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Comparison between the effects of normoxia and hypoxia on antioxidant enzymes and glutathione redox state in *ex vivo* culture of $CD34^+$ cells

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ABSTRACT

Hypoxia maintained biological characteristics of $CD34^+$ cells through keeping lower intracellular reactive oxygen specials (ROS) levels. The effects of normoxia and hypoxia on antioxidant enzymes and glutathione redox state were compared in this study. Hypoxia decreased the mRNA expression of both catalase (CAT) and glutathione peroxidase (GPX), but not affected mRNAs expression of superoxide dismutase (SOD). While the cellular GPX activities under hypoxia were apparently less than those under normoxia, neither SOD activities nor CAT activities were affected by hypoxia. The analysis of glutathione redox status and ROS products showed the lower oxidized glutathione (GSSG) levels, the higher reduced glutathione (GSH) levels, the higher GSH/GSSG ratios, and the less $O_2{\cdot}-$ and H_2O_2 generation under hypoxia (versus normoxia). Meanwhile more primary $CD34^+CD38^-$ cells were obtained when cultivation was performed under hypoxia or with N-acetyl cysteine (the precursor of GSH) under normoxia. These results demonstrated the different responses of anti-oxidative mechanism between normoxia and hypoxia. Additionally, the present study suggested that the GSH–GPX antioxidant system played an important role in HSPCs preservation by reducing peroxidation.

1. Introduction

The fate of hematopoietic stem and progenitor cells (HSPCs) is regulated by the microenvironment of the so-called "stem cells niches" (Spradling et al., 2001; Wilson and Trumpp, 2006). It is known that the oxygen saturation is around 5% in bone marrow microenvironment, where HSPCs could be localized (Harrison et al., 2002). Hypoxia has been shown to be an important parameter to preserve the progenitor characteristics of $CD34^+$ cells in vitro (Koller et al., 1992; Ivanovic et al., 2002). Recently, it has been proved that the regulation of the cd34 gene by hypoxia resulted in a delayed higher and longer antigen expression (Brunet et al., 2006). Although the influence of hypoxia on HSPCs expansion has been well studied, the anti-oxidative mechanism responded to oxygen tension has been rarely investigated.

Reactive oxygen species (ROS), such as superoxide anion radical ($O_2{\cdot}-$), hydrogen peroxide (H_2O_2) and highly reactive hydroxyl radical ($\cdot OH$) can damage cellular macromolecules, such as nucleic acids, membrane lipids, and proteins (Livingstone et al., 1993; Nordberg and Arnér, 2001; Shi et al., 2005). About 0.1–0.2% of the O_2 consumed by aerobic cells may form ROS during normal cell respiration (Fridovich, 2004). Oxidative stress occurs when radical production exceeds the cellular antioxidant capacity (Kohen and Nyska, 2002; Scandalios, 2005). It is well known that oxygen pressure (pO_2) is a critical culture parameter which can cause oxidative stress. In the presence of high pO_2, the formation of ROS is favored (Ross et al., 2001). Hypoxia is known to limit the excess production of ROS as well as reduce the oxidative damage to animal cells (Lopez-Barneo et al., 2001; Liu et al., 2004). Fan et al. (2007) demonstrated that hypoxia effectively maintained biological characteristics of $CD34^+$ cells through keeping lower intracellular ROS levels by regulating NADPH oxidase. It has been reported that hematopoietic stem cells (HSCs) expressed the phagocytic NOX2 isoform and produced constitutively ROS (Piccoli et al., 2005, 2007). In order to protect against oxidative stress, cells have developed antioxidant enzymes and nonenzymatic antioxidants to eliminate ROS (Demple, 1999). Associated with their isoforms, SOD, CAT and GPX, are easily induced by oxidative stress (Hermes-Lima and Zenteno-Savín, 2002). The SOD decomposes superoxide radicals ($O_2{\cdot}-$) and produce H_2O_2. H_2O_2 is subsequently removed to water by CAT in the peroxisomes, or by GPX oxidizing GSH in the cytosol (Dröge, 2002; Lee and Choi, 2003). The activity levels of these enzymes have been used to quantify oxidative stress in cells. Glutathione is a tripeptide (glutamate, cysteine and glycine), and commonly distributed in all eukaryotic cells (Meister, 1992). As intracellular non-enzymatic anti-oxidant, GSH status is critical to resistance of oxidative stress in cells and organisms (Lushchak and Bagnyukova, 2006; Biswas et al., 2006). Many reports suggested that the GSH/GSSG ratio might provide the indicator of oxidative stress (Avanzo et al., 2001; Kaneko et al., 2001).

The DNA-array analysis in our previous study showed that some anti-oxidation genes, such as gpx1 and gsst1, were up-regulated in

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cultured CD34$^+$ HSPCs compared to fresh ones (Li et al., 2006), which indicated the cellular oxidative stress in response to *ex vivo* environment. The present work further demonstrated that the cellular peroxide status in hypoxia were lower than that in normoxia, and detected the efficient effect of GSH–GPX antioxidant mechanisms on resisting peroxide status in HSPCs *ex vivo* cultures. Studying the response mechanism of intercellular redox state to hypoxia was essential to enrich our understanding of the effect of hypoxia on HSPCs. Moreover, this study was instructive to regulate cellular redox state for obtaining more functional HSPCs.

2. Materials and methods

2.1. Cell separation procedures

Cord blood was obtained from healthy lying-in woman. Light-density mononuclear cells (MNCs) were separated using Ficoll–Histopaque density gradient, and CD34$^+$ cells from fresh or cultured MNC were isolated with Mini MACS paramagnetic column as previously described (Li et al., 2006). The purity of isolated CD34$^+$ cells assessed by FACS was greater than 95%.

2.2. Cell culture

The environmental atmosphere for CD34$^+$ cell cultivation was 5% CO_2, 5% (for normoxic cultures) or 21% oxygen (for hypoxic cultures), and saturated with N_2. The culture medium equilibrated with the environmental atmosphere was IMDM medium (GIBCOBRL) containing 20% (v/v) fetal bovine serum (FBS) (Hyclone) and 50 U/mL gentamycin sulfate. Cytokines (PeproTech) were prepared by mixing the following ingredients: 50 ng/mL SCF, 5 ng/mL IL-3 and 10 ng/mL IL-6. The starting concentration was at 1×10^5 10^5 cells/mL. Cytokines with/without NAC (Sigma) were added to the medium as specified somewhere. Cells were cultured at 37 °C.

2.3. Flow cytometric analysis

Fresh and cultured CD34$^+$ cells were enriched by FACS. O_2.− was detected with dihydroethidium (DHE) (Beyotime, China), and H_2O_2 with 2,7-dichlorodihydrofluorescein diacetate (H2DCF-DA) (Beyotime) as previously described (Tanaka et al., 2002; Fan et al., 2007). Cell samples were analyzed for expression of CD34 and CD38 using immunofluorescence. In brief, after washed with PBS, the cells were re-suspended in 50 μL PBS, and then incubated with 20 μL phycoerythrin (PE)-CD34 and 20 μL fluorescein isothiocyanate (FITC)-CD38 monoclonal antibodies (Beckman–Coulter) at 4 °C for 30 min. The samples were washed with PBS, and detected by FACS (Becton–Dickinson).

2.4. Reverse transcription-polymerase chain reaction

Total RNAs isolated by Trizol reagent (Invitrogen) were reverse transcripted to total cDNAs with oligo-dT. Total cDNAs were used as the templates for PCR assay. To ensure the amounts of total cDNAs were identical, the samples were first assayed by PCR with primers 5′-GTCTTCACCACCATGGAGAAGCT-3′ and 5′-CATGCCAGTGAGCTTCCCGTTCA-3′ specific for GAPDH, a house keeping gene. PCR primers and conditions for SOD1, SOD2, SOD3, CAT, GPX1 and GPX4 were same as described (Piccoli et al., 2007). After electrophoresis on 1% agarose gel, PCR products were visualized by ethidium bromide staining and quantified by image analysis.

2.5. SOD, CAT and GPX activities assay

The cells were harvested with anti-CD34 linked paramagnetic resin and re-suspended in 75% normal saline, and then were lysed using supersonic wave (power: 200 W). The supernatant liquid was obtained for determinations after centrifugation of the whole-homogenized lysate at 4 °C for 20 min at 3000 *g*. SOD, CAT and GPX were detected using spectrophotometric methods with available kits (Jiancheng Bioengineering Institute, China).

2.6. GSH and GSSG measurements

The determination of GSH and GSSG were performed using DTNB method with kits (Beyotime).

2.7. Statistical analysis

All data were expressed as mean values±S.D. for $n\geq3$. Student's *t*-test was used for comparison of pairs, or ANOVA was used for different groups followed by Student–Newman–Keuls test for determination of significant differences ($P<0.05$). Statistical analysis was performed with SPSS 13.0 software.

3. Results

3.1. mRNA expressions of antioxidant enzymes

The mRNA levels of antioxidant enzymes under normoxic and hypoxic conditions were detected and compared. Fig. 1A showed that CD34$^+$ cell samples expressed CAT, the mitochondrial and cytosolic

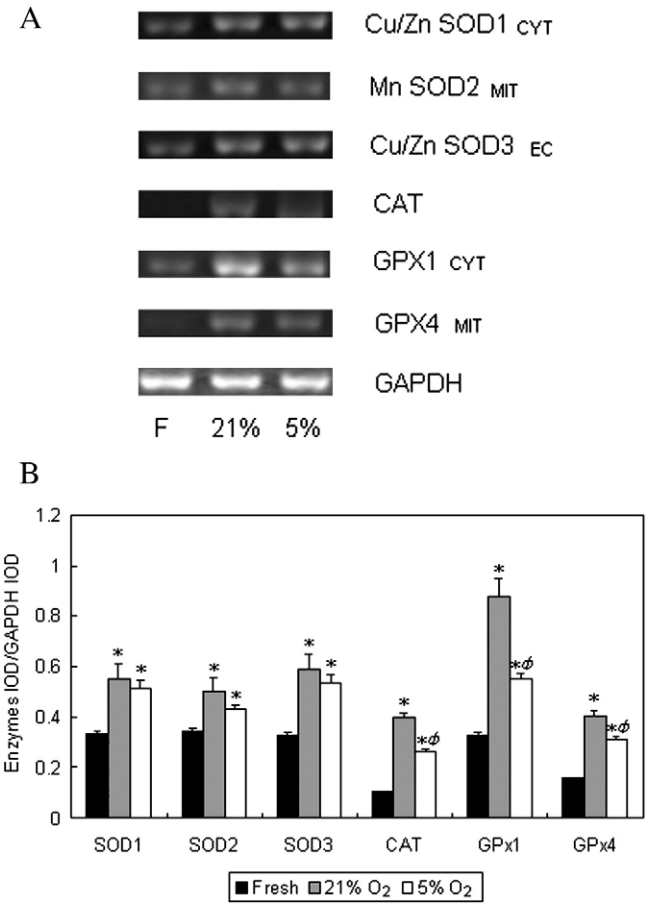

Fig. 1. (A) Representative electrophoresis images of RT-PCT products for antioxidant enzymes. Effect of oxygen tension on mRNA expression of SOD isoforms (SOD 1–3), CAT and GPX isoforms (GPX1 and GPX4) was tested after 7 days cultures. (B) Densitometric analysis of antioxidant enzymes were normalized to GAPDH (the integrated optical density (IOD) ratio of antioxidant enzymes and GAPDH). * Indicates significant difference from the fresh group. Φ Indicates the hypoxic group significantly difference from the normoxic group. (Student's *t*-test, $P<0.05$, $n=3$).

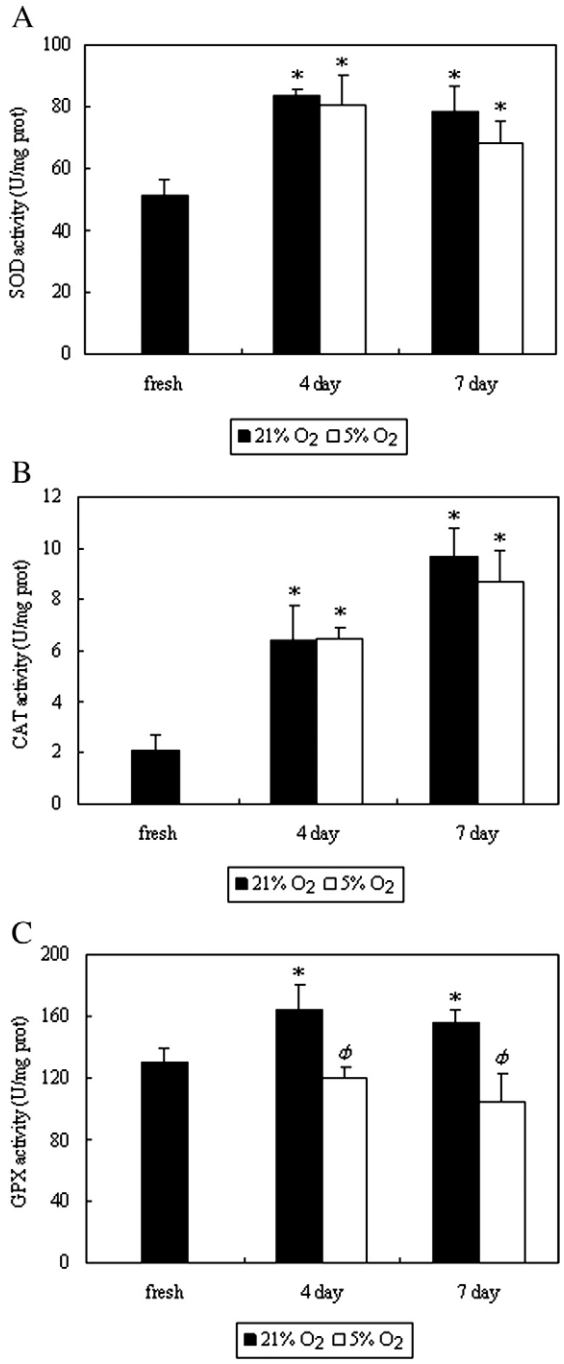

Fig. 2. Effect of oxygen tension on cellular SOD (A), CAT (B) and GPX (C) activities in 7 days cultures. The activities of antioxidant enzymes were expressed per milligram of soluble protein. * Indicates significantly difference from the fresh group. Φ Indicates the hypoxic group significantly difference from the normoxic group. (Student's *t*-test, $P<0.05$, $n=3$).

isoforms of GPX and SOD together with the extracellular SOD3 isoform. mRNAs of antioxidant enzymes were affected by oxygen tension. Apparently, more mRNAs of either CAT or GPX was observed at 21% pO_2 than those at 5% pO_2. However, the effect of hypoxia on mRNA levels of SOD was not significant (Fig. 1B). These results demonstrated that rather than O_2-scavenger SOD, the gene expression of H_2O_2 scavenger CAT and GPX were easier increased by normoxia.

3.2. Antioxidant enzyme activities

To further demonstrate the of enzymatic activity level, the SOD, CAT and GPX activities were measured. As it shown in Fig. 2A, B, SOD activities and CAT activities was continuously increased during 7 days cultures, but neither of them was not affected by oxygen tension. The most marked difference between normoxic group and hypoxic group was seen in GPX activity (Fig. 2C). Normoxic cultures increased GPX activity, however hypoxia exposure kept the GPX activity value near or even below the fresh level. The results provided the evidence that antioxidant enzyme activities were stimulated in $CD34^+$ cells *ex vivo* cultures. Higher activity of GPX was detected when cultivation under normoxia, and it was identified to mRNA assay.

3.3. Glutathione redox status

The present study indicated that GPX played an important role in cellular anti-oxidative system. Furthermore, GSH, GSSG and GSH/GSSG ratio were measured to understand the glutathione redox status changes in $CD34^+$ cells *ex vivo* cultures. NAC was used to increase GSH content under normoxia. As shown in Fig. 3, after cells were cultured in the both oxygen patterns, the decrement of GSH content and the

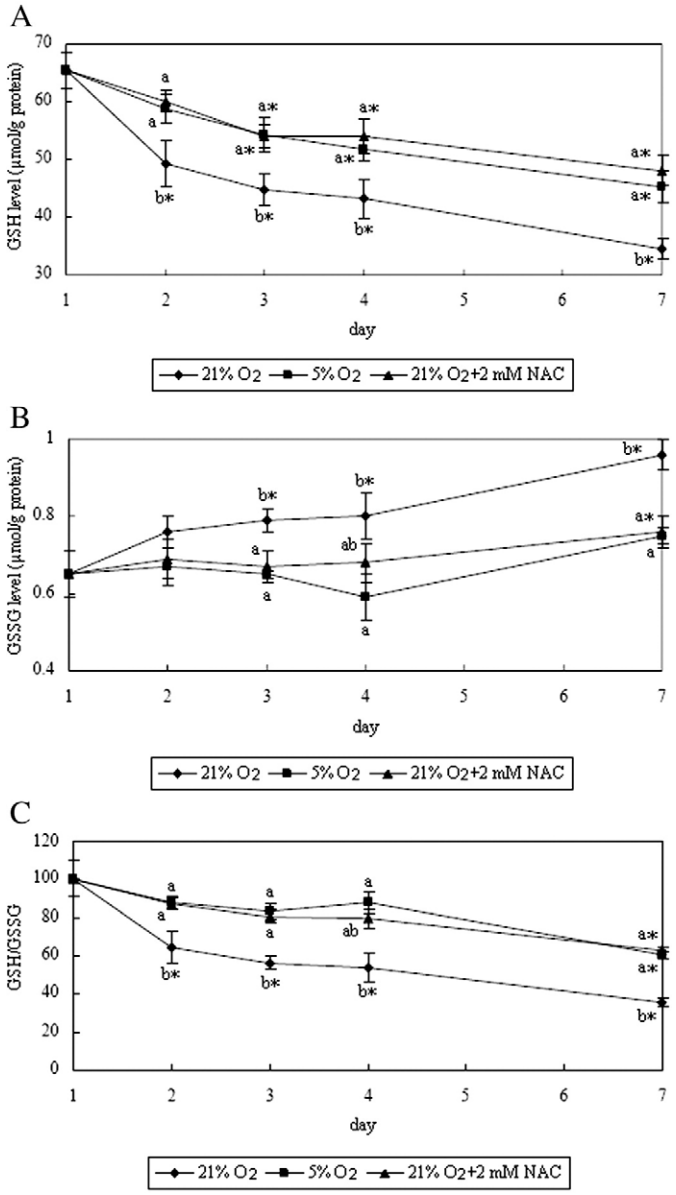

Fig. 3. Changes of the glutathione redox status in 7 days cultures. (A) GSH level, (B) GSSG level and (C) GSH/GSSG ratio. * Indicates significantly difference from the fresh group. (Student's *t*-test, $P<0.05$, $n=3$). Where letters differ (a, b), significant differences among normoxic group, hypoxic group and NAC group within the same day. (*N–K* post hoc test, $P<0.05$, $n=3$).

increment GSSG content were observed, while GSH/GSSG ratios tended to decline progressively. These findings suggested an oxidized cellular environment in *ex vivo* cultures. Nevertheless, hypoxic condition or additional NAC displayed a significant increase in GSH levels and GSH/GSSG ratios, whereas GSSG levels were reduced. This effect showed that the peroxide status of hypoxic cells were lower than normoxic cells. NAC was a stable precursor of GSH, so it provided the evidence that cellular redox status could be regulated by glutathione system.

3.4. $O_2^{\cdot-}$ and H_2O_2 generation

The $O_2^{\cdot-}$ and H_2O_2 generation of $CD34^+$ cells under different oxygen tension or with NAC were detected at day 7 and compared in

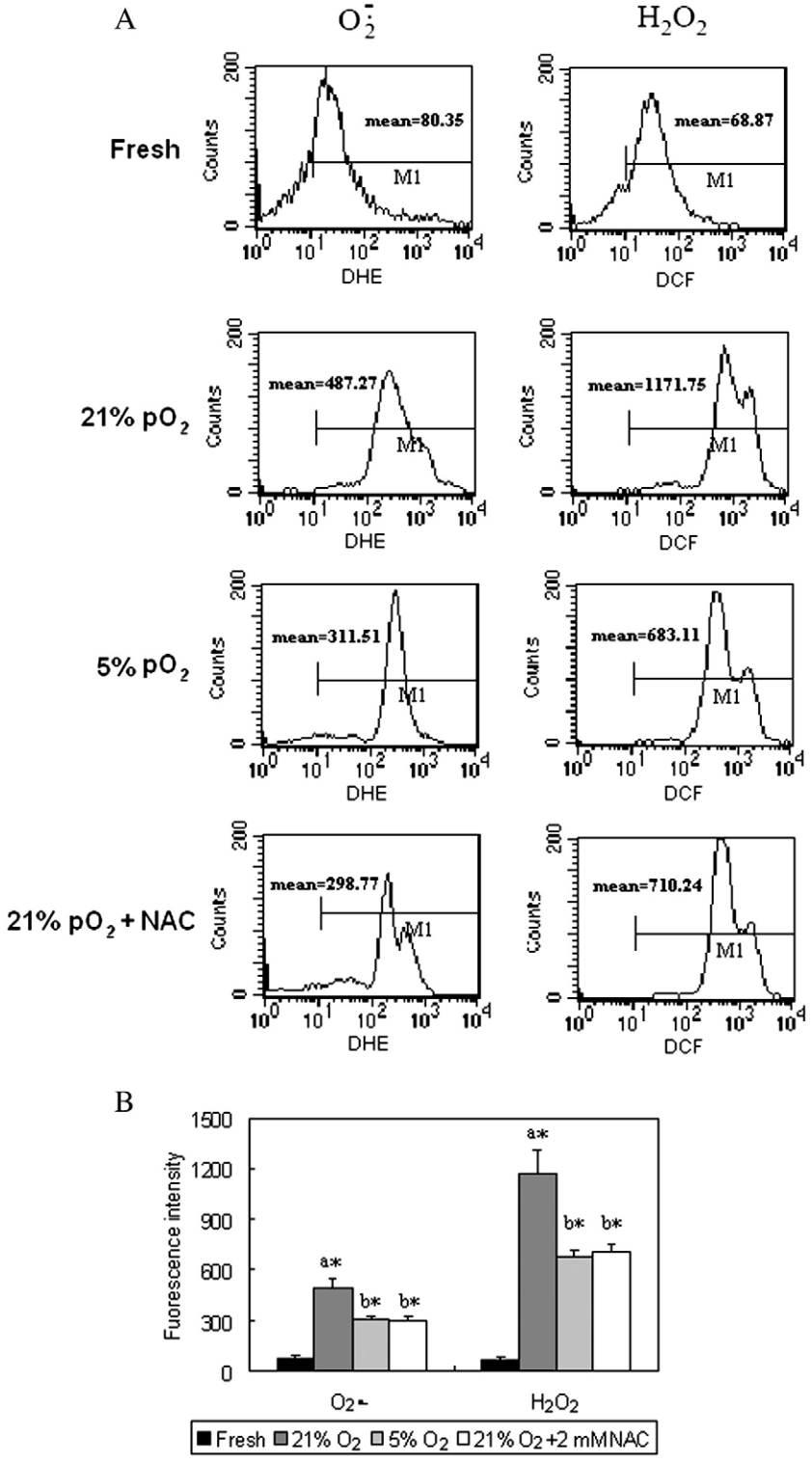

Fig. 4. (A) Representative flow cytometric images for $O_2^{\cdot-}$ and H_2O_2 in $CD34^+$ cells. Effects of hypoxia or NAC on $O_2^{\cdot-}$ and H_2O_2 generation of $CD34^+$ cells were tested by FACS at day 7. (B) Fluorescence intensity analysis. * Indicates significantly difference from the fresh group. (Student's *t*-test, $P<0.05$, $n=4$). Where letters differ (a, b), significant differences among normoxic group, hypoxic group and NAC group at day 7. (*N–K* post hoc test, $P<0.05$, $n=4$).

Table 1
Effects of 5% pO_2 and NAC on the expansion characteristics of HSPCs

Treatment		Fold expansion of the cells		Percentage of the subsets (%)	
		$CD34^+$	$CD34^+CD38^-$	$CD34^+$	$CD34^+CD38^-$
Freshly prepared HSPCs		1	1	96.3±2.98	81.0±2.50
Cultured cells	21% pO_2	7.34±0.96*	$0.70\pm0.03^{a,*}$	$41.2\pm3.31^{a,*}$	$3.61\pm0.83^{a,*}$
	5% pO_2	8.01±0.95*	$1.06\pm0.04^{b,*}$	$57.3\pm0.83^{b,*}$	$7.26\pm0.59^{b,*}$
	21% pO_2 + 2 mM NAC	8.10±1.69*	$1.00\pm0.06^{b,*}$	$58.8\pm3.20^{b,*}$	$7.01\pm0.75^{b,*}$

Subsets of cultured cells were analyzed at day 7. *Indicates significant difference from the fresh group. (Student's *t*-test, $P<0.05$, $n=4$). Where letters differ (a, b), significant differences among normoxic group, hypoxic group and NAC group at day 7. (*N–K* post hoc test, $P<0.05$, $n=4$).

Fig. 4. When cells were cultured in the medium with SCF/IL-3/IL-6, both $O_2\cdot-$ and H_2O_2 generation were observed, which were greatly affected by oxygen tension. The more oxygen, the more $O_2\cdot-$ and H_2O_2 were generated. However, the increased ratio of H_2O_2 generation was higher than that of $O_2\cdot-$ generation under both hypoxic and normoxic conditions, especially under the latter. Furthermore, it was also noted that when medium contained 2 mM NAC, the levels of $O_2\cdot-$ and H_2O_2 were close to the levels under hypoxia. These results provided the evidence that the intercellular ROS of cultured HSPCs can be regulated simply by NAC.

3.5. HSPCs preservation

The present study indicated the positive effects of GSH–GPX antioxidant system on reducing peroxidation under normoxia. To determine effects of 5% pO_2 and NAC on the expansion characteristics of $CD34^+$ cells, $CD34^+$ cells were cultured in three different conditions (with or without NAC at 21% pO_2, or without NAC at 5% pO_2), and the cells were counted and harvested for flow cytometric analysis at day 7. As shown in Table 1, the $CD34^+$ cell numbers at 5% pO_2 or with NAC were close to those at 21% pO_2, but the $CD34^+CD38^-$ cells at 5% pO_2 or with NAC were more than those at 21% pO_2, suggesting that more primary HSPCs were obtained. In $CD34^+$ cells *ex vivo* cultures, the percentage of $CD34^+$ subset and $CD34^+CD38^-$ subset were decreased with HSPCs maturation. If glutathione redox related with progenitor cells preservation, adding NAC could exert the same positive effects as hypoxia. When the cells were cultured at 5% pO_2 or with NAC, the percentages of $CD34^+$ subsets and $CD34^+CD38^-$ subsets were increased significantly compared to those at 21% pO_2, which reflected that HSPCs subsets could be preserved through increasing GSH content under normoxia.

4. Discussion

It has been well established that oxygen tension could regulate HSPCs expansion. Nevertheless, the effect of oxygen tension on cellular redox-sensitive molecular in HSPCs *ex vivo* cultures has been revealed yet. In this work, we linked oxygen tension to altered mRNA expression of SOD, CAT and GPX. Compared to hypoxia, normoxia resulted in more $O_2\cdot-$ generation that are not reflected by the transcription of SOD. So the suitability of SOD mRNA expression as a biomarker of normoxia-induced oxidative stress in cultured $CD34^+$ cells may be limited. However the mRNA levels of CAT and GPX were higher in normoxia exposure cells compared to those in hypoxia exposure cells. As higher oxygen concentration resulted in more H_2O_2 generation in the present study, it was implied that the increase of H_2O_2 levels could interact with more efficiently transcription of CAT and GPX, which suggested that ROS may serve as subcellular messengers in redox-sensitive gene regulatory.

The analysis of antioxidant enzyme activities showed that either SOD, CAT or GPX activity was the useful biomarker for oxidative stress in response to *ex vivo* cultures environment. The increment of antioxidant enzyme activities indicated that cellular anti-oxidative system was triggered to resist oxidative damage. Furthermore, unlike the SOD or CAT activity, which was not affected by oxygen tension markedly, the GPX activity was oxygen-sensitive.Nakata et al. (2004) demonstrated that NAC and thioredoxin X (TRX) but not Mn(III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP) efficiently cancelled IκBSR-enhanced apoptosis, which suggested that H_2O_2 were more toxic to normal hematopoietic stem/ progenitors cells than $O_2\cdot-$. Meanwhile new evidences proved that hematopoietic progenitors multiplied and became quiescent in mouse bone marrow culture using CAT to eliminate H_2O_2 (Gupta et al., 2006). SOD removed superoxide radicals and produced hydrogen peroxide. To eliminate H_2O_2, there was cooperation effect between CAT and GPX: the enzyme-generating H_2O_2 was generally eliminated by CAT in the peroxisomes, while H_2O_2 generated in the mitochondria or the cytosol was usually eliminated by GPX (Blokhina et al., 2003). GSH–GPX antioxidant system has the capacity to protect the integrity of unsaturated bonds of membrane phospholipids by eliminating ROS (Meister and Anderson, 1983; Deleve and Kaplowitz, 1991). Our data showed that compared to CAT activity, GPX activity was easier activated under normoxia. The results implied the different response of intracellular anti-oxidative systems to H_2O_2 regulated by oxygen tension, which may be due to H_2O_2 formation and localization in $CD34^+$ cells (Scandalios, 2005).

For more insight evidences in changes of redox state in $CD34^+$ cells expansion procedures, glutathione redox status was analyzed. *Ex vivo* cultures caused a loss of GSH, a rise in GSSG level and a decline in GSH/GSSG ratio in $CD34^+$ cells. GSH is the most abundant non-protein thiol to detoxification of ROS (Sies, 1999). The GSH/GSSG decrease indicated the cellular peroxide status in vitro environment. Moreover, compared to normoxia, hypoxia exposure displayed a relatively lower degree of oxidative stress, as reflected by the GSH, GSSG content and GSH/GSSG ratio. Traditionally, NAC is supposed to increase the pool of reduced GSH when intercellular oxidative stress occurred. The effects of additional NAC under normoxia on the glutathione redox status and the HSPCs subsets preservation were similar to hypoxia, which further confirmed that as mitochondrial and cytosolic H_2O_2 scavengers, glutathione redox system was important in the resistance to induced oxidative stress under high oxygen tension.

In conclusion, our findings provided the evidence that intracellular GPX and GSH redox system were regulated by oxygen tension, which may be a normoxic oxidative stress mechanism in $CD34^+$ cells *ex vivo* cultures. A further understanding of the cellular redox state to *ex vivo* culture environment will aid in designing new strategies to reduce the oxidative damage in HSPCs *ex vivo* cultures.

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