Original Article

Evaluation of relationship between serum homocysteine and Vitamin B12 levels in oral submucous fibrosis patients using chemiluminescence immunoassay

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Abstract

Background: Serum homocysteine (Hcy), a nonessential amino acid, is considered as a helpful indicator of vitamin status for its strong correlation with Vitamin B12. Although Hcy levels in oral submucous fibrosis (OSMF) have been studied, the relationship between Hcy and Vitamin B12 has not been studied yet. This study is the first one to compare and correlate the levels of serum Hcy and Vitamin B12 in OSMF patients.

Materials and Methods: The study group comprised 60 patients. Serum Hcy and Vitamin B12 estimation were done by chemiluminescence immunoassay. These levels in OSMF patients were compared and correlated with corresponding levels in healthy controls.

Results: Hcy levels were elevated in OSMF and were found to be statistically significant ($P = 0.014$) as compared to healthy controls. On the contrary, although Vitamin B12 levels were found to be higher in healthy controls, the difference was statistically nonsignificant ($P = 0.657$). A significant correlation was found ($P = 0.01$) between Hcy and Vitamin B12 in both groups, i.e., decreased Vitamin B12 levels led to elevated Hcy levels but vice versa was not found. No correlation was found between levels of Hcy and the severity of OSMF ($P = 0.806$).

Conclusion: Chronic inflammation in OSMF leads to hyperhomocysteinemia, which may also be seen in cases of Vitamin B12 deficiency and certain systemic disorders. Thus, while serum Hcy could be used as biomarker for OSMF, Vitamin B12 deficiency and certain systemic disorders should be ruled out.

Keywords: Chemiluminescence immunoassay, homocysteine, oral submucous fibrosis, serum, Vitamin B12

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INTRODUCTION

Oral submucous fibrosis (OSMF), first described by Schwartz in 1952, is a collagen metabolic disorder and a chronic premalignant condition of the oral mucosa.[1] Pindborg and Sirsat defined OSMF 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 juxtaepithelial inflammatory reaction followed by fibroelastic change of the lamina propria, with epithelial atrophy leading to stiffness of the oral mucosa and causing trismus and inability to eat."[2]

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Amino acids and their derivatives can be useful disease markers as they reflect the protein metabolism, problems related to dietary uptake and aid in understanding the metabolic derangements that occur during the pathological processes induced by the potentially malignant disorders (PMDs).[3]

Homocysteine (Hcy) found in humans is a nonessential, sulfur-containing amino acid with a molecular weight of 135.2 Da. Hcy is not contained in the protein or DNA, but it is a metabolic intermediary derived from the essential sulfur-containing amino acid – methionine, during its metabolism in the methionine cycle. In plasma, 80% of Hcy is protein bound.[4,5]

Hcy is a valuable indicator for factors such as exercise, coffee drinking, smoking, vitamins and cholesterol. Only a handful of studies have shown an association of serum Hcy in PMDs of the oral cavity and elsewhere, with a few reports showing elevated Hcy levels.[6-8]

Hence, the present study was undertaken to evaluate the serum levels of Hcy and Vitamin B12 in OSMF patients as compared to healthy controls. This study also aimed at assessing the correlation between the levels of serum Hcy and Vitamin B12 in both the groups and to find if there is any correlation between the levels of serum Hcy and the severity of OSMF.

MATERIALS AND METHODS

This comparative study was carried out in the Department of Oral and Maxillofacial Pathology and Microbiology for a period of 1 year, after obtaining approval from the Institutional Ethical Committee and Review Board (MPDC_113/OPATH-27/17).

The study group comprised 60 participants and was divided into two groups. Group 1 comprised 30 patients, clinically diagnosed with OSMF, and grading was done according to the criteria given by Ranganathan *et al*.[9] Group 2 comprised 30 healthy controls.

With regard to the first group, gender matching was done in the second group to avoid the bias. A written informed consent was taken from each of the patient before enrolling in the study.

Diagnostic criteria used for selection of cases

Inclusion criteria included patients between the age range of 20 and 60 years and only those patients who gave consent to participate. Thirty patients clinically diagnosed with OSMF and 30 healthy individuals without any habit of areca nut chewing and tobacco were included in the study. Diseases such as diabetes mellitus, osteoporosis, coronary artery disease, Parkinson's disease, smoking, alcoholism, peripheral vasculitis, deep-vein thrombosis and Alzheimer's disease are known to alter the serum Hcy profile. Hence, patients suffering from such diseases were excluded from the study. Furthermore, patients undergoing any drug therapy capable of altering Hcy levels were not included.

Methodology

Venous blood samples were collected with aseptic precautions using spirit-soaked cotton. A volume of 5 ml of blood was drawn from the antecubital vein (cephalic vein) using disposable syringe, and without any delay, the blood was transferred to the vacutainer. Cold centrifugation (15–17° C) at 4000 rpm was done immediately to separate the serum from the cells, and this separated serum was then subjected to chemiluminescence immunoassay (CLIA) for the estimation of Hcy and Vitamin B12.

This immunoassay is based on the specific binding of an antibody toward the Hcy enzyme conversion product, S-adenosyl-Hcy. The quantification thus achieved is through construction of a standard curve with multiple known concentrations of Hcy calibrators.

Statistical analysis

The statistical analysis was done using Statistical Package for Social Science version 16.0 Software (SPSS Inc., Chicago, IL, USA) by setting the significance value at $P < 0.05$. The difference between the serum Hcy levels in OSMF patients and healthy controls was estimated using independent *t*-test, whereas for Vitamin B12, the difference was estimated using Mann–Whitney U-test. Furthermore, Pearson's correlation test was used to find the correlation between serum Hcy and Vitamin B12 in OSMF patients and healthy controls and also to find the correlation between the levels of serum Hcy and the severity of OSMF.

RESULTS

A total of 60 patients were taken which included 30 clinically diagnosed OSMF cases and 30 healthy controls. Patients between the age range of 20 and 60 years participated in the study. Furthermore, gender matching was done to avoid the bias.

For adults, the biological reference range of serum Hcy is 5–15 μmol/L, whereas for Vitamin B12, the range is 200–900 pg/ml.

In our study, the mean Hcy levels were elevated in OSMF (46.6 ± 26.9 μmol/L) and were found to be statistically significant ($P = 0.014$) as compared to healthy controls (31.1 ± 20.4 μmol/L) [Table 1].

On the contrary, although mean Vitamin B12 levels were found to be higher in healthy controls (187.6 ± 145.5 pg/ml) as compared to OSMF (141.3 ± 89.6 pg/ml), the difference was statistically nonsignificant ($P = 0.657$) [Table 2].

Furthermore, a significant correlation was found ($P = 0.01$) between serum Hcy and Vitamin B12 in both the groups [Table 3], i.e., decreased Vitamin B12 levels led to elevated Hcy levels, but vice– versa was not found; that means, there was a one-way inverse proportional relationship.

However, no correlation ($P = 0.806$) was found between the levels of serum Hcy and the severity of OSMF [Tables 4 and 5].

DISCUSSION

OSMF, a PMD, has a multifactorial etiology although chewing of areca nut and tobacco are chiefly associated with this disorder in the South East Asian populations. It causes significant morbidity and has a malignant transformation rate of about 7%–13%.[10] The nutritional deficiencies such as iron, folates, Vitamin B12 and Vitamin B6 might not play a primary role in the etiopathogenesis, but it could synergize the symptomatology by contributing to epithelial atrophy.[11,12]

OSMF patients either present with the complaint of reduced mouth opening or with burning sensation, which results in difficulty in consumption of normal diet leading to poor nutrition. Deficiency of iron and Vitamin B complex, other trace elements and lipids, could possibly initiate anemia, alter the cell-mediated immunity and generate free radicals and reactive oxygen species from the peroxidation of lipids and induce DNA damage.[13,14] The psychological stress due to burning, pain and reduced mouth opening may lead to reduced intake of food, leading to the nutritional deficiency, consequently leading to an increase in Hcy levels.[15]

Elevated Hcy concentrations are also associated with specific pathological conditions, including cancer development, autoimmune diseases, vascular dysfunction and neurodegenerative disease. Moreover, Vitamin B12, folate and B6 are needed in the Hcy remethylation pathway and transsulfuration pathway.[16]

In earlier studies conducted by Goel *et al*. in 2014[3] and Jaganath *et al*. in 2016,[17] Hcy was determined using high-performance liquid chromatography; but in the present research, CLIA was used, which is much more advanced and sophisticated method used nowadays.

In our study, the mean Hcy levels were elevated in OSMF (46.6 ± 26.9 μmol/L) and were found to be statistically significant ($P = 0.014$) as compared to healthy controls (31.1 ± 20.4 μmol/L). This is in accordance with the studies conducted by Bais *et al*. in 2013[6] and Narang *et al*. in 2014[7] on OSMF patients. In these studies, no healthy controls were taken, and it was observed that serum Hcy level was increased in all the patients irrespective of gender and age.

Moreover, the mean Vitamin B12 levels in our study were found to be higher in healthy controls (187.6 ± 145.5 pg/ml) as compared to OSMF (141.3 ± 89.6 pg/ml); but, the difference was statistically nonsignificant ($P = 0.657$).

These results obtained are in congruence with the study done by Chen *et al*. in 2015;[8] in which the Hcy concentrations and Vitamin B12 levels of oral lichen planus (OLP) patients were measured and compared with the corresponding levels in healthy controls. OLP patients showed a significantly higher mean Hcy level than healthy controls and a lower mean Vitamin B12 level when compared to healthy controls. It was found in their research that OLP patients had a significantly higher frequency of Vitamin B12 deficiency and had an abnormally elevated Hcy level than the control group.

Table 1: Difference between homocysteine levels in oral submucous fibrosis patients and healthy controls

	Independent samples test								
	Levene's test for equality of variances		*t*-test for equality of means						
	F	Significant	*t*	df	Significant (two-tailed) *P*	Mean difference	SE difference	95% CI of the difference	
								Lower	Upper
Hcy									
Equal variances assumed	4.046	0.049	2.525	58	0.014	15.58333	6.17220	3.22833	27.93833
Equal variances not assumed			2.525	53.980	0.015	15.58333	6.17220	3.20871	27.95795

SE: Standard error, CI: Confidence interval, Hcy: Homocysteine

Table 2: Difference between vitamin B12 levels in oral submucous fibrosis patients and healthy controls

Test statistics[a]	
	Vitamin B12
Mann-Whitney U	420.000
Wilcoxon W	885.000
Z	−0.444
Asymptotic significant (two-tailed) *P*	0.657

[a]Grouping variable: Group (OSMF patients and Healthy Controls)

Table 3: Correlation between serum homocysteine and Vitamin B12 in oral submucous fibrosis patients and healthy controls

Correlations		
	Hcy	Vitamin B12
Hcy		
Pearson correlation	1	−0.558**
Significant (two-tailed) *P*		0.000
n (Number)	60	60
Vitamin B12		
Pearson correlation	−0.558**	1
Significant (two-tailed) *P*	0.000	
n (Number)	60	60

**Correlation is significant at the 0.01 level (two-tailed).
Hcy: Homocysteine

Table 4: Mean homocysteine levels of different grades of oral submucous fibrosis

OSMF grade	Mean Hcy levels	n (Number)	SD	Median	Minimum	Maximum
II	45.7333	21	28.35068	40.2000	11.30	98.90
III	49.0500	6	24.56288	46.5000	19.10	93.30
IV	48.4000	3	31.15301	44.3000	19.50	81.40
Total	46.6633	30	26.97017	43.0500	11.30	98.90

OSMF: Oral submucous fibrosis, Hcy: Homocysteine, SD: Standard deviation

Table 5: Correlation between the levels of serum homocysteine and severity of oral submucous fibrosis

Correlations		
	Hcy OSMF	OSMF Grade
HCY OSMF		
Pearson correlation	1	0.047
Significant (two-tailed) *P*		0.806
n (Number)	30	30
OSMF Grade		
Pearson Correlation	0.047	1
Significant (two-tailed) *P*	0.806	
n (Number)	30	30

OSMF: Oral submucous fibrosis, Hcy: Homocysteine

A research conducted by Nacci *et al*. in 2008[18] showed decreased Vitamin B12 levels and elevated Hcy levels in patients with laryngeal cancer. Furthermore, Rasool *et al*. in 2012[19] did a study and demonstrated an inverse relationship between serum levels of Vitamin B12 and Hcy in patients with functional dyspepsia. Likewise, Sun *et al*. in 2012[15] and Lin *et al*. in 2013[20] evaluated an intimate association of deficiency of Vitamin B12 and high blood Hcy level in patients with atrophic glossitis and burning mouth syndrome, respectively.

Another similar study conducted by Wang *et al*. in 2014[21] also showed deficiency of Vitamin B12 and abnormally high blood Hcy levels in patients with antithyroid autoantibodies. Similar results were also found in the researches conducted by Sun *et al*. in 2015[22] and Narang *et al*. in 2016[23] in patients with recurrent aphthous stomatitis and metabolic syndrome, respectively.

According to the current literature search, the relationship between serum Hcy and Vitamin B12 in OSMF has not been studied till now; therefore, this study was undertaken to compare and correlate the levels of serum Hcy and Vitamin B12 in OSMF.

In this study, a significant correlation ($P = 0.01$) was obtained between serum Hcy and Vitamin B12 in both the groups, i.e., decreased Vitamin B12 levels led to elevated Hcy levels, but vice– versa was not found; that means, there was a one-way inverse proportional relationship.

This proves that Vitamin B12 deficiency can indicate hyperhomocysteinemia (elevated Hcy levels), but hyperhomocysteinemia does not necessarily indicate Vitamin B12 deficiency. Elevated serum Hcy levels can be associated with chromosome damage even in the absence of Vitamin B12 deficiency. Moreover, hyperhomocysteinemia can indicate any systemic disease, especially vascular disease or any pathological condition.

Besides, due to skewed distribution of data, our study showed no correlation ($P = 0.806$) between the levels of serum Hcy and the severity of OSMF, which is in consonance to the studies done by Bais *et al*. in 2013[6] and Narang *et al*. in 2014,[7] in which no statistically significant correlation was found when comparing Hcy levels with the clinical staging of OSMF.

Hcy levels >15 μmol/L bring about a copper-dependent oxidative damage to cellular and isolated DNA, as seen in our study. In patients with betel quid chewing habit, the betel nut within the quid acts as a source of high levels of soluble copper, which gets acted on by lysyl oxidase, a copper-dependent enzyme, which is vital for collagen synthesis and cross-linkage of fibers in OSMF. Thus, the increased availability of copper and Hcy doubles the chance of DNA damage.[24]

However, the mechanism of chromosomal damage by Vitamin B12 deficiency might be due to reduced methylation of uracil to thymine, leading to subsequent incorporation of uracil into human DNA.[25]

CONCLUSION

OSMF is a chronic inflammatory disease which results in oxidative stress (oxidative DNA damage) leading to hyperhomocysteinemia which in turn exerts its deleterious effects through induction of acute and chronic inflammation pathways such as endothelial adhesion and leukocyte adhesion.

Thus, chronic inflammation in OSMF leads to hyperhomocysteinemia, which can be used as a potential biological marker for the early detection and treatment of the disease.

However, hyperhomocysteinemia may also be seen in cases of Vitamin B12 deficiency and certain systemic disorders such as cardiovascular disease. Thus, while serum Hcy levels could be used as biomarker for OSMF, Vitamin B12 deficiency and certain systemic disorders should be ruled out.

The present research revealed that OSMF patients have significantly elevated Hcy concentrations and decreased Vitamin B12 levels as compared to healthy controls. Additionally, an interesting correlation was found between serum Hcy and Vitamin B12, that is, a one-way inverse proportional relationship exists between serum Hcy and Vitamin B12.

Furthermore, no correlation was found between the levels of serum Hcy and the severity of OSMF. Hence, studies with equal sample size are needed to reinforce this finding of our study.

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Conflicts of interest

There are no conflicts of interest.

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