

# Molecular Mechanism of Oral Submucous Fibrosis induced by Arecoline: A Literature Review

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## ABSTRACT

Oral Submucous Fibrosis (OSMF) is an oral mucosal disease with lamina propria collagen hyperplasia as the main pathological change, which belongs to Oral Potentially Malignant Disorders (OPMDs). Areca nut is identified as a class I carcinogen with complex components. Arecoline is the main component of areca nut which plays an important role in the pathogenesis of OSMF. Through the review of literature, it is shown that arecoline can lead to the change in level of cytokines in oral mucosa, which further leads to the imbalance of collagen metabolism. This article reviews the molecular mechanism research progress of arecoline in promoting the pathogenesis of OSMF in recent years, including Transforming Growth Factor- $\beta$  (TGF- $\beta$ ) and Reactive Oxygen Species (ROS) associated with increased collagen production, Matrix Metalloproteinase (MMP) and Tissue Inhibitor of Metalloproteinase (TIMP) associated with reduced collagen degradation, hypoxia and microvascular related factors and in order to provide reference for the treatment of the disease at the molecular level.

**Keywords:** Areca nut, Aetiology, Potentially malignant disorders, Transforming growth factor-beta

## INTRODUCTION

World Health Organisation (WHO) definition of OPMDs is “clinical presentations that carry a risk of cancer development in the oral cavity, whether in a clinically definable precursor lesion or in clinically normal mucosa” [1]. OSMF was modified to OPMDs by WHO and included in the fourth edition of the World Health Organisation for head and neck tumours [1,2]. Pindborg, in 1966, defined OSMF as “an insidious chronic disease affecting any part of the oral cavity and sometimes pharynx. It is associated with juxta-epithelial inflammatory reaction followed by fibroelastic changes in the lamina propria layer, along with epithelial atrophy which leads to rigidity of the oral mucosa proceeding to trismus and difficulty in mouth opening.” [3]. It is more common in South and East Asia, including India, Pakistan, Bangladesh, southern China and the Pacific Islands. Most patients present with an intolerance to spicy food, rigidity of lip, tongue and palate leading to varying degrees of limitation of opening of the mouth and tongue movement [4]. Various other names has also been given to OSMF by different authors in literature such as idiopathic scleroderma of the mouth, juxta-epithelial fibrosis, idiopathic palatal fibrosis, submucous fibrosis of the palate and pillars, sclerosing stomatitis and diffuse OSMF. The reported prevalence rate of OSMF in India is 0.5% [5], while in Hunan province of China is between 0.1% and 1.9% [6]. OSMF shows a malignant transformation rate as high as 5.2%, and the annual rate of cancer evolution is 0.98% [7]. It appears from current evidence that a combination of various factors could best explain the pathogenesis of OSMF. These include genetic alterations, infectious agents associated with oral cancer including Herpes Simplex Viruses (HSV) and Human Papilloma Virus (HPV) infection, carcinogens such as tobacco and areca nut, nutritional factors including iron depletion and immunologic factors, all of which also have been implicated in the development of oral cancer. The genetic susceptibility to OSMF is due to genomic instability, which is characterised by loss of heterozygosity related to OSMF grading [8]. Genetic factors are the cause of immune abnormalities. It has been reported that the serum levels of IgG, IgA and IgM are increased in OSMF patients. Cys-X-Cys ligand 9 (CXCL9) is an important factor in inducing effector neutrophils and lymphocytes in immune response. It was found that the level of CXCL9 in OSMF increased significantly, indicating that the intensity of immune response increased during the pathogenesis of OSMF.

[9]. However, it is a well-established fact in many scientific literatures that areca nut is the most important etiologic factor of OSMF.

Areca nut (or betel nut) has been identified as a class carcinogen by the WHO and is the fourth largest addictive substance in the world after tobacco, alcohol and caffeine [10]. About 600 million people around the world chew areca nut. Areca nut chewers are mainly distributed in India, south of China, Pacific Rim islands and Africa, Europe and North America [11], which is basically consistent with the areas with high prevalence of OSMF. The components of areca nut are very complex, and the main alkaloids are arecoline, arecaidine, guvacoline, guvacine, arecaidine-D5,5 and arecaidine-D5,6 [12]. Arecoline has carcinogenicity, mutagenicity and genotoxicity. Compared with those who did not chew betel nut, the risk of OSMF among people who ate areca nut increased by 32 to 109.6 times, with a significant dose-dependent relationship [13]. It is confirmed through the invitro study on the stimulation of human fibroblasts with areca nut extract, arecoline could promote the proliferation of fibroblast resulting in collagen production. Arecoline interferes with the deposition and degradation of Extracellular Matrix (ECM) collagen-related molecules and accelerates local oral mucosal ischemia and hypoxia resulting in changes in local tissue microenvironment, which in turn leads to oral mucosal lamina propria fibrosis.

In this article, various etiological factors behind pathophysiology of OSMF have been reviewed.

### A) Increased Collagen Synthesis

Many studies have shown that OSMF is the result of the dynamic imbalance between the synthesis and degradation of ECM. Collagen is the most important component of ECM. Collagen can be secreted by fibroblasts, Endothelial Cells (EC) and some epithelial cells. TGF- $\beta$  powerfully stimulates the production and deposition of ECM. Arecoline can induce and activate epithelial TGF- $\beta$  and act on fibroblasts which induce the expression of other fibrogenic cytokines. These changes may lead to excessive ECM deposition of OSMF [14]. TGF- $\beta$  significantly increases the collagen production by activating procollagen genes such as COL1A2, COL3A1, COL6A1, COL6A3 and COL7A1, simultaneously, it also upregulates the level of procollagen protease and Lysyl Oxidase (LOX) for cross-linkage of collagen fibers.

TGF- $\beta$  also strongly promotes the expression of LOX at mRNA and protein levels [15]. The functional activity of LOX depends on copper. Areca nut has been shown to contain high copper content, which stimulates fibrosis by upregulating the activity of LOX. It has been found that arecoline can upregulate the expression of  $\alpha v \beta 6$  integrin in Keratinocyte (KC) and promote the activation of TGF- $\beta 1$  through M4 acetylcholine receptor [16]. In addition,  $\alpha v \beta 6$ -dependent TGF- $\beta 1$  activation can induce oral fibroblasts to differentiate into myofibroblasts and upregulate genes related to tissue fibrosis [17,18]. On the other hand, Bone Morphogenetic Protein-7 (BMP7) has been shown to reverse TGF- $\beta$ -mediated collagen expression in mouse models. The role of BMP7 in preventing/reversing fibrosis has been shown to reduce the accumulation of SMAD2 in the nucleus [19]. The up-regulation of fibrogenic TGF- $\beta$  and the down-regulation of anti-fibrotic BMP7 may be the markers in the pathogenesis of OSMF which disturbed the dynamic balance of ECM production in OSMF.

There is a close relationship between ROS and TGF- $\beta$  in fibroblasts. Arecoline has been shown to produce ROS through self-oxidation or metabolic activation in cultured cells [20]. When chewing areca nut, ROS may be produced by self-oxidation in saliva or activated by intracellular metabolism [21]. A series of further experiments confirmed that arecoline could induce ROS, within 30 minutes of treatment of HaCaT. Arecoline activates c-Jun N-Terminal Kinase (JNK) through M receptor and ROS, then after JNK phosphorylation, it activates activating transcription factor 2 (ATF2) and TGF- $\beta$ . These two transcription factors activate the typical TGF- $\beta$  pathway. It was suggested that arecoline completes the induction and activation of TGF- $\beta 2$  through the JNK pathway, which contributes to a continuous autocrine cycle of TGF- $\beta$  and promoted the occurrence of OSMF [22]. Arecoline can also induce human Buccal Mucosa Fibroblasts (BMFs) to synthesise Connective Tissue Growth Factor (CTGF) and Early growth response-1 (Egr-1). CTGF and Egr-1 whose synthesis is mediated by ROS are important mediators for the fibrotic response of OSMF to TGF- $\beta$  [23,24]. In a study the level of activated TGF- $\beta 1$  in culture medium was about 2.5 times higher than that in culture medium at 0.2 mmol/l arecoline concentration. The effect of TGF- $\beta 1$  receptor inhibitor on CTGF was detected. The results showed that TGF- $\beta$  neutralisation antibody could completely inhibit the synthesis of CTGF and Egr-1 in BMF induced by arecoline. At the same time, different kinds of ROS scavengers were used to stimulate BMFs and it was found that they could completely inhibit the activation of TGF in it. These results suggested that mitochondrial-derived ROS participated in arecoline-induced potential TGF- $\beta 1$  activation and then induced the synthesis of CTGF and Egr-1 in human buccal fibroblasts and promotes the increase of collagen in the lamina propria of oral mucosa [25,26]. In addition, the expression of Egr-1 was increased when BMF was stimulated by arecoline alone [24]. Because arecoline can lead to excessive increase of middle ROS, the oral mucosa may suffer cellular DNA damage. The damage of cellular DNA caused by different promoters may be one of the mechanisms of OSMF malignant transformation [27].

Transglutaminase-2 (TGM-2), also known as tissue transglutaminase, belongs to the calcium-dependent enzyme family. Thangjam GS et al., confirmed that arecoline in normal gingival fibroblasts, TGM-2 is highly resistant to protease degradation through cross-linking, which leads to the accumulation of ECM and fibrosis [28]. Lee SS et al., confirmed that arecoline can stimulate the production of ROS in human BMF to enhance the expression of TGM-2, suggesting that arecoline-induced TGM-2 expression in human BMF may be partially mediated by ROS. The addition of N-Acetyl-L-Cysteine (NAC) and Epigallocatechin-3 Gallate (EGCG) can inhibit the expression of TGM-2 induced by arecoline, suggesting that NAC and EGCG may play an antioxidant role in the decrease of TGM-2 expression induced by arecoline [29].

Cyclooxygenase (COX) is an endooxidase reductase of prostaglandins (PG), which plays an important role in inflammation

and tumourigenesis. When oral fibroblasts were treated with 80  $\mu$ g/mL arecoline, it was found that the expression of COX-2 was upregulated as early as half an hour, suggesting that this is the early response of cells to arecoline at the transcriptional level. Immunohistochemical detection showed that the expression of COX-2 was increased in moderate fibrosis and disappeared in late fibrosis. This finding is consistent with the histology of the disease because there is no inflammation in the advanced disease [30]. Thrombin is a multifunctional serine protease that promotes platelet aggregation by converting soluble fibrin to insoluble fibrin. It can activate the receptor Proteinase-Activated Receptors (PARs) through specific cell surface receptors called proteases. In addition to the procoagulant effect of PAR-1, PAR-1 has been proved to be the main receptor mediating mitosis, fibrosis, inflammation and ECM remodeling by thrombin [31]. Thrombin exerts its fibrotic effect by inducing CTGF and other secondary mediators [32]. BMFs were treated with arecoline and the expression of PAR-1 in BMFs was observed. It has been mentioned that arecoline can stimulate BMFs to produce CTGF. This suggests that one of the pathogenic mechanisms of OSMF should be that resident cells directly or indirectly trigger the synthesis of PAR-1 expression of CTGF in the response to betel nut stimulation. Further experiments showed that COX-2 inhibitors could inhibit PAR-1 expression stimulated by arecoline, suggesting that COX-2 signal transduction pathway may be involved in PAR-1 expression [33]. Arecoline can activate ROS to stimulate the expression of COX-2 or the production of Prostaglandin E (PGE) in gingival keratinocytes. COX-2-derived PGE-2 is likely to mediate the migration and ECs that drive angiogenesis. The persistent presence of COX-2-derived PGE-2 may stimulate the proliferation of epithelial and ECs, which may contribute to the formation of malignant transformation. Previous studies have shown that COX-2 plays a certain role in the process of local invasion and metastasis of tumour. The increased expression of COX-2 in oral squamous cell carcinoma is related to high recurrence rate, poor radiotherapy response and poor prognosis after treatment [34].

## B) Decreased Collagen Degradation

TGF- $\beta$  regulates collagen degradation by activating Tissue Inhibitor of Metalloproteinase gene (TIMPs) and Plasminogen Activator Inhibitor (PAI) gene. TIMPs is a biological regulator of ECM turnover and a specific inhibitor of matrix Metalloproteinase (MMPs). MMP is composed of a series of structure-related degradation proteases. Their main function is tissue remodeling through the degradation of ECM. The balance between MMPs and TIMPs is disrupted, which may lead to continuous deposition of ECM. When normal fibroblasts and OSMF fibroblasts were cultured with arecoline, OSMF fibroblasts produced more TIMP-1 protein than normal fibroblasts, and TIMP-1 mRNA expression was also higher. Arecoline affects the deposition of ECM by increasing the production of TIMP-1, which is enhanced when fibroblasts are co-cultured with keratinocyte [35]. Another recent study reported that MMP-2 and MMP-9 secreted by oral fibroblasts were rarely found in OSMF. The study further showed that arecoline decreased the secretion of MMP-2 and increased the level of TIMP-1, thus increasing the deposition of collagen in ECM [36]. Plasminogen activator (PLG) is an extracellular proteolytic system that plays an important role in tissue remodeling [37]. Plasmin plays an important role in the activation of pro-MMP, as plasmin promotes the formation of active MMPs, they promote the degradation of collagen. As an inhibitor of PLG, the process of PLG activation is inhibited in OSMF. TGF- $\beta$  has been shown to stimulate the secretion of PAI-1 in various cell lines and *invivo*, inhibit existing collagenase and reduce the production of active collagenase, resulting in a significant reduction of collagen degradation and collagen accumulation in OSMF [38]. Maintaining the dynamic balance of connective tissue is very important for the normal function of the tissue. Various cytokines, growth factors and enzymes are considered to play a key role in regulating ECM remodeling. The dynamic balance between MMPs and matrix

metalloproteinase TIMPs is one of the decisive factors to maintain the balance and integrity of ECM. With the severity of OSMF histopathology, the expression of MMP-2 increases, which confirms its role in disease progression and malignant trends [39].

### C) Hypoxia

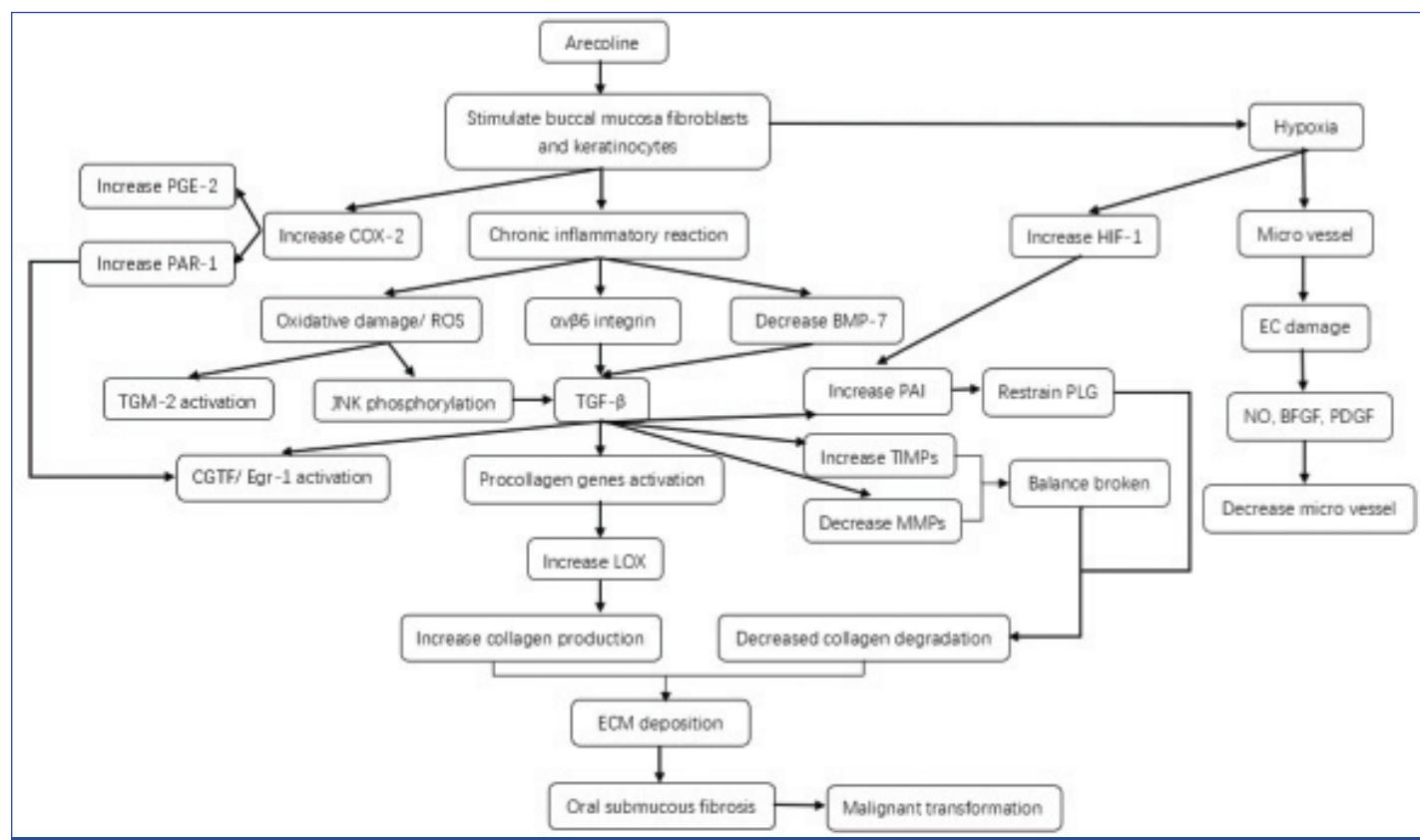
Hypoxia has been considered as an important microenvironmental factor in the development of tissue fibrosis. Hypoxia-Inducible Factor-1 (HIF-1) is a kind of  $\alpha$  subunit regulated by O<sub>2</sub> which is induced by various stimuli is the key mediator of cell adaptation to hypoxia [40]. Hypoxia promotes fibrosis through HIF-1-mediated ECM-modified factors such as PAI-1 [41]. No matter at the mRNA level or at the protein level, arecoline significantly increased the expression of PAI-1 mRNA and protein in BMFs. The results showed that HIF-1 $\alpha$  inhibitor could significantly inhibit the expression of PAI-1 protein under anoxic condition, and arecoline could upregulate the expression of PAI-1 protein under anoxic condition than that under normoxic condition. Hypoxia through HIF-1 $\alpha$  may promote the formation of fibrosis by stimulating PAI-1 to promote the accumulation of ECM in oral submucosa. It is suggested that HIF-1 $\alpha$  may play its role in promoting fibrosis by upregulating the accumulation of PAI-1 protein induced by arecoline, and there is local hypoxia in OSMF tissue [42]. Tissue fibrosis in OSMF may lead to local hypoxia by blocking blood vessels, thus stimulating the upregulation of HIF-1 $\alpha$ . In addition, the degree of cell dysplasia is related to the expression of HIF-1 $\alpha$ , suggesting that tissue hypoxia plays an important role in the malignant transformation of fibrosis [8].

### D) Micro Vessel

Arecoline toxicity may lead to the decrease of blood vessels in OSMF. With the progress of fibrosis, mucosal blood vessels decrease, and microvascular changes are the adaptive response of mucous membrane to hypoxia induced by progressive fibrosis [43]. Arecoline has cytotoxic and growth inhibitory effect on vascular ECs which is caused by cell cycle arrest and apoptosis. Long-term

exposure to low concentration of arecoline is also toxic to EC [44]. EC is the basic structure of microvessels the target cell and effector in the pathological process of the occurrence and development of OSMF. The cytokines secreted after EC injury can also stimulate the proliferation of smooth muscle cells, thicken the vascular wall, narrow the lumen, tissue ischemia and hypoxia, insufficient oxygen supply of blood perfusion, and aggravate tissue fibrosis. In one of the study, immunohistochemistry showed that the expression of Basic Fibroblast Growth Factor (BFGF) and Platelet-Derived Growth Factor (PDGF) increased in the tissues affected by OSMF [45]. Low concentration of arecoline had obvious cytotoxic effect on EC and cell proliferation was significantly inhibited. It has been confirmed that arecoline possessed cytotoxic and growth inhibitory effect on vascular ECs. Due to the cell cycle arrest and apoptosis of EC induced by arecoline, long-term exposure to low concentration of arecoline is also toxic to vascular ECs. BFGF has strong proliferative activity on fibroblasts and ECs, because of lack of conventional secretory signal sequence, then which is considered playing an important role in the occurrence and development of many fibrotic diseases including OSMF [46]. Data shows that arecoline-induced vascular BFGF combines with endothelial necrosis by increasing the expression of growth factor, thus promoting fibroblast proliferation and the occurrence of OSMF [47]. Studies have shown that arecoline can stimulate ECs to secrete Nitric Oxide (NO), to dilate blood vessels. NO in ECs is produced by L-arginine as substrate under the action of Nitric Oxide Synthase (NOS). NO is an important vasodilator, which leads to vasodilation due to the decrease of free Ca<sup>2+</sup> concentration. Arecoline inhibits the damage of vascular endothelial function induced by high glucose. The mechanism may be related to arecoline activating M receptor on ECs, increasing the production of NO and arecoline reducing the production of lipid peroxides and inhibiting oxidative stress [48].

As one of the main pathogenic factors of OSMF, arecoline affects the changes of molecular level in the pathways related to the increase of collagen production, the decrease of collagen degradation and



**[Table/Fig-1]: Molecular Mechanism Associated with Arecoline of Oral Submucous Fibrosis.**

Arecoline can stimulate buccal mucosa fibroblasts and oral keratinocytes, cause chronic inflammation and hypoxia reaction. Chronic inflammation can induce ROS,  $\alpha$ v $\beta$ 6 and BMP7 to activate Transforming Growth Factor- $\beta$  (TGF- $\beta$ ), which leads to the change of fibrosis related factors and increase collagen production. At the same time, TGF- $\beta$  can also destroy the balance between TIMP and MMP, which reduce collagen degradation. Oral mucosal hypoxia can lead to microvascular changes in oral mucosa; These change leads to excessive deposition of ECM and occurrence of OSF in oral mucosa

hypoxia through long-term chronic stimulation of oral mucosa related lesions of OSF. The changes of molecular mechanism of arecoline in OSMF and the relationship between them are shown in [Table/Fig-1].

## Molecular-Based Therapy

In view of the important role of TGF- $\beta$  in OSMF and its malignant transformation, different components of TGF- $\beta$  signaling pathway provide a potential attractive therapeutic target for the treatment of OSMF. Targeting TGF- $\beta$  and its receptors and following the downstream steps of its signal pathway, a new therapy for OSMF may be found. Glabridin is extracted from licorice root. It is a kind of isoflavone or natural phenolic compound with antioxidant and anti-inflammatory properties. It can inhibit the production of TGF- $\beta$  in human buccal fibroblasts [49].

Epigallocatechin-3-Gallate (EGCG) is the most abundant catechin in tea. It is an antioxidant that inhibits intracellular ROS. Invitro studies have shown that EGCG inhibits several fibrogenic genes, such as Egr-1, CTGF-2 and TGM-2. EGCG completely blocks arecoline-induced Egr-1 and gel contraction of bone marrow stromal cells [26].

Hyperbaric Oxygen Therapy (HBOT) increases the oxygen tension and transport of hypoxic tissue and can be used as an adjuvant therapy for fibrosis involving hypoxia. HBOT inhibits the activity of fibroblasts and has the properties of anti-inflammation and antioxidation, so it has the therapeutic effect of OSMF [50]. Tea pigment can reduce high blood viscosity, improve microcirculation and relieve local ischemia. Tea pigment can significantly increase the degree of mouth opening in patients with OSMF with hyper viscosity. The efficacy of peripheral vasodilator bufenin hydrochloride is similar to that of tea pigment. Experimental studies have found that bufenin hydrochloride can quickly improve the subjective symptoms of patients with OSMF [51].

## CONCLUSION(S)

The existing literature shows that the main pathogenic factor of OSMF is arecoline the main component of areca nut. By interfering with the molecular process of deposition and degradation of ECM, the main related upstream and downstream factors of TGF- $\beta$  can be affected, resulting in the imbalance between collagen synthesis and degradation in the lamina propria of oral mucosa which can induce OSMF and its malignant transformation. Through strengthening clinical and basic research in the future, the relationship between molecules as a marker and targeted drug for the diagnosis of the disease can be explored, to provide ideas for OSMF treatment.

## Acknowledgement

This work was funded by the China Hunan Provincial Science and Technology Program (2018GK51001).

**Author's contributions:** LC designed the study and drafted the manuscript. XC collected data, and drafted figures. JH revised article for important intellectual content. HL analysed and interpreted the data. ZY corrected the content of the article critically.

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#### AUTHOR DECLARATION:

- Financial or Other Competing Interests: None
- Was informed consent obtained from the subjects involved in the study? NA
- For any images presented appropriate consent has been obtained from the subjects. NA

#### PLAGIARISM CHECKING METHODS: [\[Jain H et al.\]](#)

- Plagiarism X-checker: Mar 09, 2020
- Manual Googling: May 08, 2020
- iThenticate Software: Jun 26, 2020 (18%)

#### ETYMOLOGY: Author Origin

Date of Submission: **Mar 08, 2020**  
 Date of Peer Review: **Mar 28, 2020**  
 Date of Acceptance: **Jun 01, 2020**  
 Date of Publishing: **Jul 01, 2020**