AJDS LAZHAR Al-Azhar Journal of Dental Science
Vol. 27- No. 3- 357:366- July 2024
Print ISSN 1110-6751 | online ISSN 2682 - 3314
https://ajdsm.journals.ekb.eg
Oral Biology, Medicine & Surgical Sciences Issue *(Oral Biology, Oral Pathology, Oral Periodontology, Medicine, Oral & Maxillofacial Surgery)*

REVERSING GINGIVAL SYMPTOMS OF VITAMIN A DEFICIENCY BY RED PALM OIL SUPPLEMENTATION

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ABSTRACT

Objectives: To evaluate the influence of red palm oil (RPO) on gingival tissues in rats and compare it to the effects caused by vitamin A deprivation, and to examine the repercussions of vitamin A deprivation specifically on gingival tissue. **Subjects and Methods:** Gingival tissue specimens were excised from male albino rats subjected to vitamin A deprivation (white flour diet) for 60 days. Red palm oil (RPO) was used to alleviate vitamin A deficiency by oral administration of gavage with a dose (2mg/kg/day) for 4 weeks. Serum retinol level was assessed two times after 2 months from the experiment and at the end of the experiment using High-performance liquid chromatography (HPLC). Gingival tissue specimens of control and experimental groups were assessed using Hematoxylin and eosin staining. Collagen synthesis was detected using Masson Trichrome staining. The proliferative capacity was evaluated via an Immunofluorescent Ki-67 antibody (epithelial marker). The results were evaluated statistically. **Results:** Gingival tissues of VAD rats showed the most damaging effect regarding keratin thickness of epithelium, collagen synthesis, and epithelium proliferation. While gingival tissues of RPO rats showed better results approaching those of the control group. **Conclusion:** Gingival tissues are adversely affected by vitamin A deficiency, leading to a statistical increase in keratin thickness and a reduction in collagen synthesis. Conversely, red palm oil alleviates the consequences of vitamin A deficiency by elevating serum retinol levels.

KEYWORDS: Vitamin A deficiency; Gingiva; Red palm oil.

INTRODUCTION

The fruit of the oil palm tree termed (Elaeis guineensis) is a unique source of red palm oil which is rich in antioxidants. This fruit can produce two types of oil, namely palm oil from the fibrous mesocarp (which has a shining, deep orange-red pulp) and palm kernel oil (which looks like coconut oil) from the nucleus. Red palm oil has a special taste and aroma and is rich in phytonutrients such as carotenes (which give the oil a deep orange-red color), phytosterols, vitamin E, squalene, and coenzyme Q10, all possessing nutritional attributes and oxidative stability [(1)].

There is a suggestion that red palm oil (RPO) is an excellent origin for provitamin A and accordingly demonstrates high effectiveness in enhancing vitamin A status and preventing or treating vitamin A deficiency (VAD) disease in populations exposed to the risk of VAD [(2)].

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DOI: *10.21608/AJDSM.2024.283190.1531*

On the other hand, Vitamin A deficiency (VAD) stands as a significant global public health concern. The World Health Organization (WHO) reported that 190 million preschool children faced exposure to VAD worldwide. The prevalence of VAD remained notably high on a global scale in 2019. Insufficient consumption of vitamin A-rich foods emerges as the primary cause of VAD [3,4].

Vitamin A deficiency leads to mucosal issues and degenerative changes in epithelial tissues, causing keratinizing metaplasia. The crucial role of vitamin A in maintaining the epithelium is highlighted, as epithelial tissues serve as a primary barrier against microbial invasion. Experimental studies in animals suggest that a deficiency in vitamin A may result in increased hyperkeratosis and hyperplasia in the gingiva affecting its attachment to the tooth surface, potentially elevating the risk of developing periodontal pockets [5].

The human gingiva plays an important role as a mechanical barrier against bacterial invasion and a part of the innate immune response to infectious inflammation in periodontal tissue [6].

SUBJECTS AND METHODS

The experiment was accomplished in compliance with the guidelines set forth by the Ethics Council, the Faculty of Dentistry, Ain Shams University. The ethical approval code is **(FDASU-Rec IM012242).**

Subjects:

- ***Animals***

Twenty-one adult male albino rats, two weeks old, were utilized in the present research. The animals were kept in wire mesh cages, 7 rats each, in the Medical Research Institute, Faculty of Medicine, Ain Shams University, under the supervision of a specialized veterinarian. Rats were kept under good ventilation, 14 rats were fed only white flour while the rest of them were fed a standardized diet and tap water ad libtium, as revised and confirmed by the institution instructions of Ain Shams University Ethical Council.

100% Pure unrefined Red palm olein oil was purchased in the form of bottles from (Walmart, California, U.S.A); obtained from the manufacturers, and used before the "best before date".

Methods

- ***Experimental design***

The 21 rats were randomly assigned into three groups, with each group consisting of seven rats, as outlined below:

Group I (Control group): Each rat received a normal diet for 60 days.

Group II (VAD group): Each rat received a white flour diet for 60 days to induce vitamin A deficiency[7].

Group III: (VAD+RPO): Each rat received a white flour diet for 60 days and then received red palm oil by oral administration of gavage with a dose (2mg/kg/day) for 4 weeks [8,9].

- ***Serum Retinol Level Assessment***

Serum retinol level was assessed two times, blood samples of 2 ml were withdrawn from the tail vein 2 months after the experiment and at the end of the experiment [10,11].

Determination of serum retinol by HPLC (high-performance liquid chromatography) [12].

- ***Samples preparation***

At the end of the 88-day study period, all rats were euthanized, and gingival tissue was excised and prepared for examination. Half of the specimens underwent processing for H&E examination and were fixed in 10% buffered formaldehyde for 48 hours (buffered in pH 7.2 phosphate buffer saline), followed by washing and decalcification in ethylene diamine tetra-acetic acid. These specimens were then stained with hematoxylin and eosin (H&E)

as well as Masson Trichrome. The remaining half was prepared for examination under an immunofluorescence microscope using a Ki-67 antibody. These specimens were fixed with 4% formaldehyde for 10 minutes at room temperature, washed, permeabilized with 0.1% TX-100/PBS for 15–20 minutes, and washed again.

- ***H&E stain***

Following the decalcification process, tissue specimens underwent rinsing under running water, followed by dehydration using increasing alcohol concentrations, and then were transferred to xylene. Subsequently, the specimens were penetrated, fixed in the central region of paraffin wax blocks, and sectioned utilizing a microtome to have 4 mm thick sections. These specimens were then moisturized and stained with H&E stain [13].

- ***Masson Trichrome***

Sections were mordanting in Bouin's solution at 60°C for an hour, followed by a 5-minute wash in municipal water to eliminate pieric acid. Subsequent steps encompassed the application of Weigert's working hematoxylin for 10 minutes, staining with light green for 5 minutes, and rinsing in pure water. Additional stages involved Biebrich scarlet application, rinsing, treatment with phosphotungstic/phosphomolybdic acid, direct transfer into light green, and a final step of 1% acetic acid immersion for 1 minute, followed by rinsing in distilled water. This process was crucial for preparing the specimens for subsequent examination and analysis [13].

- ***Ki-67 Immunofluorescent stain***

Slides are initially washed in phosphate-buffered saline (PBS) for 5 minutes, and any remaining PBS is carefully eliminated. To prevent drying, the section is kept moist. The slide is then flooded with the primary or conjugated antibody and left to incubate for 30 minutes. Afterward, a 10-minute wash in PBS is done, and the remaining PBS is removed from the slide. This washing step is repeated for an additional 10 minutes. Subsequently, the section undergoes a second-stage conjugated antibody flood with a 30-minute incubation period. Another 10-minute PBS wash follows. Finally, all slides are mounted in buffered glycerol with plate glass and reserved at 4°C until another review [13].

Sections were inspected and shot at 100x and 400x magnifications utilizing a light microscope (Olympus BX 60, Tokyo, Japan).

- ***Morphometric analysis***

The assessment of new collagen area percentage in Masson Trichrome stained sections was conducted using image analysis software (Image J, version 1.53e, NIH, USA). For each rat in the studied groups, five representative fields were selected from each slide, and the area of each field was measured within a standard frame area of 5.04 × 106 μm2 at a magnification of x400 for the gingiva. Following grey adjustment, the picture transformed to a grey outlined picture, featuring a red binary color indicating the percentage area of new collagen. In the case of H&E-stained sections, examination was performed using high-power fields (x400) under a light microscope, and keratin thickness was measured in micrometers at three different points on each slide for each rat in the studied groups.

Statistical analysis

The histomorphometric information underwent analysis using the Statistical System for Social Sciences, version 23.0 (SPSS Inc., Chicago, Illinois, USA). Quantitative data were presented as mean ± standard deviation along with the ranges. Normality checks were produced using the Kolmogorov-Smirnov and Shapiro-Wilk Tests. Significance comparisons between two means were assessed using the independent-sample t-test, while the Mann-Whitney U test was applied for two-group comparisons in non-parametric data. The confidence interval was established at 95%, and the allowed border of error was set at 5%. A p-value of 0.05 or less was considered statistically significant.

RESULTS

H&E, Masson Trichrome, and Statistical results

Histological examination of the gingival tissue of the rats of group II (VAD group) showed histological changes in the control group. The covering epithelium appeared with apparent hyperkeratosis with an apparent reduction in the size and prominence of rete pegs, and the basement membrane appeared less defined. The lamina propria showed an obvious decrease in collagen bundles, thinning of the wall of blood vessels with extravasated blood, and areas of disintegration within the reticular layer of the lamina propria. Statistical analysis revealed a significant difference ($p<0.003$) comparing keratin thickness in group II and the control group.

Histochemical analysis confirmed the histological examination, where Masson Trichrome staining revealed a decreased stained blue new-to-stained red old collagen ratio. Statistical analysis showed a significant difference ($p<0.0001$) comparing the area percent of new collagen in group II that in the control group.

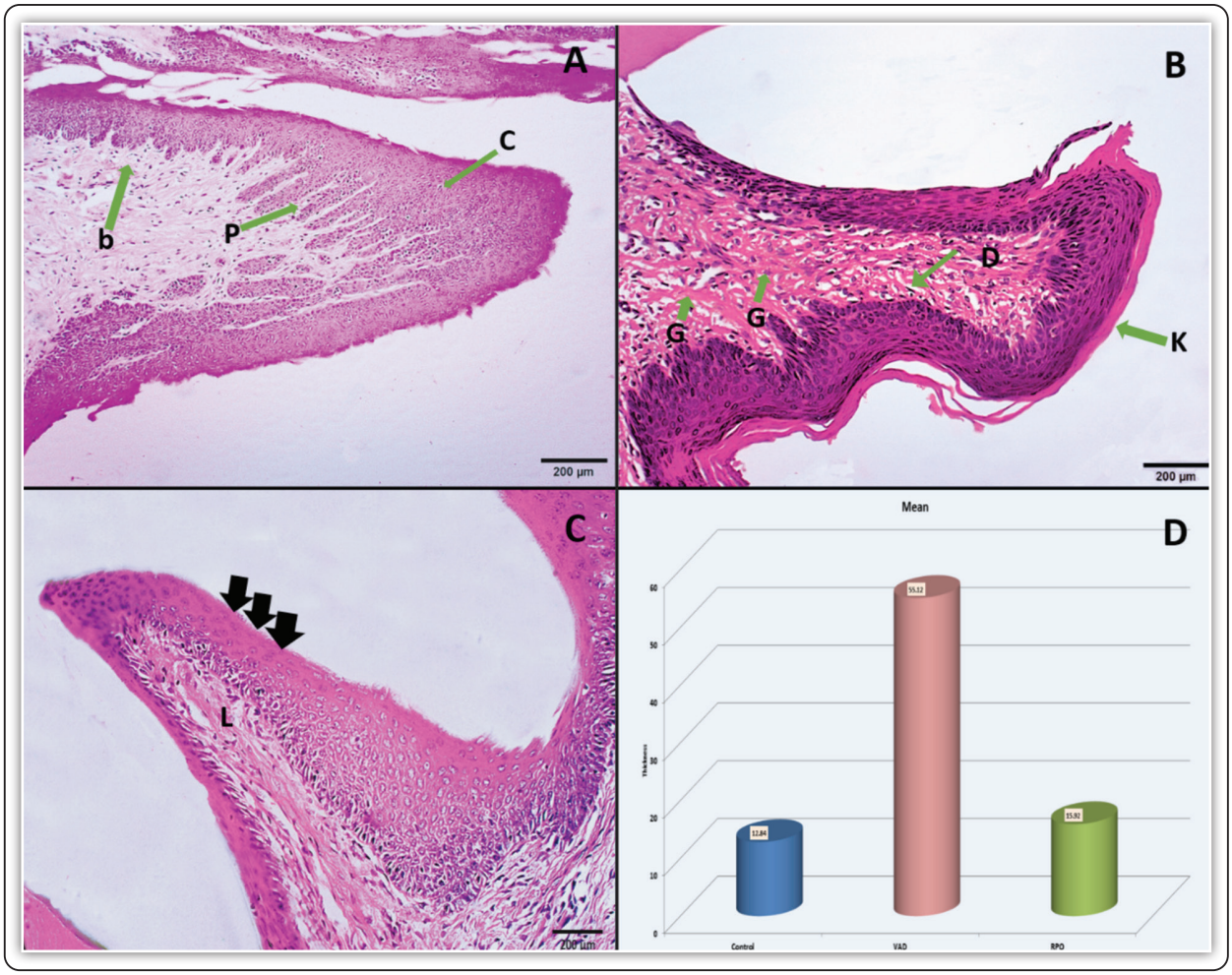

FIG (1) **(A):** A photomicrograph showing gingival tissues in Group I with keratinized stratified squamous epithelium. Regular eosinophilic keratin layer, clear cells (C). long and slender c.t papillae within Dense and fibrous lamina propria (P) and normal blood vessels lining (b). **(B):** a photomicrograph of gingival tissue in Group II showing an increase in thickness of the keratin layer (K). areas of disintegration within the reticular layer of the lamina propria (D), along with irregular collagen bundles(G). **(C):** A photomicrograph of gingival tissue in Group III showed an apparent reduction in the thickness of the keratin layer (black arrowheads), and the lamina propria exhibited a structural arrangement (L). (H&E x100). **(D):** Statistical analysis regarding keratin thickness revealed significant differences between the two groups.

Histological examination of the gingival tissues of the rats of group III (RPO group) showed improvement in histological features of the VAD group approaching that of the control group. The covering epithelium revealed a significant decrease in the keratin layer with a noticeable change in rete pegs to be approaching that of the control group. The lamina propria showed a noticeable enhancement in collagen bundles, an improvement in the histological picture of the extracellular matrix, and a notable reduction in areas of degeneration, however, a slight hyalinization was distinguished. Statistical analysis revealed a significant difference ($p<0.003$) comparing keratin thickness in group III and the control group.

Masson Trichrome staining for specimens of group III revealed an increase in the ratio of new to old collagen. Statistical analysis showed an insignificant difference ($p<0.0001$) comparing the area percent of new collagen in group III to that in the control group.

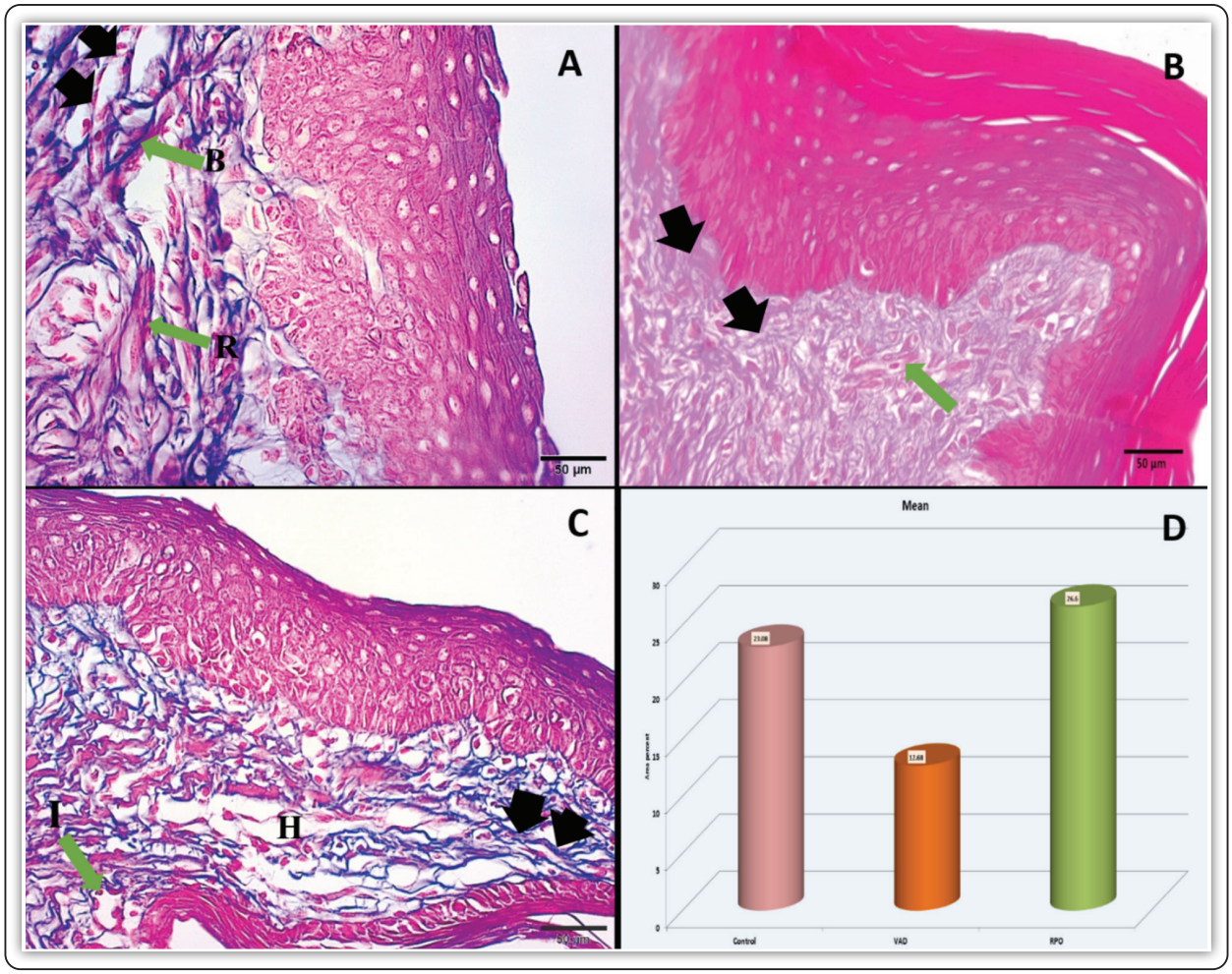

FIG (2) **(A):** a photomicrograph of gingival tissue in Group I illustrates that the lamina propria showed new collagen appearing as a blue stain (B) and older collagen displaying a red hue (R), red blood cells can be observed within the blood vessels (black arrowheads). **(B):** A photomicrograph of gingival tissue in Group II revealed a higher amount of old collagen (red) (black arrowheads), along with the presence of extravasated red blood cells (green arrow). **(C):** A photomicrograph of gingival tissue in Group III showed an apparent increase in new (blue) collagen (arrowheads), the integrity of the vessel walls showed improvement (I) and slight hyalinization was observable (H). (Masson Trichrome x400). **(D):** Statistical analysis revealed significant differences between all groups but no significant difference between control and RPO groups.

Immunofluorescent results

Immunofluorescent examination of the gingival tissue of Group I regarding the expression of Ki-67 antigen revealed antigen-positive nuclei in the basal layer, while a negative nuclear Ki-67 immunoreaction was found in the most superficial cell layers.

Gingiva of group II revealed a positive nuclear ki-67 immuno-expression in the basal, suprabasal cells. Immunofluorescence examination of the surface epithelium of the gingiva of group III revealed the basal and suprabasal layer with apparently less number of positively stained cells to Ki-67.

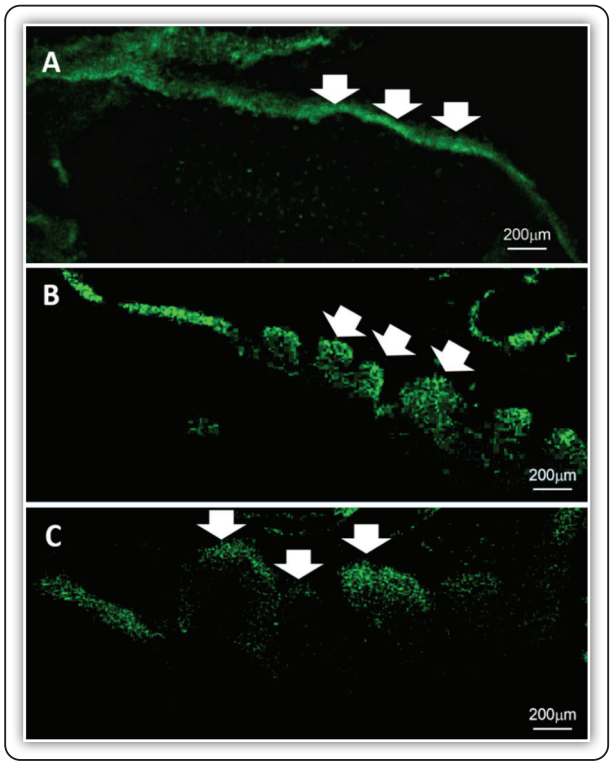

FIG (3) Photomicrographs showing: **(A):** Gingiva of the control group showing a positive nuclear Ki-67 immunoreaction in the basal layer (arrowheads). **(B):** Gingiva of the VAD group showing a positive reaction of all the basal calls, most of the parabasal cells, and sparse cells in the prickle cell layer (arrows). **(C):** Gingiva of RPO group 3 showing a positive reaction of few basal cells to Ki-67 (arrowheads). (Ki-67 antibody x200).

DISCUSSION

Vitamin A deficiency or insufficient retinol levels represents a prevalent public health issue and is recognized as the most widespread nutritional deficiency globally[14]. Investigating vitamin A deficiency is crucial because it can have serious health consequences, particularly in developing countries where deficiency rates can be high[15].

The scope of this research was to examine how the utilization of Red Palm Oil could mitigate the risks of vitamin A deficiency in the gingival tissue of rats.The primary reason for employing red palm oil in this study is its distinctive characteristic, which sets it apart from other oils, and its remarkable stability. Zeb &Lutfullah, 2005 have observed that red palm oil, when exposed to gamma radiation, demonstrates a notable level of stability, mainly attributable to its abundant β-carotene content, in contrast to soybean oil [16]. Based on existing literature, it is suggested that palm oil or red palm oil (RPO) could serve as a superior matrix for maintaining the stability of vitamin A[17]. Furthermore, according to [18], the findings indicate that red palm oil (RPO) is highly effective in enhancing vitamin A levels and preventing or treating vitamin A deficiency (VAD) in populations exposed to high threat of VAD.

In the present study, histochemical examination of the gingival tissues of the rats of the control group (Group I) by Masson Trichrome staining was utilized to detect connective tissues specifically collagen, in tissue sections. This came in accordance with [19] who used Masson trichrome staining to detect gingival new collagen fibers, [20] reported that the animal deficient in vitamin A experiences a swift decline in growth rate and a gradual transformation of most epithelia into a keratinized state. This phenomenon is attributed to alterations in the mitotic activity of epithelial cells and the pace of cellular replacement in these tissues.

Also,[21] reported that available evidence suggests that a deficiency in vitamin A can lead to the development of oral hyperkeratosis. This phenomenon is linked to the lack of vitamin A, which

causes the transformation of cuboidal, columnar, and transitional epithelia into a stratified, squamous, keratinized epithelium. This transformation appears to occur through a sequence of events, involving a reduction in the mitotic activity of epithelial cells, leading to epithelial atrophy and a compensatory mechanism that ultimately results in keratinization.

Gingival tissue of the rats of group II (VAD group) exhibited a statistically significant increase in keratin thickness as compared to other groups. This came per [5] who found that rats deprived of vitamin A displayed increased cellular growth and elevated keratinization in the gingival epithelium. Moreover, there was notable expansion of the junctional epithelium along with a slowdown in the healing of gingival wounds, [22] stated that retinoids regulate the growth and differentiation of different cell types, and their insufficiency results in aberrant epithelial keratinization. This phenomenon is linked to the actions of retinoids, which are accomplished through two families of nuclear receptors within the steroid hormone receptor superfamily—retinoic acid receptors (RARs) and retinoid X receptors (RXRs). Both RARs and RXRs bind and activate specific response elements within genes essential for preserving differentiation and proliferation in epithelial tissues. The ligand that activates these receptors is retinoic acid.

Also,[23] mentioned Another mechanism by which Retinoic acid (RA) and its receptors enact a pivotal part in governing the differentiation and proliferation of keratinocytes by acting as antagonists of activating protein-1 (AP-1), a well-established regulator of keratinocyte differentiation. Consequently, the expression of keratin genes is intricately influenced by retinoids in various complex ways.

Gingival tissues of the rats of the VAD group showed the lamina propria with a statistically significant decrease in collagen bundles in comparison to the control group regarding the area percent of new collagen. This came in accordance with [24] who mentioned that male rats were exposed to a vitamin A-deprived diet until each rat exhibited vitamin A deficiency, it was found that such deficiency led to a significant reduction in collagen levels by 40–60%. These findings were corroborated through semiquantitative analyses, as indicated by a 2-way ANOVA with a significance level of less than 0.05. The explanation provided attributes this outcome to the suppressive effect of retinoids on the synthesis of matrix metalloproteinases, which play a role in the degradation of the extracellular matrix.

Furthermore, according to[22], a decrease in collagen synthesis rates and the cross-linking of newly formed collagen is accompanied by vitamin A deficiency. These effects are explained by the regulatory influence of retinoids on the activity of the alpha-2 type I procollagen promoter. Furthermore, retinoids were identified as contributors to the increased production of extracellular matrix components such as collagen type I and fibronectin. Additionally, retinoids were observed to activate the proliferation of keratinocytes and fibroblasts, while concurrently reducing the levels of degrading matrix metalloproteinases.

The gingiva of the rats from the VAD group showed thinning of the wall of blood vessels with extravasated blood. This was explained by[24] who associated alterations in the expression of extracellular matrix components with the role of retinoids in governing cell adhesion and the synthesis of extracellular matrix proteins in the gastrointestinal tract.

The Gingiva of the rats from the VAD group showed regions of disintegration within the reticular layer of the lamina propria.[25] Confirmed the participation of retinoid signaling in the expression of extracellular matrix (ECM) proteins, such as collagen, laminin, elastin, and proteoglycans, is evident. Furthermore, retinoic acid exerts an influence on the expression of ECM receptors located on the cell membrane.

In this study, red palm oil administration, the richest naturally occurring source of beta-carotene, a carotenoid that the body can transform into functional vitamin A (retinol), was accompanied by a significant decrease in the keratin layer. This was confirmed by our statistical analysis which revealed a significant increase in the RPO group expressed lower keratin thickness as compared to the VAD group. This was verified by [23], who reported a positive response to oral therapy with retinol, based on the overarching hypothesis that retinol plays a normalizing role in keratinocyte differentiation.

Furthermore,[26] attributed the amelioration in connective tissue architecture to the observed reduction in the levels of pro-inflammatory cytokines (TNFα, IL-1β, and IL-6) in rats' liver, indicating the possible anti-inflammatory effects of red palm oil. Also, [27] reported that a deficiency in Vitamin A leads to the down-regulation of the Th2 response, resulting in an elevation of pro-inflammatory cytokines that foster a Th1-type inflammatory response. Simultaneously, the production of cytokines that inhibit inflammation, including IL-4 and IL-10, is diminished.

The RPO group revealed a statistically significant augmentation in collagen bundles comparable to the control group. This aligns with[28] and[29] who noted that retinol stimulates the synthesis of collagen type 1 and glycosaminoglycans (GAGs), improves elasticity by removing degenerated elastin fibers, and fosters angiogenesis. These effects were attributed to retinol's ability to stimulate fibroblasts for collagen fiber synthesis (enhancing fibroblast activity and increasing their number through TGF-β). Additionally, retinol inhibits collagenase and matrix metalloproteinases (MMPs) while promoting the synthesis of tissue inhibitors of metalloproteinases (TIMPs).

RPO group showed improvement in vessel wall integrity and a reduction in the presence of extravasated red blood cells as explained by[14] *and*[29] where RPO caused a significant reduction in plasma tissue plasminogen activator antigen, a fibrinolytic risk marker for cardiovascular disease.

Positive cells revealed by the Ki-67 antibody stain were restricted to the basal layer of the epithelium in the control group. This aligns with the findings of[30] who explained this observation by noting that the gingival tissue is a somatic tissue with a normal turnover rate compared to other tissues in the body.

Ki-67 antibody stain in the VAD group revealed an obvious increased positive expression in the basal, suprabasal cells. These findings were in accordance with [31] who reported that there is an evident rise in the number of PCNA (proliferating cell nuclear antigen) positive cells in the basal layer of VAD. They clarified that deprivation in vitamin A derivatives, such as retinol, leads to a lack of regulation over cell proliferation, leading to hyperplasia. This is attributed to a rise in the quantity of mRNA encoding the transcription factor under conditions of vitamin A deficiency.

In agreement with [32] Ki-67 antibody stain in the RPO group showed the basal and suprabasal layer with a slight decrease in the amount of positively stained cells to Ki-67. These effects are consistent with an inhibition of NF-ϰB (Nuclear factor kappa B) and repression of key cellular controllers including cyclin D1.

CONCLUSION AND RECOMMENDATIONS

Conversely, red palm oil mitigated the impact of vitamin A deprivation by increasing serum retinol levels. Future objectives involve scrutinizing ultra-structurally the gingival tissues of rats in the Vitamin A deficiency (VAD) and red palm oil (RPO) groups using a transmission electron microscope. Additionally, there is a necessity for further investigations to identify the optimal dosage of RPO, aiming to prevent carotenoderma.

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