Safety of umbilical cord blood-derived mesenchymal stem cells (MSCs) following 5-azaserine induction and inhibition of human cardiac myocyte apoptosis by MSCs

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ABSTRACT

الأهداف: إجراء المزيد من الدراسات على السلامة وما إذا كان تحويل الحبل السري (UCB) يؤثر على مضادات انتهاء المرض على الزراعة المشتركة مع (HCM).

الطريقة: تم الحصول على (UCB) في وقت الولادة مع العينة الحاصلة من 10 متبرعين. تمت معالجة تحويل (UCB) بواسطة 5–(AZA) والمزيد من التغيرات على خلايا القلب. تم فحص نشاط تيلوموريس ونماذج ربط–جي للربط الكروموزومي النووي. وتشكل الورم في عقد الفئران وRT–PCR وتثبيط انتهاء المرض لتحويل الحبل السري لخلايا جذع النسيج المتوسطي. أجريت هذه الدراسة في مختبر مستشفى شيجيتان وقسم مركز الأبحاث –المعهد الطبي الصيني خلال الفترة ما بين يوليو 2005م إلى ديسمبر 2007م– بيجنج – الصين.

النتائج: تم تمييز تحويل (MSCs) من (UCB) في خلايا القلب، ونشاط تيلوميريس بعد تلقي التحفيز بعقار(5– AZA) ولم يتم ملاحظة أنماط كروموزوميه نووية غير طبيعية. كان ظهور (p53) و(cyclin A) و (CDK2) و(تفاعل بيتا) و(fos– c) و (h-TERT) و (myc–c) مشابهة في (MSCs) قبل وبعد تلقي المعالجة بعقار 5–AZA). كما لم تبين وجود تشكل ورمي محتقن في عقد الفئران. ثبط تحويل (MSCs) (UCB) بشكل ملحوظ انتهاء مرض الخلايا القلبية البشرية.

خاتمة: يعتبر تحويل (MSCs) (UCB) مصدر فعال وآمن وقيم في معالجة زراعة الخلايا ويمكن أن يثبط انتهاء المرض لدى خلايا القلب البشرية في الزراعة المشتركة.

Objectives: To further study the safety and effect of the umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) on apoptosis of human cardiac myocyte (HCM).

Methods: The UCB was collected at the time of delivery with informed consent obtained from 10 donors. The UCB-derived MSCs was treated with 5-azaserine (5-AZA), and further introduced differentiation into cardiomyocytes. The telomerase activity, G-banding patterns of chromosomal karyotypes, tumor formation in nude mice, reverse transcription polymerase chain reaction (RT-PCR), and the inhibited apoptosis of UCB-derived MSCs were further investigated. This study was carried out in the laboratory of Beijing Shijitan Hospital, Beijing, China and Inheritance Research Section of Chinese Medical Institute, Beijing, China from July 2005 to December 2007.

Results: The MSCs-derived from UCB were differentiated into cardiomyocytes *in vitro*, possessed telomerase activity after 5-AZA induction, and no abnormal chromosomal karyotypes were observed. Expression of p53, cyclinA, cdk2, ß-actin, C-fos, h-TERT and c-myc were similar in MSCs before and after 5-AZA treatment. There was no tumor formation injected into nude mice. The UCB-derived MSCs significantly inhibited apoptosis of human cardiomyocytes.

Conclusion: Umbilical cord blood-derived MSCs are safe and effective source of cell-transplantation treatment, and can inhibit the apoptosis of human cardiomyocytes in co-cultured.

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The need to obtain mesenchymal stem cells (MSCs) from alternative sources is still an expanding field of research at present.[1] It is well accepted that blood from the umbilical cord contains hematopoietic stem cells, and obtaining these cells after delivery of a fetus is very easy. It was reported[2-4] that there was multi-lineage differentiation ability of cells isolated from umbilical cord blood (UCB). Safety is one of the major concerns of using MSC-differentiated cells. Therefore, the safety and ability of these MSCs from UCB to be expanded and differentiated *in vitro* through chronic 5-azaserine (5-AZA) treatment were tested in this study. As well-known, apoptosis occurs in a wide variety of cardiovascular disorders, and is a fundamental process that contributes to the deterioration of cardiac function. Stem cell homing and engraftment to the heart can result in new tissue formation, potentially capable of replacing the lost myocardium, and inhibiting apoptosis of myocardial cells.[5-8] But the ability of MSC and human cardiac myocyte (HCM) co-culture to inhibit apoptosis of HCM has not been reported in the setting of cellular cardiomyoplasty. So, the effect of UCB-derived MSCs on apoptosis of HCM was investigated in this study.

Methods. This study was carried out in the Laboratory of Shijitan Hospital, Peking University, Beijing, China, and the Inheritance Research Section of Chinese Medical Institute, Beijing, China, from July 2005 to December 2007. The UCB was collected at the time of delivery with informed consent obtained from 10 donors (the ethical approval is not necessary in our study at that time. A certification from the Chair of the local Research Ethics Committee confirmed that the ethics approval was not needed). The UCB mononuclear cells obtained were allowed to adhere to the flasks overnight. Medium changes were performed every 3 days, with the expansion medium containing: DMEM/F12 (DMEM:F12=1:1; Hyclone) plus 20% fetal bovine serum (FBS), (Lanzhou National Hyclone Bio-Engeering Co, Ltd, Lanzhou, Gansu, China) supplemented with 10 ng/mL HbFGF (TECR Incorporated, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. To induce the differentiation of UCB-derived MSCs into cardiomyocytes, third to fifth passage cells were treated with DMEM/F12 plus 10 ng/mL 5-AZA (Sigma Chemical Co., St. Louis, MO, USA) for 24 hours. The UCB-derived MSCs were further purified by flow cytometry with fluoresceinated antibodies CD34, CD44 and CD90 (BD Pharmingen, New Jersey, USA). The presence of cardiomyocytes was assessed 4 weeks later. To detect telomerase activity, the third to fifth passage cells were induced ($1x10^6$) using a Telomerase Detection Kit (Chemicon International Inc., CA, USA) according to the manufacturer's instructions. To detect the presence of pro-oncogenes of MSC, such as p53, cyclinA, cdk2, C-fos, h-TERT, c-myc, and so forth, the RNA was extracted from the third to fifth passage cultured and differentiated cells (1×10^6) using the Trizol (Invitrogen, Life Technologies Corp., CA, USA) technique as per the manufacturer's instructions.The mRNA was reverse transcribed into cDNA using a cloned AMV first-strand cDNA synthesis Kit (Invitrogen, Life Technologies Corp., CA, USA) as per manufacturer's instructions, and cDNA was amplified using GeneAmp PCR System (Applied Biosystems Inc.,, CA, USA) at 94°C for 40 seconds, 56°C for 50 seconds, and 72°C for 60 seconds, with a total of 35 cycles after initial denaturation. Primers used for amplification are listed in **Table 1**. To detect G-banding patterns of chromosomal karyotpes, the third to fifth passage MSCs and MSCs treated with 5-AZA (5×10^6) were grown in DMEM/F12 plus 0.3 μg/mL colchicine (Sigma, Beijing, China) for 4 hours. Metaphase chromosome spreads were prepared. Then, standard G-banding with trypsin-Giemsa staining was performed. The chromosomes were arranged to create a karyotype. To detect the possibility of tumor-formation of MSC, nude mice test was carried out *in vivo*. All procedures performed on animals were approved by the Animal Care and Use Committee of the National Laboratory Animal Center, Shanghai Branch, Shanghai, China. Male BALB/c nude mice, 6 weeks old, weighing 16-20g were obtained from the National Laboratory Animal Center, Shanghai Branch. The mice were housed under specific pathogen-free conditions. Third to fifth passage MSCs and MSCs treated with 5-AZA were resuspended in aseptic phosphate buffered saline (PBS) (pH 7.4) solution at a concentration of 1×10^7 cells/mL. A 0.2 mL cell suspension was innoculated subcutaneously into the right anterior limb of each nude mouse, with 6 mice per cell type.

For the detection of antigen phenotyping of each cell surface, third to fifth passage cells were detached and stained with fluorescein or phycoerythrin-coupled antibodies, and analyzed with FACS Calibur (Becton-Dickinson, Beijing, China). For apoptosis detection, cells were subjected to analysis after AnnexinV and PI double staining. To detect the inhibited apoptosis of UCB-derived MSCs, UCB-derived MSCs and HCM (ScienCell, San Diego, CA, USA) were co-cultured in 6-well plate, 12 mm glass coverslips (glass coverslips must be sterilized for TUNEL analysis of apoptosis) were placed on the bottom of the 6-well plate. An upper chamber containing a suspension of MSCs from UCB was separated by a membrane from a lower chamber containing a medium with the above cells. Control cells were cultured in the original 6-well plate. Apoptotic cells on 12 mm glass coverslips were visualized using

Table 1 - Primers used for reverse transcriptase-polymerase chain reaction.

Primer	Sequence	Product
ß-actin	Sense: 5'-CATGTACGTTGCTATCCAGGC-3'	250 bp
	Antisense: 5'-CTCCTTAATGTCACGCACGAT	
h-TERT	Sense: 5'-TCTACCGGAAGAGTGTCTGGAGCAA-3'	151 bp
	Antisense: 5'-GCGCCCACGACGTAGTCCATGTTCA-3'	
C-myc	Sense: 5'-TGGTCGCCCTCCTATGTTG -3'	151 bp
	Antisense: 5'-CCGGGTCGCAGATGAAACTC-3'	
P53	Sense: 5'-TGGTTGCAGTCTAGCGACC-3'	223 bp
	Antisense: 5'-TCAGGAAGACAAGCATCTGGG -3'	
C-fos	Sense: 5'-CCACCCGAACAGTCTCTCCT-3'	115 bp
	Antisense: 5'-AGAAGCCCAAAAGCCATAGGT-3	
Cdk2	Sense: 5'-GGCCCGGCAAGATTTTAGTA-3'	102 bp
	Antisense: 5'-CTATCAGTCGAAGATGGG-3'	
cyclinA	Sense: 5'-GCATTGCAGCAGACGGCGCT-3'	451 bp
	Antisense: 5'-TGGCTGTTTCTTCATGTAACCCAC-3'	

the CardioTACS *in situ* apoptosis detection kit (R&D Systems, Minneapolis, USA). To discriminate apoptotic cells from necrotic cells, the samples were counter-stained to aid in the morphological verification of apoptosis.

The statistical analysis was performed in the study utilizing t-test with SPSS software.

Results. The UCB mononuclear cells, and UCB-derived MSCs is shown in **Figure 1** (arrows) (Hematoxylin & Eosin [H & E] x200). The cardiomyocytes of UCB-derived MSCs induction with 5-AZA for 21 days (H&E x200) is shown in **Figure 2.** The start of spindle formation as well as bi-nucleation was noted. The telomerase activity and the presence of pro-oncogenes of MSC were shown in **Figures 3 and 4.** Telomerase activity of the third to fifth passages of UCB-derived MSCs was similar to that of UCB-derived MSCs after 5-AZA induction (**Figure 3**). There was almost no detectable telomerase activity in UCB mononuclear cells (**Figure 3**, lane 3). **Figure 4A** shows total RNA from long-term cultured MSCs (left panel) and mRNA expressions of related genes in 5-AZA treated MSCs cells (**Figure 4B**) (right panel). The 5-AZA treated MSCs were probed for various signaling cascades. Expressions of p53, cyclinA, cdk2, β-actin, C-fos, h-TERT and c-myc were similar in MSCs after (top), and before (bottom) 5-AZA treatment, as shown in the individual lanes. The G-banding patterns of chromosomal karyotypes in MSCs were not shown here. The UCB-derived MSCs and differentiated cells induced with 5-AZA have normal female diploid chromosomes karyotypes. The results of immunophenotypic characterization and purification of UCB-derived MSCs had not changed before, and after 5-AZA induction. Fibroblast-like, rapidly dividing

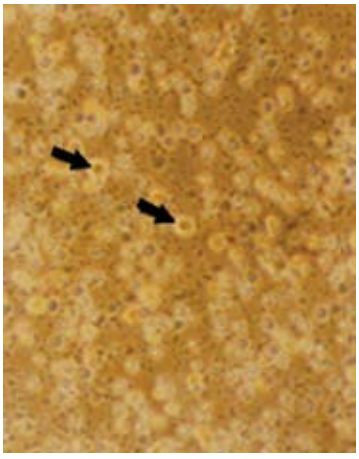

Figure 1 - Umbilical cord blood mononuclear cells (arrows) visualized under light microscopy (Hematoxylin & Eosin x200).

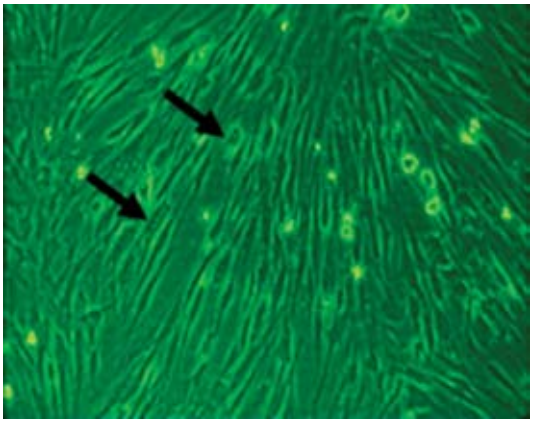

Figure 2 - The cardiomyocytes of umbilical cord blood-derived mesenchymal stem cells induction with 5-azaserine for 21 days (Hematoxylin & Eosin x200), Note the start of spindle formation as well as binucleation (arrows).

cells were extensively expanded and characterized by flow cytometry, revealing that the cells were negative for CD34 (leukocyte common antigen), and positive for matrix receptor CD44 (hyaluronate receptor), CD90 (Thy-1). The tumor formation in nude mice *in vivo* after transplantation of MSCs is not shown here. Following 10 weeks after transplantation of MSCs, the nude mice remained alive and healthy. No solid tumors were detected anywhere near the innoculation site. Paraffin tissue sections were prepared and stained with hematoxylin and eosin stain, showing normal subcutaneous tissue. The result of inhibition of HCM apoptosis by MSCs is shown in **Figure 5**. As shown, the apoptosis of HCM was inhibited significantly by co-culture of HCM with MSC.

Discussion. The MSCs are valuable sources of tissue regeneration, especially for cellular cardiomyoplasty.[9-11]

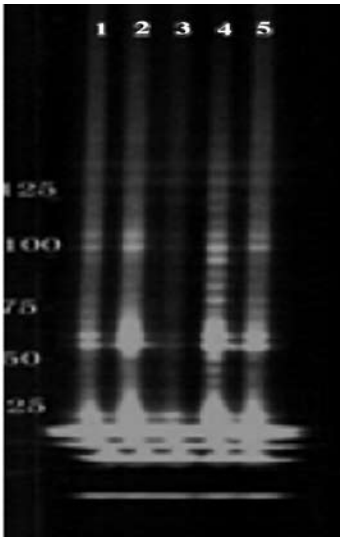

Figure 3 - Telomerase activity of umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs). Lanes 1 and 2: third to fifth passage detached UCB-derived MSCs; Lane 3: UCB mononuclear cells; Lanes 4 and 5: UCB-derived MSCs after induction of 5-azaserine.

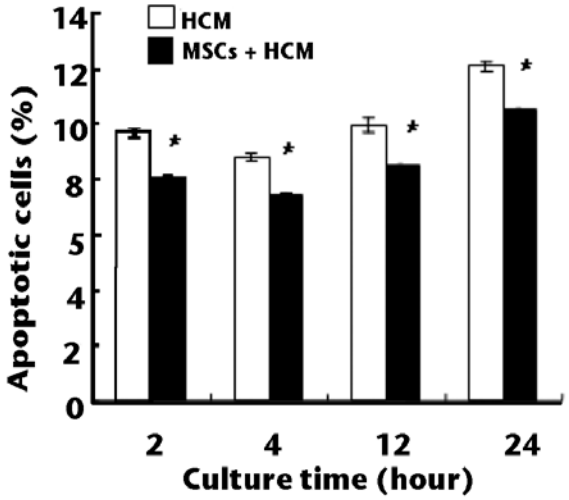

Figure 5 - The addition of mesenchymal stem cells (MSCs) inhibited apoptosis of human cardiac myocyte (HCM) in co-culture. Data are expressed as mean ± SD of the 3 independent experiments. *p<0.05 versus HCM.

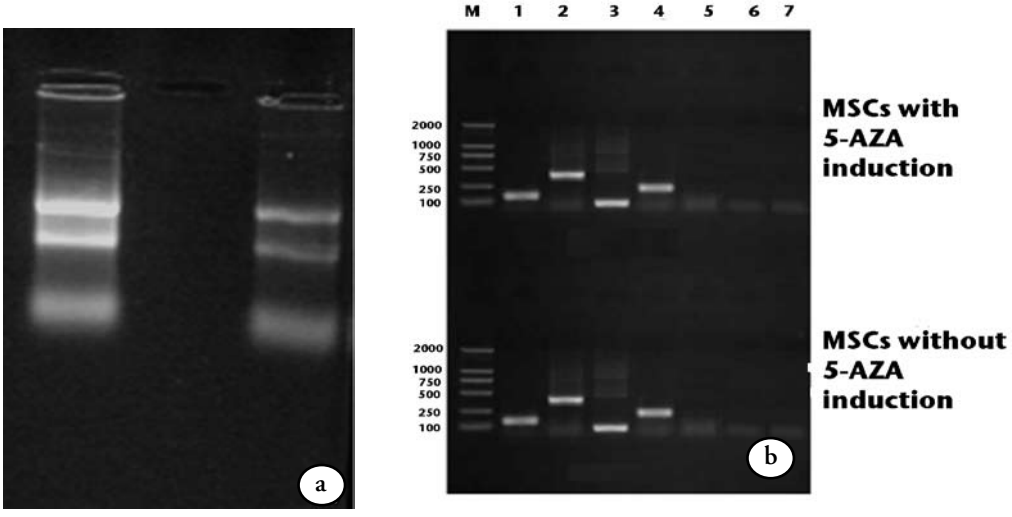

Figure 4 - Detection of RNA in umbilical cord blood (UCB)-derived mesenchymal stem cells (MSCs) cells. **a)** Total RNA of UCB-derived long-term cultured MSCs; **b)** mRNA expressions of related genes in 5-azaserine (5-AZA) induced (top) and without induced (bottom) MSCs cells. Lane 1: p53; Lane 2: cyclinA; Lane 3: cdk2; Lane 4: ß-actin; Lane 5: C-fos; Lane 6: h-TERT; Lane 7: c-myc

However, the best source of these cells has not been determined. Most important for obtaining MSCs are the abilities for proliferation and differentiation, however these abilities decrease with age,[12,13] whereas the MSCs from UCB are mostly from young donors, so these cells could be an excellent source of MSCs. In the present study, we further proved that the UCB-derived MSCs could be a new valuable source of MSCs. Compared with obtaining bone marrow-derived MSCs, we found that UCB-derived mononuclear cells (**Figure 1**) is easily obtained, furthermore, the affliction of bone marrow puncture was avoided. Also, induced with 5-AZA, the successful differentiation rate of cardiomyocytes (**Figure 2**) from UCB-derived MSCs was seemingly high and easy, compared to bone marrow-derived MSCs from our previous study,[14] and there may be a co-relationship with young donors of the UCB-derived MSCs.[15,16] Our results demonstrated that in the telomerase activity (**Figure 3**), there was no difference between the UCB-derived MSCs lineages with and without 5-AZA induction. Telomerase activity has been shown to be specifically expressed in immortal cells, such as cancer and germ cells, where it compensates for telomere shortening during DNA replication, and thus, stabilizes telomere length. Therefore, the expression of telomerase activity in pluripotent stem cells may be a necessary, and essential step in the maintenance and differentiation of those cells.[17] Following 5-AZA induction, USB-derived MSCs showed apparent telomerase activity, implying their pluripotency and ability for further amplification, but with no significant difference compared with un-induced, and it may be conclusion of the safety of 5-AZA induction. The cardiomyocytes from UCB-derived MSCs induction have been obtained, but whether it is safe for clinical transplantation treatment,[18,19] especially for the utilization of cellular cardiomyoplasty. We further tested the presence of pro-oncogenes of MSC with and without introduction: the expressions of p53, cyclinA, cdk2, C-fos, β-actin, h-TERT and c-myc, also the chromosomal karyotypes, tumor formation in nude mice were investigated. Our results showed that there was no difference of the presence of pro-oncogenes of MSC with and without induction (**Figure 4**), and no abnormal chromosomal karyotypes in UCB-derived MSCs after 5-AZA induction compared with un-induced MSCs. Chromosomes are visible and analyzed only during active cell division when the chromosomal material is condensing. Normally the number, shape, and structures of chromosomes is stable, however various factors can influence karyotypes changes, with most tumor cells having abnormal chromosome karyotypes. Additionally, transplantation of 5-AZA treated MSCs did not form any tumors in nude mice, all of these results suggest that UCB-derived MSCs are safe for transplantation treatment.

We had determined the surface molecular markers of UCB-derived MSCs in previous study.[20] The flow cytometry was used to further purify these MSC cells. Flow cytometry applying fluoresceinated antibodies can be used to determine surface molecules present in the expanded cell population, in both positive and negative surface molecules. Flow cytometric analysis showed that these cells were negative for CD34 HSCs markers, but positive for human MSC markers CD44 and CD90, and is consistent with that reported in the literature for the bone marrow counterpart, indicating the MSC nature of these UCB-derived cells.

It was proven that stem cell homing and engraftment to the heart can result in new tissue formation, potentially capable of replacing the lost myocardium, and inhibiting apoptosis of myocardial cells, ultimately improving the functional performance of the damaged heart.[5-8] Interestingly, our results demonstrated that the apoptosis of HCM could be inhibited when it is co-cultured with UCB-derived MSCs (**Figure 5**). This could be a paracrine mechanism, whereby myocyte-released factors are quenched by MSCs, thereby inhibiting the formation of apoptotic signals,[21,22] also by enhancing survival signaling cascades leads to inhibition of apoptosis,[23,24] as well as, controlling myocardial cell losses. These results could prove a new mechanism and provide a valuable new therapeutic tool in the treatment of debilitating illnesses, such as myocardial infarction, and a useful strategy for slowing the development of heart failure. Since apoptosis is a known mechanism in the formation of cardiovascular disease, therefore, UCB-derived MSCs could be another viable source of somatic cells that could potentially be used to treat cardiovascular disease. However, the data is inconclusive and this area warrants further study to characterize the ability of these cells to actively differentiate into other cell types, as well as the mechanism(s) involved in this potential inhibiting apoptosis.

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