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ADVANCED REGENERATIVE MEDICINE FOR MUSCULOSKELETAL HEALTH



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01

ARTICLE

Tissue regeneration: an overview from stem cells to micrografts

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Tissue regeneration: an overview from stem cells to micrografts

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Abstract

Regenerative medicine represents a major challenge for the scientific community. The choice of the biological sources used, such as stem cells and grafts, is crucial. Stem cell therapy is mainly related to the use of mesenchymal stem cells; however, clinical trials are still needed to investigate their safety. The micrografting technique was conceived by Cicero Parker Meek in 1958. It is based on the principle that by increasing the superficial area of skin grafts and reducing the size of its particles, it is possible to cover an area larger than the original donor site. Stem cells are pluripotent cells that have the capacity to differentiate into all cell types and are self-renewing, whereas micrografts derive from a small fragment of an autologous tissue and exhibit limited differentiative potential compared with stem cells. Therefore, stem cells and micrografts cannot be considered equivalent, although in some cases they exhibit similar regenerative potential, which is the focus of this review. Last, stem cell therapies remain limited because of complex and costly processes, making them not very feasible in clinical practice, whereas obtaining micrografts is generally a one-step procedure that does not require any advanced tissue manipulation.

Keywords

Mesenchymal stem cell, micrografts, tissue regeneration, autologous, wound management, ulcer

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Introduction

The correct histological structure of a given tissue plays a pivotal role in allowing appropriate function and optimizing the activities to which that tissue is predisposed. Unfortunately, many factors can alter the

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physiology of a tissue. Dermal tissue is the first line of defense of the human body and it is exposed to many risks; therefore, acute or chronic lesions of the skin greatly affect the quality of life of the patient. Tissue repair and regeneration represent a major challenge for the scientific community, which is continually seeking new strategies able to promote this process, especially for tissues in which the regenerative potential is very low, such as heart, nerve, and cartilage.¹⁻³

The percentage success for regenerative medicine is closely related to the biological sources used, such as stem cells, scaffolds, growth factors, and grafts. The main role of regenerative medicine is to replace damaged tissue while maintaining its original function or, alternatively, to stimulate regeneration of the tissue itself, respecting the original histological hierarchy.

The aim of stem cell therapy is to replace a damaged or aged tissue, restoring healthy, functioning cells. In practice, stem cell therapies are based mainly on the use of mesenchymal stem cells (MSCs), which are multipotent cells with unique biological properties.^{4,5} Several *in vitro* and preclinical studies have reported that MSCs might be promising in cell therapy because of their ability to differentiate into several cell types and secrete bioactive molecules capable of stimulating recovery of injured cells through a paracrine effect of inflammation inhibition. MSCs also show a lack of immunogenicity and can exert immunomodulatory functions.⁶ The use of MSCs has been evaluated under different conditions, such as ischemic cardiovascular diseases, critical limb ischemia, bone and cartilage regeneration, and neural diseases.⁷

Tissue engineering is an emerging interdisciplinary field based on the combined use of scaffolds and biologic mediators such as stem cells and growth factors, providing a new tool for regenerative medicine. An example of tissue engineering is represented

by bioengineered dermal substitutes, which not only repair wounds, but also supply growth factors, antibiotics, and anti-inflammatory drugs, which help hasten the wound healing process.⁸

Another approach is skin grafting, the success of which depends on the engraftment rate into the recipient site. Skin grafting is the most ancient healing technique used worldwide by surgeons to close wounds and, although less ideal than flap closures, can produce a good aesthetic result.⁹ Although this approach offers a rapid and temporary solution, it can be extremely expensive, especially when the defects are wide, because the availability of donor tissue is always limited.

A valid alternative to the classical skin graft is the micrografting technique, which allows a large defect to be covered using a minimal amount of donor skin by expanding it. However, although micrografting is an appealing strategy for wound coverage, additional studies are needed to identify its true potential and pitfalls.¹⁰

Stem cells and micrografts cannot be considered equivalent entities; however, in some cases, they exhibit similar regenerative potential even though some differences exist. Stem cells are self-renewing pluripotent cells with the capacity to differentiate into all cell types; in contrast, micrografts derive from a small fragment of an autologous tissue and exhibit limited differentiative potential compared with stem cells. Recent scientific evidence has shown that micrografts derived by mechanical tissue disaggregation with a cut-off of 80 μm (largest aggregates) are enriched in progenitor cells expressing MSC-like markers and have strong regenerative potential.¹¹ A progenitor cell, like a stem cell, can differentiate into a specific cell type but its differentiation is more specific and committed to its “target” cell. The most important difference between stem cells and progenitor cells is that stem cells can replicate

indefinitely, whereas progenitor cells can divide only a limited number of times.¹²

In the following sections, we will elucidate the main applications, benefits, and limitations of stem cells and micrografts.

Stem cells: definitions, therapy, and limitations

The concept of stem cells was introduced at the end of the last century to account for the regenerative capabilities showed by some tissues. “Stemness” is a defining characteristic of cells able to differentiate into more specialized cells because of pluripotency and self-renewal properties. Pluripotency is the ability to differentiate into all three germ layers: endoderm, mesoderm, and ectoderm,¹³ whereas self-renewal is the capability of unlimited cell replications while maintaining the same differentiative stages.¹⁴ Therefore, stem cells can duplicate practically limitlessly and, to different extents, differentiate into various lineages, making their use appealing when the aim is regenerating damaged tissue in complex injuries.^{5,15–17} The clinical significance of these populations of stem cells led to 30 years of research in which the greatest challenge was identifying human stem cells and determining how to isolate them. Today, stem cells are classified as embryonic stem cells, adult stem cells, MSCs, and, finally, induced pluripotent stem cells.¹⁸ A significant milestone was the pioneering work by Owen and Friedenstein, who first described a population of clonogenic, non-hematopoietic stem cells that were able to differentiate into several lineages, including cartilage, bone, and adipocytes.¹⁹ Because of their multipotency, they were named mesenchymal stem cells.²⁰ Contradictory findings about their origins, development, and biological function have prompted the scientific community to better investigate the role of MSCs; however, despite

the inadequate understanding of MSCs, ongoing efforts are being made to use these cells in a clinical setting.²¹

MSCs are applied in different fields of medicine, including orthopedics, wound healing, neurology, oncology, and many others. MSCs are the most investigated stem cell population because of their biological and immunomodulatory properties in addition to ease with which they can be collected from many tissues. A recent review summarized MSC-based clinical trials, either complete or ongoing in the database of the United States National Institutes of Health, including trials for hematological disease, graft-versus-host disease (GvHD), organ transplantation, diabetes, and other diseases involving liver, kidney, lung, cardiovascular system, bone, cartilage, neurological, and autoimmune. The authors of these studies concluded that, in most cases, MSC therapy was efficient and promising in terms of safety, indicating that MSC infusion and administration are well tolerated.⁷ However, the potential risk of MSC transplantation should be considered based on long-term observations. Other studies focused on MSC paracrine properties, including the release of extracellular vesicles containing mRNAs and regulatory micro-RNAs and secretion of a large number of regulatory substances.^{22,23} However, if the paracrine effect of MSCs represents a risk, it can also offer an opportunity to develop new therapeutic approaches combining nanomedicine and stem cell therapy to create a novel class of next-generation drug delivery systems. By far, the most widely used source of MSCs is adult bone marrow followed by adipose tissue and, in rare cases, discarded tissues, such as umbilical cord and placental cells.²⁴ In this regard, human adult adipose tissue may be a suitable alternative source of MSCs because adipose stem cells can be largely extracted from subcutaneous adipose tissue and used

in surgical practice for tissue remodeling.^{25,26} In fact, adipose-derived stem cells can undergo multi-lineage differentiation and secrete growth factors that enhance wound-healing processes by promoting angiogenesis, and hence increase local blood supply.²⁷

In recent years, the first stem cell-based therapy—named Holoclar—received market authorization by the European Medicines Agency (EMA).²⁸ This therapy can efficiently replace damaged corneal epithelial cells and is based on the use of ocular limbal cultured stem cells. The most important characteristic of a new therapy for wound healing or epithelial graft is the maintenance of an adequate number of stem cells or progenitors to ensure engraftment of the injured area and increase the success rate of treatment.²⁹

Despite these efforts, very few of the 900 clinical trials officially registered have reached clinical significance (<https://clinicaltrials.gov/>); among 19 reported industry-sponsored phase III clinical trials, only 10 were completed and even fewer had publicly available data. Even though those clinical studies using “mesenchymal stem cells” or “medicinal signaling cells” are indicated as safe, results for the majority of listed trials are unavailable.

Among the completed studies, three have been disclosed allowing for public consideration: allogenic marrow MSCs for GvHD, autologous marrow MSCs for heart diseases, and allogenic adipose MSCs for Crohn’s fistula diseases.²⁷ Osiris Therapeutics (Columbia, MD, USA) received Health Canada approval via a Notice of Compliance with Conditions for Prochymal for the treatment of children with refractory GvHD. Prochymal is a cryobanked product containing marrow-derived MSCs sourced from healthy volunteers. Subset analysis showed that children with GvHD were responsive to MSCs.³⁰

MSCs were the first approved allogeneic regenerative medicine product in Japan. In 2003, JCR Pharmaceuticals (Hyogo, Japan), using the licensed technology of Osiris Therapeutics, developed TEMCELL for acute GvHD, which was approved in 2015 by the Japanese Ministry of Labour. The first unambiguously successful use of MSCs resulted from the TiGenix-sponsored trial, NCT01541579. It demonstrated that allogeneic stem cells were significantly and substantially superior to placebo in treating perianal fistulas associated with Crohn’s disease. In that trial, MSCs were retrieved from cryo storage and cultured for a few days before being administered to patients.³¹

The reason for major concern is not the contradictory research on stem cells, that has led to very few market approvals, but the increasing number of unregistered treatments largely in private clinics around the world that lack approval from regulatory bodies. There is an urgent need to educate patients, doctors, and researchers about MSCs and what the therapeutic opportunities truly are.³²

From a regulatory perspective, any treatment or procedure that involves manipulation of stem cells is classified as an advanced therapy medicinal product, which is subject to strict criteria from the regulatory agencies.³³ In the last 20 years, the scientific community has experienced exponential growth in clinical trials involving such cells, but unfortunately the scientific rationale and clinical efficacy are unclear, highlighting the need for a different approach to hasten their translational application. Cells should be described not only based on their origin tissue but primarily on their differentiation capacity, as established by rigorous assays.

In summary, despite advances in stem cell therapy, very few clinical results have been achieved until recently. Large-scale clinical trials are necessary to investigate

the safety of stem cells, especially immediately and years after transplantation.

Micrografting: theory and applications

The micrografting technique was conceived by Cicero Parker Meek at the University of South Carolina Aiken in 1958.³⁴ Micrografting is based on the concept that by increasing the superficial area of a skin graft by cutting the graft into smaller “micrografts,” it is possible to cover a wound larger than the original donor site. Micrografting was first applied to the treatment of burns because of a lack of available donor sites for skin grafting. The original Meek technique was complex because the micrografts were placed with the dermal side down to achieve optimal survival. Because this requirement of dermal orientation was impracticable, especially with very small graft fragments, the technique did not gain widespread clinical application, and it was eclipsed in 1964 after the introduction of mesh skin grafts by Tanner et al.³⁵ Subsequently, several modifications were made to overcome the limitations; in 1993, Kreis et al. modified Meek’s technique by using a dermatome running on compressed air to obtain widely expanded postage stamp autografts. This modification, combined with the use of cultured grafts or allografts, improved the treatment of severe burns, reaching a percentage of wound coverage of 75%.³⁶ A recent literature review of Meek and mesh techniques indicated that micrografting can be used in burns of >30% total body surface area (TBSA), even with inadequate donor sites and in patients with comorbidities, such as in patients with diabetes with very low vascularity and low metabolic demand.³⁷ To date, the Meek micrografting technique is often used for extensive burn management,

and the micrografting concept is strongly related to this context.^{38,39}

Micrografting has subsequently been applied in other clinical settings, such as hair transplantation, hair restoration of the scalp, and regeneration of the face after burn injuries, particularly eyebrow reconstruction.^{40,41} The micrografting approach can be also used for treatment of vitiligo by melanocyte transplant or autologous punch grafting, resulting in rapid and satisfactory pigmentation.^{42,43}

In recent years, a new clinical approach has been developed related to the micrografting concept, called “Rigenera micrografting technology” (Human Brain Wave LLC, Turin, Italy). The objective of this technology is to mechanically disaggregate autologous tissue, with a calibrated size of 80 μ m, collecting autologous micrografts enriched in progenitor cells, growth factors, and particles of extracellular matrix derived from the patient’s own tissue. This technology was developed after several years of experimental and clinical research on stem cell isolation from dental pulp⁴⁴ to obtain highly viable calibrated micrografts that express MSC markers. In vitro studies have shown that micrografts obtained mechanically by selecting particles with a cut-off of 80 μ m are positive for MSC markers such as CD73, CD90, CD115, and CD146 and negative for hematopoietic markers such as CD34 and CD45^{14,45–47} only. Additionally, these studies have reported cell viability ranging from 70% to 90%. These data support the regenerative potential of micrografts, and several studies have reported the ability of micrografts to differentiate into chondrocytes, osteocytes, and adipocytes.^{47,48}

In vitro studies have shown that micrografts exhibit a fibroblast-like morphology when cultured and have confirmed the expression of MSC markers. When combined with collagen sponges, micrografts can form a viable and proliferative

bio-complex, enhancing their regenerative potential.⁴⁹

Clinically, these micrografts have been applied in different fields of medicine such as dentistry, dermatology, orthopedics, and especially wound care. The first clinical application of autologous micrografts was reported in the dentistry field, showing that micrografts derived from human dental pulp or periosteum promote periodontal regeneration⁵⁰ and bone regeneration in the atrophic maxilla⁵¹ and can preserve the alveolar socket after tooth extraction by reducing bone resorption and increasing new bone formation.⁵² Furthermore, a recent study reported the ability of autologous micrografts to promote sinus lift augmentation.⁵³

In contrast, the first application of micrografts in wound care was reported 5 years ago, in which the authors described two clinical cases of complex postoperative wounds. In the first case, after abdominoplastic surgery, a woman developed necrosis at the end of the wound flaps and was initially treated with vacuum-assisted closure (VAC) therapy without any improvement of wound margins with respect to skin surface. The application of autologous skin micrografts showed a gradual improvement of the wound's margins with leveling to the skin surface.⁵⁴ In the second case, a man underwent numerous different surgical interventions due to the presence of adhesions on the colon, ascites, an entero-cutaneous fistula, and other complications. He was first treated with VAC therapy and then with autologous micrografts, resulting in progressive improvement of wound surface and size reduction.⁵⁴

One advantage of a micrograft suspension obtained by mechanical disaggregation is that the suspension can be used alone or in combination with different scaffolds or biomaterials to form a bio-complex. Micrografts have been combined with a collagen sponge to treat a post-traumatic

lesion previously treated with two radical debridements and negative pressure therapy; the authors reported improvements of both the re-epithelialization process and softness of the lesion.⁴⁶

Micrografts obtained by mechanical disaggregation of autologous tissue have also been widely used in the management of post-surgical dehiscence in a set of patients. One case report described an oncological and immunocompromised patient who underwent decompressive spinal laminectomy and vertebral fixation, after which a dehiscence occurred. The patient was treated with advanced dressings without any benefit and started a cycle of chemotherapy. Subsequently, the patient was treated with negative wound pressure therapy for 2 months, which resulted in a reduction in diameter and depth of the wound dehiscence but not complete re-epithelialization. After application of micrografts, complete re-epithelialization was achieved within 70 days despite the patient's comorbidities.⁵⁵ A similar result on wound dehiscence was achieved in another case that showed good remission of the wound within 1 month, on average, of micrograft application and a complete re-epithelialization after 1 year.⁵⁶

Chronic wounds represent a major challenge for wound care specialists in terms of management and time, as well as representing an economic burden for the healthcare system.⁵⁷ Among chronic wounds, non-healing ulcers, especially venous ulcers in the lower extremity, occur very frequently; these wounds reduce the quality of life of affected patients and represent a social issue due to reduced mobility and social isolation. The treatment of chronic venous ulcer includes several approaches, such as compression therapy and debridement when necessary, even if these ulcers do not completely heal in some cases, even after several months.⁵⁸

The role of micrografts in the treatment of ulcers has been demonstrated in several

studies in which micrografts promoted healing of chronic leg ulcers of different etiologies, including venous, diabetic, and post-traumatic ulcers. In the study of Trovato et al., the authors reported the appearance of granulation tissue 3 weeks after micrograft application and the formation of new tissue covering the area around the lesion after 4 weeks. The authors also reported no signs of inflammation in the skin around the wound and a reduction of pain after micrograft application.⁵⁹ Similar results were observed in another independent study in which the authors evaluated the ability of dermal micrografts to improve the healing of venous, diabetic, pressure, and post-traumatic ulcers. Reductions in wound size, increased granulation, and reduced exudation were reported.⁴⁹ Another study reported that 15 patients showed wound reduction of $37.33 \pm 19.35\%$ after 2 weeks, 9 patients were healed after 8 weeks, and 13 patients were

healed 16 weeks following micrografting. The quality of scars was good, and they did not deteriorate at the 6-month follow-up.⁶⁰ In a recent study, the Rigenera micrografting technique was used in 70 cases of traumatic wounds of the lower and upper limbs; in 69 cases, complete healing of the wounds was achieved in a period between 35 and 84 days. This clinical trial was registered at <https://clinicaltrials.gov/ct2/show/NCT04030832>. On day 0, the mean surface area of the wounds was 14 cm^2 ; on day 14, the mean surface area was $9.3 \pm 1.6 \text{ cm}^2$, representing a reduction of $33.6\% \pm 11.4\%$. In the same clinical trial, patients using a visual analog scale to self-assess pain, used the Vancouver Scar Scale to evaluate the aesthetic characteristics of the lesions, and the Wound Bed Score to track the progress of the wound; all scored significant results with optimum outcomes.⁶¹

Micrografts have also been used for other non-healing wounds, such as those

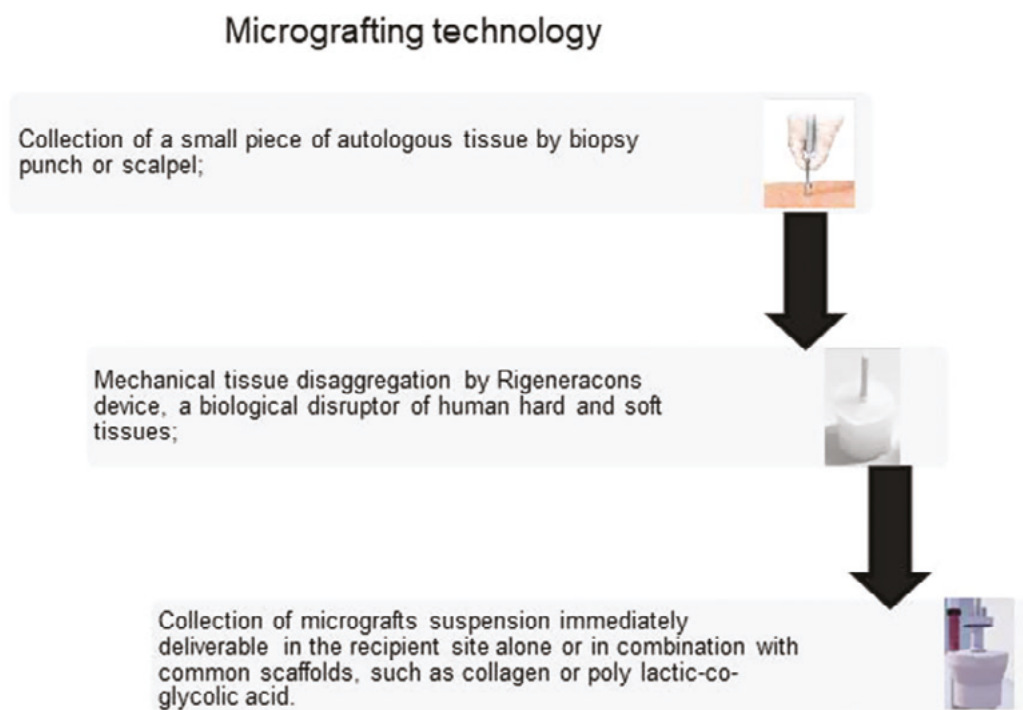


Figure 1. Representative diagram of micrografting technology.

developing following Fournier's gangrene or chronic osteomyelitis, showing a reduction in wounds after approximately 1 month of treatment.^{62,63} The efficacy of micrografts has also been reported in repairing cartilage defects, for cartilage regeneration in patients affected by external nasal valve collapse,⁶⁴ and the treatment of function-limiting and painful knee chondral injuries.⁶⁵ An in vitro study showed that autologous micrografts influenced chondrocyte differentiation, increasing deposition of glycosaminoglycans and the presence of collagen II in primary human cells cultured in the presence of micrografts, which supported the formation of chondrogenic micro-masses and acted as a scaffold for chondrocytes.⁶⁶

Finally, a recent clinical trial reported good outcomes in terms of safety and feasibility using autologous micrografts derived by atrial appendages during on-pump coronary artery bypass graft surgery for epicardial transplantation.⁶⁷ Figure 1 shows the different steps to obtain autologous micrografts by mechanical disaggregation. From a biological point of view, a recent in vivo and in vitro study showed that micrografts improve skin re-epithelialization by accelerating the migration of fibroblasts and keratinocytes by activation of the extracellular signal-regulated kinase (ERK) signaling pathway. Specifically, micrograft-treated wounds showed an improvement in granulation tissue formation, organized collagen content, and newly formed blood vessels.⁶⁸

In summary, good clinical evidence supports the role of autologous micrografts in different medical settings of tissue repair and regeneration, such as bone and cartilage regeneration, cardiac applications, and wound care management.

Conclusions

Despite recent progress in stem cell therapy, few clinically significant results have been

reported. The clinical applications of stem cell therapy are reduced by the complexity of the technique and the enormous expense, making this approach time consuming and not very feasible. There is a growing need for more practical and reasonable approaches, especially for diseases that are not life threatening. In this context, micrografts represent an innovative tool for clinicians because of their ability to stimulate tissue regeneration. Additionally, from a regulatory perspective, micrografts are considered a normal graft and do not require extensive manipulation before application, resulting in a greater safety profile.

Declaration of conflicting interest

All authors belong to the Scientific Division of Human Brain Wave SRL (Turin, Italy), the owner company of the Rigena technology cited in this review and developed to obtain autologous micrografts.

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ARTICLE

In Vitro and In Vivo Studies of Alar-Nasal Cartilage Using Autologous Micro-Grafts: The Use of the Rigenera® Protocol in the Treatment of an Osteochondral Lesion of the Nose

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Article

In Vitro and In Vivo Studies of Alar-Nasal Cartilage Using Autologous Micro-Grafts: The Use of the Rigenera[®] Protocol in the Treatment of an Osteochondral Lesion of the Nose

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Abstract: Cartilage defects represent a serious problem due to the poor regenerative properties of this tissue. Regarding the nose, nasal valve collapse is associated with nasal blockage and persistent airway obstruction associated with a significant drop in the quality of life for patients. In addition to surgical techniques, several cell-based tissue-engineering strategies are studied to improve cartilage support in the nasal wall, that is, to ameliorate wall insufficiency. Nevertheless, there are no congruent data available on the benefit for patients during the follow-up time. In this manuscript, we propose an innovative approach in the treatment of cartilage defects in the nose (nasal valve collapse) based on autologous micro-grafts obtained by mechanical disaggregation of a small portion of cartilage tissue (Rigenera[®] protocol). In particular, we first analyzed in vitro murine and human cartilage micro-grafts; secondly, we analyzed the clinical results of a patient with pinched nose deformity treated with autologous micro-grafts of chondrocytes obtained by Rigenera[®] protocol. The use of autologous micro-graft produced promising results in surgery treatment of cartilage injuries and could be safely and easily administrated to patients with cartilage tissue defects.

Keywords: Rigenera[®] protocol; autologous micro-grafts; chondrocytes; tissue engineering approaches; nasal valve collapse

1. Introduction

Osteochondral pathologies may result from trauma or sports injuries or be the consequence of disorders associated with cartilage degeneration, such as chondromalacia and osteoarthritis [1,2]. Irrespective of the etiology of the osteochondral pathology, the loss of cartilage, evidenced in magnetic resonance images (MRI) as a thickening of the cartilage line, causes pain and physical disability [3–5]. In addition, regarding nasal cartilage, obstruction at the level of the nasal valve is a common and long-recognized cause of nasal blockage [6,7]. It is estimated that about 60% of nasal obstruction is

limited by the lateral nasal wall and internal valve in some way. The surgical treatment of nasal valve collapse has proven to be difficult over the years, either resulting in unfavorable cosmetic results or resulting in persistent airway blockage [8].

In recent years, many efforts have been made in developing complementary strategies aimed at regenerating the damaged cartilage or shortening the natural regeneration process. In addition to standard surgical techniques based on the use of “spreader” grafts to reconstruct the internal nasal valve [9], various approaches have been proposed to stimulate the production of new cartilage. Biostimulation techniques have included physical approaches, such as the use of high-frequency biostimulation during surgery [10] or low-power laser targeting the osteochondral defect [11], and biological approaches, such as autologous chondrocyte implantation [12] and the use of platelet-rich plasma to stimulate the production of new cartilage [13].

Stem cells play a pivotal role in tissue repair and regeneration in response to injury. The finding of stem-like cell populations with multipotent features in various tissues has motivated the use of these cells for regenerative purposes [14,15]. In fact, mesenchymal stem cells are widely studied in tissue engineering therapeutic approaches [16–18]. In particular, for the correction of joint defects, even if Mesenchymal stem cells (MSCs) were derived from different mesoderm tissues, the availability of ready-to-use methods for a safe and effective isolation of mesenchymal stem cells during surgery has been a bottleneck for the clinical use of multipotent cells. In addition, the identification of stem-like cells in many other tissues suggests additional uses of autologous micro-grafts preparation and transfer [19]. Tissue engineering approaches aim to develop new strategies in order to improve interfacial interactions between biomaterials and cells, and to design novel surface modifications to control these interactions in diagnostic systems, medical devices, and for tissue-regenerative purposes. In this regard, electrospraying-related technologies, such as electrospinning, have been extensively studied to create scaffolds used in cell proliferation analysis. Several *in vitro* studies showed that living cells could be directly electrospun successfully on different organic polymers [20,21]. Although these techniques have been used in *in vivo* animal models, they cannot be still applied to clinical procedures on humans.

The Rigenera[®] protocol is a novel strategy for tissue mechanical disaggregation, which allows obtaining autologous micro-grafts enriched with stem cells during surgery [22,23]. Compared to enzymatic techniques, mechanical disaggregation has claimed to be faster and safer. So far, the Rigenera[®] protocol has shown clinical efficacy in the management of complex wounds [24,25] and for the regeneration of the bone in periodontal surgeries [26]. In this preliminary study, we initially analyzed micro-grafts obtained from human and murine auricular cartilage tissues with *in vitro* tests in order to characterize the population of cells obtained by the mechanical disaggregation. Secondly, we described the clinical and functional outcomes of cartilage autologous micro-grafting obtained with the Rigenera[®] protocol in a patient affected by external and internal nasal valve collapse, so-called pinched nose deformity.

2. Results

2.1. Chondrocyte Murine Micro-Grafts Analysis

Hoechst 33342 staining of suspension obtained with Rigenera[®] protocol from murine auricular cartilage showed a population of single cells and clusters of micro-grafts containing small groups of intact cells. Morphology and brightness of nuclei together with the ability of the dye to pass the cell membranes indicate that the majority of cells were living cells (Figure 1A,B). The single cell portion of the suspension was found positive by FACS (fluorescence-activated cell sorting) analysis for surface markers CD44 (25%), CD90 (30%), and CD117 (5%), and concomitantly negative for hematopoietic marker CD45 (<2%), thus confirming their mesenchymal origin. RNA from the micro-grafts was quantified (34.5 ng/ μ L) and subsequently analyzed with quantitative real-time PCR (qRT-PCR) (Figure 1C). Our results indicate that murine micro-grafts expressed CD44, CD90, and CD117 surface

genes, even if their levels were lower with respect to murine MSCs, thus indicating the mesenchymal phenotype of the micro-grafts. In addition, cartilage murine micro-grafts showed the expression of Sox-9 and Collagen II (COL2A1) genes, key genes involved in articular cartilage differentiation. In fact, Sox-9 is identified as a regulator of the chondrocyte lineage [27], while COL2A1 encodes the alpha-1 chain of type II collagen, a fibrillary collagen found in cartilage [28]. The expression of Sox-9 and COL2A1 were expressed at the same level with respect to murine MSCs in proliferative medium, indicating that the micro-grafts obtained from auricular cartilage contained chondrocyte-progenitor cells with a mesenchymal genotype (Figure 1C).

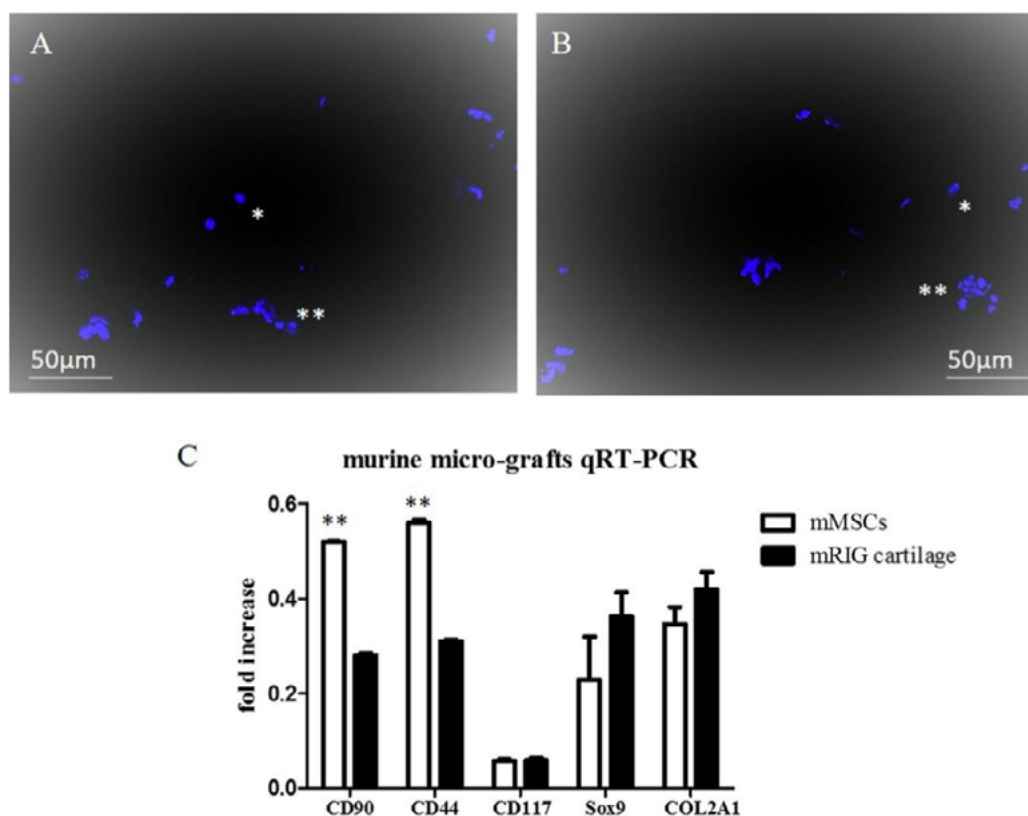


Figure 1. (A,B): Hoechst 33342 staining on micro-grafts suspension obtained from murine auricular cartilage. (*) represents single nuclei cells, while (**) represents clusters of micro-grafts containing small groups of living cells. Magnification at 20 $\times$. (C): Gene expression analysis of the indicated specific markers as determined by qRT-PCR. qRT-PCR analyses were performed on murine MSCs cultivated in proliferative medium for 7 days and on micro-grafts obtained from murine auricular cartilage. The graph shows the fold induction of gene expression expressed in arbitrary units. Statistical significance values are indicated as *: $p < 0.05$, **: $p < 0.01$.

2.2. Chondrocyte Human Micro-Grafts Analysis

Hoechst 33342 staining of suspension obtained with the Rigenera[®] protocol from human auricular cartilage showed similar results to murine micro-grafts; in fact, single cells and clusters of micro-grafts were observed as in murine Hoechst staining (Figure 2A,B), indicating that the majority of the cells were living cells. FACS analysis demonstrated that human cartilage micro-grafts were positive for surface markers CD44 (35%), CD90 (45%), and CD117 (5%), and concomitantly negative for hematopoietic marker CD45 (<2%). Total RNA from the human micro-grafts was quantified (38.5 ng/ μ L) and analyzed with qRT-PCR. Results displayed that the cartilage micro-grafts expressed in a similar manner mesenchymal genes (CD90, CD44 and CD117) with respect to MSCs, and also tissue cartilage markers, such as Sox-9 and COL2A1 (Figure 2C).

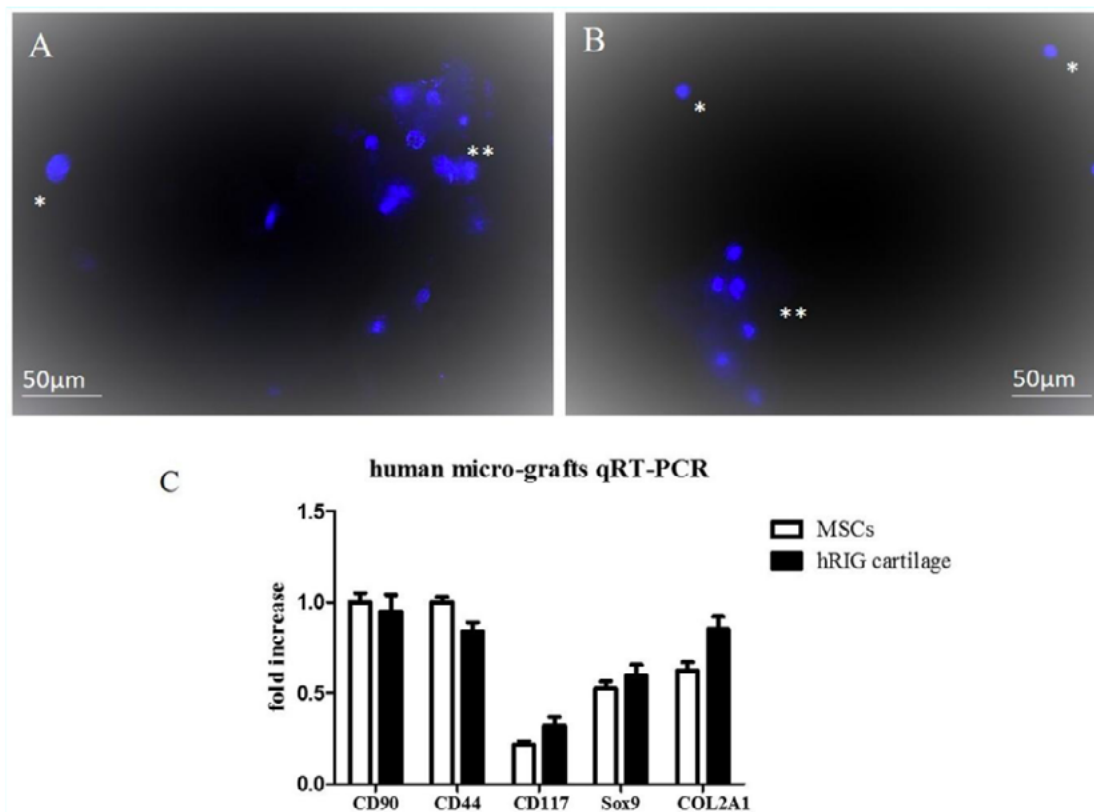


Figure 2. (A,B): Hoechst 33342 staining on micro-grafts suspension obtained from human auricular cartilage. (*) represents single nuclei cells, while (**) represents clusters of micro-grafts containing small groups of living cells. Magnification at 20 $\times$. (C): Gene expression analysis of the indicated specific markers as determined by qRT-PCR. qRT-PCR analyses were performed on human MSCs cultivated in proliferative medium for 7 days and on micro-grafts obtained from auricular cartilage. The graph shows the fold induction of gene expression expressed in arbitrary units. Statistical significance values are indicated as *: $p < 0.05$, **: $p < 0.01$.

2.3. In Vivo Micro-Grafts

The aim of Rigeneracons[®] was to disaggregate a small piece of tissue (septum cartilage strips) and opportunely select a cell population with a size of 50 μ m. Chondrocytes obtained were suitable to form autologous micro-grafts, which could be used alone or in combination with Platelet Rich Plasma (PRP) as published [29,30], and as reported in a patient analyzed in this work, to obtain a biocomplex ready to be implantable in subjects in need of such intervention. PRP was prepared with the approval of the transfusional service from a small volume of blood. The composite graft obtained (suspension of chondrocytes mixed into PRP in solid form), was applied on the external nasal valve collapse, in the alar cartilage side by fixing with absorbable stitches, improving the soft tissue volume in the site of defects. This construct, subcutaneously injected, resulted in a persistent cartilage tissue with appropriate morphology, adequate central nutritional perfusion without central necrosis or ossification, and further augmented nasal dorsum without obvious contraction and deformation. In addition, microscopic analysis of excisional fragments showed the persistence of healthy cartilage tissue with the formation of new capillaries penetrating into the cartilage as previously reported [29,30]. Here, we demonstrated that the Rigenera[®] System is a useful method to isolate human chondrocytes when cells were injected with PRP in vivo in patients affected by nasal valve collapse and pinched nose deformity or cartilage defects.

2.4. Patients and Clinical Procedure

A patient affected by pinched nose deformity (Figure 3A), selected by a group of study treated at the department of Plastic and Reconstructive Surgery of University of Rome “Tor Vergata”, Italy, with chondrocyte micro-grafts gently poured onto PRP in solid form was analyzed. Postoperative follow-up evaluation has shown optimal aesthetic results (Figure 3B) and the improvement of nasal obstruction. These composite grafts provide functional support to the alar cartilages, usually collapsed because of excessive resection during previous surgery. Trans-columellar open-tip access was necessary to allow for better visualization of the valve collapse, alar cartilage, and for the fixation of the cartilaginous structures, to allow for the placement of unexposed absorbable stitches. In the CT (Computed Tomography) scans, the pre-operative situation (Figure 4A) is shown in comparison with the regenerated site in the post-operative image. In particular, CT scans performed after 12 months (Figure 4B) show soft tissue volume improvement and the correction of the nasal septum deviation.

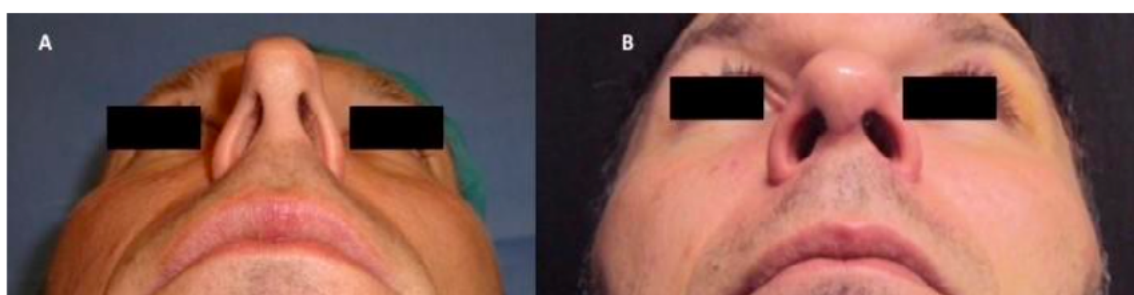


Figure 3. (A) Pre-operative situation of patient affected by bilateral external and internal nasal valve collapse with nasal obstruction produced by excessive resection during previous surgery. (B) Post-operative situation after 12 months of the same patient treated with chondrocyte micro-grafts obtained by Rigeneracons® (CE certified Class I) mixed with platelet-rich plasma (PRP) in solid form. The authors used septum cartilage cut in the strips, processed by Rigenera® Centrifuge. These composite grafts provided functional support to the alar cartilages. Trans-columellar open-tip access was necessary to allow for better visualization of the valve collapse, alar cartilage, and for the fixation of the cartilage.



Figure 4. (A) CT scans show the pre-operative situation with bilateral soft tissue defect of nasal tip and cartilage collapse. In addition, nasal septum deviation was detected. (B) CT scans of the same area after 12 months show the regenerated site in the post-operative image with soft tissue volume improvement and the correction of the nasal septum deviation.

3. Discussion

Chondral injuries are a challenging scenario for surgical reconstruction. In particular, nasal valve collapse caused by aging, iatrogenic causes, congenital or secondary injuries after trauma is associated with airway obstruction and significant effects on patient's life. Despite the good results observed with surgical and prosthetic resurfacing, there is increasing interest in developing safe and effective procedures for biologic resurfacing of cartilage tissues, thanks to the important knowledge of regenerative medicine [16–18]. Moreover, articular cartilage is an avascular structure and for this reason exhibits significant problems to repair. Regenerative-tissue engineering approaches combines chondrogenic cells and scaffolds [31]. Regarding chondrocytes, they are isolated from different cartilage tissues, such as articular cartilage, nasal septum, ribs, or ear cartilage [32]. Nevertheless, it is very difficult to grow chondrocytes *in vitro*, because of their instability in monolayer culture that also causes chondrogenic phenotype loss. Regarding scaffolds for cartilage repair, collagen-based matrices or collagen sponges are the most-used biomaterials [33]. Other scaffolds employed for cartilage repair include agarose, alginate, hyaluronic acid, or cellulose alone or in combination with artificial matrices composed by polylactic acid (PLA) and polyglycolic acid (PGA) or hydrogel [34]. Several techniques have been developed to colonize organic and inorganic polymers, such as cell electrospinning. These jet-based techniques represent a high-resolution method for the manipulation of single cells in order to create biocompatible scaffolds as composite microthreads [20,21]. Frequently, these bio-complexes composed by scaffolds and cells are enriched with platelet-rich plasma (PRP), an autologous concentrated cocktail of growth factors and inflammatory mediators that seems to have a strong positive effect on chondrocyte proliferation *in vitro* [35]. It has been demonstrated that when adult chondrocytes are cultured in alginate beads in the presence of 10% PRP, the chondrocytes proliferation is higher with respect to chondrocytes grown in classical proliferation medium [35]. However, these different cell-based strategies have not given positive results, probably because their protocols are still far from regenerating a functional and stable cartilage tissue. In addition, many studies with these technologies have passed all *in vitro* and *in vivo* mouse models, but they are not clinical-grade methods yet.

The rationale behind the Rigenera[®] protocol lies in the multipotent properties of mesenchymal stem cells, which may promote cell differentiation and subsequent new cartilage formation [23–25]. The Rigenera[®] protocol opens new surgical strategies especially for tissue repair. Using the Rigenera[®] protocol, in just one surgical time, the patient is the donor and the acceptor of calibrated micro-grafts. Several authors demonstrated that: (i) Rigenera[®] improves the healing of bone lesions as suggested by the use of micro-grafts obtained by periosteum in combination with collagen sponge [36]; (ii) Rigenera[®] is effective in the process of wound healing, improving the superficialization of the wound and reducing wound size [25]; (iii) Rigenera[®] opens new surgical strategies for rhinoplasty [29,30]. In this manuscript, we demonstrated with *in vitro* tests that micro-grafts isolated from murine and human auricular cartilage consisted of living single cells and clusters with mesenchymal properties. In addition, gene expression profiling showed similarities between human and murine micro-grafts obtained with the Rigenera[®] protocol at the transcriptional level, thus confirming their mesenchymal and chondrogenic origins. In order to understand and manage the results, we should consider the ecto-mesenchymal origin of the cranial-facial cartilage samples used in the study. The ecto-mesenchymal origin makes the head cartilage different from the other collectable cartilage tissues in the rest of the body, and this is recognizable in the perichondrium layer maintaining vascularization and a high number of progenitors, mechanically isolated by Rigenera[®]. In fact, *in vivo* treatment of a patient with pinched nose deformity with chondrocyte micro-grafts isolated from perichondria cartilage during open-tip rhinoplasty, gently poured onto PRP, showed soft tissue volume improvement and the correction of the nasal septum deviation.

In summary, our experience shows that the transfer of autologous micro-grafts using the Rigenera[®] protocol is safe and has promising results in the treatment of cartilage defects, such as pinched nose deformity. These results suggest the suitability of further prospective, controlled studies with larger

cohorts to confirm the therapeutic properties of this intervention. Finally, the Rigenera[®] protocol could be potentially used in others clinical and regenerative medicine fields, including plastic surgery and orthopedics.

4. Materials and Methods

4.1. Study Subjects

A 45-year-old male patient affected by pinched nose deformity, selected by a group of study treated at department of Plastic and Reconstructive Surgery of University of Rome “Tor Vergata”, Italy, underwent bilateral nasal alar reconstruction with chondrocyte micro-grafts gently poured onto PRP gel in solid form. The preoperative study included a complete clinical examination of the nasal pyramid and of the nasal cavities through anterior rhinoscopy, a photographic examination in three projections, and computed tomographic scans (CT). Post-operative evaluation included a clinical and a photographic examination, and at a 12-month follow-up, CT scans were conducted. Informed written consent was obtained from patients in order to use their data for research purpose. In particular, nasal obstruction was very marked in the patient analyzed; external nose analysis showed pinched nose deformity, external nasal valve collapse, and a deficit of supra-tip projection (Figure 4A). Anterior rhinoscopy and CT scans (Figure 3A) showed a left deviation of the nasal septum and stenosis of the internal valves with nasal obstruction produced by excessive resection during previous rhinoplasty.

4.2. Analysis of Micro-Grafts from Auricular Murine Cartilage

This study was conducted under EU guidelines for the care and use of laboratory animals in accordance with Italian and European legislation (D.lgs. 116/92, European Directives 86/609/EE for the protection of animals used in scientific and experimental studies and 2010-63UE) and was approved by the Ethical Committee recognized from University of Pavia.

Micro-grafts of murine auricular cartilage were obtained by Rigenera Protocol[®] (CE certified Class I). Auricular cartilage from two ears of C57BL/6 mouse were cut in strips (2 mm × 0.5 mm). The strips were subsequently cut in smaller pieces and dissociated using the Rigeneracons[®] System (HBW srl, Turin, Italy) in 1.2 mL of physiological solution. After one minute of disaggregation at 80 RPM, the suspension was harvested from the system, filtered through a 40-µm strainer and processed for FACS analysis, Hoechst staining, and RNA extraction. Cytofluorimetric analysis was performed as previously described [37] with some modifications. Briefly, micro-grafts suspension was resuspended in 0.5% BSA in PBS (FACS buffer cells) and incubated with FITC-PE monoclonal antibodies for the following CD antigens: CD44, CD117, and CD45 for 30 min at RT. Analysis was performed on a FACScan (BD Biosciences, San Jose, CA, USA). In addition, micro-grafts were centrifuged at 1200 RPM and stained with Hoechst 33342 (2 µg/mL) for nuclei labeling and samples were observed with a confocal fluorescence microscope (Nikon Eclipse80, ViCo, Nikon, Tokyo, Japan). Hoechst staining was normally used to determine the number of nuclei and to assess gross cell morphology.

Total RNA from micro-grafts was extracted by Trizol[®] reagent (Invitrogen), according to the manufacturer's instructions, and retrotranscribed into cDNA with the iScript cDNA Synthesis Kit (BioRad Laboratories) as previously reported [38]. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) analysis was performed in a 48-well optical reaction plate using a MiniOpticon Real-Time PCR System (BioRad Laboratories). Oligonucleotide primers were designed with gene sequences published in GenBank and are indicated in Table 1. Gene expression was normalized to the phosphoglycerate kinase (PGK) housekeeping gene expression. Each sample was analyzed in triplicate and correlated against a standard curve.

4.3. Analysis of Micro-Grafts from Auricular Human Cartilage

Six perichondrial tissue samples surrounding healthy ear cartilage were collected during routine surgeries and cut in strips (3 mm × 0.5 mm). The strips were subsequently cut in smaller pieces

and dissociated using Rigeneracons® System (HBW srl, Turin, Italy) in 1.5 mL of physiological solution. After one minute of disaggregation at 80 RPM, the suspension was harvested from the system, filtered through a 40-µm strainer and processed for the same analysis of murine ear cartilage (FACS, Hoechst staining, and molecular gene expression). Oligonucleotide primers were designed with gene sequences published in GenBank and are indicated in Table 1. Gene expression was normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene expression. Protocols for gene expression analysis and characterization were the same as those for mouse micro-grafts cartilage.

Table 1. Primers used for qRT-PCR on murine/human cartilage micro-grafts.

Genes	Forward Sequences	Reverse Sequences
mCD90	5' AAG TCG GAA CTC TTG GCA CC 3'	5' CCA GGC GAA GGT TTT GGT TC 3'
mCD44	5' CGA ACC AGG ACA GTG GAG TG 3'	5' TCT GCC CAC ACC TTC TCC TAC TAT 3'
mCD117	5' TGA ACG GTA ACA TGG CTG CAT T 3'	5' ACC ACC GTA AAT GTG TCC CC 3'
mSOX-9	5' AGA CTC ACA TCT CTC CTA ATG CT 3'	5' ACG TCG GTT TTG GGA GTG G 3'
mCOL2A1	5' GGC TCC CAA CAC CGC TAA C 3'	5' GAT GTT CTG GGA GCC CTC AGT 3'
*mPGK	5' CAA AAT GTC GCT TTC CAA CAA G 3'	5' AAC GTT GAA GTC CAC CCT CAT C 3'
hCD90	5' CAG CGG AAG ACC CCA GT 3'	5' CGT TAG GCT GGT CAC CTT CT 3'
hCD44	5' TTA CAG CCT CAG CAG AGC AC 3'	5' TGA CCT AAG ACG GAG GGA GG 3'
hCD117	5' GCA CAA TGG CAC GGT TGA AT 3'	5' GGT GTG GGG ATG GAT TTG CT 3'
hSOX-9	5' AGG AGA ACC CCA AGA TGC AC 3'	5' GAG GCG TTT TGC TTC GTC AA 3'
hCOL2A1	5' AGG ACT GAC CAA GAT GGG AA 3'	5' AGG GGA GCT GGC TAC TTC TC 3'
*hGAPDH	5' AGC CTC AAG ATC ATC AGC AAT GCC 3'	5' TGT GGT CAT GAG TCC TTC CAC GAT 3'

4.4. Chondrocyte Micro-Grafts Preparation for in Vivo Treatment

Autologous micro-grafts of chondrocytes for immediate clinical use were prepared using a medical device called Rigeneracons® (CE certified Class I, HBW srl; Turin, Italy). After the extraction of the perichondrial cartilage during open-tip rhinoplasty, the tissue was cut into strips (2 × 2 mm). The strips were gently collected and disaggregated under sterile conditions (vertical laminar flow hood) by Rigeneracons® (HBW srl; Turin, Italy) in 1.2 mL of physiologic solution. After 60 s of centrifugation at 80 RPM per minute, the cell suspension was collected from the system and gently infiltrated onto PRP gel in solid form. Autologous PRP was performed with specific protocol and was centrifuged, during the same procedure, according to the transfusional service, harvesting a small volume of blood from the peripheral vein of the patient. The product obtained (PRP in solid form mixed with chondrocytes) was applied on the defect in external nasal valve collapse.

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Author Contributions: Maria Gabriella Cusella De Angelis designed the research, and was responsible for the correctness of the biological analyses, and contributed to writing and editing the manuscript; Gabriele Ceccarelli performed all the in vitro biological analysis; in addition, Gabriele Ceccarelli contributed to the writing of the introduction, results and discussion sections; Pietro Gentile and Marco Marcarelli performed all the in vivo experiments. All authors approved the final manuscript.

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03

ARTICLE

Preclinical Evidence of Intra-Articular Autologous Cartilage Micrograft for Osteochondral Repair: Evaluation in a Rat Model

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Abstract

Objective. The search for an effective and long-lasting strategy to treat osteochondral defects (OCD) is a great challenge. Regenerative medicine launched a new era of research in orthopaedics for restoring normal tissue functions. The aim of this study was to test the healing potential of Rigenera micrografting technology in a rat model of OCD by investigating 2 cartilage donor sites. **Methods.** Full-thickness OCD was bilaterally created in the knee joints of rats. Animals were randomly divided into 2 groups based on the anatomical site used for micrograft collection: articular (TO) and xiphoid (XA). Micrograft was injected into the knee via an intra-articular approach. The contralateral joint served as the control. Euthanasia was performed 2 months after the set-up of OCD. Histological evaluations foresaw hematoxylin/eosin and safranin-O/fast green staining, the modified O'Driscoll score, and collagen IAI and 2AI immunostaining. Kruskal-Wallis and the *post hoc* Dunn test were performed to evaluate differences among groups. **Results.** Histological results showed defect filling in both autologous micrografts. The TO group displayed tissue repair with more hyaline-like characteristics than its control ($P < 0.01$). A fibrocartilaginous aspect was instead noticed in the XA group. Immunohistochemical assessments on type 2AI and type I collagens confirmed the best histological results in the TO group. **Conclusions.** TO and XA groups contributed to a different extent to fill the OCD lesions. TO group provided the best histological and immunohistochemical results; therefore, it could be a promising method to treat OCD after the validation in a larger animal model.

Keywords

osteochondral defects, autologous micrograft, osteochondral repair, immunohistopathology, rat model

Introduction

OCD of the knee is among the most frequent musculoskeletal disorders that impair the patients' quality of life with a great burden on the health care system.¹ To date, there are several approaches to repair osteochondral injuries after a traumatic event. Conventional debridement and various methods of penetrating subchondral bone often lead to fibrocartilage formation, which lacks the typical biochemical and biomechanical properties of hyaline articular cartilage.² Several therapeutic strategies, including autologous chondrocyte implantation (ACI) and periosteum, perichondrium or osteochondral grafting, are already used in clinics for many years.^{3,4}

To address some problems about the use of the ACI procedure, a second-generation technique was introduced

by exploiting the intrinsic potential of suitable scaffolds to drive matrix synthesis and tissue organization.⁵ This approach, based on autologous matrix-induced chondrogenesis (AMIC)⁶ or matrix-induced autologous chondrocyte

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implantation (MACI), was successful to treat large cartilage defects.⁷ Nowadays, ACI is in its fourth generation and can be regarded as an effective tissue engineering procedure.⁸

Despite the wide range of therapeutic strategies for OCD,¹⁻⁸ there is a need for identifying novel perspectives for cartilage repair to overcome current limitations and ensure a long-lasting repair.⁹ Employment of 1-step techniques is fast rising in the orthopedic field thanks to different point-of-care medical devices. This approach exploits the multipotency and paracrine abilities of progenitor cells from different tissue sources, like bone marrow or adipose tissue, thanks to their direct processing in the surgical room.¹⁰⁻¹⁴ Among 1-step strategies, the use of minced cartilage is fast emerging, based on the employment of small graft fragments to promote tissue repair by covering tissue lesions.¹⁵⁻¹⁷ The surgical technique of slicing viable cartilage into small pieces and direct reimplantation is not novel. Cicero Parker Meek conceived it in 1958 to treat skin burns.¹⁸ Then, micrografting technology was used in different clinical settings, including oral and maxillofacial surgeries, hair loss,¹⁹ several kinds of wounds,^{20,21} and bone defects.²²⁻²⁵

When referring to OCD, the feasibility of using cartilage fragments as a regenerative biological solution could benefit from a precious source of articular chondrocytes and progenitor cells entrapped in an extracellular matrix. Recently, some authors have demonstrated that primary human chondrocytes cultured with micrografts, obtained with an innovative protocol (Rigeneracons, Human BrainWave Srl, Torino, Italy),²⁶ maintain their phenotypical characteristics and promote collagen and glycosaminoglycans synthesis.²⁷ This technology, based on the use of a specific class I (CE) medical device, performs the mechanical microfragmentation of human autologous tissues in micrografts of 80 μ m in sterile saline solution by overcoming the use of enzymatic treatments. In the present study, we hypothesized that the use of micrografts, thanks to their biological properties, could have beneficial effects to treat OCD. Therefore, the purpose of this preclinical investigation was to test the efficacy of intra-articular injection of autologous micrografts got from 2 anatomical sites (articular cartilage and xiphoid appendix) in a rat model of OCD. Histological and immunohistochemical analyses were carried out to evaluate tissue architecture and typical cartilaginous markers after the micrografting technology.

Materials and Methods

Animal Experimental Design

The present study was approved by the Ethics Committee on the Use of Animals, Federal Rural University of Pernambuco

(UFRPE) with license 126/2017. The experiments followed international, national, and/or institutional guidelines for animal treatment and complied with relevant legislation. Twelve male adult albino Wistar rats (subspecies: *Rattus norvegicus*, body weight: 300 ± 50 g) were used (**Fig. 1**). The animals were kept in suitable cages under controlled illumination and temperature (12-hour light-dark cycle and approximately 23 °C), feed and water ad libitum.

At the time of surgery, rats were anaesthetized using oxygen in a flow of 3 L/min and 3% isoflurane. The pre-anesthetic action was performed by intramuscular tight administration of meloxicam 0.2% (dose of 2 mg/kg), volume of 0.4 mL; morphine 1% (dose of 5 mg/kg), volume of 0.2 mL. The maintenance was performed in an anesthetic mask diluted in oxygen 3 L/min, at an isoflurane concentration of 2.5%. Full-thickness trochlear osteochondral defects of 2.5 mm diameter and 2.0 mm depth were created bilaterally in all 12 rats (24 articular joints). Briefly, a Bard-Parker type scalpel cable mounted with blade no. 11 was used and an initial puncture of the bone with the aid of punch, in contra-angle and at right angles to the cortical bone involved were performed. Then, enlargement and deepening of the bone were created using a 2.5 mm punch (**Fig. 2A**). Once the knee joint was exposed, the joint capsule was cut for intra-articular access, the patella was displaced, allowing access to the trochlea and the extraction of a small fragment. During the injury, the region was irrigated with saline to avoid necrosis. The animals were randomly divided into 2 groups. In the first group tissue samples for preparing micrografts were collected from the knee following the procedure described above (TO group), while in the second group tissue samples were collected from the xiphoid appendix (XA group).

For the XA group, micrografts were generated from a tissue sample collected from the xiphoid appendix. Animals were positioned in dorsal decubitus to allow surgical access to the sternum and the knee. A Bard-Parker scalpel cable mounted with blade no. 11. Once the musculature of the area was exposed, the fascia was sectioned in the same direction as the cutaneous incision. Using a pair of scissors, a fragment of the xiphoid appendix (approximately 1 cm) was extracted (**Fig. 2B**). In both control groups CTR (TO) and CTR (XA), the process of the articular defect in the right knee was described above then treated only with saline solution.

After the surgical procedure, the animals were sutured with absorbable wires and kept in recovery cages until they stabilized and returned to the individual cages where they received the following recovery medication: meloxicam 0.2%: 0.2 mL, (subcutaneously [SC]) one a day, for 3 days; tramadol 5%: 0.08 mL (SC) twice an day, and enrofloxacin 2.5%: 0.08 mL (SC) twice a day, for 5 days all animals were recovered with the medication.

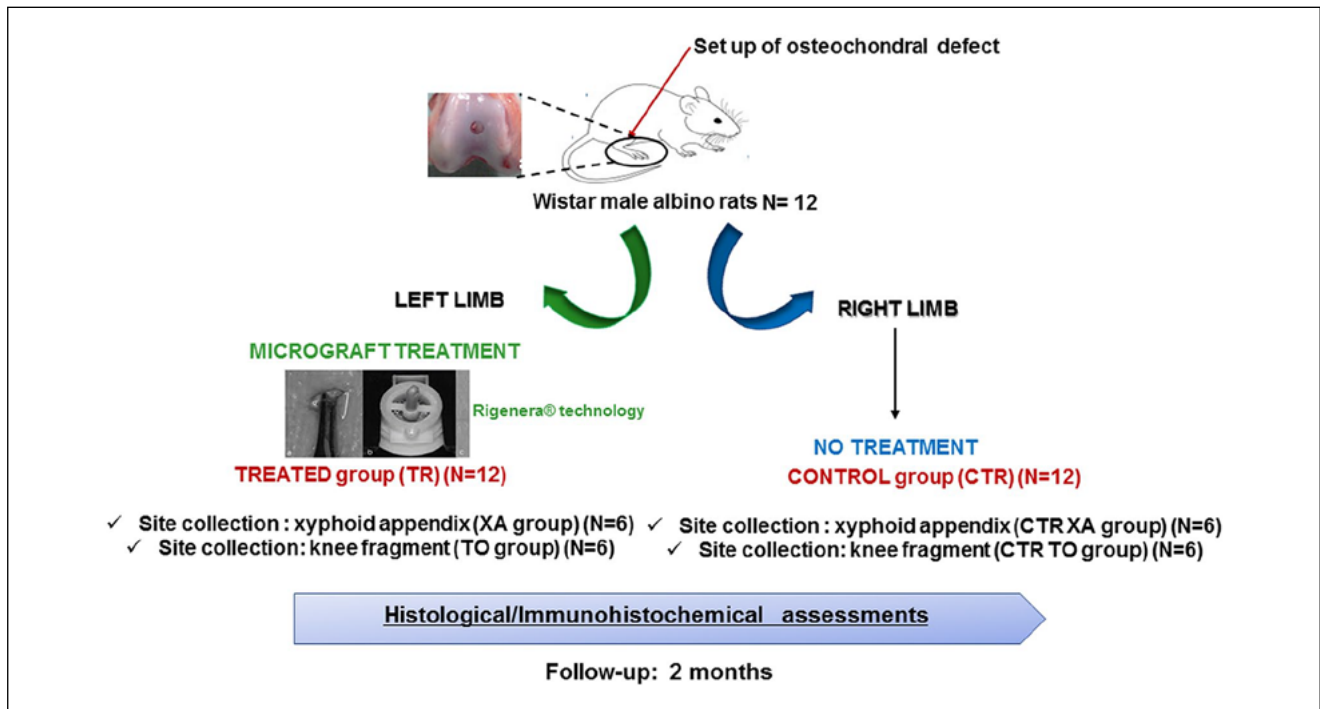


Figure 1. Graphical scheme of the experimental design. Twelve Wistar male albino rats underwent bilateral osteochondral defects. Animals were divided into 4 groups: (i) group treated with micrograft got from the xiphoid appendix (XA) ($n = 6$; right knee); (ii) untreated group after collecting the XA, CTR (XA) ($n = 6$; left knee); (iii) group treated with micrograft got from the knee fragment (TO) ($n = 6$; right knee); (iv) untreated group after collecting the TO, CTR (TO) ($n = 6$; left knee). Animals were euthanized at 2 months of follow-up. Histological and immunohistochemical analyses were carried out.

Autologous Micrografts Preparation and Grafting

To generate autologous micrografts from the collected tissues, we followed the method established by Ceccarelli *et al.*²⁸ The procedure is based on the use of a class IIa CE medical device named Rigeneracons (**Fig. 2C**), composed of (1) grid with hexagonal holes, each hole is equipped at the edges with microblade and (2) a rotating helix. Through this device, the rotating helix repeatedly pushes the tissue sample on the blades allowing the formation of microfragments particles called “micrografts.” The tissues collected during the surgery (articular cartilage for TO group and xiphoid cartilage for XA group) described above were inserted in the devices along with sterile saline solution. Tissue fragmentation was carried out in 4 minutes at room temperature. After processing, the tissues were completely disaggregated in micrometer-sized particles composed of cell clusters (micrografts) in sterile saline solution. The micrograft suspension was intra-articularly injected into the articular lesions in the treated groups (**Fig. 2D**).

Histological Assessments

Animals were euthanized by using intramuscular anaesthesia with ketamine and xylazine, followed by CO₂ in a

chamber until death at 2 month follow-up. An authorized veterinarian carried the euthanasia process out following the protocol recommended by the Brazilian Board for Animal Experimentation. After reaching the bone plane and locating the defect, the femur was disarticulated and removed (**Fig. 2E and F**). The soft tissues were removed by dissection and the samples were placed in containers with 10% buffered formalin solution for fixation at room temperature and then bones were decalcified in 12.5% formic acid solution and 20% sodium citrate for 20 days. After the decalcification, tissue samples were first washed in running water and then processed in a gradual series of alcohol and xylene for tissue dehydration and diaphanization up to their embedding with paraffin. Microscopic slides (thickness 7 μ m) were stained with Gill III hematoxylin/eosin (Sigma Aldrich, St. Louis, MO) and 0.1% safranin O/0.03% fast green (Sigma Aldrich) to assess tissue morphology and the proteoglycan content in the extracellular matrix.

A modified O’Driscoll (mO’Driscoll) score was used for providing a semiquantification of histological features in the control and experimental groups (TO and XA groups). This score considers 8 parameters: nature of the repair tissue, matrix staining, structural integrity, surface regularity, filling of the defect, bonding to host tissue, degenerative



Figure 2. Schematic representation of surgical and processing procedures for preparing the autologous tissue micrograft in the rat model. **(A)** The set-up of an osteochondral defect of 2.5 mm in diameter and 2.0 mm depth in the left knee. **(B)** Fragment extraction from the xiphoid appendix. **(C)** Image of a medical device called Rigeneracons® (CE certified class I) to provide autologous micrografts from tissue disaggregation. **(D)** Intra-articular delivery of autologous liquid micrografts suspension in the animal's left knee. At 2 months of follow-up, femur extraction of left **(E)** and right **(F)** limb for each animal and tissue processing for histological assessment.

changes of the repair tissue, degenerative changes of the adjacent cartilage.²⁹ It has a range from 0 (no repair) up to 21 (osteochondral repair). Six microscopic fields, spaced in

5 sections, were assessed for each specimen by 2 blinded investigators with the Eclipse 90i microscope (Nikon, Melville, NY).

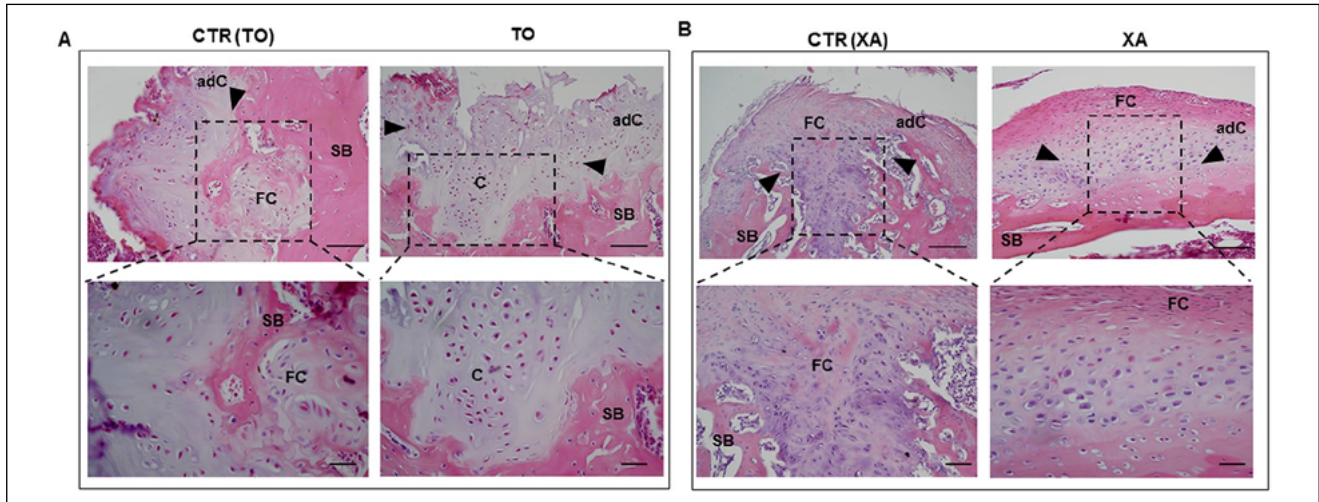


Figure 3. Histological staining with hematoxylin and eosin. Representative images of hematoxylin/eosin staining in the TO (knee fragment), XA (xiphoid appendix), CTR (TO), and CTR (XA) groups (**A** and **B**). Dashed rectangular represents the repaired tissues in the different groups with indications of the adjacent cartilage (adC), fibrocartilage (FB), and bone areas (SB). The upper panel shows representative micrographs with low magnification (scale bar: 100 μ m) whereas the lower panel has high magnification (scale bar: 50 μ m).

Immunohistochemical Assessments

Analyses for type 1 and type 2 collagens were carried out. After deparaffinization, the antigen retrieval was performed incubating the tissue sections with 0.1% proteinase (Sigma Aldrich) and 0.1% hyaluronidase (Sigma Aldrich) at 37 °C for 15 minutes, respectively. After washing steps with phosphate-buffered saline (PBS), sections were blocked with 1% bovine serum albumin (BSA) at room temperature for 20 minutes. Then, the incubation with monoclonal anti-mouse collagen type 2 (2 μ g/mL; Abcam, Cambridge, UK) and collagen type 1 (5 μ g/mL; Sigma Aldrich) was carried out at room temperature for an hour. Ready-to-use anti-mouse biotinylated secondary antibody and alkaline-labeled streptavidin (Biocare Medical, Pacheco, CA) were used. The histochemical detection was performed with Fast Red Substrate Kit (Biocare Medical). Specific negative controls were carried out by using an isotype-matched control or omitting the primary antibodies. The nuclear component was stained with Gill III hematoxylin. Three microscopic fields (100 $\times$ magnification) were assessed for each sample by a blinded investigator with a semiquantitative method.

We segmented and selected the neo-formed tissues for each marker by avoiding the surrounding adjacent native tissues. Image acquisition and processing were carried out with the Eclipse 90i microscope (Nikon) and NIS-Elements through the hue/saturation/intensity (HSI) system. We set the threshold value for H was set for positive pixels between 225 and 255. Ranges from 0 to 155 were threshold values for S and I. Positive cells/ area for each marker of neo-formed tissue were measured (10 $\times$ objective lens) and

expressed as a percentage of positive cells/ area on a scale from 0 (no protein expression) to 100 (the highest protein expression).

Statistical Analysis

Statistical analysis was performed with GraphPad Prism software. The Kolmogorov-Smirnov test was used to test the data distribution. We used the Kruskal-Wallis followed by the *post hoc* Dunn's test to assess differences in the control and treated groups. Data were expressed as the 95% confidence intervals (CI) of the mean $\pm$ standard deviation (SD). $P \leq 0.05$ was considered statistically significant.

Results

Histological Evaluations

Histological evaluation showed that the treatment with autologous micrografts promoted defect filling to a different extent in both TO and XA groups. In the CTR (TO) group, the defect was filled with fibrocartilage which displays impaired tissue integrity, some blood vessels, empty lacunae and a poor content of proteoglycan. Moderate hypocellularity with cell clusters and little bone remodeling were further histological features observed in the defect area in the CTR (TO) group (**Figs. 3A** and **4A**). Conversely, the defect area from the TO group showed a repair tissue with hyaline-like histological features. This neoformed tissue displayed a partial bonding with the host tissue, islets of chondrons dispersed in the extracellular matrix and several

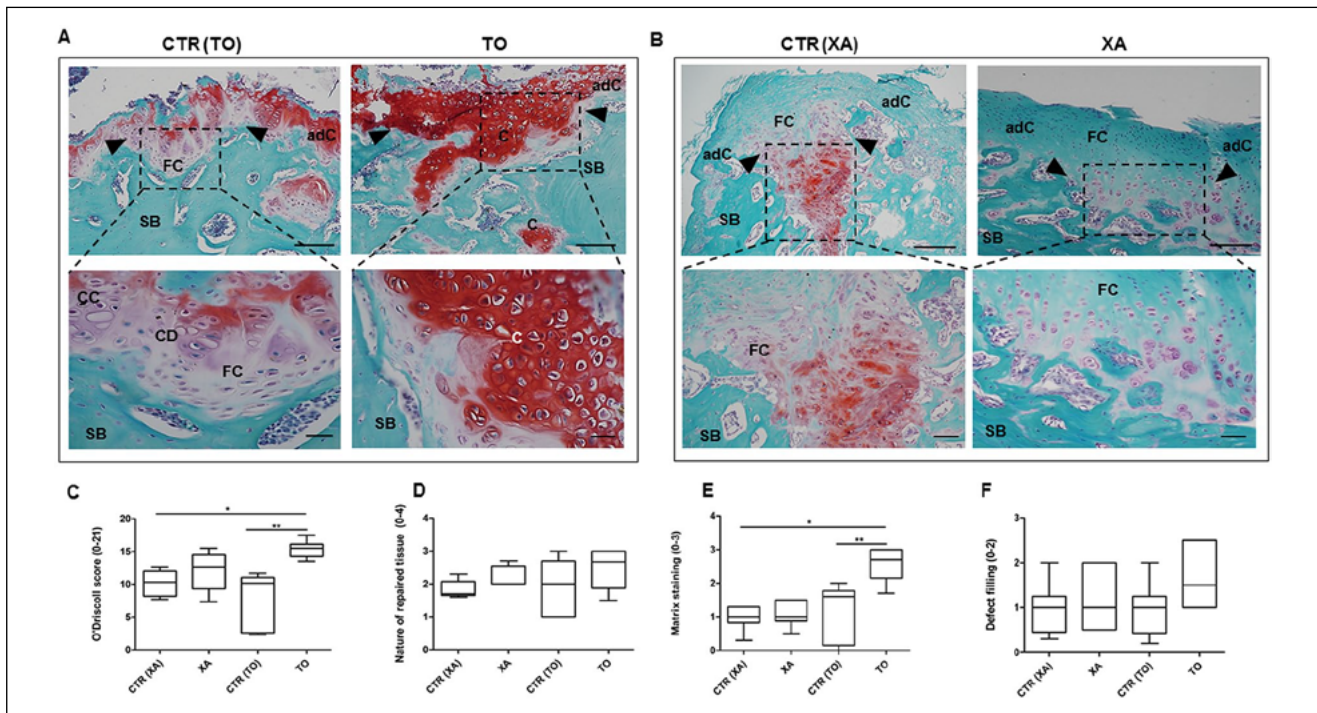


Figure 4. Histological staining with safranin O/fast green. Representative images of safranin O/fast green staining (red staining, proteoglycan content; green staining, collagen content) in the TO (knee fragment), XA (xiphoid appendix), CTR (TO), and CTR (XA) groups. (**A** and **B**) Dashed rectangular represents the repaired tissues in the different groups with indications of adjacent cartilage (adC), fibrocartilage (FB), cartilaginous areas (C), and bone areas (SB). The upper panel shows representative micrographs with low magnification (scale bar: 100 μ m), whereas the lower panel has high magnification (scale bar: 50 μ m). (**C**) Graphical representation of modified O'Driscoll score in the TO, CTR (TO), XA, and CTR (XA) groups; $**P < 0.01$: TO versus CTR (TO) group; $*P < 0.05$: TO versus CTR (XA) group. (**D**) Graphical representation of the repaired tissue, a subparameter of mO'Driscoll score, in all control and experimental groups. (**E**) Graphical representation of the matrix staining, a subparameter of mO'Driscoll score, in all groups. Data are expressed as 95% confidence intervals (CIs) of the mean $\pm$ standard deviation (SD), $**P < 0.01$: TO versus CTR (TO) group; $*P < 0.05$: TO versus CTR (XA) group. (**F**) Graphical representation of the defect filling, a subparameter of the score, in all groups.

processes of osteochondral ossification (**Figs. 3B** and **4B**). Good structural integrity and normal cellularity with several round cells surrounded by proteoglycans were further histological features noticed in the TO group (**Figs. 3B** and **Fig 4B**). The CTR (XA) group showed an abnormal tissue formation with an altered tissue architecture, several fibrillations, low proteoglycan content, and immature bone formation (**Figs. 3B** and **Fig.4B**). The OCD lesions were filled with fibrocartilage in the XA group, which displayed a slight cellular positivity for safranin O staining in the deep zone. The repaired cartilage tissue was immature with no tidemark and the bone displayed several spaces with osteoprogenitors (**Figs. 3B** and **Fig. 4B**).

The semiquantification of histological features was performed with the mO'Driscoll score. The TO group showed a higher mO'Driscoll score (15.36 ± 0.5) than the XA group (12.07 ± 1.3) (**Fig. 4C**). In particular, the TO group (15.36 ± 0.5) displayed a higher score than its control, CTR (TO) (7.9 ± 1.7) ($**P < 0.01$) and CTR (XA) (10.14 ± 0.89) ($*P < 0.05$). The XA group showed a higher

mO'Driscoll score than its control, but with no statistical evidence (**Fig. 4C**). Among the histological parameters within the mO'Driscoll score, the TO group (2.6 ± 0.2) showed a higher matrix staining than CTR (TO) (1.2 ± 0.3) ($P < 0.01$) and CTR (XA) (0.98 ± 0.1) ($P < 0.05$) groups (**Fig. 4E**).

Immunohistochemical Assessments

To further evaluate the quality of the neoformed tissues in the control and experimental groups, we performed immunostaining for type 2A1 and 1 collagens. Both TO and XA groups showed strong positivity for type 2A1 collagen at the cellular and extracellular levels (**Fig. 5A**). They displayed a higher protein expression for type 2A1 than their respective controls. In particular, the TO group showed a higher significant value for type 2A1 than the CTR (TO) ($P < 0.01$) and CTR (XA) ($P < 0.05$) groups (**Fig. 5B**). Regarding type 1 collagen, both groups treated with the micrografts showed lower protein values at a cellular level

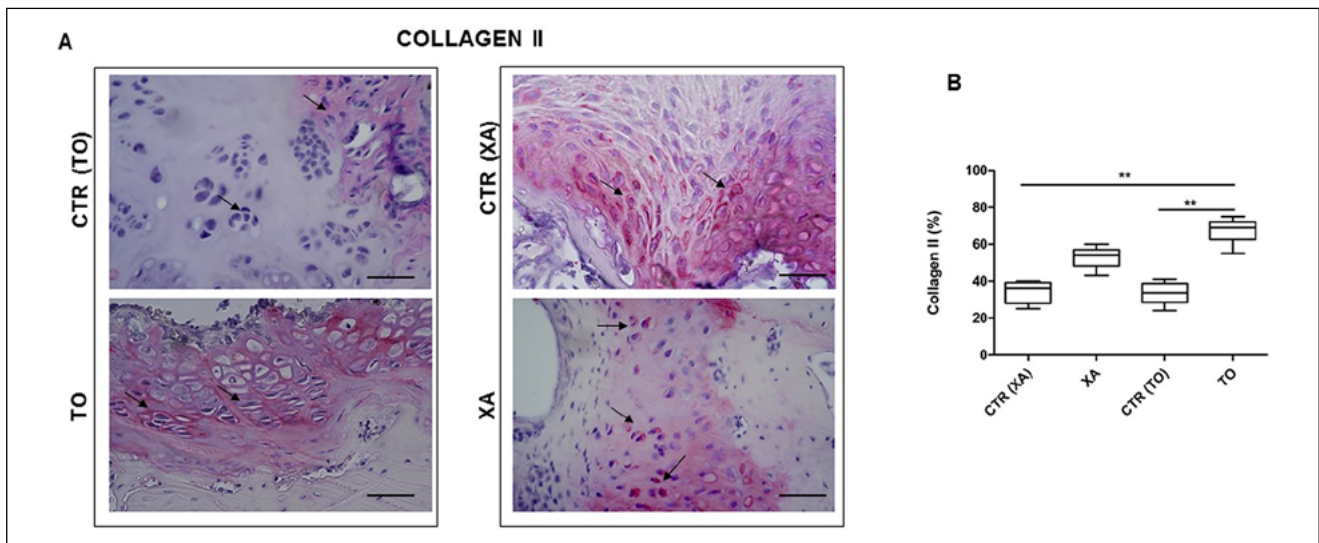


Figure 5. Immunohistochemical analysis: type 2A1 collagen. **(A)** Representative images for the immunohistochemical analysis for type 2A1 collagen in the TO (knee fragment), XA (xiphoid appendix), CTR (TO), and CTR (XA) groups. **(B)** Graphical representation of protein expression for type 2A1 collagen with hue/saturation/intensity (HSI) system with software NIS-Elements in all groups. Data are expressed as 95% CI of the mean $\pm$ SD. ** $P < 0.01$: TO versus CTR (TO) group; * $P < 0.05$: TO versus CTR (XA) group.

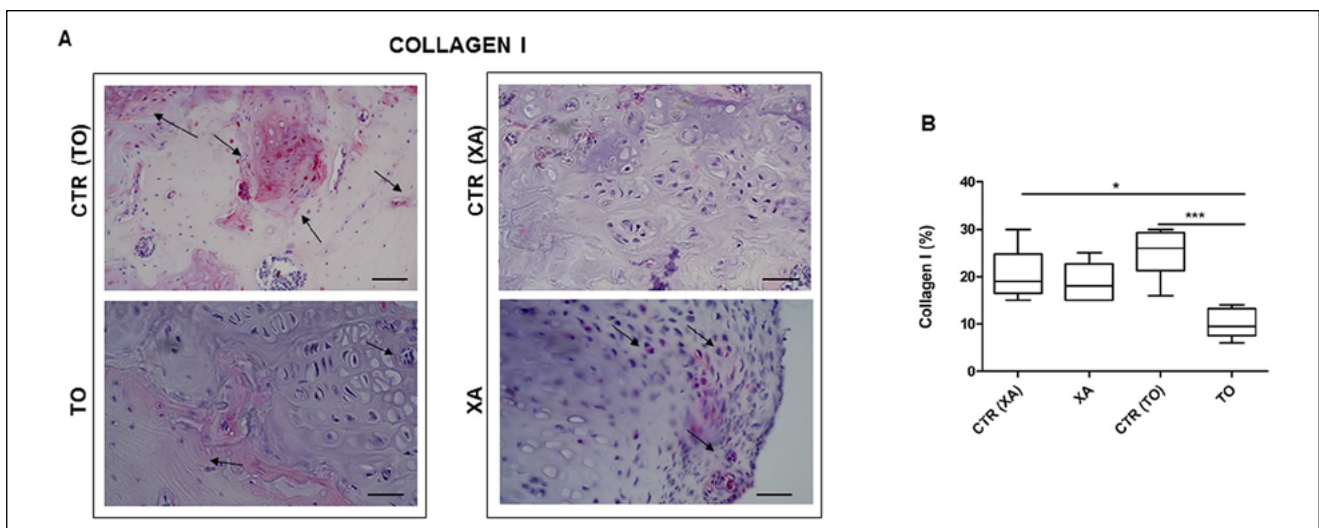


Figure 6. Immunohistochemical analysis: type I collagen. **(A)** Representative images for the immunohistochemical analysis for type I collagen in the TO (knee fragment), XA (xiphoid appendix), CTR (TO), CTR (XA). **(B)** Graphical representation of protein expression for type I collagen after image analysis with hue/saturation/intensity (HSI) system with software NIS-Elements in all groups. Data are expressed as 95% CI of the mean $\pm$ SD. ** $P < 0.01$: TO versus CTR (TO) group; * $P < 0.05$: TO versus CTR (XA) group.

than their respective controls (**Fig. 6A**). The TO group showed a lower protein expression for type 1 collagen than CTR (TO) ($P < 0.01$) and CTR (XA) ($P < 0.05$) (**Fig. 6B**).

Discussion

The main finding of this preclinical *in vivo* study is that the intra-articular delivery of autologous cartilage micrograft

can be a promising mini-invasive 1-step approach to treat OCD. Employing autologous micrograft as a biological solution can offer significant benefits, allowing to overcome current limitations of therapeutic approaches, like invasiveness, donor site morbidity, cell death and allogeneic response. Albrecht³⁰ introduced the concept of cartilage chips in 1983. Early preclinical and clinical studies provided promising results on the use of various minced

cartilage techniques through different shaver medical devices, delivery systems and the enrichment with bioactive molecules for chondrocyte activation.³¹⁻³⁵ In particular, Lind and Larsen³⁶ provided preclinical evidence on the equal repair capacity of 1-step minced cartilage under collagen scaffold to standard ACI technique in sheep. Mostly, studies on cartilage micrograft investigated its combination with bioactive molecules and/or biomimetic scaffold and implantation into the defect site by arthroscopy.³¹⁻³⁵ To date, full knowledge of the role of micrografts in musculoskeletal tissue regeneration, and especially for the repair of OCD, is still scarce.^{15,16,37}

The main aim of this study is to raise the level of knowledge of autologous micrograft to treat OCD in a rat model via an intra-articular approach by testing its effectiveness after tissue fragmentation with a novel medical device (CE-certified class I), named Rigeneracons. This system produces small fragments of 80 µm, capable of being incorporated into the native tissue to promote chondrocyte activation and tissue engraftment. In-depth investigation clarified the influence of two harvesting sites, articular cartilage and xiphoid appendix, to promote tissue repair. Preliminary *in vitro* studies showed that this technology does not affect cell viability in articular cartilage and the chondrogenic potential.²⁷ Herein, we investigated the efficacy of micrograft and some critical characteristics conditioning tissue repair. Our study showed that the autologous micrografts from articular cartilage show attractive properties to treat OCD with important perspectives in clinics. The micrograft from xiphoid appendix displayed some repair processes with immature fibrous features. The different biological responses from the two tissue harvesting sites could depend on the different anatomical architectures,³⁸ which do not guarantee proper biomechanics. Immunohistochemical analyses showed that the TO group displayed various islets of chondrocytes embedded in the extracellular matrix in the defect area. These cell islets showed a high positivity for type 2 collagen and low levels of type 1 collagen, promoting a repair tissue with typical hyaline-like features. Regarding the bone component, this group exhibited tissue restoration, which is a critical factor during osteochondral defect healing as it ensures adequate nutrient and growth factor supply to cartilage tissue.³⁹ The potential of this technology could derive from the ability to obtain ready-to-use micrografts containing viable chondrons, the morpho-functional units of articular cartilage. The feasibility of implanting instructive “cell niche” could enhance endogenous tissue regeneration by recruiting progenitor cells from the surrounding tissues in the lesion site. Indeed, using the native extracellular matrix scaffold can restore typical spatial organization of the articular cartilage at the treatment site, thus ensuring suitable mechanical tissue properties and cellular functions. Besides the chondrocyte population, micrograft contains progenitor cells, which may further promote tissue repair thanks to their

biological properties.²⁶ Progenitor cells could act as “growth factors secreting factories” and exert a therapeutic effect through the release of soluble mediators.^{26,40-42} This study provides the first preliminary interesting concepts on the role of micrografts to treat OCD via an intra-articular approach. However, it presents some limitations due to (1) the use of a small animal model, (2) the small sample number, and (3) short-term follow-up. In conclusion, our data show that this micrografting technology could be a potential low-cost strategy to treat OCD since it promotes a repair tissue with typical hyaline-like features. Further studies are, however, needed to validate the potential biological relevance of this micrograft in a larger animal model before considering its clinical application.

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

The study was carried out after being approved by the Ethics Committee on the Use of Animals, Federal Rural University of Pernambuco (UFRPE) with License 126/2017.

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ARTICLE

Biostimulation of Knee Cartilage Using Autologous Micro-Grafts: A Preliminary Study of the Rigenera Protocol in Osteochondral Lesions of the Knee

Agustín Dorta Fernández and Ana Baroni Luengo

Biostimulation of Knee Cartilage Using Autologous Micro-Grafts: A Preliminary Study of the Rigenera Protocol in Osteochondral Lesions of the Knee

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Abstract: Osteochondral pathologies are associated with cartilage loss. Autologous micro-grafts, obtained from various tissues containing mesenchymal stem cells, has proven regenerative properties in various tissues. The study included patients with osteochondral pathology not responding to conventional therapies and considered suitable for treatment with autologous micro-graft. Fifteen days before the intervention, patients started treatment with chondroitin sulfate and supplementary vitamin C and zinc. Autologous micro-grafts were obtained from ear skin and cartilage, disaggregated using the Rigenera system, and injected into the knee under local anesthesia. After the intervention, patients underwent six-week rehabilitation program based on non-weight bearing exercises. Nine patients were enrolled into the study. The autologous graft transfer was successful in all patients, and no severe complications were recorded, including aesthetical defects in the donor site. Eight patients (88.9%) experienced an improvement in all subdomains of the WOMAC scale. The median (IR) improvement in the WOMAC scale was 22.0 (10.0 – 40.0) points. The MRI examination revealed the absence of space-occupying lesions in the knee and the surrounding soft tissues in all patients. An edema reduction was observed in 8 of 9 patients (88.9%); 3 patients (33.3%) showed a thickening of the cartilage line. Autologous micro-graft transfer using the Rigenera system is safe and has promising results in the treatment of chondral injuries associated with pain and function limiting.

Keywords: Rigenera, Osteoarthritis, Osteochondral Regions, Mesenchymal Stem Cells, Tissue Regeneration

1. Introduction

Osteochondral pathologies may result from trauma or sports injuries or be the consequence of disorders associated with cartilage degeneration, such as chondromalacia and osteoarthritis [1, 2]. Irrespective of the etiology of the osteochondral pathology, the loss of cartilage, evidenced in magnetic resonance images (MRI) as a thinning of the cartilage line, causes pain and physical disability [3–5]. In addition to surgery, the traditional management of degenerative cartilage diseases is based on physical therapy and pain relief. However, some patients do not respond well to pain relievers, and the regeneration of the damaged cartilage may last for a long time, thus compromising the

complete functional recovery.

In the recent years, many efforts have been made in developing complementary strategies aimed at either regenerating the damaged cartilage or enhancing the natural regeneration process. In addition to the intra-articular use of hyaluronic acid, various approaches have been proposed to stimulate the production of new cartilage. Biostimulation techniques include physical approaches, such as the use of high-frequency biostimulation during surgery [6] or low-power laser targeting the osteochondral defect [7, 8], and biological approaches, such as autologous chondrocyte implantation [9] and the use of platelet-rich plasma to stimulate the production of new cartilage [10].

The finding of stem-like cell populations with multipotent features in various tissues has motivated the use of these cells

for regenerative purposes [11, 12]. However, the availability of ready-to-use methods for a safety and effective isolation of mesenchymal stem cells during surgery has been a bottleneck for the clinical use of multipotent cells. The Rigenera protocol is a novel strategy for tissue mechanical disaggregation which allows obtaining autologous micro-grafts enriched with mesenchymal stem cells during surgery [13, 14]. Compared to enzymatic techniques, mechanical disaggregation has claimed to be faster and safer. To date, the Rigenera protocol has shown clinical efficacy in the management of complex wounds [15, 16] and the regeneration of the dental pulp in periodontal surgeries (Periodontal tissue generation) [17]. However, the identification of stem-like cells in many other tissues suggests additional uses of autologous micro-graft preparation and transfer [12]. In this preliminary study, the clinical and functional outcomes of cartilage autologous micro-grafting (obtained with the Rigenera protocol) in patients with knee osteoarticular manifestation of cartilage injury or degeneration is described.

2. Materials and Methods

2.1. Study Subjects

In this prospective study, patients with mechanical and/or degenerative osteochondral pathology who did not respond to conventional therapies and were considered suitable for being treated with the autologous micro-grafts in an experimental setting were included. Patients were recruited and treated by the same surgeon between January 2016 and April 2016.

In addition to the complete blood count and the erythrocyte sedimentation rate, all patients were screened for immune system diseases and metabolic abnormalities, including vitamin D3 and B12, triglycerides, cholesterol, glycated hemoglobin, and thyroid hormone levels. Testosterone levels were also assessed in male subjects. Patients with immune or inflammatory diseases such as familial osteoarthritis, history of rheumatic inflammatory diseases, and those with a positive antinuclear antibody test, the presence of rheumatoid factor, and anti-streptolysin O greater than 150 were excluded. Patients with morbid obesity, diabetes mellitus, and other metabolic diseases – including those treated with statins and/or hypolipidemic agents – were also excluded. All patients signed the informed consent to undergo the experimental procedure.

2.2. Autologous Micro-Grafts Transfer

15 days before the intervention, patients started treatment with chondroitin sulfate and supplementary vitamin C and zinc. Patients with low levels of vitamin D3 and B12 were also supplemented with these vitamins. Non-steroidal anti-inflammatory drugs were not allowed during the 15 days preceding the intervention. Six hours before the intervention, patients started treatment with azithromycin 500 mg (maintained during the two days following the intervention)

and fasted until intervention.

The cartilage graft was obtained from the auricle cartilage of the ipsilateral ear to the target knee using x3.5 magnification Zeiss lens in sterile file. To expose the auricle cartilage, an eye-shaped skin graft of 2 x 1.2 cm (approximately 120 mm²) was removed. The cartilage graft was excised with a scalpel (No. 11) and microsurgery scissors. The perichondrial tissue surrounding the cartilage gap was scratched and added to the sample. The wound was rinsed with a 1:1 solution of 3% hydrogen peroxide and saline and sutured with 6/0 subcutaneous Vicryl and 2-to-3 transverse stitches.

Approximately 0.1 cm³ of the dermis and subcutaneous tissue were detached from the skin graft and mixed with 0.25-to-0.3 cm³ of auricle cartilage and perichondrial tissue. The sample was mechanically disaggregated using the Rigenera protocol according to the manufacturer's specifications during two cycles of 2 minutes, obtaining a cellular graft of 4 cm³. The final number of cells obtained was not determined. However, according to previous experiences with the Rigenera system, the nasal cartilage yielded 9088.30 ± 788.86 chondrocytes per mL of digested tissue [22]; hence, the sample was expected to yield 2-to-3x10³ chondral cells. Likewise, the phenotype of dermal cells obtained using the Rigenera system was previously analyzed by Svolacchia et al., who found the following percentages of mesenchymal stem cells markers: 82% of CD34, 82% of CD90 (Thy-1), and 53% of CD105 [23]. The authors reported a yield of 80x10³ cells – 70-80% of them mesenchymal – with 92% of viability from 2 mm³ of dermal tissue. Considering that dermal sample was approximately 120 mm³ (120-mm² surface and 1-mm thickness), to obtain nearly 4.8x10⁵ cells was expected.

Disaggregated cells were finally injected into the knee by using a patellar injection under local anesthesia. Passive knee movement was applied to evenly distribute the sample inside the knee.

2.3. Rehabilitation Protocol and Assessments

Full weight-bearing without any orthosis was allowed six weeks after intervention. During this period, patients were advised to perform non-weight bearing exercises involving the hamstring and quadriceps, as well as stationary cycling without resistance. Upon healing of the ear wound, patients were allowed to swim. Normal gait was achieved through progressive weight bearing for six additional weeks. Patients were not allowed to take non-steroidal anti-inflammatory drugs during the entire follow-up.

The progress of osteochondral injury was assessed by MRI tests, which were performed before and twelve weeks after the intervention. Functional and pain assessments were performed using the WOMAC scale (Spanish version) [24, 25]. Data is presented as raw and as the median and interquartile range (IR, corresponding to percentiles 25 and 75) or the mean and standard deviation (SD).

3. Results

3.1. Patients and Procedure

The autologous graft transfer was performed in nine patients meeting all the selection criteria. The mean (SD) age was 51.7 (6.2), 56% were male, and none of them were active smokers. In all cases, the procedure was successful, and none of them experienced infections or severe complications after the procedure. Two patients experienced joint effusion, manifested at a few days after the procedure. No aesthetical defects were observed at the donor site in any patient and none of them complained about the result on the donor site. One patient with a history of blood dyscrasias experience retroauricular bruising which healed without other interventions than vitamin K supplementation and any aesthetical defect.

3.2. Symptoms and Functional Outcome

Except one patient who experienced edema associated with cell infiltration, all patients referred a remarkable pain decrease during the few days following the procedure. At the end of the follow-up all patients returned to their activities performed before the worsening of the cartilage injuries. Table 1 shows the details of the functional and pain assessment at baseline and the end of the follow-up. Eight patients (88.9%) experienced an improvement in all subdomains of the WOMAC scale. Median (IR) total WOMAC score in the study population was 39.0 (19.5 – 59.0) before intervention and 9 (3.0 – 23.5), a median improvement of 22.0 (10.0 – 40.0) points. Patient 3 improved her stiffness and functional impairment but worsened pain associated with her knee condition. Overall, the median (IR) pain, stiffness, and function improvement were 5.0 (3.3 – 9.0) on a 20-point scale, 3.0 (1.3 – 3.8) on an 8-point scale, and 19.5 (9.3 – 30.0) on a 68-point scale, respectively.

The MRI examination revealed the absence of space-occupying lesions in the knee and the surrounding soft tissues in all patients. An edema reduction was observed in 8 of 9 patients (88.9%) (Figure 1A). The woman in case 9 displayed more edema in the post-intervention than in the pre-intervention MRI. Chondromalacia showed a partial regression in 5 of 7 cases (71.4%) in which the progress of the chondromalatic signs was reported. Three patients (33.3%) showed a thickening of the cartilage line (Figure

1B). Of 8 patients with visible cartilage tear at baseline, 4 maintained the injury, 1 showed a remarkable improvement, and 2 showed no signs of the injury at the post-intervention MRI. Three patients showed a Baker's cyst at baseline: one remained unchanged, one reduced the cyst size, and one had no visible cysts at post-intervention MRI. The patient who experienced a worsening in knee stiffness had a cartilage injury before the procedure which was maintained after the procedure.

Figure captions

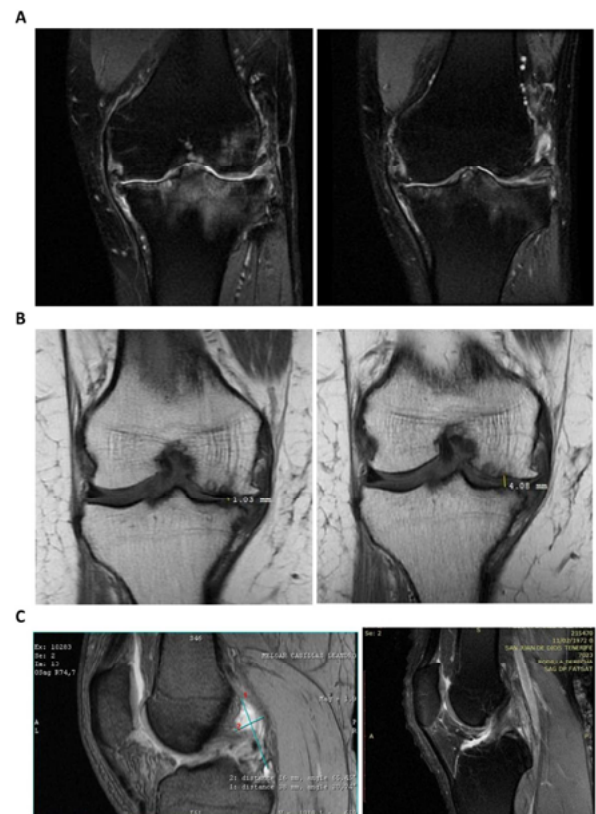


Figure 1. Highlights of MRI findings before and after procedure. A: case 4 at presentation (left) and sixteen weeks after (right); the images show an edema reduction and an increase in the definition of the cartilage line. B: case 7 at presentation (left) and twelve weeks after the intervention (right); the cartilage thickness experienced an increase of 2.78 cm. C: case 1 at presentation (left) and 17 weeks after; the Baker's cyst of 36 mm length x 16 mm wide identified at presentation showed a complete remission 17 weeks after the procedure.

Table 1. Patient characteristics and results of the WOMAC scale.

Patient no.	Sex	Age (years)	WOMAC scores* pain stiffness function limitation	
			Before the intervention	After the intervention
1	male	44	4 1 8	0 0 0
2	male	44	2 3 4	0 0 2
3	female	54	3 5 19	7 2 15
4	male	63	11 5 45	6 2 18
5	male	50	7 2 30	4 2 17
6	male	48	12 6 29	3 2 4
7	female	56	18 8 63	1 2 4
8	female	56	12 5 40	3 3 9
9	female	50	6 3 17	1 0 3

*Score ranges for the assessment of pain, stiffness, and functional limitation are 0-20, 0-8, and 0-68, respectively.

4. Discussion

We present here in 9 cases of patients with cartilage injuries who experienced an improvement in pain and function – some of them with changes visible at MRI examination – after intra-articular injection of an autologous micro-graft obtained with the Rigenera protocol.

Chondral injuries in the knee are a challenging scenario for surgical reconstruction. Despite the good results observed with prosthetic resurfacing, there is increasing interest in developing safety and effective procedures for biologic resurfacing and joint preservation [18]. The rationale behind the Rigenera protocol lies in the multipotent properties of the mesenchymal stem cells which may promote cell differentiation and subsequent new cartilage formation [13, 14]. In our experience, the use of autologous grafts enriched in mesenchymal cells resulted in healing or improvement of cartilage defects visible at MRI examination. After graft transfer intervention, all patients except one showed an edema reduction. However, considering the function and pain improvement in this patient, the post-intervention edema was attributed to the normal healing process. Most patients had a visible cartilage tear or decrease in the cartilage line at baseline. The technical limitations of the MRI assessment did not allow for a quantification of interesting parameters – such as the cartilage thickness or the size of cartilage defects – in all patients. Despite this limitation, three patients showed a remarkable increase in cartilage thickness (for 1.03 to 4.08 cm in the best case observed) at the examinations before and after the intervention. It is noteworthy that one patient had a 36-cm length Baker's cyst at baseline examination, which was not visible at MRI examination after the intervention, indicating a significant reduction of the inflammation.

Regardless of the MRI findings, all patients except one showed an improvement in pain, stiffness, and function, and returned to their regular physical activity twelve weeks after the intervention. In some cases, the cartilage defect had progressed to a severe function limitation associated with intense pain which prevented patients from performing their activities of daily living, including work and normal gaiting. Pain and function were assessed by means of the Spanish version of the WOMAC scale, which has been extensively used in research and in routine clinical practice for assessing the severity of knee injuries [19]. The results of the WOMAC assessment in studies investigating the efficacy of other biostimulation techniques are heterogeneous, with studies reporting significant improvement and other reporting a slight worsening at 2 or 3 months [10, 20]. Also, the different study designs and etiologies hamper reliable comparisons between studies. However, without losing sight of these limitations, in the study patients showed a greater trend towards functional and pain improvement than those treated with platelet-rich plasma preparations and assessed with the same scale. Furthermore, although the number of included

patients does not allow for a correlation analysis, pain improvement concurred with an improvement in function and stiffness, and the WOMAC scores were overall consistent with the MRI findings, as expected, considering the correlation between pain and cartilage defects visible at MRI examination [21].

The strict selection criteria limited the number of patients included in the study. However, as this was an exploratory test of safety and effectiveness of the Rigenera protocol in the management of knee cartilage conditions, we considered the exclusion of immune and inflammatory diseases an important condition to achieve the best performance of the transferred cells and to assess safety independently. Another important limitation was the heterogeneity of the MRI information, which precluded a quantitative analysis of the MRI findings. However, MRI assessments provided valuable input on the physiological effects of cartilage biostimulation.

In summary, this study shows that the transfer of autologous micro-grafts using the Rigenera protocol is safe and has promising results in the treatment of cartilage injuries associated with pain and functional limiting. These results suggest the suitability of further prospective, controlled studies with larger cohorts to confirm the therapeutic properties of this intervention.

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ARTICLE

Cartilage Micrografts as a Novel Non-Invasive and Non-Arthroscopic Autograft Procedure for Knee Chondropathy: Three-Year Follow-Up Study

Marco Marcarelli, Marcello Zappia, Lorenzo Rissolio, Chiara Baron, Carlo Astarita, Letizia Trovato and Antonio Graziano



Article

Cartilage Micrografts as a Novel Non-Invasive and Non-Arthroscopic Autograft Procedure for Knee Chondropathy: Three-Year Follow-Up Study

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Abstract: (1) Background: Focal chondral defects of the knee can significantly impair patient quality of life. Although different options are available, they are still not conclusive and have several limitations. The aim of this study was to evaluate the role of autologous cartilage micrografts in the treatment of knee chondropathy. (2) Methods: Eight patients affected by knee chondropathy were evaluated before and after 6 months and 3 years following autologous cartilage micrografts by magnetic resonance imaging (MRI) for cartilage measurement and clinical assessment. (3) Results: All patients recovered daily activities, reporting pain reduction without the need for analgesic therapy; Oxford Knee Score (OKS) was 28.4 ± 6 and 40.8 ± 6.2 and visual analogue scale (VAS) was 5.5 ± 1.6 and 1.8 ± 0.7 before and after 6 months following treatment, respectively. Both scores remained stable after 3 years. Lastly, a significant improvement of the cartilage thickness was observed using MRI after 3 years. (4) Conclusions: Autologous cartilage micrografts can promote the formation of new cartilage, and could be a valid approach for the treatment of knee chondropathy.

Keywords: cartilage micrografts; medical device; regenerative medicine



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1. Introduction

Articular cartilage defects remain a challenging clinical issue for orthopedic surgeons and, if left untreated, can progress to degenerative osteoarthritis, knee pain, and ultimately loss of function [1]. The limited self-healing ability of articular cartilage is due to its avascular nature and slow extracellular matrix turnover, along with limited capacity of resident chondrocytes to migrate to damaged areas [2].

Joint preservation is the preferred treatment strategy for knee chondropathologies in young and active patients to improve pain, restore activity, and delay arthroplasty. To date, there have been several approaches that aim the repair cartilage defects, even if the natural course of such lesions is still not well known. Among these, bone marrow stimulation techniques, cartilage or chondrogenic tissue-based repair, and pharmacological approaches have been widely discussed in the literature [3]. Osteochondral autologous transplantation (OAT) is used for small and medium focal articular cartilage defects [4], while autologous chondrocyte implantation (ACI) can be considered for the treatment of early osteoarthritis affecting younger patients [5]. Different generations of ACI and matrix-induced autologous chondrocyte implantation (MACI) techniques have been studied both in long-term follow-ups and in several randomized trials, and these techniques

lead to satisfactory results [6]. Although the above-mentioned therapeutics represent an option today, all come with limitations: (i) the need for cell expansion, (ii) high cost, (iii) invasiveness, and (iv) formation of fibrocartilage often occurring by de-differentiation of chondrocytes during cell expansion [7,8]. Pharmacological treatments for knee or hip osteoarthritis include non-selective non-steroidal anti-inflammatory drugs, as well as intra-articular injections of corticosteroids, focusing on pain and inflammation reduction without addressing the underlying causes [9,10].

The aim of this study was to evaluate the clinical effects of autologous cartilage micrografts, delivered with a non-arthroscopic procedure—injection in the articular chamber—on eight patients affected by knee cartilage degeneration and evaluated by magnetic resonance imaging (MRI) and clinical assessment before and after 3 years following treatment.

For this specific aim, we used the Rigeneracons medical device, an innovative technology that allowed us to mechanically disaggregate autologous cartilage, collected from the patient, to obtain a micrografts suspension ready to use without the need for cell expansion *in vitro* and able to regenerate damaged tissues. To date, the Rigenera[®] micrografting technology has demonstrated its efficacy in different clinical settings. It is widely used with dermal tissue samples for the treatment of complex wounds such as dehiscences, chronic ulcers, and burns [11–16], for dermatological applications such as hypertrophic scars [17], and for the management of androgenetic alopecia [18,19], with cartilage samples for the management of chondropathy [20,21], with bone samples for the treatment of the osteonecrosis of the femoral head [22].

2. Experimental Section

2.1. Research Design

The study protocol was in accordance with the Declaration of Helsinki of 1975 and European regulations. All patients provided written informed consent before participating in the study. Eight patients were enrolled. Their ages were between 43 and 69 years old affected by knee degenerative chondral lesions, pain for more than 3 months following conservative treatment, and no bone axial defect. Patient diagnosis was accomplished with the Outerbridge classification [23]. Patient inclusion criteria were knee chondropathy grade I to III (pre-arthritis). The exclusion criteria were chondropathy grade IV, several osteophytosis, traumatic or degenerative meniscal lesions, and systemic disease such as rheumatoid arthritis and psoriatic arthritis.

All patients reported in Table 1 were treated with a single intra-articular injection of autologous micrografts obtained with the Rigenera[®] micrografting technology. The Oxford Knee Score (OKS) and the visual analogue scale (VAS) were used before and after 6 months following micrografts treatment to perform a global assessment of patient satisfaction, pain decrease, and functional status [24].

Table 1. Diagnosis and type of treatment of the patients included in the study.

Patients	Sex, Age (y)	Diagnosis	Treatment
1	F, 66	Chondropathy grade III	Micrografts collection from auricular cartilage and application on left knee
2	F, 53	Chondropathy grade III	Micrografts collection from auricular cartilage and application on left knee
3	F, 60	Chondropathy grade III	Micrografts collection from auricular cartilage and application on left knee
4	M, 54	Chondropathy grade III	Micrografts collection from auricular cartilage and application on both right and left knee
5	F, 55	Chondropathy grade II	Micrografts collection from auricular cartilage and application on right knee
6	M, 69	Pre-arthritis	Micrografts collection from auricular cartilage and application on both right and left knee
7	M, 43	chondropathy grade III	Micrografts collection from auricular cartilage and application on right knee
8	M, 50	chondropathy grade III	Micrografts collection from auricular cartilage and application on right knee

MRI of the knee was performed in patients before and after 3 years following single micrografts application to evaluate the status of cartilage degeneration/regeneration.

To measure the thickness of cartilage, DP, weighted or intermediate-weighted, was used with fat saturation sequences (serial checks always made on the same sequence). The slice thickness was 3 or 4 mm. The measurement of the cartilaginous thickness of the central portion of the condyles was done on the coronal plane (in the middle of the condyle on the sagittal plane).

The measurement of the posterior portion of the condyles was made on the sagittal plane at 1cm from the end of the cartilage. The point at which cartilage is thickest was chosen and measured. Moreover, if bone edema or focal lesions of cartilage were detected, it would be reported.

2.2. Technique

A small piece of auricular cartilage was mechanically disaggregated immediately by the Rigeneracons medical device (Human Brain Wave, Turin, Italy), which can generate a micrografts suspension with a cut-off of 80 microns, making it possible to deliver such graft suspension with a simple syringe into the synovial capsule. More specifically, an autologous auricular cartilage sample (one for each knee) is easily collected by a 3 mm punch biopsy (Figure 1A,B); the autologous cartilage is then placed into the medical device, adding 4 mL of sterile saline solution. Afterwards, the Rigeneracons is activated with a specific motor for 4 min, allowing the rotation of the inner blades responsible for the micro-fragmentation of the cartilage autologous sample (Figure 1C); such cartilage micro-fragments (or cartilage micrografts) are now in suspension with sterile saline and are easily collected from the device with a syringe (Figure 1D). Lastly, the cartilage micrografts suspension is injected with a syringe by anterolateral approach in the knee of the patient (Figure 1E).

2.3. Statistical Analysis

Statistical analysis was performed by using GraphPad Prism software. Data were expressed as the mean $\pm$ standard deviation (SD). The difference between parameters was examined using an independent-samples or paired-samples t-test. A $p \leq 0.05$ was considered statistically significant.



Figure 1. Use of the Rigeneracons medical device to generate autologous cartilage micrografts: (A) The autologous cartilage sample is collected with a punch; (B) Size of the autologous cartilage micrografts after collection; (C) The tissue, along with sterile saline solution, is inserted in the Rigeneracons device, which is activated by its motor; (D) After the cartilage fragmentation, the micrografts suspension is directly collected; (E) The cartilage micrografts suspension is directly injected into the knee of the patient with a syringe.

3. Results

In all cases, the procedure was successful, and none of the patients experienced infections or severe complications after the procedure. No aesthetic defects were observed at the donor site, and none complained about the results. All patients reported a pain resolution and a good recovery of daily activities (Table 2).

Table 2. Clinical outcomes of patients included in the study.

Patients	Clinical Outcomes
1	Complete pain resolution, recovery of daily activity
2	Complete pain resolution, general improvement about 6 months
3	Complete pain resolution, recovery of daily activity
4	Pain resolution, general improvement, and recovery of daily activity after 1 month following treatment
5	Complete pain resolution, general improvement, and recovery of physical and daily activity after 1 month following treatment
6	Complete pain resolution, general improvement, and recovery of physical and daily activity after 6 months following treatment
7	Complete pain resolution
8	Complete pain resolution, general improvement, and recovery of physical activity

3.1. Oxford Knee Score (OKS) and the Visual Analogue Scale (VAS)

From a clinical point of view, all patients recovered their daily activities without the need for analgesic therapy as indicated by OKS and VAS (Table 3). The mean preoperative and postoperative OKS was 28.4 ± 6 (range, 19–36) and 40.8 ± 6.2 (range, 30–47), respectively.

Table 3. Clinical outcomes of patients included in the study.

Patients	OKS Pre	OKS Post	VAS Pre	VAS Post
1	19	30	5	3
2	22	33	6	2
3	25	40	7	2
4	28	41	8	2
5	31	45	4	1
6	32	44	3	1
7	34	46	5	2
8	36	47	6	1

The VAS was 5.5 ± 1.6 and 1.8 ± 0.7 before and after 6 months, respectively. Both OKS and VAS values remained stable also after 3 years.

3.2. Magnetic Resonance (MRI) and Cartilage Measurements

To confirm the positive clinical outcome, as reported by the OKS and VAS score, we evaluated the cartilaginous thickness of the patients undergoing the micrografts application by MRI, which was performed at baseline and after 3 years. Interestingly, we observed an increase in thickness of the femoral lateral condyles on the sagittal and coronal plane (Figures 2–4). The mean measurements of femoral condyle on the sagittal plane were 1.2 mm (± 0.2) at baseline and 1.3 mm (± 0.1) after 3 years, showing an increase of 10% ($p = 0.006$), as reported in Table 4. For the coronal plane, the mean length of femoral condyle was 1.2 mm (± 0.3) at baseline and 1.8 mm (± 0.2) after 3 years showing an increase of 40% ($p = 0.025$), as shown in Table 5.

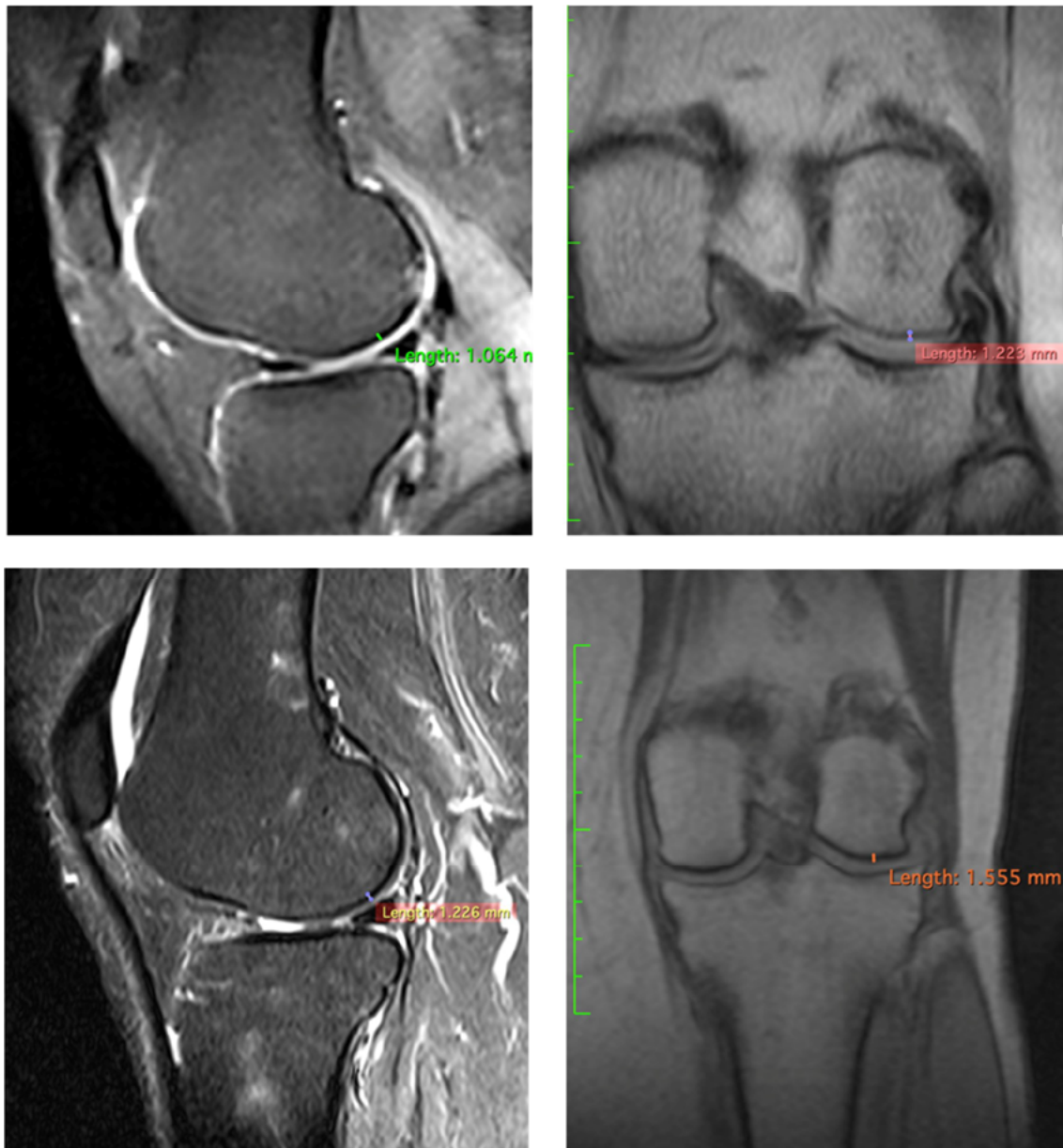


Figure 2. Representative images of MRI of patient no. 1 at 3 years. Images of femoral lateral condyles and measurement of thickness cartilage. We state that different MRI machines and different sequences were used for each patient.

Table 4. MRI results of cartilaginous thickness of femoral lateral condyles on the sagittal plane.

Patients	Pre/Femoral Lateral Condyles	3 Years/Femoral Lateral Condyles	Difference
1	1.064 mm	1.226 mm	+15.22%
2	1.339 mm	1.472 mm	+9.32%
3	1.068 mm	1.263 mm	−14.20%

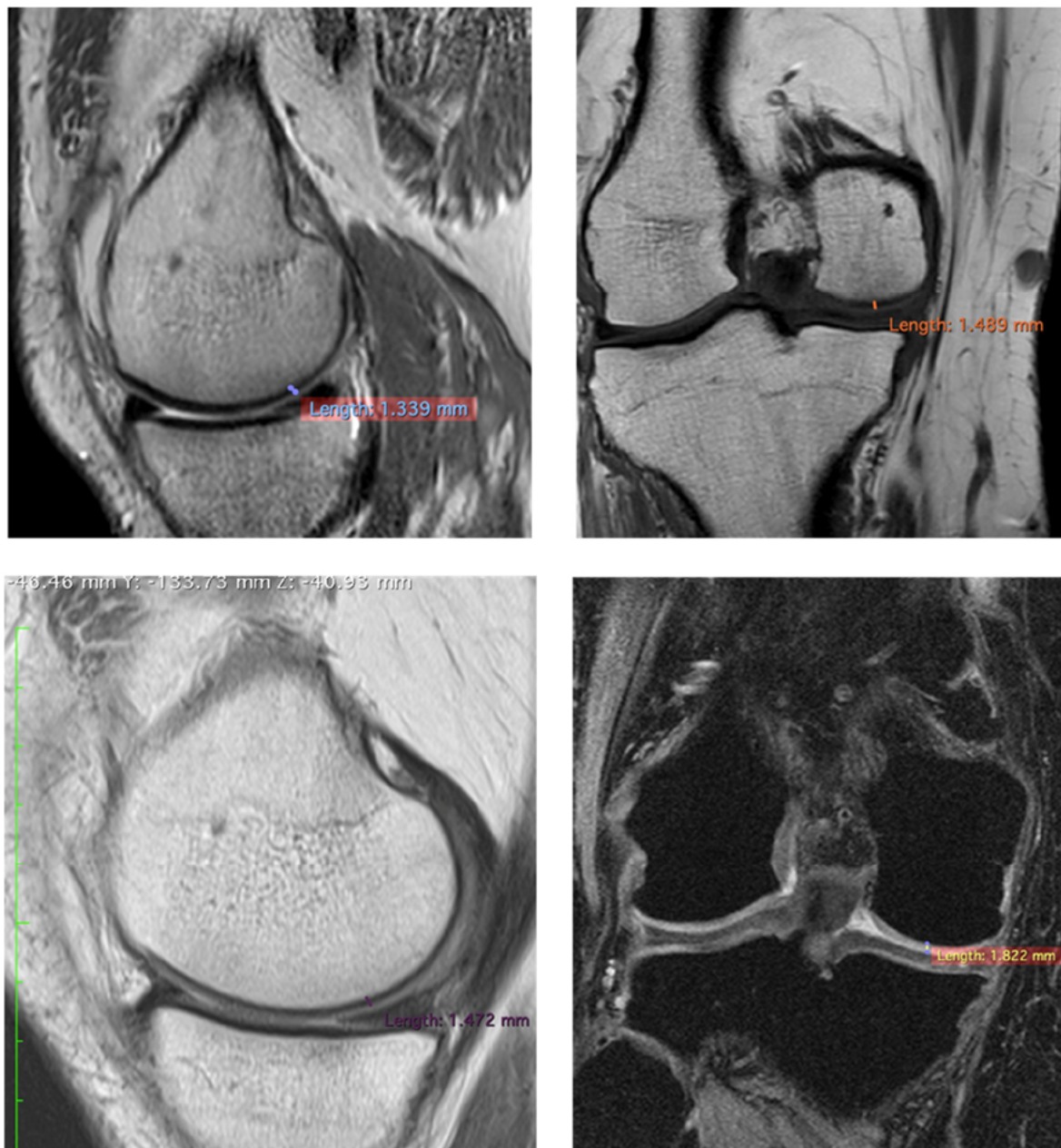


Figure 3. Representative images of MRI of patient no 6 at 3 years. Images of femoral lateral condyles and measurement of thickness cartilage. We state that different MRI machines and different sequences were used for each patient.

Table 5. MRI results of cartilaginous thickness of the femoral lateral condyles on the coronal plane.

Patients	Pre/Femoral Lateral Condyles	3 Years/Femoral Lateral Condyles	Difference
1	1.223 mm	1.555 mm	+27.14%
2	1.489 mm	1.882 mm	+26.39%
3	0.893 mm	1.914 mm	+114.33%

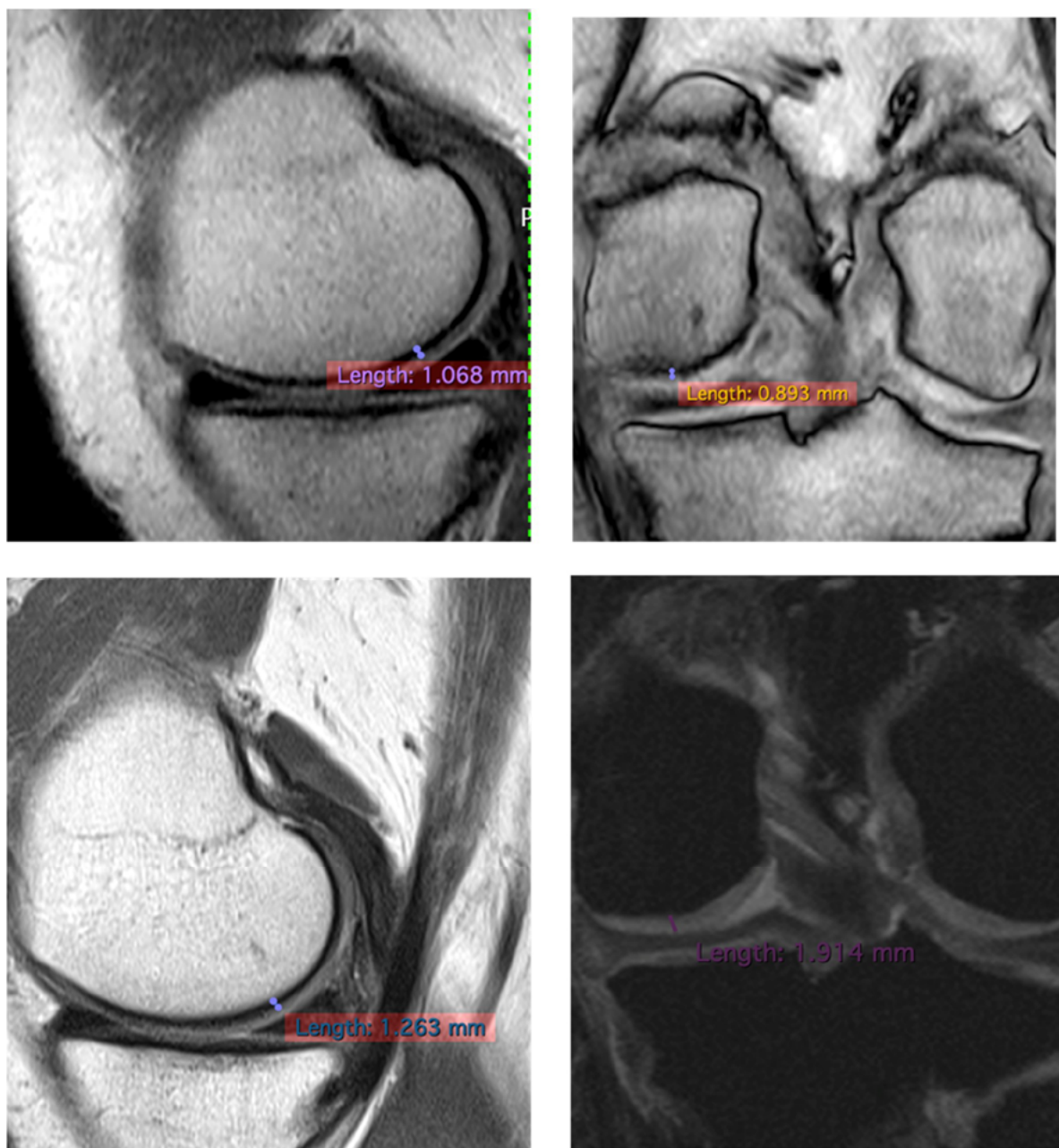


Figure 4. Representative images of MRI of patient no 2 at 3 years. Images of femoral lateral condyles and measurement of thickness cartilage. We state that different MRI machines and different sequences were used for each patient.

4. Discussion

Cartilage lesions have a wide variety of causes and may result in the persistence of important alterations in the skeletal plane. Some lesions, after the removal of the etiological agent, have the possibility to be repaired by natural biological processes. This outcome is only achievable in a few cases because of limited self-healing ability of the cartilage. On the other hand, this process could take years to complete a repair, which becomes a complicating factor for the resolution of the entire process.

Current intervention can be grouped into biologic-based therapeutics as Hyaluronic Acid (HI) or Platelet-Rich Plasma (PRP), surgical intervention, and cell-based approaches [25].

In this study, were reported preliminary data and exploited the use of cartilage autologous micrografts in the treatment of knee chondropathy, showing clinical improvement in terms of quality of life of the patients (Table 3) and, most importantly, patients who have

undergone MRI analysis show an increase in thickness of cartilage (Figures 2–4). MRI analysis was selected as the standard to evaluate the cartilaginous thickness, compared to X-ray evaluation, which would allow the discrimination of only the intra-articular space and therefore an indirect information on the cartilage status as described in the literature [26]. Even though the analysis was performed with different MRI machines, this is not an issue, since both had a 1.5 Tesla (T) scanner and the parameter analyzed was only dimensional.

The analyses here reported were evaluated by an expert radiologist, standardizing the MRI plane to evaluate the cartilaginous thickness.

The novelty of this approach is related to the technology applied, the Rigeneracons, which allowed mechanical disaggregation of the small sample of autologous cartilage, generating a cartilaginous tissue suspension ready to be grafted onto the patient with a simple syringe (Figure 1).

It should be noted that the autologous micrografts used in this study were immediately available, since the procedure lasted 10 min, without the need for culture/differentiation *in vitro*. Furthermore, previous studies have demonstrated that autologous micrografts generated from auricular cartilage were positive for cartilage progenitor cell markers such as CD44, CD90, and CD117; moreover, the micrograft-derived RNA was quantified and positive for tissue cartilage markers such as Sox-9 and COL2A1 [27]. *In vitro* studies also reported that the Rigenera Cartilage-Derived Micrografts can influence chondrocyte growth by (i) increasing glycosaminoglycans (GAGs) deposition, (ii) enhancing the production of Collagen II and (iii) the presence of IGF-1 and TGF β within the micrografts suspension [20]. The clinical application of those micrografts was also reported in another independent study where all patients reported a remarkable pain decrease during the few days following the procedure, suggesting an immunomodulatory effect mediated by micrografts even if other studies are required to demonstrate this [21].

The use of micrografts, with respect the use of cell-based techniques such as ACI or MACI, overcomes the limitations related to these methods. In fact, the ACI or MACI techniques are based on the *in vitro* cell expansion of chondrocytes, which can lead to the formation of fibrocartilage due to the de-differentiation of chondrocytes during cell expansion [28]. Additionally, the proliferative rate of chondrocytes decreases with the age of the donor, limiting the applicability of ACI or MACI techniques [29].

Another promising approach in cartilage repair is represented by mesenchymal stem cell (MSC) application due their high index of differentiation and facility in collection from different sources including bone marrow, adipose tissue, synovial membrane, and others [30,31]. MSCs also have an extensive potential to differentiate into chondrocytes, exerting anti-inflammatory and immunomodulating properties, offering a promising tool in cartilage repair [32]. However, the transplantation of MSCs often gives rise to a mixture of hypertrophic, cartilaginous, and fibrous tissues, which, in the long run, leads to a loss of repair tissue [33].

OAT (mosaicplasty) is a tissue-based option suitable for patients with lesions of a substantial size ($>3\text{ cm}^2$). In this procedure, which can be performed arthroscopically, grafts are collected from the periphery of the non-weight-bearing trochlea, then a drill guide is inserted perpendicular to the joint surface in the defect to allow graft sockets to be reamed. The OAT has shown encouraging results for up to 10 years after surgery; however, the additional effect over traditional microfracture treatment is reduced over time [34].

Lastly, compared to non-surgical intervention (PRP or Hyaluronic Acid) the autologous cartilage micrografts retain the same simplistic approach in terms of delivery (injections in the articular space); however, being autologous living tissue, the cartilage grafts represent the conjunction with surgical-based intervention (OAT), up to now rarely used because of its extreme invasiveness.

5. Conclusions

The use of the Rigenera micron-sized (80 micron) micrografts delivered with a simple syringe is increasingly expanding in different clinical settings with the possibility for use in

different tissues. In the context of knee chondropathy (grades I to III), a single application of autologous cartilage micrografts improved the quality of life of patients, since no analgesic therapy was needed and, most importantly, it promoted the formation of new cartilage. This novel autograft procedure represents an innovative and accessible approach for the management of chondropathy; however, considering the small sample size, more studies are necessary to validate such clinical application. Lastly, this approach could be expanded to different joints over the human body, as our group have successfully treated the hip joint and ankle joint by using the same criteria here reported. However, for other cartilaginous pathologies (i.e., arthritis) more in vitro analysis should be performed to monitor if and how the micrografts have a positive outcome, possibly reducing the chronic inflammation in this different clinical context. The major limitations of this study are: (i) the lack of standardization for MRI; and (ii) the absence of a control group. However, it should be defined what would a suitable control group, if non-surgical (palliative) approaches such as PRP and HI or tissue-based approaches, surgical approach as OAT are acceptable. A suitable comparison would be with the surgical/tissue-based approaches such as OAT; however, such procedures are rarely preferred, as they are extremely invasive.

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ARTICLE

Prospective Observational Study of a Non-Arthroscopic Autologous Cartilage Micrografting Technology for Knee Osteoarthritis

Dimitrios Tsoukas, Ilie Muntean, Christos Simos and Ruben Sabido-Vera

Article

Prospective Observational Study of a Non-Arthroscopic Autologous Cartilage Micrografting Technology for Knee Osteoarthritis

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Abstract: Autologous micrografting technology (AMT[®]) involves the use of autologous micrografts to stimulate/enhance the repair of damaged tissue. This study assessed the efficacy and safety of the AMT[®] procedure in patients with early stages of knee osteoarthritis. Briefly, the AMT[®] procedure involved extraction of auricular cartilage, disaggregation using the Rigeneracons[®] SRT in 4.0 mL of saline solution, and injection of the disaggregated micrografts into the external femorotibial compartment area of the affected knee. Ten patients (4 men, 6 women; age range: 37–84 years) were included in the study. In all patients, there was a steady improvement in knee instability, pain, swelling, mechanical locking, stair climbing, and squatting at 1- and 6-months post-procedure. Improvement in mobility was observed as early as 3 weeks post-procedure in 2 patients. Significant improvements were seen in mean scores of all five subscales of Knee Injury and Osteoarthritis Outcome Score (KOOS [KOOS symptoms, KOOS pain, KOOS ADL, KOOS sport and recreation, and KOOS quality-of-life]) between pre-procedure and 1- and 6-months post-procedure (all $p \leq 0.05$). Autologous auricular cartilage micrografts obtained by AMT[®] procedure (using Rigenera[®] technology) is an effective and safe protocol in the treatment of early stage knee osteoarthritis. These encouraging findings need to be validated in a larger patient population and in a randomized clinical trial (RCT).

Keywords: autologous; intra-articular; knee osteoarthritis; micrograft technology; pain management; regenerative medicine



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1. Introduction

Knee osteoarthritis (OA) is a common progressive joint disease, characterized by chronic pain and functional disability [1]. The pooled global prevalence of knee OA in the year 2020 was 16.0% in individuals above 15 years of age and 22.9% in those aged 40 years and over [2]. The prevalence and incidence of knee OA increases with age and is higher in women than in men [2]. It represents a substantial and increasing health burden with considerable personal, economic, and societal toll [1,3].

OA is characterized by the degradation of articular cartilage and bone matrix components [4]. The aim of treatment is to preserve joints in order to improve pain, restore activity, and delay arthroplasty [5]. Bone marrow stimulation techniques, cartilaginous or chondrogenic tissue repair, osteochondrogenic autologous transplantation, and autologous chondrocyte implantation [5] are the most widely used options for treating OA. In addition, injection of corticosteroids, hyaluronic acid into the intra-articular region, and subchondral injections of platelet-rich plasma are other widely used techniques in OA patients [6]. However, none of the aforementioned treatment options effectively arrest structural deterioration of cartilage and bone or successfully reverse existing structural defects [3,4,7]. They also do not enable regeneration of the articular tissue with its distinct

functional characteristics [8]. As OA is a highly heterogeneous disease, targeting a single joint tissue for treatment may not be effective [4].

Regenerative medicine is currently considered a promising therapy for the treatment of OA in humans. This innovative method involves the use of biological sources such as stem cells and grafts that allow the regeneration and replacement of cells, tissues or organs in order to restore the original structure and physiological functions [4,9]. Autologous micrografting technology (AMT[®]) involves the use of autologous micrografts to enhance regeneration of an impaired or damaged tissue. The key strengths of AMT[®] are a good safety profile, non-rejection of the injected micrografts, and specificity to recover normal functioning of tissues through specific signaling pathways [10]. Use of autologous micrografts can overcome some of the current limitations of other therapeutic approaches, like invasiveness, donor site morbidity, cell death, and allogeneic response [11]. The AMT[®] procedure is not considered an advanced therapy medicinal product as it falls under the category of non-substantially manipulated cells or tissues used for the same essential function, which means that the cells when removed from their original environment in the human body are used to maintain the original function(s) in the same anatomical or histological environment under allogeneic or autologous conditions [12].

The Rigenera[®] technology (Regenera Activa Worldwide S.L., Barcelona, Spain, and Human Brain Wave SRL, Torino, Italy) is a novel strategy for tissue mechanical disaggregation, which allows obtaining autologous micrografts [13,14] enriched in progenitor cells expressing MSC-like markers and having strong regenerative potential [15]. The AMT[®] procedure using Rigenera[®] technology has been used worldwide for more than ten years since its development in 2012 to stimulate and enhance self-regenerative processes for multiple conditions. It has shown clinical efficacy in the management of complex wounds [16–18], for the regeneration of the bone in periodontal surgeries [19], for pinched nose deformity [20–22], for improving hair density in patients affected by androgenetic alopecia [23–26], for treatment of scars [27], for treatment of osteochondral lesions of the knee [28], and for the treatment of knee chondropathy [5]. The procedure has also shown promise for treatment of osteochondral defects in a preclinical study [11].

The aim of this study was to determine the clinical effect and outcomes of the AMT[®] procedure in patients with early stages of knee OA.

2. Materials and Methods

This was a prospective, open-label study conducted between June 2022 to November 2022.

2.1. AMT[®] Procedure

The AMT[®] procedure used in this study was like the one used in previous studies in knee-joint-degeneration patients [5,24,27]. The tools used in the AMT[®] procedure are Rigeneracons[®] SRT (7945RS), Sicurdrill[®] 2.0, Sicurlid and Sicurstick (Regenera Activa Worldwide S.L., Figure 1a–d).

The Rigeneracons[®] SRT is a sterile disposable class IIa medical device designed to mechanically disaggregate solid human tissue to obtain soluble autologous micrografts, which are injectable (the AMT[®] solution). Rigeneracons[®] SRT comprises a grid with 100 hexagonal holes, each containing 6 calibrated microblades; a helix that rotates through an internal metal ring at a constant 80 revolutions per minute to disaggregate tissues with a cut off of 80 µm without causing cellular disruption and maintaining cell viability; and 3 arms. The Sicurdrill[®] 2.0 is a class I medical device with a motor that provides the electromechanical impulse to rotate the helix of the Rigeneracons[®] SRT. The Sicurdrill[®] 2.0 operates in 1-min cycles, each cycle started by pressing the frontal button. It is specially designed to be easily transportable. The Sicurlid and Sicurstick are specific adapters to secure the Rigeneracons[®] SRT to the Sicurdrill[®] 2.0. The procedure is shown in Figure 2 and described below:

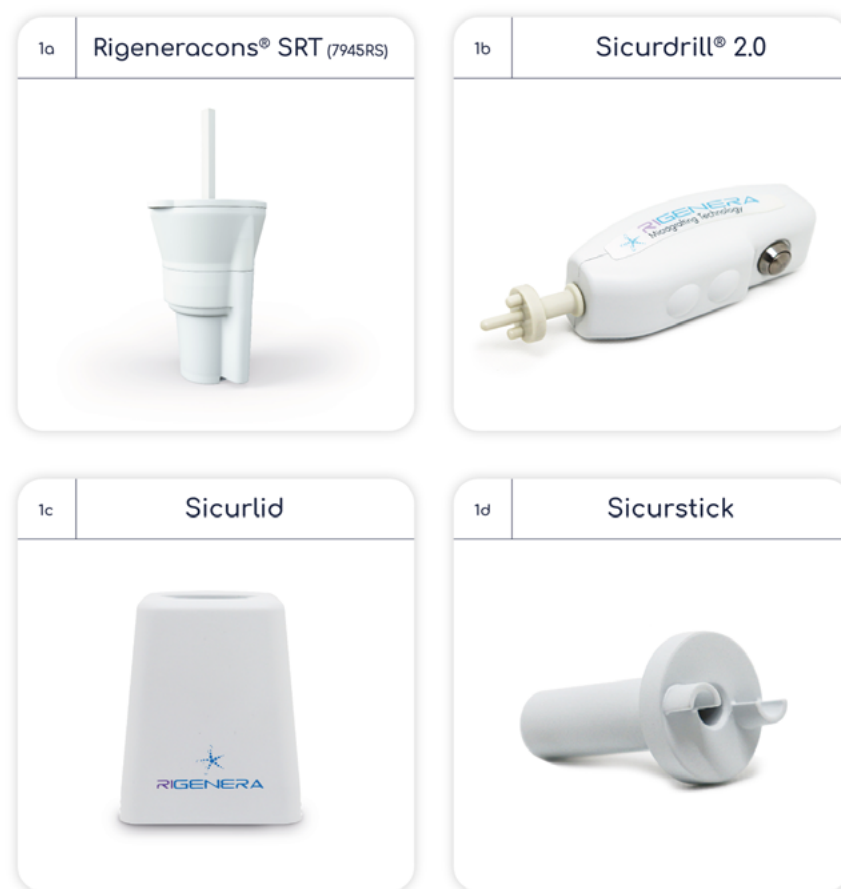


Figure 1. Tools used in autologous micrografting procedure.

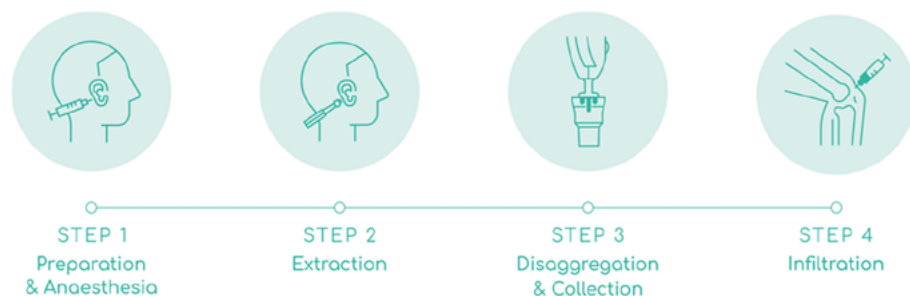


Figure 2. Steps involved in autologous micrografting procedure. The figure shows AMT[®] procedure in 4 steps. STEP 1: preparation and anesthesia of donor's area. STEP 2: extraction of 3 auricular cartilage biopsies. STEP 3: mechanical disaggregation of the biopsies and collection of AMT[®] solution. STEP 4: infiltration of AMT[®] solution into the knee joint.

Preparation and anesthesia: The auricular concha of the ear was disinfected with chlorhexidine and the area for biopsy extraction was marked with a dermal marker. The area was anesthetized with 2.5 mL of 2% lidocaine without vasoconstrictors; 0.5 mL in the retroauricular nerve to anesthetize and the rest on both sides of the auricular concha to hydro separate the skin from the cartilage. Adrenaline was used if the bleeding did not stop after applying 5 min of hemostatic pressure.

Extraction: Three biopsy punches were made in the marked area with a 2.5 mm dermal punch. The three biopsies were placed on the grid of the Rigeneracons[®] SRT, and manually rotated to cover them under the helix. The donor area in the ear was covered with a band-aid.

Disaggregation and collection: Approximately 4.0 mL of saline solution was added through the extraction hole of the Rigeneracons® SRT with a Luer Slip syringe, until the biopsies were slightly embedded in the solution. Then the cap was closed, the Sicurstick inserted in the Rigeneracons® SRT and placed into the Sicurlid. The device was then attached to the Sicurdrill® 2.0. The frontal button on the Sicurdrill® was pressed to start the tissue disaggregation, which was indicated by the yellow flashing LED on the Sicurdrill®. The Sicurdrill® was operated for six consecutive cycles, each cycle of 1-min duration. At the end of six cycles, 4.0 mL of the AMT® solution was collected with a Luer Slip syringe.

Infiltration: The external femorotibial compartment area of the affected knee was disinfected with chlorhexidine. The 4.0 mL of AMT® solution was injected with a 21G $\frac{1}{2}$, 0.8×40 mm needle into the external femorotibial compartment. The joint was mobilized to distribute the injected AMT® solution evenly across the treated area.

All steps were carried out under sterile conditions and the full Rigeneracons® SRT kit which is intended for single use was safely disposed of after use in accordance with local guidelines.

Joint inflammation, if any, in the first 24–72 h post-procedure was treated with analgesics (other than nonsteroidal anti-inflammatory drugs which may interfere with the effectiveness of the micrograft). Patients were advised to rest in positions that do not overstrain the treated joint for at least 7 days post-procedure. To ensure healing of the biopsy site in the ear, patients were advised to not shower or have a sauna bath in the first 24–48 h post-procedure, to change the dressing of the biopsy site after 24 h and to keep the biopsy site clean and dry.

The study was conducted in accordance with the Declaration of Helsinki. Good Manufacturing Practices rules for processing, Good Clinical Practices for the clinical application and ethics committee approval did not apply to the study as it fell under the category of “autologous use in one step surgery, minimal manipulation, monofunctional use (used for the same essential function in the recipient as in the donor), and manipulation with devices in aseptic conditions” [24,25].

All patients provided written informed consent before the start of the procedure.

2.2. Eligibility Criteria

Patients included in the study were at the age between 35 and 75 those diagnosed with OA grade 2–3 (except for two patients with OA grade 3–4) on Kellgren and Lawrence scale [29], meaning that the joint preserved at least part of the articular cartilage.

Patients excluded were those with any autoimmune diseases including rheumatoid arthritis; those with any concomitant uncontrolled metabolic diseases; patients undergoing systemic and/or local corticosteroids treatment; hyaluronic acid infiltration within the previous six months; as well as those with grade 4 advanced OA.

2.3. Assessments

Magnetic resonance imaging (MRI) and X-ray were performed pre-procedure. The Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire [30] recommended by the International Knee Documentation Committee was used for assessing pain, stiffness, and function pre-procedure and at 1- and 6-months post-procedure. The KOOS is a self-reported outcome measure assessing the patient’s opinion about the health, symptoms, and functionality of their knee. It is a 42-item questionnaire, including 5 subscales: symptoms (seven items), pain (nine items), function in daily living (ADLs [17 items]), sports and recreation function (five items), and quality of life (four items). Each item has five possible answer options scored from 0 (No Problems) to 4 (Extreme Problems) and each of the five scores is calculated as the sum of the items included. Scores are transformed to a 0–100 scale, with zero representing extreme knee problems and 100 representing no knee problems. An aggregate score of all subscales is not calculated since it is regarded desirable to analyze and interpret the five dimensions separately [30].

Incidence of adverse events was monitored during the study. The biopsied area in the ear was checked for signs of infection and for any abnormality in healing. A clinical evaluation and safety assessment was carried out at 12 months post-procedure.

2.4. Statistical Analysis

Data were analyzed with one-way analysis of variance and posterior multiple comparison Bonferroni test. Data were expressed as the mean $\pm$ standard deviation (SD). A $p \leq 0.05$ was considered statistically significant. RStudio software (RStudio Team (2020). RStudio: Integrated Development for R. RStudio PBC, Boston, MA, USA) was used for statistical analysis.

3. Results

The study included 10 patients, 4 men and 6 women; all except one patient were aged 53 years and above (Table 1). Patients reported pain in their affected knee for the past <1 year to 10 years prior to start of study.

Table 1. Patients' baseline demographics and disease characteristics.

Patient Number	Age (Years)	Sex	BMI (kg/m ²)	Diagnosis Based on KL Scale	Clinical History	Past Medications	Past Procedures
1	63	Male	25	OA grade 3; chondropathy in left knee	Pain for the past 4 years and stiffness which became worse around a month before enrollment into study. Medial meniscus posterior horn tear 10 years prior to study enrollment which was not treated	None	None
2	84	Female	23	OA grade 3–4	Pain in right knee from past 10 years	None	None
3	64	Female	22	OA grade 3–4	Left knee pain for 8 years, worsening over time. Gonarthrosis for 8 years	NSAIDs	PRP injections in 2020 and 2021
4	37	Male	25	Chondropathy grade 3	Gonarthrosis	NSAIDs and paracetamol	Physiotherapy and PRP
5	74	Female	23	OA grade 3	Gonarthrosis. Pain in affected right knee for the past 10 years that became worse after an accident nearly 7 months prior to study enrolment	None	None
6	73	Female	22	OA grade 2	Pain for past 2 years	NSAIDs for 3 months	Physiotherapy
7	73	Female	22	OA grade 1-early 2	Very mild pain for the past less than 1 year	None	None
8	64	Female	23	OA grade 1 early 2	Pain for the past one year. Fibromyalgia and depression	None	None
9	53	Male	31	OA grade 1 early 2	Pain, reduced mobility and grinding sensation (crackling)	NSAIDs, education about weight control	Physiotherapy
10	53	Male	31	OA grade 3 symptomatic	Mild pain and reduced mobility	None	None

BMI: body mass index; KL: Kellgren–Lawrence; NSAIDs: nonsteroidal anti-inflammatory agents; OA: osteoarthritis; PRP: platelet-rich plasma therapy. Note: Patient number 4 was an ex-professional basketball player; Patient number 5 was a very active person, bicycling for 40 years, but had stopped it about a year before enrollment into study.

3.1. MRI and X-ray Findings

MRI at baseline revealed subchondral bone edema, chondropathy patella, and medial compartment narrowing; Baker cyst was noted in one patient (Table 2). X-ray revealed presence of osteophytes and medial compartment narrowing (Table 2).

Table 2. Magnetic resonance imaging and X-ray findings pre-procedure.

Patient Number	MRI Findings	X-ray Findings
1	Subchondral bone edema medial femoral condyle with small cartilage lesion, medial meniscus degenerative tear. Chondropathy patella grade III.	Standing X-ray: medial compartment narrowing
2	Chondropathy patella II–III and medial femoral condyle grade III–IV.	Osteoarthritis KL scale grade 3–early 4
3	Medial compartment narrowing with chondropathy both condyles. Baker cyst 4 cm × 9 cm	Osteophytes present; KL scale grade 3 to 4 of osteoarthritis with medial compartment narrowing
4	Patella alta 3rd degree chondropathy patella and cartilage wear 1.7 cm × 1.5 cm at the femoral trochlea	Patella alta
5	Chondropathy with diffuse cartilage defects	Osteoarthritis grade 3
6	Chondropathy patella grade III with cartilage defects	Osteoarthritis grade 2
7	Diffuse cartilage wear Outerbridge grade II	Osteoarthritis grade 1–2
8	Three-plane study of the left knee. Internal meniscus of preserved morphology and signal. External meniscus with increased horizontal signal that also extended to the lower articular surface compatible with meniscal tear and associated with the presence of small loculated cystic images that extended anteriorly with a larger component that crossed the lateral retinaculum which might correspond to the palpable swelling, compatible with parameniscal cyst. Conclusion: Rupture of the external meniscus body with parameniscal cyst extending anteriorly, crossing the lateral retinaculum. Slight decrease in the thickness of the patellar cartilage in its superior and central portion.	Osteoarthritis KL grade 1–2
9	Chondropathy wear lateral tibial condyle	Osteoarthritis of the femorotibial joint left knee KL grade 1–2
10	Cartilage wear medial femoral condyle Outerbridge grade 2 and medial tibial condyle grade 2–3.	Osteoarthritis KL grade 3

KL: Kellgren–Lawrence; MRI: magnetic resonance imaging; NA: not available.

3.2. AMT[®] Procedure Outcomes

In all patients, the wound in the ear completely healed within one week without any need for stitches. None of the patients needed adrenaline to stop the bleeding from the biopsied area in the ear. There were no cases of infection or signs of abnormal healing of the donor area like “cauliflower ear”. The patients strictly followed physician’s aftercare recommendations for the healing of the donor area.

The AMT[®] procedure was successful in all 10 patients. Pain resolution and good recovery of daily activities was seen in all 10 patients, with improvement in mobility being observed as early as 3 weeks post-procedure in 2 patients (Table 3).

Table 3. Clinical outcomes within 12 months following AMT[®] procedure.

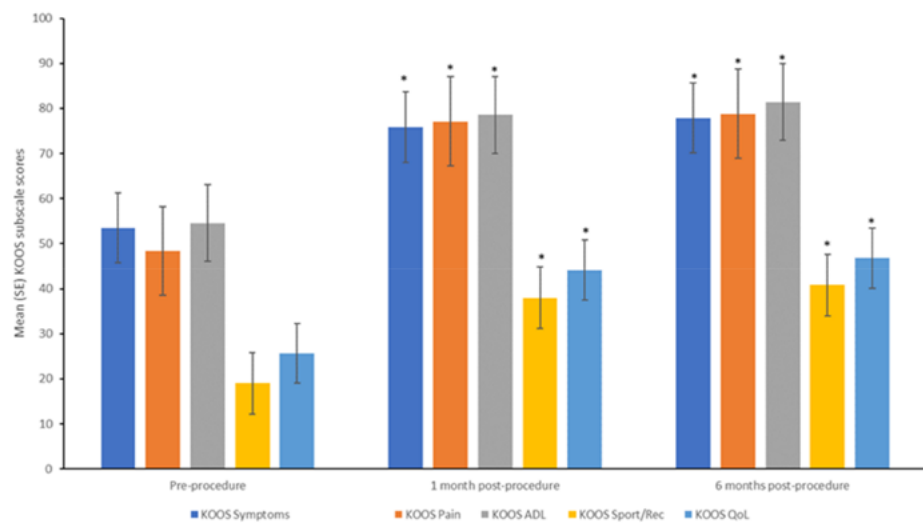
Patient Number	Description of Clinical Condition
1	No pain and good mobility at 10 months post-procedure
2	Lateral pain and good mobility as early as 3 weeks post-procedure
3	Less pain and better mobility as early as 3 weeks post-procedure
4	Impressive improvement in mobility and pain reduction from within 2 months post-procedure. The patient started bicycling post-procedure, which was stopped earlier due to pain
5	Improvement in clinical outcomes as early as 6 weeks post-procedure The patient started running at 2 months and playing basketball at low intensity at 3 months
6	Able to do daily activities without any pain. Clinical improvement observed was maintained even after 11 months post-procedure
7	No pain while carrying out daily activities, while pre-procedure the patient could not be on foot for more than 2 h The patient was able to walk for longer periods and was also able to take the stairs instead of the elevator Clinical improvement observed was maintained even after 1-year post-procedure
8	No pain and good mobility which was maintained even after 10 months post-procedure
9	Reduced pain and ability to walk long distances of more than 10 km with only minimal pain The patient lost some weight, but not sufficient to significantly help with the overall body inflammation and biomechanics in the joint
10	Reduced pain and improved mobility

AMT: autologous micrografting technology.

Physical examination at 1- month and 6-months post-AMT[®] procedure demonstrated a steady improvement in all patients in knee instability, pain, swelling, mechanical locking, stair climbing and squatting.

3.3. KOOS Subscale Scores

All 10 patients completed the assessments at 1- and 6-months post-procedure. Significant improvements were seen in the mean scores of all five subscales of KOOS (KOOS symptoms, KOOS pain, KOOS ADL, KOOS sport and recreation, and KOOS quality-of-life) between pre-procedure and 1- and 6-month post-procedure (Figure 3, all $p \leq 0.05$). Between 1- and 6-months post-procedure, significant improvements were observed in the mean scores of the subscales of KOOS symptoms, KOOS pain, and KOOS ADL (all $p \leq 0.05$), while for the subscales of KOOS sport and recreation, and KOOS quality-of-life, the improvements observed at 1-month were maintained at 6-months. Each one of the KOOS subscales was analyzed and passed the Bonferroni test.



ADL: activities of daily living; KOOS: Knee Injury and Osteoarthritis Outcome Score; SE: standard error; Sport/Rec: sport and recreation function; QoL: quality of life * $p \leq 0.05$ versus pre-procedure

Figure 3. KOOS subscale scores pre-procedure and at 1- and 6-months post-procedure.

3.4. Safety and Long-Term Follow-Up

Clinical evaluation at 12 months post-procedure showed that all 10 patients had continued good mobility and minimal or no pain in the affected knee.

The AMT[®] procedure showed a good tolerability profile since none of the patients reported any adverse event during the study.

4. Discussion

Use of biological strategies such as endogenous stem cells or tissue-specific progenitor cells to enable patient's own cartilage regeneration ability represents an important advance in the treatment of cartilage defects and OA [31,32].

Chondrocytes, which are highly specialized cells in the articular cartilage of the knee constitute approximately 1% of total volume [33] and supply the extracellular matrix (ECM) with the elements such as collagen and proteoglycans that constitute the cartilage function [34]. Chondrocytes are responsible for replacement of ECM molecules lost through degradation over the years. However, aging decreases the ability of chondrocytes to replace ECM molecules resulting in progressive degeneration of articular cartilage leading to joint pain and dysfunction, clinically identified as OA [35]. Also, while articular cartilage can withstand intensive and repetitive physical stress, impact forces caused by falls, sports injuries and road accidents lead to substantial damage [8], but the articular cartilage has limited ability to repair and self-renew as it is an avascular tissue [8,36,37]. Autologous chondrocytes are an effective cell therapy source for repair of chondral defects, but the challenges of using them are the paucity of donor sites, de-differentiation during expansion in culture and early apoptosis [38]. MSCs are another treatment option with a potential for cartilage regeneration but their use is limited by their tendency to differentiate into various cell lineages and the need for specific induction manipulation to promote chondrogenesis [38].

Perichondrial tissue, which is present in all adult cartilage tissues except for articular and fibrocartilage [39], has the potential to produce hyaline-like cartilage [40]. The clinical relevance of perichondrium was recognized more than a century ago [39], and it is possibly among the first tissues to attract clinical interest for its regenerative potential, with initial reports on its role in cartilage regeneration made as early as the 19th century [40–42]. Interest in the perichondrium was revived in recent years owing to its possible role as a microenvironment containing stem and chondrogenic progenitor cells [38,39]. Findings from multiple animal and human studies both in vivo and in vitro indicate that perichon-

drium can facilitate and is essential for cartilage regeneration and has the clinical potential to treat various cartilage-associated diseases and traumas [39]. Perichondrium contains a pool of mesenchymal progenitor cells called chondroblasts. Chondroblasts contribute to tissue homeostasis by replicating and differentiating into chondrocytes. An in vitro study showed that the progenitor cells in perichondrium had paracrine effects on prolonging the lifespan and promoting the proliferation and cartilaginous expression of chondrocytes [38]. Subcutaneous implantation of progenitor cells and chondrocytes together in nude mice was found to promote formation of cartilaginous tissue [38].

Auricular cartilage as a source of perichondrial progenitor cells has the advantages of ease of harvest and minimal donor-site morbidity compared with other sources of cartilage with perichondrium like the nasal cartilage [38]. Animal studies have demonstrated that autologous micrografts generated from auricular cartilage were positive for cartilage progenitor cell markers such as CD44, CD90, and CD117, and the ribonucleic acid derived from micrografts was positive for tissue cartilage markers such as Sox-9 and COL2A1 [22]. In comparison with murine MSCs, these levels indicate presence of chondrocyte-progenitor cells with a mesenchymal phenotype in auricular micrografts [22]. Autologous micrografts from human auricular cartilage cultured in vitro with human chondrocytes were found to positively influence chondrocyte differentiation as shown by both increased glycosaminoglycans deposition and the presence of collagen II and stimulate secretion of cartilage trophic factors such as insulin-like growth factor 1 and transforming growth factor β , without affecting chondrocyte viability [31]. The principle behind AMT[®] procedure with auricular cartilage for knee OA is to transfer the signaling potential of chondroprogenitor cells, chondrocytes and signaling factors together with ECM components to enhance the restoration of joint function and reduce pain and stiffness [5].

In this study, use of AMT[®] procedure with auricular micrografts was found to be effective and safe in the treatment of early stage and moderate knee OA. Post-procedure, all patients reported minimal or no pain. Improvement in mobility was observed as early as 3 weeks post-procedure, which was maintained even after 12 months post-procedure, indicating early benefits maintained over the long-term. These observations were supported by the significant improvements observed in all five subscales of KOOS, a patient-reported outcome (PRO) measure, at 1- and 6-months post-procedure. PROs are increasingly recognized by regulators, clinicians, and patients as valuable tools to collect patient-centered data [43,44]. The KOOS scale, an extension of the Western Ontario and McMaster Universities Arthritis Index (WOMAC) is mainly focused on the knee and is better adapted for younger, more active populations [43]. It has adequate internal consistency, test-retest reliability and construct validity in young and old adults with knee injuries and/or OA [45]. A change of 8–10 points is suggested to represent the minimal perceptible clinical improvement of the KOOS [43]. In this study, the change in KOOS subscale scores from pre-procedure ranged between 20 and 30 points at 6-month post-procedure.

The findings from this study in patients with early to moderate stage of knee OA strengthen the observation from in vitro and animal studies on the use of autologous auricular micrografts for joint pain management. The observations from this study and that of Marcarelli et al. [5] suggest a potential role for AMT[®] procedure in the treatment of various articular cartilage defects as well as cartilage defects in other joints in the body. Future studies in larger patient populations are needed to validate such clinical application. Limitations of the study are the small sample size, absence of a control group, lack of long-term follow-up and findings being based on a single AMT[®] procedure.

5. Conclusions

Autologous auricular cartilage micrografts obtained by AMT[®] procedure (using Rigena[®] technology) is an effective and safe protocol in the treatment of early stage knee OA and could be a valid treatment approach. The findings need to be validated in a larger patient population and in a randomized clinical trial (RCT).

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki. Ethical review and approval were waived for this study as it fell under the category of “autologous use in one step surgery, minimal manipulation, monofunctional use (used for the same essential function in the recipient as in the donor), and manipulation with devices in aseptic conditions”.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Data are contained within the article.

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07

ARTICLE

Autologous grafts in the treatment of avascular osteonecrosis of the femoral head

Marco Marcarelli, Marco Fiammengo, Letizia Trovato, Vincenzo Lancione, Elvio Novarese, Pier Francesco Indelli and Salvatore Risitano

ORIGINAL ARTICLE

Autologous grafts in the treatment of avascular osteonecrosis of the femoral head

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Summary. *Background:* Osteonecrosis of the femoral head (ONFH) is a frequent orthopedic disease leading to destruction of the hip joint and disabling arthritis. Several procedures have been developed to treat the joint deterioration in case of osteonecrosis, trying to avoid or delay an intervention of total hip replacement, especially in young patients. The aim of this study was to analyze the use of autologous bone micrografts derived from cancellous bone in the management of avascular ONFH. The treatment described was implemented using the Rigenera® protocol to obtain autologous micrografts: small fragments of cancellous bone collected by femoral neck, disaggregated and injected in the necrotic area using an empty screw. *Materials and methods:* Twenty adult patients affected by avascular ONFH were enrolled in this study; all patients reported a preoperative intermittent coxo-arthritis and limited function of intra and extra rotation of the hip. Inclusion criteria were an Oxford Hip Score between (OHS) 20 and 39, a Harris hip score (HHS) showing pre-operative poor results (lower than 70 points) and a stage II-III A and IIIB according with the classification proposed by the Association Research Circulation Osseous (ARCO). *Results:* Using an MRI evaluation, after six months, the authors observed a complete regression of necrotic area and the restoration of osseous structure. Clinical outcome has been evaluated at 6-12 and 24 months follow-up. At the final F.U. the HHS rised from poor to good results (mean value at final F.U of 84) while the OHS improved significantly already after 21 days from micrografts injection (mean 35.4 ± 7.5) with an increasing trend until to two-year final FU (mean 37.4 ± 9.5). The full recovery of daily and mild sport activities was reached after 20 and 90 days from intervention, respectively. *Conclusion:* The results of this study are suggestive for a new approach in the treatment of avascular ONFH assuming a process of bone regeneration based on a dual mechanism of action, biological and mechanical, induced by micrografts and injected using an empty screw as vehicle. (www.actabiomedica.it)

Key words: autologous, micrografts, bone regeneration, conservative treatment, osteonecrosis, femoral head

Introduction

Aseptic, non-traumatic osteonecrosis of the femoral head (ONFH) is a rare, but disabling orthopaedic disease that usually results in cellular necrosis of the femoral head leading to bone collapse and hip joint destruction (1).

Etiology and pathogenesis of ONFH are still unclear. Several risk factors have been proposed in its development and progression such as trauma, chronic use of corticosteroids, alcohol consumption, coagulation disorders and different pathogenic mechanisms are supposed to be related with ONFH, including ischemia due to vascular interruption, thrombosis, sickle

cell occlusion, or direct cellular toxicity due to pharmacological agents or oxidative stress (2-3). Furthermore, because of lack of specific symptoms, especially in its early stage, the diagnosis of ONFH can be extremely difficult.

Standard anteroposterior and lateral frog-leg radiographs and magnetic resonance imaging (MRI) are effective diagnostic tools (4). Anteroposterior and lateral frog-leg radiographs allow to identify both necrosis around sclerotic bone as well as segmental collapse, while MRI has a high sensitivity and specificity visualizing necrotic areas also in absence of subchondral fractures (4).

Two different approaches are usually adopted in the management of ONFH: a joint-preserving strategy or a joint-replacing treatment. Conservative treatments are usually based on pharmacological agents, biophysical therapy and multiple surgical approaches, classified as femoral head sparing procedures (FHSP) as core decompression (5), osteotomies, not vascularized (6) and vascularized bone grafting (7). These are usually indicated to address a pre-collapse stage and they are also finalized to delay as much as possible the time of total hip arthroplasty (THA). Joint-replacing treatments are commonly applied managing a post-collapse stage of ONFH.

Several bone grafting procedures have been proposed in the literature and performed using not vascularized or vascularized bone grafts to fill the necrotic area (8). The objective of this study was to evaluate subjective patient satisfaction (PROMs) as well as objective clinical and radiological outcomes in a series of patients affected by ONFH and undergoing a novel FHSP procedure where a classic core decompression surgery was implemented using autologous bone micrograft. A new system (Rigenera® technology) has been used obtaining fast and easily viable bone micrografts avoiding in vitro cells expansion or other biochemical manipulations (9). The clinical effectiveness of micrografts has already been demonstrated in different clinical areas including dentistry, dermatology or management of chronic wounds, showing the capacity to promote bone or dermal regeneration (10-11). The current authors hypothesized that autologous micrografts could play a significant role in the treatment of avascular ONFH supporting the subchondral bone

and articular cartilage in the immediate post-operative stage and secondarily they could improve the function of the hip stimulating a rapid bone regeneration. None of the authors have conflict of interest in connection with this study.

Materials and methods

The study protocol used in this report complied with the Declaration of Helsinki of 1975, the European regulations and the current authors proceeded to the data collection after concession of written consent for personal data collection and biological sampling by each patient.

Twenty consecutive male patients affected by idiopathic, aseptic and avascular ONFH were enrolled in this study. The mean age was 51.5 years (range from 40 to 63 years). The inclusion criteria for the study were an Oxford Hip Score (OHS) between 20 and 39, a Harris hip score (HHS) showing a pre-operative poor (lower than 70 points) score and a stage II-IIIA and IIIB according to the classification proposed by the Association Research Circulation Osseous (ARCO) and documented by an MRI. The exclusion criteria were the following: chronic steroid therapy, previous serious traumatic hip injuries, previous surgical hip treatments, rheumatoid or other immunological arthropathies.

Clinical and radiographic assessments

All patients were clinically assessed preoperatively and at 7-14 - 21 days, 2 months, 1 year and 2 years follow-up (F.U) according to the Harris Hip Score (HHS) and the Oxford Hip Score questionnaire (OHS) (12) as patient reported outcomes measurement systems (PROMs) and as overall validated measurement instruments.

For radiographic assessment, standard anteroposterior weight-bearing and lateral frog-leg radiographs were performed in all patients preoperatively, at 6 months and final 2 years FU; all patients underwent MRI evaluation pre-operatively and at 6 months FU. All radiographs were reviewed by an orthopedic surgeon not part of the surgical team (SR), blinded to

the clinical outcomes results: the radiological evaluation was performed according to ARCO classification system (13) for disease staging, necrotic lesions size and position and healing process.

All surgical procedures were performed by a single surgeon from January 2015 to December 2015 following spinal anesthesia with the patient in the lateral decubitus position. Following radiographic planning, a Kirschner wire (2,5 mm) was introduced in the femoral neck (Fig. 1A-B) in order to drive the insertion of a 6-mm cannulated cutter. Subsequently, a small piece of cancellous bone from the lateral edge of femoral neck was collected (Fig. 1C) and mechanically disaggregated according to the Rigenera[®] protocol using a class-I medical device (Rigeneracons. Human Brain Wave, LLC, Turin, Italy) (14).

The Rigeneracons is a biological disruptor of human tissues, composed by a grid made by 100 exagonal

holes which are embraced by six micro-blades designed for efficient cutting of both hard and soft tissues, able to filter and select a specific cell population of 50-micron size enriched progenitor cells and expressing markers typical of mesenchymal stem cells (9).

The fragments of cancellous bone were then mixed into the rigeneracons, adding 1.5 ml of sterile saline solution and then disrupted for 5 minutes by a pure mechanical rotation process. After this, the cell suspension was collected from a needle-free syringe for a total of 10 ml of micrograft disaggregating multiple bone fragments for each patient

The femoral head was then prepared with a 8 mm cutter (Fig. 1D); at this time, an cannulated screw was implanted and used as carrier to inject the micrografts directly in the necrotic camera (Fig. 1E). Finally, the screw head was obliterated with a pin to reinforce the fixation system and creating a closed camera.

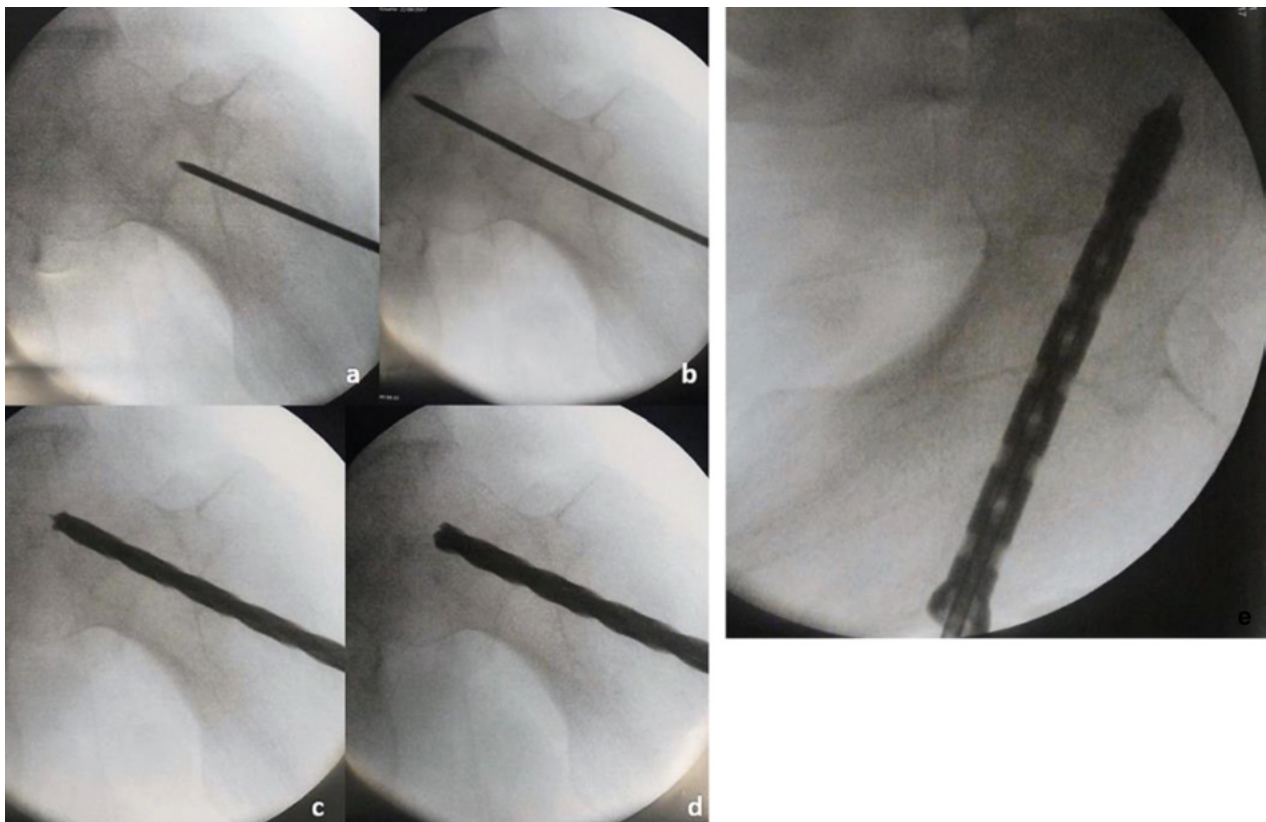


Figure 1. Surgical procedure: a Kirschner wire (2,5 mm) was first introduced in the femoral neck (A-B) and a small fragment of cancellous bone from the lateral edge of femoral neck was collected (C); the femoral head was then prepared with a 8 mm cutter (D); finally, a cannulated screw was implanted and the micrografts injected in the necrotic camera using the screw as a carrier (E)

All interventions were performed as outpatient and all patients were discharged few hours after injection. Patients were maintained at toe-touch weight bearing with crutches for two days: after that, weight-bearing as tolerated was allowed.

Results

A continuous improvement in the Harris hip score in 19/20 patients was noted after 7, 14, 21, 60 days, and 1 year and two years FU. HHS was calculated pre-operatively (mean value of 68) and post-operatively (table 2) showing a significant improvement in function and pain relief: at the final FU the HHS rise from poor to good results (mean, 84 points). Micrografts application failed only in one patient: a 58 Years old gentleman who subsequently underwent THA one year after the index procedure.

The average preoperative OHS was 28.1 ± 6.5 , while the postoperative OHS improved significantly starting 21 days from micrografts injection (mean 35.4

± 7.5), with an increasing trend to improvement after 60 days and all the way to one- and two-year FU (mean 37.4 ± 9.5). (table 1)

The full recovery allowing complete activities of daily activities was reached after 20 days from intervention, while sports activities were not allowed before the 90 days landmark.

All hips underwent MRI evaluation at the six months FU, using an artefacts suppression technique: the authors observed the complete regression of the necrotic area accomplished by restoring of osseous structure suggesting an autologous micrografts-induced bone regeneration in 19 out of 20 patients (Fig. 2).

Discussion

Osteonecrosis of the femoral head is a disabling orthopedic disease which represents an important economic burden on the public health care system affecting young and middle-aged subjects. It can be

Table 1. Oxford Hip Score evaluation before and after 7, 14, 21, 60 days and 1, 2 years of follow up. The results are expressed as average of all patients

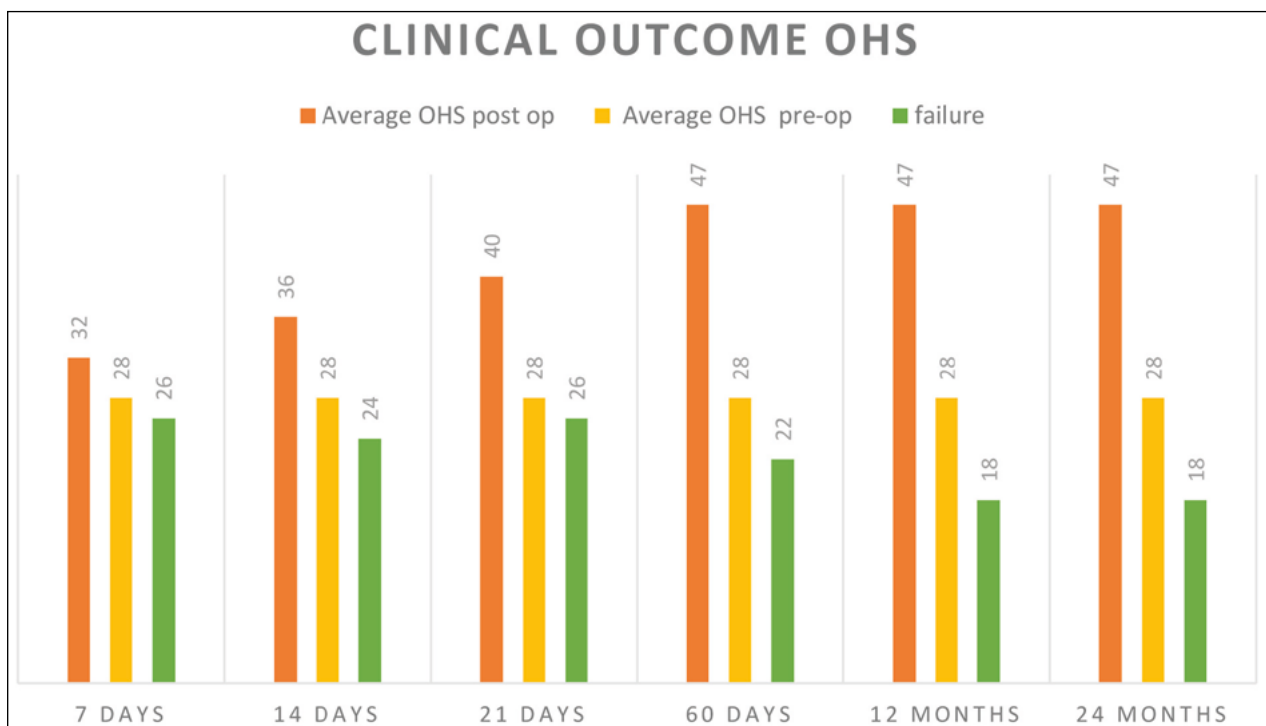
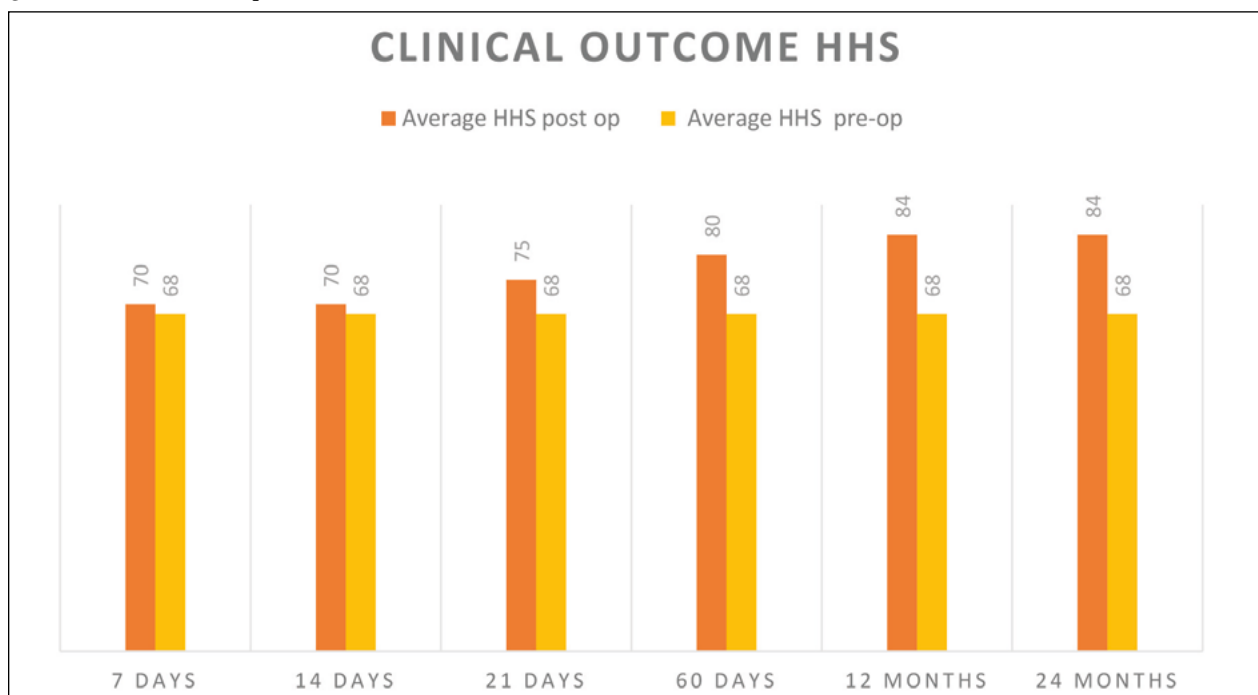


Table 2. Harris Hip Score evaluation before surgery and after 7, 14, 21, 60 days and 1, 2 years of follow up. Pre-operative evaluation showed poor results indicating a moderate to severe hip arthritis and disability. The improvement at the final F.U. was from poor to good results (mean of 84 points)



asymptomatic until the progression to a symptomatic osteonecrotic phase, leading to femoral collapse. For these reasons, new approaches to manage this pathological condition become mandatory in order to preserve the joint and delay as much as possible the THA intervention.

In this study, we reported the positive role of autologous micrografts on the treatment of ONFH, confirming the hypothesis of the study. We observed a clinical improvement according to the HHS and OHS; regression of the necrotic area and new bone regeneration were both observed following MRI after six months from application.

The goal of the treatment of ONFH is to prevent rapid joint degeneration. The ideal approach should consider patients age, stage and location of the disease and the amount of bone affected and even if several options have been previously described, unfortunately there is not consensus regarding the ideal approach (15). The current authors assume that a pure and isolated mechanical action of core decompression (16) might be a limited approach to start the healing

process: a combined mechanism of action, biological and mechanical, induced respectively by the use of micrografts and a cannulated screw as a delivery method could be adopted to increase the success rate.

To support the role of micrografts in the treatment of ONFH, previous studies have reported their application in the management of chronic and complex wounds such as ulcers of different etiology (17-19), wound dehiscence (14, 20) and pathological scars (11). More recent studies have demonstrated the efficacy of micrografts also for cartilage (21-22) and cardiac regeneration (23). To date, the efficacy of autologous micrografts in bone regeneration is more evident in the dentistry applications including periodontal regeneration (24), sinus lift augmentation (10, 25) and alveolar socket preservation (26).

The use of autologous bone grafts in the treatment of osteonecrosis has been already reported but more clinical studies are mandatory to evaluate the real advantages of this approach. A recent study showed that the outcomes of autologous concentrated bone marrow grafting for advanced osteonecrosis of the hu-

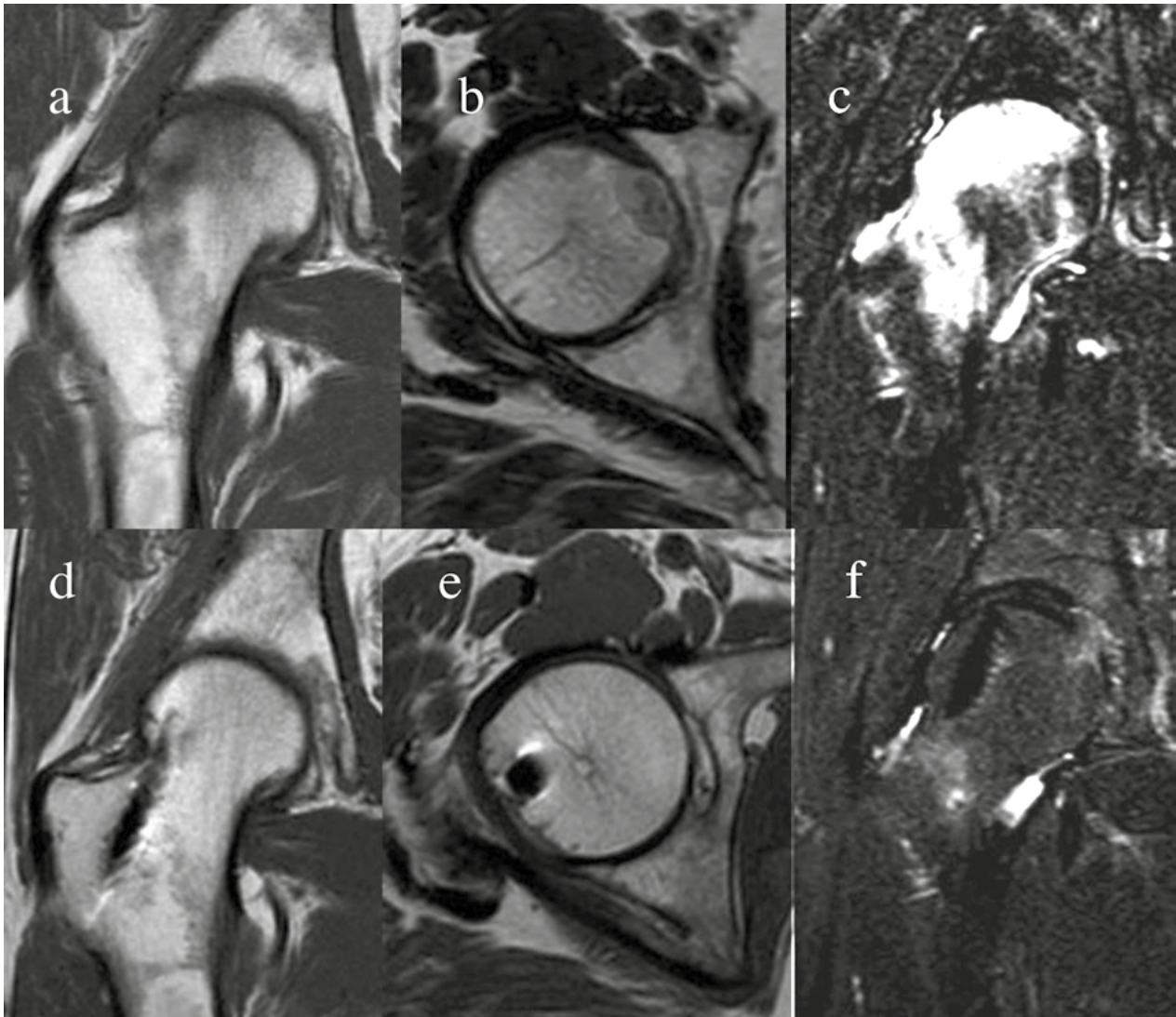


Figure 2. Magnetic resonance imaging (MRI) before and after micrografts treatment. The femoral head MRI was performed using a standard technique for artefact suppression. A complete regression of the necrotic area accomplished by restoring of osseous structure suggesting an autologous micrografts-induced bone regeneration

meral head are variable, concluding that further investigations are needed to determine their effectiveness (27). Other authors, analyzing the factors related to the clinical failure of bone grafting in the treatment of avascular ONFH, suggested that the advanced stage of the disease increases the risk of failure after bone graft surgery (28). According to our results, only patients belonging to ARCO stage II and IIIA showed a positive response to this treatment.

With the increasing interest for stem cell research and regenerative medicine, several authors consider

the ONFH as a result of an imbalance between osteocytes formation and apoptosis suggesting that cytotrapy could represent a new approach (29-30). Pre-clinical studies in animal models have confirmed the positive effects of stem cells injection in the treatment of ONFH (31-32) and promising results were also obtained in few clinical reports showing as bone marrow cells (BMCs) transplantation reduces volume of necrosis in patients in the pre-collapse stage (33-34-35). These evidences are in line with the results described in our study: in fact, micrografts are able to maintain

great regenerative properties similar to MSCs (36), leading to expression of several markers of mesenchymal stem cells, such as CD73, CD90 and CD105 (9, 37). Conversely, the current literature is heterogeneous in terms of the choice of cells, method of cell processing, cell characterization, quantitative and qualitative assessment of the cells used, and finally surgical methods of cell delivery (38-39). Despite cellular therapies are certainly a promising tool for restoring local cell populations after an injury or disease and encouraging data are reported also in the current study, more clinical trials, including a control group when possible, are required for a better understanding about the role of micrografts in the treatment of ONFH.

Conclusions

The results of this study confirm the authors hypothesis on the clinical efficacy of micrografts in the treatment of aseptic, avascular and non-traumatic ONFH: this technology appears to improve the quality of life of patients at least at a short F.U. and offers a valid alternative tool among those already used in the clinical practice.

Conflict of interest: Each author declares that he or she has no commercial associations (e.g. consultancies, stock ownership, equity interest, patent/licensing arrangement etc.) that might pose a conflict of interest in connection with the submitted article

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ARTICLE

Rationale and pre-clinical evidences for the use of autologous cartilage micrografts in cartilage repair

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Alberto Magi, Andrea Toto, Giuseppe Peretti and Laura de Girolamo

RESEARCH ARTICLE

Open Access



Rationale and pre-clinical evidences for the use of autologous cartilage micrografts in cartilage repair

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Abstract

Background: The management of cartilage lesions is an open issue in clinical practice, and regenerative medicine represents a promising approach, including the use of autologous micrografts whose efficacy was already tested in different clinical settings. The aim of this study was to characterize in vitro the effect of autologous cartilage micrografts on chondrocyte viability and differentiation and perform an evaluation of their application in racehorses affected by joint diseases.

Materials and methods: Matched human chondrocytes and micrografts were obtained from articular cartilage using Rigenera® procedure. Chondrocytes were cultured in the presence or absence of micrografts and chondrogenic medium to assess cell viability and cell differentiation. For the pre-clinical evaluation, three racehorses affected by joint diseases were treated with a suspension of autologous micrografts and PRP in arthroscopy interventions. Clinical and radiographic follow-ups were performed up to 4 months after the procedure.

Results: Autologous micrografts support the formation of chondrogenic micromasses thanks to their content of matrix and growth factors, such as transforming growth factor β (TGF β) and insulin-like growth factor 1 (IGF-1). On the other hand, no significant differences were observed on the gene expression of type II collagen, aggrecan, and SOX9. Preliminary data in the treatment of racehorses are suggestive of a potential in vivo use of micrografts to treat cartilage lesions.

Conclusion: The results reported in this study showed the role of articular micrografts in the promoting chondrocyte differentiation suggesting their potential use in the clinical practice to treat articular lesions.

Keywords: Micrografts, Cartilage repair, Regenerative medicine, Cartilage defects, Rigenera

Background

The management of cartilage lesions still represents a challenge for surgeons, due to the limited regenerative ability of cartilage, given its avascularity and hypocellularity. Additionally, cartilage defects can lead to the pathogenesis of osteoarthritis, resulting in pain and disability with a high economic and social impact in many developed countries [1]. Depending on the type of cartilage defect, different methodologies for cartilage repair and regeneration can be applied today, such as arthroscopic

debridement [2], bone marrow stimulations [3], osteochondral autografts, or allograft [4]. Beyond these methodologies, autologous chondrocyte implantation (ACI) or matrix-induced autologous chondrocyte implantation (MACI) can represent valid approaches in the treatment of cartilage defects, to promote the formation of hyaline or hyaline-like cartilage and improve the pain and functional outcomes in most of treated patients. However, nowadays, the use of autologous chondrocytes is limited by the need to perform two-stage procedures and by the long recovery time required after surgery [5].

Recently, regenerative medicine approaches, based on cell therapy or tissue engineering, gather increasing interest, representing possible therapeutic alternatives.

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Indeed, the use of live cells with appropriate scaffold and growth factors could allow for restoration of physiological tissue within small or large defects. Nevertheless, recent studies demonstrated that progenitors cells from cartilage are the preferred cell type for this kind of approach, since other cell types, such as bone marrow-derived mesenchymal stem cells, are not able to generate hyaline cartilage [6].

The Rigenera® procedure is an innovative clinical protocol to obtain autologous articular micrografts, containing both live cells and fragments of hyaline cartilage matrix, ready to use alone or in combination with the most common scaffolds. Previous studies have been reported that autologous micrografts are enriched of progenitor cells and maintain regenerative properties [7, 8]. The autologous micrografts are already used in dentistry [9, 10] and wound healing [11–15], reporting satisfactory results in terms of bone and dermal regeneration. Furthermore, some recent papers showed the effectiveness of micrografts also in the cartilage and cardiac regeneration [16, 17].

In this study, we provide an in vitro characterization of cartilage autologous micrografts properties on chondrocytes viability and differentiation, together with an evaluation of their pre-clinical application in racehorses affected by joint diseases, where autologous micrografts were used in combination with autologous platelet-rich plasma (PRP) to better vehicle the micrografts in the injured site and obtain a biocomplex ready to be used.

Joint diseases represent the main cause of reduced athletic function for racehorses and are characterized by a degenerative process involving several components of the joints including cartilage, subchondral bone, and articular capsule [18]. Anti-inflammatory and analgesic drugs represent the standard treatment for mild defects, while articular cartilage curettage, osteophyte removal or surgical arthroscopy, and arthrodesis can be indicated for severe cartilage and bone degeneration [19]. Nevertheless, while all these therapies effectively reduce symptoms, they are not able to restore the physiological conditions in cartilage tissue. Regenerative therapy for racehorses is assuming a growing interest for the significant economic impact on the horse industry, and racehorses can be a valuable large animal model for the evaluation of new therapies due to the interspecies similarities with humans in the thickness of the non-calcified cartilage of the stifle joint [20].

Materials and methods

Isolation of human primary chondrocytes and culture with micrografts

Human primary chondrocytes were isolated from eight samples of articular cartilage of femoral head of donor patients undergoing total hip arthroplasty. All individuals

provided informed consent as per the Institutional Review Board approved procedure (M-SPER-014.ver7). Primary chondrocytes were isolated from articular cartilage (0.6–1.2 mg) using overnight incubation at 37 °C with 0.15% w/v type II collagenase (Worthington, NJ, USA) solution in DMEM (Sigma Aldrich, MO, USA) + 5% fetal bovine serum (FBS, Hyclone, Thermo-Fisher Scientific, MA, USA). Cells were then seeded at 5,000 cell/cm² for expansion. The autologous micrografts were obtained by Rigenera protocol after mechanical disaggregation using a medical disposable Rigeneracons (Human Brain Wave srl, Turin, Italy) [9]. Briefly, 200 mg of each sample was inserted in the Rigeneracons and minced for 5 min in a total of 5 ml of DMEM. The primary chondrocytes isolated by collagenase were cultured in four different conditions: DMEM supplemented with 10% FBS (control medium), control medium plus 10% v/v autologous micrografts, DMEM supplemented with 1% FBS and chondrogenic factors (chondrogenic medium), and chondrogenic medium plus 10% v/v autologous micrografts. For cell viability assay, only control medium and control medium with 10% v/v autologous micrografts were tested. Particles obtained after disaggregation with Rigenera ranged from 50 to 70 µm.

Cell viability

Cell viability was assessed at 1, 4, 7, and 14 days of incubation with the different media by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma-Aldrich] assay. Cells at passage 3 were cultured in 96-well plates at the density of 3.0×10^3 cells/cm²; to perform the assay, a final concentration of 0.5 mg/mL MTT was added to the culture medium and incubated for 4 h at 37 °C; the medium was removed and 100% DMSO was added to each well to solubilize the precipitate. Absorbance was read at 570 nm.

Chondrogenetic differentiation assay

For chondrogenic differentiation, 5.0×10^5 cells were centrifuged at 250g for 5 min to obtain pellets. The pellets were cultured in four different media: control medium, DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine, 1 mM sodium pyruvate, 1.25 mg/ml human serum albumin (HAS; Sigma-Aldrich), and 10% FBS; chondrogenic medium, consisting of DMEM supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 0.29 mg/ml L-glutamine, 1 mM sodium pyruvate, 1.25 mg/ml human serum albumin (HAS; Sigma-Aldrich), 1% ITS+1 containing 1.0 mg/ml insulin from bovine pancreas, 0.55 mg/ml human transferrin, 0.5 µg/ml sodium selenite, 50 mg/ml bovine serum albumin and 470 µg/ml linoleic acid (Sigma-Aldrich), 0.1 µM dexamethasone, 0.1 mM L-ascorbic acid-2-phosphate, and 10 ng/ml

TGF- β 1 (PeproTech, Rocky Hill, NJ, USA) (Lopa S); control medium plus 10% *v/v* autologous micrografts; chondrogenic medium plus 10% *v/v* autologous micrografts. The medium was replaced every 3 days and cells cultured at 37 °C under a 5% CO₂ atmosphere for 4 weeks before the following evaluations.

Histology and immunohistochemistry

For the histological analysis, representative pellets from each sample and treatment ($n = 32$) were fixed in 10% neutral buffered formalin (Bio-Optica Milano SpA, Milan, Italy), embedded in paraffin blocks, and cut into 4- μ m-thick sections. To detect sulfated glycosaminoglycans (GAGs), sections were stained with standard Alcian blue protocol (Bio-Optica). Briefly, slides were deparaffinized and rehydrated then stained with Alcian blue (pH 2.5; according to Mowry) for 30 min. The sections were then immersed in a sodium tetraborate solution for 10 min and counterstained with Mayer's hematoxylin, dehydrated, and mounted.

For immunohistochemical localization of collagen type I (COLL I) and collagen type II (COLL II), the sections were dewaxed and rehydrated, and a heat-induced antigen retrieval was applied using a microwave treatment for 5 min at 400 W in citrate buffer pH 6.0 (Thermo Fisher Scientific, Waltham, MA, USA). Then, the slides were treated with 3% H₂O₂ in absolute methanol for 10 min to quench endogenous peroxidases and successively with 3% *w/v* bovine serum albumin (BSA) in PBS for 30 min to inhibit non-specific reactivity. Biotinylated anti-COLL I (10 μ g/ml; #7026, Chondrex Inc., Redmond, WA, USA) and biotinylated anti-COLL II (10 μ g/ml; #7049, Chondrex Inc.) antibodies were applied overnight at 4 °C in a humid chamber upon sections. The primary antibodies were diluted in PBS with 1% *w/v* BSA and 0.3% *v/v* Tween 20 (Thermo Fisher Scientific). At the end of incubation, biotinylated antibodies were detected with streptavidin conjugated to horseradish peroxidase (Abcam, Cambridge, UK) and then with HIGHDEF® yellow IHC chromogen (Enzo Life Sciences Inc., Farmingdale, NY, USA). All sections were finally weakly counterstained with Mayer's hematoxylin, dehydrated, and mounted. For negative control, the primary antibody was omitted. Photomicrographs were taken with an Olympus IX71 light microscope and an Olympus XC10 camera (Japan).

GAGs deposition

Glycosaminoglycans (GAG) content was evaluated by dimethylmethylene blue (DMMB) assay. Briefly, the pellets were digested at 60 °C for 16 h in PBE buffer (100 mM Na₂HPO₄, 10 mM Na EDTA, pH 6.8) containing 1.75 mg/ml L-cysteine (Sigma-Aldrich) and 14.2 U/ml papain (Worthington, Lakewood, NJ, USA). The obtained extracts were incubated with 16 mg/l

dimethylmethylene blue (Sigma-Aldrich), and absorbance was read at 500 nm (Perkin Elmer Victor X3 microplate reader). For normalization purposes, DNA content evaluation was performed on each sample by CyQUANT Kit (Life Technologies), following manufacturer's instructions. Data are presented as microgram of GAGs per microgram of DNA.

Quantitative real time-PCR

Total RNA was isolated from cells using TriReagent (Life Technologies) and cDNA was synthesized from 1 μ g of total RNA by reverse transcription (RT) reaction. The expression of type II collagen (*COL2A1*), SOX9, and aggrecan (*ACAN*) mRNAs was measured by real-time RT-PCR, using TaqMan reagents (Life Technologies). The calculations of the results were carried out according to the 2 ^{Δ Ct} methods. GAPDH was used as an internal control for data normalization [21].

Growth factors measurement

Immediately after micrograft production with the Rigena protocol, an aliquot of the micrograft suspension was frozen at -20 °C. Transforming growth factor β (TGF β) and insulin-like growth factor 1 (IGF-1) concentrations in the micrograft suspensions were measured by commercially available ELISA kit, according to the manufacturer's instructions (PeproTech, UK).

Horses

Three horses (1 gelding and 2 thoroughbreds) aged from 3 to 5 years (4.4 ± 1.5) with intra-articular lesions were treated. Their characteristics, before and after treatment, are provided in Table 1.

Preparation of equine platelet-rich plasma (PRP)

Autologous PRP was prepared as previously described [22]. Briefly, two units of 450 ml of blood are collected from the horses through a standard triple-bag system, a method that allows easily the removal of 450–900 ml of blood. Sampling was done from the jugular vein after trichotomy and disinfection of the area. Blood was centrifuged at 1450 rpm for 10 min at 20 °C, in order to obtain the separation of red blood cells from plasma containing platelets and the factors that lead to the formation of a clot. Plasma is then centrifuged at 3000 rpm for 20 min at 20 °C, thus obtaining the separation of a platelet pellet and platelet-poor plasma (PPP). The platelets are then re-suspended in 30–35 ml of PPP in order to have a PRP with a platelet concentration of about 1×10^6 platelets/ μ l. The bag containing the PRP is placed on a platelet agitator under constant agitation at room temperature and after about 2 h transferred under a sterile hood to dispense the platelet concentrate into

Table 1 Baseline characteristics of horses before and after treatment

Horses	Sex and breed	Diagnosis	AAEP lameness scale (before treatment)	AAEP lameness scale (after treatment)
1	Gelding, quarter horse	Middle-carpal joint arthrosis, severe cartilage erosions, and detachment on radial and third carpal bone, radial bone fragments	3	0
2	Male, thoroughbred	Severe cartilage damage to the metacarpal-phalangeal joint, joint space reduction, early signs of bone proliferation associated with degenerative osteoarthritis at early stage	3	0
3	Female, thoroughbred	Cartilage ulcer of the dorsomedial eminence of the first phalanx. Linear erosions of metacarpal condyles. Cartilage thinning	2	1

AAEP American Association of Equine Practitioners

sterile tubes (Monovette, Sarstedt). The PRP product is stored at -20°C until use.

Use of equine autologous micrografts and PRP in arthroscopy interventions

Equine autologous micrografts were prepared as previously described using Rigena protocol. Briefly, a small piece of intra-articular cartilage (weight 0.0230 g) was collected by endoscopic procedure and disaggregated by Rigenacons medical device for 5 min for three times adding 1.5 ml of sterile physiological solution (Fig. 1a–d). The chondrocyte-derived micrografts were then mixed to 10 ml of PRP (Fig. 1d) and after an arthroscopic curettage injected in the articular lesions of horses (Fig. 1e). After the procedure, the skin is disinfected with iodine product and dressed with cotton gauze and a Vetrap-type bandage

strip. The dressing remains in situ for 48 h. A clinical follow-up was performed every week, while the radiographic follow-up was performed between 4 and 6 months after arthroscopy.

Statistical analysis

All data are reported as means $\pm$ SD. Statistical difference between two groups was determined by one-way ANOVA or *t* test, when appropriate. The significance was established for a *p* value ≤ 0.05 .

Results

Effect of autologous micrografts on chondrocyte differentiation and viability

The effect of micrografts on cell viability was evaluated after 10 days of culture, and no significant interference

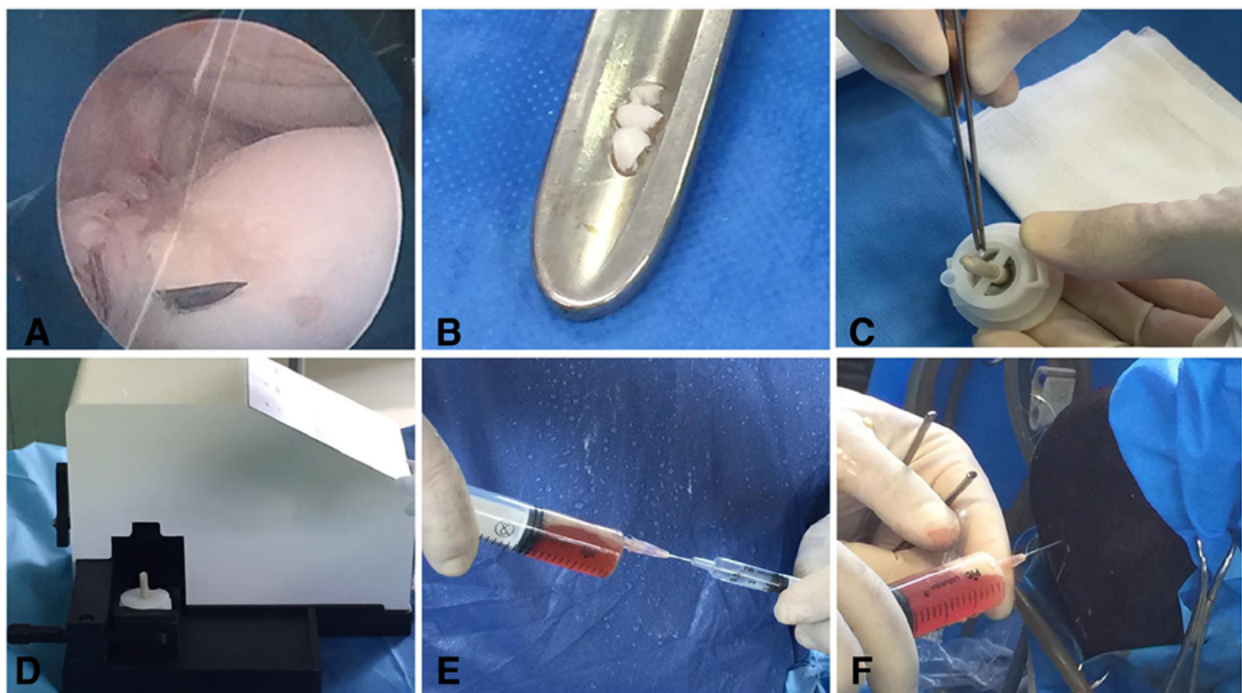


Fig. 1 Collection of equine autologous micrografts. **a, b** During the arthroscopy intervention, a small piece of intra-articular cartilage was collected by endoscopic procedure. **c, d** The intra-articular cartilage was inserted in Rigenacons disposable and disaggregated for 2 min by a rotation process triggered by Rigena machine. **e, f** The micrografts obtained were mixed to autologous PRP and directly injected on the lesion

was observed comparing cells cultured in the presence and absence of cartilage micrografts (Fig. 2a). The chondrocyte differentiation was evaluated by histology and immunohistochemistry on human primary chondrocyte pellets cultured in the presence or absence of autologous micrografts. The micromasses cultured in the presence of micrografts showed higher dimension and positivity

to Alcian blue staining with respect to control samples. When cultured in a chondrogenic medium, these differences were less obvious, but the action of micrografts in favoring cell harboring was confirmed (Fig. 2b). In addition, a DMMB assay revealed that GAGs content was significantly increased in the cells cultured with micrografts in standard culture conditions ($p < 0.01$)

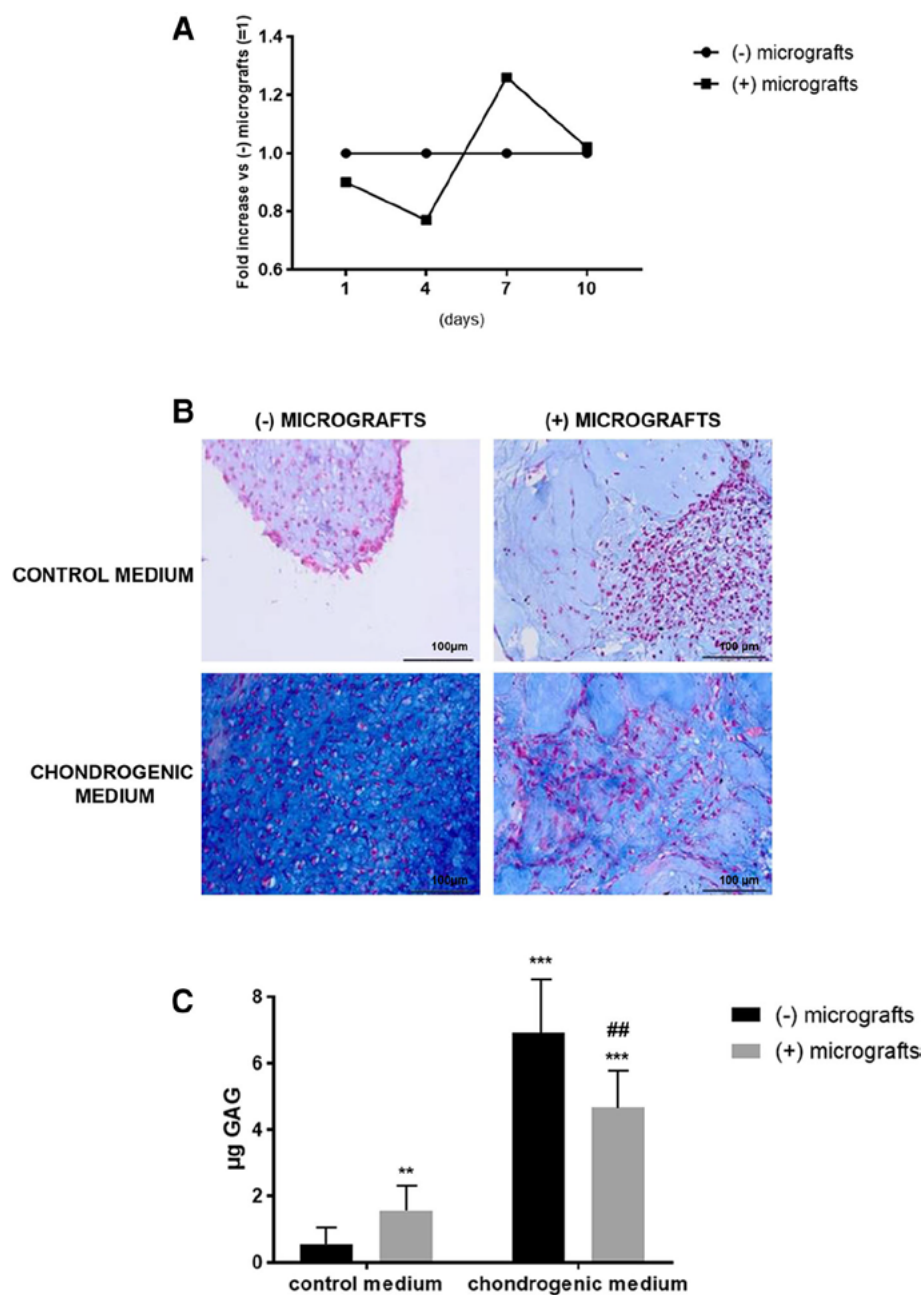


Fig. 2 Chondrogenic differentiation. **a** Cell viability evaluated after 10 days of culture in the presence or absence of autologous micrografts. The results are expressed as fold increase with respect to chondrocytes cultured without micrografts (= 1). **b** Alcian blue staining to evaluate the chondrogenic differentiation of cells cultured in the control or chondrogenic medium with or without autologous micrografts (magnification $\times 20$). **c** GAGs deposition in cells cultured in the control or chondrogenic medium with or without autologous micrografts (** $p < 0.01$, *** $p < 0.001$ vs cells in the control medium without micrografts; ## $p < 0.01$ vs cells in the chondrogenic medium without micrografts)

(Fig. 2c). Nevertheless, a decrease was observed in chondrogenic medium plus micrografts cultured pellets with respect to samples maintained in chondrogenic medium alone. Despite this difference resulted statistically significant ($p < 0.01$), the differentiation ability was not prevented as demonstrated by the great increase in GAGs content observed in these pellets when compared to control cells ($p < 0.001$) (Fig. 2c).

Finally, immunohistochemistry analysis revealed a strong presence of type II collagen in the chondrocytes plus micrografts with respect to those without micrografts, both in the control or chondrogenic medium (Fig. 3). As expected, the cells are negative for type I collagen (Fig. 4), confirming the lack of trans-differentiation events.

Evaluation of the cartilage-specific gene expression

Type II collagen and aggrecan are critical components for cartilage structure. We evaluated their expression in human primary chondrocytes showing that in the chondrogenic medium, the expression of these markers increases with respect to samples cultured in control medium, in the presence or absence of autologous micrografts. The expression of the cartilage-specific transcription factor SOX9 resulted more expressed in samples treated with chondrogenic medium and micrografts, with respect to all the other culture conditions. Nevertheless, due to the high inter-donor variability, no statistically significant differences were found in the gene expression of these markers (Fig. 5a–c).

Autologous micrografts suspension contains cartilage trophic factors

The suspension of the autologous micrografts was assayed for cartilage trophic factors TGF β and IGF-1. The mean content of TGF β in all micrografts samples was 81.4 ± 88.2 pg/ml, while IGF-1 was 676.3 ± 212.7 pg/ml. All samples showed the presence of IGF-1, while only six out of eight samples showed the presence of TGF β (Fig. 5d).

Use of autologous micrografts plus PRP for arthroscopic intervention in the sport racehorses

Autologous micrografts and PRP were used for arthroscopic intervention in sports racehorses affected by joint disease causing lameness. In the first horse, a severe cartilage erosion was observed (Fig. 6a) and the RX pre-intervention showed the presence of both middle-carpal arthrosis and an osteophyte on the dorsomedial edge of the radial bone with an articular fragment close to the third carpal bone (Fig. 6b). After 4 months from intervention, an improvement of articular, dorsomedial, and inter-carpal edge can be observed (Fig. 6c). In the second horse, a severe cartilaginous damage was

observed (Fig. 6d) and the RX pre-intervention showed a reduced articular space and an early stage of degenerative arthrosis (Fig. 6e). After 4 months from micrografts plus PRP injection, an increase of articular and peri-articular proliferation was observed (Fig. 6f). For the third horse, the diagnosis of cartilaginous damage on the fetlock was confirmed by clinical evidences but not at a radiographic level showing no difference before and after the treatment (data not shown). In two cases, a complete resolution of lameness which allowed the recovery of sports race activity was observed (Additional files 1 and 2).

Discussion

The possibility to use biological strategies to enhance the cartilage regeneration ability in a one-step surgery would represent an important advance in the treatment of cartilage defects. Indeed, while the most used techniques are nowadays limited to microfractures for bone marrow stimulation or two-step surgeries for articular chondrocyte transplantation, the use of Rigenera® procedure would improve the feasibility of biological treatments in the field. The in vitro results reported in this study demonstrate that autologous micrografts do not affect chondrocyte viability and influence chondrocyte differentiation, as shown by both increased GAGs deposition and the presence of collagen II in primary human cells cultured in the presence of micrografts supporting the formation of chondrogenic micromasses and acting like a scaffold for chondrocyte harboring. From our experiments, the presence of IGF-1 and TGF β in the product obtained by cartilage processing with Rigenera protocol also emerged; both factors were able to enhance cartilage repair in vivo by increasing proteoglycan synthesis and stimulating mesenchymal stem cell differentiation into chondrocytes, stimulate matrix synthesis, and reverse the catabolic effects of pro-inflammatory cytokines [23, 24].

Previous in vitro studies reported that micrografts maintained a high cell viability after mechanical disaggregation of different types of human tissues such as dental pulp, periosteum, and cardiac atrial appendage [7] and that they are able to differentiate in osteocytes, adipocytes, and chondrocytes [8].

In addition to in vitro data, the study provides positive preliminary results in the treatment of racehorses affected by joint diseases, suggesting an in vivo application of cartilage micrografts associated with PRP. The efficacy of micrografts in the cartilage repair was reported in previous human studies where the authors described the combined use of autologous chondrocyte-derived micrografts and PRP to reconstruct not hyaline articular cartilage and to promote cartilage regeneration in patients affected by external nasal valve collapse. In fact,

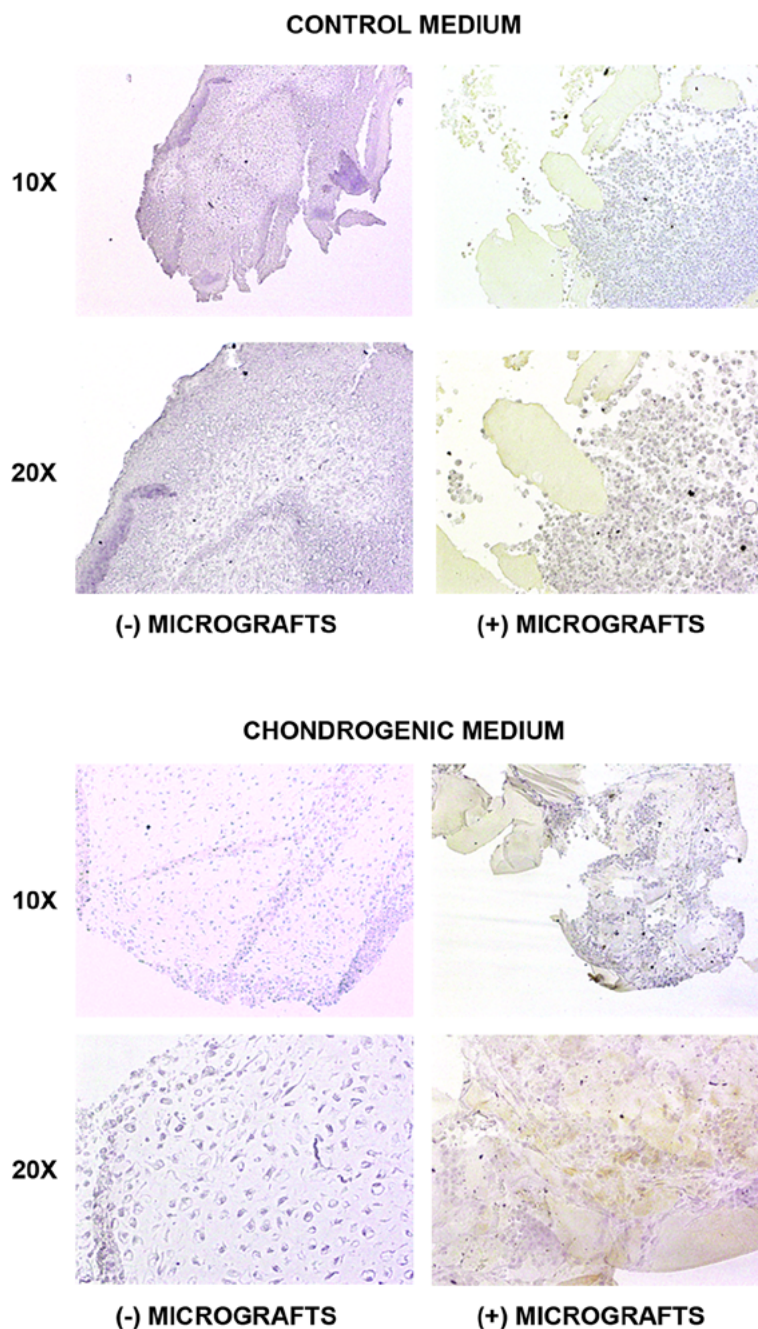


Fig. 3 Type II collagen expression. The type II collagen expression was evaluated by immunohistochemistry in the cells cultured both in the control or chondrogenic medium in the presence or absence of autologous micrografts after 4 weeks of culture (magnification $\times 10$ and $\times 20$)

the constructs of chondrocyte micrografts-PRP resulted in a persistent cartilage tissue with appropriate morphology, adequate central nutritional perfusion without central necrosis or ossification, and further augmented nasal dorsum without obvious contraction and deformation [16, 25].

To confirm the clinical efficacy of micrografts in the tissue repair/regeneration, several case series studies were performed in different clinical areas such as dentistry,

dermatology, and wound care. To this regard, it has been reported that human dental pulp or periosteum-derived micrografts were able to promote the bone regeneration in the atrophic maxilla [9], to preserve the alveolar socket after tooth extraction by both reducing bone resorption and increasing new bone formation [10] and to promote sinus lift augmentation [26]. Autologous micrografts also improve the wound healing of complex post-operative and post-traumatic wounds [27] of post-surgical dehiscences

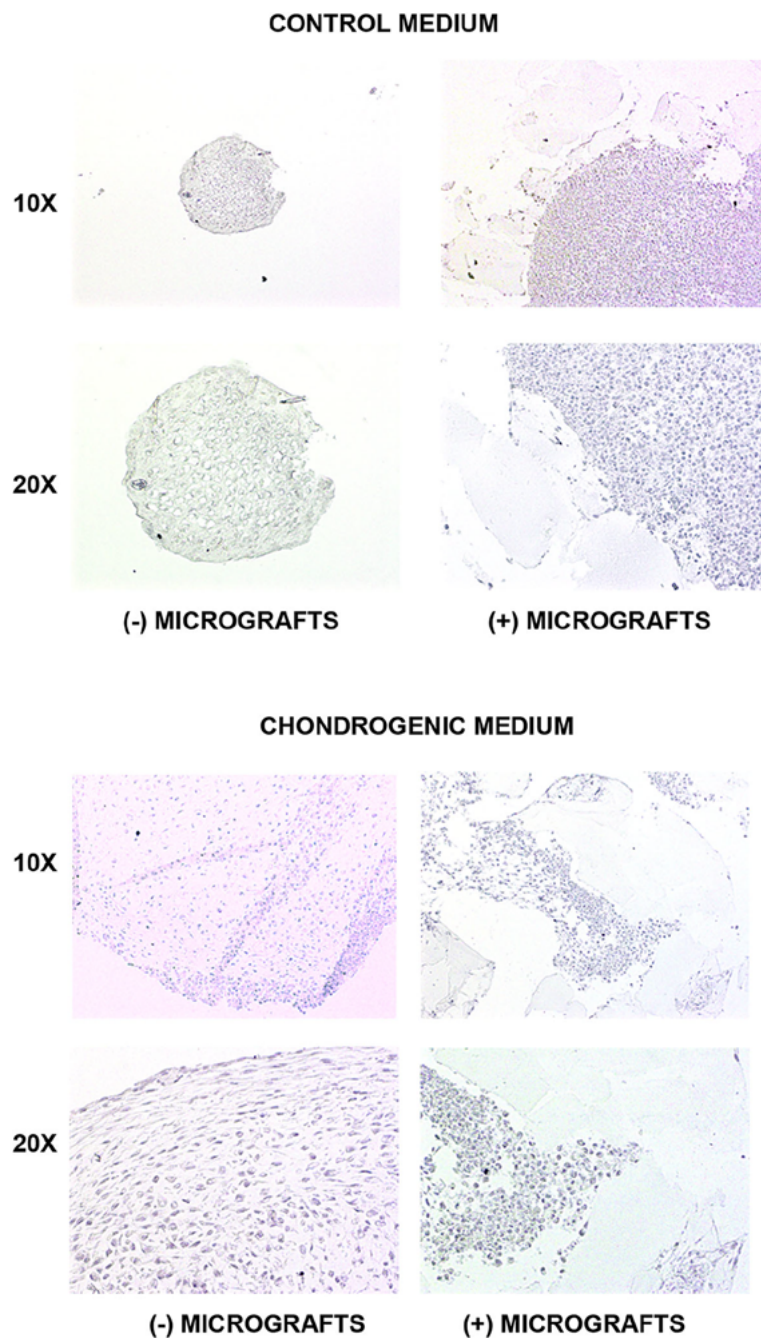


Fig. 4 Type I collagen expression. The type I collagen expression was evaluated by immunohistochemistry in the cells cultured both in the control or chondrogenic medium in the presence or absence of autologous micrografts after 4 weeks of culture (magnification $\times 10$ and $\times 20$)

[11, 12] and chronic ulcers [13, 14]. Furthermore, micrografts were also used in the treatment of pathological and hypertrophic scars restoring the structural layers immediately below the epidermis and promoting the horizontal realignment of collagen fibers in the papillary dermis [15]. Finally, a very recent paper showed the ability of autologous micrografts to induce cardiac regeneration [17].

Joint healing can be improved by supplying stem cells, growth factors, and growth substrates at the site of injury. For example, several studies demonstrate that mesenchymal stem cells were effective in the bone and cartilage regeneration due their capacity to differentiate into osteocytes and chondrocytes and stimulate the synthesis of the chondrocyte extracellular matrix [28]. In previous studies, we showed that cells contained in the

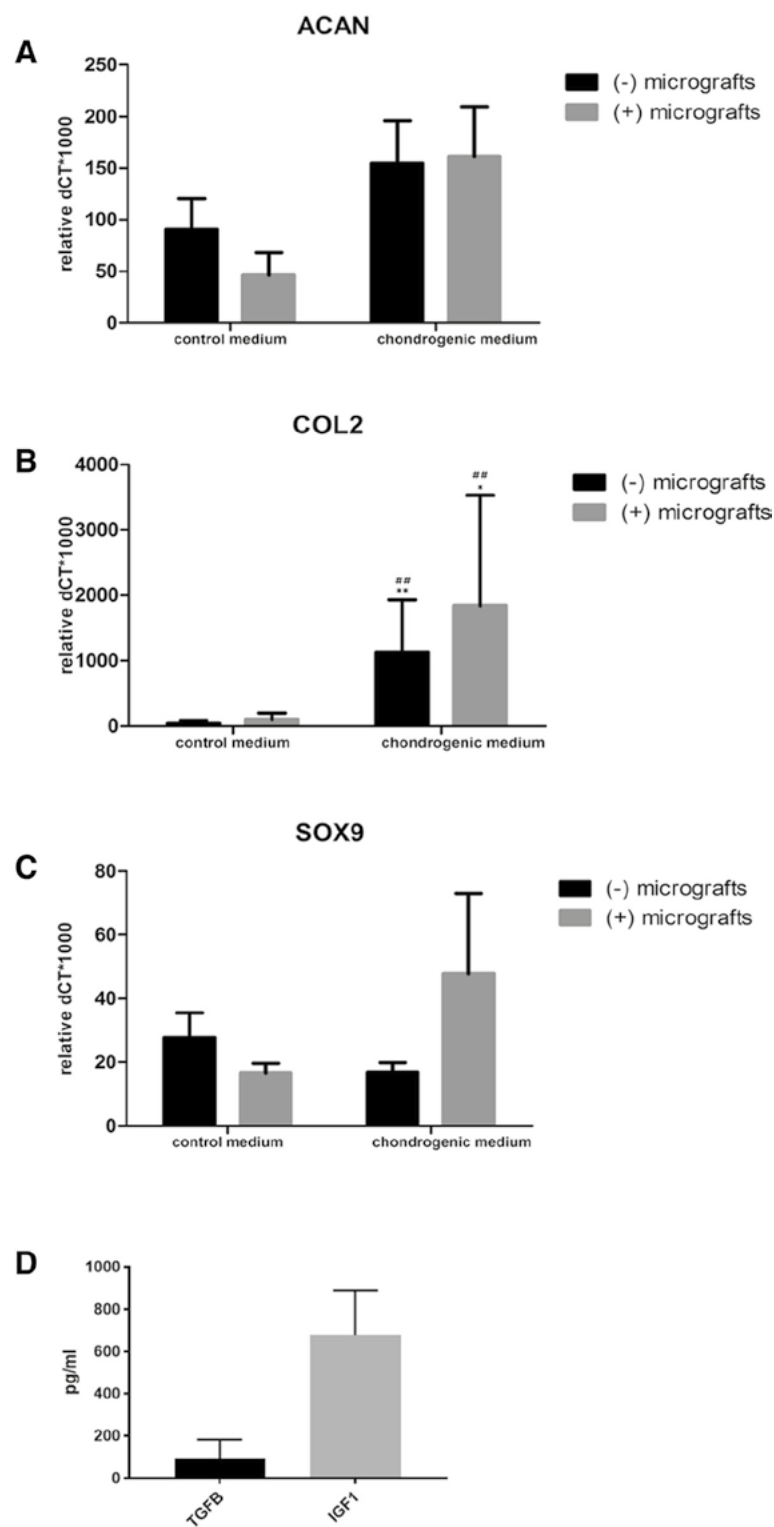


Fig. 5 Gene expression of ACAN, COL2, and SOX9 and measurement of cartilage trophic factors TGFβ and IGF-1. **a–c** mRNA expression of ACAN (aggrecan), COL2 (type II collagen), and SOX9 was evaluated in the cells cultured both in the control or chondrogenic medium in the presence or absence of autologous micrografts after 4 weeks of culture. The results are expressed as dCt vs. GAPDH. **d** The levels of cartilage trophic factors TGFβ and IGF-1 were measured by ELISA in the autologous micrografts suspension. * $p < 0.05$, ** $p < 0.01$ vs control medium (–) micrografts; ^{##} $p < 0.01$ vs control medium (+) micrografts

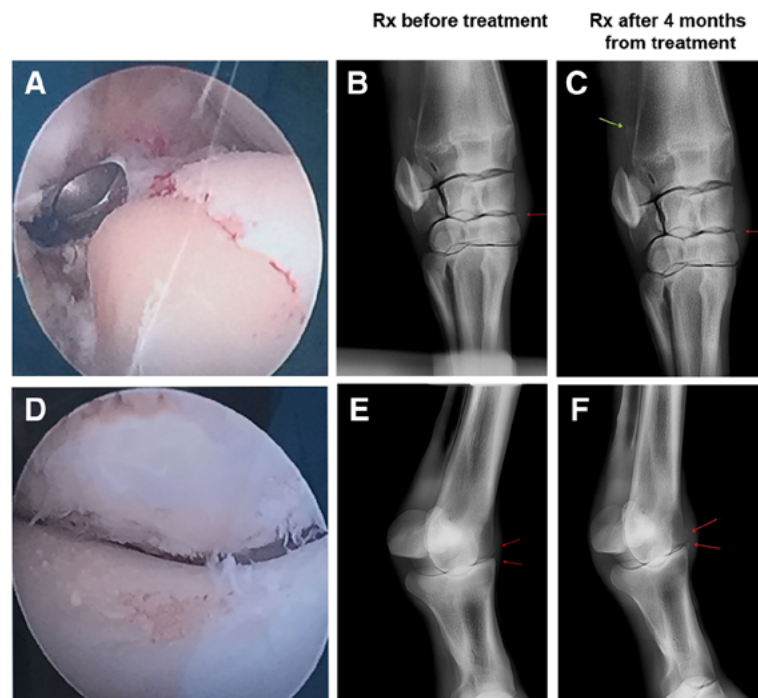


Fig. 6 Application of equine autologous micrografts in arthroscopy intervention. **a** Endoscopic image of left intercarpal joint showing the surface of the radial bone of the carpus after curettage and removal of the large portion of damaged cartilage. **b, c** Right front carpus, dorsolateral palmaro-medial X-ray pre- and post-intervention which evidences the radio-carpal joint, the middle carpal joint, and the proximal extremities of the metacarpal bones. Osteophyte on the dorsomedial edge of radial bone (yellow arrow) and articular fragment close to the third carpal bone (red arrows). **d** Endoscopic image of right metacarpal-phalangeal joint showing a severe damage to the articular cartilage. At the bottom, at the level of the lateral condyle of third metacarpal bone, and at the top, on the lateral portion of the proximal side eminence of the phalanx. **e, f** Right fetlock, dorsolateral palmaro-medial X-ray pre- and post-intervention which reports the metacarpal-phalangeal joint. Articular space and signs of bone proliferation before and after treatment (red arrows)

micrografts derived from different tissues express mesenchymal stem cells (MSCs) markers, such as CD90, CD73 and CD105, CD117, and CD44 [7, 8, 25], suggesting the presence of MSCs or tissue-specific progenitors within micrografts, as a possible explanation of their regenerative potential. Behind the stem cells, also the PRP containing both PDGF and TGF- β 1 was shown to be effective in cartilage regeneration by promoting chondrocyte proliferation and synthesis of proteoglycan and type II collagen [29]. We reported in this study the pre-clinical application of autologous micrografts combined with PRP in the cartilage repair in racehorses, suggesting that a combined action of both these factors would be able to promote the osteochondral regeneration. In fact, PRP alone resulted effectively in the treatment of cartilage lesions only at short-term [30, 31], and it has been suggested that the combination with other approaches would allow for the stabilization of its beneficial effects [29]. Even if the known short-term effect of PRP may have masked the benefits of autologous cartilage micrografts, our *in vivo* data provide the first proof of concept for the use of the combination of these techniques in cartilage defects. Moreover, the use

of osteochondral autografts and allograft is already a well-established practice in the management of horse injuries, allowing the treatment of large defects, thanks to the immediate reconstruction of the articular surface by transfer of mature intact hyaline cartilage and the underlying subchondral bone. The success of this technique depends on the viability of chondrocytes in the graft and on the mechanical stability of the host-graft interface [32]. Furthermore, the use of osteochondral grafts transfer is limited by donor site availability in the autologous approach or joint congruency and host response in the case of allogeneic tissue [19]. The use of autologous micrografts overcomes some of these limitations, given that their collection is scarcely invasive, reducing donor site morbidity without influencing the grafts viability and overcoming the possible rejection issue related to allogeneic grafts.

Conclusion

Taken together, these results showed that autologous cartilage micrografts may promote cartilage repair, favoring chondrocytes harboring and growth, and

they suggest their potential in the treatment of articular lesions in combination with PRP. However, further studies are needed to confirm the effectiveness of micrografts on cartilage repair/regeneration both alone or in combination with different approaches.

Additional files

Additional file 1: Resolution of lameness after micrograft application. Videos showing the activity of horses 1 and 3 (referring to Table 1) after 4 months from micrografts application. (MP4 2050 kb)

Additional file 2: Resolution of lameness after micrograft application. Videos showing the activity of horses 1 and 3 (referring to Table 1) after 4 months from micrografts application. (MP4 10901 kb)

Abbreviations

ACAN: Aggrecan; ACI: Autologous chondrocyte implantation; BSA: Bovine serum albumin; COL2A1: Type II collagen; COLL I: Collagen type I; COLL II: Collagen type II; DMMB: Dimethylmethylene blue; DMSO: Dimethyl sulfoxide; FBS: Fetal bovine serum; GAGs: Glycosaminoglycans; HAS: Human serum albumin; IGF-1: Insulin-like growth factor 1; MACI: Matrix-induced autologous chondrocyte implantation; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PPP: Platelet-poor plasma; PRP: Platelet-rich plasma; TGFβ: Transforming growth factor β

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Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Authors' contributions

MV, IT, and AC participated in developing the experimental design, data interpretation, and analysis. LT drafted the manuscript. MS, AM, and AT performed in vivo experiments on horses. LdG and GP contributed to the developing of experimental design and editing of the manuscript. All authors read and approved the final submitted manuscript.

Ethics approval and consent to participate

Experimental research on horses comply with institutional, national, or international guidelines. For primary chondrocytes isolation, all individuals provided informed consent as per the Institutional Review Board approved procedure (M-SPER-014.ver7).

Consent for publication

Not applicable

Competing interests

The author Letizia Trovato is a consultant of the Scientific Division of Human Brain Wave, the company owner of Rigenere™ technology. The other authors declare that they have no competing interests.

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ARTICLE

Efficacy of Autologous Micrografting Technology in Managing Osteoarthritis Pain: A Pilot Study

Camilo Partezani Helito, Valeria Pessei, Cecilia Zaniboni and Ilie Muntean.

Article

Efficacy of Autologous Micrografting Technology in Managing Osteoarthritis Pain: A Pilot Study

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Abstract: Osteoarthritis (OA) is one of the most common joint diseases worldwide, predominantly present in elderly people. Being a major source of pain for patients, it is debilitating and leads inevitably to a reduction in quality of life. The management of OA needs a personalized and multi-dimensional approach, resulting in the emergence of new regenerative and non-invasive methods, such as the use of micrografts. In this pilot study, Rigenera[®] Technology was employed to obtain micrografts of cartilage tissue to be injected into the knees of 10 patients with osteoarthritic pain. To assess the efficacy of the treatment concerning pain reduction at this site, patients were asked to complete KOOS and WOMAC questionnaire and a VAS test before and after the procedure. The results presented in this article show how Rigenera[®] treatment can potentially improve OA symptoms, alleviating pain in patients.

Keywords: osteoarthritis (OA); intra-articular injection; knee osteoarthritis; micrograft; pain management; regenerative medicine



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1. Introduction

Osteoarthritis (OA) is a widespread condition that affects over 500 million people globally, making it one of the most common musculoskeletal disorders [1,2]. The prevalence of OA increases with age, with an estimated prevalence of 10% of men and 18% of women over 60 years of age [3,4]. Patients affected by OA show symptoms such as pain, stiffness, swelling, and limitations to motion in the affected area, thus reducing quality of life and having consequences on people's mental health [5,6].

From a clinical point of view, OA is a degenerative inflammatory joint disease characterized by the progressive disintegration of cartilage, synovial fluid modifications, and bone erosion, leading to a change in the whole joint structure [7]. Based on etiology, it is possible to distinguish primary and secondary OA [8]. Primary OA, also known as idiopathic OA, is usually related to aging and genetic factors, while secondary OA is caused by an underlying condition or factor such as joint injuries or trauma, obesity, inflammatory diseases such as rheumatoid arthritis, and metabolic disorders [9,10].

The management of OA requires a multidimensional approach tailored to the individual patient's needs and the severity of their condition. In the early stages, lifestyle changes such as weight reduction, regular and low-impact physical activity, and a more balanced diet rich in anti-inflammatory foods could improve the situation by increasing mobility and reducing pain [11,12]. However, in advanced cases, the standard procedures include pharmacological treatments such as analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), intra-articular injections of corticosteroids, or hyaluronic acid [13,14]. Although these interventions provide short-term benefits, they can result in significant

adverse complications for patients, including cardiovascular risks and gastrointestinal disorders [15]. Moreover, these treatments are not resolutive, often leading the physician to the only available solution for joint replacement, an artificial prosthesis [16,17]. In this context, researchers are investigating new therapeutic methods to reduce pain and improve patients' quality of life through the employment of biological and regenerative therapies involving growth factors or cytokine inhibitors, platelet-rich plasma (PRP), and others. Specifically, in this study, a procedure based on the use of micrografts [18] will be investigated.

Rigenera[®] Technology is an approach that can obtain autologous micrografts through the mechanical disaggregation of a small biopsy of the patient's tissue, typically from the auricular cartilage [19–21]. Recently, the efficacy and safety of this procedure in patients with early-stage knee osteoarthritis was assessed by a study that showed significant improvements in knee stability, pain, swelling, mechanical locking, and overall mobility by using Rigenera[®] Technology [22]. Overall, Rigenera[®] technology offers a potentially effective approach to managing osteoarthritis, with encouraging preliminary results. The aim of the present study is to evaluate the effect of Rigenera[®] treatment in the modulation of pain in patients with OA.

2. Materials and Methods

2.1. Clinical Study and Patients

This study was conducted from August 2022 to March 2023 and enrolled 10 patients (5 males and 5 females) with an average age of 55 years. Six patients were suffering from primary osteoarthritis, two were suffering from post-traumatic osteoarthritis, and the other two were suffering from patellofemoral pain (Table 1).

Table 1. Baseline characteristics: demographics and disease profiles of the patients.

Patient Number	Age	Sex	BMI (kg/m ²)	Diagnosis	Kellgren and Lawrence Scale Mean Score	Past Relevant Medications—Type and Treatment Period
1	64	F	31	Primary Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (1 time), HA Infiltration (2 times)
2	59	M	28	Post-Traumatic Osteoarthritis	4	Anti-Inflammatory Drugs, Corticoid Infiltration (3 times), HA Infiltration (3 times)
3	39	F	35	Patello-femoral Pain/Chondral Injuries	2	Anti-Inflammatory Drugs, Corticoid Infiltration (1 time), HA Infiltration (4 times)
4	66	F	25	Primary Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (5 times), HA Infiltration (4 times)
5	45	M	24	Post-Traumatic Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (3 times), HA Infiltration (3 times)
6	54	M	34	Primary Osteoarthritis	4	Anti-Inflammatory Drugs, Corticoid Infiltration (2 times), HA Infiltration (4 times)

Table 1. Cont.

Patient Number	Age	Sex	BMI (kg/m ²)	Diagnosis	Kellgren and Lawrence Scale Mean Score	Past Relevant Medications—Type and Treatment Period
7	64	F	32	Primary Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (1 time), HA Infiltration (1 time)
8	47	F	30	Patello-femoral Pain/Chondral Injuries	2	Anti-Inflammatory Drugs, Corticoid Infiltration (3 times), HA Infiltration (2 times), Genicular Nerve Block
9	55	M	28	Primary Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (3 times), HA Infiltration (2 times)
10	63	M	31	Primary Osteoarthritis	3	Anti-Inflammatory Drugs, Corticoid Infiltration (2 times), HA Infiltration (3 times), Genicular Nerve Block

2.2. Eligibility Criteria

The study included patients with knee pain diagnosed with primary or secondary OA grades 2 to 4 based on the Kellgren and Lawrence scale [10], all of whom had previously received both corticoid and hyaluronic acid infiltrations. Patients were excluded if they had any autoimmune diseases, including rheumatoid arthritis, uncontrolled concomitant metabolic diseases, or the presence of a prior knee infection.

2.3. Rigenera® Protocol

Autologous micrografts were collected using the Class II-a CE medical device Rigeneracons (Human Brain Wave s.r.l, Turin, Italy) (Figure 1), while the rotation of Rigeneracons was triggered using Sicurdrill® 2.0, Sicurlid, and Sicurstick (Figure 1).

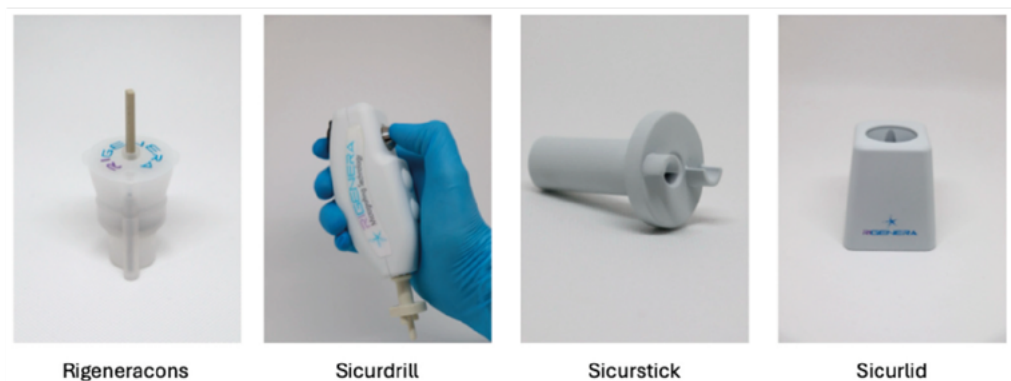


Figure 1. Rigenera® devices used during the procedure.

First, three biopsy samples were obtained from the retro-auricular zone using a 2.5 mm dermal punch. The area for biopsies was first cleaned with an antiseptic solution; 2.5 mL of Lidocaine 2% without vasoconstrictors was applied around the tissue to be biopsied. Biopsy samples were transferred into the medical device, and 4 mL of saline solution (0.9% Sodium Chloride injection B.P.) was added to the Rigeneracons. The samples were processed

for 6 min with controlled rotations of 80 rpm. Afterward, the micrograft suspension was aspirated from the hole using syringes and injected into the area with a 5 mL Luer Lock Syringe with a needle of 21G 1/2, 0.8×40 mm [22]. The entire procedure was conducted under sterile conditions, and the Rigeneracons, intended for single use, were safely disposed of following local guidelines (Figure 2).

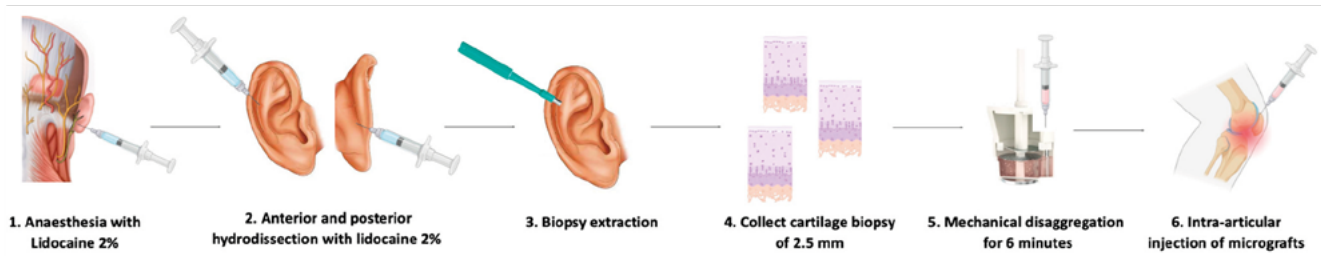


Figure 2. Rigenera[®] technology protocol. The procedure begins with local anesthesia using 2% lidocaine (1), followed by the anterior and posterior hydro-dissection of the auricular cartilage (2). Cartilage biopsies (2.5 mm) are then extracted from the ear (3,4). The biopsies are mechanically disaggregated for 6 min (5) to obtain micrografts. Finally, the micrografts are injected intra-articularly into the target joint (6). Created with Biorender.com.

2.4. Assessment

X-rays were performed before the procedure. The Knee Injury and Osteoarthritis Outcome Score (KOOS) questionnaire [23], recommended by the International Knee Documentation Committee, was used to assess pain, stiffness, and function before the procedure and at 1, 6, and 12 months post-procedure. The KOOS is a 42-item self-reported measure that evaluates the patient's perception of knee health, symptoms, and functionality. Each item offers five possible answers scored from 0 (no problems) to 4 (extreme problems), and scores are transformed to a 0–100 scale, where 0 indicates severe knee issues and 100 indicates no problems. Additionally, the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) questionnaire [24] was administered before the procedure and at 1, 6, and 12 months afterward. This widely used index assesses pain, stiffness, and physical function related to daily activities such as walking, climbing stairs, and sitting down and standing up from chairs. WOMAC scores are also transformed to a 0–100 scale, where 0 represents no knee problems and 100 indicates significant knee pain or functional limitations. Furthermore, the Visual Analog Scale (VAS) [25,26] was used to evaluate pain intensity before the procedure and at 1, 6, and 12 months post-procedure. Patients rate their pain on a scale from 0 (no pain) to 10 (awful pain), providing a numeric score to quantify osteoarthritis-related pain severity. Throughout the study, adverse events were monitored. The biopsied area on the ear was regularly examined for signs of infection or abnormal healing.

2.5. Statistical Analysis

The count data were summarized as absolute numbers and proportions. Additionally, means and standard deviations were calculated for continuous variables. Data were collected and analyzed using Microsoft Excel for MAC (Microsoft, Washington, DC, USA).

3. Results

3.1. Pain Management

The mean VAS score at baseline was 8.3 ± 0.94 , indicating severe-to-awful pain among the participants. The distribution of VAS scores is shown in Figure 3. After 1 month post-procedure, the mean VAS score significantly decreased to 2.4 ± 0.51 . This substantial reduction in pain was sustained over time, with mean VAS scores recorded at 2.7 ± 0.67 after 6 months and 3.3 ± 0.82 after 12 months, as illustrated in Figure 3.

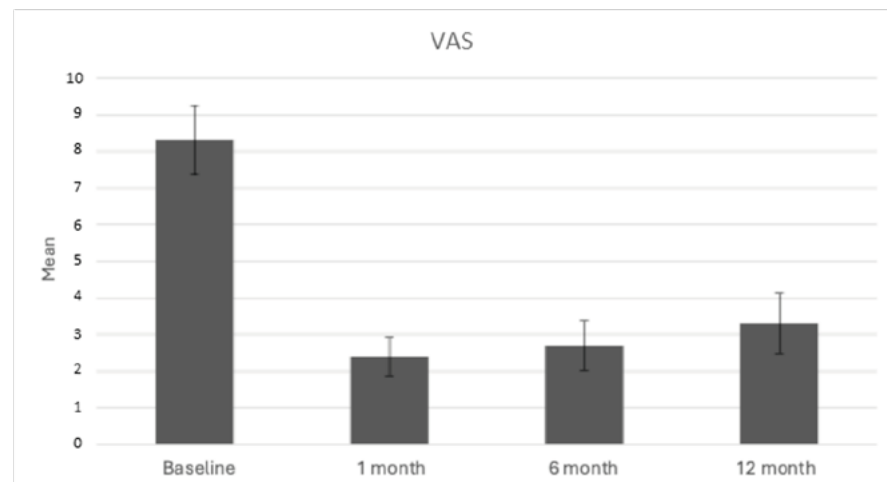


Figure 3. Mean VAS scores recorded at baseline and 1 month, 6 months, and 12 months post-treatment, illustrating the changes in pain perception over time.

3.2. Assessment of Joint Function and Symptoms: KOOS and WOMAC Scores

All 10 patients completed assessments at 1, 6, and 12 months post-operation. Improvements in the mean scores of both the KOOS total and WOMAC questionnaires revealed significant progress in managing pain and enhancing quality of life for the patients. The mean KOOS total score showed significant improvement over time, starting from 35 ± 5.18 at baseline and increasing to 63.3 ± 7.27 at 1 month, then to 66.7 ± 6 at 6 months, and then remaining stable even 12 months post-procedure (Figure 4). In parallel, the mean WOMAC total score demonstrated significant improvement over time, decreasing from 54.8 ± 14.36 at baseline to 29.6 ± 9.24 at 1 month. At the 6- and 12-month follow-ups, the mean WOMAC score decreased further with values of 25.5 ± 8.31 and 24.4 ± 7.2 , respectively (Figure 5). These improvements reflect significant reductions in pain, stiffness, and physical dysfunction, indicating enhanced mobility and daily functioning for the patients. Overall, the WOMAC results underscore the effectiveness of the procedure in reducing symptoms and improving quality of life for patients over the 12-month follow-up period.

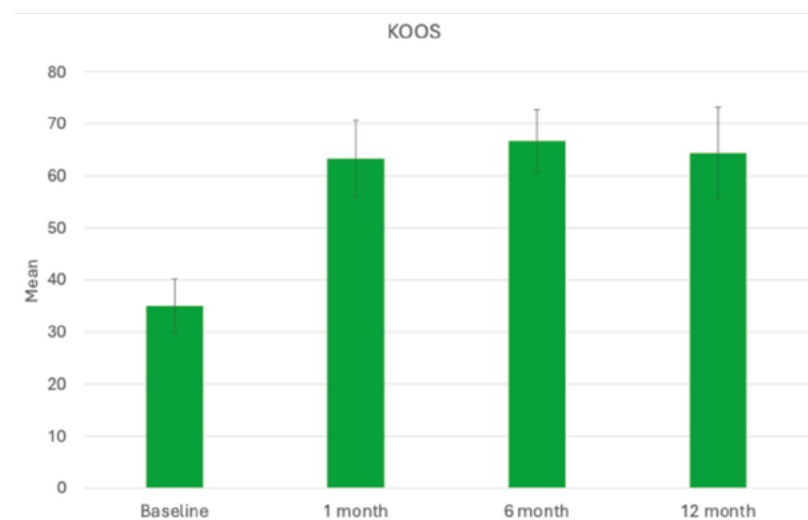


Figure 4. Mean KOOS scores recorded before the procedure, and at 1, 6, and 12 months post-procedure, highlighting changes in knee function and symptoms over the course of the follow-up period.

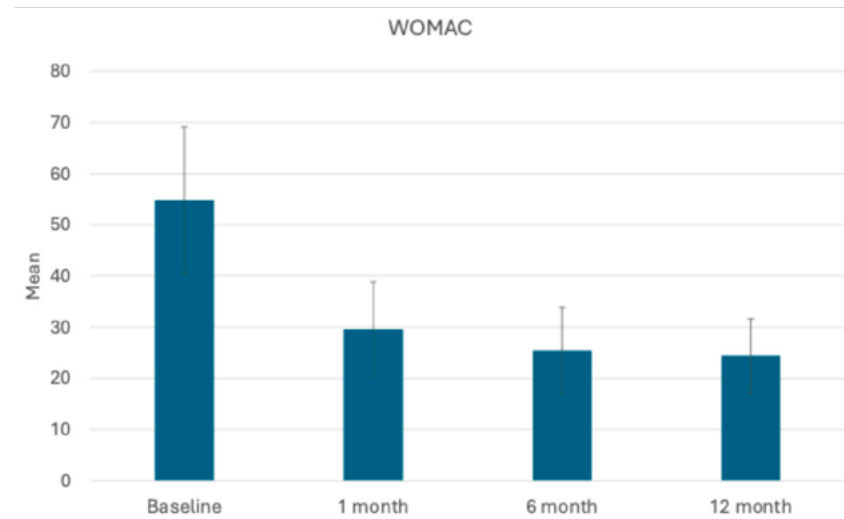


Figure 5. Mean WOMAC scores recorded before the procedure and at 1, 6, and 12 months post-procedure, demonstrating the improvements in knee function and symptoms during the follow-up period.

4. Discussion

In patients with OA, pain is a complex and multifaceted symptom that significantly affects quality of life [27,28]. This pain has multiple facets, including inflammatory and neuropathic characteristics [29]. It initially results from the degradation of the cartilage, which exposes the underlying bone and creates friction within the joint [30,31]. The disruption of the articular cartilage, which normally provides a smooth and lubricated surface for movement, causes a release of fragments and decomposition products from the matrix into the joint space, triggering an inflammatory response. The subsequent activation of cytokines sensitizes pain receptors and promotes the release of other pain-inducing substances [32,33]. This combination of factors makes OA pain management challenging, often requiring a comprehensive approach that includes medication, lifestyle changes, and surgical interventions [13,34]. Specifically, newly studied orthobiologic treatments may play a role in pain control associated with OA.

Some of these treatments utilize biological substances to modulate the inflammatory response and try to promote tissue repair. In this way, they try to modify the disease process and improve management of the symptoms, potentially offering long-term benefits for patients with OA [16,35]. Rigenera[®] Technology is a procedure designed to harness the body's regenerative capabilities to treat various conditions in the fields of dermatology, dentistry, orthopedics, and wound care. This technology focuses on the use of micrografts to reduce inflammation and promote tissue healing [36]. The role of micrografts has been explored in depth in previous in vitro and in vivo studies for the treatment of osteochondral defects [37,38], and the results of the present study indicate that treatment led to a significant reduction in pain in patients with knee osteoarthritis. In fact, the mean scores on the VAS decreased by 71% after 1 month post-procedure compared with the baseline, and these improvements were maintained for up to 12 months after the procedure. Similar results were obtained with KOOS scores, in which an increase of 80% was observed after the first month of follow-up compared to baseline, reaching a score of 83% one year after the procedure. In parallel, in the WOMAC scores, we also observed a reduction of 55% one year post-procedure. These data suggest that Rigenera[®] Technology could represent an effective and safe therapeutic option for patients with knee osteoarthritis, especially in those who do not respond well to conventional treatments. In all patients, the ear wound was fully healed within 7 days of the procedure, with no adverse events reported either during the procedure or in the subsequent weeks and months. This approach could reduce the need for invasive surgery and improve the quality of life for patients.

This study presents some limitations, including the small number of patients treated, the short-term follow-up and the absence of a control group. However, due to the results shown, further studies with a statistically significant number of patients and two-year follow-up should be carried out to confirm the results presented.

5. Conclusions

This study indicates that treatment with autologous micrografts using Rigenera® Technology is a safe and effective method for reducing pain and improving function in patients with knee osteoarthritis. These encouraging results highlight the need for further research to validate and expand the potential clinical applications of this therapeutic approach, promoting its broader use in clinical practice.

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NOTES

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