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Resuscitation Using Hypertonic Saline, Effect on Muscle Performance and Wound Healing in a Rat Model of Burn

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Abstract

Muscle wasting is a serious burn complication. The possible protective impact of hypertonic saline vs. ringer lactate on muscle wasting and wound healing in a rat burn model was investigated.

Rats were divided into; control rats received normal saline, untreated burn rats, ringer lactate treated burn rats & hypertonic saline treated burn rats received normal saline, ringer lactate or hypertonic saline, respectively after induction of burn. After 18 hours, serum levels of substance P, tumor necrosis factor- alpha (TNF-α), interleukin-1beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), and interleukin-10 (IL-10) were measured. Wound healing was assessed after 2, 4, and 6 weeks. At the end of the 6th week, gastrocnemius muscle weight and performance, transforming growth factor (TGF-β), collagen type I, forkhead box transcription factors O (FOXO) gene expression and histopathological examination were done. Hypertonic saline treatment significantly lowered substance P, TNF-α, IL-1β, IL-6, IL-2 with elevated IL 10, improved muscle weight and performance, improved wound healing, reduced TGF ß, collagen I and FOXO genes compared with ringer lactate treatment. Histopathological findings supported these results.

Early use of hypertonic saline in burn showed a more promising effect on the improvement of muscle performance and skin regeneration than ringer lactate solution does.

Keywords: Hypertonic saline, ringer lactate, muscle performance, wound healing, burn.

1. Introduction

Burn is a common accident in our daily life, particularly in developing countries. It represents the third cause of death in children and adolescents. An immunosuppressed condition frequently complicates thermal injury, making patients more susceptible to sepsis, systemic inflammatory response syndrome, and distant organ dysfunction and damage [1].

Inflammatory cytokines secreted by macrophages as TNF-α and IL-6 are peaked in serum during the first hours after injury and their levels were directly proportionate to the burned area size in many experimental animal models [2]**.** Other inflammatory mediators that helper T lymphocytes can produce as IL-4, IL-10, and TGF-β are also increased. These inflammatory mediators, in addition to vascular dilation and increased vascular hydrostatic pressure, are the leading causes of systemic microvascular leakage seen in burn trauma. Generalized venoushyperpermeability, endothelial cell contraction,tight junction damage, and kinins that are created at the burn area increasemicrovascular permeability [3]**.**

When vascular permeability increases, a new osmotic gradient is generated that will draw more fluid into the interstitium, increasing tissue edema. In 40% burn, the body can lose up to half of its blood plasma within 2 to 3 hours**.** So, hypovolemia and hemoconcentration usually occur within the first day after burns [4]**.**

In association, after a severe burn, hypermetabolism causes oxidative stress and protein degradation. Skeletal muscles are reservoirs for endogenous amino acids that share the liver in the synthesis of acute-phase reactant proteins [5]**.** Rapid changes of mitochondrial function in skeletal muscle after burn are other possible mechanisms underlying burn-induced muscle wasting [6].

Fluid therapy in a large amount is the cornerstone of burn management. Early following a burn, crystalloids

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can be given to replace fluid loss and maintain patient hemodynamics. This is followed by adding colloids to the fluid regimen [7]**.** The crystalloid of choice for the initial resuscitation in burn patients may be Ringer's acetate since it appears to protect the electrolytic balance in large replacements [8]**.**

Hypertonic saline is one of the crystalloids that are known to rapidly increase the plasma osmotic pressure, facilitate the rapid return of fluid from the interstitial into the blood vessels and restore blood circulation [9]**.** In addition, hypertonic saline can promote osmotic diuresis, reduce intracranial pressure and improve brain perfusion**.** Moreover, it has an anti-inflammatory effect and can regulate immune function [10]**.**

Dong et al [11] showed that cecal ligation and puncture-induced sepsis in rats may be treated with hypertonic fluids. It was shown that hypertonic saline dextran intravenously injected can alleviate hypoperfusion at early sepsis that was linked to decrease inflammatory interleukins, intracellular adhesion molecules, and TNF-α**.** Additionally, hippocampal apoptosis was suppressed by infusion of hypertonic saline in a rat model of cardiopulmonary resuscitation [12]**.**

Hypertonic saline impact on burn-triggered muscle wasting, to our knowledge, has not been studied up until now. We aimed to analyze the potential role of hypertonic saline treatment on muscle wasting and the rate of muscle and skin regeneration in cases of burn-induced in rats in comparison with ringer lactate solution as well as to investigate its possible underlying anti-inflammatory and anti-apoptotic effects through its influence on substance P, TNF-α, IL1β, IL-2, IL-6, IL-10 serum levels, and tissue expression of TGF-β, collagen type I and FOXO genes.

2. Methods

2.1 Fluids used

Hypertonic saline solution (Each 100 mL contains sodium chloride 3gm) (Otsuka Pharmaceutical, 10th of Ramadan city, Cairo - Egypt). Ringer's lactate solution (Each 100 mL contains sodium chloride 600 mg, sodium lactate 310 mg, potassium chloride 30 mg, and calcium chloride dihydrate 20 mg in distilled water) (Otsuka Pharmaceutical, 10th of Ramadan city, Cairo - Egypt).

2.2 Experimental Design

2.2.1 Animals

After obtaining the approval from ethical committees of animal experimentation of Cairo University (CU III F 39 22); thirty-two male albino rats, each weighing about 200- 220g were kept in properly ventilated clean cages (2 rats per cage) and received a balanced diet (containing 40% carbohydrate, 20% protein, 5% fiber, together with 3.5% fat, 6.5% ash, and a vitamin mixture) and free access to water supply. The experimental protocol was approved by the institutional licensing committee. Also, the care of animals and all steps of the experiment were carried out according to the rules and regulations laid down by the institutional ethical committees of animal experimentation with a ref no, CU III F 39 22.The study was conducted in accordance with the ARRIVE (Animal Research: Reporting of *in vivo* Experiments) guidelines.

2.2.2 Animal grouping

Rats were divided into four groups (eight rats in each group)

Group I: Normal control group: rats received 4 ml/kg normal saline (0.9% sodium chloride in distilled water) i.p. once after placing in water at 37°c for 10 seconds.
Group II: Untreated burn group (Model of burn without resuscitation by fluids): rats received 4 ml/kg normal saline i.p. once immediately after induction of burn.
Group III: Ringer lactate treated burn group (Burned rats resuscitated with ringer lactate fluid therapy): rats received 4ml/kg i.p. ringer lactate solution once immediately after induction of burn as group II [13].
Group IV: Hypertonic saline-treated burn group (Burned rats resuscitated with hypertonic saline fluid therapy): rats received 4 ml/kg i.p. hypertonic saline solution once immediately after induction of burn as group II [14].

2.3 Rat model of burn

A 30% TBSA (total body surface area) full-thickness burn model was created. All rats were anesthetized by administrating ketamine/xylazine; 100/10 mg/kg i.p. (Sigma-tec. Pharmaceutical Industries, 6th of October City, Egypt). Dorsal hair backs and the right hind leg were shaved and removed completely. The anesthetized rats were hold in a special design plastic box, with polyethylene tubing fixed along the length of its opening, in order to seal out the hot water preventing burning of the whole rats' skin.

About 25 cm^2 of their back were exposed with protrusion of their right hind legs. The exposed rats' back and the right hind leg were immersed in a hot water path was set at 95 °c for 10 seconds for producing an intermediate-sized burn in the back and right hind limb. An immediate injection of fluids was administered following the schedule of experimental design.

The back wound and the right hind leg were then treated with 1% tincture of iodine and kept dry to prevent infection. The animals were observed for signs of pain and treated with buprenorphine (0.05 mg/kg) (Sun

Pharmaceutical Company, Stockley Park, Uxbridge, UK) SC if needed. The rats in the control group were exposed to all steps applied to the burned rats except being immersed in a 37°c water path instead of 95 °C [15].

2.4 Measurement of concentrations of substance P, TNF-α, IL-1β, IL-2, IL-6, and IL-10

After 18 hours from burn induction, retro-orbital venous plexus samples were obtained under ketamine anesthesia to assess serum levels of substance P, TNF-α, IL-1β, IL-2, IL-6, and IL-10. The enzyme-linked immunosorbent assay (ELISA) technique was followed according to kit instructions to assess serum levels of substance P (ab133029) and inflammatory cytokines TNF-α(ab236712), IL-1β (ab255730), IL-2 (ab221834), IL-6 (ab100772) and anti-inflammatory cytokine IL-10 (ab214566) all obtained from abcam Official website, USA.

2.5 Assessment of Wound healing after 2, 4, and 6 weeks from burn induction

Assessment of Wound healing after 2, 4, and 6 weeks was done grossly by general examination of the wound, and photographic images were taken for the wound of the back in studied groups. The percent of wound healing was assessed as:
Wound healing rate = (original wound area - specific day wound area)/Original wound area × 100% [16].

2.6 Assessment of gastrocnemius muscle performance after 6 weeks from burn induction

The skeletal muscle's ability to contract in normal circulation entire and at body temperature was studied. Surgery was performed on all rats to expose their right sciatic nerve, while they were under general anesthesia with urethane (IM) injection (Sigma-tec. Pharmaceutical Industries, 6th of October City, Egypt). Cuts were made in the superficial muscle layer once the skin has been removed from the underlying connective tissue.
To separate the gastrocnemius muscle from other tissues, the right hind leg of a rat was dissected bluntly. The Achilles tendon was pushed out from under the plantaris tendon, exposing the gastrocnemius tendon. The tendon of the plantaris was pulled out from under the Achilles tendon, tied exposed tendon of gastrocnemius that will be connected by fiber thread to isometric force transducer MLT0201/RAD**:** (5 mg to 25 g) (Panlab, Spain Model number: TRI201), which connected to the amplifier of the power lab (ADinstruments, Australia Model number: ML221). Power lab was connected to an Isolated Tissue stimulator (FE155) (Dunedin, New Zealand). A platinum electrode for nerve stimulation was connected to stimulator.
A platinum electrode was applied around the sciatic nerve for the transmission of electrical stimulation. After the application of the electrode to the sciatic nerve, the rats were examined for electrophysiological assessment by performing repetitive nerve stimulation by electrical stimulation of the sciatic nerve at frequency 1 hz, pulse duration 0.1 milliseconds, amplitude 20 mA. Measurement of muscle-developed tension (expressed in grams), muscle performance ΔP/ΔT (expressed in gram/sec).
The 9:1 ratio was taken as an objective marker of the severity of the decrement response. As it is the ratio of the amplitudes of the ninth to the first compound muscle action potential (CMAP) where the decrement response is characterized by a progressive fall in the amplitude of the successive CMAPs elicited by a train electrical stimuli delivered to the sciatic nerve [17].

2.7 Assessment of gastrocnemius Weight after 6 weeks from burn induction

The weight of the gastrocnemius muscle was measured in all rats and was used as an indicator for the degree of muscle atrophy [18].

2.8 Measurement of quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) for transforming growth factor (TGF-β), collagen type I, and forkhead box transcription factors O (FOXO) genes after 6 weeks from burn induction

To extract total RNA, 25 µg of injured skin tissue from each group was homogenized in 300 µl of lysis buffer, following the instructions of the Gene JET Kit (Thermo Fisher Scientific Inc., Germany, #K0732). Reverse transcription was then performed using 5 µl of total RNA from each sample. Bioline'sSensiFASTTM SYBR R Hi-ROX One-step Kit (catalog number PI-50217 V) was used to amplify the RNA in a 48-well plate using the Step-one instrument (Applied Biosystems, Foster City, U.S.A.). The thermal profile was as follows: 45 °C for 15 minutes in one cycle for cDNA synthesis, followed by 10 minutes at 95 °C to inactivate the reverse transcriptase enzyme. Then, 40 cycles of PCR amplification were performed, each cycle consisting of 10 seconds at 95 °C, 30 seconds at 60 °C, and 30 seconds at 72 °C. The expression of the genes of interest was normalized relative to the mean critical threshold (CT) values of *β-actin*, a housekeeping gene, using the Ct method. The sequences of the primers used for the genes of interest are listed in (table I).

Table I: Primers sequence for studied genes; transforming growth factor (TGF-β), collagen type I and forkhead box transcription factors O (FOXO)

Gene	Sequence	NCBI reference number	Amplicon size (bp)
β-actin (housekeeping gene)	Forward: GACGTTGACATCCGTAAAGACC Reverse: CTAGGAGCCAGGGCAGTAATCT	NM_031144.3	113
TGF-β	Forward: TAATGGTGGACCGCAACAACG Reverse: GGCACTGCTTCCCGAATGTCT	NM_021578.2	100
Collagen type I	Forward: TACAGCACGCTTGTGGATGG Reverse: CAGATTGGGATGGAGGGAGTT	NM_053304.1	194
FOXO	Forward: CTACGAGTGGATGGTCAAGAGC Reverse: CCAGTTCCTTCATTCTGCACACG	NM_002015.4	138

TGF-β: transforming growth factor, FOXO: forkhead box transcription factors O

2.9 Histopathological assessment after 6 weeks from burn induction

At the end of 6 weeks after burn induction, excision of the middle parts of the right gastrocnemius muscle was carried out for animals of all groups. In addition, full-thickness skin specimens, including about 0.5 cm around the healing wound were taken from all animal groups. Both skin and muscle specimens were fixed in 10% buffered formalin solution for 24 hours, dehydrated in ascending grades of ethanol, and embedded in paraffin. Serial sections at 7 um thicknesses were cut using a microtome and mounted on glass slides. Paraffin sections of both skin and muscle biopsies were stained by: Hematoxylin and Eosin (H&E) stain for histological evaluation and Masson's Trichrome stain for collagen fibers [19].

Muscle sections were subjected to immunohistochemical staining [20] using anti-Myogenin antibodies (Mouse monoclonal anti-myogenin antibody used for immune-histochemical staining (ab212667, Abcamplc, Cambridge Biomedical Campus, Cambridge, England) to detect early differentiating myogenic precursor cells.

The bound primary antibody was immunodetected using the labeled avidin-biotin-peroxidase complex (Histostain SP kit, Zymed Laboratories Inc, San Francisco, USA). Diaminobenzidine (DAB) was used as a chromogen and Meyer's hematoxylin as a counterstain. To establish immunohistochemical staining specificity, negative control serial sections were processed by omitting the primary antibody while all other steps were performed in the same manner. Positive control was a known case of human rhabdomyosarcoma with a positive nuclear reaction.

2.10 Morphometric image analysis

10 different regions were selected from each group of samples to measure: a) Cross-sectional area of skeletal muscle fibers in H&E-stained sections x100, b) Area percent of collagen fibers stained with Masson's trichrome stain x100. c) The number of cells stained with myogenin antibody (10 non-overlapping fields) x400. This was done using Leica Qwin-500 LTD-software image analysis computer system (Cambridge, England).

2.11 Statistical method

Data were analyzed using SPSS (Statistical Product for Services Solutions) software version 22 and presented as mean and standard deviation (SD). The differences between groups were compared using analysis of variance (ANOVA). Post hoc tests were used to adjust for multiple comparisons. All tests were two-tailed. A *p-value* of less than 0.05 was considered statistically significant [21].

3. Results

3.1 Serum levels ofsubstance P, TNF-alpha, IL-1beta, IL-2, IL-6 and IL 10 and in studied groups:

The levels of serum substance p, TNF-α, IL-1β, IL-2, and IL-6 were significantly elevated in the untreated burn group (Group II) compared to the control group (Group I) ($p<0.001$). This elevation was significantly decreased after treatment of burn animals with ringer lactate (Group III) or hypertonic saline (Group IV) compared with the untreated burn group (Group II) ($p<0.001$). Better improvement was detected in the burn group treated with hypertonic saline when compared with the burn group treated with the ringer lactate group ($p<0.05$) returning the elevated measuring level near normal (no significant difference with the normal control group; $p>0.05$) (Table II).

The level of serum of IL10 cytokine was significantly decreased in the untreated burn group compared to the control group ($p<0.001$). After treatment of burn groups with hypertonic saline (Group IV) or ringer lactate (Group III), IL-10 serum level showed significant improvement in group IV compared with group III ($p<0.05$) (Table II).

Table II: Serum levels of substance P, tumor necrosis factor alpha (TNF-α), interleukin-beta (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6) and interleukin-10 (IL-10) in different studied groups: (Mean ±SD) (each group = 8 rats)

Studied parameter (pg/ml)	Normal control group	Untreated burn group (Model Group)	Ringer lactate treated burn group	Hypertonic saline treated burn group
Substance p	12.25±1.44	46.72±2.11[a]	22.1±2.52[ab]	14.52±2.01[bc]
TNF-α	25.35±3.86	89.07±11.52[a]	32.08±5.41[ab]	25.32±3.06[bc]
IL1β	58.67±5.79	169.48±12.19[a]	90.57±3.36[ab]	70.9±6.11[bc]
IL2	30.57±4.39	109.08±7.88[a]	55.75±8.82[ab]	38.83±3.74[bc]
IL6	56.5±7.97	161.67±13.6[a]	108.83±7.47[ab]	63±6.07[bc]
IL10	93.32±5.13	27.02±3.34[a]	35.62±7.42[ab]	52.83±8.95[abc]

TNF-α: tumor necrosis factor alpha, IL-1β: interleukin-beta, IL-2: interleukin-2, IL-6: interleukin-6, IL-10: interleukin-10
a: Significant compared with normal control group (p<0.05)
b: Significant compared to untreated burn model group (p<0.05)
c: Significant compared with ringer lactate treated burn group (p<0.05)

3.2 Transforming growth factor (TGF-β), collagen type I, and FOXO gene expression in different studied groups

Studied gene expressions were significantly induced in the untreated burn group compared to the control group (p<0.001). Genes expressions were significantly repressed after treatment of burn animals with ringer lactate or hypertonic saline (p<0.001) with better improvement in burn group treated with hypertonic saline compared with burn group treated with ringer lactate (p<0.05) (Figure 1).

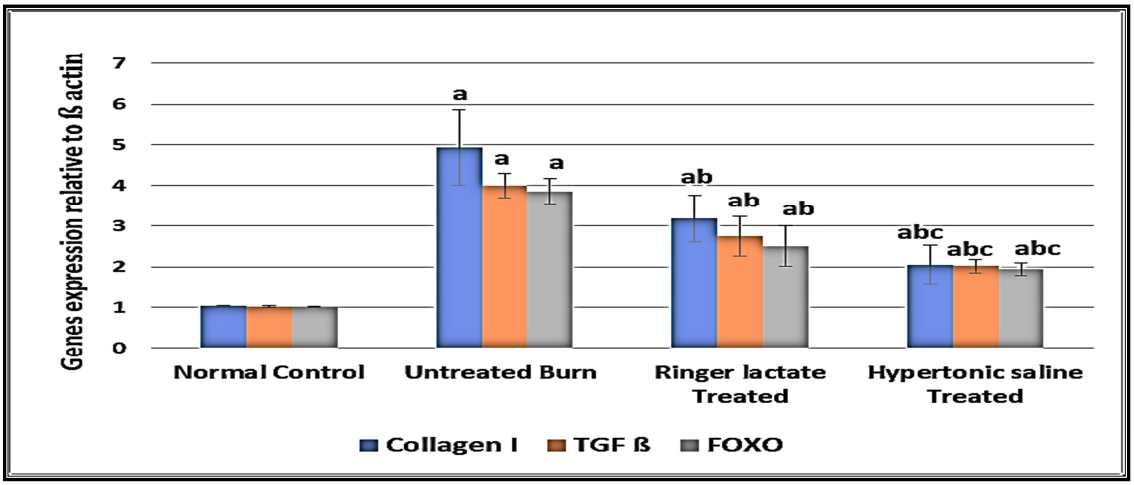

Figure (1): Quantitative reverse transcriptase- polymerase chain reaction (RT-PCR) of *collagen type I, transforming growth factor (TGF-β) and forkhead box transcription factors O (FOXO)* genes in different studied groups: (Mean ±SD) (each group = 8 rats)
a: Significant compared with normal control group (p<0.05)
b: Significant compared to untreated burn model group (p<0.05)
c: Significant compared with ringer lactate treated burn group (p<0.05)

3.3 Wound healing after 2, 4, and 6 weeks of induction of burn

The percentage of wound healing was significantly improved in burn rats treated with ringer lactate solution or hypertonic saline solution compared with untreated burn rats at the end of the 2nd, 4th, and 6th weeks after induction of burn (p<0.05). Percent of healing recorded was significantly better in burn rats treated with hypertonic saline compared with those treated with ringer lactate (p<0.05) (Table III).

Table III: Percent of wound healing after 2, 4 and 6 weeks of burn induction in rats in different studied groups (Mean ±SD) (each group = 8 rats)

	Untreated burn group (ModelGroup)	Ringer lactate treated burn group	Hypertonic saline treated burn group
Percent of wound healing after 2 weeks	12.2 ±0.8	34.4±2.3[a]	45.1 ±4.4[ab]
Percent of wound healing after 4 weeks	33.4 ±1.2	57.5 ±4.5 [a]	75.7 ±4.9[ab]
Percent of wound healing after 6 weeks	67.8 ±4.5	82.6 ±6.2 [a]	93.8 ± 5.4[ab]

a: Significant compared to burn model group (p<0.05)
b: Significant compared with ringer lactate treated burn group (p<0.05)

3.4 Muscle performance in response to the ninth response

Regarding gastrocnemius muscle repeated stimulation, the amplitude of the first & ninth responses showed a significant decrease (decrement response) in the untreated burn group compared to the normal control group (p< 0.001). Ringer lactate and hypertonic saline treated groups showed a non-significant difference in the amplitude of 1st muscle contraction (p>0.05) while the amplitude of muscle contraction at 9th contraction showed a significant increase in the treated groups compared with the corresponding results in untreated burn group (p<0.05). Decrement response was significantly improved in groups treated with ringer lactate or hypertonic saline compared with the untreated burn group (p<0.05). Significant improvement in calculated muscle performance (Δ P/ΔT) of ninth contraction was evident in the hypertonic saline treated burn group compared to the untreated burn model group and ringer lactate treated burn group (p<0.05) (Table IV, Figure 2).

Table IV: Gastrocnemius muscle **weight, Amplitude of muscle contraction at 1st and 9th contraction, Muscle performance** (Δ P/ΔT) at 1st and 9th contractions and 9:1 ratio **in different studied groups:** (Mean ±SD) (each group = 8 rats)

	Normal control Group	Untreated burn group (Model Group)	Ringer lactate treated burn group	Hypertonic saline treated burn group
GastrocnemiusWeight (g)	1.7±0.07	1.1±0.14[a]	1.3±0.06[ab]	1.4±0.08[ab]
Amplitude 1st contraction (g)	1.5± 0.18	0.8 ±0.3[a]	0.8 ± 0.09 [a]	1.1 ± 0.08 [a]
Amplitude 9th contraction (g)	1.4 ± 0.8	0.2 ± 0.01 [a]	0.6 ±0.04[ab]	0.8 ± 0.04[ab]
Muscle performance (Δ P/ΔT) at 1st contraction (g/sec)	1.1 ± 0.2	1 ± 0.09	1 ± 0.1	1.1± 0.1
Muscle performance (Δ P/ΔT) at 9th contraction (g/sec)	1.1 ± 0.21	0.5 ± 0.07 [a]	0.7±0.1[a]	1.1 ± 0.04[bc]
9: 1 ratio	0.8	0.2[a]	0.5[b]	0.8[bc]

a: Significant compared with normal control group (p<0.05)
b: Significant compared to untreated burn model group (p<0.05)
c: Significant compared with ringer lactate treated burn group (p<0.05)

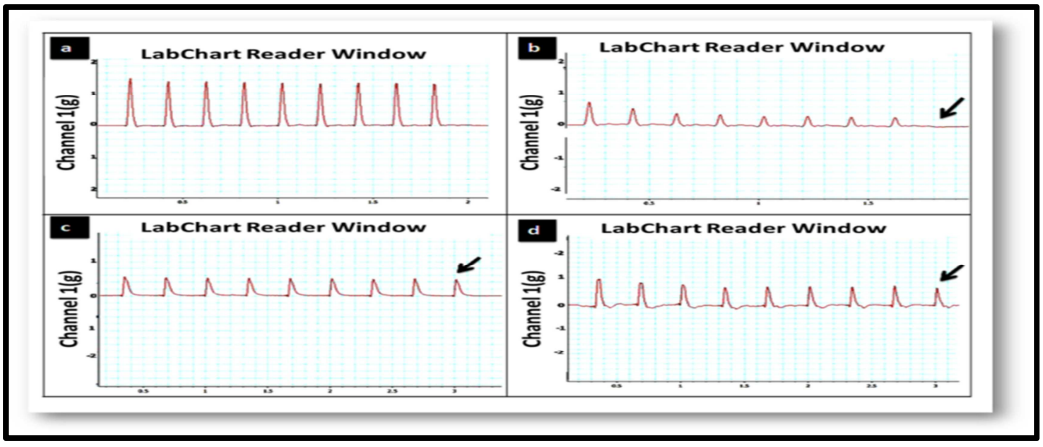

Figure (2): The amplitude of gastrocnemius muscle contractions after repeated sciatic nerve stimulation in different studied groups after 6 weeks of burn induction:
-a: Normal control group: normal muscle response to nine repeated electrical impulses at 1 Hz, 0.1 ms pulse duration and 20 mA amplitude.
-b Untreated burn group: progressive decrease in the amplitude of muscle contraction until absence of the ninth response is depicted by the black arrow
-c: Ringer lactate treated burn group: improvement in muscle amplitude with reappearance of the amplitude of the ninth response
-d: Hypertonic saline treated burn group: improvement in muscle amplitude with reappearance of the amplitude of the ninth response

3.5 The weight of gastrocnemius muscle

The weight of the gastrocnemius muscle showed a significant decrease in the untreated burn model group compared to the normal control group (p<0.05). Meanwhile, groups treated with either ringer lactate or hypertonic saline showed significant improvement in muscle weight (p<0.05) compared to the untreated burn model group. In addition, hypertonic saline showed a greater increase in the weight of the muscle compared to the ringer lactate-treated group (Table IV).

3.6 Histopathological results

3.6.1 Skin sections stained with H&E

The control group (Group I) had a thin epidermis with a basal layer resting on a clear wavy basement membrane with the superficial layer of the epidermis composed of keratin filaments. The dermis was densely packed with hair follicles and sebaceous glands with an evident superficial papillary layer, a deep reticular layer, and underlying hypodermis. The untreated group (Group II) in the burnt area shows failure of skin regeneration with lost whole epidermis and part of the dermis with destruction in hair follicles and congested vessels. Disarrangement of collagen fibers was seen in the reticular layer of the dermis. The papillary layer showed dense collagen bundles with excessive growth extending beyond the epidermal surface of the adjacent normal skin. Mononuclear cell infiltration and some fibrocytes, Ringer lactate treated burn group (Group III) exhibited thick horizontally arranged parallel collagen bundles. However, the hypertonic solution-treated burn group (Group IV) exhibited apparently normally arranged collagen fibres and apparent hair follicles (Figure 3).

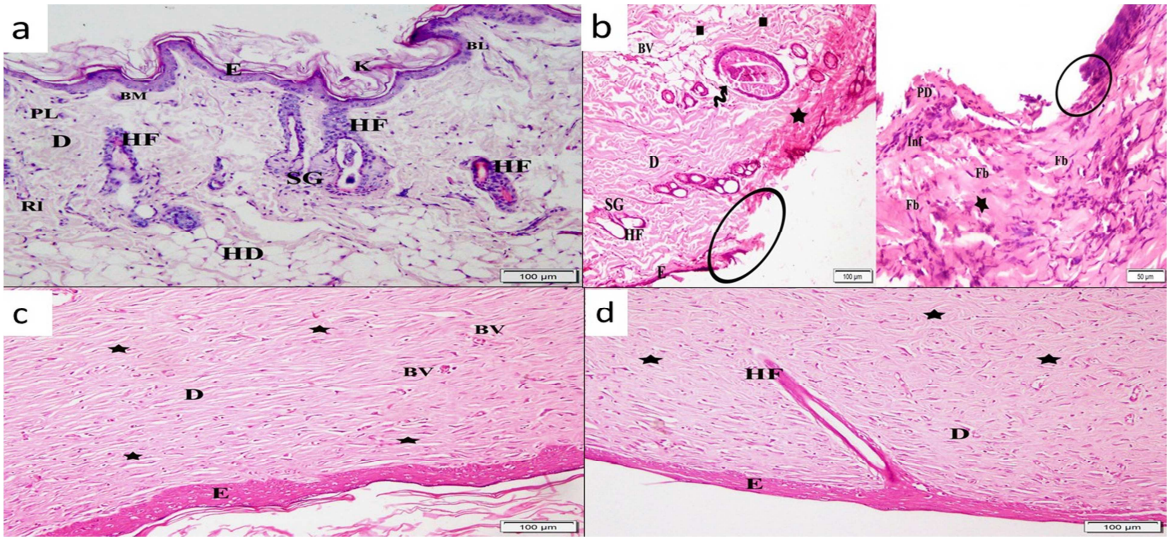

Figure (3): Photomicrograph of H & E stained skin section in different studied groups (x 100):
-a: Normal control group showing epidermis (E), basal layer (BL), basement membrane (BM), dermis (D), superficial papillary layer (PL) and deep reticular layer (RL), hair follicles (HF), sebaceous glands (SG), hypodermis (HD) and keratin filaments (K)
-b: Untreated burn group on the left side showed a junction between the healthy skin and the burnt area (circle); epidermis (E), dermis (D), hair follicles (HF), and sebaceous glands (SG) at the healthy side while the burnt area shows destruction in hair follicles (spiral arrow), congested vessels (BV), disarrangement in collagen fibers (square) in the reticular layer of the dermis and apparently dense collagen bundles (star) in the remaining part of the papillary layer. The right side shows a junction between the healthy skin and the burnt area (circle), thickened papillary dermis (PD) with abnormally thick densely packed collagen bundles (star), cellular infiltration (Inf), and fibroblasts (Fb).
-c: Ringer lactate treated burn group exhibits epidermis (E), dermis (D) with thick horizontally arranged parallel collagen bundles (star); congested blood vessels (BV) and absent hair follicles and sebaceous glands
-d: Hypertonic solution-treated burn group exhibits epidermis (E), dermis (D) with thick collagen bundles (star). Note hair follicles (HF).

3.6.2 Masson trichrome stained skin sections

Skin sections obtained from the control group (Group I) showed normal skin appearance. The burnt area sections from the model group (Group II) showed failure of skin regeneration with loss of the whole epidermis and a considerable part of the dermis with disarrangement of collagen bundles with excessive growth of the papillary layer. Partially regenerated skin with horizontally arranged parallel, blue-stained collagen bundles was detected in the ringer lactate-treated burn group (Group III). However, almost regenerated skin was detected in the context of the hypertonic saline-treated burn group (Group IV) (Figure 4).

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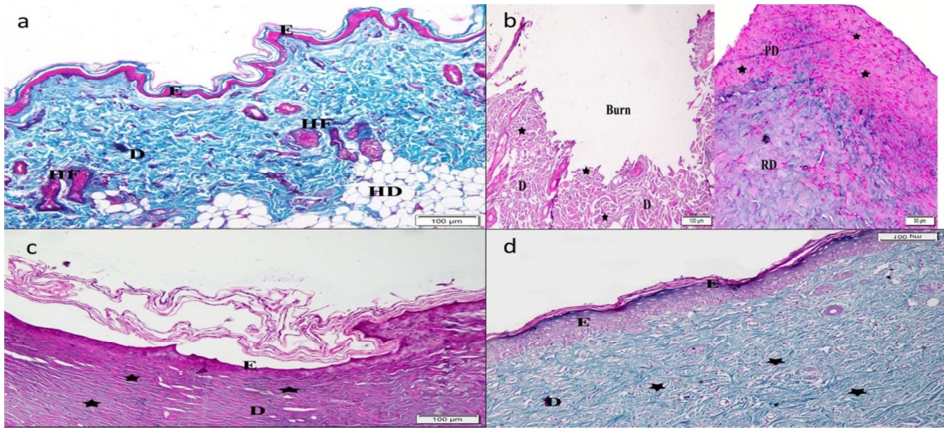

Figure (4): Photomicrograph of Masson trichrome stained skin section in different studied groups (x 100):
-a: Normal control group showing epidermis (E), dermis (D), hypodermis (HD), many hair follicles (F).-b: Untreated burn group at the left side showing lost whole epidermis and a considerable part of the dermis (D) with disarranged collagen bundles (star). The right side showed lost epidermis, thickened papillary layer (PD), and abnormally thick densely packed collagen bundles (star) within the reticular dermis (RD).-c: Ringer lactate treated burn group showed regenerated epidermis (E), regenerated dermis (D), thick horizontally arranged parallel collagen bundles (star).
-d: Hypertonic saline-treated burn group showed fully regenerated epidermis (E), regenerated dermis (D), thick collagen bundles arranged in different directions (star).

3.6.3 H&E-stained skeletal muscle sections:

Examination of skeletal muscle of the normal control group (Group I) showed muscle fascicles. Each fascicle is composed of polyhedral myofibers with acidophilic cytoplasm and peripherally located nuclei and little intervening endomysial connective tissue in between. The fascicle is wrapped by a layer of perimysium. Muscle fibres in the untreated burn group (Group II) showed retention of the cellular outlines with pale mostly structureless sarcoplasm devoid of myofibrils with markedly diminished endomysium and perimysium. Most Fibres in the ringer lactate-treated burn group (Group III) have rounded contours with pale mostly structureless sarcoplasm and few Scattered regenerating fibres while the fibres in the hypertonic saline solution treated burn group (Group IV) are mostly polyhedral with the majority having normal appearance (Figure 5).

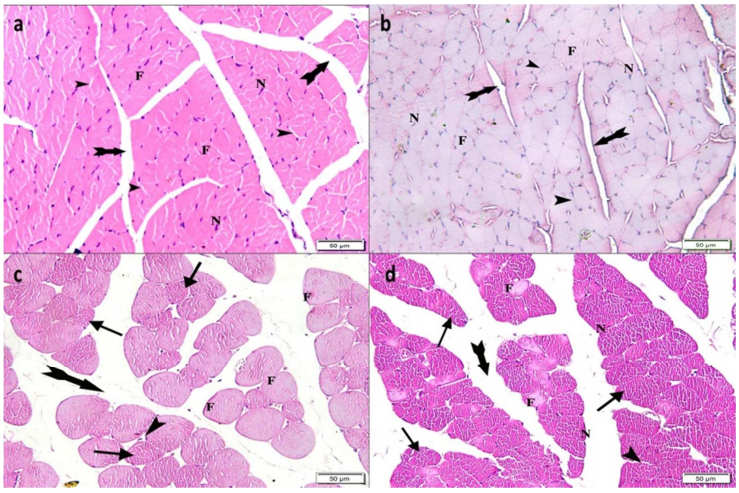

Figure (5): Photomicrograph of H&E-stained transverse section in gastrocnemius muscle in different studied groups (x 200):
-a: Normal control group showing muscle fibres (F) with peripheral nucleus (N), endomysium (arrowhead), and perimysium (arrow with bifid end)-b: Untreated burn group showing pale acidophilic sarcoplasm devoid of striation (F) with peripheral nucleus (N), diminished CT endomysium(arrowhead), and perimysium (arrow with bifid end)-c: Ringer lactate treated burn group showing fibres (F) with rounded contour exhibiting pale mostly structureless sarcoplasm. Nuclei are peripherally located (N). Expanded endomysium (arrowhead) and perimysium (arrow with bifid end), scattered regenerating fibres (smaller, darker cytoplasm, and larger nuclei) were detected (arrow)-d: Hypertonic saline-treated burn group showing mostly polyhedral myofibers with the majority showing myofibrils in their cytoplasm (arrow). Few myofibers still exhibit homogenous pale cytoplasm with no myofibrils (F). Nuclei are peripherally located (N). Normal endomysium (arrowhead) and expanded perimysium were detected (arrow with bifid end).

3.6.4 Masson trichrome stained skeletal muscle sections:

Skeletal muscle fibers of the normal control group (Group I) showed normal apparent polyhedral muscle fibres surrounded by connective tissue endomysium with perimysium surrounding the muscle fascicles. Deficient endomysium and perimysium were seen in burn group (Group II)). However, most muscle fibres and bundles in the ringer lactate treated group (Group III) appeared widely separated with connective tissue endomysium and perimysium enclosing congested blood vessels. On the other hand, muscle fibres of the hypertonic saline treated group (Group IV) showed a normal appearance surrounded by apparently normal endomysium and perimysium (Figure 6).

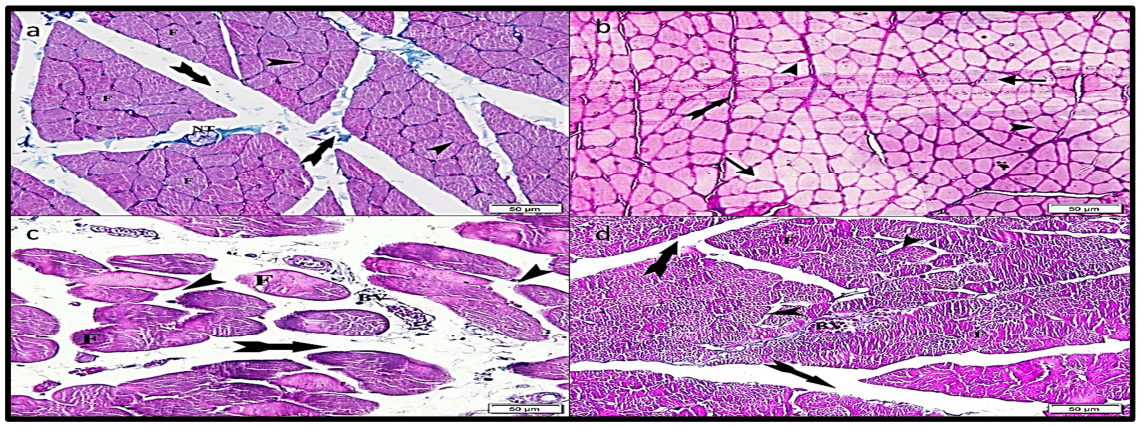

Figure (6): Photomicrograph of Masson trichrome stained transverse section in gastrocnemius muscle in different studied groups (x 200):
-a: Normal control group showing polyhedral muscle fibres (F) surrounded by endomysium (arrowhead), and perimysium (arrow with bifid end) surrounding the fascicles and nerve trunk (NT).-b: Untreated burn group showing polyhedral muscle fibres with pale structureless sarcoplasm devoid of myofibrils (arrow), most of them surrounded by deficient endomysium (arrowhead) and perimysium (arrow with bifid end).-c: Ringer lactate treated burn group showing muscle fibres most of them exhibiting smooth rounded edges rather than being polyhedral (F), The muscle fibres appear widely separated in some bundles (arrowhead). Connective tissue perimysium (arrow with bifid end) shows congested blood vessels (BV).-d: Hypertonic saline-treated burn group showing polyhedral skeletal muscle fibres (F) surrounded by apparently normal endomysium (arrowhead), connective tissue perimysium (arrow with bifid end) showing blood vessels (BV).

3.6.5 Immuno-histochemicalmyogenin stained skeletal muscle sections:

The untreated burn group (Group II) showed faint positive immune reactivity.Sections obtained from ringer Lactate treated burn group (Group III) showed strong positive nuclear immune reactivity. On the opposite side, the hypertonic saline-treated burn group (Group IV) showed negative nuclear staining (the same as the control group (Group I)) (Figure 7).

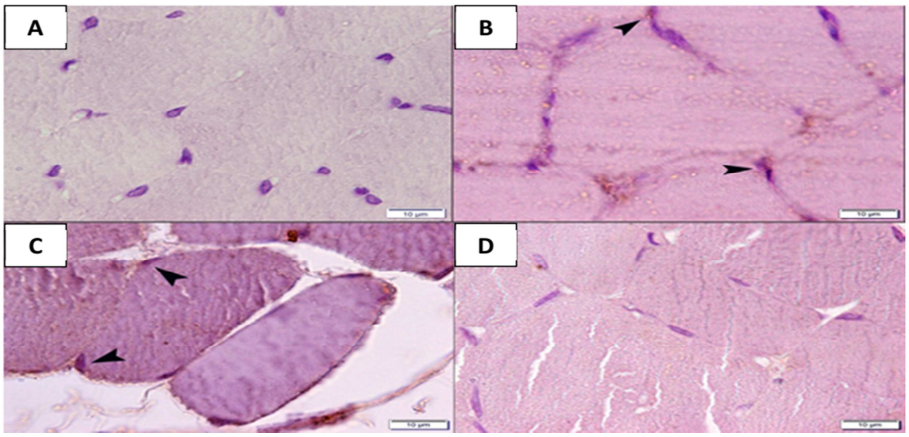

Figure (7): Photomicrograph of Myogenin stained transverse section in gastrocnemius muscle in different studied groups (x 1000):
-a: Normal control group showing negative nuclear immunoreactivity to myogenin -b: Untreated burn group showed faint positive nuclear immunoreactivity to myogenin (arrow).-c: Ringer lactate treated burn group showing strong positive nuclear immunoreactivity to myogenin-d: Hypertonic saline-treated burn group showed negative nuclear immunoreactivity to myogenin

3.6.6. Morphometric results

Regarding the mean area % of trichrome-stained collagen fibers in both skin sections and skeletal muscle fibers sections and the mean cross-sectional area of transverse skeletal muscle fibers showed a significant reduction in the untreated burn group (Group II) compared with the control group (Group I) (p<0.05). This reduction was significantly improved in the ringer lactate-treated burn group (Group III) and the hypertonic saline-treated burn group (Group IV) (p<0.05). The recorded improvement was more in group IV compared with group III. The mean number of myogenin-positive cells in group III was significant to all other groups (p<0.05) (Table V).

Table V: Morphometric results in different studied groups: (Mean ±SD) (each group = 8 rats)

Item	Normal control Group	Untreated burn group (ModelGroup)	Ringer lactate treated burn group	Hypertonic saline treated burn group
Mean area % of trichrome stained collagen fibres in skin sections (μm² %)	34.2±12	11.9±1.6[a]	23.7±3[ab]	31.7±5[bc]
Mean area % of trichrome stained collagen fibres in skeletal muscle fibres sections (μm² %)	6 ±1.9	2.4±0.9 [a]	4.4 ±1.1[ab]	4.7 ±2.2[b]
Mean cross sectional area of transverse skeletal muscle fibres	852.6±27	688±34 [a]	824±35 [b]	837.9±28 [b]
number of myogenin positive cells	1.3±0.9	2.3±1.3 [a]	3.5±1.4[ab]	1.4±1[bc]

a: Significant compared with normal control group (p<0.05)
b: Significant compared to untreated burn model group (p<0.05)
c: Significant compared with ringer lactate treated burn group (p<0.05)

4. Discussion

Skeletal muscle wasting is one of possible complications after burn trauma. The occurrence of sepsis leads to improper wound healing and muscle dysfunction [22]. Post-burn immobilization can be another contributing risk factor to the development of muscle atrophy [23].

Hypertonic salt solutions are effective in the treatment of burn shock by decreasing fluid shifts, and rapid stabilization of heart rate and blood [24]. In addition, hypertonic fluids inhibit the inflammatory response to shock, that occurs with the use of other isotonic fluidsand enhance host defense against bacteria. Use hypertonic saline glucose free solution could be valuable in resuscitation of diabetic patients [25].

Previous studies have investigated the impact of numerous pharmacological and nutritional interventions on post-burn skeletal muscle wasting. However, as far as we know, no previous research has examined the impact of fluid selection on post-burn skeletal muscle wasting. The present work was designed to investigate the effects of using hypertonic saline on wound healing and muscle wasting versus the conventional use of ringer lactate for fluid resuscitation in post-burn management.

In the present study, after 18 hours of burn induction, rats resuscitated by hypertonic saline solution immediately after burn injury showed a significant decrease in serum substance p, TNF-α, IL-1β, IL-2, IL-6 compared with untreated burn group and with rats resuscitated with ringer lactate solution. While serum IL10 level was significantly increased in both treated groups with hypertonic saline or ringer lactate with significant improvement with hypertonic saline solution. Wound healing is important to guard against infection and maintain homeostasis. The healing process starts immediately after injury and occurs in 3 phases: inflammation, proliferation, and maturation. Inflammation involves the recruitment of neutrophils and macrophages to the site of injury. They produce IL-1β, IL-2, IL-6, TNF-α, and TGF-α which are essential for chemotaxis of keratinocytes, endothelial cells, and fibroblasts [26].

Increased cytokines are demonstrated by Kim et al [27] who recorded the highest levels of IL-6 and IL-8 in patients with 15-30% TBSA burn injuries during the first day. In skeletal muscle, these cytokines promote muscle proteolysis, resulting in muscular atrophy as TNF-α and its downstream target nuclear factor- kappa B (NF-κB) have been shown to decrease protein synthesis and potentiates protein degradation. Ozdemir et al [28] showed elevated TNF-α and NF-κB in rat model of severe burn.

The results of the present study are consistent with that of Horton et al [29] and Oliynyk et al [30] who showed that administration of hypertonic saline immediately after burn significantly lowered TNF-α, IL-1β, and IL-6. Also, Yuan et al[31] revealed that resuscitation using hypertonic saline inhibited TNF-α and IL-1β release alleviated oxidative stress and improved renal histopathology.

Moreover, Sun et al[32] stated that hypertonic saline use reduced intestinal injury, decrease serum IL-1β concentrations, and improved intestinal histopathology

caused by severe burns. In this sepsis model, hypertonic saline attenuated significantly the increases in neutrophil activity and macrophage migration. Mahung et al[33] showed that the level of cytoprotective IL-10 is associated with better outcomes in patients with burns.

Hypertonic saline or ringer lactate treatment after burn in the present study significantly reversed burn-induced fibrosis, asTGF-β, collagen type I, and FOXO genes were significantly decreased compared with untreated burn group with better improvement with burn group treated with hypertonic saline compared with that treated with ringer lactate.

In the epidermis, FOXO inhibits wound healing and re-epithelialization processes through its effect on matrix metalloproteinases (MMPs) that degrade collagen and extracellular matrix (ECM) during wound healing[34]. TGF-β1 induces the expression of integrin in keratinocytes (KCs) resulting in complete epithelialization. But excessive TGF-β1 expression induces pathological hypertrophic scar and has a negative regulator of skeletal muscle size and induces muscle fibrosis [35].

The present results agreed with Liu et al [36] who detected that FoxO3a depletion accelerates wound healing. Brightwell et al [37] described an increased level of TGF-β1 after severe burn-induced in mice and suggested its responsibility for the development of muscle fibrosis and dysfunction. An increased level of collagen after injury and in the early phases of wound healing was demonstrated by Shomita et al[38].

In the present study, the untreated burn group showed grossly obvious hypertrophic skin scarring which was consistent with the microscopic findings that revealed excessive and dysregulated deposition of collagen fibers with abundant fibroblasts in the dermis. This correlates with the increased TGF-β and collagen I gene expression detected in the skin specimens resulting in disfigurement and deformity and matched with results of Merritt et al [39] who detected that use of anti- TGF-β reduced scar formation without hindering wound healing in animal models.

Some sections of the untreated burn group in the present work showed failure of re-epithelialization as uncontrolled MMP expression by excessive cytokines results in degradation of newly formed matrix, aberrant cell–cell contacts of the migrating keratinocytes, and an increased rate of cell death of wound edge keratinocytes. Ringer lactate-treated burn group showed a better degree of regeneration with thick newly formed epidermis and parallel horizontally arranged collagen fibers in the dermis but with failure of skin appendages regeneration. Almost completely regenerated skin was detected in the hypertonic saline-treated burn group, with apparently normal epidermal thickness and normal orientation of collagen fibers in the dermis. In addition, some hair follicles started to appear. These findings were coinciding with the gross picture, serum cytokines, and gene expression in skin specimens.

Kinsky et al [40] reported the beneficial effect of hypertonic saline in wound healing and concluded that early infusion of hypertonic saline dextran solution (HSD) in a sheep model of burn led to a reduction in skin edema compared with the use of ringer lactate. Campbell [41] and Samidah et al [42]stated that hypertonic saline-soaked dressing in animal models facilitated wound healing.

In the present study, there was a significant improvement in calculated muscle performance of the ninth contraction in the hypertonic saline-treated burn group compared to ringer lactate treated burns with the disappearance of decrement response detected in the untreated burn group. Moreover, the weight of the gastrocnemius muscle showed a significant improvement in the hypertonic saline-treated group compared to the ringer lactate-treated group.

Deleterious effects of burn on neuromuscular function were detected by Higashimori et al [43] who showed that burn animal model with 20% of the TBSA reduced tibial nerve conduction and Schaeffer et al [44] who recorded that third-degree hindpaw burn injury in rats' results in muscle damage and degeneration of sensory and motor neuron fibers of sciatic nerve.

Wu et al [45] showed that muscle weight, and muscle contractile properties were reduced in the rat burn model. Mizushima and Komatsu, [46] showed loss of muscle mass of gastrocnemius muscles of a mammal after burn injury that may be mediated through FoxO3-medicated muscle loss. Salvinelli et al [47] showed that nasal administration of saline solution can enhance neurons survival through release of nerve growth factor.

Wang et al [48] provided evidence from *in* vivo and *ex*-vivo burn injuries induced significant failure of musculoskeletal membrane repair and skeletal muscle damage. The protective effect of hypertonic saline in protection against muscle injury after skeletal muscle ischemia-reperfusion injury in a rodent model was detected by Dillon et al[49].

Muscle fibers in the untreated burn group in the current study showed degeneration with irregular contours. Most muscle fibers in the ringer lactate-treated burn group appeared with rounded contours with partial loss of myofibrils indicating incomplete regeneration. While in the hypertonic treated burn group, the fibers exhibited polyhedral contour with complete restoration of myofibrils. This picture is matched with the corresponding serum level of measured cytokines in each group. Masson trichrome stained muscle sections revealed reduced endomysium and perimysium in the untreated burn group. There was wide endomysium and perimysium with congested blood vessels in the ringer lactate-treated burn group. Moreover, in the hypertonic saline-treated burn group the endomysium appears normal with less enlarged perimysium in the majority of muscle fibers suggesting a better chance for structural and hence functional recovery.

After muscle injury, the regeneration process starts with the stimulation of dormant myogenic cells called satellite cells, situated between muscle sarcolemma and the basement membrane. Once activated, satellite cells are converted to myoblasts that replicate repeatedly and then fuse together forming multinucleated myotubes which then turn into adult myofibers [50]. However, exhaustion of satellite cells reduces muscle regenerative potentials with consequent muscle deterioration. Myogenin is a specific skeletal muscle gene encoding a transcription factor related to muscle repair. It is responsible for the induction of terminal differentiation of myoblasts and expression of skeletal muscle proteins such as creatine kinase and myosin heavy chain [51].

Our investigation indicated that myogenin faint negative immunoreactivity in the untreated burn group may be attributed to dysregulation of the satellite cells with subsequent reduction in their ability to repair skeletal muscle damage precipitated by thermal injury. The ringer lactate-treated burn group displayed positive immunoreactivity suggesting that the process of regeneration is still in progress. While negative immunoreactivity in the hypertonic saline-treated burn group may be explained by the consumption of satellite cells during the regeneration process. That confirms complete healing occurred at the end of the 6th week in this group returning the immune reactivity to the same as the control group.

These results are matched with Mann et al [52] who revealed that diminished endomysium and perimysium in the burn model may be the cause of the failure of muscle regeneration after burn. Yousuf et al [53] suggested that burn-induced muscle atrophy may be treated by targeting satellite cells. Corrick et al [54] detected that incubation of human muscle cells with serum obtained from burn patients showed impaired myogenesis, reduced myotube size, and reduced number of nuclei per myotube. These results indicate the effect of circulating cytokines in impaired muscle recovery following burn injury. Clark et al [55] showed that severe burns in adult patients induced skeletal atrophy that was associated with increased cellular apoptosis and the inhibition of satellite cell activation and differentiation.

5. Conclusions

The results of the present study indicate that immediate resuscitation of burn rats with hypertonic saline solution showed significantly lower levels of substance P, TNF-α, IL-1β, IL-6, IL-2, and higher IL-10 levels, better improvement of muscle weight and degree of performance after repeated muscle stimulation, better gross and microscopical wound healing, better rate of skin re-epithelialization and less expression of *TGF-β, collagen I and FOXO* genes compared with burn rats resuscitated by ringer lactate solution. So, we can suggest the preferable use of hypertonic saline solution over ringer's lactate solution in the resuscitation of a patient with a burn. However, we were limited by a lack of prior research studies on the topic which limited comparison with other studies. Further research is recommended to show the effect of different doses and types of IV fluids. The protective effect of hypertonic saline solution must be evaluated in other animal models and in other degrees of burn. Safety of resuscitation with hypertonic saline on vital organs, especially in the presence of burns needed. Also, the safety of its use in different patients with different ages and co-morbidities should be evaluated.

6. Availability of data and material

The data supporting the findings of the study are available within the article. The data supporting the findings of the article is available from the 1st author upon a reasonable request.

7. List of Abbreviations:

Δ P/ΔT: Muscle performance, ANOVA: Analysis of variance, Bcl-2: B cell lymphoma, CMAP: Compound muscle action potential, CT: Critical threshold, DAB: Diaminobenzidine, ECM: Extracellular matrix, ELISA: Enzyme-linked immunosorbent assay, first compound muscle action potential (CMAP), *FOXO: forkhead box transcription* factors O, H & E: Haematoxylin and Eosin stain, HSD: Hypertonic saline dextran solution, i.p.: Intraperitoneal, IL-10: interleukin-10, IL-1β: interleukin-1beta, IL-2: interleukin-2, IL-6: interleukin-6, IM: Intramuscular, KCs: Keratinocytes, MMPs: Matrix metalloproteinases, NF-κB: Nuclear factor-kappa B, RNA: Ribonucleic acid, RT-PCR: Reverse transcriptase- polymerase chain reaction, SC: Subcutaneous, SD: Standard deviation, TBSA: Total body surface area, *TGF-β: transforming growth factor*, TNF-α: Tumor necrosis- alpha, VHMNs: Ventral horn motor neurons.

8. Conflict of interest

We declare that we have No Conflict of Interest.

9. Acknowledgments

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