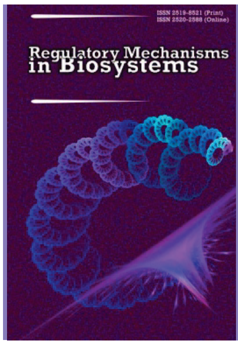

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Influence of autological growth factors on activation of regenerative processes of the superficial digital flexor tendon of horses

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Injury of the superficial digital flexor tendon is known for slow and functionally incomplete recovery, which is a problem in the equestrian horse farming. In the clinical studies, we used platelet-rich plasma, obtained from horse blood, and a specially designed scheme of rehabilitation after physical loads for the treatment of injuries of the superficial digital flexor tendon of horses. We analyzed four clinical cases of injuries of the superficial digital flexor of horses, of which 3 cases were treated using platelet-rich plasma, injected under ultrasound-diagnostic control, and one case that was treated without its administration. Ultrasound studies of the superficial digital flexor tendon revealed the following changes: increase in the volume of the tendon, damage to the fiber structure, and absence of fibrosity on the longitudinal images, and hypo-echogenic or anechogenic structure on transverse and longitudinal images. The results of the platelet-rich-plasma treatment of the horses were compared with the control (without utilization of platelet-rich plasma). As seen on the ultrasound images during the intermediate-control stage (2.5–4.0 months after injury), the horses that had received injection of platelet-rich plasma and had undergone the complex of physical exercises were forming new fibers of the superficial digital flexor tendon, which had distinct structure and alignment. This indicated the process of their physiological development. Four and a half months after being injured, the horse that had only received a symptomatic treatment combined with a complex of rehabilitating physical loads had poorly structured fibers in the damaged area, with their disordered alignment, as revealed by the longitudinal images. This suggested the formation of a scar. Transverse images showed the anechogenic areas of the injury suffered by this horse. The method of preparing and administration of platelet-rich plasma is relatively cheap, given the equipment and a qualification of a veterinary doctor. Taking into account its identified efficacy for recovery of the superficial digital flexor tendon, it would be promising to conduct further clinical trials using platelet-rich plasma for treating other injured tendons of horses, which can notably increase the quality of recovered connective tissues.

Keywords: growth factors; platelet-rich plasma; tendon injury; ultrasound diagnostics; rehabilitation.

Introduction

During training and competitions, equestrian horses are subject to heavy loading on the musculoskeletal system, which may result in acute and chronic injuries. Horse parts that are sensitive to a mechanical loading the most are the superficial digital flexor tendons (SDFT) and also the deep digital flexor tendons (DDFT) (Kalisiaketal., 2012; Holroydetal., 2013). The superficial digital flexor tendon (SDFT) of horses is an elastic structure working at the verge of its biomechanical safety during heavy physical loading. The function of the superficial digital flexor is transferring load on the muscles onto the skeleton of the limb. Moreover, SDFT is a specific accumulator of energy deriving from the tendon's elastic elongation, returning it to the previous state (spring effect) (O'Brien et al., 2021). Therefore, fast gallop running leads to stretching of SDFT in 10–15% horses on average (Stephens et al., 1989). Specifics of this tendon affect its ability to adapt to stress factors and proneness to progressing degeneration (Dowling et al., 2000). Foal SDFT is more adaptive than such of adult horses, and therefore an optimal regime of exercises helps developing a functionally adaptive tendon. Grazing the young on pastures promoted the development of a stronger and more elastic tendon than young horses that just trained according to the training program, being a guarantee of a better adaptation to physical loading when reaching maturity (Dowling et al., 2005).

Degenerative alterations occur because a part of SDFT-accumulated energy transforms into thermal energy and, during blood-circulation impairments, the tendon does not cool, leading to local hyperthermia. Those processes are especially expressed during multiple loads on the tendons, when there is not enough time for homeostasis to recover between the training sessions, even those of average intensity (O'Brien et al., 2021). According to the data from different researchers, potential risk factors of a SDFT injury include inconsistencies in the frequency and intensity of training, qualitative characteristics of the surfaces of running tracks and training grounds, mechanical injuries, and individual peculiarities of an animal – blood circulation in the limbs, elasticity and tensileness of the tendons, and also age, breed, and sex (Perkins et al., 2005; Kalisiak et al., 2012).

The healing process in the tendons is comprised of three phrases: inflammation, proliferation (lasts for weeks), and remodeling (lasts for months/years). The tendons heal slowly and regenerate poorly, and therefore the recovery process is complex and requires participation of both intrinsic and extrinsic cell populations (Voleti et al., 2012). According to the authors (Bruns et al., 2000; Yang et al., 2013), the damaged tissue of a tendon only partially regenerates its biomechanical properties, and its biochemical and ultrastructural characteristics are inferior to the normal even a year after recovery.

The healing of a tendon is characterized by the structural changes similar to those observed during its development. During the proliferation and remodeling phases, fibroblasts synthesize proteoglycans, collagens, and other components of the extracellular matrix. Collagen is being organized in mature fibers, reorganization of which takes place in the modeling phase. This process is regulated by fibroblasts of the tendon indirectly through the growth factors, cytokines, and matrix metalloproteinases of a tendon (Voleti et al., 2012).

Interest of the scientists in the mechanisms of repair of collagen structures led to the study of the efficiency of using various growth factors when treating injured tendons (Dowling et al., 2005).

Growth factors are cell-secreted peptides and proteins that affect the cell functions, in particular differentiation and growth of cells, including repair of the tissues (Hsu & Chang, 2004). Among the main growth factors that play the key role in healing of the tendons, the following are designated (Molloy et al., 2003): insulin-like growth factor-I (IGF-I), transforming growth factor beta (TGFbeta), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and the basic fibroblast growth factor (bFGF).

The greatest problem when injuring the ligaments and tendons is that they will no longer have the former elasticity and strength. A rupture of collagen fibers causes bleeding, leading to the formation of hematoma inside a tendon, which in turn is accompanied by granulation of the tissues and formation of a scar. Such a healing method does not always allow the tendon to recover from its post-traumatic functional abilities to its prior condition and often leads to reinjuries (Henninger, 1994; Dyson, 2004).

The degree and intensity of an inflammation when healing are the determining factors influencing parameters of a formed scar such as its size and quality of a novel tissue. That is, healing of tendons should not be a repair of the tissues but rather their regeneration. Growth factors can play an important role in providing the natural healing. They include, in particular, platelet-rich plasma (PRP) (Everts et al., 2020).

The first article about a successful use of the PRP came out in 1998 (Marx et al., 1998). The authors presented the positive effects of PRP on the regeneration of bones after transplantation. Platelet-rich plasma is an autological product that has no side effects like for example corticosteroids, and causes no immune reaction.

Therefore, the objective of our study was identifying the influence of PRP on the intensity and quality of regeneration of the injured superficial digital flexor tendon of horses.

Materials and methods

All the studies described in this paper were carried out following the ethical principles and recommendations of the ARRIVE (https://arriveguidelines.org); owners of all animals involved in the study gave an informed consent. We analyzed 4 clinical cases of partial tear of the SDFT of the male horses of various ages. The diagnosis was made based on a collected anamnesis (we identified when and in what circumstances the animals began limping, how they got injured, and the load regime), general clinical and orthopedic examinations (presence of edema, hyperthermia in the injured place; lameness of various degrees during all horse gaits; pain on palpitation of the affected area of the SDTF), and also ultrasonographic study (presence of hypoechogenic structures in the SDTF) using a SonoScape device (S6V), China.

Preparing platelet-rich plasma was done right before its administration. The PRP was prepared following the rules of aseptics and antiseptics. Before drawing blood, we carefully shaved the puncture region, the area of which was washed with the Dr Seidel Laboratorium Dermapharm (Poland) shampoo containing chlorhexidine and treated with antiseptic Sterillium Hemi GmbH (Germany) with 5 min and 15 s exposures, respectively.

Platelet-rich plasma was obtained using the two-step method of blood centrifugation. In three-component 60 mL sterile plastic syringe, we drew 6 mL of sterile sodium citrate and with the same syringe drew 54 mL of blood from the jugular vein using a 1.2 mm needle. After this, the blood was mixed with sodium citrate by circular movements to prevent an early activation of platelets. Stabilized blood was transferred into six 10 mL sterile centrifuge test tubes without filler and centrifuged for 7 min at 270 g rate. The centrifugation was carried out using an UNICO centrifuge (USA).

By the end of centrifugation, first fraction of plasma (FPF) had formed in the test tubes. A thin layer of buffy coat (BC) appeared between FPF and packed cell volume (PCV). After the first centrifugation, around 30 mL of FPF was attained.

The first fraction of plasma was put into two sterile 15 mL centrifuge test tubes and centrifuged for 10 min at 900 g rate. The division of FPF by lower fraction results in platelet-rich plasma, whereas the division by upper fraction results in platelet-depleted plasma, PDP.

To gather pure platelet-rich plasma, we used a sterile syringe and a pencil-type needle for spinal anesthesia, 120 mm long. Prior to drawing PRP into syringe, it was delicately mixed using a needle tip in order to elevate platelets that had settled on the bottom of test tube.

As a result, from 54 mL of dense blood, we obtained 2–3 mL of prepared platelet-rich plasma. The amount of blood drawn at the beginning depends on amount of platelet-rich plasma that is needed for injection, which in turn depends on the area of damaged tendon fibers. Platelet-rich plasma was injected 1.0–1.5 weeks after injury (reduction of inflammation process). During the inflammation phase, we took the following measures: the movement of the animals was limited (24 h maintenance in a loosebox for a horse); local application of cold to the injury area twice a day for 30 min (ice boots, dousing in cold water); in hard cases and great pain we employed non-steroid anti-inflammatory compounds based on phenylbutazone, according to the manufacturers' instruction (5–10 days on average). After the said measures, when the signs of reduction of the inflammatory process appeared (decrease in edema, hyperthermia, and pain), we decided on application of platelet-rich plasma. The region for the injection was shaved using an electric shaver, washed with a shampoo with chlorhexidine, and treated with the Sterillium solution.

The animals received an intravenous injection of detomidine-based sedative drug Ekvisedan (Farmaton Company, Ukraine) or Domosedan (orion Pharma, Finland) in the dose of 0.1 mL/100 kg of live weight of horse. After sedation under the ultrasound control, we injected platelet-rich plasma into the injured area, evenly distributing it where the injury localized. To the puncture area, we applied a bandage with a sterile gauze swab, fixated by elastic bandage. Injection of platelet-rich plasma into the injured region was single. After injection, each animal was held for 7 days in a loosebox for horse. Further rehabilitation of the animals was performed according to the following scheme (Table 1).

Table 1
Approximate plan of returning a horse to its usual training regime

Weeks	Load (per day)
1	ultrasound studies, PRP injection
2	All-day loosebox maintenance
3	10 min walk
4	15 min walk
5–12	20 min walk; control ultrasound study
13–16	30 min walking and 5 min trotting
17–20	30 min walking and 10 min trotting
21–24	30 min walking and 25 min trotting; control ultrasound study
25–26	25 min walking and 25 min trotting
27–28	20 min walking and 25 min trotting
29–30	15 min walking and 30 min trotting
31–32	10 min walking and 35 min trotting
33–48	Starting running gallop; gradual return to the regular load level
48+	Regular training regime

The scheme was corrected depending on how actively the damaged tissues were healing. When the training regime changed, the diet of animals was corrected, in particular, concentrated feeds in it. For a comparison, we demonstrate the case of treating a tear of the superficial digital flexor tendon following the anatomical scheme with limiting the horse's movement and changing the training regime as given in Table 1, but without administration of platelet-rich plasma.

Results

Gelding, 6 years, purebred riding horse, show jumper. During training on soft soil, the owners noted an insignificant limp. A detailed examination found first-degree lameness in the left front limb during all horse gaits; large edema in the proximal part of the metacarpus; local hyperthermia in the region of superficial digital flexor; during palpation, there was pain and thickening of the tendon. No such changes were seen on the collateral ligament. Transverse and longitudinal ultrasound images of the SDTF of the left front limb displayed increase in the volume of the tendon. Moreover, the longitudinal images showed impaired structure of the fibers and transversal images revealed that the body of the tendon had a hypo-echogenic structure (edema). On the longitudinal image, absence of fibers on the tissue was ~ 30% of the area of the entire tendon (Fig. 1).

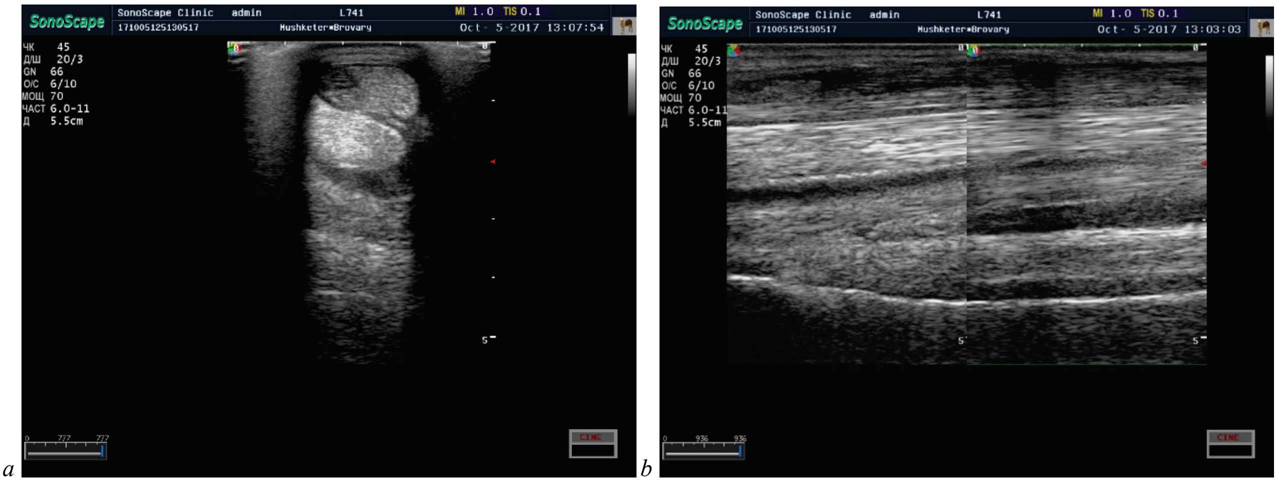


Fig. 1. Ultrasound study of SDTF of the left front leg after injury: *a* – transversal image; *b* – longitudinal image

Platelet-rich plasma was injected into the injured zone in the volume of 2 mL on the 10th day after the injury occurred. The next day, local edema increased, and on the fourth day decreased to its initial level, i.e. such observed prior to the injection. On the 14th day, we gradually started the recovery training regime according to the plan given in Table 1.

Four months after the date of PRP injection, we made a second monitoring of the horse. No edema, pain, and hyperthermia in the SDTF area were observed. Neither was lameness. The tendon was somewhat thickened. The ultrasound revealed structural improvements in fiberness – collagen fibers became aligned, more parallel, the hypo-echogenicity reduced, and new connective tissue in was growing in the injured area.

On the 41st week of the treatment, the animal returned to complete physical loads. No signs of SDTF reinjury were seen.

Gelding, Ukrainian Riding Breed, 15 years. The horse began limping after a walk in an open area outside the stable. The lameness was pronounced, even when walking. In a distal part of the metacarpus on the plantar side, there were an edema, local hyperthermia and pain in the region of the superficial digital flexor tendon. On day of the injection, day 16 since the animal had been injured, an ultrasound image revealed a SDTF tear. Transverse imaged (Fig. 2a) shown anechogenic structure (contours are marked with a yellow line), which took up about 25% of the area of transversal section of the tendon. At the same time, we observed significant edema and the fiber tear itself. On the longitudinal image (Fig. 2b), we saw lesions of the structure of fibers of the tendon – anechogenic longitudinal structure with various degrees of anechogenicity (ruptures of single fibers and tears of others). Anechogenic condition was more expressed in the center of the area. Into the injured region, under ultrasound control, we injected 2 mL of platelet-rich autological plasma.

Intermediate ultrasound control, carried out 2.5 months after the PRP injection, indicated significant decrease in anechogenic areas and their intensity on both the transverse (Fig. 2c) and the longitudinal images (Fig. 2d). On the transverse image, the tear region was still visible, but anechogenicity was expressed only in the central zone of the lesion, where individual fibers were torn the most. Their structure was well manifested, and the positions were aligned – located in parallel one to another. Such a location of the fibers evidenced their respective anatomical process of formation. Six and a half months after the injection of platelet-rich plasma, we clinically observed complete absence of inflammation, local hyperthermia, pain, and lameness. Duration of trotting per day exceeded 30 min and caused no lameness or other signs of deterioration of the tendon condition. This suggests faster regenerative processes in damaged tissues and less time necessary for rehabilitation. No relapse of SDTF lesion was observed.

Gelding, 16 years, Westphalian Riding Horse. The horse was injured when training in overcoming obstacles on a soft and viscous soil. We observed 2/5-degree lameness in the left front limb. Locally, in the region below the carpus on the palmar side, there was a significant edema, hyperthermia and pain on palpation of the superficial digital flexor tendon. Five days after the injury, ultrasound images were made (Fig. 3a), the longitudinal image revealing a significant lesion, which varied 0.46 to 0.66 mm place to place. The transverse image revealed an anechogenic region of around 1.52 cm^2 area. The tear occupied around 25% of the area of the transverse section of the tendon.

Figure 3 visualizes the tear of the superficial digital flexor tendon and its location in relation to other structures of the metacarpus, in particular, the deep digital tendon, DDTF. Into the injury region, under the ultrasound control, we injected 2.5 mL of platelet-rich plasma. The control ultrasound study was performed 5.5 months after injecting platelet-rich plasma into the tendon (Fig. 3b). On the longitudinal image, we observed formation of new tendon fibers, located in parallel one to another, on almost the entire length. Small anechogenic sites were still present.

On the transverse image, we saw contours of localization of the injured region, but anechogenic state was practically absent. This means that new fibers have formed in the tear region. Such a result can be explained by the fact that the injury was quite large. After the rehabilitation, horse returned to the normal training and competitions. Unfortunately, the animal was reinjured 2 years later during a competition, and has never engaged in sports again.

Horse, 5 years, Ukrainian Riding Breed. The injury was incurred while training, exercising on a soft soil. The horse was observed to have lameness (2/5). Examination of the limb found edema, local hyperthermia, pain on palpation of the superficial digital flexor tendon in the middle of the metacarpus of the left front limb. The horse received treatment according to the classic method, without PRP injection.

On the ultrasound images made 4 days after the injury (Fig. 4a), we saw areas of tears of fibers of the superficial digital flexor tendon. The margins of the tendon were uneven, and the achenogenic zone had no notable margins. The general area of the tears accounted for around 30% of the general area of the transverse section of tendon. The longitudinal image revealed a significant lesion in the tendon structure. The margins of the fibers were indistinct. A large edema was present in the injured area.

On the longitudinal images taken 34 days after the previous study (Fig. 4b), we observed increase in echogenicity in the injured area, which indicated the formation of the connective tissue. Formation of the fibers was not observed.

The images made 4.5 months after the injury of the tendon (Fig. 4c) visualize increase in echogenicity of the pathological region almost to the level of normal tendon and emergence of individual fiber-like structures. However, the ultrasound picture indicates the formation of the scar tissue, i.e. no new collagen fibers grew in the tendon. It has to be noted that during that period, the horse was observed to have mitigation of the clinical signs and pain alleviation in the tendon region, and lameness in this limb disappeared.

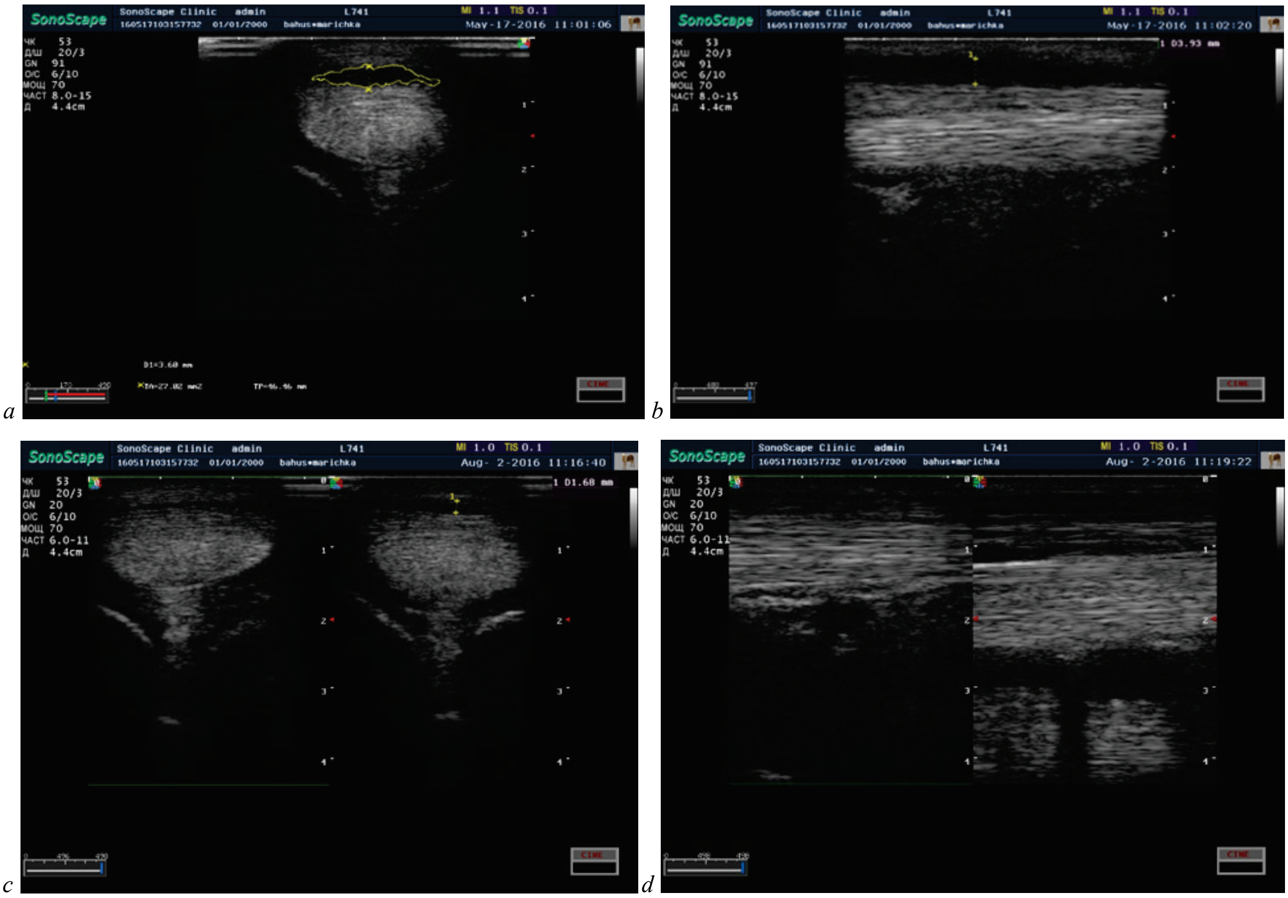


Fig. 2. Ultrasound study of SDTF: transverse (*1*) and longitudinal images (*2*); *a*, *b* – on the 16th day after injury; *c*, *d* – 2.5 months after injecting PRP

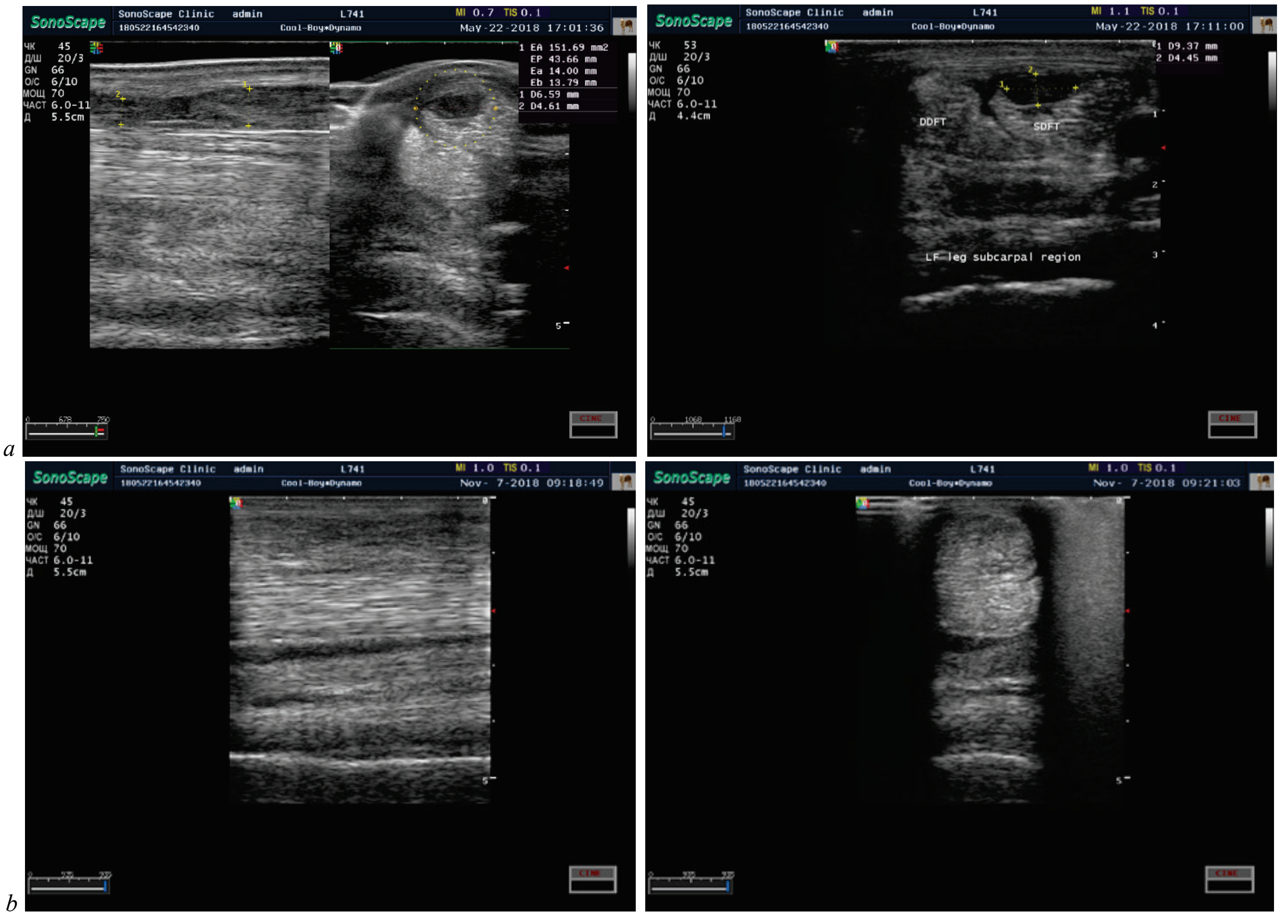
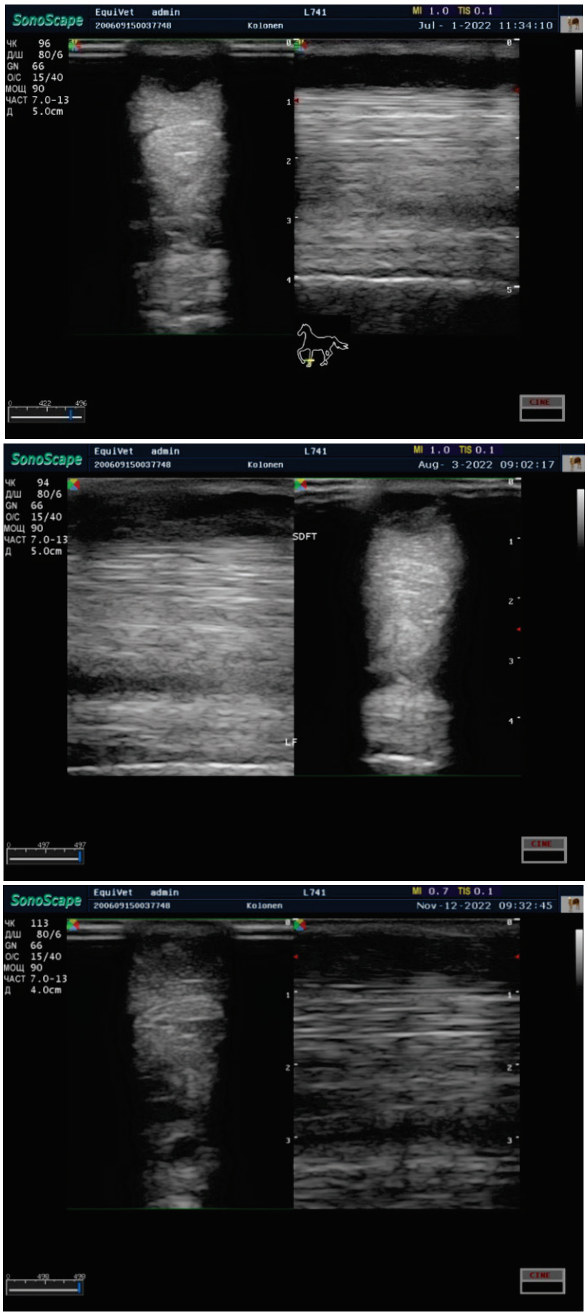


Fig. 3. Ultrasound study of the SDTF (longitudinal and transverse images): *a* – on the 5th day after injury; *b* – 5.5 months after PRP injection



a
b
c

Fig. 4. Ultrasound study of the superficial digital flexor tendon: *a* – 8 days after the injury (transverse and longitudinal images); *b* – 34 days after the injury (longitudinal image); *c* – 4.5 months after the injury (transverse and longitudinal images)

Discussion

Platelet-rich plasma is a source of various growth factors and cytokines from α-granules, present in platelets. Platelets were found to have a large amount of cytokines: insulin-like growth factor (IGF-I, IGF-II), transforming growth factor β (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor, and fibroblast growth factor (VEGF). All the said cytokines partake in the cellular proliferation, chemotaxis, cellular differentiation, and antiogenesis. A peculiarity of PRP is that all the mentioned cytokines (unlike exogenous recombinant cytokines) are present in the normal biological ratios, which likely influences the effectiveness of the processes of tissue healing (Foster et al., 2009).

The first results of using PRP were reported in a study back in 1998 (Marx et al., 1998), after which the studies in this sphere became popular in the world of science (Camargo et al., 2002; de Mos et al., 2008; Kajikawa et al., 2008; Alsousou et al., 2009; Everts et al., 2020). As of now, PRP is actively used in orthopedics for enhancing healing of injuries of skeletal muscles, tendons, and ligaments, even despite a number of aspects regarding PRP application being in the need of elaborations, in particular: volume of injection/usage; most effective drug; bufferizaton/activation; injection technique (depot 1 against several depots); timing of injection to injury; one application against a series of injections; and the most efficient rehabilitation protocol to follow after a PRP injection (Paoloni et al., 2011).

Active and, mostly, effective usage of PRP in medicine has led to its broad implementation in veterinary medicine on various animals with different pathologies: medical conditions of the locomotor apparatus (tendinopathy, sesamoiditis, osteochondritis dissecans, bone fractures), endometritis (Camargo Garbin et al., 2021), skin wounds (Iacopetti et al., 2020), corneal ulcers (Farghali et al., 2021), and other.

As known, SDFT injuries require long rehabilitation with the risk of unpredictable results and high reinjury rate. Priority tasks in treating SDF tendonitis are reducing inflammation and performing procedures that would maximally drive the recovery of the tendon structure. According to the data of a number of researchers, various efficacy in treatment of SDFT injuries is attained by tendon splitting (decreases pressure of hemorrhage, promotes vascularization); desmotomy of accessory ligament of the superficial digital flexor tendon; use of β-aminopropionitrile fumarat, polysulfated glycosaminoglycans, and sodium hyaluronate; mesenchymal, embryonal and induced pluripotent stem cells; and isolated exogenous growth factors (insulin-like growth factor 1 and transforming growth factor-β) (Dyson, 2004; Witte et al., 2016).

As a drug for treating SDF tendonitis, PRP is popular among practicing veterinarians because of its simplicity and economic benefits. However, inconsistency in the results of PRP studies raises concerns.

Some of the discussed issues are concentration of growth factors in PRP samples and estimating a required dose for the usage in veterinary practice. Advantage of *in vitro* studies is a clear control of their parameters and fast study results. Some studies indicate that efficacy of PRP action depends on its dose, and high concentrations of platelet-derived growth factors do not always contribute to the cellular-growth processes and can have a harmful impact (Vahabi et al., 2017). One of the reasons may be that number of receptors of cellular membranes is limited and when the number of platelet-derived growth factors significantly exceeds the number of receptors, they negatively affect the cell function (Everts et al., 2020).

According to the results of the monitoring, dose of injected PRP should be chosen based on scales and area of damaged tissue of the superficial digital flexor tendon, visualized using ultrasound, though the most important moment is a clear control of the so-called therapeutic amount of platelets (over $10^6/\mu L$) and aseptic conditions when obtaining PRP.

Analysis of results of the clinical trials of PRP, given in this study, is optimistic, because the treatment results, corroborated by ultrasound control, indicate its efficacy during proliferation, producing good results of recovery of the structure of superficial digital flexor tendon with the area of tear of around 25–30% of the area of the transverse section.

The results we obtained are consistent with the data from other authors, who have had positive results of treating injuries of the superficial digital flexor tendon of horses using PRP on the experimental model, obtained surgically (Bosch et al., 2010). The authors came to the conclusion that PRP enhances the metabolic activity and definitely fosters the maturation of tendon tissues, as compared with untreated injuries, suggesting that PRP can be useful in treatment of clinical cases of tendon injuries.

Conclusions

Autological plasma, enriched by platelets, is a source of mixed endogenous cytokines and has undeniable benefits, and also is economically practical. Despite discussions over PRP usage, based on our clinical trials with long monitoring period, we may state the efficacy of this method of treatment of the superficial digital flexor tendinopathy, which in a complex with the designed plan of physical loads gave positive results of recovery of the tendon structure. Perhaps, controlled exercises during rehabilitation play an important role in the recovery of the superficial digital flexor tendon after injuries. It has to be noted that the efficacy of this method directly depends on a rigid adherence to the aseptic rules, and the main source of bacterial infection during the application of PRP drugs is insufficient sanitation of the skin of an animal, the hands of a doctor, and also contamination of an injection region with microorganisms from the environment.

The authors declare no conflict of interests.

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