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Improved isolation protocol for equine cord blood-derived mesenchymal stromal cells

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Background aims

A robust methodology for the isolation of cord blood-derived multipotent mesenchymal stromal cells (CB-MSCs) from fresh umbilical cord blood has not been reported in any species. The objective of this study was to improve the isolation procedure for equine CB-MSCs.

Methods

Pre-culture separation of red and white blood cells was done using either PrepaCyte?-EQ medium or Ficoll-Paque? PREMIUM density medium. Regular FBS and MSC-qualified FBS were compared for their ability to support the establishment of putative primary MSC colonies.

Results and conclusions

Our results indicate that PrepaCyte®-EQ medium is superior to Ficoll-Paque™ PREMIUM density medium for the isolation of putative equine CB MSC and that MSC-qualified FBS may improve the isolation efficiency.

Keywords

Cord blood, horse, isolation, mesenchymal stromal cells.

Introduction

Consistent and reproducible isolation of cord blood (CB)-derived multipotent mesenchymal stromal cells (MSC) from fresh umbilical cord blood has not been reported in any study regardless of the species concerned. The highest reported isolation percentage has been 63% on selected cord blood samples. Developing a robust isolation method for these progenitor cells is crucial for the widespread acceptance of cord blood as a cell source for autologous use later in life. The objective of this study was to improve the isolation procedure for equine CB MSC by evaluating different cell separation methods and fetal bovine serum (FBS) batches. Our results indicate that PrepaCyte®-EQ medium is superior to Ficoll–Paque™ PREMIUM density medium for the isolation of putative equine CB MSC and that MSC-qualified FBS may improve the isolation efficiency.

Methods

Study design and cell line establishment

Each of five fresh CB samples was split into three groups of 42 mL, from which the nuclear or mononuclear cell (MNC) fraction was isolated by one of three methods: PrepaCyte®-EQ medium (PEQ; BioE Inc., St Paul, MN, USA), Ficoll-Paque™ PREMIUM medium (1.077 g/mL; GE Healthcare, Mississauga, ON, Canada) loaded with undiluted whole blood (FUD) and Ficoll-Paque™ PREMIUM medium loaded with diluted whole blood (FD). The manufacturer's guidelines were followed using A and B salt solutions (Figure 1). The cell suspension from each of the three separation methods was split into two groups to allow assessment of two different batches of FBS, one being regular FBS and one being 'MSC-qualified' FBS.

The CB was collected as described previously [1]. The PEQ protocol was based on the manufacturer's guidelines using 7 mL whole blood mixed with 7 mL PEQ in 15-mL tubes. In the FUD and FD groups, 7 mL sample were loaded on to 6 mL Ficoll-Paque PREMIUM density medium (1.077 g/mL) in 15-mL tubes (six tubes total) prior to cell separation, as per the manufacture's guidelines. The nucleated cells in each culture group were suspended in 10 mL isolation medium (low-glucose Dulbecco's modified Eagle medium; DMEM; Lonza, Wakersville, MD, USA), 30% regular FBS (Invitrogen, Burlington, ON, Canada) or

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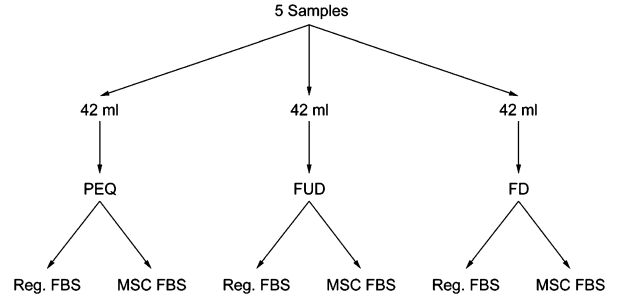

Figure 1. *Study design. Each of five fresh CB samples was split into three groups of 42 mL and the nuclear or MNC fraction was isolated using the PEQ, FUD or FD protocol. Cell suspensions from each of the three separation groups were split further into two groups to allow assessment of two different batches of FBS, regular FBS (Reg. FBS) and MSC-qualified FBS (MSC FBS).*

MSC-qualified FBS (Invitrogen), with low dexamethasone (10^{-7} M; Sigma, Oakville, ON, Canada), penicillin (100 IU/mL; Invitrogen), streptomycin (0.1 mg/mL; Invitrogen) and L-glutamine (2 mM; Sigma). One milliliter was submitted for automated differential cell counting. As most reported seeding densities are based on manual cell counts, which do not discriminate between granulocytes and monocytes, seeding densities were based on the total white blood cell count. Cells were seeded in six-well polystyrene plastic culture plates and incubated at 38.5°C in humidified atmosphere containing 5% CO_2 in air.

Population doubling time (PDT) was calculated from passage (P) 2 onwards as follows: PDT = (log(number of cells obtained at subculture per cm^2/[cell seeding density per cm^2)/log 2)/d], where d is the number of days in culture.

Trilineage differentiation studies

Three cryopreserved cell lines from the PEQ group were assessed for adipogenic, chondrogenic and osteogenic potency, as demonstrated previously for cells derived using Ficoll medium separation [1].

For adipogenesis, each cell line was cultured for 10 days in either continuous expansion culture medium (regular FBS), as defined above, or continuous adipogenic induction medium (BulletKit®; Lonza). The induction medium consisted of 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 10 μg/mL recombinant human (rh) insulin, 0.2 mM indomethacin and 10% fetal calf serum (FCS) in DMEM. The fetal calf serum included the commercial medium kit for adipogenic induction was discarded and substituted with 15% rabbit serum (Sigma). Oil Red O staining was done as described previously except rinsing and staining volumes of 200 μL were used because of the smaller well size [1]. The AdipoRed™ assay (Lonza) was used according to the manufacturer's protocol for 96-well plates with the exception of a 520-nm emission filter instead of 572 nm or 535 nm. The suitability of the 520-nm emission filter was discussed with the manufacturer's scientific product adviser prior to use. The total protein content of the AdipoRed assayed wells was determined as described previously using a DC Bio-Rad protein method (DC protein assay reagents package; Biorad, Mississauga, ON, Canada) [1].

Chondrogenic differentiation was performed using a micromass culture system [1,2]. Undifferentiated cells (2.5×10^5 cells) were cultured for 2 and 4 weeks in 0.5 mL complete chondrogenic differentiation medium (Lonza) containing 10 ng/mL transforming growth factor-beta 3 (TGF-β3). Pellets were fixed in 10% formalin, imbedded in paraffin blocks and sectioned into 5-μm sections. Hematoxylin and eosin (Sigma) as well as Safranin O (Sigma) and Alcian Blue (Sigma) staining was used to evaluate chondrogenic differentiation histologically.

Osteogenesis was induced using osteogenic induction medium (100 nm dexamethasone and 10 mM β-glycerophosphate; Sigma), 0.05 mM L-ascorbic acid-2-phosphate (Fluka Biochemika, Sigma) and 10% FBS in low-glucose DMEM (Lonza) for 10 days and compared with control cultures exposed to regular expansion medium. Osteogenic differentiation was evaluated qualitatively using Alizarin Red S staining and semi-quantitatively by alkaline phosphatase, calcium and protein assays, as reported elsewhere [1].

Statistical analysis

Data were analyzed using ANOVA, Box Cox transforms, linear modeling and contrast methods as applicable.

Results and Discussion

Significantly more MNC were isolated in the PEQ group compared with the FUD and FD groups (Figure 2A). The average total numbers of MNC seeded from each of the processed 42 mL cord blood within each group were as follows: PEQ, $4.5.0 \times 10^8$ cells (range $2.43 \times 10^8 - 8.91 \times 10^8$); FUD, 2.88×10^7 cells (range $9.0 \times 10^6 - 5.4 \times 10^7$); FD, 2.52×10^7 cells (range $9.0 \times 10^6 - 5.4 \times 10$). Significantly more colonies were established in the PEQ group