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Original article

Influence of Radial Pressure Wave Therapy (RPWT) on collagenase-induced Achilles tendinopathy treated with Platelet Rich Plasma and Autologous Adipose Derived Stem Cells

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Abstract

Tendinopathy treatment poses a current challenge for sport medicine due to unique physiology and biomechanics of tendons. The goal of this work was to compare the efficacy of the addition of the radial pressure wave therapy (RPWT) treatment to injection of autologous Adipose Derived Stem Cells (ADSCs) or Platelet Rich Plasma (PRP) in the therapeutic procedure for collagenase induced Achilles tendinopathy in sheep. 14 sheep (aged 5 and 6 years, Polish Mountain Sheep breed, weight 60-70 kg) were injected bacterial collagenase type 1A-S (*Clostridium histolyticum,* C-5894, Sigma Aldrich, Poznań, Poland) bilaterally to Achilles tendons. Subsequently, the animals were injected with PRP (7 sheep) or ADSCs (7 sheep) to previously induced tendinopathy foci. Left limbs of all the animals were additionally treated with RPWT focused above the tendinopathy origins. Treatment progress was controlled by ultrasound scans, and tendon samples were taken on the 126th day of the experiment. Tendon samples taken from the sheep treated with RPWT+ADSCs showed lower cellularity and the highest number of thick collage fibers. Samples taken from the sheep treated with RPWT+PRP showed an elevated rate of neovascularization. Addition of the RPWT to ADSCs injections in the treatment of induced Achilles tendinopathy in sheep resulted in good quality of the tissue regeneration. Dual therapy with RPWT+PRP injection can lead to neovascularization in the tendon tissue.

Introduction

The treatment of tendinopathy has been a challenge in medicine, despite such a significant diagnostic and therapeutic progress in the treatment of these diseases. An injection of stem cells of various origins and platelet-rich plasma is now considered to be a procedure which improves the prospects of full physical recovery in humans and animals (Gomes et al. 2015). These treatment methods are often combined with different rehabilitation techniques. One of them is the use of the already known influence of mechanical waves on living organisms as a factor inducing desirable effects in cells and tissues. Despite numerous experiments investigating this type of interaction, the combination of shock or pressure wave action with common

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healing practices of tendon or ligaments using intralesional growth factor injections has not been studied.

Materials and Methods

The experiment was carried out with the consent of the 2nd Local Ethics Committee for Experiments on Animals in Wrocław (resolution No. 3/2015 of 21 January 2015).

Experimental animals

The study used 14 five-year old sheep, with a body weight of 61-72 kg (average 65.4 kg), of the Polish Mountain Sheep breed. The animals were kept in groups of 3-5 in appropriate boxes with unrestricted access to water, mineral licks and hay. All sheep went through a two-week quarantine and a thorough clinical examination combined with an ultrasound examination of the Achilles tendon in order to exclude the presence of changes in the tendon structure prior to the start of the experiment.

The animals were divided into three groups:

Control group 0 - 1 sheep subjected to focal tendinopathy of the Achilles tendon of the left pelvic limb. This sheep was not treated during the course of the experiment.

Experimental group 1 - 7 sheep subjected to focal induction of tendinopathy of both Achilles tendons, treated with the injections of Platelet Rich Plasma (PRP) (both limbs) and radial pressure wave therapy (RPWT) (left limbs). The right limb was a negative control for the use of RPWT.

Experimental group 2 - 7 sheep subjected to focal induction of tendinopathy of both Achilles tendons, treated with the injections of autologous Adipose Derived Stem Cells (ADSCs) (both limbs) and RPWT (left limbs). The right limb was a negative control for the use of RPWT.

Stages of experiment

The sheep were subjected to intramuscular premedication (Butomidor 10mg/ml, Austria at a dose of 0.25 mg/kg body weight (bw) and Midanium 5mg/ml, Poland at a dose of 0.5 mg/kg bw intramuscularly in a 2 ml syringe, with a 22G needle) and an ultrasound examination was carried out of tendon structures of the pelvic limbs (a Mindary M5 ultrasound scanner). Then the sheep had a 17G catheter inserted into the external jugular vein and were introduced into the basic sleep (Scanofol 10 mg/ml, Poland, 1 ml/kg i.v.) and provided with analgesia (Finadyne Solution 50 mg/ml, Holandia 1.1 mg/kg bw – single intravenous administration, metamizole sodium 40mg/kg, once a day, in an intramuscular injection) and antibacterial protection (Shotapen, 10 g+10 g+16.4 million IU, 1 ml/25 kg bw, every 48 hours in an intramuscular injection). In the animals from group 2 the fat tissue was collected from the interscapular area in a volume of approximately 1 cm^3 in order to culture autologous stem cells. Then, all sheep were injected with a sterile collagenase solution (respectively by group to one or both pelvic limbs) of a bacterial origin (*Clostridium histolyticum*) of type 1A - S (C-5894, Sigma Aldrich, Poznań, Poland) in a volume of 0.2 ml, containing 100 IU of the enzyme. The enzyme injection was performed with ultrasound monitoring using a 23G needle placed each time in the central part of the Achilles tendon at a distance of about 2 cm from the calcaneal tuber from the lateral side. During the injection, the limb was held in flexion at the ankle joint with an angle of about 90 degrees. A single node made of non-absorbable suture material (Dafilon, 0) was placed at the puncture site in order to permanently determine the exact location of the defect.

On day 14 of the experiment, all the sheep were subjected to premedication (Sedazin, Polska, 14 mg/kg intramuscularly), control ultrasound scans and an injection of platelet-rich plasma (PRP), prepared on the day of application (group 1) or a solution of ADSCs (group 2) to the previously induced focal point of tendinopathy of both pelvic limbs. Then, the left pelvic limbs of the sheep from both groups underwent RPWT ("Rosetta ESWT" CR Technology, South Korea - 0.15 mJ/mm^2, 8 Hz, 1000 impulses divided into the dorsal, medial and lateral sides of the Achilles tendon area). The RPWT procedure was then repeated twice more at 7 day intervals. A control ultrasound examination was performed on the sheep from groups 1 and 2 once a month from the end of the combination therapy to the end of the experiment (Mindray M5 ultrasound scanner). On day 126 of the experiment, the sheep were premedicated and introduced into a basic sleep (Scanofol, 10 mg/ml, Polska, at a dose of 2-8 mg/kg bw - according to the effect), and then killed by intravenous injection of a lethal dose of a combination of substances registered for animal euthanasia (Morbital preparation, 133.3 mg/ml + 26.7 mg/ml, Polska, at a dose of 100 mg/kg bw), administered intravenously to the cephalic vein of a forearm (*v. Cephalica antebrachii*). Samples were taken for macroscopic, histopathological and immunohistochemical tests.

PRP preparation

To prepare the platelet-rich plasma solution from the sheep of group 2, blood from the external jugular vein was collected into four 5 ml tubes containing 0.5 ml of sodium citrate. The samples were centrifuged

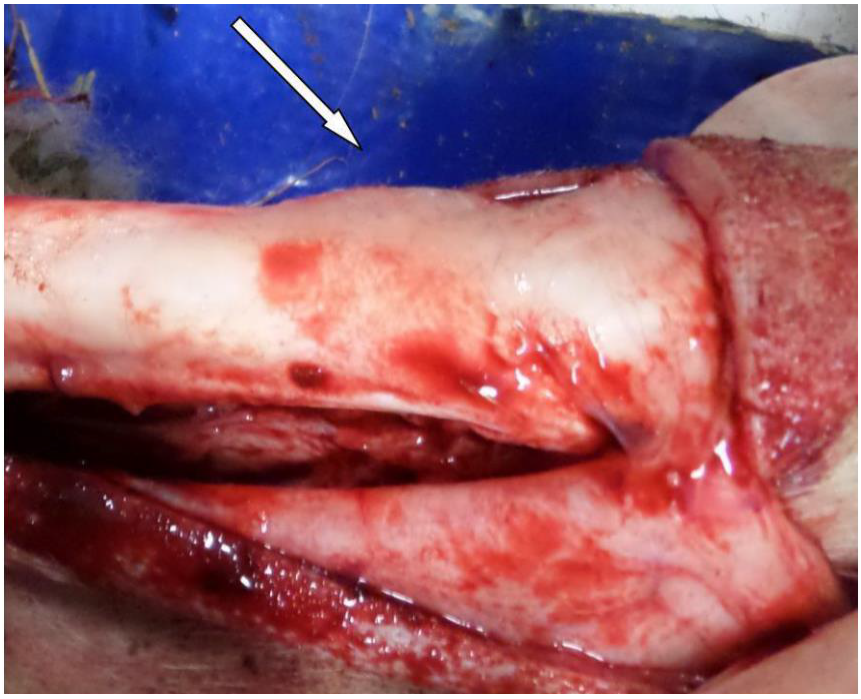


Fig. 1. Macroscopic view of Achilles tendon after the end of the experiment. The white arrow points collagenase injection site.

at the rate of 300 x g for 20 minutes. The supernatant was then collected into dry 2ml tubes and centrifuged for 10 minutes at 800 x g. 2/3 of the upper part of the obtained plasma, i.e. the platelet-poor plasma, was collected. 1/3 of the lower part of the plasma (i.e. the platelet-rich plasma) was collected in sterile 2 ml syringes. A suspension containing 898 +/- 100 x 10^3 plates/µl in the volume of 0.4 ml was obtained.

Preparation of stem cell suspension

The adipose tissue in a volume of about 1 cm^3 was surgically collected from the interscapular area of the sheep from group 2 and suspended in a sterile Hank's Balanced Salt Solution (HBSS). ADSCs were isolated in aseptic conditions according to the protocol of Marycz (Nicpoń et al. 2013). The adipose tissue samples were washed in the HBSS, enriched with a 1% antibiotic and antimycotic solution (Penicillin/Streptomycin/Amphotericin B), and cut with surgical scissors into smaller parts. The extracellular matrix was subjected to etching with type 1 collagenase (1 mg/ml) for 40 minutes at 37°C and in the presence of 5% CO_2. The homogenate was then centrifuged at the rate of 1200 x g for 10 minutes at room temperature (IEC CL31R, ThermoScientific). The supernatant was removed, and the obtained cell pellets were resuspended in the medium. This solution was transferred to a cell culture bottle. The culture was aseptically carried out in an incubator with constant conditions (37°C, 5% CO_2 and humidity of 95%). The first cultures were placed in a t-25 bottle and cultured on Dulbecco's Modified Eagle's Medium (DMEM) with Ham's F-12 nutrient solution, 10% fetal bovine serum (FBS) and 1% PSA solution. The DMEM, containing 4500 mg/L of glucose with the addition of 10% FBS and 1% PSA, was also used in the secondary culture. The media were changed every other day, and the cells were passaged with a trypsin solution (TrypLE Express, Life Technologies, Poland) after reaching 80% confluence. The cells were passaged three times.

Microscopic and macroscopic examinations

On day 126 of the experiment, after the euthanasia of the animals, samples were taken for macroscopic, histopathological and immunohistochemical examinations. The macroscopic examination consisted in performing the autopsy of Achilles tendons of all the sheep. The following items were evaluated: general appearance of tendons, their thickness, color and the presence of inflammatory features of the subcutaneous tissue around the intratendinous injection sites, as well as the homogeneity of tendon-building structures in the cross-section. Photographic documentation was produced during the autopsy (Figs. 1, 2).

Microscopic examination

Histopathological examination (hematoxylin-eosin (HE) and Sirius Red staining)

For the microscopic tests, Achilles tendon samples of 1 cm^3 were taken from the area under examination and fixed in a 4% formalin solution. The samples

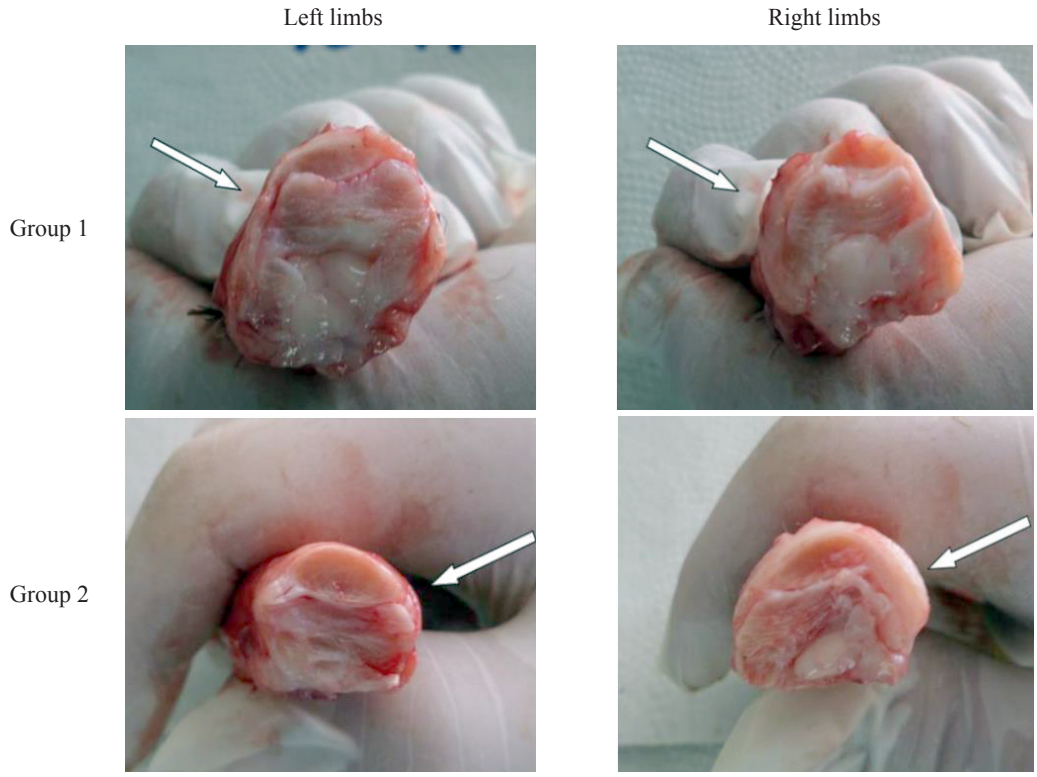


Fig. 2. Macroscopic view of transverse cut through the Achilles tendon after the end of the experiment. The white arrow points collagenase injection site.

were stained with HE according to the Feldman and Wolfe protocol (2014) and with Sirius Red according to the protocol described by Puchtler et al. (1973), followed by Junqueira et al. (1979). The Sirius Red staining made it possible to color the collagen fiber network in tissue (Lattouf et al. 2014). Microphotographs of the examined sections were subjected to a computer-aided analysis of the image using a computer equipped with the cell^A software (Olympus Soft Imaging Solution GmbH, Germany) coupled with an Olympus BX53 optical microscope equipped with a digital Color View IIIu camera (Olympus, Japan).

Immunohistochemical examination (Anti-col 1)

The immunohistochemical examination was performed according to the procedure of the manufacturer of the Anti-Collagen I antibody [COL-1] (ab6308) kit (made by Abcam company).

Statistical analysis

The statistical analysis was carried out using the Statistica 12 PL program (StatSoft, Krakow). Data normality was checked using the Shapiro-Wilk test. The differences among groups were assessed using the ANOVA, Kruskal-Wallis and Mann-Whitney tests. The statistical significance was assumed for $p<0.05$.

Results

Macroscopic examination

The skin around the site of intratendinous injections and procedures using the radial pressure wave was evenly covered with hair without visible scars and inflammatory features. After removing the skin, no inflammatory features of the surrounding soft tissues were observed in all the examined sheep, only a small post-inflammatory infiltration at the site of the collagenase solution injection caused by regeneration after the lysis of collagen fibers of the epitenone, a small area of the adjacent extracellular matrix and subcutaneous tissue (Fig. 1). This effect was clinically and scientifically insignificant. It was a complication after an iatrogenic induction of tendinopathy. The tendons had a light cream and pink color and a homogeneous surface, with no visible protuberances. The thickness of the tendons subjected to injections was slightly greater than the thickness of the healthy tendon. The tendon structure in the cross-section in the preparations collected from the sheep of group 2 resembled the structure of a healthy Achilles tendon. Small reactions at the site of the collagenase solution injection did not affect the regeneration of the tendon tissue (Fig. 2).

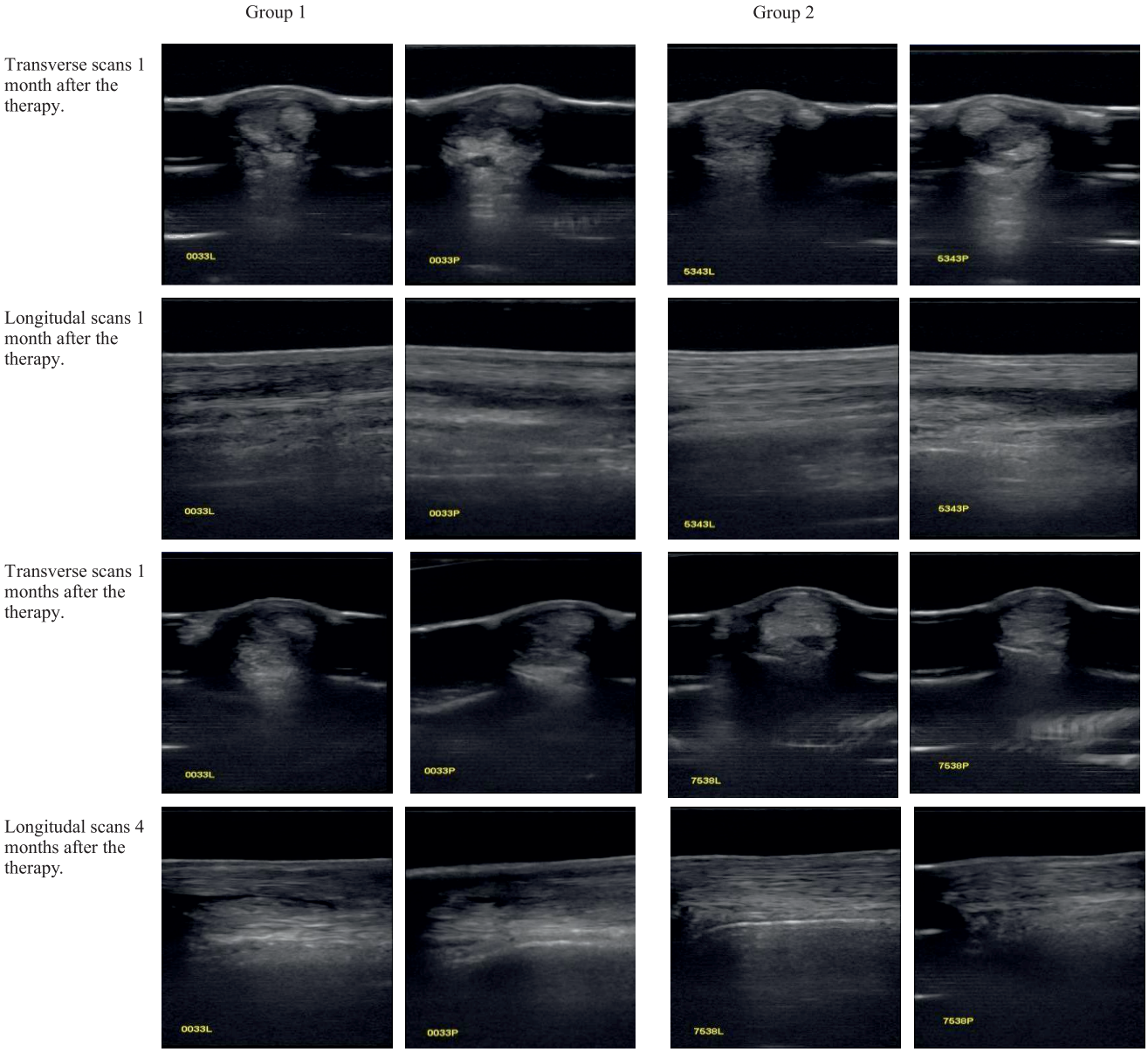


Fig. 3. Achilles tendon USG scans after the first and fourth month from the end of the therapy.

Ultrasound examination

Ultrasonographically, tendons of the sheep from group 1 in the cross-section and longitudinal section showed few hypoechogenic areas with obliteration of a normal tissue structure and hyperechogenic areas (indicative of tendon scars creation). In group 2, the tendons in the ultrasound image resembled a normal tendon tissue with a regular echogenicity and a slight enlargement of the cross-sectional area in transverse scans. In some sheep, hypoechogenic and hyperechogenic areas were also observed (Fig. 3).

Microscopic examination

The evaluation of the results of histopathological and immunohistochemical examinations was made on the basis of the modified Nixon's protocol (Table 1), which had been developed to assess the effectiveness of ADSCs application in the therapy of collagenase-induced tendinopathy (Nixon 2008). The examined tendons in the sheep from both groups obtained a total mean score of 3.73 for the left limbs and 3.57 for the right limbs (group 2) and 3.45 for the left limbs and 3.43 for the right limbs (group 1). The tendons of the sheep from group 2, subjected to a combination treatment, i.e. injection of ADSCs, followed by the RPWT, were similar to healthy tendons in the histological image. Fibroblasts were shaped like mature tenocytes; they were elongated and mostly distributed parallel to the collagen fibers. The number of cells was the smallest among all studied groups of sheep. This group showed slightly more new blood vessels compared

Table 1. Nixon's protocole – author's modification.

Parametr	Characteristics
Cell shape	1 – oval
	4 - elongated
Fibroblasts count	>75% (1)
	50-75% (2)
	25-50% (3)
	<25% (4)
Neovascularization	Severe (1)
	Moderate (2)
	Mild (3)
	Minimal (4)
Inflammatory cells infiltration	Severe (1)
	Moderate (2)
	Mild (3)
	Minimal (4)
Thick collagen fibers	<25% (1)
	25-50% (2)
	50-75% (3)
	>75% (4)
Parallel arrangement of collagen fibers	<25% (1)
	25-50% (2)
	50-75% (3)
	>75% (4)
Collagen type I expression	>75% (1)
	50-75% (2)
	25-50% (3)
	<25% (4)

to right limb tendons (treatment only with ADSCs), which is consistent with the expected direction of tissue reparation after the application of shock waves. In this group of samples, the tendons also had the largest amount of type I thick collagen fibers (at a low expression of type I collagen), arranged in a more parallel manner (H&E and Sirius Red staining). In the tendon samples taken from the right limbs of group 2 sheep, i.e. the limbs subjected only to ADSCs injections, similar results were obtained with a slightly smaller network of new blood vessels (Table 2).

Statistical analysis

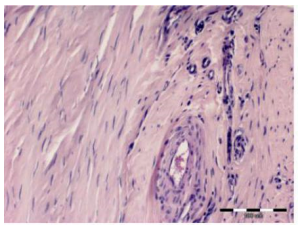
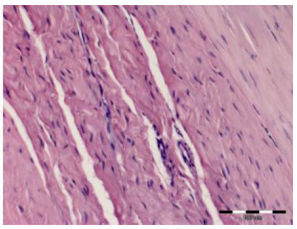
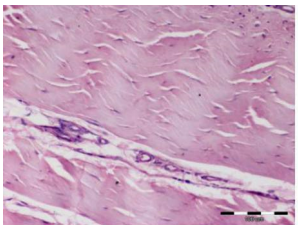
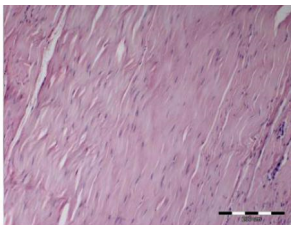
Based on the analysis of all the examined groups (PRP, PRP+RPWT, ADSCs, ADSCs+RPWT), no significant differences were found in the total points for all the test groups (Kruskal-Wallis ANOVA analysis, $p > 0.05$) (Fig. 4).

The comparison of results obtained in group 1 did not show significant differences in the total points in the PRP group as compared to the PRP+RPWT group (Mann-Whitney test, $p > 0.05$) (Fig. 4). The comparison of results obtained in group 2 did not show significant differences in the total points in the ADSC group compared to the ADSCs+RPWT group (Mann-Whitney test, $p > 0.05$) (Fig. 4). There were no statistically significant differences in the total points between the PRP+RPWT group and the ADSCs+RPWT group (Mann-Whitney test, $p > 0.05$) (Fig. 4).

Discussion

Tissue engineering methods are currently focused, among others, on an early stimulation of type I collagen production, in exchange for type III collagen, which leads to the acceleration of regeneration and reduces the risk of tendon scar formation (Maffulli 2002). During the regeneration of the tendon, the collagen type I expression decreases to low values observed in healthy tendons (Martinello et al. 2013). In this research, the low expression of type I collagen mole-

Table 2. Microphotographs of Achilles tendons samples from group I and II. Line A – hematoxyline and eosin staining, line B – Siriur Red staining, line C – immunohistochemical staining.

Group 1			Group 2	
Left limbs	Right limbs		Left limbs	Right limbs
		line A		
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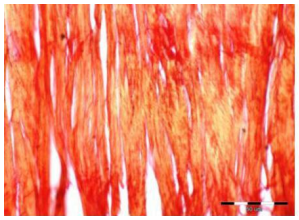
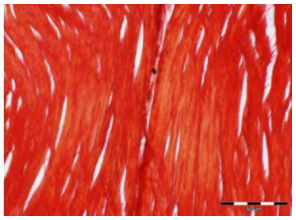
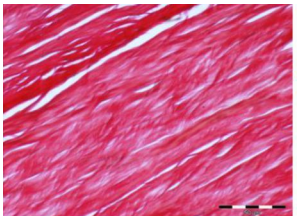
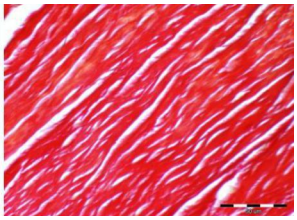
		line B		
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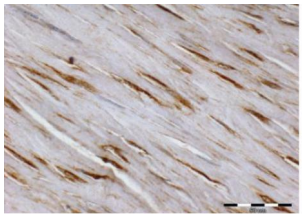
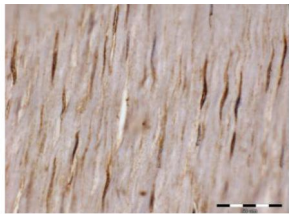
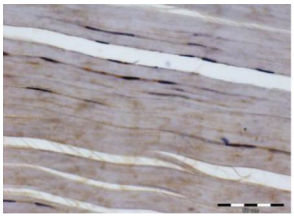
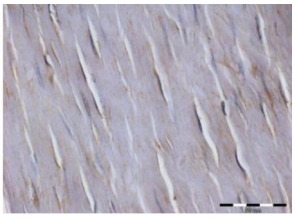
		line C		
1C	2C		3C	4C

cules in all the tested tendon samples can be considered as a positive effect of the selected forms of the treatment (Table 2). At the same time, it should be mentioned that the low expression of collagen type I occurs not only in the healthy tendon, but also in the tendon with tendinopathy (Maffulli and Ewen 2000).

The results in the form of optimization of the tendon healing process or treatment of tendinopathy by injections of autologous adipose derived stem cells were already obtained in research and described in the scientific literature (Gimble et al. 2007, Ruzzini et al. 2012). The application of these types of cells has also gained popularity in recent years in comparison with bone marrow derived cells. This is due to an easier access to the site of material collection, a greater possibility of collecting a convenient quantity, a lower risk of damage to vital tissues and an easier performance of the collection procedure. In addition, the cells are characterized by the same ability to differentiate into mesenchymal lines as is attributed to the bone marrow cells (Gimble et al. 2007). In a study based on a rabbit model of the tendon defect, even a higher rate of proliferation of ADSCs from tenocytes was demonstrated, with similar values of collagen type I production (Kryger et al. 2007). ADSCs placed in a hyaluronic acid based mixture (Hialonect) and subjected to mechanical stress were able to produce a vascularized tendon-like tissue (Vindigni et al. 2013). The rabbit Achilles tendons, in which a mixture of ADSCs and rich platelet plasma were introduced into the lesions, showed an increase in strength and collagen type I content, compared to those injected only with platelet rich plasma (Uysal et al. 2012). Similar results were obtained by Martinello et al. (2013) in a study on the healing of induced tendinopathy of the Achilles tendon in sheep. The experiment involved 18 sheep, in which collagenase type 1A-induced tendinopathy in both Achilles tendons was treated using autologous platelet-rich plasma (groups 1 and 4), plasma mixtures with autologous stem cells derived from the peripheral blood (groups 2 and 5 stem cells (groups 3 and 6). Sheep from groups 1-3 were euthanized 30 days after the start of the treatment, while the sheep from groups 4-6 on day 120. In groups 1-3, the analysis of cellular parameters and the degree of the ECM ordering did not show significant differences. The tendons in each group were characterized by an image typical of the active tissue regeneration process. Interestingly, however, there was an increase in vascularization in the group treated with the PRP compared to the other groups, including placebo. Neovascularization was visible in groups 4 and 5 even 120 days after the initiation of the treatment,

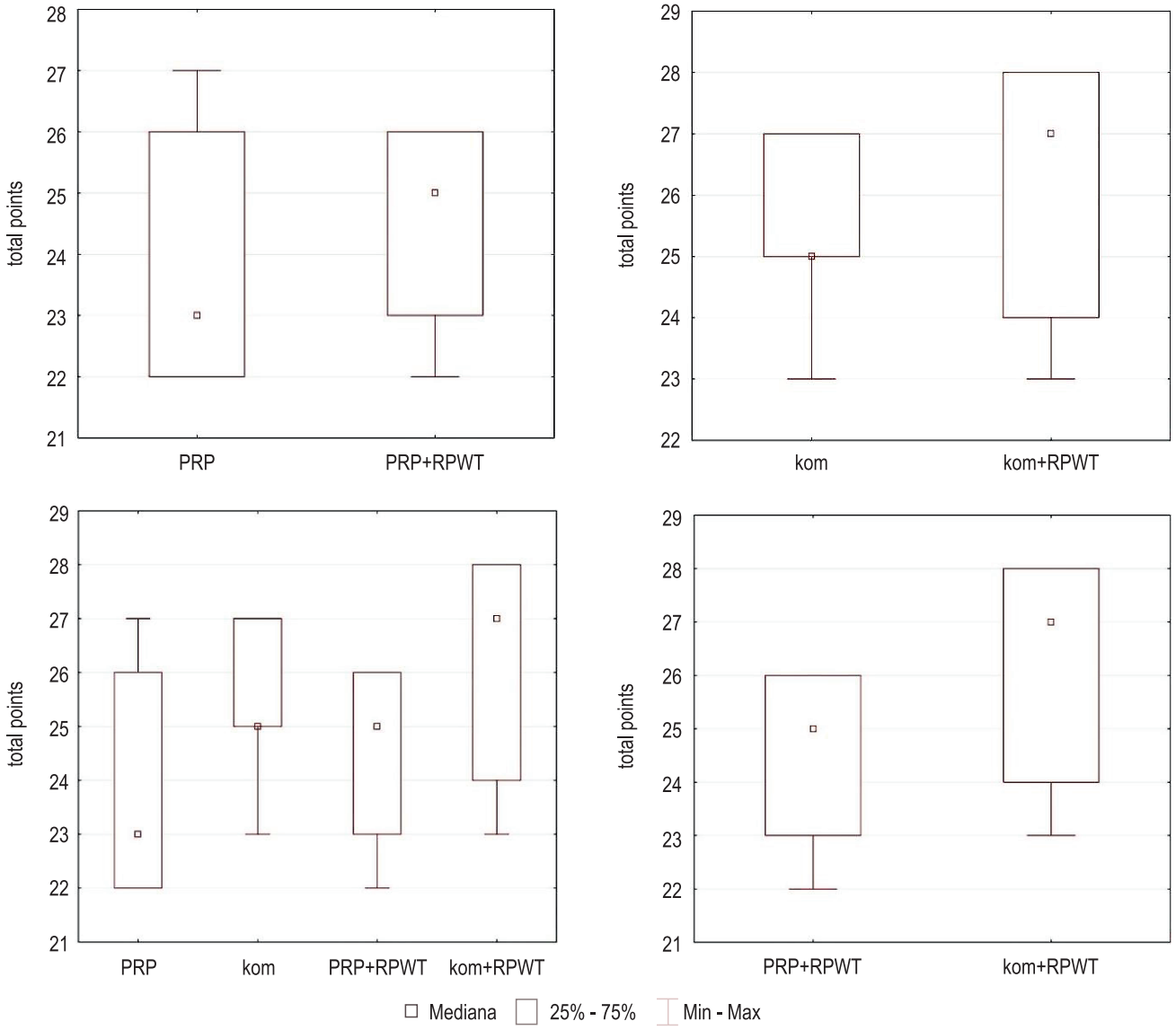


Fig. 4. Results of statistical analysis.
Graph showing the sum of points achieved by left and right limbs of animals in group I (PRP or PRP/RPWT treatment) according to modified Nixon's scale.

but its level decreased compared to that observed on day 30. According to the authors, the large amounts of growth factors contained in the platelet rich plasma contributed to the activation of neovascularization and recruitment of cells in groups 1 and 2 in comparison with group 3. Also in the present study, a similar phenomenon was observed (group 1), although the effect of radial pressure wave itself is conducive to the formation of new blood vessels, which was observed while analyzing the results of the histological examination of the right limbs tendons of all the sheep. In scientific works investigating the impact of using different types of shock wave at low energies, tissue effects in the form of a stimulation of neovascularization and an induction of low-intensity inflammation associated with an increased blood flow in tendon sites affected by chronic tendinopathy have already been shown. This effect, along with the reduction of pain through a change in the permeability of cell membranes of neurons, was assessed as a cause of relieving pain symptoms associated with tendinopathy (Furia 2006, Al-Abbad and Simon 2013). The clinical value of this effect is, however, a questionable issue. Poor tendon vascularization is considered to be a limiting factor for their reparative potential; however, hypervascularization is seen as a pathology characteristic of chronic tendinopathy (Beck et al. 2011). The authors of the work cited (Martinello et al. 2013) also observed a significantly lower cellular density in group 6 (tendons treated with an injection of stem cells) in relation to groups 4 and 5 (tendons treated with PRP injections and a mixture of PRP with stem cells).

The histopathological analysis, also based on the assessment of the cell shape, the levels of neovascularization and the ECM ordering, confirmed the positive effect of stem cells on tendon healing. In the present study, similar beneficial effects were observed in group 1. However, no strong synergy was demonstrated between RPWT and the injection of ADSCs in the therapy of induced tendinopathy of the Achilles tendon. In the work of Martinello et al. (2013), it was additionally observed that exclusively in group 4 (tendinopathy treated with PRP injections), an elevated level of expression of ECM elements continued, including collagen type I, indicating the active regeneration phase process. Similar results were observed in the present study, where the expression of collagen type I was slightly higher in group 1, compared to the low values observed in group 2.

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