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Effect of Intra-Medullar and Intra-Venous Infusions of Mesenchymal Stem Cells on Cell Engraftment by *In-Vivo* Cell Tracking and Osteoinductivity in Rabbit Long Bones: A Pilot Study

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Abstract

Objective: Stem cell therapy can be an efficacious treatment option for bone fragility disorders (eg, osteogenesis imperfecta, disuse osteopenia, and osteoporosis), and successful cell therapy application may be dependent on optimal cell engraftment in target bones. The objective of this study was to compare the efficiency of intra-medullar and intra-venous delivery of mesenchymal stem cells (MSC) to improve cell engraftment rate, bone mineral density, and micro-architecture.

Methods: By using six healthy juvenile New Zealand White rabbits, MSC were isolated from cancellous bone harvests and confirmed to have osteogenic capacity by inducing ectopic bone formation. The MSC were cultured, transduced by foamy viral vectors with marker genes for in vivo cell tracking, and expanded. All rabbits had one randomly selected limb receive intra-medullar infusion of 3×10^7 to 1×10^8 autologous MSC in the distal femur or the distal femur and proximal tibia. Two of six rabbits also received an intra-venous MSC infusion. At 28 days, MSC bone engraftment was assessed by PCR and the bone density and microstructure assessed by computed tomography and histomorphometry.

Results: The intra-medullar-infused MSC were detected in epiphysis or diaphysis of the distal femurs and/or proximal tibiae. Infused MSC comprised 0.01 to 0.3% of all cells in the bone tissues. The intra-venous-infused MSC were not detected in any location. Neither intra-medullar nor intra-venous MSC infusion altered bone volume, bone mineral density, or cortical bone porosity/thickness. Systemic biodistribution of intra-medullar-infused MSC was not evident.

Conclusions: Our results indicated that intra-medullar infusion can be an effective cell delivery route for stem cell therapy potentially for orthopedic disorders, in preference to systemic administration. Further research is warranted to demonstrate an efficacy of intra-medullar MSC infusion on bone density and micro-architecture using animal models of bone disorders.

Keywords: Mesenchymal stem cells; Cell engraftment; Intra-medullar infusion; Intra-venous infusion; Bone fragility disorder; Rabbit; Biodistribution

Introduction

Stem cell therapy can be an efficacious treatment option for bone fragility disorders including osteogenesis imperfecta (OI), disuse osteopenia, and osteoporosis [1], and successful cell therapy application may be dependent on optimal cell engraftment in target bones. A clinical trial of OI patients who received intra-venous administration of mesenchymal stem cells (MSC) exhibited an increase in growth rate [2]. Also, intra-venous application of MSC transduced with osteogenic transcription factors showed the restoration of bone mass and strength in a mouse model of glucocorticoid-induced osteoporosis [3]. Conversely, another study showed that the level of MSC engraftment following systemic transplantation is extremely low and thus its therapeutic efficacy remains questionable [4,5]. A previous work demonstrated that MSC infused into immune-compromised mouse femurs were sufficiently engrafted and contributed to bone formation [6]. In an immune-competent large animal model, direct injection of dermal fibroblasts into the bone tissues has shown to be an effective cell delivery strategy [7,8]. Therefore, even though the systemically administered MSC can have a homing effect to congregate into active bone-forming sites [9], local administration of MSC directly into the target bones could be an alternative cell delivery route and may improve cell engraftment and enhance efficacy.

The objectives of this study were to compare the efficiency of intra-medullar and intra-venous delivery of autologous MSC on cell engraftment rate, bone mineral density, and bone micro-architecture. We hypothesized that intra-medullar MSC infusion would achieve greater cell engraftment in target bone than intra-venous infusion, and potentially improve bone mineral density and micro-architecture.

Materials and Methods

Experimental design

All procedure was approved by the Institutional Animal Care and Use Committee of The Ohio State University. Intra-medullar and intra-venous infusion were compared using autologous MSC in 6 rabbits (Figure 1A, 1B and 1D). At 28 days after MSC administration, MSC engraftment was assessed by PCR and the bone density

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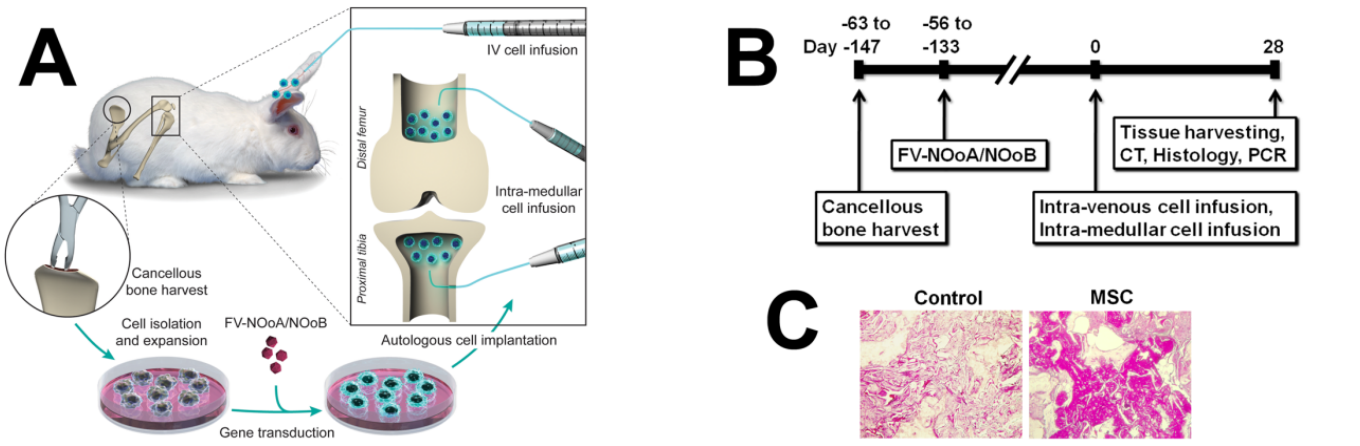

D

Animal ID	Systemic cell delivery	Local cell delivery			
		One randomly assigned hindlimb		Contralateral hindlimb	
		Distal femur	Proximal tibia	Distal femur	Proximal tibia
#01 - #04	N/A	Intra-medullar MSC infusion	Intra-medullar MSC infusion	Untreated	Untreated
#05 - #06	Intra-venous MSC infusion	Intra-medullar MSC infusion	Untreated	Untreated	Untreated

Figure 1: Experimental models (A), protocol (B), ectopic bone formation by MSC (C), and assignment table (D) of the in vivo studies. Six rabbits received intra-venous infusion and intra-medullar infusion of autologous mesenchymal stem cells (MSC), transduced by Foamy viral (FV) vector carrying 2 marker genes (NOoA or NOoB), as illustrated. C: The ectopic bone formation was induced by subcutaneous MSC implantation with collagen matrix in NOD/SCID mice ("MSC"), but not by empty matrix ("Control").

and microstructure assessed by computed tomography (CT) and histomorphometry. The MSC harvested from 3 rabbits were confirmed to be osteoinductive by histologic evidence of ectopic bone formation after subcutaneous implantation with collagen matrix in NOD/SCID mice (Figure 1C).

Intra-medullar and intra-venous infusions of autologous MSC

Six healthy juvenile New Zealand White rabbits (6-month-old) were used to compare intra-medullar and intra-venous infusions of MSC (Figure 1A, 1B and 1D). In each of six rabbits, the cancellous bone was harvested from the iliac crest at day -60 to -90 under general anesthesia with isoflurane to isolate MSC [10]. Autologous MSC were cultured with Dulbecco's Modified Eagle Medium (37-degree, 5%-CO_2), transduced by foamy viral vectors with marker genes (NOoA/NOoB) at 2 moi (multiplicity-of-infection) at day -30 to -60, and expanded, as described before [10,11]. In brief, recombinant foamy viral vectors with the marker genes were produced by calcium phosphate transfection of 293T cells with four plasmids (i.e., pΔϕFPF, pCiGSΔPsi, pCiPS, and pCiES), purified by filtration and ultracentrifugation, and the infectious titer was determined by transducing HT1080 cells with flow cytometry [10,11]. Two different marker genes (NOoA/NOoB) were used, so that the intra-medullar-infused MSC and intra-venous-infused MSC can be distinguished within the same animal. The rabbits were re-anesthetized and received intra-medullar or intra-venous MSC infusions at day 0. Rabbits and limbs received treatments as described in Figure 1D. In brief, 6 rabbits had one randomly selected limb receive intra-medullar MSC infusion in the distal femur (n=6) or the distal femur and proximal tibia (n=4) by passing a tube through the unicortical diaphyseal drill hole (2.38 mm diameter) to the metaphysis. Limbs were hung dependently to use gravity force to settle the cells into the metaphyseal regions. The MSCs were infused slowly over 5 minutes to limit backwash of the cells from the drill hole. In 4 of these rabbits, the contralateral limb served as the untreated control. The two other rabbits also received an intra-venous MSC infusion performed by venous catheter placed in the lateral marginal ear vein; hence, the contralateral limb served as the intravenous treated femur. All MSC infusions were made in 1 mL total volume with PBS containing a range of 3×10^7 to 1×10^8 autologous MSCs.

Evaluation of the effects of intra-medullar/venous MSC infusions

At day 28, the rabbits were euthanized with Pentobarbital, and both hindlimbs were harvested and scanned by CT (Picker PQS Helical CT Scanner, Philips Medical Systems, N.A., Bothell, Washington). The epiphysis, metaphysis, and diaphysis (<1 cm, 1-3 cm, 3-6 cm from the knee joint, respectively) of femurs and tibiae were quantitatively evaluated for bone volume (mm^3), bone mineral density (mg/ml), and drill hole filling (%) using image analysis software (Mimics; Materialise, Ann Arbor, MI) [8]. The femurs and tibiae were fixed with formalin, decalcified with alcohol, paraffin-embedded, sectioned at 5-μm, and stained with Masson's Trichrome. The five representative sites of epiphysis, metaphysis, and diaphysis in each femur and tibia were histomorphometrically evaluated for cortical bone porosity (%), cortical bone thickness (mm), and trabecular bone area (%) [8]. To determine the cell engraftment, genomic DNA was extracted from the epiphysis, metaphysic, and diaphysis regions of each femur and tibia, and several organs/tissues (i.e., Liver, Spleen, Lung, Gonad, and Muscle) using QIAamp Mini Kit (Qiagen,Valencia, CA) and analyzed

by PCR to detect the percentages of NOoA/NOoB-transduced cells, as described before [11].

Statistical analysis

Repeated-measure analysis of variance (ANOVA) (SAS Institute Inc., Cary, NC) was used to evaluate the effects of MSC engraftment and osteoinductivities with the post-test multiple comparisons between the treatment groups using Proc Mixed statistical models. Repeated variables were considered to be nested within animal, and the distribution of data was assessed by use of a subset of normality tests [7,8]. Significance level was set at $p<0.05$ for all analyses.

Results

On the PCR analysis, the intra-medullar-infused MSC were detected in epiphysis or diaphysis of the distal femurs or proximal tibiae for 67% (4/6) of animals (Table 1). The MSC were taken up into the recipient long bones in proportions of 0.01 to 0.3% of all cells in the bone tissues (Table 1). The intra-medullar-infused MSC were not detected in the contralateral hind limbs or organs/tissues, and the intra-venous-infused MSC were not detected in any location of the animal (Table 1). On the quantitative CT, the intra-medullar/venous MSC infusion animals did not show any improvement on bone volume, bone mineral density, or drill hole filling in any regions of the distal femur or proximal tibia (Figure 2A and 2B). Similarly, on the histomorphometric evaluation, the intra-venous/medullar MSC infusion animals did not show any improvement on cortical bone porosity, cortical bone thickness, or trabecular bone area in any regions of distal femurs or proximal tibiae (Figure 2C and 2D).

Discussion

Intra-medullar infusion can be an effective cell delivery route and was confirmed in 67% of our animals to engraft stem cells in metaphyseal bone (Table 1) and may be effective in stem cell therapy for orthopedic disorders. Even though the survival rate of intra-medullar-infused cells was unknown, the infused MSC comprised 0.01 to 0.3% of all cells in the bone tissues (Table 1). There were no correlation between the number of MSC infused and cell engraftment rate, and performing in-vivo cell tracking at multiple time points would further elucidate the mechanism of MSC engraftment. In a recent work using orthotopic implantation of stem cells into the bone defect, small portion of target tissue was appeared to comprise the implanted cells on the histologic evaluation, yet it still induced a significant bone formation [12]. On the other hand, in our work, we were unable to identify any engraftment with systemic intravenous infusion; thus, an intra-medular infusion may be in preference to systemic administration. For future study, radiologic ablation or physical aspiration of bone marrow materials prior to intra-medullar MSC infusion may further increase a degree of cell engraftment. Our finding of a lack of detectable engraftment of intra-venous-infused MSC in bones or organs/tissues (Table 1) is in an agreement with previous work reporting low level of cell engraftment by systemic MSC administration [4,5]. Our study supports a superior engraftment capability in cells infused directly into the medullary cavity of target bones, compared to an intra-venous cell infusion that is dilutional and relies on the homing effect of stem cells. Clearly, small sample size is a major drawback of current work, and additional experiments using larger number of animals would validate our findings.

Intra-medullar infusion method could facilitate autologous stem cell therapy for OI. Previously, MSC from OI patients were successfully altered by either suppressing the disease causing mutations of type I collagen gene [13,14] or over-expressing the normal type I collagen gene [15]. By engrafting these genetically-corrected MSC, optimal strength of OI patients' bones might be restored by healthy collagen deposition. Further research is warranted to demonstrate an efficacy of intra-medullar cell infusion using animal models of OI. In addition, the same treatment strategy can be utilized in a stem cell therapy for

Treatment	Sample tissue			Number of animals with intra-venously injected MSC detected	Number of animals with intra-medullary infused MSC detected	Proportion of intra-medullary infused MSC detected (%)
Intra-medullar infusions into femur and tibia	*MSC -treated hindlimb*	Distal femur	Diaphysis	NA	0 / 4	
			Metaphysis	NA	3 / 4	0.02, 0.04, 0.05
			Epiphysis	NA	0 / 4	
		Proximal tibia	Diaphysis	NA	1 / 4	0.01
			Epiphysis	NA	2 / 4	0.03, 0.001
	Contra-lateral hindlimb	Distal femur		NA	0 / 4	
		Proximal tibia		NA	0 / 4	
Intra-venous injection and intra-medullar infusion into femur	*MSC -treated hindlimb*	Distal femur	Diaphysis	0 / 2	1 / 2	0.3
			Metaphysis	0 / 2	1 / 2	0.02
			Epiphysis	0 / 2	0 / 2	
		Proximal tibia	Diaphysis	0 / 2	0 / 2	
			Epiphysis	0 / 2	0 / 2	
	Contra-lateral hindlimb	Distal femur		0 / 2	0 / 2	
		Proximal tibia		0 / 2	0 / 2	
	Organs	Liver		0 / 2	0 / 2	
		Spleen		0 / 2	0 / 2	
		Lung		0 / 2	0 / 2	
		Gonad		0 / 2	0 / 2	
		Muscle		0 / 2	0 / 2	

Table 1: Quantitative PCR detection of mesenchymal stem cells (MSC) with foamy-virally transduced 2 marker genes (NOoA and NOoB) using the HT1080 cell standards (single NOoA/NOoB copy per cell; Fig 2A right) in bone and organ tissue from rabbits after intra-venous and intra-medullar MSC infusions (Fig 1A, 1C, 1E). The contralateral hindlimbs were untreated or intravenous treated. The bones (femur and tibia of both hindlimbs) and organ tissues (liver, spleen, lung, gonad, muscle) were harvested at 4-weeks after MSC-injection/infusion. Detection limit was <0.001%. N/A: non-applicable.

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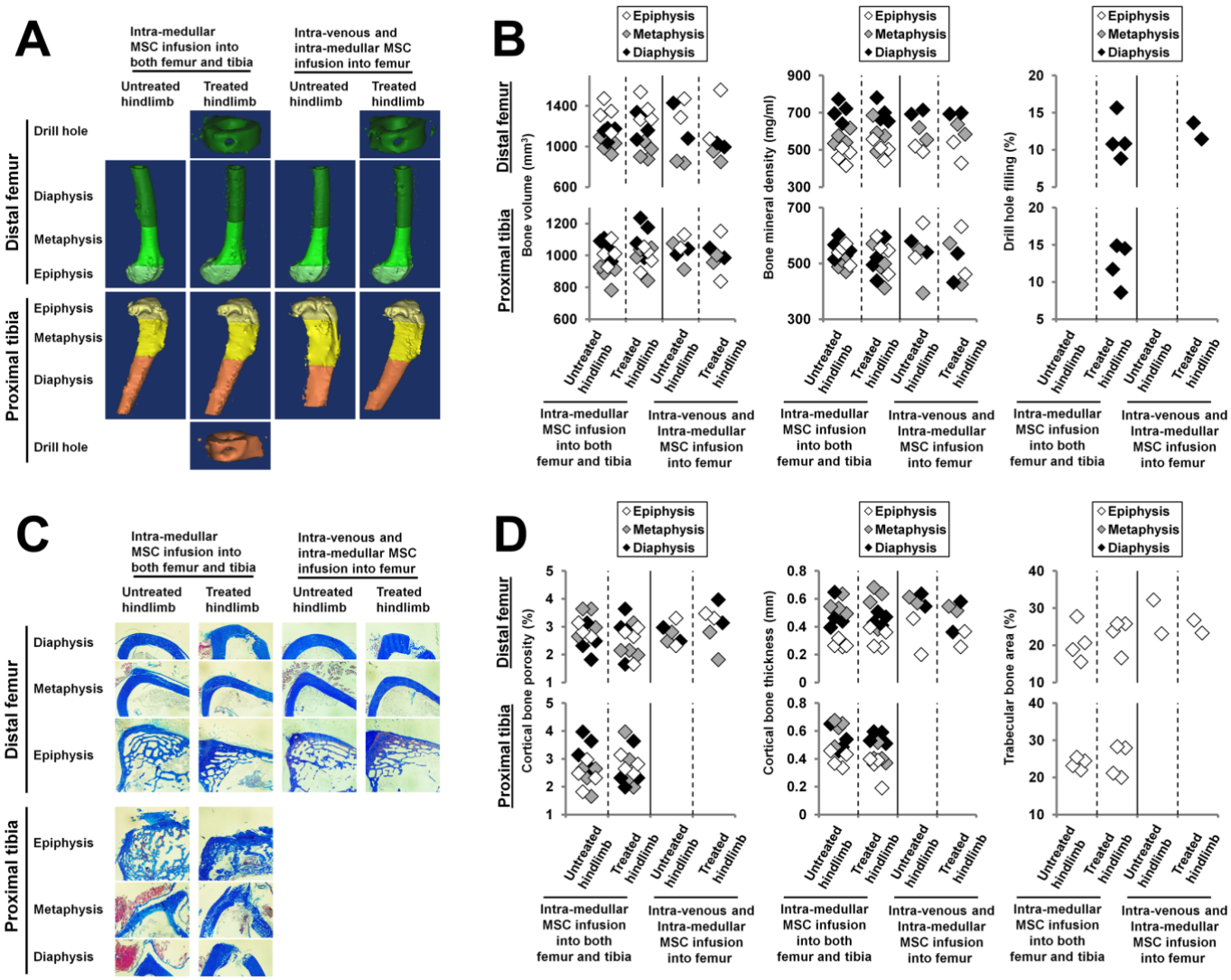

Figure 2: Computed tomography of distal femurs and proximal tibiae (A) of rabbits that received intra-venous and intra-medullar infusion of autologous mesenchymal stem cells (MSC). Uninjected contralateral limbs served as the untreated or intravenous treated limbs (Fig 1A, 1B, and 1D). There were no significant changes on bone volume, bone mineral density, or drill hole filling in all regions of the femurs and tibiae (B). Histomorphometric evaluation of the distal femurs and proximal tibiae (C) showed no significant changes on cortical bone porosity, cortical bone thickness, or trabecular bone area in all regions of the femurs and tibiae (D).

osteoporosis. By genetically-modifying MSC to secrete osteogenic growth factors such as bone morphogenetic proteins, the MSC could be engrafted by intra-medullar infusion in the osteoporotic long bones in vivo, and the bone mineral density and micro-architecture may be improved by a paracrine effect of the secreted growth factors, as shown in our previous work [16]. This may translate to humans with OI or osteoporotic fractures. As a result, the physical strength of recipient bones may be significantly increased to prevent painful bone fracture in osteoporotic individuals.

In our study, intra-medullar or intra-venous MSC infusions did not significantly alter the bone volume, bone mineral density, and cortical bone porosity/thickness of the femurs and tibiae. This may be due to insufficient numbers of MSC at the bone site, lack of the capability of MSC alone to enhance bone parameters in immune-competent large animals, or that healthy rabbits were used. In our study, the MSC were not augmented with bone forming genes. In prior work with large animal models, cells (dermal fibroblasts) [7,8] or MSC alone without genetic engineering [17] were unable to improve bone density without being engineered to express bone morphogenetic protein 2. Also, the engrafted MSC might be regulated by host local tissues and not prompted for over-physiologic osteoinduction. Once again, further studies using animal models of bony disorders will be required to investigate the effects of stem cell infusions on the density or structure of recipient bone tissues.

Systemic biodistribution of intra-medullary and intravenous infused MSC was not evident in this study, because the MSC were not detected in any distant organs/tissues (Table 1). In the authors' previous work, intra-medullar-infused fibroblasts were also not detected in any distant organs, except in the regional lymph nodes at very low level [16]. It is thus possible that the intra-medullar-infused cells generally remain within the marrow cavities, and the small portion of cells that are leaked/migrated out would be cleared in the local lymphatic systems. If intra-medullar-infused MSC resulted in no/negligible systemic biodistribution, use of allogeneic stem cells could

be a potential therapeutic strategy, as it enables a more quick and inexpensive cell therapy application and may avoid immune-rejection or systemic inflammatory responses.

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