

ARTICLE

Decreased osteogenesis of adult mesenchymal stem cells by reactive oxygen species under cyclic stretch: a possible mechanism of age related osteoporosis

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Age related defect of the osteogenic differentiation of mesenchymal stem cells (MSCs) plays a key role in osteoporosis. Mechanical loading is one of the most important physical stimuli for osteoblast differentiation. Here, we compared the osteogenic potential of MSCs from young and adult rats under three rounds of 2 h of cyclic stretch of 2.5% elongation at 1 Hz on 3 consecutive days. Cyclic stretch induced a significant osteogenic differentiation of MSCs from young rats, while a compromised osteogenesis in MSCs from the adult rats. Accordingly, there were much more reactive oxygen species (ROS) production in adult MSCs under cyclic stretch compared to young MSCs. Moreover, ROS scavenger N-acetylcysteine rescued the osteogenic differentiation of adult MSCs under cyclic stretch. Gene expression analysis revealed that superoxide dismutase 1 (SOD1) was significantly downregulated in those MSCs from adult rats. In summary, our data suggest that reduced SOD1 may result in excessive ROS production in adult MSCs under cyclic stretch, and thus manipulation of the MSCs from the adult donors with antioxidant would improve their osteogenic ability.

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INTRODUCTION

Bone is one of the most important tissues both for maintenance of architectural integrity and metabolic activity,¹ which undergoes continuous remodeling.² The microstructure of bone depends on genetic determinants and on the continuous response of the skeleton to mechanical cues. With increased amounts of mechanical loading, osteogenesis occurs. In contrast, there is significant bone loss during immobilization or in space.³ Mechanical stress not only influences bone homeostasis in post-natal remodeling processes,⁴ but also the continuous osteogenesis in the whole life.⁵

Human aging is associated with a progressive decline in bone mass and an accumulation of marrow fat, suggesting that osteogenesis defect might be the reason.⁶ Mesenchymal stem cells (MSCs) is one of the keys *in vivo* progenitors for osteogenesis under mechanical stretch both *in vivo* and *in vitro*.^{7–11} In view of the well-known role of MSCs in osteogenesis, more information about

age-related changes of MSC osteogenic differentiation under mechanical stretch is essential for under mechanical forces. As far as the differentiation and proliferation capacity alteration of MSCs with age being considered, there is conflicting evidence: with some groups reporting no change, while others finding an age-related decrease.¹² The main cause for the inconsistency might be due to the different osteogenic models used.

The aim of this study was to assess the effect of cyclic stretch on the proliferation and differentiation of MSCs from different aged rats, and to identify the mechanism underpinning the differences between the young and adult MSCs.

MATERIALS AND METHODS

Isolation and growth of MSCs

Ten rats at the age of postnatal 5 week (five rats) and 1 year (five rats) were included for MSCs isolation. Bone marrow was harvested by flushing the femurs and tibiae of

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male SD rats Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Carlsbad, CA, USA). Nucleated cells were isolated with a density gradient (Ficoll-Paque; Amersham-Pharmacia, Piscataway, NJ, USA) and resuspended in complete culture medium. The culture medium was supplemented with 10% fetal calf serum (Gibco) and antibiotic/antimycotic (100 U·mL⁻¹ penicillin, and 0.1 mg·mL⁻¹ streptomycin; Invitrogen, Carlsbad, CA, USA). All of the cells were plated in 15 ml of medium in a 75-cm² culture flask (Corning, St Louis, MO, USA) and maintained in a humidified atmosphere with 5% CO₂ at 37 °C. After 24 h, non-adherent cells were discarded, and adherent cells were thoroughly washed twice with PBS. The culture medium was changed twice a week, during which non-adherent cells were discarded. Fluorescence activated cell sorter analysis on cells at passage 3 verified that the cell population expressed high levels of the MSC surface marker CD90 (92.1%±0.7%) but not CD45, in agreement with previously published data.¹³

Differentiation induced by osteogenic medium

To induce the MSCs toward osteogenic differentiation, cells were switched to the osteogenic medium(OS), in which the DMEM culture medium was supplemented with 10% FBS containing 50 µg·mL⁻¹ ascorbic acid (for appropriate collagen and extracellular matrix production) and 5 mmol·L⁻¹ β-glycerophosphate (for appropriate mineralization) and 0.1 µmol·L⁻¹ dexamethasone.

Mechanical stretch application

MSCs of passage 3 were trypsinized and seeded on bottom-flexible six-well plate (Type I collagen coated, Flex I; Flexcell International, McKeesport, PA, USA) at a density of 1×10⁵ cells per well. Mechanical loading was applied on MSCs Flexercell Tension Plus system 3000 (Flexcell International, McKeesport, PA, USA). Since cyclic stretch extension over 5% is lethal to mesenchymal stem cells,¹⁴ MSCs were subjected to mechanical stress at a rate of 1 Hz with 2.5% elongation, 2 h·d⁻¹, for 3 days in this study.

Reverse transcription polymerase chain reaction

Total cellular RNA was extracted from MSCs by Trizol (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed to cDNA by M-MLV (Promega, Madison, WI, USA). Primers for target genes were listed in Table 1, with GAPDH as an internal control.

Table 1. Primers for the analysis of the target genes by RT-PCR

Primer name	Forward	Reverse
<i>Runx2</i>	CACTTGGCAAAGAGCTGGAG	CAAGGGACTTGAAGTTAGCAG
<i>Col 1a</i>	GACACAGAGGTTTCAGTGGT	GTTTCCAGCAGGACCAGCAG
<i>SOD1</i>	TACAGGATTAAGTGAAGGCG	CAGATGAGCTGAGACTCAG
<i>GAPDH</i>	TCTACATGTTCCAGTATGAC	GCATTGCTGACAATCTTGAG

Abbreviation: RT-PCR, reverse transcription polymerase chain reaction.

Western blot

To assess protein expression level, cells were lysed in buffer (25 mmol·L⁻¹ HEPES, 5 mmol·L⁻¹ MgCl₂, 5 mmol·L⁻¹ EDTA, 5 mmol·L⁻¹ dithiothreitol, 0.1 mmol·L⁻¹ PMSF, 5 µg·mL⁻¹ pepstatin A, 2 µg·mL⁻¹ leupeptin, 2 µg·mL⁻¹ aprotinin, 2 µg·mL⁻¹ sodium orthovanadate, pH 7.4). Protein concentration was determined using the PIERCE BCA assay in accordance with the manufacturer's instructions. Samples were then heated in the boiling water for 5 min. Proteins (60 µg per lane) were separated by electrophoresis on a 12% polyacrylamide gel, transferred to nitrocellulose membrane, and immunoblotted with anti-superoxide dismutase 1 (SOD-1) (Santa Cruz, CA, USA) and secondary antibody (anti-mouse IgG; 1:1 000) conjugated to horseradish peroxidase. Bands were visualized by Chemiluminescence (Supersignal, Pierce, Leiden, The Netherlands). Immuno-blots of β-actin (Santa Cruz) served as an internal control.

3-(4, 5-methylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide assay

To examine the cell proliferation ability, cells were seeded at the density of 5 000 per well and cultured in the growth medium for indicated periods. Cell number at each time-point was counted with 3-(4, 5-methylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide assay. Briefly, 3-(4, 5-methylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide was added (100 µg per well) to each well of the 96-well plate and incubated in 37 °C for 4 h. Formazan products were solubilized with DMSO, and the optical density was measured at 490 nm.

ALP activity

ALP activity was measured according to the manual instruction. Briefly, cells were washed once with PBS before trypsinization. Then, cells were centrifuged at low speed, and the supernatant was discarded. The pellet was lysed with the same buffer as the Western blot at 4 °C. The activity of alkaline phosphatase was measured by the ALP kit (Jiancheng, Nanjing, China) according to the manual instructions. Fold induction of the enzyme activities are calculated.

Reactive oxygen species (ROS) detection

ROS measurement was done as described previously.¹⁵ Cells were harvested and washed twice with PBS. Then cells were divided into two parts equally: one for cell number count and the other for ROS measurement. For ROS measurement, cells were incubated for 30 min at 37 °C in the dark with the oxidation-sensitive probe 2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes, Eugene, Oregon, USA). After washing twice with PBS, PBS was added and fluorescence from the plates were read in a

Microplate Fluorescence Reader FL600 (Bio-Tek, Winooski, VT, USA) using a wavelength of 486 nm for excitation and 530 nm for emission. ROS levels per 5000 cells of each group were calculated. Unstained cells were applied as background and the data were normalized to values obtained from the control group.

Statistical analysis

Data were reported as the mean \pm s.d. Statistical analysis was carried out by SPSS 13.0 software to determine whether significant differences existed between (among) the populations. $P < 0.05$ was considered statistically significant.

RESULTS

No significant differences of osteogenic differentiation ability in the OS

Since there is conflicting evidence about the proliferation and differentiation potential of MSCs from donors of different ages,^{12,16} we first wanted to compare the proliferation and differentiation capacities of MSCs from both the young and adult rats. The passage 3 MSCs were used for

the analysis of cell proliferation and differentiation under OS. As shown in Figure 1a, both the young MSCs and the adult MSCs displayed similar proliferation ability in the common culture medium. In addition, there were no significant differences between the young and adult MSCs in osteogenic differentiation capacity under differentiation medium, as seen from the ALP activity and Runx2 and Col1a expression (Figure 1b and 1c).

Decreased osteogenesis of adult MSCs under cyclic stretch compared to young MSCs

To further explore the potential intrinsic factors of the MSCs responsible for the clinical findings that the adult and aged patients have a poorer prognosis after distraction osteogenesis, we next included the *in vitro* cyclic stretch model to mimic the *in vivo* distraction osteogenesis reliably. Consistent with previous findings,^{9,17–19} cyclic stretch with 2.5% elongation for 10 days increased the ALP activity significantly both in the young and adult MSCs (Figure 2a), though the level was less than that induced by OS. In addition, the induced ALP activity was much lower in the adult MSCs than that in the young MSCs (Figure 2a). In

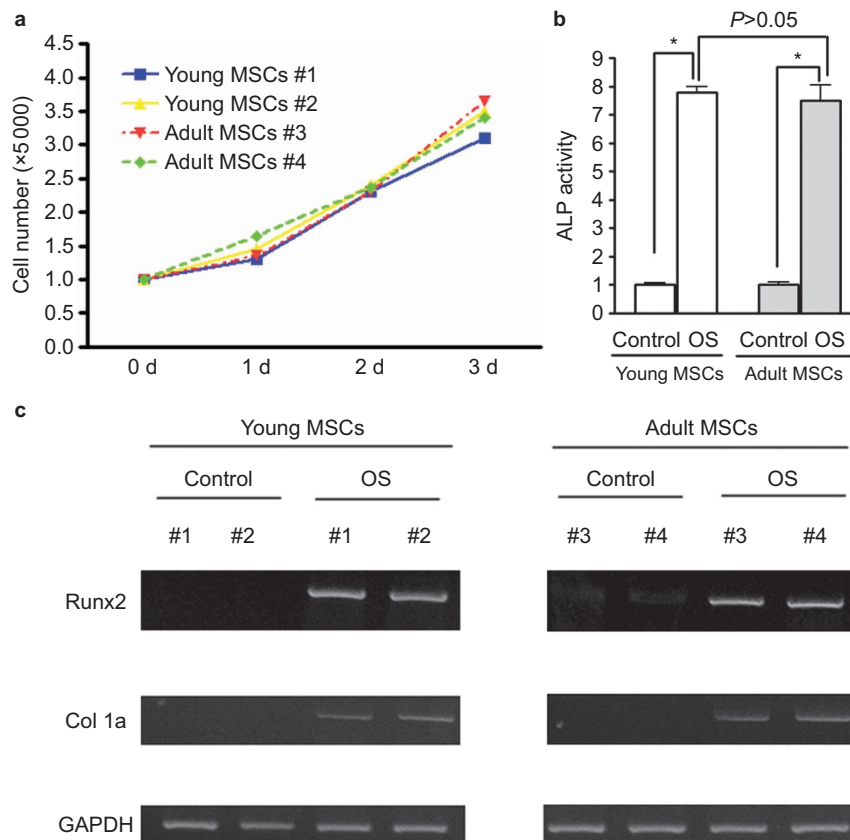


Figure 1. The proliferation ability of MSCs from rats of different ages and their differentiation potential induced by OS. (a) Representative cell growth curves of the MSCs from rats 1, 2 (young rats), 3, 4 (adult rats). No significant differences of cell proliferation between adult and young rat MSCs. (b) ALP activity were increased to a similar level by OS (osteogenic supplement) both in MSCs from young and adult rats. All data were presented as mean \pm s.d. ($N=5$). (c) Runx2 and Col1a expression were significantly induced by OS, and no significant differences were found between the MSCs from both the young and adult rats. * $P < 0.05$, compared with the indicated control.

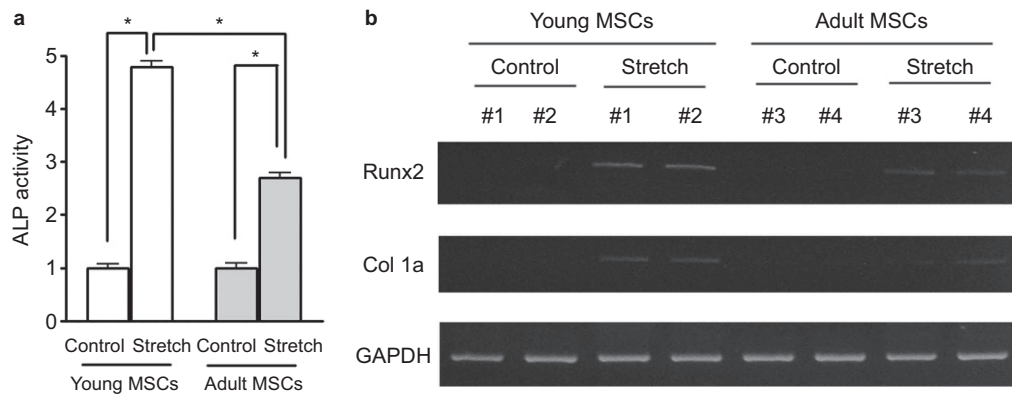


Figure 2. Impaired osteogenic differentiation of adult MSCs under mechanical stretch. (a) ALP activity increased when the young MSCs were subjected to 3-day cyclic stretch, while ALP activity in the adult MSCs was much lower, though ALP increased compared with the control. * $P < 0.05$, compared with the indicated control. (b) The expression of Runx2 and Col1a in the MSCs with indicated treatments. A 3-day cyclic stretch increased the expression of Runx2 and Col1a in the young MSCs, while the extent was much lower in the MSCs from the adult rats. Data presented here were a representative of three independent experiments.

accordance with the ALP activity, Runx2 expression and Col1a expression under cyclic stretch displayed a similar trend as the ALP activity (Figure 2b). All of these suggested adult MSCs had a deficient differentiation potential under mechanical strain.

Adult MSCs produced more ROS under mechanical strain. From the above data, we next wanted to see the mechanism of the impaired osteogenic differentiation of adult MSCs. We previously revealed that ROS generation played an important role in the cyclic stretch.¹⁵ To this end, we examined the ROS production both in young and adult MSCs after cyclic stretch. 2.5% stretch increased the endogenous ROS production in both adult and MSCs. In the young MSCs, ROS production was about 1.12-fold of the endogenous level in the non-stretched control MSCs. In contrast, there was nearly 1.65-fold ROS induction in the adult MSCs by stretch (Figure 3a). Furthermore, expression of SOD1 in adult MSCs under cyclic stretch was much lower

than that in the young MSCs both at mRNA and protein level (Figure 3b), suggesting the insufficient production of antioxidant enzyme might be involved in the deficiency of osteogenic differentiation of MSCs under mechanical stretch.

Inhibition of the endogenous ROS by 1 mmol·L⁻¹ NAC rescued the ALP activity induction of adult MSCs under cyclic stretch (Figure 4), suggesting that excessive ROS production in the adult MSCs should be the one of the main reasons for their insufficient osteogenic differentiation potential.

DISCUSSION

Through *in vitro* model, our study here has revealed that the age related alteration of the differentiation potential of MSCs is dependent on the external differentiation cues.²⁰ For the OS induced differentiation, no obvious changes were found in the MSCs from both young and adult rats. In contrast, under mechanical stretch, MSCs from the adult donors displayed significant deficiency in osteogenic differentiation compare to young MSCs.

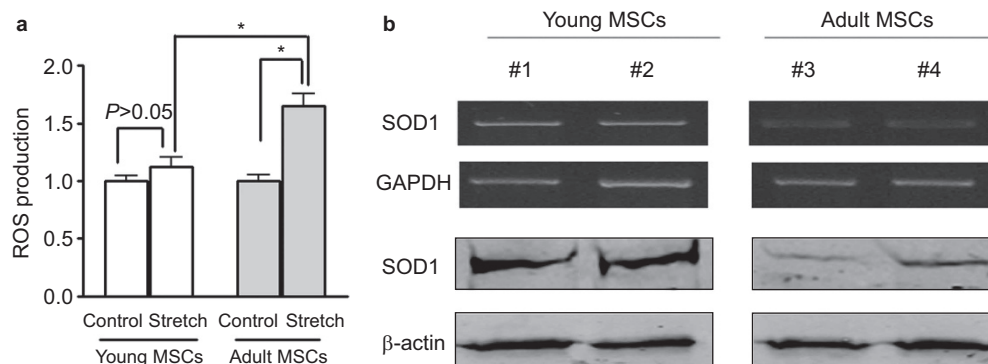


Figure 3. Increased ROS production is responsible for the osteogenesis defect in adult MSCs undergoing mechanical stretch. (a) Cyclic stretch increased the endogenous ROS production both in young and adult MSCs, and the increased ROS in adult MSCs are much more significant than those in the young MSCs. (b) Expression of SOD1 in adult MSCs under 2.5% extension stretch. Lower expression of SOD1 at mRNA level in adult MSCs (upper panel), compared with that in the young MSCs. Lower expression of SOD1 at protein level in adult MSCs (lower panel). Data presented here were the representative of three different experiments.

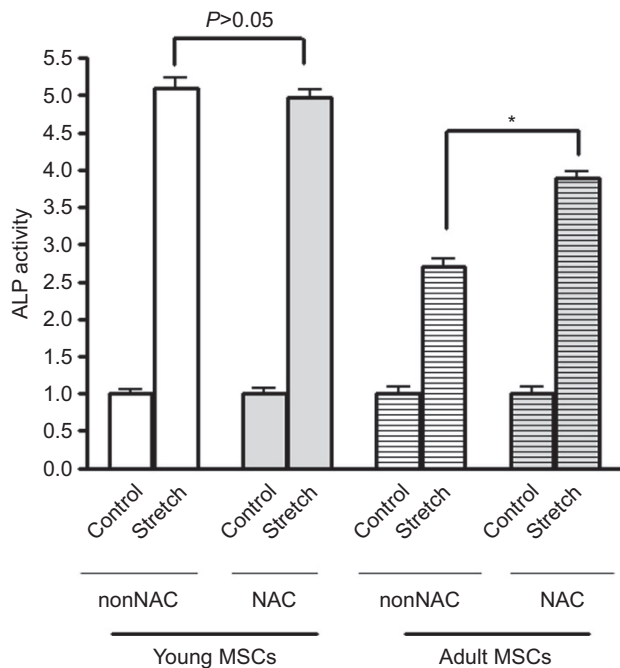


Figure 4. Inhibition of ROS rescued the osteogenic defect of adult MSCs. Inhibition of the endogenous ROS by $1 \text{ mmol} \cdot \text{L}^{-1}$ NAC rescued the ALP activity induction of adult MSCs under tensile strain.

Although previous studies have revealed that mechanical stretch induces osteogenic differentiation of MSCs,^{9,17–19} our study here for the first time reveals that MSCs from older rats have defective osteogenic differentiation potential only under mechanical stretch compared to young MSCs.

As to the apparent differences of the differentiation potential under OS induction and mechanical stretch, one explanation is the different mechanisms MSCs used for osteogenesis under the two contexts. In other words, MSCs from adult rats just have defects in certain pathways, which is bypassed by *in vitro* OS induction while could not be bypassed by mechanical stimuli. However, the mechanical stimuli mimic the Distraction Osteogenesis faithfully, which may represent the *in vivo* data better. Importantly, our study here should give an alternative explanation to the inconsistent findings about the decreased osteogenic differentiation ability of adult MSCs. It was postulated early in 1971 by Meunier *et al.*²¹ that MSCs from adult rats loses osteogenic potential and gain adipogenic potential—termed ‘adipogenic switch’,²² leading to senile osteoporosis. However, osteogenic differentiation potential is well maintained even in the adult rats according to some recent reports.^{23–25} In other words, there are some defects in certain pathways regulating the osteogenesis in the adult MSCs. Our study here provides the evidence why the aged people are inclined to osteoporosis, which could unify all the inconsistent data about the effects of age on the osteogenic ability of MSCs. In addition, Distraction Osteogenesis addresses one of today’s hottest topics in orthodontics

and oral and maxillofacial surgery.^{26–27} However, clinical studies revealed that age was one of the key factors affecting the prognosis of Distraction Osteogenesis,²⁸ which could be partially explained by the defect of the osteogenic differentiation ability of adult MSCs under mechanical stretch.

As to the mechanism for the deficient osteogenic differentiation of MSCs from adult rats under mechanical stretch, we mainly focused on the role of ROS. Different from the myoblast cell C2C12, which is much more resistant to mechanical strain,¹⁵ MSCs are much more sensitive to mechanical stretch induced apoptosis.¹⁴ In addition, ROS are essential for mechanical stretch induced apoptosis,¹⁵ suggesting a role of ROS in the mechanical stretch induced changes in the adult MSCs. Recently, ROS are found to be fundamental in maintaining the full differentiation potential of stem cells. ROS are the main mediators of the age related intrinsic or external stress associated changes of stem cells.²⁹ Luca Vanella *et al.*⁴ found that osteoblast differentiation of MSCs is positively regulated by HO-1 expression, which was associated with a reduction of ROS, suggesting an essential role of ROS balance in osteogenic differentiation. Chen *et al.*⁶ found that antioxidant enzymes were concurrently upregulated to prevent the accumulation of intracellular ROS during osteogenic differentiation of hMSCs. What’s more, antioxidants can reduce oxidative DNA damage and ROS levels in MSCs and increases the MSC yield which can rescue age-related bone loss.^{30–31} Our study here pointed out that mechanical strain induced much more ROS in adult MSCs, which is at least one of the main causes of the deficient osteogenic differentiation potential of adult MSCs as ROS blocker NAC partially rescues the defect. However, how excessive ROS suppress the osteogenic differentiation is still largely unknown. It might be due to that excessive ROS induced cell damage¹² which in turn arrested the cell differentiation process. Genome-wide analysis of the gene expression changes under cyclic stretch might shed light on how excessive ROS impaired the MSCs osteogenic differentiation. Our study also revealed that SOD1, one of the key antioxidant enzymes is greatly reduced in adult MSCs, which should be responsible for the increased ROS under mechanical stretch.

In conclusion, our findings provide important information on the role of mechanical stretch on the MSCs from rats with different ages. We have also provided new data regarding the mechanisms how MSCs from adult rats displayed deficient osteogenic differentiation. These results emphasize the robust antioxidant ability in the MSCs is essential for the stem cell ability, especially their differentiation potential. Our study suggests that *in vivo* or *in vitro* modulation of the antioxidant capacity of the elderly MSCs would at least help the bone regeneration, which is of clinical importance both in age-related osteoporosis and the distraction osteogenesis based therapies in adult rats.

Competing interests

The authors declare no conflict of interest.

Acknowledgements

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