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Histopathological Assessment for the Effect of Allogenic Stem Cells Based Therapy in Stifle Joint for Rabbit Model of Osteoarthritis

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Abstract

Background: Osteoarthritis (OA) is the most common form of arthritis and cause physical disability. The ability of autologous mesenchymal stem cells to regenerate lost articular cartilage in OA been clearly confirmed.

Objectives: The aim of this study was to estimate the allogeneic stem cells as treatment for OA by histopathological evidence.

Materials and Methods: Twenty-four (24) males New Zealand white rabbits were used in this study. They were divided into four groups (n=6); Rabbit stem cell-treated group (RSTG), Sodium Hyaluronate-treated group (SHTG), Media stem cell-treated group (MSTG) and Normal saline-treated group (NSTG). OA was induced by a single intra-articular injection of monosodium iodoacetate (MIA) 2.5 mg/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 1.5X106 cells / 0.3 ml media, while the (SHTG) was treated with four injections of 0.3 ml 0.1% sodium hyaluronate at weekly intervals starting 4 weeks post OA induction. Lastly, 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 20 weeks post-OA induction then histopathology images were assessed.

Results: 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 followed by SHTG, which are restricted to pain relief, delayed progression of the disease, and improving general mobility while the MSTG and NSTG showed the worst scores.

Conclusion: In conclusion, a single intra-articular injection of allogeneic stem cells could promote the regeneration of damaged articular cartilage in OA as evidenced by improved histopathological outcomes.

Keywords: Osteoarthritis; Stifle joint; Rabbit; Histopathology; Allogenic stem cells; Stem cells therapy

Introduction

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 [1]. Osteoarthritis is the most common form of arthritis, and mostly results in physical disability. OA affects major weight bearing joints leading to pain, physical incapacity and reduced quality of life [2]. Other researchers conform to the view that the disease is primarily degenerative in nature and the inflammatory changes are secondary [3]. The chondrocytes of articular cartilage play an important role in the early stages of the disease development. OA were classified 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 that have an apparent predisposing factor. OA is multifactorial in origin and normally does not have a single cause or factor that could be used to explain why OA does not behave in the same way over the world [4]. Both groups of researchers; 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 [5,6]. The previous authors added that, poor health and nutritional awareness are other factors that might affect both the occurrence and clinical presentation of OA. 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 [7]. Wise et al. suggested an association between deteriorated measures of mental health and OA pain and risk of pain flares [8]. General mental health is a modifiable component of health and maybe a new avenue for preventing outbreaks of OA pain. 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 [9]. OA drug treatments include non-steroids such as diclofenac, ibuprofen, naproxen, and ketoprofen, as well as corticosteroids, narcotic as morphine and hyaluronic acid [10]. The efficiency of these treatments is still controversial because unfavorable gastrointestinal complications have been reported [11]. Pharmacological treatment options for OA are still very limited, making the search for more options worthwhile [12]. Recently, 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 [13].

Bajada et al. reported that replacement of either lost or defective tissues can be achieved with the assistance of regenerative medicine when current therapies are inadequate [14]. 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.

Joanne et al., 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 [15]. 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 [16]. Transplantation of MSCs to affected discs in stifle joints of rabbits showed proliferation and differentiation into desired cells resulting in regeneration of affected joints [17]. 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 and xenogeneic stem cells as therapy for OA to study healing of the joints and articular cartilage following experimentally induced OA. Successful use of both allogeneic and xenogeneic 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. The main objective of this work is to evaluate the usefulness of rabbit bone marrow derived mesenchymal stem cell (allogeneic stem cell) therapies in comparison with sodium hyaluronate in the replacement of degraded articular cartilage through histopathological examination.

Materials and Methods

Experimental animals

Twenty four male New Zealand white rabbits, aged 6 months and weighing between 2.0 and 2.5 kg, were used in this study. All rabbits were certified clinically healthy following physical and blood profile examinations. The rabbits were housed in individual cages, fed on commercial diet (Cargill) and drinking water was

provided addlibitum. Physical examination was done weekly during the study period, which included rectal temperature, pulse and respiratory rate to ensure that they were in healthy state. 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 rabbit bone marrow-derived mesenchymal stem cells (BM-MSCs)

The isolation of MSCs was performed on euthanized rabbits as illustrated by Braga-Silva et al. [18]. This included anesthetizing the rabbits with ketamine-xylazine and subsequently euthanizing them with sodium pentobarbital (Dolethal). An incision was then made through the skin on the cranial thigh region and all muscles attached to the femur were removed to allow for a brief immersion of the femoral bone in 70% alcohol. The femoral bone was later placed in a 50 ml falcon tube containing media and both ends of the epiphysis were cut using a bone cutter. Finally, bone marrow was flushed out into a 15ml falcon tube with 5ml media.

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 [19,20]. 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 37^0C in 5% CO_2 for 3 days in a CO_2 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 di amine 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 trypsination 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, Auterian). 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 [21]. At 1st passage the stem cells were preserved using liquid nitrogen N2. 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, [22] and Linch et al., [23]. 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 based on eyes and intra-digital reflexes, heart rate, respiratory rate and response to stimuli. The hair over the left stifle joint was clipped and the skin was aseptically prepared as is routine using chlorhexidine scrub, 70% alcohol and tincture iodine. A 26–gauge 1 ½ 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. Any resistance to injection was taken as evidence that the needle was not being introduced into the joint cavity, and in such cases the needle was re-positioned before attempting to administer the MIA. The needle was withdrawn when the injection was complete. Care was taken not to have any evidence of leakage through the needle tract [24,25].

Protocol of treatment

The current methods of treatments using both allogeneic and xenogeneic MSCs were explored for their potential to regenerate damaged tissues by OA in MIA-induced model of OA in rabbit's stifle joint. Four 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 1.5X106 cells / 0.3 ml media (the cells were in the second passage, and were derived from an anesthetized rabbit and cryopreserved at -20 ^{0}C). 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 sodium hyaluronate-treated group (SHTG) was treated with four injections of 0.3 ml 0.1% sodium hyaluronate at weekly intervals starting 4 weeks post OA induction. Lastly, The fourth 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.

Note: All rabbits were exercised for 10 mins daily during the whole study period, except for the first month after initiation of treatments, in which case the rabbits were rested.

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 dehydrated through an ascending series of ethanol, followed by clearing with xylene and finally, impregnation in paraffin using an automated tissue processing apparatus. After embedding in paraffin blocking, sectioning was achieved using microtome, and the slides were stained with Hematoxylin-Eosin (H and E) and Safranin O stains. Then, histological images were captured using a microscope image analyzer (OLYMPUS) According to Bancroft, [26]. Histopathological changes of the articular cartilage and subchondral bone were evaluated after staining with H and E stain using the scoring system described by Kobayashi et al. [27]. Briefly, the degree of changes were graded as no changes (normal): 0, slight changes (mild): 1, moderate changes (moderate): 2 and severe changes (severe or very severe): 3. The grading items included chondrocyte cells loss, chondrocyte cloning and hypertrophy, chondrocyte disorganization, surface irregularity of articular cartilage, fibrillation of articular cartilage surface, Safranin O stain reduction, degeneration/necrosis, marginal osteophyte formation and subchondral changes (Table 1).

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.

Table 1: *Histological grading scale.*

Grading Scales Histological OA feature	Grade (0)	Grade (1)	Grade (2)	Grade (3)
Chondrocyte Loss	Normal	Mild	Moderate	Severe
Chondrocyte Cloning & Hypertrophy	Normal	Mild	Moderate	Severe
Chondrocyte Disorganization	Normal	Mild	Moderate	Severe
Surface Irregularity of Articular Cartilage	Normal	Mild	Moderate	Severe
Fibrillation of Cartilage Surface	Normal	Mild	Moderate	Severe
Safranin O Stain Reduction	Normal	Mild	Moderate	Severe
Degeneration/Necrosis	Normal	Mild	Moderate	Severe
Marginal Osteophyte Formation	Normal	Mild	Moderate	Severe
Subchondral Changes	Normal	Mild	Moderate	Severe
Total Histological OA Score	0 – 27			

Ethical consideration

The Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, University Putra Malaysia (UPM) approved the use of animals, on 9th April 2010 (Ref. UPM/FRV/PS/3.2.1.551/ AUP-R94).

Statistical analysis

According to Duncan, [28], nonparametric statistical methods were used to analyze the data from the study using SPSS version 16 for the window software package. P value < 0.05 was considered statistically significant. Data was expressed as mean ± standard deviation (SD) of mean. Kruskal-Wallis and Mann Whitney (one tail) tests were conducted on histopathological scoring among all groups.

Results

Histopathological evaluation was done on stifle joints after 20 weeks of OA induction (16 weeks after application of different treatments). Right (normal) joint samples were observed under 200µm powered objective and showed smooth articular cartilage surface with the underneath layer of flattened chondrocytes in

tangential zone. Chondrocytes were normally arranged in parallel rows in the transitional and radial zones of the articular cartilage while the subchondral bone revealed normal distribution of trabeculae composed of osteocytes and canaliculli surrounding bone marrow filled with blood forming elements. Additionally, intercellular matrix were deeply and uniformly stained with Safranin O fast green stain in the non-calcified part (region extending from articular surface to tidemark) and to a lesser extent in the calcified region (Figure 1).

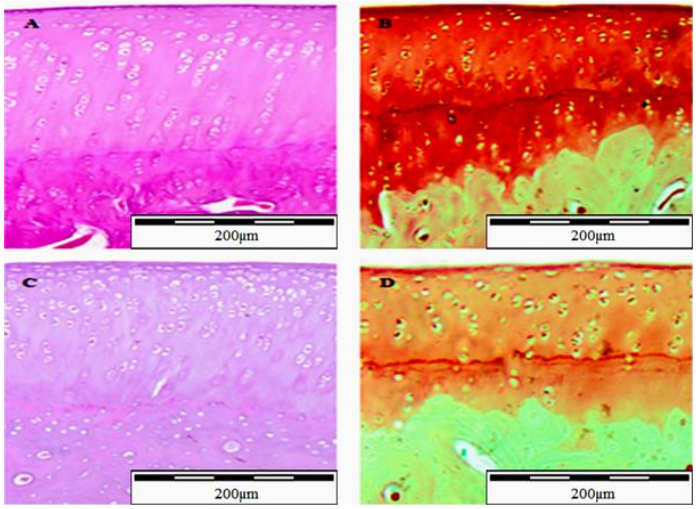

Figure 1: *Right (normal) articular cartilages and subchondral bones of the femoral condyle and tibial plateau observed under 200 µm powered objective. (A) H and E staining of the femoral condyle; (C) H and E staining of the tibial plateau. Both A and C revealed smooth articular cartilage surface with underneath flattened chondrocytes of the tangential zone. Chondrocytes were distributed in parallel rows in transitional and radial zones. 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 uniformly normal staining of the extracellular matrix.*

The histopathological observations for left OA stifle joint (articular cartilage and subchondral changes) showed loss of chondrocyte cells, cloning and hypertrophy of chondrocytes, chondrocyte disorganization, surface irregularity of articular cartilage, and fibrillation of articular cartilage surface, reduction in intensity of Safranin O stain, degeneration, necrosis and marginal osteophyte formation. These observations have been described according to the different groups as follows:

- RSTG: Left (OA) articular cartilages and subchondral bones of the femoral condoyle and tibial plateau were observed under 200µm powered objective after staining with H and E stain revealed smooth articular cartilage surfaces with mild chondrocyte loss in the tangential zone. Chondrocytes were distributed in parallel rows in transitional and radial zones but besides that there were no pathological changes detected in the subchondral bone. Also, there was mild loss of staining with Safranin O fast green stain of the intercellular matrix. The total histopathological score for this group was 9.16 ± 0.56 derived from the summation of 3.83 ± 0.28 (femoral condyle) and 5.33 ± 0.28 (tibial plateau) (Figure 2, 3) (Tables 2 and 3).

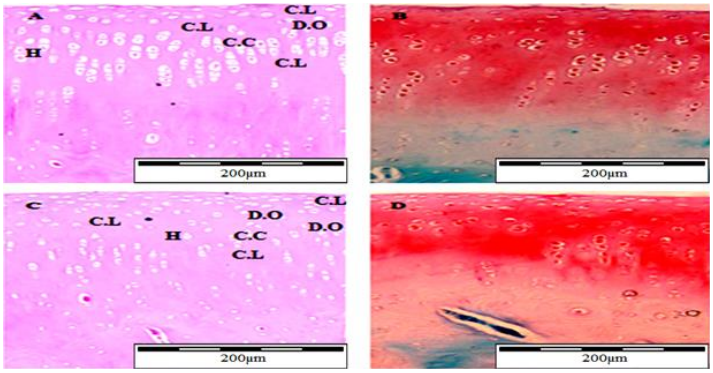

Figure 2: *Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the RSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle and (C) H and E staining of the Tibial plateau; both A and C revealed smooth articular cartilage surfaces 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.*

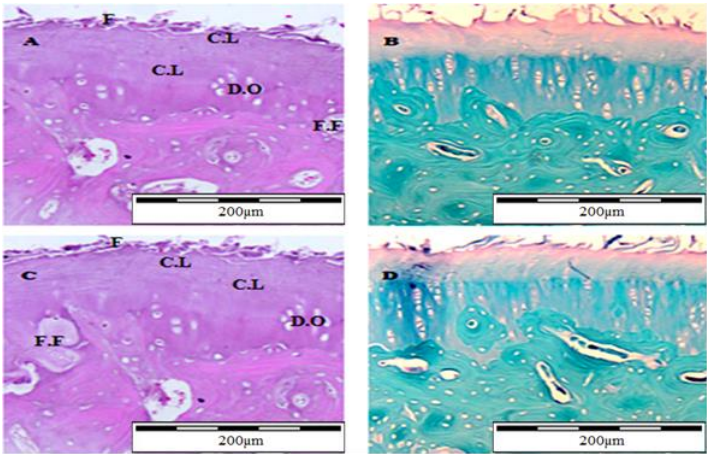

Figure 3: *Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the SHTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle; (C) H and E staining of the Tibial plateau; both A and C revealed moderate to severe fibrillation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

Table 2: *Histopathology scoring of left (OA) stifle joint (Femur) for different groups at week 20.*

Observations	RSTG	SHTG	MSTG	NSTG
Chondrocyte Loss	1 1 0 0 0 0	2 2 2 3 2 2	3 2 2 3 3 3	3 3 3 3 2 3
Average Pathology Score	0.33	2.17	2.67	2.83
Chondrocyte Cloning and Hypertrophy	1 0 2 0 1 1	3 2 2 2 2 3	2 3 3 3 3 3	2 3 2 2 3 3
Average Pathology Score	0.83	2.33	2.83	2.50
Chondrocyte Disorganization	0 0 1 1 1 1	3 2 2 2 2 2	3 2 3 2 3 3	3 3 3 3 2 3
Average Pathology Score	0.67	2.17	2.67	2.83
Surface Irregularity of Articular Cartilage	0 0 2 1 0 0	2 2 2 3 2 1	3 3 2 2 2 3	3 3 3 2 3 2
Average Pathology Score	0.50	2.00	2.50	2.67
Fibrillation of Cartilage Surface	0 0 0 2 0 0	1 2 2 2 2 2	2 3 3 3 3 2	3 2 3 3 3 3
Average Pathology Score	0.33	1.83	2.67	2.83
Safranin O Stain Reduction	1 1 1 0 1 1	2 3 2 2 3 2	3 3 3 3 3 3	3 3 3 3 3 3
Average Pathology Score	0.83	2.33	3.00	3.00
Degenerative/Necrosis	0 0 0 1 0 0	2 2 3 2 2 2	3 3 3 3 2 3	3 3 3 3 2 2
Average Pathology Score	0.17	2.17	2.83	2.67
Marginal Osteophyte Formation	0 0 0 0 0 0	3 3 2 2 2 2	2 2 3 3 3 3	3 2 2 3 3 2

Average Pathology Score	0.00	2.33	2.67	2.50
Subchondral Changes	0 1 0 0 0 0	2 2 2 2 1 1	2 2 3 3 3 3	3 2 3 3 3 3
Average Pathology Score	0.17	1.67	2.67	2.83
Total Averages Pathology Scores ± SD	3.83± 0.28	19.00± 0.22	24.51± 0.13	24.66± 0.16
0: No Change, 1: Mild, 2: Moderate, 3: Severe				

Table 3: *Histopathology scoring of left (OA) stifle joint (Tibia) for different groups at week 20.*

Observations	RSTG	SHTG	MSTG	NSTG
Chondrocyte Loss	1 1 0 0 0 0	2 2 2 3 2 3	2 3 3 3 3 3	3 3 3 3 3 3
Average Pathology Score	0.33	2.33	2.83	3.00
Chondrocyte Cloning and Hypertrophy	1 0 2 0 2 1	3 2 2 3 2 3	3 3 3 3 3 3	3 3 3 3 3 2
Average Pathology Score	1.00	2.50	3.00	2.83
Chondrocyte Disorganization	0 0 1 1 2 1	3 3 2 2 2 2	2 3 3 3 3 3	3 3 3 3 3 3
Average Pathology Score	0.83	2.33	2.83	3.00
Surface Irregularity of Articular Cartilage	0 1 2 1 0 0	2 2 2 3 2 2	3 3 2 2 3 3	3 3 3 2 3 3
Average Pathology Score	0.67	2.17	2.67	2.83
Fibrillation of Cartilage Surface	1 0 1 2 0 0	2 2 2 2 2 3	3 3 3 3 3 2	3 3 3 3 3 3
Average Pathology Score	0.67	2.17	2.83	3.00
Safranin O Stain Reduction	1 1 1 0 2 1	2 3 2 2 3 3	3 3 3 3 3 3	3 3 3 3 3 3
Average Pathology Score	1.00	2.50	3.00	3.00

Degenerative/Necrosis	0 0 1 1 0 0	2 2 3 2 3 2	3 3 3 3 2 3	3 3 3 3 3 2
Average Pathology Score	0.33	2.33	2.83	2.83
Marginal Osteophyte Formation	0 0 0 0 0 1	3 3 2 2 3 3	3 2 3 3 3 3	2 3 3 3 3 3
Average Pathology Score	0.17	2.67	2.83	2.67
Subchondral Changes	0 1 0 0 0 1	2 2 2 2 1 2	3 3 3 3 3 2	3 3 3 3 3 3
Average Pathology Score	0.33	1.83	2.83	3.00
Total Averages Pathology Scores ± SD	5.33± 0.28	20.83± 0.23	25.65± 0.09	26.32± 0.11
0: No Change, 1: Mild, 2: Moderate, 3: Severe.				

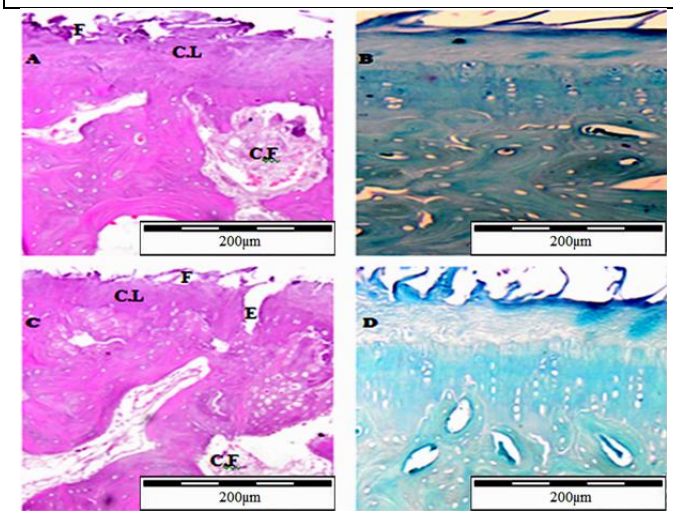

Figure 4: *Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the MSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle; (C) H and E staining of the Tibial plateau; both A and C revealed very severe fibrillation (F). They 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.*

- SHTG: Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau were observed under 200µm powered objective after staining with H and E stain revealed moderate to severe fibrillation and chondrocyte loss in tangential zone. Also, there were moderate to severe cellular loss in tangential and radial zones with moderate to severe chondrocyte colonies, hypertrophy, necrosis and disorganization. Besides that, subchondral structures showed some changes including replacement of bone marrow elements with fibrous tissue, with accompanying cyst formation in some joints. Also, it revealed moderate to severe loss of staining with Safranin O fast green stain of the intercellular matrix. The total histopathological score for this group was 39.83 ± 0.45 derived from the summation of 19.00 ± 0.22 (femoral condyle) and 20.83 ± 0.23 (tibial plateau) (Figure 4).
- MSTG: Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau were observed under 200µm powered objective after staining with H and E stain revealed severe to very severe fibrillation and chondrocyte loss in tangential zone. Also, it showed moderate cellular loss in tangential and radial zones with severe to very severe chondrocyte colonies, hypertrophy, necrosis and disorganization. Besides that, the subchondral bone showed mild subchondral fibrosis with small subchondral cyst formation and marginal osteophyte formation. Also, there was severe to very severe loss of staining with Safranin O stain of the intercellular matrix. The total histopathological score for this group was 50.16 ± 0.22 derived from the summation of 24.51 ± 0.13 (femoral condyle) and 25.65 ± 0.09 (tibial plateau) (Figure 5,6).

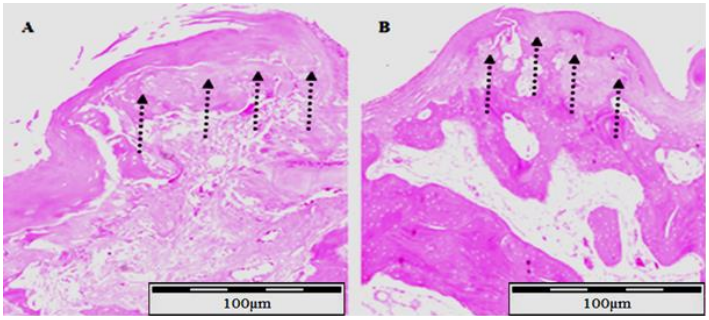

Figure 5: *Picture of marginal osteophyte formation on the femoral condyle (A) and tibial plateau (B) of the MSTG observed under 100 µm powered objective.*

- NSTG: Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau were observed under 200µm powered objective after staining with H and E stain revealed severe to very severe fibrillation and chondrocyte loss in tangential zone. Also, it showed moderate cellular loss in tangential and radial zones with severe to very severe chondrocyte colonies, hypertrophy, necrosis and disorganization. Besides that, subchondral structures showed some changes including replacement of bone marrow elements with fibrous tissue. There was accompanying cyst formation in some joints and marginal osteophyte formation. Additionally, it showed severe to very severe loss of staining with Safranin O stain of the intercellular matrix. The total histopathological score for this group was 50.98 ± 0.27 derived from the summation of 24.66 ± 0.16 (femoral condyle) and 26.32 ± 0.11 (tibial plateau) (Figures 7 and 8; Tables 2 and 3). Overall, there were significant differences at $P < 0.05$ among different treated groups for the average histopathological observations (Figure 7).

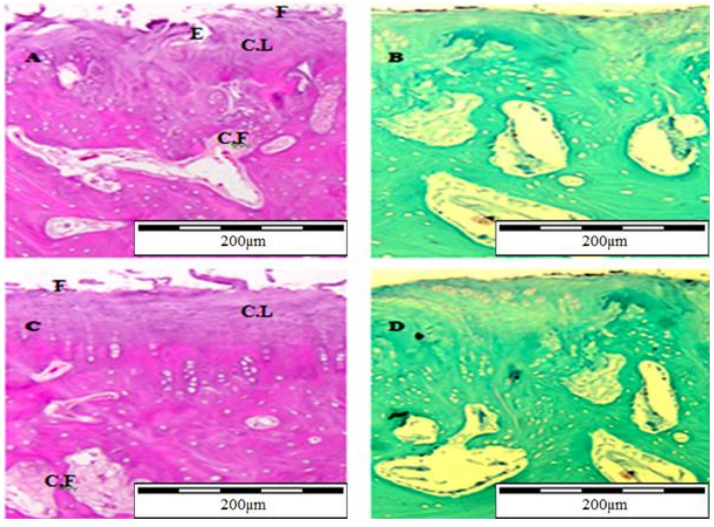

Figure 6: *Left (OA) articular cartilages and subchondral bones of the femoral condyle and tibial plateau of the NSTG observed under 200 µm powered objective. (A) H and E staining of the Femoral condyle; (C) H and E staining of the Tibial plateau; both A and C revealed very severe fibrillation (F) and 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 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.*

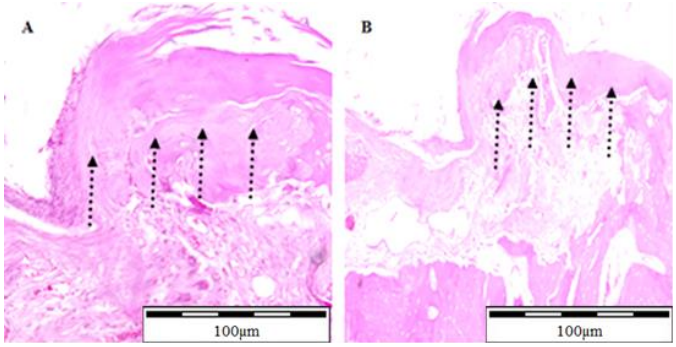

Figure 7: *Picture of marginal osteophyte formation on the femoral condyle (A) and tibial plateau (B) of the NSTG observed under 100 µm powered objective.*

Discussion

The present work on the histopathological observations in stifle joints indicated that there were significant differences among the different groups after 20 weeks of OA induction (16 weeks after the start of treatments). The right normal joints revealed no detectable histopathological changes in the joint structures (articular cartilage and subchondral changes), and there were no significant differences among the different groups. Histopathological changes in the left OA-induced stifle joint were detected and were significantly differences among different groups after 20 weeks of OA induction. In the present work, the allogeneic stem cell-treated rabbits (RSTG) showed regeneration ofarticular cartilage in the left OA induced stifle joint and the histological appearance showed normal to mild changes in joint structures. That might be due to allogeneic stem cell secretions which activate the residual stem cell and help rebuild cartilage in affected articular cartilage. This is in agreement with previous works in which it was proposed that MSCs are able to adhere to articular cartilage surfaces in the absence of other substrates in human and rabbit [29,30]; in other previous studies, also consistent with the present work, MSCs cultured with TGF-β3 and IGF-1 have been successfully used to repair articular cartilage defects in-vitro [31,32] and in-vivo [33-35]. Other previous studies using histological observation found that the knee joint treated with autologous MSCs clearly demonstrated the repair of articular cartilage, meniscal tissue regeneration and retardation of the progressive destruction normally seen in those models of OA [16,36-38]. In yet another recent study, it was suggested that allogeneic MSC-based cartilage repair over generations are

feasible and may also validate the use of immature porcine models as clinically relevant to test the feasibility of synovial MSCs-based therapies in chondral lesions [39]. Also, a recent study conducted in the rabbit model of OA treated with allogeneic MSCs revealed lesser grade of cartilage degeneration, formation of osteophyte and subchondral sclerosis than the control group. The quality of cartilage was significantly better in the cell-treated group compared to controls. One possible reason for this may be the genetic similarity of laboratory rabbits. However, large groups and longer periods of the study may provide additional support for the use of this therapeutic approach as a new way of engineering cartilage [40]. All the previous studies mentioned earlier are consistent with the present work. However, a subsequent equine study performed in equine model developed carpal osteochondral fragment, the results of which showed no response to treatment with bone marrow-derived cells histologically. It seems that the outcome of this technique can be greatly enhanced by the timely administration of MSCs into the joint space [41]. Several studies using cell-labeling techniques have shown that, after intra-articular injection, MSCs preferentially localize to structures of articular soft tissue with little or no adhesion of the injected cells to the cartilage surface [16,30, 42,43] and little or no retention in cartilage defects [30,42-46]. In light of these results, the beneficial effects of intra-articular administered MSCs on articular cartilage are probably mediated primarily by effects on other joint tissues or by soluble factors or by cells attraction to chemokines [47,48], like subchondral mesenchymal progenitor cells, which are sensitive to these chemotactic signals. In addition, OA articular chondrocytes secrete morphogenetic factors that stimulate chondrogenic differentiation of MSCs with the phenotypic characteristics of joint, as opposed to populations of endochondral chondrocytes [49]. Previous studies mentioned earlier are in contrasts to the current study perhaps because some of these studies were on aged animal models or because of the varied effects of MSC therapy on different cartilage defects. In the present study, sodium hyaluronate (SHTG) showed reduced OA progression compared with control, and revealed moderate to severe histopathological changes in the joint structures likely as a result of sodium hyaluronate ability to decline OA progression in affected OA stifle joint structure. It is agree with recently studies illustrated by Kim et al., [25] which explored the effect of growth hormone in OA therapy compared to the effect of hyaluronic acid in rabbit MIA model of OA. Another work was Similar to present research, which concluded that the HA therapy was used in the treatment of high quality cases with OA and there was no cure [50]. While disagree with research published by Mihara et al., [51] that tested the effect of intra-articular injections of high molecular weight sodium hyaluronate (HA) and NSAID on affected joints in a model of OA-induced rabbit with partial meniscectomy, and showed that in the HA group, the damaged cartilage area decreased and cartilage degeneration was ameliorated. Disagree may be due to different way of OA induction, or could be due the type of hyaluronate that used in each research. Also, another work in a rabbit model of OA induced by ACLT concluded by Amiel et al., [52] that was un likely with current work which indicated that the repeat courses of HA can reduce the degree of articular degeneration observed over a 26-week follow-up period compared with no treatment, this contrast might be due to the different repeated courses of HA lead to different result. In the current study, the left stifle joint induced with OA from the MSTG or NSTG showed increased severity of OA progression and revealed severe to very severe microscopic appearance in joint structures. It is likely that both treated groups did not respond to the treatments, which may have resulted in the progression of OA with severe consequences. These findings are in accordance with previous studies in which severe histopathological lesion of OA were observed in the stifle joint injected with basal medium only [37,39,40]. In this aspect, another study illustrated that normal saline showed more severity in histopathological lesions compared to other groups [25,51,52]. Generally, there is limited information on the use of xenogeneic stem cells approach for OA therapy. This limitation in data may be due to the fact that greater antigenic differences exist between different species than within the same species, and so the immune response to xenografts is much stronger than allograft. Thus, induction of tolerance is essential to the success of clinical xenotransplantation. The ability to induce tolerance across highly disparate xenogeneic barriers remains poorly studied. The genetic incompatibility between species can also affect the induction of xenograft tolerance by mixed chimerism. While we are still far from achieving tolerance in clinical xenotransplantation, recent studies using a transgenic mouse model have demonstrated the principle that mixed hematopoietic chimerism can induce mouse and human T cell tolerance to xenografts from pig. The induction of tolerance through mixed chimerism depends on the successful engraftment of donor hematopoietic cells in the hosts and the ability of the chimeric cells to eliminate the development and/or decline the xenoreactive T cell function [53].

Overall, the histopathology results for articular cartilage and subchondral bone from the current study indicate that the allogeneic treatments have the best therapeutic effect on OA, followed by xenogeneic therapy which showed significant improvement in the histopathology of the articular cartilage and subchondral. Sodium hyaluronate on the other hand delayed the progression of OA, while the media of stem cells and normal saline showed the most severe histopathological changes in the articular cartilage and subchondral. These appear to mirror findings from previous researches [37,39,40].

Conclusions

Overall, the current study evaluated the usefulness of rabbit BM-MSCs (allogeneic stem cells) therapies in comparison with sodium hyaluronate in the replacement of degraded articular cartilage through histopathological scores for articular cartilage and subchondral bone of the stifle joint were evaluated, which indicated that the treatment with rabbit BM-MSCs was the most effective therapy, followed by Sodium hyaluronate therapy. 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).

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