Original Article

A Histomorphometric study on the Effect of Consumption of Caffeinated Energy Drinks on the Aorta of the Adult Male Albino Rats

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ABSTRACT

Introduction: Energy drinks intake has been increasing since its introduction commercially. Research on the impacts of energy drinks on individuals has also been progressing ever since. Recently, the description of the contents of such drinks has not been well proven despite the fact that caffeine is considered the most reliable ingredient in all of its types.
Aim of Work: To investigate the microscopic alterations in the ascending aorta associated with ingestion of Red Bull.
Material and Methods: Twenty adult male albino rats weighing 150 grams were used and divided into.: Control Group: had free access to food and water; Experimental Group: given 3.75 ml/kg BW Red Bull through oral gavage. Experiment lasted for 4 weeks. Then, animals were sacrificed, their ascending aortae were excised, and processed for examination.
Results: The tunica intima showed areas of partial loss of its endothelium with projection of the underling media into the lumen of the aorta. A tear in the tunica intima extending towards the superficial part of the tunica media was also detected. The tunica media showed disorganization of its control pattern with distortion of the regularly organized elastic fibers. Sections stained with Masson's Trichrome showed increased accumulation of collagen fibers. Elastic fibers distribution was declined when stained with Orcein in the three tunica of the aortic wall.
Conclusion: Energy drinks could be considered as a aggravating factor for acute aortic dissection. More research on the reversibility of these effects in case of withdrawal and stoppage are recommended.

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Key Words: Aorta; energy drinks, red bull; tunica media.

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INTRODUCTION

Caffeinated energy drinks use has continued to grow popular everywhere. These drinks are being commercialized for youth as a natural healthful options that help improve mood as well as physical and intellectual functioning[1].

They are enriched non-alcoholic cocktails with more levels of nutritive enhancements. However, these vary from other soft beverages in that they contain excessive amounts of caffeine and sugars[2]. New preparations of these drinks are always being aimed to reach new customers, which might include women and dieters relying mainly on carbohydrates, in addition to, teenagers[3].

Red Bull; as an example of widely consumed energy drinks; sells under a promotion phrase of "Giving you wings"[4]. Unfavorable consequences of Red Bull usage on important body organs have been reported[5]. Caffeine is known to cause toxic impacts if its limit exceeds 3 mg/kg/day which can be easily surpassed with one serving of caffeinated drink[6]. Two of the major reported risks with the extra usage of such drinks was obesity and metabolic syndrome which could be the result of increasing the activity of the intestinal bacteria[7]. On the other hand, researchers proved that adequate amounts of caffeine-including drinks helped to enhance awareness, retention of memory, facilitate alertness, and improve spoken logic[8].

Insulin and glucose levels were elevated in the blood of experimental animals when they were subjected to consumption of several types of energy drinks[9]. In male Wister rats, continuous consumption had drastically elevated the serum glucose, the total protein levels and the alanine transferase and aspartate transferase liver enzymes[10].

Additionally, several microscopic alterations like fatty infiltration of liver cells, degeneration of kidney glomeruli, pyknosis of the brain nerve cells, and changes in the cerebellar Purkinje fiber layer[11].

Prolonged use of these drinks may be linked with headache, weak intellectual health, tachycardia, gasping, polyuria, metabolic and renal dysfunctions as well as obesity[12]. A healthy individual may suddenly suffer from a thrombus in the coronary artery after drinking of more than 3 cans of these drinks if combined with alcohol[13]. Moreover, several cases of myocardial infarction in healthy individuals with age range 17 to 19 years old has

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been attributed to consuming different amounts of energy drinks[14]. Other articles have reported a relationship between overconsumption of energy drinks on one hand and different vascular manifestation on the other hand[15].

Reviewing the literature, most researchers studied the physiological or biochemical changes in the aortic tissue in response to the use of energy drinks, however; few literature explained the microscopic variations in experimental animals. Accordingly, it was important to make further studies, particularly pointing out the microscopic consequences to broaden the spectrum of their impacts on the vascular system.

MATERIAL AND METHODS

Experimental Animals

Twenty adult male albino rats were used, average age 4 months and weight 150 grams. Rats were purchased from Medical Ain Shams Research Institute (MASRI), Faculty of Medicine. Rats were housed in adequate ethical conditions with free access to food and water. All rats were kept under the same circumstances throughout the experiment.

Ethical considerations

The study conforms with the guidelines adopted by the Committee of Animal Research Ethics (CARE). Ethical approval number of Research Ethics committee Ain Shams Faculty of Medicine FMASU MS350/2021.

Substance used

Red Bull® cans (each 250 ml) as an example of caffeinated energy drinks[16], was bought from a local Egyptian market.

Study Design

Rats were divided into two groups: ten rats each according to the sample size checklist done by community department, Ain Shams university.

Group I (Control): did not undergo any procedures.

Group II (Experimental): were infused orally with 3.75 ml/kg of Red Bull every day for four weeks[17].

Tissue preparation

At the end of the experiment, all animals were sacrificed by intraperitoneal injection of sodium thiopental (25mg/kg BW). The thoracic cages were opened through a midline incision, the heart and ascending aortae were dissected; rinsed in saline then the ascending aortae were dissected from the heart to be processed for preparation of paraffin sections, staining with H & E, Masson's Trichrome and orcein followed by light microscopic examination[18].

Statistical and morphometric analysis

All measurements were done using the image analyzer (TS view ® program) in the Anatomy Department, Faculty of Medicine, Ain Shams University.

Concerning ascending aorta, its mean total thickness was measured in sections stained with H & E. Additionally, the mean thickness of the tunica media was measured in sections stained with orcein. The mean percentage area of collagen fibers deposition was measured in sections stained with Masson's Trichrome. All measures were taken in both groups using high power magnification of light microscope (x400).

The SPSS program was used to conduct the statistical analysis (version 13.0). Unpaired t-test was selected for comparison of the observed microscopic and morphometric data in both studied groups. Values gained were stated as means ± standard deviation (SD) and differences with $P<0.05$ were considered statistically significant[19]. Using MS Excel 2013, tables and charts were used to display the data.

RESULTS

Light Microscopic results

Control group

Hematoxylin and eosin (H&E) staining of ascending aorta serial sections in the control group revealed the presence of three different layers or tunics, namely the tunica intima, tunica media, and tunica adventitia (Figure1).

Squamous endothelial cells with flattened nuclei made up the Tunica intima, which lined the interior surface of the aorta. The ascending aorta's tunica media, the thickest of the three layers and the primary component of its wall, was made up of smooth muscle cells (SMCs), elastic fibers and collagen fibers. The SMCs had single oval nuclei. Intermingled among these SMCs were the elastic fibers which appeared as regularly arranged and distributed, parallel lamellae (Figure 2).

Tunica adventitia was the outermost layer that consisted of closely packed wavy connective tissue with abundant collagen fibers (Figure 2). Small blood vessels (vasa-vasorum) could be detected in the adventitia (Figure 2).

Masson's Trichrome staining revealed a very thin sub endothelium layer made up of loose connective tissue in the sections. Moreover, the elastic lamellae in the tunica media were separated by narrow interlamellar spaces occupied by scanty collagen fibers (Figures 3,4). Abundant collagen fibers were detected in the tunica adventitia (Figure 5).

The distribution of elastic fibers in the three layers of the aortic wall was clearly visible upon examination of orcein stained sections (Figure 6). The concentric wavy elastic lamellae in the tunica media were clearly detected. Elastic fibers were also noticed in the tunica adventitia (Figure 7).

Experimental group

Serial sections of the adult male albino rats from the experimental group's ascending aorta were examined, and they were stained with hematoxylin and eosin. These showed obvious and considerable histopathological

changes of the three layers of the aorta as compared to the control group.

Regarding the tunica intima, there were areas of focal loss of its endothelial flat regular smooth lining with bulging and protrusion of the underling tunica media into the lumen of the ascending aorta (Figure 8). Moreover, areas of discontinuation and desquamation of the linear endothelial cells alternating with areas of normal intact tunica intima were also observed (Figure 9).

Additionally, a discontinuity in the tunica intima extending down to the superficial part of the tunica media was detected (Figure 10). Changes in the nuclei of the endothelial cells could be detected as well; some were darkly stained, small in size and even pyknotic, others were seen protruding into the lumen of the ascending aorta losing their flat normal shape (Figures 10,11).

In some sections, an interesting finding was the presence of a dome shaped structure attached to the inner wall of the ascending aorta and protruding into the lumen, appearing as an intraluminal bulge (Figure 12).

Concerning the tunica media, various light microscopic changes resulting in disorganization of its normal architecture were observed. The characteristic finding was the arrangement of the nuclei of most of the smooth muscle cells with perinuclear cytoplasmic vacuolations (Figures 9,10). A lot of the smooth muscle cells' nuclei were bizarre in shape; small, darkly stained, irregular, or even pyknotic. In other areas, muscle nuclei were aggregated together and sometimes existed in linear colonies. Moreover, few fat-laden macrophages taking the form of foam cells were noticed in the tunica media (Figures 9,11).

Another noticeable finding was the distortion and the disorganization of the regularly arranged elastic fibers of the tunica media in comparison to the control group with areas of elastic fibers disruption (Figure 11). The tunica adventitia showed loosely arranged and dispersed connective tissue (Figure 10).

Sections stained with Masson's Trichrome showed that both the tunica media and the subendothelial layer of the tunica intima had an apparent increase in collagen deposition (Figure 13). In contrast to the control group, there were many collagen fibers encroaching on the tunica media's muscle layer. (Figure 14). Loosely dispersed connective tissue with decreased collagen was observed in the tunica adventitia (Figure 13).

In general, the distribution of elastic fibers in the three tunics of the aorta wall appeared to be decreasing in orcein stained sections (Figure 15). Fragmentation, thinning and variable degrees of separation of the concentric elastic lamellae of the tunica media could be clearly observed. Elastic fibers were hardly detected in the tunica adventitia (Figures 15,16).

Results from morphometry and statistics

The mean thickness of the ascending aorta

The thickness of the ascending aorta in both the control and experimental groups was statistically analyzed in H&E stained sections (under a high power magnification of light microscope x400), and the results revealed a statistically significant increase in thickness in the experimental group compared to the control group (Table 1, Chart 1).

The mean thickness of the tunica media of the ascending aorta:

In sections stained with Orcein of the ascending aorta from the control and experimental groups, statistical examination of the thickness of the tunica media revealed a statistically significant increase in thickness in the experimental group when compared to the control group. (Table 2, Chart 2).

The ascending aorta's mean percentage area of collagen fiber deposition is as follows:

In sections stained with Masson's Trichrome of the ascending aorta from the control and experimental groups, statistical analysis of collagen fiber deposition revealed a statistically significant increase in collagen deposition in the experimental group compared to the control group. (Table 3, Chart 3).

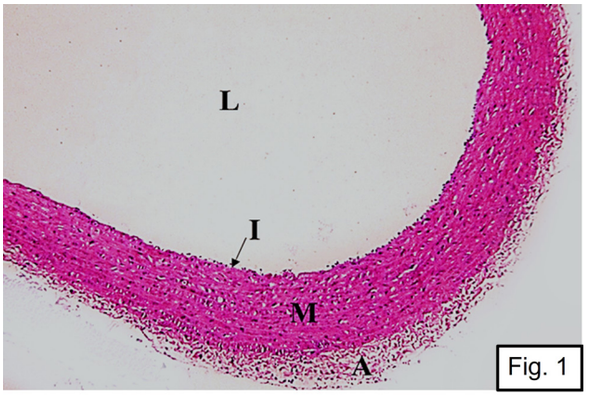

Fig. 1: A photomicrograph of a transverse section of the ascending aorta from the control group, displaying the three layers of the wall: innermost thin tunica intima (I), middle-to-outermost thick tunica media (M), and tunica adventitia (A). Observe the lumen (L) (H&E X100)

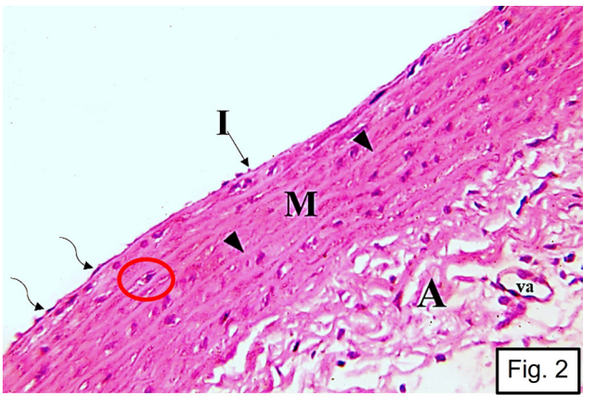

Fig. 2: A photomicrograph of a part of a transverse section of the ascending aorta from the control group showing the innermost thin tunica intima (I), the thick tunica media (M) in the middle and the packed, wavelike connective tissue in the tunica adventitia (A). Observe the tunica intima's (I) flat endothelial cells with flattened nuclei (angled arrow). Also note the presence of the regularly arranged elastic fibers (arrow head) and smooth muscle cells having oval nuclei (red oval) in the tunica media and the vasa vasorum (va) in the tunica adventitia (A). (H&E X400)

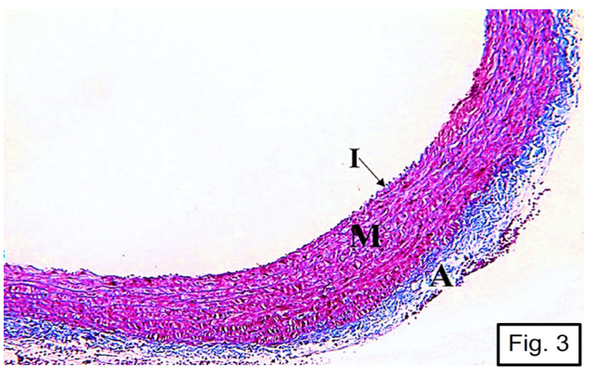

Fig. 3: A photomicrograph of a part of a transverse section of the ascending aorta presenting data from the control group revealing the distribution of collagen fibers in the three layers of the aorta, the innermost thin tunica intima (I), scanty in interlamellar space of the tunica media (M), packed and abundant in the outermost tunica adventitia (A). (Masson's Trichrome X100)

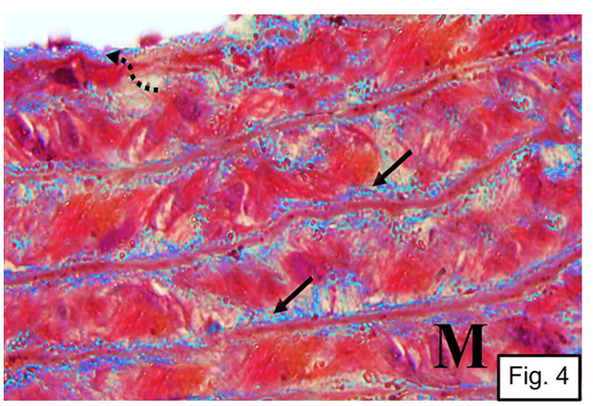

Fig. 4: A photomicrograph of a part of a transverse section of the ascending aorta from the control group showing scanty collagen fibers (black arrows) in the interlamellar space of the tunica media (M) and the thin subendothelial collagen fibers (dotted arrow). (Masson's Trichrome X1000)

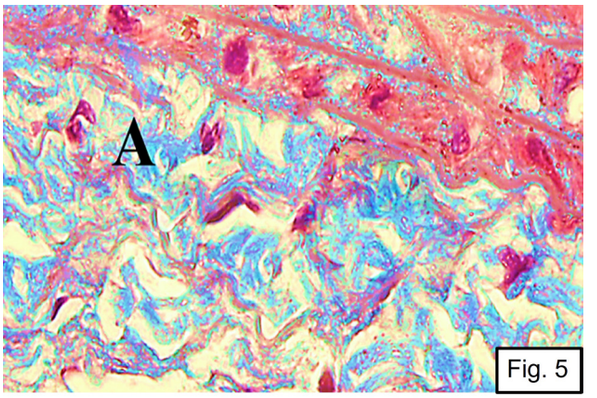

Fig. 5: A photomicrograph of a part of a transverse section of the ascending aorta from the control group showing the packed wavy abundant collagen fibers in the tunica adventitia (A). (Masson's Trichrome X1000)

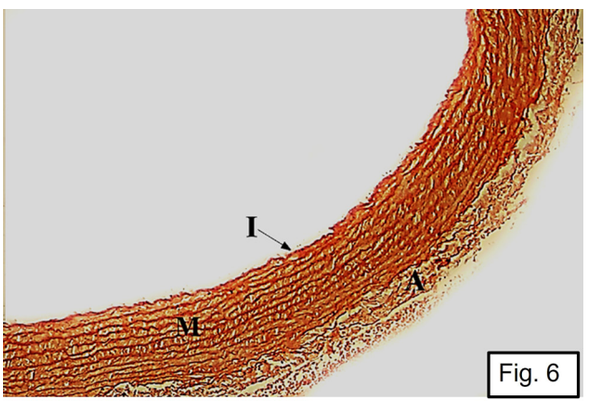

Fig. 6: A photomicrograph of a part of a transverse section of the ascending aorta from the control group showing the distribution of elastic fibers in the three layers of the aorta, the innermost thin tunica intima (I), the middle thick tunica media (M), and the outermost tunica adventitia (A). (Orcein X100)

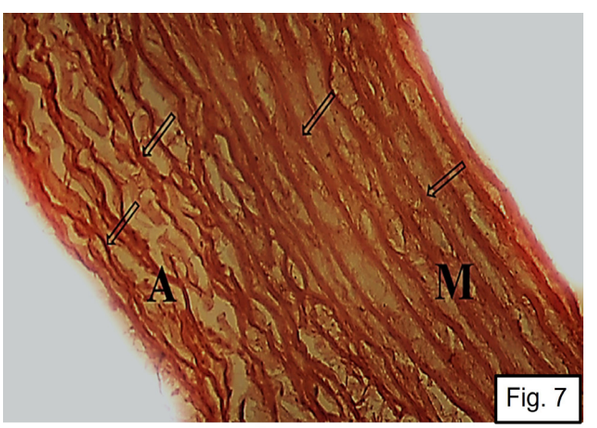

Fig. 7: A photomicrograph of a part of a transverse section of the ascending aorta from the control group showing parallel lamellae of elastic fibers (hollow arrows) in tunica media (M). Notice the elastic fibers (hollow arrows) in the tunica adventitia (A). (Orcein X400)

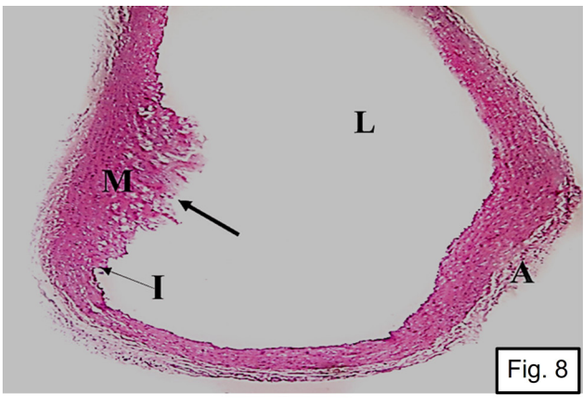

Fig. 8: A photomicrograph of a transverse section of the ascending aorta from the experimental group showing focal loss (thick arrow) of the tunica intima (I) with bulging of the underling tunica media (M) into the lumen (L). Notice the tunica adventitia (A). (H&E X40)

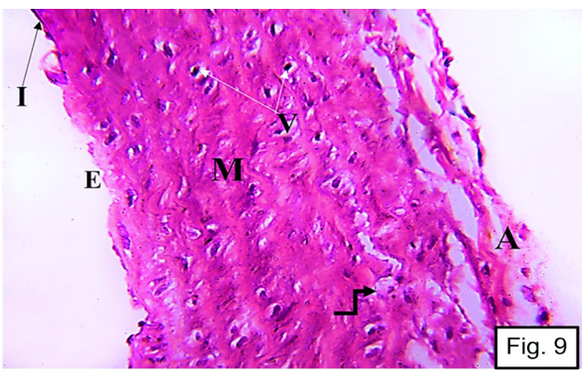

Fig. 9: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group showing focal discontinuity of the linear endothelial cells (E) of the tunica intima (I). Notice the small darkly stained nuclei with perinuclear cytoplasmic vacuolations (V), the foam cells (angled arrow) in the tunica media (M) and the loose connective tissue of the tunica adventitia (A). (H&E X400).

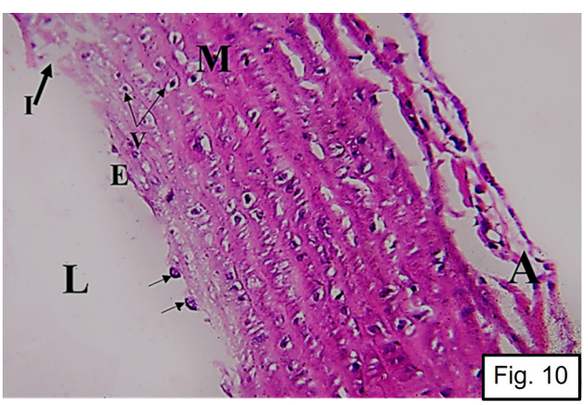

Fig. 10: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group showing focal desquamation (E) of the tunica intima's endothelial cells with the endothelial nuclei (arrows) protruding into the lumen (L). Notice the discontinuity in the tunica intima (I) extending down to the superficial part of the tunica media (M). Also note the arrangement of smooth muscle cells in rows in the tunica media (M) with perinuclear cytoplasmic vacuolations (V) and the tunica adventitia's loose connective tissue (A). (H&E X400).

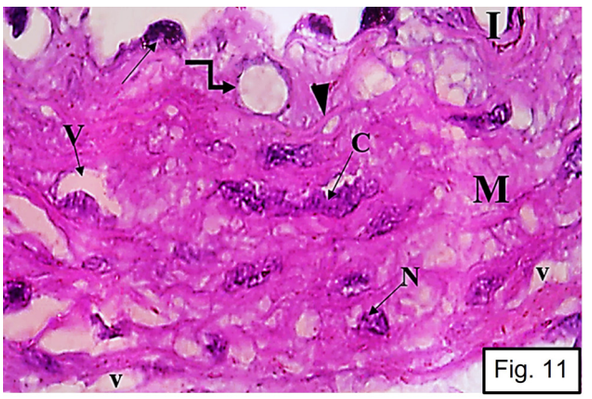

Fig. 11: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group showing darkly stained nuclei (arrow) of the tunica intima (I). Some smooth muscle cells nuclei existed in colonies (C) in the tunica media (M). Notice the presence of foam cells (angled arrow), vacuoles (v), perinuclear cytoplasmic vacuolations (V), and distorted elastic fibers (arrow head) in the tunica media (M). Also note the irregularly shaped nuclei (N) of the tunica media's smooth muscle cells. (H&E X1000).

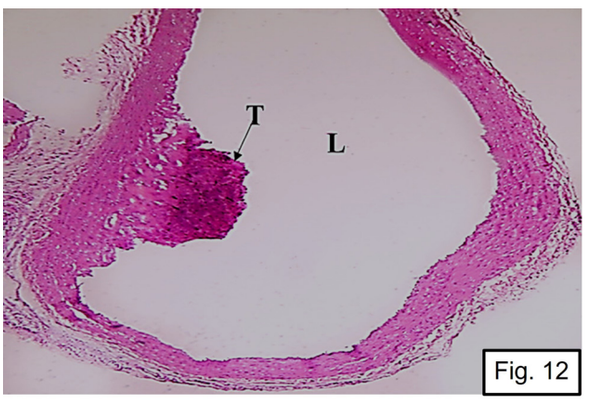

Fig. 12: A photomicrograph of a transverse section of the ascending aorta from the experimental group showing the presence of an intraluminal mass (T) protruding into the lumen (L). (H&E X40)

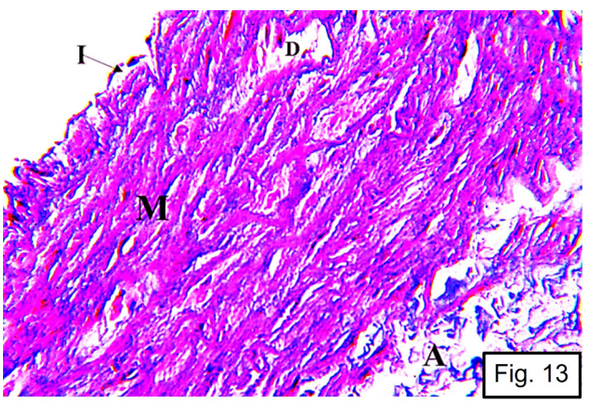

Fig. 13: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group Showing apparent increase in collagen deposition in tunica intima (I) and media (M). Notice the areas of degeneration (D) in the tunica media (M). Also note the loosely dispersed collagen in the tunica adventitia (A). (Masson's trichrome x400)

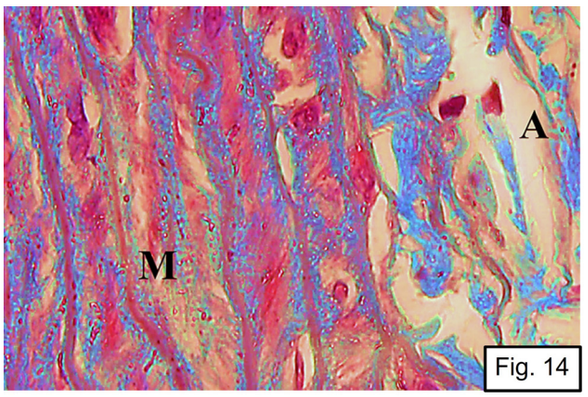

Fig. 14: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group displaying connective tissue that is dispersed throughout the tunica adventitia (A). Observe the amount of collagen in the tunica media (M). (Masson's Trichrome x1000)

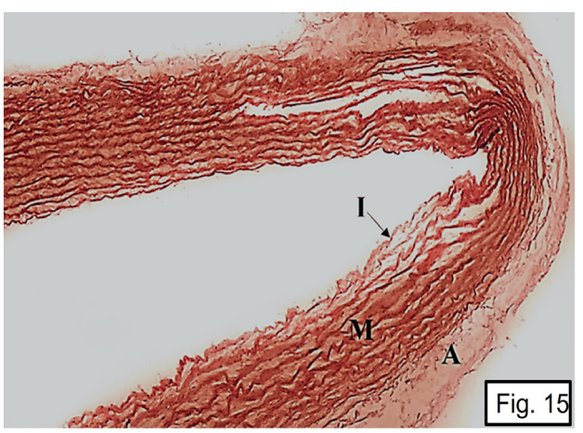

Fig. 15: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group showing the distribution of elastic fibers in the three layers of the aorta, the tunica intima (I), the tunica media (M), and the tunica adventitia (A).(Orcein x100)

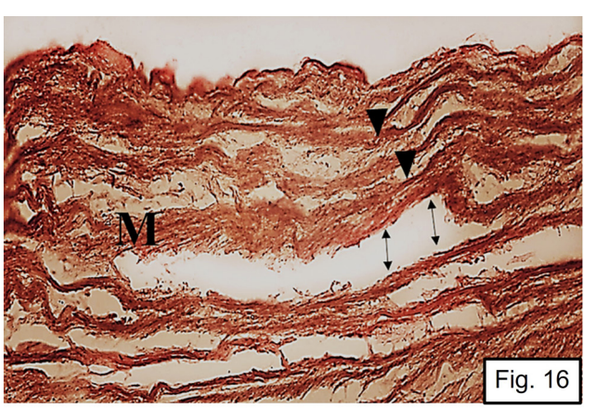

Fig. 16: A photomicrograph of a part of a transverse section of the ascending aorta from the experimental group showing the elastic fibers are widely spaced (double head arrows), and distorted (arrow heads) in the tunica media (M). (Orcein X400)

Table 1: The mean thickness of the ascending aorta between the two groups

Groups	Mean (µm)	±SD	*P value*
Control group	313.1	±58.7	0.00198*
Experimental group	394.7	±66.6	

*Probability (*P*) value was considered statistically significant if ≤ 0.05.
SD: Standard deviation.

Table 2: The mean thickness of the tunica media of the ascending aorta between the two groups

Groups	Mean (µm)	±SD	*P value*
Control group	150.89	±15.012	0.0075*
Experimental group	182.1	±42.52	

*Probability (*P*) value was considered statistically significant if ≤ 0.05.
SD: Standard deviation.

Table 3: The mean percentage area of collagen fibers deposition in the ascending aorta between the two groups

Groups	Mean (µm)	±SD	*P value*
Control group	19.55	±1.488	0.0029*
Experimental group	24.22	±2.51	

*Probability (*P*) value was considered statistically significant if ≤ 0.05.
SD: Standard deviation.

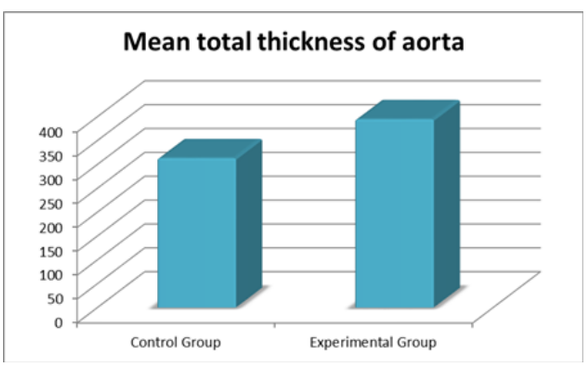

Chart 1: The mean thickness of the ascending aorta between the two groups

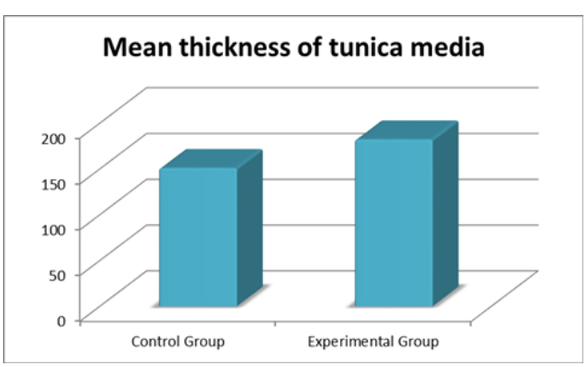

Chart 2: The mean thickness of the tunica media of the ascending aorta between the two groups

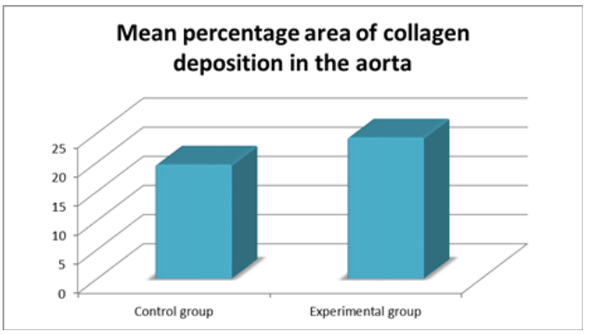

Chart 3: The mean percentage area of collagen fibers deposition in the ascending aorta among the two study groups

DISCUSSION

Previous research have clarified the toxic effects of consumption of energy drinks on different body organs[20]. The present work was carried out to detect the possible histopathological changes that are likely to occur in the ascending aorta after ingestion of caffeinated energy drinks using the albino rat as an experimental model. In the current study, Red Bull has been used as an example of a widely consumed energy drink in various parts of the world[21]. Added to that; it is the most widespread and accessible energy drink in the Egyptian market[22].

The current study targeted the group of people who are more likely to encounter the hazardous effects of such drinks. Consequently, rats aged 4 months old were chosen as this age corresponds in human to youths (average age 15-20 years old)[23]. Thirty to fifty percent of teenagers now take energy drinks., with more than 31% of the ages 12–19 years reporting frequent use[6]. Some of the factors that specifically draws the attention of this group of consumers are successful product advertising, peer pressure and minimal understanding of the possible toxic effects[24]. They are also sometimes augmented with other banned substances such as amphetamines and marijuana[25].

The choice of the ascending aorta in the current study was mostly reliable on the fact that cases reported of aortic dissection after energy drinks intake, were either type I (including the ascending aorta, aortic arch, and descending aorta) or type II (confined to the ascending aorta)[26].

As observed in the current work; a discontinuity in the tunica intima extending down to the superficial part the tunica media. This might indicate an early manifestation of aortic dissection[27]. The potential trigger for such aortic dissection could be the hypertension known to be induced by these caffeine containing drinks[28]. Systemic hypertension is commonly observed in association with cases of aortic dissection which might suggest a vascular effect as a causal factor in addition to hypertension induced fragility of the aorta[29].

Light microscopic findings of the present study also exhibited areas of discontinuation and desquamation of the linear tunica intima endothelial cells. Regulation of the vascular resistance is normally dependent on the normal function and morphology of endothelial cells. This contributes to blood coagulation, smooth muscle cell development, and barrier function, among other things[30].

However; this endothelial dysfunction can be generally explained as an inequality between factors causing vascular protection and deterioration produced by the endothelial cells[31]. Authors clarified that prolonged energy drinks consumption influences the chronic endothelial dysfunction[32]; which is manifested by vasoconstriction, poor vascular reactivity, thrombosis, adhesions and inflammation[33].

Under typical physiological circumstances platelets spread closely without adhesion to the blood vessel's endothelial lining[34]. However, due to certain pathological conditions, platelets rapidly act in response to alterations in the endothelial cell membrane and firmly affix themselves to the damage site. Cardiovascular illnesses also cause platelets to aggregate[35]. This could explain the dome shaped structure observed in the current research, attached to the internal wall of the ascending aorta and protruding into the lumen covered by a thin endothelial layer.

Morphometric and statistical results in the current study clarified an increase in the whole thickness of the ascending aorta with specific increase in the diameter of the tunica media. Arterial hypertrophy has been explained by smooth muscle cells proliferation especially in hypertensive animals[36].

Another interesting finding observed in the present research, was the existence of vacuolated foam cells in the tunica media with the appearance of perinuclear vacuolations. This changes were reported before as senile changes happening in the aorta[13]. These foam cells might be the spot of cholesterol buildup and their presence leads to the development of atherosclerotic patches reported before in mice[37]. Endothelial cells produce free radicals that might share in oxidizing the low density lipoproteins derived from these foam cells which in turn are the resultant of both macrophages and smooth muscle cells[38]. Also; the L-carnitine added to some energy drinks, can be processed to a molecule that stimulates formation of atherosclerosis through its interaction with lipid metabolism[39].

Another characteristic result in the present work was the disorganization of the normal architecture of the tunica media with the presence of smooth muscle cells having bizarre shaped nuclei. These findings were reported by other researchers as senility changes in the aorta of rats[13].

Orcein stained sections in the current study demonstrated distortion and decrease in the distribution of elastic fibers in the whole layers of the aorta despite that there was statistically significant increase in the mean thickness of tunica media in the experimental group compared to the control group. As consumption of energy drinks might raise the risk of developing arterial hypertension[40]; then; any increase in blood pressure, authors suggested that there

should be thickened media layer of blood vessels due to increasing of the size of the smooth muscle layer[41].

Furthermore, in contrast to the control group, Masson's Trichrome sections of the ascending aorta revealed numerous collagen fibers encroaching on the tunica media muscle layer. This was further demonstrated by the finding of an overall increase in collagen deposition in the ascending aorta in the morphometric and statistical analyses. Such deposition of collagen fibers may lead to diminished arterial wall compliance that considerably impacts the increase in systolic blood pressure in different individuals[42]. Authors previously confirmed that excessive use of these energy drinks is linked to increased blood pressure either systolic or diastolic especially in wholesome adolescents and children[43].

CONCLUSION

As a conclusion, the current study showed that Red Bull, as an example of an energy drink, had degenerative effects on the adult male albino rat's ascending aorta histological structure. These degenerative effects indicate that energy drinks could be considered a provoking factor for acute aortic dissection in young healthy adults. However, more research on different types of energy beverages and their possible specific harmful histopathological effects on different tissues and organs are recommended.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة نسيجية مورفومترية حول تأثير استهلاك مشروبات الطاقة التي تحتوي على الكافيين على الشريان الأورطي للفئران البيضاء الذكور البالغين

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المقدمة: يتزايد تناول مشروبات الطاقة منذ تقديمه تجاريا. كما تتقدم الأبحاث حول آثار مشروبات الطاقة على الأفراد منذ ذلك الحين. في الآونة الأخيرة ، لم يثبت وصف محتويات هذه المشروبات جيدا على الرغم من حقيقة أن الكافيين يعتبر المكون الأكثر موثوقية في جميع أنواعه.

الهدف: التحقيق في التغيرات المجهرية في الشريان الأورطي الصاعد المرتبط بابتلاع Red Bull.

المواد والأساليب: تم استخدام عشرين من ذكور الفئران البيضاء البالغة التي تزن ١٥٠ جراما وتقسيمها إلى.: المجموعة الضابطة: كان لديها حرية الوصول إلى الطعام والماء. المجموعة التجريبية: أعطيت ٣,٧٥ مل / كجم BW ريد بل من خلال gavage عن طريق الفم. استمرت التجربة لمدة ٤ أسابيع. بعد ذلك ، تم التضحية بالحيوانات ، وتم استئصال الشريان الأورطي الصاعد ، ومعالجتها للفحص.

النتائج: أظهرت الغلالة الداخلية مناطق فقدان جزئي لبطانتها مع إسقاط الوسائط السفلية في تجويف الشريان الأورطي. كما تم الكشف عن تمزق في السترة الداخلية يمتد نحو الجزء السطحي من وسائط الغلالة. أظهرت وسائط السترة عدم تنظيم نمط التحكم الخاص بها مع تشويه الألياف المرنة المنظمة بانتظام. أظهرت الأقسام الملطخة بتريكروم ماسون زيادة تراكم ألياف الكولاجين. انخفض توزيع الألياف المرنة عندما تلطخت مع Orcein في السترة الثلاثة لجدار الأبهر.

الاستنتاج: يمكن اعتبار مشروبات الطاقة عاملا مشددا لتسلخ الأبهر الحاد. يوصى بإجراء المزيد من الأبحاث حول إمكانية عكس هذه الآثار في حالة الانسحاب والتوقف.