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ORIGINAL ARTICLE

Ameliorative Effect of L-carnitine VS L-carnitine with Rifaximin on Skeletal Muscle Changes in Experimentally Induced Liver Cirrhosis in Adult Male Albino Rats (Biochemical and Histological Study)

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ABSTRACT

Background: Sarcopenia and liver cirrhosis are two prevalent worldwide diseases. The current work aims to compare the effect of L-carnitine alone to that combined with Rifaximin on the skeletal muscle of cirrhotic rats.

Methods: Forty adult male rats were divided equally into four groups. Group I (Control group), group II (Cirrhotic group) cirrhosis was induced by bile duct ligation for 5 weeks. Group III (Cirrhotic+ L-carnitine), rats were given L-carnitine 100 mg/kg orally daily for 5 weeks from the first day of induction of liver cirrhosis. Group IV (Cirrhotic+ L-carnitine+ Rifaximin) rats were received L-carnitine (100 mg/kg) with Rifaximin (100 mg/kg) orally daily for 5 weeks from the first day of induction of cirrhosis. Skeletal muscle tissue was prepared for H&E staining, immunohistochemical staining for Bax and CD34 and electron microscope examination. Blood samples were collected for serum alanine aminotransferase (ALT) and albumin measurement. Plasma and muscle ammonia, serum levels of lipopolysaccharide (LPS) and IL-6 measurements were also assessed.

Results: H&E-stained sections showed disorganized fibers with congested blood vessels in group II while electron photomicrographs showed areas of myofibrillar loss. Some muscle fibers appeared organized and others disorganized in group III. Most of the muscle fibers in group IV appeared nearly normal. Regarding LPS, IL6, ammonia, myostatin and TNFα expression, the highest mean values were observed in group II while the least mean values were observed in group IV.

Conclusion: Combination of L-carnitine and Rifaximin has a more ameliorative effect on the histological and biochemical changes induced by liver cirrhosis on the skeletal muscle of adult male albino rats.

Key words: L-carnitine; Rifaximin; Skeletal muscle; Liver cirrhosis; Albino rats.

INTRODUCTION

Liver cirrhosis, a global disease, is primarily caused by fatty liver disease, excessive alcohol consumption, viral hepatitis, autoimmune disorders, and high iron or copper levels. [1].

Compensated cirrhosis is an asymptomatic stage, followed by decompensated cirrhosis, which requires

hospitalization, reduces quality of life, and increases mortality rates [2]. The condition starts with inflammation, transforming the liver into fibrotic tissue and regenerating nodules, affecting various body systems, including the musculoskeletal system [3].

Skeletal muscle wasting caused by liver cirrhosis has a complex pathophysiology that includes malnutrition, hyperammonemia, alterations in hormone levels, particularly insulin growth factor-1 (IGF-1), persistent inflammation, elevated resting consumption of energy, and reduced levels of physical activity [4].

Satellite cells, which are located near the skeletal muscle fibers, are required for their lifelong preservation. They act as stem cells and are activated by growth signals or physical damage. Satellite stem cells divide symmetrically to increase in quantity or asymmetrically to produce progenitors after being activated. Myogenic progenitors multiply and finally differentiate by joining together or by fusing with injured fibers to restore the integrity and functionality of the fiber [5].

L-carnitine, also known as hydroxy-g-N-trimethyl aminobutyric acid, is an endogenous chemical that participates in the process of oxidation of fatty acids and is produced by hepatocytes, kidneys, and the brain from l-lysine amino acids that prevents the loss of skeletal muscle because of its anti-inflammatory and antioxidant effects. Advanced research has shown that L-carnitine may slow the progression of skeletal muscle loss by reducing hyperammonemia in cirrhotic individuals [6].

Rifaximin is a poorly absorbed antibiotic used to reduce hyperammonemia in cirrhosis and hepatic encephalopathy cases, with broad-spectrum activity against Gram-positive and negative bacteria [7].

Kumar et al. demonstrated that Rifaximin's reduction in ammonia levels could prevent skeletal muscle loss [8]. Also, Murata et al. suggested that combination therapy of L-carnitine and Rifaximin would benefit patients with cirrhosis-related muscle loss [9]. The study aimed to compare the ameliorative effects of L-carnitine alone or in combination with Rifaximin on skeletal muscle histological alterations in cirrhotic adult male albino rats.

METHODS

Forty adult male rats weighing 200–250 gm were used. They were acquired from Zagazig University Faculty of Medicine's Breading Animal House. The protocol was authorized by the medical research ethics committee of Zagazig University in Egypt with approval number ZU-IACUC/3/F/117/2023. Rats were kept in standard conditions during the experiment.

Drugs:

-L-carnitine (Carnitol 500 mg capsule): purchased from Global Napi Company for pharmaceutical and chemical industries

-Rifaximin (550 mg tablet): purchased from Sigma, a company for the pharmaceutical and chemical industries.

The animals were divided equally into 4 groups (ten rats for each group):

Group I (control group):

-Subgroup Ia (negative control): Rats were given normal saline orally once daily for 5 weeks.

-Subgroup Ib (positive control): Rats were given L-carnitine (100 mg/kg body weight) dissolved in 2 ml saline and given orally by oro-gastric tube daily for 5 weeks.

-Subgroup Ic (positive control): Rats received Rifaximin (100 mg/kg body weight) dissolved in saline and were given orally by oro-gastric tube daily for 5 weeks.

Group II (cirrhotic group):

Bile duct ligation was used to produce cirrhosis within 5 weeks [10].

Rats were anaesthetized with phenobarbital (40 mg/kg body weight) by I.P. injection, then the common bile duct was exposed and ligated by two surgical sutures. The first suture was made below the hepatic duct junction, and the second one was made above the pancreatic duct entry. The common bile duct was then resected between the ligatures. The abdominal incision was closed with two layers of sutures [10]. Then the rats were returned to their cages. The procedure was carried out at Zagazig University's Faculty of Medicine's Animal House.

Group III (cirrhotic +L-carnitine group):

Rats received L-carnitine 100 mg/kg body weight (about 20 mg/rat/day) dissolved in 2 ml saline and were given orally by oro-gastric tube daily from the first day of duct ligation for 5 weeks [11].

Group IV (Cirrhotic+ L-carnitine+ Rifaximin group):

Rats were co-treated with L-carnitine (100 mg/kg) and Rifaximin (100 mg/kg) (10 ml/kg) dissolved in saline and given orally via an oro-gastric tube daily from the first day of duct ligation for 5 weeks [12].

At the end of the experiment, rats were euthanized using pentobarbital (40 mg/kg) [13]., blood samples were drawn from the femoral vein, and muscle specimens were taken from the right gastrocnemius

muscle for biochemical and histological procedures. Also, liver specimens were taken for histological purposes only.

Light microscope technique

● H&E-stained sections:

The specimens were fixed in 10% formol saline for 24 hours and then processed to prepare 5 µm-thick paraffin sections and then stained with H&E stain [14].

● Immunohistochemical stained sections for detection of:

1- Bax: to detect apoptosis.

2-CD34: to detect satellite cells differentiation.

The **5µm**-thick sections were treated with primary then secondary antibodies. For Bax, the primary antibody is rabbit polyclonal anti-Bax antibody and the secondary antibody is Goat anti Rabbit IgG (Fc): Biotin, Catalog: AF4117. For CD34, the primary antibody is monoclonal mouse CD34 antibody and the secondary antibody is a biotinylated goat-anti-mouse IgG, Catalog: AF820. Immunoreactivity was visualized using 3,3′-diaminobenzidine-hydrogen peroxide, with Mayer's hematoxylin as a counterstain. Negative control sections were created. An Olympus microscope was used for examination, and digital cameras were used for pictures. Scale bars were applied to photomicrographs [15].

Transmission electron microscope technique:

For electron microscopic studies, tissue specimens were cut into small pieces (0.5–1.0 mm^3), prefixed in 2.5% glutaraldehyde for 2 hours, then postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4 and 4 °C for 2 hours. Specimens were then dehydrated and embedded in epoxy resin to obtain resin blocks. Semithin (1µm) and ultrathin (60–90 nm) sections were cut using a Leica ultracut (Glienicker, Berlin, Germany). Semithin sections were stained with toluidine blue (1%) and examined by a light microscope. Ultrathin sections were inspected under a light microscope, and pictures were taken at the Agricultural Faculty at Mansoura University in Egypt, which utilized a JEOL JEM 2100 EXII electron microscope [16].

Histo-morphometric analysis

Measurements were made with a "Leica Qwin500" image analyzer computer system in the pathology department of the Dentistry Faculty at Cairo University of the mean area percentage of muscle fiber and immunoreactivity for Bax and CD34. In each group of five randomly selected rat slides, measurements were taken in ten non-overlapping fields at a magnification of 400 [17].

Biochemical investigations

Collection and preparation of samples

Rat blood samples were collected, preserved at 4 °C, and samples were washed with ice-cold saline, cut into smaller fragments, weighed, and homogenized with 50 mM phosphate buffer (pH 7.4). Centrifugation was done at 10,000 g for 20 min at 4 °C, and the supernatant was preserved at −80 °C for various biochemical assays [18].

Measurement of serum ALT and albumin

Serum ALT and albumin levels were detected using a Rat Alanine Aminotransferase ELISA kit (cat. no. ab285264, Abcam) and a Rat Albumin ELISA kit (cat. no. ab108789, Abcam).

Measurement of plasma and muscle ammonia

Ammonia levels in plasma and muscle were measured by the Ammonia Assay Kit (cat. no. ab83360, Abcam).

Measurement of lipopolysaccharide (LPS), IL-6 serum levels

Analysis was done using ELISA kits from R&D Systems (Minneapolis, MN, USA). Optical density values were detected at 450 nm using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA).

TNFα and myostatin gene expression in muscle tissue by quantitative real-time PCR (qRT-PCR)

Total RNA extraction was done from tissue homogenate using Trizol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. To evaluate the quality of RNA, the A260/A280 ratio was done using the NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies; Wilmington, Delaware, United States). The steps of reverse transcription and complementary DNA (cDNA) formation were done using HiSenScriptTMRH [-] for cDNA Synthesis Kit, INtRON Biotechnology, China. Gene expression analysis was evaluated by qRT-PCR using 5 uL of the cDNA, 10 pmol/uL of each primer (1 uL each), and 10 uL of SYBR Green 2x Master Mix Green (QuantiTect SYBR Green PCR Kits,Qiagen). The real-time RT-PCR was performed in a Mx3005P Real-Time PCR System (Agilent Stratagene, USA) following the manufacturer's instructions. Primers used for real-time PCR were as follows: rat TNF-α; forward primer 5′-AAATGGGCTCCCTCTATCAGTTC-3′ and reverse primer 5′-TCTGCTTGGTGGTTTGCTACGAC-3′; and myostatin forward primer 5′-CCTGGAAACAGCGCCTAACA-3′ and reverse primer 5′-ATGGTGGTGAAGACGCCAGTA-

3′. Gene relative expression was normalised to GAPDH expression with a forward primer (5′-GGCACAGTCAAGGCTGAGAATG-3′) and a reverse primer (5′-ATGGTGGTGAAGACGCCAGTA-3′) and measured by the $2^{-\Delta\Delta Ct}$ equation, which was then established as fold change compared with controls [19].

Statistical analysis:

The IBM SPSS 19.0 programme was used [20]. We expressed all obtained data as the mean ± standard deviation (SD). ANOVA was used, followed by least significant difference (LSD) for comparison between groups. $P<0.05$ was considered a significant difference.

RESULTS

General observation:

No rats lost their lives or displayed symptoms of gangrene or limb loss during the observation period.

Five days following the development of cirrhosis, rats in the experimental group were observed with limbing, which gradually improved with L-carnitine or L-carnitine + Rifaximin treatment. Control rats walked normally through the experiment.

Histological results:

1) Light microscopic results:

H&E- stained liver sections (Figure 1):

Hepatocytes arranged into hepatic cords radiate from the central vein with rounded vesicular nuclei and acidophilic cytoplasm in the control group. Between hepatocyte cords, blood sinusoids were observed. Hepatic arteries and bile ducts were seen in the portal area (Figure 1a & 1b).

A dilated central vein with a detached endothelial lining appeared in the cirrhotic group. Hepatic lobules showed hepatocytes with vacuolated cytoplasm and darkly stained nuclei. Portal area with bile duct proliferation and dilated congested portal vein were observed (Figure 1c & 1d).

H&E- stained skeletal muscle sections (Figure 2):

Control group sections showed gastrocnemius muscle's long, cylindrical, unbranched muscle fibers, multiple peripherally flattened nuclei, and acidophilic cytoplasm. Perimysium appeared separating muscle bundles from each other. (Figure 2a).

The cirrhotic group Cirrhotic group revealed distorted wavy muscle fibers. Some muscle fibers displayed distorted organization and lightly stained foci. Cellular infiltration, and accumulated fat cells in-between, excessive connective tissue (CT) and congested blood vessels were also observed (Figure 2b & 2c).

L-carnitine group sections showed cylindrical muscle fibers with peripherally enlarged vesicular nuclei and acidophilic cytoplasm. There were a few centronucleated muscle fibers. (Figure 2d).

In Cirrhotic+ L-carnitine+ Rifaximin group, most of muscle fibers appeared normal and there were few centronucleated muscle fibers. Narrow spaces were detected (Figure 2e).

Immunostaining for detection of Bax (Figure 3):

The control group displayed no cytoplasmic immunoreaction to Bax in skeletal muscle fibers (Figure 3a). The cytoplasm of skeletal muscle fibers in the cirrhotic group showed an intense positive immunoreaction for Bax (Figure 3b). The cirrhotic+ L-carnitine group showed a moderately strong positive immunoreaction for Bax in the cytoplasm of several muscle fibers (Figure 3c). In the Cirrhotic+ L-carnitine + Rifaximin group, there were few skeletal muscle fibers that showed a weak positive immunoreaction for Bax (Figure 3d).

Immunostaining for detection of CD34 (Figure 4):

Few CD34-positive cells were found in the control group's peripheral fibers (Figure 4a). The cirrhotic group had CD34-positive spindle-shaped cells within muscle fibers (Figure 4b). Cirrhotic+ L-carnitine group displayed some CD34-positive spindle-shaped cells near muscle fiber's periphery (Figure 4c). Multiple positive CD34 spindle-shaped cells were seen in the Cirrhotic+ L-carnitine+ Rifaximin group (Figure 4d).

2) **EM Results** (Figure 5):

Electron photomicrographs of longitudinal sections of the control group's gastrocnemius muscle (I) revealed a myocyte with parallel myofibril organization and narrow inter-myofibrillar spaces. The dark and light bands were alternating. The light band was split in half by the Z line. Z lines were surrounded by pairs of small mitochondria, with peripherally larger ones. There was a euchromatic nucleus (Fig. 5a). A large nucleolus and flattened peripheral euchromatic nucleus below the sarcolemma were seen (Fig. 5b).

Cirrhotic group II showed areas of myofibrillar loss with wide spaces between myofibrils. Aggregated mitochondria and a large autophagic vacuole with heterogenous content were noticed (Fig. 5c). A heterochromatic nucleus with an indented nuclear envelope, fat droplets in between myofibrils and dilated mitochondria with destroyed cristae were seen. There was a congested blood vessel (Fig. 5d).

Cirrhotic+ L-carnitine group (III) showed a myocyte with some disorganized and other normal myofibrils.

Mitochondria, collagen fibrils, and autophagic vacuoles were noticed (Fig. 5e).

Cirrhotic+ L-carnitine + Rifaximin group (IV) showed an apparently normal myocyte with normal regular organization of myofibrils containing consecutive Z lines and reduced inter-myofibrillar gap. There was a focal area of degeneration. Apparently normal mitochondria around the Z line, glycogen granules, and collagen fibrils were noticed (Fig. 5f).

Morphometric results (Table 1& Figure 6).

- There was a highly statistically significant difference in the mean area percent of muscle fiber among the four groups. There was a statistically significant decline in the cirrhotic group compared to the control group. While the L-carnitine group represented a significant decline over the control group, However, there was no statistically significant difference between the control group and the L-carnitine+ Rifaximin group.

-There was a highly statistically significant difference in the mean area% of Bax in the studied groups. The cirrhotic group showed a statistically significant rise compared to the control group. Comparing the cirrhotic group to the L-carnitine group, a significant reduction was seen. The difference between the control and L-carnitine and Rifaximin groups was not statistically significant.

- There was a highly statistically significant difference in the mean CD34 area%. The cirrhotic group showed a statistically significant decline as compared to the L-carnitine and rifaximin groups. In comparison to the control group, there was a highly statistically significant rise in the L-carnitine + Rifaximin group. The difference between the cirrhotic group and the control group was statistically significant. In comparison to the control and cirrhotic groups, a statistically significant increase was observed in the L-carnitine group.

Biochemical results:

Serum Alanine transaminase enzyme (ALT) levels in all studied groups:

A significant difference was recognized in the serum ALT level of rats in different groups; the highest mean value of ALT level in serum was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of serum ALT was observed in the control group and the Rifaximin+L-carnitine group. The results were statistically significant ($p \leq 0.05$). A comparison between groups revealed a statistically significant increase in serum ALT level in group 2 compared to groups 1 and 4. Also, there was a statistically significant increase in serum ALT level in group 3 compared to groups 1 and 4. However, there was no significant difference between group 2 and group 3 (Table 2).

Serum Albumin levels in all studied groups:

A significant difference ($p \leq 0.05$) was found in the serum albumin level of rats in different groups. The highest mean value of serum albumin was observed in the control group and the Rifaximin+L-carnitine group, while the least mean value of serum albumin was observed in the cirrhotic group and the L-carnitine-treated group. A comparison between groups revealed a statistically significant increase in serum albumin level in group 1 compared to groups 2, 3, and 4. Statistically significant increase in serum albumin level in group 4 compared to group 2, group 3, and group 1. However, there was no significant difference between group 4 and group 3 (Table 2).

Serum lipopolysaccharide (LPS) levels in all studied groups:

A significant difference ($p \leq 0.05$) was found in the serum LPS level of rats in different groups; the highest mean value of serum LPS was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of serum LPS was observed in the Rifaximin+L-carnitine and control groups. A comparison between groups revealed a statistically significant increase in serum LPS level in group 2 compared to groups 1, 3, and 4. There was a statistically significant increase in serum LPS level in group 3 compared to group 4 and group 1 (Table 2).

Serum IL 6 levels in all studied groups:

A significant difference ($p \leq 0.05$) was found in the IL-6 serum level of rats in different groups; the highest mean value of IL-6 serum level was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of IL-6 serum level was observed in the Rifaximin+L-carnitine group and the control group. Comparison between groups revealed a statistically significant increase in IL6-Serum level in group 2 compared to group 1, group 3, and group 4. Statistically significant increase in IL-6 serum level in group 3 compared to group 4 and group 1 (Table 2).

Plasma Ammonia levels in all studied groups:

A significant difference ($p \leq 0.05$) was found in the plasma ammonia level of rats in different groups; the highest mean value of plasma ammonia level was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of plasma ammonia level was observed in the control group and the Rifaximin+ L-carnitine group. A comparison between groups revealed a statistically significant

increase in plasma ammonia levels in group 2 compared to groups 1 and 4. Statistically significant increase in plasma ammonia level in group 3 compared to group 4, and group 1. Also, there was a significant difference between group 2 and group 3 (Table 3).

Muscle Ammonia levels in all studied groups:

A significant difference ($p \leq 0.05$) was found in the MS ammonia level of rats in different groups. The highest mean value of the MS ammonia level was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of the MS ammonia level was observed in the control group and the Rifaximin+ L-carnitine group. A comparison between groups revealed a statistically significant increase in MS ammonia levels in group 2 compared to groups 1, 3, and 4. Statistically significant increase in MS ammonia level in group 3 compared to group 4 and group 1 (Table 3).

Muscle myostatin gene expression levels in all studied groups:

A significant difference ($p \leq 0.05$) was detected in the myostatin expression fold change level of rats in different groups. The highest mean value of myostatin expression level was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of myostatin expression level was observed in the control group and the Rifaximin+ L-carnitine group. Comparisons between groups revealed a statistically significant increase in myostatin expression level in group 2 compared to groups 1, 3, and 4. Statistically significant increase in myostatin expression level in group 3 compared to group 4, and group 1. However, there was no significant difference between group 1 and group 4 (Table 4).

Muscle TNF α expression levels in all studied groups:

A significant difference ($p \leq 0.05$). was detected in the TNF α expression fold change level of rats in different groups; the highest mean value of TNF α expression level was observed in the cirrhotic group and the L-carnitine-treated group, while the least mean value of TNF α expression level was observed in the control group and the Rifaximin+L-carnitine group. There was a statistically significant increase in TNFα expression level in group 2 compared to groups 1, 3, and 4. Statistically significant increase in TNF α expression level in group 3 compared to group 4, and group 1. Also, there was a significant difference between group 1 and group 4 (Table 4).

Table 1: Mean values (± SD) of morphometric parameters in the studied groups**:**

	Control group	**cirrhotic group**	**l-carnitine group**	**l-carnitine rifaximin + group**	**F**	**P value**
Mean area% of muscle fiber	81.2±6.2	68.6±6.04*	75±6.7	79.4±7.9	**32.25**	****0.001>**
Mean area % of Bax	23.9±15	67.4±17.5*	47.01±15.4	25.9±8.01	**19.78**	****0.001>**
Mean area% of CD34	10.8±0.4	2.5±0.03*	20.9±0.8	25.8±0.6	**50.09**	****0.001>**

highly statistically significant difference **0.001> ****
statistically significant difference 0.001 *

Table (2): Mean values (± SD) of serum ALT, albumin, LPS and IL-6 in the studied groups:

Groups	Group 1 Normal) (group	Group 2 Diseased) (group	Group 3 (L-carnitine treated group)	Group 4 (*Combined treatment (Rifaximin+L-carnitine)* group)	f	P value	LSD
	mean±SD range	mean±SD range	mean±SD range	mean±SD range			
Serum ALT	3±54.10 (60-50)	7.82±239.90 (250-224)	8.08±237.20 (248-221)	4.82±209.10 (217-203)	1970.5	**0.001>	**P1<0.001 **P2<0.001 **P3<0.001 P4=0.344 **P5<0.001 **P6<0.001
Serum Albumin	0.14±3.81 (4-3.6)	0.14±2.59 (2.8-2.4)	0.14±3.57 (3.8-3.4)	0.08±3.66 (3.8-3.5)	189.5	**0.001>	**P1<0.001 **P2<0.001 *P3=0.012 **P4<0.001 **P5<0.001 P6=0.123
Serum LPS	0.16±2.70 (2.9-2.5)	0.16±4.34 (4.6-4.1)	0.11±3.42 (3.6-3.2)	0.09±2.89 (3-2.8)	308.8	**0.001>	**P1<0.001 **P2<0.001 *P3=0.003 **P4<0.001 **P5<0.001 **P6<0.001
IL 6-Serum	1.62±162.8 (165-160)	3.24±195.6 (200-190)	2.72±185.6 (190-182)	1.27±166.5 (168-165)	440.6	**0.001>	**P1<0.001 **P2<0.001 *P3=0.001 **P4<0.001 **P5<0.001 **P6<0.001

One way ANOVA test LSD (least significant difference) * *highly significant
P1 group 1 vs Group 2 P2 group 1 vs Group 3
P3= group 1 vs Group 4 P4= group 2 vs Group 3
P5= group 2 vs Group 4 P6= group 3 vs Group 4

Table (3): Plasma and muscle ammonia levels in the studied groups:

Groups	Group 1 Normal) (group	Group 2 Diseased) (group	Group 3 (L-carnitine treated group)	Group 4 (*Combined treatment (Rifaximin+L-carnitine)* group)	f	P value	LSD
	mean±SD range	mean±SD range	mean±SD range	mean±SD range			
Plasma Ammonia	3.21±89.10 (95-85)	2.69±164.4 (170-161)	2.57±160.2 (163-157)	6.3±110.68 (120-100)	830.8	0.001> **	**P1<0.001 **P2<0.001 **P3<0.001 *P4=0.035 **P5<0.001 **P6<0.001
MS Ammonia Spectro	0.13±0.43 (0.6-0.2)	0.15±3.78 (4-3.6)	0.17±3.3 (3.5-3)	0.18±2.28 (2.5-2)	907.3	0.001> **	**P1<0.001 **P2<0.001 **P3<0.001 **P4<0.001 **P5<0.001 **P6<0.001

One way ANOVA test LSD (least significant difference) * *highly significant

P1 group 1 vs Group 2 P2 group 1 vs Group 3

P3= group 1 vs Group 4 P4= group 2 vs Group 3

P5= group 2 vs Group 4 P6= group 3 vs Group 4

Table (4): Myostatin and TNF α relative expression levels in the studied groups:

Groups	Group 1 Normal) (group	Group 2 Diseased) (group	Group 3 (L-carnitine treated group)	Group 4 (*Combined treatment (Rifaximin+L-carnitine)* group)	f	P value	LSD
	mean±SD range	mean±SD range	mean±SD range	mean±SD range			
Myostatin expression	0.03±1 (1.04-0.97)	0.13±2.86 (3-2.6)	0.30±2.59 (3-2)	0.02±1 (1.03-0.98)	384.2	**0.001>	**P1<0.001 **P2<0.001 P3=0.945 *P4=0.001 **P5<0.001 **P6<0.001
TNF α expression fold change	0.01±1 (3-0.97)	0.17±4.71 (5-4.5)	0.11±4.32 (4.5-4.2)	0.15±1.24 (1.5-1)	2449.6	**0.001>	**P1<0.001 **P2<0.001 **P3<0.001 **P4<0.001 **P5<0.001 **P6<0.001

One way ANOVA test LSD (least significant difference) * *highly significant

P1 group 1 vs Group 2 P2 group 1 vs Group 3

P3= group 1 vs Group 4 P4= group 2 vs Group 3

P5= group 2 vs Group 4 P6= group 3 vs Group 4

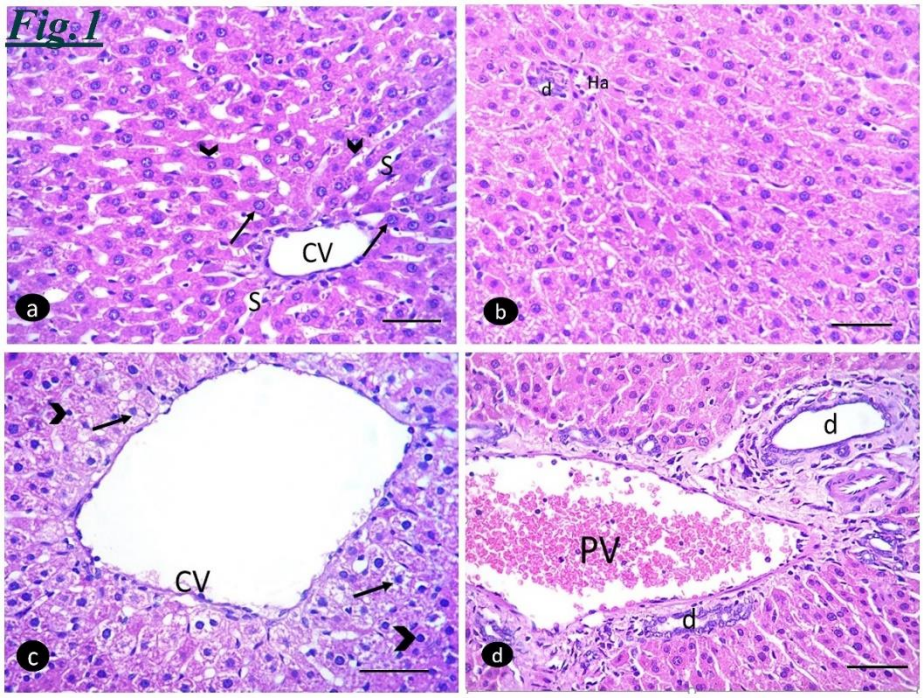

Fig.

(1): Photomicrograph of sections stained by H&E of rats' liver: (a) The control group demonstrates hepatocytes (arrow) radiating from the central vein (CV) with rounded vesicular nuclei and acidophilic cytoplasm. Blood sinusoids are seen, along with their endothelial lining (arrowhead). (b) Another control group section reveals the hepatic artery (Ha) and bile duct (d) in the portal area. (c) The cirrhotic group exhibits a dilated central vein with the endothelial lining detached (CV). Hepatocytes with vacuolated cytoplasm (arrow) and darkly colored nuclei (arrowhead) are seen. (d) Another cirrhotic group section showing bile duct proliferation (d) and dilated congested portal vein (PV) apparent in the portal area.

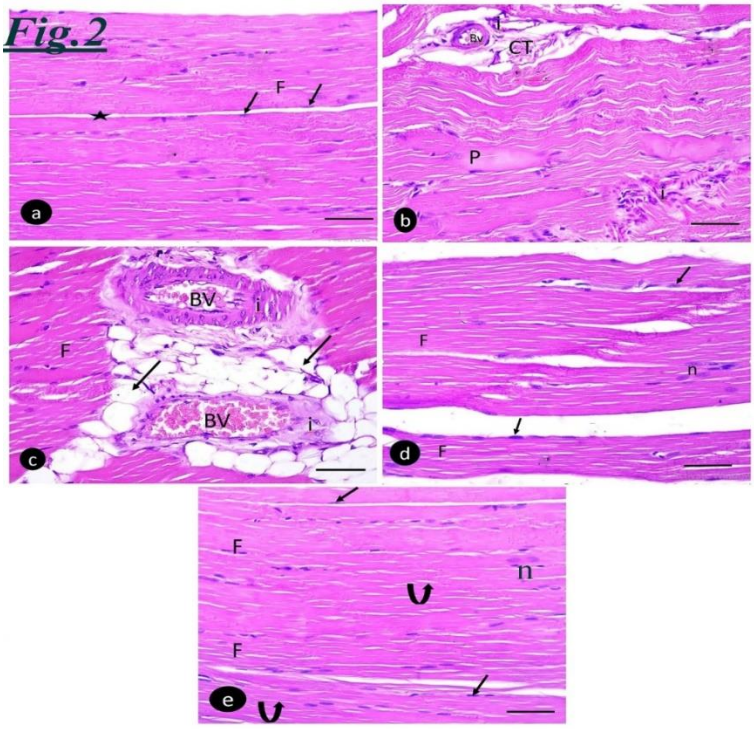

Fig (2): Photomicrograph of sections stained by H&E of rats' muscle: (a) The control group reveals gastrocnemius muscle's long, cylindrical, unbranched muscle fibers (F), many peripherally flattened nuclei (arrow), as well as acidophilic cytoplasm. The perimysium (asterisk) separates muscle bundles from each other. (b) The cirrhotic group reveals distorted, wavy muscle fibers. Some muscle fibers display cellular infiltration (i) and lightly stained foci (P). Connective tissue (CT) in the perimysium surrounding congested blood vessels (Bv) is also observed. (c) Another section of the cirrhotic group showing distorted muscle fibers (F) with extensive accumulation of fat cells in-between (arrow). Cellular infiltration (i) and some dilated, congested blood vessels (Bv) are visible. (d) The L-carnitine group shows cylindrical muscle fibers (F) with peripherally enlarged vesicular nuclei (arrow) and acidophilic cytoplasm. There are few centronucleated muscle fibers (n). (e) L-carnitine + Rifaximin group showing majority of muscle fibers appear normal (F) with peripheral flat nuclei (arrow) and there are few centronucleated muscle fibers (n). Little spaces are seen (curved arrow).
(H&E X400, scale bar 30 μm).

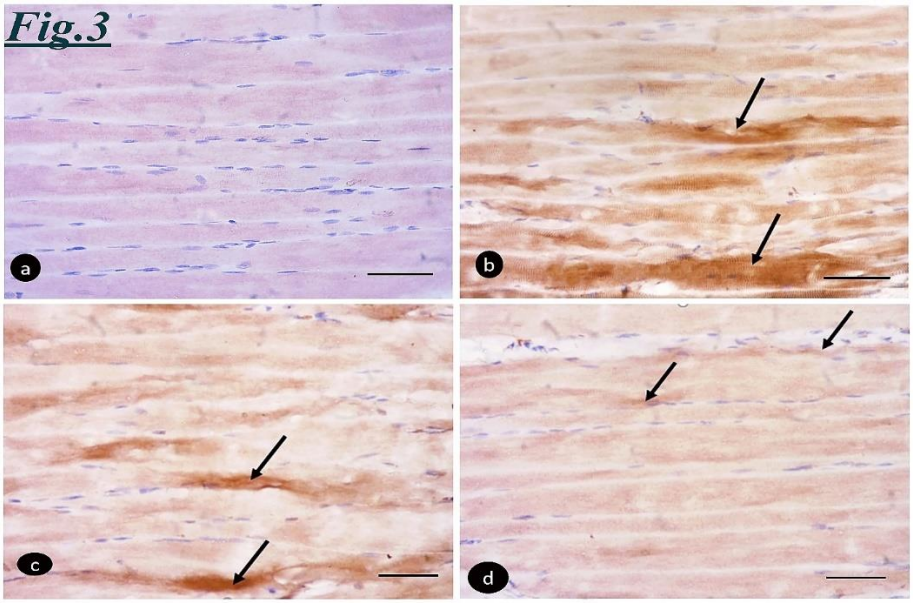

Fig. (3) Photomicrograph of Bax immunostained sections of rats' muscle: (a) The control group shows negative Bax immunoreactivity in the cytoplasm of skeletal muscle fibers. (b) The cirrhotic group reveals an intense positive immunoreaction for Bax (arrow) in the cytoplasm of skeletal muscle fibers. (c) The L-carnitine group shows a moderate positive immunoreaction for Bax (arrow) in the cytoplasm of some muscle fibers. (d) L-carnitine + Rifaximin group reveals a weak positive immunoreaction for Bax (arrow) in the cytoplasm of a few skeletal muscle fibers.
(Immunoperoxidase technique for Bax X 400, scale bar 30µm**).**

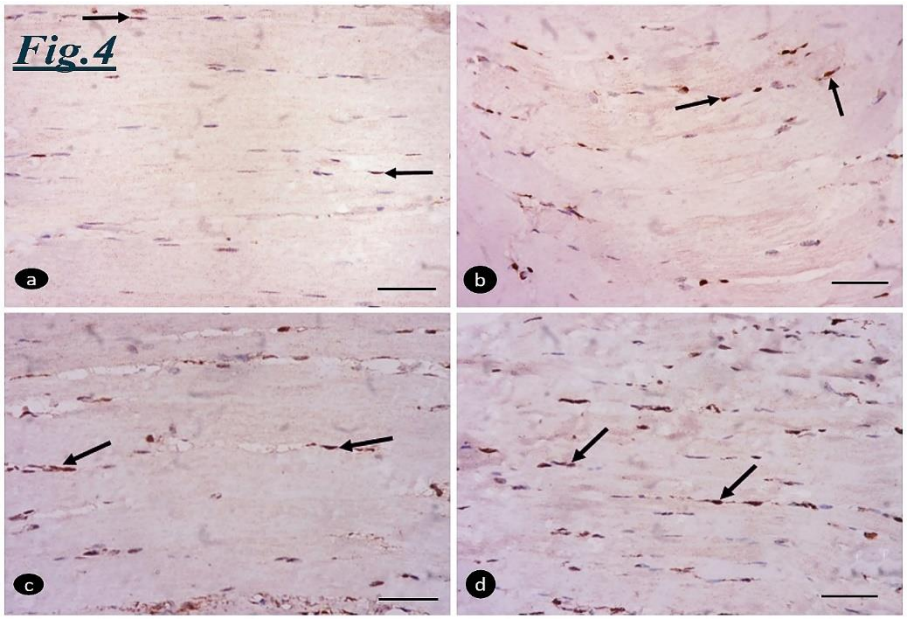

Fig. (4). Photomicrograph of CD34 immunostained sections of rats' muscle: (**a) The control group** reveals few CD34-positive cells at the periphery of some muscle fibers (arrow). **(b) Cirrhotic group** displaying sporadic CD34 spindle-shaped cells that are positive (arrow) at the periphery of muscle fibers **(c) The L-carnitine group** shows some positive CD34 spindle-shaped cells (arrow) at the periphery of the muscle fibers. **(d) L-carnitine**

+ Rifaximin group showing multiple positive CD34 spindle-shaped cells (arrow) at the periphery of muscle fibers.

(Immunoperoxidase technique for CD34 X 400, scale bar 30µm).

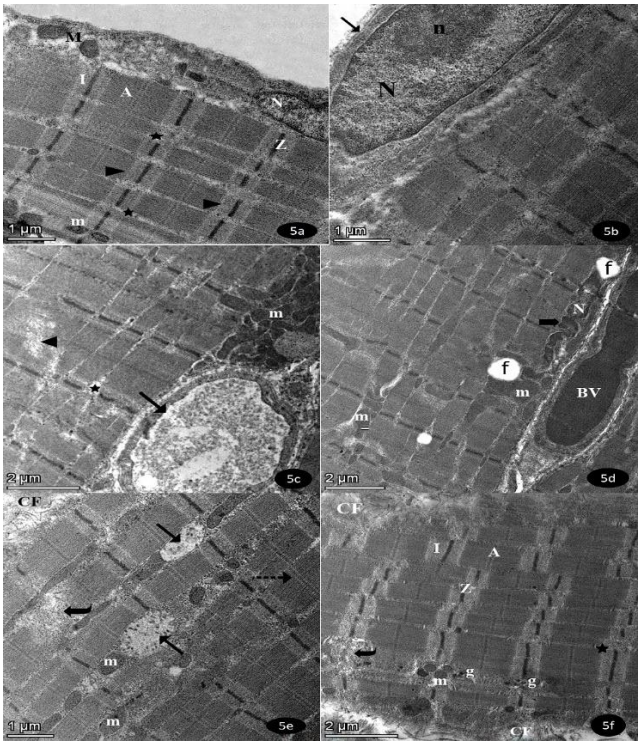

Fig.(5): Electron photomicrographs of longitudinal sections in the gastrocnemius muscle of **the control group** show **(a)** a myocyte with a parallel arrangement of myofibrils (arrow head) and narrow inter-myofibrillar spaces (asterisk). There are alternating light (I) and dark (A) bands. The light band is divided by the Z line. Around the Z lines, little mitochondria (m) are paired, whereas some larger mitochondria (M) are located peripherally. An euchromatic nucleus is seen (N). (TEM; X 12.000). **(b)** Another section showing a flattened peripheral euchromatic nucleus (N) with a prominent nucleolus (n) just beneath the sarcolemma (arrow) (TEM; X 14.000). **Cirrhotic group** showing **(c)** areas of myofibrillar loss (arrow head) and wide spaces between myofibrils (asterisk) Aggregated mitochondria (m) are seen. Notice a large autophagic vacuole (arrow) with heterogeneous content. (TEM; X 10.000). **(d)** A heterochromatic nucleus (N) with an indented nuclear envelope (thick arrow) is observed. There is a congested blood vessel (BV) with RBCs inside it. Fat droplets (f) in between myofibrils and dilated mitochondria with destroyed cristae (m) are seen. (TEM; X 9000).

L-carnitine group showing **(e)** a myocyte with some myofibrils that appear disorganized (curved arrow), while other myofibrils appear normal (knotted arrow). Mitochondria (m), collagen fibrils (CF), and autophagic vacuoles (arrow) are noticed. (TEM; X 12.000)

L-carnitine + Rifaximin group showing **(f)** an apparently normal myocyte having a normal regular arrangement of myofibrils with light (I) and dark (A) bands and clear successive Z lines (Z) and decreased intermyofibrillar space (asterisk). There is a focal of degeneration (curved arrow). Apparently, normal mitochondria (m) are seen around the Z line. Note glycogen granules (g) and collagen fibrils (CF). (TEM; X 10.000).

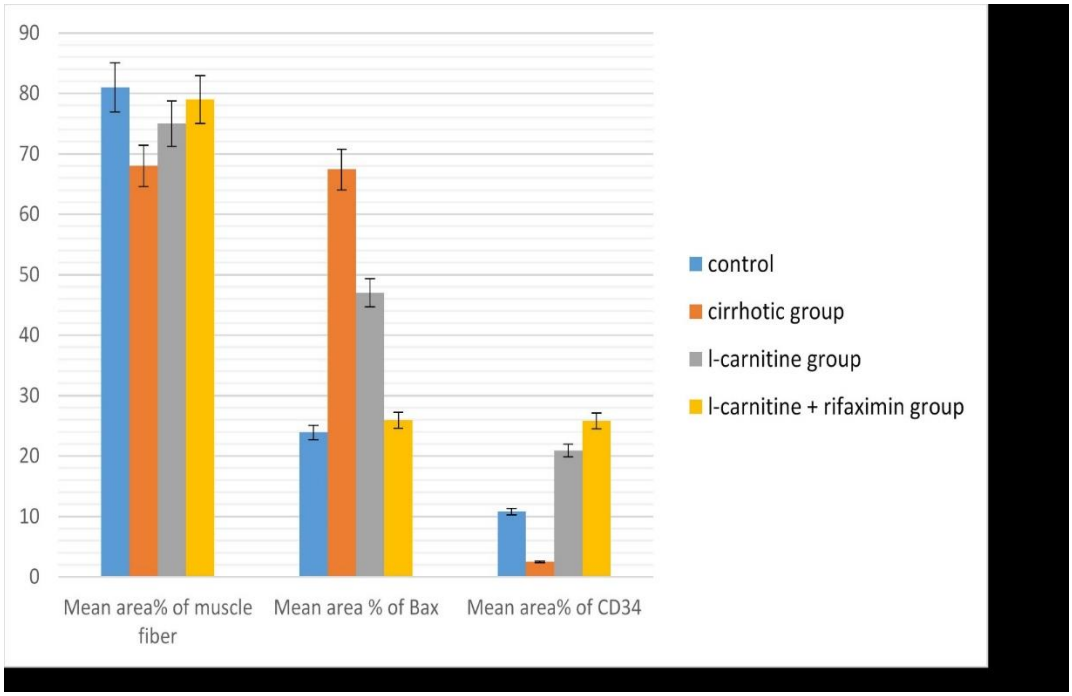

Fig. (6): Mean values of area percent of muscle fibers, Bax and CD34 immune reactions in different studied groups.
(** P< 0.001 highly statistically significant difference between different groups, * P< 0.001 statistically significant difference compared to cirrhotic group).

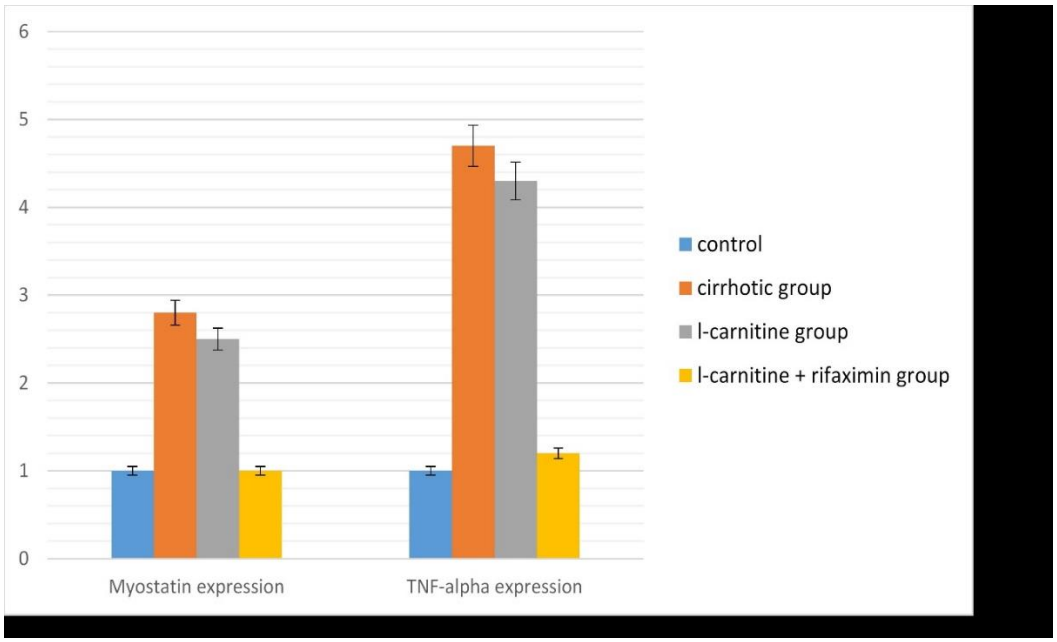

Fig. (7): Myostatin and TNF-α relative expression levels in the studied groups.
(** P< 0.001 highly statistically significant difference between different groups, * P< 0.001 statistically significant difference compared to cirrhotic group).

DISCUSSION

The major comorbidity associated with liver cirrhosis is sarcopenia, or skeletal muscle loss. So, this work aimed to compare the effects of L-carnitine alone and combined with Rifaximin on the histological changes in the skeletal muscles of cirrhotic adult male albino rats [21].

By examination of H&E specimens, the cirrhotic group revealed disrupted muscle fibers. It was confirmed by electron photomicrographs which showed areas of myofibrillar loss, wide spaces between myofibrils and disruption of Z lines. This can be explained as cirrhotic individuals experience increased blood ammonia levels due to a defective urea cycle, which inhibits protein synthesis in skeletal muscles by phosphorylating the mTORC1 signal (mammalian target of rapamycin complex 1) [22].

Cell infiltration and centrally dark-stained nuclei were detected in the cirrhotic group. Beyer et al. stated that the ubiquitin-proteasome pathway is activated in cirrhosis, triggering inflammatory cells and promoting the production of inflammation mediators like TNF-α and IL-1 [23].

Extensive accumulation of adipocytes between distorted muscle fibers in the cirrhotic group sections was detected. Walter et al. reported that liver cirrhosis disrupts L-carnitine storage in liver and skeletal muscle cells, disrupting fat metabolism and accumulation and affecting L-carnitine's role in lipid breakdown [24].

A heterochromatic nucleus with an indented nuclear envelope and vacuoles were seen in the cirrhotic group. Nakanishi et al. reported that liver cirrhosis causes a L-carnitine shortage in skeletal muscle cells, disrupting fat breakdown and ATP synthesis, which serves as an energy source. ATP deficiency leads to malfunction of calcium adenosine triphosphatase pumps with increased intracellular calcium levels, fluid disruption with vacuolation in skeletal muscle cells and membrane destruction [25].

The L-carnitine group showed mild improvement in comparison to the cirrhotic group in H&E sections. Some myofibrils appeared disorganized with degenerated parts and the loss of Z lines, while other myofibrils appeared normal in electron photomicrographs. Montesano et al. stated that L-carnitine enhances myogenin production and regulates IGF-1, promoting myotube differentiation and muscle hypertrophy, thus preventing muscle degeneration [26].

The L-carnitine-group revealed few centronucleated muscle fibers. Cadot et al. stated that muscle injury triggers the activation, pr oliferation and myoblast differentiation of satellite cells.

The production of the repaired myofibers is aided by the new myoblasts.

Myotubes, multinucleated syncytia formed by the f usion of myoblasts, develop into myofibers [27].

During the creation of myofibers, distinct nuclear motions and positioning events take place. Follo wing fusion, the fusing myoblasts' nuclei rapidly tr avel towards the centre of the myotube, a process k nown as nuclearcentration. Nuclear spreading then occurs as

nuclei move from the fiber's centre to its periphery as the myotube develops into a myofiber, where th ey become uniformly dispersed [28].

Supplementation with L-carnitine significantly improves energy metabolism and lowers systemic inflammation in people with liver cirrhosis. Additionally, L-carnitine administration increases liver metabolic activities like gluconeogenesis, fatty acid metabolism, albumin synthesis, and ammonia reduction through the urea cycle [29].

Numerous studies have evaluated L-carnitine's role and potential efficacy in many physiological and pathophysiological situations, including physical activity, cardiac disease, ageing, managing weight, and brain function. Skeletal muscle, heart, and neurological systems all operate significantly worse when L-carnitine levels are low [30,31].

The mean area percent of Bax immunoreaction in the cirrhotic group exhibited a highly statistically significant increase. Beyer et al. reported that an inflammatory state linked to liver cirrhosis results in apoptosis, increasing myostatin levels, which hinder muscle satellite cell proliferation and development [32].

The mean area percent of Bax in the L-carnitine group showed a considerable decline when compared to the cirrhotic group but showed a significant rise when compared to the control. Studies show that L-carnitine has anti-apoptotic properties due to its antioxidant properties, which reduce the harmful effects of free radicals on tissue and prevent apoptotic stages [29].

The mean CD34 area% showed a statistically significant difference between the studied groups. The cirrhotic group showed a statistically significant decline as compared to the L-carnitine and rifaximin groups. The L-carnitine group

showed a highly statistically significant rise compared to the control group.

Alfaro et al. proved that CD34 has many roles, including homing receptor and pro-adhesive receptor, in the regeneration process in skeletal muscle, which demonstrates an amazing ability to regenerate quickly while fully restoring its mass and function after being injured. In order to repair the damage, muscle stem cells, known as satellite cells, arise, give rise to myoblasts, commit to terminal differentiation, then fuse with one another or with preexisting myofibers. During regeneration, satellite cells move in both directions, from the viable area to the injured zone and from a necrotic portion to the periphery [32].

L-carnitine stimulates satellite cells by modulating mitochondrial biogenic gene expressions, promoting the passage of long-chain fatty acids for beta-oxidation and gene expression modulation [33].

L-carnitine controls satellite cell progression through regulatory myogenic factors, e.g., Myf5, to direct them towards muscle cell proliferation. Also, it inhibits genes in the ubiquitin proteasome system, which leads satellite cells to differentiate [34].

Cirrhotic+ L-carnitine + Rifaximin group revealed the majority of muscle fibers appear normal with peripheral flat nuclei. Rifaximin enhances the L-carnitine effect in the improvement of sarcopenia in cirrhotic rats by inhibiting the higher levels of inflammatory mediators, e.g., TNF, in skeletal muscle and blocking their harmful effects on increasing myostatin and stimulating autophagy [35]. Also, it lessens hyperammonemia, which causes skeletal muscle wasting associated with hepatic cirrhosis [36].

According to Murata et al., the benefits of L-carnitine and Rifaximin on wasting skeletal muscle were achieved without any drug toxicity, and both drugs are clinically available for people with chronic liver illness. These findings suggested that this combined regimen may be clinically advantageous for cirrhosis-related sarcopenia [11].

Regarding biochemical results, serum levels of ALT significantly increased in cirrhotic rats in comparison with controls. L-carnitine induced a mild decrease in the level of ALT, but the combination of Rifaximin and L-carnitine significantly decreased the level towards normal. However, Murata et al. showed that ALT levels were not affected by neither Rifaximin nor L-carnitine [11]. Also, Han et al. who compared cirrhotic rats and rats after injection of Rifaximin, found that there was no difference in the level of ALT and albumin between the two groups [37]. Nakano et al. found no significant difference in the level of ALT after treating chronic liver disease patients with L-carnitine [38].

The present study revealed improved albumin levels in rats after injection of L-carnitine and Rifaximin. This goes hand in hand with Ohara et al. who showed that albumin levels significantly improved after taking L-carnitine in patients with liver cirrhosis. Sarcopenia and loss of muscle mass may occur by several mechanisms; one of them is decreasing protein synthesis [39].

Ammonia levels were quantified. Many studies found that hyperammonemia is a consistent abnormality in cirrhosis, combined with impaired skeletal muscle protein synthesis and breakdown (proteostasis). However, after the intake of effective ammonia-lowering therapies, lowering ammonia neither restores proteostasis nor reverses muscle mass [10]. In our study, the combination of Rifaximin and L-carnitine effectively lowered the level of ammonia.

Results showed a decrease in myostatin expression after combination therapy with L-carnitine and Rifaximin. Supporting our results, Kumar et al. demonstrated increased myostatin skeletal muscle expression with increased levels of ammonia [10]. Myostatin is a myokine that regulates muscle mass. Nakano et al. showed that myokines have various biological functions through stimulation of receptors on the liver, bone, fat, brain, heart, and muscle. In particular, serum levels of myostatin in patients with liver cirrhosis are known to be significantly higher compared with healthy controls. Also, elevated serum myostatin levels were associated with elevated ammonia levels and muscle atrophy in patients with liver cirrhosis [38].

Inflammatory cytokines have been proven to impair intramuscular mitochondrial biogenesis. In our study, results revealed a decrease in TNF-α expression after supplementation with L-carnitine and a significant decrease towards normal after combination therapy with Rifaximin [11]. Murata et al. reported that L-carnitine significantly decreased the sequence of muscle degradation induced by TNF-α stimulation compared to Rifaximin, which did not directly affect the myotubes [11].

Also, our study focused on the Rifaximin effect on the level of endogenous LPS, which may cause

impaired intestinal barrier integrity. L-carnitine improved both levels of LPS and IL-6, but after adding Rifaximin, levels of LPS and IL-6 greatly decreased. Our results were in line with a previous study, which revealed that Rifaximin inhibits the hepatic LPS/TLR4 signaling pathway that causes the blunting of intestinal hyperpermeability and suppresses the proinflammatory response and fibrogenesis [11].

CONCLUSIONS

The combination of L-carnitine and rifaximin has a more ameliorative effect on the histological and biochemical changes induced by liver cirrhosis on the skeletal muscle while lowering the levels of ammonia and inflammatory mediators.

Recommendations:

Many clinical studies are needed to investigate the benefit of the L-carnitine and Rifaximin combination in improving the hepatic cirrhotic effects on skeletal muscle.

Conflicts of interest: The authors declare that they have no conflicts of interest.

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