Glycine Improves Biochemical and Biomechanical Properties Following Inflammation of the Achilles Tendon

CRISTIANO PEDROZO VIEIRA,[1]* LETÍCIA PRADO DE OLIVEIRA,[1] FLÁVIA DA RÉ GUERRA,[2] MARCOS DOS SANTOS DE ALMEIDA,[1] MARIA CRISTINA CINTRA GOMES MARCONDES,[1] AND EDSON ROSA PIMENTEL[1]

[1]Department of Structural and Functional Biology, Institute of Biology, State University of Campinas 13083-863 CP 6109, Campinas, SP, Brazil

[2]Department of Anatomy, Institute of Biological Sciences, Federal University of Alfenas, Minas Gerais, Brazil

ABSTRACT

Tendinopathy of the Achilles tendon is a clinical problem that motivates the scientific community to search for treatments that assist in restoring its functional properties. Glycine has broad biological effects, acting as a modulator of the inflammatory cascade, and is the predominant amino acid in collagen. A 5% glycine diet provided beneficial effects against toxicity and inflammation since glycine may restructure the collagen molecules faster due to its broad anti-inflammatory effects. The purpose was analyze the effects of a 5% glycine diet in rats as a treatment for the inflammatory process. The experimental groups were as follows: C (control group), G1 and G3 (inflammatory group), and G2 and G4 (glycine + inflammatory group). G1 and G2 were euthanized 8 days following injury, and G3 and G4 were euthanized 22 days following injury. The concentrations of hydroxyproline, noncollagenous proteins, and glycosaminoglycans, as well as the activity of MMP-2 and -9 were analyzed. Biomechanical and morphological tests were employed. Higher concentrations of hydroxyproline and glycosaminoglycans were found in G4 and an increased activity of MMP-2 was found in G2. Higher birefringence was noted in group G2. The biomechanical results indicated that the tendon was more resistant to loading to rupture upon treatment with a glycine diet in group G4. Glycine induced the synthesis of important components of the tendon. A rapid remodeling was noted when compared with the inflamed-only groups. These data suggest that glycine may be a beneficial supplement for individuals with inflammation of the Achilles tendon. Anat Rec, 298:538–545, 2015. © 2014 Wiley Periodicals, Inc.

Key words: tendinopathy; achilles tendon; glycine; inflammatory process; extracellular matrix

Tendon injuries often occur in tendons that are exposed to high mechanical forces and that undergo more extensive matrix remodeling, such as the Achilles, supraspinatus and patellar tendons (Benjamin et al., 2008; Wang et al., 2012). The biomechanical properties of tendons depend on the structural arrangement of the collagen fibrils, fiber diameter, and the molecular aggregation of the collagen. The fibrils aggregate into collagen fibers that are responsible for the resistance of the tendon, and the efficiency of this mechanism depends on the parallel arrangement of these fibers in the direction of the tension forces (James et al.,

Grant sponsor: National Council for Scientific and Technological Development (CNPq - Brazil).

*Correspondence to: Mr. Cristiano Pedrozo Vieira, Department of Structural and Functional Biology, Institute of Biology, CP 6109, State University of Campinas – UNICAMP, 13083-863 Campinas, SP, Brazil. Fax: 0055-19-35216111.
E-mail: cristianobio@gmail.com

Received 21 May 2014; Accepted 23 July 2014.

DOI 10.1002/ar.23041

Published online 23 August 2014 in Wiley Online Library (wileyonlinelibrary.com).

2008; Wang et al., 2012; Aro et al., 2012). However, biomechanical properties of the tendons are often compromised and the tendon may undergo significant changes following injuries (Maganaris et al., 2004).

The incidence of Achilles tendon lesions is common, indeed increasing in sports, comprising 30–50% of the total number of injuries (Järvinem et al., 2005; Ruschel et al., 2009; Dario et al., 2010; Fung et al., 2010). Each year in the United States, there are about 16.4 million cases of tendon and ligament injuries, and about 100,000 cases that involve the Achilles tendon (Järvinem et al., 2005). The use of some antibiotics and medications is associated with induction of these injuries (Kader et al., 2002; de Oliveira et al., 2013). Tendon injuries are difficult to repair surgically and are typically slow to heal. Furthermore, is known that after the tendon ruptures, it does not return to its preinjury condition (Jozsa and Kannus, 1997).

The extracellular matrix (ECM) components of the tendons undergo structural and biochemical changes during injuries caused by the local inflammation (Vieira et al., 2012, 2013) and with partial ruptures (Almeida et al., 2012; Guerra et al., 2013). In the inflammatory process, macrophages, monocytes and neutrophils are primarily responsible for the action of enzymes and subsequent degradation of ECM components. Various types of collagenases are involved in inflammation in addition to the matrix metalloproteinases (MMP) that include MMP-2 and MMP-9. A balance between MMPs and their inhibitors is important to maintain and prevent excessive tissue degradation (Gill and Parks, 2008; Clutterbuck et al., 2010). Furthermore, the molecular mechanisms of tendinopathy are still unclear, and therefore, the results of current treatments are largely empirical and often less effective for these types of injuries (Wang et al., 2006).

Glycine is an amino acid with a simple molecular structure, and it displays important biological activities by acting as a modulator of the systemic inflammatory cascade, improving the microcirculation and assisting in the inhibition of TNF-α and IL-1β (Hartog et al., 2007; Figueiredo et al., 2009). Many studies have proposed glycine as a useful treatment for many types of inflammatory processes (Hartog et al., 2007; Weeler et al., 2009; Carmans et al., 2010; Stoffels et al., 2011). In addition, glycine is used in the prevention and treatment of cancers such as melanomas (Weeler et al., 2009). A 5% glycine diet has provided beneficial effects against liver toxicity and inflammation in rats (Li et al., 2001; Mikalauskas et al., 2011). The metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis, thus dietary sources are important for normal metabolism (Meléndez-Hevia et al., 2009).

The hypothesis that was tested in this study was whether a 5% glycine diet could accelerate recovery of the biochemical and biomechanical properties of tendons following inflammation. The collagenase-induced inflammation model in rats was employed to test this hypothesis. The main objective of this study was to evaluate glycine supplementation as a viable alternative or supplementary treatment for Achilles tendon inflammation.

MATERIAL AND METHODS

Experimental Design

Animal care was in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, and it was consistent with the ethical principles of animal experimentation adopted by the Brazilian College of Animal Experimentation. The protocol was approved by the Ethics Committee on Animal Experiments of the State University of Campinas (UNICAMP), SP, Brazil, and filed under no. 2307-1.

In this study, young adult (60 days) male Wistar rats, weighing on average 300 g, were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) of the State University of Campinas. The rats were housed two per cage under a 12-h light/dark cycle at 23°C with free access to standard rat chow and water prior to the treatment with glycine and the control diet (without glycine). The animals were divided into five groups: C, control group; G1 and G3, inflamed group; and G2 and G4, inflamed and supplied with a glycine diet. Groups G1 and G2 were euthanized on day 8 after induction of inflammation, and groups G3 and G4 were euthanized on day 22 after induction of inflammation.

To induce inflammation, the animals were anesthetized with isoflurane (Forame) and given an intratendinous injection in the right Achilles tendon of 10 μL of collagenase (10 mg mL^{-1}; Sigma, C6885) dissolved in sterile buffered saline (PBS) (Lake et al., 2008). After induction of tendinitis, the animals in the treatment groups (G2 and G4) received a diet containing 5% glycine. The animals in the induced tendinitis groups G1 and G3 and the control group (C) control received the control diet without glycine. The treatments lasted 7 days for groups G1 and G2 and 21 days for groups G3 and G4. The entire right tendon was taken after euthanasia. Five animals in each group were used for biochemical studies and the other five were used for morphological analysis. Six animals per group were used in biomechanical analyses. The left tendon was not used.

A diet containing 5% glycine (AJINOMOTO, BRAZIL) was provided by the Institute of Biology, Animal Physiology, State University of Campinas, following the guidelines of AIN-93M (Reeves et al., 1993). The diet was administered to the animals and the consumption was recorded daily.

Extraction Procedures

The Achilles tendon was removed and treated with 50 volumes of 4 M guanidine hydrochloride (GuHCl) containing 20 mM EDTA and 1 mM PMSF in 50 mM sodium acetate buffer, pH 5.8, for 24 h at 4°C with stirring (Heinergard and Sommarin, 1987). Then, the material was centrifuged (13,000*g*, 25 min, 4°C), and the supernatant was used for the quantification of non-collagenous protein and glycosaminoglycans (GAGs).

Quantification of Proteins and Sulfated GAGs

The extracts collected from the experimental groups were used to quantify the relative amounts of protein according to the method of Bradford (1976). Bovine serum albumin was employed as a standard. The quantification of GAGs was performed using the dimethylmethylene blue method (Farndale et al., 1986) with chondroitin sulfate as the standard. The absorbance was measured at 595 nm for proteins and at 540 nm for

GAGs using an ASYS Expert Plus Microplate Reader (Biochrom, Holliston, MA).

Hydroxyproline Quantification

Fragments of the tendon were hydrolyzed in 6 N HCl (1 mL/10 mg of tissue) for 4 h at 130°C. Then, the lysate was treated with 1.41% chloramine-T solution and 15% *p*-dimethylaminobenzaldehyde, as described by Stegemann and Stalder (1967). After incubation for 15 min at 60°C, the hydroxyproline solution was cooled, and the absorbance was measured at 550 nm using an Ultrospec 2100 Pro spectrophotometer (Amersham Biosciences, England).

Zymography for MMP-2 and -9

The tendons were treated according to the method described by Marqueti (2006). For protein extraction, the fragments of the tendon were immersed in a solution of 50 mM Tris-HCl (Synth) pH 7.4, 0.2 M NaCl, 10 mM CaCl2 (Ecibra, São Paulo, SP, Brazil), and 0.1% Triton X-100 (Nuclear) with 1% of a protease inhibitor cocktail (Sigma) at 4°C for 2 h. After the first extraction, the samples were incubated with 1/3 of the volume of the solution described above at 60°C for 5 min. Then, 20 μg of the protein extract was loaded onto the gel. The protein samples were electrophoresed at 4°C on a 10% polyacrylamide gel containing 0.1% gelatin, and after completion of electrophoresis, the gel was washed with 2.5% Triton X-100 (Nuclear) and incubated for 21 hr in a solution of 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and 0.03% sodium azide (Sigma) at 37°C. The gel was stained with Coomassie brilliant blue R-250 for 1 hr. After staining, the gels were washed with a solution containing 50% methanol and 10% acetic acid to observe negative bands of proteins corresponding to enzymatic activity. As a positive control, 20 mM EDTA was used in the incubation buffer. EDTA inhibits the activity of gelatinase and confirms the identification of MMPs in the gels. The bands in the negative image were quantified by densitometry using Scion Image software Alpha 4.0.3.2 (Scion Corporation, Frederick, MD).

Light Microscopy Analysis and Morphometry

The tendons were fixed in a solution containing 4% formaldehyde in Millonig buffer (0.13 M sodium phosphate, NaOH 0.1 M, pH 7.4) for 24 hr at room temperature. The tendons were then washed for 6 hr in tap water, dehydrated with a sequence of increased ethanol concentrations, diaphonized with xylene and embedded in paraffin (Histosec, Merck), according to Neto et al. (2003). Longitudinal serial sections of 7 μm in thickness were prepared for microscopic analysis. To visualize the overall structure of the tissue, some sections were stained with toluidine blue (TB) (0.025%) in McIlvaine buffer (0.03 M citric acid, 0.04 M sodium phosphate dibasic, pH 4.0).

Digital images were observed with a camera coupled to a light microscope (Olympus BX 60) and then were used to perform all morphometric analyses using Image-Pro Plus 6.0 software. Ten random images (200× magnification) were used to measure the thickness of the epitenon and the same number of images was used (400× magnification) to count the cells. Ten images were used per animal (n = 5).

Birefringence: Image Analysis and Measurements

Birefringence properties were studied using an Olympus BX51-P BX2 polarizing microscope with an image analyzer (Image-Pro Plus 6.3, Media Cybernetics, Silver Spring, MD). Because birefringence appears visually as brilliance, this phenomenon was measured with the image analyzer and expressed as gray average (GA) values in pixels, after its calibration (8 bits = 1 pixel). The major tendon axis was positioned at 45° to the crossed analyzer and polarizer during the measurements. Considering that collagen bundles exhibit two kinds of birefringence: intrinsic birefringence (Bi) and form or textural birefringence (Bf) (Vidal and Volpe 2005; Vidal and Mello 2010), the total birefringence (sum of Bi and Bf) was used in this study. The measurements of the transected region of the tendons in each experimental group were made after immersing the sections in water.

Biomechanical Tests

The biomechanical tests were performed using a TAXT2 texturometer (Stable Micro Systems, from the Department of Engineering, Faculty of Food Engineering, UNICAMP), according to Almeida et al., (2009). We used six tendons in each experimental group for this analysis. The tendons were maintained in saline solution until the time of testing. Prior to testing, the lengths, widths and thicknesses of the tendons were measured with a caliper, and the latter two parameters were determined at the midpoint of the tendon; the cross-sectional area was calculated from these measurements. The adapters that were encased in the machine secured the tendons by their ends.

During the test, the tendons were subjected to a gradually increasing load at a constant displacement rate of 1 mm sec^{-1}, using a load of 5 N until the tendons ruptured. The maximum stress was calculated from these data. The stress (MPa) was calculated using the ratio between the load (N) and the cross-sectional area (mm^2). The maximum displacement was determined as the load that the tendon could withstand prior to rupture.

Statistical Analysis

The data were presented as the mean ± SD of the results obtained from five animals per group. To compare the data, statistical analysis was performed using one-way analysis of variance (ANOVA) with the Tukey post hoc test. The Mann–Whitney test was used only for analysis of the birefringence measurements. A value of $P < 0.05$ was considered statistically significant, and the statistical program GraphPad PrismVR, version 3.0, was used for all analyses.

RESULTS

Biochemical analysis via quantification of hydroxyproline and an indirect quantification of collagen showed a greater concentration of hydroxyproline in G4 ($P < 0.05$) in comparison to the other inflamed and treated groups. In addition, the amount of hydroxyproline in G4 was

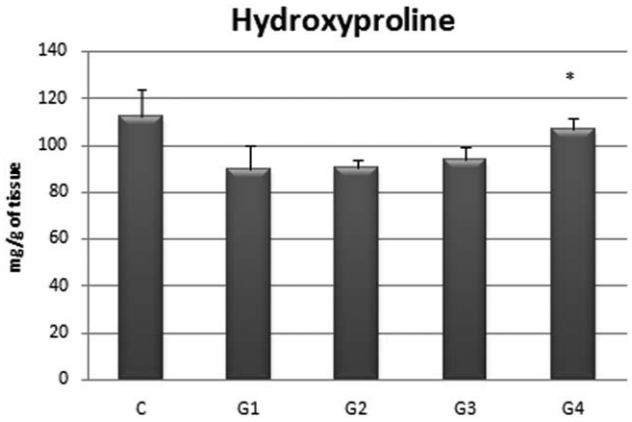

Fig. 1. Concentration of hydroxyproline (mg g^{-1} dry tissue) in different groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. G4 showed values similar to those of C and higher than the other groups ($P < 0.05$*).

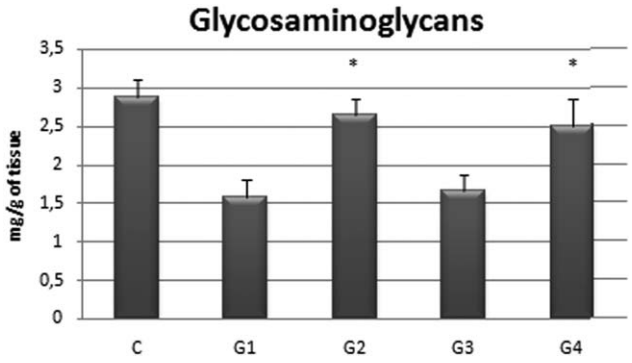

Fig. 2. Concentration of sulfated glycosaminoglycans (mg g^{-1} wet tissue). C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. Note the higher concentration in G2 and G4 compared to G1 and G3 ($P < 0.05$*). Both treated groups presented values similar to those of C.

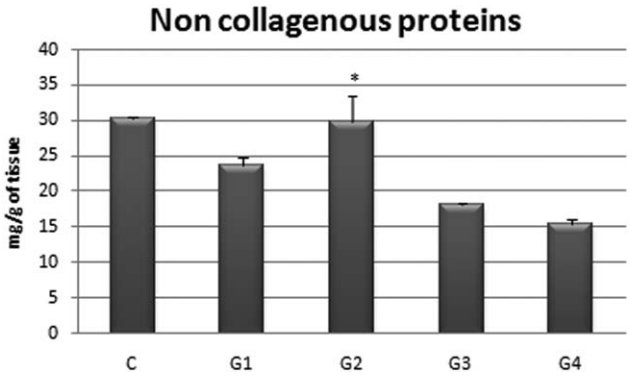

Fig. 3. Concentration of non collagenous proteins. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. Observe a higher concentration of proteins in G2 when compared to G1 ($P < 0.01$*). There were not differences between G3 and G4. Note the concentration in G2 was similar the found in C.

TABLE 1. Densitometry of the bands corresponding to zymography of the MMP-2 and MMP-9 isoforms

Groups	pro-MMP-2 (72 and 68 kDa)	MMP-2 active (62 kDa)	MMP-9 active (82 kDa)
C	160.9 ± 30.20[a]	28.76 ± 9.2	0
G1	84.6 ± 12.0	40.43 ± 14.3	29.51 ± 9.1
G2	66.76 ± 21.04	62.40 ± 4.1	49.91 ± 12.9
G3	94.71 ± 8.9	31.11 ± 7.2	27.70 ± 8.7
G4	75.87 ± 9.6	36.12 ± 6.9	54.27 ± 23.6

[a]$P < 0.05$; the activity of pro-MMP-2 was elevated in the C group; a trend increase was observed for the active MMP-2 in G2 when compared to the other groups, but the difference was not significant; It was not observed differences among the groups in relation to MMP-9. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

similar to that found in control group, C (Fig. 1). Apparently, the action of glycine on the hydroxyproline concentration occurred within 7–21 days, as there were no changes in the first 7 days of treatment.

In the quantification of total sulfated GAGs (Fig. 2), a higher concentration was observed in G2 ($P < 0.01$) and G4 compared to G1 and G3. In G2 and G4, the concentrations of GAGs were similar to that found in group C.

Moreover, quantification of the non-collagenous proteins showed a greater concentration in G2 when compared to G3 and G4 ($P < 0.001$). G4 showed a lower concentration of proteins compared to all other groups (Fig. 3).

Regarding the activity of metalloproteinases in the zymography for MMP-2 (Table 1), a greater concentration of pro-MMP-2 was observed in the control group (C) compared with the other groups. However, the active isoform of MMP-2 was greater G2 relative to the other groups; however, this result was not significantly different among the groups. Densitometry of the gel zymography bands showed no significant difference in the activity of MMP-9 among the groups. As expected, the C group did not show the presence of MMP-9 (Fig. 4).

The biomechanical characteristics of the Achilles tendon presented interesting results in the animals that received glycine as a treatment for inflammation (Table 2). In the maximum load analysis graph, G4 withstood a larger load (N) than the other groups that were inflamed ($P < 0.05$), and this value remained close to that observed in the control group. Considering the maximum stress (MPa), no differences were found among the groups; however, the values of this parameter had a tendency to increase in G4 compared to G3. Regarding the displacement and cross-sectional area, there were no significant differences among the groups.

The birefringence images showed a high birefringence of collagen molecules in G2 when compared to G1 (Figs. 5 and 6). There were no differences among the groups in 21 days. The epitenon was thicker in G2 (Fig. 7) when compared to the others groups ($P < 0.05$). However, there were no differences among the groups, in relation to the number of cells (Table 3).

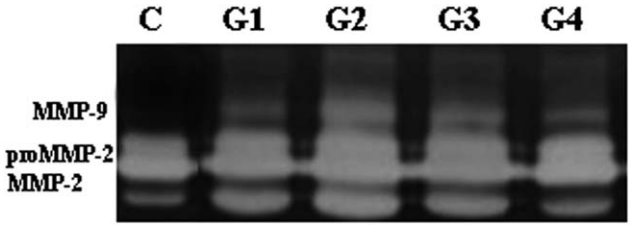

Fig. 4. Zymography for MMP-2 and 9. The activity of MMP-2 showed a trend of increase in G2. However, these results were not significant. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

TABLE 2. Biomechanical properties of the experimental groups

Groups	Maximum load (N)	Maximum displacement (mm)	Maximum stress (MPa)	Cross-sectional area (mm^2)
C	53.2 ± 7.0	1.40 ± 0.5	57.3 ± 6.3	1.01 ± 0.4
G1	33.2 ± 4.7	1.10 ± 0.2	52.1 ± 9.4	0.66 ± 0.1
G2	33.0 ± 7.1	1.20 ± 0.1	51.0 ± 19.0	0.76 ± 0.1
G3	35.2 ± 6.5	1.08 ± 0.4	39.6 ± 12.2	0.90 ± 0.2
G4	42.5 ± 4.3*	1.25 ± 0.4	54.6 ± 11.9	0.72 ± 0.1

The maximum load were higher in G4 ($^*P < 0.05$). These values were similar to those of C. The maximum stress was smaller in inflamed tendons (G3) but this result was not differences significant. The cross-sectional area and displacement were similar in all groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.

DISCUSSION

The inflammatory process applied to the Achilles tendon was remediated via its biochemical and biomechanical properties after treatment with a 5% glycine diet. In this study, biochemical components of the ECM that are involved directly in the remodeling process after the induction of a lesion were analyzed. In addition remarkable changes were observed in the endurance of the tendon after submitting it to a biomechanical test.

Glycine is predominant (~35%) in the collagen molecule, and some studies have utilized it for therapeutic purposes for inflammatory processes and for tumor and hepatotoxic conditions (Yamashina et al., 2007; Hartog et al., 2007; Stoffels et al., 2011; Mikalauskas et al., 2011). The results from the present study on the benefits of a glycine-rich diet on the biochemistry, structural and biomechanics of the inflamed Achilles tendon also suggest potential new therapeutic approaches for this and related injuries.

The collagen molecule includes hydroxyproline, proline and glycine, which assist in the stabilization of the triple helix structure of collagen (Piez and Reddi, 1984). The glycine-rich diet provided to the animals with inflamed tendons resulting in levels of hydroxyproline that reached the values observed in the control, despite the degradation and disorganization of collagen fibers associated with the inflammatory process (Clutterbuck et al., 2010; Guerra et al., 2013). These observations suggest that the higher levels of hydroxyproline may help maintain the integrity of collagen fibers, perhaps making them more resistant to degradation.

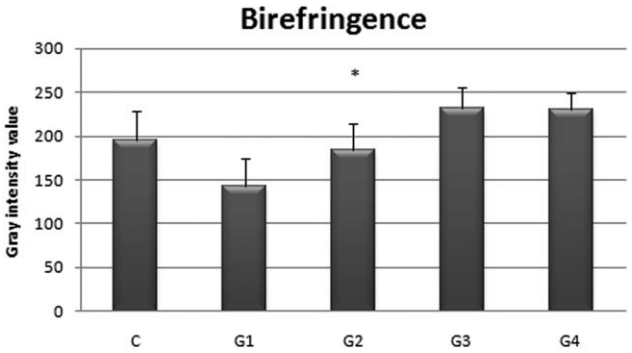

Fig. 5. Birefringence measurements of different groups of tendon. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. The measurements of G2 was higher than G1 (*). G3 and G4 showed similar measurements.

Concentrations of GAGs are decreased in inflamed tendons (Vieira et al., 2012). In the present study, GAGs concentrations were observed to return to normal levels in tendons of animals treated with a diet rich in glycine, from 8 to 22 days following the induction of inflammation. GAGs are part of the proteoglycans molecules, which are integral to the regulation of many cellular events, including cell proliferation, migration and adhesion (Jozsa and Kannus, 1997; Banos et al., 2008). The high concentration of GAGs may promote a more rapid remodeling of the extracellular matrix after inflammation. It is also known that the small proteoglycan, decorin, is involved directly with the fibrillogenesis of collagen molecules and is responsible for controlling the diameters of these fibers (Banos et al., 2008; James et al., 2008).

During the inflammatory process, signaling molecules and proteins of the ECM are cleaved (Vieira et al., 2012). Regarding the increase of non-collagenous proteins in G2, the data suggests that glycine may promote or facilitate protein synthesis during inflammation.

Previous studies have shown that glycine contributes to the inhibition of TNF-α and IL-1β (Hartog et al., 2007; Figueiredo et al., 2009). These inflammatory molecules are involved in the induction of apoptosis (Kim et al., 2010) and the activation of metalloproteinases involved in the excessive degradation of matrix components (Tsuzaki et al., 2003). MMP-2 is the enzyme responsible for extracellular matrix remodeling, and it shows high activity during pathological processes such as injury and partial rupture of the tendon (Almeida et al., 2012; Guerra et al., 2013). A greater trend towards increased MMP-2 activity was observed in 7 days in the present study. It is known that this enzyme is required to restore the tissue after injury, and it has beneficial effects in the remodeling process (Clutterbuck et al., 2010). The trend toward increasing activity may promote tissue remodeling in the ECM and thus more effectively restoring tendon biomechanical properties.

Cells and their associated enzymes recruited during the initiation of inflammation can induce disorganization

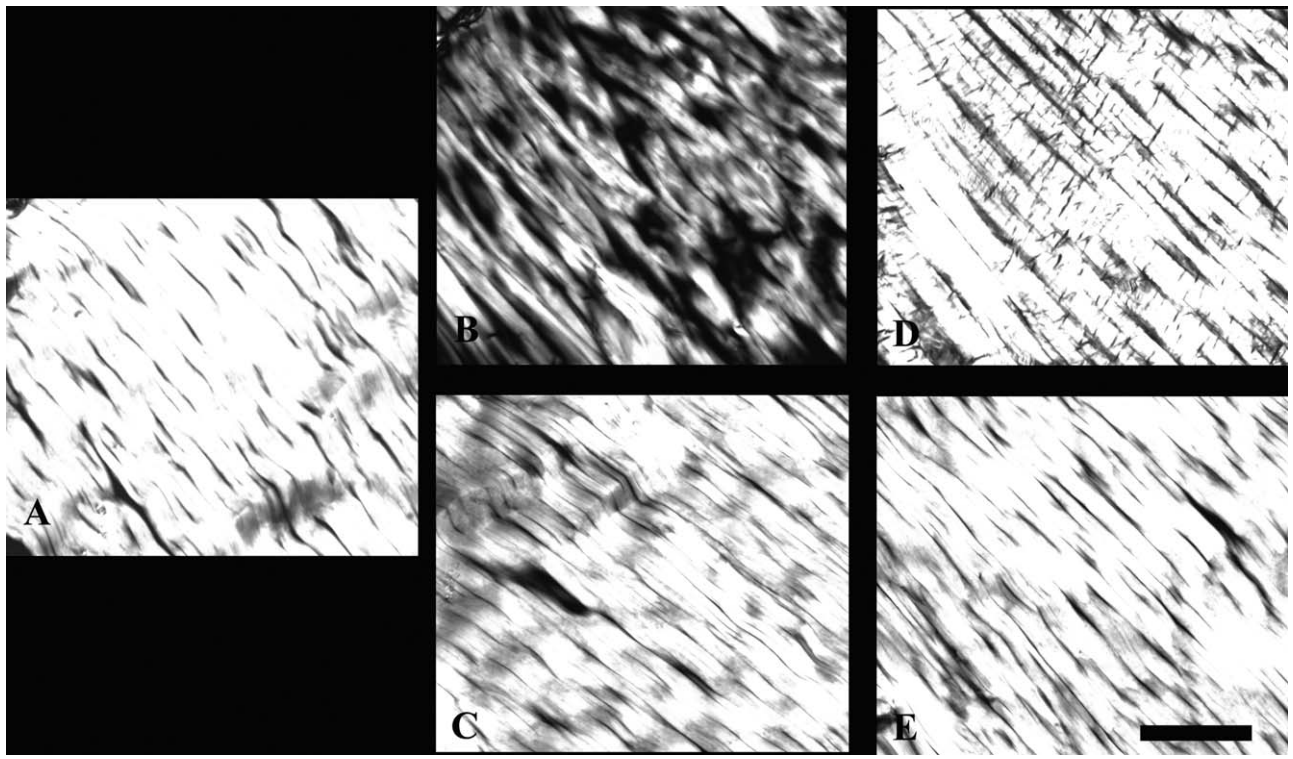

Fig. 6. Birefringences of longitudinal sections of tendons from the different groups, observed by polarization microscopy. The largest axis of the tendon is positioned at 45° in relation to the crossed polarizers. A: C group; B: G1; C: G3; D: G2; E:G4. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days. It is possible to note the high birefringence of collagen molecules in G2 when compared to G1. In G3 and G4 the organization of collagen fibers was the same. Bar: 20 μm.

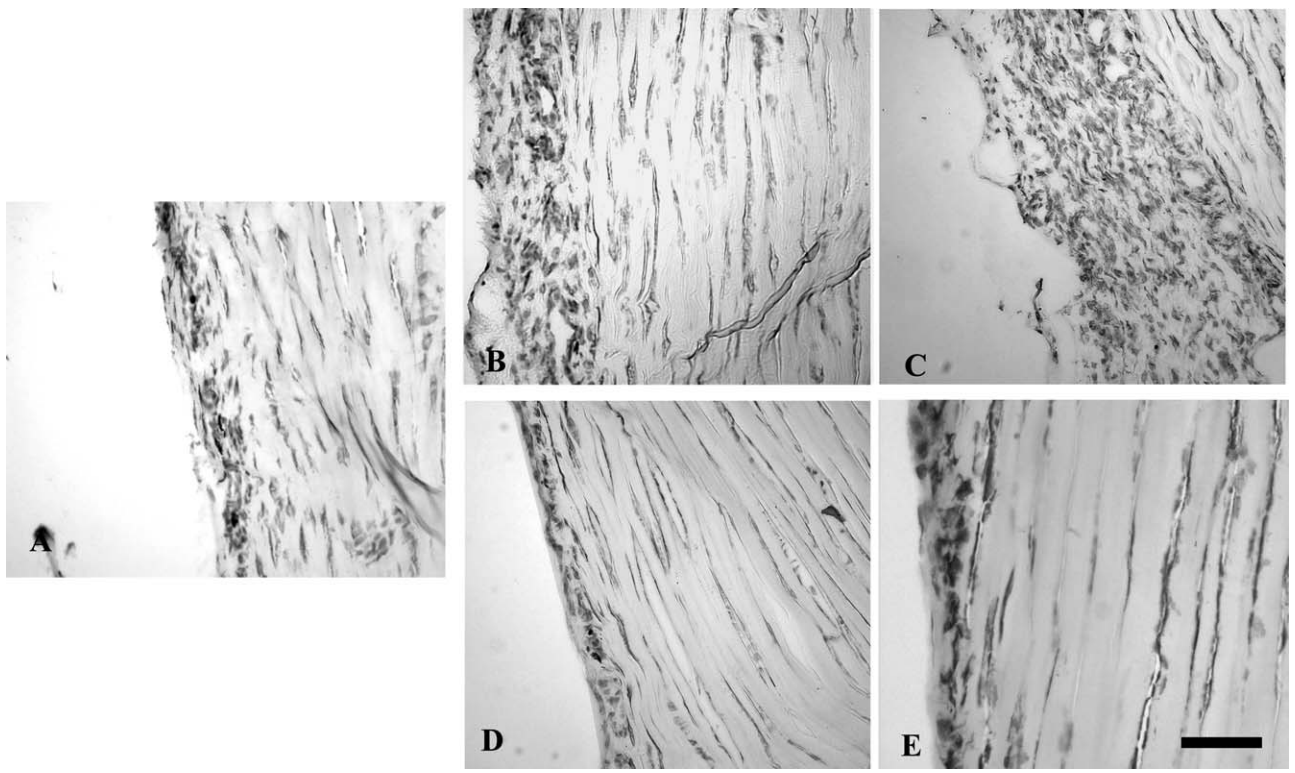

Fig. 7. Histological sections stained with toluidine blue. A: C group; B: G1; C: G2; D: G3; E: G4. Note the epitenon thicker in G2 compared to the other groups. Bar: 40 μm.

TABLE 3. Morphometry of tendon sections stained with Toluidine Blue represented by the mean and standard deviation

Parameters	C	G1	G2	G3	G4
Cells/$\mu m^2(10^{-4})$	8.0 ± 0.78	6.0 ± 0.8	7.0 ± 0.8	6.0 ± 0.8	5.0 ± 0.8
Epitenon (μm)	93.2 ± 25.2	233.7 ± 45.0	268.7 ± 44.0*	111.7 ± 23.2	138.0 ± 22.2

There were no differences observed in the amount of cells of tendon. In relation to epitenon, it was noted thicker in G2 compared to the others experimental groups. C: control group; G1: inflamed group in 7 days; G2: inflamed and supplied with a glycine diet in 7 days; G3: inflamed group in 21 days; G4: inflamed and supplied with a glycine diet in 21 days.
*$p < 0{,}05$.

of the collagen bundles and promote degradation of ECM components, which may lead to severe chronic pathologic processes. This process may result in a reduction in the tensile strength of the tendon and predisposes the tendon to rupture (Sharma and Maffulli, 2006; Den Hartog, 2009; Silva et al., 2011). The biomechanical analyses showed that G4 sustained a higher biomechanical load compared to the other inflamed groups. The profile found in G4 remained closer to the control group. The tendons from this group more resistant to rupture after treatment with glycine were compared to G1, G2, and G3. These data were corroborated by the high concentrations of glycosaminoglycans and hydroxyproline found in G4. In case of tendinopathy, the weakening of the tendon causes morbidity and disability among athletes and people in general (Tallon et al., 2001). The biomechanical characteristics of the Achilles tendon are primarily responsible for the ability to withstand large muscular forces with minimum deformation (Maganaris et al., 2002; Casalechi et al., 2012). The addition of dietary glycine in the present study was associated with a higher biomechanical resistance in the Achilles tendon after the induction of inflammation. It is known that glycine is present in the triple helix of the collagen molecule in large quantity and the addition of greater dietary glycine may have promoted collagen synthesis.

High birefringence noted in G2 showed that glycine supplementation resulted in better organization of collagen fibers after induced inflammation compared with the non-supplemented groups. G3 and G4 showed similar measurements in the birefringence images. However, the biomechanics properties noted in G4 were significantly better compared to the inflamed group in the same period. The biomechanical properties of tendons change during physical activity, stretching and pathological processes (Marqueti et al., 2006; Wang et al., 2012). The improvement or maintenance of the biomechanical properties of tendons after a pathological event, such as inflammation, suggests that glycine supplementation improved or maintained effective connective tissue remodeling processes.

The epitenon was thicker in G2. This result suggests a greater cellular infiltration in this region. During inflammation, the tissue becomes weaker due to the cascade of events arising from the injury, such as release of cytokines that are involved in the infiltration of macrophages and neutrophils from the blood stream, as well as the imbalance between MMPs and their inhibitors (TIMPs). The MMP-2 and -9 can be secreted by neutrophils (Clutterbuck et al., 2010; Casalechi et al., 2012). During the recovery time, the tendon is more susceptible to further injuries. Glycine apparently functions on the active isoform of MMP-2 in the first 7 days of treatment, and no further effect was observed after 21 days. This enzyme may be involved for the rapid remodeling of collagen fibers and arrangement of proteoglycans, as well as for improving the functionality of the tendon to withstand higher loads in 21 days, when compared to animals that had inflammation in the tendon.

This study demonstrated effects of a glycine-rich diet on inflammation of the Achilles tendon. The glycine diet stimulated the synthesis of hydroxyproline, glycosaminoglycans, non-collagenous proteins and appeared to maintain or improve the organization of collagen molecules. The biomechanical results indicated that the tendon was more resistant to mechanical loading upon treatment with a glycine diet. Glycine also induced a rapid remodeling of tissue when compared with the groups without treatment. The data from this study suggest that dietary glycine supplementation may be a useful therapeutic adjunct for individuals with inflammatory injuries of tendons, such as Achilles tendon injuries, and perhaps other types of connective tissue injuries and inflammatory events.

ACKNOWLEDGEMENTS

The authors thank Francisco A. Mallattesta for his technical assistance.

LITERATURE CITED

Almeida FM, Tomiosso TC, Nakagaki WR, Gomes L, Matiello-Rosa SMG; Pimentel ER. 2009. Effects of passive stretching on the biochemical and biomechanical properties of calcaneal tendon of rats. Connect Tissue Res 50:279–284.

Almeida Mdos S, De Aro AA, Guerra FdaR, Vieira CP, De Campos Vidal B; Pimentel ER. 2012. Electroacupuncture increases the concentration and organization of collagen in the tendon healing model in rats. Connect Tissue Res 53:542–547.

Aro AA, Vidal BC, Pimentel ER. 2012. Biochemical and anisotropical properties of tendons. Micron 43:205–214.

Banos CC, Thomas AH, Kuo CK. 2008. Collagen fibrillogenesis in tendon development: current models and regulation of fibril assembly. Birth Defects Res C Embryo Today 84:228–244.

Benjamin M, Kaiser E, Milz S. 2008. Structure-function relationships in tendons: a review. J Anat 212:211–228.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Carmans S, Hendriks JJA, Thewissen K, Van den Eynden J, Stinissen P, Rigo JM, Hellings N. 2010. The inhibitory neurotransmitter glycine modulates macrophage activity by activation of neural amino acid transporters. J Neurosci Res 88:2420–2430.

Casalechi HL, Leal-Junior ECP, Xavier M, Silva JA, Jr, de Carvalho Pde T, Aimbire F, Albertini R. 2012. Low-level laser therapy in experimental model of collagenase-induced tendinitis in rats: effects in acute and chronic inflammatory phases. Lasers Med Sci 28:989–995.

Clutterbuck AL, Harris P, Allaway D, Mobasheri A. 2010. Matrix metalloproteinases in inflammatory pathologies of the horse. Vet J 183:27–38.

Dario BES, Barquilha G, Marques RM. 2010. Lesões esportivas: um estudo com atletas de basquetebol Bauruense. Rev Bras Cienc Esporte 31:205–215.

de Oliveira LP, Vieira CP, Guerra Fda R, de Almeida Mdos S, Pimentel ER. 2013. Statins induce biochemical changes in the Achilles tendon after chronic treatment. Toxicology 311:162–168.

Den Hartog BD. 2009. Insertional achilles tendinosis: pathogenesis and treatment. Foot Ankle Clin N Am 14:639–650.

Farndale RW, Buttle DJ, Barret AJ. 1986. Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethyleneblue. Biochim Biophys Acta 883:173–177.

Figueiredo JA, Petroianu A, Correia MITD, Castro Júnior HA, de Speschit W, Silveira R de OP, Nunes CB, Abrantes MM. 2009. Efeito da suplementação nutricional com glicina e glutamina, por via oral, na cicatrização colônica em coelhos. Rev Col Bras Cir 36: 148–151.

Fung DT, Want VM, Andarawis-Puri N, Basta-Pljakic JB, Li Y, Laudier DM, Sun HB, Jepsen KJ, Schaffler MB, Flatow EL. 2010. Early response to tendon fatigue damage accumulation in a novel in vivo model. J Biomech 43:274–279.

Gill SE, Parks WC. 2008. Metalloproteinases and their inhibitors: regulators of wound healing. Int J Biochem Cell Biol 40:1334–1347.

Guerra FdaR, Vieira CP, Almeida MS, Oliveira LP, De Aro AA, Pimentel ER. 2013. LLLT improves tendon healing through increase of MMP activity and collagen synthesis. Lasers Med Sci 28:1281–1288.

Hartog A, Leenders I, van der Kraan PM, Garssen J. 2007. Anti-inflamatory effects of orally ingested lactoferrin and glycine in different zymosan-induced inflammation models: evidence for synergistic activity. Int Immunipharmacol 7:1784–1792.

Heinergard D, Sommarin Y. 1987. Isolation and characterization of proteoglycans. Methods Enzymol 144:319–373.

James R, Kesturu G, Balian G, Chhabra B. 2008. Tendon: biology, biomechanics, repairs, growth factors and evolving treatment options. J Hand Surg 33A:102–112.

Järvinem TAH, Kannus P, Maffulli N, Khan KM. 2005. Achilles tendon disorders: etioloy and epidemiology. Foot Ankle Clin N Am 10:255–266.

Jozsa L, Kannus P. 1997. Human tendons. Champaign (IL): Human Kinetics. p 1–576.

Kader A, Saxena A, Movn T, Maffulli N. 2002. Achilles tendinopathy: some aspects of basic science and clinical management. Br J Sports Med 36:239–249.

Kim JJ, Lee SB, Park JK, Yoo YF. 2010. TNF-alpha-induced ROS production triggering apoptosis is directly linked to Romo1 and Bcl-XL. Cell Death Differ 17:1420–1434.

Lake SP, Ansorge HL, Soslowsky LJ. 2008. Animal models of tendinopathy. Disabil Rehabil 30:1530–1541.

Li X, Bradford BU, Wheeler MD, Stimpson SA, Pink HM, Brodie TA, Schwab JH, Thurnan RG. 2001. Dietary glycine prevents peptidoglycan polysaccharide-induced reactive arthritis in the rat: role for glycine-gated chloride channel. Infect Immunol 69: 5883–5891.

Maganaris CN, Paul JP. 2002. Tensile properties of the in vivo human gastrocnemius tendon. J Biomech 35:1639–1646.

Maganaris CN, Varici MV, Almekinders LC, Maffulli N. 2004. Biomechanics and pathophysiology of overuse tendon injuries—ideas on insertional tendinopathy. Sport Med 34:1005–1015.

Marqueti RC, Parizotto NA, Chriguer RS, Perez SEA, Selistre-de-Araujo HS. 2006. Androgenic-anabolic steroids associated with mechanical loading inhibit matrix metallopeptidase activity and affest the remodeling of the achilles tendon in rats. Am J Sports Med 34:1274–1280.

Meléndez-Hevia E, De Paz-Lugo P, Cornish-Bowden A, Cárdenas ML. 2009. A weak link in metabolism: the metabolic capacity for glycine biosynthesis does not satisfy the need for collagen synthesis; J Biosci 34:853–872.

Mikalauskas S, Mikalauskiene L, Bruns H, Nickkholgh A, Hoffmann K, Longerich T, Strupas K, Büchler MW, Schemmer P. 2011. Dietary glycine protects from chemotherapy induced hepatotoxicity. Amino Acids 40:1139–1150.

Neto AGF, Rodrigues CI, Tolosa EMC, Behmer AO. 2003. Manual de técnicas para histologia normal e patológica. 2nd ed. São Paulo: Editora Manole.

Piez KA, Reddi AH. 1984. Extracellular matrix biochemistry. In: Piez KA, Reddi AH, editors. Molecular and aggregate structures of the collagens. New York, NY: Elsevier. p 1–40.

Reeves PG, Nielsen FH, Fahey J. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76 rodent diet. J Nutr 123:1939–1951.

Ruschel C, Menezes FS, Haupenthal A, Hubert M, Schutz GR, Cerutti PR, Pereira SM, Roesler H. 2009. Incidência de lesões em velejadores brasileiros de diferentes níveis técnicos. Rev Bras Med Esporte 15:4–10.

Sharma P, Maffulli N. 2006. Biology of tendon injury: healing, modeling and remodeling. J Musculoskelet Neuronal Interact 6: 181–190.

Silva RD, Glazebrook MA, Campos VC, Vasconcelos AC. 2011. Achilles tendinosis—a morphometrical study in a rat model. Int J Clin Exp Pathol 4:683–691.

Stegemann H, Stalder K. 1967. Determination of hydroxyproline. Clin Chim Acta 18:267–273.

Stoffels B, Türles A, Schmidt J, Nazir A, Tsukamoto T, Moore BA, Schunurr C, Kalff J, Bauer AJ. 2011. Anti-inflammatory role of glycine in reducing rodent postoperative inflammatory Ileus. Neurogastroenterol Motil 23:76–28.

Tallon C, Maffulli N, Ewen SW. 2001. Ruptured achilles tendons are significantly more degenerated than tendinopathic tendons. Med Sci Sports Exerc 33:1983–1990.

Tsuzaki M, Guyton G, Garrett W, Archambault JM, Herzog W, Almekinders L, Bynum D, Yang X, Banes AJ. 2003. IL-1beta induces COX2, MMP-1, −3 and −13, ADAMTS-4, IL-1beta and IL-6 in human tendon cells. J Orthop Res 21:256–264.

Vidal BC, Volpe PLO. 2005. Differental scanning calorimetry and optical properties of collagen-dichroic azo Ponceau SS Complexes. Braz J Morphol Sci 22:49–153.

Vidal BC, Mello MLS. 2010 Optical anisotropy of collagen fibers of rat calcaneal tendons: an approach to spatially resolved supramolecular organization. Acta Histochem 112:53–61.

Vieira CP, Aro AA, Almeida MS, De Mello GC, Antunes E, Pimentel ER. 2012. Effects of acute inflammation induced in the rat paw on the deep digital flexor tendon. Connect Tissue Res 53:160–168.

Vieira CP, De Aro AA, Da Ré Guerra F, De Oliveira LP, De Almeida Mdos S, Pimentel ER. 2013. Inflammatory process induced by carrageenan in adjacent tissue triggers the acute inflammation in deep digital flexor tendon of rats. Anat Rec (Hoboken) 296: 1187–1195.

Wang JHC, Guo Q, Li B. 2012. Tendon biomechanics and mechanobiology—a minireview of basic concepts and recent advancement. J Hand Ther 25:133–141.

Wang JHC, Iosifidis MI, Fu FH. 2006. Biomechanical basis for tendinopathy. Clin Orthop Relat Res 443:320–332.

Weeler MD, Ikejema K, Enomoto N, Stacklewitz F, Seabra V, Zhong Z, Bradford B, Thurman RG. 2009. Glycine: a new anti-inflammatory immunonutrient. Cell Mol Life Sci 56:843–856.

Yamashina S, Ikejima K, Rusyn I, Sato N. 2007. Glycine as a potent anti-angiogenic nutrient for tumor growth. J Gastroenterol Hepatol 22 (Suppl 1):S62–S64.