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Factors influencing adverse skin responses in rats receiving repeated subcutaneous injections and potential impact on neurobehavior.

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Abstract

Repeated subcutaneous (s.c.) injection is a common route of administration in chronic studies of neuroactive compounds. However, in a pilot study we noted a significant incidence of skin abnormalities in adult male Long-Evans rats receiving daily s.c. injections of peanut oil (1.0 ml/kg) in the subscapular region for 21 d. Histopathological analyses of the lesions were consistent with a foreign body reaction. Subsequent studies were conducted to determine factors that influenced the incidence or severity of skin abnormalities, and whether these adverse skin reactions influenced a specific neurobehavioral outcome. Rats injected daily for 21 d with food grade peanut oil had an earlier onset and greater incidence of skin abnormalities relative to rats receiving an equal volume (1.0 ml/kg/d) of reagent grade peanut oil or triglyceride of coconut oil. Skin abnormalities in animals injected daily with peanut oil were increased in animals housed on corncob versus paper bedding. Comparison of animals obtained from different barrier facilities exposed to the same injection paradigm (reagent grade peanut oil, 1.0 ml/kg/d s.c.) revealed significant differences in the severity of skin abnormalities. However, animals from different barrier facilities did not perform differently in a Pavlovian fear conditioning task. Collectively, these data suggest that environmental factors influence the incidence and severity of skin abnormalities following repeated s.c. injections, but that these adverse skin responses do not significantly influence performance in at least one test of learning and memory.

Keywords: adverse skin reaction, foreign body reaction, learning and memory, neurobehavior, peanut oil, subcutaneous injection

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Introduction

Repeated subcutaneous (s.c.) injection is a common route of administration in chronic studies of neuroactive compounds. While peanut oil is often used as a vehicle for s.c. administration of lipophilic compounds, scattered reports indicate that repeated s.c. injections of peanut oil can leave deposits, introduce impurities or induce local irritation [1]. Whether this reaction is unique to peanut oil and how environmental factors known to influence sensitization alter the incidence and severity of skin irritation subsequent to repeated s.c. injections of peanut oil is not known. Also unknown is whether these peripheral immune reactions interfere with behavioral outcomes. The latter is a plausible concern in light of experimental evidence demonstrating that the immune system can modulate central nervous system function via stress or neuroendocrine signals [2,3]. The cells of the immune and nervous systems generate and respond to the same neurotransmitters and cytokines, thus, changes in the activational status of the peripheral immune system can alter neural signaling pathways [2]. As one example, the cytokine, interleukin-1, has been implicated in impaired contextual fear conditioning in Sprague-Dawley rats [3].

Here, we conducted studies to determine whether skin abnormalities initially observed in Long-Evans rats injected for 21 d with food grade peanut oil (1.0 ml/kg/d s.c.) were influenced by the type of oil used as the vehicle, the bedding material on which rats were housed, the barrier facility from which animals were imported or a

combination of these factors. Additionally, we tested if these skin abnormalities interfered with performance in a learning and memory task. Our findings indicate that all the environmental factors we tested contributed to the incidence and severity of skin abnormalities following repeated s.c. injections, but that these adverse skin responses do not significantly influence learning and memory in a Pavlovian fear conditioning task.

Materials and Methods

Chemicals

Food grade peanut oil (Planters® 100% peanut oil) was purchased from a local grocery store (Davis, CA, USA). Reagent grade peanut oil (Sigma catalog #P2144, CAS: 8002-03-7) and reagent grade ethanol were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). Laboratory grade triglyceride of coconut oil, Neobee M-5 (Catalog #N1328, CAS: 73398-61-5), was purchased from Spectrum Chemical Manufacturing Corporation (Gardena, CA, USA).

Animals

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California-Davis, and conformed to the Guide for the Care and Use of Laboratory Animals set forth by the United States National Institute of Health. All animals were treated humanely and with regard to minimizing pain and distress. Male Long-Evans rats (5 weeks old, 250-350g) were obtained from either Charles Rivers (Portage, Michigan, USA) or Harlan (Indianapolis, IN, USA) and were housed two per cage on 1/8″ corncob (Harlan Teklad, Madison, WI, USA) or paper bedding (Carefresh®, Higby's Country Feed Store, Dixon, CA, USA). All subjects were housed under controlled environmental conditions with a 12 h light/dark cycle and a temperature of 72-78°F. Rats received rodent chow (2018 18% Protein Rodent Diet, Harlan Tekland, USA) and tap water *ad libitum*. Bedding and food were autoclaved prior to use.

Injections

Rats were acclimated for 7 d prior to administration of vehicle. Animals received daily s.c. injections of vehicle (1.0 ml/kg) between the shoulder blades for 21 d. Fur was not shaved and disinfectants were not applied to the site of injection. Vehicle solutions (in 10% ethanol) were made daily. After vortexing for 2 min followed by sonication for 1 min, vehicle solutions were wrapped in foil to prevent exposure to light and kept at room temperature. All animal were injected in the afternoon at approximately the same time of day (± 1 h) using sterile, disposable syringes with 25 G PrecisionGlide® needles (BD Biosciences, Franklin Lakes, NJ, USA).

Analyses of skin abnormalities

Rats were scored daily as to the presence and type (swelling, irritation, alopecia, scabbing, or abrasions) of grossly evident skin abnormalities. In a subset of studies, the severity of the skin reaction was categorized as mild, moderate, or marked based on the reaction diameter (<3mm, 3-6mm, and ≥7mm, respectively). All rats were checked by a board-certified laboratory animal veterinarian every 2-3 d to confirm scoring by laboratory personnel. Two rats with the most visibly severe reactions at the conclusion of a 21-d injection study (both from the same barrier facility, injected with reagent grade peanut oil and housed on corncob bedding) were submitted for necropsy at the Comparative Pathology Laboratory in the UC Davis School of Veterinary Medicine, Davis, California, USA. Complete sets of tissue samples, including kidneys, spleen, pancreas, heart, lungs, brain, stomach, duodenum, jejunum, cecum, colon, eye, Harderian gland, salivary glands, reproductive tract, skin and subcutis from affected areas were collected and submersion-fixed in 10% neutral-buffered formalin. The tissue samples were routinely processed, paraffin-embedded, sectioned and stained with hematoxylin and eosin. Tissue sections were evaluated by a board-certified veterinary anatomic pathologist.

Contextual Fear Conditioning

Following the last injection, subjects were exposed to a contextual fear conditioning paradigm [4] to assess learning and memory. During fear conditioning, rats learn to associate the context of the chamber and a white noise cue with a foot shock. If they remember the association, rats will exhibit freezing behavior when challenged again with the context or cue. Freezing behavior is defined as a lack of movement other than breathing. Rats were placed in a chamber with a metal floor grid (Med Associates, St. Albans, VT, USA) with house lights on and a 10% acetic acid scent cue. After 2 min, mice were subjected to the conditioned stimulus (CS), an 85 decibel white noise cue coinciding with a 2 sec foot shock (unconditioned stimulus, US) of 0.35 mA. The rat remained in the chamber for 30 sec with no noise or shock following the CS-US pairing. Chambers were cleaned thoroughly with 70% ethanol between subjects. Memory of the context and of the auditory cue was tested 24 h after conditioning. For the context test, rats were placed back in the chamber with the same environmental cues (acetic acid scent cue, lights on and metal grid floor) for 300 sec. For the cue test, the grid floor was covered with paper towels and plexiglass, the house lights turned off and the scent cue changed to lemon. Rats were placed in the silent chamber for 120 sec followed by the white noise cue for 180 sec but without the foot shock and ended with a 60 sec period of silence.
All trials of fear conditioning were video recorded. Freezing was scored by an observer blinded to experimental group.

Statistical Analyses

The severity and incidence of skin abnormalities were analyzed using R 3.1.1 software suite (Vienna, Austria). For the subset of rats (N = 12) in which severity of the abnormality was recorded, the severity was scored from 0 to 3 corresponding to none, mild, moderate, or marked. These rats came from different barrier facilities and were exposed to different bedding conditions but the same reagent-grade oil. For the subset of rats (N = 12) in which only the incidence of abnormalities was recorded, the incidence was recorded as 0 (absent) or 1 (present). These rats were exposed to different vehicle oils and different bedding conditions. The two subsets of rats were analyzed separately.

Because each rat received a score every day for 21 d, the observations were not independent but rather were correlated within rats. To address this correlation, a measure was created for each rat that summarized the score across all days. First, generalized linear models were fitted with severity or incidence as the outcome (identity link for severity; logit link for incidence) and day-specific fixed effects as predictors. Pearson (standardized) residuals were obtained for each animal for each day. These residuals were then averaged across all days in order to obtain an average daily Z-score for each rat. This score summarizes a rat's incidence or severity relative to the average. Positive values indicate a greater incidence or severity of abnormalities and negative values indicate the opposite.

The rat-specific average daily Z-scores were used as outcomes in two-way ANOVA analyses. Two such analyses were conducted, one for each subset of rats, in which the predictor variables were respectively (a) barrier facility of origin and bedding condition, or (b) vehicle oil type and bedding condition. Given the small sample size (12 observations in each ANOVA), the parametric assumptions underlying the p-values were questionable. Therefore permutation testing was used to verify the p-values and thus the inferences from confidence intervals. In the event that permutation testing failed to corroborate the ANOVA p-values, confidence interval estimates were obtained by small-sample bias-corrected bootstrapping (R package "bootstrap" version 2014.4 by Rob Tibshirani). Statistical analysis results are presented as the difference (contrast) between average Z-scores of two different experimental conditions (graphics produced with R package "ggplot2" version 1.0.0 by Hadley Wickham). In the relevant figures, the point estimate of the contrast is plotted as a dot with the 95% confidence interval extending above and below. If the interval crosses zero, there is said to be no significant difference. If the interval is entirely above (below) zero, the difference is significantly greater (smaller) than zero at the 5% level. Any value within the 95% confidence interval is considered to be plausible for the contrast.

Behavioral data were recorded as the percentage of time the animal spent immobile during the context and cue test. The data were analyzed using standard one-way ANOVA methods (GraphPad Prism software Version 5.0).

Results

Grossly evident skin abnormalities ranging from minor skin inflammation, swelling, dermatitis, scabbing, and abrasions were observed along the neck, shoulders and/or ears of male Long-Evans rats injected with food grade peanut oil at 1.0 mg/kg/d s.c. (Fig. 1A-F). Skin abnormalities typically began to manifest during the first week of injections and became progressively more severe with increasing number of injections. Pruritus and self-trauma was evident as injected rats were observed to scratch the subscapular region proximal to the site of injection. Alopecia was also noted and may have been due to local edema that caused hair follicles to be more widely dispersed and thus appear sparser.

Two animals with the most severe skin lesions were submitted to necropsy. Histopathologic evaluation of the skin lesions revealed pyogranulomatous cellulitis characterized by variable numbers of foamy macrophages, neutrophils, lymphocytes and plasma cells surrounding large clear vacuoles (Fig. 2). The intralesional clear vacuoles were interpreted to be the injected peanut oil. Nonspecific changes (acanthosis and compact hyperkeratosis) were observed in the overlying epidermis. Together, the histopathologic changes were consistent with a foreign body reaction to the injected peanut oil and chronic self-trauma. Splenic marginal zone expansion was also noted in both animals and interpreted as a systemic inflammatory response (data not shown). The histology of additional tissues collected at necropsy was unremarkable.

In the initial pilot study in which we noted skin abnormalities in animals injected with peanut oil, two male Sprague Dawley rats obtained from the same barrier facility and housed under the same conditions but injected daily with saline (1 ml/kg/d s.c.) for 8 days (N=2) did not exhibit any notable skin response (data not shown). This suggested that the peanut oil, rather than the injection itself, was triggering the adverse inflammatory response. To test this hypothesis, we compared the incidence of skin abnormalities between male Long-Evans rats injected daily for 21 d with food grade peanut oil versus reagent grade peanut oil versus Neobee M-5, a triglyceride of coconut oil (1.0 ml/kg/d s.c.). Skin abnormalities manifested earlier and with higher incidence on any given injection day in rats injected s.c. with food grade peanut oil

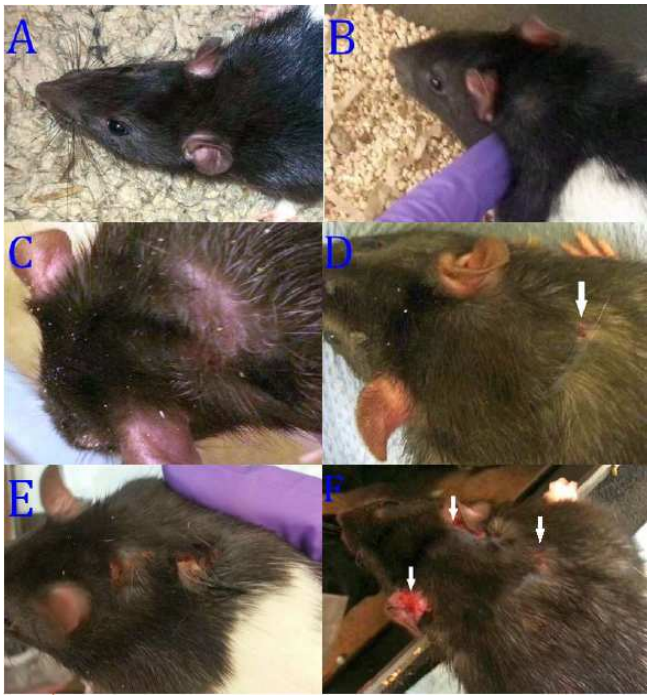

Figure 1: Representative images of skin lesions in male Long-Evans rats injected with peanut oil (1 ml/kg/d, s.c.) for up to 21 d. (A)
Normal animal with no visible skin abnormalities on injection day 21. ***(B)*** *Example of swelling around the injection area on injection day 21.* ***(C)*** *Demonstration of dermatitis on injection day 21.* ***(D)*** *Animal with clinically insignificant abrasion on injection day 13.* ***(E)*** *Illustration of a clinically significant abrasion on the neck/shoulder region on injection day 11.* ***(F)*** *Animal with clinically significant abrasions on the ears and neck on injection day 21.*

relative to rats injected with either reagent grade peanut oil or Neobee M-5 (Fig. 3). In the food grade peanut oil treatment group, skin reactions were noted as early as injection day 4; whereas in rats injected with reagent grade peanut oil, skin reactions were not observed until injection day 8. All animals in the food grade peanut oil treatment group exhibited skin abnormalities during the 21 d injection period whereas only one-third of the rats injected with reagent grade peanut oil developed skin abnormalities during the study. Notably, none of the animals injected with Neobee M-5 exhibited skin abnormalities on the subcutis at any time during the study.

Since skin abnormalities were only observed in the animals injected with peanut oil, we next examined the effect of bedding material by comparing corncob bedding versus paper bedding in rats injected with either food grade or reagent grade peanut oil (1.0 ml/kg/d s.c.) for 21 d. The number and severity of skin abnormalities in rats housed

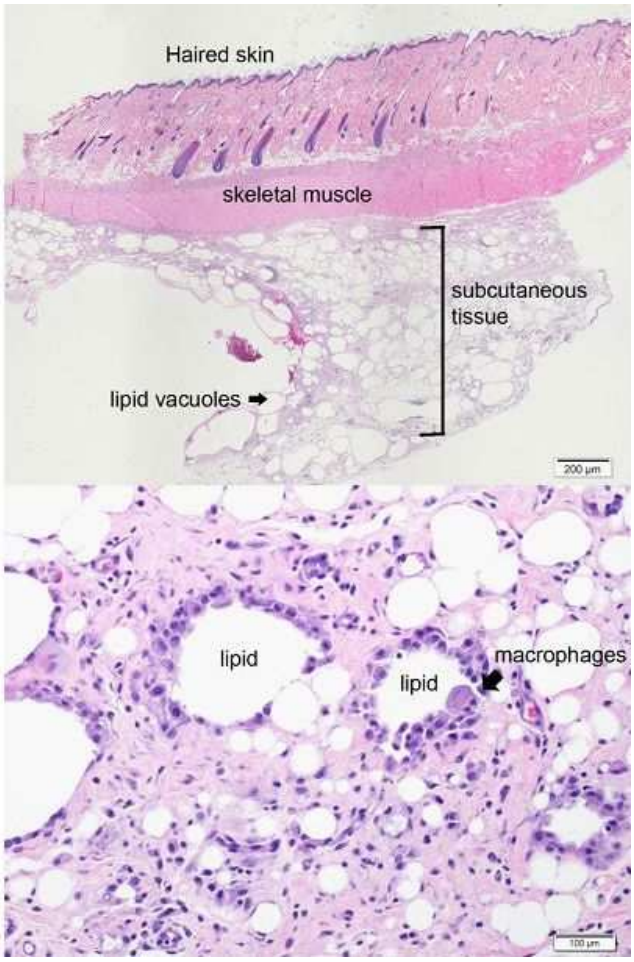

Figure 2. Histological analyses of skin lesions from rats injected with reagent grade peanut oil (1.0 ml/kg/d s.c.) for 21 d. (A)
Hematoxylin and eosin staining of the dorsal haired skin and subcutis. Deep to the dermis and skeletal muscle, the subcutaneous adipose tissue is expanded by large clear lipid vacuoles and pyogranulomatous inflammation, 20X. Bar = 200 µm. ***(B)*** *Higher magnification of the subcutis demonstrating large clear lipid vacuoles surrounded by foamy uninucleate to multinucleated macrophages, 200X. Bar = 100 µm.*

on corncob bedding was significantly increased relative to those housed on paper bedding (Fig. 4). Comparison of the effects of the vehicle type indicated that repeated injections with either food grade or reagent grade peanut oil increased the incidence of skin abnormalities relative to Neobee M-5 (Fig. 4).

To determine the influence of the animal's prior environmental experiences (e.g., potential differences in sensitization) on skin reactions triggered by repeated s.c. injections of peanut oil, we compared the severity of skin abnormalities between rats imported from different barrier facilities that were injected with reagent grade peanut oil

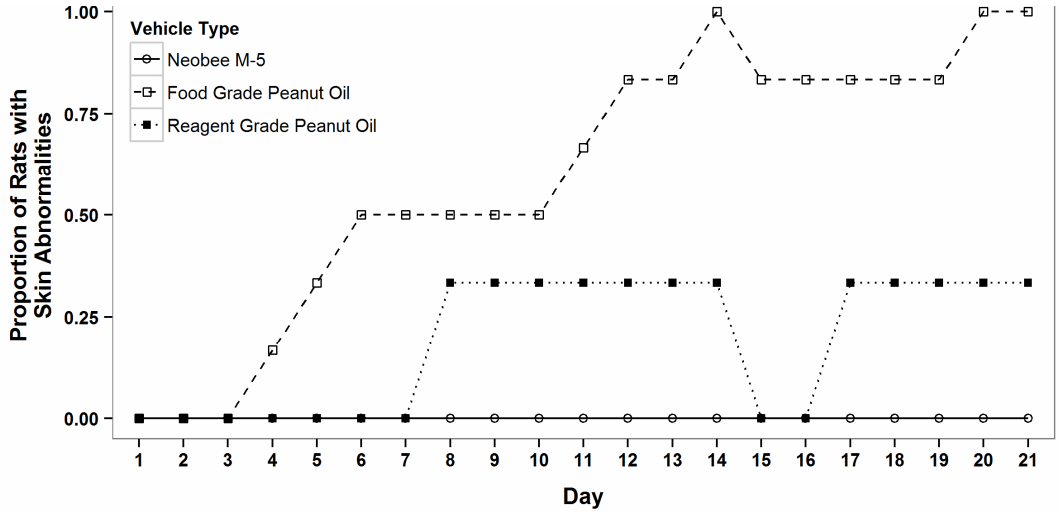

Figure 3. Variable incidence of skin abnormalities depending on the vehicle injected. *Rats were housed on paper bedding and injected daily with food grade peanut oil, reagent grade peanut oil or Neobee M-5 (1.0 ml/kg/d s.c.) for 21 d. The percentage of rats in each treatment group exhibiting skin abnormalities (swelling, irritation and scabbing) was recorded each day. Animals injected with food grade peanut oil (open boxes, N=6) had an earlier onset and greater incidence of skin abnormalities relative to animals injected with either reagent grade peanut oil (closed boxes, N=3) or Neobee M-5 (open circles, N=3).*

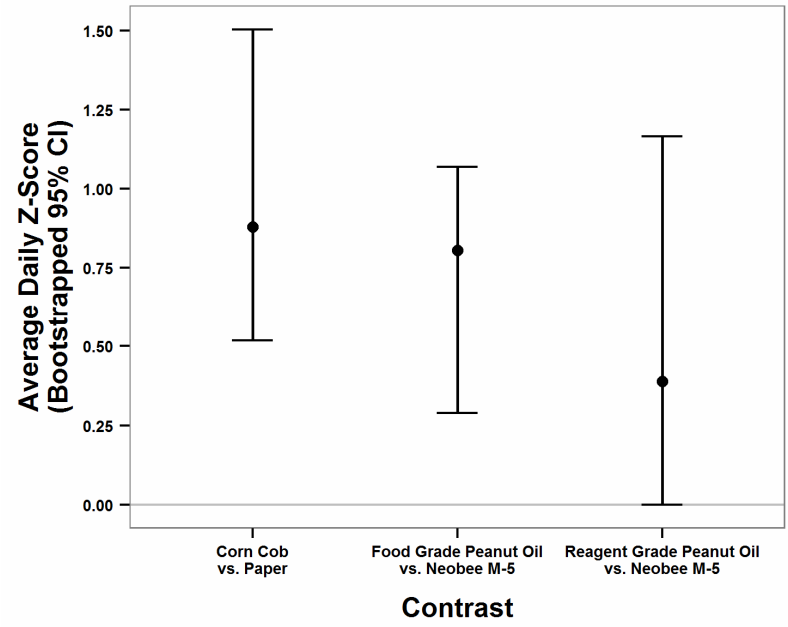

Figure 4. Peanut oil grade and bedding type significantly influence the incidence of skin abnormalities triggered by repeated s.c. injection with peanut oil. *Rats were housed on either corn cob or paper bedding throughout the 21 day testing period and were injected with food grade peanut oil, reagent grade peanut oil, or Neobee M-5 at 1.0 ml/kg/d s.c. The incidence of skin abnormalities was recorded daily. These data were analyzed using a logistic regression model, yielding Pearson residuals that were used to generate a standardized measure of how large or small the observed skin abnormality was relative to the day's average. For each animal, the Pearson residuals were averaged across all days to obtain an average daily Z-score for each rat. A positive Z-score indicates a high propensity to exhibit skin abnormalities whereas a negative Z-score indicates resistance to skin abnormalities relative to the average. Significant differences between treatment groups were identified using 2-way ANOVA (N=3-6 per treatment group) and are presented as a contrast comparing the mean of one factor level to the mean of another. The dot represents the point estimate of the contrast between the means of the two factors; the whiskers represent the bootstrapped 95% confidence interval. Confidence intervals that lie entirely above or below zero indicate that the contrast is significant at the 5% level.*

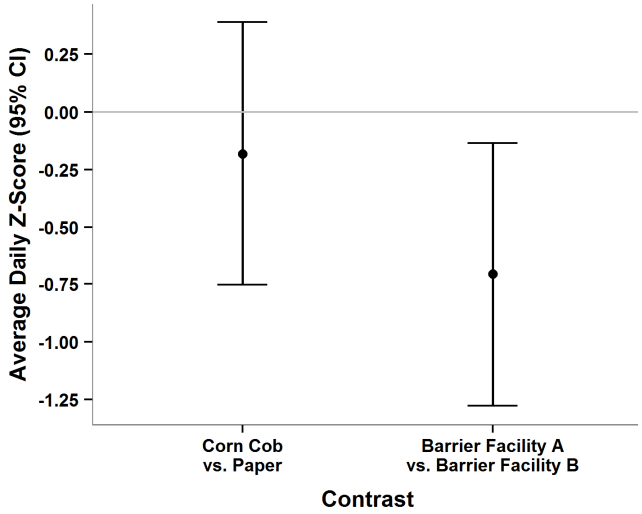

Figure 5: Prior environmental exposures influence the development of skin abnormalities in response to injection of reagent grade peanut oil (1.0 ml/kg/d s.c.) for 21 d. *Rats of the same strain, sex and weight were obtained from 2 different barrier facilities and allowed to acclimate for 7 days prior to beginning injections. The severity of skin abnormalities were scored daily according to the diameter of the lesion with 0 = no abnormality, 1 = mild abnormality (< 3 mm), 2 = moderate abnormality (3-7 mm) and 3 = marked abnormality (≥ 7 mm). These data were analyzed using a standard linear regression model, yielding Pearson residuals that were used to generate a standardized measure of how large or small the observed skin abnormality was relative to the day's average. For each animal, the Pearson residuals were averaged across all days to obtain an average daily Z-score for each rat. A positive Z-score indicates a high propensity to exhibit skin abnormalities whereas a negative Z-score indicates resistance to skin abnormalities relative to the average. Significant differences between treatment groups were identified using 2-way ANOVA (N=6 per treatment group) and are presented as a contrast comparing the mean of one factor level to the mean of another. The dot represents the point estimate of the contrast between the means of the two factors; the whiskers represent the 95% confidence interval. Confidence intervals that lie entirely above or below zero indicate that the contrast is significant at the 5% level.*

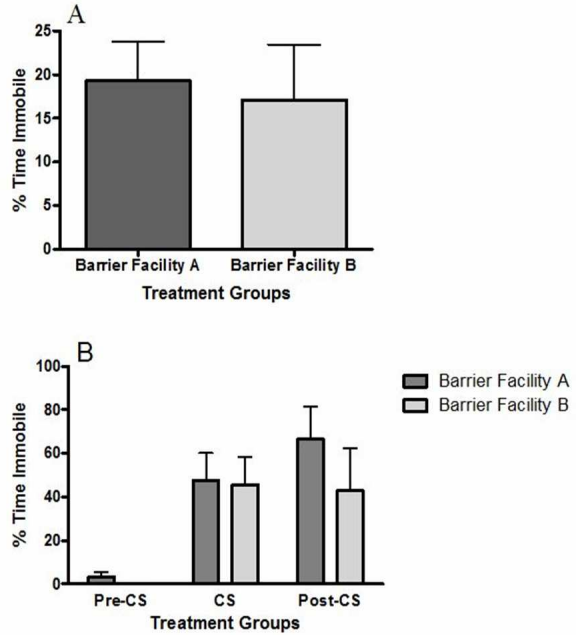

Figure 6: The barrier facility from which rats were obtained did not significantly influence Pavlovian fear conditioning. *Long-Evans rats from two different barrier facilities were injected daily for 21 days with reagent grade peanut oil (1.0 ml/kg/d s.c.). At the end of the injection period, learning and memory were assessed using Pavlovian fear conditioning. There were no statistically significant differences between experimental groups in either the context test* ***(A)*** *or the cue test* ***(B)****. Data presented as the mean ±S.E. (N=6 animals per treatment group). Data were analyzed by one-way ANOVA with significance set at p<0.05.*

Discussion

Collectively, our data indicate that skin abnormalities observed in adult male Long Evans rats receiving repeated s.c. injections are vehicle-dependent. Animals injected with either food grade or reagent grade peanut oil, but not those injected with Neobee M-5 (triglyceride of coconut oil), exhibited adverse skin reactions. The incidence and severity of the inflammatory skin responses elicited by s.c. injections of peanut oil were strongly influenced by the grade of the peanut oil, the bedding material and by the barrier facility from which the animals were imported, with the most significant adverse responses observed in rats injected with food grade peanut oil that were housed on corncob bedding. The most severe skin lesions were characteristic of a foreign body reaction and were associated with a systemic immune response as evidenced by splenic marginal zone expansion. However, this immune response did not alter learning and memory as assessed in a Pavlovian fear conditioning task.

The fact that we did not observe gross skin abnormalities across all treatment groups or amongst all animals injected with reagent grade peanut oil strongly suggests that the skin abnormalities we observed were not caused by repetitive insertion of the needle. Moreover, the histopathology was consistent with a foreign body reaction, which is a chronic state of inflammation that develops and persists as long as the exogenous stimulus resists degradation [5,6]. Foreign body reactions can be triggered by bubbles or adulterants in the vehicle [5]. These seem unlikely causes of the foreign body reactions observed in this study because vehicle solutions were sonicated and confirmed to be clear of bubbles prior to injection, and because food grade peanut oil is tightly regulated by the U.S. Food and Drug Administration and, therefore, unlikely to contain adulterants. Rather, the most likely cause of the foreign body reactions we observed is degradation-resistant lipid deposits in the subcutis. It has previously been reported that peanut oil can persist at the injection site for up to months following a single injection [1], and that peanut oil deposits in the skin can recruit macrophages, lymphocytes, and fibroblasts to the injection site [7]. Our histopathologic analysis of the skin lesions in rats injected with peanut oil were consistent with these previous reports in that they suggested that unabsorbed oil was trapped between the skin and muscle layer and that these oil deposits were surrounded by inflammatory cells. Thus, our findings extend previous reports demonstrating that injection of exogenous lipids can generate foreign body reactions, resulting in chronic inflammation [8].

Peanut oil has also been reported to be a skin irritant in rodent species [1,7]. For example, 3 dermal applications of technical grade peanut oil across 72 hours was noted to cause skin irritation in male Wistar rats [1]. Peanut oil has also been demonstrated to increase the skin fold thickness of rats up to 25% by 4 hours after a single s.c. injection, and to cause a fivefold increase in inflammation compared to saline [1]. Interestingly, differences in the supplier reportedly alters the degree of irritation observed following peanut oil administration [9]. Thus, peanut oil likely also was the cause of the earlier skin abnormalities we observed, such as redness and swelling, and the different suppliers likely explains the differences in incidence and severity noted in animals injected with food grade versus reagent grade peanut oil.

Vegetable oils other than peanut oil, including sesame and olive oils, have also been reported to cause foreign body reactions [10,11], and s.c. injection of olive oil was associated with both subcutaneous and intraperitoneal lipogranulomas in Sprague-Dawley rats [12]. An interesting finding of our studies was that the incidence and severity of skin abnormalities varied significantly depending on the vehicle that was injected. The reason(s) for the differing skin responses to different oils are not known but likely reflect differences in the fatty acid composition of the oils and/or the presence of antioxidants, both of which determine the irritant and inflammatory properties of oils [13-15] as well as their resistance to degradation [16]. For example, the *in vivo* degradation of lipid fatty acids depends in large part on their carbon chain length [17]. Long-chain fatty acids degrade at a slower rate than short-chain fatty acids [17], and the ratio of long-chain to short-chain fatty acids varies significantly between peanut oils derived from different peanut crops, and between oils derived from peanuts *versus* other botanical species [18-20]. An increase in unsaturated bonds increases susceptibility of a fatty acid to oxidation, which is associated with free radical formation, lipid peroxidation, and chronic inflammation [21]. Peanut oil has a significantly higher proportion of unsaturated fatty acids than many other vegetative oils [20,22], and as a result, the fatty acids in peanut oil oxidize significantly faster than those in corn, salmon or rice bran oil as determined using the Rancimat method [23]. Interestingly, relative to Planters® peanut oil in which >90% of the fatty acids by weight have unsaturated bonds, <10% of the fatty acids in coconut oil have unsaturated bonds [20,22]. This difference likely contributes to our observation that in contrast to either food grade or reagent grade peanut oil, Neobee M-5 did not cause skin abnormalities in animals receiving repeated s.c. injections.

The different skin responses triggered by repeated s.c. injection of food grade versus reagent grade peanut oil likely reflects differences in production and/or storage of the oils. Exposure to heat and light promotes oxidation of fatty acids, and there is experimental evidence that heating peanut oil alters how it interacts with biological systems. For example, daily oral administration for 6 d of 15% (by weight) peanut oil that had been heated in the

laboratory prior to administration caused dermatitis in male weanling rats while littermates administered the same amount of fresh unheated peanut oil exhibited no adverse skin effects [24]. The oxidation of fatty acids is also influenced by the presence of antioxidants. Tocopherols are natural antioxidants endogenous to 100% peanut oil, but the amount of endogenous antioxidant capacity that is lost during processing can vary from 19.8% to 51.2% [25]. While exogenous tocopherols are often added to inhibit autoxidation and thereby prolong shelf-life of peanut oil [25,26], neither the food grade nor the reagent grade peanut oil we used in our studies contained exogenous antioxidants. Nonetheless, since levels of endogenous antioxidants are influenced by the botanical taxon, age, and harvest season of the parent plant as well as the manufacturing, handling and storage practices, differences in the fatty acid and antioxidant composition can differ significantly between different lots of peanut oil [18,27]. This is the likely explanation of the differential skin responses we observed in animals injected s.c. with food grade versus reagent grade peanut oil.

While the vehicle was the predominant factor in determining skin responses to s.c. injections, these responses were significantly influenced by environmental conditions, including the bedding on which animals were housed during the testing period. Although different types of bedding are known to alter liver enzymes [28] as well as intestinal and mucosal immune responses and stress responses [29-31] in rodents, little has been reported regarding the effect of bedding on skin responses to repeated s.c. injections of oil. Corncob bedding is thought to be one of the least toxic choices for nesting [28]; however, corncob bedding has recently been associated with altered endocrine function, which is known to influence host immunology [30-32]. This is consistent with our finding that housing on corncob bedding exacerbated the skin abnormalities in animals with repeated s.c. injections of food grade peanut oil.

Another environmental factor we found that influenced the severity of skin lesions in rats injected with peanut oil was the barrier facility from which the animals were imported. Long-Evans rats are an outbred strain that were originally crossed nearly a century ago. Although it is possible the different responses observed in rats from different barrier facilities reflect genetic drift over many generations, the more likely explanation of the barrier effect is differences in the rearing environment. This is supported by a study in which male C57BL/6 mice from 5 different vendors, but not their first generation offspring, were found to exhibit significantly different airway hyperresponsiveness [33]. The influence of the rearing environment on sensitivity to environmental immune triggers likely arises from differences in husbandry practices, such as bedding material. For example, corncob bedding had a strong influence on stress and anxiety measures in adulthood Long-Evans rats when introduced during their early development, but not when introduced during adulthood, suggesting that bedding materials modulate developmental programming [29].

Behavioral tests that measure learning and memory in rodents are highly sensitive to environmental influences. Differences in stress factors, housing conditions and testing location are examples of environmental factors that are known to impact animal performance in learning and memory tasks. Inflammatory cytokines have been reported to impair several behavioral tests, including contextual fear conditioning [3]. Interleukin-6, a cytokine secreted by macrophages, is a critical mediator of the foreign body reaction [6,34] and has been found to alter learning and memory consolidation [35]. Such observations raise concerns that skin abnormalities triggered by repeated s.c. injections might influence learning and memory behavior in rats. However, performance in a Pavlovian fear conditioning task was not significantly different between rats imported from different barrier facilities who exhibited significant differences in the severity of skin lesions triggered by repeated s.c. injections of peanut oil. This observation suggests that the systemic peripheral immune response triggered by these skin abnormalities did not produce levels of inflammatory mediators in the brain that interfered with neurobehavioral function at least in this particular task of learning and memory.

In conclusion, our findings indicate that vehicle, bedding and the rearing environment significantly influence the incidence and severity of skin abnormalities following repeated s.c. injections, but that these adverse skin responses do not significantly influence performance in at least one test of learning and memory. Nonetheless, it is recommended that Neobee M-5 be used as the vehicle of choice for repeated s.c. injections in studies of CNS function.

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