Review

Oral Submucous Fibrosis: A Review on Biomarkers, Pathogenic Mechanisms, and Treatments

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Abstract: Oral submucous fibrosis (OSF) is a collagen deposition disorder that affects a patient's oral function and quality of life. It may also potentially transform into malignancy. This review summarizes the risk factors, pathogenic mechanisms, and treatments of OSF based on clinical and bio-molecular evidence. Betel nut chewing is a major risk factor that causes OSF in Asia. However, no direct evidence of arecoline-induced carcinogenesis has been found in animal models. Despite identification of numerous biomarkers of OSF lesions and conducting trials with different drug combinations, clinicians still adopt conservative treatments that primarily focus on relieving the symptoms of OSF. Treatments focus on reducing inflammation and improving mouth opening to improve a patient's quality of life. In conclusion, high-quality clinical studies are needed to aid clinicians in developing and applying molecular biomarkers as well as standard treatment guidelines.

Keywords: biomarkers; epidemiology; oral submucous fibrosis (OSF); pre-malignant disorders; therapeutic interventions

1. Epidemiology of OSF

Oral submucous fibrosis (OSF) is a common oral precancerous lesion in Asian countries, especially in areas with a culture of chewing betel nuts. OSF is caused by abnormal collagen deposition in the connective tissues and affect mouth functions. Although there is no immediate danger with a diagnosis of OSF, it seriously affects the quality of life of patients. OSF interferes with a patient's quality of life because of annoying symptoms, such as ulceration, xerostomia, a burning sensation, and limitation in mouth opening. Moreover, OSF is a pre-malignant disorder with the potential for malignant transformation. Therefore, it is necessary to understand its clinical features, prevalence, malignant transformation rate, and risk factors. In Asia, the major risk factor for OSF is betel nut chewing. To reduce the occurrence of OSF, it is important to discover the pre-malignant disorder in the early stage, and understand the pathologic mechanism and treatment options. This review introduces OSF from both clinical and molecular perspectives and focuses on the epidemiology, diagnostic biomarkers, mechanisms of OSF induction and transformation, and aggressive therapeutic interventions.

1.1. Clinical Features of OSF

The oral mucosa can be divided into masticatory, specialized, and lining mucosa based on their function and histology. OSF occurs on all three types of mucosae, and most frequently occurs in the buccal mucosa [1,2], retromolar area, and the soft palate sites. The symptoms of OSF include

dry mouth, pain, taste disorders, restricted tongue mobility, trismus, dysphagia, and changed tone movability. In addition to the oral cavity, the fibrosis even involves the pharynx and esophagus. In OSF cases, the soft and pink oral mucosa initially becomes inelastic and slightly blanched. Subsequently, the mucosa becomes markedly inelastic and opaque, with white blanching, and appears papery white and tough on palpation, with a firm vertical band, which can be felt just opposite the premolar region. In the later stages, the lips and palate are also involved with lesions occurring on one or more sites. Patients with OSF experience a severe burning sensation in their mouths after ingesting spicy food. Finally, the patients' abilities to open their mouths become limited and their oral mucosae become hardened; moreover, they have poor wound healing, and their cheeks and lips become tightly held against their teeth.

1.2. Prevalence of OSF

OSF is a chronic oral disease that produces scar and tissue fibrosis. It is a pre-malignant disorder that may eventually lead to oral cancer. This disease contributes significantly to mortality because of its high malignant transformation rate (1.5–15%) [3]. OSF occurrence differs with ethnicity and region and is closely associated with diet, habits, and culture [4–6]. South and South-East Asia have the highest prevalence of OSF patients [5,7,8]. Additionally, South Africa also has a high prevalence of OSF patients because of a large proportion of Indian immigrants. The prevalence of OSF varies among South-East Asian countries. The prevalence is reported to be 0.9–4.7% in China [9], 0.62–6.42% in India, 0.15–14.6% in Vietnam [10], and 0.086–17.6% in Taiwan [11]. Based on the World Health Organization statistics, there are more than 5 million OSF patients globally [12,13]. The ages of OSF patients range from 8 to 80 years [14], with varying mean age across different studies.

1.3. The Malignant Transformation Rate of OSF

OSF is widely recognized as condition precancerous to oral cancer. The malignant transformation rate of OSF ranges from 1.2 to 23% worldwide [15–17]. In China, epidemiologic and clinical studies have reported that the overall malignant transformation rate of OSF is 1.2 to 2.2% [9]. In India, around 7.6% of OSF patients develop oral cancer [8,18]. Based on our survey of articles available in PubMed, Google Scholar, and Medline, we found no information on the malignant transformation rate of OSF in Vietnam. The malignant transformation rate of OSF in Taiwan is about 3.27–23% [3,17,19,20]. The varying malignant transformation rate of OSF may be due to the different ages, sex, tracking period, risk factors, and pathological diagnosis of the studies. However, these studies show that OSF patients are at risk of developing oral cancers. Previous studies have proven that the duration of OSF and the degree of worsening of symptoms directly correlate with the progression to oral cancer. According to statistics, OSF generally progresses to oral cancer 3–16 years after the OSF diagnosis [15,21].

1.4. Risk Factors of OSF

Immunologic causes (inflammation and autoimmunity) contribute to OSF, along with nutritional factors (vitamin B, C, and iron deficiency), carcinogenic causes (chewing tobacco and betel nut), alcohol, consumption of spicy food, epigenetic regulation, and genetic predisposition [22–27]. Overconsumption of chili-containing food irritates the oral mucosa that may cause an inflammatory response to induce OSF [28]. However, in Mexico and America where chili is widely used, OSF is not found [29]. Regarding nutritional deficiency, OSF patients show significantly lower levels of serum β-carotene [30], iron [31], vitamin C [25] and, zinc [32] in a grade dependent manner; all these factors are known to negatively affect the wound healing process. Conversely, the patients also have higher serum levels of copper [32] that enhance the lysyl oxidase (LOX) activity of cross-linking collagen fibers and elastin [33]. The increase in salivary copper concentration is reported to be associated with increasing clinical grade [34]. Epigenetic alteration had been observed in Wnt inhibitory factor-1 (WIF1) [35] and p16 [36] genes of the buccal cells in OSF patients. Hypermethylation of these two genes also contributes to the potential malignancy of OSF.

Current epidemiological studies and evidence indicate that betel nut chewing is one of the most significant risk factors for OSF [37,38]. Unfortunately, the commercially modified areca nut is cheap, sweet, and readily available in India. Moreover, the low oral health literacy of parents and children furthers the risk of addiction in young children, which consequently increases the burden of OSF [39,40]. Arecoline is the major compound in betel nut that initiates the OSF process [41–43]. Low doses of arecoline enhance the cell proliferation rate, while high doses of arecoline induce total reactive oxygen species (ROS), DNA damage, and LOX activity [44]. The ataxia-telangiectasia mutated activated DNA repair is also inhibited by arecoline [45]. LOX is overexpressed in oral cancer and upregulated by arecoline treatment of cells [46–48].

Among OSF patients in China, 62.3–99.85% have a habit of chewing betel nuts [9,49,50]. Men had a significantly higher OSF prevalence than women. Betel quid is composed of halved betel nut with dried flavoring substances. Bags of different brands of betel quid are sold in the markets of China [9]. In India, areca nut-based chewing substrates were used by patients in 219 of 220 OSF cases (99.55%) [5] and the incidence of OSF was more common in women than in men, possibly because of the significant differences in chewing habits between men and women (with men chewing gutkha, mawa, and kharra, and women chewing only areca nut) [51]. Another study demonstrated that the male-to-female ratio in OSF was 4.9:1. OSF occurred in a significantly younger age group among men than that in women [51]. Betel quid chewing is still prevalent in Vietnam, but there is no detailed information on its association with OSF. The average male-to-female ratio among OSF patients was 1.05. The prevalence of oral pre-cancerous lesions and other oral mucosal diseases was significantly higher in the betel quid chewing group (80.4%) [10]. In Taiwan, where betel quid does not have the tobacco additive, 85.4–100% OSF patients have betel nut chewing habits [52,53], and 73.4% patients with betel nut chewing-induced OSF swallow the juice of betel quid during the chewing process [53]. The average male-to-female ratio among OSF patients was 8.52. Men had a significantly higher OSF prevalence than women because most of the betel nut users in Taiwan are men [11].

In addition to chewing betel nut, some studies reported that habits such as chewing tobacco, smoking, and drinking alcohol increase the probability of developing OSF [24,54]. An observational study of 1000 cases in India showed that a high percentage of men chewed areca nut combined with tobacco [51]. A study in Taiwan indicated that a high proportion of betel quid chewers are also smokers (86%) or drinkers (74%) [9]. The combination of chewing betel nut and tobacco has led to an obvious increase in the frequency of OSF [55]. Other studies have also confirmed that consuming and chewing betel nut have an additive effect on the development of OSF [9,52].

2. Differential Diagnosis of OSF

Understanding the progression of the OSF can help to determine the appropriate treatment. Because of advancements in biotechnology, the research on OSF biomarkers has become more and more vigorous, which is helpful for the diagnosis of OSF and malignant transformation.

2.1. OSF Classification by Function

Various staging/grading classification systems of OSF have been documented in the past, such as clinical, functional, and histological staging/classification of OSF. Most systems include three staging/grading classifications according to the mucosa status, fibrous bands, and mouth opening [55–57]. Some of the staging systems are used by doctors in the clinic to diagnose or treat OSF [22,58]. According to clinical staging/classification, patients with early OSF show stomatitis and vesiculation; those with moderate OSF show a marble-like appearance and palpable fibrous bands; and those with severe OSF show leukoplakia and erythroplakia in the lesion. The maximum average mouth opening of 18–30 years is 56.60 mm for men and 51.04 mm for women in healthy individuals [59]. On functional staging/classification of OSF, the maximum interincisal mouth openings are divided into >20 mm, 11–19 mm, and <10 mm for stage 1 to stage 3, respectively (Table 1) [55].

Table 1. Oral submucous fibrosis (OSF) staging/classification system.

Clinical Stage	Functional Stage
Stage I: faucial bands only	Stage A: mouth opening >20 mm
Stage II: faucial and buccal bands	Stage B: mouth opening 11–19 mm
Stage III: faucial, buccal and labial bands	Stage C: mouth opening <10 mm

2.2. OSF Classification by Histology

It is worth noting that the OSF can transform to oral squamous cell carcinoma (OSCC), and that solid biopsy is needed for clinical diagnosis and treatment. In histological staging/classification, the amount and distribution of fibroblasts, collagen fibers, inflammatory cells, and blood vessels is used to determine the various stages of OSF [60]. Generally, OSF is classified into four histopathological stages (Table 2) [61,62]. The pathological characteristics are chronic inflammation and excessive collagen deposition in the connective tissues below the oral mucosal epithelium, accompanying local inflammation in the lamina propria or deep connective tissues, and degenerative changes in muscles. Generally, epithelial atrophy [62,63], and loss of rete pegs [64] are also reported. The sub-epithelium of patients with OSF show fibrosis with chronic inflammatory cells, such as lymphocytes, monocytes, plasma cells, and macrophages. Dense collagen bundles caused hyalinized areas in the connective tissue, and the vascularity and lumen were reduced in the connective tissue.

Table 2. Histological staging of oral submucous fibrosis [62].

Group	Histological Features
Group I-very early	Fine fibrillar collagen network interspersed with marked edema, blood vessels dilated and congested, large aggregate of plump fibroblasts with abundant cytoplasm, inflammatory cells mainly PMN with few eosinophils. Epithelium normal, with occasional hyperplasia
Group II-early	Juxta-epithelial hyalinization with collagen present as thickened but separate bundles, blood vessels dilated and congested, moderate number of young fibroblasts, inflammatory cells mainly PMN, eosinophils, and occasional plasma cells. Epithelium shows flattening/shortening of rete pegs with varying degree of keratinization
Group III-moderately advanced	Juxta-epithelial hyalinization is present. Faintly discernible collagen bundles separated by very slight, residual edema. Muscle fibers interspersed within collagen fibers reveal the beginning of degeneration and irregularity of striae. Blood vessels constricted, mature fibrocytes with scanty cytoplasm, and spindle-shaped nuclei. Inflammatory cells, mainly lymphocytes and plasma cells. Epithelium markedly atrophic with total loss of rete pegs
Group IV-advanced	Collagen hyalinized as a smooth sheet eliminating all evidence of individual bundles. Extensive fibrosis obliterating the mucosal blood vessels and eliminating melanocytes. Fibroblasts markedly absent within hyalinized zones. Extensive degeneration of muscle fibers. Total loss of rete pegs with mild-to-moderate atypia

PMN: Polymorphonuclear.

2.3. Biomarkers of OSF

Some underlying molecular differences exist between normal, oral, and potentially malignant disorders and early and late cancer stages. In recent years, biomarkers have been developed through existing biology techniques; cytological features, promoter methylation, polymorphism, mRNAs, microRNA, non-coding RNAs, and protein and trace elements in a solid biopsy, liquid biopsy from serum, and saliva have been used as potential biomarkers for OSF (Table 3) [65]. The use of cytology, tissue, serum, and saliva samples for analysis has unique advantages. It is easy to collect and observe mucosal cells with appropriate staining methods. Although histology is the most accurate method for examination, it is invasive, with long waiting times for test reports and low patient acceptance. Recently, the development of body fluid biopsy testing showed less invasiveness, shorter waiting times for testing reports, and high patient acceptance, although more supporting data are needed to establish accuracy. There are more biomarkers in typical solid biopsy studies than in liquid biopsy currently. These biomarkers have not been widely used in OSF stage classification. Larger sample sizes are needed to establish the accuracy of most novel biomarkers. At present, clinical symptoms and pathological examination are still the mainstay of OSF diagnosis. Combining instrumentation with biomarkers is a more effective method to save time and

reagents in OSF diagnosis. In addition to the diagnosis of OSF, single or multiple biomarkers expression levels may also be used as a new OSF staging method for improvement of OSF evaluation index, evaluate OSF transformation to malignant tumor index, and for gene and targeted therapy.

Table 3. Biomarkers in OSF specimens.

Specimens		Upregulation	Downregulation
Cells	Cytology	micronuclei in exfoliated buccal cells [66]	
Tissues	DNA	hyper-methylated loci reported in three or more studies included p16, p14, MGMT and DAPK [67]	
		Wnt inhibitory factor-1 promoter methylation [35]	
		secreted frizzled-related proteins (SFRP-1) and SFRP-5 [68]	
		Matrix metalloproteinases-3 (MMP3) polymorphism [69]	
	RNA	transforming growth factor beta receptor (TGF-βR1) and TGF-βR2 [70]	miR-200b [71]
		LncRNA LINC00974 [72,73]	miR-200c [74]
		miR-199-5p [75]	miR-203 [76,77]
		miR-1246 [78]; miR10-b [79]	LncRNA GAS5-AS1 [80]
	Protein	TGF-βR1 and TGF-βR2 [70]	β-catenin [81]
		proliferating cell nuclear antigen (PCNA) [82]	Wnt inhibitory factor-1 (WIF1) [35]
		cyclophilin A [83,84]	SFRP-1 and SFRP-5 [68]
		nuclear coactivator 7 (NCOA7) [85]	
		hypoxia-inducible factor 1α (*HIF*-1α) and plasminogen activator inhibitor-1 (PAI-1) [86]	
		endoglin (CD105) [87]	
		Ki67, cyclin D1, mesenchymal-epithelial transition factor (c-Met), insulin-like growth factor II mRNA binding protein 3 (IMP3) [81]	
		β-catenin [68]	
		α-enolase [88]	
		Zinc finger E-box-binding homeobox 1 (ZEB1) [74]	
		ZEB2 [71]	
Serum	Cytology	sister chromatid exchange in lymphocytes [89]	
		DNA damage in lymphocyte [90]	
	Protein	lactate dehydrogenase (LDH) [91,92]	superoxide dismutase (SOD) and glutathione peroxidase (GPx) [93]
		malondialdehyde (MDA) [90]	serum protein, globulin [94]
	Others	Copper [95]	β-carotene [96]
Saliva	RNA	S100A7 [97–99]	
	Protein	LDH [91,92,100]	GPx and SOD [101]
	Others	8-hydroxy-2-deoxyguanosine (8-OHdG) and MDA [102]	vitamin C and vitamin E [102]

3. The Mechanism of OSF Pathogenesis and Malignant Transformation

OSF is prevalent in Asia, and the main risk factor is betel nut chewing. The clinical biomarkers indicated the inflammation reaction and collagen deposition disorder contribute to OSF. Clinical data showed identical biomarkers in late-stage OSF and malignant transformation tissues. It may interpret some parts of the mechanisms of how OSF transform into malignancy. In this section, we integrated the clinical findings and animal studies to have a profound understanding of the etiology.

3.1. Pathogenesis of OSF

OSF is mainly induced by areca nut chewing in Asia. The main components of areca nut contain 31.1% phenols, 18.7% polysaccharides, 14% fat, 10.8% fiber, and 0.5% alkaloids [103]. Arecoline is the main alkaloid that causes the pathogenesis of the OSF [38–40]. Arecoline stimulates the fibroblast cells to express growth factors and cytokines that enhance the collagen deposition and repress the collagen degradation. Clinical studies reported transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), beta fibroblast growth factor (bFGF) [104], alpha-smooth muscle actin (α-SMA) [105], tumor necrosis factor-α (TNF-α) [106], serum c-reactive protein [107], ROS level [108],

matrix metalloproteinases (MMP), and the tissue inhibitors of metalloproteinases (TIMP) [109] were expressed abnormally in OSF group.

Arecoline activates the oral tissue express TNF-α that stimulates cell inflammation. Cell inflammation will activate the wound healing reaction, which decreases MMP and increases TIMP expression. This TIMP and MMP expression profile is also found in the oral tissue of OSF patients [109]. The function of MMP is to degrade the extracellular matrix protein, and TIMP inhibits this process. This contributes to the abnormal collagen deposition on the lesion. Inflammation reaction also stimulates the cell express bFGF and TGFβ-1. Continuously overexpressing bFGF in oral cells contributes to the collagen deposition disorder in OSF [104]. TGFβ-1 stimulates fibroblasts to transform to myofibroblasts, which are mainly responsible for collagen production [110,111] and wound contraction. Normally, myofibroblasts undergo apoptosis after finishing the mission of wound healing [112]. However, this mechanism is disrupted in OSF. Arecoline also increases the ROS level in OSF patients' serum [108]. Serum ROS attacks the structure of the blood vessels in endothelial cells, induced cell senescence [113], and DNA double-stranded breaks [114]. The decrease in blood flow around the oral mucosa finally causes one of the pathological symptoms - epithelial atrophy. The cell inflammation reaction and ROS attack stimulate the cell to activate the TGF-β signaling pathways. The TGF-β signaling is responsible for ceasing the cell cycle and promoting apoptosis in the unrepaired damage cells while the cells are damaged by stimulants. TGF-β also activates the downstream gene, CTGF, expression [115] to promote the fibroblast-mediated production of extracellular matrix deposition [116].

In addition, copper participates in the cross-linking of collagen [117]. It enhances the hardness of the oral submucosa tissue and exacerbates the limitation of mouth opening and trismus [118]. The commercial areca nut was reported to contain a significantly higher level of copper than the raw areca nut [117]. Higher serum copper was also found among OSF patients [119] and was deemed as one of the factors that induce OSF.

3.2. Transformation of OSF to Malignancy

OSF is deemed as a pre-malignant phenotype of OSCC. However, a cross-sectional multi-central study in Pakistan, from 2004 to 2012, showed that among 1774 patients, 26.6% had malignant transformation of OSF to OSCC, and 30.27% had OSCC without clinically visible OSF [120]. Another study reported the potential malignant rate of OSF to be 7–30% [121]. This reveals that one-third of OSF cases have a chance to turn into malignancy. The mechanism underlying the transformation of OSF to OSCC is still unclear. Researchers conducted a clinical observational study to identify molecular biomarkers from patient specimens and detect a possible link between OSF and OSCC. The common molecular markers in late-stage OSF and OSCC were cysteine proteinase inhibitor [122], TGF-β1 [123], hypoxia-inducible factor 1α (HIF-1α) [124], DNA damage phenotype [125], MMP and TIMP [126], and Cytokeratin [127] at the lesion site and elevated serum immunoglobulin G (IgG) [128]. Common alterations of the epigenetic regulation are also reported in late-stage OSF and OSCC. The hypermethylation of WIF1, p16, which was mentioned in Section 1.4, and the expression of long-noncoding RNA HCG22, RP11-397A16.1, LINC00271, CTD-3179P9.1, and ZNF667-AS1 [129] have been reported and may contribute to OSF malignant development.

Wound healing is a process that helps the human body repair tissue damage. However, persistent inflammation, collagen deposition, growth factors, and cytokine secretion induced by arecoline may lead to malignancy. TGF-β activation in late-stage cancer can promote tumorigenesis, including metastasis and chemoresistance [130]. CTGF is involved in epithelial-mesenchymal transition and angiogenesis [116]. TNF activates distinct signaling pathways to decide the cell fate [131]; the Nuclear factor-κB (NF-κB) pathway contributes to cell survival, and the c-Jun N-terminal kinase pathway contributes to cell death. TNF-α is reported to stimulate cancer cell growth, proliferation, invasion, and metastasis [83]. MMPs have been reported as one of the main factors of cancer progression and metastasis formation [132].

Carcinogenesis has four main phases, including initiation, promotion, progression, and metastasis. Normal cells are exposed to carcinogens that induced DNA damage, as well as dysregulate cellular

proliferation, survival, differentiation, and the DNA repair function [133]. Areca nut, tobacco, and alcohol are the three main carcinogens in oral cancer. In vitro and in vivo studies have revealed that arecoline and areca alkaloid induced mutagenicity in vitro and carcinogenicity in vivo [134]. A clinical observational study showed the potential for malignant transformation of OSF cases in individuals consuming both areca nut and tobacco consumption [51]. The promotion stage is a lengthy and irreversible process. This is a phase between a pre-malignant lesion and the development of malignant tumors. Once the patient suffers from OSF, limitation of mouth opening and burning sensation compel them to seek medical help. Medical intervention and eliminating the use of the carcinogens effectively interrupts the malignant promotion process. This may explain why the clinical malignant transformation rate of OSF is at most 30% rather than 100%. Clinical studies also proved that prolong areca nut and tobacco use induced malignant transformation [51].

From the other point of view, change of the microenvironment around the fibrosis tissue is also a malignant promotion factor. While the collagen deposition alters in oral mucosa, the compact tissue oppresses the capillaries and block the blood flow that produces a hypoxic environment suitable for the promotion of malignant cell growth [135]. Virus infection may be one factor that induces OSF malignancy [136]. Human papillomavirus types 16 and 18 are well-known viruses that cause oral cancer. OSF patients have a higher infection rate of human papillomavirus (HPV) than the normal group, which could explain the potential malignancy of OSF [137].

3.3. OSF and Malignancy Formation-The Evidence on Animal Models

Building an animal model is crucial for investigating the mechanism of OSF formation and malignant transformation. Early in 1997, Huan et al. established an animal model using Sprague-Dawley (SD) rats; they either injected or applied aqueous areca nut extracts (AANE) to buccal mucosa and successfully induced the collagen deposition in the buccal mucosa [138]. The common animal species used in OSF animal models are SD rats and BALB/C mice. Usually, males are used to avoid the effects of hormone fluctuations. All the studies use chemicals in areca nut as a material, including areca nut water extract and arecoline (Table 4). These studies are all conducted in Asia. Some studies inject the water extract into the subcutaneous region, while others topically applied the water extract onto the surface of buccal mucosa followed by fasting for 2 hrs. Other studies added the water extract into drinking bottles. The dosage depends on the purity of the compound and the method of administration. Topical arecoline applied at 8 mg/mL could effectively induce OSF. If the AANE is administered through a water bottle, the concentration is much higher.

The animal models successfully mimic the phenotype of human OSF, including limitation of mouth opening [139] and the elevation of biomarkers TGF-β1 [140] and type I and type III collagen [139]. However, we have still not observed malignant transformation in arecoline-induced OSF animal models, possibly because of insufficient dosage or treatment duration or owing to the biological differences between humans and mice/rats. Numerous studies have effectively induced malignancy formation using arecoline combined with 4-Nitroquinoline-1-oxide or benzo a pyrene [141,142]. This type of animal model mimics a clinical patient who consumes both the areca nut and tobacco.

Moreover, oral intake of areca nut powder had been confirmed a rapid systematic absorption of areca nut alkaloids in human [134]. We have known that some metabolic disease like diabetes is associated with betel nut chewing [143]. The arecoline-induced OSF animal model does not merely provide the synergism test of arecoline with other carcinogens like 4-Nitroquinoline-1-oxide and benzo a pyrene but also an in-depth exploration of arecoline-induced systematic disease for future studies. At present, there is no direct evidence to indicate arecoline-induced carcinogenesis in rat/mice oral mucosa, which makes clinical drug discovery for target therapy of arecoline-induced potential malignancy challenging. The scientists need to develop better in vitro cell models and in vivo animal models to study the key factors of OSF malignant transformation, and more samples and evidence are needed to support them. These studies will be the translational medical basis for future OSF drug development, gene therapy and targeted therapy.

Table 4. Summary of the arecoline-induced OSF animal models.

Author/Year/Country	Animal Type	Methods	Outcomes	Ref.
		Drug, Dosage, Control, Observed Time Point	**Phenotypes or Markers**	
M.W. Sumeth Perera/2007/Sri Lanka	Female BALB/c 10–12 weeks of age Weighing 28–30 g	Topical application of aqueous areca nut extracts 256 mg/mL on buccal mucosa twice daily 6 days per week Control group: apply 50 mM NaCl. Observed at 300th, 350th, 450th and 600th day	Epithelial atrophy, Increased cellularity of fibroblasts, Fibrosis of connective tissue, Focal infiltration of inflammatory cells, Muscle atrophy.	[144]
M.H. Chiang/2016/Taiwan	Specific pathogen-free male BALB/C mice 6-week weeks of age Weighing 20 g	Subcutaneous (SC) injection of 100μL ANE, 10mg/mL and 20 mg/mL on mice shaving back once per 2 day. Control group, PBS injection Observed on 3rd, 7th, 14th,30th day	Increased dermal thickness Collagen deposition, Elevated biomarkers: α-SMA, and connective tissue growth factor (CTGF)	[145]
Shilpa Maria/2016/India	Sprague-Dawley rats Weighing 120–150 g	Inject o.2ml supernatant of o.2 g/6 mL water areca nut extract to buccal mucosa every alternate day for 48 weeks Control group: inject 0.2 mL saline Observed duration: every 6 weeks	OSF-like lesions: Atrophic epithelium, Inflammation and accumulation of dense bundles of collagen fibers Upregulation of TGF-β1	[140]
Qi-Tao Wen/2017/China	Male BALB/c mouse (6 weeks old)	Mice were allowed to drink distilled water containing1000 mg/mL arecoline Control group: drinking distilled water Replace water bottle once a week Observed every 2 weeks to 20 weeks	Epithelium atrophy, Elevated collagen type I and angiogenesis	[146]
Bo Yang/2019/China	Male SD rat Weighing 325.4 ± 22.7 g	Arecoline 0.5, 2, 8 mg/mL dissolved in normal saline and wiping on oral mucosa with cotton bud plus mechanical stimulation Control group, w/o mechanical stimulation Once per 2 days Observed at 16th week.	Mouth openings were significantly reduced, and the expression levels of type III collagen and TGF-β1 were significantly increased. Mechanical stimulation can increase the three indexes of mucosa, no pathological change and difference in the mouth opening was observed.	[139]

4. Treatment Strategy

OSF contributes to the hardness of submucosa tissue. It jointly affects the muscles, bones, and joints movement below the submucosa tissue, which will eventually affect the degree of mouth opening and results in trismus. Limitation of the mouth opening makes it difficult in daily routine oral cleaning, speaking, and eating. Combining the annoying symptoms like burning sensation and xerostomia, patients' quality of life is low. The goals of clinical treatments are relieving the annoying symptoms and improving mouth opening to elevate the patients' quality of life. The current treatment methods for OSF are mainly divided into three categories: drug treatment, mouth opening exercising, and elective surgery. However, there is no standardized treatment protocol for clinicians. In this section, we summarize the commonly used drugs, types of exercise devices, and types of surgeries mentioned in the recent literature.

4.1. Clinical Drug Treatments of OSF

As mentioned in Section 3, the main clinical phenotypes of OSF are inflammation and fibrosis on the oral mucosa. Clinical drug treatment mainly focuses on resolving the inflammation and fibrosis in the oral tissue. Some adjuvants, such as vitamins, minerals, and vasodilators, help ameliorate the sign and symptoms.

Corticosteroids [147], such as hydrocortisone, triamcinolone, dexamethasone, and betamethasone, as well as anti-inflammatory cytokines, such as interferon-gamma (IFN-γ) [148], ameliorate inflammation and decrease collagen formation. Enzymatic drugs, such as collagenase, hyaluronidase, and chymotrypsin [2], have been used in OSF treatment. These enzymes reduce the viscosity of the extracellular matrix and show good improvement in patients with trismus and fibrosis [149,150]. Supplementation of vitamins and minerals are reported to effectively ameliorate the burning sensation and ulceration [151]. Table 5 summarizes the commonly used drugs.

We were able to find numerous articles investigating the drug treatment effect of OSF in online databases. Most of them aim to relieve the symptoms of mouth opening limitation and burning sensation. Studies are attempting different combinations of corticosteroids with an adjuvant to investigate the OSF treatment effect. However, the combination treatment may present risks. Many clinical studies examined the conventional treatment of combining steroid injections and hyaluronidase with topical vitamin A, steroid applications, and oral iron preparations found. These studies found a hazardous effect where only the conservative treatment of the steroid injections steroids in combination with hyaluronidase was found to be safe [152]. To our knowledge, no articles provided a standard guideline and advised the most appropriate drug combination for the management of OSF. We agree with the point of view of Prof. Hu (2010) [153]. High-quality clinical studies are needed to help clinicians develop a standard treatment guideline for OSF drug treatment.

Table 5. The common drugs in clinical drug treatment of OSF.

Drug Name	Drug Type	Effect	Reference
Hydrocortisone, Triamcinolone, Dexamethasone Betamethasone	Corticosteroid	Anti-inflammation	[147]
IFN-γ	Cytokines	Anti-inflammation	[148]
Collagenase, Hyaluronidase	Enzymes	Breaking down the ground substance in connective tissue	[149] [150]
Pentoxifylline	Vasodilator	Hampered mucosal vascularity	[154]
Isoxsuprine			
Vitamin A, B, C, D, E	Adjuvant	Deactivate free radicals	[151]
Iron,	Adjuvant	Relief the symptom	[25]

4.2. Mouth Exercising Devices

Therapeutic conventional exercise [155,156] is a commonly used and non-invasive treatment method for patients with OSF. The devices can be prefabricated like EZBite or custom-made like an oral stent. At present, most devices are designed for vertical oral movement. The devices developed by P. G. Pati and S. P. Patil in 2012 squeeze/stretch the cheek to increase elasticity and blood circulation of the oral mucosa [157]. Mouth exercising devices can significantly improve the mouth opening by approximately 10.5 mm and maintain these results for 12 weeks to 6 months [158]. Other commercially available devices such as TheraBite®, Malmö, Sweden Jaw Motion, Rehabilitation System™ (TheraBite), and Dynasplint Trismus System® (DTS), have been reported to increase mouth opening up to 14 mm [159]. EZBite designed by Li et al. in 2019, provides users a clear and simple protocol and continuously trains patients to open their mouths to a certain quantitative value each time to achieve maximal interincisal opening from 15.7 to 29.7 mm [156,160].

Although various types of devices are available for this treatment option, there is still no consensus standard for the frequency or duration of use, or how much strength should be given to the fibrotic tissue. However, these mouth exercising devices are not suitable for individuals with periodontal disease and with a partially edentulous ridge on the anterior arches, which causes soft tissue injuries [158] and pain [161].

4.3. Elective Surgery

Surgery is required for patients with severe OSF whose mouth opening is less than 20 mm. Clinicians use scalpel blades, electrocautery, and lasers to cut the fibers that restrict mouth opening, and coronoidectomy to reconstruct soft tissue to increase mouth opening. The fat flap, nasolabial flap, tongue flap, mandibular mucoperiosteal flap, palatal flap, and platysma myocutaneous flap [162] are used for soft tissue reconstruction. The flap must be derived from well-vascularized tissue close to the defect, with minimum donor site morbidity to prevent OSF recurrence. A total of 150 patients in 1995 underwent buccal fat pad grafting; it was particularly successful in diminishing scarring after two years as compared with split-thickness skin and fresh human amnion grafts [163]. Medical treatment with a vasodilator with antioxidant, anti-inflammatory, and immune-modulatory properties was used after flap transplantation [164].

4.4. Comparison of Treatment Methods

Treatment of OSF with clinical drugs primarily includes injecting the treatment direction to the local lesion to infiltrate the drugs deep into the tissue. The efficacy of oral medication may interfere with the patient's other prescription drugs. The efficacy of oral ointment may interfere with the constitution of saliva. For mild symptoms, drugs are the recommended first-line prescription, and the patient's history of medication must be taken into consideration [165].

Mouth exercising is the most acceptable treatment method for patients owing to its low cost, convenience, and non-invasiveness. However, treatment efficacy depends on the patient's self-motivation. Self-motivation can increase by using biofeedback app, which tracks changes in patients and increases their motivation [166]. While patients choose using mouth exercising devices, the intensive exercise protocol and fitting problems between the patient and the devices must be evaluated first [159]. It can be constructed by 3D printing technology, oral scanning equipment, and CT images to provide a customized instrument.

For OSF patients undergoing complex surgery in severe late stages, scalpel blades, electrocautery, and laser therapy are alternative choices for the treatment of OSF. Laser therapy provides a choice that will not disappoint clinicians or patients. It limits the collagen damage up to 5 μm that makes a bloodless therapy, minimizing tissue shrinkage, without damage teeth, nerves and no wound suturing [162]. Treatment by laser, Diode, KTP 532, and ErCr: YSGG, for OSF management produce good results with no limitations on age, gender, ethnicity, or socioeconomic status of the participants.

The most important thing is that the use of lasers can increase cheek flexibility. However, continuous mouth opening exercises are needed to maintain the treatment effects postoperatively. Sixty-two percent of individuals in a study underwent surgical excision with fresh amnion graft, which decreased inter-ID in the range of 5–10 mm after a two-year follow-up [163]. We summarized the comparison outcomes of treatment methods in Table 6.

Table 6. Comparison of different treatment methods for OSF.

Items	Drug Treatments	Mouth Exercising Devices	Elective Surgery
Long recovery time for wound	+	+	+++
Increase mouth opening	+	++	+++
Side effect	++	+	+++
Cost	++	+	+++
Need for patient cooperation	+	+++	++

Score sort: +: Lowest ++: Medium +++: Highest.

5. Conclusions

The prevalence of OSF and the rate of malignant transformation are different among countries. Quitting betel nut chewing is the best strategy to prevent OSF and potential malignancy. Regardless of the strategy, clinical diagnosis and treatment are still based on conservative methods. The treatment must improve the elasticity of the oral mucosa and mouth opening distance. This ensures that patients have normal oral functions like speaking and eating to improves the patient's quality of life and provides an adequate nutritional intake. High-quality clinical studies are needed to help clinicians to develop and apply molecular biomarkers and to formulate standard treatment guidelines for OSF.

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