Effect of Amino Acid L-leucine On the Musculo-Skeletal Changes during Cast-Immobilization in Adult Male Albino Rats. Physiological and Histological study

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Abstract: The relation between muscle atrophy and bone osteoporotic changes due to immobilization are still not completely understood. This study tried to throw more light on this association and to probe the ability of amino acid L-leucine, to limit these changes in a trial to accelerate rehabilitation. Adult male Albino rats weighing 180-210 g were used and classified into three groups, I-control group, II-cast-immobilized group and III-immobilized-L-leucine treated group. Right hind-limb cast immobilization was performed for 15 days in groups II&III, while L-leucine, was given by oral gavage in a dose 0.7g/kg/day concomitant with immobilization in group III. The initial and final body weights were determined. Blood samples were used for determination of serum levels of total calcium, CPK, ADH, TNFα and Cortisol as well as for plasma MDA and glucose level. In the immobilized right hind-limb after removing the cast, the gastrocnemius muscle was identified, dissected, weighed. Then the right gastrocnemius muscle was prepared for light and transmission electron microscopic studies and the right tibia was prepared for both decalcified and un-decalcified light microscopic studies. Cast-immobilized group II showed significantly increased serum calcium, LDH, CPK, cortisol, TNFα and plasma MDA with non-significant change in blood glucose level. Also immobilization resulted in significantly reduced body weight, reduced gastrocnemius body weight ratio and resulted microscopically in both skeletal muscle atrophy in the gastrocnemius muscle and osteoporosis in the tibia cancellous bone compared with control group. Immobilized -Leucine treated group-III exhibited significantly reduced LDH, CPK, MDA and glucose levels but the levels of calcium was non-significantly altered compared to immobilized non treated group. Although serum cortisol and TNFα levels in leucine treated group were reduced non-significantly compared to immobilized non treated group, microscopically Leucine administration to cast-immobilized rats of group III markedly prevented skeletal muscle atrophy and partially prevented cancellous bone osteoporosis. It is concluded that increased MDA, Cortisol, TNFα with immobilization may explain in part the associated changes in muscle and bone. Leucine prevented these changes which could be attributed to its direct anabolic effect or its ability to reduce oxidative stress and /or its ability to counteract the effect of Cortisol and TNFα rather than reducing their levels. Overall, these data suggesting that leucine intake may represent a nutritional strategy for limiting muscle and bone protein loss as a consequence of immobilization.
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Key words: leucine, immobilization, osteoporosis, muscle atrophy and TNFα
Abbreviations: LDH (lactate dehydrogenase), CPK(Creatine phoshokinase), MDA(malondialdehyde).

1. Introduction

Skeletal muscle is a highly differentiated tissue that has the capacity to adapt to extreme fluctuations in its functional state. Clinical studies had confirmed that skeletal muscle atrophy and strength reduction, secondary to cast-immobilization, cannot always be returned to normal levels despite extensive rehabilitation **(1)**.

As the duration of immobilization has a significant impact on the duration and intensity of rehabilitation **(2)** and the process of musculo-skeletal tissue restructuring seems to require more time than that needed to cause atrophy by immobilization **(3 & 4)** so any intervention that accelerate rate of healing and accelerate rehabilitation is very valuable.

Advances in cell biology have progressed our understanding of those factors that contribute to muscle atrophy. Understanding the molecular mechanisms behind disuse muscle atrophy is important to develop countermeasures in order to prevent muscle wasting and to preserve its function **(5)**. Taking in consideration that immobility per se is a stressful condition and hypercortisolemia was suggested to represent the predominant hormone initiating muscle protein catabolism and bone demineralization **(6 & 7).** Also, Muscle atrophy due to immobilization was attributed to the possible role of inflammatory cytokines **(8)**, or to excess formation of reactive oxygen species (ROS**) (9).**

Muscle is closely related to bone and contains highly osteo-inducible cellular populations that have been implicated to have roles in bone formation and repair. Adjacent muscle acting as a “secondary periosteum” is able to contribute progenitors that can be reprogrammed to form bone **(10)**. Skeletal development and subsequent maintenance of bone mass and morphology during adulthood is greatly influenced by viable muscle function. On the other hand, degraded muscle function, arising by disease or age, is clearly accompanied by diminished bone mass and morphology. Thus, increased muscle function not only has the potential to generate

anabolic mechanical signals for bone, but normal muscle function is required for maintaining a healthy skeleton **(11)**.

In fact, weak muscles compromise the activities of daily living and consequently reduced the quality of life **(12).** Moreover, osteoporosis constitutes a major worldwide public health burden characterized by enhanced skeletal fragility **(13)**.

Protein and amino acids are of the most popular additives given to endurance athletes and body builders to cover the need to synthesize new muscle or to repair muscle damage with heavy training. On many occasions, amino acids are frequently used, since they are easier to absorb than proteins **(14)**. Amino acids is preferable than proteins in some instance where protein may influence the balance between osteoblastic and osteoclastic activity as it increases urinary calcium excretion due to the associated decrease in pH with its excess ingestion **(15)**. Prolonged periods of skeletal muscle inactivity lead to a loss of muscle protein and strength, thus more specific interventions can be designed for the attenuation of protein loss **(5)**.

Leucine is one of the branched-chain amino acids (BCAA); it is an essential amino acid with a role in protein synthesis and may influence maintenance of muscle mass during weight loss **(16)**. Leucine is an anticatabolic agent **(17).** The impact of ingesting extra leucine on muscle protein synthesis and muscle loss in immobilized human muscle has not specifically examined **(18)**.

So the aim of this study was to evaluate both the ability of amino acid leucine to prevent the immobility associated changes in muscle and bone when it is given during immobilization as well as its effects on the changes in MDA, cortisol and TNF-α. The later is to identify its possible mechanism in preventing skeletal muscle atrophy and cancellous bone osteoporosis induced by cast-immobilization.

2. Material and Methods

2.1Experimental animals:

This study was carried out on adult male albino rats weighing 180-210g (n=24). Rats were purchased from Military Animal Farm (Cairo) and maintained in the hold facilities in Physiology Department, Faculty of Medicine, Ain Shams University, under standard conditions of boarding. Water and food were provided *ad libitum.*

2.2 Experimental protocol:

Experimental animals were randomly allocated into three equal groups, 8 rats each:

Group I: Control group.

Group II: Cast-immobilized group.

Group III: Cast-immobilized leucine treated group.

2.3-Experimental procedure:

At the start of the study, the initial body weight was determined then application of the cast was done.

Unilateral cast-immobilization technique:

Following anesthesia induced by Ether inhalation, the right hind–limbs of groups II & III were immobilized by applying several layers (4-5) of moistened plaster of Paris strips (GIBSON, supplied by Egyptian Medical Group CO.) from mid thigh to the toes. The cast was applied according to **Nascimento *et al.*, (19)** with little modification. A pad of cotton was applied over the right hind-limb with knee in extension and the ankle in plantar flexion to produce marked atrophy of gastrocnemius muscle as well as to avoid weight bearing on this limb. The dorsum of the toes was left uncovered to check for edema or circulatory problems. The cast was covered by a wire mesh to prevent the rats from eroding the cast **(20)**. The casting procedure let the muscle innervations remain intact. The rats were left immobilized for 15days and they were monitored on a daily basis for chewed plaster, abrasions and venous occlusion, and problems which may require replacement of the cast.

Amino acid treatment:

The powder of L-Leucine was dissolved in distilled water, shacked well and leucine supplementation ~0.7 g/kg/day over a period of 15 days, starting from the first day of immobilization, was given by oral gavage. This dose is considered to be a moderate dose **(21)**. The control and immobilized -non leucine treated group, both were given distilled water at the same route, volume and frequency as the treated group. (L-Leucine powder, B.D.H, Laboratory chemicals, England, was supplied by Biochemistry Department, Faculty of Medicine, Ain Shams University).

On the day of sacrifice, overnight fasted rats with free access to water were anaesthetized by intra-peritoneal injection of thiopental sodium in a dose of 40mg/kg body weight. After careful removal of the cast in the immobilized groups, the final body weight was determined and then the rats were subjected to the following studies:

I) Biochemical study:

At the end of the experimental period, midline abdominal incision was done and the abdominal aorta was cannulated for collection of blood samples. The first sample collected in dry tube, then centrifugation was done at 3000 rpm for 15 min to separate serum for measuring the levels of total calcium, CPK, LDH, cortisol and TNFα. Another blood sample was taken in heparinized tube and centrifuged at 4000 rpm to separate plasma for determination of glucose and MDA levels. Serum was kept at -80^{o}C until the day of analysis

-Estimation of fasting plasma glucose was done by enzymatic colorimetric methods, using kit provided by Beckman Insturments, Inc., Brea, USA **(22).**

- Measurements of malondialdehyde (MDA) as an indicator of lipid peroxidation were carried out using thiobarbituric acid (TBA) **(23).**

-Serum cortisol was measured according to the manufacture's instructions by the use of Immulite Cortisol kit supplied by Diagnostic products corporation (DPC) depending on competitive immunoassay method.
-TNFα was estimated by ELISA technique according to the manufacture's instructions. TNFα kit was supplied by Biosource International, Inc., California, USA (purchased from Gamma – Trade CO.).
-Creatine phosphokinase activity (CPK) and Lactate dehydrogenase activity (LDH) were determined by colorimetric method at 340nm wave length, using kits supplied by Biodiagnostic- Egypt.
-Total serum calcium was determined using the colorimetric method at wave length 570 nm. Kit was supplied by Teco Diagnostics, Anaheim. **(24)**.

II)- Muscle weight Study:

The right hind-limbs gastrocnemius muscles were excised, gently trimmed of neighboring tissue, wet-weighed. Wet-weights of the muscles were recorded as ratios of total final body weights.

III)-Histological study:

A) Skeletal muscle:

The gastrocnemius muscles of right hind-limbs were longitudinally divided into medial and lateral halves. The medial halves were fixed in 10% formalin and processed for light microscopic study (LM), while the lateral halves were processed for transmission electron microscopic study (TEM). The left hind-limb was not used as a control limb because overloading on it may induce hypertrophy and its muscle may be affected by the changes in blood chemistry due to immobilization. So, separate control animals of the same average weight were used.

For LM study, 10% formalin fixed muscle slices were processed to form paraffin blocks. Serial longitudinal and transverse sections, 5µm thick, from the central part of the medial halves of the gastrocnemius muscle were prepared. Then, the sections were stained with hematoxylin and eosin stain (H&E) **(25)** and Mallory stain **(26)**.

For TEM study, phosphate buffered gluteraldehyde fixed small pieces (1-2mm thick) from the central part of the lateral halves of the muscle were processed to form capsules. Semi-thin longitudinal sections were cut at 1µm in thickness using glass knife, stained by 1% toluidine blue in 1% borax and examined by the light microscope. Ultra-thin sections (50-60 nm in thickness) were cut using ultra-microtome. Then sections were mounted on copper grids and stained with saturated solution of uranyl acetate **(27)** followed by lead citrate **(28)**. Ultra-thin sections were examined and photographed by JEM-1200EXII transmission electron microscope in Faculty of Science, Ain Shams University.

B) Cancellous bone:

The tibias of rats' right hind-limbs were carefully dissected and then immediately fixed in neutral buffered formaldehyde for 2 days. After fixation, halves of the proximal metaphysis of the right tibias were processed for preparation of decalcified specimens, and the other halves were processed for preparation of un-decalcified specimens. Decalcification was performed by using the chelating agent, ethylene-diamine-tetra-acetic acid (EDTA) in the form of its disodium salt (5.5 g ethylenediaminetetraacetic acid in 90 ml distilled water and 10 ml formaldehyde 37–40%). Decalcification was carried out for 4 weeks, during which time the decalcifying solution was changed every day **(29).** The decalcified specimens were dehydrated and processed to form paraffin blocks. Serial longitudinal sections, 5µm thick were prepared. Then, the sections were stained with H&E **(25).**

The un-decalcified specimens were cut longitudinally into small pieces, 3–5mm thick, dehydrated in ascending grades of alcohol, then in one change of acetone for 15 minutes. Acrylic resin embedding medium were freshly prepared and were applied to the specimens with acetone in 1:1 ratio. Then the specimens were embedded in freshly prepared acrylic resin-embedding medium of medium consistency that was formed by mixing 20ml EM bed-812, 16ml dodecenyl succinic anhydride, 8ml nadic methyl anhydride and 0.77ml dimethylamino methyl phenol. The specimens were left in a shaker for 1 day, and then they were transferred into special capsules, one specimen in each capsule. Freshly prepared acrylic resin embedding medium was applied. Capsules were then placed in an oven at 58^{o}C for 2 days. When cooled, the capsules were broken and trimmed to a suitable shape for the microtome. Serial longitudinal semi-thin sections were cut at a thickness of 1–2µm using a glass knife and an ultramicrotome **(30)**. Sections were then stained using modified Von Kossa's technique **(29).**

IV- Histomorphometric and Statistical Study:

All histomorphometric studies of both skeletal muscle and cancellous bone were performed using Image Analyser (Olympus Image J, NIH, 1.41b, USA) in the Oral pathology Department, Faculty of Dentistry, Ain Shams University.

A) Skeletal muscle:

The mean fiber cross-sectional area of 100 muscle fibers, from randomly chosen fields in the central region per muscle section stained by H&E of each gastrocnemius muscle, was performed. The reason for choosing quantification based on cross sectional area of muscle fibers is that this parameter is not affected by edema or relative increase in connective tissue **(31)**.

B) Cancellous bone:

The histomorphometric parameters for bone were defined according to the report by the American Society for Bone and Mineral Research committee **(32)**.

- **Trabecular Bone Volume (%):** percentage of cancellous bone area occupied by trabeculae and

expressed as percentage of total measured area (area of trabeculae and bone marrow space) **(33)**.

- **Osteoid thickness (µm):** mean thickness of the osteoid layer overlying the bone trabeculae **(34)**.
- **Relative Osteoid Surface (%):** percentage of cancellous bone surface covered with osteoid **(33)**.

$$\frac{\text{Length of trabecular surface covered by osteoid tissue \%}}{\text{Total length of trabecular surface}}$$

- **Relative Bone Resorption Eroded Surface (%):** percentage of cancellous bone surface with areas of resorption **(33)**.

$$\frac{\text{Length of eroded trabecular surface \%}}{\text{Total length of trabecular surface}}$$

All trabecular bone histomorphometric measurements were performed, beginning at more than 1mm from the growth plate–metaphysial junction to exclude the primary spongiosa and thus restrict measurements to the secondary spongiosa of proximal tibia metaphysis. The secondary spongiosa extending between 1.0 and 1.9 mm from the epiphyseal growth plate, 3.7 mm wide, centered on the long axis of the bone. For each group of rats, slides from studied animals were examined and eight fields were analyzed for a total metaphyseal area of 1.7 mm^2 **(33)**. The mean values of 40 fields, [8 different fields from five serial sections], were estimated.

Statistical studies:

The means of all measured parameters (biochemical, weight, histomorphometric) were performed and the standard error of mean (SEM) was calculated and statistical analysis was done using SPSS statistical program version 17. Data were evaluated by using one way analysis of variance test (ANOVA). As regards the probability, the least significant level used was at $p < 0.05$.

3. Results

I- Biochemical Results

As shown in table (1) & figure (1), immobilization in group II was associated with significantly increased ($p<0.05$) plasma level of malondialdehyde (MDA) compared to control rats, while leucine treatment concomitants with immobilization in group III significantly ($p<0.05$) reduced plasma MDA level compared to immobilized group. However the plasma MDA level with leucine still significantly ($p<0.05$) higher compared to control rats (Fig. 1A).

While serum levels of total calcium was significantly ($p<0.05$) increased in immobilized group II compared to control rats, it was non-significantly differ between leucine treated group and both of control rats and immobilized group II (Fig. 1B).

Immobilization in group II, also caused significant ($p<0.05$) increase in both serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) levels compared to control group. On the other hand leucine treatment with immobilization was associated with significant reduction in both of CPK and LDH compared to immobilized group, but their levels were still significantly higher compared to control (Figs. 1C & 1D).

Although plasma level of glucose with immobilization in group II was non-significantly differ compared to control, it was significantly ($p<0.05$) reduced in leucine treated group compared to immobilized group. However its value was non-significantly differing from that of control (Fig. 1E).

Immobilization in group II was associated with significantly increased serum cortisol and serum TNFα. ($p<0.05$ for both) compared to control rats. On the other hand, in leucine treated group, the serum levels of both cortisol and TNFα were reduced though non-significantly compared to immobilized group. However, cortisol level still higher significantly ($p<0.05$) compared to control and TNFα still higher non-significantly compared to control (Figs. 1F & 1G).

II- Body Weight and Gastrocnemius Muscle Weight Results

As shown in table (2), immobilized group rats exhibited significant ($p<0.05$) reduction in final body weight and significant reduction in absolute weight of gastrocnemius muscle as well as its weight to body weight ratio ($p<0.05$ for both). Meanwhile, in leucine treated group the final body weight was non-significantly differ from control, but it was significantly ($p<0.05$) higher compared to immobilized non treated group II.

Leucine treatment associated with significant ($p<0.001$) increase in absolute weight of gastrocnemius muscle as well as its ratio to body weight compared to immobilized non treated rats.

III- Histological Results

A) Skeletal muscle:

1. Group I (Control group):

As shown in figure (2), the gastrocnemius skeletal muscle of control group in transverse sections stained with Mallory showed collagenous connective tissue surrounding the bundles of muscle fibers within the gastrocnemius muscle, the perimysium. Each muscle fiber itself was surrounded by a delicate layer of connective tissue, the endomysium (Fig. 2A). In H&E stained transverse sections, the gastrocnemius muscle appeared consisted of muscle fibers collected in bundles. The myofiber oval nuclei were usually found at the periphery of the cell under the cell membrane (Fig. 2B). The sarcoplasm was filled with long cylindrical bundles of multinucleated myofibrils, which run parallel to the long axis of the muscle fiber. The entire muscle fiber exhibited a characteristic pattern of cross-striations of alternating light (I-bands) and dark bands (A-bands) as shown in longitudinal H&E and semi-thin sections stained by toluidine blue (Figs. 2C & 2D). In-addition TEM revealed that the I-band appeared bisected by a dark transverse line, the Z-line. The repetitive subunit of

the contractile apparatus, the sarcomere, extended from Z-line to Z-line. The A-band showed the presence of a lighter zone in its center, the H-band, bisected by the M-line. Oval myonucleus with finely dispersed chromatin throughout the nucleoplasm, with only a small amount of margination was observed and the nuclear membrane showed small indentations in its contour. Glycogen was obvious in the sarcoplasm in the form of coarse granules and mitochondria were also normally observed (Figs. 2E & 2F).

2. Group II: cast-immobilized group

As shown in figure (3), cast-immobilized gastrocnemius muscle showed apparent increased content in the collagenous connective tissue in both the endomysium and the perimysium compared with control in Mallory stained transverse sections (Fig. 3A). Moreover, transverse sections stained with H&E showed apparent widening of the interstitial spaces between muscle fibers with apparent decrease in most fiber cross-section area compared with control. Some myofibers showed central core-like lesions and some fibers exhibited central nuclei (Fig. 3B). Hypercontraction areas in some muscle fibers were obvious in longitudinally H&E stained sections (Fig. 3C). Moreover, undulating sarcolemma and small vacuoles in the myofibers were noticed in semi-thin sections stained by toluidine blue (Fig. 3D). In-addition, many electron-lucent vacuoles were noticed in TEM examination (Figs. 3E, 3F & 3G). The myofibrils showed severely disturbed contractile structure with loss of sarcomere organization and indistinguishable A-band, I-band, and irregular and distorted Z-line with disruption of myofilaments (Fig. 3E). Irregularly shaped markedly shrunken myonuclei with clumped and marginated chromatin with nearby electron-lucent vacoules were also noticed (Fig. 3F). Moreover, the amount of glycogen granules appeared less compared with the control with unapparent normal mitochondria (Fig. 3G).

3. Group III: cast-immobilized leucine treated group

As shown in figure (4), cast-immobilized leucine treated gastrocnemius muscle showed nearly normal content of collagenous connective tissue in the perimysium and the endomysium when compared with control in Mallory stained transverse sections (Fig. 4A). Transverse sections stained with H&E showed muscle fibers collected in bundles with oval nuclei that were usually found at the periphery of the cell under the sarcolemma (Fig. 4B). Moreover, longitudinal semi-thin sections stained by toluidine blue showed the peripherally placed oval nuclei and revealed the characteristic pattern of transverse striations (Fig. 4C). In addition, TEM showed preservation of the contractile banding structure of muscle fibers in which the myofilaments were oriented parallel to the long fiber axis nearly similar to control. The oval myonuclei showed evenly dispersed peripheral chromatin lying under the nuclear membrane (Fig. 4D). Plenty of glycogen granules in the sarcoplasm with nearby intact mitochondria were observed compared with group II (Fig. 4E).

B) Cancellous bone:

1- Group I (control group):

As shown in figure (5), H&E stained longitudinal decalcified sections of the proximal tibia metaphysis of control rats revealed that its cancellous bone secondary spongiosa consisted of a network of branching and anastomosing bone trabeculae separated by bone marrow spaces. The bone marrow was formed of hematopoietic tissue, scattered adipocytes, and blood sinusoids (Fig. 5A). The bone trabeculae consisted of irregular bone lamellae and contained lacunae housing osteocytes in between bone lamellae. The bone trabeculae were covered with endosteum showing flat osteoprogenitor cells and cuboidal osteoblasts (Fig. 5B). The matrix of some trabeculae showed more basophilic stainability (Fig. 5A), and cement lines were observed as basophilic lines (Fig. 5B). In-addition, longitudinal un-decalcified semi-thin sections stained by modified Von Kossa's technique showed a red zone of unmineralized bone matrix (osteoid) overlying the mineralized bone, which appeared black (Fig. 5C).

2- Group II: cast-immobilized group

As shown in figure (6), the cancellous bone of secondary spongiosa of the proximal tibia metaphysis of this group in longitudinally decalcified H&E stained sections appeared as thin trabeculae with small pieces of bone spicules. Widening of bone marrow spaces and apparent increased numbers of adipocytes were noticed compared with the control group (Fig. 6A). Resorption areas on bone surface containing multinucleated osteoclasts showing their characteristic acidophilic cytoplasm were obvious (Fig. 6B). Moreover, longitudinal un-decalcified semi-thin sections stained by modified Von Kossa's technique showed multiple resorption areas and apparently thin red zone of osteoid (unmineralized bone) on the mineralized bone that appeared black compared with control (Fig. 6C).

3- Group III: cast-immobilized leucine treated group

As shown in figure (7), H&E stained decalcified longitudinal sections of the proximal tibia metaphysis secondary spongiosa of this group revealed that the cancellous bone trabeculae appeared thicker compared with group II with less apparent widening of bone marrow spaces, which also contained apparently less adipocytes than in group II (Fig. 7A). The bone trabeculae were covered with endosteum which showed flat osteoprogenitor cells and cuboidal osteoblasts on one surface which appeared smooth; whereas the other bone surface appeared irregularly eroded (Fig. 7B). In-addition longitudinal un-decalcified semi-thin sections stained by modified Von Kossa's technique showed

resorption areas and a red zone of unmineralized bone matrix (osteoid) covering the mineralized bone, which appeared black (Fig. 7C).

IV) Histo-morphometric and Statistical Results

A) Skeletal muscle:

As shown in table (3), cast-immobilized gastrocnemius muscle of group II showed significant decrease ($p<0.05$) in the mean fiber cross-sectional area compared with control group. On the other hand, leucine treatment to cast-immobilized rats of group III showed significant increase ($p<0.05$) in the mean fiber cross-sectional area compared with group II and non-significant decrease ($p>0.05$) compared with group I.

B) Cancellous bone:

As shown in table (3), both cast-immobilized tibia of group II and cast-immobilized leucine treated tibia of group III showed significant decrease ($p<0.05$) in the mean trabecular bone volume, mean osteoid thickness and mean percentage of relative osteoid surface compared with control group. On the other hand, leucine treatment to cast-immobilized rats of group III showed significant increase ($p<0.05$) in these previous parameters compared with group II. Moreover, both cast-immobilized non-treated tibia in group II and cast-immobilized leucine treated tibia in group III showed significant increase ($p<0.05$) in the mean relative resorption eroded surface compared with control group. On the other hand, leucine treatment to cast-immobilized rats of group III showed significant decrease ($p<0.05$) in the mean percentage of relative resorption eroded surface compared with group II.

Table (1): Showing the biochemical measured parameters in the different studied groups.

	Control	Immobilized	Immobilized + L-leucine
MDA(Umol/L)	3.21 ± 0.03 (8)	4.32 ± 0.03[a] (8)	3.51± 0.04[ab] (8)
Plasma Calcium (mg/dl)	10.3 ± 0.38 (8)	11.54 ± 0.41 [a] (8)	10.97 ± 0.38 (8)
CPK(U/L)	240.9 ± 4.8 (8)	307.1 ± 6.3 [a] (8)	279.3 ± 3.7[ab] (8)
LDH(U/L)	295.0 ± 3.7 (8)	417.8 ± 5.7[a] (8)	327.6 ± 6.6[ab] (8)
Glucose (mg/dl)	98 ± 2.1 (8)	102.5 ± 0,45 (8)	95.6 ± 1.2[b] (8)
Cortisol (ug/dl)	2.0 ± 0.09 (6)	2.35 ± 0.1 [a] (6)	2.2 ± 0.04[a] (6)
TNF-α(pg/ml)	11.5 ± 0.36 (8)	12.9 ± 0.44 [a] (8)	12.0 ± 0.45 (8)

- Values are mean ± SEM. - Number in parenthesis indicates the number of rats.
- **a:** significance of difference by LSD from control group at least $p<0.05$.
- **b:** significance of differences by LSD from immobilized group at least $p<0.05$.

Table (2): The changes in body weight, absolute and relative weight of gastrocnemius muscle in the different studied groups

	Control	Immobilized	Immobilized + L-leucine
Final Body weight(gm)	229.7 ± 3.2 (8)	201.9 ± 3.0 [a] (8)	222.0 ± 1.59 [b] (8)
Weight of Gastrocnemius muscle (mg).	514.4 ± 5.84 (8)	415.0 ± 4.12[a] (8)	495.4 ± 1.04 [ab] (8)
Gastrocnemius/ body weight ratio.(mg/gm)	2.24 ± 0.01 (8)	2.06 ± 0.04[a] (8)	2.22 ± 0.01[b] (8)

- Values are mean ± SEM. - Number in parenthesis indicates the number of rats
- **a:** significance of difference by LSD from control group at least $p<0.05$.
- **b:** significance of differences by LSD from immobilized group at least $p<0.05$.

Table (3): Showing all different histomorphometric muscle & bone parameters in all different studied groups

	Control	Immobilized	Immobilized + L-leucine
Cross-sectional area of muscle fiber (µm2)	3596.38 ± 57.67 (8)	2212.13 ± 178.93[a] (8)	3419.38 ± 72.45[b] (8)
Trabecular bone volume (%)	52.9 ± 2.19 (8)	20.7 ± 0.51[a]	40.9 ± 1.64[ab]

		(8)	(8)
Osteoid thickness (µm)	6.1 ± 0.20 (8)	2.6 ± 0.06**a** (8)	4.3 ± 0.08**ab** (8)
Relative osteoid surface (%)	59.7 ± 0.72 (8)	25.6 ± 0.69**a** (8)	40.3 ± 0.79**ab** (8)
Relative bone resorption surface (%)	8.6 ± 0.31 (8)	19.8 ± 0.90**a** (8)	12.4 ± 0.61**ab** (8)

- Values are mean ± SEM. - Number in parenthesis indicates the number of rats.
- **a:** significance of difference by LSD from control group at least $p<0.05$.
- **b:** significance of differences by LSD from immobilized group at least $p<0.05$.

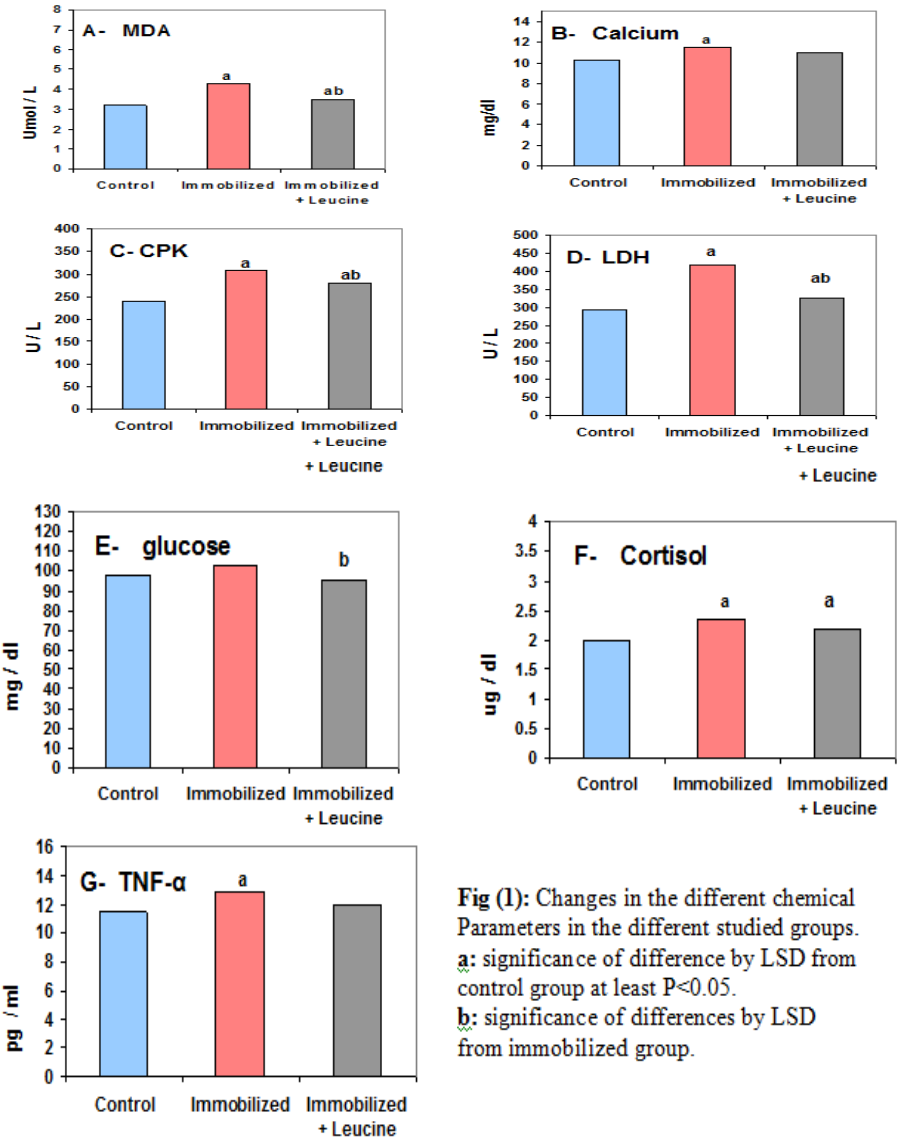
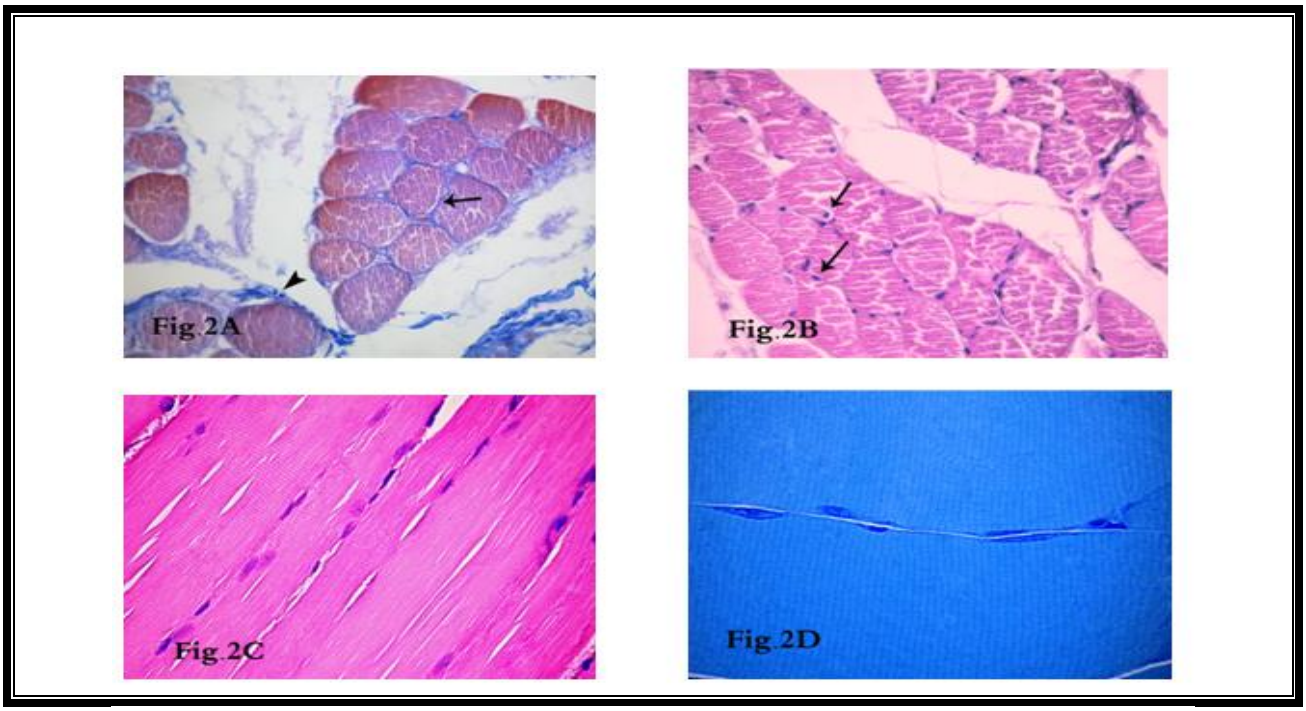


Fig (1): Changes in the different chemical Parameters in the different studied groups.
a: significance of difference by LSD from control group at least $P<0.05$.
b: significance of differences by LSD from immobilized group.



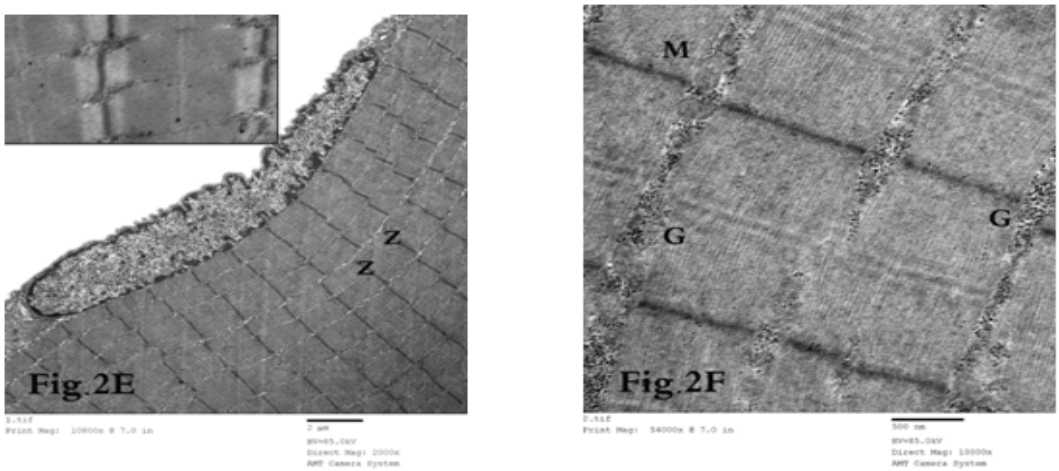


Fig. (2A): A photomicrograph of a transverse section of rat gastrocnemius muscle showing perimysial (▲) collagenous connective tissue surrounding bundle of myofibers and a delicate endomysium (↑) is surrounding each muscle fiber. Group I (Mallory x 640)

Fig. (2B): A photomicrograph of a transverse section of rat gastrocnemius muscle showing bundles of muscle fibers with peripheral oval nuclei (↑). Group I (H&E x 640)

Fig. (2C): A photomicrograph of a longitudinal section of rat gastrocnemius muscle showing long cylindrical bundle of myofibrils running parallel to the long axis of the muscle fiber, exhibiting a characteristic pattern of transverse striations. Group I (H&E x 640)

Fig. (2D): A photomicrograph of a longitudinal semi-section of rat gastrocnemius muscle showing long cylindrical multinucleated cells that show cross-striations of alternating light I-bands and dark bands A-bands. Group I (Toluidine blue x 1000)

Fig. (2E): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing oval myonucleus with finely dispersed chromatin throughout the nucleoplasm, with only a small amount of margination. Regular sarcomeres extending from Z-line (Z) to Z-line are obvious. Notice the Inset, it shows that the dark Z-line is bisecting the I-bands. Group I (TEM x 10800 / inset x 40500)

Fig. (2F): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing A bands with a lighter zone in their centers, the H bands, bisected by the M lines. Notice the glycogen granules (G) and the mitochondria (M). Group I (TEM x 54000)

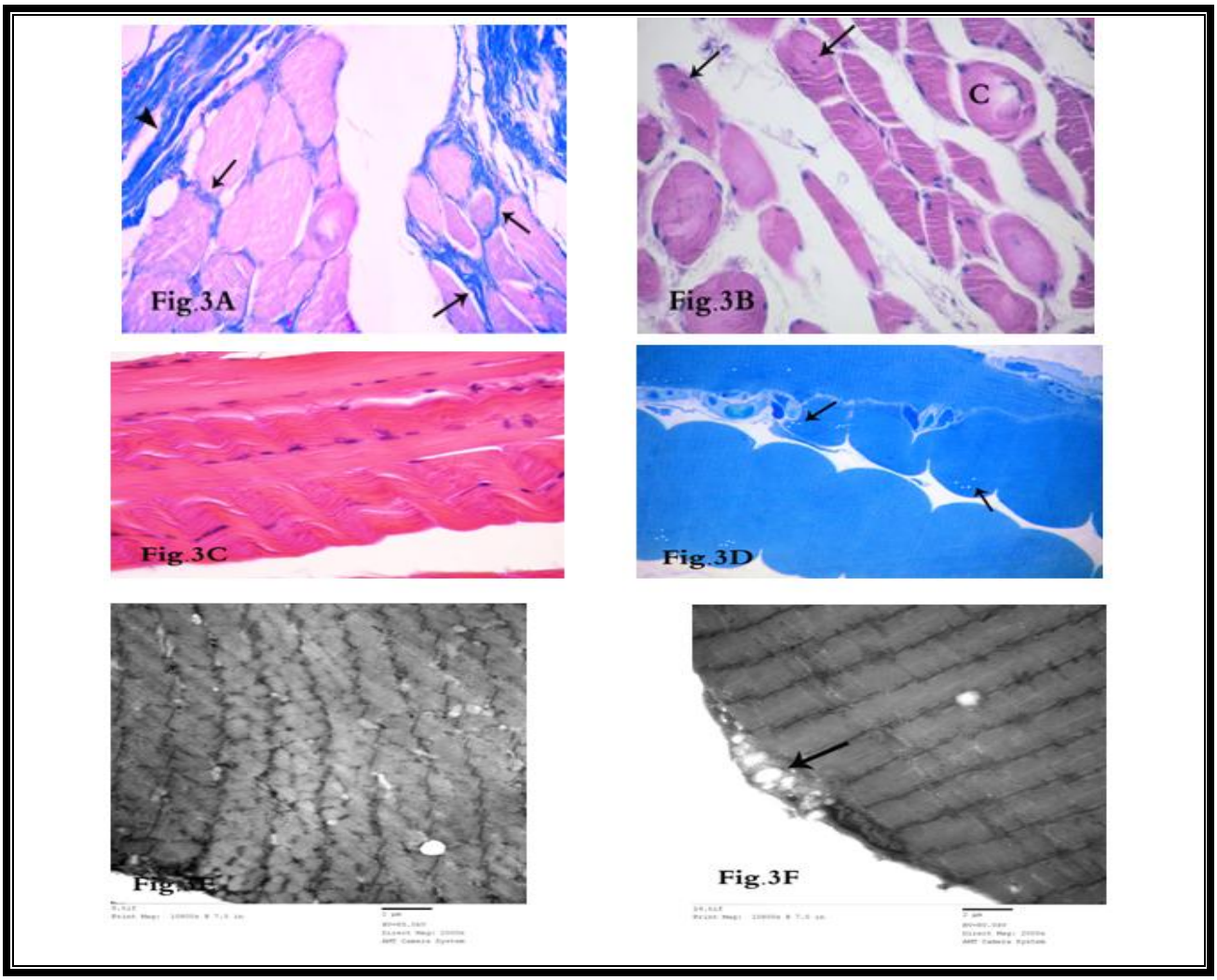


Fig. (3A): A photomicrograph of a transverse section of rat gastrocnemius muscle showing apparent increase content in the collagenous connective tissue in both the endomysium (↑) and the perimysium (▲) compared with control. Group II (Mallory x 640)

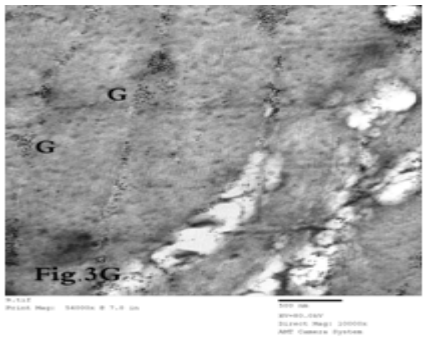
Fig. (3B): A photomicrograph of a transverse section of rat gastrocnemius muscle showing apparent widening of the interstitial spaces between muscle fibers. Most myofibers show apparent decrease in their cross-section area compared with control. Some fibers show central core-like lesions (C). Notice the central nuclei in some muscle fibers (↑). Group II (H&E x 640)

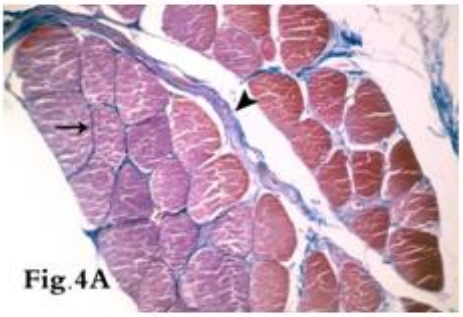
Fig. (3C): A photomicrograph of a longitudinal section of rat gastrocnemius muscle showing hypercontraction areas in some muscle fibers. Group II (H&E x 640)

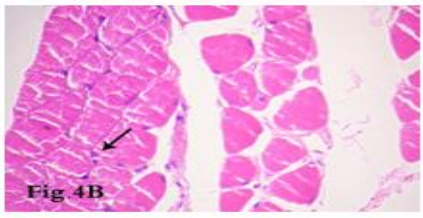
Fig. (3D): A photomicrograph of a longitudinal semi-section of rat gastrocnemius muscle showing undulating sarcolemma. Notice small vacuoles in the myofibers (↑). Group II (Toluidine blue x 1000)

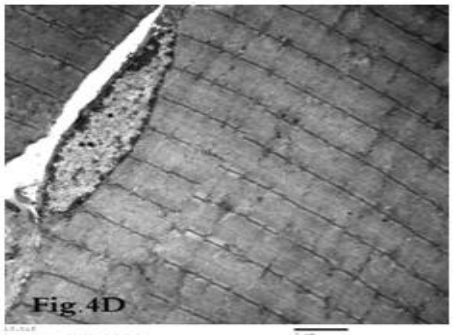
Fig. (3E): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing severely disturbed contractile structure with loss of sarcomere organization and indistinguishable A-band, I-band, and irregular and distorted Z-line with disruption of myofilaments. Notice electron-lucent vacuoles. Group II (TEM x 10800)

Fig. (3F): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing irregularly shaped markedly shrunken myonuclei with clumped and marginated chromatin with nearby electron-lucent vacuoles (↑). Group II (TEM x 10800)









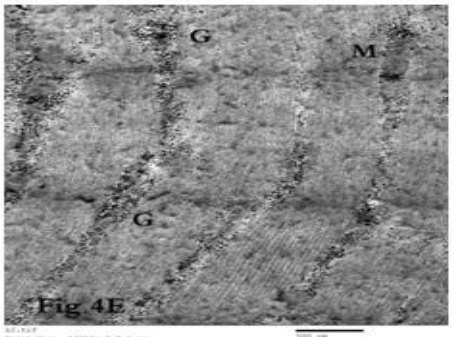


Fig. (3G): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing apparent decrease amount of glycogen granules (G) compared with the control. Notice electron-lucent vacuoles. Group II (TEM x 54000)

Fig (4A): A photomicrograph of a transverse section of rat gastrocnemius muscle showing nearly normal content of collagenous connective tissue in the perimysium (▲) and the endomysium (↑) compared with control. Group III (Mallory x 640)

Fig. (4B): A photomicrograph of a transverse section of rat gastrocnemius muscle showing bundles of muscle fibers with peripheral oval nuclei (↑) nearly similar to control. Group III (H&E x 640)

Fig. (4C): A photomicrograph of a longitudinal semi-section of rat gastrocnemius muscle showing the characteristic pattern of transverse striations. Notice the peripheral oval myonuclei. Group III (Toluidine blue x 1000)

Fig. (4D): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing preservation of the contractile banding structure of muscle fibers. Notice the oval myonucleus with evenly dispersed peripheral chromatin. Group III (TEM x 10800)

Fig. (4E): An electron-micrograph of a longitudinal section of rat gastrocnemius muscle showing plenty of glycogen granules (G) with nearby intact mitochondria (M). Group III (TEM x 54000)

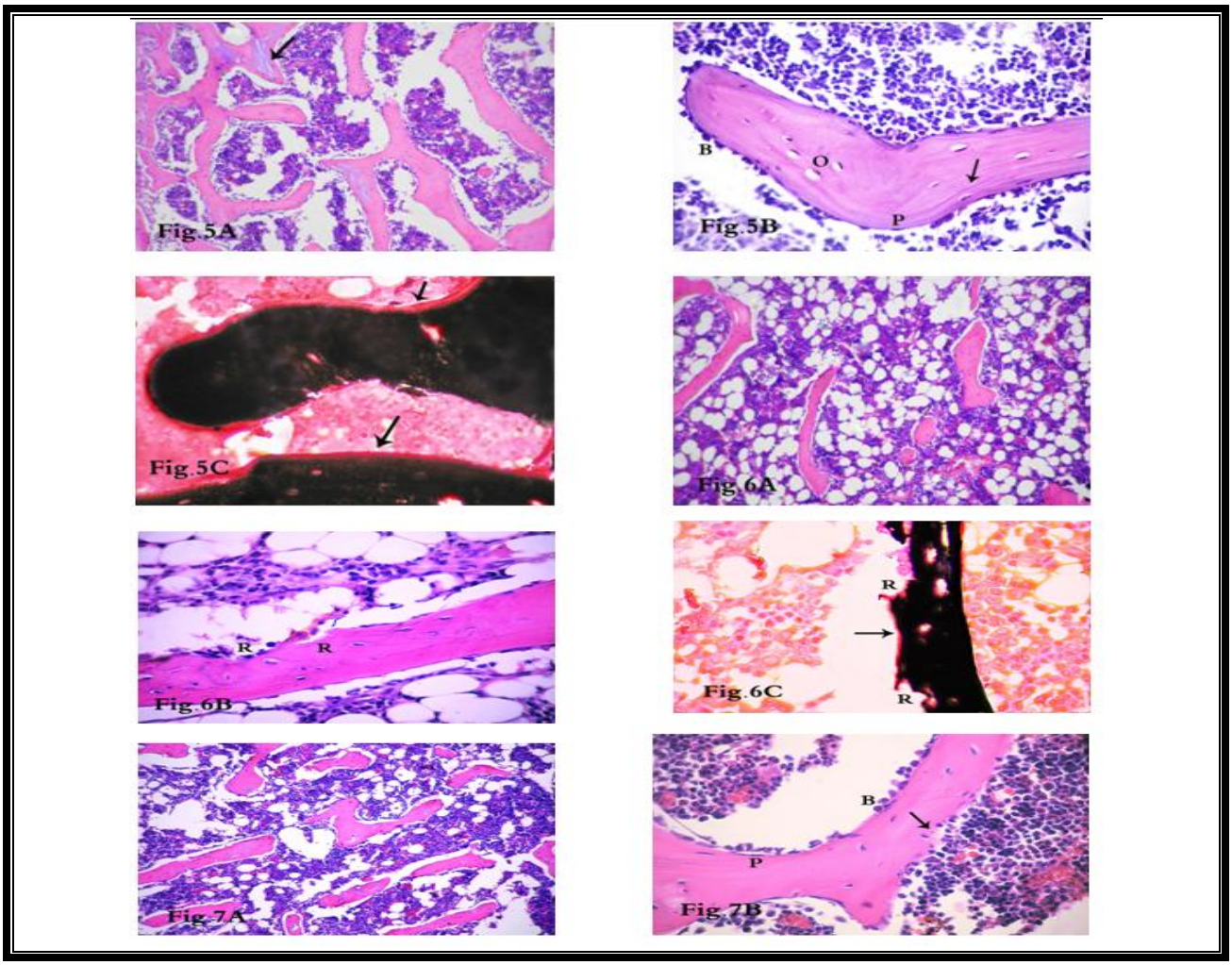


Fig. (5A): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing a network of branching and anastomosing bone trabeculae separated by bone marrow spaces formed of hematopoietic tissue, scattered adipocytes, and blood sinusoids. The matrix of some trabeculae showed more basophilic stainability (↑). Group I (H&E x 250)

Fig. (5B): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing bone trabeculae covered with endosteum showing flat osteoprogenitor cells (P) and cuboidal osteoblasts (B). Osteocytes (O) are seen present inside their lacunae in-between bone lamellae. Notice basophilic cement lines (↑). Group I (H&E x 640)

Fig. (5C): A photomicrograph of an un-decalcified longitudinal semi-section of rat proximal tibia metaphysis secondary spongiosa showing red zone of unmineralized bone matrix (osteoid) (↑) overlying the mineralized bone, which appears black. Group I (Modified Von Kossa x 640)

Fig. (6A): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing thin bone trabeculae with small pieces of bone spicules. Widening of bone marrow spaces and apparent increased numbers of adipocytes compared with the control group is obvious. Group II (H&E x 250)

Fig. (6B): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing resorption areas (R) on bone surface. Group II (H&E x 640)

Fig. (6C): A photomicrograph of an un-decalcified longitudinal semi-section of rat proximal tibia metaphysis secondary spongiosa showing thin red zone of osteoid (unmineralized bone) (↑) on the mineralized bone that appears black compared to control. Notice multiple resorption areas (R). Group II (Modified Von Kossa x 640)

Fig. (7A): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing cancellous bone trabeculae apparently thicker compared with group II. Less apparent widening of bone marrow spaces containing less adipocytes than in group II are obvious. Group III (H&E x 250)

Fig. (7B): A photomicrograph of a longitudinal section of rat proximal tibia metaphysis secondary spongiosa showing bone trabeculae covered with endosteum which shows flat osteoprogenitor cells (P) and cuboidal osteoblasts (B) on one surface which appears smooth; whereas the other bone surface appears irregularly eroded (↑). Group III (H&E x 640)

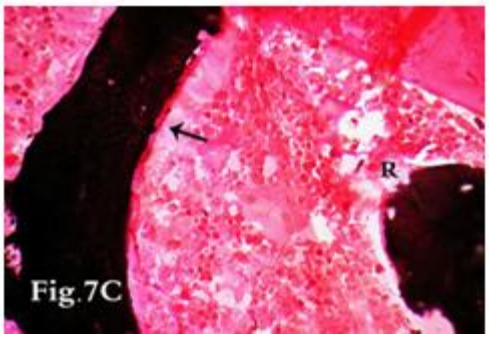


Fig. (7C): A photomicrograph of an un-decalcified longitudinal semi-section of rat proximal tibia metaphysis secondary spongiosa showing red zone of unmineralized bone matrix (osteoid) (↑) covering the mineralized bone, which appears black. Notice the resorption area (R). Group III (Modified Von Kossa x 640)

4. Discussion

The results in this study demonstrated that cast-immobilized group II showed significantly decreased body weight which was noted previously in other studies with immobilization **(35)**. Also immobilized rats in group II exhibited significant decrease in both absolute and gastrocnemius body weight ratio. These findings were observed before with cast-immobilization and explained by the imbalance between protein synthesis and proteolysis and also between apoptosis and regeneration processes **(36).**

On the other hand, leucine treatment significantly increased the weight of gastrocnemius muscle and its ratio to body weight compared to immobilized group which dictate the ability of leucine in ameliorating the atrophic changes due to immobility. Leucine reported to have greater stimulatory effect for muscle protein synthesis **(37)** as it stimulates the activity of protein kinases in muscle with a cascade of anabolic signaling molecules resulting in translation of pre-existing mRNA and translation of specific mRNA coding for regulatory proteins in the muscle **(38 & 39).**

Cast-immobilized right hind-limb rats of group II in this study resulted histologically and histomorphometrically in both skeletal muscle atrophy in the gastrocnemius muscle and osteoporosis in the cancellous bone secondary spongiosa of the proximal tibia metaphysis compared with control group. Similarly, it was previously reported that skeletal muscle atrophy is a morphological adaptation that normally occurs in response to conditions of disuse as immobilization. Both decrease in protein synthesis and increase in protein degradation had been contributed to muscle protein loss due to disuse **(40)**. Moreover, it was previously noticed that skeletal disuse resulted in loss of cancellous bone volume in the proximal tibia **(41)**.

Cast-immobilized gastrocnemius skeletal muscle of group II in this study, revealed widening in the interstitial space. This finding was suggested by **Ferreira *et al.* (42)** to be a result of myofiber edema. They also noticed sarcoplasmic vacuolization in their study on soleus muscle hindlimb-suspended animals and revealed these to be consisted of mitochondrial swelling. Moreover, disrupted mitochondria surrounding irregularly shaped myonuclei was noticed in a previous study on immobilized muscles **(43)**. This coincided with the observation of irregularly shaped markedly shrunken myonuclei with clumped and marginated chromatin by TEM with nearby electron lucent spaces in the cast-immobilized gastrocnemius muscle in group II in this study. Moreover, LM transverse sections of cast-immobilized gastrocnemius muscle of group II in this study showed centrally located nuclei in some myofibers. This was in line with the previously noticed in the skeletal muscle fibers of two weeks hind-limb unloaded rats that resulted in fiber atrophy **(44)**.

Cast-immobilized gastrocnemius muscle fibers in group II in this study presented undulating sarcolemma, hypercontraction areas, central core-like lesions in the myofibers and small vacoules by LM. Moreover, TEM revealed many electron-lucent vacuoles, myofilament loss in-addition to the loss of sarcomere organization and indistinguishable A-band, I-band, and irregular and distorted Z-line; all these led to the severe disturbed contractile structure of the myofibers. These observations in the immobilized muscles might be attributed to be signs of the various stages of the atrophy process, as a result of promoting the disassembly of myofibrillar proteins that anchor myofilaments to the Z-line and maintain sarcomeric alignment **(31)**. Moreover, sarcomere disruption, myofilament loss and the vacuolated myofibers were suggested to be a main component of muscle degeneration in the gastrocnemius muscle of rats immobilized in plaster-of-Paris casts **(45).**

The increased serum creatine phosphokinase (CPK) activity in this study with immobilization is an indicator of muscle damage and so its release. In a previous study it was showed that hind-limb cast-immobilization for 4 weeks reduced the muscle creatine phosphokinase content by about 40% **(46)**.

Also, the increased serum lactate dehydrogenase (LDH) with cast-immobilization in this study may be attributed to the increased proteolysis with immobilization and release of such muscle enzyme.

Immobilization thought to induces insulin resistance and a catabolic state in human skeletal muscle **(47 & 48).** In addition, increased LDH could be possibly due to the effect of immobilization in causing adaptive metabolic transformation of oxidative muscle fiber to glycolysis, and increased lactate formation and hence LDH activity. In line with this explanation immobilization reported to induce prooxidative-to-glycolytic fiber type switching causing increased muscle fatigability **(49).**

On the other hand, the leucine treated group showed a significant decrease in serum levels of LDH and CPK compared to non treated group which may be attributed to its ability to decrease proteolysis. Leucine reported to decrease the increased plasma LDH due to proteolysis with resistance-exercise training **(50).**

Moreover, microscopic examination in this study showed that the gastrocnemius muscle of leucine supplemented cast-immobilized rats (group III) did not reveal atrophic changes that were well pronounced in rats of group II. These observations might be explained according to the reported ability of leucine to interact with the insulin signaling pathway resulting in maintenance of muscle protein by stimulating protein synthesis and reversing the catabolic conditions **(51)**.

Coinciding, group III gastrocnemius muscle revealed that leucine administration preserved the banding structure showing the characteristic pattern of transverse striations and preserved the contractile structure of muscle fibers as the myofibrils were oriented parallel to the long fiber axis nearly similar to control. Thus leucine in this study markedly prevented skeletal muscle atrophic changes. This was in line with what was noted by **Baracos and Mackenzie (52)** that myofibrillar proteins are composed of approximately 18% Branched chain amino acid (BCAA) including leucine. They added that leucine alone could improve protein balance and lean body mass because of its role in regulation of protein synthesis and degradation. Moreover, the unique treatment by BCAA and specifically leucine was hypothesized to provide an important signal of dietary quality for skeletal muscle since dietary BCAAs reach the blood and skeletal muscle virtually unaltered and in direct proportion to dietary intakes **(51)**. In-addition, leucine was reported to stimulate skeletal muscle growth, repair, and regeneration by stimulating activation of myogenic satellite cells in skeletal muscle through mammalian target of rapamycin (mTOR) pathway **(53)**.

Moreover, in this study the plasma MDA level was increased significantly in cast-immobilized group compared to control rats and it could be suggested to play a role in the atrophic changes which were observed in this study. In line with this result suspended muscles as a method for immobilization for 14 days showed to have greater (29% more) content of malondialdehyde (MDA) compared to control rats **(54)**. Mitochondria have been shown to be an important source of ROS production in skeletal muscle during inactivity **(55 & 56).** Several lines of evidence suggested that disuse-induced oxidative stress in skeletal muscle contributes to muscle atrophy by activation of one or more proteolytic pathways **(9).** In addition, the increased MDA level may explain the osteoporotic changes in bone, as oxidative stress reported to induce cancellous bone loss with musculoskeletal disuse **(57).**

On the other hand, leucine supplemented cast–immobilized rats showed significantly lower MDA compared to non treated immobilized group. In line with this result, the role for amino acids supplements in controlling the antioxidant defense system and reducing the oxidative stress was concluded in diabetic skeletal muscle **(58).**

Although, amino acids supplemented group still also exhibiting a high level of cortisol compared to control group and non-significant lower level compared to immobilized, but it showed less catabolic changes. Thus it could be suggested that the direct effect of amino acids in stimulating protein synthesis was able to overcome or to prevent the suspected catabolic effect of cortisol. It is of interest to mention that amino acids is one of positive regulator, while corticosteroids is one of the negative regulator of mammalian target of rapamycin (m-TOR) signaling for protein synthesis in skeletal muscle **(39 & 59).** In addition, the dose of leucine, 0.7g/kg, which used in this study, is considered to be relatively enough as Leucine was reported in a dose 0.1g/kg able to stimulate protein synthesis when it was orally administrated as a single bolus **(21).**

Moreover, as leucine has the same transporter of phenylalanine and tryptophane, amino acids essential for synthesis of catecholamine, and as they compete for uptake via the same transporter **(14)**, it could be suggested that leucine ameliorated the catabolic changes due to stress and catecholamine.

The suggested effect of leucine in counteracting the catabolic changes despite of cortisol level is in line with a previous finding, where the deleterious effects of bed rest on human skeletal muscle which were exacerbated by hypercortisolemia were ameliorated by dietary supplementation of amino acids **(60).** In contradiction with suggestion, dietary branched chain amino acids reported before not to prevent skeletal muscle atrophy in aged rats injected with glucocorticoid **(61)** but the variability in model age as well as the increase of cortisol in this study model is completely intrinsic response without injection may explain this discrepancy.

Casting was mentioned previously to induce disuse loss of muscle mass and atrophy indicated by inducing loss of muscle fiber cross-sectional area **(62).** This coincided with the significant decrease in the mean muscle fiber cross-sectional area in cast-immobilized gastrocnemius muscle in group II in this study compared with control resulting in disuse muscle atrophy. Similarly, **Tipton (18)** stated that muscle may be lost during immobilization through increased negative muscle protein balance mediated by decreased basal levels of muscle protein synthesis, as well as less positive net muscle protein balance due to the decreased

response of muscle protein synthesis to nutritional anabolic stimuli. They added that nutritional interventions should aim at ameliorating muscle loss during injury-induced immobilization. Moreover, extra-leucine was reported to overcome the attenuated response of muscle protein synthesis to nutritional anabolic stimuli in the elderly as they suffer decline in skeletal muscle mass **(63)**.

Coinciding, this study showed that muscle mass loss and atrophy was prevented by leucine administration to rats of group III in which their mean muscle fiber cross-sectional area showed non-significant change compared with control as well as it showed significant increase compared with group II. Similarly, **Baptista *et al.* (64)** noticed that leucine supplementation attenuated muscle mass loss in rats driven by immobilization. In accordance, **Rieu *et al.* (65)** stated that dietary leucine supplementation may represent a useful nutritional tool for maintenance of muscle mass and prevention of muscle atrophy and it may be considered as a good alternative to high protein diets, which could have deleterious effects on renal functions particularly in the elderly.

Cast-immobilized gastrocnemius muscle of group II in this study showed decreased content of glycogen granules compared with control as observed in TEM study. This finding was previously noticed with immobilization **(66).** On the other hand, leucine administration in group III showed apparent increase in glycogen granules and intact nearby mitochondria compared with those of group II. These changes might be explained on basis of the reported ability of leucine to modulate the insulin signal and glucose use by skeletal muscle **(51).**

Although blood glucose level was increased non-significantly with immobilization in group II which may be correlated to effect of associated stress and /or higher cortisol, leucine supplementation in group III showed a significant decrease in plasma glucose level compared to immobilized group to be non-significantly differ from its level in control group. The reduced glucose level with leucine may be attributed to the effect of leucine in stimulating insulin secretion and inhibiting glucagon secretion especially in high glucose state **(67).**

This decrease in blood glucose with leucine supplementation in group III may explain the apparent increased glycogen in gastrocnemius muscle compared with the observed decreased glycogen content with cast-immobilization in group II. Moreover, the reduced glucose level with leucine treatment in group III in spite of the level of cortisol in this group is non-significantly reduced, dictate the impact of leucine interaction with other hormones.

Mallory stained sections of group II cast-immobilized gastrocnemius muscles in this study showed increased content of collagen fibers in both endomysial and perimysial connective tissues compared with group I. These findings are in line with **Järvinen *et al.* (68)** who noticed that the amount of collagenous connective tissue was dramatically increased in the endomysium and perimysium of an immobilized gastrocnemius muscle, with complete disorganization in the perimysial connective tissue. They concluded that intramuscular fibrosis contributed to the deteriorated function and biomechanical properties of the immobilized atrophied skeletal muscle.

On the other hand, leucine administration to rats of group III did not reveal increase in collagen content compared with either control or group II. These results could be explained according to the notes reported by **Babraj *et al.* (69)** in which collagen synthesis rates in skeletal muscle did not respond to increased amino acid levels as leucine because they did not stimulate fibroblast collagen synthesis in skeletal muscle in vivo.

Significant increased serum calcium level was noticed in cast-immobilized group II in this study. Hypercalcemia was attributed to impairment of bone mineralization due to the local effect of casting **(70).**

Moreover, group II in this study showed that cast-immobilization induced osteoporosis proved by the significant decrease in the mean trabecular bone volume, in mean osteoid thickness and in mean percentage of relative osteoid surface and significant increase in the mean percentage of the relative bone resorption eroded surface compared with control; resulting in bone loss demonstrated as small pieces of bone spicules of thin cancellous bone trabeculae showing resorption areas. **Jee and Yao (71)** found that casting-immobilization in rats induced osteoporosis resulting in 60% trabecular bone loss associated with a statistically significant increase in bone resorption and a decrease in bone formation and was significant as early as 14 days from immobilization. They added that immobilization-induced tibial metaphysic model is an appropriate model to test anabolic agents in the prevention and treatment of osteoporosis in an adult.

Widening of bone marrow spaces and apparent increased numbers of adipocytes compared with the control group was also noticed in group II in this study. **Elabd *et al.* (13)** noted that osteoblasts and adipocytes share the same precursor cell, and there is an inverse relationship exists between the two lineages. Thus increase in bone resorption is accompanied by increased bone marrow adiposity.

This study showed that leucine administration to cast-immobilized rats of group III partially prevented osteoporosis since their cancellous bone showed significant increase in the mean trabecular bone volume, in mean osteoid thickness and in mean percentage of relative osteoid surface and significant decrease in the mean percentage of the relative bone resorption eroded surface compared with group II; resulting in protection of the cancellous bone trabeculae of secondary spongiosa of the proximal tibia metaphysis. Coinciding, **Eneroth *et al.* (72)** noted that protein supplementation enhanced recovery from hip fracture surgery and decreased fracture-related complications. Moreover, leucine was previously reported to markedly suppress

proteolysis and inhibit whole-body protein degradation *in vivo* **(73)**. In-addition **Babraj *et al.* (69)** noted that bone collagen synthesis which is an important aspect of bone healing, responded to increased amino acid levels. This is in sharp contrast to the effect of leucine on skeletal muscle collagen. They suggested that lack of response in skeletal muscle and the acute stimulation in bone of collagen synthesis might be indicative of the different roles that collagen plays in the tissues of the musculoskeletal system.

The increased serum tumor necrosis factor alpha (TNFα) in immobilized group II may indicate a condition of inflammation and also may be implemented in the process of muscle atrophy as well as the osteoporotic changes. Tumor necrosis factor reported to inhibit myogenesis through redox-dependent and -independent pathways **(74, 75 & 76).** Moreover, immobilization is commonly associated with increased circulating inflammatory cytokines that was suggested to stimulate osteoclastogenesis and to suppress osteoblast recruitment **(8, 77 & 78).** TNF-α is expressed by T-lymphocytes but both the stromal cell and osteoclast express its receptor. TNF-α promote osteoclast formation and also it has potent antiapoptotic effects on osteoclasts, prolonging their lifespan **(79 & 80).**

In leucine treated group, although the value of serum level of TNFα decreased though non-significantly compared to immobilized non treated group II, and it was still non-significantly higher compared to control group, the skeletal muscle atrophic changes were markedly prevented and the osteoporotic bone changes were partially prevented as demonstrated histologically and histomorphometrically. This is in contradiction with **Lang *et al.* (76)** who mentioned that a cooperative interaction between both TNF-alpha and glucocorticoids during sepsis and inflammation induces leucine resistance and failure of leucine to perform its effect efficiently. Thus from the results in this study, it appeared that there is no resistance which might be attributed to absence of sepsis and or inflammation. However, substitution of the methionine residue by leucine in TNFα –converting enzyme, which mediate the release of multiple membrane proteins signaling the effect of TNFα, showed to inactivate TNFα **(81)** and this may explain the ability of leucine in preventing the effects of TNFα.

This study showed that leucine administration to rats of group III partially prevented osteoporosis secondary to cast-immobilization compared with group II. However, leucine administration to rats of group III did not completely prevent the secondary spongiosa osteoporosis of the proximal tibial metaphysis as the case in the gastrocnemius muscle atrophy compared with group I. There were still significant decrease in the mean trabecular bone volume, in mean osteoid thickness and in mean percentage of relative osteoid surface and significant increase in the mean percentage of the relative bone resorption eroded surface in group III compared with control. **Iwaniec *et al.* (82)** noted that cancellous bone formation rate was significantly affected by loading status and weight bearing. Moreover, it was previously reported that complete recovery from disuse may never occur except with return to normal weight bearing **(41)**.

Thus bone recovery depends on muscle integrity so the results of this study suspected that the apparently healthy non atrophied leucine treated muscle in group III appearing similar to control will enhance and complete the recovery of the residual bone effects of immobilization after cast removal and remobilization. On the other hand, the effect of the atrophied muscle on osteoporotic bone in group II will take more time in recovery if it will occur completely.

Conclusion

Increased MDA, Cortisol, TNFα with immobilization may explain in part the associated changes in musculoskeletal system. Leucine markedly prevented skeletal muscle atrophy and partially prevented cancellous bone osteoporosis which could be attributed to its direct anabolic effect or its ability to reduce oxidative stress and /or its ability to counteract the effect of Cortisol and TNFα rather than reducing their levels. Overall, these data suggesting that increasing leucine availability may represent a nutritional strategy for limiting muscle and bone protein loss as a consequence of immobilization.

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