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Energy drinks and oral non-keratinocytes (Histological & Immunohistochemical rat study)

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Aim: To study the effect of the chronic consumption of Red-bull energy drink and its withdraw on oral non-keratinocytes in middle-aged adult albino rats.
Materials and Methods: 24 adult male albino rats were divided into 3 groups (6 rats each). Group I (control) divided into Ia, Ib subgroups received 1.5ml/100g/day distilled water for 4 and 8 weeks respectively, group II (Red-bull group) received 1.5ml/100g/day RB orally for 4 weeks. Group III (withdrawal) received the above dose of RB for 4 weeks and was left for 4 weeks without any treatment. Paraffin sections of buccal mucosa were processed for H&E and immuno-histochemical examination of Melanocytes (HMB-45), Langerhans cells (CD1a) and Merkel cells (CK20).
Results: Compared to control and withdrawal groups, H&E sections of Red-bull group displayed apparent increase of inflammatory and degenerative changes. H&E and immunohistochemical sections of group II revealed a significant increase in Langerhans cells, Merkel cells and melanocytes compared to group III and group I respectively. In group III, a significant decrease in Melanocytes, Langerhans cells and Merkel cells respectively was detected compared to group II. A significant increase in Langerhans's cells was observed in all groups followed by Merkel cell then melanocytes.
Conclusion: There was a significant increase in oral non-keratinocytes after chronic consumption of Red-bull drink while a significant decrease was revealed the withdrawal group despite the histopathological changes of the oral epithelium were not entirely restored to normal. Oral non-keratinocytes have a substantial role in immune and inflammatory reactions besides the renovation of oral epithelium.

Keywords: Redbull, HMB-45, CD1a, CK20, Oral non-keratinocytes.

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Introduction

Energy drinks (EDs) consumption and abuse has been increasing worldwide in recent years. Red Bull (RB) is considered as the most popular energy drink. These drinks are commonly used to improve physical and cognitive performance.[1] EDs are lightly carbonated, non-alcoholic beverages with a number of energy-enhancing ingredients.[2] The stimulant effects of EDs are often due to the high concentration of caffeine and other gradients as sucrose, taurine, glucuronolactone, and B-vitamins. It is essential to highlight the potential adverse effects of energy drinks. Serious adverse effects of energy drinks have been reported like seizures, cardiac abnormalities, diabetes, or behavioral disorder.[3,4]

Non keratinocytes are a group of cells that exhibit unique structural features, perform different functions and do not participate in the epithelial maturation in the oral mucosa. They include Melanocytes (ML), Merkel cells (MKs), Langerhans cells (LCs) plus Lymphocytes and they constitute 10 % of the oral epithelial cells.[5]

MLs are a heterogeneous group of cells derived from neural crest cells produce melanin and aid in physiologic pigmentation. MLs are classified into cutaneous, follicular and extra-cutaneous MLs. The latter type executes various extra-pigmentary functions. Furthermore, melanocytes secrete signaling molecules that regulate epithelial homeostasis. [6,7]

MKs are acknowledged by Merkel in 1875 as "Tastzellen" (touch cells) and are endocrine cells associated with nerve fibers in the basal epithelial layers of animal and human oral mucosa as well as epidermis. Several reports have studied the distribution of MKs in normal and pathological conditions in skin and oral mucosa. [8]

LCs, the dendritic, antigen-presenting cells reside suprabasally in the stratified squamous epithelium of skin and oral mucosa. The dynamic LCs arise from bone marrow, constitute 2-8% of the oral epithelium and form a reticuloepithelial trap for antigens. They are initially able to stimulate naïve T-cells conditional on their subset and the type of stimulus received, and can also drive a secondary immune response by stimulating memory T cells. [9,10]

To our knowledge, no published research examined the direct effect of EDs on the occurrence of non-keratinocytes in the oral epithelium. Therefore, the aim of this study was to investigate the direct effects of RB consumption on the occurrence of oral non-keratinocytes in the oral epithelium of rats especially stratified squamous epithelium of the buccal mucosa.

Materials and methods

Ethical clearance

This study was performed according to the regulations of the Research Ethics Committee (FDASU-REC 2304) of the Faculty of Dentistry, Ain Shams University, Egypt.

Animals

18 middle-aged adult male albino rats (230-250 gm body weight aged 7-8 months) were housed under controlled temperature, humidity and dark-light cycle. This was done under supervision of specialized veterinarian since their housing till getting rid of sacrificed bodies in Medical Waste of the Animal House, Faculty of Medicine, Cairo University. The rats were kept under good ventilation and adequate stable diet consisting of fresh vegetables, dried bread and tap water throughout the experimental period.

Materials

Red Bull Cans were purchased from Egyptian markets. Each 100 ml of Red Bull comprises of caffeine C8H10N4O2 (32mg), sucrose and glucose (11.3 g), taurine (400

mg), gluconolactone (240 mg), B12 (0.4mcg), B6 (0.8 mg), B2 (0.64 mg), niacin (7.2 mg), panthenol (2.4 mg), inositol (20 mg), Carbonated water and artificial flavoring (Kassab and Tawfik, 2018).[11]

Sample size calculation

The sample size was calculated using Minitab computer program for three groups. The calculation was based on the data of reference to Hulail et al., 2020. [12]Analysis of Variance with max difference of means 2.58 and 0.16 estimated standard deviation. Two cases per group is required to achieve a power of 80% and an alpha error 0.05. Four cases were added to each group to make a total of 6 cases per group.

Experimental design

The animals were kept in acclimatization period for one week then were randomly divided into three groups (six rats each) as follows:

Group I (Control): consisted of 6 adult male albino rats and received 1.5 ml/100g distilled water by oral gavage. [11,13,14]

Group Ia: consisted of 3 animals and were sacrificed after 4 weeks.

Group Ib: consisted of 3 animals and were sacrificed after 8 weeks.

Group II (Red Bull group): consisted of 6 adult male albino rats and received Red Bull by oral gavage with a daily dose of 1.5ml/100g. Animals were sacrificed after 4 weeks. [11,13,14]

Group III (withdraw group): consisted of 6 adult male albino rats and received the aforementioned dose of Red Bull by oral gavage for the same period. Then, rats were left for another 4 weeks without any treatment. Animals were sacrificed after 8 weeks. [11,12,13,14]

Getting rid of sacrificed rats' bodies was done according to ethical committee rules in the incinerator of Cairo University Hospital.

Tissue preparation for light microscopic examination

Specimens for light microscope (LM) examination were fixed in 10 % neutral buffered formalin for 48 hours. Each specimen was rinsed in a water beaker and then placed in capsules carrying certain codes for each rat to be dehydrated in ascending concentrations of ethanol, infiltrated then embedded in paraffin wax and processed. Next, 5 µm thick sections were executed for routine histological examination using hematoxylin and eosin (H & E) stain.[15]

Immuno-histochemical examination

Immune-histochemical examination for immunoreactivity of Langerhans cells (LCs) to CD1a [9], HMB-45 for melanocytes (MLs) [16] and CK20 for Merkel cells (MKs) [7]. 4-5 µm thick paraffin sections were obtained from each group and mounted on positively charged slides. The immuno-stained sections were examined under the light microscope, where the positive reaction appeared as brown membranous, cytoplasmic, and/or nuclear staining.[17,18]

Histomorphometric analysis

H & E and immuno-stained sections were examined by digital light microscope (Carl Zeiss Microscopy Gmbh, Primostar 3 , FULL-K, bi, cam, Fov22, 5 pos, Axiocam 208 color camera, LED 3W white light, 5600k , made in Germany). At x400 magnification, the density of immunoreactive cells in the oral epithelium was assessed by analyzing the surface area of positive cells per mm^2 of epithelial sheet. The images analysis was implemented by ImageJ v 1.54j (2024) using processing tools that segment pixels in a digital image based on color or density. Blinding was done during histomorphometric analysis to ensure unbiased results.

Statistical Analysis

All data were calculated, tabulated, and statistically analyzed using one way ANOVA (Analysis of variance) to compare between the groups under study. Bonferroni post hoc test was performed for pair wise comparisons among the groups. P value ≤ 0.05 is considered statistically significant. Statistical analysis was performed using the computer program SPSS software for windows version 26.0 (Statistical Package for Social Science, Armonk, NY: IBM Corp) at significant levels<0.05 (P- Value).

Results

Histopathological results

Group I (Control): Results of subgroups Ia and Ib were almost identical. In rats, the keratinized oral mucosa of the control group (middle aged) showed oral epithelium with few basal and suprabasal oval or round clear cells and small hyperchromatic nuclei. The clear halo was mostly caused by cytoplasmic shrinkage occurred during processing. Likewise, few degenerative signs included nuclear pyknosis and karyrrhexis were seen in basal, spinous and granular keratinocytes. Subepithelial degenerative areas, few congested blood vessels, along with inflammatory cells were also observed in the lamina propria (Fig.1a).

Group II (Redbull group): Inflammatory and degenerative signs were observed in some areas of this group. Apparent increase of clear cells was noticed in basal and suprabasal cell layers. Most specimens presented basilar epithelial hyperplasia, binucleated cells, cloudy swelling and apparent pale eosinophilic cytoplasm in some keratinocytes in different epithelial layers. Apparent increase of apoptotic changes such as nuclear pyknosis, karyorrhexis and cell vacuolation were also perceived. Moreover, apparent increase in the epithelial and keratin thickness was displayed in some areas. Underlying lamina propria presented apparent increase of subepithelial degeneration and inflammatory cells along with congested blood vessels (Fig. 1b).

Group III (Withdrawal group): Restoration of inflammatory changes to some extent was distinguished in this group. Most of the examined H&E sections revealed apparent decrease of clear cells in oral epithelium and reduced degenerative signs. Congested and lengthened blood vessels were demonstrated in some areas (Fig.1c).

Immunohistochemical Results

Immunohistochemical Results of subgroups Ia and Ib were almost alike.

Immunohistochemical results for CD1a immunoreactivity

Group I (control): Langerhans cells (LCs) were examined in the rat oral mucosa using CD1a+ immunomarker. The oral epithelium of this group showed almost negative reactivity to CD1a+ (Fig.2a).

Group II (Redbull group): Comparing to control group, most specimens displayed a significant increase in CD1a positive LCs with strong expression in the basal and suprabasal layers of the oral epithelium (membranous & cytoplasmic expression). Sub-epithelial immunoreacted cells in underlying lamina propria were also revealed. This immunoreaction was demonstrated as numerous large brown dendritic LCs that were variable in length and number of dendrites. In addition, some positive non-dendritic oval or round LCs in basal and suprabasal layers were also detected (Figs.2b).

Group III (Withdraw group): Oral epithelia of this group compared to Redbull group showed a significant decrease in the positively reacted LCs but a significant increase still identified in this group comparing to control group. LCs appeared with reduced dendrites (Fig.2c).

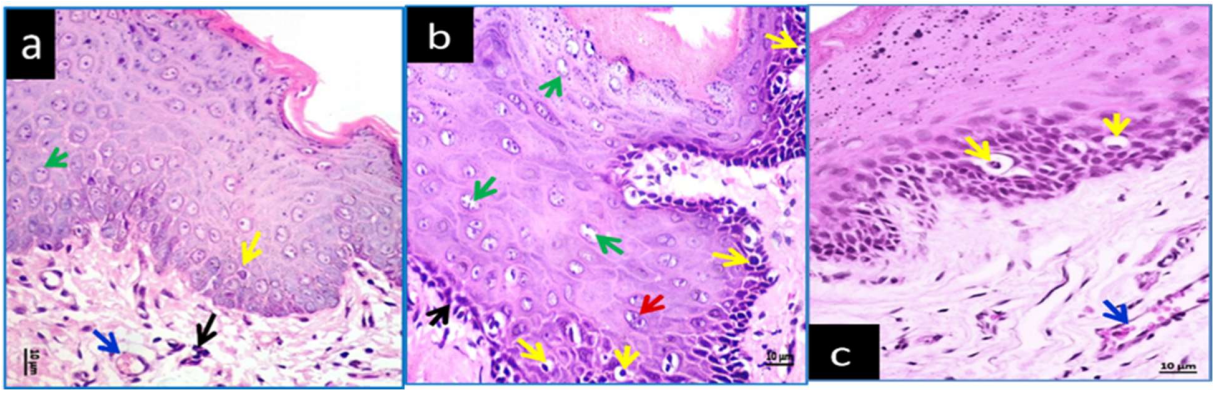

Figure 1: Photomicrographs of rat oral mucosa showing (a) control group with few basal and suprabasal clear cells , few apoptotic keratinocytes and some degenerative areas in the lamina propria, inflammatory cells with few congested blood vessels, (b) Redbull specimens presented apparent increase in clear cells, basilar hyperplasia, binucleated cells (red arrow), cloudy swelling of keratinocytes, apoptotic keratinocytes with cell vacuolation, pyknosis &karyorrhexis, in addition to subepithelial degeneration and some inflammatory cells, (c) Withdrawal sections with apparent decrease in clear cells and degenerative signs & congested lengthened blood vessels, Original magnification (H&E, X400). Clear cells (yellow arrows), apoptotic keratinocytes (green arrows), inflammatory cells (black arrows), congested blood vessels (blue arrows). (Original magnification x400).

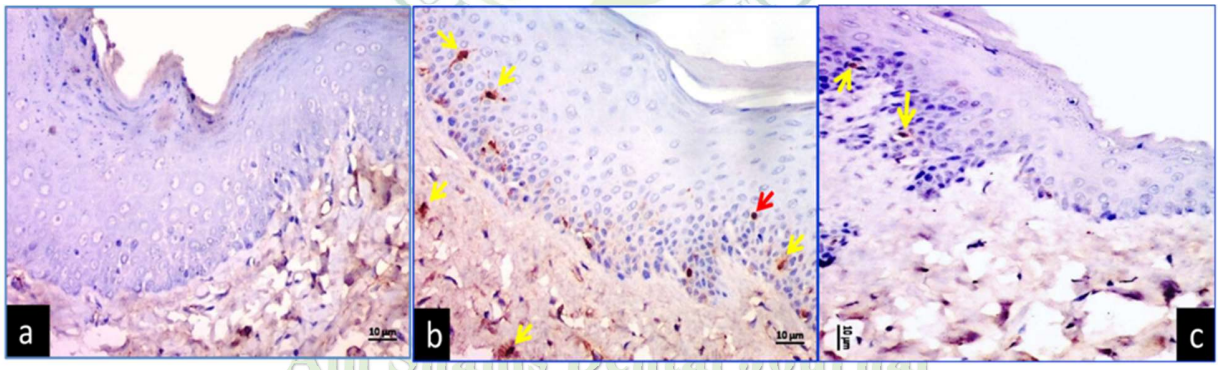

Fig. 2: Photomicrographs of rat oral mucosa showing the immunohistochemical reaction to CD1a (a) Control group (I) presenting the negative reaction of the oral epithelium, (b) Redbull group (II) displaying a significant immunoexpression of dendritic (yellow arrows) and non-dendritic (red arrow) LCs in basal & suprabasal epithelial layers as well as in lamina propria, (c) Withdraw group (III) showing a significant decrease in CD1a positive cells. (Original magnification x400)

Immunohistochemical results for CK20 immunoreactivity

Group I (control): Merkel cells (MKs) were examined in the rat oral mucosa using CK20 immunomarker. Normal oral epithelium exhibited negative immunoreactivity in some oral mucosal areas while some areas presented single oval or round CK20 immunoexpressed MKs in the basal cell layer (Fig.3a).

Group II (Redbull group): Compared to the control group, the oral epithelium of this group exhibited a significant increase in immunoreactive MKs. Most MKs were oval or spherical in shape while some cells appeared polygonal dendritic with cytoplasmic processes of various lengths and number. Few ectopic MKs resided in prickle cell layer. MKs presented with focal &/or diffuse cytoplasmic expression (Fig.3b).

Group III (Withdraw group): Oral epithelium of this group compared to the Redbull group displayed a significantly decreased CK20- positive MKs in the basal epithelial layer. However, the significant increase in the immunoexpressed MKs compared to the control group was also elucidated (Fig.3c).

Immunohistochemical results for HMB-45 immunoreactivity

Group I (control): Oral active/stimulated melanocytes (MLs) were examined in the rat oral mucosa using HMB-45 immunomarker. The oral epithelium displayed very few immunoreacted MLs to HMB-45 in the basal cell layer (Fig.4a).

Group II (Redbull group): In comparison to the control group, the oral epithelium of this group displayed a significant increase of immunoreacted active/stimulated melanocytes. This expression was manifested as a strong , focal cytoplasmic reaction in some areas, while it appeared as a moderate and diffuse reaction in other areas (Fig.4b).

Group III (Withdraw group): Oral epithelium of this group compared to the Redbull group displayed a significant decrease in the positively reacted MLs. However, the significant increase in positive MLs still revealed when compared to the control group. Active/stimulated MLs showed a mild to moderate (focal/diffuse) cytoplasmic expression in the basal epithelial layer (Fig.4c).

Statistical Results

The density of the immunoreaction in the oral epithelium (CD1a for Langerhans cells, CK20 for Merkel's cells and HMB-45 for Melanocytes) was measured by analysing the surface area of positive cells per mm^2 of the epithelial sheet in all studied groups. A significant increase (P value<0.001) in the expression of CD1a, CK20 and HMB-45 in group II (Redbull) was noticed when compared to group I (control) and group III (withdrawal). The highest significant mean value in the Redpull group was perceived in CD1a (LCs) followed by CK20 (MKs) and then HMB-45 (MLs) respectively. Group III (withdrawal) presented a significant decrease (P value <0.001) in the reactivity of CD1a, CK20 and HMB-45 compared to group II (Redbull). But a significant increase (P value<0.001) in group III (withdrawal) was still noted in contrast to the control group. The least significant mean value in the withdrawal group was observed in HMB-45 (MLs) followed by CD1a (LCs) and then CK20 (MKs) respectively. Comparing the results of CD1a, CK20 and HMB-45 immunoexpression in all studied groups, statistically non-significant differences (P value = 0.158) were detected in the control group while significant differences were shown in the Redpull group (P value=0.001) as well as the withdrawal group (P value <0.001) (Table1 and fig.5).

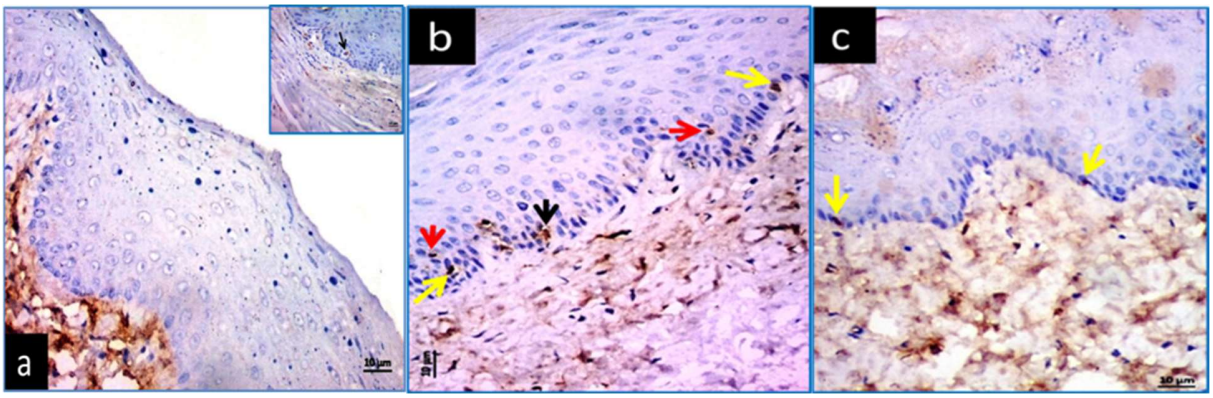

Figure 3: Photomicrographs of rat oral mucosa showing immunohistochemical reaction to CK20 (a) Control group (I) showed negative expression in some areas of the oral epithelium, single immunoreacted MK cell (black arrow) in the basal cell layer in other areas (inset; x400), (b) Redbull group (II) displayed a significant increase of round or oval MKs (yellow arrows) as well as polygonal dendritic MKs (black arrows), ectopic MKs in prickle cell layer (red arrows), (c) Withdraw group (III) exhibited a significant decrease of CK20 positive MKs (yellow arrows) in the basal cell layer. (Original magnification x400)

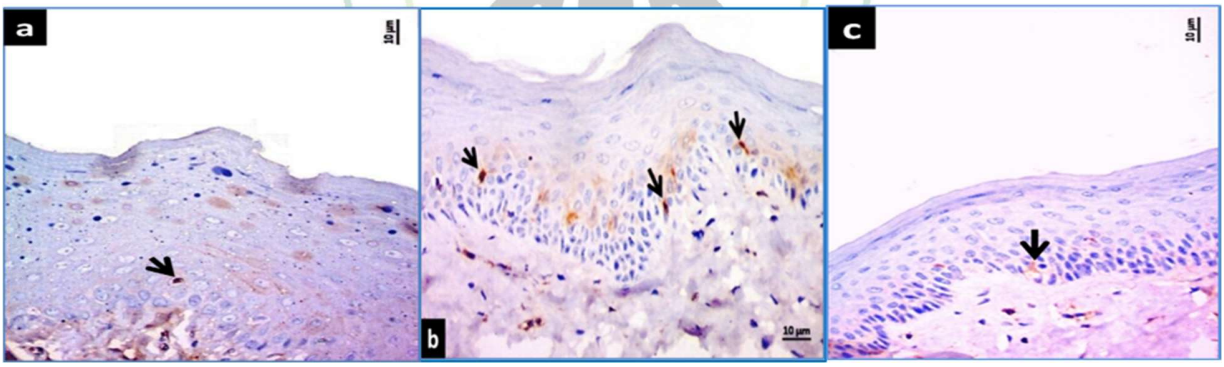

Figure 4: Photomicrographs of rat oral mucosa showing the immunohistochemical reaction to HMB-45 (a) Control group (I) with a positively stained melanocyte (black arrow) in the basal cell layer, (b) Redbull group (II) displayed a significant increase of melanocytes with focal (black arrow) as well as diffuse immunoreaction, (c) Withdraw group(III) exhibited a significant decrease of HMB-45 positive melanocytes (black arrow). (Original magnification x400).

Table 1: Showing surface area/mm² mean values and standard deviations (SD) of immunoexpression for markers of Langerhans cells (CD1a), Merkel's cells (CK20) and Melanocytes (HMB-45) in all studied groups

	CD1a		CK20		HMB-45			
	Mean	SD	Mean	SD	Mean	SD	F test	P value
Control	11.06$^{c\ B}$	2.23	12.82$^{c\ A}$	3.32	10.27$^{c\ C}$	1.22	2.045	0.158
Red bull	163.89$^{a\ A}$	13.81	144.45$^{a\ B}$	14.62	129.18$^{a\ C}$	11.93	17.90	0.001**
withdraw	115.53$^{b\ B}$	5.35	123.83$^{b\ A}$	7.48	40.66$^{b\ C}$	9.38	255.491	<0.001**
F test	571.05		1185.241		345.61			
P value	<0.001**		<0.001**		<0.001**			

**means significant difference at P<0.05
Small superscript letters at the same column means significant difference between groups.
Capital superscript letters at the same row means significant difference between markers.

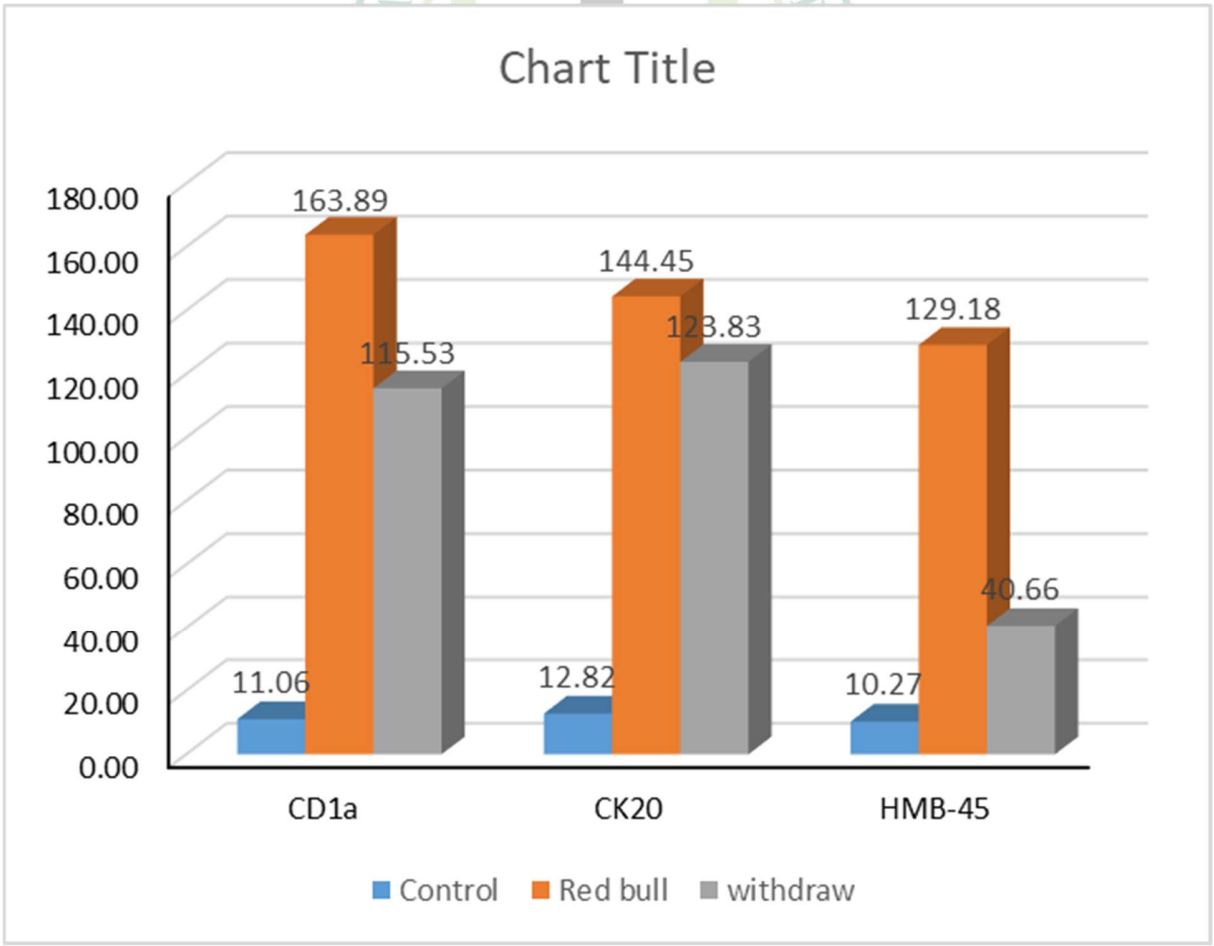

Figure 5: Bar chart showing CD1a, CK20 and HMB-45 immunoreaction (surface area of positive cells per mm2 of the epithelial sheet) in all studied groups.

Discussion

EDs are mostly consumed on a daily basis to increase alertness and boost athletic performances. The active ingredients of Redbull ED are; caffeine, taurine, glucose and different groups of vitamin B.[19] Non-Keratinocytes (clear cells) present in the oral epithelium are Melanocytes (MLs), Langerhans cells (LCs), Merkel cells (MKs) and inflammatory cells. [5]

The present study aimed to histologically and immunohistochemically assess the effect of chronic consumption of Redbull ED on the occurrence of non-keratinocytes (MLs, MKs and LCs) in the keratinized oral mucosa of male albino rats. It should be mentioned that the current study to our knowledge is the first study on the effect of Redbull ED on oral non-keratinocytes.

Non-keratinocytes were labelled immunohistochemically in the current study using antibodies that target intracellular glycoproteins; CD1a (cluster of differentiation1a), CK20 (cytokeratin 20) and HMB-45 (homatropine methyl bromide-45) to evaluate the density of non-keratinocytes in the study groups. CD1a marker has been used to detect LCs that play an important role in inflammatory conditions and regulation of immune responses. [9] CK20 marker has been employed to identify MKs in squamous epithelium which are considered as slowly adapting mechanoreceptors type I.[20] It was reported that HMB-45 marker selected in this study is the most sensitive marker for melanocytes.[21]

In the present study, H & E and immunohistochemical results of subgroups Ia and Ib were almost identical.The H & E results of untreated middle-aged adult rats (control group) were paralleled to those of Ali et al [22] investigation. The authors accredited the few deteriorative changes explored in the intestinal mucosa of middle aged rats (8 months) to the "aging free radical theory," of the aging process.

The Red-bull group of this study revealed an apparent increase of clear cells (non-keratinocytes) compared to group III (withdrawal) and group I (untreated control adult rats). This increase was associated with the increase of the degenerative changes included apparent increase of apoptotic signs such as nuclear pyknosis, karyorrhexis and cell vacuolation in oral keratinocytes along with increased inflammatory cells in the underlying lamina propria. These results were consistent with Abdelwahab et al [23] and Brizuela and Winters [5] findings. Graneri et al [24] declared that chronic use of energy drinks leads to an inflammatory response and cell apoptosis. Shaikh and Rajeh [25] attributed these deleterious inflammatory effects of the increased EDs consumption to the increase of the reactive oxygen species and oxidative stresses together with the significant decrease in the antioxidant enzymes in epithelial cells. Thus, the resulting disturbance in cell membrane permeability, volume and homeostasis could be related to the increase of artificial sweeteners and sucrose in EDs, high insulin availability and finally the increased the catabolic rate. Moreover, Al Sammak et al [26] supported the results of the existing study as taurine in Red-bull plays a role in lipid digestion of the cell membrane which leads to marked cellular degeneration and apoptosis.

The distinguished cell binucleation as well as the increased epithelial thickness in the Red-bull group can be explained by the fact that with chronic consumption of EDs, endomitosis for DNA duplication in oral keratinocytes together with increased mitotic figures represents an adaptive response and an attempt at cellular repair. [11]

The H & E stain results of group II (Red-bull) in the existing study were supported by the significant increase of the immune-positive reaction and area % of CD1a, CK20 and HMB-45 expression by LCs, MKs and MLs respectively compared to group III

(withdrawal) and group I (control). LCs showed the highest mean values in all study groups compared to MKs and MLs reflecting its role in the regulation of immune reactions.[9]

In accordance to LCs results of the untreated group of adult middle-aged rats in the current study, Mathew et al [27] declared that keratinized epithelium involves less LCs than non-keratinized epithelium. Non-dendritic LC is the typical form if explored in normal mucosa than the dendritic ones. Wang et al [28] stated that LCs reside within the basal and suprabasal forming a link between the oral mucosa and the immune system.

The significant increase of CD1a+ LCs in the Red-bull group in the present study could be ascribed to the inductive effect of inflammatory cytokines (like tumour necrosis factor) released by oral keratinocytes as an inflammatory response to excessive consumption of EDs. Oral CD1a+ LCs have a pivotal role in oral pathologic and inflammatory conditions. LCs mobilize and mature in response to inflammatory cytokines that can affect the immunological activity of LCs. [29,30] Notably, Attia and Nasr[31] reported that EDs increase inflammatory response with increased lymphocytic motility and cytotoxicity. In addition, excessive intake of carbonated drinks especially those with high caffeine (Domínguez et al) [32] increases cortisol level due to the increase of stress hormones resulting in inflammation.[33] Additionally, citric acid, magnesium and so cadmium bicarbonate in Red-bull can increase the density of macrophages to reduce inflammation. [34, 35, 36]

In agreement with the apparent prominence of dendritic LCs in the Red-bull group of the ongoing study, Ferrisse et al[9] reported the increase of dendritic CD1a + LCs for the surveillance of a wide epithelial area reflects their biological trap function for antigens. LCs transform the antigen proteins into antigenic peptides, which are consequently presented to CD8+ T-cells including T helper-17 cells that produce high levels of interleukin-17. Thus, oral LCs exhibit a high stimulatory capacity.

In parallel to the findings of CK20+ MKs of this study, Tachibana et al [37] and Righi et al [7] informed that the incidence of MKs was relatively low in normal mucosa. MKs have been explored as a single basally located cell within oral keratinocytes in areas frequently subjected to masticatory trauma. In rodents and human, the polymorphic oval/ round and dendritic MKs have been detected in the oral mucosa as well as non-innervated and innervated MKs denoting the functional heterogeneity of the cells. Furthermore, MKs act as mechanoreceptors, changing mechanical stimuli to action potential in the afferent nerve fibers. Previous reports stated that this specialization as a neural receptor in oral MKs is not as high as that of cutaneous MKs. [38]

Concerning the results of Red-bull group in the current study, the significant increase of CK20 immunoexpressed MKs could be accredited to the chronic inflammation caused by the excessive consumption of EDs. In concurrence with this result, Tachibana et al [37] and Righi et al [7] disclosed the increase of MKs within the inflamed oral mucosa in human and rodents with a higher incidence of oval/ round and non-innervated MKs alongside the occurrence of ectopic MKs. Through the neuroendocrine function, the neuropeptides (growth factors) produced by MKs have a modulatory effect on immunocytes, promote cell proliferation and differentiation of keratinocytes, endothelial cells besides fibroblasts and can also induce pluripotent stem cells.[20] These indicate the cell role in the regeneration and reparative processes via its endocrine-paracrine function.[39] Moreover, the inflamed oral mucosa exhibited greatly reduced innervation and innervated MKs indicating peripheral neural damage. This is clarified by Yarım et

al [40] and Bano et al [41] who found that caffeine present in EDs caused neuronal cell death by inhibiting adenosine receptors leading to interstitial inflammation, and ROS generation. However, it was stated by Yarım et al [40] that the taurine amino acid in the Red-bull act as a neurotransmitter which regulate nerve cell activity and maintain their calcium levels and their development.

Regarding the negatively HMB-45 immunoreacted MLs in the control adult rats in the present study, Thomas and Erickson[42] reported that MLs are dendritic melanin-producing cells located in the basal layer of the oral epithelium. Aiiltors et al[43] testified that oral mucosa in rats normally displayed no melanocytes. Yet, it was stated that melanocyte release signaling molecules that act as regulators maintaining epithelial homeostasis.

In harmony with the significant increase of HMB-45 immunopositive MLs in the Red-bull group of the existing study, Feller et al[44] and Xiao et al[45] postulated that oxidative stresses and inflammatory conditions induced by EDs could trigger melanogenic activity via increasing number of oral MLs and oral epithelial melanin that act as a defense barrier scavenging the free radicals. [46,47] In chronic inflamed mucosa, increased expression of HMB-45 by metabolically active MLs may relate to the generation of stimulatory cytokines and mitogenic factors for MLs such as melanocyte-stimulating hormone released by infiltrating neutrophils. Consecutively, oral MLs function as neuroendocrinal cells producing neurotransmitters, neuropeptides besides hormones. Additionally, acting as stress-sensors they react and generate immunomodulatory cytokines as well as growth factors in inflammatory conditions. Phagocytic and antigen presenting function of MLs in inflammation was also elucidated. [44,48] Furthermore, Zhou et al [49] declared that citric acid in Red-bull increases melanin content in mice and decrease melanin content in human. In addition, Brescoll and Daveluy[50] and Katsuyama et al [51] reported that vitamin B6 and B12 boost melanin production.

Regarding withdrawal group (III), the current study revealed a statistically significant decrease of MLs, LCs and MKs respectively with the markedly renovated histological architecture of oral keratinocytes compared to Red-bull group (II). This finding could result from the above-mentioned attempts at repair and regeneration such as increased mitotic figure. These attempts began with chronic intake of EDs and continued after elimination of EDs for 4 weeks, resulting in attenuation of oxidative stresses. This ensued in ceasing the disturbances in the synthesis of proteins that regulate the cell cycle, lower the stress hormone level and preserve the cell membrane phospholipids from apoptosis. [11, 23, 26, 52] Furthermore, (Akande and Banjoko)[2] believed that the deteriorating changes caused by excessive intake of caffeinated energy drinks could be reversed. However, the withdrawal group in the ongoing study still elucidated a significant increase in non-keratinocytes as well as an apparent increase in inflammatory changes compared to the untreated control group.

Conclusion

There was a significant increase in oral non-keratinocytes after the chronic consumption of the Red-bull energy drink as proved in the current study. However, Red-bull withdrawal exhibited a significant decrease in oral non-keratinocytes but the histopathological changes detected in the oral epithelium were not entirely restored to normal. Oral non-keratinocytes play a substantial role in immune and inflammatory reactions as well as in the regeneration of surrounding keratinocytes. Accordingly, we have concerns about the safety of using energy drinks. So, it is recommended to

eliminate the use of the energy drinks and replace them with fresh, natural drinks.

Conflict of interest

There are no conflicts of interest.

Funding

Self –financed research.

Data availability

All the data regarding the results of the present study are original and available as a part of the article and no additional sources data are required.

Ethical Statement

This study was performed according to the regulations of the Research Ethics Committee (FDASU-REC 2304) of the Faculty of Dentistry, Ain Shams University, Egypt.

Declarations and competing interests

The authors state that, there is no known challenging financial benefits or personal relationships that would have seemed to affect the work described in the study.

References

1. Kaminer Y. Problematic use of energy drinks by adolescents. Child and adolescent psychiatric clinics of North America. 2010;19(3):643-650.
2. Akande IS, Banjoko OA. Assessment of biochemical effect of "Power Horse" energy drink on hepatic, renal and histological functions in Sprague Dawley rats. Annual Review & Research in Biology. 2011;1(3):45-56.
3. Ragsdale FR, Gronli TD, Batool N, Haight N, Mehaffey A, McMahon EC, Nalli TW, Mannello CM, Sell CJ, McCann PJ, Kastello GM. Effect of Red Bull energy drink on cardiovascular and renal function. Amino acids. 2010;38:1193-200.
4. Mora-Rodriguez R, Pallares JG. Performance outcomes and unwanted side effects associated with energy drinks. Nutrition reviews. 2014;72(1):108-120.
5. Brizuela M, Winters R. Histology, Oral Mucosa. StatPearls Publishing. 2024.
6. Kamble RG and Minchekar VS. Non keratinocytes of oral mucosa - A brief review. International Journal of Current Research. 2018;10(12):75880-75882.
7. Righi A, Betts CM, Marchetti C, Marucci G, Montebugnoli L, Prati C, Eusebi LH, Muzzi L, Ragazzini T, Foschini MP. Merkel cells in the oral mucosa. International journal of surgical pathology. 2006;14(3):206-211.
8. Natesan SC, Ramakrishnan BP, Krishnapillai R, Thomas P. Biophysiology of oral mucosal melanocytes. Journal of Health Sciences & Research. 2019;10(2):47-51.
9. Ferrisse TM, de Oliveira AB, Palaçon MP, da Silveira HA, Massucato EM, de Almeida LY, Léon JE, Bufalino A. Immunohistochemical evaluation of Langerhans cells in oral lichen planus and oral lichenoid lesions. Archives of oral biology. 2021;124:105027.
10. Barrett AW, Cruchley AT, Williams DM. Oral mucosal Langerhans' cells. Critical Reviews in Oral Biology & Medicine. 1996;7(1):36-58.
11. Kassab AA, Tawfik SM. Effect of a caffeinated energy drink and its withdrawal on the submandibular salivary gland of adult male albino rats: A histological and immunohistochemical study. Egyptian Journal of Histology. 2018; 41(1):11–26.
12. Hulail ME, Qenawy NM, Abdel-Kareem RH, Mohamed GA. The toxic effect of energy drinks on the structure of pancreas of adult male albino rats. Zagazig University Medical Journal. 2020;26(6):1110-1117.
13. Ugwuja E. Biochemical effects of energy drinks alone or in combination with alcohol in normal albino rats. Advanced pharmaceutical bulletin. 2014;4(1):69.
14. Khayyat L, Essawy A, Al Rawy MM, Sorour JM. Comparative study on the effect of energy drinks on haematopoietic system in Wistar albino rats. Journal of Environmental Biology. 2014;35(5):883-891.
15. Kiernan J. Histological and histochemical methods. Scion publishing ltd. 2015.
16. Robinson L, Bunn BK, Blumenthal R, Bernitz H. The hypopigmented bitemark a clinical and histologic appraisal. International Journal of Legal Medicine. 2023;137(1):99-104.
17. McIlwain DR, Berger T, Mak TW. Caspase functions in cell death and disease. Cold Spring Harbor perspectives in biology. 2013; 5(4):a008656.
18. Kim SW, Roh J, Park CS. Immunohistochemistry for pathologists: protocols, pitfalls, and tips. Journal of pathology and translational medicine. 2016; 50(6):411–418.

19. Bawazir AE. Effects of Energy Drink (Red bull) on some neurotransmitters content and histological structure in the hippocampus region in male albino rats. International Journal of Pharmaceutical Research & Allied Sciences. 2017;6(2):263-276.
20. Tong CK, Moayedi Y, Lumpkin EA. Merkel cells and keratinocytes in oral mucosa are activated by mechanical stimulation. Physiological Reports. 2024;12(2):e15826.
21. Honwad S, Ravi SV, Donoghue M, Joshi M. Immuno-histochemical and quantitative study of melanocytes and melanin granules in oral epithelial dysplasia. Journal of Clinical and Diagnostic Research. 2017;11(7): 56–58.
22. Ali, R.A.M., Abdelrahima, E.A., Ahmed, Y.A.G. and Abdel-lateif, S.M. Effect of Aging on the Histological Structure of the Duodenum Wall in Male Albino Rats. SVU-IJMS. 2020; 3(1): 42-47.
23. Abdelwahab W, Elsayed S, Afify A, Mohammed A, Abd AlRahman R. Study of the biochemical, histological and cytogenetic effects of two different energy drinks (EDs); Red bull and power horse; on brain of adult male Albino rats and to determine the possible protective role of omega-3 on the adverse effects of EDs. Journal of Recent Advances in Medicine. 2020; 1(2):55–66.
24. Graneri L, D'Alonzo Z, Lam V, Mamo J, Dhaliwal S, Takechi R. Chronic Consumption of a Commercial Energy Drink Reduces Blood Pressure in Normotensive Wild-Type Mice. Frontiers in Nutrition. 2019; 6(111):1–7.
25. Al-Shaikh TM, Rajeh NA. Ameliorating effect of blueberry consumption on energy drink-induced testicular damage in rats: histological and immunohistochemical study. The Journal of Basic and Applied Zoology. 2023; 84(1):1-9.
26. Al Sammak M, Qassim AH, Hamdi OR. Histological Changes of Stomach and Intestine Induced by Energy Drink (Tiger) in Adult Male Rats. Open Access Macedonian Journal of Medical Sciences. 2021;9:735-740.
27. Mathew JK, Pandian RMK, Gaikwad P, Rabi S, Nadu TT. Non-dendritic Langerhans cells: A new entity in normal and malignant buccal mucosa. European Journal of Anatomy 2019; 23 (5): 383-388.
28. Wang YP, Chen IC, Wu YH, Wu YC, Chen HM, Chang JY. Langerhans cell counts in oral epithelial dysplasia and their correlation to clinicopathological parameters. Journal of the Formosan Medical Association. 2017;116(6):457-463.
29. Hasseus B, Dahlgren U, Bergenholtz G, Jontell M. Antigen presenting capacity of Langerhans cells from rat oral epithelium. Journal of oral pathology & medicine. 1995 Feb;24(2):56-60.
30. Upadhyay J, Upadhyay RB, Agrawal P, Jaitley S, Rhitu ShekhaR. Langerhans Cells and Their Role in Oral Mucosal Diseases. North American Journal of Medical Sciences. September 2013;5(9).
31. Attia AM, Nasr HM. Evaluation of the protective effect of omega-3 fatty acids and selenium on paraquat intoxicated rats. Slovak Journal of Animal Science.2009;42(4):180-187.
32. Domínguez GR, Mateos RM, Sancho LAM, Cortés GJJ, Cuevas CM, Cots RJA, Segundo C, Schwarz M.: Synergic effects of sugar and caffeine on insulin-mediated metabolomic alterations after an acute consumption of soft drinks. Electrophoresis. 2017;38(18):2313-2322.
33. Ariffin H, Chong XQ, Chong PN, Okechukwu PN. Is the consumption of energy drink beneficial or detrimental to health: a comprehensive review?. Bulletin of the National Research Centre. 2022;46(1):163.
34. Abdel-Salam OME, Youness ER, Mohammed NA, Morsy SMY, Omara EA and Sleem AA Citric acid effects on brain and liver oxidative stress in lipopolysaccharide-treated mice. Journal of medicinal food. 2014; 1; 17(5): 588–598.
35. Kawakami T, Koike A, Maehara T, Hayashi T, Fujimori K. Bicarbonate enhances the inflammatory response by activating JAK/STAT signalling in LPS+ IFN-γ-stimulated macrophages. The journal of biochemistry. 2020;167(6):623-631.
36. Vida C, Carracedo J, de Sequera P, Bodega G, Pérez R, Alique M, Ramírez R. Increasing the magnesium concentration in various dialysate solutions differentially modulates oxidative stress in a human monocyte cell line. Antioxidants. 2020;9(4):319.
37. Tachibana T, Kamegai T, Takahashi N and Nawa T. Evidence for polymorphism of Merkel cells in the adult human oral mucosa. Archives of histology and cytology. 1998;61(2): 115-124.
38. Nikai, H., Rose, G.G. and Cattoni, M. Merkel cell in human and rat gingiva. Archs oral Bid. 1971; 16:835-843.
39. Caballero GL, Caneiro J, Gándara M, González Ortega N, Cepeda Emiliani A, Gude F, Collado M, Gallego R. Merkel cells of human oral mucosa express the pluripotent stem cell transcription factor Sox2. Histology and histopathology. 2020; 35(9): 1007–1012.
40. Yarım G, Gökçeoğlu A, Yarım M. The effects of taurine on central nervous system. Harran Üniversitesi Veteriner Fakültesi Dergisi. 2020;9(2):214-219.

41. Bano SS, Shafqat F, Shafi S, Hussain A, Bukhari S. Effects of caffeinated energy drinks on cerebellum of Male albino rats. MedERA- Journal of CMH LMC. 2022; 4(1):1–8.
42. Thomas AJ, Erickson CA. The making of a melanocyte: the specification of melanoblasts from the neural crest. Pigment Cell Melanoma Res. 2008;21(6):598-610.
43. Aiiltors EE, Laksson PA and Berostresser PR. Langerhans cell surface densities in rat oral mucosa and human buccal mucosa. Journal of Oral Pathology. 1985; 14:390-397.
44. Feller L, Masilana A, Khammissa RA, Altini M, Jadwat Y, Lemmer J. Melanin: the biophysiology of oral melanocytes and physiological oral pigmentation. Head & face medicine. 2014 Dec;10:1-7.
45. Xiao L, Mochizuki M, Nakahara T, Miwa N. Hydrogen-generating silica material prevents UVA-ray-induced cellular oxidative stress, cell death, collagen loss and melanogenesis in human cells and 3D skin equivalents. Antioxidants. 2021;10(1):76.
46. Tian T, Zhang WY, Zhou HY, Peng LJ, Zhou X, Zhang H, Yang FQ. A catechol-meter based on conventional personal glucose meter for portable detection of tyrosinase and sodium benzoate. Biosensors. 2022;12(12):1084.
47. Simila CSA, Isaac Joseph TI, Prasanth T, Girish KL. Quantitative Analysis of Apoptotic Cells in Normal Mucosa, Oral Epithelial Dysplasia and Oral Squamous Cell Carcinoma Using Methyl Green. International Journal of Health Sciences & Research. 2018; 8 (9):52-56.
48. Masilana A, Khammissa RA, Lemmer J, Feller L. Oral medicine case book 66: Physiological/racial oral melanin hyperpigmentation. South African Dental Journal. 2015;70(1):28-9.
49. Zhou S, Sakamoto K. Citric acid promoted melanin synthesis in B16F10 mouse melanoma cells, but inhibited it in human epidermal melanocytes and HMV-II melanoma cells via the GSK3β/β-catenin signaling pathway. PLOS One. 2020;15(12):e0243565.
50. Brescoll J, Daveluy S. A review of vitamin B12 in dermatology. American journal of clinical dermatology. 2015;16:27-33.
51. Katsuyama Y, Hiyama K, Sawamura A, Kawase I, Okano Y, Masaki H. Pyridoxine Has a Potential to Prevent the Appearance of Pigmented Spots: Effects on the Phagocytosis and Differentiation of Keratinocytes. Biological and Pharmaceutical Bulletin. 2022;45(9):1378-1384.
52. Denewer SA, Elsabaa H. Effect of caffeine versus hydrogen peroxide on human skin fibroblast cell line cytotoxicity, cell cycle phases, and apoptosis. Ain Shams Dental Journal. 2024; 34(2):152-158.