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ORIGINAL ARTICLE

Biochemical Study on the Role of Muscle-Specific Ubiquitin Ligase Atrogin-1 in Dexamethasone-Induced Muscle Atrophy in Rats

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ABSTRACT

Background: Skeletal muscle atrophy is defined as a loss of muscle mass resulting from an imbalance between protein breakdown and synthesis, which can be triggered by conditions such as starvation, aging, and other factors like immobilization and nerve damage. Characterized by lower muscle fiber cross-sectional area, reduced protein content, and diminished muscle strength, along with heightened fatigability. The primary objective of this research was to investigate the function of muscle-specific ubiquitin ligase atrogin-1 in dexamethasone-induced muscle atrophy in an animal model.

Methods: The study was carried out on 30 male albino rats, divided randomly into two identical groups, each consist of 15 rats, as follows: Group 1 (the control group), and Group 2 (the group treated with dexamethasone). The animal's body weight and muscle strength were evaluated. Serum creatine kinase concentration, Muscle tissue reduced glutathione and nitric oxide levels were measured using colorimetric assay. Atrogin 1 in muscle tissue was detected by ELISA. Histopathological examination and immunohistochemistry of caspase -3.

Results: The results of dexamethasone treated group when compared with control group revealed significant reduction of body weight, muscle strength, tissue nitric oxide level, in addition to histopathological changes indicative of muscle atrophy, while there was significant increase in tissue atrogin1and serum creatinine kinase level and immunoreactivity of caspase-3.

Conclusions: In view of the assessed data, it can be concluded that dexamethasone induced muscle atrophy in experimental rats was evidenced by modulation in the biochemical indices and muscle histopathological changes.

Keywords: Muscle Atrophy; Dexamethasone; Atrogin1

INTRODUCTION

Skeletal muscles constitute the main protein reservoirs in the body, which comprise approximately 40% of total body weight, are essential for energy metabolism and physical exercise. This system is accountable for physical functions, encompassing movement, postural support, and daily living activities. Furthermore, skeletal muscles are also responsible for serving as the primary site for energy metabolism and play a role in the absorption, use, and storage of energy metabolism substrates like glucose, lipids, and amino acids [1]. Muscle waste can result in weakness, which in turn limits activity, impairs an individual's quality of life, and increases the risk of falls and fractures. Advanced stages of muscle atrophy result in severe disability and the loss of one's ability to live independently [2].

Muscle wasting is characterized by enhanced expression of the ubiquitin ligases atrogin-1 and MuRF1, leading to increased ubiquitin-proteasome-dependent protein degradation [3]. Atrogin-1 and MuRF1 play a key role in regulating muscle mass in numerous catabolic situations. Their activity is specifically related to the

ubiquitination and proteasome-mediated degradation of proteins [4].

Muscle atrophy is linked to oxidative stress, although research suggests that ROS may be both the cause and effect of muscle waste [5]. Glucocorticoids are commonly used in treating inflammatory conditions [6]. The synthetic glucocorticoid dexamethasone causes muscle atrophy by promoting protein breakdown and inhibiting overall protein production.

Consequently, dexamethasone-induced muscle atrophy in animal models can been used to understand the mechanism underlying muscle atrophy [7]. This study aimed to determine the role of muscle -specific ubiquitin ligase atrogin-1 in dexamethasone-induced muscle atrophy.

METHODS

Chemicals

Dexamethasone (as sodium phosphate): Ampoules (8mg/2ml), were purchased from AMRIYA Pharmaceuticals Industries Egypt

Animals

This study involved 30 male albino rats weighing approximately 120-150 grams and aged between 6-8 weeks, sourced from the experimental animal facility at Tanta University. Throughout the study, animals were kept in wire mesh cages, received a standard chow diet, and had unrestricted access to water. The specimens were maintained under a consistent environmental setup with a temperature range of 23°C ± 2 and a 12-hour dark to 12-hour light cycle.

This research was conducted at the Medical Biochemistry Department within the Faculty of Medicine at Tanta University in Egypt, according to Ethical Committee of Medical Research as (approval code 33063/04/19).

Experimental design

Rats were divided into 2 groups (15 for each) as follows.

The control group, designated as Group 1, received subcutaneous injections of saline daily for a period of 2 weeks.

(Dexamethazone treated group), designated as Group 2, was administered dexamethasone via subcutaneous injection at a dosage of 2mg per kilogram per day for the duration of two weeks.

Body weight

The body weights of the rats were documented both at the start and the end of the study. The percentage of body weight change was determined using the following formula:

$$\textit{Body weight change \%} = \frac{\text{Final BW} - \text{Initial BW Body weight}}{\text{Initial BW}} \times 100$$

Muscle strength Assessment

Wire hanging test

Muscle strength was evaluated on day 13 through wire hanging which was conducted as follows.

A 55 cm wide and 2 mm thick metallic wire was fixed between two vertical stands, and it was kept 40 cm above a layer of bedding material to avoid injury to the animal in the event of it falling. The wire was securely fastened to the frame to prevent movement or unwanted shifting of the wire when dealing with the animals or during the measurement process, as these unwanted consequences would disrupt the animal's performance. Animals were initially grasped by the forelimb, though it was permissible to hold them using all four limbs, then timing began after the hind limb was released and continued until the animal either fell or held the wire for a maximum of 120 seconds. Each animal was permitted to attempt the task three times, with a five-minute interval between trials.

Biological specimen collection: Blood and muscle tissue sampling

In the last part of the study, all animals were sacrificed via cervical decapitation. Serum was separated using dry clean Pasteur pipettes in Eppendorf tubes, and the samples were labelled and stored at -20 °C until analysis. Gastrocnemius muscles were dissected meticulously, rinsed with ice-cold saline, and placed on ash-free filter paper. The muscles were placed on ice and one portion was preserved in a 10% formalin solution for histopathological examination. The opposite side was covered in aluminium foil and held at a temperature of minus 80 degrees Celsius until it was used.

Preparation of muscle tissue homogenate

Muscle tissue samples were processed by a Potter-Elvenhjem tissue homogenizer, undergoing 20-30 up and down strokes, in a 50 mM cold potassium phosphate buffer solution at pH 7.4, at a concentration of 1 part tissue to 5 parts buffers (w/v). The crude homogenate was centrifuged at 4000 revolutions per minute at 4 degrees Celsius for 20 minutes. The supernatant was isolated and preserved at minus 80 degrees Celsius until further use.

Biochemical analysis.

Determination of serum creatinine kinase (CK) activity levels

Colorimetric analysis was conducted using a commercial kit provided by the Bio diagnostics company based in Cairo, Egypt.

Assessment of muscle nitric oxide levels and reduced glutathione levels

Colorimetric analysis was performed using a commercial kit obtained from Bio Diagnostics Company in Egypt.

Measurement of skeletal muscle tissue atrogin-1

The concentration of Atrogin 1 in muscle tissue homogenate was quantified via an enzyme-linked immune-sorbent assay (ELISA) using the ELISA kit with the catalogue number 201-11-1762 provided by Sun Red Biotechnology Co., Shanghai, China. This is a solid phase sandwich technique where atrogin 1 either in the standard or in the tissue homogenate samples is sandwiched between two antibodies, the first antibody is a monoclonal antibody FBXO32 which is coated on the wall of a microplate wells, and the second antibody is a polyclonal antibody that is labeled with biotin combined with Streptavidin-HRP to form immune complex. The antigen-antibody complex is determined by using tetramethylbenzidine (TMB) substrate solution, which develops a blue color after addition of the chromogenic solutions. After addition of the stop solution, the color is changed from blue to yellow color and this color change was measured at a wavelength of 450 nm.

Microscopic tissue examination

Muscle tissues that had been preserved in formalin were prepared and sections of five-µm thickness were cut, treated with a haematoxylin and eosin stain, and x200 magnification photographs were taken.

Caspases-3 immunohistochemical expression

Assessment of caspase 3 expression in the gastrocnemius muscle by immunohistochemistry was performed with Rabbit Anti-Human caspase 3 Monoclonal Antibody (Clone EP36) sourced from MASTER DIAGNOSTICA in Granada. The slides were examined under magnification provided by a light microscope.

Statistical Analysis

The statistical presentation and analysis of the current study's outcomes were undertaken, and the data were displayed as mean ± standard deviation (SD). SPSS V20 software was used to conduct Student t-tests, paired t-tests, and calculate linear correlation coefficients. Values of less than 0.05 were deemed statistically significant.

RESULTS

The body weights of the rats in grams used in this study showing a percent increase of almost 29.43±6.25% in control group, while rats of group II (Dexamethasone treated group)showed percent decrease of almost -27.02±4.92%, which was statistically significantly lower than that of the control group .there was a statistically significant decrease in both Hang time (SC) and Holding impulse (S*G) in dexamethasone treated group when compared to the control group. (Table 1)

Oxidative stress parameters including nitric oxide (µmol/ mg protein) and reduced glutathione (GSH) (mg/mg protein) level in gastrocnemius muscle tissue in different studied groups showed that there was a statistically significant decrease in nitric oxide level (µmol/ mg protein) in dexamethasone treated group when compared to the control group, while Reduced glutathione was statistically insignificant. There was statistically significant increase in serum creatine kinase (CK) activity in dexamethasone treated group when compared to control group (Table 2).

In Atrogin1 levels there was statistically significant increase in the dexamethasone treated group when compared to the control group (t=8.875p<0.001). (Figure 1)

In the histopathology examination of muscle tissue from rats in group II, which received dexamethasone treatment, showed significant catabolic muscle atrophy. This atrophy was characterized by muscle fiber diminishment, micro vacuolation, and focal fibrosis within the muscle bundles. Furthermore, the mean diameter of the muscle fibers was found to be substantially reduced as a result of the dexamethasone treatment. The study revealed an increase in the proportion of muscle bundles occupied by collagen fibers (Figure 2).

Immunohistochemistry, there was significant increase in caspase-3 immunoreactivity in immunostained muscle sections of dexamethasone treated group which appeared as dense brown cytoplasmic coloration and increased color intensity of caspase-3. (Figure 3)

Table 1: Comparison between body weight changes and Muscle strength assessment tests) changes in different studied groups

		Group I (n=15)			Group II (n=15)		
Initial body weight (gm)	**Range**	158	–	200	156	–	195
	Mean ± SD	173.53	±	13.96	174.27	±	12.96
	t. test	0.149					
	p. value	0.883					
Final body weight (gm)	**Range**	195	–	259	111	–	155
	Mean ± SD	224.27	±	16.51	127.20	±	13.13

		Group I (n=15)			Group II (n=15)		
	t. test	17.819					
	p. value	0.001*					
Percent weight change%	Range	18.18	–	38.36	-34.71	–	-19.69
	Mean ± SD	29.43	±	6.15	-27.02	±	4.92
	t. test	27.762					
	p. value	0.001*					
Hang time (SC)	Range	59.5	–	69.98	19.03	–	37.56
	Mean ± SD	56.75	±	15.08	27.22	±	5.87
	t. test	7.070					
	p. value	0.001*					
Holding impulse (S*G)	Range	8587	–	12920	2050.4	–	3845.3
	Mean ± SD	10934.13	±	1513.44	3048.23	±	606.28
	t. test	18.733					
	p. value	0.001*					

*Group I: Control group. * Group II: Dexamethasone treated group.

Table 2: Comparison between oxidative stress parameters in gastrocnemius muscle tissue and serum creatine kinase (CK) activity (U/L) in both studied groups

		Group I (n=15)			Group II (n=15)		
GSH (mg/mg protein)	Range	2.361	–	3.322	2.229	–	3.125
	Mean ± SD	2.74	±	0.39	2.58	±	0.23
	t. test	1.288					
	p. value	0.208					
NO (µmol/ mg protein)	Range	1.19	–	1.46	0.69	–	0.86
	Mean ± SD	1.32	±	0.09	0.76	±	0.05
	t. test	21.514					
	p. value	0.001*					
CK (U/L)	Range	40.522 – 58.232			66.546 – 90.845		
	Mean ± SD	48.06 ± 5.45			78.61 ±7.53		
	t. test	12.739					
	p. value	0.001*					

*Group I: Control group. * Group II: Dexamethasone treated group.

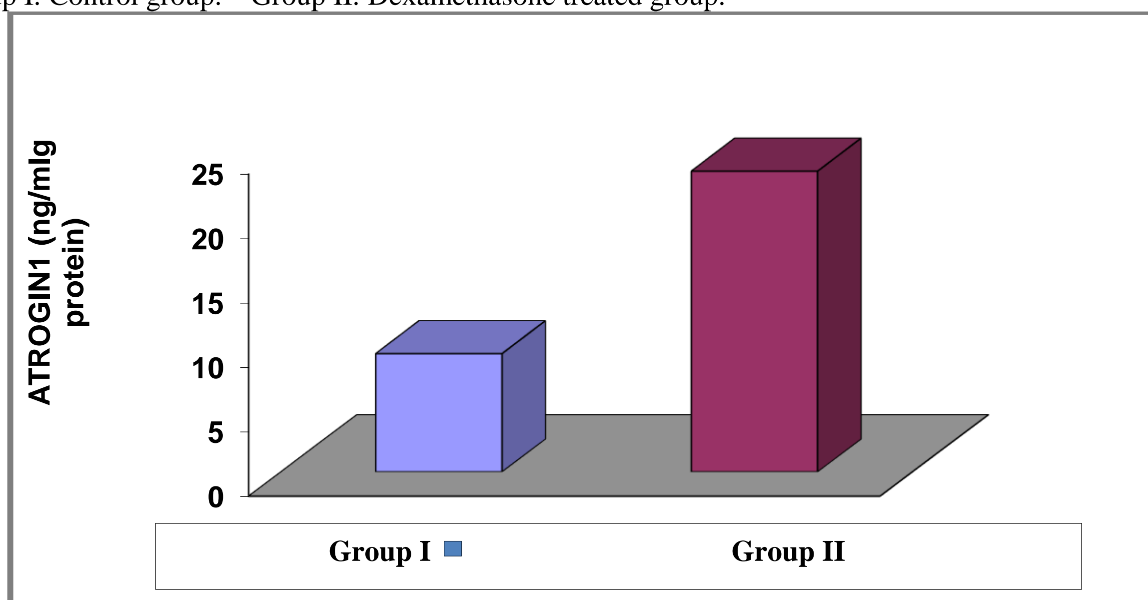

Figure1: Mean values of muscle tissue Atrogin 1(ng/mg protein) in control group (group I) and Dexamethazone treated group (group II)

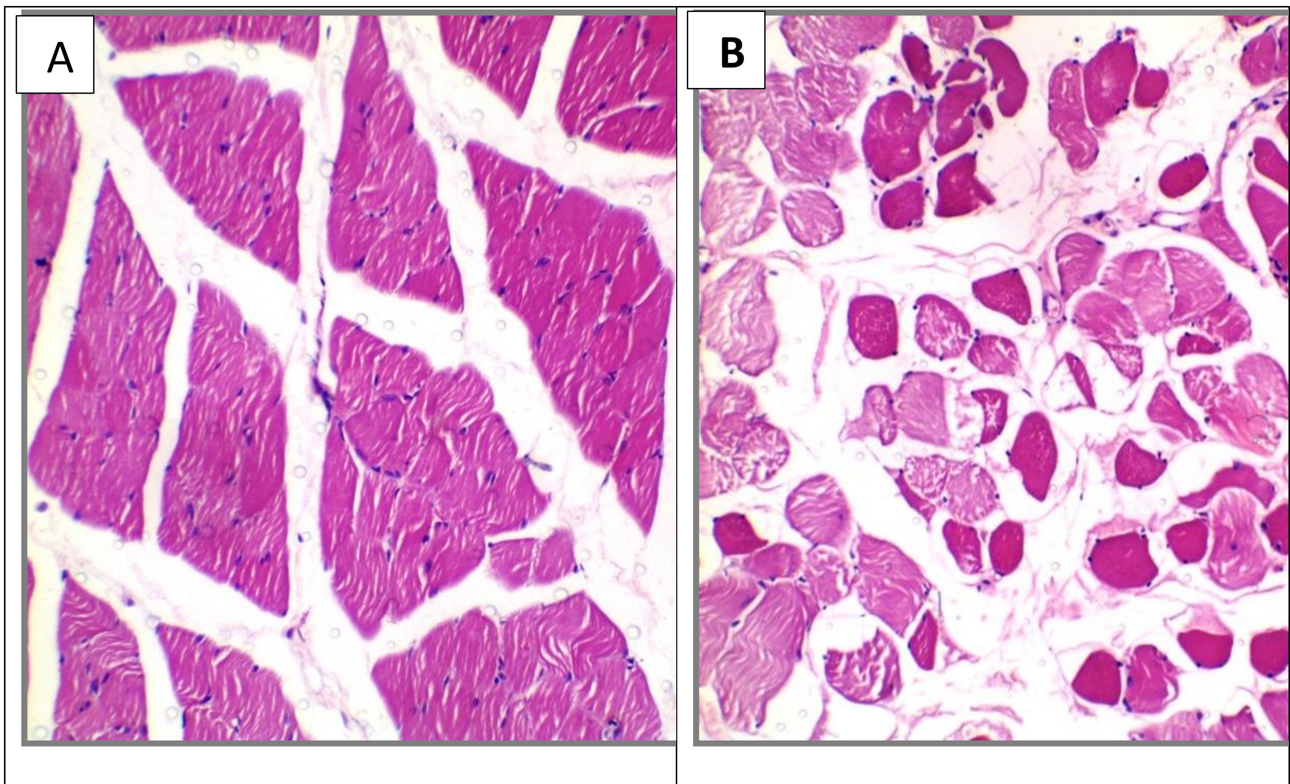

Figure 2: Micrograph of H&E-stained section from muscle tissue (H.& E. 10x). A: Group I with normal architecture. B: Group II show hypereosinophilic muscle fibers of reduced diameter and crowded nuclei

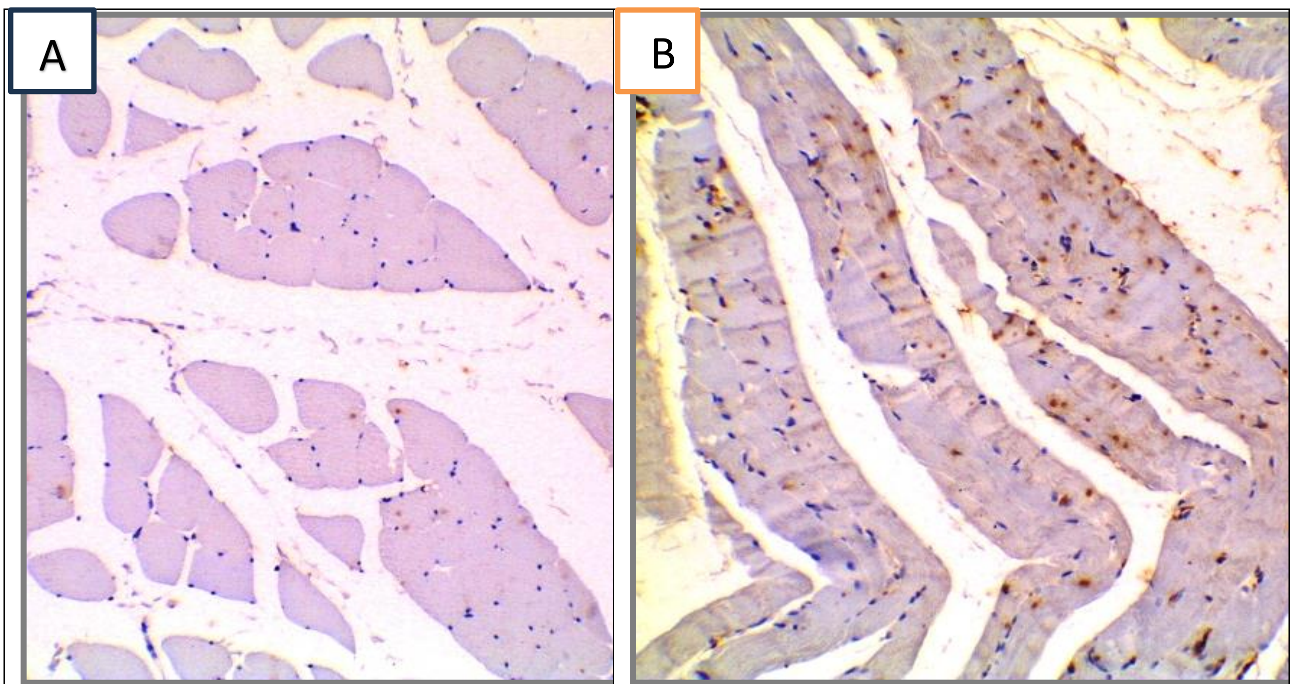

Figure 3: Immunoreactivity of caspase-3 in muscle section.A: Group I with normal architecture. B: Group II showing strong positive brownish immunoreactions for caspase-3 filling cytoplasm of many muscle fibers

DISCUSSION

Muscle wasting, or skeletal muscle atrophy, is characterized by a reduction in muscle mass resulting from an imbalance between protein breakdown and protein formation in response to different conditions. Glucocorticoids cause muscle atrophy not only when used as medications [8], but also when they are excessively produced internally in response to different stress conditions [9]. Glucocorticoids, both natural and synthetic varieties, are commonly prescribed for managing and treating numerous inflammatory and autoimmune conditions due to their strong anti-inflammatory and immunosuppressive effects [10].

In the present study the results of dexamethasone treated group when compared with control group revealed significant reduction of body weight, muscle strength, tissue nitric oxide level, in addition to histopathological changes indicative of muscle atrophy, while there was significant increase in tissue atrogin1and serum creatinine kinase level.

In the current study, dexamethasone caused a significant decrease in body weight and muscle strength as compared to control group. Administration caused significant weight loss which reached maximum at the end of experiment (27% of initial weight).A finding which is supported by many other studies[11,12].This weight loss may be attributed mainly to the catabolic state and partially to anorexia induced by dexamethasone as demonstrated by the study conducted by Figueiredo et al.[13].

In this study, the muscle wasting effect of dexamethasone was assessed using the hanging wire test, which evaluates hang time and holding impulse, and the results indicated a substantial decrease in the strength of the forelimb muscles. The test has been utilized in various studies to evaluate muscle strength in diverse animal species. Previous research by Kumar et al. [14] showed that dexamethasone administration reduces muscle strength. Muscle strength diminished from the breakdown of muscle proteins through the activation of various proteolytic pathways and the suppression of anabolic processes.

The current research found that the level of nitric oxide had a considerable drop in the dexamethasone-treated group as opposed to the control group. The reduced NO level may be attributed to the inhibition of inducible nitric oxide synthase (iNOS).This outcome aligns with findings reported by Nakada et al. [15]

Reduced Glutathione (GSH) plays a substantial role as an in vivo antioxidant and free radical scavenger, and its levels are a critical factor in determining the body's antioxidant capacity. Glutathione is capable of directly reacting with a wide variety of ROS and serves as a co-factor for glutathione peroxidase (GPx), which is known to catalyse the reduction of toxic hydrogen peroxide (H2O2) and hydroperoxides.

In the present study, the existing atrophy can be explained by the effect of dexamethasone on the tissue redox status[16].These results are consistent with results of previous studies, as Kadoguchi et al.[17] that demonstrated the effect of dexamethasone on oxidative stress in muscle tissue and the effectiveness of different antioxidants in reversing it.

Serum CK is one of the intracellular enzymes abundant in skeletal muscles, brain and cardiac muscles. An increase in its level suggests the occurrence of tissue damage. In this study, serum creatine kinase was significantly increased in dexamethasone treated group compared to control animals, and this is consistent with common features of muscle disease and previous findings of dexamethasone induced catabolic state [18,19].

The current study found that the Atrogin-1 level was substantially higher in the dexamethasone-treated group than in the control group. Elevated levels of atrogin1 may be caused by a surge in the breakdown of muscle protein. These results are consistent with results obtained by Mirzoev et al.[20]. Also, in another study carried out by Clavel et al. [21] using Western blotting revealed that atrogin 1 expression was increased by involvement of the ubiquitin-proteasome pathway in sarcopenia of fast-twitch muscle that indicates important role of atrogin -1in regulation of muscle mass.

Examination of gastrocnemius muscle sections, stained with H& E, revealed significant shrinkage of muscle bundles, considerable fibre size variation, chaotic nuclear arrangement, loss of the characteristic polygonal myofiber morphology, and an inflammatory infiltrate, which are indicative of classical atrophic changes. These findings are consistent with the histological features of muscle atrophy induced by glucocorticoid treatment in different studies such as the one done by Jhuo et al.[22].

The contribution of apoptosis to the observed muscle atrophy in our study was supported by a substantial increase in caspase-3 immunoreactivity in muscle sections from animals treated with Dexamethasone, which displayed dense brown cytoplasmic staining and heightened caspase-3 color intensity. Our findings are consistent with those reported by You et al.[23]. Caspase-3 facilitates muscle breakdown by cleaving action from its attachment to cross-bridges, thereby enabling subsequent ubiquitination.

Conclusions

it can be concluded that dexamethasone injection in a dose of 2 mg/kg/day by subcutaneous route has induced muscle atrophy which is proved by body weight loss, reduction of muscle strength in addition to histopathological findings indicative of atrophic changes and this raises the value of developing drugs that targets the glucocorticoid atrophy pathway as it will revert this undesirable adverse drug effect as well as be useful in partly, reversing other types of atrophy. This study can consider atrogin1as one of sensitive marker of muscle protein degradation by ubiquitin proteasomal system.

conflicts of interest: None
Financial disclosure: None

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