CHEMICAL-PHYSICAL PROPERTIES AND IN VITRO CELL CULTURING OF A NOVEL BIPHASIC BIO-MIMETIC SCAFFOLD FOR OSTEO-CHONDRAL TISSUE REGENERATION

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The requirements for a successful regeneration of an osteo-chondral defect could effectively be met by using a bi-layered composite scaffold, able to support proliferation and differentiation of mesenchymal stem cells, while providing a biochemical environment promoting the formations of the two distinct tissues. The novel strategy here presented consists of developing a bio-mimetic scaffolds obtained by the combination of two integrated organic compounds (type I collagen and chitosan) with or without bioactive Mg-doped hydroxyapatite (Mg-HA) nanoparticles, depending on the specific layer, reproducing cartilaginous or subchondral bone tissue. An innovative patented methodology for scaffolds production, called “pH-dependent 3-phasic assembling”, allowed to development of a highly homogenous and chemically stable scaffold, presenting a very good integration among all three components, as confirmed by extensive SEM and thermogravimetric analyses. A preliminary in vitro evaluation was also carried out by seeding bi-layered scaffold with human bone narrow stromal cells (h-MSCs), by giving particular emphasis to cell viability and distribution at day 0, 7 and 14. Cells were viable and uniformly colonized the whole scaffold until day 14, indicating that the scaffold contributed to the maintenance of cell behaviour.

ELECTRICALLY CONDUCTIVE SURFACE MODIFICATIONS OF THREE-DIMENSIONAL POLYPROPYLENE FUMARATE SCAFFOLDS

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Polypropylene fumarate (PPF) scaffolds fabricated by rapid prototyping were surface modified by solution deposition of electrically conductive polypyrrole coatings with or without hydroxyapatite. Scaffolds were electrically conductive with resistivity as low as 2\(\Omega\). Scaffold characterization by Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy and thermo gravimetric analysis shows both polypyrrole and hydroxyapatite are present. Cell viability, attachment, proliferation, and differentiation were analyzed using human fetal osteoblast cells. These studies show that surface modification using hydroxyapatite improved cell attachment and proliferation of osteoblasts onto the PPF scaffolds. Alkaline phosphatase activity as a marker for osteogenic differentiation of cell to mature osteoblasts was analyzed. Our data reveal that osteoblasts maintained their phenotype on PPF scaffolds with and without coatings. Thus, these scaffolds could be appropriate candidates for our future in vivo studies.
In the next few years research on stem cells is expected to redefine the boundaries of regenerative medicine, and possibly offer alternative therapies for a variety of diseases, including heart failure, Parkinson's disease, amyotrophic lateral sclerosis, spinal cord injury, burns, and diabetes. Among different stem cell types, mesenchymal cells (MSCs) are the most studied, as they are an easily available cell source and possess a rather good self-renewal capacity in vitro (1). Nonetheless, the debate on how to identify "real" MSCs is still ongoing, as no unique markers for these cells have been so far identified (2). Interestingly MSCs, which were initially found only in the bone marrow (BMSCs), can be now isolated from several sources, including adipose tissue (ASCs, Adipose-derived Stem cells) (3, 4). These progenitor cells possess a great therapeutic potential for tissue repair for their remarkable "plasticity", that is the capability of differentiating into a variety of different cell types. Actually, ASCs may represent a valuable alternative to BMSCs, as they are abundantly available from lipoaspirates and can be harvested with minimal morbidity. However, there are still some issues that need to be properly addressed before MSCs can be safely used for human therapy. One problem, which has been often underestimated, is that the number of MSCs that actually differentiate into the desired phenotype, upon proper chemical and/or physical stimulation, is often low, especially in the case of skeletal or cardiac muscles (3, 5). Furthermore, there is little or no evidence in the literature about the fate of those cells remaining undifferentiated (or undesirably differentiated), which may pose critical safety issues when MSCs would be employed in cell therapy (6). However, the possibility of MSCs degeneration into tumors seems to be quite remote, although somewhat controversial (7). Thus, finding new ways to increase the differentiation potential of MSCs is still a crucial matter. Along this line, an original approach has been proposed by P.G. Shultz and S. Ding (8), who synthesized a purine derivative, reversine, capable of increasing the plasticity of several adult cells (9-14) by inducing their de-differentiation. In the current study we tested reversine effects on both BMSCs and ASCs to increase their differentiation toward osteoblasts, smooth and skeletal muscle cells. Reversine pre-treatment, at very low concentration (50 nM), caused a marked increase in the differentiation yields of both BMSCs and ASCs.

**REVERSINE INCREASES MULTIPOTENT HUMAN MESENCHYMAL CELLS DIFFERENTIATION POTENTIAL**

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Among different human stem cell sources, adult mesenchymal stem cells from bone marrow (BMSCs), and more recently from adipose tissues (ASCs), have shown their capability to differentiate into a variety of different cell types, including osteoblasts, adipocytes, and muscle cells. However, mesenchymal stem cell differentiation toward certain cell types (including skeletal and cardiac muscle), while shown to be achievable, still suffers of low yields and needs to be greatly improved before any therapeutic application could be foreseen. A possible way of achieving this goal is by using a chemical-pharmacological approach to increase stem cell plasticity. Along this line, we envisioned the possibility of pre-treating BMSCs and ASCs with reversine, a synthetic purine that has been shown to induce adult cells de-differentiation. In the current study we tested reversine effects on both BMSCs and ASCs to increase their differentiation toward osteoblasts, smooth and skeletal muscle cells. Reversine pre-treatment, at very low concentration (50 nM), caused a marked increase in the differentiation yields of both BMSCs and ASCs.
ENHANCED BIOLOGICAL PERFORMANCE OF HUMAN ADIPOSE-DERIVED STEM CELLS CULTURED ON TITANIUM-BASED BIOMATERIALS AND SILICON CARBIDE SHEETS FOR ORTHOPAEDIC APPLICATIONS

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It is well known that the surface properties of biomaterials may affect bone-healing processes by modulating both cell viability and osteogenic differentiation. In this study we evaluated proliferation and osteogenic differentiation of human adipose-derived stem cells (hASCs) cultured on three prototypes of titanium disks and on thin layers of silicon carbide (SiC-PECVD), a material characterized by a high hardness and wear-resistance. Our data indicated that all the tested surfaces supported cell growth, in particular, hASCs seeded on both titanium treated by a double-step etching process (TIT) and titanium modified by two Anodic Spark Deposition processes (TAA) grew better respect to the ones cultured on titanium obtained by KOH alkali etching process on TAA (TAAK). Furthermore, hASCs well colonized SiC-PECVD surface, showing a quite similar viability to cells cultured on plastic (PA). TIT and TAA better supported osteogenic differentiation of hASCs compared to PA, as shown by a marked increase of both alkaline phosphatase activity and calcified extracellular matrix deposition; in contrast TAAK did not positively affect hASCs differentiation. SiC-PECVD did not alter osteogenic differentiation of hASC cells: indeed, ALP and calcium deposition levels were comparable to those of cells cultured on plastic. Furthermore, we observed similar results testing hASCs either pre-differentiated for 14 days in osteogenic medium or directly differentiated on biomaterials. Our study suggests that modifications of titanium surface may improve osteo-integration of implant devices and that SiC-PECVD may represent a valid alternative for the coating of prosthetic devices to reduce wear and metallosis events.
In recent years the discovery of resident cardiac progenitor cells (CPCs) in the adult heart has provided conclusive proofs against the paradigm of the heart as a post-mitotic organ (1-3). These cells represent a promising tool towards the clinical translation of autologous cardiac cell therapy for heart diseases. In fact, despite many preclinical and clinical models have assessed the therapeutic potential of stem/progenitor cells of extra-cardiac origin in post-ischemic heart diseases, the elicited temporary functional beneficial effects have

THROMBIN AND THROMBIN-DERIVED PEPTIDES PROMOTE PROLIFERATION OF CARDIAC PROGENITOR CELLS IN THE FORM OF CARDIOSPHERES WITHOUT AFFECTING THEIR DIFFERENTIATION POTENTIAL

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Many studies demonstrated that human adult cardiac progenitor cells in the form of cardiospheres (CSps) could represent a powerful candidate for cardiac cell therapy. To achieve the clinical translation of this biotechnological product, the development of well-defined culture conditions is required to optimize their proliferation and differentiation. Thrombin, a serine protease acting through the protease-activated receptor 1 (PAR-1) signalling to modulate many cellular functions such as proliferation and differentiation in several cell types, is one of the factors included in the CSps medium. Therefore, the assessment of the effective dependence of the thrombin-related cellular effects from PAR-signalling is strategic both for understanding the biological potential of these cells and for the GMP translation of the medium formulation, using synthesised analogs. In this study the effects of thrombin on human CSps and their potential relationship with the specific proteolytic activation of PAR-1 have been investigated in different culture conditions, including thrombin inhibitor hirudin and PAR-1 agonist/antagonist peptides TFLLR and MUMB2. In this study we show that, in the presence of thrombin and TFLLR, CSps, in which PAR-1 expression was evidenced by immunofluorescence and western blot analysis, increase their proliferative activity (BrdU assay). Such increased proliferative rate was consistently associated with a higher phosphorylation level of the cell cycle inhibitor GSK3. Concerning the assessment of the potential effects of thrombin and its agonist on differentiation, both western blot and real-time PCR analysis for “stemness”, cardiac and vascular markers (such as cKit, cx43 and KDR) showed that CSps commitment was substantially unaffected, except for GATA4 mRNA, whose transcription was down-regulated in the presence of the natural protease, but not after treatment with TFLLR. In conclusion, activation of PAR-1-dependent signalling is important to support CSps proliferative potential, keeping unaltered or at best stable their differentiation properties. The availability of thrombin agonists, such as TFLLR, able to guaranty the required growth effect without affecting CSps lineage commitment, could represent a technological improvement for cost-effective, easy-to-handle and GMP-translatable synthetic media.

Key words: thrombin, cardiac progenitor cells, protease activated-receptor 1, cardiospheres, cell therapy

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EVALUATION OF CHONDROCYTE BEHAVIOR IN A NEW EQUINE COLLAGEN SCAFFOLD USEFUL FOR CARTILAGE REPAIR

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Association of biomaterials with autologous cells can provide a new generation of implantable devices for cartilage repair. An ideal scaffold should possess a preformed three-dimensional shape, fix the cells to the damaged area and prevent their migration into the articular cavity. Furthermore, the constructs should have sufficient mechanical strength to facilitate handling in a clinical setting and stimulate the uniform spreading of cells and a phenotype re-differentiation process. The aim of this study was to verify the ability of an equine collagen membrane to support the growth of human chondrocytes and to allow the re-expression of their original phenotype. This ability was assessed by the evaluation of collagen type I, II and aggrecan mRNA expression by Real-Time PCR. Immunohistochemical analyses were performed to evaluate collagen type I, II and proteoglycans synthesis. Electron microscopy was utilized to highlight the structure of the biomaterial and its interactions with the cells. Our data indicate that human chondrocytes seeded onto a collagen membrane express and produce collagen type II and aggrecan and downregulate the production of collagen type I during the experimental times analyzed. These results provide an in vitro demonstration for the therapeutic potential of autologous chondrocyte transplantation by an equine collagen membrane as a delivery vehicle in a tissue-engineered approach towards the repair of articular cartilage defects.

BIODEGRADABLE MICROCARRIERS AS CELL DELIVERY VEHICLE FOR IN VIVO TRANSPLANTATION AND MAGNETIC RESONANCE MONITORING

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Microcarrier culture systems offer an attractive method for cell amplification and as delivery vehicle. At the same time, super paramagnetic iron oxide (SPIO) nanoparticles represent a unique in vivo tracking system, already approved for clinical use. In our study, we tested the combination of clinically approved microcarriers and SPIO nanoparticles for cell-construct delivery and subsequent tracking after implantation. In order to mimic better a clinical setting, biodegradable macroporous microcarriers were employed as an alternative approach to expand human primary chondrocytes in a dynamic culture system for subsequent direct transplantation. In addition, cell-seeded microcarriers were labeled with SPIO nanoparticles to evaluate the benefits of cell-constructs tracking with magnetic resonance. In vivo subcutaneous implants were monitored for up to 3 weeks and orthotopic implantation was simulated and monitored in ex vivo osteochondral defects.
Tendon injuries, in particular superficial digital flexor tendon lesions, are common clinical problems in racing horses due to the poor regeneration capability of tendon tissue with an incidence of 43% in Thoroughbred (1, 2, 3). Injured tendons heal slowly and frequently they incur in scar tissue and adhesion formation. The poor tendon regeneration after damages is related to their diminished blood supply and cell content (2, 4). Moreover, during the healing period, cells within the tendon produce excess of collagen type III instead of typical collagen type I produced in healthy tendon (5). Standard treatments are conservative and tendons require long time to heal with high risk of re-injury during athletic performances (6). In the last few years, strategies to improve tendon regeneration have focused on injecting bone marrow derived progenitor cells and growth factors (7, 8, 9). Mesenchymal stem cells are multipotent precursor cells of connective tissues and play an important role in orthopaedic disease treatments both in animal and human beings (10, 11). They can be isolated from different tissues and organs (9, 12, 13, 14, 15, 16). Bone marrow has long been considered the gold standard adult stem cell source in regenerative medicine. Bone marrow mesenchymal stem cells (BM-MSCs) have been extensively investigated, becoming the reference point for comparison studies with other stem cell sources (17). Traditionally, tendons were thought to consist of tenocytes that repair tendon tissue when damaged. However, population of adult stem cells, called tendon stem/progenitor cells (TSPCs) were recently identified in human and mouse tendons and in just one study on horse (18, 19, 20, 21). To date, most published studies have used BM-MSCs for tendon repair reporting promising results.

CHARACTERIZATION AND DIFFERENTIATION OF EQUINE TENDON-DERIVED PROGENITOR CELLS

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Mesenchymal stem cells have been recently investigated for their potential use in regenerative medicine. Population of adult stem cells were recently identified in human and lab animal tendons, but no detailed investigations have been made in the equine species. The aim of our study is to identify a progenitor cell population from tendon tissue (TSPCs) in the horse superficial digital flexor tendon that are able to be highly clonogenic, to grow fast and to differentiate in different induced cell lineages as well as bone marrow derived progenitor cells (BM-MSCs). The hypothesis that TSPCs possess a mesenchymal stem cell behavior opens a new prospective for tendon regenerative medicine approaches. TSPCs were expanded more rapidly and showed higher plating efficiency when compared with BM-MSCs. Both cell lines expressed identical stem cell markers in vitro and they were able to differentiate towards osteogenic and adipogenic lineages as demonstrated with cytochemical staining and mRNA gene expression. TSPCs showed a positive but limited chondrogenic differentiation compared with BM-MSCs as demonstrated by histological and biochemical analyses. According to our results, equine TSPCs have high clonogenic properties and proliferating potential, they express stem cell markers and have the capability to be multipotent as well as BM-MSCs. These findings suggest that TSPCs may represent a good model for stem cell biology and could be useful for future tendon regenerative medicine investigations.

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