THERAPEUTIC EFFECTS OF LASER ON THE STIFFNESS OF THE ACHILLES TENDON MAMMALIAN MUSCLES

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Abstract. The purpose of this study was to investigate the effects of therapeutic laser energy on the repair of Achilles tendons in rats. The experiments were done on rats. Using an appropriate surgery, a sharp transverse cut was done on the Achilles tendons of the animals. Wound was treated with 650 nm diode laser at a dose of 9 J/cm^2 for a week until the wound healed completely. The treatment of injured animals was done by YAG solid laser 650 nm (150 mW power) or by classical surgery. The irradiation with laser was given for 1 min daily for one week. The tendons were tested for the biomechanical index of healing. There was a significant effect of laser energy on the index of healing, the maximum strain is higher in the healing groups than in the control (the value for the laser treated group being the highest), and the Young modulus is higher in both of healing groups compared with the control. Using polymerase chain reaction (PCR) measurements, it was found that rats receiving laser therapy showed a marked increase in collagen production, which probably improved the mechanical properties of the Achilles tendon.

Key words: Achilles tendons, biomechanical index of healing, laser therapy.

INTRODUCTION

Tendons are able to respond to mechanical forces by altering their structure, composition and mechanical properties, a process called tissue mechanical adaptation. Actins and myosin are present in tenocytes, and the tendon itself may have an active contraction-relaxation mechanism, which could regulate the transmission of force from muscle to bone [22]. Low energy laser therapy is often used in the treatment of musculoskeletal conditions [4, 18]. Some studies have shown that laser enhances skin wounded recovery [9, 17], treats pain and controls inflammation, while some other studies have not found any significant improvement in similar conditions and tissues [3, 5].

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During tendon repair, several growth factors are involved in the activation and regulation of the cellular responses. These factors or cytokines bind to specific receptors that are present on the cell surface and activate specific signaling events within the cell. This initiates a cascade of pathways leading to the transcription of specific regulatory genes (collagen I(αI) type I, collagen III(αI) type I, tenomodulin, aggrecan, decorin, tenascin-C and scleraxis genes) [6–8, 11]. The initiation or release of these factors is stimulated by cells that are located at the site of injury and, during the remodeling phase, by mechanical loading of the injured tendon. These polypeptide factors regulate the expression of specific genes that are found in common among tendons and ligaments, and the expression of these genes serves as markers of tendinogenesis [12, 14, 15, 23, 25]. The healing of connective tissues comprises three phases, namely inflammation, repair, and remodeling. Each phase has unique function and characteristics in preparation of the next phase. We are interested in the active repair and early remodeling phase because the strength of the repairing tissue increases in these two stages.

This study aimed to find the effect of laser irradiation on the stiffness of the Achilles tendon of rats during the recovery after injury.

MATERIALS AND METHODS

MATERIALS

24 rats of either sex, 6–8 weeks of age, weighing 40–50 g were used. The animals were housed in standard cages, maintained at room temperature, and fed with ordinary food. Use of experimental animals in the study protocol was carried out in accordance with the ethical guidelines of the Medical Research Institute, Alexandria University (Appendix 2, Guiding Principles for Biomedical Research Involving Animals, 2011).The animals were randomly assigned to two groups, one group (8 rats) serving as non treated controls, and the other group (16 rats) was wounded as shown: the skin overlying the Achilles tendon was shaved, and a longitudinal incision was made lateral to the visible outline of the tendon. After separating the tendon from the surrounding tissue, sharp transverse cuts were made. This last group was divided into two subgroups; one was left for normal healing, while the other group was irradiated with YAG laser for 1 min daily for one week. Biomechanical testing was performed post operatively. For measurement of the biomechanical properties of the tendons tensile testing machine (force meter BG500, USA) was used. A sample was spaced 40 mm between the jaws of the machine and stretched to failure by the tensile testing machine. Load-deformation curve, tensile strength, strain and Young's modulus of elasticity were recorded by computer program.

The method of irradiation with laser was standardized before the experiment. The non contact method (6 mm distance from the wound surface) was found to be accurate for irradiation in wound healing. The incision wound was treated by a low power diode laser (650 nm, total output of 150 mW, Photon Comp., Egypt) with a constant spot of 1 cm^2. The dosage of laser was calculated using the following formula:

$$D = \frac{P \cdot t}{A} \tag{1}$$

where: D is the dose measured in J/cm^2, P is the laser output in watts, t is the treatments time in seconds and A is the area of the wound measured in cm^2.

During our present study, the dose of laser beam was 9 J/cm^2.

BIOCHEMICAL MEASUREMENTS

The expression for collagen I(αI) type I, collagen III(αI) type I, tenomodulin, aggrecan, decorin, tenascin-C and scleraxis genes was assessed by reverse transcription PCR (RT-PCR) using the procedure described in the followings.

RNA extraction

RNA was extracted from the serum of rats using QIAamp RNA tissue kit, purchased from QIAGEN, USA according to the manufacturer's instructions. Preparation of full-length first strand cDNA from RNA template was done using RevertAid^{TM} First Strand cDNA Synthesis Kit.

Reverse transcription

Reverse transcription reaction was carried out in a 20 µL reaction mixture by using RevertAid^{TM} First Strand cDNA Synthesis Kit (K1621) and cDNA Synthesis Kit (K1622), both purchased from MBI Fermentas, Lithuania, according to manufacture's instruction.

RT-PCR amplification

To each PCR tube the followings were added: 5 µL (0.25 µg) template – cDNA of each gene (see Table 1 for details), 10 µL Taq TM Green PCR Master Mix (2X) (dNTPs (0.4 mM of each dATP, dCTP, dGTP, dTTP), 0.05 u/µL Taq DNA polymerase and reaction buffer, (K1081) purchased from MBI Fermentas, Lithuania), 1.5 µL forward primer for each gene (Table 1), 1.5 µL reverse primers for each gene (Table 1) and deionized-RNase free water to final volume 20 µL. The reaction mixtures were gently vortexed, briefly centrifuged to collect all drops to the bottom of the tubes, and then were placed in the thermal cycler (Little Genius, Bioer Co). The PCR mixture was subjected to 35 amplification cycles.

PCR conditions were as follows: an initial denaturation (94 °C, 2 min), followed by 35 cycles of denaturation, annealing, and extension, with a final extension according to each gene amplification profile (Table 1).

Table1

Primer sequence and thermal cycler amplification profile

Gene	Primer	Amplification
Collagen I (αI) type I	5'-AGG CTT TGA TGG ACG CAA TG-3'	Denaturation (94 °C, 2 min) Annealing (54 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-GCG GCT CCA GGA AGA CC-3'	
Collagen III (αI) type I	5'-AGG CTT TGA TGG ACG CAA TG-3'	Denaturation (94 °C, 2 min) Annealing (54 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-GCG GCT CCA GGA AGA CC-3'	
Tenomodulin	5'-GGA CTT TGA GGA GGA TGG-3'	Denaturation (94 °C, 2 min) Annealing (55 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-CGC TTG CTT GTC TGG TGC-3'	
Aggrecan	5'-ACC CGA CAA TTT CTT TGC-3'	Denaturation (94 °C, 2 min) Annealing (55 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-GGT CTC ATC GTC CGC TTC-3'	
Decorin	5'-TGG CAG TCT GGC TAA TGT-3'	Denaturation (94 °C, 2 min) Annealing (53 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-ACT CAC GGC AGT GTA GGA-3'	
Tenascin-C	5'-GCT ACT CCA GAC GGT TTC-3'	Denaturation (94 °C, 2 min) Annealing (53 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-TTC CAC GGC TTA TTC CAT-3'	
Scleraxis	5'-CGA AGT TAG AAG GAG GAG GGT-3'	Denaturation (94 °C, 2 min) Annealing (55 °C, 1 min) Extension (72 °C, 1 min) Final extension (72 °C, 7 min)
	5'-CGC TCA GAT CAG GTC CAA AG-3'	

To verify the successful preparation of mRNA and as positive controls, samples were detected for the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA – forward primer: 5'-AGG CCG GTG CTG AGT ATG TC-3', reverse primer: 5'-TGC CTG CTT CAC CAC CTT CT-3'. Reaction tubes containing no cDNA control template and no cDNA sample were included as negative controls for each PCR reaction.

Detection

The amplicons were analyzed with 2% (wt/vol) ethidium bromide stained agarose gel. The bands were visualized on a 302 nm UV transilluminator (BIO-RAD, USA).The gel was examined for bands of 78 bp, 153 bp, 128 bp, 345 bp, 130 bp, 199 bp and 108 bp for each gene respectively as determined by the molecular weight marker (Gene Ruler TM 100 bp plus DNA Ladder, ready-to-use, #SM0323, purchased from Fermentas, Lithuania) runs at the same time and then photographed using digital camera (SONY Super Steady Shot DSC-W300).

RESULTS

BIOMECHANICS OF THE ACHILLES TENDON

The normal dependence of stress against strain for a tendon is presented in Figure 1. The wavy lines indicate the wavy configuration of the tendon at rest, straight unbroken lines indicate the effect of tensile stresses, one or two broken lines indicate that the collagen fibers are starting to slide past one another as the intermolecular cross-links fail, and the set of completely broken lines indicate macroscopic rupture due to the tensile failure of the fibers and the interfibrillar shear failure**.**

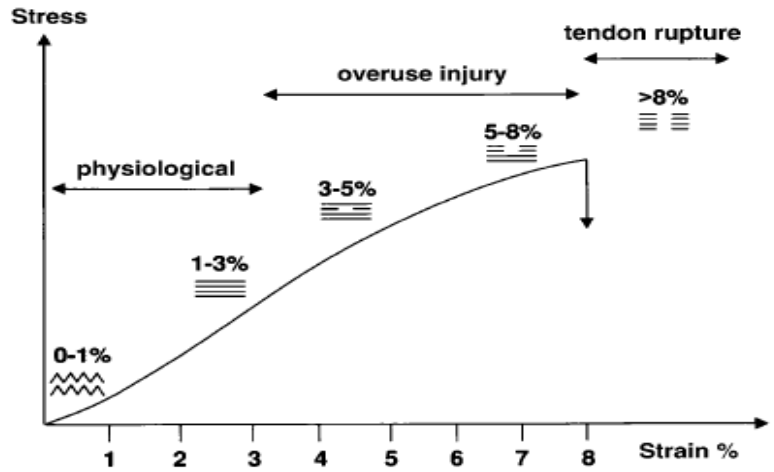


Fig. 1. Normal dependence of stress against strain for a tendon.

Biomechanical testing was performed on the 14th day postoperatively, this day was selected in order to leave the muscle to be healed without laser therapy. At

sacrifice, a visual assessment of the healed tendon was made and bilateral Achilles tendons were reseated, preserving the attachment to the calcareous.

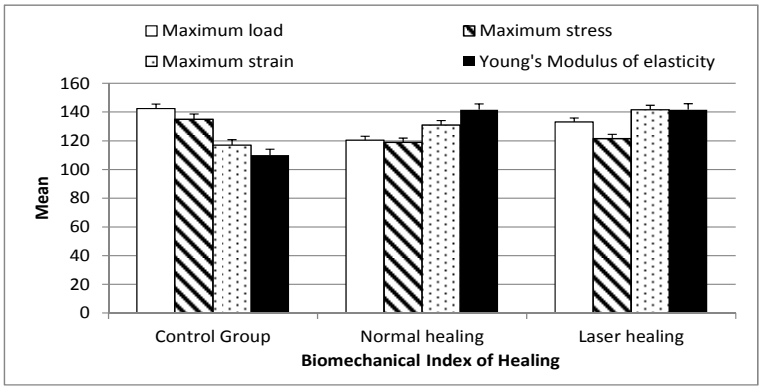


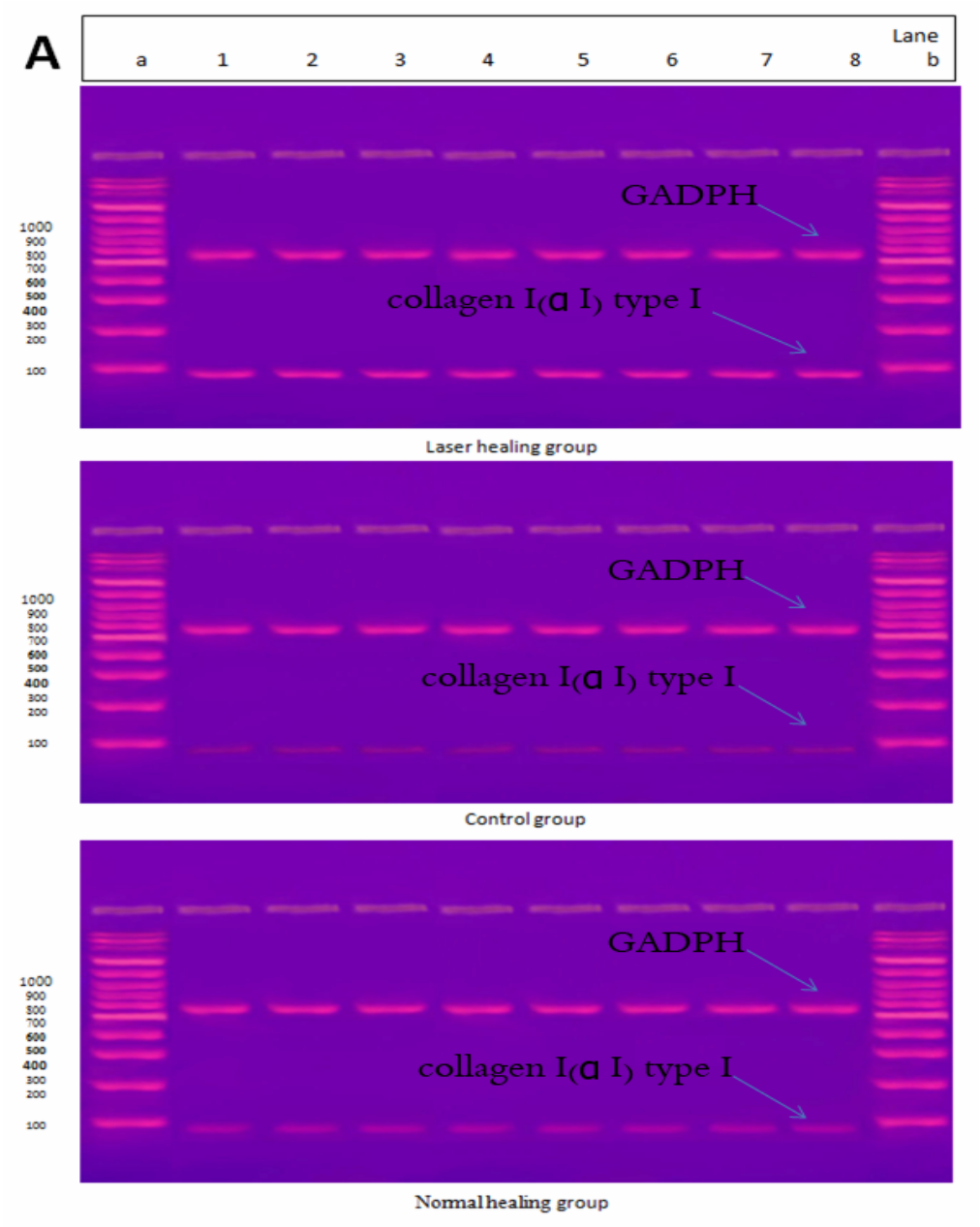
Fig. 2. Biomechanical index of healing for all groups.

We tested the hypothesis that the photo stimulation laser therapy would further accelerate healing of experimentally tenotomized and repaired rats Achilles tendons. The measurement of the biomechanical properties of the tendons revealed that: (i) the maximum load is smaller in the case of healing groups than for the control; however the laser treated group shows a bit higher value than the normal healing group, (ii) the maximum stress is smaller in the healing groups than in the control, (iii) the maximum strain is higher in the healing groups than in the control (the value for the laser treated group being the highest), (iv) the Young modulus is higher in both of healing groups comparing with the control.

These changes are statistically significant as revealed by the multivariate analysis of variance (MANOVA) test ($F = 1.77$, $p = 0.05$).

RT-PCR AMPLIFICATION RESULTS

The expression of genes that serve as markers of tendinogenesis was further followed. A molecular study of collagen I(αI) type I, collagen III(αI) type I, tenomodulin, aggrecan, decorin, tenascin-C and scleraxis genes expression as molecular markers for tendon development and repair was done for all the experimental groups. RT-PCR measurements showed a significant increase in the expression of collagen I(αI) type I – mRNA, collagen III(αI) type I – mRNA, tenomodulin – mRNA, aggrecan – mRNA, decorin – mRNA, tenascin–C – mRNA and scleraxis – mRNA genes at laser healing group than the normal healing group (Fig. 3, A–G). The differences have been revealed and approved by gel analyzer computer program based on the same starting amount of cDNA for each gene.


A
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GADPH
collagen I(α I) type I
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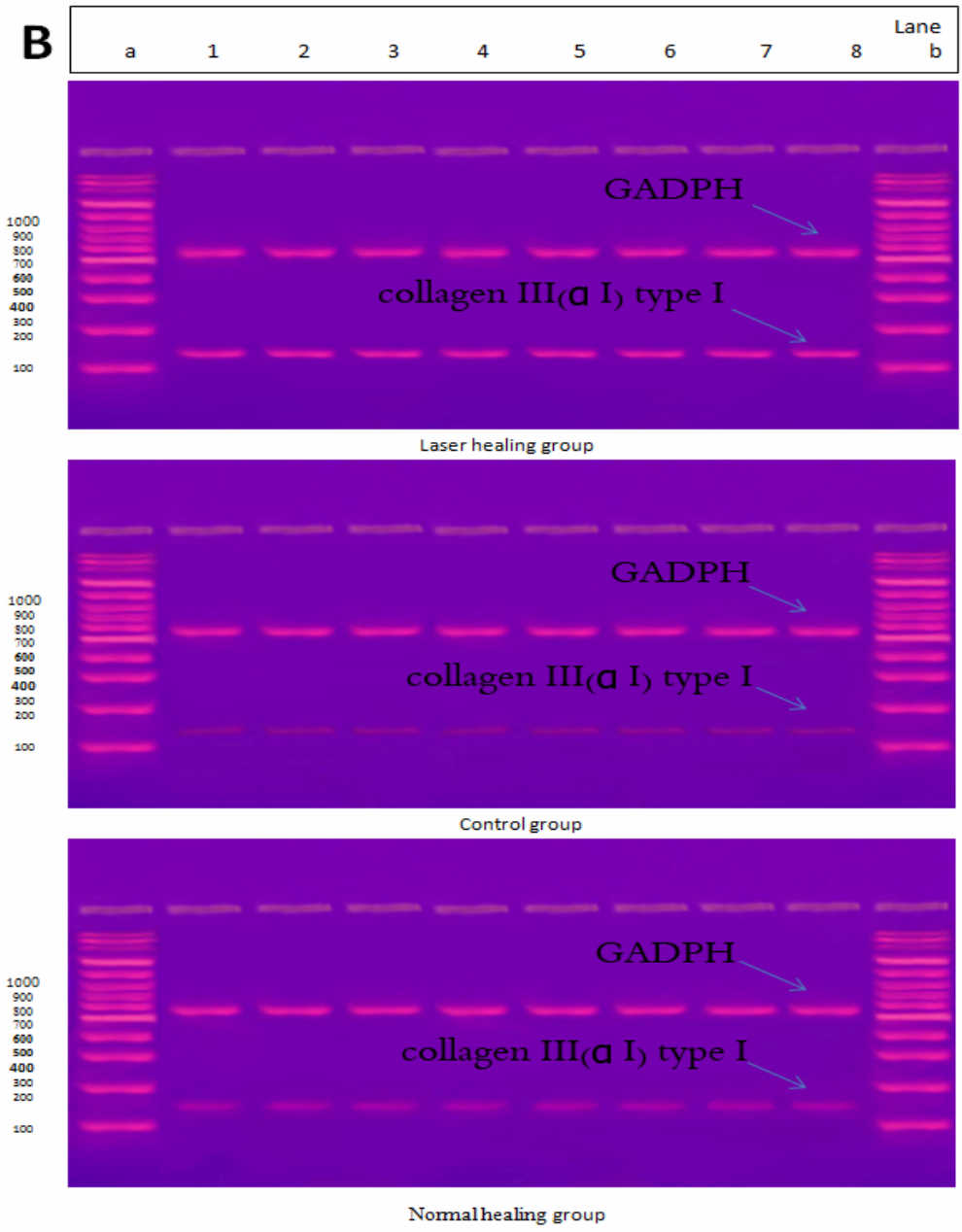
GADPH
collagen I(α I) type I
Control group
GADPH
collagen I(α I) type I
Normal healing group


B
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GADPH
collagen III(α I) type I
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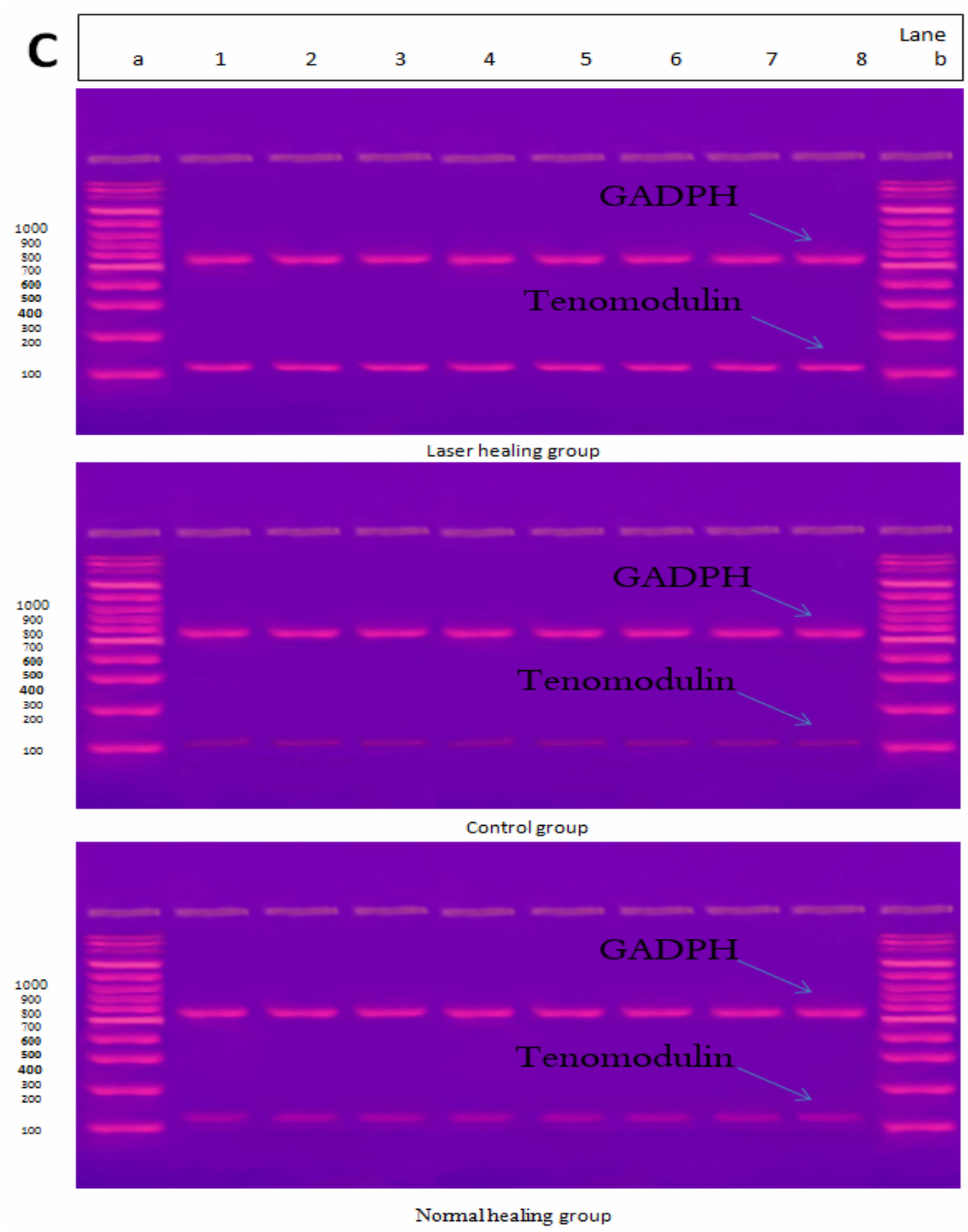
Laser healing group
Control group
Normal healing group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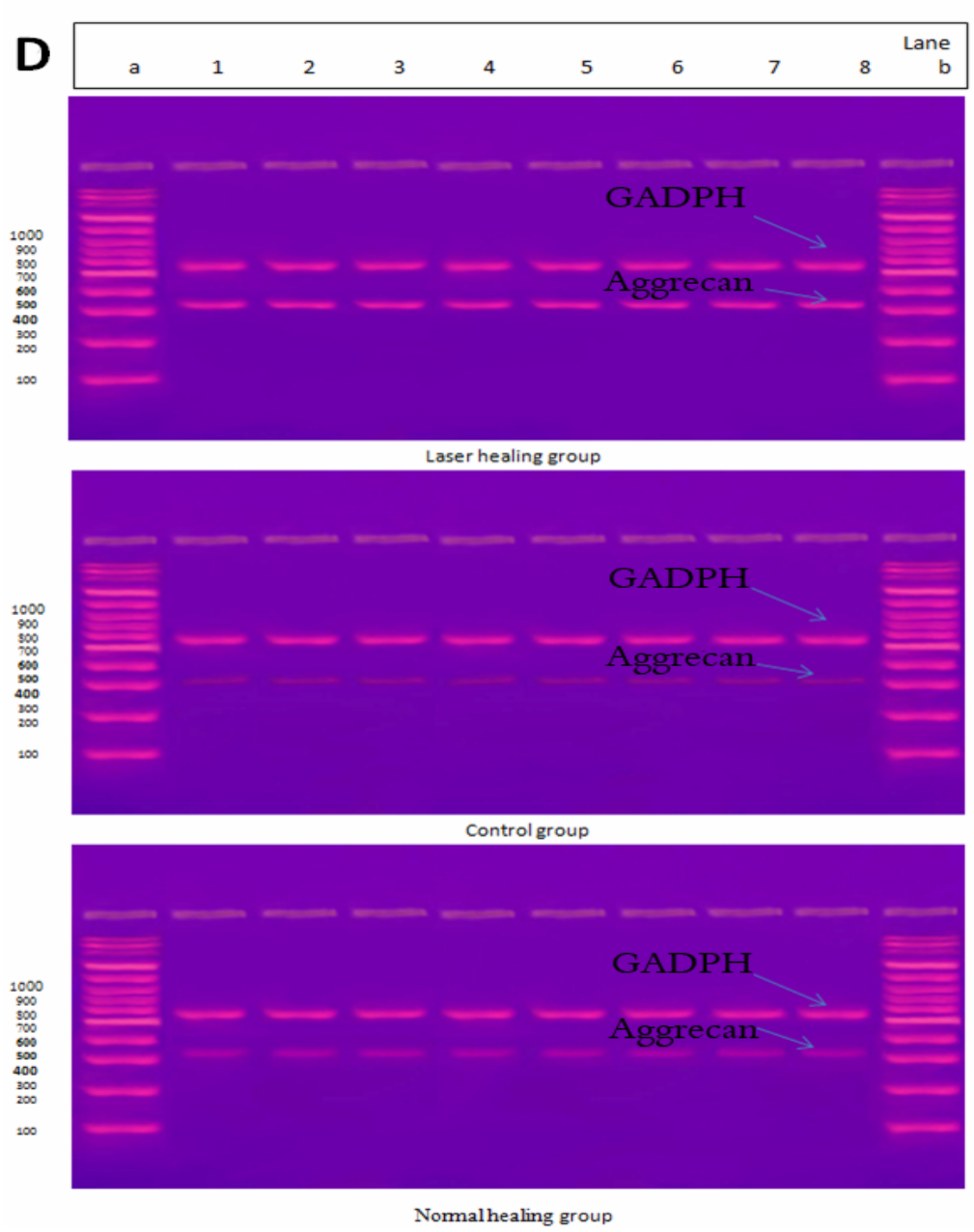
Laser healing group
Control group
Normal healing group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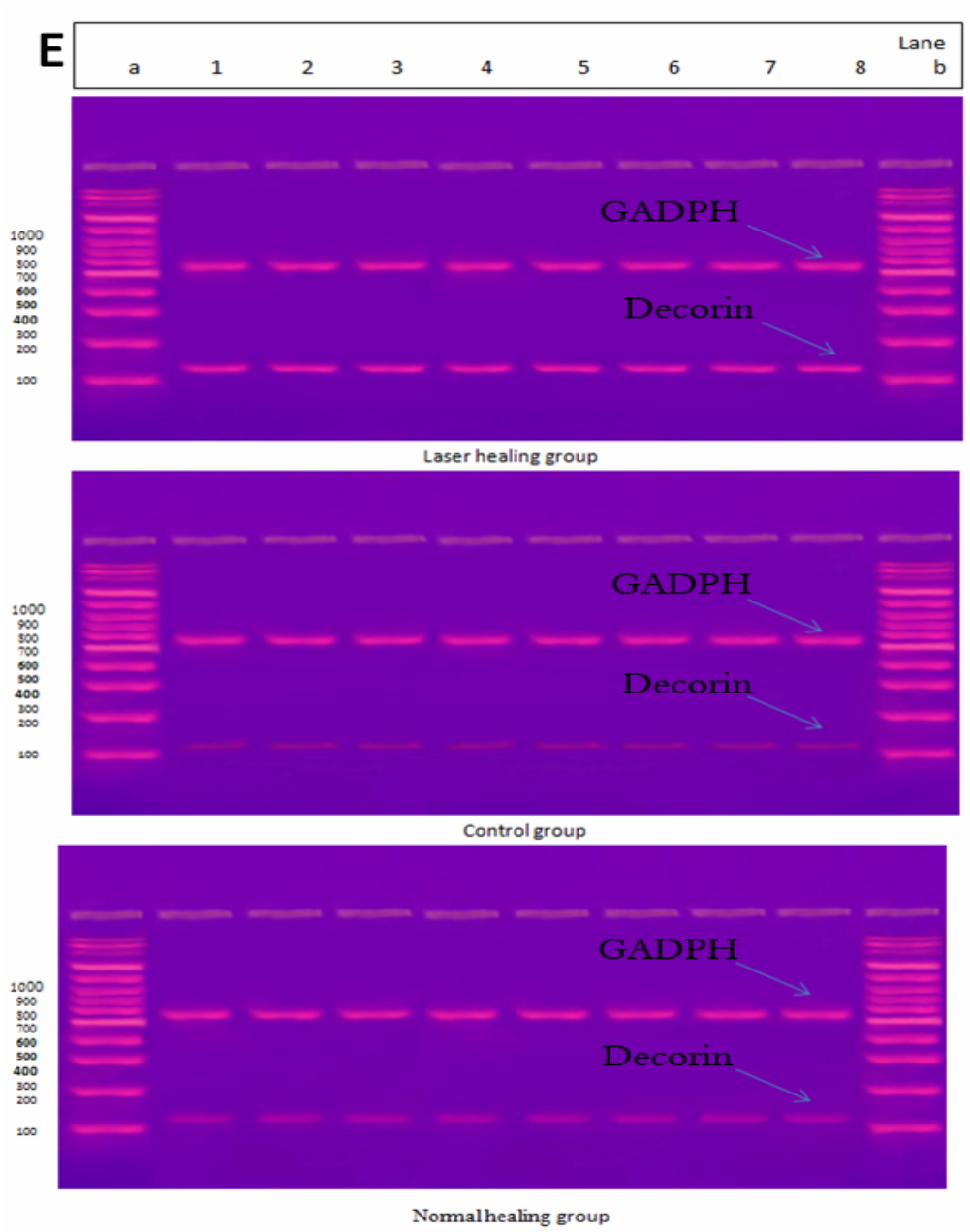
Control group
Normal healing group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Decorin
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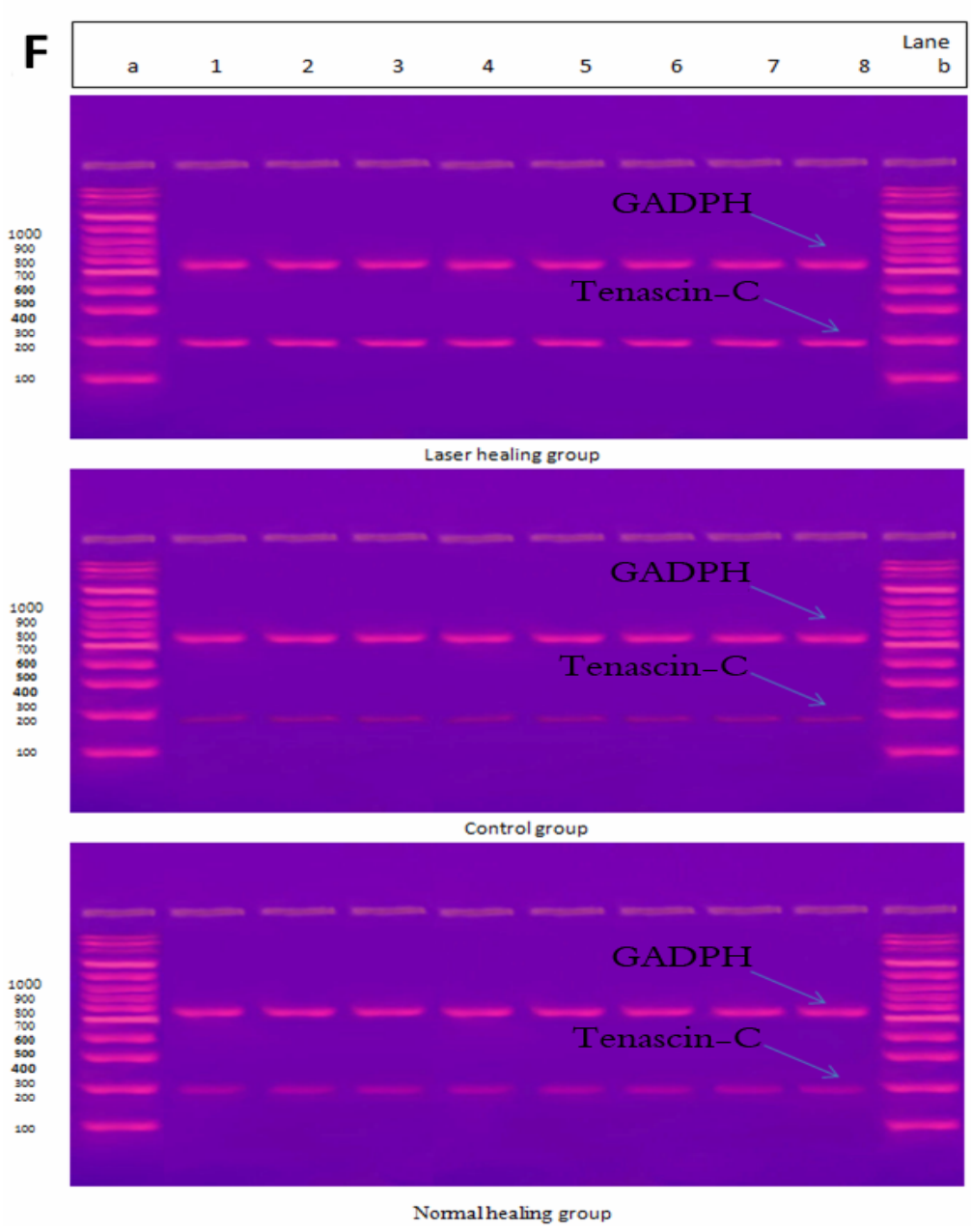
GADPH
Decorin
Normal healing group


F
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Tenascin-C
Normal healing group

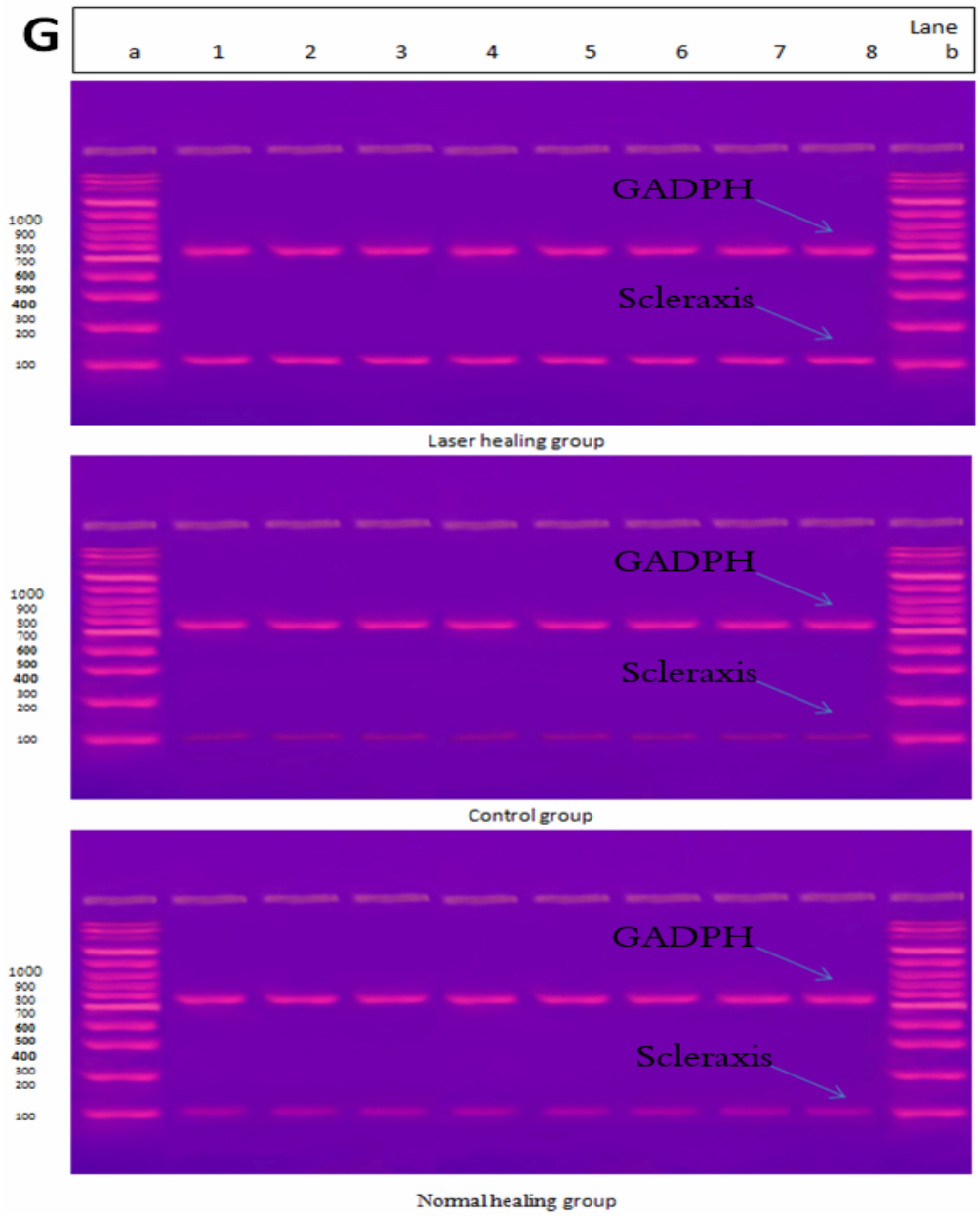

Fig. 3. Ethidium bromide stained agarose gel showing bands of amplified PCR products of A: collagen I (αI) type I, B: collagen III (αI) type I, C: tenomodulin, D: aggrecan, E: decoorin, F: tenoscin-C, G: scleroxis genes of laser healing, control, normal healing groups respectively: Lanes a and b marked the DNA marker, lanes 1–8 marked positive cases showing amplified bands (530 bp representing GADPH gene as positive control).

DISCUSSION

The initial background which prompted the use of low intensity lasers to promote the healing process of soft tissues arose from the works of Mester *et al*. [16] and Hardy [10]. Hardy *et al*. reported a fivefold increase in the number of fibroblasts exposed to four 10 J/cm doses of pulsed laser when compared to controls that were not treated with laser light. Subsequent studies have shown that the increased proliferation of fibroblasts results in increased collagen synthesis *in vitro* as well *in vivo* [1].

Roux's law of functional adaptation suggests that tendons and other connective tissues adapt themselves structurally to functional demands. The sensitivity of tendons to load enhancement or load deprivation is exemplified by the fact that intact and healing tendons are strengthened by physical activities and weakened by inactivity [4, 22]. These and other findings indicate that repaired tendons may heal faster when subjected to functional loads. Therefore, earlier in our studies, we implemented minimal (short duration) cast immobilization to facilitate functional loading and rapid repair of experimental rats.

The application of laser energy to fuse tendons could prove a useful tool in many reconstructive procedures. Tendons are unique in their structure and function. Tendons are composed of densely packed, highly organized collagen bundles able to withstand significant tensile loads while gliding through surrounding tissues. Previous studies have shown that after laser therapy the collagen content was higher in those receiving laser therapy than in those who received no treatment, which is consistent with our finding in relation to the laser treated group [13]. The exact mechanism by which laser energy influences tissue welding is not clear. *In vitro* studies suggest that laser photo stimulation promotes nucleic acid synthesis and cell division in cultures of human fibroblasts [2, 13, 24]. A similar increase in ATP production has been found in rat liver mitochondria after photo stimulation with He-Ne laser *in vitro* [20, 21].

There is evidence that laser may both reduce inflammation in the area and stimulate the body's natural healing process – helping to trigger the synthesis of collagen fibers in the tendon [11]. Our results confirm this assumption proving a significant increase in the expression of collagen I(αI) type I – mRNA, collagen III(αI) type I – mRNA, tenomodulin – mRNA, aggrecan – mRNA, decorin – mRNA, tenascin-C – mRNA and scleraxis – mRNA genes at laser healing group than at normal healing group. We consider that: collagen I(αI) type I , collagen III(αI) type I, tenomodulin, aggrecan, decorin, tenascin-C and scleraxis genes expression may be used as a set of molecular markers involved with tendon development and repair.

CONCLUSIONS

The laser therapy is efficient in improving the recovery of the mechanical properties of injured tendons, and this could be related to the stimulation of synthesis of some proteins included in the structure of tendon.

The results suggested that, for soft tissue surgery, YAG lasers may lead to shorter operative times, and fewer postoperative adverse events.

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