	Day(s) Relative to Start Date			
Group 2	-4	0	10	14
1004	2.7	2.8	2.9	-
1005	2.6	2.9	-	3.1
1006	2.8	2.9	-	3.3
Mean	2.70	2.87	2.90	3.20
SD	0.10	0.06	-	0.14

Sex: Male Bodyweight (kg)

Group: 3	Day(s) Relative to Start Date			
Group 3	-4	0	10	14
1007	2.5	2.7	-	3.0
1008	2.6	2.8	-	3.1
1009	2.6	2.7	2.9	-
Mean	2.57	2.73	2.90	3.05
SD	0.06	0.06	-	0.07

Sex: Male Bodyweight (kg)

Group: 4	Day(s) Relative to Start Date			
Group 4	-4	0	10	14
1010	2.6	2.7	2.9	-
1011	2.6	2.9	-	3.3
1012	2.7	2.7	2.8	-
Mean	2.63	2.77	2.85	3.30
SD	0.06	0.12	0.07	-