Original Article

Hepatic Changes under the Effect of Red Bull Energy Drinks and its Withdrawal in Adult Male Albino Rats (Histological and Immunohistochemical Study)

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ABSTRACT

Background: Red bull (RB) energy drinks became popular especially among young adults believing that they improve the level of performance and giving them more energy.

Objectives: One of the organs most susceptible to the toxicity of red bull is the liver. Therefore, the goal of our study was to assess the effects of RB ingestion on the livers of adult male albino rats as well as the consequences of its withdrawal.

Materials and Methods: Thirty six adult male albino rats were divided into 3 groups; control group, Red bull given group (received red bull in a dose 1.5 ml/100g b.wt daily orally for 4 weeks) and withdrawal group (received red bull as in Red bull group and left for 4 weeks without treatment). Specimens of the liver were taken from all groups and processed for the light, electron microscopic and immunohistochemical study. Morphometric measurements and statistical analyses were done.

Results: Red bull given group displayed a significantly higher serum level of liver enzymes in comparison to the control group. Loss of the typical liver architecture was noticed through histopathological analysis in the same group; most hepatocytes were degenerated with pyknotic nuclei and cytoplasmic vacuolations.Lipid droplets infiltration was noticed between hepatocytes. Leucocytic infiltration surrounding central vein and vessels of portal area with eosinophilic exudate within and between portal area vessels were seen. The immunohistochemical results demonstrated a significant increase in alpha smooth muscle actin (α-SMA) .The ultrastructural assessment confirmed these changes. However, cessation of RB intake showed improvement in the liver histopathological and biochemical changes depicted previously.

Conclusion: Hepatocytes affected by ingestion of caffeinated energy drinks. But as seen by the withdrawal group's blood and histopathology study results, these harmful effects were reversible.

Key Words: α-SMA, liver, Red bull, ultrastructure, withdrawal.

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INTRODUCTION

The consumption of energy drinks has grown in popularity across the globe. These beverages are sold to young people as healthy substitutes that boost enjoyment and enhance cognitive and physical abilities like focus, attention, and alertness[1].

Energy drinks are non-alcoholic, frequently gently carbonated beverages that contain a variety of substances that increase energy. Young people frequently utilize them when studying, participating in sports and travelling long distances. The companies suggest that using their products will provide them more energy and improve their physical and mental performance[2]. RB ingredients commonly include caffeine which is often combined with taurine, glucuronolactone,otherplant-based stimulants guarana and vitamins B complex[3,4,5].

RB is well-known throughout many people as a healthful beverage. Energy drinks' chemical makeup, however, has the potential to have numerous negative impacts, including negative behavioral repercussions. Energy drinks typically contain caffeine, which is linked to fluid-electrolyte balance and diuresis, whereas taurine is linked to detoxification and bile acid conjugation[6].

Also, many side effects and even deaths were reported, as a consequence of energy drinks consumption. Arrhythmia, cardiac arrest and hepatitis are some of the reported side effects. Consuming caffeinated energy drinks also increased creatinine levels and had negative effects on liver cells[7]. Taurine, however, has conflicting effects on the kidney and liver functions[8,9].

Despite, these drinks are popular; their effects on consumers' health are still controversial and there is not enough research on energy drinks' safety has been conducted yet. So, the present study aimed to assess biochemical, histological and immunohistochemical changes in the liver of adult male albino rats after intake of Red bull energy drink and the possible improvement of these changes after its withdrawal.

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MATERIALS AND METHODS

I- Materials:

1. Experimental animals:

A temperature-controlled environment (24°C) with an alternate 12 h light-dark cycle was maintained for the thirty six mature male albino rats, which were 10 weeks old, weighed 200-250gm, and were housed in hygienic stainless-steel cages (5 rats per cage). They received conventional rat food, were acclimated to the lab environment, and had unlimited access to water. The Animal House at the Faculty of Medicine at Zagazig University in Egypt served as the site for this study. The Medical Research Ethics Committee of Zagazig University in Egypt approved the protocol with permission number ZU-IACUC/3/F/202/2022 and it complied with the National Institutes of Health's criteria for the handling and use of laboratory animals in all cases.

2- Red bull:

(Red Bull GmbH, 5330 Fushl am see, Austeria) is offered in the shape of 250 ml cans in the Egyptian market. Labelled ingredients of the product company on the cans revealed its composition as follow water, sucrose, glucose, sodium citrate, carbon dioxide, taurine (0.4%), caffeine (0.03%), gluconolactone (0.24%), inositol, niacin (8 mg), pantothenic acid (2 mg), vitamin B6 (2 mg), vitamin B12 (0.002 mg), caramel, riboflavin, and a blend of artificial and natural flavouring and colouring agents are all contained in each 100 ml

II. Experimental design:

The animals were divided into 3 groups as follows:

Group I (control group): included eighteen rats subdivided equally into two subgroups:

Subgroup Ia (negative control group): nine animals received no treatment only regular diet and water for 8 weeks.

Subgroup Ib (positive control group): nine animals received distilled water via oral gavage for 8 weeks.

Group II (RB given group): nine animals of this group received 1.5 ml/100gmb.wt Red Bull via oral gavage daily for 4 weeks[10].

Group III (withdrawal group): nine animals of this group orally received RB as the same previous dose in group II for 4 weeks and then were given only distilled water daily by oral gavage for another 4 weeks[11].

At the end of the experiment, at designated time, rats of all groups were subjected to the followings methods.

III. Methods:

1- Chemical study:

Enzyme assays: The cardiac puncture procedure was used to obtain blood samples from each rat in each group, which were then allowed to clot. By centrifuging the sample at 3000g for 10 min in a Beckman Model 6 chilled centrifuge, the serum was quickly separated and processed for analysis. Sera were stored at -20°C until assayed for the biochemical parameters. Serum levels ofAlanine amino transferase (ALT), Aspartate aminotransferase (AST) and Alkaline phosphatase (ALP) were measured using a biochemistry autoanalyzer (Olympus AU2700, Japan).

2- Histological study:

Animals were anesthetized using ether inhalation, sacrificed, carefully dissected and specimens from liver were processed for light microscope, immunohistochemical stains and electron microscopic examination.

Light microscopicstudy: The liver specimens were immersed in 10% neutral-buffered formalin, washed, dehydrated, cleared, and embedded in paraffin. Sections of 5 µm thickness were stained with H&E[12].

Immunohistochemical study: Sections of the liver tissue (5µm thickness) weredewaxed, rehydrated, and washed with PBS. The sections were then incubated overnight in a humid chamber with the primary antibody at 4°C [primary antibodies: monoclonal anti-mouse antibody for α-SMA a marker of hepatic stellate cells (HSCs) showing brown cytoplasmic deposits. α-SMA antibody was obtained from Sigma Biochemical (St. Louis, Missouri, USA). The sections were then rinsed and incubated with the secondary antibody. Streptavidin peroxidase was added then rinsed again three times in phosphate buffer saline (PBS). Immunoreactivity was visualized using 3,3′-diaminobenzidine-hydrogen peroxide as a chromogen. The sections were counterstained with Mayer's hematoxylin. We applied positive control on sections of colon showing positive cytoplasmic deposits staining smooth muscle cells. Negative control specimen of the liver was processed in the same way omitting the steps of primary antibodiy[13].

Transmission electron microscope study: In Electron Microscope Research Laboratory, Faculty of Agricultural, Mansoura University, Egypt. Liver specimens were first fixed in 2% buffered glutaraldehyde, then cleaned in PBS, fixed in 1% osmium tetroxide, dehydrated in alcohol, and finally embedded in epoxy resins. Ultrathin sections (70-90 nm) were stained with uranyl acetate and lead citrate, and semithin sections (1mm thick) were prepared and stained with 1% toluidine blue and viewed under a light microscope[14].Then, ultrathin sections were examined and photographed by JEOL JEM 2100 EXII Electron Microscope (Jeol Ltd).

3- Morphometric analysis:

Area percentage (area %) for α-SMA immunoreaction was measured using the image analyzer computer system Leica Qwin 500 (Leica Ltd, Cambridge, UK) at the Faculty of Dentistry, Cairo University, Egypt. Using the interactive measure option, the area % was measured. The measuring frame of a standard area equal to 118476.6 mm2 was chosen so that the measurement's blue binary color would cover the brown positive immunological reaction. Ten readings from five distinct sections from each rat in all groups were examined.

STATISTICAL ANALYSIS

The mean and standard deviation (SD) were used to represent all experiment results. To assess the variations between the groups, a one-way analysis of variance (ANOVA) was employed, followed by a post hoc least significant difference (LSD) test. P value 0.05 was taken into account as a significant difference for all comparisons. The IBM SPSS 19.0 softwarewas used for all analyses.

RESULTS

Chemical results:

Liver functions enzymes: Statistical comparison among all studied groups as regards liver functions tests revealed a non- significant difference between control and withdrawal groups. While, in the RB given group, serum levels of liver enzymes Alanine transferase (ALT), Aspartatetransferase(AST), AlkalinePhosphatase (ALP) were significantly increased, compared to control and withdrawal groups ($P < 0.001$) (Table.1).

Light Microscopic Results:

H&E-stained sections from rats' liver of control group revealed hepatocytes with rounded vesicular nuclei, central veins and normal appearance of portal area and sinusoids (Fig.1a, b). Concerning RB group central veins were wide, most hepatocytes increased in size with rarefied cytoplasm and vacuolations, their nuclei were pyknotic. Lipid droplets infiltrations were noticed between hepatocytes. Also, leucocytic infiltrations surrounding central veins and vessels of portal area were seen (Fig.2a, b).Moreover, in RB group, Leucocytic infiltrations surrounding central veins and vessels of portal area with eosinophilic exudate within and between portal area vessels were seen. Thickening of portal area vessels were also noticed (Fig.2c). Examination of withdrawal group sections showed apparently normal hepatocytes, hepatic lobules, and central veins. Few wide spaces between the hepatic strands, less degenerated hepatocytes with pyknotic nuclei, mild leukocytic infiltrations surrounding the slightly dilated central veins and mild thickening of portal area vessels were seen. Few cytoplasmic vacuoles within hepatocytes were also noticed (Fig.3a, b).

Immunohistochemical examination of α- SMA stained liver sections of control rats showed weak positive immune-stained reaction of α- SMA in the smooth muscle fibers of the tunica media of the central veins, the blood vessels in the portal area and in spindle shaped cells surrounding the sinusoidal wall and within the fibrous septa (Fig.4a, b). In RB group, there was strong positive immune-stained reaction of α- SMA (its expression was significantly higher as compared to that in control group) (Fig.4c, d). Withdrawal group showed decreased immune-stained reaction of α- SMA in comparison to RB group (Fig.4e, f)

Electron microscopic examination of control liver sections revealed hepatocytes with euchromatic nuclei, prominent nucleoli, abundant mitochondria and RER appeared as parallel tubules around nuclei and between mitochondria. Bile canaliculi between adjacent hepatocytes were noticed (Fig.5a, b).In RB group, hepatocytes showed heterochromatic nuclei with irregular nuclear envelope and dilated, irregularly distributed RER in between mitochondria. Some vacuoles were noticed within the cytoplasm (Fig.5c).Examination of adjoining hepatocytes with space of disse in between revealed hepatocytes microvilli, blood sinusoids and interstitial cells. Hepatocytes had heterochromatic shrunken nuclei with irregular nuclear envelope, numerous fat droplets, vacuolated and degenerated mitochondria, numerous accumulated smooth endoplasmic reticulum and multiple vacuoles of variable shapes and sizes within the cytoplasm (Fig.6a,b). Examination of two neighboring hepatocytes revealed damaging of their cell membranes, heterochromaic nuclei and wide intercellular spaces with numerous accumulated collagen fibers. Vacuoles within cytoplasm and mitochondria were noticed (Fig.6c). In withdrawal group, Examination of neighboring hepatocytes showed normal intercellular space and cytoplasm was less vacuolated compared to the previous RB group. One nucleus appeared rounded with euchromatin and prominent nucleolus. Most mitochondria were less vacuolated and RER tubules were in close association with their and around the nuclei. Lipid droplets were also seen (Fig.6d).

Morphometrical and statistical analysis:

Area percentage of α-SMA positive immunoreaction in the RB given group showed a highly significant increase compared to the control and withdrawal groups, while withdrawal group showed non-significant increase compared to the control group (Table 2).

Table 1: Liver functions enzymes among all studied groups in rats by one-way ANOVA test.

Groups	(ALT)(U/ml)	(AST) (U/ml)	(ALP)(U/ml)	*P- value*
Control	58.4 ± 20.580	142.15 ± 25.46	30.75 a ± 5.32	
Red bull	142.0 ± 10.98*	217.77 ± 27.48*	73.01 ± 18.40*	*<0.001**
Withdrawal	63.7 ± 0.70	149.63 ± 0.33	36.23 ± 2.86	

Values are expressed as mean ± standard deviation (SD).

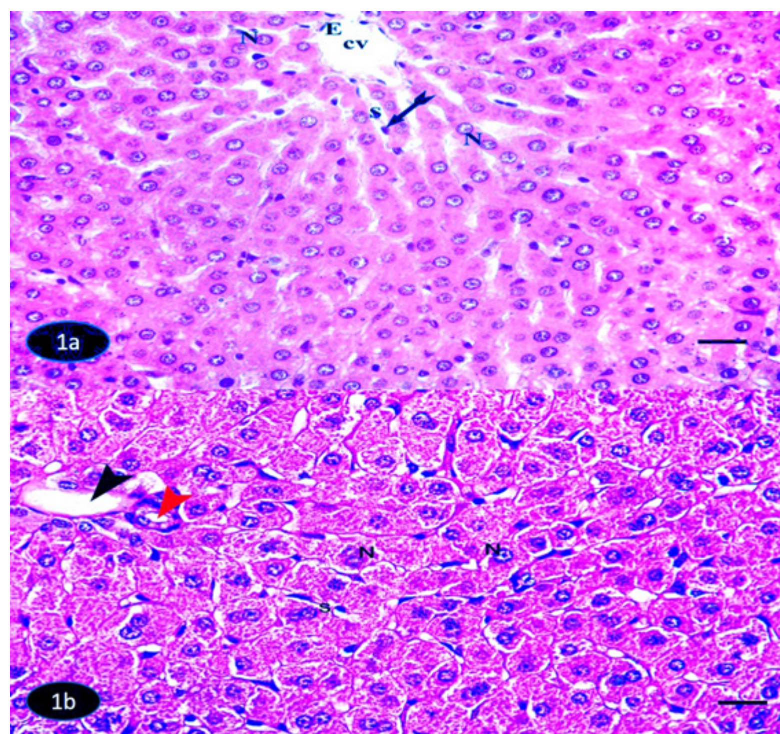

Fig. 1 (a-b): A photomicrograph of H&E-stained sections in rat liver of control group: (a) showing cords of hepatocytes withacidophilic stippled cytoplasm and central vesicular nuclei (N). Central vein (CV) lined with endothelial cells (E) and normal sinusoids (S) with Kupffer cell (bifid arrow). (b) Cords of hepatocytes with acidophilic stippled cytoplasm and centralvesicular nuclei (N), separatedby normal sinusoids (S). Normal lining of portal vein (black arrow head) and bile ductulus (red arrow head) are noticed.

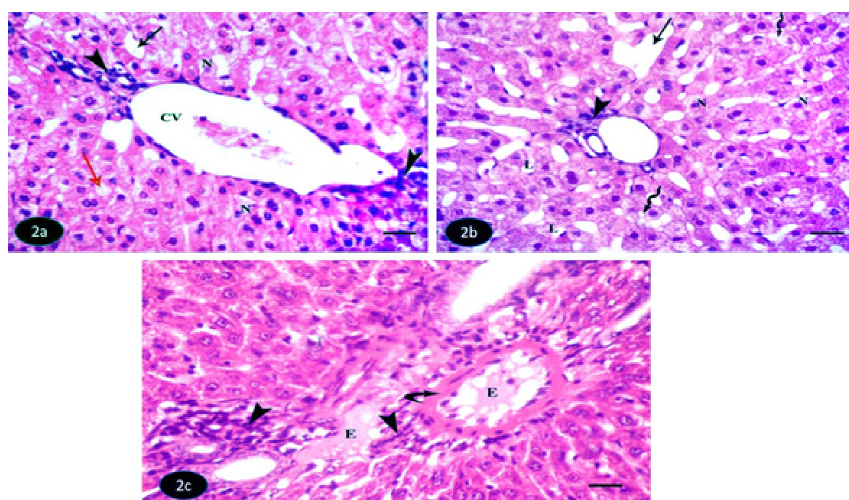

Fig. 2 (a-c): A photomicrograph of H&E-stained sections in rat liver of RB given groups: (a-b) RB given group, there is wide space between the hepatic strands (black arrow), large degenerated hepatocyte with pyknotic nuclei (N), rarefied cytoplasm (red arrow) and dilated central vein (CV). Lipid droplets (L) are seen. Leucocytic infiltration (arrow head) surrounding the dilated central vein (CV). Cytoplasmic vacuoles (zigzag arrow), leucocytic infiltration (arrow head) surrounding portal area vessels. (c) RB given group, eosinophilic exudate within and between blood vessels (E). Massive leucocytic infiltration (arrow head) surrounding thickened blood vessels of portal area (curved arrow) (H&E x400 scale bar 30 µm).

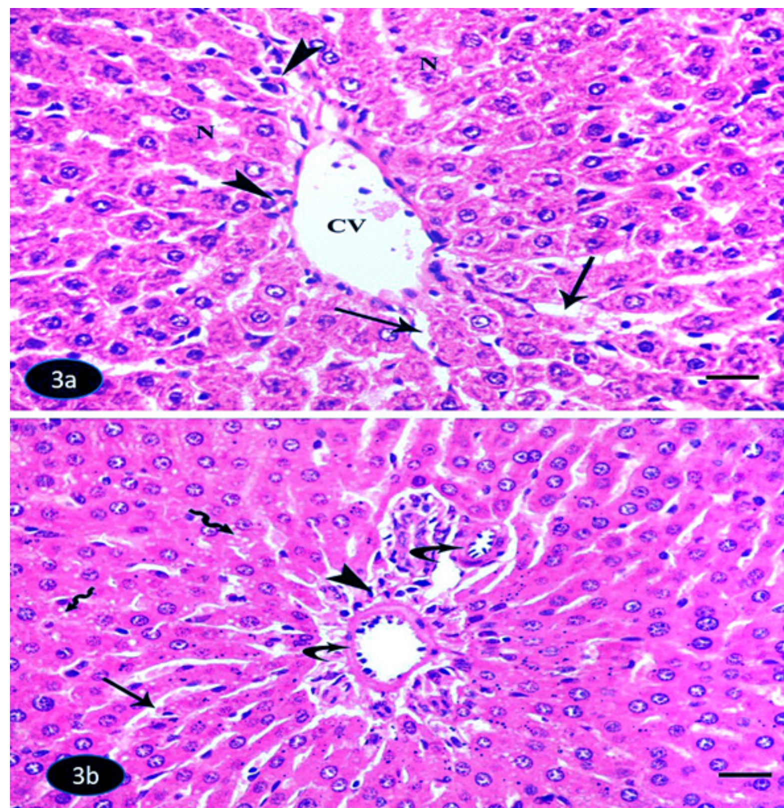

Fig. 3: A photomicrograph of H&E-stained sections in rat liver of withdrawal group : (a-b) withdrawal group shows few wide spaces between the hepatic strands (arrow), few degenerated hepatocytes with pyknotic nuclei (N), fewleucocytic infiltration (arrow head) surrounding the slightly dilated central vein (CV).Few cytoplasmic vacuoles (zigzag arrow) and mild leucocytic infiltration (arrowhead) surrounding apparently normal blood vessels (curved arrow) of portal area (H&E x400 scale bar 30 µm)

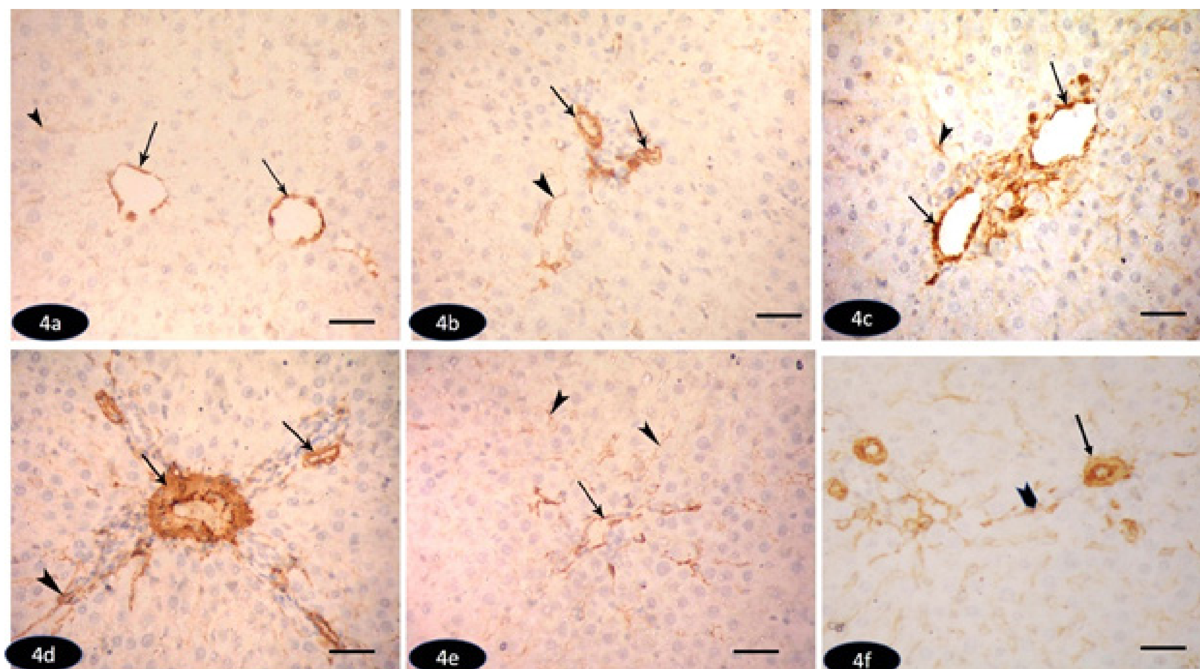

Fig. 4 (a–f): Immunolocalization of -αSMA in liver specimens of all studied groups: (a-b) Control group showing weak positive -αSMA reaction in smooth muscle fibers of the tunica media in the central vein and vessels of portal area (arrow), and in spindle shaped cells surrounding the sinusoidal wall (arrow head). (c-d) RB given group showing markedly increased reactions in smooth muscle fibers of the tunica media in the central vein and vessels of portal area (arrow) and also in spindle shaped cells surrounding the sinusoidal wall (arrow head).(e-f)In withdrawal group the immunoreactions decreases in comparison to RB group in the tunica media in the central vein and vessels of portal area(arrow), and in spindle shaped cells surrounding the sinusoidal wall (arrow head) (α-SMA immunostaining, X400 scale bar 30 µm).

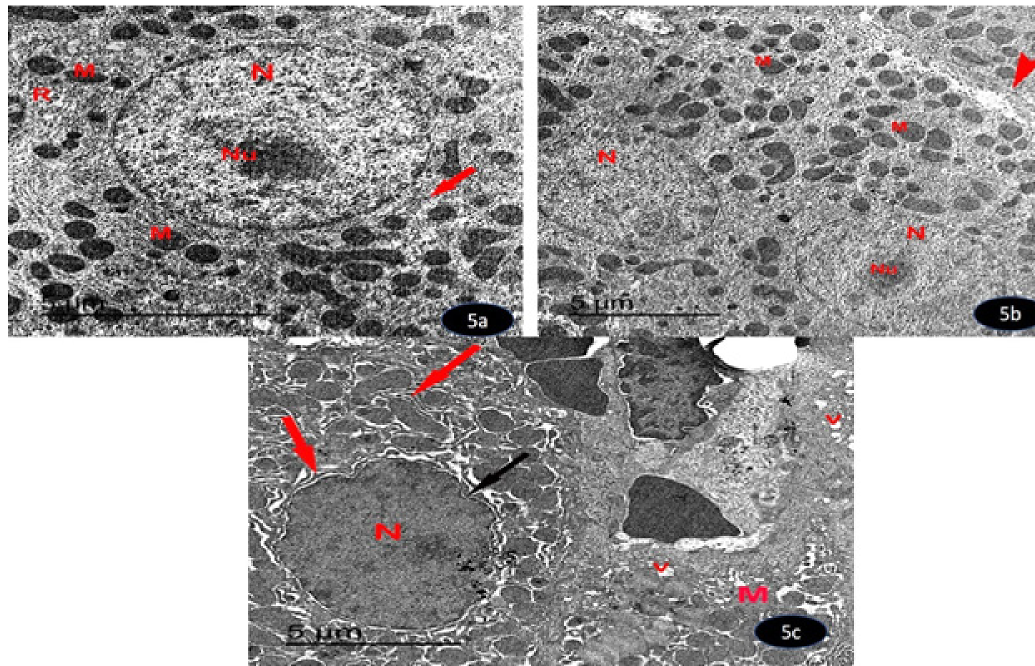

Fig. 5: Ultrathin sections in rat liver of control group (a-b) and RB group (c): Control group (a-b) showing part of hepatocytes with euchromatic nuclei (N) and prominent nucleoli (Nu), abundant mitochondria (M), RER(arrow), many ribosomes (R)and bile canaliculi (arrow head) in between hepatocytes. RB given group (c(reveals heterochromatic nucleus (N) with irregular nuclear envelope (black arrow) and dilated irregularly distributed RER (red arrow) in between mitochondria (M).Some vacuoles were noticed within the cytoplasm (V) (TEM a X4800& b X3200)(TEM c X3000).

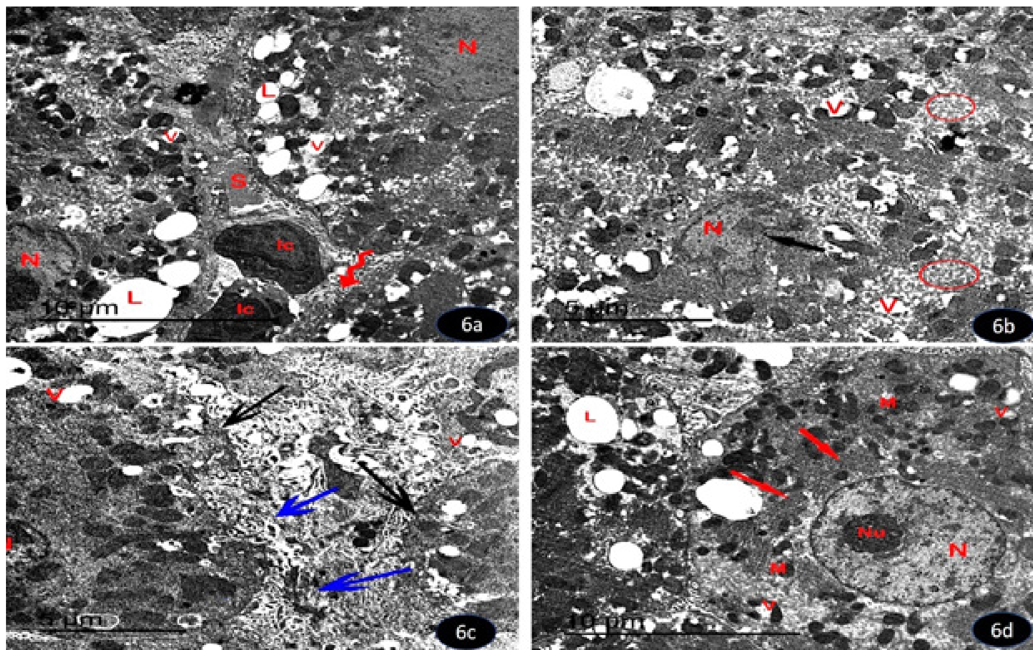

Fig. 6: Ultrathin sections in rat liver of RB given group(a-c) and withdrawal group (d): (a) showing adjoining hepatocytes with space of disse in between reveals hepatocytes microvilli (zigzag arrow), blood sinusoids (S) and interstitial cells (Ic). Hepatocytes have heterochromatic nuclei (N), numerous lipid droplets (L) and multiple vacuoles (V) of variable shape and size within the cytoplasm(TEM X2400). (b) Hepatocytes have nucleus (N) with clumped chromatin and irregular nuclear envelope (black arrow), numerous accumulated smooth endoplasmic reticulium (circle) and numerous vacuoles (V) of variable size and shape (TEM X3000).(c) Neighboring hepatocytes showing disruption of their cell membranes (black arrow), heterochromatic nucleus (N)and wide intercellular space with numerous accumulated collagen fibers (blue arrow). Vacuoles (V) within the cytoplasm and mitochondria (M) of variable shape and density are noticed (TEMX3000). (d) In withdrawal group the adjoining hepatocytes appear with normal intercellular space. The hepatocyte has few vacuoles(V)within cytoplasm and mitochondria (M)with nearly normal shape and distribution. The nucleus (N) appears rounded,euochromatic with prominent nucleolus (Nu). RER tubules (red arrow) are in close association with mitochondria and the nucleus. Few lipid droplets (L) are also seen . (TEMX2700)

Table 2: Area percentage of α-SMA immunoreaction among all studied groups by one-way ANOVA test.

Parameter	Control group	RB group	Withdrawal group	*P- value*
Area percentage of α-SMA	12.86±1.79	35.82±3.87*	18.02±1.21	*<0.001**

Values are expressed as mean ± standard deviation (SD).

DISCUSSION

Several warning statements have been made about the potential negative consequences of energy drinks (EDs)[15]. Over 500 different brands of EDs were released around the world in 2006[5]. Their output is rising and sales in 2012 totaled $12.5 billion. Concerns among the general public and the scientific community have grown as a result of rising use and accompanying health effects[16]. One of the most popular energy beverages is Red Bull, caffeine, taurine, glucuronolactone and B vitamins are the primary components of it.

Red bull energy drink intake caused significant changes in the normal histological structure and biochemical results of albino rats' liver in our study. These manifested themselves as a lack of normal liver architecture and a widening of the hepatic strands. These findings are consistent with recent research that found that caffeinated energy beverages have negative effects on hepatocytes[17]. Others remarked that the effect of energy drinks varies depending on the amount consumed[6].

When compared to the control group, the red bull given group (Group II) revealed significant histological and ultrastructural changes in hepatic cells in the current study. While, there was an improvement in the induced structural modifications in the withdrawal group (Group III), which could indicate attempts to return to normal structure.

Most hepatocytes in this study displayed pyknotic nuclei andintracytoplasmic vacuolation, as well as lipid droplets infiltration. Mansy *et al* & Elsayed *et al*.[18,19] observed the same results. Furthermore, our findings are consistent with those of Afify *et al*.[20], who explained hepatic cytoplasmic vacuolations as a result of the existence of lipid droplets, which were linked to deterioration in hepatocytes. Also, Ayuob and ElBeshbeishy[21] studied the effects of Power Horse, an energy drink, on the pancreas and the fundic mucosa of the stomach in adult male albino rats. The fundic gastric mucosa displayed degenerative histological alterations, according to their findings. They reported that Caffeine-induced elevations of tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) caused an oxidant/antioxidant imbalance in these cells.

Other study proposed that the nuclear alterations could be generated by preservatives such as sodium benzoate, which, when combined with ascorbic acid, another frequent ingredient in energy drinks, could produce the carcinogenic chemical benzene[22].

In addition, many chemicals in energy drinks may be responsible for oxidative repercussions and liver damage, according to Reis *et al*.[23], but these effects are unlikely to be attributable to a single ingredient in energy drinks. It could be owing to a synergistic interaction of multiple substances with strong antioxidant capacity, or it could be due to an enhanced oxidative effect. In consistent with Ekaluo *et al*.[24], the decrease in tissue sensitivity to insulin, decreased glucose metabolism and increased stress hormone release are all plausible pathways involved in ED-induced oxidative stress. As a result, blood glucose levels will rise, as will lipolysis and gluconeogenesis. Glycation of cell membrane phospholipids or even glycation of the cell membrane occurs as a result of the hyperglycemic environment.

There was leucocytic infiltration around the wide central vein and portal area vessels, as well as eosinophilic exudate within and between portal area vessels. Kassab and Tawfik[11] found similar findings in rat submandibular salivary glands, as well as in the rat stomach and duodenal mucosa[25]. Consumption of energy drinks has also been associated with a variety of cardiovascular diseases, increased platelet aggregation, and decreased endothelial function. These findings were the result of varied reactions between the taurine and caffeine in the Red Bull energy drink[15, 26].

In the current study, statistical analysis of the mean values of area percentage of α -SMA immunoreaction in the RB group revealed a highly significant increase compared to the control and withdrawal groups, while withdrawal group showed non-significant increase compared to the control group. Hepatic stellate cells (HSCs), which have been identified as the major cell type that mediates fibrogenesis, were found to express α-SMA as a marker for their activity[27]. To detect the activity of HSCs, immune-staining of α-SMA was used. The increase in α-SMA mean area percentage corresponded to an increase in HSC activity.

Also, after exposure to energy drinks, Zarobkiewicz *et al*.[28] revealed enhanced collagen deposition in the zona glomerulosa and zona fasciculata of the adrenal cortex of adult male rats. Abdel Moneim and El Deeb[29] found higher fibrin depositions on the underlying connective tissue in their investigation on the impact of caffeine on rat gingival wound healing, which showed increased fibrin depositions. These changes disappeared in the recovery group as a result of the energy drinks' unpleasant, harmful effect being eliminated[17]. In addition, Tek *et al*.[30] used a

rat model to assess the effect of an energy drinks' healing effects on soft tissue wounds. They noticed an increase in collagen deposition in the wound and attributed this to the active chemicals in these beverages inhibiting the A2A adenosine receptor, which protects normal tissues from inflammatory damage. Interstitial inflammation and fibrosis develop as a result.

The development of accumulating smooth endoplasmic reticulum and dilated and irregularly distributed RER cisternae were the most notable ultrastructural changes brought on by RB administration. According to Sato *et al*.[31] dilated RER is a marker for damaged hepatocytes, whereas enlarged sER, the site of action, serves as a repository for crucial cellular enzymes and a location for detoxification[32]. Also demonstrating worsened mitochondrial function was a disturbance in mitochondrial structure[33].

Majority of these effects were reversible after the energy drink was stopped, according to the current study. El Desouky *et al*.[34] found similar results when they looked at the histological and histochemical effects of energy drinks on the pancreas of adult albino rats. This was reinforced by Akande and Banjoko[17], who elucidated that the harm caused by excessive use of caffeinated energy drinks is reversible, as evidenced by blood chemistry analysis and histological examination of the organs of recovery group animals.

After intake each of the energy drinks, liver function enzymes such as ALT, AST, and ALP were shown to be raised in the sera of rats. This is consistent with the findings of Akande and Banjoko[17], who revealed that rats treated with power horse had higher serum AST, ALT, and ALP levels. In addition, Ebuehi *et al*.[35] discovered that power horse and red bull have a substantial effect on the activity of liver enzymes in rabbits. Furthermore, a link between the consumption of high-energy drinks and the concentration of liver enzymes in normal and hyperglycemic mice has been proved[36]. Energy drinks tend to contain 80-141 mg of caffeine per 8 ounces (1 cup), which is the same amount of caffeine as five ounces of coffee. In this regard, it was noted that the level of AST and ALT in rats' serum increased significantly after caffeine intake[10&37].

CONCLUSION

This experiment proved that RB is toxic to the liver. Future studies are required to show the negative effects of RB and energy drinks over a longer period of time and on other body organs. Strong restrictions on available com-

mercially energy drinks are also necessary.

CONFLICT OF INTEREST

There are no conflicts of interest.

REFERENCES

1. Kaminer Y. Problematic use of energy drinks by adolescents. Child Adol. Psych. Clin. North Am. 2010;19(3) 643-650.

2. Ragsdale FR, GronliTD, BatoolN, HaightNA, Mehaffey EC, McMahon TW, Nalli CM, Mannello CJ, Sell PJ, McCann GM, Kastello T, Hooks T, Wilson, T. Effect of Red Bull energy drink oncardiovascular and renal function. J. Amino Acids. 2010;38: 1193-1200

3. Alford C, Cox H, Wescott R. The effects of red bull energy drink on human performance and mood. Amino Acids 2001; 21:139–150.

4. Malinauskas BM, Aeby VG, Overton RF, Carpenter-AebyT,Barber-Heidal K. A survey of energy drink consumption pattern among college students. Nutr. J., 2007;6: 1-7.

5. Reissig CJ, Strain EC, Griffiths RR.Caffeinated energy drinks-a growing problem. Drug Alcohol Depend., 2009;99(1-3):1-10

6. Salih NA, Abdul-Sadaand IH,Abdulrahman NR. Histopathological Effect of Energy Drinks (Red Bull) on Brain, Liver, Kidney, and Heart in Rabbits. Med J Babylon. 2018;15:16-20.

7. Ballard SL, Wellborn-Kim JJ, Clauson KA. Effects of commercial energy drink consumption on athletic performance and body composition. The Physician and sportsmedicine. 2010; 1; 38(1):107-17.

8. Vivekan and arajah A, Ni S, Waked A. Acute hepatitis in a woman following excessive ingestion of an energy drink: a case report. Journal of medical case reports. 2011;5(1):227.

9. Burrows T, Pursey K, Neve M, Stanwell P. What are the health implications associated with the consumption of energy drinks? A systematic review.Nutr Rev 2013;71:135–48 .

10. KhayyatL, Sorour J, Al Rawi M Essawy. A. Histological, Ultrastructural and Physiological Studies on the Effect of Different Kinds of Energy Drinks onthe Liver of Wistar albino Rat.Journal of American Science. 2012; 8(8):688-697.

11. Kassab A ,Tawfik S. Effect of a caffeinated energy drink and its with drawal on thesubmandibular salivary gland of adult male albino rats: Ahistological and immunohistochemicalstudy. Egypt J of Histol. 2018;41.(1):11-26.

12. Bancroft JD, Gamble A.Theory and practice of histological techniques. 8th ed. New York, London:Churchil, Livingstone.2018.

13. Ramos-Vara JA. Technical aspects of immunohistochemistry. Veterinary pathology. 2005; 42(4), pp.405-426.

14. Glauert AM, Lewis PR. Biological specimen preparation for transmissionelectron microscopy. 1st ed. William Street, Princeton, New Jersey,USA:Princeton University Press. 1998

15. Berger AJ, Alford K. Cardiac arrest in a young man following excess consumption of caffeinated "energy drinks". The Medical Journal of Australia2009;190(1): 41-43.

16. ZucconiS, VolpatoC, AdinolfiF, GandiniE, Gentile E , Loi A. Gathering Consumption Data on Specific Consumer Groups of Energy Drinks. European Food Safety Authority (EFSA). EFSA Supporting Publications2013; 10(3):1–190. Available online on www.efsa.europa.eu/publication

17. Akande I, Banjoko O. Assessment of biochemical effect of Power Horse energy drink on hepatic, renal and histological functions in Sprague Dawley rats. Annual Review and Research in Biology 2011;1(3): 45-56.

18. Mansy W, Alogaiel D, Hanafi M, Zakaria E. Effects of chronic consumption of energy drinks on liver and kidney of experimental rats. Tropical Journal of Pharmaceutical Research2017;16 (12): 2849-2856.

19. Elsayed S, Afify A, Mohammed A, Abdelwahab W, AbdAlRahmanR.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.

20. Afify A, AbdElwhab W, Mohammed A, Abd Al-Rahman R, El-Sayed S. Study of the protective role of omega 3 on hepatotoxicity induced by different energy drinks in adult male albino rats. Zagazig Journal of Forensic Medicine 2019; 17(2), 46-60.

21. Ayuob N,ElBeshbeishy R. Impact of an energy drink on the structure of stomach and pancreas of albino rat: Can Omega-3 provide a protection?. PLoS One 2016; 11(2): e0149191.

22. Mubarak M. Effect of red bull energy drink on rats submandibular salivary glands (light and electron microscopic study). J Am Sci 2012;8(1):366-72.

23. Reis R, Charehsaz M, Sipahi H, Ekici A, Macit C, Akkaya H,Aydın A.Energy Drink Induced Lipid Peroxidation and Oxidative Damage in Rat Liver and Brain When Used Alone or Combined with Alcohol. Journal of Food Science 2017; 82 (4): 1037:1043.

24. Ekaluo UB, Uno UU, Edu NE, Ekpo PB, Etta SE. Effect of Trevo dietary supplement on caffeine induced oxidative stress in albino rat models. The Pharmaceutical and Chemical Journal 2016; 3(2):92-7

25. Mohamed RA, Ahmed AM, Al-Matrafi T, AlRoalle AH, Alfayez MA, Al-OkaielDM, El Fouhil A,Atteya M. Energy drinks induce adverse histopathological changes in gastric and duodenal mucosae of rats. International Journal of Advanced and Applied Sciences2018;5(2): 81-89

26. Grasser EK, Yepuri G, Dulloo AG, Montani J. 2014. Cardio- and cerebrovascular responses to the energy drink Red Bull in young adults: a randomized cross-over study. Eur J Nutr. 53: 1561-1571.

27. Cheung PY, Zhang Q, Zhang YO, Bai GR, Lin MC, ChanB, Fong CC, Shi L, Shi YF, Chun J, Kung HF, Yang M.Effect of WeiJia on carbon tetrachloride induced chronicliver injury. World J Gastroenterol 2006;12:1912-1917.

28. Zarobkiewicz MK, Mateusz M, Wo´zniakowski MM, Gawda EW, Sławinski MA, Halczuk P, Korolczuk A, Jedrych BJ. Decrease in Lipid Droplets in Adrenal Cortex of Male Wistar Rats after Chronic Exposure to Energy Drinks. Medicina 2018; 54(90):1-9.

29. Abdel Moneim RA and El Deeb M. Measurement of saliva flow rate and miRNA-132 expression in relation to the histological structure of submandibular salivary gland following administration of antiobesity agent (an animal study). Int. J. of Adv. Res 2018; 710-729

30. Tek M, Toptas O, Akkas I, Kazancioglu HO, Firat T, Ezirganli S,Ozan F. Effects of Energy Drinks on Soft Tissue Healing. J Craniofac. Surg 2014; 25: 1–5.

31. Dai W, Sato S, Asano G. The protective effect of hepatocyte growth-promoting factor (pHGF) against carbon tetrachloride-induced acute liver injury in rats. II. Protective effects on cell membrane injury. J Nippon Med Sch. 2001;68(2):154-64. Japanese. doi: 10.1272/jnms.68.154. PMID: 11301361.

32. Tasci I, Mas N, Mas MR, Tuncer M, Comert B. Ultrastructural changes in hepatocytes after taurine treatment in CCl4 induced liver injury. World Journal of Gastroenterology: WJG 2008; 14(31), 4897.

33. Balaban RS, Nemoto SF,Finkel T. Mitochondria, oxidants and aging. Cell.2005; 120: 483-495.

34. El Desouky A, Zaid A, El Saify G, Noya D. Ameliorative Effect of Omega-3 on Energy Drinks – Induced Pancreatic Toxicity in Adult Male Albino Rats. Egypt J of Histol 2019; 42 (2):324-334

35. Ebuehi A, Ajayl O, Onyeulor A, Awelimobor D. (2011): Effects of oral administration of energy drinks on blood chemistry, tissue histology and brain acetylcholine in rabbits.J. Nigerian quarterly journal of hospital medicine 2019;21:29-34

36. Bukhari H, ElSawyN, Header E. Biological Effect of High Energy Drink on Normal and Hyperglycemic Rats. Pakistan Journal of Nutrition.2012;11 (4): 301-309

37. Alsunni AA.Energy Drink Consumption: Beneficial and Adverse Health Effects. International journal of health sciences 2015; 9(4), 468–474.