


Treatment of Monolateral Legg-Calvé-Perthes Disease with Autologous Bone Marrow Mononuclear Cells in 32 Dogs

Alberto Maria Crovace¹ Luca Lacitignola² Francesco Staffieri² Edda Francioso² Giacomo Rossi³
Antonio Crovace²

¹IRCSS Saverio De Bellis, Castellana Grotte (Bari), Italy

²Dipartimento dell'Emergenze e trapianti di Organo, Università degli Studi di Bari, sez Cliniche veterinarie e p.a., Bari, Puglia, Italy

³University of Camerino School of Bioscience and Veterinary Medicine, Camerino, Marche, Italy

Address for correspondence Luca Lacitignola, DVM, PhD,

Dipartimento dell'Emergenze e trapianti di Organo, Università degli Studi di Bari, sez Cliniche veterinarie e p.a., Bari 70010, Puglia, Italy (e-mail: luca.lacitignola@uniba.it).

VCOT Open 2020;3:e1–e10.

Abstract

Objective In the present study, we report our results of the use of autologous bone marrow mononuclear cells (BMMCs) as a minimally invasive treatment for Legg-Calvé-Perthes in dogs.

Study Design In accordance with Ljunggren's scale, inclusion criteria were determined by clinical condition and radiographic features of the disease, resulting in 32 dogs enrolled in this retrospective study from 2007 to 2019. Bone marrow was collected from each dog from the iliac crest and the mononuclear fraction was separated with density gradient centrifugation. The mean number of BMMCs was $104.7 \pm 46.5 \times 10^6$ cells. The mean BMMC colony-forming units were $71.6 \pm 51.9 \times 10^2/\text{mL}$.

Cells were suspended in fibrin glue before BMMC administration and implanted via transcutaneous injection under computed tomography or radiographic guidance, using a Jamshidi needle inserted through the femoral head and neck.

Results A progressive reduction of pain within 3 weeks after BMMC administration was observed in 28 patients, with gradually increased weight bearing on the affected limb. In four dogs, however, pain and lameness persisted and at 3 months post-BMMC injection, femoral head and neck resection was performed. Histological and immunohistochemical studies were done on samples from those four dogs, which showed evidence of formation of new cartilage and subchondral bone in the area where cells were implanted.

Clinical Significance Based on these results, BMMC therapy may be considered as effective and minimally invasive treatment option for LCPD in dogs.

Keywords

- ▶ Legg-Calvé-Perthes disease
- ▶ dog
- ▶ bone marrow mononuclear cells
- ▶ femoral head

Introduction

Legg-Calvé-Perthes disease (LCPD) was first identified in children by Legg, Calvé and Perthes in 1910^{1–3}; in dogs, the disease was first described by Tutt in 1935.⁴ In canines, the average time of disease onset is at 7 months of age, with no gender differences. In 10 to 15% of cases, LCPD is bilateral; the incidence is 2.8 cases per 1000.⁵

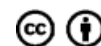
Legg-Calvé-Perthes disease has well-defined clinical and radiologic characteristics, although the aetiology and pathogenesis have not yet been completely revealed. The pathological features of LCPD are typical for avascular necrosis of bone tissue, followed by attempts to revascularization and bony remodelling of the femoral head. The most recent epidemiologic hypothesis in LCPD involves the expression

received
July 2, 2019
accepted after revision
December 4, 2019

DOI <https://doi.org/10.1055/s-0040-1701470>.
ISSN 2625-2325.

© 2020 Georg Thieme Verlag KG
Stuttgart · New York

License terms



of a defect in endochondral ossification and considers the disease to be a type of osteochondritis.⁶

Several treatment methods have been proposed in LCPD aimed at promoting blood supply, such as core decompression, or conservative approach such as physiotherapy to reduce pain, and femoral head and neck resection as treatment of choice.^{7,8}

Recently, many authors researching treatment options for avascular necrosis of the femoral head (ANFH) in humans have proposed the use of bone marrow (BM)^{9–11} and BM-derived cells.^{12–18} Bone marrow-derived cells have been employed in dogs, to verify their efficacy as a translational model for ANFH in humans.^{19,20} Several favourable biological characteristics make bone marrow mesenchymal stem cells (BMSCs), an ideal therapeutic agent for bone regeneration.^{21–24} The aim of this study was a retrospective evaluation of dogs with LCPD we treated with autologous bone marrow mononuclear cell (BMMC) implantation during the past 12 years.

Materials and Methods

Population

Clinical records of 32 dogs affected by monolateral LCPD and treated with BMMC implantation between 2007 and 2019 were included.

All treatments were performed according to the Italian guidelines on the use of stem cells for clinical applications in veterinary medicine (Gazzetta Ufficiale (Italy) serie generale n. 277 del 26/11/2013) and each dog owner provided informed consent.

Clinical Examination

In preoperative and postoperative evaluations, we considered hip pain during passive movement of the coxofemoral joint, the degree of lameness, gait and findings of radiologic and computed tomography (CT) examinations. The diagnosis of LCPD was based on the following clinical findings: monolateral hip pain, lameness and radiographic lesions graded 1 to 3 based on the Ljunggren scale.⁸ Cases of Ljunggren grade 4 and 5 were not included because of the severe modifications of the femoral head and neck.

Bone Marrow Harvesting

After sedation with acepromazine (0.02 mg/kg) and methadone (0.3 mg/kg), induction and maintenance of general anaesthesia were obtained with propofol (5 mg/kg). The hip region was prepared for aseptic surgery. Bone marrow was harvested from the iliac crest using an 18-gauge Jamshidi needle or, a 24-gauge epidural needle. Aspiration of bone marrow was performed using a 20 mL syringe containing heparin (2500 IU/20 mL) (→ Fig. 1). Ten mL of BM was considered as the minimum volume for the procedure. If this amount was not reached with a single site, then another harvesting was performed in the opposite iliac crest.

Bone Marrow Processing and BMMC Isolation

Bone marrow was diluted 1:1 in phosphate buffered saline (PBS), then layered 1:1 on Biocoll separation medium (Ficoll density gradient 1.077 g/mL; Biochrom, Berlin, Germany) and centrifuged at 2000 rpm × 30 minutes. The separated cells were counted using nuclear staining (0.1% methyl violet in 0.1 M citric acid). The cells were rinsed twice with PBS and then diluted in an adequate amount of fibrin glue (Tissucol; Baxter Spa, Rome, Italy).

To evaluate the capacity of the cells to form colony-forming unit fibroblasts (CFU-f), 100 µL of cells were seeded before and after density gradient centrifugation in two Petri dishes (100 mm diameter) in complete medium (Coon's F-12 medium, 10% foetal bovine serum, 100 IU/mL penicillin and streptomycin, 5% of a 200 µM solution of L-glutamine; Biochrom), and incubated at 37°C in a humid 5% CO₂/air (carbogen) atmosphere for 2 weeks, with the medium replaced twice a week. The cells were then washed with PBS, pH 7.2, fixed with 4% buffered formalin and stained with 1% methylene blue in borate buffer (10 mM, pH 8.8).

BMMC Injection

The dogs were re-anaesthetized using propofol (5 mg/kg). For injection of the BMMCs into the femoral head and neck, the BMMCs were suspended in 1 mL of fibrinogen. After injection of BMMCs + fibrinogen, 0.3 mL of thrombin was immediately injected using the same needle, to clot the suspension and avoid the backflow. This procedure was



Fig. 1 Legg-Calvé-Perthes disease in dogs. (A) Grade 1, (B) grade 2, and (C) grade 3 (Ljunggren scale).

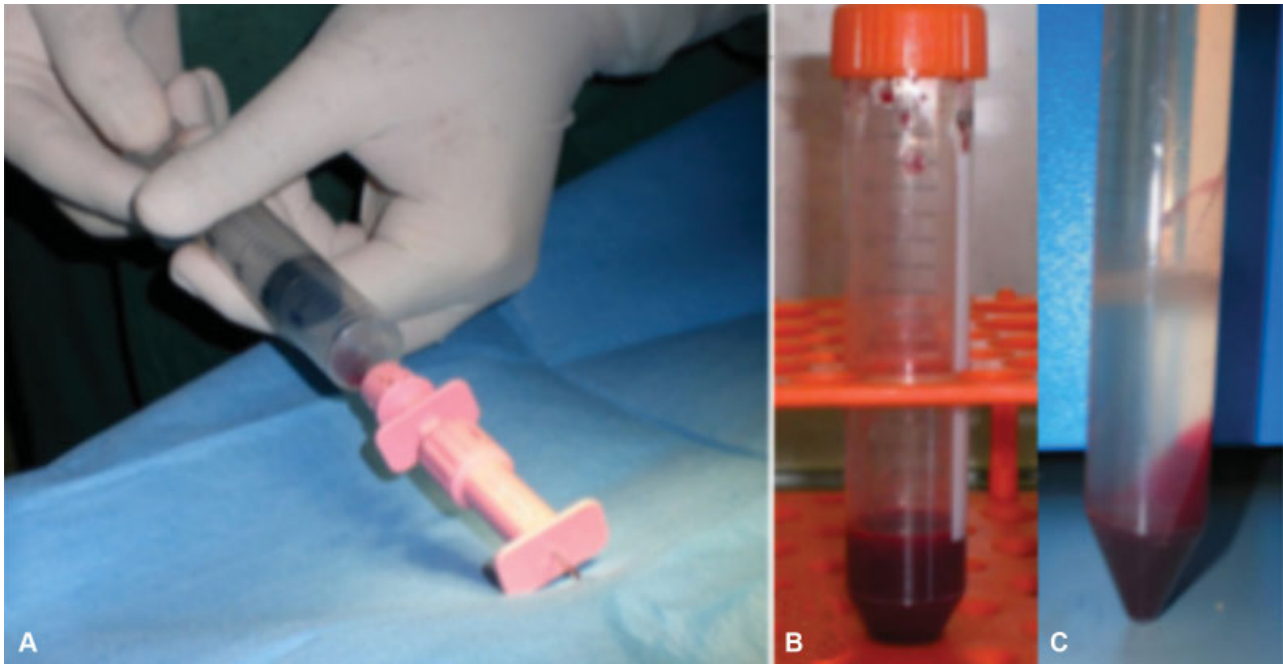


Fig. 2 Bone marrow harvesting. (A) Bone marrow harvested from iliac crest. (B) Harvested bone marrow. (C) Bone marrow mononuclear cells after Ficoll separation.

conducted under radiographic imaging or CT guidance using an 18-gauge Jamshidi needle for insertion into the femoral head via an intertrochanteric injection or directly into the femoral head (► Fig. 2).

Follow-Up

In the postoperative period, in each dog antibiotic medications were administered for 7 days (Amoxicillin/Clavulanic acid 25 mg/kg q 12h). Nonsteroidal anti-inflammatory drugs were avoided post-surgically to exclude any possible interference with the BMMC effect. Orthopaedic, radiographic and CT examinations were performed at 3 weeks and 3 months after the procedure in all cases. Long-term radiographic follow-up at 1, 2, 4 and 6 years was available in selected cases.

Histology, Histochemistry and Immunohistochemistry

Among the total number of dogs treated, four had persistent pain and lameness at 3 months after treatment, indicating the need for femoral head and neck ostectomy. In these cases, the femoral head and neck were harvested and fixed in 10% buffered formalin, and then decalcified in a solution of 10% ethylenediamine-tetra-acetic acid in 0.1 M phosphate buffer, pH 7–8, for ~8 weeks, changing the solution once a week. Ethylenediamine-tetra-acetic acid decalcification is optimal for proteoglycan preservation and is highly recommended for sections destined for immunohistochemistry (IHC)²⁵ After paraffin inclusion, the same tissues were cut into 4- μ m thick sections, placed on pre-treated slides (Bio-Optica, Milan, Italy) to promote adhesion, and dried overnight at 37°C

To obtain controls, we retrieved paraffinized tissue samples from untreated dogs with LCPD who previously underwent femoral head and neck resections; for this analysis, femoral head-neck samples from three male dogs (a 7-month-old

Papillon and two Yorkshire Terriers aged 9 and 10.5 months) with a history of severe clinical signs of pain and lameness were selected from the Veterinary Pathology Unit archives, University of Camerino. These samples had been obtained immediately following a surgical procedure in dogs that presented for elective surgery (femoral neck resection controls, FNRC).

Eight non-consecutive sections, obtained at 100- μ m intervals, were measured per each femoral head (in both the histological study group [HSG] and FNRC groups). The following parameters were evaluated at the central level of the femoral head: cartilage thickness, proteoglycan content ratio based on safranin O staining intensities (PC), chondrocyte density (CD) and subchondral bone staining intensities (BI). The degree of user interaction varied from manually tracing objects to nearly complete computer automation. For cartilage parameters, particularly evaluation of the new cartilage regeneration process inside the femoral head, four sections were stained with safranin O²⁶ and counter-stained with light green. For bone parameters, four additional non-consecutive sections were stained using Herovici polychrome dye,²⁷ to evaluate the different tissues and for pre-collagen and collagen differentiation. All slides were stained with haematoxylin and eosin (H&E) for general morphological evaluation. Finally, the presence of probably derived BMMCs was immunohistochemically evaluated using mouse monoclonal antibody anti-CD34 (Clone BL-35C; Zymed Inc., San Francisco, California, United States). Specific primary antibody substituted with Tris buffered saline or non-immune serum was used as the negative control in immunohistochemical techniques. For IHC tests, sections were treated as described elsewhere.²⁸ Immunohistochemical evaluation was made for the presence of CD34+ cell expression in cartilaginous areas and interspersed or localized in perivascular fashion throughout the damaged area of the femoral head, assessed at 100 \times magnification.

A score of 0 (absence of antigen expression), 1 (weak and spotted antigen expression), 2 (weak but diffuse antigen expression throughout the entire specimen) and 3 (diffuse and strong antigen expression) was assigned in a semiquantitative evaluation of the immunohistochemical reaction. Computed tomography, expressed in micrometres, was measured at 20× magnification, using an image-analysis system consisting of a light microscope (Carl Zeiss, Jena, Germany) attached to a Javelin JE3462 high-resolution camera and a personal computer equipped with a Coreco-Oculus OC-TCX frame grabber and high-resolution monitor. Computerized colour-image analysis was performed using Image-Pro Plus software (Media Cybernetics; Rockville, Maryland, United States). After manual delimitation of the cartilage between two reference marks (1 and 2) and the subchondral bone, the resulting area of cartilage was automatically segmented (using a threshold for black and white extraction with a green image), and the CT was calculated as the mean length of all the segments generated from each pixel situated on the border of the corresponding area of the cartilage.

The PC was approximated by quantification of the safranin O staining intensity (SOI) of the histological section. The area of measurement was centrally positioned between reference marks 1 and 2, and segmentation of the cartilage was automatically generated for measurement of the CT. The intensity was evaluated in the superficial (S) and deep (D) zones of the cartilage with manual delimitation at the upper margin of the D zone. Intensity was automatically measured as the optical density calculated on a greyscale obtained using the red component of the light passing through the section. To minimize the differences in safranin O staining between the sections and because PC varies differentially during experimental optics acquisition in the S and D zones, the following ratio was calculated: SOI in the S zone divided by SOI in the D zone; this justifies expression of the PC as a ratio.

The CD was evaluated at 200× magnification with manual counting of the cells divided by automatic calculation of the cartilage surface. Chondrocyte density is expressed in number of cells/mm². Cartilage parameters (CT and CD) were successively measured in each of the four non-consecutive sections stained with safranin O, and the bone parameter (BI) was measured in the four non-consecutive sections stained with Herovici polychrome dye and evaluated in terms of O.D. of different red colour variations. Finally, for CD34+ cells, the count was performed in four randomly selected cartilaginous fields, as follows: the number of IHC-positive cells was expressed as the number of stained cells in 100 randomly counted cells, as a percentage. For each dog, the data of each parameter were the mean of the four measurements obtained from the four sections. All sections were read blindly.

Statistical Analysis

Using MedCalc 14.8.1, parametric data were evaluated for normal distribution, and a comparison between the HSG and FNRC groups was performed with a one-way analysis of variance test. Nonparametric data were compared with a Kruskal–Wallis test. Significance was set at $p < 0.05$.

Results

Study Population

In this retrospective study, we enrolled the following 32 dogs of different breeds who were affected by monolateral LCPD: Poodle ($n = 6$), Pinscher ($n = 6$), Jack Russell Terrier ($n = 4$), Yorkshire Terrier ($n = 3$), Pomeranian ($n = 2$), Fox Terrier ($n = 2$), Scottish Terrier ($n = 1$), Papillion ($n = 1$), Russian Toy ($n = 1$), Chihuahua ($n = 1$) and mixed breed ($n = 5$); males ($n = 23$) and females ($n = 9$), average age 9.5 ± 2.5 months, average weight 4.7 ± 1.3 kg.

Dogs presented with different degrees of lameness, myoatrophy and shortening of the affected legs. Physical examination showed hip pain during passive movement of the coxofemoral joint, particularly during leg abduction. Radiographic and CT examinations confirmed the diagnosis and allowed staging in LCPD grade 1 ($n = 3$), grade 2 ($n = 11$) and grade 3 ($n = 18$) according to the Ljunggren scale (► **Figs. 3 and 4**).

No major complications or infections were seen neither after bone marrow harvesting nor after intra-osseous injection of BMMCs. For the latter, laterolateral X-rays or CT guidance were helpful to allow accurate injection into the necrotic and perinecrotic area and to avoid the potential risk of damage to the retinacular vascular blood supply of the femoral head; in fact, the dorsal and ventral retinacular artery remained throughout unaffected by trajectory of the needle. We harvested a mean of 14.3 ± 5.2 mL bone marrow. The number of BMMCs obtained after Ficoll separation was $104.7 \pm 46.5 \times 10^6$ /mL. The total CFUs from bone marrow were $71.6 \pm 51.9 \times 10^2$ /mL.

Follow-Up

A progressive and clear reduction of pain upon passive abduction and weight bearing and an improvement in gait was observed in 28 (87.5%) of the treated dogs within 3 weeks.

In all dogs, at 3 weeks, radiography did not show any radiographic changes, as compared with preoperative examinations. In four of the treated dogs (12.5%), we observed persistent hip pain and lameness.

After 3 months, the 28 improved dogs showed no signs of pain and normal gait. Radiographic examination at this follow-up showed an increase in sclerosis of the femoral head and a reduction in radiolucent zones with only mild periarticular changes. However, radiography and CT scan also revealed persistent collapse of the femoral head and incongruence of the coxofemoral joint (► **Fig. 5**). The long-term follow-ups on selected cases revealed normal gait despite the radiographic persistence of the articular collapse and joint incongruence (► **Fig. 6**) In 4 of the 32 dogs, pain and lameness persisted; therefore, femoral head and neck resection was performed.

Histology, Histochemistry and IHC

Morphological examination of the femoral head and neck of control dogs not subjected to stem cell therapy, revealed the presence of large erosive–necrotic processes, particularly at the level of the neck and in some areas of the head (► **Fig. 7A**), associated with a complete lack of safranin O staining, indicating a loss of proteoglycan content in the articular cartilage and

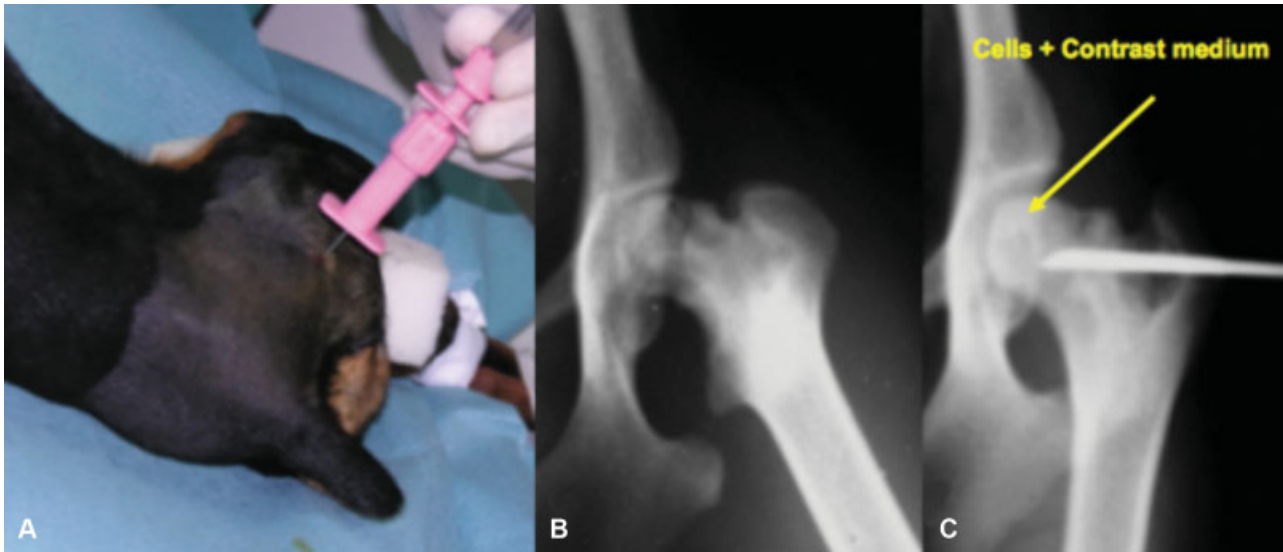


Fig. 3 Bone marrow mononuclear cell (BMMC) injection. (A) BMMCs injected in the femoral head and neck with a Jamshidi needle. (B) X-ray image before BMMC injection. (C) Injection of BMMCs mixed with contrast medium. In this specific case, the contrast medium was added to cells to detect the distribution of injected suspension in the necrotic areas.

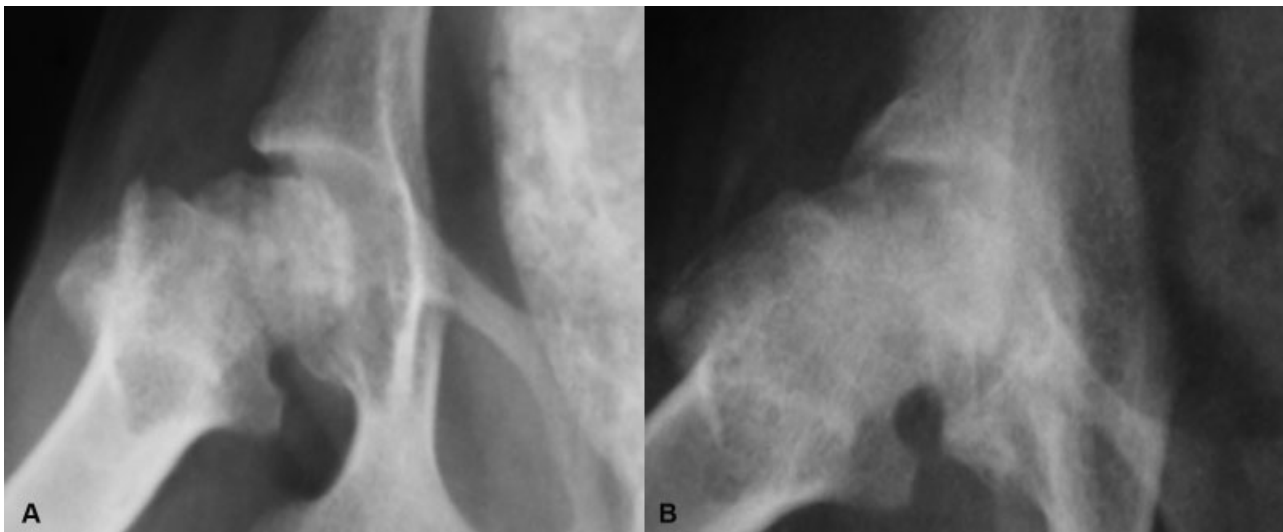


Fig. 4 Representative radiologic images show persistence of collapsed femoral head and incongruence of coxofemoral joint. (A) Before treatment and (B) 90 days after treatment.

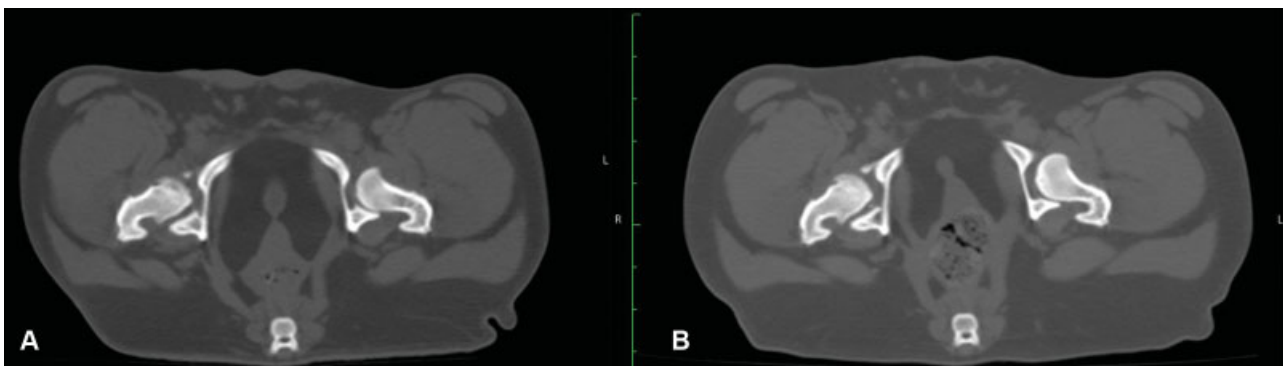


Fig. 5 Representative computed tomography images of a case (A) before treatment and (B) 90 days after treatment.



Fig. 6 Legg-Calvé-Perthes disease representative X-rays in ventrodorsal view before treatment (upper row series) and at different follow-up times (bottom row series). In particular (A) Grade III case with severe necrotic area of the femoral head. (A1) Follow-up at 6 years post bone marrow mononuclear cell (BMMC) treatment. Note sclerotic femoral head and neck with moderate articular modification. (B) Grade II case with small necrotic foci in the femoral head and neck. (B1) Follow-up at 2.5 years post BMMC treatment. Note the sclerosis of femoral head, shortening and widening of the neck and a good joint congruency. (C) Grade III case with severe modification of femoral head and joint incongruence. (C1) Follow-up at 1 year after treatment; note sclerosis with severe modification of the femoral head and neck shape and severe articular incongruence. (D) Grade III case with necrotic areas in the dorsal aspect of femoral head, with incongruent joint. (D1) Follow-up at 4 years after BMMC treatment. Sclerosis of the femoral head and neck with modification of the dorsal neck, moderate articular incongruence. (E) Grade II case with small necrotic foci and articular modification and incongruence. (E1) Follow-up at 2 years after BMMC treatment. Sclerosis of femoral head and large modification of neck with severe incongruence of articular surfaces.

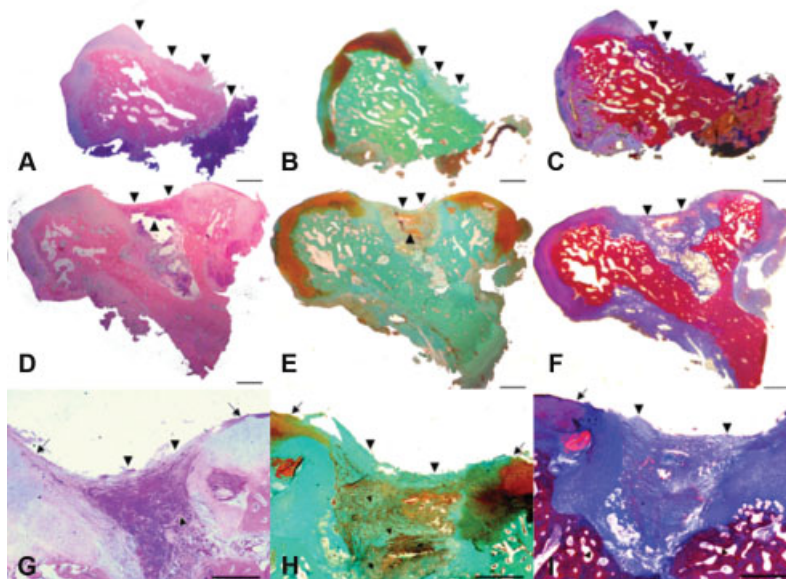


Fig. 7 Consecutive histological sections of femoral head and neck from untreated control dogs (femoral neck resection controls [A–C], and from bone marrow mononuclear cell (BMMC)-injected dogs (histological study group, HSG [D–I]), at 3 months after post-treatment. The femoral head is partially eroded and a large erosive–necrotic process is present on the neck (arrowheads) of a control dog, as revealed with haematoxylin and eosin (H&E) stain (A). In the same areas, a complete loss of proteoglycan content in the articular cartilage (arrowheads) and growth plate (normally stained in orange) is observed with safranin O stain (B). At the same level, Herovici polychrome stain reveals diffuse secondary ossification (bright red stain, arrowheads) and a notable lack of newly formed collagen or neo-angiogenesis or signs of regeneration in the same area (C). Note the presence of necrotic merged-colour-stained tissue at the end of the femoral neck. By contrast, the femoral head and neck of an HSG dog shows new fibrocartilage deposition in BMMC-treated and inflamed areas (arrowheads) in H&E-stained sections (D and G) and new nuclei of cartilage formation (G, arrows); at the same level, safranin O stain reveals proteoglycan synthesis and deposition (arrowheads, E and small arrowheads, H). Note intense orange safranin O staining of the new nuclei of chondrification (arrows, H). Finally, Herovici polychrome stain demonstrates large extracellular matrix deposition in the area of BMMC injection, composed of immature collagen, most likely corresponding to collagen type III (light-blue stained; F and D, arrowheads) and associated with neo-angiogenesis. H&E; safranin O; Herovici polychrome stain. Scale bar = 3.5 mm (A–F); scale bar = 7 mm (G–I).

growth plate (►Fig. 7B). In the head and neck of all controls, Herovici polychrome stain evidenced diffuse secondary ossification (►Fig. 7C, arrowheads) and a notable lack of newly formed collagen, lack of neo-angiogenesis and of signs of regeneration. Morphological and histochemical staining was performed on the HSG group that had not clinically recovered by 3 months post-treatment; evidence was found of an inflammatory process and new fibrocartilage deposition in the BMMC-injected area (►Fig. 7D and 7G). In particular, nuclei of cartilage formation (►Fig. 7G, arrows) and proteoglycan synthesis and deposition (arrow heads, ►Fig. 7E and small arrow heads, ►Fig. 7H) were stained orange with safranin O. In HSG dogs, Herovici polychrome staining revealed large areas of extracellular matrix deposition around the point of BMMC injection (►Fig. 7F and 7D, arrow heads), associated with neo-angiogenesis. Quantification of CD34+ cells in histological sections of the femoral head and neck from HSG group and control FNRC animals revealed a high number of CD34+ cells, mainly aggregated around capillaries in areas of new fibrocartilage deposition (►Fig. 8A insert). Computed tomography PC, CD and BI were compared between the group HSG and control FNRC group. All analysed parameters are summarized in

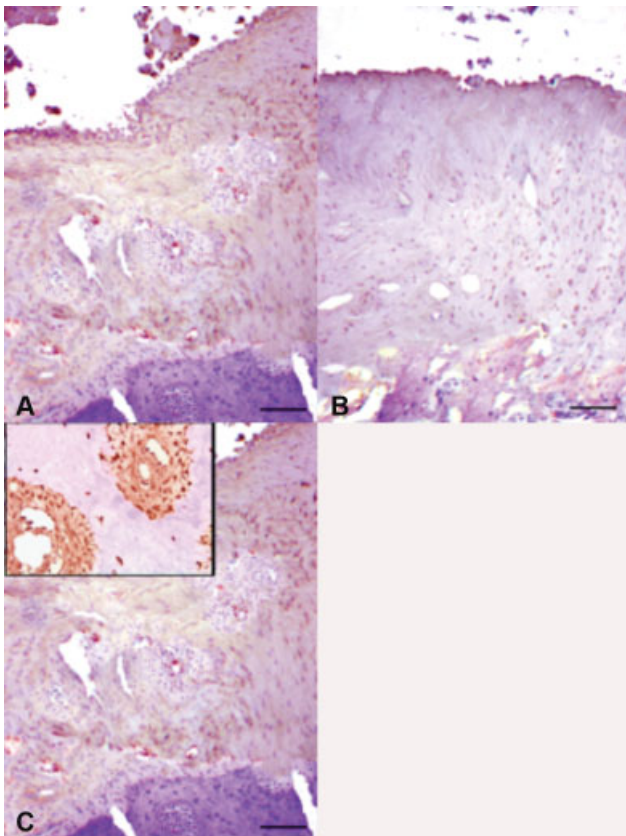


Fig. 8 Representative images for immunohistochemical quantification of CD34+ cells in histological sections of the femoral head and neck from bone marrow mononuclear cell (BMMC)-injected dogs (histological study group) (►Fig. 8A) 3 months after treatment, in comparison with untreated control dogs (bone marrow mononuclear cell) (►Fig. 8B). A high number of CD34+ cells were only observed in sections from BMMC-injected dogs. Cells were mainly aggregated around capillaries (►Fig. 8A insert). Immunohistochemistry avidin-biotin Complex method, Meyer haematoxylin counterstain. Scale bar = 700 µm.

Table 1 Mean and standard deviation for cartilage thickness, proteoglycan content ratio based on safranin O staining intensities, chondrocyte density and subchondral bone staining intensities. Histological study group (consisting of bone marrow mononuclear cell-treated femoral heads and necks) showed significantly higher parameters than femoral neck resection controls (consisting of untreated femoral heads and necks obtained after ostectomy in dogs with Legg-Calvé-Perthes disease)

	CT	PC	CD	BI	
FNRC	874.83	0.38	409.08	0.25	Mean
	155.60	0.08	80.37	0.06	SD
HSG	1414.00	0.53	747.06	0.54	Mean
	208.48	0.07	121.39	0.09	SD

Abbreviations: BI, subchondral bone staining intensities; CD, chondrocyte density; CT, computed tomography; PC, proteoglycan content ratio based on safranin O staining intensities.

Note: Significance level $p < 0.05$.

►Table 1 and ►Fig. 9. In the HSG, the CT, PC, CD and BI were significantly higher than the FNRC group. CD34+ cells were only found in stem cell-injected femoral heads and were completely absent in controls (►Fig. 8A).

Discussion

The aim of this retrospective study was to evaluate the use of BMMCs as a minimally invasive treatment for LCPD in dogs. The results showed that this technique gives good results in terms of swift recovery from pain and also leads to gradually increasing weight bearing on the affected limb, up to a complete remission of clinical signs at 3 months post-stem cell injection. Moreover, our data show that in case of failure of the BMMCs treatment (12.5%), dogs can still be safely treated with the classical femoral head and neck excision.

The clinical effectiveness of the method described in this study has been shown previously in human medicine, but the role of transplanted cells remains unclear. Our histological and IHC findings showed an increased articular cartilage and subchondral bone thickening, with a marked neoangiogenesis in treated samples, but complete absence thereof in the control group. Moreover, the presence of CD34+ cells, a marker found in BMMCs, could have been responsible for the regenerative effect, directly or indirectly through cell recruitment and chemotaxis, as found in other mesenchymal tissues. It has been documented that BMMCs secrete a wide spectrum of factors with immunomodulatory, anti-inflammatory, antiapoptotic, proangiogenic, proliferative or chemoattractive capacities. Pain improvement is probably owing to the anti-inflammatory capacity of stromal cells. Indeed, mesenchymal stromal cells have therapeutic potential for regenerative and anti-inflammatory therapy,^{29–31} and bone marrow-derived MSCs could suppress T cell proliferation.³² Currently, several soluble factors that are either produced constitutively by BMSCs or as the result of cross-talk with target immune cells, have been attributed to the immunomodulatory properties of BMSCs.^{33–35}

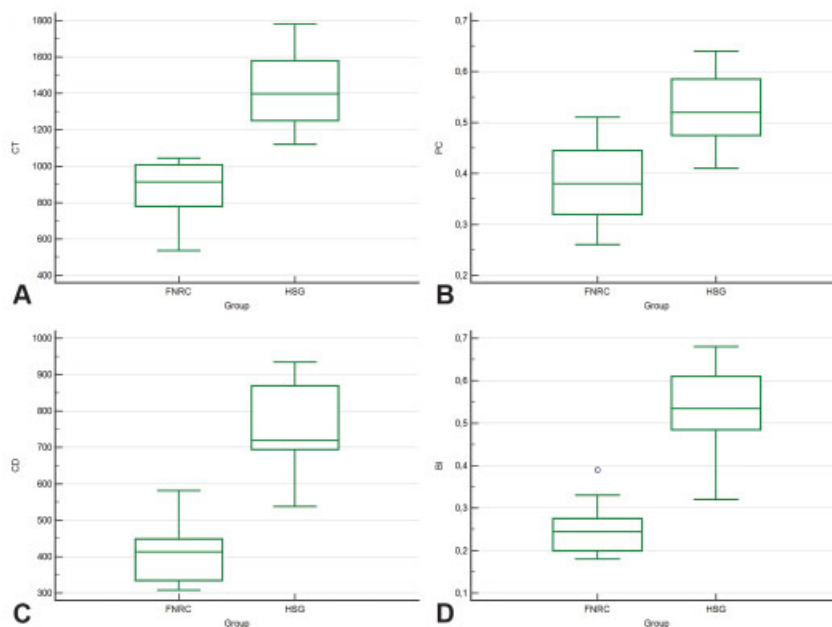


Fig. 9 Box and whisker plots for (A) cartilage thickness (CT), (B) proteoglycan content ratio based on safranin O staining intensities (PC), (C) chondrocyte density (CD), (D) and subchondral bone staining intensities (BI). Histological study group (HSG, consisting of bone marrow mononuclear cell-treated femoral heads and necks) showed significantly higher parameters than femoral neck resection controls (FNRC, consisting of untreated femoral heads and necks obtained after osteotomy in dogs with Legg-Calvé-Perthes disease). Significance level $p < 0.05$.

The amount of the injected BMSCs was very variable among our patients ($104.7 \pm 46.5 \times 10^6$ /mL), since the technique used to obtain the cells (for density gradient centrifugation) is depending on several factors including the original quality of harvested bone marrow, its volume and patient-related individual factors. Although some authors suggest that there is a direct correlation between the number of implanted cells and the treatment efficacy, at the moment, a clear reference target number is not available and the current literature suggests using the largest number of cells available.³⁶

Our good clinical results also seem related to increased precocious bone sclerosis, which is normally the last outcome of the disease, proven by radiographic and CT findings of persistent collapse of the femoral head and incongruence of the coxofemoral joint. Considering the minimally invasive and conservative approach of the proposed treatment, we suppose that a re-injection of the BMSCs could be an option in dogs not responding to the first treatment and/or with a recurrence of the symptoms. Further studies are required to confirm this hypothesis.

It has been considered that the reason for insufficient substitution in bone remodelling after osteonecrosis in the femoral head may be the small number of progenitor cells present in the femoral head.^{37,38} Others suggested that the effectiveness of bone marrow cells may be related to the availability of stem cells endowed with osteogenic properties in the femoral head, which increase after bone marrow implantation.^{39–42} Another possible explanation for the therapeutic effect of bone marrow implantation is that injected marrow stromal cells secrete angiogenic cytokines, resulting in increased angiogenesis and subsequent improvement in osteogenesis. Hernigou and colleagues stated that BMSCs can

elicit the formation of new blood vessels via the presence of endothelial cell progenitors or haemangioblasts. This may be owing to both the supply of progenitor cells and to angiogenic cytokines produced by bone marrow cells. Endothelial progenitors can actively engage in angiogenesis in tissue devoid of vessels and in neo-angiogenesis from pre-existing capillaries via the angiopoietin-1/Tie2 pathway. In addition, local ischaemia that activates HIF1A signalling and mobilization of circulating progenitors via the SDF1-dependent pathway may provide permanent stimuli for blood vessel repair, as well as a supply of new cells for bone regeneration with chemotaxis or differentiated or undifferentiated resident cells for functional tissue restoration.⁴³ In a study on cartilage repair using freshly isolated BMSCs as controls, Jiang and colleagues stated that cells obtained from *in vitro* culture and expansion are not recommended for cell-based therapy and that fresh BMSCs are the best choice.⁴⁴ The absence of a clinical control group can be considered a limitation of this study.

The present results confirmed clinically what has already shown in experimental models on dogs. Indeed, Yan and colleagues investigated the survival and differentiation of MSCs transplanted in skeletally mature mongrels in which osteonecrosis of the femoral head was surgically induced; patients were treated with either saline (control) or autologous BMSC transplantation. These previous results demonstrate that transplanted autologous BMSCs can survive, proliferate and differentiate into osteoblasts directly, which contributes to acceleration of the repair process.⁴⁵ Hang and colleagues and Liu and colleagues evaluated the efficacy of vascular endothelial growth factor 165 in transgenic BMSCs for the repair of early-stage osteonecrosis of the femoral head in mature mongrel dogs. After core decompression, transgenic and non-transgenic

autologous BMSCs were implanted and therapeutic efficacy was obtained, with new bone formation and neovascularization in the femoral head confirmed in collateral examinations.^{46,47} Jin and colleagues investigated the effects of autologous BMSCs administered via arterial perfusion on vascular repair and angiogenesis in an experimental model of osteonecrosis of the femoral head in dogs. Three weeks after establishment of ischaemia in the femoral head, a good therapeutic effect was obtained, with BMSCs promoting vascular repair and angiogenesis.¹⁹

In conclusion, on the basis of our results, we consider the technique described as minimally invasive, with no disadvantages, and that implantation of autologous bone marrow cells can be a valid alternative approach to the classical surgical treatment of LCPD with femoral head and neck excision; in case of non-response to cell treatment, surgery may still be performed successfully.

Conflict of Interest

None declared.

References

- Calvé J. On a particular form of pseudo-coxalgia associated with a characteristic deformity of the upper end of the femur. 1910. *Clin Orthop Relat Res* 2006;451(451):14–16
- Legg AT. An obscure affection of the hip joint. 1910. *Clin Orthop Relat Res* 2006;451(451):11–13
- Perthes G. The classic: on juvenile arthritis deformans. 1910. *Clin Orthop Relat Res* 2012;470(09):2349–2368
- Tutt J. Tuberculosis of the hip joint in a cairn terrier. *Vet Rec* 1935; (47):428–431
- Nunamaker DM. Legg-Calvé-Perthes disease. In: Newton CD, Nunamaker DM, eds. *Textbook of Small Animal Orthopaedics*. Chapter 82. Philadelphia: Lippincott; 1985:949–952
- Roperto F, Papparella S, Crovace A. Legg-Calvé-Perthes disease in dogs - histological and ultrastructural investigations. *J Am Anim Hosp Assoc* 1992;28:156–162
- Lee R, Fry PD. Some observations on the occurrence of Legg-Calvé-Perthes' disease (coxaplasia) in the dog, and an evaluation of excision arthroplasty as a method of treatment. *J Small Anim Pract* 1969;10(05):309–317
- Ljunggren G. Legg-Perthes disease in the dog. *Acta Orthop Scand* 1967(Suppl 95):95, 1–79
- Gangji V, Hauzeur J-P, Matos C, De Maertelaer V, Toungouz M, Lambermont M. Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. A pilot study. *J Bone Joint Surg Am* 2004;86(06):1153–1160
- Gangji V, Hauzeur J-P. Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. Surgical technique. *J Bone Joint Surg Am* 2005;87(Pt 1, Suppl 1):106–112
- Kørbling M, Estrov Z. Adult stem cells for tissue repair - a new therapeutic concept? *N Engl J Med* 2003;349(06):570–582
- Cabrolier J, Molina M. [Is instillation of bone marrow stem cells at the time of core decompression useful for osteonecrosis of the femoral head?] *Medwave* 2016;16(02, Suppl 1):e6406
- Gao F, Sun W, Guo W, Wang B, Cheng L, Li Z. Combined with bone marrow-derived cells and rhBMP-2 for osteonecrosis after femoral neck fractures in children and adolescents: a case series. *Sci Rep* 2016;6(01):30730
- Liu Y, Liu S, Su X. Core decompression and implantation of bone marrow mononuclear cells with porous hydroxylapatite composite filler for the treatment of osteonecrosis of the femoral head. *Arch Orthop Trauma Surg* 2013;133(01):125–133
- Rackwitz L, Eden L, Reppenhagen S, et al. Stem cell- and growth factor-based regenerative therapies for avascular necrosis of the femoral head. *Stem Cell Res Ther* 2012;3(01):7–15
- Sun Y, Feng Y, Zhang C. The effect of bone marrow mononuclear cells on vascularization and bone regeneration in steroid-induced osteonecrosis of the femoral head. *Joint Bone Spine* 2009;76(06): 685–690
- Tabatabaee RM, Saberi S, Parvizi J, Mortazavi SM, Farzan M. Combining concentrated autologous bone marrow stem cells injection with core decompression improves outcome for patients with early-stage osteonecrosis of the femoral head: a comparative study. *J Arthroplasty* 2015;30(9, Suppl):11–15
- Wang B-L, Sun W, Shi Z-C, et al. Treatment of nontraumatic osteonecrosis of the femoral head with the implantation of core decompression and concentrated autologous bone marrow containing mononuclear cells. *Arch Orthop Trauma Surg* 2010;130 (07):859–865
- Jin H, Xia B, Yu N, et al. The effects of autologous bone marrow mesenchymal stem cell arterial perfusion on vascular repair and angiogenesis in osteonecrosis of the femoral head in dogs. *Int Orthop* 2012;36(12):2589–2596
- Wang M, Liao Q, Zhou B, Qiu ZQ, Cheng LM. Preliminary study of influence of bone tissue from osteonecrosis of femoral head on the proliferation and differentiation of canine bone marrow mesenchymal stem cells [article in Chinese]. *Zhonghua Yi Xue Za Zhi* 2013;93(11):856–859
- Crovace A, Favia A, Lacitignola L, Di Comite MS, Staffieri F, Francioso E. Use of autologous bone marrow mononuclear cells and cultured bone marrow stromal cells in dogs with orthopaedic lesions. *Vet Res Commun* 2008;32(Suppl 1):S39–S44
- Crovace A, Lacitignola L, Francioso E, Rossi G. Histology and immunohistochemistry study of ovine tendon grafted with cBMSCs and BMMNCs after collagenase-induced tendinitis. *Vet Comp Orthop Traumatol* 2008;21(04):329–336
- Crovace A, Lacitignola L, Rossi G, Francioso E. Histological and immunohistochemical evaluation of autologous cultured bone marrow mesenchymal stem cells and bone marrow mononucleated cells in collagenase-induced tendinitis of equine superficial digital flexor tendon. *Vet Med Int* 2010;2010:250978
- Lacitignola L, Crovace A, Rossi G, Francioso E. Cell therapy for tendinitis, experimental and clinical report. *Vet Res Commun* 2008;32(Suppl 1):S33–S38
- Schmitz N, Laverty S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. *Osteoarthritis Cartilage* 2010;18 (Suppl 3):S113–S116
- Busoni V, Rossi G, Denoix J-M. Arthrite septique du boulet: corrélation entre imagerie et examen anatomo-pathologique. *Prat Vét Equine* 1996;28:121–124
- McInnes IB, Buckley CD, Isaacs JD. Cytokines in rheumatoid arthritis - shaping the immunological landscape. *Nat Rev Rheumatol* 2016; 12(01):63–68
- Camplejohn KL, Allard SA. Limitations of safranin 'O' staining in proteoglycan-depleted cartilage demonstrated with monoclonal antibodies. *Histochemistry* 1988;89(02):185–188
- Amorin B, Alegretti AP, Valim V, et al. Mesenchymal stem cell therapy and acute graft-versus-host disease: a review. *Hum Cell* 2014;27(04):137–150
- El-Jawhari JJ, El-Sherbiny YM, Jones EA, McGonagle D. Mesenchymal stem cells, autoimmunity and rheumatoid arthritis. *QJM* 2014; 107(07):505–514
- Wang D, Zhang H, Liang J, et al. Allogeneic mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus: 4 years of experience. *Cell Transplant* 2013;22(12): 2267–2277
- Mattar P, Bieback K. Comparing the immunomodulatory properties of bone marrow, adipose tissue, and birth-associated tissue mesenchymal stromal cells. *Front Immunol* 2015;6:560–566

- 33 Najar M, Raicevic G, Boufker HI, et al. Mesenchymal stromal cells use PGE2 to modulate activation and proliferation of lymphocyte subsets: Combined comparison of adipose tissue, Wharton's Jelly and bone marrow sources. *Cell Immunol* 2010;264(02):171–179
- 34 Sato K, Ozaki K, Oh I, et al. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007;109(01):228–234
- 35 Yoo KH, Jang IK, Lee MW, et al. Comparison of immunomodulatory properties of mesenchymal stem cells derived from adult human tissues. *Cell Immunol* 2009;259(02):150–156
- 36 Hernigou P, Guerin G, Homma Y, et al. History of concentrated or expanded mesenchymal stem cells for hip osteonecrosis: is there a target number for osteonecrosis repair? *Int Orthop* 2018;42(07):1739–1745
- 37 Hernigou P, Beaujean F, Lambotte JC. Decrease in the mesenchymal stem-cell pool in the proximal femur in corticosteroid-induced osteonecrosis. *J Bone Joint Surg Br* 1999;81(02):349–355
- 38 Hernigou P, Trousselier M, Roubineau F, et al. Stem cell therapy for the treatment of hip osteonecrosis: a 30-year review of progress. *Clin Orthop Surg* 2016;8(01):1–8
- 39 Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells* 2001;19(03):180–192
- 40 Conway EM, Collen D, Carmeliet P. Molecular mechanisms of blood vessel growth. *Cardiovasc Res* 2001;49(03):507–521
- 41 Friedenstein AJ, Piatetzky-Shapiro II, Petrakova KV. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;16(03):381–390
- 42 Murphy MB, Moncivais K, Caplan AI. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp Mol Med* 2013;45(11):e54
- 43 Hernigou P. Bone transplantation and tissue engineering, part IV. Mesenchymal stem cells: history in orthopedic surgery from Cohnheim and Goujon to the Nobel Prize of Yamanaka. *Int Orthop* 2015;39(04):807–817
- 44 Jiang T, Xu G, Wang Q, et al. In vitro expansion impaired the stemness of early passage mesenchymal stem cells for treatment of cartilage defects. *Cell Death Dis* 2017;8(06):e2851
- 45 Yan Z, Hang D, Guo C, Chen Z. Fate of mesenchymal stem cells transplanted to osteonecrosis of femoral head. *J Orthop Res* 2009;27(04):442–446
- 46 Hang D, Wang Q, Guo C, Chen Z, Yan Z. Treatment of osteonecrosis of the femoral head with VEGF165 transgenic bone marrow mesenchymal stem cells in mongrel dogs. *Cells Tissues Organs* 2012;195(06):495–506
- 47 Liu CH, Zhao DW, Wang BJ. Effects of different stress force stimulations on the expression of vascular endothelial growth factor in Beagle dogs in the repairing process of osteonecrosis of the femoral head [article in Chinese]. *Zhonghua Yi Xue Za Zhi* 2012;92(01):40–44