

# Evidence of the Effect of Intraepidermic Vitamin C Injection on Melanocytes and Keratinocytes in Gingival Tissues: *In Vivo* Study

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## Abstract

**Objective:** The purpose of this study is to evaluate the immediate effect of vitamin C injection on the physiologic hyperpigmented gingiva of native black goats

**Design:** Fifteen black goats were enrolled in this study. Different doses of vitamin C were injected in the hyperpigmented tissues. The examined animals were divided into 3 groups; Gp I (control) injected with saline, Gp II and III were injected with 10 mm and 30 mm of vitamin C respectively. Incisional biopsies were taken after injection. The specimens were examined histologically and immunohistochemically.

**Results:** The histological results revealed a marked decrease in melanin pigment and an increase in the number of cells with the perinuclear haloing in both groups II and III as compared to the control one.

**Conclusions:** It could be concluded that vitamin C has a potent effect on melanocytes. Vitamin C affected the melanocytes function and their quantitative productivity, as well as decreasing the cell-cell contact between melanocytes and keratinocytes. Increasing the vitamin dose augmented its depigmenting effect.

**Keywords:** Vitamin C; Pigmented Gingiva; Melanocytes; Keratinocytes; HMB-45; E-Cadherin

## Introduction

Although physiologic melanin hyperpigmentation is not a pathological condition, it is one of the most common esthetic problems. It usually occurs as a result of either over production or unequal distribution of melanin. Up till now, the total excision of the epithelial and the papillary connective tissue layers is the most common effective technique through which gingival depigmentation could be achieved. Several techniques depend on such concept as scalpel technique [1], bur technique [2], laser ablation [3], cryosurgery [4] and electrosurgery [1,5,6]. On the other hand, the non-surgical techniques as using phenol or ethanol depend on the same mechanism but by uncontrollable induction of necrosis of the epithelial cell layer [7]. However the efficiency of such techniques, the morphology of the basement membrane, thickness of the epithelial layer and the gingival biotype remains the main obstacles behind achievement of good results for long time [8].

In the past, it was thought that the high recurrence rate is more related to the deep color intensity of gingival tissues. Unfortunately, inadequate evidence based trails were reported till now. The literature depends on reporting such clinical trials in case reports. The literature depends on reporting such clinical trials in case reports. The process of melanin production is a physiologic condition which is controlled by the genetic background and the local tissue response towards continuous irritation. These factors seem to controls the activity and the type of melanocytes.

In human beings, melanin is essential for other several functions rather than giving our skin its color. Melanin pigment, presence of hair and keratinization are the main protective barriers for skin. Melanin is usually present in keratinocytes as supranuclear caps during the exposure to the ultraviolet rays [9]. While in mucosa including the oral tissues, there are other protective mechanisms as the high cellular turn over and secretions. Oral mucosa is a non-classical location for melanotic melanocytes.

Melanotic melanocytes are the main productive cells for melanin which are derived from neural crest cells. Non-melanotic melanocytes are commonly found in the oral cavity. They are mainly located in the basal and suprabasal cell layers. Their activity is the main factor that controls the degree of pigmentation [10]. During normal conditions, melanocytes have a fibroblast like pattern with two dendrites. The shape of melanocyte changes according to its activity [11,12]. Lower activity of the oral melanotic melanocytes usually detected in comparison to the dermal one.

The dermatologists discovered the role of several natural agents as vitamins, anti-oxidants and etc. in preserving the original skin color through their anti-oxidant potential, induction of collagen production and enhancement of blood supply [13,14]. Vitamin C is one of the potent anti-oxidants that play an important role in skin depigmentation [12].

On the contrary to other mammals, vitamin C (Vit.C) could not be synthesized in humans due to mutation of the gene that responsible for the production of L-glucono-gamma lactone oxidase enzyme. Most plants and animals are able to synthesize vit. C. Citrus fruits and dark

green vegetables are its main sources [15,16]. Vitamin C is a powerful antioxidant as it neutralizes the reactive oxygen species and prevents their damaging effect on the tissues. It keeps the structural integrity of the host and immune cells [13]. The depigmenting effect of vit. C depends mainly on its anti-oxidant property. The usage of vit. C in the treatment of physiologic or pathologic dermal problems such as hyperpigmentation, aging and dryness was promising [12,14].

No enough data were detected about the immediate effect of vitamin C towards the different cellular components of the pigmented dermal or mucosal tissues. The purpose of this study was to detect the immediate effect of single intraepidermic vit. C injection with two different doses on the melanocytes and keratinocytes in the gingival tissues of native black goats.

## Material and Methods

### Experimental procedure

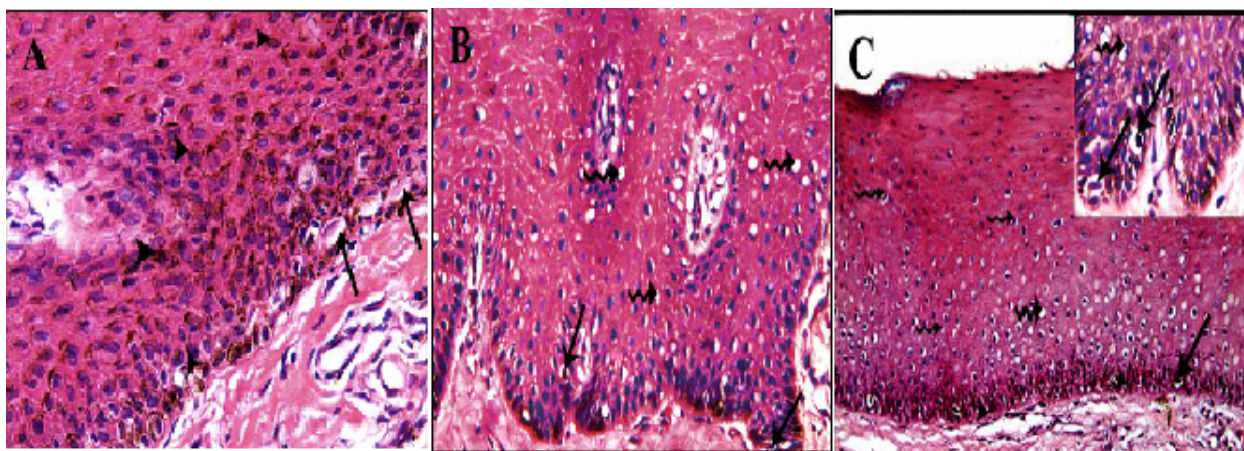
Fifteen adult male Egyptian native black goats with physiological gingival hyperpigmentation and average weight 25-30 kg were used in this study. The study was conducted in between 2012-2013. The animals were housed seven days before the experiment in individual cages at a controlled room temperature (25-28°C), light conditions (12 hours light-dark cycles) and relative humidity of 70-80%. They were fed standard diet and water. The study protocol was reviewed and approved by the Ethics Committee in the Faculty of Oral and Dental Medicine, Cairo University, Egypt.

The enrolled animals were systemically free. They were randomly divided into 3 groups, 5 goats per each. Local anesthesia was necessary prior the introduction of 2 different doses of sterile ascorbic acid (Cevaryl ampoules, Memphis Company, Egypt) were injected intraepidermic in relation to the upper anterior region. The used dosage was calculated according to Loscher et al. to avoid the local irritation accompanying the higher doses [17]. Group I (negative control group) animals received an equivalent volume of saline, group II were given 0.1 ml of vit.C and group III were given 0.3 ml of vit. C. After 15 min of injection, about 5 × 5 × 1 mm biopsies were taken. There was no need for animal sacrifice.

All the gingival tissue samples washed using saline solution and fixed in 4% buffered formalin. The specimens were dehydrated in ascending grades of ethyl alcohol, cleared in xylene and embedded in paraffin. Sections of 4-5 µm in thickness were obtained and collected on positively charged microscope slides. Tissue sections were deparaffinized and rehydrated before histological staining and immunolabeling.

### Light microscopic examination

Sections from the three groups were stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA) according to the conventional method and examined using light microscope equipped with digital camera for histological evaluation of morphological changes.



**Figure 1:** A photomicrograph of the gingiva from: Gp I showing melanocytes at the basal cell layer (arrows), melanin pigment at the basal and parabasal cell layers (arrowheads) (A); Gp II showing perinuclear halos in some of parabasal and prickle cells (zigzag arrows). Melanocytes (arrows) (B); Gp III showing increase in the number of cells with nuclear vacuolization in the basal, parabasal, prickle as well as granular cell layers (zigzag arrows). Melanocytes (arrows) with marked decrease in the amount of melanin pigment in the basal cell layer (arrowheads) (C and inset). (Hx and E, orig mag 200x) (Inset, 400x).

### Immunohistochemical examination

The sections were incubated for 2 hours at 56°C, deparaffinized in xylene and rehydrated by ethanol series ending with pure H<sub>2</sub>O (Millipore Corporation, Temecula, CA, USA). After 5-minute incubation in PBS, sections were incubated in 0.05 mg/ml proteinase K in 0.05 M Tris-HCl, 0.01 M EDTA, and 0.01 M NaCl, pH 7.8 for 10 minutes at 37°C. After two washes with PBS, unmasking of the antigens was carried out using antigen retrieval citrate buffer solution

for 10 min in boiling water. Then, the sections were placed in a humid chamber and the endogenous tissue peroxidase was blocked with 3% hydrogen peroxide for 5 min. Incubation with bovine serum albumin for 20 min was performed to reduce unwanted nonspecific reactions. Without washing, the sections were incubated with the Primary antibodies overnight at 4°C. The Primary antibodies used were polyclonal anti-human melanoma black 45 (anti- HMB-45) (1:50; Thermo Fisher Scientific Inc., IL, USA) and finally, anti-E-Cadherin (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA). In the next day,



after washing in PBS, the sections were incubated with secondary universal antibody and then with the Avidin-Biotin complex (ABC) (Vectastain Universal Elite ABC kit, Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol. The substrate DAB was applied until development of desired brown color (2-10 min). Finally, the sections were counter-stained with Mayer's hematoxylin (Sigma, St. Louis, MO, USA) for 30 sec. to visualize tissue topography. The negative control was obtained by omitting the primary antibody from the protocol outlined above.

## Results

### Clinical observation

Immediate clinical fainting occurred following the injection of vit.C while after 10-15 minutes, the tissue became darker.

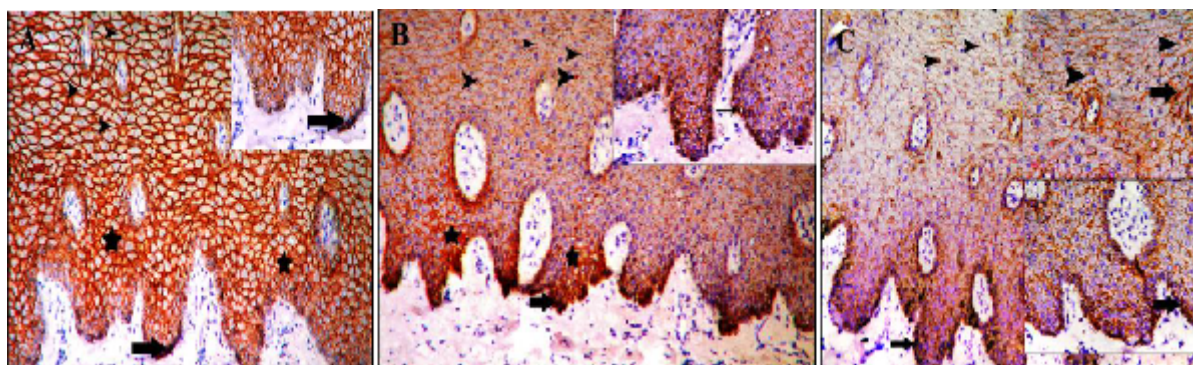
### Histological results

Examination of Gp I specimens (control group) revealed normal gingival epithelial structure with melanocytes at the basal cell layer. Large amount of melanin pigment could be detected at the basal and parabasal cell layers. The melanin formed a supranuclear cap and located along the cell membranes of the surrounding keratinocytes.

The melanocytes appeared as large clear cells but the dendrites could not be distinguished (Figure 1A). In group II, significant changes were detected in the related keratinocytes (melanin containing and non-containing cells). Unequal distribution of the vacuolated cells in relation to the basal and prickle cell layer was clearly detected (Figure 1B). On the other hand, samples of group III showed equal distribution of vacuolated cells along the basal and prickle cell layers. These changes were directly proportional with increasing the dose (Figure 1C).

### Immunohistochemical results

**HMB-45:** The basal cell layer including melanocytes revealed strong reaction for anti HMB-45 antibody (Figure 2A-2C). Group II showed strong HMB-45 immunoreaction in relation to the basal cell layer. Pycknotic nuclei with few melanin granules surrounding the nuclei clearly appeared in relation to the basal and parabasal layers (Figures 3A, 4B and 4C). Marked reduction of such positivity was detected in group III (moderate reaction). On the other hand, higher incidence of relatively small sized melanin granules were uniformly dispersed at the basal, parabasal and prickle cell layers (Figure 4A-4C). This dispersion was markedly decreased or even absent towards the surface layer (Figure 4C).



**Figure 2:** A photomicrograph of the gingiva from: Gp I showing strong membranous E-cadherin immunoreaction, some cells in the parabasal and prickle cell layers revealed cytoplasmic reaction (stars). Melanin granules were identified in the surface cell layer (arrowheads) (A) Some of the basal cells revealed very strong E-cadherin immunoreaction (thick arrow) (A and inset); Gp II showing strong E-cadherin immunoreaction in some of the basal cells (thick arrow) and in melanocytes (arrow) (B and inset) while moderate reaction was revealed in the prickle cell layer (stars). Numerous melanin granules were dispersed in the parabasal as well as in granular cell layer in this group (arrowheads) (B); Gp III showing a mild to moderate membranous E-cadherin immunoreaction. A marked decrease in the amount of melanin granules in the basal cell layer, small sized melanin granules were present in the prickle cell layer (arrowheads). Few basal and prickle cells showed strong reaction (thick arrows) (C and inset). (DAB, orig mag 200x) (Inset, 400x).

**E-cadherin:** Specimens of the control group revealed strong positive reaction while the positivity gradually decreased with the other two groups. On the contrary to the parabasal layers, the basal cell layer and melanocytes were revealed strong reaction in the control group (Figure 5B). However, the immunoreaction was strongly membranous, some of the cells in the parabasal and prickle cell layers revealed cytoplasmic reaction (Figure 5A). A mild to moderate membranous reaction was detected in Gp III. Few basal and prickle cells showed strong reaction (Figure 5C).

As doses administered orally exceed 200 mg, absorption decreases, urine excretion increases and its bioavailability is reduced. In contrast,

because intravenous injection bypasses the intestinal absorption system, it results in higher plasma concentrations [22,23].

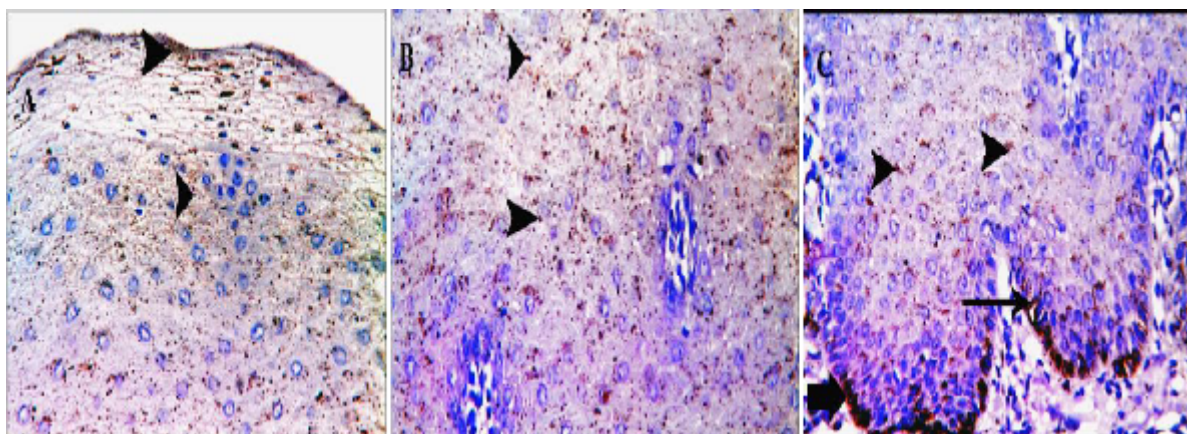
## Discussion

Great demands were behind the presence of minimally invasive technique treating the gingival hyperpigmentation without aggressive surgical intervention. Therefore, depigmentation using anti-oxidants is the hope to achieve simpler technique. In comparison with group I, 3 main differences were detected in the other groups. Firstly, cellular vacuolization including both melanocytes and keratinocytes was clearly detected. Secondly, the difference of specimens' intensity especially in H&E staining that faded away with higher doses. Finally,

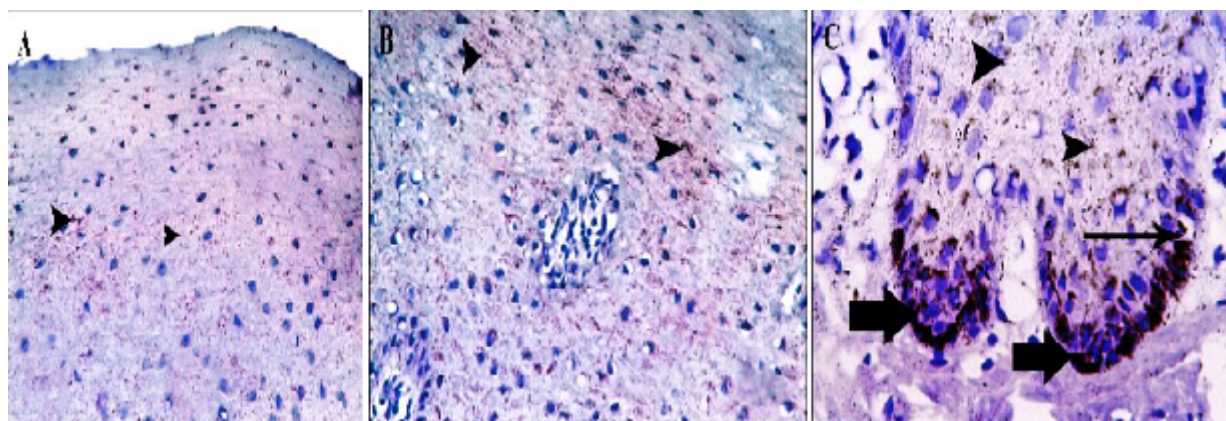
melanin granules were detected extracellularly. On examination of H&E specimens, cellular vacuolization was directly increased with the higher doses. The main question is shall this phenomenon usually related to the injection technique or vit.C. In accordance with Kimura et al. [19], higher percentage of cellular vacuolization immediately detected after the intradermal injection which revealed the ability of the anesthetic agent to infuse intracellularly more efficiently. It is related to the dose, higher vacuolization was detected in group III in comparison to group II. Such cellular changes were detected equally along the melanin containing or non- containing cells.

These results were minimally detected in the control group (intraepidermal saline injection). Such changes could be attributed to the combined effect of the used technique with high dosage of vit.C. The higher percentage of vacuolization may be explained by the increased affinity between vit.C and melanin. Melanin acts as a store for calcium, copper, iron and also acts as a ROS scavenger [20-22].

In the current study, HMB-45 was preferred due to its higher specificity to stimulated melanocytes and melanin [24,25]. Highly positive immuno-reaction was detected in group I & II which reveals the high activity of melanocytes. On the other hand, such positivity reduced to its moderate level with marked detection of the dispersed melanin granules extracellularly through the entire thickness of the basal and prickle cell layer in group III. These changes were attributed to the effect of the higher dosage also. The relationship between the melanocytes and keratinocytes is considered as bi-directional induction to the melanogenesis process [26]. Cadherins are calcium-dependent transmembrane glycoproteins that mediate cell-cell adhesion [27-29]. In our study, it was clearly demonstrated an inversely proportional relationship between the E-cadherin expression and the dose of the vit.C. On the other hand, directly proportional relationship was detected between the intensity of pigmentation and the E-cadherin expression. Such vacuolization could not be achieved with intact intercellular junction.

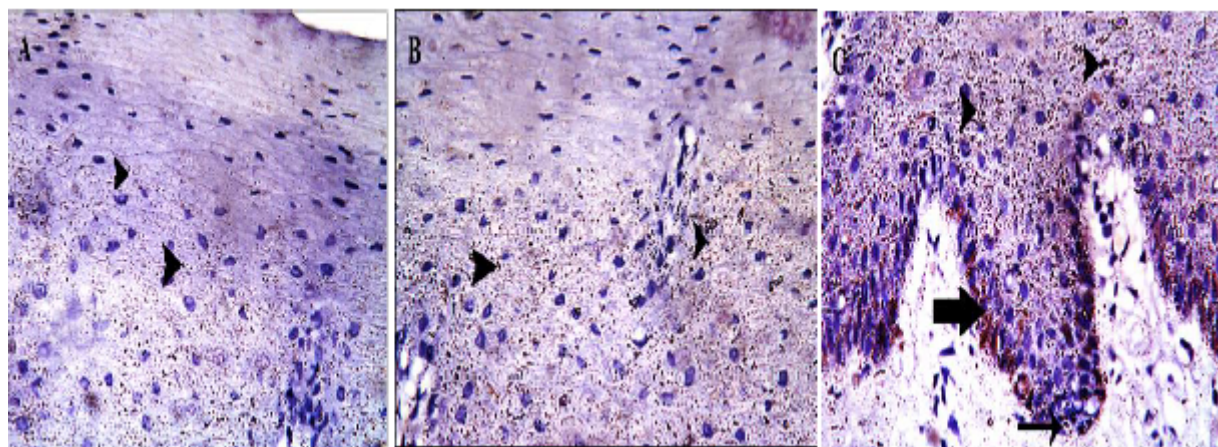


**Figure 3:** (A) photomicrograph of the gingiva from Gp I showing strong HMB-45 immunoreaction in the melanocytes (arrow) and in the basal cell layer (thick arrow) (C); Melanin granules were dispersed along the whole epithelial thickness (arrowheads) (A,B&C). (DAB, orig mag 200x).



**Figure 4:** A photomicrograph of the gingiva from Gp II showing strong HMB-45 immunoreaction in the basal cell layer (thick arrows), melanocytes with pyknotic nuclei (arrow) (C) dispersed melanin granules at the prickle cell layer (arrowheads) (B); and to a lesser degree of melanin granules at the surface cell layers (arrowheads) (A). (DAB, orig mag 200x).





**Figure 5:** A photomicrograph of the gingiva from Gp III showing small sized melanin granules at the basal (arrowheads), parabasal (C) and prickle cell layers (B) while they were decreased or even absent at the surface cell layers (A). Melanocytes with pyknotic nuclei and without dendrites (arrow), few keratinocytes revealed moderate HMB-45 reaction (thick arrow) (C). (DAB, orig mag 200x)

Several studies reported the effect of vit.C on the E-cadherin junctions between melanocytes and keratinocytes. Regnier et al. [12] reported that supplementation of the culture medium of keratinocyte-melanocyte co-cultures with Vit.C reversibly affects this relation which in turns affects the melanocyte morphology and activity. They added that melanocytes didn't show any morphological changes when exposed to comparable doses of Vit.C, indicating that keratinocytes are implicated in the mediation of such morphological changes on melanocytes.

The intercellular junction is mediated through cadherin junctions. Li et al. [28] reported that decrease in the E-cadherin expression could be referred to the switch from E-cadherin to N-cadherin in some melanocytes and keratinocytes. The disturbance of melanocyte-keratinocyte relationship affects the quality and quantity of the produced melanin. Such immediate disturbance of E-cadherin junctions could be due to the injection technique only and not the effect of vit.C. This couldn't be proved through our results. From the previous analytical study we can conclude that the direct effect of vit.C could be due to the affinity of melanin to react with it which in turns affect the cellular junctions with the melanin contacting cells (causing the immediate fainting) and force such cells to spell out its content of melanin leading to tissue darkening after a while. Further investigations and studies are recommended to detect its long-term effect on the melanocytes and keratinocytes.

## Disclosure

This paper's contents are solely the responsibility of the authors.

## Competing Interests

None of the authors have any competing interests with respect to this paper.

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## References

1. Anoop S, Abraham S, Ambili R, Mathew N (2012) Comparative evaluation of gingival depigmentation using scalpel and diode laser with 1 year follow up. *International Journal of Laser Dentistry* 2: 87-91.
2. A Karydis, P Bland, J Shiloah (2012) Management of oral melanin. *J Tenn Dent Assoc* 2: 10-17.
3. S Suthprasertporn (2007) Treatment of gingival melanin hyperpigmentation by Er,Cr:YSGG laser: report of 2 cases. *Thai J Periodont* 1: 46-55.
4. S Kumar, G Bhat, K Bhat (2013) Comparative evaluation of gingival depigmentation using tetrafluoroethane cryosurgery and gingival abrasion technique: two years follow up. *J Clin Diagn Res* 7: 389-394.
5. B Bhusari, S Kasat (2011) Comparison between scalpel technique and electrosurgery for depigmentation: A case series. *J Indian Soc Periodontol* 15: 402-405.
6. S Chandna, S Kedige (2015) Evaluation of pain on use of electro surgery and diode lasers in the management of gingival hyperpigmentation: A comparative study. *J Indian Soc Periodontol* 19: 49-55.
7. I Hirschfeld, L Hirschfeld (1951) Oral pigmentation and method of removing it. *Oral Surg Oral Med Oral Pathol* 4: 1012-1016.
8. M Liebart, C Fouque-Deruelle, A Santini, F Dillier, V Monnet-Corti, et al. (2004) Smile line and periodontium visibility. *Perio* 1: 17-25.
9. A Slominski, D Tobin, S Shibahara, J Wortsman (2004) Melanin pigmentation in mammalian skin and its hormonal regulation. *Physiol Rev* 84: 1155-1228.
10. N Le Douarin, S Creuzet, G Couly, E Dupin (2004) Neural crest cell plasticity and its limits. *Development* 131: 4637-4650.
11. S Jungbauer, R Kemkemer, H Gruler, D Kaufmann, J Spatz (2004) Cell shape normalization, dendrite orientation, and melanin production of normal and genetically altered (haploinsufficient NF1)-melanocytes by microstructured substrate interactions. *Chemphyschem* 5: 85-92.
12. M Regnier, C Tremblay, R Schmidt (2005) Vitamin C affects melanocyte dendricity via keratinocytes. *Pigment Cell Res* 18: 389-390.
13. Y Shindo, E Witt, D Han, W Epstein, L Packer (1994) Enzymic and non-enzymic antioxidants in epidermis and dermis of human skin. *J Invest Dermatol* 102: 122-124.
14. L Espinal-Perez, B Moncada, J Castaneda-Cazares (2004) A double-blind randomized trial of 5% ascorbic acid vs.4% hydroquinone in melisma. *Int J Dermatol* 43: 604-607.

16. J Dover, M. Alam (2008) Procedures in Cosmetic Dermatology Series: Cosmeceuticals. Z. D. Draelos (Ed.). Elsevier Health Sciences.
17. P Telang (2013) Vitamin C in dermatology. *Indian Dermatol Online J* 4: 143-146.
18. W Loscher, G Jaeschke, H Keller (1984) Pharmacokinetics of ascorbic acid in horses. *Equine Vet J* 6: 59-65.
19. S Traikovich (1999) Use of Topical Ascorbic acid and its effects on Photo damaged skin topography. *Arch Otorhinol Head Neck Surg* 125: 1091-1098.
20. S Kimura, A Hirai, H Shimizu (1981) Epidermal Vacuolation: An Artifact Due to Injection of Local Anesthetics. *Arch Dermatol Res* 270: 413-9.
21. C Huh, K Seo, J Park, J Lim, H Eun, et al. (2003) A Randomized, Double-Blind, Placebo-Controlled Trial of Vitamin C Iontophoresis in Melasma. *Dermatology* 206: 316-320.
22. S Matsuda, H Shibayama, M Hisama, M Ohtsuki, M Iwaki (2008) Inhibitory effects of novel ascorbic derivative VCP-IS-2Na on melanogenesis. *Chem Pharm Bull* 56: 292-297.
23. Z Draelos (2007) Skin lightening preparations and the hydroquinone controversy. *Dermatol Ther* 20: 308-313.
24. P Farris (2005) Cosmeceutical vitamins: vitamin C. *Procedures in cosmetic dermatology series: Cosmeceuticals*. Philadelphia 2005: 51-6.
25. B Smoller, N McNutt, A Hsu (1989) HMB-45 recognizes stimulated melanocytes. *J cutan Pathol* 16: 49-53.
26. N Mahmood, W Lee, M Linden, S Nathanson, T Hornyak, et al. (2002) Diagnostic Value of HMB-45 and Anti-Melan A Staining of Sentinel Lymph Nodes with Isolated Positive Cells. *Mod Pathol* 15: 1288-1293.
27. Fitzpatrick, A Breathnach (1963) The epidermal melanin unit system. *Dermatologische Wochenschrift* 147: 481-489.
28. N Haass, K Smalley, M Herlyn (2005) Adhesion, migration and communication in melanocytes and melanoma. *Pigment Cell Res* 185: 150-159.
29. G Li, K Satyamoorthy, M Herlyn (2001) N-cadherin-mediated intercellular interactions promote survival and migration of melanoma cells. *Cancer Res* 61: 3819-3825.