The Possible Protective Role of Sesame Oil on Skeletal Muscle Regeneration in Induced Red-Bull Injury: Experimental Study

Original Article

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ABSTRACT

Background: Energy drinks (Eds), including Red Bull, were introduced to United States markets in 1997. Consumption has increased worldwide mainly consumed by athletes and teenagers as they are seeking for increase their energy level. Seasme oil (SO) has been known to have anti-inflammatory and antioxidant properties.

Aim: The current study was designed to elucidate study the possible protective role of SO on skeletal muscle regeneration in Red Bull experimentally induced injury.

Materials and Methods: Twenty adult albino rats were divided into four groups, control, sham control, RB and RB+SO. Skeletal muscle sections were subjected to histological, morphometric, biochemical and statistical studies.

Results: RB induced various histological changes in skeletal muscle in the form of congestion, atypical fibre, multiple fibroblast and dark nuclei. RB also caused a sig increase in area % collagen, area % of caspase3 immunoexpression (IE), count of alpha smooth muscle actin IE(no I SMA) and count of +ve CD34 IE were recorded. Sig increase in the activity of interleukin-6(IL-6) and PI3K and AKT proteins. Concomitant administration with SO improved sig the previously mentioned changes.

Conclusion: It can be concluded that SO enhanced skeletal muscle regeneration in RB induced muscle injury expressed as a definite ameliorating effect to the induced inflammatory and degenerative changes.

Key Words: Antioxidant, : muscle, red bull, regeneration, sesame oil.

Revised: 28 August 2019, **Accepted:** 2 September 2019.

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ISSN:2536-9172, December 2019, Vol. 3, No. 2

INTRODUCTION

Energy drinks (Eds), including Red Bull, were introduced to United States markets in 1997. Consumption has increased worldwide mainly consumed by athletes and teenagers as they are seeking for increase their energy level (Totarro *et al.*, 2018). They contain high level of caffeine ranged from 50-550 mg /each can according to the brand, compared to 95 mg for an eight-ounce cup of coffee (Kassab *et al*., 2018). Although moderate caffeine intake (up to 400 mg/day for adults and 100 mg/day for teens) is considered safe, higher volumes may increase the possibility of various health problems. Energy drinks contain also higher level of sugar to increase glucose energy level as well alertness to overcome fatigue, which might increase the risk for obesity, diabetes and dental caries (Salih *et al*., 2018).

The plant sesame is a well-known medicinal plants that is used in alternative medicine in treatment of many diseases (Alsallami and Alaauldeen, 2017). Seasme oil (SO) has been known to have anti-inflammatory and antioxidant properties, which makes it effective for reducing atherosclerosis and the risk of cardiovascular disease.

Recent studies indicate that quadriceps muscle weakness and dysfunction is directly related to oxidative stress and SO is a natural product with excellent antioxidative property (Hsu *et al*., 2016).

Owing to the lack of sufficient data on the effect of neither Red Bull nor Sesame Oil on skeletal muscle regeneration. Therefore the aim of the present study was to study the possible protective role of SO on skeletal muscle regeneration in Red Bull experimentally induced injury.

MATERIALS AND METHODS

Animals:

The current study was carried out on 20 adult male albino rats aged three to five months and weighing about 150-200 grams. The rats were obtained from Animal House of Kasr -Alainy, Faculty of Medicine, Cairo University. Rats were housed for one week for environmental adaptation under standard laboratory conditions at 22-24°C with 12 hours light\dark cycle. They were fed on a constant adequate nutrition diet and allowed free access to drinking water ad libitum. The experimental work was conducted in accordance with the guidelines of the Animals Committee at Cairo University. They were housed in cages, five

 DOI: 10.21608/jmh.2019.16388.1065

rats/cage. The rats were divided into four groups as follow (five rats each):

- **Group I (Control group):** The rats received 7.5 ml saline using a gastric tube daily for four weeks.

- **Group II (SO group):** The rats received SO (Seed Crop Department, Ministry of Agriculture, Giza, season 2018) by a gastric tube, at a dose of 4 ml/kg daily for four weeks (Hsu *et al*., 2016).

- **Group III (RB group):** Rats received RB, one of the commonly used Eds available in local market (11-g sucrose, 32-mg caffeine, 400-mg taurine, 8-mg niacin, 2-mg pantothenic acid, 2-mg vitamin B6 and 2-µg vitamin B12 per 100 ml) (Roldán *et al*., 2017) at a dose (equivalent to 5ml) daily for four weeks using gasrtic tube (Ayoub and Elsherbiny, 2016). This dose for rats was equivalent to the human dose conversion tables (Akande *et al*., 2011).

- **Group IV (RB +SO Group):** The rats received RB at a dose (equivalent to 5ml) daily and SO, at a dose of 4 ml/kg by a gastric tube daily for four weeks.

Methods:

Chemicals: Red bull is one of the commonly used EDs available in local market was used in this study. Ingredients: (11-g sucrose, 32-mg caffeine, 400-mg taurine, 8-mg niacin, 2-mg pantothenic acid, 2-mg vitamin B6 and 2-µg vitamin B12 per 100 ml)[8].

SO (Seed Crop Department, Ministry of Agriculture, Giza, season 2018).

All rats were sacrificed by cervical dislocation (Iranpour and Kheiri 2016) using IP injection of phenobarbitone sodium (60 mg/kg) (Ozmen *et al*. 2002). Gastrocnemius muscle was exposed and muscle specimens were placed in 10% formol saline. for 48 hours. Paraffin blocks and 5µm thick sections were prepared. Sections were subjected to:

Histological study:

Hematoxylin and eosin (H&E) stain (Kiernan 2001) and Masson's trichrome stain (Bancroft and Gamble 2008).

Immunohistochemical Study:

- **Caspase 3 immunostaining (Bressenot *et al*., 2009)**: the marker for apoptosis.7ml of rabbit polyclonal Ab (RB-1197-R7) (Lab Vision Corporation, USA) was prediluted ready to use solution stored at 2-8°C. The +ve tissue control was a specimen of human tonsil. Caspase 3 +ve cells showed cytoplasmic reaction. On the other hand, one of the muscle sections was used as a negative control by passing the step of applying the primary antibody.

- **Anti-alpha smooth muscle actin (α-SMA) immunostaining (Elia *et al*., 2012)**: The marker for this antibody stains smooth muscle cells in vessel walls, gut wall, myometrium and myoepithelial cells. Anti-alpha smooth muscle actin antibody (Rabbit polyclonal antibody) (ab5694) was used at a concentration 0.5-2 µg/ml. Perform heat mediated antigen retrieval before commencing with IHC staining protocol. Glioma tissue sections were used as positive control specimens. Cellular localization is the cytoplasm. On the other hand, one of the muscle sections was used as a negative control by passing the step of applying the primary antibody.

- CD34 immunostaining (Pasut *et al*., 2012) is the marker for hematopoietic progenitor cells, small vessel endothelium (15) and satellite cells of skeletal muscle (16) of a variety of tissues. CD34goat polyclonal Ab (Sigma-Aldrich Chemie Corporation laboratories, Germany, catalogue ID SAB4300690). The sections were treated with CD 34, at 5-15 µg/ml ready to use at room temperature. Cellular localization is the cell membrane. Tonsil sections were used as positive control specimens. On the other hand, one of the muscle sections was used as negative control by passing the step of applying the primary antibody.

Morphometric study:

The area% of collagen fibers, that of caspase3 IE, α-SMA IE and count of CD34+ve cells were measured in Masson's trichrome and immunostained sections. These measurments were done in 10 high power fields (HPF) in control and experimental groups using interactive measurements menu.

Biochemical parameters:

IL-6 estimation: Tissue of skeletal muscle from the gastrocnemius muscle of the different groups were stored at −80 °C and were homogenized in 10–20 volumes with a buffer containing 1% sodium dodecylsulfate (SDS), 100 mmol/L Tris HCl (pH6.8), 1 mmol/L phenylmethyl sulfonyl fuoride (PMSF), and 0.1mmol/L β-mer-captoethanol. IL-6 level was assessed in muscle tissue homogenate using RayBio1 Rat IL-1β and RayBio1 Rat IL6 ELISA Kits (Ray Biotech, Inc., USA). The steps were carried out according to the manufacturer's instructions (Ali 2016).

Western blotting analysis: Tissue homogenate was centrifuged at 12000×g for 10min at 4 °C and supernatant A was collected. The supernatant was centrifuged again at 4°C, 40000×g for 10min, with the proper amount of membrane protein extraction reagent to obtain the supernatant after high-speed mixing using a vortex mixer after 5 seconds. Then, the sample was placed in an ice bath precipitation for 5–10 minutes, which was repeated 3 times to obtain the full extraction of protein. At 4°C, 40000×g/15 min, supernatant B for PM GLUT4 was collected for the membrane protein solution. The protein concentration was determined by the BCA protein assay. For each sample, proteins were separated by electrophoresis via 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-

PAGE). The gel was transferred to a nitrocellulose (NC) membrane (Bio-Rad Instruments, CA, USA) in transfer bufer containing 25mmol/L Tris, 192mmol/L glycine, and 20% methanol. The NC membranes were blocked using PBS containing 2% bovine serum albumin (BSA) and 0.05% Tween-20 at 4°C for 1 h at room temperature and incubated with primary antibody overnight. The following proteins were used for the supernatant A: Akt protein and Phosphoinositide 3-kinase (PI3-K) protein (Cell Signaling Technology, Danvers, MA) (Glass.2010).

Statistical study:

Quantitative data were summarized as means and standard deviations and compared using one-way analysis-of-variance (ANOVA). Any significant ANOVA was followed by post hoc Tukey test to detect which pairs of groups caused the significant (sig) difference. *P-values <0.05* were considered statistically sig (Emsley). Calculations were made on Statistical Package of Social Science (SPSS) software version 16.

RESULTS

Histological changes:

Haematoxylin and Eosin (H&E) stained sections: Longitudinal sections in skeletal muscle of control rats in group I showed cylindrical fibers arranged in a parallel pattern exhibiting peripheral pale nuclei and regular sarcoplasmic striations (Fig 1a). RB group revealed congested vessels surrounded by atypical fibers (Fig 1b), multiple fibroblasts between fibers exhibiting dark nuclei (Fig 1c). Other sections showed atypical muscle fibers exhibiting some striations in sarcoplasm, in addition to some fibroblasts among the fibers (Fig 2a). Transversely cut fibers demonstrated vacuolations and dark nuclei (Fig 2b). Few swollen transversely cut fibers were detected exhibiting a wide disorganized area of the sarcoplasm and dark nuclei (Fig 2c). RB+SO group showed less congestion, few fibroblasts in CT surrounding muscle fibers exhibiting separated myofibrils and striations (Fig 3a), multiple muscle fibers exhibited regular striations (Fig 3b) and transversely cut fibers exhibited few dark nuclei. Noncongested vessels were noticed (Fig 3c).

Masson's trichrome stained sections: In group I fine collagen fibers were seen between the muscle fibers (Fig 4a), in RB group widely distributed dense collagen fibers were detected between the muscle fibers (Fig 4b) and in RB+SO group focal less dense collagen fibers were found between the muscle fibers (Fig 4c).

Immunohistochemical changes:

- **Caspase 3 IE:** In group I –ve IE was evident (Fig 5a), in group II multiple fibers demonstrated obvious +ve IE (Fig 5b) and in group III some fibers revealed less obvious +ve IE (Fig 5c).

- **Alpha smooth muscle actin IE:** In group I +ve IE was found in the wall of vessels and few flat cells (Fig 6a), in group II +ve IE was seen in the wall of multiple vessels and in few flat cells (Fig 6b) and in group III +ve IE was obvious in the wall of some vessels and in multiple flat cells (Fig 6c).

- **CD34 IE:** In group I -ve IE was found (Fig 7a), in group II few +ve flat cells were observed at the periphery of the fibers (Fig 7b) and in group III multiple +ve flat cells were mainly detected at the periphery of the fibers (Fig 7c).

Morphometric results:

The mean of area% of collagen fibers and +ve alpha smooth muscle actin IE was significantly increased in group II compared to groups I and III, while in group III a significant increase was found compared to group I. The mean area% of +ve caspase 3 and +ve CD34 IE was found to be significantly increased in group II compared to group III (Table 1).

Biochemical changes:

- Inflammatory marker parameter changes (IL-6): In RB group a sig increase was found compared to the other two groups. In RB+SO group a sig decrease was detected compared to RB group.

- PI3-K and AKT: Sig increase was found in both PI3-K and AKT proteins expression in RB group compared to the other two groups. While, a sig decrease was found in RB+SO group compared to Ed group.

Table 1: Mean area% of collagen fibers, +ve caspase3, +ve alpha smooth muscle actin and +ve CD34 IE.

Group	Area% of collagen fibers	Count of +ve caspase3 IE	Count of +ve alpha smooth muscle actin IE	Count of +ve CD34 IE
Group I (control)	0.19± 0.03	-	0.32±0.05	-
Group II(RB)	11.34±3.21*	35.11±8.27*	1.33±0.14*	2.31±0.04*
Group III (RB+SO)	0.36±0.09^	7.28±1.05	2.19±0.25^	7.92±1.33

- sig P≤0.05
- *compared to groups I and III
- ^compared to groups I and II

Table 2: Mean values of IL-6, PI3-K and AKT proteins in different groups:

Group	IL-6	PI3-K	AKT
Group I (control)	1.02 ± 0.2	1.00 ±0.02	1.03± 0.09
Group II(RB)	6.3±1.57*	4.6±1.02*	6.5±0.54*
Group III (RB+SO)	2.5± 0.09^	2.06± 0.07	3.4± 0.21^

- *sig compared to all groups.
- ^ sig compared to RB group.

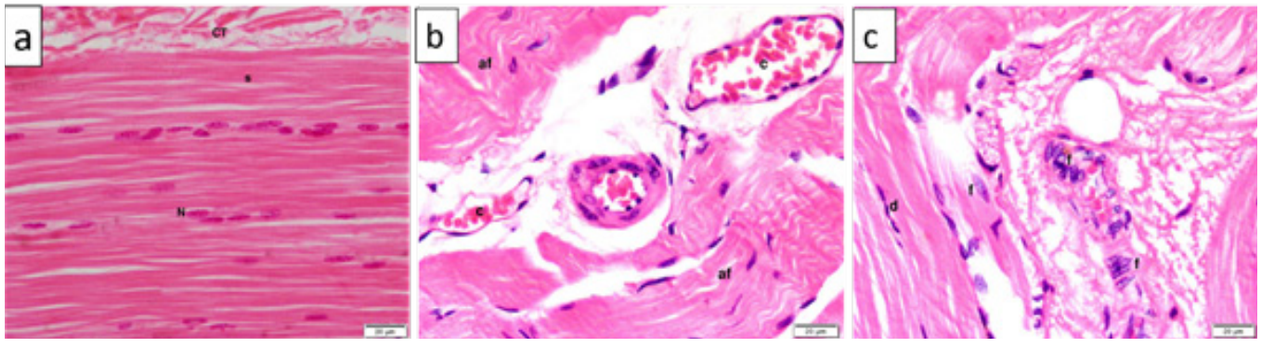

Fig. 1: Section in the skeletal muscle of a rat in control group stained by H&E showing: (a) fibers exhibiting peripheral nuclei (N) and obvious striations (s) in sarcoplasm. Note connective tissue (CT) around the muscle fibers. (b) RB group showing congested vessels (c) surrounded by atypical fibers (af). (c) RB group showing multiple fibroblasts (f) between fibers exhibiting dark nuclei (d). (H&E,x 400).

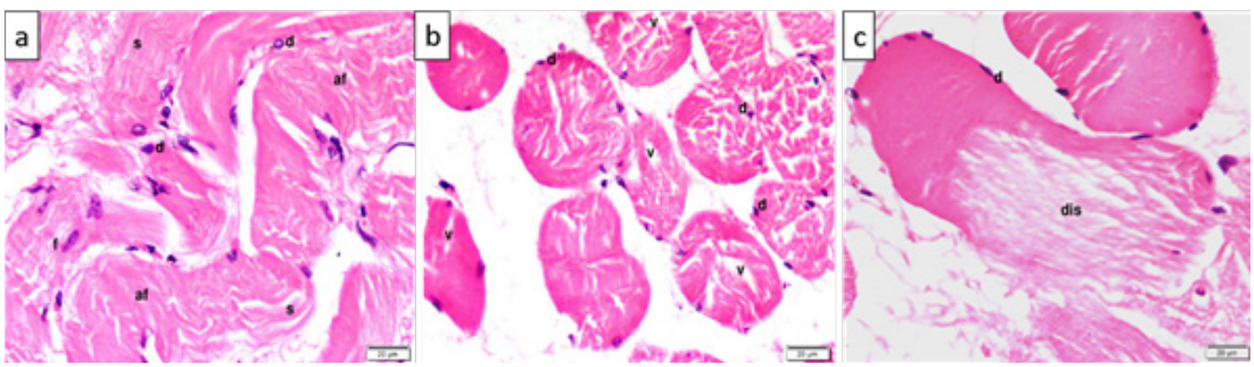

Fig. 2: Section in the skeletal muscle of rats in the RB group stained by H&E showing: (a) atypical muscle fibers (af) exhibiting some striations (s) in sarcoplasm and dark nuclei (d), in addition to some fibroblasts (f) among the fibers. (b) transversely cut fibers exhibiting vacuolations (v) and dark nuclei (d). (c) a swollen transversely cut fiber exhibiting a wide disorganized area of the sarcoplasm (dis) and dark nuclei (d). (H&E, x 400).

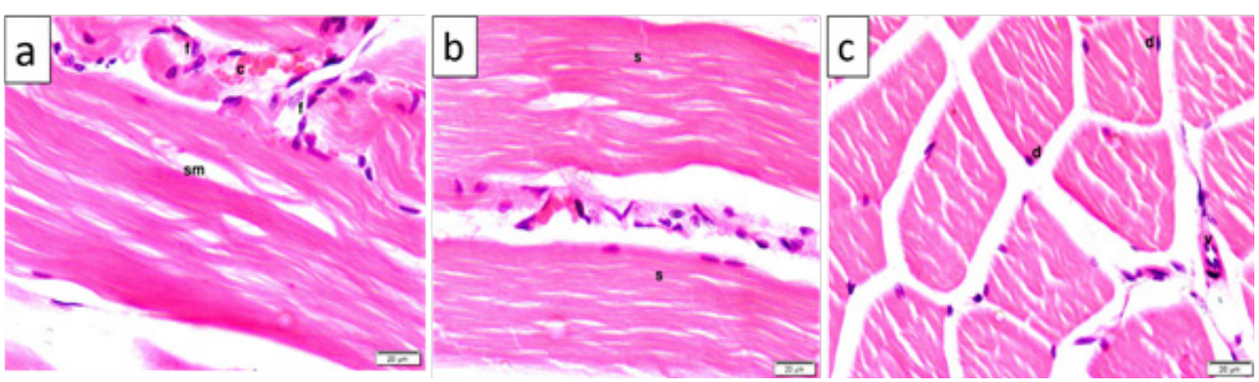

Fig. 3: Section in the skeletal muscle of rats in RB+SO group stained by H&E showing: (a) less congestion (c), few fibroblasts (f) in CT surrounding a muscle fiber exhibiting separated myofibrils(sm) and striations.(b)two muscle fibers exhibiting regular striations(s). (c) transversely cut fibers exhibiting few dark nuclei (d). Note a non congested vessel (v). (H&E, x 400).

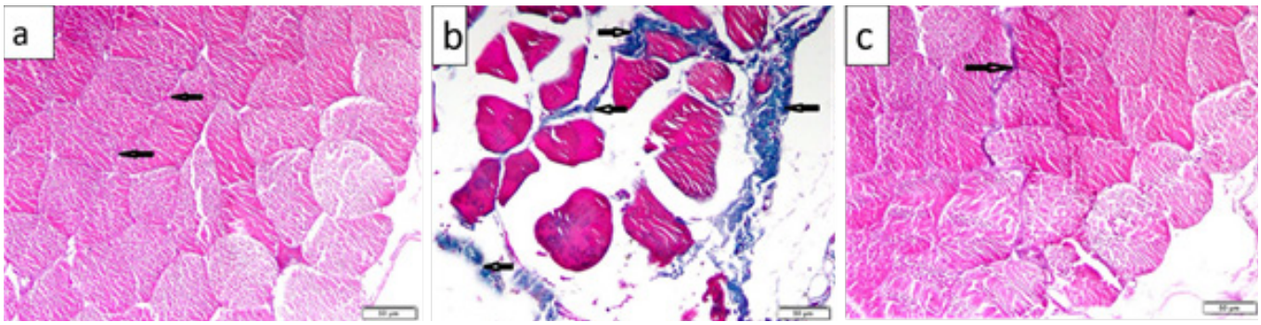

Fig. 4: Section in the skeletal muscle of a rat in Masson stained section showing: (a) control group showing fine collagen fibers (arrows) between the muscle fibers. (b) RB group showing widely distributed dense collagen fibers (arrows) between the muscle fibers. (c) RB+SO group showing focal less dense collagen fibers (arrows) between the muscle fibers. (Masson's trichrome, x 200).

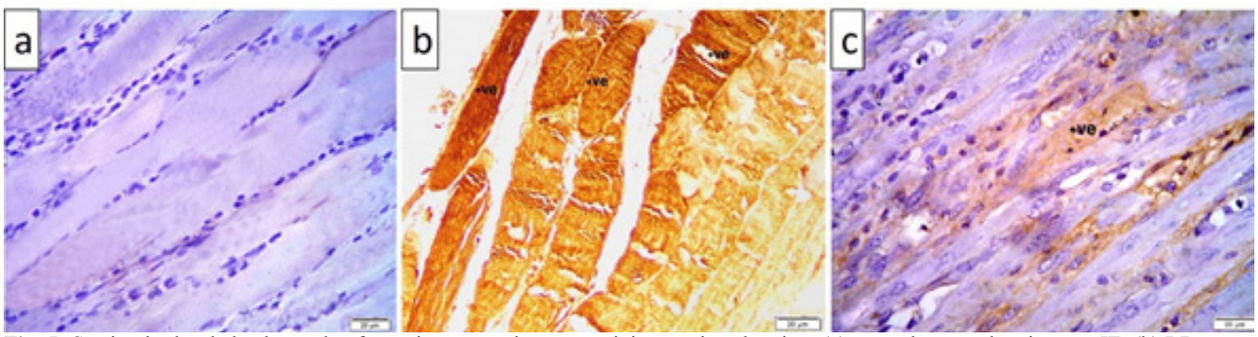

Fig. 5: Section in the skeletal muscle of a rat in caspase immunostaining section showing: (a) control group showing –ve IE. (b) RB group showing multiple fibers demonstrating obvious +ve IE (+ve). (c) RB+SO group showing some fibers demonstrating less obvious +ve IE (+ve). (Masson's trichrome, x 200).

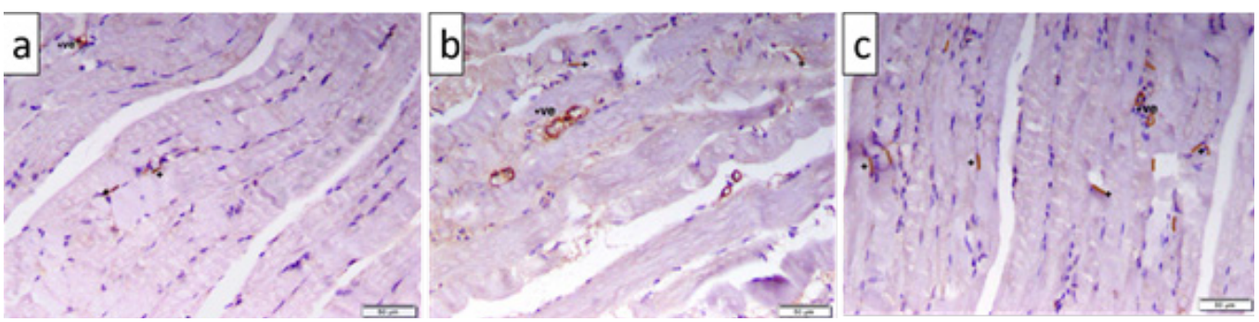

Fig. 6: Section in the skeletal muscle of a rat in α SMA immunostaining section showing: (a) control group showing +ve IE in the wall of a vessel and few flat cells (+ve). (b) RB group showing +ve IE in the wall of some vessels and in few flat cells (+ve). (c) RB+SO group showing +ve IE in the wall of few vessels and in multiple flat cells (+ve). (α SMA immunostaining, x 200).

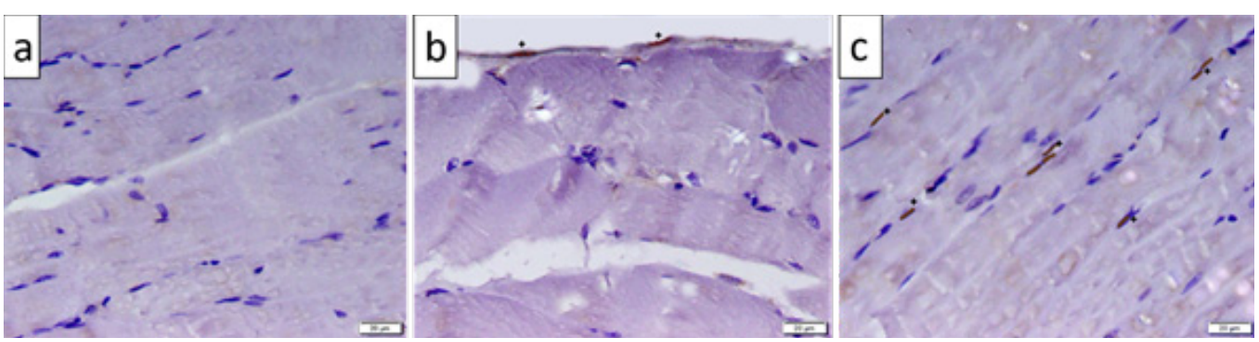

Fig 7: Section in the skeletal muscle of a rat in CD34 immunostaining section showing: (a) control group showing -ve IE (b) RB group showing few +ve flat cells (+) at the periphery of the fiber. (c) RB+SO group showing multiple +ve flat cells (+) mainly at the periphery of the fiber. (CD34 immunostaining, x 400).

DISCUSSION

The aim of the present study was designed to elucidate the possible ameliorative role of SO on the skeletal muscle regeneration in RB induced skeletal muscle injury of adult male albino rat. Amelioration of skeletal injury was evidenced by histological and immunohistochemical results and was confirmed by morphometric and biochemical results.

In the present study, in the RB group revealed various histological changes in the form of vacuolations, separation of myofibrils, swollen muscle fibres and congestion. These previous changes were indicative of degenerative changes. The latter changes might be due to the inadequate oxygen supply and the exchange of metabolites and other inflammatory mediators resulting in enlarged and hypertrophied fibers (Hassan *et al*., 2009). In accordance, the histopathological changes induced by chronic consumption of Power Horse one of the energy drinks for 4 weeks on the structure of pancreas and fundic mucosa of stomach in adult male albino rats was studied, and they attributed these changes to caffeine effect as it elevates levels of both tumor necrosis factor alpha (TNF-α) and inducible nitric oxide synthase (iNOS) and increased oxidant stress (Ayuob and ElBeshbeishy, 2016).

Swollen muscle fibres existence can be related to, a sig higher expression of the PI3K/AKT proteins was recorded in the RB group. The PI3K/Akt signaling pathway plays an important role in regulating muscle metabolism. Activation of the PI3 kinase pathway can induce skeletal muscle hypertrophy, defined as an increase in skeletal muscle mass. Akt is a serine-threonine protein kinase that can induce protein synthesis and block the transcriptional upregulation of key mediators of skeletal muscle atrophy and subsequently increase in the muscle size (Glass. 2010).

In the current work many fibroblasts, dark nuclei and congestion were noticed in the RB group, and that was confirmed by sig increase in IL-6 as inflammatory marker. Congestion observed in the present work could be related to the acute inflammatory response, followed by degenerative changes (Gillani *et al*., 2012). Tissue injury as a result of inflammatory response could lead to tissue death, followed by replacement of necrotic tissue by fibrous tissue (Youssef *et al*., 2015).

Dark nuclei which was obvious in RD group, and confirmed both by +ve IE in caspase3 IE and morphometrically by sig increase in the area% of +ve caspase IE. Going with, activation of an apoptotic process might affect the viability of the entire fiber (Sandari *et al*., 1998).

Dense collagen fibres were found in RB group which was confirmed by sig increase in area% of collagen fibers, which can be explained by the direct toxic effect of caffeine (Mubark *et al*., 2012). Recently, Munteanu *et al* (2018) studied the occurrence of negative effects of RD on the myocardium, and he correlated it to collagen accumulation in the myocardium.

In the present work, increased +ve αSMA IE and few CD34 +ve cells were detected in RB group, αSMA IE is an indicator of an attempt at muscle regeneration in response to injury. CD34 is considered the marker for muscle progenitor cells and satellite unipotant stem cells, which was supported by (Alfaro *et al*. 2011).

Concerning changes in SO treated group, regular muscle striations with less congestion were found compared to RB group, that was confirmed and related to the sig decrease in IL-6 value. In support, it was postulated that protective role of SO might be due to its potent ability to suppress oxidative stress, inflammation and antiapoptotic role (Woo., 2019). However, still some dark nuclei and separation of the myofibrils were residual, but less obvious +ve caspase IE and sig decrease in +ve caspase IE. That might be related to the dose and duration issues, as four weeks duration seemed to be insufficient for SO supplementation to start gaining complete recovery.

Minimal collagen fibers were detected in the SO group confirmed by a sig decrease in the mean area% of collagen fibers, and Pi3/AKt proteins expression compared to RD group. This confirmed the antifibrotic role of SO which was in agreement with other authors, who investigated the antifibrotic potential role of SO. They found that SO inhibiting cardiac fibroblast proliferation and blocking transforming growth factor (TGF-β1) signaling cascade as transforming growth factor-β1 (TGF-β1) is one of the strongest pro-fibrotic factors that can induce fibroblasts proliferation, differentiation and collagen synthesis (Zaho *et al*., 2015).

In the present work, increased +ve αSMA IE and multiple CD34 +ve cells were detected in SO group. Results showed statistically sig increase in αSMA IE and count of CD34+ve cells in SO group compared to RB group denoting activated regeneration in SO group, which might be explained by enhanced migration of SCs to muscle tissue exposed to injury (Alfaro *et al*. 2011). It was considered that CD34 in addition of being a stem cell marker might play an important role in the stem cell activity. It was added that satellite cells within adult skeletal muscle are an enriched population of CD34+ve cells (Passut *et al*., 2012). It was postulated that activation of muscle precursor cells is an important determinant for the efficiency of muscle regeneration. It was added that the main source of muscle precursor cells are satellite cells, which proliferate and migrate to the injured site (Mu *et al*., 2010). Therefore, local SCs were documented as a promising treatment for different diseases (Zaho *et al*., 2013).

CONCLUSION

In can be concluded that SO enhanced skeletal muscle regeneration in RB induced muscle injury expressed as a definite ameliorating effect to the induced inflammatory and degenerative changes.

CONFLICT OF INTEREST

There are no conflicts of interest.

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الملخص العربى

الدور الوقائي المحتمل لزيت السمسم على تجديد العضلات في الإصابة المستحثة بمشروب الطاقة: دراسة تجريبية.

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مقدمة: مشروبات الطاقة مثل الريد بُول ظهرت فى أسواق الولايات المتحدة في عام 1997. وقد زاد الإستهلاك في جميع أنحاء العالم بشكل أساسي من قبل الرياضيين والمراهقين واللذين يسعون لزيادة مستوى الطاقة لديهم. زيت السمسم له خصائص مضادة للإلتهابات ومضادة للأكسدة.

الهدف من الدراسة: تم تصميم الدراسة الحالية لدراسة الدور الوقائي المحتمل لزيت السمسم على تجديد العضلات الهيكلية في إصابة ريد بُول التجريبية.

المواد والطرق: تم تقسيم عشرون فأرا بالغة إلى أربع مجموعات: مجموعتين مراقبة , ومجموعة الريد بول ومجموعة الريد بول مع زيت السمسم .وفى نهاية التجربة تم أخذ عينات من العضلات وتم اعداد شرائح مجهرية وصبغها بالهيماتوكسلين والإيوسين ومن ثم القياسات المورفومترية والكيميائية الحيوية والإحصائية.

النتائج: التغيرات النسيجية المختلفة الناجمة عن الريد بُول في العضلات ظهرت في شكل احتقان و ظهورالألياف غير التقليدية ، والألياف الليفية متعددة والنواة الداكنة. تسبب الريد بول أيضًا في حدوث زيادة كبيرة في نسبة الكولاجين في الخلايا، وتم تسجيل نسبة المناعة في بعض الصبغات المناعية. زيادة ملحوظة في نشاط بعض الإنزيمات والجينات. االعلاج بزيت السمسم أدي الي تحسين التغييرات المذكورة سابقا.

الخلاصة: يمكن أن نستنتج أن زيت السمسم يساعد علي تجديد العضلات الهيكلية في إصابة العضلات الناجم عن الريد بُول معبرعنه بتخفيف واضح للتغيرات الإلتهابية المستحثة.