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REVIEW

Therapies intended for joint regeneration in the horse

Terapias con potencial regenerativo articular en el caballo

JA Sandoval, C López, JU Carmona*

Grupo de Investigación Terapia Regenerativa, Departamento de Salud Animal,
Universidad de Caldas, Manizales, Caldas, Colombia.

RESUMEN

Las cojeras constituyen la principal causa de consulta en clínica equina y la osteoartritis (OA) representa cerca del 60 % de los casos. Durante la última década se han presentado avances significativos en nuestro entendimiento de la fisiopatología de la OA. Sin embargo, se ha avanzado muy poco en el desarrollo de tratamientos efectivos que no solo alivien el dolor y la inflamación asociados al problema, sino que también limiten los cambios degenerativos o incluso promuevan la regeneración de los tejidos articulares afectados por esta enfermedad crónica inflamatoria. Por otra parte, estos tratamientos deberían ser libres de efectos adversos como trastornos gastrointestinales, daño renal e inmunosupresión. El actual conocimiento de proteínas y células comprometidas en la biología de las enfermedades musculo-esqueléticas, como la OA, han permitido el desarrollo de nuevos enfoques terapéuticos desde el punto de vista experimental y clínico. El objetivo de esta revisión es presentar estado del arte de la terapia regenerativa articular en el caballo.

Palabras clave: caballo, osteoartritis, medicina regenerativa.

SUMMARY

Lameness is the main cause of consultation in the current equine clinic, and osteoarthritis (OA) represents about 60% of cases. During the last decade, there have been significant advances in our understanding of the pathophysiology of OA. However, there has been little progress in the development of effective treatments that not only relieve pain and inflammation associated with the problem, but also limit degenerative changes, or even promote regeneration in joint tissues affected by this chronic and inflammatory disease. These treatments should be free of side effects such as gastrointestinal disorders, kidney damage and immunosuppression. Current knowledge about the proteins and cells involved in the biology of musculoskeletal diseases, such as OA, has allowed the development of new therapeutic approaches from an experimental and clinical viewpoint. The aim of this review is to present the state of the art for regenerative therapy of joints in the horse.

Key words: horse, osteoarthritis, regenerative medicine.

INTRODUCTION

Recent advances in our knowledge about the cellular and molecular biology related to the pathophysiology of chronic musculoskeletal diseases in different animal species, including humans and equines, have led to the discovery of some suggesting possibly efficacious novel treatments for these diseases (McIlwraith 2009).The identification of key genes and proteins involved in the development of joint disease has opened a new window to achieving more effective and safer treatments with affordable costs (Sutter 2007). The current challenge is to develop therapeutic options that slow down the progress of degenerative changes in articular cartilage and other joint structures, while avoiding the adverse effects associated with conventional therapy (McIlwraith 2009). However, economic, legal and ethical constraints restrict the use of biological therapies (biopharmaceuticals) that most probably could be used successfully in these diseases.

Osteoarthritis (OA) has always been treated symptomatically in the horse using conventional therapies such as non-steroidal anti-inflammatory drugs (NSAID) and corticosteroids (CS). In recent years, there has been a growing interest in the use of nutraceuticals, such as chondroitin sulfate and glucosamine, amongst others (Neil *et al* 2005, Coudry *et al* 2007, Byron *et al* 2008, Gough *et al* 2010). These substances are intended to slow down the progression of joint damage and reduce the need for NSAID and CS (Shoemaker 2004, Carmona and Giraldo 2007, McIlwraith 2009), chronic use of which frequent occurrence of intestinal bleeding, hemorrhagic ulcerative colitis, nephropathy and immunosuppression (Malone 2002).

Equine OA and the pathological conditions associated with this disease show similar biochemical and clinical manifestations as in humans (Frisbie 2005, McIlwraith

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* Calle 65 No 26-10, Manizales, Caldas, Colombia; carmona@ucaldas.edu.co

2008, Fox *et al* 2010). OA can be classified as either primary or secondary. Primary OA is a rare *per se* genetic disorder that has been rarely reported in the horse (Reynard and Loughlin 2012, Sandell 2012). Secondary OA may appear as a sequel of joint infection or osteochondrosis (Carmona and Prades 2009, Carmona *et al* 2010). However, this disease can also be triggered by factors such as exaggerated joint overload, joint instability, single or continued trauma, hypoxia, overweight and aging, amongst others. All of the aforementioned factors or pathologic conditions can be responsible for dysregulating the expression of several genes, which leads to the overproduction of certain catabolic cytokines, mainly interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF-α) and, subsequently, the activation of pro-inflammatory nuclear factors (e.g: nuclear factor kappa beta -NFKb-) that induce joint destruction, mediated and ultimately perpetuated by matrix metalloproteinases (MMP), eicosanoids and free radicals. All of these molecules contaminate the joint space, and may either lead to chondrocyte death or decrease the metabolic capacity of these cells. In these conditions, the capacity of the extracellular matrix (ECM) to respond properly to challenges is impaired and a vicious cycle is started in which the damage to the joint increases (Argüelles *et al* 2005, Carmona and Giraldo 2007, McIlwraith 2009). Clinical signs of the disease include synovial effusion, limited joint mobility and joint deformation in chronic cases (Patan-Zugaj and Edinger 2009, Carmona *et al* 2010, Sellam and Berenbaum 2010).

There is no doubt that the era of symptomatic therapy for chronic musculoskeletal diseases such as OA is fading away and that new therapeutic ideas are heralding a new era of regenerative therapy (medicine) (Sutter 2007). However, considerable research efforts are still necessary before the actual expectations of these new therapies can be substantiated. Double-blind randomized clinical studies and strictly controlled research using animal models for musculoskeletal disease will be necessary to show the translational value of these techniques and their potential for application in human beings, so overcoming international legal frameworks that actually limit the clinical use of these therapies (Frisbie 2005, Kofron and Laurencin 2005, McIlwraith 2008). This review addresses the main regenerative treatments (experimental and clinical) that have been proposed as regenerative therapies for joint disease in the horse.

JOINT REGENERATIVE THERAPY

The main goal of articular regenerative medicine is to improve the anatomical or functional condition of diseased joints (Carmona *et al* 2011). Regenerative joint therapy aims to restore the normal structure and physiology of the articular surface, subchondral bone, ligaments, menisci, synovial membrane and other structures surrounding the affected joints, damaged either by trauma, degenerative or inflammatory processes (Fortier and Smith 2008, McIlwraith 2009). To achieve these goals, several biotechnological approaches have been proposed, including: a) gene therapy, b) the use of recombinant or autologous growth factors (GFs), c) the use of cells, d) employing procedures involving synthetic biological matrices, e) the use of cells embedded in synthetic matrices; or a combination of two or more of these therapeutic approaches at the same time (Sutter 2007, Fortier and Smith 2008, McIlwraith 2009).

GENE THERAPY

Gene therapy has not been evaluated in naturally occurring joint disease in horses. However, this species has been employed as a research model to understand the clinicopathological and biological effects of gene therapy in experimental osteoarthritis based on an osteochondral defect model (Frisbie *et al* 2002).

Gene therapy intends to introduce genes into joint cells that are most associated with the disease process, for instance chondrocytes, synoviocytes and leukocytes (Frisbie 2005, Kofron and Laurencin 2005, Zachos and Bertone 2005). Theoretically, the introduction of appropriate genes into these cells will allow an increase of the production of anabolic molecules such as GFs, especially insulin-like growth factor I (IGF-I), bone morphogenetic protein 8 (BMP-8), and anti-inflammatory cytokines such as, IL-1 receptor antagonist (IL-1ra), IL-10 and IL-4, among others (Evans *et al* 2004, Kofron and Laurencin 2005). Anabolic proteins are used for increasing the metabolic rate of the chondrocytes and subsequently to increase the production of ECM. On the other hand, anti-inflammatory cytokines are used for inducing direct antagonism of IL-1 catabolic effects or for blocking higher inflammation ways, such as the down-regulation of NF-kb.

The introduction of one or more therapeutic genes into a cell or target tissue can be achieved by direct administration of the gene into the tissue (introduction *in vivo*), or indirectly through its propagation into the tissue (cells) *ex vivo*. Genes can be introduced by viral vectors (transduction) or by chemical or physical agents (transfection) (Giannoudis *et al* 2006). The main goal in either case is that the therapeutic gene (cDNA) penetrates the target cell membrane, avoiding the lysosomal degradation machinery, and enters the nucleus, where it may integrate into a chromosome or function as an episome and eventually begin the process of transcription. Finally, the resulting mRNA is translated into the peptide or protein of interest (Giannoudis *et al* 2006).

Ex vivo transduction is the most efficient method for gene therapy, but is technically more complicated when the intention is to insert a therapeutic gene into a target cell. The viral vectors tested to date include retroviruses, adenoviruses, herpesviruses associated-adenoviruses, and

lentiviruses (Kofron and Laurencin 2005). These vectors should have the ability to infect cells without affecting their division process, have the capacity to accommodate the cDNA encoding the protein of interest, be easy and inexpensive to produce, be safe and easy to titrate, and they should not provoke an immune response (Kofron and Laurencin 2005, Giannoudis *et al* 2006).

Transfection *in vivo* or *ex vivo* has been developed as an alternative to employing viral agents and their associated problems (Evans *et al* 2004, Kofron and Laurencin 2005). Non-viral vectors include plasmids, peptides, cationic liposomes (lipofection), DNA-ligand complexes that are recognised and consumed by cellular receptors, high-speed cellular bombardment with DNA-coated gold and cell microinjection (Kofron and Laurencin 2005, Evans *et al* 2006).

GROWTH FACTORS

Recombinant growth factors. Growth factors are basically signaling proteins that regulate the metabolism of other cells (Zachos and Bertone 2005, Fortier and Smith 2008). Different *in vitro* studies performed in explants of equine articular cartilage and chondrocyte cultures have shown that equine recombinant IGF-I represented one of the most promising proteins for further research in animal models and subsequent clinical use in the horse (Fortier *et al* 2002, Davenport-Goodall *et al* 2004). In addition, GFs such as basic fibroblast growth factor (bFGF) and transforming growth factor beta 1 (TGF-β_1) have also been evaluated (Fox and Stephens 2010, Fox *et al* 2010). It has been observed in an equine osteochondral defect model that IGF-I accelerates the repair of articular defects (Fortier *et al* 2002). However, to date, according to the information reviewed by the authors, their use has not been routinely extended to equine practice. It is necessary to clarify that during naturally occurring osteoarthritis, IGF-I synovial fluid concentrations are increased in comparison with levels in healthy joints. However, chondrocytes from OA-affected joints show a decreased biological response to this GF, since IGF-I binding proteins (IGFBP) are also increased in these diseased joints. The IGFBP sequester the IGF-I and prevent its anabolic effect on joint tissues (Zachos and Bertone 2005).

Autologous platelet concentrates (APC) or platelet rich plasma (PRP). Platelets are cytoplasmic fragments derived from bone marrow megakaryocytes. Platelets are a source of several GFs and other proteins that stimulate tissue repair, reduce inflammation, induce chemotaxis in mesenchymal cells, cause cell proliferation and cell differentiation, and favor neovascularization and ECM deposition (Argüelles *et al* 2006, Fortier and Smith 2008, Carmona *et al* 2009, DeRossi *et al* 2009, Maia *et al* 2009, McIlwraith 2009, Bosch *et al* 2010). The main growth factors released by platelets include TGF-β_1, TGF-β_2, growth factors derived from platelets (PDGF), IGF-I, epidermal growth factor (EGF), hepatocyte growth factor (HGF), among others (Carson and Roach 2002, Anitua *et al* 2004, Argüelles *et al* 2006, Carmona *et al* 2011). Technically, an APC is considered a plasma concentrate with platelets and leukocytes. Normally, an APC contains between 300 and 400 x10^3 platelets per mL of plasma, and its leukocyte concentration is similar to that of whole blood (Argüelles *et al* 2006), or lower.

There is scarce information about the complex molecular mechanism which PRP acts in a positive fashion in patients with OA. However, such as it was above mentioned, GFs contained in PRP, such as VEGF, HGF, PDGF, TGF-β_1 and IGF-I have beneficial effects for cartilage and synovial membrane afflicted with OA. Angiogenic GFs, such as VEGF, HGF and, PDGF increase the vascularization of these tissues with the subsequent increase of the metabolic rate of the constitutive cells. PDGF and TGF-β_1 are chemotaxic for stem cells, particularly from synovial membrane and, this later protein, also induces differentiation of these cells to chondrocytes. Finally, TGF-β_1, PDGF and IGF-I acts as anti-inflammatory and anabolic proteins by direct down-regulation of NF-kb (Carson and Roach 2002, Anitua *et al* 2004, Argüelles *et al* 2006, Carmona *et al* 2011).

The first study in which horse platelets and consequently their GFs were concentrated by the tube method was described by Arguelles *et al.* (Argüelles *et al* 2006). In another study, Carmona *et al.* (2009) described the treatment of 4 horses with OA in different joints with APC. The results were promising, as the degree of lameness and synovial effusion were reduced for more than 8 months. This study did not include a control group, therefore the authors could only conclude that intra-articular injection of APC in horses with degenerative joint disease was safe (Carmona *et al* 2009).

A study by Abellanet (2009) evaluated the clinical effects of intra-articular injection of APC in horses with OA (30 treated horses and 12 untreated). Clinical improvement was observed in 75% of treated horses vs. 0% in the control group. The recurrence rate in this study was 30%. The horses that relapsed had severe radiographic abnormalities, primarily free bone fragments greater than 4 mm (Abellanet 2009). All these studies confirm the positive results found in humans with platelet concentrates as a treatment for OA in its early stages (Sánchez *et al* 2012, Spaková *et al* 2012).

Moreover, new reports show that the clinical use of APC acts positively on other tissues such as tendons, ligaments, muscles and skin with naturally occurring damage or experimentally induced lesions (Abellanet 2009, DeRossi *et al* 2009, Maia *et al* 2009, Bosch *et al* 2010, Lopez *et al* 2010). The approach has won great acceptation among equine practitioners, since platelet related products can be obtained from the same patient (autologous) by using different procedures or devices un-

der field conditions and are themselves free of adverse effects (Schnabel *et al* 2007, Bosch *et al* 2010). The use of APC opens a new era in the treatment of musculoskeletal diseases in the horse. To date, several manual protocols (DelBue *et al* 2007, Textor *et al* 2011, Torricelli *et al* 2011) and semi-automated devices (Schnabel *et al* 2007, DeRossi *et al* 2009, Monteiro *et al* 2009) for concentrating equine platelets have been described and some of them have been used clinically in horses with musculoskeletal disease (Monteiro *et al* 2009, Bosch *et al* 2010, Textor *et al* 2011). However, more research focused on the cellular and molecular effects of these autologous biopharmaceuticals is needed.

Autologous Conditioned Serum (ACS). IL-1 appears to be the most important catabolic protein in the development of OA (Dinarello 2011). Therefore, blockade of the cellular receptor for IL-1 might mitigate the signs of this disease. Within the IL-1 family there exists a protein that antagonises this function, which is IL-1 receptor antagonist (IL-1ra) or IRAP (Malemud 2010). Recently, the effect of autologous conditioned serum (with glass-chromium beads) was evaluated in an experimental model of equine OA (Wehling *et al* 2007). It has been observed that equine or human serum conditioned with chromium stimulates supraphysiological production of IL-1ra and other GFs and cytokines (IL-10, IL-1, TNF-α, IGF-1, and TGF-β_1, amongst others) (Baltzer *et al* 2009, Hraha *et al* 2011).

In theory, IL-1ra blocks the catabolic effect of IL-1 in the joint space (Frisbie 2005). In one investigation it was concluded that horses treated with ACS did better clinically and showed a greater degree of joint repair than the control group (Frisbie *et al* 2005, Wehling *et al* 2007). However, the clinical results of the study were less positive than those observed in the same animal model in which IL-1ra gene therapy was evaluated (Frisbie *et al* 2002). In other studies, 4 doses (at 7-day intervals) were found to be sufficient to reduce the degree of lameness and decrease synovial hyperplasia thanks the increased level of IL-1ra; no adverse effects of this treatment were seen (Frisbie *et al* 2007).

Some equine practitioners believe that this therapy, which is very expensive because it requires sterile disposable kits, an incubator and a specialized centrifuge (Hraha *et al* 2011), should be reserved for cases where conventional therapy has failed (Fox and Stephens 2010). In the market, some of the products available are Orthokine®[1] (Wehling *et al* 2007, Baltzer *et al* 2009), IRAP®[2] and IRAP II®[3] (Hraha *et al* 2011).

[1] Orthogen AG, Düsseldorf, Germany

[2] Dechra, Düsseldorf, Germany

[3] Arthrex, Bonita Springs, Florida USA

CELL-BASED THERAPIES

Allogenic and autologous chondrocyte transplantation. A research team from Cornell University created a bank of chondrocytes obtained from horses euthanized for reasons other than joint disease (Nixon *et al* 1992, Hendrickson *et al* 1994). Chondrocytes are obtained by digestion of the surrounding extracellular matrix with collagenase and can be expanded *in vitro*. These cells have been used experimentally in the horse, embedded in fibrin matrices (Frisbie 2005). These researchers recommended the use of allogenic chondrocytes, since these cells have low immunogenicity and a high capacity for replication. However, it needs to be said that more than 80% of the chondrocytes implanted in the joint defects died or disappeared from the defect site over a period of 6-8 months (Nixon *et al* 1992, Sams and Nixon 1995). It is believed that the main effect of these cells is immunomodulatory and that they accelerate joint repair mechanisms by stimulating resident cells (Sams and Nixon 1995).

In the mid-90s, a surgical technique was introduced in humans called autologous chondrocyte implantation (ACI), involving the removal of healthy chondrocytes and reinsertion after expansion outside the body into joint defects in the same patient (Peterson *et al* 2002). Frisbie and his team used this technique in horses (Frisbie *et al* 2008). Autologous cartilage from the lateral trochlear ridge of the femur was arthroscopically harvested in 15 horses aged 3 years. Chondrocytes were isolated, expanded and cultured on a collagen membrane (porcine small intestinal submucosa), and then reinserted into articular defects of 15 mm^2 on the medial trochlear ridge of the femur in the opposite femoropatellar joint. The histological and immunohistochemical results showed that the ACI treated group scored overall subjectively better compared with horses in the untreated group, or the group treated with collagen membrane alone after 12 and 18 months (Frisbie *et al* 2008).

In another study using a similar equine model, it was noticed that the use of this technique also enhanced the content of proteoglycans and collagen type II, as well as improving the appearance of the cartilage surface (Nixon *et al* 2011). Studies *in vitro* have shown differences in both cellular and structural characteristics in zones at various depths of the articular cartilage. It is thought that implants with a zonal structure could improve the quality of autologous implants by improving the structure of the newly formed cartilage and by avoiding the complications of classic ACI, such as formation of a fibrous repair tissue rather than hyaline cartilage and separation of the periosteal flap from the surrounding cartilage (Schuurman *et al* 2009).

Mesenchymal stem cells. The use of mesenchymal stem cells (MSC) to treat degenerative diseases has been one of the most controversial topics in clinical medicine and

bioethics in recent times, especially when these cells are applied in humans (Barry and Murphy 2004, Colleoni *et al* 2009). MSC can differentiate into almost any type of adult cell (multidifferential potential), such as neurons and hepatocytes. However, these cells have an inherent ability to transform into chondrocytes, osteoblasts, fibroblasts and adipocytes, amongst others (Baksh *et al* 2004, Barry and Murphy 2004, Sutter 2007, Fortier and Smith 2008, Colleoni *et al* 2009, Fox *et al* 2010).

MSC can be found in any tissue, but abound in blood, adipose tissue, umbilical cord, and especially in bone marrow (Barry and Murphy 2004, Richardson *et al* 2007, Colleoni *et al* 2009, Fox *et al* 2010). Currently, MSC have also been found in others tissues such as embryonic tissue (Paris and Stout 2010), amniotic fluid and Wharton´s jelly (Iacono *et al* 2012). In the horse, as well as in other species, mesenchymal cells can be obtained from any of these sites. However, these cells must meet certain characteristics, such as having the potential for self-renewal, the ability to generate identical copies of themselves through periods of mitotic division as well as the potential for growth *in vitro* (Baksh *et al* 2004, Fox *et al* 2010). Once obtained, the MSC are expanded *in vitro* and used clinically. It is believed that the tissue environment in which these cells are applied induces their differentiation (Smith *et al* 2003, Richardson *et al* 2007, Guest *et al* 2008, Crovace *et al* 2010).

The differentiation of MSC into chondrocytes requires that these cells be challenged with TGF-β_1 and other GFs, such as IGF-I and bFGF (Fox *et al* 2010). Additionally, it is important that these cells be cultured in a 3D matrix to avoid their dedifferentiation to fibroblasts. The most commonly used matrices include those formed with fibrin or polydioxanone. It must be noted that donor age, tissue type, medium and culture time all influence the quality and quantity of the obtained cells (Colleoni *et al* 2009).

The majority of clinical experience with the use of MSC in horses comes from the treatment of tendon and ligament injuries (Smith *et al* 2003, Richardson *et al* 2007, Guest *et al* 2008, Crovace *et al* 2010, Guest *et al* 2010, Watts *et al* 2011, Marfe *et al* 2012).

MSC have also been studied and employed for articular cartilage repair in horses (Wilke *et al* 2007, Frisbie *et al* 2009). However, there are only a few and disappointing studies in this regard. In one study it was concluded that at short notice stem cells produced an improvement of the induced articular defects. However, this therapeutic effect was not observed when long-term evaluations (8 months) were performed (Wilke *et al* 2007).

In another study, 24 horses with induced carpal OA were treated with adipose-derived stromal vascular fraction and bone marrow-derived MSC. The authors found that these cells produced increased concentrations of PGE_2 in synovial fluid in both groups of horses. Further, in the animals treated with cells derived from adipose tissue increased concentrations of TNF-α were found in the synovial fluid. The authors concluded that under the conditions of their animal model, the use of these cells as a treatment for OA could not be recommended (Frisbie *et al* 2009).

McCarthy *et al* (2012) compared equine articular cartilage progenitor cells (ACPC) with equine bone marrow-derived stromal cells (BMSC) cultured in an *in vitro* system. ACPC and BMSC have demonstrated functional equivalence in their multipotent differentiation capacity. Chondrogenic induction of BMSC resulted in hypertrophic cartilage with limited repair capacity that was predisposed to mineralisation. These changes were not produced by ACPC. According to these findings, ACPC could be considered superior to BMSC with respect to the production of cartilage with functional repair capacity (McCarthy *et al* 2012).

A recent study found that joints from healthy horses responded similarly to the injection of autologous and allogenic MSC. Injection of allogenic MSC did not generate a systemic response. Intra-articular injection of both types of MSC elicited synovial swelling, but there were no significant differences between the synovial effects of allogenic or autologous cells. These results open a new avenue for the treatment of joint diseases with allogenic stem cell therapies (Carrade *et al* 2011).

DISCUSSION AND CONCLUSION

Joint regeneration is a long-term clinical goal/working paradigm that is likely decades away from being accomplished, if ever. Certainly, the horse is one of the best models for understanding the biochemistry of the main arthropathies affecting human beings and their response to regenerative treatments. However, very little data have been obtained through controlled experimentation in equine patients with natural disease. Various conditions related to the owner and the patient itself limit rational experimentation on naturally occurring arthropathies in the horse. Not all patients have the same degree of joint disease and it is nearly impossible to acquire a group of horses with joint disease with a minimum level of clinical and biochemical homogeneity. Nevertheless, all researchers who are passionate about the topic of equine arthropathies and similar diseases should be open for data from patients truly suffering any degree of joint disease, as they may lead to better understanding of the responses to different kinds of regenerative treatments.

The use of gene therapy and recombinant growth factors has lost popularity, due to their difficulty in preparation, high costs and possible side effects. The potential employment of APC (PRP) should be heavier researched, since there is a plethora of methods for producing these substances. Each of these will produce a product with a different cellular and growth factor profile that, consequently, will produce a different joint response. Fur-

ther, proteomic and genomic studies will be necessary for elucidating the complete mechanism of action of this substance, since to now only small pieces of the puzzle become to explain the effects of PRP in patients with degenerative arthropaties.

With regard to MSC, another debate is ongoing: "the use of autologous vs. allogenic cells." Autologous stem cells carry potentially less risk of tumor development, such as teratomas, in treated tissue (Guest *et al* 2010) and incite less or no response by the body against the applied cells. The use of allogenic stem cells is very advantageous both time wise and cost wise. In addition, when allogenic stem cells are used, donor site morbidity associated with invasive procedures in the host does not occur, compared with the autologous approach (Shimomura *et al* 2010). Furthermore, it is as yet unknown whether MSC directly applied to an environment full of pro-inflammatory and catabolic substances function successfully or die (Zhang *et al* 2005). Other inevitable questions emerge when MSC are considered: do the cells need to be injected, or just the GFs released from the MSC, or even the culture media? (Kurazumi *et al* 2011, Cargnoni *et al* 2012, Oshimori and Fuchs 2012, Ryu *et al* 2013).

Finally, in spite of the booming interest and research effort in regenerative therapy of recent years, we have to conclude that the large amount of information collected in the last decade still is insufficient and further research is needed in this exciting field.

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