Hyaluronic acid injections protect patellar tendon from detraining-associated damage

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Summary. Introduction: Having previously demonstrated that detraining affects patellar tendon (PT) proteoglycan content and collagen fiber organization, we undertook the present study with two aims: to improve knowledge on the adaptation of PT and its enthesis to detraining from a histological and histomorphometric point of view, and to investigate the hypothesis that repeated peri-patellar injections of hyaluronic acid (HA) on detrained PT may reduce and limit detrained associated-damage.

Methods: Twenty-four male Sprague-Dawley rats were divided into 3 groups: Untrained (n=6), Trained (n=6) (10 wks-treadmill) and Detrained (n=12). In the detrained rats, the left tendon was untreated while the right tendon received repeated peri-patellar injections of either HA or saline (NaCl). Structure and morphology of PTs (modified Movin score, tear density, collagen type I and III) and enthesis (cell morphology, chondrocyte cluster formation, tidemark integrity, matrix staining and vascularization) were evaluated.

Results: The left PT and enthesis of the Detrained groups showed altered structure and morphology with the highest Movin score values, the highest percentage of collagen III and the lowest of collagen I; the lowest score values were observed in the Trained and Detrained-HA groups. Detrained-NaCl PTs showed the highest collagen III and the lowest collagen I values with respect to Detrained-HA PTs.

Conclusion: This study strengthens previously published data showing the alteration in tendon and enthesis morphology due to discontinuation of training, and provides new data showing that treatment with HA is effective in the maintenance of the structural properties of PT and enthesis in Detrained rats. Such beneficial effects could play a significant role in the management of conservative and rehabilitation strategies in athletes that change type, intensity and duration of training.

Key words: Tendon, Enthesis, Detraining, Hyaluronic acid, Rats

Introduction

Tendons have a critical role in musculoskeletal system by transferring tensile loads from muscle to bone so as to enable joint motion and stabilization (Rees et al., 2009; Tresoldi et al., 2013). Their complex response to loading allows for multi-axis bending - tendons are essential for movement - movement is also essential for tendons - and this adds to the stress concentration in the region where they attach to bone, i.e. the “enthesis” (Benjamin et al., 2002, 2004, 2006). The enthesis is a transition tissue which progressively turns from tendon, to fibrocartilage, to calcified fibrocartilage, and finally to bone. Functionally, the enthesis provides strong and
stable anchorage that promotes musculoskeletal movement with the necessary, concomitant joint integrity (Benjamin et al., 2004, 2006). However, the importance of tendon and enthesis extends beyond this, as they are also the target organ in collection of pathological changes. In fact, a number of studies show that up to 50% of the injuries suffered by athletes who exercise daily involve tendons, tendon sheaths and enetheses (Leppilahti et al., 1991; Rantanen and Orava, 1999; Buchanan and Marsh, 2001; Tallon et al., 2001; Cook et al., 2004; Firth, 2006).

More than 28 million patients in the United States have tendon damage annually (Edelstein et al., 2011). Several conditions such as training, aging, estrogen deficiency and drugs can affect the biological and anatomo-physiological characteristics of the tendon and its enthesis (Nakama et al., 2005; Franchi et al., 2013; Frizziero et al., 2013a,b; Malliaras et al., 2013; Moerch et al., 2013; Torricelli et al., 2013). Additionally, recent preclinical and clinical studies have also examined the effect of detraining on tendon and its enthesis showing alteration in proteoglycan content and collagen fiber organization (Frizziero et al., 2011; Kubo et al., 2012), and an increase in tendon elongation (Kubo et al., 2012). However, restoration of function in tendon and its enthesis following the damage associated with sudden detraining has never been analyzed.

The main goals of any therapeutic regimen for damage to the tendon are to prevent collagen degradation, enhance cell collagen synthesis and finally improve tissue maturation with the aim of improving the rate and quality of healing beyond natural repair (Sharma and Maffulli, 2005). Various pharmacological agents have been used in an attempt to reduce tendon disability. One of the most promising among these agents is hyaluronic acid (HA), which is a high molecular-weight glycosaminoglycan that has a ubiquitous distribution in the extracellular matrix with high concentration in soft connective tissues (Laurent and Fraser, 1992). HA has several physiologic and biologic functions, such as space filling, lubrication, and providing a hydrated matrix through which cells can migrate. The use of HA for the treatment of tendon injuries in animals and humans has been widely investigated showing a beneficial effect that could facilitate structural organization and may result in improved mechanical properties (Muneta et al., 2012; Ozgenel and Etöz, 2012), but has never been considered for the treatment of detraining-associated degenerative changes of the tendon.

The present study takes advantage of our previous experience on the same experimental model (Frizziero, 2011), with the aim of affording two hypotheses: (a) to understand how training, untraining and sudden detraining may affect the PT and its enthesis; and (b) to evaluate how repeated peri-patellar injections of HA (Hyalgan®, Fidia Farmaceutici, Abano Terme, Padua) may reduce and limit damage on detrained PT and its enthesis. Briefly, Sprague-Dawley rats were divided into three groups, Untrained, Trained, Detrained, where the left tendons were untreated, while the right tendons of the Detrained group were treated with repeated peri-patellar injections of HA or with physiologic solution (NaCl). In fact, since clinical evidence suggests that tendon injuries are more frequent in athletes that change type, intensity and duration of training (Andersen et al., 2005) we hypothesized that HA injections might accelerate restoration of the tissue integrity and function; results obtained in the rat model might be translated into viable clinical application for humans. Results may be of interest for both sports medicine practitioners and orthopedic surgeons, wishing to prevent the pathological or degenerative modification that affect these structures.

Materials and methods

Animal model

The study was performed in accordance with the European and Italian Law on animal experimentation and the principles stated in the “NIH Guide for the Care and Use of Laboratory Animals”. The research protocol on animals was approved by the Ethical Committee of Rizzoli Orthopaedic Institute (Protocol: Tendine Rotuleo; Approved: December 15th, 2010).

A total of 24 male Sprague-Dawley rats (Charles River Italia SpA, Lecco) aged 8 weeks, 280±40 g body weight, were placed in standard cages and fed standard diet without limitations (Laboratorio Dottori Piccioni SRL, Gessate, Milano); room temperature was kept at 20.5±0.5°C. After 1 week of quarantine, 18 rats were randomly chosen to run on a treadmill 1 hr a day, three times a week. The speed was gradually increased to reach 25 m/min in 5 weeks, which corresponds to ~ 65-70% VO₂ max, and was then maintained constant for a further 5 weeks (Wisloff et al., 2001). The other six rats underwent no training and they were euthanized under general anesthesia - ketamine 87 mg/kg (Imalgene 1000, Merial Italia S.p.A., Italy) and xylazine 3 mg/kg (Rompun, Bayer S.p.A., Italy) - with i.v. injection of Tanax (Hoechst Roussel Vet GmbH, Wiesbaden, Germany) after 10 weeks (Untrained Group). At the end of the 10-week training, under general anesthesia 6 trained rats were randomly chosen for immediate euthanasia (Trained group), whereas 12 rats were caged without exercise for a further 4 weeks before being euthanized (Detrained group). In 6 of the 12 rats of the Detrained group at the end of the I, II, III and IV week without exercise a peri-patellar infiltration (Hamilton Syringe, Labocest, Bologna, Italy) of 300 μl of HA at a concentration of 20 mg/2 ml (Hyalgan®, Fidia Farmaceutici, Abano Terme, Padua, Italy) was injected in the right PT (Detrained-HA group). The remaining 6 rats, control sham-treated group, at the end of I, II, III and IV week without exercise, at the level of the right PT, received a peri-patellar infiltration of 300 μl of physiological saline solution (Fresenius Kabi, Isola Della Scala, VR, Italy) (Detrained-NaCl group).
After the euthanasia, PTs and entheses were explanted from each animal; all the left PTs and entheses and 4 out of the 6 right PT and entheses were used for the current study, while the remaining 2 right tendons and entheses were used for a correlate in vitro study (data not shown).

Staining procedure

Left and right PTs and entheses were fixed in 10% neutral buffered formalin and decalcified with a 4% HCl, 5% formic acid decalcificant solution for about 7 days. After decalcification, all the samples were embedded in paraffin and cut longitudinally at 5 μm (Microm HM 340E, Microm International GmbH, Heidelberg, Germany). Finally, sections were stained with Hematoxylin-Eosin (HE) (Movin score, tendon tears density and enthesis score) or Picrosirius-Red (collagen I and III), and examined under white light or polarized light microscopy, respectively. All the analyses were performed by three blinded examiners.

Tendon assessments

For each left and right PT, 3 slides were randomly selected and examined with a digital scanner (Aperio Scanscope CS System, Aperio Technologies, Vista, CA - USA) at 20x magnification. These slides were analyzed using the modified semi-quantitative Movin grading scale (Movin et al., 1997; Maffulli et al., 2000a,b, 2004), which assessed various features of tendineus tissue. The variables included in the score were 1) fiber structure, 2) fiber arrangement, 3) rounding of the nuclei, 4) regional variation of cellularity, 5) increased vascularity, 6) collagen stainability, 7) hyalinization. Each variable is scored between 0 and 3, with 0 being normal, 1 slightly abnormal, 2 abnormal and 3 markedly abnormal. Overall, the total score could vary between 0 (normal tendon) and 21 (most abnormal appearance detectable).

Additionally, densitometric quantification of tendon tearing, accomplished with the image analyzer Leica QWin standard (Leica Microsystems Ltd, Switzerland), was carried out by considering all tears of sizes 3-300 μm²; smaller or larger tears were considered artifacts and were not included in the analysis. The following measure for each examined region of interest (ROI) was calculated:

\[
\text{Tear Density (Tears/mm}^2\text{)} = \frac{\text{Tears}}{\text{Tendon Area}}
\]

Picrosirius Red, an anionic composite that distinguishes the thickness and density of collagen fibers through coloration emitted under polarized light, was used to estimate the percentage of reticular fibers within the extracellular matrix. While the thin dissociated fibers typical of type III collagen are greenish, the thickest and strong associated fibers of type I collagen emit colors with bigger length wave such as red and yellow (Zimmermann et al., 2009). Densitometric quantification of reticular fibers, accomplished with the image analyzer Leica QWin standard, was achieved by image binarization using an hexadecimal RGB value (RGB): the green color, corresponding to type III collagen, was defined as [R:0-115, G:55-140 e B:10-50], whereas the red and yellow colors, corresponding to type I collagen, were defined as [R:120-255, G:30-190 e B:0-125]. The following formula was used to quantify the areas:

\[
\sum \frac{\text{RGB}}{\text{Tendon Area}} \times 100
\]

Enthesis assessments

For each left and right enthesis 3 slides were randomly selected and examined with a digital scanner (Aperio Scanscope CS System) at 20x magnification. The assessment of the structure and morphology of the PT enthesis was performed by using a semi-quantitative score. Since there is no validated specific score for the enthesis in scientific literature, an enthesis score was developed by us, based on the five most widely used

Table 1. Histological semi-quantitative score for the assessment of the structure and morphology of the PT enthesis.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patellar enthesis structure</td>
<td>0</td>
<td>Smooth and intact</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight irregularity, &lt; 25%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate irregularity, 25%-50%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe irregularity, 50%-100%</td>
</tr>
<tr>
<td>Cell morphology (O'Driscoll, 1986; Wakitani, 1994; Sellers, 1997; Fortier, 2002; Pineda, 1992) in calcified cartilage</td>
<td>0</td>
<td>Normal cellularity (hypocellularity)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight hypercellularity</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate hypercellularity</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe hypercellularity</td>
</tr>
<tr>
<td>Chondrocyte cluster formation in calcified cartilage</td>
<td>0</td>
<td>No clusters</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&lt;25% of the cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25-50% of the cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50-100% of the cells</td>
</tr>
<tr>
<td>Cell morphology (O'Driscoll, 1986; Wakitani, 1994; Sellers, 1997; Fortier, 2002; Pineda, 1992) in non-calcified cartilage</td>
<td>0</td>
<td>Normal cellularity (longitudinal columns between collagen bundles)</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Slight hypercellularity</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Moderate hypercellularity</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe hypercellularity</td>
</tr>
<tr>
<td>Chondrocyte cluster formation (O'Driscoll, 1986) in non-calcified cartilage</td>
<td>0</td>
<td>No clusters</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>&lt;25% of the cells</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>25-50% of the cells</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>50-100% of the cells</td>
</tr>
<tr>
<td>Tidemark integrity (Sellers, 1997; Fortier, 2002) between calcified and non-cartilage</td>
<td>0</td>
<td>Complete</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>75 to 90% complete</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>50 to 74% complete</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25 to 49% complete</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>&lt;25% complete</td>
</tr>
<tr>
<td>Matrix staining (O'Driscoll, 1986; Wakitani, 1994; Sellers, 1997; Pineda, 1992)</td>
<td>0</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Reduced staining</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Significantly reduced staining</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Faint staining</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>No stain</td>
</tr>
<tr>
<td>Vascularization</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Present</td>
</tr>
</tbody>
</table>
histological scores for the evaluation of cartilage (Pineda, Wakitani, O’Driscoll, Sellers, Fortier score) (Orth et al., 2012). This semi-quantitative score, reported in Table 1, evaluated several parameters such as patellar enthesis structure; cell morphology in calcified cartilage; cell morphology in non-calcified cartilage; chondrocyte cluster formation in calcified cartilage; chondrocyte cluster formation in non-calcified cartilage; tidemark integrity between calcified and non-calcified cartilage; matrix staining; vascularization. Patellar enthesis structure, cell morphology in calcified and non-calcified cartilage, chondrocyte cluster formation in calcified and non-calcified cartilage were scored 0 to 3; tidemark integrity between calcified and non-calcified cartilage and matrix staining were scored 0 to 4, while vascularization was scored 0 to 1. Total score ranges from 0 to 24, where 0 corresponds to healthy enthesis while 24 to severe alteration of the enthesis structure.

Statistical analysis

Statistical evaluation was performed using the software package SPSS v.12.1 (SPSS Inc., Chicago, IL USA). Data were reported as mean ± standard deviation (SD) at a significance level of p<0.05. After checking normal distribution (Shapiro-Wilk test) and homogeneity of variance (Levene test), a one-way ANOVA was performed for comparison between groups. Finally, Sidak post-hoc multiple comparison tests were performed to detect significant differences between groups.

Results

Histology

Tendon

In the left and right PTs of the Untrained group, rare rounded nuclei interspersed between fibers with moderate changes of collagen fiber arrangement were found (Fig. 1a); in Trained rats collagen fibers were arranged closely and parallel to each other with occasional elongated nuclei interspersed between the fibers (Fig. 1b). In untreated PTs (left PTs) of Detrained rats the parallel arrangement of collagen fibers was altered and an increased waviness and separation of fibers were found (Fig. 1c). In addition, seven out of ten examined tendons from Detrained rats showed the presence of adipose and connective tissue strictly connected to the outer surfaces of PTs. Detrained-HA PTs (right PTs) highlighted collagen fibers arranged parallel to each other, similar to that observed in the Trained group but with less evidence of tears (Figure 1d). Finally, regarding Detrained-NaCl PTs (right PTs) an increased waviness and separation of fibers and presence of an initial hyalinization was observed (Figure 1e, f). No evidence of increased angiogenesis could be detected in any groups.

Enthesis

In left and right entheses of the Untrained group, cartilaginous cells at the enthesis level were dispersed in fibrous tissue and the tidemark, i.e. the basophilic line separating the areas of non-calcified and calcified cartilage, was difficult to distinguish (Fig. 2a). On the contrary, in the entheses from the Trained group, the tidemark clearly separates the areas of calcified cartilage from that of non-calcified (Fig. 2f1) and the cartilaginous cells were arranged in longitudinal rows between parallel bundles of collagen fibers (Fig. 2b,f1). In the Detrained groups (left entheses) the entheses showed the highest level of structural and morphological disorganization in both the calcified and the non-calcified cartilage (Fig. 2c). Furthermore, the tidemark was not recognizable in any of the examined histological sections. Regarding the entheses of the Detrained-HA group (right entheses) cells in the non-calcified cartilage showed the typical columnar structure with collagen fibers arranged longitudinally and with a strongly basophilic tidemark (Fig. 2d). Finally, in the Detrained-NaCl enthesis (right entheses) a considerable loss in structure and morphology of the calcified and non-calcified cartilage was observed (Fig. 2e) and the tidemark was not recognizable (Fig. 2f2).

Histomorphometry

Tendon

Histomorphometric results of evaluation of tendon

| Table 2. Histomorphometric results of PT for each experimental group (Mean ± SD). |
|---------------------------------|---------------------------------|
| Untrained Trained Detrained-HA Detrained-NaCl Untrained Trained Detrained-HA Detrained-NaCl |
| Tear Density (Tears/mm²) | 1177±628 | 2232±539* | 1239±847 | 1610±598 | 1087±887 | 2276±308a | 1045±533 | 1363±404 |
| Collagen III (%) | 30.2±8.4 | 14.7±3.1**,*** | 32.2±8.1 | 31.6±4.1 | 36.7±12.0 | 15.2±4.2abc | 13.4±16.8a,b | 31.7±4.1 |
| Collagen I (%) | 14.7±7.3 | 26.3±4.7**,*** | 11.5±3.7 | 12.3±4.4 | 16.3±6.9 | 29.5±4.2ab | 38.1±10.2 | 12.3±4.2ab |

Sidak multiple comparison test: Left: Trained versus Untrained (*, p<0.05; **, p<0.005) and Detrained-HA and Detrained-NaCl (***, p<0.0005; ****, p<0.0005). Right: Trained versus Untrained (a, p<0.05; b, p<0.005), Detrained-HA (a, p<0.05), Detrained-NaCl (c, p<0.05; f, p<0.005); Detrained-HA versus Detrained-NaCl (d, p<0.005) and Untrained (e, p<0.0005).
modified Movin score, Tear Density, Collagen III and Collagen I, are reported in Fig. 3 and in Table 2.

The modified Movin score for the left PTs was significantly lower in the Trained group than in the Untrained and Detrained groups (p<0.0005) (Fig. 2). The highest score value was observed in the Detrained groups, which fared worse than the Untrained one (p<0.005) (Fig. 2). Regarding the right PTs, modified

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**Fig. 1.** Representative histological sections of the longitudinal axis of the central region of the PT of Untrained, Trained, Detrained, Detrained-HA and Detrained-NaCl groups (5 µm thickness, Hematoxylin-Eosin). In Detrained groups, right PT was injected with either HA or NaCl, whereas left PT was not injected at all. 

- **a.** Untrained group - rounded nuclei interspersed between fibers with moderate changes of collagen fibers arrangement.  
- **b.** Trained group - collagen fibers arranged closely and parallel to each other.  
- **c.** Detrained group - the parallel arrangement of collagen fibers was altered with an increased waviness and separation of fibers.  
- **d.** Detrained-HA - Collagen fibers run in parallel arrays, in a manner that is very similar to the orientation of collagen fibers seen in Trained PT.  
- **e.** Detrained-NaCl group - increased waviness and separation of fibers and f) presence of an initial hyalinization were observed. x 20
Fig. 2. Representative histological sections of the longitudinal axis of the left and right articular patellar enthesis of Untrained, Trained, Detrained, Detrained-HA and Detrained-NaCl groups (5 µm thickness, Hematoxillin-Eosin). In Detrained groups, right PT was injected with either HA or NaCl, whereas left PT was not injected at all. a. Untrained group - cartilage cells were dispersed in a highly disorganized fibrous tissue and the tidemark was difficult to recognize. b. Trained group - a typical fibrocartilaginous enthesis with cartilage cells arranged in longitudinal rows between parallel bundles of collagen fiber and f1 with the tidemark that separates the zone of calcified and non-calcified cartilage (white arrows). c. Detrained group - morphological disorganization in both the calcified and the non-calcified cartilage. d. Detrained-HA group - a typical fibrocartilaginous enthesis, cells in non-calcified cartilage show the typical columnar structure with collagen fibers arranged longitudinally and with a basophilic tidemark. e. Detrained-NaCl group - considerable loss in morphology of calcified and non-calcified cartilage seen and f2) tidemark was difficult to recognize. f. Detail of the enthesis tidemark shown in Figure 2b and 2e. x 20
Movin score was significantly lower in Trained and Detrained-HA groups in comparison to the Untrained and Detrained-NaCl groups (Fig. 2).

The statistical analysis of Tear Density of the left PTs showed a significantly higher value in the Trained group in comparison with the Untrained one (p<0.05); in the right PTs, significantly higher value of Tear Density was found in the Trained group with respect to the

![Fig. 3. Stacked histogramms of histological modified Movin semi-quantitative score of the left (Mean ± DS, n=6) and right (Mean ± DS, n=4) PT enthesis of Untrained, Trained, Detrained-HA, Detrained-NaCl groups. In Detrained groups, right PT was injected with either HA or NaCl, whereas left PT was not injected at all. Sidak test: Left: a, Trained versus Untrained, Detrained-HA (n=5), Detrained-NaCl (n=5) (p<0.0005); b, Detrained-HA and Detrained-NaCl versus Untrained (p<0.005). Right: a, Trained versus Untrained (p<0.0005); b, Detrained-HA versus Untrained (p<0.0005); c, Detrained-NaCl versus Untrained (p<0.005), Trained, Detrained-HA (p<0.0005).]

![Fig. 4. Stacked histogramms of histological semi-quantitative score of the of the left (Mean ± DS, n=6) and right (Mean ± DS, n=4) PT enthesis of Untrained, Trained, Detrained-HA, Detrained-NaCl groups. In Detrained groups, right PT was injected with either HA or NaCl, whereas left PT was not injected at all. Sidak test: Left: a, Trained versus Untrained (p<0.0005); b, Detrained-HA (n=5), Detrained-NaCl (n=5) versus Untrained (p<0.005); c, Detrained-HA and Detrained-NaCl versus Trained (p<0.0005). Righ: a, Trained versus Untrained (p<0.0005); b, Detrained-HA versus Detrained-NaCl and Untrained (p<0.0005); c, Detrained-NaCl versus Trained (p<0.0005).]
Untrained and the Detrained-HA groups (p<0.05) (Table 2).

Collagen III showed significantly higher percentages in left PTs of Untrained (p<0.005) and Detrained groups (p<0.0005) in comparison with the Trained one (Table 2). Conversely, Collagen I showed significantly higher percentages in the left PT of the Trained group in comparison with Untrained (p<0.005) and Detrained groups (p<0.005) (Table 2). Finally, in the right PTs, collagen III was significantly lower in Trained and Detrained-HA groups in comparison to Untrained and Detrained-NaCl groups, whereas collagen I showed a significantly higher value in Trained and Detrained-HA groups in comparison to Untrained and Detrained-NaCl groups (Table 2).

Enthesis

The highest score value for the left PT enthesis was observed in the Detrained groups. Scores were significantly higher when compared to both Untrained (p<0.0005) and Trained groups (p<0.005). In the right PT enthesis, the lowest score values were observed for Trained and Detrained-HA group, which showed significantly higher values when compared to Untrained and Detrained-NaCl groups (p<0.0005) (Fig. 4).

Discussion

Following our previous observation that detraining may affect PT properties, involving alteration in proteoglycan content and collagen fiber organization (Frizziero et al., 2011) the first aim of this study was to evaluate how training, untraining and sudden detraining may affect PT and its enthesis from a histological and histomorphometric point of view. Whether repeated peri-patellar injections of HA on Detrained PT and its enthesis might reduce and limit damage of sudden detraining was then tested.

The present results suggest that, in the adopted experimental setting, moderate physical activity alters the PT and enthesis structure and morphology in such a way as to increase their capacity to endure stresses and strains. On the other hand, discontinuing such activity has the opposite effect and alters intra-tendinous tendon and enthesis morphology in the short term. Both the untreated PT and the enthesis of the Detrained groups showed altered structure and morphology in comparison with those of Trained and Untrained rats. These results were confirmed by the fact that the Detrained groups displayed the highest modified Movin score, providing novel evidence that discontinuing intense muscular activity alters tendon structure and morphology much more than the untrained condition. In addition, the untreated (left) PTs of Detrained groups also showed the highest percentage of collagen III and the lowest percentage of collagen I, which means less resistance to stress, and a related increased risk of rupture (Maffulli et al., 2000b).

To our knowledge, no studies investigated the presence of tears smaller than 500 μm² in PT following sudden detraining; tears larger than 1 cm² were observed in tendinosis using high-resolution ultrasound, MR imaging, and 3D-volume-rendered images from multidetector computer tomography as reported by Nakama and coworkers (La et al., 2003; Cvitanic et al., 2004). A background amount of tears occurring normally with normal physical activities (Nakama et al., 2005), together with experimental artifacts occurring during histological processing, were observed in all groups also in the present study. Nevertheless, tear density was higher in the Trained group when compared with the Untrained one.

Finally, we propose here a new tool for enthesis evaluation, similar to the modified Movin score for tendons. This new semi-quantitative score gave the highest value to the Detrained groups. This highlights the high vulnerability of the enthesis, which is the point where mechanical stress concentrates at the hard-to-soft tissue interface.

Repeated peri-patellar injections of HA on Detrained PT and its enthesis were able to preserve morphology and collagen composition, whereas PTs and entheses of the Detrained-NaCl group showed the worst morphology. On the other hand, Detrained-HA PTs displayed structural similarity with Trained group PTs. In fact, modified Movin score revealed the highest score value in the Detrained-NaCl group with major alterations in the arrangement and structure of collagen fibers, which were markedly abnormal when compared with the other groups. The alterations observed in untreated or NaCl-treated PTs and entheses from Detrained rats might result from the fact that sudden interruption of training induces a decrease in tenocyte synthetic activities and a sudden increase of metalloproteinases (MMPs) (Hae Yoon et al., 2003). In fact, following the cessation of physical activity, proteoglycan synthesis is reduced, while MMPs, which had been synthesized during the training phase to help the fiber remodeling processes, persist and contribute to the disruption of bundle organization (Frizziero et al., 2011). Contrary to the Detrained-NaCl group, in the Detrained-HA group the collagen fibers were arranged parallel to each other, similarly to those of the Trained group, suggesting that the peri-tendinous injections of HA might limit the damage associated with detraining. This result underscores the fact that HA injection is an effective conservative procedure during detraining, which might accelerate restoration of tissue integrity and function by preserving the original collagen fibers.

Collagen type I is the main component of tendon collagen fibers, and is the major contributor to the transmission of mechanical strength (Magnusson et al., 2003). The histomorphometrical analysis demonstrated significantly higher amounts of Collagen I in the Trained and Detrained-HA groups in comparison to the other groups; on the other hand, collagen type III had the opposite trend. This suggests a higher tissue and metabolic efficiency in the Trained and Detrained-HA groups. An improved collagen I biosynthesis in
Detained-HA in the absence of tissue inflammation, as in our findings, is indicative of the increased efficiency of tissue adaptation to the new mechanical and functional requests, while the increase of collagen III in the Detained-NaCl group is indicative of a decreased resistance of tendon tensile forces, and may therefore predispose the tendon to spontaneous rupture (Maffulli et al., 2000a,b).

Our histomorphometrical results revealed higher tear density values in the Trained group in comparison to the Untrained and Detained-HA groups. Particularly, the Detained-HA group showed the lowest tear density. Nakama et al proposed a possible explanation for tear formation, stating that it might be pathognomonic of a degenerative pathway involving tear formation and possibly the release of MMPs or cytokines; in turn, MMPs may further degrade the tendon’s matrix, leading to the formation of additional tears (Nakama et al., 2005). However, the only data available in the literature for tear evaluation are related to tendinosis (Nakama et al., 2005; Silva et al., 2011). Our results should therefore be interpreted with caution, since very little is known about tear formation.

Finally, the assessment of the histological characteristics, structure and morphology of PT enthesis showed the most serious damage to occur in the calcified and non-calcified cartilage of Detained-NaCl enthesis, which showed the highest score, along with Untrained enthesis. Thus, the peri-patellar injections of HA during detraining seems to be an effective procedure not only for tendon, but also for keeping the integrity and function of the enthesis. These results are very important because enthesis injuries are a common occurrence, but rarely heal because of the complex structure involved: a gradation that consists of tendon, fibrocartilage, mineral fibrocartilage and bone. This complex attachment creates a particularly difficult challenge to effectively respond to an injury and achieve reconstruction following surgery.

Despite the promising and innovative results obtained in the present research, some limitations of the study should be considered. Firstly, the rat animal model may not be representative of human conditions. However, invasive analyses in animals permit in depth investigation for the advancement of knowledge of many aspects on tendon response to mechanical stimulation and to the development of novel therapeutic targets. Rat and rodents are the most used animals when mechanical load with treadmill running is used (Warden, 2009; Lui et al., 2011). The results of our studies demonstrated that the adopted running protocol did not induce tendinopathy or other pathologic changes in hindlimbs. Secondly, all morphometric parameters were measured by 2D image analysis, while other investigation methods, such as micro-MRI, may allow a more in-depth understanding of PT and enthesis structure. Third, the tissue preparation, involving formalin fixation and paraffin-embedding, required heat treatment and dehydration of the tissue, which could have altered tissue architecture, as suggested by Nakama et al. Finally, the adopted semiquantitative score, which assesses the five most important histological parameters used in cartilage analysis (Orth et al., 2012), was adapted to evaluate enthesis structure (calcified and non-calcified cartilage); such a score should be further validated in other models.

In conclusion, notwithstanding the above-mentioned limitations, the present study confirms previous observations and adds new data demonstrating that discontinuing training activity alters tendon and enthesis morphology in the short term; moreover, it shows that a cycle of peri-patellar infiltrations of HA at the end of the I, II, III and IV week without exercise was completely effective in maintaining the structural and functional properties of tendon and enthesis. Preservation of structure and arrangement of collagen fibers in PT, together with the maintenance of matrix staining, tidemark integrity and cell morphology in non-calcified and calcified cartilage of PT enthesis, were the main positive findings in Detained-HA animals compared to Detained-NaCl ones. Therefore, it could be concluded that treatment with HA was completely effective in the maintenance of the structural and functional properties of detrained PT and its enthesis in rats, thus highlighting its efficacy in many cellular processes such as cell migration, proliferation, differentiation and regulation of matrix organization. These findings may play a significant role in the management of conservative strategies in tendon disorders for the recovery of tendon structural integrity. In particular, new information is here provided, which suggests that after a period of sudden detraining, physical activity should be restarted with caution and appropriate loads. Moreover this study demonstrates that hyaluronan peritendon injection therapy can represent an effective option for treatment of patellar tendinopathy related to sudden detraining.

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