

Systematic assessment of quaternary ammonium compounds for the potential to elicit developmental and reproductive effects

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Abstract

Introduction: Quaternary ammonium compounds (QUATs) are commonly found in cleaning products, disinfectants, hand sanitizers, and personal care products. They have been used for >50 years and are considered safe when used according to directions. Recent papers report reduced fertility and neural tube defects in rodents after low-level exposures. To determine if QUATs interfere with mammalian reproduction and development, we conducted a methodical assessment of all available data.

Methods: A systematic literature search identified 789 potential articles. Review of titles and abstracts found eight relevant studies, including two dissertation chapters; to these, 10 unpublished, guideline-compliant developmental and reproductive toxicity (DART) studies of QUATs (alkyldimethylbenzylammonium chloride [ADBAC] and dialkyldimethylammonium chloride [DDAC]) were added. ToxRTool was utilized to evaluate all 18 studies for data quality.

Results: Six studies were scored as “reliable without restriction”; four studies were considered “reliable with restriction” (mainly due to small rabbit group sizes). No test article-related, adverse DART endpoints were reported in these studies. ToxRTool scored the remaining eight studies as “not reliable.” The unreliable studies failed to fully describe methods and/or endpoints, did not quantify (and in some cases, did not verify) exposures, utilized non-standard test methods, reported endpoints incorrectly, and assessed endpoints at inappropriate times.

Some (not all) unreliable studies reported adverse effects after 7.5 mg QUATs/kg/day (mice), but these results were inconsistent. The reliable studies tested exposures ≥ 100 mg/kg/day (rats) with no effects.

Conclusions: The available weight of evidence indicates no adverse DART effects after QUATs exposures at anticipated concentrations and normal use.

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KEYWORDS

ADBAC, BAC, benzalkonium chloride, DDAC, development, neural tube defects, NTDs, QAC, reproduction

1 | INTRODUCTION

Quaternary ammonium compounds (QUATs) are a diverse set of permanently charged cationic surfactants. These molecules contain derivatives of the ammonium (NH_4^+) ion in which the hydrogens have been replaced by alkyl or aryl substituents (IUPAC, 2014). QUATs possess antimicrobial and virucidal activities, are stable and resistant to varying pH levels, and remain effective on surfaces for long periods of time (Fu, McCue, & Boesenberg, 2007). Consequently, QUATs are commonly found as active ingredients in cleaning products, disinfectants, hand sanitizers, hand wipes, other personal care products, and pesticides. Although QUATs have been used for more than half a century, use of these products has increased due to the recent pandemic. When used according to directions these compounds have been reported to possess a good safety profile (Luz, DeLeo, Pechacek, & Freemantle, 2020).

The QUATs most commonly used in disinfectants are alkyldimethylbenzylammonium chloride (ADBAC; also known as benzalkonium chloride [BAC] and cocobenzyltrimethylammonium chloride [BKC]) and dialkyldimethylammonium chloride (DDAC) (Fu et al., 2007). Due in part to their low vapor pressures at room temperatures, these substances are relatively persistent on hard surfaces, which contributes to their effectiveness as surface disinfectants. Since their introduction many decades ago, the potential toxicities of both ADBAC and DDAC have been assessed multiple times by regulatory agencies in the United States and Europe to support their continued registration for use as contact antimicrobials and pesticides. Consequently, registrants have submitted dossiers of toxicity and safety data generated through the conduct of testing performed according to both good laboratory practices (GLP) and established regulatory testing guidelines. To address potential developmental and reproductive toxicity—the subject of this review—test guidelines have been published by the US Environmental Protection Agency (OPPTS 870.3700 [USEPA, 1998a]; OPPTS 870.3800 [USEPA, 1998b]) and the Organisation for Economic Cooperation and Development (OECD Test Guideline 414 [OECD, 2018a]; OECD Test Guideline 416 [OECD, 2001]; OECD Test Guideline 443 [OECD, 2018b]) to ensure that test designs are standardized and capable of detecting potential health hazards.

The most recent evaluations completed by the USEPA for ADBAC and DDAC considered multiple

guideline-compliant developmental and reproductive studies. USEPA concluded that these studies and others in the regulatory submission package showed no evidence of reproductive harm related to the use of these compounds; these conclusions were published in the Reregistration Eligibility Decision (RED) documents for ADBAC (USEPA, 2006a) and DDAC (USEPA, 2006b). More recently, the European Chemicals Agency (ECHA, 2020a, 2020b, 2020c) completed updated, detailed assessments of the potential toxicity of ADBAC and DDAC in 2020. Based on this review, ECHA came to similar conclusions as the USEPA of no reproductive or developmental harm and approved both ADBAC and DDAC for multiple uses, including the disinfection and cleaning of animal quarters. While protective gear was recommended to mitigate dermal contact during dilution and use due to the corrosive nature of the QUATs, no restrictions due to reproductive harm and no classification for reproductive toxicity were required for either ADBAC or DDAC (ECHA, 2020a, 2020b, 2020c).

As of late, a series of papers from a single investigatory group has been published asserting that exposure to QUATs is responsible for reduced fertility and the production of neural tube defects (NTDs) in mice (e.g., Hrubec et al., 2017; Melin et al., 2014). These adverse findings conflict with the safety profile supported by guideline-compliant developmental and reproductive tests of ADBAC and DDAC conducted over the past 30 years. Furthermore, the novel observations challenge the conclusions of regulatory bodies that have considered the potential risks to health due to QUATs exposure when these products are used appropriately.

Given the broad usage of QUATs, it is important to determine if the new findings have uncovered a previously unrecognized health risk that has been missed by current safety paradigms and overlooked by the community of regulatory toxicologists. To that end, a Teratology Working Group with expertise in developmental and reproductive toxicology was empaneled and tasked by the Household and Commercial Products Association (HCPA) with performing a systematic review of the available data to resolve whether QUATs interfere with reproductive and developmental processes in mammals. This review concentrates on ADBAC (BAC) and DDAC.

2 | METHODS

2.1 | Literature search

A search of the published scientific literature was conducted to identify studies that reported exposures to QUATs and assessment of developmental or reproductive outcomes. This search of Medline (<https://www.nlm.nih.gov/bsd/medline.html>) employed the ProQuest DIALOG search service and PubMed (<https://www.ncbi.nlm.nih.gov/pubmed>) using the National Library of Medicine's database website. The search was completed on October 26, 2020, employing the following search terms:

“Quaternary ammonium compounds” OR
Quats OR “Didecyl dimethyl ammonium
chloride” OR DDAC OR “Alkyl dimethyl
benzyl ammonium chloride” OR ADBAC
OR “Benzalkonium chloride”

OR

(RN(7173-51-5 OR 61789-71-7 OR 8001-54-5
OR 68424-85-1))

AND

Gestation OR Pregnancy OR Prenatal OR
Fetal OR Embryonic OR Maternal OR
“Developmental toxicity” OR Reproduction
OR “Neural tube defect”

A system algorithm was used to remove duplicate citations. The search results were culled (see flow diagram in Figure 1) to a body of potentially relevant *in vivo* studies conducted in mammalian species through a review of titles and abstracts which was conducted independently by two authors. Factors considered in determining relevant articles included: QUATs exposure; use of mammalian species; exposure prior to or during reproductive and/or developmental periods; and evaluation of products of conception/offspring. Studies in non-mammalian model systems such as zebrafish were not included. Although the search was limited to papers published in English, a Japanese paper (Momma et al., 1987) that included an English language abstract and data tables were identified; because it was of direct relevance to the assessment, the paper was professionally translated into English and included in our evaluation. All other articles were published in English. To these studies, unpublished developmental and reproductive toxicology studies of ADBAC and DDAC obtained from HCPA and the European Quats Consortium were added.

2.2 | Data quality assessment

No relevant human studies were located. The experimental animal studies relevant to developmental and/or

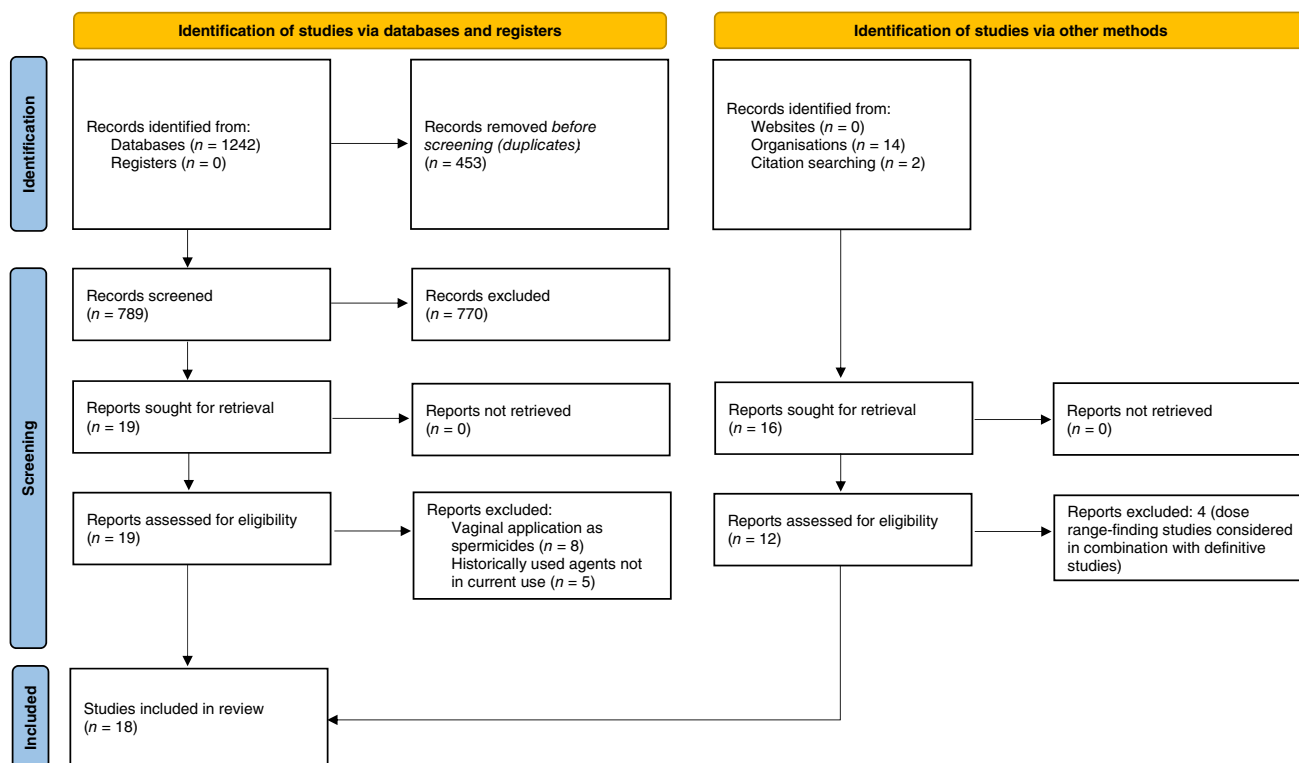


FIGURE 1 PRISMA 2020 flow diagram to describe culling of literature (Page et al., 2021)

reproductive toxicity, whether published in the scientific literature or described in unpublished safety assessment reports, were assessed for data quality using the Toxicological data Reliability Assessment Tool (ToxRTool) (Schneider et al., 2009; <https://ec.europa.eu/jrc/en/scientific-tool/toxrtool-toxicological-data-reliability-assessment-tool>). The ToxRTool is a quality assessment application that was developed by the European Centre for the Validation of Alternative Methods (ECVAM) based on the Klimisch, Andreae, and Tillmann (1997) scoring criteria. Studies were scored 1 through 3 (1, reliable without restrictions; 2, reliable with restrictions; or 3, not reliable) based on the reported study methods and level of data documentation.¹ Each study was evaluated independently by a team of two authors using the ToxRTool. Any discrepancies in scoring were discussed such that the authors came to agreement on the overall score assigned to each study.

2.3 | Studies summation and data synthesis

Each of the studies included in the assessment was briefly summarized. Information was collected regarding the exact test compound and dosages; the animal model; dosing route, vehicle and duration and/or specific window of exposure; the endpoints assessed and when and how these data were collected; the statistical analyses applied; the results reported; the conclusions of the study authors, and the strengths and weaknesses of the study. Within each quality score category, and across the overall body of papers, the studies were considered with an emphasis on the consistency of the reported results, the potential influence of any confounding factors on those results, and the overall strengths and weaknesses of the body of data. Discrepancies in results reported across studies were considered and addressed in the discussion.

3 | RESULTS

3.1 | Literature search and data quality assessment

The literature search for studies that reported exposures to QUATs and assessment of developmental or reproductive outcomes was conducted in October 2020. The initial search identified 789 published articles. Some of these papers were not primary laboratory studies, but rather, published letters to the editor, interviews, news pieces, or review articles of relevance to the assessment conducted herein. Because these secondary sources did not present primary study data, they were not included in the overall

assessment; however, references in these papers were assessed to determine whether relevant primary experimental studies were cited.

The literature search results were reviewed to identify those studies that were of potential relevance to the assessment presented herein. Some papers identified in the literature search described the investigation of ADBAC/BAC via direct vaginal application as a contraceptive agent or for the prevention of HIV transfer to the offspring during birth. Because these uses are not relevant to the types of exposures to people expected as a result of using disinfectants, the papers were excluded from the analysis. The one exception was a paper by Buttar (1985) that studied potential embryotoxicity after vaginal application in early pregnancy. Another study by Herron et al. (2019) was excluded from our assessment because, although conducted in mice, evaluations were done at 3 min to 3 hr post-dosing and involved multi-omics investigation of neonatal brain tissues; this study was thus considered a mechanistic evaluation and of secondary value to the analysis. An additional study (Palmer, Bottomley, Edwards, & Clark, 1983) used topical application to pregnant rats of three QUATs that are rarely, if ever, used commercially: dimethyldistearylammonium chloride, benzyldimethylstearyl ammonium chloride, and trimethylstearyl ammonium chloride. Because the bulk of studies available for examination involved ADBAC and DDAC, the QUATs most commonly used in commercial and household settings, the study by Palmer et al. (1983), which reported an absence of embryotoxic effects, was not further considered.

As part of the literature retrieval and review of papers, an academic dissertation (Melin, 2015) was also found freely accessible via the internet. Two chapters of the dissertation had been published and were identified as separate papers in the literature search (Melin et al., 2014, 2016). Two additional chapters from the dissertation (Melin & Hrubec, 2015; Melin, Melin, Dessify, Nguyen, & Hrubec, 2015) reported on studies that were not located in the published literature. It was determined by the Working Group that these two chapters should be included in the body of literature to be assessed in the systematic review. Based on the review of titles and abstracts, the body of published scientific studies was reduced to eight *in vivo* laboratory studies conducted in mammalian species with ADBAC or DDAC (Table 1). In addition, the Working Group was granted access to the developmental and reproductive safety studies (10 definitive studies and four dose range-finding studies) performed in support of the regulatory registration of ADBAC/BAC and DDAC. For the purposes of our review, the dose range-finding studies were considered in combination with their associated definitive studies.

Using the ToxRTool a quality score of 1 (reliable without restriction), 2 (reliable with restrictions), or 3 (not reliable) was assigned to each study based on the review

TABLE 1 Experimental animal studies of QUATs exposure and developmental and/or reproductive outcomes included in the assessment

	Paper	References	Quality score
1	DDAC—Two-generation study (reproduction and fertility effects) by dietary admixture in rats (unpublished)	Chevalier, 2008	1
2	BKC—Two-generation study (reproduction and fertility effects) by dietary admixture in rats (unpublished)	Foulon, 2008	
3	Two-generation reproduction study in Sprague–Dawley (CD [®]) rats with alkyl dimethyl benzyl ammonium chloride (ADBAC) administered in the diet (unpublished)	Neeper-Bradley, 1990	
4	Two-generation reproduction study in Sprague–Dawley (CD [®]) rats with didecyldimethylammoniumchloride administered in the diet (unpublished)	Neeper-Bradley, 1991a	
5	Developmental toxicity evaluation of didecyldimethylammoniumchloride administered by gavage to CD [®] (Sprague Dawley) rats ^a (unpublished)	Neeper-Bradley, 1991b	
6	Developmental toxicity evaluation of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered by gavage to CD [®] rats ^b (unpublished)	Neeper-Bradley, 1992	
7	BKC—Prenatal developmental toxicity study by oral route (gavage) in rabbits (unpublished)	Gaoua, 2005	2
8	DDAC—Prenatal developmental toxicity study by oral route (gavage) in rabbits (unpublished)	Chevalier, 2005	
9	Developmental toxicity evaluation of alkyl dimethyl benzyl ammonium chloride (ADBAC) administered by gavage to New Zealand white rabbits ^c (unpublished)	Neeper-Bradley & Kubena, 1992	
10	Developmental toxicity study of didecyldimethylammoniumchloride administered by gavage to New Zealand white rabbits ^d (unpublished)	Tyl, 1989	
11	Embryotoxicity of benzalkonium chloride in vaginally treated rats	Buttar, 1985	3
12	Ambient and dosed exposure to quaternary ammonium disinfectants causes neural tube defects in rodents	Hrubec et al., 2017	
13	Teratogenicity study of didecyldimethylammonium chloride in mice	Inoue & Takamuku, 1980	
14	Exposure to common quaternary ammonium disinfectants decreases fertility in mice	Melin et al., 2014	
15	Quaternary ammonium disinfectants cause subfertility in mice by targeting both male and female reproductive processes	Melin et al., 2016	
16	Disinfectant compounds ADBAC + DDAC exhibit concentration and temporally dependent reproductive toxicity in-vitro and in-vivo (unpublished)	Melin & Hrubec, 2015	
17	Direct and in-utero exposure to quaternary ammonium disinfectants alters sperm parameters and RNA expression of epigenetic enzymes in the testis of male mice (unpublished)	Melin et al., 2015	
18	Effects of benzalkonium chloride on pregnant mice	Momma et al., 1987	

Note: ToxRTool Scores: 1 = reliable without restrictions; 2 = reliable with restrictions; or 3 = not reliable.

^aThe dose range-finding study of Neeper-Bradley (1993) was considered in combination with the definitive study of Neeper-Bradley (1991b).

^bThe dose range-finding study by Chun and Fisher (1993) was considered in combination with the definitive study of Neeper-Bradley (1992).

^cThe dose range-finding study by Chun and Neeper-Bradley (1993) was considered in combination with the definitive study of Neeper-Bradley and Kubena (1992).

^dThe dose range-finding study of Tyl (1988) was considered in combination with the definitive study of Tyl (1989).

of the study's methods and level of data documentation provided. Any discrepancies in scoring were resolved through discussion and critical review of reported information. Six studies were given a quality score of 1, four were assigned a quality score of 2, and eight studies received a quality score of 3 (Table 1).

3.2 | Study evaluations

3.2.1 | ToxRTool Quality 1 studies (reliable without restriction)

Six studies were found to be reliable without restriction using the ToxRTool (quality score of 1). These included one rat developmental toxicity study each for ADBAC and DDAC as well as two rat multi-generation reproductive toxicity studies for each test agent ADBAC (called BKC in one of these studies) and DDAC (Table 2). All of these studies were conducted under GLP and according to the regulatory test guidelines available at the time. In the prenatal toxicity studies for both ADBAC and DDAC, no compound-related developmental effects were reported in any dose group, including the highest dose groups tested at which maternal toxicity was observed. In the reproductive toxicity tests, both ADBAC and DDAC caused offspring systemic effects at the highest doses tested, which were also associated with maternal effects (reduced food consumption, body weights, and/or body weight gains, accompanied by some histopathology findings in those studies that included such investigations). In the original studies, results of decreased offspring body weights and body weight gains were reported—likely consequent to the pups beginning to consume feed toward mid- to late lactation. In a more recent ADBAC study, the most sensitive offspring finding was a delay in puberty, which was considered to have occurred secondary to the reduced pup growth. In a more recent DDAC study, the most sensitive offspring finding was reduced spleen weights. No adverse effects on reproduction were reported with either ADBAC or DDAC. Concerning the more recent reproductive studies, the concentration of the test articles was relatively low; thus, it is possible that some of the effects observed in these studies could be due to exposure to an impurity rather than the test article itself.

The six Quality 1 category studies are briefly described below and summarized in Table 2.

3.2.2 | ADBAC rat embryofetal development study (Neeper-Bradley, 1992)

This guideline-compliant rat developmental toxicity study was conducted under GLP according to the USEPA

Office of Pesticide Programs Guidance document 83-3, which is generally in agreement with the current USEPA 870.3700 (1998), and OECD No. 414 (1981) test guideline. ADBAC (81% pure) was administered to pregnant female Sprague Dawley rats ($n = 25/\text{group}$) by gavage at 0, 10, 30, or 100 mg/kg/day (doses adjusted for % ADBAC in test article) on gestational days (GD) 6–15. These dose levels were selected based on results of a dose range-finding study in which treatment-related maternal deaths were observed at dose levels of ≥ 200 mg/kg/day. The most frequent treatment-related clinical sign observed in the definitive rat study was perioral wetness in 14 of 21 dams (67%) at 100 mg/kg/day. Audible respiration during and after the treatment period was also observed in two and three dams at 30 and 100 mg/kg/day, respectively; additional clinical signs were noted in at least one dam at each of these dose levels. No effect of treatment was seen on gestational body weight and body weight gain although maternal food consumption was transiently reduced in the GD 6–9 interval at both 30 and 100 mg/kg/day. No effects of treatment were observed on the mean number of corpora lutea/implantations, post-implantation loss, viable fetuses, sex ratios, or fetal body weights. There was no treatment-related increase in fetal external, visceral, or skeletal malformations or variations. Based on this study's results, the maternal NOAEL was determined to be 10 mg/kg/day, and the developmental NOAEL was considered 100 mg/kg/day, the highest dose tested.

Strengths and weaknesses

The dosages for this study were selected properly based upon results of a dose range-finding study. There was verification of test article concentration in the gavage solutions. Toxicity endpoints assessed were standard for this study design, and the effect levels were reliably determined. This study is also compliant with the OECD 414 Guideline revised and adopted June 2018 with the exceptions that anogenital distance was not assessed, maternal thyroid hormone levels were not evaluated, and the period of dosing was only through organogenesis. Nevertheless, the endpoints assessed were appropriate and sufficient detail was provided in the study report.

3.2.3 | DDAC rat embryofetal development study (Neeper-Bradley, 1991b)

This rat developmental toxicity study was conducted in accordance with GLP and the USEPA Guideline 83-3 (which is in general agreement with the current USEPA 870.3700 [1998] guideline) and OECD No. 414 (1981) test guideline. DDAC (81% pure) was given to pregnant

TABLE 2 Quality Category 1 studies—reliable without restriction

Test agent	Species	Doses	Route	N/sex	Maternal effects			Developmental effects			Citation	Comment	
					LOAEL	NOAEL	Basis	LOAEL	NOAEL	Basis			Reproductive NOAEL
Developmental toxicity study													
ADBAC	SD rat	0, 10, 30, 100 mg/kg/day (GD 6–15)	Oral gavage in water	25/F	30	10	Clinical signs	—	100 (HDT)	No effects reported	—	Neeper-Bradley, 1992	Compliant with USEPA 83-3, OECD 414 (1981)
DDAC	SD rat	0, 1, 10, 20 mg/kg/day (GD 6–15)	Oral gavage in water	25/F	10	1	Clinical signs, ↓ BW, ↓ FC	—	20 (HDT)	No effects reported	—	Neeper-Bradley, 1991b	Compliant with USEPA 83-3, OECD 414 (1981)
Reproductive toxicity study													
ADBAC	SD rat	0, 300, 1,000, 2000 ppm (high dose = 122.7 mg/kg/day) ^a	Dietary	28/M, 28/F	2000	1,000	↓ BW/ BWG, ↓ FC	2000	1,000	↓ BW/ BWG, ↓ FC	2000 (HDT)	Neeper-Bradley, 1990	Compliant with USEPA 83-4, OECD 416 (1981)
SD rat	SD rat	0, 250, 1,000, 2000 ppm (high dose = ~154–269 mg/kg/day) ^a	Dietary	25/M, 25/F	250	<250	↓ BW/ BWG, ↓ FC, intestinal findings	2000	1,000	Delayed puberty secondary to ↓ BW	2000 (HDT)	Foulon, 2008	Compliant with USEPA OPPTS 870.3800 (1998), OECD 416 (2001)
DDAC	SD rat	0, 300, 750, 1,500 ppm (high dose = 112.6 mg/kg/day) ^a	Dietary	28/M, 28/F	1,500	750	↓ BW, ↓ FC	1,500	750	↓ BW/ BWG, ↓ FC	1,500 (HDT)	Neeper-Bradley, 1991a	Compliant with USEPA 83-4, OECD 416 (1981)
SD rat	SD rat	0, 203, 608, 1,620 ppm (high dose = ~154–269 mg/kg/day) ^a	Dietary	25/M, 25/F	1,620	608	↓ BWG, ↓ FC; adrenal cortical hypertrophy	1,620	608	↓ spleen wts	1,620 (HDT)	Chevalier, 2008	Compliant with USEPA OPPTS 870.3800 (1998), OECD 416 (2001)

Abbreviations: BW, body weight; BWG, body weight gain; F, female; FC, food consumption; GD, gestational day; HDT, highest dose tested; LOAEL, lowest observed adverse effect level; M, male; NOAEL, no observed adverse effect level; SD, Sprague Dawley.

^aDose as calculated and stated in the study report.

female Sprague Dawley rats ($n = 25/\text{group}$) by gavage on GD 6–15 at dosages of 0, 1, 10, or 20 mg/kg/day (doses adjusted for % DDAC in test article). These dose levels were selected based on results of a dose range-finding study in which maternal deaths and clinical signs were noted at dosages of ≥ 25 mg/kg/day and maternal weight gain was reduced at doses of ≥ 12.5 mg/kg/day. In the definitive rat study, pregnancy was not affected in any treatment group. The most frequently observed treatment-related clinical signs were audible respiration and gasping at 20 mg/kg/day and audible respiration at 10 mg/kg/day. At necropsy, two dams at 20 mg/kg/day presented with stomach ulcerations and gas-filled intestines. Mean maternal body weight/body weight gain and food consumption were described as reduced at 20 mg/kg/day but were not statistically confirmed. No effects were observed on corpora lutea, post-implantation loss, live fetuses, sex ratios, or fetal body weights. Additionally, no treatment-related increases in fetal external, visceral, or skeletal malformations or variations were observed. Based on these results, the maternal NOAEL was determined to be 1 mg/kg/day, and the developmental NOAEL was 20 mg/kg/day, the highest dose tested.

Strengths and weaknesses

The dose levels for this guideline-compliant study were determined correctly based upon data collected from a dose range-finding study. Concentrations of the test chemical in the gavage solutions were analytically verified. Toxicity endpoints assessed were standard for this study design, and the effect levels were reliably determined. The study report is clearly written and provides adequate detail for an evaluation. However, some endpoints specified in the current test guideline, including anogenital distance and maternal thyroid hormone levels, were not included and dosing was only through the period of organogenesis.

3.2.4 | ADBAC rat multigeneration study (Neeper-Bradley, 1990)

This GLP-compliant multi-generation study of ADBAC was done in accordance with the USEPA Guidance document 83-4 (which is in general agreement with the current USEPA 870.3800 [1998] guideline) and the OECD No. 416 (1981) test guideline. ADBAC (81% pure) was administered in the diet to two generations of Sprague-Dawley rats ($n = 28/\text{sex per group}$) at concentrations of 0, 300, 1,000, or 2,000 ppm (dietary preparations adjusted for % ADBAC in test article) beginning 10 weeks before mating of the F0 generation and continuing to weaning of the F2 pups. Treatment-related

toxicity was observed in both parental generations at the highest dietary concentration (2000 ppm). The effects were limited to sporadic, statistically significant reductions in body weight, body weight gain, and/or food consumption in one or both sexes in both generations. Offspring body weights were statistically significantly reduced at postnatal day (PND) 28 at 2000 ppm, likely due to the pups beginning to consume the feed toward the end of lactation. Of relevance to this assessment, no treatment-related adverse changes were observed in either generation with respect to histopathology of the reproductive organs of parental males (testes, epididymides, seminal vesicles, prostate) or parental females (vagina, uterus, ovaries).

Additionally, there were no treatment-related effects on any of the reproductive indices, including male/female mating and fertility indices, the gestational index and duration, litter size, the number of live pups, pup sex ratio, or pup survival (Days 4, 14, and 21). Reproductive organ weights were not assessed, and sperm endpoints were not evaluated because neither was required when the study was performed. Due to the body weight/weight gain changes and reduced food consumption in both generations, the NOAEL for both parental and offspring systemic toxicity was determined to be 1,000 ppm. The reproductive NOAEL was 2,000 ppm, the highest dietary concentration tested.

Strengths and weaknesses

The range of dose levels tested was appropriate because toxicity was observed but not excessive, and the concentrations of test chemical in the diet were analytically verified. In addition, the numbers of animals per group were sufficient to provide adequate statistical power. Toxicity endpoints were standard for this study design, and the effect levels were reliably determined. The study report is clearly written and provides adequate detail for an evaluation. Although compliant with the test guidelines available at that time, specific endpoints required by today's standards (OECD No. 416 [2001]) were not assessed; these include sperm endpoints, estrous cyclicity, ovarian follicle counts, pup anogenital distance, and pubertal development (preputial separation and vaginal opening).

3.2.5 | ADBAC rat multigeneration study (Foulon, 2008)

This multi-generation study of ADBAC (referred to as BKC in the study report) was done according to GLP in compliance with the USEPA 870.3800 (1998) and OECD No. 416 (2001) test guidelines. The test material, which was reported to contain 49.9% ADBAC, was administered

in the diet to two generations of Sprague–Dawley rats ($n = 25/\text{sex}$ per group) at concentrations of 0, 500, 2,000, or 4,000 ppm beginning 10 weeks before mating of the F0 generation and continuing to weaning of the F2 pups. These dietary concentrations correspond approximately to ADBAC levels of 0, 250, 1,000, and 2,000 ppm, respectively. The dose levels were selected based on results of a previous 90-day repeat dose rat study. Decrements in body weight, body weight gain, and/or food consumption were observed for the F0 females in the 4,000 ppm group (2,000 ppm ADBAC), F1 females at $\geq 2,000$ ppm (1,000 ppm ADBAC), and for F0 and F1 males at all doses; these effects were thought to be related to diet palatability issues. Absolute and/or relative liver weights were also reduced at these doses. At necropsy, distension of the intestinal tract was observed in both generations at the top dose in the absence of histopathologic changes. In offspring, no effect of treatment was observed on pup weights at birth or throughout most of the lactation period. By the time of weaning, however, pup body weights were lower than control at 4,000 ppm (2,000 ppm ADBAC) (statistically significant for F1 generation); pup body weight gains also were reduced for F1 pups at $\geq 2,000$ ppm ($\geq 1,000$ ppm ADBAC) between PND 14–21 and for F2 pups at 4,000 ppm (2,000 ppm ADBAC). Although F1 pubertal attainment was significantly delayed at 4,000 ppm (2,000 ppm ADBAC), the difference was not statistically significant when evaluated using body weight as a covariate. The finding was thus considered to be a secondary effect of the reduced body weights at this dose level. At necropsy, statistically reduced spleen weights were observed in F1 and F2 pups at 4,000 ppm (2,000 ppm ADBAC). Importantly, there were no effects of treatment on F0 or F1 mating or fertility indices, estrous cyclicity, sperm, ovarian primordial follicles, precoital interval, duration of gestation, litter size, pup viability, or pup sex ratio. Although the number of implantations and litter size were decreased ($\sim 13\%$) for the F1 parental generation at 4,000 ppm (2,000 ppm ADBAC), the findings were within the range of the laboratory's historical control data, while those of the control were on the high end of the range. Histology of the testis, epididymis, and prostate, coagulating glands, and seminal vesicles in males and of the ovaries, uterus, and vagina in females was not affected by treatment, and examination of the ovaries did not show treatment-related alterations. Although relative weights of the seminal vesicles were increased in F1 (but not F2) males at $\geq 2,000$ ppm ($\geq 1,000$ ppm ADBAC), all other reproductive organ weights/relative weights were unaffected by treatment. Additionally, there were no treatment-related gross abnormalities in the pups. Due to the body weight/weight gain changes and reduced food consumption at

all dose levels in both generations, the NOAEL for parental systemic toxicity was considered to be < 250 ppm ADBAC. The NOAEL for offspring effects was considered to be 1,000 ppm ADBAC. The reproductive NOAEL was 2,000 ppm ADBAC, the highest dietary concentration tested.

Strengths and weaknesses

The range of doses tested was appropriate based on the observation of toxicity that was not excessive, and the concentrations of test chemical in the diet were analytically verified. The numbers of animals per group were sufficient to provide adequate statistical power. Toxicity endpoints were standard for this study design and consistent with the current guideline requirements. The study report is clearly written and provides adequate detail for an evaluation. Because of issues with palatability of the diets, it is not clear whether the effect levels for parental systemic toxicity (and possibly those for offspring effects) were reliably determined. The effect levels for reproductive toxicity appear to be reliable. However, because of the low concentration of the test article, it cannot be discounted that some of the reported findings may be due to exposure to an impurity in the dietary preparations.

3.2.6 | DDAC rat multigeneration study (Neeper-Bradley, 1991a)

This two-generation rat study was conducted under GLP according to the USEPA Guidance document 83–4 (which is in general agreement with the current USEPA 870.3800 [1998] guideline) and the OECD No. 416 (1981) test guideline. DDAC (81% pure) was administered to Sprague–Dawley rats (28/sex per group) in the diet at concentrations of 0, 300, 750, or 1,500 ppm (dietary preparations adjusted for % ADBAC in test article) beginning 10 weeks prior to mating of the F0 animals and continuing through weaning of the F2b litters (two litters produced per generation). During the pre-breeding period, F0 and F1 parental male and female body weights, body weight gains, and food consumption were significantly reduced at 1500 ppm compared to controls. There was no impairment of reproduction in F0 or F1 parental animals for either litter per generation, as determined by mating and fertility indices, gestation lengths, litter sizes, and pup sex ratios, which were unaffected by treatment. None of the litters (F1a, F1b, F2a, and F2b) showed adverse effects on survival or gross necropsy findings. However, decreases in pup body weights compared to control were observed in the 1,500 ppm group. The weight reductions, which likely occurred due to the pups beginning to consume the feed, reached statistical significance for the F1a

and F1b litters on PND 21 and 28; for the F2a pups, beginning PND 14; and for the F2b pups beginning on PND 7. Based on reductions in body weight and food consumption at 1500 ppm, the NOAEL for both parental and offspring systemic toxicity was 750 ppm. The NOAEL for reproductive toxicity was 1,500 ppm, the highest dietary concentrations tested.

Strengths and weaknesses

The dietary concentrations administered were well-selected to produce some, but not excessive, toxicity at 1,500 ppm, and the concentrations of test chemical in the diet were verified analytically. The number of animals per group was sufficient to provide adequate statistical power. Toxicity endpoints were standard for this study design, and the effect levels were reliably determined. The study was performed and reported in a careful manner. Although compliant with the guidelines in force at the time, compared to the current OECD No. 416 test guideline that has been in effect since 2001, certain endpoints that are required by today's standards were not assessed, including sperm endpoints, estrous cyclicity and ovarian follicle counts, pup anogenital distance, and pubertal development (preputial separation and vaginal opening).

3.2.7 | DDAC rat multigeneration study (Chevalier, 2008)

This multi-generation study of DDAC was conducted according to GLP in compliance with the USEPA 870.3800 (1998) and OECD No. 416 (2001) test guidelines. The test material, which was reported to contain 40.5% DDAC at study initiation, was administered in the diet to two generations of Sprague–Dawley rats ($n = 25/\text{sex}$ per group) at concentrations of 0, 500, 1,500, or 4,000 ppm beginning 10 weeks prior to mating of the F0 generation and continuing to weaning of the F2 pups. These dietary concentrations correspond approximately to DDAC levels of 0, 203, 608, 1,620 ppm, respectively. The dose levels were selected based on results of a previous 90-day repeat dose rat study. Decrements in body weight, body weight gain, and/or food consumption were observed for the F0 and F1 parental animals in the 4,000 ppm group (1,620 ppm DDAC). At this same dose level, absolute and/or relative liver weights and thyroid weights (depending on sex and generation) were reduced. Relative adrenal weights were significantly increased in F0 females (absolute weights as well) and F1 males at 4,000 ppm (1,620 ppm DDAC) and in F2 females at $\geq 1,500$ ppm (608 ppm DDAC). Absolute pituitary weights were significantly reduced in F1 females at the

high dose level. At necropsy, intestinal distention in F0 males and F1 females and increased prevalences of adrenal cortical cell hypertrophy were observed in F0 females and F1 males and females at 4,000 ppm (1,620 ppm DDAC). In offspring, no effect of treatment was observed on F1 or F2 pup weights at birth. By the time of weaning, however, pup body weights and weight gains were lower than control at 4,000 ppm (1,620 ppm DDAC) (statistically significant for F1 pups), likely as a secondary effect of the pups beginning to consume feed toward the end of lactation. Although F1 pubertal attainment was significantly delayed at 4,000 ppm (1,620 ppm DDAC), the difference was not statistically significant when evaluated using body weight as a covariate. Further, for males, the delay appeared to be mainly due to two animals with extremely low body weight. The finding was thus considered to be a secondary effect of the reduced body weights at this dose. At necropsy, statistically reduced spleen weights were observed in F1 and F2 pups at 4,000 ppm (1,620 ppm DDAC). No effects of treatment were observed on reproductive endpoints of either generation, including mating or fertility indices, estrous cyclicity, sperm, ovarian primordial follicles, precoital interval, duration of gestation, litter size, pup viability, or pup sex ratio. Histologic examination of the testis, epididymis, and prostate, coagulating glands, and seminal vesicles in males and of the ovaries, uterus, and vagina in females revealed no effect of treatment. Absolute, but not relative, ovarian (F0 and F1) and seminal vesicle (F0) weights were significantly reduced at 4,000 ppm (1,620 ppm DDAC). A reported uterine weight increase at this dose level was not confirmed statistically. The ovarian and uterine findings were considered to be a result of minor fluctuations in hormonal status, as indicated by uterine tissue variations and a lower number of corpora lutea observed at the microscopic evaluation. All other reproductive organ weights were unaffected by treatment. There were no treatment-related pup gross abnormalities. Due to the reductions in body weight/weight gains and/or food consumption, as well as some organ weight changes and adrenal cortical hypertrophy at 4,000 ppm (1,620 ppm DDAC) in both generations, the NOAEL for parental systemic toxicity was considered to be 1,500 ppm (608 ppm DDAC). The NOAEL for offspring effects was also 1,500 ppm (608 ppm DDAC). The reproductive NOAEL was 4,000 ppm (1,620 ppm DDAC), the highest dietary concentration tested.

Strengths and weaknesses

The range of doses tested was appropriate based on the observation of toxicity that was not excessive. The concentrations of test chemical in the diet were analytically verified. The numbers of animals per group were

sufficient to provide adequate statistical power. Toxicity endpoints were standard for this study design and consistent with the current guideline requirements. The study report is clearly written and provides adequate detail for an evaluation. While the effect levels for parental and offspring systemic toxicity as well as reproductive toxicity were reliably determined, because of the low concentration of the test article, it cannot be discounted that some of the reported findings may be due to exposure to an impurity in the dietary preparations.

3.3 | ToxRTool Quality 2 studies (reliable with restrictions)

Four unpublished rabbit developmental toxicity studies of ADBAC and DDAC were scored using the ToxRTool as reliable with restriction (Table 3). Although both of the original studies were conducted according to GLP and in compliance with the guidelines for testing that were in effect at the time, the numbers of animals per group were too few according to current guidance. In addition, a substantial number of does died at the highest dose tested in Tyl (1989), which further affects the statistical power of the study. The later rabbit developmental toxicity studies were conducted according to current test guidelines; however, only 16 (instead of all available) litters were evaluated at each dose level. Moreover, the concentration of the test compounds used in these studies was relatively low; thus, the possibility that some of the observed effects may be related to exposure to an impurity cannot be discounted. In the ADBAC studies, limited maternal toxicity and no offspring effects were observed. In contrast, DDAC exposure was associated with some maternal deaths, clinical signs, and reduced maternal body weights and/or body weight gains. Developmental effects (reduced fetal weights and fetal deaths/increased post-implantation losses) were observed only at the highest DDAC doses tested.

The four Quality 2 category studies are briefly described below and summarized in Table 3.

3.3.1 | ADBAC rabbit embryofetal development study (Neeper-Bradley & Kubena, 1992)

This rabbit developmental toxicity study was conducted according to GLP and in compliance with the USEPA Office of Pesticide Programs Guidance document 83-3 (generally in agreement with the current USEPA 870.3700 [1998] guidance), and OECD No. 414 (1981). ADBAC (81% pure) was administered to pregnant

New Zealand white (NZW) rabbits ($n = 16/\text{group}$) by oral gavage with dosages of 0, 1, 3, or 9 mg/kg/day (doses adjusted for % ADBAC in test article) on GD 6–18. These dose levels were selected based on the results of a dose-range finding study in which body weight losses and clinical signs were observed at 10 mg/kg/day and deaths resulted at high dose levels. In the definitive study, at 9 mg/kg/day, one doe presented with hypoactivity and labored respiration and another doe with audible respiration; no other does exhibited clinical signs. No treatment-related changes in maternal body weight or food consumption, corrected body weight, gravid uterine weight, or liver weight were observed. The numbers of corpora lutea and total, viable, and nonviable (dead, early/late resorptions) implantations were not affected by treatment. No effect of treatment was observed on fetal body weight or fetal sex ratios. There was also no increase in external, visceral, or skeletal malformations or variations in any dose group. Although the study investigators called the maternal NOAEL at 3 mg/kg/day, we consider the NOAEL to be 9 mg/kg/day based on the limited nature of these findings. Because unequivocal maternal toxicity was observed at 10 mg/kg/day in the dose-range-finder study (which had fewer than half as many animals), the 9 mg/kg/day dose level is likely to be close to the LOAEL. The developmental NOAEL was ≥ 9 mg/kg/day, the highest dose tested.

Strength and weaknesses

Because the study did not meet current requirements for an adequate sample size, we considered it reliable with restrictions. The dose levels were selected appropriately based on the results of the dose-range-finding study and ADBAC concentrations in the dosing solutions were verified analytically. Toxicity endpoints assessed were standard for this study design. The developmental effect levels were appropriately chosen; however, based on the limited findings observed and the group sample sizes, questions exist regarding the maternal effect levels. In addition, dosing was only through the period of organogenesis rather than to the end of gestation. Finally, the starting number of animals per group was low compared to current (OECD, 2018a) recommendations (20 vs. 16 per group), and the number of surviving rabbits per group at term was even smaller.

3.3.2 | ADBAC rabbit embryofetal development study (Gaoua, 2005)

This study was conducted in compliance with GLP and current regulatory test guidelines (USEPA 870.3700 [1998] and OECD 414 [2001]). ADBAC (referred to as

TABLE 3 Quality Category 2 studies—reliable with restriction

Test agent	Species	Dose	Route	N/sex	Maternal effects			Developmental effects			Citation	Comment
					LOAEL	NOAEL	Basis	LOAEL	NOAEL	Basis		
Developmental toxicity study												
ADBAC	NZW rabbit	0, 1, 3, 9 mg/kg/day (GD 6–18)	Oral gavage in water	16/F	— ^a	9 th (HDT)	Clinical signs	—	9 (HDT)	No effects reported	Neepier-Bradley & Kubena, 1992	Compliant with USEPA 83-3, OECD 414 (1981) (downgraded due to low number of pregnant rabbits per current guidance)
	NZW rabbit	0, 3, 10, 30 mg/kg/day (GD 6–28)	Oral gavage in water	22/F	10	3	Liver, lung & stomach, effects	—	30 (HDT)	No effects reported	Gaoua, 2005	Compliant with USEPA OPPTS 870.3700 (1998), OECD 414 (2001) (downgraded for evaluation of litters from only 16 pregnant rabbits/group)
DDAC	NZW rabbit	0, 1, 3, 10 mg/kg/day (GD 6–18)	Oral gavage in water	16/F	3	1	Clinical signs, ↓ BW/ BWG, early deaths	10	3	↑ fetal deaths; ↓ fetal BW	Tyl, 1989	Compliant with USEPA 83-3, OECD 414 (1981) (downgraded due to low number of pregnant rabbits per current guidance)
	NZW rabbit	0, 4, 12, 32 mg/kg/day (GD 6–28)	Oral gavage in water	22/F	12	4	Clinical signs, ↓ BWG, intestinal findings	32	12	↑ post-implantation loss, fetal deaths	Chevalier, 2005	Compliant with USEPA OPPTS 870.3700 (1998), OECD 414 (2001) (downgraded for evaluation of litters from only 16 pregnant rabbits/group)

Abbreviations: BW, body weight; BWG, body weight gain; F, female; GD, gestational day; HDT, highest dose tested; LOAEL, lowest observed adverse effect level; NOAEL, no observed adverse effect level; NZW, New Zealand White. ^aThe effect levels shown here are those based on our own assessment; the study investigators called the LOAEL to be 9 mg/kg/day and the NOAEL to be 3 mg/kg/day based on two does showing clinical signs.

BKC in the study report; concentration of 49.9%) was administered by oral gavage to mated female NZW rabbits ($n = 22/\text{group}$) at dose levels of 0, 3, 10, or 30 mg/kg/day (adjusted based on % concentration of the test article) on GD 6–28. The dose levels were selected based on the results of a dose range-finding study in which marked clinical signs of toxicity and a high number of deaths occurred at dose levels of ≥ 50 mg/kg/day. In the definitive study, transiently reduced maternal body weight gain (GD 9–12) and maternal deaths (three found dead; two euthanized early) were observed in the high dose group (30 mg/kg/day). At terminal necropsy, 5 and 8 does at 10 and 30 mg/kg/day, respectively, presented with hepatic changes, stomach mucosal deposits, dilated gall bladders, and reddish-brown foci in the lungs. There were, however, no changes noted in the placentae, in litter endpoints, fetal weights, or external, visceral, or skeletal fetal findings. Based on the presence of maternal findings in the liver at 10 mg/kg/day, the NOAEL for maternal toxicity was considered to be 3 mg/kg/day. In the absence of fetal effects, the NOAEL for developmental toxicity was 30 mg/kg/day, the highest dose tested.

Strength and weaknesses

Although the study meets current guideline requirements, we considered it reliable with restrictions because only the first 16 litters per dose group were evaluated instead of all litters. The remaining litters in excess of 16 were discarded without evaluation; thus, a total of 15 litters (5 control; 10 treated) were not examined for visceral or skeletal findings. The dose levels were selected appropriately based on the results of the dose-range-finding study (although the extent of maternal toxicity at the high dose level was greater than anticipated). Concentrations of ADBAC in the dosing solutions were verified analytically. The toxicity endpoints assessed were standard for this study design. The study report is well documented and provides adequate detail for an evaluation. In addition, effect levels were reliably determined. However, because of the low concentration of the test article, it cannot be discounted that some of the reported findings may be due to exposure to an impurity in the dosing solutions.

3.3.3 | DDAC rabbit embryofetal development study (Tyl, 1989)

This rabbit developmental toxicity study of DDAC was performed according to USEPA Office of Pesticide Programs Guideline 83-3 (generally in compliance with the current EPA 870.3700 (1998) guideline) and OECD 414 (1981) test guideline. DDAC (81% pure) was

administered to mated female NZW rabbits ($n = 16/\text{group}$) by gavage at 0, 1, 3, or 10 mg/kg/day on GD 6–18. These dose levels were selected based on results of a dose-range finding study, the results of which were not described. Clinical signs of toxicity (increased audible respiration and hypoactivity) and statistically significantly reduced maternal body weight gains were observed at ≥ 3 mg/kg/day. At 10 mg/kg/day, four pregnant does died before GD 13; these animals exhibited sloughing of the lining of the esophagus; sloughing, hemorrhage, and distention of the glandular portion of the stomach; and sloughing and hemorrhage of the non-glandular part of the stomach. Two and one does delivered early at 1 and 10 mg/kg/day, respectively; no does aborted. At necropsy, there were no effects of treatment on most litter endpoints, including the numbers of corpora lutea, implantations, early and late resorptions, live fetuses, and fetal sex ratio. However, at 10 mg/kg/day, the percent dead fetuses was statistically increased (0.7% vs. 0.1% in control), and fetal body weights reported as reduced (36.5 vs. 40.5 g), although not statistically confirmed. No treatment-related increases in fetal external, visceral, or skeletal malformations or variations were observed. Based on these results, the NOAEL for maternal toxicity was 1 mg/kg/day; the NOAEL for developmental toxicity was 3 mg/kg/day.

Strength and weaknesses

Because the study did not meet current requirements for an adequate sample size (20 rabbits versus 16 per group), we considered it reliable with restrictions. Based on the loss of 25% of the does at 10 mg/kg/day, the top dose level was too high. However, the range of dose levels assessed was adequate to identify maternal and developmental effect levels. Toxicity endpoints evaluated were standard for this study design, and DDAC concentrations in the dosing solutions were verified analytically. Dosing was only through the period of organogenesis rather than to the end of gestation. The low number of animals per group was compounded by the maternal losses observed at 10 mg/kg/day.

3.3.4 | DDAC rabbit embryofetal development study (Chevalier, 2005)

This rabbit developmental toxicity study was conducted in compliance with GLP and current regulatory test guidelines (USEPA 870.3700 [1998] and OECD 414 [2001]). In it, DDAC (41% pure) was administered by oral gavage to mated female NZW rabbits ($n = 22/\text{group}$) at 0, 4, 12, and 32 mg/kg/day (dosages adjusted based on % purity in the test article) on GD 6–28. These dose levels

were selected based on the results of a dose range-finding study in which one maternal death occurred, and body weights and food consumption were reduced at 32 mg/kg/day. In the definitive study, two does died at 32 mg/kg/day: one on GD 22 (found with an aborted fetus) and one on GD 25. Another female at this dose level was sacrificed on GD 22 due to poor health. Two additional deaths occurred at the low dose (4 mg/kg/day). One female in the 12 mg/kg/day group was reported to have aborted (or more likely, delivered early) on GD 28 after experiencing a significant reduction in food consumption and body weight losses; another doe aborted in the control group. Clinical signs of toxicity (colored urine, soft feces, and soiled urogenital region) were seen at 32 mg/kg/day; a single doe at 12 mg/kg/day also exhibited colored urine. Maternal body weights and body weight gains were significantly reduced at dose levels ≥ 12 mg/kg/day. At necropsy, intestinal findings (brownish contents, dilation, and/or distension) were observed in all treated groups; additional findings were noted in the stomach and gall bladder at 32 mg/kg/day. There were no effects of treatment on placentae, mean number of corpora lutea, fetal body weights and sex ratios, or on the incidences of fetal external, visceral, and skeletal malformations and variations. However, statistically significant increases in mean post-implantation loss and the number of dead fetuses (resulting in a significantly reduced number of live fetuses and mean litter size) were observed at 32 mg/kg/day. Based on these findings, the NOAEL for maternal toxicity was 4 mg/kg/day and the NOAEL for developmental toxicity was 12 mg/kg/day.

Strength and weaknesses

Although the study meets current guideline requirements, we considered it reliable with restrictions due to evaluation of only the first 16 litters per dose group instead of all litters. The remaining litters in excess of 16 were discarded without evaluation; thus, a total of 15 litters (4 control, 11 treated) were not examined for visceral or skeletal findings.

The dose levels were selected appropriately based on the results of the dose-range-finding study, and concentrations of DDAC in the dosing solutions were verified analytically. The toxicity endpoints assessed were standard for this study design. The study report is well documented and provides adequate detail for an evaluation. The effect levels were reliably determined. However, because of the low concentration of the test article, it cannot be discounted that some of the reported findings may be due to exposure to an impurity in the dosing solutions.

3.4 | ToxRTool Quality 3 studies (not reliable)

Among the studies that received a quality score of 3 (Table 4), most were performed in mice using variable experimental designs and methods and involved assessment of a wide range of endpoints. None of the studies were conducted according to GLP, none followed accepted EPA or OECD guidelines for reproductive or developmental toxicity testing, and information important to study interpretation frequently was missing. Further, the endpoints evaluated in these studies often were not those typically assessed or were evaluated using non-standard study designs or test methods.

The eight Quality 3 category studies are summarized in Table 4 and are described briefly below.

3.4.1 | Single-day treatment of pregnant mice (Inoue & Takamuku, 1980)

Pregnant JCL-ICR mice ($n = 7-11$ /group) received a subcutaneous dose of DDAC (97.5% pure) of 50 or 200 mg/kg on a single day in gestation (GD 7, 9, 11, 13, or 15). The dose levels were based on the dam's body weight at conception and not adjusted as the animals gained weight throughout pregnancy. Thirty control animals injected on different days of the experiment were combined into a single control group (results for the different days were not shown). On GD 18, the dams were sacrificed, and all fetuses examined externally; half of each litter was then allocated to visceral or skeletal examination using standard methods. Although occasional findings were statistically different from control, these did not show a pattern, and the authors concluded that DDAC exposure on a single day had no effect on litter endpoints (implantations, litter size, fetal weight, sex ratio) or the percentage of fetuses with external, visceral or skeletal malformations. However, a general increase in the incidence of fetuses with some variations of the cervical vertebrae and sternbrae was noted, however, with "a strong litter bias" (meaning that all the sternbral findings were found in multiple fetuses from only a few litters).

Strengths and weaknesses

Subcutaneous dosing does not represent a relevant human exposure route for the current risk assessment, and the dose volume (10 mL/kg) may have been painful for the animals. If compared to the intravenous LD₅₀ for DDAC in mice of 27 mg/kg (Henderson, 1992), the doses were extremely high. Dosages also were not verified

TABLE 4 Quality Category 3 studies—not reliable

Test agent	Species	Doses	Route	N/sex	Reported findings	Citation	Comment
DDAC	JCL-ICR mouse	0, 50, 200 mg/kg (GD 7, 9, 11, 13 or 15)	Subcutaneous injection	7–11/F	No effect on litter endpoints or fetal findings; ↑ skeletal variations with a “litter bias”	Inoue & Takamuku, 1980	Irrelevant route for current human risk assessment; no analytical verification of dosing solutions; dose levels extremely high; numbers of litters/group low; improper statistical analysis
ADBAC	Wistar rat	0, 25, 50, 100, 200 mg/kg (GD 1)	Intravaginal instillation	10/F	Fetal losses & ↑ skeletal variations at ≥100 mg/kg; ↓ implantations & fetal BW at 200 mg/kg	Buttar, 1985	Irrelevant route for current human risk assessment; no information on test material purity; no analytical verification of dosing solutions; numbers of litters/group low; improper statistical analysis; sternebral finding not detailed
ADBAC	JCL-ICR mouse	0, 3, 10, 30 mg/kg/day (GD 0–6)	Oral gavage in water	9–12/F	No maternal or fetal effects on GD 13; possibly reduced pregnancy rate	Momma et al., 1987	Unclear if doses adjusted for 50% purity of test article; no analytical verification of dosing solutions; methods of fetal examination poorly described; fetal examinations on GD 13 and low animal numbers in experiments 1 & 2; inadequate dose levels in experiments 2 & 3
		0, 0.001, 0.05, 0.1 mg/kg/day (GD 0–6)	Oral gavage in water	5–7/F	No maternal or fetal effects on GD 13		
		0.001, 0.05 mg/kg/day (GD 0–18)	Oral gavage in water	20/F	No maternal or fetal effects on GD 18		
ADBAC and DDAC disinfectant	CD-1 mouse	0, 60, 120 mg/kg/day (continuous)	Dietary	10/M + F	Clinical toxicity resulting in maternal deaths at ≥60 mg/kg/day; ↑ time to first litter & ↓ total pregnancies over breeding period at 120 mg/kg/day; other anecdotal findings reported	Melin et al., 2014	Anecdotal findings reported; mouse colony maintained using brother–sister pairings; use of disinfectant formulation; dietary concentrations not reported; no analytical verification of diets; non-standard methods for assessment of reproductive performance; questionable data presented

TABLE 4 (Continued)

Test agent	Species	Doses	Route	N/sex	Reported findings	Citation	Comment
ADBAC and DDAC	TM4 mouse	0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1% (72 hours)	In culture medium	—	Cytotoxicity at $\geq 0.005\%$; reduced barrier resistance at $\geq 0.01\%$	Melin & Hrubec, 2015	Adjustment for purity of dosing components not reported; no analytical verification of dosing solutions; low number of animals/group per time point; limited methods description for IVF study; im proper statistical analysis of IVF data
	Sertoli cells	0, 30 mg/kg/day (7 days)	Oral gavage in saline	10/M	No effect on sperm concentration, motility or IVF fertilization rate with “unrested” sperm; \downarrow IVF fertilization rate with “rested” sperm		
ADBAC and DDAC	CD-1 mouse	—	Ambient exposure	8–11 breeding pairs across 3 generations	\uparrow mating & pregnancy indices with relocation to QUATs-free facility	Melin et al., 2015	No controls for ambient exposure study; treatment with disinfectant formulations; drinking water concentrations not reported; no analytical verification of drinking water solutions; missing information on standard toxicity endpoints important for interpretation; possible technical difficulties with sperm analysis; mRNA results possibly due to chance; low numbers of animals per group (especially for mRNA analyses); possibly pooled samples for RNA analyses
	disinfectant	0, 120 mg/kg/day (F0 only; 8+ weeks pre-mating)	Drinking water	8–10/M	F0: \downarrow sperm concentration, motility, altered mRNA expression for 3 genes F1: \uparrow sperm concentration, \downarrow motility, altered mRNA expression for 1 gene (different than genes affected in F0 mice) F2: No effects		
ADBAC and DDAC	CD-1 mouse	0, 120 mg/kg/day (2 or 8 weeks)	Dietary or drinking water	6–10/F	2 weeks: \downarrow implantations; \downarrow estrous cycles 8 weeks: \downarrow corpora lutea & implantations; \downarrow GD 10 embryos; no effect on % resorptions	Melin et al., 2016	Controls housed separately from treated animals; treatment with disinfectant formulation; dietary and drinking water concentrations not reported; no analytical verification of dosing formulations; exposure method (dietary or drinking water) for different experiments not reported; gavage vehicle & volume not
	disinfectant	0, 7.5 mg/kg/day (8 days) OR ambient exposure (7 weeks)	Oral gavage (vehicle unknown) OR ambient exposure	4–7/M	\downarrow sperm counts/ concentration & motility; no effect on sperm viability		

(Continues)

TABLE 4 (Continued)

Test agent	Species	Doses	Route	N/sex	Reported findings	Citation	Comment
ADBAC and DDAC disinfectant	CD-1 mouse	—	Ambient exposure	9–13/F	↓ open neural tubes in GD 10 mouse embryos with relocation to QUATs-free facility	Hrubic et al., 2017	Incorrect designation of early open neural tubes as being neural tube defects; treatment with disinfectant formulation; dietary & gavage dosing solutions concentrations not reported; no analytical verification of dosing formulations; gavage vehicle & volume not identified; missing information on some methods and standard toxicity endpoints important for interpretation; males counted 3x times in the statistical analysis due to 3:1 mating ratio; experimental procedures not conducted in a random fashion across groups
	SD rat	—	Ambient exposure	9–20/F	↓ open neural tubes in GD 11 rat embryos with relocation to QUATs-free facility		
	CD-1 mouse	0, 60, 120 mg/kg/day (8+ weeks)	Dietary exposure	10–12/F	GD 10: ↑ open neural tubes in mouse embryos GD 18: ↓ fetal BW at ≥60 mg/kg/day, ↑ late resorptions at 120 mg/kg/day; no gross malformations		
	CD-1 mouse	—	Ambient exposure	12–15/F	↓ open neural tubes in GD 10 embryos with relocation from one QUATs-free facility to another QUATs-free facility		
	CD-1 mouse	0, 120 mg/kg/day (8+ weeks)	Dietary exposure	5/M, 15/F	↓ litter percentage of open neural tubes in GD 10 embryos if both parents were exposed to QUATs versus if only one parent was exposed		
	CD-1 mouse	M: 0, 30 mg/kg/day (every other day for 10 days) F: 0, 15 mg/kg/day (GD 8)	Oral gavage (vehicle unknown)	10/M, 15/F	↑ litter percentage of open neural tubes in GD 9.5 embryos if both parents were exposed to QUATs versus if only one parent		

TABLE 4 (Continued)

Test agent	Species	Doses	Route	N/sex	Reported findings	Citation	Comment
	CD-1 mouse	M: 0, 7.5 mg/kg/day (every other day for 10 days) F: 0, 7.5 mg/kg/day (GD 8)	Ambient exposure with or without oral gavage (vehicle unknown)	5/M, 15/F	was exposed versus control ↑ litter percentage of open neural tubes in GD 9.5 embryos if parents ambiently exposed and gavage dosed with QUATs versus if parents only ambiently exposed versus control		

Abbreviations: BW, body weight; F, female; GD, gestational day; IVF, in vitro fertilization; M, male; SD, Sprague Dawley.

analytically. The number of animals per dose group was generally inadequate. The combining of control animals regardless of day of treatment may have decreased the validity of the comparisons between treated and control animals. Statistical procedures were not described, but the results tables suggest per-fetus analysis (rather than based on the litter), which inflates the degrees of freedom in statistical testing and may lead to spurious results. The fact that the authors reported a “strong litter bias” in skeletal variations suggests that the lack of per litter analysis may have influenced the outcome. Few developmental data were shown.

3.4.2 | Vaginal benzalkonium chloride in pregnant rats (Buttar, 1985)

This study evaluated the potential effects of benzalkonium chloride (ADBAC) when used as a spermicidal agent. Wistar rats ($n = 10/\text{group}$) were injected intravaginally with ADBAC (purity not reported) at 0, 25, 50, 100, or 200 mg/kg on GD 1; the dosing volume (1 mL/kg) was administered under ether anesthesia. Clips were applied to the labia for up to 24 hours thereafter to reduce potential loss of the applied material from the vagina. On GD 21, the dams were necropsied, and the fetuses evaluated for external, visceral (1/3 of each litter), and skeletal anomalies (2/3 of each litter). Reduced maternal body weight gains and watery discharge from the vagina were observed at 100 and 200 mg/kg; corrected body weights (without uterine contents) were similar to control at these dose levels. Increased embryofetal death and resorptions were observed at ≥ 100 mg/kg, live litter size was reduced at ≥ 50 mg/kg, and the mean number of implantations and fetal weights were significantly lower at 200 mg/kg. There was no increase in malformations at any dose level. Variations of the sternebrae were reported to be increased at 100 and 200 mg/kg/day.

Strengths and weaknesses

This study provided a straightforward assessment of vaginal exposure to benzalkonium chloride (ADBAC) during pregnancy such as might occur with use of a spermicide in an undiagnosed human pregnancy; however, the route is not relevant for addressing the potential adverse effects associated with use of ADBAC in household disinfection products. There was a wide range of dose levels, and standard methods were used to assess fetuses. However, doses were not verified analytically; the purity of the test article was not specified; a low number of animals was included in each dose group; and insufficient detail was provided on the

sternebral findings. Some of the results were analyzed on a per fetus basis rather than on a litter basis.

3.4.3 | Benzalkonium chloride in pregnant mice (Momma et al., 1987)

Pregnant JCL-ICR mice ($n = 9\text{--}12/\text{group}$) were treated by oral gavage with a commercial benzalkonium chloride (ADBAC) product (50% concentration) at 0, 3, 10, or 30 mg/kg/day on GD 0–6. The dams were evaluated on GD 13 for litter outcome and fetal malformations. No maternal toxicity was observed; however, the pregnancy rate was reduced from 91.7% in the control group to 70% at 10 mg/kg/day and 60% at 30 mg/kg/day. No adverse effect of treatment was seen on mean number of implantations, litter size, % resorptions, fetal weights, or the prevalence of malformations. In another experiment, pregnant dams ($n = 5\text{--}7/\text{group}$) were treated with lower dose levels of 0, 1, 50, or 100 $\mu\text{g}/\text{kg}/\text{day}$ (0, 0.001, 0.05, or 0.1 mg/kg/day) on GD 0–6 and again examined on GD 13. No maternal or fetal toxicity was seen, but the rate of pregnancy in the control group was only 71.4% (similar to the rates observed with treatment in the first experiment) and 80–100% in the treated groups. In the third experiment, pregnant mice ($n = 20/\text{group}$) were treated with 1 or 50 $\mu\text{g}/\text{kg}/\text{day}$ (0.001 or 0.05 mg/kg/day) throughout pregnancy (GD 0–18) and examined on GD 18 for litter outcome and fetal malformations. There were no effects of treatment on dam weight, food consumption, or the rate of pregnancy. There were no effects of treatment on numbers of implantations, live fetuses, dead or resorbed fetuses, fetal body weight, or fetal malformations.

Strengths and weaknesses

The design of the study was reasonable in an evaluation of possible preimplantation and total pregnancy exposure to ADBAC; however, the methods were poorly described, particularly those related to fetal examinations. For example, the only malformation specifically mentioned was open eye, raising the possibility that fetuses were only evaluated for external appearance. It is unclear if the dose levels were adjusted to account for the 50% concentration of the test article and analytical verification of dosing solution concentrations was not reported. The general lack of effects seen in the first experiment indicates the inadequacy of the dose levels selected for evaluation in the second and third experiments, which were three orders of magnitude lower. Because fetal development continues to the end of gestation, examination on GD 13 is inappropriate. Due to the extremely small size of the fetuses at this stage ($\sim 0.15\text{--}$

0.18 g), it would be difficult to assess fetal structure adequately.

3.4.4 | ADBAC + DDAC in breeding mice (Melin et al., 2014)

The impetus for this study was an observation of changes in breeding performance in two different mouse colonies that reportedly coincided with use of disinfectants containing ADBAC and DDAC. In the first case in the colony comprised of C57BL/6J mice, the change occurred around the time the laboratory relocated from one university to another. Anecdotal data on pregnancy rates and total pups born/weaned at different times were presented in the paper and increased dystocia rates were noted; the levels of DDAC in cage washings after “extensive cleaning and fogging” of animal rooms (due to a pinworm outbreak) were also shown. However, no data from controlled breeding experiments were provided.

In the second circumstance at a different university, the breeding declines were noted in a colony of CD-1 mice at around the time HWS-256 disinfectant (containing 10.14% DDAC, 6.76% ADBAC, and 83.10% inert ingredients) began to be used in a “liberal” fashion, and improvements in performance were reported after disinfectant use was discontinued. To investigate further, CD-1 mice ($n = 10/\text{group}$) were exposed over a continuous breeding period of 6 months to HWS-256 added to a gel diet at levels estimated (based on an assumed daily food consumption rate of 28% of body weight) to produce ADBAC + DDAC doses of 0, 60, and 120 mg/kg/day. Although actual dietary concentrations were not reported, assuming an average body weight of 30 g, we calculated that the dose levels were equivalent to dietary concentrations of ADBAC+DDAC 0, 214, and 429 ppm.² These treatments resulted in 1 and 4 mice euthanized early due to clinical signs of toxicity (inappetence, reduced activity, cyanosis, rough haircoat; at 120 mg/kg/day, dystocia and vaginal hemorrhage were also reported). In addition, time to first litter was increased and the number of total pregnancies over the breeding period was reduced at the high dose.

Strengths and weaknesses

Largely anecdotal observations are reported. The colony of C57BL/6J mice was reported to be maintained through brother–sister matings, which may have affected their breeding performance. These mice were kept under different conditions at the two university sites (e.g., fed different diets and kept in open wire-topped cages versus enclosed microisolator cages). Estimates of DDAC on the walls of the cages were made by HPLC analysis of the

methanol rinses from several cages previously inhabited by CD-1 mice; details regarding the procedure were limited. The relationship between DDAC on the surface of the caging and exposure of the animals was inferred but not demonstrated. With regard to the continuous breeding trial, a formulation rather than the active ingredients was used, and actual concentrations of the test article in the diet were neither reported, nor verified analytically. Data to support the claim of no effect of the treated diets on body weights were absent and female body weights apparently were not collected. Although the protocol used to assess reproduction appears similar to the Reproductive Assessment by Continuous Breeding (RACB) protocol developed by the National Toxicology Program (Lamb IV, 1985), the methods description is not sufficient to evaluate adherence to this or any other standard protocol. Moreover, the RACB design does not allow discernment of effects on individual matings. The cohabitation schedule and time between weaning and the next mating (which influence mating preparedness) were not disclosed. Although the mice were cohabited for 180 days, only data related to litters produced over the first 100 days were analyzed and presented. Additionally, at least some of the data presented are not plausible. For example, the control group was reported to have a minimum of seven pregnancies per dam in the first 100 days of exposure, which translates to a gestation period of no more than 14.3 days (if the litters are not weaned and the mice immediately mated again); however, the typical length of a mouse pregnancy is 20 days (DeSesso, 2012).

3.4.5 | ADBAC + DDAC on male reproduction (Melin & Hrubec, 2015)

Chapter 4 of a doctoral dissertation (Melin, 2015)³ is an unpublished manuscript that examined the potential effects of ADBAC and DDAC on the male reproductive system using both in vitro and in vivo experiments. For the in vitro study, a stock solution of ADBAC+DDAC was prepared in culture medium to replicate the alkyl chain length ratios and concentrations of these compounds as found in a commercial disinfectant. The solution was added to culture medium in order to expose TM4 mouse Sertoli cells at final concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, and 1% for 72 hr. Cytotoxicity was observed at concentrations $\geq 0.005\%$. Using flow cytometry, disruption of the G1 cell cycle phase at 0.1 and 1% was identified. Using a two-compartment model to evaluate transepithelial resistance as a measurement of the integrity of the tight junctions that characterize the blood-testis barrier, reduced transepithelial resistance

was observed at $\geq 0.01\%$, concentrations already reported to cause cytotoxicity.

In an in vivo study, male CD-1 mice ($n = 10/\text{group}$) were treated for 7 days by gavage with ADBAC + DDAC dose levels of 0 or 30 mg/kg/day in saline; the dosing volume was not given. After exposure, five males/group were immediately killed for standard evaluation of epididymal sperm concentration and motility, which were unaffected by treatment. Although not specifically stated in the study methods, it appears that the other five males/group were sacrificed after a 10-day rest period. Sperm from both “unrested” and “rested” mice were then used for in vitro fertilization. There was no effect of treatment on in vitro fertilization rates using sperm from “unrested” males, but the fertilization rate for sperm from “rested” males was lower than that of controls.

Strengths and weaknesses

The concentration of the ADBAC components was not indicated and there was no mention of adjusting the components in the dosing solutions to compensate for their percent concentration. The working solutions were not assayed for stability or concentration. The effects on epithelial barrier function were only observed at concentrations shown to be cytotoxic. The methods for capacitation of the sperm and super-ovulation of oocytes were not described. The in vitro fertilization results were not properly analyzed because the male, and not the oocytes, should have been the experimental unit and the basis of the statistical testing. Additionally, the putative decrease in fertilization capacity after 10 days without treatment is not consistent with the lack of an effect on sperm concentration and motility. The number of “rested” and “unrested” animals assessed ($n = 5/\text{group}$) was low.

3.4.6 | ADBAC + DDAC exposure over two generations of mice (Melin et al., 2015)

This unpublished manuscript is Chapter 5 of the doctoral dissertation (Melin, 2015). CD-1 mice ($n = 8$ breeding pairs) raised in an animal facility routinely cleaned using a disinfectant containing 6.76% ADBAC and 10.1% DDAC were moved at weaning to a QUATs-free facility, then mated 8 weeks thereafter to produce the F1 generation. The F1 mice were similarly cohabited at 6–8 weeks of age ($n = 11$ breeding pairs) to produce the F2 mice ($n = 10$ breeding pairs), which were also bred at 6–8 weeks of age. For the F0 generation, the mating index and pregnancy rate were calculated to be 75 and 78%, respectively; both indices were 92% for the F1 generation and 100% for the F2 generation. In a second experiment, mice were exposed to the disinfectant in drinking water at a target dose of 120 mg/kg/

day from 8 weeks pre-mating, through mating and gestation. The concentration in water was not reported, but assuming water consumption was 10% of body weight (as reported in the paper) and a body weight of 30 g, this dose level equates to an ADBAC+DDAC drinking water concentration of 1,200 ppm.⁴ On GD 19, the dams were moved to a QUATs-free facility and, after birth, the F1 pups were fostered to control dams. At 8–10 weeks of age, the F1 males ($n = 8–10$) were bred to control females to produce the F2 generation. Epididymal sperm concentration was lower in F0 treated males than F0 controls, but higher in F1 treated males compared to F1 controls; in F2 males, there was no difference with treatment. Sperm motility was also lower than control in F0 and F1 treated males, but not in F2 treated males. In a related investigation, the mRNA expression of two genes involved in chromatin remodeling was increased and expression was decreased for one gene in the testis tissues of F0 males; a different chromatin remodeling gene was down-regulated in the F1 males. There were no expression changes in F2 males.

Strengths and weaknesses

For the ambient exposure study, the length of the cohabitation period was not reported, and no control group was included in the experiment. In the drinking water study, a formulation was used rather than the active ingredients. The actual concentration of the test article in the drinking water solution was neither reported, nor verified analytically. Additionally, no information regarding clinical condition, body weights, or food consumption was provided; however, in the dietary exposure study discussed above, 4 of 10 pregnant mice at 120 mg/kg/day had to be euthanized early due to extreme clinical signs of toxicity. Thus, general systemic toxicity may be a confounding factor in the reported findings. Further, the increase in sperm concentration in the F1 generation is not explained and suggests possible technical problems with the analysis. mRNA analyses were done using only three males per group and whether the samples were analyzed on an individual animal basis or the samples were pooled per group is not reported. The reported findings could be unrelated to treatment as there were no consistent findings across generations, and evaluation of the data using a p -value of .05 with this number of endpoints would be expected to result in at least four comparisons being identified as statistically significant by chance alone.

3.4.7 | ADBAC + DDAC on mouse reproduction (Melin et al., 2016)

In this study, CD-1 mice were raised for two generations in a QUATs-free facility. Offspring of the second generation

included untreated controls (which remained in their original location) and treated mice (which were transferred to another facility). Treated mice were administered a disinfectant containing 10.1% DDAC and 6.76% ADBAC either in a gel diet or in drinking water at a dose level of 120 mg/kg/day for 2 or 8 weeks and throughout breeding. The diet and drinking water concentrations were not reported but are assumed to be similar to those estimated for the other studies discussed herein that used this same dose and routes of exposure. Using intravenous administration of pontamine blue for visualization of ovarian and uterine findings on GD 6, the mean number of corpora lutea with 2 weeks of exposure was not different from control ($n = 6$ /group). Still, it was significantly reduced with 8 weeks of treatment ($n = 9–10$ /group), and the mean number of implantations was reported to be non-significantly reduced at both intervals. The number of estrous cycles over 20 days was significantly reduced by ~50% with 2 weeks of disinfectant exposure ($n = 8$ /group). For dams fed the disinfectant in the diet for 8 weeks and sacrificed on GD 10, the mean number of embryos present was ~20% fewer than in controls, but treatment had no effect on the percent resorptions. In another experiment, male mice either ambiently exposed to QUATs (7 weeks) or gavage dosed with 7.5 mg/kg/day of the disinfectant solution (8 days) both exhibited a 20–24% decrease in epididymal sperm counts and a 10–16% reduction in motility compared to controls. Sperm viability, however, was unaffected. Only four mice were included in both the control and ambiently exposed groups and seven in the gavage-dosed group for these analyses.

Strengths and weaknesses

The fact that controls were housed in a separate facility, and thus exposed to different environmental conditions than the treated animals, confounds the study's interpretation. Because moving animals to a different room or building can be stressful, a more appropriate design would have included moving of the control animals to a different facility. Animals were dosed with a disinfectant formulation rather than the active ingredients ADBAC and DDAC and the dosing formulations were not analyzed to verify their concentration or stability. Exposure was through either diet or drinking water; however, based on the study report text, it is often difficult to determine from what exposed groups (diet or drinking water) the different data were derived. In the evaluation of post-implantation losses, there is no indication of whether or not the males were also treated. The reduction in numbers of corpora lutea without a concomitant reduction in implantation sites is not a coherent finding, although the small number of animals may have limited the ability to show a difference in implantation sites. While the

authors indicated that viable GD 10 embryos were staged by somite count, branchial arches, and extent of the heart, limbs, and lens pit, data on these endpoints were not shown. Food consumption was said to be unaffected by exposure, but no data were shown. Mating procedures were not described, and data on body weights and clinical conditions were not reported. This last omission is critical, because in the earlier study, this dosage was associated with substantial toxicity necessitating early termination of 40% of the dosed animals. In the experiments with male mice, the dosing vehicle and volume for the gavage studies were not reported, only a single control group was included (raising the question of whether the control group was gavaged with vehicle), the number of animals per group was low, and the ages of mice at the time of assessment (to ensure that all were at the same stage of development) were not reported.

3.4.8 | Open neural tubes in early embryos in rats and mice (Hrubec et al., 2017)

An anecdotal observation was reported of open neural tubes in GD 10 CD-1 mouse embryos (mean litter percent of ~15%; $n = 13$ litters) and GD 11 SD rat embryos (mean litter percent of ~5%; $n = 20$ litters) from a laboratory animal room (Rm. A1) in which a QUATs disinfectant was regularly used. This finding was incorrectly referred to as a NTD. After these animals were moved to a separate QUATs-free room (Rm. A2) and bred for two generations, the mean litter percentages decreased to ~6% for GD 10 mice and ~1% for GD 11 rats ($n = 9$ litters each). To further investigate the role of QUATs in this phenomenon, mice from room A2 were fed gel diets meant to deliver doses of a QUATs disinfectant (containing 6.76% ADBAC and 10.1% DDAC) of 0, 60, or 120 mg/kg/day for 8 weeks, then bred (one male to three females; eight breeding units per dose group). The females were maintained on their respective diets until necropsy on either GD 10 or GD 18 ($n = 10$ – 12 /group per time point). The mean litter percent of open neural tubes in GD 10 embryos were ~6, ~8, and ~20% in the 0, 60, and 120 mg/kg/day dose groups, respectively. When evaluated on GD 18 instead, both fetal and placental weights were significantly reduced with treatment and late resorptions were significantly increased at 120 mg/kg/day; however, no gross malformations were observed in treated litters. Methanol washes of the cages yielded ADBAC residues of 10, 15, and 55 ppb for exposures to dietary dose levels of 0, 60, and 120 mg/kg/day, respectively.

In another experiment done because of concerns that Rm. A2 had residual QUATs present, mice from Rm. A2 were moved to a new QUATs-free room (Rm. B) and

compared to those remaining in Rm. A2. The litter prevalence of open neural tubes in GD 10 mouse embryos from Rm. A2 was reported to be ~15% (compared to the previously reported ~6% incidence) while that for embryos from Rm. B dropped over successive generations from ~10 to ~5% to 0% ($n = 12$ – 15 litters per generation). To assess whether the observed findings were the result of either maternal or paternal exposure, mice were dosed with 0 or 120 mg/kg/day of the disinfectant in gel diet for 8 weeks, then bred (one male to three females) to either treated or untreated mice of the opposite sex; another group involved treated males bred to treated females that were continued on treated diet in gestation. In this instance, the litter percentage of open neural tubes in GD 10 embryos was ~5% if either the male or female was singly dosed and ~2–2.5% if both parents were dosed (including when the females continued to be dosed into gestation). These percentages are well below the ~20% incidence reported in the earlier dietary dosing experiment. The experiment was repeated using gavage exposure of the males to either 0 or 30 mg/kg/day of the disinfectant prior to mating (every other day for 10 days); the females were treated with a single gavage dose of 0 or 15 mg/kg/day of the disinfectant on GD 8. This time, the GD 9.5 embryos from untreated mice had no open neural tubes, matings in which only one parent was dosed showed litter percentages of 1–1.5%, and those from matings in which both parents were dosed had a litter percentage of ~3%.

In a final experiment, mice were moved from a QUATs-free room (Rm. B) to another room (Rm. C) in which QUATs were routinely used and ambiently exposed for 10 days before breeding. Half of the males were also gavage dosed with 7.5 mg/kg/day of disinfectant (every other day for 10 days); half of the females received a single gavage dose of disinfectant of 7.5 mg/kg on GD 8. Compared to those that were not moved to Rm. C (which had a 0% litter incidence of open neural tubes on GD 9.5), the litter prevalence for those exposed only to ambient disinfectants or to ambient disinfectants plus gavage was ~6–7%; there was no difference between ambient exposure and ambient + gavage exposure.

Strengths and weaknesses

Throughout the study, the open neural tubes observed in early gestation are referred to as NTDs; this terminology, however, is incorrect and misleading. NTDs are malformations observed at the end of gestation due to failure of the neural tube to close. However, because the neural tube undergoes closure during embryogenesis, it will be found in an open stage in early gestation. Because GD 9.5 and GD 10 embryos are not typically evaluated for the presence of open neural tubes, it is not possible to put the

reported findings in perspective of what is the normal background range, although the study reports a control range of 0–15% across the different experiments. Further, provided photographs suggest that some of the affected embryos were developmentally behind the controls, which may have reflected the stage at which viability was impaired. Certainly, the dose-related decrease in fetal weight on GD 18 is consistent with developmental delay in the treated fetuses. There were no NTDs observed in fetuses examined close to term. The increase in resorptions with evaluation at term in Experiment 2 is consistent with impaired viability of the embryos rather than disruption of neural tube closure.

Because the mice used in this study were moved to multiple rooms throughout the study, they were exposed to different experimental conditions throughout (e.g., different lighting conditions, temperatures, room set-ups, and technicians), which confounds the study's interpretation. Also, because moving can be stressful, a more appropriate design would have included moving of the control animals. In the dosing experiments, a disinfectant formulation was used rather than the active ingredients ABDAC and DDAC, and the dosing formulations were not analyzed to verify their concentration or stability. As in previous studies, the dietary concentrations of the test material were not reported, and identification of gavage vehicle and volume are missing. Length of the breeding period also was not reported. Information on maternal effects is missing, except for the statement that food intake was not affected by incorporation of the disinfectant in the diet. In a previous study by this group, 120 mg/kg/day was associated with substantial maternal toxicity, which could account for the decrease in fetal weight and viability observed. While the dam/litter was the statistical unit of treatment, in studies involving treated males, the 3:1 mating ratio effectively counts each treated male three times in the statistical analysis. Finally, in Experiment 4, the lower prevalence of open neural tubes after treatment of both parents compared to one parent was attributed to discontinuation of QUATs disinfectant use in the animal room between the single parent only treatments and the matings in which both parents were exposed, demonstrating that the procedures were not conducted randomly. Throughout the paper, the authors varied multiple parameters from one experiment to the next (instead of one at a time), which precludes straightforward interpretation of their erratic data.

4 | DISCUSSION

We performed a systematic review of the worldwide scientific literature to identify publications that investigated

the potential developmental or reproductive effects caused by exposure to QUATs. This investigation concentrated on ADBAC (BAC/BZK) and DDAC—both because these are the most commonly used QUATs compounds, but also because they are the ones for which the most data are available. In our literature search, we identified eight published studies that met our inclusion criteria. To these studies, 10 guideline-compliant developmental and reproductive toxicity safety studies that have been submitted to regulatory authorities in the US or Europe were added. Each of these studies (published or unpublished) was evaluated to determine its quality for risk assessment purposes using the ToxRTool (Schneider et al., 2009). Six studies were determined to be reliable without restriction (quality Category 1); four studies were considered to be reliable with restrictions (quality Category 2); and the remaining eight studies were determined to be unreliable (quality Category 3).

None of the six studies considered reliable without restriction reported developmental or reproductive toxicity due to exposure to ADBAC or DDAC. In all cases, the developmental and reproductive NOAELs were set at the highest doses tested, above levels at which maternal/parental systemic toxicity was observed. In the rat developmental toxicity studies, no adverse developmental effects were observed, and the NOAELs were set at the highest doses tested. In the reproductive studies, the offspring NOAELs were established based on indications of general systemic toxicity. Although pubertal development was reported to be delayed in one rat reproductive study of ADBAC, this finding was considered to be secondary to the reductions in offspring body weights.

The four available rabbit developmental toxicity studies of ADBAC and DDAC were considered reliable with restriction. No adverse developmental toxicity was observed with ADBAC exposure, including at dose levels that were maternally toxic. With DDAC exposure, increased post-implantation losses/fetal deaths and reduced fetal body weights were reported at the same dose levels at which maternal toxicity was observed.

Of the eight studies that were classified as being unreliable, six reported adverse effects of QUATs on reproduction and/or development. One of these studies, which involved intravaginal application of ADBAC for use as a spermicidal agent, is not useful for assessing the risks associated with regular use of ADBAC in household and commercial disinfectants. The other five studies are from a single group that primarily evaluated exposure to a commercial disinfectant formulation containing ADBAC and DDAC in the reproduction and development of mice. These studies suffer from poorly described experimental designs, lack of confirmation of exposure under “ambient” conditions, inadequate information

regarding dosing, failure to collect or report important maternal, uterine, and litter data relevant to the interpretation of study findings, and the misuse of terminology such that the severity of the reported findings was greatly overstated.

4.1 | Design flaws

Some methods and endpoints reported in the unreliable studies are non-standard, not reported, not well-described, or incorrectly calculated. For example, in Melin and Hrubec (2015), the methods by which capacitation of the sperm and super-ovulation of the oocytes were carried out to evaluate *in vitro* fertilization rates were not described, and in Melin et al. (2015), it is not stated whether the sperm were pooled for mRNA analysis or whether these analyses were done on sperm from individual males. In some of the studies, the cohabitation schedule and time between weaning and the next mating were not disclosed. This omission is important as these factors can influence mating preparedness. In Melin et al. (2014), limited results are provided from a 6-month continuous breeding period instead of data from controlled single- or two-generation matings. Further, the average number of pregnancies over the breeding period was calculated based on 10 animals per group although one and four animals were euthanized early at 60 and 120 mg/kg/day, respectively. Thus, it would seem that the number of pregnancies for animals that did not survive the full breeding period were included in the calculation. From information reported in Hrubec et al. (2017), it is evident that some of the breeding trials (e.g., matings between singly dosed parents versus those of parents that were both dosed) were not conducted in a random manner. It is not known to what extent procedures were done in a non-random fashion across all the studies reported.

4.2 | “Ambient” exposure

The experiments described as using “ambient” exposures are anecdotal observations of reduced reproductive performance in animals that were housed in rooms that were cleaned or disinfected with solutions that contained ADBAC and DDAC. The authors were unaware of when the subject cleaning solutions were first used in the animal rooms or when use was suspended. Neither substance (ADBAC or DDAC) was identified or measured in maternal tissues (e.g., blood, liver), placentae, or embryos. The route of exposure was not identified. Inhalation exposure is unlikely because neither substance has

an appreciable vapor pressure (Table 5), and the authors used disposable cages that had lids with openings for HEPA filters. The authors inferred animal contact with ADBAC and DDAC based on methanol washes of the caging used to group-house animals.

Often in the ambient exposure experiments, either no controls were included for comparison or animals were moved to a different animal room, which would have environmental conditions that vary from those in the initial room (e.g., different temperatures, room set-ups, background noise levels, technicians). Because the process of relocation can be stressful, the better procedure would have been to also move the “unexposed” animal group as well. Finally, how the study authors defined “ambient” exposure is not clear. For example, in Melin et al. (2014), “extensive cleaning and fogging” of rooms with a QUATs-based disinfectant after a pinworm outbreak was described. However, aerosolization-type uses are inconsistent with label directions for these types of products. Therefore, it is unclear how representative these “ambient” conditions are of the types of exposures expected with approved uses.

4.3 | Dose calculations

In the dietary and drinking water studies, the disinfectant concentrations administered in either the diet or drinking water were not reported. The study investigators reported that their mg/kg/day doses were based on assumed food and water intake levels of 28 and 10% of body weight, respectively. However, the weights of the mice frequently were not reported, and whether the dose levels were adjusted for weight changes throughout the studies was not reported. Based on the information provided, we assume that the reported doses in these studies were for the amount of the (ADBAC+DDAC) incorporated into either the diet or water. However, whether this assumption is correct is not clear from the study reports, and it is possible that the dose levels represent the amount of sanitizer formulation present in the dosing media instead.

TABLE 5 Vapor pressure of water and QUATs at 1 Atm and 25°C

Chemical	Vapor pressure (mm Hg)	Reference
Water	23.8	Virtual Chemistry, 2021
ADBAC	3.53×10^{-12}	USEPA, 2006a
DDAC	2.33×10^{-11}	USEPA, 2006b

The numbers of mice per cage were not always reported, although group housing was noted in some cases.

Assuming a mouse body weight of 30 g, we calculated the concentrations of test material (ADBAC+DDAC) in the diet to be approximately 214 and 429 ppm. The drinking water concentration has been estimated at 1200 ppm. These concentrations appear to be comparable to the lowest dietary concentrations administered in the Quality 1 (reliable without restriction) studies. In both cases, the concentrations of the sanitizer formulation in the diet or drinking water would have been higher and the presence of other unspecified ingredients in the formulation must be considered as a potential confounding factor that may account for some of the observed effects.

4.4 | Missing data

Important maternal, uterine, and litter data relevant to the interpretation of study findings are frequently not reported. Melin et al. (2014) clearly reported that dosages of the disinfectant formulation in the gel diet of 60 and 120 mg/kg/day resulted in significant clinical signs of toxicity, including inappetence, reduced activity, cyanosis, and rough haircoat; additional findings were observed at 120 mg/kg/day. These clinical signs of toxicity were substantial enough to require early termination of one and four animals (of 10 in each group) at 60 and 120 mg/kg/day, respectively. Despite the obvious systemic toxicity associated with these doses, the study investigators failed to report on these and other standard measures of toxicity at the same dose levels in later studies. Many of the findings on which they report in these studies could be mediated secondary to general systemic effects.

Some of the data reported also are questionable. For example, in the 6-month continuous breeding period, the control group was reported to have a minimum of seven pregnancies per dam in the first 100 days of exposure—i.e., a gestation period of ≤ 14.3 days; however, the typical length of a mouse gestation is 20 days. This estimate further assumes no time for lactation and weaning and no break before the animals are remated.

4.5 | Incorrect terminology

NTDs comprise both osseous and membranous structures that develop late in gestation; thus, they are findings classified at term. In Hrubec et al. (2017), however, the findings designated as “NTDs” were described in GD 9.5–10 mouse embryos or GD 11 rat embryos, not at term. Because the time of observation was around the time at

which neural tube closure is occurring (the anterior neural tube generally closes early on GD 10 in mice [Juriloff, Harris, Tom, & MacDonald, 1991; Copp, 2005; DeSesso, 2012; DeSesso & Williams, 2018]), the finding of an open cranial neural tube at this early timepoint cannot be classified as an NTD. It may be, however, an indication of normal developmental variation, developmental delay (i.e., the process of neurulation was not yet complete), or of an embryo that may be resorbed as intrauterine development proceeds. In the former two cases, the condition would likely resolve by the time of parturition; in the latter case, the finding at term would be an early resorption. None of those conditions would be an NTD. An increase in NTDs was not detected in fetuses that were allowed to continue to term in either this or other studies reported by the same group or in regulatory-compliant study reports. Classifying open neural tubes on GD 10 as NTDs is both incorrect and misleading.

5 | CONCLUSION

We performed a systematic literature review to identify published articles concerning potential developmental and reproductive findings regarding QUATs focusing on ABDAC (BAC) and DDAC. To the relevant literature identified, we added guideline-compliant safety studies that had been submitted to regulatory agencies for registration. The data quality for risk assessment purposes was evaluated for each study using the ToxRTool and the studies were placed into one of three categories: Category 1 (reliable without restriction); Category 2 (reliable with restrictions); or Category 3 (unreliable). We note that there were no test article-related developmental or reproductive findings in studies involving rats or rabbits among the 10 combined unpublished (Category 1 and Category 2) studies. The one possible exception is a slight increase in dead fetuses and reduced fetal weights at the highest DDAC dose tested (10 mg/kg/day) in the rabbit (Tyl, 1989); however, the high dose caused significant maternal toxicity (including death) and the findings in offspring were generally without dose-response, and/or not statistically significant. Notably, in a more recent rabbit study (Chevalier, 2005), the findings did not repeat at a dose of 12 mg/kg/day, although maternal toxicity and increased post implantation loss was observed at 32 mg/kg/day.

The only adverse findings in offspring were reports among those studies considered unreliable (Category 3). The subset of unreliable studies generally used small group sizes; unconventional experimental designs and methods; anecdotal (rather than verified) doses; did not report data necessary for determining dosages; and used

incorrect terminology to describe findings. The shortcomings in the unreliable studies make their findings uninterpretable.

Taken together, the body of available information does not indicate a risk of potential developmental and reproductive effects with ABDAC and/or DDAC at levels of exposure anticipated with normal use.

NOTE ADDED IN PROOF

We are aware of two papers describing the DART effects of QUATs that appeared in the literature during the review/processing of our manuscript:

Hostetler, K. A., Fisher, L. C., Burruss, B. L. (2021). Prenatal developmental toxicity of alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride in CD rats and New Zealand white rabbits, *Birth Defects Research*, 113, 925-944. <https://doi.org/1002/bdr2.1889>

Hostetler, K. A., Fisher, L. C., Burruss, B. L. (2021). Reproductive toxicity assessment of alkyl dimethyl benzyl ammonium chloride and didecyl dimethyl ammonium chloride in CD rats, *Birth Defects Research*. <https://doi.org/1002/bdr2.1952>

The data in these papers strengthen our conclusions that QUATs do not pose a risk of reproductive harm to humans under expected exposure conditions when used as directed.

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ENDNOTES

¹ ToxRTool provides a transparent method for assessing the quality of primary toxicological data. The tool asks 21 yes/no questions (criteria) related to five domains: test substance identification; test organism; description of study design; documentation of study results; and plausibility of the design and results. The number of "yes" answers is summed to provide the score (see EU Science Hub website listed above for details).

² Calculated as $[(60 \text{ or } 120 \text{ mg/kg/day}) \times 0.030 \text{ kg bw}] / (\text{daily feed consumed of } 0.030 \text{ kg} \times 0.28)$.

³ The chapter is noted to be intended for submission to *Toxicology in vitro*, but we were not able to locate a published version of the manuscript.

⁴ Calculated as $[(120 \text{ mg/kg/day}) \times 0.030 \text{ kg bw}] / (\text{daily water consumed of } 0.030 \text{ kg} \times 0.10)$.

DATA AVAILABILITY STATEMENT

Many data that support the findings of this analysis are available in the open literature as cited in the References and are identified by DOI. The remainder of the data is from confidential safety studies that are owned by private companies. Confidential data that support the findings of our analysis can be requested from the corresponding author and may be available pending permission from the owners of the data.

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