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Effect of Energy drink and its withdrawal on Dorsal Surface of Albino Rat tongue (Histological & Immuno-histochemical study)

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Aim: To study the effect of intake and withdrawal of EDs on the histological structure of dorsal surface of the tongue of adult male albino rats.
Materials and Methods: 30 adult male albino rats were divided into 3 groups; 10 rats each. Group I (control group), group II (Redbull group), the rats received 2 ml/200gm of Redbull twice daily orally for 4 weeks. Group III (Withdrawal group) the rat received Redbull in the same dose and duration as group II then sacrificed after further 4 weeks without treatment. Paraffin sections of dorsal surface of the tongue were stained by H & E, Masson trichrome and Caspase-3 immuno-histochemical study. Statistical analysis on area% of collagen fibers and apoptotic cells outcomes was performed.
Results: H & E results of group II revealed atrophied blunt ended filiform papillae with hyperplastic changes and elongated rete ridges. Focal areas of dysplastic changes were detected in the epithelium. Loss of taste buds occurred in both fungiform and circumvallate papillae. Lamina propria showed mononuclear cellular infiltration in some areas. Group III regained the normal appearance of tongue papillae. However, some hyperplastic and dysplastic changes in the epithelium remained. Significant decrease in area % of collagen fibers was detected in group II compared with other groups. Whereas Caspase-3 positive reaction showed significant increase in group II compared with other groups.
Conclusion: Redbull induced structural changes in the dorsal surface of albino rats' tongue which were partially improved by its withdrawal. Excessive intake of Redbull considered as health hazard.

Key words: Caspase-3, Masson Trichrome, Redbull, Rat tongue papillae.

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Introduction

The Food and Drug administration (FDA) defines energy drinks (EDs) as a class of products in liquid form that typically contain caffeine with or without other added ingredients.[1]

Energy drinks (EDs) are widely spread all over the world. It was obvious that EDs consumption was recently shifted from athletes to adolescents to increase their physical performance without knowledge of their health hazards. However, insomnia, nervousness, restlessness, gastric irritation, nausea, vomiting, tachycardia, tremors and anxiety were also detected.[2] The active ingredient of all EDs is caffeine with high concentration. EDs always have additives such as sugar, amino-acids (taurine or carnitine). As well as various forms of vitamin B and herbal extracts.[3]

The major cause of life-threatening EDs side effects is the increased concentration of caffeine which has a stimulating effect on cardio-vascular system. It also stimulates neurological systems which may result in Caffeine toxicity leading seizures, strokes, and gonadotoxic effects. In addition, a significant rise in the incidence of infertility in albino rats of adult males and adult females using EDs were also observed.[4] Kassab and Tawfik[5] claimed that, submandibular salivary glands of albino rats upon intake of EDs induced structural changes which were partially ameliorated by its withdrawal. So, excessive intake of the caffeinated EDs should be considered as a health hazard for humans. Furthermore, Abdelwahab *et al* [6] and Salem *et al* [7] reported that EDs induced oxidative stresses which plays a crucial role in carcinogenesis and induction of inflammatory response.

However, there was little literature regarding the effect of EDs on oral mucosa so the aim of the current work was to study the effect of intake and withdrawal of Redbull (RB) energy drink on the histological structure of dorsal surface of tongue of adult male albino rat.

Materials and methods

Sample size Calculation

The sample size calculation was carried by One-way ANOVA with Minitab software version-16. The calculation was based on the data of reference to El-Haddad and El-Faramawy.[8] For the present calculation we considered the maximum difference of means 16.66 and the estimated standard deviation of sample size equals 3 cases per group is the minimum required number. Thus, 30 adult male albino rats were used, 10 for each group.

Animals

30 adult male albino rats (200-220 gm body weight aged 5-6 months) were enrolled in the present study. All rats were kept the same dietary and environmental circumstances in the experimental animal house of Faculty of Medicine-Cairo University-Egypt according to the Five Freedoms of Farm Animal Welfare Council.[9] The rat population was divided into 3 groups, 10 rats each; according to the Three Rs guiding principles for the use of animals in research. The rats were housed in polypropylene cages (two per cage) with free access to water and standard nourishment. The animals were kept in a chamber with a temperature of 22-24°C and were exposed to 12:12 hour light-dark cycles. The rat colonies were health monitored in respect with the Federation of European Laboratory Animal Science Associations' recommendations.[10] The protocol for the present was approved by Ain-Shams ethics committee with number *FDASU- Rec IR112305*.

Energy drink administration

Red Bull cans (250 ml) [Red Bull GmbH, 5330 Fuschl am See, Austria](RB) were purchased from a local market in Cairo-Egypt. Each 100 ml of the Red Bull contains

caffeine (0.03%), taurine (0.4%), gluconolactone (0.24%), niacin (8 mg), vitamin B6 (2 mg), B12 (0.002 mg), pantothenic acid (2 mg) together with a mixture of water, glucose, sucrose, sodium citrate, carbon dioxide, inositol, riboflavin, caramel, natural and artificial flavoring and coloring agents.[5]

RB was administered with a total daily dose 4ml/200 gm (2ml/12 hours). This dose was chosen.[11, 12]

Grouping

Animals were divided into three groups

Group I (Control): Include 10 adult male albino rats that received 2ml/200 gm distilled water by oral gavage twice daily. This group was further subdivided into:

Subgroup Ia: Consisted of 5 animals and will be sacrificed after 4 weeks.

Subgroup Ib: Consisted of 5 animals and will be sacrificed after 8 weeks.

Group II (Red Bull group): Included 10 adult male albino rats that received Redbull by oral gavage with 2ml/200 gm twice daily for 4 weeks.

Group III (withdrawal group): Included 10 adult male albino rats that 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 will be sacrificed after 8 weeks.

The animals were anesthetized using pentobarbital (35 mg/kg) given intraperitoneally and then sacrificed.[13, 14] The tongues were excised and washed. Each tongue at posterior 1/3 was resected horizontally on the midline to detect rat circumvallate papilla as rats have only one circumvallate papilla placed posteriorly on the midline of the tongue.[15] Then the anterior 2/3 of each tongue was incised sagittally along the midline into 2 halves to detect the filiform and fungiform papillae along the dorsal surface of the tongue.

Tissue preparation for light microscopic examination

Specimens for light microscope (LM) examination were fixed in 10 % neutral buffered formalin for 48 hours. Getting rid of sacrificed rats bodies will be done according to ethical committee rules in the incinerator of Cairo University Hospital-Egypt.

Each specimen will be 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. Then, 4 µm thick sections were done for histological routine hematoxylin and eosin (H & E) stain, Masson trichrome special stain which stains green for collagen.[16]

Immuno-histochemical examination

Immune-histochemical examination for apoptotic changes in epithelial cells of filiform papillae was carried by Caspase-3 immune reaction stain. Whereas, paraffin sections (5 µm thickness) obtained from each group were mounted over positively charged slides. The immune-histochemical marker used was a caspase-3 antibody. Immuno-stained sections were examined under the light microscope, where the positive reaction appeared as brown membranous, cytoplasmic, and/or nuclear staining [17, 18] at Research lab- Faculty of Medicine- Azhar Baneen University-Egypt).

Histomorphometric analysis

H & E, Masson trichrome and caspase-3 immune-reaction stained samples were examined by digital light microscope Leica DM3000 LED, S.N 346986, camera DFC295, S.N 0705530414, made in Germany. Image analysis was performed at the middle part of the sagittal section of anterior 2/3 of tongue. The measurement of collagen fibers area % in lamina propria and core papillae stained by Masson trichrome

were obtained at magnification power (X 200). Whereas, area % of apoptotic cells gave IMH caspase-3 positively reacted brown stained cells in the epithelium of filiform papillae at magnification power (X 400) at middle part of the tongue (lingual protuberance). Both area % were analyzed by the Image analysis system of Leica Qwin images processing and analysis software. Part no. 872705, Version V3.5.1, Leica microsystems LTD.CH 9435 Herbrug (Switzerland). Blinding was done during histomorphometric analysis to ensure unbiased results. Ten fields were measured per case. This was done at the Research Laboratory Center, Faculty of Dentistry, Modern Science and Art University (6 October-Giza- Egypt).

Statistical Analysis

The area % of collagen fibers in lamina propria just beneath the epithelium of the dorsal surface of the tongue were statistically analyzed. Also area % of immune-reaction for caspase-3 in the epithelial cells were statistically analyzed. All Data were collected, tabulated and subjected to statistical analysis. Statistical analysis was performed by SPSS in general (version 20), while Microsoft office Excel was used for data handling and graphical presentation. Quantitative variables are described by the Mean, Standard Deviation (SD), the Range (Minimum – Maximum), Standard Error (SE) and 95% confidence interval of the mean. The Shapiro-Wilk test of normality is used to test the normality hypothesis of all quantitative variables for further choice of appropriate parametric and non-parametric tests. All the variables except one are found normally distributed allowing the use of parametric tests. One way analysis of variance ANOVA is used to compare the mean values of the three groups. Multiple comparisons are carried out by applying Bonferroni method. Significance level is considered at $P < 0.05$ (S); while for $P < 0.01$ is considered highly significant (HS). Two Tailed tests are assumed throughout the analysis for all statistical tests.

Results

Histopathological results

H & E stained histological sections of the *control group (I)* revealed that the anterior part of the dorsal surface of the tongue consisted of many filiform papillae and one fungiform papilla. Each papilla was formed of connective tissue core covered by stratified squamous keratinized epithelium. Stratified squamous keratinized epithelium was formed of stratum basale, stratum spinosum, stratum granulosum and stratum corneum. Filiform papillae were thread-like with pointed end and heavily keratinized surface that lacked taste buds (fig.1-a, b). The fungiform papilla was mushroom-like with taste buds on its dorsal surfaces (fig.1-c). The posterior part of the dorsal surface of the rat tongue showed circumvallate papillae surrounded by troughs with numerous taste buds along its lateral walls (fig.2-d,e). VonEbner's glands consisted of serous acini that opened into the trough of circumvallate papilla by duct (fig.2-d). As well as we go more towards posterior 1/3 of the tongue pure mucous acini of lingual salivary glands were observed (Weber's mucus salivary glands) (fig.2- f). Skeletal muscle fibers of different directions formed the bulk of rat tongue were detected beneath the tongue papillae (fig 2-a, f).

While in the *Redbull group (II)*, various changes in filiform and fungiform were observed. Some filiform papillae were atrophied with blunt ended tips and hyper-keratinized surface. Fungiform papillae showed loss of taste buds. Increased proliferation (hyperplasia) of cells in the basal layer of epithelium were seen. Loss of skeletal muscle fibers in some areas with the appearance of many fat cells in the superficial layer of lamina propria.

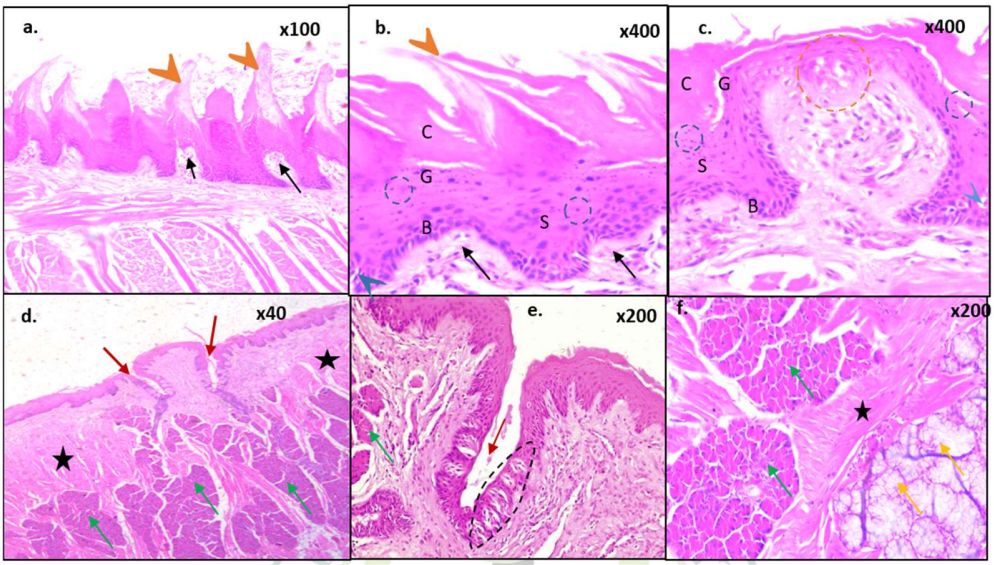

Figure 1: Photomicrograph plate of control group (I) showing; filiform papillae (orange arrow heads) (a, b). Fungiform papillae with intact taste bud (orange circle) (c). Layers of epithelial lining; stratum basale (B), stratum spinosum (S), stratum granulosum (G) and stratum corneum (C) with dispersed blue keratohyaline granules (blue circle) and few clear cells (non-keratinocytes) (blue arrow heads) (b, c). Circumvallate papillae surrounded by trough (red arrows) and its lateral wall with many taste buds (black oval) (d, e). Skeletal muscle fibers of the tongue in different directions (black stars). Notice, Von Ebner's pure serous acini (green arrows) (d, f) and Weber's pure mucous acini (yellow arrows) (f). H & E original magnification (a) (X 100); (b, c) (X 400); (d) (X 40); (e, f) (X 200)

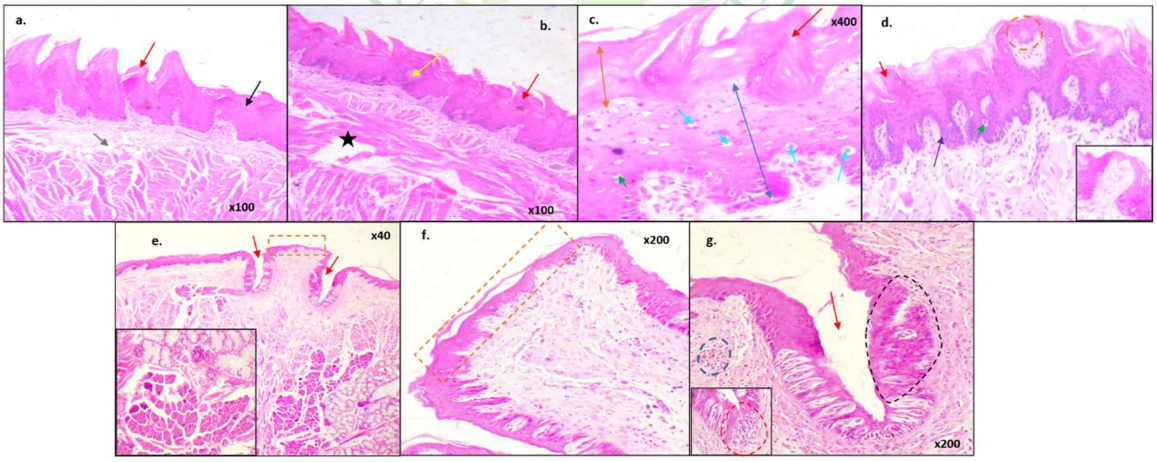

Figure 2: A photomicrograph plate of RB group (II) showing; some atrophied filiform papillae (black arrows) with blunt ended tips (red arrows) (a). Hyperplasia of cells in the basal layer of epithelium (yellow arrows). Loss of skeletal muscle fibers in some areas (black stars) and presence of some fat cells in the lamina propria (grey arrows) (b). Hyper-keratinization of filiform papillae (orange double arrow) .Lamina propria with numerous inflammatory cells. Loss of epithelial stratification (dark blue double arrow). Vacuolated cells with abnormal shape and size with many nucleoli (light blue arrows) (c). Loss of taste buds (orange circle) of fungiform papillae. Increase in number and height of rete ridges with hyper-proliferation of basal cells of epithelium (violet arrows). Many clear cells in rete ridges (green arrows). Inset shows loss of taste bud in fungiform papilla (Black Square) (d). Circumvallate papilla displaying; atrophied outer epithelium (orange rectangle) (e, f). Inset shows Von Ebner's gland with widely separated acini and some degenerative changes (e). Troughs (red arrows) (e, g) lined by epithelium carrying degenerated taste buds (black oval), Aggregations of inflammatory cells (blue oval). Inset shows cellular inflammation (red circle) (g). H & E original magnification (a, b) (X 100); (c) (X 400);(e) (X 40); (d, f, g) (X 200); (Inset X 400)

Increase in number and height of rete pegs with hyper-proliferation of basal cells of epithelium were detected (fig.2- a, b). Presence of a large number of vacuolated cells with abnormal shape and size was detected. Vacuolated cells contain many nucleoli (fig. 2-c). Many clear cells were present in rete pegs. Lamina propria contained numerous blood vessels and inflammatory cells. In some areas epithelial cells in the dorsal surface of the tongue showed loss of epithelial stratification. The nuclei of the basal layer lie away from the basement membrane with loss of cellular connection (fig. 2- c, d).

Circumvallate papillae showed apparent decrease in thickness and keratinization of its dorsal surface. Its lateral surface showed Loss of taste buds in some areas. Epithelium around taste buds showed focal areas of basement membrane loss and subsequent appearance of inflammatory cells inside the epithelium. Lamina propria in the core of circumvallate papillae showed inflammatory cells. Von Ebner's gland showed widely separated acini. Some acini appeared atrophied with hyperchromatic nuclei. Degenerative changes were also detected in skeletal muscle fibers (fig. 2- e, f, g).

Whereas in the *withdrawal group (III)*, filiform papillae regained its normal conical-shape architecture in some areas while other areas showed loss or atrophic papillae. The rete ridges still showed an increase in their length and number. Rete ridges showed the presence of many clear cells. Mitotic figures were detected in the basal cell layer. The epithelium at the surface still showed some vacuolated cells with many multiple nucleoli. Lamina propria contains spindle shaped cells (fibroblasts). Apparent increase in thickness of dorsal surface of fungiform papilla with abnormal appearance of taste buds were detected (fig. 3- a, b, c).

Withdrawal group (III) showed normal appearance of circumvallate papillae with its associated glands. However, fragmentation between skeletal muscle fibers was noticed in between serous acini (fig.3- d, e). Cellular infiltration of lamina propria was seen invading epithelium surrounding taste buds (fig. 3-f).

Histopathological Results of Masson' Trichrome stain

Masson's trichrome stain in group (I) revealed normal appearance of collagen fibers (lamina propria) in core papillae and in between muscles (fig. 4 a, d). Apparent decrease in collagen fibers in lamina propria with an increase in collagen fibers in between muscles was observed in group (II) (fig. 4 b, e). On the other hand, collagen fibers were more or less similar to the control group (III) (fig 4 c, f).

Immuno-hitochemical Caspase-3 stain

Immuno-histochemical Caspase-3 stain showed absence of apoptotic cells in epithelium of filiform papillae of group (I). Whereas, group (II) displayed an apparent increase in positive Caspase-3 reaction. In contrast, group (III) had few cells with caspase-3 positive reaction (fig. 4- g, h, i).

Statistical Results

The statistical results of the area % of collagen fibers revealed a significant decrease in group II compared to other groups. Group (III) showed significant increase compared to group II but didn't reach control level (Table- 1 & fig.5-a). Area % of caspase-3 positive cells revealed a significant increase in group II compared with other groups. Group (III) showed significant decrease compared to group II but didn't reach control level (Table- 1 and fig.5-b).

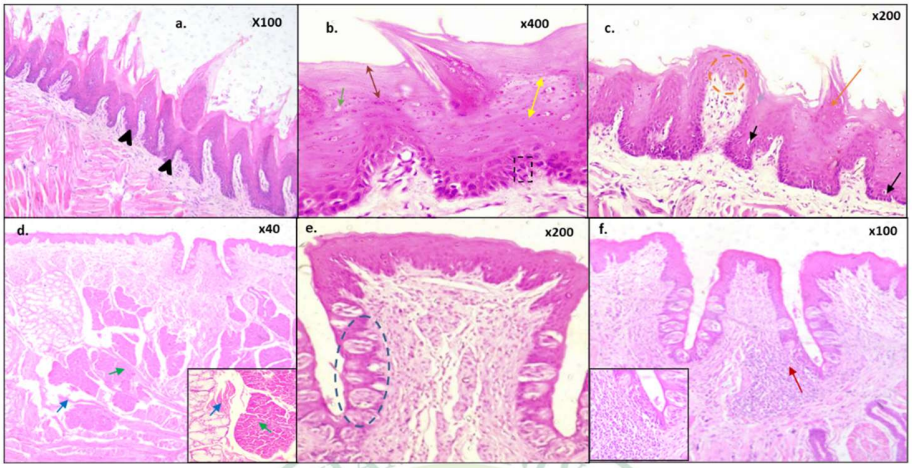

Figure 3: A photomicrograph plate of withdrawal group (III) showing; (a) normal filiform papilla and numerous elongated rete ridges (black arrow heads). (b) mitotic figure in the basal layer (black square) with many clear cells (black arrows), Vacuolated cells of abnormal shape (green arrow) and size with multiple nucleoli with loss of normal epithelial stratification (yellow arrow). Areas of hyperkeratinization (red double arrow). Lamina propria with many spindle shaped cells (fibroblasts) around blood vessels (c) fungiform papilla with taste bud (orange circle). (d) Fragmentation of skeletal muscle fibers (blue arrows) in between Von Ebner's gland (green arrow) (Inset). (e) Normal appearance of circumvallate papillae with many taste buds in its lateral wall (blue oval). (f) Lamina propria with mononuclear cellular infiltration (red arrow) and degenerated taste buds near circumvallate papillae (Inset). H & E original magnification (a,f) (X 100); (b) (X 400); (c,e) (X 200); (d) (X 40); Inset (X 400)

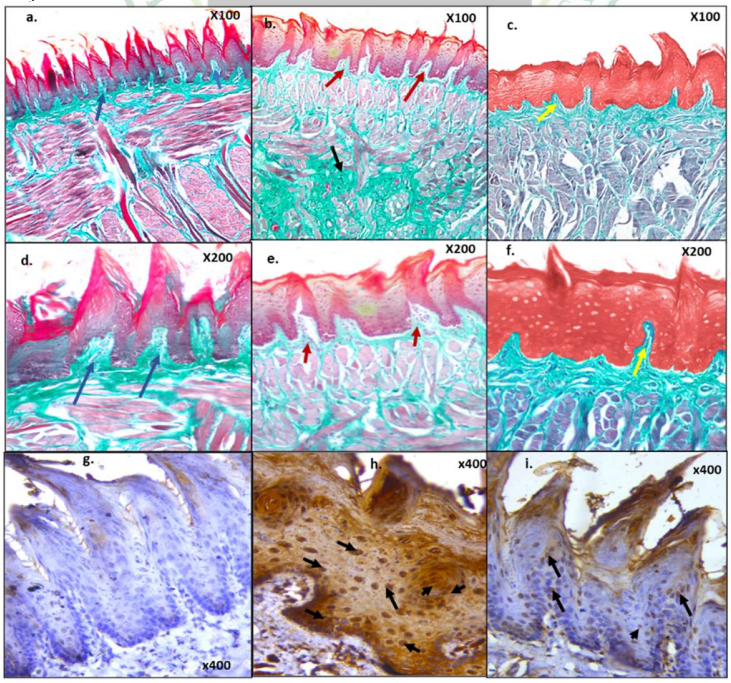

Figure 4: A photomicrograph of the dorsal surface of the rat tongue showing; (a, d) Normal content of collagen fibers in core of papillae (blue arrows) in group (I). (b, e) Moderate decrease in collagen fibers in lamina propria (red arrows) in group (II). Notice an apparent increase in collagen fibers in between skeletal muscle fibers (black arrows) in group II. (d, f) Mild decrease in collagen fibers in group (III) (yellow arrows). (g) Caspase-3 negative reaction in epithelial cells of the control group. (h) An apparent increase in Caspase-3 positive reaction in many epithelial cells of group II. (i) Few cells with caspase-3 positive reaction in group III. Positive reaction in cells (black arrows). Masson Trichrome original magnification (a, b, c) (X 100); (d, e, f) (X 200). Caspase-3 immuno-histochemical stain original magnification (g, h, i) (X 400)

Table 1: showing; area % of collagen fibers and Caspase-3 immuno-reaction in different groups

Area % of collagen fibers in different groups						
				95% Confidence Interval for Mean		
	N	Mean	SD	Lower Bound	Upper Bound	P Value
Control (Group I)[a]	10	17.38	1.16	16.55	18.21	P < 0.001
Redbull (Group II)[b]	10	8.66	3.23	6.34	10.97	
Withdrawal (Group III)[c]	10	14.46	1.90	13.10	15.82	
Area % of Caspase-3 positive cells in different groups						
				95% Confidence Interval for Mean		
	N	Mean	SD	Lower Bound	Upper Bound	P Value
Control (Group I)[a]	10	0.32	0.24	0.15	0.49	P < 0.001
Redbull (Group II)[b]	10	8.02	1.09	7.24	8.80	
Withdrawal (Group III)[c]	10	2.72	0.79	2.16	3.29	

Any two variables with different superscript are statistically significantly different

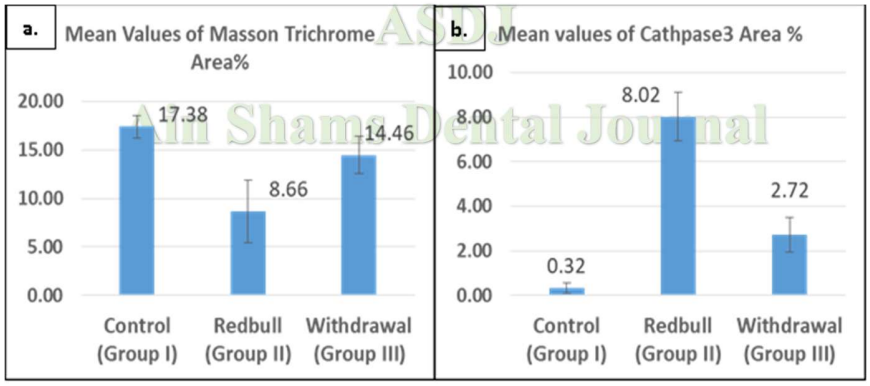

Figure 5: Graph bar showing; area % of collagen fibers in different studied groups (a). Area % of Caspase -3 positive cells in different groups (b).

Discussion

EDs consumption has increased since the introduction of RB in the USA in 1997. Despite the worldwide spread of EDs USA doesn't regulate their ingredients.[2] The aim of the present work was to evaluate the effect of intake & withdrawal of RB on the dorsal surface of rat tongue. So to the best of our knowledge, the current study is the first histological and immunohistological examination of RB on the dorsal surface of the tongue.

The present study, group II revealed atrophic changes in the filiform papillae. They appeared flattened with variable inclination and loss of keratinized surface. Osman *et al* [19] declared that filiform papillae had high metabolic activity, any enzymatic disturbance or nutritional deficiency resulted in papillae atrophy. They added that the filiform papillae were the earliest papillae to undergo damage and degeneration. Fungiform papillae of group II showed loss of central taste buds. These results were in accordance with Lieder *et al* [20] who claimed that fungiform papillae chemosensory function was reduced with taste dysfunction upon consumption of a sugar-sweetened soft drink with a high sugar-high fat diet for a long time.

Circumvallate papillae (group II) showed apparent decrease in the epithelial thickness and keratinization of the dorsal surface. Their lateral surface showed focal loss of taste buds with focal disruption of basal lamina of epithelial cells surrounding the taste bud. Inflammatory cells were detected in the epithelium surrounding taste buds. Also, degenerative change in some acini of Von Ebners serous glands was seen. These results were in agreement with Vlădescu *et al* [21] who postulated that when carbonated drinks come in contact with oral tissues, CO2 forms cavities that limit the saliva flow. Thus increasing the rate of friction within oral tissues. They added that carbonated drink decreased lubricative effect by removing salivary pellicle which could explain the atrophied outer epithelium of circumvallate papillae.

The loss of taste buds in circumvallate and fungiform papillae of group II could be related to caffeine in EDs which caused neural cell death by inhibiting adenosine receptors leading to interstitial inflammation, insulin resistance and formation of reactive oxygen species.[22]

The dorsal surface of the tongue (group II) showed hyperplasia of basal cells. The epithelium was folded into projection with a central connective tissue core. Epithelial hyperplasia and presence of numerous elongated rete ridges (acanthosis) was seen. Xiong *et al* [23] and Shen *et al* [24] hypothesized that Rete ridges are epithelial extensions that project into underlying connective tissue of lamina propria. The morphological change in rete ridges was attributed to the presence of internal pushing forces derived from keratinocyte division. This force pushed stem cells and progenitor cells to migrate in the opposite direction. Elongation of rete ridges into lamina propria was similar to invasion of malignant cells. This occurred due to remodeling of lamina propria by activation of metalloproteinases (MMPs) enzymes. Moreover, Shen *et al* [25] revealed that rete ridges are formed during wound healing of oral mucosa for better mechanical resistance and nutritional support.

Some areas of oral mucosa (group II) showed many cells with abnormal shape and size. These cells have clear cytoplasm and multiple nucleoli in stratum granulosum. Basal cells appeared irregular in shape with loss of cellular cohesion and epithelial stratification (dysplastic changes). Simila *et al* [26] and Abdelwahab *et al* [6] suggested that the cause of dysplastic changes in epithelium was due to oxidative stresses induced by EDs with marked increase in reactive oxygen

species (ROS) and DNA damage. Oxidative stresses play a crucial role in carcinogenesis and induction of inflammatory response. Moreover, Hankinson *et al* [27] and Natarajan [28] declared that these changes are precancerous. They recorded the presence of DNA damage in these mutated cells.

An apparent increase in clear cells (non-keratinocytes) was noticed in group II. Non keratinocytes in the present study might be Merkel cells, Langerhans cells, or melanocytes. An increase in Merkel cells may be attributed to decreased receptors for touch sensation due to loss of filiform papillae .An increase in Langerhans cells was related to immune-reaction. An increase in melanocytes was related to their role in protection of tissues from reactive oxygen species and free radicals.[29, 30, 31]

The present study (group II) revealed marked increase in caspase-3 positive immune-reaction in some epithelial cells of the dorsal surface of the tongue. Al-Shaikh and Rajeh [4] reported the presence of epithelial cells vacuolization, and nuclear karyolysis in response to EDs. These results were similar to the study of Ku *et al* [32] who stated that caffeine caused DNA damage and inhibited DNA damage repair. It causes cell cycle arrest at G0/G1 phase thus promoting keratinocytes apoptosis. Huang *et al* [33] stated that taurine component of RB plays a role in lipid digestion including the lipid present in the cell membrane which leads to marked cellular degeneration and apoptosis.

In the group II of the present study, lamina propria beneath the dorsal surface of the tongue showed focal area of cellular infiltration. Graneri *et al* [34] stated that, EDs are filled with artificial colors and high amounts of sugar which when regularly consumed can lead to chronic inflammation. They added that EDs cause an increase in the level of TNF- α due to the presence of sugar. Moreover, Díaz *et al* [35] declared that chronic use of energy drinks leads to an inflammatory response, oxidative stress, and cell death by apoptosis.

In group II of the present study, collagen fibers in lamina propria just beneath the dorsal surface of the tongue showed significant decrease in area %. Suragimath *et al* [36] declared that carbonated drinks caused decrease proliferation in fibroblasts .This was attributed to the high PH 7.2–7.5 of EDs which is not suitable for viability of fibroblasts which need acidic PH. Fisher *et al* [37] and Doyle *et al* [38] stated that EDs consumption leads to increase in activity of MMPs enzyme. This caused fragmentation of collagen fibers. As fibroblasts cannot attach to fragmented collagen fibers so they are unable to receive mechanical information and undergo collapse. Donejko *et al* [39] stated that caffeine inhibits collagen biosynthesis through inhibition of prolidase activity which is responsible for recycling of proline. Skeletal muscle fibers below the dorsal surface of tongue (group II) showed area of muscle fibers loss with fat cells in lamina propria. Jang *et al* [40] reported that caffeine promotes autophagy in skeletal muscle fibers.

Group III (Withdrawal group) of the current study revealed improvement of all lingual papillae structure, their taste buds and Von Ebner salivary gland in some areas. However, some areas showed acanthosis and dysplastic changes. This was confirmed by the presence of mitotic figures in the basal layer of epithelium.[41, 42]

The present study (group III) revealed significant decrease of caspase-3 immuno-reaction in some epithelial cells of the dorsal surface of the tongue compared to group II but did not reach control level. Lamina propria still showed inflammatory changes in some areas while area % of collagen fibers were less or more similar to the control group. This denoted the regain of the activity of wound healing of fibroblasts. There was marked preservation of the skeletal muscle fibers. [4, 5, 43, 44]

Regarding the mechanism of action of EDs, Simila *et al* [26] stated that EDs induced oxidative stresses due to high caffeine content. Denewer & Elsabaa [45] conveyed that caffeine disturb regulatory proteins of the cell cycle inducing apoptosis. Abdelwahab *et al* [6] declared that EDs decreased sensitivity to insulin and impaired glucose mechanism. So, withdrawal of the drug can partially improve the oxidative stresses. Complete regain of the structure of the dorsal surface of the tongue structure might need longer time than the time used in the current study.

Conclusion

Redbull ED induced structural changes in the dorsal surface of albino rats tongue. These changes were partially improved by its withdrawal. Thus, excessive intake of Redbull should be considered as a health risk hazard for oral tissues in human.

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.

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.

Ethical approval and consent to participate

All stages of the current work were revised and accepted by Ethical Committee of Faculty of Dentistry-Ain Shams University with approval number *FDASU-Rec IR112305.*

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