



# EVALUATION OF ALLOGENEIC STEM CELL-BASED THERAPY THROUGH HISTOPATHOLOGY IN A RABBIT MODEL OF OSTEOARTHRITIS



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## Abstract

**Osteoarthritis (OA)** is the most common form of arthritis and mostly results in physical disability. The ability of autologous mesenchymal stem cells to regenerate lost articular cartilage in OA has clearly been proven.

The aim of this study was to estimate the allogeneic stem cells as treatment for OA by microscopic pathological anatomy evidence.

Eighteen male New Zealand white rabbits were used in this study. They were divided into 3 groups (n=6); Rabbit stem cell-treated group (RSTG), Media stem cell-treated group (MSTG) and Normal saline-treated group (NSTG). OA was induced by a single intra-articular injection of 2.5 mg of monosodium iodoacetate (MIA) / 0.3 ml normal saline (NS). After 4 weeks of OA induction the (RSTG) was given a single intra articular injection of rabbit bone marrow-derived mesenchymal stem cells (BM-MSCs) at a density of 1.5X10<sup>6</sup> cells / 0.3 ml, while both the (MSTG) & (NSTG) received an injection of the same volume of medium without cells & normal saline as respectively. Rabbits were euthanized by intravenous injection of sodium phenobarbital (Dolethal) 100mg/kg at 16 weeks post-treatment then histopathology images were assessed.

The results showed that there were significant differences among all groups in histopathological scoring of the stifle joints evaluation at week 20. The RSTG showed the best histopathological scoring while the MSTG and NSTG showed the worst scores.

In conclusion, single intra-articular injection of rabbit bone marrow-derived stem cells (allogeneic stem cells) could promote the regeneration of damaged articular cartilage in OA as evidenced by improved histopathological outcomes.

**Key Words:** Osteoarthritis, histopathology, Stem Therapy

## Introduction

OA affects a large number of humans and animals at different ages; it commonly affects horses, dogs, and cats. At least, 80% of joint problems are classified as degenerative joint disease (McDougall, 2006). The Osteoarthritis (OA) is a degenerative disease of the joint characterized by the degradation of articular cartilage with loss of matrix and also cyst and osteophyte formation (Ehrlich, 1972). It affects major weight bearing joints leading to pain, physical incapacity and reduced quality of life (Wolfe et al., 1990). This disease is primarily degenerative in nature and the inflammatory changes are secondary, also the chondrocytes of articular cartilage play an important role in the early stages of the disease development (Thompson et al., 2007).

Thompson et al., (2007) and Farooqi, (2008) classified the OA into primary and secondary. Primary degenerative joint disease refers to those cases which have no apparent predisposing factor and commonly occur in older humans or animals but the secondary degenerative joint disease refers to those cases which have an apparent predisposing factor. Moreover, Chopra and Billampelly, (1997) & Muirden, (2005) found that the occurrence and clinical presentation of degenerative joint disease vary between the developed and developing countries due to geo-ethnic differences in lifestyle and many other factors such as nutritional, genetic, gender, cultural and occupational. The previous authors added that, poor health and nutritional awareness are other factors that might affect both the occurrence and clinical presentation of OA.

There are several drug classes available for OA management in both humans and animals. Most of these drugs are limited to pain control, symptom alleviation, delayed progression of the disease, and improving general mobility and exercise tolerance as well as eliminating the risk factors (Felson et al., 2000b). OA drug treatments include non-steroids such as diclofenac, ibuprofen, naproxen, and ketoprofen, as well as corticosteroids, narcotic as morphine and hyaluronic acid (Taylor et al., 2008). The efficiency of these treatments is still controversial because unfavorable gastrointestinal complications have been reported (Papaioannou et al., 2007). Pharmacological treatment options for OA are still very limited (Baltzer et al., 2009), making the search for more options worthwhile.

Joanne et al. (2004) reported that autologous adult stem cells are a much better potential source of cells than mature chondrocytes, because of their better compatibility and less likelihood of provoking an immune rejection. Furthermore, mesenchymal stem cells (MSCs) have been shown to treat degenerative joint disease, influence regeneration of articular cartilage and slow the progression of the disease (Murphy et al., 2003). Transplantation of MSCs to affected discs in stifle joints of rabbits showed proliferation and differentiation into desired cells resulting in regeneration of affected joints (Daisuke et al., 2005).

Bajada et al. (2008) reported that replacement of either lost or defective tissues can be achieved with the assistance of regenerative medicine when current therapies are inadequate. Regenerative medicine comprises the use of tissue engineering and stem cell technology as the stem cells are suitable and effective biological agents that can help damaged tissues to regenerate because of their ability to renew themselves and differentiate into several types of body tissues such as bones, heart, liver, muscles, etc. under the influence of growth factors but by a process yet undefined and also their differentiation capacity depend on its type either embryonic or non embryonic stem cells. Recently, the attention has been focused on agents that could stimulate the endogenous production of cytokines that can arrest the disease and, in some cases, help rebuild the cartilage in joints that have been damaged by the disease (Selfe and Innes, 2009). Bone marrow-derived MSCs could be promising cell sources for the treatment of OA; neither stem cell culture nor scaffolds are absolutely necessary for a favorable outcome (Singh, et al., 2014). Using of these cells in the treatment of joints & musculoskeletal problems have been investigated in numerous pre-clinical studies in animals (Li et al., 2013).

Thus, it is evident that previous studies on management of degenerative joint disease using autologous stem cells have shown promising results. In this study, we investigated the possibility of using allogeneic stem cells as therapy for OA to study healing of the joints and articular cartilage following experimentally induced OA. The main objective of this work is to evaluate the usefulness of rabbit bone marrow derived mesenchymal stem cell (allogeneic stem cell) therapy in the replacement of degraded articular cartilage through histopathological examination.

## Materials & Methods

### Experimental animals:

Eighteen clinically healthy male New Zealand white rabbits, aged 6 months and weighing between 2.0 and 2.5 kg, were used in this study. The use of animals was approved by the Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, University Putra Malaysia, on 9th April 2010 (Ref. UPM/FRV/PS/3.2.1.551/AUP-R94). Prior to induction of degenerative joint disease, radiographs of both stifle joints were taken to rule out any possible joint disease.

### Isolation and characterization of mesenchymal stem cells from rabbit bone marrow (BM-MSCs):

The isolation of MSCs was performed on euthanized rabbits as illustrated by Braga-Silva et al. (2006). The collected bone marrow was immediately mixed with 5 ml of 83% Dulbecco's Modified Eagle's Medium Ham's F12 (DMEM F12) that contained high glucose supplemented with 15% fetal bovine serum (FBS), 1% penicillin/streptomycin (antibiotic) and 1% amphotericin B (fungi zone) (GIBCO®, USA) as previously described (Al-Timmemi et al., 2011b; Cuevas et al., 2004). Ten ml of previously prepared media was placed in a T75 tissue culture flask and bone marrow suspension was added. The flask was incubated at 37°C in 5% CO<sub>2</sub> for 3 days in a CO<sub>2</sub> incubator. Non-adherent cells were removed together with the old medium and replaced with a fresh medium. After 12 days of incubation, the culture reached the semi-confluent stage (P0) and the monolayer cells were washed twice with 2 ml of phosphate buffer saline (PBS) (pH 7.2). Then, two ml 0.2% trypsin in ethylene diamine tetra-acetic acid (EDTA) (Sigma, USA) was added to the flask and gently mixed for equal distribution in the tissue culture flask for 2 minutes in order to separate adhered cells from the culture flask. The cells were examined under an inverted microscope (Olympic, Japan) until the cells appeared rounded and the trypsin solution was then discarded. DMEM F12 medium containing 10% FBS was added and gently tapped to detach the cells from the flask. The trypsinization process was repeated for another three consecutive sub-cultures.

The cells were harvested by discarding the medium, washing with PBS and addition of trypsin to the tissue culture flask in order to detach the cells. The trypsin solution was then replaced with 10 ml of fresh DMEM F12. The medium and cells were collected in a test tube, centrifuged (Hettich, Germany) at 1800 revolutions per minute (rpm) for ten minutes and the supernatant was decanted to allow for resuspension of the pellet in 2 ml DMEM F12.

The number of cells in each culture flask was quantified using a haemocytometer (Neubaur, Haemocytometer, Hawksleyand son.Ltd, England). Cell suspension (0.1 ml) was removed in a sterile manner and added to a dilution tube containing 0.8 ml of DMEM F12 and 0.1 ml of 0.4% Trypan Blue stain. The mixture was gently mixed at room temperature and a small drop of the stained cell suspension was transferred onto the haemocytometer and cover slip placed on top. A small drop of the cell suspension was removed aseptically using a Pasteur pipette and placed on one side of the haemocytometer and examined under the inverted microscope (Leica, Aulerian). The total number of viable cells in each four corners of the haemocytometer was counted. The total number of cells harvested from the tissue culture flasks was determined using the following equation: NCxDx104/Q, where NC=number of count vital cells (non-vital cell is stained blue), D=sample dilution (10) and Q=number of squares used in haemocytometer (Freshney, 2001).

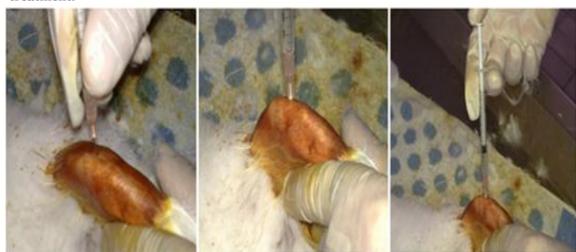
At 1st passage the stem cells were preserved using liquid nitrogen N<sub>2</sub>. Since freezing can be lethal to cells due to the effects of damage by ice crystals, alteration in the concentrations of electrolytes, dehydration and changes in pH, a typical freezing medium containing 90% serum and 10% Dimethyl sulfoxide (DMSO) was used, as reported by Fleming and Hubel, (2006) and Linch et al., (1982). The isolated cells were pre-characterized by their morphology, multipotency and immunophenotyping characters of stem cells to ensure the isolated cells were mesenchymal stem cell (MSCs) in nature.

### Induction of Osteoarthritis:

Rabbits were anaesthetized using intra-muscular injection of Ketamine hydrochloride - xylazine hydrochloride - acepromazine at the dose rate of 40 mg / kg, 5 mg / kg and 1 mg / kg respectively. Adequate anesthesia depth was monitored then the hair over the left stifle joint was clipped and the skin was aseptically prepared. A 26-gauge 1 1/2 inches hypodermic needle was used to inject 2.5 mg MIA / 0.3 ml NS intra-articularly. The needle was inserted into the mid-line and advanced between the femoral epicondyles and menisci. Care was taken not to have any evidence of leakage through the needle tract (Holland et al., 2000; Kim et al., 2008).

### Protocol of Treatment:

The current method used the allogeneic MSCs that explored for their potential to regenerate damaged tissues by OA in MIA-induced model of OA in rabbit's stifle joint. Three treatment groups were used in this study (Six rabbits were used in each group). The first group, the rabbit stem cell-treated group (RSTG) was given a single intra articular injection of rabbit bone marrow-derived MSCs at a density of 1.5X10<sup>6</sup> cells / 0.3 ml media. The second group, the media stem cell-treated group (MSTG) received an injection of the same volume of medium without cells into the osteoarthritic stifle joints. The third group, the control group, the NS-treated group (NSTG) was given a single intra-articular injection of 0.3 ml NS in the affected stifle joints. Rabbits were euthanized by intravenous injection of sodium phenobarbital (Dolethal) 100mg/kg at 16 weeks post-treatment.



### Histopathology Evaluation:

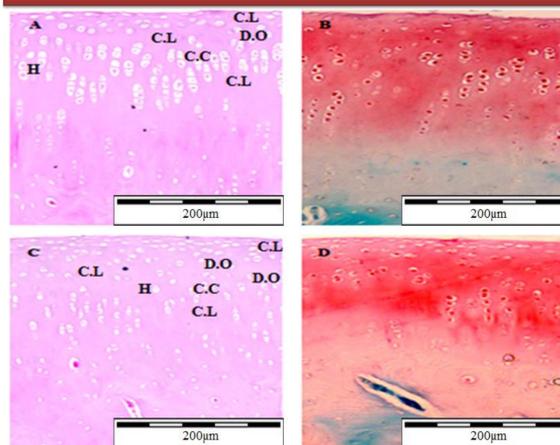
The stifle joints of both legs were fixed in 10 % formalin for about two months, followed by decalcification with EDTA + 12% hydrochloric acid for about one month. The decalcification solution was changed twice per week. Samples of both tibia and femur were firstly separated into medial and lateral parts and further subdivided into two parts. The samples were prepared by the ordinary histological technique then stained with Hematoxylin-Eosin (H & E) and Safranin O stains. The histological images were captured using a microscope image analyzer (OLYMPUS) According to (Bancroft, J., 1990).

According to Kobayashi et al. (2003); Histopathological changes of the articular cartilage and subchondral bone were evaluated after staining with H and E stain, normal (no changes) indicates absence of OA lesion in articular cartilage and subchondral bone. Mild changes denote only small or focal area (less than 50%) of the articular cartilage or subchondral bone showing changes. Moderate changes showed that about 50% of articular cartilage or subchondral region was affected. Severe changes indicated histopathological changes of large area (more than 50%) of articular cartilage or subchondral region.

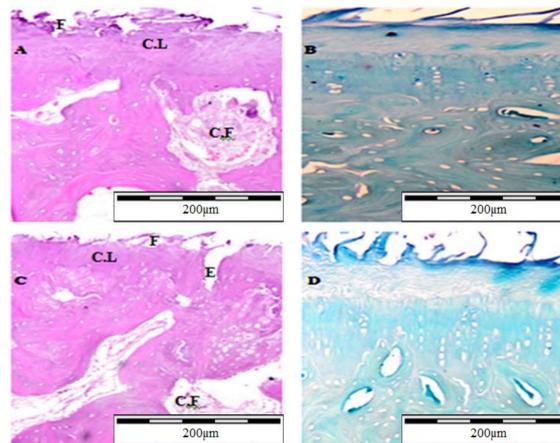
### Statistical Analysis

The mean histopathological scores were calculated with standard deviations. The difference between groups was analyzed using the Mann-Whitney U test. Statistical analyses were performed using SPSS 16 (IBM, New York, United States) and a p-value < 0.05 was considered to indicate statistical significance.

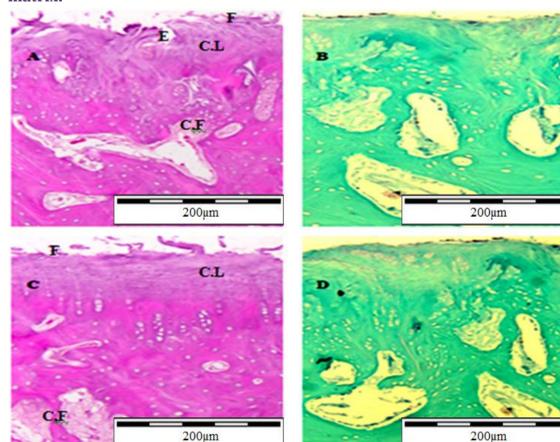
## Results & Discussion



**Plate 1:** Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the RSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle and (C) H and E staining of the Tibial plateau; both A and C revealed smooth articular cartilage surfaces with mild chondrocyte loss (C.L) in the tangential zone. They also showed mild cellular loss (C.L) in transitional and radial zones with mild chondrocyte colonies (C.C), hypertrophy (H) and chondrocyte disorganization (D.O). Besides that, there was no pathological change detected in the subchondral bone. (B) Safranin O staining of the Femoral condyle; (D) Safranin O staining of the Tibial plateau; both B and D revealed mild loss of staining of the intercellular matrix.



**Plate 2:** Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the MSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle; (C) H and E staining of the Tibial plateau; both A and C revealed very severe fibrillation (F). They also showed severe to complete cellular loss in tangential, transitional and radial zones. Besides that, the subchondral bone showed severe fibrosis and cyst formation (C.F). (B) Safranin O staining of the Femoral condyle; (D) Safranin O staining of the Tibial plateau; both B and D revealed severe to complete loss of staining of the intercellular matrix.



**Plate 3:** Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the NSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle; and D revealed severe to complete loss of staining of the intercellular matrix. (C) H and E staining of the Tibial plateau; both A and C revealed very severe fibrillation (F) and erosion (E). They also showed severe to complete cellular loss in tangential, transitional and radial zones. Besides that, the subchondral bone showed severe fibrosis and cyst formation (C.F). (B) Safranin O staining of both B and D revealed severe to complete loss of staining of the intercellular matrix.

## Conclusion

Overall, the current study evaluated the usefulness of rabbit BM-MSCs (allogeneic stem cells) in the replacement of degraded articular cartilage through histopathological scores for articular cartilage and subchondral bone of the stifle joint. The findings indicated that the treatment with rabbit BM-MSCs was an effective therapy in correction of OA. Both media without cells and normal saline treatments produced the most severe histopathological changes and proved that these agents had no remedial effect on degenerative joint disease (OA). According to this study results and the previously published papers, more advanced investigations should be performed for confirmation of stem cell therapy in this field (OA).

In conclusion that successful use of both allogeneic stem cells therapies to replace degraded articular cartilage will provide an opportunity to reduce the cost, time and effort that are involved currently in the treatment of OA in humans and large animals such as sport horses, which are susceptible to joint disease.

Finally, We hypothesized that the replacement of degraded articular cartilage using stem cells therapy provides a useful alternative approach in the treatment of degenerative joint disease.

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