Evaluation of role of drinking water copper in pathogenesis of oral submucous fibrosis:

a prospective case control study in Yadgir district of northeast Karnataka, India

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DOCTORAL THESIS

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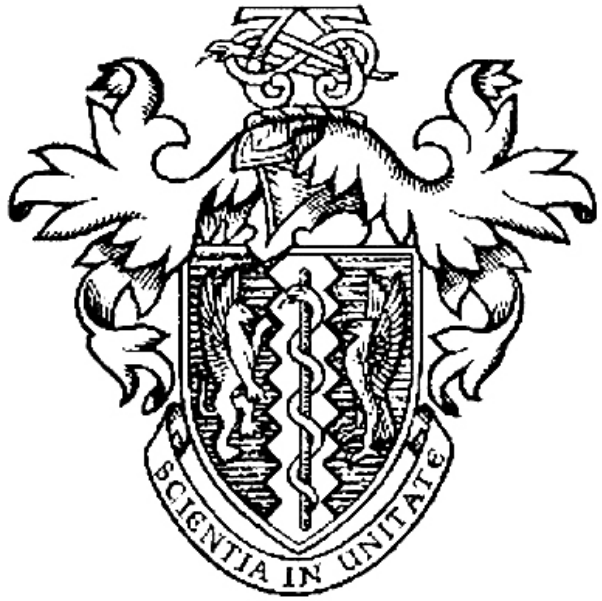
BAOMS

British Association of Oral and and Maxillofacial Surgeons



This thesis is dedicated to my parents
Smt. Anusya Arakeri and Sri. Palaxhi Arakeri
For their endless love, support and encouragement

Contents

Chapter 1

Introduction

'The chains of habits are too weak to be felt until they are too strong to be broken' -Dr.Samuel Jones

The growth and development of civilisation and industrialisation subjected Human beings to varied amount of stress and tension. Most of them adapt psycho-stimulating and euphoria-inducing habits such as smoking, alcoholism, areca nut chewing etc. as stress relieving methods. These habits are highly addictive and affect human body with many crippling mental and physical disorders. Oral Submucous fibrosis (OSMF) is one of the devastating conditions resulting from these tension relieving habits.

OSMF is a chronic, debilitating condition of the oral cavity causing progressive scarring of the oral mucosa.[1] In ancient Indian medicine Shushrutha described a condition which he named it as "Vidari" presenting symptoms of progressive narrowing of the mouth, depigmentation of oral mucosa and pain on taking food. These features precisely fit in with symptomatology of OSMF.[2]

Schwartz J (1952)[3] described a fibrosing condition of the oral cavity in five female Indian immigrants to Kenya and termed it as "Atrophica Idiopathica (Tropica) Mucosae Oris". (Schwartz J. Atrophia idiopathica (tropica) mucosae oris.Presented at the Eleventh International Dental Congress, London, 1952)

Joshi SG[4] (1953) and Lal D[5] (1953) were the first Indian workers to describe this condition and termed it as "Sub Mucous Fibrosis of Palate & Pillars" and "Diffuse Oral Submucous Fibrosis" respectively.

Other names suggested in literature include:

- Idiopathic scleroderma of mouth[6]
- Submucous fibrosis of the palate[7]
- Submucous fibrosis of palate and cheek[8]
- Idiopathic palatal fibrosis[9]
- Oral submucous fibrosis[10]

However, the most accepted term is "Oral Submucous Fibrosis" which was suggested by Pindborg JJ & Sirsat SM (1966)[10] describing the nature of the condition in a simplified form. They defined the condition on the basis of clinical and histopathological findings as- "an insidious chronic disease affecting any part of the oral cavity and

sometimes the pharynx. Although occasionally preceded by and /or associated with vesicle formation, it is always associated with a juxtaepithelial inflammatory reaction followed by a fibro elastic change of the lamina propria with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat".[10]

Buccal mucosa is the commonest site followed by labial mucosa, retromolar pads, soft palate and floor of the mouth. Rarely fibrotic changes of the pharynx, esophagus and paratubal muscles of eustachian tubes have also been observed.[11] Initially OSMF presents with burning sensation, hypersalivation/xerostomia and mucosal blanching with marble like appearance.[1] Later on, the mucosa becomes leathery and inelastic with palpable fibrous bands resulting in restricted mouth opening. Eventually, OSMF leads to difficulty in swallowing, speech & hearing defects and defective gustatory sensation.[11] Paymaster in 1956 described the development of a slow-growing squamous cell carcinoma in one third of OSMF cases seen in the Tata Memorial Hospital, Bombay[12] and postulated it as a precancerous condition.[12,13] Later studies by Pindborg substantiated this report.[14-16] The WHO definition for an oral precancerous condition - "a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer," accords well with the characteristics of OSF.[12] The precancerous potential was also emphasized by other authors,[17-21] based on clinical and epidemiological grounds.[12] What makes it more sinister is the malignant transformation rate, which has been reported to be around 7.6% over a 17-year period.[18,22,23] Perhaps what is most distressing about the condition is most of the time difficult to diagnose is clinically owing to its malignant transformation associated with severe trismus.[1] Surveys in various cancer hospitals in India reveal a 15-20% frequency of oral cancer among all cancers.[24] The finding of a high frequency of OSMF among oral cancer patients in India (e.g., 40 among 100 oral cancer patients) has strengthened the postulated link between the two.[12] Until recently, it was thought to be localized to the Indian subcontinent, China and other regions of South East Asia but is now considered to be of global importance due to large numbers of migrant populations also demonstrating the condition.[1]

In the 1960s the prevalence figures observed in 35,000 Indians visiting dental colleges were - Lucknow 0.5%, Bombay 0.7%, Bangalore 0.2% and Trivandrum 1.2%.[25] In 1968, a survey was conducted among 50,915 villagers to know the prevalence of OSMF in rural India and the prevalence figures were-Gujarat 0.2%, Kerala 0.4%, Andhra Pradesh 0.04%, Singhbum 0% and Darbhanga 0.7%.[26] In Maharashtra, the prevalence is 0.3% among 101,761 villagers.[27] In Ernakulam district of Kerala, the yearly incidence for males and females combined was 13 per 100,000 person-years.[12]

In 1992, the prevalence of OSMF in Trivandrum and Quilon were found to be 0.32% and 0.27% respectively.[11,28] The prevalence of OSMF has increased over the past

four decades from 0.03% to 6.42%.[11, 26,29] In the Indian population in South Africa, the prevalence of OSMF was between 0.5%-1.2%.[27] In 2002, the statistics of OSMF for India alone was 5 million people.[30] The disease has also been reported among local population in Sri Lanka, Myanmar, Singapore, Thailand, China, South Vietnam, Fiji, Papua New Guinea, and Saudi Arabia and sporadically among Europeans.[31] In 2011 survey, OSMF amounted to almost 4.38% in the span of 16 years in an Indian teaching hospital alone. It was also observed areca nut/betel nut was associated with almost 78% of the patients with OSMF, while smoking alone leading to OSMF was seen in a very small percentage of patients (2.43%) and was especially associated with beedi smoking. Multiple habits, i.e., areca nut, smoking, alcohol was observed in 6.82% people.[23] The prevalence of OSMF in a recent north Indian survey by Nigam et al. (2013) was 6.3% (63/1000). It was also found that gutkha chewing was the most common abusive habit (42/63) amongst OSMF patients. The prevalence found was more in urban patients and severity of disease was more in rural population.[11]

OSMF occurs over a wide age range. The youngest to be reported was a 4 year old Indian immigrant in Canada[31] and the oldest patient more than 80 years. However, majority of the patients diagnosed with OSMF are between the age 20-40 years.[10,22,32] OSMF is thought to have a female preponderance.[10,33,34] However, the reports of sex ratio vary. In a report of the prevalence of OSMF among 50,915 Indian villagers, the female: male ratio was 3:1.13 In Ernakulam, the yearly incidence was reported to be 9 for males and 20 for females per 100,000 person years.[35] A study by Ranganathan et al.[36] had revealed male predisposition 9.9:1. There was a high male predilection, almost to the ratio of 11:1 in a recent study[22] which is in accordance with others studies.[11] The higher involvement of males in all studies, reflects their easy access to the abusive habits when compared with females.[11]

Over the years, many theories linking oral submucous fibrosis to various risk factors have been proposed. The Indian habit of repeatedly insulting the oral mucosa with very spicy, pungent foods and irritant like supari (areca nut), pan (betel leaves), tobacco (chewed & smoked) over a period of years have all been incriminated by various authors as causative agents.[22] Systemic factors like nutritional deficiency, genetic predisposition and autoimmunity were also proposed in pathogenesis of OSMF.[1,22]

However the precise aetiology of OSMF is still unknown.[1] Till date no conclusive evidence has been found despite many extensive investigations on factors implicated.[1,22] Most of the ideas proposed have been derived from existing clinical and epidemiological data.[12]

Areca nut chewing is strongly correlated with OSMF. This observation was made after it was seen that OSMF did not occur in populations who did not chew area nut. In

South Africa it was observed that OSMF was more common among Indians and not seen among Blacks as areca nut chewing is more common among the Indian population there.[26,37] A high correlations has been found in Taiwan between areca nut chewing and oral submucous fibrosis.[35] In an Indian study with 275 cases there was increase of OSMF was seen in 5 year period. The trend corresponded to an increase in areca nut (mawa) chewing habit in that area.[23]

In vitro studies have shown that arecoline and arecaidine content of aracanut stimulate proliferation of fibroblasts and enhance the synthesis of collagen.[38,39] However in an experimental set up, 2% arecoline was applied to the palatal surfaces of Wistar rats. Only two rats showed a staining pattern similar to that seen in classic human submucous fibrosis of the palate. The investigators concluded that arecoline probably did not play a role in causation of OSMF.[40]

The high concentration of copper in areca-nut also has been found to stimulate lysyl oxidase activity, an enzyme essential to the final cross-linking of collagen fibres.[1] Increased copper has been seen in mucosa affected by OSMF, which supports its role in fibrogenesis by enhancing lysyl oxidase activity.[1]

The pathogenesis of the disease is not well-established, but the cause of OSMF is believed to be multifactorial. Various mechanisms were suggested for the etiopathogenesis of OSMF[41,42], including: 1) clonal selection of fibroblasts with a high amount of collagen production during long-term exposure to areca nut ingredients[43] 2) stabilization of collagen structure by catechin and tannins from betel quid decreases the secretion of collagenase[44] 3) production of stable collagen (type I trimer) by OSMF fibroblasts[45] 4) increase in collagen cross-linking by up-regulation of lysyl oxidase[46] 5) deficiency in collagen phagocytosis and effect of fibrogenic cytokines secreted by activated macrophages and T lymphocytes[47,48] and 6) deficiencies in micronutrients and vitamins.[41,42]

The incidence of OSMF is on the rise in the younger age group in India. OSMF is silently spreading like wildfire throughout in India, as pan masala and gutkha are easily available in most part of the country even to young children at a very low price.[1,50] We are observing a big epidemic of OSMF across India and are afraid that we will witness an epidemic of cancer in near future. But unfortunately, the complex pathophysiology of this condition is still obscure and there are no reliable diagnostic parameters, which can indicate the irreversible nature and/ or chance of malignant transformation.[1, 50] Also to date, there is no method which can be seen as the definitive treatment for oral submucous fibrosis.

"Behind a restricted mouth lies a restricted personality." This is more or less true for the patients suffering from OSMF, a highly perplexing disease. Unfortunately the

condition is seen mostly in developing countries and is failed to get attentions from international health organisation owing to its remoteness.[49] Eventually we see more number of patients every year due to lack of awareness and treatment. Hence the condition demands more understanding of pathogenesis and its predisposing factors to develop prevention modalities. In this context, we consider systemic predisposition and pre-conditioning of the oral mucosa is a promoting factor responsible for development of OSMF.

In the present prospective case control study, an attempt has been made to know the association between water copper ion concentration and pathogenesis of OSMF. In chapter 2 we will review and discuss all general components of OSMF, including the terminology, presentation, aetiology, and pathogenesis, and provide a brief overview of its management. In chapter 3 we postulate the novel biological pathway through which copper is thought to predispose oral mucosa to OSMF. The hypothesis explains various unexplored aspects of the disease. Chapter 4 include pilot investigation on 50 patients with clinically and histologically diagnosed OSMF. The preliminary study investigates the role of copper in drinking water in the pathogenesis of OSMF. In chapter 5 the association of drinking water copper and OSMF will be further investigated in a heterogeneous population in Hyderabad-Karnataka, India. The study will evaluate 3 groups, each of 100 patients: those with OSMF who chewed gutkha, those who chewed gutkha but did not have OSMF and healthy controls who did not chew gutkha. In chapter 6 we will investigate association of pattern of saliva pooling and distribution of OSMF in an attempt to postulate a possible mechanism for the sporadic incidence of the OSMF. In chapter 7 we will discuss about all studies with their impact, emerging challenge and preventive measures.

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Chapter 2

Oral submucous fibrosis: an overview of the aetiology, pathogenesis, classification, and principles of management

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Abstract

Oral submucous fibrosis (OSMF) is a complex, debilitating, and precancerous condition. Formerly confined to the Indian subcontinent, it is now often seen in the Asian populations of the United Kingdom, USA, and other developed countries, and is therefore a serious problem for global health. The well-known causative agent of the disease, areca-nut is now recognised as a group one carcinogen. We review and discuss all components of OSMF, including the terminology, presentation, aetiology, and pathogenesis, and provide a brief overview of its management.

Keywords
Oral submucous fibrosis; Areca-nut; Gutkha; Aetiology; Pathogenesis; Review

Introduction

Oral submucous fibrosis (OSMF) is a chronic, debilitating disease characterised by juxtaepithelial fibrosis of the oral cavity. It is regarded as a precancerous and potentially malignant condition.[1,2] The most widely accepted definition of the disease by Pindborg and Sirsat[3] is one of an insidious, chronic disease that affects any part of the oral cavity and sometimes the pharynx. Although occasionally preceded by, or associated with, formation of vesicles, it is always associated with a juxtaepithelial inflammatory reaction followed by fibroelastic change of the lamina propria and epithelial atrophy that leads to stiffness of the oral mucosa and causes trismus and an inability to eat.[3]

The definition by the World Health Organization (WHO) of an precancerous oral condition: “a generalized pathological state of the oral mucosa associated with a significantly increased risk of cancer” fits well with the characteristics of OSMF.[4,5] The condition is thought to be multifactorial in origin with a high incidence in people who chew areca-nut,[6] and a significant malignant transformation rate (7–30%)[7] poses global problems for public health. The physical effects, which include a burning sensation in the mucosa and progressive trismus, can also have psychological and social implications for patients.

Terminology

In 1952, Schwartz described a condition in 5 Indian women that he called “atrophia idiopathies (tropica) mucosae oris” (Schwartz J. Atrophia idiopathica (tropica) mucosae oris. Presented at the Eleventh International Dental Congress, London, 1952); Joshi coined the term “submucous fibrosis of the palate and pillars”.[8] Other names suggested include “diffuse oral submucous fibrosis”, “idiopathic scleroderma of the mouth”, “idiopathic palatal fibrosis”, and “sclerosing stomatitis”.[3,4] Pindborg and Sirsat used the term “submucous fibrosis” although they suggested that a more appropriate name would be “juxtaepithelial fibrosis”.[3] Its premalignant nature was first described by Paymaster in 1956.[9]

Clinical presentation

Clinical presentation depends on the stage of the disease.[10] Initially, most patients present with a burning sensation or intolerance to spicy food, and they may have vesicles, particularly on the palate. Ulceration and dryness of the mouth is later followed by fibrosis of the oral mucosa, which leads to rigidity of the lips, tongue, and palate, and trismus.[10]

Petechiae, in the absence of blood dyscrasias or systemic disorders, are found in

about 22% of patients with OSMF, and occur most often on the tongue followed by the labial and buccal mucosa.[4,11]

A useful clinical sign is pain on palpation in the sites where submucosal fibrotic bands are developing, [11] and trismus is caused mostly by fibrosis in the dense tissue around the pterygomandibular raphae.[11] Fibrosis of the eustachian tube may lead to deafness.[7, 12] When the fibrosis involves the nasopharynx or oesophagus, patients may experience referred pain to the ear, a nasal voice, and dysphagia to solids; usually these are features of more advanced disease.[7,11]

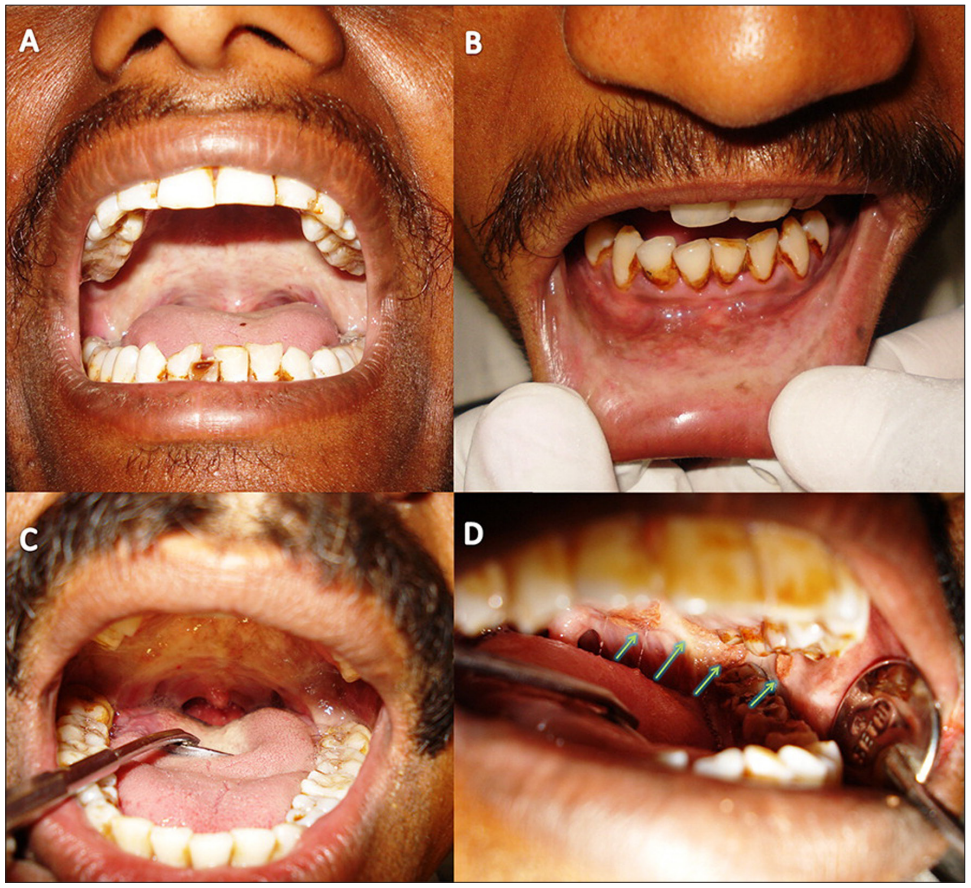

Fig. 1. Clinical appearance of oral mucosa in oral submucous fibrosis (A–C) and malignant changes in the condition (marked with arrows in D).

The most obvious clinical signs include blanched, opaque oral mucosa with palpable fibrous bands (Fig. 1).[10,13,14] Furthermore, the overlying epithelium may become dysplastic and malignant. Restricted mouth opening interferes with examination of the oral mucosa, and makes early diagnosis of cancer a daunting task.[10,15-17]

Epidemiology

Geographically, OSMF has a specific distribution and affects predominantly Asians (and particularly Indians) from the southern states, and Taiwanese.[10,18] Other series of OSMF in Europe, the Far East, and the Pacific Rim have been reported.[7,10,18] An estimate from 1996 indicated that globally, about 2.5 million people have OSMF,[7] but studies have found that over 5 million people are affected in India alone (0.5% of the Indian population).[1,7 ,19] It is also estimated that up to 20% of the world's population use betel nut in some form, so the incidence of OSMF is likely to be much higher than current estimates suggest, and it is regarded as a public health issue in the Indian subcontinent, the UK,[1,20] and South Africa.[1,7,21] It is predominantly seen in the second or third decade, and recent data suggest a male predominance; however, both sexes are equally at risk.[10,18] Oral cancer that arises in those who chew betel quid is one of the most common malignancies in south and southeast Asian countries,[1,22] and with immigration from the Indian subcontinent to the UK, USA, and South Africa, oral and maxillofacial surgeons in these countries are likely to encounter the disease more often in future.[7]

Aetiology

At first, OSMF was thought to be idiopathic, but it was later concluded to be multifactorial in origin, and possible aetiological factors include capsaicin in chillies, iron, zinc, and deficiencies in essential vitamins.[6,18,23,24] Various autoantibodies and specific human leucocyte antigens (HLA) in some patients have indicated an autoimmune role as well as a genetic predisposition for the disease.[25] However, various epidemiological studies, large cross-sectional surveys, case–control studies, and cohort and intervention studies have provided overwhelming evidence that areca-nut is the main aetiological factor in OSMF.[21,25-34] The nut is the endosperm of the fruit of the Areca catechu palm tree.[7] A range of case–control studies have given convincing evidence that there is a definite dose-dependent relation between areca-nut and causation of the disease, and it is well known that the onset of the disease is directly proportional to the concentration, incidence, and duration of chewing the nut (without tobacco).[25,29,32,34] Generally, younger patients develop clinical features of OSMF within 3.5 years from onset of the habit while in older patients it takes 6.5 years.[25,27]

Currently, in India, Pakistan, and Bangladesh, betel quid and gutkha are the most commonly used commercially freeze-dried areca-nut products. Gutkha (also spelled gutka or guthka, thought to be derived from Hindi meaning "a shred or small piece") is a light brown, grainy powder available in compact storable sachets (Fig. 2). It consists mainly of areca-nut, tobacco, and flavours, and is typically taken to relieve stress. When chewed it dissolves quickly in saliva and provides central stimulation, which is said to be more intense than tobacco.

Fig. 2. Picture showing betel leaf (A), a typical betel quid (B), contents of a tobacco betel quid with areca-nut (C), compact sachets of gutkha (D) and contents (E). Note the increased load of areca-nut compared with betel quid

Gutkha has replaced most of the commercial areca-nut preparations, and contains the nut in high concentrations along with tobacco. Betel quid commonly contains areca-nut (incorrectly known as betel nut), slaked lime, and A. catechu with or without tobacco, and is typically wrapped in betel leaf (from the Piper betle, a pepper shrub).[7] Although it is still often chewed (usually after meals and thought also to be an appetiser) in India, its consumption as an addictive habit has reduced. However, consumption of the addictive gutkha is increasing rapidly and its increased popularity may be because it is easily accessible, and because of effective changes in price and marketing strategies.

Unlike pan masala, which has to be freshly prepared before use, gutkha is available in compact sachets, which are easy to handle, and allow it to be consumed at any point during the day. The habit often starts among young people, usually as a fashion or status symbol, because of peer pressure, or to imitate parents. It is often used by adults (such as taxi drivers or merchants) to cope with irregular meals, to relieve stress, or to stay awake during shift work, and even to relieve toothache.[35] It is more highly addictive than ordinary chewing tobacco and is exported to well over 22 countries.[35]

Gutkha is usually placed into the buccal or labial vestibule (and sometimes beneath the tongue), and is chewed for up to an hour until the nut softens and dissolves completely in saliva. The excess is either spat out or swallowed. It is typically done several times and more in those who are addicted. Many patients place it in the buccal vestibule at night while they sleep.

Pathogenesis

OSMF is essentially a disease of collagen metabolism,[7] but despite research spanning more than 3 decades, its pathogenesis is still not fully understood. There is compelling evidence that the areca-nut has a primary role in the development of OSMF (Fig. 3), but it has yet to be elucidated.[10,25] However, it seems that changes that occur in the extracellular matrix are likely to have a key role.[25] These studies have focused on increased synthesis, or reduced degradation, of collagen, as possible mechanisms in the development of the disease[25]; there are changes in the normal collagen metabolism at different stages.

Areca-nut contains alkaloids, flavonoids, and copper, which all interfere with homeostasis of the extracellular matrix. Four alkaloids – arecoline (most potent), arecaidine, guvacine, and guvacoline – are known to stimulate fibroblasts to produce collagen.[25] Flavonoids (tannins and catechins) inhibit collagenase, stabilise the collagen fibrils, and render them resistant to degradation by collagenase.[10,25]

The localised mucosal inflammation caused by areca-nut or gutkha results in the recruitment of activated T-cells and macrophages that lead to an increase in cytokines and tumour growth factor beta (TGF-β).[7] The latter considerably increases the production of collagen by activating procollagen genes, and upregulating procollagen proteinase enzymes and lysyl oxidase activity.[7,36]

Simultaneously, TGF-β inhibits collagen degradation by activating the tissue inhibitor of matrix metalloproteinase (TIMP) genes and plasminogen activator inhibitor (PAI).[7] The high concentration of copper in areca-nut has been found to stimulate lysyl oxidase activity, an enzyme essential to the final cross-linking of collagen fibres.[1,7] Increased copper has been seen in mucosa affected by OSMF, which supports its role in fibrogenesis by enhancing lysyl oxidase activity.[10,37-40]

Continually chewing areca-nut leads to increased activity of the masticatory muscles, depletion of glycogen, and muscle fatigue. The reduced blood supply following fibrosis further promotes muscle fatigue and causes extensive degeneration and fibrosis in the muscles.[41]

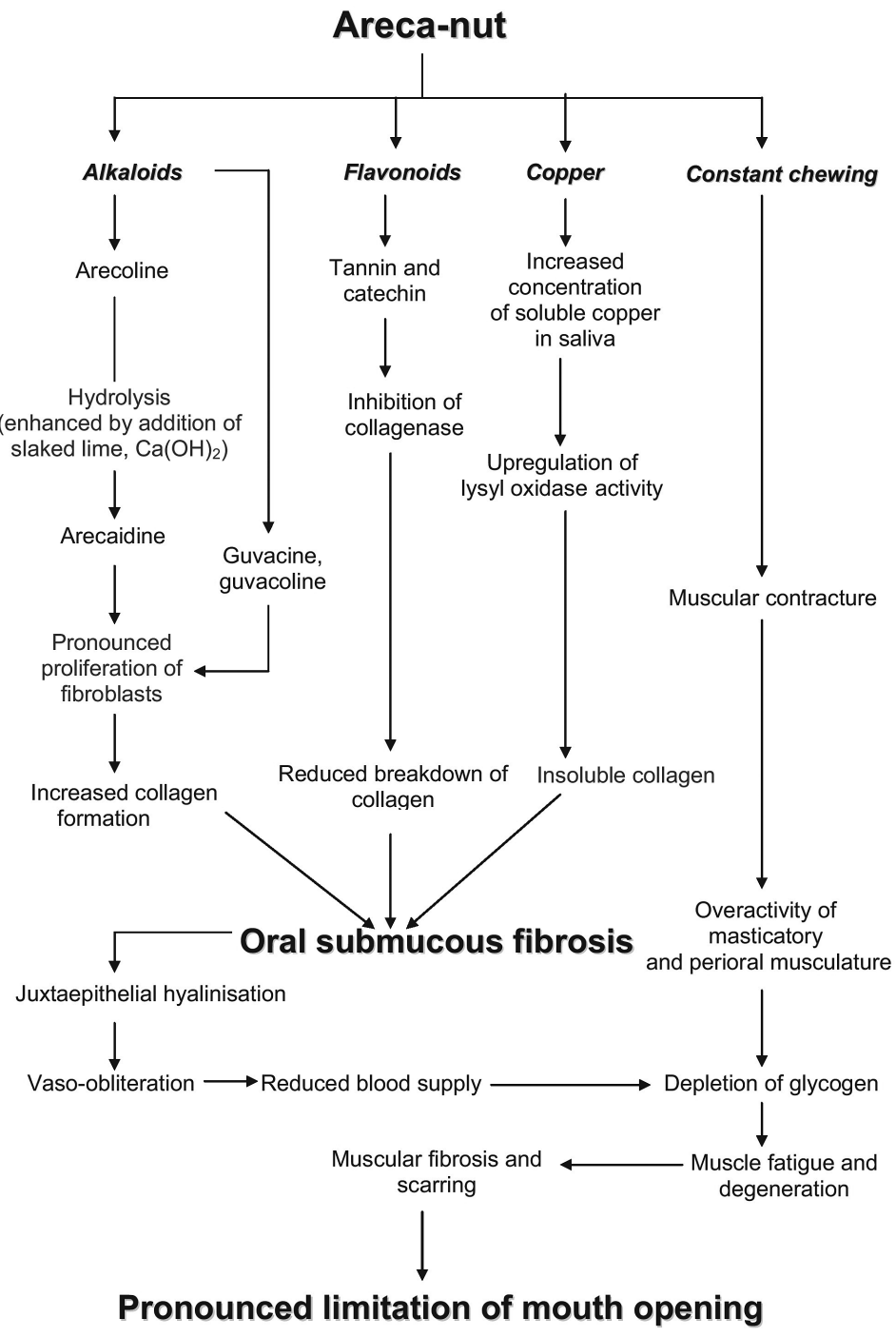

Fig. 3. Role of areca-nut in the pathogenesis of oral submucous fibrosis.

As previously mentioned, another two possible overlapping mechanisms are autoimmune factors and genetic predisposition.[10] This has been substantiated by the presence of circulating immune complexes, immunoglobulins, and autoantibodies in some patients with OSMF, as well as altered cellular and humoral responses.[10,25,42-47] Genetic susceptibility is supported by raised HLA-A10, -B7, and DR3 in OSMF patients compared with normal controls.[11] The familial occurrence of the disease has been reported from India and South Africa.[10,25,48-51]

It seems likely that OSMF is a multifactorial disease with initiators, promoters, and other modifying cofactors. But the tenet – loss of equilibrium of extracellular matrix and continuous deposition of extracellular matrix – in OSMF is currently well accepted.[10]

Classification schemes for OSMF

Several classification and staging systems based on different aspects of the disease have been suggested.[52] The earliest classification by Pindborg and Sirsat[3] in 1966 was based on histopathological features and was updated in 2005 by Utsunomiya et al.[36] The main disadvantage of them is the absence of any description of the epithelial component of the disease.[52] Wahi et al.[53] presented the first clinical classification (of 3 groups) based on symptoms, and several others have now been proposed.[11,12,54-60] However, one well known and respected staging system (Khanna and Andrade[41]) has successfully combined histopathological and clinical features of the disease, and was developed mainly to aid in surgical management.

Malignant transformation and molecular markers

OSMF has a malignant transformation rate of 7–30% (Fig. 1D).[7,12] Pathogenesis is thought to be multifactorial. The carcinogenic effects of tobacco acting in synergy with areca-nut is well known, but the second report on betel quid by the International Agency for Research on Cancer (IARC) identified areca-nut as a “group one carcinogen”.[10,61,62] Its genotoxic and mutagenic effects are attributed to polyphenols, alkaloids, and areca-nut-specific nitrosamines such as N-nitrosoguvacoline, N-nitrosoguvacine, 3-(N-nitroso-methylamino)propionaldehyde, and 3-(N-nitrosomethylamino)propionitrile.[10, 57] Various studies have been conducted in an attempt to identify molecular markers that could be used to predict malignant change in OSMF. Recently, a loss of heterozygosity in 23 “hotspot” loci which alter genes that control the cell cycle has been recognised as an important molecular marker for malignancy in OSMF.[10, 63]

Overview of management principles for OSMF

Patients with OSMF characteristically complain of two problems: an inability to open the mouth, which impedes function, and a burning sensation and intolerance to spicy

foods (intolerance to normal diet in severe cases). Management aims to reverse or alleviate these signs and symptoms, stop the disease progressing, and minimise the risk of malignant transformation.[64]

The current protocol for the management of OSMF can be divided into 3 broad groups: surgical, physical, and medical treatments (Table 1). Surgical treatment, used mainly to manage trismus, involves incising and releasing the fibrotic areas, and leads to further scarring and fibrosis. The introduction of remote tissue (pedicled, such as a buccal fat pad,[65] nasolabial or platysmal flaps,[66] or free tissue transfer) in an attempt to release fibrosis[67,68] is one approach but results are variable.

Physical treatment attempts to influence the remodelling of tissue by using movement – for example, exercises and physiotherapy,[58, 69] various splints or other devices to improve mouth opening,[70,71 ,72] or localised heat (such as with microwave diathermy[56,73,74]). Medical treatment includes dietary supplements (vitamins, antioxidants), the use of anti-inflammatory drugs (principally corticosteroids), proteolytic agents (such as hyaluronidase), anticytokines, and other agents that are not available in the UK. They can be given orally, topically, or by submucosal injection.[58,64,75-90]

Operation is generally reserved for established cases of OSMF while physical treatment is usually combined with all other interventions. However, Kerr et al.[64] recently hypothesised that cessation of the habit alone may have a considerable effect – more on the symptoms of OSMF than on reversing fibrosis.

Conclusion

As management of OSMF aims to slow the progression of the disease, better legislation to govern the availability and sale of areca-nut is recommended, although implementation of this may not be practical at present.[10]

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Table 1: Management of oral submucous fibrosis.

Surgical therapy	**Conservative therapy**
Rationale: incision (incorrectly termed as excision) or surgical release of fibrous bands followed by forceful opening of the mouth (widening of the incised tissue or region), and covering of surgical defects using various flaps or synthetic biological material	***Physical therapy***
	Physical exercise regimen
	Splints or other mouth opening devices
	Microwave diathermy
Extraoral flaps	***Medical therapy***
Split thickness skin graft	**Modulators of inflammation:**
Superficial temporal fascia pedicled flap	Steroids
Temporalis pedicled flap	Interferon gamma
Nasolabial flap	Placental extracts
Amnion graft	Immunised milk
Platysma myocutaneous muscle flap	**Modulators of vascularity or relief of ischaemia:**
Intraoral flaps	Pentoxiphylline
Tongue flap	Buflomedil hydrochloride
Palatal island flap	Nylidrin
Buccal fat pad	**Nutritional support and to combat reactive oxygen species:**
Microvascular free flaps	Beta-carotene
Radial forearm free flap	Lycopene
Anterolateral thigh flap	Vitamins
Alloplasts	Micronutrients
Collagen membrane	Fibrinolysis:
Artificial dermis	Collagenase
	Hyaluronidase
	Chymotrypsin
	Ayurvedic treatment:
	Turmeric (*Curcuma longa* L.)
	Tea pigments
	Oxitard

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Chapter 3

Dietary copper: A novel predisposing factor for oral submucous fibrosis?

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Abstract

Oral submucous fibrosis (OSMF) is known devastating disorder commonly seen in South Asian developing countries. It is directly linked to areca nut chewing and the contents of areca are subjected to multitude of investigations. Among all the contents of areca nut, the copper element has been extensively studied. Most of the published studies have validated its association with OSMF because of its local action. In this paper we postulate a novel biological pathway through which copper is thought to predispose oral mucosa to OSMF. The hypothesis is instructive in explaining various unexplored aspects of the disease.

Introduction

Oral submucous fibrosis (OSMF) is a chronic debilitating and a premalignant condition affecting the oral cavity, pharynx, and upper digestive tract of the oral cavity. The characteristic pathophysiology of the disease is submucosal fibrosis characterized by juxta-epithelial inflammatory reaction followed by chronic change in the fibroelasticity of the lamina propria and associated with epithelial atrophy.[1-6]

OSMF's morbidity/mortality is associated with limitation of mouth opening (trismus) which leads to significant masticatory dysfunction and discomfort.[7] Once, the disease has developed, there is neither regression nor any effective treatment. It is considered as a pre-malignant stage of oral cancer and reported risk of malignant transformation is up to 7.6% over a 17-year period.[2,8,9]

The etiology of OSMF is unknown. The various hypotheses proposed suggest a multifactorial origin for this condition.[10] Many studies have implicated various environmental factors as likely etiological factors. These include capsaicin in chillies, micronutrient deficiencies, immunological, and genetic predisposition.[3,1,11] There is also clinical and experimental evidence of presence of circulating immune complexes, immunoglobulin contents, and circulating auto-antibodies associated with specific HLA antigens in patient's sera and alteration in cellular and humoral responses suggesting an autoimmune etiology and genetic propensity.[1,13-19] However, the existing scientific literature at present makes it apparent that areca (betel) nut is the major etiological factor.[1,20,21]

While a clear association exists between areca chewing and OSMF, the precise mechanism still remains elusive and controversial.[1] There has been a recent interest in the role of copper content of areca nut as a possible etiological factor in the development of this disorder. The association between copper and OSMF has been linked on the basis that excess copper is found in the tissue of other fibrotic disorders such Wilson's disease, Indian childhood cirrhosis and primary biliary cirrhosis (PBC).[13,22] However, the nature and characteristics of copper in this diseases is not been defined clearly.[10,22] Though the solubility of certain copper complexes in saliva has not fully evaluated, preliminary investigations have found that increasing copper is found in saliva during chewing of areca products. [10,22]

Copper in pathogenesis of OSMF

Areca nut has a high copper content (302 nmol/g), a substantial amount of which is released into saliva while chewing.[23,24] The role of copper in the pathogenesis of OSMF is not clearly understood although it is known that the copper dependent enzyme, lysyl oxidase, secreted by the fibroblasts, facilitates the cross-linking of collagen, thereby inhibiting its degradation.[23]

The possible role of copper as a mediator of fibrosis is supported by the demonstration of up regulation of lysyl oxidase in OSMF biopsies and in OSMF fibroblasts compared to normal fibroblasts from oral mucosa grown in culture. Copper added at various concentrations in vitro has also been shown to increase proliferation of fibroblasts in culture. The fibroblasts also followed specific growth characteristics of cell doubling time of 3.2 days for OSF and 3.6 days for normal fibroblasts.[13,25-27]

The nature and characteristics of copper compounds in the areca is as yet unknown, though there is evidence to suggest that the metal–matrix binding of copper in plants is associated with lectins and glycoproteins. The solubility of these complexes in physiological fluids as saliva has not been fully evaluated either, but studies have shown that soluble copper is extracted into saliva following chewing areca products.[10,27]

Several factors may influence the bioavailability and subsequent absorption of copper by the oral mucosa. These factors include binding to non-soluble complexes, such as hemicellulose, dietary carbohydrates, dietary fats, the presence of amino acids, other mineral elements and the pH of the oral environment .[27,28] This would suggest that the composition of the quid or pan masala may have a significant effect on the availability of copper in the oral cavity. The mechanisms that regulate the uptake of copper by cells of the oral mucosa are also not fully understood. At a cellular level, there is evidence to support the role of membrane-bound copper transporting adenosine triphosphates (Cu-ATPase) in the uptake of copper by cells.

The exact mechanism for this is unclear, but probably the copper binding sites form an extended polypeptide chain at the N-terminus of the trans-membrane domain. Recently in vitro kinetic studies using human skin fibroblasts have demonstrated that fibroblasts possess an effective system for the uptake of copper from the culture medium.[27,29] Intracellular copper may possibly form tissue complexes that then facilitate the upregulation of collagen synthesis and subsequent cross-linking via the lysyl oxidase pathway.[27]

Copper may also bind to p53 protein causing aberrations in the oral keratinocytes.[10,22] Studies have also found an increased concentration of copper in fibrotic tissue in unilateral OSMF patients. Thus it was postulated that the site on which patient habitually kept the quid had increased the copper levels suggesting the local action of copper on oral mucosa.[22,30]

The hypothesis

Various hypotheses put forward to date have not been proven conclusively and the precise mechanism of copper mediated pathogenesis of fibrosis still remains elusive. Most studies on OSMF have emphasized only on the oral copper availability and its local mucosal action. An equally important second aspect which needs to be considered is the pre-conditioning of the oral mucosa by a prolonged, chronic copper exposure. Considering the results of existing studies questions can be asked as to why there is often disease progression even after the cessation of the areca chewing habit, and why there is recurrence even after medical therapy in absence of areca chewing.

We postulate that the chronic exposure to subtle high normal copper diet (food, copper potable water) predisposes the oral mucosa to OSMF. This may be considered through two overlapping pathways, namely alteration of normal salivary copper concentrations by increased secretion and secondly preconditioning of the oral mucosa through chronic exposure to salivary copper. When the preconditioned oral mucosa and saliva faces a copper challenge during areca nut, chewing fibrosis is triggered in the mucosal tissue. The presence of other additional factors (systemic factor, other local factors like chillies) might determine the onset and its severity. Once the disease is established, the tissue suffers from hypoperfusion due to fibrosis. Subsequently the copper clearance is further delayed in the tissue thereby increasing the local tissue copper concentration.

The initial symptoms (burning oral mucosa) often result in poor oral intake and malnutrition which potentiate tissue fibrosis. The condition enters a vicious cycle when the mouth opening reduces progressively predisposing the patient to psychological as well as physical stress and further fibrosis. It is also speculated that the chronic exposure alters the absorption threshold of gut and increases the body resistance to the subtle higher level of copper. It is also showed that the drinking water with higher copper concentration may be well tolerated without any gastrointestinal symptoms.[31] Hence systemic adaptation to the marginal increase in copper level can be expected which leads to preconditioning the oral mucosa.

Testing the hypothesis

To test the hypothesis a careful investigation of copper level of potable water (community and home) and food is recommended. The concentrations can be correlated with serum and saliva copper levels. Additionally serum ceruloplasmin can validate the systemic predisposition.

Discussion

Among many trace elements associated with OSMF, role of copper and iron have been extensively studied and have been the subject of a multitude of investigations.[32] There are many studies which showed the local action of copper in patho-

genesis of OSMF. However, its systemic role is not been investigated. We postulate systemic exposure of copper from diet as an etiopathogenic factor. Though most of the studies have shown a normal blood and faecal values for copper in OSMF,[13] still there are some studies which demonstrated increased systemic copper values in OSMF and in other fibrotic conditions.[32] There is also an experimental evidence that increased dietary copper intake is linked to a direct increase in lysyl oxidase activity.[10,33] Furthermore, the presence of copper consumed from other sources, such as copper cooking utensils, would be expected to produce at least sporadic cases of OSMF.[34] This is supported by reports of Indian infantile cirrhosis, an ecogenetic copper-related fibrosis, which is not only independent of OSMF but is also found in Austrians who use similar copper made cooking vessels.[34] Hence the role of systemic copper can be speculated in OSMF and our hypothesis is instructive in explaining the typical features of the disease. Based on the hypothesis it can be concluded that the preconditioned oral cavity is prone to bilateral fibrosis whereas no conditioning could lead to unilateral fibrosis only. It may also explain why there are reports of progression of disease even after cessation of areca chewing habit or medical treatment. After the cessation of the habit or medical therapy the compromised oral mucosa is still exposed to residual copper from diet (which is absorbed due to an altered threshold) which is sufficient to precipitate the fibrosis further as bioavailability is relatively increased due to tissue hypoperfusion.

Conclusion

In spite of continued research the pathogenesis of OSMF is still obscure. Though there are various treatment modalities are available for the disease, recurrence remained as major challenge. Hence it is advised to emphasize the identification and elimination of the causative factors of OSMF. This present hypothesis proposes a new predisposing factor which if proved promises a new horizon in helping to prevent this devastating disease which is widespread in the Indian subcontinent and a prospective study is currently in progress.

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Chapter 4

Evaluation of the possible role of copper ions in drinking water in the pathogenesis of oral submucous fibrosis: a pilot study

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Abstract

We aimed to investigate the concentration of copper ions in drinking water and to assess whether copper has a role in the pathogenesis of oral submucous fibrosis (OSMF). We studied 50 patients with clinically and histologically diagnosed OSMF from the Yadgir district of Karnataka in India. Fifty healthy people matched for age and sex were used as controls. In both groups concentrations of copper ions in serum, saliva, and home drinking water were measured using atomic absorption spectroscopy and intelligent nephelometry technology. Serum ceruloplasmin concentrations were also estimated in both groups. The mean (SD) concentration of copper in the home drinking water of patients with OSMF was significantly higher (764.3 (445.9) µmol/L) than in the controls (305.7 (318.5) µmol/L) ($p < 0.001$). Patients with OSMF also had significantly higher copper concentrations in serum and saliva, and serum ceruloplasmin than controls ($p < 0.001$). For the first time these data have shown a positive association between copper concentrations in home drinking water and OSMF. It raises the possibility that increased copper in drinking water contributes to the development of OSMF, and adds to that ingested when areca nut is chewed.

Keywords

Oral submucous fibrosis; Pathogenesis; Copper; Home drinking water

Introduction

Oral submucous fibrosis (OSMF) is a debilitating disease of the oral cavity that causes serious functional morbidity and an increased risk of malignancy.[1,2] It was first described in 5 Indian women and was termed atrophia idiopathies (tropica) mucosae oris (Schwartz J. Atrophia idiopathica (tropica) mucosae oris. Paper presented at the Eleventh International Dental Congress. London, July 1952), submucous fibrosis of the palate and pillars,[3-5] and later, submucous fibrosis.4 Its premalignant nature was first described by Paymaster in 1956.[6]

The disease is characterised by juxtaepithelial inflammation, fibroelasticity of the lamina propria, and epithelial atrophy. It can cause varying degrees of debility because of a burning mucosa, restricted mouth opening, and limited intake of food.[7-9] The signs and symptoms depend on the stage and site of involvement,[7] and malignancy has been noted among 7–30% of patients over a 17-year period.[2,4,7] It is predominantly seen in South Asian developing countries, among Asian immigrants in the UK, and in south and east Africa,[10] and consequently is considered a problem for global public health.[2,11] In India the prevalence varies from 0.2% to 0.5% and a high percentage is found in the south of the country.[12] It is mostly seen in the second or third decade, and recent data suggest a male predominance, however, both sexes are equally at risk.[7]

The exact cause is not known and is therefore the subject of speculation. Most authorities suggest that it does not have a single cause but is multifactorial[13]; many different factors combine to induce disease and influence outcomes – for example, chewing betel nut or tobacco, smoking, eating chillies, malnutrition, vitamin deficiency, autoimmunity, and genetic predisposition.[13] However, there is growing evidence that areca nut is the primary aetiological factor.[10,14]
Recently there has been interest in the role of copper in the pathogenesis of OSMF. Several clinical and experimental investigations have provided evidence of a casual relation between the copper found in areca nut and its association with OSMF.[7,15-18] It has been suggested that chewing areca nut significantly raises the concentration of soluble copper in saliva and thereby upregulates local lysyl oxidase activity in the oral mucosa, which promotes fibrogenesis by the cross-linking of collagen fibres.[15,16]

Copper is a trace metal essential for the function of several key enzymes involved in the human metabolism.[16, 19] They include cytochrome-c oxidase, superoxide dismutase, metallothionein, and lysyl oxidase.16 Genetic disorders such as Wilson disease, or environmental contamination that leads to the accumulation of copper in childhood cirrhosis and pulmonary fibrosis in India, can cause abnormalities in the absorption, metabolism, and excretion of copper, and result in it being deposited in several sites in the body.[16,20] As high concentrations are seen in OSMF, it has be-

come a subject of interest in the field of head and neck oncology. While most studies that involve copper have emphasised its local action on the oral mucosa, its systemic effect in OSMF has also been shown.[10,17,18]

We aimed to investigate whether copper in drinking water has a role in the pathogenesis of OSMF.

Patients and methods

We obtained approval from the local ethical committee for a prospective case control pilot study. It was conducted in the Yadgir district of the Hyderabad–Karnataka region in India. Healthy patients native to the Yadgir district who had no serious medical history, and who had lived and worked in the same area since birth, were included. Those who spent time away from home (including long distance drivers because they did not use their regular water supply at home), and those who had previously had operations for OSMF and had secondary oral changes, were excluded.

For the study group we recruited 50 patients with standard clinical symptoms of OSMF[4] confirmed with oral biopsy examination, and matched them by age and sex with 50 healthy people who did not chew areca nut. Written consent was obtained. Samples of serum and saliva were collected in both groups to measure copper concentrations, and concentrations of ceruloplasmin were also measured. To avoid contamination by areca nut, all patients in the study group were asked to avoid chewing it in any form for one hour, and a sterile plastic container was used to collect the unstimulated saliva. One of the authors collected and refrigerated samples of drinking water (most came from bore holes and wells) from each patient's home.

Copper in water was analysed using flame atomic absorption spectroscopy after the equipment was calibrated with standards for copper. According to World Health Organization (WHO), United States Environmental Protection Agency (USEPA), and Indian standard specification (IS 10500), the desirable concentration of copper in water is 31.85 µmol/L, and the standard limit is 828.03 µmol/L (data provided by University Agriculture Sciences, Raichur, India).

To estimate copper concentrations in serum and concentrations of ceruloplasmin we collected 5 ml of blood by venipuncture in a plain tube and froze the serum for transportation. To collect mixed saliva samples patients rinsed their mouths with ultrapure water and spat into individual sterile plastic containers, which were then frozen for transportation. The volume of each whole mixed saliva sample was accurately measured, wet washed with 3.5 ml 60% nitric acid (HNO3) and 0.2–0.5 ml 60% perchloric acid (HClO4), then adjusted to a constant volume of 2.5 ml with the most pure water available. All chemicals were of the highest available purity. A beaker with nitric acid and a beaker of perchloric acid were prepared for each sample to test for purity and accidental contamination. We analysed serum, salivary copper, and serum ceruloplasmin using intelligent nephelometry technology (MISPA-i, Agappe diagnostics, Ernakulum, Kerala, India) with quality control (QC Passed no. 2160110056) smart card calibration, and verified the assay performance. Data were expressed in mean, standard deviation (SD), and percentage.

Comparisons between the two groups were done using Student's t-test, z-test for proportion, and Pearson's correlation coefficient. Probabilities of less than 0.05 were considered significant. Data were analysed using SPSS version 16.0 (IBM).

Results

The study included 100 male patients. The mean (SD) age of patients in the study group was 28.0 (6.9) years, and in the control group was 28.1 (8.1) years. The difference between ages in the 2 groups was not significant. There was a significant difference between the groups in the mean (SD) concentration of copper in water measured by atomic absorption ($p < 0.001$), and there were also significant differences in mean (SD) concentrations of serum copper, salivary copper, and ceruloplasmin between the groups ($p < 0.001$) (Table 1).

Group	Mean (SD)		95% CI of difference	*p*-Value
	OSMF group	Control group		
Serum ceruloplasmin (mg/L)	$57.3 \times 10^{-5}(16.6 \times 10^{-5})$	$41.1 \times 10^{-5}(18.2 \times 10^{-5})$	9.24×10^{-5} to 23.07×10^{-5}	<0.001
Serum copper (µmol/L)	1.34 (0.57)	0.89 (0.39)	0.29–0.67	<0.001
Salivary copper (µmol/L)	4.65 (1.59)	2.74 (1.66)	1.25–2.54	<0.001
Copper in drinking water (µmol/L)	764.3 (445.9)	305.7 (318.5)	261.2–598.7	<0.001

Table 1: Comparison between groups for serum ceruloplasmin, serum copper, salivary copper, and copper concentration in drinking water.

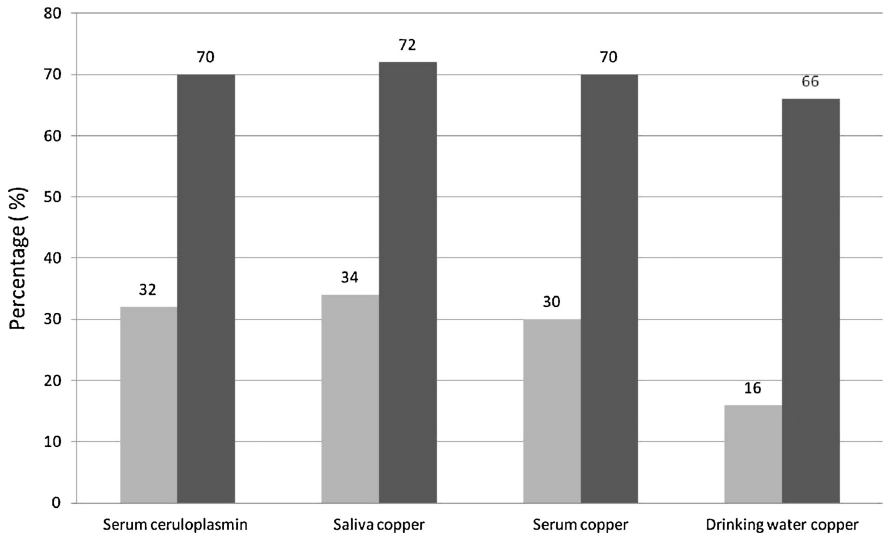

Fig. 1. Percentage of higher than average copper concentrations in OSMF and control groups (dark grey = OSMF; light grey = control)

Copper concentrations were high in the drinking water taken from the homes of 33 patients (66%) in the OSMF group and 8 patients in the control group (16%). Serum ceruloplasmin and copper concentrations were also high in 35 patients in the OSMF group (70%). Copper concentrations in saliva were higher than normal ranges in 36 patients (72%) (Fig. 1). Serum ceruloplasmin, and concentrations of copper in serum and saliva were above the normal range in 16 (32%), 15 (30%), and 17 (32%) patients with OSMF, respectively (Table 2). There was a positive correlation between concentrations of copper in water and serum as well as in salivary copper in both groups (Table 3).

Table 2: Number (%) of patients with high copper concentrations.

	OSMF group (*n* = 50)	Control group (*n* = 50)
Serum ceruloplasmin	35 (70)	16 (32)
Serum copper	35 (70)	15 (30)
Salivary copper	36 (72)	17 (34)
Copper in drinking water	33 (66)	8 (16)

Table 3: Pearson's correlation of copper concentrations in water with serum ceruloplasmin, serum copper, and saliva copper.

	r-Value		*p*-Value
	OSMF group	Control group	
Copper in water and serum ceruloplasmin	0.90	0.90	<0.001
Copper in water and serum	0.80	0.88	<0.001
Copper in water and saliva	0.79	0.82	<0.001

Discussion

Among many trace elements associated with OSMF, the role of copper ions has been studied extensively and has been the subject of many investigations.[18] Copper is an essential trace element. The average daily intake of copper in adults in developed countries is between 0.6 and 1.6 mg, of which 35–70% is absorbed from the gastrointestinal tract.[15]

The intake of copper comes almost exclusively from food and water although a small amount comes from skin contact with substances that contain it,[21] and its balance is normally controlled by efficient homeostatic mechanisms.[19,21] Anecdotal reports from isolated occurrences in humans suggest that when drinks or drinking water are contaminated with copper it either causes no symptoms or causes abdominal pain, nausea, vomiting, and diarrhoea. However, the threshold for gastrointestinal symptoms caused by excessive ingestion of copper has not been established in controlled prospective studies.[21,22]

Local mucosal exposure to copper by chewing areca nut is causally linked to OSMF.[23] The role of copper in OSMF is not clearly understood although it is known that the copper dependent enzyme lysyl oxidase, which is secreted by fibroblasts, facilitates the cross-linking of collagen, and inhibits its degradation.[23, 24]

Systemic exposure to copper through drinking water has been implicated in the pathogenesis of several fibrotic conditions,[25] but its role in OSMF has not been examined previously. Trivedy et al.[15] observed increased salivary copper and normal systemic copper concentrations in patients with OSMF. Based on the data they hypothesised that chewing areca nut is the main source of local concentrations of copper in the oral cavity and in salivary secretions.[15] Nevertheless some studies have shown raised concentrations of copper and ceruloplasmin in the serum of patients with oral premalignant and malignant lesions, and OSMF.[18,26,27]

While there is a clear association between chewing areca nut, concentrations of salivary copper, and OSMF, the mechanism of generalisation of oral mucosal fibrosis and its heterogeneous pattern of occurrence have yet to be elucidated. It is interesting to know the sporadic trend of occurrence of the condition and its diverse mucosal incidence irrespective of how often areca nut is chewed or where it is placed in the mouth. Trivedi et al.[15] noted that mucosal fibrosis and increased salivary and mucosal copper concentrations developed at the site where patients habitually placed the quid. They hypothesised that the copper concentration is raised in tissue that is in contact with the quid and this results in fibrosis of the oral mucosa.[15] However, it does not explain the bilateral incidence of OSMF in unilateral chewers, and early isolated fibrous bands that are seen around the lip (rima oris), tongue, and retromo-

lar or pterygomandibular tissue without involvement of the buccal mucosa (where the quid is commonly placed).[2,4,28]

It is possible that raised concentrations of systemic copper caused by regularly drinking water that contains high concentrations of copper ions has an important role in the pathogenesis of OSMF as suggested by our results. Chronic drinking or ingestion of copper may alter the normal threshold for the absorption of copper by the gut and may also change the body's resistance to copper. When exposed to additional copper in areca nut, the cumulative effect could compound OSMF. This hypothesis might also help to explain the sporadic incidence and appearance of fibrosis in the oral mucosa, and could also explain bilateral disease in patients who chew only on one side. Our data have shown that the drinking water of patients with OSMF had a significantly higher copper concentration than that of the control group, and that patients with OSMF also had significantly higher mean serum copper and ceruloplasmin concentrations.

Eight volunteers (16%) in the control group had raised copper concentrations in their drinking water, serum, and saliva. It could be postulated that they might have a high risk of developing OSMF if they started to chew areca nut, but further work is needed to understand the role of systemic copper in the pathogenesis of the condition. Further research in this area is in progress to find the association in a large heterogeneous population and to ascertain whether the amount of water drunk is proportional to the total copper concentration in serum, or whether it has a lesser role than the copper contained in areca nut.

Ethical approval: Institutional research ethical committee (Human) of Navodaya medical college and Hospital Raichur.

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Conflicts of interest: None.

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Chapter 5

Role of drinking water copper in pathogenesis of oral submucous fibrosis: a prospective case control study

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Abstract

Although oral submucous fibrosis (OSMF) is thought to be multifactorial in origin, the chewing of areca nut is thought to be the main cause. Alkaloids and tannins in areca nut are responsible for fibrosis, but recent evidence has suggested that copper ions are also an important mediator, and in a small pilot study we recently found that OSMF was significantly associated with a raised concentration of copper in drinking water. We have further investigated this association in a heterogeneous population in Hyderabad-Karnataka, India, a region with a high incidence of the condition. We evaluated 3 groups, each of 100 patients: those with OSMF who chewed gutkha, those who chewed gutkha but did not have OSMF, and healthy controls who did not chew gutkha. The difference between the groups in the mean concentration of copper in water measured by atomic absorption spectrometry was significant ($p < 0.001$). There were also significant differences between the groups in mean concentrations of serum copper, salivary copper, and ceruloplasmin ($p < 0.001$). Our results confirm that copper in drinking water contributes to the pathogenesis of OSMF, but ingestion of copper is unlikely to be the sole cause.

Keywords

Oral submucous fibrosis; Pathogenesis; Copper; Home drinking water

Introduction

Oral submucous fibrosis (OSMF) is a potentially malignant disease that is seen predominantly in the Asian subcontinent.[1] Its prevalence varies from 0.2% to 0.5% with a high percentage found in the southern part of India,[2] and it affects more women than men, although reports of the sex ratio vary. The highest incidence occurs between the ages of 20 and 40 years.[3] Many factors such as tobacco, smoking, pan masala, chili, malnutrition, vitamin deficiency, autoimmunity, and genetic predisposition, have been thought to contribute to the aetiology[4,5] but areca nut is thought to be the main factor.[4- 9]

Gutkha is a commercial areca nut preparation containing the nut in high concentrations along with tobacco and slaked lime. Over the last 10 years it has replaced other products that contained areca nut and tobacco. Consumption of the addictive gutkha is increasing rapidly, possibly because it is easily accessible and because of effective changes in price and marketing strategies.[4] Alkaloids, tannins, and the copper content of areca nuts are thought to be responsible for mucosal fibrosis.[4]

Copper may be the primary mediator of fibrosis because of up regulation of lysyl oxidase in both tissue biopsy specimens10 and OSMF fibroblasts.[11] In vitro studies have found increased lysyl oxidase activity, as well as specific growth factors such as interleukin 1 (IL-1), transforming growth factor beta (TGF-b), insulin-like growth factor (IGF), and epidermal growth factor (EGF). The role of copper is also supported by the fact that patients with OSMF have raised concentrations in tissue, serum, and saliva.[4,12- 15]

A recent pilot study provided evidence that copper ions in drinking water have a role in the pathogenesis of OSMF, and the authors proposed that chronic ingestion of copper could lead to a systemic increase in its concentration and predispose the oral mucosa to OSMF.[16] We have further investigated this in a large heterogeneous population from the Hyderabad-Karnataka (involving 6 districts) region of Karnataka, India.

Patients and methods

The Institutional Research Ethical Committee (Human) of Navodaya Medical College and Hospital, Raichur, Karnataka, India approved this prospective case control study. Healthy patients native to the region with no serious medical history, and who had lived and worked in the same area since birth, were included. Those who spent time away from home (including long distance drivers because they did not use their regular water supply at home), and those who had previously had operations for OSMF and had secondary oral changes, were excluded. We advertised and organ-

ised several clinics across the Karnataka district for patients with OSMF to attend. For the study group we recruited 100 patients with standard clinical symptoms of OSMF[17] confirmed by clinical examination (OSMF group), and matched them for age and sex with 100 healthy people who chewed gutkha but did not have OSMF (non-OSMF group) and 100 healthy people who did not chew gutkha (control group). Written consent was obtained from all patients. Serum and saliva samples were collected in all groups to assay concentrations of copper and ceruloplasmin. To avoid contamination by areca nut, all patients were asked to avoid chewing it in any form for one hour before saliva was collected. Patients rinsed their mouths with ultra-pure water and spat into individual sterile plastic containers, which were then frozen for transportation.

To estimate the concentrations of copper and ceruloplasmin, 5 ml of venous blood was collected in a plain tube and frozen for transportation. One of the authors collected and refrigerated samples of drinking water (most originating from bore holes, municipal taps, and wells) from each patient's home.

Laboratory analysis

Copper in water was analysed using flame atomic absorption spectroscopy after the equipment was calibrated with standards for copper. According to the World Health Organization (WHO), the United States Environmental Protection Agency (USEPA), and Indian standard specification (IS 10500), the desirable concentration of copper in water is less than 31.85 µmol/L and the maximum limit is 828.03 µmol/L (data provided by University Agriculture Sciences, Raichur, India).

The volume of each whole mixed saliva sample was accurately measured, wet washed with 3.5 ml 60% nitric acid and 0.2–0.5 ml 60% perchloric acid, then adjusted to a constant volume of 2.5 ml with pure water. Serum and salivary copper, and serum ceruloplasmin were analysed using intelligent nephelometry technology (MISPA-i, Agappe diagnostics, Ernakulum, Kerala, India) with quality control (QC Passed no. 2160110056) smart card calibration, to verify the performance of the assay.

The mean (SD) and percentages for each group were recorded. Comparison between groups was calculated using ANOVA for parametric distribution and a post hoc Dunnett's test. Pearson's rank correlation coefficient (r) was used to assess association between variables within each group. Probabilities of less than 0.05 were considered significant. Data were analysed with the help of SPSS version 19.0 (IBM Corp).

Results

The study included 300 male patients. We encountered men only in our clinics because local social circumstances made women reluctant to attend. Mean (SD) ages are shown in Table 1.

The mean copper concentration in water measured by atomic absorption differed significantly between the groups ($p < 0.001$). Significant differences between the groups were also found in mean

Table 1. Comparison of age between groups. Data are mean (SD).

	OSMF group (Gutkha with OSMF)	Non-OSMF group (Gutkha without OSMF)	Control group	*p* value
Age (years)	29 (8)	29 (7)	28 (7)	0.59

concentrations of serum copper, salivary copper, and ceruloplasmin ($p < 0.001$) (Table 2). Copper concentrations were higher than normal in the home drinking water of 33 patients in the OSMF group and 8 in the control group (Fig. 1). Serum copper and ceruloplasmin concentrations were also higher than normal (normal range 0.48–0.92 µmol/L; 20 × 10−5 and 60 × 10−5 mg/L, respectively) in the OSMF group (37% and 35%, respectively) and the control group (19% and 17%, respectively) (Fig.1). A total of 85 patients in the OSMF group and 8 in the control group also had higher than normal concentrations of copper in saliva (Fig. 1).

All those in the non-OSMF group had values within standard limits for serum ceruloplasmin and for concentrations of copper in serum and water, but they all had higher than normal concentrations of copper in saliva (n = 100) (Fig. 1).

There was a positive correlation between copper concentrations in water and serum and salivary copper in the OSMF group and the control group (Table 3) ($p< 0.001$).

Discussion

Our previous pilot study[16] showed a significant association between OSMF and copper in drinking water, and serum and salivary copper. The present study conducted on a larger series of 300 patients has confirmed this. It is thought that copper stimulates the activity of lysyl oxidase, an enzyme essential to the final cross-linking of collagen fibres. Increased concentrations of copper have been seen in mucosa affected by OSMF, and this enhancement of lysyl oxidase activity supports its role in fibrogenesis.[10,11]

Table 2. Comparison between the groups for all variables. Data are mean (SD).

	OSMF group (Gutkha with OSMF)	Non-OSMF group (Gutkha without OSMF)	Control group	*p* value
Serum copper (µmol/L)	1.02 (0.53)**	0.34 (0.15)**	0.60 (0.41)	<0.001
Serum cerulo-plasmin (mg/L)	51.43 × 10^{-5} (16.1 × 10^{-5})**	26.3 ×10^{-5} (8.63 × 10^{-5})	37.69 × 10^{-5}(16.74 × 10^{-5})	<0.0001
Salivary cop-per (µmol/L)	4.81 (1.17)**	4.41(0.401)**	2.41 (1.41)	<0.0001
Water copper (µmol/L)	388.53 (229.23)**	203.82 (82.80)*	267.51 (165.60)	<0.004

* p < 0.05 compared with control group.

**p < 0.01 compared with control group.

Dunnett›s test or Dunn›s test used after analysis of variance (ANOVA).

Copper is found in natural deposits in ores that contain other elements.[18] It is a nutrient essential for the function of several key enzymes involved in the human metabolism[4] including cytochrome-c oxidase, superoxide dismutase, metallothionein, and lysyl oxidase.[16] It is rarely found in water sources, but drinking water in the home can often be contaminated by the corrosion of copper piping and cannot be directly detected or removed.[18] Mining and smelting operations, and municipal incineration may also contaminate supplies.[18] This may be one reason why OSMF is typically found in low socioeconomic populations which may be exposed to contaminated water, and it also explains the high incidence of OSMF in developing countries where standards and regulations for the quality of drinking water are low.

Table 3. Pearson's correlation (r-value) of copper in water with serum ceruloplasmin, salivary copper, and serum copper.

	OSMF group (Gutkha with OSMF)	Non-OSMF group (Gutkha without OSMF)	Control group
Serum cerulo-plasmin	0.727**	−0.037	0.715**
Salivary copper	0.419**	0.122	0.708**
Serum copper	0.854**	−0.14	0.828**

**p < 0.001.

In vitro work has found that raised copper concentrations increase the proliferation of fibroblasts.[12] If this were the case then raised concentrations would be expected to cause mucosal fibrosis in all who chew gutkha, but many do not develop the condition.

Recently, the concept of "mucosal pre-conditioning"[16] was introduced to explain why this might be the case. Chronic exposure to higher than normal concentrations of copper in drinking water might predispose the oral mucosa to OSMF, alter the normal threshold for its absorption, and change the cellular resistance to copper. When

exposed to the additional copper in areca nut, the cumulative effect could compound the condition.[16]

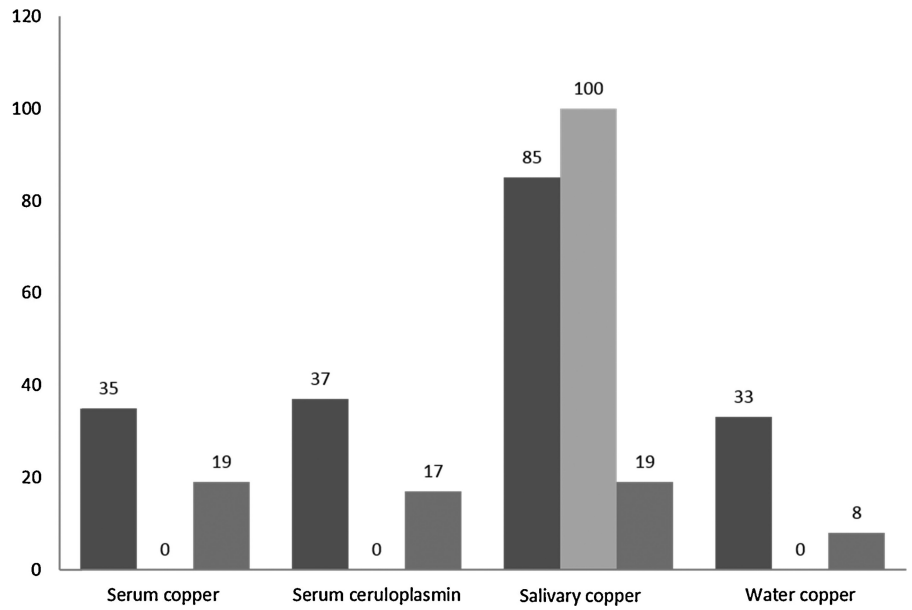

Fig. 1. Number of patients (n = 100) with high concentrations of copper (dark grey = OSMF group, medium grey = non-OSMF group, light grey = control group).

Local exposure of the mucosa to copper when areca nut is chewed is causally linked to OSMF,[4] and systemic exposure from drinking water with high concentrations of copper has been implicated in the pathogenesis of several fibrotic conditions.[19] Some studies have found that concentrations of serum copper are directly proportional to the increase in the severity of OSMF,[15,20] although Trivedy et al.[19] reported increased salivary copper and normal systemic concentrations in patients with OSMF. It seems that raised concentrations of copper and ceruloplasmin are found in some patients with premalignant and malignant oral lesions, and OSMF.[21-23]

We found that the mean concentration of copper in water was higher in the OSMF group than in both the other groups (Fig. 2) although it was at the upper end of normal, which might explain the absence of any toxic effects.

We noticed that concentrations of copper in drinking water were lowest in the non-OMFS group (Fig. 2). One possibility is that low concentrations of total body copper spare the oral mucosa from pre-sensitisation (or pre-conditioning) to OSMF. The mean concentration of serum ceruloplasmin was highest in the OSMF group, which again may be because of the raised concentrations of serum copper in patients with

OSMF who chewed gutkha (Table 2). Concentrations of salivary copper were higher than normal in both groups that chewed gutkha (85% of the OSMF group and all those in the non-OSMF group) (Fig. 1), which suggests that copper from the gutkha or areca nut constantly leaches into the saliva, but the oral mucosa responds differently.

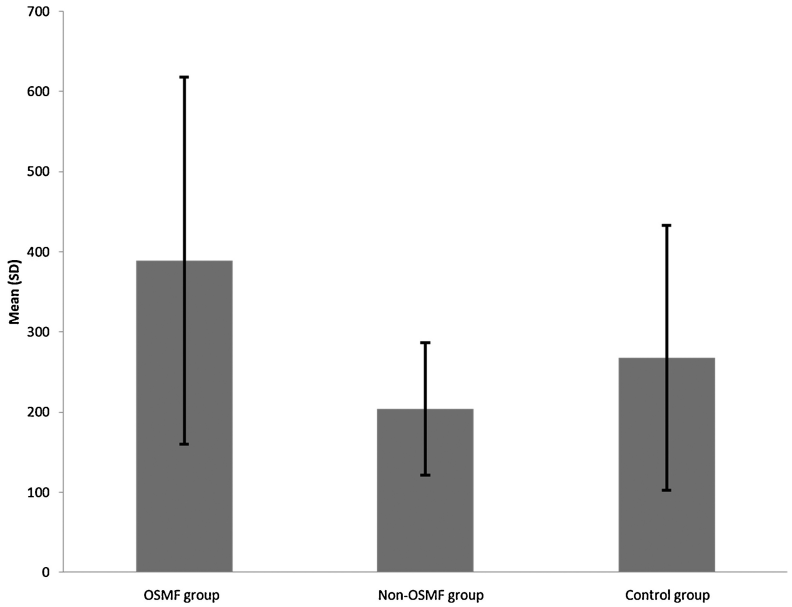

Fig. 2. Comparison of copper concentrations in water between groups

We can speculate that the oral mucosa in patients with OSMF may have been presensitised or conditioned by chronic raised concentrations of copper in water, or that a threshold of total copper is needed to trigger the condition. Concentrations of salivary copper were within normal ranges in the control group as there was no source of local copper (gutkha or areca nut) in the oral cavity (Fig. 3). It can be inferred that chronic ingestion of copper has a vital role in the pathogenesis of OSMF. As far as we are aware, this is the first study to evaluate serum and salivary copper concentrations in people who chew gutkha but do not have OSMF, which are likely to be the best controls in research into this condition. They had normal serum values and copper in drinking water caused no problem. However, concentrations of salivary copper were persistently higher than normal (Fig. 1), which indicates that the copper content of gutkha is not the only cause of oral mucosal fibrosis.

Conclusion

This large study shows a positive correlation between the incidence of OSMF and concentrations of copper in drinking water. It also provides evidence that locally

available copper in saliva is not the only cause of OSMF but may affect sensitised mucosa. This could explain why some people who constantly chew gutkha or areca nut do not develop the condition. Undoubtedly, other factors are necessary to initiate fibrosis, and copper may be just one variable. OSMF may affect people in developing countries and those of low socioeconomic status because drinking water can be contaminated with copper (and other trace elements) which we consider to be an important contributing factor. To reduce its incidence, high standards of drinking water must be maintained, and we recommend that all public water supplies meet those set out in the National Primary Drinking Water Regulations.[24]

Ethics statement/confirmation of patient permission: Institutional research ethical committee (Human) of Navodaya medical college and Hospital Raichur.

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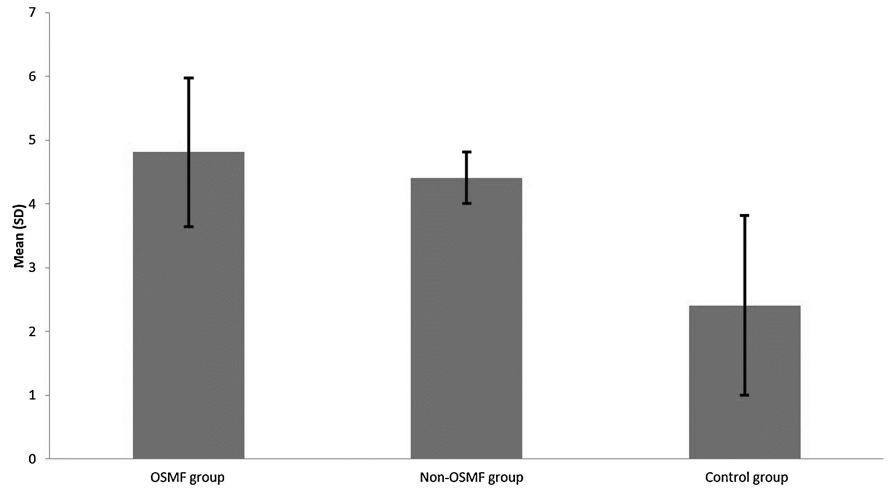

Fig. 3. Comparison of copper concentrations in saliva between groups.

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Chapter 6

Salivary pooling: is it specific to particular regions in oral submucous fibrosis?

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Abstract

Despite extensive research, the pathophysiology of oral submucous fibrosis (OSMF), a premalignant condition that primarily affects the mucosa, is still unclear, although the chewing of areca nut is known to be the primary cause. While a clear association exists between areca nut and OSMF, very little has been published on the reason for its sporadic incidence in the mouth. Many authors have suggested the site where quid is habitually placed, but this fails to explain multiple sites in those who chew on one side. We hypothesised that the pattern of salivary pooling might affect the distribution of OSMF by carrying the chemicals responsible for mucosal damage. In our study of 174 patients, we evaluated the sites where quid was habitually placed and the areas of salivary pooling, and their association with the incidence of OSMF. Most chewers (136/174, 78%) placed the quid in the buccal vestibule, although other sites were also used including the vestibule of the lip, tongue, and floor of the mouth. The standardised residuals suggested significant associations ($p < 0.001$) between salivary pooling and OSMF, and indicated that salivary pooling affects the mucosal surfaces where it occurs. Our results show that the quid is not the only cause of OSMF. Salivary pooling also has an important role and provides a possible mechanism for the sporadic incidence of the condition. To our knowledge this is the first study to evaluate salivary pooling as a contributory factor in OSMF, and it may help to explain the pattern of distribution. Further work is needed in this area to understand the association more fully

Keywords

Oral submucous fibrosis; Saliva pool; Gutkha; Areca nut; Pathogenesis

Introduction

Oral submucous fibrosis (OSMF) is a complex, debilitating, and precancerous condition that is associated with abnormal metabolism of collagen.[1] Despite more than 3 decades of research, its pathogenesis is still not fully understood,[1,2] and although it is thought to be multifactorial with many influencing factors, areca nut is considered the primary cause.[3–8]

Areca nut contains alkaloids, flavonoids, and copper, which interfere with homeostasis of the extracellular matrix.[1] Many studies provide evidence of a casual association between OSMF and copper in areca nut and drinking water,[3,8–12] and it has been suggested that chewing arecanut substantially raises the concentration of soluble copper in saliva, and as a consequence upregulates local lysyl oxidase activity in the oral mucosa, and promotes fibrosis by the cross-linking of collagen fibres.[8]

In south Asia, the most commonly used, commercially freeze-dried areca nut products are betel quid and gutkha, the latter having replaced most areca nut preparations. Gutkha contains high concentrations of areca nut along with tobacco,[1] and when chewed, dissolves quickly in saliva and provides central stimulation, which is reported to be more intense than tobacco alone.[1] It is usually placed in the buccal or labial vestibule, and is sometimes placed sublingually, and is chewed for up to an hour until the nut softens and dissolves in saliva. The excess is then spat out or swallowed. Some patients have been known to place it in the buccal vestibule while they sleep. As increased copper concentrations have been found in fibrotic tissue in patients with unilateral OSMF, it has been postulated that chewing at the site where the quid is habitually placed raises the local levels of copper sufficiently to cause fibrosis.[13,14]

While there is a clear association between the chewing of gutkha or areca nut and the incidence of OSMF, [10,11] we know of little that has been published on the cause of its sporadic pattern of distribution in the oral cavity. Persistent chewing of gutkha quid at a specific site has been suggested as the reason for the diverse distribution of OSMF. According to this hypothesis unilateral chewers will have unilateral OSMF. However, this postulation is based on experimental data rather than clinical observation.[9-11]

It has been proposed that proximity of the gutkha or areca quid to the oral mucosa is responsible for the local development of OSMF, and experimentally, exposure of the oral mucosa to saliva containing dissolved products of the quid has resulted in OSMF.[10,11] Based on these findings, it was postulated that gutkha quid and saliva containing its chemicals are the primary cause of OSMF because they enable the chemicals to be absorbed into the oral mucosa.[10, 11] However, this fails to explain the

incidence of OSMF in multiple sites among unilateral chewers, and why some surfaces are not affected.

The prolonged exposure of the oral mucosa to saliva containing dissolved products of the quid, which occurs when the saliva pools in a specific area, could be an explanation. It could therefore be speculated that the pattern of pooling affects the distribution of OSMF. We recorded the sites where gutkha was chewed and where the saliva pooled to find out whether they were associated with the site of OSMF.

Material and methods

The local ethics committee approved the study which was conducted in the Yadgir district of the Hyderabad–Karnataka region in India. We randomly recruited 174 patients who chewed gutkha or areca nut, and had had OSMF confirmed by clinical examination by one of the authors of the study (GA). We avoided histopathological examination because the pain that results from the biopsy procedure can change the patterns of chewing and salivary pooling. We therefore followed the standard protocol for clinical diagnosis by Khanna and Andrade.[15]

Those previously operated on for OSMF and those who had OSMF but were not currently chewing gutkha, were excluded. Patients were informed only during the examination to avoid influencing the site of salivary pooling. They were given a detailed explanation about the placement of gutkha quid and sites of salivary pooling in their native language. Salivary pooling was described as the collection of pooled saliva under pressure in one part of the oral cavity during the process of chewing, and that the surface where this occurred contained a high concentration of the gutkha ingredients. For convenience we divided the oral mucosal surfaces into 6 categories: right buccal mucosa, left buccal mucosa, tongue, lip, floor of the mouth, and back of the mouth.

Each patient (holding the gutkha quid for 5 min) was shown the 6 areas in a healthy volunteer and questioned about where they placed the quid and about the surfaces affected by salivary pooling. These were recorded by one of the authors (GA) and a panel of 3 examiners (medical physiology staff) examined patients repeatedly to assess the sites. Histopathological examination of all the surfaces involved in this large group was beyond the scope and aims of the study.

We recorded the patient's age and occupation, and recorded isolated cases of OSMF separately with the site where quid was placed and surfaces where saliva pooled. Any secondary malignant changes in the mucosal surfaces were confirmed histopathologically and recorded separately for each site involved.

Statistical methods

The nominal (categorical) data were analysed and assessed for association between the site where quid was habitually placed, site of salivary pooling, and clinically affected area. The chi-square test with Yates' continuity correction for 2 × 2 contingency tables was used to analyse association between groups. The chi square test is an approximate test, and Yates' correction for continuity should make it more exact, specifically in small samples. It makes the result more conservative, which is thought to be beneficial because of the interdependence of the cells in a 2 × 2 table. The correction subtracts 0.5 from the difference between each served and expected value and therefore reduces the size of the test statistic and increases the probability value. While its use for large samples makes little difference to the outcome of the chi square test, it has been applied here to show that significant associations were identified with the most conservative application of the test. Probabilities of less than 0.05 were considered significant. Data were analysed using SPSS Statistics for Windows (version 20.0, IBM Corp, Armonk, USA).

Results

A total of 174 men, mean (SD) age 26.0 (8) years were included. Quid was most commonly placed in the left buccal mucosa (79/174, 45%) followed by the right buccal mucosa (57/174, 33%), the lower labial vestibule (23/174, 13%), ventral surface of the tongue or lingual sulcus (8/174, 5%), and anterior two-thirds of the tongue (7/174, 4%) (Table 1). While salivary pooling occurred mostly at each placement site, other sites were also found (Table 2).

The clinical diagnosis of OSMF followed the pattern of salivary pooling in all patients (Table 1) and showed a significant association ($p < 0.001$). All 22 cases of isolated OSMF were associated with areas where the quid was placed and where the saliva pooled, which indicated that the site of chewing site was a primary site of OSMF (Table 3).

In 21 patients (12%) who were concerned about their appearance and needed communication skills as part of their occupation (including doctors and software engineers) we often found salivary pooling and OSMF in the posterior surface of the oral cavity. A total of 5 patients presented with histologically confirmed malignant transformation.

Table 1: Association between occurrence of oral submucous fibrosis (OSMF) and site where quid is placed, and site of salivary pooling. Data are number (%).

Mucosal surfaces	Site where quid is placed (one/patient) (*n* = 174)	No. of patients with each site of pooling (multiple sites in most patients)	Submucous fibrosis (*n* = 174)		Chi square value with 1 df*	*p* value	Chi square value with 1 df*	*P* value
			Site of pooling	Without pooling				
Right buccal mucosa	57 (33)	115	115	0	169.57	<0.001	169.49	<0.001
Left buccal mucosa	79 (45)	146	143	0	147.32	<0.001	169.99	<0.001
Tongue	7 (4)	43	39	0	147.97	<0.001	98.56	<0.001
Lip	23 (13)	33	25	0	118.66	<0.001	165.39	<0.001
Floor of the mouth	8 (5)	22	17	0	121.56	<0.001	151.95	<0.001
Back of mouth	–	73	70	9	125.84	<0.001	–	–

*Degree of freedom.

Discussion

Our results show that the sites where areca nut is chewed and saliva collects are important factors in the distribution of OSMF. Particles of gutkha are reduced in size by chewing and softened by saliva. The effects of salivary pooling depend on the permeability and absorptive capacity of the oral mucosa, which is not generally considered to be an effective barrier to the penetration of substances.[16] Its permeability is related to the thickness and degree of keratinisation and from the sublingual to the buccal and palatal mucosa, permeability decreases.[16,17]

Table 2: Placement of quid and sites of salivary pooling.

Placement of quid	No. of patients (*n* = 174)	Site of pooling						
		Right buccal mucosa	Left buccal mucosa	Tongue	Lip	Floor of mouth	Back of mouth	Total
Right buccal mucosa	57	57	44	6	11	4	20	142
Left buccal mucosa	79	43	79	4	10	5	34	175
Tongue	7	4	7	7	1	3	7	29
Lip	23	7	9	23	5	2	8	54
Floor of the mouth	8	4	7	3	6	8	4	32
Total	174	115	146	43	33	22	73	432

Table 3: Isolated oral submucous fibrosis (OSMF), placement of quid, and salivary pooling.

Isolated OSMF	No. of patients	Placement of quid	Salivary pooling
Left buccal mucosa	14	Left buccal mucosa	Left buccal mucosa
Right buccal mucosa	6	Right buccal mucosa	Right buccal mucosa
Lip	2	Lower lip	Lower lip, left buccal mucosa

As the buccal mucosa is less permeable than the sublingual mucosa, it is a favourable route of sustained delivery of gutkha ingredients. It is also the most convenient site to place the gutkha,[16] and can therefore be considered the most vulnerable surface for OSMF. The rapid absorption of gutkha in the lingual sulcus results in an early loss of its taste so the site might not be preferred by the patient subconsciously.[16] Placing betel quid in other sites than the buccal mucosa may hinder speech and interestingly, patients who preferred these less common sites (lip, tongue, and floor of the mouth) had occupations that required little use of speech (garage workers, drivers, night duty watchman, and carpenters). In those with occupations that required a good appearance and verbal communication skills, saliva pooled in the posterior surface to mask the appearance of chewing, and in them OSMF affected the soft palate, and facial and retromolar tissue.

In our study, fibrosis most commonly affected the site where the quid was placed and was also more severe (in terms of number of fibrotic bands, burning sensation, erosion and fragility) than in other areas. It is likely that the pool of saliva containing concentrated gutkha grains initiates an early fibrotic reaction.

However, OSMF was also found where salivary pooling was absent, but these patients gave an earlier history of salivary pooling in these areas before fibrosis had occurred. It could be speculated that the pattern of pooling changed when the mucosal surface became fibrosed, which would reduce the absorption of the quid, and the change continued until all the oral surfaces had been affected by OSMF.

There was no isolated fibrosis in the mucosa of the upper lip, but fibrosis was always preceded by OSMF of the lower lip. This may be caused by gravity, which makes it a favourable site to place the gutkha, and the upper lip may be spared because it is protected by the upper incisors when present.

Conflict of interest: We have no conflicts of interest.

Ethics statement/confirmation of patient permission: Institutional research ethics committee (Human) of the medical college and hospital.

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Chapter 7

Summary, Conclusions and future perspectives

Summary

This thesis is based on studies that evaluated the role of copper concentrations in drinking water in the pathogenesis of oral submucous fibrosis (OSMF), and also the role of saliva pooling in the sporadic incidence of OSMF on oral mucosa. The research was conducted in the northeastern region of Karnataka, India through the support of endowment grants by the British Association of Oral and Maxillofacial Surgery (BAOMS), London, UK under the supervision of Professor Matthias A.W. Merkx from Radboud University of Nijmegen, The Netherlands, and Professor Peter A. Brennan from the University of Portsmouth, UK. The undertaken research is premised on the fact that OSMF affects only few areca nut-chewing individuals, who exhibit only sporadic incidences irrespective of the chewing site in the oral cavity. The hypothesis was that raised concentrations of systemic copper, caused by the regular consumption of drinking water that contains high concentrations of copper ions, plays an important role in the pathogenesis of OSMF. It was also postulated that the pattern of salivary pooling affects the distribution of OSMF.

The study objectives were:

- To review the existing literature on OSMF, and discuss the various aspect of the condition
- To hypothesize possible mechanisms of OSMF development through dietary copper
- To evaluate the possible role of copper in drinking water in the pathogenesis of OSMF through a pilot study
- To assess the relationship between copper in drinking water and OSMF through a prospective case control study
- To analyze salivary pooling as a contributory factor in OSMF pathogenesis, and to assess its role in the pattern of the disease distribution

Chapter 1 presents the rationale of the study. Oral sub mucous fibrosis (OSMF) is a devastating condition of the oral cavity causing progressive scarring of the oral mucosa, which results in progressive reduction of mouth opening. Eventually, patients experience difficulties in swallowing, which then severely affect body nutrition. As the disease progresses, it may cause speech & hearing defects as well as defective gustatory sensations. The fibrous tissue in the faucial pillars diverges from a slight submucosal accumulation in both pillars to a dense fibrosis extending deep into the pillars, with strangulation of the tonsils. It is this dense fibrosis involving the tissue around the pterygomandibular raphae that causes varying degrees of difficulty in mouth opening. Until recently, it was thought to be locally restricted to the Indian subcontinent, China and other regions of South East Asia, but is now considered to be of global importance due to large numbers of migrant populations also demonstrating the condition. Moreover, the incidence of OSMF is on the rise in a younger age group in India, thereby spreading through in India in a rapid and seemingly uncontrollable manner. It is considered as a pre-malignant stage of oral cancer and reported as a significant risk of malignant transformation (7.6%).

Although six decades have passed after the condition was first described, there is still no apparent cure that has been proven as effective beyond doubt. In spite of relentless research, there are ongoing controversies regarding pathogenesis, and certain aspects of existing treatment modalities still need to be considered as questionable. This may be due to the fact that the disease is complex and multifactorial in origin. Existing research data can currently explain only some aspects of this multifaceted condition, and there may be many other factors which are presently overlooked and need to be elucidated.

Without doubt, OSMF is a crippling disease albeit without immediate life-threatening consequences. However, it does pose a great long-term threat as large segments of society could be afflicted by oral cancer in the future.

As a result of the above mentioned considerations, the following research questions were formulated:

- Why does OSMF frequently progress even after the cessation of the areca-chewing habit?
- Why does OSMF recur even after medical therapy, and in the absence of areca -chewing?
- Why is the condition of OSMF predominantly witnessed among populations with low socioeconomic status, and geographically distributed across a belt of developing countries?
- What is the extent of the possibility that the copper in the areca nut may be participating in the pathogenesis of OSMF?
- If the copper contained in the areca nut is the primary cause of OSMF, why are not all areca nut-chewers affected by the condition?
- Is there any possibility of oral mucosa being exposed to copper through a systemic route?
- Why do some areca nut -chewers develop OSMF in an early phase of their habit?
- What is the reason for the occurrence of OSMF in multiple sites among unilateral chewers?

Chapter 2 reviews the current literature on OSMF. It reviews and discusses all components of OSMF, including the terminology, presentation, aetiology, and pathogenesis, and provides a brief overview of its management. OSMF has been documented

since 1952 and is thought to be multifactorial in origin, with a high incidence in people who chew areca nut; moreover, with a significant malignant transformation rate (7–30%), it poses global challenges for public health. Formerly confined to the Indian subcontinent, it is now often seen in the Asian populations of the United Kingdom, the USA, and other developed countries, therefore presenting a serious problem for global health. There is compelling evidence that the areca nut plays a primary role in the development of OSMF. A range of case–control studies have given convincing evidence that there is a definite dose-dependent correlation between consumption of areca nut and causation of the disease. The onset of the disease is directly proportional to the concentration, incidence, and duration of chewing the nut. The high concentration of copper in areca nut has been found to stimulate lysyl oxidase activity, an enzyme essential to the final cross-linking of collagen fibres. Increased copper has been observed in mucosa affected by OSMF, which supports its role in fibrogenesis by enhancing lysyl oxidase activity. However the hypothesis, loss of equilibrium of extracellular matrix and continuous deposition of extracellular matrix in OSMF is currently well accepted. It seems that changes occuring in the extracellular matrix are likely to play a key role. Nevertheless, oral submucous fibrosis (OSMF) seems to be surrounded by unending enigma. Despite research spanning more than three decades, its pathogenesis is still not fully understood. There is consequently an immediate and urgent need to wisely invest in research to precisely identify the various contributing or promoting factors of OSMF which also affect disease progression and treatment outcomes.

Chapter 3 presents a hypothesis to explain preconditioning of the oral mucosa, which may play an important role in pathogenesis of OSMF. Various hypotheses put forward to date to elucidate the pathogenesis of OSMF have not been proven conclusively, and the precise mechanism of copper-mediated pathogenesis of fibrosis still remains elusive. Most studies on OSMF have focused only on the oral copper availability and its local mucosal action. An equally important second aspect to be considered is the pre-conditioning of the oral mucosa by a prolonged, chronic copper exposure. The results of existing studies in the literature raise the questions of why the disease progresses even after the cessation of the areca-chewing habit, and why there is a recurrence even after medical therapy and in the continued absence of areca chewing. This present study postulates that the chronic exposure to a diet containing a high-normal copper concentration (through food or copper-contaminated potable water) predisposes the oral mucosa to OSMF. This was explained through two overlapping pathways, namely the alteration of normal salivary copper concentrations by increased secretion, and the preconditioning of the oral mucosa through chronic exposure to salivary copper. Mucosal fibrosis may be initiated when the preconditioned oral mucosa face additional copper challenges from areca nut-chewing. The presence of additional factors (such as systemic factors, or other local factors like chillies) might determine the onset and its severity. Once the disease is established, the tissue suffers from hypoperfusion due to fibrosis. Subsequently, the copper clearance may be further delayed in the tissue, thereby increasing the copper concentration in the local tissue, causing progression of the fibrosis. The condition enters a vicious cycle when the mouth opening reduces progressively, subsequently predisposing the patient to psychological as well as physical stress and further fibrosis. To test the hypothesis, a careful investigation of the copper concentration in drinking water (community and home) and food is recommended. The concentrations can be correlated with serum and saliva copper levels. Additionally, serum ceruloplasmin may validate the systemic predisposition.

Chapter 4 describes a pilot investigation to assess whether copper in drinking water plays a role in the pathogenesis of OSMF. The pilot sample was comprised of 100 individuals from the Yadgir district of Karnataka in India, where the study was conducted. This included 50 patients who were clinically and histologically diagnosed with OSMF, and therefore served as the study group. For the control group, 50 healthy people matched by age and sex, were recruited. Both groups were tested for copper concentration in serum and saliva. Copper ion concentration in drinking water was measured using atomic absorption spectroscopy and intelligent nephelometry technology. The mean (SD) concentration of copper in the home drinking water of patients with OSMF was significantly higher (764.3 ± 445.9 µmol/L) than in the controls (305.7 ± 318.5 µmol/L) ($p < 0.001$). Patients with OSMF also had significantly higher copper concentrations in serum and saliva, and serum ceruloplasmin than controls ($p < 0.001$). The data showed a positive association of copper in drinking water with OSMF. It raised the possibility that the high-normal copper in drinking water may contribute to the development of OSMF, and adds to that level of ingestion when areca nut is chewed. Noticeably, eight volunteers (16%) in the control group had raised copper concentrations in their drinking water, serum, and saliva. It was postulated that they might have a high risk of developing OSMF if they started to chew areca nut, but further work was recommended to understand this association in a large heterogeneous population, and in order to ascertain whether the amount of consumed water is proportional to the total copper concentration in serum, or whether it has a lesser role than the copper contained in the areca nut.

Chapter 5 covers a prospective case control study conducted in response to significant results of the pilot study, which provided evidence that copper ions in drinking water play a role in the pathogenesis of OSMF. The association was further investigated in a large heterogeneous population from the Hyderabad- Karnataka (involving 6 districts) region of Karnataka, India. The study evaluated 3 groups, each consisting of 100 patients: those with OSMF who chewed gutkha, those who chewed gutkha but did not have OSMF, and healthy controls who did not chew gutkha. All three groups were subjected to investigations for copper concentration in serum, saliva and also copper concentration in drinking water. This was the first study to

evaluate serum and salivary copper concentrations in people who chew gutkha but do not have OSMF, which are likely to be the best controls in research into this condition. The mean concentration of copper in water was higher (388.53 ±229.23 µmol/L) in the OSMF group than in both the other groups. The concentrations of copper in drinking water were lowest in the non-OMFS group (203.82± 82.80 µmol/L). Significant differences between the groups were also found in mean concentrations of serum copper, salivary copper, and ceruloplasmin ($p < 0.001$). Serum copper and ceruloplasmin concentrations were higher than normal in the OSMF group (37% and 35%, respectively) and the control group (19% and 17%, respectively). All non-OSMF areca nut chewers had values within standard limits for serum ceruloplasmin and for concentrations of copper in serum and water, but they all had higher-than-normal concentrations of copper in saliva (n=100). There was a positive correlation between copper concentrations in water and serum and salivary copper in the OSMF group, and the control group. The study conducted on a larger series of 300 patients has confirmed the significant association between OSMF and copper in drinking water, which supported the concept of "mucosal pre-conditioning". Chronic exposure to high-normal concentrations of copper in drinking water might alter the normal threshold for its absorption, change the cellular resistance to copper, and predispose the oral mucosa to OSMF. When exposed to the additional copper in areca nut, the cumulative effect could compound the condition. It was also found that concentrations of salivary copper were higher than normal in both groups that chewed gutkha (85% of the OSMF group and all those in the non-OSMF group), which suggests that copper from the gutkha or areca nut constantly leaches into the saliva, but that the oral mucosa responds differently. It may be possible that the oral mucosa in patients with OSMF may have been preconditioned by chronic raised concentrations of copper in water, or that a threshold of total copper is needed to trigger the condition. On the whole, this large study showed a positive correlation between the incidence of OSMF and concentrations of copper in drinking water. It also provided a hint that locally available copper in saliva might not be the only cause of OSMF but may affect sensitized mucosa. This could explain why some people who constantly chew gutkha or areca nut do not develop the condition.

Chapter 6 demonstrates a study with the objective of assessing the role of saliva pooling in sporadic pattern of distribution of OSMF in the oral cavity. The studies from previous chapters provided sufficient data about mucosal preconditioning and its vulnerability to OSMF when exposed to areca nut challenge. However, the phenomenon of preconditioning was less instructive in explaining the incidence of OSMF in multiple sites among unilateral chewers, and why some surfaces are not affected. Hence the study was aimed to elucidate the reason for the diverse distribution of OSMF. It was postulated that prolonged exposure of the oral mucosa to saliva containing dissolved products of the quid, which occurs when the saliva pools in a specific area, could cause of fibrosis. It could therefore be speculated that the pattern of

pooling affects the distribution of OSMF. The study included 174 OSMF patients of Yadgir district of the Hyderabad–Karnataka, India. Each patient (holding the gutkha quid for 5 min) was shown the 6 areas in a healthy volunteer, and questioned about where they placed the quid and about the surfaces affected by salivary pooling. These were recorded by one of the authors, and a panel of 3 examiners (medical physiology staff) examined patients repeatedly to assess the sites. The sites where gutkha was chewed and where the saliva was pooled, were recorded to find out whether these were associated with the site of OSMF. The clinical diagnosis of OSMF followed the pattern of salivary pooling in all patients and showed a significant association ($p < 0.001$). Isolated OSMF were associated with areas where the quid was placed and where the saliva pooled, which indicated that the chewing site was also a primary site of OSMF. Results showed that the sites where areca nut is chewed and saliva collects, are important factors in the distribution of OSMF. It is likely that the pool of saliva containing concentrated gutkha grains initiates an early fibrotic reaction. Furthermore, it was speculated that the patterns of pooling change when the mucosal surface fibroses, which would reduce the absorption of the quid, with the change continuing until all the oral surfaces had been affected by OSMF. It can therefore be inferred that OSMF develops when the preconditioned mucosa is faced with the challenge presented by copper through saliva pooling.

Conclusions

The results of the thesis supported the hypotheses that: (1) Copper in drinking water is associated with pathogenesis of OSMF, (2) Copper in the areca nut is not the only cause of mucosal fibrosis, and (3) Saliva pooling is responsible for the sporadic incidence of OSMF on different oral mucosal tissue. Based on the results obtained in the variously performed studies, the following overall conclusions can be made:

1. OSMF is a crippling disorder that is considered to be multifactorial in origin. With a high incidence in people who chew areca nut, and a significant malignant transformation rate (7–30%), it poses a global challenge to public health.
2. The literature review on OSMF identified the copper content of areca nut as the primary mediator of fibrosis because of upregulation of lysyl oxidase in both tissue biopsy specimens and OSMF fibroblasts
3. Most studies on OSMF have focused only on the oral copper availability and its local mucosal action. An equally important second aspect to consider is the pre-conditioning of the oral mucosa by a prolonged, chronic copper exposure through diet.
4. Mucosal pre-conditioning may occur due to chronic exposure to sublethal copper diet (food, potable water), which predisposes the oral mucosa to OSMF.
5. The association of copper in drinking water with pathogenesis of OSMF as a co-factor is confirmed by the results of both pilot and prospective studies.

6. As suggested by the results of both pilot and prospective studies, it is possible that raised concentrations of systemic copper caused by the regular consumption of water that contains high concentrations of copper ions, plays an important role in the pathogenesis of OSMF. Chronic drinking or ingestion of copper may alter the normal threshold for the absorption of copper by the gut, and thereby alter the limits of metabolic tolerance to copper. When exposed to additional copper through the areca nut, the cumulative effect could compound OSMF.
7. The data also confirmed the presence of copper-preconditioned individuals who had raised copper concentrations in their drinking water, serum, and saliva. It can be postulated that they might have a higher risk of developing OSMF if they started to chew areca nut.
8. Serum and water values of non-OSMF areca nut-chewers were within standard limits. But salivary copper concentration was found to be consistently higher in all individuals. The data derived from this novel investigation confirmed the presence of co-factors other than areca nut, which are also necessary for the development of OSMF.
9. The results provided evidence that the contamination of drinking water with copper plays a promoting role in pathogenesis of OSMF. This may be one reason why OSMF is typically found in low-socioeconomic populations who are exposed to contaminated water, and it also explains the high incidence of OSMF in developing countries, where standards and regulations for the quality of drinking water are low.
10. Salivary pooling was described as the collection of pooled saliva under pressure in one part of the oral cavity during the process of chewing, and that the surface where this occurred contained a high concentration of the gutkha ingredients. The prolonged exposure of the oral mucosa to saliva containing dissolved products of the quid, which occurs when the saliva pools in a specific area, could therefore be an explanation for the incidence of OSMF in multiple sites among unilateral chewers, and also elucidate why some surfaces are not affected.

Future Perspectives

The results obtained in this thesis show that copper-contaminated drinking water plays a subtle, but crucial role in the pathogenesis of OSMF. A very important lesson derived from these studies was that the diseases were commonly observed among population with low socioeconomic status, who are prone to consume contaminated water. It is therefore likely that additional contaminants in drinking water may also be causing or promoting fibrosis of oral mucosa.

Drinking water is derived from two basic sources: surface waters, such as rivers and reservoirs, and groundwater. All water contains natural contaminants, particularly

inorganic contaminants that arise from the geological strata through which the water flows and, to a varying extent, anthropogenic pollution by both microorganisms and chemicals. In general, groundwater is less vulnerable to pollution than surface waters. Also, the natural impurities in rainwater, which replenishes groundwater systems, are removed while infiltrating through soil strata. But, in developing countries like India, where groundwater is used intensively for irrigation and industrial purposes, a variety of land and water-based human activities are causing the pollution of this precious resource. Pollution of groundwater due to industrial effluents and municipal waste in water bodies is another major concern in many cities and industrial clusters in India. Consequently, through relative industrialization and urbanization, the water pollution of heavy metals has become an issue of concern, especially in view of their toxicity to human and other biological systems. Drinking water may for example be contaminated with copper from water pipes and plumbing fixtures, especially when the ph level of the water is below 7. Copper salts are sometimes purposely added in small amounts to water supply reservoirs in order to suppress the growth of algae. Organic and inorganic compounds of copper have also been used extensively in agricultural pesticides sprays. The element is therefore likely to be more readily available for solution in surface and ground water than its low average abundance in rocks might have implied. The lower concentrations of copper are readily explainable as a result of co-precipitation by oxides or absorption on mineral surfaces. It follows that, much more research is needed to assess the possible role of various other contaminants (e.g. fluoride) found in drinking water for OSMF.

The studies in this thesis assessed a population in north-east Karnataka, and further research is needed to confirm the present findings in various other parts of the country, and also elsewhere on the South Asian (sub-) continent. Hence comparative studies between different states within one country, and between different countries should be considered.

Another aspect requiring improvement is the standardization of the drinking water source in regards to the investigation of copper ion concentrations. In this study, one of the authors collected and refrigerated samples of drinking water (most came from bore holes and wells) from each patient's home. The method was adapted to assess the water source at the primary level and included individuals who had lived and worked in the same area since birth. Those who spent time away from home (including long-distance drivers because they did not use their regular water supply at home) were excluded. As a follow-up step to this research, it is suggested to assess the copper concentration of drinking water at various levels of the community supply, and test its association with regional epidemiological data of OSMF.

This study points to the possibility of copper also influencing dietary sources other than water in terms of disease incidence and progression. A critical laboratory analy-

sis of all regional dietary sources for copper concentrations (eg. food, tea, coffee etc) is recommended.

For the first time, the studies undertaken as part of this thesis evaluated serum and salivary copper concentrations in people who chew gutkha, but do not have OSMF; these are likely to be the best controls in research into this condition. They had normal serum values, and the copper concentration of their drinking water was within normal limits. However, concentrations of salivary copper were persistently higher than normal, which indicates that the copper content of gutkha is not the only cause of oral mucosal fibrosis. Further clinical and laboratory studies of non-OSMF areca-chewers and their tissues are required, including comparisons with OSMF patients and healthy individuals. All laboratory parameters should be studied in non-OSMF areca-chewers and their near relatives, as well as members of the same ethnic group.

The results of the thesis supported the concept of mucosal preconditioning in pathogenesis of OSMF. It is surmised that the effect of areca nut on the oral mucosa may be secondary to the initial preconditioning of oral tissue. Chronic exposure to sublethal concentrations of copper in drinking water might alter the normal threshold for its gut absorption, and might precondition the oral tissues with systemic copper exposure. This may change the cellular resistance to copper, and when exposed to the additional copper present in the areca nut, the cumulative effect could compound the condition. This is an important finding of this study in the context of prevention and treatment of OSMF. The disease incidence can be controlled effectively by preventing preconditioning of oral tissue, which necessitates measures to prevent and cure groundwater quality deterioration. This may also effectively prevent recurrence or disease progression after treatment, which may be because of persistent preconditioning of oral tissues. Hence, a well-designed animal experiment to demonstrate preconditioning of oral mucosa with copper in drinking water is recommended.

Treatment follow-up studies of OSMF that observe the possibility of recurrence or progression, and concomitant monitoring of copper values (in serum, saliva and drinking water) are recommended to demonstrate the role of persistent copper preconditioning in treatment failure.

The potential for the development of squamous cell carcinoma among OSMF patients should also be further studied through investigations of drinking water, serum and saliva copper concentration. It is apparent that in many cases of oral carcinoma, the serum level of copper is found to be elevated, which may be correlated to the copper concentration of drinking water. In the severe stages of OSMF, trismus and burning sensations restrict solid food ingestion. This triggers an increased water intake in patients, which may exceed metabolic tolerance, alter systemic levels of copper, and drive the initiation of malignant change.

There is an obvious need for public awareness regarding water purification. Additionally, there is a dire need for medical and dental training, as well as for the continuing education of dental therapists, government dental and medical officers and dental surgeons working in the developing districts and regions. This may be accomplished through additional instructional courses emphasizing the importance of ‘preconditioning and drinking water’ in preventing and treating OSMF.

Finally, to reduce the incidence of OSMF, high drinking water standards must be maintained. Stringent preventive and curative measures are recommended against the pollution and contamination of groundwater.

Chapter 8

Samenvatting en conclusies en Perspectieven voor de toekomst

Samenvatting

Deze proefschrift is gebaseerd op studies naar de rol van koperconcentraties in drinkwater bij de pathogenese van orale submuceuze fibrose (OSF), evenals de rol van verhoging van koperconcentratie in het speeksel bij de sporadische incidentie van OSF in de orale mucosa (mondslijmvlies). Het onderzoek is uitgevoerd in het noordoosten van Karnataka, een regio in India, met de steun van subsidies van de British Association of Oral and Maxillofacial Surgery (BAOMS, Britse vereniging voor mond- en kaak- en aangezichtschirurgie), Londen, Verenigd Koninkrijk, onder leiding van professor Matthias A.W. Merkx van de Radboud Universiteit Nijmegen (Nederland) en professor Peter A. Brennan van de University of Portsmouth (Verenigd Koninkrijk). De vooronderstelling van het onderzoekswerk is gebaseerd op het feit dat OSF enkel voorkomt bij een klein aantal betelnoot kauwers en verder slechts een sporadische incidentie vertoont, ongeacht het kauwgebied in de mondholte. De hypothese was dat verhoogde systemische koperconcentraties een belangrijke rol speelden bij de pathogenese van OSF. Dit kwam door het regelmatig drinken van water met hoge concentraties koperionen. Ook werd gesteld dat het patroon van koperstapeling in het speeksel vaak de locatie spreiding van OSF beïnvloedt.

De onderzoeksdoelen waren:

- De bestaande literatuur over OSF evalueren en de diverse aspecten van de aandoening bespreken.
- Hypothesen bespreken voor de mogelijke mechanismen van de ontwikkeling van OSF door koper in de voeding.
- De mogelijke rol van koper in drinkwater bespreken bij de pathogenese van OSF aan de hand van een pilot-onderzoek.
- De relatie van koper in het drinkwater en OSF beoordelen met een prospectief patiëntcontrole-onderzoek.
- Koperstapeling in het speeksel onderzoeken als bijdragende factor bij de pathogenese van OSF, en de rol ervan beoordelen bij het distributiepatroon van de ziekte.

In **Hoofdstuk 1** wordt de rationale van het onderzoek gepresenteerd. Orale submuceuze fibrose (OSF) is een ernstige aandoening in de mondholte. Het veroorzaakt een progressieve littekenvorming van de orale mucosa. Dit leidt tot een progressieve beperking van de mondopening. Uiteindelijk krijgen de patiënten functionele problemen met eten en slikken. Dit heeft een sterk nadelige invloed op de voedingsstatus van het lichaam. Naarmate de ziekte voortschrijdt, kunnen er spraak- en gehoorproblemen en een gebrekkige smaakzin ontstaan. Het bindweefsel in de pharynxbogen varieert van een licht submuceuze verlitteking in beide bogen, tot een dichte fibrose die zich uitstrekt tot diep in beide bogen en de tonsillen beknelt. Deze dichte fibrose van het weefsel rond de raphe pterygomandibularis veroorzaakt problemen in diverse gradaties bij het openen van de mond. Tot voor kort werd gedacht dat het een lokaal probleem was en alleen op het Indische subcontinent, China en andere regio's in Zuidoost-Azië voorkwam. Door de grote aantallen migranten die deze aandoening ook hebben, wordt het nu echter gezien als een wereldwijd probleem. De incidentie van OSF stijgt onder de jongeren in India, en verspreidt zich geruisloos en razendsnel door heel India. Het wordt gezien als een premaligne stadium van mondkanker, en er is een significant risico te zien van maligne transformatie (7,6%).

Hoewel er zes decennia voorbij zijn gegaan nadat de aandoening voor het eerst werd aangetoond, is er nog steeds geen duidelijke remedie gevonden die zich onomstotelijk als zodanig heeft bewezen. Ondanks het niet-aflatende onderzoek blijven er controverses bestaan wat betreft de pathogenese en bepaalde aspecten van de bestaande behandelmethoden die nog steeds twijfelachtig zijn. Dit kan komen doordat de aandoening complex en de oorsprong multifactorieel is. Bestaande onderzoeksgegevens kunnen slechts enkele aspecten verklaren van de veelzijdige aandoening, en er kunnen andere factoren meespelen die over het hoofd zijn gezien en nog moeten worden opgehelderd.

OSF is zonder twijfel een invaliderende ziekte. Het is niet levensbedreigend, maar het vormt een grote bedreiging op de lange termijn omdat een groot deel van de bevolking mondkanker kan krijgen.

Als gevolg van bovenstaande overwegingen zijn de volgende onderzoeksvragen geformuleerd:

- Waarom is er vaak een progressie van OSF, zelfs nadat is gestopt met het kauwen op betelnoten?
- Waarom recidiveert de OSF, zelfs na een medische behandeling en zonder dat er nog wordt gekauwd op betelnoten?
- Waarom komt de aandoening OSF zo vaak voor in de lagere sociaaleconomische lagen van de bevolking, en is deze in geografisch opzicht verspreid in de gordel van ontwikkelingslanden?
- Hoe groot is de kans dat koper in de betelnoten een rol speelt bij de pathogenese van OSF?
- Als koper in de betelnoot de hoofdoorzaak is van OSF, waarom zijn dan niet alle betelnoot kauwers getroffen door de aandoening?
- Is er een mogelijkheid op blootstelling aan koper van de orale mucosa via de systemische route?
- Waarom krijgen sommige mensen al in een vroeg stadium OSF als ze beginnen met het kauwen op betelnoten?
- Wat is de reden van de incidentie van OSF op meerdere plekken bij eenzijdige betelnoot kauwers?

Hoofdstuk 2 evalueert de huidige literatuur over OSF. Hierin worden alle componenten van OSF geëvalueerd en besproken, inclusief de terminologie, presentatie, etiologie en pathogenese. Ook geeft het hoofdstuk een kort overzicht van de behandeling. OSF is beschreven sinds 1952. Het zou multifactorieel zijn met een hoge incidentie onder mensen die kauwen op betelnoten, en het significante percentage van maligne transformatie (7–30%) vormt wereldwijd problemen voor de volksgezondheid. Het werd vroeger beperkt tot het Indische subcontinent, maar het komt nu ook vaak voor onder de Aziatische bevolkingsgroepen in het Verenigd Koninkrijk, de Verenigde Staten en andere westerse landen. Het is daarom wereldwijd een serieus probleem voor de volksgezondheid. Er zijn overtuigende bewijzen dat de betelnoot een belangrijke rol speelt bij de ontwikkeling van OSF. Diverse patiënt controle-onderzoeken hebben overtuigende bewijzen geleverd dat er zeker een dosisafhankelijke relatie is tussen het kauwen van betelnoten en het ontstaan van de ziekte. De aanvang van de ziekte is direct evenredig met de concentratie, incidentie en duur van het kauwen op deze noot. De hoge concentratie van koper in betelnoten stimuleert de werking van lysyl oxidase, een enzym dat essentieel is voor de uiteindelijke crosslinking van collageen vezels. Er is een verhoogd gehalte aan koper aangetroffen in slijmvliezen die zijn aangetast door OSF. Dit ondersteunt de rol van fibrogenese bij het vergroten van de activiteit van lysyl oxidase. Wat de basis ook is, een verstoring van het evenwicht in de extracellulaire matrix en de voortdurende depositie van extracellulaire matrix bij OSF zijn momenteel algemeen geaccepteerd. Het lijkt erop dat de veranderingen die optreden in de extracellulaire matrix waarschijnlijk een sleutelrol spelen. Desondanks blijft het raadsel rond orale submuceuze fibrose (OSF) nog steeds bestaan. Ondanks onderzoeken die al meer dan drie decennia omvatten, is de pathogenese nog steeds niet volledig doorgrond. We moeten nu verstandig investeren in het onderzoek, zodat we de diverse factoren exact kunnen identificeren die bijdragen aan het ontstaan van OSF of de ontwikkeling ervan stimuleren, en die ook van invloed zijn op de ziekteprogressie en de behandelresultaten.

Hoofdstuk 3 geeft een hypothese om de preconditionering van de orale mucosa uit te leggen, welke een belangrijke rol kan spelen bij de pathogenese van OSF. Diverse hypothesen die tot nu toe naar voren zijn gebracht om de pathogenese van OSF op te helderen, zijn nog niet als zodanig bewezen. De exacte rol van koper bij de pathogenese van fibrose blijft nog steeds onduidelijk. De meeste onderzoeken naar OSF hebben alleen nadruk gelegd op de orale beschikbaarheid van koper en de lokale inwerking op de slijmvliezen. Een tweede aspect dat net zo belangrijk is en ook moet worden bekeken, is de preconditionering van de orale mucosa door een langdurige, chronische blootstelling aan koper. Resultaten van bestaande studies in de literatuur roepen de vraag op waarom er vaak een ziekteprogressie is, zelfs nadat de gewoonte om op betelnoten te kauwen is gestaakt, en waarom er recidieven zijn zelfs na een medische behandeling en er niet meer op betelnoten wordt gekauwd. In het huidige onderzoek wordt gesteld dat de chronische blootstelling aan de in-

name van een sublethale koperconcentratie (via voeding, koper in drinkwater) de orale mucosa gevoelig maakt voor OSF. Dit werd verklaard aan de hand van twee overlappende paden, namelijk wijziging van de koperconcentraties in speeksel door een verhoogde secretie en de preconditionering van de orale mucosa via chronische blootstelling aan koper vanuit het speeksel. Muceuze fibrose kan beginnen als de gepreconditioneerde orale mucosa extra wordt blootgesteld aan koper bij het kauwen op betelnoten. De aanwezigheid van aanvullende factoren (systemische factoren of andere lokale factoren zoals voedsel met chilipepers) kunnen het ontstaan en de ernst bepalen. Zodra de afwijking aanwezig is, is er sprake van hypoperfusie van de weefsels door fibrose. De klaring van koper uit het weefsel kan vervolgens verder worden vertraagd. Hierdoor nemen de koperconcentraties in het weefsel toe, waardoor de fibrose verergert. De aandoening komt in een vicieuze cirkel terecht. De mondopening wordt steeds verder beperkt, waardoor de patiënt vatbaar wordt voor geestelijke en lichamelijke stress en verdere fibrosering. Om de hypothese te testen, wordt een zorgvuldig onderzoek naar de koperconcentraties in drinkwater (binnen de gemeenschap en thuis) en in het voedsel aangeraden. De concentraties kunnen samenhangen met het kopergehalte in het serum en het speeksel. Bovendien kan ceruloplasmine in het serum de systemische predispositie valideren.

Hoofdstuk 4 beschrijft een pilot-onderzoek om te bepalen of koper in het drinkwater een rol speelt bij de pathogenese van OSF. De onderzoeksgroep voor de pilot bestond uit 100 mensen uit het Yadgir-district in Karnataka, India. Aan het pilot-onderzoek namen 50 patiënten deel die klinisch en histologisch waren gediagnosticeerd met OSF. Zij vormden de onderzoeksgroep. Voor de controlegroep werden er 50 gezonde mensen gerekruteerd die qua leeftijd en geslacht overeenkwamen met de onderzoeksgroep. Beide groepen werden onderzocht op koperconcentraties in het serum en het speeksel. De koperionenconcentratie in drinkwater werd gemeten via atomaire absorptiespectrometrie en intelligente nefelometrie. De gemiddelde koperconcentratie in het drinkwater thuis bij de patiënten met OSF was significant hoger (764,3 ± 445,9 µmol/l) dan bij de controlegroep (305,7 ± 318,5 µmol/l) ($p < 0,001$). Patiënten met OSF hadden ook een aanzienlijk hogere koperconcentratie in hun serum en speeksel, en ceruloplasmine in het serum dan de controlegroep ($p < 0,001$). De gegevens toonden een positief verband van koper in het drinkwater en OSF. Het kan dus zijn dat een hoognormaal kopergehalte in het drinkwater bijdraagt aan de ontwikkeling van OSF, bovenop het koper dat vrij komt bij het kauwen op betelnoten. Opmerkelijk genoeg hadden acht vrijwilligers (16%) in de controlegroep verhoogde koperconcentraties in hun drinkwater, serum en speeksel. Gesteld werd dat ze misschien een hoger risico hadden om OSF te krijgen als ze op betelnoten zouden gaan kauwen, maar verder onderzoek werd aanbevolen om het verband te begrijpen binnen een grote heterogene groep en om er zeker van te zijn dat de hoeveelheid water die is gedronken proportioneel is aan de totale koperconcentratie in serum, of dat de rol ervan kleiner is dan de rol van koper in betelnoten.

Hoofdstuk 5 beschrijft een prospectief patiëntcontrole-onderzoek, uitgevoerd als reactie op de significante resultaten van het pilot-onderzoek waarin bewijzen zijn gegeven dat koperionen in drinkwater een rol spelen bij de pathogenese van OSF. Het verband werd nader onderzocht in een grote, heterogene populatie uit de regio Hyderabad-Karnataka (bestaande uit 6 districten) in Karnataka, India. Bij het onderzoek werden 3 groepen bestudeerd, elk van 100 patiënten: degenen met OSF die gutka kauwden, degenen die gutka kauwen maar geen OSF hadden, en gezonde controlepersonen die geen gutka kauwden. Alle drie de groepen werden onderworpen aan onderzoeken op koperconcentraties in het serum, speeksel en het drinkwater. Dit was het eerste onderzoek naar de koperconcentraties in het serum en speeksel van mensen die gutka kauwden maar geen OSF hadden. Zij zijn waarschijnlijk de beste controlepersonen bij het onderzoek naar deze aandoening. De gemiddelde koperconcentratie in het water was hoger (388,53 ±229,23 µmol/l) in de OSF-groep dan in de twee andere groepen. De concentraties van koper in het drinkwater waren het laagst in de groep zonder OSF (203,82± 82,80 µmol/l). Significante verschillen tussen de groepen werden ook aangetroffen in de gemiddelde concentraties van koper in het serum en in het speeksel, en het gehalte aan ceruloplasmine ($p < 0,001$). Concentraties van serumkoper en het ceruloplasmine waren hoger dan normaal in de OSF-groep (respectievelijk 37% en 35%) en de controlegroep (respectievelijk 19% en 17%). Alle kauwers op betelnoten zonder OSF hadden waarden binnen de standaardlimieten voor het serum ceruloplasmine en voor concentraties van koper in het serum en het water, maar ze hadden allemaal hogere koperconcentraties dan normaal in het speeksel (n=100). Er was een positieve correlatie tussen de koperconcentraties in het water, serum en speeksel in de OSF-groep en de controlegroep. Het onderzoek dat werd uitgevoerd onder een grotere groep van 300 patiënten bevestigde het significante verband tussen OSF en koper in drinkwater. Dit ondersteunt het idee van ‘muceuze preconditionering’. Een chronische blootstelling aan hoognormale concentraties van koper in drinkwater kan de normale drempel voor de absorptie aanpassen, de cellulaire weerstand voor koper wijzigen en de orale mucosa vatbaar maken voor OSF. Bij een blootstelling aan extra koper in de betelnoot zou het cumulatieve effect de ziekte kunnen verergeren. Ook werd ontdekt dat de concentraties van koper in het speeksel hoger waren dan normaal in beide groepen die gutka kauwden (85% van de OSF-groep en iedereen in de groep zonder OSF). Dit suggereert dat koper uit gutka of betelnoten zich constant in het speeksel stapelt, maar dat de orale mucosa anders reageert. Het kan ook zijn dat de orale mucosa bij patiënten met OSF is gepreconditioneerd door chronisch verhoogde koperconcentraties in water, of dat er een drempel nodig is voor het totale kopergehalte om de aandoening te triggeren. In zijn geheel toonde deze omvangrijke studie een positieve correlatie aan tussen de incidentie van OSF en de concentraties van koper in drinkwater. Het gaf ook een aanwijzing dat lokaal beschikbaar koper in speeksel niet de enige oorzaak is van OSF, maar wel gevoelig gemaakte slijmvliezen kan aantasten. Dit kan verklaren waarom sommige mensen die constant kauwen op gutka of betelnoten de ziekte niet krijgen.

Hoofdstuk 6 beschrijft een onderzoek met het doel om de rol van speekselophoping te beoordelen in een sporadisch distributiepatroon van OSF in de mondholte. De onderzoeken uit eerdere hoofdstukken gaven genoeg gegevens over muceuze preconditionering en de kwetsbaarheid voor OSF bij blootstelling aan het kauwen op betelnoten. Het fenomeen van preconditionering was echter minder nuttig bij het verklaren van de incidentie van OSF op meerdere plekken onder mensen die unilateraal kauwden, en waarom sommige gebieden niet zijn aangetast. Het onderzoek was dus bedoeld om de reden te verklaren voor de gevarieerde distributie van OSF. Gesteld werd dat een voortdurende blootstelling van de orale mucosa aan speeksel met opgeloste stoffen uit het gutka pruimtabak fibrose kan veroorzaken. Dit treedt op wanneer het speeksel zich ophoopt in een bepaald gebied. Veronderstelt kan dan ook worden dat het patroon van ophoping de distributie van OSF beïnvloedt. Het onderzoek omvatte 174 patiënten met OSF uit het Yadgir-district in Hyderabad–Karnataka, India. Elke patiënt (die 5 minuten lang de gutka in zijn mond hield) kreeg de 6 plaatsen in de mondholte te zien van een gezonde proefpersoon, en de vraag waar ze het pruimtabak hielden en welke vlakken zijn aangetast door speekselophoping. Deze werden vastgelegd door één van de auteurs. Een panel van 3 onderzoekers (medisch team) onderzocht de patiënten herhaaldelijk om de mondholte te beoordelen. De plaatsen in de mondholte waar gutka werd gekauwd en waar de speekselophoping was, werden vastgelegd om te zien of ze een verband hadden met de locaties van OSF. De klinische diagnose van OSF volgde het patroon van de speekselophoping bij alle patiënten, en toonde een significant verband ($p < 0,001$). Geïsoleerde OSF werd in verband gebracht met gebieden waar het pruimtabak was geplaatst, en waar het speeksel zich ophoopte. Dit toonde aan dat de kauwplaats de belangrijkste locatie was van het ontstaan van OSF. De resultaten toonden aan dat de plekken waar betelnoten werden gekauwd en speeksel zich ophoopt belangrijke factoren zijn bij de distributie van OSF. Waarschijnlijk veroorzaakt de ophoping van speeksel met geconcentreerde gutka-korrels een vroege reactie van fibrose. Verder werd gespeculeerd dat het patroon van ophoping verandert bij fibrose van het slijmvliesoppervlak, waardoor er minder stoffen uit het pruimtabak wordt geabsorbeerd. De veranderingen gaan door tot alle orale vlakken zijn aangetast door OSF. Geconcludeerd kan worden dat OSF zich ontwikkelt wanneer gepreconditioneerde slijmvliezen een extra koperblootstelling krijgen via speekselophoping.

Conclusies

De resultaten van de scriptie ondersteunen de hypothesen dat: (1) koper in het drinkwater verband houdt met de pathogenese van OSF, (2) koper in de betelnoot niet de enige oorzaak is van muceuze fibrose en (3) speekselophoping verant-

woordelijk is voor de sporadische incidentie van OSF op verschillende orale muceuze weefsels. Op basis van de verkregen resultaten uit de uitgevoerde onderzoeken, kunnen de volgende algemene conclusies worden getrokken:

1. OSF is een invaliderende ziekte met een multifactoriële oorsprong en een hoge incidentie onder mensen die op betelnoten kauwen. Een significante maligne transformatie (7–30%) veroorzaakt wereldwijd problemen voor de volksgezondheid.
2. Het literatuuronderzoek naar OSF identificeert het kopergehalte van de betelnoot als primaire mediator van fibrose vanwege de opregulatie van lysyl oxidase in weefselbiopten en OSF-fibroblasten.
3. De meeste onderzoeken naar OSF hebben alleen nadruk gelegd op de orale koperbeschikbaarheid en de lokale inwerking op de slijmvliezen. Een even belangrijk tweede aspect dat moet worden beschouwd, is de preconditionering van orale slijmvliezen door een langdurige, chronische blootstelling aan koper via de voeding.
4. Muceuze preconditionering kan optreden door een chronische blootstelling aan een sublethale koperconcentratie in het eetpatroon (voeding en drinkwater) die de orale mucosa predisponeert voor OSF.
5. De relatie van koper in het drinkwater met de pathogenese van OSF als cofactor is bevestigd aan de hand van de resultaten van de pilot-onderzoeken en prospectieve onderzoeken.
6. Zoals gesuggereerd aan de hand van de resultaten van de pilot-onderzoeken en prospectieve onderzoeken is het mogelijk dat een verhoogde concentratie van systemisch koper, veroorzaakt door het regelmatig drinken van drinkwater met hoge concentraties koperionen, een belangrijke rol speelt bij de pathogenese van OSF. Het chronisch drinken of innemen van koper kan de normale drempel veranderen voor de absorptie van koper door de darmen, en de grenzen veranderen van de metabole tolerantie voor koper. Bij blootstelling aan extra koper in de betelnoot kan het cumulatieve effect bijdragen aan OSF.
7. De gegevens bevestigden ook de aanwezigheid van door koper gepreconditioneerde personen die verhoogde koperconcentraties in hun drinkwater, serum en speeksel hadden. Gesteld kan worden dat ze een hoger risico hebben om OSF te krijgen als ze gaan kauwen op betelnoten.
8. De koperconcentratie in serum en water onder betelnoot kauwers zonder OSF waren binnen de standaardlimieten. Maar de koperconcentratie in het speeksel was bij alle personen consequent hoger. Gegevens van dit nieuwe onderzoek bevestigden de aanwezigheid van cofactoren naast de betelnoten die nodig zijn voor de ontwikkeling van OSF.
9. De resultaten bewijzen dat de watervervuiling met koper de pathogenese van OSF mede kan bevorderen. Dit kan een reden zijn waarom OSF vooral voorkomt in de lagere sociaaleconomische klassen, die vaker blootgesteld worden aan vervuild water. Tevens verklaart het de hoge incidentie van OSF in ontwikkelingslanden, waar de normen en richtlijnen voor de kwaliteit van drinkwater niet streng zijn.
10. Speekselophoping wordt omschreven als een verzameling van opgehoopt speeksel onder druk in een bepaald deel van de mondholte tijdens het kauwproces. Het oppervlak van die plaats bevatte een hoge concentratie van de gutka-ingrediënten. De langdurige blootstelling van de orale mucosa aan speeksel met opgeloste producten van het pruimtabak (dit gebeurt wanneer het speeksel zich ophoopt in een bepaald gebied) kan een uitleg zijn voor de incidentie van OSF op meerdere plekken onder unilaterale pruimtabak kauwers en ook waarom sommige oppervlakten niet zijn aangetast.

Perspectieven voor de toekomst

Resultaten van dit onderzoek hebben aangetoond dat drinkwater vervuild met koper een subtiele maar cruciale rol speelt bij de pathogenese van OSF. Een erg belangrijke les is dat de aandoeningen vaak voorkomen onder populaties uit lagere sociaaleconomische klassen, omdat deze vaker vervuild water consumeren. Het is waarschijnlijk dat er nog andere vervuilende stoffen in het drinkwater fibrose van de orale mucosa veroorzaken of de ontwikkeling ervan stimuleren.

Drinkwater komt uit twee basisbronnen: oppervlaktewater, zoals rivieren en bassins, en grondwater. Al het water bevat natuurlijke vervuilende stoffen, vooral anorganische vervuilende stoffen uit de geologische lagen waardoor het water stroomt, en in variabele mate antropogene vervuiling door micro-organismen en chemicaliën. Over het algemeen is grondwater minder kwetsbaar voor vervuiling dan oppervlaktewater. De onzuiverheden in regenwater dat het grondwater aanvult, worden bovendien verwijderd als het door de bodemlagen sijpelt. Maar in ontwikkelingslanden zoals India, waar grondwater intensief wordt gebruikt voor de irrigatie en industriële doeleinden, veroorzaken diverse land- en wateractiviteiten van de mens vervuiling van deze kostbare hulpbron. Vervuiling van het grondwater door industrieel en stedelijk afvalwater in waterbassins is een andere belangrijke bron van zorg in veel steden en industriegebieden in India. Bij een relatieve industrialisatie en urbanisatie is de vervuiling van water met zware metalen daarom een bron van zorg geworden, vanwege de toxiciteit van die stoffen voor mensen en andere biologische systemen. Drinkwater kan vervuild zijn met koper uit de waterleidingen en rioleringen, vooral bij een pH-waarde lager dan 7. Koperzouten worden soms expres in kleine hoeveelheden toegevoegd aan kleine waterbassins om de groei van algen tegen te gaan. Organische en anorganische verbindingen van koper worden daarnaast extensief gebruikt in spuitbussen met landbouwpesticiden. Koper is daarom waarschijnlijk meer beschikbaar voor de oplossing in het oppervlaktewater en grondwater dan het lage gemiddelde gehalte in rotsen zou doen vermoeden. De lagere

concentraties van koper zijn gemakkelijker te verklaren als gevolg van coprecipitatie door oxiden en de absorptie op minerale oppervlakken. Daarom moet er veel meer onderzoek worden gedaan om de mogelijke rol van diverse andere vervuilende stoffen (bijv. fluoride) in drinkwater op OSF te onderzoeken.

De onderzoeken in deze studie zijn gehouden onder de bevolkingsgroep in het noordoosten van Karnataka en nadere onderzoeken moeten deze bevindingen bevestigen voor andere delen in het land en het Zuid-Aziatische continent. Dan kan er een vergelijking worden overwogen van de onderzoeken binnen het land zelf en tussen verschillende landen.

Een ander aspect dat verbetering behoeft, is dat drinkwater standaard wordt onderzocht op de concentratie van koperionen. In dit onderzoek heeft een van de auteurs monsters van drinkwater verzameld en gekoeld bewaard (de meeste monsters kwamen uit putten en bronnen) uit de huizen van de patiënten. De methode was aangepast om de waterbron op primair niveau te onderzoeken, en er namen personen deel die al sinds hun geboorte in dat gebied woonden en werkten. Mensen die een tijd van huis waren geweest, werden uitgesloten (zoals chauffeurs voor lange afstanden, omdat ze niet hun gebruikelijke waterbron thuis hadden gebruikt). Als verdere stap in dit onderzoek wordt geadviseerd om de koperconcentraties in drinkwater te onderzoeken op diverse niveaus van de gemeentelijke watervoorziening, en het verband met regionale epidemiologische gegevens van OSF te testen.

Dit onderzoek wijst op de mogelijke invloed van koper uit een andere voedingsbron (anders dan water) op de incidentie en progressie van de ziekte. Een kritisch laboratoriumonderzoek van alle regionale voedingsbronnen voor koperconcentraties (bijv. voeding, thee, koffie, enz.) wordt aanbevolen.

Voor het eerst wordt in deze studie onderzoek gedaan naar de koperconcentratie in het serum en speeksel van mensen die gutka kauwden, maar geen OSF hadden. Zij zijn waarschijnlijk de beste controlepersonen voor het onderzoek naar deze aandoening. Ze hadden normale serumwaarden, en het kopergehalte in het drinkwater viel binnen de normale waarden. Echter, de concentraties van koper in het speeksel waren consequent hoger dan normaal, wat erop duidt dat het kopergehalte van gutka niet de enige oorzaak is van orale muceuze fibrose. Verdere klinische onderzoeken en laboratoriumonderzoeken van betelnoot kauwers zonder OSF en hun weefsels zijn nodig, inclusief een vergelijking met OSF-patiënten en gezonde mensen. Alle laboratoriumparameters moeten worden onderzocht bij betelnoot kauwers zonder OSF en hun naaste familieleden, evenals leden uit dezelfde etnische groep.

De resultaten van deze studie ondersteunen het concept van muceuze preconditionering in de pathogenese van OSF. De interpretatie hiervan is dat het effect van

betelnoten op de orale mucosa secundair kan zijn aan de eerdere preconditionering van het orale weefsel. Een chronische blootstelling aan sublethale koperconcentraties in drinkwater kan de normale drempel voor de absorptie in de darmen wijzigen, en de orale weefsels kunnen worden gepreconditioneerd door de systemische koperblootstelling. Dit kan de cellulaire weerstand tegen koper veranderen en bij blootstelling aan extra koper in de betelnoot kan het cumulatieve effect bijdragen aan het ontstaan van de aandoening. Dit is een belangrijke bevinding van het onderzoek wat betreft de preventie en behandeling van OSF. De incidentie van de ziekte kan effectief worden gecontroleerd door preconditionering van de orale weefsels te voorkomen. Hiervoor zijn er maatregelen nodig om een verslechtering van de grondwaterkwaliteit te voorkomen en de kwaliteit juist te verbeteren. Dit kan ook recidieven of een progressie effectief voorkomen na een behandeling, die mogelijk door een aanhoudende preconditionering van orale weefsels worden veroorzaakt. Daarom is het aan te raden om de preconditionering van orale slijmvliezen door koper in drinkwater te onderzoeken met goed opgezette dierproeven.

Vervolgonderzoeken met behandeling van OSF waarbij wordt gekeken naar de mogelijkheid van recidieven of progressie en bijkomende bewaking van koperwaarden (in serum, speeksel en drinkwater) zijn aanbevolen om de rol van aanhoudende preconditionering door koper aan te tonen bij het falen van de behandeling.

De mogelijke ontwikkeling van een mondholte carcinoom onder patiënten met OSF moet verder worden onderzocht door de koperconcentraties in het drinkwater, serum en speeksel te meten. Het is duidelijk dat in veel gevallen van orale carcinomen het kopergehalte in het serum is verhoogd, wat verband kan houden met de koperconcentratie in het drinkwater. Bij ernstige gevallen van OSF kan door trismus en een brandend pijnsensatie de inname van vast voedsel beperkt zijn. Hierdoor zal de patiënt meer water drinken, waardoor de metabole tolerantie misschien wordt overschreden, de systemische gehalten van koper worden veranderd en het ontstaan van maligne veranderingen wordt beïnvloed.

Het is duidelijk nodig de overheid bewust te maken van de noodzaak van waterzuivering. Ook moeten er medische en tandheelkundige opleidingen komen en moeten er meer mondhygiënisten, (tand)artsen in overheidsdienst worden opgeleid, die in onderontwikkelde districten en regio's aan het werk gaan. Met onderwijs gericht op het belang van 'preconditionering en drinkwater', kan OSF mogelijk beter voorkomen en behandeld worden.

Tot slot moet de kwaliteitseisen van het drinkwater hoog blijven om de incidentie van OSF te verminderen. Strikte preventieve en curatieve maatregelen worden aanbevolen tegen vervuiling en verontreiniging van het drinkwater.

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