Study of Relation between Vitamin D Serum Level and Biomarkers of Human Sinonasal Fibroblast Proliferation in Patient with Chronic Rhinosinusitis with Nasal Polyposis

Ali Reza Lotfi¹, Nikzad Shahidi²*, Tala Pourlak³, Ladan Noori Bayat³

¹Associate professor, Department of Otolaryngology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
²Assistant professor, Department of Pathology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
³Resident of Otolaryngology, Department of Otolaryngology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

Corresponding Author: Nikzad Shahidi, E-mail: nikzadsh@yahoo.com

INTRODUCTION

Chronic rhinosinusitis (CRS) is an inflammatory disease of paranasal sinuses and upper airways for more than 12 weeks and requires mucosal inflammatory evidence for diagnostic establishment (1-2-3-4-5). The prevalence of this disease varies between 2-27% in different parts of the world (6-7-8). It reduces the quality of life and overall physical health of involved patients (9-10-11). The disease is divided into three subgroups of CRS with NPs (CRSwNP), CRS without NPs (CRSsNP) and allergic fungi (12).

CRSwNP is defined as the presence of bilateral polyposis in both middle meatuses of the nose, mostly due to T2 helper cells, and it often involves patients with immune dysfunction (13-14). Mucosal tissue biopsy indicates eosinophilic infiltration in these patients (13-14-15).

Not only Vitamin D has a role in calcium and bone homeostasis, but also it regulates many cellular functions, including anti-inflammatory and immune-modulatory functions (16-17-18).

Vitamin D status is defined properly by evaluating the concentration of serum 25(OH)D level (19-20). The active form of vitamin D is 1,25-dihydroxyvitamin D. 1,25-dihydroxyvitamin D is an immune system modulator, and the lack of it increases the risk of autoimmune diseases. Many epidemiologic studies confirm that serum level of vitamin D less than 20 ng/ml increases the risk of cancers, infectious diseases, cardiovascular diseases and autoimmune diseases.

Fibroblast cells have a complex cytokinetic role in the regulation of the immune system. There is increasing evidence relative role of fibroblasts in the accumulation of inflammatory cells in chronic rhinosinusitis.

Ki67 is a protein that is related to cell cycle. this antigen is a nuclear antigen and is find only in proliferating cells. Ki67 presents during specific stages of cell cycle. It is practical marker of cell proliferation.

Vimentin is a protein that is find in mesenchymal cells and is one of major cytoskeletal parts of mesenchymal cells. Epithelial cells can convert to fibroblasts under certain con-
ditions and Vimentin is a indicator for this conversion. Also this protein is a marker for fibroblast proliferation.

In-vitro studies have shown 1,25VD3 reduces the release of eosinophilic recruiting chemokines from sinonasal fibroblasts in CRSwNP.

Regarding the fact that CRS reduces the quality of life and decreases the overall physical health of patients, and so far no study has been conducted in the region, we decided to investigate the relationship between serum vitamin D level with proliferation biomarkers of sinonasal fibroblasts in patients with CRSwNP and its therapeutic effect.

**MATERIAL AND METHOD**

This case-control study was approved by the ethic committee of Tabriz University of Medical Sciences and written informed consent were obtained from all enrolled subjects. The sinonasal tissue sample was obtained during surgery from control group and patient with chronic rhinosinusitis with nasal polyposis(inflammatory and allergic polyps).

This study was performed in ENT ward of Imam Reza educational hospital of Tabriz University of Medical Sciences during 2017. The case group was collected from patients with sinonasal polyposis admitted for surgery. The control group was collected from patients with non-inflammatory conditions admitted for other sinonasal surgeries such as CSF leak repair, septoplasty, and non-secretory tumors of hypophysis. Inclusion criteria for case group was patients with CRSwNP admitted for polypectomy and nasal surgery, and inclusion criteria for control group was patients with non-inflammatory conditions undergoing endoscopic and non-endoscopic surgery such as cerebrospinal fluid leakage repair and non-secreted tumors of the pituitary.

The exclusion criteria was use of systemic steroids (including long acting steroids), use of antibiotics and immune modulatory agents 14 days before surgery, consuming drugs that accelerate the metabolism of vitamin D, acute upper respiratory tract infections, kidney disease, asthmatic patients, patients with samter triad, digestive diseases, endocrine diseases, bone disease, idiopathic pulmonary fibrosis, cystic fibrosis, ciliary dyskinesia, immune system disorders (multiple myeloma, rheumatoid arthritis, Immune deficieny, etc.), pregnancy, obesity (BMI>30), non-sufficient tissue samples for microscopic evaluation or bleeding and necrosis and inappropriate lamellae quality (inappropriate staining and fixation). Local steroids was ceased at least 14 days before surgery.

**Tissue Processing and Analyze**

Sinonasal tissue biopsies were taken from sinus tissue and dissociated, prepared, and stored as defined by the EPOS-2012. Tissue from a total of 60 patients was used for flow cytometric analysis.

The proliferation of Human sinonasal fibroblast (HSNFs) was evaluated by Ki67 and Vimentin proliferation factors using immunohistochemistry (IHC) method. All blood and pathology samples were reviewed at Imam Reza Hospital’s lab.

Immunohistochemical study of these specimens was performed with two 3 micron cuts using Ki67 and Vimentin monoclonal antibodies. All sections were initially dewaxed and dehumidified in a fresh solution of Citrate HCl Buffer 10MM with pH of 6 for 10 minutes in the microwave.

After cooling at room temperature, they were washed with PHOSPHATE BUFFERED SALIN (PBS) and incubated with anti-Ki67 and anti-Vimentin antibodies at 10% dilution for 1 hour.

The sections were then washed with PBS and incubated with Piotinylated antibodies for 30 minutes followed by Pbs washing and peroxidase-labeled streptavidin incubation for 30 minutes and then washed again with Pbs and adjacent with 3.3 diaminobenzidine hydrochloride chromogene, which results in a brown-colored reaction product. Then the sections were stained with ethyl-green and re-dehumidified and finally covered with lamell. independent sample t-test was utilized to determine significance. For nonparametric data, Kruskal-Wallis followed by Mann-Whitney U test was used. Correlations for parametric and nonparametric data were examined using a Pearson or Spearman correlation analysis, respectively. The p-values less than 0.05 were statistically considered significant. Plasma levels of vitamin D were simultaneously evaluated with other preoperative laboratory tests. Plasma 1,25(OH)2D levels were measured using an enzyme immunoassay (RIA kit, IDS) using 0.5 mL of serum EDTA and heparin plasma, according to the manufacturer’s instructions. The assay’s range was 0 to 210 pg/mL. All data were collected and analyzed using IBM® SPSS® release 23 software.

**RESULTS**

All of 60 patients (30 control and 30 with CRSwNP) had sinonasal tissue biopsy available for fibroblast proliferation analysis. Thirty percent of each group were female (M:F ratio=7:3). The average age for control and CRSwNP patients were 29.8 and 44.9 years respectively. The average BMI of control subjects was 24.5 and 25.4 in the CRSwNP patient. The prevalence of smoking was 10% in the case and 16.7% in control group.

The average serum level of 1,25(OH)2D was 23.1 ng/mL in CRSwNP patients while it was 31.2 ng/mL in control patients (P=0.008, t=-4.35).

The fibroblast proliferation determined by analysis of sinonasal tissue explants flow cytometry. The proliferation of HSNFs was identified by Vimentin and Ki67 staining.

We observed that patients in case group had a higher expression of Ki67 proliferation marker compared to controls, (90% vs 0%, P=0.007).

We also observed higher Vimentin expression (proliferation marker) compared to controls, (100% vs 20%, P=0.009).

An independent sample T-test was used to investigate the relationship between vitamin D level and the proliferation of HSNFs in CRSwNP patients by Vimentin proliferation marker. This indicated a low level of vitamin D in the Vimentin positive group (proliferation of fibroblasts) (P<0.01).
These findings are in line with the Ki67 results demonstrated an inverse correlation between vitamin D level and Ki67 positivity, with regard to One Way ANOVA test (P <0.01).

DISCUSSION
Vitamin D deficiency is one of the major concerns in public health and is exacerbated by obesity, lack of adequate sunlight and excessive consumption of sunscreens. Recent study showed that patients with CRSwNP have lower levels of serum vitamin D than those with chronic rhinosinusitis without nasal polyposis. Comparing the results of this study with previous ones (21, 22, 23), it can be concluded that vitamin D levels have been reduced in patients with CRSwNP. This confirms the immunomodulatory role of vitamin D, which acts upon attachment to vitamin D receptors in the nucleus of cells such as monocytes, macrophages, dendritic cells, and immune cells including B and T cells. The reduction of this vitamin causes inflammation and pathogenesis of the CRSwNP.

Vitamin D deficiency is associated with many upper and lower respiratory diseases (25-26) such as chronic rhinosinusitis with nasal polyps (22-23-24-25-26-27).

Variety of cells have VDR on their surface, including fibroblasts (28-29). The fibroblasts are responsible for the eosinophils recruitment, one of the dominant cells in the inflammatory response and the development of symptoms in patients with CRSwNP (30). The results indicate that these cells were elevated with respect to the Ki67 and Vimentin proliferation markers increase in patients with CRSwNP, consistent with Carroll et al. study, which showed high levels of fibroblasts in patients with CRSwNP (23). These results indicate an increase in fibroblasts in CRSwNP, which are the dominant cells responsible for the production of extracellular matrix in the connective tissue and asthma. These cells also play an important role in immune system regulation, and at least part of the inflammatory response in CRSwNP is due to them, and their number greatly adds to this response.

Further studies on the relationship between vitamin D levels and the proliferation of sinonasal fibroblasts should be undertaken. Therefore, this study evaluates the relationship between vitamin D levels and the proliferation of sinonasal fibroblasts in patients with CRSwNP. The results of this study showed an inverse relationship between sinonasal fibroblast proliferation and systemic vitamin D levels. These results are consistent with the results of studies performed by Rostkowski (34), Ling-Feng (35), Takahashi H (36), and Carroll (23), resulting in an increase inflammatory response in CRSwNP. Further researches on the effects of HSNFs and Vitamin D on CRSwNP pathophysiology are necessary.

The proliferation of fibroblasts is just one way to increase the number of these cells in the target tissue. Fibrocytes are bone marrow-derived and blood-transfused cells that represent a modest mesenchymal differentiation and may migrate into tissues and form fibroblasts (31-32). Also, fibroblasts may be formed by epithelial-mesenchymal transfer (EMT), in which epithelial cells exhibit fibroblast features (33). Recent research suggests that EMT can also play an important role in the pathogenesis of CRS. Further studies are needed to understand all the mechanisms involved in increasing the number of fibroblasts in the CRS.

Limitations and Suggestions
There were some limitations in the current study, including the small sample size and the design of the study. Additionally, there may be changes in HSNF levels based on the anatomical site of the biopsy. Despite these limitations, the current study provides compelling data to evaluate the role of HSNF and Vitamin D in CRSwNP. Further microscopic studies are needed to fully understand the role of vitamin D and its supplementation benefit for patients with CRSwNP.

CONCLUSION
The results of this study showed that vitamin D deficiency is associated with the sinonasal fibroblasts proliferation and, consequently, the development of chronic rhinosinusitis with nasal polyposis. Therefore, vitamin D supplementation may have therapeutic and preventive effects in patients with chronic rhinosinusitis with nasal polyposis.

REFERENCES
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