Hypoxia preconditioned adipose tissue derived mesenchymal stem cells improve the survival of mouse neural stem cell in vitro injury models

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석사과정을 진학할 때부터 순탄치 않았던, 그리고 끝이 보이지 않을 것만 같았던 저의 석사과정의 기간도 이제 막바지에 접어들었습니다. 먼저 석사과정 동안의 큰 버팀목이 되어주셨던 윤도흠 교수님과 김긍년 교수님께 그리고 자문의원 교수님이신 이원택 교수님께 감사의 말씀을 전합니다. 또한 매주 있었던 랩미팅 때마다 제게 많은 자극을 주셨던 하윤 교수님께도 감사의 말씀을 전합니다. 교수님의 그런 따끔한 충고가 있었기에 지금과 같은 결실을 맺을 수 있었습니다. 감사드립니다.

석사과정을 하면서 가장 힘들고 괴로웠던 2008년 겨울... 졸업을 하기위한 논문 제출 자격도 얻지 못해 망연자실 했던 그때와, 노력한 끝에 마침내 자격을 얻을 수 있었던 그때 겨울이 가장 기억에 남습니다. 아마도 그 당시의 시련이 좋은 밑거름이 되었는지 2009년 1학기는 처음으로 재미를 느끼면서 공부했던 과정이 되었고 제 짧은 인생에 있어서 열정을 갖고 임했던 시기였으며 그로인해 지금의 이런 기회가 오지 않았나 생각합니다.

의사소통의 어려움 때문에 깊은 대화는 나누지 못했지만 곁에서 많은 관심과 지도를 해주었고 연구자가 갖추어야 할 마음가짐을 일깨워준 Liu, 홍련누나, 성수, 성록형 새로운 식구가 된 선영, 지영, Momin과 용섭형, 재형형, 동규, 진형, 다운, 희진, 상헌, 이경희 선생님, 김은정 선생님, 명훈형, 영천형, 그리고 혁진, 원종, 오랜 기간 동안 함께 곁에 있어준 유나...마지막으로 우리 가족...아버지, 어머니 그리고 동생 민재와 함께 이 기쁨을 함께 하고 싶습니다.

어떤 분야에 있어서 천재적인 재능을 갖고 있는 사람마저도 이겨내지 못하는 사람은 그 분야에 흥미를 갖고 임하는 사람이라고 합니다. 이제 그 흥미를 뛰어 넘어 이 분야에 프로가 되기 위해 더욱 정진할 것을 다짐하고 또 다짐하며 이글을 마칩니다.

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ABSTRACT

Hypoxia preconditioned adipose tissue derived mesenchymal stem cells improve the survival of mouse neural stem cell in vitro injury models

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Hypoxic stem cells have resistance of hypoxia condition and hypoxic preconditioning can increase their tolerance of hypoxia condition after

transplantation into damaged tissue accompanying with ischemia. In order to improve survival of the therapeutic stem cells carrying a reporter DsRed Gene after transplantation into the injured spinal cord, this study examined the effect of a novel strategy that co-culture the mouse neural stem cells expressing DsRed (mNSC-DsRed) with hypoxic preconditioned adipose tissue derived mesenchymal stem cells(HP-MSC). Hypoxia preconditioning MSC that cultured for 1 day in hypoxia condition expressed the surface marker CD90 and CD105 like normal conditioned MSC (N-MSC). It suggest that hypoxia preconditioning not change the nature caracterization of AT-MSC. In the hypoxia and serum starvation injury model, the viability of mNSC-DsRed was clearly increased in by co-culture with HP-MSC or with normoxic conditioned MSC (N-MSC), compared with mNSC-DsRed alone. Espesially HP-MSC showed better protective effect on mNSC-DsRed survival than N-MSC. MSC co-clture system could inhibit the early apoptosis of mNSC compare to control group in serum deprivation and oxidative stress injury. These findings suggest that the strategy of hypoxic preconditioning stem cells and gene delivery platform using stem cells may represent a more effective stem cell therapy.

Key Words: Hypoxia preconditioning AT-MSC (HP-MSC), mouse neural stem cell expressing DsRed (mNSC-DsRed), co-culture, serum deprivation, oxdative stress

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I. Introduction

In the last few years, the efforts to exert for stem cell therapy have

been made a transplantation using bone marrow-derived stem cells [1,2], adult neural stem cells [3], adipose tissue derived mesenchymal stem cells(AT-MSC) [4].

Adipose tissue has been identified as possessing a population of multipotent stem cell [5,6]. And the phenotypic and gene expression profiles of ADSC are similar to BMSC [7]. Also human have abundant subcutaneous fat deposits and ADSC and easily be isolated by conventional liposuction procedures [8]. AT-MSC is multipotential stem cell that can secrete many cytokines or growth factors and can also differentiate to other lineages such as bone, cartilage like BMSC. And transplanted AT-MSCs can secrete bioactive factors such as IL-1Rα, IL-6, IL-8, G-CSF, GM-CSF, MCP-1, NGF and HGF more than BMSCs and improved liver function by trophic action of AT-MSC [4]. Transplantation of neural stem cell in spinal cord injury model contributed to promote the locomotor recovery and presented the opportunity for clinical application [9]. Neural stem/progenitor cells co-transplantation with primary fibroblasts in injured spinal cord provided a cellular guiding and enhanced the outgrowth of corticospinal axons in lesion site [10].

Although a transplantation of neural stem cells has beneficial effect to induce the recovery in host tissue, the most of transplanted cells die few days after transplantation [11]. Because protection of stem cells death from apoptosis is critical factor for successful stem cell therapy, cell survival strategies need to promote the survival of transplanted cells and increase the efficiency of stem cell therapy.

The induction of neurotrophic factors, anti-apoptotic genes into the cells [12,13] and the co-transplantation with different stem cells has been used to improve the transplanted cells. The mixed transplantation of neural stem cell and NT-3 modified schwann cells promoted the differentiation of neural stem cells into neuron-like cells and enhanced repairing of neural stem cells in spinal cord injury [14]. Human AT-MSC improved the differentiation potential and cell migration of mouse neural stem cells [15] and mouse BMSC also promoted neuronal differentiation of mesencephalic neural stem cells [16]. It suggested that these neuronal cell survival and regeneration and differentiation were stimulated by some secreted factors of mesenchymal stem cells [17,18].

Also hypoxic preconditioning of stem cells improved their neuroprotective ability in ischemic brain injury [19]. It suggests that protection effect is base on up-regulation of endogenous pro-survival mechanisms and intracellular signaling pathway under hypoxia conditions [20]. Finally stem cells may be express hypoxia inducible factors such as hypoxia-inducible factor-1α (HIF-1α), erythropoietin (EPO), VEGF [21,22]. EPO plays a critical role in cell survival by serum deprivation induced cell death in vitro [20]. Other group examined whether culturing MSC in hypoxic conditions improves their tissue regenerative potential, mice that had received hypoxic preconditioned MSC recovered faster than the normoxic MSC or control groups in hind limb injury model [23]. Hypoxic preconditioned embryonic stem cells promoted their cell survival and functional benefits after transplantation into the ischemic rat brain [10].

In present study, we hypothesized that co-culture with hypoxia preconditioning AT-MSC played main role to promote the viability of DsRed expressing mouse neural stem cells that transfected by non-viral vector in serum deprivation and hydrogen peroxide injury models.

II. MATERIALS AND METHODS

1. Cultivation of adipose tissue derived mesenchymal stem cell and hypoxia preconditioning

The adipose tissue derived mesenchymal stem cells (AT-MSC) was donated from the laboratory of the plastic surgery. The AT-MSC was suspended in Dulbecco's modiied Eagle's medium (DMEM, GIPCO) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (100 unit/ml, invitrogen, UK) and plated in flask. Cultures were maintained at 37℃ in a humidified atmosphere containing 5% CO_2. And we confirmed the identity of AT-MSC by FACS scan flow cytometer using a FITC conjugated CD90 and CD105 antibody.

For hypoxia preconditioning, When the AT-MSC grew to 80% confluence, we changed the fresh media containing 10% FBS and then transfer to 1% O_2 hypoxic incubator and maintained for 24 hrs.

2. Transfection and neural stem cell culture

We established the stable cell lines which express the DsRed protein. mNSC (2x105) was suspended in DMEM/F12 supplement with 10% fetal bovine serum (Hyclone), 1 % penicillin and streptomycin (Invitrogene) and plated in cell culture dish. The culture was maintained at 37℃ in a humidified atmosphere containing 5% CO_2.

pBudCE4.1-DsRed vector (Clontech) was transfected into normal mouse neural stem cells using lipofectamine (Gibco-BRL). 24 hours after transfection, 100mg/ml Geneticin (Zeocin, invitrogene) was added to the culture medium. Single Zeocin-resistant cells were isolated by cloning rings and expanded. Cell populations with intense DsRed expression were used for experiment. We performed a process like this. Step 1: plasmid DNA pBudCE4.1-DsRed(1ug/ul) were mixed with 100ul opti-MEM (GIPCO). Step 2: 2.5ul Lipofectamine 2000 (Invitrogene) mixed with 100ul opti-MEM and were maintained for 5 mins on room temperature. Step 3: solution of step 1 and step 2 were mixed for 20 mins on room temperature. Step 4: mNSC was washed with PBS and were detached with 0.25% trypsine/EDTA (Invitrogene). mNSC was suspended in MEM (GIPCO) supplement with 10% fetal bovine serum and 1% P/S and step 3 solution. After plate in dish, culture was maintained at 37℃ in a humidified atmosphere containing 5% CO_2 for 24 hrs.). 24 hours after transfection, Zeocin, (100mg/ml, invitrogene) was added to the culture medium. We changed media supplemented with Zeocin (100ug/ml) every 2 days until form the colony. Single Zeocin-resistant cells were isolated by cloning rings and expanded. Cell populations with intense DsRed expression were used for experiment.

3. Establishment of in vitro injury model

In the present study, we investigated whether co-culture system with hypoxia preconditioning MSC can improve the survival of mNSC-DsRed in serum deprivation and H2O2 injury which two components of ischemia. Group 1: mNSC($3x10^5$) co-culture with hypoxia preconditioned MSC($3x10^5$), Group2: mNSC($3x10^5$) co-culture with normoxia preconditioned MSC($3x10^5$), Group3: mNSC alone($3x10^5$). The hypoxia preconditioned and normoxic MSC, mNSC-DsRed seeded in 6 MTP respectively, then maintained in 37℃ 5% CO_2 incubator for 24 hrs. And then cells were washed with PBS and changed the fresh media containing with hydrogen peroxide (500uM) or serum free media and maintained 37℃, 5% CO_2 for 24 hrs. Each plate was taken photographs of random five areas under 556nm fluorescence microscope, and then examined the optical density of mNSC-DsRed uing software in each of samples. The scheme of present study display in table-1.

4. FACS analysis

We confirmed whether MSC co-culture system can protect the early apoptosis of mNSC under three different injury models. We performed FACS analysis using ApoScan ™ Annexin V FITC apoptosis detection Kit (BioBud; Aposcan). In brief, cells were harvested using trypsin/EDTA after injury. Cell pellets washed with ice cold PBS and then added 500ul 1X binding buffer. Annexin-V solution (1.25ul) added

to the 1X binding buffer and then incubated for 15 min at room temperature in dark. Because the mNSC-DsRed cells express the red color, we not performed the propidine iodide (PI) staining. After finish the incubation of Annexin-V, we analyzed using FACS( )and Cell Quest software. Number of analyzed cells was $1x10^4$cells in each of the samples.

5. Immunocytochemistry

For immunofluorescence staining, cells on plate were fixed 4% formaldehyde in PBS for 10 min at room temperature. After wash with ice cold PBS, incubated with 0.5% triton X-100 in PBS for 15 min at room temperature. Samples were blocked with 3% BSA in PBS for 1 hour at room temperature. Plates were incubated with Rabbit anti-Bax antibody (Chemicon; 1:2000) for 1 hour, then washed with PBS, and incubated with FITC-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch; 1:250) for 1 hour at room temperature. Cells were washed with ice cold PBS three times and then covered the cover slide after dropping with Vectashield mounting medium with DAPI (Vector; H-1200). We analyzed the samples under a fluorescence microscope.

6. Statistics analysis

The t-test was performed for comparison of two experimental groups. Data are expressed as the mean ± standard error of mean.

III. Results

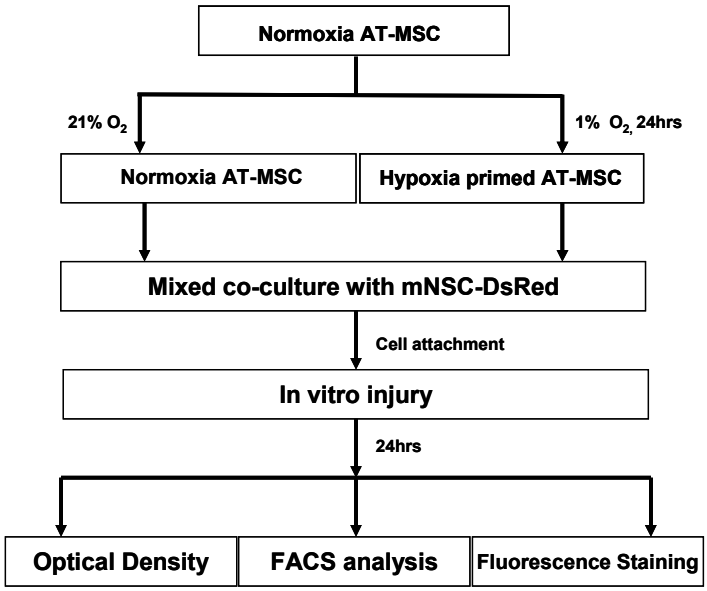


Figure. 1 scheme of present study

Diagram indicates in vitro experimental procudure.

2. Characterization of adipose tissue derived mesenchymal stem cell

To confirm whether primary cultured cells are AT-MSC, we performed FACS scan using CD 90 and CD 105 antibody before use the AT-MSC in this experiment. Both hypoxia preconditioning MSC and normoxia MSC have similar morphology and characterization (Figure 2). FACS analysis of normoxia and hypoxia AT-MSC showed the high positive of CD 90 and CD 105 surface marker, we couldn't found any difference features between hypoxia MSC and normoxia MSC. This suggests that nature characterization of AT-MSC is not change by hypoxia preconditioning (1% O_2).

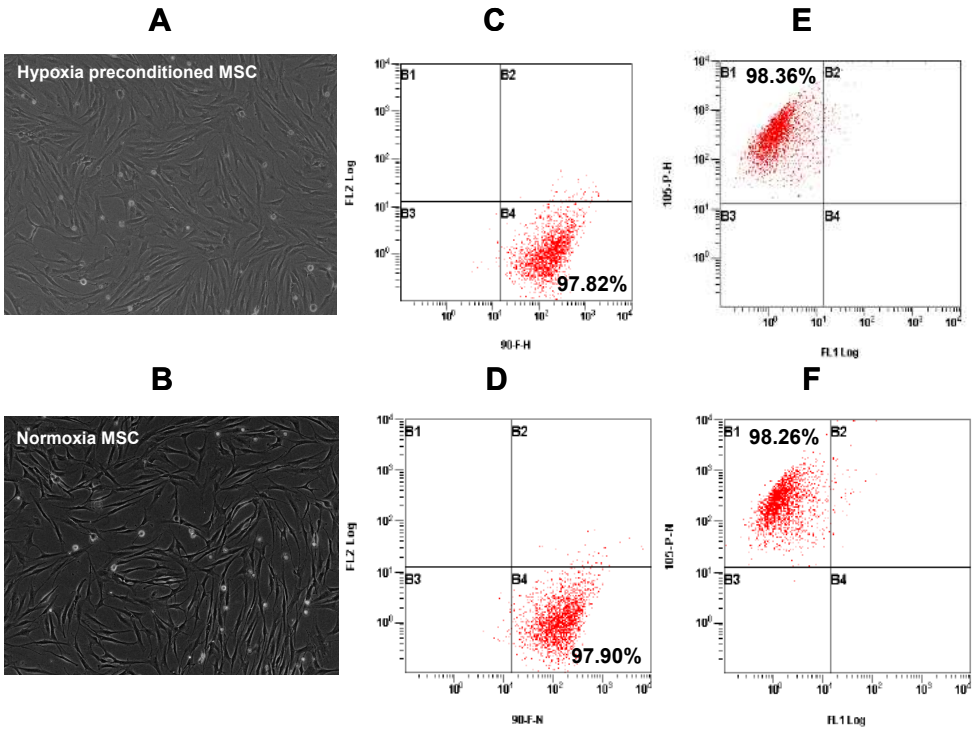


Figure 2. Characterization of adipose tissue derived mesenchymal stem cell. Phase contrast micrograph displayed a typical fibroblast morphology of plated MSC. Both hypoxia(A) and normoxia(B) MSC have similar morphology. The cells were photographed by phase microscopy at x100 magnification. (C-F) Immuno phenotype analysis of hypoxia preconditioning MSC(C) and (E) and normoxia MSC(D) and (F). Cells were trypsinized, labeled with CD90, CD105 and then analyzed by flow cytometry. Flow cytometric characterization of MSC revealed that cells expressed positively for MSC markers CD105 and CD90. It suggest that characterization of AT-MSC not change by hypoxia preconditioning.

3. Characterization of DsRed expressing mouse neural stem cell

To check whether mNSC-DsRed still has similar features like normal neural stem cells, we confirmed the growth speed and morphology of both mNSC-DsRed and normal NSC. A growth speed of DsRed expressing neural stem cell was slowed little compare to normal NSC (Figure 3). However, this difference was not significant, and then mNSC-DsRed also had doubling time at 12hrs similar to normal NSC. And our neural stem cells could express their reporter gene continuously, and then was difficult to distinguish the morphology of both cells (Figure 3-D).

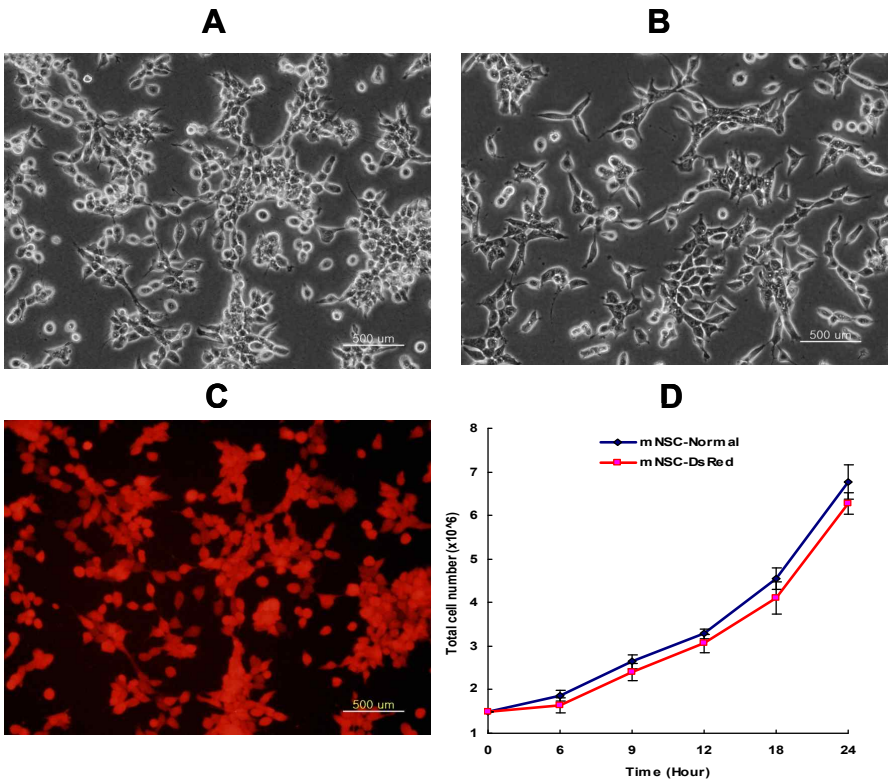


Figure 3. mNSC-DsRed still has the similar growth speed and morphology like normal mouse neural stem cell

Phase contrast micrograph displayed a similar morphology between mNSC-DsRed (A) and normal mouse neural stem cell (B). mNSC-DsRed express the red color at 556nm under flurosecence microscope (C). mNSC-DsRed has similar growth speed compare to normal NSC (D). Doubling time of mNSC-DsRed and normal NSC is around twelve hours. Scale bar, 500um (A-C)

4. The viability of mNSC-DsRed in serum deprivation or oxidative suppress

We confirmed that the hypoxia preconditioning MSC can protect the mNSC death by serum deprivation or hydrogen peroxide injury. At 24 hrs after injury, number of mNSC in control group was decreased compare to co-culture with AT-MSC. In contrast to, co-culture with AT-MSC could protect the mNSC death under serum deprivation or oxidative suppress injury. In addition to, the viability of mNSC-DsRed co-culture with hypoxia preconditioning MSC was increased significantly compare to normoxia MSC co-culture group. Also the co-culture with hypoxia preconditioning MSC could protect significantly the death of mNSC-DsRed under oxidative suppress injury (Figure 4).

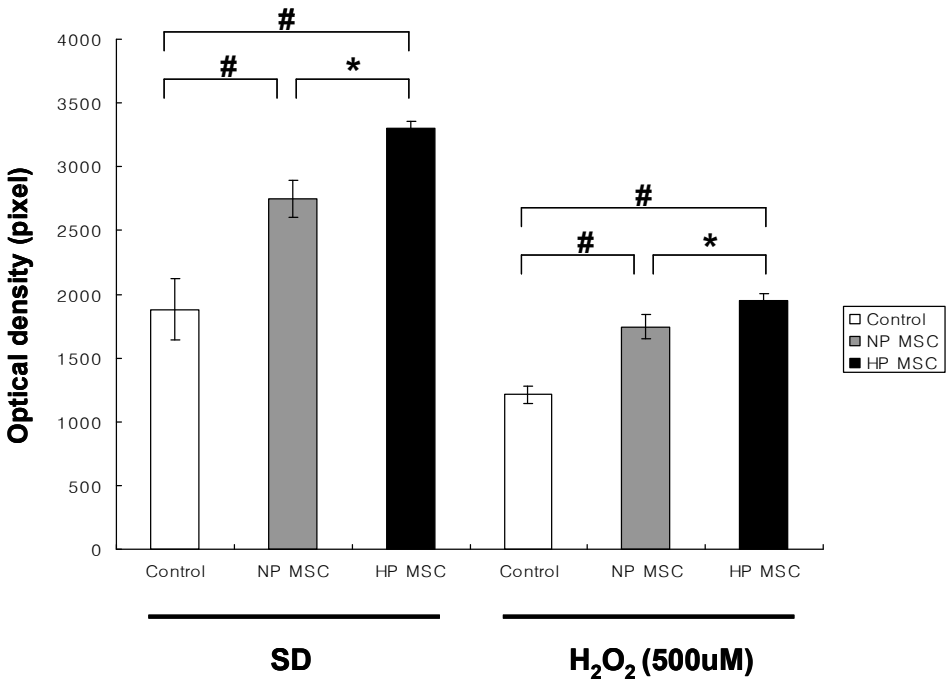


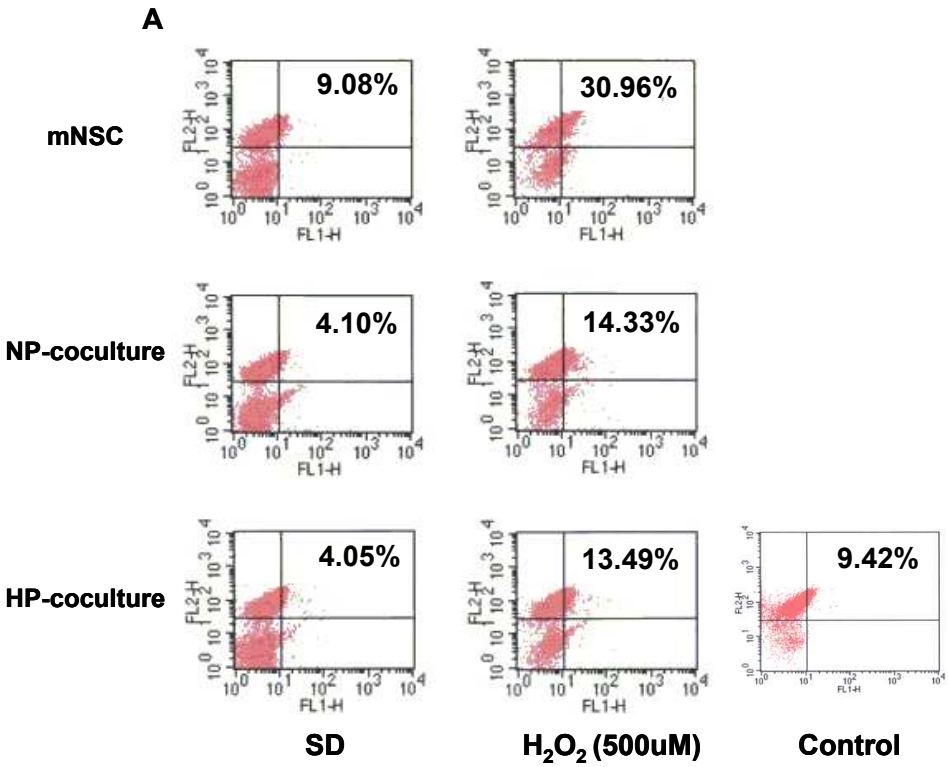
Figure 4. Viability of mNSC-DsRed in serum deprivation (SD) and oxidative stress (H_2O_2)

mNSC-DsRed of each group incubated for 24 hrs in SD and H_2O_2 injury. The data suggests that MSC co-culture system can improve the viability of mNSC-DsRed in SD and H_2O_2 injury. Especially co-culture with hypoxia preconditioning MSC (HP-MSC) can improve significantly the viability of mNSC-DsRed compare to co-culture with normoxia MSC (NP-MSC).

* $p<0.05$ compare to Normoxia MSC (NP-MSC); # $p<0.05$ compare to control group

5. Apoptosis of mNSC-DsRed in serum deprivation and oxidative stress

We confirmed whether MSC coculture system can inhibit the early apoptosis of mNSC-DsRed in vitro injury models. The number of Annexin-V positive cells showed decrease tendency in coculture with hypoxia preconditioning MSC group. But hypoxia MSC coculture system couldn't decrease significantly the early apoptosis of mNSC compare to normoxia MSC (Figure 5-A). However, MSC coculture system could decrease around 50% the early apoptosis of mNSC by serum deprivation and oxidative suppress in each of injury model (Figure 5-B).


A
mNSC
NP-coculture
HP-coculture
9.08%
30.96%
4.10%
14.33%
4.05%
13.49%
9.42%
FL2-H
FL1-H
SD
H2O2 (500uM)
Control

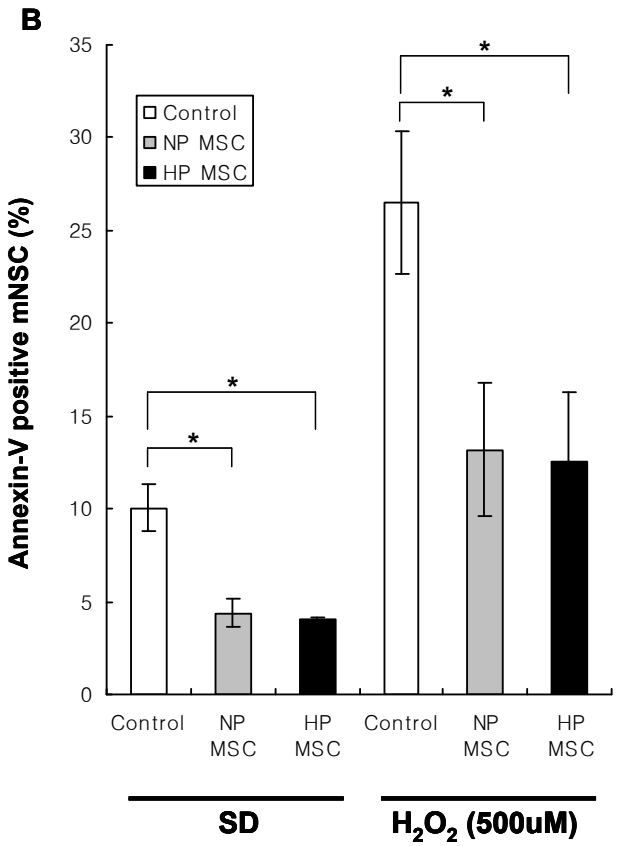


Figure 5. Quantification for Apoptic mNSC in serum deprivation (SD) and oxidative stress (H_2O_2)

mNSC-DsRed of each group incubated for 24 hrs in SD and H_2O_2 injury. The data indicate the early apoptosis of mNSC-DsRed by FACS analysis after staining with Annexin-V FITC only. Co-culture with hypoxia preconditiong MSC show the decrease tendency of Annexin-V positive compare to co-culture with normoxia MSC and mNSC alone (A). The number of $DsRed^+/Annexin\text{-}V^+$ was decrease about 50% after serum deprivation and oxidative injury in MSC co-culture system (B).

* $p<0.05$ compare to control group

6. Immunocytochemistry

To confirm the expression of Bax, pro apoptosis factor, we performed the immunoflurosecence staining using primary antibody rabbit anti-Bax (Chemicon; 1:2000) and secondary antibody FITC-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch; 1:250) after serum deprivation and oxidative stress injury for 24 hrs. The expression of Bax showed increase pattern in mNSC alone and reduced in hypoxia and normoxia MSC co-culture group after serum deprivation (Figure 6-A) and oxidative stress (Figure 6-B) injury .

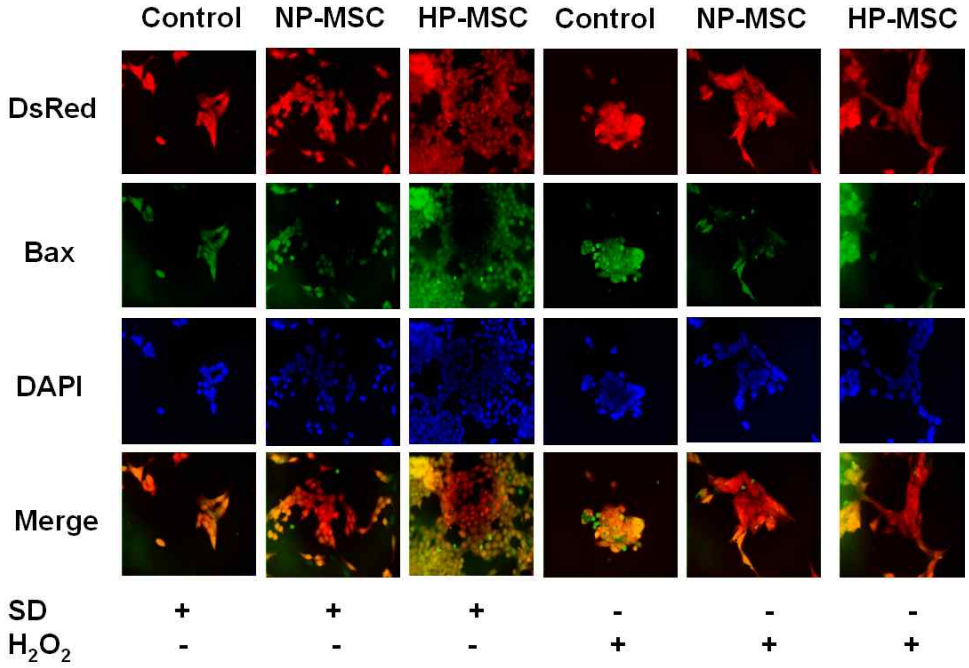


Figure 6. Expression of Bax, pro apoptosis factor, in injured mNSC-DsRed

Immunocytochemistry showed the expression of pro apoptosis marker Bax in mNSC-DsRed after injury. Images indicate that AT-MSC co-culture system can inhibit the early apoptosis of mNSC-DsRed after serum deprivation and oxidative stress injury for 24 hrs. The cells were stained with rabbit anti-Bax antibody and FITC-conjugated secondary antibody. Images were obtained by fluorescence microscopy at x400 magnification. control; mNSC-DsRed alone, NP-MSC; non-preconditioning MSC, HP-MSC; hypoxia preconditioning MSC

IV. Discussion

In present study, first objective is to establish the gene delivery platform using neural stem cells transfected by non-viral vector. Survival improving of transplanted cells is critical factor in stem cell therapy. Before induction the therapeutic gene such as Bcl-2 or other useful growth factors, we needed to confirm whether reporter gene expressing mNSC still has similar ability like normal neural stem cells or not. The method to check characterization of cells is to confirm their morphology and growth speed and differentiation capability. In our results, DsRed expressing neural stem cells had similar features like normal neural stem cells. Therefore it suggests that our gene delivery platform using neural stem cells may be able to use for stem cell therapy with gene therapy.

Our second objective is to improve the survival of mNSC-DsRed after transplantation into the injured spinal cord. Before start in vivo experiment, we need to confirm whether MSC co-culture system can protect the mNSC death by in vitro serum deprivation or oxidative stress injury which two components of ischemic condition. Also co-culture with hypoxia MSC could promote the proliferation and adhesion of mNSC compare to control group. Before use the SD and oxidative injury model, we performed that mNSC-DsRed incubated under hypoxia and normoxia conditions time dependently. mNSC could proliferate quickly under hypoxia and looks like resist to hypoxia condition.

the co-culture with hypoxia preconditioning MSC could improve the

viability of mNSC-DsRed in serum deprivation and oxidative stress injury models (Figure 3). But we couldn't found significant positive effects of co-culture with the hypoxia MSC that inhibits the early apoptosis of mNSC-DsRed compare to Normoxia MSC co-culture group.

V. Conclusion

These study demonstrated that co-culture with hypoxia preconditioning AT-MSC can promote significantly the viability of mNSC-DsRed in vitro injury models. The characterization of AT-MSC didn't changed nature features by hypoxia preconditioning. DsRed expressing mNSC has similar features like normal neural stem cells. MSC co-culture system can inhibit the early apoptosis of mNSC in serum deprivation and oxidative stress injury. In further study, we need to examine the mixed transplantation of HP-MSC and mNSC-DsRed into the injured spinal cord.

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ABSTRACT (IN KOREAN)

저산소 상태를 적응시킨 지방유래 간엽줄기세포와의 합동배양 시스템은 마우스유래 신경줄기세포의 생존을 향상시킨다.

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저산소 상태의 적응훈련을 받은 줄기세포의 이식은 허혈을 동반하는 손상받은 조직으로 이식 한 후에 저산소증에 대한 저항력을 더욱 상승 시킬 수 있다. DsRed라는 유전자의 운반체 역할을 하는 줄기세포를 손상 받은 척수에 이식 후 신경줄기세포의 생존을 향상시키기 위한 실험에 앞서, 본 실험에서는 저산소 상태에서 한번 적응과정을 거친 지방유래 간엽줄기세포와 DsRed를 발현하는 마우스 유래 신경줄기세포와의 합동배양이라는 새로운 전략의 효과를 평가하였다. 저산소 상태에서 하루 동안 배양된 간엽줄기세포는 정상상태에서 배양된 간엽줄기세포와 동일하게 표면항원 마커인

CD90 과 CD105에 양성을 나타내었다. 이것은 저산소 상태가 간엽줄기세포의 본질적인 특징을 변화시키지 않는다는 것을 나타낸다. 산화적 억제와 영양분의 차단으로 인한 시험관 손상모델에서, 간엽줄기세포와의 합동배양을 한 그룹에서의 신경줄기세포의 생존력은 실험군과 비교 하였을 때 확연히 증가 하였다. 특히 저산소 상태의 적응훈련을 마친 간엽줄기세포는 정상상태에서 배양되어 왔던 간엽줄기세포보다 신경줄기세포의 생존에 더욱 뛰어난 효과를 보였다. 간엽줄기세포와의 합동배양은 실험군과 비교하였을 때 신경줄기세포의 세포사멸을 억제했다. 이러한 결과는 줄기세포를 이용한 유전자 전달 시스템과 저산소 상태의 적응훈련을 마친 줄기세포의 복합 전략이 더욱더 효과적인 줄기세포치료를 선사할 수 있다는 것을 나타낸다.

핵심이 되는 말: 저산소에 적응된 지방유래 간엽줄기세포, 마우스유래 신경줄기세포, 합동배양, 영양분 공급 차단, 산화적 억제