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REVIEW ARTICLE

Molecular pathogenesis of oral submucous fibrosis – a collagen metabolic disorder

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Oral submucous fibrosis (OSF) is a chronic debilitating disease and a premalignant condition of the oral cavity. It is characterized by a generalized submucosal fibrosis. The pathogenesis of the disease is not well established. Epidemiological evidences strongly indicate the association of the betel quid (BQ) habit and OSF. Various findings indicate the disease to be a consequence of disturbances in the homeostatic equilibrium between synthesis and degradation of extracellular matrix (ECM), wherein collagen forms a major component, thus can be considered as a collagen-metabolic disorder. Transforming growth factor-beta (TGF-β) is a potent stimulator of production and deposition of the ECM. The objectives of this review are to highlight the molecular events involved in the overproduction of insoluble collagen and decreased degradation of collagen occurring via exposure to BQ and stimulation of the TGF-β pathway, and elucidate the cell signaling that is involved in the etiopathogenesis of the disease process.
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Keywords: collagen; lysyl oxidase; oral submucous fibrosis; signaling pathway, transforming growth factor-beta

Introduction

Oral submucous fibrosis (OSF) is an oral disease first described three decades ago by Pindborg and Sirsat (1). It is regarded as a pre-cancerous condition (2). The age and sex distribution varies. It is characterized by a juxtaepithelial inflammatory reaction followed by fibroelastic change in the lamina propria and associated epithelial atrophy. This leads to a restricted mouth opening, resulting as trismus leading to restriction of food consumption, difficulty in maintaining oral health, as well as impairs the ability to speak (1). The fibroelastic changes are almost entirely due to abnormal accumulation of collagen in the sub epithelial layers (3, 4), resulting in dense fibrous bands in the mouth.

The pathogenesis of the disease is believed to be multifactorial. A number of factors trigger the disease process by causing a juxtaepithelial inflammatory reaction in the oral mucosa. Factors include areca nut chewing, ingestion of chilies, genetic and immunologic processes, nutritional deficiencies, and other factors. The chewing of betel quid (BQ) (containing areca nut, tobacco, slaked lime or other species) has been recognized as one of the most important risk factors for OSF as supported by the epidemiological evidence (5, 6) as well as from its histopathological effects on fibroblasts and keratinocytes (7–11).

Worldwide estimates in 1996 indicate that 2.5 million people were affected by the disease (12). In 2002, the statistics for OSF from the Indian continent alone was about 5 million people (0.5% of the population of India) (13). This indicates that the worldwide estimate will be much higher in recent times. Hence, OSF is considered as a public health issue in many parts of the world, including the UK (3), South Africa (11), and many Southeast Asian countries (14). Most of the affected people are BQ chewers. In an epidemiological study on oral cancer and precancerous lesions in a rural Indian population, the malignant transformation rate of OSF was 7.6% (5 of 66) over a 17-year period (median observation, 10 years) (15). 'BQ chewers' oral cancer' is one of the most common malignancies in South and Southeast Asian countries (16).

The important histopathological characteristic of OSF is the deposition of collagen in the oral submucosa (17). The areca nut (betel nut) component of BQ, especially an alkaloid called arecoline, plays a major role in the pathogenesis of OSF by causing an abnormal increase in the collagen production (7). The exact mechanism is not known. Similarly, the flavonoid components of areca nut have been found to have some direct influence on collagen metabolism. It has been found that alkaloid exposure of buccal mucosal fibroblasts results in the accumulation of collagen (18). A decreased degradation of collagen due to increased cross-linking of the fibers and reduced collagenase activity are found in OSF mucosa compared to normal

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oral mucosa (19). This evidence implies that OSF may be considered a collagen-metabolic disorder resulting from exposure to areca nuts.

Collagen is the major structural component of the connective tissues and its composition within each tissue needs to be maintained for proper tissue integrity. The synthesis of collagens is influenced by a variety of mediators, including growth factors, hormones, cytokines, and lymphokines. A prominent mediator is transforming growth factor-beta (TGF-β); TGF-β1, in particular, seems to be the one that plays a major role in wound repair and fibrosis. This growth factor has also been implicated in the development of many fibrotic diseases (20). It causes the deposition of extra cellular matrix (ECM) by increasing the synthesis of matrix proteins like collagen and decreasing its degradation by stimulating various inhibitory mechanisms. Although TGF-β is essential for healing, overproduction leads to scar tissue, and fibrosis (21). As in other fibrotic diseases, TGF-β signaling pathway might be critical for pathogenesis of OSF. The direct influence of areca nut components and molecular events occurring in the disease via the TGF-β pathway are elucidated in this review.

Initial events of the disease

'Quid' has been defined as 'a substance, or mixture of substances, placed in the mouth or chewed and remaining in contact with the mucosa, usually containing one or both of the two basic ingredients, tobacco and/or areca nut, in raw or any manufactured or processed form' (22). In most areas, BQ consists of a mixture of areca nut (betel nut), slaked lime, catechu, and several condiments according to taste, wrapped in a betel leaf. Areca nut is the endosperm of the fruit of the *Areca catechu* tree. There are many reasons for chewing betel; it causes euphoria, increases salivation, satisfies hunger, relieves tooth pain, etc. The major areca nut alkaloids are arecoline, arecaidine, arecolidine, guyacoline, and guacine (23). The important flavonoid components of areca nut are tannins and catechins. Arecoline is the most abundant alkaloid. These alkaloids undergo nitrosation and give rise to N-nitrosamines, which might have a cytotoxic effect on cells (24). Arecoline has been demonstrated to promote collagen synthesis (3).

The chewing habit varies among individuals, but usually the BQ is placed in the buccal vestibule for about 15 min to an hour and repeated five to six times a day. There is constant contact between the mixture and oral mucosa. The alkaloids and flavonoids from the BQ are absorbed and undergoes metabolism. These constituents and their metabolites are a source of constant irritation to oral tissues (Fig. 1). In addition to the chemical irritation from BQ constituents and their metabolites, the coarse fibers of areca nut also cause mechanical irritation to the oral mucosa. Furthermore, the microtrauma produced by the friction of coarse fibers of areca nut also facilitates the diffusion of BQ alkaloids and flavonoids into the subepithelial connective tissue, resulting in juxtaepithelial inflammatory cell infiltration (25). Any external factor, which causes any form of injury to tissue, can elicit a protective inflammatory process. Over a period of time, due to persistent habit, chronic inflammation sets in at the site (Fig. 1). Initial irritation leads to further atrophy and ulceration of the mucosa.

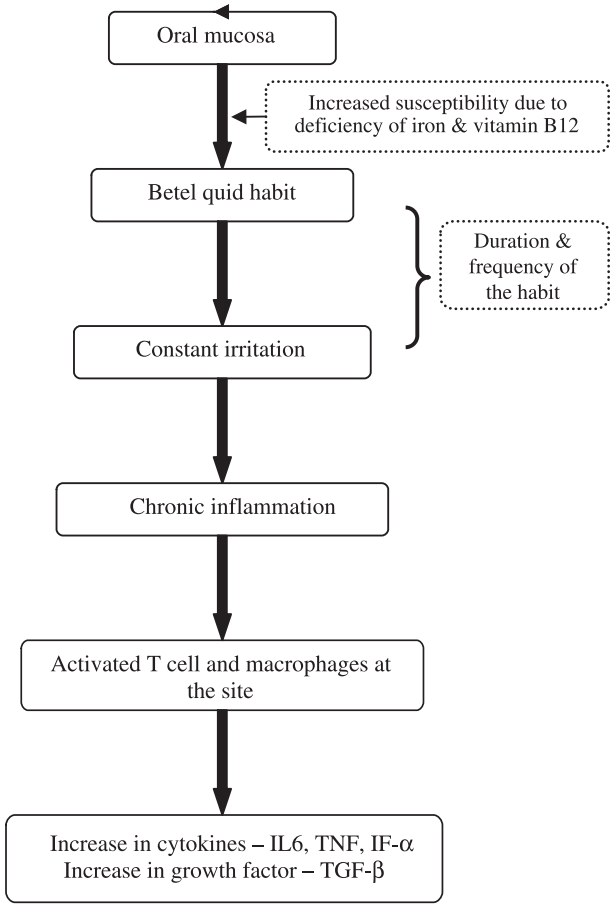

Figure 1 Initial events of the disease process: oral mucosa, which is in direct contact with BQ due to the habit, is the site of constant irritation. This results in a chronic inflammatory process characterized by the presence of inflammatory cells like T cells and macrophages. These cells release and/or stimulate the synthesis of various cytokines and growth factor. IL6: interleukin 6; TNF: tumor necrosis factor; IF-α: interferon alpha; TGF-β: transforming growth factor-beta.

Inflammation is characterized by the presence of activated T cells, macrophages, etc. (Fig. 1). There is an elaboration of various chemical mediators of inflammation, especially prostaglandins (PGs) plays an essential role (26–28). PGs secretion by oral keratinocytes in response to areca nut extract (ANE) has been shown (8, 9). Aberrant and persistent tissue inflammation is crucial for the occurrence of cancer and tissue fibrosis (26). Thus, it can be considered that induction of oral mucosal inflammation by BQ ingredients to be a critical event in the pathogenesis of OSF. Cytokines like interleukin 6, tumor necrosis factor (TNF), interferon α, etc. (10) and growth factors like TGF-β are synthesized at the site of inflammation (29) (Fig. 1). Increased susceptibility among individuals who are anemic due to

iron or vitamin B12 deficiencies has been demonstrated (30). This could be due to increased fragility of the mucosa by which there is more BQ absorption.

TGF-β1 is a key regulator of ECM assembly and remodeling. The action of TGF-β on the genes implicated in the formation and degradation of the ECM is mostly exerted at the transcriptional level through ill-defined intracellular pathways. The molecular events are discussed in this review in two main sections: collagen production pathway and collagen degradation pathway, as regulated by TGF-β and the flavonoids present in areca nut.

Collagen production pathway

The three main events that are modulated by TGF-β, which favors the collagen production are: (1) activation of procollagen genes; (2) elevation of procollagen proteinases levels: (a) procollagen C-proteinase (PCP)/ bone morphogenetic protein1 (BMP1) and (b) procollagen N-proteinase (PNP); (3) up-regulation of lysyl oxidase (LOX) activity (Fig. 2).

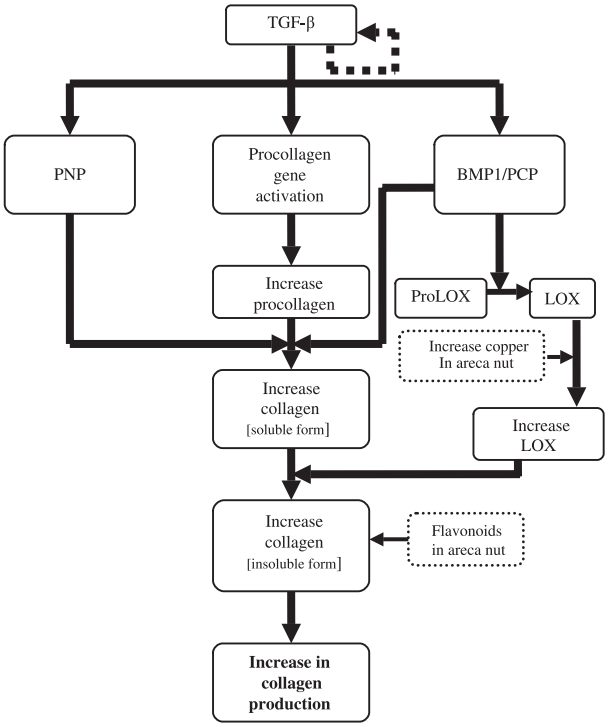

Figure 2 Collagen production pathway as regulated by TGF-β: TGF-β is a growth factor, which has autocrine activity. This activates the pro-collagen genes, resulting in production of more pro-collagen. It also induces the secretion of PCP and PNP, both of which are required for the conversion of pro-collagen to collagen fibrils. In OSF, there is increased cross-linking of the collagen, resulting in increased insoluble form. This is facilitated by increased activity and production of a key enzyme – LOX. PCP/BMP1 and increased copper (Cu) in BQ stimulate LOX activity, a key player in the pathogenesis of this disease. The flavonoids increase cross-linking in the collagen fibers. These steps results in increased collagen production. Pro-LOX: pro-lysyl oxidase; LOX: lysyl oxidase; PNP: pro-collagen N-proteinase; PCP: pro-collagen C–proteinase; BMP1: bone morphogenetic protein 1.

Activation of procollagen genes

Collagen is the most abundant protein in the human body and it plays a critical role as a structural element of connective tissue. About 27 types of collagen have been recognized, which can be grouped into seven broad classes. Major class is fibrillar collagen, among them types I, III, and VI form a major part of connective tissue. Collagen type VII forms the anchoring fibrils of oral mucosa. The distinguishing feature is a unique type of triple helix, stabilized by unusual cross-links. The processing of fibrillar collagen occurs in a stepwise manner.

Procollagen genes are transcribed and translated to form procollagen monomeric chains (procollagen precursor) (Fig. 2). Three of these monomers assemble into a trimer triple helix. This is aided by disulphide bridge formation. Trimeric procollagen chains are then acted upon by N- and C-terminal proteases (PCP and PNP), to cleave the terminal domains (Fig. 2). After this cleavage the collagen units form spontaneously into fibrils. The newly formed fibrils are then covalently stabilized through cross-linking to form a stable mature structure of collagen.

The genes COL1A2, COL3A1, COL6A1, COL6A3, and COL7A1 have been identified as definite TGF-β targets (Fig. 2). These are early induced genes in fibroblasts. They were identified by differential hybridization of cDNA array (31). The transcriptional activation of types I and VII collagen gene expression by TGF-β has been demonstrated (32, 33). This transcriptional activation of procollagen genes by TGF-β is causing an increased expression of procollagen genes and hence contributing to increased collagen level in OSF.

Elevation of procollagen proteinases levels

Procollagen proteinases play an essential role in processing of procollagen precursors into collagen fibrils, which are soluble. There are two types of proteinases that cleave the N- and C-terminal, respectively – PNP and PCP (Fig. 2) (34).

PCP

The PCP and BMP1 have been shown to be the same protein that cleaves the C-terminal of procollagen precursor (Fig. 2) (35, 36). TGF-β1 has been found to induce BMP1 at the transcriptional and translational levels in different cell types such as the osteosarcoma cells and fibrogenic cell cultures (37).

PNP

It cleaves the N-propeptide of procollagen precursor (Fig. 2) (39). There are two types of PNPs, PNP I and III, they are classified based on the type of procollagen fibers on which they act (38–40). TGF-β-treated cells have been shown to have an elevated level of PNP (37).

Thus not only is procollagen gene expression increased by TGF-β, but also their processing into fibrils is enhanced by increased levels and activities of the N- and C-procollagen proteinases.

Up-regulation of LOX

The LOX is an essential enzyme for final processing of collagen fibers into a stabilized covalently cross-linked mature fibrillar form that is resistant to proteolysis (Fig. 2).

The LOX is dependent on copper for its functional activity (41). Removal of copper leads to a catalytically inactive apoenzyme. The LOX is synthesized as prolysyl oxidase and conversion of this precursor into an active LOX is mediated by BMP1 and takes place in the extra cellular space (Fig. 2) (42). During the biosynthesis of LOX, copper is incorporated into LOX (43). Apart from copper, LOX also contains another co-factor, a covalently bound carbonyl prosthetic group – lysine tyrosylquinone (LTQ) (44). The LTQ is essential for the reaction mechanism of LOX, i.e. in the formation of cross-links in the collagen fibers (45). Copper has been suggested to play a structural role in stabilizing the LTQ (46). During the process of cross-linking, copper plays an important role in reoxidizing the reduced enzyme facilitating the completion of the catalytic cycle (44). Areca nuts have been shown to have a high copper content, and chewing areca nuts for 5–30 min significantly increases soluble copper levels in oral fluids. This increased level of soluble copper could act as an important factor in OSF by stimulating fibrogenesis through up-regulation of LOX activity (Fig. 2) (47, 48).

Apart from this, the flavonoids that are present in areca nut have been implicated in the process of enhancing the cross linking of collagen fibers. *In vitro* studies have demonstrated the presence of catechin to raise LOX activity (49, 50). They might be oxidatively converted to quinones and hence, might resemble LTQ, which is an important co-factor for LOX activity. This could be a possible explanation for enhancing LOX activity (51). Apart from this process, the *in silico*, molecular modeling experiments have revealed that the direct interaction of flavonoids with collagen facilitates the cross linking of collagen fibers (52).

The expression of LOX is regulated by various factors, among which TGF-β is considered to be an important factor (Fig. 2). TGF-β has been found to strongly promote the expression of LOX both at the mRNA and protein levels in various cell lines (53, 54). The exact mechanism underlying this is not yet fully understood. This could be indirectly via the elevation of BMP1 by TGF-β, as it mediates the biosynthetic processing of LOX, i.e. conversion of prolysyl oxidase to active LOX (42).

The LOX activity is important for formation of insoluble collagen due to cross-linking. The process of cross-linking gives tensile strength and mechanical properties to the fibers as well as makes the collagen fibers resistant to proteolysis. Increased levels and activity of LOX due to increased BMP and copper levels, and further enhancement of its activity by LTQ-like flavonoids present in BQ, causes increased cross-linking of the collagen fibers, tilting the balance towards a fibrotic condition as present in OSF.

Collagen degradation pathway

There are two main events modulated by TGF-β, which decreases the collagen degradation: (i) activation of tissue inhibitor of matrix metalloproteinase gene (TIMPs); and (ii) activation of plasminogen activator inhibitor (PAI) gene (Fig. 3).

Activation of TIMP gene

Matrix metalloproteinases (MMPs) constitutes a set of structurally related matrix degrading proteases. They are endopeptidases that play an essential role in tissue remodeling by degrading ECM, both in health and disease (55). The MMPs are of many types but MMP1, MMP8, and MMP13 are referred to as collagenases. They are synthesized as zymogens (pro-MMPs) and are activated by endoproteolytic cleavage (55). Under normal physiological conditions, the activities of MMPs are regulated at the level of transcription, activation of the zymogens, and inhibition by endogenous inhibitors (55). The flavonoids have been shown to inhibit the collagenase activity (56).

TIMPs are specific inhibitors of MMPs that play an essential role in controlling their local activities in tissues

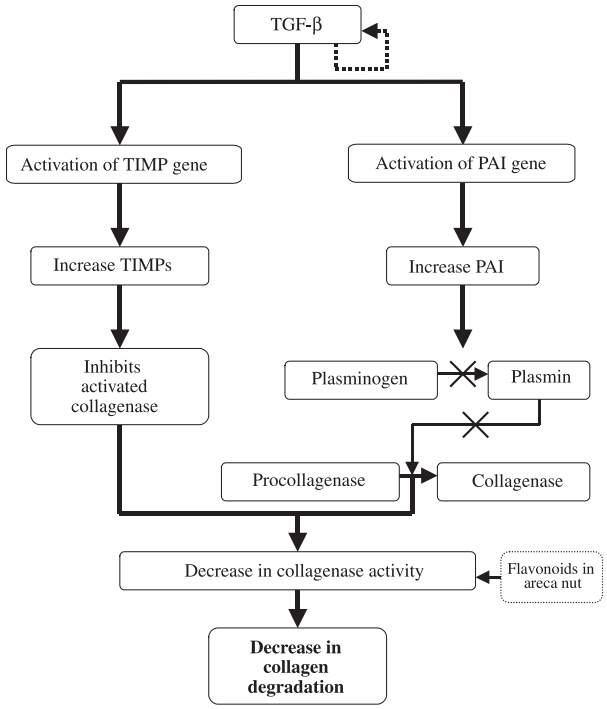

Figure 3 Collagen degradation pathway as regulated by TGF-β: TGF-β activates the genes for TIMPs; thereby more TIMP is formed. This inhibits the activated collagenase enzyme that is necessary for the degradation of collagen. It also activates the gene for PAI, which is an inhibitor of plasminogen activator, thus there is no plasmin formation. Plasmin is required for the conversion of pro collagenase to active form of collagenase and absence of plasmin results in the absence of active collagenase. The flavonoids inhibit the collagenase activity. A reduction in the activity and levels of collagenase results in a decrease in collagen degradation. TGF-β: transforming growth factor-beta, PAI: plasminogen activator inhibitor; TIMP: tissue inhibitor of matrix metalloproteinase.

(Fig. 3) (57). Four types (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) have been identified in vertebrates (57). TIMP1 inhibits most of the MMPs and is reported to have diverse effects on cell growth and apoptosis (58). TIMPs are thought to act as biological regulators of the turnover of ECM, a process that occurs in normal tissues during development and wound healing, as well as in inflamed tissues during rheumatoid arthritis and during tumor invasion and metastasis (59). Increase in TIMPs expression has been demonstrated in OSF (60, 61), thereby inhibiting collagenase and decreasing collagen degradation.

TGF-β induces TIMP1 gene expression (Fig. 3) and it has been identified as one of the definite early induced TGF-β target gene in fibroblasts (31). The exact mechanism of regulation is not known. Thus TGF-β decreases the collagen degradation by activating TIMP gene, thereby enhancing its level resulting in inhibition of the activated collagenases.

Activation of PAI gene

The plasminogen (Plg) activation system is an extra cellular proteolytic system, which plays an important role in tissue remodeling (62). The main event of this system is conversion of zymogen, Plg, to the active serine protease, plasmin (Fig. 3). Plasmin is generated by proteolytic cleavage of Plg by tissue plasminogen activator (tPA) bound to fibrin and urokinase plasminogen activator (uPA) bound to a specific cell surface receptor. These activators are regulated by two plasminogen activator inhibitors, PAI1 and PAI2 (63).

Plasmin is important in the activation of pro-MMPs. Studies have demonstrated that plasmin contributes to the proteolytic activation of pro-MMP-1, -3, -7, -9, -10, and -13 *in vitro* (64). Activated MMPs can participate in processing other MMPs. As plasmin promotes the formation of active MMPs (Fig. 3), they facilitate the degradation of collagen. In OSF, the Plg activation process is inhibited, as there is an increase in PAI1 (Fig. 3) (65). The PAI1 may be regulated by different cytokines, among, which TGF-β plays an important role. TGF-β has been shown to stimulate PAI1 secretion in various cell lines and *in vivo* (Fig. 3) (66).

The inhibition of the existing collagenase and decreased generation of active collagenase together results in a marked decrease in collagen degradation and a resultant build up of collagen in OSF.

Conclusions

As described in this review, OSF can be regarded as a disease of collagen-metabolic disorder. Overall increased collagen production and decreased collagen degradation results in increased collagen deposition in the oral tissue, leading to fibrosis (Fig. 4). This is further aggravated by the autoregulatory process of TGF-β, which is the main trigger for both the increased collagen production and decreased degradation pathways.

Understanding of the molecular events helps in better therapeutic intervention of the disease. Some of the possible interventions suggested based on the pathway involved are as follows (Fig. 5):

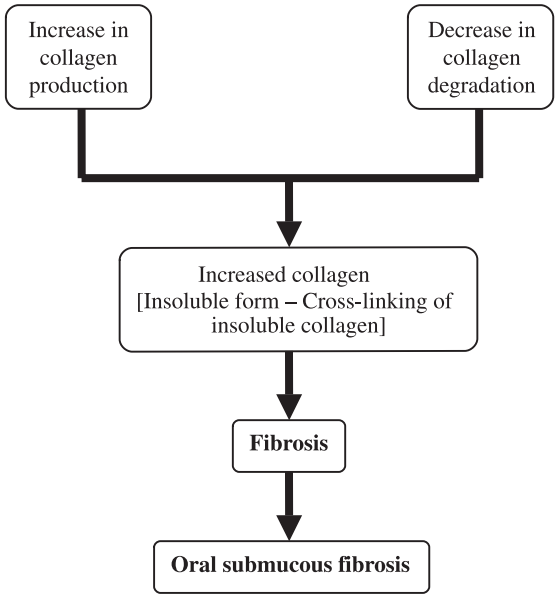

Figure 4 Overall effect of activated TGF-β pathway. There is an increase in collagen production and cross-linking (insoluble form) along with a decrease in collagen degradation. This produces an increased collagen deposition in the subepithelial connective tissue layer of the oral mucosa leading to OSF.

1. As inflammatory process is the main factor that leads to the fibrosis, anti-inflammatory/immuno-modulatory drugs such as colchicines and steroids can be effective. Colchicine is an anti-inflammatory drug that suppresses collagen synthesis and/or stimulates collagenase. Glucocorticoids are an immunosuppressive drug. Presently this is one of the important groups of drugs used in the treatment of OSF.
2. TGF-β is an important cytokine involved. The entire pathway that is invoked by it can be suppressed by its inhibition. Anti-TGF-β drugs can be in the form of antibodies or peptide mimetics. These drugs are in the development process and are currently being experimented with the treatment of liver fibrosis. An antibody to TGF-β inhibits its action by interacting with it. Peptide mimetics can be in the form of soluble TGF-β receptors, which competitively inhibit TGF-β.
3. We consider LOX to be a key enzyme tilting the balance in the collagen metabolism toward fibrosis. Curtailing the activity of LOX either by using a copper chelator or through another inhibitory mechanism may help in reducing the fibrosis by reducing cross-linking of the collagen fibers. In the case of Wilson's disease, D-penicillamine, a copper chelator has been used for treatment by systemic route of administration. The same drug has been thought to have some antifibrotic activity wherein it inhibits LOX and directly binds with collagen fibrils, preventing cross-linking into stable collagen fibers. In case of OSF, a local use of this drug might be useful.

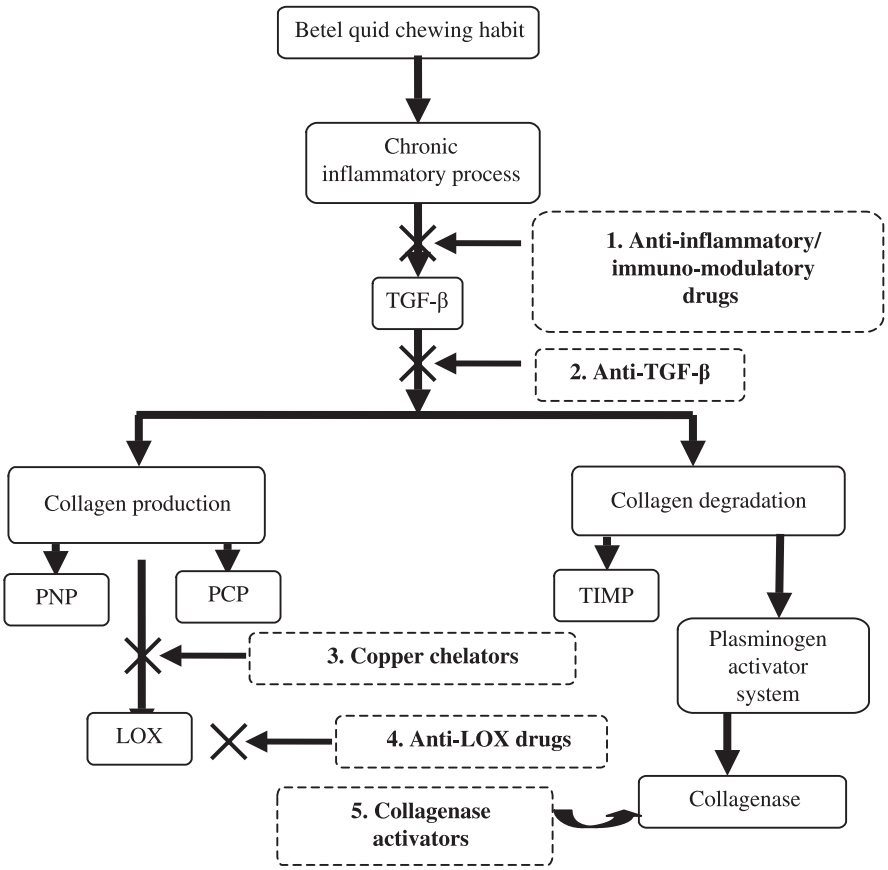

Figure 5 Possible therapeutic interventions for OSF. Some of the possible interventions suggested based on the pathway involved include: (1) blocking the chronic inflammatory process by anti-inflammatory/immuno-modulatory drugs; (2) blocking TGF-β action by anti-TGF-β antibodies or peptide mimetics of soluble TGF-β receptors; (3) copper chelators like penicillamine to block LOX activity and prevent cross-linking; (4) other anti-LOX drugs that prevent its action; (5) collagenase activators like colchicine to promote collagen degradation. Probably a combinational therapy of the above mentioned drugs thereby intervening at multiple points along the pathway might be useful for the successful treatment of OSF.

4. Collagenase activators can be helpful in activating the procollagenases thus improving the collagen degradation process.

Presently hyaluronidase (that breaks down the components of connective tissue) intralesional injection along with steroid has been used for OSF therapy. Interferon-γ, an antifibrotic cytokine has also been used. Probably a combinational therapy of the above mentioned drugs might be useful in the therapy. 'Prevention is better than cure', cessation of BQ chewing habit is very important for any of the treatment modalities.

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