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Research Paper

Cell multiplication, apoptosis and p-Akt protein expression of bone mesenchymal stem cells of rat under hypoxia environment

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Abstract

Objective: To elucidate whether cell multiplication, apoptosis, glucose intake and p-Akt protein expression of bone Mesenchymal Stem Cells(MSCs) of rats is influenced by a hypoxic environment *ex vivo*. **Methods:** Passage 3 of bone marrow MSCs taken from Wistar rats, were cultured in a culturing chamber with 94%N_2, 1%O_2, 5%CO_2 at 37℃. At different hypoxia time points, 0, 0.5, 1, 4 and 8 h, glucose uptake was assayed by using radiation isotope ^{3}H-G, Apoptotic Rate(AR) and dead rate(DR) were analyzed by flow cytometry(FCM) after Annexin V/PI staining, cell multiplication(by MTT methods) and p-Akt protein by immunocytochemistry and western blot. **Results:** Assay for $CD29^+$, $CD44^+$, $CD71^+$, $CD34^-$, Tn T^+(after 5-azacytidine agent inducing) and ALP^+(after bone differentiation agent inducing) suggested these bone-derived cells were MSCs. The ^{3}H-G intaking ratio(CPM/flask value: 157 ± 11, 110 ± 11, 107 ± 13, 103 ± 10, 100 ± 9 and 98 ± 10) of MSCs at different hypoxia time points, significantly decreased compared to that of normoxia($P < 0.01$) and tended to descend slowly with hypoxia time duration, for which there was no statistical significance($P > 0.05$). The AR(0.09 ± 2.03%, 12.9 ± 1.72%, 13.7 ± 2.26%, 13.8 ± 3.01%, 14.1 ± 2.78% and 14.7 ± 4.01% at 0, 0.5, 1, 4 and 8 h, respectively, $P < 0.01$) and DR (0.04 ± 1.79%, 0.93 ± 1.85%, 3.11 ± 2.14%, 4.09 ± 2.36%, 4.72 ± 2.05% and 4.91 ± 3.72% at 0, 0.5, 1, 4 and 8 h, respectively, $P < 0.05$) at different hypoxia time points significantly increased compared to those time in normoxia; The AR further went up with time ($P < 0.05$), however there was no statistical significance ($P > 0.05$) for the DR. Optical absorption value of MTT methods at different hypoxia time points significantly decreased compared to those with a corresponding normoxia time ($P < 0.01$) and degraded with time (in an hypoxic environment –$P < 0.01$). IOD of p-Akt protein of MSCs at different hypoxia time points significantly increased (0.367 ± 0.031, 0.556 ± 0.023, 0.579 ± 0.013, 0.660 ± 0.024, 0.685 ± 0.039 and 0.685 ± 0.011, respectively) compared to their equivalents in normoxia (P<0.05), however, there was no statistical significance ($P > 0.05$) for different hypoxia time points. Hypoxia may result in ultramicrostructure changes, such as defluvium of Microvilli, apoptotic body, "margination" and so on and are further aggravated with hypoxia time stretching. **Conclusion:** Hypoxia may lead to a depression of MSCs intaking glucose, creep of cell multiplication, upregulation of p-Akt protein and apoptosis of MSCs *ex vivo*.

Keywords: bone marrow mesenchymal stem cells of rat; hypoxia; glucose uptaking; apoptotic ratio; p-Akt protein

INTRODUCTION

Nowadays, the properties of mesenchymal stem cells (MSCs), such as autoreduplication, non-specific (poor) immunogenicity, and multipotency differentiating in various embryonic layers (myocardium, endothelium) and so on, are increasingly coming to light, and is currently a hot topic in the cardiovascular area[1-6] throughout the world. However, whether the hypoxic condition[7] of infarction and the ischemic region (after myocardial infarction) influences survival, or proliferation and differentiation of

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MSCs after cellular cardiomyoplasty (CCM) has been seldom reported. Therefore this study examines third generation of fostered Passage 3, P_3 MSCs, while interventing measurements taken (in hypoxia -94% N_2, 1% O_2 and 5% CO_2) serve to elucidate whether hypoxia influences glucose uptake, and /or the apoptosis and proliferation of MSCs. The aim explained the doubtful points, of why it is difficult for MSCs to exist in the infarction (ischemic region) in many reported pictures.

MATERIALS AND METHODS

The animal studies were approved by the Animal Care and Use Committee of China Medical University(CMU). Wistar rats [SCXK (Provence Liao-ning) 2003-0009], with body weights of 150~200 g, were purchased from the animal experiment center of CMU.

The test utilized Standard Fetal Bovine serum (sFBS, Hyclone Ltd); L-DMEM and H-DMEM (Gibco Ltd); The plastic culture flask (Corning Ltd); A polyclone antibody of rabbit-anti-rat (for CD29, CD34, CD44, CD71), Tn T and p-Akt, SABC coloring reagent kits of rabbit Ig G, caprine anti-rabbit antibody marked by Horseradish peroxidase (Wuhan boster biological technology Ltd); Annexin V FITC/PI kit (Boehringer Mannheim biological technology Ltd); Trypase, MTT, DMSO, dexamethasone (DEX), β-Glycerophosphate disodium (β-GP), ascorbic acid (AsA), 5-azacytidine agent (5-aza) [Sigma Ltd]; Hyzone labeling glucose (^{3}H-G, Atomic Energy Research Establishment of China); POP and POPOP (KOCH-LIGHT, Shanghai chemical agent Ltd); Superclean bench (Ibemotol Ltd); Cell incubation cabinet (Heal Force Development Co. Ltd); Refrigerated centrifuge (Hitachi Ltd); Flow cytometer (FCM, FACSC alibur, B&D Ltd); Hypoxia incubation cabinet, transmission electron microscope (TEM, Olympus Ltd.); Liquid scintillation counter (LKB-1214, LKB Ltd.) and so on.

Cell Isolation and Culture

Wistar rats were each sacrificed by certebrae colli luxatio methods, after 10% chloral hydrate intraperitoneal injection anesthetizing. After 15~20 min, the femoral and tibial bones were dislodged and dealt with cautiously under sterile conditions. Bone marrow aspirates were passed through a density gradient (Percoll gradient being 1.074 g/mL and 1.070 g/mL, respectively) to derive boundary layer cells. Those cells, after being washed (100 g, 10min, two times) with 10 mL phosphate-buffered saline (PBS, pH 7.2~7.4 in order to remove the Percoll) were suspended in 10 ml L-DMEM (containing 10%FBS)s and then plated in two culture flask and cultured under normoxia (37℃, 5% CO_2). Nutrient liquid was totally substituted for 2~3 days to wash away hematopoietic cells, fibroblasts, and other nonadherent cells during medium changes, and then the remaining purified MSCs population was further expanded in culture, to be used *in vitro* studies.

Immunocytochemistry Assays for Receptor of CD29, CD34, CD44 and CD71

P_2 cells were dissociated and innoculated in six-well plates and cultured continuously. When cells reached about 80~90% confluence, cells were assayed immunocytochemically. Culture fluid was removed and cells were washed three times with PBS for 5 mins/rinse, and were then fixed in 4% paraformaldehyde (PFA) in PBS (pH 7.2) for 20 mins at 4℃ and washed twice with PBS for 15 mins/rinse. According to the instructions of CD29 (1 ∶ 200), CD34 (1 ∶ 200), CD44 (1 ∶ 200) and CD71 (1 ∶ 200) kits, respectively primary antibodies were incubated overnight at room temperature. Immunocytochemical assays were finished by means of SAB colouration (PBS as negative control) and the cells were photographed. Buffy grains appearing on the cell membrane suggested that they were positive, otherwise assumed negative.

Immunocytochemical Assays for Differentiation Capability

P_2 cells were inoculated in six-well plates, and cultured continuously until about 70~80% confluence. In one of two plates, the cells were incubated with L-DMEM containing 5-aza (10 μmol/L) for 24 hours and then washed with PBS and incubated routinely. After two weeks, the cultured cells were immunocytochemically stained to expose cardiac-specific troponin T (Tn T, Buffy grains appearing were classified as above). In the other, the cells were incubated with H-DMEM containing DEX (100 nmol/L), β-GP (10 mmol/L) and AsA (0.25 mmol/L), the liquid was totally substituted for 3 days. The cultured cells were stained with ALP (according to Gomori methods-reformed, 1 well without above DEX, β-GP and AsA as control) and after two weeks photographed as above.

Hypoxia Intervention[8,9]

P_2 cells were dissociated and inoculated in culture flasks and culture dishes. Except cells in the ninety-six-well plate (which reached about 70~80% confluence), all cells reached about 80~90% confluence. The cells were put into the hypoxia incubation cabi-

net (94% N_2, 1% O_2 and 5% CO_2) at the same time point, and then taken out at 0.5 h, 1 h, 2 h, 4 h and 8 h to detect as follows.

Radiation Isotope ^{3}H-G Detecting Glucose Uptaking of MSCs

The cells (at different hypoxia time points), were dissociated and washed with PBS and numbered in each sample (1×10^6/mL, 1 mL per sample, 3 samples per time point), respectively. Then ^{3}H-G was put into each sample (final concentration: 1.0 uci/mL) and incubated continuously under hypoxic conditions (normoxia culturing cells, were similarly treated under normoxic conditions) for 30 mins. Afterwards, 0.2 mL from each sample was drawn and mixed fully with 0.2 mL dehydrated alcohol and each put into scintillation vial, with 2 mL scintillation fluid (POP 4.0 g and POPOP 0.1 g in 1000mLdimethyl benzene). After 4℃ overnight, CPM/flask value was harvested by Liquid scintillation counter.

FCM Analyzing Apoptosis and Dead Rate of MSCs

The cells (at different hypoxia time points), were dissociated, washed with PBS and adjusted for each sample (1×10^6/mL), as previously. These were handled according to the directed usage of the Annexin Ⅴ FITC/PI kits, and handled FCM as thus (wavelength of excitation light 488 nm, pass-band filtrum wavelength 515 nm -detecting FITC fluorescence, pass-band filtrum wavelength detecting PI fluorescence). More than 99% of the non-specific fluorescence of control pipe was put on a background and shown with 2D point lattice map. In a double variances scatterplot, left inferior quadrant represented living cells ($FITC^-/PI^-$), right superior represented non-(ie dead cells, $FITC^+/PI^+$) and right inferior quadrant represented apoptosis cells($FITC^+/PI^-$).

MTT Analyzing Cell Proliferation of MSCs

After 20 μl MTT was put into each respective well, the cells were incubated under normoxic conditions for 4 hrs, cleaned up gently with supernatant fluid, and put into 150 ul DMSO wells (without innoculating cell as control), and then shaken for 10 mins. The optical absorption value (OAV) was recorded by Enzyme-linked immunosorbent assay analyzer, in wavelength 540nm and cell a growth curve was drawn (abscissa axis representing time points and longitudinal axis representing OAV).

Immunocytochemical Assays for p-Akt Protein Detection

After routinely washing, fixing and then re-washing, we prepared the immunocytochemical assays, according to the p-Akt (1 ∶ 200) kits and SAB directions. We used coloured PBS(as negative control) and then photographed(Again buffy grains appearing in the cytoplasm suggested that they were positive, otherwise negative).

Western Blot Analysis for p-Akt Protein Detection

The cells at different hypoxia time points were dissociated, washed with PBS and harvested. The cells was schizolysissed prechilly, homogenated on ice for 15 minutes, centrifuged (12,000 g, 15 mins, 4°C), and the supernatant was collected. The protein concentration in the supernatant was determined and adjusted to the same level, 50 μg of protein per lane was subjected to SDS polyacrimide gel electrophoresis(PAGE), and the protein was transferred to 0.45 μm nitrocellulose filters. Filters were blocked and incubated overnight with a primary polyclonal rabbit p-Akt (1 ∶ 500) antibody in Tris-buffered saline[10 mM Tris·HCl(pH 7.2) and 0.15 M NaCl] that contained 5% nonfat dry milk and 0.1% Tween 20. This was washed, and then incubated for 1 h at room temperature with the appropriate peroxidase-conjugated secondary antibody (1 ∶ 2,000 dilution). The blots were washed, the chemiluminescent signals(ie, Integral optical density, IOD) were detected by SCION Image software, and the signals were normalized (β-actin as interior reference) to account for protein loading, and transfer variability. Experiments were conducted three times with samples from different time point.

TEM Sample Preparation and Observation

The cells (at different hypoxia time points) were dissociated, washed with PBS three times, fixed with 2.5% glutaraldehyde over night at 4℃, and we proceeded as per the above routine. Finally, an extra thin section was made and observed with TEM.

Statistical Analysis

Data was expressed as mean±SD. All statistical analyses were performed by SPSS 12.0. Comparisons of variables between >2 groups was performed by 1-way ANOVA. The critical α-level for these analyses was set at $P < 0.05$.

RESULTS

Expression of Receptors(CD29, CD71, CD44 and CD34) and Protein(Tn T, ALP)

The bone marrow P_3 mononuclearcell showed positive staining of CD29, CD44 and CD71 (***Fig 1a***, ***1b*** and ***1c***), negative staining of CD34 (***Fig 1d***), ex-

pressed Tn T protein(***Fig 1e***, differentiating cardiac-like myocyte). The results after 5-aza (sclerotomal cell liquid) inducing and ALP (***Fig 1f***, -differentiating sclerotomal-like cell) Looking at the data all those suggested bone marrow mononuclearcell, were MSCs.

^{3}H-G Uptaking of MSCs at Different Hypoxia Time Point

The ^{3}H-G intake ratio(CPM/flask value) of MSCs was 157 ± 11,110 ± 11,107 ± 13,103 ± 10,100 ± 9 and 98 ± 10 at normoxia, and 0.5,1,2,4 and 8 h, in the hypoxia group respectively. Compared to normoxia, the CPM/flask value ($P < 0.01$) tended to descend slowly over time under hypoxic conditions, however there was no statistical significance ($P > 0.05$) for different hypoxia time points.

Apoptotic and Dead Rate of MSCs at different hypoxia time point

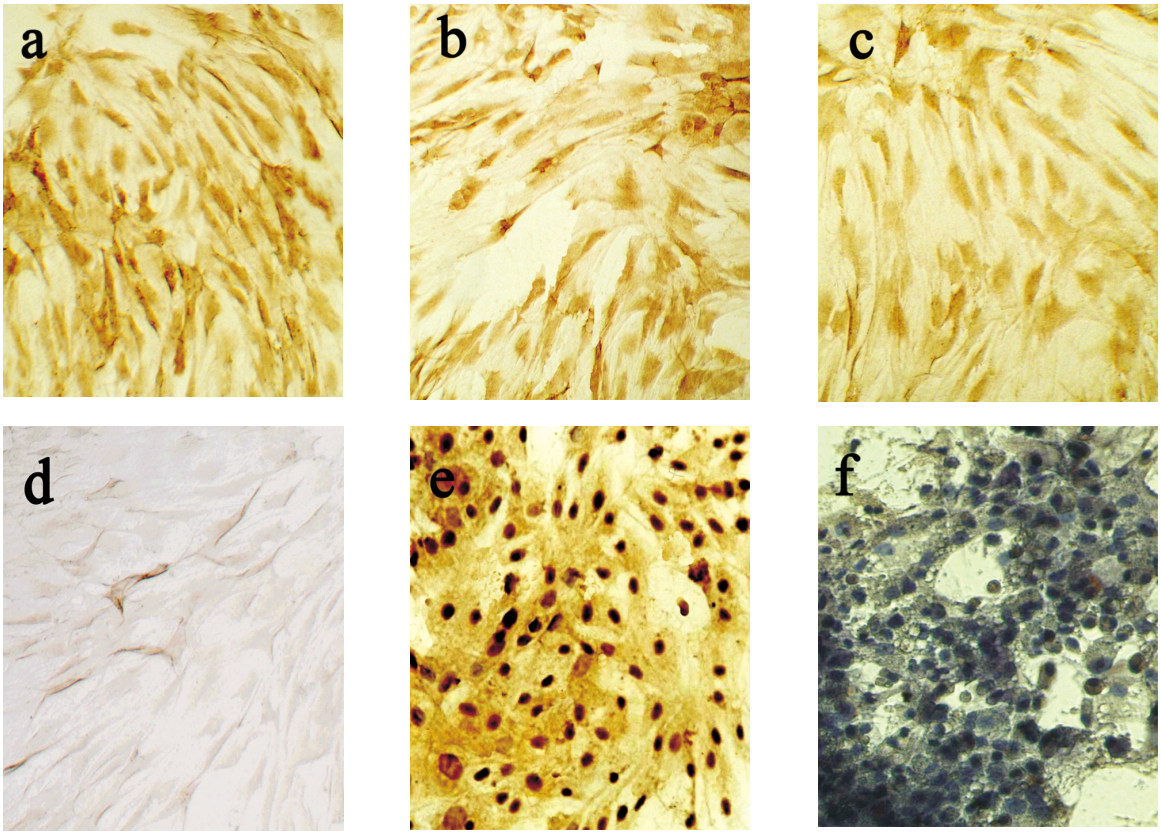
The apoptotic Rate(AR) of the MSCs was 0.09 ± 2.03%,12.9 ± 1.72%,13.7 ± 2.26%,13.8 ± 3.01%, 14.1 ± 2.78% and 14.7 ± 4.01% respectively(***Fig 2***), and the dead rate (DR) was 0.04 ± 1.79%,0.93 ± 1.85%,3.11 ± 2.14%,4.09 ± 2.36%, 4.72±2.05% and 4.91 ± 3.72% respectively (***Fig 2***). Compared to normoxia, AR significantly increased at different hypoxia time points ($P < 0.01$) and further increased over time($P < 0.05$). However, DR significantly increased at different hypoxia time points ($P < 0.05$) compared to normoxia, and there was no statistical significance($P > 0.05$) owing to differing time points.

Proliferation of MSCs at Different Hypoxia Time Point

Compared to the same normoxia time point, the hypoxic condition group degraded differently over time ($P < 0.01$);There was a slow significant ascension trend in the OAV at each hypoxia time point compared to hypoxia(***Tab 1 Fig 3***).

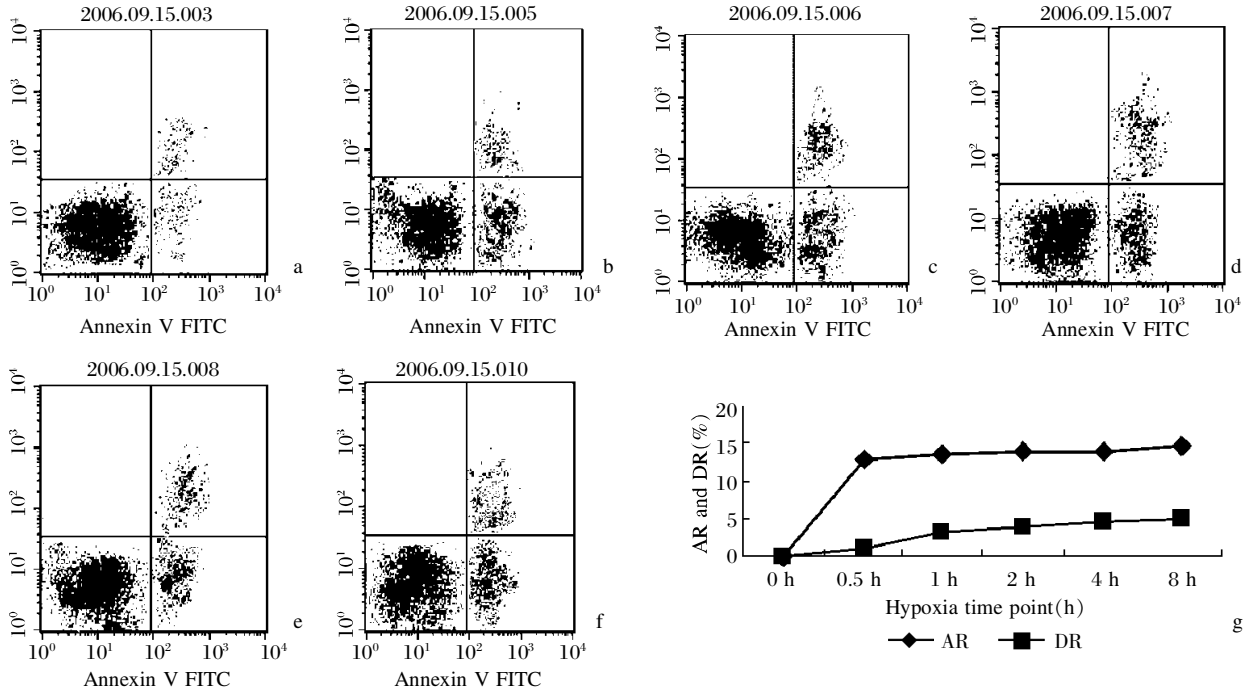
p-Akt Protein Expression

The immunocytochemical assays of p-Akt protein (***Fig 4a***) showed positive for normoxia and hypoxia. IOD of p-Akt protein (in MSCs) at normoxia and different hypoxic time points were 0.367 ± 0.031, 0.556 ± 0.023,0.579 ± 0.013,0.660 ± 0.024, 0.685± 0.039 and 0.685 ± 0.011,respectively. IOD of p-Akt protein significantly increased at different time points as compared to the normoxic group ($P < 0.05$), however, there was no statistical significance ($P > 0.05$) for different hypoxia time points.



a:CD29^{+}(200 ×), b:CD44^{+}(100 ×), c:CD71^{+}(100 ×), d:CD34^{-}(100 ×), e:Tn T^{+}(100 ×), f:ALP^{+}(100 ×)

Fig 1 Immunocytochemical staining of P_3 bone marrow mononuclear cells for CD29,CD44,CD71,CD34,Tn T and ALP



A: normoxia, b: hypoxia 0.5 h, c: hypoxia 1 h, d: hypoxia 2 h, e: hypoxia 4 h, f: hypoxia 8 h, g: the polygram of AR/DR at different hypoxia time points, h: the polygram of AR/DR of MSCs at different hypoxia time points.

Fig 2 AR/DR of MSCs at different hypoxia time points

Tab 1 **Proliferation of MSCs at different hypoxia time point**

	0 h	0.5 h	1 h	2 h	4 h	8 h
Normoxia	0.017 ± 0.023	0.0200 ± 0.019	0.0240 ± 0.027	0.0250 ± 0.011	0.0260 ± 0.031	0.0260 ± 0.015
Hypoxia	0.017 ± 0.023	0.0175 ± 0.034	0.0178 ± 0.025	0.0181 ± 0.014	0.0185 ± 0.017	0.0191 ± 0.031
P value	–	0.037	0.013	0.001	0.000	0.000
**P* value	–	0.043	0.043	0.044	0.043	0.042

Note: *Compare of OAV between each hypoxia time point and normoxia at 0 h.

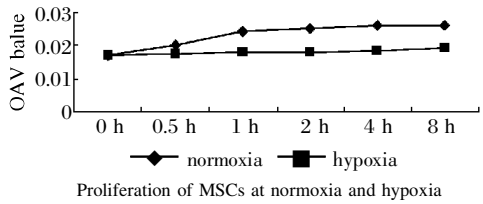
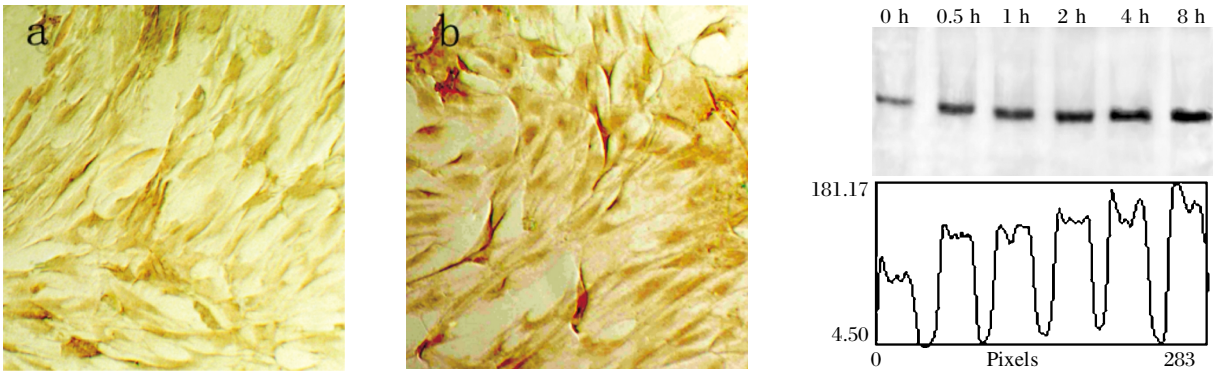


Fig 3 The polygram of OAV value of MSCs at different normoxia/hypoxia time points(MTT methods)

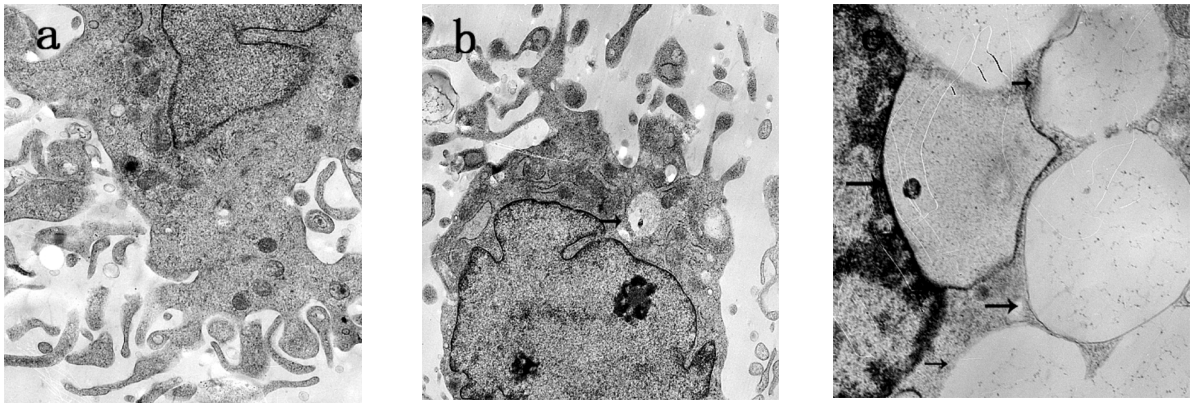
The Change of Ultrastructure of MSCs

In the normotoxic state, there was abundant microvilli on the surface (***Fig 5a***). In the endoplasmic reticulum and free ribosome there was little chondrosome in the cell. The microvilli of MSCs defluvium at hypoxia(at 0.5 h) and apoptotic body at hypoxia 2 h went continuously up over a longer hypoxia time duration(***Fig 5b***) and "margination" appeared(***Fig 5c***) at hypoxia 8 h.



a: normoxia and b: hypoxia (0.5 h), Immunocytochemical staining(100 ×); c: Western blot for p-Akt(60kd).

Fig 4 The expression of p-Akt in MSCs at different hypoxia time points



a:under normal state,aboundance of microvilli on the surface of MSCs (6,000 ×), b:microvilli of MSCs defluvium, apoptotic body appearance at 4 h after hypoxia(5,000 ×), c:"margination" appearance and apoptotic body obviously added at 8 h after hypoxia(10,000 ×).

Fig 5 The change of ultrastructure of MSCs at different hypoxia time points

DISCUSSION

MSCs,which acted as basic cells in cellular transplantation, have increasingly been concerned in cardiovascular, cerebral vascular (and other) areas, however whether MSCs would survive under poor blood and poor oxygen transplanted regions is as yet pended [7], therefore, this study was dedicated to research the problem. In view of P_3 MSCs tendency to be stable, meeting with clinical needs (and more passage promoting aging *in vitro* [10]), we chose P_3 and intervention with hypoxia. In order to avoid ischemia/reperfusion injury, MSCs were dealt with on the 4℃ layer at once.

The results show CPM/flask values significantly decreased at different hypoxia time points as compared to normoxia, however there was no statistical significance among each hypoxia time point. This suggested that hypoxia might lead to degradation in the glucose uptake (of MSCs) and downregulate MSCs' metabolic level. The phenomenon,(ie hibernation phenomenon) may be a kind of self-adjusting mechanism, protecting MSCs from apoptosis. The tendency of CPM/flask value to descend slowly (with hypoxia time stretching), may be owing to the retention of glucose intake in apoptosis cells, but this awaits further research.

AR and DR significantly increased at different hypoxia time points as compared to normoxia and further increased over time, which suggested that hypoxia may induce MSCs apoptosis and death. However, the results states that AR quickly and significantly increased at hypoxia 0.5 h and then further (slowly) increased over time,(with hypoxia) suggesting that MSCs can not adapt to hypoxia at once, but adapt step by step with hypoxia over time, "death" may be one of later apoptotic manifestations. The mechanism of apoptosis of MSCs may be as follows:(1)Low cells uptake of glucose, Krebs' cycle and/or glycolysis may not be enough to satisfy with the survival of MSCs;(2)Hypoxia may be activated directly and/or indirectly on the chondrosome pathway and/or dead receptor pathway, and then be activated on the sequent caspase pathway, which lead cells to die.

The results, namely OAV of MSCs having significantly decreased in hypoxiac conditions as compared to the corresponding normoxic time point (and its tendency to ascend compared to hypoxia). This no doubt, suggest that hypoxia inhibit severely the proliferation of MSCs, droping cells almost into total "retention".

Akt gene, which is located in the center of PI3K/Akt signal conduction pathway, codes a kind of 56KD Ser/THr protein kinase, which produces an Akt protein (a signaling molecule downstream of PI3K.) Once its two sites are deemed phosphorylate (primarily mediated by PI3K activation) [11], ie by the phosphorylation of Akt protein (p-Akt). P-Akt, which act as the medium[12,13],becomes an indispensable factor for surviving cells (as a kind of activated molecule), and plays a key role in promoting cell survival, proliferation and so on[14] and its continuous existence may protect the cell from injury[15]. The results of which show that hypoxia may significantly promote the expression of p-Akt, as compared to normoxia, and that hypoxia may strongly enhance p-Akt, although accompanied by that hypoxia we may see the apoptosis and slow proliferation of MSCs. These results also suggest that hypoxia may switch on the self-defense mechanism "p-Akt", however, the relationship between p-Akt and proliferation/apoptosis remains ambiguous until research fur-

ther research is undertaken.

In conclusion, our study suggests the following: (1)That hypoxia may result in the downregulation of glucose intake, promote apoptosis, slow down proliferation and enhance p-Akt protein expression of MSCs; (2)The "hibernation phenomenon"(reflected by low glucose uptake) and a defense protein (such as p-Akt protein) induced by hypoxia, may protect MSCs from apoptosis during hypoxic conditions. All above results may explain partly why it was difficult for MSCs to be seen in the hypoxic transplantation area, after CCM.

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