

Effects of areca nut consumption on cell differentiation of osteoblasts, myoblasts, and fibroblasts

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Key words: Areca nut, Cell differentiation, Osteoblast, Myoblast, Fibroblast

Abstract: Areca nut is used worldwide as a hallucinogenic addicting drug along the tropical belt. Arecoline, a toxic compound, is the most important alkaloid in areca nuts. The adverse effects of oral uptake and chewing of areca nut are well known. For example, the possibility of cancer caused by chewing areca nuts is widely discussed. Chewing areca nut has other adverse effects on other organs, including abnormal cell differentiation, oral cancer, and several other diseases. The use of areca nut is also associated with low birthweight. Skeletal musculature is the largest organ in the body and is attached to the bones. During embryo development, the differentiation of bone and muscle cells is critical. In this article, we reviewed the effects of areca nut and arecoline on embryonic cell differentiation, particularly osteoblasts, myoblasts, and fibroblasts.

Introduction

Areca or betel nut is used worldwide by 600 million people, with India and Southeast Asian countries being the major consumers of areca nut (Arora and Squier, 2019). Arecoline (methyl 1-methyl-3,6-dihydro-2H-pyridine-5-carboxylate) is the most important alkaloid and the main toxic compound in areca nuts (Chen *et al.*, 2021). Most studies have examined the role of areca nut and arecoline on the pathogenesis of oral lesions (Das and Giri, 2020). However, areca nut also affects most organ systems, such as reproductive organs, heart, brain, gastrointestinal tract, and lungs. Areca nut consumption also increases the risk of cardiac arrhythmias, asthma, metabolic syndrome, myocardial infarction, neuronal injury, hepatotoxicity, type II diabetes, central obesity, and hyperlipidemia (Garg *et al.*, 2014). The above-mentioned diseases, such as cardiac arrhythmias, asthma, and metabolic syndrome, involve dysregulated cell differentiation. Arrhythmogenic cardiomyopathy is characterized by myocardial dysfunction and fibrofatty replacement of myocardial tissue. It may result from abnormal differentiation of cardiac progenitor cells to adipocytes (Lombardi *et al.*, 2011). Asthma is an inflammatory disease. The differentiation of Th2/Th17 cells

is increasingly dysregulated and is associated with asthma severity (Vroman *et al.*, 2015). Obesity is a risk factor for the development of metabolic disorders. Subcutaneous-preadipocyte differentiation is associated with metabolic syndrome (Park *et al.*, 2012). Cancers are considered developmental disorders that disrupt the normal cell differentiation (Tiwari, 2012). Long-term chewing of betel nut causes sperm reduction, asthma, uterine, mouth, and esophageal cancers (Chen *et al.*, 2021). A systematic review showed that areca nut chewing also increases the risk of developing liver diseases (Khasbage *et al.*, 2022). The risk of oral cancer increases with the daily number of quids consumed and the number of years of chewing betel nut in a dose-responsive manner (Warnakulasuriya and Chen, 2022). A systematic review showed that the use of areca (betel nut) is significantly associated with low birthweight (de Silva *et al.*, 2019); consumption of high doses of areca nut leads to an arrest of endothelial cell differentiation and subsequent dysfunction of the fetus (Al-Rmalli *et al.*, 2011).

In this review, we focused on the effects of areca nut on cell differentiation.

Osteoblast cell differentiation

The skeletal muscle is the largest organ in the body (Pedersen, 2013). The main function of bone is to support the attachment of muscles. Bone remodeling is regulated by the balance between osteoclasts and osteoblasts (Kim *et al.*, 2020).

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Received: 28 July 2022; Accepted: 28 August 2022



Decreased bone formation by osteoblasts and increased bone resorption by osteoclasts result in alveolar bone loss (Mori *et al.*, 2007). Osteoclasts are differentiated from monocyte/macrophage lineage cells. Hematopoietic stem cells (HSCs) are capable of self-renewal and have the pluripotent ability to differentiate into all hematopoietic cell types (Yahara *et al.*, 2020). DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) can be used to trace short periods of osteogenic differentiation of mesenchymal stem cells (Ocarino *et al.*, 2008). HSCs give rise to oligopotent progenitor cells, such as common myeloid progenitor cells (CMPs), upon differentiation, which in turn, differentiate into macrophages and osteoclasts (Ono and Nakashima, 2018). Macrophage precursors differentiate into marked monocytes and then into tissue-specific macrophages. These tissue-specific macrophages can fuse to provide osteoclasts, or sometimes they fuse into multinucleated giant cells to further differentiate into osteoclasts (Yao *et al.*, 2021). Multinucleated osteoclasts work for bone resorption as induced by the receptor activator of nuclear factor- κ B ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Suda *et al.*, 1992).

RANKL is a key regulator in osteoclastogenesis. It plays indispensable and irreplaceable functions in the osteoclast differentiation program by stimulating several osteoclastogenic pathways, such as NF- κ B, mitogen-activated protein kinases (MAPK), and immunoreceptor tyrosine-based activation motif signals, as well as suppressing inhibitory molecules, such as tumor necrosis factor receptor-associated factor 3, p100, retinol binding protein-J, interferon regulatory factor 8, MAF bZIP transcription factor B, and B-cell lymphoma 6 (Takayanagi, 2021). RANKL is a membrane-associated cytokine expressed by osteoblasts. Osteoclast precursors express RANK, a RANKL receptor, to recognize RANKL by cell-cell interaction and differentiate into osteoclasts. Inhibition of RANKL-RANK signaling results in increased bone mass by preventing osteoclastic-regulated bone resorption (Udagawa *et al.*, 2021). Osteoclasts can be generated *in vitro* by inducing macrophage lineage cells with RANKL treatment (Kurotaki *et al.*, 2020). When primary bone marrow macrophages were treated with CSF and RANKL, 50–100 μ M arecoline reduced the development of multinucleated osteoclasts by suppressing the expression of osteoclast differentiation-related genes through interference with the AKT, MAPK, and NF- κ B activation pathways (Liu *et al.*, 2020). Ling *et al.* (2005) showed that ripe areca nut extracts (rANE) can modulate the expression of RANKL. The expression of RANKL mRNA and protein in osteoblast-like MG63 cells is stimulated by rANE in a dose-dependent manner. However, the viability of these cells was reduced with rANE treatment.

Bone marrow stromal cells can be differentiated into osteoblast-like cells (Rodriguez *et al.*, 2009). KUSA/A1 cells (bone marrow stromal cell line) differentiated into osteoblast-like cells and induced bone tissue by both *in vitro* cell culture and intra-abdominal diffusion chambers implanted in SCID mice.

Muscle cell differentiation

Arecoline exposure results in a decrease in locomotor activity caused by defective somatic skeletal muscle development and

mitochondrial dysfunction. In arecoline-exposed zebrafish embryos, reduced locomotor activity and swimming ability impairment were observed (Peng *et al.*, 2015). Immunofluorescent staining and ultrastructural observations revealed a defective arrangement of myosin heavy chains and abnormal myofibril arrangement; arecoline also inhibited the embryonic development of mice. Arecoline could decrease the number of implanted embryos in mice at early pregnancy (Liu *et al.*, 2011) and also inhibit the expansion of trophoblast outgrowth of blastocysts. Taken together, arecoline was harmful to mouse embryos as early as the peri-implantation stage (Liu *et al.*, 2011). C2C12, a myoblast cell line, is a good material to study skeletal muscle cell differentiation. However, high passage numbers induce resistance to apoptosis (Pronsato *et al.*, 2013). High (>60) passage numbers of C2C12 cells were found depleted of mitochondrial DNA (mtDNA) and resistant to H₂O₂ induction of apoptosis. We used low (<20) passage numbers of C2C12 for our studies. It was shown that arecoline could inhibit the myogenic C2C12 cell differentiation by reducing the activated signal transducer and activator of transcription 3 (STAT3). At 0.4 mM concentration of arecoline, apoptosis increased, and the viability of C2C12 cells decreased (Chang *et al.*, 2012). Myogenic differentiation markers such as myosin heavy chain and myogenin were inhibited by arecoline. Furthermore, arecoline inhibited the activated form of phosphorylated STAT3 during myotube formation. The clustering of acetylcholine receptors (AChRs) at the neuromuscular junction (NMJ), initiated by the protein agrin was important for developing muscular function. It was demonstrated in this study that arecoline could inhibit the formation of agrin-induced AChR clusters as well as destabilize agrin-induction spontaneously. These results demonstrate the adverse effects of arecoline from areca nut on muscle development (Chang *et al.*, 2013). Arecoline was reported to generate reactive oxygen species (ROS) as well as to induce apoptosis (Yen *et al.*, 2011). Oxidative stress is important for the pathological development of skeletal musculature (Musarò *et al.*, 2010). To prevent the damage caused by oxidative stress on differentiating myoblasts, N-acetyl-cysteine (NAC), a scavenger of ROS, is involved in the generation of hepatic glutathione (GSH) to remove drugs (Yan *et al.*, 2018). It can decrease oxidative stress and glutathione-dependent damage in cells, such as rat pancreatic Rin-5F cells in the presence of high concentrations of glucose and fatty acids, and increase the viability of rat C6 astroglia-like cells treated with cocaine (Alnahdi *et al.*, 2019; Badisa *et al.*, 2015). Our recent study showed that when C2C12 cells were treated with arecoline, NAC regenerated a decreased number of myotubes as well as nuclei in each myotube (Li *et al.*, 2022). In addition, there was a minor improvement in NAC-mediated expression of myogenin and MYH, two myogenic markers, caused by arecoline. MEK/ERK signaling was shown to participate in the maintenance of myogenic progenitor cells (Miyake *et al.*, 2020). Knockdown of ERK1/2 inhibits C2C12 cell differentiation (Feng *et al.*, 2013). Recently, one study showed that NAC could restore the arecoline-induced decrease in the expression of p-ERK1/2 (Li *et al.*, 2022). Areca nut is significantly associated with low birthweight in human studies (de Silva *et al.*, 2019; Senn *et al.*, 2009).

Fibroblast differentiation

Fibrosis development is a response to inflammation and epithelial injury resulting in the accumulation of extracellular matrix and myofibroblast activation (Edeling *et al.*, 2016). Myofibroblasts differentiating from fibroblasts and other cell types, contribute to tissue repair during wound healing. Differentiated myofibroblasts are characterized by increased production of extracellular matrix (ECM) proteins. However, they can impair organ function when the contraction and ECM protein secretion become excessive, such as in Dupuytren's disease, scleroderma, hypertrophic scars, and heart and kidney fibrosis (Hinz *et al.*, 2007). Betel quid chewing can increase the risk of oral submucous fibrosis OSF, a collagen deposition (Shen *et al.*, 2020). Areca nuts contain the components considered the main causative factors in the OSF. The trauma caused by coarse areca nut fiber and alkaloids induces tissue inflammation, fibroblast proliferation, collagen deposition, and myofibroblast differentiation to finally develop into OSF and oral cancer. During these processes, ECM turnover changes. The rearrangement of ECM is regulated by molecules such as plasminogen activator inhibitor-1 (PAI-1), transforming growth factor (TGF)- β 1, lysyl oxidase, cystatin, metalloproteinases, and tissue inhibitors of metalloproteinases (Cheng *et al.*, 2020). Slug is an EMT inducer associated with fibrogenesis and carcinogenesis. Arecoline increases the expression of Slug in normal fibrotic buccal mucosal fibroblasts (fBMFs). The inhibition of Slug can prevent arecoline-induced myofibroblast activation. The binding of Slug to the E-box of type I collagen promoter enhances the expression of type I collagen. These results showed the importance of arecoline-induced fibrogenesis (Fang *et al.*, 2019). The expression of microRNA (miR)-21 was induced by arecoline treatment in a dose-dependent manner in BMFs through TGF- β signaling. The myofibroblast characteristics, such as higher cell motility and collagen gel contractility, induced by arecoline were suppressed by the miR-21 inhibitor. Moreover, miR-21 and myofibroblast marker, smooth muscle actin- α (α -SMA), were positively correlated. Therefore, the overexpression of miR-21 may be caused by the activation of areca nut components through the TGF- β pathway (Yang *et al.*, 2021). Areca nut water extract induced p-SMAD2, an effector of TGF- β signaling, and TGF- β downstream targets, such as transglutaminase 2 (TGM2), transmembrane prostatic androgen-induced RNA (TMEMPAI), thrombospondin 1, and TGF- β 1) in human epithelial keratinocyte HaCaT cells. These results suggest the important role of TGF- β induced by areca nut in OSF progression (Khan *et al.*, 2012). Profibrotic long noncoding RNAs (lncRNAs) H19 have been reported as overexpressed in several fibrotic diseases. With arecoline treatment, the expression of H19 was dose-dependently upregulated in BMFs. The results further demonstrated that arecoline stimulated the upregulation of H19 through the TGF- β pathway (Yu *et al.*, 2021). Copper was reported in tissue fibrogenesis to cross-link collagen through the copper-dependent enzyme lysyl oxidase. The mean tissue copper level was higher in the OSF specimens compared with the non-areca chewing controls among patients with OSF. This finding suggests that copper is an initiating factor in OSF to stimulate fibrogenesis by increasing lysyl oxidase activity

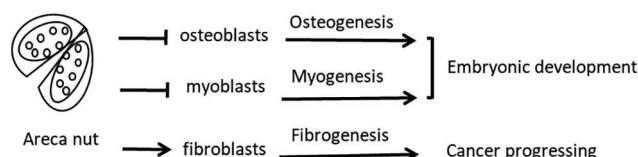


FIGURE 1. The effects of areca nut on cell differentiation. Consumption of areca nut can inhibit the differentiation of osteoclasts and myoblasts and stimulate fibrogenesis of fibroblasts.

(Trivedy *et al.*, 2000). TGF- β 1 is the primary inducer of fibrosis and plays an important role in myofibroblast formation. The activation of myofibroblasts includes rapid synthesis of ECM components, such as collagen and fibronectin, during the repair of pathological tissues (Shu and Lovicu, 2017). Arecoline can induce wound healing capacities, invasion, migration, and collagen gel contractility of BMFs. Arctigenin, a lignan extracted from *Arctium lappa*, is capable of abolishing these myofibroblast characteristics of fBMFs through TGF- β /Smad signaling (Lin *et al.*, 2019). Areca quid can cause OSF via ROS generation. TGM-2 is a matrix protein related to several fibrotic disorders and can be induced by ROS. Fibroblasts derived from OSF were found to have higher TGM-2 expression than normal BMFs. TGM-2 and ROS induced by arecoline were found in BMFs. These results suggest that arecoline induces ROS generation and upregulates TGM-2 expression, leading to ECM accumulation in OSF tissues of areca nut chewers (Lee *et al.*, 2016). Hypoxia-inducible factor-1 (HIF-1) is a heterodimer containing α and β subunits as key mediators of cellular adaptation to low oxygen. It can regulate several profibrogenic genes related to tissue fibrosis. Arecoline was found capable of enhancing the expression of HIF-1 α protein dose-dependently. PAI-1 is important for the inhibition of plasmin-dependent ECM degradation to accumulate ECM, and hypoxia can increase arecoline-induced PAI-1 protein expression. These data showed that the level of HIF-1 α protein increased in OSF tissues from areca quid chewers (Tsai *et al.*, 2015). Production of collagen of the ECM and induction of EMT is associated with renal cell fibrosis, and E-cadherin, N-cadherin, and vimentin are markers of EMT. TGF- β was shown to mediate the progression of renal fibrosis. Arecoline caused a dose-dependent decrease in E-cadherin expression and increases in α -SMA, vimentin, N-cadherin, and collagen in cultured human kidney (HK2) cells. In addition, arecoline also increases the expression of the phosphorylated extracellular signal-regulated kinase (ERK), suggesting that arecoline is important for inducing the EMT and fibrogenesis in renal tubule cells through ERK-mediated signaling pathways (Hsieh *et al.*, 2020).

Conclusion

The adverse effects of areca nut have been the subject of several studies recently. In addition to oral diseases, areca nut also inhibits the differentiation of different cell types, such as osteoblasts and myoblasts, and promotes the fibrogenesis of fibroblasts. Areca nut is significantly associated with low birthweight in human studies. For embryonic development, the differentiation of bone and muscle cells is critical. Skeletal muscle is the largest organ in

the body, and the primary function of the bones is to support the attachment of muscles; besides, muscle strength increases with the increase in muscle mass. These results will be helpful in understanding the mechanisms behind adverse effects, such as oral diseases, cancer progression, fibrosis of different organs, and ill effects on embryonic development caused by the consumption of areca nut (Fig. 1).

Acknowledgement: I thank the constant support provided by Kaohsiung Medical University (Taiwan) for this study.

Author Contribution: The author confirms sole responsibility for the following: article conception and manuscript preparation.

Ethics Approval: Not applicable.

Funding Statement: I acknowledge the funding provided by the Ministry of Science and Technology, Taiwan (108-2314-B-037-075) and the Kaohsiung Medical University Research Foundation (KMU-M103001, KMU-M104003, KMU-TP104PR16).

Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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