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Disruption of Laboratory Experiments Due to Leaching of Bisphenol A from Polycarbonate Cages and Bottles and Uncontrolled Variability in Components of Animal Feed

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Mammalian embryonic development is epigenetic in that hormonal signals not only control the timing of gene expression but also set the activity of genes and thus the functioning of organs and homeostatic systems for the remainder of life. Variation in endogenous hormones (e.g., estradiol and testosterone), which regulate the development of organs (vom Saal 1989), or disruption of the activity of these hormones during development by chemicals can lead to permanent changes in organ structure and function. Adult exposure to endocrine-disrupting chemicals can lead to transient changes in organ function that can disrupt experiments.

Polycarbonate cages and water bottles are manufactured by polymerizing the chemical bisphenol A, which was initially considered for use as an estrogenic drug before being used to manufacture polycarbonate in the 1950s (Dodds and Lawson 1936). More than 50 published studies have shown effects of developmental as well as adult exposure to bisphenol A on a wide variety of traits in mollusks, insects, fish, frogs, rats, and mice. Polycarbonate cages have been commonly used to house rodents and aquatic animals in laboratory experiments. What was not appreciated by scientists using these cages until recently is that after repeated washings the rate of leaching of bisphenol A increases dramatically and can reach levels that can alter traits in animals. Howdeshell and coworkers reported that a small but detectable amount of bisphenol A leached out of new polycarbonate animal cages into water at room temperature, and the rate of leaching was more than 1000 times greater (> 300 μg/L) in old, visibly

worn (scratched and discolored) polycarbonate cages (Howdeshell and others 2003).

Hunt and colleagues reported that an adverse effect of exposure to very low doses of bisphenol A leaching from polycarbonate animal cages and water bottles is profound disruption of chromosomes during meiosis in oocytes in female mice. Specifically, there was a dramatic increase in the incidence of abnormal alignment of chromosomes during the first meiotic division in oocytes, which was caused by the leaching of bisphenol A from the polycarbonate cages washed with a harsh detergent (Hunt and others 2003; Koehler and others 2003). Abnormal alignment of chromosomes results in aneuploidy, or abnormal numbers of chromosomes in oocytes, which can lead to abnormal development such as occurs in Down's syndrome. These authors thus refer to bisphenol A as a "potent meiotic aneugen." Aneuploidy is thought to be a major cause of embryonic mortality in humans. Hunt and coworkers reported that severe oocyte chromosome abnormalities increased in peripubertal female mice in the following proportions: from a baseline frequency of 1.8% in control animals (not housed in damaged cages) to 20% due to housing females in damaged polycarbonate cages; 30% due to the use of damaged polycarbonate water bottles; and 41% due to combined use of both damaged cages and water bottles. In a subsequent experiment, the researchers intentionally accelerated the normal aging process associated with repeated washing of polycarbonate cages and water bottles by washing them different numbers of times in a harsh detergent. The polycarbonate water bottles were found by gas chromatography/mass spectrometry analysis to release between 100 (mild damage) and 260 μg/liter (severe damage) of free bisphenol A into water placed into the bottles, resulting in daily exposure of the female mice ranging between 15 and 72 μg/kg. When peripubertal female mice housed in undamaged new cages were fed bisphenol A once daily at the very low doses of 20, 40, and 100 μg/kg to simulate exposure within the range released by the polycarbonate, there was a significant dose-related increase in the incidence of chromosomal damage beginning even at the lowest dose.

Based on studies in which bisphenol A was found to have limited binding to the plasma proteins that serve as a barrier to the movement of estrogen from blood into tissues (Nagel and others 1997), we had predicted that doses of bisphenol A as low as 20 μg/kg/day would disrupt development in mice. This dose is below the predicted "safe" or reference dose for human exposure of 50 μg/kg/day, which was calculated based on old studies that examined only very high doses of bisphenol A, when the lowest dose administered (50 mg/kg/day) had resulted in adverse effects (IRIS 2002).

Taken together, the large number of independent findings concerning adverse effects of very low doses of bisphenol A suggest that the use of

polycarbonate to manufacture animal cages and water bottles can alter the results of laboratory animal research. In fact, due to greater resistance to heat and alkaline detergents, many facilities have switched from polycarbonate to polysulfone animal cages. The ether bond in the polysulfone co-polymer is more resistant to heat and alkaline detergents relative to the ester bond in polycarbonate. We have always used polypropylene cages and glass water bottles, because these cages do not leach biologically active amounts of estrogenic chemicals (Howdeshell and others 2003).

Some components of feed used in laboratory experiments (e.g., phytoestrogens and mycotoxins) have hormonal activity that can interfere with experiments involving outcomes that are sensitive to these hormone-mimicking chemicals. There is wide variation in phytoestrogen content in different types of commercial rodent feed. Both the amount of phytoestrogens and metabolizable energy in different feeds were sources of phenotypic variation (specifically body weight, uterine growth, and age at vaginal opening) in prepubertal CD-1 female mice (Thigpen and others 2003). Thigpen and colleagues selected one soy-based commercial feed (Purina 5002) and examined the consequences of using five batches of this diet with different mill dates. They first measured the amounts of the soy phytoestrogens genistein and daidzein in the five different batches, which ranged from 159 to 431 μg/g. It is well known that one of the effects of the estrogenic drug diethylstilbestrol (DES) is to accelerate vaginal opening, and although a 4-ppb dose of DES accelerated vaginal opening in female CD-1 mice fed the batch of feed with 159 μg/g of genistein and daidzein, there was no accelerating effect of DES in females being fed the batch of feed with 431 μg/g of genistein and daidzein. Administration of even this potent estrogenic drug could not further accelerate this process (Thigpen and others 2003). A similar finding had previously been reported for prepubertal female rats fed different batches of feed produced by another feed manufacturer (Boettger-Tong and others 1998). Together, these studies reveal that the issue of variation in phytoestrogen content in batches of feeds is one that is a general problem and not just restricted to Purina 5002 feed, because any closed-formula diet can contain variable amounts of phytoestrogens due to the source as well as the amount of soy isoflavones.

It is important to emphasize that the isoflavones genistein and daidzein are only two of many naturally occurring compounds that could be sources of estrogenic activity in feed, and even casein-based feeds show variation in total estrogenic activity (Thigpen and others 2003). For example, we have found significant variation in estrogenic activities in different batches of casein-based feeds, and none of these estrogens were genistein or daidzein (unpublished observation). Simply screening for these two isoflavones will thus not guarantee the lack of variability in other potential endocrine-disrupting contaminants in a feed. Feed

manufacturers need to develop new approaches to reduce variability in endocrine-disrupting activity in different batches of feeds to attain levels that will not disrupt research results.

We have also found that there are components of some batches of commercial mouse feeds, such as soy-based Purina 5002 certified diet, that, relative to other feeds (Purina 5008 soy-based pregnancy diet), dramatically increase endogenous estradiol in CD-1 mouse fetuses (unpublished observation). This increase is associated during later life in both males and females fed Purina 5002 throughout life with an increase in postnatal rate of growth, accelerated onset of puberty in females, and an increase in the amount of abdominal fat. Male mice fed Purina 5002 diet also evidenced differences in reproductive organs, such as an increase in prostate size and a decrease in daily sperm production, relative to males whose mothers were fed Purina 5008 during pregnancy and lactation, followed by soy-based Purina 5001 after weaning. An interesting additional finding is that oral administration of DES to pregnant mice of a low dose (0.1 μg/kg/day) and a high dose (50 μg/kg/day) resulted in a dose-related decrease in daily sperm production in adult male offspring on the Purina 5008/5001 regimen, whereas males from the Purina 5002 regimen showed no effect of DES, even at the high dose (unpublished observation). Most investigators are aware of the marked effects that different types of feed can have on the phenotype of their animals. Of great concern, however, is that variability between batches of some feeds is a potential source of uncontrolled variability in research results.

Another variable of concern in laboratory studies is water quality. Copper pipes are used inside the building that houses our mice, and water is provided to the mice in glass bottles. We purify the water by ion exchange and a series of carbon filters. These measures are important to remove contaminants such as phthalates, which can enter water from PVC water pipes and herbicides. These potential contaminants, as well as other solutes that can affect an animal's physiology, are an issue particularly in agricultural areas.

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