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**21 DAY REPEATED DOSE DERMAL TOXICITY STUDY OF
AIRCRAFT ENGINE OILS USING SPRAGUE DAWLEY RATS
(*RATTUS NORVEGICUS*): EFFECTS ON ORGANS,
HEMATOLOGICAL PARAMETERS AND CHOLINESTERASE
ENZYME ACTIVITIES IN TISSUES AND BLOOD**

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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 managers for HJF were David R. Mattie, PhD and Armando Soto (711 HPW/RHB, formerly RHXJ). David R. Mattie, PhD was also the technical manager for this project.

The repeated dose dermal toxicity study of aircraft engine oils 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) 870.3200, 21/28-Day Dermal Toxicity 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.

The repeated dose dermal toxicity study protocol, “21 Day Repeated Dose Dermal Toxicity Study of Aircraft Engine Oils using Sprague Dawley Rats (*Rattus norvegicus*)” was approved as F-WA-2018-0173-A by the Wright-Patterson AFB Installation Animal Care and Use Committee (IACUC). 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.

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1.0 SUMMARY

There is little data available for the toxicity of used aircraft engine oils relative to their unused (new) versions. This study was conducted to determine if two MIL-PRF-7808 aircraft engine oils (grade 3 (G3) and grade 4 (G4)) in their new states (G3-N and G4-N) and their used versions (G3-U and G4-U) have the potential to induce toxicity via dermal application. Male and female Sprague Dawley rats were dermally exposed to water (control), new and used versions of G3 and G4 oils to determine the oil sub-chronic toxicity potentials. A volume of 300 microliters (μL) of undiluted oil was applied to the pad of the Hill Top Chamber System[®]. Then the chamber was attached to a fur-free test site located at the back of the rat for 6 hours (h)/day for 5 consecutive days/week for 21 days (15 total exposures). Recovery rats exposed the same were kept for 14 days post-exposure to screen for reversibility, persistence, or delayed occurrence of toxic effects. A pilot study, with rats receiving the same treatment, was conducted to characterize electrophysiological effects on hippocampal neurons associated with exposure to these oils. Oil analysis by Phosphorus-31 nuclear magnetic resonance (^{31}P NMR) indicated that G3 oils contained 4 isomers of tri-cresyl phosphate (TCP) while G4 comprised phenol isopropylated phosphate (3:1) (PIP) and tri-phenyl phosphate (TPP). While both versions of G3 contained 4 isomers of TCP at detectable levels, two were more predominant. The concentration for TCP in G3-U decreased by 26 percent (%) relative to G3-N while amounts of TPP and PIP in G4-U decreased by 22% and 6.4%, respectively, relative to G4-N. Both G3 and G4 oils had a significant impact on the weight of male and female reproductive organs: testes weights for recovery rats exposed to G3-N significantly decreased (12%) relative to controls; G3-N and G3-U decreased uterus weights by 23% and 29%, respectively; G4-N decreased uterus weights by 32% but were resolved at the end of the recovery period; G4-N increased the weight of the adrenals and spleen for females by 34% and 27%, respectively, during the recovery period. G3 and G4 induced more changes in female blood indices than in those for males. Of all versions of oils, G4-N induced the most changes in profiles of female blood. G4-N significantly decreased the white blood cells, lymphocytes, neutrophils, eosinophils and increased the mean platelet volumes. Interestingly, males were not affected by exposure to G4-N oil. While G3-N decreased the white blood cells and lymphocytes for females it slightly increased those for males. In general, the unused versions of G3 and G4 oils induced large effect sizes (Cohen's d ; $d \geq |0.8|$) for blood indices relative to used versions of these oils, regardless of gender. In brain tissues, both versions of G3 and G4 oils significantly ($p \leq 0.05$) decreased (32% to 41%) the Acetylcholinesterase (AChE) activity for females. Only G3-N and G4-N significantly lowered (33%) the AChE activity for males. All these effects resolved by end of the recovery period. All oils had no impact on AChE activity in the liver, regardless of gender. In whole blood samples, only G3-U significantly decreased AChE by 29% for females at end of exposure period, persisting during the recovery period. Blood AChE activity for males was not affected. Butyrylcholinesterase (BChE) in plasma for males exposed to G4-N decreased by 29%, but resolved during the recovery period. An initial limited electrophysiology study revealed that hippocampal synaptic plasticity properties were affected by G3 and G4 oils. However, effects on synaptic transmission and spontaneous activity are thus far inconclusive. The overall repeated dermal toxicity study highlights the health risks that aircraft maintenance workers may be exposed to if precautions are not taken to minimize exposure to these oils.

2.0 INTRODUCTION

Human toxicity associated with organophosphate compounds has been known at least since 1899, when it was reported that they induce neurotoxicity (**Echobion**). Reports for various studies indicate that people who are chronically exposed to a low level of organophosphate compounds develop neuropathy and neuropsychiatric problems (**Singh and Sharma, 2000; Davies et al., 2000a; Davies et al., 2000b; Jamal et al., 2002; Arun and Palimar, 2008**). Currently, the use of organophosphate compounds is not just limited to pesticides used to control pests on crops in fields, but they are also used in aircraft engine oils and hydraulic fluids as additives, and as flame retardants as well. In recent decades, concerns have arisen regarding aircraft crew members who have developed various symptoms consistent with exposure to organophosphates and toxic fumes (**Liyasova et al., 2011; Abou-Donia et al., 2014; Harrison and Mackenzie Ross, 2016**). Concerns have also been raised regarding the possibility that the aircraft maintenance workers may be exposed to organophosphate esters that are in aircraft engine oils through inhalation and/or dermal contact (**Singh and Sharma, 2000; Winder and Balouet, 2002; Denola et al., 2011**). Organophosphate compounds are capable of producing a range of subacute or chronic neurological syndromes and neuropsychological effects beside their well-known inhibition of cholinesterase activities (**Rosenstock et al., 1991; De Bleecker et al., 1992; Leon-S et al., 1996; Steenland, 1996**).

Toxicity of organophosphorus compounds is generally derived from their inhibition effects on the esterase enzymes. Enzymes that are most particularly affected are cholinesterases and neurotoxic esterases (**Aldridge, 1954; Barrett and Oehme, 1994; Carletti et al., 2013**). The class of cholinesterase enzymes includes AChE (EC 3.1.1.7) and the pseudocholinesterase or BChE (EC 3.1.1.8). AChE hydrolyses the neurotransmitter acetylcholine yielding choline and acetic acid molecules while BChE catalyzes the breakdown of butyrylcholine generating choline and butyric acid products. The specific mechanisms of enzyme inhibition have been well established and include the phosphorylation events in the enzyme's catalytic sites (**Earl and Thompson, 1952; Carletti et al., 2011; Liyasova et al., 2012**). Because AChE breaks down endogenous acetylcholine, a neurotransmitter molecule acting throughout the cholinergic nervous system, an inhibition of this enzyme results in an abnormal buildup of this molecule in the synaptic cleft. The overflow of acetylcholine stimulates the muscarinic, nicotinic and central nervous system receptors leading to patients experiencing clinical complications (**Indu et al., 2016**). Muscarinic stimulation leads to acute cholinergic syndrome while acetylcholine-induced desensitization of the nicotinic receptors leads to muscular twitching, neuromuscular paralysis and central nervous system manifestations such as tremor, anxiety, giddiness and coma (**De Bleecker et al., 1992; Paudyal, 2008; Girish et al., 2010; Indu et al., 2016**). Organophosphate compounds are regarded as irreversible AChE inhibitors since the amount of time necessary for the enzyme inhibition to be reversed exceeds the time required for the synthesis of new AChE (**Lionetto et al., 2013**). Studies have indicated that clinical symptoms are generally seen when the level of AChE in the brain is decreased to 20-50% of normal activity (**Lotti, 2001**). AChE inactivation between 60 and 90% yields moderate symptoms that include diarrhea, sweating, vomiting (**Maroni et al., 2000**). Death from cardiac or respiratory failure can occur following a 90-100% inhibition of AChE (**Maroni et al., 2000**). On the other hand, organophosphate-mediated inactivation of neuropathy target esterase (NTE) can lead to neuropathological condition of neuronal damage known as organophosphorus-induced delayed neuropathy (**Baron, 1981; Metcalf, 1982**).

Since the 1960s, ingredients for aircraft lubricating oils have barely changed and their compositions include approximately 95% synthetic esters with roughly 1% phenyl- α -naphthalamines and 3% TCP (**Winder and Balouet, 2002**). Other organophosphate ingredients of aircraft engine oils that potentially pose health risks include PIP and TPP (**Winder and Balouet, 2002**). Although some of these ingredients such as TCP are known to induce health effects (**Carletti et al., 2011; Liyasova et al., 2012; Hausherr et al., 2014; Duarte et al., 2017; Hausherr et al., 2017**), the manufacturers for jet engine oils have been reluctant to modify their ingredient formulations and substitute the toxic TCP additives with non-toxic ingredients. They continue to use TCP isomer mixtures because they perform so well in critical applications (**Mackerer et al., 1999; Winder and Balouet, 2002**). A TCP molecule comprises three cresyl (methylphenyl and hydroxyl) groups linked to a phosphate group. The methyl group attached to each cresol can be in the ortho-, meta- or para- positions relative to the hydroxyl group on the cresol and the combination gives rise to 10 structural TCP isomers. Megson *et al.* (**Megson et al., 2016**) analyzed fresh and used aircraft engine lubricants (Mobil Jet Oil II) and found that only four TCP isomers (tri-meta-TCP; para-di-meta-TCP; meta-di-para-TCP and tri-para-TCP) were present at detectable levels in both versions of oils. Although all TCP isomers have different properties and can induce hazardous effects to a certain degree, tri-ortho-cresyl phosphate (TOCP) is considered to be the most notable toxicant (**Winder and Balouet, 2002**) and its proportions in lubricants have been reduced in the recent decades (**Craig and Barth, 1999**). Megson *et al.* (**Megson et al., 2016**) reported that the formulation of Mobil Jet Oil II does not contain TOCP at concentrations higher than 0.0005%. The TOCP metabolism has been examined in chickens (**Abou-Donia et al., 1990**), rats (**Casida et al., 1961; Abou-Donia and Nomeir, 1986; Somkuti and Abou-Donia, 1990**) and cats (**Nomeir and Abou-Donia, 1986**). Its metabolic pathway involves multiple steps in which a reactive cyclic phosphate residue is formed (**Eto et al., 1962**). The final product is o-hydroxy-benzoic acid, which is excreted in the urine as a free or conjugated molecule (**Eto et al., 1962**). The formation of the reactive cyclic phosphate residue (which cannot be formed from meta- or para- cresol residues) has been proposed as a critical step in the ultimate development of neurological events of axonopathy (**Jortner and Ehrich, 1987**). Jortner and Ehrich (**Jortner and Ehrich, 1987**) administered cyclic phenyl saligenin phosphate directly to White Leghorn chickens and noted that this molecule was more neurotoxic than the parent TOCP.

Exposure to TCP has been associated with various adverse health effects (**Rosenstock et al., 1991; Leon-S et al., 1996; Steenland, 1996**) that include toxicity to the reproductive system (**Carlton et al., 1987; Chapin et al., 1988; Asmathbanu and Kaliwal, 1997; Dhondup and Kaliwal, 1997; Sortur and Kaliwal, 1999**). Animal studies have provided limited details about TCP effects on the rat's reproductive system (**Carlton et al., 1987; Somkuti et al., 1991; Latendresse et al., 1994a; Latendresse et al., 1994b; Latendresse et al., 1995**). Several studies have suggested that the mechanisms by which organophosphates in general induce reproductive toxicity in animals involve alterations in the release of neurotransmitters, thus impairing hypothalamic-pituitary gonadal regulation of reproduction (**Muller et al., 1977; Baligar and Kaliwal, 2002**). Histopathologic changes in testes, epididymides and ovaries have been observed in rats exposed to TCP (**Carlton et al., 1987**). Latendresse *et al.* (**Latendresse et al., 1994a; Latendresse et al., 1994b; Latendresse et al., 1995**) reported that exposure to TCP significantly decreased testicular and epididymal weights, increased ovarian weights, caused degeneration of the seminiferous tubular epithelium and produced severe lesions in the ovaries of F344 rats.

Somkuti *et al.* (Somkuti *et al.*, 1991) have shown that male F344 rats exposed to the TOCP isomer of TCP have decreased sperm density and increased necrotic spermatids.

TCP and other organophosphate ingredients in lubricants such as hydraulic fluids and extreme pressure fluids are used as anti-wear and anti-corrosive additives designed to enhance the load bearing properties and improve tolerance to high temperatures (Solbu *et al.*, 2010). While aircraft engine oils are known to contain organophosphate toxic ingredients at a very low level, little is currently known about oil transformations occurring in running engines. The oil in a jet engine may be heated to several hundred degree Celsius (Ramsden, 2013). The extremely high temperatures in running engines may be conducive to the breakdown of oil's ingredients and/or the release of worn engine particles into the oil, thus, altering the composition of the original oil and yielding a cocktail of chemicals that could potentially change the oil's properties. This could also increase the oil's hazard profile. Currently, there is no data available on toxicity associated with exposure to used aircraft engine oils relative to their unused (new) versions. In this study, we dermally exposed male and female Sprague Dawley (SD) rats with unused (new) and used versions of grade 3 and 4 aircraft engine oils over a period of 21 days (total 15 exposures) and assessed their potential effects on organ weights, hematological indices and cholinesterase activities in blood and tissues since these oils contain organophosphate additives that are known to induce adverse health conditions. A pilot study was also conducted to characterize the electrophysiological effects of repeated dermal exposures to these aircraft engine oils. Measurements under this study included synaptic transmission of rat hippocampus, short and long term synaptic plasticity and spontaneous activity of hippocampal neurons.

The objective of this study was to assess potential toxicity effects of aircraft engine oils in both their new (unused) and used states following a 21day repeated dose dermal exposure to male and female SD rats. The U.S. Air Force (USAF) engine oils involved in this study were MIL-PRF-7808 Grades 3 and 4 used in the majority of USAF aircraft from legacy aircraft to 5th generation fighters. The hypothesis of this proposed study was that an exposure to used aircraft engine oils is toxic as compared to the new (unused) oils. The dermal exposure was selected because the skin is a route of human exposure. This study was intended to provide information on the health hazards likely to arise from a sub-chronic dermal exposure to engine oils.

3.0 MATERIALS AND METHODS

3.1 Engine Oils and Control Substance

Grade 3 and 4 aircraft engine oils in their new states were obtained from the Air Force Petroleum Office, Wright-Patterson Air Force Base (WPAFB), Ohio. The used version of grade 3 oil was removed from a C-17 aircraft at WPAFB, Ohio. The used version of grade 4 oil was removed from F-22 aircraft at Langley Air Force Base, Virginia. These oils were kept in a chemical cabinet at the room temperature (22-23 °C). The reverse osmosis deionized (RODI) water used as a control substance was produced in the laboratory at WPAFB, Ohio.

3.1.1. Test Substance 1

Identification:	Grade 3 (Used)
Batch (Lot) No./Source:	Unknown; removed from aircraft
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)
Batch (Lot) No./Source:	2017202525
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

3.1.2. Test Substance 2

Identification:	Grade 4 (Used)
Batch (Lot) No./Source:	Unknown; removed from aircraft
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
Receipt Date:	21 Feb2018
Expiration Date:	April 2020
Physical Description:	Red liquid
Storage Conditions:	Kept in a controlled room temperature area

3.1.3. Control Substance

Identification:	RODI Water
Physical Description:	Clear liquid

3.2 Supplies

FS-PAK multispecies reagent kit (Product No. 200106; ERBA Diagnostics, Inc., Miami, Florida, USA), MULTI-TROL quality control standards (Product No. 600070; ERBA Diagnostics, Inc., Miami, Florida, USA) and MD Calibration kit (Product No. CDC0317; ERBA Diagnostics, Inc., Miami, Florida, USA) were used to measure hematological parameters. Neuronal Protein Extraction Reagent (N-PER™, Cat. # 87792; ThermoFisher Scientific, Waltham, MA, USA) and Halt Protease Inhibitor single-use cocktail (Cat. # 78430; ThermoFisher Scientific, Waltham, MA, USA) were used in tissue homogenization. 1.5 milliliter (mL) sterile microcentrifuge RINO™ tubes (TUBE1R5-S; Next Advance, Inc., Troy, NY, USA) and zirconium oxide beads (ZrOB20; 2.0 millimeter (mm) diameter; Next Advance, Inc., Troy, NY, USA) were used in tissue homogenization. Deuteriochloroform (CDCl₃) (Sigma-Aldrich, St. Louis, MO, USA) was used in lipid extraction. Acetylcholinesterase activity kits (Cat. # MAK119; Sigma-Aldrich, St. Louis, MO, USA) were used in acetylcholinesterase enzyme activity assays. Butyrylcholinesterase activity kits (Cat. #K516-100; BioVision Inc., Milpitas, CA, USA) were used in butyrylcholinesterase enzyme activity assays.

3.3 Animals

All male and female SD rats (*Rattus norvegicus*) involved in this study were obtained from Charles River Laboratories (640 N. Elizabeth Street, Spencerville, OH 45887). They were 6-7 weeks old on their arrival. The SD strain of rats was chosen as the test animal for this study because of their larger size which provide more surface area for the treatment application system. All rats were housed individually in polycarbonate cages with cell sorb bedding. The animals were acclimated to their designated housing for 14 days prior to the start of the study. The room was maintained under standard environmental conditions of 12:12 h light:dark cycle, an ambient temperature of 25-28 degrees (°C) and with a relative humidity of 40% to 60%. Formulab chow (#5008; Cincinnati Lab Supply Inc., Cincinnati, OH, USA) for rats was provided *ad libitum* throughout the study, except during designated procedures (skin shaving and treatment application). Food intake was measured weekly. At the end of exposure and recovery period, all rats were fasted for 12 hours prior to their blood draw and gross necropsy by withdrawing food at the appropriate time on the day before euthanasia. Reverse osmosis drinking water was freely available to each rat via a chew-proof clear water glass bottle, except during skin shaving and treatment application. There was no restriction of water. Water intake was measured weekly.

3.4 Experimental Design and General Procedures

3.4.1. Animal Grouping

For treatments and necropsy procedures to be performed in a timely manner, the study was carried out in phases. Details of these phases are shown in [Tables 1-3](#). Phase 1 involved a total of 75 rats that comprised 50 rats assigned to the main study and 25 rats allocated to the recovery groups. Phase 2 involved a total of 85 rats that included 50 rats assigned to the main study, 25 rats for the recovery groups and 10 rats allocated to electrophysiology measurements. In phase 1, each treatment was repeated three times (25 rats/replicate). The schedules for the start of the replicates in each session were spaced by one day.

Table 1. Details for rat allocation to treatment groups in phase 1

Phase	Test Articles	Oil Status	Replicate #	Dose (μL)	Main Study		Recovery Groups	
					# of males	# of females	# of males	# of females
1	Control		1	300	1	2	1	1
			2	300	2	1	1	1
			3	300	2	2	0	1
	Grade 4	Unused (G4-N)	1	300	1	2	1	1
			2	300	2	1	1	1
			3	300	2	2	0	1
		Used (G4-U)	1	300	1	2	1	1
			2	300	2	1	1	1
			3	300	2	2	0	1
			Grade 3	Unused (G3-N)	1	300	1	2
	2	300			2	1	1	1
	3	300			2	2	0	1
	Used (G3-U)	1		300	1	2	1	1
		2		300	2	1	1	1
		3		300	2	2	0	1
	Total				25	25	10	15

Phase 1 of the project required a large number of personnel to be conducted in a timely manner. To address this issue, we performed phase 2 of this study in two sessions (Phase 2A and Phase 2B; [Tables 2 & 3](#)). Phase 2A involved 41 rats (30 rats assigned to the main study and 11 rats allocated to the recovery groups) and each treatment was repeated three times (three replicates). Phase 2B comprised 44 rats (20 rats assigned to the main study, 14 rats allocated to the recovery groups and 10 rats for electrophysiology measurements) and each treatment was repeated four times (four replicates). The schedules for the start of the replicates in each session were spaced by one day.

Table 2. Details for rat allocation to treatment groups in phase 2A

Phase	Test Articles	Oil Status	Replicate #	Dose (µL)	Main Study		Recovery Groups		Electrophysiology Groups	
					# of males	# of females	# of males	# of females	# of males	# of females
2A	Control		1	300	1	1	1	0	0	0
			2	300	1	1	0	1	0	0
			3	300	1	1	0	0	0	0
	Grade 4	Unused (G4-N)	1	300	1	1	1	0	0	0
			2	300	1	1	0	0	0	0
			3	300	1	1	1	1	0	0
		Used (G4-U)	1	300	1	1	0	1	0	0
			2	300	1	1	1	0	0	0
			3	300	1	1	0	0	0	0
	Grade 3	Unused (G3-N)	1	300	1	1	0	1	0	0
			2	300	1	1	1	0	0	0
			3	300	1	1	0	0	0	0
		Used (G3-U)	1	300	1	1	0	0	0	0
			2	300	1	1	0	1	0	0
			3	300	1	1	1	0	0	0
Total				15	15	6	5	0	0	

Each replicate in all phases involved both males and females randomly assigned to one of five treatment groups consisting of control, grade 3 (G3) and 4 (G4) engine oils in their new states (G3-N and G4-N) and their used versions (G3-U and G4-U). Each treatment group involved 10 rats in total per sex for the main study, except for G3-N and G3-U groups for males which involved 9 and 11 rats, respectively. For the recovery groups, each treatment involved 5 rats per sex.

Table 3. Details for rat allocation to treatment groups in phase 2B

Phase	Test Articles	Oil Status	Replicate #	Dose (µL)	Main Study		Recovery Groups		Electrophysiology Groups		
					# of males	# of females	# of males	# of females	# of males	# of females	
2B	Control		4	300	0	1	0	0	0	0	
			5	300	1	0	0	1	0	0	
			6	300	0	1	1	0	0	0	
			7	300	1		1	0	1	1	
	Grade 4	Unused (G4-N)	4	300	1	1	0	1	1	0	
			5	300	0	1	1	0	0	0	
			6	300	1	0	0	0	0	0	
			7	300	0	0	0	0	0	1	
		Used (G4-U)	4	300	1	0	1	0	0	0	
			5	300	1	1	0	0	1	0	
			6	300	0	0	1	0	0	1	
			7	300	0	1	0	1	0	0	
		Grade 3	Unused (G3-N)	4	300	1	0	1	0	0	0
				5	300	0	1	0	0	0	1
				6	300	1	0	0	1	1	0
				7	300	0	1	1	0	0	0
	Used (G3-U)		4	300	0	0	0	1	0	1	
			5	300	1	0	1	0	0	0	
			6	300	0	1	0	0	0	0	
			7	300	1	1	1	0	1	0	
	Total				10	10	9	5	5	5	

3.4.2. Electrophysiology Measurements

We also conducted a pilot study to examine the potential neurological effects associated with aircraft engine oil after dermal exposure. Electrophysiology recordings were performed on brain tissue slices. At the end of phase 2B (Table 3) two rats (male and female) from each treatment group were used to assess the synaptic function, plasticity and spontaneous spiking activity of neurons within the hippocampus which is a brain region that is critical for cognition function.

3.4.3. Assigning Animals to Cages and Treatment Groups

At the start of each phase, animals were randomly assigned to cages and treatment groups. Rats were randomly placed one per cage by the vivarium staff. They did not know which treatment each rat would receive when they assigned them to cages. The cage cards identifying the treatment groups were randomly assigned to cages by the principle investigator. He did not know which rat was in each cage when he assigned the rats to groups. Each animal was identified by a cage card and a tattoo (identifying the animal number and treatment) on the tail applied using an indelible marker. The information on the cage card included the group, the gender and the tattoo number.

3.4.4. Justification for Dose Level

A dose of 300 µL of engine oil used in this study was chosen based on (1) the results of the dermal irritation study (Sibomana *et al.*, 2019) that showed only a slight irritation to acute dermal

exposure and (2) the volume capacity of Hill Top Chamber System© used as a dermal treatment delivery system.

3.4.5. Method of Administration of Treatments

The Hill Top Chamber System© (full system, Hill Top Research, St. Petersburg FL) was chosen as the dermal treatment delivery system (**Figure (Fig) 1**). A Hilltop chamber system© consists of a circular plastic chamber 25 mm in diameter, 300 µL chamber capacity with a square piece of hypoallergenic adhesive tape around the outside (tape size of 4.5 cm x 4.5 cm).



Figure 1. Hill Top Chamber System© Used to Apply Treatment on Rat Skin

25 mm chamber diameter, 300 µL chamber capacity used to apply treatment on rat skin.

3.4.6. Animal Preparation

To facilitate the dermal applications, the testing site on each animal (**Fig. 2**) was clipped free of hair approximately 24 hours before the test using a small animal clipper (a No. 40 clipper blade). The clipped area on each animal constituted approximately 10% of the total body surface area. In order to be able to apply the exposure chamber, the area starting at the scapulae (shoulders) and extending caudally 4-5 centimeters (cm) was shaved. Repeated animal clippings were performed at weekly intervals. Before each round of oil exposure, all rats were inspected for dermal abnormalities (rash, clipper abrasion, cut, scab, etc.). Care was always taken to avoid abrading the skin during the clipping procedure.

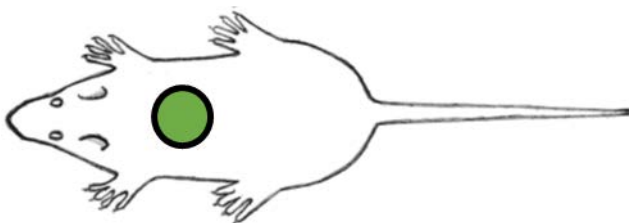


Figure 2. Location of Treatment Site on Back of Each Rat

3.4.7. Animal Treatments

After removing the backing from the adhesive tape of the Hill Top Chamber System©, a volume of 300 µL of undiluted engine oil was evenly dropped into the pad of the chamber and the chamber was placed on the clipped skin at the back of the rat (Fig. 2). The tape was gently pushed down to attach the chamber to the hairless skin. For controls, a volume of 300 µL of reverse osmosis deionized water was applied to the pad of the Hill Top chamber. The Hill Top Chamber System© was flexible enough to conform to the contour of the skin for a close fit to the skin surface. As the hypoallergenic adhesive tape on the chamber was not strong enough to hold it to the rat skin, an additional surgery tape was placed on the top of the chamber and around the rat trunk to secure the chamber in place.

3.4.8. Cleaning of Treatment Site and Dermal Observations

After 6 hours of treatment exposure each day, the chamber was removed and treatment site delineated using an indelible marker. Each treatment site was gently cleansed as thoroughly as possible using a soft pad soaked in Dawn® soapy water to eliminate the residual engine oil. The site was then rinsed with a water-soaked soft pad to remove the soap. Dawn® soap has been used by the wildlife animal rescue workers worldwide to clean wild animals affected by oil pollution.

3.4.9. Duration of Treatment Exposure

On each treatment day, application of the chambers on the rat's test sites started at 7 am and exposure lasted for 6 hours. The treatment was always applied to the same spot delineated by indelible marker. All the treatment procedures were repeated every day for 5 consecutive days a week (except weekends) for a period of 21 days (15 total exposures). Rats in the recovery groups also received similar treatments and were kept for 14 days post-exposure to screen for reversibility, persistence, or delayed occurrence of toxic effects.

3.4.10. Mortality/Moribundity Checks

Throughout the study, animals were observed for general health/mortality and moribundity twice daily (morning and afternoon). Cage side observations were performed once every 30 minutes to make sure that the Hill Top Chambers were still attached to treatment sites.

3.4.11. Body Weights

Body weight was taken for rats on their arrival at the animal facilities in Building 838, Area B, WPAFB. The initial body weight for each rat was determined before the first exposure (day 0). It was followed by weekly body weight measurements and at the end of the study prior to necropsy.

3.4.12. Necropsy

Animals were euthanized in accordance with current American Veterinary Medical Association (AVMA) guidelines (AVMA, 2013).

Blood samples: Blood was drawn following deep anesthesia. Whole blood samples from each rat were placed in two BD vacutainer blood collection tubes with spray-coated anticoagulant ethylene diamine tetra-acetic acid (K₂EDTA). The 2 mL tube (Cat. # 02-687-98) was used to collect a whole blood sample. A portion of blood in this tube was assigned to hematological

analyses and the other portion used to generate plasma. Immediately following blood collection the 2 mL tube was inverted 8-10 times and placed on a platform rocker at room temperature until hematological analysis was performed (within 5 minutes of blood draw). The remaining portion of blood collected in the 2 mL tube was centrifuged at 1,500 relative centrifugal force (x g) for 20 minutes at room temperature to obtain plasma. The plasma samples were transferred into cryovials on ice and stored at -80 °C for later use in clinical chemistry analyses. Blood in the 6 mL tube (Cat. #13-680-61) was used for acetylcholinesterase and butyrylcholinesterase enzyme activity assays. An aliquot of 500 µL of blood was transferred from each 6 mL tube to a 0.5 mL Eppendorf tube and immediately frozen in liquid nitrogen before being stored at -80 °C for later use in the acetylcholinesterase enzyme assays. The remaining blood in the 6 mL tube was centrifuged at 1,500 x g for 20 minutes at room temperature to obtain plasma. Plasma samples were transferred to cryovials on ice and stored at -80 °C for later use in butyrylcholinesterase enzyme activity assays.

Tissues: Organs of interest were collected, weighed and stored in 10% neutral buffered formalin together with a standard list of tissues for histopathological analysis. The organs of interest included brain, testes, epididymis, uterus, ovary, liver, right and left kidneys, adrenals, spleen, thymus, and heart. Brain and liver samples were also collected into 1.5 mL Eppendorf tubes, immediately frozen in liquid nitrogen, and stored at -80 °C until they could be analyzed for AChE, NTE and phospholipid analyses.

3.5 Hematological Parameters

Blood samples were analyzed immediately after collection. Hematological parameters determined included white blood cells (WBC#), neutrophils (NE# and NE%), lymphocytes (LY# and LY%), monocytes (MO# and MO%), eosinophils (EO# and EO%); basophils (BA# and BA%); total red blood cells (RBC), hemoglobin (Hgb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW%), platelets (PLT) and mean platelet volume (MPV). Each blood sample was analyzed using a Drew Scientific (ERBA) HEMAVET 950 Multispecies Hematology Analyzer and multispecies software (Drew Scientific). Each day of analysis, quality control was assessed according to the manufacturer's protocols.

3.6 Acetylcholinesterase Enzyme Activities in Blood and Tissues

3.6.1. Preparation of Brain and Liver Tissue Lysates

Thawed brain and liver tissue samples were homogenized in the lysis buffer made of Neuronal Protein Extraction Reagent (N-PER™, Cat. # 87792; Thermo Scientific), Halt Protease Inhibitor single-use cocktail (Cat. # 78430; Thermo Scientific) and EDTA mixture using a Bullet Blender® (BB24-AU, Next Advance, Inc., Averill Park, NY, USA) homogenizer. The N-PER™ reagent is designed to optimize the removal of neuronal proteins from neuronal tissues and primary neuronal cells. Brain tissues and liver tissues were thawed, weighed (≈ 50 mg/tissue sample) and placed into the pre-chilled 1.5 mL sterile microcentrifuge RINO™ tubes (TUBE1R5-S), each containing four zirconium oxide beads (ZrOB20; 2.0 mm diameter). Appropriate volumes of the mixture of N-PER™ Neuronal Protein Extraction Reagent (1 mg of tissue/10 µL of N-PER™ reagent), Halt Protease Inhibitor (1 µL of protease inhibitor/100 µL of N-PER™ reagent) and 5

mM EDTA were transferred into the RINO™ tubes. After the sample homogenization in the Bullet Blender® for two minutes, homogenates were incubated on ice for 10 minutes, then centrifuged at 10,000 x g for 10 minutes at 4 °C to pellet the debris. Supernatants were collected into 1.5 mL Eppendorf tubes and stored at - 80 °C for future use in AChE enzyme activity assays.

3.6.2. Acetylcholinesterase Activity Assays in Whole Blood, Brain and Liver Tissues

Whole blood samples were thawed and dilutions (1/40; v/v) were prepared using ddH₂O. The activity of AChE in whole blood, brain and liver tissue lysates was determined using the MAK119 AChE activity assay kit. This assay is an optimized version of the Ellman method ([Ellman *et al.*, 1961](#)) which provides procedure for assessing the AChE levels in a variety of samples (blood, plasma, serum, etc.). In this optimized assay, AChE cleaves thiol ester acetylthiocholine to generate thiocholine and acetate molecules. Then, thiocholine reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form 2-nitro-5-thiobenzoate (TNB), a colorimetric product that can be quantified at 412 nanometers (nm) and the amount is proportional to the AChE activity present in the sample.

The activity of AChE in whole blood, brain and liver tissue lysates was assessed as follows. A total of 10 µL of whole blood sample or tissue lysate (brain or liver) was added in duplicate to a 96-well plate. Then, 190 µL of the freshly prepared working reagent was added to all sample wells; the content of each well was mixed by briefly tapping the plate. This working reagent was prepared by dissolving 2 mg of MAK119C (Cat. # MAK119C) in 200 µL of the assay buffer (Cat. # MAK119A). Water (ddH₂O) and calibrator (Cat. # MAK119B) in a volume of 200 µL each were transferred to separate wells in duplicate to serve as blank and standard samples, respectively. After incubating samples at room temperature for 2 minutes, the initial absorbance was taken at 412 nm [(A₄₁₂)_{initial}] using the FlexStation 3 microplate reader. The final absorbance [(A₄₁₂)_{final}] was measured 10 minutes later. The AChE activity in each sample was calculated using the following equation,

$$\text{AChE activity (Units/L)} = \frac{(A_{412})_{\text{final}} - (A_{412})_{\text{initial}}}{(A_{412})_{\text{Calibrator}} - (A_{412})_{\text{Blank}}} \times D \times 200 \text{ (U/L)}$$

Where U/L = unit(s)/liter, (A₄₁₂)_{final} = the absorbance of the sample at 10 minutes, (A₄₁₂)_{initial} = the absorbance of the sample at 2 minutes, (A₄₁₂)_{Calibrator} = the absorbance of the calibrator (standard) at 10 minutes, (A₄₁₂)_{Blank} = the absorbance of ddH₂O (blank sample) at 10 minutes, D = the dilution factor for the sample and 200 = the equivalent activity (Units/L) of the calibrator when the assay is read at 2 minutes and 10 minutes. One unit of AChE corresponds to the amount of AChE enzyme that catalyzes the production of 1 µmole of thiocholine in a minute at pH of 7.5 at room temperature.

3.6.3. Butyrylcholinesterase Activity Assays in Plasma

The activity of BChE in plasma was determined using BioVision BChE activity assay kit. This assay is based on the ability of BChE to cleave the substrate producing thiocholine and intermediate product. Then, thiocholine reacts with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) to form TNB, a colorimetric product that can be quantified at 412 nm and the amount is proportional to the BChE activity present in the sample. The activity of BChE in plasma samples was assessed as follows. Each sample was briefly vortexed before loading it into the 96-well plate. A total volume of 20 µL of undiluted or diluted (3 to 5-fold dilution) plasma sample was loaded in

duplicate into a 96-well plate. Then, 10 μL of diluted BChE solution (50-fold dilution) was loaded in duplicate into the plate wells to serve as a BChE positive control. A volume of 95 μL of BChE assay buffer was also loaded in duplicate into the plate wells to serve as the background control. The volume for the wells containing the test samples and BChE positive controls was adjusted to 95 μL using BChE assay buffer. The TNB standard curve was generated by adding in duplicate the volume of 0, 2, 4, 6, 8, 10 and 12 μL of the 2.5 millimolars (mM) TNB standard into separate wells of the 96-well plate to yield 0, 5, 10, 15, 20, 25 and 30 nanomoles (nmol) of TNB in these wells. The final volume in the wells containing the TNB standard was brought to 200 μL using BChE assay buffer (we added 200, 198, 196, 194, 192, 190 and 188 μL , respectively). Also, a volume of 5 μL of DTNB solution was added to each well containing the test sample, background control and the BChE positive control, bringing the final volume in each well to 100 μL . At this step, the plate was wrapped in aluminum foil for protection against light and incubated for 10 minutes at room temperature to achieve temperature equilibrium and complete the reaction of sample proteins's sulfhydryl groups" with DTNB. After the incubation, a volume of 100 μL of diluted BChE substrate (120-fold dilution) was added to each well containing the test sample and the BChE positive control to bring the total volume to 200 μL in each well. The volume in the wells assigned to the background control was brought to 200 μL by adding 100 μL of BChE assay buffer. The content in each well was mixed by briefly and carefully tapping the plate prior to the start of read-out. The initial absorbance (OD1) was immediately taken (at t_1) and the final absorbance (OD2) was measured at 10 minutes later (at t_2). Both readings were taken at 412 nm using the FlexStation 3 microplate reader at 412 nm. In order to generate the TNB standard curve, the reading obtained with 0 μL of TNB standard was subtracted from absorbance readings for all other wells that contained this standard (wells that received 2, 4, 6, 8, 10 and 12 μL of the 2.5 mM TNB standard). The TNB standard curve was then generated by plotting these absorbance readings against the concentrations of TNB in these wells (0, 5, 10, 15, 20, 25 and 30 nmol, respectively). The absorbance reading for each sample following 10 minute incubation period (ΔOD) was obtained by subtracting the final absorbance (OD2) from the initial absorbance (sample background reading; OD1). The ΔOD was applied to the TNB standard curve to obtain B nmol of TNB generated during 10 minute reaction time (Δt) and the BChE activity (U/mL/min) in each sample was calculated using the following equation,

$$\text{Sample BChE Activity (nmol/min/mL} = \text{mU/mL)} = \frac{(\text{B test sample} - \text{B sample control})}{\Delta t \times V} \times D$$

where nmol/min/mL = nanomole(s)/minute/liter, mU/mL = milliunit(s)/milliliter, B = TNB amount from standard curve (nmol), Δt = reaction time (min.), V = sample volume (mL) loaded into the reaction well and D = dilution factor. One unit of BChE corresponds to the amount of BChE that generates 1.0 μmol of thiocholine per minute at a pH of 7.4 at room temperature.

3.6.4. Electrophysiology Measurements

3.6.4.1 Hippocampal Slice Preparation

The brain was kept viable after removal by maintaining it in ice-cold artificial cerebrospinal fluid (ACSF) that was kept continuously oxygenated with 95% oxygen (O_2) and 5% carbon dioxide (CO_2). ACSF consisted of (in mM): 124 sodium chloride (NaCl), 3 potassium chloride (KCl), 1.25 monopotassium phosphate (KH_2PO_4), 10 D-Glucose, 1 magnesium sulfate (MgSO_4), 36 sodium bicarbonate (NaHCO_3) and 2 calcium chloride (CaCl_2) (pH $\sim$ 7.4). For hippocampal slices, cerebellum and approximately 1 cm of frontal cortex were removed and the remaining brain

was sectioned at 350 μm thick using a vibratome (VT1000S Leica Microsystems) in the transverse plane, at approximately 30° laterally off the horizontal axis. Brain slices were maintained in warmed (37°C) oxygenated ACSF and allowed to recover for at least 60 min prior to recording. A new batch of ACSF was prepared each morning of experimentation and continuously oxygenated with 95% O₂ and 5% CO₂.

3.6.4.2 Electrophysiology Recordings

All electrophysiology data were obtained using AlphaMed's MED64 Quad II systems (Automate, Berkeley, CA). Probes were initially pretreated with 0.1% polyethylenimine (PEI) in 25-mM borate buffer for at least 24 hours prior to use. A hippocampal slice was placed onto the pre-coated microelectrode array and positioned such that the neurons from the CA1 region were recorded. A perfusion cap was used to circulate fresh oxygenated warmed ACSF into the probe and prevent the slice from drying. The flask containing the ACSF solution was continuously kept in a 37°C water bath. Flow rates of ACSF were adjusted such that the brain slice was constantly maintained in a liquid-air interphase. Typical flow rates range between 0.5 -1.0 ml/min. In order to deliver warmed, humidified oxygen to the brain slice, a line carrying 95% O₂ and 5% CO₂ was passed through a heated water-containing flask before entering the MED64 probe.

Data acquisition and stimulation protocols were performed using Mobius software (Automate, Berkeley, CA). A biphasic stimulating current of 10-120 μA was applied to the Schaffer collateral of the hippocampus to obtain an input-output relationship curve. Evoked field potentials mainly consisting of inward deflections were recorded from neurons within the CA1 region of the hippocampus at a sampling frequency of 20 kilohertz (kHz) using the Quad II multichannel amplifier, digitized and graphically displayed using Mobius software. Paired pulse facilitation (PPF) were obtained by delivering sets of two consecutive stimuli of equal intensity that were 40 milliseconds (ms) apart. Degrees of PPF were quantified by calculating the paired pulse (PP) ratio in which the response amplitude from the second stimulating pulse was divided by the response amplitude from the first pulse. Thus, a PP ratio of >1 indicates PPF whereas a PP ratio of <1 indicates paired pulse depression (PPD). Spontaneous spiking activity of neurons within the CA1 region were recorded for 10 minutes and frequency of firing was calculated using the Mobius software. A threshold value of 5 times the standard deviation was used to calculate spike frequency.

For long term potentiation (LTP) experiments we use the input-output function to determine the size of the stimulating current that resulted in half of the maximal output response. Typically, a stimulating current size of 30-50 μA induced a half-maximal response and thus was used for our LTP experiments. Baseline recording was obtained for each slice for at least 20 min prior to LTP measurements. LTP was induced by delivering 3 trains of theta burst stimulation (TBS), consisting of 10 repeats of 4 high frequency stimulations (100 hertz (Hz)) every 200 ms to the Schaffer collateral regions. Evoked responses in the forms of field excitatory postsynaptic potentials (fEPSP) and population spikes were monitored at 12 second intervals for at least 20 minutes following LTP induction. Percent potentiation was calculated by computing the percent difference in population spike amplitude or fEPSP slope at 30 min following LTP induction by TBS from baseline. Averages of 10 data points were calculated to obtain baseline and LTP values.

3.7 Phosphorus-31 NMR (³¹p NMR) of Grade 3 and 4 Aircraft Engine Oils

Aircraft engine Oil samples, both new and used, were diluted with deuteriochloroform (CDCl₃) and the final composition of CDCl₃:oil ratio in NMR tube was set to 60:40. A 280 µL aliquot of undiluted oil was mixed with 320 µL of CDCl₃ and the mixture was transferred into a 5 mm NMR tube. A 100 µL aliquot of phosphatidylcholine (PC) solution (34 mM of PC) prepared using CDCl₃ was transferred to the NMR tube containing oil to serve as a chemical shift reference and an internal standard for quantification of the phosphorus metabolites. The ³¹P NMR spectra of oils were acquired using a 5 mm broadband probe operating at 242.8 MHz on a Varian Inova 600 NMR Spectrometer, applying a 90° pulse (10.4 µsecond pulse width) and 0.8 second acquisition time with a 15.8 second interpulse delay (which was long enough to allow full T1 relaxation). The acquisition signal was averaged for more than 2 h to achieve a spectrum with sufficient signal-to-noise ratio (S/N). No proton (¹H) decoupling was needed. Spectral ³¹P data were Fourier transformed and the baseline corrected (flattened) using a 5th order polynomial and spline peak-fitting routine. The chemical shifts were referenced to PC set at 0.0 parts per million (ppm). NMR peaks were fit using a Lorentzian function fitting routine (deconvolution tool) in the Varian software. Quantitative measures of each organophosphate were determined based on the peak area of PC and on its known concentration in each ³¹P NMR run sample.

3.8 Statistical Analysis

Levine's and Welch's tests were performed to assess the equality of variances between experimental groups for each measurement using JMP® software, version 11.0 for Windows (SAS Institute, Cary, NC, USA). If Levine's test was significant (probability (*p*) less than or equal to (≤) 0.05), then a Welch's test was conducted to assess if there were significant differences in the mean values between groups for each measurement. If the Levine's test was not significant, then the data were considered to have equal variances (Welch's test was not applicable), and significance was tested using one-way analysis of variance (ANOVA). If both Levine's and Welch's tests were significant (*p* ≤ 0.05), then a pairwise Welch test was performed for all pairs of groups. Results shown in figures and tables are expressed as mean plus or minus (±) standard error of the mean (S.E.) and are considered statistically significant at *p* ≤ 0.05.

For hematology values, we also calculated the effect size or Cohen's *d* (magnitude of change) (Cohen, 1988) induced by each treatment by subtracting the values obtained for the control group from those obtained for the oil treated groups and assessing the difference relative to the pooled standard deviations for oil treated groups and control group as shown in Equation 1,

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

where (X)_T and (X)_C are the blood parameter values for oil treated and control groups, respectively, while (σ)_T and (σ)_C are the standard deviations values for the means in oil treated and control groups, respectively. Effect size values were graded as large (*d* = |0.8|) based on Cohen's effect size (*d*) classification (Cohen, 1988).

Analyses for electrophysiology endpoints were performed using Mobius software. The downward responses (negative, inward deflections) were used to calculate the degree of evoked response. In the input-output, paired pulse and LTP analyses, amplitudes and initial slopes of

population spikes and field potentials were calculated to quantitate the size of evoked response. Amplitude and slope calculations were performed using Mobius software, Excel and Igor. Neuronal activity was recorded from typically 14 microelectrodes within the MED64 probe chamber. For any given slice, data obtained from multiple microelectrodes covering one defined brain region (eg. CA1) were averaged together to yield the response value for that particular slice. Because we only had one animal per experimental group, we did not perform statistical analysis among the different groups.

4.0 RESULTS

4.1 Organophosphate Contents of Aircraft Engine Oils

The ^{31}P NMR analyses were performed on unused and used versions of grade 3 (G3-N and G3-U) and grade 4 (G4-N and G4-U) aircraft engine oils and the results of these analyses are shown in [Fig. 3](#) and [Table 4](#). With four different TCP isomers present at detectable levels in both versions of grade 3 oil, two were more predominant ([Fig. 3A](#)). The concentration for all of the four TCP isomers combined in G3-N was 30.0 mM while that in G3-U amounted to 22.2 mM, representing a 26% decrease relative to G3-N ([Table 4](#)). Both versions of grade 4 contained TPP and PIP ([Fig. 3B](#)). The concentration of TPP in G4-N was 12.7 mM while that in G4-U was 9.9 mM, representing a 22% decrease relative to G4-N ([Table 4](#)). G4-N contained 37.6 mM of PIP while G4-U comprised 35.2 mM, representing a 6.4% decrease relative to G4-N. These data show that TPP in G4-U was depleted to a greater extent than PIP.

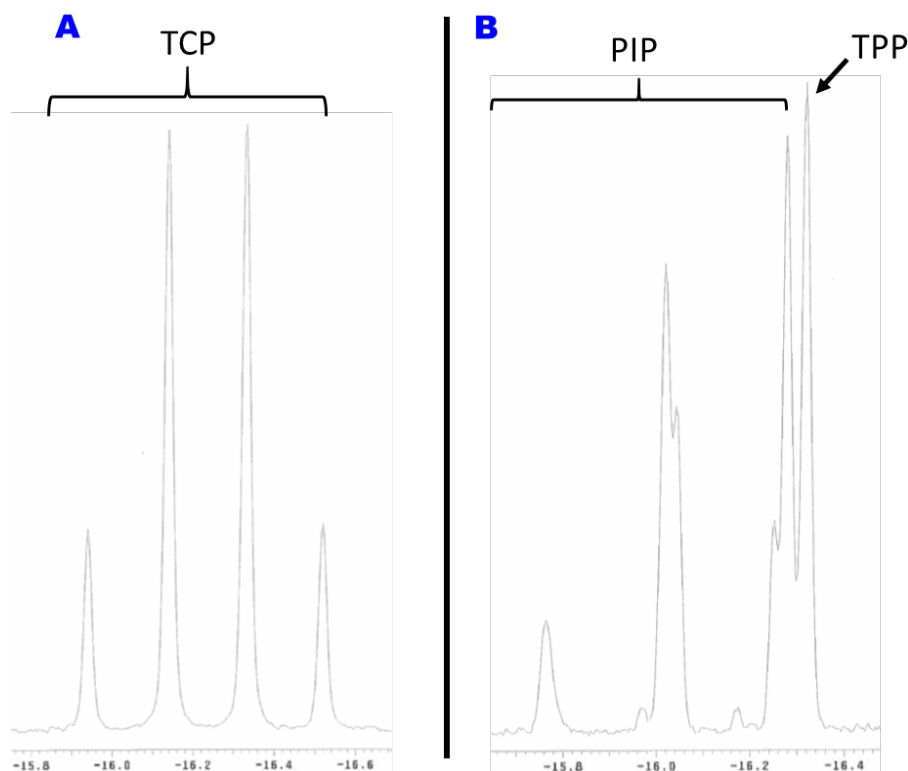


Figure 3. ^{31}P NMR profiles of grade 3 and grade 4 aircraft engine oils

^{31}P NMR profiles of (A) grade 3 and (B) grade 4 aircraft engine oils. TCP, tricresyl phosphates; PIP, phenol isopropylated phosphate (3:1); TPP, triphenyl phosphate.

Table 4. Organophosphate contents of grade 3 and 4 of aircraft engine oils

Organophosphates	Versions of grade 3 and 4 aircraft engine oils			
	G3-N	G3-U*	G4-N	G4-U*
TCP (mM)	30.0	22.2 (-26.0%)	Not found	Not found
TPP (mM)	Not found	Not found	12.7	9.9 (-22.0%)
PIP (mM)	Not found	Not found	37.6	35.2 (-6.4%)

*Changes (%) relative to new oils; TCP isomers were not found in G4 oils; TPP and PIP were not found in G3 oils.

4.2 Effects of Grade 3 and 4 Aircraft Engine Oils on Food Intake for SD Rats

Figures 4 & 5 show data on the weekly food intake for male and female rats exposed to grade 3 and 4 engine oils. It is worth noting that exposure to these oils did not significantly impact the weekly measured food intake, regardless of sex and oil's state.

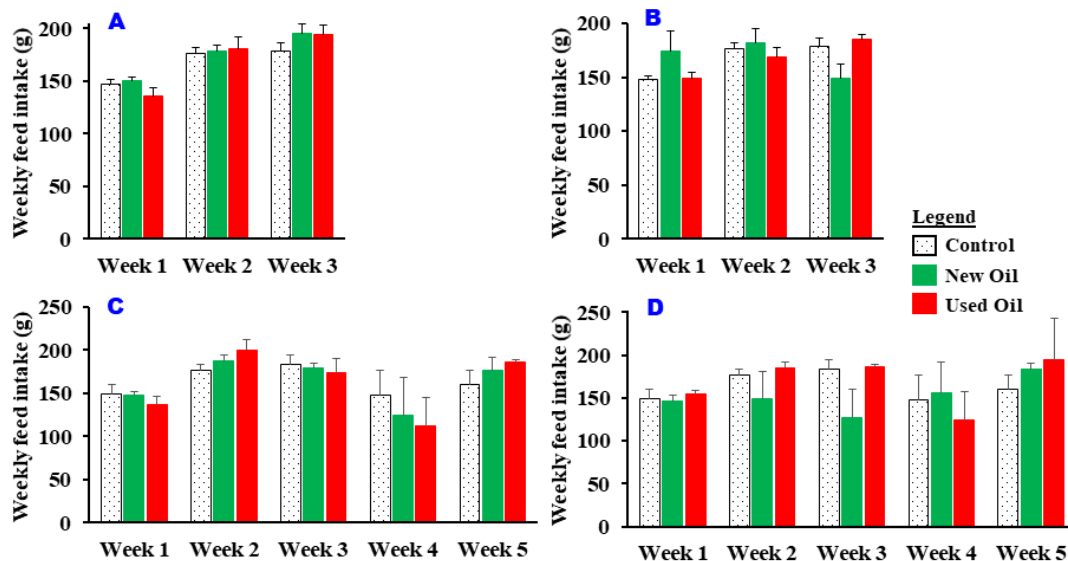


Figure 4. Weekly food intake for male rats exposed to grade 3 and 4 aircraft engine oils

Male rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Males exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 9-11$). Males in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$).

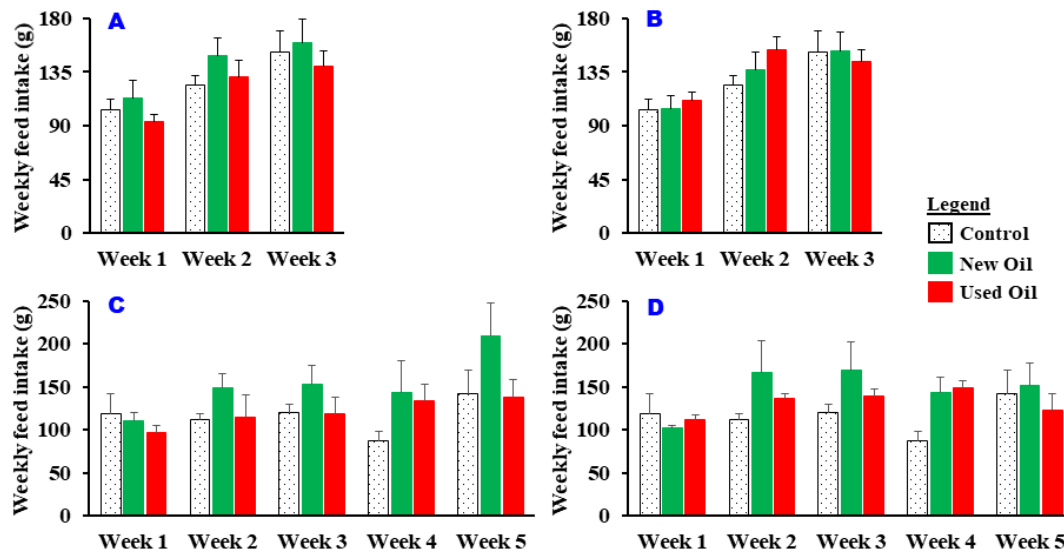


Figure 5. Weekly food intake for female rats exposed to grade 3 and 4 aircraft engine oils

Female rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Females exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 10). Females in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5).

4.3 Effects of Grade 3 and 4 Aircraft Engine Oils on Water Consumption for SD Rats

As shown in **Fig. 6 & 7**, exposure to G3 and G4 engine oils had no effect on the rat's water consumption, regardless of sex and oil's state.

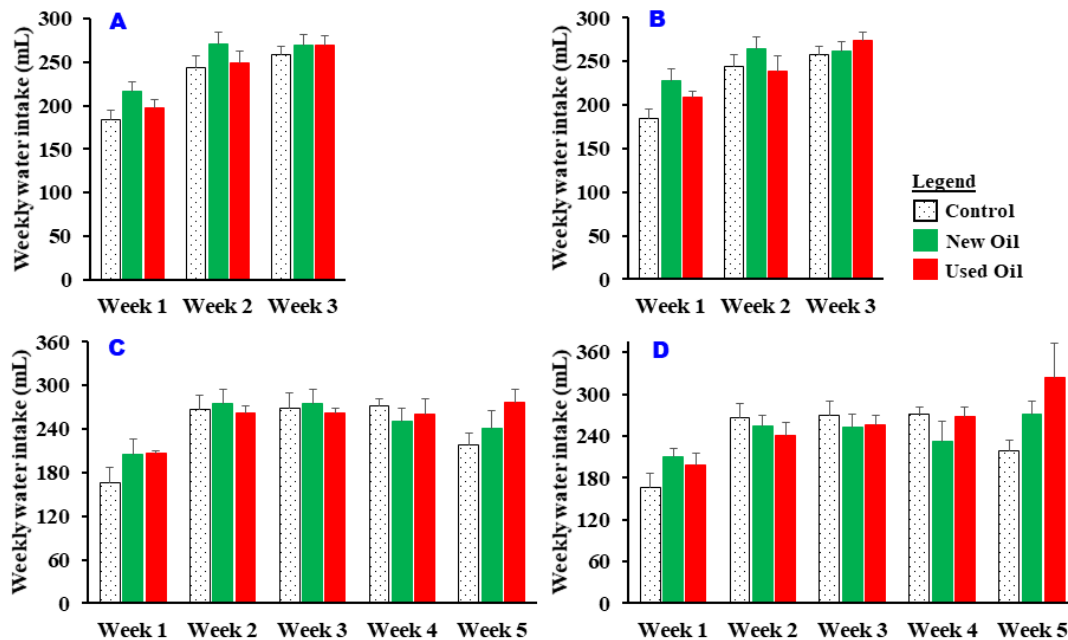


Figure 6. Weekly water intake for male rats exposed to grade 3 and 4 aircraft engine oils

Male rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Males exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 9-11). Males in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5).

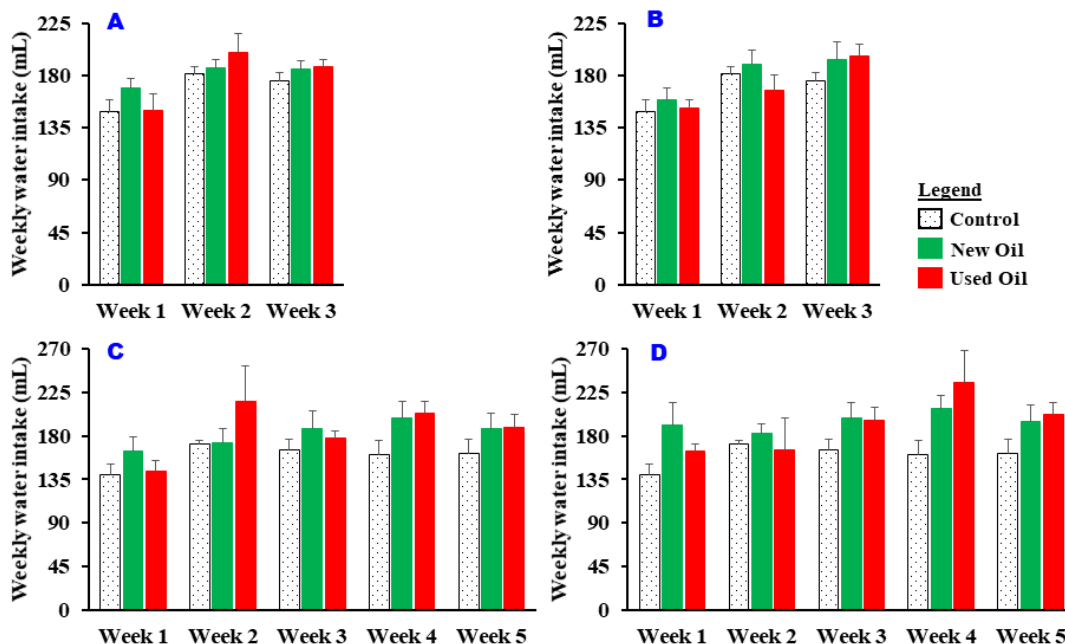


Figure 7. Weekly water intake for female rats exposed to grade 3 and 4 aircraft engine oils

Female rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Females exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 10). Females in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5).

4.4 Effects of Aircraft Engine Oils on Body Weights for SD Rats

Prior to oil exposure, rats involved in this study were 8-9 weeks old. As shown in **Figs. 8A-B & 9A-B**, the initial weights for male and female controls were 322.0 ± 6.8 grams (g) and 196.6 ± 7.6 g, respectively. At the end of the treatment period, they were about 11-12 weeks old and weighed 417.8 ± 7.6 g and 234.9 ± 4.8 g, respectively. The initial weights for control rats assigned to the recovery assessment were 324.8 ± 4.2 g and 203.7 ± 10.7 g, respectively (**Figs. 8C-D & 9C-D**). At the end of the recovery period, they were about 13-14 weeks old and weighed 460.7 ± 19.9 g and 252.5 ± 10.8 g, respectively. Exposure to grades 3 and 4 aircraft engine oils in both new and used states over a period of 21 days (15 total exposures) did not have a significant impact on the body weights for both male and female rats (**Figs. 8A-B & 9A-B**). Over the three week period, male rat controls gained an overall mean $\pm$ SE of $27 \pm 4\%$ relative to their initial body weight (prior to treatments). Males exposed to G3-N gained $25 \pm 3\%$ in body weight. Similarly, those exposed to G3-U gained $25 \pm 3\%$ in body weight while those exposed to G4-N and G4-U increased their weights by $29 \pm 2\%$ and $25 \pm 3\%$, respectively. Female control rats gained $14 \pm 3\%$ in body weight during the three week period. Female rats treated with G3-N and G3-U increased their weights by $13 \pm 3\%$ and $9 \pm 5\%$, respectively. Those exposed to G4-N gained $13 \pm 2\%$ in body weight as did those exposed to G4-U. It is noteworthy to highlight that rats exposed to these oils and fasted for 12 h tended to lose more weight when compared to controls, regardless of their sex

(Figs. 8A-B & 9A-B). Interestingly, this trend was not observed for rats assigned to the recovery groups and subjected to similar treatments (Figs. 8C-D & 9C-D). Over a period of five weeks, male control rats in the recovery group gained $42 \pm 5\%$ of their initial body weights. Males exposed to G3-N and G3-U gained $43 \pm 5\%$ and $48 \pm 4\%$, respectively. Those exposed to G4-N and G4-U increased their body weight by $44 \pm 2\%$ and $47 \pm 2\%$, respectively. Female control rats gained $24 \pm 3\%$ in body weight during the five weeks period. Females exposed to G3-N and G3-U gained $29 \pm 2\%$ and $22 \pm 2\%$ of their initial body weights, respectively. Those exposed to G4-N and G4-U gained $21 \pm 7\%$ and $25 \pm 3\%$, respectively. Both males and females did not show any delayed effect on body weights that could be associated with exposure to these oils (Figs. 8C-D & 9C-D).

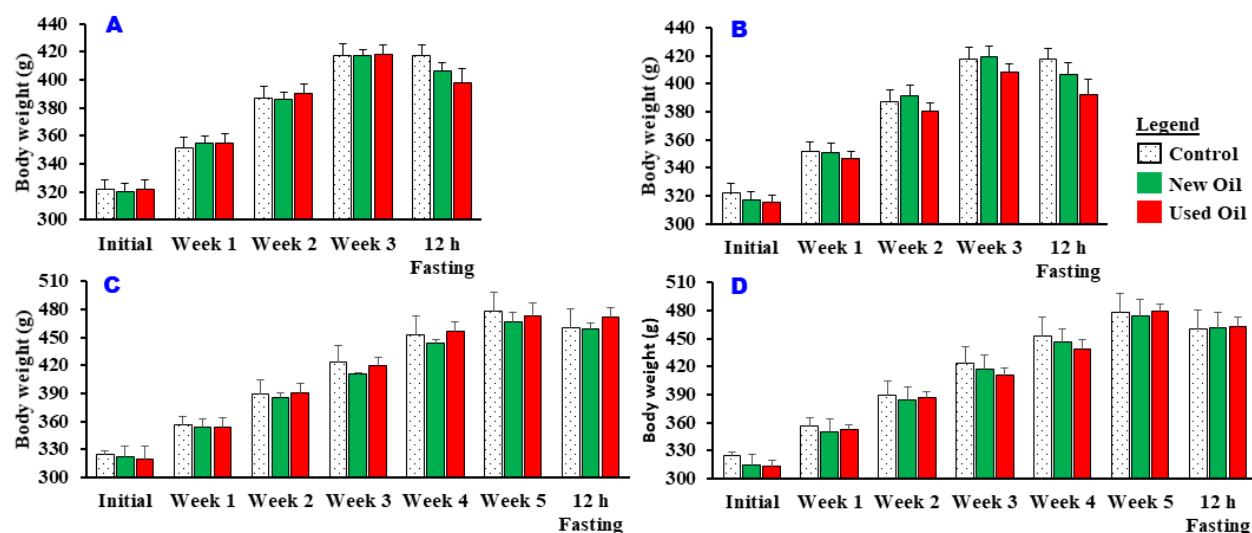


Figure 8. Body weights for male rats exposed to grade 3 and 4 aircraft engine oils

Male rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Males exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 9-11$). Males in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$).

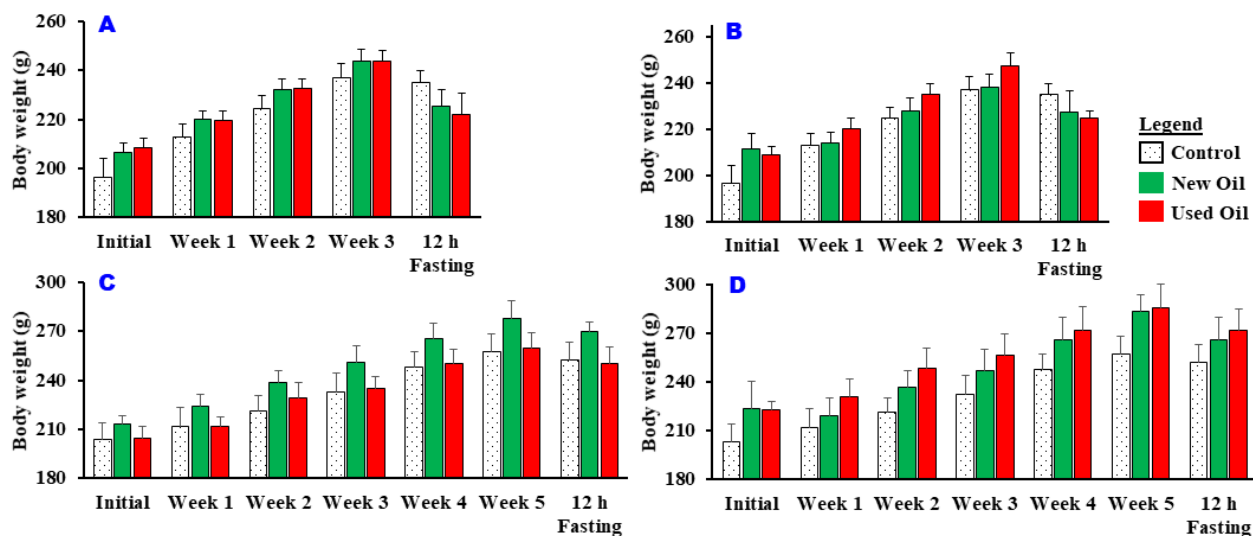


Figure 9. Body weights for female rats exposed to grade 3 and 4 aircraft engine oils

Body weights for female rats dermally exposed to unused (new; green bars) and used (red bars) versions of grade 3 and grade 4 aircraft engine oils. Body weights for females exposed to (A) grade 3 and (B) grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Body weights for females in the recovery groups exposed to (C) grade 3 and (D) grade 4 engine oils over a period of 21 days (15 total exposures) and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$).

4.5 Effects of Aircraft Engine Oils on Organ Weights for SD Rats

4.5.1. Grade 3 Aircraft Engine Oil Impacts the Reproductive Organ Weights of SD Rats

As shown in Fig. 10A, exposing male rats to both versions of G3 (G3-N and G3-U) oil over a period of 21 days did not impact the brain weights. Additionally, keeping these rats for 14 days post-treatment did not indicate any delayed treatment effect on brain weights (Fig. 10D). Similar results were also obtained for female rats (Figs. 11A & 11D). Therefore, all organ weights were normalized to brain weights and the data are herein shown as organ/brain weights (Figs. 10 & 11; Tables 5-6; see Appendices A-D for non-normalized organ weights).

Data obtained at the end of 21-day treatment period indicate that exposure to both versions of grade 3 oil did not affect testes and epididymis weights (Figs. 10B & 10C). A delayed treatment effect observed during the recovery period was only associated with exposure to G3-N (Fig. 10E). Male rats treated with this oil had significantly decreased testes weights relative to controls. Although the testes weights for rats exposed to G3-U were similar to those obtained for the controls (Fig. 10E), they were statistically higher relative to those for G3-N-treated rats. No delayed effect on epididymis weights was observed with G3 treated rats (Fig. 10F).

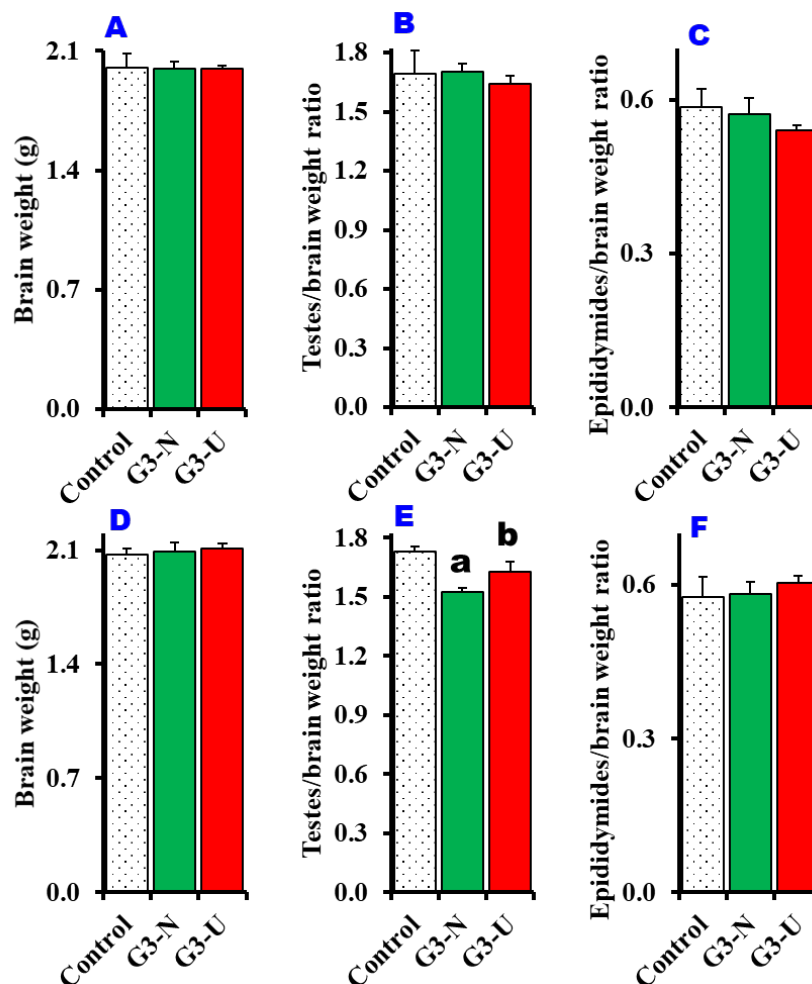


Figure 10. Brain weights and reproductive organ weight/brain weight ratios for male rats exposed to grade 3 aircraft engine oils

Male rats assigned to main study and recovery groups and dermally exposed to grade 3 aircraft engine oils. (A) brain weights, (B) testes/brain weight and (C) epididymis/brain weight ratios for male rats exposed to unused (green bars) and used (red bars) versions of grade 3 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 9-11$). (D) brain weights, (E) testes/brain weight and (F) epididymis/brain weight ratios for male rats in the recovery groups that received similar treatments and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between groups are indicated by letters: a, differs from control group; b, differs from G3-N treated group; $p \leq 0.05$.

As shown in Fig. 11B, all versions of G3 oil significantly ($p \leq 0.05$) decreased the uterus weights at the end of 21 days of exposure period relative to controls. During the recovery period, the uterus weights for rats treated with G3-N were resolved. Those for rats exposed to G3-U remained significantly ($p \leq 0.05$) decreased as compared to controls group (Fig. 11E). Data obtained at the end of 21-day treatment period and at two weeks post exposure indicate that both versions of grade 3 oil did not affect ovary weights (Figs. 11C & 11F).

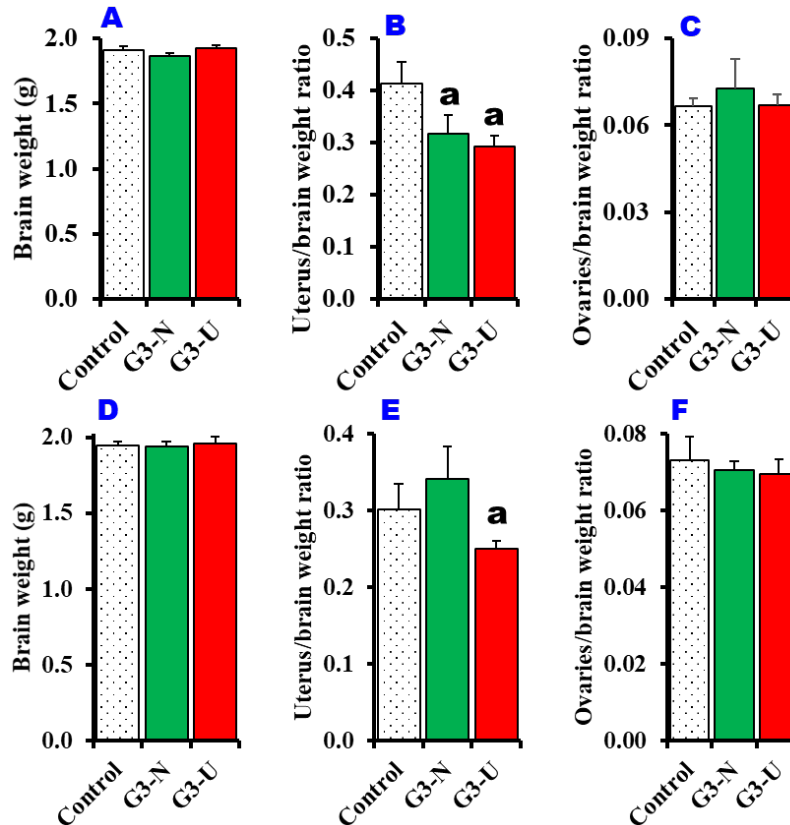


Figure 11. Brain weights and reproductive organ weight/brain weight ratios for female rats exposed to grade 3 aircraft engine oils

Female rats assigned to main study and recovery groups and dermally exposed to grade 3 aircraft engine oils. (A) brain weights, (B) uterus/brain weight and (C) ovary/brain weight ratios for female rats exposed to unused (green bars) and used (red bars) versions of grade 3 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 10). (D) brain weights, (E) uterus/brain weight and (F) ovary/brain weight ratios for female rats in the recovery groups that received similar treatments and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5). Significant differences between groups are indicated by letter “a”; $p \leq 0.05$.

4.5.2. Grade 4 Aircraft Engine Oil Impacts the Reproductive Organ Weights of SD Rats

Data shown in Fig. 12A indicate that exposing male rats to both versions of G4 oil over a period of 21 days did not impact their brain weights. Additionally, keeping these rats for 14 days post-treatment did not indicate any delayed treatment impact on brain weights (Fig. 12D). Similar results were also obtained for female rats exposed to these oils (Figs. 13A & 13D). Therefore, all organ weights were normalized to brain weights and the data are herein shown as organ/brain weights (Figs. 12 & 13; Tables 7-8; see Appendices A-D for non-normalized organ weights).

Data obtained at the end of 21-day treatment period indicate that exposure to both versions of grade 4 oil did not have an effect on the weights for testes (Fig. 12B). However, a delayed treatment

effect was observed at the end of the recovery period and was only associated with exposure to G4-N (Fig. 12E). Male rats treated with this oil had significantly decreased testes weights relative to controls. No delayed effect on testes weights was observed with G4-U treated rats (Fig. 12E). Although both versions of G4 oil significantly ($p \leq 0.05$) decreased epididymis weights during the 21-day exposure period relative to those obtained with control group (Fig. 12C), this effect was reversed at the end of two weeks of the recovery period (Fig. 12F).

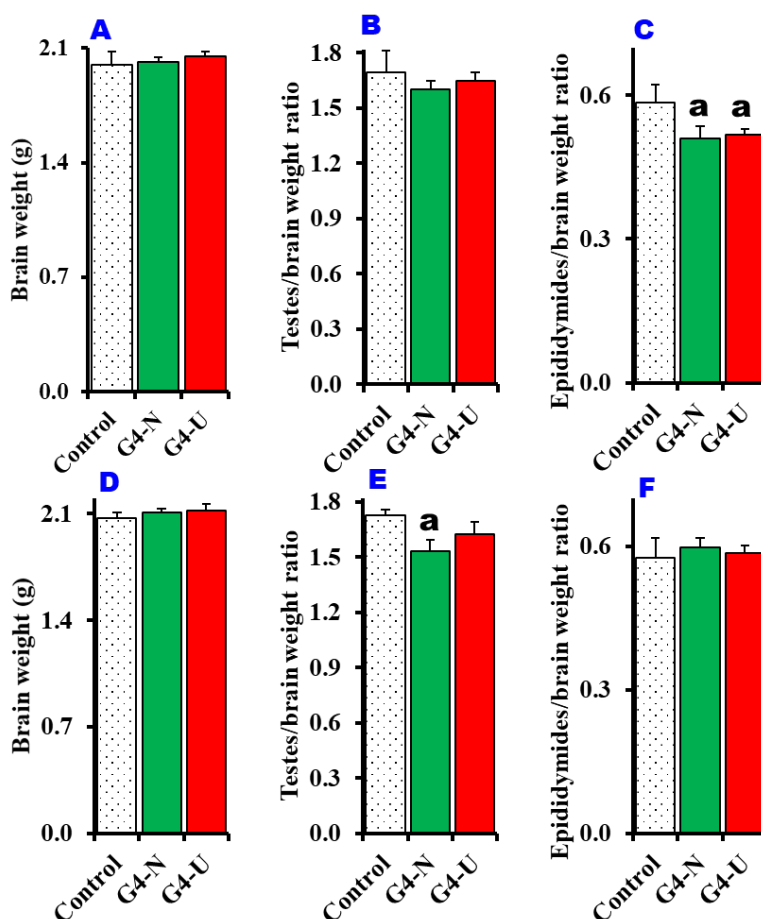


Figure 12. Brain weights and reproductive organ weight/brain weight ratios for male rats exposed to grade 4 aircraft engine oils

Male rats assigned to main study and recovery groups and dermally exposed to grade 4 aircraft engine oils. (A) brain weights, (B) testes/brain weight and (C) epididymis/brain weight ratios for male rats exposed to unused (green bars) and used (red bars) versions of grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). (D) brain weights, (E) testes/brain weight and (F) epididymis/brain weight ratios for male rats in the recovery groups that received similar treatments and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between groups are indicated by letter “a”; $p \leq 0.05$.

Fig. 13B shows that the unused version of G3 oil significantly ($p \leq 0.05$) decreased the uterus weights at the end of 21 days of treatment period relative to controls. However, this effect was resolved at the end of the recovery period (**Fig. 13E**). G4-U did not have an effect on the uterus weights (**Figs. 13B & 13E**). Exposure to both versions of G4 oil had no impact on ovary weights, regardless of the time of data collection (**Figs. 10C & 4F**).

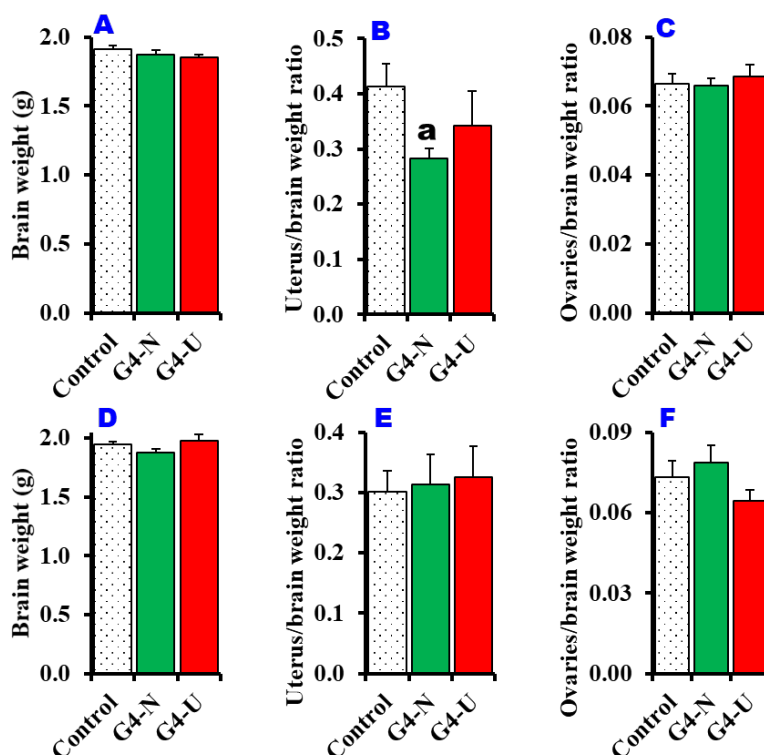


Figure 13. Brain weights and reproductive organ weight/brain weight ratios for female rats exposed to grade 4 aircraft engine oils

Female rats assigned to main study and recovery groups and dermally exposed to grade 4 aircraft engine oils. (A) brain weights, (B) uterus/brain weight and (C) ovary/brain weight ratios for female rats exposed to unused (green bars) and used (red bars) versions of grade 4 engine oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). (D) brain weights, (E) uterus/brain weight and (F) ovary/brain weight ratios for female rats in the recovery groups that received similar treatments and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between groups are indicated by letter “a”; $p \leq 0.05$.

4.5.3. Aircraft Engine Oils Affect Weights of Liver, Adrenals and Spleen of SD Rats

The weights for liver, right and left kidneys, adrenals, spleen, thymus and heart were also taken at the end of 21 days of exposure to G3 and G4 oils and at the end of the 14 days recovery period (**Tables 5-8**). Data obtained at the end of the treatment period indicate that G3 and G4 oils did not significantly influence the weights for these organs when compared to controls, regardless of sex (**Tables 5-8**). It is worthy to point out that G4-U slightly increased the weight of the adrenals

for females during treatment period as compared to those obtained for G4-N group (Table 8). There was no delayed effect associated with exposing males to G3 and G4 oils (Tables 5 & 7). However, exposure to G3-N elevated the liver weights for females during the recovery period compared to both control and G3-U groups (Table 6). During this period, G4-N increased the weight of the adrenals and spleen for females relative to those obtained for controls (Table 8). The adrenal weights for G4-N group were also elevated when assessed against those obtained with G4-U group. G4-U exposure yielded an increase in spleen weights relative to those obtained for controls (Table 8).

Table 5. Brain weights and organ weight/brain weight ratios for male rats exposed to grade 3 aircraft engine oils

Organs	Main study groups			Recovery groups		
	Control (n = 10)	G3-N* (n = 9)	G3-U* (n = 11)	Control (n = 5)	G3-N (n = 5)	G3-U (n = 5)
Liver	6.00 ± 0.37	5.82 ± 0.21	5.97 ± 0.22	6.63 ± 0.61	6.52 ± 0.42	6.34 ± 0.27
L. kidney	0.73 ± 0.02	0.70 ± 0.02	0.73 ± 0.02	0.75 ± 0.05	0.72 ± 0.03	0.76 ± 0.03
R. kidney	0.75 ± 0.03	0.71 ± 0.02	0.75 ± 0.02	0.77 ± 0.05	0.73 ± 0.04	0.79 ± 0.03
Adrenals	0.027 ± 0.002	0.025 ± 0.002	0.024 ± 0.002	0.023 ± 0.003	0.023 ± 0.002	0.024 ± 0.002
Spleen	0.42 ± 0.02	0.37 ± 0.01	0.38 ± 0.02	0.42 ± 0.03	0.41 ± 0.02	0.39 ± 0.02
Thymus	0.26 ± 0.02	0.23 ± 0.03	0.23 ± 0.02	0.19 ± 0.04	0.26 ± 0.03	0.23 ± 0.03
Heart	0.60 ± 0.03	0.59 ± 0.02	0.59 ± 0.02	0.61 ± 0.05	0.65 ± 0.06	0.59 ± 0.02

*Sample sizes for G3-N and G3-U groups were 9 and 11 rats, respectively.

Organ weight to brain weight ratios for male SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G3-N) and used (G3-U) versions of grade 3 of the aircraft engine oils over a period of 21 days. Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean ± S.E.).

Table 6. Brain weights and organ weight/brain weight ratios for female rats exposed to grade 3 aircraft engine oils

Organs	Main study groups			Recovery groups		
	Control (n = 10)	G3-N (n = 10)	G3-U (n = 10)	Control (n = 5)	G3-N (n = 5)	G3-U (n = 5)
Liver	3.61 ± 0.11	3.88 ± 0.20	4.00 ± 0.22	3.92 ± 0.19	4.44 ± 0.10 ^a	3.96 ± 0.13 ^b
L. kidney	0.46 ± 0.01	0.47 ± 0.02	0.48 ± 0.02	0.45 ± 0.01	0.49 ± 0.02	0.48 ± 0.01
R. kidney	0.47 ± 0.01	0.48 ± 0.02	0.50 ± 0.02	0.49 ± 0.02	0.52 ± 0.02	0.50 ± 0.01
Adrenals	0.035 ± 0.002	0.032 ± 0.002	0.034 ± 0.002	0.032 ± 0.002	0.036 ± 0.003	0.034 ± 0.003
Spleen	0.27 ± 0.02	0.29 ± 0.01	0.29 ± 0.02	0.26 ± 0.02	0.33 ± 0.03	0.27 ± 0.03
Thymus	0.20 ± 0.02	0.22 ± 0.02	0.21 ± 0.03	0.18 ± 0.03	0.27 ± 0.04	0.20 ± 0.02
Heart	0.41 ± 0.01	0.43 ± 0.02	0.41 ± 0.01	0.43 ± 0.01	0.45 ± 0.01	0.41 ± 0.02

^asignificant differences relative to control group ($p \leq 0.05$); ^bsignificant differences relative to G3-N group ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data.

Organ weight to brain weight ratios for female SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G3-N) and used (G3-U) versions of grade 3 of the aircraft engine oils over a period of 21 days. Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean ± S.E.).

Table 7. Brain weights and organ weight/brain weight ratios for male rats exposed to grade 4 aircraft engine oils

Organs	Main study groups			Recovery groups		
	Control (n = 10)	G4-N (n = 10)	G4-U (n = 10)	Control (n = 5)	G4-N (n = 5)	G4-U (n = 5)
Liver	6.00 ± 0.37	6.30 ± 0.40	5.48 ± 0.26	6.63 ± 0.61	6.84 ± 0.76	6.56 ± 0.46
L. kidney	0.73 ± 0.02	0.70 ± 0.02	0.68 ± 0.03	0.75 ± 0.05	0.72 ± 0.03	0.76 ± 0.05
R. kidney	0.75 ± 0.03	0.74 ± 0.03	0.69 ± 0.02	0.77 ± 0.05	0.73 ± 0.04	0.79 ± 0.05
Adrenals	0.027 ± 0.002	0.024 ± 0.001	0.025 ± 0.002	0.023 ± 0.003	0.026 ± 0.002	0.030 ± 0.002
Spleen	0.42 ± 0.02	0.40 ± 0.02	0.38 ± 0.02	0.42 ± 0.03	0.41 ± 0.02	0.41 ± 0.02
Thymus	0.26 ± 0.02	0.23 ± 0.02	0.22 ± 0.03	0.19 ± 0.04	0.23 ± 0.03	0.20 ± 0.04
Heart	0.60 ± 0.03	0.58 ± 0.01	0.51 ± 0.05	0.61 ± 0.05	0.61 ± 0.01	0.57 ± 0.02

Organ weight to brain weight ratios for male SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days. Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean ± S.E.).

Table 8. Brain weights and organ weight/brain weight ratios for female rats exposed to grade 4 aircraft engine oils

Organs	Main study groups			Recovery groups		
	Control (n = 10)	G4-N (n = 10)	G4-U (n = 10)	Control (n = 5)	G4-N (n = 5)	G4-U (n = 5)
Liver	3.61 ± 0.11	3.75 ± 0.16	3.72 ± 0.25	3.92 ± 0.19	4.47 ± 0.27	4.13 ± 0.15
L. kidney	0.46 ± 0.01	0.46 ± 0.02	0.48 ± 0.01	0.45 ± 0.01	0.51 ± 0.02	0.47 ± 0.01
R. kidney	0.47 ± 0.01	0.48 ± 0.02	0.49 ± 0.02	0.49 ± 0.02	0.54 ± 0.01	0.50 ± 0.01
Adrenals	0.035 ± 0.002	0.030 ± 0.002	0.036 ± 0.002 ^b	0.032 ± 0.002	0.042 ± 0.004 ^a	0.033 ± 0.002 ^b
Spleen	0.27 ± 0.02	0.28 ± 0.01	0.28 ± 0.02	0.26 ± 0.02	0.33 ± 0.02 ^a	0.34 ± 0.04 ^a
Thymus	0.20 ± 0.02	0.20 ± 0.02	0.18 ± 0.02	0.18 ± 0.03	0.19 ± 0.02	0.24 ± 0.03
Heart	0.41 ± 0.01	0.41 ± 0.01	0.43 ± 0.02	0.43 ± 0.01	0.45 ± 0.01	0.46 ± 0.03

^asignificant differences relative to control group ($p \leq 0.05$); ^bsignificant differences relative to G4-N group ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data.

Organ weight to brain weight ratios for female SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days. Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean ± S.E.).

4.6 Effects of Grade 3 and 4 Aircraft Engine Oils on Hematological Parameters of SD Rats

Data describing hematological indices for male and female SD rats following a 21-day exposure to G3 and G4 engine oils and 14 days recovery period are shown in [Table 9-12](#). To more clearly illustrate the levels of changes in blood indices induced by exposure to these oils, we calculated the effect size or Cohen's d (magnitude of change induced by each treatment relative to control). Contrary to p values that are dependent on the sample size and are less likely to be statistically significant when the sample size is small and more likely if the sample size is large, effect sizes are not influenced by sample size and provide information about the direction (decrease or increase) and strength of the relationship between variables ([Berben et al., 2012](#)). The equation used for calculation of effect sizes for each blood parameter is shown in the Methods and [Tables 9-12](#) show effect size results in parentheses.

4.6.1. Effects of Grade 3 Aircraft Engine Oils on Blood Indices for SD Rats

Hematology data for male SD rats following a 21-day exposure to G3 engine oils and those obtained after 14 days recovery period are shown in [Table 9](#). G3-N significantly increased ($p \leq 0.05$) the levels of eosinophil count and eosinophil % for males compared to those for controls ([Table 9](#)). As shown in parentheses for this table this treatment induced large effect sizes for eosinophils ($d = 1.1$ and $d = 1.0$ for eosinophil count and eosinophil %, respectively), hematocrit ($d = 0.8$) and platelets ($d = -0.8$). The impact of G3-U exposure on blood parameters for male rats was relatively moderate as compared to that induced by G3-N treatment. G3-U did not induce significant changes in blood parameters and no large effect size ($d \geq |0.8|$) was obtained with this treatment. Although the levels of all blood parameters in G3-U treated group were similar to those obtained for controls, eosinophil levels were significantly decreased ($p \leq 0.05$) relative to those

obtained for male rats exposed to G3-N. During the recovery period, the levels of eosinophils and basophils for G3-N group fell below the instrument's limit of detection and were not measured (**Table 9**). As shown in this table, G3-N treatment induced a delayed effect of significant increase ($p \leq 0.05$) in white blood cell and lymphocyte counts for males. This treatment also induced large effect sizes for the numbers of white blood cells, lymphocytes and monocytes ($d = 2.0$, $d = 2.7$, and $d = 0.9$, respectively). Although males exposed to G3-U did not show a significant delayed impact on their hematological indices when compared to controls, this treatment induced large changes for white blood cells ($d = 1.0$), lymphocyte count ($d = 0.9$) and eosinophil % ($d = 0.9$) cells. Additionally, the numbers of lymphocytes under this treatment was significantly reduced ($p \leq 0.05$) relative to that obtained for G3-N group.

Hematology data for female SD rats following a 21-day exposure to G3 engine oils and those obtained after 14 days recovery period are shown in **Table 10**. Females exposed to G3-N had significantly decreased ($p \leq 0.05$) levels of white blood cells, lymphocyte count and hematocrit relative to controls following a 21-day treatment period (**Table 10**). Under this treatment, six blood indices were characterized by large effect sizes. These included white blood cells ($d = -1.6$), neutrophil count ($d = -0.8$), lymphocyte count ($d = -1.5$), red blood cells ($d = -0.9$), hemoglobin ($d = -0.8$) and hematocrit ($d = -1.0$). However, all the blood indices that were affected by G3-N treatment returned to normal at the end of the recovery period (**Table 10**). Delayed effects associated with this treatment included large effect sizes that were observed for mean corpuscular hemoglobin concentration ($d = 0.9$) and platelets ($d = -1.1$). Females exposed to G3-U significantly increased ($p \leq 0.05$) the levels of monocyte count and monocyte % relative to controls (**Table 10**). Under G3-U treatment, these blood indices also had large effect sizes ($d = 1.2$ and $d = 1.5$, respectively) together with lymphocyte % ($d = -1.3$) and mean platelet volume ($d = 1.1$). It is worth noting that the numbers of white blood cells, neutrophils, lymphocytes and monocytes, as well as monocyte % and hemoglobin were statistically elevated ($p \leq 0.05$) for G3-U treated group relative to those obtained for G3-N group. In general, G3-N treatment reduced the levels of most blood indices for females compared to G3-U (**Table 10**). Under G3-N treatment, twelve blood parameters scored negative effect sizes compared to only five that were recorded for G3-U treatment. Although the levels of all measured blood indices for both versions of G3 oil were statistically similar to those obtained for the control group during the recovery period, large effect sizes were observed for mean corpuscular hemoglobin concentration ($d = 0.9$) and platelets ($d = -1.1$) for G3-N treated group and neutrophil % ($d = -1.4$), lymphocyte % ($d = 1.0$), hematocrit ($d = 0.9$) and platelets ($d = -1.3$) for G3-U group (**Table 10**).

Table 9. Hematological indices for male rats exposed to grade 3 aircraft engine oils

Blood parameters	Main study groups			Recovery groups		
	Control (n = 6)	G3-N (n = 6)*	G3-U (n = 7)*	Control (n = 4)	G3-N (n = 4)*	G3-U (n = 4)*
WBC# (x 10 ³ cells/μL)	11.90 ± 2.11	12.79 ± 1.49 (0.2)	11.54 ± 0.94 (-0.1)	10.24 ± 0.77	13.96 ± 1.09 (2.0) ^a	11.82 ± 0.76 (1.0)
NE# (x 10 ³ cells/μL)	2.43 ± 0.45	2.13 ± 0.14 (-0.4)	2.37 ± 0.26 (-0.1)	2.43 ± 0.50	3.01 ± 0.60 (0.5)	2.72 ± 0.37 (0.3)
LY# (x 10 ³ cells/μL)	8.65 ± 1.70	9.65 ± 1.39 (0.3)	8.28 ± 0.67 (-0.1)	7.44 ± 0.38	10.27 ± 0.64 (2.7) ^a	8.51 ± 0.77 (0.9) ^b
MO# (x 10 ³ cells/μL)	0.81 ± 0.16	0.98 ± 0.14 (0.4)	0.88 ± 0.12 (0.2)	0.36 ± 0.10	0.52 ± 0.06 (0.9)	0.53 ± 0.21 (0.5)
EO# (x 10 ³ cells/μL)	0.008 ± 0.002	0.022 ± 0.007 (1.1) ^a	0.007 ± 0.001 (-0.1) ^b	0.013 ± 0.001	-	0.039 ± 0.017
BA# (x 10 ³ cells/μL)	-	0.005 ± 0.003	-	0.003 ± 0.001	-	-
NE%	20.39 ± 3.53	17.57 ± 1.77 (-0.4)	20.47 ± 1.45 (0)	23.17 ± 3.15	21.22 ± 2.94 (-0.3)	23.16 ± 3.32 (0)
LY%	72.89 ± 3.86	74.14 ± 2.88 (0.1)	71.88 ± 1.13 (-0.1)	73.06 ± 2.24	74.07 ± 3.66 (0.2)	71.74 ± 2.86 (-0.3)
MO%	6.63 ± 0.80	8.10 ± 1.28 (0.6)	7.59 ± 0.63 (0.5)	3.63 ± 1.07	3.77 ± 0.59 (0.1)	4.58 ± 1.84 (0.3)
EO%	0.08 ± 0.02	0.15 ± 0.04 (1.0) ^a	0.06 ± 0.01 (-0.6) ^b	0.12 ± 0.02	-	0.32 ± 0.16 (0.9)
BA%	-	0.037 ± 0.024	0.009 ± 0.006	0.03 ± 0.01	-	-
RBC (x 10 ⁶ cells/μL)	7.72 ± 0.29	7.99 ± 0.16 (0.5)	7.74 ± 0.23 (0)	8.14 ± 0.16	8.06 ± 0.29 (-0.2)	8.15 ± 0.33 (0)
Hgb (g/dL)	15.50 ± 0.51	16.10 ± 0.31 (0.6)	15.69 ± 0.47 (0.1)	15.75 ± 0.38	15.29 ± 0.28 (-0.7)	16.05 ± 0.59 (0.3)
HCT%	48.41 ± 1.49	50.59 ± 0.55 (0.8)	48.19 ± 1.23 (-0.1)	47.03 ± 1.83	46.68 ± 1.12 (-0.1)	47.38 ± 1.77 (0.1)
MCV (fL)	62.89 ± 1.41	63.45 ± 0.98 (0.2)	62.38 ± 0.88 (-0.2)	57.79 ± 1.95	58.13 ± 2.37 (0.1)	58.26 ± 1.95 (0.1)
MCH (pg/cell)	20.10 ± 0.26	20.19 ± 0.46 (0.1)	20.29 ± 0.34 (0.2)	19.38 ± 0.59	19.01 ± 0.51 (-0.3)	19.71 ± 0.24 (0.4)
MCHC (g/dL)	32.02 ± 0.40	31.82 ± 0.43 (-0.2)	32.52 ± 0.24 (0.6)	33.59 ± 0.75	32.79 ± 0.46 (-0.6)	33.91 ± 0.80 (0.2)
RDW%	16.15 ± 0.42	16.31 ± 0.10 (0.2)	16.59 ± 0.23 (0.5)	16.66 ± 0.38	16.86 ± 0.56 (0.2)	16.85 ± 0.35 (0.3)
PLT (x 10 ³ cells/μL)	924.9 ± 22.7	852.6 ± 55.6 (-0.8)	906.1 ± 37.3 (-0.2)	968.0 ± 11.6	873.2 ± 140.8 (-0.5)	873.2 ± 138.3 (-0.6)
MPV (fL)	6.24 ± 0.33	6.16 ± 0.15 (-0.1)	5.93 ± 0.10 (-0.5)	6.11 ± 0.20	6.29 ± 0.22 (0.4)	6.18 ± 0.45 (0.1)

*Changes (effect sizes) relative to controls; “-” indicates a missing value because the level of the parameter in the sample was below the instrument’s limit of detection; ^asignificant differences relative to control ($p \leq 0.05$); ^bsignificant differences relative to G3-N group ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data. The sample sizes in the table are different from those indicated in Materials and Methods because not all blood samples were analyzed due to a malfunction of the hematology analyzer.

Hematological indices for male SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G3-N) and used (G3-U) versions of grade 3 of the aircraft engine oils over a period of 21 days (Mean ± S.E.; effect sizes or Cohen’s *d* (Cohen, 1988) (magnitude of changes) estimated relative to controls are shown in parentheses). Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects.

Table 10. Hematological indices for female rats exposed to grade 3 aircraft engine oils

Blood parameters	Main study groups			Recovery groups		
	Control (n = 7)	G3-N (n = 7)*	G3-U (n = 7)*	Control (n = 3)	G3-N (n = 4)*	G3-U (n = 3)*
WBC# (x 10 ³ cells/μL)	10.39 ± 0.54	7.37 ± 0.86 (-1.6) ^a	11.42 ± 1.23 (0.4) ^b	7.83 ± 1.24	7.07 ± 0.51 (-0.5)	8.83 ± 1.11 (0.5)
NE# (x 10 ³ cells/μL)	1.91 ± 0.27	1.35 ± 0.29 (-0.8)	2.33 ± 0.27 (0.6) ^b	1.22 ± 0.03	1.34 ± 0.32 (0.3)	1.09 ± 0.19 (-0.5)
LY# (x 10 ³ cells/μL)	7.86 ± 0.28	5.59 ± 0.76 (-1.5) ^a	7.96 ± 0.86 (0.1) ^b	6.37 ± 1.30	5.40 ± 0.53 (-0.6)	7.48 ± 0.87 (0.6)
MO# (x 10 ³ cells/μL)	0.57 ± 0.10	0.41 ± 0.10 (-0.6)	1.06 ± 0.20 (1.2) ^{ab}	0.22 ± 0.08	0.27 ± 0.07 (0.3)	0.24 ± 0.04 (0.2)
EO# (x 10 ³ cells/μL)	0.044 ± 0.019	0.018 ± 0.006 (-0.7)	0.059 ± 0.026 (0.2)	-	0.055 ± 0.039	-
BA# (x 10 ³ cells/μL)	0.016 ± 0.009	0.004 ± 0.003 (-0.7)	0.013 ± 0.007 (-0.2)	-	-	-
NE%	17.93 ± 1.81	18.98 ± 3.25 (0.2)	20.45 ± 0.98 (0.7)	16.29 ± 2.15	18.85 ± 4.24 (0.4)	12.22 ± 0.93 (-1.4)
LY%	76.10 ± 2.14	75.13 ± 4.32 (-0.1)	69.69 ± 1.67 (-1.3)	80.35 ± 3.57	76.42 ± 4.73 (-0.5)	84.93 ± 1.19 (1.0)
MO%	5.44 ± 0.86	6.13 ± 0.94 (0.3)	9.29 ± 1.08 (1.5) ^{ab}	3.33 ± 1.43	3.85 ± 1.09 (0.2)	2.73 ± 0.21 (-0.3)
EO%	0.39 ± 0.17	0.30 ± 0.14 (-0.2)	0.47 ± 0.19 (0.2)	-	-	0.10 ± 0.10
BA%	0.15 ± 0.08	0.13 ± 0.08 (-0.1)	0.11 ± 0.05 (-0.2)	-	0.11 ± 0.09	-
RBC (x 10 ⁶ cells/μL)	7.59 ± 0.17	7.05 ± 0.25 (-0.9)	7.42 ± 0.15 (-0.4)	7.49 ± 0.25	7.18 ± 0.42 (-0.5)	7.77 ± 0.38 (0.5)
Hgb (g/dL)	14.97 ± 0.30	14.21 ± 0.37 (-0.8)	15.37 ± 0.40 (0.4) ^b	14.23 ± 0.37	13.93 ± 0.77 (-0.3)	14.73 ± 0.75 (0.5)
HCT%	47.26 ± 0.75	44.39 ± 1.28 (-1.0) ^a	46.78 ± 1.01 (-0.2)	42.53 ± 0.84	40.63 ± 2.74 (-0.5)	44.47 ± 1.51 (0.9)
MCV (fL)	62.40 ± 0.83	63.05 ± 0.65 (0.3)	63.05 ± 0.84 (0.3)	56.80 ± 0.93	56.56 ± 1.80 (-0.1)	57.37 ± 0.83 (0.4)
MCH (pg/cell)	19.75 ± 0.35	20.18 ± 0.26 (0.5)	20.75 ± 0.63 (0.7)	19.00 ± 0.21	19.41 ± 0.41 (0.6)	18.97 ± 0.07 (-0.1)
MCHC (g/dL)	31.69 ± 0.49	32.02 ± 0.30 (0.3)	32.91 ± 0.91 (0.6)	33.43 ± 0.48	34.35 ± 0.55 (0.9)	33.10 ± 0.57 (-0.4)
RDW%	14.53 ± 0.29	14.24 ± 0.19 (-0.4)	14.66 ± 0.30 (0.2)	15.47 ± 0.22	15.16 ± 0.31 (-0.6)	15.37 ± 0.33 (-0.2)
PLT (x 10 ³ cells/μL)	735.3 ± 121.1	693.3 ± 83.2 (-0.2)	770.1 ± 99.3 (0.1)	928.0 ± 26.9	809.8 ± 81.4 (-1.1)	769.3 ± 94.6 (-1.3)
MPV (fL)	5.69 ± 0.08	5.89 ± 0.29 (0.4)	0.14 (1.1)	5.80 ± 0.06	6.31 ± 0.60 (0.6)	5.87 ± 0.29 (0.2)

*Changes (effect sizes) relative to controls; “-” indicates a missing value because the level of the parameter in the sample was below the instrument’s limit of detection; ^asignificant differences relative to control ($p \leq 0.05$); ^bsignificant differences relative to G3-N group ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data. The sample sizes in the table are different from those indicated in Materials and Methods because not all blood samples were analyzed due to a malfunction of the hematology analyzer.

Hematological indices for female SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G3-N) and used (G3-U) versions of grade 3 of the aircraft engine oils over a period of 21 days (Mean ± S.E.; effect sizes or Cohen’s *d* (Cohen, 1988) (magnitude of changes) estimated relative to controls are shown in parentheses). Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects.

4.6.2. Effects of Grade 4 Aircraft Engine Oil Exposure on Blood Indices for SD Rats

Hematology data for male SD rats following a 21-day exposure to G4 engine oils and those obtained after 14 days recovery period are shown in [Table 11](#). All hematological parameters for males were not significantly influenced by G4-N. Although G4-N induced considerable increases in eosinophil count and eosinophil % levels compared to controls, these changes were not statistically significant due to large variations in data. Eosinophil count and eosinophil % were also the only blood parameters associated with large effect sizes ($d = 1.2$ and $d = 1.0$, respectively). Male rats exposed to G4-U significantly increased ($p \leq 0.05$) hematocrit levels (by 7.2%) relative to controls ([Table 11](#)). Under this treatment, hematocrit and hemoglobin were the only blood parameters that were associated with a large effect sizes ($d = 1.0$ and $d = 0.8$, respectively). Although the levels of all hematological indices for both versions of G4 oil were statistically similar to those obtained for the control group during the recovery period ([Table 11](#)), large effect sizes were observed for monocyte count ($d = 0.8$) and eosinophil count ($d = -1.3$), eosinophil % ($d = -0.8$), mean corpuscular volume ($d = 0.8$) and platelets ($d = -2.3$) for G4-N treated group and white blood cells ($d = -0.8$), lymphocytes ($d = -0.8$), monocyte % ($d = 0.8$) and eosinophil % ($d = -1.2$) for G4-U group.

Hematology data for female SD rats following a 21-day exposure to G4 engine oils and those obtained after 14 days recovery period are shown in [Table 12](#). Female rats exposed to G4-N had significantly decreased ($p \leq 0.05$) levels of white blood cells, neutrophil count, lymphocyte count, eosinophil count, eosinophil % and increased levels of mean platelet volume compared to controls ([Table 12](#)). All these blood parameters scored large effect sizes ($d = -1.5$; $d = -1.2$; $d = -1.4$; $d = -1.0$; $d = -0.9$ and $d = 1.2$, respectively) together with red blood cell distribution width ($d = 0.8$) and platelets ($d = 0.8$). Although changes induced by G4-N were all resolved during the recovery period, large effect sizes were observed for hemoglobin ($d = 0.8$), hematocrit ($d = 1.0$), mean corpuscular volume ($d = 0.9$), mean corpuscular hemoglobin ($d = 1.4$), red blood cell distribution width ($d = -1.2$) and mean platelet volume ($d = -1.2$) at the end of the recovery period. During oil exposure period, G4-U significantly decreased ($p \leq 0.05$) the levels of eosinophils and eosinophil % relative to those obtained for controls ([Table 12](#)). These blood parameters also scored large effect sizes ($d = -1.1$ and -0.9 , respectively) together with mean platelet volume ($d = 0.8$). The level of red blood cell distribution width measured for G4-U group was statistically decreased ($p \leq 0.05$) compared to that obtained for G4-N group. Although changes induced by G4-U were all resolved during the recovery period ([Table 12](#)), a large effect size was observed for mean platelet volume ($d = 1.3$). Females exposed to G4-U had significantly higher ($p \leq 0.05$) levels of mean platelet volume (3.4% increase) compared to those obtained for G4-N group ([Table 12](#)).

Table 11. Hematological indices for male rats exposed to grade 4 aircraft engine oils

Blood parameters	Main study groups			Recovery groups		
	Control (n = 6)	G4-N (n = 7)*	G4-U (n = 7)*	Control (n = 4)	G4-N (n = 3)*	G4-U (n = 4)*
WBC# (x 10 ³ cells/μL)	11.90 ± 2.11	12.11 ± 0.34 (0.1)	10.73 ± 0.65 (-0.3)	10.24 ± 0.77	11.38 ± 0.98 (0.7)	8.73 ± 1.03 (-0.8)
NE# (x 10 ³ cells/μL)	2.43 ± 0.45	2.51 ± 0.27 (0.1)	2.27 ± 0.16 (-0.2)	2.43 ± 0.50	2.69 ± 0.20 (0.3)	1.90 ± 0.26 (-0.7)
LY# (x 10 ³ cells/μL)	8.65 ± 1.70	8.58 ± 0.25 (0)	7.60 ± 0.77 (-0.3)	7.44 ± 0.38	8.09 ± 1.13 (0.4)	6.40 ± 0.83 (-0.8)
MO# (x 10 ³ cells/μL)	0.81 ± 0.16	1.00 ± 0.21 (0.4)	0.83 ± 0.12 (0.1)	0.36 ± 0.10	0.59 ± 0.19 (0.8)	0.44 ± 0.06 (0.4)
EO# (x 10 ³ cells/μL)	0.008 ± 0.002	0.019 ± 0.005 (1.2)	0.015 ± 0.006 (0.7)	0.013 ± 0.001	0.007 ± 0.003 (-1.3)	-
BA# (x 10 ³ cells/μL)	-	0.003 ± 0.002	0.006 ± 0.004	0.003 ± 0.001	-	0.003 ± 0.001 (0)
NE%	20.39 ± 3.53	20.61 ± 1.86 (0)	21.66 ± 2.01 (0.2)	23.17 ± 3.15	23.86 ± 2.45 (0.1)	21.76 ± 1.81 (-0.3)
LY%	72.89 ± 3.86	71.04 ± 2.05 (-0.2)	69.95 ± 2.90 (-0.3)	73.06 ± 2.24	70.46 ± 4.31 (-0.4)	72.95 ± 1.55 (0)
MO%	6.63 ± 0.80	8.17 ± 1.59 (0.5)	8.18 ± 1.45 (0.5)	3.63 ± 1.07	5.59 ± 2.12 (0.6)	5.20 ± 0.87 (0.8)
EO%	0.08 ± 0.02	0.16 ± 0.04 (1.0)	0.15 ± 0.05 (0.7)	0.12 ± 0.02	0.07 ± 0.04 (-0.8)	0.06 ± 0.03 (-1.2)
BA%	-	0.027 ± 0.018	-	-	-	-
RBC (x 10 ⁶ cells/μL)	7.72 ± 0.29	7.88 ± 0.09 (0.3)	8.21 ± 0.28 (0.7)	8.14 ± 0.16	8.01 ± 0.22 (-0.4)	8.19 ± 0.09 (0.2)
Hgb (g/dL)	15.50 ± 0.51	15.84 ± 0.33 (0.3)	16.36 ± 0.28 (0.8)	15.75 ± 0.38	16.03 ± 0.32 (0.4)	15.63 ± 0.30 (-0.2)
HCT%	48.41 ± 1.49	48.89 ± 0.71 (0.2)	51.89 ± 1.15 (1.0) ^a	47.03 ± 1.83	48.08 ± 0.91 (0.4)	46.94 ± 1.39 (0)
MCV (fL)	62.89 ± 1.41	62.04 ± 0.67 (-0.3)	63.41 ± 1.12 (0.2)	57.79 ± 1.95	60.08 ± 0.52 (0.8)	57.24 ± 1.10 (-0.2)
MCH (pg/cell)	20.10 ± 0.26	20.12 ± 0.36 (0)	20.02 ± 0.43 (-0.1)	19.38 ± 0.59	20.03 ± 0.55 (0.6)	19.09 ± 0.27 (-0.3)
MCHC (g/dL)	32.02 ± 0.40	32.39 ± 0.50 (0.3)	31.57 ± 0.48 (-0.4)	33.59 ± 0.75	33.35 ± 0.65 (-0.2)	33.31 ± 0.50 (-0.2)
RDW%	16.15 ± 0.42	16.41 ± 0.31 (0.3)	15.98 ± 0.33 (-0.2)	16.66 ± 0.38	16.13 ± 0.74 (-0.5)	17.01 ± 0.57 (0.4)
PLT (x 10 ³ cells/μL)	924.9 ± 22.7	976.6 ± 55.4 (0.5)	880.6 ± 51.0 (-0.5)	968.0 ± 11.6	889.7 ± 24.4 (-2.3)	961.0 ± 85.8 (-0.1)
MPV (fL)	6.24 ± 0.33	6.69 ± 0.60 (0.4)	6.25 ± 0.41 (0)	6.11 ± 0.20	5.92 ± 0.16 (-0.6)	6.09 ± 0.29 (-0.1)

*Changes (effect sizes) relative to controls; “-” indicates a missing value because the level of the parameter in the sample was below the instrument’s limit of detection; ^asignificant differences relative to control ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data. The sample sizes in the table are different from those indicated in Materials and Methods because not all blood samples were analyzed due to a malfunction of the hematology analyzer.

Hematological indices for male SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days (Mean ± S.E.; effect sizes or Cohen’s *d* (Cohen, 1988) (magnitude of changes) estimated relative to controls are shown in parentheses). Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects.

Table 12. Hematological indices for female rats exposed to grade 4 aircraft engine oils

Blood parameters	Main study groups			Recovery groups		
	Control (n = 7)	G4-N (n = 7)*	G4-U (n = 6)*	Control (n = 3)	G4-N (n = 4)*	G4-U (n = 3)*
WBC# (x 10 ³ cells/μL)	10.39 ± 0.54	7.65 ± 0.81(-1.5) ^a	9.28 ± 1.22 (-0.5)	7.83 ± 1.24	7.40 ± 0.88 (-0.2)	7.45 ± 1.11 (-0.2)
NE# (x 10 ³ cells/μL)	1.91 ± 0.27	1.24 ± 0.14 (-1.2) ^a	1.64 ± 0.08 (-0.5)	1.22 ± 0.03	1.26 ± 0.08 (0.3)	1.32 ± 0.35 (0.2)
LY# (x 10 ³ cells/μL)	7.86 ± 0.28	5.94 ± 0.65 (-1.4) ^a	6.98 ± 1.11 (-0.4)	6.37 ± 1.30	5.94 ± 0.85 (-0.2)	5.98 ± 0.78 (-0.2)
MO# (x 10 ³ cells/μL)	0.57 ± 0.10	0.46 ± 0.08 (-0.5)	0.64 ± 0.10 (0.3)	0.22 ± 0.08	0.19 ± 0.03 (-0.3)	0.15 ± 0.02 (-0.7)
EO# (x 10 ³ cells/μL)	0.044 ± 0.019	0.006 ± 0.002 (-1.0) ^a	0.004 ± 0.003 (-1.1) ^a	-	-	0.013 ± 0.003
BA# (x 10 ³ cells/μL)	0.016 ± 0.009	-	-	-	-	-
NE%	17.93 ± 1.81	16.32 ± 1.14 (-0.4)	19.16 ± 2.29 (0.2)	16.29 ± 2.15	17.52 ± 1.55 (0.4)	17.01 ± 2.10 (0.2)
LY%	76.10 ± 2.14	77.55 ± 1.80 (0.3)	73.66 ± 2.77 (-0.4)	80.35 ± 3.57	79.43 ± 2.34 (-0.2)	80.61 ± 1.65 (0.1)
MO%	5.44 ± 0.86	6.01 ± 0.95 (0.2)	7.08 ± 0.93 (0.7)	3.33 ± 1.43	2.92 ± 0.98 (-0.2)	2.14 ± 0.50 (-0.6)
EO%	0.39 ± 0.17	0.11 ± 0.03 (-0.9) ^a	0.09 ± 0.04 (-0.9) ^a	-	0.09 ± 0.06	0.22 ± 0.06
BA%	0.15 ± 0.08	-	-	-	0.05 ± 0.03	-
RBC (x 10 ⁶ cells/μL)	7.59 ± 0.17	7.43 ± 0.09 (-0.4)	7.44 ± 0.21 (-0.3)	7.49 ± 0.25	7.51 ± 0.06 (0.1)	7.52 ± 0.16 (0.1)
Hgb (g/dL)	14.97 ± 0.30	14.49 ± 0.29 (-0.6)	14.83 ± 0.37 (-0.2)	14.23 ± 0.37	14.66 ± 0.20 (0.8)	14.40 ± 0.51 (0.2)
HCT%	47.26 ± 0.75	46.32 ± 0.74 (-0.5)	47.30 ± 1.08 (0)	42.53 ± 0.84	43.94 ± 0.74 (1.0)	43.27 ± 1.70 (0.3)
MCV (fL)	62.40 ± 0.83	62.32 ± 0.77 (0)	63.66 ± 0.87 (0.6)	56.80 ± 0.93	58.54 ± 1.13 (0.9)	57.57 ± 1.59 (0.3)
MCH (pg/cell)	19.75 ± 0.35	19.53 ± 0.43 (-0.2)	19.94 ± 0.19 (0.3)	19.00 ± 0.21	19.51 ± 0.19 (1.4)	19.17 ± 0.29 (0.4)
MCHC (g/dL)	31.69 ± 0.49	31.34 ± 0.66 (-0.2)	31.34 ± 0.32 (-0.3)	33.43 ± 0.48	33.44 ± 0.96 (0)	33.30 ± 0.67 (-0.1)
RDW%	14.53 ± 0.29	15.05 ± 0.21 (0.8)	14.34 ± 0.12 (-0.3) ^b	15.47 ± 0.22	14.98 ± 0.21 (-1.2)	15.80 ± 0.40 (0.6)
PLT (x 10 ³ cells/μL)	735.3 ± 121.1	907.3 ± 58.1 (0.8)	868.3 ± 35.6 (0.6)	928.0 ± 26.9	907.0 ± 45.0 (-0.3)	859.0 ± 74.9 (-0.7)
MPV (fL)	5.69 ± 0.08	6.84 ± 0.55 (1.2) ^a	5.91 ± 0.14 (0.8)	5.80 ± 0.06	5.69 ± 0.04 (-1.2)	6.00 ± 0.12 (1.3) ^b

*Changes (effect sizes) relative to controls; “-” indicates a missing value because the level of the parameter in the sample was below the instrument’s limit of detection; ^asignificant differences relative to control ($p \leq 0.05$); ^bsignificant differences relative to G4-N group ($p \leq 0.05$). See Materials and Methods for details on statistical analyses performed on the data. The sample sizes in the table are different from those indicated in Materials and Methods because not all blood samples were analyzed due to a malfunction of the hematology analyzer.

Hematological indices for female SD rats assigned to the main study and recovery groups. All rats were dermally exposed to new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days (Mean ± S.E.; effect sizes or Cohen’s *d* (Cohen, 1988) (magnitude of changes) estimated relative to controls are shown in parentheses). Rats in the recovery groups were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects.

4.7 Effects of Grade 3 and 4 Aircraft Engine Oils on Acetylcholinesterase Activity in Tissues and Whole Blood for SD Rats

4.7.1. Grade 3 and 4 Aircraft Engine Oils Decrease Acetylcholinesterase Activity in Brain Tissues for SD Rats

The AChE activity in brain tissues for control rats was similar for males and females assigned to the main study (**Fig. 14A**) and the recovery groups (**Fig. 14D**). This activity was 0.28 ± 0.04 U/mg tissue/min for males in the main study vs. 0.37 ± 0.05 unit/milligram (U/mg) tissue/min for females. The activity in the recovery groups was 0.20 ± 0.05 U/mg tissue/min for males vs. 0.17 ± 0.01 U/mg tissue/min for females. These data show that the AChE activity decreased as the rats age, regardless of sex. The level of AChE activity for females significantly ($p \leq 0.05$) decreased by 55% when females assigned to the main study were compared to those in the recovery groups. The same comparison for males shows a 28% decrease, although this was not significant.

The G3 and G4 oils affected the AChE activity in brain tissues for male rats only when they were dermally applied in their unused states (**Figs. 14B-C**). The AChE activity was significantly ($p \leq 0.05$) decreased by 33% for males assigned to the main study and exposed to G3-N oil relative to controls (**Fig. 14B**). This effect was resolved at the end of the recovery period (**Fig. 14E**). Similar results were obtained with male rats exposed to G4 oil. The AChE activity was statistically ($p \leq 0.05$) decreased by 32% for males exposed to G4-N oil in the main study (**Fig. 14C**) when compared to their controls. However, the AChE activity in brain tissues for these rats returned to normal during the recovery period (**Fig. 14F**).

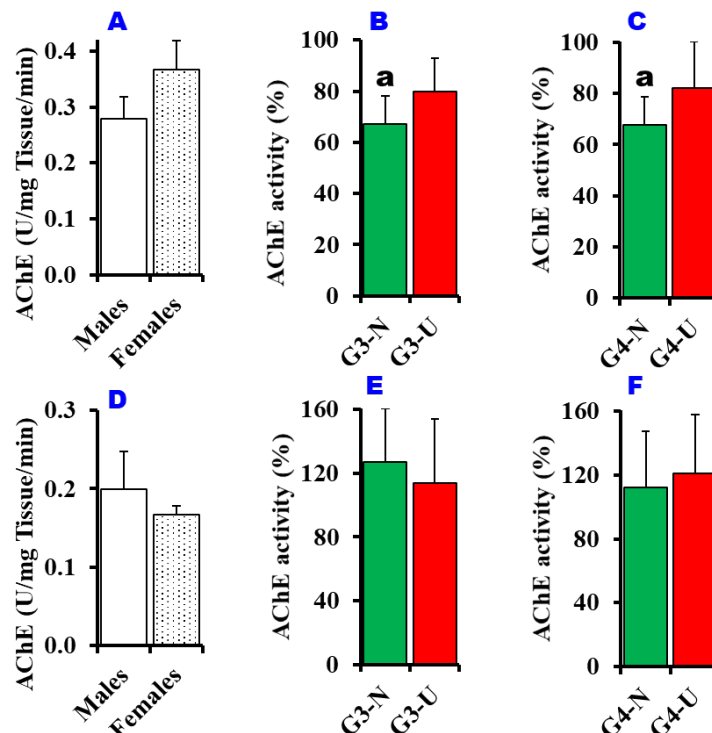


Figure 14. AChE activity in brain tissues for male and female controls and male rats exposed to grade 3 and 4 aircraft engine oils

AChE activity (Mean $\pm$ S.E.) in brain tissues for (A) control rats in the main study and (D) recovery groups. Normalized (to controls) AChE activity (%) in brain tissues for males in the main study exposed to (B) grade 3 and (C) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Males in the recovery groups that were exposed to (E) grade 3 and (F) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between control and engine oil treated groups are indicated by letter "a"; $p \leq 0.05$.

Contrary to males that showed decreased AChE activity in brain tissues when they were only exposed to unused versions of G3 and G4 oils, the activity of this enzyme in brain tissues for females was affected by both unused and used versions of these oils (Fig. 15). Treating females with G3-N and G3-U significantly ($p \leq 0.05$) decreased the AChE activity by 32% and 41%, respectively when compared to controls (Fig. 15A). Exposing females to G4-N and G4-U significantly ($p \leq 0.05$) decreased the activity of this enzyme by 38% and 37%, respectively, relative to controls (Fig. 15B). However, AChE activity returned to normal during the recovery period, regardless of oil state (Figs. 15C-D).

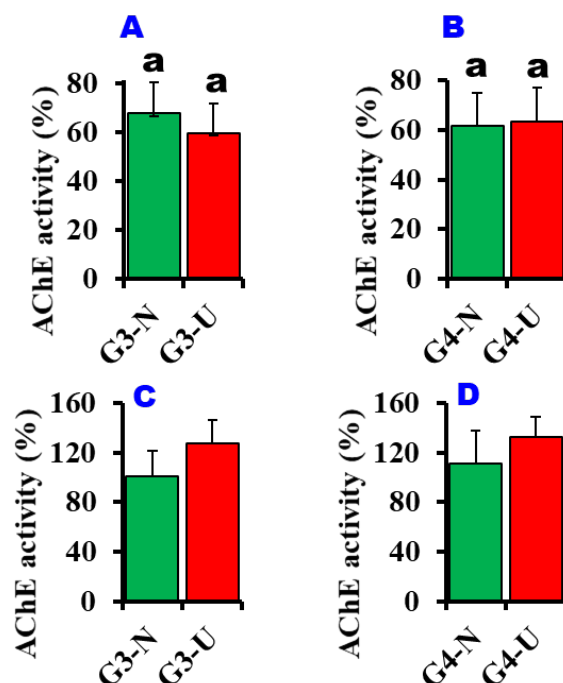


Figure 15. AChE activity in brain tissues for female rats exposed to grade 3 and 4 aircraft engine oils

Normalized (to controls) AChE activity (%) in brain tissues for female rats assigned to main study and recovery groups and dermally exposed to unused (green bars) and used (red bars) versions of grade 3 and 4 aircraft engine oils. Females in the main study were exposed to (A) grade 3 and (B) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Females in the recovery groups that were exposed to (C) grade 3 and (D) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between control and engine oil treated groups are indicated by letter “a”; $p \leq 0.05$.

4.7.2. Grade 3 and 4 Aircraft Engine Oils have no Impact on Acetylcholinesterase Activity in Liver Tissues for SD Rats

Our data for AChE activity in liver tissues for control rats indicate that males and females displayed differences in activity (Figs. 16A & 16D). The AChE activity obtained for males assigned to the main study was significantly ($p \leq 0.05$) lower (0.47 ± 0.04 U/mg tissue/min) than that obtained for females (0.82 ± 0.08 U/mg tissue/min) in this study (Fig. 16A). Similar observations were also noted for the rats assigned to the recovery groups (0.47 ± 0.02 U/mg tissue/min vs. 1.01 ± 0.11 U/mg tissue/min for males and females, respectively) (Fig. 16D).

As shown in Figs. 16B-C & 16E-F, G3 and G4 oils did not have a significant impact on the AChE activity in liver tissues, regardless of the oil state.

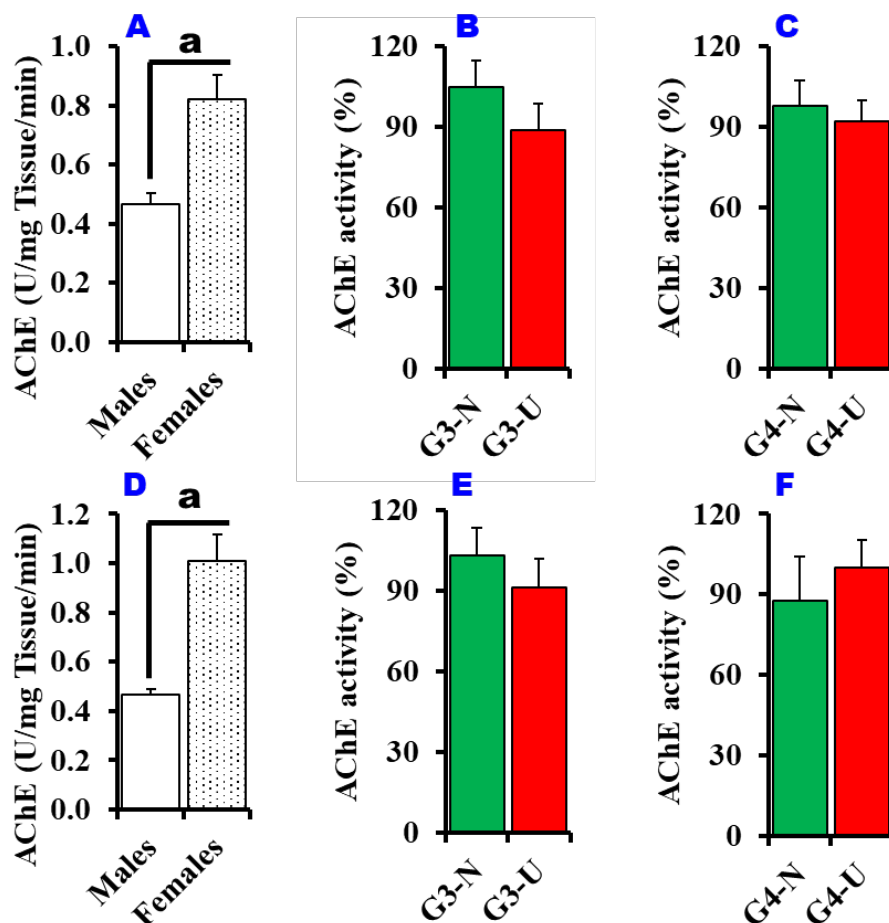


Figure 16. AChE activity in liver tissues for male and female controls and male rats exposed to grade 3 and 4 aircraft engine oils

AChE activity (Mean $\pm$ S.E.) in liver tissues for (A) control rats in the main study and (D) recovery groups. Normalized (to controls) AChE activity (%) in liver tissues for males in the main study exposed to (B) grade 3 and (C) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Males in the recovery groups that were exposed to (E) grade 3 and (F) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant differences between male controls and female controls are indicated by letter “a”; $p \leq 0.05$.

Data on the activity of AChE in liver tissues from female rats exposed to G3 and G4 oils are shown in Fig. 17. These oils did not have a significant effect on the activity of this enzyme in the liver, regardless of the oil state.

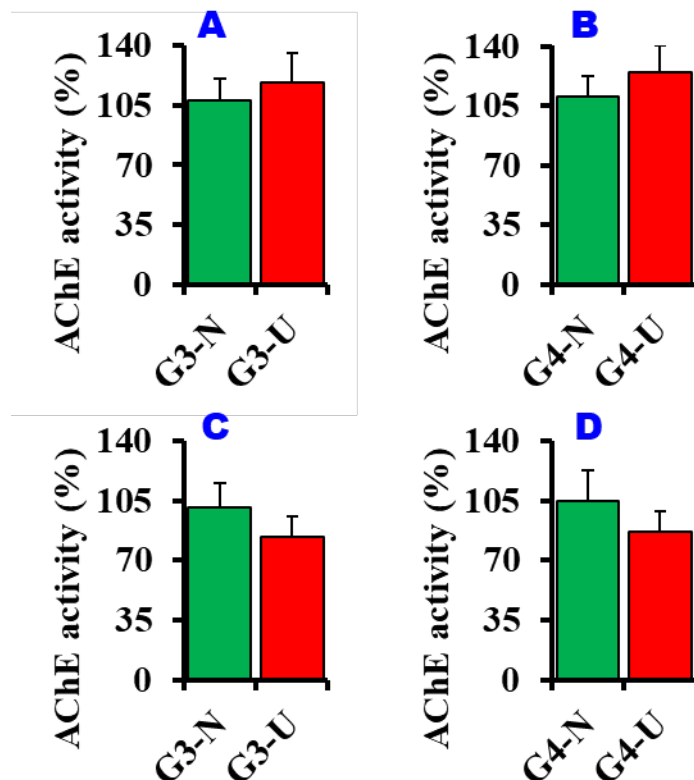


Figure 17. AChE activity in liver tissues for female rats exposed to grade 3 and 4 aircraft engine oils

Normalized (to controls) AChE activity (%) in liver tissues for female rats assigned to main study and recovery groups and dermally exposed to unused (green bars) and used (red bars) versions of grade 3 and 4 aircraft engine oils. Females in the main study were exposed to (A) grade 3 and (B) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 10). Females in the recovery groups that were exposed to (C) grade 3 and (D) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5).

4.7.3. Grade 3 Aircraft Engine Oil Alters Acetylcholinesterase Activity in Whole Blood for SD Rats

The AChE activity in whole blood samples for control rats was similar for males and females assigned to the main study (Fig. 18A). This activity was 0.14 ± 0.02 U/mL/min for males in the main study vs. 0.15 ± 0.02 U/mL/min for females. However, results for samples collected from the recovery groups indicate that males and females displayed differences in AChE activity (Fig. 18D). The AChE activity in whole blood samples collected from males was significantly ($p \leq 0.05$) lower (0.09 ± 0.01 U/mL/min) than that obtained for samples collected from females (0.15 ± 0.01 U/mL/min) (Fig. 18D).

As shown in Figs. 18B-C & 18E-F, exposing rats to G3 and G4 oils did not have a significant impact on the AChE activity in whole blood, regardless of the oil state.

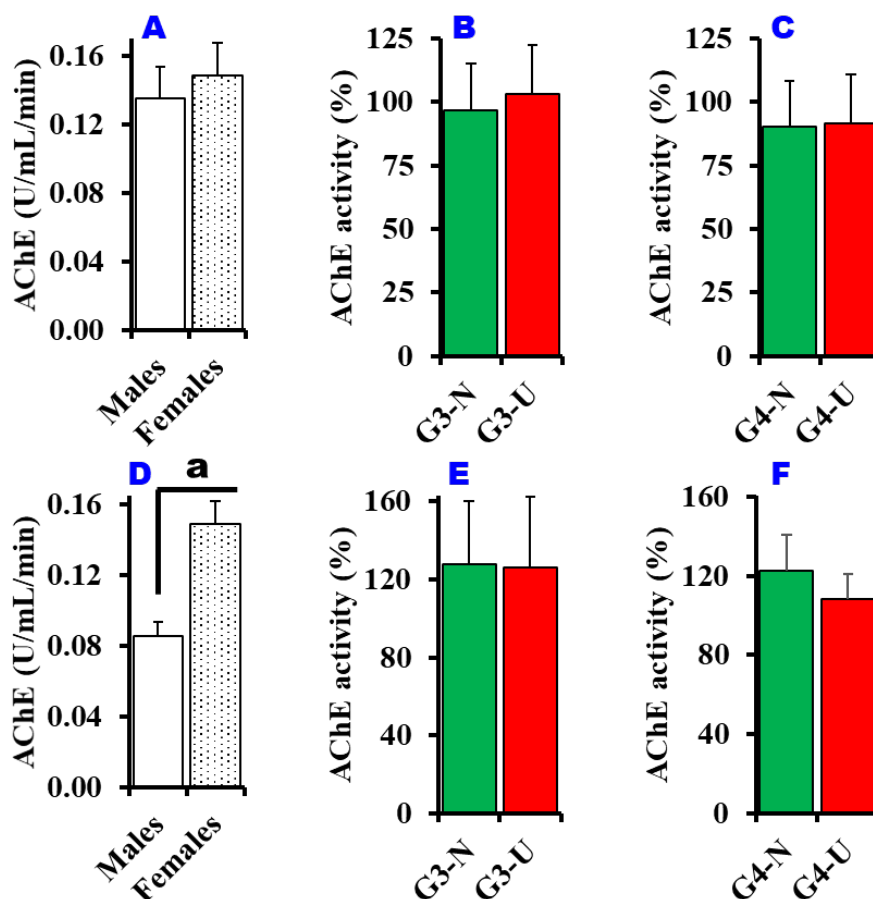


Figure 18. AChE activity in whole blood for male and female controls and male rats exposed to grade 3 and 4 aircraft engine oils

AChE activity (Mean $\pm$ S.E.) in whole blood for (A) control rats in the main study and (D) recovery groups. Normalized (to controls) AChE activity (%) in whole blood for males in the main study exposed to (B) grade 3 and (C) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Males in the recovery groups that were exposed to (E) grade 3 and (F) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant difference between male controls and female controls is indicated by letter "a"; $p \leq 0.05$.

Contrary to males from which the whole blood AChE activity was not affected by exposure to G3 and G4 oils (Fig. 18), treating females with G3-U significantly ($p \leq 0.05$) decreased the AChE activity in whole blood by 29% during the main study (Fig. 19A) and by 32% at the end of the recovery period (Fig. 19C) when they were compared to their controls. Exposure to G3-N has no impact on the activity of this enzyme in whole blood. Similarly, exposing female rats to G4 oils did not affect AChE activity in whole blood, regardless the oil state (Figs. 19B & 19D).

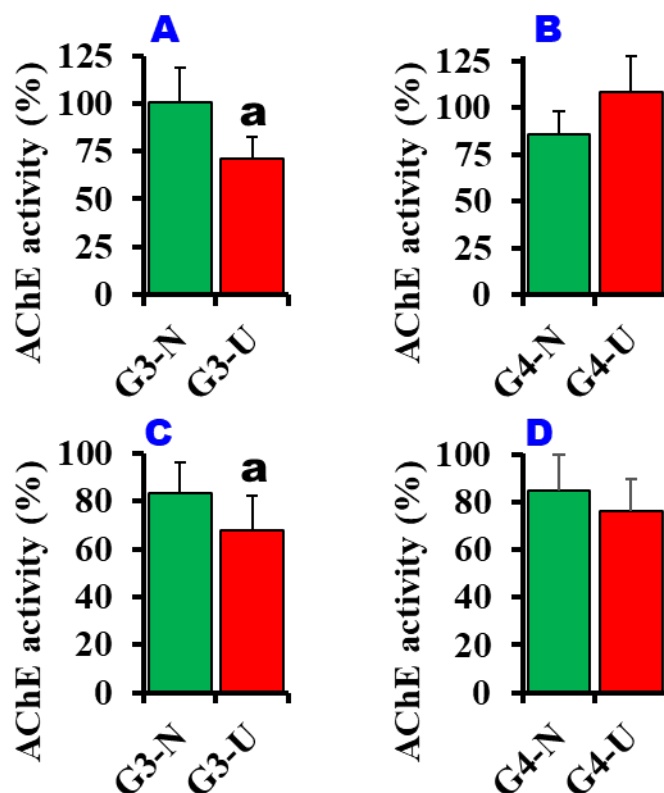


Figure 19. AChE activity in whole blood for female rats exposed to grade 3 and 4 aircraft engine oils

Normalized (to controls) AChE activity (%) in whole blood for female rats assigned to main study and recovery groups and dermally exposed to unused (green bars) and used (red bars) versions of grade 3 and 4 aircraft engine oils. Females in the main study exposed to (A) grade 3 and (B) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; N = 10). Females in the recovery groups that exposed to (C) grade 3 and (D) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; N = 5). Significant differences between control and engine oil treated groups are indicated by letter “a”; $p \leq 0.05$.

4.8 Effects of Grade 3 and 4 Aircraft Engine Oils on Butyrylcholinesterase Activity in Plasma for SD Rats

4.8.1. Grade 4 Aircraft Engine Oil Decreases Butyrylcholinesterase Activity in Plasma for Female SD Rats

Results for BChE activity in plasma for control rats indicate that males and females displayed differences in activity of this enzyme (Figs. 20A & 20D). The BChE activity in plasma for males assigned to the main study was significantly ($p \leq 0.05$) lower (0.07 ± 0.01 U/mL/min) than that obtained for females (0.18 ± 0.04 U/mL/min) in this study (Fig. 20A). Similar observations were also noted for the rats assigned to the recovery groups (0.06 ± 0.01 U/mL/min vs. 0.33 ± 0.05 U/mL/min for males and females, respectively) (Fig. 20D).

As shown in **Figs. 20B-C & 20E-F**, G3 and G4 oils did not have a significant impact on the BChE activity in plasma, regardless of the oil state.

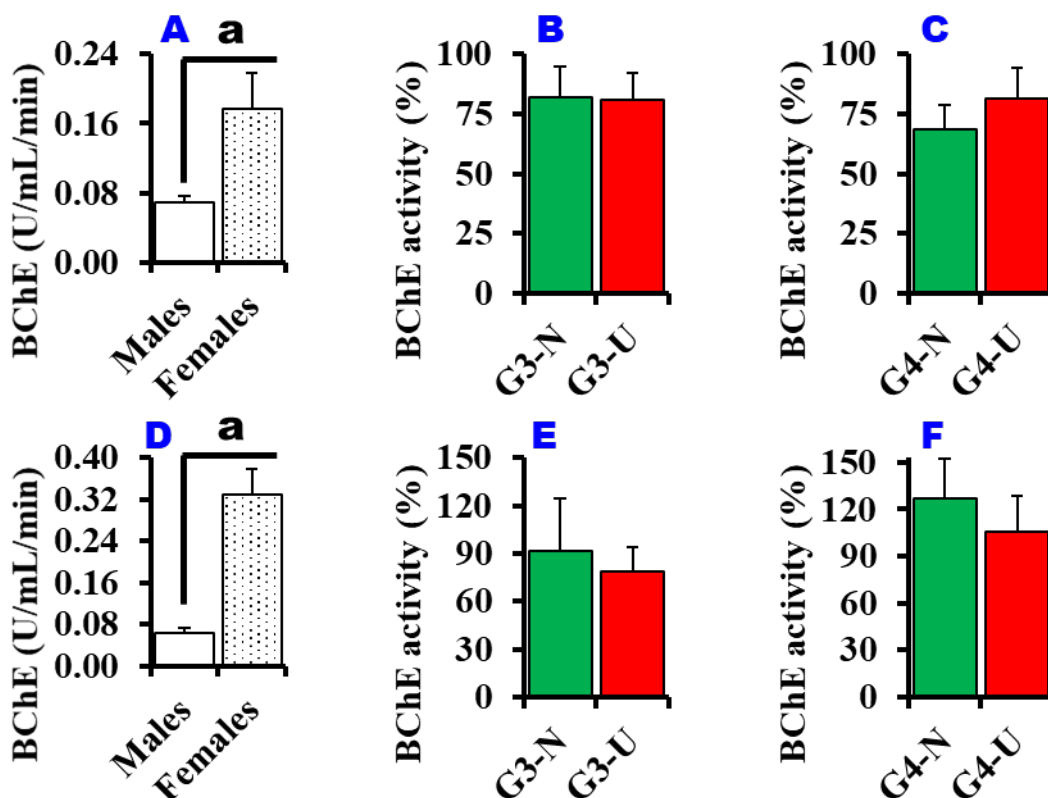


Figure 20. BChE activity in plasma for male and female controls and male rats exposed to grade 3 and 4 aircraft engine oils

BChE activity (Mean ± S.E.) in plasma for (A) control rats in the main study and (D) recovery groups. Normalized (to controls) BChE activity (%) in plasma for males in the main study exposed to (B) grade 3 and (C) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean ± S.E.; N = 10). Males in the recovery groups that were exposed to (E) grade 3 and (F) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean ± S.E.; N = 5). Significant difference between male controls and female controls is indicated by letter “a”; $p \leq 0.05$.

Although both versions of G3 (**Figs. 21A & 21C**) and the used version of G4 (**Figs. 21B & 21D**) oils did not have an effect on BChE activity in plasma for females (**Fig. 20A**), females exposed to G4-N showed significant ($p \leq 0.05$) decreased activity of this enzyme in plasma (48% decrease) during the recovery period when compared to the controls (**Fig. 21D**).

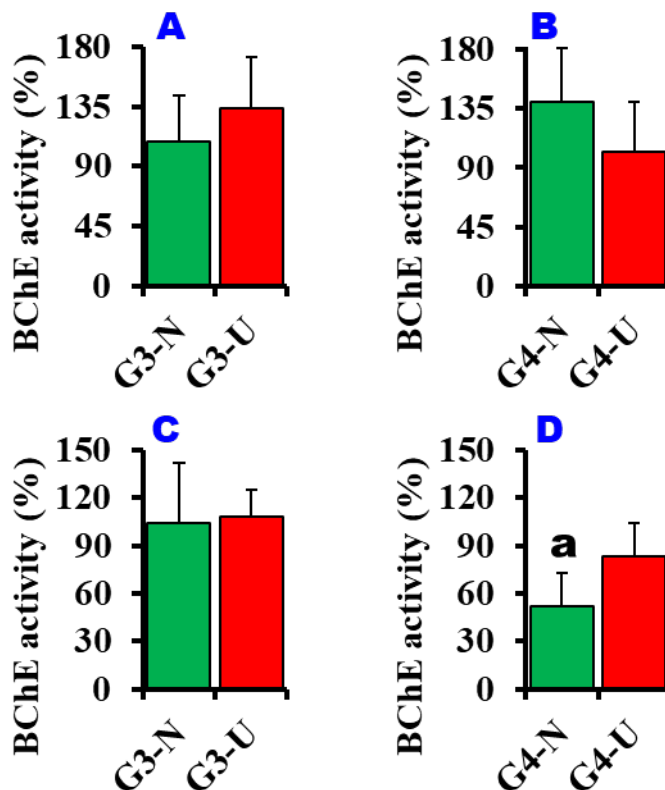


Figure 21. BChE activity in plasma for female rats exposed to grade 3 and 4 aircraft engine oils

Normalized (to controls) BChE activity (%) in plasma for female rats assigned to main study and recovery groups and dermally exposed to unused (green bars) and used (red bars) versions of grade 3 and 4 aircraft engine oils. Females in the main study exposed to (A) grade 3 and (B) grade 4 of the oils over a period of 21 days (15 total exposures) (Mean $\pm$ S.E.; $N = 10$). Females in the recovery groups that exposed to (C) grade 3 and (D) grade 4 over similar period as the main study and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (Mean $\pm$ S.E.; $N = 5$). Significant difference between the control and engine oil treated groups is indicated by letter "a"; $p \leq 0.05$.

4.9 Effects of Aircraft Engine Oils on Electrophysiology Measurements

Based on reports indicating that organophosphates induce changes in hippocampal synaptic transmission (Speed *et al.*, 2012; Lee *et al.*, 2016), a pilot study was conducted to assess the effect of G3 and G4 oil exposure on (1) synaptic transmission in hippocampus, (2) spontaneous activity of hippocampal neurons, (3) short and (4) long term synaptic plasticity of SD rats. Results for this pilot study are shown in Figs. 22-26.

4.9.1. Effects of Aircraft Engine Oils on Efficacy of Synaptic Transmission in Rat Hippocampus

Figure 22 shows the effect of G3 and G4 oil exposure on the field responses of CA1 hippocampal neurons for male (Fig. 22A) and female (Fig. 22B) rats. Although statistical analyses were not

performed for these data due to a small sample size for each treatment ($n = 1/\text{sex}$), males exposed to unused version of G4 and both versions of G3 tended to display different field responses of neurons within the CA1 region relative to control (**Fig. 22A**). Similar observations were noted for the females exposed to the unused version of G3 (**Fig. 22B**).

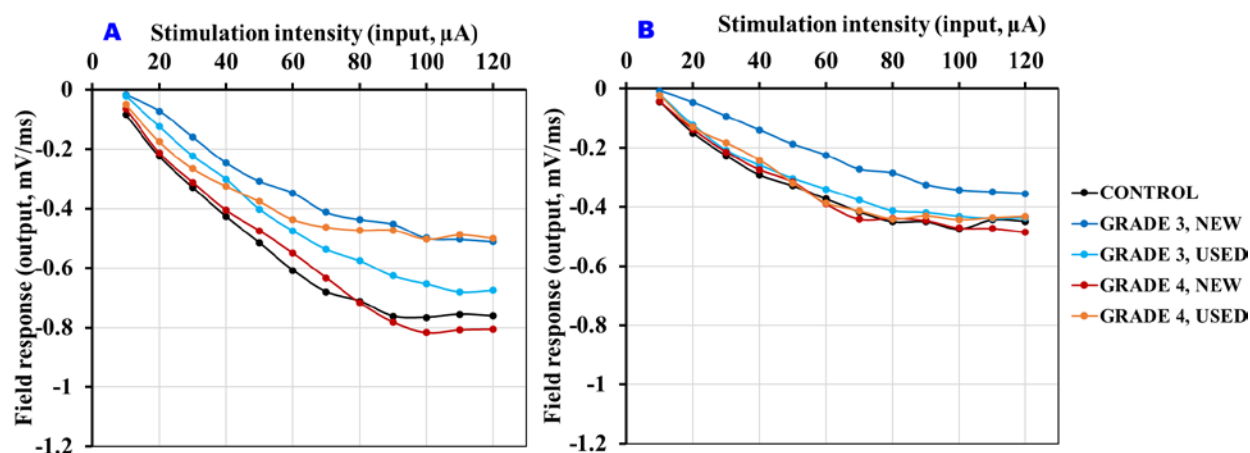


Figure 22. Input-output function from CA1 hippocampal neurons of male and female rats exposed to grade 3 and 4 aircraft engine oils

Input-output function from CA1 hippocampal neurons of (A) male and (B) female rats exposed to unused and used versions of G3 and G4 aircraft engine oils, calculated by the slope of evoked responses. Data are shown as mean across multiple slices per rat ($n = 4-8$ slices).

4.9.2. Effects of the Aircraft Engine Oils on Short Term Synaptic Plasticity

Short term synaptic plasticity was also assessed following G3 and G4 oil exposure. As shown in **Figs. 23A** and **23B**, rats exposed to these oils did not show any alteration in paired pulse facilitation regardless of gender.

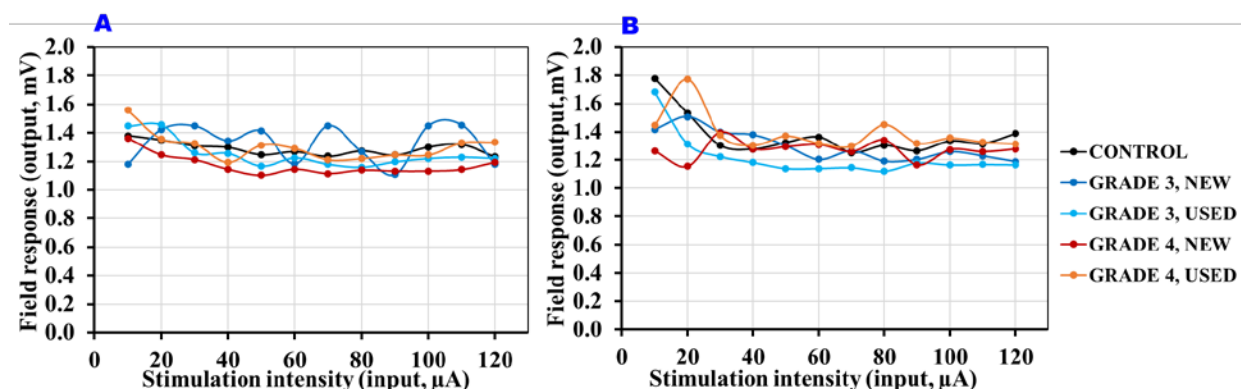


Figure 23. Paired pulse facilitation from CA1 hippocampal neurons of male and female rats exposed to grade 3 and 4 aircraft engine oils

Paired pulse facilitation from CA1 hippocampal neurons of (A) male and (B) female rats exposed to unused and used versions of G3 and G4 aircraft engine oils, calculated using the amplitude of evoked responses. Data are shown as mean across multiple slices/rat ($n = 4-8$ slices).

4.9.3. Effects of the Aircraft Engine Oils on Long Term Synaptic Plasticity

Data in **Figures 24 & 25** show results of long term synaptic plasticity in rats following exposure to G3 and G4 engine oils. These data do not show any trend that may suggest exposure to these oils can alter the long term potentiation from CA1 hippocampal neurons of male (**Figs. 24A, 25A**) and female (**Figs. 24B, 25B**) rats.

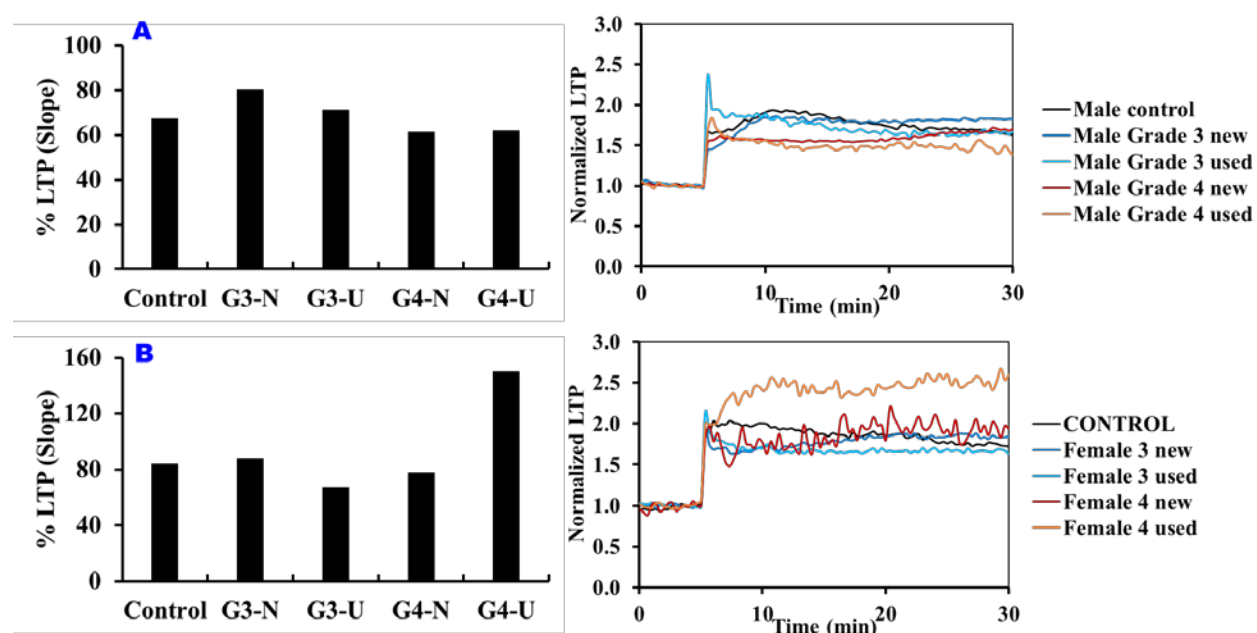


Figure 24. Long term potentiation from CA1 hippocampal neurons of male and female rats exposed to grade 3 and 4 aircraft engine oils, calculated using the slope of evoked responses

Long term potentiation from CA1 hippocampal neurons of (A) male and (B) female rats exposed to unused and used versions of aircraft engine oils, calculated using the slope of evoked responses. Graphs of averaged normalized trace across multiple slices per rat are shown to the right. Data in bar graphs are shown as mean across multiple slices per rat ($n = 3-6$ slices).

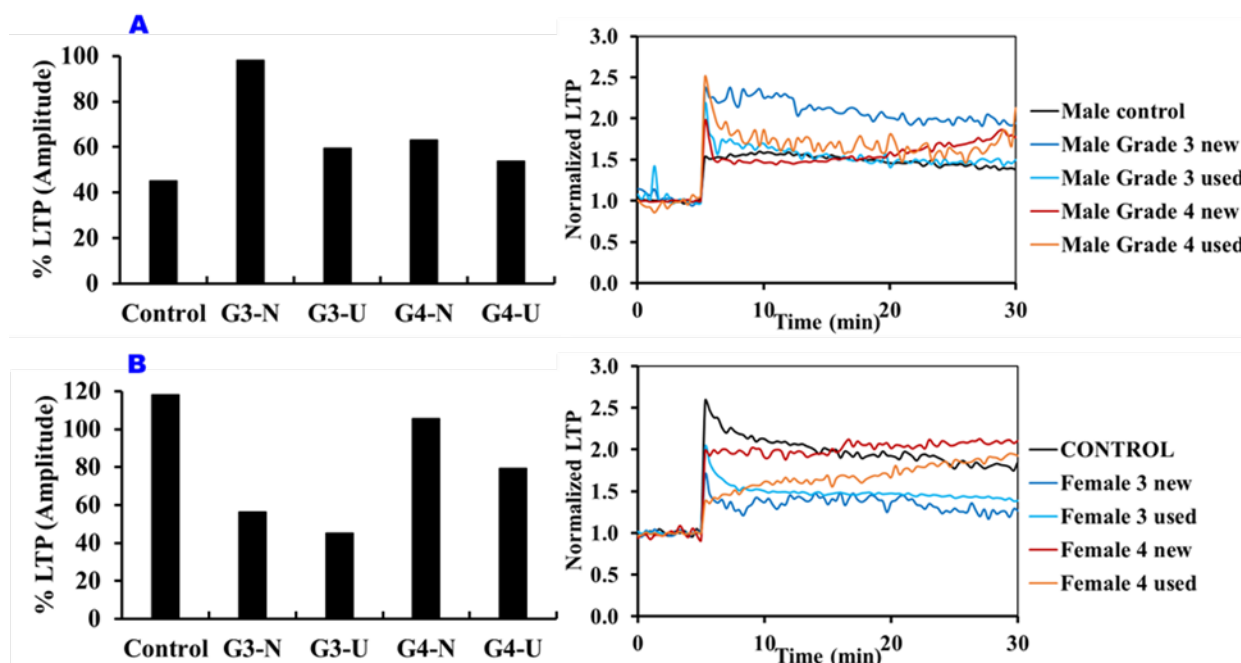


Figure 25. Long term potentiation from CA1 hippocampal neurons of male and female rats exposed to grade 3 and 4 aircraft engine oils, calculated using the amplitude of evoked responses

Long term potentiation from CA1 hippocampal neurons of (A) male and (B) female rats exposed to unused and used versions of aircraft engine oils, calculated using the amplitude of evoked responses. Graphs of averaged normalized trace across multiple slices per rat are shown to the right. Data in bar graphs are shown as mean across multiple slices per rat ($n = 3-6$ slices).

4.9.4. Effects of the Aircraft Engine Oils on Spontaneous Activity of Hippocampal Neurons

Data for the effect of G3 and G4 engine oil exposure on the spontaneous activity of hippocampal neurons in rats are shown in Fig. 26. While both versions of G4 oil tend to show an increased spontaneous activity of hippocampal neurons for males relative to control (Fig. 26A), similar trend is noted for females exposed to both versions of G3 relative to control (Fig. 26B).

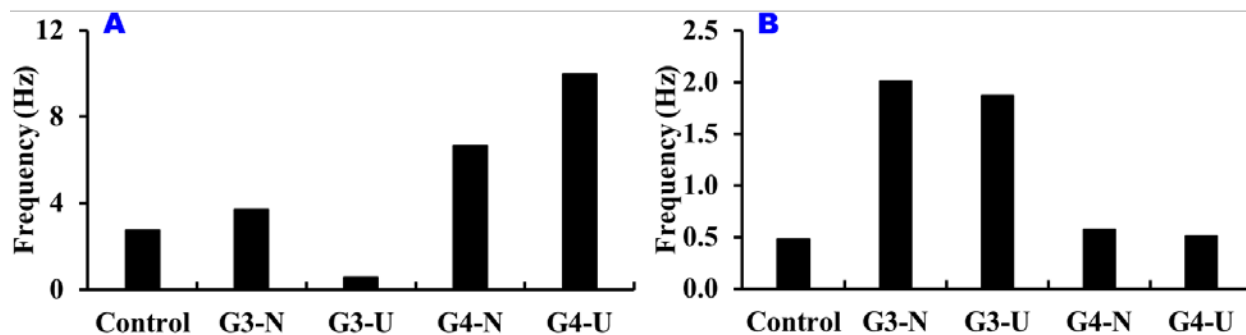


Figure 26. Frequency of spontaneous activity of neurons within the CA1 region of male and female rats exposed to grade 3 and 4 aircraft engine oils

Frequency of spontaneous activity of neurons within the CA1 region of (A) male and (B) female rats exposed to unused and used versions of G3 and G4 aircraft engine oils. Data in bar graphs are shown as mean across multiple slices per rat (n = 4-8 slices).

5.0 DISCUSSION

To the best of our knowledge, this is the first study to assess the potential dermal subchronic toxicity of grade 3 and 4 aircraft engine oils in unused states relative to their used versions. G3 and G4 oils are chemical mixtures. Since there may be synergistic interactions between ingredients that can enhance their hazardous status, the study assessed their potential toxicity as whole products instead of evaluating their individual components.

5.1 Effects of Aircraft Engine Oils on Rat's Body Weights, Feed and Water Intake

This study indicated, as expected, that males gained more body weight during three weeks of treatments and two weeks of recovery as compared to females. Exposure to G3 and G4 oils did not significantly influence body weight of rats, regardless of sex. However, it was interesting to note that rats exposed to oils tended to lose more body weight at the end of the treatment period following a 12 h fasting when compared to controls. Fasting is known to induce body weight loss in rodents (Dietze *et al.*, 2016). Our data suggest that the fasting-induced body weight loss may have been associated with treatment effects. There may have been minor changes occurring in body weights due to treatments which were probably unmasked due to fasting-induced stress. Upon fasting, females tended to lose more body weight than males. Female controls and those exposed to G3-N and G3-U oils lost 0.9%, 8% and 9% of their body weight, respectively, while males subjected to similar treatments lost 0%, 3% and 5%, respectively. Females exposed to G4-N and G4-U oils lost 4% and 9%, respectively, while males subjected to these treatments lost 3% and 4%, respectively. Moreover, the body weights for rats exposed to new oils tended to be less affected by treatments as compared to those exposed to their used versions, regardless of sex (Figs. 7 & 8). Although we did not measure their fecal and urinary outputs during the fasting challenge, fasting may have increased the rat's metabolic functions, thus increasing their fecal and urinary excretion and contributing to weight loss. Food deprivation or restriction for animals has been associated with increasing metabolic and psychological stress (Toth and Gardiner, 2000). Limiting water intake and/or increasing fecal and urinary outputs can have a noticeable effect on an animal's body weight. The weekly food and water consumption measurements taken during treatments and before fasting indicate that treatments did not influence the food and water intake. Although we did not assess the water consumption during the fasting period, we cannot rule out that oil treated rats reduced their water intake, which may also have contributed to the body weight loss trend observed at the end of the treatment period. Studies have indicated that rats exposed to organophosphates can significantly lose weight. The USA National Toxicology Program has reported that male and female F344/N rats that received (by gavage) doses of mixed isomer preparation of 79% TCP esters (consisting of 21% tri-*meta*-cresyl, 4% tri-*para*-cresyl, 1% tri-*ortho*-cresyl and other unidentified tri-cresyl phosphate esters) ranging between 1,450 milligrams per kilogram (mg/kg) and 5,800 mg/kg body weight, 5 days per week, for a total of 13 or 14 doses in a 16-day period have mean body weights significantly lower than those of the controls. It has also been shown that exposure to TPP can alter the rat's body weight gain (Sutton *et al.*, 1960). When male Holtzman rats were fed with 0-0.5% TPP (equivalent to 0-3632 mg/kg body weight) for 35 days, the body weight gain for rats that received the highest dose decreased by 7% relative to controls (Sutton *et al.*, 1960). Taib and colleagues (Taib *et al.*, 2013) reported that exposing (orally by gavage) male SD rats to a 20 mg/kg body weight of an organophosphate compound (fenitrothion) for 28 days induced significant adverse effects, including a decrease in body weight gain and attributed this decrease to systemic toxicity. Thus, the organophosphates in the oil

mixtures may also have contributed to a decrease in body weight that was only manifested after the stress of fasting. It is interesting to note that the trend in fasting-induced body weight loss observed for rats exposed to G3 and G4 oils at the end of the treatment period was not observable with rats in the recovery groups. This may suggest that the treatment residues were fully or sufficiently cleared from the rat's body systems.

5.2 Alteration of Reproductive Organ Weights of SD Rats

Our findings indicate that only G3-N decreased the weights for testes. We analyzed the organophosphate contents of these oils and observed that both versions of G3 oils contained a mixture of four TCP isomers. The concentration of these isomers combined in G3-U decreased by 26% relative to those in G3-N ([Table 4](#)), suggesting that the potential toxicity of TCP in G3 oil is weakened as the oil ages. This may explain the observation that the unused version of G3 affected the weights of testes while the used version of this oil did not significantly affect this reproductive organ. These results are in agreement with the findings of Latendresse and colleagues ([Latendresse et al., 1994a](#); [Latendresse et al., 1994b](#)) who reported that F344 rats exposed to TCP decreased testicular weights. The mechanisms of testicular degeneration caused by TCP exposure are not well known. Thus, more studies are needed to elucidate the TCP-induced testis weight decrease.

Results for this study show that both versions of G3 and the unused version of G4 oils reduced uterus weights relative to controls. Since our study did not control the estrous cyclicity for females, we cannot rule out that it may have contributed to the alteration of uterine weight. None of the oils had a significant impact on epididymis and ovarian weights. The effects of organophosphate compounds on testes, epididymis, uterus and ovaries have been extensively studied in animals. Some of the results for these studies are in agreement with our findings. For instance, Choudhary and colleagues ([Choudhary et al., 2008](#)) reported a significant reduction in the weight of testes for male Wistar rats orally dosed with malathion ranging from 50 mg/kg to 250 mg/kg body weight per day for 60 days. A study carried out by Dhondup and Kaliwal ([Dhondup and Kaliwal, 1997](#)) indicates that the IP administration of 5 mg/kg body weight of methyl parathion to normal hemicastrated virgin rats for 15 consecutive days induced a decrease in uterine weight. Although G3 and G4 oils did not have significant effects on epididymis and ovarian weights, other investigators have reported an alteration in their weights following rat exposure to organophosphates ([Latendresse et al., 1994a](#); [Latendresse et al., 1994b](#); [Latendresse et al., 1995](#); [Dhondup and Kaliwal, 1997](#); [Prashanthi et al., 2006](#); [Choudhary et al., 2008](#)). Although our study did not investigate the effects of G3 and G4 oils on rat fertility, other studies have highlighted the adverse effects of organophosphates on the fertility of mice and rats ([Ngoula et al., 2007](#); [Mitra and Maitra, 2018](#)). Mathew and colleagues ([Mathew et al., 1992](#)) reported an increase in sperm shape abnormalities for Swiss albino mice after 1 week and 5 weeks following a single acute oral exposure to four different doses of methyl parathion, an organophosphate pesticide used in agriculture. A relatively small dose of this compound (i.p. injection of 0.5-1.5 mg/kg/day for 12-25 days) induced the formation of abnormal sperms of male Wistar rats and a reduction in the sperm count ([Narayana et al., 2005](#)). Exposing rat females to organophosphate compounds is associated with a decrease in healthy follicles, follicular dynamics, estrous cycle and reproductive performances ([Asmathbanu and Kaliwal, 1997](#); [Dhondup and Kaliwal, 1997](#); [Sortur and Kaliwal, 1999](#); [Choudhary et al., 2008](#)). Kaur and Dhanju ([Kaur and Dhanju, 2005](#)) have also reported that exposure to a low dose of methyl parathion for 90 days decreased the phospholipids, protein, total lipids and cholesterol levels in the ovaries for albino

rats. All these observations highlight various adverse effects associated with exposure to organophosphate compounds. Although the rats in our study were dermally exposed, we cannot rule out that changes in the reproductive organ weights that we observed for male and female SD rats exposed to G3 and G4 engine oils were inherent to toxicity associated with the oil's organophosphates or any other ingredients.

5.3 Alteration of Liver, Adrenal and Spleen Weights of Female SD Rats

Data taken at the end of both treatment and recovery periods indicate that the weights for brain, liver, left kidney, right kidney, adrenals, spleen, thymus and heart of male Sprague-Dawley rats were not affected by G3 (Table 5) and G4 (Table 7) oils. Similar observations were noted with females only at the end of the treatment period (Tables 6 & 8). During the recovery period, G3-N elevated the liver weights for females (Table 6) while the unused version of G4 increased adrenal and spleen weights (Table 8). Other researchers have also observed changes in liver weights for animals exposed to organophosphate compounds (Sutton *et al.*, 1960; Xu *et al.*, 2016). Sutton and collaborators (Sutton *et al.*, 1960) reported a 25% increase in liver weight for male Holtzman rats exposed to a sub-chronic low dose of TPP. Our data suggest that females are more likely to experience adverse effects resulting from exposure to G3 and G4 oils. Hinton and colleagues (Hinton *et al.*, 1987) reported that when male and female SD rats were fed with a diet containing TPP doses ranging from 0-1% daily for 120 days, these treatments did not alter the weights for their thymus and spleens. Although the route of administration of TPP and doses applied in the study of Hinton and colleagues (Hinton *et al.*, 1987) and ours were different, their findings are in agreement with our data for G4 oil which contains TPP and did not impact the weights for thymus. However, these data raise two questions that remain to be answered: (1) why the delayed treatment effects were only observed with females and (2) which molecular mechanisms are involved in altering the female's weights of liver, adrenals and spleen.

5.4 Alteration of Hematological Parameters of SD Rats

Hematological assessment provides useful information about the extent of toxicity of drugs or toxicants. Alterations in hematological parameters are considered as crucial biomarkers of physiological stress (Akinrotimi *et al.*, 2009). In this study, we report the profiles of hematological parameters for male and female SD rats dermally exposed to new and used versions of grade 3 and 4 aircraft engine oils over a period of 21 days (total 15 exposures). We found noticeable changes in hematological profiles, regardless of rat gender (Tables 9-12). In general, new oils induced more changes in blood parameters for both males and females when compared to the performance of their used versions. Overall, females were more susceptible to oil exposure than males. Female response to G4 oils was more pronounced relative to G3 performance. All effects induced by exposure to G3 and G4 oils were resolved during the recovery period (Tables 10 & 12), suggesting that alterations in hematological indices for females were transient and reversible.

It is interesting to note that all the blood parameters that were affected by G3 and G4 oils in males were increased while those affected by these treatments in females were decreased except mean platelet volume which was elevated for G4-N. Both unused versions of G3 and G4 oils significantly influenced the levels of white blood cells regardless of sex. G3-N increased the levels of white blood cells in males which decreased in females. However, this effect in males was not observed until the end of the recovery period while the response in the females was noticeable at

the end of treatments. The differences in response to oil exposure between males and females may derive from their differences in hormones. White blood cells, neutrophils, eosinophils, monocytes, basophils and lymphocytes are essential for the immune system involved in body's protection against infections and foreign invaders. Any adverse effect on these parameters might be regarded as an alteration of the immune system. Kumar and colleagues (Kumar *et al.*, 2018) have suggested that leukocyte levels in blood could serve as a simple marker for assessment of organophosphorus poisoning. Although the mechanisms by which aircraft engine oils significantly altered the levels of white blood cells, neutrophils, lymphocytes, eosinophils, eosinophil % and monocyte % in SD rats are not yet known, it is worthy to suggest that organophosphate components of these oils may have been a key player in the alteration process. This assertion is supported by various studies which reported adverse changes in blood indices for farm workers occupationally exposed to organophosphate pesticides (Al-Sarar *et al.*, 2009; Andreadis *et al.*, 2013; Fareed *et al.*, 2013; Nassar *et al.*, 2016). Hematocrit is another blood parameter that was affected by engine oil exposure. Our data on this parameter in males exposed to G4-U oil are in disagreement with Lox's (Lox, 1983) findings who reported a decrease in hematocrit and platelet counts for SD rats which were treated with 1 ml of malathion (99% pure), 1750 ppm given orally via gavage. Nevertheless, Lox's findings corroborate with our data for females exposed to G4-N oil. This oil also elevated mean platelet volume levels in females. Mean platelet volume is the most accurate measure of size of platelets and is inversely associated with platelet count (Levin and Bessman, 1983; Thompson and Jakubowski, 1988). An increased mean platelet volume is an indicator of larger and more reactive platelets resulting from an elevated turnover (Martin *et al.*, 1983) which represents a risk factor for overall vascular mortality (Slavka *et al.*, 2011). Some studies have reported a decrease in mean platelet volume levels for farm workers exposed to pesticides (Varol *et al.*, 2014; Nassar *et al.*, 2016). Although it may be worthy to insinuate that male rats resisted the G4-N-induced biochemical events that led to alteration of mean platelet volume levels in females, the mechanisms at play remain to be elucidated. Although the results show only limited toxicity they suggest the need for additional more focused studies. Additional endpoints are still being evaluated and will be reported in the future.

In general, new oils induced more changes in blood parameters for both males and females when compared to the performance of their used versions, suggesting that the potential toxicity associated with the unused versions of the oils was reduced as they aged. A possible explanation could be that during the aircraft engine operations, the extremely high temperatures in running engines may have been conducive to the breakdown of the oil's ingredients into products that were less toxic relative to their parents. This assertion is supported by our data on organophosphate contents of oils which decreased their levels in used oils relative to those measured in their unused versions (Table 4). The observed decrease in organophosphate contents of used aircraft engine oils relative to new oils corroborates with Johnson and colleagues' report (Johnson *et al.*, 2013) which shows that when three lubricants containing various amounts of TCP were tested on two different stainless steels at varying temperatures ranging from 300 °C to 350 °C, a significant breakdown of the lubricant's phosphate ester additive was noted with a film containing phosphorus deposited onto the stainless steel substrate. Overall, females were more susceptible to oil exposure than males. These differences may have resulted from sex difference and/or treatment dose since females weighed less than males and all animals were exposed to the same dose of 300 µL of oil regardless of their weights. In other words, females may have received a higher dose compared to males. There probably was a difference in plasma exposure between males and females. Although it would be nice to know the concentration of organophosphates in the blood

of the rats, it would be difficult to measure because the small percent of each organophosphate compound in the oil slowly being absorbed through the skin is then diluted into the total blood volume of the rat. It may only be possible to detect in blood by using a radioactive organophosphate. The use of radiotracers were beyond the scope of this study. The justification of applying the same dose of oil to all animals regardless of sex and body weights was to mimic what may happen in a real world environment where male and female aircraft maintenance workers may similarly be exposed to engine oils. Further studies of this nature in which sex and body weights are considered when dosing animals and determining extrapolation to humans are needed. The purpose of this study was limited to assessing the potential toxicity of G3 and G4 oils in their new and used states to organ weights and hematology parameters. In the future studies, we will include other endpoints and report about the full consequences for the animals' health and how the results translate to the occupational setting. Conducting detailed analytical assessment of both versions of these oils may throw light on the status of their ingredient composition, especially the organophosphate degradation by-products such as xylenyl and ethylphenyl phosphates which have been reported to exhibit a similar toxicity to ortho substituted TCP isomers (Bondy *et al.*, 1960; Winder and Balouet, 2002).

5.5 Cholinesterase activities

Studies have reported the existence of significant relationship between exposure to organophosphate compounds in occupational environments and cholinesterase inhibition in exposed workers (Nigg and Knaak, 2000; Hernández *et al.*, 2004; Hernández *et al.*, 2005; Mourad, 2005; Aardema *et al.*, 2008; Masson and Lockridge, 2009; Remor *et al.*, 2009). In our study, we also assessed the activity of AChE in whole blood, brain and liver tissues and BChE in plasma samples because G3 and G4 oils contain some of these compounds (Table 4) and the aircraft maintenance workers, mostly those who maintain the engines may be exposed through inhalation and/or dermal contact. Although inhalation may be a route of occupational exposure, we conducted the dermal exposure, as this route has been reported as the main route of exposure for organophosphate compounds in occupational (Durham *et al.*, 1972; Fenske *et al.*, 2012) and operational settings (Hardos *et al.*, 2019).

This study indicates that control males and females have similar AChE activity in brain (Figs. 13A & 13D). In liver, control females displayed higher activity as compared to males (Figs. 15A & 15D). The activity of this enzyme in whole blood samples collected from females and males of 13-14 weeks (main study groups) of age was also similar (Fig. 17A). However, at age of 15-16 weeks (recovery groups), assayed AChE activity in whole blood for females was significantly increased as compared to males (Fig. 17D). Although there is no major difference in general morphology of the brain cholinergic system, subtle functional differences related to gender have been reported (Giacobini and Pepeu, 2018). Sex hormones exert effects on the cholinergic system (McEwen and Milner, 2017). Female animals possess some cholinergic neurons that express estrogen alpha receptors while in males androgens are expressed (Giacobini and Pepeu, 2018). Our data do not indicate gender differences in brain AChE activity. However, our findings are in disagreement with the report of Das and colleagues (Das *et al.*, 2001) which indicates a significant increase in AChE activity when AChE was assayed in a detergent soluble fraction of cerebral cortex, hippocampus, hypothalamus, thalamus and cerebellum in female Sprague-Dawley adult (3 months of age) rats as compared to males of the same age. These differences in our findings and their data may derive from the samples examined and assay preparation since they used detergent and salt soluble fractions for each brain sample while our assays were conducted for whole brain

lysates that combined the results of detergent and salt soluble fractions. For Das and collaborators, the detergent soluble fraction consisted of predominantly G4 (globular tetrameric) molecular isoform of AChE while the salt soluble fraction consisted of mainly the G1 isoform. Meneguz and colleagues (Meneguz *et al.*, 1992), who investigated the distribution of these molecular forms of AChE in hypothalamus, hippocampus, cerebellum, cerebral cortex, striatum and medulla-pons areas of brain for SD rats, reported variations in their expression in these brain regions. This suggests that our data reflect the combined activity of both G4 and G1 molecular isoforms of AChE while in Das and colleagues' report the activities of these isoforms are reported separately. Our differences may also derive from tissue homogenization protocols since we sampled the whole brain while Das and colleagues sampled different brain regions. The level of AChE activity for control females assigned to the main study significantly ($p \leq 0.05$) decreased by 55% as compared to those in the recovery groups, suggesting a decrease in AChE activity when the rats age. The same comparison for males shows a 28% decrease, although this was not significant. A decrease in AChE activity associated with animal aging was also reported by other studies in different brain regions of SD rats (Meneguz *et al.*, 1992; Das *et al.*, 2001). Das and collaborators observed that females of 18-22 months of age decreased AChE activity by 20-40% in striatum, thalamus, medulla, cerebellum, cerebral cortex and frontal cortex as compared to those with 3 months of age. For males of similar age, the AChE activity decreased by 40-45% in hypothalamus, striatum, frontal cortex and pons. It has been reported that the activity of AChE decreases with age (Pradhan, 1980; Meneguz *et al.*, 1992) and the loss of activity of this enzyme may indicate functional decline in the neuron population (Rani and Panneerselvam, 2001).

Our data indicate that the activity of AChE in the brain for males only significantly decreased when they were exposed to unused versions of G3 and G4 engine oils (Figs. 13B & 13C). However, both unused and used versions of these oils impacted the activity of this enzyme in the brain for females (Figs. 14B & 14C), suggesting that females were more sensitive to engine oil exposure than males. This assertion is supported by the observation that the activity of AChE in blood for males was not affected by oil exposure (Fig. 17) while the used version of G3 significantly decreased its activity in blood for females (Fig. 18A & 18C). This differential response to engine oil exposure between males and females may be associated with gender related functional differences such as sex hormones (McEwen and Milner, 2017; Giacobini and Pepeu, 2018). This engine oil-induced alteration in AChE activity in brain that was observed following three weeks exposure was reversed at the end of two weeks post exposure, regardless of sex (Figs. 13E-F; Figs. 14C-D), suggesting that it was a transient effect. The observation that the impact of the used version of G3 oil on AChE in blood for females was not resolved at the end of two week recovery period suggests that more time is needed for the compounds to be cleared from blood or inactivated. This may also suggest that more time is needed for the blood to replace the AChE inactivated by organophosphate poisoning. It has been reported that organophosphate-induced AChE inhibition is associated with a lower recovery rate (Kamel and Hoppin, 2004). A rapid recovery of AChE activity may involve the reactivation of inhibited forms (Nigg and Knaak, 2000) and the synthesis of new enzymes. Damodaran and colleagues (Damodaran *et al.*, 2003) reported that exposure to organophosphates can increase the overall expression of AChE in the brain of male Sprague-Dawley rats. These findings suggest that a rapid recovery from organophosphate-induced decrease in AChE activity may also derive from increase in biosynthesis of this enzyme to replace the loss. Our data tend to support this assertion since the activity of AChE in brain for both males and females was decreased following the three weeks exposure to G3 and G4 oils but the effect was reversed at the end of the recovery period with a slight increase in

activity although this was not statistically significant. In fact, the activity of this enzyme increased by 27% during the recovery period for males exposed to G3-N while females exposed to G3-U and G4-U showed a 28% and 32% increase, respectively. A trend in increasing the AChE activity following exposure to organophosphate containing engine oils may not be surprising since these compounds are known to alter the relative expression of AChE-R and AChE-S in mammalian adult brain (Salmon *et al.*, 2005).

It is interesting to note that while exposure to G3 and G4 oils had a significant impact on the AChE activity in the brain and blood for male and female rats, these oils did not adversely affect its activity in the liver, regardless of sex. It may not be surprising that this enzyme was more protected in the liver than in the brain and blood as the liver has been shown to be the principal organ mediating biotransformation of thiono-organophosphates before their elimination (Neal, 1972; Selmi *et al.*, 2018). These differences between AChE in brain and liver may be related to differences in rate of metabolizing the poisoning compounds. It is possible that the brain had lower metabolic activity towards engine oil contained organophosphates allowing them to accumulate and cause more harms. Conversely, the liver may have had a higher metabolic activity towards these compounds allowing it to quickly convert them into less harmful products. It is known that the rate of dermal penetration and absorption varies greatly between organophosphate compounds and these differences can be reflected in their toxicokinetics once absorbed through the skin (Voorhees *et al.*, 2017). With dermal exposure, the rate of AChE inhibition may be correlated with the amount of organophosphates that absorb through the skin and achieve the system dosing. Organophosphate absorption through the skin, which is the primary route of occupational exposure (Buchanan *et al.*, 2001), is relatively slow in comparison to intraperitoneal injection or oral exposure (Tomokuni and Hasegawa, 1985; Garfitt and Jones, 2002). Chronic exposure may increase the likelihood of toxicity when the poisoning compounds accumulate into the system. In our study, the liver may have been able to handle the amount of organophosphates that penetrated the skin and amounted to the systemic dose. The amount of organophosphate compounds in the engine oil that reached the liver may have been too low to significantly impact the AChE activity. The observation that this enzyme was not affected by the oil exposure in liver does not eliminate the possibility of non-cholinesterase effects which may not translate into toxicity. Although the main mechanism of toxicity for organophosphate compounds invoke the AChE inhibition, several lines of evidence suggest the existence of other mechanisms through which they induce toxicity (Berríos *et al.*, 2015). Ray and Richards (Ray and Richards, 2001) have reported that low doses that do not result in cholinergic effects have been shown to initiate a variety of adverse effects in animal settings ranging from slowed nerve conduction to enhanced maze learning. Proteins other than AChE may be sensitive to low levels of some organophosphates as evidence of this nature is known to exist in the brain (Ray, 1998).

BChE is another enzyme targeted by organophosphate compounds. Although this enzyme is expressed in several tissues such as heart, lungs, brain, gut, liver and others, it is mainly synthesized by liver and secreted into the plasma (Šišková *et al.*, 2017). Therefore, BChE activity in plasma reflects primarily the level of its production in the liver. Its level in plasma can serve as a good indicator for organophosphate-induced BChE inhibition. We assessed the levels of BChE in plasma for rats and observed strong gender related differences in BChE activity, with females exhibiting higher activity as compared to males (Figs. 19A & 19D). Sex differences in BChE activity in plasma have been reported (Edwards and Brimijoin, 1983). The regulation of BChE activity may be influenced by sex hormones since gender related functional differences exist (McEwen and Milner, 2017; Giacobini and Pepeu, 2018) and sex hormones exert effects on the

cholinergic system (McEwen and Milner, 2017). We also investigated the effect of G3 and G4 oil exposure on rat's BChE activity in plasma. The activity of this enzyme in plasma for males was not affected by any version of these oils. Although exposure to both versions of G3 oils did not have an impact on this enzyme in plasma for females, the unused version of G4 significantly decreased its activity, two weeks post exposure (Fig. 20D). The G4-N induced delayed effect on BChE activity remains a mystery that requires further investigation. It has been reported that alteration in BChE activity does not reflect the inhibition activity of organophosphate in the nervous system (He, 1999). A decrease in its activity is generally regarded as the consequence of liver damage (Prohanka, 2013). Although data for the three weeks engine oil exposure indicate that BChE for females may be more sensitive to G4 oil than males, we cannot rule out that a more prolonged exposure can also have an adverse impact on males.

Although our findings in this study indicate that some adverse effects observed on AChE and BChE following three weeks exposure to G3 and G4 engine oils were reversed within the two weeks of the recovery period, we cannot conclude that the effects observed in rats are also reversible in humans. The aircraft maintenance workers may be chronically exposed to low levels of engine oil's organophosphate contents which may accumulate over time in their system, leading to a variety of health problems. Additionally, since these workers are not exactly exposed to the same amount of these compounds, they may display different toxicity profiles and severity of symptoms. As Voorhees and collaborators (Voorhees *et al.*, 2017) reported, there is evidence suggesting that repeated exposure to lower organophosphate levels that do not invoke cholinergic symptoms are capable of posing serious risks to people such as neurodegenerative diseases and sensorimotor deficits. In several countries, the conventional method of assessing the impact associated with an occupational exposure to organophosphates is subjecting workers to a periodic statutory medical surveillance of their cholinesterase levels in blood (Ng *et al.*, 2009). It has been reported that after a chronic exposure to these compounds, evaluating the AChE activity gives more sensitive indication of their inherent toxicity than using the BChE (Lionetto *et al.*, 2013), as the AChE inhibition is currently known as the main mechanism of organophosphate-induced toxicity (Berríos *et al.*, 2015). Thus, assessing AChE inhibition has been regarded as a useful biomarker for adverse effects related to organophosphate exposure (Lionetto *et al.*, 2013). Although monitoring AChE activity in workers is a good idea, there may be variation in the baseline activity of the enzyme, and its decrease may not correlate well with observed manifestations or the symptoms may persist longer after the level of this enzyme has returned to baseline (Rohlman *et al.*, 2011; Rohlman *et al.*, 2016).

5.6 Electrophysiology Measurements

Electrophysiology endpoints were measured in a preliminary pilot study to characterize the electrophysiological effects associated with repeated exposures to unused and used versions of G3 and G4 aircraft engine oils on the hippocampus. Hippocampus is critical for the learning process as well as memory formation and has been implicated in other cognitive functions. We initiated this pilot study based on several reports that highlight the adverse effects of organophosphate exposure on neuronal cells. These reports show that organophosphates induce changes (1) in hippocampal neuropeptides and synaptic transmission (Speed *et al.*, 2012; Lee *et al.*, 2016), (2) in neurite and axonal lengths, neurite number and dendritic branching (Howard *et al.*, 2005; Yousefpour *et al.*, 2006; Yang *et al.*, 2008; Chen *et al.*, 2013; Hausherr *et al.*, 2014; Pizzurro *et al.*, 2014a; Pizzurro *et al.*, 2014b; Berríos *et al.*, 2015; Duarte *et al.*, 2017; Hausherr *et al.*, 2017), (3) alter neurotransmission (Jameson *et al.*, 2007; Slotkin and Seidler, 2010) and (4)

modulate neuroinflammation (Mense *et al.*, 2006; Banks and Lein, 2012; Banks, 2015; Binukumar *et al.*, 2111). In this pilot study, we measured the effects of G3 and G4 exposure on (1) synaptic transmission in rat hippocampus, (2) spontaneous activity of hippocampal neurons, (3) short and (4) long term synaptic plasticity.

We must first note here that this pilot study carries a drawback of using a small sample size for each treatment group ($n = 1$ rat/sex or $n = 2$ if we combine male and female values for each group). However, there are sufficient differences in synaptic properties between male and female animals such that we cannot combine data across gender and thus data are presented as one animal per experimental group. Since organophosphates have an impact on neurotransmitters (Torres-Altoro *et al.*, 2011; Judge *et al.*, 2016), exposures to G3 and G4 oils were expected to induce changes in the size of evoked synaptic responses within hippocampus of rats. Although statistical analyses were not performed on these data since we have only one sample for each treatment, males exposed to unused version of G4 and both versions of G3 tended to display different field responses of neurons within the CA1 region relative to control (Fig. 22A). Similar observations were noted for the females exposed to the unused version of G3 (Fig. 22B). We also expected the engine oil treatments to impact the PPF and LTP since (1) organophosphates alter cholinergic inputs (Barrett and Oehme, 1994; Costa, 2006; Mwila *et al.*, 2013; Costa, 2018) and (2) these inputs have a direct effect on the hippocampal synaptic plasticity (Gu and Yakel, 2011). PPF is the short term potentiation of response during two consecutive stimuli (paired stimuli) delivered within tens of milliseconds apart and it is believed to derive from a build-up of calcium ions resulting from the paired stimuli delivered very close together (Katz and Miledi, 1968; Wu and Saggau, 1994). Although our findings did not show any trend for engine oil-induced alteration in PPF and LTP parameters, females exposed to both versions of G4 oils showed a trend in increasing the spontaneous activity of hippocampal neurons (Fig. 26B). Similar observations were noted with males exposed to unused version of G4 (Fig. 26A). Since there was a trend in increasing spontaneous activity, the lack of alterations in synaptic plasticity (PPF and LTP) following engine oil exposure may suggest that changes to synaptic plasticity inherent to treatment were small and could not be detected.

Engine oils did not have significant impact on measured electrophysiology properties of hippocampal neurons when using one animal per experimental group. There were some noticeable differences in the size of evoked response as reported by the input-output function as well as in the frequency spontaneous activity, but the differences detected may be due to animal variation. A more detailed study using a large sample size per experimental group is needed to assess whether the detected differences in AChE and BChE activity resulting from engine oil exposures can induce measurable changes in synaptic transmission and spontaneous activity of hippocampal neurons. On the other hand, there were no noticeable differences in the synaptic plasticity parameters (PPF, LTP) following exposures to G3 and G4 oils. However, we cannot rule out the possibility that exposing these rats to engine oils for a period longer than three weeks and increasing the sample size per treatment group could reveal changes in synaptic plasticity. Further electrophysiological investigations are needed to elucidate the effects that exposure to G3 and G4 oils may have on neuronal function.

6.0 CONCLUSION

This study revealed that a subchronic exposure to G3 and G4 oils in both their new or used states altered the weights of the reproductive organs, hematological indices, AChE and BChE activities for male and female SD rats. Some of these alterations are similar to changes that have been observed with agricultural workers occupationally exposed to organophosphate pesticides. All these observations resulting from just three weeks of exposure to G3 and G4 oils raise concerns about the magnitude of impact related to prolonged exposure in real world environments; aircraft maintenance workers may be exposed for many years.

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LIST OF ACRONYMS

%	Percent
±	Plus or minus
≤	Less than or equal to
μL	Microliter(s)
°C	Degree Celsius
ΔOD	Difference in optical density (absorbance)
Δt:	Reaction time
³¹ P NMR	Phosphorus-31 nuclear magnetic resonance
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
AChE	Acetylcholinesterase
ACSF	Artificial cerebrospinal fluid
AFB	Air Force Base
AFRL	Air Force Research Laboratory
BA#	Basophil counts
BA%	Percentage of the basophils
BChE	Butyrylcholinesterase
CaCl ₂	Calcium chloride
cm	Centimeter(s)
CO ₂	Carbone dioxide
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
EO#	Eosinophil counts
EO%	Percentage of the eosinophils
EPA	Environmental Protection Agency
fEPSP	Field excitatory postsynaptic potentials
Fig(s)	Figure(s)
g	Gram(s)
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
h	Hour(s)
HCT	Hematocrit;
Hgb	Hemoglobin
HJF	Henry M. Jackson Foundation for the Advancement of Military Medicine
Hz	Hertz
IACUC	Installation Animal Care and Use Committee
KCl	Potassium chloride
kg	Kiligram(s)

KH ₂ PO ₄	Monopotassium phosphate
kHz	Kilohertz
L	Liter(s)
LY#	Lymphocyte counts
LY%	Percentage of the lymphocytes
LTP	Long term potentiation
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
mg	Milligram(s)
mg/kg	Milligram(s) per kilogram
MgSO ₄	Magnesium sulfate
mHz	Megahertz
min.	Minute(s)
mm	Millimeter(s)
mM	Millimolar
MO#	Monocyte counts
MO%	Percentage of the monocytes
MPV	Mean platelet volume
ms	Millisecond(s)
mU/mL	Milliunit(s) per milliliter
NaCl	Sodium chloride
NaHCO ₃	Sodium bicarbonate
NE#	Neutrophil counts
NE%	Percentage of neutrophils
nm	Nanometer(s)
nmol	Nanomole(s)
nmol/min/mL	Nanomole(s) per minute per milliliter
NRC	National Research Council
NTE	Neuropathy Target Esterase
O ₂	Molecular oxygen
OD	Optical density (absorbance)
OECD	Organization for Economic Cooperation and Development
OPPTS	Office of Prevention, Pesticides, and Toxic Substances
OROC	Office of Research Oversight & Compliance
p	Probability
PIP	Phenyl Isopropylated Phosphate (3:1)
PLT	Platelets
PPD	Paired pulse depression
PPF	Paired pulse facilitation
ppm	Parts per million
RBC	Total red blood cells
RDW%	Red blood cell distribution width percentage
RODI	Reverse Osmosis Deionized Water
SD	Sprague Dawley rats
SDS	Safety Data Sheet

S.E	Standard error of the mean
S/N	Signal-to-noise ratio
TBS	Theta burst stimulation
TCP	Tri-Cresyl Phosphate
TNB	2-nitro-5-thiobenzoate
TOCP	Tri-Ortho-Cresyl Phosphate
TPP	Tri-Phenyl Phosphate
U	Unit(s)
U/mg	Unit(s) per milligram(s)
USAF	U.S. Air Force
V	Volume
WBC	White blood cells
x g	Relative centrifugal force (g force)

APPENDIX A: DEVIATIONS

Only one deviation occurred during the study. Although the sample size for animals assigned to the main study was set to 10 males and 10 females per treatment group, G3-N and G3-U groups included 9 and 11 rats, respectively.

APPENDIX B: NON-NORMALIZED ORGAN WEIGHTS

Table 1. Organ weights (mg) for male Sprague-Dawley rats dermally exposed to water (control), new (G3-N) and used (G3-U) versions of grade 3, new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days (N = 9-11).

Treatment	Organ weights (mg)									
	Liver	Brain	Left kidney	Right kidney	Adrenals	Spleen	Thymus	Heart	Testes	Epididymides
Control	9.925	2.000	1.437	1.387	0.053	0.844	0.431	1.030	3.635	1.214
Control	9.885	2.026	1.391	1.444	0.049	0.702	0.283	1.050	3.720	1.152
Control	9.816	2.194	1.510	1.568	0.063	0.657	0.592	1.104	2.689	1.060
Control	12.754	2.163	1.546	1.586	0.053	1.099	0.521	1.417	3.165	1.105
Control	12.669	2.321	1.460	1.412	0.049	0.752	0.803	1.371	3.502	1.142
Control	10.158	1.364	1.181	1.214	0.054	0.627	0.304	1.063	3.555	1.158
Control	11.729	2.033	1.606	1.557	0.042	0.769	0.541	1.253	2.980	1.095
Control	13.374	2.050	1.424	1.510	0.053	1.072	0.770	1.355	3.136	1.461
Control	12.881	1.989	1.452	1.594	0.052	0.904	0.545	1.111	3.335	1.057
Control	15.140	1.859	1.470	1.579	0.062	0.893	0.514	1.133	3.332	1.045
G3-N	10.944	2.094	1.559	1.610	0.047	0.854	0.278	1.370	3.477	1.089
G3-N	10.718	1.890	1.402	1.429	0.065	0.638	0.428	1.210	3.423	1.076
G3-N	12.325	2.069	1.369	1.429	0.063	0.814	0.623	1.183	3.589	1.191
G3-N	10.757	2.162	1.465	1.380	0.064	0.751	0.176	1.108	3.166	1.048
G3-N	12.031	1.927	1.193	1.310	0.047	0.742	0.221	1.062	3.253	1.040
G3-N	11.921	1.835	1.472	1.431	0.028	0.733	0.528	1.116	3.337	1.109
G3-N	13.234	1.988	1.298	1.340	0.032	0.693	0.638	1.282	3.642	1.586
G3-N	10.388	2.086	1.244	1.279	0.048	0.776	0.584	1.023	3.189	1.123
G3-N	11.865	1.929	1.527	1.494	0.06	0.635	0.677	1.142	3.41	1.011
G3-U	12.107	1.964	1.446	1.496	0.028	0.757	0.525	1.220	3.323	1.090
G3-U	12.215	2.101	1.455	1.477	0.064	0.772	0.401	1.200	3.023	1.091
G3-U	11.350	1.970	1.400	1.484	0.049	0.625	0.464	1.044	3.099	1.114
G3-U	10.564	1.988	1.395	1.391	0.077	0.781	0.363	1.010	3.546	1.136
G3-U	10.934	2.021	1.194	1.250	0.024	0.550	0.293	1.222	3.149	1.113

G3-U	10.503	1.942	1.472	1.533	0.045	0.847	0.567	1.105	2.902	1.038
G3-U	12.180	2.007	1.390	1.440	0.060	0.954	0.625	1.468	3.402	1.085
G3-U	15.436	2.017	1.609	1.593	0.051	0.996	0.465	1.281	3.205	1.083
G3-U	10.46	1.914	1.426	1.475	0.054	0.616	0.486	1.133	3.362	0.926
G3-U	11.699	2.04	1.444	1.485	0.036	0.702	0.498	1.018	3.312	1.023
G3-U	13.802	2.005	1.792	1.839	0.045	0.809	0.353	1.204	3.723	1.188
G4-N	11.724	2.110	1.279	1.288	0.057	0.710	0.283	1.178	3.254	1.014
G4-N	11.699	2.033	1.280	1.326	0.047	0.687	0.683	1.233	2.672	0.703
G4-N	14.330	1.909	1.488	1.548	0.047	0.841	0.221	1.164	3.417	1.108
G4-N	15.939	1.947	1.512	1.696	0.058	0.705	0.352	1.134	3.369	1.172
G4-N	9.365	2.121	1.318	1.472	0.055	0.841	0.646	1.127	3.133	0.979
G4-N	14.075	1.867	1.398	1.466	0.039	0.745	0.428	1.113	3.024	0.989
G4-N	11.457	2.005	1.351	1.408	0.057	0.797	0.501	1.172	3.442	1.135
G4-N	12.828	2.116	1.484	1.626	0.042	1.043	0.592	1.215	3.391	1.023
G4-N	12.043	2.03	1.518	1.497	0.036	0.81	0.471	1.137	3.267	1.081
G4-N	14.322	-	1.513	1.485	0.046	0.915	0.515	1.432	4.223	1.164
G4-U	10.621	2.042	1.371	1.353	0.062	0.716	0.274	0.212	3.481	1.109
G4-U	10.351	2.127	1.437	1.439	0.047	0.777	0.260	1.085	3.358	1.092
G4-U	10.067	2.127	1.153	1.200	0.041	0.730	0.886	1.225	3.221	1.146
G4-U	12.519	2.093	1.583	1.613	0.032	1.123	0.202	1.250	3.579	1.079
G4-U	10.522	2.134	1.396	1.371	0.056	0.666	0.382	1.000	3.514	1.152
G4-U	9.621	1.915	1.071	1.180	0.048	0.559	0.410	1.011	3.258	1.119
G4-U	10.858	2.143	1.490	1.534	0.044	0.789	0.445	1.157	3.359	1.113
G4-U	10.341	1.976	1.425	1.502	0.051	0.934	0.479	1.138	2.904	0.938
G4-U	12.525	1.902	1.454	1.459	0.064	0.838	0.609	1.16	3.723	0.900
G4-U	14.874	2.07	1.616	1.565	0.064	0.758	0.606	1.18	3.428	1.001

“-” indicates a missing value

Table 2. Organ weights (mg) for female Sprague-Dawley rats dermally exposed to water (control), new (G3-N) and used (G3-U) versions of grade 3, new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days (N = 10).

Treatment	Organ weights (mg)									
	Liver	Brain	Left kidney	Right kidney	Adrenals	Spleen	Thymus	Heart	Uterus	Ovaries
Control	5.935	1.911	0.795	0.784	0.064	0.425	0.306	0.795	0.662	0.104
Control	7.179	1.779	0.861	0.796	0.058	0.563	0.360	0.823	0.381	0.142
Control	6.896	1.986	0.807	0.852	0.083	0.468	0.571	0.868	1.058	0.135
Control	8.472	1.988	0.997	0.983	0.060	0.438	0.366	0.870	0.945	0.124
Control	7.164	1.897	0.877	0.889	0.065	0.481	0.377	0.786	0.692	0.153
Control	6.461	1.804	0.839	0.885	0.087	0.666	0.536	0.810	0.769	0.131
Control	6.861	1.916	1.014	1.023	0.066	0.662	0.473	0.751	1.201	0.101
Control	6.191	1.930	0.787	0.858	0.055	0.409	0.247	0.749	0.479	0.116
Control	6.207	1.868	0.842	0.857	0.064	0.516	0.215	0.692	0.962	0.13
Control	7.600	2.034	0.892	1.025	0.066	0.497	0.449	0.672	0.778	0.13
G3-N	6.261	1.842	0.848	0.859	0.071	0.627	0.370	0.799	0.999	0.125
G3-N	9.572	1.885	1.145	1.091	0.079	0.621	0.506	1.016	0.407	0.143
G3-N	7.396	1.747	0.750	0.723	0.044	0.561	0.439	0.773	0.470	0.275
G3-N	6.587	1.814	0.836	0.902	0.038	0.400	0.474	0.766	0.662	0.109
G3-N	6.986	1.958	1.061	1.084	0.065	0.593	0.213	0.792	0.480	0.105
G3-N	7.430	1.943	1.072	1.107	0.072	0.547	0.511	0.878	0.453	0.160
G3-N	5.780	1.879	0.758	0.779	0.061	0.540	0.191	0.653	0.412	0.100
G3-N	5.826	1.815	0.617	0.649	0.045	0.407	0.456	0.696	0.770	0.083
G3-N	8.228	1.855	0.863	0.839	0.064	0.545	0.429	0.755	0.743	0.121
G3-N	8.248	1.908	0.869	0.919	0.066	0.631	0.456	0.841	0.505	0.127
G3-U	7.916	1.858	0.912	0.907	0.071	0.609	0.772	0.858	0.546	0.129
G3-U	6.062	1.864	0.791	0.775	0.058	0.426	0.383	0.716	0.617	0.104
G3-U	6.374	1.895	0.827	0.889	0.054	0.526	0.513	0.761	0.332	0.109
G3-U	7.095	2.016	0.965	0.986	0.076	0.689	0.199	0.851	0.483	0.160
G3-U	7.998	1.910	1.011	1.068	0.056	0.660	0.446	0.732	0.555	0.139
G3-U	6.387	2.016	0.916	0.971	0.066	0.395	0.167	0.792	0.704	0.114

G3-U	9.172	2.053	0.921	1.017	0.068	0.570	0.312	0.897	0.749	0.134
G3-U	8.236	1.894	1.178	1.229	0.084	0.518	0.401	0.889	0.501	0.172
G3-U	7.770	1.92	0.775	0.856	0.059	0.553	0.351	0.704	0.456	0.12
G3-U	9.936	1.848	0.996	0.982	0.067	0.724	0.410	0.79	0.693	0.112
G4-N	5.785	1.836	0.754	0.789	0.059	0.514	0.282	0.671	0.453	0.118
G4-N	6.169	1.828	0.748	0.770	0.040	0.459	0.437	0.824	0.704	0.128
G4-N	9.242	2.067	1.100	1.100	0.037	0.521	0.492	0.795	0.575	0.128
G4-N	7.555	1.803	0.825	0.890	0.057	0.656	0.400	0.693	0.583	0.128
G4-N	7.461	1.976	1.161	1.112	0.072	0.556	0.364	0.876	0.526	0.145
G4-N	7.147	1.766	0.803	0.839	0.066	0.468	0.439	0.780	0.453	0.130
G4-N	5.944	1.833	0.642	0.709	0.054	0.486	0.577	0.788	0.474	0.102
G4-N	5.746	1.891	0.832	0.913	0.054	0.466	0.311	0.680	0.644	0.123
G4-N	7.544	1.785	0.931	0.962	0.05	0.548	0.233	0.812	0.514	0.123
G4-N	7.766	1.952	0.824	0.839	0.063	0.492	0.144	0.701	0.349	0.11
G4-U	6.901	1.824	0.888	0.917	0.073	0.518	0.442	0.779	0.421	0.128
G4-U	8.523	1.826	1.036	1.094	0.054	0.488	0.295	0.807	0.416	0.139
G4-U	7.766	1.828	0.930	0.939	0.084	0.599	0.346	0.976	0.523	0.160
G4-U	7.719	1.693	0.884	0.853	0.067	0.598	0.532	0.871	0.855	0.124
G4-U	7.001	1.830	0.863	0.910	0.083	0.700	0.456	0.719	0.470	0.139
G4-U	5.687	1.851	0.765	0.771	0.047	0.347	0.216	0.728	1.481	0.114
G4-U	6.290	1.910	0.869	0.884	0.072	0.483	0.293	0.770	0.402	0.105
G4-U	6.557	1.973	0.945	0.894	0.065	0.511	0.362	0.797	0.951	0.143
G4-U	8.289	1.910	0.83	0.846	0.065	0.463	0.190	0.745	0.383	0.101
G4-U	3.921	1.852	0.838	0.881	0.055	0.519	0.162	0.647	0.416	0.111

Table 3. Organ weights (mg) for male Sprague-Dawley rats dermally exposed to water (control), new (G3-N) and used (G3-U) versions of grade 3, new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (N = 5).

Treatment	Organ weights (mg)									
	Liver	Brain	Left kidney	Right kidney	Adrenals	Spleen	Thymus	Heart	Testes	Epididymides
Control	16.424	1.989	1.656	1.748	0.034	0.945	0.310	1.417	3.460	1.232
Control	10.738	2.155	1.356	1.387	0.032	0.886	0.227	1.039	3.671	1.257
Control	14.084	2.079	1.653	1.695	0.061	0.983	0.590	1.424	3.555	0.876
Control	11.974	2.146	1.311	1.402	0.056	0.653	0.209	1.088	3.560	1.307
Control	14.960	1.981	1.722	1.718	0.052	0.878	0.570	1.327	3.630	1.287
G3-N	14.017	2.138	1.598	1.556	0.048	0.856	0.407	1.222	3.308	1.232
G3-N	14.881	2.115	1.594	1.633	0.049	1.046	0.634	1.429	3.107	1.195
G3-N	13.298	2.233	1.423	1.410	0.045	0.839	0.515	1.243	3.412	1.219
G3-N	10.987	2.073	1.339	1.353	0.038	0.896	0.485	1.201	3.099	1.151
G3-N	14.772	1.907	1.54	1.664	0.059	0.681	0.655	1.694	3.014	1.281
G3-U	11.725	2.162	1.463	1.580	0.062	0.729	0.435	1.110	3.147	1.200
G3-U	14.119	2.077	1.779	1.765	0.041	0.726	0.342	1.245	3.366	1.229
G3-U	14.202	2.132	1.501	1.593	0.039	0.894	0.750	1.373	3.750	1.338
G3-U	13.644	2.012	1.586	1.570	0.053	0.837	0.464	1.246	3.435	1.281
G3-U	13.091	2.171	1.724	1.859	0.055	0.926	0.407	1.300	3.468	1.315
G4-N	13.300	2.154	1.521	1.589	0.068	0.968	0.403	1.246	3.412	1.265
G4-N	13.455	2.115	1.669	1.743	0.046	0.850	0.517	1.371	3.686	1.202
G4-N	13.089	2.119	1.661	1.662	0.048	0.729	0.679	1.270	3.120	1.266
G4-N	21.029	2.141	1.334	1.339	0.058	1.019	0.414	1.387	3.023	1.201
G4-N	11.368	2.011	1.401	1.375	0.056	0.774	0.395	1.185	2.950	1.348
G4-U	12.023	2.284	1.392	1.505	0.066	0.836	0.616	1.244	3.878	1.468
G4-U	14.567	2.031	1.799	1.872	0.056	1.010	0.367	1.351	3.237	1.168
G4-U	12.439	2.128	1.509	1.481	0.052	0.781	0.673	1.200	2.959	1.222
G4-U	16.390	2.098	1.635	1.779	0.060	0.894	0.328	1.155	3.434	1.150
G4-U	13.914	2.076	1.650	1.666	0.079	0.842	0.202	1.140	3.745	1.227

Table 4. Organ weights (mg) for female Sprague-Dawley rats dermally exposed to water (control), new (G3-N) and used (G3-U) versions of grade 3, new (G4-N) and used (G4-U) versions of grade 4 of the aircraft engine oils over a period of 21 days and were kept for 14 days post exposure to screen for reversibility, persistence, or delayed occurrence of toxicity effects (N = 5).

Treatment	Organ weights (mg)									
	Liver	Brain	Left kidney	Right kidney	Adrenals	Spleen	Thymus	Heart	Uterus	Ovaries
Control	8.131	1.930	0.824	0.836	0.061	0.458	0.166	0.827	0.689	0.122
Control	8.325	2.051	0.956	0.987	0.076	0.625	0.410	0.862	0.830	0.146
Control	8.420	1.922	0.963	1.111	0.055	0.476	0.343	0.856	0.458	0.139
Control	6.657	1.886	0.815	0.887	0.054	0.423	0.419	0.721	0.450	0.181
Control	6.596	1.928	0.831	0.933	0.061	0.574	0.451	0.902	0.523	0.123
G3-N	8.149	1.986	0.999	1.071	0.086	0.922	0.865	0.819	0.763	0.145
G3-N	8.724	1.934	0.842	0.940	0.070	0.549	0.397	0.847	0.665	0.130
G3-N	8.705	2.019	0.905	0.974	0.052	0.588	0.532	0.960	0.602	0.154
G3-N	8.836	1.938	1.084	1.141	0.074	0.618	0.466	0.937	0.909	0.141
G3-N	8.555	1.820	0.895	0.915	0.067	0.512	0.345	0.764	0.387	0.117
G3-U	7.345	1.885	0.907	0.996	0.071	0.485	0.477	0.824	0.436	0.157
G3-U	7.069	1.953	0.869	0.875	0.072	0.449	0.365	0.821	0.548	0.140
G3-U	9.290	2.120	1.091	1.076	0.068	0.722	0.366	0.947	0.486	0.140
G3-U	7.709	1.907	0.957	1.002	0.076	0.612	0.406	0.778	0.517	0.128
G3-U	7.468	1.938	0.892	0.958	0.045	0.400	0.316	0.678	0.465	0.116
G4-N	9.077	1.882	0.920	0.957	0.087	0.663	0.248	0.816	0.627	0.129
G4-N	7.930	1.853	1.025	1.057	0.068	0.549	0.336	0.879	0.570	0.150
G4-N	10.078	1.890	0.962	1.060	0.079	0.736	0.304	0.933	0.513	0.169
G4-N	8.235	1.974	1.058	1.095	0.107	0.624	0.449	0.849	0.335	0.188
G4-N	6.765	1.796	0.828	0.896	0.058	0.559	0.478	0.783	0.868	0.106
G4-U	8.019	2.106	0.982	1.062	0.052	0.526	0.507	0.803	0.904	0.123
G4-U	7.663	1.918	0.925	0.978	0.074	0.892	0.667	1.077	0.409	0.151
G4-U	8.370	2.024	0.914	0.962	0.067	0.647	0.354	0.787	0.496	0.139
G4-U	9.596	2.039	1.039	1.004	0.068	0.618	0.369	1.013	0.958	0.118
G4-U	7.174	1.798	0.794	0.896	0.065	0.623	0.443	0.816	0.486	0.106