Original Article

The Role of Omega-3 on Red Bull Induced Skeletal Muscle Injury in Adult Albino Rat. A Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Energy drinks (EDs), are kind of beverages containing stimulant substances, with caffeine as integral part. The most popular EDs in Egypt are Red Bull (RB) and Power Horse.

Aim of the Work: The aim of the current study was done to study the role of omega-3 on RB induced skeletal muscle injury in rat.

Materials and Methods: Thirty adult albino rats, were divided equally into three groups: control, RB, and RB & Omega-3. Group I (control group): rats were subdivided into subgroup IA: the rats received 5 ml saline, by gastric tube daily, for 4 weeks, and subgroup IB: rats received Omega-3 at dose 300 mg/kg/day (60mg/rat = 0.5ml syrup/rat) orally, by gastric tube for 4 weeks. Group II (RB group): the rats received RB at dose of 5ml (l0 mg/kg/day) orally, by gastric tube, for 4 weeks. Group III (RB & omega-3 group): the rats received RB, and omega-3 concomitantly for 4 weeks. Animals were sacrificed, and the skeletal muscles were dissected out, and prepared to be stained with hematoxylin and eosin, Masson's trichrome, and immunohistochemical stain (Caspase-3). Morphometric measurements, and statistical analysis were done.

Results: RB induced various histological changes, in the form of mononuclear inflammatory infiltration, congested blood capillaries, disrupted non-striated sarcoplasm in some areas and splitting of myofibrils. Other muscle fibers appear with dark sarcoplasm, dark nuclei, and rows of centrally located oval nuclei in some fibers. RB group revealed significantly increased collagen fibres, and many myofibers showed strong cytoplasmic brownish reaction to Caspase-3 detecting apoptosis. Concomitant administration with omega-3 markedly attenuated these changes with significant decrease in collagen fibres, and weak cytoplasmic brownish reaction in some myofiber.

Conclusion: It can be concluded that omega-3 enhance skeletal muscle protection, and regeneration in RB- induced muscle injury, and has an ameliorating effect on the induced inflammatory changes.

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Key Words: Caspase-3, omega-3, rat, red bull, skeletal muscle.

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INTRODUCTION

Energy drinks (EDs) are type of beverages that includes stimulanting substances, with caffeine as integral part[1]. EDs were first introduced in Asia and Europe in the l960s[2]. In Egypt, the most popular energy drinks are Red Bull (RB) and Power Horse[3].

The stimulant components in RB consist of caffeine, B vitamins, panthenol, simple sugars, niacin, glucuronolactone, and taurine amino acids, along with taste-like suppliers like ginseng, ephedrine, and ginkgo[4].

The effects of RB drinking are attributed to excessive caffeine that may lead to sleep disorders, anxiety, effects on gastrointestinal system, and other cardiac and neurological symptoms as tachycardia, seizures[5]. Moreover, RB contains high level of sugar that might elevate the risk for obesity, insulin resistance, and dental caries[6].

Omega-3, a crucial polyunsaturated fatty acid (PUFA) found in fish oil, has both an antioxidant, and anti-inflammatory effect[7]. Dietary intake of PUFAs, particularly Omega-3 FAs, is believed to regulate immune system functions[8]. The omega-3 FAs contain three primary acids, α-Linolenic acid (ALA), Docosahexaenoic acid (DHA), and Eicosa-pentaenoic acid (EPA)[8].

Omega-3 FAs might reduce the release of pro-inflammatory cytokines and decrease production of reactive oxygen species, while enhancing phagocytic activity[9]. These immunomodulatory properties are thought to be due to the regulation of intracellular signaling pathways, like the NF-κB, and PPAR-γ pathways[10].

Benefits of omega-3 FAs are due to different biological mechanisms, including their crucial roles in the cell membrane structure, control gene expression, and acting as precursors to hormones that control blood coagulation,

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arterial wall contraction, and relaxation. Also, regulating cell division, growth, and inflammation[11].

This work was done to study the role of Omega-3 on RB-induced skeletal muscle damage in rats.

MATERILS & METHODS

Chemicals

Red Bull (RB) is commonly used ED that is available in the local market. Each can of RB contains the following: 1000 mg of taurine, 80 mg of caffeine, 600 mg of glucuronolactone, 18 mg of niacin, 6 mg of pantothenic acid, 2 mg of vitamin B6, vitamin B2, vitamin Bl2, inositol, and other component such as carbonated water, sucrose, glucose (27 g of sugar), citric acid and flavors[12].

Omega-3 is present in the form of syrup 120 ml, each 5ml contains 640 mg of high Docosahexaenoic acid (DHA) fish oil, 213 mg of Rigel evening primrose oil g, Dl-alpha tocopherol acetate, 0.40 mg thyme oil, and 7.82 mg vitamin E (purchased from Arab Co, Egypt).

Animals

The study was performed on thirty male adult albino rats. Aged 4 to 5 months, and weighing approximately 180-200 gm. The animals were got from the animal house at the Faculty of Medicine, Ain Shams University. They were housed at the Medical Research Centre of the same faculty for a week to adapt to their environment. They were kept under standard laboratory conditions at 22-24°C with l2 hours light\dark cycle. The experimental work was done according to the Animal Care guidelines set by the Scientific Research Ethical Committee of Ain Shams University. The rats were kept in plastic cages with mesh wire covers, provided with a constant, and sufficient nutritional diet, with unrestricted access to drink water ad libitum (approval number: No. FWA 000017585).

Experimental design

Rats were randomly divided into three groups, with ten rats in each:

Group I (control group): Rats were further divided into 2 subgroups, each with 5 rats:

- Subgroup IA: the rats were given 5 ml of saline, via a gastric tube, daily for four weeks.
- Subgroup IB: the rats were administered omega-3 at a dose of 300 mg/kg/day (60 mg/rat = 0.5ml syrup/rat)[7] orally, via gastric tube, for four weeks.

Group II (RB group): Rats were given Red Bull (RB) at dose of 5ml/rat (l0 mg/kg/day) orally, via gastric tube, for four weeks[13]. The dose for rats is equal to the human dose conversion table[14].

Group III (RB & omega-3 group): Rats were given RB at dose of 5 ml/rat (l0 mg/kg/day) as in group II and omega-3 at dose 300 mg/kg/day (60 mg/rat= 0.5 ml syrup/rat)[7] orally, via gastric tube, concomitantly for four weeks.

Histological study

At the end of the experiment, all rats were anesthetized with ether, and then sacrificed. Gastrocnemius muscle was exposed, and samples were gathered, and fixed using l0% formalin to prepare paraffin blocks. Paraffin sections of 5µm thickness were got and then subjected to:

Light Microscopic Study

- Hematoxylin and eosin (H&E) staining[15].
- Masson's Trichrome staining to visualize collagen fibers[16].
- Immuno-histochemical staining for Caspase-3[17] to detect apoptotic cells.

For caspase-3 staining, 7 ml of rabbit polyclonal antibody (primary antibody) (RB-1197-R7) from Lab Vision Corporation, USA, was pre-diluted (1:100), and was ready for use, stored at 2-8°C. Positive control used was a human tonsil specimen, where Caspase-3 positive cells exhibited a brownish cytoplasmic reaction. In contrast, one of the muscle sections used as the negative control, by replacing primary antibody by phosphate buffered saline.

Morphometrical and statistical studies

1. The mean area percentage of collagen fibers in sections stained with Masson's Trichrome.
2. The mean optic density of Caspase-3 in immune-stained sections.

These measurements were done by LEICA Q win Image Analyzer at the Histology Department, Faculty of Medicine, Ain Shams University. The parameters were assessed in 5 distinct, non-overlapping fields from 5 sections of each rat, with 5 rats per group (the magnification of the lens used x 40). The results were presented as mean ± standard deviation (SD).

Statistical analysis was done by SPSS software (Version 20, USA), using one-way ANOVA to compare the means of all groups (post-Hoc least significant difference to compare between groups). *P value* of < 0.05 was considered significant.

RESULTS

Histological and immuno-histochemical results of all subgroups of the control group were parallel, so they were collectively referred to as the control group (group I).

Hematoxylin and Eosin (H&E) staining

H&E-stained longitudinal sections of gastrocnemius muscle of Group I (control) revealed regularly arranged myofibers with acidophilic regularly striated sarcoplasm, rod shaped peripheral nuclei and flat nuclei of fibroblast between them (Figure 1a).

Examination of group II (RB group) showed mononuclear inflammatory infiltration, congested blood capillaries, and disrupted nonstriated sarcoplasm in some

areas (Figure 1b). Also, excessive deposition of connective tissue in the perimysium (Figure 1c) with distorted muscle fibers, pale sarcoplasm and splitting of myofibrils were found. other muscle fibers with dark acidophilic sarcoplasm, pyknotic nuclei and rows of centrally located elongated nuclei in some fibers (Figure 1d).

Examination of group III (RB&omega-3 group) showed regularly arranged myofibers with regular striations, normally apparent blood capillary with few connective tissues in between the myofibers, many oval pale nuclei appeared (Figures 1e,f). Few inflammatory infilteration was found (Figure 1f).

Masson's Trichrome Staining

Masson's Trichrome stained longitudinal sections of gastrocnemius muscle of group I (control) revealed fine collagen fibers in-between myofibers (Figure 2a). Group II revealed apparent increase in collagen fibers in-between myofibers (Figure 2b). Group III revealed few collagen fibers in-between myofibers as compared to control (Figure 2c).

Immunostaining for Caspase-3

The immunohistochemical stained longitudinal section of rat skeletal muscle Caspase-3 showed that in the control group (Group I), there was negative cytoplasmic reaction (Figure 3a). In RB group (Group II) there was strong positive brownish reaction in many myofibers (Figure 3b) while in RB&omega-3 group (Group III) there was weak positive brownish reaction in some myofibers (Figure 3c).

Morphometric results

The mean area percentage of collagen fibers was significantly increased in G2 comparable to G1 and G3, meanwhile in G3, non-significant increase was present comparable to G1 (Table 1, Histogram1). The mean optical density of Caspase-3 was found to be significantly increased in G2 comparable to both G 1 and G3. Moreover, G 3 was present to be significantly increased as compared to G 1, while showing significant decrease as compared to G 2 (Table 2, Histogram 2).

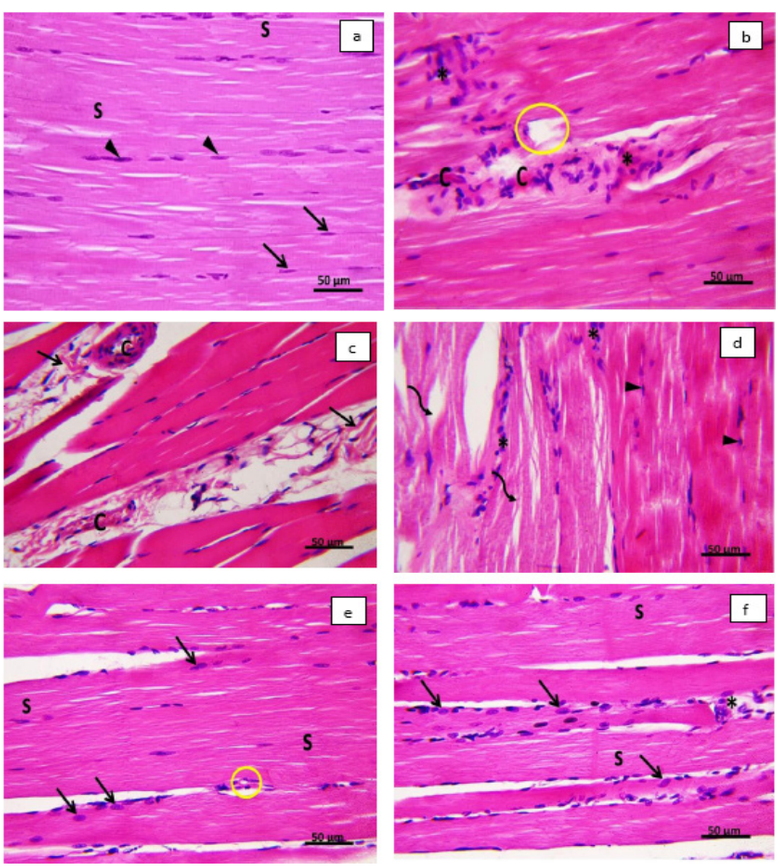


Fig.1: Photomicrographs of longitudinal section in a rat gastrocnemius muscle. (a): Group I (control) showing regularly arranged myofibers with acidophilic regularly striated sarcoplasm (s), rod shaped peripheral nuclei (▲) and flat nuclei of fibroblast between them (↑). (b): Group II showing mononuclear inflammatory infiltration (*), congested blood capillaries (C) and disrupted nonstriated sarcoplasm in some areas (yellow circle). (c): Group II showing congested blood vessels (C), excessive deposition of connective tissue (↑) in the perimysium. (d): Group II showing distorted muscle fibers with pale sarcoplasm and splitting of myofibrils (curved arrow). Notice, other muscle fibers with dark acidophilic sarcoplasm, pyknotic nuclei (▲) and rows of centrally located elongated nuclei in some fibers (*). (e): Group III showing regularly arranged myofibers with regular striations (S), normally apparent blood capillary with few connective tissues (yellow circle) in between the myofibers, many oval pale nuclei appeared (may be of activated satellite cells) (↑). (f): Group III showing regularly arranged myofibers with regular striations (S), many oval pale nuclei appeared (↑), few inflammatory infilteration (*). (H&E x 400).

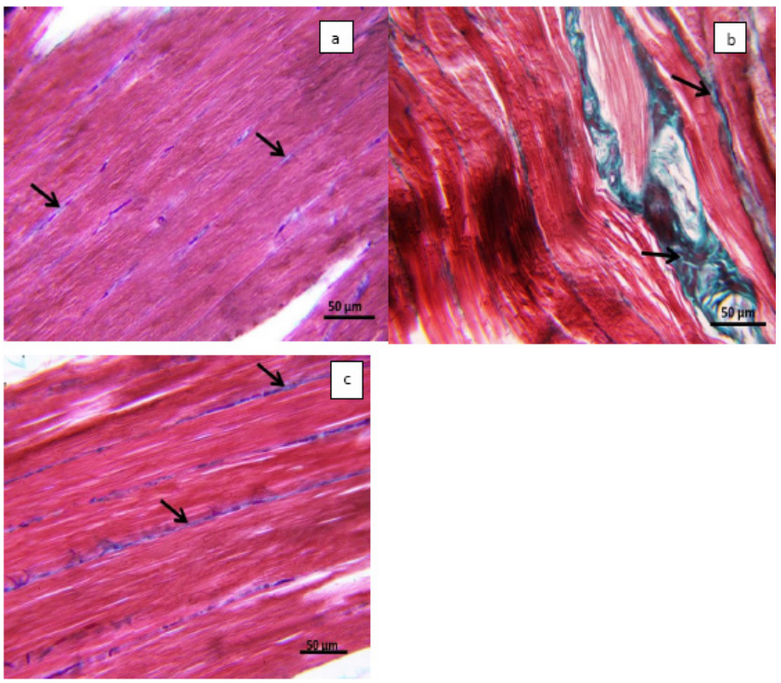


Fig. 2: Photomicrographs of longitudinal section in a rat gastrocnemius muscle. (a): group I (control) showing fine collagen fibers in-between myofibers (↑). (b): group II showing apparent increase in collagen fibers in-between myofibers (↑). (c): group III showing few collagen fibers in-between myofibers (↑). (Masson's Trichrome x 400).

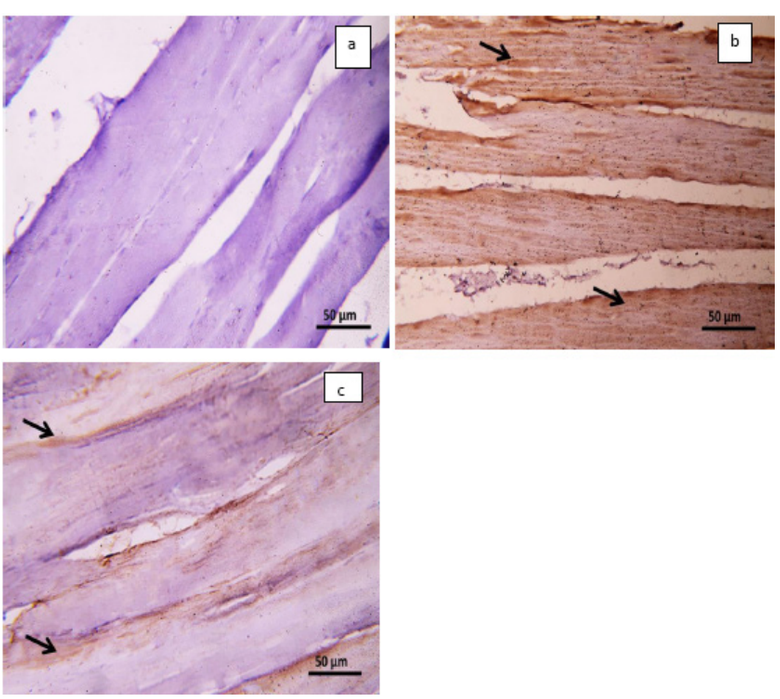


Fig.3: Photomicrographs of longitudinal section in a rat gastrocnemius muscle. (a): group I (control) showing negative cytoplasmic reaction. (b): group II showing strong positive brownish reaction (↑) in many myofibers. (c): group III showing weak positive brownish reaction (↑) in some myofibers. (Caspase-3 immunostaining x 400).

Table 1: The mean area percentage of collagen fibers in different experimental groups

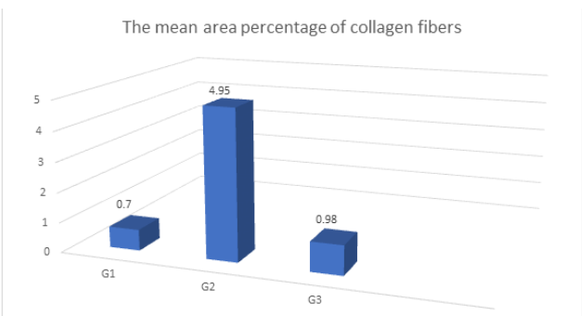
	G1 (control) n=10	G2 (RB) n=10	G3 (treated) n=10
The mean area percentage of collagen fibres	0.70± 0.21	4.95±1.17 a^{*} b^{*}	0.98±0.23 a^{ns}

Data are expressed as mean ± standard deviation (SD), n: number of rats/ groups. ns: non-significance. Significance at * P<0.05. (a: vs. G1 and b: vs. G3)

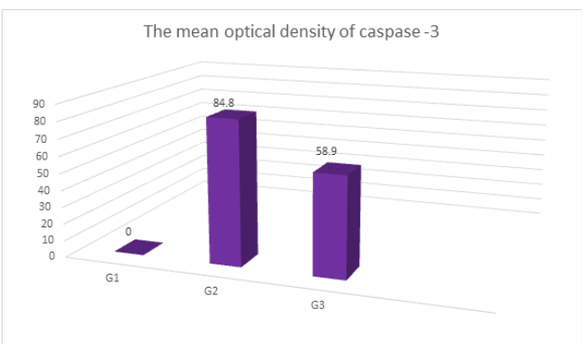
Table 2: The mean optical density of caspase-3 of rats in the different experimental groups

	G1 (control) n=10	G2 (RB) n=10	G3 (treated) n=10
The mean optical density of caspase-3	0.00± 0.00	84.80±3.79 a^{*} c^{*}	58.29±5.38 a^{*} b^{*}

Data are expressed as mean ± standard deviation (SD), n: number of rats/ groups. ns: non-significance. Significance at * $P < 0.05$ (a: vs. G1, b: vs. G2 and c: vs. G3)



Histogram 1: The mean area percentage of collagen fibers among the different study groups



Histogram 2: The mean optical density of caspase- 3 among the different study groups

DISCUSSION

The current study was conducted to study the effects of EDs on the structure of the skeletal muscles and to evaluate the role of omega-3 against these effects. The high doses of caffeine (3 mg/kg) might cause impaired glucose tolerance, digestive disorders, anxiety, irritability, nausea, and tachycardia[5]. The effects of EDs have been attributed to the presence of caffeine that induces a pro-oxidant environment[18]. Caffeine may exert its effects through different mechanisms, including reducing insulin sensitivity in tissues, impairing glucose metabolism, stimulating release of stress hormones, such as cortisol, and adrenalin, which cause hyperglycemia[18]. Hyperglycemia can result in the glycation of phospholipids in organelles or cell membranes, leading to oxidative stress, and lipid peroxidation in tissues[19]. Also, excessive sugar content results in obesity and diabetes, whereas the disturbances in the homeostasis of taurine amino acid which is another ingredient of EDs might affect brain, cardiac and skeletal system[20]. Some researchers[18] added that high sugar concentration in addition to niacin in EDs might change carbohydrate metabolism causing high level of glucose, and insulin in the blood, and further insulin resistance.

In our study, histological examination of skeletal muscle of rats of group II (RB) revealed disrupted muscle fibers, splitting of myofibrils, loss of striation, pyknotic nuclei and dark, acidophilic cytoplasm. These findings were similar to those reported in another study[21] that found the same results in skeletal muscle injury induced by RB in albino rats. These findings were linked to the high caffeine-content and its interaction with taurine, an ingredient in EDs[22]. Additionally, previous study[23] reported that these changes were attributed to the preservatives in energy drinks like sodium benzoate. Excessive ingestion of caffeine and taurine in EDs has been reported to produce ischemia by inducing vasospasm[24]. Moreover, components of EDs as caffeine, taurine, and guarana were stated to decrease production of free radicals such as superoxide dismutase, and catalase in *vitro*[25].

Moreover, there are congestion of blood capillaries and infiltration of leucocytes. Researchers[13] explained these alterations to excessive caffeine, which might elevate levels of both tumor necrosis factor alpha (TNF-α), and inducible nitric oxide synthase (iNoS), with increased oxidative stress. The toxic effect of caffeine causes congestion of blood capillaries and pyknosis of nuclei. This toxicity was attributed to caffeine that causes inhibition of immune system or possibly a form of "protection for muscle tissue", where some cells are lost to protect the rest of cells[26]. Degenerated muscle produces various inflammatory materials, including reactive oxygen and nitric oxide, which express the genotype of adhesion molecules like vascular cell adhesion molecule. These molecules stimulate the inflammatory process, leading to further inflammation and muscle damage[27]. Moreover, muscle degeneration and apoptosis due to EDs containing caffeine causes an increase in the incidence of necrosis by 14%[28].

Previous study[29] explained that the centrally located oval nuclei (might be activated satellite cells) were attributed to tissue regeneration by satellite cells. This would lead to formation of myoblasts that fuse to themselves or to the injured myofibers. This study[29] added that satellite cells, which are the primary source of muscle precursor cells, proliferate and migrate to the site of injury.

Furthermore, an increased area percentage of collagen fibers in Group II could be attributed to caffeine toxic effects[26,27]. This increase in collagen fibers was confirmed by significant increase in area percentage of collagen fibers. Also, there was strong positive caspase-3 in Group II could be attributed to the effect of the excessive caffeine content in EDs on oxidative stress-induced apoptosis[13].

Concerning group III (omega-3), regular muscle striations with less congestion, less infiltration, weak positive caspase-3, and few collagen fibers were found in comparable to Group II. The protective role of omega-3 was attributed to antioxidant and anti-inflammatory properties[30]. This study reported that omega-3 was effective in preventing apoptosis induced by oxidative stress by suppressing gene expression of apoptosis, and reducing DNA fragmentation. Moreover, another study[31] reported that omega-3 might reduce susceptibility of inflammation and decrease the inflammatory response by inhibiting cytokine production. The anti-inflammatory effect of omega-3 was reported in another study[32]. Other studies stated that Omega-3 regulates inflammation, and oxidative stress, decreases production of reactive oxygen species, and enhances expression of anti-oxidative factors[33,34,35].

CONCLUSION

It can be concluded that omega-3 enhance skeletal muscle protection, and regeneration in RB-induced muscle injury, and has an ameliorating effect on the induced inflammatory changes.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دور اللأوميجا-٣ على التلف المستحث بالريد بول في العضلات الهيكلية في الجرذ الأبيض البالغ. دراسة هستولوجية وهستومناعية كيميائية

نيبال جمال عبد الوهاب، فاتن عبدالظاهر محمود

قسم الهستولوجيا - كلية الطب - جامعة عين شمس - مصر

مقدمة: مشروبات الطاقة هي نوع من المشروبات التي تحتوي على مواد منشطة مع الكافيين كجزء أساسي منها. وتعد الأكثر شعبية منها في مصر الريد بول والباور هورس.

الهدف من الدراسة: الهدف من الدراسة الحالية هو دراسة دورالأوميجا٣ على التلف العضلي الهيكلي المستحث بالريد بول في الجرذان.

المواد والطرق: تم تقسيم ثلاثون فأرا بالغ إلى ثلاث مجموعات: مجموعة مراقبة، ومجموعة الريد بول ومجموعة الريد بول مع أوميجا٣. المجموعة الأولى (المجموعة الضابطة): تم تقسيم الفئران إلى المجموعة الفرعية الأولى أ: تلقت الفئران ٥ مل من محلول ملحي بواسطة أنبوب المعدة يومياً لمدة ٤ أسابيع والمجموعة الفرعية الثانية ب: تلقت الفئران أوميجا ٣ بجرعة ٣٠٠ مجم / كجم / يوم (٦٠ مجم /جرذ=٠,٥ مل شراب /جرذ) عن طريق الفم عن طريق أنبوب المعدة لمدة ٤ أسابيع. المجموعة الثانية (مجموعة الريد بول): تلقت الفئران الريد بول بجرعة تعادل ٥ مل (١٠مجم/ كجم/يوم) عن طريق الفم عن طريق أنبوب المعدة لمدة ٤ أسابيع. المجموعة الثالثة (مجموعة الريد بول مع الأوميجا٣): تلقت الجرذان الريد بول وأوميجا ٣ بشكل متزامن لمدة ٤ أسابيع. تم التضحية بالحيوانات وتم تشريح العضلات الهيكلية وتهيئتها لصبغة الهيماتوكسيلين والأيوسين، وصبغة الماسون ثلاثية الألوان والصبغة الهستوكيميائية مناعية (caspase-٣) وتم عمل القياسات المورفومترية والتحليل الإحصائي.

النتائج: التغيرات النسيجية المختلفة الناتجة عن الريد بُول ظهرت في شكل تخلل خلوي أحادي النواة، إحتقان الشعيرات الدموية، ظهورسيتوبلازم غير منتظم بدون خطوط واضحة في بعض المناطق وإنقسام الألياف العضلية. أيضا ظهرت ألياف عضلية أخرى ذات سيتوبلازم داكن وأنويه داكنة وظهرت صفوف أنويه بيضاوية الشكل في منتصف بعض الألياف. وقد أظهرت مجموعة الريدبول عن ارتفاع ذو دلالة إحصائية في ألياف الكولاجين، وقد أظهرت العديد من الألياف العضلية تفاعل بني قوي في السيتوبلازم لصبغة ال caspase-٣ للكشف عن موت الخلايا المبرمج. وقد أدى التناول المتزامن مع الأوميجا٣ إلى تحسن هذه التغيرات بشكل ملحوظ مع انخفاض ذو دلالة إحصائية في ألياف الكولاجين، وتفاعل بني ضعيف في سيتوبلازم بعض الألياف العضلية.

الخلاصة: يمكن الاستنتاج أن الأوميجا- ٣ساعد على وقاية وتجديد التلف المستحث بالريدبول في العضلات الهيكلية حيث إنه يخفف التغيرات الإلتهابية المستحثة.