The potential of intra-articular injection of chondrogenic-induced bone marrow stem cells to retard the progression of osteoarthritis in a sheep model

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A B S T R A C T

In recent years, the use of bone marrow mesenchymal stem cell (BMSC) implantation has provided an alternative treatment for osteoarthritis. The objective of this study is to determine whether or not an intra-articular injection of a single dose of autologous chondrogenic induced BMSC could retard the progressive destruction of cartilage in a surgically induced osteoarthritis in sheep. Sheep BMSCs were isolated and divided into two groups. One group was cultured in chondrogenic media containing (Ham’s F12:DMEM, 1:1) FBS+5 ng/ml TGF-β3 + 50 ng/ml IGF-1 (CM), and the other group was cultured in the basal media, FBS + 10% PBS (BM). The procedure for surgically induced osteoarthritis was performed on the donor sheep 6 weeks prior to intra-articular injection into the knee joint of a single dose of BMSC from either group, suspended in 5 ml FD at density of 2 million cells/ml. The control groups were injected with basal media, without cells. Six weeks after injection, gross evidence of retardation of cartilage destruction was seen in the osteoarthritic knee joints treated with CM as well as BM. No significant ICRS (International Cartilage Repair Society) scoring was detected between the two groups with cells. However macroscopically, meniscus repair was observed in the knee joint treated with CM. Severe osteoarthritis and meniscal injury was observed in the control group. Interestingly, histologically the CM group demonstrated good cartilage histoarchitecture, thickness and quality, comparable to normal knee joint cartilage. As a conclusion, intra-articular injection of a single dose of BMSC either chondrogenically induced or not, could retard the progression of osteoarthritis (OA) in a sheep model, but the induced cells indicated better results especially in meniscus regeneration.

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1. Introduction

Osteoarthritis (OA) is the most common joint disease in middle aged and older people and is among the most frequent and symptomatic health problems for this age demographic (Schaap et al., 2011; Brooks, 2002). It is characterized by the degeneration of the articular cartilage, leading to loss of matrix, fibrillation, formation of fissures, and ultimately completely loss of the cartilage surface. To date, the common treatment focuses on alleviating the pain and thus allows the patient to move and reduces morbidity (Moe et al., 2011; Magill et al., 2011; Brooks, 2002; Altman, 2009). More recently, pharmacological treatment with drugs such as chondroitin sulfate, even though they cannot reduce the symptoms quickly, has proven to have some structure-modifying effects on the disease area in the long term (Imada et al., 2010; Uebelhart, 2008). Surgical intervention, including arthroscopic surgery to remove and stimulate joint repair, and prosthetic joint replacement, is also a treatment option (Magill et al., 2011; Steadman et al., 2007, 2003). However, despite the multiple modalities of treatment, the outcome is still poor and focuses on temporary measures to alleviate the symptoms rather than treating the pathogenesis of the disease or focusing on reversing the process of osteoarthritis.

Since the development of tissue engineering, cartilage cells, better known as chondrocytes, are one of the first such treatments being studied and manipulated (Langer and Vacanti, 1993; Saim et al., 2000; Kamili et al., 2002). The first clinical application of tissue engineering and cell-based therapy to treat joint and bone defects is the implantation of autologous chondrocytes (Horas et al., 2003).
The most recent clinical studies have shown that implantation of chondrocytes with bioinert biomaterial has a potential in treating osteoarthritis (Vijayan et al., 2010; Kreuz et al., 2009; Hollander et al., 2006; Ossendorf et al., 2007).

Even though the clinical application of chondrocyte implantation has been quite popular and has been aggressively pursued in recent years, other potential cells, such as the mesenchymal stem cells (MSCs) and in particular BMSC, became more popular (Madry and Cucchiariini, 2011). The interest in these cells came from a study reported by Murphy et al. (2003), where intra-articular injection of BMSCs was used in an OA goat model. Since then, several clinical trials have been performed using a similar technique and have shown promising results (Maumus et al., 2011; Centeno et al., 2008; Wakeitani et al., 2002; Csaki et al., 2008).

The use of BMSC not only provides an alternative cell candidate, but may also allow for manipulation to provide the best and most ideal cells for implantation. This manipulation was initiated by examining the induction media. It has been shown that induction using growth factors, such as transforming growth factor (TGF) and insulin like growth factor (IGF), significantly influences the quality of the chondrocytes (Huang et al., 2012; O’Sullivan et al., 2011; Indrawattana et al., 2004; Chua et al., 2007, 2005, 2004). MSCs cultured with TGF-β3 for 2 to 3 weeks were shown to stimulate proteoglycan and collagen type II synthesis, both of which are characteristic of hyaline articular cartilage (Indrawattana et al., 2004). On the other hand, many of the components of a cartilaginous extracellular matrix are deposited by differentiating MSCs exposed to TGF-β3, including aggrecan link protein and type II collagen (Mi et al., 2000). Chondrogenic differentiation occurs when MSCs are grown under conditions that include a three-dimensional culture format, serum-free nutrient medium and the addition of TGF-β3 (Ng et al., 2008; Cao et al., 2012). When these conditions are met, the cells rapidly lose their fibroblastic morphology and begin to initiate the expression of a number of cartilage-specific extracellular matrix components (Lum et al., 1996). During differentiation in the presence of TGF-β3, MSCs synthesize aggrecan link protein, fibromodulin, cartilage oligomeric matrix protein, decorin, type II collagen and chondroadherin, all of which are components of the normal articular cartilage matrix (Moulharat et al., 2004).

With the advancement of growth factors and the manipulation of in vitro induction, it is important to study the effect of these factors in a big animal study. In this study the induction using chondrogenic medium was prepared according to our previous study (Al-Faqeh et al., 2011). After inducing OA in sheep model, the chondrogenic induced BMSCs were injected intra-articularly. The sheep were scheduled for a required exercise regime and sacrificed after 6 weeks. The results will be observed via histological examination of the affected joints and compared to control. We do hope this study will be a reference to our future first phase clinical trial.

2. Material and methods

2.1. Osteoarthritis induction in sheep

The study was conducted after an approval from the Universiti Kebangsaan Malaysia Animal Ethical Committee. A total of sixteen (Siamese long tail) (Universiti Putra Malaysia) sheep were involved in the study. Each was aged between 11 and 12 months old with an average weight of 25 kg. The sheep were randomized into three groups of six animals each, with the exception of the control group that consisted of 4 sheep. No differences were observed between the control group and the test groups with respect to age and weight. For the induction of osteoarthritis (OA) the sheep underwent a bone marrow aspiration for cell preparation on the same day of the surgical procedure. The surgical protocol was conducted according to previous methods described by Murphy et al. (2003) with some modification. In the anesthetic procedure, the animals were induced by intravenous injection of xylazine (0.1 mg/kg) and ketamine (5 mg/kg) and maintained with isoflurane (1.5%) in oxygen, delivered by an endotracheal tube (Troy Laboratories Australia).

A lateral parapatellar skin incision was made beginning at a level of 2 cm proximal to the patella and extending to the level of the tibial plateau. The subcutaneous tissue was incised, and the lateral fascia was separated for 1 cm from the joint capsule in either direction from the incision. The lateral aspect of the vastus lateralis and the joint capsule were incised, and the patella was luxated medially to expose the trochlear groove and medial and lateral condyles of the distal femur. ACL excision was performed by first excising its attachment on the medial aspect of the lateral femoral condyle. The proximal attachment was brought forward and the entire ligament was excised from its tibial attachment. The stifle was moved in a drawer test to ensure that the entire cruciate ligament had been excised.

In the medial meniscus removal, meniscus was removed by sharp excision. The caudal horn of the meniscus was grasped with a hemostat, and its axial (lateral) attachment was excised from its tibial attachment. Working from caudal to lateral and then cranial, the meniscus was excised from its attachments until it was completely removed. Postoperatively the sheep were observed and monitored for 24 h in an animal intensive area. All the vital signs were observed and after 24 h the sheep were placed in an animal house.

After a recovery period of 3 weeks, all animals were made to run on a hard surface for a distance of 100 m, once daily for 3 weeks. At
all other times, the animals were allowed to move freely in an unconfined environment.

2.2. Cell isolation and culture

Five to ten milliliters of bone marrow aspirate was drawn from the iliac crest of a sheep (n = 12) using a Jamshidi-bone marrow aspiration needle (Cardinal Health Inc, USA) in a 20 ml heparinized sterile syringe (Cringe, Malaysia). The BMSCs were isolated from the aspirated bone marrow via gradient centrifugation (Universal 32 R, Germany) over a Ficoll-Paque PLUS layer (Healthcare, Sweden), at 3000 rpm equivalent to 1400 g for 30 min, and subsequently washed twice with phosphate buffered saline (PBS) (Sigma, USA). Cells were resuspended in standard culture medium FD (Ham’s F12:high glucose DMEM 1:1; GIBCO, USA) supplemented with 10% fetal bovine serum (GIBCO), 1× antibiotic–antimycotic (GIBCO), 1× GlutaMAX-1 (GIBCO), 50 μg/ml l-ascorbic acid (Sigma), 0.02 M HEPES buffer (GIBCO), and plated in 25 cm²/plastic culture flasks. The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. The medium was changed every 2 to 3 days. On days 8 to 10, the cells reached confluence and were then detached with 0.25% Trypsin/0.1% EDTA (GIBCO) and recultured as the first passage with FD +10% fetal bovine serum (FD/FBS). When the cells reached 80% confluence, they were trypsinized and cryopreserved. Briefly, the cell pellet was suspended in 1 ml of DMSO + FBS 20% medium, stored at −80 °C overnight, and then stored in liquid nitrogen at −196 °C.

Fig. 2. Gross evaluation of the OA knee joint 6 weeks after intra-articular injection. Gross morphology of the control (basic media) group showed severe OA lesion without any evidence of repair (2A). The meniscus was entirely damaged in the control group (2B). Joint injected with BM showing some evidence of repair and healing of the cartilage surface (2C). The meniscus showed evidence of scarring in BM group (2D). Joint injected with CM showing a smaller lesion with smooth edges (2E). A significant amount of regeneration of the meniscus like tissue was observed in CM group (2F). Normal contralateral knee joint (2G and 2H).
2.6. Statistical analysis

Comparisons of the histology scores between groups were made using the Mann–Whitney test as it is the most suitable descriptive and non-parametric test. A p value of less than 0.05 between groups was considered significant.

3. Results

3.1. Morphology observation of sheep BMSC in in vitro culture

The BM group showed obvious spindle bipolar to polygonal cells attached to the flask after 4 days of culture. After day 12, the culture reached confluency and formed a dense monolayer of adherent cells (Fig. 1A). In contrast, the CM group grew as cell aggregates and after day 12 formed larger aggregates (Fig. 1B).

3.2. Clinical observations

There was no evidence of infection in any of the animals before and after the experiments. All animals bore weight after surgery. The animals tolerated the cell injection well, and there was no evidence of local inflammation or immobilization of the joint from the cell injection.

3.3. Macroscopic observations post surgery

3.3.1. Cartilage lesion

Changes to the femoral condyle in CM, BM and control groups after 6 weeks of exposure, were independently assessed with regards to articular lesions using the ICRS grading system. Quantitatively, all femoral distal heads and many of the medial plateaus from the meniscectomy knees of the sheep had osteoarthritis. The highest lesion score, 3.44 ± 0.38 points, was observed in the control group (Fig. 2A). On the other hand, the knee joints that received BM showed a smaller lesion of the femoral condyle compared to the control group, demonstrating scores of 1.22 ± 0.89 points (Fig. 2E). The less cartilage lesion scores of 0.8 ± 0.35 points were observed for the knee joints that received CM, the lesion was shallow and exhibited smooth edges (Fig. 2C).

The ICRS scores were compared by analysis of variance and reveal no significant difference between the CM and BM groups (Fig. 3). However, these two groups have a significant difference when compared to the control group (Fig. 3).

3.3.2. Meniscus lesion

The BM and control samples showed a damage and absence of meniscus, respectively (Fig. 2B and D). However in the CM group there is an appearance of meniscal-like tissue formation at the surgical site (Fig. 2F).

Fig. 3. The grading of the joints using the ICRS grading system. The control group scored 3.4 ± 0.38 points, the CM group scored 0.8 ± 0.35 points and the BM group scored 1.22 ± 0.89 points. Scores were compared by analysis of variance. There were significant differences between CM and control, ** p<0.05 and between BM and control, * p<0.05. No significant difference was detected between both groups treated with cells (CM and BM).

3.4. Intra-articular injection of BMSC

After 3 weeks in culture (passage 2), the cells were trypsinized, washed with PBS and suspended in FD at a density of 2×10^6 cells/ml. The sheep were anesthetized, intubated, and placed in dorsal recumbency. Macroscopic observations were done in accordance with the International Cartilage Repair Society (ICRS) cartilage repair assessment system. Using the above system the assessment was recorded using cell culture flasks. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and the medium was changed every 3 days.

2.3. Chondrogenic induction of BMSC

Frozen cells at passage 2 were thawed 3 weeks before intra-articular injection by immediately placing them in a water bath at 37 °C. They were then washed twice with a phosphate buffer solution (1× PBS) containing antibiotic/antimycotic. The cells were then divided into two groups. Group CM was suspended in FD, 1% FBS, 1% ITS (Gibco), 50 μg/ml ascorbic acid-2 phosphate, 40 ng/ml l-praline, 1×10^-4 mM dexamethasone, 5 ng/ml transforming growth factor beta 3 (TGF-β3), and 50 ng/ml insulin like growth factor 1 (IGF-1) according to a previous study (Al-Faqeh et al., 2011). Group BM was suspended in FD supplemented with 10% FBS and plated in T-75 cm² cell culture flasks. All cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂ and the medium was changed every 3 days.

2.4. Intra-articular injection of BMSC

2.5. Gross and histological analysis

After 3 weeks post surgery the animals were sacrificed. The knee of the experimental sheep and contra-lateral joints were dissected and examined macroscopically by two independent (blinded) examiners. Macroscopic observations were done in accordance with the International Cartilage Repair Society (ICRS) cartilage repair assessment system. Using the above system the assessment was recorded using macroscopic examination on the surface of the cartilage. Overall repair assessment was scored and later graded, from normal (grade 1) to severely abnormal (grade 4).

The femoral condyle and proximal tibia were bisected in sagittal plane sections of the operated medial and contra-lateral head of the femur and the distal head of the tibia, using a diamond saw. All specimens were fixed in 10% formalin buffer at 4 °C for 48 h and then decalcified in 0.5 EDTA containing 8% hydrochloric acid for 3 to 4 weeks, with the decalcification solution changed every week. The specimens then underwent automated tissue processing for 24 h using a tissue processor (Leica TP-1020, Germany). Sections were imbedded in paraffin and cut into 5 μm slices. Each section was then stained with haematoxylin and eosin (H&E) and safranin O.

2.6. Statistical analysis

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3.4. Histological analysis

3.4.1. Cartilage

The joints that were injected with BM exhibited cartilage loss almost exposing the bony layer, and showing no evidence of regeneration, (Fig. 4A and B). In this group there was substantial fibrillation of the articular surface with loss of the extracellular matrix as well as large areas of osteophytic remodeling. The BM group displayed reasonable cartilage regeneration when compared to normal contralateral knee joint, (Fig. 4C and D). The CM group also demonstrated reasonable cartilage surface comparable to normal contralateral knee joint (Fig. 4E and F). The superficial area was smooth without any evidence of inflammation and almost similar cartilage thickness to the normal knee, (Fig. 4G and H).

3.4.2. Meniscus

The H&E staining showed presence of cells and fibrous tissue in the meniscus-like tissue of the CM group and this feature resembles fibrocartilage as in native meniscus even though the cell looks immature with large nucleus (Fig. 5).

4. Discussion

Mesenchymal stem cells have become a promising and fascinating biological approach for cartilage repair. The present study evaluated the utility of stem cell therapy in retarding the progression of osteoarthritic lesions that occur following knee joint injury. Six weeks after total medial meniscectomy and resection of the ACL, CM and BM were delivered by an intra-articular injection. In this study, no biomaterial or additional chemicals were used to ensure that clear results from the stem cell therapy in the osteoarthritic knee joint could be observed.

For the induction of osteoarthritis, the ACL ligament and meniscus must be excised. Postoperatively the sheep must undergo normal movement to simulate the continuous trauma and repair as observed in OA. The ICRS grading system is used to evaluate our study as it is

![Fig. 4.](image-url)
the most consistent method used to evaluate current clinical studies on OA treatment utilizing cell therapy (Van den Borne et al., 2007).

The effect of osteoarthritis was not only seen on the cartilage surface, but also on the meniscus site. In the CM group, the results showed a formation of meniscus-like structure at the site of surgery. The histology showed cartilage-like cells with big nucleus resembling an immature tissue and possibly given a longer time it might turn into a mature fibro cartilage similar to native meniscus. At present, the surgical repair of the meniscus is via the removal of the damaged area with or without a graft (Jarit and Bosco, 2010; Stärke et al., 2009; Gomoll et al., 2009; Beaufils et al., 2009). All of the grafts used were either synthetic or non-synthetic without any regenerative advantage. With the injection of CM, as shown in this study, it is possible to regenerate the meniscus.

For the osteocartilage surface, both groups that were treated with cells demonstrated potential regeneration of cartilage and thus retarding the progress of OA. Even though the CM group did not demonstrate any significant differences compared to the BM group, the ICRI score is slightly higher.

Previous in vitro studies have shown that the induction media containing bioactive factors, such as transforming growth factor family (TGF-β), insulin like growth factor 1 (IGF-1) and dexamethasone, promote the synthesis of proteoglycans without significantly affecting inflammation or cartilage breakdown (Chubinskaya et al., 2011; Mi et al., 2000; Lum et al., 1996; Mouharat et al., 2004). TGF-β is capable of increasing proteoglycan synthesis by neutralizing interleukin-1β (IL-1β), which is responsible for the suppression of proteoglycan synthesis (Cheng et al., 2010; Lum et al., 1996). This effect was seen in our in vitro culture experiments, in which BMSC grown in chondrogenic medium containing TGF-β3, IGF-1 and dexamethasone for 3 weeks began to form colonies and aggregated, in contrast to cells seeded in basic medium, which formed multilayered cells.

The concept of injecting BMSC to treat OA is not new and has been the subject of many clinical trials for the last decade (Madry and Cucchiarini, 2011; Centeno et al., 2008). The results are quite promising, but they usually depend on the institution (Pak, 2011; Kasemkijwattana et al., 2011). BMSCs have the ability to suppress immunity and prevent rejection by suppressing the local immune response and preventing scar formation (Caplan and Dennis, 2006). Interestingly, studies have shown that BMSCs derived from a patient with OA are able to produce matrix and even able to express the collagen type II marker upon induction with TGF-β3 (Kafienah et al., 2007; Ringe et al., 2007). A worldwide multicenter clinical trial reported encouraging results on BMSC implantation (Wakitani et al., 2002; Maruccci et al., 2007; Verbruggen et al., 2007; Pak, 2011; Kasemkijwattana et al., 2011). Unfortunately it is quite difficult to standardize the outcome. Most of the studies above were done using cell therapy using undifferentiated BMSC together with surgical treatment. This is an important oversight, since BMSCs are multipotent cells that can potentially create uncontrolled cell regeneration in cells such as osteoblast, fibroblast or even neuroblast if injected without induction to a specific cell lineage. This is very possible in a damaged joint, such as one with traumatic osteoarthritis, which contains multiple growth factors (Barthel et al., 2009). It might be the explanation for why the clinical outcome differs from one center to another. In this study, no surgical intervention was done, only cell therapy. To date, only one case that has been reported by Centeno et al. (2008) relied solely on a percutaneous injection of BMSC expanded in vitro with normal basal medium. From the finding of our study there is an advantage in using induced BMSC over BMSC alone especially after seeing the effect on the meniscus and the possibilities of reducing the morbidity of OA patient.

The next step would be the first phase clinical trial. We do understand that it would be a daunting task as we would be facing a stringent ethical guideline and expensive in vitro cell preparation requirement. Nevertheless, this study has shown the potential of BMSC injection for the treatment of OA, with the induction media displaying better results on meniscus repair. Most importantly, with the increase in worldwide human clinical trials, we hope that this study will serve as a reference for the patients and clinicians involved.

5. Conclusion

From our findings it is suggested that an intra-articular injection into OA knee joints of sheep with autologous bone marrow stem cells cultured in chondrogenic medium has the possibility of retarding the progression of OA.

Abbreviations

BMSC bone marrow mesenchymal stem cells
OA osteoarthritis
TGF transforming growth factor
IGF insulin like growth factor
ACL anterior cruciate ligament
ICRS International Cartilage Repair Society
FBS fetal bovine serum
PBS phosphate buffered saline
CM chondrogenic induced bone marrow stem cells
BM bone marrow stem cell induced in basal media

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

HF, NM, CC, AS and RI participated in animal study, and data acquisition as well as data analysis. HF, AS and RI contributed to the drafting of the manuscript and the study design, and coordination. RI is the principal investigator and grant holder, and gave the final approval for the version to be published. All authors read and approved the final manuscript.

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The text provided is a collection of references from a scientific paper, likely discussing the impact of osteoarthritis and methods for its treatment. The references cover a range of topics including cartilage repair, stem cell therapy, and the use of growth factors and scaffolds in regenerative medicine. The references are cited in the text, indicating a detailed review of the scientific literature on the subject. The references are formatted in a citation style typical of scientific journals, with authors, titles, and publication details. The text does not contain additional commentary beyond the cited references.