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Models of Toxicity of Diacetyl and Alternative Diones

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Abstract

Diacetyl (DA; 2,3-butanedione), with the chemical formula $(CH_3CO)_2$ is a volatile organic compound with a deep yellow color and a strong buttery flavor and aroma. These properties have made DA a particularly useful and common food flavoring ingredient. However, because of this increased occupational use, workers can be exposed to high vapor concentrations in the workplace. Despite being listed by the USFDA to be 'generally regarded as safe' (GRAS), multiple lines of evidence suggest that exposure to high concentrations of DA vapor causes long-term impairments in lung function with lung function testing indicating evidence of either restrictive or obstructive airway narrowing in affected individuals. A growing number of preclinical studies have now addressed the short and long-term toxicity associated with DA exposure providing further insight into the toxicity of DA and related diones. This review summarizes these observations.

Keywords

diacetyl; 2-,3-pentanedione; 2-,3-hexanedione; popcorn lung; occupational lung disease; bronchiolitis obliterans

1. Introduction

The ability to appreciate flavor is largely dependent on aroma (Mozell, Smith et al. 1969). The study of aroma and aroma perception is in its infancy, but is of increasing interest to science as well as the perfume and food industries (Silva Teixeira, Cerqueira et al. 2016). Aromas arise largely from the volatilization of chemicals that are then perceived by olfactory receptors in the nares (Buck and Axel 1991) and are a point of emphasis in marketing, highlighting their economic importance. Because the scale of food production requires enormous amounts of raw materials, workers may now be exposed occupationally to high concentrations of volatile food flavoring ingredients. Examples of such food flavoring ingredients include isoamyl acetate (banana), benzaldehyde (bitter almond or cherry), cinnamaldehyde (cinnamon), limonene (orange), methyl salicylate (wintergreen) and 2-,3-butanedione (butter). Because of the retrospective nature of hazard regulations in

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the US, the effects of occupational exposure to such food flavoring ingredients on workers is often not appreciated until after many years of use and occupational exposures. Such is the case with 2,3-butanedione.

2,3-butanedione (diacetyl; DA) is a naturally occurring α-diketone that is produced during fermentation. In its pure form, DA is a yellowish liquid with a strong buttery aroma. DA has a relatively high vapor pressure that renders small quantities particularly pungent (Starek-Swiechowicz and Starek 2014). DA is present in varying concentrations in a variety of foodstuffs including wine, dairy products, roasted coffee and beer (Shibamoto 2014). Because DA imparts a buttery aroma and flavor it has been used in a variety of applications including the manufacture of microwave popcorn. Finally, DA is among those food additives classified by the United States Food and Drug Administration as ‘generally regarded as safe’ (GRAS).

The earliest mention of diacetyl we could find in the literature is from 1911 (Harden and Norris 1911) in which it was used as a fluorescent indicator of the Voges and Proskauer reaction in which the presence of acetoin, an intermediate step in DA synthesis, may be detected in bacterial broth culture. In 1946, the likely chemical synthesis pathway for DA through the dehydrogenation of pyruvic acid was described in Nature (Suomalainen and Jannes 1946) and was followed over 20 years later by a more mechanistic report also published in Nature in 1968 (Suomalainen and Ronkainen 1968). In 1933, it was noted that “butter cultures with satisfactory character contained considerable quantities of acetylmethylcarbinol and diacetyl.” (Michaelian, Farmer et al. 1933). Oddly, in 1951 a patent was issued that described the use of DA in the manufacture of synthetic latex rubber (Messer and Reynolds 1951). This manufacturing process was out-competed by developments in the petrochemical sector, but during a later oil crisis in the late ‘70’s interest in large scale laboratory production of DA was revived briefly (Gupta, Yadav et al. 1978). In 1969 the oral and intraperitoneal dose determined to cause 50% mortality (LD50) in both male and female rats was determined (Colley, Gaunt et al. 1969). A key finding of that study was that “The no-effect level (90 mg/kg/day) is equivalent to about 500 times the estimated daily intake by man (0.17 mg/kg).” that may, in part, account for the GRAS designation. The 1970’s saw advances in understanding of DA chemistry and furthered the use of DA in laboratory tests for urea in biological samples. In 1979, Bjaldanes and Chew (Bjeldanes and Chew 1979) recognizing that DA is present in a wide variety of foods reported on the mutagenic effects of DA. In 1979 it was recognized that DA could alter the binding of proteins to their known receptors and it was speculated that this could be due to direct protein damage (Rome and Miller 1980). The 1980’s and early 1990’s saw the isolation and characterization of diacetyl reductase that converts DA to acetoin, while analytic methods for the detection of DA and its derivatives improved. In 1996, Sengupta et al. (Sengupta, Chou et al. 1996) identified a DA-specific odorant receptor in C. elegans that is used to affect chemotaxis towards or away from food sources (Langa, Martin-Cabrejas et al. 2014). Notably, Linda Buck who first cloned this family of receptors in 1991 (Buck and Axel 1991) was awarded the Nobel Prize in Physiology or Medicine for this fundamental observation. Interestingly, the DA-specific receptor does not sense the closely related 2-,3-pentanedione (PD) that is now being used as a popcorn flavoring alternative to DA. A confirmation of the same DA-specific odorant receptor in mammalian systems was reported

in 1997 by Zhang et al. (Zhang, Chou et al. 1997). In 1998, Eriksson et al. (Eriksson, Fontaine et al. 1998) reported that DA can modify arginines, essentially confirming the report by Rome and Miller (Rome and Miller 1980) and suggesting a mechanism by which DA may be toxic to cells and tissues. In a key observation in 2002, Kreiss et al. (Kreiss, Gomaa et al. 2002) noted the development of respiratory symptoms including spirometric evidence of airway obstruction as well as reduced forced vital capacity (FVC) measurements, used to evaluate restrictive and obstructive lung disease, in otherwise healthy popcorn factory workers. These changes were independent of smoking history. The aggregate of these symptoms were suggestive of bronchiolitis obliterans (BO). BO is a rare lung disease that is most commonly associated with the aftermath of lung or bone marrow transplantation. Therefore, this observation published in the New England Journal of Medicine was of considerable note. This report was followed by a number of others characterizing the full spectrum of the effects of DA on the human respiratory tract including cough, shortness of breath and wheezing, and biopsies showed histological and morphological changes in the lung that are consistent with bronchiolitis obliterans (Akpinar-Elci, Travis et al. 2004, Kanwal, Kullman et al. 2006, van Rooy, Rooyackers et al. 2007, Kreiss, Fedan et al. 2012, Kreiss 2014).

Because of these observations, alternatives to DA are now being used as food flavoring ingredients. These include 2-,3-pentanedione (PD) and possibly 2-,3-hexandione (HD). There are a number of excellent reviews on the subject of DA toxicity that include descriptions of how DA is synthesized in biological systems and its concentrations in different foods (Shibamoto 2014), while others touch on chemical properties and summarize clinical reports of occupational exposures (Starek-Swiechowicz and Starek 2014). Still others discuss physico-chemical properties of DA as a way of understanding its toxic effects on cells and tissues (Kovacic and Cooksy 2010). The purpose of the present review is to synthesize what is known about the relative toxicities of DA, PD and HD with an emphasis on their hypothesized modes of action in in vitro cell culture and in vivo animal models.

1. *In vitro* toxicity

1.1 The Epithelium

DA, with the chemical formula $(CH_3CO)_2$, is an α-diketone, meaning that in this four carbon molecule the carbons double bonded to oxygens are directly adjacent. In this configuration the sharing of electrons between the adjacent carbonyl groups is thought to render it particularly reactive as a nucleophile (Wondrak, Cervantes-Laurean et al. 2002). PD and HD are also α-diketones and may thus be expected to have similar modes of action against similar substrates. It is thought that because of these chemical features α-diketones including DA can affect protein structure and function as a consequence (Miller and Gerrard 2005). There are four representative in vitro studies that address the possible nature of the cellular injury that DA may cause (Zaccone, Goldsmith et al., Fedan, Dowdy et al. 2006, More, Raza et al. 2012, Kelly, Sun et al. 2014). For example, it has been shown that exposure to 10 mM DA increased epithelial layer permeability as determined by decreased measures of transepithelial resistance (Fedan, Dowdy et al. 2006). This observation has been supported by another study in which exposure of differentiated airway epithelial cells in air

liquid interface cultures to DA for six hours at concentrations of 60 ppm and above caused a complete loss of transepithelial resistance (Zaccone, Goldsmith et al.). In yet another study, it was shown that DA forms guanosine adducts (More, Raza et al. 2012) that the authors state contributes to DNA uncoiling and ultimately to cell death although the mechanism by which this occurs isn't clear. Finally, this laboratory has previously shown that exposure of NCI-H292 cells in liquid culture or primary human airway epithelial cells in air liquid interface culture induces robust shedding of the epidermal growth factor (EGFR) receptor ligand amphiregulin (Areg) (Kelly, Sun et al. 2014). In that study it was shown that inhibition of tumor necrosis factor-α converting enzyme (TACE) effectively abrogated Areg shedding, thereby providing a plausible mechanism through which the epithelium could be affecting the mesenchymal response to DA exposure in vivo, thereby leading to development of fibroproliferative airway lesions. Thus, in vitro studies have shown a loss of barrier function, formation of DNA adducts, protein modification and shedding of the EGFR ligand Areg. These observations are consistent with epithelial injury, which in vivo studies support after exposure to higher concentrations of DA.

1.2 Extrapulmonary toxicity in vivo and in vitro

The first reference we could find for the industrial use of DA as a food flavoring ingredient comes from a 1964 publication (Jenner, Hagan et al. 1964) in which its large scale use provided the rationale for determining its LD50 in rats, which was identified to be between 1310–1920 mg/Kg. In 1969 Colley et al. (Colley, Gaunt et al. 1969) determined this dose to be somewhat higher, reporting the oral LD50 for males at 3.4 g/Kg and for females at 3 g/Kg. The intraperitoneal LD50 for males was reported at 0.4 g/Kg for males and 0.64 g/Kg for females. In that follow-up report at a high dose of 540 mg/Kg/day in chronic studies the authors describe significant hematological alterations including increased hemoglobin concentrations, increased numbers of circulating neutrophils and lymphocytes, curiously only observed in females at the highest tested dose while circulating monocytes were increased at the highest dose only in males. At the highest dose tested the absolute weight of the brain, heart, liver, spleen, kidneys, adrenal glands and thyroid as well as terminal body weight were all significantly altered in male rats. All organs except the kidneys weighed significantly less than at baseline, while the mass of the adrenal glands increased significantly. Similar observations were made in the females with the difference being that the heart and spleen masses were unchanged. At the next highest dose tested (90 mg/Kg/day) no changes in organ weights were observed.

Methylglyoxal is closely related to DA in chemical structure and reactivity and has been hypothesized to play a role in development of Alzheimer's by virtue of its ability to cross the blood-brain barrier and to form adducts with amyloid-β peptides that increase their propensity to form aggregates that go on to become plaques in the brain (More, Vartak et al. 2012). To begin to understand the neuronal toxicity of DA, the authors showed that DA altered amyloid-β peptides and potentiated their growth inhibitory effects on cells in culture. Carbonyl scavengers attenuated this effect. The study also reports that DA effectively crosses an in vitro model of the blood brain barrier and that the enzyme that inactivated methylglyoxal is ineffective against DA. In another study in human liver cells it was determined that DA is highly mutagenic (Whittaker, Clarke et al. 2008) as measured by the

L5178Y mouse lymphoma assay (Lloyd and Kidd 2012) supporting the observation made by More et al. (More, Raza et al. 2012) in epithelial cells that DA causes guanosine adduct formation.

Prior to 2002, such toxicological studies were justified as screens for the identification of potential mutagens. After 2002 all reports of the toxicology of DA refer back to the Kreiss finding that DA exposure causes BO in popcorn factory workers (Kreiss, Gomaa et al. 2002).

2 *In vivo* pulmonary toxicity

2.1 Rats

The USFDA has designated that diacetyl may be ‘generally recognized as safe’ (GRAS). According to federal regulations “the use of a food substance may be GRAS either through scientific procedures or, for a substance used in food before 1958, through experience based on common use in food.’ It is likely that consumption of DA in the vanishing quantities required to add a buttery flavoring to foods is actually quite safe. However, the GRAS designation does not account for the possibility that workers involved in preparing foods with diacetyl may be exposed to high concentrations of the food flavoring ingredients that do not involve direct and intentional consumption. For example, in the Kreiss study (Kreiss, Gomaa et al. 2002) both the degree of airway obstruction and the proportion of workers with irregular spirometry correlated with increasing cumulative exposure to DA. These observations led directly to the first study of the toxicity of DA in rats (Hubbs, Battelli et al. 2002). Given the findings of the Kreiss study, the study by Hubbs et al. (Hubbs, Battelli et al. 2002) is very important in terms of how we consider the toxicity of food flavoring compounds and the GRAS designation more generally. In that study, Ann Hubbs and co-authors examined the hypothesis that inhalation of artificial butter flavoring containing DA causes airway epithelial injury (Hubbs, Battelli et al. 2002). That careful study showed that inhalation of a combination of vapors released from a proprietary mixture of commercially available artificial butter flavoring that included DA at 285 ppm or greater for 6 hours resulted in histopathologic abnormalities in the nasal epithelium at all concentrations of DA vapor and airway epithelial necrosis at all but the lowest DA concentration one day after exposure (Hubbs, Battelli et al. 2002). In a follow-up study, the authors showed effectively that the component of artificial butter flavoring that was most closely associated with the observed epithelial injury was diacetyl itself (Hubbs, Goldsmith et al. 2008). In that study, rats were exposed to DA vapor of up to 365 ppm in a time weighted average for 6 hours and the animals were sacrificed 18–20 hours later. DA exposure at levels well short of the maximum at 365 ppm caused significant damage to the nasal epithelium including dose dependent necrosuppurative rhinitis. In the lungs, epithelial injury was observed in only two of six animals exposed to the highest concentration of DA. That epithelial injury was characterized by SEM to show flattening of the airway epithelial cells accompanied by loss of cilia and the formation of gaps in the epithelial layer. However, it is worthwhile to note that the epithelium of four of the six rats in this group was grossly unaffected. These observations introduce a consistent theme in understanding the effects of DA and alternative

diones on the lung, namely that there appears to be a threshold exposure required for epithelial injury to occur.

In the first report to show a definitive connection between DA exposure and airway lesion development in rats, Palmer et al. (Palmer, Flake et al. 2011) showed that intratracheal instillation of 125 mg/Kg of DA in rats causes profound injury to the airway epithelium, altered lung function, airway neutrophilia and lesions characteristic of BO. In that study it was shown that at one and three days after DA exposure, the airway epithelium was ablated in many airways and fully repaired by day 7. In that report it was also shown that mRNA expression of club cell secretory protein (CC10; CCSP), the definitive marker for airway secretory cells is significantly reduced at day 7 post-exposure. This observation is supported by immunofluorescent staining of histological sections in which prominently reduced CCSP immunoreactivity was shown. These observations support the notion that there is a rapid burst of epithelial repair after DA-induced injury and further suggest that the composition of the repaired epithelium is fundamentally altered at this stage in disease development. A follow up study published by Kelly et al. (Kelly, Sun et al. 2014) and discussed earlier showed that DA vapor exposure induces robust shedding of the epidermal growth factor receptor (EGFR) ligand amphiregulin (Areg). In that study it was shown that DA vapor exposure of immortalized epithelioid NCI-H292 cells as well as normal human bronchial epithelial cells grown at air liquid interface culture shed significantly increased quantities of Areg into the culture media in a TNF-α converting enzyme (TACE)-dependent manner. Areg is of considerable interest because it is known to participate in epithelial regeneration after injury in other organ systems including the small intestine (Shao and Sheng 2010). These observations taken together provide a plausible connection between exposure to DA vapor, epithelial injury and repair and airway lesion development that may be EGFR dependent.

At roughly the same time as the Palmer report in PLoS One (Palmer, Flake et al. 2011), two separate reports characterized the dosimetry of inhaled DA in rats and compared this with theoretical deposition fractions in humans using computational modeling (Morris and Hubbs 2009, Gloede, Cichocki et al. 2011). These reports showed that a larger fraction of inhaled DA vapor would reach the smaller airways of a human lung than in a rat lung because of the convoluted complexity of rat nares.

Altered lung function is a key feature of BO that is recapitulated by day 7 in rats exposed to DA via intratracheal instillation (Palmer, Flake et al. 2011). Similarly, early denudation of the airways of epithelial cells is also now recognized as an established feature of DA exposure. Previous reports have shown that removing the epithelium increases the contractile response to bronchoprovocative agents such as acetylcholine (Hay, Farmer et al. 1986, Spina 1998). Therefore, investigators began to address whether DA exposure causes acute changes in lung function. For example, in 2013 Zaccone et al. (Zaccone, Thompson et al. 2013) exposed rats to 100, 200, 300 or 360 ppm DA for 6 hours and the animals were sacrificed for analysis 18 hours later. Counter-intuitively, DA inhalation by itself had little to no effect on airway reactivity 18 hours after DA exposure. Contrary to a previous report in which it was shown that guinea pig tracheas had increased contractility in response to DA exposure (Fedan, Dowdy et al. 2006), it was observed in denuded rat tracheas that exposure to DA increased contraction slightly followed by an overall net increase in relaxation

(Zaccone, Thompson et al. 2013). Overall, DA exposure decreased airway contractility and had no net effect on dynamic compliance in the lungs.

Another study that focused on how DA alters lung physiology observed that diacetyl inhalation dose dependently increases sensory innervation that can project into airway epithelium (Goravanahally, Hubbs et al. 2014). Substance P is important in the development of neurogenic inflammation and contributes to coughing and wheezing (Pernow 1985, Barnes 1990, Nieber, Baumgarten et al. 1992). That study reported on the significant airway epithelial injury observed after 356 ppm DA vapor exposure and further showed prominent substance P staining in neurons that innervate the airway epithelium. Similarly, this study shows increased innervation in the tracheal epithelium.

In yet another study, rats and mice were administered radiolabeled DA via intratracheal instillation to evaluate the ability of DA to move from the lung into the systemic circulation and to subsequently bind to cellular macromolecules. In this study it was shown that DA forms protein adducts with hemoglobin and albumin (Fennell, Morgan et al. 2015). The authors suggest that such adducts could be used as markers of exposure.

Finally, in a report comparing the pulmonary effects of DA with some alternative diones, it was shown again that only the higher concentration vapor exposures caused airway epithelial injury and BO-like lesions (Morgan, Jokinen et al. 2016). Rats were exposed to 0, 100, 150 or 200 ppm of DA for six hours per day, five days per week for two weeks + two days or for two weeks followed by two weeks of recovery before evaluation. At 100 ppm DA, the exposed rats did not lose any weight, which is a consistent feature of vapor concentrations that cause epithelial injury and airway lesions. At 150 and 200 ppm the rats lost significant amounts of weight and had epithelial injury and airway lesions further supporting a general observation that there is a threshold exposure above which these vapors are highly toxic. Additionally, rats exposed to 200 ppm of DA developed parenchymal lesions consistent with pulmonary interstitial fibrosis. Rats exposed to 150 ppm did not have altered lung function after the end of the two weeks' exposure, but when evaluated two weeks after the end of the exposure had developed increased resistance and decreased compliance. All vapor concentrations of DA induced inflammation in the airways, the parenchyma and in the nares at the end of the exposure. This was largely resolved in the airways by the end of the two weeks' recovery period, but inflammation persisted through the recovery period in the parenchyma and the nares.

2.2 Mice

A central theme in the rat studies described above is that below a certain threshold of exposure the epithelium remains grossly unaffected by DA exposure. Associated with this is the observation that only above those exposure levels, in which the epithelium is injured, do the animals develop airway lesions consistent with BO. That mice are different from rats is almost a mantra in animal models of disease. Despite this, we do not have a clear understanding of what those differences are that lead to differences in responses to similar exposures. In this review we have separated out the discussion of rats and mice because of a fundamental difference in their responses to exposure to DA and alternative diones. The

studies to be described below feature prominent epithelial injury and repair, but the lesions that develop are not consistent with BO.

The first report of mouse exposures to DA comes from Morgan et al. in 2008 (Morgan, Flake et al. 2008) in which it was reported that C57BL/6 mice were exposed to either: 0, 200 or 400 ppm DA vapor for six hours per day for five consecutive days; 0, 100, 200 or 400 ppm DA vapor for one hour per day for two or four weeks; to 0 or 1200 ppm DA vapor for 15 minutes per exposure twice daily for four weeks; to 0, 25, 50 or 100 ppm DA vapor for six hours per day, five days per week for 12 weeks; or finally to 400 mg/kg DA delivered by intratracheal instillation. This represents a truly comprehensive investigation of the effects of DA inhalation on the respiratory system in which the experimental design was to model relevant workplace exposures in the microwave popcorn industry. In this study it was determined that significant nasal and bronchial epithelial injury, peribronchial lymphocytic inflammation and fibrohistiocytic lesions in the terminal bronchioles occur after the higher exposure concentrations. The conclusion of the study for the purposes of this review may be that despite significant epithelial injury and inflammation, C57BL/6 mice simply do not develop BO-like lesions after DA exposure. Because human subjects exposed to DA have altered lung function and constrictive and obstructive airway lesions and because rats develop similar lesions to humans, we must consider mice to be a less than ideal model of occupational DA exposure.

In another study of DA exposure in mice by Fennell et al. (Fennell, Morgan et al. 2015) it was shown that, as with rats, less than 1% of orally administered DA entered the bloodstream, but that when it did a significant portion of that radiolabel was bound to hemoglobin and albumin. Also, as with the rats exposed to DA, protein-DA adducts were observed in both the bound hemoglobin and the albumin. Most recently Hubbs et al. (Hubbs, Fluharty et al. 2016) have shown in mice that DA injury of the respiratory epithelium is characterized by accumulation of ubiquitin, involved in protein degradation, and sequestosome-1, a marker of autophagy, as indicators of protein damage, which has been shown to be highly toxic to cells (Goldberg 2003).

3. Pentanedione

3.1 Rats

In 2012, Hubbs et al. (Hubbs, Cumpston et al. 2012) reported on experiments in which rats were exposed to 120, 240, 320 or 360 ppm of PD for 6 hours and evaluated for up to 20 hours later. As with rats exposed to DA, described above, there was significant dose and time dependent injury to the nasal, trachea and mainstem bronchus epithelium in rats exposed to PD. In that study it was also shown that nasal epithelial cells that were olfactory marker protein (OMP) positive indicating that they are nasal epithelial neuroendocrine cells were also positive for activated caspase 3 by immunofluorescence, suggesting a mechanism by which there could be changes in epithelial innervation after PD exposure. Notably, this study reports alterations in cytokine and growth factor mRNA expression in diverse regions of the brain after PD exposure.

In 2015, Morgan et al. (Morgan, Merrick et al. 2015) exposed male Wistar Han rats to 200 ppm PD or air for 6 hours per day, 5 days per week for 2 weeks and performed laser capture microdissection on both exposed and affected airways and exposed and unaffected airways. RNA from the microdissected airways was isolated for microarray analysis. This study identified over 3800 differentially expressed genes in fibrotic bronchi. In pathway analysis, the authors identify that genes associated with vascular development were the most highly enriched followed by genes associated with extracellular matrix remodeling, internal cytoskeletal organization and cell adhesion. Perhaps not surprisingly, TGF-β family proteins in the pathway analysis, with TGF-β2 being the most significantly differentially expressed member of the family. The authors point out that this is consistent with previous reports that TGF-β2 is the primary family member expressed in airways after development (Tschumperlin, Shively et al. 2003, Balzar, Chu et al. 2005, Thompson, Mih et al. 2006)

3.2 Mice

In another comprehensive report, Morgan et al. (Morgan, Jokinen et al. 2012) show that both male and female mice exposed to 0, 50, 100 or 200 ppm PD vapor for six hours per day five days a week for up to two weeks develop a wide range of epithelial lesions in the nares at the two highest vapor concentrations. Similar observations were made in the larynx and in the mainstem bronchus. Interestingly, in that report only male mice exposed to the 200 ppm PD vapor for the full two-week period had any change in the lavageable cell differential in the lung with ~50% of the cells being neutrophils.

4. Hexanedione

4.1 Rats

The first report in the literature of a rat HD exposure was in 1984, well before it was appreciated that DA and possibly alternative diones could cause BO. In that study by Iwasaki and Tsuruta (Iwasaki and Tsuruta 1984) the goal was to determine the neurotoxicity of hexane indirectly. In that study, rats were exposed to 2-,3-hexanedione, 2-,4-hexanedione and 2-,5-hexanedione by gavage with each study animal receiving 4 mL of a 0.5% solution and sacrificed up to 24 hours later. Concentrations of each form of HD were measured both in the blood and in neuronal tissue. The authors made the observation that the 2-,3- form of HD had the lowest retention time in neuronal tissue of any of the three forms.

More recently, as part of a comparative study between the effects of DA, PD and HD on rats, Morgan et al. (Morgan, Jokinen et al. 2012) exposed male rats to 0, 100, 150 or 200 ppm HD vapor and demonstrated that HD vapor exposure induced airway lesions in exposed rats only at 200 ppm and only in 2/12 rats in the exposed group. There was significant mortality in the DA and PD groups but none in the HD exposed group. Consistent with this the HD exposed rats did not lose weight in contrast with the rats exposed to either DA or PD. HD had no effect on measured lung function parameters including resistance and compliance, and caused considerably less epithelial injury.

4.2 Mice

To the best of our knowledge there are no studies that examine the effects of inhaled HD vapor on the mouse respiratory tract.

5 Conclusions

Two central themes have emerged from the body of literature we have reviewed. First, there appears to be a threshold of exposure, below which the nasal and airway epithelium appear essentially unchanged after exposure. This observation holds true for both rats and mice, currently the only study animals we are aware of that have been exposed to DA or alternative diones experimentally. Notably, while current OSHA guidelines require additional labeling stating that DA can cause damage to respiratory tract and lungs if inhaled, as recently as April 1 of 2016, the GRAS status of DA was reaffirmed. These observations, taken together, strongly suggest that the GRAS definition must be expanded to include other likely routes of exposure. While high concentration occupational exposures are encountered by a significantly smaller proportion of the general population, the effects of such exposures may be catastrophic. Therefore, a new metric that accounts for the likely route of exposure in industrial settings must be established.

The second theme that has emerged is that, while mice experience nasal and airway epithelial injury to the same general degree that rats do, they do not develop BO-like lesions. It is currently not known what the molecular differences between the responses of rats and mice to DA might be. Understanding this difference may have therapeutic value.

In summary, DA vapor causes toxic injury to and necrosis of the nasal, tracheal and bronchial epithelium. This has been demonstrated in the literature repeatedly since the initial observation in 2002 that DA exposure is associated with development of BO in popcorn factory workers. Recently, e-cigarette manufacturers have begun using DA as a flavoring ingredient in e-cigarettes with marketing campaigns that highlight the ‘fun’ of products such as fruit and candy-flavored ‘vape juice’ (Farsalinos, Kistler et al. 2015, Allen, Flanigan et al. 2016). These products are now ostensibly marketed to adults, but with flavor names such as ‘Fruity Fun Cereal’ and ‘Bubblegum’ it is hard to see how these would not be appealing to younger consumers. This raises the possibility that exposure to DA or related diones used in e-cigarettes could also induce pulmonary epithelial injury and increase the risk for development of BO in a cohort of young users of e-cigarettes. Given the rapid growth of e-cigarette usage, further research into the toxic effects of DA and related diones is greatly needed to protect individuals from the potential consequences of these exposures.

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