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Reconstruction of Alar Nasal Cartilage Defects Using a Tissue Engineering Technique Based on a Combined Use of Autologous Chondrocyte Micrografts and Platelet-rich Plasma: Preliminary Clinical and Instrumental Evaluation

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Background: Developing cartilage constructs with injectability, appropriate matrix composition, and persistent cartilaginous phenotype remains an enduring challenge in cartilage repair. The combined use of autologous chondrocyte micrografts and platelet-rich plasma (PRP) is an alternative that opens a new era in this field.
Methods: At the Department of Plastic and Reconstructive Surgery, University of Rome Tor Vergata, Italy, 11 patients underwent nasal alar reconstruction with chondrocyte micrografts gently poured onto PRP in solid form. A computed tomographic scan control was performed after 12 months. Pearson's Chi-square test was used to investigate difference in cartilage density between native and newly formed cartilages.
Results: The constructs of chondrocyte micrografts–PRP that were 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.
Conclusion: This report demonstrated that chondrocyte micrografts derived from nasal septum poured onto PRP in solid form are useful for cartilage regeneration in patients with external nasal valve collapse. *(Plast Reconstr Surg Glob Open 2016;4 :e1027; doi: 10.1097/GOX.0000000000001027; Published online 26 October 2016.)*

Chondrogenesis is the process by which cartilage is formed from condensed mesenchymal tissue. For therapeutic cartilage regeneration, the use of chondrocyte micrografts mixed with platelet-rich plasma (PRP) has not been studied for recapitulating chondrogenesis. PRP extracted from blood provides an autologous source of various growth factors (GFs), and it has been demonstrated to be effective in the treatment of soft-tissue defects and pattern hair loss[1–3]; moreover, the incomparable biocompatibility and thrombin-stimulated clotting enabled PRP to be a promising cell carrier for tissue engineering.[4] It has been demonstrated that 3-dimensional culture system in type I collagen scaffold and the addition of multiple GFs contained in PRP, such as transforming growth factor β(TGFβ), insulin-like growth factor 1, and fibroblast growth factor 2 (FGF-2), in the culture medium induced proliferation and a robust chondrogenesis of adult stem cells in vitro.[5,6] Unfortunately, owing to poor mechanical stability and rapid degradability, direct mixing of chondrocytes with PRP leads to shrinking and deformed cartilage formation in vivo.[7] Hence, the use of biomaterials loaded with chondrocytes into the defect site is highly desirable for cartilage repair; in particular, the use of chondrocyte micrografts represents a microinvasive procedure, and grafts are more flexible to fill the lesions

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with various shapes. Today, the main problem of translating experimental protocols of tissue engineering to the routine clinical practice is the identification of accessible sites where an adequate amount of stem cells are collected.[8,9] In addition, the need to specifically define technical procedure and its safety is an essential factor, like in the case of stem cell application in breast reconstruction and soft-tissue defects.[10–13]

Although within the adult human body there are several "loci" or "niches" inhabited by a significant number of stem cells,[14–16] often these loci are not easy to access and have high residual morbidity of the anatomical site.

The nasal septum represents a niche housing chondrocytes that display plasticity and multipotential capability. This niche is easily accessible, and there is limited morbidity of the anatomical site after collection of the micrografts. In a previous study, Ba et al[17] developed the cell bricks technique, in which a chondrocyte sheet was cultured and such a cell–extracellular matrix (ECM) complex was cut into multiple small fragments (cell bricks). They found that chondrocyte bricks significantly inhibited vascular infiltration into PRP gels and slowed their degradation, thus maintaining the framework and shape of the PRP grafts.[18] They hypothesized that the cell brick–enriched PRP gel could be an ideal injectable niche for adults stem cells, which is expected to regenerate biological cartilage tissues with persistent cartilaginous phenotype, less deformation, and uniform histological structure.[18]

In this study, we investigated the in vivo performance of chondrocytes in cell brick–enriched PRP gels and evaluated the persistence of a stable chondrogenic phenotype.

Chondrocytes can be cultured by 2 methods. The first is the enzyme-digestion method in which the cartilage tissue is collected under sterile conditions and digested with appropriate enzymes, and then the resulting cell suspensions are seeded in culture dishes containing a special medium supplemented with necessary additives and then incubated. Finally, the resulting colonies are subcultured before confluence, and the cells are stimulated to differentiate. The second method for isolating chondrocytes is the mechanical centrifugation of cartilage (Rigenera method).

With this innovative approach, the cartilage is treated as any other connective tissue subjected to grafts, with a phase of collection and a phase of mechanical disaggregation of the tissue without manipulating the matrix. Rigenera System produces millions of viable micro-grafts and filters them with a cutoff of 50 µm, to promote the discharge of old differentiated cells and the enrichment of young progenitors cells contained within the cartilage.

MATERIALS AND METHODS

Patients

A total of 11 patients aged 23 to 67 years with external nasal valve collapse the so-called pinched nose deformity, were treated from January 2014 to September 2015 at the Plastic and Reconstructive Surgery Department of Tor Vergata University, Rome.

The preoperative study included a complete clinical examination of the nasal pyramid and of the nasal cavities through anterior rhinoscopy, a photographic examination in 3 projections, and computed tomographic scans. Postoperative evaluation included a clinical and a photographic examination at 1 month, 3 months, 6 months, and 1 year. After 12-month follow-up, radiographic examinations were done using computed tomography scans. Informed written consent approved by the local ethics committee was obtained from patients to use their data for research purpose.

The authors cut the septal cartilage into strips during open tip rhinoplasty. The strips were gently collected and dissociated using Rigenera System (HBW srl; Turin, Italy) in 1.2 ml of physiologic solution. After a specific centrifugation, the cellular suspension was collected from the system and gently infiltrated into PRP gel.

The product obtained (PRP gel mixed with chondrocytes) was applied on the defect in external nasal valve collapse. The postsurgical course was uneventful.

PRP Preparation

PRP was prepared from a small volume of blood (18 ml) according to the method of the Cascade-Selphyl-Esforax procedure (Aesthetic Factors, LLC; Wayne, N.J., http://www.selphyl.com), with modifications, and from 60 ml of blood according to the P.R.L. Platelet Rich Lipotransfert system (CORIOS Soc. Coop; San Giuliano Milanese, Italy, http://www.corios.it), with modifications, using PRP alone (C-Punt, Biomed Device; Modena, Italy, http://www. biomeddevice.it) without the combined use of stromal vascular fraction cells. Briefly, blood was taken from a peripheral vein using sodium citrate as an anticoagulant (ACD sodium citrate/citric acid/Glucose SALF SpA; Italy). The current procedures for preparing platelet concentrations use various centrifuges (in the Cascade-Selphyl-Esforax procedure, we used 1100*g* for 10 min; in the C-Punt procedure, we used 1200 RPM for 10 min). PRP was prepared in all cases with approval of the transfusional service.

Although the method of preparation is not selective and may include leukocytes, the final aim is to obtain a platelet pellet. GFs are secreted once the platelet activation begins, which, in turn, is stimulated by calcium chloride ($CaCl_2$). Autologous PRP, not activated, obtained after first centrifugation (9 ml), was switched with a second container extracted by Cascade system containing 1 mM $CaCl_2$ and centrifuged for a second time at high speed (>1450*g*) for 15 minutes to produce the platelet-rich fibrin matrix (PRFM) membrane.

Autologous PRP, not activated, obtained by the C-Punt procedure after centrifugation (23 ml), was inserted in the support of the separation machine, where platelets and plasma were automatically pushed into a separate bag under the control of an optical sensor so as to prevent any buffy coat contaminations, thus minimizing the number of leukocytes. The bag was subsequently positioned in the centrifuge basket for a second centrifugation (1900 RPM for 15 min). At this step, the platelet concentrate (PC) was at the bottom of the bag. Part of

poor platelet plasma, 4 ml, was removed using 10-ml syringe with needle. PC and the remaining plasma were mixed gently by massaging the bag. Thus, the leukocyte-poor PRP was obtained.

PRP Technology and Biomolecular Aspects

Conventional PRP is plasma with a platelet concentration above the "normal" physiologic levels found in blood. Increased concentration of platelets also yields an increase in the concentration of GFs stored in the α-granules of platelets.[19,20] Currently, several methods are available for PRP preparation, most producing a liquid end product. PRP is clinically used in liquid or gel form to promote tissue repair. Because of poor mechanical properties, conventional PRP is often difficult to handle in clinical settings that require secure implantation in a specific site or where released GFs could be washed out during an operation. The physical properties of PRP can be changed if plasma and platelets are stimulated, usually by the addition of $CaCl_2$ and thrombin, to produce a fibrin network. However, this method leads to a clot with high concentrations of red and white blood cells and is associated with an almost total platelet activation, degranulation, and GF release.[19,20]

The Cascade system produces a PRFM, prepared as an easy-to-apply membrane without the use of exogenous thrombin. Instead of producing a PC and platelet-poor plasma, as in conventional PRP systems, a thixotropic separator gel is employed in a low-speed (1100*g*) radial (swing bucket) centrifugation to rapidly isolate both the platelets and fibrinogen-containing plasma from the packed red and white cell fraction. In vitro studies[19–23] demonstrated that platelets isolated from blood using the PRFM membrane are essentially intact and inactivate (cluster designation 62p, a membrane-associated glycoprotein expressed by activated platelets expression <5%). In contrast, 5-minute exposure to 100 U/ml bovine thrombin resulted in >95% activation and >65% degranulation of exposed platelets.

Lucarelli et al[24] studied the ultrastructural and physical characteristics of the PRFM membrane describing it ''as a translucent yellow-white disk of 0.105–0.021 mm thickness and 33 mm in diameter." PRFM membrane showed significant tensile properties with elasticity, peak breaking strength, and elongation to break equivalent to intact aorta and far greater than conventional thrombin-generated PRP clots. Confocal scanning fluorescence microscopy and scanning electron microscopy revealed a dense highly cross-linked fibrin matrix with high concentrations (>100×) of intact platelets localized on one side (upper side) of the membrane and not on reverse side.[24] In this study, using a "washed-out" protocol, where the media are replaced after each time point, the authors demonstrated that the conditioned media from in vitro PRFM cultures (37°C) produced high levels of platelet-derived growth factors (PDGFs) with maximal release in the first 24 hours (PDGF, 28 ng/ml; vascular endothelial growth factor (VEGF), 240 pg/ml; and TGFβ, 10 ng/ml) and decreasing levels out to day 7. Furthermore, the authors showed that media supplemented with conditioned media from the PRFM cultures (20% v/v) were able to support proliferation of human mesenchymal stem cells to a level significantly greater than that achieved with 20% fetal bovine serum supplementation.[24]

One important distinction is whether a PRP preparation is leukocyte-rich or leukocyte-poor. Because leukocytes are known to produce VEGF and have antimicrobial properties, they may play an important role in further enhancing the tissue repair processes, but they may also lead to increased local inflammation.[22]

Several studies[25,26] have demonstrated a difference in the platelet and GF concentrations in PRP produced by platelet separation systems. However, these analyses included either expensive cell separator systems not practical for clinical use or manual protocols with unreliable reproducibility. The studies also focused on comparing PRP PC and yield and did not analyze the difference in PRP leukocyte or fibrinogen concentrations, which are important components of PRP.

Filardo et al[27] in a multicenter study compared 2 different PRP preparations: high-concentrate leukocyte-rich PRP versus low-concentrate leukocyte-free PRP. In the sample of 144 patients, treated and evaluated for up to 6 months, similar positive results were reported for both treatments.

Chondrocyte Micrograft Preparation

Autologous micrografts of chondrocytes for immediate clinical use were prepared using an innovative medical device called Rigeneracons (CE certified Class I, HBW srl; Turin, Italy). After the extraction of the nasal septum during rhinoplasty, the authors cut the septum 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 seconds of centrifugation at 80 RPM per minute, the cell suspension was collected from the system and gently infiltrated onto PRP gel. In addition, the cell suspension obtained was cultured and subsequently characterized by cytospin and Alcian-PAS staining to identify the chondrocytes.

The goal standard to Rigeneracons was to disaggregate a small piece of tissue (septum cartilage strips) and opportunely select a cell population with a size of 50 µm.

Chondrocytes obtained were suitable to form autologous micrografts, which can be used alone or in combination with biomaterials (PRP in our work) to obtain a biocomplex ready to be implantable in the subjects in need of such intervention.

Cytospin and Alcian-PAS Staining

Chondrocytes, isolated by Rigenera System (HBW srl; Turin, Italy), were made to adhere to a glass slide by cytospin, and then Alcian-PAS staining was performed (Ventana-Roche Diagnostics; Milan, Italy). Positive cells were counted in the total area under a light microscope (Eclipse E600, Nikon; Japan) and microphotographs captured by DXM1200F Digital camera (Nikon) using ACT-1 software (Nikon).

Chondrocyte Isolation and Culture

Using Rigenera System (HBW srl; Turin, Italy), chondrocytes were isolated by centrifugation and disaggregation of cartilage septum strips from patients undergoing rhinoplasty as previously described. Chondrocytes were plated at a density of 0.25×105 cells/cm^2 in 12-well tissue culture plates and cultured for 24 hours in a humidified atmosphere at 37°C with 5% CO_2 in Dulbecco modified Eagle medium (Sigma; St. Louis, Mo.) without fetal bovine serum to permit them to adhere to the wells.

The incubation period was 7 days, during which time the culture medium was not changed. At the end of the incubation time (7 d), culture supernatants were collected and maintained at −80°C until their use in enzyme-linked immunosorbent assay tests, whereas chondrocytes were used for proliferation assay and then lysed for RNA extraction.

Chondrocyte growth was evaluated through the Alamar Blue test. Briefly, the cells were incubated with 10% of Alamar Blue for 3 hours, and the fluorescence was measured with use of a microplate reader (CytoFluor 2350, Millipore, Mass.). The results were expressed as a percentage of Alamar Blue reduction as indicated by the manufacturer's data sheet (AbD Serotec; Oxford, United Kingdom). The expression of specific genes by chondrocytes was assayed with real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). Interleukin (IL)-1β, IL-6, IL-8/CXCL8, tumor necrosis factor–α, IL-10, metalloproteinase-13, tissue inhibitor of metalloproteinase (TIMP)-1, VEGF, TGFβ1, FGF-2, HGF, hyaluronic acid synthases 2, aggrecan, collagen II, and Sox-9 were determined.

Histological Evaluation

Excisional tissue was obtained from 4 randomly selected patients 6 months after surgery. Microscopic evaluation of routinary hematoxylin–eosin-stained paraffin-embedded sections[28] was performed to morphologically analyze cartilage tissue.

RESULTS

Clinical and Instrumental Observation

In this study, we reported the results obtained using composite autologous chondrocyte micrografts with PRP, as exemplified by the following case. Nasal obstruction was very marked in these patients; external nose analysis showed external nasal valve collapse, deficit of supratip projection. Anterior rhinoscopy showed a deviated nasal septum and bilateral stenosis of the internal valves. The composite graft was applied on the external nasal valve collapse, in the alar cartilage side by fixing with absorbable stitches. Postoperative follow-up evaluation has shown optimal aesthetic results and improvement of nasal obstruction. These composite grafts provide functional support to the alar cartilages, usually collapsed because of excessive resection during previous surgery. Transcolumellar open-tip access was necessary to allow for better visualization of the valve collapse and alar cartilage and for fixation of the cartilaginous structures to allow for placement of unexposed absorbable stitches.

Histological Observation

Chondrocytes isolated by Rigenera System were stained with Alcian-PAS and counted under a light microscope. The mean of cell yield was 9088.30 + 788.86 cells/ml of digestion.

In addition, microscopic analysis of excisional fragments, in 2 patients, showed the persistence of healthy cartilage tissue surrounded by a fibrous connective tissue layer in which newly formed capillaries spread and penetrate into cartilage.

DISCUSSION

The aim of nasal valve reconstruction is the anatomical and functional correction of the superior airways. The main surgical techniques include the use of "composite grafts" made up of skin and cartilage, or mucosa and cartilage, and "spreader grafts." Spreader grafts are made up of a small bar of cartilage (generally from the septum) attached to the sides of the dorsal margin of the quadrangular cartilage.[29] A spreader graft exhibits the following relationships: medially, it is in contact with the cartilaginous tissue of the septum, and laterally, it rests on the septal perichondrium. It is mandatory that it is contained in the integral extramucosal space. Therefore, correcting the collapse of the lateral walls while keeping them apart permits the widening of the internal nasal valve, thus correcting the patency of the nasal airway spaces.

These grafts provide a functional support to the lower part of the lateral cartilages, which, in most cases, collapse because of excessive resection during previous surgical operations (the cartilages collapse during inspiration), giving support to the external nasal valve.[29] They are implanted through what is known as "open-tip" access, which permits a wide exposure of the dorsum and a broad view of the osteocartilaginous structures to be reshaped, and allows the nonabsorbable sutures that are not exposed on the surface (to avoid them being touched from the outside) to perfectly stabilize the graft. From a biologic point of view, the graft nourishes itself through imbibition, because of the establishment of new microvascular bridges between the graft and the receiving bed.

Therefore, if the graft is not stable, the irroration will not be perfect over time, and dehydration and reabsorption could occur. In most cases, a composite graft that is more than 5 mm from the vascular bed is at risk of necrosis.[29]

The tissue engineering opens new challenges for rhinoplasty. Developing an injectable approach for cartilage regeneration could meet today's demand for microinvasive surgery.[30] Different from solid scaffold-based tissue engineering, injectable cartilage constructs require fluidity of components and instant establishment of a chondrogenic niche. Zhu et al[18] proved that fragmented chondrocyte macroaggregates (cell bricks) could stabilize the PRP gel efficiently in vivo and support chondrogenesis with stable morphology.[18] In this study, they found that such

an injectable complex could support chondrogenesis of bone marrow mesenchymal stem cells (BMSCs) in vivo and demonstrated that such a complete biological graft could meet the requirement of nasal augmentation, thus holding future promise in craniofacial reconstruction.

The most important finding in this study is that BMSCs embedded in chondrocyte brick–enriched PRP gel underwent persistent chondrogenesis, and hypertrophic translation was prevented.[18] In addition, BMSCs are capable of promoting angiogenesis through the secretion of GFs, in particular VEGF.[31] Angiogenesis is a crucial event for tissue regeneration[32] and tumor growth.[33,34] This finding warranted reduction of the donor cartilage compared with conventional chondrocyte transplantation and provides a microinvasive approach for cartilage regeneration, indicating a promising use of cell bricks in clinical application in the future. For clinical translation, donor cartilage could be harvested from the nasal septum.

The important advantage of PRP gel is that it could initiate faster tissue remodeling than synthetic polymers and stimulate the wound-healing process. A surprising phenomenon presented in the study of Ba et al[17] is that BMSCs mixed with CB-PRP gel promoted angiogenesis in BMSC regions and prevented central necrosis of the whole graft. In their previous experiment, the chondrocytes mixed with CB-PRP gels resulted in necrosis of the interior of constructs, which could be attributed to the robust antiangiogenic potential of chondrocytes. Their results confirmed that BMSCs in CB-PRP gels presented higher VEGF expression than chondrocytes in CB-PRP gels, which was regarded to contribute to the angiogenesis in BMSCs regions in PRP grafts.[17]

Many studies recently published[35] have investigated the effect of PRP and insulin treatment on adipose-derived stem cells (ASC) chondrogenic/osteogenic differentiation in 3D collagen scaffold cultures. After 21 days, Alcian blue staining and the DMMB assay documented an increase of chondrogenic induction after PRP treatment and even more with insulin ($P < 0.01$ and $P < 0.001$, respectively). Combined treatment also significantly enhanced ASC chondrogenesis in 3D collagen scaffolds ($P < 0.0001$). RT-PCR and real-time PCR analysis for Agcan and COL2A1 transcripts confirmed the histochemical data.[35] Von Kossa staining documented that PRP favors the osteogenic differentiation of ASCs more than insulin alone, and the combined treatment enhanced osteogenic differentiation.[35] Ultrastructural analysis confirmed abundant cell membrane–invested vesicles containing material (presumptive matrix) released in the extracellular space; large amounts of thick fibrils of ECM can be also observed and black deposits similar to hydroxyapatite/calcium crystals also can be observed in both cell cytoplasm and the ECM areas.[35] X-ray microanalysis performed on these deposits confirmed the presence of both calcium and phosphorus. Results of RT-PCR and real-time PCR analyses for ALP and OCN transcripts were in line with the histochemical and ultrastructural results.

These histochemical and biomolecular analyses demonstrated that chondrogenic/osteogenic differentiation was increased in ASC-populated 3D collagen scaffolds compared with 2-dimensional plastic dish culture. Chondrogenic/osteogenic differentiation was further enhanced in the presence of combined PRP (5% v/v) and insulin (100 nM) treatment.[35]

These findings underline that 3D collagen scaffold culture in association with PDGFs and insulin favors the chondrogenic/osteogenic differentiation of ASCs, suggesting new translational applications in regenerative medicine for the management of osteochondral defects.

Roffi et al[36] reported that fresh and frozen PRPs did not differ in their ability to induce cell proliferation or ECM production and secretion in both chondrocytes and synoviocytes. It was found using gene expression analysis that chondrocytes cultured with both PRPs showed similar results for collagen II, aggrecan, and Sox-9, thus indicating that frozen PRP did not lose or reduce its ability to enhance chondrocyte anabolism. Albeit with no statistical significant difference with respect to frozen PRP, IL-1β, IL-6, FGF-2, and TIMP-1 were highly induced by fresh PRP. It could be speculated that their amounts might be attributed to leukocytes in fresh PRP.[36] In fact, it has been reported that leukocytes may be responsible for the increased expression of IL-1β, IL-6, FGF-2, and TIMP-1,[36] and this might explain why their presence was not so marked in frozen PRP, in which freeze/thawing caused leukocyte destruction.

Here, we demonstrated that it is possible to isolate human chondrocytes without enzymatic digestion with the Rigenera System and construct combined micrografts with autologous fresh PRP in vivo in patients with external nasal valve collapse.

The constructs of chondrocyte micrografts–PRP that were subcutaneously injected resulted in a persistent cartilage tissue with appropriate morphology and 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 cartilage.

The use of appropriate biomaterial scaffolds combined with selected GFs can significantly improve the survival and differentiation of the transplanted stem/progenitor cells.[6,37–42]

Brunelli et al[43] demonstrated that the micrografts derived from dental pulp poured onto collagen sponge are useful for bone regeneration in atrophic maxilla.

Dental stem/progenitor cells (DPSCs) collected from dental pulp can be differentiated in vitro and then transplanted with biomaterial scaffolds into the host without immunologic rejection.[44,45] Graziano et al[46] observed DPSCs performances on different scaffolds, such as PLGA or poly(lactic-co-glycolic acid) 85:15, hydroxyapatite chips (hyaluronic acid), and titanium. Results showed that stem cells exerted a different response, depending on the different types of textured surface. Actually, stem cells challenged with concave surfaces differentiated quickly and showed nuclear polarity, an index of secretion, cellular activity, and matrix formation. Moreover,

bone-specific proteins were significantly expressed, and the bone tissue obtained was of significant thickness. Thus, cells cultured on the concave-textured surface had better cell–scaffold interactions and were induced to secrete factors that, because of their autocrine effects, quickly lead to osteogenic differentiation, bone tissue formation, and vascularization.

The quality and quantity of regenerated bone formed by DPSCs were demonstrated in in vitro and in vivo experiments using stem cells and biomaterials.[16,44,45,47]

In conclusion, chondrocytes, ASC, DPSCs, and BMSCs could be considered an interesting and potentially important sources of autologous stem/progenitor cells that are ready for use for therapeutic purposes, such as the repair/regeneration of craniofacial bones.

With the Rigenera procedure, the septal cartilage tissue of the nose can have a new clinical use and open new surgical strategies during the rhinoplasty.

Although this is only a preliminary report, its results are extremely encouraging for rhinoplasty and new therapeutic approaches can be developed by translating experimental protocols into clinical practice using the enormous regenerative potential of the human tissue.

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