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# Tendon injury healing with G-90 in a rabbit model: biomechanical and histopathological evaluation

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**ABSTRACT**

Tendon injuries are one of the most common and disabling acute orthopedic disorders. Several techniques are used to restore the mobility of patients, but all have significant limitations. In some studies utilization of biomaterials has been investigated in the healing of tendons, skin and nerves. The present study was designed to investigate the effects of G-90, as a stimulating factor agent, on the healing of the superficial digital flexor tendon (SDFT) of rabbits after 35 days post tenotomy and surgical repair. Twenty male rabbits, 12 months old and weighing 2.0 ± 0.5 kg were used in this study. All rabbits were anesthetized, and the superficial flexor tendons of both hind limbs were cut transversely and then sutured with a Bunnel-Mayer suture pattern. After suturing tendons and skin, 0.5 mL of normal saline was injected in the injured tendon area of the left leg and 0.5 mL earthworm extract (EW) G-90 was injected at a concentration of 10 mg/mL into the injured tendon area of the right leg. Every two weeks 4 rabbits were euthanized, and samples were collected and sent for histopathological and biomechanical evaluation. In the histopathological evaluation less inflammation, more maturity of fibrocytes and more aggregation of collagen fibers were observed in tendons treated with G-90 in comparison with untreated tendons. In the biomechanical evaluation the ultimate strength of tendons treated with G-90 was superior to untreated tendons. The findings of the present experiment clearly show that administration of G-90 could enhance the structural and biomechanical properties of the experimentally tenotomized SDFT in rabbits.

**Key words:** earthworm extract, G-90, tendon healing, rabbit model

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## Introduction

Tendons are anatomic structures interposed between muscles and bones that transmit the force created in the muscle to the bone, and make joint movement possible (KANNUS, 2000). Tendons vary in form, and may be rounded cords, strap-like bands or flattened ribbons. When healthy they appear brilliant white and have a fibroelastic texture. Structurally, a tendon is composed of tenoblasts and tenocytes, lying within the network of an extracellular matrix (ECM) (SHARMA and MAFFULLI, 2006). When tendons are subjected to high physiological loads, these tissues are commonly injured and fail to heal optimally, due to their low cellularity and vascularity (STRAUSS et al., 2007). Tendon injuries produce substantial morbidity, and at present there are only a limited number of scientifically proven management modalities (SHARMA and MAFFULLI, 2005). Tendons are at the highest risk for rupture if tension is applied quickly and obliquely, and the highest forces are seen during eccentric muscle contraction (SHARMA and MAFFULLI, 2005). Growth factors are short sequences of amino acids, which usually transmit signals between cells and thereby modulate their activities (CHAN et al., 1997; McGEACHIE and TENNANT, 1997). They regulate cell activity by a number of mechanisms, such as: mitogenic activity, cell differentiation, cell migration and gene regulation, and they play important roles in cell chemotaxis, proliferation, matrix synthesis, and differentiation. The use of growth factors to enhance tendon healing remains largely experimental and has been restricted to *in vitro* studies and animal models (SHARMA and MAFFULLI, 2005; McGEACHIE and TENNANT, 1997).

G-90 was obtained from the tissue homogenate of the earthworm *Eisenia foetida* (phylum Annelida, family *Lumbricidae*). Earthworms, which possess antibacterial activity, have been widely used in traditional Chinese medicine (HRZENJAK et al., 1992; POPOVIC et al., 2001; EL-KAMALI, 2000). It has been shown that G-90 is neither an allergen nor a toxin, and it possesses antibacterial activity which aids wound healing (POPOVIC et al., 1998; HRZENJAK et al., 1992; HRZENJAK et al., 1993; HRZENJAK et al., 1998). The G-90 mixture contains the growth factors of the insulin superfamily, adhesion of the immunoglobulin superfamily, and proteolytic enzymes of the trypsin family (GRDISA et al., 2001). Therefore, the present study was designed to investigate the effects of G-90, as a stimulating factor agent, on the healing of the superficial digital flexor tendon (SDFT) of rabbits, after 35 days post tenotomy and surgical repair.

## Materials and methods

*Animals.* Twenty male rabbits, 12 months old and weighing 2.0 ± 0.5 kg, were used in this study. Before the experiment, they were kept in their new location for 20 days for adaptation and stress removal. The rabbits were kept in individual standard rabbit cages and were maintained on a standard rabbit diet, with no limitation of access to food

or water. The experimental protocol was approved by the Animal Care and Experiment Committee of the University, in accordance with the ethics standards of the "Principles of Laboratory Animal Care".

*Preparation of G-90 complex.* To prepare this compound, Hrzenjak et al.'s method was used (HRZENJAK et al., 1992). 200 earthworms of the species *Eisenia foetida* were washed with warm water several times to remove impurities, and then the worms were immersed in 10 % sodium chloride solution for one hour at room temperature until they expired. After this period, the worms were washed again, cut into pieces with scissors and homogenized with homogenizer machine. The mixture was transferred to a beaker, ethanol and chloroform in a 1:1 ratio were added to the solution, and it was left at 4 °C overnight, after which distilled water was added to produce a final volume of 200 mL of mixture. After stirring, it was filtered several times, until the mixture was light brown in appearance. The mixture was centrifuged at 4000 rpm for at least 10 minutes in 50 mL Falcon® tubes (Corning Life Sciences, Corning, NY, USA). After centrifugation, 3 layers developed in each tube: The top layer was a clear, light brown-colored liquid, the middle layer was a brown-colored solid, and the bottom layer was a straw colored liquid. The solid middle layer was placed on a filter paper until the remaining liquid slowly evaporated, the pellet dried, and the brown color was clearly visible. The discs were transferred into 1000 mL balloons and freeze dried at -50 °C. The resultant powder (G-90) was placed under UV light for 30 minutes.

*Surgical procedure.* All the rabbits in the present study were sedated using acepromazine (0.02 mg/kg i.m., Alfasan, Woerden, Holland), the caudal parts of both hind limbs between the stifle and hock joint were clipped and prepared aseptically, and the limb was draped with sterile drapes. Anesthesia was induced using ketamine (30 mg/kg, IM, Alfasan, Woerden, Holland). An incision was made directly over the Achilles tendon, the superficial digital flexor tendon was exposed, cut transversely and then sutured with nylon (Supa, Tehran, Iran) 2/0 in a Bunnel-Mayer suture pattern. The skin was sutured routinely with silk suture. After suturing the tendon and skin, 0.5 mL of normal saline was injected in the injured tendon area of the left leg and 0.5 mL earthworm extract G-90 was injected at a concentration of 10 mg/mL into the injured tendon area of the right leg. Postoperatively, the antibiotic [enrofloxacin(Bytril®, Bayer, Germany) 10 % at a dose of 5 to 10 mg/kg body weight] was injected subcutaneously for 3 days.

*Sampling.* At the 1$^{st}$, 2$^{nd}$, 3$^{rd}$, 4$^{th}$ and 5$^{th}$ postoperative weeks 4 rabbits were euthanatized with anesthetic overdosing for pathological and biomechanical evaluation. The treated tendons were excised and removed.

*Biomechanical evaluation.* Freshly harvested specimens were submitted to tensile strength measurement using a biomechanical analyzer (Instron, Canton, MA). The harvested tendons were clamped in the upper jaw and in the distal jaw. Each tendon

was loaded by elongating it at a displacement rate of 10 mm/s until a 50 % decrease in load was detected. During tensile testing no slippage was noted. Load and cross-head displacement data were recorded at 1500 Hz, and load-deformation and stress-strain curves were generated and biomechanical markers, including ultimate strength, stiffness and stress, were measured.

*Histopathological evaluation.* Immediately after the biomechanical tests, samples were fixed using formalin solution (10 %) and transported to the pathology laboratory. The formalin solution was changed after 24 hours and then after 10 days, tissue samples were sectioned, stained with the H&E method, and observed under light microscopy. For histopathological evaluation, samples were scored qualitatively and semi-quantitatively based on a modified Rosenbaum et al and Oryan et al scoring system (Table 1) (ROSENBAUM et al., 2010).

Table 1. Histopathological scoring system

| Marker | Scores |
|---|---|
| Inflammation degree | 0, 1, and 2 (qualitative) |
| Fibroblast maturation | 0, 1, and 2 (qualitative) |
| Aggregation of connective fibers | 0, 1, and 2 (qualitative) |
| Connective fibers alignment | 1, 2, 3, and 4 (semi-quantitative)<br>(1-25 %: 1), (25-50 %: 2), (50-75 %: 3), and (75-100 %: 4) |
| Vasularization rate | 1, 2, and 3 (semi-quantitative)<br>Average number of vascular sections in 5 microscopic fields (×40). (0-5: 1), (5-10: 2), and (>10: 3) |

*Statistical analysis.* Biomechanical test driving data were analyzed by the t-student test ($P<0.05$ was considered significant). Histopathological driving data were analyzed by Mann-Whitney U test and $P<0.05$ was considered significant (SPSS version 20 for Windows, SPSS Inc, Chicago, USA).

**Results**

There was no intraoperative and postoperative death during the study. None of the rabbits sustained any tendon rupture in the injured area. The tendons treated with G-90 on the right legs showed less peritendinous adhesions, less hyperemia and better general appearance in comparison with the other legs at the time of necropsy.

*Biomechanical findings.* Biomechanical data are presented in Table 2 as Mean ± standard deviation (M ± SD). There was no significant difference between biomechanical markers, except for ultimate strength, which was statistically higher in the tendons treated with G-90 in comparison with the untreated tendons ($P<0.05$).

*Histopathological findings.* In histopathological evaluation, some criteria such as: severity of inflammation, maturation of fibrocytes, the rate of aggregation of connective fibers, connective fiber alignment and vascularization rate, were studied. Histopathological evaluation after the first week showed significantly less inflammation in the tendons treated with G-90 in comparison with the untreated tendons (Fig. 1, Table 3, P = 0.03). Moreover, there were significant differences (Table 3, P = 0.013) between the two groups in the rate of maturation of fibrocytes, where tendons treated with G-90 were superior to the untreated tendons after the first week (Figs 2 and 3). Table 2. Results of biomechanical assessment (Mean ± SD)

Table 2. Results of biomechanical assessment (Mean ± SD)

| Biomechanical Criterion | Ultimate strength (N) (n = 4) | | Stiffness (N/mm) (n = 4) | | Stress N/mm$^2$ (n = 4) | |
|---|---|---|---|---|---|---|
| Post injury week | Treated tendons | Untreated tendons | Treated tendons | Untreated tendons | Treated tendons | Untreated tendons |
| 1st | 6.6 ± 0.3 | 5.53 ± 0.4 | 8.85 ± 0.2 | 8.92 ± 0.4 | 2.38 ± 0.2 | 0.79 ± 0.05 |
| 2nd | 22.9 ± 0.1 | 23.01 ± 0.2 | 5.64 ± 0.56 | 5.72 ± 0.7 | 3.38 ± 0.2 | 3.26 ± 0.3 |
| 3rd | 39.5 ± 0.1 | 38.75 ± 0.2 | 5.11 ± 0.9 | 4.26 ± 0.3 | 4 ± 0.44 | 4.46 ± 0.4 |
| 4th | 37 ± 0.1 | 30 ± 0.2 | 1.3 ± 1.0 | 1.3 ± 1.4 | 4.4 ± 0.2.14 | 4.71 ± 0.2 |
| 5th | 57 ± 0.4[a] | 35.9 ± 0.1 | 3.67 ± 2.92 | 3.09 ± 6.1 | 5.2 ± 2.42 | 4.8 ± 0.1 |

[a] Tendons treated with G-90 showed significant difference (P<0.05), in comparison with untreated tendons

Table 3. The results of histopathological evaluation at postoperative week intervals (n = 4)

| Histopathological criteria | | | Med (Min-Max) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Postoperative week | Inflammation | | Fibroblast maturation | | Aggregation connective fibers | | Connective fiber alignment | | Vasularization rate | |
| | Treated tendons | Untreated tendons | Treated tendons | Untreated tendon | Treated tendons | Untreated tendons | Treated tendons | Untreated tendons | Treated tendons | Untreated tendons |
| 1st | 1 (0-2) | 2 (2-2)[a] | 1 (1-2) | 1 (1-1)[b] | 2 (1-2) | 1 (1-2) | 1 (1-1) | 1 (1-1) | 2 (1-2) | 2 (2-2) |
| 2nd | 1 (1-2) | 1 (1-2) | 2 (1-2) | 2 (2-2) | 2 (2-2) | 2 (2-2) | 2 (1-2) | 2 (1-2) | 2 (1-2) | 2 (1-2) |
| 3rd | 0 (0-2) | 1 (0-2) | 2 (2-3) | 2 (1-2) | 3 (3-3)[c] | 2 (1-2) | 3 (2-3) | 1 (1-3) | 1 (1-1) | 1 (1-2) |
| 4th | 0 (0-0) | 0 (0-0) | 3 (2-3) | 3 (2-3) | 3 (3-3) | 3 (2-3) | 3 (3-4) | 3 (3-3) | 1 (1-1) | 1 (1-1) |
| 5th | 1 (0-1) | 0 (0-0) | 3 (2-3) | 2 (2-3) | 3 (2-4) | 2 (2-3) | 3 (2-3) | 2 (2-3) | 1 (1-1) | 1 (1-1) |

[a] significantly less inflammation in tendons treated with G-90 in comparison with untreated tendons (P = 0.03); [b] there were significant differences (P = 0.013) between the two groups in the rate of maturation of fibrocytes, where tendons treated with G-90 were superior to untreated tendons after the first week; [c] significant differences in the rate of aggregation of connective fibers between tendons treated with G-90 and untreated tendons where treated tendons showed superior aggregation of connective fibers in comparison of the untreated tendons (P = 0.013).

After the third postoperative week there was a significant difference in the rate of aggregation of connective fibers between tendons treated with G-90 and untreated tendons, where the treated tendons showed superior aggregation of connective fibers in comparison with the untreated tendons (Figs 4 and 5, Table 3, P = 0.013).

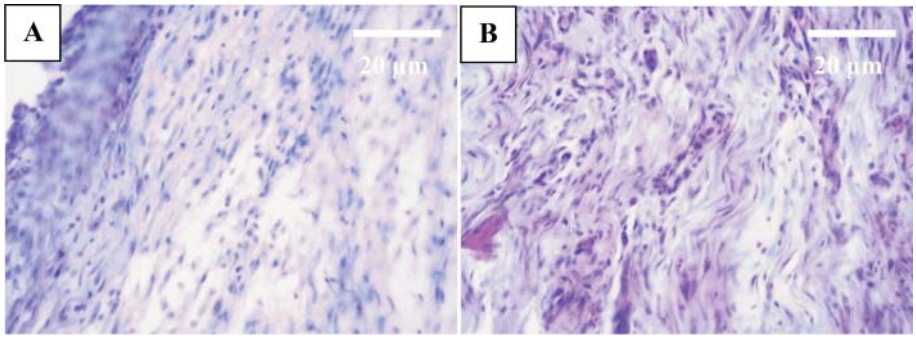


Fig. 1. Note the connective tissue formation with large numbers of fibroblasts and fibrocytes in the treated tendon (A) and untreated tendon (B) after the first week. Note the large number of inflammatory cells in the untreated tendon (H&E, ×40)

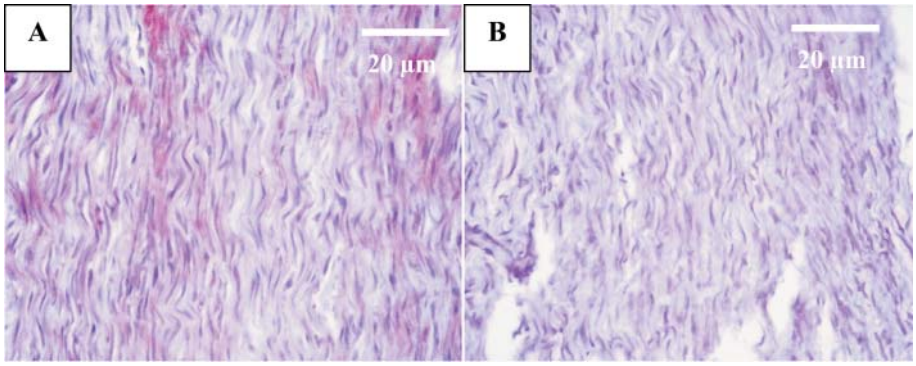


Fig. 2. Incision space filled by strands of connective tissue with more fibroblasts and proper orientation of connective fibers in the treated tendon (A) and untreated tendon (B) at the second week (H&E, ×40)

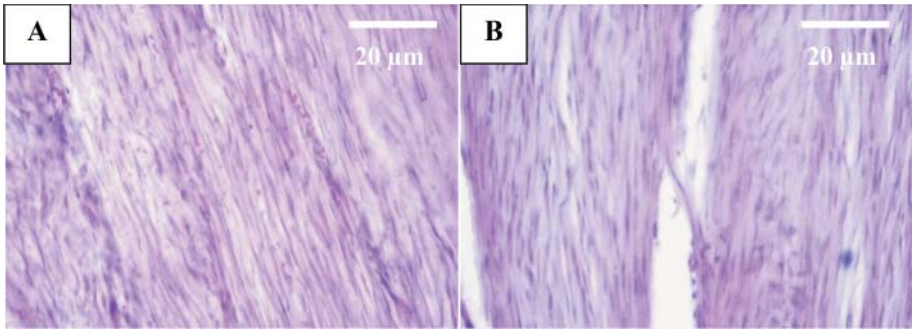


Fig. 3. The incision space occupied by strands of connective tissue with proper orientation of connective fibers in the treated tendon (A) compared with untreated tendon (B) after the third week (H&E, ×40)

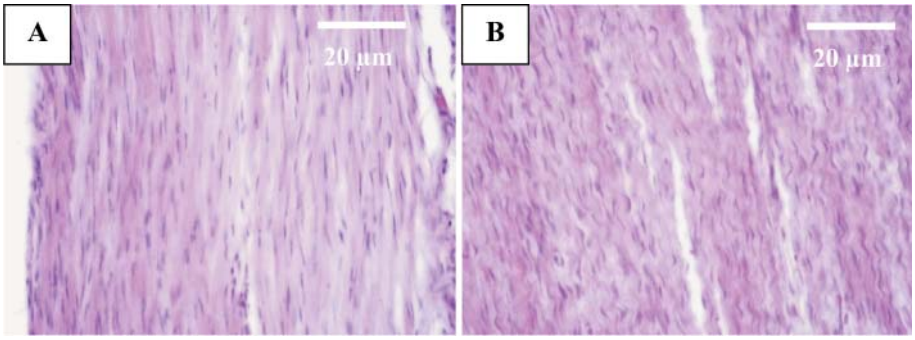


Fig. 4. Aggregation of connective fibers with proper orientation of connective fibers and increased number of fibrocytes to fibroblasts in the treated tendon (A) compared with the untreated tendon (B) after the fourth week (H&E, ×40)

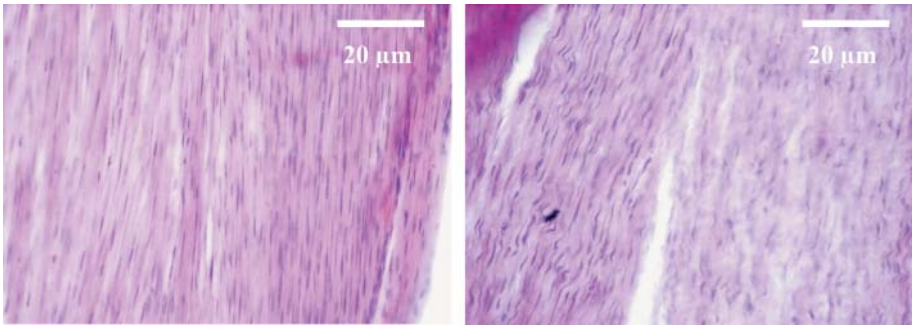


Fig. 5. The organization of the connective fibers to the tendon at day 35 in the treated tendon was more pronounced than in the untreated tendon (H&E, ×40)

**Discussion**

This experiment was designed on the basis of the theory that G-90 can stimulate maturation of tenoblasts, initiate earlier collagen formation and maturation, and result in the improved biomechanical performance of the treated injured tendon. These hypotheses were morphologically and biomechanically tested 35 days post-injury on a completely sectioned superficial digital flexor tendon in rabbits. The proximal section of the SDFT has been selected as the tissue of choice by many investigators as an extra-synovial model because of its accessibility and easier exposure (BIGHAM et al., 2011; ORYAN et al., 2008; ORYAN et al., 2011; ORYAN et al., 2009; ORYAN et al., 2010). In addition, it simulates the hand flexor tendon of humans and SDFT injuries in horses (OSULLIVAN, 2007; SHARIFI et al., 2009). Superficial digital flexor tendonitis is a common career injury in sport horses, and when severe or with recurrence it can result in early retirement or destruction of the affected horse (DYSON, 2004).

Tendon injuries are an important clinical issue for orthopedic surgeons. There are obstacles on the path to healing tendon injuries as follows: 1. Low blood supply: the tendon healing period is considerably longer than other types of connective tissue such as bones (CHAN et al., 2000; YOUNG, 2012). 2. Healing does not lead to a normal histological structure, i.e. healing occurs by forming scar tissue, whose quality is less than that of normal tendon. Therefore, a healed tendon does not have normal function and may suffer recurrent injury (GULOTTA et al., 2011; LACITIGNOLA et al., 2008; LEEA and HUI, 2006). 3. These tissues are susceptible to adhesion due to excess fibrous tissue formation (GULOTTA et al., 2011).

Despite progress in surgical techniques and rehabilitation, early complications, such as rupture of the repaired area and postoperative adhesions, may occur (KHANNA et al., 2009; SHARMA and MAFFULLI, 2005). Adhesion formation after intrasynovial tendon injury poses a major clinical problem. Synovial sheath disruption at the time of injury or surgery allows granulation tissue and the tenocytes from surrounding tissue to invade the repair site. Exogenous cells are predominant over endogenous tenocytes, allowing the surrounding tissue to attach to the repair area and thus create adhesion (SHARMA and MAFFULLI, 2006). The most common complications, after repair of flexor tendon rupture, are tendon adhesion and joint contracture (ROUHANI et al., 2013). Flexor tendon healing leads to complications in the form of soft tissue adhesions to the surrounding sheath (HAKIMI et al., 2012). In the present study, necropsy evaluation showed that there were fewer tendon adhesions to the surrounding tissues in tendons treated with G-90 in comparison to the untreated tendons. This phenomenon in the treated tendons may be related to the fibrinolytic property of G-90 which was mentioned in previous studies. The first extract of fibrinolytic enzymes from the earthworm *Eisenia foetida*, was reported in 1983 (MIHARA et al., 1983). Similar enzymes extracted from different species, such

as *Eisenia foetida* and *Lumbricus bimastus,* indicate that these earthworm species have fibrinolytic properties (HU et al., 2005; ZHAO et al., 2005; MIHARA et al., 1990; LI et al., 2011).

In the present study, histopathological evaluation showed less inflammatory response after the first postoperative week in tendons treated with G-90 in comparison with the untreated tendons. This finding is in accordance with the previous study by BALAMURUGAN et al. (2009), where the anti-inflammatory and anti-pyretic properties of G-90 were proven in a hind paw inflammation model in Wistar albino rats (BALAMURUGAN et al., 2009). In addition, histopathological evaluation in our study showed superior fibroblast maturation and superior aggregation of connective fibers in the tendons treated with G-90 in comparison with untreated tendons. Earlier investigations of a G-90 glycolipoprotein mixture showed that this mixture is capable of stimulating various activities, such as mitogenesis, as well as stimulating the synthesis of transforming growth factor and epithelial growth factor, which could all contribute to the speed of wound healing (HRZENJAK et al., 1993). The mitogen activity of G-90 (HRZENJAK et al., 1993; GRDISA et al., 2004) could be responsible for the proliferation of fibroblasts cells, contributing in that manner to the celerity of the wound-healing process (POPOVIC et al., 2005).

The influence of G-90 on the structural organization of the tendon, including improved tissue alignment and crimp formation, enhanced cell maturation, increased collagen fibril differentiation and maturation with decreased peritendinous adhesion, are possibly the most significant effects of G-90 on the tendon healing. Therefore, due to the enhanced hierarchical organization, improved biomechanical parameters were expected to be seen in the treated animals in the present study. The tensile strength of the tendons is co-related to the total collagen content, type of collagen, diameter and unimodal or multimodal distribution pattern of the collagen fibrils, the quality of the cross links of the collagen fibrils, and the quantity and quality of the non-collagenous material of the ground substance (ORYAN and MOSHIRI, 2011). The findings of the present study are in agreement with those of HAMADA et al. (2006) who showed that, after placement of a nylon monofilament coated with bFGF in the injured area of the flexor tendon of rabbits, the treated animals showed significantly enhanced ultimate strength, compared to those of the control, after 21 days post injury (HAMADA et al., 2006). TANG et al. (2008) also found that injured SDFT treated with bFGF showed a higher tensile strength at 2, 4, and 8 weeks post injury (TANG et al., 2008). On the other hand, it has also been reported that bFGF affected the initial events of tendon healing on the cell proliferation, but had no significant effect on the ultimate stress of the injured tendon during the first two weeks post injury, in a rat patellar model (CHAN et al., 2000).

Previous studies have shown that G-90 could enhance epithelialization recovery, collagen production, fibroblast proliferation and angiogenesis in the wound area (GRDISA et al., 2004). The most common growth factors are EGF and FGF, which are also involved in the wound healing process. GRDISA et al. (2004) measured EGF and FGF activity on intact skin, physiological wounds or healing and wounds treated with G-90. In the second and third groups after the first 24 hours, EGF and FGF activity was measured, and in both cases, in the first 6 hours of restoration, enhancement was observed. Compared with normal skin, the concentration of EGF was ten times higher, and the concentration of FGF was 5 times higher in the group treated with G-90. In physiological conditions, EGF doubled and FGF increased one point five (1.5) times. The macromolecules of earthworms stimulate the process of creating EGF and FGF which assumes the role of epithelization during mouse skin wound healing. EGF stimulates proliferation of epithelial cells and causes rapid healing of the wound. FGF is effective in the angiogenesis and induction of fibroblast growth (GRDISA et al., 2004).

The findings of the present experiment clearly show that administration of G-90 could enhance the structural and biomechanical properties of experimentally tenotomized SDFT in rabbits. Many factors, such as: less peritendinous adhesion, less inflammation, maturity and proper organization of the collagen fibers, and good biomechanical performance of the healing tendon, were observed in this study.

## Conclusion

In the present study, G-90 treatments of tendons led to significant differences in some biomechanical factors and enhanced tissue strength. Histopathology showed that G-90 reduces the severity of inflammation and increases aggregation of connective fiber.

Further biochemical and molecular studies are needed to elucidate other aspects of the mechanism of the action of this reagent on the structural and functional performance of tendon injuries.

## Conflict of Interest

There are no conflicts of interest related to this study

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**BIGHAM-SADEGH, A., M. ARABI, E. KARIMI, I. KARIMI, A. ORYAN: Cijeljenje tetivnih ozljeda uporabom G-90 na modelu kunića: biokemijska i histopatološka procjena. Vet. arhiv 86, 407-420, 2016.**

**SAŽETAK**

Ozljede tetiva predstavljaju jedan od najčešćih akutnih ortopedskih poremećaja što dovode do njihove funkcionalne oslabljenosti. Razvijeno je nekoliko postupaka za ponovnu uspostavu pokretnosti pacijenata, ali svi imaju znatna ograničenja. U nekim istraživanjima opisana je uporaba biomaterijala za cijeljenje ozljeda tetiva, kože i živaca. U ovom su radu istraženi učinci G-90, kao stimulacijskog čimbenika cijeljenja tetive površinskog digitalnog fleksora kunića 35 dana nakon tenotomije i kirurškog liječenja. U pokus je bilo uzeto 20 kunića u dobi od 12 mjeseci, tjelesne mase 2,0 ± 0,5 kg. Svim su kunićima pod anestezijom bile poprečno prerezane tetive površinskog digitalnog fleksora stražnjih nogu te potom spojene Bunnel-Mayer-ovim šavom. Nakon šivanja tetive i kože, 0,5 mL fiziološke otopine bilo je ubrizgano u područje tetivne ozljede lijeve noge, a 0,5 mL iscrpka G-90 kišne gujavice u koncentraciji od 10 mg/mL bilo je ubrizgano u područje tetivne ozljede desne noge. Svaka dva tjedna bila su eutanazirana 4 kunića te su im bili uzeti uzorci za patohistološke

i biokemijske pretrage. Patohistološkom pretragom tetiva kunića kojima je bio primijenjen G-90 ustanovljena je slabija upala, veća zrelost fibrocita i veće nakupljanje kolagenih vlakana u usporedbi s tetivama kojima nije bio primijenjen G-90. Kod biokemijske procjene konačna čvrstoća tetiva obrađenih s G-90 bila je veća u odnosu na one neobrađene. Rezultati ovog pokusa jasno su pokazali da primjena G-90 može osnažiti strukturu i biokemijska svojstva tetiva kunića nakon tenotomije i kirurškog liječenja.

**Ključne riječi:** iscrpak kišne gujavice, G-90, cijeljenje tetiva, kunić