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Mechanism of DNA damage induced by arecaidine: The role of Cu(II) and alkaline conditions

Weijun Chen [a], Xiaoling Liu [b], Junling Shi [b,*], Yajun Zheng [a], Songlin Zhao [a,**], Jiankang Liu [c]

[a] Coconut Research Institute, Chinese Academy of Tropical Agriculture Sciences, Wenchang, Hainan 571339, China
[b] School of Food Science and Engineering, Northwest A&F University, Yangling, Shaanxi 712100, China
[c] Institute of Mitochondrial Biology and Medicine, Department of Biology and Engineering, The Key Laboratory of Biomedical Information Engineering of Ministry of Education, Xi'an Jiaotong University School of Life Science and Technology, Xi'an, Shaanxi, 710049, China

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ABSTRACT

Betel quid chewing is a widely prevalent habit correlated with a high incidence of oral cancer. However, the underlying mechanism of the carcinogenicity of betel quid chewing is poorly understood. In the present study, the carcinogenic mechanism of action of betel quid chewing was examined by determining DNA damage induced by arecaidine and Cu(II). It was found that arecaidine alone had no significant effect on inducing DNA damage, but it caused significant DNA double stand breaks in the presence of Cu(II) ions under alkaline conditions. Further studies showed that reactive oxygen species were generated and Cu(I) was formed in the reaction. The arecaidine anion exhibited a lower IP and a higher HOMO energy than arecaidine itself, suggesting an increased ability to donate electrons under alkaline pH conditions. These results suggest that the presence of Cu(II) and alkaline conditions are two essential factors in arecaidine-induced DNA damage, a contributing factor to oral cancer occurrence.

1. Introduction

Betel (*Areca catechu* L.) quid (BQ) chewing is a widely prevalent oral habit in Taiwan, India, Papua New Guinea, South Africa and other Southeast Asian countries (Thomas & Maclennan, 1992). Betel is the fourth most commonly used drug worldwide, after tobacco, alcohol, and caffeine. It is reported that there are about 600 million BQ-chewers living in different regions of the world (Sharan, 1996). In China, betel has long been used in traditional Chinese medicine, mainly as an anthelmintic for parasites of the intestinal tract. BQ chewing was probably introduced into China during the Ming Dynasty (A.D. 1368–1644); today the main areas of consumption are in the southern provinces of Mainland China, including the provinces of Hunan, Hainan, and Yunnan. Reported prevalence of BQ chewing in Hunan Province is as high as 64.5–82.7% (Zhang & Peter, 2007). The BQ habit in Hunan consists of chewing the dried husks and nuts, while the fresh nut is chewed in Hainan Province.

Recently, epidemiological studies have correlated BQ chewing with a high incidence of oral leukoplakia, oral submucous fibrosis, and oral cancer (Ko, Chiang, Chang, & Hsieh, 1992). However, the molecular mechanism underlying the carcinogenicity of areca nut is poorly understood. Hung et al. (2009) suggests that Asb6 upregulation by areca nut extract could act as a prognostic marker for oral cancer, and Lee (Lee et al., 2008) finds that HO-1 expression and HSP70 are also significantly upregulated in oral squamous cell carcinomas occurring in areca quid chewers. More and more studies have pointed out that areca nut extracts (ANE) and lime induce DNA damage *in vitro* by generating reactive oxygen species (ROS) (Chang et al., 2001; Chen, Chi, & Liu, 2002; Liu, Chen, & Chi, 1996; Manashi et al., 2002), and this process can be enhanced by the presence of metal ions (Nair et al., 1987). However, whether the arecaidine, the major biocompound in betel quid, bears a part in DNA damage caused by ANE is unclear. Evidence indicates that, although other chemical compounds have been identified in the areca nut (IARC, 1985; Van der Bijl, Stockenström, & Vismer, 1996; Van der Bijl, Stockenström, Vismer, & Van Wyk, 1997), the two major alkaloids present, arecoline and arecaidine, may play an important role in the cancer pathogenesis. In addition, there has been recent interest in the role of copper as a possible etiological factor in the development of oral cancer because areca nut products are found to contain a high level of copper (Trivedy, Baldwin, Warnakulasuriya, Johnson, & Peters, 1997); areca-chewing may raise salivary levels of soluble copper (Kishor, Vinay, Timothy, & Saman, 2007), an ion reported to be involved in DNA damage (David, 2002). In this paper, DNA damage induced by arecaidine was studied by agarose gel electrophoresis; the roles of Cu(II) ion and alkaline conditions were investigated. The interaction between arecaidine and Cu(II), the

* Corresponding author. Tel.: +86 029 87092486.
** Corresponding author. Tel.: +86 0898 63331201.
E-mail addresses: chenwj@nwu.edu.cn (W. Chen), sjlshi2004@yahoo.com.cn (S. Zhao).

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generation of ROS, and the reduction of Cu(II) were measured to clearly assess their ability to induce DNA damage. Density functional theory (DFT) calculations were performed to explain the variation in electron-donating ability of arecaidine at different pHs. The results show that Cu(II) and alkaline conditions play important roles in the plasmid pBR322 DNA damage induced by arecaidine. The electron-donating ability of arecaidine anion is higher than that of the neutral molecule; this explains why alkaline pH is required for induction of DNA damage by arecaidine and Cu(II).

2. Materials and methods

2.1. Materials

Arecaidine hydrochloride (Alfa Aesar, Ward Hill, MA, USA); neocuproine, 2-deoxy-D-ribose, and pBR322 plasmid DNA were purchased from Sigma (St. Louis, MO, USA); agarose (Gene Tech, Shanghai, China); Electrophoresis System (DYCP-32A, Beijing, China). Cupric chloride and other chemicals were of the highest quality available form local market.

2.2. Detection of plasmid pBR322 DNA damage by agarose gel electrophoresis

The DNA damage induced by arecaidine was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA to open circular and linear forms by gel electrophoresis (Zheng et al., 2006). To assay this damage, reaction mixtures (25 μl) containing 10 mM Tris–HCl buffer (pH 9.5 or 5.5), pBR322 DNA (0.3 μg), Cu(II) (100 μM), and arecaidine in various concentrations were prepared. After incubation at 37 °C for 6 h, the samples were mixed with 5 μl of gel loading buffer (0.25% bromophenol blue and 40% (w/v) sucrose); then the reaction mixtures were loaded onto a 1% agarose gel stained with GoldView, and immediately electrophoresed in a horizontal slab gel apparatus in TAE buffer (40 mM Tris, 20 mM sodium acetate and 2 mM ethylenediaminetetraacetic acid) at 100 V for 50 min. The gels were photographed on a UV transilluminator (Sanfu TU1002, Shanghai, China).

2.3. ROS assay

The production of ROS by arecaidine in the presence of Cu(II) ions was detected according to the method of Quinlan and Gutteridge (1987). Deoxyribose was used as substrate and the malondialdehyde generated from deoxyribose was assayed. The reaction mixtures (5.0 ml) contained 10 mM Tris–HCl buffer (pH 9.5), 28 mM 2-deoxy-D-ribose (0.6 ml), copper (200 μM) and arecaidine at various concentrations. After incubation at 37 °C for 6 h, the samples were mixed with 0.6 ml 1% (w/v) thiobarbituric acid (TBA) and then boiled at 100 °C for 20 min. The absorbance was measured at 532 nm after the samples had cooled down.

2.4. Detection of Cu(II) reduction

Cupric chloride (100 μM) in 10 mM Tris–HCl buffer (pH 9.5) was mixed with various concentrations of arecaidine and neocuproine (final concentration 400 μM) in a total volume of 3.0 ml. The neocuproine–Cu(I) complex was determined by measuring the absorbance at 450 nm after incubation at 37 °C for 6 h .

2.5. UV–visible spectra measurements

UV–visible spectra were measured with a Shimadzu 2450 Spectrophotometer (Kyoto, Japan). A solution with a final volume of 3 ml consisting of 40 μM arecaidine and 0–100 μM Cu(II) in 10 mM Tris–HCl (pH 9.5) was prepared and subjected to spectral analysis.

2.6. The ionisation potential (IP) and highest occupied molecular orbital (HOMO) analysis of arecaidine and its anion

DFT (Kohn & Sham, 1965) implanted in Gaussian 03 software (Frisch et al., 2003) was used to optimise the low energy structures of arecaidine and its anion utilising Becke's (1983) three parameter hybrid functional and Lee, Yang, and Parr's (1988) correlation functional B3LYP. Minima and saddle points were identified through frequency calculations performed at the same level of theory. Geometry optimisation was followed by single-point calculations using the B3LYP/6-311++G (2d, 2p) basis set. The total molecular energy includes the single-point energy and zero-point vibrational energy. IP and HOMO analyses were performed to explain the differences in electron-donating ability between arecaidine and its anion.

2.7. Statistical analysis

All data were analysed by one-way analysis of variances ($p < 0.05$) using SAS statistical program version 9.1.3. Duncan multiple range test was used for mean discrimination. A confidence level superior to 95% ($p < 0.05$) were considered as significant. All experiments were repeated at least three times.

3. Results

3.1. DNA damage induced by arecaidine with or without Cu(II) at different pHs

The DNA damage at different pH was assessed by measuring the conversion of the supercoiled pBR322 plasmid DNA to open circular by gel electrophoresis (Zheng et al., 2006). At pH 5.5 (Fig. 1a), the arecaidine shows little effect on DNA damage whether or not Cu(II) at present because the there are no obvious changes has been observed between line 1 and line 7. At pH 9.5 (Fig. 1b), the arecaidine or Cu(II) alone shows little effect on the DNA structure because the line 2 and line 3 has no obvious changes compared with line 1, while in the line 4, the open circular has been observed, also the amount of open circular is increased with arecaidine concentration (line 4 to line 7), indicating that the arecaidine and Cu(II) could work cooperatively to break the DNA molecule. Based above results, the pH and Cu(II) are two major factors for DNA damage caused by arecaidine.

3.2. ROS generation from the reaction of arecaidine in the presence of Cu(II)

The capacity of arecaidine to generate ROS in the presence of Cu(II) was explored to explain the course of DNA damage. ROS react with deoxyribose to form a free radical intermediate that decomposes to form an aldehyde, which in turn gives an adduct with TBA. Fig. 2 shows that increasing concentrations of arecaidine led to generation of progressively increasing amounts of ROS.

3.3. Reduction of Cu(II) by arecaidine

A possible mechanism for DNA cleavage would involve the formation of ROS and the reduction of Cu(II) to Cu(I). For this purpose, neocuproine, a Cu(I)-specific sequestering agent, was used to measure the reduction of Cu(II) by arecaidine. The absorbance of the neocuproine–Cu(I) complex increased with increasing arecaidine

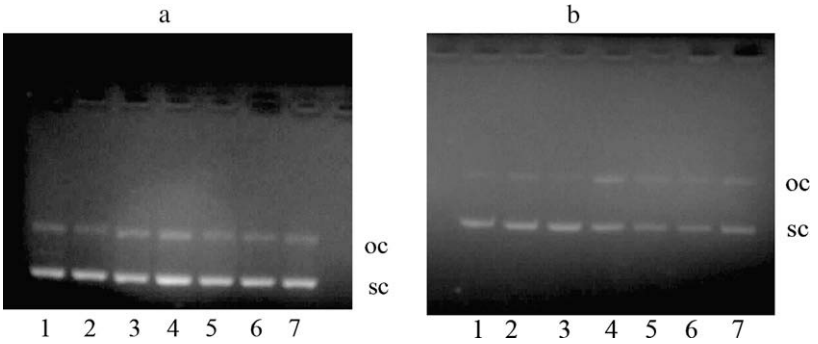


Fig. 1. DNA damage induced by arecaidine at pH 5.5 (a) and pH 9.5 (b). Lane 1: DNA alone; lane 2: DNA + 100 μM Cu(II); lane 3: DNA + 400 μM arecaidine; lanes 4–7: DNA and 100 μM Cu(II) + 400, 200, 100 or 50 μM arecaidine, respectively. sc = supercoiled; oc = open circular.

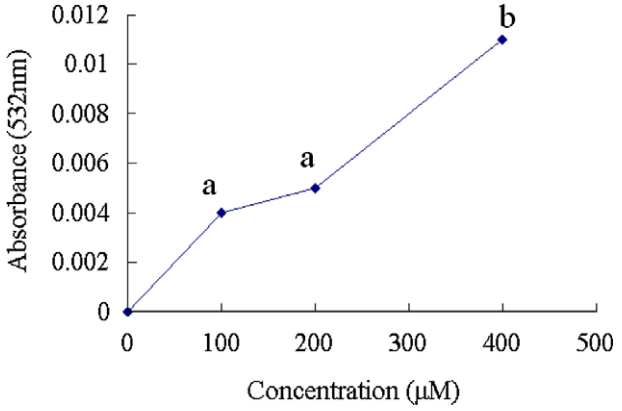


Fig. 2. ROS generation as a function of arecaidine concentration. Reaction conditions were as described in Section 2.3; the concentrations of arecaidine shown are final reaction concentrations; the final Cu(II) concentration was 200 μM. All values are expressed as mean ± SE from three independent experiments and different letters are significantly different at $p < 0.05$; $n = 3$.

concentration (Fig. 3), suggesting that arecaidine can reduce Cu(II) to Cu(I) in a concentration-dependent manner.

3.4. Interaction of Cu(II) and arecaidine

UV spectra were recorded to investigate the interaction between arecaidine and Cu(II) concentrations from 0 to 100 μM (Fig. 4). The absorbance of arecaidine changes with Cu(II) concentration between 230 and 320 nm, and a new peak appears. The results showed that arecaidine was oxidised by Cu(II) to form a new oxide which has an absorbance at 280 nm.

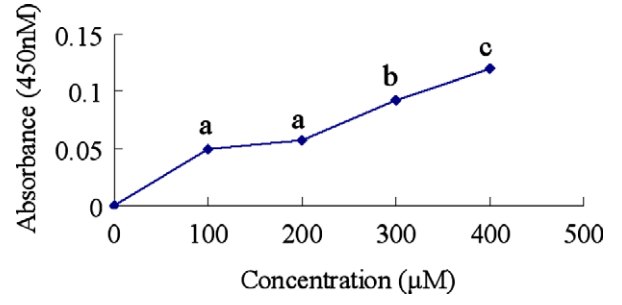


Fig. 3. Reduction of Cu(II) to Cu(I) by arecaidine . The mixture solution contained various concentrations of arecaidine. The final concentrations of Cu(II) and neocuproine in Tris–HCl buffer (pH 9.5) were 100 and 400 μM, respectively. The results are representative of three independent experiments and mean values from three experiments are plotted. Different letters are significantly different at $p < 0.05$; $n = 3$.

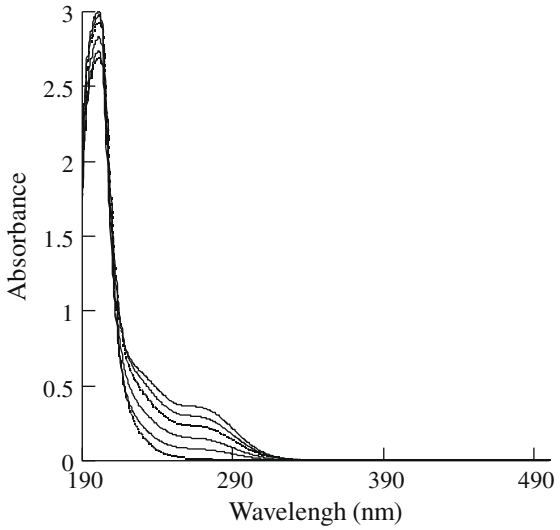


Fig. 4. Effect of Cu(II) ion (0, 20, 40, 60, 80 and 100 μM) on the absorption of arecaidine in Tris–HCl buffer (pH 9.5).

3.5. IP and HOMO analysis

Based on above results, the DNA damage caused by arecaidine is main due to its electron-donating ability, thus the electron-donating ability of arecaidine and its anion has been studied by DFT method to explore molecular mechanism of DNA damage. Both the IP and the HOMO energy are used to determine the electron-donating ability of a molecule: a low IP or a high HOMO energy reflects a strong ability to donate electrons. According to our calculated results, the IP of arecaidine anion is 76.69 kcal/mol (Table 1), which is significantly lower than that of arecaidine (176.18 kcal/mol); also the HOMO energy of arecaidine anion is −0.05397 hartree, much higher than that of arecaidine (−0.22735 hartree), indicating that the electron-donating ability of arecaidine anion is higher than that of arecaidine. Considering that arecaidine is inclined to exist as an anion at alkaline pH, the difference in electron-donating ability between arecaidine and arecaidine anion can be used to explain why arecaidine induces DNA damage in alkaline solution.

4. Discussion

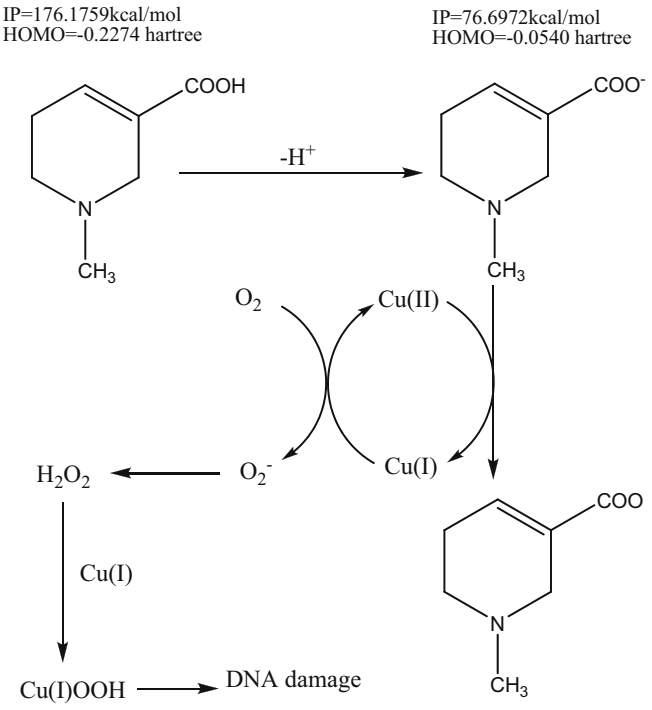
In this study, the effect of arecaidine on DNA damage has been studied in both alkaline and acid conditions in the presence of Cu(II). These results showed that alkaline conditions play an

Table 1
The zero-point vibrational energy (ZPVE), single-point energy (SPE), total energy (TE), ionisation potential (IP) and the highest occupied molecular orbital (HOMO) energy of arecaidine (A-COOH) and its anion (A-COO$^-$) calculated with B3LYP/6-311CG(2d, 2p) level DFT in gas phase at 298.15 K.

Species	ZPVE (hartree)	SPE (hartree)	TE (hartree)	IP (kcal/mol)	HOMO (hartree)
A-COOH	0.1768	−478.7034	−478.5266	176.1759	−0.2274
A-COO$^-$	0.1628	−478.1467	−477.9839	76.6972	−0.0540

important role in DNA damage induced by arecaidine in the presence of Cu(II), because DNA was damaged only under alkaline conditions, not under acid conditions. Under alkaline conditions, the carboxyl group (–COOH) of arecaidine will be deprotonated to form the anion, which has a lower IP, but a higher HOMO energy compared with deprotonated arecaidine (Table 1), indicating that its electron-donating ability is improved. Another important factor in the DNA damage induced by arecaidine is Cu(II). Cu(II) is an important structural metal ion in chromatin; however, it is reported to be an important factor in DNA damage induced by many organic compounds (Quinlan & Gutteridge, 1987; Saurabh, Asad, Ahmad, Khan, & Hadi, 2001; Zheng et al., 2006). In our experiment, the DNA damage only occurs in the presence of Cu(II), which was found to interact with arecaidine anion to give a new peak at 280 nm (Fig. 4). Also, the content of Cu(I) increases with increased arecaidine concentration (Fig. 3).

Based on the above results, a possible mechanism of DNA damage induced by arecaidine in the presence of Cu(II) can be described (Scheme 1). The arecaidine is deprotonated in alkaline solution to form its anion, which has a higher electron-donating ability and can reduce Cu(II) to Cu(I). The Cu(II)/Cu(I) redox cycle will help O_2 to form O_2^-, which produces hydrogen peroxide by dismutation. The hydrogen peroxide is reported to be readily converted to hydroxyl radical by the Fenton reaction, resulting in oxidative DNA damage.



Scheme 1. Possible mechanism of arecaidine-induced DNA damage.

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