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Original Research

Mesenchymal Stem Cells Derived From Subcutaneous Fat and Platelet-Rich Plasma Used in Athletic Horses With Lameness of the Superficial Digital Flexor Tendon

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ABSTRACT

Tendinitis of superficial digital flexor tendon (SDFT) represents a major cause of injury in the equine athlete. Although numerous treatments have been described, few are effective and a great potential remains for recurrence and, in certain cases, an abrupt end to the horse's athletic career. Recently, several experiments have focused on the therapeutic potential of mesenchymal stem cells (MSCs) in cases of tendon lesions. The aim of this study was to evaluate the possible clinical application of equine adipose tissue–derived MSCs (AD-MSCs) and autologous platelet-rich plasma (PRP) for the treatment of acute injuries of tendons in sport horses. Nine athlete horses with an injury of the SDFT were enrolled. Subcutaneous fat from each horse was collected, and AD-MSCs were isolated, characterized, and injected with autologous PRP in tendon injury. The evolution of tendinopathy healing was assessed by ultrasound. Horses underwent to a 6-month rehabilitation program. The ultrasound findings have shown indicative signs of a reparative process that led to the formation of tissue morphologically comparable with healthy tissue. Recurrences observed in only two of the nine horses treated occurred for reasons not related to treatment. In fact, in horse, the lesion occurred in the same tendon but at a different point from the first; another recurrence was caused by failure to comply with the rehabilitation protocol. Our study showed that therapy with AD-MSCs and PRP for treatment of tendon injuries in the athlete horse seems to be promising. However, the postoperative treatment of the patient is an essential support for the proper remodeling of the tendon.

1. Introduction

Mesenchymal stem cells (MSCs) are nonhematopoietic (NH) multipotent stem cells of particular interest for basic research and increasingly used as therapeutical aid for the orthopedic injuries in the horse. Mechanical stresses cause tendon lesions of different degree (partial tears) in equine athletes. In horse, the most frequently area affected is the superficial digital flexor tendon (SDFT) [1–3]. Tendinopathy is most common in racehorses and event horses but can occur in horses used for any discipline. Tendinitis is an important cause of lameness and diminished performance in equine athletes because of its high incidence, prolonged recovery period, and high rate of recurrence. Possible

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explanations for the slow healing of tendons and, in most cases, the resulting formation of mechanically inferior extracellular matrix are probably because of the fact that tendon is a minimally vascularized tissue, presents cells that exhibit diminished mitotic activity, and the presence of few progenitor cells in the tissue.

Recurrences are related to the nonregeneration of tendon tissue and the production of a matrix of collagen fibrils that are smaller in diameter and of inferior quality, showing a reduced number of cross-links. Knowledge concerning tendon repair and its implications for the athletic capacity of the individual horses affected by tendinitis has stimulated research regarding new therapies applied to this tissue. Cell therapy using MSCs has shown promising results in several published works [4–6]. The isolation of MSCs has been described in several species and from different tissues [7,8]. Particularly, in horse, MSCs have also been isolated and differentiated from sources as umbilical cord blood, amniotic fluid, bone marrow, and adipose tissue [9–11]. Mesenchymal stem cells derived from bone marrow (BM-MSCs) and adipose tissue–derived MSCs (AD-MSCs) are the most highly characterized and are considered comparable [12]. Both have demonstrated broad multipotency with differentiation into different cell lineages, including adipocytic, osteocytic, and chondrocytic lineages [13,14]. However, the easy and repeatable access to adipose tissue, the simple isolation procedure, and the greater numbers of fresh MSCs derived from equivalent amounts of fat versus bone marrow provide a clear advantage in using AD-MSCs over BM-MSCs [11,15,16]. Adipose tissue–derived MSCs can be isolated, expanded in vitro, and then inoculated into the damage tissue [10] showing their utility for the treatment of musculoskeletal and osteoarticular diseases in several animal species [7,17,18]. Because these cells are readily accessible in large quantity in the horse, they have received increasing attention for the possible application for the treatment of tendon and ligament diseases. In particular, the use of AD-MSCs and their migration was evaluated after tendinitis therapy in equine [11,19].

Recently, platelet-rich plasma (PRP), an autologous concentrate of blood platelets, has been introduced as possible new therapy for the treatment of tendon injuries because it seems to represent a temporary therapy that provides important biological effects such as the increase in production of type I collagen, the proliferation of tenocytes, neovascularization, the increase of resistance, and the organization and alignment of the fibers [20–22]. In fact, the field of AD-MSC and PRP therapies in regenerative medicine is a rapidly growing area of research, and stem cell therapy is being used for tendon regeneration, given the frequency of tendon injuries worldwide together with the technical difficulty often encountered when repairing or augmenting tendons. Considering that tendons connect bone to muscle and are essential for transmitting forces to produce joint movement, tendon injury is a major cause of population morbidity.

Based on that the aim of this study was to evaluate the production of equine AD-MSCs and PRP autologous and the possible clinical application of cellular therapy for the treatment of acute injuries of tendons in horse. Particularly, to assess whether cellular therapy can replace drug therapy, the clinical effect of a single intratendinous injection of AD-MSCs and autologous PRP was evaluated in athletic horses with spontaneous and acute lameness of the SDFT.

2. Materials and Methods

2.1. Animals

Nine athletic horses (three males, three females, and three geldings), ranging in age from 3 to 12 years and with mean body weight of 450 ± 60 kg, were recruited based on the presence of lesion in SDFT in the forelimb (Table 1). To be eligible, the horses had be cared for by attentive owners who agreed by informed consent to participate in this clinical study, to follow a set schedule of veterinary appointments and to observe their horses for the entire study period. Before enrollment, all horses underwent routine clinical chemistry and hematologic evaluation to ensure overall health. The clinical examination revealed the presence of pain, edema, and swelling of the affected metacarpal region. Tendinitis was evaluated by ultrasound examinations using MicroMaxx portable ultrasound system with 7.5-MHz linear probe provided with gel pad; the Sony printer provided the ultrasound images. Lesion sites were identified according to the Davis and Smith [23] model. The degrees of echogenicity (transverse section) and fiber alignment (longitudinal section) of each lesion were recorded to assess the severity of tendon injuries (Reef [24]). All horses were treated with regenerative therapies as AD-MSCs and PRP, defined as the alternative therapy useful to reduce the degeneration of tendinitis.

Table 1
Summary of clinical cases treated.

Horses	Age	Gender	Breed	Attitude	Lesion	Lameness	Relapse
1	4	Female	Sella Italiana	Jumping	Recurrence, right, SDFT	Yes	No
2	15	Gelding	Sconosciuta	Jumping	Primary, left, SDFT	Yes	No
3	5	Female	Quarter Horse	Barrel racing	Primary, left, SDFT	No	No
4	12	Gelding	Holsteiner	Jumping	Primary, right, SDFT	Yes	Yes
5	8	Male	Quarter Horse	Barrel racing	Recurrence, right, SDFT	Yes	No
6	8	Male	Quarter Horse	Barrel racing	Primary, left, SDFT	Yes	Yes
7	3	Male	Quarter Horse	Raining	Primary, left, SDFT	No	No
8	5	Gelding	Sella Italiana	Jumping	Primary, left, SDFT	No	No
9	7	Female	Sella Italiana	Jumping	Recurrence, left, SDFT	Yes	No

Abbreviation: SDFT, superficial digital flexor tendon in the forelimb.

2.2. Adipose Tissue Collection

Adipose tissue samples were taken from the gluteal region subcutaneous layer of each horse. Before performing the collection, horses were shaved and disinfected with povidone-iodine (Betadine; Meda Pharma S.p.A., Milano, Italy) and sedated with detomidine hydrochloride (0.02 mL/kg IV; Domosedan; Orion Pharma, Milano, Italy). An inverted L pattern of local anesthetic infiltration, with 5 mL of mepivacaine hydrochloride, was used for regional desensitization. Adipose tissue (10 g) collected from each horse was stored into antibiotic-phosphate-buffered saline (PBS) containers and carried to the laboratory to obtain a suitable amount of cell to inject.

2.3. Culture and Expansion of AD-MSCs

The AD-MSCs were obtained collecting subcutaneous adipose tissue from the gluteal region of each horse. Each adipose tissue sample was weighed and digested for 3–4 hours at 37°C with 0.2% collagenase type IA (Gibco, Monza, Italy) prepared in sterile PBS (Sigma-Aldrich, Milano, Italy), supplemented with 1% antibiotics (penicillin, streptomycin, and amphotericin). The collagenase type IA activity was neutralized by adding 10% fetal bovine serum (FBS; EuroClone, Pero Milano, Italy). After centrifugation (300*g* for 10 minutes) and washing of the pellet, cells were incubated (about 2 g of tissue per 25 cm^2 flask) in Dulbecco's modified Eagle's medium (D-MEM) low glucose (Gibco) with 20% FBS, in an incubator supplied with humidified air and 5% CO_2. Unattached cells were removed the next day by washing with Hank's balanced salt solution (HBSS; Gibco), supplemented with 1% antibiotics (penicillin, streptomycin, and amphotericin; Sigma-Aldrich). The medium was renewed every 3 days. Adherent cells, grown to semiconfluence, were harvested, quantified, and subcultured. For harvesting viable AD-MSCs, a small volume of sterile and warm HBSS was added to the flasks. HBSS was replaced with 500 μL of 0.5% trypsin and/or EDTA solution (Sigma-Aldrich). The medium containing the suspended cells was transferred from the flask to a sterile tube of 15 mL and centrifuged at 300*g* for 5 minutes. The supernatant was aspirated, and the cells were suspended in a small volume of culture medium. An aliquot of cells was diluted in trypan blue (1:2 dilution) for cell counting, and cells were counted using the cell counter (Cellometer Auto T4; EuroClone). Cells were then replated in new flasks. Cells were also cryopreserved for further studies in D-MEM low glucose medium with 80% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich).

The identity of AD-MSCs was verified by their ability to attach to the plastic surface of culture flasks, to form colony-forming units (CFUs) and to differentiate in cells derived from mesodermal lineages: chondrocytes, adipocytes, and osteocytes.

2.4. CFU Assay

The colony-forming efficiency on plastic was assayed by plating isolated cells at three different seeding densities (150, 60, and 30 cells/cm^2) in six-well plates in D-MEM low glucose with 5% FBS. Cells were incubated for 2 weeks in incubator supplied with humidified air and 5% CO_2. The colonies were then stained with Giemsa solution and scored.

2.5. Differentiation of AD-MSCs (Osteogenesis, Chondrogenesis, and Adipogenesis)

Cells, derived from subcutaneous fat and expanded in the D-MEM low glucose medium with 20% FBS, were used for differentiation studies. These MSCs were cultured in appropriate differentiation media to obtain the differentiation into three mesodermal lineages (osteogenic, chondrogenic, and adipogenic). All studies were carried out with the same number of controls.

2.6. Osteogenesis

Cells were plated at the seeding density of 4,500 cells/cm^2 in six-well plates and treated with the NH OsteoDiff medium (Miltenyi Biotec, Calderara di Reno, Bologna, Italy) for 3 weeks with medium change once in every 3 days. Von Kossa staining was carried out to detect calcified extracellular matrix deposits.

2.7. Chondrogenesis

Micromass cultures of cells (1×10^6 cells/mL) were incubated in 15-mL test tubes with NH ChondroDiff medium (Miltenyi Biotec) for 30 days, with medium change once in every 3 days. To observe cells and matrix formed, paraffin-embedded sections of micromasses were stained with toluidine blue and hematoxylin and eosin (H & E).

2.8. Adipogenesis

Cells were plated at the seeding density of 7,500 cells/cm^2 in six-well plates and treated with the Complete MesenCult Adipogenic Medium (STEMCELL Technologies, Peschiera Borromeo, Milan, Italy) for 21 days with medium change once in every 3 days. Oil Red O staining was done to examine the lipid droplet formation.

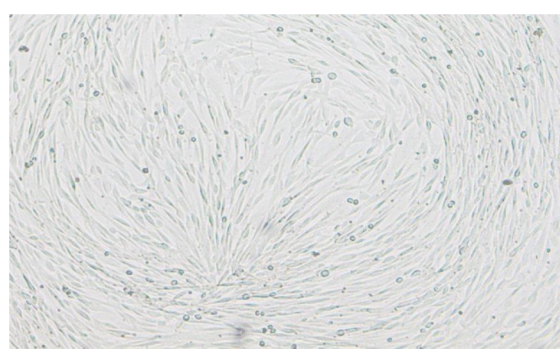

Fig. 1. Fibroblast-like cells. Adipose-derived mesenchymal stem cells are spindle-shaped cells with a fibroblast-like morphology, which attach to tissue culture plates. These characteristics are well preserved during repeated subculture.

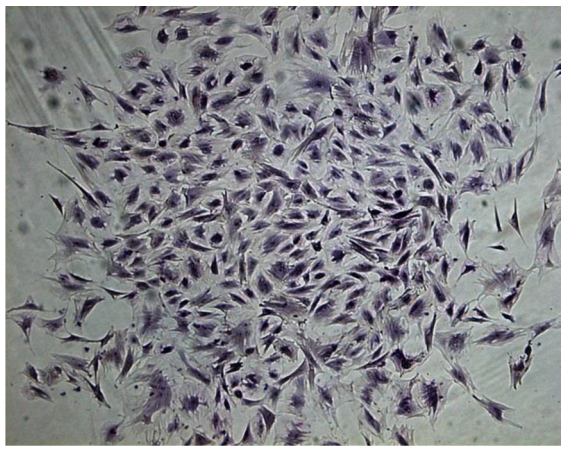

Fig. 2. Colony-forming units. Cells isolated from subcutaneous fat have ability to form clusters and those of more than 50 cells were considered colonies.

2.9. Microbiological Control of AD-MSCs and Reagents

Mesenchymal stem cells were tested for a putative contamination during the steps of production. These quality controls include tests for bacteria and fungi detection—samples are inoculated into liquid- and solid-specific media; mycoplasma detection—culture in solid and liquid media, indirect DNA staining (Hoechst 33258), polymerase chain reaction (PCR); and virus detection—a panel of tests to detect pathogens and endogenous and adventitious viruses (culture in permissive monolayer cultured cells, PCR, real-time PCR, hemoagglutination, enzyme-linked immunosorbent assay, sieroneutralization, electronic microscopy, and immunoenzymatic tests).

2.10. Tumorigenic Control of AD-MSCs

Tumorigenic control of AD-MSCs was assessed in vitro and in vivo. The AD-MSCs, the cell line Vero (negative control) and the cell line Hep-2 (positive control) were cultured in vitro into three different flasks. Later, each cell culture was inoculated in six-well plates, which contains the solid medium, and incubated at 37°C. They were observed at inverted microscope for 3 weeks. After 7–10 days, the tumorigenic cells started to replicate producing multicellular agglomerates. The negative cells showed atrophy.

The AD-MSCs, Vero cells, and Hep-2 cells were inoculated in vivo by subcutaneous injection each in groups of 10 mice without thymus gland (genotype nu/nu). Animals were observed, and any new formation of nodules in the injection area was checked. The neoplasia was measured to evaluate the growth. The test is considered valid if at least nine mice inoculated with the control positive cells (Hep-2) produce neoplasia.

2.11. PRP Preparation

Horse blood samples (250 mL) were aseptically collected from the jugular vein using sterile bags containing 3.8% sodium citrate solution and then cooled samples were carried to the laboratory to prepare the autologous PRP.

Platelet-rich plasma was obtained by centrifuging the blood at 180*g* for 10 minutes. The upper two-thirds of the PRP was carefully removed, using a plastic transfer pipette, and was transferred into plastic containers to analyze the concentration of platelets. Then, it was centrifuged at 1,200*g* for 10 minutes to separate platelet concentrate (CP) from platelet-poor plasma (PPP). The CP was resuspended

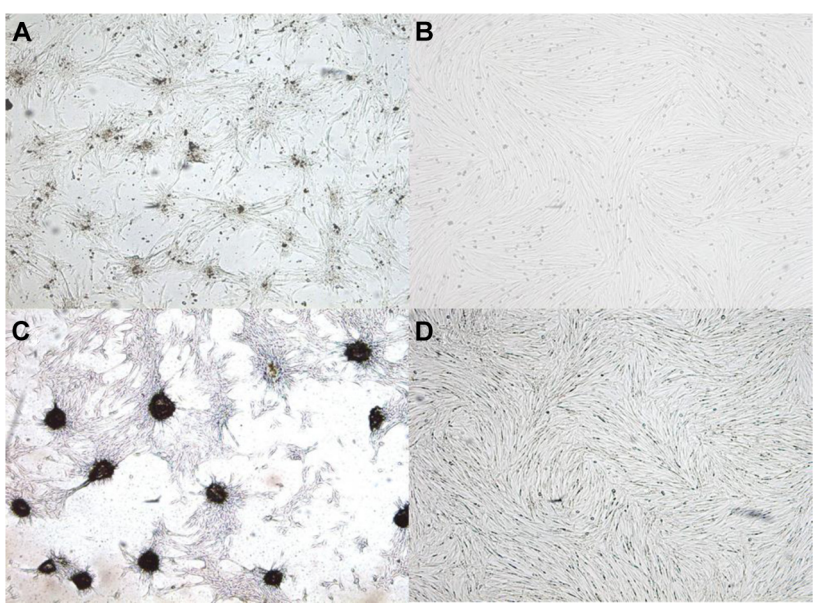

Fig. 3. Osteogenic differentiation of mesenchymal stem cells. Deposition of calcified extracellular matrix occurred in treated cells that formed cell aggregates (A), but not in monolayer cells (B), as was revealed by von Kossa staining (C and D).

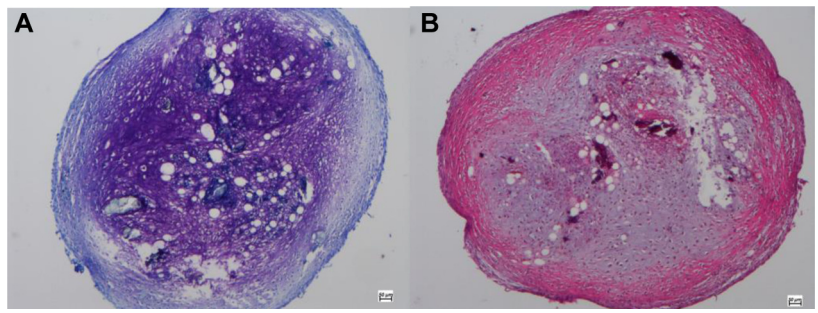

Fig. 4. Chondrogenic differentiation of mesenchymal stem cells. Cartilage-like matrix deposition was stained with toluidine blue (A) and hematoxylin and eosin (B).

in an adequate volume of PPP to obtain a final platelet concentration of 1×10^9 platelets/mL.

All the hemocomponent production phases were performed in a sterile environment with a hazard cabinet.

2.12. Treatment and Follow-up

Before performing the injection, horses were sedated with detomidine hydrochloride 0.02 mL/kg IV (Domosedan; Orion Pharma, Espoo, Finland) and anesthetic blocks of lateral and medial palmar nerves were performed with 5 mL of mepivacaine hydrochloride 2%. The affected area of the forelimb was shaved and disinfected with povidone-iodine (Betadine; Meda Pharma S.p.A.) and alcohol. Then, maintaining the forelimb in 90° flexion, ultrasound-guided injection of AD-MSCs and PRP was performed by using a 20 ga × 1½-inches needle. The amount of AD-MSCs and PRP inoculated for each horse was of 1×10^6 cells in 5–10 mL (depending on the severity and extent of the lesion) of PRP with a concentration of 1×10^9 platelets/mL of plasma. After a short massage to improve the AD-MSCs and PRP distribution, a sterile bandage was applied and kept for at least 2 days. No anti-inflammatory or antibiotic therapy was performed after the injection. Horses were on stall rest for 48 hours before starting the rehabilitation program. During the first week of physical therapy, horses were subjected to passive exercises involving flexion and extension of the treated forelimb twice a day; in addition, corrective horse shoeing was performed to reduce the tensile stress. Since the second week, horses were hand walked on hard surface for 15 minutes each day, with the time progressively extended up to 30 minutes in the coming 2 weeks. After 2 months, the rehabilitation program continued with riding at the walk (10 minutes per die) and trot (15 minutes per die) on hard surface. From the fourth month onward, horses started exercising at walk, trot, and gallop on soft surface after 10 minutes warm-up on hard surface. After this period, brief sessions of canter were introduced up to a gradual and complete return to training and competition. The treated horses were subjected to clinical examinations for 6 months during the rehabilitation program.

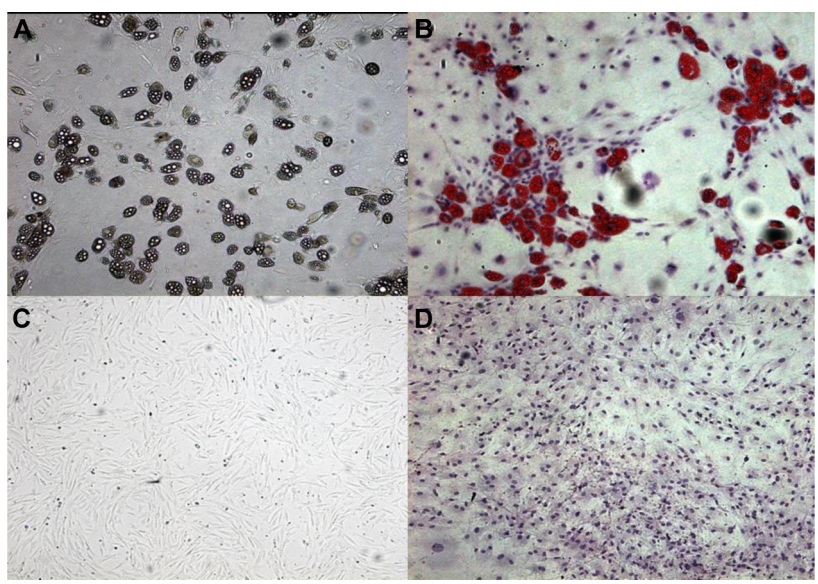

Fig. 5. Adipogenic differentiation of mesenchymal stem cells. Fat globules appeared within 4–5 days of treatment with differentiation medium, which continued to increase in size (A) and stained positive with Oil Red O (B). No differentiation was observed in the untreated cells (C and D).

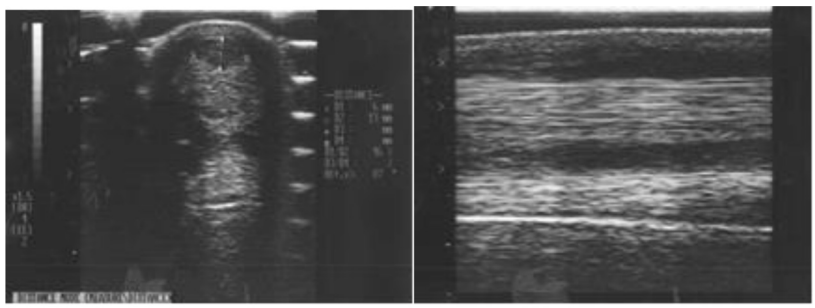

Fig. 6. Ultrasound evaluation of the initial tendon injury, before inoculation of mesenchymal stem cells and platelet-rich plasma. Alignment of grade 3 fiber according to Reef classification.

3. Results

The finding showed that AD-MSCs were successfully isolated from subcutaneous fat of all horses becoming semiconfluent in 25 cm^2 flasks in 1 week. As shown in Fig. 1, fibroblast-like cells, observed in primary culture, maintain this phenotype and expanded in culture.

As observed in Fig. 2, the cells isolated from subcutaneous fat were apparently similar in terms of their ability to form colonies on a plastic surface in (D-MEM) low glucose with 5% FBS. Subcutaneous fat cells showed differentiation potential in all the three lineages tested, and the results were evaluated by staining evaluation. On induction with NH OsteoDiff medium, the cells became more cuboidal-like in phenotype and continued to proliferate actively and formed cell aggregates that would roll in a sheet and easily detach (Fig. 3A). Deposition of calcified extracellular matrix was evident in treated cells that formed cell aggregates (Fig. 3B) but not in monolayer cells as revealed by von Kossa staining (Figs. 3C and 3D). Cell phenotype did not change in untreated cells. Twenty-four hours after seeding in micromass culture of the AD-MSCs, three-dimensional aggregates were observed in tubes treated with NH ChondroDiff medium but not in control tube. Cartilage-like matrix deposition was stained with toluidine blue and H & E (Figs. 4A and 4B). Fat globules were noted within 4–5 days of treatment with differentiation medium (Fig. 5A), which continued to increase in size, and stained positive with Oil Red O (Fig. 5B). No differentiation was observed in the untreated cells (Figs. 5C and 5D).

Microbiological quality controls performed on cells and PRP ensured their safety. Tumorigenicity assays performed on cells were negative. Cells were also cryopreserved for possible further treatments, studies, and allogeneic implantations.

Before implanting the AD-MSCs, SDFT was clinically evaluated, and all horses showed a lesion of the forelimb of third degree according to Reef (Fig. 6). After 20 days, the implantation has been carried. Immediately after implanting the AD-MSCs, the site showed a small tumefaction that disappeared after 48 hours of bandage. Thirty days after inoculation, the ultrasound examination showed an increase in echogenicity, intralesional, and an array of grade 2 according to the tendon fibers by Reef. The size of the lesion appeared almost unchanged (Fig. 7). After 60 days, the alignment of tendon fibers was grade 1 in according to Reef with decrease in the size of the lesion (Fig. 8). At 120 days, a full alignment of tendon fibers was evident (Fig. 9). Seven horses resumed their normal competitive activity after 7 or 9 months of therapeutic treatment with AD-MSCs and PRP, the animals have shown no recurrence. Two horses had relapsed; one horse returned to activity after about a month after surgery resulted in the relapse, in the portion of tendon treated with AD-MSCs and PRP. The owner had not complied with the recovery time determined by the rehabilitation

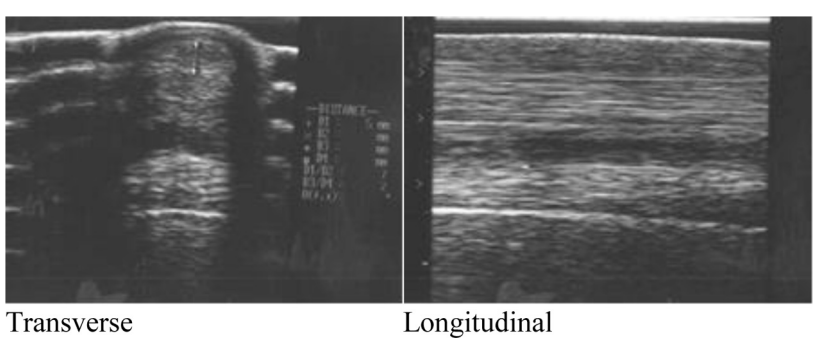

Fig. 7. Ultrasound evaluation after 30 days of surgery. Increase of intralesional echogenicity according to Reef classification. Lesion size unchanged. Alignment of grade 2 fiber according to Reef classification.

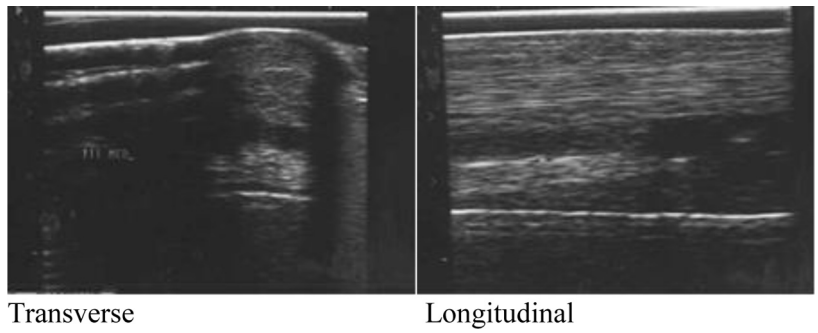

Fig. 8. Ultrasound evaluation after 60 days of surgery. Alignment of grade 1 fiber according to Reef classification.

protocol. In another horse, the SDFT injury occurred after about a year after resumption of athletic activities but in a different portion of the tendon respect to portion treated with regenerative therapy.

4. Discussion

In recent years, regenerative medicine has become a fairly common practice in veterinary medicine because the inoculation of MSCs at the level of tendons or ligaments damaged can stimulate the healing of the lesion [4,5,11,25,26]. So, in this study, SDFTs of athletic horses were treated with regenerative therapy using MSCs taken from subcutaneous fat and autologous PRP. The withdrawal of the fat at the base of the tail for the isolation of stromal fraction has been proven safe and effective.

The amount of cells obtained after isolation and the calculation of the ultrasound lesion size, cross-sectional and longitudinal, allowed inoculation of the volume of biological material (AD-MSC and PRP) in accordance to other authors [11,27,28].

No reactions of postimplant have been verified, and the inoculation of AD-MSC and PRP under ultrasound guidance allowed us to minimize the damage to the injured tissue and carefully route the cell suspension in the lesion. After treatment, all horses were subjected to a period of rehabilitation of gradually increasing intensity and duration before resuming normal competitive activity. During this period, the healing mechanism was monitored by ultrasounds to programmed intervals.

The ultrasound findings have shown signs indicative of a reparative process that led to the formation of tissue morphologically comparable, in terms of size and alignment of the fibers, with healthy tissue.

Recurrences observed in only two of the nine horses treated, occurred independent of the therapy used; in fact, in one horse, the lesion recurred in the same tendon but at a different point from the first; another recurrence was caused by failure to comply with the rehabilitation protocol.

The postoperative treatment of the patient called rehabilitative physiotherapy is considered to be an essential support for the proper remodeling of the tendon; in fact, it has been demonstrated that rehabilitation time less than 6 months would increase the incidence of recurrences [2,25,29].

In terms of our results and reviewed literature, it becomes obvious that the vast number of available therapies in the treatment of equine tendons and ligaments diseases, is probably the significant evidence that none of them is potentially able to get a real “restitution ad integrum” with morphofunctional characteristics and elasticity of the original tissue [4,25,30,31], whereas the use of MSCs and PRP play an important role for tendinitis therapy.

5. Conclusions

On the basis of clinical evaluation by ultrasound and of the results obtained in the short and long term, therapy

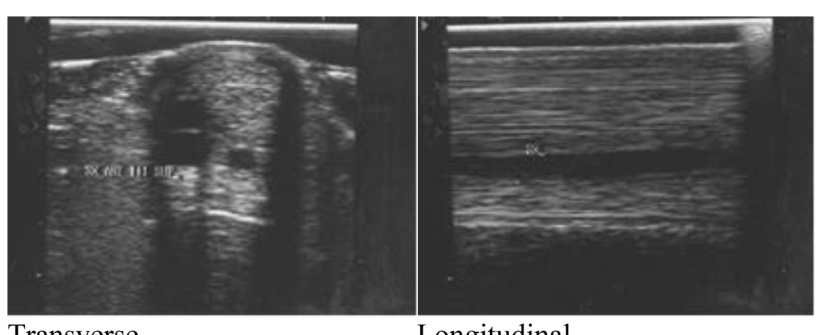

Fig. 9. Ultrasound evaluation after 120 days of surgery. Restitutio ad integrum and total alignment of fiber.

with MSC and PRP for treatment of tendon injuries in the athlete horse seems to be promising.

Moreover, a frequent ultrasound monitoring and a proper rehabilitation remain fundamental for the restoration of the biomechanical function of the structures involved. In conclusion, our results may help to improve the application of MSCs and PRP in horse outlining new aspects with regard to tissue engineering.

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