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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 pre-clinical 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.

1. Introduction

The ability to appreciate flavor is largely dependent on aroma (Mozell 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 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 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 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 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 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

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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 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 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 et al., 1997). In 1998, Eriksson et al. (Eriksson 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 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 et al., 2004; Kanwal et al., 2006; van Rooy et al., 2007; Kreiss 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.

2. *In vitro* toxicity

2.1. 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 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 et al., 2015; Fedan et al., 2006; More et al., 2012a,b; Kelly 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 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 et al., 2015). In yet another study, it was shown that DA forms guanosine adducts (More et al., 2012a,b) 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 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.

2.2. 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 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 and 1920 mg/kg. In 1969 Colley et al. (Colley 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 et al., 2012a,b). To begin to understand the

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