

Adipose stem cell combined with plasma-based implant bone tissue differentiation *in vitro* and in a horse with a *phalanx digitalis distalis* fracture: a case report

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ABSTRACT: A horse suffering from an undetected hoof bone fracture was diagnosed three weeks after injury. The formation of callus tissue was detected at the fracture site. Standard orthopaedic screw application was augmented by a novel method, a combination of stem cells and plasma components. For experimental therapy, fat tissue and blood samples were collected from the patient to isolate stem cells and plasma proteins. The obtained and characterised mesenchymal stem cell population was applied to the wound area, together with an implant prepared from plasma, wrapped over the orthopaedic screw. Additionally, cells with implant were differentiated *in vitro* into bone tissue, to evaluate if cells could successfully produce extracellular matrix in such material. Three weeks after application, the hoof was significantly regenerated, and after three months – the bone was completely rebuilt. The *in vitro* experiment also gave positive results, with completely differentiated cells after three weeks. Our data show that enriching the standard orthopaedic material with mesenchymal stem cells adds therapeutic value to the treatment of refractory bone fractures.

Keywords: hoof bone fracture; fat tissue; plasma implant; *in vitro* differentiation; horse

MSC = mesenchymal stem cells, **PPA** = plasma protein adhesive, **HBSS** = Hank's balanced salt solution, **FBS** = foetal bovine serum, **DMEM** = Dulbecco modified Eagle's medium

Sport horses, due to their physical exertions and significant mechanical overload of the locomotive system, are at particular risk of traumatisation. Besides tendon and ligament damage, fractures of the *phalanx digitalis distalis* are the most common contusions (Ross and Dyson 2003). With regard to the character of hoof breaks, these have been split into seven major types (Vanderweerd et al. 2009). Among the causes of hoof breaks predominate wrong horse usage, overly hard top layers and impact with hard objects when on the course. Standard treatment includes junction of the hoof bone with an orthopaedic screw, often with an application of special horseshoes, which amortise biomechanical overloads within the broken bone. Most fracture types have a positive prognosis; however, the healing course is prolonged and connected with a long convalescence period lasting up to eight

months (Keegan et al. 1993; Vanderweerd et al. 2009; Rijkenhuizen et al. 2012). Screw application is also connected with inflammatory processes, which prevents rapid regeneration. Also, prolonged immobilisation of the limb imposes many negative consequences. The most relevant are deformations of hooves and degradation of hoof joints or coronary joints. Unhealed bone ruptures can cause permanent lameness, excluding the horse from further sport activities (Sherman et al. 2007; O'Neill and O'Meara 2010). All types of *phalanx digitalis distalis* ruptures have a positive prognosis, but in case of significant tissue loss or comminuted fractures, the regeneration process is severely limited. However, tissue engineering methods have resulted in the development of novel therapeutic strategies (Dimitriou et al. 2011). Autologous mesenchymal stem cells injection represents an alternative to the

traditional bone rupture treatment methods. Their presence in fat tissue, as well as beneficial therapeutic influence of their application in locomotive system disorders has been proven by many researchers (Black et al. 2008; Zhu et al. 2008; Frisbie and Smith 2010). Their multipotential and anti-inflammatory properties provide scar-free tissue regeneration, helping to restore an organ functionality *ad integrum* (Charbord et al. 2011). The efficiency of stem cell application is greatly dependent on the type of scaffold in which cells are suspended prior to implantation (Bootle-Wilbraham et al. 2001; Osathanon et al. 2008). In bone regeneration, fibrin-derived autologous implants are among the most promising materials. These contain a natural web, including many endogenous growth factors (such as transforming growth factor beta, platelet derived growth factors or vascular endothelial growth factors) that together activate and accelerate the rebuilding processes, promoting neovascularisation or decreasing the local inflammatory process. They also represent a suitable environment for mesenchymal stem cell (MSCs) growth (Bolta 2007). The efficiency of bone formation in this kind of biomaterial was previously shown by simultaneous *in vitro* differentiation of MSCs-growth plasma proteins adhesive (PPA) (Zuk et al. 2002). The present case report describes the experimental treatment of a horse suffering from unhealed hoof bone rupture, with combined autologous adipose-derived mesenchymal stem cell (AD-MSC)/PPA therapy. The effectiveness of treatment in this case was confirmed using X-ray examination, three and twelve weeks after the procedure. We show that the addition of stem cell-containing plasma implants accelerates the regeneration process, restoring the animal's health.

Case description

Ethical and owner approval

The experiment, as an autologous application, did not need to be approved by the Bioethical Commission, as stated by the Second Local Bioethical Commission at the Department of Biology and Animal Breeding, at University of Environmental and Life Sciences in Wrocław, Chelmonskiego 38C (dec. number 177/2010 from 11.15.2010). The horse owner's agreement to the experimental therapy was approved in writing.

Patient – a horse with a *phalanx digitalis distalis* fracture

The patient subjected to this treatment was a standard bred horse, nine years old. It had been diagnosed with a *phalanx digitalis distalis* fracture three weeks after the injury emerged. This damage was caused by mechanical impact. X-ray pictures were taken during the first examination after the accident.

Cell isolation and blood collection

The patient's tail base area was disinfected and incised with a sterile surgical blade. Next, a sample (2 g) of fat tissue was dissected and placed into sterile normal saline; then the wound was sutured. Additionally, 250 ml of whole venous blood was collected into a blood container, containing anti-coagulant (Ravimed, Warsaw, Poland).

Cell isolation

The tissue sample was processed under a laminar flow hood. Traces of blood were removed by extensively washing in Hank's balanced salt solution (HBSS) and the tissue was then cut into small pieces. After that, it was put into digestion buffer comprised of collagenase and trypsin (0.2% collagenase; 0.25% trypsin, both from Sigma) and placed in an incubator for 40 min at 37 °C. Next, the tube with digested tissue was centrifuged for 10 min at 1200 × g at room temperature. The supernatant was discarded, and the cell pellet on the bottom of the tube was suspended in high glucose (4.8mM/l) Dulbecco's modified Eagle medium (DMEM) with 10% foetal bovine serum (FBS) and cultured at 37°C/5% CO₂, 95% humidity. After two passages (about one week), cells were used for therapy application and for control *in vitro* experiments. All procedures were performed on the basis of previously published experimental protocols (Zhu et al. 2008).

Osteogenic differentiation

For differentiation into bone tissue, experimental implants with cells and monolayers grown in plastic cell culture vessels (24-well plates) were treated with osteogenic medium, containing 0.1µM dexamethasone, 50µM ascorbate-2-phosphate and

10mM β -glycerophosphate (Zuk et al. 2002). The medium was changed every two days.

Plasma protein isolation

Plasma protein isolation was carried out based on a method published by Glynn and William (1980) with minor modifications. In brief, whole blood was centrifuged for 10 min at $300 \times g$ to obtain a clear plasma layer; this was then collected, frozen and stored at a temperature below -80°C . Just before the planned therapeutic application (about one week, when the cells were ready to use) the plasma was thawed at 4°C . After thawing the plasma was centrifuged at $2000 \times g$ for 15 min at 4°C to pellet the particulate fraction. The upper half of the plasma was then discarded, and the pellet was re-suspended in the remaining volume of plasma, to generate the fibrin glue.

Plasma protein adhesive scaffold preparation

The plasma was mixed with high glucose DMEM with 10% FBS containing 5×10^5 AD-MSCs (3 : 2), and 102mM CaCl_2 was then added. Preparations were incubated for 15 min at $37^\circ\text{C}/5\% \text{CO}_2$, in 6-well plates. After plasma clotting, CaCl_2 was removed by three washes with PBS. Finally, high glucose DMEM with 10% FBS was added and plates were cultured at $37^\circ\text{C}/5\% \text{CO}_2$, 95% humidity. All cultures were checked daily with an inverted microscope. After seven days of culture, and after fixation in paraformaldehyde and permeabilisation with Triton X-100, fluorescence staining with green-labeled FITC-phalloidin and DAPI was performed to visualise the actin cytoskeleton and nuclei.

Preparation of plasma protein adhesive biofilm

3×10^6 AD-MSCs were mixed with 10 ml of fibrin glue and sterile filtered 102mM CaCl_2 was then added before transfer to a 15 cm diameter culture dish and overnight incubation at $37^\circ\text{C}/5\% \text{CO}_2$. On the next day, the biofilm was extensively washed, scrapped and wound around an orthopaedic screw. Additionally, a suspension of 6×10^6 AD-MSCs in 2.5 ml of PPA solution was prepared for the therapeutic application.

Evaluation of the efficiency of osteogenic differentiation

For histological examination, implants were taken after 1, 7, 14 and 21 days of culture in osteogenic conditions. They were extensively washed in HBSS, fixed in 10% formalin and embedded in paraffin. Next, samples were cut into 20 μm sections, deparaffinised and stained with Alizarin Red S dye for 3 min at room temperature. After washing in distilled water, they sections were observed by means of light microscopy (Axio Imager A1, Zeiss). Alizarin Red staining was also performed on the osteo-induced monolayer, with respect to non-treated implants and cellular monolayer controls. Cells were fixed in 10% formalin on the 21th day. After washing in distilled water, cells were stained with Alizarin Red S for 5 min, than washed again. Finally, they were observed under an inverted microscope (Axio Observer A1, Zeiss) with the DIC option enabled.

SEM examination

Additional SEM-EDX analyses of cultured bio-scaffolds were performed to show the quantity and topography of stored calcium and phosphorus. For elemental analyses, samples were fixed in 2% paraformaldehyde, and dried in alcohol chain with critical point drying. Elemental maps were made with scanning electron microscope Zeiss Evo LS 15 with EDX Quantax Bruker hardware.

Course of therapy

The patient was first put into general anaesthesia (isofluran, 4%, 10 min). After routine disinfection of the hoof and application of sterile foil, a hole (1 cm diameter) was drilled with an electrical drill under X-ray control. Next, the frontal fragment of the hoof was drilled with a 0.45 cm drill, followed by distal fragment drilling with a 0.32 cm drill. Then, the hole was threaded. After that, 6×10^6 AD-MSCs suspended in unclotted PPA was injected into the gap with a 1.2 mm syringe needle. Finally, the prepared screw (0.45cm) wrapped in AD-MSCs/PPA material was screwed into the drilled hole. The hoof was then dressed with an aseptic bandage, and the limb was secured with a cast bandage up to the proximal part of the metatarsus level. The adherence of connected fragments appeared not to be satisfactory,



Figure 1. Orthopedic screw wrapped with AD-MSC/PPA biofilm

because of the time elapsed since the injury. After the operation, the horse was managed with antibiotics (intravenously, gentamycin) for seven days. After fourteen days, the bandage was changed, and only the cast bandage was applied to the hoof. This bandage was kept in place for eight weeks, and was changed every two weeks. X-ray examinations were performed three and twelve weeks after operation.

RESULTS

Plasma protein adhesive scaffold

The bioscaffold obtained from fresh frozen plasma was formed into a disk shape and showed elastic and plastic properties (visco-elastic). It was also permeable to all substances required

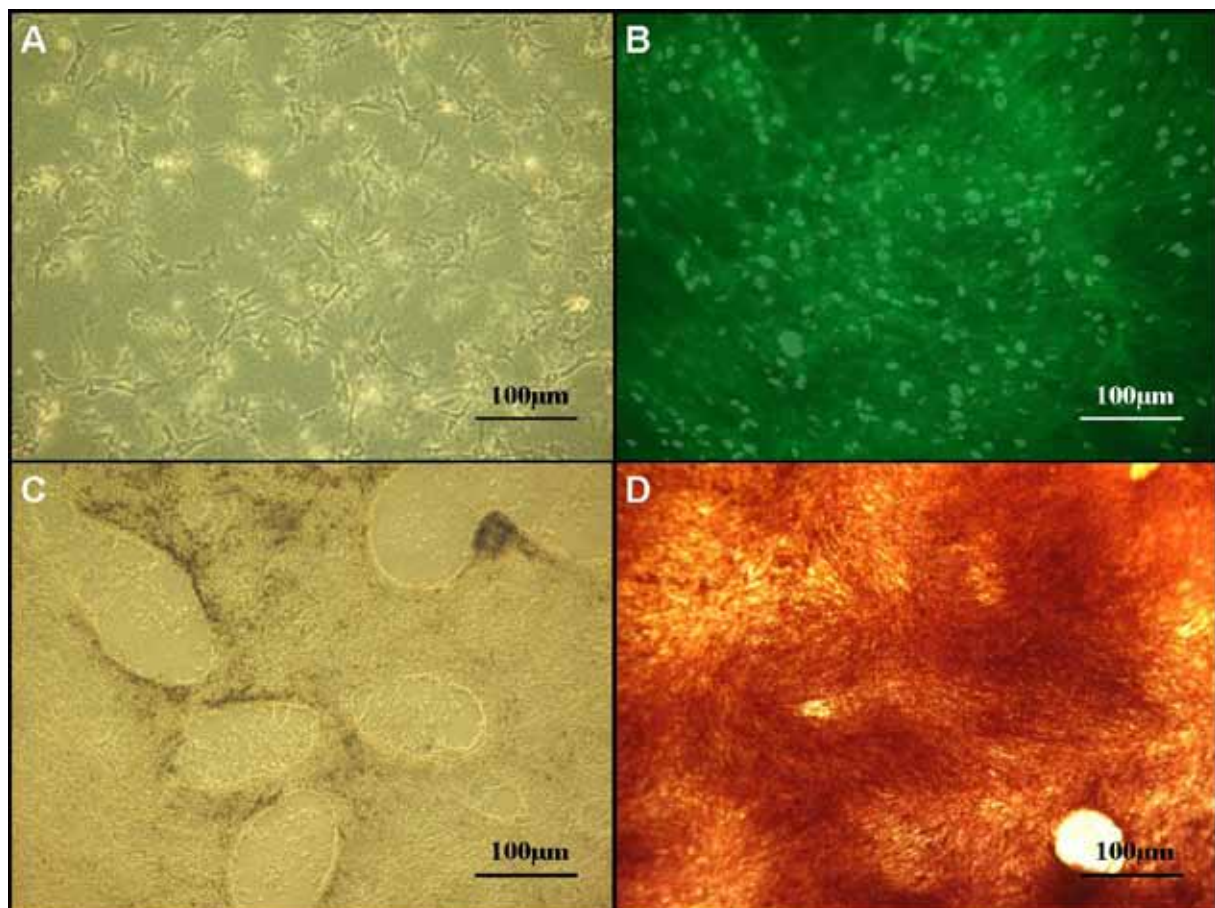


Figure 2. Evaluation of osteogenic differentiation efficiency in cultured scaffolds. Cells showed normal morphology in fibrin glue disc (A = phase contrast microscope), developed and concentrated cell-cell connections (B = fluorescence: f-actin filaments – green, nuclei – white). Changes in growth pattern in monolayer culture at 14th day (C = phase contrast microscope) were prominent. On 21th day, cells were almost completely differentiated (D = DIC/alizarin red staining)

in the regeneration process and growth of cells. This structure kept its properties also after it was woven around the orthopaedic screw as a thin biofilm (Figure 1).

Cell culture and osteogenic differentiation

After the cell isolation process, beside the adherent cells there were also red blood cells present. These were discarded after 48 h by a washing step and a medium change. After two passages the cells were used for therapeutic application and control *in vitro* experiments. Cells that were seeded into PPA showed optimal morphology, forming contacts with other cells (Figure 2A). Fluorescence staining demonstrated that they grew in a three-dimensional pattern (Figure 2B). Cells cultured in PPA, as well as cells grown in a single monolayer were successfully differentiated into bone-forming cells (Figures 2C, 2D). The structure of the fibrin glue underwent changes during this process manifested by gradual implant mineralisation. Successive changes in the morphology of the implant were also clearly visible by the naked eye (Figures 3A–D). Histological

examinations performed after 1, 7, 14 and 21 days showed increasing calcium deposits in the cultured implant (Figures 3E–H). These light microscopy findings were also confirmed by SEM-EDX map analysis, which additionally demonstrated increased phosphorus levels (Figures 3I–L).

Patient treatment

The patient's bone fracture was diagnosed three weeks after injury, following the signs of aggravating lameness. Bone callus tissue was noted, which was intercalating and prevented the adherence of the bone fragments (Figure 4A). After implantation of a pulling screw prepared with an AD-MSC bioscaffold, the gap was filled with cells mixed with PPA. After operation and during the whole healing period the animal weighted down on the limb properly and the whole post-operation period was without complications. After three weeks, X-ray pictures showed prominent gap filling with bone callus (Figure 4B), and after twelve weeks the bone structure was rebuilt, despite the width of the initial fracture (Figure 4C).

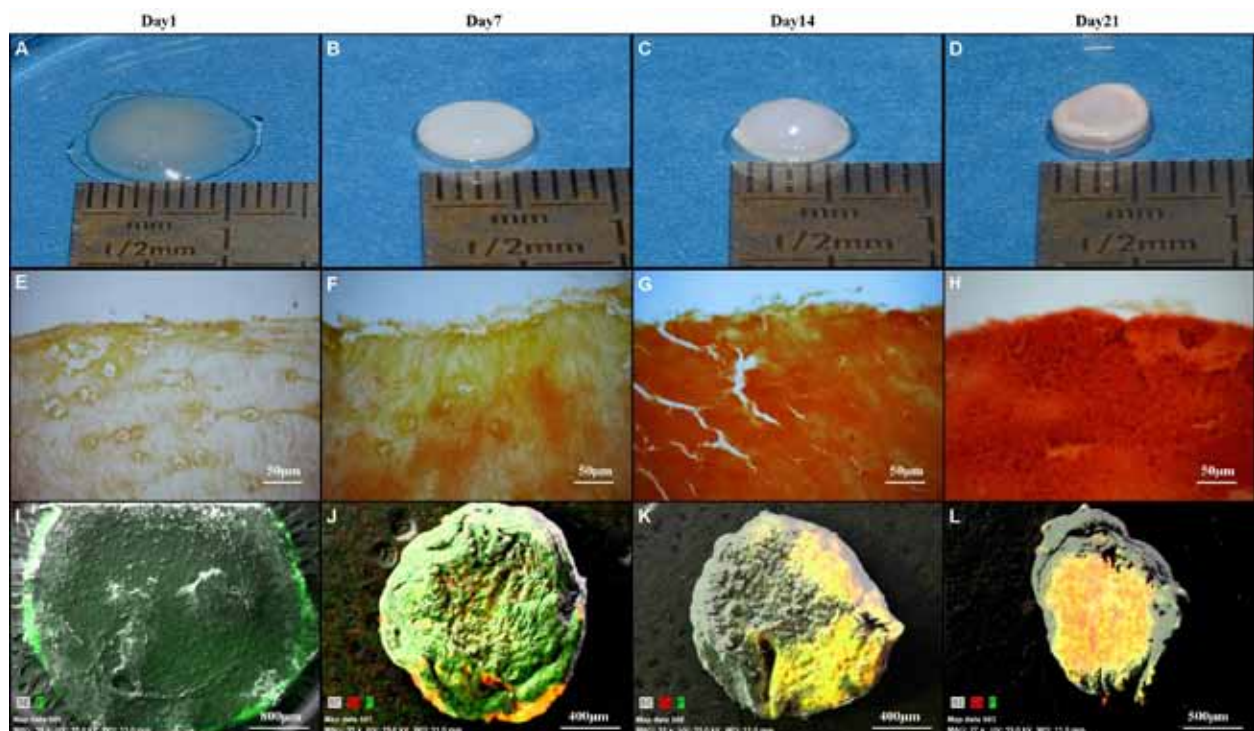


Figure 3. Implant formed from plasma protein adhesive on consecutive days (A–D). On 14th and 21th day mineralising process is visible by naked eye. Progressive calcium deposition is showed on histological cuts of scaffolds (E–H, alizarin red). SEM-EDX maps also shows gradual calcium and phosphorus accumulation. Initially, there was no calcium (I). During the next days its deposition increased (J–L).

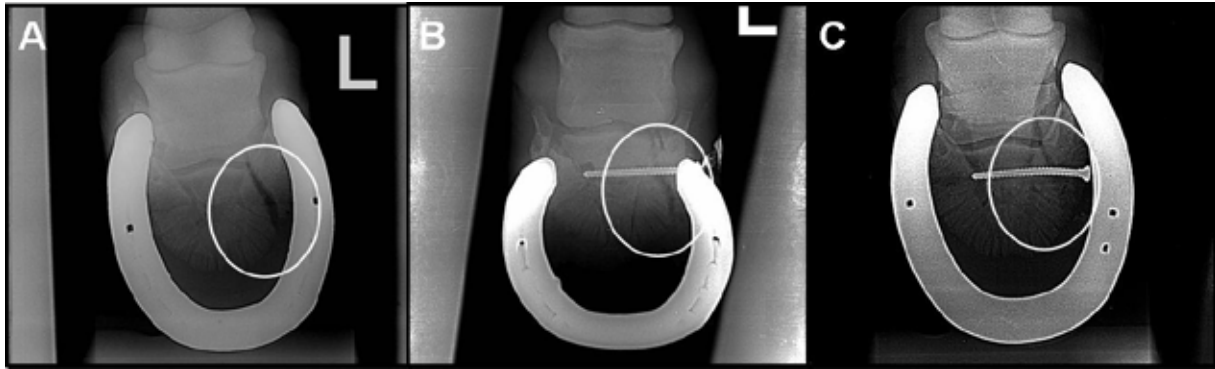


Figure 4. X-ray pictures of ruptured bone before treatment (A); three weeks after treatment, with visible filling in of bone space (B); three months after treatment, with completely regenerated bone (C)

DISCUSSION AND CONCLUSIONS

Autologous stem cells injections are one of the important new forms of locomotive disorder therapy and hold great promise (Frisbie and Smith 2010). The multilineage potential of stem cells, as well as immunomodulatory properties makes them an attractive tool for medicine (Discher et al. 2009). Indeed, the beneficial influence of stem cell application in orthopaedic diseases, mainly in animals, has recently been reported. Their uniform characteristics have allowed the establishment of general criteria for the phenotype of MSCs used for therapeutic application, in accordance with which the cells used in this experiment have been selected. This assured both the cells' origin and properties (Zhu et al. 2006, 2008).

There have also appeared many reports regarding the beneficial influence of plasma protein adhesive. This technology is often used for connecting tissue fragments during orthopaedic reconstructions; due to its natural bacteriostatic capabilities and viscoelastic properties, this method has now become widely used (Falanga et al. 2007; Kalbermatten et al. 2008; O'Ceirbheill et al. 2010; Seybold et al. 2010). When prepared correctly, this technique creates a natural biodegradable scaffold for cell growth and differentiation, (Bansa et al. 2003). Obtaining the PPA using a fresh plasma freezing method gives invaluable biomaterial, which can be used immediately or stored at low temperatures for future use. Depending on the technique, the cells can be transformed into sponges, biofilms, disks and others (Bansa et al. 2003; Rowe et al. 2007; Zhao et al. 2009). Additionally, fibrin implants completely integrate with the wound site, limiting the adverse

reactions. It was reported that mesenchymal stem cells cultured within a fibrin scaffold can be successfully differentiated into bone tissue, attesting to the stimulating abilities of fibrin itself (Bansa et al. 2003; Seybold et al. 2010).

In this study, the fracture of the *phalanx digitalis distalis* in the horse was diagnosed three weeks after the initial injury, during X-ray examination undertaken due to the prolonged lameness. X-ray imaging showed the presence of a bone callus, suggesting an old, undetected injury. The present callus tissue also prevented the healing of the broken bone, leaving a free space. In this case, bone regeneration could last longer than two months, and the regenerated tissue might gain insufficient mechanical properties. Also, prolonged healing time could bring about permanent lameness and hoof capsule deformation (Sala 2009; Vanderweerd et al. 2009; Rijkenhuizen et al. 2012) and the remaining gaps, before they overgrow with callus, could cripple the structure of the bone, resulting in renewed ruptures. Our therapy, a combined addition of AD-MSC/PPA, successfully filled out the pathologic spaces with bioactive components, resulting in the generation of a bone mass which healed the broken bone. X-ray pictures showed that cells with a bioscaffold can differentiate into normal tissue after three weeks. This gap filling typically takes over two months from the time of operation, if standard procedures are applied.

The *in vitro* experiments demonstrated the suitability of the fibrin bioscaffold for stem cell growth and differentiation into bone (Bolta 2007; Linnes et al. 2008; Seybold et al. 2010). In our study, during the first days of osteogenic incubation, calcium and phosphorus became deposited

on the scaffold's margin. After three weeks, the accumulation of these elements took place more centrally and in higher quantities. In a limited way, this image could reflect the regeneration mechanism taking place in the animal's injured tissue. Growth factors naturally present in fibrin glue activate the regeneration process by increasing cell migration, neovascularisation and tissue reorganisation (Bolta 2007). It has been shown that also the mesenchymal stem cells themselves are sources of growth factors. Upon application into damaged tissue stem cells undergo specific changes leading to differentiation into tissue and healing of the wound (Zhu et al. 2006). The introduction of an orthopaedic screw may cause inflammatory processes and induce tension as a reaction against the presence of a foreign body (Sherman et al. 2007). A combination of stem cells/fibrin glue thin biofilm, superposed on the screw could limit the inflammation, making the artificial material of the screw more acceptable. Combining these insights allowed its use on a suffering animal, which proved the immense effectiveness of these kinds of therapies. The patient's health significantly improved and normal limb function was restored. Thus, in conclusion, we here report the successful therapy of a difficult case of a bone fracture in horse, utilising the combination of two recently developed techniques, using stem cells and fibrin glue, as effective therapeutic agents.

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