ISSN: 2581-5989
PubMed - National Library of Medicine - ID: 101738774

International Journal of Dental Science and Innovative Research (IJDSIR)

IJDSIR : Dental Publication Service

Available Online at: www.ijdsir.com

Volume – 4, Issue – 5, October - 2021, Page No. : 134 - 143

Quantitative analysis of serum retinol in oral squamous cell carcinoma - A case control study

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Citation of this Article: Dr.Veda Priya, Dr.Vijay Srinivas, Dr. Anuradha, Dr.Vignatha, "Quantitative analysis of serum retinol in oral squamous cell carcinoma- A case control study ", IJDSIR- October - 2021, Vol. – 4, Issue - 5, P. No. 134 – 143.

Type of Publication: Original Research Article

Conflicts of Interest: Nil

Abstract

Oral squamous cell carcinoma is the most common cancer of the oral cavity with a higher incidence in the tongue, buccal mucosa and the palate. The main etiological factors for the occurrence of the oral squamous cell carcinoma are tobacco smoking, alcohol consumption, micronutrient deficiencies and various other environment factors. Micronutrients though required in small quantities are essential organic compounds to maintain the fundamental functions of the body. Lower levels of vitamin A (Retinol) can be one of the etiological factors for the occurrence of the oral squamous cell carcinoma. Increasing the level of retinol will help in prevention of malignant transformation of potentially malignant disorders like Leukoplakia. Supplementation of retinol and its synthetic derivatives helps in the prevention of the second primary tumors of the oral cavity.

Keywords: Oral Squamous Cell Carcinoma, Micronutrients, Vitamin A, Retinol, Essential organic compounds

Introduction

Cancer is a condition where a normal cell transforms into an abnormal cell with an inherent capacity to divide continuously without any check and finally end up in development of solid tumors or lumps[1]. Cancer development is promoted by some of the infections and non-infectious agents. Environmental pollutants such as chemical, industrial effluents, therapeutic drugs and mutagenic agents including ionizing radiation can increase the incidence of cancer. Some cancers occur due to the habitual induction, such as due to diet, tobacco chewing and smoking, alcohol consumption and exposure to industrial toxins[2, 3].The term oral cancer is used to describe the malignancy which arises from oral tissues. The main etiological factors for oral squamous cell carcinoma are tobacco and alcohol consumption, betel quid, Human Papilloma virus, microorganisms like Streptococci, Candida, Neisseria, Dietary factors, Vitamin and Mineral deficiencies, Compromised Immune status, Environmental pollutants, Occupational exposures and

Heritable conditions [4, 5].The major risk factors for oral squamous cell carcinoma are smoking and alcohol consumption and 10 to 15% are because of micronutrient deficiency.[6,7] The importance of diet and nutrition in oral squamous cell carcinoma has been mentioned in various epidemiological studies. According to the available literature, decreased levels of the Vitamin A can act as one of the etiological agent for the occurrence of the oral and pharyngeal carcinomas. Considering it as preventive supplementation, in combination with Retinol or its synthetic analogues have proven to inhibit the progression of the Oral Leukoplakia[8, 9]. Retinol can be supplemented as synthetic retinoid i.e. 13-Cis- Retinoic acid, which is useful in inhibiting the progression of the oral Leukoplakia and reduces the occurrence of the second primary cancers in patients with head and neck cancers. Reduction in the retinol level can lead to cancer as it is shown in many studies; hence estimation of the serum retinol level can be considered as a diagnostic tool to assess the risk of occurrence of the oral cancer [10, 11, 12]. Vitamin A deficiency is considered as one of the etiological factors for the occurrence of oral squamous cell carcinoma along with other major risk factors such as smoking, tobacco and alcohol consumption. Considering the role of Vitamin A in the squamous cell carcinoma, the present study was undertaken to evaluate the role of vitamin A, which was analysed by measuring serum retinol and serum retinol binding protein level with ELISA method.

Materials and Methods

The present study was carried out in the department of Oral and Maxillofacial Pathology, St. Joseph Dental College, Duggirala, Eluru. The study population and the control group were drawn from the patients attending the outpatient department of Good Samaritan Cancer Hospital, Vangayagudem, Eluru, St. Joseph Dental College and Hospital, Duggirala, Eluru and Surya Hospital, Koppaka, Eluru. The Majority of the subjects were from the nearby villages of Vangayagudem, Duggirala, Pinakadimi and Koppaka.

A total of 120 subjects were included in the study. They were classified into 2 groups. Group-I: Study group contains sixty clinically diagnosed Oral squamous cell carcinoma patients. Group- II: Control Group contains sixty clinically healthy subjects. The importance and need for the study was explained to each individual and after clinical examination, the relevant data was entered into the proforma and an informed consent was obtained from all 120 subjects. 3ml intravenous blood was collected from the subject. The collected blood was allowed to clot in the room temperature. After two hours the blood was centrifuged at 2000-3000 rpm for 15-20 minutes and transferred to the plain vaccutainer and kept at -4^0 c and was subjected to ELISA. The result was considered by optical density of micro plate reader as standard. The standard density as the horizontal, the optical density value for the vertical, obtain the standard curve, then the corresponding density was identified according to the sample optical density value, and multiplied by the dilution multiple.

Results

The values obtained were tabulated and statistically evaluated using independent t test. The mean serum retinol level in squamous cell carcinoma was **5.371±5.325** and in controls it was **25.78±12.14.** The mean difference between the groups was statistical significant. **(P<0.0001****)** (Table: 1, Graph-I)

To know the effect of age on serum retinol level, the mean serum retinol level was compared between age groups of 30-50 (group-I) and 50-70 (group-II) in both squamous cell carcinoma and controls.

In squamous cell carcinoma, the mean serum retinol level in group-I was **5.82± 6.139** and in group-II was **4.846±4.255.** Thus no statistical significance was seen between the groups **(P=0.0781)** (Table: 2, Graph: 2)

In controls, the mean serum retinol level in group–I was **27.13±11.45** and in group-II was **24.26±12.93.** No statistical significance was seen between the groups. **(P=0.05369)** (Table: 3, Graph: 3)

To know the effect of gender on serum retinol level, the mean serum retinol level was compared between males and females in both squamous cell carcinoma and control group. In squamous cell carcinoma, the mean serum retinol level in males was **(5.441±5.717)** and in females was **(5.331±4.63).** Thus no statistical significance was seen between both genders. **(P=0.3554)** (Table: 4, Graph: 4)

In controls, the mean serum retinol level in males was **27.88±11.93** and in females was **22.99±13.33.** Thus no statistical significance was seen between the genders. **(P=0.5611)** (Table: 5, Graph: 5)

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Inference

- Serum retinol level in Oral squamous cell carcinoma decreased when compared with controls.
- There was no influence of age on serum retinol level.
- There was no influence of gender on serum retinol level.

Discussion

Vitamins and minerals are required for growth and protection of human body. The essential vitamins are Vitamin B complex, A, D, K and E. Vitamin A cannot be synthesized by animals but obtained only through the diet in the form of retinol. The body obtains vitamin A from two sources; they are Pre-formed vitamin A such as Retinol and its esterified form Retinyl ester. The other source is Provitamin A i.e. Carotenoids such as β-carotene, α-carotene and β-Cryptoxanthin. Pre-formed vitamin A is found in butter, eggs, animal products and grains. Provitamin A Carotenoids are generally found in pigmented vegetables like carrots, squash and yams[13, 14.] The body converts carotenoids into vitamin A. Both provitamin A and Preformed vitamin A must be metabolized intracellulary to retinal and retinoic acid, the active form of vitamin A to support the vitamins biological functions. Both forms of vitamin A are solubilised into micelles in the intestinal lumen and absorbed by duodenal mucosal cells. They are then converted to retinol which is oxidisized to retinal and then retinoic acid. Most of the body's vitamin A is stored in the liver in the form of retinyl esters. Retinoids are essential for the proper development and maintenance of mucous membrane and surface epithelia. Various studies have shown that the prevention or reversal of carcinogen-induced tissue changes and inhibition of growth of neoplastic cell lines can be fostered by Retinoids. The natural and synthetic retinoids can effectively inhibit the carcinogen-induced hyperplasia, metaplasia and proliferation especially prostate organ cultures and also transformation of fibroblast cultures by chemical and physical transforming agents was inhibited by retinoids[15, 16]

Vitamin A and its analogues comprise a major role in head and neck carcinogenesis. According to the available literature lower levels of serum retinol level is one of the predisposing or etiological factors for oral squamous cell carcinoma. In the present study we have tried to estimate the serum retinol levels in squamous cell carcinoma using ELISA. In this study serum retinol level in oral squamous cell carcinoma patients was comparatively lower than that of controls. Normally the retinol which is present within

the serum is utilized by the epithelial cells and helps in development of the epithelial linings throughout the body. When the vitamin A intake is less than the normal amount, stored vitamin A is utilized to meet the metabolic demands in the body. It results in the retinol insufficiency, which persists for a short duration[17]

If deficiency condition continues for the prolonged period of time, complete depletion of retinol is seen. If the serum retinol level drops there is major impairment in the cellular function which causes abnormal differentiation, physiological consequences and clinical manifestations of deficiency such as anaemia and impaired resistance to infections. We have discerned decreased serum retinol levels in oral squamous cell carcinoma patients, but we are not precise whether the decrease befalled prior to carcinogenesis or whether it has occurred after development of cancer. If it has eventualized after carcinogenesis it can be explained by the fact that there is increased utilization of retinol present in the serum and its reserves by neoplastically transformed leading to deficiency[18, 19].

Lower serum retinol levels associated with head and neck squamous cell carcinoma, lung cancers, gastrointestinal tract cancers and second primary tumors are reported in similar studies[17]. Now the question is how do the cancer cells utilize vitamin A? Cancer cells form high levels of reactive oxygen species (ROS) from increased metabolic activity, mitochondrial dysfunction, peroxisome activity, increased cellular receptor signalling, oncogene activity, increased activity of oxidases, cyclooxygenases, lipoxigenases and thymidine phosphorylase or through crosstalk with infiltrating immune cells. Growth factors such as TNFα, PDGF, EGF, TGFβ and angiotensin and cytokines such as IFNγ and IL-1 stimulate the production of ROS to revert their diverse biological effects in cancer[20].

Two principle mechanisms of action have been proposed for antioxidants. The first is a chain-breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the systems. The second mechanism involves removal of ROS/reactive nitrogen species initiators (secondary anitoxidants) by quenching chain initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms including electron donation, metal ion chelation, co-antioxidants, or by gene expression regulation.

The antioxidant activity of vitamin A and carotenoids conferred by the hydrophobic chain of polyene units can quench singlet oxygen, neutralize thiyl radicals and combine with and stabilize peroxyl radicals. In general, the longer the polyene chain, the greater the peroxylradical stabilizing ability. Because of their structures, vitamin A and carotenoids can autoxidize when Oxygen tension increases, and thus are most effective antioxidants at low oxygen tensions that are typical of physiological levels found in tissues. Vitamin A not only counters free radicals and but also inhibits the C-Kinase activity. C-Kinase is believed to be the membrane receptor for phorbol esters, some hormones, growth factors and oncogene products. It is thought that it mediates the effects of esters upon cellular metabolism. Hence this probably might be the primary mechanism involved in the protective effects of vitamin A and its derivatives against cancer [21, 22].

In contrariety Vitamin A deficiency may precede the inception of cancer. Though not systemically, a local Vitamin A deficiency may occur because of chronic exposure to carcinogens or inflammatory mediators like cytokines[23]**.** The reduced levels could be on behalf of its antioxidant activity or due to mutation in one or more of the key retinoid signalling genes probably endorsed by the same carcinogens. Due to altered gene expression there

could be impaired function of specific receptors on the cell surface and nucleus for the vitamin A complex or its active metabolites such as retinoic acid. This impairment affects the expression of several other genes and subsequently perturbs cell differentiation and vitamin A dependent functions. Few studies have mentioned that retinoids can modify the gene expression through the mediation of the intracellular binding proteins and also the nuclear receptors. Other genes which are indirectly regulated by vitamin A are Hox genes, nur77, TR, VDR, bcl_2, EGFR[19].

Vitamin A and its metabolites help in enhanced immunity by potentiating antibody responses to T- cell dependent antigens, increase lymphocyte proliferation, responses to antigens and mitogens, inhibit apoptosis and restore the integrity and function of mucosal surfaces[28]**.** In case of HPV associated squamous cell carcinomas, HPV-immortalized cells undergo growth inhibition by all-trans-retinoic acid and are associated with reduction in EGFR levels and EGF signalling which suggest that retinoids may affect HPV transcription or replication. Also supplementing vitamin A may increase or prolong the expression of HPV antigens allowing the natural host clearance of HPV-dependent skin lesions. It has been suggested to use a prophylactic dose of systemic retinoid for post-transplant patients at risk of developing squamous cell carcinoma**.**[24] Retinoic acid can induce the cell-mediated cytotoxicity to allogenic tumour cells[25]**.**

In the present study both the study group and control group showed marginally high serum retinol levels in subgroup-I i.e. 30-50 years when compared to subgroup-II i.e. 50-70 years, but the difference was statistically insignificant. Physiologically low retinol levels in older age group could be sequential to low energy intake or low nutrient density of the diet, slower gastric emptying, altered hormonal responses, decreased basal metabolic rate and altered taste and smell. From the results it can be assumed that age related changes in serum retinol level are meagre and may not play a significant role in carcinogenesis.

According to the results of our study, there is no gender related difference in serum retinol. Males showed marginally more concentration of retinol when compared to females, but the difference was not statistically significant. The possible reason for the low levels in females could be due to hormonal disturbances, long standing anaemia, obesity, menstrual cycles and malnourishment [26, 27].

Vitamin A also helps in substantial regression of potentially malignant disorders and certain cancers. This regressive or preventive effect could be due to its ability to interact with highly reactive oxygen radicals to prevent cellular damage. It also enhances immune response and restores the cytotoxic capacity to macrophages and even returns the number of langerhans cells to normal level. It decreases the tumour size by enhancing the immune surveillance[28].

Serum retinol concentration reflects a person's vitamin A status. The general factors which lower the serum retinol level are infections, protein status, adequacy of other nutrients and organ disease. The serum retinol is usually assessed by High Performance Liquid Chromatography (HPLC) or otherwise by the ELISA, spectrophotometry, Fluorometry ELISA and nephelometry. In the present study Enzyme-linked Immunosorbant assay (ELISA), was used to analyse the serum RBP. In this method an enzyme is attached to an antibody that changes the colour of a substrate. The intensity of the colour change depends on the amount of RBP bound. ELISA was preferred because of it is cost effective and much simpler than HPLC. Serum handling is also easier than HPLC because Retinol

Binding Protein is more stable than retinol with respect to light and temperature[29].

Thus a substantial body of evidence from subsequent laboratory and epidemiologic investigations indicates that dietary carotenoids are inhibitors of epithelial carcinogenesis. As in our study inverse associations between vitamin A intake and risk of oral, laryngeal, lung and gastrointestinal tract cancers is established. The results of the study limelight the imperative role of vitamin A in carcinogenesis and maneuvers in tumour progression. Serum retinol acts as an illustration tool in the cancer diagnosis and progression.

Conclusion

The present study was a novel study which tried to assess the levels of the serum retinol level in the oral squamous cell carcinoma. And the levels were compared with the healthy individuals without any systemic illness. The study was attempted to assess the role of retinol concentrations in cancer proliferation and prevention. The results of this study have shown reduction in the level of the serum retinol concentration in the persons suffering from oral squamous cell carcinoma. It may be due to the fact that increased utilization of retinol reserves than the normal during the process of carcinogenesis.

The levels of the retinol concentration in both squamous cell carcinoma and in control group were appeared to be indifferent in our study. Hence it was suggested that there will be no influence related to age and gender in oral squamous cell carcinoma. Based on these results, we conclude that reduction in the serum retinol concentration in oral squamous cell carcinoma may prove that it can be one of the evaluationary tools for assessment of cancer and also aid in the treatment planning as by increasing the serum retinol concentration will help in the prevention of the second primary tumours.

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Legend Figure and Table

Graph 1: Comparison of serum retinol in cases and controls by using unpaired t test

CASE

CONTROL

Table 1: Comparison of mean values in case and control group

Groups	Mean	Sd	F Value Dfn, Dfd	P Value	Highly Significant
Cases (Group-I)	5.371	5.325	5.195 52,51	<0.0001****	
Control (Group-Ii)	25.78	12.14			

Statistical analysis: Unpaired T test. Statistically significant if p <0.05**** - Highly significant

Graph 2: Comparison of serum retinol in Group-I and Group-II cases by using unpaired t test.

Table 2: Comparison between the mean values of cases of Group-I (30-50) and Group-II (50-70) using unpaired t test

Groups	**Mean**	**Sd**	**F Value Dfn, Dfd**	**P Value**	
Sub group-I (30-50)	5.82	6.139	2.082 27,23	0.0781	Not significant
Sub group-II (50-70)	4.846	4.255			

Statistical analysis: Unpaired T test. Statistically significant if p <0.05

Graph: 3 Comparison of serum retinol in Group-I and Group-II controls by using unpaired t test.

Table 3: Comparison between the mean values of controls of Group-I (30-50) and Group-II (50-70) using unpaired t test

Groups	Mean	Sd	F Value Dfn, Dfd	P Value	
Sub Group-I (30-50)	27.13	11.45	1.276,24,27	0.5369	Not Significant
Sub Group-Ii (50-70)	24.26	12.93			

Statistical analysis: Unpaired T test. Statistically significant if p <0.05

Graph 4: Comparison of serum retinol in male and female cases by using unpaired t test.

Table 4: Comparison between the mean values of cases among males and females using unpaired t test

Groups	Mean	Sd	F Value Dfn, Dfd	P Value	
Males	5.441	5.717	1.525, 35,17	0.3554	Not significant
Females	5.331	4.63			

Statistical analysis: Unpaired T test. Statistically significant if p <0.05

Graph 5: Comparison of serum retinol in male and female controls by using unpaired t test.

Table 5: Comparison between the mean values of Controls among males and females using unpaired t test

Groups	Mean	Sd	F Value Dfn, Dfd	P Value	
Males	27.88	11.93	1.249,17,35	0.5611	Not significant
Females	22.99	13.33			

Statistical analysis: Unpaired T test. Statistically significant if p <0.05

Figure 1: Armamentarium for Vitamin A ELISA Method

Figure 2: Armamentarium for ELISA Method. (ELISA Analyzer)

Figure 3: Microelisa strip plate with blank well setted and testing sample well

Figure 4: Armamentarium for ELISA Method. Processing of the sample by setting of the wells on the ELISA analyzer

Figure 5: Final result of Micro plate reader as a standard, reference wavelength is 630nm.

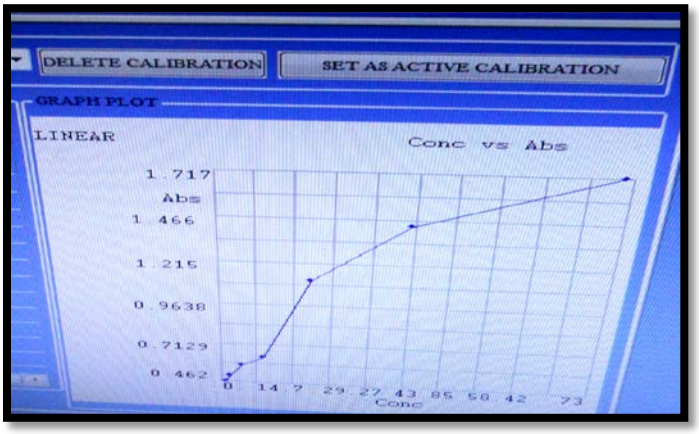

Figure 6: Standard Curve for each set of samples assayed. Standard density on horizontal, the OD value on vertical. Corresponding density obtained according to the sample OD value, and multiplied by the dilution multiple.