Original Article

Effects of Combined Nicotine and Caffeine on the Rat Skeletal Muscles: A Histological and Immunohistochemical Study

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Abstract

Background: Nicotine and caffeine are pharmacologically active substances that consumed widely in the whole world. Most of the nicotine users also consume caffeine. Smokers tend to drink more coffee than nonsmokers. It is important to characterize these substances with regard to their effects on the histological and immunohistological structure. **Objectives:** The objective of the study is to assess the impact of combined administration of nicotine and caffeine on histological structure of the skeletal muscle tissue in the adult male Wistar rats. **Materials and Methods:** Twenty adult male Wistar rats with an average weight of 200–250 g were randomly divided into four equal groups: control, nicotine, caffeine, and combined (nicotine + caffeine). The diaphragm muscle was processed and stained with hematoxylin and eosin (H and E) stain, histochemically by periodic acid–Schiff (PAS) and immunohistochemically by anti-CD68 antibodies. **Results:** After injected nicotine, thick basement membrane with apparent increase in the positive CD68 macrophages inbetween the diaphragm muscle fibers. After injected caffeine, there was an apparent accumulation of mononuclear cells around some fibers with decrease in the PAS positive fibers. Combined injected (nicotine + caffeine) group, some fibers exhibited deep acidophilic cytoplasm with flat peripheral nuclei and apparent increase of the CD68 positive cells. There was an increase in PAS positive material around fibers appearing as a thick basement membrane. **Conclusions:** The present study proved that caffeine and nicotine either taken alone or in combination have many negative impacts on the active type of skeletal muscles like diaphragm leading to degenerative changes that may affect their function.

Keywords: Caffeine, CD68, histology, nicotine, periodic acid–Schiff, skeletal muscles

Introduction

Nicotine and caffeine are addictive substances that widely consumed in the whole world.[1] Approximately 80%–97% of nicotine users also consume caffeine and smokers tend to drink more coffee than nonsmokers.[2] Moreover, caffeine was known to have the potential of interaction with smoking which is known to increase the rate of caffeine metabolism in humans.[3] Nicotine, 3-(1-methyl-2-pyrrolidinyl) pyridine, is the most common addictive psychostimulant.[4] It is an alkaloid which derived from tobacco plant known as *Nicotiana tabacum*.[5]

Cigarette smoking is one of the most critical problems that threaten human health; it kills approximately six million individuals per year; the annual death rate could increase to more than eight million by 2030.[6] Once nicotine is entered the blood, it is rapidly transported to the brain, where it reacts with neuronal acetylcholine receptor. Nicotine is metabolized into its main metabolites (cotinine, trans-3-hydroxycotinine, nicotine-N-oxide, and cotinine-N-oxide).[4] Moreover, one of the nicotine effects at the cellular level is that it leads to oxidative stress.[5]

Caffeine (1, 3, 7-trimethylxanthine) is a plant alkaloid found in coffee, tea, cocoa, and cola soft drinks.[3,7,8] According to statistics from European and North American, ~90% of the adults are coffee users with an average consumption of 200 mg (about two cups of coffee/day).[4] Caffeine is well known to act as a neuromodulator with associative effect

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on cognitive function, motor behavior, and information processing.[8]

Muscle is one of the major tissues by weight in the body, conferring adaptive functions such as mobility. It is a high consumption organ, and due to its demanding physiology throughout a normal lifespan, skeletal muscle requires lifelong regenerative capacities.[9]

CD68, the human homolog of macrosialin, is commonly used as a selective marker for human monocytes and macrophages. Its expression is regulated by a macrophage-specific promoter.[10]

Nicotine and caffeine are popular-consumed substances over the world since ancient times; therefore, it is important to characterize these substances and their effects on body organs such as muscle tissue since muscle is considered as a major tissue and high consumption organ. However, few literature were focused on their impact on skeletal muscles and most concentrate on physiological aspects. Hence, the main objective of the current study was to assess the impact of a combined administration of nicotine and caffeine on the histological structure of skeletal muscle tissue in male Wistar rats.

Materials and Methods

Materials

Drug preparation

1. Nicotine hydrogen tartrate had purchased from Sigma, USA, and was freshly prepared in normal saline. The prepared solutions were stored in foil-wrapped glass bottle at 4°C for no longer than 10 days
2. Caffeine anhydrous extra pure (99%) (CA0150) had purchased from Scharlau Chemie, Spain. The dissolved caffeine was filtered through a disposable sterile filter membrane (Corning Incorporated, USA) immediately before injection.

Experimental animals

Twenty adult male Wistar rats (200–250 gm) were obtained from the Animal House of King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia. Rats were randomly divided into four equal groups each one including five rats and were housed in an animal room with 12-h light/dark cycle and free access to water. The experiment was conducted at 23°C–25°C the experimental procedure was evaluated and approved by the King Abdulaziz National Committee of the ethical approval and conducted according to their guidelines.

Methods

Animals grouping and drug administration

Rats were divided into four groups, five male rats each:

- Group I: Control group where rats received normal saline subcutaneously (SC) three times/week through intraperitoneal route (IP) daily.
- Group II (nicotine group): Rats were injected with pure nicotine; each rat was injected SC as 10-mg/kg body weight three times/week to produce a chronic plasma level of nicotine according to a previous study[11]
- Group III (caffeine group): Rats were injected with caffeine. The animals were treated by IP injection of 100-mg/kg body weight caffeine dissolved in saline daily for 30 days. The choice of this dose was dependent on the protective results of a biochemical study[12]
- Group IV (nicotine + caffeine): Rats were injected with combination of nicotine and caffeine.

Histological and immunohistochemical studies

After 30 days, animals were euthanized by cervical dislocation under deep anesthesia. The diaphragm muscles were dissected carefully from their attachment site. For light microscopic examination, muscle samples were fixed in 10% buffered formalin, dehydrated, cleared, and embedded in paraffin. Serial 5-µm sections of the diaphragm muscles were stained with hematoxylin and eosin (H and E) for routine histological study and periodic acid–Schiff stain (PAS) for detection of mucopolysaccharides.[13] From each muscle, a transverse and longitudinal section was obtained and examined.

Immunohistochemical staining for anti-CD68 antibody detection was done in diaphragm muscle specimens using Avidin-Biotin detection system (Ventana, Tucson, AZ, USA), following the manufacturer's instructions. Sections were counterstained with hematoxylin.[14] All stained slides were examined and photographed using a compound light microscope (Olympus 51, Japan).

Results

Hematoxylin and eosin staining

Examination of H- and E-stained longitudinal sections of diaphragm muscle of control group revealed that the muscle appeared to be formed of longitudinally parallel-arranged muscle fibers. The fibers were cylindrical in shape with nearly constant diameter. Each muscle fiber was multinucleated and possessed acidophilic sarcoplasm. The nuclei appeared oval in shape and are peripherally located just beneath the sarcolemma. Cross striations of the fibers appeared also as characteristic features of striated muscles [Figure 1]. Muscle fibers demonstrated a polygonal shape, and they are fitted together in a mosaic pattern. They showed peripheral nuclei. The muscle fibers are arranged in bundles which were invested by a minimal amount of connective tissue (perimysium) [Figure 2]. Examination of Group II (nicotine only). Longitudinal sections showed some fibers with pale sarcoplasm and patchy loss of transverse striations. Crowded nuclei scattered along its length [Figures 3-5]. Multiple inflammatory cells appeared between the muscle fibers [Figure 5]. In transverse section, some fibers showed variations in size and shape, while most fibers appeared large with homogenous acidophilic and widely separated from each other [Figure 6]. Group III (caffeine only) variation in the size and shape of the muscle fibers within the same bundles associated with mononuclear cell infiltration [Figures 7 and 8]. Histological evaluation of muscle in Group IV receiving a combination of nicotine and caffeine longitudinal section showed variation in the size and shape of

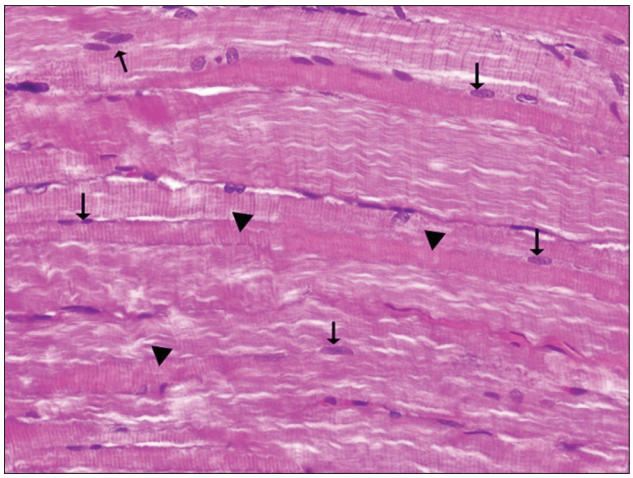

Figure 1: A photomicrograph of a longitudinal section in diaphragm muscle of the control group showing parallel multinucleated muscle fibers, having peripheral oval nuclei just beneath the sarcolemma (↑). Note the well-defined transverse striations across the muscle fibers (▲) (H& E x 40)

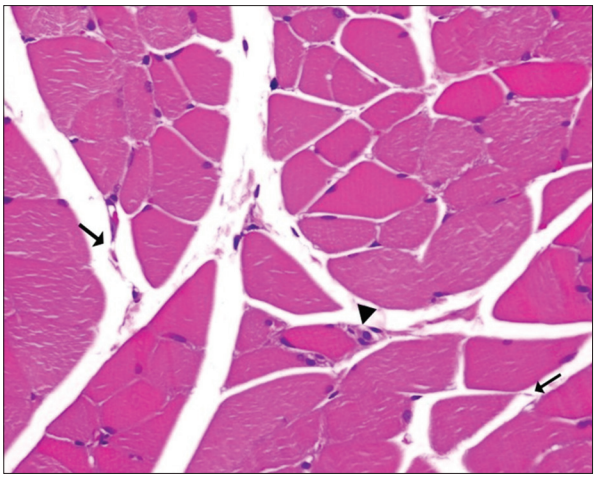

Figure 2: A photomicrograph of a transverse section in a rat diaphragm muscle of the control group. The muscle bundles are separated by few the delicate connective tissue surrounding individual fibers (endomysium) (↑). Note a muscle spindle bounded by a thin connective tissue capsule (▲). It contains the intrafusal muscle fibers (H& E x 40)

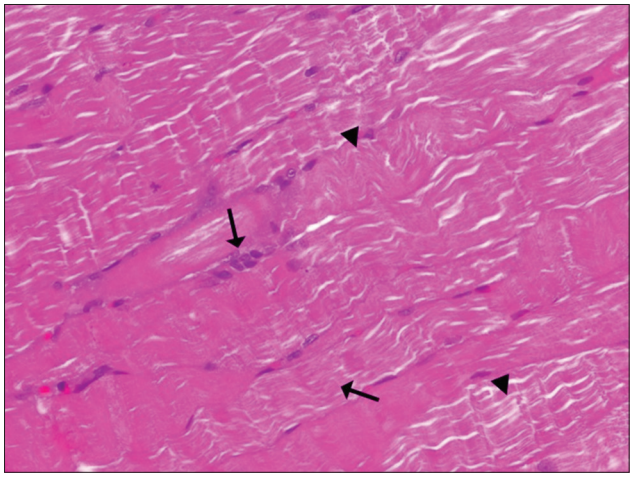

Figure 3: A photomicrograph of a longitudinal section in a rat diaphragm muscle of experimental Group II (nicotine only), showing some fibers with pale sarcoplasm and crowded nuclei (↑). Other fibers are wavy with loss of transverse striations (▲) (H& E x 40)

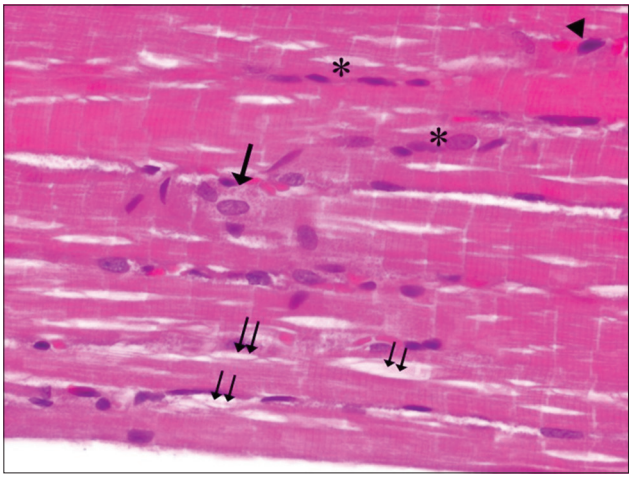

Figure 4: A photomicrograph of a longitudinal section in a rat diaphragm muscle of experimental Group II (nicotine only), showing some fibers with pale sarcoplasm and crowded nuclei (↑). Some of the peripheral-flattened nuclei are pyknotic (▲), and striation is lost in some muscular tissues. Notice centrally located nuclei (*) with appearance of splitting of the muscle fibers (↑↑) (H& E x 40)

muscle fibers within the same bundle with peripheral nuclei. Some fiber exhibits deep acidophilic cytoplasm and showed many nuclei scattered along its length with centrally located nuclei in some fibers [Figure 9]. Transverse sections showed distorted muscle fibers with loss of their architecture. Some fibers have pale sarcoplasm and others showed variations in muscle fiber sizes. Some muscle fibers appeared large with homogeneous acidophilic sarcoplasm and related to nearby congested blood vessels [Figure 10].

Periodic acid–Schiff staining

In the control group, PAS-positive reaction was observed in most muscle fibers [Figure 11a]. Most fibers in nicotine-treated Group II showed PAS-positive thick basement membrane [Figure 11b]. In caffeine Group III, there was an apparent decrease in the PAS-positive fibers [Figure 11c]. In Group IV (nicotine + caffeine), there was an increase in PAS-positive material around fibers that represented thickened basement membrane [Figure 11d].

Immunostaining for CD68

The immunohistochemical-stained longitudinal section of rat skeletal muscle CD68 showed that in the control group (Group I), there were few cells with positive CD68 expression [Figure 12a]. After nicotine injection (Group II), there was an apparent increase in cells with positive CD68 expression [Figure 12b]. In caffeine group (Group III), few positive CD68 cells were also seen. These cells showed a weak-positive reaction similar to the expression of CD68 in control sections [Figure 12c]. After combined administration of (nicotine + caffeine) (Group IV), an

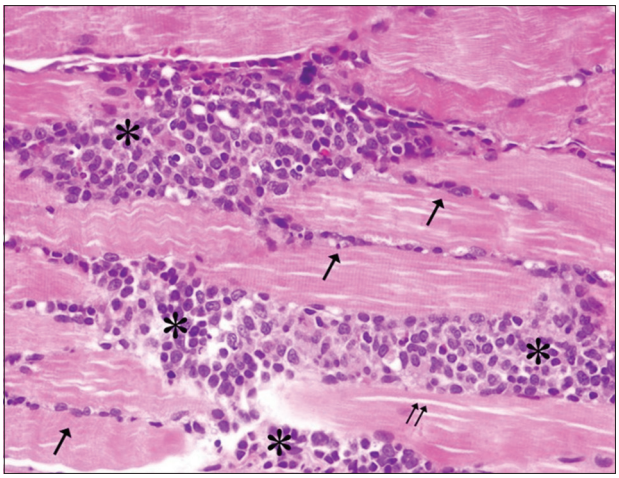

Figure 5: A photomicrograph of a longitudinal section in a rat diaphragm muscle of experimental Group II (nicotine only), showing distorted, interrupted muscle fibers with loss of their architecture. Some fibers with pale sarcoplasm, the fibers contain many nuclei scattered along its length, and loss of transverse striations is seen (↑). Notice multiple inflammatory cells (*) with appearance of splitting of the muscle fibers (↑↑) (H& E x 40)

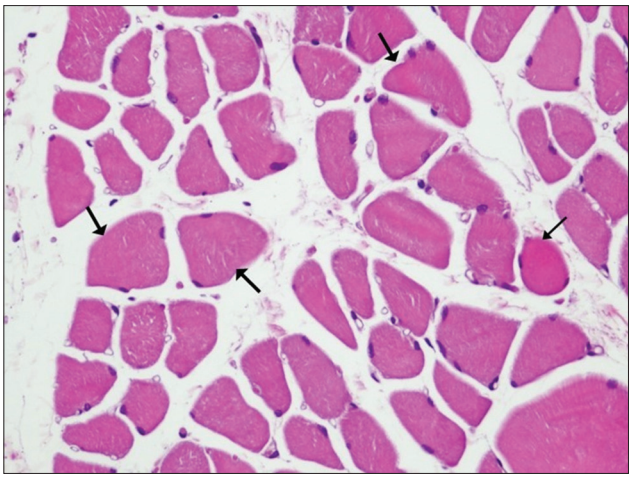

Figure 6: A photomicrograph of a transverse section in a rat diaphragm muscle of experimental Group II (nicotine only), showing variations in muscle fiber sizes. Some muscle fibers appear large with homogenous acidophilic sarcoplasm and widely separated from each other (↑) (H& E x 40)

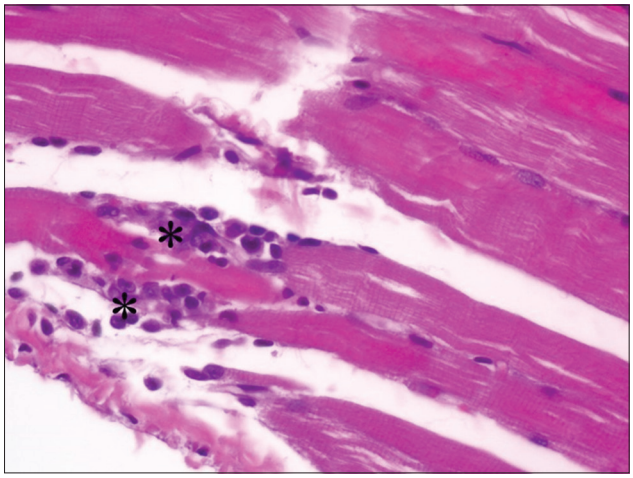

Figure 7: A photomicrograph of a longitudinal section in a rat diaphragm muscle of experimental Group III (caffeine only), showing accumulation of mononuclear cells around some fibers (*) (H& E x 40)

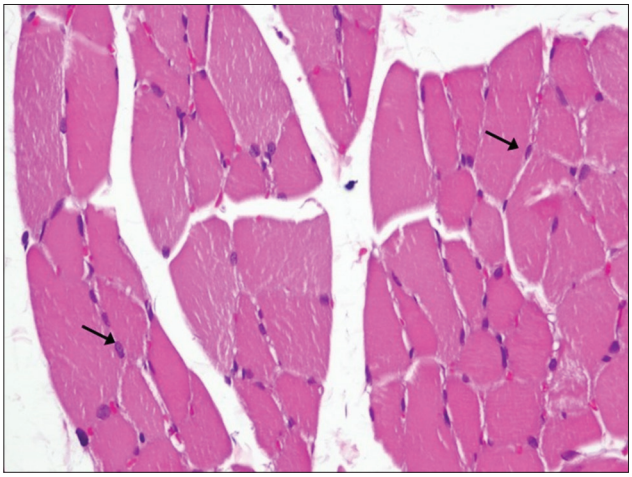

Figure 8: A photomicrograph of a transverse section in a rat diaphragm muscle of experimental Group III (caffeine only), showing variation in the size and shape of muscle fibers within the same bundle with flat peripheral nuclei (↑) (H& E x 40)

apparent increase of the positive CD68-expressing cells was observed [Figure 12d].

Discussion

Caffeine and nicotine are widely consumed psychoactive drugs in the world. Caffeine has proven to have a direct effect on muscle through maintaining electrolyte homeostasis, enhancing sarcoplasmic reticulum calcium release or exert an impact on muscular tissue through its effect on the central nervous system.[15] Caffeine contributes to the dietary antioxidants that protect neurons and other cells from free radical-induced oxidative damage and consequently can reduce the risk of chronic degenerative diseases. It also enhances the rate of cerebral glucose utilization that participates in cognitive functions.[8] In the current study, the nicotine-injected group showed muscles with peripheral-flattened dark pyknotic nuclei and patchy loss of transverse striation. In caffeine-injected rats, muscle tissue showed accumulation of mononuclear cells around some fibers. In the combined group, similar findings were observed in addition to longitudinal splitting of some fibers that also showed dark pyknotic nuclei. Such observation is concomitant with Carmo-Araújo *et al.*[16] who described that muscle cells looked damaged when show signs of necrosis, such as acidophilic cytoplasm, loss of striation, loss of nucleus, and pyknotic nuclear changes. Deformed muscle outlines or degradation by phagocytes is among morphological changes associated with muscle damage. In the present study, the nicotine group showed split muscle fibers and multiple nuclei located in a central location, suggesting tissue repair by satellite cells. This result concomitant with El-Gamal and Ahmed *et al*.,[17] who observed and explained that those centrally

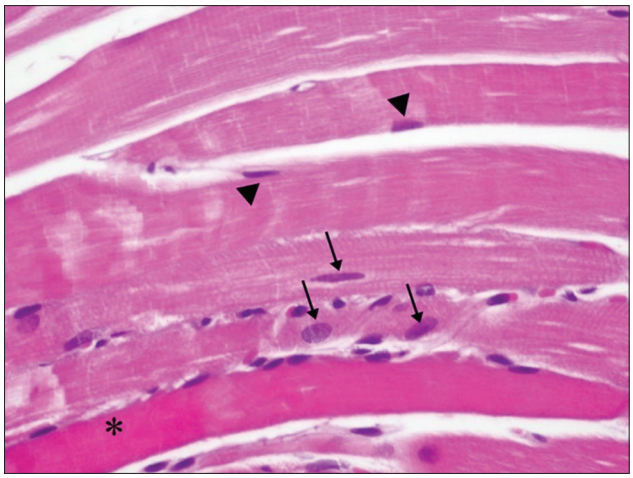

Figure 9: A photomicrograph of a longitudinal section in a rat diaphragm muscle of experimental Group IV (nicotine and caffeine), showing variation in the size and shape of muscle fibers within the same bundle with flat peripheral nuclei (▲). Some fiber appears deep acidophilic cytoplasm which contains many nuclei scattered along its length (*). Notice centrally located nuclei (↑) (H& E x 40)

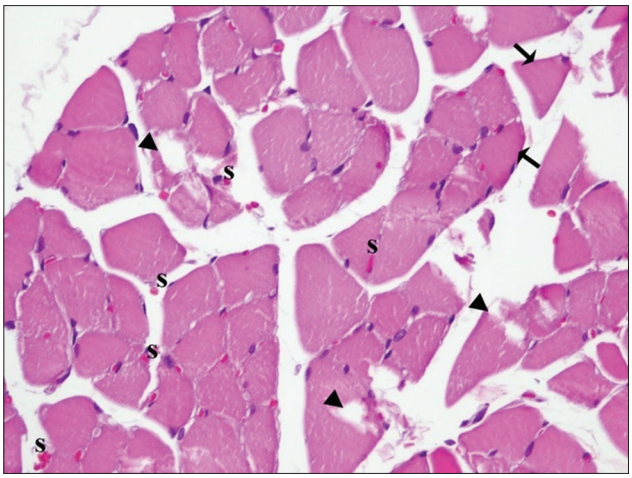

Figure 10: A photomicrograph of a transverse section in a rat diaphragm muscle of experimental Group IV (nicotine and caffeine), showing distorted muscle fibers with loss of their architecture. Some fibers with pale sarcoplasm and some with variations in muscle fiber sizes (▲). Some muscle fibers appear large with homogenous acidophilic sarcoplasm and surrounded by a wide space (↑). Notice congested blood vessels(s) (H& E x 40)

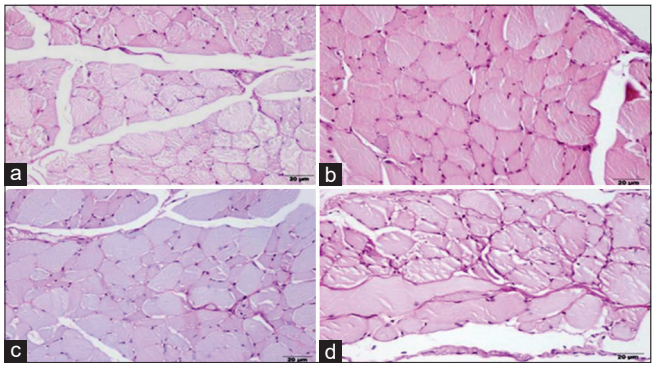

Figure 11: Transverses cut section of diaphragm in (a) Control group showing PAS-positive reaction in most muscle fibers. (b) Nicotine injected, showing a thick basement membrane. (c) Caffeine injected, showing apparent decrease in the periodic acid–Schiff-positive fibers. (d) Combined injected (nicotine and caffeine) group, showing an increase in periodic acid Schiff-positive material around fibers appearing as a thick basement membrane (PAS X40)

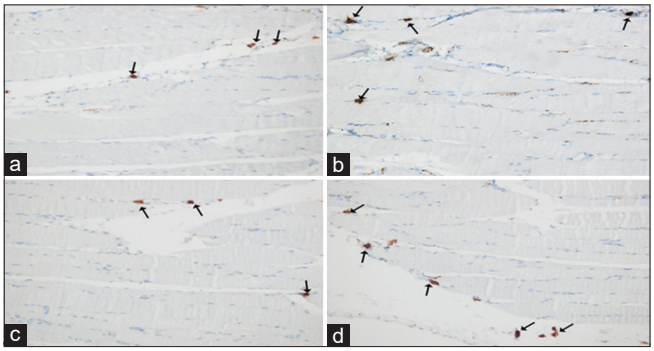

Figure 12: longitudinal section of diaphragm muscle showing in: (a) Control group few cells (↑) with positive CD68 expression. (b) Nicotine-injected group an apparent increase in cells with positive CD68 expression (↑). (c) Caffeine-injected Group III a similar expression of CD68 (↑) to control. (d) Group IV an apparent increase in positive CD68-expressing cells (↑) (IHC for CD68 × 20)

located nuclei are due to tissue repair by satellite cells. This would lead to the formation of new muscle fibers or myoblasts that fuse either to themselves or to the damaged myofibers. In addition, the migration appearance of macrophages at the vicinity of damaged fibers is benefice for removal of cell debris beside playing role in signaling proliferation of fibroblasts resulting in the production of new temporary extracellular matrix,[18] which in the present study was represented by the increase of intermuscular PAS-stained material.

Cellular damages mostly attributed to impaired oxidative metabolism in chronically stressed animals.[19] A special gene can induce mitochondrial metabolism and may result in the reduced serum lactate and creatine kinase activity as well as the relatively low number of degenerated muscle fibers and inflammatory cells observed in trained animals compared with those in the sedentary groups.[20] Some authors have suggested a disordered synthesis of myofibrillar proteins or cytoskeletal disorders responsible for the specific arrangement of myofibrils.[21] On the other hand, da Costa Santos *et al*.[20] mentioned that the levels of caffeine that have been confirmed to outcome in enhanced muscle efficiency *in vivo* were found to have no essential effect *in vitro*, showing that the repercussions seen in personal athletes may be due to the results of attributed caffeine.

Most of the fibers in nicotine-treated Group II showed thickened PAS-positive basement membrane. In caffeine Group III, there was an apparent decrease in PAS-positive fibers. In Group IV, there was an increase in PAS-positive material around fibers as compared to the control group. The mechanism of caffeine action can be explained in view of what was reported by Souissi *et al*.,[15] who mentioned

that caffeine increased muscle glycogen accumulation. The satellite cell involvement in the muscle regeneration is the main contributor in the collagen I and III secretion and the deposition in the epimysium and perimysium,[17] which explained the appearance of PAS-positive stained thickened membrane.[17]

Using immunohistochemical staining in the current study revealed that nicotine-injected Group II showed an apparent increase in positive CD68. After caffeine injection, there was also an apparent similar expression of CD68 compared to control sections. After combined administration of nicotine + caffeine, there was more increase in the positive CD68-expressing cells. Acute caffeine intake could increase the muscle tissue injury, which is approved by signs of the muscle injury in the blood as the lactate dehydrogenase, the creatine kinase and the inflammatory cells spreading among the elite football players after a comprehensive exercise.[22] In contradict to such finding, some studies, have suggested that chronic caffeine intake decreases inflammatory injury and chronic inflammation in the liver and the brain.[21] Caffeine is a well-known competitive antagonist of adenosine receptors. Chronic caffeine intake results in physiological and behavioral adaptations due to change in expression and sensitivity of adenosine receptors.[23,24] Activation of adenosine receptors in neutrophils and macrophages decreases chemotaxis, the generation of reactive oxygen species, and the production of pro-inflammatory cytokines.[25,26] These effects may protect muscle cells from oxidative stress and inflammatory damage. Chronic intake of caffeine in rats results in physiological and behavioral adaptations due to altered expression and sensitivity of adenosine receptors.[23,24] This explained that caffeine has a weak-opposing effect with nicotine.

Conclusions

The present study proved that both caffeine and nicotine have many negative impacts on the diaphragm as an example of the active skeletal muscles eliciting degenerative changes in its fibers.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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