

## Lipogems, a New Modality of Fat Tissue Handling to Enhance Tissue Repair in Chronic Hind Limb Ischemia

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### ABSTRACT

**Background:** Human adipose-derived stem cells (hASCs) may represent an easy-to-harvest tool for cell therapy of peripheral artery disease. In most clinical trials, stem cells undergo prolonged *ex vivo* expansion, with significant senescence, and decline in multipotency, leading to clinical results below expectations.

**Methods:** We have developed a non-enzymatic method yielding a microfractured fat tissue (Lipogems), harboring intact stromal-vascular niche and pericyte/mesenchymal stem cells. Human Lipogems, Lipogems-derived hASCs preconditioned with or without a vasculogenic mixture, including hyaluronan, butyric and retinoic acids (H+B+R), were transplanted into the *gracilis* muscle of 16 rats subjected to chronic hind limb ischemia. After two weeks, tissue rescue was assessed by a perivascular flow probe and immunohistochemistry. Coculture of HUVECs with Lipogems or Lipogems-derived hASCs was performed. Vasculogenic gene transcription and secreted cytokines were assessed.

**Results:** Xenogeneic transplantation of human Lipogems elicited significantly higher muscle tissue repair and lower inflammatory infiltration than expanded Lipogems-derived hASCs, even preconditioned with H+B+R. Lipogems action involved a significantly higher arteriogenic re-

sponse than that elicited by untreated or preconditioned hASCs.

Although transplanted Lipogems bordered within the external side of collating primary fibers, it led to remarkably better preservation of muscular fiber size than untreated or preconditioned hASCs. Lipogems-derived hASCs responded to vasculogenic agents with increased VEGF, KDR, and HGF transcription, associated with early and long-lasting secretion of the encoded cytokines. In coculture with HUVECs, Lipogems enhanced endothelial cell proliferation remarkably more than untreated or preconditioned hASCs.

**Conclusion:** Lipogems emerges as a transplantable immunomodulatory fat tissue product with remarkable arteriogenic and paracrine properties for the rescue of ischemic limb.

### INTRODUCTION

Vascular diseases are a major cause of mortality and disabilities worldwide, and vascular complications of diabetes are now emerging as a pandemic scenario<sup>1</sup>. Although surgical intervention may prove rewarding to support *district* revascularization, it fails to rescue cutaneous and subcutaneous trophism in severe diabetic or atherosclerotic vascular diseases. Within this context, the transplantation of skin flaps for reconstructive purposes offers inconsistent and often poor outcomes due to an extremely low grafting efficiency, with bare perspective for ulcer healing. At worst, the local progression of vascular

complications may lead to amputation. Delays and/or deficiencies in treatment are ultimately expected to inevitably increase healthcare costs in the future<sup>2,3</sup>.

Regeneration of occluded arteries following autologous stem cell transplantation appears to be a possible innovative modality for no-alternative-option patients with peripheral artery disease (PAD). So far, a number of clinical trials have been conducted in patients with PAD, using bone marrow-derived mononuclear cells (BMMNCs) or peripheral blood-derived mononuclear cells (PBMNCs) mobilized with granulocyte-colony stimulating factor G-CSF<sup>4</sup>.

On the whole, these trials appear to be safe<sup>4</sup>, although a noticeable deficiency is still the poor understanding of the mechanism(s) underlying the potential beneficial effect(s) of cell therapy in treating PAD. It is now emerging that effective therapeutic revascularization of ischemic tissues requires a combination of arteriogenesis and angiogenesis<sup>5,6</sup>. Arteriogenesis relates to the outward remodeling and enlargement of collateral arterioles, a mechanism affording increased blood flow to distal vascular beds<sup>5-7</sup>. To this end, aside the large number of studies on angiogenic mechanisms for tissue repair, a growing body of investigations with human adult stem cells, including mesenchymal stem cells (hMSCs), is currently focusing on the attempt to achieve effective arteriogenic tissue rescue.

Albeit hMSCs can be harvested from an increasing number of tissue sources, they are particularly scarce in the body. In most clinical trials, stem cells are subjected to long-term expansion *ex vivo*, in order to yield a large number of viable elements or to precondition stem cells with various strategies (i.e. chemical or physical agents) optimizing their commitment and/or paracrine release prior to transplantation. Analysis of currently available cell therapy protocols reveals that hMSCs are transplanted at high doses usually ranging between 10 and 400 million hMSCs per treatment ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). As a consequence, these cells need to be expanded *in vitro* for multiple passages and prolonged time in culture – 8-12 weeks – before implantation. Stem cell culturing for multiple passages has been shown to represent both a risk of, and a well-established model for cell senescence *in vitro*<sup>8,9</sup>. Human adipose-derived stem cells (hASCs), due to the ease of fat tissue harvesting and their robust multipotency *in vitro*, have also been used as a tool to afford peripheral revascularization in limb ischemia both in experimental an-

imal models, as well as in few, small-size human studies. Nevertheless, expanded hASCs also undergo significant senescence after multiple passages in culture<sup>8,10,11</sup>, raising a cautionary note for the safety of their clinical applications.

Within this context, the availability of a tissue product that can be readily transplanted to afford significant tissue repair, avoiding the needs for cell expansion and the cumbersome requirements for Advanced Therapy Medicinal Products (ATMPs) expanded cells, would have relevant biomedical implications.

We have recently developed a microfractured human fat tissue product by the aid of an innovative device and method, named Lipogems<sup>12</sup>. This tissue graft was shown to improve clinical results from orthognathic surgery, enhancing post-operative healing<sup>13</sup>. The Lipogems product was shown to maintain an intact stromal vascular niche harboring cellular elements with mesenchymal stem cell and pericyte characteristics<sup>12</sup>. Lipogems-derived stem cells expressed transcriptional profiles characterized by self-renewal/stemness patterning, along with a set of genes orchestrating commitment towards the neurogenic lineage<sup>14</sup>. Interestingly, the Lipogems product can be reliably cryopreserved, without losing its niche structure and the viability of its embedded stem cells. Further analysis<sup>12</sup> have revealed that hASCs residing within the Lipogems product are significantly more responsive than enzymatically dissociated hASCs to a mixture of hyaluronic, butyric and retinoic acid, previously shown to induce stem cell expression of vasculogenic genes<sup>15</sup>. Moreover, exposure to physical energy, such as asymmetrically conveyed radio electric fields, induced a significantly higher yield of commitment along the endothelial, myocardial, skeletal muscle and neural lineages in Lipogems-derived, as compared to enzymatically dissociated hASCs<sup>16</sup>.

In the current study, we investigated whether the human Lipogems product may be used as a tool affording efficient revascularization and tissue rescue in a rat model of chronic hind limb ischemia. In the same model, we also performed a comparative analysis of the effects yielded by Lipogems-derived hASCs that had been expanded in culture prior to transplantation.

## METHODS

### CELL CULTURE AND TREATMENTS

According to the policies approved by the Institutional Review Boards for Human Studies local ethical committees, all tissue samples were obtained after informed consent. Human subcutaneous adi-

pose tissue samples were obtained from lipospiration procedures and processed by using the Lipogems device, as previously described<sup>12</sup>.

A volume of 1.5 ml of Lipogems product was seeded in a T75 flask previously coated with human fibronectin (0.55  $\mu\text{g}/\text{cm}^2$ ) (Sigma-Aldrich) and human collagen I-III (0.50  $\mu\text{g}/\text{cm}^2$ ) (ABCCell-Bio), cultured in  $\alpha$ -MEM medium supplemented with 20% heat-inactivated FBS, antibiotics (200 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin), L-Glutamine (1%), and incubated at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Medium was changed every 4 days, but the Lipogems product was maintained in culture for ten days, then it was eliminated. After two weeks, at confluence, released cells were detached by treatment with trypsin-EDTA (Sigma-Aldrich), and subcultured. Experiments were performed at passage 3-5. Lipogems-derived hASCs were cultured in the absence or presence of a vasculogenic mixture<sup>15</sup> containing 10-20 kDa hyaluronic (H) (2 mg/ml, Lifecore Biomedical), butyric (B) (5 mM, Sigma-Aldrich), and retinoic acid (R) (1  $\mu\text{M}$ , Sigma-Aldrich), to investigate whether cell exposure to the mixture (H+B+R) may affect the angiogenic or antiapoptotic cell properties.

#### ANIMAL MODEL OF HIND LIMB ISCHEMIA

Sixteen Sprague Dawley rats weighing 175-200 g (Charles River Laboratories, Lecco, Italy) were housed in a temperature and moist controlled room with a 12-hour light-dark cycle and given free access to tap water and standard pelleted diet (Mucedola s.r.l., Milan, Italy).

All surgical procedures were performed under anesthesia induced by intraperitoneal (IP) injection of Zoletil (1 ml/kg i.p., Virbac, Milan, Italy). A perivascular flow probe (Transonic system) was placed in the distal portion of the saphenous artery in order to monitor the vascular flow before and after ischemia, then the proximal portion of the femoral artery and the distal part of the saphenous artery were clamped and then ligated through a 7-0 silk ligature. The remaining arterial branches between the femoral and saphenous artery were closed and excised. The left hind limb was kept intact and used as control. All procedures were conducted according to the guidelines for the care and use of laboratory animals approved by our Institution.

#### EXPERIMENTAL PROTOCOL

Twenty-four hours after ischemia, rats were randomized into four groups. In each group the ischemic hind limb was injected with 500  $\mu\text{l}$  ( $1 \times 10^6$

cells) Lipogems-derived hASCs [control cells (CTR CELLS) group] (n=4), 500  $\mu\text{l}$  ( $1 \times 10^6$  cells) Lipogems-derived hASCs treated with H+B+R [treated cells (H+B+R CELLS) group] (n=4), 500  $\mu\text{l}$  ( $\sim 2 \times 10^4$  cells) Lipogems (LG group) (n=4), or 500  $\mu\text{l}$  of phosphate buffered saline solution (PBS group) (n=4). Five separate 100- $\mu\text{l}$  injections were administered intramuscularly into the *gracilis* muscle.

Hind limb perfusion was assessed at the paw level by a surface probe (Transonic system) the day of surgical procedure, 24 hours later contextually at the injections (day 0), and at 7 and 14 days after treatment. Perfusion values used for statistical analysis represent the mean of three consecutive perfusion measurements on the plantar side of the paw. As perfusion measurements can vary based on limb position, paws were precisely positioned and each measurement was evaluated after flow stabilized.

Two weeks after induction of critical limb ischemia, rats were sacrificed. Adductor, gracilis, gastrocnemius and flexor were excised from ischemic and non-ischemic limb, sampled, fixed in 10% formaldehyde solution and embedded in paraffin for histopathological analysis.

For light microscopy examination, three  $\mu\text{m}$ -thick sections were stained with Hematoxylin & Eosin (H&E) for histopathological assessment.

For immunohistochemical analysis, additional muscle sections were labeled with a monoclonal antibody directed against  $\alpha$ -smooth muscle actin (ASMA) to assess the arteriogenic patterning. Briefly, paraffin embedded specimens were dewaxed, rehydrated in graded ethanol (from 100% to 70%) and rinsed in distilled water. Antigenicity was restored with an antigen retrieval treatment using citrate buffer pH 6.0 at 120°C, 1 atm for 21 minutes. After cooling and washing, the endogenous peroxidase activity was quenched for 10 minutes at room temperature (RT) with 3% hydrogen peroxide solution in absolute methanol in the dark. Then, the slides were processed according to the non-biotin-amplified method (NovoLink™ Polymer Detection System, Novocastra Laboratories Ltd., Newcastle Upon Tyne, UK). After being rinsed in TBS 1X, the samples were blocked with Novocastra™ Protein Block for 5 minutes in a humid chamber to reduce the non-specific binding of primary antibody, and rinsed again with TBS 1X. Ischemic muscle tissue sections were subsequently stained using  $\alpha$ -smooth muscle actin (ASMA, 1:9000 dilution, Sigma-Aldrich) monoclonal antibody in 1% Bovine Serum

Albumin in PBS overnight at 4°C. After washing, samples were treated with Novocastra™ Post Primary Block to enhance penetration of the next polymer reagent, rinsed in TBS 1X and incubated with NovoLink™ Polymer for 30 minutes at RT each reagent. Antigen-antibody reactions were visualized using the chromogenic substrate 3,3'-diaminobenzidine (DAB) prepared from Novocastra™ DAB Chromogen and NovoLink™ DAB Substrate Buffer. After that, specimens were rinsed in distilled water, counterstained with Gill's hematoxylin, dehydrated, cover-slipped and viewed in a light microscopy using the Image-Pro Plus® 6 software (Media Cybernetics, Inc., Bethesda, MD, USA). Negative control consisted of omission of primary antibody from sections.

Morphometry was performed on 150 cross-sectioned fibers that were randomly selected on the whole surface of each tissue section; digitalized images were acquired at 25X magnification. The diameter of muscle fibers was calculated as the half-sum between the largest and the shortest cross-sectioned diameter. To quantify arteriogenesis, the ASMA positive area was evaluated in 10 different fields on digitalized images taken at 10X magnification. Computer-assisted image analysis (Image-Pro Plus software; Media Cybernetics, www.mediacy.com) was employed to perform counts and density calculations.

#### GENE EXPRESSION

Total RNA from cells treated with H+B+R for 1, 3, and 6 days (n=3) was extracted using RNeasy Micro Kit (QIAGEN), and 1 µg was reverse-transcribed into cDNA in a 21-µl reaction volume with SuperScript™ III Reverse Transcriptase (Life Technologies). Two-µl cDNA was amplified by real time PCR using Lightcycler system (Roche Applied Science) and the SYBR Green I FastStart kit (Lightcycler® FastStart DNA MasterPLUS SYBR Green I), following the manufacturer's instructions. Obtained Ct values were normalized against GAPDH. Relative gene expression was determined by using the "delta-delta method" for comparing relative expression results between treatments<sup>17</sup>.

Primers used (0.25 µM) were as follows: VEGF forward 5'-AGAAGGAGGAGGGCAGAATC-3' and reverse 5'-ACACAGGATGGCTTGAAGATG-3'; HGF forward 5'-ATTTGGCCATGAATTTGACCT-3' and reverse 5'-ACTCCAGGGCTGACATTTGAT-3'; KDR forward 5'-CTGCAAATTTGGAAACCTGTC-3' and reverse 5'-GAGCTCTGGCTACTGGTGATG-3';

GAPDH forward 5'-CAGCCTCAAGATCATCAGCA-3' and reverse 5'-TGTGGTCATGAGTCCTTCCA-3'.

#### ELISA

Supernatants were collected from control and H+B+R treated cells at 1-2-3-6 days (n=3), and VEGF and HGF concentrations (pg/ml) were measured by enzyme immunoassay (Human VEGF/HGF ELISA Kit, Boster Biosciences Co., LTD). For each sample, total cell protein content was extracted by TCA precipitation/NaOH lysis, quantified by Bradford assay, and used for normalization. Data are expressed as pg of secreted factor per µg of protein at the time of harvest.

#### TRANSWELL COCULTURE TO EVALUATE THE EFFECT OF CYTOKINE SECRETION

Human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in Endothelial Growth Medium containing 2% FBS (EGM-2, Lonza) until confluence. They were then trypsinized, centrifuged at 350 x g for 5 minutes, and labeled with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich). Briefly, HUVECs were resuspended in 100 µl Diluent C. Separately 100 µl Diluent C was mixed with 4 µl PKH26. The cell suspension was mixed with the staining solution (PKH26 final concentration: 20 µM), and incubated for 3 minutes in the dark. The labeling reaction was stopped by adding an equal volume of FBS. Labeled HUVECs were centrifuged, washed with culture medium, centrifuged again, and seeded in the bottom of 24-well plates at a density of 9,000 cells/cm<sup>2</sup> in Endothelial Basal Medium (EBM-2, Lonza) containing 2% FBS. In the corresponding transwell cell culture inserts (0.4 µm pore size, Falcon BD) we seeded Lipogems in α-MEM/5% FBS, or Lipogems-derived hASCs (40,000 cells/cm<sup>2</sup>) which had been previously cultured for 3 days in the absence or presence of H+B+R.

After seven days, HUVEC images were taken at 40X magnification using an inverted optical microscope equipped with a digital camera and fluorescence filters (Nikon). Five different fields on digitalized images were chosen, and computer-assisted image analysis was employed to perform cell number counts (freely available software ImageJ, <http://imagej.nih.gov/ij/>).

#### DATA PRESENTATION AND STATISTICS

Data are presented as mean ± standard error mean (SEM). Statistical comparison between two groups was carried out by unpaired Student's *t* test for in-



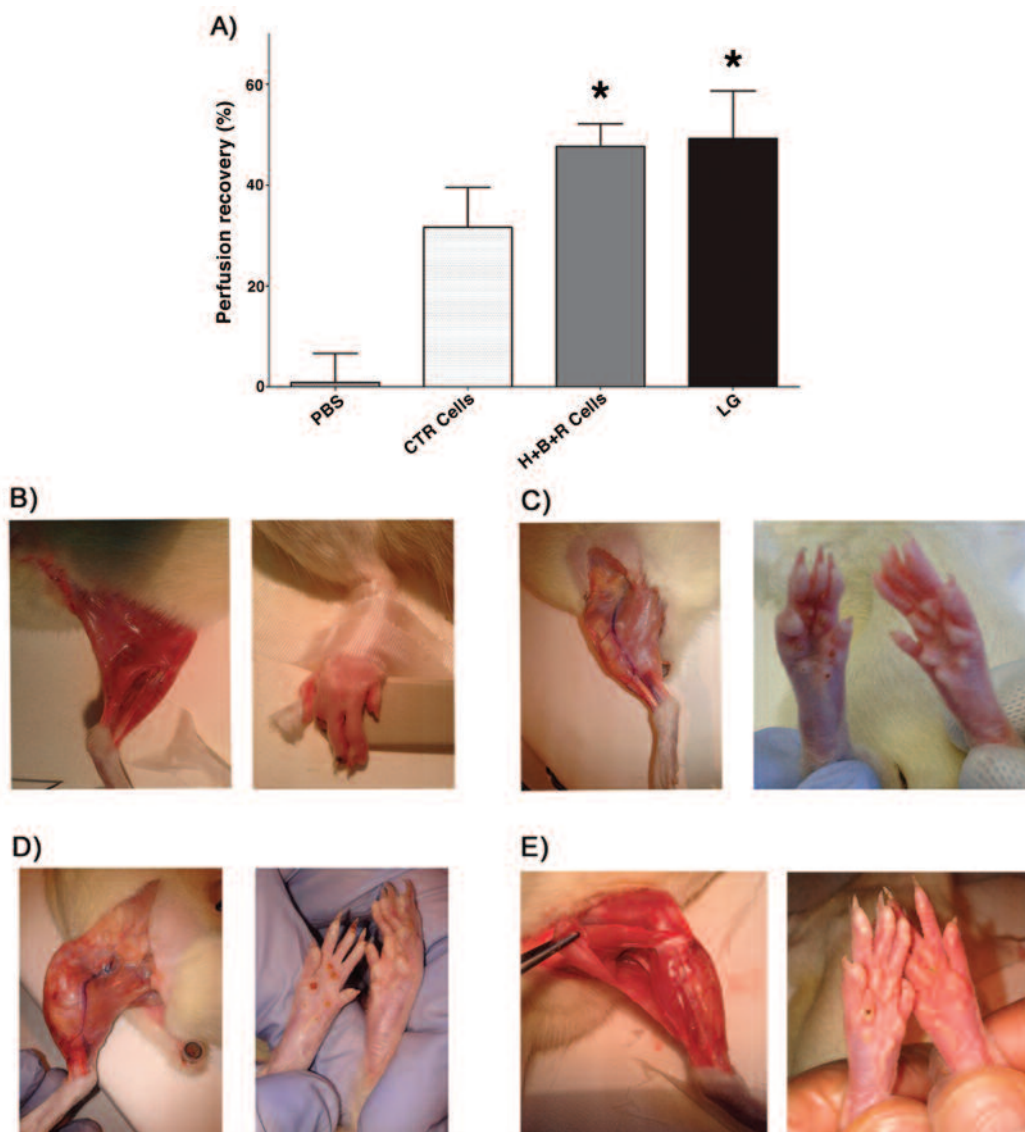
dependent samples (GraphPad Prism 4.0 software). Comparison of more than two groups was performed by one-way ANOVA, followed by Bonferroni post hoc test. All tests were two-tailed and a  $p$  value less than 0.05 was assumed as the limit of significance.

## RESULTS

### HIND LIMB PERFUSION RECOVERY

At each time point the perfusion of ischemic paw was normalized to the perfusion of non-ischemic paw that was used as internal control. Hind limb perfusion recovery was assessed by subtracting the

normalized perfusion of ischemic paw at day 0 from the normalized perfusion at day 14. As showed in Figure 1, after 14 days rats treated with PBS showed no recovery in terms of paw blood flow. In contrast ischemic paw blood flow from rats that received control cells, treated cells or Lipogems was increased up to fifty percent. The percentage of limb reperfusion was significantly higher in the H+B+R CELLS and LG groups, as compared with the PBS group (Figure 1A).



**Figure 1.** *Perfusion recovery.* A, Graph shows the percentage of perfusion recovery as evaluated at the paw level comparing measurements at day 0 and 14 days after injection ( $n=4$  for each group, mean  $\pm$  SEM) (\*indicates statistical significance  $p < 0.05$ ). Paw perfusion in rats receiving control cells, treated cells or Lipogems was increased up to 50% at 14 days after intramuscular injection. B-E, Pictures show the effect of chronic hind limb ischemia on muscular degeneration, and on rat paws: B) PBS group; C, CTR CELLS group; D, H+B+R CELLS group; E, LG group.

**Abbreviations:** PBS, phosphate buffered saline; CTR CELLS, control cells; H+B+R CELLS, cells treated with a mixture of hyaluronic, butyric and retinoic acids; LG, Lipogems.

At gross examination, the ischemia provoked severe damages in rat paw and the inadequate perfusion caused a muscular degeneration. This effect was particularly evident in the PBS group, where the muscle mass was dramatically reduced, and the paw was characterized by necrotic areas, that often lead to the auto-amputation of some fingers (Figure 1B). Transplantation of control cells slightly reduced the damage (Figure 1C), but if the cells were pretreated with H+B+R there was an improvement in tissue healing (Figure 1D). Noteworthy, the best results were obtained in rats transplanted with the Lipogems product, that showed a good muscular tone, and a few and very small necrotic areas in the paw (Figure 1E).

#### HISTOLOGICAL EXAMINATION OF NON-ISCHEMIC AND ISCHEMIC LIMB TISSUES

Muscle fibers morphometry, tissue necrosis and inflammatory infiltrate analysis were assessed in each group by H&E.

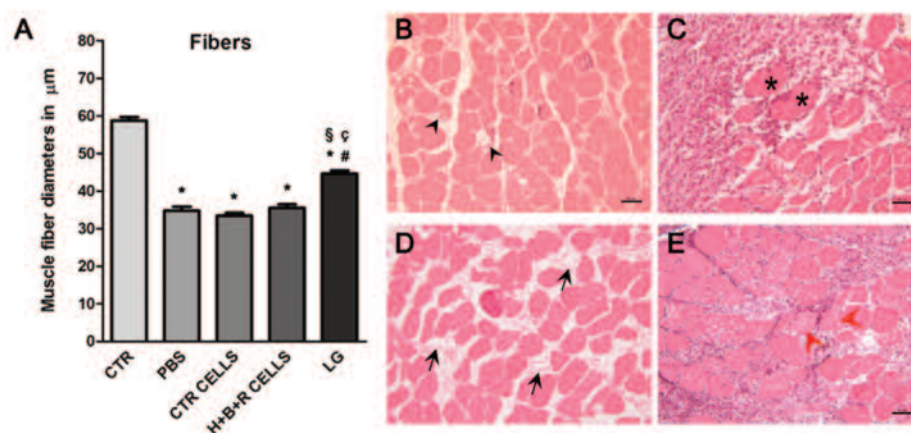
The morphometric quantification revealed an overall decrease of muscle fiber diameters in all groups versus the contralateral non-ischemic tissue (CTR group). Noteworthy the fiber size decreased less in the LG group, whose average size was statistically greater than that measured in muscle tis-

sues treated with PBS, CTR CELLS and H+B+R CELLS (Figure 2A). Changes in fiber size were associated in ischemic muscles with degenerated and necrotic fibers. Figures 2B-E illustrate the major pictures of fiber degeneration; pale necrotic fibers (Figure 2B); myophagocytosis (Figure 2C); empty-looking, ghost fibers (Figure 2D), and moth eaten fibers (Figure 2E). These aspects predominated in the PBS group.

In non-ischemic limb tissues (CTR group), there was preservation of the primary and secondary fascicular structure. Muscle fibers showed a polygonal shape with minimal variation in size and subsarcolemmal nuclei (Figure 3A).

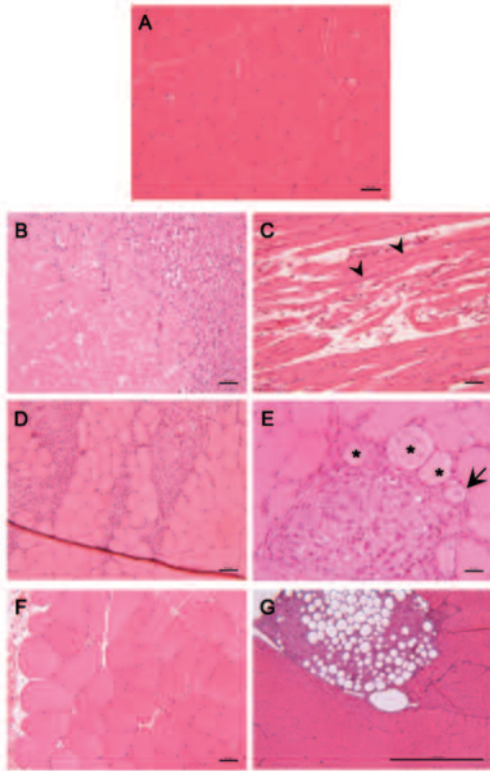
In ischemic muscle treated with PBS, we observed large areas of compact necrotic tissue surmounted by an inflammatory infiltrate rich in polymorphonuclear cells (Figure 3B). Additional findings were fibers with internal nuclei that, in longitudinal sections, appeared as nuclear chains (Figure 3C).

In ischemic muscle with untreated Lipogems-derived hASCs (CTR CELLS), we noted a fibrovascular thickening of the perimysium that dissociated the adjacent muscular fascicles (Figure 3D). In the contact area, mixed aspects of regeneration and degeneration, i.e., polymorphic, variously-sized, round muscle fibers with internal nuclei having light stained chromatin and pale pink cytoplasm, were seen (Figure 3E).



**Figure 2.** Muscle fiber evaluation. A, Morphometric analysis of muscle fiber diameters (25X magnification, 150 fibers on randomly selected fields). The graph shows the statistical differences among groups. Each value represents the mean  $\pm$  SEM of separate experiments (n=4). \*  $p < 0.05$  vs. CTR, #  $p < 0.05$  vs. PBS, §  $p < 0.05$  vs. CTR CELLS, ç  $p < 0.05$  vs. H+B+R CELLS. B-E, Histopathological micrographs of cross-sections of muscle tissue stained with H&E. The examination reveals (B) pale, necrotic fibers (black arrowheads), (C) myophagocytosis (\*), (D) ghost fibers (arrows), and (E) moth eaten fibers (red arrowheads). Scale bars = 50  $\mu$ m.

**Abbreviations:** CTR, non-ischemic limb tissue; PBS, phosphate buffered saline; CTR CELLS, control cells; H+B+R CELLS, cells treated with a mixture of hyaluronic, butyric and retinoic acids; LG, Lipogems.



**Figure 3.** Morphological evaluation of ischemic limb tissues. A, CTR tissue. B, Necrotic area with intense inflammatory reaction in PBS group. C, In longitudinal sections, internal nuclei look like nuclear chains (arrowheads). D, Perimysial fibro-vascular septa dissociating muscle fascicles. E, Regeneration (\*) and degeneration aspects of fibers muscle (arrow). F, Split fibers, and small sized fibers yielding dimensional polymorphism in H+B+R CELLS group. G, LG lying on the collating primary fibers. Scale bar = 1 mm. Representative images of separate experiments (n=4). A-F scale bars = 50  $\mu$ m. Abbreviations, as in Figure 2.

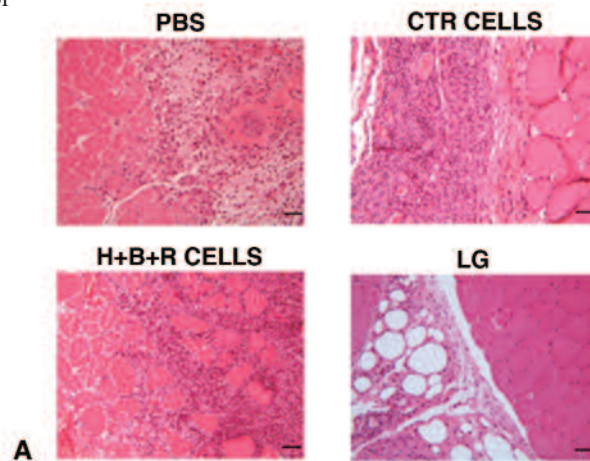
In ischemic muscles of the remaining groups, necrotic areas were smaller and associated with presence of round, large, split fibers, and small sized fibers (Figure 3F). In muscle tissues of the LG group, Lipogems was clearly visible on the external side of the collating primary fibers (Figure 3G).

**INFLAMMATORY REACTION**

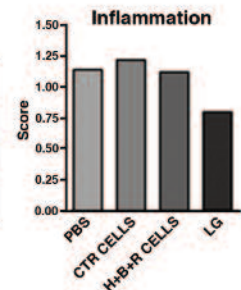
The degree of inflammatory process was evaluated scoring the entire sections of ischemic muscle tissues. Lipogems transplantation reduced the inflammatory infiltrate while, the groups with cells, treated or not with H+B+R, showed an overall degree of inflammation similar to that of the PBS group (Figure 4).

**ARTERIOGENESIS**

Histological signs of arteriogenesis were identified by the presence of ASMA positive cells (Figure 5A). The quantitative analysis revealed that arteriolar density was significantly increased in all ischemic groups, as compared to the non-ischemic contralateral tissue (CTR group). In particular, Lipogems-induced arteriogenesis significantly exceeded the response elicited by hASCs that had been cultured in the absence or presence of H+B+R (Figure 5B).



Score	Degree of cell infiltration
0	No inflammatory infiltration
1	Inflammatory infiltration in correspondence of necrosis and/or close to the site of cell or LG injection
2	Inflammatory infiltration in apparently healthy tissue distant from necrosis and/or site of cell or LG injection



**Figure 4.** Inflammatory process in ischemic muscle tissues. A, Representative histological images of inflammatory infiltrate seen in the different animal groups. Scale bars = 50  $\mu$ m. B, The analysis of inflammatory infiltrate was performed in each group by attributing a score from 0 to 2 according to the degree of cell infiltration.



VASCULOGENIC PATTERNING FROM LIPOGEMS-DERIVED hASCs CAN BE ENHANCED IN VITRO BY A MIXTURE OF NATURALLY OCCURRING MOLECULES

Real-time PCR confirmed that stem cell preconditioning with the H+B+R mixture modulates the transcription of genes that play a crucial role in the orchestration of angiogenic signals. Cell exposure to H+B+R for 1-6 days remarkably increased the expression of VEGF (Figure 6A), HGF (Figure 6B), and KDR (Figure 6C), encoding a major VEGF receptor, as previously shown<sup>12</sup>. Here we show that gene expression is associated with an increased secretion of the respective cytokines, starting at 24 hours, and maintaining higher than untreated cells until 6 days (Figure 6 D,E).

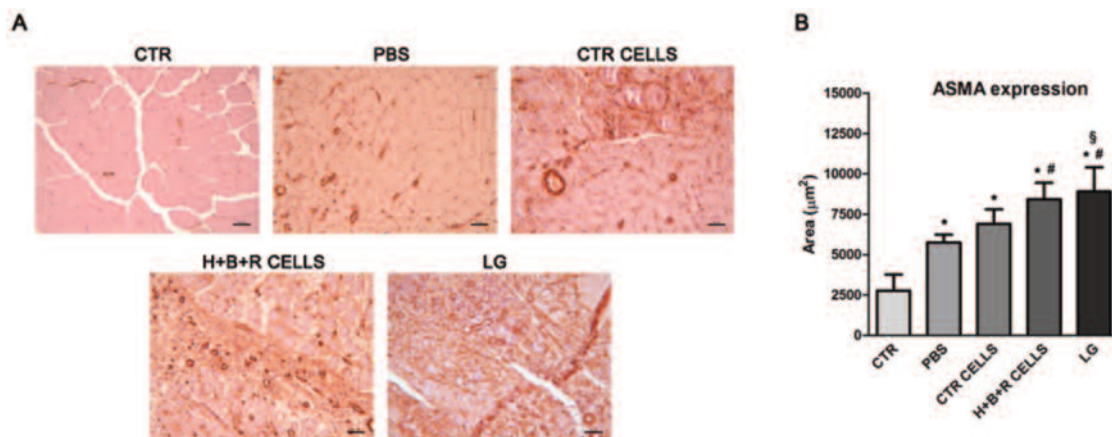
THE LIPOGEMS PRODUCT INCREASES HUVEC PROLIFERATION BY PARACRINE SECRETION

To assess whether the Lipogems product secretes proangiogenic/prosurvival cytokines able to increase tissue regeneration by endothelial cell proliferation, we employed transwell cell culture chamber inserts to restrict interactions between cell types to soluble factors. HUVECs were cultured in a basal medium with 2% FBS, but without any of the factors they need to survive and proliferate. After a week of coculture with untreated Lipogems-derived hASCs, only a few HUVECs survived (Figure 7A). HUVEC number was higher when they were cocultured with hASCs preconditioned for 3 days with the H+B+R mixture (Figure 7B), but it dramatically increased

when the Lipogems product was cocultured with them (Figure 7C,D). This result evidences that Lipogems product secretes soluble factors that stimulate HUVEC survival and proliferation.

DISCUSSION

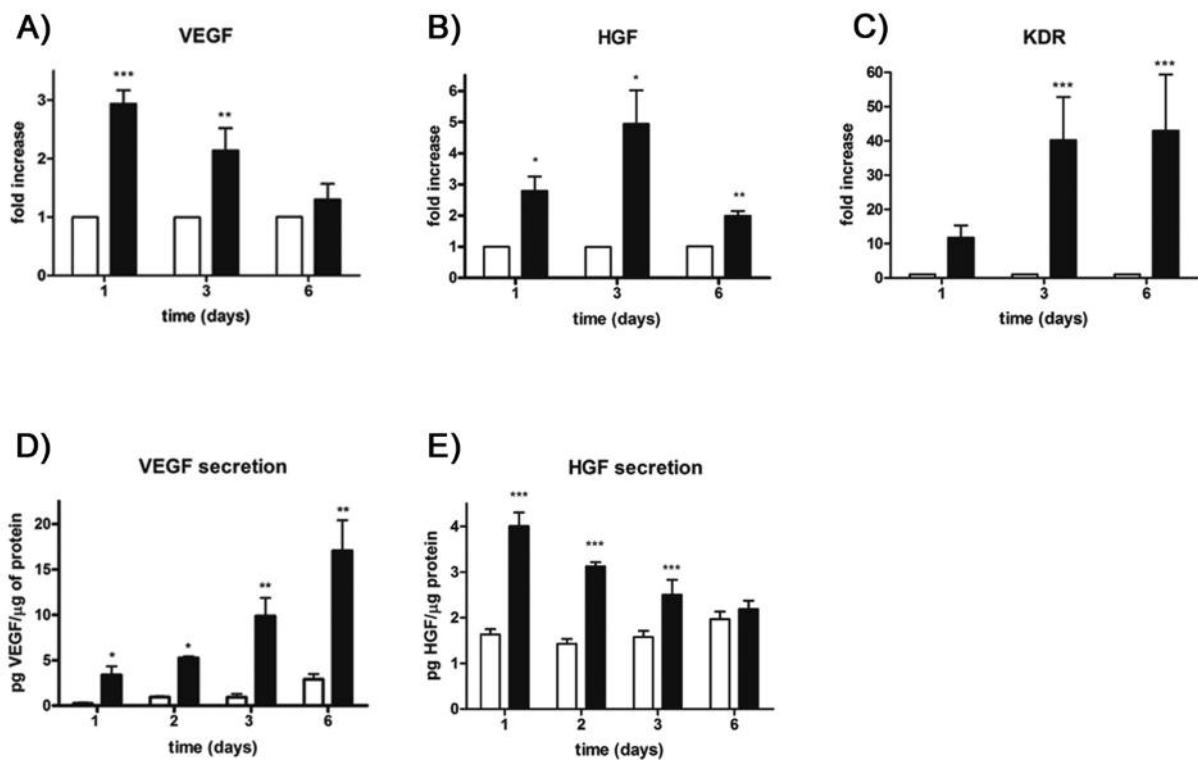
The present study shows that in a rat model of chronic hind limb ischemia xenogeneic transplantation of human non-expanded Lipogems product was capable to elicit a significantly higher perfusion recovery than that induced by expanded hASCs treated in the absence or presence of H+B+R. Despite the xenogeneic transplantation, there was no need for administration of immunosuppressant agents and even a reduction of inflammatory infiltration was evident at the immunohistochemical analysis in the Lipogems-transplanted tissue, as compared with all the other experimental groups. This observation suggests a remarkable immunomodulatory action of the Lipogems product and is the subject for ongoing mechanistic studies. The total number of injected hASCs ( $1 \times 10^6$  cells) was based upon our previous experience in hMSC transplantation in infarcted rats, and was consonant with the average number of transplanted hMSCs/hASCs in murine models of hind limb ischemia<sup>18,19</sup>. Given the optimization of the whole injection volume (500  $\mu$ l) with respect to the volume of recipient tissue, and considering the concentration limits imposed by the density of the Lipogems product, its injection could only provide a two-order of magnitude lower number of cells ( $2 \times 10^4$ ), as compared with number of injected hASCs.



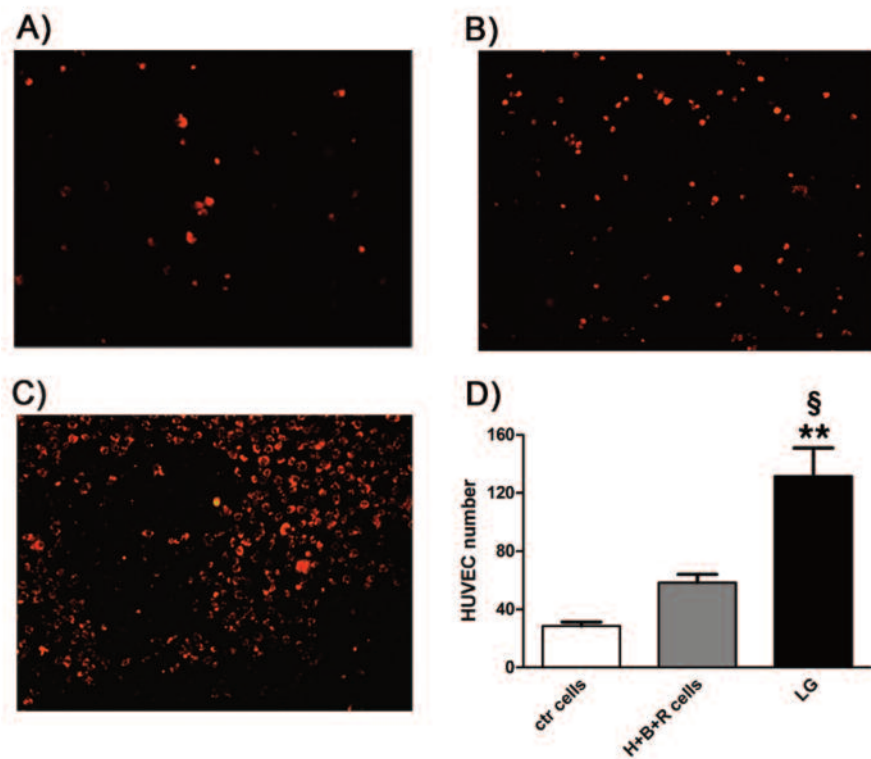
**Figure 5.** Arteriogenesis in ischemic muscle tissues. *A*, Immunohistochemical staining of arterioles was identified by the ASMA antigen. Scale bars = 50  $\mu\text{m}$ . *B*, Quantification of the total area of ASMA expression. The bar graph showed statistical difference between groups. Representative images of separate experiments ( $n=3$ ). Values are means  $\pm$  SEM. \* $p < 0.05$  vs. CTR, # $p < 0.05$  vs. PBS, § $p < 0.05$  vs. H+B+R CELLS.

**Abbreviations:** CTR, non-ischemic limb tissue; PBS, phosphate buffered saline; CTR CELLS, control cells; H+B+R CELLS, cells treated with a mixture of hyaluronic, butyric and retinoic acids; LG, Lipogems.





**Figure 6.** Enhanced angiogenic gene expression and secretion in H+B+R treated hASCs. Cells were cultured in the absence (white bar) or presence (black bar) of H+B+R for 1-6 days. The expression of genes was assessed by real time PCR. The quantity of each mRNA in control cells was defined as 1, and the amounts of VEGF (A), HGF (B), and KDR (C) mRNA from treated cells are plotted relative to that value. Time-course analysis of VEGF (D) and HGF (E) secretion was assessed by ELISA. Values are means  $\pm$  SEM of separate experiments (n=3). \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001.



**Figure 7.** Lipogems exerted paracrine properties *in vitro*. Seven days after setting up the cocultures, images of HUVECs (red, seeded in the bottom of the well plates) were taken. Into the transwell inserts were: control Lipogems-derived hASCs (ctr cells) (A), treated Lipogems-derived hASCs (H+B+R cells) (B), or the Lipogems product (LG) (C). Original magnification, 40X. D, Quantification of HUVEC number (\*\* $p$  < 0.01 vs. ctr cells; § $p$  < 0.05 vs. H+B+R cells).

Nevertheless, the tissue rescue observed following the injection of the Lipogems product proved to be superior to the recovery induced by control expanded cells, and even higher than the repair promoted by Lipogems-derived hASCs that had been exposed to H+B+R. This observation clearly shows that the chance for efficient cell therapy is not solely dependent upon the number of injected stem cells, and prompts further studies to dissect the mechanism(s) underlying the rescuing ability of Lipogems.

Of particular interest is the observation that in the ischemic limbs the transplanted Lipogems did not extend beyond the boundaries of the external side of the collating primary fibers. However, Lipogems-induced tissue repair involved a remarkably better preservation of muscular fiber size than did both untreated and hASCs pretreated with H+B+R. This finding indicates that the action of the Lipogems product may have involved the secretion of trophic mediators delivering instructive messages that may help creating a more compliant “regenerative environment” within the recipient tissue, as compared with the paracrine action induced by expanded cells. Considering the Lipogems product as a fluid tissue harboring remarkable paracrine properties is supported by the ability of Lipogems-derived hASCs to respond to a vasculogenic mixture with a transcriptional increase in VEGF, HGF, and KDR, which was associated with an early and long-lasting secretion of the encoded cytokines. The paracrine features of Lipogems are further inferred from the results of coculture experiments in the presence of HUVECs, showing the capability of the Lipogems product to enhance endothelial cell proliferation at a remarkably higher extent than control hASCs or hASCs pretreated with H+B+R. We have previously shown that, compared to the unprocessed lipoaspirate, the Lipogems product retained an intact stromal vascular niche, embedding elements with pericyte and mesenchymal stem cell characteristics<sup>12</sup>. Both pericytes enveloping microvessels and adventitial cells surrounding larger arteries and veins have been described as possible MSC forerunners. In particular, it has been described that hMSCs originate from two types of perivascular cells, namely pericytes and adventitial cells, and contain the *in situ* counterpart of MSCs in developing and adult human organs, which can be prospectively purified using well defined cell surface markers<sup>20-22</sup>. We have also shown that the

Lipogems product, besides expressing CD90<sup>+</sup>/CD29<sup>+</sup>/CD34<sup>-</sup> elements, unambiguously identifying a hMSC population, also encompassed cells exhibiting a CD146<sup>+</sup>/CD90<sup>+</sup>/CD34<sup>-</sup> pattern<sup>12</sup>, identifying cells with pericyte identity<sup>23</sup>, as well as a consistent percentage of CD146<sup>+</sup>/CD34<sup>+</sup>. This expression pattern has been found to identify a pericyte subset that may be transitional between pericytes and supra-adventitial adipose stromal cells, and/or a set of endothelial (progenitor) cells<sup>24,25</sup>. Now that MSCs have been shown to be perivascular (pericytes) *in vivo*, there is increasing evidence that within an injured tissue MSCs are released from their perivascular location, become activated, and establish a regenerative microenvironment by secreting bioactive molecules<sup>22</sup>. Moreover, we may hypothesize that pericytes/hMSCs in the Lipogems product may reside within a highly responsive niche, that makes its embedded cells particularly able to sense the cues emerging from the recipient injured tissue.

Further dissection of the sequelae of events elicited by Lipogems *in vitro* and *in vivo* may provide novel clues on the mechanisms by which pericytes/hMSCs residing within this product may act to promote a regenerative environment.

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#### CONFLICT OF INTEREST

Carlo Tremolada has invented and patented the Lipogems device (PCT/IB2011/052204).

The other Authors declare no conflict of interest

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