Evaluation of injectable Vitamin C as a depigmenting agent in physiologic gingival melanin hyperpigmentation: A clinical trial

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Abstract: Objective: Evaluate the efficiency of vitamin C as a depigmenting agent in patients with physiologic gingival hyperpigmentation and assess its clinical and histopathologic effect on the patients' gingival health. **Background data:** Gingival melanin hyperpigmentation is an aesthetic problem which concern many individuals. Various surgical and non-surgical approaches have been reported for its management but most of them are associated with hazardous effects. **Methods:** Forty patients suffering from physiologic gingival melanin hyperpigmentation was included. Intraepidermal injection of vitamin C was repeated once per week until no visible pigmentation. The patients were recalled after 1, 3 and 6 months after treatment for follow up. **Results:** There is a decrease in pigmentation indices scores and reduction in the area with a significant statistical difference between pre-operative visit and both injection as well as follow-up visits. Regarding gingival health, the results revealed a reduction in mean SBI scores after vitamin C injection and statistical significant improvement of the gingival biotype with shift from the thin gingival biotype to the thick one. **Conclusion:** Vitamin C injection is a safe, minimally invasive non-surgical depigmenting technique which improves health of gingival tissues.
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1. Introduction:

Cosmetic dentistry is a fast growing field that involves not only aesthetic restorative procedures but also the appearance of the gingiva. Oral melanin pigmentation has been observed in a wide scale of people affecting many locations and sites in the oral cavity [1, 2]. The gingiva is the most frequently affected intraoral tissue [3]. The great majority of oral melanin hyperpigmentation is physiologic [4]. Many non-surgical approaches as well as surgical intervention have been suggested for the management of melanin hyperpigmentation including 90% phenol, 95% ethanol solutions, free gingival grafts, gingivectomy and de-epithelialization by bur abrasion, scalpel, laser and cryosurgery [2].

However, most of these modalities cause harmful effects such as chemical burn, high incidence of repigmentation and relapse, prolonged healing, excessive pain, alveolar bone loss, difficulty to control depth of de-epithelization[5, 6]. New therapeutic modalities were introduced after well understanding of regulation of melanogenic pathway. They can interfere with the melanogenesis and inhibit melanin production by acting on one or more steps. Linoleic acid, soy, licorice, methimazole, mulberry, niacinamide (vitamin B3) and vitamin C are examples of these agents [7-9].

Vitamin C has many variable biological, pharmaceutical and dermatological functions that include promotion of collagen biosynthesis [10, 11], photo-protection, skin strengthening [12], enhancement of the immunity (anti-virus effect), cancer therapy and melanin reduction [13, 14]. There is increased awareness about the important role of vitamin C in health and maintaining physiological status. New therapeutic uses are being investigated daily. In dermatology, vitamin C is used in treatment of various skin problems including depigmentation of hyperpigmented spots [15, 16].

The present study evaluates the efficiency of vitamin C as a depigmenting agent in patients with physiologic gingival hyperpigmentation and assesses its clinical and histopathologic effect on the patients' gingival health.

2. Materials and Methods:

Patient selection:

Forty patients ranging from 20 to 44 years of age suffering from physiologic gingival melanin hyperpigmentation was included. They were selected from the pool of patients seeking periodontal treatment in Oral Medicine, Periodontology and Diagnosis Department, Faculty of Oral and Dental Medicine – Cairo University.

Inclusion criteria were: Gingival melanin hyperpigmentation should be present in upper and lower esthetic regions, Patients should be free from any systemic diseases according to modified Cornell Medical index [17], Patients with esthetic concern and Patients with fair oral hygiene. On the other hand, exclusion criteria included: Patients with systemic autoimmune disease or endocrine disorders, smokers or addictives, drug induced gingival pigmentation, pregnant and lactating mothers and patients suffering from chronic and aggressive periodontitis.

The esthetic region (upper or lower anterior gingiva) to which we injected ascorbic acid was randomly determined using random.org and allocation concealment was done using numbered opaque sealed envelopes. The entire procedure was explained to the patients and a written consent was obtained before starting the procedure.

Protocol of work:

All enrolled patients received thorough supragingival scaling using ultrasonic scaler and hand instruments with two weeks sessions and one week apart. Patients were asked to follow self performed plaque control measures by modified bass brushing technique using soft tooth brush; plain non-irritant tooth paste and anti-inflammatory mouth wash (benzydamine hydrochloride, Epico Company, Egypt). No further gingival therapy was provided during the injection visits [18].

Patients were instructed to avoid acidic food, spicy food, salty food, coloring food and drinks and coloring or irritating mouth washes. A 200 -300 mg (1-1.5 ml) ascorbic acid (Ascorbic acid ampoule-Memphis Company) was injected in the tissues once per week until no visible pigmentation.

Digital photographs were obtained for all patients in the same dental unit with the same position (45^0 position with the head supported) at 8 o'clock morning with fixed magnification and distance.

Clinical evaluation:

A- Gingival health assessment:

Patients' gingival health was evaluated by assessing O'Leary plaque index (PI) [19], sulcus bleeding index (SBI) [20] and gingival tissue biotype.

Claffey & Shanley categorized the periodontal tissues into: Thin scalloped: ≤ 1.5mm and Thick flat: ≥ 2mm. The gingival tissue biotype was assessed via the transgingival probing using endodontic file size 25 with rubber stopper [18, 21].

B- Pigmentation assessment:

The color of gingival melanin pigmentation was evaluated by Dummett pigmentation index [22]. The surface area of the pigmentation [from the left and right mid cervical region of the canine] was traced and measured using the image analyzer computer system using software Leica Qwin 515 system (England) from the clinical photographs.

C- Patients' satisfaction assessment:

Patients' satisfaction was recorded according to the modified McGill pain questionnaire [23, 24].

Histopathological evaluation:

Pre-operative and post-operative gingival biopsies were obtained from non-esthetic region before and after ascorbic acid application. Biopsy material was immediately fixed then processed for preparing a paraffin block. Tissue sections w ere then cut and stained with conventional Hematoxyline & Eosin (H& E), silver impregnation and s100. Histopathological sections were examined by the image analyzer computer system using software Leica Qwin 515 system (England).

Statistical analysis:

All statistical calculations were done using computer program SPSS (StatisticalPackage for the Social Science; SPSS Inc., Chicago, I L, USA) release 15 forMicrosoft Windows (2006).

3. Results:

I- Descriptive data:

Age of the enrolled patients' ranges from 20 to 44 years, with the average age is about 25 years. As regards the gender, 35 (87.5%) out of the 40 selected patients are females and 5 (12.5) are males. The difference in the enrolled frequency and percent reflects the over care of female patients to esthetic procedures. The skin color indices and smile line classes of these patients are shown in table (1).

II- Clinical evaluation:

1- Gingival health assessment:

The number of patients with thick gingival biotype increased from 2 to 6 after vitamin C injection. On the other hand, the patients with thin gingival tissue biotype decreased from 36 to 25. Concerning the PI and SBI, both of them decrease following injection of vitamin C. Table (2) illustrates the comparison between mean gingival tissue biotype, PI and SBI of the pre-operative and postoperative visits.

2- Pigmentation assessment:

The mean value of Dummett index in the preoperative visit was 1.062. There was decrease in the mean values throughout the injection visits from 0.8 to 0.15.During the follow up period, the mean

value reduced from 0.17 in the first month visit to 0.07 in the 3 month and 6 month visit. When the treatment progression was compared by Tukey - Kramer multiple comparison tests, it showed significant statistical difference between the injection visits but no significant difference was detected between the follow-up visits [Table 3 & 4] (figures 1&2).

Regarding surface area, statistically significant reduction is clearly detected after vitamin C injection. The pigmented area ranged from 1.5 to 15 cm^2 with mean6.87cm2 ± 4.053. After vitamin C injection, the scores ranged from 0 to 3 cm2 with mean 0.84 cm ±2 0.737.

3- Patients' satisfaction assessment:

The patients' satisfaction was clearly reflected on their attitude and oral hygiene habits, 30 patients were totally satisfied, other patients were less satisfied. The treatment outcome met the expectations of 38 patients. At the end of treatment, 37 of the treated patients asked for repeating the treatment for the other pigmented regions, 3 were annoyed due to the continuous needling or the long term treatment.

No clear adverse effects were detected between the injection visits or after finishing treatment. Mild itching was a common sign for few hours in the first day in all treated patients, while only 1 patient suffered from mild pain during vitamin C injection.

II- Histopathological evaluation:

1- H & E stained sections:

The Microscopic examination of the pre-operative H and E stained sections revealed pigmented parakeratinized stratified squamous epithelium with normal thickness. Numerous melanin containing cells with coarse melanin granules were observed related to the basal cell layer. Rounded to polygonal melanin containing cells with round nuclei were arranged in cell nests. The underlying connective tissue is totally free of any melanocytes as well as inflammatory cells with well formed collagen bundles and blood capillaries (Fig. 3).

Examination of the post-operative sections revealed parakeratinized hyperplastic stratified squamous epithelium. Numerous epithelial archades were detected indicating epithelial proliferation. The basal cell layer was almost free of melanin containing cells. The higher turnover of epidermis detected due to increasing the granular cell layer and parakeratin thickness. The observed epidermal hyperplasia and cellular proliferation was explained by the rich blood supply of the treated tissues. In the connective tissue, newly formed collagen bundles and newly formed capillaries (angiogenesis) were detected (Fig. 4).

2- Silver impregnation stained sections:

The pre-operative sections showed positive reaction for silver stain in the form of supra nuclear caps in the upper portion of the cytoplasm of the basal melanin containing cells indicating presence of melanin granules (Fig.5).On the other hand, the post-operative sections showed negative reaction reflecting absence of melanin granules (Fig. 6).

3- s100 stained sections:

The immunopositive reaction for s100 appeared as moderate to strong stain in the basal cell layer indicating the presence of melanocytes (Fig.7). Mild positive reaction was detected in the prickle cell layer representing langerhans' cells. As regards to post-operative sections, moderate to weak immuno-positive reaction of the basal cell layer indicates decrease in number of melanocytes in comparison to the pre-operative specimens (Fig. 8).

Table 1: Descriptive data of the selected patients

Skin color indices	Frequency	Percent	Smile line classes	Frequency	Percent
Very white(Type I)	0	0	Very high (class I)	18	45.0
White (Type II)	5	12.5	High (class II)	12	30.0
White to olive(Type III)	11	27.5	Average (class III)	10	25.0
Brown (Type IV)	14	35	Low (class IV)	0	0
Dark brown(Type V)	6	15			
Black (Type VI)	4	10.00			
Total	40	100.0	Total	40	100.0

Table 2: Comparison between mean gingival tissue biotype, PI and SBI before and after vitamin C injection

	Pre-operative		Post-operative		*p*-value
	Mean	SD	Mean	SD	
Gingival tissue biotype	1.1	0.34	1.56	0.43	0.0001*
O'Leary plaque index	13.03	4.23	12.1	2.97	0.023*
Sulcus bleeding index	1.66	0.69	1.28	0.59	0.0001*

* Significant at $P \leq$ 0.05

Table 3: Comparison between mean Dummett pigmentation indices of the preoperative and injection visits

	Pre-operative		1st visit		2nd visit		3rd visit		4th visit		*P*-value
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Dummett pigmentation index	1.06 [a]	0.34	0.8 [b]	0.27	0.55 [c]	0.23	0.33 [d]	0.19	0.15[e]	0.2	0.0001*

* Significant at $P \leq 0.05$

Different superscripts denotes significant difference between groups according to Tukey-Kramer multiple comparison test

Table 4: Comparison between mean Dummett pigmentation indices of the preoperative and follow up visits

	Pre-operative		**1 month**		**3 months**		**6 months**		***P*-value**
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	
Dummett pigmentation index	1.06 a	0.34	0.17 b	0.22	0.07b	0.12	0.07b	0.12	0.0001*

* Significant at $P \leq 0.05$

Different superscripts denotes significant difference between groups according to Tukey-Kramer multiple comparison test

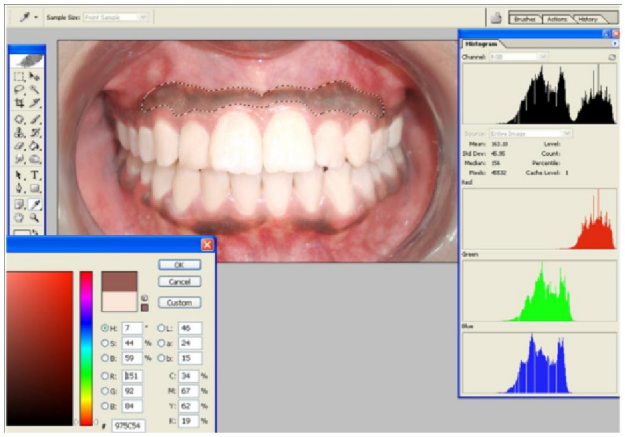

Figure 1: Pre-operative photograph revealed Dummett gingival hyperpigmentation score 2 135x66mm (300 x 300 DPI).

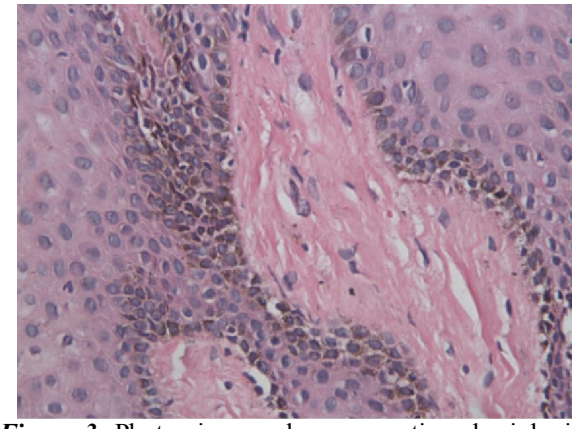

Figure 3: Photomicrograph pre-operative physiologic pigmentation revealed numerous melanin containing cells related to the basal cell layer (H&E X400) 173x130mm

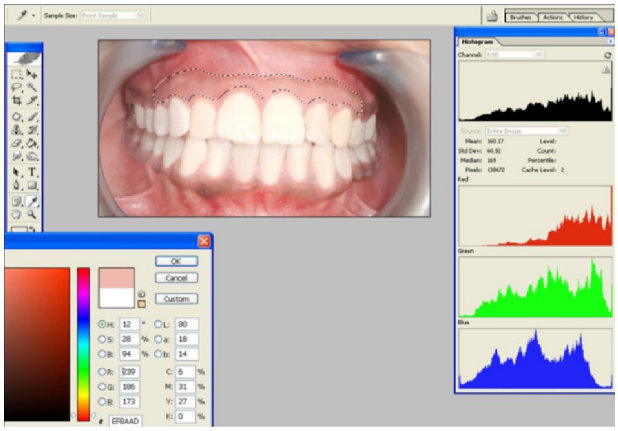

Figure 2: Post-operative photograph revealed Dummett gingival hyperpigmentation score 0 113x60mm (300 x 300 DPI)

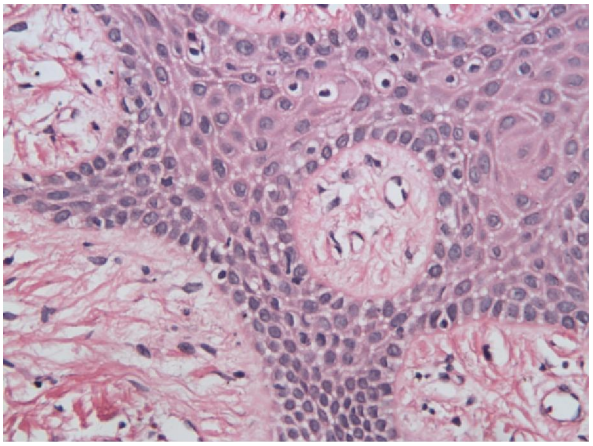

Figure 4: Photomicrograph POST-operative physiologic pigmentation revealed epithelium free of melanin containing cells (H&E X400) 173x130mm

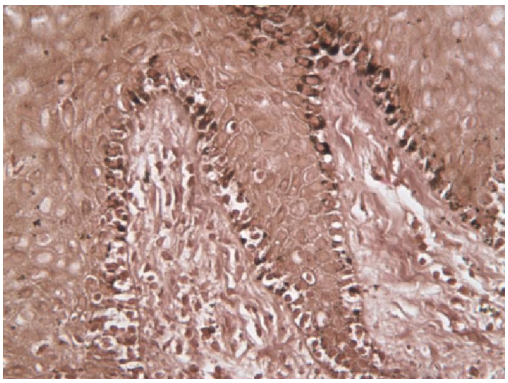

Figure 5: Photomicrograph pre-operative physiologic pigmentation revealed superanuclear caps of silver stained melanin (Silver stain x 400) 173x130mm

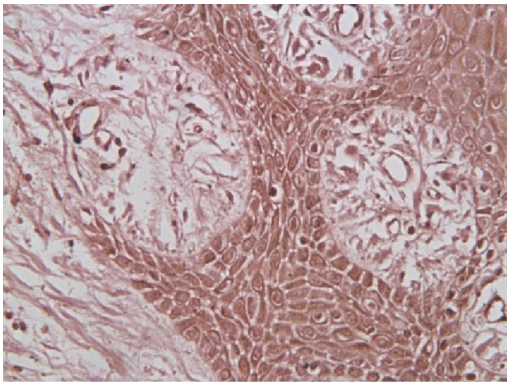

Figure 6: Photomicrograph post-operative physiologic pigmentation revealed absence of silver stained basal cells (Silver stain x 400) 173x130mm

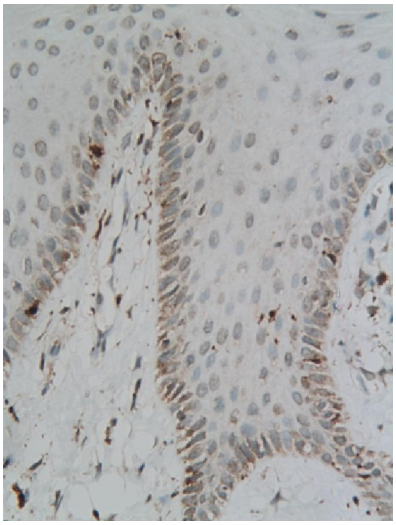

Figure 7: Photomicrography pre-operative physiologic pigmentation revealed positive s100 protein immunoexpression in the numerous cells along the basal cell layer of the surface epithelium indicating presence of numerous melanocytes (s100 stain x 400) 173x130mm

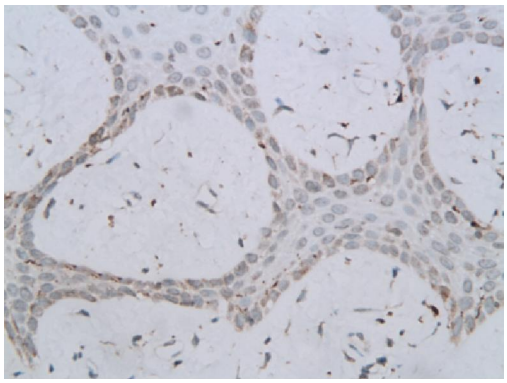

Figure 8: Photomicrography post-operative physiologic pigmentation revealed weak positive s100 protein immunoexpression in the basal cell layer showing few stained cells denoting decrease in number of melanocytes at the basal cell layer of surface epithelium (s100 stain x 200) 173x130mm

4. Discussion:

All enrolled patients were free from any systemic diseases according to Modified Cornell Medical Index [(17)]. Smokers, pregnant ladies and lactating females were also excluded from the study to avoid their influence on the level of melanin production and bring about gingival hyperpigmentation. Only patients with fair oral hygiene were included as any inflammatory process may result in increased melanin production and could mask the outcomes. For that reason, all patients were subjected to supra gingival debridement with two weeks sessions and one week apart.

The efficiency of vitamin C in the management of gingival melanin hyperpigmentation was evaluated in the present study by various clinical methods including Dummett pigmentation index [(22)], surface area analysis as well as histopathological evaluation by H & E, silver impregnation and s100 staining.

Concerning the pigmentation indices and area analysis, there is a decrease in indices' scores and reduction in the area with a significant statistical difference between pre-operative visit and both injection as well as follow-up visits, confirming the efficiency of vitamin C as a depigmenting agent. These results were in agreement with **Shimada et al. (2009)** and **Sheel et al. (2015)**. **Shimada et al. (2009)** confirmed the potential role of ascorbic acid in treatment of gingival melanin pigmentation by their placebo-controlled clinical trial [(25)]. **Sheel et al. (2015)** reported the ancillary role of vitamin C along with surgical scalpel excision in their case report study [(26)]. Statistical significant difference was found between mean scores of injection visits. On the other hand, no statistical significant difference was obtained on comparing post-operative follow-up visits. This was in accordance with **Shimada et al. (2009)** who evaluated the depigmenting effect of vitamin C by measuring the

L* value (lightness). They revealed that there is more increase in the L* value every 4 weeks, but no significant difference was apparent between weeks 8 and 12 [25].The results of the current study and **Shimada et al. (2009)** may reflect the early depigmenting effect of topical vitamin C which appeared during the injection visits and/ or the early weeks, respectively. Another explanation may be the narrow range of pigmentation indices that was an obstacle in the evaluation of the treatment progression especially slow progress during follow-up visits.

An interesting observation was the higher efficiency of vitamin C in cases with darker pigmentation. **Ancans et al. (2001)** documented that the pH of melanosomes control tyrosinase activity and melanogenesis where neutral pH is the optimal for enzyme activity **(27)**. In fair skinned individuals, melanosome pH is acidic which suppress tyrosinase activity. However, neutral pH in dark skinned individuals promotes tyrosinase activity [28].

By screening the histopathological findings, we observed that the basal cell layer revealed almost free of melanin containing cells in the post-operative H & E stained sections and negative reaction in the silver impregnation stained sections reflecting absence of melanin granules. These results may be attributed to the inhibitory effect of vitamin C on the enzymatic activity of tyrosinase enzyme [29]. It was confirmed that vitamin C inhibit melanin production in many steps including reduction of o-dopaquinine, blocking 5, 6-DHICA oxidation and preventing free radicals required for melanin production [29, 30].The immunohistochemical reaction of tissues to s100 protein ranged from moderate to weak reaction after vitamin C injection indicating decrease in the number of melanocytes. This was in agreement with **Maedea & Fucuda (1991)** who demonstrated that ascorbic acid reduces the viability of cultured human neonatal melanocytes [31]. Another remarkable finding was significant statistical reduction in the mean surface area and roundness of melanocytes after vitamin C injection. The size of the melanocyte increases with the increase in the enzymatic activity and melanin production [32].

In the present study, SBI was useful approach to assess early gingival changes because bleeding on probing is an early sign of gingivitis [33]. The results revealed a reduction in mean SBI scores after vitamin C injection with a significant statistical difference between pre-operative and post-operative visits; this may be due to one of the following causes or synergistic effect of all/some of them. The first cause may be patient adherence to the oral hygiene instructions this was evident by the significant statistical difference obtained on comparing mean PI scores of the pre-operative and post-operative visits. Another reason is the role of vitamin C in collagen production, tissue healing and angiogenesis [34, 35]. In the present study, the post-operative H&E sections showed newly formed collagen bundles and capillaries in the connective tissue.

Ascorbic acid enhances the periodontal ligament maturation and renewal by induction of the collagen formation especially collagen III (young collagen) and keeps the balance between collagen I (mature collagen) and III for tissue maturation. It also modifies the rate of fibroblast proliferation. In angioneogenesis, ascorbic acid acts as a cofactor in hydroxyproline synthesis to produce collagen type IV and improves endothelial cell vitality and function. It was suggested the inclusion of ascorbic acid in the periodontal treatment has a great role in tissue healing, formation of the new periodontal ligament and resistance of periodontal disease [34, 35].

One more cause is the statistical significant improvement of the gingival biotype with shift from the thin gingival biotype to the thick one after vitamin C injection. **Abraham et al. (2014)** stated that the switch of the gingival tissue to the thick biotype is important in gingival and periodontal health together with esthetic treatment outcome. They also confirmed that the thick gingival tissue biotype which is more resistant to inflammation and usually associated with periodontal health [36].

The last reason is that vitamin C modifies and enhances both innate and adaptive immune response. It neutralizes the bacterial toxins especially endotoxins by blocking the essential signal for lipopolysaccharides (LPS) formation [37]. Furthermore, it improves the phagocytic properties and activity of various immune cells [38].Thus, while being used as a depigmenting agent, vitamin C improves the health of the gingival tissues. This dual action (depigmenting effect & gingival health improvement) of vitamin C will give better aesthetic results in management of gingival melanin hyperpigmentation than other depigmenting agents that can by itself cause signs of inflammation including cryosurgery, electrosurgery and chemical agents such as phenol [5, 39, 40].

Conclusion:

Vitamin C injection is a safe, minimally invasive non-surgical depigmenting technique which improves health of gingival tissues.

Conflict of interest:

There is no conflict of interest

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