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Biological Baseline of Joint Self-Repair Procedures

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Abstract: Gel-Repairer is a biomaterial composed of Polydeoxyribonucleotides (Pdrn), Heat Shock Proteins (Hsps) and a thickening substance. It works as a local mesenchymal stem cells (MSCs) stimulator, finally generating connective tissue renewal.

Our research is within the field of regenerative medicine and has historically built its foundation from the studies carried out on non-vital amnion and placental membranes.

Our end point is the activation and stimulation of the local mesenchymal stem cells (MSCs) for the structural recovery of the joint involved in the degenerative process.

Since 2003, we have been applying the Gel Repairer over morethan 1200 patients, most of them elderly, affected by Degenerative Joint Disease (DJD). After 10 years of clinical experience, the results are really impressive, including the absence of toxicity, adverse reactions or side effects.

Our clinical findings allowed the presentation of a clinical preliminary study performed on a large group of patients from 2003 to 2009 and recently published [1].

The following article is aimed at looking into the mechanism of action of the Joint Self-Repair procedure; furthermore some new technical opportunities are presented on tissue engineering advances in this fast evolving sector.

Keywords: Regenerative medicine, Polydeoxyribonucleotides (Pdrn), Heat Shock Proteins (Hsps), Growth Factors (GFs), Joint Stem-Cells, Mesenchymal Stem-Cells (MSCs), self-repair, tissue engineering, scaffolds, tissue renewal and Degenerative Joint Disease (DJD).

INTRODUCTION

In the field of biology, regeneration is the progression of renewal that makes it possible for genomes, cells, organs, organisms to be resilient to natural changes or events that cause damage or disturbance. Regeneration is mediated by the molecular processes of DNA synthesis. Every species is capable of regeneration, from the simplest organisms such as bacteria to those as complex as humans. However, regeneration in biology mainly means the morphogenic processes that differentiate the phenotypic plasticity of cellular traits allowing multi-cellular organisms to repair and maintain the stability of physiologic and morphologic states [2].

Regenerative medicine investigates the processes of replacing or regenerating human cells, tissues or organs to restore or establish normal function. This field holds the promise of regenerating damaged tissues and organs in the body by replacing damaged tissues and/or by stimulating the body's own repair mechanisms to heal previously irreparable tissues or organs.

The term "Regenerative Medicine" was first introduced in 1992 by Leland Kaiser, who intended regenerative medicine as ‘‘a new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems” [3, 4].

Regenerative Medicine refers to a group of biomedical approaches to clinical therapies that may involve the use of stem cells or progenitor cells (cell therapies); the induction of regeneration by biologically active molecules administered alone or as a secretion by infused cells or their by-products (immunomodulation therapy); and transplantation of *in vitro* grown organs and tissues (tissue engineering) [5-7].

Our research is focused on an innovative biomaterial (Gel-Repairer) composed of Polydeoxyribonucleotides, Hsps and a thickening, scaffolding substance, aimed at stimulating local-joint mesenchymal stem cells (MSCs) to finally generate connective tissue renewal.

Gel-Repairer plays its role in the extracellular microenvironment. This space is usually deemed to be outside the plasma membranes, and occupied by fluid or matrix.

The composition of the extracellular space includes metabolites, ions, various proteins and non-protein substances (i.e. DNA, RNA, lipids, microbial products etc.) that might affect cellular functions. For example, hormones, growth factors (GFs), cytokines and chemokines act by crossing the

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extracellular space towards biochemical receptors on cells. Other proteins that are active outside the cell are various enzymes, including digestive enzymes (Trypsin, Pepsin), extracellular proteinases (Matrix metalloproteinases, ADAMTSs, Cathepsins) and antioxidant enzymes (extracellular Superoxide dismutase). Often, proteins present in the extracellular space are stored outside the cells by attaching to various extracellular matrix components (Collagens, Proteoglycans, etc.). In addition, Extracellular matrix proteolytic products are also present in the extracellular space, especially in tissues undergoing remodelling [8].

EXTRACELLULAR MATRIX

Extracellular matrix (ECM) is a substance secreted by cells in the extracellular space. It is the main component of the human body. Collagen fiber - a basic component of extracellular matrix material - represents 25 to 30% of the total protein mass of our body. It serves to reinforce the body structure and plasma membranes so that we can withstand gravity and tension, and plays a key role for being an adhesive substance and a signalling molecule for cells. This material has many constituents such as Fibrous Components, Glycosaminoglycans, Proteoglycans and Glycoproteins [9, 10].

Materials: Gel-Repairer Composition and Properties

1) ***Polydeoxyribonucleotides*** **(Pdrn).** They have an average molecular weight of 350kd, and are heat resistant (can resist at 121C° for over 15').

Historical observations have shown that Pdrn has the ability to induce the formation of Platelet-Fibronectin complexes on wound sites [11, 12].

Nucleic acids, nucleosides, and nucleotides derived from cell fragmentation, physiologically diffuse into the extracellular environment as a result of cell lyses (following cellular death), possibly providing a local stimulus for tissue regeneration [13, 15].

Polydeoxyribonucleotides, introduced in the site of injury are subjected to enzymatic splitting. This generates nucleosides, nucleotides, purine and pyrimidinebases.

How they work:

- as cell activators, through the Purinergic System A2 receptors – P1 subclass-[11, 16-19];
- Increase the cell's reactivity to GFs action [15, 20];
- Stimulate the ECM turnover and renewal [21];
- Activate cellular proliferation [11, 17-19];
- Increase local micro-vascularization through VEGF [22, 23];

They trigger the energetic Salvage pathways for the neosynthesis of Nucleic Acids [21, 24, 25];

- Facilitating cell growth and protein synthesis;
- Energy saving metabolic pathways;
- Increasing speed of tissue regeneration compared to the metabolic pathways *ex novo*.

It has been demonstrated *in vitro* and *in vivo* that nucleotides and nucleosides act as growth promoters for fibroblasts, osteoblasts (Fig. **1** and **2**), endothelial cells and collagen matrix production [11, 20, 21, 26-33].

A very recent *in vitro* study investigated the effect of polydeoxyribonucleotides (Pdrn) on cartilage protection and degradation. Chondrocytes treated with Pdrn showed a physiological deposition of extracellular matrix. Pdrn was able to inhibit proteoglycan degradation in cartilage explants. In addition the activities of matrix metalloproteinases 2 and 9 were reduced in all Pdrn-treated samples. This research indicates that Pdrn issuitable fora long-term cultivation of *in vitro* cartilage and has therapeutic effects on chondrocytes by protecting cartilage [34].

It has also been demonstrated that nucleic acids, being diffused in the extracellular environment, act in synergy with different growth factors like PDGF, FGF, IGF,EGF, TGF-β, BMP (bone morphogenetic proteins) and cytokines on various cellular lineages. Furthermore, they have the ability to promote GFs production. Nucleic acids even influence immunologic responses [20, 25, 35-38].

2) ***Heat shock proteins*** (Hsps) are a class of functionally related proteins expression of which is increased when cells are exposed to elevated temperatures or other environmental stressors [39]. The mechanism by which "shock factors"

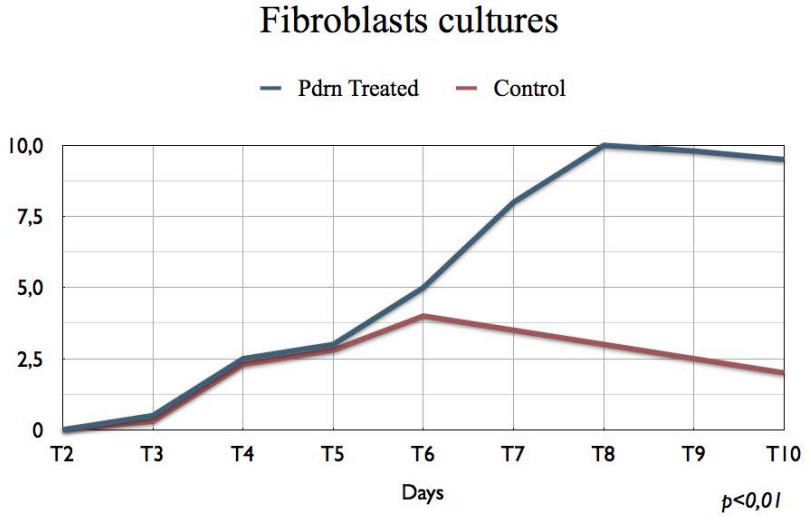

Fig. (1). Growth curves of primary cultures of human fibroblasts with and without Pdrn (n° of cells x 10^4).

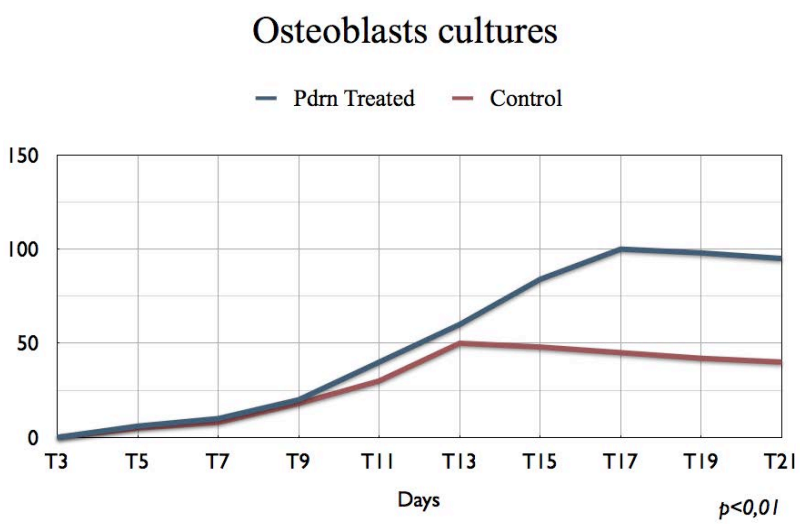

Fig. (2). Growth curves of primary cultures of human osteoblasts with and without Pdrn (n° of cells x 10^4).
*The graphs are developed with the data presented by Sapelli P. at the 2° world congress of osteogenesis and osseointegration (Rome 1996).

activate Hsps production has still not completely been determined. However, some studies suggest that an increase in damaged or abnormal proteins activates Hsps [40].

The functions of Hsps are well characterized in differentiated cells, whereas their role in stem cells remains unclear.

It has been demonstrated in differentiated cells, that Hsps function as molecular chaperones in the stabilization of intracellular proteins, repairing damaged proteins, and assisting in protein translocation [41, 42].

Hsps, in non-stressful conditions, help monitor old proteins to the cell's "recycling bin" and so promote newly synthesized proteins fold properly. This function is generally called "housekeeping".

Hspsalso play a role in cellular immunity. Extracellular matrix and membrane bound heat-shock proteins, especially Hsps70 are involved in binding antigens and presenting them to the immune system [43].

Stem cells exhibit an increased stress tolerance and concomitant high levels of some Hsps expression [44]. Wang observed that over expression of Hsp20 protected mesenchymal stem cells against cell death triggered by oxidative stress *in vitro.* The mechanisms contributing to the beneficial effects of Hsp20 were associated with enhanced Akt activation and increased secretion of growth factors such as VEGF, FGF-2, and IGF-1 [45].

Various kinds of stem cells (embryonic stem cells, adult stem cells, or induced pluripotent stem cells) have to maintain their stemness and, under certain circumstances, undergo stress. Hsps, mostly those from 8 to 20 Kd, seem to have an important influence on stem cells biology [46] as:

- they play important roles in maintaining stem cell dormancy, proliferation and differentiation;
- they have a protective function in the survival of the transplanted and local activated stem cells;
- they possibly regulate stem cell aging.

Gel-Repairer Production [Patent Pending]

Gel-repairer is prepared with distressed (low and high temperature) processed blood, Pdrn, and a thickening, scaffolding substance.

The processing blood method makes it sterile and devoid of antigenic potential. This biomaterial is aimed at taking advantage of the combining effects of Polydeoxyribonucleotides, Hsps and local GFs (trauma induced) on clusters of joint stem cells.

Mechanism of Action

The Gel-repairer procedure fosters tissue regeneration in which, the constituents of the Gel act, thanks to the biologic cascade of events that are triggered by the minimally invasive surgical trauma.

The surgical trauma operatesto trigger the process. Trauma necrosis induces modification in the biological microenvironment that is often capable of generating some basic reparative transformation. Products of necrosis and O2 deficiency lead to an increased production and activation of local GFs. Specific microenvironmental cues regulate self-renewal and differentiation capabilities.

Trauma necrosis and hypoxia cause a local increase of some important GFs, such as VEGF, FGF 1-2, TGF-β1. In fact, they can promote a rise in vascular permeability and endothelial cell proliferation, fibroblast chemotaxis, neoangiogenesis, and matrix deposition [47] (Fig. **3**).

TGF-β1 is involved in granulocyte, macrophage, lymphocyte, fibroblast, and smooth muscle cell chemotaxis. It is also implicated in tissue inhibitors of metalloproteinase synthesis (TIMPS) and matrix metalloproteinase production inhibition.

Most recently, TGF-β1 has been associated with regeneration of articular cartilage [48].

Furthermore, increasing local concentrations of basic fibroblast growth factor (bFGF), fibroblast growth factor 2

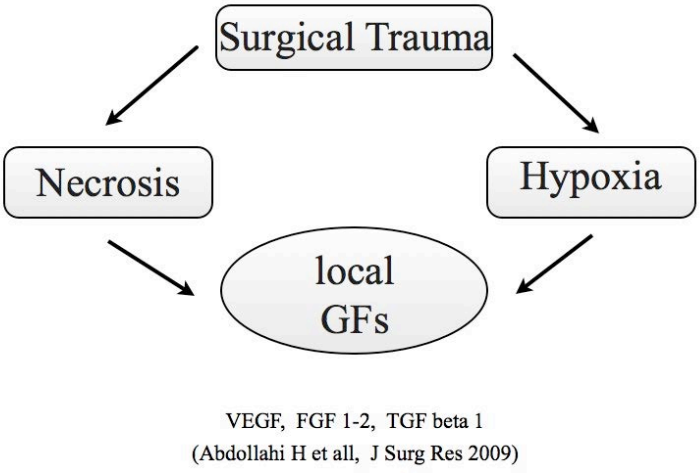

Fig. (3). Biological role of trauma necrosis and hypoxia.

(FGF2), has been associated with increased proliferation of mesenchymal stem cells [49].

A new hypoxia-inducible factor (HIF) modulator has recently been identified, that demonstrated effects over stem cell differentiation status. The biology of the alpha subunits of hypoxia-inducible factors (HIF-α) has expanded from their role in angiogenesis to their current position in the self-renewal and differentiation of stem cells [50].

For the Gel-repairer mechanism of action, we developed the following hypothesis, coherent with biochemical, *in vitro* and experimental research. This knowledge has been supported by clinical evidence when it has been applied to Degenerative Joint Disease (DJD). In this process:

- The Gel allows a prolonged stimulating action by Polydeoxyribonucleotides, Hsps and GFs on joint stem cells clusters;
- The Gel is introduced with minimally invasive surgery, which leads to a local traumatic necrosis, hypoxia and GFs response;
- This favourable microenvironment activates Gel's substances enhancing its performance;
- The Gel's conformation can act as a scaffold for the adherence and survival of stimulated stem cells.

In other words, we can create a long lasting regenerative microenvironment over local joint stem cells formed by a surgical pocket rich of necrosis products and deficient in oxygen. This conducive condition activates local reparative mechanisms through GFs such as VEGF, FGF1-2, TGF-beta 1, HIF-alpha, PDGF. The surgical pocket must be then filled with the Gel-Repairer, that is a concentrated mixture of Pdrn and Hsps, that works in unison with stress induced GFs (Figs. **4** and **5**).

Tissue Renewal Through Joint MSCs Clusters

The Gel-repairer procedure is aimed at clinically improving the articulation involved in DJD through the activation of resident MSCs, finally resulting in tissue regeneration.

Clusters of MSCs are located in different spots of the joint tissue. There are increasing reports that MSCs can be isolated from various adult mesenchymal tissues such as synovium, periosteum, skeletal muscle, and adipose tissue in addition to bone marrow. These MSCs have been assumed to be similar irrespective of their original tissue source since

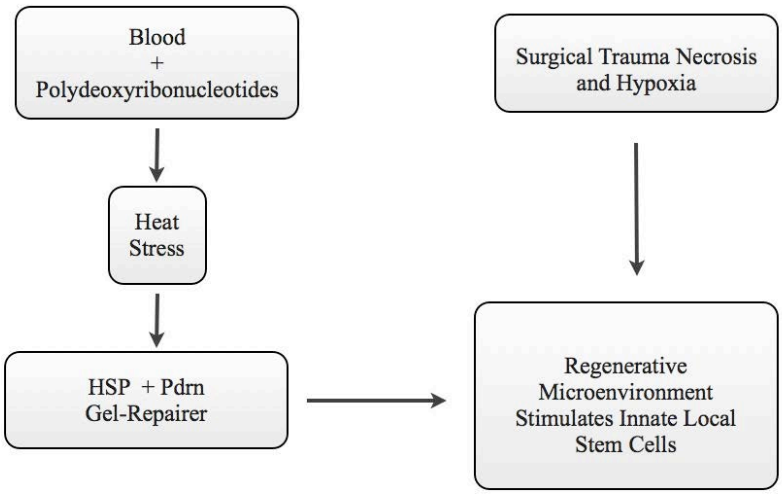

Fig. (4). Hypothesis about tissue regeneration mechanism.

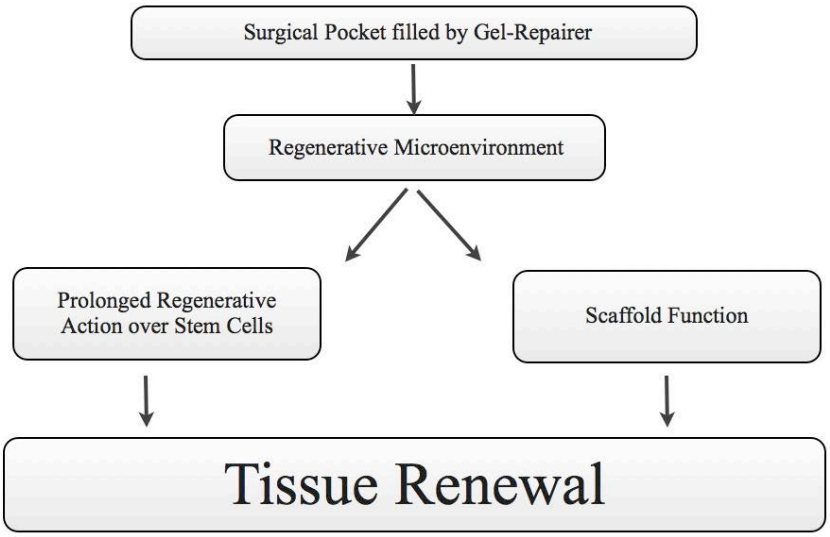

Fig. (4). Hypothesis about tissue regeneration mechanism.

they all have self-renewal and multi-differentiation potential with common surface epitopes (Fig. **6**).

The superiority of synovium as a potential source of MSCs for clinical applications has lately been claimed by some authors [51].

Recently, it has been picked out in the Hoffa's fat pad clonogenic cells that meet the criteria for MSCs and produce multipotent cultures that maintain their long-term chondrogenesis [52].

The synovial membrane is a tissue that lines the joint cavity of synovial joints and consists of a lining layer of macrophage-like (type A) and fibroblast-like (type B) synoviocytes and a loose sub-lining tissue. In the healthy joint, type A synoviocytes act as innate immunologic defence and support adaptive immunity, while type B synoviocytes regulate the release of nutrients and molecules, including hyaluronic acids, into the synovial fluid.

In response to injury of various types, including trauma, the synovial membrane rapidly becomes hyperplastic. It is commonly believed that synovial hyperplasia is sustained mainly by stromal cells including type B (fibroblast-like) synoviocytes, also called synovial fibroblasts, with infiltration of blood-borne inflammatory and immune cells, particularly in inflammatory joint diseases such as rheumatoid arthritis.

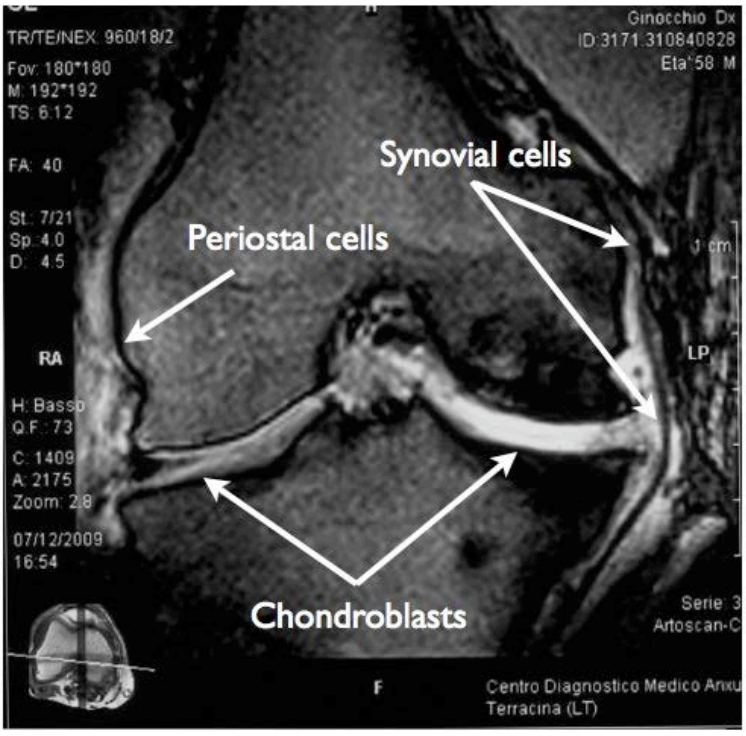

Fig. (6). Localization of joint MSCs clusters.

In vitro was found joint MSCs defined as fibroblast-like cells that undergo sustained growth and have the capacity to form mesenchymal tissues such as cartilage and bone.

In 2011, Kurth TB *et al.*, provided the first evidence of the existence, *in vivo*, of resident MSCs in the knee joint synovium that undergo proliferation and chondrogenic differentiation following injury *in vivo*. They found, within postnatal knee joint synovium, stromal stem cells with a phenotype are compatible with MSCs. These cells proliferated after articular cartilage injury and differentiated into chondrocytes in areas of cartilage metaplasia within the synovium [53].

Similar evidences were proved in entheses (i.e., insertion sites, osteotendinous junctions, osteoligamentous junctions) that are sites of stress concentration in the region where tendons and ligaments are attached to the bone. The ability of entheses for self-repair is emphasized in regenerative medicine through the PDGF stimulating procedure [54].

Gel Repairer Procedure and Tissue Renewal

The Gel-repairer exerts a structural repairing effect over tissues.

The hypothesis, as mentioned before, is that a prolonged action of proliferative and differentiating stimulus of Polydeoxyribonucleotides, Hsps and other local growth factors- trauma induced- on resident MSCs, together with a scaffold function that the Gel might have on the activated stem cells, produces a structural change of the joint.

The Gel is placed over the ligament bursa tissue with the aim of activating clusters of MSCs located in different spots of the joint tissue.

The activation and differentiation of MSCs are now evident in literature, and data are reported *in vitro* and in experimental studies. Lines of fibroblasts, osteoblasts and chondroblasts have been found in various conditions of joint MSCs stimulation. The process of cell differentiation generates new proteins such as collagen (I-II type), elast in and healthy ECM production.

Tissue modifications result in an increased flexibility of the articular-capsule and ligaments. An improvement of the compliance of the bursa leads to a reduction in the intra-articular pressure and, consequently, of pain. Another effect of the Gel probably occurs on the sub-periosteal cluster of MSCs, inducing proliferation and cartilage-bone repair (Fig. **7**).

Our Clinical Experience

From 2003 until 2009 we applied gel-repair procedure over 948 patients. Our research was focused on two groups of patients, both affected by Degenerative Joint Disease (DJD). The first one composed of over-eighties while the second group was of 45 to 55 year old patients. The first group had high surgical risk and both had been non-responders to currently adopted conservative therapies.

The first group was composed of 86 ultra-octogenarian with severe osteoarthritis (OA) of the hip and/or knee, and the second group was composed of 90 young patients (around fifty year old) affected by the same disease.

Treated patients have been clinically and radiologically evaluated with a follow-up of 6 to 48 months. Results show a statistically significant improvement in terms of pain and joint mobility, sometimes coupled with a clear radiological improvement. Follow-up shows encouraging data in terms of clinical stability over time. During the study we encountered virtually no side effects, adverse reactions or toxicity.

This study suggests a new methodological approach for treating and structurally improving articulations affected by DJD based on tissue regeneration and restoration resulting in clinical resolution [1].

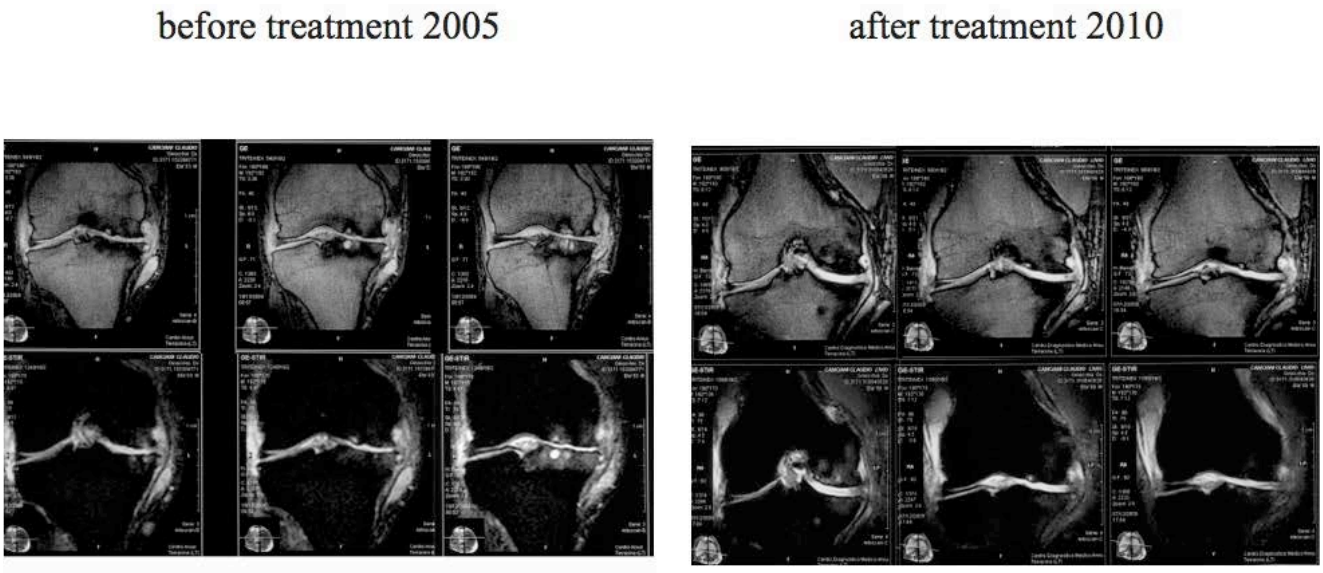

Fig. (7). Comparative MR of a 53-year-old patient before and after gel-repairer treatment. These images show the right knee of a man who underwent our procedure. Comparing MR imagery, they show evidence of the recovery of thickness in the soft tissue layer and great improvement over bone damage through recovering erosion and pseudo-cyst marrow.

DISCUSSION

Our clinical experience using nonviable amnios and Pdrn over DJD has led to the development of a new substance effective at stimulating innate articular stem-cells in the attempt to regenerate consumed tissue. We tried to create a long-lasting regenerative microenvironment over resident stem cells formed by a surgical pocket rich in necrosis products and deficient in oxygen. These conditions are useful for activating local reparative mechanisms through GFs and other biochemical activators (VEGF, FGF 1-2, TGF-beta1, HIF-alpha, PDGF). The surgical pocket was filled with gel-repairer that is a concentrated mixture of Hsps and Pdrn. This gel matrix, through its active components in unison with the increased concentration of local GFs, triggers the activation of resident MSCs, finally resulting in tissue regeneration.

Since their emergence in the mid-1980s, tissue engineering and regenerative medicine have continued to evolve as stimulating multidisciplinary fields aimed at developing biological substitutes to restore, replace or regenerate defective tissues. Cells, scaffolds and growth-stimulating signals are generally referred to as the key components of engineered tissues. In practice different approaches are focused to repair or replace portions or whole tissues (i.e., bone, cartilage, blood vessels, skin, muscle etc.).

There is a great debate about scaffold constitution and functions. Scaffolds in engineered tissues serve to imitate the ECM in native tissues, at least partially. Expectedly, their functions should mimic the ECM of the target tissue. In the recent years four major scaffolding approaches have been developed, namely by implanting cell-seeded pre-made porous scaffolds, cell-seeded decellularized allograft or xeno graft ECM or laminated cell sheets with secreted ECM, or injecting cell encapsulated self-assembled hydrogels. Each approach has its advantages and disadvantages and different tissue engineering applications [55].

Scaffolds, typically made of polymeric biomaterials, provide the structural support for cell attachment, surviving and subsequent tissue development.

Cells are often implanted, seeded or locally stimulated into an artificial structure capable of supporting three-dimensional tissue formation. These structures, called scaffolds, usually serve at least one of the following purposes:

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and local biochemical mediators
- Exert certain mechanical and biological influences to modify the behaviour of the stem cells proliferation and differentiation

To achieve the goal of tissue reconstruction, scaffolds must meet some specific requirements. A *high porosity* and an adequate pore size are necessary to facilitate cell seeding and diffusion throughout the whole structure of both cells and nutrients. *Biodegradability* is often an essential factor since scaffolds should preferably be absorbed by the surrounding tissues without the necessity of a surgical removal. The rate at which degradation occurs has to coincide as much as possible with the rate of tissue formation: this means that while cells are fabricating their own natural matrix structure around themselves, the scaffold is able to provide structural integrity within the body and eventually it will break down leaving the neo-tissue.

Many different materials (natural and synthetic, biodegradable and permanent) have been investigated. Most of these materials have been known for surgical applications before the advent of tissue engineering as a research topic, being already employed as bio-resorbablesutures.

New biomaterials have been engineered to have ideal properties and functional customization: injectability, synthetic manufacture, biocompatibility, non-immunogenicity, transparency, nano-scale fibers, low concentration, and resorption rates.

Ispolylactic acid (PLA)is a commonly used synthetic material. This is a polyester, which degrades within the human body to form lactic acid, a naturally occurring chemical which is easily removed from the body. Similar materials are polyglycolic acid (PGA) and polycaprolactone (PCL): their degradation mechanism is similar to that of PLA, but they exhibit a faster and a slower rate of degradation compared to PLA.

§Scaffolds may also be constructed from natural materials: in particular different derivatives of the extracellular matrix have been studied to evaluate their ability to support cell growth. Proteic materials, such as collagen or fibrin, and polysaccharidic materials, like chitosan or glycosaminoglycans (GAGs), have all proved suitable in terms of cell compatibility, but some issues with potential immunogenicity still remain. Among GAGs hyaluronic acid, alone or in combination with cross-linking agents, is one of the possible choices as scaffold material.

Another form of scaffold under investigation is decellularized tissue extracts whereby the remaining extracellular matrix acts as a natural scaffold. A recent proposal has been suggested: decellularized omentum as biological scaffold in regenerative surgery [56].

Hydrogels consist of elastic networks with interstitial spaces that contain as much as 90–99% of water. They are prepared by chemical polymerization or by physical self-assembly of man-made or naturally occurring building blocks. Most commonly, these building blocks are macromolecules in a variety of architectures, including cross-linked polymers, entangled fibrillar networks, or colloidal assemblies.

Nanobiomaterials such as bioactive hydrogels are designed to deliver biomolecules and instructive ligands at close proximity to the cell for better uptake or exposure. Biodegradable hydrogels provide transient scaffolding support for therapeutic cell settlement while gradually degrading in response to physical or enzymatic stimuli. In addition, biomechanical stimuli from hydrogels can induce mutual beneficial responses on cells [57, 58].

Scaffolding systems are a key factor to achieve tissue renewal. Concerning our procedure, the identification of the

appropriate scaffold might be the corner-stone knowledge to improve the joint self-repair procedure.

CONFLICT OF INTEREST

The authors declare that they have no financial or non-financial competing interests.

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ABBREVIATIONS

DJD	=	Degenerative Joint Disease
OA	=	Osteoarthritis
Pdrn	=	Polydeoxyribonucleotides
Hsps	=	Heat shock proteins
MSCs	=	Mesenchymal stem cells
GFs	=	Growth factors
ECM	=	Extracellular matrix.

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