

A Review on Role of Arecoline and Its Metabolites in the Molecular Pathogenesis of Oral Lesions with an Insight into Current Status of Its Metabolomics

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Received June 5, 2018; Accepted November 9, 2020.

Key words: Arecoline – Molecular pathogenesis – Metabolism of arecoline – Arecoline N-oxide – Oral submucous fibrosis – Oral cancer

Abstract: Areca nut consumption is a popular habit in Southeast Asian countries. One of the important biologically active alkaloids of areca nut is arecoline, which plays a role in mediating the development of several pathologies of the primary exposure site, the oral cavity. Studies on the metabolism of arecoline revealed the formation of several metabolites which themselves might be toxic. Moreover, polymorphisms in genes encoding enzymes involved in the metabolism of arecoline might predispose an organism towards the development of oral cancer. The present review tries to accumulate all the relevant existing literature and then elucidate the molecular mechanism by which arecoline plays a role in the development of oral submucous fibrosis and oral cancer. Existing information regarding arecoline metabolism, enzymes involved in the metabolic process and biological effects of the metabolites of arecoline have also been compiled and compared to study the toxicity of metabolites with its parent compound arecoline and whether they play any role in the pathogenesis of oral cancer mediated by areca nut consumption. A repertoire of molecular targets has come up in the discussion whose expression profile is perturbed by arecoline. Construction of induction cascade from existing literature has given an idea about the process of molecular pathogenesis. The summarized and analysed data can help to determine the molecular mechanism and drug targets,

This study was supported by the University Grants Commission (UGC), India, under UGC Junior Research Fellowship scheme.

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<https://doi.org/10.14712/23362936.2020.19>

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which in turn could be helpful in the prevention or treatment of these pathological conditions. It also brings into light areas where further research needs to be directed.

Introduction

Mortality is a severe issue in the case of oral cancer, as indicated by the International Agency for Research in Cancer (IARC) report, GLOBOCAN series, 2012. According to this data, globally, 300,000 cases of oral cancer and 145,000 cases of death were reported in the year 2012 (Ferlay et al., 2015). As this form of cancer majorly develops from the squamous cell region of the oral cavity, it is also called oral squamous cell carcinoma (OSCC) (Rivera, 2015). The metastatic form is preceded by several pre-malignant stages, including leukoplakia, erythroplakia, oral lichen planus, and oral submucous fibrosis (OSF). These lesions show different rates of transformation to the cancerous stage: 3.6 to 17.5% in leukoplakia, 70.3% in proliferative verrucous leukoplakia, 14 to 50% in erythroplakia, 0.04 to 1.74% in oral lichen planus and 7 to 13% in oral submucous fibrosis (Reichart and Philipsen, 2005; Tilakaratne et al., 2006; Kademani, 2007).

Areca nuts have been classified as a Class I carcinogen by IARC (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004). The chemical composition of areca nut comprises alkaloids, flavonoids, tannins, along with carbohydrates, proteins, fats, crude fibre, and elements (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Peng et al., 2015). Of these, an important constituent is the alkaloid arecoline (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004). In a study by Cox et al. (2010), most of the habitual areca nut consumers were found to have a baseline concentration of arecoline in saliva reaching up to 2.4 µg/ml (0.01 mM). Apart from saliva, arecoline was detected in blood plasma, hair samples and breast milk samples from areca nut consumers (Marchei et al., 2005; Pellegrini et al., 2007; Wu et al., 2010). It was also detected in the excreta collected from new-borns, whose mothers had consumed areca nut during their gestation period (Pichini et al., 2003). Therefore, the effects induced by arecoline might be due to its stability in the system apart from direct exposure.

The metabolic profile of arecoline has brought into light the existence of several metabolites (Nery, 1971; Giri et al., 2006). Few of these metabolites have been detected in body fluids of areca nut consumers opening up another area of research involved in investigating the role of these metabolites in areca nut mediated oral pathologies.

In this review, we try to (1) create a metabolic map of arecoline from existing literature, (2) discuss the molecular mechanisms and pathways induced by arecoline that are responsible for the development of oral submucous fibrosis (OSF) and oral squamous cell carcinoma (OSCC), (3) describe the biological effects of arecoline metabolites and thereby assess if the metabolites are more toxic than the parent compound itself and play a part in the pathogenesis of OSF and OSCC, and finally

(4) understand the mechanism of metabolism of arecoline and arecoline N-oxide by gathering knowledge about enzymes involved, which in turn may enable to estimate predisposition of an individual towards the development of oral cancer in areca nut consumers.

Metabolism of arecoline

The metabolic profile of arecoline might play a role in areca nut mediated pathogenesis of OSF and OSCC. This brings out the need to delve into the depths

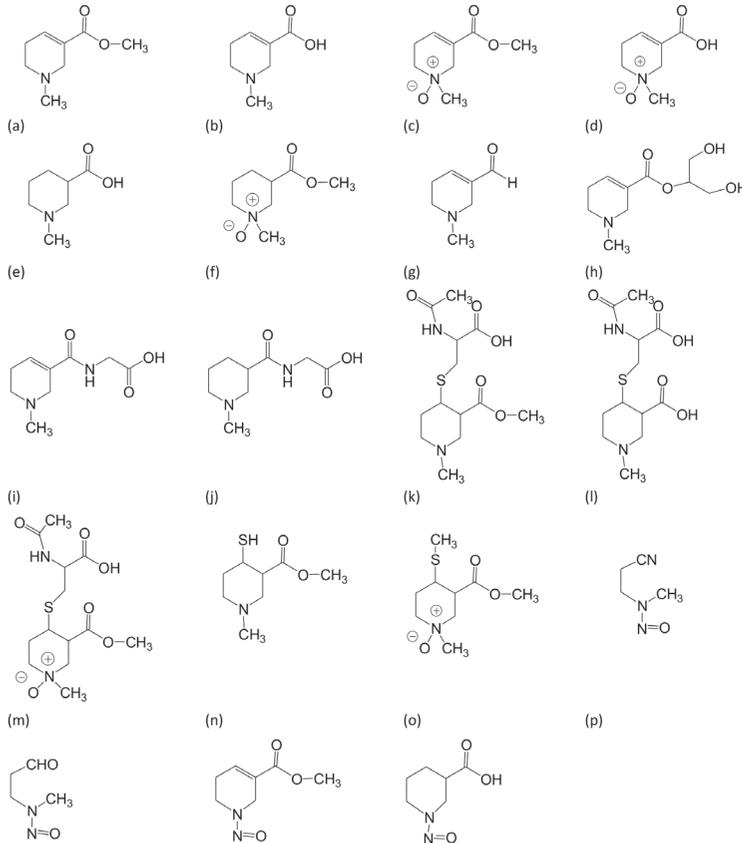


Figure 1 – Chemical structure of arecoline and metabolites.

(a) arecoline; (b) arecaidine; (c) arecoline N-oxide; (d) arecaidine N-oxide; (e) N-methylnipecotic acid; (f) 1-methylnipecotic acid 1-oxide methylester; (g) 1-methyl-3,4-dehydropiperidine-3-carboxaldehyde; (h) arecaidinylglycerol; (i) arecaidinylglycine; (j) N-methylnipecotylglycine; (k) mercapturic acid of arecoline; (l) mercapturic acid of arecaidine; (m) mercapturic acid of arecoline N-oxide; (n) 4-mercapto-1-methylnipecotic acid methylester; (o) 4-methylmercapto-1-methylnipecotic acid 1-oxide methylester; (p) 3-methylnitrosaminopropionitrile; (q) 3-methylnitrosaminopropionaldehyde; (r) N-nitrosoguvacoline; (s) N-nitrosonipecotic acid.

(b, c, d, e, h, i, j, k, l, m, p, q, r – metabolites of arecoline; d, e, h, i, j, l – metabolites of arecaidine; a, b, f, g, k, m, n, o – metabolites of arecoline N-oxide; s – metabolite of N-nitrosoguvacoline)

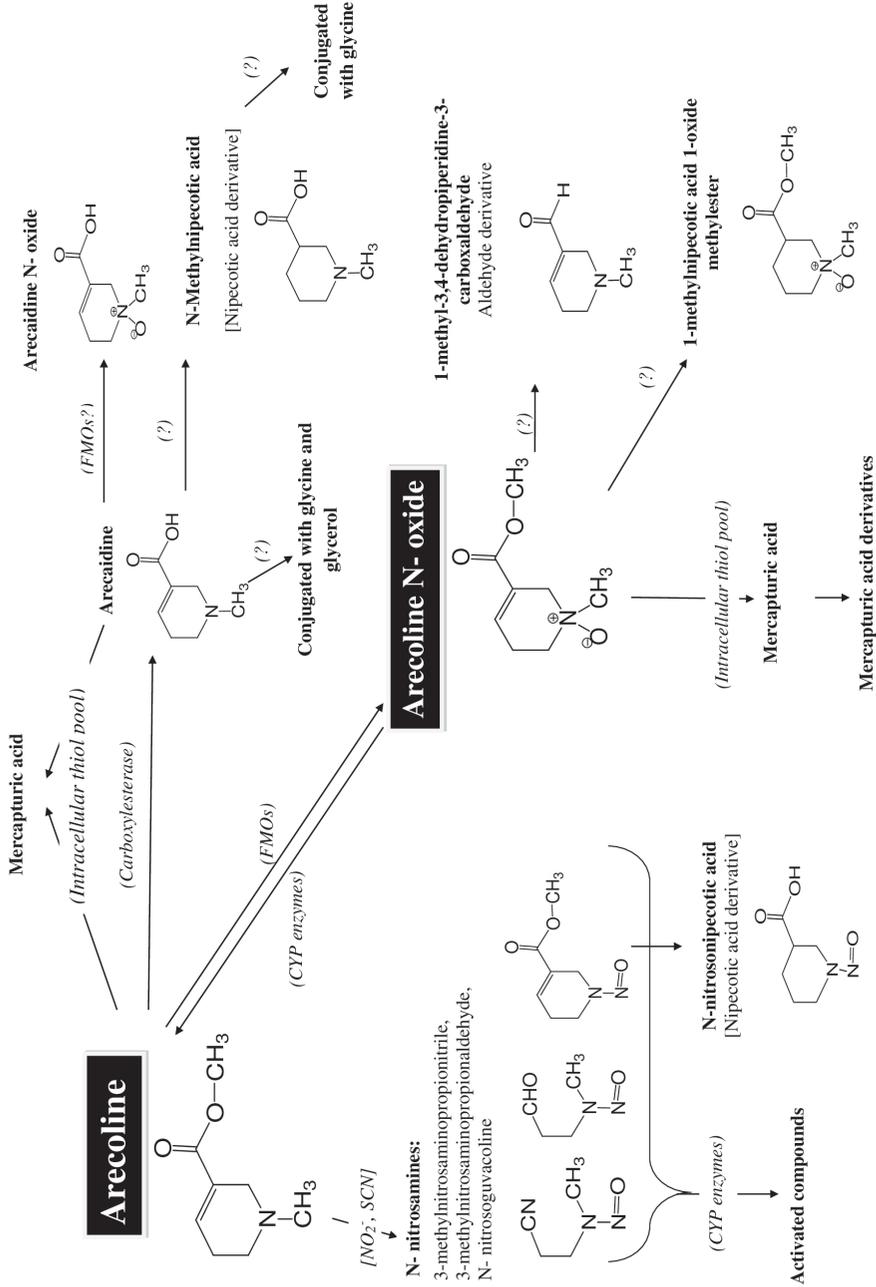


Figure 2 – Metabolic map of arecoline and its metabolites. Pathway adapted from Wenke and Hoffman (1983), Ohshima et al. (1989), Miyazaki et al. (2005), Giri et al. (2006, 2007). The symbol “?” represents the unavailability of knowledge about the enzyme involved. Enzyme involved in each step has been mentioned in italics.

of the metabolomics of arecoline and explore its potential towards the development of oral pathology. Metabolism of arecoline starts in the oral cavity itself. Both nitrite and thiocyanate (catalyst for nitrosation reaction) are present in human saliva (Boyland et al., 1971; Shivapurkar et al., 1980; Wenke et al., 1984a). When arecoline was incubated with sodium nitrite with or without sodium thiocyanate, the formation of three compounds was observed: 3-(methylnitrosamino)propionaldehyde (MNPA), 3-(methylnitrosamino)propionitrile (MNPN) and N-nitrosoguvacoline (NGL) (Wenke and Hoffmann, 1983). Of these, NGL and MNPN were detected in the saliva of betel quid chewers (without tobacco) in the range of 2.2–9.5 µg/l and 0.5–11.4 µg/l, respectively (Wenke et al., 1984a; Prokopczyk et al., 1987). These findings indicate that nitrosation of arecoline does take place in the oral cavity of areca nut consumers, and thereby, buccal cells do get exposed to these nitrosated metabolites on chewing areca nut. In a mammalian test system, N-nitrosonipecotic acid (NNIP) was detected as a product of metabolism of NGL (Ohshima et al., 1989). MNPA has not been detected in saliva samples of areca nut consumers. However, as the formation of MNPA from arecoline takes place under *in vitro* conditions, fast metabolism of MNPA to its metabolites *in vivo* might explain this finding. The metabolome of arecoline, arecaidine and arecoline N-oxide *in vivo* was investigated by Nery (1971) and Giri et al. (2006, 2007). This research revealed several unknown and novel metabolites (Figure 1). Of these, arecaidine and N-methyl nipecotic acid were detected in the urine of areca nut consumers (Hu et al., 2010).

Figure 2 depicts a metabolic map of arecoline.

Role of arecoline in induction of oral pathologies

Genotoxic effects of arecoline were proved in mice using tests evaluating the formation of chromosomal aberrations and micronucleus (Shirname et al., 1984; Deb and Chatterjee, 1998). At the molecular level, arecoline induces a DNA damage response cascade involving phosphorylation of ataxia-telangiectasia (ATM) kinase and its downstream targets checkpoint kinase 1/2 (Chk1/2), p53 and Nbs1, leading to a G2/M cell cycle arrest. However, the overall expression of p53 is down-regulated by arecoline, which is followed by suppression of p53 mediated DNA repair activities and expression of its downstream target p21^{WAF1} (Tsai et al., 2008). Arecoline has also been found to induce decreased expression of p21 and p27 via a p53 independent process that includes reactive oxygen species (ROS) and activation of mammalian target of rapamycin complex-1 (mTORC1) pathway (Ji et al., 2012). According to Ji et al. (2012), down-regulation of these inhibitors of cell cycle might lead to erroneous DNA replication as the cells escape the G1/S checkpoint. Arecoline also disturbs the fluidity of polymerisation-depolymerisation kinetics of α -tubulin by favouring their polymerisation. It leads to a disfigurement of the mitotic spindle and an erroneous arrangement of chromosomes, thereby inducing the pro-metaphase cell cycle arrest (Wang et al., 2010). Apart from these alterations,

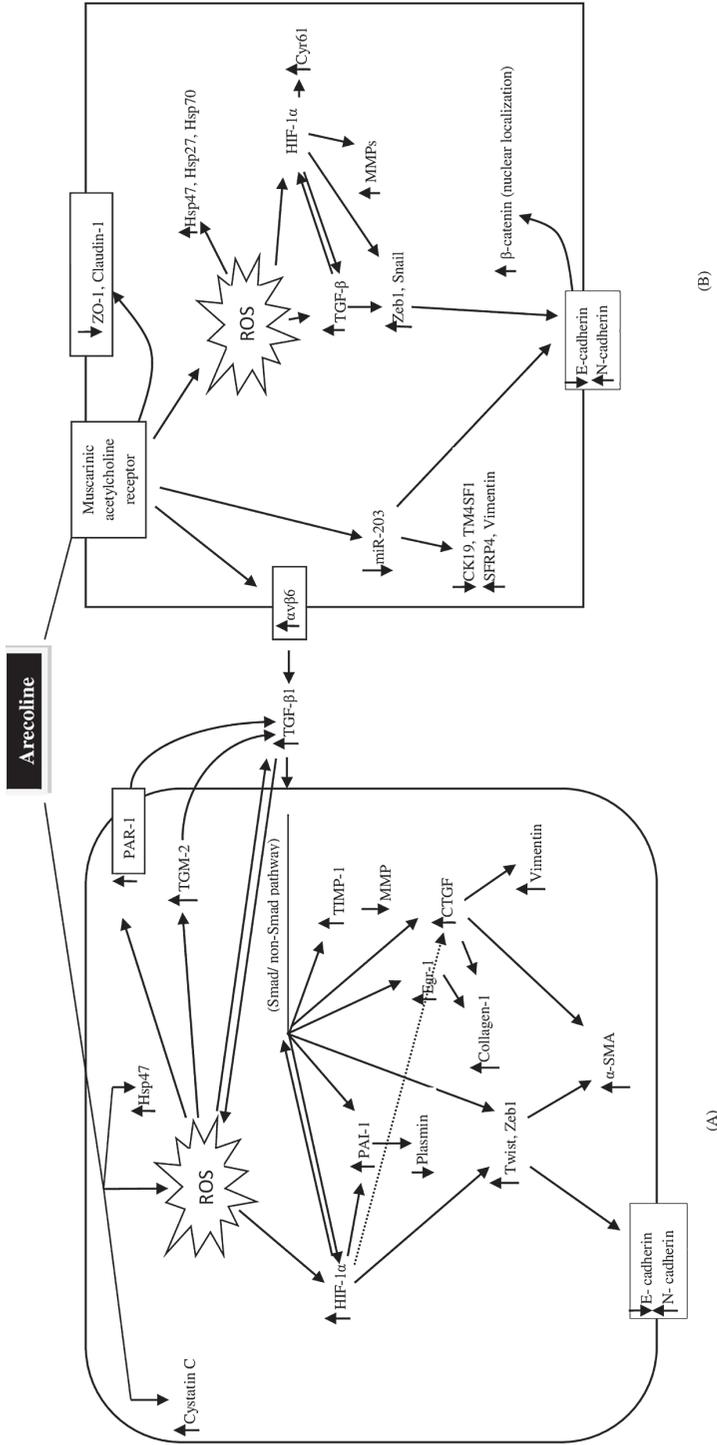


Figure 3 – Induction cascade induced by arecoline in fibroblast (A) and epithelial cell/carcinoma cell of epithelial origin (B) facilitating development of oral pathologies. All abbreviations have been elaborated in text except: N-cadherin – neural-cadherin; CK19 – cytokeratin 19; TM4SF1 – transmembrane 4 L six family member 1; SFRP4 – secreted frizzled related protein 4. Both hard dashed arrows and dotted arrow indicate the same.

protein expression of several other cell cycle regulatory molecules like cdc25c in basal carcinoma cells (Huang et al., 2012), cyclin B1 and Wee-1 in KB epithelial cells (Lee et al., 2006) and cyclin D1, cyclin A, cyclin E, CDK4, and CDK2 in HaCaT keratinocytes (Zhou et al., 2013) have been found to be modulated by arecoline.

Arecoline treatment leads to the down-regulation of the immune system in mice (Dasgupta et al., 2006; Wen et al., 2006). By contrast, Hung et al. (2011) reported the production of ROS in endothelial cells on exposure to arecoline, which resulted in an up-regulated expression of adhesion molecules (intercellular adhesion molecule – ICAM, and vascular cell adhesion molecule – VCAM). This effect increased adhesion between mononuclear cells and endothelial cells, which might play a role in augmenting the inflammation. Additionally, several inflammatory cytokines have also been found to be induced by arecoline, such as interleukin-1 α , prostaglandin E-2, and cyclooxygenase-2 in fibroblasts (Jeng et al., 2003; Tsai et al., 2003; Thangjam and Kondaiah, 2009).

DNA damage and impaired DNA repair, along with chronic inflammation, can be emphasized as the main causes of arecoline induced oral pathologies. Moreover, expression profiles of several extracellular matrix (ECM) proteins, enzymes, growth factors, and transcription factors are altered under the effect of arecoline (Figure 3). All these factors work in an association as indicated by several studies involving fibroblasts, epithelial cells and cancer cell lines (Tables 1 and 2).

Oral submucous fibrosis (OSF) is a pre-cancerous condition that develops from an abnormal wound healing process under continuous exposure to the components of areca nut (Angadi et al., 2011). Inhibition of elements involved in the degradation of extracellular matrix (ECM) or enhanced stability and synthesis of matrix components disturbs the homeostasis of ECM, which can give rise to disease conditions like fibrosis. Arecoline works positively in both these aspects. It enhances the expression of several inhibitors of proteinases, including tissue inhibitors of metalloproteinases (TIMPs), plasminogen activator inhibitors (PAI-1) and cysteine proteinase inhibitor cystatin C in fibroblasts (Chang et al., 2002a; Yang et al., 2003; Tsai et al., 2007) along with induction of factors that enhance the stability of ECM, such as heat shock protein-47 (Hsp-47) and transglutaminase-2 (TGM-2) (Yang et al., 2008; Lee et al., 2015). Moreover, arecoline has been found to decrease the phagocytosis of collagen by fibroblasts (Shieh et al., 2004).

An analysis of the repertoire of moieties affected by arecoline (data compiled in Tables 1 and 2) has brought into light several mediators of the effects induced by arecoline of which three, ROS, transforming growth factor- β 1 (TGF- β 1) and hypoxia-inducible factor-1 α (HIF-1 α), might play key roles in the pathogenesis of OSF and OSCC.

Of the several isoforms of TGF- β (TGF- β 1/2/3), TGF- β 1 is a critical mediator of oral pathologies. Its overexpression has been observed in OSF tissue samples (Kamath et al., 2015). Up-regulated expression of α v β 6 integrin on keratinocytes under arecoline's affect supports the activation of latent TGF- β 1 present in ECM that

Table 1 – A collective representation of various studies conducted *in vitro* to assess the effect of arecoline on the expression of proteins whose expression profile is perturbed in oral submucous fibrosis (OSF) cases

| Serial no. | Cell type and dose of arecoline | Effects | ROS/receptor/ pathways involved | Expression in OSF specimen (compared to normal mucosal specimen) | Reference |
|------------|------------------------------------------|-----------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------|
| 1. | Buccal mucosal fibroblasts (0–200 µg/ml) | Arecoline induced upregulation of vimentin expression. | – | Moderately advanced/advanced OSF specimen showed higher expression of intermediate filament vimentin in cytoplasm of fibroblasts in the connective tissue. | Chang et al. (2002b) |
| 2. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced cyclooxygenase-2 expression, the induction subsided after sometime. | Decreased thiol content involved in induction. | Moderate cases of OSF showed higher expression of cyclooxygenase-2 in epithelial cells, fibroblasts and inflammatory cells whereas advanced cases showed weak expression. | Tsai et al. (2003) |
| 3. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of plasminogen activator inhibitor-1 (PAI-1) expression. | – | Moderately advanced/advanced OSF specimen showed higher expression of plasminogen activator inhibitor-1 in fibroblasts, endothelial cells and inflammatory cells. | Yang et al. (2003) |
| 4. | Buccal mucosal fibroblasts (0–80 µg/ml) | Arecoline induced upregulation of insulin like growth factor-1 expression. | – | Moderately advanced/advanced OSF specimen showed higher expression of insulin like growth factor-1 in fibroblasts, endothelial cells and inflammatory cells of connective tissue. | Tsai et al. (2005a) |
| 5. | Buccal mucosal fibroblasts (0–80 µg/ml) | Arecoline induced upregulation of keratinocyte growth factor-1 expression. | – | Moderately advanced/advanced OSF specimen showed higher expression of keratinocyte growth factor-1 in the epithelial cells and to a lesser degree in fibroblast, endothelial and inflammatory cells. | Tsai et al. (2005b) |
| 6. | Buccal mucosal fibroblasts (0–80 µg/ml) | Arecoline induced upregulation of cystatin C expression. | – | Connective tissue of moderately advanced/advanced OSF specimen showed higher expression of cystatin C in fibroblasts, endothelial cells and inflammatory cells. | Tsai et al. (2007) |
| 7. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of heat shock protein 47 (Hsp-47) expression. | PI3K, Cox-2, MEK pathways involved. Decreased intracellular thiol content involved in induction. | OSF specimen showed higher expression of heat shock protein 47 mRNA. | Yang et al. (2008) |

| Serial no. | Cell type and dose of arecoline | Effects | ROS/receptor/ pathways involved | Expression in OSF specimen (compared to normal mucosal specimen) | Reference |
|------------|------------------------------------------|--------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------|------------------------|
| 8. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of hemeoxygenase-1 (HO-1) expression. | – | OSF specimen showed higher expression of hemeoxygenase-1 in fibroblasts, epithelial cells and inflammatory cells. | Tsai et al. (2009) |
| 9. | Buccal mucosal fibroblasts (0–0.4 mM) | Arecoline induced upregulation of connective tissue growth factor (CTGF) expression. | NF-κB, JNK, p38 pathways involved. Decreased intracellular thiol content involved in induction. | OSF specimen showed higher expression of connective tissue growth factor in fibroblasts, endothelial cells and epithelial cells (in some cases). | Deng et al. (2009) |
| 10. | HaCaT keratinocyte (0–50 µg/ml) | Arecoline induced expression of transforming growth factor-β2. | Muscarinic acetylcholine receptor – intracellular [Ca ²⁺] rise-PKC activation pathway involved. Decreased intracellular thiol content involved in induction. | OSF specimen showed higher expression of transforming growth factor β2 mRNA. | Thangiam et al. (2009) |
| 11. | Keratinocyte (5–10 µg/ml) | Arecoline induced upregulation of αvβ6 integrin expression. | Muscarinic acetylcholine receptor involved. | OSF specimen showed higher expression of αvβ6 integrin. | Moutasim et al. (2011) |
| 12. | Buccal mucosal fibroblasts (0–20 µg/ml) | Arecoline induced upregulation of S100A4 expression. | NF-κB, ERK, mTOR pathways involved. | OSF specimen showed higher expression of S100A4. | Yu et al. (2013) |
| 13. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of protease activated receptor-1 (PAR-1) expression. | ERK, PI3K, Cox-2, TK pathways involved. Decreased intracellular thiol content involved in induction. | Moderately advanced/advanced OSF specimen showed higher expression of protease activated receptor-1 in fibroblasts and inflammatory cells. | Tsai et al. (2013) |
| 14. | Buccal mucosal fibroblasts (0–20 µg/ml) | Arecoline induced ZEB1, αsmooth muscle actin (αSMA) and vimentin expression. | – | OSF specimen showed higher expression of ZEB1 localised in nucleus of fibroblast and αSMA in fibroblast and blood vessel. | Chang et al. (2014) |

| Serial no. | Cell type and dose of arecoline | Effects | ROS/receptor/ pathways involved | Expression in OSF specimen (compared to normal mucosal specimen) | Reference |
|------------|------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|
| 15. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of hypoxia inducible factor-1 α expression. | – | Moderately advanced/advanced OSF specimen showed higher expression of hypoxia inducible factor-1 α in fibroblasts, epithelial cells and inflammatory cells. | Tsai et al. (2015) |
| 16. | Buccal mucosal fibroblasts (0–0.4 mM) | Arecoline induced upregulation of early growth response-1 (Egr-1) expression. | JNK, ERK pathways involved. Decreased intracellular thiol content involved in induction. | OSF specimen showed higher expression of early growth response-1 in epithelial cells, fibroblasts, inflammatory cells. | Hsieh et al. (2015) |
| 17. | HaCaT keratinocytes (0–0.16 mM) | Arecoline induced downregulation of miR-203, downregulation of cytokeratin CK19 and E-cadherin; upregulation of N-cadherin and vimentin, increased expression of SFRP4 and decreased expression of TM4SF1. | – | OSF specimen showed lower expression of miR-203 and transmembrane 4 L six family member 1 (TM4SF1) and higher expression of secreted frizzled related protein 4 (SFRP4). | Zheng et al. (2015) |
| 18. | Buccal mucosal fibroblasts (0–20 µg/ml) | Arecoline induced upregulation of Twist. | – | OSF specimen showed higher expression of Twist mRNA and protein. | Lee et al. (2016) |
| 19. | Buccal mucosal fibroblasts (0–160 µg/ml) | Arecoline induced upregulation of transglutaminase-2 (TGM-2) expression. | Reactive oxygen species involved in induction. | Moderate/advanced OSF specimen showed higher expression of transglutaminase-2 in fibroblasts. | Lee et al. (2016) |

ROS – reactive oxygen species; PI3K – phosphatidylinositol 3-kinase; Cox-2 – cyclooxygenase-2; MEK – mitogen activated protein kinase; JNK – c-Jun NH₂-terminal kinase; ERK – extracellular signal-regulated protein kinase; PKC – protein kinase C; TK – tyrosine kinase; NF- κ B – nuclear factor-kappa B; mTOR – mechanistic target of rapamycin

in turn triggers the myofibroblastic transformation of fibroblasts (Moutasim et al., 2011). After being activated via integrin, plasminogen activated receptor-1 (PAR-1) or TGM-2, TGF- β 1 binds to its receptor and sets on a cascade of signalling events that induce the expression of growth factors, cytokines and transcription factors in resident fibroblasts thereby transforming them into myofibroblasts (Figure 3) (Eickelberg et al., 1999; Griffin et al., 2002; Samarakoon et al., 2008; Lin et al., 2013; Yang et al., 2013, 2016; Chang et al., 2014; Lamouille et al., 2014; Chen et al., 2016; Hsieh et al., 2017). Myofibroblasts are cells specialized for wound healing with capacity for the secretion of ECM materials and cellular contraction (Micallef et al., 2012). TGF- β 1 stimulates myofibroblasts to produce modulators of ECM proteins (PAI-1, TIMP-1, CTGF, etc.) and transcription factors involved in epithelial-to-mesenchymal transition (EMT) (Twist, Zeb, etc.) (Figure 3). Up-regulated expression of TGF- β 1 has also been observed in cancer tissue samples (Lu et al., 2004).

Another important mediator of arecoline induced effects is HIF-1 α . Apart from being induced by arecoline itself in fibroblasts and epithelial cells (Lee et al., 2010; Tsai et al., 2015), it plays a part in the induction of several downstream factors that overlaps the repertoire induced by TGF- β 1 (Figure 3) (Higgins et al., 2004; Tsai and Wu, 2012). Hypoxia prevails in fibrotic as well as tumour conditions, which prevents degradation of HIF-1 α . Under the hypoxic condition, stabilized HIF-1 α dimerizes with HIF-1 β and, in association with co-activators, it participates in the transcription of genes with hypoxia-responsive element (HRE) (Tsai and Wu, 2012; Eckert et al., 2016; Rankin and Giaccia, 2016). As reviewed by Tsai and Wu (2012) and Rankin and Giaccia (2016), several genes involved in metastasizing are regulated by HIF-1 α , including transcription factors involved in EMT (Twist, Snail, Aeb1/2, etc.), enzymes like matrix metalloproteinases (MMP 1/3), matricellular proteins (cysteine rich protein 61 [Cyr61]), and angiogenic factors (vascular endothelial growth factor).

Several of the pro-fibrotic or carcinogenic factors induced by arecoline are coupled with a decrease in intracellular thiol content and show reversible expression after treatment with antioxidants (Tables 1 and 2). This emphasizes ROS generation by arecoline to be a “cause” of the various arecoline driven “effects” that trigger OSF or OSCC. Interplay can be observed between ROS and TGF- β 1 as well as HIF-1 α . For instance, ROS plays a part in the activation of latent TGF- β 1 complex tethered to the ECM (Jobling et al., 2006). In turn, TGF- β 1 acts to increase ROS production via the activity of its downstream target NADPH oxidase-4 (NOX-4) along with suppression of the antioxidant defence system of exposed cells (Richter and Kietzmann, 2016). Apart from sharing a common array of downstream targets, TGF- β 1 and HIF-1 α augment each other’s expression too. HIF-1 α supports transcription of TGF- β 1 under hypoxic conditions, whereas, under normoxic conditions, TGF- β 1 enhances the stability of HIF-1 α by decreasing expression of HIF-1 α inhibitor prolyl hydroxylase 2 (PHD2) (McMohan et al., 2006; Hung et al., 2013). ROS induces HIF-1 α stability and thereby its transcriptional activity via an adenosine monophosphate-activated protein kinase (AMPK) pathway (Jung et al., 2008).

Table 2 – A collective representation of various studies conducted *in vitro* to assess the effect of arecoline on the expression of proteins whose expression profile is modulated in oral squamous cell carcinoma (OSCC) cases

| Serial no. | Cell line and dose of arecoline ($\mu\text{g/ml}$) | Effects | ROS/receptor/ pathways involved | Association of expression with oral squamous cell carcinoma (OSCC) | Reference |
|------------|------------------------------------------------------|----------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------|
| 1. | OECM-1, CE81T/VGH cells (200 $\mu\text{g/ml}$) (LT) | Arecoline exposure induced expression of matrix metallo proteinase-1 (MMP-1). | – | Higher expression of matrix metallo proteinase-1 mRNA in OSCC samples. | Lee et al. (2008) |
| 2. | GNM epithelial cells (0–160 $\mu\text{g/ml}$) | Arecoline induced higher expression of heat shock protein 70 (Hsp-70). | AP-1, ERK, PKC pathway. Decreased intracellular thiol content involved in induction. | Higher expression was seen in OSCC cases of poor differentiation. In between OSCC specimen, association found between low grade expression and lymph node metastasis. | Lee et al. (2008a) |
| 3. | GNM epithelial cells (0–160 $\mu\text{g/ml}$) | Arecoline induced higher expression of metallothionein-1 transcriptionally. | Decreased intracellular thiol content involved in induction. | Higher expression was seen in OSCC cases. Association found with lymph node metastasis. | Lee et al. (2008b) |
| 4. | GNM epithelial cells (0–160 $\mu\text{g/ml}$) | Arecoline induced higher expression of hemeoxygenase-1 (HO-1) transcriptionally. | Decreased intracellular thiol content involved in induction. | Higher expression was seen in OSCC cases. Association found with lymph node metastasis. | Lee et al. (2008c) |
| 5. | OC2 epithelial cells (0–160 $\mu\text{g/ml}$) | Arecoline induced higher expression of heat shock protein 47 (Hsp-47). | ERK, Cox-2, PI3K, tyrosine kinase pathways. Decreased intracellular thiol content involved in induction. | Higher expression was seen in OSCC cases. In between OSCC specimen, association found between low grade expression and lymph node metastasis. | Lee et al. (2011) |
| 6. | Human oral keratinocytes (0–160 $\mu\text{g/ml}$) | Arecoline induced higher expression of heat shock protein 27 (Hsp-27). | Cox-2, ERK, p38 pathways. Decreased intracellular thiol content involved in induction. | Higher expression was seen in OSCC cases. In between OSCC specimen, association found between low grade expression and lymph node metastasis. | Lee et al. (2012a) |

| Serial no. | Cell line and dose of arecoline ($\mu\text{g}/\text{ml}$) | Effects | ROS/receptor/ pathways involved | Association of expression with oral squamous cell carcinoma (OSCC) | Reference |
|------------|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 7. | Human oral keratinocytes, OECM-1 cancer cells (0–160 $\mu\text{g}/\text{ml}$) | Arecoline induced higher expression of Snail. | ROS involved in induction. | Higher expression is seen in cases of OSCC samples. Association found with lymph node metastasis and poor differentiation of OSCC samples. | Lee et al. (2013) |
| 8. | S-G epithelial cells, FaDu carcinoma cells (0–20 $\mu\text{g}/\text{ml}$) | Arecoline induced higher expression of zinc finger E-box binding homeobox-1 (ZEB1). | – | Higher expression of ZEB1 mRNA was seen in recurrent cases of OSCC than in initial stages. | Ho et al. (2015) |
| 9. | S-G epithelial cells, FaDu carcinoma cells (0–20 $\mu\text{g}/\text{ml}$) | Arecoline induced higher expression of cell lineage abnormal 28 B (Lin28B) protein. | – | Higher expression of Lin28B mRNA is seen in cases of OSCC samples. Association found with lymph node metastasis. | Lin et al. (2015) |
| 10. | S-G epithelial cells OECM-1 cancer cells (0–20 $\mu\text{g}/\text{ml}$) | Arecoline induced higher expression of S100A4. | PI3K, JNK pathway, Hypoxia inducible factor 1 α involved. | Higher expression seen in cases of OSCC samples, especially those that were moderately or poorly differentiated. Association found with lymph node metastasis. | Hu et al. (2015) |

LT – long-term; ROS – reactive oxygen species; PI3K – phosphatidylinositol 3-kinase; Cox-2 – cyclooxygenase-2; JNK – c-Jun NH₂-terminal kinase; ERK – extracellular signal-regulated protein kinase; PKC – protein kinase C; AP-1 – activator protein-1

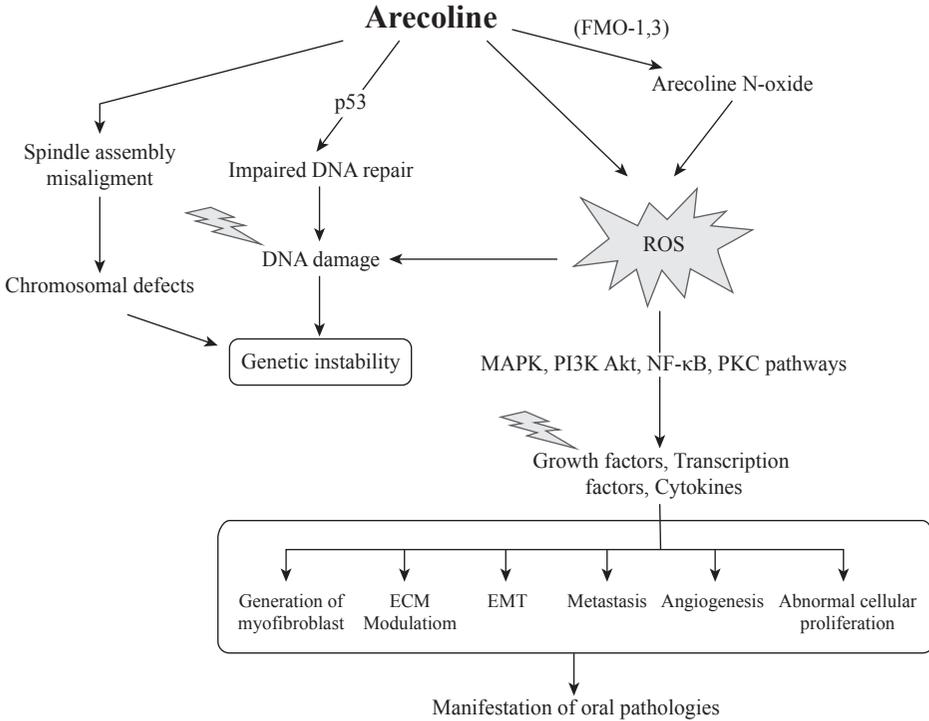


Figure 4 – A hypothetical mechanistic pathway of arecoline mediated oral pathologies. Induction indicated by the symbol: ⚡

ROS involvement in cancer includes induction of pathways like mitogen-activated protein kinase (MAPK), phosphatidylinositol-3-kinase (PI3K)/Akt and nuclear factor kappa-light chain enhancer of activated B-cells (NF-κB) (Liou and Storz, 2010). Analysis of the data compiled in Tables 1 and 2 also indicates these pathways to be involved in the up-regulation of pro-fibrotic or carcinogenic factors in cell systems affected by arecoline. Targeting these pathways might be a promising tool in the therapy of ROS induced OSF and OSCC.

Figure 4 depicts hypothetical mechanistic pathways where ROS induced DNA damage along with perturbation of expression profile of growth factors, transcription factors and ECM proteins drive the development of areca nut mediated oral pathologies.

As mentioned earlier, OSF can develop into a malignant phenotype. Therefore, it is important to understand how arecoline triggers the expression of effectors that mediate this transition. Under continuous exposure to hypoxic conditions and HIF-1α activity, the transformation from fibrotic to cancer condition might take place, thereby highlighting arecoline’s role as a tumour promoter. Arecoline induced stabilisation of HIF-1α and activation of TGF-β1 play roles in the regulation of

several transcription factors, such as ZEB-1, Snail, Twist, that induce both type 1 and type 2 EMT leading to fibrosis and cancer, respectively (Lee et al., 2013; Chang et al., 2014; Ho et al., 2015; Lee et al., 2016).

Another interesting aspect of arecoline, which was discovered during the study, is its differential activity to induce ECM modulators in fibroblasts and cancer cells. Exposed fibroblasts up-regulate TIMP-1, whereas MMP-2 is inhibited (Chang et al., 2002a). In cancer cells, MMP-9 is induced, but TIMP-1 is inhibited (Chang et al., 2013a). TGF- β 1 has been found to regulate the expression of MMP-9 via Snail transcription factor in cancer cells (Sun et al., 2008). After exposure of cells from cancer cell line to arecoline for an extended period of time, increased expression of metalloproteinases MMP-8 and MMP-1 was observed (Liu et al., 2007; Lee et al., 2008). This might indicate that under already initiated and promoted tumour conditions, arecoline might play a role in the progression of areca nut induced carcinogenesis.

Junctional protein disruption is an essential phenomenon by which cells can acquire a metastatic phenotype (Parker et al., 2001). Giri et al. (2010) found arecoline to down-regulate tight junctional protein zona occludens-1 (ZO-1) and claudin-1 along with delocalisation of both ZO-1 and E-cadherin. Down-regulation of E-cadherin during junctional protein disruption leads to the dislocation of β -catenin from the plasma membrane. Under the influence of Wnt signalling in cancer conditions, degradation of cytoplasmic β -catenin is also inhibited. Both junctional and cytoplasmic β -catenin then moves to the nucleus and acquires transcriptional control over genes, leading to abnormal cell proliferative activity (Kam and Quaranta, 2009; Camilli and Weeraratna, 2010; Liu and Millar, 2010). Arecoline exposure induces elevated expression of β -catenin in epithelial cells (Lee et al., 2012b). Signalling cascades have been constructed using the accumulated data in Figure 3.

Role of arecoline metabolites in areca nut induced oral pathologies

Formation of these metabolites *in vitro* or in *in vivo* systems indicates the probability of exposure of humans to these metabolites. An assessment of the biological effects of these metabolites is, therefore, necessary to understand the areca nut mediated pathogenesis of OSF or OSCC.

The N-nitrosated metabolites, NMPA, NMPN and NGL, induce DNA single-stranded breaks in epithelial cells where NMPA was the strongest while NMPN and NGL were weak inducers (Sundqvist et al., 1989). Of the *in vivo* metabolites, genotoxic effects were displayed by arecaidine in mice via induction of sister chromatid exchanges (SCE) in bone marrow cells (Panigrahi and Rao, 1984). Arecoline N-oxide was also found to be genotoxic in both mice model and fibroblasts (Kuo et al., 2015). The genotoxic potential of the other metabolites of arecoline is unknown.

Both MNPA and MNPN were found to be carcinogenic in rats (Wenke et al., 1984b; Nishikawa et al., 1992). NGL was neither found to be a strong mutagen in

the bacterial test system nor a strong carcinogen in the murine test system (Rivenson et al., 1988; Miyazaki et al., 2005). Activated N-nitrosamines can cause alkylation of DNA base pairs (Miyazaki et al., 2005). Hence, activated areca nut derived nitrosamines can initiate carcinogenicity via alkylation of DNA, as indicated by the study where MNPN was observed to induce methylation and cyanoethylation of guanine residues in rats, especially in the genetic material obtained from nasal mucosa, esophagus and liver (Prokopczyk et al., 1987, 1988). The role of arecaidine as a pro-fibrotic agent remains unclear as indicated by contradictory reports in *in vitro* models (Harvey et al., 1986; Tsai et al., 1999; Chang et al., 2013b).

The most important metabolite of arecoline might be arecoline N-oxide. It was found to be mutagenic in bacteria without any metabolic activation (Lin et al., 2011). In mice, the compound induced increased collagen deposition in the tongue along with hyperplasia. Several pro-fibrotic genes (TGF- β 1, IL-6, S100A4, and fibronectin) were induced by the compound in fibroblasts along with suppression of E-cadherin (Kuo et al., 2015). Therefore, fibrosis induced by areca nut chewing can be mediated partially by arecoline N-oxide. In another study, N-oxide induced sub-lingual hyperplastic lesions in mice along with the up-regulated expression of caspase-8, which, instead of producing a pro-apoptotic effect, enhanced cell survival and proliferation (Ko et al., 2018). 8-hydroxydeoxy guanosine level in fibroblasts cultured in the presence of arecoline N-oxide indicates oxidative stress induced DNA damage (Kuo et al., 2015). Similarly to arecoline, supplementation of thiol-containing agents can reverse the mutagenic property of the compound (Lin et al., 2011). These facts indicate oxidative stress to be an important factor for both arecoline and arecoline N-oxide induced pathologies. Hence, the consumption of antioxidants can be a preventive factor against the development of fibrosis and areca nut driven oral cancer.

Table 3 summarizes the various effects of arecoline metabolites. It also provides an assessment of the potential harmfulness of the metabolites based on the possibility of involvement in areca nut induced pathologies. Although arecoline N-oxide has been discovered as a possible candidate mediating areca nut effects, in the *in vivo* studies mentioned above, arecoline N-oxide was administered via oral brushing. On the other hand, Lin et al. (2011) observed that the compound lost its mutagenicity after metabolic activation by S9 fraction of rat liver. Hence, the specific role of the compound *in vivo* remains uncertain because the knowledge about direct exposure through areca nut is still unknown.

Involvement of enzymes in the metabolism of arecoline and arecoline N-oxide

A study of arecoline metabolism along with metabolism of its metabolites arecaidine and arecoline N-oxide has revealed basic routes that these compounds undergo. It involves de-esterification, N-oxidation and reduction of the double bond leading to the formation of metabolites, including mercapturic acids, mercapturic acid derivatives and nipecotic acid derivatives (Nery, 1971; Giri et al., 2006, 2007).

According to Patterson and Kosh (1993), cytochrome P450 (CYP450) family of enzymes do not play a significant role in arecoline metabolism because most of arecoline was metabolized by mice liver homogenate even in the presence of nonspecific CYP450 inhibitor. However, these enzymes might be involved in the metabolic activation of N-nitrosamine compounds formed from arecoline. This is supported by a study conducted in a bacterial test system where metabolic activation of MNPN, MNPA and NGL was observed to be carried out by human CYP450 enzymes, especially by members of family CYP2A and CYP1A1 (Miyazaki et al., 2005). CYP enzymes activate N-nitrosamines and the products formed, thereby lead to alkylation of nucleic acid base pairs (Miyazaki et al., 2005). Genetic polymorphisms of CYP enzyme encoding genes (CYP2A6 and CYP1A1) have been found to be associated with oral cancer (Kao et al., 2002; Topcu et al., 2002).

In contrast to CYP450, flavin-containing monooxygenases participate in the metabolism of arecoline to its N-oxide (Giri et al., 2007). No association study has been found so far between the FMO polymorphism and oral cancer. As arecoline N-oxide is biologically active and might have a prominent role in areca nut mediated fibrotic disorders, the existence of polymorphic variants of the gene encoding this enzyme in the general population might develop predisposition towards development of areca nut mediated fibrosis. FMO-1 carries out the process most efficiently of all the other isozymes (Giri et al., 2007). It is abundantly expressed in the kidney (Zhang and Cashman, 2006). An association has been found between betel quid chewing and chronic kidney disorder in a population-based study. However, the association was influenced by several other factors (Hsu et al., 2011). In addition, a portion of the formed N-oxide undergoes reduction and forms the parent compound arecoline. CYP450 family of enzymes plays a role in this type of deoxygenation (Krueger and Williams, 2005; Montellano, 2013).

Carboxylesterases are involved in the metabolism of arecoline to arecaidine (Patterson and Kosh, 1993). *In vitro*, mercapturic acid formation from arecoline does not require any enzymatic assistance (Boylard and Nery, 1969). Therefore, under *in vivo* condition, mercapturic acid formation from the parent compound and metabolites might involve reaction with glutathione without the involvement of any enzymes. Although glutathione S-transferases (GSTs) participate in the phase 2 metabolism of xenobiotics, producing mercapturic acids (Hayakawa, 1977), the involvement of GSTs in arecoline metabolism is not known.

Apart from the study conducted by Patterson and Kosh (1993) and Giri et al. (2007), direct involvement of enzymes in the metabolism of arecoline in mammals has not been studied. In a study carried out by Chiang et al. (2007), arecoline has been found to suppress the expression of several phase I and phase II xenobiotic-metabolizing enzymes, which might indirectly affect the metabolism of arecoline itself. Moreover, mechanism of formation of other metabolites like nipecotic acid derivatives and the aldehyde derivative of arecoline (1-methyl-3,4-dehydropiperidine-3-carboxaldehyde) has not been studied yet.

Table 3 – Summarizes the biological effects of the metabolites of arecoline

| Serial no. | Metabolites | Biological activity | Risk assessment |
|------------|-------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|
| 1. | Arecaidine | <p>Mutagenic in bacterial tester strains (TA100, TA 1535, TA 98, TA 1538) (Shirname et al., 1983).</p> <p>Induced sister chromatid exchange in mice bone marrow cells (Panigrahi and Rao, 1984).</p> <p>Weakly induced DNA single strand break in cultured human epithelial cells (Sundqvist et al., 1989).</p> <p>Induced both collagen formation by fibroblasts and collagen phagocytic ability reduction of fibroblasts (Harvey et al., 1986; Tsai et al., 1999).</p> <p>Had no effect on the myofibroblastic transdifferentiation of fibroblasts (Chang et al., 2013b).</p> <p>Induced senescence, DNA double stranded breaks along with transforming growth factor-β and matrix metalloproteinase-2 expressions in fibroblasts (Rehman et al., 2016).</p> | Possibly harmful |
| 2. | Arecoline N-oxide | <p>Mutagenic in bacterial tester strains (TA100, TA 98) (Lin et al., 2011).</p> <p>Induced DNA damage in both mammalian test system and cultured fibroblasts (Kuo et al., 2015).</p> <p>Induced pro-fibrotic genes in cultured fibroblasts (Kuo et al., 2015).</p> <p>Induced caspase-8 activation which rather had a cell proliferative effect (Ko et al., 2018).</p> | Possibly harmful |
| 3. | 3-methylnitrosamino-propionitrile | <p>Induced tumour in several organ of exposed rats (Wenke et al., 1984b).</p> <p>Induced modification of DNA base pair in exposed rats (Prokopczyk et al., 1988; Rivenson et al., 1988).</p> <p>Weak inducer of DNA single strand breaks in cultured human epithelial cells (Sundqvist et al., 1989).</p> | Possibly harmful |
| 4. | 3-methylnitrosamino-propionaldehyde | <p>Induced DNA single strand break in cultured human epithelial cells (Sundqvist et al., 1989).</p> <p>Induced tumour in several organ of exposed rats (Nishikawa et al., 1992).</p> | Possibly harmful |

| Serial no. | Metabolites | Biological activity | Risk assessment |
|------------|-------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------|
| 5. | N-nitrosoguvacoline | Weakly carcinogenic in rats (Rivenson et al., 1988). Weak inducer of DNA single strand breaks in cultured human epithelial cells (Sundqvist et al., 1989). Weakly mutagenic in bacterial tester strains (TA100, TA 98) (Wang and Peng, 1996). | Possibly harmless |
| 6. | Arecaidine N-oxide | Unknown | – |
| 7. | N-methylnipecotic acid | Unknown | – |
| 8. | 1-methylnipecotic acid 1-oxide methylester | Unknown | – |
| 9. | 1-methyl-3,4-dehydropiperidine-3-carboxaldehyde | Unknown | – |
| 10. | Arecaidinylglycerol | Unknown | – |
| 11. | Arecaidinylglycine | Unknown | – |
| 12. | N-methylnipecotylglycine | Unknown | – |
| 13. | Mercapturic acid of arecoline | Unknown | – |
| 14. | Mercapturic acid of arecaidine | Unknown | – |
| 15. | Mercapturic acid of arecoline N-oxide | Unknown | – |
| 16. | 4-mercapto-1-methylnipecotic acid methylester | Unknown | – |
| 17. | 4-methylmercapto-1-methylnipecotic acid 1-oxide methylester | Unknown | – |
| 18. | N-nitrosonipecotic acid | Unknown | – |

CYP450 and FMO mediated metabolism of arecoline N-oxide has been found to take place in the mitochondria of liver cells in a study conducted by Wang et al. (2018). Mitochondrial metabolism of arecoline N-oxide by the above-mentioned enzymes might be responsible for the generation of ROS, which mediates the toxic effects of the compound.

Conclusion

Apart from arecaidine and arecoline N-oxide, the biological effects of other metabolites also need to be elucidated. Some of them might be promptly modified by enzymes in a manner similar to mercapturic acids so that their excretion is facilitated, and subsequently, they pose a lesser threat for carcinogenic activity. Other compounds as well as metabolites that possess a high toxic potential might

also be present in areca nut and oral cells might be directly exposed to them on the consumption of the nut. For example, both arecaidine and N-methylnipecotic acid are also present in areca nut (IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2004; Hu et al., 2010). But as the toxicity of N-methylnipecotic acid is not known, its effect on the site of exposure cannot be determined.

Knowledge about the enzymes and genes that encodes them can provide an important insight into the metabolism of these xenobiotics. This will open another field of research correlating the differential expression and polymorphisms of these genes to an individual predisposition to oral cancer in betel nut consumers. Even the metabolites might be more potent in causing hazardous effects than the parent compound, as seen in the case of arecoline N-oxide.

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