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Dr. John L. Butenhoff
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Re: *ToxSci* 01-002

Dear Dr. Beierschmitt:

Thank you for submitting your manuscript "Investigation of the No Observable Effect Level for Perfluorooctanesulfonic Acid Potassium Salt in Cynomolgus Monkeys after Twenty-Six Weeks of Oral Dosing and One Year of Recovery" for publication in *Toxicological Sciences*. Your manuscript has been reviewed by two members of our editorial board and myself. Unfortunately your manuscript has been **not recommended for publication.** The major concern is that there is too little new information to justify publication. I hope this will not prevent you from submitting manuscripts to *Toxicological Sciences* in the future.

Sincerely,

Michael L. Cunningham, Ph.D., D.A.B.T.
Associate Editor

cc: Editorial Office

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Manuscript Number: 01-002
Rev 1

Comments to the Author: Recently, 3M announced that it would cease manufacturing most products derived from or containing PFOS due to concerns about its toxicity, biopersistence, and widespread exposure to human populations. PFOS is found in the serum of workers and the general public, as well as in wildlife. While PFOS produces a range of toxicological effects at relatively low doses, few reports have been published in peer reviewed journals. Publication of any well-performed study on PFOS would significantly increase the available database for what is potentially a significant public health issue.

However, I have a number of concerns about the manuscript submitted by Seacat et al. (Tox Sci 01-002). My concerns include the design and conduct of the study, the manner in which the results are presented, and the interpretation of the results. These concerns need to be addressed before the manuscript is considered for publication.

The Introduction fails to mention that "chemistries" related to PFOS are being withdrawn from commerce. The Introduction should also include a more complete description of the health effects of PFOS, including studies with lithium or other salts of PFOS. The purity of the test compound (86.9%) is low and there were significant errors in the dose levels (+/- 25 to 35 percent). While I realize that all the details of a subchronic study cannot be included in a journal article, I cannot recall ever seeing the phrase "data not shown" so many times in one paper, and there are numerous instances where it was omitted. In some cases, major conclusions of the study, such as the reversibility of effects and the lack of peroxisome proliferation, are presumably supported by data that are not included in the paper. To the extent that space allows, additional details should be included, such as photomicrographs of the livers of recovery phase animals, HDL and hormone levels in the recovery phase animals, and the results of the palmitoyl CoA oxidase assay.

The authors also need to explain more fully why effects in the low and mid dose groups were not considered significant. Cholesterol and hormone levels were affected in a dose-dependent manner in both sexes. Statistically significant effects were seen at the mid dose and, in some cases, at the low dose. These are clearly dose-related and, in my view, the low dose should be considered the NOAEL. Finally, in describing the adverse effects of PFOS as reversible, the authors seem to have forgotten that two high-dose animals died on test.

Although there are concerns about the study itself as well as the manuscript, a revised and expanded paper would provide useful toxicological data on a ubiquitous environmental contaminant. Therefore, I recommend that the manuscript by Seacat et al. (Tox Sci 01-002) be published with major revisions. Specific comments are as follows.

Abstract:

Page 2, line 6. There are no data to support the conclusion that there was no peroxisome proliferation.

Page 2, lines 6 to 7. I disagree with the statement that "No toxicologically significant effects were observed in the low- and mid-dose groups." Cholesterol and hormone levels were affected in a dose-dependent manner. Statistically significant effects were seen at the mid dose and, in some cases, at the low dose levels.

Page 2, lines 10 to 12. "Other effects of questionable toxicological significance..." This statement should be amended to say that some of these effects were observed at the mid and low doses. As explained above, I think that these effects should be regarded as toxicologically significant. Furthermore, to say that these are of QUESTIONABLE toxicological significance is inconsistent with the statement on lines 6 to 7 that NO toxicologically significant effects were observed at the low and mid dose levels.

Page 2, lines 12 to 13. The statement that reduced cholesterol is the most SENSITIVE effect is not supported by the data. Other effects occur at the mid and low dose. This statement is inconsistent with the statement on p. 26 (under Conclusions) that reduced cholesterol is the EARLIEST effect.

Include the doses along with the serum levels.

Page 2, last sentence. "The adverse effects of PFOS ... and were reversible." Mortality is not reversible. Rephrase the sentence as follows: Histopathological effects and changes in clinical chemistry values (data not shown [why not include the data]) were reversible in surviving animals.

Introduction:

Page 4, first paragraph. Include a statement that the manufacturer is voluntarily stopping production of PFOS due to concerns about biopersistence and widespread exposure to humans. The opening paragraph should include what these chemicals are used for. It is also noteworthy that the U.S. EPA proposed regulating new uses of PFOS and related chemicals (Federal Register, 65: 62319-62333).

Page 4, first paragraph, last sentence. While environmental transport and routes of exposure are not well understood, the ability of PFOS to bioaccumulate suggests food as a possible source of environmental exposure. According to the U.S. EPA exposure from water is also possible (ibid.). PFOS is also used to manufacture a variety of household products (water and stain repellants) and food packaging, which are other potential sources of exposure to the general public.

Page 4, second paragraph, lines 6 to 9. What was the range of PFOS serum levels in workers?

Page 4, Introduction, pages 4 to 6. The Introduction should not be limited to studies with the potassium salt of PFOS. It should include studies with lithium or any other salts, such as: S.M Henwood et al. (1994) Developmental toxicity with lithium perfluorooctanesulfonate in rabbits. Teratology, 49: 398 and S.M Henwood et al. (1994) Developmental toxicity with lithium perfluorooctanesulfonate in rats. Teratology,49: 398.

Page 5, middle paragraph. What are the lethal and no observed effect levels in rats? What was the duration of the rat study?

Page 5, last paragraph, first 2 lines. There are no rat data in the previous paragraph to support this statement.

Page 6, last paragraph. What other tests were done to monitor the employees' health? Were any adverse effects observed at higher serum levels, even if they are not statistically valid?

Materials and Methods:

Page 7, line 2. The purity (86.9 %) is rather low. What are the major impurities? Are the same impurities likely to be present in occupational or environmental sources of PFOS? Are they likely to contribute to the toxic effects? If they are partially fluorinated analogues, are they more toxic than PFOS?

Page 7, last sentence, continuing on page 8. Was the water tested for PFOS?

Page 8, first complete paragraph. Why were there fewer animals at the low dose?

Page 10, last paragraph, under Necropsy. There were only 4 animals at the low dose. Sacrificing 4 leaves none for recovery.

Page 11, line 4. For consistency, insert "(males)" after epididymis.

Page 11, lines 6 to 13. Apparently livers were also examined by light microscopy (see page 18 and Figures 3A and 3B), but liver was omitted from the list of organ sites examined. How many liver sections per animal were viewed by light microscopy? By EM? Did they include sections from each lobe?

Page 12, Palmitoyl CoA oxidase determinations. Peroxisome proliferation may vary among different liver lobes in rodents. Why was only one liver lobe tested? Why didn't the authors assay peroxisome number and size, as well?

Page 12, Immunohistochemistry for Cell Proliferation. Is it Pathology AssociatED International or Pathology AssociatES International?

Page 12, Regulatory Compliance. Are there any other regulatory guidelines, such as OECD guidelines, that are applicable to this study?

Results:

Page 14, first paragraph. Were there other cases of respiratory infection during the test?

Page 14, second paragraph. What about organ to body weight ratios?

Page 14, last paragraph. "... qualitatively lowered food consumption." Why was food consumption not measured? How much weight did they lose? What were the body weights of the animals that died?

Pages 14-20, under Results. What were the results of the palmitoyl CoA oxidase assay? I could not find them.

Page 15, Analysis of PFOS levels in the Dose, Serum, and Liver." Is 0.03 mg/kg the actual dose or the target dose? Please clarify. Is this due to an error in

compounding the test material? If yes, say so. How frequently were the dose mixtures prepared during the study? Are the errors standard deviations? Do they reflect analytical error, weekly variations in the dose, differences in body weight?

This paragraph states that "the mean daily intake of PFOS was calculated on a mole percent basis as 80% of the nominal KPFOS dose for each dose group." I'm not sure what this means. By my math, potassium is about 7% of the mass of KPFOS, which means that 93% of the dose is PFOS anion. The compound is 86.9% pure. Do you mean 0.869x0.93 = 80.6? If the impurities are related compounds which contribute to the toxicity of PFOS, then why make this adjustment?

Was PFOS measured in the feed and water? Is food the likely source of background levels in the test animals? The authors should explain why PFOS was present in control animals.

Is PFOS found at any other organ sites? Has anyone looked?

Page 15, Hematology. Define "N-SEG" and include it in the Methods section. Can the hematology data be presented in tables? At least list ALL statistically significant changes in the text. Explain why the hematology changes are not considered adverse or biologically significant.

Page 16, first full paragraph. Why was HDL not tested prior to dosing?

Page 16, last paragraph. "Significant changes in clinical chemistry were seen only in the high dose group..." Do you mean biologically significant? Toxicologically? Clinically? Please qualify the word significant, since there were statistically significant effects at the mid- and low- doses.

This paragraph does not mention the statistically significant decrease in cholesterol on days 62 and 182 and HDL on days 153 and 182 (consecutive tests) in the low dose males (Table 2A). Include this in the results and explain why it is, or is not, biologically significant.

The first sentence of this paragraph lists the "most notable" effects. Were there any other statistically significant effects?

Page 17, last sentence of paragraph continued from page 16. "...HDL was significantly lower in the 0.75 mg/kg-d dose group following 153 and 182 days of

dosing." HDL was also lower at 0.03 mg/kg-d in males and 0.15 mg/kg-d in females on the same days.

Page 17, first full paragraph. "No notable changes in enzymes used to assess liver function..." Were there any statistically significant changes? Liver is a target organ. These data should be presented in a table with statistical analyses, or at least insert the phrase "(data not shown)" at the end of the sentence.

Page 17, middle. "Urinalysis was unremarkable (data not shown)." Were there any statistically significant changes?"

Page 17, under Hormone Analysis. "Estradiol levels in the 0.75 mg/kg-d males were ... signifcantly lowered following 182 days of dosing." In Table 3, none of the estradiol values has an asterisk indicating statistical significance.

Pages 17-18. (Last sentence on 17, which continues on 18). "The T3 values in the mid dose groups were not significantly different than their pre-dose values and were not considered adverse effects..." Include the pre-dose values or insert the phrase "(data not shown)". In any case, there clearly are dose-dependent, statistically significant changes in hormone levels in both sexes at 26 weeks. These changes are clearly treatment-related and, in my opinion, should be regarded as adverse effects.

Page 18, under Replicative DNA Synthesis. "PFOS had no effect on..." Do you mean no statistically significant effect?

Page 18, under Histopathology. What about the other tissues examined, especially the thyroid. If there were no treatment-related effects, then say so.

Page 18, last sentence. The term "no OBSERVED effect level" is preferred. Before concluding that the NOEL/NOAEL for hepatocellular vacuolation is 0.15 mg/kg-d, say what you observed at this dose.

Page 19, first paragraph. What was the half-life at low and high dose? What were the standard deviations? The text expresses time as weeks after the cessation of dosing, but Figure 6 expresses time as weeks after the start of dosing. This is confusing. The text should include the liver PFOS levels at week 27 (1 week post-dose) as well as week 56 (29 weeks post-dose). The PFOS levels in the females apparently do not drop during the first 29 weeks post-dose. Consider put the liver data in a table instead of a figure; it would be easier to read.

Discussion:

Page 20, first sentence under Discussion. Do you have any information on what the "environmental sources" might be? Is PFOS found in food? Water? Are food packaging or household products significant sources of exposure? Maybe you don't know exactly how PFOS is transported through the environment, but it comes from products made by 3M.

Page 20, first paragraph under Discussion. Throughout the manuscript, doses are variously expressed as daily dose (mg/kg-d), cumulative dose (mg/kg), or serum level (ppm). This tends to be confusing. Most authors would use the daily dose, although, due to its biopersistence, cumulative dose is arguably more appropriate for PFOS. For consistency, it may be best to routinely give the daily dose, with the cumulative dose in parentheses where appropriate. It would also be helpful to have the cumulative doses in parentheses following the corresponding serum PFOS concentrations.

Page 20, first paragraph under Discussion, lines 7-9. "Weight loss occurred ... when serum levels..." But we don't have the data on weight loss over time.

Same paragraph, line7. Following the sentence, "Also found, but of less obvious clinical significance...and decreased estradiol in males only" insert the following:

"Some small but statistically significant changes in cholesterol, HDL, T3, and/or TSH" were observed at the mid dose in females and in the low or mid dose in males, but these were not considered adverse effects." [Unless you agree that some of these are adverse effects.]

Page 20, first paragraph under Discussion, lines 9-12. "The initial lowering of cholesterol..." This sentence compares cholesterol levels on day 62 with serum levels on day 56. This is confusing. Why were they tested on different days? Can you estimate the serum level on day 62 from the graph?

Page 20, first paragraph under Discussion, lines 12-13. "Thus, lowered cholesterol appars to be a more sensitive indicator ..." How do you mean? Is the percentage change in cholesterol greater than the percentage change in body weight. What are the respective percent changes? Or do you mean that the cholesterol changed earlier in time? We don't have the appropriate body weight or hormone level data for comparison? The data presented do not support this conclusion.

Page 21, lines 4 to 6. "Deaths occurred at cumulative doses that ..." Did deaths occur in the 100 mg/kg-d and 300 mg/kg-d? They should have occurred after one or two doses.

Page 21, lines 4 to 13. Deaths were observed at a daily dose as low as 0.75 mg/kg (cumulative dose ~100 mg/kg), whereas the NOAEL in this study was 0.15 mg/kg-d (cumulative dose 13.5 mg/kg). Thus, there is a small range--5-fold based on daily dose and 10-fold based on cumulative dose--between the NOAEL and lowest lethal dose. This seems noteworthy to me; it should be included in the Discussion.

Page 22, line 3. "It is interesting to note that HDL levels in female cynomolgus monkeys IS under the influence of estrogen..." Change "is" to "are".

Page 22, lines 3-7. Were any histopathological effects seen in the ovaries of PFOS-exposed females in this or any other study?

Page 22, lines 7 to 9. Delete the sentence "Given these factors, the significant reductions..." It adds nothing to the discussion.

Page 22, second paragraph. The authors fail to mention the statistically significant decreases in cholesterol (on days 62 and 182) and HDL (days 153 and 182) in low dose males (Table 2A).

In the same paragraph, last sentence. "No biological significance was given to ... due to factors mentioned in the previous paragraph." What factors? Because they are within the reference range?

Page 24, first complete paragraph, lines 12-13. "T4 reference values for rhesus monkeys..." The units for T4 in the text (ug/mL) should match the units in Table 3 (ng/dL).

Page 25, first complete paragraph. "Although not considered of clinical significance..." If it's not considered clinically significant, use this space to include another table. This is complete conjecture, anyway.

Page 25, last paragraph. "Liver, pancreas, and testis ..." Where are the data?

Page 26, first complete paragraph. Include the results of the palmitoyl CoA oxidase measurements. Was there any evidence of peroxisome proliferation?

In the same paragraph, "PFOS caused effects on body weight, lever weight, and cholesterol at RELATIVELY low cumulative doses." I would characterize these as EXCEPTIONALLY low doses.

Conclusions:

Page 26, last paragraph, lines 1-2. The conclusion that reduced cholesterol is the earliest effect is not supported by the data, because time-dependent data are not provided for the other endpoints. It is also inconsistent with the Abstract (see above).

Page 26, last paragraph, lines 2-4. What doses lead to a serum level of 100 ppm?

Page 26, last paragraph, lines 4 to 5. "The results of the primate study indicate a NOAEL of 0.15 mg/kg-d." I conclude that the changes are cholesterol, HDL, and hormone levels are 0.15 mg/kg-d are compound related and may be considered adverse effects (see above). I consider 0.03 +/-0.11 for 90 days to be a more appropriate NOAEL.

Whichever NOAEL is used, the significant errors in measuring the doses should be included.

Furthermore, the Discussion suggests that cumulative dose correlates more closely with the adverse effects of PFOS than the daily dose. Would the authors predict that adverse effects would be observed at 0.15 mg/kg-d day if the study extended beyond 26 weeks? With any toxicant, it is possible that the NOAEL in a chronic study would be lower than a study of shorter duration. However, in the case of PFOS, this is almost certain. Therefore, it would be misleading to describe 0.15 mg/kg-d as a NOAEL without qualification. The authors should include the cumulative dose and state that the adverse effects are likely to be dependent on the cumulative dose or, alternatively, qualify the NOAEL by stating that it applies to exposure durations up to 90 days.

Page 26, last paragraph, lines 5-7. I agree that the deaths were probably compound related. Why were they dismissed in the Abstract?

Page 27, lines 4 to 6. "With the exclusion of hepatocellular peroxisome proliferation observed in rodents..." The results of the peroxisome proliferation studies were not discussed.

Page 27, lines 6-7. Explain why the thyroid hormone effects were not considered clinically significant.

Page 27, last paragraph. Insert the following phrase at the beginning of the first sentence: Although two of 6 males at the high dose died or were found moribund, ...the recovery group animals ...

References:

Page 33, Kennedy et al. The correct abstract # is 1828.

Tables:

Table 1. In footnote #1, why adjust the cumulative dose, but not the daily dose?

The mean serum PFOS and mean liver PFOS are many times greater than the control values. Shouldn't they have an asterisk if they are (statistically) significantly greater than the control values?

Does footnote #3 (it's hard to read my copy) apply to the mean liver PFOS value in the high dose males?

Table 2A, cholesterol levels in males. Footnote b states that day 91 is the earliest significant difference from pre-dose cholesterol values, which occurs in the high dose in males. However, the cholesterol levels in low dose males were significantly elevated on day 62.

Table 2B, cholesterol levels in high dose females. Footnote b states that day 62 is the earliest significant difference from pre-dose cholesterol values, which occurs in the high dose in females. Should this value (127 +/- 18.9) also have an asterisk or asterisks to indicate statistical significance?

Figures:

Figures 1 and 5. Figure 5 is essentially a continuation of Figure 1. Figure 1 gives average PFOS levels, while Figure 5 gives data for individual animals. Thus, it appears that PFOS is higher at 0 days post-treatment than it was on the last day of treatment. For consistency, the data in both figures should be presented in the same way.

The controls had detectable serum PFOS. Did it remain constant throughout the test or did it vary?

Figure 2. Do these data include recovery group animals? The figure legend should indicate the range of the duration of treatment. What happened to the Controls?

Manuscript Number: 01-002
REV 2

Comments to the Author: This manuscript describes investigation of biochemical and histological effects of perfluoroctanesulfonic acid (PFOS) in cynomolgus monkeys. The authors report that adverse effects were observed only at high levels of exposure, and were reversible.
In general, this study seems to be well designed. The only concern in this regards is the timing at which the authors looked at liver mitotic indices. Some similar compounds have been shown to cause a burst of cell proliferation shortly after the initiation of treatment, and return to normal
levels at later time points. The authors might have missed such a phenomenon in this study
because of their late timepoint. A more significant weakness however is the inference that PFOS does not appear to be harmful at levels encountered occupationally. This conclusion can't be supported by the findings of the study.
1. PFOS is poorly eliminated from the body and hence a career-long exposure may indeed result in significant serum/tissue concentrations. Exposure to PFOS for 26 weeks, regardless of dose, does not come close to simulating occupational exposure even at much lower levels, but for a much longer time.
2. Although medical surveillance of occupationally-exposed 3M employees showed serum
PFOS concentrations of less than 6 ppm, with no detectable clinical changes, the authors report
that a "few employees had serum PFOS levels >= 6 ppm", but gave no information on data gathered from these employees, and is referenced in the manuscript.
3. One of the two high-dose male monkeys died as a result of "an acute flare-up of recurring
pulmonary infection". It's therefore of great importance to examine the effect of this chemical
on the immune system. Workers who are immunologically challenged may be at a greater
risk than others. Some of the results presented elude to an immune effect, but without a
discussion on their relevance.
4. The authors state that "lowered cholesterol appears to be a more sensitive indicator of PFOS
than weight loss", yet they go on to contradict this assertion in the very next sentence by
stating that in the recovery phase "Serum PFOS and cholesterol lowering did not

directly correlate with each other". In an occupational exposure situation, how would one know if a worker is being exposed, or is recovering to make a valid conclusion?
5. On two occasions (the second paragraph of page 24 and the second paragraph of page 25) the authors refer to changes in thyroid hormones as being "not of clinical significance". The reason for this conclusion is not clear, especially in light of the fact that they state that observed
effects "resembled some aspects of non-thyroidal (sick euthyroid) syndrome, of which lowered T3 values are the most common abnormality".

The conclusions drawn from this study need to be modified such that they do not give even an
impression that PFOS is decided

Manuscript Number: 01-002
REV 3

Comments to the Author: In this study Seacat et al. have examined the toxicity of PFOS in a dose-response study, and the recovery from PFOS toxicity. The study appears to have been carefully carried out. It is not clear from the paper what this study found that an earlier study from the 1970's had not already shown. The authors need to more clearly emphasize the novel findings from this paper (if they exist). Other changes are suggested below:

Introduction

What is the commercial use of PFOS?

From the intro, it is not clear what further information this study will add that the 1978 primate studies have not already shown.

The authors do not seem to have a hypothesis for their study. Their hypothesis should be clearly stated in the Introduction.

Methods

Animals seem to have been exposed to a wide range of temperatures: 18-29°. Was there some reason for this? Was there a target temperature that animals were supposed to be exposed to?

No references are listed for any of the methods performed on serum or urine. These need to be added; otherwise, it would be impossible for readers to interpret the paper or to repeat the analyses.

Results

Many of the values in the tables have been replaced by NA: not applicable. It is not clear what this means. If the analyses simply were not done, this should be stated (e.g. ND).

Figure 5: What was the apparent half-life in the high dose group?

The use of very short paragraphs should be avoided (e.g. "Urinalysis was

unremarkable"). Also, the frequently-used construction ". . . ; whereas, . . ." is grammatically incorrect.

Discussion

Is it possible that altered excretion of PFOS could contribute to the plateauing of serum values? Has this been studied?
