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Regular paper

Muscle adaptation to sleeve gastrectomy: Potential role of nutritional supplementation and physical exercise

Entesar Ali Saber[1*], Neven Makram Aziz[2*✉], Mohammed Mustafa Abd El Aleem[3*], Maha Yehia Kamel[4], Seham A Abd El-Aleem[1], Fatma Farrag Ali[2], Doaa Mohamed Elroby Ali[5] and Amira Fathi Behery[1]

[1]Department of Histology and Cell Biology, Minia University Faculty of Medicine, Minia, Egypt; [2]Department of Medical Physiology, Minia University Faculty of Medicine, Minia, Egypt; [3]Department of Anatomy, Minia University Faculty of Medicine, Minia, Egypt; [4]Department of Pharmacology, Minia University Faculty of Medicine, Minia, Egypt; [5]Department of Biochemistry, Sohag University, Faculty of Pharmacy, Sohag, Egypt

Skeletal muscle is metabolically and functionally flexible and contractile under normal conditions. Obesity is a risk factor that causes metabolic disorders and reduces muscle contractility. Sleeve gastrectomy (SG) has been used for surgical correction of obesity. This work aimed to investigate how obesity and its surgical correction affects skeletal muscle and the possible role of nutritional supplementation and physical exercise. Adult male albino rats were randomly divided into five groups, 8 rats per group: group Ia (control non-obese), group Ib (control obese), group II (post-operative, SG), group III (post SG + nutritional supplementation) and group IV (post SG + nutritional supplementation + physical exercise). SG resulted in cellular and metabolic degenerative disorders in the muscle including wasting, weakness and fibrosis with elevated inflammatory, oxidative and injury markers. Nutritional supplementation induced the post SG muscle regeneration indicated by high expression of insulin growth factor-1 (IGF-1) and myogenin and low expression of transforming growth factor beta 1 (TGF-β1). Interestingly, it improved the metabolic state of the muscle by reducing the oxidative stress, inflammatory and muscle injury markers and delaying the onset of fatigue. What is more, physical exercise along with nutritional supplementation resulted in further improvement of the muscle metabolic state and function. In conclusion, nutritional supplementations together with physical exercise after SG are essential for preserving muscle mass and contractility and improving its metabolic and functional status.

Keywords: gastrocnemius muscle, sleeve gastrectomy, nutritional supplementation, physical exercise, insulin growth factor-1, myogenin.

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✉e-mail: emadmax71@yahoo.com
*Delegated to Deraya University, New Minia City
Abbreviations: IGF-1, insulin growth factor-1; TGF-β1, transforming growth factor beta 1; SG, sleeve gastrectomy

INTRODUCTION

Obesity is one of the most important risk factors causing metabolic disorders that predispose to heart diseases, hypertension and diabetes (Gobl *et al*., 2017). Obesity affects muscle functions and with aging increases morbidity and mortality (Tallis *et al*., 2018).

Obese individuals usually tend to use mineral and vitamin supplementation to get a balanced diet (Ali *et al*., 2019). Calcium and vitamin D supplementation for correcting obesity-related vitamin D deficiency improves muscle mass and has an appetite-suppressing effect that helps reduce weight (Petroni *et al*., 2019).

Tallis and others (Tallis *et al*., 2018) reported that 6-weeks dietary intervention reversed the adverse changes in muscle function caused by essential fatty acids deficient diet. In contrast, a similar dietary intervention failed to reverse obesity-related changes in skeletal muscle function although improved the metabolic state (Seebacher *et al*., 2017). These treatment regimens reduce body weight, improve insulin sensitivity and control of blood sugar level, thus, are effective over a short time. Therefore, they are difficult to maintain as lifestyle changes and often unrealistic to manage the disease in the long term. Recently, surgical interventions that alter intestinal physiology were found to be more effective. Though its durability remains unclear, the approach may be the best one for successful, long-term management of body weight and obesity-related chronic diseases. Currently, the two most popular bariatric surgeries are gastric bypass surgery (RYGB) and gastrectomy (SG). Both approaches result in significant weight loss and blood sugar control in humans and animals (Sinclair *et al*., 2018).

Despite the multiple clinical benefits, a number of surgical and digestive complications can occur after bariatric procedures. The potential complications include undernutrition that can develop as a result of reduced intake of macronutrients and micronutrients, with subsequent development of anemia and protein malnutrition (Lupoli *et al*., 2017).

Physical exercise is nowadays an indispensable component of a healthy lifestyle able to reduce the risk of developing cardiovascular, endocrine, muscular and immunity disorders. Regular physical activity can counteract the negative effects and toxicity of oxidative stress that develops with aging (Simioni *et al*., 2018).

The aim of this study was to investigate the molecular, cellular and physiological adaptation of the gastrocnemius muscle to SG. Moreover, we studied the possible role of nutritional supplementation and physical exercise in improving the muscle state following SG. The muscle was assessed using biochemical markers, electrical stimulation response technique, gene expression study, histological and immunohistochemical techniques.

MATERIAL AND METHODS

Animals

Sixty Sprague Dawley rats (3 weeks old, 95–100 g body weight) from the National Research Centre (Giza, Egypt) were kept under standard laboratory conditions with natural day/night cycle. Rats were housed in cages measuring 45 × 40 × 65 cm, fed commercial rat chow and left freely wandering for one week before the onset of the study. After one week, the body weight and the food consumed (initial food intake) were measured weekly for 6 weeks. The choice of the duration in the present experiment was based on the previous finding of (Brinckerhoff *et al*., 2013) who reported that six weeks in rats are equivalent to 3 years of human life.

Rats were divided into two categories: normal non-obese rats (10 rats) received a standard diet and the obese group (50 rats) received a high-fat diet (HFD). After the 6 weeks, Lee index was calculated for each rat according to the formula:

$$Lee\ index = \sqrt[3]{\frac{Body\ weight\ in\ gram}{Naso-anal\ length\ in\ mm}}\ x\ 10000$$

Only forty-five rats fed with HFD had a Lee index ≥0.3 and were considered obese (Campos *et al*., 2008) and, subsequently, divided into four groups plus control non-obese group (8 rats each after calculating the mortality rate) for further 6 weeks.

Finally, the rats were divided into the following groups:

Group **Ia** (10 rats): control non-obese rats fed a commercial standard diet.

Group Ib (10 rats): control obese rats fed an HFD.

Group II (15 rats): obese rats subjected to sleeve gastrectomy "SG" and then fed a standard diet with no further treatment. Only 8 rats survived the surgery (survival rate=50%).

Group III (10 rats): obese rat which undergone SG fed a standard diet supplemented with an oral daily dose of sodium selenite (0.05 mg/kg, Sigma) (Zhang *et al*., 2018) plus "vitamin B complex" (9.036 mg/kg, Amriya Pharmaceuticals, Egypt) (Ibrahim *et al*., 2017), vitamin D (2000 IU, Sigma), and calcium (500 mg/kg, Minapharm Pharmaceuticals) (Alatawi *et al*., 2018). In addition, each animal was gavaged orally with protein/essential amino acids (rich in leucine, 1.35 g/kg body weight) (Crozier *et al*., 2005). Two rats died post-operation (survival rate=80%).

Group IV (10 rats): obese rats which underwent SG and were fed a standard diet with the same supplementation as group III. In addition, equipped cages were used for housing post-operative rats until the end of the experiment (physical exercise equipment) according to the method of Makowska and Weary (Makowska & Weary, 2016) with modification. Two rats died post-operation (survival rate=80%).

Diet protocol

The standard diet (g/kg diet) formula was prepared as in Davidson and others (Davidson *et al*., 2012). HFD contained 20 g of fat/100 g of the diet and provided 4.1 kcal/g of the diet (Hariri & Thibault, 2010). Standard diet and HFD constituents were purchased from El-Gomhoria Company, Cairo, Egypt.

Body mass index (BMI)

BMI was done with light ether according to the method of Rabiu and others (Rabiu *et al*., 2017). It was calculated weekly according to the formula: BMI = body weight (g)/length^2 (cm^2).

Sleeve gastrectomy

Thirty-five obese rats were abstained from solid food for 12 hours and allowed access to water the day prior to the surgery. Rats subjected to SG were anaesthetized with an intra-peritoneal injection of sodium pentobarbital (60 mg/kg of body weight) according to the method of Valenti and others (Valenti *et al*., 2011). Post SG, rats were fed a liquid diet (5% glucose + 0.9% normal saline) for two days. On the third postoperative day, animals resumed regular rat chow and water *ad libitum* and treated groups were given the supplementation (Teive *et al*., 2012).

Electric stimulation of the right gastrocnemius muscle

After 6 weeks of the treatment and following overnight fasting, rats were anesthetized with urethane (1.5 g/kg; i.p.) and fixed to a dissection board as previously described by MacIntosh and others (MacIntosh *et al*., 2011). Force of contraction was recorded on a calibrated chart of oscillograph (Bioscience, USA). The maximal force (F) reached was determined from the calibrated chart. The time till 50% fatigue was determined according to the speed of the chart recorded by the oscillograph.

Biochemical analysis

After electrical stimulation forty rats were decapitated, blood samples were collected and sera were separated and stored at –80°C and both gastrocnemius muscles were excised for further analysis.

Serology

Glucose concentration was determined using enzymatic colorimetric methods (Bio-Diagnostic kits, Egypt).

Insulin concentration was assayed using an enzyme-linked immunosorbent assay (ELISA) kit (Calbiotech, USA).

Triglycerides (TGs), low-density lipoprotein cholesterol (LDL-c) and high-density lipoprotein cholesterol (HDL-c) levels were determined using enzymatic colorimetric methods (Bio-Diagnostic kits, Egypt). The atherogenic index was calculated according to Ikewuchi and others (Ikewuchi *et al*., 2014).

Albumin concentration was determined using the enzymatic colorimetric method (Spinreact SAU, Spain).

Ca^{+2} concentration was determined by enzymatic colorimetric method, using kits purchased from BioSystems, Spain.

25-hydroxyvitamin D was assayed using enzyme-linked immunosorbent assay (ELISA) kit (ALPCO, USA).

Molecular assessment of the muscle

The known weight of the right stimulated and its corresponding left gastrocnemius muscle were homogenized in phosphate buffer saline, centrifuged and the supernatants were used for determination of malondialdehyde (MDA) concentration as previously described Ohkawa and others (Ohkawa *et al*., 1979); tumor necrosis factor-α (TNF-α) concentration was estimated by ELISA kit (Lab Vision Corporation, USA) according to the manufac-

Table 1. The primers sequences for IGF-1and reference gene.

Gene title	Forward primer 5′-3′	Reverse primer 5′-3′
IGF-1	5′-AGC TGA GAT AGT GTT TCC CAA AGG-3′	5′-TTC CAA ACG CGA AAT GAA TG-3′
GAPDH	5′–CCT TCC GTG TTC CTA CC-3′	5-′AGG ATG CCC TTT AGT GG-3′

turer's instructions and muscle creatine kinase (CK) concentration using colorimetric kit (ElabScience, China). In addition, muscle glycogen level was determined by sulfuric acid method adopted by Ramu and others (Ramu *et al.*, 2016).

Assessment of muscle Insulin Growth Factor (IGF-1) RNA

Total RNA was isolated from the known weight of the left gastrocnemius muscle specimens using RNA isolation kit (RiboZol™ RNA Extraction Reagents, AMRESCO, LLC, Solon, Ohio, USA). High-quality RNA (A260/280 ≥ 1.6) was selected and used for quantitative real-time PCR and IGF-1 (Insulin growth factor-1, regeneration marker) and GAPDH genes were selected as target and internal reference gene, respectively. The sequence of the gene of interest was obtained from NCBI database and primers were designed via Thermo Fisher Scientific. Quantitative real-time PCR was performed using the One-Step TaqProbe qRT-PCR Kit (Applied Biological Materials, Canada). The sequence of the primers used in this study were summarized in Table 1. TaqMan qRT-PCR gene expression assays were conducted in 0.1 ml fast tubes according to the manufacturer's instructions, in a final volume of 50 µl. Thermal cycling was performed in the Bioer thermocycler using the following cycling conditions: 50°C for 1 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min.

Histological and Immunohistochemical Examination

The remaining part of the gastrocnemius muscle of the left hind-limb of each rat was fixed in 10% formal saline and processed for histological and immunohistochemical evaluation. Paraffin sections were stained with hematoxylin and eosin (H&E), Masson trichrome, Glees method (for nerve fibers) and immunohistochemical markers: myogenin (Halevy *et al.*, 2004), and transforming growth factor beta 1 (TGF-β1) (Delaney *et al.,* 2017). The second piece of the muscle was rapidly transferred to the cryostat for histochemical staining of succinate dehydrogenase as an indicator of the mitochondrial oxidative capacity (Chinopoulos, 2013) and acetylcholine esterase (AChE) as an important component of all cholinergic synapses in the central and peripheral nervous system (Bancroft & Gamble, 2008).

Morphometric analysis

For the morphometric analysis, an unbiased sampling procedure was applied. Four blocks/animal were used for cross and longitudinal sectioning. Blocks were randomly selected for serial sectioning and H&E stained for the light microscope examination (Olympus CX-31, Tokyo, Japan, equipped with ImageJ software). Morphometric analysis was performed on a cross and longitudinal section for each block on three randomly selected microscopic fields/section and the following parameters were obtained:

- The mean number of muscle fibers (the muscle mass).
- The mean number of cells expressing myogenin protein.
- The volume density (Vv) of the three types of muscle fibers and of collagen fibers.
- The cross sectional area of the muscle fibers (CSA).

Statistical analysis

Data were represented as means (for 8 rats after omitting the dead rats) ± standard error of the mean (S.E.M.). Statistical analysis was performed and the significancy of differences between the groups was assessed by one-way ANOVA followed by Tukey-Kramer posthoc test for multiple comparisons with a value of $P<0.05$ considered statistically significant .

RESULTS

Survival rate

The survival rate for the SG group (group II) was 50% due to marked loss of appetite resulting in wasting and death. Deaths occurred in post SG treated groups (III and IV) during anesthesia, surgery, or the first hours following surgery due to sepsis.

Assessment of the BMI and food intake

As shown in Table 2, the present study revealed that the initial BMI of all experimental groups were significantly higher compared with that of group Ia. In addition, group Ib showed the highest significance while group II showed the lowest significance of the final BMI as compared with the all experimental groups and with its initial values. The final BMI of group III showed a

Table 2. BMI and food intake in all experimental groups.
Data are expressed as mean± S.E.M. of 8 rats per group. BMI: Body mass index. a: significantly different from group Ia, b: significantly different from its corresponding group, c: significantly different from the final value of group Ia, $P\leq0.05$.

Parameters Groups	BMI		Food intake (g/d)	
	Initial	Final	Initial	Final
Group Ia	0.55±0.01	0.62±0.03	15.1±1.3	13.9±0.24
Group Ib	0.69±0.02[a]	0.88±0.02[bc]	19.9±1.4[a]	13.5±0.2[b]
Group II	0.67±0.01[a]	0.44±0.01[bc]	18.9±0.9[a]	9.1±0.4[bc]
Group III	0.70±0.02[a]	0.53±0.01[bc]	19.2±1.1[a]	11.3±0.1[bc]
Group IV	0.73±0.02[a]	0.65±0.01[b]	19.6±1.2[a]	12.6±0.1[bc]

Table 3. The serum parameters in the different experimental groups.
Data are expressed as mean ±S.E.M. of 8 rats per group. a: significantly different from group Ia, b: significantly different from group Ib, c: significantly different from group II, d: significantly different from group III, $P \leq 0.05$. TGs: Triglycerides; HDL: High-density lipoproteins; LDL: Low-density lipoproteins.

Groups Parameters	Group Ia	Group Ib	Group II	Group III	Group VI
TGs (mg/dl)	44.4±1.6	98.3±2.1^{a}	42.5±1.5^{b}	45.1±1.7^{b}	44.5±1.8^{b}
HDL-c (mg/dl)	56.5±1.4	32.4±1.3^{a}	54.8±1.4^{b}	55.8±1.5^{b}	62.8±1.7^{ab}
LDL-c (mg/dl)	24.5±1.2	50.2±1.7^{a}	21.5±1.3^{b}	26.5±1.3^{b}	21.5±1.3^{b}
Atherogenic index	–0.1±0.001	0.5±0.007^{a}	–0.1±0.007^{b}	–0.09±0.009^{b}	–0.15±0.005^{ab}
Fasting glucose (mg/dl)	98.1±0.7	210.2±2.1^{a}	90.6±0.5^{ab}	99.3±0.9^{bc}	96.4±0.8^{bc}
Insulin (mIU/l)	7.4±0.21	9.3±0.15^{a}	6.4±0.11^{ab}	7.1±0.24^{bc}	7.7±0.31^{bc}
Albumin (g/dl)	4.2 ± 0.1	3.6 ± 0.2^{a}	2.9 ± 0.1^{ab}	4.1 ± 0.1^{bc}	4.5 ± 0.2^{bc}
Ca^{+2} concentration (mg/dl)	9.5 ± 0.16	8.1 ± 0.11^{a}	7.5 ± 0.12^{ab}	9.2 ± 0.14^{bc}	9.8 ± 0.15^{bc}
25-hydroxyvitamin D (ng/dl)	26.5 ± 0.3	19.7 ± 0.1^{a}	18.5 ± 0.2^{ab}	24.5 ± 0.4^{bc}	28.5 ± 0.5^{bc}

lower significance level as compared with groups Ia and Ib and with its initial values. While the final BMI of group IV showed a lower significance from its initial BMI but the remaining showed insignificant difference when compared with final BMI of group Ia. This was accompanied with a significantly higher initial food intake of all experimental groups as compared with group Ia but the final food intake showed significantly lower values in all experimental groups except group Ib that was still insignificant compared to final food intake of group Ia.

Assessment of the serum parameters

As shown in Table 3, group Ib showed atherogenic serum lipid profile. There were significant higher values in the TGs and LDL-c with a significant lower value in HDL-c level and a significant higher value in the atherogenic index as compared with all experimental groups. Contrary, groups II and III showed insignificant differences in all serum lipid profile as compared to group Ia while group IV showed a significant higher value in HDL-c with insignificant difference in TGs and LDL-c levels.

As regard serum fasting glucose and insulin, group Ib showed significant highest values and group II showed the lowest values among the all experimental groups. While the group III and IV showed insignificance differences as compared with group Ia. On the other hand, group II showed the lowest values of albumin, Ca^{+2} and 25-hydroxyvitamin D among all the experimental groups followed by group Ib. While, groups III and IV showed insignificance differences as compared with group Ia.

Assessment of the gastrocnemius muscle parameters

In the resting state, muscle TNF-α and MDA levels showed a significant increase and CK and glycogen

Table 4. Mechanical and biochemical parameters of the right (stimulated) and left (resting) gastrocnemius muscles in the different experimental groups.
Data are expressed as mean ±S.E.M. of 8 rats per group. (Rest.: left resting, Stim.: right stimulated) a: significantly different from the resting limb, b: significantly different from the corresponding limb of the control group, c: significantly different from the corresponding limb of group Ib, d: significantly different from the corresponding limb of group II, e: significantly different from the corresponding limb of group III, $P \leq 0.05$. MDA: malondialdehyde; TNF-α: tumor necrosis factor α; CK: creatine kinase.

Groups Parameters		Group Ia		Group Ib		Group II		Group III		Group IV	
		Rest. muscle	Stim. muscle	Rest. muscle	Stim. muscle	Rest. muscle	Stim. muscle	Rest. muscle	Stim. muscle	Rest. muscle	Stim. muscle
Force (gm tension)			58.63± 1.97		15.75± 1.24^{b}		7.13± 1.1^{bc}		30± 1.30^{bcd}		60± 1.73^{cde}
Duration in seconds (till 50% fatigue)			12.13± 1.16		6.25± 0.75^{b}		4± 0.46^{b}		8± 0.80^{bd}		15.88± 1.16^{bcde}
At 50% Fatigue	TNF-α (Pg/mg)	20.2± 0.7	25.7± 0.9^{a}	38.6± 1.3^{b}	44.4± 1.1^{ab}	45.1± 1.2^{bc}	51.2± 1.3^{abc}	22.6± 1.1^{cd}	27.9± 0.8^{acd}	18.6± 0.9^{cde}	21.8± 0.7^{abcde}
	MDA (µmol/gm)	11.7± 0.8	23.6± 1.2^{a}	19.7± 0.6^{b}	44.6± 1.6^{ab}	22.7± 0.4^{bc}	49.9± 1.3^{abc}	13.2± 0.5 ^{cd}	26.1± 1.1^{acd}	11.2± 0.5^{cde}	18.3± 1.1^{abcde}
	CK (U/gm)	34.5± 1.4	51.3± 1.5 ^{a}	17.4± 0.7 ^{b}	22.1± 0.8^{ab}	14.7± 0.9^{bc}	18.4± 1.2^{abc}	31.5± 1.2^{cd}	45.5± 1.7^{abcd}	38± 1.3^{cde}	59.2± 1.4^{abcde}
	Glycogen (mg/gm)	4.3± 0.1	3.4± 0.2^{a}	3.1± 0.1^{b}	2.09± 0.1^{ab}	2.5± 0.1^{bc}	1.7± 0.09^{abc}	4.0± 0.2^{cd}	3.3± 0.1^{acd}	4.7± 0.2^{cde}	4.1± 0.2^{abcde}

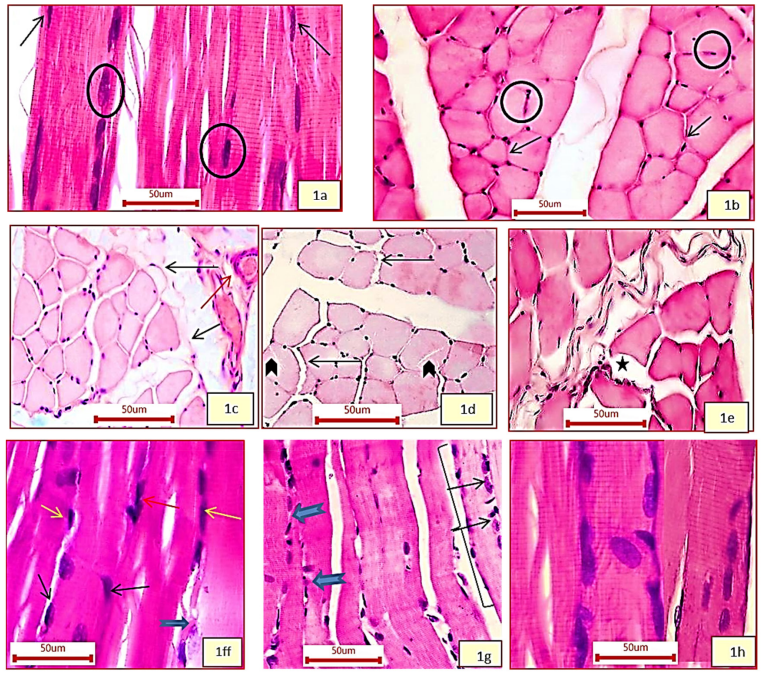

Figure 1. Photo-micrographic plates of muscles.
1a, 1b) Control group, showing L.S (1a) and T.S (1b) of muscle fibers. Polygonal muscle fibers exhibiting various diameters, mosaic distribution, peripheral nuclei (circles) and satellite cells (arrows). **1c)** Group Ib, showing small aggregates of adipocytes between muscle fibers (black arrows), close to blood vessels (red arrows). **1d-1f)** SG group, showing **1d)** enlarged, split (arrows) and fragmented faintly stained (arrowheads) fibers; **1e)** increased connective tissue density and prominent fibroblasts (asterisks); **1f)** myofibers with vesicular linearly arranged nuclei surrounded by satellite cells (black & yellow arrows), other fibers showing rounded, central (arrowhead) or irregular clumped nuclei (red arrow), and inflammatory cells infiltration (tailed arrow). **1g, 1h)** Post SG treated groups (III and IV) showing some fibers with multiple peripheral oval nuclei (tailed arrows), regenerating fibers (brace) small size basophilic with large nuclei (black arrows). Notice that in group IV (1h) muscle fibers appeared more or less normal with distinct striation and peripheral nuclei. H&E staining, x40 magnification.

showed a significant decrease in group II when compared to group Ia. However, groups III and IV showed insignificant changes (Table 4).

In the stimulated muscle, the parameters significantly changed as compared to its corresponding left resting muscle. SG group (II) showed a significant increase in TNF-α and lipid peroxides levels and a significant decrease in CK and glycogen levels. The profile remained the same in post SG treated groups.

Both the resting and the stimulated muscle showed a significant decrease in IGF-1 expression in groups II and Ib and a significant increase in groups III and IV when compared to group Ia (Fig. 8).

Histological and Immunohistochemical assessment

H&E sections:

In the control group (groups Ia, Ib), "L.S" and "T.S" showed the normal histological appearance of skeletal muscles (Fig. 1a and 1b). Intramuscular fat, either scattered or as small aggregation, was observed between muscle fibers in group Ib. Occasionally, it was seen in association with blood vessels (Fig. 1c).

In the SG group (group II), most of the muscle fibers showed normal appearance but some were hypertrophied (Fig. 1d), others atrophied with increased connective tissue density and fibroblasts (Fig. 1e). Occasionally, fibers were clumped with inflammatory cell infiltration (Figure 1f).

In group III, regenerating fibers with distinct transverse striations and multiple peripheral nuclei were observed (Fig. 1g).

In group IV, fibers appeared normal with peripheral nuclei. Linear central nuclei and widening of the endomysium were only seen in few muscle fibers**.** Fibroblast cell activation, leading to increased synthesis of extracellular matrix and fibers was clearly observed (Fig. 1h).

Histochemical assessment

Succinic dehydrogenase (SDH): Group Ia was mostly formed by moderately stained type IIA fibers (intermediate fibers), followed by weakly stained type IIB fibers (large fibers) and type I fibers (small and heavily stained fibers) which were occasionally observed (Fig. 2a). In group Ib, fast type IIB fibers were predominant (Fig. 2b). However, group II showed selective atrophy of type II fibers with loss of the staining, while type I fibers were not affected (Fig. 2c). In groups III & IV, muscle fibers consisted of a large population of oxidative fibers (type IIA and type I) and the lowest population of type IIB fibers (Fig. 2d and 2e).

Glees histochemical staining for nerve fibers: Group Ia showed intact nerves with hen leg appearance of myoneural junctions (Fig. 3a). In group Ib, nerve fibers appeared thinner (Fig. 3a). In group II, nerve fibers showed breakup and thinning (Fig. 3c, 3d). In group III, myelin sheaths and axons of regenerating nerves were significantly increased along the repaired zone (Fig. 3e).

Acetylcholine esterase (AChE) activity: AChE staining was observed in the neuro-muscular junctions (NMJ). In the control group Ia, the NMJs were visible as intensely stained oval, round or elliptical structures, compact and distributed along the border of the muscle fibers (Fig. 4a). In Ib and II group, they were lightly stained, smaller in size and less compact when compared to group Ia but more pronounced (Fig. 4b, c). In group III, the NMJs ap-

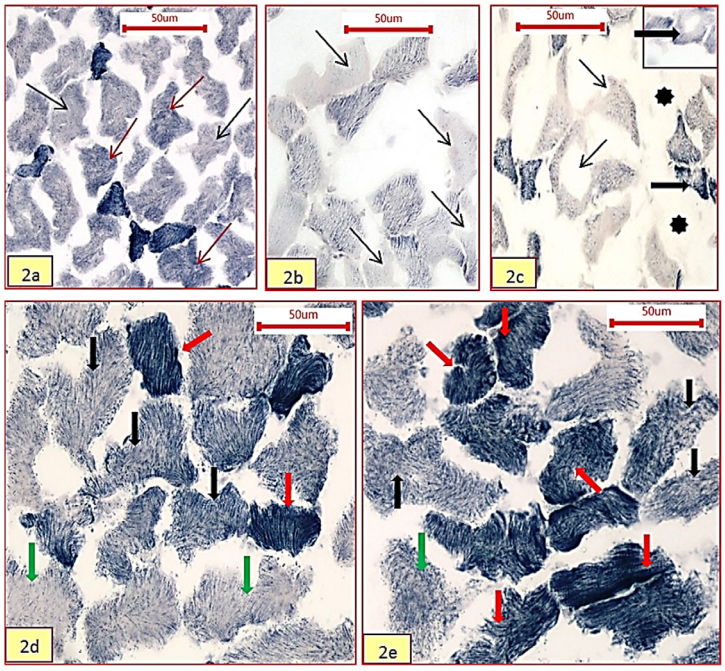

Figure 2. Succinic dehydrogenase enzyme (SDH) activity in the muscle.
2a) Control group Ia with type IIA fibers (brown arrows) being the most abundant and the pale IIB type (black arrows) the least abundant. **2b)** Group Ib with type IIB fibers being predominant (arrows). **2c)** Group II showing selective atrophy of type II fibers with central and eccentric loss of staining (arrows) and loss of staining (asterisks). Type I fibers show eccentric loss of staining (thick arrows). **2d, 2e)** Post SG treated groups III and IV showing hypertrophied fibers – mostly oxidative fibers type IIA (black arrows) and type I (red arrows) with few type IIB pale fibers (green arrow). Notice that group IV (2e) showed abundant type I dark fibers. SDH staining, x400 magnification.

peared the same as that of the control group Ia while in group IV they appeared more compact (Fig. 4d, e).

Masson trichrome staining of collagen: Collagen fibers were stained intensitylyn group Ib (Fig. 5a) and in the muscular tissue of group II. Collagen was approximately twofold greater in the interstitial space between fibers in the operated groups compared to the group Ia (Fig. 5b, c and d). The amount of intramuscular collagen was apparently maintained in group IV compared to the remaining groups. Groups III and IV showed fibroblast cells activation, leading to increased synthesis of collagens (Fig. 5e and 5f).

Myogenin expression: Group Ia showed an absence of myogenin expression while group Ib showed weak expression (Fig. 6a, b, c). The operated group II exhibited occasional expression in specialized myogenic cells in between the polyhedral muscle fibers (Fig. 6d). However, groups III and IV showed a high expression (Fig. 6e).

TGF-β1 expression: TGF-β1 was detected in the control groups (Ia & Ib), around blood vessels in the connective tissues and fibroblasts (Fig. 7a, b). Group II showed higher TGF-β1 expression (Fig. 7c, d). In groups III & IV lower expression was observed (Fig. 7e and 7f).

Morphometric analysis using the light microscope

Mean number of myofibers (MNF): There was an insignificant decrease in MNF in group Ib and group II compared to that of group Ia. There was an insignificant increase in the MNF in nutrition-supplemented groups III & IV and the increase was slightly higher in group IV as compared to the group II (Table 5).

Mean number of myogenin immunopositive cells (Myo. CN): The mean number of myogenin positive cells in group Ia was negligible. Group II showed a significant increase compared to group Ia. Further increase was seen in groups III and IV with the highest significance in group IV (Table 5).

Volume density (Vv.) of muscle fibers (Vv. of different types of muscle fibers) and Vv. col for

Table 5. The mean number of myofibers, myogenin immune-positive cells and the volume density of type I, IIA, and IIB muscle fibers and of collagen fibers of the gastrocnemius muscle in the different experimental groups.
Data are expressed as mean ±S.E.M. of 8 rats per group. a: significantly different from group Ia, b: significantly different from group Ib, c: significantly different from group II, d: significantly different from group III, $P \leq 0.05$. MNF: mean number of myofibers; Myo. CN: myogenin immune-positive cells; Vv: volume density.

Parameters Groups	MNF	Myo. CN	Vvs			
			type I fiber%	type IIA fiber%	type IIB fiber%	Vv col %
Group Ia	98±2.09	0.01±0.09	5.68±0.22	20.09±0.61	70.23±0.51	5.89±0.58
Group Ib	96.77±2.01	0.5±0.05^{a}	5.02±0.41	18.00±0.97	69.46±0.46	6.65±0.70
Group II	96.01±3.08	0.5±0.03^{a}	4.64±0.1^{a}	15.15±0.91^{a}	68.07±0.62	10.32±0.16^{ab}
Group III	100±2.11	6.32±0.4^{abc}	6.29±0.23^{bc}	29.64±0.55^{abc}	55.45±0.78 ^{abc}	9.32±0.36^{abc}
Group IV	102.13±1.12	8.91±0.5^{abcd}	6.50±0.31^{bc}	35.58±0.76^{abcd}	49.88±0.19 ^{abcd}	9.67±0.06^{abc}

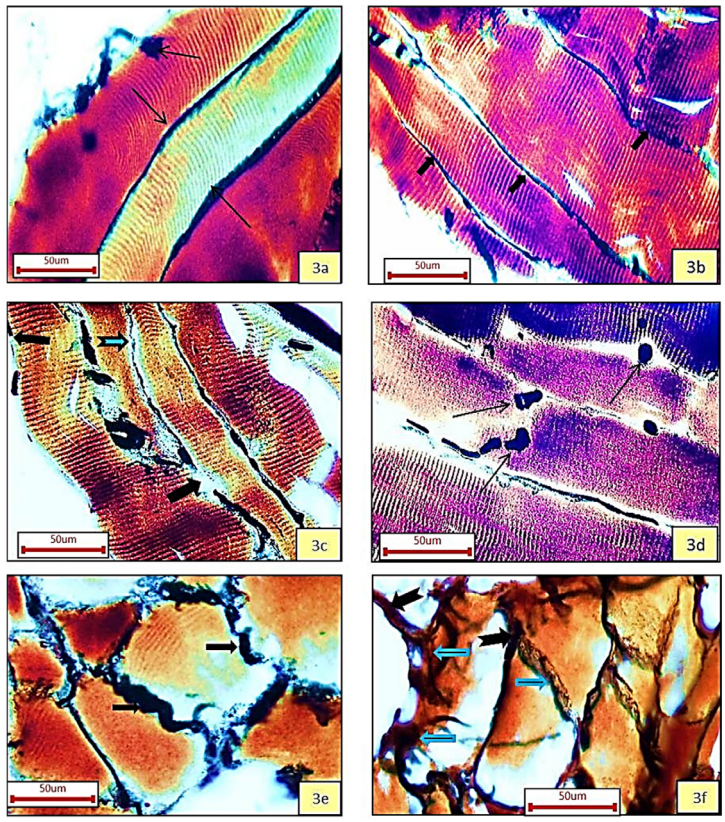

Figure 3. Glees staining for nerve fibers.
3a) The control group Ia showing intact nerve fibers with a continuous uniform impregnation of silver stain with hen leg appearance of nerve terminals on the surface of the muscle fibers (arrows). **3b)** Group 1b with thinner nerve fibers (yellow arrows) and shrinkage of the nerve terminals (black arrow). **3c)** SG group II with neurodegenerative changes visible as interrupted nerve fibers (yellow arrows), ghosts (tailed arrow), silver droplets around the degenerated nerves (**3d**, red arrows). **3e, f)** Thick myelin sheath in groups III (**3e**) and IV (**3f**) (arrows). Group IV showed extensive nerve terminal branching on the surface of muscle fibers (tailed arrow). Glees histochemical staining, x400 magnification.

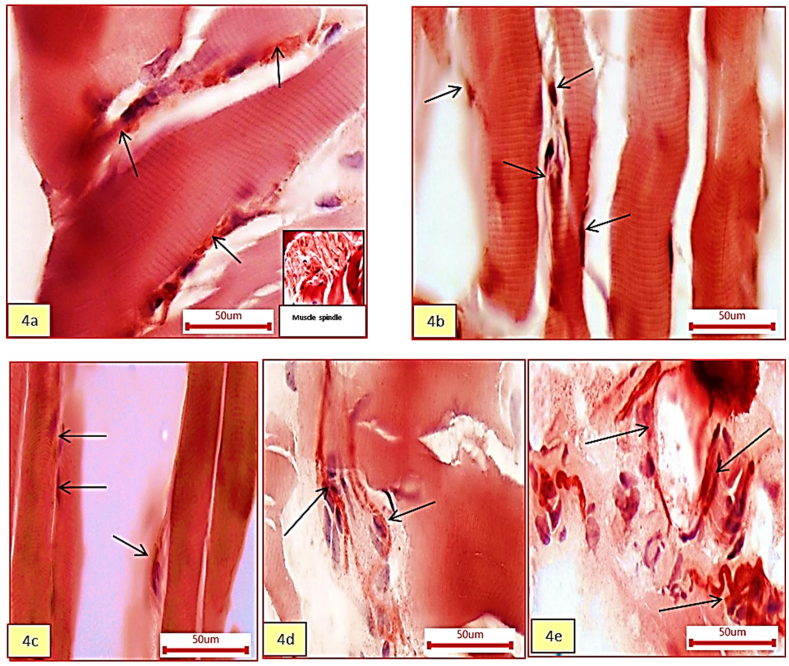

Figure 4. Neuromuscular junctions (NMJ) of the muscle.
4a) Control group Ia showing brown, oval, round or elliptical compact NMJs, distributed along the border of the muscle fibers (arrows). **4b)** Group Ib showing lightly stained NMJs. **4c)** SG group II exhibiting smaller and less compact NMJs composed of numerous spot-like contacts on the muscle surface (arrows). **4d)** Group III showing NMJs' appearance more or less the same as that of group Ia. **4e)** Group IV with compact NMJs (arrows). Acetylcholine esterase histochemical staining, x400 magnification.

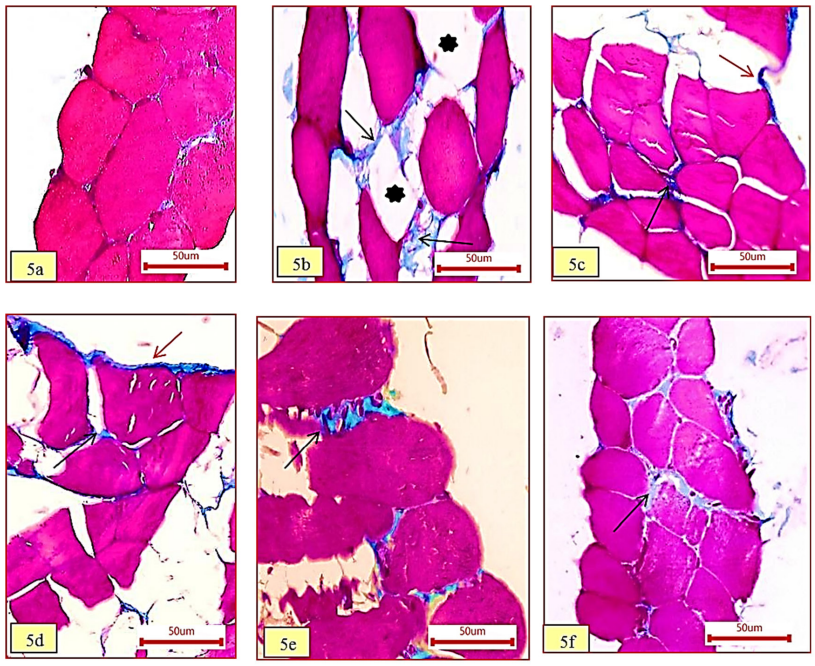

Figure 5. Masson trichrome collagen staining. Increased collagen amount between the accumulated intra-muscular fat cells (asterisks) in group Ib (5b) by comparison to group Ia (5a).
5c, d) SG group exhibiting prominent deposition of collagen in endomysium and perimysium (black & red arrows, respectively). **5e, f)** Post SG treated groups III and IV showing preservation of the collagen contents. Masson trichrome staining, x400 magnification.

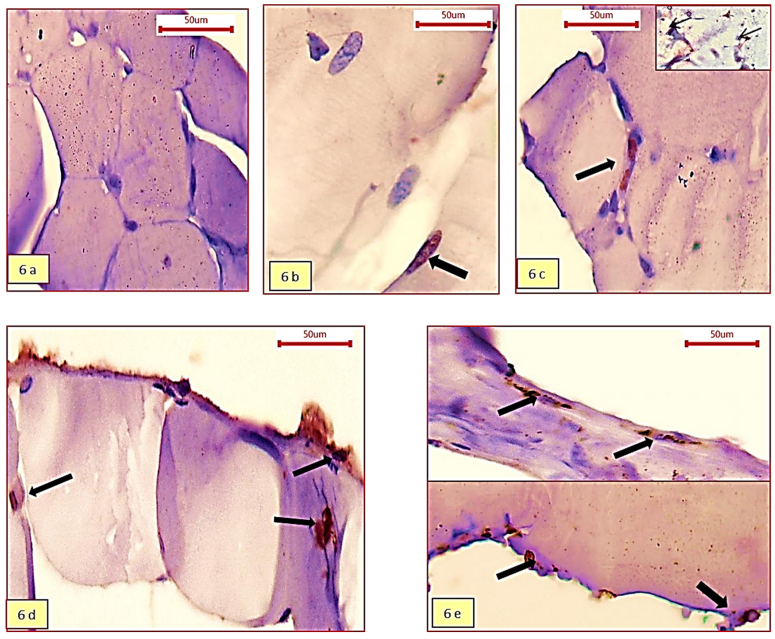

Figure 6. Immunostaining of myogenin.
6a, Control group Ia showing no immunoreactivity. **6b)** Group Ib showing weak immunoreactivity (arrow). **6c)** SG group II showing nuclear immunoreactivity in myogenic cells in-between the polyhedral muscle fibers (arrow). **6d)** Group III showing many immunoreactive myogenic cells. **6e)** Group IV showing marked increase in myogenin immunoreactivity (arrows). Myogenin immunostaining, x1000 magnification.

collagen fibers: Group Ia showed predominance of type IIB fibers, followed by type IIA and type I. Groups Ib and II showed insignificant decrease in Vv. of type IIB fibers. On the other hand, groups III and IV showed a significant decrease. In the latter groups, there was a significant muscle remodeling characterized by an increase in the volume density of oxidative fibers (type I and type IIA). The increase was significant for type I and highly significant for IIA fibers. There were nearly identical values of volume density of collagen fibers "Vv. col" in group Ib and group Ia, but in groups III and IV, there was an insignificant increase compared to group Ib. On the other hand,

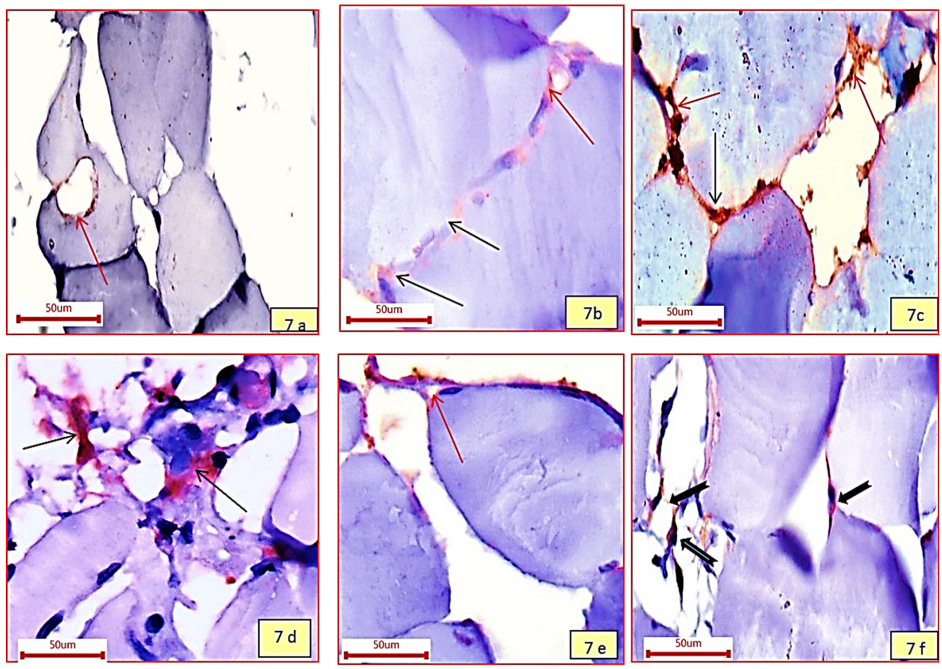

Figure 7. Immunostaining of TGF-β.
7a) Control group Ia showing TGF-β expression around blood vessels. **7b)** Group Ib showing weak expression in the endomysium (black arrows) and near the blood vessels (red arrows). **7c, d)** Group II showing upregulation of TGF-β expression around the blood vessels (red arrows) primarily in the endomysium and in the connective tissue (black arrows). **7e, f)** Post SG treated groups III and IV showing down-regulation of TGF-β in the endomysium (red arrow) and in the fibroblasts (tailed arrow). TGF-β immunostaining, x1000 magnification.

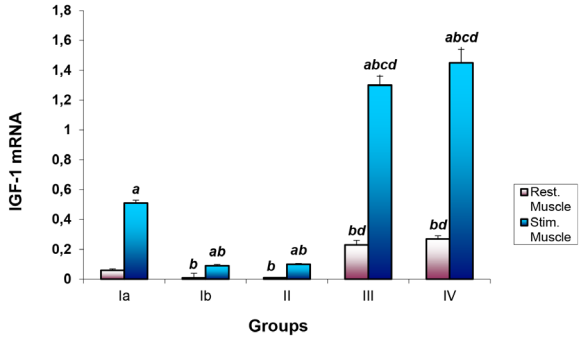

Figure 8. Quantitative analysis of IGF-1.
Data are expressed as mean ±S.E.M. of 6 rats per group (Rest.: left resting, Stim.: right stimulated). a: significantly different from group Ia, b: significantly different from group Ib, c: significantly different from group II, d: significantly different from group III. $P \leq 0.05$. IGF-1: Insulin growth factor-1.

Table 6. Cross-sectional area of myocytes (μm²) (CSA) of the gastrocnemius muscle according to the fiber types.
Data are expressed as mean ±S.E.M. of 8 rats per group. a: significantly different from group Ia, b: significantly different from group Ib, c: significantly different from group II, d: significantly different from group III, $P \leq 0.05$.

Parameters Groups	Type I (μm²)	Type IIA (μm²)	Type IIB (μm²)
Group Ia	2202.40±31.87	3089.62±61.45	4136.22±57.77
Group Ib	2146.49±35.07	2768.20±67.85[a]	4090.87±29.33
Group II	2079.14±42.21[a]	2329.90±66.10[ab]	3909.01±25.28[a]
Group III	2438.92±50.78[abc]	4558.30±79.43[abc]	3265.75±81.66[abc]
Group IV	2520.35±72.50[abc]	6086.96±59.94[abcd]	2702.12±35.48[abcd]

group II showed higher values of "Vv. col" when compared to all other groups (Table 5).

Cross-sectional area of muscle fibers (CSA): In group Ib and II, type IIB fibers has insignificantly lower area compared to group Ia. Groups III and IV showed a significant decrease in the CSA of type IIB fibers compared to that in the group Ia. Moreover type IIA and type I fibers (oxidative fibers) showed a clear remodeling reflected by a significant increase of the CSA of type I fibers and a highly significant increase of type IIA fibers when compared to group Ia (Table 6).

Right gastrocnemius muscle response

Group Ib showed a significantly reduced peak force upon electric stimulation associated with shortened time till 50% decline of peak force indicating accelerated fatigue in comparison to group Ia. On the contrary, group II showed a significantly lowered peak force with an insignificant change of the time till 50% fatigue as compared to group Ib. Introducing supplementation to SG rats resulted in a significant increase in the peak force when compared to both Ib and II group and prolongation of the time till 50% fatigue compared to group II, but these parameters were still significantly lower compared to group Ia. On the other hand, physical exercise with supplementation introduced in SG rats led to significant improvement of muscle contraction, regarding peak force and the time till 50% fatigue, as compared to groups Ib, II and III, but in comparison with group Ia, the peak force was not significantly different while the time till 50% fatigue was significantly increased (Fig. 9 and Table 4).

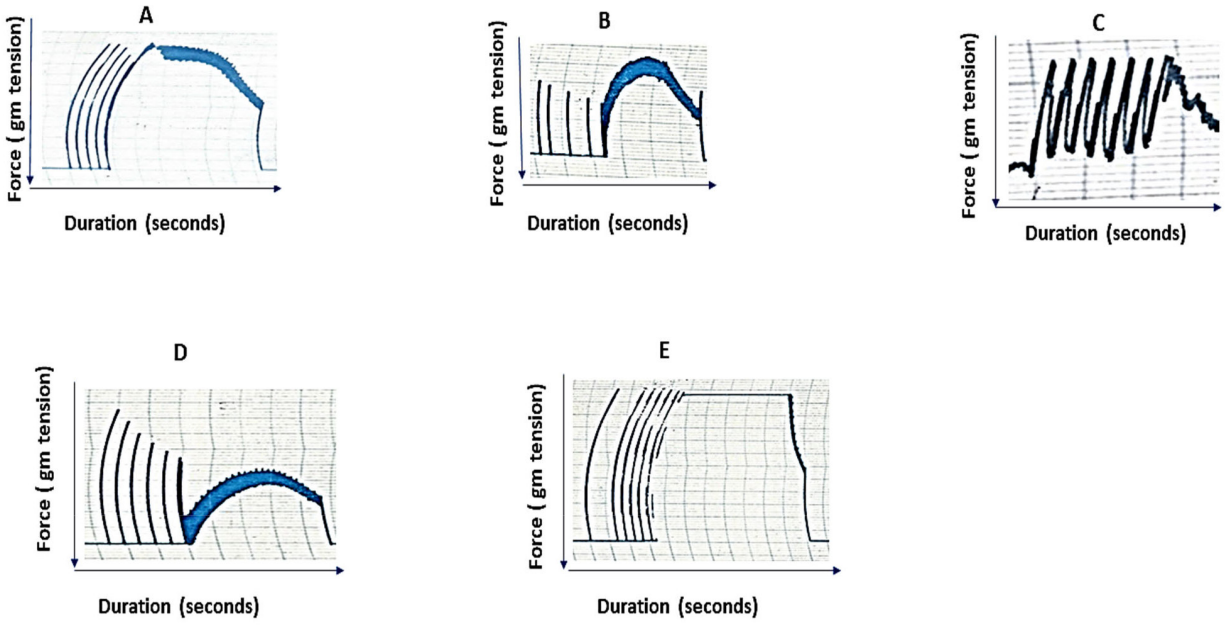

Figure 9. Right gastrocnemius muscle response of the different experimental groups represented by a vertical line (Force, gm tension); one big vertical square = 5 g tension, and horizontal line (the time (t) till 50% decline from peak force); one big horizontal square = 2 seconds duration.
A: group Ia, B: group Ib, C: group II, D: group III, E: group IV. $P \leq 0.05$.

DISCUSSION

Obesity is one of the metabolic disorders that affects skeletal muscle function (Tallis *et al*., 2018). Skeletal muscle is the largest metabolic organ performing carbohydrate and fat oxidation followed by the liver, thus, it is affected by nutritional disorders (Müller *et al*., 2014). It is responsible for 85% of insulin-mediated glucose clearance and maintaining whole-body glucose homeostasis. Metabolic disorders of the muscle and disruption of mitochondrial activity, fatty acids oxidation, and glycolysis cause failure of glucose homeostasis (Abu-Remaileh *et al*., 2019).

Gastrocnemius muscle received attention in research because it consists of type I or slow-twitching muscle fibers and type II or fast-twitching muscle fibers (Ibrahim *et al*., 2016). Furthermore, this study showed that electric stimulation-induced metabolic, molecular and cellular changes in the muscle. Some evidence at the cellular level suggests that electrical stimulation increases reactive oxygen species (ROS) production (Dong *et al*., 2018). In addition, it can affect the regeneration process as well as the oxidative stress of satellite cells (Di Filippo *et al*., 2017).

HFD group being the model obese group showed increased BMI, possibly due to the high caloric content of satiety pellets (Lipinski *et al.,* 2017). It showed a disturbed lipid profile and the highest atherogenic index. Obesity is associated with metabolic disorders: »dyslipidemia, atherosclerosis and type II diabetes» as a result of energy impairment and increased caloric intake compared to the calories loss (Orsolic *et al.,* 2019). The hyperinsulinemia and hyperglycemia sparing glucose are attributed to the higher free fatty acid content in the HFD that compete with glucose for mitochondrial oxidation, hence and promoting lipid oxidation; and lipid metabolites interfere with insulin function resulting in the development of insulin resistance (Ibrahim *et al.,* 2017).

Hyperglycemia increases the activity of mitochondrial NADPH oxidase and mitochondrial biogenesis with an excessive generation of ROS as detected by the increase in MDA levels (Hurrle & Hsu, 2017). Reduction in plasma albumin level could be attributed to the decreased insulin action and the associated chronic inflammation (Chang *et al*., 2019). Moreover, insulin resistance may account for obesity-related hyper-filtration, glomerulomegaly, and albuminuria (Martínez *et al*., 2019). The reduction in serum 25-hydroxy vitamin-D and Ca^{+2} may be caused by vitamin D metabolic disorder associated with obesity including lower dietary intake, reduced cutaneous synthesis or altered metabolism with the subsequent decrease in Ca^{+2} absorption (Ali *et al*., 2019).

HFD group showed histopathological changes with a decrease in both volume density and cross-sectional area of all types of muscle fibers. Thus, the muscle becomes metabolically inflexible, probably due to inefficient oxidation of carbohydrates and lipids leading to progressive intracellular deposition of lipid droplets, ATP deficiency, and inflammatory cellular infiltration (Abu-Remaileh *et al*., 2019) that result in progressive muscle fatigue (Wu & Ballantyne, 2017). Furthermore, it was reported that long-term HFD leads to excessive accumulation of fat tissue in skeletal muscles and muscle atrophy by activating the atrophy-pathway proteins such as TNF-α (Ferretti *et al*., 2018). Moreover, the predominance of type IIB fast fibers might account for the inefficient oxidation of the muscle; in line with this, it was reported that obese patients' skeletal muscles exhibit low oxidative capacity by transiting from slow oxidizing muscle fibers to rapidly twitching fibers (Tallis *et al*., 2018).

The functional impairment of skeletal muscle in the HFD group demonstrated by the reduced force and accelerated fatigue could be attributed to dysfunctional mitochondrial oxidation with subsequent reduction in ATP generation, glycogen storage and creatine kinase (CK)

activity (Hamrick *et al*., 2016). CK ensures adequate energy supply for the skeletal muscle upon rest and during activity (Baird *et al*., 2012). The significantly higher CK level in the gastrocnemius muscle of the stimulated limb in the control group in this study suggests continuous supply of ATP. However, CK activity can also contribute to muscle fatigue especially under oxidative stress conditions when the breakdown of CK results in excessive accumulation of inorganic phosphate (Pi) (Wan *et al*., 2017).

Although a number of growth factors have the potential to influence muscle repair, there is strong evidence that insulin-like growth factor-1 (IGF-1) is a powerful enhancer of tissue regeneration and coordinates the regeneration of skeletal muscles by stimulating the muscle satellite cells.The increased expression of IGF-1 in muscle injury accelerates the inflammation as reported by (Tonkin *et al*., 2015). HFD causes mitochondrial dysfunction that leads to insulin resistance and reduced muscle mass by lowering protein levels of IGF-1 as shown in the present study (Ferretti *et al*., 2018).

Sleeve gastrectomy (SG) is considered a restrictive measure to reduce oral intake by reducing stomach size (White *et al*., 2019). It is considered a highly effective treatment of obesity and improvement of the quality of life by sustaining weight loss, reducing comorbid diseases and deaths related to obesity. In the present work, we aimed to study the molecular, cellular and physiological adaptation of the gastrocnemius muscle to SG. SG resulted in a significant decrease in BMI, changes in lipid profile and glucose level as compared to the obese rats. This can be attributed to the reduced intake of food that was also observed in the postoperative groups (Arble *et al*., 2018).

SG group showed a significant reduction in serum insulin, serum albumin, Ca^{+2} and 25-hydroxy vitamin-D levels. Insulin levels reduction could be attributed to the hormonal mechanisms as the levels of YY peptide (PYY) and Glucagon-like peptide 1 (GLP-1) secreted by L-cells of the hindgut appeared to increase after SG. The increase in PYY and GLP-1 levels is due to the acceleration of gastric emptying and early contact of the chyme with L-cells (Catoi *et al*., 2016) compared to all other experimental groups. Albumin, Ca^{+2} and 25-hydroxy vitamin-D levels reduction could be attributed to the decreased food intake and the physiological effect of the anatomical changes caused by the surgery (Donadelli *et al*., 2012). Generally, one of the complications of the surgical correction of obesity is under-nutrition, a condition of excessive catabolism that leads to loss of both fat and protein (Sharma *et al*., 2019).

Physiologically, the SG group showed a reduction in peak force generation indicating a reduction in muscle power, and a shortening of the time till half relaxation indicating fatigue acceleration. Muscle power reduction and easy fatigability could be attributed to: 1) the histopathological changes seen in the muscle fibres including degenerative, inflammatory and fibrotic changes (Saclier *et al*., 2013); 2) inflammatory cell infiltration into the muscle, especially macrophages (Desgeorges *et al*., 2019) which could be the source of high TNF-α level noticed in the present study; 3) the molecular and metabolic changes in the muscle as a consequence of malnutrition following SG due to restriction of food intake; 4) metabolic disorders may be greater in type II compared to type I fibers due to the faster rate of degradation of phosphocreatine and anaerobic breakdown of glucose and hence accumulation of lactate and H^+ (Brzobohatý *et al*., 2015). They added that the daily food intake restriction is able to induce fiber loss with a higher share of type IIB muscle fibers.

SG group showed a reduction in glycogen and CK levels due to a decrease in food intake that results in malnutrition, decreased energy sources and acceleration of fatigue. ROS production leads to the oxidation of proteins, fatty or nucleic acids accompanied by a marked decrease in the antioxidant capacity, which ultimately leads to fatigue (Wan *et al*., 2017). It was also proven in the current study through a significant increase in the MDA.

Muscle growth, development and regeneration occur throughout the life of vertebrates and the processes are characterized by the proliferation of precursor cells, followed by the expression of genes for muscles, and finally the merging of differentiated muscle cells into myotubes (Murphy & Kardon, 2011). Myogenin is a skeletal muscle-specific gene that encodes a myogenic regulator essential for skeletal muscle formation and repair (Bentzinger *et al*., 2012). Following muscle injury, satellite cells are activated into muscle precursors, multiply, migrate toward each other and merge into muscle fibers (Zammit, 2017). This process accounts for the increase in the cross-sectional area of muscle fibers and the increase in the mean number of myogenin-positive cells in the SG group.

Moreover, the current study revealed a significant decrease in the expression of IGF-1 mRNA in the skeletal muscle both in the resting state and even upon stimulation in the SG group. IGF-1 concentration is usually affected by multiple factors; apart from genetics and hormones, especially nutrition is considered to be the key factor for IGF-1 production (Ban & Zhao, 2018). Malnutrition can alter the growth hormone/IGF-1 axis at multiple levels including decreasing its gene expression, accelerating IGF-1 decomposition, and reducing the biological activity of serum IGF-1. Therefore, in the present study, we rejected the concept that the competing processes of cellular proliferation, differentiation and increased protein synthesis required for muscle repair or hypertrophic adaptation in the SG group were regulated by the expression of IGF-1.

SG group showed an upregulation of pro-inflammatory factor TGF-β1 which is an important cytokine for extracellular matrix synthesis and fibrosis (Delaney *et al*., 2017). Thus, the resulting fibrosis could weaken the muscle function, negatively affect muscle regeneration and increase the susceptibility of muscles to re-injury (Mahdy, 2019).

In post-SG treatment, increased protein intake may have impressive effects on appetite, metabolic rate and weight loss (Arentson-Lantz *et al*., 2015). Moreover, vitamin D affects the muscular system by stimulating muscle cells proliferation and differentiation (Zadka *et al*., 2018). Vitamin B complex is involved in many important physiological functions such as carbohydrates and fatty acids metabolism, cell proliferation, and cell membrane permeability (EL-Toweissy *et al*., 2013). Leucine supplementation is an effective nutritional strategy for increasing muscle mass (Amaral *et al*., 2015). It improved HGH resistance in malnourished rats by enhancing IGF-1 production, reducing IGF-1 degradation, and facilitating the synthesis of HGH receptors in the liver (Gao *et al*., 2015)).

Compiling evidence has shown that selenium (Se) is a trace mineral that is essential to human health (Mistry *et al*., 2012). It is known for its antioxidant properties responsible for its antiviral and anti-cancer capabilities. Moreover, it is important for normal muscle function.

Musculoskeletal disorders that appear as muscle pain, fatigue, weakness, and high serum creatine kinase (CK) were reported in patients with selenium deficiency (Bodnar *et al*., 2016).

Post-SG treated groups (group III and IV) showed marked improvement in biochemical parameters as compared to the obese and SG groups. They showed insignificant differences in all the resting gastrocnemius muscle parameters compared to the SG group. However, group VI showed significantly lower TNF-α and MDA levels and significantly higher CK and glycogen levels in stimulated right gastrocnemius if compared to group Ia and III. This could be explained by the fact that selenium supplementation is important for the body's antioxidant defense mechanism and suppresses the production of interleukins and TNF-α (Dasgupta & Aly, 2016). It is also important for the improvement of skeletal muscular dystrophy due to the down regulation of myogenin and related cytokines (Bodnar *et al*., 2016).

Skeletal muscles are a very dynamic tissue, capable of continuous reconfiguration in response to various environmental stimuli as proved by a significant increase in the myogenin-positive cells in post-SG treated groups compared to that of the SG group. There was a correlation between protein availability and satellite cells proliferation, differentiation, and myonuclear accretion. Muscle tubes in muscle fibers and ECM undergo remodeling, which has a major role in transferring strength, maintenance and repair of muscle fibers (Zammit, 2017). Furthermore, there was a significant increase in the IGF-1 mRNA expression in the post SG treated group which could be attributed to selenium supplementation (Alehagen *et al*., 2017). IGF-1 production in group IV which underwent physical exercise could be attributed to the mechanical activity (Ichinose *et al*., 2014). TGF-β1 localization at sites targeted for regeneration such as in the endomysium fibroblasts around the muscle fibers could be for regulating muscle repair by activating satellite cells, forming connective tissues, as well as regulating the intensity of the immune response. (Delaney *et al*., 2017).

In post-SG groups, muscle fibers exhibited signs of regeneration that might be attributed to enhanced metabolic activity of protein synthesis to restore the damaged cells (Roman & Gomes, 2018). In addition, volume density "Vv" of collagen fibers showed a non-significant increase. This might be a consequence of fibroblast cells activation, leading to increased synthesis of ECM and fibers that form a structural network holding myofibers together and providing them with blood and nerve supply.

Therefore, in the post-SG group muscle fibers showed cellular and molecular changes indicating regenerative activity which indeed could improve muscle contractility. This was confirmed by demonstrating that the peak force and the time till 50% fatigue improved. It could be explained that the NMJ (Neuromuscular junction) in the post-SG groups restored its normal appearance suggesting an improved quality of axons. In addition, the muscle fibers showed apparent hypertrophy that was emphasized in group IV consisting of a large population of oxidative fibers (type IIA and type I) and the lowest population of type IIB fibers. It could be said that nutritional supplementation increased the rate of myofibrillar protein synthesis and the exercise-stimulated rate of muscle protein synthesis to an even greater extent (Trabal *et al*., 2015).

Interestingly, physical exercise along with nutritional supplementation following SG resulted in improvement in the muscle, probably because the anabolic and muscle pathways are strongly influenced by physical exercises. Regular training improves muscle mass and strength by increasing protein synthesis, the number of fibrous fibers, and the cross-sectional area of the fibers. Exercise increases IGF-1 levels with the subsequent induction of protein synthesis. Moreover, the exercises increase the muscle fiber protein by activating the satellite cells and reduce the fat leakage into the muscles. In addition to stimulating muscle metabolism, exercise prevents protein degradation, and this effect may be mediated by low levels of oxidative stress after training (Gomes *et al.,* 2017). Physical activity improves antioxidant defense and lowers lipid peroxide levels. Therefore, consuming enough high-quality protein with physical activity appears to be a promising strategy to prevent or treat muscle weakness and muscle atrophy (Simioni *et al.,* 2018).

CONCLUSION

It is evident that malnutrition due to food restriction following SG is a major cause of muscle atrophy with metabolic and functional deterioration. Besides, nutritional supplementation together with physical exercise are necessary to maintain muscle mass and strength, along with synchronistic new muscle fibers innervation by the surviving nerve terminals. Due to the difficulties in performing such a study in humans, the present study conclusions should be reflected in the recommendations for patients after SG surgery.

Limitations

One of the drawbacks of the present study was the short follow-up of 6 weeks. However, this period is equivalent to 3 years of human life (Brinckerhoff *et al*., 2013). Another limitation was the smaller groups due to the high complication rate in the post-operative rats.

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Declaration of interest:

The authors declare that there is no conflict of interest that could be perceived as violating the impartiality of the research reported.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed according to the guidelines of the Animal Care and Committee of Faculty of Medicine, Minia University, Minia, Egypt.

Author Contribution Statement

All authors' contribution to the paper was almost equal including interpretation of the results and writing of the manuscript. First, third, fifth and eighth author contributed to the histopathologic examination.

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