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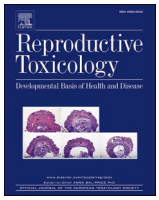

Letter to the editor

Decamethylcyclopentasiloxane developmental neurotoxicity testing

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A paper by Yi et al. on the putative developmental toxicity of decamethylcyclopentasiloxane (D5) contains methodological errors that detract from the reliability of the conclusions [1]. To support studying D5, Yi et al. state that octamethylcyclotetrasiloxane (D4) is an endocrine disrupting chemical and was associated with developmental neurotoxicity in a previous study. The cited support for identifying D4 as an endocrine disruptor was a study in which rat pituitary cells exposed *in vitro* to D4 up-regulated calcium-binding protein 9k, a response shared with 17β-estradiol and blocked by fulvestrant [2]. However, D4 in this assay was at least four orders of magnitude less potent than 17β-estradiol. Moreover, an *in vivo* uterotrophic assay in immature rats was negative with D4 at 1000 mg/kg, suggesting that D4 has no estrogenic activity *in vivo*. The authors admitted that D5 has no estrogenic activity but that possible developmental neurotoxicity should be investigated notwithstanding. The Yi et al. paper described an *in vitro* study and an *in vivo* study with D5.

The *in vitro* study used a mouse neural progenitor cell line indicated as 46 C, source not given. This cell line may be derived from embryonic stem cells. The transcription factor Sox1 was labeled with green fluorescent protein as a marker for differentiation. Cells were incubated for 48 h with D5 (source not given) in an unspecified diluent. The D5 concentrations were 10^{-9} to 10^{-2} M without an explanation for how the range was selected. Fluorescence spectroscopy was used to measure green fluorescence, which was characterized as an indicator of viability in one part of the methods and an indicator of proliferation in another part of the methods.

Cell viability was evaluated with a "CCK assay." I assume this reference is to a commercial CCK-8 assay, but no information was given in the methods or results on how the assay was performed. The median lethal concentration was compared to the median inhibitory concentration for proliferation, which appears to have been interpreted as green fluorescence in the cultures. The values were used in a discriminant function that is not described in this paper but is referenced by citation to a paper appearing in *Reproductive Toxicology* [3]. In the referenced paper mouse embryonic stem cells with knocked-in *sox1-gfp* were induced toward the neuroprogenitor pathway. Cell viability was assessed using a CCK-8 assay. The discriminant function developed using 11 positive and four negative compounds was $1.1280674 \times \log_{10}IC_{50} - 0.2027356 \times \log_{10}ID_{50} + 2.811444$ where IC_{50} is the median lethal concentration and ID_{50} is the median inhibitory concentration for proliferation. A negative number was taken as an indication of developmental neurotoxicity.

This discriminant function cannot be accepted because:

1. Labeling compounds as toxic or nontoxic ignores the importance of exposure level on toxicity. The dichotomization of chemicals as toxic or nontoxic has been rejected for the development of alternative testing in developmental toxicity [4,5].
2. The development of a discriminant function *a priori* does not validate a test strategy. The 15 chemicals used here would be called the "discovery set" and validation of the assay requires application of the technique to an experimental set, that is, to chemicals that have unknown toxicity or to chemicals with concealed names and properties. The predictive value of the model can be calculated based on how often "positives" in the test represent a gold-standard assessment of developmental neurotoxicity.

For these two reasons, the conclusions by Yi et al. that D5 is a developmental neurotoxicant based on the *in vitro* assay cannot be accepted.

The *in vivo* study used 12-week-old C57BL/6 N males and females that were mated in the authors' facility on a 12-hour light schedule. Vaginal plugs the morning after mating indicated E0. Dams were treated with D5 in corn oil by gavage at 0, 3, 6, or 12 mg/kg body weight/day from E10.5 through PND 7. Pups were weaned at four weeks of age. Dose selection was described as based on "Health & Council, 2006," which is not otherwise referenced but may refer to an unpublished ENVIRON evaluation produced for the Silicone, Environmental, Health, and Safety Council. According to the authors, 6 mg/kg bw/day is a "standard dose," but the authors do not further specify what this term means. Starting at postnatal week 6, pups were subjected to eight behavioral tests, apparently sequentially. It is not clear whether each mouse pup was subjected to each test, but somewhat different numbers of animals were given in the footnotes to the data figures, so perhaps there was some variation in the pups included. There is additional uncertainty from the cryptic statement that mouse offspring were killed one week after being tested "for the stabilization of the mouse conditions." Animals were also killed at postnatal week 17. The authors do not indicate how many

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litters or how many pups there were in the experiment.

The data were analyzed using one-way ANOVA with post-hoc Dunnett test to compare each group to the control group within each endpoint. The Dunnett test will protect the overall *P* value within each endpoint inasmuch as comparing each group to the control would give three comparisons per endpoint, but there was no adjustment for the evaluation of endpoints from eight behavioral tests. Analysis appears to have used the pup as the statistical unit whereas the litter of origin should have been the statistical unit as recommended by OECD [6] and a decade earlier by the U.S. Environmental Protection Agency [7]. There was no adjustment for or mention of effects on maternal weight or weight gain, pup or litter weight or weight gain, or viability, any of which can confound behavioral testing.

The *in vivo* study evaluated grooming, marble burying, preference for a stranger mouse in a three-chamber test, social interaction, immobility time in a tail-suspension test and in a forced-swim test, novel object recognition, and a Morris water maze. The authors interpreted the test animal interactions with other mice as showing impaired social interactions analogous to autism. The increased time grooming and the increase in marble burying were interpreted as repetitive behaviors, also analogized to autism. The increased immobility time in the tail suspension and forced swim tests were considered signs of depression, and the small prolongation of escape latency in the Morris water maze was considered impaired learning.

Although these interpretations of behavioral test findings are not novel, the authors failed to consider how the deficiencies of their study and analytic design may have created spurious findings. This paper has two main deficiencies.

1. Failure to adjust for multiple comparisons: In the *in vivo* study, there were eight tests some of which had multiple subtests. The social interaction test had two subtests (preference for a stranger mouse vs. an empty cylinder and preference for a stranger mouse vs. a familiar mouse). The social interaction test rated four behaviors. The Morris water maze gave results for each of nine days; that is, there was a repeated measures design without an appropriate repeated measures analysis plan [6]. The multiple comparisons could have been adjusted, but they were not. The authors might argue that some of their comparisons were significant at $P < 0.001$, which might be expected to compensate for the multiple comparisons and that many of their findings showed dose-relationship, which would be unlikely to arise by chance. However, the second deficiency invalidates these arguments, as discussed below.
2. Failure to report or adjust for litter effects: Animals within the same litter are more likely to behave similarly than animals from different litters [8]. Moreover, there may have been treatment-related impairment of maternal health, maternal caretaking, and pup health including nutrition and weight gain. This paper failed to indicate how many pups came from how many litters and whether there were potentially important differences based on litter of origin. Offspring from dams with impaired nurturing ability may perform differently than offspring from dams with normal nurturing ability. The failure to consider the litter as the unit of treatment and analysis exacerbated the lack of adjustment for multiple comparisons. Using the pup as the statistical unit inflates the degrees of freedom in the statistical analysis and can give rise to spuriously low *P* values.

Although the authors have used standard rodent behavioral tests, the failure to account for litter size and effects and the misuse of statistical methods invalidate the conclusions they have drawn from their experiments. The *in vitro* test has not been appropriately validated and adds nothing to our understanding of D5 effects on development.

CRediT authorship contribution statement

Anthony R. Scialli: Writing – review & editing, Writing – original draft, Methodology, Formal analysis.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Anthony Scialli reports financial support was provided by American Chemistry Council Silicones Environmental Health and Safety Center. Anthony Scialli reports a relationship with American Chemistry Council Silicones Environmental Health and Safety Center that includes: consulting or advisory.

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