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EFFECT OF BETAMETHASONE ADMINISTRATION ON SKELETAL MUSCLES OF PREGNANT RATS AND THE AMELIORATIVE ROLE OF CURCUMIN

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ABSTRACT

Using the skeletal muscle as a target organ, the present study involved two integrated aspects. The first aspect has dealt with investigating the possible toxic side effects of maternal exposure to one of the glucocorticoids, namely, betamethasone for ten days i.e. from the 6th to the 15th day of gestation in female Wister rats. However, the second aspect examined the possible ameliorative role of curcumin against the toxicity induced by betamethasone administration. Three integrated approaches i.e. histological, histo-morphometric, and molecular were utilized. The drug group exhibited evident histological changes compared with the control group in the form of wide splitting of the skeletal muscle fibers in association with fibrillolysis and nuclear internalization. Furthermore, muscle fibers showed irregular variation in size with rounding of some of them. At the molecular level, SDS-PAGE for protein in the muscles exhibited a marked lose in most protein bands as compared with the control group. Administration of curcumin after betamethasone caused an evident amelioration at the three utilized approaches.

KEYWORDS: Betamethasone; Skeletal muscle; Curcumin; Pregnancy; Histology, Histo-morphometric; Molecular; toxicity.

INTRODUCTION

Muscle atrophy is associated with an increase in circulating glucocorticoid (GC) levels, suggesting that GCs might have a role in this concern (Hattori *et al.*, 2013). The fluorinated steroids such as betamethasone, dexamethasone and the like seem more likely to produce myopathy (Lee *et al.*, 2005). Indeed, administration of high doses of these GCs induces muscular atrophy in both animals and humans (Shin *et al.*, 2000; Viguerie *et al.*, 2012). Other studies have suggested that GCs inhibit protein synthesis and stimulate protein degradation in skeletal muscle (Pereira and Freire de Carvalho, 2011). An effect of GCs on the breakdown of specific skeletal muscle proteins has been described in some studies (Schakman *et al.*, 2008). In addition, typical findings of steroid myopathy are selective atrophy of type II muscle fibers and necrotic changes. Furthermore, it has been suggested that GC-induced mitochondrial damage which in turn can lead to muscle fiber necrosis (Kelly *et al.*, 1986; Polsonetti *et al.*, 2002). In a relatively recent review, it has been demonstrated that glucocorticoids, either exogenous or endogenous, produce oxidative stress in multiple tissue, including bone and muscle (Klein, 2015). However, it has been reported that glucocorticoids can exert not only negative but also positive effects on the cardiac muscle as for example, they improve contractile performance of the heart and inhibits cardiomyocyte apoptosis triggered by ischemia, cytokines, and cardiotoxic drugs (Kuropka, et al., 2017). It has been also reported that curcumin administration enhances mitochondrial biogenesis in cardiac muscles (Sahebkar et al., 2017).

The impairment of muscle function caused by damage and subsequent inflammatory responses could reduce the ability to perform body activities. Thus, it is important to prevent or at least minimize muscle damage by attenuating inflammatory responses. One of many measures for this purpose could be an oral intake of natural anti-inflammatory substances (Connolly et al., 2003; Rondanelli et al., 2016). It has been recently reported that oral curcumin ingestion suppresses oxidative stress, reducing hydrogen peroxide in skeletal muscle following downhill running-induced muscle damage in mice (Kawanishi et al., 2013; Tanabe et al., 2015). Moreover, Thaloor et al. (1999) investigated the kinetic and extent of muscle regeneration in vivo after trauma following systematic administration of curcumin in mice. Curcumin acted directly on cultured muscle precursor cells to stimulate both cell proliferation and differentiation. The striking effects of curcumin on myogenesis suggested therapeutic application for treating muscle injuries. This study aimed to examine the possible hazard effect of betamethasone administration for ten days during gestation upon skeletal muscles of albino rats. Furthermore, owing to its characters, curcumin has been used to ameliorate this adverse effect.

MATERIALS AND METHODS

Animals and grouping

All the experiments were done in compliance with the guide for the care and use of laboratory animals approved by Faculty of Science, Menoufiya University, Egypt. Healthy mature virgin females and fertile males of Wistar albino rats (*Rattus norvegicus*), weighing 135 ± 15g and aged 17±1 weeks, were obtained from Hellwan Animal Breeding Farm, Ministry of Health, Cairo, Egypt. They were kept in the laboratory for at least one week before initiation of the experiments for acclimatization. They were housed in specially designed plastic rodent cages and maintained at 25 ± 2ºC in 12h light: 12h dark cycle. Free access of water and standard diet composed of 50% ground, barely, 20% ground yellow maize, 20% milk and 10% vegetables were supplied. A total of 60 rats were used for the present study.

The female rats were divided equally (15 in each group) into four groups as follows:

1- Control group, administrated distilled water.
2- Curcumin administrated group given oral injection of curcumin (15.75 mg/kg).
3- Experimental betamethasone group given subcutaneous injection of betamethasone (0.3 mg/kg).
4- Betamethasone injected and curcumin administrated group, received subcutaneous injection of betamethasone first followed by oral injection of curcumin one hour later.

Betamethasone administration

Betasone tablets were manufactured in the Memphis company for pharmaceutical and chemical industries, Cairo, Egypt and purchased from a local pharmacy in Shebeen El-Koom, Menoufiya, ground and dissolved in distilled water, subcutaneously administrated daily by insulin syringe for ten days during the organogenesis phase of gestation i.e. starting from the 6^{th} day and ending at the 15^{th} day of gestation. The applied dose was 0.3 mg /kg body weight which is equivalent to the human dose (McDonald *et al.*, 2003)

Water extraction of curcumin

Dry turmeric rhizomes of the plant *Curcuma longa* were purchased from a local market at Shebeen El-Koom, Menoufiya, Egypt. One-kilogram fresh *Curcuma longa* were crushed into powder, macerated in distilled water, filtered and orally given daily at a dose of 15.75 mg/kg body weight (Hashem *et al*., 2011).

Investigated parameters

A- Body weight

The weight (g) of female rats of control and experimental groups was recorded daily during the time span of the experiment.

B- Histological investigation

At the time of experiment termination, the pregnant rats were anaesthetized using ether, sacrificed and skeletal muscles were removed from the thigh region. For light microscopical examination, skeletal muscles from the control and experimental groups were taken and fixed by immersion in 10% neutral formalin for 24 hours at room temperature and washed under running tap water for 12 hours. The specimens were dehydrated in an ascending series of alcohol, cleared in butanol and embedded on molten paraffin.

Sections of 5µm thickness were cut using a rotatory microtome (Leica, Model Rm 2125, Germany) and mounted on albumin-coated slides. Staining was performed with Ehrlich's hematoxylin and counter-stained with aqueous eosin. Histological sections were subjected to microscopical examination and when necessarily photographing using Olympus microscope.

C- Histo-morphometric parameters

- Estimation of skeletal muscle fibers

An estimation of the number of maternal skeletal muscle fibers was performed by counting the number of fibers in histological sections under X10 magnification (n=15 rats per group).

D- Sodium Dodecylsulfate (PolyAcrylamide Gel Electrophoresis) SDS-PAGE

SDS (Sodium dodecylsulfate)-PAGE of denatured proteins of the skeletal muscle was carried out in 15% polycarylamide gels pH8.8, in a discontinous buffer according to Maziel and Jr, (1971). Photograph of the gel was taken using Sony digital camera.

E- Determination of DNA fragmentation

As a measure of apoptotic DNA fragmentation, the presence of DNA ladder was determined according to Woldek *et al*., (1991). Extraction of DNA was done according to the method of Aljanabi and Martinez, (1997). The DNA was visualized and photographed with illumination under ultraviolet (uv) light using a photo documentation hood (Fisher Scientific, Pittsburg PA, USA) equipped with a polaroid 667 film with an orange filter (Kodak, Rochester, NY, USA). The UV reacts with the ethidium bromide to show the DNA fragments. Apoptotic bands appeared and located at 200 bp and its multiples.

Data evaluation and statistical analysis

All data sets were expressed as mean ± standard error of the mean (SEM). The data were analyzed statistically for normal distribution (student't test) and homogeneity of variances (Levene test) using statistical package of social sciences (SPSS) software for windows, version 11. Differences were considered insignificant whenever $P>0.05$. The significances of the obtained data were classified into three categories, *i.e.* $P<0.0001$, $P<0.001$ and $P<0.05$ according to P values.

RESULTS

A- Body weight

Fig. (1), summarize the changes in body weights of both control and experimental groups. Pregnant rats which received curcumin exhibited a gradually progressive increase in body weights similar to that of the control group. There was an evident and gradual reduction in body weights of betamethasone group till the end of injection at the 15th day of gestation then the weight reduced slowly until the 18th day after which the body weight gradually increased in lowest values until the 20th day (-1.66 ± 0.556; -10.5 ± 0.50; -12.83 ± 0.30; -9.33 ± 0.84; -3.83 ± 0.31; 3 ± 0.45; 11.5 ± 0.22). Pregnant rats of betamethasone and curcumin group exhibited a gradual increase in the body weights but in low values as compared with the control group.

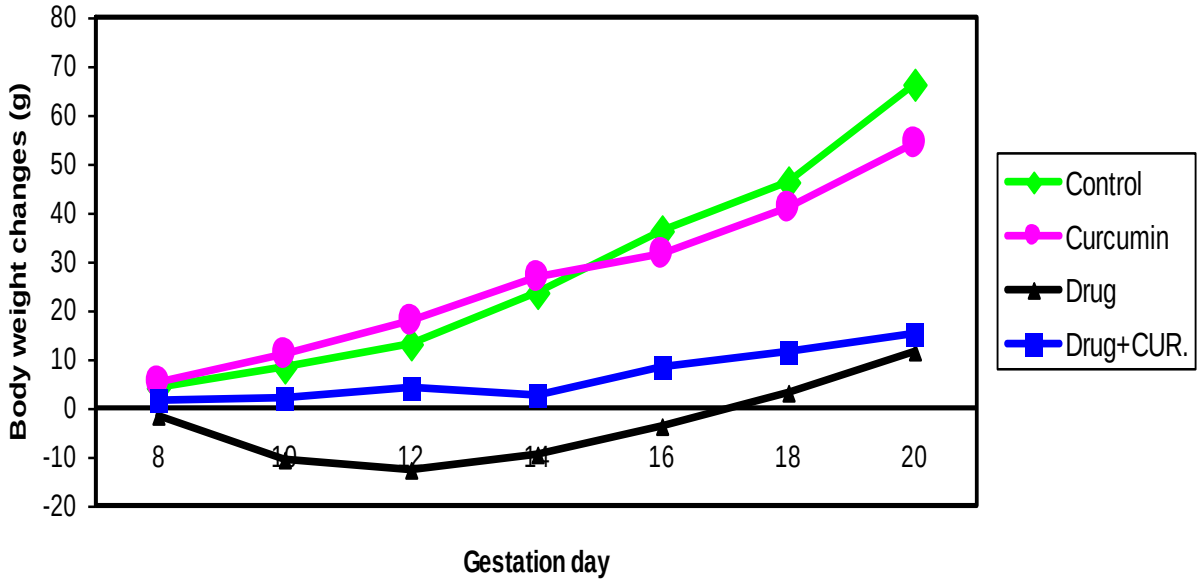

Figure 1: Graph showing changes in the body weight in different groups.

B- Histological observation

Control group

Light microscopic examination of the histological sections of control group displayed normal structure of skeletal muscle. It was formed of bundles of muscle fibers separated by connective tissue (perimysium). The fibers were connected together by connective tissue (endomysium). Some blood vessels were seen in the connective tissue partitions of the muscle. In cross section, skeletal muscle fibers appeared polygonal (Fig. 2A). However, in longitudinal section, the skeletal muscle fibers appeared parallel, long, cylindrical and multinucleated with minimal variation in fiber size. The sarcoplasm of the muscle fibers appeared acidophilic and crossly striated. The nuclei were elongated and peripheral in position under the sarcolemma (Fig. 2B).

Curcumin group

Skeletal muscles of the curcumin group exhibited histological picture nearly similar to its counterpart of the control group. In the transverse section, the muscle fibers appeared polyhedral with flattening of adjacent cells and peripheral location of nuclei (Fig. 2C). The longitudinal section showed elongated muscle fibers with numerous flattened peripheral nuclei (Fig. 2D).

Betamethasone group

Skeletal muscles of the betamethasone group showed evident histological changes in the form of wide splitting of the skeletal muscle fibers in association with fibrillolysis and nuclear internalization. Muscle fibers showed irregular variation in size with rounding of some of them. Nuclei were internal in position rather than peripheral and some appeared rounded in shape rather than oval. Blood vessels were dilated and congested (Fig. 2E&F). Mononuclear cellular infiltration was observed (Fig. F). Splitting of some fibers was also observed which appeared as a transverse invagination or complete separation. In addition, few muscle fibers appeared atrophied. Some muscle fibers were completely degenerated and replaced by debris of the damaged tissue. In longitudinal sections, the degeneration was segmental, involving only part of the fiber with loss of striations in the affected regions (Fig. 2G).

Betamethasone + curcumin group

Skeletal muscles of betamethasone and curcumin group displayed an evident ameliorative effect i.e., the muscle fibers appeared more or less similar to that of the control group. The sections displayed mild focal histological changes. Some muscle fibers showed variations in size (Fig. 2H). Splitting of some fibers were also observed (Fig. 2I).

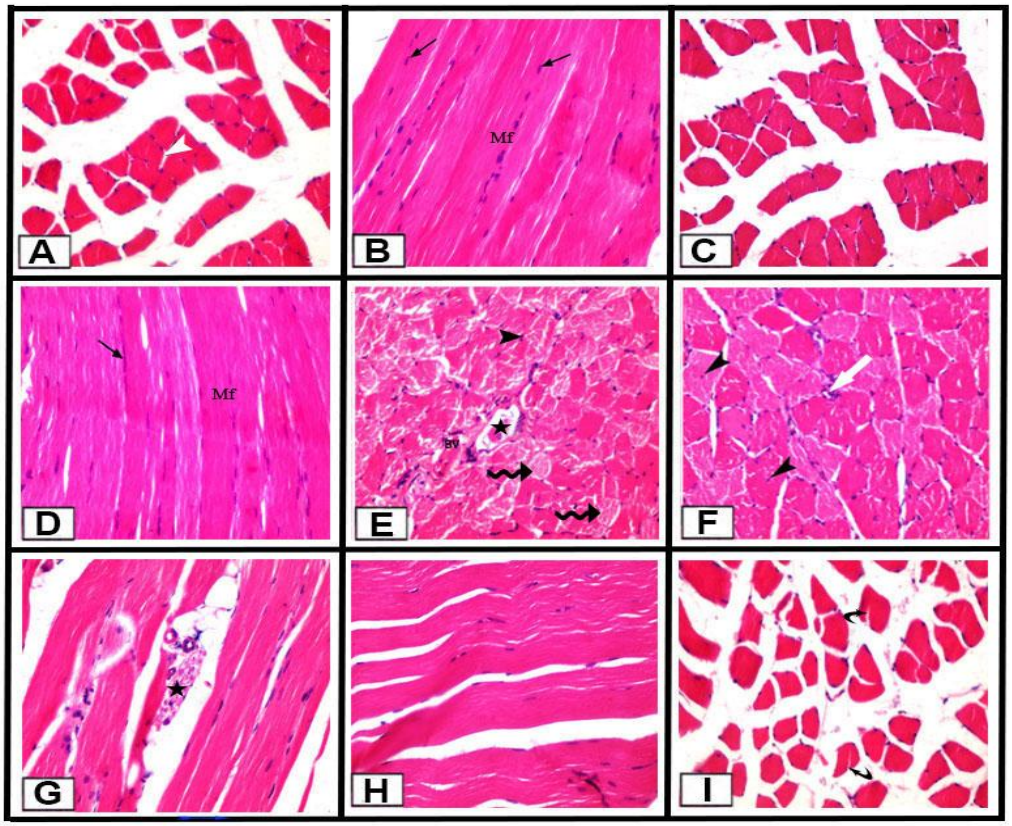

Figure 2: Photomicrographs of sections in the skeletal muscle showing: (A) control, transverse section. (B) control, longitudinal section. (C) curcumin, transverse section. (D) curcumin, longitudinal section. (E&F) betamethasone, transverse sections. (G) betamethasone, longitudinal section. (H) betamethasone + curcumin, longitudinal section. (I) betamethasone + curcumin, transverse section. Muscle fiber (Mf), blood vessel (Bv), endomysium (white arrow head), peripheral nuclei (black arrows), rounded myonuclei (black arrow head), degenerated muscle fiber (star), rounded muscle fiber (wavy arrow), macrophage infiltration (white arrow), splitting of some muscles fibers (curved arrow).

C- Histo-morphometric observation

Number of muscle fibers

Fig. (3) illustrate the changes in the number of muscle fibers in different groups. No significant differences were observed in the number of muscle fibers between the control and curcumin groups. On the other hand, there was a highly significant reduction in the maternal muscle fibers injected with betamethasone. Administration of curcumin after betamethasone led to a marked amelioration in the number of the muscle fibers as compared with betamethasone group and a low significant increase compared with control (7.5 ± 0.223; 6.33 ± 0.210; 8.5 ± 0.223 for betamethasone + curcumin, betamethasone and control groups respectively).

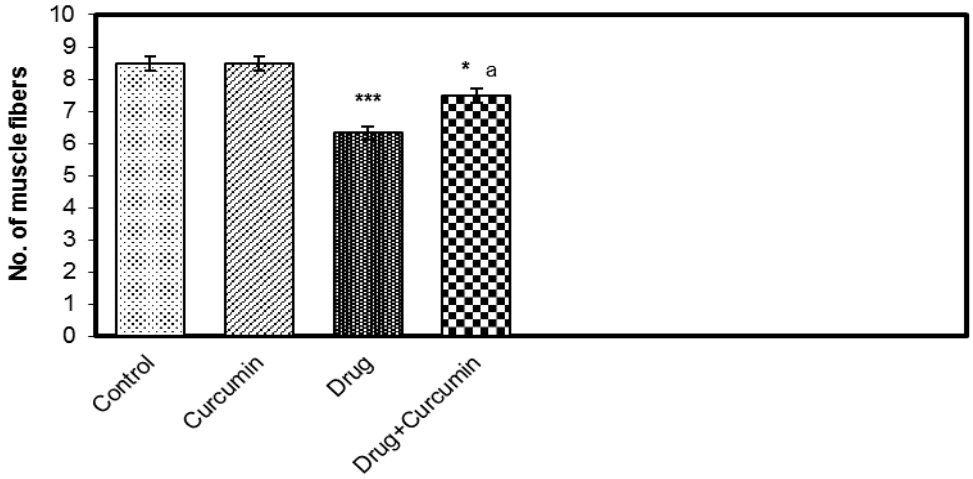

Figure (3): Graph showing changes in the number of muscle fibers of control and experimental groups.

D-Total protein (SDS- PAGE) analysis

Total protein isolation and identification for skeletal muscles are illustrated in Figure (4). After fractionation of skeletal muscles protein by polyacrylamide gel electrophoresis no differences in pattern were observed between curcumin and control groups. However, severe decrease in the number of expressed protein bands were noted in betamethasone group which lacked expression of all protein bands except at 10 KDa. Administration of curcumin after betamethasone caused an evident increase in the skeletal muscles protein bands as compared with betamethasone group.

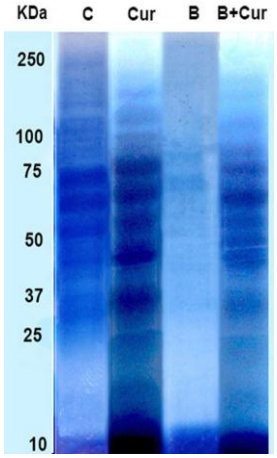

Figure 4: Changes in protein banding patterns of skeletal muscles of different groups (control (C), curcumin (Cur), betamethasone (B) and betamethasone + curcumin (B + Cur)) using SDS-PAGE.

E- DNA fragmentation

Figure (5) shows that the administration of curcumin during gestation period had no effect on DNA of muscle fibers compared with the control group. The muscle fibers of the control and curcumin injected mothers possessed intact DNA without any fragmentation. Highest incidence of genomic DNA fragmentation was markedly increased in muscle fibers of betamethasone injected group. On the other hand, the maternal administration of curcumin after betamethasone exhibited less genomic DNA fragmentation compared with betamethasone group.

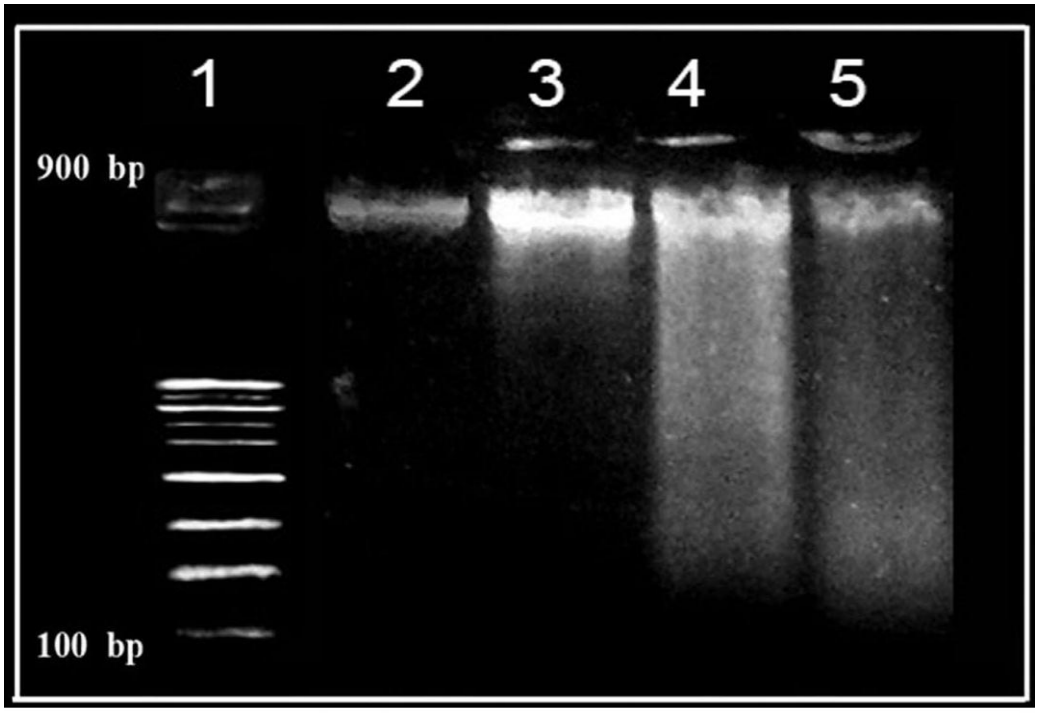

Figure (5): Agarose gels showing evident variations of DNA fragmentation in extracts from skeletal muscles, marker (Lane 1), control (Lane 2), curcumin (Lane 3), betamethasone (Lane 4) and betamethasone + curcumin (Lane 5).

DISCUSSION

As observed in the present study, betamethasone was found to cause a gradual reduction in maternal body weights during the period of injection which lasted for ten days. This is in agreement with previous findings in rats injected with different GCs during late pregnancy by injection, ingestion, and continuous infusion (Swolin-Eide *et al*. 2002; Scheepens *et al*., 2003; O'Regan *et al*., 2004; Woods, 2006). Rotschild *et al*. (1997) showed that intramuscular injections of doses of 0.6 mg/kg bw/day triamcinolone acetonide in the Sprague-Dawley rats on gestational day 12-14 reduced maternal weight gain. The reduced maternal weight gain during betamethasone treatment in the current study was correlated not only to the mother weight but also to the fetuses' weight (Badawy et al., 2018).

Long-term administration of GCs in rats has been reported to induce steroid myopathy (Lee *et al*., 2005). Although any of the commonly available GC preparations can cause myopathy, the fluorinated steroids, e.g., triamcinolone, betamethasone and dexamethasone seem more likely to produce muscle weakness (Lee *et al*., 2001). It has been suggested that GC-induced cellular changes can lead to cell death (Lee *et al*., 2005). The present study showed that betamethasone injection for 10 days during gestation induced myopathy expressed as degenerative changes of maternal skeletal muscles. The histological changes were in the form of wide splitting of the skeletal muscle fibers in association with fibrillolysis and nuclear internalization. Blood vessels were dilated and congested. Some muscle fibers were completely degenerated and replaced by debris of the damaged tissue. Mononuclear cellular infiltration was observed. On the other hand, there was a highly significant reduction in the maternal muscle fibers of betamethasone group. This is in agreement with Lee *et al*. (2001) who showed that steroid-induced myopathies in rats were developed by daily intraperitoneal injections of triamcinolone acetonide for 9 days. Variation in fiber size and shape was seen in the of triamcinolone acetonide - injected soleus muscles. Atrophic fibers were noted occasionally and some fibers had pale-staining cytoplasm. Necrotic fibers invaded by numerous macrophages were seen. Another study by Steiss *et al*. (1989) demonstrated that dexamethasone injection to rats during 7 days can induce skeletal muscles degeneration. The connective tissue elements between muscle cells increased and some destructive myonuclei were also observed in inter-cellular spaces. Degenerated and disorganized muscle fibers were also observed. Moreover, Griffin *et al*. (1992) explained pathological features include diffuse muscle weakness, generalized fiber atrophy or focal/diffuse necrosis after short-term high-dose steroid administration. Similarly, Kaasik *et al*. (2012) reported that administration of dexamethasone to male Wistar rats caused myopathy where the muscle fibers are thinner and have wide distances between myofibrils. Also, Rezaie *et al*. (2005) showed that after dexamethasone injection the myocyte cytoplasm became more condensed. In some of the myocytes, degenerative changes were found including small foci of myofibrolysis. Komamura *et al*. (2003) showed that high levels of corticosteroids are frequently associated with loss of muscle mass. In rabbits injected with certain glucocorticoids from days 1 to 9 after birth, the skeletal muscles had decreased fiber diameters and satellite cell degeneration (Jirmanova *et al*., 1982). Shah *et al*. (2000) demonstrated that dexamethasone dose- and time-dependently induces loss of body and muscle weights.

In skeletal muscle, GCs decrease the rate of protein synthesis and increase the rate of protein breakdown (Lofberg *et al*., 2002) contributing to atrophy. The inhibitory effect on protein synthesis results from different mechanisms. First, GCs inhibit the transport of amino acids into the muscle, which could limit the protein synthesis. Secondly, GCs inhibit the stimulatory action of insulin, insulin-like growth factor-I (IGF-I), and amino acids (in particular leucine), on the phosphorylation of eIF4Ebinding protein 1 (4E-BP1) and the ribosomal protein S6 kinase 1 (S6K1), two factors that play a key role in the protein synthesis machinery by controlling the initiation step of mRNA translation (Liu *et al*., 2004). Finally, there is also evidence that GCs cause muscle atrophy by inhibiting myogenesis through the down regulation of myogenin, a transcription factor mandatory for differentiation of satellite cells into muscle fibers (Carballo-Jane *et al*., 2004). Moreover, GCs has been reported to increase the cytoplasmic protease activity in muscles, leading to myofibrillar destruction (Shah *et al*., 2000).

The results of the present study demonstrate that betamethasone is capable of inducing extensive adverse effect on DNA and total protein in the tissues under investigation. The cells of betamethasone injected mothers showed extensive DNA fragmentation compared with control group. However, SDS-PAGE for protein of the tissues in betamethasone injected group exhibited a marked loses in most protein bands compared with the control group. Similar to the outcome of the present results, an earlier study by Lofberg *et al*., 2002 revealed that GCs decrease the rate of protein synthesis and increase the rate of protein breakdown in skeletal muscle.

The present study assessed whether the toxic effects caused by betamethasone administered can be ameliorated by treatment with curcumin. It also ascertained that, there is no significant difference between control and curcumin extract groups. These results are in agreement with observations of previous workers who reported that curcumin is safe and well tolerated (Devasena *et al*., 2002). Indeed, the present study showed that curcumin caused marked improvements against the adverse effects caused after betamethasone administration and this is in agreement with our previous studies (Badawy et al., 2016 & 2017).

The histological results under consideration revealed that the muscles of the combined-treatment group showed an evident ameliorative outcome with the exception that some fibers showed splitting and variations in size. Also, the Histomorphometric parameters showed that administration of curcumin after betamethasone led to a marked amelioration in the number of muscle fibers as compared with betamethasone group.

Earlier study by Thaloor et al. (1999) showed a beneficial effect of curcumin on muscle regeneration after trauma that was linked to its anti-inflammatory activity. Another study by Mark Davis *et al*. (2007) explained a benefit of curcumin on whole body exercise performance following exercise-induced muscle damage. Soebadi and Pawana (2008) found that the addition of oral curcumin significantly reduces atrophy of soleus muscle in rats immobilized for 2 weeks.

Curcumin, has been reported to alleviate the symptoms of muscular dystrophy in mdx mice (Pan *et al*., 2008) and decrease the expression of inflammatory mediators involved in muscle injury (Pinniger *et al*., 2012). Therefore, curcumin could provide a new avenue for the treatment of muscle disorders (Pan *et al*., 2008; Pinniger *et al*., 2012). Moreover, Avci *et al*. (2012) demonstrated that curcumin had protective effects against skeletal muscle ischemia-reperfusion injury. Also, Sumbilla *et al*. (2002) showed that curcumin has desirable effects on muscle damage and regeneration.

A recent study, by Kawanishi *et al*. (2013) has clarified properties of curcumin after downhill running-induced muscle damage in mice. They were able to underline how curcumin has an anti-oxidant effect in mice following downhill running –induced muscle damage. In addition, Tanabe *et al*. (2015) suggested that curcumin intake has some beneficial effects on recovery of eccentric exercise-induced muscle damage.

According to the present study, curcumin had ameliorative effects on DNA fragmentation and total protein in the maternal skeletal muscle. A study by Li *et al*., (2003) found that green tea, curcumin, grape stone and resveratrol caused protective potential effects on DNA against nitrobenzene-induced DNA adductions. Cheng *et al*. (2003) investigated the inhibitory effects of curcumin, garlic squeeze, grape seed extract, tea polyphenols, vitamin C and vitamin E on nicotine-DNA adduction *in vivo*. They suggested that these dietary constituents are beneficial to prevent the harmful adduct formation and thus to block the potential carcinogenesis induced by nicotine.

Siddique *et al*. (2010) declared that, curcumin inhibits the generation of ROS that are responsible for DNA damage. This action of curcumin was explained by Piwocka *et al*. (2001) who stated that curcumin leads to attenuated DNA fragmentation due to the elevation of GSH. More recently, it has also been reported that curcumin can be beneficial in malnutrition cases (Ahmed-Farid et al., 2017).

Similar to our results, Sarvalkar *et al*. (2014) demonstrated that the electrophoretic alterations in protein content in mice was significantly reduced during aging, after curcumin treatment in protective and curative groups, it was again increased significantly. Thus, curcumin is able to ameliorate the stress induced changes in protein profile during aging. Ameliorative effect of curcumin in the investigated animals, so far, might be due to its antioxidative property. Many other investigators have also reported a decrease in protein content in skeletal muscle, heart, liver and kidney of aflatoxin-fed animals (Jha *et al*., 2012).

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