Systemic inflammatory response to the Radial Pressure Wave Therapy (RPWT) in collagenase-induced Achilles tendinopathy treated with Adipose Derived Stem Cells or Platelet Rich Plasma

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Abstract

Novel tendinopathy treatment protocols should be assessed for safety. The goal of this work was to compare differences in selected systemic inflammatory marker concentrations after two treatment protocols for collagenase induced Achilles tendinopathy in sheep. 14 sheep (aged 5 and 6 years, Polish Mountain Sheep breed, weight 60-70kg) were injected with bacterial collagenase type 1A-S (Clostridium histolyticum, C-5894, Sigma Aldrich, Poznań, Poland) bilaterally to Achilles tendons. Subsequently, the animals were injected with Platelet Rich Plasma (7 sheep) or Adipose Derived Stem Cells (7 sheep) to induced tendinopathy foci. Left limbs of all sheep were additionally treated with Radial Pressure Wave Therapy (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. Serum Amyloid A (SAA) concentration showed mild elevation before the experiment (2 sheep from group I, 4 sheep from group II) and two days after the intratendinous growth factors injection (4 sheep from group I, 4 sheep from group II) combined with RPWT (mean 22,63 mg/L and 53, 6 mg/L respectively). Haptoglobine (Hp) concentration increased from 0 to 0,01 g/L in 2 animals from group I two days after injection. These values declined to 0 during the course of the treatment. Fibrinogen (Fb) concentrations were within reference levels throughout the research, although mild elevation was observed before the treatment course in 6 sheep from group I and 1 sheep from group II. In conclusion, addition of RPWT to growth factors injections in the treatment of yatrogenic Achilles tendinopathy in sheep did not induce systemic inflammatory response.

Key words: Radial Pressure Wave Therapy, collagenase, Achilles, tendinopathy, Platelet Rich Plasma, Adipose Derived Stem Cells

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Introduction

Overload tendon injuries accounted for 30-50% of all injuries recorded among athletes in the twentieth century (Lui et al. 2011). However, in veterinary medicine, e.g. in racehorses, tendon injuries account for about 50% of diagnoses in the group of orthopedic diseases (Ely et al. 2009). The unique structure and biomechanics as well as the physiology of the tendon tissue contribute to the occurrence of difficulties in reparative processes. They result in frequent adhesions within the tendon sheath, reduced tendon strength to mechanical loads, reduced elasticity of the resulting scar and predisposition to further injuries in the future (Lui et al. 2011). Recent years have brought a development of techniques for the treatment of diseases from the borderline of medicine and rehabilitation. One of them is the Shock Wave Therapy. The operation of wave generators is based on the production of acoustic waves that propagate in the tissues, applying the laws of physics characteristic of mechanical waves. At the border of tissues with different acoustic resistance, some waves are reflected, and some pass further, resulting in the creation of kinetic energy (Kearney et al. 2010). The expression “shock wave” refers to the generation of high values of positive pressure in a short time, after which the tissue is subjected to negative pressure, which in turn leads to tension and sometimes also the formation of cavities filled with gas, or the phenomenon of cavitation. The kinetic energy of shear forces generated at the border of tissues with different acoustic resistance and the phenomenon of cavitation are responsible for the biological effects of shock wave therapy on tissues (Cleveland et al. 2007).

The clinical application refers to waves of varying power, expressed as Energy Flux Density (EFD), given in mJ/mm², i.e. an energy unit operating on the surface of 1 mm² during each impact (Smith et al. 2006). On this basis, shock waves are divided into those with low (<0.08 mJ/mm²), medium (0.08-0.27 mJ/mm²) and high power (0.28-0.60 mJ/mm²) (Albert et al. 2007). Some authors, however, use a simplified classification, based on a limit value of 0.28 mJ/mm², defining the waves with high or low energy (Shaheen 2011). Due to the way the waves propagate in tissues, they can be divided into focused, soft-focused and radial ones (Girolamo et al. 2014). Traditionally, focused waves were produced electrohydraulically, now also electromagnetically and piezoelectrically, while radial waves - pneumatically (Cleveland et al. 2007). The focal and radial shock waves differ in many aspects. Apart from the ability to concentrate energy in the optimal place inside the body, focused waves, whose history derives from their use in breaking up kidney stones, can generate a large positive pressure - from 30 up to about 110 MPa - in a short time (20-45 ns) at the border of tissues with different acoustic resistance (Cleveland et al. 2007). They penetrate tissues depending on selected parameters to a depth of approximately 12 cm. High and quick peaks of positive pressure were the basis for the term “shock waves”, which was then expanded also for radial wave therapy. The latter, however, produce much lower positive pressure - about 8 MPa (Cleveland et al. 2007) in a much longer time - about 600 ns (Chitnis and Cleveland 2006). The highest values of positive pressure are obtained to a depth of about 2-4 cm into the tissue, and the energy stream density decreases to the squared distance from the wave generation point, i.e. the point of contact of the applicator with the skin surface, which results from the physical properties of acoustic waves (Cleveland et al. 2007). The radial wave is determined by: energy density (mJ/mm²) or pressure (Bar, Torr, or MPa), the number of pulses and frequency (Hz) (Foldager et al. 2012).

Both types of waves also induce negative pressure of values from – 5 MPa to about -15 MPa for a focused wave (depending on the parameter settings of the equipment) and up to approximately -6 MPa for a radial wave. The negative pressure phase lasts about 5 microseconds (focused wave) and about 20 microseconds (radial wave). Considering the facts mentioned above, shock wave characteristics, such as an increase of a high positive pressure in a short time, affecting the tissue and non-linearity of propagation, are typical of only a focused wave type (Yamaya et al. 2014). Therefore, Worp et al. (2013) have suggested that the more appropriate name for a radial wave is Radial Pressure Wave (RPW). In literature, however, both terms, RPW and RSW, i.e. Radial (Unfocused) Shockwave, function equally well.

The last significant difference between the focused and radial waves is the character of applicators that produce them. For focused waves they are larger, filled with liquid and with a concave concentrating surface inside, while the heads of radial wave generators are much smaller and cylindrical. The liquid (water) environment inside the focused wave applicator allows minimizing energy loss due to reflection - water has a similar acoustic resistance to live tissues (Yamaya et al. 2014).

The work from year 2013 (Raabe et al.) on the impact of shock wave on the activation and proliferation of equine adipose derived stem cells in vitro showed a positive correlation between the studied phenomena. In another experiment, osteoblasts enriched with Platelet Rich Plasma (PRP) treated with Extracorporeal Shockwave Therapy ESWT (0.17 mJ/mm, 500 pulses)
after 48 hours of culture showed an increase in the expression of insulin-like growth factor binding protein (IGFBP-3) and transcription factor 2 (RUNX2) as well as after 72 hours, exhibited an increase in the synthesis of type I collagen, osteocalcin, IGF-1 and IGFBP-3 (Notarnicola et al. 2011). ESWT also increases the proliferative and migration activity of human tenocytes in vitro (Leone et al. 2012). So far, no attempts have been made to conduct research in vivo on these phenomena or on the effect of the shock wave on the PRP administered earlier to the focal point of tendinopathy. In addition, the aforementioned works are based on ESWT and do not focus on safety of such treatment.

The present study investigated general immunological response to experimental tendinopathy therapeutic protocol which comprises a combination of Radial Pressure Wave Therapy and intrasosional injections.

**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:

Experimental group 1 - 7 sheep subjected to focal induction of tendinopathy of both Achilles tendons, treated with the injections of PRP (both limbs) and 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.

The RPWT protocol for left pelvic limbs of the sheep consisted of - 0.15 mJ/mm², 8 Hz, 1000 impulses divided into the dorsal, medial and lateral sides of the Achilles tendon area. Device used was the “Rosetta ESWT” (CR Technology, South Korea). 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).

**Blood tests**

Control blood tests in all the animals were done before the experiment (blank tests), 2 days after each RPWT procedure and 7 days after the end of therapy. The concentrations of selected acute phase proteins (APPs) were determined: SAA, Hp and Fb. Blood was collected by puncturing the external jugular vein with a 23G needle, after prior shaving and disinfection of the puncture site (SkinseptPur, Ecolab, Germany).

**Determination of serum amyloid A concentration**

The measurement was made possible due to a commercial ELISA Kit (TP-802, Tridelta Development Ltd.). The determination was made according to the manufacturer’s protocol using the sera of the tested sheep. The determination of SAA concentration was performed according to the manufacturer’s scheme (Tridelta Development Ltd.):

1. Dilution of a sample diluent and calibrator with distilled water at a ratio of 1:10.
2. Dilution of serum samples in the diluent at a ratio of 1:500.
3. Placing 50 μl of anti-SAA conjugate in each well of a 96-well plate for ELISA determinations.
4. Placing 50 μl of the test sample and a standard solution in the wells of a 96-well plate (to determine the standard curve for the correct reading of extinction by the ELISA measuring apparatus). Preparation of the standard solution according to the manufacturer’s instructions.
5. Incubation of the covered plate for 1 hour at 37°C.
6. Rinsing the wells four times with a washing buffer solution previously diluted according to the manufacturer’s instructions.
7. Application of 100 μl of the TMB substrate to each well of the plate.
8. Incubation in a dark room at room temperature for 15 minutes.
9. Application of 100 μl of the STOP solution to each well.
10. Immediate reading of results using a BIOTEK ELISA test reader at 450 nm and 630 nm of wave lengths. The samples were tested according to the SAA standard curve.
11. Conversion of the received extinction to the SAA concentration in g/ml.
Determination of haptoglobin concentration

Haptoglobin (Hp) was determined by a guaiacol method according to Jones and Mould (1984). This method is based on the measurement of peroxidase activity of Hp complexes with methemoglobin, where the amount of decomposed hydrogen peroxide is directly proportional to the amount of Hp. The colorless guaiacol oxidizes to yellow tetraguaiacol under the influence of decomposing hydrogen peroxide.

The determination was performed on Medlab flat-bottom 96-well plates. 10 μl of the tested sera was inserted in each well and supplemented up to 50 μl with a phosphate buffered saline (PBS) solution (pH 7.3). At the same time, (reagent and serum) blank tests were performed and 50 μl of human haptoglobin standard solution (Hp 2-2, Sigma Aldrich) in the 20-200 μg/ml range was applied to each well. Then 50 μl of 0.05% equine methemoglobin diluted in distilled water was added to each well. The mixture obtained in this way was
incubated for 10 minutes at room temperature. After incubation, 150 μl of guaiacol reagent (0.08 M guaiacol in 0.2 M acetic acid/sodium hydroxide pH 4.0 buffer) and 50 μl of 0.02 M hydrogen peroxide were added to each well. The absorbance was read after 5 minutes at a wavelength of 492 nm (QuantBioTek). The reagent blank test included all the reagents except the tested samples (containing Hp). The serum blank test on the other hand comprised a mixture of all components with the exception of methemoglobin. The Hp concentration was read from the standard curve in mg% (5, 10, 15 and 20 mg %) and converted into g/L, by multiplying by dilution.

**Determination of fibrinogen concentration**

Fibrinogen was determined by the method of Millar et al. (1971).

A hematocrit capillary was filled with the tested whole blood and centrifuged in the hematocrit centrifuge (MPW 2012H) for 3 minutes at a rate of 1300 rpm. Then, the capillary was placed in a water bath at a temperature of 56°C for 3 consecutive minutes to precipitate the fibrinogen. After removing and drying the capillary, it was centrifuged for 3 minutes in the same centrifuge, which allowed the fibrinogen to be deposited above the surface of the blood cells. The result (in %) was read using a reader.

**Results**

In the experiment, an elevated SAA concentration level was observed in 6 sheep in the blank test and a slightly elevated Fb level in 9 sheep compared to the determinations performed after the end of the experiment. On the second day after the intratendinous injections, an increase in SAA was observed in about half of the animals (4 sheep from group 2 and 3 sheep from group 1). In subsequent determinations, no increase in selected acute phase proteins was found (Table 1, 2).

**Discussion**

The shock wave is repeatedly used for *in vivo* and *in vitro* tests to obtain data to assess its impact on tissues and living organisms. There are many reports about its impact on tissues with difficult reparative capabilities. In rat studies, the positive effect of the focused form of the wave on neuronal regeneration has been demonstrated (Lee et al. 2015), including regeneration of spinal cord lesions with the improvement in motor functions and stimulation of vascular endothelial growth factor (VEGF) along with its Ft-1 receptor (Yamaya et al. 2014). Lee et al. (2014) came to other interesting conclusions in their work on the use of shock-wave to change the target microenvironment for transplanted stem cells. In the research involving a group of 36 rats in which a chronic spinal cord injury was induced, some of them received a shock wave therapy immediately prior to an intravenous mesenchymal stem cell injection. This treatment, compared to the other rats treated with only the injection of cells or only shock waves, brought more beneficial therapeutic effects in the form of a larger number of settled stem cells at the site of injury, without any observed side effects. The *in vitro* application of an acoustic wave impact directly to human and rat adipose derived stem cells showed that, after the therapy, they not only retain their multipotent character, but also limit the tendency to apoptosis and increase proliferative, migration and differentiating abilities, especially towards osteogenic and adipogenic lineages and cells morphologically similar to Schwann cells (Suhr et al. 2013, Schuh et al. 2014). In another study, adipose derived stem cells of horses were subjected to the focused shockwave therapy, in which their enhanced activity was observed due to the increased phosphorylation, and the enhanced proliferation, without features of increased targeting to a specific type or cell line (Raabe et al. 2013). Zhang et al. (2014) compared the effects of the therapy connecting shock waves and the endothelial progenitor cell implant with each of these therapies separately for revascularization of the ischemic area of the skin in rats. They showed that the first procedure had the best clinical effect. A recently introduced new wave type, soft-focused, proved particularly useful in studies on isolated cells thanks to the possibility of focusing waves on a larger surface (Girolamo et al. 2014). In this way human tendon cells were examined, gaining a positive modulation of their viability, proliferative abilities, expression of tendon-specific markers and the release of anti-inflammatory cytokines (Girolamo et al. 2014). The aforementioned experiments offer real perspectives for the future uses of the shockwave therapy in regenerative medicine. Therefore, based on current achievements described in the literature, the present work has been intended to create an experimental model of *in vivo* conditioning of both stem cells and platelet rich plasma. This is a solution that goes beyond the current scientific reports which have used shock wave therapy to condition the tissue prior to the injection of growth factors or to condition stem cells *in vitro*.

**Collagenase injection**

An intratendinous injection of bacterial collagenase produced by the *Clostridium histolyticum* strain is a popular experimental method of iatrogenic...
induction of tendinopathy in the scientific literature (Soslowsky et al. 1996, Chen et al. 2004). Collagenase injection caused transient inflammatory effect within the tendon tissue (Lui et al 2011). Due to the complex structure of the Achilles tendon, which consists of the tendon of the flexor digitorum superficialis muscle, the tendon of the gastrocnemius muscle and the tendons of the soleus muscle, biceps femoris muscle and semimembranosus muscle, in the present study it was not possible to avoid a slight outflow of the solution between the aforementioned tendon bands and under the paratenon, and a small degree of degradation of collagen fibers at the injection site in the subcutaneous tissue, which had no effect on the course of the experiment and the results obtained. The histopathological ultrasound examination revealed an almost complete damage of collagen fibers within the application area of the collagenase solution.

**Acute phase proteins**

The experiment tested the reaction of selected acute phase proteins on the impact of RPWT on the examined area of the Achilles tendon, treated with two types of intratendinous injections. The acute phase reaction is the first nonspecific response of an organism to various forms of homeostatic disturbances, caused by infections, injuries, carcinogenesis or immunological disorders (Petersen 2004). Acute phase proteins (APPs) are usually decomposed in a few days or weeks, but they can also be detected in the peripheral blood much longer if the factor stimulating their production is not removed (Mackiewicz 1997). Changes in the concentrations of acute phase proteins enable an objective assessment of the animal’s health status or the intensity of a disease and are used to detect sub-clinical inflammations (Petersen 2004). It has been shown that the course of the acute phase reaction mechanism is similar in all animal species; however the share of individual APPs and changes in their concentrations are specific for individual species (Petersen 2004, Cray 2012).

The examination of the level of selected acute phase proteins has been specifically applied in monitoring the health of cattle in which inflammation is not always associated with leukocytosis. The usually low ratio of neutrophils to lymphocytes (e.g. 0:5 in cows) compared to other species (e.g. 3:5 in dogs and 1:8 in cats) may predispose to myelopoiesis delay and, consequently, to the delayed increase in the amount of leukocytes in the peripheral blood as well (Taylor 2006). Additionally, in cattle, poorly expressed clinical symptoms and free-range farming contribute to a delayed detection of inflammation. Hence the key role of acute phase proteins in this species is to allow an early and precise detection of inflammation (Kent 1992) and, indirectly, also other stressors for the organism (Kostro and Gliński 2003). For this purpose, the most commonly determined levels concern the following APPs in cattle: Hp, SAA and Fb, but also ceruloplasmin, alpha 1-antitrypsin and alpha 1 acid glycoprotein (AGP) (Horadagoda et al. 1999). In sheep, stress in the form of electroshock or excessive flock density is a factor stimulating the production of another acute phase protein - Fb (Kostro and Gliński 2003). Therefore, in this animal species the measurements of Hp and Fb concentrations have a special diagnostic significance, not only in the detection of inflammation, but also for animal welfare. In the present study, the concentrations of three positive acute phase proteins, i.e. SAA, Hp and Fb, were determined. In the literature and clinical practice, changes in SAA and Hp concentrations are used to distinguish between acute and chronic inflammations (Horadagoda et al. 1999). A significant increase in SAA concentration was demonstrated in cattle suffering from acute inflammation compared to Hp concentration, which in turn showed higher values in the case of chronic inflammation (Horadagoda et al. 1999). In clinical practice, the determination of APPs concentrations has been widely used and, currently, their levels are being investigated for diagnostic purposes as well as for prognosis and evaluation of the effectiveness of the applied treatment in common diseases of cattle, such as mastitis, uteritis, placental retention, complicated childbirth, abdominal diseases and diseases of limbs, fat cow syndrome and stress (Kostro and Gliński 2003).

**Acute phase proteins in sheep - reference values**

The reference level of SAA for healthy sheep shows, according to some authors, the values of 0 - 6.46 mg/L (Iliev and Georgieva 2016). The levels considered to be the norm for Hp, as in the case of SAA, are defined differently by various authors. The Hp level in healthy animals is usually undetectable (“0”) or, in the vast majority of animals, it falls below 0.1g/dm³, as demonstrated in numerous screening tests of herds, despite the accepted value of 0.2 g/l setting the upper standard limit (Kostro and Gliński 2003).

The concentration of Fb in healthy sheep varies from 1.32 g/dm³ to 5.54 g/dm³ and the average is 2.78 g/dm³ - 3.7 g/dm³ (Kostro and Gliński 2003, Iliev and Georgieva 2016).

In the case of stimulation of acute phase proteins production, Hp and Fb levels increase 1-5 days after the initiation of inflammation/infection, while an increase in SAA level is noted 4-8 hours after the occurrence of an inflammatory stimulus (Stefaniak 2000, Kostro and Gliński 2003). Three periods of peripheral blood
collection were determined in the experiment. The first collection was performed just before the PRP or ADSCs intratendinous injection combined with the RWPT (blank test – test 0), and then two days after each RPWT procedure and 7 days after the end of the therapy. The increase in SAA concentration in the blank test, revealed by the experiment, could indicate that some sheep had an active inflammatory process of an unknown etiology and location, possibly related to the induction of the focal point of tendinopathy. On the second day after intratendinous injections, an increase in SAA was observed in about half of the animals (4 sheep from group 2 and 3 sheep from group 1), which could indicate the initiation of a small degree of inflammation due to the intratendinous injection or RPWT. The lack of increase in SAA and Hp concentrations in further determinations confirmed the effect of the RPWT alone on the induction of inflammation in sheep organisms, predicted by the author (Table 1). The initial increase was, therefore, due to the intratendinous injections. The few leukocytes remaining in PRP were the probable cause of this phenomenon in a larger number of sheep from group 2. It was shown that the PRP deprived of leukocytes has an anti-inflammatory in vitro activity due to the release of the hepatocyte growth factor (HGF) (Zhang et al. 2013). In contrast, the PRP containing leukocytes may enhance the expression of proinflammatory factors (IL-1beta, IL-8 and FGF-2) and reduce the expression of HGF (Asirelli et al. 2015). Similar anti-inflammatory effects are attributed to ADSCs (Mora et al. 2015), due to the secretion of anti-inflammatory mediators, including interleukin-10 (Young 2012). Other presumed factors might have been the few bacteria or the volume of injection itself, which had an expanding effect on tissues. All the aforementioned mechanisms might have co-occurred at the same time, which contributed to the increase in SAA on the second day after the intratendinous injections. The analysis of Fb concentration changes dynamics confirms the lack of the RPWT influence on the concentration of this acute phase protein. In the blank test, nine animals showed a slight increase of the fibrinogen level, but far below the mean value for this species (about 2.5 g/dm$^3$ - 4.5 g/dm$^3$), probably due to the induction of tendinopathy. After treatment, the level of this acute phase protein oscillated around the low values characteristic of healthy animals (Dellenback and Shu Chien 1970) (Table 2).

In conclusion, an important finding of the experiment is that no systemic inflammatory response was observed during and after treatment with RPWT in both groups of sheep, which was confirmed by the analysis of acute phase protein concentrations.

References


