Fasudil Hydrochloride Induces Rat Bone Marrow Mesenchymal Stem Cells Differentiating into Neuron-like Cells

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ABSTRACT: OBJECTIVE: To explore the possibility of Rho-kinase inhibitor fasudil hydrochloride to induce rat bone marrow mesenchymal stem cells (MSCs) differentiating into neuron-like cells, and study the role of Rho-kinase inhibitor during the proliferation and development of MSCs. **METHODS:** Rat MSCs were isolated from Wistar rats and cultured to 15-18 passages.We used fasudil hydrochloride (200 μmol/L) and β-Mercaptoethanol as inducers to differentiate MSCs respectively, and then observed morphologic changes of differentiated cells,stained with AO/EB mixed fluorescent dye to estimate the apoptosis of MSCs, identified them by detecting NF200, NSE and GFAP by immunofluorescence. **RESULTS:** Both fasudil hydrochloride and β-Mercaptoethanol can induce MSCs differentiate into neuron-like cells.However,fasudil hydrochloride can induce MSCs more rapdily and efficiently.After 120 min of induction, most MSCs turned neuron-like apperance and connected to network especially apparent in fasudil hydrochloride (200 μmol/L) group, the survival rate gradually decreased as the induction time extend. Immunofluorescence suggested the expression of NSE, NF200 increased but GFAP decreased apparently as the induction time extend. **CONCLUSION:** Rho-kinase inhibitor fasudil hydrochloride can rapidly and efficiently induce rat bone marrow derived MSCs differentiating into neuron-like cells.
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Key Words: Fasudil Hydrochloride; β-Mercaptoethanol; MSCs;Induce;Neuron

1. Introduction

Mesenchymal stem cells (mesenchymal stem cells, MSCs) are mesoderm-derived multipotent differentiation capacity of stem cells, multipotent differentiation into bone cells, cartilage cells, tendon cells, muscle cells, stem cells, fat cells and hematopoietic cells and capacity for self-renewal. Moreover, in vivo and in vitro transdifferentiation certain conditions into neurons and glial cells [1]. Rho / Rho kinase signaling pathway is an important signaling pathway in vivo, it is through the regulation of intracellular actin cytoskeleton polymerization status while playing a "molecular switch" role of cytoskeletal proteins involved in regulation of the synthesis, degradation, movement and shrinkage, on cell division, shrinkage, adhesion, migration, secretion activity has an important regulatory role [2,3]. The study found that Rho / Rho kinase (ROCK) inhibitor fasudil hydrochloride in vitro rapid, efficient MSCs induced into neuron-like cells, reports are summarized below.

2.Materials and methods

2.1 Materials

2.1.1 Animals Adult male Wistar rats weighing 150-200 g, of either sex, provided by the Experimental Animal Center of Zhengzhou University.

2.1.2 Reagents high glucose DMEM were purchased from Gibco, fetal bovine serum FBS was purchased from Hyclone, fasudil hydrochloride injection provided by Tianjin Pharmaceutical Co., Ltd. red, β- mercaptoethanol were purchased from Sigma Chemical Co., NSE, NF200, GFAP polyclonal rabbit anti-rat antibody and Cy3 immunofluorescence staining kit rabbit anti-rat were purchased from Santa Cruz, import or other biochemical reagents were analytical grade.

2.2 Methods

2.2.1 Isolation and culture of rat MSCs, purification and amplification take purebred male Wistar rats were sacrificed by cervical dislocation after 75% ethanol for 5 min, separated on both sides of the femur and tibia of rats under sterile conditions, cut the femur and tibia both ends of the exposed bone marrow cavity with 5 ml syringe volume fraction of DMEM medium containing 15% FBS repeated flushing the marrow cavity, 3 000 r / min centrifugation 2 min, the supernatant was discarded, the cells were resuspended in culture medium, pipetting system into a single cell suspension with a cell density of 1 × 105/ml were seeded in plastic culture flask 25cm2 of saturated humidity at 37 ℃, the volume fraction of 5% CO2 incubator thermostat. 48 h half the amount was changed. Wall stickers to be completely fused cells, the mass fraction of 0.25 %

trypsin / 0.04% EDTA digestion and passage, by 1 × 104 / mL density inoculation was changed every 48 h semi- fluid, dynamic inverted microscope to observe the growth of MSCs. Spread to 15 generations (P15) reserve.

2.2.2 MSCs induced differentiation of preliminary experiments in this study was induced by high glucose DMEM containing four different concentrations of fasudil hydrochloride (final concentration 50,100,200,400 μmol / L), and found 200μmol / L group induced effects is ideal. After this experiment, take 15-18 generations MSCs, with 2 × 104 / mL in 24 -well plates were seeded, 24 h after the medium was changed, and discard non-adherent cells, cultured 3-4 d, when the degree of cell fusion reaches 70% -80%, the experimental group was divided into A, B two groups. A group with a final concentration of 200 μmol / L induced by high glucose DMEM solution, and set up four wells. Group B β-mercaptoethanol induced, ie, the first containing 1 mmol / L β- mercaptoethanol serum-containing medium pre- induction 24 h after removal of the pre-induction medium, then add containing 5 mmol / L β-mercaptoethanol No serum-free medium induced 5 h, set up four wells.

2.2.3 cell morphology was observed under an inverted microscope dynamic changes in cell morphology before and after induction of MSCs. After fasudil hydrochloride induced 30 min induced group started under an inverted microscope after observation once every 30 min. After the induction of β- mercaptoethanol group every 6 h, after pre-induction induction observed once every 1 h.

2.2.4 AO-EB staining to identify cell survival acridine orange (AO), ethidium bromide (EB) were dissolved in PBS to 100 μg / mL of the working solution before use 1:1 mix. When the morphology change significantly after the induction at different time points after induction of each group, AO-EB were added to the mixture solution. Were counted under the fluorescence microscope, five non-overlapping visual field (× 100), cell viability was calculated for each group.

2.2.5 immunofluorescence identified in 4% formalin-fixed cells after induction, 4 ℃ overnight, TBSTx washed three times. 5% BSA containing the TBSTx closed 60 min. Removal of the blocking solution, respectively, dropping a polyclonal antibody anti-rabbit anti-rat NSE (titer 1:100), NF200 (titer 1:100), GFAP (titer 1:100), washed 4 ℃ overnight. Join Cy3 -labeled secondary antibody effect 1 h, washed observed under a fluorescence microscope, were counted five non-overlapping field of view (× 200), calculated NSE, NF200 and GFAP -positive cells were counted as a percentage of the total number of cells in the visual field.

2.3. Statistical analysis using SPSS 13.0 software, data are expressed as mean ± standard deviation ($\bar{x}$ ± s). The groups were compared using analysis of variance measurement data (One-way ANOVA), P <0.05 statistically significant difference.

3.Results

3.1 rat MSCs cultured and purified

After inoculation 24 ~ 48 h, MSCs appear spindle change, which began adherent growth, with the incubation time, adherent cells increased significantly, and proliferation. 7 ~ 10 d cell body enlargement, extending varying lengths, various forms of uneven thickness projections, cluster -like cells were grown unevenly distributed, showing colony growth trend. After three to four passages, to form a more typical MSCs (Figure 1).

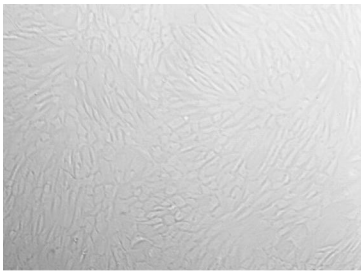
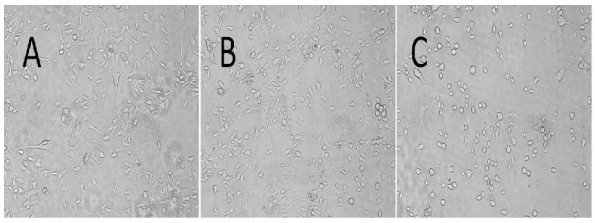
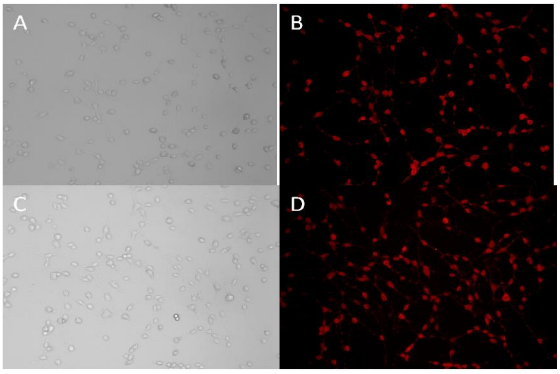


Fig1 18 passages of rat MSCs(×200)

3.2 MSCs induced differentiation of morphological observation post

Fasudil hydrochloride group after induction were induced into neuron- like cells, 30 min after induction of cell morphology began to change, the performance of the cell body shrinkage, cell gap widened. After the induction of 120 min, morphological changes is ideal. The performance of the cell body shrinkage into spherical or conical refraction enhancement, halos around some cells, there are more long processes, and connect a network-like structure. Continue to extend the induction time, neuron-like cells increased significantly, but a small amount of cell loss, death (Figure 2). β- mercaptoethanol induced group pre-induction after 24 h, some cells smaller size, cytoplasmic shrinkage, conical or spherical cell body, the contraction of the surrounding cytoplasm protrusions forming cells, a small amount of cell loss, death. 5 h after induction, the vast majority of cells tapered cell processes can be further extended, three-dimensional sense, there is a halo around the part of the cell protrusions interwoven into a network of interconnected, but shedding dead cells increased significantly.





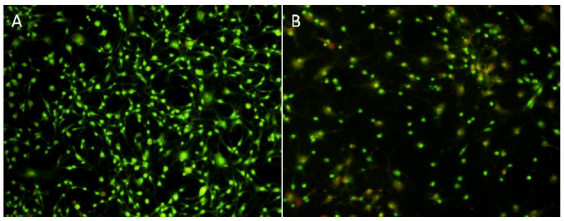


Fig 3 AO/EB mixed fluorescent dye in fasudil hydrochloride group(A) and β-Mercaptoethanol group(B)

Tab 1 Survival rate of MSCs after induction by AO/EB mixed fluorescent dye

Group	Inducing Time	Survival Rate (%)
Fasudil Hydrochloride	30 min	96.7±2.2
	60 min	95.3±1.9
	90 min	93.8±1.8
	120 min	92.5±2.1
	180 min	90.1±1.3
β-Mercaptoethanol	5 h	68.6±3.1

3.4 induction immunofluorescence

Fasudil hydrochloride induced group, with the induction of prolonged, NSE, NF200 expression was significantly increased, fewer GFAP expression. Induced 60,90,120,180 min, each group NSE, NF200-positive rate gradually increased (Figure 7-10), GFAP expression in each group were less than 5%. β-mercaptoethanol group, 5 h after induction, NSE positive rate was 58.7% ± 3.7%, NF200-positive rate of 64.8% ± 2.8%, GFAP-positive rate of 34.1% ± 2.4%. Contrast with fasudil hydrochloride induced group, a significant difference (P <0.01), the results in Table 2.

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4.Discussion

MSCs to differentiate into neural cells in vitro using a variety of cytokines present, chemically-induced gene transfection and the like various methods. But the first two induction a long time, to get a lower percentage of neuron-like cells. Previous studies use more the latter. In this study, conventional chemical inducer of β-mercaptoethanol and Rho kinase inhibitor fasudil hydrochloride were induced MSCs, the results show that both methods can be induced in vitro, MSCs differentiate into neuron-like cells. After induction cells mainly express NSE and NF200, GFAP expression less, mainly to prove to the MSCs induced neuronal cell differentiation, but not to glial cells. AO-EB staining and immunofluorescence showed that after induction efficiency fasudil group and induction of cell survival rate was significantly higher than β-mercaptoethanol group; and fasudil induction process is continuous, 2 h after induction, significant changes in cell morphology have been omitted from the pre-induction period, so that the induction time is greatly reduced.

Recent studies show, MSCs differentiation through a series of signal transduction pathway. MSCs differentiate into nerve cells involved sox, pax, notch, delta, frizzled and erbB and other signals, multi-channel approaches [4,5]. Rho / Rho kinase signaling pathway in vivo is a very important signal

transduction pathways. It has three main components: a small G proteins (mainly Rho), connected with Rho Rho kinase (mainly ROCK) and effector molecule Rho kinase. Rho GDP by GTP-bound form and bound form of the molecule plays a conversion switch, and regulation of many intracellular signaling pathways. By activating Rho Rho kinase and its downstream target molecules regulate contraction cell adhesion, migration, proliferation, apoptosis, and other biological behavior and function. Myosin phosphatase is activated Rho kinase substrate receiving Rho / Rho kinase phosphorylation activation signal, and its own inactivation; inactivated myosin phosphatase, myosin light chain can be (MLC) dephosphorylation, making pulp cells to enhance the level of phosphorylation of MLC, actin - myosin cross-linking increases, thereby promoting actin cytoskeleton of actin polymerization, the impact of the contraction cell adhesion, proliferation, apoptosis, migration and other biological school behavior and function [3,6].

2006, Emilie et al reported that the combination of CoCl2 ROCK inhibitor Y-27632 promotes MSCs into dopaminergic neuron-like cells [7]. In the same year, Yoichi, etc. in the study of human neuroblastoma GOTO cells found, Rho extracellular enzyme inhibitor C3 transferase and Rho kinase inhibitor fasudil can induce neurite formation, promotion GOTO cells into neuron-like cells, speculate GOTO cell differentiation and Rho / Rho kinase signaling pathway is closely related to [8]. Fasudil hydrochloride is currently the only application to clinical Rho kinase inhibitor, through competitive inhibition of ROCK activity of ATP [9]. Fasudil hydrochloride is not only at the cellular level regulating cell proliferation, migration and adhesion, cytoskeletal rearrangement, cytoplasmic moving sports inflammatory cells, gene regulation at the molecular level but also inflammation, thrombosis, and related multi-oxidation fibrosis kinds of factors [10].

Differentiation of MSCs directed primarily involve changes in cell morphology, cell morphology depends on the continued renewal of the cytoskeleton, actin cytoskeleton is the main component. This change in the basic cell biology and cellular actin cytoskeleton proteins are closely related. Cytoskeletal function not only has the support and maintenance of cell morphology and cell movement, while participating in the exchange of information within and outside the cell in the process. Rho kinase actin cytoskeleton is important regulatory molecule, as many signal molecules involved in cytoskeleton and cell signaling. This is the first confirmed Rho / Rho kinase (ROCK) inhibitor fasudil hydrochloride in vitro can quickly and efficiently induced MSCs differentiate into neuron-like cells in. Speculated that the mechanism may be as fasudil inhibits Rho kinase activity, leading to cytoskeletal remodeling, cell differentiation process started, through the interference between the Rho / Rho kinase signaling pathway and other signaling pathways have been relatively stable in the balance environment, close or activate the downstream pathway genes associated with cell differentiation, thereby promoting MSCs into neuron-like cells. Provides for us to further study the feasibility of Rho kinase inhibitor MSCs proliferation, differentiation and development role.

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