From the Editor’s Desk

One of the admirable aspects of science is that its practitioners accept that advancing the field is an ongoing process. There is always more to be discovered. I view scientific publishing in a similar light. Last month, we instituted several changes to the journal, and I look forward to receiving your feedback. I anticipate some of the changes will be welcomed while others will not. ToxSci relies on the feedback from our readers, authors, and reviewers to assure that we are serving the research community to the highest degree. The editorial staff is dedicated to producing a high-quality journal that features outstanding science like that seen in this issue. In their Forum article, Wu and coworkers describe how increasing dietary diversity can reduce exposure to foodborne toxins. This is an important public health observation that can help mitigate many of the toxic effects described in the pages of the ToxSci. You will also note that we are using a new format for our Letters to the Editor. Rather than appearing at the end of the journal they immediately follow the highlights below. In this issue, the formulation of carboxyfullerenes is the topic of discussion. I invite you to Look Inside ToxSci for the best original research in the field of toxicology. —Gary W. Miller

Editor's Highlights

Avoiding porphyria in antiepileptic use: Many antiepileptics have serious side effects making the development of new compounds critical in the treatment of epilepsy. Drugs that interact with the synaptic vesicle glycoprotein 2A (SV2A) have proven to be excellent compounds with reduced toxicity compared to older medications. Porphyria occurs when enzymes in the heme biosynthetic pathway are impaired. Toxicity studies revealed that a new SV2A drug candidate induced the rather rare side effect of porphyria in dogs, including distinctive Maltese cross configurations. Here, Nicolas and colleagues demonstrate that the observed toxicity was due to a rare metabolite that is formed in dogs, but not in rats or humans. This suggests that the observed canine toxicity may not preclude further testing in humans. (p. 354)

Paraquat and multidrug resistant protein in the kidney: Paraquat is a widely used herbicide. The primary target organs of paraquat toxicity are the lung and kidney. One of the key determinants of toxicity is the transport of paraquat to and from its site of action. While OCT2 and other transporters have been demonstrated to transport paraquat from the kidneys, there are many other transporters in the kidney that could play a role. Wen et al. demonstrate that the multidrug resistant protein (MDR1) also is involved in the removal of paraquat from the kidney and the protection of proximal tubule cells from injury. Using a variety of molecular tools, from siRNA to mice with a genetic deletion of MDR1, the group showed that MDR1 plays a crucial role in preventing paraquat-induced toxicity to renal cells, primarily by enhancing its elimination from the organ. (p. 476)

Cadmium persistence in the kidney: The heavy metal cadmium has been estimated to have a biological half-life in the kidneys of over 10 years. Since the kidney is the primary compartment for cadmium storage, it is essential to understand the kinetics in this key excretory organ. Fransson and colleagues performed Markov-chain Monte Carlo analysis to develop a toxicokinetic model of cadmium. In order to examine the various parameters in humans, they obtained samples from living kidney donors. This allowed the authors to measure cadmium levels, not only in the bloodstream and urine, but in the kidney itself. By collecting these data in humans, their toxicokinetic model provides a more accurate estimation of cadmium kinetics in humans. Their data suggest that the half-life of cadmium in the kidney is closer to 45 years. (p. 366)

Self-regulating actions of bile: Bile acids act as biological detergents aiding the solubilization of lipids and various nutrients. Various enzymes influence the synthesis of the bile acids in our bodies and transporters play a critical role in their disposition in the liver and gut. Bile acids have been observed to participate in their own homeostasis by feeding back through the farnesoid X receptor. Unfortunately, there are notable differences in how bile acids are handled among different species, such that models based on non-human species may fail to capture key components of the regulatory pathway. This underscores the need for a human-based in vitro model to study this important process. In this issue, Liu and coworkers utilized primary human hepatocyte cultures to study the differential regulatory effects of the primary bile acids in humans. The authors were able to identify the comparative self-regulatory actions of the key human bile acids in this model that faithfully recapitulated the processes that occur in humans. (p. 539)

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Letters to the Editor

Study Examining Fullerene Toxicity Raises Questions as to the Purity of the Nanomaterials and Erroneous Experimental Conclusions

To the Editor,

We are compelled to comment on a recent article in your journal “C₆₀ Exposure Augments Cardiac Ischemia/Reperfusion Injury and Coronary Artery Contraction in Sprague Dawley Rats; 138(2), 365–378” by Thompson¹ in which various conclusions were made concerning the toxicity of C₆₀. In this study, insoluble C₆₀ was mixed with polyvinylpyrrolidone (PVP) and used to challenge rats which were then surgically induced to examine various cardiovascular toxicity parameters. Both intravenously (IV) and intratracheally (IT) exposure to the C₆₀/PVP mixture resulted in expansion of myocardial infarction in male and female rats following I/R injury, elevated inflammatory cytokines, and augmented vasoconstriction of coronary arteries.

Previous studies in which the starting material used for the experiments consisted of uncharacterized and impure fullerene-containing mixtures, have led to erroneous results suggesting fullerenes, rather than the impurities, large particle sizes, and many isomers, induced the toxicological effects.²³ Similarly, in this study insoluble C₆₀ is mixed with PVP and this mixture is used for the in vitro and in vivo studies. Unfortunately, although the manuscript suggests that one can find “more information about our formulation of C₆₀ in the Supplementary materials,” no such data are presented. Thus, the relevance of the conclusions interpreted using the test materials is unclear for several reasons.

First, the exact nature of the starting material is not clear. For example, is the C₆₀ inside the PVP or coated with it? Does the C₆₀ stay bound to PVP in serum? If not, then C₆₀ will simply precipitate out in the blood. Although the title and the manuscript continuously state C₆₀ (not C₆₀ plus PVP) as the substance being studied the authors are not observing data revealed through C₆₀ exposure; rather they are observing data from a mixture of insoluble C₆₀ plus PVP which together make a completely new molecular entity or entities. The authors fully acknowledge that the insolubleness of C₆₀ “due to its hydrophobicity” was the reason it was formulated with PVP.

Second, proper controls were not included. Although controls for PVP were performed, there were no controls performed to conclude it was something specific about the nature/structure of the C₆₀ as any amorphous carbon nanoparticle milieu with the same incredibly large size (up to 800 nm particles) would in all likelihood invoke the same response indicated in these studies.

Third, in addition to the difficulties of injecting large, amorphous, aggregates directly into the circulatory or respiratory systems, the fact that C₆₀ particles are not water soluble raises toxicological issues that should not be surprising to anyone; ingestion of insoluble material causes various toxicological stresses. Thompson et al.’s study reveals no evidence that the induced inflammation and exacerbated cardiac injury is a result of fullerenes, however, suggests a correlation between inflammation and cardiac injury induced via large agglomerates of particles. This is further supported by the author’s citations within the introduction.⁴⁻⁶

Given the questionable starting material used, we refute the misleading conclusion that “C₆₀ exposure of Sprague Dawley rats resulted in deleterious cardiovascular consequences” as PVP is not mentioned and no control for the C₆₀ (e.g., carbon black mixed with PVP) was used.

In detail, the experimental data reveal that the vehicle control has a z-average size of approximately 35 nm and the C₆₀/PVP aggregate has a z-average of approximately 370 nm. After 30 min, no size could be observed using DLS in the vehicle (PVP alone) whereas the aggregate PVP/C₆₀ was still significantly larger (resulting in a sample nearly 1,000% larger than the vehicle control). The investigators show that these physical characteristics of PVP and C₆₀/PVP are stable for over 38 min (the indicated length of the experiments). Although these stability results are important, a more comparable sized control is critical to accurately assess the impact of the C₆₀ fullerenes. Notwithstanding problems with experimental controls, a majority of the data presented by Thompson et al. provide readers with very little statistically significant results and some of the comparisons evaluated seem arbitrarily selected. For instance, one significant measurement describes the effect on protein concentration from PVP/C₆₀ administered IT versus IV, a more substantial finding would be whether these results were significant against naive or vehicle controls, which seems to have not been evaluated. However, in subsequent experiments the investigators do evaluate the significance between either PVP control or C₆₀/PVP agglomerates versus naive (as opposed to the IT vs. IV statistics examined earlier). Despite the seemingly selective statistical analysis, in most cases where significant results are revealed with C₆₀/PVP the same outcomes are observed in PVP control versus naive, revealing a somewhat convincing argument that PVP is inducing the observed deleterious consequences.

The authors mention the potential for using fullerenes in medicine among many other applications. Indeed our group has published several studies using these molecules as therapeutics and diagnostics. We have demonstrated that highly purified, water soluble fullerene derivatives are not acutely toxic, are cleared from the body within 48 h, and in general have anti-inflammatory properties.⁷⁻¹² The likelihood that such a concoction of fullerenes used in the article by Thompson et al. would ever be used for any medical application (and injected at particle sizes used in this study) is non-existent. The FDA requires that every new chemical entity (NCE) must be evaluated separately; extrapolating toxicity (or non-toxicity) by categorizing compound mixtures and making generalizations about classes of compounds (as is the case in this study with fullerenes) with many different isomers is not acceptable to the FDA. Many studies have revealed that even extremely similar molecules can have different biologic activities, where two very similar isomers have completely different biological behavior. This applies to the studies with any molecule being evaluated for potential medical applications where even extremely small changes can result in the NCE having completely different biological properties. In potential fullerene derived therapies, any modifications to the core carbon structure (through the addition of side-chain moieties, coatings, etc.) must be thoroughly studied and evaluated to establish safety profiles. This has been demonstrated repeatedly by several laboratories, which highlight the difficulty in interpreting data gathered from extrapolating findings between even very similar compounds. Thompson et al. describe results from directly injecting insoluble material with millions of different aggregate sizes and isomers of C₆₀ and PVP which make extrapolation into any medical application difficult.

The concerns of toxicity have slowed the initial enthusiasm that surrounded the discovery of fullerenes. Although there are over 11,000 peer-reviewed publications on the NIH’s National Library of Medicine (www.pubmed.gov) using fullerenes and over 4,000 granted fullerene patents according to the US Patent and
Trademark Office (www.uspto.gov), no health-related diagnostic, therapeutic, or theranostic compound using fullerenes has reached the market in the United States. Only those studies using well-characterized, single species molecules, be it fullerenes or otherwise, can provide meaningful information regarding potential toxicological effects. Such studies are increasingly critical as the state of nanomedicine-based research today with fullerenes is shaped by studies that address the observation “that extrapolation across similar nanoparticles will be dependent upon surface chemistry and concentration which may affect the degree of agglomeration and thus biological effects.” 13 Contributing to the confusion in the field is the continuous findings that C60-derivatives significantly extend the life of mammals and improve cognition.14,15 It is difficult for scientists and non-scientists to interpret how a material that significantly extends the life of an animal can simultaneously be toxic as suggested here. As more studies make erroneous conclusions such as those presented here, the likelihood that the benefits of these materials could impart on human health will never be realized.

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The Need for Reflective Consideration of an Integrative Understanding of Cardiovascular Consequences to PVP Formulated C60 Exposure

To the Editor,

We appreciate the discussion raised in the LTE “Study examining fullerene toxicity raises questions as to the purity of the nanomaterials and erroneous experimental conclusions”. We acknowledge the potential for fullerenes and other engineered nanomaterials to have benefits, medical or otherwise. It is clear that the scientific and lay communities are excited about the possibilities that these types of materials invoke, but may need further studies if we are to understand how the body discriminates between their unique properties. We have dedicated substantial effort to studying how this fullerene, a suspension of PVP formulated C60 in saline, and other engineered nanomaterials may impact cardiovascular physiology.

Our work investigates how exposure to xenobiotic particulate matter can elicit little to no overt toxicological response but alters the background physiology to promote a robust injury response following a secondary insult, as in a model of cardiac ischemia/reperfusion (I/R) injury. Despite the similarity in the magnitude of I/R injury following exposure to carbon based particles (i.e., C60, carbon nanotubes, and carbon black), the physiology that promotes the expansion of I/R may be disparate. We hypothesize exposure to these types of materials, while not overtly toxic, sensitizes an organism’s repair mechanisms to a secondary injury. The full mechanistic explanation of our hypothesis remains in question but may reflect a second order systems level integration of toxicological responses to an otherwise presumed nontoxic material.

We worked with the Journal to ensure that all materials we provided with our original submission have been made available in the Supplementary material and have been characterized as 99% C60 without significant contamination of amorphous carbon or other forms of graphene, abrogating the argument that impurities were responsible for the effects.

The LTE authors suggest that dose formulation resulted in insoluble C60 and PVP and raises an important question as to whether C60 is coated with PVP. Possible inner-structures within some C60/PVP formulated particles have been characterized by transmission electron microscopy (TEM).1 Our study did not specifically focus on answering this question. However, we have worked with the Journal to provide details on the formulation of C60 in both the radiolabeled and non-radiolabeled forms. Our data demonstrate our dosage formulary, a suspension of PVP formulated C60 in saline, was not a mixture of PVP and insoluble C60. Dynamic Light Scattering (DLS) measurements of C60 dose formulation maintained a constant size distribution distinctly different from the size distribution observed for PVP formulation in saline. The DLS measurements of the C60 dose formulary exhibited a size distribution of >80% hydrodynamic diameters in the range of 100–500 nm, with an average hydrodynamic diameter of 370 nm with <10% as either large agglomerates (diameter 500–800 nm) or small agglomerates (diameter <100 nm). Additionally, the physical size as measured by TEM was 115 nm. We at no time make the supposition we were administering a strictly nano-sized material and went to lengths to provide a characterization of the material at the time of administration.

The assumption the C60 dose formulary precipitated out in the blood is unsubstantiated as there was no immediate pulmonary ischemic embolism, nor were there signs of respiratory distress when the material was delivered. In fact, the rats remained asymptomatic for as long as 30 days post administration of the material. Additionally, published2 and currently unpublished data, on the biodistribution of uniformly radiolabeled C60 dose formulary demonstrates the majority is recovered in spleen and liver by 24 h after administration. In aggregate, we argue our C60 dose formulation remained dispersed at the time of delivery and was capable of distributing throughout the body.

Regarding the lack of a carbon-based control data, our lab reported Printex 90 instillation results in a similar level of myocardial injury (38 ± 5% of the Area at Risk). The vehicle from that Printex 90 study did not exacerbate injury resulting in a 20% infarction, like the PVP vehicle and to the same extent of injury as observed in naïve animals. Not all materials induce the same I/R injury, e.g., the instillation of cerium oxide nanoparticles expanded I/R injury (26 ± 4% of the Area at Risk) smaller than that of C60 or MWCNT.3,5 We acknowledge limitations exist in any study attempting to unravel toxicity associated with insoluble particles in a physiological system. The broad purpose of our study was to determine if expansion of I/R injury was unique to a pulmonary exposure, or if similar infarct expansion would occur approaching the pulmonary interface intravenously. We found expansion of I/R injury occurred after both intratracheal and intravenous administration. The extent to which there was any significant inflammatory response by either route is limited to the intravenous...
administration and could be associated with the delivery of the sham PVP formulation in saline.

The issue raised that the large agglomerates of C60 dose formulary should have produced inflammation that would explain the expansion of I/R injury requires a liberal interpretation of our results. Intravenously administration of the C60 dose formulary, did yield a minimal pulmonary and circulatory inflammatory profile, and also manifested an expansion of the I/R injury. However, one cannot ignore the results showing that pulmonary/ circulatory inflammation was non-existent 24 h after C60 dose formulary was instilled into the lungs, yet expansion of I/R injury remained. We also extensively investigated C60 inflammatory responses in both endothelial and epithelial cell types and found no cytotoxicity or inflammatory responses despite cellular uptake of the PVP formulated C60.

While the authors of the LTE express an interest in developing C60 as a therapeutic agent, we are not aware if they provided appropriate disclosure of any conflict of interest with regard to commercial development and use of C60 as a parent material. We would like to emphasize that the purpose of toxicological studies, is not to hinder/decelerate the development of nanotechnology or its applications. These studies aimed at identifying potential mechanisms and side effects within the specific exposure scenario. We expect reporting these findings and their proper interpretation might stimulate the design/application of alternative particle formulations, routes of administration and novel applications.

Toxicity indicates the degree to which a substance is detrimental to biological structure and function. The material itself does not have to interact with a specific molecule but may influence the biological environment resulting in an abnormal response. In recent years, the methods estimating the degree of risk posed by materials has come under scrutiny and a discussion continues on how best to identify/define the detrimental effects of selected materials. Although, one can naively declare some substances harmless, the basic principle of Toxicology is any substance can be toxic, it is the dose that determines toxicity. We would advocate the publishing of such studies in a leading toxicology journal, that was peer reviewed, has merit even if we may not presently fully appreciate nuances of formulation, dosimetry and tissue targeting. We are optimistic about the utility of a variety of purposefully manufactured materials and would gladly open the doors of collaboration to elucidate any cardiovascular outcomes.

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