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REVIEW ARTICLE

Genetic toxicology and toxicokinetics of arecoline and related areca nut compounds: an updated review

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Abstract

Areca nut (AN) is consumed by more than 600 million of individuals, particularly in some regions of South Asia, East Africa, and tropical Pacific, being classified as carcinogenic to humans. The most popular way of exposure consists of chewing a mixture of AN with betel leaf, slaked lime, and other ingredients that may also contain tobacco named betel quid (BQ). Arecoline is the principal active compound of AN, and, therefore, has been systematically studied over the years in several in vitro and in vivo genotoxicity endpoints. However, much of this information is dispersed, justifying the interest of an updated and comprehensive review article on this topic. In this sense, it is thus pertinent to describe and integrate the genetic toxicology data available as well as to address key toxicokinetics aspects of arecoline. This review also provides information on the effects induced by arecoline metabolites and related compounds, including other major AN alkaloids and nitrosation derivatives. The complexity of the chemicals involved renders this issue a challenge in genetic toxicology. Overall, positive results in several endpoints have been reported, some of them suggesting a key role for arecoline metabolites. Nevertheless, some negative genotoxicity findings for this alkaloid in short-term assays have also been reported in the literature. Finally, this article also collates information on the potential mechanisms of arecoline-induced genotoxicity, and suggests further approaches to tackle this important toxicological issue.

Keywords Arecoline · Areca nut · Toxicokinetics · Genotoxicity · Cancer

Introduction

Arecoline (*N*-methyl-1,2,5,6-tetrahydropyridine-3-carboxylic acid methyl ester) is considered the major natural alkaloid present in areca nut (AN; Fig. 1). AN corresponds to the dried seed of the fruit derived from the Areca palm (*Areca catechu* L, *Arecaceae*). This tree is endemic in many regions in Asia (especially in the South and South-East), East Africa, and Western Pacific (Kumpawat et al. 2003; Volgin et al. 2019). The use of AN in these areas of the world is very ancient, being employed in traditional folk medicine, in culinary and for religious and social purposes. Notably, its use is still very pervasive nowadays in different countries from these regions, including India, Taiwan, Nepal, Malaysia, Indonesia, Pakistan, and China—Hunan province and Papua New Guinea. Moreover, AN is also used by some communities in other parts of the world, such as USA, UK, South and East Africa, and Australia (Sharan et al. 2012; Volgin et al. 2019) as a consequence of immigration.

AN is commonly referred to as betel nut (BN), although some authors stated that this designation can be misleading

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Fig. 1 Areca nut (AN) is the dried seed of the fruit derived from the Areca palm (*Areca catechu*), which is a tree endemic in many regions in Asia (especially in the South and South-East), East Africa, and Western Pacific. It is a widely chewed natural product with estimated 600 million users across the world. AN is also referred to as the betel nut (BN), although this latter designation should be avoided. Betel or Betel quid refers to a combined preparation of the areca nut and slaked lime (calcium hydroxide) rolled into a leaf of betel pepper (*Piper betle*), and may also contain tobacco. Calcium hydroxide favors hydrolyzation of arecoline and guvacoline to arecaidine and guvacine, respectively

and should be avoided, being the terminology AN more appropriate (Patidar et al. 2015). There are several available forms of presentation of AN, named with common or traditional designations that vary according to the geographical location and among the cultural groups/individuals that consume them. The most popular form consists of chewing a mixture named betel quid (BQ), that contains AN, betel leaf (BL), as well as slaked lime paste and different flavoring agents and additives. In some countries, tobacco is also incorporated in the mixture (IARC 2012). A detailed description of the different chewing items and their respective traditional names (e.g., *pan* or *paan*, *gutkha*) is beyond the scope of this review and can be found elsewhere (IARC 2012; Sharan et al. 2012; Patidar et al. 2015; Mehrtash et al. 2017). Also, it should be noted that there is significant confusion regarding the exact terminology reported for these products which hinders the adequate characterization of the habit and consequent health effects (Patidar et al. 2015).

It is generally estimated that BQ is consumed by about 10–20% of the world's population and that approximately 600 million persons chew AN (IARC 2012; Volgin et al. 2019; Arora and Squier 2019). These estimates alert us to the impressive number of users that are exposed to arecoline, often at a daily basis. In view of this, BQ chewing is considered by many authors as the fourth most common psychoactive and addictive habit worldwide, after tobacco (nicotine), ethanol, and caffeine (Giri et al. 2006; Chou et al. 2008; Volgin et al. 2019). Moreover, it should be noted that BQ, either with or without added tobacco, as well as AN, have been classified by the International Agency for Research on Cancer (IARC) as Carcinogenic to humans, belonging to Group 1 (IARC 2004, 2012). The oncological diseases for which sufficient evidence in humans exists comprise cancers of the oral cavity, esophagus, pharynx (BQ with tobacco), and a positive association for liver cancer (BQ without tobacco). The impact of AN in human health is not restricted to cancer. In fact, other harmful effects associated with AN exposure include cardiovascular disease, cerebrovascular disorders, metabolic syndrome, diabetes, and asthma, among others (Javed et al. 2010; Mehrtash et al. 2017). The systemic effects of AN have also been reviewed by Garg et al. (2014) that organized the various toxic effects observed towards each of the major organs/systems. In addition, AN also displays deleterious effects in the fetus upon maternal use during the pregnancy. Clearly, these reviews and several other studies show that this habit may affect almost all organs of the human body, triggering or aggravating several disorders. Notably, the use of AN or BQ is a neglected global emergency in terms of public health that requires a closer attention from the scientific community and policy makers (Mehrtash et al. 2017).

Arecoline has not yet been evaluated by the IARC monograph program, although this assessment can be anticipated, since this alkaloid was recently mentioned on the Report of the Advisory Group that recommend priorities for the timeframe 2020–2024 (IARC 2019). In fact, arecoline, as the primary active ingredient of AN, was given the high priority recommendation status. As stated in the IARC (2004) monograph

and summarized in IARC (2019), there is limited evidence for the carcinogenicity of arecoline in experimental animals with positive findings for lung adenocarcinomas, stomach squamous cell carcinomas, and liver haemangiomas in male mice treated with arecoline by gavage, although negative results were also observed in other studies with different administration routes. Importantly, no human studies specifically focusing on arecoline are known (IARC 2019).

As abovementioned, arecoline has indeed a pivotal role in the context of AN or BQ use not only, since it is usually reported as the most abundant alkaloid present, constituting about 85–95% of the total alkaloid fraction, but also because it is generally recognized to be the most active AN alkaloid involved in the psychoactive and other toxic effects observed in AN chewers. Despite all the existing knowledge on the carcinogenic effects of BQ and AN, there are still many gaps concerning the mode of action (MoA) of these agents as human carcinogens. Moreover, the precise role of arecoline in this context is still a matter of discussion. There is growing evidence pointing out to non-genotoxic events induced by arecoline (Jeng et al. 1994; Chang et al. 1998). Epigenetic effects have been described and considered in the context of AN-induced cancers (Lin et al. 2011; Lai et al. 2014; Chuerduangphui et al. 2018). Nonetheless, the broad spectrum of genotoxic effects induced by arecoline per se as well as by its major metabolites and related compounds has been observed in different experimental models, being known, for instance, the mutagenic potential of arecoline in the Ames test since the early 1980s. In view of this, the present work primarily aims at reviewing the available data on the genotoxic potential of arecoline, describing the deleterious effects observed in complementary endpoints and correlating them to toxicokinetic aspects of arecoline, especially to its metabolic activation and nitrosation. The innovative aspects of this comprehensive review also include genetic toxicology information concerning other major AN alkaloids (i.e., arecaidine, guvacine, and guvacoline) and additional chemicals present in BQ.

This review also intends to gather the scattered information concerning the possible mechanisms of genotoxicity, identifying throughout the article different approaches used to modulate the responses observed in the genotoxicity tests which may be important to support a genotoxic MoA for arecoline. Finally, some gaps in this research field are recognized and some suggestions provided towards a better comprehension of this complex issue that affects so many individuals.

Methodology

A comprehensive literature search in English was performed in PubMed, without any timeframe, to identify and select articles (i.e., original research and reviews) to be included in this review. The keywords systematically used were "arecoline", "areca nut","betel nut" or "betel quid" plus "genotoxicity", or plus a specific genetic damage endpoint (e.g., "gene mutations", "Ames test", "chromosomal aberrations"(CA) "micronuclei" (MN), "Sister-chromatid-exchanges" (SCE), "Comet assay", "DNA damage"). "Toxicokinetics" and "metabolism" were also used as keywords combined with "arecoline". Electronic copies of the articles were obtained, being further examined to identify additional relevant publications addressing the genotoxicity induced by arecoline, arecoline metabolites, or arecoline-related compounds.

Toxicokinetics of arecoline

As stated above, arecoline, arecaidine, guvacine, and guvacoline are the major alkaloids isolated from AN, the fruit of *Areca catechu* palm (Shih et al. 2010). Despite the prevalence of AN use and potential human carcinogenicity, more studies focusing on the toxicokinetics of its primary toxic/active alkaloid, arecoline, in humans or in animals are needed (Gupta et al. 2020). Arecoline (Fig. 2) toxicokinetics is fully reviewed below. Nevertheless, it is also important to investigate the toxic effects of the two active metabolites, i.e., arecaidine and arecoline N-oxide (AO, also known as arecoline 1-oxide), to better understand their function in arecoline-induced toxicology (Pan et al. 2018). Moreover, it is also important to remember that the pharmacokinetics studies of the extracts of this plant are most likely different from the results regarding its isolated alkaloids such arecoline.

Hayes et al. (1989) developed a gas chromatography–mass spectrometry (GC–MS) technique for quantitative analysis of arecoline in blood plasma, in the concentration range 1–50 ng/mL, which was used on plasma samples from healthy volunteers who had received transdermal doses at

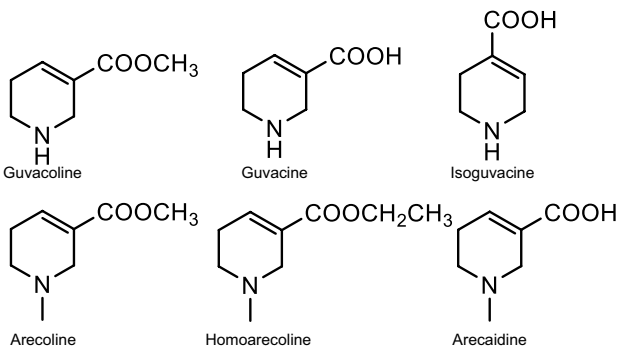

Fig. 2 Structures of the major areca alkaloids found in areca nut. In addition to arecoline, the other three main alkaloids are arecaidine, guvacine, and guvacoline, being these three alkaloids related. Arecaidine is the free carboxylic acid derivative of arecoline; guvacine is the *N*-desmethyl derivative of arecaidine; and guvacoline is the *N*-desmethyl derivative of arecoline

3 mg/h. The time–concentration profile showed a maximum plasma concentration of 4–5 ng/mL at 5–10 h after dermal application. Moreover, the oral mucosal absorption of arecoline was rapid, with blood plasma concentrations increasing with dose and time (Strickland et al. 2003).

Arecoline easily crosses the blood–brain barrier with a brain/plasma concentration ratio close to unity (Doucette et al. 1986; Perry et al. 1981). Other studies have shown that arecoline readily crosses the blood–brain barrier after its intravenous administration to subjects with Alzheimer's disease (Asthana et al. 1996), confirming a very short plasma $t_{1/2}$ values in rats (Soncrant et al. 1989) and other human studies (Shetty et al. 1991). Indeed, arecoline has deep brain penetration as evidenced by its numerous central nervous system effects (Gupta et al. 2020). The mean volume of distribution was 2.55 ± 2.05 L/kg (range, 0.63–6.1 L/kg) with a clearance of 13.6 ± 5.8 L/min. Arecoline was found in the placenta of a mother using AN whose child was affected by neonatal withdrawal syndrome (López-Vilchez et al. 2006), a sign that may possibly underlie the addictive potential of the AN.

Arecoline is rapidly metabolized to arecaidine mainly in the liver, but also in the kidney (Patterson and Kosh 1993). An overview of the metabolic pathways of both compounds is depicted in Fig. 3. Hydrolysis in plasma was also reported (Pan et al. 2017). Interestingly, the hydrolysis of ester moiety of arecoline to arecaidine was completely inhibited using 5% formic acid as a stabilizer, which was immediately added to freshly collected rat plasma samples (Pan et al. 2017). Arecoline was not metabolized by either blood or brain homogenates to any significant degree. Giri et al. (2006) administered arecoline and arecaidine to mice, and their urinary metabolite profiles were analyzed by using ultra-performance liquid chromatography-coupled time-of-flight mass spectrometry (UPLC-TOFMS). Eleven metabolites of arecoline were identified, including arecaidine, AO, arecaidine *N*-oxide,

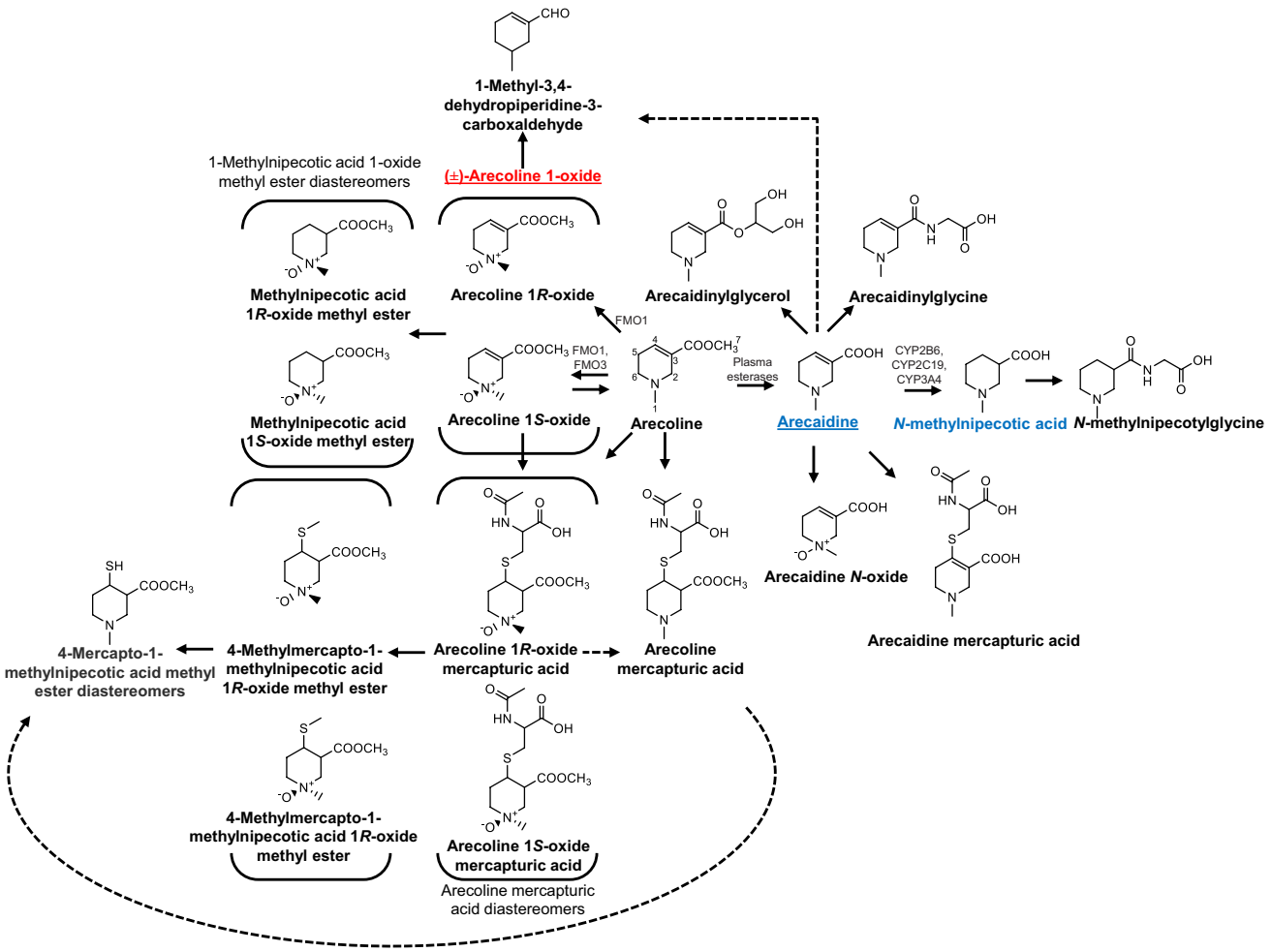

Fig. 3 The metabolic pathway of arecoline and arecaidine. Arecaidine and *N*-methylnipecotic acid represent the major metabolites of arecoline. Underlined compounds denote the two active metabolites of arecoline, being arecoline 1-oxide (also known arecoline *N*-oxide) critical in toxicity. Arecoline hypothetical metabolic routes are presented with dashed lines

N-methylnipecotic acid, *N*-methylnipecotylglycine, arecaidinylglycine, arecaidinylglycerol, arecaidine mercapturic acid, arecoline mercapturic acid, and arecoline *N*-oxide mercapturic acid, together with nine unidentified metabolites. Results also evidenced that the major metabolite of both arecoline and arecaidine was *N*-methylnipecotic acid, which is the result of C=C double-bond reduction (Boyland and Nery 1969; Nery 1971). These metabolic findings were recently corroborated after detailed in vivo analysis of absorption and metabolism of AN extracts by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) (Li et al. 2017). Arecaidine, formed by arecoline hydrolysis in liver, is a Michael acceptor like arecoline and can react with proteins, generating protein-toxic adducts (Chou et al. 2012).

Since the administration of AO to rats produce a broadly similar metabolic picture as arecoline itself, it was suggested the reduction of AO back to arecoline and then its subsequent metabolism (Nery 1971). This process of N-oxide reduction and re-synthesis has been termed "metabolic retroversion" from the study of trimethylamine N-oxide in volunteers with a trimethylamine N-oxidation pharmacogenetic deficiency (Al-Waiz et al. 1987). It was suggested that N-oxides may undergo metabolic reduction in vivo, perhaps by the gut flora, before being N-oxidized back to the parent compound (Giri et al. 2007). It was possible to visualize the "metabolic retroversion" during the administration of trimethylamine N-oxide (TMAO) to two patients with an inherited trimethylamine N-oxidation deficiency, known as fish-odor syndrome. While unaffected subjects excreted > 94% of the administered TMAO apparently unchanged, with only 4% as the free base trimethylamine (TMA), the two affected patients excreted 35 and 51% of dose as TMA, and two heterozygous subjects excreted 12 and 16% of dose as TMA. Overall, these findings suggested that 40–60% of an oral dose of TMAO undergoes retroverted metabolism (Al-Waiz et al. 1987) and approximately 80% of the urinary metabolites are N-oxides.

Subsequently, Giri et al. (2007) studied the metabolism of one of the two major urinary metabolites of arecoline in the mouse, i.e., AO, after oral administration of a dose of 20 mg/kg. A total of 16 mass/retention time pairs yielded 13 metabolites of AO, most of them novel. The principal pathways of metabolism of AO were mercapturic acid formation, with catabolism to mercaptan and methylmercaptan metabolites, apparent C=C double-bond reduction, carboxylic acid reduction to the aldehyde, N-oxide reduction, and de-esterification, together with various combinations of these pathways. Furthermore, four of these metabolites were detected as diastereomers, making a total of 14 urinary metabolites, including the arecoline. Arecoline was converted to AO in vitro by human flavin-containing monooxygenases FMO1 and FMO3, but not by FMO5 or any of 11 human cytochromes P450 tested (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11). As discussed below, this metabolite has moderate mutagenicity as the ultimate carcinogen and plays an important role in arecoline-induced oral carcinogenesis (Lin et al. 2011). The relative abundance of each isozyme of FMO1 and FMO3 will determine which isozyme is preferentially utilized in any given tissue. The FMOs catalyze the NADPH-dependent oxidation of a variety of xenobiotics which contain nucleophilic heteroatoms, typically nitrogen, sulfur, or phosphorus (Shephard and Phillips 2010; Ziegler 1993). These enzymes are efficient two-electron oxygenating enzymes for N-oxidation, unlike P450s, which generally use sequential one-electron transfer chemistry (Guengerich et al. 1997). The microsomal FMO enzyme family is comprised of five isozymes, i.e., FMO1–FMO5, whose expression is tissue-specific (Phillips and Shephard 2017). FMO1 is predominantly expressed in human kidney, and FMO2 in lung and kidney (Cashman and Zhang 2006; Klaassen 2019; Phillips and Shephard 2017). FMO3 is the prominent isozyme in adult human liver, FMO4 is more broadly distributed in liver, kidney, small intestine, and lung, and FMO5 is expressed in human liver, lung, small intestine, and kidney (Cashman and Zhang 2006; Klaassen 2019; Phillips and Shephard 2017).

1-Methylnipecotic acid 1-oxide methyl ester is a metabolite arising from the reduction of the 3,4-double bond of AO (Giri et al. 2007). Such a metabolic reaction was also reported for the biotransformation of arecoline to 1-methylnipecotic acid in the mouse (Giri et al. 2006). Perusal of the structure of 1-methylnipecotic acid 1-oxide methyl ester reveals chiral centers at positions N1 and C3 and, thus, the possibility of diastereomers. The two trans-diastereomers are 1-(R), 3-(R) and 1-(S), 3-(S), and the cis-diastereomers are 1-(R), 3-(S), and 1-(S), 3-(R) [cis/trans relative to the oxide]. Both arecoline and arecaidine have been reported to form mercapturic acids in the rat (Boyland and Nery 1969) and, therefore, presumably undergo glutathione conjugation. Arecoline 1-oxide mercapturic acid exhibits asymmetry at positions *C*3 and *C*4, meaning that arecoline 1-oxide mercapturic acid exists as two pairs of diastereomers, 3-(*R*), 4-(*S*) and 3-(*S*), 4-(*R*) [both *cis*-] together with 3-(*R*), 4-(*R*) and 3-(*S*), 4-(*S*) [both *trans*-]. These undergo cysteine *S*-conjugate β-lyase activity producing 4-methylmercapto-1-methylnipecotic acid 1-oxide methyl ester, which are likely precursors of the thiol metabolites. The aldehyde 1-methyl-3,4-dehydropiperidine-3-carboxaldehyde was also identified as metabolite of AO**.**

The rapid hydrolysis of arecoline by plasma esterases results in the short plasma $t_{1/2}$ of arecoline and reduced excretion unchanged by the kidneys (Sethy and Francis 1988). In the study of Giri et al. (2007) approximately 50% of the urinary metabolites corresponded to unchanged AO, 25% to other N-oxide metabolites, while approximately

30% corresponded to mercapturic acids or their metabolites. Many metabolites, principally mercapturic acids and their derivatives, were excreted as diastereomers that could be resolved by UPLC-TOFMS. In another study of the same research group, unchanged arecoline comprised 0.3–0.4%, arecaidine 7.1–13.1%, AO 7.4–19.0%, and *N*-methylnipecotic acid 13.5–30.3% of the dose excreted in 0–12 h urine after arecoline administration (Giri et al. 2006). In a study of 15 Alzheimer patients receiving 5 mg intravenous arecoline, the mean terminal plasma $t_{1/2}$ of arecoline was approximately 9.3 min with plasma arecoline concentrations returning to baseline within approximately 1 h (Asthana et al. 1996). Relatively longer $t_{1/2}$ were obtained for arecaidine (4.3 h) and *N*-methylnipecotic acid (7.9 h) in five healthy nonchewers after oral administration of AN water extracts (Hu et al. 2010). In an interesting study, the ultra-high-performance liquid chromatography system coupled to tandem mass spectrometry method was developed to quantify arecoline, arecaidine, guvacoline, and guvacine in hair as proof of long-term exposure to BN (Gheddar et al. 2020). Of note, some authors were unable to detect any alkaloids in the female hair samples. This was claimed to be due to the chemical hair treatments or excessive use of hair products, since all other factors regarding AN chewing habits were similar to the male volunteers (Franke et al. 2020).

Arecoline psychoactive effects

As already mentioned, AN is the fourth most common abused drug after nicotine, ethanol, and caffeine, especially among people of lower socioeconomic status (Kumar Srivastava 2014; Lee et al. 2012; Ni et al. 2013; Shah et al. 2002; Wang et al. 2005). In several communities, AN is used as a socializing food substance and in religious festivities (Osborne et al. 2011; Ujváry 2014).

The effects are described as pleasurable and generally stimulating, inducing a sense of well-being, euphoria, heightened alertness, a warm sensation throughout the body, and an increased capacity to work. Psychosis due to AN consumption was first described in Papua New Guinea in 1977 by Burton-Bradley, and was associated with high intake as part of a ceremony with traditional healers (Burton-Bradley 1978). Since then, this condition has been rarely described in literature. Indeed, clinical studies currently available show that AN consumers do not have an increased risk to develop schizophrenia (Bales et al. 2009; Coppola and Mondola 2012; Sullivan et al. 2007).

Dependence is positively correlated with the frequency of chewing per day and the ability to stop is highly dependent on the period and frequency of consumption (Bhat et al. 2010; Lin et al. 2006; Winstock 2002). Although dependence originates mild symptoms, the proportion of dependence is relatively high, ranging from 20 to 90% among current users (Ko et al. 2020). Acute toxic psychosis as characterized by auditory hallucinations, grandiose, or persecutory delusions has not been widely observed following AN use. Although available reports are very limited, AN psychosis appears to be reversible and there are no reports of "bad trips" following excessive chewing. There is also some evidence that pure arecoline may enhance working memory in Alzheimer's disease patients. However, the results are unpredictable and inconclusive (Raffaele et al. 1996). Ko et al. (2020) highlighted that AN increased both brain serotonin and noradrenaline levels, whereas arecoline displayed monoamine oxidase-A (MAO-A) inhibitor-like properties. MAO-A inhibitors prevent neurotransmitter breakdown and increase dopamine and serotonin concentrations in the brain. A significant increase in brain dopamine levels was observed after arecoline administration through injection to mice (Molinengo et al. 1986). Moreover, the nonselective muscarinic and nicotinic receptor agonist effects of arecoline, responsible for the parasympathomimetic effects of AN preparations, should also not be discharged, since cholinergic system plays a key role in the development of cocaine dependence (Coppola and Mondola 2012). More recently, it was demonstrated that individuals chronically exposed to BQ have higher functional connectivity than controls of the orbitofrontal cortex, and inferior temporal and angular gyri (Sariah et al. 2020).

A withdrawal syndrome has been described for the AN, and it is characterized by mood swings, anxiety, irritability, reduced concentration, sleep disturbance, and increased appetite (Lee et al. 2012; Wiesner 1987; Winstock et al. 2000). Symptoms typically begin within 48 h and tolerance was also reported (Winstock et al. 2000).

Genotoxicity of arecoline and related compounds

The genetic toxicity of arecoline has been evaluated in different in vitro and in vivo models, and some information concerning the possible mechanisms involved is available in the literature. Some of these data have been reported in original articles or summarized either in review articles or in the IARC publications (2004, 2012, 2019). Moreover, there are also reports on the genotoxicity of AN extracts, reflecting not only arecoline's inherent genotoxicity but also the potential contribution of the other genotoxic alkaloids and chemicals present. This fact renders the assessment of the genotoxic burden induced by arecoline in the extracts far more complex, being also important to mention that other phytochemicals present may even counteract the noxious effects of the abovementioned alkaloids. Nevertheless, there is a significant amount of data concerning the genetic toxicology of arecoline per se and some information regarding the genotoxic effects of arecoline's major metabolites, particularly AO. Since arecoline and other AN alkaloids can

react with nitrites forming nitrosamines, these derivatives have also been studied, especially those found in the saliva of BQ chewers. In view of this, we organize the information available in separate subsections, summarizing the results of arecoline and related compounds in: (i) gene mutation assays, (ii) DNA damage and repair assays, and (iii) cytogenetic endpoints in mammalian systems. Some authors included the S9 metabolic activation mixture (S9) in their studies. Moreover, some of the genotoxicity results were achieved not only in vitro but also in in vivo animal models (i.e., rat and mouse). An overview of the genetic toxicology data for arecoline, its metabolites, and related AN alkaloids is depicted in Fig. 4. Some of the main key findings of these studies are also present in this figure in a simplified manner, i.e., identifying positive (+ve), weak positive (weak + ve), and negative (−ve) results, according to the conclusions of the authors in their original publications. This figure does not intend to characterize in depth the responses observed but rather to provide a holistic view of the genetic toxicology information available. The results obtained with AN or BQ extracts are not present in Fig. 4, although some results concerning these extracts are mentioned below in this section.

(i) Gene mutations

Bacterial reverse mutations The mutagenicity of arecoline in prokaryotes was described in the early 1980s by Shirname et al. (1983). In this work, the mutagenic activity of BQ extracts and major alka-

Fig. 4 Overview of the genetic toxicology data on arecoline, arecoline metabolites, and related areca nut alkaloids in different endpoints in vitro and in vivo. *+ve* positive result, *weak +ve* weak positive result, *–ve* negative result, *w/o* without, *mut* mutation, *UDS* unscheduled DNA synthesis, *HPRT* hypoxanthine phosphoribosyltransferase, *NMPN* 3-(*N*-nitrosomethylamino)propionitrile, *NMPA* 3-(*N*-nitrosomethylamino)propionaldehyde, *CA* chromosomal aberrations, *SCE* sister chromatid exchange, *MN* micronuclei, *PBLs* peripheral blood lymphocytes. References: [a]Shirname et al. (1983), [b]Wang and Peng (1996), [c]Lin et al. (2011), [d]Wang et al. (2018), [e]Shirname et al. (1984), [f]Shih et al. (2020), [g]Wu et al. (2012), [h]Kevekordes et al. (1999), [i]Shakya and Siddique (2018), [j]Sinha and Rao (1985a), [k]Sharan and Wary (1992), [l]Jeng et al. (1999), [m]Wary and Sharan (1988), [n]Rehman et al. (2016), [o]Kuo et al. (2019), [p]Kuo et al. (2015), [q]Tsai et al. (2008), [r]Huang et al. (2016), [s]Jeng et al. (1994), [t]Sundqvist et al. (1989), [u]Panigrahi and Rao (1982), [v]Panigrahi and Rao (1983), [w]Deb and Chatterjee (1998), [x]Chatterjee and Deb (1999), [y]Kumpawat et al. (2003), [z]Sinha and Rao (1985b), [aa]Panigrahi and Rao (1984), [ab]Stich et al. (1981), [ac]Dave et al. (1992), [ad]Lee et al. (1996), [ae]Kevekordes et al. (2001), [af]Wang et al. (2010)

loids were evaluated in the classical mutagenicity Ames test, using four *Salmonella typhimurium* strains (TA100, TA 1535, TA98, and TA 1538) in the presence or absence of S9. The aqueous extracts of BQ, BQ plus tobacco, and BN were mutagenic in TA100 and TA1535 strains, while the aqueous extract of BL displayed negative results in all four strains. The most sensitive strain was TA100, indicating base-paired substitutions upon extract incubation. Importantly, arecoline (hydrochloride form) and arecaidine were also mutagenic in all the four tester strains, being the former clearly the most mutagenic ingredient. In fact, arecoline revealed to increase the number of revertants/plate at doses ≥ 10 µg/plate, both with and without S9. At lower doses of 1 and 5 µg/plate, arecoline gave negative results, while the maximum revertant colony level was achieved with arecoline at 200 µg/plate with S9 (613 revertants/plate). The sensitivity was consistently higher in the presence of S9 either for isolated alkaloids tested or for the extracts. Moreover, the impact of metabolic activation was more pronounced for lower mutagenic doses of arecoline. For instance, for arecoline at 10 µg/plate, the number of revertants was 242 with S9 vs 17 without S9, whereas for 200 µg/plate, the number of revertants was 613 with S9 vs 375 without S9.

Wang and Peng (1996) also studied the mutagenic potential of arecoline. In this report, they evaluated the effects of arecoline free base, as well as of *N*-nitrosoguvacoline (NG) and crude alkaloid extracts. Arecoline was not mutagenic in TA98, although an increased mutation frequency was observed in TA100 in the presence or absence of S9. Conversely to the results abovementioned from Shirname et al. (1983), the mutagenic effect of arecoline was more pronounced without S9. In view of this, the authors concluded that the discrepancies between studies should be ascribed to the type of arecoline evaluated, i.e., arecoline hydrochloride vs free base (oily). Moreover, Wang and Peng (1996) also mentioned that the crude alkaloid extract, mainly constituted by arecoline (~ 75%), but also containing guvacoline (~ 13%), guvacine (~ 11%), and arecaidine (1%), is a weak mutagenic agent in TA100 in the presence of S9, with no response in TA98, possibly indicating a base-paired mechanism upon metabolic activation. Another aspect of this study was the evaluation of the mutagenic activity of NG in both tester strains TA98 and TA100. NG was the only *N*-nitrosation product of arecoline found in the saliva of Taiwan BQ chewers. This compound revealed to be a weak direct acting mutagen in both tester strains, with a reduction in the mutagenic activity after S9 incubation.

Other authors were also interested in the assessment of the mutagenic potential of BQ-derived *N*-nitrosoamines. In fact, Miyazaki et al. (2005) using a set of genetically engineered *Salmonella typhimurium* strains YG7108, co-expressing one human cytochrome P450 (i.e., CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2A13, CYP2D6, or CYP3A4) and the NADPH-cytochrome P450 reductase, found that the human CYP2A subfamily members have determinant roles in the bioactivation and consequent mutagenic activity of all the specific BQ-derived nitrosoamines. NG, considered the most relevant nitrosation product of arecoline, was shown to be activated by CYP2A13 and CYP2A6, revealing to be much less mutagenic than 3-(*N*-nitrosomethylamino)propionaldehyde (NMPA; mainly activated by CYP2A13 and also activated by CYP2A6, CYP1A1, CYP1A2, and CYP1B1) or 3-(*N*-nitrosomethylamino)propionitrile (NMPN; primarily activated by CYP2A6 and also activated by CYP1A1 and CYP1B1).

The quest for the identification of the ultimate mutagen responsible for the carcinogenic activity of BQ/AN in human populations (chewers) using the Ames test was resumed by Lin et al. (2011). In this study, not only arecoline (hydrobromide) but also its metabolite AO was evaluated in TA98 and TA100 tester strains in the presence or absence of S9. The results obtained in both strains have a similar pattern and did not indicate mutagenic effects of arecoline without S9, pointing out, however, to a low mutagenic effect in the presence of S9. The response obtained for arecoline at 1 µg/plate in TA100, revealed an increase in the mean number of revertants from 103 (without S9) to 210 (with S9), at this dose level. Likewise, an increase from 11 (without S9) to 25 (with S9) was obtained for arecoline at 1 µg/plate in TA98. The effect of AO was studied without S9, and the authors considered that this metabolite exerted a moderate mutagenic effect in both strains with a clear dose-effect relationship in both tester strains. In fact, the mean number of revertants in the background increased from 101 in TA100, and 10 in TA98 to 964 and 86, respectively, at the highest concentration tested. The presence of sulfhydryl compounds (i.e., GSH, *N*-acetylcysteine, and cysteine) and the N-oxide-reducing reagent titanium trichloride markedly reduced AO mutagenicity. Overall, these authors claimed that AO is possibly the ultimate mutagen. They suggested that AO should be further studied in view of its inher-

ent mutagenicity, because it is considered the main metabolite of arecoline.

More recently, Wang et al. (2018) also studied arecoline and AO in complementary in vitro assays, including the Ames test. The tester strains TA98 and TA 100 were selected and the experiments performed in the absence of S9-mix at concentrations up to 1000 µM. In general, arecoline led to small and non-significant increases in the number of revertants/plate. Conversely, AO elicited much higher rises in the frequency of revertants, rendering significant responses, albeit not for all the concentrations tested. The maximum response was achieved with AO 1000 µM, with increases of the number of revertants/plate from 14 in TA98 and 168 in TA100 (background) to 53 and 618, respectively. While comparing the mutagenic effects induced by both compounds, the authors used the term "strikingly" to describe the higher response displayed by AO.

Mammalian gene mutations. A few reports addressed the formation of gene mutations in mammalian cells in vitro and in vivo. The induction of 8-azaguanine resistant mutations in V79 Chinese hamster cells was observed in a dose–response manner for arecoline, extracts of BN, as well as for extracts of BQ plus tobacco. Significant increases in the number of mutant colonies were present with and without S9. While arecaidine also induced forward mutations with S9 but in a much lower frequency, BQ extract per se revealed to be nonmutagenic (Shirname et al. 1984).

The mutagenicity of arecoline was very recently reported in CHO-K1 cells using the hypoxanthine phosphoribosyltransferase (HPRT) forward mutation assay (Shih et al. 2020). In fact, arecoline (100 and 200 µg/mL) significantly increased the mutation frequency of these cells. Moreover, the authors also described that the co-treatment of melatonin (1 mM) with arecoline significantly reversed the mutagenic potential of this alkaloid.

The mutations at the HPRT locus were also evaluated in cultured human keratinocytes (HaCaT cells) treated with AN extracts (Lai and Lee 2006). The short-term treatment (24 h) resulted in the increase in the mutation frequency for extract concentrations ≥ 40 µg/mL. The long-term protocol (35 passages) revealed that AN extract, at a sublethal concentration of 20 µg/mL, but not 5 µg/mL, markedly increased the mutation frequency. Intracellular reactive oxygen species (ROS) and other genotoxicity biomarkers were also increased, specifically the formation of 8-hydroxydeoxyguanine (8-OHdG) and MN. These two endpoints will be further discussed in this section in the context of experiments performed with arecoline.

An in vivo approach was described in Wu et al. (2012) using *gpt* delta transgenic mice (male) to assess the mutagenic effects of arecoline in organ-specific sites, i.e., oral and hepatic tissues. Two weeks after oral exposure to arecoline (hydrobromide) via drinking water (300 and 700 µg/mL, 6 week treatment), the average mutation frequency in these tissues was not significantly different when compared to the mutagenicity observed in unexposed mice. Nevertheless, the authors emphasized that in some individual mice treated with arecoline, the mutation frequency was 2.5 higher than the average of control mice and a unique mutation pattern was present. Notably, in these arecoline-treated mice, G:C → T:A transversions (more frequent), G:C → A:T transitions, and G:C → C:G transversions were found in contrast to G:C → A:T transitions, the main spontaneous mutations present.

(ii) DNA damage and repair endpoints

Arecoline and related compounds have been studied in diverse reports performed to assess the formation of DNA lesions and their repair. Some experiments have been performed using non-mammalian assays, for instance the induction of bacterial SOS functions as a measure of the primary response to DNA damage (SOS Chromotest) or the comet assay using transgenic Drosophila melanogaster. Nevertheless, most of the studies were performed with mammalian cells in vitro, often resorting to cells derived from oral tissues either from benign or malignant origin. A few reports mentioned below have also described in vivo experiments with rodent models to assess DNA damage and repair.

Non mammalian assays Kevekordes et al. (1999). assessed the genotoxicity of several naturally occurring xenobiotics, including arecoline in *Escherichia coli* PQ37 (SOS chromotest). In contrast to the Ames test positive results mentioned, arecoline gave negative results in this colorimetric test either in the presence or absence of S9. The close correlation between both bacterial tests and their comparable results has been recognized, since most of the mutagenic compounds are also SOS inducers in the *E. coli* strains. In view of this, arecoline could be included in the group of genotoxic chemicals that display discrepant results between the two tests.

Recently, Shakya and Siddique (2018) used a transgenic fly strain of *Drosophila melanogaster*, which has been recognized as an alternative in vivo model in Toxicology. The third instar larvae of *Drosophila melanogaster (hsp70-lacZ) Bg*^{9} were exposed via diet

to arecoline (up to 80 µM) and a set of toxicological assays were performed, including the comet assay and oxidative stress indicators. For the lower concentrations of 5 and 10 µM, an absence of adverse effects was noticed. Significant increases in the tail length were observed in a concentration-dependent manner for arecoline concentrations ≥ 20 µM. At these levels, increases in parameters associated with oxidative stress (e.g., protein carbonyl content, and lipid peroxidation) as well as decreases in the antioxidant defense (GSH content) were also present. Overall, the authors suggest that an oxidative stress-based mechanism is relevant for arecoline's toxicity.

Mammalian assays The unscheduled DNA synthesis (UDS) assay was initially employed by Sinha and Rao (1985a) in the early spermatids of Swiss albino mice treated with arecoline. UDS occurs when the DNA damage inflicted by a given genotoxicant results in repair DNA synthesis. In these experiments, arecoline at doses of 20–80 mg/kg led to dose–response increases in the unscheduled incorporation of tritiated thymidine into the DNA of the early spermatids. The authors speculated that the justification of the UDS data could be the result of the binding reaction of arecoline to DNA. Morphological alterations in the shape of sperm heads were also observed, and in this case, non-disjunction events in the germ cells were suggested to be involved.

In addition, an in vitro study (Sharan and Wary, 1992) reported that arecoline and different BN extracts induced UDS in Hep-2 cells (originally thought to be derived from an epidermal carcinoma of the larynx and then identified as established via HeLa contamination, as described in ATCC). In relative terms, the aqueous and acetic acid BN extracts were more effective in this endpoint than arecoline, hydrochloric acid, and ethanol extracts. Conversely, Jeng et al. (1999) using human gingival keratinocytes (GK) reported negative results for arecoline (concentrations up to 1.6 mM). Inflorescence of piper betle (IPB) extracts also gave negative results, whereas AN extract elicited an increase in UDS in GK up to 5.5-fold comparing with untreated controls.

The formation of DNA breaks is an important genotoxic feature that has been evaluated by complementary assays in the last decades in the context of arecoline or AN extracts exposure. Wary and Sharan (1988) compared the ratio of single-stranded DNA/double-stranded DNA (ssDNA/dsDNA) recovered after exposure to arecoline (10 µg/mL) or to an aqueous extract of BN from North-East India (100 µg/mL) in cultured kidney cells from Swiss albino mice. Using the alkaline DNA-unwinding technique, arecoline-exposed cells led to large increase in the ssDNA/dsDNA ratio with time, with a small reduction after the 3rd day culture. The BN extract also significantly increased DNA damage but in lesser magnitude when compared to arecoline alone. Conversely, comparing to arecoline, the aqueous BN extract (100 µg/mL) enhanced the rate of cell proliferation in a higher extent, being this finding highlighted in the report.

The in vitro effects of AN extracts and the four major AN alkaloids (i.e., arecoline, arecaidine, guvacoline, and guvacine) in human buccal epithelial cells were studied by Sundqvist et al. (1989) using the Alkaline elution assay. These authors also included in this report data on the nitrosation products of the alkaloids, i.e., NG, *N*-nitrosoguvacine, NMPA, and NMPN. AN extract (300 µg/mL) caused DNA single-strand breaks (SSBs) and DNA protein crosslinks. Both DNA lesions increased in a concentration-dependent manner with AN extracts. In contrast, arecoline as well as the other individual alkaloids did not significantly increase the level of SSBs. The same negative response occurred for the nitrosated derivatives, except for NMPA. In view of this, the authors highlighted the potential importance of this nitrosation product in the induction of tumors in BQ chewers.

DNA-strand breakage was also evaluated using the DNA precipitation in arecoline-treated human oral mucosal fibroblasts derived from the normal oral mucosa (Jeng et al. 1994). While this alkaloid induced concentration-dependent cytotoxic effects, no DNA damage was observed even at a high concentration level (up to 600 µg/mL). This absence of genotoxicity of arecoline contrasts with the positive results obtained with the extracts of BN and IBP, although at cytotoxic concentrations.

The histone H2AX phosphorylation assay has been used by several authors to evaluate the induction of DSBs by arecoline and related compound types. In this context, arecoline (0.3 mM; 24 h) induced DNA damage in different human cell lines in vitro. The effect of arecoline in terms of generation of γ-H2AX was concentration- and time-dependent (immunofluorescence assay), being also observed a cell cycle arrest at G2/M (Tsai et al. 2008). Accordingly, arecoline represses the expression of p53 and respective p53 target genes, and inhibits UV-induced DNA repair, which might lead to an increase in DNA damage and gene alterations. Tsai et al. (2011) studied the expression profile of microRNA (miRNA) upon AN extract (800 µg/mL) or arecoline (100 µg/mL) treatment as compared with untreated human gingival fibroblasts (HGF cells). The authors identi-

fied eight miRNAs that were differentially expressed in a consistent manner after AN extract or arecoline exposure. Moreover, the miR-23a overexpression in AN extract-treated cells was correlated with the increase in γ-H2AX expression.

Another study addressed the in vitro induction of DNA damage by arecoline and AO using the phosphorylation of histone H2AX as the endpoint (Kuo et al. 2015). The effect of both compounds was evaluated in confluent normal human gingival fibroblasts (HGF-1 cells) in a 7-day continuous exposure protocol. Arecoline (200 and 400 μM) weakly increased γ-H2AX expression. In contrast, at equimolar concentrations, AO led to a significant four-to-fivefold increase. The quantification of cellular 8-OHdG by ELISA revealed that AO induced larger oxidative DNA damage levels comparing with arecoline. Nevertheless, all concentrations tested of both chemicals were significantly different than untreated controls, and the magnitude of the higher effect of AO was not as evident as with the γ-H2AX assay. The authors further studied the induction of γ-H2AX in vivo by AO using the immunodeficient NOD/SCID mice model. Sublingual tongue sections revealed an approximately 11-fold increase in the expression of γ-H2AX (immunohistochemistry analysis) when compared with control mice. In a subsequent study (Kuo et al. 2019), this group recently used the same endpoint to reinforce the importance of the metabolite AO and highlighted the impact of human flavin-containing monooxygenase FMO3 in this context. In fact, AO led to higher DNA damage activity in terms of γ-H2AX comparing to arecoline in HGF-1 cells, and the knockdown of FMO3 by siRNA elicited the downregulation of H2AX in HSC3 cells treated with arecoline.

The comet assay was also used in experiments performed with Hep-2 cells to evaluate the concurrent impact of arecoline with benzo(a)pyrene (BaP) in terms of DNA damage and repair (Huang et al. 2016). In this study, subtoxic concentrations of arecoline (0.1 mM) or BaP in a 7-day treatment protocol increased genotoxicity, leading to comets characterized by mild DNA damage. Notably, the extended co-exposure to both chemicals caused moderate-to-severe DNA damage observed in 60% of the cells. In terms of DNA repair, BaP at short-term treatment enhanced the Nucleotide Excision Repair (NER) activity, although in a prolonged co-exposure with arecoline, XPD expression was repressed leading to the accumulated of unrepaired DNA damage and consequent genomic instability. The authors highlighted the impact of the concomitant exposure to both deleterious chemicals in BQ and tobacco smoke.

Recently, Wang et al. (2018) addressed the genotoxicity, ROS production and cytotoxicity of arecoline and AO in rat liver clone 9 cells. Arecoline did not induce significant DNA breaks as evaluated by the alkaline comet assay (mean tail moment) at concentrations up to 500 μM (2 h treatment). Conversely, a marked increase in DNA damage was observed in a concentration-dependent manner for AO, being this genotoxic effect counteracted in the presence of NAC. The authors also measured the formation of ROS by AO and showed that this pro-oxidant effect can be reduced using diverse chemical strategies (e.g., NAC, Trolox, and penicillamine).

The fraction of cells containing large 53BP1 foci, a surrogate indicator of irreparable DNA-strand breaks, was evaluated in two human oral fibroblast lines (NHOF-1 and NHOF-5) exposed for 48 h to arecoline (100 and 300 μM) and arecaidine (30 and 100 μM) (Rehman et al. 2016). Both alkaloids induced DNA damage at all the concentrations tested, with increases of approximately three-to-fourfold comparing to untreated controls. It should be mentioned that comparing the equimolar concentration of 100 μM, the genotoxic effect for arecaidine was higher than the observed for arecoline. In this report, the authors also studied other biomarkers, including $p16^{INK4A}$, a specific marker of senescence in oral fibroblasts, being this parameter also increased for both alkaloids.

(iii) Cytogenetic studies

In vitro experiments The genotoxic effects displayed by arecoline and related alkaloids per se and combined with other compounds present in BQ extracts have been often reported using different in vitro cytogenetic assays. In the early 1980s, the clastogenic effects of arecoline in vitro were initially reported (Stich et al. 1981). An exposure period of 3 h to arecoline increased the frequency of metaphases containing chromosome aberrations in Chinese Hamster Ovary (CHO) cells. The clastogenicity of arecoline increased upon concurrent exposure with other components of BQ, i.e., quercetin, chlorogenic acid, or eugenol. Using the same cell line, the in vitro genotoxic effects induced by arecoline and aqueous AN extract were reported in terms of CA and SCE (Dave et al. 1992). Two types of exposure schedules were addressed for both agents in the absence of S9 metabolic activation, i.e., a continuous treatment with low concentration and a pulse short-term treatment. Concentration-dependent increases in the cytogenetic damage indicators were observed for both assays and treatment protocols either for arecoline or AN extract, being the effects more

severe in the case of low concentration and longer exposures. This experimental design tends to better mimic the chronic exposure that might occur in AN long-term consumers. Significant increases in CA/cell were observed for arecoline at 50 and 75 µg/mL for continuous treatment, and at 200 and 250 µg/mL for pulse treatment. SCEs' induction revealed to be a very sensitive biomarker, with significant results of SCE/cell for arecoline at a lower concentration level, i.e., at concentrations ≥ 12.5 µg/mL for continuous exposure, and ≥ 100 µg/mL for short-term treatment. In addition, the genotoxicity of arecoline combined with nicotine was further evaluated in CHO resorting to the same CA and SCE assays (Trivedi et al. 1993). This study was considered pertinent by the authors, since tobacco is mostly chewed with AN. Overall, the results, obtained with concentrations of arecoline and nicotine in the same range of those found in the saliva of BQ chewers, pointed out to a significant cytogenetic damage in CHO cells.

The induction of MN after exposure to arecoline and sodium fluoride (NaF, component of BQ) in the CHO cell line was also studied (Lee et al. 1996). After exposure to arecoline (up to 6 µM) a significantly increase in micronucleated cells was observed in a concentration-dependent effect. The prolongation of the cell cycle duration was also observed. NaF also showed to be genotoxic, increasing the frequency of MN. As abovementioned, BQ has a complex composition. Among other substances, glycyrrhizin and catechin are present. The combined exposure of arecoline (6 µM) with glycyrrhizin (10 µM) or catechin (10 µM) led to significantly reductions in the number of micronucleated cells as well as delays in the cell cycle duration when compared to arecoline alone. At a high concentration, glycyrrhizin (100 µM) also significantly increased the frequency of MN, whereas catechin was not genotoxic. The authors inferred from the results of the combined exposure to arecoline and catechin that the genotoxicity of arecoline might be mediated by the production of ROS.

The induction of MN was further studied in CHO-K1 cells (Shih et al. 2020). Arecoline (100 and 200 µg/mL) significantly induced the formation of MN in a concentration-dependent manner. Melatonin co-treatment resulted in a decrease in the frequency of MN, in accordance with the HPRT results from the same study. The authors suggested that melatonin activates the DNA repair activity of the cells, reducing these deleterious effects induced by arecoline.

The in vitro genotoxic potential of arecoline was also evaluated in cells of human origin. In fact, significant increases in the number of MN in human peripheral blood lymphocytes (PBLs) in the presence and absence of S9 as well as in the human hepatoma cell line Hep-G2 were reported by Kevekordes et al. (2001). In addition, the induction of CA and SCE by arecoline as well as by raw BN extract was also observed in human PBLs using a 72-h exposure protocol (Kumpawat et al. 2003). This report also explored mechanistic clues, namely the influence of superoxide dismutase (SOD) and GSH on the genotoxic effects observed, as well as the role of anoxic vs air incubation conditions in the % aberrant cells and SCE/metaphase. Importantly, the authors concluded that the formation of ROS by arecoline partially contributes to the induction of CA, since the clastogenicity of this alkaloid decreased with SOD post-treatment as well as in anoxic conditions. The depletion of GSH by buthionine sulfoximine (BSO) resulted in small increases in the % aberrant cells. In contrast for SCE formation, another mechanism is proposed involving p53-dependent delayed cell kinetics which may be relevant to allow more time for DNA repair.

In an attempt to gain mechanistic insights on the carcinogenicity of AN, Wang et al. (2010) showed that arecoline arrests Hep-2 and KB cells (considered HeLa derivative) at prometaphase with the presence of large quantities of misaligned chromosomes. Arecoline was also able to stabilize the mitotic spindle assembly and deregulate mitosis genes. As a consequence, arecoline-exposed cells exhibit chromosomal instability and a higher probability of aneuploidy and MN formation.

In vivo experiments In vivo cytogenetic experiments have been performed since the 1980′s addressing the genotoxicity of arecoline and related compounds. Arecoline was considered to exert in vivo weak chromosome breaking effects in Swiss albino mice, increasing in a dose–response manner the frequency of chromosome aberrations in bone-marrow cells (Panigrahi and Rao 1982). The pattern of the aberrations present included chromatid and chromosome breaks, rings, as well as cells with pulverized chromosomal components and cells with multiple breaks. In subsequent works, these authors reported the formation of SCE by arecoline (Panigrahi and Rao 1983), arecaidine (Panigrahi and Rao 1984), and BN extract (Panigrahi and Rao 1986) in bone-marrow cells using this in vivo animal model. Arecoline also revealed additive genotoxic effects in terms of SCEs in the presence of caffeine (Panigrahi and Rao 1983).

Shirname et al. (1984) reported increases in the frequency of MN in polychromatic erythrocytes (MNPCE) from male Swiss mice treated with arecoline, as well as with extracts of BN and of BQ with tobacco. Negative results were observed for arecaidine, extract of BQ alone, or BL. Moreover, arecoline induced the formation of MNPCE in fetal blood because of the transplacental exposure that occurred in pregnant Swiss albino mice during the late gestation phase (Sinha and Rao 1985b). Accordingly, the authors concluded that the placenta does not constitute an efficient barrier to arecoline.

Deb and Chatterjee (1998) evaluated the effect of arecoline (intraperitoneal administration, i.p.) alone as well as combined with BSO or GSH in mouse bone-marrow cells in vivo (Swiss albino mice). Either arecoline or BSO per se induced CA and SCE, but GSH alone only induced

SCE. Arecoline combined with BSO-mediated GSH depletion (20 mg/kg; 10 h) revealed an increase of CA and SCE frequencies when compared with arecoline alone. GSH (400 mg/kg) revealed to reduce the formation of CA by arecoline, although it was ineffective in the reduction of SCE. These authors also further studied the in vivo effects of arecoline in bone-marrow cells from Swiss albino mice following oral and intraperitoneal (i.p.) administration (Chatterjee and Deb 1999). At both administration routes, arecoline induced CA, SCE, and delays in the cell cycle. This alkaloid caused a higher level of CA 1 day after ip treatment as compared with the clastogenicity observed after 5 or 15 days (both similarly genotoxic). The oral treatment with arecoline induced chromosomal damage, but no differences among the three periods evaluated were detected. Cell cycle delay was observed 15 days after oral exposure to arecoline, with a greater percentage of first-cycle metaphases and a higher delay than after ip administration. Arecoline i.p. administration induced a delay in the cell cycle kinetics and there was a tendency of SCE towards a non-Poisson distribution. In view of this, it was considered that the induction of CA and the impairment of cell cycle kinetics could result from the formation of DNA adducts which mediated the cell division delay and induced SCEs. However, for oral administration of arecoline, induced delay in cell cycle progression was considered by these authors to probably result as a consequence of DNA damage repair. No linear increase was observed for i.p. treatment, although the SCE frequency was higher after 5- and 15-day exposure than after 1 day. Conversely, for oral treatment, there was a linear increase in SCEs frequency from day 1 to day 15. The authors considered that a higher DNA damage by arecoline administered orally can be explained by its metabolic conversion to potential carcinogens/mutagens that does not occur after i.p. administration. In this study, the addition of NAC revealed protection against cytotoxicity induced by arecoline through an increase of endogenous thiol levels. In addition, Kumpawat et al. (2003) studied not only the cytogenetic damage induced by arecoline and BN extracts in human PBLs in vitro as described above, but also the impact of these agents in mouse bone-marrow cells in vivo. The genotoxic effects of arecoline in this model were in the same line as with PBLs, although with some differences in terms of magnitude.

Mechanistic insights, conclusions, and future perspectives

The impact of AN and of its major constituents undoubtedly constitutes a pertinent issue in the global human health. Figure 5 displays many aspects related with AN and arecoline exposure and toxicological issues that should be taken into account. Moreover, the genetic toxicity of arecoline is clearly a complex topic that must be addressed considering all metabolites and nitrosation products as well as the interaction with other AN alkaloids and chemicals present in the AN or BQ extracts. Most of the authors cited herein concluded that arecoline per se or upon metabolic bioactivation displays a genotoxic potential that should be considered. This was demonstrated in multiple endpoints related to the formation of gene mutations (bacteria and mammalian cells), and different types of DNA lesions (DNA breaks and oxidative DNA damage), as well as in terms of cytogenetic damage observed in complementary endpoints (CA, MN and SCE) (Fig. 4). Nevertheless, it should also be mentioned that in some endpoints and upon certain experimental conditions, arecoline failed to reveal this significant genotoxic burden. In addition to these contradictory outcomes, in some reports, the positive genotoxic response of arecoline or arecoline's metabolite was considered slight. Conversely, in some studies with negative (non-significant) results in global terms, minor increases in the genetic damage could be observed (Wang et al. 2018) or a unique pattern of arecoline-induced mutations could be disclosed (Wu et al. 2012). It is also important to note that the magnitude of the positive responses observed varied among the endpoints and experimental conditions of the studies performed. Importantly, it is critical to understand the MoA of arecoline in the context of AN- or BQ-induced carcinogenesis. The possibility that arecoline may also exert its effects by non-genotoxic mechanisms cannot be excluded. These mechanisms should also be carefully regarded, although they are beyond of the scope of the present review.

In what concerns the exploration of the underlying mechanisms of genotoxicity of arecoline, several strategies have been described in the literature. The first key aspect is related to the impact of the metabolites and the classification of arecoline as a direct- or indirect-acting genotoxic compound. In this context, the relevance of some metabolites is obvious, particularly the active chemical AO. In addition, the genotoxicity of some nitrosation products, particularly NMPA has been highlighted. Moreover, the clear mutagenic responses upon metabolic activation, particularly in the Ames test, also support the key role of the metabolome. Nevertheless, arecoline is also able to induce significant genotoxic insults in diverse assays without external metabolic activation. This fact, along with the notion that the metabolite (i.e., AO) or the exogenous metabolic activation (S9) experiments usually originate larger yields of genetic damage than those observed for arecoline per se, suggests that this alkaloid displays features of both direct and indirect-acting genotoxic agents, predominantly from the latter.

The next question is how arecoline and/or its active metabolite(s) exert genetic toxicity. As depicted in Fig. 4 and above mentioned in this section, positive results can be found for almost all endpoints studied, suggesting genetic

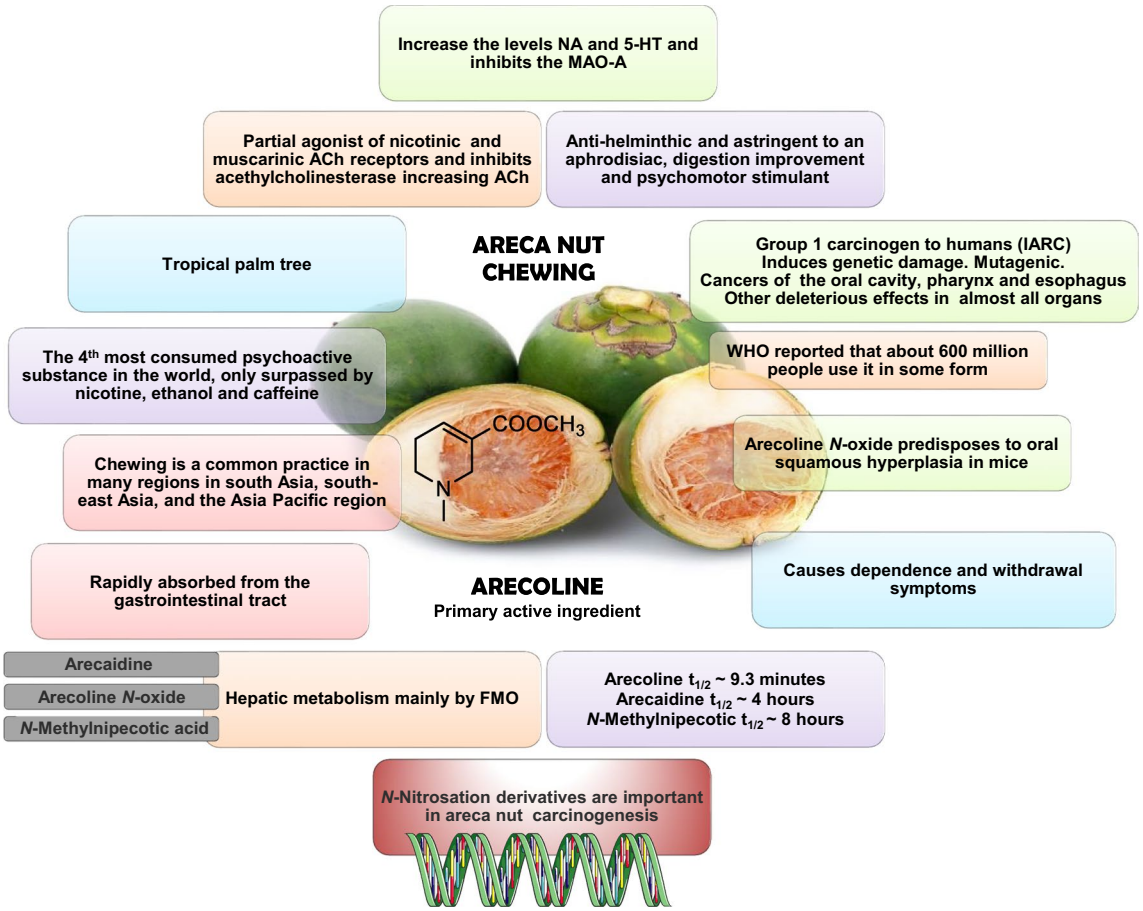

Fig. 5 Overview of key toxicological aspects associated with areca nut chewing and arecoline

damage at different levels and possibly various mechanisms of action involved. The conclusions from several publications focused in understanding arecoline's MoA reinforce this argument. Some mechanistic studies address only, or mainly, specific genotoxicity endpoints, while others are interested in additional deleterious features of arecoline or AN extracts towards malignant transformation. Overall, different mechanisms have been proposed in the literature. Previous studies suggested that arecoline is a biological alkylating chemical due to its ability to undergo addition reaction through its reactive ethylenic bond (Boyland and Nery 1969; Nery 1971). Therefore, and in view of the close correlation between DNA adducts and SCE formation, it was suggested the involvement of DNA adduction as a putative mechanism for arecoline's genotoxicity. As discussed by Chatterjee and Deb (1999), this alkaloid could lose one of its methyl groups and may bind to a nucleic acid and protein. The possible relevance of arecoline–DNA adducts is also present in other reports (Deb and Chatterjee 1998; Kumpawat et al. 2003; Chiang et al. 2007). Importantly, information relative to the impact of DNA adducts formed by electrophilic arecoline nitrosation products is also available. The generation of these nitrosamine-DNA adducts has been reported in different contexts (Prokopczyk et al. 1987; Chung et al. 1994; Bhattacharjee and Sharan 2008; IARC 2012). This topic was further reviewed by Sharan and Choudhury (2010), considering the formation of this particular type of adducts as a possible key event in the context of AN-induced carcinogenesis.

Additional mechanisms reported include the role of oxidative stress and ROS in the genotoxicity of arecoline and related compounds (Lai and Lee 2006; Shih et al. 2010; Shakya and Siddique, 2018). Some of the arguments in favor have already been mentioned and involve the generation of ROS, the depletion of cellular GSH, the induction of oxidative DNA damage, and increases in other oxidative stress biomarkers. In some studies, the authors modulated features associated with oxidative stress and gathered relevant mechanistic data (e.g., resorting to the use of GSH synthesis inhibitor BSO, enzymatic and non-enzymatic antioxidants, and anoxic conditions) (Chang et al. 2001; Kumpawat et al. 2003; Lai and Lee 2006; Wang et al. 2018; Shih et al. 2020).

In addition, some indications obtained from the UDS assay revealed that arecoline may lead to alterations in DNA

repair. Moreover, the impact of arecoline in DNA repair have been reported in different studies (Tsai et al. 2008, 2011; Lee et al. 2013; Huang et al. 2016; Tu et al. 2019; Shih et al. 2020) and should also be considered in a mechanistic perspective. Finally, another possibility already noted is that arecoline could induce alterations in the mitotic apparatus, being this possibly associated with aneuploidy events. Wang et al. (2010) reported that arecoline interferes with genome stability through the deregulation of spindle assembly and mitosis regulatory genes. Accordingly, arecoline could be able to arrest cells at prometaphase with many misaligned chromosomes that in turn may result in chromosome lagging, i.e., an aneugenic effect. Very recently, Li et al. (2020) also addressed this topic associating it to arecoline-induced mitochondrial dysfunction. The authors showed that the treatment of mouse oocytes with arecoline results in the disruption of actin filament dynamics, altering the spindle assembly and the kinetochore-microtubule attachment stability. Consequently, aneuploidy and oocyte meiosis arrest can be observed, clearly interfering with the quality of oocytes.

This review also aims at proposing novel strategies and intends to identify further aspects that warrant elucidation on the role of arecoline and related compounds in the carcinogenic events that occur in individuals that regularly consume AN or BQ. Physiologically relevant concentrations of AN alkaloids (i.e., similar to those found in the saliva of chewers) should be considered as well as long-term treatment protocols. The diversification of the cell models selected for the in vitro studies and experimental animal models for the in vivo studies is also important, since AN or BQ consumption may constitute increasing risk factors for other cancers than those that arise from the oral cavity and esophagus (e.g., hepatocellular carcinoma, lung cancer). In this context, we propose herein some topics considered pertinent to be explored: (i) Genotoxicity of arecoline or its major metabolite(s) in combination with other alkaloids and derivatives present in AN extracts, as well as with other oral cancer risk factors. These in vitro or in vivo experiments will allow to address the uncertainty of co-exposure scenarios; (ii) Systematic analysis of the endometabolome (intracellular metabolites) and exometabolome (metabolites secreted in the culture medium) of normal and oral cancer cell lines in vitro using LC–MS–MS techniques; (iii) Comprehensive cytome analysis, with particular emphasis in the MN evaluation, performed either with buccal cells or PBLs. The characterization of the content of the MN is also important for the discrimination between a clastogenic and aneugenic MoA for arecoline; (iv) Genotoxicity assays and related endpoints performed in more physiologically relevant culture systems, particularly in three-dimensional (3D) cultures. These in vitro experiments can be performed using normal human cells from oral mucosa as well as cells from other target organs or, alternatively, can be done using co-cultures of oral cancer cells in the presence of additional cell types of the tumor microenvironment (e.g., fibroblasts). Since the liver is also thought to be an organ at risk in the context AN or BQ exposure (Wang et al. 2018), advanced models, such as human stem cell-derived hepatocyte-like cells (Cipriano et al. 2017a, b, 2020) could also be very informative; (v) Further elucidation on the potential formation of DNA adducts in cultured cells exposed to AN, BQ, arecoline, and related chemicals; (vi) Migration and invasion experiments performed to assess the impact of arecoline and related compounds in more aggressive phenotypes. The information obtained should be integrated with data from genotoxicity endpoints; and finally, (vii) Additional DNA repair mechanistic insights, a topic somehow overlooked, although some more recent studies have been published (e.g., Huang et al. 2016; Tu et al. 2019; Shih et al. 2020). Nevertheless, further information could be obtained in vitro with pharmacological inhibitors for different DNA repair pathways that can be used as mechanistic tools or using siRNA technology towards key DNA repair enzymes. These aspects of arecoline's genetic toxicology and toxicokinetics along with an integrated view of other arecoline-induced health effects, including the unraveling of the hallmarks of cancer associated with AN exposure, would be determinant for a better understanding of the impact of this complex toxicological issue.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

References

Al-Waiz M, Ayesh R, Mitchell SC, Idle JR, Smith RL (1987) Disclosure of the metabolic retroversion of trimethylamine N-oxide in humans: a pharmacogenetic approach. Clin Pharmacol Ther 42(6):608–612. https://doi.org/10.1038/clpt.1987.207

Arora S, Squier C (2019) Areca nut trade, globalisation and its health impact: perspectives from India and South-east Asia. Perspect Public Health 139(1):44–48. https://doi.org/10.1177/1757913918785398

Asthana S, Greig NH, Holloway HW et al (1996) Clinical pharmacokinetics of arecoline in subjects with Alzheimer's disease. Clin Pharmacol Ther 60(3):276–282. https://doi.org/10.1016/s0009-9236(96)90054-5

Bales A, Peterson MJ, Ojha S, Upadhaya K, Adhikari B, Barrett B (2009) Associations between betel nut (Areca catechu) and symptoms of schizophrenia among patients in Nepal: A longitudinal

study. Psychiatry Res 169(3):203–211. https://doi.org/10.1016/j.psychres.2008.06.006

Bhat SJ, Blank MD, Balster RL, Nichter M, Nichter M (2010) Areca nut dependence among chewers in a South Indian community who do not also use tobacco. Addiction 105(7):1303–1310. https://doi.org/10.1111/j.1360-0443.2010.02952.x

Bhattacharjee C, Sharan RN (2008) Aqueous extract of betel nut-induced adducts on pMTa4 DNA acquires stability in the presence of Na^+ and K^+ ions. Mol Med Rep 1(3):435–441. https://doi.org/10.3892/mmr.1.3.435

Boyland E, Nery R (1969) Mercapturic acid formation during the metabolism of arecoline and arecaidine in the rat. Biochem J 113(1):123–130. https://doi.org/10.1042/bj1130123

Burton-Bradley BG (1978) Betel chewing in retrospect. P N G Med J 21(3):236–241

Cashman JR, Zhang J (2006) Human flavin-containing monooxygenases. Annu Rev Pharmacol Toxicol 46:65–100. https://doi.org/10.1146/annurev.pharmtox.46.120604.141043

Chang YC, Tai KW, Cheng MH, Chou LS, Chou MY (1998) Cytotoxic and non-genotoxic effects of arecoline on human buccal fibroblasts in vitro. J Oral Pathol Med 27(2):68–71. https://doi.org/10.1111/j.1600-0714.1998.tb02096.x

Chang MC, Ho YS, Lee PH et al (2001) Areca nut extract and arecoline induced the cell cycle arrest but not apoptosis of cultured oral KB epithelial cells: association of glutathione, reactive oxygen species and mitochondrial membrane potential. Carcinogenesis 22(9):1527–1535. https://doi.org/10.1093/carcin/22.9.1527

Chatterjee A, Deb S (1999) Genotoxic effect of arecoline given either by the peritoneal or oral route in murine bone marrow cells and the influence of N-acetylcysteine. Cancer Lett 139(1):23–31. https://doi.org/10.1016/s0304-3835(98)00364-4

Chiang SL, Jiang SS, Wang YJ et al (2007) Characterization of arecoline-induced effects on cytotoxicity in normal human gingival fibroblasts by global gene expression profiling. Toxicol Sci 100(1):66–74. https://doi.org/10.1093/toxsci/kfm201

Chou WW, Guh JY, Tsai JF et al (2008) Arecoline-induced growth arrest and p21WAF1 expression are dependent on p53 in rat hepatocytes. Toxicology 243(1–2):1–10. https://doi.org/10.1016/j.tox.2007.09.003

Chou C-H, Chuang L-Y, Tseng W-L, Lu C-Y (2012) Characterization of protein adducts formed by toxic alkaloids by nano-scale liquid chromatography with mass spectrometry. J Mass Spectrom 47(10):1303–1312. https://doi.org/10.1002/jms.3083

Chuerduangphui J, Ekalaksananan T, Chaiyarit P et al (2018) Effects of arecoline on proliferation of oral squamous cell carcinoma cells by dysregulating c-Myc and miR-22, directly targeting oncostatin M. PLoS ONE 13(1):e0192009. https://doi.org/10.1371/journal.pone.0192009

Chung FL, Krzeminski J, Wang M, Chen HJ, Prokopczyk B (1994) Formation of the acrolein-derived 1, N2-propanodeoxyguanosine adducts in DNA upon reaction with 3-(N-carbethoxy-N-nitrosamino)propionaldehyde. Chem Res Toxicol 7(1):62–67. https://doi.org/10.1021/tx00037a009

Cipriano M, Freyer N, Knöspel F et al (2017a) Self-assembled 3D spheroids and hollow-fibre bioreactors improve MSC-derived hepatocyte-like cell maturation in vitro. Arch Toxicol 91(4):1815–1832. https://doi.org/10.1007/s00204-016-1838-0

Cipriano M, Correia JC, Camões SP et al (2017b) The role of epigenetic modifiers in extended cultures of functional hepatocyte-like cells derived from human neonatal mesenchymal stem cells. Arch Toxicol 91(6):2469–2489. https://doi.org/10.1007/s00204-016-1901-x

Cipriano M, Pinheiro PF, Sequeira CO et al (2020) Nevirapine biotransformation insights: an integrated in vitro approach unveils the biocompetence and glutathiolomic profile of a human hepatocyte-like cell 3D model. Int J Mol Sci 21(11):3998. https://doi.org/10.3390/ijms21113998

Coppola M, Mondola R (2012) Potential action of betel alkaloids on positive and negative symptoms of schizophrenia: a review. Nord J Psychiatry 66(2):73–78. https://doi.org/10.3109/08039488.2011.605172

Dave BJ, Trivedi AH, Adhvaryu SG (1992) In vitro genotoxic effects of areca nut extract and arecoline. J Cancer Res Clin Oncol 118(4):283–288. https://doi.org/10.1007/BF01208617

Deb S, Chatterjee A (1998) Influence of buthionine sulfoximine and reduced glutathione on arecoline-induced chromosomal damage and sister chromatid exchange in mouse bone marrow cells in vivo. Mutagenesis 13(3):243–248. https://doi.org/10.1093/mutage/13.3.243

Doucette R, Fisman M, Hachinski VC, Mersky H (1986) Cell loss from the nucleus basalis of Meynert in Alzheimer's disease. Can J Neurol Sci 13(S4):435–440. https://doi.org/10.1017/S0317167100037070

Franke AA, Li X, Custer LJ, Lai JF (2020) Chemical markers for short- and long-term areca nut exposure. Subst Use Misuse 55(9):1395–1402. https://doi.org/10.1080/10826084.2019.1630442

Garg A, Chaturvedi P, Gupta PC (2014) A review of the systemic adverse effects of areca nut or betel nut. Indian J Med Paediatr Oncol 35(1):3–9. https://doi.org/10.4103/0971-5851.133702

Gheddar L, Ricaut FX, Ameline A et al (2020) Testing for betel nut alkaloids in hair of papuans abusers using UPLC-MS/MS and UPLC-Q-Tof-MS. J Anal Toxicol 44(1):41–48. https://doi.org/10.1093/jat/bkz045

Giri S, Idle JR, Chen C, Zabriskie TM, Krausz KW, Gonzalez FJ (2006) A metabolomic approach to the metabolism of the areca nut alkaloids arecoline and arecaidine in the mouse. Chem Res Toxicol 19(6):818–827. https://doi.org/10.1021/tx0600402

Giri S, Krausz KW, Idle JR, Gonzalez FJ (2007) The metabolomics of (+/-)-arecoline 1-oxide in the mouse and its formation by human flavin-containing monooxygenases. Biochem Pharmacol 73(4):561–573. https://doi.org/10.1016/j.bcp.2006.10.017

Guengerich FP, Vaz ADN, Raner GN, Pernecky SJ, Coon MJ (1997) Evidence for a role of a perferryl-oxygen complex, FeO^{3+}, in the N-oxygenation of amines by cytochrome P450 enzymes. Mol Pharmacol 51(1):147–151. https://doi.org/10.1124/mol.51.1.147

Gupta AK, Tulsyan S, Thakur N, Sharma V, Sinha DN, Mehrotra R (2020) Chemistry, metabolism and pharmacology of carcinogenic alkaloids present in areca nut and factors affecting their concentration. Regul Toxicol Pharmacol 110:104548. https://doi.org/10.1016/j.yrtph.2019.104548

Hayes MJ, Khemani L, Bax M, Alkalay D (1989) Quantitative determination of arecoline in plasma by gas chromatography chemical ionization mass spectrometry. Biomed Environ Mass Spectrom 18(11):1005–1009. https://doi.org/10.1002/bms.1200181109

Hu CW, Chang YZ, Wang HW, Chao MR (2010) High-throughput simultaneous analysis of five urinary metabolites of areca nut and tobacco alkaloids by isotope-dilution liquid chromatography-tandem mass spectrometry with on-line solid-phase extraction. Cancer Epidemiol Biomark Prev 19(10):2570–2581. https://doi.org/10.1158/1055-9965.Epi-10-0483

Huang JL, Lu HH, Lu YN et al (2016) Enhancement of the genotoxicity of benzo[a]pyrene by arecoline through suppression of DNA repair in HEp-2 cells. Toxicol In Vitro 33:80–87. https://doi.org/10.1016/j.tiv.2016.02.007

IARC (2004) Betel-quid and areca-nut chewing and some areca-nut derived nitrosamines. IARC Monogr Eval Carcinog Risks Hum 85:1–334

IARC (2012) Personal habits and indoor combustions. Volume 100 E. A review of human carcinogens. IARC Monogr Eval Carcinog Risks Hum 100(Pt E):1–538

IARC (2019) Report of the advisory group to recommend priorities for the IARC monographs during 2020–2024. Available from: https://monographs.iarc.fr/wp-content/uploads/2019/10/IARCMonographs-AGReport-Priorities_2020-2024.pdf

Javed F, Bello Correra FO, Chotai M, Tappuni AR, Almas K (2010) Systemic conditions associated with areca nut usage: a literature review. Scand J Public Health 38(8):838–844. https://doi.org/10.1177/1403494810379291

Jeng JH, Kuo ML, Hahn LJ, Kuo MY (1994) Genotoxic and non-genotoxic effects of betel quid ingredients on oral mucosal fibroblasts in vitro. J Dent Res 73(5):1043–1049. https://doi.org/10.1177/00220345940730050501

Jeng JH, Hahn LJ, Lin BR, Hsieh CC, Chan CP, Chang MC (1999) Effects of areca nut, inflorescence piper betle extracts and arecoline on cytotoxicity, total and unscheduled DNA synthesis in cultured gingival keratinocytes. J Oral Pathol Med 28(2):64–71. https://doi.org/10.1111/j.1600-0714.1999.tb01998.x

Kevekordes S, Mersch-Sundermann V, Burghaus CM et al (1999) SOS induction of selected naturally occurring substances in Escherichia coli (SOS chromotest). Mutat Res 445(1):81–91. https://doi.org/10.1016/s1383-5718(99)00141-2

Kevekordes S, Spielberger J, Burghaus CM et al (2001) Micronucleus formation in human lymphocytes and in the metabolically competent human hepatoma cell line Hep-G2: results with 15 naturally occurring substances. Anticancer Res 21(1A):461–469

Klaassen CD (2019) Casarett & Doull's toxicology: the basic science of poisons, 9th edn. McGraw-Hill Education, New York

Ko AM, Lee CH, Ko AM, Ko YC (2020) Betel quid dependence mechanism and potential cessation therapy. Prog Neuropsychopharmacol Biol Psychiatry 103:109982. https://doi.org/10.1016/j.pnpbp.2020.109982

Kumar Srivastava V (2014) To study the prevalence of premalignancies in teenagers having betel, gutkha, khaini, tobacco chewing, beedi and ganja smoking habit and their association with social class and education status. Int J Clin Pediatr Dent 7(2):86–92. https://doi.org/10.5005/jp-journals-10005-1243

Kumpawat K, Deb S, Ray S, Chatterjee A (2003) Genotoxic effect of raw betel-nut extract in relation to endogenous glutathione levels and its mechanism of action in mammalian cells. Mutat Res 538(1–2):1–12. https://doi.org/10.1016/s1383-5718(03)00048-2

Kuo TM, Luo SY, Chiang SL et al (2015) Fibrotic effects of arecoline N-oxide in oral potentially malignant disorders. J Agric Food Chem 63(24):5787–5794. https://doi.org/10.1021/acs.jafc.5b01351

Kuo TM, Nithiyanantham S, Lee CP et al (2019) Arecoline N-oxide regulates oral squamous cell carcinoma development through NOTCH1 and FAT1 expressions. J Cell Physiol 234(8):13984–13993. https://doi.org/10.1002/jcp.28084

Lai KC, Lee TC (2006) Genetic damage in cultured human keratinocytes stressed by long-term exposure to areca nut extracts. Mutat Res 599(1–2):66–75. https://doi.org/10.1016/j.mrfmmm.2006.01.005

Lai ZL, Tsou YA, Fan SR et al (2014) Methylation-associated gene silencing of RARB in areca carcinogens induced mouse oral squamous cell carcinoma. Biomed Res Int 2014:378358. https://doi.org/10.1155/2014/378358

Lee CH, Lin RH, Liu SH, Lin-Shiau SY (1996) Mutual interactions among ingredients of betel quid in inducing genotoxicity on Chinese hamster ovary cells. Mutat Res 367(2):99–104. https://doi.org/10.1016/0165-1218(95)00081-x

Lee CH, Ko AM, Yen CF et al (2012) Betel-quid dependence and oral potentially malignant disorders in six Asian countries. Br J Psychiatry 201(5):383–391. https://doi.org/10.1192/bjp.bp.111.107961

Lee SS, Tsai CH, Yu CC, Ho YC, Hsu HI, Chang YC (2013) The expression of O(6) -methylguanine-DNA methyltransferase in human oral keratinocytes stimulated with arecoline. J Oral Pathol Med 42(8):600–605. https://doi.org/10.1111/jop.12037

Li L, Luo Z, Liu Y et al (2017) Screening and identification of the metabolites in rat plasma and urine after oral administration of *Areca catechu* L. nut extract by ultra-high-pressure liquid chromatography coupled with linear ion trap-orbitrap tandem mass spectrometry. Molecules 22(6):1026. https://doi.org/10.3390/molecules22061026

Li WD, Zang CJ, Yin S, Shen W, Sun QY, Zhao M (2020) Metformin protects against mouse oocyte apoptosis defects induced by arecoline. Cell Prolif 53(7):e12809. https://doi.org/10.1111/cpr.12809

Lin CF, Wang JD, Chen PH, Chang SJ, Yang YH, Ko YC (2006) Predictors of betel quid chewing behavior and cessation patterns in Taiwan aborigines. BMC Public Health 6:271. https://doi.org/10.1186/1471-2458-6-271

Lin KH, Lin CY, Liu CC, Chou MY, Lin JK (2011) Arecoline N-oxide: its mutagenicity and possible role as ultimate carcinogen in areca oral carcinogenesis. J Agric Food Chem 59(7):3420–3428. https://doi.org/10.1021/jf104831n

López-Vilchez MA, Seidel V, Farré M, García-Algar O, Pichini S, Mur A (2006) Areca-nut abuse and neonatal withdrawal syndrome. Pediatrics 117(1):e129–e131. https://doi.org/10.1542/peds.2005-0281

Mehrtash H, Duncan K, Parascandola M et al (2017) Defining a global research and policy agenda for betel quid and areca nut. Lancet Oncol 18(2):e767–e775. https://doi.org/10.1016/S1470-2045(17)30460-6

Miyazaki M, Sugawara E, Yoshimura T, Yamazaki H, Kamataki T (2005) Mutagenic activation of betel quid-specific N-nitrosamines catalyzed by human cytochrome P450 coexpressed with NADPH-cytochrome P450 reductase in *Salmonella typhimurium* YG7108. Mutat Res 581(1–2):165–171. https://doi.org/10.1016/j.mrgentox.2004.12.002

Molinengo L, Cassone MC, Orsetti M (1986) Action of arecoline on the levels of acetylcholine, norepinephrine and dopamine in the mouse central nervous system. Pharmacol Biochem Behav 24(6):1801–1803. https://doi.org/10.1016/0091-3057(86)90525-3

Nery R (1971) The metabolic interconversion of arecoline and arecoline 1-oxide in the rat. Biochemical J 122(4):503–508. https://doi.org/10.1042/bj1220503

Ni LF, Dai YT, Su TC, Hu WY (2013) Substance use, gender, socioeconomic status and metabolic syndrome among adults in Taiwan. Public Health Nurs 30(1):18–28. https://doi.org/10.1111/j.1525-1446.2012.01039.x

Osborne PG, Chou TS, Shen TW (2011) Characterization of the psychological, physiological and EEG profile of acute betel quid intoxication in naïve subjects. PLoS ONE 6(8):e23874. https://doi.org/10.1371/journal.pone.0023874

Pan H, Huang L, Li Y, Zhou X, Lu Y, Shi F (2017) Liquid chromatography-tandem mass spectrometric assay for determination of unstable arecoline in rat plasma and its application. J Chromatogr B Anal Technol Biomed Life Sci 1070:112–116. https://doi.org/10.1016/j.jchromb.2017.10.026

Pan H, Li Y, Huang L, Zhou X, Lu Y, Shi F (2018) Development and validation of a rapid LC-MS/MS method for simultaneous quantification of arecoline and its two active metabolites in rat plasma and its application to a pharmacokinetic study. J Pharm Biomed Anal 154:397–403. https://doi.org/10.1016/j.jpba.2018.03.033

Panigrahi GB, Rao AR (1982) Chromosome-breaking ability of arecoline, a major betel-nut alkaloid, in mouse bone-marrow cells in vivo. Mutat Res 103(2):197–204. https://doi.org/10.1016/0165-7992(82)90029-x

Panigrahi GB, Rao AR (1983) Influence of caffeine on arecoline-induced SCE in mouse bone-marrow cells in vivo. Mutat

Res 122(3–4):347–353. https://doi.org/10.1016/0165-7992(83)90018-0
Panigrahi GB, Rao AR (1984) Induction of in vivo sister chromatid exchanges by arecaidine, a betel nut alkaloid, in mouse bone-marrow cells. Cancer Lett 23(2):189–192. https://doi.org/10.1016/0304-3835(84)90153-8
Panigrahi GB, Rao AR (1986) Study of the genotoxicity of the total aqueous extract of betel nut and its tannin. Carcinogenesis 7(1):37–39. https://doi.org/10.1093/carcin/7.1.37
Patidar KA, Parwani R, Wanjari SP, Patidar AP (2015) Various terminologies associated with areca nut and tobacco chewing: a review. J Oral Maxillofac Pathol 19(1):69–76. https://doi.org/10.4103/0973-029X.157205
Patterson TA, Kosh JW (1993) Elucidation of the rapid in vivo metabolism of arecoline. Gen Pharmacol 24(3):641–647. https://doi.org/10.1016/0306-3623(93)90224-l
Perry EK, Blessed G, Tomlinson BE et al (1981) Neurochemical activities in human temporal lobe related to aging and Alzheimer-type changes. Neurobiol Aging 2(4):251–256. https://doi.org/10.1016/0197-4580(81)90032-4
Phillips IR, Shephard EA (2017) Drug metabolism by flavin-containing monooxygenases of human and mouse. Expert Opin Drug Metab Toxicol 13(2):167–181. https://doi.org/10.1080/17425255.2017.1239718
Prokopczyk B, Rivenson A, Bertinato P, Brunnemann KD, Hoffmann D (1987) 3-(Methylnitrosamino)propionitrile: occurrence in saliva of betel quid chewers, carcinogenicity, and DNA methylation in F344 rats. Cancer Res 47(2):467–471
Raffaele KC, Asthana S, Berardi A et al (1996) Differential response to the cholinergic agonist arecoline among different cognitive modalities in Alzheimer's disease. Neuropsychopharmacology 15(2):163–170. https://doi.org/10.1016/0893-133x(95)00179-h
Rehman A, Ali S, Lone MA et al (2016) Areca nut alkaloids induce irreparable DNA damage and senescence in fibroblasts and may create a favorable environment for tumour progression. J Oral Pathol Med 45(5):365–372. https://doi.org/10.1111/jop.12370
Sariah A, Guo S, Zuo J et al (2020) Acute and chronic effects of betel quid chewing on brain functional connectivity. Front Psychiatry 11:198. https://doi.org/10.3389/fpsyt.2020.00198
Sethy VH, Francis JW (1988) Regulation of brain acetylcholine concentration by muscarinic receptors. J Pharmacol Exp Ther 246(1):243–248
Shah SM, Merchant AT, Luby SP, Chotani RA (2002) Addicted schoolchildren: prevalence and characteristics of areca nut chewers among primary school children in Karachi, Pakistan. J Paediatr Child Health 38(5):507–510. https://doi.org/10.1046/j.1440-1754.2002.00040.x
Shakya B, Siddique YH (2018) Evaluation of the toxic potential of arecoline toward the third instar larvae of transgenic *Drosophila melanogaster* (hsp70-lacZ) Bg^9. Toxicol Res 7(3):432–443. https://doi.org/10.1039/c7tx00305f
Sharan RN, Wary KK (1992) Study of unscheduled DNA synthesis following exposure of human cells to arecoline and extracts of betel nut in vitro. Mutat Res 278:271–276. https://doi.org/10.1016/s0165-1218(10)80007-2
Sharan RN, Choudhury Y (2010) Betel nut and susceptibility to cancer. In: Roy D, Dorak MT (eds) Environmental factors, genes, and the development of human cancers. Springer-Verlag, New York, pp 401–428
Sharan RN, Mehrotra R, Choudhury Y, Asotra K (2012) Association of betel nut with carcinogenesis: revisit with a clinical perspective. PLoS ONE 7(8):e42759. https://doi.org/10.1371/journal.pone.0042759
Shephard EA, Phillips IR (2010) The potential of knockout mouse lines in defining the role of flavin-containing monooxygenases in drug metabolism. Expert Opin Drug Metab Toxicol 6(9):1083–1094. https://doi.org/10.1517/17425255.2010.503705
Shetty HU, Daly EM, Greig NH, Rapoport SI, Soncrant TT (1991) An automatic reaction control chemical ionization technique in ion trap detector for quantitative plasma profding of arecoline in treated alzheimer patients. J Am Soc Mass Spectrom 2(2):168–173. https://doi.org/10.1016/1044-0305(91)80011-u
Shih YT, Chen PS, Wu CH, Tseng YT, Wu YC, Lo YC (2010) Arecoline, a major alkaloid of the areca nut, causes neurotoxicity through enhancement of oxidative stress and suppression of the antioxidant protective system. Free Radic Biol Med 49(10):1471–1479. https://doi.org/10.1016/j.freeradbiomed.2010.07.017
Shih YH, Chiu KC, Wang TH et al (2020) Effects of melatonin to arecoline-induced reactive oxygen species production and DNA damage in oral squamous cell carcinoma. J Formos Med Assoc. https://doi.org/10.1016/j.jfma.2020.07.037
Shirname LP, Menon MM, Nair J, Bhide SV (1983) Correlation of mutagenicity and tumorigenicity of betel quid and its ingredients. Nutr Cancer 5(2):87–91. https://doi.org/10.1080/01635588309513783
Shirname LP, Menon MM, Bhide SV (1984) Mutagenicity of betel quid and its ingredients using mammalian test systems. Carcinogenesis 5(4):501–503. https://doi.org/10.1093/carcin/5.4.501
Sinha A, Rao AR (1985a) Induction of shape abnormality and unscheduled DNA synthesis by arecoline in the germ cells of mice. Mutat Res 158(3):189–192. https://doi.org/10.1016/0165-1218(85)90083-7
Sinha A, Rao AR (1985b) Transplacental micronucleus inducing ability of arecoline, a betel nut alkaloid, in mice. Mutat Res 158(3):193–194. https://doi.org/10.1016/0165-1218(85)90084-9
Soncrant TT, Holloway HW, Greig NH, Rapoport SI (1989) Regional brain metabolic responsivity to the muscarinic cholinergic agonist arecoline is similar in young and aged Fischer-344 rats. Brain Res 487(2):255–266. https://doi.org/10.1016/0006-8993(89)90830-5
Stich HF, Stich W, Lam PP (1981) Potentiation of genotoxicity by concurrent application of compounds found in betel quid: arecoline, eugenol, quercetin, chlorogenic acid and Mn^{2+}. Mutat Res 90(4):355–363. https://doi.org/10.1016/0165-1218(81)90058-6
Strickland SS, Veena GV, Houghton PJ, Stanford SC, Kurpad AV (2003) Areca nut, energy metabolism and hunger in Asian men. Ann Hum Biol 30(1):26–52. https://doi.org/10.1080/0301446021015744 8
Sullivan RJ, Andres S, Otto C, Miles W, Kydd R (2007) The effects of an indigenous muscarinic drug, Betel nut (*Areca catechu*), on the symptoms of schizophrenia: a longitudinal study in Palau, Micronesia. Am J Psychiatry 164(4):670–673. https://doi.org/10.1176/ajp.2007.164.4.670
Sundqvist K, Liu Y, Nair J, Bartsch H, Arvidson K, Grafström RC (1989) Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. Cancer Res 49(19):5294–5298
Trivedi AH, Dave BJ, Adhvaryu SC (1993) Genotoxic effects of nicotine in combination with arecoline on CHO cells. Cancer Lett 74(1–2):105–110. https://doi.org/10.1016/0304-3835(93)90051-A
Tsai YS, Lee KW, Huang JL et al (2008) Arecoline, a major alkaloid of areca nut, inhibits p53, represses DNA repair, and triggers DNA damage response in human epithelial cells. Toxicology 249(2–3):230–237. https://doi.org/10.1016/j.tox.2008.05.007
Tsai YS, Lin CS, Chiang SL, Lee CH, Lee KW, Ko YC (2011) Areca nut induces miR-23a and inhibits repair of DNA double-strand breaks by targeting FANCG. Toxicol Sci 123(2):480–490. https://doi.org/10.1093/toxsci/kfr182

Tu HF, Chen MY, Lai JC et al (2019) Arecoline-regulated ataxia telangiectasia mutated expression level in oral cancer progression. Head Neck 41(8):2525–2537. https://doi.org/10.1002/hed.25718

Ujváry I (2014) Psychoactive natural products: overview of recent developments. Ann Ist Super Sanita 50(1):12–27. https://doi.org/10.4415/ann_14_01_04

Volgin AD, Bashirzade A, Amstislavskaya TG et al (2019) DARK classics in chemical neuroscience: arecoline. ACS Chem Neurosci 10(5):2176–2185. https://doi.org/10.1021/acschemneuro.8b00711

Wang CK, Peng CH (1996) The mutagenicities of alkaloids and *N*-nitrosoguvacoline from betel quid. Mutat Res 360(3):165–171. https://doi.org/10.1016/s0165-1161(96)90013-8

Wang YC, Lee CM, Lew-Ting CY, Hsiao CK, Chen DR, Chen WJ (2005) Survey of substance use among high school students in Taipei: web-based questionnaire versus paper-and-pencil questionnaire. J Adolesc Health 37(4):289–295. https://doi.org/10.1016/j.jadohealth.2005.03.017

Wang YC, Tsai YS, Huang JL et al (2010) Arecoline arrests cells at prometaphase by deregulating mitotic spindle assembly and spindle assembly checkpoint: implication for carcinogenesis. Oral Oncol 46(4):255–262. https://doi.org/10.1016/j.oraloncology.2010.01.003

Wang TS, Lin CP, Chen YP, Chao MR, Li CC, Liu KL (2018) CYP450-mediated mitochondrial ROS production involved in arecoline N-oxide-induced oxidative damage in liver cell lines. Environ Toxicol 33:1029–1038. https://doi.org/10.1002/tox.22588

Wary KK, Sharan RN (1988) Aqueous extract of betel-nut of north-east India induces DNA-strand breaks and enhances rate of cell proliferation in vitro. Effects of betel-nut extract in vitro. J Cancer Res Clin Oncol 114(6):579–582. https://doi.org/10.1007/BF00398180

Wiesner DM (1987) Betel-nut withdrawal. Med J Australia 146(8):453

Winstock A (2002) Areca nut-abuse liability, dependence and public health. Addict Biol 7(1):133–138. https://doi.org/10.1080/13556210120091509

Winstock AR, Trivedy CR, Warnakulasuriya KA, Peters TJ (2000) A dependency syndrome related to areca nut use: some medical and psychological aspects among areca nut users in the Gujarat community in the UK. Addict Biol 5(2):173–179. https://doi.org/10.1080/13556210050003766

Wu M, Xing G, Qi X et al (2012) Assessment of the mutagenic potential of arecoline in gpt delta transgenic mice. Mutat Res 748(1–2):65–69. https://doi.org/10.1016/j.mrgentox.2012.07.001

Ziegler DM (1993) Recent studies on the structure and function of multisubstrate flavin-containing monooxygenases. Annu Rev Pharmacol Toxicol 33(1):179–199. https://doi.org/10.1146/annurev.pa.33.040193.001143