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Efficient method for isolation of human umbilical cord mesenchymal stem cells★

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Abstract

BACKGROUND: The theoretical value of the mesenchymal stem cells from human umbilical cord isolated by the traditional methods is far away from that of the mesenchymal stem cells from human umbilical cord contained in the human umbilical cord tissues, which lead to the great waste of the umbilical cord tissues and prevent the large-scale preparation and culture of mesenchymal stem cells.

OBJECTIVE: To optimize and develop a new efficient method for the isolation of human umbilical cord mesenchymal stem cells in order to provide the technical solutions for the clinical application of mesencymal stem cells.

METHODS: The experiment was divided into four groups. The control group was treated with traditional enzymatic method (trypsin and collagenase type II), on this basis, the collagenase type Ⅳ, hyaluronidase and DNase were added into the umbilical cord tissues for digestion. The indicators obtained through different isolation methods and different operation time were compared, including the amount of the cells, cells activity, time for the occurrence of the primary cell adherent and fusion time, as well as the proliferative capacity, surface markers and differentiation capacity of the passage 3 umbilical cord mesenchymal stem cells.

RESULTS AND CONCLUSION: Collagenase type II, collagenase type IV, trypsin, hyaluronidase, and DNAase were used to digest the umbilical cord, thus cell count reached up to (12.47±0.16)×10^6/cm (P < 0.01) in 4 hours; cell activity was (75.00±5.07) (P < 0.01); CD105 positive cell rate was 41.1%; and CD29 positive cell rate was 83.1%, these measurement indicators in the experimental groups were higher than those in the control group; the obtained cells got adherent and associated fusion, and the time in the experimental groups was shorter than that in the control group (P < 0.01); the cells gradually got uniform shape, the cell morphology of the third-passage was spindle-shaped and swirling. The positive rates of the specific markers CD44, CD105 and CD29 of passage 3 human umbilical cord mesenchymal stem cells were higher than those in the control group, and the positive rate of CD34 in the experimental groups was lower than that in control group. After induction to fat cells for 4 weeks, differentiation results were assayed by oil red O staining, showing that a large number of lipid droplets were observed in cells; the differentiation capacity to osteoblasts was measured by alizarin red staining, and visible positive calcium deposition was observed. The experiment demonstrated that the combination of collagenase type Ⅱ, collagenase type Ⅳ, hyaluronidase, trypsin and DNAase for digestion of umbilical cord for 4 hours could significantly improve the yield, viability and proliferation ability of mesenchymal stem cells and shorten the time of isolation. In addition, the time for primary mesenchymal stem cells to adhere and grow to confluence was shortened by using the combined digestion method than common digestion method. And the ratio of mesenchymal stem cells in the primary mesenchymal stem cells and the purity of the passage three mesenchymal stem cells obtained using combined digestion method are much better than those isolated using common digestion method.

INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent stem cells that have the abilities of self-renewal and differentiation into a variety of cell types[1], including muscle cells, myocardial cells and nerve cells, and have shown great promise in regenerative medicine[2-3]. The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy has proposed the following minimal criteria to define human MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must highly express the specific markers of CD44, CD73, CD105 and CD29, and lowly express CD14, CD19, CD31 or CD45 and CD34. Third, MSCs must be able to differentiate into osteoblasts, adipocytes and chondroblasts *in vitro*[1, 4]. MSCs can be isolated from a variety of tissues or organs such as bone marrow[5], adipose tissue[6], skin and hair follicle, and the MSCs have the great ability of proliferation and differentiation. Recent studies found that the MSCs also can be isolate from periosteum,

trabecular bone, synovium, skeletal muscle, teeth, pancreas, lung, liver, amniotic fluid, placenta and umbilical cord blood[7], as well as the umbilical cord. Therefore, alternative sources of MSCs are in urgent need. Recent evidence suggested that umbilical cord contains more MSCs than umbilical cord blood[8-10]. Umbilical cord is extra-embryonic tissue and the fetal waste after the delivery. It is easily available without ethical controversy, and restriction on donor age and gender[11]. Moreover, MSCs are abundant in umbilical cord tissue. Therefore, umbilical cord is the ideal source of MSCs[5, 12].

Wharton's jelly is an important component of umbilical cord and is a rich source of fetus-derived stem cells[13-14]. Wharton's jelly is composed of collagen fibers and proteoglycans[15]. Collagen Ⅰ, Ⅲ, Ⅳ and Ⅴ are all present in Wharton's jelly and 70% of collagen is collagen Ⅳ, while 70% of proteoglycans is hyaluronic acid[16]. Currently, different protocols have been developed to isolate MSCs from umbilical cord, including explant-culture method, single enzyme digestion method (collagenase[17], collagenase Ⅳ[5] or collagenase Ⅰ[18]), double enzyme digestion method (collagenase and trypsin[19]), and three enzyme digestion method (collagenase, hyaluronidase and trypsin[20]. Explant culture method is simple and easy to operate, but the yield of MSCs is low and the operation is time-consuming[21]. Enzyme digestion method also has some disadvantages: the selection and the digestion time for the enzymes are not standardized, the viscosity of the digested tissue is high, it is not easy for the cells to be isolated and adhered for the culture, and the culture time is long.

In this study we aimed to optimize the protocol for the isolation of MSCs from umbilical cord. We found that the combination of collagenase Ⅱ, collagenase Ⅳ, hyaluronidase, trypsin and DNAase can led to the completely digestion of umbilical cord tissue and reduce the viscosity, and human umbilical cord mesenchymal stem cells (HUCMSCs) can be effectively isolated and cultured from umbilical cord.

MATERIALS AND METHODS

Design

Single sample observation.

Time and setting

The experiment were accomplished at Department of Biochemistry and Molecular Biology, Shanxi Medical University from 2010-09 to 2012-03.

Materials

Human umbilical cord (20 cm long) was aseptically collected from infants delivered by full-term normal labor at Second Affiliated Hospital of Shanxi Medical University. The pregnant women gave informed consent.

Methods

The umbilical cord was treated as described previously[17] and then divided into different experimental groups for different enzyme digestion. After the digestion the cells were cultured in α-minimum essential medium (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS, Sijiqing Co, Hanzhou), 100 U/mL penicillin and 100 g/mL streptomycin in the incubator with 95% humidity at 37 ℃, containing 5% (v/v) CO_2. The medium was changed every 2 days during the culture. The cells were passaged when they reached 70%-80% confluence[22].

Experimental groups

For enzyme digestion, the experiments were divided into four groups (Table 1). Collagenase Ⅱ, collagenase Ⅳ, hyaluronidase, trypsin and DNAase were purchased from Sigma.

Table 1 Different enzyme digestions in each experimental group

Agent	Control group	Experimental group 1	Experimental group 2	Experimental group 3
Collagenase type Ⅱ	0.2%	0.2%	0.2%	0.2%
Collagenase type Ⅳ	-	0.2%	0.2%	0.2%
Hyaluronidase	-	-	0.1%	0.1%
	Incubated for different time at 37 ℃			
Trypsin	0.1%	0.1%	0.1%	0.1%
DNAase	-	-	-	15 UI/mL
	Incubated for 30 min at 37 ℃			

Evaluation of the yield and viability of HUCMSCs isolated from umbilical cord

After digestion, the digested tissues were washed extensively with phosphate buffered saline (PBS) and resuspended. The amount of the cells was counted under microscope and the viability of the cells was evaluated by 0.4% trypan blue staining. The dead cells were stained as light blue while live cells were not stained as light blue.

The yield and viability of the cells were calculated as follows: the yield (cells/cm) = (density of cells × volume of cell suspension)/length of the tissue; the viability (%) = (amount of live cells/amount of total cells) ×100.

Flow cytometry analysis of isolated HUCMSCs

The isolated MSCs were resuspended at the density of 1×10^6 cells/mL and incubated with phycocrthrin (PE) labeled CD29, CD105, CD34 and CD44 antibodies (BD) for 30 minutes at 4 ℃ in the dark. Then the cells were washed 3 times with PBS and fixed in 10 g/L

paraformaldehyde and subjected for flow cytometry analysis. The assays were repeated for 3 times.

Analysis of colony-forming ability of isolated HUCMSCs

The isolated MSCs from different groups were diluted and incubated into 6-well plates at the density of 1×10^4 cells, 2×10^4 cells or 3×10^4 cells per well. After cultured for 14 days, the cells were stained with crystal violet and the colonies were counted. The assays were repeated for 3 times.

Analysis of the differentiation ability of isolated MSCs

The differentiation of isolated MSCs into adipocytes and osteoblasts was examined as described previously[23]. Briefly, for adipogenic differentiation, the isolated MSCs from different groups were incubated into 6-well plates at the density of 2×10^4 cells per well and cultured in adipogenic stimulatory medium (Cyagen, USA) following the manufacture's protocols. After cultured for 3 weeks, the cells were fixed in 10% formalin for 15 minutes, and stained with oil red O. For osteogenic differentiation, the isolated MSCs from different groups were incubated into 6-well plates at the density of 3×10^3 cells per well and cultured in osteogenic medium (Cyagen, USA) following the manufacture's protocols. After cultured for 2−3 weeks, the cells were fixed in 70% ethanol for 30 minutes at 20 ℃, and subjected to alizarin red S staining.

Statistical analysis

Data were expressed as the mean ± standard deviation and analyzed using the Statistical Product and Service Solutions version 12 statistical analysis package (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was accepted as statistically significant.

RESULTS

The yield of MSCs in different groups

The yield of MSCs isolated from umbilical cord was shown in Figure 1. The results demonstrated that the yield was significantly increased with the addition of 0.2% collagenase Ⅳ, 0.1% hyaluronidase, and 15 UI/mL DNAase. The combination of collagenase Ⅱ and Ⅳ, hyaluronidase, trypsin and DNAase led to the highest yield, and the optimal time for digestion was 4 hours.

The viability of HUCMSCs in different groups

The viability of HUCMSCs was shown in Figure 2. The results demonstrated that the viability of HUCMSCs was significantly increased with the addition of 0.2% collagenase Ⅳ, 0.1% hyaluronidase (HAase), and 15UI/mL DNAase. The combination of collagenase Ⅱ and Ⅳ, hyaluronidase, trypsin and DNAase led to the highest viability, and the optimal time for digestion was 4 hours.

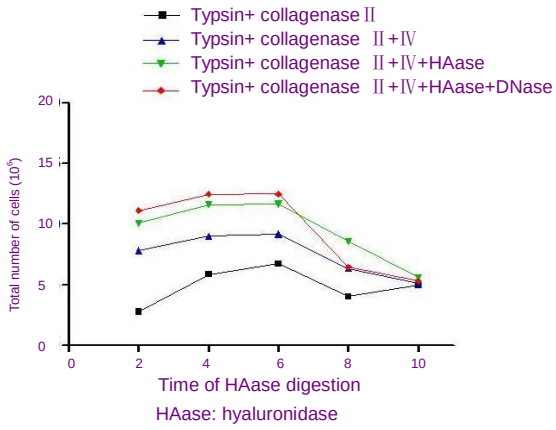


Figure 1 Yield of human umbilical cord mesenchymal stem cells digested by different methods

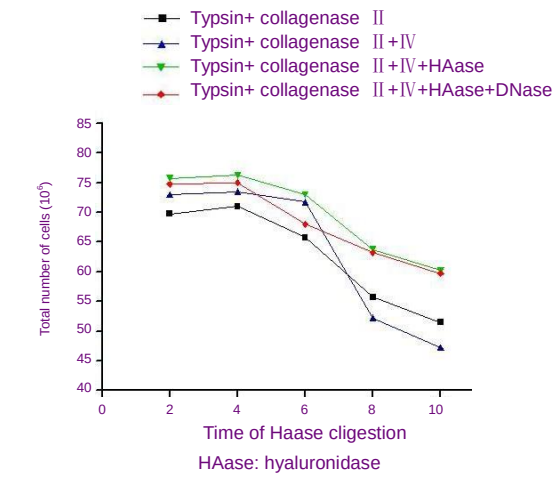
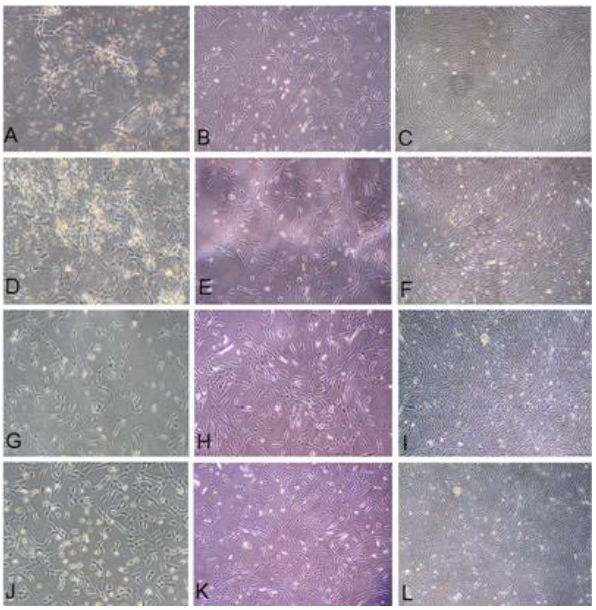


Figure 2 Viability of human umbilical cord mesenchymal stem cells in experimental group 3 digested by different methods

Morphology of HUCMSCs (Figure 3)

HUCMSCs adhered to the walls and exhibited a fibroblast-like, spindle-shaped morphology. In control group and experimental group 1, HUCMSCs exhibited spindle-shaped morphology on the 5th day after culture. After passage, the cells grew to 40%−50% confluence on the 7th day and grew to 90%−100% confluence on the 9th day. In experimental group 2 and 3, HUCMSCs exhibited spindle-shaped morphology on the 3rd day after culture. After passage, the cells grew to 60%−70% confluence on the 5th day and grew to 90%−100% confluence on the 7th day. Compared with control group, it took 2 days less for the HUCMSCs isolated from experimental group 3 to adhered and grew to confluence.

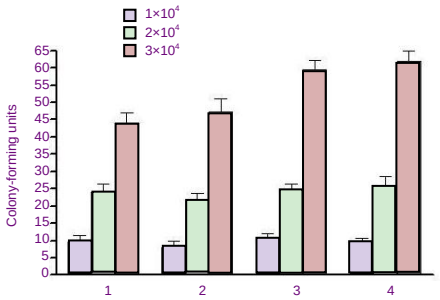


A, B, C: Morphology of HUCMSCs in control group after cultured for 5, 7 and 9 d; D, E, F: Morphology of HUCMSCs in experimental group 1 after cultured for 5, 7 and 9 d; G, H, I: Morphology of HUCMSCs in experimental group 2 after cultured for 3, 5 and 7 d; J, K, L: Morphology of HUCMSCs in experimental group 3 after cultured for 3, 5 and 7 d

Figure 3 Morphology of human umbilical cord mesenchymal stem cells (HUCMSCs) in each experimental group (×100)

The colony-forming ability of HUCMSCs in different groups

The colony-forming ability of HUCMSCs was shown in Figure 4. The results demonstrated that the colony-forming ability of HUCMSCs was significantly increased with the addition of 0.2% collagenase Ⅳ, 0.1% HAase, and 15 UI/mL DNAase. The combination of collagenase Ⅱ and Ⅳ, hyaluronidase, trypsin and DNAase led to the isolation of HUCMSCs with the highest colony-forming ability.



1: Typsin+ collagenase Ⅱ; 2: Typsin+ collagenase Ⅱ+Ⅳ; 3: Typsin+ collagenase Ⅱ+Ⅳ+HAase; 4: Typsin+ collagenase Ⅱ+Ⅳ +HAase+DNase

Figure 4 Colony-forming ability of human umbilical cord mesenchymal stem cells digested by different methods

Characterization of HUCMSCs

To characterize the HUCMSCs, first we examined the expression of several defined surface markers of HUCMSCs. After the digestion of umbilical cord tissue with different combinations of enzymes, the crude cells suspensions were subjected to flow cytometry analysis. The results showed that for the cells isolated from control group, 30.2% were positive for CD29 and 38.8% were positive for CD105 and 16.6% were positive for CD44 (Figures 5a, c, e). In contrast, 82.8% of cells isolated from experimental group 3 were positive for CD29 and 41.1% of them were positive for CD105 and 17.1% of them were positive for CD44 (Figures 5b, d, f).

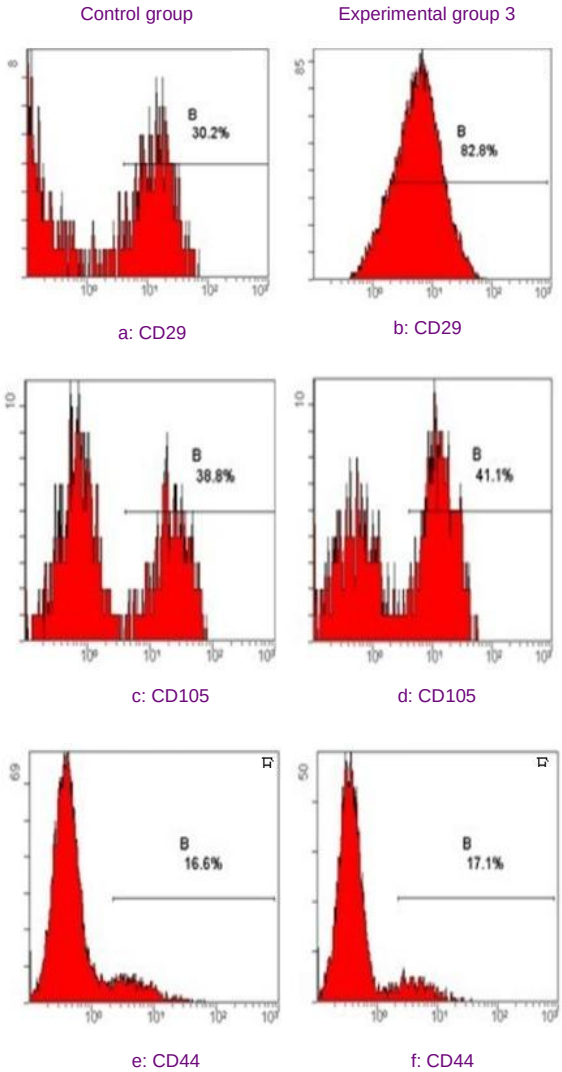


Figure 5 Flow cytometry analysis of surface markers of mesenchymal stem cells isolated from umbilical cord

Furthermore, we analyzed the HUCMSCs of the 3rd generation after passage, the results showed that for the HUCMSCs from control group, 80.9%, 95.3%, 66.9% and 2.6% were positive for CD105, CD29, CD44

and CD34, respectively (Figures 6a, c, e, g). While 85.3%, 97.6%, 95.8% and 1.5% HUCMSCs from experimental group 3 were positive for CD105, CD29, CD44 and CD34, respectively (Figures 6b, d, f, h). Taken together, these data suggested that HUCMSCs isolated using combined enzyme digestion method had higher purity than those isolated using common digestion method.

Finally, we investigated the differentiation potential of HUCMSCs. After cultured in specific differentiation media, we found that HUCMSCs isolated using combined enzyme digestion method could differentiate into adipocytes and osteoblasts (Figure 7).

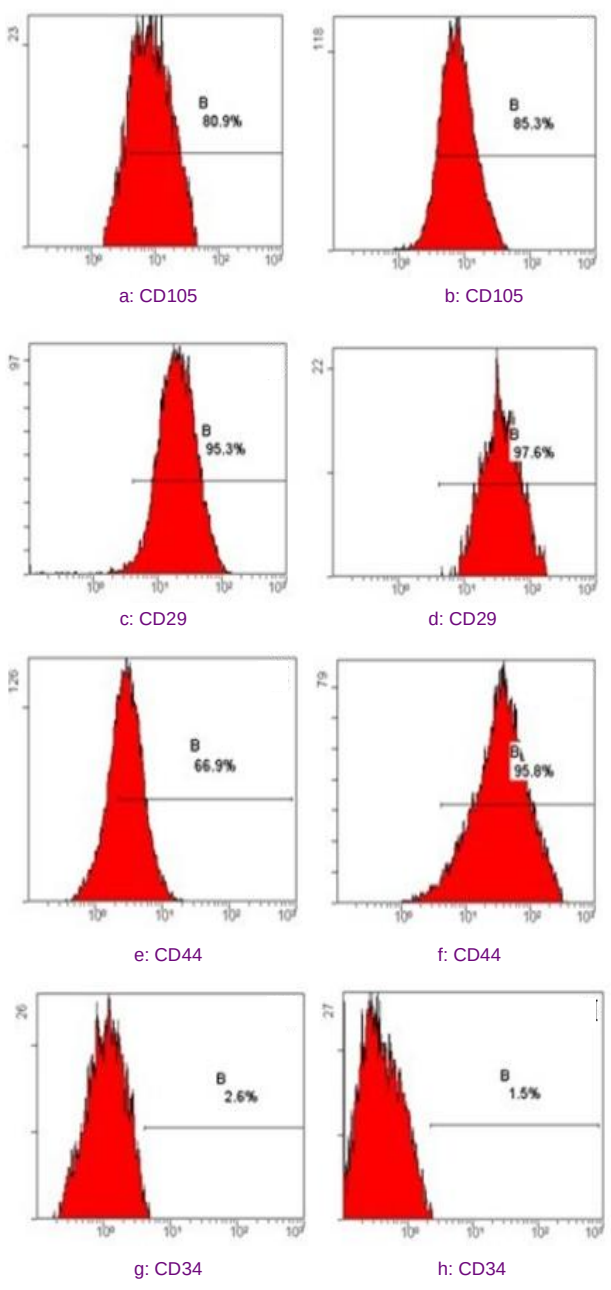


Figure 6 Flow cytometry analysis of passage 3 human umbilical cord mesenchymal stem cells

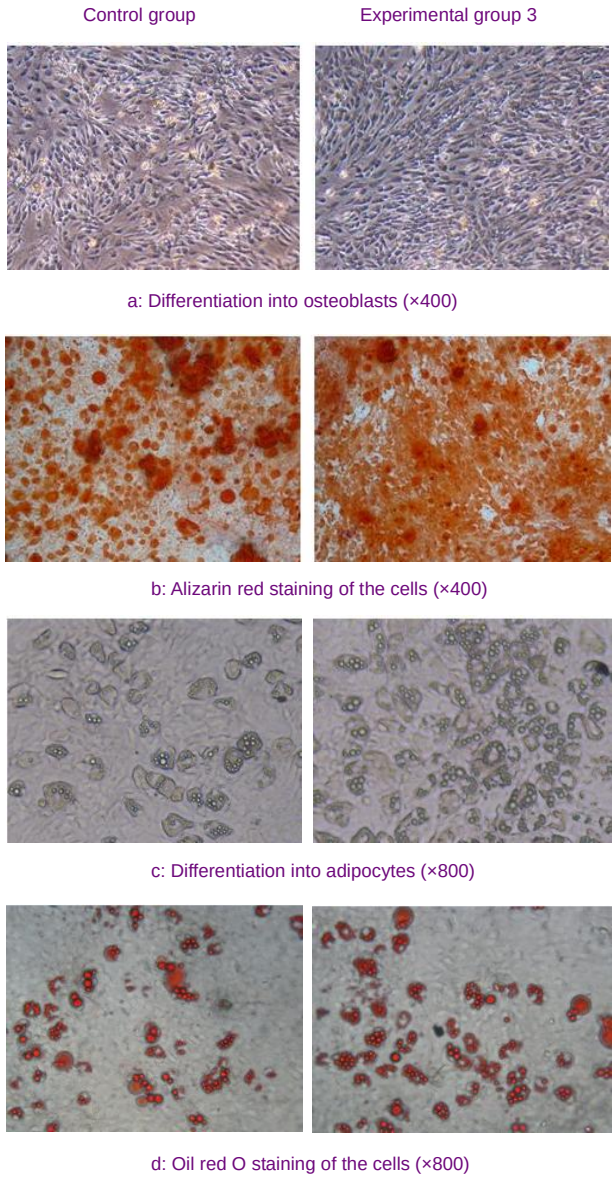


Figure 7 Differentiation of human umbilical cord mesenchymal stem cells (HUCMSCs) into adipocytes and osteoblasts

DISCUSSION

Although HUCMSCs were first isolated from bone marrow, HUCMSCs are very rare in bone marrow[17]. In addition, isolation of HUCMSCs from bone marrow is unfavorable due to the highly invasive donation procedure and the decline in number and differentiation potential with increasing age[24-25]. More recently, umbilical cord blood emerges as an alternative source for HUCMSCs because it is attainable by a less invasive method[26]. However, only 36% of HUCMSCs can be recovered from umbilical cord blood and the success rate of culture of HUCMSCs is only 10%[9]. A normal human umbilical cord from neonates is about 20 cm long and 40 g weight, and contains about 500×10^6 cells. The

concentration of HUCMSCs is about 11×10^6/g[27]. About 1.75×10^5 cells/g could be obtained after cultured for 10 days using explants culture method[18], while 5.3×10^5 cells/g was obtained using collagenase method[18]. To facilitate the application of HUCMSCs in the clinic, effective methods of harvesting HUCMSCs are urgently needed.

Therefore, in this study we employed collagenase Ⅱ and collagenase Ⅳ and hyaluronidase to digest the collagen and hyaluronic acid. In addition, we used trypsin to break the covalent bond between the N/C terminal lysine and hydroxyl lysine of the collagens to increase the solubility of collagen fibers. DNAase could further digest DNA released from the cells and decrease the viscosity of the digested mixtures to improve the efficacy of the collagenases. Our result showed that the combination of collagenase Ⅱ and Ⅳ, hyaluronidase, trypsin and DNAase which used to digest umbilical cord could significantly improve the yield, viability and colony-forming ability of HUCMSCs. In addition, the time for HUCMSCs to adhere and grow to confluence is less by using our combined digestion method than common digestion method. Further characterization of HUCMSCs demonstrated that the purity and differentiation potential of HUCMSCs isolated using combined digestion method were much better than those of MSCs isolated using common digestion method.

In conclusion, in the present study we optimized and developed a new combined digestion method for the isolation of MSCs from umbilical cord. Compared with common method, this method is more efficient and less time-consuming, and is suitable for large-scale *in vitro* expansion of MSCs and the application in regenerative medicine.

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人脐带间充质干细胞高效分离的方法*

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文章亮点：实验证明了Ⅱ型胶原酶、Ⅳ型胶原酶、胰蛋白酶、透明质酸酶和 DNAase 四酶联合消化脐带组织作用 4 h 的方法，缩短了分离细胞时间，并显著提高了细胞产量、细胞活力以及细胞增殖能力，同时缩短原代细胞贴壁时间和融合时间。

关键词：脐带；脐带间充质干细胞；Ⅱ型胶原酶；Ⅳ型胶原酶；透明质酸酶；DNAase；高效

摘要

背景：目前利用传统分离提取细胞的方法所获取的人脐带来源的间充质干细胞与脐带组织中所含人脐带来源的间充质干细胞的理论值相差甚远，造成脐带组织的极大浪费，阻碍了间充质干细胞的规模化制备培养。

目的：建立一套高效分离人脐带间充质干细胞的方法，为间充质干细胞应用于临床提供技术方案。

方法：实验分为 4 组，以传统酶法(胰酶与Ⅱ型胶原酶)为对照组，在此基础上，针对脐带结构分别逐一加入Ⅳ型胶原酶、透明质酸酶、DNase 来对脐带组织进行消化，各实验组设不同作用时间组，比较不同分离方法、不同作用时间所获得细胞数量、细胞活性、原代细胞贴壁出现时间、融合时间及所获得人脐带来源的间充质干细胞第 3 代细胞在增殖能力、表面标记与多向分化能力方面的差别。

结果与结论：经Ⅱ型胶原酶、Ⅳ型胶原酶、胰蛋白酶、透明质酸酶和 DNAase 联合作用于脐带组织，4 h 细胞计数达到最高 $(12.47\pm 0.16)\times10^6$/cm($P < 0.01$)；活力为 $(75.00\pm 5.07)\%$($P < 0.01$)；收获的混合细胞中 CD105 阳性率及 CD29 阳性率均高于对照组；细胞贴壁及细胞团融合时间均较对照组缩短($P < 0.01$)；伴随细胞的传代，形态逐渐均一，第 3 代细胞形态基本达到统一，呈梭形漩涡状生长；实验组 P3 人脐带来源的间充质干细胞特异性标记 CD44，CD105，CD29 阳性率阳性率均高于对照组，CD34 阳性率低于对照组。向脂肪细胞诱导 4 周后，油红 O 染色鉴定可见细胞内大量脂滴；向成骨细胞诱导后，茜素红染色鉴定，大体观察可见阳性钙沉积。实验证明了Ⅱ型胶原酶、Ⅳ型胶原酶、胰蛋白酶、透明质酸酶和 DNAase 四酶联合消化脐带组织作用 4 h 的方法缩短了分离细胞用时并显著提高了细胞产量、细胞活力以及细胞增殖能力，同时缩短原代细胞贴壁时间、融合时间。提取得到的原代细胞中间充质干细胞比例，第 3 代间充质干细胞细胞纯度较传统方法均有提高。

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文章概要：

文章要点：建立一套高效分离人脐带间充质干细胞的方法。

关键信息：采用Ⅱ型胶原酶、Ⅳ型胶原酶、胰蛋白酶、透明质酸酶和 DNAase 四酶联合消化脐带组织作用 4 h 的方法提取得到的原代细胞中间充质干细胞比例，第 3 代间充质干细胞细胞纯度较传统方法均有提高。

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