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Rationale for Using Direct Bone Marrow Aspirate as a Proliferant for Regenerative Injection Therapy (Prolotherapy)

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Abstract: Adult mesenchymal stem cells (MSCs) obtainable from autologous bone marrow aspirates have generated tremendous interest in the medical and scientific communities in the last two decades and are currently being investigated by a of interested physicians for use in point-of-care stem cell therapies due to their great potential to differentiate into multiple cell lineages such as bone, cartilage, muscle, tendon, and nerve. However, as these stem cells are found in very low numbers in adult tissue, centrifugal concentration or expansion through *in vitro* culturing has been pursued to obtain higher numbers of efficacious regenerative therapeutic applications. More recently, some physicians and scientists have chosen to explore use for direct injection of un-fractionated, native whole bone marrow aspirate as a strategy in regenerative treatment regimes. This review examines the potential merits and disadvantages of using either concentrated and culture expanded MSCs versus native whole bone marrow aspirate as key proliferant in direct regenerative injection therapy (RIT). Results from a number of published investigations have clearly shown high potential of various deleterious effects on manipulating MSCs obtained from native bone marrow aspirate either by centrifugal forces or expansion through *in vitro* culturing; moreover, currently used centrifugal concentration techniques do not significantly concentrate MSCs from bone marrow aspirate, thus, defeating the purpose of this manipulative step. On the other hand, preliminary results and observations of using un-fractionated whole bone marrow injection for treatment of various musculoskeletal joint diseases (for example, osteoarthritic joints) suggest that the procedure is safe and potentially efficacious, with no known deleterious effects as yet reported.

Keywords: Regenerative injection therapy (RIT), degenerative joint disease, mesenchymal stem cells (MSCs), stem cells, bone marrow, autologous whole bone marrow aspirate, concentrated MSCs, culture expanded MSCs.

INTRODUCTION

Degenerative joint disease represents a major and growing cause of disability and healthcare costs. Osteoarthritis (OA), the most common joint disease, affects approximately 14 percent of adults aged 25 and older and approximately 33.6 percent of those older than 65 years; an estimated 27 million U.S. adults [1]. It has been estimated that arthritis and related conditions, such as OA, cost the U.S. economy nearly $128 billion annually in medical care and indirect expenses, including lost wages and productivity [2]. A major goal of therapy in degenerative joint disease is the stimulation of regenerative processes in the joint that will facilitate the restoration of degenerated cartilage to a healthy state. The need for effective cell-based therapies is increasing due to a rise in the ageing population and the associated increase in the prevalence of musculoskeletal disorders. The development of percutaneous interventions that potentially enhance regenerative processes has improved the prospect for nonsurgical treatments that may produce durable improvement in pain and function [3]. One approach to regenerative therapy is to supply affected joints with either autologous chondrocytes or chondrogenic bone marrow-derived mesenchymal stem cells (BMSCs), prepared as a buffy coat fraction of bone marrow with or without *ex vivo* expansion. Recent preliminary studies support the investigation of these therapies for OA [4-11].

Intra-articular injection of whole tibial bone marrow is being explored by some physicians to treat patients displaying degenerative joint ailments. Whole bone marrow (WBM) injection potentially captures elements of several regenerative strategies in contrast to prior BMSCs therapies. With WBM injection, marrow is not fractionated and potentially supportive chondrogenic components in marrow plasma are retained in addition to BMSC thus, mimicking the bone marrow natural niche microenvironment with retention of all the key cells in their natural ratios, regenerative, cellular viability and proliferative potentials. An additional potential benefit is that tibial marrow represents a rich source of marrow adipocytes. Marrow adipocytes share properties [12] with brown fat adipocytes that have been linked to endochondral bone formation, via a mechanism thought to involve adipocyte-dependent generation of a chondrogenic microenvironment [13]. WBM injection, therefore, represents a novel modification of regenerative therapy for degenerative joint disease.

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Table 1. Results of Cytological Analysis of Bone Marrow Aspirate and Bone Marrow Concentrate

	Bone Marrow Aspirate*	Bone Marrow Concentrate*	Absolute Change*	Relative Change†	P Value
Platelet count ($x\ 10^3/\mu L$)	31.1	208.3	177	8.7	0.002
White blood-cell count ($x\ 10^3/\mu L$)	36.5	267	230	7.4	0.0007
Red blood-cell count ($x\ 10^3/\mu L$)	6774	3156	-3617	0.5	<0.0001

*These values are presented as the mean and standard deviation. N=10. †The relative change is presented as the mean with the 95% confidence interval.

Bone marrow stromal cells (BMSCs) include cells with multidirectional differentiation potential such as mesenchymal stem cells (MSCs), multipotent adult progenitor cells such as endothelial progenitor cells (EPCs), and marrow-isolated adult multilineage inducible cells such as hematopoietic stem cells (HSCs) and BMSC-derived multipotent cells have been used extensively in various fields of regenerative medicine, due to their tissue regenerative and repair capabilities. Two of these cell types have received much attention, namely HSCs and MSCs. Bone marrow derived MSCs are known for their ability to self-renew, undergo clonal expansion, differentiate into multiple musculoskeletal tissues (such as osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and adipocytes), support hematopoietic stem cells (HSCs), and regulate the immune system in response to various local cellular, tissue, injury or disease signaling cues and pathways [14-21]. Adult stem cells make up only a small percentage of cells in a tissue and are surrounded by mature cells that have reached the end of the differentiation process and do not have the capacity to proliferate or differentiate [22]. The use of adult stem cells in clinical application is currently undergoing rigorous investigation due to restrictions and ethical and religious issues surrounding the use of embryonic stem cells.

Their ability to differentiate into many cell types is one of the advantageous characteristics that have highlighted MSCs importance for use in cell-based therapies; as a result, physicians and scientists are currently harvesting these cells for diverse preclinical and clinical studies and/or applications. Autologous bone marrow aspirate (commonly referred to as BMA) percutaneously obtained from a patient's iliac bone crest provides a cell suspension from the patient's own body that can be readily used for direct injection as a proliferant for regenerative injection therapy (RIT). Autologous bone marrow aspirates (BMA) have been a major source for obtaining MSCs; although, BMA is commonly withdrawn from the iliac crest, it can also be aspirated from the tibia, clacaneal, femur and humerus.

Bone marrow is a rich source of hematopoietic and osteogenic adult stem cells [23]. Many surgeons currently use unprocessed BMA for implantation; however, in order to gain the maximum potential of BMA, it has been suggested that the BMA be processed in a way that concentrates the stem cells and isolates them away from the other cells within bone marrow in order to obtain sufficient amount of MSCs to provide an effective environment for healing [24,25]; this is due to the fact that the amount of MSCs within bone marrow is very limited. The range of concentration of MSCs in the literature is 7 to 33 MSCs per 1,000,000 nucleated cells within native bone marrow [24]. Culturing MSCs isolated from bone marrow aspirate is another approach employed by some investigators to expand the cells to higher numbers.

Multiple research groups have attempted to determine whether the concentration of MSCs would provide a more effective environment for healing. Thus, preclinical and clinical studies of the use of autologous bone marrow for musculoskeletal and bone tissue repair have focused on preparations in which MSCs are enriched and expanded, with the assumption that the quantity of delivered MSCs is critical [4, 6, 7, 26-28]. In this regard, new products have recently been developed and are currently making the claim that stem cells can be isolated and concentrated for point-of-care usage; these products are known as bone marrow concentrate (BMC) systems.

DO CURRENT CENTRIFUGAL CONCENTRATION METHODS REALLY CONCENTRATE MSCS EFFICIENTLY FROM BONE MARROW ASPIRATE?

In the work reported by Fortier *et al*. [29], they compared the approximate numbers of red blood cells in whole bone marrow aspirate and concentrated or centrifuged bone marrow. The figures in Table **1** shows that there is still a considerable number of red blood cells in concentrated bone marrow (only approximately 50% less than in whole bone marrow aspirate). In other words, the concentration or centrifugation techniques are not very efficient. The results also imply that the presence of erythrocytes may not be as detrimental to the healing efficacy of whole bone marrow aspirate as reported by some authors.

Indeed, the assumption regarding delivery of a certain minimum quantity of MSCs for effective healing as claimed by certain investigators, remains untested and recent studies, including the use of MSCs in a goat OA model [30], suggest that the chondrogenic action of MSCs may depend more on the trophic function than on the chondrocytic differentiation and structural incorporation of these cells [31,32]. In this case, delivered MSC concentration may be less important than the micro-environmental context of delivery, and complex preparations, including whole marrow, are potentially more advantageous. Another recent publication compared the effects of injecting centrifuged and not centrifuged whole bone marrow cells on the healing of the meniscal wound in a dog model [33]; the results suggest that the group of dogs injected with whole bone marrow cells (not centrifuged)

healed slightly better than the centrifuged group, and the authors recommended that there is no need for bone marrow centrifugation prior to injection, due to the potential for contamination and infections. A recent review article analyzed a number publications and claims of concentrating MSCs from aspirated bone marrow to higher numbers for clinical applications [24]. The conclusion of the analysis is that current bone marrow concentration systems and related centrifuge-based claims fail to accomplish the feat of significantly increasing the numbers of MSCs in the so-called bone marrow concentrates. The result of this analysis is generally consistent with an earlier observation by Connolly *et al*. that there is no statistically significant difference between the data for centrifuged marrow and uncentrifuged marrow [34]. Moreover, while a correlation has been observed between marrow MSC concentration and the efficacy of grafted marrow for osteogenic repair of non-unions [35], early studies demonstrated efficacy of non-union treatment using direct, immediate injection of unprocessed and non-concentrated whole bone marrow without MSCs enrichment [36, 37].

OPTIMAL NUMBERS OF MSCS FOR CLINICAL APPLICATIONS

Moreover, a recent review article by Fossett and Khan analyzed results from several publications in an attempt to determine if a specific number of MSCs is optimal for achieving clinical efficacy [38]. The authors examined MSCs seeding density and proliferation/expansion capacity. The analysis of seeding density studies showed consistent results from numerous MSCs sources (e.g., bone marrow, adipose, skeletal muscle, synovial fat pad), favoring lower seeding densities in order to achieve higher rates of MSCs proliferation. As noted by Fossett and Khan, "Finding the optimum seeding density for maximal expansion is useful in both laboratory investigations as well as potential clinical applications as the cell culturing procedure can be less time consuming, decreasing the risk of cell culture contamination, infection or loss of biological characteristics in cell culture, in addition to making the process more cost effective. These studies show that rapid expansion to reach a sufficient number of cells for clinical applications can be achieved by using lower seeding densities." Thus, the results of these studies bring into question the current concept pursued by some investigators of specifically concentrating MSCs from autologous bone marrow aspirate to obtain "higher number of cells" prior to injection in order to "provide more effective" healing environment in the joint.

POTENTIAL ADVERSE EFFECTS OF CENTRIFUGAL FORCE, CELL CULTURE MEDIA COMPOSITION AND CONDITIONS ON MSCS

Several published scientific investigations have discussed short comings or potential negative effects on the viability and proliferative ability of cells associated with the use of centrifugal force to concentrate MSCs from native bone marrow cells or to expand the number of MSCs through *ex vivo* culturing techniques [15, 39-49]; other related concerns include potential for genetic/epigenetic alterations, transfer of zoogenic diseases to recipient host, contamination and loss of critical key cells present in the original naturally balanced bone marrow niche microenvironment [49-51]. Thorough understanding of the impact of various culture media compositions and culturing parameters (e.g., temperature, seeding densities, and type of mechanical agitation during culturing) on the viability and proliferative status of bone marrow MSCs that will be used for regenerative medicine applications is very important. For example, some published reports indicate that depending on the cultivation technique, significant differences in both gene and protein expression can result from culturing MSCs [39]. Other reports have indicated that long term *in vitro* expansion alters the biology of adult MSCs (for example, functional analysis of genes that were differentially expressed in human bone marrow MSCs revealed that pathways involved in cell cycle, cell cycle checkpoints, protein-ubiquitination, and apoptosis were altered [40].

The culturing and subsequent transplantation of undifferentiated precursor stem cells from bone marrow are complex and involve costly procedures and methodologies that are restricted to large research centers [15]. Some published reports in the scientific literature have clearly stated that optimal culture media compositions and culturing conditions are yet to be clearly determined/defined [41, 42]. Authors of these publications maintain that it is important not to underestimate the potential health risk of using xenogenic compounds (for example, fetal calf serum, human serum, plasma, and blood derivatives) and point out the possibility of immunological reactions to the xenogeneic compounds used in the culture once stem cells are transplanted. Furthermore, they state that the results obtained with these compounds are controversial, and recommend a careful examination of the pros and cons of serum-free and ad hoc formulation strategies need to be made - regarding the compositions and conditions of culture media currently in use due to the potential interference with the self-renewal and differentiation processes of MSCs, and hence the effects on long-term therapeutic efficacy and safety [41, 42].

There are also legitimate concerns regarding the potential impact of centrifugal hydrodynamic forces, centrifugation time and temperature of the process used to separate and concentrate MSCs from whole bone marrow cells on cell loss and damage/rewarming injury to the characteristics (proliferative capacity, pluripotency and differentiation status) of the resulting concentrated MSCs. Some published results indicate that centrifugal processing of MSCs from whole bone marrow could potentially alter some biological characteristics of stem cells [43-48]. For example, changes in gene expression without a change in DNA sequence can occur due to epigenetic forces. With respect to stem cells, there is an increasing body of evidence for an epigenetic basis for pluripotency and differentiation potential [49-51]. As the precise epigenetic mechanisms are currently not well understood, it is important to recognize and possibly identify the centrifugal/bioprocess conditions that can significantly influence the epigenome, and thus affect the safety and efficacy of MSCs used for injection regenerative therapy.

HETEROGENEITY OF BONE MARROW STEM CELLS AND MESENCHYMAL STEM CELLS (MSCS)

Although, preclinical and clinical studies of the use of autologous bone marrow for musculoskeletal and bone tissue repair have focused on preparations in which MSCs are enriched and expanded in order to obtain certain amount or number of mesenchymal cells (presumably to provide an effective environment for healing), there is growing understanding that the whole bone marrow milieu or "niche" may be a more viable option. Whole bone marrow contains other stem and nonstem cell types and other bone marrow components (e.g. hematopoietic stem cells, microvesicles) that carry out biochemical functions and interact dynamically and synergistically together to assure effective biological regulation and biofunctionality within the bone marrow microenvironment, which affect tissue regeneration and/or repair [52-56].

Bone marrow derived MSCs display biochemical heterogeneity in phenotypes and functions – with potentially incredible biological and medical benefits and implications due to their known involvement in tissue repair, immunomodulation and inflammation [57-61]. MSCs populations exhibit considerable intra- and inter-population heterogeneity. It has been suggested that heterogeneous populations reflect the complexity of the stromal system in the bone marrow and the varied functions it performs to regulate tissue homeostasis and its contribution to the demonstrated functionality of MSCs *in vivo* [57,59]. As of present, it is still unclear which cell(s) types are the *in vivo* precursor cells that expand in culture *in vitro* as MSCs. Thus, it cannot be claimed that the MSCs populations expanded in cultures definitively reproduce or generate functionally equivalent MSCs populations found *in vivo* in native bone marrow microenvironment.

REGENERATIVE POTENTIAL OF MSCS AND PARACRINE ACTIVITIES

When musculoskeletal tissues are injured, a healing response is initiated in an attempt to repair the damage. Healing requires a coordinated interplay among cells, growth factors, and extracellular matrix (ECM) proteins through a three-stage overlapping healing process (hemorrhage with inflammatory, matrix and cellular proliferation and finally, remodeling and maturation). Central to this process are the endogenous mesenchymal stem cells (MSCs) from bone marrow or other sources, which coordinate the repair response by recruiting other host cells and secreting growth factors and matrix proteins. In addition, MSCs have a role in each of the three-stage injury healing responses (inflammatory, proliferative, and remodeling), and their presence supports healthy physiologic functioning towards successful healing. For example, MSCs regulate immune and inflammatory responses, and possess powerful tissue protective and reparative effects through paracrine signaling by releasing biologically active molecules that affect cell migration, proliferation, and survival/remodeling of the cells surrounding the site of injury [62-67].

Published data indicate the importance of MSC anti-inflammatory and immunomodulatory activities in injury healing. MSCs in vivo have been shown to migrate to sites of injury in response to chemotactic signals modulating inflammation, repairing damaged tissue, and facilitating tissue regeneration. Differentiation and paracrine signaling have both been implicated as mechanisms by which MSCs improve tissue repair. MSC differentiation contributes by regenerating damaged tissue, whereas MSC paracrine signaling regulates the local cellular responses to injury. Although, the molecular mechanisms of MSCs involvement in healing response are not yet fully understood, MSC paracrine signaling is thought to be the likely primary mechanism for the beneficial effects of MSCs on injury healing, that is, to reduce inflammation, promote angiogenesis, and induce cell migration and proliferation [62-67].

ADVANTAGES OF WHOLE BONE MARROW ASPIRATE FOR DIRECT INJECTION REGENERATIVE THERAPY

The direct use of native, uncultured, non-volume reduced and freshly isolated autologous bone marrow aspirate stem cells as a proliferant for regenerative injection therapy is highly desirable and has several advantages, including the following:

- Avoids the problem of tissue rejection
- Facilitates point-of-care usage for patients
- Native composition or heterogeneous populations of the bone marrow cells are not altered, and thus, all the different types of stem cells and associated cells in the native bone marrow aspirate are fully available to work together synergistically for tissue repair and/or regeneration; no critically useful stem and other cells are lost through processing or centrifugation
- Rich source of marrow adipocytes, extracellular matrix, and growth factors which all coordinate interplay among the various cell types, including mesenchymal stem cells, to coordinate the repair response
- Reduces ethical issues such as those associated with use of embryonic stem cells
- Does not require *ex vivo* culture expansion of bone marrow stem cells which could result in potential alterations and instability in the genetic/epigenetic makeup of the cells, and thus impacts negatively on the maintenance of key bone marrow cells "stemness"
- Avoids the concerns raised about the lack of standardized protocols for stem cell preparations (which could interfere with their self-renewal and differentiation processes) – for example, stem cells culturing in fetal calf serum for clinical purposes raises concerns related to possible contaminations, immunization and transmission of zoonoses or immunological reaction towards xenogeneic compounds
- Has cost saving benefits, as it does not involve use of any equipment for volume reduction of the bone marrow aspirate or *in vitro* culture expansion of MSCs before use
- Reduces regulatory issues as the approach satisfies the key requirement of FDA for such human cells in terms of minimal manipulation and/or processing before use

Table 2. Results from Published Literature of Use of Whole Bone Marrow for Regenerating Nonunion Fracture

	Ahmed HH (2002) [68]	Connolly *et al.*, (1991) [69]	Garg *et al.*, (1993) [70]
Healed	13 (92.8%)	18 (90%)	17 (85%)
Nonunion	1 (7.2%)	2 (10%)	3 (15%)
Total	14	20	20

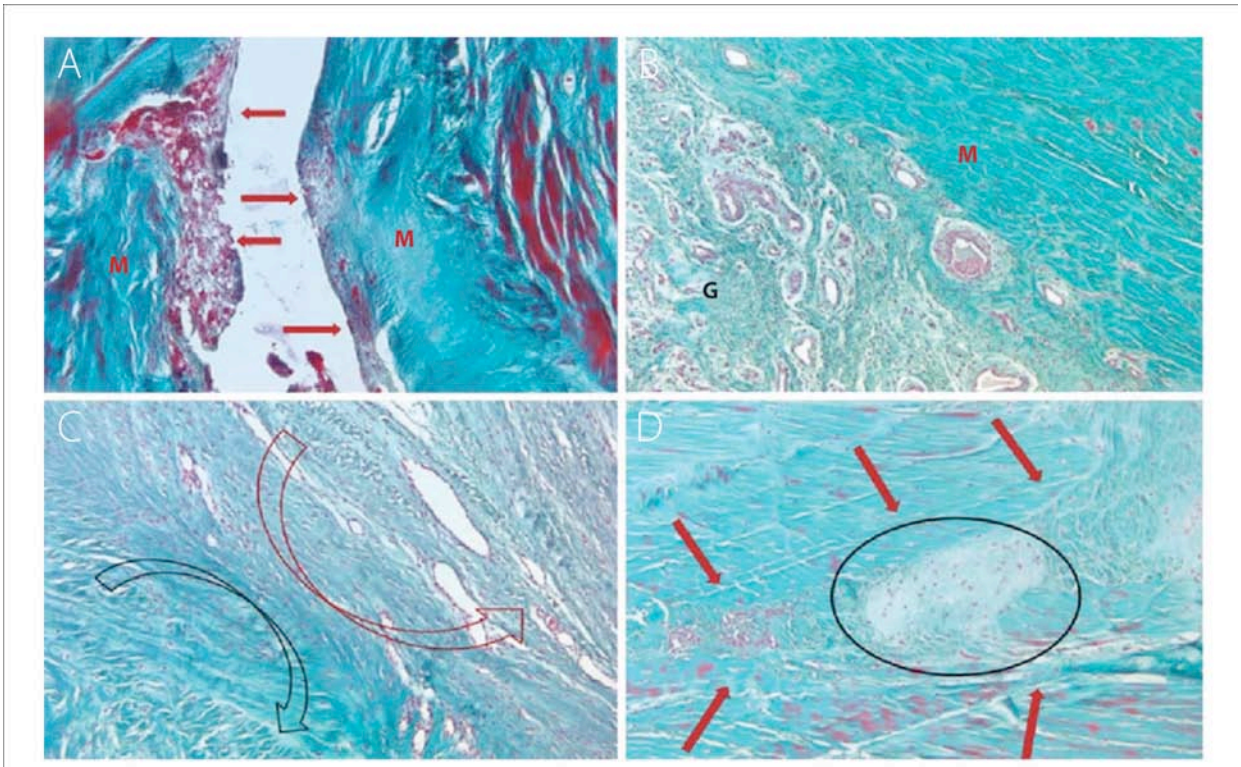

Fig. (1). Effects of autologous bone marrow aspirate on healing of a full-thickness mensical tear. (**A**) A light microscopy sample of meniscal tears treated with bone marrow aspirate. Granulation tissue, formed by migrating and proliferating cells, are marked with red arrows. M = meniscus. D4 x200 Masson's trichrome. (**B**) G: Granulation tissue, in which increase of cellular infiltration, vascularization and collagen formation can be seen at the deeper sites of wedge-like tear. M: meniscus. D4 x100 Masson's trichrome. (**C**) A micrograph of experiment (treated) group. Black arrow: meniscus, Red arrow: granulation tissue can be seen. D2a x100 Masson's trichrome. (**D**) In the wedge-like tear site (red arrows), newly formed cartilage islets within the granulation tissue is marked with black circle. D2 x100 Masson's trichrome.

Adapted and used with permission from: Duygulu F, et al. Effects of intra-articular administration of autologous bone marrow aspirate on healing of full-thickness meniscal tear: an experimental study on sheep. *Acta Orthop Traumatol Turc*. 2012;46(1):61-67.

Fig. (1). Effects of autologous bone marrow aspirate on healing of a full-thickness meniscal tear.

EXAMPLES OF CLINICAL APPLICATIONS OF WHOLE BONE MARROW CELLS

The use of whole bone marrow for regenerating nonunion fracture is shown in the table below:

Furthermore, the work reported by Abdel-Hamid *et al.* [33] compared the healing efficacy of whole bone marrow versus centrifuged bone marrow injected to repair meniscal wounds in an animal model as shown in Table **2**. The results demonstrate a slightly better healing for whole bone marrow compared to centrifuged bone marrow. In addition to the slightly better performance of the whole bone marrow reported by Abdel-Hamid *et al.* [33], we also emphasized in the review article the fact that the use of whole bone marrow injection is a simple, safe and cost-effective technique. Also, the work reported by Duygulu *et al.* [71] clearly demonstrated that injection of bone marrow into the meniscus tear site improved healing in a meniscal tear model as shown by both light and electron microscope findings (Fig. **1** and **2**).

CONCLUDING REMARKS

Autologous bone marrow aspirate mesenchymal stem cell (MSC) based regenerative injection therapy (RIT) opens the door to a very exciting and promising field. This review

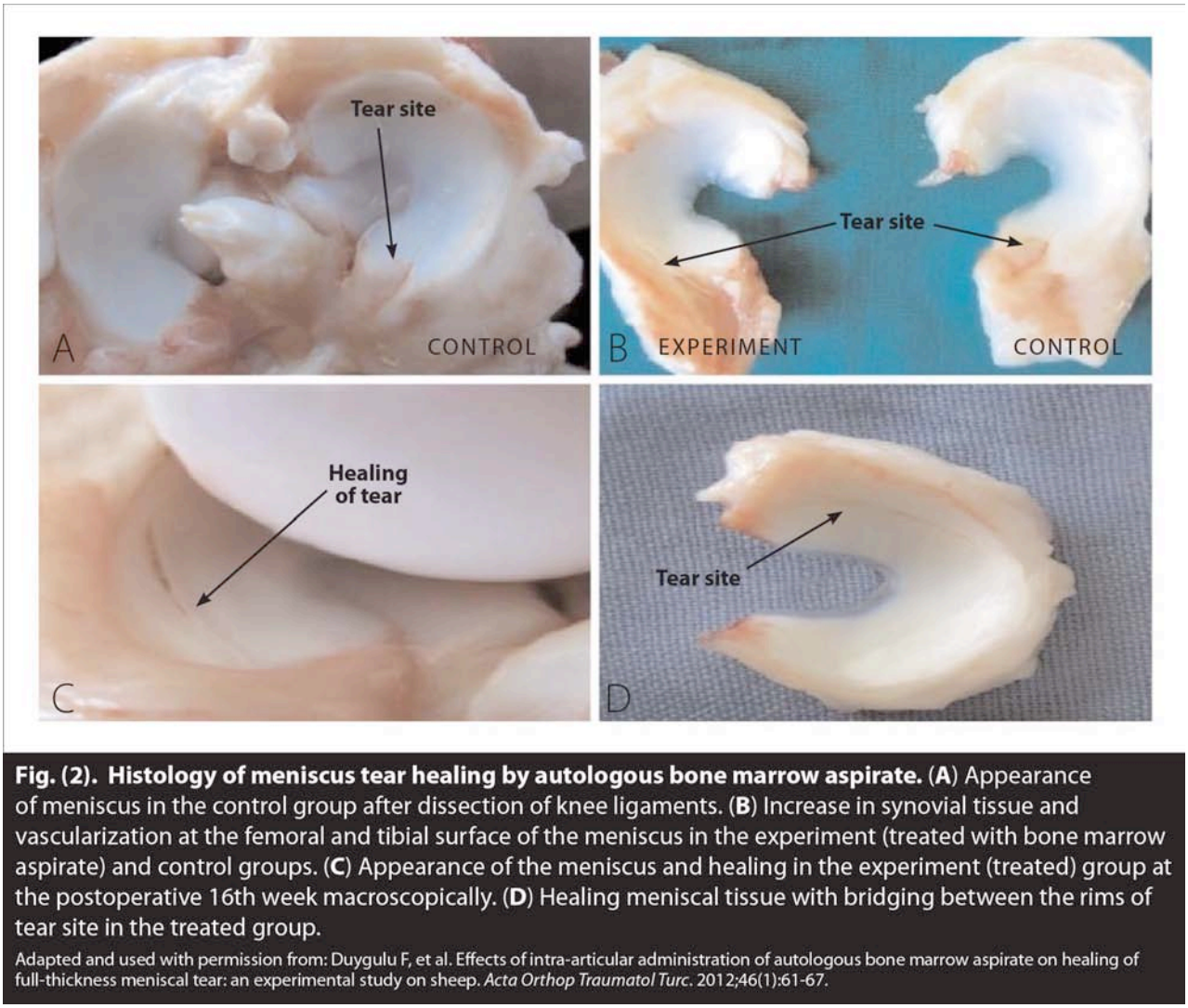

Fig. (2). Histology of meniscus tear healing by autologous bone marrow aspirate. (**A**) Appearance of meniscus in the control group after dissection of knee ligaments. (**B**) Increase in synovial tissue and vascularization at the femoral and tibial surface of the meniscus in the experiment (treated with bone marrow aspirate) and control groups. (**C**) Appearance of the meniscus and healing in the experiment (treated) group at the postoperative 16th week macroscopically. (**D**) Healing meniscal tissue with bridging between the rims of tear site in the treated group.

Adapted and used with permission from: Duygulu F, et al. Effects of intra-articular administration of autologous bone marrow aspirate on healing of full-thickness meniscal tear: an experimental study on sheep. *Acta Orthop Traumatol Turc*. 2012;46(1):61-67.

Fig. (2). Histology of meniscus tear healing by autologous bone marrow aspirate.

has looked at the potential merits and disadvantages of using either concentrated and culture expanded MSCs versus native whole bone marrow aspirate as key proliferant in direct regenerative injection therapy (RIT). Results from a number of studies have found that currently used centrifugal concentration techniques do not achieve significant concentration of MSCs from bone marrow aspirate and therefore, do not offer the advantage of larger concentrations or numbers of MSCs as proliferant for better regenerative therapeutic outcomes. In addition, studies seeking to find optimal numbers of human MSCs for clinical applications from a number of published sources have consistently shown that rapid expansion to attain a sufficient number of cells can be achieved by using lower numbers of cells – that is, MSCs show a faster rate of proliferation/population doubling at lower seeding numbers. Furthermore, results from several studies have clearly shown a number of deleterious effects due to manipulating bone marrow aspirate or obtaining MSCs from either centrifugal force or *in vitro* culture using varying culture media and growth conditions. Although MSCs have been observed to home to sites of injury *in vivo*, there is currently a scarcity of data regarding the concentration of MSCs at such sites of injury. Thus, research to determine the number or concentration of MSCs that home to sites of injury *in vivo* is needed to provide evidence of the effective number of MSCs that may be required for use in regenerative injection therapy to achieve favorable therapeutic outcomes.

Conversely, preliminary results and observations of using unfractionated whole bone marrow injection for treatment of various musculoskeletal joint diseases (for example, osteoarthritic joints) suggest that the procedure is safe and potentially efficacious, with no known deleterious effects as yet reported. However, to further verify these initial results, it would be beneficial for physicians and scientists to work together very closely to design better clinical trials. It is known that one major reason for the current increasing need for effective cell-based therapies is due to rise in the aging population, increasing the occurrence of musculoskeletal disorders. Thus, it is important that research of how age and gender may affect the therapeutic outcomes when using native bone marrow aspirates in RIT be carefully designed – making sure that patients are matched by age, gender, social factors, medical history, and any chronic illness to ensure that all results obtained have taken into account potential confounders.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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Both RH and EE made substantive intellectual contributions to the conception and development of this article. RH provided the outline of the paper, clinical review, and expertise and participated in writing the final version of the paper. EE conducted literature reviews and synopses, wrote the first draft of the paper and participated in writing the final version of the paper.

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