Histological, Ultrastructural and Physiological Studies on the Effect of Different Kinds of Energy Drinks on the Liver of Wistar albino Rat

Latifa khayyat
Biology Department
Faculty of Applied Sciences
Umm Al-Qura University
Makkah, Saudi Arabia
likhayyat@uqu.edu.sa

Amina Essawy
Biology Department
Faculty of Applied Sciences
Umm Al-Qura University
Makkah, Saudi Arabia
amina_essawy@yahoo.com

Jehan Sorour
Biology Department
Faculty of Applied Sciences
Umm Al-Qura University
Makkah, Saudi Arabia
Jehansorour@hotmail.com

Maisaa Al Rawi
Biology Department
Faculty of Applied Sciences
Umm Al-Qura University
Makkah, Saudi Arabia
mmalrawi@uqu.edu.sa

Abstract— Three kinds of energy drinks (Power horse, Red bull and Code red) were used to study their histological, ultrastructural and physiological effects on Wistar albino rat liver. Forty male Wistar albino rats were divided into four groups. Group 1 was the control, while Groups 2, 3 and 4 were each orally administered with a type of the energy drinks daily for 4 weeks. After two and four weeks of treatment, five animals from each group were killed and dissected. The liver was removed, cut and fixed quickly to carry out light and electron microscopic preparations. Blood samples were collected from each rat via Cardiac puncture method for enzyme determination. The histopathological and ultrastructural results indicated mild hepatotoxicity of Power horse, Red bull and Code red. The alterations in liver ultrastructure were almost similar to each other; however the necrotic areas and the pyknotic nuclei were more obvious in Power horse and Red bull than that of Code red. Moreover, the present study showed that the energy drinks induced an elevation of liver enzymes AST, ALT and ALP after two and four weeks of treatment. The data illustrated that power horse was more effective in its action on liver enzymes, followed by red bull and to less extend code red. The different action of the energy drinks on liver function could be attributed to the different mixture of their ingredients.

Keywords: energy drinks, rat, liver, histopathology, ultrastructure, liver enzymes

I. INTRODUCTION

Energy drinks are non-alcoholic, often lightly carbonated beverages that are designed to give energy by the addition of a number of energy enhancing ingredients. They are widely used by youth while studying, playing sports and driving long distances.

Energy drinks commonly include caffeine, other plant based stimulants (guarana, ephedrine, yerba mate), simple sugars (glucose, fructose), amino acids (taurine, carnitine, creatine), herbs (various forms of ginseng, ginkgo biloba), maltodextrin, inositol, glucuronolactone (anaturally occurring glucose metabolite) and vitamins B complex. [1, 2]

Caffeine is the main ingredient of energy drinks associated with diuresis and fluid-electrolyte balance, while taurine is related to detoxification and bile acid conjugation. Caffeinated energy drinks consumption showed damaging effects on the hepatocytes and increasing on creatinine, [3, 4], aspartate transferase (AST), alanine amino transferase (ALT) and alkaline phosphatase (ALP) on serum of rats [5, 6]. However, taurine has inconclusive effects on renal and liver functions [1,7, 8 ,9]. Other studies indicated that oral administration of the energy drinks, especially power horse and red bull, affected blood chemistry and liver enzymes activities. On the other hand no obvious histopathological abnormalities of the brain and liver were recorded [10].

The present study aims to investigate the histopathological, ultrastructure and physiological effects of three kinds of energy drinks (Power horse, Red bull and Code red) on liver of rats.

II. MATERIAL AND METHODS

A.Experimental animals:

Forty male Wistar albino rats 10 weeks old, weighting 120 ± 10 gm were used, conditioned in standard metallic cages (5 rats per cage) and kept in a temperature-controlled environment (24 ± 2 0C) with an alternating 12 h light – dark cycle. They were acclimatized to the laboratory conditions, fed standard rat chow and water was available *ad libitum*.

Animals were maintained and experimental procedures complied with the guide for care and use of laboratory animals [11].

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B.Experimental design:

Several cans of three types energy drinks:

Power horse, Red Bull and Code red were used in this experiment. The animals were divided into 4 groups of 10 rats each and treated as follows:

Group 1: Animals of this group were given 3 ml distilled water and served as control.

Group 2: Animals of this group were orally administered with 1.5 ml/100g b.wt of Power horse daily for 4 weeks.

Group 3: Animals of this group were orally administered with 1.5 ml/100g b.wt of Red bull daily for 4 weeks.

Group 4: Animals of this group were orally administered with 1.5 ml/100g b.wt of Code red for 4 weeks.

After two and four weeks of treatment, five animals from each group were killed by cervical dislocation, quickly dissected and liver was removed.

C. Histological and Ultrastructural study:

Liver were removed from the dissected rats and fixed in buffered formalin, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Sections were stained with haematoxylin and eosin and examined with light microscope. For transmission electron microscopy: small slices of liver were immediately fixed in 4F1G in phosphate buffer (pH7.2) for 3 hours at 4°C and postfixed in 2% OSO4 in the same buffer at 4°C for 1-2 hours .The specimens were dehydrated through graded series of ethanol, embedded in epon-araldite mixture and polymerized at 60°C. Ultrathin (50 nm) sections from selected areas were cut with glass knives on LKB ultramicrotome. They were double stained with uranyl acetate and lead citrate and examined by jeol 100CX electronmicroscope.

D. Physiological study

Enzyme assays:

For enzyme determination, blood samples were collected from each rat via cardiac puncture method and allowed to clot. The serum was rapidly separated by centrifuging the clotted blood at 3000g for 10 min in a Beckman Model T-6 refrigerated centrifuge and processed for determination in to clean and dry tubes. Sera were stored at -20 0C until assayed for the biochemical parameters.

1. Alanine amino transferase:

Alanine amino transferase catalysed was measured with the spectrophotometer according to method of Steven [12].

2. Aspartate aminotransferase :

Aspartate aminotransferase catalysed was measured with the spectrophotometer according to method of steven [12].

3.Alkaline phosphatase:

Alkaline phosphatase catalyzed was monitored with a recording spectrophotometer according to method of Juliet and John [13].

E. Statistical analysis:

Data were expressed as mean ± SD of five replicates and were subjected to one way analysis of variance(ANOVA) followed by student,s T test. Results were considered statistically significant at $P<0.05$.

III. RESULTS

A.Histopathological results:

Examined liver sections of control rats showed the normal appearance of hepatic lobules, bile ductule, central vein and hepatocytes (Fig.1).

Concerning the liver tissue of rat given Power horse drink for 2 weeks, there were no obvious changes except widening between the hepatic strands and appearance of mitotic division (Fig.2). After 4 weeks the liver showed leucocytic infiltration and congestion of blood sinusoids (Fig.3). Most hepatocytes lost their polyhedral shape and appeared with lipid contents and pyknotic nuclei (Fig.4). Several mitotic cells were also noticed.

Liver sections of animals given Red bull drink were illustrating some signs of distortion in tissue after 2 weeks (Fig.5)

However, there was clear loss of the normal liver architecture with marked vacuolization and necrosis of most hepatocytes after 4 weeks (Fig.6). Moreover, marked inflammation as well as accumulation of lipid droplets could be observed in some parts (Fig.7).

Histological examination of liver tissue of rats given Code red showed mild architectural distortion after 2weeks. On the other hand, after 4 weeks the hepatocytes have increased in size and showed intracytoplasmic vacuolization (Fig.8). Less severe morphological lesions of the liver tissue ,as exhibited in few fatty droplets and mild inflammation were also detected (Fig. 9).

B.Ultrastructural Results:

Electron micrographs of liver from animals of the control group revealed characteristic normal hepatocyte ultrastructure. The nucleus appeared rounded and surrounded by parallel cisternae of rough endoplasmic reticulum (rER).The mitochondria were found in close association with the rER, varying from circular to elongated form. Some lipid droplets were present in the cytoplasm which was mainly filled with glycogen (Fig.10).

Various ultrastructure alterations were observed in the rat hepatocytes after giving the animals Power horse for 4 weeks. The cytoplasm lacked its compartimentation and appeared to be necrotic in most hepatocytes (Figs.11 & 12). Numerous lipid droplets (lipidosis) were found in the cytoplasm of many hepatocytes (Fig .13). In addition , the cisternae of rough endoplasmic reticulum (rER) were dilated and divided into vesicles , while the smooth endoplasmic reticulum (sER) was developed (Fig. 12). Several mitochondria showed abnormalities such as dumbbell-shaped , pear-shaped and horseshoe-shaped (Fig.11), while others appeared vacuolated (Fig.12). Throughout the cytoplasm , a random dispersion of free ribosomes , glycogen particles , lysosomes and myelin – like bodies could be observed (Figs.11,13 & 14). Concerning the nuclei, they were almost spherical in shape with two nucleoli (Figs.12&14) , while others appeared to have irregular outlines or were pyknotic (Figs.11&13). Moreover, leucocytes infiltration could be observe in the

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blood sinusoids (Fig.14). Several abnormalities could be noted in the rat liver hepatocytes after giving the animals Red bull for 4 weeks . The cytoplasm of some hepatocytes seemed to be vacuolized and almost devoid of its components (Fig.15). Besides, there were various – sized lipid droplets present throughout the cytoplasm .On the other hand , dilatation and vesiculation of rough endoplasmic cisternae (rER) were most prominent with partial loss of ribosomes from their membranes (Fig.16). Various cisternae of smooth endoplasmic reticulum (sER) as well as vacuolated and swollen mitochondria were also observed (Fig.17). Widening of intercellular space between the hepatocytes (Fig .18), Golgi bodies , primary lysosomes , myelin – like membrane structures , glycogen and free ribosomes could be noticed.

Further, many nuclei showed shrinkage and deformation and the nucleoli were displaced to the periphery of the nucleus or disappeared (Figs.18&19). Degenerative alterations were observed in cytoplasmic organelles as well as in the nuclei of the rat hepatocytes after giving the animals Code red for 4 weeks. In this group, the cytoplasm of some hepatocytes appeared diffused (Fig.20), while others showed several lipid droplets (Fig.21). The rough endoplasmic reticulum was dilated, fragmented or appeared swirling and some mitochondria were irregular in shape. In addition a few nuclei exhibited obvious irregular outlines (Fig.21) while others retained the spherical shape and appeared normal. It is clear that alterations in the liver ultrastructure of rat giving power drinks were almost similar to each other, however the necrotic areas and the pyknotic nuclei were more obvious in Power horse and Red bull than that of Code red. The histopathological and ultrastructural results indicated mild hepatotoxicity of the power drinks and they may affect the overall health of the animal.

C. Liver function enzymes:

Data in table (1) showed that there was a significant elevation ($P<0.05$) in AST and ALT after 2 and 4 weeks of treatment with power horse and red bull compared to the control.

Administration of power horse induced insignificant increase in ALP after the second week followed by a significant increase after 4 weeks. However, treatment with red bull for 2 weeks resulted in an insignificant decrease in ALP level followed by a significant elevation after 4 weeks . On the other hand, animals treated with code red induced insignificant elevation in AST, ALT and ALP after 2 weeks of treatment with respect to controls. A significant increase in the level of AST and ALT and an insignificant decrease in ALP were recorded in animals treated with cod red for 4 weeks.

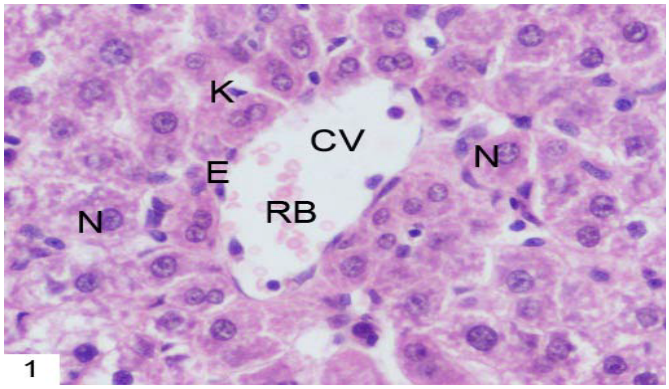


Fig.(1): Light micrograph of liver section from control rat showing the normal histological structure of hepatocytes with central spherical nucleus(N). Central vein (CV) lined with endothelial cells (E), Kupffer cells (K) and contained red blood cells (RB). H&E. x400

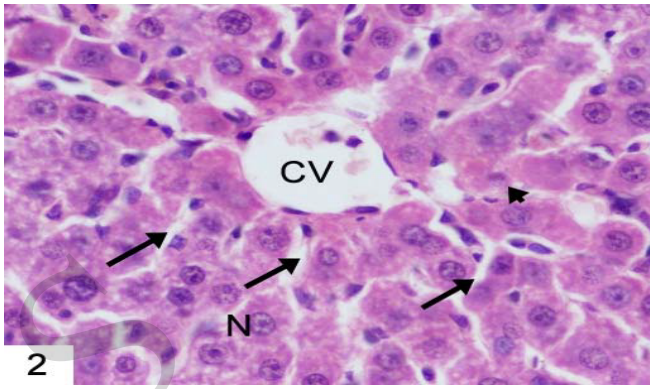


Fig.(2): Light micrograph of liver section from rats given power horse for 2 weeks. Note the wide space between the hepatic strands (arrows),mitotic division (head arrow). N, nucleus; CV, central vein. H&E. x400

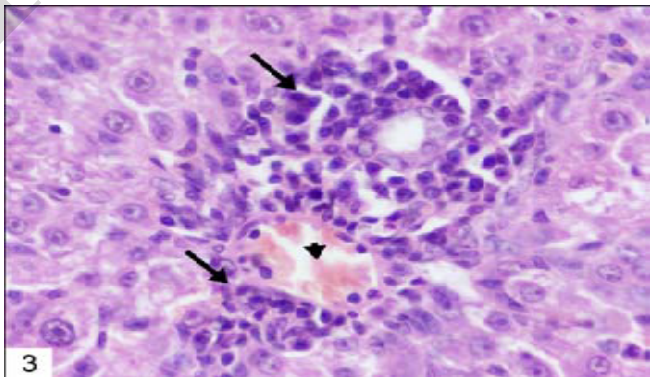


Fig.(3): Light micrograph of liver section from rats given power horse for 4 weeks showing leucocytic infiltration (arrows) and congestion of blood vessel (head arrow). H&E. x400

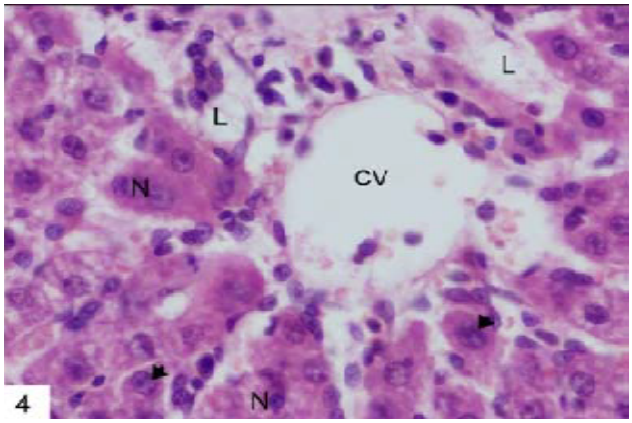


Fig.(4): Light micrograph of liver section from rats given power horse for 4 weeks. Note the presence of lipid droplets (L), mitotic division (head arrows), pyknotic nuclei (N). central vein, CV. H&E. x400

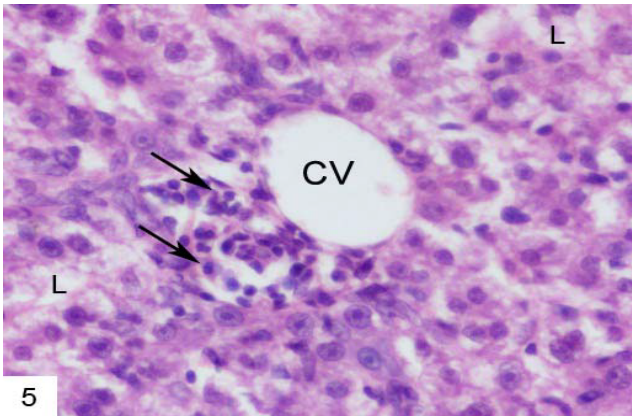


Fig.(5): Light micrograph of liver section from rats given red bull for 2 weeks showing disorganization of the hepatic structure. Central vein (CV); leucocytic infiltration (arrows); lipid droplets, L. H&E. x400

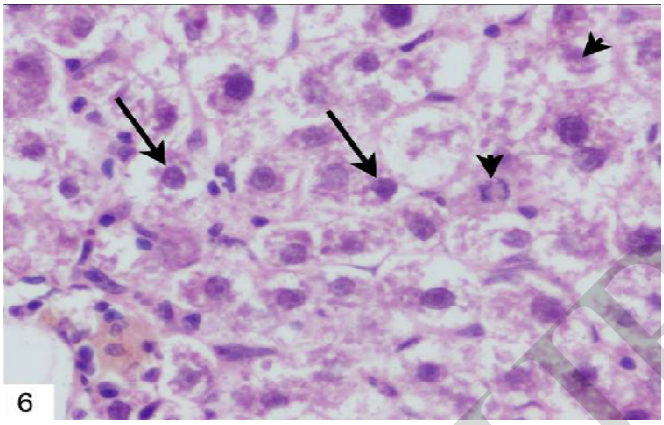


Fig.(6): Light micrograph of liver section from rats givenred bull for 4 weeks. Notice the necrosis of most hepatocytes (arrows) and marked cytoplasmic vacuolization. Mitotic division (head arrow). H&E. x400

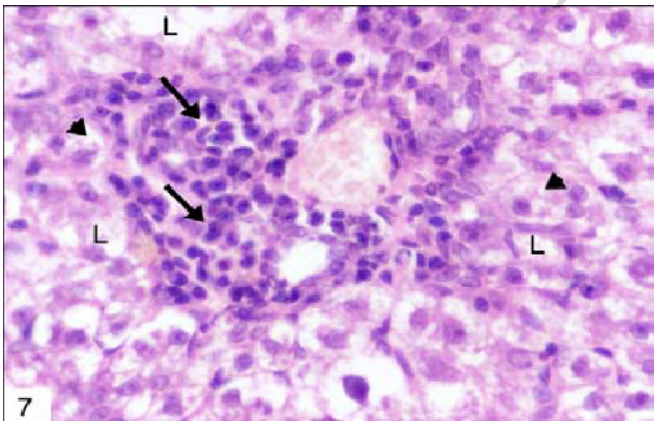


Fig.(7): Light micrograph of liver section from rats given red bull for 4 weeks showing marked inflammation (arrows), accumulation of lipid droplets (L) and necrotic hepatocytes (arrowheads). H&E. x400

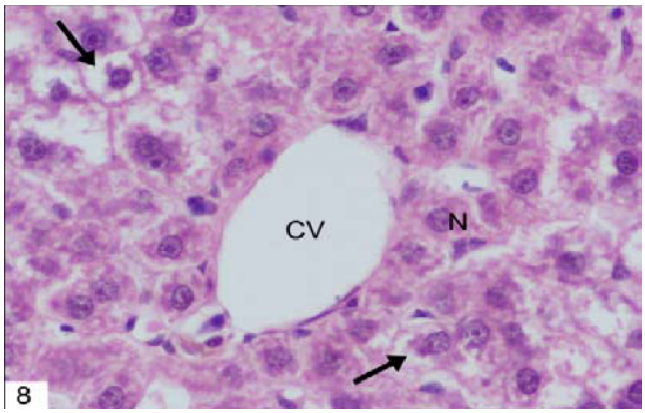


Fig.(8): Light micrograph of liver section from rats rats given code red for 4 weeks. The hepatocytes increased in size and showed intracytoplasmic vacuolization (arrows). Central vein, CV ; Nucleus, N. H&E. x400

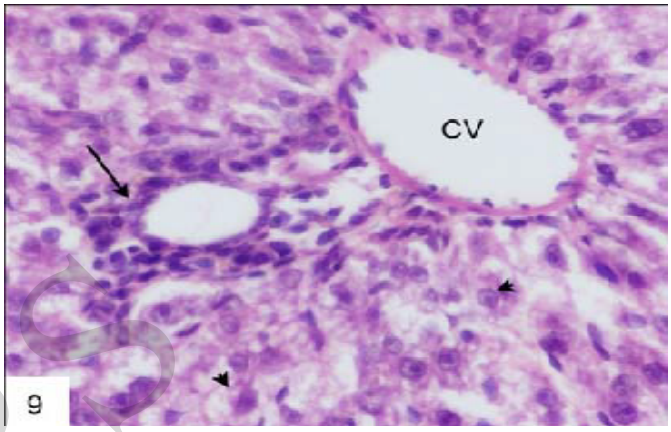


Fig.(9): Light micrograph of liver section from rats given code red for 4 weeks showing less inflammation (arrow) and degenerated hepatocytes (arrow head). CV: central vein . H&E. x400

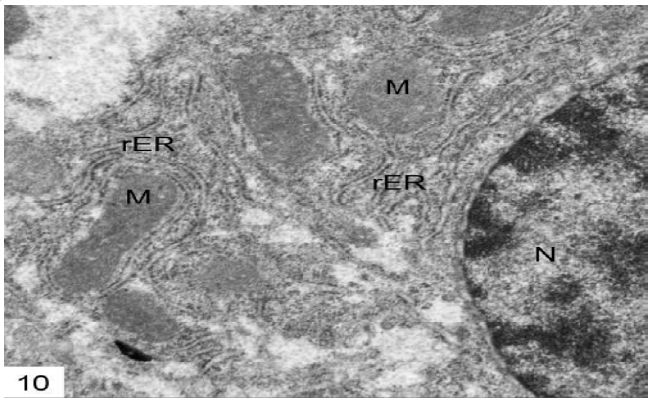


Fig. (10): Electron micrograph of liver rat from control group showing part of the hepatocyte with nucleus (N), parallel cisternae of rough endoplasmic reticulum (rER) and numerous mitochondria (M). x7500

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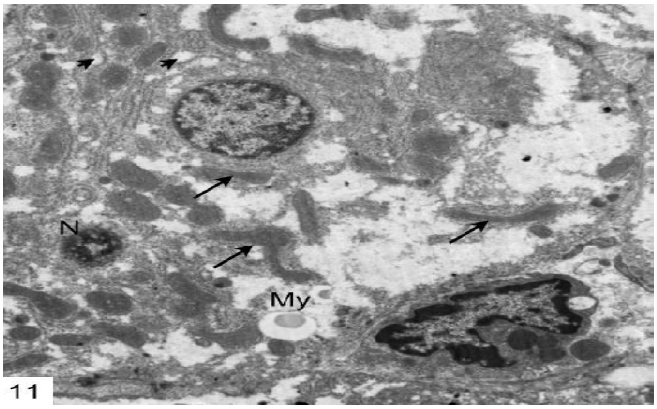


Fig.(11): Electron micrograph of liver from rats given power horse for 4 weeks showing degenerative hepatocytic cytoplasm, pyknotic nucleus (N) and dilatation of rough endoplasmic reticulum (head arrows). Variable-shaped mitochondria ,arrows; myelin- like bodies, My. X2500

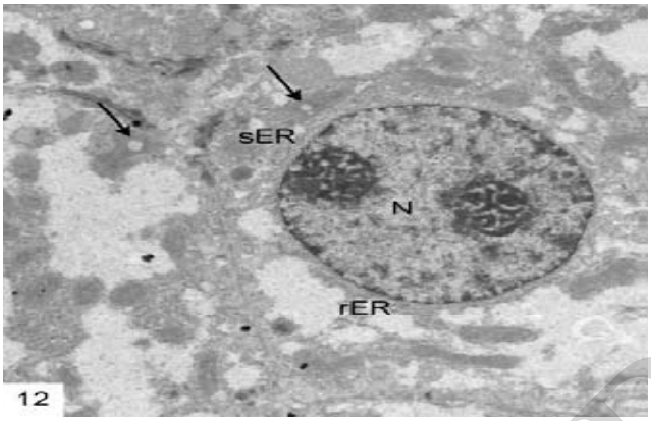


Fig.(12): Electron micrograph of liver from rats given power horse for 4 weeks showing vacuolized cytoplasm, nucleus(N) with two nucleoli fragmented rER, developed sER and abnormal, vacuolated mitochondria (arrows). X3000

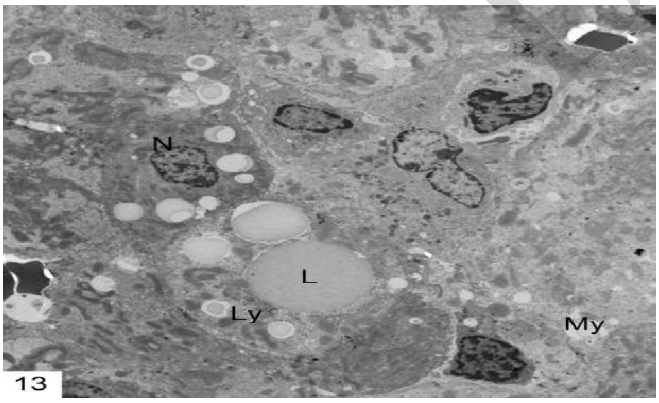


Fig.(13): Electron micrograph of liver from rats given power horse 4 weeks. Note the presence of numerous lipid droplets (L), irregular nucleus (N), myelin-like bodies (My) and lysosomes (Ly). X1300

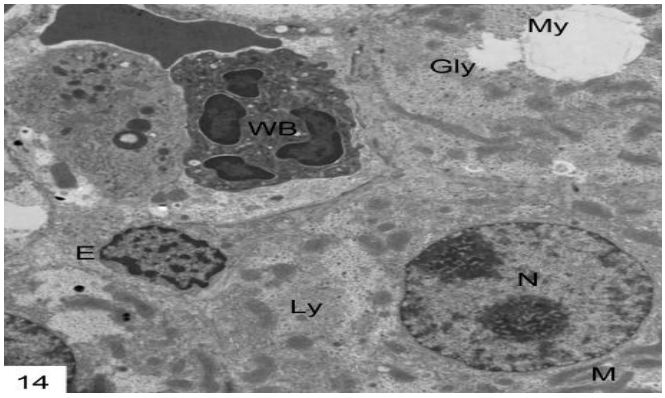


Fig.(14): Electron micrograph of liver from rats given power horse for 4 weeks. Note the leucocytes infiltration (WB) in blood vessel, endothelial cell (E) and nucleus(N) with two nucleoli . Glycogen particles, Gly; lysosomes, Ly; mitochondria,M; myelin-like bodies, My. X2000

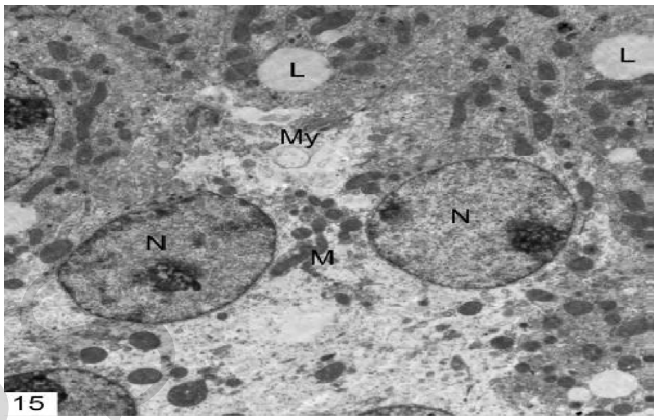


Fig.(15): Electron micrograph of liver from rats given red-bull for 4 weeks. The cytoplasm of hepatic cells seemed to be vacuolized and devoid of its components. Lipid droplets, L ; mitochondria, M ; myelin-like bodies, My;
nucleus, N. X2000.

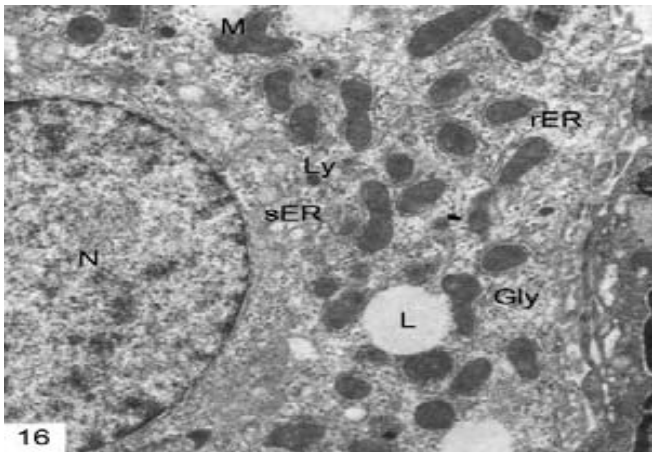


Fig.(16): Electron micrograph of liver from rats given red-bull for 4 weeks showing dilatation of rER cisternae, developed sER and abnormal mitochondria (M). Glycogen particles, Gly lipid droplets, L ; lysosomes, Ly. X4000

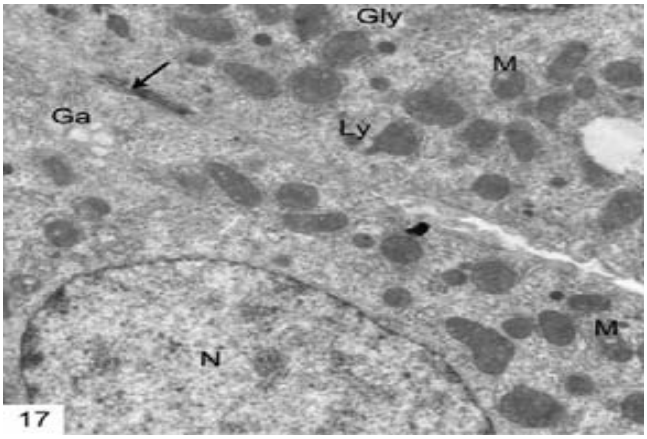


Fig.(17): Electron micrograph of liver from rats given red-bull for 4 weeks. Note vacuolized mitochondria (M), Golgi bodies (Ga), primary lysosomes (Ly), glycogen particles (Gly) and desmosomes in the cell boundary between two hepatocytes (arrow). X4000

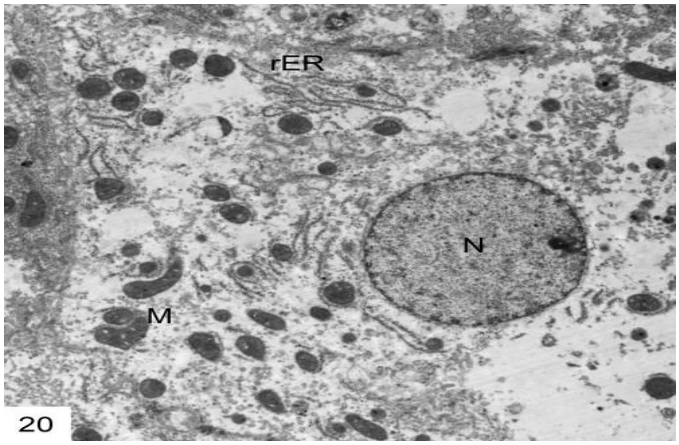


Fig.(20): Electron micrograph of liver from rats given code-red for 4 weeks showing diffusion of cytoplasm, fragmentation of rER cisternae and irregular shaped mitochondria (M). X 2500

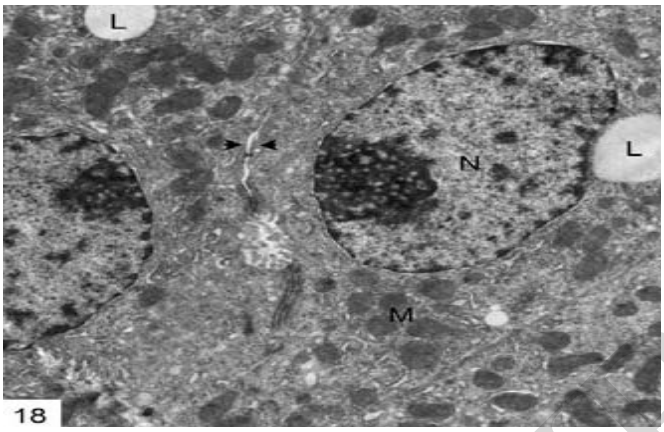


Fig.(18): Electron micrograph of liver from rats given red-bull for 4 weeks showing widening of the intercellular space between the hepatocytes (head arrows). The nucleolus displaced to the periphery of the nucleus (N). Lipid droplets, L; mitochondria, M. X3000

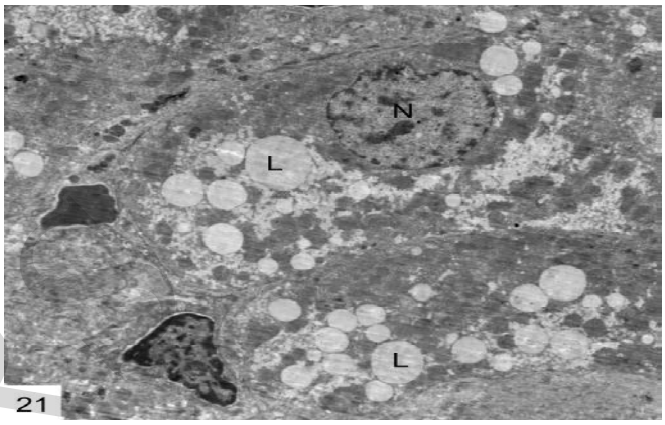


Fig.(21): Electron micrograph of liver from rats given code-red for 4 weeks. The hepatic cytoplasm contains numerous lipid droplets (L) and irregular nucleus (N). X1500

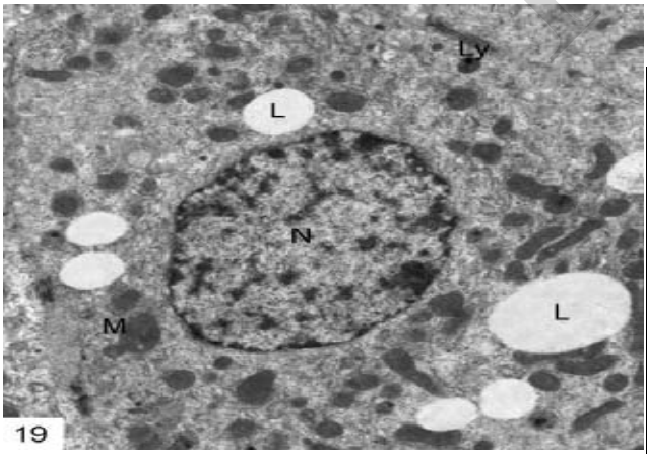


Fig.(19): Electron micrograph of liver from rats given red-bull for 4 weeks. Notice an irregular nucleus (N) and margination of chromatin on the innersurface of the nuclear envelope. Lipid droplets, L; lysosomes, Ly; mitochondria, M. X3000

Table (1): Effect of administration of power horse, red bull and red code on aspartate aminotransferase(AST), alanine aminotransferase(ALT) and alkaline phosphatase (ALP) in Wistar rats

Time	Control	Power horse	Red bull	Code red
AST 2wks (u/ml)	139.2a ± 19.77	224.2b ± 23.78	197.7bc ± 28.65	169.25ac ± 14.24
4wks	140.2a ± 35.85	239.0b ±28.65	220.0b ± 39.0	196.75b ± 48.0
P	0.010*	0.344	0.046*	0.005*
ALT 2wks (u/ml)	58.75 a ± 10.87	89.0 b ± 8.83	84.0 b ± 18.92	78.25 ab ± 14.17
4wks	59.75 a ± 11.59	165.0 b ± 10.98	175.50 b ± 19.36	155.25 b ± 38.43
p	<0.001*	0.0360*	0.071	<0.001*
ALP 2wks (u/ml)	32.0 ac ± 3.46	38.50 ab ± 7.85	23.75 c ± 3.20	33.75 ab ± 5.68
4wks	30.75 a ± 5.32	83.0 b ± 18.40	74.0 b ± 6.84	45.50 a ± 15.35
p	<0.001*	0.448	0.065	0.002*

Data represented as mean ± S.E. (n=5)
p : p value for student t-test between the two periods of treatment in each group
Different superscripts within each raw indicate statistical significant differences between groups at 0.05
*: Statistically significant at $p \leq 0.05$

IV. DISCUSSION

The ultrastructural findings and their correlation with histopathological results proved that oral administration of energy drinks induced alterations in the hepatic cells being more obvious after 4 weeks.

Almost many investigators are in agreement with the adverse effects of energy drinks on liver [6, 14]. However, Ebuehi *et al.* [10] reported that power horse and red bull affected blood chemistry, liver enzymes activities but not significantly affect the histopathology of the brain, heart and liver of the rabbits. In the present study, the hepatic cells cytoplasm of rats giving energy drinks appeared vacuolized with presence of lipid droplets, which might be attributed to degenerative changes within the hepatocytes. Such observations were previously reported by Mubarak [15] in rat submandibular salivary glands induced by Red Bull for 8 weeks.

The most significant ultrastructural alterations with energy drinks administration were dilatation and fragmentation of rough endoplasmic reticulum cisternae (rER) and development of smooth endoplasmic reticulum (sER). The dilated rER is representative of damaged hepatocytes [16], while increased sER which is the place of action provide a storage of important cellular enzymes and a site for detoxification [17, 18].

In addition, there was some disruption in mitochondrial structure indicating deteriorated function of mitochondria [19]. The hepatocytic nuclei showed irregular outlines and pyknosis. Numerous mitotic figures were also detected. These results could be sign s of toxicity. Mubarak [15] pointed out that such changes might be due to the preservatives added to energy drinks such as sodium benzoate, and to the toxic action of the caffeine content of them. Light and electron microscopic results revealed congestion of blood vessels and infiltration of leucocytes through the hepatocytes. This might be due to different reaction of taurine associated with other active ingredients of the energy drinks as caffeine. The combination of excessive ingestion of caffeine and taurine – containing energy drinks can produce myocardial ischaemia by inducing coronary vasospasm [20]. On the other hand, it is well known that taurine conjugates with bile acids and aids digestion of lipids. It has also several important regulatory actions as detoxification, membrane stabilization, osmoregulation and modulation of cellular calcium levels [21].

The present study showed that administration of energy drinks, power horse, red bull and code red caused hepatotoxicity including an elevation of liver enzymes and alterations in the structure of liver tissue. Liver function enzymes, ALT, AST and ALP were elevated in the sera of rats after treatment with each of the energy drinks . This is in agreement with the results of Akande and Banjoko [6] who reported that there is an increase in the serum AST, ALT and ALP in rats treated with power horse. Also Ebuehi *et al.*,[10], found that, power horse and red bull significantly affect liver enzyme activities in rabbits. Moreover, Bukhar *et al.*,[14] mentioned that, there is a relation between high energy drink administration and concentration of liver enzymes in normal and hyperglycemic rats. Energy drinks typically contain 80-141mg of caffeine per 8 ounces, the equivalent of five ounces of coffee or two 12- ounce cans of caffeinated soft drink [22].

In this respect Cheul Do *et al*., [5] reported thatcaffeine administration caused a significant increase in the level of AST, ALT in serum of rats. On the other hand, Ruhl and Everhart[23] and Cadden *et al* .,[24] mentioned that, caffeine treatment leads to decrease in serum ALT.

The data of the current study showed that power horse was more effective in its action on liver enzymes, followed by red bull. The different action of the energy drinks could be attributed to the different mixture of their ingredients.

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