The study of testosterone and tacrolimus roles on gastrocnemius muscle following experimental sciatic nerve injury in rats

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Abstract

Peripheral nerve damage is a critical disorder causing disability of locomotion. The aim of the study was to clarify the effects of testosterone and tacrolimus on the gastrocnemius muscle following sciatic injury. The study was done on 20 rats (n = 5 in each group) whose left sciatic nerve was crushed for 10 s. The sham group (S) of animals received no medicine; the testosterone group (Tes) received testosterone (5 mg/kg, s.c.); the tacrolimus group (Tac): received tacrolimus (5 mg/kg, p.o.); the testosterone and tacrolimus group (Tes+Tac) received testosterone (5 mg/kg, s.c.) and tacrolimus (5 mg/kg, p.o.) daily for four weeks. The gastrocnemius was assessed by gross observation of the plantar surface of paws; the pelvic limb mass muscle and the muscle diameter ratio of the left pelvic limb to the right one by ultrasonography. The gastrocnemius muscle index (GMI) of the left and right pelvic limb, muscle colour, and pathologic changes were also studied. Pathology study of the gastrocnemius included fatty infiltration, muscle atrophy, presence of inflammatory cells and fibrosis formation. Heel redness and swelling were seen in group Tac. No significant difference was found in the GMI between the Tes and S groups ($P > 0.01$); its value was higher than in the Tes+Tac and Tac groups ($P < 0.01$). One rat in group Tes had fatty infiltration grade II. Inflammatory cells were grade I in group Tes but fibrosis formation was grade I in group Tes+Tac. Our results show that tacrolimus and testosterone administration may shorten sciatic nerve regeneration time. Testosterone may diminish gastrocnemius muscle atrophy after sciatic nerve crush.

Peripheral nerve, neurogenic atrophy, gonadotrophic hormones, immunosuppressive

There are situations of muscle loss where physical activity is not always a suitable way for muscle strength in patients with fractures and multiple injuries with peripheral nerve damage (Watson and Dyck 2015). The trauma may cause partial or complete damage to the peripheral nerve (Watson and Dyck 2015). Denervation usually is a sequel to trauma in humans and animals (Bodine-Flower et al. 1996). Denervation causes musculoskeletal change and is followed by a decrease in muscle mass, its force and physiologic function, named denervation atrophy and ultimately, nerve atrophy (Bodine-Flower et al. 1996). Denervation atrophy, also known by the misnomer neurogenic atrophy, is not uncommon in clinical practice of veterinary medicine (Valentine et al. 2011).

As mentioned above, a significant decrease in muscle mass and fibre size occurs routinely within one week after denervation (Bodine-Flower et al. 1996). One of the most common nerve dysfunctions by trauma in humans and animals is sciatic injury and transection of its continuing (Kobayashi et al. 1997). Transection of the sciatic nerve impairs the function of all groups of muscles it supplies which causes disability of the patient to ambulate and change posture of limbs (Kobayashi et al. 1997). Even though nerve function can be restored medically or surgically, muscle recovery may take a long time even with the help of all the main and complementary therapies (Langer et al. 2018). Muscle change

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starts with the connective tissue formation in the perimysium and its insufficient ability to perform its function (Verdú et al. 2000; Fernandes et al. 2005). Once this occurs, mass reduction has been started and the process of atrophy becomes ongoing (Langer et al. 2018).

Researches have long focused on restoring muscle function after nerve injury for prevention of the patient's disability (Langer et al. 2018). Recent studies have focused on finding a suitable solution for the above concern (Langer et al. 2018). Some types of medication such as by sexual hormones, immunosuppression, stimulating growth factors, supplement therapy, conservative treatment as a combination of physical therapy and medical therapy have been successful but there are still doubts about their definite efficacy (Fargo et al. 2008; Glaus et al. 2011; Langer et al. 2018). It should be kept in mind that side effects and unsatisfactory results are common depending on the type of therapy. Recent studies have paid more attention to the role of gonadotrophic hormones because of their anabolic and likely neuroprotective effects (Fargo et al. 2008). Among sexual hormones, testosterone and its metabolites have two distinctive roles in muscle and nerve. The effect of testosterone on muscle in promotion of muscle strength has been well known. Testosterone also has a neuroprotective effect with enhancement of axonal regeneration that plays a main role in return to function (Fargo et al. 2008). The immunosuppressant tacrolimus has been well used in patients receiving grafts and organ transplantations (Glaus et al. 2011). The positive role on peripheral nerve regeneration via neurotrophic pathway was shown in experimental studies but still the role of each one solely or in combination needs more investigation in the field of healing of nerve lesions and post-traumatic muscle atrophy (Glaus et al. 2011).

The present study aimed to show the efficacy of testosterone and tacrolimus in the healing process, early back to the normal function of limbs, preserving or restoring muscle strength and mass volume and improvement of clinical signs (paralysis or paresis) of an experimentally crushed sciatic nerve of the rat.

Materials and Methods

Ethical Statement

All protocols were approved by the animal experimental ethics committee of IAU (Approval No. IR.IAU.ARB.REC.1400.072). All rats were kept and sacrificed under conditions of welfare. All trials were carried out in a humane manner.

Animals

A total of 20 adult male Wistar rats (weighing 250–300 g, age 8 weeks) were purchased from the Pasteur Institute of Iran (Tehran, Iran). Rats were randomly divided into four groups by five animals in each group: rats of the sham group (S) did not receive any medicine; rats of the testosterone group (Tes) received testosterone (5 mg/kg, subcutaneously [s.c.]); rats of the tacrolimus group (Tac) received tacrolimus (5 mg/kg) by oral gavage; rats of the testosterone and tacrolimus group (Tes+Tac) received testosterone (5 mg/kg, s.c.) and tacrolimus (5 mg/kg) by oral gavage daily for four weeks. All the rats had free access to water and food (Behparvar Co, Tehran, Iran) and were maintained at constant temperature (23 ± 1 °C) and stable air humidity (40%–50%) under a 12-h light/dark cycle in an animal house of the Shahid Beheshti University of Medical Sciences. The rats were acclimated preoperatively during an eight-day period after transport.

Surgical procedure

The rats of each group were anaesthetized with an injection of medetomidine (90 µg/kg, intramuscularly [i.m.], Laboratories Syva S.A.U., León, Spain) and ketamine 10% (20 mg/kg, i.m., Bremer Pharma GmbH, Warburg, Germany). The body temperature of rats was maintained at 37 °C with an electric thermal blanket and oxygen therapy with anaesthesia workstations (Leon plus, Bad Ems, Germany). Following induction, left legs of rats were shaved and washed with a scrubbing solution before positioning for surgery. A 2 cm long straight skin incision was made on the lateral aspect of the thigh, from the major trochanter to the knee. Then the left sciatic nerve was exposed through a muscle fascia splitting under an operating microscope (Topcon OMS 90, Tokyo, Japan). The nerve was then crushed at 10 mm proximal to the trifurcation of the sciatic nerve with sterile fine haemostat forceps (Aesculap Inc, Pennsylvania, United States) at 2.0 mm width for 10 s (Sun et al. 2009). The presence of a translucent band across the nerve and thin crush was seen after forceps were removed (Feng and Yuan 2015).

On the crushed site, a marker made of nylon suture material 6-0 (Ethicon Inc, New Jersey, United States) was placed on the facia of the biceps femoris. The incision was then closed in a muscle layer with polyglactin 910 suture material 5-0 (Ethicon Inc) and skin with nylon suture material 5-0 (Ethicon Inc) with simple continuous and simple interrupted sutures, respectively. In the sham group, the sciatic nerve was exposed but not crushed and was sutured. A dose of meloxicam (1 mg/kg, s.c., Razak Pharmaceutical Labs Co., Tehran, Iran) and enrofloxacin (10 mg/kg, i.m., Laboratorios HIPRA, Girona, Spain) was injected postoperatively to all animals. The medications were used once daily for 28 days till end of study in each group.

Ultrasound assessment

On the last day of study all rats were anaesthetized with the explained method and the gastrocnemius muscle of the left and the right limbs of each animal was studied by B-mode ultrasound Portable ultrasound (S9, SonoScape, Düsseldorf, Germany). Before the examination, both legs were shaved with a blade. Animals were restrained in a prone position and longitudinal ultrasonographic images were obtained in mentioned positions using acoustic gel (Polygel, Parsteb Co, Esfahan, Iran) and a 10 MHz linear probe. The muscle diameter ratio (MDR) of the gastrocnemius muscle of left and right legs was measured by an investigator blinded to the groups by electronic cursors which were set at the anatomic borders of the muscle (Faustino-Rocha et al. 2013). By evaluation of the sagittal plane, both muscle thickness and cross-sectional area were assessed. After identifying three points including the fibula, fibulae nerve, and junction of the distal point of the semitendinosus muscle and gastrocnemius muscle, a line from the third point and perpendicular to the fibular nerve and fibula bone was traced and the thickest point at the maximum diameter was recorded (Nijhuis et al. 2013). After investigation of the thickest diameter for three times, the average was analysed (Nijhuis et al. 2013).

Heel assessment

Varying degrees of ulceration in the rats' left and right heels were observed and recorded (He et al. 2017).

Gastrocnemius muscle index (GMI) and colour

Following the ultrasonography evaluations, the animals were killed with an overdose of the anaesthetic. Consequently, the gastrocnemius muscle was precisely and carefully excised from left and right limbs and wet muscle weight was measured immediately using a digital scale (Sartorius, Goettingen, Germany). Values were expressed as a ratio of the wet weight of the gastrocnemius muscle of the operated side to the wet weight of the normal side (He et al. 2017; Feng and Yuan 2015). All measurements were made by a blind observer. Therefore, the gross shape of the operated side muscle and the contralateral leg muscle were compared (Nijhuis et al. 2013). In addition, the colour of gastrocnemius muscle tissue from operated and unaffected patients was carefully considered and studied (He et al. 2017).

Histological assessment

The gastrocnemius tissues of both the affected and the contralateral side of all rats were removed and embedded in paraffin and cut into thin 5 µm sections. The sections were stained using haematoxylin and eosin (H&E) and also Masson's trichrome. The slides were observed under a light microscope (DM500, Leica, Wetzlar, Germany). Then fatty infiltration distribution, muscle atrophy and muscle inflammation grading were studied with H&E staining and also muscle fibrosis grading in Masson's trichrome staining by a blinded pathologist. The H&E staining allowed semiquantitative assessment of the degree of fat infiltration and muscle atrophy within the affected muscle. The assessment of fat infiltration was based on a four-stage scale, in a manner consistent with the Goutallier classification: stage 0 = a completely normal muscle; stage 1 = muscle containing some fatty streaks; stage 2 = still less fatty infiltration than muscle; stage 3 = as much muscle as fat present; stage 4 = more fat than muscle present (Abdou et al. 2019). In addition, H&E stained slides were graded semiquantitatively for muscle atrophy as follows: normal = 0, mild atrophy = 1, moderate = 2 and severe = 3 (Abdou et al. 2019). Fat infiltration and muscle atrophy grades were arranged with parameters such as an angular shape of muscle fibres as opposed to a round shape, decreased muscle fibre size, and decreased distance between myonuclei and centralized myonuclei (Abdou et al. 2019). The number of inflammatory cells per ten high-power fields (HPF) was recorded to identify the inflammation grade. Based on this method, inflammatory cells were graded as follows: 0 = no inflammation, equivalent to no inflammatory cell/HPF; 1 = mild inflammation, equivalent on average to 25 inflammatory cells/HPF; 2 = moderate inflammation, equivalent on average to 26–50 inflammatory cells/HPF; and 3 = severe inflammation, equivalent on average to more than 50 inflammatory cells/HPF (Abdou et al. 2019; Meuten et al. 2016). In Masson's trichrome staining, five fibrosis grades were identified that included 0 = no fibrosis; 1 = mild perimuscular fibrosis reaction; 2 = easily detected thick bands; 3 = well-developed dense bands of collagen; and 4 = a severe fibrotic response replacing large areas (Sannad et al. 2017).

Statistical analysis

The experimental data were expressed as means ± SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) protocols followed by multiple comparisons tests using Tukey's method to analyse the differences. Statistical analyses were performed using SPSS version 25.0.x (SPSS, Inc., Chicago, Illinois, USA).

Results

All rats recovered after anaesthesia and survived till the end of study, except one rat from group Tes+Tac which aspirated during gavage at 15 days postoperatively. Then the rat was replaced by another which had been kept for 28 days in the same environmental conditions. No wound infection and dehiscence were seen. Plegia was recorded for all rats of groups Tes, Tac, and Tes+Tac once they recovered from anaesthesia. Swelling and redness of the hindpaw skin were observed in all rats of group Tac and were only mild or absent in the others (Plate X, Fig. 1). Gross atrophy of left pelvic limb musclesin contrast to the right one was revealed in standing position and also in prone position after shaving for ultrasonography assessment.

Muscle diameter ratio

There was a marked difference in MDR between left and right legs of group Tac which showed the decrease of MDR of the affected limb (Plate X, Fig. 2). The ratio was near one in the sham group; however, it was about 0.6 in group Tac (Fig. 3). MDRs of groups Tes and Tes+Tac were nearer one, being 0.8 and 0.7, respectively, and they were lower than in the sham group (Fig. 3). There was no significant difference between any of the four experimental groups.

Gastrocnemius muscle index and colour

The results of GMI evaluation showed that the wet weight ratio of the C, Tes, Tac and Tes+Tac groups were 1, 0.8, 0.6 and 0.7, respectively. These results stated that there was no significant difference between groups C and Tes ($P > 0.01$) and there was a significant difference between groups C and Tac, and between group C and Tes+Tac ($P < 0.01$) (Fig. 4).

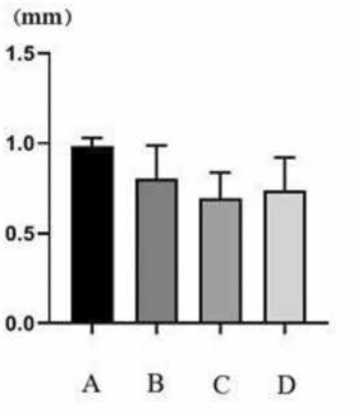

Fig. 3. Muscle diameter ratios of left and right pelvic limbs of the rats in all groups on 28 days postoperatively

A - Sham group; B - Tes group; C - Tac group; D - Tes+Tac group.

Tes group diameter of the left leg showed better recovery than the Tes+Tac and Tac groups. Differences were not significant.

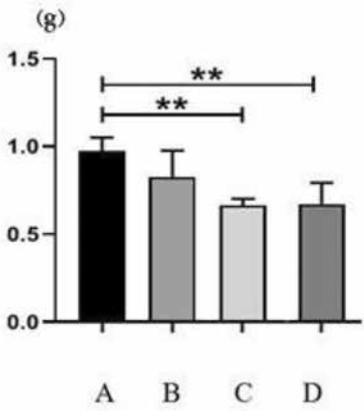

Fig. 4. Wet weight ratio of the gastrocnemius muscle of left and right pelvic limbs of the rats in all groups on 28 days postoperatively

A - Sham group; B - Tes group; C - Tac group; D - Tes+Tac group.

Weight recovery of Tes+Tac followed by Tac group was less than Sham and Tes group, respectively (**$P < 0.01$).

Gross evaluation revealed muscle atrophy of the left pelvic limb, where it was greater than in the right pelvic limb in all groups except for the sham group. Atrophy was not clearly observed in the sham group. No marked colour change was observed among the groups during our study for 28 days (Plate X, Fig. 5).

Histology

Grading of fatty infiltration

Histological study demonstrated that fatty infiltrations in all groups were of grade 0 except for one left limb of a rat in group Tes which was of grade 2 (Plate XY, Fig. 6, B-1).

Muscle atrophy grade

As previously, this grading was based on the centralization of myonuclei and rounded muscle fibres. The grade of muscle atrophy was zero in both left and right gastrocnemius muscles of the sham animals. It shows a few fibres with numerous small rounded fibres. Although group Tac shows atrophy of grade 3 in the left limb, the grade of group Tes was 2, and both groups revealed grade 2 atrophy in right legs. Also group Tes+Tac was better than groups Tac and Tes, showing grade 2 atrophy for the affected limb and grade 1 for the right limb. Overall, group Tes+Tac showed less atrophy than groups Tes and Tac, after 28 days post operation (Plate XI, Fig. 6).

Grading of inflammatory cells

Group Tac showed grade 3 inflammation of the left pelvic limb and grade 0 in the one. In this group, more inflammation was evident than in the other groups and it showed severe inflammatory phase with degenerated myofibres. Group Tes showed mild inflammatory cell infiltration of grade 1 for both left and right legs. In contrast to the atrophy and fatty infiltration, group Tes+Tac had more inflammation than the sham and Tes groups, at grade 2 for the left leg and 0 for the right one (Plate XI, Fig. 6).

Grading of fibrosis

In Masson's trichrome staining, group Tac showed marked collagen deposition and fibrosis. It was of grade 2 for the left and grade 1 for the right leg. Group Tes was also grade 2 with less collagen for the left leg and grade 1 for the right leg. Fibrosis was not seen in the sham group but the Tes and Tac group showed moderate collagen deposition and it showed grade one of fibrosis for left leg (Plate XII, Fig. 7).

Discussion

Treatment of peripheral nerve injury and subsequent muscle atrophy is one of the unresolved problems until this day. We studied changes in the gasterocnemius muscle following nerve crushing via gross pathology, histopathology and morphometry, and its consequences on the fast muscle for four weeks with treatment by an anabolic hormone and an immunosuppressant. Anabolic hormones such as testosterone (Kadi 2008) are one of the motivating factors in muscle preservation volume and strength. Testosterone acts at one step or more additional steps on myofibres of muscles and muscle mass as myogenic and/or adipogenic pathways. The first one is the direct effect of steroids on muscle (Kadi 2008). Testosterone directly modulates protein synthesis, satellite cell replication, myoblast fusion and myogenic progression to differentiate fibre and cellular proliferation (Kadi 2008). Testosterone has one or more processes in the adipogenic differentiation pathway (Kadi 2008). In our study, we found fat infiltration in one rat in group Tes. This finding is a mark of the adipogenic pathway due to androgen administration.

The results of our study have shown that testosterone has a substantial effect on muscle volume and mass. The difference between the sham and Tes group was non-significant ($P > 0.01$) but the difference between the sham and Tac group, and the sham and Tes+Tac group, was significant ($P < 0.01$). In the study by Hanson et al. (2020), reduction of the gastrocnemius muscle mass and function and finally muscle atrophy following orchiectomy and decreasing testosterone level occurred in the short term after immobilization and diminishing of activity (Hanson et al. 2020). Their results show that testosterone deficiency had a negative effect on muscle (Hanson et al. 2020). According to Brown et al. (1999), gonadal steroid accelerates axonal regeneration, shortens functional recovery time, and increases cytoskeletal protein production and muscle recovery function (Brown et al. 1999). Their findings demonstrated early recovery of axonal lesions following testosterone administration, as well as testosterone binding to androgen receptors in motor neurons and regeneration (Brown et al. 1999). Therefore, administration of testosterone for 28 days in rats with a crushed sciatic nerve improved the motor neuron function, and testosterone also preserves cytoskeletal proteins through nitrogen retention and production, leading to weight gain and unchanged GMI in groups Tes and S compared to the other groups (Brown et al. 1999). The GMI value in groups Tes and S in our study confirmed the role of the anabolic hormone after disuse atrophy due to a nerve crush in four weeks. MDR was different among the experimental groups but not significantly. These findings pointed out that the gasterocnemius muscle did not undergo significant changes in the diameter during the four weeks. The present research claims that differences among studied groups in MDR are due to the role of anabolic action of testosterone in preservation of the cytoskeletal content and its volume but its role was not statistically significant. The discrepancy between GMI and MDR as the morphometry finding using ultrasonography at the end of the study (4 weeks) could be explained by two main factors. Ultrasonography records in 2D, while muscle weight is associated with 3D (Nijhuis et al. 2013). Another reason is that the muscle diameter is not correlated with muscle density (Nijhuis et al. 2013). The density of the muscle is defined as the ratio between the cell volume of muscle fibres and the amount of collagen; and atrophy is defined as the muscle cell volume decreasing and the collagen deposition increasing (Nijhuis et al. 2013). Another less important cause is likely inaccuracy in ultrasonography measurement of the muscle diameter and/or some inadvertent erroneous determination of the diameter by the sonologist, as well as our small sample size. Therefore it should be noted that MDR does not have the sensitivity and specificity of GMI.

The immunosuppressant tacrolimus is used to diminish the immune response following transplantations. Many researchers have focused on and demonstrated the two mechanisms of action of tacrolimus: immunosuppressive and neurotrophic (Zheng et al. 2020). Investigating sciatic nerve regeneration, He et al. (2017) showed that nerve regeneration of the gastrocnemius muscle during tacrolimus administration for four weeks after surgery was not significant, however, regeneration was significant at 8 and 12 weeks after surgery (He et al. 2017). Farahani et al. (2022) revealed that administration of combined oestrogen and tacrolimus were effective with acceptable results in nerve regeneration in male mice after 4 weeks (Farahani et al. 2022). They found a significant difference between the oestrogen+tacrolimus group and the oestrogen group, and the oestrogen+tacrolimus group and the tacrolimus group in male mice (Farahani et al. 2022). Our study was consistent with their investigation. Zheng et al. (2020) showed more muscle atrophy and marked reduction of GMI in the quadriceps muscle than in the gasterocnemius muscle (Zheng et al. 2020). The red muscle is sensitive to disuse but white muscle is sensitive to starvation. The results of their study were not confirmed by He et al. (2017) who revealed greater recovery of the gasterocnemius muscle after receiving tacrolimus in an experimental nerve crush (He et al. 2017). The reason could be that the fast muscle is more sensitive to starvation than disuse

and tacrolimus helps in maintaining the muscle mass (He et al. 2017; Sartori et al. 2021). We found marked atrophy of the gastrocnemius muscle of the left limb compared to the right one after four weeks in group Tac. Our findings are in disagreement with the study by He et al. (2017). GMI was significantly different in group Tac compared to groups Tes and Tes+Tac, and MDR showed non-significant differences between Tac to the other test groups. The reduction of GMI and MDR in group Tac was due to the insufficiency of tacrolimus in eliminating inflammation and fibrous tissue formation. Atrophy in the fast muscle is started 10 days after reinnervation (Zheng et al. 2020). Group Tac had more redness, ulceration, collagen deposition, and severe inflammation of the affected limb than the other groups, but this was not evaluated statistically. Redness and ulceration was due to paralysis of the left limb. Therefore, the effect of tacrolimus on nerve regeneration was not prominent and ultimately induced muscle atrophy and pressure sores in the affected limb due to nerve palsy (He et al. 2017). The marked infiltration of inflammatory cells in the muscle tissue following administration of an immunosuppressive drug could be explained by the facts that conventional administration of tacrolimus is insufficient to eliminate the presence of T-cells in the muscle tissue and their resistance against the immunosuppressant. Therefore, tacrolimus was unable to reduce the inflammation process in the muscle. Tacrolimus minimizes inflammation in the site of surgery, inhibits transformation of fibroblasts and their proliferation, and prevents collagen deposition and migration (Zheng et al. 2020). It has been known that tacrolimus is a risk factor because it decreases insulin secretion, induces insulin resistance and imbalance of energy metabolism that causes skeletal muscle atrophy (Zheng et al. 2020).

As known, inflammation leads to fibrous formation in tissue, where more collagen deposition occurs, and the adhesion of collagen bundles to epineurium interferes with nerve impulse conduction. This process causes disuse atrophy.

The current study indicates that tacrolimus likely had a suppressive effect on testosterone in group Tes+Tac. In our study we found that GMI in group Tes+Tac was significantly lower than in the sham and Tes groups, and tacrolimus had an inhibitory effect on the group that received testosterone. Tai et al. showed in 1994 that testosterone was reduced significantly in rats receiving tacrolimus only (Tai et al. 1994). They also indicated that the main testosterone product and response of Leydig cells to hormone therapy (human chronic gonadotropin) was not significantly altered in rats that received tacrolimus (Tai et al. 1994). Their study showed that tacrolimus has an adverse effect on Leydig cell function in rats. The level of testosterone synthesis reduced the subsequent negative effect of tacrolimus on the Leydig cells (Tai et al. 1994). Canegium et al. (2009) suggested the alteration of the epithelial layer of seminiferous tubules after using tacrolimus in rats. Srinivas et al. (1998) and Masuda et al. (2003) indicated testicle weight loss and loss of epithelial thickness in seminiferous tubules following tacrolimus administration in rats. Another study showed tacrolimus-induced testicular toxicity in rats (Poormoosavi et al. 2021). It reported that tacrolimus decreases the level of angiotensin-converting enzyme 2, is responsible for destructive toxicity on the testicle tissue, and decreases testosterone level (Poormoosavi et al. 2021). The above findings are in accordance with the results of the current study. In our study, receiving tacrolimus could likely decrease the testosterone product from Leydig cells in male rats. Finally, we conclude that the group receiving testosterone only suffered less muscle weight loss compared to groups Tac and Tes+Tac, and the GMI of group Tes was similar to the sham group.

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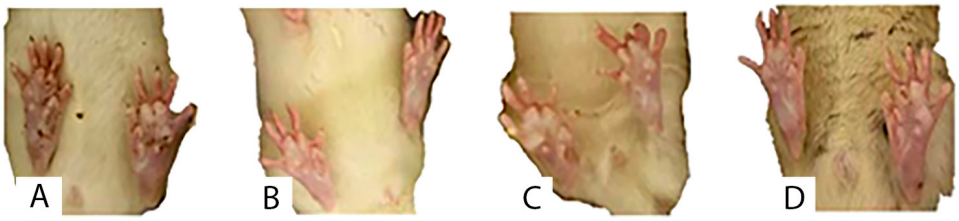

Fig. 1. Swelling and redness of the hindpaw on 28 days postoperatively

A - Sham group (did not receive any medicine): not seen; B - Tes group (received testosterone): not seen; C - Tac group (received tacrolimus): all rats showed severe ulceration, redness and swelling; D - Tes+Tac group (received testosterone and tacrolimus): showed mild redness.

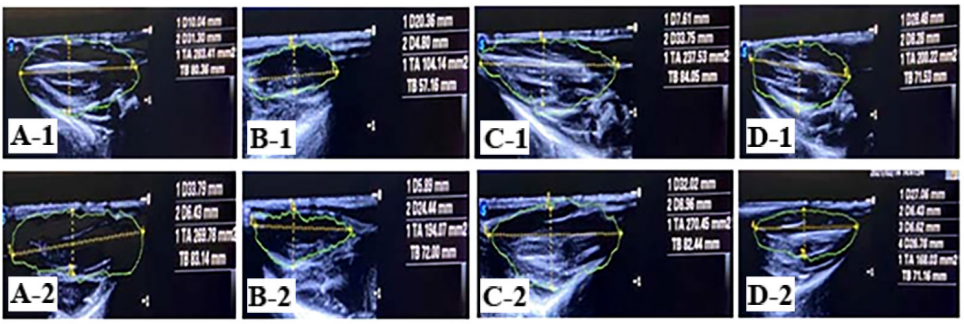

Fig. 2. Central field used to visualized whole gastrocnemius muscle and muscle diameter ratio was measured with left to right gastrocnemius diameter

A - Sham group: A-1 left, A-2 right ; B - Tes group: B-1 left, B-2 right; C - Tac group: C-1 left, C-2 right; D - Tes+Tac group: D-1 left, D-2 right

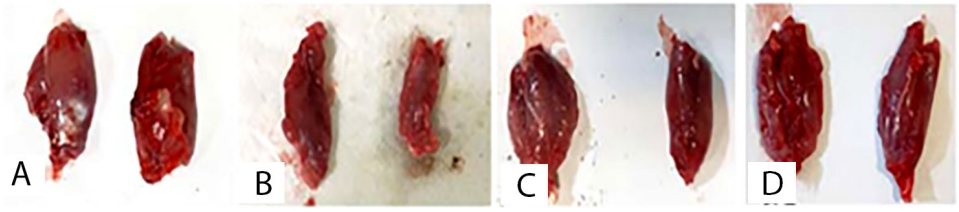

Fig. 5. Gross assessment and colour change

A - Sham group; B - Tes group; C - Tac group; D - Tes+Tac group

Gross atrophy of the left gastrocnemius compared to the right one was revealed.
Significant colour change was not seen between gastrocnemius muscles of legs with the crushed nerve and the non-affected side.

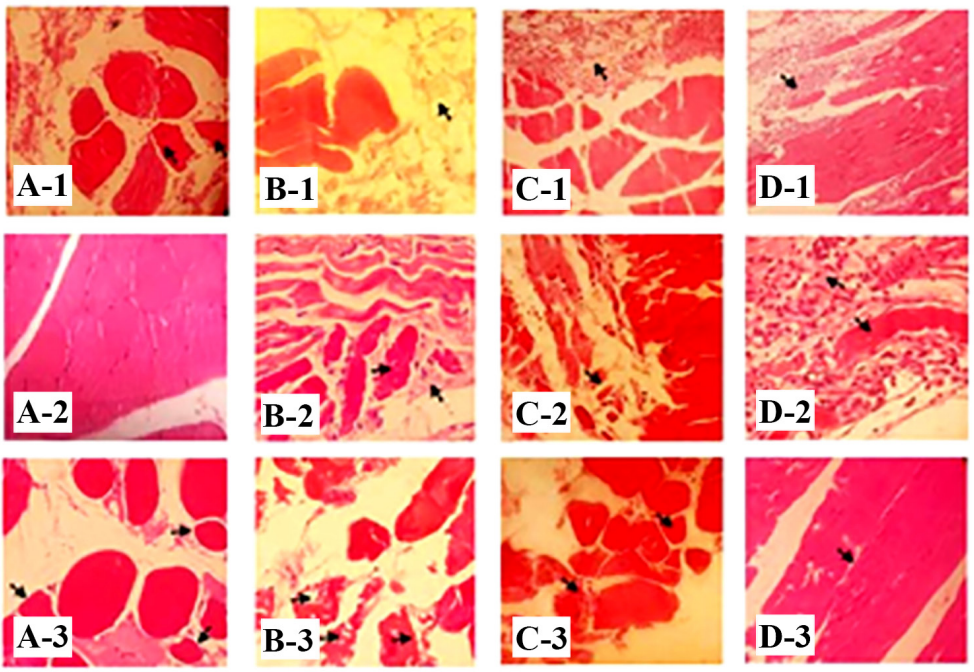

Fig. 6. Haematoxylin-eosin staining of gastrocnemius of left side with crushed sciatic nerve and right side with intact siatic nerve

A - Sham group. A-1 left: a few fibres with internal nuclei and numerous small rounded fibres (arrows) (× 400); A-2 left: normal myofibres (× 400); A-3 right: moderate myofibre atrophy (arrows) (× 400).

B - Tes group. B-1 left: fat infiltration fewer than muscle (arrows) (× 400); B-2 left: myofibres degenerated and atrophy with fibrotic change (arrows) (× 400); B-3 right: myocytes swelling and vacuolation, hyalinization, and fragmentation of sarcoplasm (arrows) (× 400).

C - Tac group. C-1 left: shows severe inflammatory phase, degeneration of myofibres (arrows) (× 100); C-2 left: severe inflammatory phase, degeneration of myofibres (arrows) (× 100); C-3 right: numerous hypereosinophilic small round muscle fibres with internal nuclei show severe inflammatory phase, degeneration of myofibres (arrows) (× 400).

D - Tes+Tac group. D-1 left: (arrows) (× 100) and D-2 left: (arrows) (× 400) severe inflammatory response with myofibre necrosis and degeneration; D-3 right: normal myofibres (arrows) (× 100).

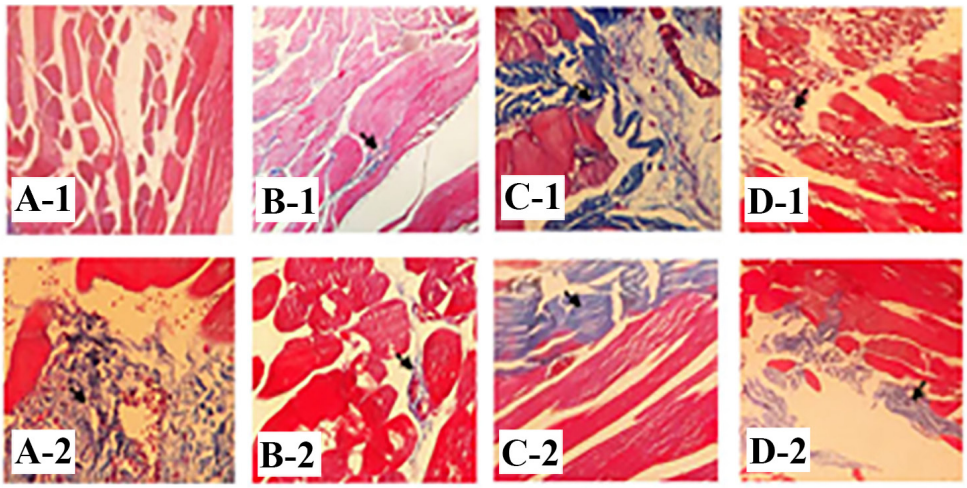

Fig. 7. Masson's trichrome staining of gastrocnemius of the left side with a crushed sciatic nerve and the right side with an intact sciatic nerve

A - Sham group. A-1 left: normal myofibres (arrows) (× 100); A-2 right: moderate fibrotic areas (arrows) (× 400).

B - Tes group. B-1 left: mild collagen deposition and fibrosis (arrows) (× 100); B-2 right: mild fibrosis (arrows) (× 400).

C - Tac group. C-1 left: marked collagen deposition (arrows) (× 400); C-2 right: moderate collagen deposition (arrows) (× 400).

D - Tes+Tac group. D-1 left: moderate collagen deposition and fibrosis (arrows) (× 400); D-2 right: mild collagen deposition (arrows) (× 100).