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

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Abstract:

To the Editor,

We are compelled to comment on a recent article in your journal "C_{60} Exposure Augments Cardiac Ischemia/Reperfusion Injury and Coronary Artery Contraction in Sprague Dawley Rats; 138(2), 365–378" by Thompson[1] in which various conclusions were made concerning the toxicity of C_{60}. In this study, insoluble C_{60} 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 C_{60}-PVP mixture resulted in expansion of myocardial infarction in male and female rats following I/R injury, elevated inflammatory cytokines, and augmented vasocontraction of coronary arteries.

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resulted in expansion of myocardial infarction in male and female rats following I/R injury, elevated inflammatory cytokines, and augmented vasocontraction 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.[2,3] Similarly, in this study insoluble C_{60} 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 C_{60} in the Supplementary Data," 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 C_{60}inside the PVP or coated with it? Does the C_{60} stay bound to PVP in serum? If not, then C_{60} will simply precipitate out in the blood. Although the title and the manuscript continuously state C_{60} (not C_{60} plus PVP) as the substance being studied the authors are not observing data revealed through C_{60} exposure; rather they are observing data from a mixture of <u>insoluble</u> C_{60} plus PVP which together make a completely new molecular entity or entities. The authors fully acknowledge that the insolubleness of C_{60} "due to its hydropho-bicity" 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 C_{60} 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 C_{60} 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 "C_{60} exposure of Sprague Dawley rats resulted in deleterious cardiovascular consequences" as PVP is not mentioned and no control for the C_{60} (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 C_{60}/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/C_{60} 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 C_{60}/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 C_{60}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/C_{60} administered IT versus IV, a more substantial finding would be whether these results were significant against naïve 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 C_{60}/PVP agglomerates versus naïve (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 C_{60}/PVP the same outcomes are observed in PVP control versus naïve, 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 C_{60} 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 C_{60}-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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