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ORIGINAL ARTICLE

Histological Study on the Effect of Energy Drinks on the Cerebral Cortex of Adult Male Albino Rats and the Possible Protective Effect of Ashwagandha Extract

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Abstract

Background: Energy drinks are so popular for consumption by athletes and service members. Ashwagandha is a commonly used medical herb. This work aimed to study the effect of energy drinks on the cerebral cortex of adult male albino rats and the possible protective effect of ashwagandha extract histologically.

Methods:32 rats were divided into four groups (8 rats each): **Group I (control):** Each rat was given 1ml of distilled water | day. **Group II (Ashwagandha group):** Each rat was given 400 mg/ kg/day of Ash orally. **Group III (Energy drink group):** Each rat was given 10 ml | day of energy drink orally. **Group IV (Energy drink + Ashwagandha group):** Each rat was given both energy drink and Ashwagandha in the same dose and manner as group II and group III. All rats were given treatment for 28 consecutive days then sacrificed.

Results: Group III showed significant increase in body weight in comparison with group I. Group IV showed significant decrease in body weight in comparison with group III. Histopathological changes of cerebral cortex were seen in group III. Group IV revealed improvement in the cerebral cortical tissue.

Conclusions: This study demonstrates that energy drinks induce histopathological changes in the cerebral cortex of adult male albino rats. While, Ashwagandha extract can reduce these histopathological changes in cerebral cortex. Thus, concomitant use of Ashwagandha with energy drinks is able to protect the cerebral cortex from energy drinks induced injury.

Key words: Energy Drinks; Ashwagandha; Cerebral cortex.

INTRODUCTION

Energy drinks (ED) are commonly used by athletes, secondary school students and service members. They have a distinct taste attracting young adults to feel strong, overcome sleep, improve mental & physical performance and increase concentration during studying and driving [1,2]. Most of energy drinks contain caffeine, taurine, glucuronolactone, carbohydrates, vitamins and other herbal extracts as ginseng and guarana that represent natural sources of caffeine. 250 ml of energy drink contains 80mg of caffeine, 20mg of niacin, 5mg of vitamin B6, 27g of carbohydrate, 1.0g of taurine, 5mg of pantothenic acid and 5 µg of vitamin B12 [2,3,4].

Ashwagandha (ASH) (Withania somnifera) is a commonly used Indian medical herb due to its anti-inflammatory, neuro protective, anti-tumor and anti-diabetic effects.

Moreover, it enhances mitochondrial functions and reduces reactive oxygen species (ROS) [5]. Furthermore, it was found that ASH can cross blood brain barrier. So, it improves cognition and Alzheimer and is used in treatment of anxiety & mental disorders. Also, it normalizes subclinical hypothyroidism [6,7,8,9].

This work aimed to study the effect of energy drinks on the cerebral cortex of adult male albino rats and the possible protective effect of ashwagandha extract histologically.

METHODS

The current experiment was done on thirty-two adult male albino rats that weighed (180-220 grams each) and were collected from animal house of faculty of medicine, Tanta University, Egypt, fed on the standard laboratory diet ad libitum under the same environmental conditions. This animal experiment was carried out according to guidelines of Committee for Research and Ethical Issues, Faculty of Medicine, Tanta University, Egypt (Approval number: (36264PR1240\5\25).

A-*Chemicals*:

Energy drinks were purchased from the local market.

Dried roots of Ash extract were supplied in powder form and were purchased from Imtenan, Cairo, Egypt. The powder was dissolved in water and then the mixture was homogenized with stirrer [10].

B-Experimental design:

The rats were divided into four groups (8 rats each) as follow:

Group I (control):

Each rat was given 1ml of distilled water | day for 28 consecutive days then sacrificed at the end of the study.

Group II (Ashwagandha group):

Each rat was given 400 mg/ kg/day of Ash orally for 28 consecutive days [10, 11] then sacrificed.

Group III (Energy drink group):

Each rat was given 10 ml | day of energy drink orally using gastric tube for 28 consecutive days [12] then sacrificed.

Group IV (Energy drink + Ashwagandha group):

Each rat was given both Energy drink and Ashwagandha in the same dose and manner as group II and group III for 28 consecutive days [11,12].

Rats from all groups were given treatment for 28 consecutive days then sacrificed at the end of the study.

The duration of the study was 28 days.

After sacrifaction of the rats from different subgroups, specimens of cerebral cortex of both cerebral hemispheres of each rat were taken and prepared for histological examination.

C-Measurement of body weight

Rats were weighed 2 times; at the onset of the experiment and after 28 days before scarification.

D-Statistical Analysis:

Regarding the weight, at the end of the study, data collected were analyzed statistically. The mean, the standard deviation (S.D) and the (P) value were calculated using Statistical Package for the Social Sciences version 20. Differences were considered as ** Highly significant if P value ≤ 0.001 and significant if P-value ≤ 0.05 *.

E- Sample Preparation and Examination

At the end of the experiment, rats from different experimental groups were anaesthetized by an intraperitoneal injection of sodium pentobarbital (150 mg/kg) according to **(Laferriere &**

Pang 2020) [13] to be able to do perfusion fixation. Perfusion fixation was done by cannulation to the heart and irrigation with saline followed by 4% paraformaldehyde solution according to Zheng et al [14] and then, rats were sacrificed by decapitation to the head with part of the neck and the cerebrum was carefully dissected, immediately isolated and divided midsagittal into two halves. The right half was immediately immersed in 10% formol saline for light microscopic examination [15]. The left half was fixed in 3% glutaraldehyde in 0.1 phosphate buffer solution for transmission electron microscopic examination [16]. Sections were examined and photographed with JEOL-100 JEM (Jeol, Tokyo, Japan) in the Electron Microscopic Unit of the Faculty of Medicine, Tanta University.

RESULTS

I) Statistical study:

Body weight measurements

Initial body weight of rats from all groups ranged from 180 to 220 gm. Final body weight statistical analysis at time of scarification showed a significant increase in body weight in rats from group III (ED group) in comparison with the control group. Non significant difference was noticed between control group and group II (ASH group) or group IV (ED & ASH group). Non significant difference was seen between group II and group III. A significant decrease in the body weight was noticed in group IV in comparison with group III. Non significant difference was seen between group II and group IV.

Table 1 Final body weight (gm) from all groups.

Groups	ANOVA			Tukey (HSD) post-hoc test						
	Mean±SD	F	P Value	Group 1	Group 2	meandiff	p-adj	lower	upper	reject
G I	298.5±6.071	3.8654	<0.05	G I	G II	0.125	1	-17.5259	17.7759	FALSE
				G I	G III	17.75	0.0483	0.0991	35.4009	TRUE
				G I	G IV	-0.75	0.9994	-18.4009	16.9009	FALSE
G II	298.625±8.314			G II	G III	17.625	0.0504	-0.0259	35.2759	FALSE
				G II	G IV	-0.875	0.9991	-18.5259	16.7759	FALSE
GIII	316.25±23.26			G III	G IV	-18.5	0.0373	-36.1509	-0.8491	TRUE
G IV	297.75±4.652									

ANOVA, Analysis of variance. P>0.05, no significant difference.
* P≤0.05, significant difference. **P≤0.001, highly significant difference.

II) Histological and Ultrastructural examination:

<u>Hematoxylin and Eosin stained sections:</u>

Examination of cerebral cortex sections from group I (control group) and group II (ASH group) showed the well-organized cortex layers and normally attached pia matter. The outer layers of cerebral cortex showed granular and pyramidal cells. The acidophilic neuropil contained blood vessels with narrow perivascular spaces. The inner cortical layers showing granular and pyramidal cells with vesicular nuclei and basophilic cytoplasm with blood vessels with narrow perivascular spaces Fig. 2 (A, B & C).

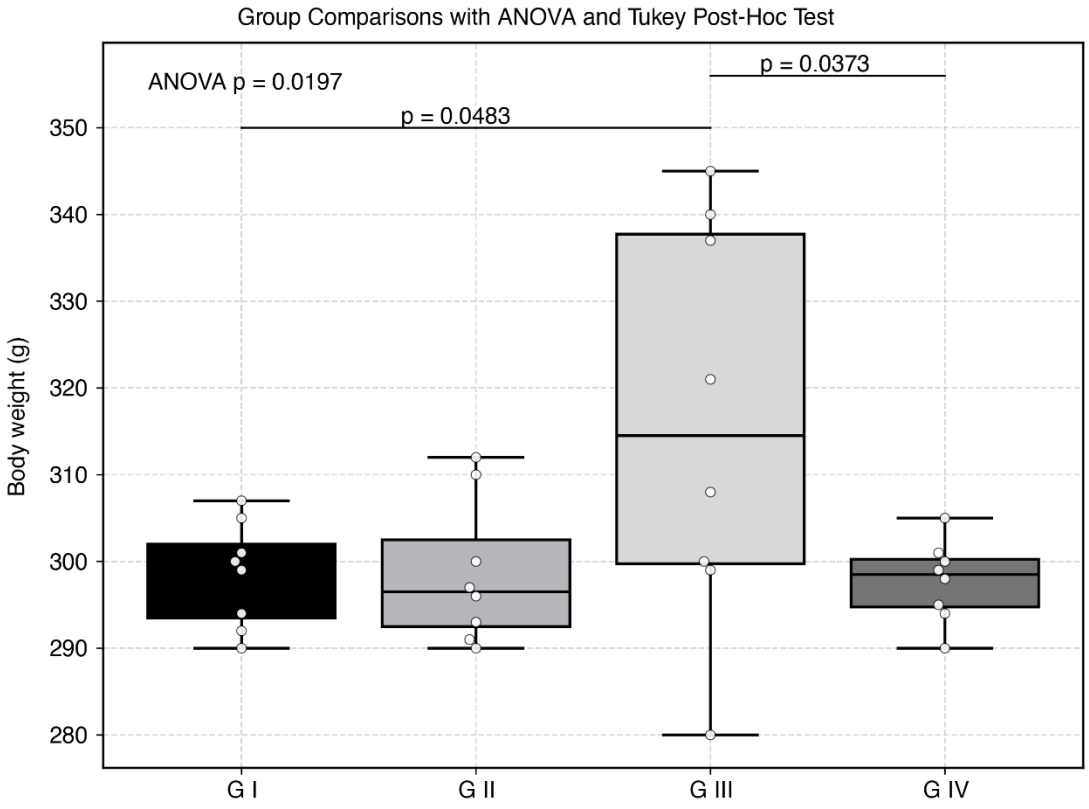

Fig. (1): A histogram showing the final body weight of rats from all groups at scarification time.

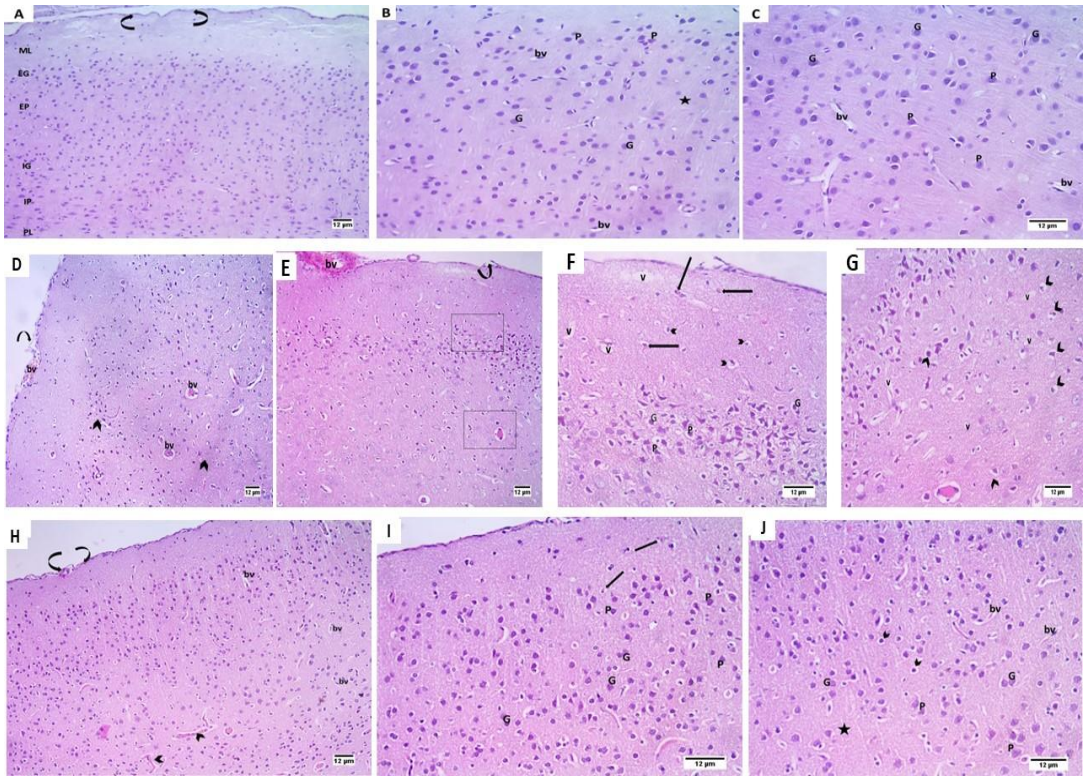

Fig. (2): Cerebral cortex Hx. & E

Sections of group I and group II show the same histological findings (control group) showing: A) Well organized regularly arranged six cortical layers from outer to inner surface: Molecular layer (ML), external granular (EG), external pyramidal (EP), internal granular (IG), internal pyramidal (IP) and polymorphic layer (PL)and normally attached pia matter (arrow) (A: Hx &E x 200). B) Higher magnification of the outer layers of cerebral cortex showing granular (G) and pyramidal cells (P). The acidophilic neuropil (star) contains blood vessels with narrow perivascular spaces (bv). C) Higher magnification of the inner cortical layers showing granular (G) and pyramidal cells (P) with vesicular nuclei and basophilic cytoplasm with blood vessels with narrow perivascular spaces (bv) (B&C: Hx &E x 400). Sections of group III (energy drinks group) showing: (D): Loss of the organization of layers (arrow heads) with separation of pia matter (curved arrow) and congested blood vessel (bv). (E): Tear in the pia mater (curved arrow) with congested blood vessel (bv). The upper square shows the outer cortex. The lower square shows the inner cortex (D & E: Hx &E x 200). (F): Higher magnification of the upper square in the outer cortex shows the molecular layer containing red neurons (arrows) and deformed neurons surrounded by perineural spaces (arrow heads). Vacuolated neuropil (v) is also seen. Granule cells (G) appear with pyknotic nuclei surrounded by perineural spaces. Pyramidal cells (P) show deeply stained nuclei and surrounded by perineural spaces. Neuropil appears vacuolated(V). (G): Higher magnification of the lower square in the inner cortex shows degenerated cortical neurons with darkly stained nuclei and surrounded by vacuolations (arrow heads). Vacuolated neuropil is also seen (F & G: Hx &E x 400). Sections of group IV (energy

drinks & ASH group) showing: H) Nearly normal arrangement of the layers of the cerebral cortex with the pia matter attached with normal blood vessel (curved arrows) are seen. Within the cortex, blood vessels appear either with narrow (arrows) or wide perivascular space (arrow heads) (H: Hx &E x 200) I) Higher magnification of the outer layers of cerebral cortex showing most granular (G) and pyramidal cells (P) are normal. Some red neurons (arrows) are noticed. J) Higher magnification of the inner cortical layers showing most granular cells with vesicular nuclei and basophilic cytoplasm (G). Normal pyramidal cells (P) are seen. Few cortical neurons still exhibit dark stained nuclei surrounded by halos (arrow heads). The acidophilic neuropil (star) contains blood vessels with narrow perivascular space (bv) (I & J: Hx &E x 400).

While, group III (ED treated) showed Loss of the organization of layers of cerebral cortex with separation of pia matter and congested blood vessel. Tear in the pia mater with congested blood vessel were also noticed. The outer cortex showed the molecular layer containing red neurons and deformed neurons surrounded by haloes. Vacuolated neuropil was also seen. Granule cells appeared with pyknotic nuclei surrounded by halos. Pyramidal cells showed deeply stained nuclei and surrounded by haloes. The inner cortex showed degenerated cortical neurons with darkly stained nuclei and surrounded by vacuolations Fig. 2 (D, E, F& G).

Regarding group IV (ED and ASH treated), Hx & E stained sections of the cerebral cortex showed nearly normal arrangement of the layers of the cerebral cortex with the pia matter attached and normal blood vessels. Within the cortex, blood vessels appeared either with narrow or wide perivascular space. The outer layers of cerebral cortex showed that most granular and pyramidal cells appeared normal. Some red neurons were noticed. The inner cortical layers showed most granular cells with vesicular nuclei and basophilic cytoplasm. Normal pyramidal cells were seen. Few cortical neurons exhibited dark stained nuclei surrounded by halos. The acidophilic neuropil contained blood vessels with narrow perivascular space Fig. 2 (H, I & J).

Ultrastructural examination of the cerebral cortex:

Transmission electron microscopic examination of sections of the cerebral cortex of group I (control group) and group II (ASH group) showed cortical neurons containing euchromatic nuclei, multiple mitochondria, rough endoplasmic reticulum and Nissl's granules. Neuronal axon sections showed normal mitochondria, myelinated nerve fibers surrounded by thick myelin sheath and non-myelinated nerve fibers. Capillaries in the cerebral cortex were seen with the basement membrane coating the abluminal surface of the endothelial cells and was in direct contact with the narrow extracellular space of the cortex Fig. 3 (A, B & C).

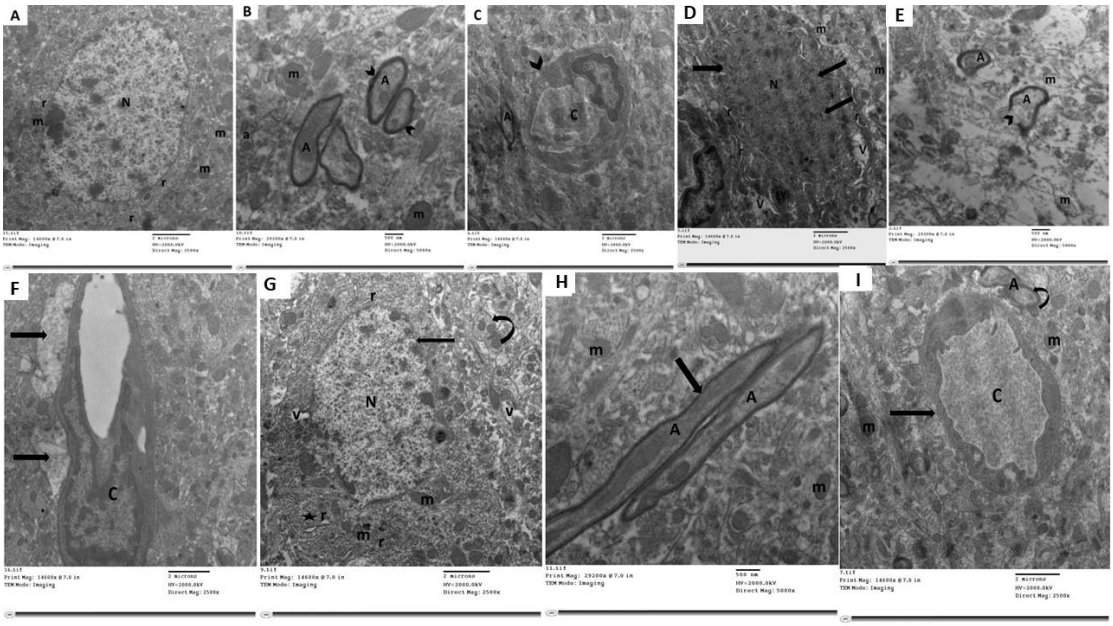

Fig. (3): Transmission electron micrographs showing the ultra-structure of the cerebral cortex of group I and group II (control group) shows the same architecture and reveals A) Cortical neuron containing euchromatic nucleus (N), multiple mitochondria (m) and rough endoplasmic reticulum (r) can be noticed. B) Neuronal axon section showing normal mitochondria (m), myelinated nerve fibers (A) surrounded by thick myelin sheath (arrow heads), non-myelinated nerve fibers (a) can be noticed. C) A capillary in the cerebral cortex is seen. A basement membrane coats the abluminal surface of the endothelial cells and is in direct contact (arrow head) with the narrow extravascular space of the cortex. myelinated nerve fibers (A) can be also noticed. group III (Energy drinks group) shows D) Cortical neuron showing nucleus (N) with irregular membrane and marked indentation (arrow). The cytoplasm shows dilated cisternae of endoplasmic reticulum (r), swollen mitochondria with destructed cristae (m), lysosomes (LY) and vacuoles (v). E) Myelinated axons show irregular outline and splitting in the myelin sheath (arrow head). Swollen mitochondria (m) with destructed cristae within the axoplasm are noticed. F) A capillary is noticed with area of rarified cytoplasm near to it (arrows). group IV (Energy drinks & ASH group) shows G) Cortical neuron showing large euchromatic nucleus (N) with regular contour (arrow). The cytoplasm contains normal mitochondria (m), vacuoles (v), rough endoplasmic reticulum (r), Nissl's granules (star) and non myelinated nerve fibers with regular contour (curved arrow). H) Neuronal axon section showing normal mitochondria (m), myelinated nerve fibers (A) surrounded by thick myelin sheath with regular contour (arrow). I) A capillary in the cerebral cortex is seen. The narrow extravascular space of the cortex (arrow) is also noticed. Myelinated nerve fibers (A) with thick myelin sheath (curved arrow) and normal mitochondria (m) within the axoplasm can be also noticed.

While, group III (ED group) showed cortical neurons that contained nucleui with irregular membrane and marked indentation. The cytoplasm showed dilated cisternae of endoplasmic reticulum, swollen mitochondria with

destructed cristae, lysosomes and vacuoles. Myelinated axons showed irregular outline and splitting in the myelin sheath. Swollen mitochondria with destructed cristae within the axoplasm were also noticed. Capillaries with widening of the extracellular space of the cortex were also seen Fig. 3 (D, E & F).

Regarding group IV **(**ASH and ED group), sections of the cerebral cortex showed cortical neurons containing large euchromatic nucleui with regular contour. The cytoplasm contained normal mitochondria, vacuoles, rough endoplasmic reticulum, Nissl's granules and non myelinated nerve fibers with regular contour. Some mitochondria showed destructed cristae. Neuronal axon sections showed normal mitochondria, myelinated nerve fibers surrounded by thick myelin sheath with regular contour. Capillaries in the cerebral cortex were seen. The narrow extracellular space of the cortex was also noticed. Myelinated nerve fibers with thick myelin sheath and normal mitochondria within the axoplasm were also seen Fig. 3 (G, H & I).

DISCUSSION

Energy drinks are accessible in local markets for all people including children and young ones [17]. However, many adverse effects of energy drinks were reported in different studies and poison centers of different countries [18]. Previous studies on Ashwagandha showed its anti-inflammatory, antioxidant, antitumor and immunomodulatory effects. Furthermore, it had useful effects on the endocrine, vascular and nervous systems. Many authors showed that ASH had anxiolytic and antidepressant properties [19,20].

Regarding the final body weight statistical analysis. group III (ED group) showed significant increase in body weight in comparison with group I. Non-significant difference was noticed between control group and group II (ASH group) or group IV (ED & ASH group). Non-significant difference was seen between group II and group III. A significant decrease in the body weight was noticed in group IV in comparison with group III. Non-significant difference was seen between group II and group IV. These results coincided with some authors [21] that noticed that caffeinated soft drinks and energy drinks raised body weight.

Moreover, it was reported that prolonged consumption of energy drinks accounted for a significant increase in body weight. This was explained by the sleeplessness effects of the caffeine component of the energy drinks. In addition, sweetening constituents of energy drinks caused high insulin levels and high rate of catabolism leading to an increase in the rate of lipid storage in the adipose tissues. Overweight and obesity might result from artificial sweeteners used in energy drinks [22].

Our results coincided with previous researches that reported that Ashwagandha modulates adipogenesis, lipid metabolism and energy expenditure. Thus, it has a strong anti-obesity effect. ASH serve as a natural therapeutic agent for obesity and metabolic diseases [23].

In contrast with our results, previous researchers reported that ED had no significant effect on the body weight. This might be due to caffeine that activates metabolism and elevates energy consumption [24]. Light and electron microscopic sections of cerebral cortex of ASH group (group II) showed the normal features similar to control group. These results coincided with previous researches that demonstrated that ASH

did not change the normal structure of the cortex of cerebrum [25].

In this work, light microscopic picture of the cerebral cortex of group III (ED group) revealed loss of the organization of layers with separation or tear of pia matter and congested blood vessels. The molecular layer contained red neurons and deformed neurons surrounded by haloes. Vacuolated neuropil with red neurons was also seen. The outer cortical layers showed granule & pyramidal cells containing pyknotic nuclei and were surrounded by halos. Degenerated cortical neurons containing darkly stained nuclei and surrounded by vacuolations were seen in the inner cortical layers. Red neurons were also seen. These findings were in accordance to other workers who suggested that Energy drinks consumption in high dose caused degeneration many brain regions [2,26].

Moreover, these results coincided with other researchers who reported that prolonged consumption of ED was associated with histopathological effects the brain tissue, stress, anxiety and depression. These changes were explained increasing the inflammatory response resulting in oxidative stress, local gliosis, and increased reactive oxygen species (ROS) levels, lipid peroxidation and nitric oxide. ED consumption led to increase in the oxidative stress markers such as superoxide dismutase, catalase, glutathione peroxidase & malondialdehyde [27]. In contrary to these results, previous work reported that the histopathological changes induced by the intake of energy drinks were dose dependent. These changes were more obvious in high dose treated rats. While Low dose treated rats exhibted moderate degree of changes under microscopic examination [28]. Furthermore, it was reported that ED treated rats showed improved attention [11].

Ultrastructural examination of sections of cerebral cortex from group III (ED group) confirmed the light microscopic results and showed cortical neurons containing nuclei with irregular membrane and marked indentation. The cytoplasm showed dilated cisternae of endoplasmic reticulum, swollen mitochondria with destructed cristae, lysosomes and vacuoles. Myelinated axons showed irregular outline and splitting in the myelin sheath. Swollen mitochondria with destructed cristae within the axoplasm were noticed. A capillary was seen with widening of the extracellular space of the cortex. These results were explained by other authors due to mitochondrial dysfunction associated with ED consumption. The mitochondria represented the target organelle for degenerative process. Abnormal activity of the mitochondrial complex I resulted from oxidative stress and ROS and this directly interfered with adenosine triphosphate ATP production leading to neuronal cell death [29].

In addition, ED caused neuronal cell death due to several molecular mechanisms including glutamate excitotoxicity, production of glycation end products and supression of Nuclear factor erythroid 2 (NRF2) which was responsible for anti-inflammation and anti-oxidative stress. Preservatives and caffeine components of energy drinks caused brain toxicity due to cellular damage by lipid peroxidation and DNA damage. All these changes resulted in alterations in brain tissue including cellular apoptosis, dark small nuclei and congested blood vessels [30,31].

Current histopathological changes coincided with the findings of **(Sayed,**

2021) who noticed extensive structural changes including severe neuropil degeneration in the cerebral cortex with darkly stained nuclei and pale cytoplasm in the groups treated with energy drinks and they demonstrated these histopathological changes resulted from oxidative stress which was detected by the increased levels of thiobarbituric acid reactive substance that produced by lipid peroxidation of cortical tissue and down regulation of mRNA Expression Levels of Nrf2 proteins which have protective role against oxidative damage [32].

In our experiment, light microscopic sections of the cerebral cortex in group IV (ED & ASH treated) revealed improvement in the histological features compared to the ED group and revealed nearly normal arrangement of cerebral cortical layers with the pia matter attached with normal blood vessels. Within the cortex, some blood vessels appeared with narrow perivascular space. Others appeared with wide perivascular space. cerebral cortical outer layers revealed that most granular and pyramidal cells appeared normal. Some red neurons were noticed. The inner cortical layers showed most granular cells containing vesicular nuclei and basophilic cytoplasm. Normal pyramidal cells were seen. Few cortical neurons still contained dark stained nuclei surrounded by halos. Blood vessels with narrow perivascular space were seen within the acidophilic neuropil. These results were in agreement with other workers who stated that ASH could relieve brain oxidative stress and improve the histopathological changes in the cerebral cortex. Also, ASH had a strong antidepressant effect [25].

Additionally, ASH had the ability to reduce the effect of hypothyroidism on the brain tissue due to its anti-inflammatory & antioxidant effects. ASH could relieve oxidative stress and improve neuroinflammation as a result of reduction of lipid peroxidation [8].

Ultrastructural examination of sections of the cerebral cortex from group IV (ED & ASH group) confirmed the light microscopic results and revealed improvement as compared to ED group and showed cortical neurons with large euchromatic nucleus and regular contour. The cytoplasm contained normal mitochondria, vacuoles, rough endoplasmic reticulum, Nissl's granules and non myelinated nerve fibers with regular contour. Some mitochondria showed destructed cristae. Neuronal axon sections showed normal mitochondria, myelinated nerve fibers surrounded by thick myelin sheath with regular contour. Capillaries in the cerebral cortex were seen with the narrow extracellular space of the cortex. Myelinated nerve fibers with thick myelin sheath and normal mitochondria within the axoplasm were also noticed. These results were in agreement with other workers who reported that ASH alleviated oxidative stress induced neurodegeneration through upregulation of Nrf2 signaling pathway and elevating glutathione levels in the hippocampus [33]. Withanolides that represented major bioactive components of ASH. They exerted neuroprotective effect through inhibition of pro-inflammatory factors and modulation of acetylcholine levels [34, 35].

Ashwagandha possessed antioxidant, anti-inflammatory, neuroprotective, endocrinological and immune modulatory effects that improve cognition. ASH was useful in treatment of bipolar disease, cognitive impairment and chronic stress. Furthermore, it improved memory, sleep quality, time of reaction and psychomotor performance in healthy people [36].

Several mechanisms described the effect of ashwagandha. ASH served as an adaptogen, therefore improved response to stress. It inhibited acetylcholinesterase,increased neurotransmission and provided neuroprotective effects. Furthermore, it affected gamma-aminobutyric acid (GABA) activity which is an inhibitory neurotransmitter that has been a target in the treatment of anxiety. ASH had similar effects as anxiolytic drugs. It has also been reported to promote the quality of sleep. ASH had the ability to decrease oxidative stress, reduce brain inflammation and improve memory in people under stress conditions [36,37,38,39].

CONCLUSION

This study demonstrates that energy drinks induce histopathological changes in the cerebral cortex of adult male albino rats. While, Ashwagandha extract can reduce these histopathological changes in cerebral cortex. Thus, concomitant use of Ashwagandha with energy drinks is able to protect the cerebral cortex from energy drinks induced injury.

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Citation

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