Bone Fracture Healing with Umbilico-Placental Mononuclear Cells: A Controlled Animal Study

Onur Polat, Gurur Polat, Sercin Karahuseyinoglu, Nüket Yörür Kutlay, Arzu Gül Tasci, Esra Erdemli, Ajlan Tukun, Mustafa Cihat Avunduk, Sükrü Küplülü, Mehmet Demirtas

Abstract

Background: Fracture healing is a significant process in orthopedics. In this controlled animal study, our aim is to expose the healing effects of cord blood umbilico-placental mononuclear cells (UPMNCs) on bone fractures.

Materials and Methods: Caesarean sections were performed on five pregnant New Zealand rabbits at term. Placentas and cords were collected. Standard closed transverse shaft fractures were created on both tibial bones of 15 baby rabbits. The right tibias were given UPMNCs; the left tibias were the control group. Histological examinations, osteoblast and osteoclast cell counts, and mechanical stabilities were compared. Anchorage of the donor cells was shown by the fluorescence in situ hybridization (FISH) technique.

Results: In the group injected with UPMNCs, histopathological fracture healing was faster, osteoblast and osteoclast counts were significantly increased, and the maximum load capacity was higher. The presence of XX and XY chromatins on the same slide revealed the anchorage of female donor cells on male tissues.

Conclusion: The effects of umbilico-placental mononuclear cells on bone healing are histopathological healing priority, increased osteoblastic and osteoclastic activities (bone turnover), and better mechanical stability.

Key Words

Bone fracture · Mononuclear · Stem cell · Cord blood · Placenta

DOI 10.1007/s00068-009-9038-8

Introduction

Fracture healing is a complex, well-organized regenerative process initiated in response to injury, which results in optimal skeletal repair and restoration of skeletal function. Delayed fracture healing is a significant problem in orthopedics. Einhorn reported that 5.6 million fractures occur annually in the United States [1]. Praemer et al. reported that 5–10% of these patients had delayed or impaired healing [2]. Researchers have arduously sought alternatives to the current treatments, with tissue engineering receiving much recent attention. Bone tissue engineering refers to the science of creating viable tissue that will replace, repair or augment diseased or defective bone tissue. To achieve successful bone tissue engineering, four critical parameters must be met for osteogenesis: osteoproduction, osteoinduction, osteoconduction, and mechanical stimulation [3]. Osteoproduction refers to the ability of a cell to produce the actual bone material (osteoid matrix).

Traditionally, osteoblasts are responsible for osteogenesis; mesenchymal stem cells can differentiate to become an osteoblast, which can subsequently form bone [4]. Mesenchymal stem cells have been shown to have osteogenic potency, and as the technology to purify and deliver these cells is perfected, it will become possible to treat diseases such as osteogenesis imperfecta and osteoporosis [5]. It has been shown that mononuclear cells derived from umbilical cord blood give rise to fibroblast-like cells that express mesenchymal stem cell related antigens [6]. The objective of this study was to evaluate the healing effects of umbilico-placental mononuclear cells (UPMNCs) on cortical bone regeneration.

Materials and Methods

Surgical Procedure

Following University Ethics Committee approval, caesarean sections were performed on five pregnant
New Zealand rabbits at term. All baby rabbits were marked to identify their mothers and the order in which they were born. Placentas and cord blood were collected in a 50 ml conic Falcon tabbed tube that contained 10 ml of cord blood stem cell feeding medium (TX-ES, Thromb-X, Leuven, Belgium).

**Separating and Freezing the Mononuclear Cells**

After 3 min of vortexing, the extract was transferred to a Falcon tube. It was then filtered to get rid of large tissue pieces. The filtered extract was transferred to another Falcon tube containing 2 ml of Ficoll. After centrifugation (at 10°C, 1,300 rpm for 30 min), the upper layer of serum was transferred to another tube for cryopreservation. The intermediate part, which was rich in mononuclear cells, was mixed with 2 ml of hydroxyethyl starch (HES) and centrifuged at 1,200 rpm for 10 min. The supernatant was separated and the bottom part was resuspended with 1 ml HES. The suspension was placed in a 4.5 ml cryo-vial and 1 ml of serum and 0.2 ml of a dimethyl sulfoxide (DMSO)-containing cryo solution were poured slowly onto the cell suspension and left in a liquid nitrogen tank for vitrification.

**Thawing Procedure**

Frozen vials were taken out of the nitrogen tank, rotated manually for 10 s, and placed into a 37°C water bath until little pieces of crystal could be seen. The thawed suspension was transferred to another tube. After 5 ml of HES solution had been slowly added, the solution was mixed by swinging the tube. The tube was then centrifuged at 1,200 rpm for 5 min. The cell pellets formed at the bottom were resuspended with stem cell culture media (TX-ES) and placed in a 5 ml syringe to be injected into the fracture area.

**Tibial Fracture Procedure and Cell Injections**

The baby rabbits that survived were nurtured for four weeks. At the end of this period, fifteen male rabbits were anesthetized with intraperitoneal ketamine 20 mg/kg and xylazine 5 mg/kg, and standard closed transverse shaft fractures were created on both tibial bones. To produce the standardized fractures, a metal rod that weighed 500 g and was pointed at one end in order to exert pressure on a single point was left to fall freely for 10 cm between two parallel rods that were 3 cm apart, and there was a hole at the bottom of the parallel rods to insert the rabbit’s leg. A tibial fracture was chosen for this study because the subcutaneous localization of the tibia makes it easy to identify the callus and provides easy access for injections near the fracture line in rabbits.

After thawing, 1 ml of the umbilico-placental fluid of the female rabbit born to the same mother were injected into the right tibias of their male siblings at the fracture line immediately after the fractures had been generated. The fluid contained $3.2 \pm 0.8 \times 10^6$ mononuclear cells, with a viability of 96.2 ± 1.9%. The same amount of TX-ES was injected near the fracture lines of the left tibias of the same males, forming the control group. A sterile wound dressing was applied to the site of injection and the fracture was immobilized in a circular cast extending well over the proximal and distal joints of the tibial bone.

To obtain a cumulative effect, the UPMNC injections into the leg were repeated three times on the study legs, with an injection performed every other day. The casts were removed and prophylactic antibiotics were injected intramuscularly 30 min before cell injection. The fracture hematoma was palpated and injections were performed through the posteromedial soft tissue near the fracture hematoma to decrease the risk of infection. The same procedure was applied and the same amount of TX-ES was injected into the control tibia.

One week after the last injections were completed, five rabbits were sacrificed for histological examination. This step was repeated every week for two weeks. At the end of the third week, three of the last five rabbits sacrificed were chosen randomly and a three-point bending test was performed on their tibias on both sides (study and controls). Cross-sectional tissue samples were also obtained from these animals for histological examination. The remaining two rabbits were sacrificed for direct histological examination. Tissue sections obtained from these five animals were prepared for fluorescence in situ hybridization (FISH) to analyze the donor cell anchoring. The weekly plans and histological examinations were conducted according to a study by Kılıçoğlu et al. [7].

**Histological Examination and Quantification of Osteoblasts and Osteoclasts**

After decalcification, tissue specimens were prepared in an autotechnicon, embedded in paraffin, and sectioned with a microtome. The sections (5 μm) were stained with hematoxylin–eosin and Mallory’s azan dyes. Stained specimens were investigated by a Nikon Eclipse E400 light microscope. After examining the histopathological healing site, four different areas were photographed for each specimen after staining using a Nikon Coolpix 5000 camera attachment. All of the images were transferred to a PC and analyzed using Clemex Vision Lite 3.5 image analysis software. The