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RESEARCH ARTICLE

Current Situation of Adipose-Derived Stem Cells Treatments for Knee Ostheoarthritis and Hypothesis for a New Protocol Based on Cryoconservation and Multiple Injections of Mechanically Fragmented Adipose Tissue Extracts

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ABSTRACT

Osteoarthritis (OA) is a widespread degenerative joint disease. The knee arthroplasty has represented for about 40 years the main treatment option, when pain and functional decrease became unbearable for the patient. The knee arthroplasty represents a challenging surgical operation, in which, based on current data available, about 20% of the patients are left totally or partially unsatisfied with the clinical result.

A recent therapeutic alternative for mild-to-moderate grade OA (Kellgren-Lawrence grade II to III) in order to delay (or ultimately avoid) the need for prosthetic surgery, is a treatment with Adipose-Derived Stem Cells (or ADSCs). The safety and efficacy in terms of functional improvement, cartilage repair and inflammation reduction were already reported for OA treatment through a single intra-articular injection of Micro-Fragmented Autologous Fat Tissue (M-FAT), which is known to be rich in ADSCs. This efficacy period appears to be ranging from several months to a few years.

Research has shown that the effect of single-dose M-FAT treatment, despite being evident in most cases, is not statistically uniform among the studies now available. This is also due to different treatment protocols, that leave to every research group the ultimate choice of preparation methods, rules and exclusion criteria.

It is possible, because of this, to hypothesize a new kind of treatment protocol based on a single surgical harvesting operation. The extract would then need to be cryopreserved and administered multiple times over a several-years period. We hope, thanks to the subsequent injections of purified extract, to prolong and boost the effect of the treatment.

We tried to elaborate, based on our experience in the field, a new protocol for this multi-injection treatment. This protocol is going to be applied to a small number of patients and evaluated over time, to elaborate the actual benefit from a treatment like this.

INTRODUCTION

OVERVIEW ON KNEE OSTEOARTHRITIS

Osteoarthritis (also indicated as OA) is a widespread degenerative disease affecting the articular cartilage and subchondral bone of the whole joint. It leads to cartilage destruction, sustained inflammation and, ultimately, degeneration of the affected joint. This process has always been considered as irreversible, with a small role for conservative therapies. Hip, knee, and shoulder are the most affected joints from this disease. It affects more than 300 million people worldwide, with a larger incidence in developed countries where life expectancy is higher [1]. OA leads to a negative impact on quality of life (QoL) and autonomy in performing the activities of daily living (ADLs), caused by persistent pain (common also during night-time) and loss of function.

In the eighties, the international orthopedic community discovered with great enthusiasm the total arthroplasty for knee degenerative OA in advanced stages of joint diffuse degeneration [2].

Since then, the rapidly growing demand for this procedure in the elderly population generated an increase in revision surgeries and overall complications; however, the numbers remained still relatively low in percentage. Despite a wide array of technological advancements and new discoveries in materials, treatment options and surgical techniques, the management of implants failures is going to become a major sanitary burden in developed countries with a dramatic increase in healthcare costs [3]. For example, the expected revision rate at 10 years for medial pivot knee implants is around 1,9% [4].

In addition to this failure risk, based on current data available, about 20% of the patients treated with TKA are left totally or partially unsatisfied with the clinical results (reduced stability perception, persistent difficulty in performing ADLs, localized - anterior, medial, or lateral - capsule pain, among others) [5].

The knee arthroplasty might also represent a big challenge during the surgical procedure and after, with possible medical complications (pulmonary thromboembolism, blood loss and possible haemorrhagic shock, among the others) [6,7].

We think, as surgeons and clinicians, that a radically new approach to OA treatment is required, especially in early stages or younger patients, to delay or deny the necessity for a joint replacement procedure.

There are, in fact, many patients affected by initial and yet very symptomatic knee OA, in which joint replacement represents an overtreatment. Nevertheless, the available non-surgical options have been regarded until now as an ineffective under-treatment, considering the growing functional requests in ADLs for over-65 patients. In addition, lifetime risk for knee OA is progressively increasing with the ageing of the population and the increase of obesity, thus cases are expected to double in the incoming decades [8].

The OA is known as a degenerative joint pathology primarily affecting the articular cartilage, but research has shown that subchondral bone and synovium (the entire joint capsule) potentially play a key role in the development and progression of the disease [9,10].

In many cases, OA first appears as a focal osteochondral lesion, usually in load-bearing areas, which subsequently can spread over the rest of the joint surface [11]. Surgical intervention in this initial phase usually aims to spare the suffering joint from further degeneration. Historically, three main typologies of "conservative" surgical treatment for initial OA have been developed and proposed over time: reparative or regenerative techniques aiming at cartilage healing or corrective strategies with the objective of correcting the underlying mechanical defect.

Cartilage repairing techniques, generally offered to young and middle-aged patients, can be summarized in bone marrow stimulations such as subchondral drilling and micro-fracturing. They were already performed in the Eighties. Anyway, the lack of mechanical support capabilities of the newly formed fibro-cartilaginous tissue to the suffering cartilage caused symptoms recurrence in more than 50% of the patients at 3 years [12,13].

Osteochondral transplant and autologous or allogenic chondrocytes implantation (ACI) demonstrated encouraging results, but the first is only possible in case of small and isolated defects while the second is of limited capabilities due to the high cost of cell expansion techniques [14].

If this initial phase is not addressed properly, the self-sustained degeneration damages progressively the chondral surfaces and causes persistent pain in association with decrease in joint function. In advanced stages, the chondral tissue is destroyed, with the sub-chondral bone surfaces coming to contact (the so-called "bone-to-bone" osteoarthritis).

Historically, OA has been classified as a non-inflammatory arthritis, caused by mechanical overload, because of the early observation of a small number of leukocytes in the osteoarthritic knee synovial fluid. However, comparing OA tissue and synovial fluid with those from "healthy" joints, an enrichment in plasma proteins, components of complement cascade and pro-inflammatory cytokines can be noticed, underlying the

inflammatory nature which takes active part into the cartilage degeneration [15].

A fundamental barrier to the restoration of cartilage integrity is its low intrinsic repair capacity, which depends on poor vascularization, low cellularity, and the presence of a dense hydrated extracellular matrix hindering cell migration to and from the site of injury. Moreover, in OA patients, local (synovial, chondral, subchondral, etc.) mesenchymal stem cells' (indicated as MSCs) vitality decreases, and these cells are unable to repair damages due to their poor differentiation and proliferative potential [16].

Until now, the commonest non-surgical pharmaceutical choices for a newly diagnosed symptomatic knee OA were either the use of oral NSAIDS or an infiltrative treatment of visco-supplementing agents, namely hyaluronic acid derivatives (referred to as HA). The local effect of visco-supplementation, mimicking the physiologic action of lubricin results in a pain decrease related to motion and, eventually, in a reduction of the inflammatory evolution of the arthritic joint [17]. High molecular weight (HMW) hyaluronans have then been introduced: their longer-lasting effect and their increased viscosity reduce the number of injections required for symptomatic relief [18]. Efficacy in easing pain with these visco-supplementing agents is progressively reduced and shortened in the more inflamed and damaged joints. In addition, they do not address the lack of regenerative capacity of the chondral surface, and they can't "reverse" in any way the degenerative process [19].

In fact, in patients affected by end-stage OA where surgical options are unavailable (because of older age or clinical pre-conditions), many doctors choose to treat the patient with an intra-articular injection of corticosteroids and hyaluronic acid as a mere local palliation treatment, in which the steroids temporarily reduce inflammation [20]. Unfortunately, if used for a long time, local steroids can activate a general catabolic effect on the whole joint, limiting their efficacy and ultimately even affecting joint integrity [21]. Other types of approach based on lifestyle changes, such as physical activity or weight loss, usually are hard to maintain and can only slow down the loss of function associated to the pathology [22,23].

In more recent years, innovative therapies, ranging from Platelet-Rich Plasma (PRP) to cell-based treatments were proposed as a solution for OA patients. PRP is an autologous extract, purified from blood samples, containing platelets that, when injected in the affected joint, behave as a carrier of cytokines and growth factors that can help reducing inflammation and create a better environment [24]. They demonstrated a good level of efficacy and a quite prolonged effect in a wide array of high level-of-evidence studies, but also a non-uniformity of validation in the literature, perhaps due to a lack of standardization in protocols and inclusion criteria [25–28]. The long-term effect is especially unclear, and BMI, Kellgren-Lawrence and age at treatment of the affected patient might negatively correlate with the result, but the evidence is not complete [24]. An association between PRP and HA has also been tested, with promising results [29].

In addition to the common intra-articular injection of biologic agents, subchondral bone has become a target for therapies in recent years: the high quantity of nervous fibers and a leading role in the first progression of OA make it a primary objective [30–32].

MESENCHIMAL STEM CELLS OPTION FOR TREATMENT AND AVAILABLE LITERATURE

The goal for an early treatment should be to control the symptoms, prevent further damage and possibly return to a normal chondral surface status, reversing the degeneration progression.

When it comes to cellular-based treatments, MSCs emerged as a potential cellular therapy "silver bullet". Among the available sources of MSCs, two received the most attention as possible therapies: adipose-derived stem cells (or ADSCs) and bone marrow stem cells (or BMSCs) [33].

The clinical use of MSCs has been strictly regulated from the beginning both in Europe and In the US, with particular attention to the ex-vivo cell expansion process. To overcome this limitation, companies developed strategies to handle the MSCs-rich extract in proximity of the operating theatre, introducing the "minimal manipulation" concept [34].

According to the literature, ADSCs are more advantageous than BMSCs in many aspects: the isolation process is simpler, the procedure is safer and less invasive; in addition, the amount of MSCs harvested through adipose tissue is larger. These ADSCs have also shown greater potential, both in terms of propagation and differentiation [35,36].

The ADSCs were initially isolated from lipoaspirate extract through enzymatic digestion (collagenase) to obtain the so-called Stromal Vascular Fraction (SVF). This approach, requiring xenogeneic substances, was not compatible with the European Good Manifacturing Practice (eGMP) Guidelines. Therefore, other methods, such as mechanical fragmentation, were developed to isolate the SVF maintaining the adipose tissue microscopic structural integrity [37]. Mechanical fragmentation preserves ADSCs in clusters, called "niches", that nest the activated ADSCs, protecting them and enhancing

their potency in the recipient environment. The adipose-derived "stromal vascular niches" can be easily harvested and handled and are known to be rich in ADSCs, as previously shown.

Several years ago, we embraced a cellular therapy based on intra-articular injections of autologous micro-fragmented adipose tissue (also known as M-FAT) aiming to treat early-to-mid phase symptomatic knee OA. M-FAT is known to be rich in ADSCs. This procedure has several advantages over previous standard OA treatments using other sources of MSCs [15].

Beside the easiness of sourcing and handling, ADSCs have the potential to differentiate *in vitro* into osteoblasts, chondrocytes and adipocytes, according to different stimuli received, thus they are an attractive therapeutic tool for the treatment of osteochondral damages [38]. In an experimental setting they displayed a significant plasticity to propagate and differentiate into mesoderm-like tissue [39]. However, once injected, they are known to survive in an inflamed joint for a period ranging from a few days to several weeks, depending on various factors [40]. Despite this, their chondroprotective and immunomodulatory effects last longer, thanks to endogenous cells stimulation. The administration of cells on a gel carrier and the use of bio-adhesive materials has been proposed to enhance their survival.

These ADSCs also have an intrinsic paracrine and immunomodulatory activity: they can boost local stem cells in the damaged joint leading them to differentiation; they can produce growth factors (GFs like TGFb, VEGF, FGF, HGF, among the others) to promote angiogenesis and proliferation, they can inhibit apoptosis in endangered local cells and interact with T-cells to avoid differentiation toward dangerous phenotypes (Th1, Th17) [15].

Exosomes are a key player in the storage and distribution of GFs: they are small extracellular vesicles secreted by cells for intercellular communication. They contain a wide array of messages encoded in nucleic acids (mRNA, miRNA), in addition to proteins and lipids that can modify the gene expression of recipient cells. The content of these granules can differ substantially between one another, with a big change in targeting, and is modulated by extracellular milieu. They seem to have a major role in promoting cartilage repair through MSCs. This could possibly lead in the future to further developments for the "off-the-shelf" cell-free treatment of OA [41].

A recent systematic review and several randomized controlled trials (RCTs) analyzed the clinical evidence of ADSCs to treat OA patients, demonstrating the safety and efficacy of intra-articular injections of ADSCs with promising results in terms of functional improvement, cartilage repair and inflammation reduction [42–46].

According to Michalek et al., the results of a multi-center non-randomized case-control study of 1128 patients with OA in several joints, treated with SVF, reported an improvement of several clinical indicators (pain, non-steroid analgesic usage, limping, joint range-of-motion) [47]. More recently, the same authors published another controlled study conducted on elderly patients, treated with SVF, which demonstrated to be beneficial even in patients older than 80 years with grade II to IV OA [48]. Nevertheless, a recent work showed that patients with stage II Kellgren-Lawrence (K-L) knee OA were most likely to benefit from intra-articular injections of ADSCs compared to the patients with stage IV K-L [49].

A study from Lee et al., in a double-blind randomized phase IIb clinical trial, demonstrated a significant clinical improvement of treatment group, if compared to placebo [44]. However, no significant thickening of the cartilage surface was detected on MRI. Recently, Hong and colleagues published results of a randomized double-blind trial, demonstrating that pure SVF provided effective improvements in both radiological and clinical outcomes (significantly superior to HA treatment) for bilateral knee joint with OA II-III stage [43].

So far, only one clinical trial (Kang-Il et al.) reported safety and efficacy of ADSCs infiltrations in a longer-term follow-up. In their cohort, however, patients underwent additional intra-articular HA injections, not allowing a clear understanding of the efficacy of MFAT treatment alone [50]. Our group recently submitted for publication a new retrospective study with a 4 years minimum follow-up and a group of 46 patients enrolled, treated with M-FAT single-dose treatment: the data available seem to show an efficacy, in terms of function improvement and pain decrease, of about 90% at one year and 65% at four years ("Autologous microfragmented adipose tissue treatment of knee osteoarthritis demonstrates effectiveness up to four-year follow-up", by F.Onorato et al.). The efficacy window of a single treatment appeared to be ranging from several months to a few years.

M-FAT SINGLE-DOSE TREATMENT PROCEDURE

Our surgical procedure for one-stage treatments with M-FAT consisted of a lipoaspirate in spinal anesthesia of about 150mL of adipose tissue solution in sterile conditions, within the operating theatre, from subcutaneous anterior lower abdomen or lateral thighs through a small skin incision (area previously irrigated with a modified Klein Ringer solution containing adrenaline 1 on 500, mostly to

control bleeding during harvesting). Harvesting was performed using mechanical aspiration from a blunt fenestrated cannula.

The preparation and purification of this solution occurred through a commercially available LipoGems® kit (LipoGems Int Spa, Milan, Italy), that mechanically fragmented adipose tissue clusters by constant irrigation with saline solution and mechanical disruption (i.e., shaking). The end-product was then injected in the affected joint (usually, hip or knee).

This end-product is not actually considered to be a true cellular SVF, as it would be with enzymatic digestion (through collagenase). It is more of a combination of cellular debris, free blood cells, pericytes and extracellular matrix components in association with several soluble factors. They are known to have anti-inflammatory, immunomodulatory and analgesic effects.

According to the recent literature, ADSCs obtained from mechanical devices showed good cell viability, specific CD markers expression and differentiation potency, if compared to those generated via enzymatic digestion. According to our research, this type of concentrated M-FAT injections contained nucleated cells ranging from $0.5x10^6$ to $3x10^6$ per gram of final product [51].

WHAT IF IT COULD LAST MORE?

By the analysis of the literature available, there were no common aspects in single-dose treatment protocols. Research has shown that the effect of ADSC treatment, despite being evident in most cases, is not statistically uniform among the studies available. This is also due to a non-standardized treatment protocol, that leaves to every research group the choice on rules and exclusion criteria. In some of the patients the effect is short-lived, not completely justifying the procedure.

Aiming to prolong the shutdown of the joint's inflammatory and degenerative processes, clinical protocols including multiple injections, administered in different moments over a short period of time, should be feasible and would hopefully obtain a longer lasting effect.

We therefore envisioned and prepared a shared protocol for a three-injections treatment over a 12-months period. This protocol would be based on a single surgical harvesting operation and multiple administrations over a 12-months period. These can be set conventionally to be at t+0, t+6 months and t+12 months. Because of this new development, adipose tissue needs to be cryopreserved, avoiding multiple liposuction procedures for each patient.

We opened a collaboration with the "Tissue and Cell Factory-Skin Bank" (TCF), which is a GMP- and GLP-compliant Tissue Facility connected to the C.T.O. Hospital (Centro Traumatologico Ortopedico) based in Turin, Italy. The TCF received a clearance to proceed with the storage of adipose tissue from AIFA, the Italian Medicines Agency.

Therefore, we previously developed a shared protocol for adipose tissue cryopreservation, characterizing ADSCs contained in the "stromal niches" in terms of viability, phenotype and multi-lineage differentiation abilities. As these extracts and the conservation process did not displace ADSCs from their own niches, their viability and their capability to differentiate should be maintained with cryopreservation up to several years [52].

CONCLUSIONS

We envision that three-doses MFAT intra-articular injections could have a better effect over a longer period, when compared to a single-dose treatment protocol. This should be achieved through an emboldened regenerative action of the M-FAT injections on the endangered joint environment. We expect a sustained gain in Quality of Life (QoL) due to the reduction of pain and functional improvement, reducing disability burden and ultimately decreasing the need for knee joint replacement surgery.

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