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RDX and miRNA Expression in B6C3F1 Mice

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In a recent issue of *EHP* Zhang and Pan (2009) reported on the effects of the explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) on the differential expression of microRNAs (miRNAs) in brain and liver of B6C3F1 mice. It is always of interest when new technologies are applied to existing toxicologic problems, with a view to increasing our understanding of the effect on, or risk to, humans. However, in the abstract of their article originally published online (but deleted from the final version), Zhang and Pan (2009) concluded that “environmental toxicant exposure alters the expression of a suite of miRNAs that in turn regulates gene expression which may lead to carcinogenesis, developmental, neuronal, and reproductive toxicity”; they reached this conclusion in the absence of any observations for dose response, clinical chemistry, histopathology, or neurotoxicity. Because their results do not support these conclusions, we felt a response was warranted.

Zhang and Pan (2009) exposed B6C3F1 female mice to RDX in food. The mouse chow was sprayed with a solution of acetone-dissolved RDX and allowed to dry; this resulted in a formulation chow containing 5 mg RDX/kg of food. At this dose of RDX, we estimated that the mice received approximately 0.75–1.5 mg/kg body weight/day, based on mouse food consumption of 3–6 g/day and an average body weight of 20 g. To put this dose in perspective, the 2-year cancer study on which RDX risk assessment was based (Lish et al. 1984) used oral doses of 0, 1.5, 7.0, 35, and 175 mg RDX/kg/day in the same mouse strain, with statistically significant liver cancers found only in the 35-mg/kg dose group. The dose used by Zhang and Pan in their 1-month study was therefore less than the lowest dose in the 2-year mouse cancer study and over 20 times lower than the only dose of RDX associated with cancer. Furthermore, given that only a fraction of the exposed animals developed cancer at the 35-mg/kg dose in the 2-year study (Lish et al. 1984), we wonder how let-7 and other miRNAs used by Zhang and Pan (2009) identify which animals could potentially get cancer at a higher dose (i.e. susceptibility), or whether all animals could develop cancer even at this low dose (i.e., overprediction).

At high oral exposures, RDX causes tonic-clonic seizure, an effect that has been well correlated with internal dose (blood RDX was

not measured in Zhang and Pan’s study). The mode of action of RDX is thought to be direct because seizures can occur within minutes of dosing. Zhang and Pan (2009) reported that brain derived miRNA 206 was increased 26-fold and brain-derived neurotrophic factor (BDNF) was computationally identified as a downstream target, with the direction of change presumably inhibitory on BDNF. Current literature shows that BDNF is actually up-regulated in response to seizure-inducing agents, such as kainite (Revuelta et al. 2005) and domoic acid (Doucette et al. 2004). Whether other presumed targets of miRNA would be up-regulated is not known, making verification of miRNA targets (mRNA) critical in the validation of this kind of study.

Although miRNAs have been used extensively to examine the profiles of small RNAs in distinct phenotypes such as cancer, their significance as predictors of toxic insult or disease has not been demonstrated. The field of miRNAs is burgeoning with publications (1,738 in 2008), many of which involve the retrospective examination of diseased tissue (tumors) for changes in the expression of miRNA species. Prospective work relating chemical exposure to changes in miRNA as predictors of imminent disease has been less successful, and a study of dioxin found miRNAs refractive (Moffat et al. 2007). More important, some reviews (Kozak 2008) caution against overinterpretation of miRNA data, especially without verification of downstream targets.

It has been said that “a difference, to be a difference, should make a difference.” We found it difficult to assess the biological significance of the suite of differentially regulated miRNAs and their computational targets culled from the study of Zhang and Pan (2009); although these miRNAs could be associated with exposure to RDX, they do not seem related to disease. In our opinion, Zhang and Pan’s results fall short of their experimental hypothesis that exposure to specific environmental agents, such as RDX, would cause alteration in miRNA expression and that “the altered miRNA expression contributes to carcinogenesis.” For innovative work of this kind, a solid model of exposure–disease is always a good starting point, coupled with the classical toxicology stalwarts of dose response and positive/negative controls, and of course, verification of putative targets. Here, we feel that poor study design, absence of phenotype, and overinterpretation of data significantly weakened a potentially informative body of work.

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RDX and miRNA Expression: Zhang and Pan Respond

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We appreciate the interests and comments from Bannon et al. regarding our recent article on RDX-induced aberrant microRNA (miRNA) expression in mice (Zhang and Pan 2009). However, we disagree with their comments based on the misunderstanding of our results and conclusions.

The objective of our study (Zhang and Pan 2009) was not to use clinical chemistry and histopathological features to diagnose neoplasm and carcinogenesis, but to investigate the effect of a low-dose RDX exposure on miRNA expression in mice. Our results show that exposure to RDX at 5 mg/kg in the diet for 28 days induced aberrant expression of miRNAs in B6C3F1 mice. Our discussion of the implications of altered miRNAs in the carcinogenic risk of RDX suggested by a previous study (Lish et al. 1984) are based on the knowledge that aberrant miRNA expression

is associated with a broad range of cancers (Zhang et al. 2007). Meanwhile, we also discussed the potential anticancer effects of RDX in our article. Results in our Figure 6 indicate that RDX exposure induced miR-206 expression, which may inhibit expression of *TNKS* (tankyrase, TRF1-interacting ankyrin-related ADP-ribose polymerase). The inhibition of *TNKS* causes telomere shortening and apoptosis, which inhibits carcinogenesis, and has thus been proposed as a potential cancer therapy (Seimiya 2006). Thereby, the miR-206 overexpression induced by RDX may provide a mechanism to prevent carcinogenesis.

Animals are more sensitive to chemical exposure at the gene level than at the physiological level, and gene expression profile is a more powerful predictor of the outcome of disease than standard systems based on clinical and histologic criteria (van de Vijver et al. 2002). However, aberrant gene expression may or may not cause carcinogenesis. Cancer pathogenesis is a complex process associated with aberrant expression of many genes. Recently identified miRNAs play critical roles in cancer development; overexpression or down-regulation of a single miRNA could influence cancer cell growth, invasion, and metastasis (Ma et al. 2007; Takamizawa et al. 2004). Also, more and more evidence demonstrates that environmental carcinogens cause aberrant expression of a suite of miRNAs. For example, Kalscheuer et al. (2008) recently demonstrated the differential expression of miRNAs in early-stage neoplastic transformation in the lungs of F344 rats chronically treated with the tobacco carcinogen NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone). Thus, carcinogen-induced aberrant expression of miRNAs may be associated with carcinogenesis.

Brain-derived neurotrophic factor (BDNF) is targeted by multiple miRNAs. Besides miR-206, two experimentally validated BDNF-targeting miRNAs, miR-30a and miR-195 (Mellios et al. 2008), were also significantly up-regulated in mouse brain after RDX exposure. Thus, it is reasonable to suggest that miRNA-mediated BDNF expression is a neurotoxic effect of low RDX exposure, given that BDNF is an important neurotrophin supporting neuronal survival and differentiation, neurite outgrowth, and synaptic plasticity.

Seizures have been induced by RDX at high acute doses but not at low doses (Burdette et al. 1988; Schneider et al. 1978). Our result is similar to that in previous reports, and we observed no seizures in our study using low doses. Both proepileptogenic and antiepileptogenic effects of BDNF have been reported (Binder et al. 2001); therefore, we did not discuss the potential inhibition of BDNF in the context of RDX-induced seizure

in our article. However, the up-regulation of BDNF reported in most chemical-induced seizures have been measured at postseizure and postlesion periods instead of pre-seizure periods (Binder et al. 2001; Nawa et al. 1995). Indeed, BDNF is naturally down-expressed in chronic epileptic stages (Shetty et al. 2003), and the reduced level of BDNF may play important roles in the proexcitotoxic effect of chronic stress (Mattson 2007). Neurotoxicants such as alcohol (Fattori et al. 2008) and polybrominated diphenyl ethers (Viberg et al. 2008) also inhibit BDNF expression. Alcohol has also been found to increase miR-335 expression at low doses but suppress miR-335 at high doses, suggesting some miRNAs respond to neurotoxicants in a dose-specific manner (Sathyan et al. 2007).

Our study (Zhang and Pan 2009) provides substantial information on miRNA expression as a phenotype in response to RDX exposure in mice. We reported many important novel findings that enlighten future research. For example, miR-206—belonging to the same family as miR-1, which regulates signal transduction at neuromuscular junctions (Simon et al. 2008)—was significantly up-expressed in our study. Therefore, elucidating the role of miR-206 in RDX-related neurotoxicity is an enormous project.

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Don't Flush the Yuck Factor

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I enjoyed Charles Schmidt's original and informative article on the "Yuck Factor" (Schmidt 2008). This phenomenon has come into play in my attempts to decide on whether to purchase a low-flow toilet for my home. My city is currently offering rebates on low-flow toilets, but only on the least consumptive models (1.6 gallons per flush). I have read on web sites and heard from friends that these models do not quite do the job. The details are important to me. If it is a question of not flushing gobs of toilet paper down, I can deal with that. But if these toilets leave stains on (or product in) the bowl, my family will not be happy. My discussions with city officials and friends who have installed these toilets invariably break down when I press for details. The "Yuck Factor" reigns supreme. The end result may be that I do not purchase a low-flow toilet.

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