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In vitro cytotoxicity of *Nicotiana gossei* leaves, used in the Australian Aboriginal smokeless tobacco known as pituri or mingkulpa

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HIGHLIGHTS

- We report on alkaloids chemistry and cytotoxicity of *N. gossei* leaves used in pituri preparation.
- The nicotine contained in extract from *N. gossei* leaves is not the source of its high cytotoxicity.
- The carcinogenic NNN and NNK in extract from *N. gossei* leaves are likely to cause high cytotoxicity.

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ABSTRACT

The Aboriginal population of Central Australia use endemic *Nicotiana* species to make a smokeless tobacco product known usually as pituri or mingkulpa. *Nicotiana* leaves are masticated with wood ash into a 'quid' that is chewed/sucked for absorption of nicotine. In addition to nicotine, smokeless tobacco products contain a spectrum of biologically active compounds that may contribute to effects on health. The objective of this study was to quantify nicotine, and related alkaloids and tobacco specific nitrosamines (TSNAs), in *Nicotiana* leaves used in pituri, and compare *in vitro* toxicity of pure nicotine with *Nicotiana* leaf extract at the same concentration of nicotine. An aqueous extract of dry leaves of *Nicotiana gossei* and a reference smokeless tobacco (CORESTA CRP2) were quantified for major pyridine alkaloids and TSNAs using HPLC-UV and LC–MS/MS. A range of extract concentrations and corresponding concentrations of nicotine standard were tested using an MTS assay to measure human lung epithelium cell (A549) survival. Cells treated for 24 h with the maximum concentration of 1.5 mg/ml of nicotine resulted in 77% viability. In contrast, extracts from *N. gossei* leaves and CRP2 containing a similar concentration of nicotine (1.3 mg/ml) resulted in remarkably lower viability of 1.5 and 6%, respectively. Comparison of cytotoxicity of pure nicotine with that of the extracts revealed that nicotine was not the source of their cytotoxicity. Other biologically active compounds such as the known carcinogens NNK and NNN, derived from nicotine and nornicotine and found to be present in the smokeless tobacco extracts, may be responsible.

1. Introduction

Tobacco plants are chewed for nicotine absorption by indigenous populations of the Americas, Africa, the Indian subcontinent and the Asia-Pacific region (Latz, 1995; International Agency for Research on Cancer, 2007). A range of endemic *Nicotiana* species are chewed by Indigenous Australian people, primarily in the central region of Australia (Latz, 1995). Pituri is the most commonly recognised word used for this (Latz, 1995; Peterson, 1979; Young, 2005; Ratsch et al., 2010), though there are a variety of synonyms such as mingkulpa (Young, 2005; Ratsch et al., 2010) also in use. Currently there is no national or international acknowledgement of the existence of chewed tobacco use by Australian Indigenous people. Australia was not mentioned in the IARC monographs on smokeless tobacco (International Agency for Research on Cancer, 2007; 2012) published by the World Health Organization, yet pituri chewing is commonplace amongst Aboriginal populations in Central Australia and other areas; 41% of Aboriginal people in Central Australia were chewing pituri according to a survey conducted in 1987 (Fleming et al., 1991) and in a recent study over 30% of Aboriginal women giving birth at a

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large hospital regularly chew pituri throughout pregnancy and lactation (Ratsch, 2011).

The two main types of smokeless tobacco products are snuff and chewing tobacco (Ebbert et al., 2004). Snuff is prepared from cured and ground leaves of *Nicotiana* spp., and can be held in the mouth and sucked or inhaled (in its dry form). Chewing tobacco consists of *Nicotiana* spp. leaves chewed in combination with substances such as lime, betel leaf or areca nut (Scollo and Winstanley, 2015). Smokeless tobacco products vary in their composition and process of production, so each smokeless tobacco product will vary in terms of chemical composition and consequently pharmacological activity (Ebbert et al., 2004; Scollo and Winstanley, 2015).

In the case of pituri, fresh or dry leaves of certain *Nicotiana* species are mixed with burnt wood ash and chewed into a 'quid' (Latz, 1995; Ratsch et al., 2010; Watson et al., 1983). Species that tend to be preferred for pituri are *Nicotiana gossei* Domin, *N. excelsior* (J.M. Black) J.M. Black and *Nicotiana rosulata* subsp. *ingulba* (J.M. Black) P. Horton (Latz, 1995) and the ash may be derived from wood of a variety of plants, including *Acacia* spp., *Eucalyptus* spp. and *Grevillea* spp. (Peterson, 1979). Once formed, the quid is held in the mouth for long periods of time and sucked rather than chewed (Ratsch et al., 2010). A quid may be shared between family and friends, may have more ash added to boost nicotine release, and storage between uses may involve contact with skin (e.g. behind the ear, under a breast, arm band or head band) (Ratsch et al., 2010). The way in which pituri is prepared and used is similar to iq'mik used by Indigenous people in Alaska (International Agency for Research on Cancer, 2007; Hearn et al., 2013) and maras from Turkey (International Agency for Research on Cancer, 2007).

Smokeless tobacco products are considered by many to be less hazardous than cigarette smoking and therefore a suitable substitute to promote harm reduction (Benowitz, 2011). Indeed, smokeless tobacco can deliver similar quantities of nicotine as smoking and avoids exposure to an extensive suite of deleterious chemicals present in smoke (International Agency for Research on Cancer, 2007). However, there is global concern about increasing the consumption of smokeless tobacco due to its adverse effects on human health. Smokeless tobacco products have different health effects than smoked tobacco, but few smokeless tobacco products have been studied for the health outcomes and diseases they may cause (Ebbert et al., 2004; Scollo and Winstanley, 2015). A short-term increase of blood pressure and heart rate is attributed to smokeless tobacco use, and users are more at risk of dying from stroke and heart disease (International Agency for Research on Cancer, 2007). Also, significant effects on the soft and hard tissues of the mouth caused by smokeless tobacco can lead to oral disease such as bad breath, tooth decay, receding gums, leukoplakia and lesions in the mouth (International Agency for Research on Cancer, 2007; Critchley and Unal, 2003). In pregnant women using smokeless tobacco, there is a high risk of low birth weight, premature birth and preeclampsia (International Agency for Research on Cancer, 2007; Ebbert et al., 2004; Critchley and Unal, 2003; Ratsch and Bogossian, 2014). In male users of smokeless tobacco, there is an increase in number of abnormal sperm, while sperm count and semen volume decrease (International Agency for Research on Cancer, 2007). Based on animal and epidemiological studies, smokeless tobacco products have been classified as carcinogenic to humans (International Agency for Research on Cancer, 2007; Hecht, 2003). Smokeless tobacco use is associated particularly with cancer of mouth tissues, though cancer of the pancreas and oesophagus have also been linked (Ebbert et al., 2004; Secretan et al., 2009). The carcinogenicity of tobacco products is mainly attributed to tobacco-specific *N*-nitrosamines (TSNAs) (Hecht, 1998), which are nitrosated derivatives of nicotine and other pyridine alkaloids present in tobacco. The main two TSNAs are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*-nitrosonornicotine (NNN), derived from nicotine and nornicotine, respectively; both have been classified as group 1 human carcinogens by the International Agency for Research on Cancer (IARC) (International Agency for Research on Cancer, 2007). Other common pyridine alkaloids present in tobacco leaves are anabasine, which gives rise to the weakly carcinogenic *N*-nitrosoanabasine (NAB), and anatabine, which produces the apparently non-carcinogenic N-nitrosoanatabine (NAT) (Hecht et al., 1983). A variety of other carcinogenic compounds have been measured in some types of smokeless tobacco, usually at lower concentrations than TSNAs, including other types of nitroso compounds, polycyclic aromatic hydrocarbons (PAHs) and volatile aldehydes (International Agency for Research on Cancer, 2007; Hecht, 1998).

In vitro studies have been used to elucidate any cell-specific effects of smokeless tobacco consumption that can indicate possible toxicity and carcinogenicity of smokeless tobacco chemicals in humans. There have been reports of cytotoxicity caused by smokeless tobacco products such as gutkha (Avti et al., 2010), khaini (Das et al., 2013), Sudanese toombak (Costea et al., 2010), American moist snuff (Misra et al., 2014), Swedish moist snuff (Costea et al., 2010, 2010; Coggins et al., 2012), Kentucky reference moist smokeless tobacco product (Lombard et al., 2010) and commercial chewing tobacco (Coppe et al., 2008). The present study is the first to report the quantity of nicotine along with related alkaloids and TSNAs in dry leaves of *N. gossei*, which is the main ingredient in pituri. It is also the first assessment of cytotoxicity induced by the extract obtained from these leaves as a means of investigating potential carcinogenicity of pituri. Direct comparison is made for *N. gossei* leaves with pure nicotine, and an official CORESTA reference chewing tobacco (CRP2) which contains only tobacco and is the closest available reference product to pituri.

2. Materials and methods

2.1. Chemicals and reagents

The solvents used for HPLC analysis were acetonitrile, from Merck (Darmstadt, Germany), and buffer prepared using ammonium formate from Sigma-Aldrich (St. Louis, MO). The water was deionised and filtered using a Milli-Q system (Millipore, Billerica, MA). Hydrochloric acid and sodium hydroxide used for adjusting pH were from Merck (Darmstadt, Germany). The analytical standards used were *N*′-nitrosonornicotine (NNN), 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK), nornicotine, myosmine and cotinine from Sigma, nicotine from Fluka (Milwaukee, WI), anabasine from Sigma-Aldrich, anatabine from Cayman Chemical Company (AnnArbor, MI), and the internal standards used were caffeine from Ajax Finechem (Sydney, Australia) for HPLC-UV analysis and *N*′- nitrosonornicotine-d4 (NNN-d4) and 4-(methylnitrosoamino)-1-(3-pyridyl-d4)-1-butanone (NNK-d4) from Toronto Research Chemicals (Ontario, Canada) for LC–MS analysis. Reagents and culture medium used for cell culture including Dulbecco's Modified Eagle Medium, nutrient mixture F-12 (DMEM/F-12), Fetal Bovine Serum (FBS) and Trypsin-EDTA (0.25%) were Gibco (Thermo Fisher Scientific, Vic, Australia). Triton X-100 solution used as positive control was from Sigma-Aldrich (St. Louis, MO). MTS assay reagent, CellTiter 96 AQueous One Solution Assay was from Promega (Madison, WI).

2.2. Preparation of extracts

Leaves of *N. gossei* were collected in Alice Springs NT, Australia and air dried, such that they were ready for mastication, by an