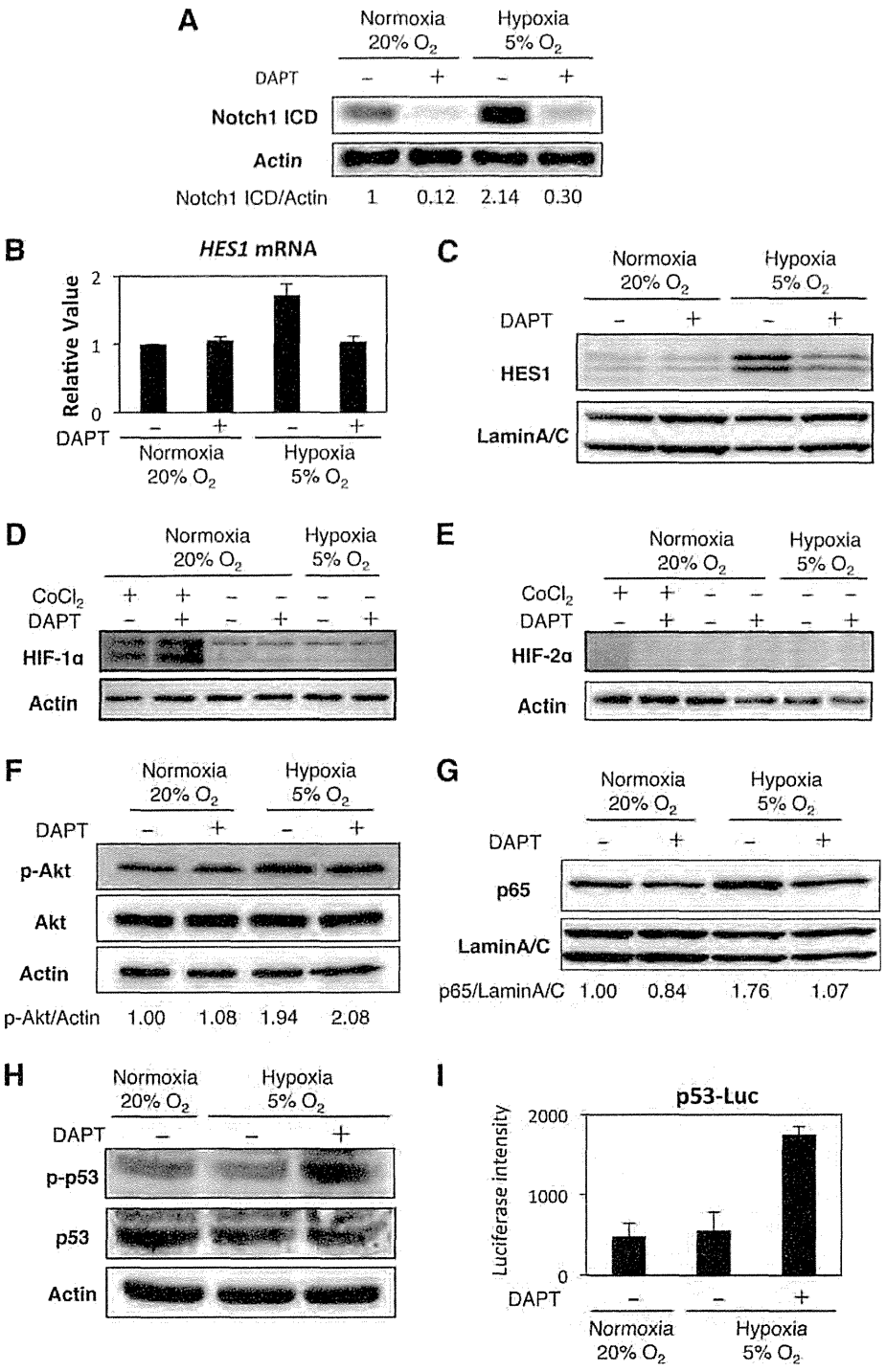


**FIG. 4.** Hypoxic culture condition activates Notch signaling but not HIF proteins. hADMPCs were expanded under normoxic (20% $O_2$) and hypoxic (5% $O_2$) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Western blot analysis of intracellular domain of Notch1 (Notch1 ICD) expression. Actin served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(B)** Q-PCR analysis of *HES1*. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(C)** Western blot analysis of HES1 in nuclear fractions of hADMPCs. Lamin A/C served as the loading control. **(D, E)** Western blot analysis of HIF-1α **(D)** and HIF-2α **(E)**. Cobalt chloride ($CoCl_2$) was added at a concentration of 100 μM to stabilize HIF proteins (positive control). **(F)** Western blot analysis of phosphorylated Akt (p-Akt) and Akt. Actin served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(G)** Western blot analysis of nuclear localization of p65. Lamin A/C served as the loading control. Numbers below blots indicate relative band intensities as determined by ImageJ software. **(H)** Western blot analysis of phosphorylated p53 (p-p53) and p53. Actin served as the loading control. **(I)** Activity of p53 was measured by the p53-luciferase reporter assay. Relative luciferase activity was determined from three independent experiments and normalized to pGL4.74 activity.

in the Hx culture condition. To inhibit Notch signaling, DAPT was added to the medium at a final concentration of 1 μM. DAPT treatment significantly decreased the PDL when hADMPCs were cultured under either 20% or 5% oxygen (Fig. 5A). Intriguingly, measurement of the DNA content in hADMPCs revealed that inhibition of Notch signaling by 1 μM DAPT significantly attenuated the decrease in apoptotic cells in the Hx condition (Fig. 5B). These data suggest that 5% oxygen increases the proliferation capacity of hADMPCs through Notch signaling by promoting their survival. To examine whether Notch signaling affects the stem cell properties of hADMPCs, differentiation into adipocyte, osteocyte, and chondrocyte lineages was analyzed at passage 8. Hx-cultured hADMPCs underwent greater differentiation into all lineages as described in Fig. 3, whereas application of a Notch inhibitor significantly decreased the differentiation capacity to all lineages (Fig. 5C–E). In addition, SA-β-Gal staining revealed that inhibition of Notch signaling by DAPT remarkably promoted senescence in both the Nx and Hx

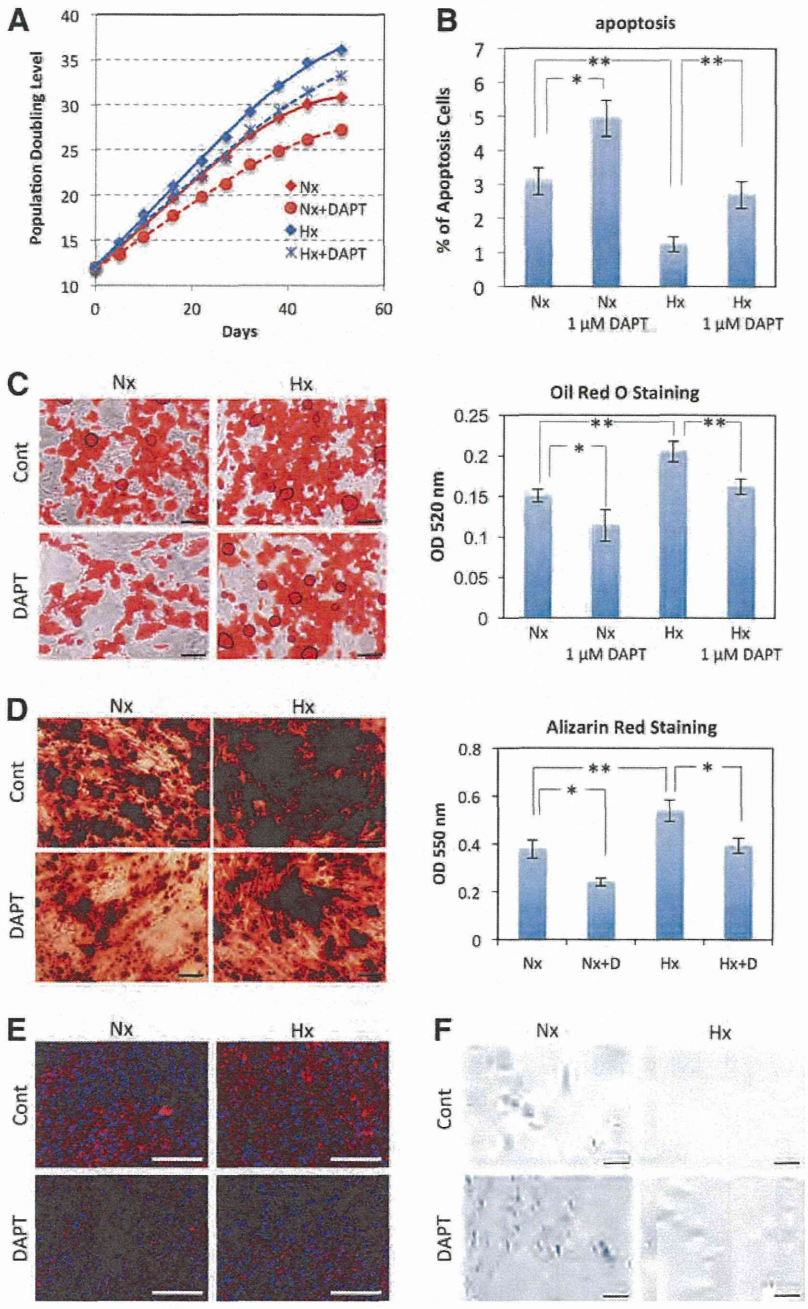


**FIG. 5.** Notch signaling is indispensable for acquisition of the advantageous properties of hADMPCs. hADMPCs were expanded under normoxic (20% $O_2$; Nx) and hypoxic (5% $O_2$; Hx) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Growth profiles of hADMPCs under Nx (*red*) and Hx (*blue*) conditions. *Solid lines* represent control cells, and *dotted lines* represent DAPT-treated cells. The number of population doublings was calculated based on the total cell number at each passage. **(B)** Percentages of apoptotic cells with sub-G1 DNA. Results are presented as the mean of three independent experiments ± SD. **(C, D)** hADMPCs at passage 8 were induced for 3 weeks to differentiate into adipocytes **(C)** and osteoblasts **(D)** and stained with Oil Red O and Alizarin Red, respectively. The stained dye was extracted, and OD values were measured and plotted as the means of three independent experiments ± SD. **(E)** hADMPCs at passage 8 were induced for 3 weeks to differentiate into chondrocytes, and an immunofluorescent analysis of collagen II (*red*) was performed. The *blue* signals indicate nuclear staining. **(F)** hADMPCs were stained with SA-β-gal. $*P<0.05$ and $**P<0.01$ indicate significant differences (independent *t*-test) between Nx and Hx. Scale bars; 100 μm. Color images available online at www.liebertpub.com/scd

culture conditions, suggesting that the suppression of replicative senescence observed in the Hx condition is mediated by Notch signaling (Fig. 5F).

### *Glycolysis is enhanced in the 5% oxygen condition through Notch signaling*

Recent studies suggest that the metabolic shift from aerobic mitochondrial respiration to glycolysis extends the life span possibly via reduction of intrinsic ROS production [18,19]. Our results demonstrate that the 5% oxygen condition reduced ROS accumulation in hADMPCs (Fig. 1F). In addition, the relationship between Notch signaling and glycolysis has been recently established [48,49]. We, therefore, considered glycolytic flux by measuring the glucose consumption and lactate production of hADMPCs in the Nx or Hx culture conditions. As shown in Fig. 6A, glucose consumption and lactate production were elevated in the Hx culture condition, indicating that a metabolic shift to glycolysis occurred when hADMPCs were cultured in 5% oxygen. In contrast, the Notch inhibitor DAPT markedly reduced glycolytic flux as assessed by glucose consumption and lactate production (Fig. 6A). To identify the genes responsible for the glycolytic change, we performed a Q-PCR analysis. As shown in Fig. 6B, *SLC2A3, TPI, and PGK1*, encoding glycolytic enzymes, were upregulated in the 5% oxygen condition; whereas these genes were suppressed by DAPT treatment. Interestingly, Hes1 transduction by an adenoviral vector markedly induced the mRNA expression of the same genes (Fig. 6E). In addition, SCO2, a positive

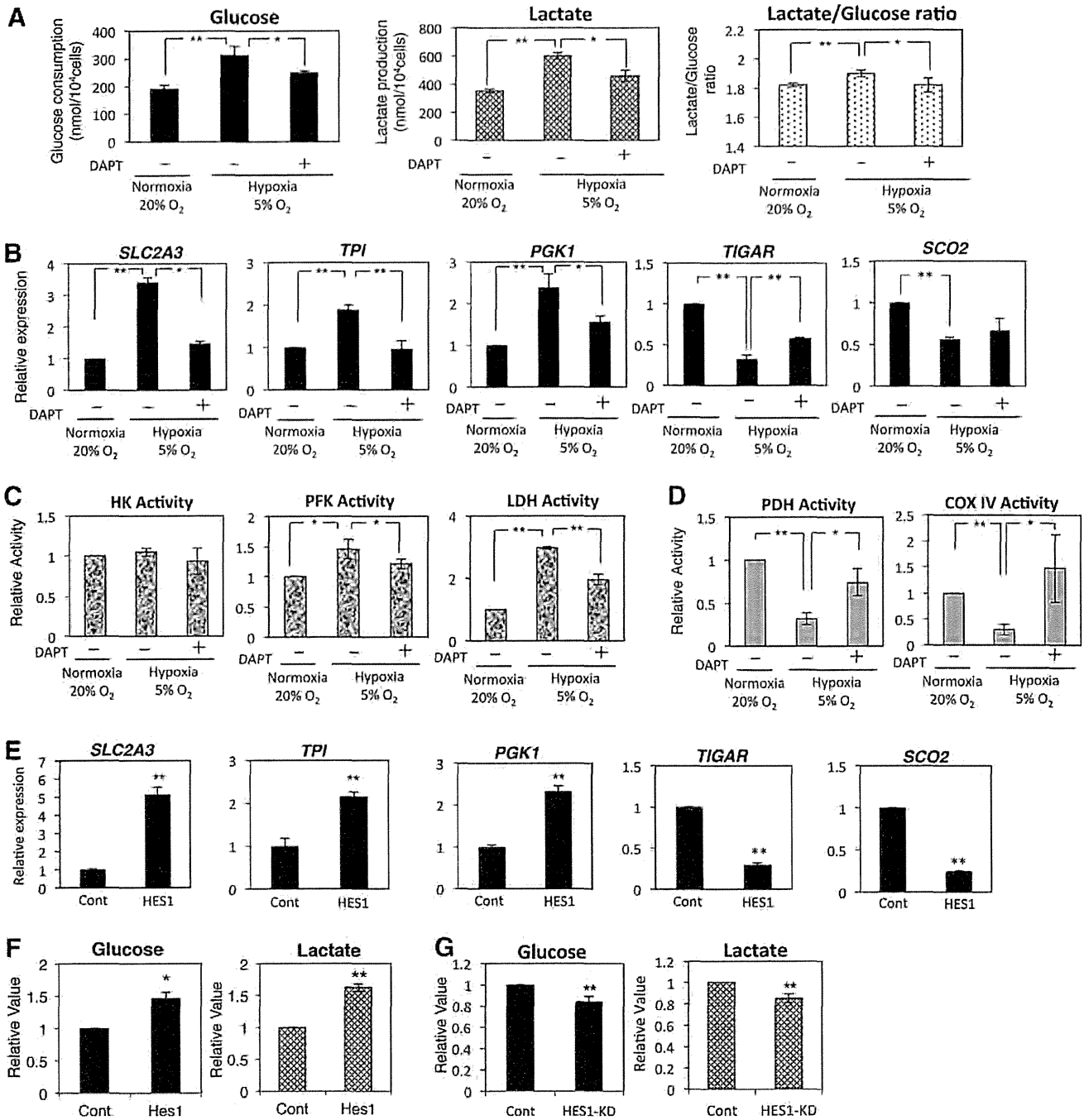


**FIG. 6.** Glycolysis is enhanced under 5% oxygen through Notch signaling. **(A–D)** hADMPCs were expanded under normoxic (20% $O_2$) and hypoxic (5% $O_2$) conditions. DAPT (1 μM) was added to inhibit Notch signaling. **(A)** Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. **(B)** Relative mRNA expression of *SLC2A3*, *TPI*, *PGK1*, *TIGAR*, and *SCO2* in hADMPCs. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(C, D)** Hexokinase (HK), phosphofructokinase (PFK), lactate dehydrogenase (LDH) **(C)**, pyruvate dehydrogenase (PDH), and Complex IV (Cox IV) **(D)** activities were measured and the value of relative activity was plotted as the means of three independent experiments ± SD. **(E, F)** hADMPCs were transduced with either mock (Cont) or HES1 and then cultured for 3 days. **(E)** Relative mRNA expression of *SLC2A3*, *TPI*, *PGK1*, *TIGAR*, and *SCO2* in hADMPCs. Each expression value was calculated with the ΔΔCt method using *UBE2D2* as an internal control. **(F)** Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. **(G)** hADMPCs were transduced with either scrambled control RNAi (Cont) or RNAi against HES1 (HES1-KD), and then cultured for 3 days. Glucose consumption and lactate production of hADMPCs were measured and plotted as the means of three independent experiments ± SD. $^{**}P < 0.01$. $^{*}0.01 < P < 0.05$.

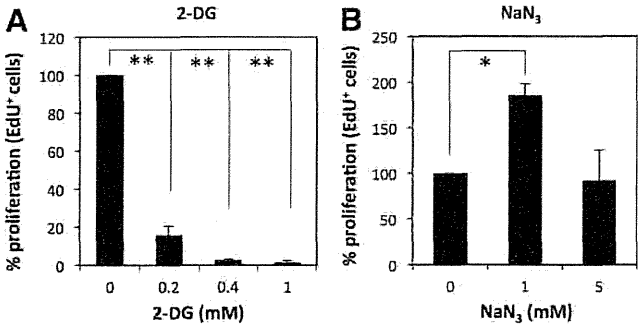


**FIG. 7.** Glycolysis supports proliferation of hADMPCs. hADMPCs were treated with 0, 0.2, 0.4, and 1 mM 2-deoxy-D-glucose (2-DG) (**A**) or 0, 1, and 5 mM sodium azide ($NaN_3$) (**B**) for 24 h. Cells were then allowed to incorporate EdU for 2 h, and the EdU-positive cells were analyzed by flow cytometry. The percentages for the 0 mM control were plotted as the means of three independent experiments ± SD. $*P < 0.05$; $**P < 0.01$.

modulator of aerobic respiration, and TIGAR, a negative regulator of glycolysis, were transcriptionally downregulated in the 5% oxygen condition; whereas DAPT treatment partially restored the expression level (Fig. 6B). Adenoviral expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Fig. 6E), which suggests that the Notch-Hes1 signaling modulates the metabolic pathway. We also measured the activities of key enzymes in glycolysis. Hexokinase activity was not changed under hypoxic conditions; however, PFK and LDH were activated in 5% oxygen condition, which was attenuated by Notch inhibition (Fig. 6C). In addition, pyruvate dehydrogenase (PDH) and cytochrome c oxidase (Complex IV) activity assays showed that mitochondrial respiration decreased under the hypoxic condition and that DAPT treatment restored it (Fig. 6D). Moreover, glycolytic flux in Hes1-expressing hADMPCs was positively correlated with the expression of these glycolytic genes as assessed by glucose consumption and lactate production (Fig. 6F). In contrast, HES1 knockdown by adenoviral transduction of *HES1* RNAi resulted in a significant reduction of glycolytic flux (Fig. 6G), demonstrating that HES1 is involved in the regulation of glycolysis.

### *Glycolysis supports the proliferation of hADMPCs*

To determine whether aerobic glycolysis is important for the proliferation of hADMPCs, hADMPCs were treated with the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) or the respiration inhibitor sodium azide ($NaN_3$). We found that hADMPCs were sensitive to treatment with 2-DG even at a low concentration of 0.2 mM (Fig. 7A). In contrast, treatment of hADMPCs with $NaN_3$ rather increased their proliferation at the concentration of 1 mM and supported their proliferation even at the concentration of 5 mM (Fig. 7B). These data suggest that the proliferation of hADMPCs is compromised when aerobic glycolysis is blocked.

## Discussion

Recent evidence suggests that hypoxic culture conditions confer a growth advantage, prevent premature senescence, and maintain undifferentiated states in ESCs, iPSCs, and somatic stem cells. However, the molecular mechanism underlying the beneficial effects of culturing these cells at low oxygen conditions remains unclear. Our findings prompted us to hypothesize that Notch signaling in physiological hypoxic conditions (5% $O_2$) contributes to these effects on hADMPCs by modulating glycolytic flux.

We found that 5% $O_2$ significantly increased the proliferation capacity, decreased apoptosis, and inhibited senescence of hADMPCs (Fig. 1). Moreover, 5% $O_2$ improved the differentiation of hADMPCs without affecting the expression of their cell surface markers (Figs. 2 and 3). Welford et al. reported that HIF-1α delays premature senescence of mouse embryonic fibroblasts under hypoxic conditions (2% $O_2$) [50]. Tsai et al. reported that hypoxia (1% $O_2$) inhibits senescence and maintains MSC properties through accumulation of HIF-1α [26]. Hypoxia was recently reported to enhance the undifferentiated status and stem cell properties in various stem and precursor cell populations via the interaction of HIF with the Notch intracellular domain to activate Notch-responsive promoters [38]. In the current study, the effects observed in 5% $O_2$ condition were independent of HIF proteins, because accumulation of HIF-1α and HIF-2α was not observed (Fig. 4). Instead, our findings suggest that 5% $O_2$ activated Notch signaling, which contributed advantageous effects of hypoxic culture on hADMPCs. A pharmacological inhibitor of Notch signaling, DAPT, abrogated the hypoxic-induced Notch activation, increased proliferation capacity and lifespan, maintenance of stem cell properties, and prevention of senescence (Figs. 4 and 5). Moreover, we also found that 5% $O_2$ enhanced glucose consumption and lactate production, and these effects were also attenuated by Notch inhibition (Fig. 6A) and knockdown of HES1 (Fig. 6G). Previously, it has been reported that Notch signaling promotes glycolysis by activating the PI(3)K-Akt pathway [48,49]. However, our results indicate that Akt signaling was not activated by Notch signaling, because DAPT did not attenuate hypoxia-induced Akt phosphorylation (Fig. 4F). Although Akt is unlikely to be regulated by Notch signaling in hADMPCs, it is obvious in our data that Akt signaling was activated by 5% $O_2$. Therefore, we could not rule out the possibility that the promotion of glycolysis in the 5% $O_2$ condition was caused by Akt signaling.

Recent evidence suggests that Notch signaling acts as a metabolic switch [48,51]. Zhou et al. demonstrated that hairy, a basic helix-loop-helix transcriptional repressor regulated by Notch signaling, was upregulated and genes encoding metabolic enzymes, including TCA cycle enzymes and respiratory chain complexes, were downregulated in hypoxia-tolerant flies. Intriguingly, they also found that hairy-binding elements were present in the regulatory region of the downregulated metabolic genes. Their work, thus, provides new evidence that hairy acts as a metabolic switch [51]. Landor et al. demonstrated that both hyper- and hypoactive Notch signaling induced glycolysis, albeit by different mechanisms. They showed that Notch activation increased glycolysis through activation of PI3K-AKT signaling, whereas decreased Notch activity inhibited mitochondrial function in a p53-dependent manner in MCF7 breast cancer cell lines [48]. Consistent with their reports, our findings that Notch signaling promoted activity of some glycolysis enzymes and inhibited mitochondrial activity

(Fig. 6) also suggest that Notch signaling functioned as a metabolic switch. While our data showed that Notch inhibition by DAPT resulted in reduced glycolysis (Fig. 6A–C), induction of mitochondrial function (Fig. 6D) and activation of p53 (Fig. 4H, I) are not consistent with the report of Landor el al. This contradiction might be explained by the expression level of endogenous Notch. Landor et al. showed that in breast cancer MDA-M-231 cells, which showed higher endogenous Notch activity, high glucose uptake, and lactate production than MCF7 breast cancer cell lines, Notch inhibition by DAPT significantly reduced glucose consumption and lactate production [48]. As shown in Fig. 4A, we observed that hADMPCs in 5% $O_2$ displayed high Notch activity. Moreover, the lactate-to-glucose ratio was 1.8–1.9 in hADMPCs, suggesting that hADMPCs largely rely on glycolysis for energy production (Fig. 6A). In addition, it was reported that hMSCs showed a higher glycolytic rate than primary human fibroblast [52]. It appears that hADMPCs cultured under hypoxic conditions might possess cell properties similar to MDA-M-231 cells or MCF7 cells, in which stable expression of constructs NICD1-GFP produces high Notch activity.

Nuclear translocation of p65 was observed in hypoxic conditions, demonstrating that NF-κB is a direct target of Notch signaling (Fig. 4G). Intriguingly, the hypoxic culture conditions in this study upregulated several genes encoding glycolytic enzymes (*SLC2A3, TPI, and PGK1*); whereas the expression of these genes was suppressed by Notch inhibition. In addition, Hes1 transduction induced mRNA expression of the same genes (Fig. 6). It was previously reported that *SLC2A3* expression was regulated by p65/NF-κB signaling, and that Notch/Hes1 is able to induce the activation of the NF-κB pathway in human T-ALL lines and animal disease models [53]. Espinosa et al. demonstrated that Hes1 directly targeted the deubiquitinase CYLD, resulting in deubiquitination and inactivation of TAK1 and IKK, degradation of IκBa, and activation of NF-κB signaling [53]. In our systems, however, we did not observe repression of *CYLD* mRNA in Hes1-overexpressing hADMPCs (data not shown). While *PGK1* mRNA has been reported to be upregulated by NF-κB, it has not clearly been shown to be controlled by NF-κB despite the presence of an NF-κB site in the promoter [54]. Although modulation of *TPI* expression by NF-κB has not been reported, we found several NF-κB binding sites on the human *TPI* promoter (data not shown). Since NF-κB is likely to be one of the responsible signals for hypoxic-induced glycolysis [53], further analysis will be required to determine the mechanism by which NF-κB signaling is induced by Notch signaling. In addition, it will be important to investigate whether NF-κB is really responsible for the observed glycolysis and whether it regulates the expression of *SLC2A3*, *TPI*, and *PGK1* in hADMPCs under 5% oxygen.

In addition, SCO2, a positive modulator of aerobic respiration, and TIGAR, a negative regulator of glycolysis, were transcriptionally downregulated in the 5% oxygen condition; whereas DAPT treatment partially restored expression (Fig. 6B). We observed some glycolysis and mitochondrial enzyme activity and found that the activities of COX IV and PFK were consistent with gene expression data (Fig. 6C, D). Adenoviral expression of Hes1 dramatically reduced *SCO2* and *TIGAR* expression (Fig. 6E), which suggests that Notch-Hes1 signaling modulates the metabolic pathway. Intriguingly, our results also indicate that Hes1 could suppress the expression of *TIGAR* and *SCO2*, a p53 target gene. It has been reported that Notch signaling suppresses p53 in lymphomagenesis [46]. Moreover, Kim et al. reported that NICD1 inhibits p53 phosphorylation and represses p53 transactivation by interacting with p53 [47]. In addition, DAPT treatment resulted in the enhancement of p53 activity in the hypoxic conditions (Fig. 4H, I). Therefore, it is possible that p53 activation was regulated by Notch signaling in hADMPCs, although we did not observe a decrease in p53 activity in hypoxic conditions in this study (Fig. 4). Further analysis will be required to determine whether p53 activity is suppressed in hypoxic conditions over a longer period of culture.

Cells undergoing active proliferation utilize large amounts of glucose through glycolysis, producing pyruvate for use in substrates (amino acids and lipids) and the pentose shunt for use in nucleic acid substrates, and also producing NADPH as a reducing agent to counter oxidative stress [18,55]. In the current study, 5% $O_2$ actually increased proliferation and decreased the accumulation of ROS, which may be involved in the reduction of senescence (Fig. 1). Since accumulation of endogenous ROS might be a major reason for replicative senescence [20], enhancing glycolysis in cultured cells may improve the quality of the cells by suppressing premature senescence. Kondoh et al. demonstrated that enhanced glycolysis is involved in cellular immortalization through reduction of intrinsic ROS production [14,18,19]. Therefore, it is possible that the extension of lifespan observed in our experimental conditions was caused by the reduction of intracellular ROS levels through enhanced glycolysis by Notch signaling. Our data indicate that aerobic glycolysis is utilized for proliferation of hADMPCs, because the glycolytic inhibitor 2-DG attenuates the proliferation rate of hADMPCs (Fig. 7A). Intriguingly, the aerobic respiration block by $NaN_3$ did not decrease the proliferation; rather, it increased proliferation at a low concentration (Fig. 7B), which may support our data indicating that the metabolic switch from mitochondrial respiration to glycolysis provides a growth advantage to hADMPCs. However, the question of whether the enhanced glycolysis really contributes to the prolonged lifespan in hADMPCs remains to be determined in this study.

In the current study, the molecular mechanism for how Notch signaling is activated in 5% $O_2$ conditions was explored. It has been reported that Notch1 activity is influenced by oxygen concentration [40,41,56]. In melanoma cells, hypoxia (2% $O_2$) resulted in increased expression of Notch1 by HIF-1α and also by Akt through NF-κB activity [41]. Similarly, in hypoxic breast cancer cells, Notch ligand JAG2 was shown to be transcriptionally activated by hypoxia (1% $O_2$) in an HIF-1α-dependent manner, resulting in an elevation of Notch signaling [40]. In contrast, in hESCs continuously cultured in 5% $O_2$, alteration of the Notch pathway seems to be independent of HIF-1α [56]. In our system, Notch1 activation was not likely dependent on HIF-1α and HIF-2α, because these proteins did not accumulate in the Hx condition. In contrast, our results indicate that the 5% $O_2$ condition activated Akt and NF-κB signaling (Fig. 4), which suggests that these molecules may activate Notch signaling in hADMPCs. NF-κB was previously shown to

increase Notch1 activity indirectly by increasing the expression of Notch ligand Jagged1 in HeLa, lymphoma, and myeloma cells [57]. In addition, Akt regulated Notch1 by increasing Notch1 transcription through the activity of NF-κB in melanoma cells [41]. Further analysis is required to clarify the mechanism underlying this phenomenon.

In conclusion, the 5% oxygen condition conferred a growth advantage through a metabolic shift to glycolysis, improved the proliferation efficiency, prevented the cellular senescence, and maintained the undifferentiated status of hADMPCs. These observations, thus, provide new regulatory mechanisms for the maintenance of stemness observed in 5% oxygen conditions. In addition, our study sheds new light on the regulation of replicative senescence, which might have an impact for quality control of hADMPC preparations used for therapeutic applications.

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## Author Disclosure Statement

The authors declare no conflict of interest. No competing financial interests exist.

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# Effectiveness of a simplified cardiopulmonary resuscitation training program for the non-medical staff of a university hospital

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## Abstract

**Background:** The 2010 Consensus on Science and Treatment Recommendations Statement recommended that short video/computer self-instruction courses, with minimal or no instructor coaching, combined with hands-on practice can be considered an effective alternative to instructor-led basic life support courses. The purpose of this study was to examine the effectiveness of a simplified cardiopulmonary resuscitation (CPR) training program for non-medical staff working at a university hospital.

**Methods:** Before and immediately after a 45-min CPR training program consisting of instruction on chest compression and automated external defibrillator (AED) use with a personal training manikin, CPR skills were automatically recorded and evaluated. Participants' attitudes towards CPR were evaluated by a questionnaire survey.

**Results:** From September 2011 through March 2013, 161 participants attended the program. We evaluated chest compression technique in 109 of these participants. The number of chest compressions delivered after the program *versus* that before was significantly greater (110.8 ± 13.0/min vs 94.2 ± 27.4/min, $p < 0.0001$), interruption of chest compressions was significantly shorter (0.05 ± 0.34 sec/30 sec vs 0.89 ± 3.52 sec/30 sec, $p < 0.05$), mean depth of chest compressions was significantly greater (57.6 ± 6.8 mm vs 52.2 ± 9.4 mm, $p < 0.0001$), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased (8.9 ± 23.2% vs 38.6 ± 42.9%, $p < 0.0001$). Of the 159 participants who responded to the questionnaire survey after the program, the proportion of participants who answered 'I can check for a response,' 'I can perform chest compressions,' and 'I can absolutely or I think I can use an AED' increased *versus* that before the program (81.8% vs 19.5%, 77.4% vs 10.1%, 84.3% vs 23.3%, respectively).

**Conclusions:** A 45-min simplified CPR training program on chest compression and AED use improved CPR quality and the attitude towards CPR and AED use of non-medical staff of a university hospital.

## Background

Bystander-initiated cardiopulmonary resuscitation (CPR) and the automated external defibrillator (AED) have major roles in the 'chain of survival' for both out-of-hospital and in-hospital cardiac arrest [1,2]. The effectiveness of the rapid response system for in-hospital cardiac arrest or critically ill patients has been reported [3,4], and in our hospital, the rapid response system was introduced in 2001. During an emergency, the rapid response team (emergency medicine doctors and nurses) are paged to rush to the stricken patient. However, education for the non-medical staff working at medical institutions, who could potentially be first responders and could activate the system, has not been established.

The 2010 Consensus on Science and Treatment Recommendations (CoSTR) Statement recommended that training should aim to ensure that learners acquire and retain the skills and knowledge that will enable them to act correctly during actual cardiac arrests, and short video/computer self-instruction courses, with minimal or no instructor coaching, combined with hands-on practice can be considered as an effective alternative to

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instructor-led basic life support (BLS) courses [5]. In September 2010, we introduced a 45-min simplified CPR training program consisting of instruction on chest compression and AED use with a personal training manikin for the non-medical staff working at our university hospital. From September 2011, the quality of CPR skills was recorded *via* a CPR skill report system, and a questionnaire survey on the participants' attitudes towards CPR and AED use was conducted before and immediately after this training program. The purpose of this study was to examine the effectiveness of a simplified CPR training program for the non-medical staff working at a university hospital.

## Methods

### Study design

This was a prospective observational study that was approved by the Ethics Committee of Osaka University Graduate School of Medicine. The institutional review board waived the need for informed consent. We surveyed participants who attended this CPR training program from September 2011 through March 2013. Those eligible to participate in this program were non-medical staff working at our university hospital.

### Simplified CPR training program

The simplified CPR training program consisted of instructions and practice on chest compressions and AED use with a personal training manikin, and the total time of this program was 45 minutes. We used the CPR Training Box APPA-KUN® obtained from the non-profit organisation Osaka Life Support Association, Osaka, Japan, as the personal training manikin (Figure 1A). Doctors and nurses who were instructors of the Immediate Cardiac Life Support (ICLS) course certified by the Japanese Association for Acute Medicine (JAAM) or instructors with equivalent qualifications and who were specially trained for this program instructed the participants. The instructor/participant ratio was 1:10–20. A photo of this training program in action is shown in Figure 2. The training program was DVD-based and could be held using a small number of instructors (at least one instructor was required). Table 1 shows the time schedule of this training program, which consisted of an opening speech; introduction containing a check of the participants' knowledge about 'check for a response', 'chest compressions', and 'AED use'; explanation of the rapid response system; simulation of an in-hospital resuscitation by DVD; practice on chest compression and AED use with the personal training manikin; and a question and answer session. We standardised the contents of the training program by using the DVD presentation.

### Evaluation of CPR skills

Before and immediately after the 45-min CPR training, CPR skills were recorded *via* the CPR skill report system APPA-KUN Pro® (Alexon, Osaka, Japan) (Figure 1B). This CPR evaluation system automatically records the number of chest compressions, interruption of chest compressions, and depth of chest compressions. The evaluation of CPR skills was performed on the participants, whose cooperation was voluntary, and the time for evaluation of CPR skills was 30 seconds due to restrictions of time and the number of CPR skill report systems available. We evaluated the participants' CPR skill at each training program attended, as well as for all programs attended.

### Questionnaire survey evaluating participants' attitudes towards CPR and AED use

Before and immediately after the 45-min CPR training, participants' attitudes towards CPR and AED use were evaluated by a questionnaire survey. The question items included 'Can you check for a response?', 'Can you

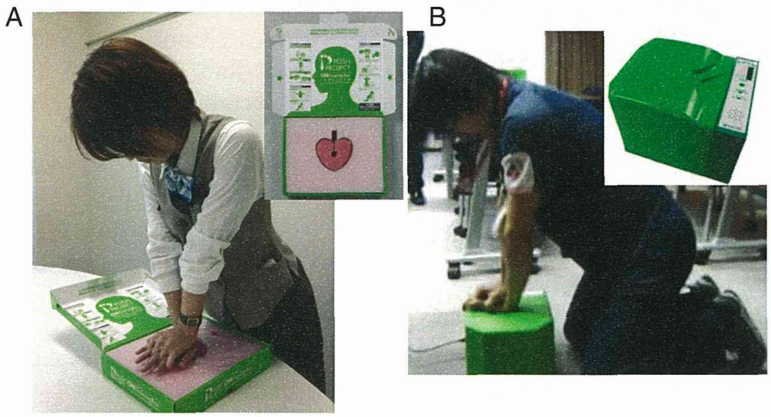


**Figure 1 The personal training kit. (A)** The CPR Training Box APPA-KUN®. **(B)** The CPR skill report system APPA-KUN Pro®.

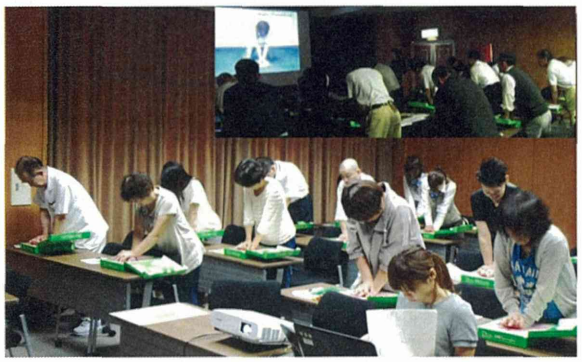

**Figure 2** The classroom scene of the simplified cardiopulmonary resuscitation training program with personal training kit.

perform chest compression?', and 'Can you use an AED?'. The questionnaire survey was given to all participants, and replies were anonymous. The participants provided one answer for each multiple choice question.

### Statistical analysis

All data are represented as mean ± standard deviation (SD). The Wilcoxon signed rank test was used to compare the differences between before and immediately after training, and the Wilcoxon rank sum test was used to compare the differences between the first-time and the second-time participants in the evaluation of CPR skills. A value of $p < 0.05$ was considered statistically significant. All statistical analyses were performed with JMP 9.0.2 for Windows (SAS Institute Inc., Cary, NC, USA).

## Results

### Evaluation of CPR skills

From September 2011 through March 2013, 161 participants attended the program, and we evaluated the chest compression technique of 109 participants due to restrictions of time and the number of CPR skill report systems available. The study group comprised 44 men and 65 women with a mean age ± SD of 42.2 ± 14.7 years.

**Table 1 Time schedule of the simplified cardiopulmonary resuscitation training program**

| Training schedule | Device used | Time (min) |
|---|---|---|
| Welcome | | 2 |
| Introduction (check of knowledge) | DVD | 6 |
| Rapid response system and simulation of an in-hospital resuscitation | DVD | 6 |
| Chest compression and AED use | Practice with a personal training manikin | 26 |
| Question and answer session | | 5 |
| Total | | 45 |

Among the 109 participants, 57 were the first-time participants of CPR training, 48 were second-time participants, and 4 were participating for the third time or more. In the analysis of the 109 participants, the number of chest compressions was significantly greater (110.8 ± 13.0/min vs 94.2 ± 27.4/min, $p < 0.0001$) (Figure 3A), the interruption of chest compressions was significantly shorter (0.05 ± 0.34 sec/30 sec vs 0.89 ± 3.52 sec/30 sec, $p < 0.05$) (Figure 3B), the mean depth of chest compressions was significantly greater (57.6 ± 6.8 mm vs 52.2 ± 9.4 mm, $p < 0.0001$) (Figure 3C), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased (8.9 ± 23.2% vs 38.6 ± 42.9%, $p < 0.0001$) (Figure 3D) after the program *versus* before the program.

We also compared CPR skill between the first and second attendance. The interval of attendance was 12.7 ± 4.3 months. Before the program, the number of chest compressions in the second-time participants was significantly greater (100.9 ± 28.4/min vs 87.7 ± 26.0/min, $p < 0.05$) (Figure 4A), and the interruption of chest compressions was significantly shorter (0.08 ± 0.58 sec/30 sec vs 1.62 ± 4.7 sec/30 sec, $p < 0.05$) (Figure 4B), compared with these values in the first-time participants. There were no significant differences between the two groups in mean depth of chest compressions (52.6 ± 8.6 mm vs 51.2 ± 10.1 mm, $p = 0.42$) (Figure 4C) and in the proportion of incomplete chest compressions of <5 cm among all chest compressions (35.9 ± 44.7% vs 43.5 ± 41.6%, $p = 0.32$) (Figure 4D). After the program as compared with before, the number of chest compressions was significantly greater (first-time participants: 109.5 ± 13.7/min vs 87.7 ± 26.0/min, $p < 0.0001$; second-time participants: 111.8 ± 12.5/min vs 100.9 ± 28.4/min, $p < 0.05$) (Figure 4A), the interruption of chest compressions was shorter (first-time participants: 0.09 ± 0.47 sec/30 sec vs 1.62 ± 4.7 sec/30 sec, $p < 0.05$; second-time participants: 0.0 ± 0.0 sec/30 sec vs 0.08 ± 0.58 sec/30 sec, $p = 1.0$) (Figure 4B), the mean depth of chest compressions was significantly greater (first-time participants: 57.9 ± 8.1 mm vs 51.2 ± 10.1 mm, $p < 0.0001$; second-time participants; 57.1 ± 5.1 mm vs 52.6 ± 8.6 mm, $p < 0.0001$) (Figure 4C), and the proportion of incomplete chest compressions of <5 cm among all chest compressions was significantly decreased (first-time participants: 10.1 ± 26.1% vs 43.5 ± 41.6%, $p < 0.0001$; second-time participants: 8.2 ± 20.3% vs 35.9 ± 44.7%, $p < 0.0001$) (Figure 4D). We excluded the participants who attended a third time or more because of the low number of participants.

### Questionnaire survey to evaluate participants' attitudes towards CPR and AED use

Responses to the questionnaire survey were obtained from 159 (98.8%) of the 161 participants. The responder

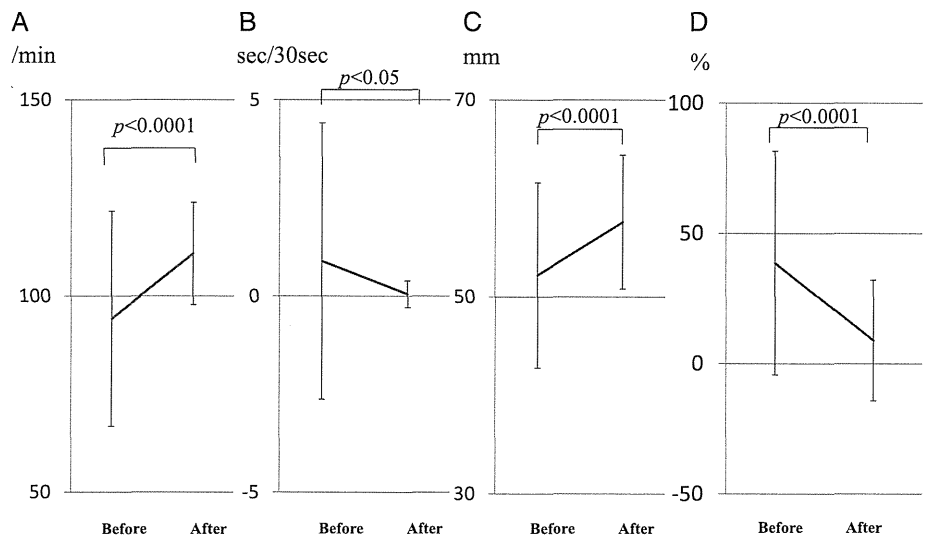


**Figure 3 Evaluation of cardiopulmonary resuscitation (CPR) skills for all participants before and immediately after the simplified CPR training program.** **(A)** Number of chest compressions, **(B)** time chest compressions were interrupted, **(C)** mean depth of chest compressions, and **(D)** proportion of incomplete chest compressions of <5 cm among all chest compressions.

group comprised 56 men and 103 women with a mean age ± SD of 42.5 ± 14.2 years, with 95 first-time, 59 second-time, and 5 third-time or more participants.

After the program as compared with before, the proportion of participants who answered 'I can check for a response,' 'I can perform chest compressions,' and 'I absolutely can or I think I can use an AED' increased (81.8% vs 19.5%, 77.4% vs 10.1%, and 84.3% vs 23.3%, respectively) (Table 2).

In the second-time participants, the proportion of participants who answered 'I can't or shouldn't check for a response' was smaller (2 [3.4%] vs 23 [24.2%]), who

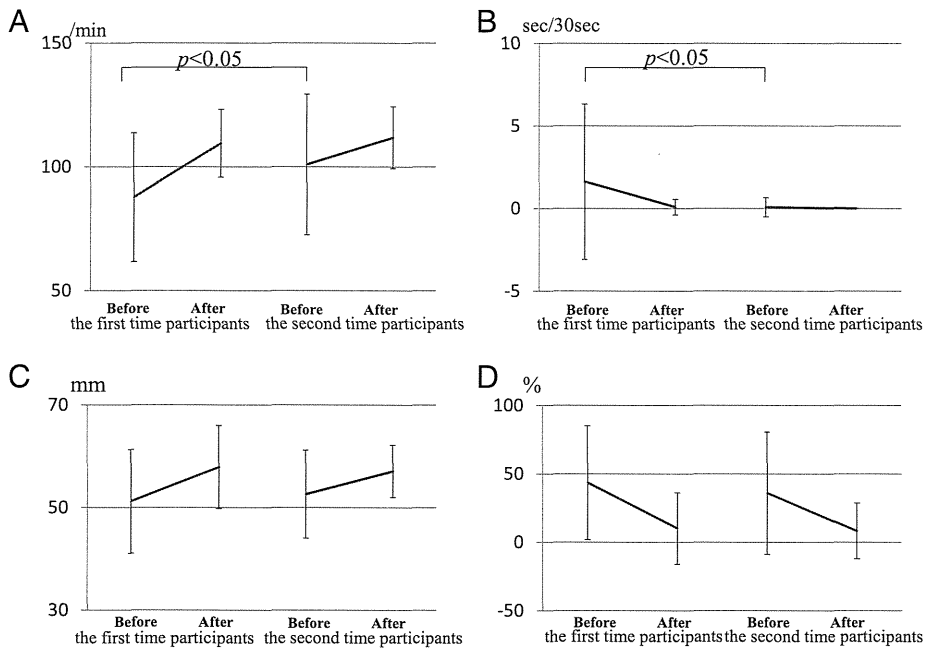


**Figure 4 Evaluation of cardiopulmonary resuscitation (CPR) skills according to first- and second-time attendance of the participants.** **(A)** Number of chest compressions, **(B)** time chest compressions were interrupted, **(C)** mean depth of chest compressions, and **(D)** proportion of incomplete chest compressions of <5 cm among all chest compressions.

**Table 2 Results of the questionnaire survey to evaluate participants' attitudes towards cardiopulmonary resuscitation and automated external defibrillator (AED) use**

| | Total (N = 159) | | First-time participants N = 95) | | Second-time participants(N = 59) | |
|---|---|---|---|---|---|---|
| | Before | After | Before | After | Before | After |
| Q. 1 Can you check for a response? | | | | | | |
| I can. | 31 (19.5%) | 130 (81.8%) | 14 (14.7%) | 80 (84.2%) | 15 (25.4%) | 47 (79.7%) |
| I don't know if I can. | 103 (64.8%) | 29 (18.2%) | 58 (61.1%) | 15 (15.8%) | 42 (71.2%) | 12 (20.3%) |
| I can't. | 21 (13.2%) | 0 (0%) | 20 (21.1%) | 0 (0%) | 1 (1.7%) | 0 (0%) |
| I shouldn't. More skillful people should. | 4 (2.5%) | 0 (0%) | 3 (3.2%) | 0 (0%) | 1 (1.7%) | 0 (0%) |
| Q. 2 Can you perform chest compression? | | | | | | |
| I can. | 16 (10.1%) | 123 (77.4%) | 8 (8.4%) | 75 (78.9%) | 7 (11.8%) | 46 (78.0%) |
| I don't know if I can. | 88 (55.3%) | 36 (22.6%) | 38 (40.0%) | 20 (21.1%) | 46 (78.0%) | 13 (22.0%) |
| I can't. | 49 (30.8%) | 0 (0%) | 43 (45.3%) | 0 (0%) | 6 (10.2%) | 0 (0%) |
| I shouldn't. More skillful people should. | 6 (3.8%) | 0 (0%) | 6 (6.3%) | 0 (0%) | 0 (0%) | 0 (0%) |
| Q. 3 Can you use an AED? | | | | | | |
| I absolutely can. | 4 (2.5%) | 27 (17.0%) | 2 (2.1%) | 17 (17.9%) | 1 (1.7%) | 9 (15.3%) |
| I think I can. | 33 (20.8%) | 107 (67.3%) | 14 (14.7%) | 67 (70.5%) | 18 (30.5%) | 38 (64.4%) |
| I don't know if I can. | 58 (36.5%) | 19 (11.9%) | 31 (32.6%) | 7 (7.4%) | 25 (42.4%) | 10 (16.9%) |
| I think I can't. | 47 (29.6%) | 4 (2.5%) | 34 (35.8%) | 3 (3.2%) | 12 (20.3%) | 1 (1.7%) |
| I absolutely can't. | 17 (10.7%) | 2 (1.3%) | 14 (14.7%) | 1 (1.1%) | 3 (5.1%) | 1 (1.7%) |

answered 'I can't or shouldn't perform chest compression' was smaller (6 [10.2%] vs 49 [51.6%]), and who answered 'I absolutely can't or I think I can't use an AED' was smaller (15 [25.4%] vs 48 [50.5%]) than that of the first-time participants. After the program as compared with before, the proportion of participants who answered 'I can check for a response' was greater (first-time participants: 80 [84.2%] vs 14 [14.7%]; second-time participants: 47 [79.7%] vs 15 [25.4%]), who answered 'I can perform chest compressions' was greater (first-time participants: 75 [78.9%] vs 8 [8.4%]; second-time participants: 46 [78.0%] vs 7 (11.8%]), and who answered 'I absolutely can or I think I can use an AED' was greater (first-time participants: 84 [88.4%] vs 16 [16.8%]; second-time participants: 47 [79.7%] vs 19 [32.2%]) (Table 2).

## Discussion

In this study, we showed the effectiveness of a simplified 45-min CPR training program for non-medical staff working at a university hospital that improved both the quality of CPR and the attitude of the staff towards CPR and AED use.

CPR and AED use by bystanders are very important in the 'chain of survival' for both out-of-hospital and in-hospital cardiac arrests to improve patient survival [1,2]. It was reported that survival to hospital discharge is still about 15% to 20% after in-hospital-cardiac arrest [6]. The non-medical staff working at a large hospital potentially can be the first responders for patients requiring CPR in most settings. To improve the 'chain of survival', especially in the first three links of the chain and to quickly activate the rapid response team, an adequate educational program is needed for these personnel.

Recently, animal and clinical research suggested that bystander-initiated cardiac-only resuscitation is at least as effective as conventional CPR for ventricular fibrillation (VF) or short periods of untreated arrest [7–10]. In addition, it was reported that cardiac-only resuscitation without mouth-to-mouth ventilation was easier to learn and perform and made it possible for the general public to perform a greater number of appropriate chest compressions than with the conventional CPR program [11,12]. Therefore, we introduced a 45-min simplified CPR training program consisting of instruction and practice in chest compression and AED use with a personal training manikin for the non-medical staff working at our university hospital because we needed to educate a number of these personnel in a short time.

In this study, we successfully demonstrated an improvement in the quality of CPR after the simplified CPR training course (Figure 3). The 2010 CoSTR Statement emphasised the need for improving the quality of CPR to increase patient survival after cardiac arrest [5]. Christenson et al. reported that the chest compression fraction appears to be an important determinant of survival from cardiac arrest [13]. It was also reported that shallower chest compressions correlated significantly with a decrease in successful defibrillation [14,15]. Now, the rescuer should give chest compressions to a depth of at least 5 cm and at a rate of at least 100 times per

minute, allow full chest recoil after each compression, and minimise interruptions in chest compression [5]. The improvement in the quality of CPR seen in the participants after the simplified CPR training may lead to an improvement in the prognosis of patient suffering in-hospital cardiac arrest in our hospital. Further analysis of serial in-hospital cardiac arrest statistics would prove this hypothesis.

In this study, we conducted a questionnaire survey to evaluate participants' attitudes towards CPR and AED use and demonstrated an improvement in their attitudes after the training (Table 2). Our result was consistent with that of a previous report that indicated that in an actual emergency setting, the participants of CPR training were more likely to perform CPR than those without the experience of CPR training [16]. However, Dwyer reported that even if the participants answered that they were confident that they could initiate CPR after CPR training, they could not perform CPR adequately in an actual emergency situation [17]. Therefore, further research as to whether the result in this study will lead to an increase in the initiation of CPR in an actual emergency situation is needed.

We found that the quality of CPR and the participants' attitude towards CPR and AED use were better in the second-time participants than in the first-time participants before CPR training (Figure 4, Table 2). BLS and advanced cardiac life support (ACLS) knowledge and skills can deteriorate in as little as 3 to six months [5,18,19]. Therefore, more frequent assessments or refresher training is recommended to maintain knowledge and skills [5]. In the present study, the interval of attendance from the last CPR training was 12.7 ± 4.3 months, and even though the CPR skills and attitude towards CPR of the second-time participants were better than those without experience of previous CPR training, they were not still sufficient (Figure 4D). As such, repeated attendance at an interval of less than 1 year would be desirable. The optimal interval between CPR training programs to maintain the quality of CPR and attitude towards CPR and AED use requires further clarification.

The 45-min time of this training program was shorter than that of conventional BLS or ACLS courses, and this training could be conducted by at least one instructor. Therefore, less burdens on time and expense exist for either the participants or the instructors. Because our program improved the quality of CPR and attitude towards CPR and AED use of the participants, this program could be considered not only in medical but also in non-medical institutions when implementing CPR and AED use.

As limitations of the present study, first, due to restrictions of time and the number of available CPR skill report systems, the evaluation of CPR skills was performed for only 30 seconds on 109 participants in whom cooperation was voluntary. Second, we did not test the practical skill of the participants on the AED due to restrictions of time. Further study is needed to evaluate the appropriate use of an AED after the training program. Third, there is no data on longer-term retention of skills. Forth, whether the participants of this CPR training could actually perform CPR in an emergency situation is unknown.

## Conclusion

A simplified 45-min CPR training program combining instruction and practice in chest compression and AED use improved the quality of CPR and the attitude towards CPR and AED use of the non-medical staff working at a university hospital. Further study to reveal the optimal interval for conducting the CPR training program to maintain the quality of CPR and a positive attitude towards CPR and AED use is needed.

### Competing interests

The authors have no conflicts of interest to declare in relation to this manuscript.

### Authors' contributions

TH, TI, and TS designed the study. TH, HM, TS, and KY collected and generated the data. TH wrote the first draft. TH, TI, HO, KY, TM, YF and TS analyzed the data and helped to draft the manuscript. All of the authors read and approved the final manuscript.

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# Presence of Neutrophil Extracellular Traps and Citrullinated Histone H3 in the Bloodstream of Critically Ill Patients

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## Abstract

Neutrophil extracellular traps (NETs), a newly identified immune mechanism, are induced by inflammatory stimuli. Modification by citrullination of histone H3 is thought to be involved in the in vitro formation of NETs. The purposes of this study were to evaluate whether NETs and citrullinated histone H3 (Cit-H3) are present in the bloodstream of critically ill patients and to identify correlations with clinical and biological parameters. Blood samples were collected from intubated patients at the time of ICU admission from April to June 2011. To identify NETs, DNA and histone H3 were visualized simultaneously by immunofluorescence in blood smears. Cit-H3 was detected using a specific antibody. We assessed relationships of the presence of NETs and Cit-H3 with the existence of bacteria in tracheal aspirate, SIRS, diagnosis, WBC count, and concentrations of IL-8, TNF-α, cf-DNA, lactate, and HMGB1. Forty-nine patients were included. The median of age was 66.0 (IQR: 52.5–76.0) years. The diagnoses included trauma (7, 14.3%), infection (14, 28.6%), resuscitation from cardiopulmonary arrest (8, 16.3%), acute poisoning (4, 8.1%), heart disease (4, 8.1%), brain stroke (8, 16.3%), heat stroke (2, 4.1%), and others (2, 4.1%). We identified NETs in 5 patients and Cit-H3 in 11 patients. NETs and/or Cit-H3 were observed more frequently in "the presence of bacteria in tracheal aspirate" group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" group (4/27, 14.8%) ($p<.01$). Multiple logistic regression analysis showed that only the presence of bacteria in tracheal aspirate was significantly associated with the presence of NETs and/or Cit-H3. The presence of bacteria in tracheal aspirate may be one important factor associated with NET formation. NETs may play a pivotal role in the biological defense against the dissemination of pathogens from the respiratory tract to the bloodstream in potentially infected patients.

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## Introduction

Neutrophils play an important role as the first line of innate immune defense [1]. One function of neutrophils, called "neutrophil extracellular traps" (NETs), has been discovered recently. NETs are fibrous structures that are released extracellularly from activated neutrophils in response to infection and also the sterile inflammatory process [2–5]. This distinctive phenomenon was first reported by Brinkmann et al in 2004 [6]. The main components of NETs are deoxyribonucleic acid (DNA) and histones H1, H2A, H2B, H3, and H4; other components such as neutrophil elastase, myeloperoxidase, bactericidal/permeability-increasing protein, cathepsin G, lactoferrin, matrix metalloproteinase-9, peptidoglycan recognition proteins, pentraxin, and LL-37 have also been reported [5–11]. The type of active cell death involving the release of NETs is called NETosis [12], which differs from apoptosis and necrosis. Because formation of NETs does not require caspases and is not accompanied by DNA fragmentation, it is believed that this process is independent of apoptosis [12]. Despite several in vitro and animal experiments that have clearly shown the biological importance of NETs, little is known about the function of NETs in the human body [13,14].

Before the discovery of NETs, several studies reported on an increase in the concentration of circulating free DNA (cf-DNA) in

the blood in various diseases including sepsis, trauma, stroke, autoimmune disorders, and several cancers [15–20]. This cf-DNA is thought to be derived from necrotic and/or apoptotic cells [21]. Recent articles have suggested that NETs and cf-DNA are related [15,16]. In these reports, cf-DNA was quantified directly in plasma, and the cf-DNA in plasma was treated the same as NETs in blood. However, it remains unknown whether cf-DNA is derived from NETs.

Citrullination of histone H3 is considered to be involved in NET formation in vitro. Neutrophils show highly decondensed nuclear chromatin structures during NETosis, and hypercitrullination of histone H3 by peptidylarginine deiminase 4 (PAD4) plays an important role in chromatin decondensation [14,22,23]. Inhibition of PAD4 prevents citrullination of H3 and NET formation [23]. Thus, measuring the presence of citrullinated histone H3 (Cit-H3) in conjunction with the presence of NETs may help clarify the kinetics of the response of NETs to systemic stress.

In preliminary studies, we recently identified NETs immunocytochemically in sputum and blood smear samples from intensive care unit (ICU) patients [24,25], whereas NETs could not be detected in blood smears from healthy volunteers [25].

In the present study, we used immunofluorescence to prospectively explore the existence of NETs and Cit-H3 in the blood of critically ill patients hospitalized in an ICU.

The respiratory tract is considered one of the most vulnerable places for bacterial invasion of the body, and NETs might start to be produced in response to pathogens before infection is completely apparent. Therefore, in this study we evaluated the presence of bacteria by Gram staining in tracheal aspirate as the preclinical stage of manifested infection to highlight its relationship with the induction of NETs in blood. The purpose of this study was to evaluate the relationships between NET or Cit-H3 and various clinical and biological parameters.

## Materials and Methods

### Patients and Setting

This study was a prospective observational study and was approved by the Ethics Committee of Osaka University Graduate School of Medicine. The institutional review board waived the need for informed consent. From April to June 2011, we examined blood samples collected from all patients who required intubation at the time of admission into the ICU of the Trauma and Acute Critical Care Center at the Osaka University Hospital (Osaka, Japan).

### Evaluation of Clinical Background and Severity of Illness

Age, sex, Acute Physiological And Chronic Health Evaluation (APACHE) II score, and Sequential Organ Failure Assessment (SOFA) score were recorded at the time of admission. Systemic inflammatory response syndrome (SIRS) was diagnosed at the time of admission on the basis of the criteria for SIRS defined by the American College of Chest Physicians/Society of Critical Care Medicine Consensus [26]. At admission, the blood samples were analyzed to obtain the following laboratory data: white blood cell (WBC) count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. WBC count was measured by an automated hematology analyzer (KX-21N; Sysmex, Hyogo, Japan). Lactate concentration was measured by a blood gas analyzer (ABL 835 Flex; Radiometer, Brønshøj, Denmark). The serum levels of IL-8 (R&D Systems, Minneapolis, MN, USA), TNF-α (R&D Systems), and HMGB1 (Shino-Test Corporation, Tokyo, Japan) were measured by enzyme-linked immunosorbent assay (ELISA) kits, and cf-DNA concentration was quantified using the Quant-iT PicoGreen dsDNA Assay kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

### Immunofluorescence Analysis to Identify the Presence of NETs and Cit-H3

For histological analysis, each blood sample collected at the time of admission to the ICU was immediately smeared in a thin layer on a glass slide. After drying, the specimens were stored at −80°C until immunostaining was performed. We confirmed that this sample preparation method did not induce additional generation of NETs or citrullination of histone H3 using neutrophils isolated from healthy donors on the smear (Fig. S1). To identify NETs, DNA and histone H3, the main components in NETs, were visualized simultaneously by immunofluorescence, and Cit-H3 was also detected using a specific antibody as follows. The sample on the glass slide was fixed with 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline (PBS) (pH 7.4), and then blocked with a solution containing 20% Block-Ace (Dainippon-Sumitomo Seiyaku, Osaka, Japan) and 0.005% saponin in PBS for 10 min. The samples were then incubated for 60 min with the primary antibody as follows: anti-human histone H3 mouse monoclonal antibody (diluted 1:100) (MABI0001; MAB Institute, Inc., Hokkaido, Japan) and anti-human Cit-H3 rabbit polyclonal antibody (1:100) (ab5103; Abcam, Cambridge, UK). After washing in PBS, each primary antibody was visualized using secondary antibodies coupled to 1:500 Alexa Fluor 546 goat anti-mouse IgG (Invitrogen) and 1:500 Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen). The primary and secondary antibodies were diluted with 5% Block-Ace and 0.005% saponin in PBS. After incubation for 60 min with the secondary antibodies, the specimens were washed with PBS, and the DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen) in PBS for 5 min. All procedures were performed at room temperature. The specimens were analyzed using a confocal laser-scanning microscope (BZ-9000; Keyence Corporation; Osaka, Japan).

The validity of immunostaining was ensured by the negative results of control experiments in which whole mouse or rabbit IgG (Abcam) was used instead of primary antibodies or primary antibodies were omitted in the procedure (Fig. S2). In addition, neutrophils stimulated with phorbol myristate acetate from healthy donors were used as a positive control for immunostaining (Fig. S3).

In the preliminary experiments, string-like structure extending from the cell body, which was positive for DNA and histone, was exclusively also positive for neutrophil elastase (Fig. S4). Hence, we considered the extracellular component that is double-positive for DNA and H3 to be a NET. The production of NETs and the specific expression of the citrullination of histone H3 in neutrophils were confirmed using anti-CD66b antibody (Fig. 1). Diff-Quik staining revealed the presence of a variety of blood cells in the smears (Fig. S5).

For the purpose of estimating the presence of NETs and the occurrence of citrullination of histone H3 concurrently, triple staining for DNA, H3, and citrullinated H3 was performed in this study. Samples were considered negative for the presence of NETs or Cit-H3 if cells harboring NETs or Cit-H3 were not identified in 300 neutrophils by immunostaining. If at least one of NETs and Cit-H3 was positive in the smear according to the definition mentioned above, the corresponding patient was classified into the "NET- and/or Cit-H3-positive" group.

### Detection of the presence of bacteria in tracheal aspirate

Aspiration is defined as the inhalation of oropharyngeal or gastric contents into the larynx and lower respiratory tract, and

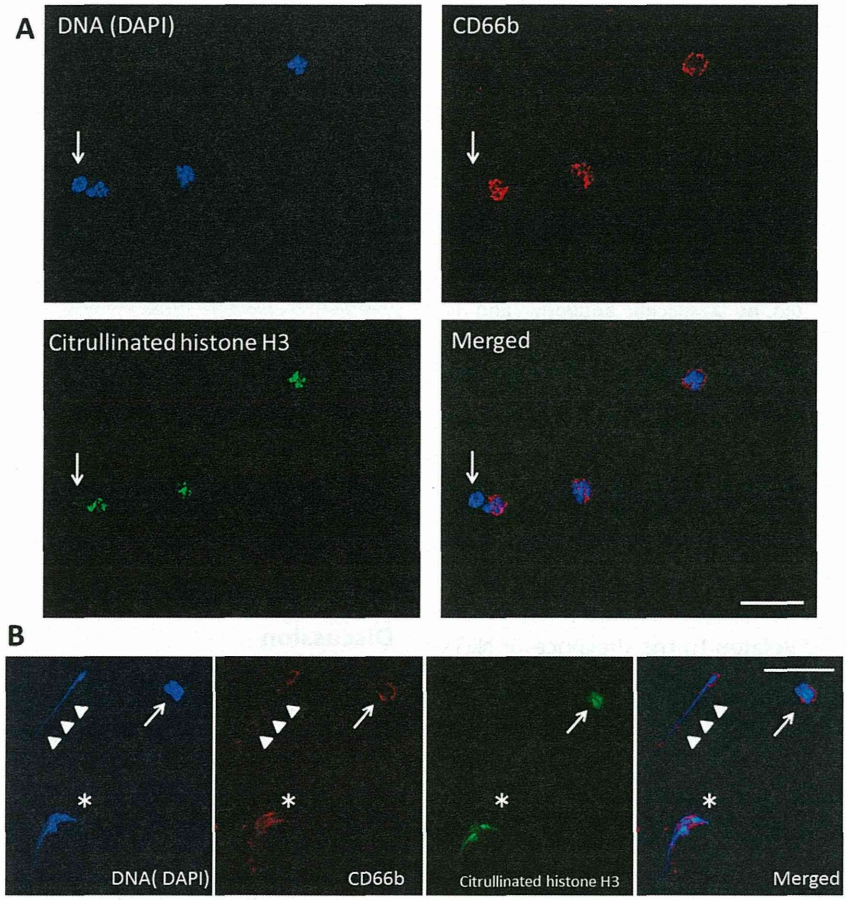


**Figure 1. Representative images of immunostaining using anti-CD66b antibody in the blood smear sample from a critically ill patient.** Triple staining by DAPI, anti-CD66b antibody, and anti-citrullinated histone H3 was performed using the blood smear sample obtained from a critically ill patient. A. The CD66b-positive cells were subjected to citrullination of histone H3 in their nuclei. Citrullination of histone H3 was not detected in the CD66b-negative cell (arrow). B. Arrow indicates the occurrence of citrullination of histone H3 in a neutrophil that had immunoreactivity against CD66b. Arrowheads indicate NETs stained with CD66b, whose appearance was of a string-like structure extending from the cell body. Asterisk indicates a neutrophil that was beginning to release NETs from its ruptured cell body. Interestingly, freshly produced NETs (asterisk) held immunoreactivity against citrullination of histone H3. In contrast, elongated NETs (arrowheads) were not stained with anti- citrullinated histone H3 antibody. Blue, DAPI; Red, CD66b; Green, citrullinated histone H3. (Magnification ×400). Scale bar; 50 μm.
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aspiration pneumonia is an infectious process caused by the inhalation of oropharyngeal secretions that are colonized by pathogenic bacteria [27]. The presence of bacteria in tracheal aspirate by Gram staining is regarded as part of aspiration that favors the development of infection. In this study, we evaluated the presence of bacteria in tracheal aspirate as the preclinical stage of manifested infection. To screen for the presence of bacteria in tracheal aspirate, an aspirated sputum smear was also prepared independently from immunostaining at the time of each patient's admission to the ICU. For Gram staining, the smear was dried, stained with crystal violet (Merck KGaA, Darmstadt, Germany) followed by iodine (Merck KGaA), washed with 99.5% ethanol (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and stained with Safranin (Merck KGaA). Images were captured on an optical microscope system (ECLIPSE 50i; Nikon Instruments Inc., Tokyo, Japan).

### Statistical Analysis

Continuous variables are presented as the median and interquartile range (IQR). The Wilcoxon rank-sum test and Pearson's chi-square test were used to compare two patient groups. Single and multiple logistic regression analyses were used to identify associations between the presence of NETs and/or Cit-H3 and the clinical and biological parameters studied. A $p$-value of $< .05$ was considered significant. All statistical analyses were performed using JMP 9.0.2 (SAS Institute Inc., Cary, NC, USA) and reviewed by a statistician.

## Results

### Patient Characteristics

During the study period, 263 patients were admitted to the ICU; 49 of these 263 patients were intubated patients and were included in this study. We excluded patients with cardiopulmonary arrest (CPA) who could not be resuscitated on admission. The patients' characteristics are shown in Table 1. The study group comprised 29 men and 20 women with a median age of 66.0 (IQR, 52.5–76.0) years. The median APACHE II score was 18.0 (IQR, 12.5–21.5), and the median SOFA score was 5.0 (IQR, 4.0–8.0). Thirty-eight patients (77.6%) were diagnosed as having SIRS, and 22 patients (44.9%) were judged as positive for "the presence of bacteria in tracheal aspirate". Thirty-six patients (73.5%)

survived and 13 patients died. The ICU mortality rate of intubated patients during this study period was 26.5%. The median WBC count was 10,900/μL (IQR, 8215–14,915/μL). The diagnoses included trauma (n = 7, 14.3%), infection (n = 14, 28.6%), resuscitation from CPA (n = 8, 16.3%), acute poisoning (n = 4, 8.1%), heart disease (n = 4, 8.1%), brain stroke (n = 8, 16.3%), heat stroke (n = 2, 4.1%), and others (n = 2, 4.1%) (Table 2).

### Presence of NETs and Cit-H3 in the Bloodstream

NETs were identified as extracellular string-like structures that were simultaneously immunoreactive for DNA and histone H3 (Fig. 2). Cit-H3 was detected by a specific antibody, and its presence was confirmed to be located inside lobulated nuclei and histone H3 (Fig. 3). In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients (Table 2). Both NETs and Cit-H3 were identified concurrently in one patient with infection. We detected the presence of circulating NETs and/or Cit-H3-positive cells in samples from patients with infection (4/14, 28.6%), resuscitation from CPA (5/8, 62.5%), acute poisoning (1/4, 25.0%), brain stroke (3/8, 37.5%), and heat stroke (1/2, 50.0%). We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4).

### Identification of Factors Related to the Presence of NETs and Cit-H3 in the Bloodstream

We tried to identify the factors that are related to the presence of NETs or Cit-H3 in the bloodstream. We first examined clinical parameters recorded at the time of admission including age, APACHE II and SOFA scores, number of patients who presented with SIRS or with the presence of bacteria in tracheal aspirate, and biological parameters such as the total WBC count and concentrations of lactate, IL-8, TNF-α, HMGB1, and cf-DNA. We also recorded the number of survivors. We compared these variables between the patients positive or negative for NETs and/or Cit-H3. The results are shown in Table 3. Among the factors evaluated in this research, only "the presence of bacteria in tracheal aspirate" differed significantly between the NET- and/or Cit-H3-positive and -negative groups ($p<.01$, Wilcoxon rank-sum test and Pearson's chi-square test). The other factors were not significantly related to the presence of NETs and/or Cit-H3. In patients classified into two groups based on the presence or absence of bacteria in tracheal aspirate, the occurrence rate of NETs and/or Cit-H3 was significantly higher in "the presence of bacteria in tracheal aspirate" (BTA (+)) group (11/22, 50.0%) than in "the absence of bacteria in tracheal aspirate" (BTA (−)) group (4/27, 14.8%) ($p<.01$) (Table S1). In patients with SIRS on admission, there was a trend toward greater expression of NETs and/or Cit-H3 ($p = .079$) (Table S2).

Logistic regression analysis was performed to identify the factors related to the presence of NETs and Cit-H3 in the bloodstream. The results of single logistic regression analysis of factors associated with the presence of NETs and Cit-H3 are shown in Table 4. Only BTA (+) at the time of intubation was a significant factor associated with the presence of NETs and Cit-H3 ($p = .0112$). Although there were indications of a trend toward an association between the presence of circulating NETs and/or Cit-H3 and the comorbid conditions of SIRS or elevated cf-DNA concentration ($p = .1093$ and.3003, respectively), these were not statistically significant. Table 5 shows the results of multiple logistic regression analysis of factors associated with the presence of NETs and/or Cit-H3 and model selection. Two methods of multiple regression analysis, backward and forward regression, yielded similar models. Again, "the presence of bacteria in tracheal aspirate" was the only factor that was significantly related to the presence of NETs and/or Cit-H3 in the bloodstream; the odds ratio for aspiration was 5.750.

## Discussion

A series of in vitro and animal experiments have uncovered a suppressive function of NETs against the dissemination of microorganisms in blood by mechanical trapping and by exploiting coagulant function to segregate these microorganisms within the circulation [28,29]. However, direct evidence remains scarce in living human systems. In this clinical study of blood smears, we attempted to identify morphologically the presence of NETs and Cit-H3 in the bloodstream of critically ill patients at the time of admission to the ICU and to characterize the factors associated with the presence of NETs and Cit-H3.

Among the 49 enrolled patients, immunofluorescence analysis revealed blood-borne NETs in five patients (10.2%), Cit-H3 in 11 patients (22.4%), and NETs and/or Cit-H3 in 15 patients (30.6%) (Table 2). These data replicate the results of our previous preliminary study in which NETs were present in patients in a critical condition [25] and show for the first time, to our knowledge, the presence of Cit-H3 in circulating blood cells. Cit-H3-positive cells possessed a multi-segmented nucleus, and most were immunoreactive for CD66b (Fig. 1), suggesting that citrullination of histone H3 occurred exclusively in neutrophils.

**Table 1.** Patient characteristics.

| Variable | Value |
|---|---|
| No. of patients (M/F) | 49 (29/20) |
| Age (years, median, IQR) | 66.0 (52.5–76.0) |
| APACHE II score (median, IQR) | 18.0 (12.5–21.5) |
| SOFA score (median, IQR) | 5 (4–8) |
| No. of patients with SIRS | 38 (77.6%) |
| The presence of bacteria in tracheal aspirate | 22 (44.9%) |
| No. of survivors | 36 (73.5%) |
| WBC (median, IQR) | 10,900 (8215–14,915) |

During the study period, 263 patients were admitted to the ICU of whom 49 were intubated and were included in this study. We excluded patients with cardiopulmonary arrest who could not be resuscitated on admission. IQR: interquartile range, APACHE: Acute Physiological And Chronic Health Evaluation, SOFA: Sequential Organ Failure Assessment, SIRS: systemic inflammatory response syndrome, WBC: white blood cell.
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**Table 2.** Diagnoses and the number of patients exhibiting neutrophil extracellular traps and citrullinated histone H3 in each diagnostic group.

| Diagnosis | NET positive (n) | Cit-H3 positive (n) | NET and/or Cit-H3 positive (%) |
|---|---|---|---|
| Trauma (n = 7) | 0 | 0 | 0/7 (0) |
| Infection (n = 14) | 3 | 2 | 4/14 (28.6) |
| Resuscitated from cardiopulmonary arrest (n = 8) | 2 | 3 | 5/8 (62.5) |
| Acute poisoning (n = 4) | 0 | 1 | 1/4 (25.0) |
| Heart disease (n = 4) | 0 | 0 | 0/4 (0) |
| Brain stroke (n = 8) | 0 | 3 | 3/8 (37.5) |
| Heat stroke (n = 2) | 0 | 1 | 1/2 (50.0) |
| Others (n = 2) | 0 | 1 | 1/2 (50.0) |
| Total (n = 49) | 5 | 11 | 15/49 (30.6) |

In the blood smears surveyed in this study, we identified NETs in 5 patients and Cit-H3 in 11 patients. Both NETs and Cit-H3 were identified concurrently in one patient with infection. We found no NETs or Cit-H3-positive cells in samples from patients with trauma (0/7) or heart disease (0/4). NETs: neutrophil extracellular trap, Cit-H3: citrullinated histone H3.

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Citrullination of histone H3 is considered an important process in the release of NETs through decondensation of chromatin [14,22,23]. Interestingly, the occurrence ratio of Cit-H3 was twice that of NETs. In vitro experiments imply that a substantial period of time is necessary to expel NETs extracellularly after the initiation of cell death by a stress stimulus [12,30,31]. However, it is still not clear how much time is required in vivo for NETs to appear intravascularly. The number of patients who exhibited circulating NETs in this study was lower than anticipated. We collected blood samples on admission to the ICU, and the timing might have been too early to detect NETs after the onset of a critical illness. The 11 Cit-H3-positive patients could be considered to have been in an early stage of NET formation. The change in the appearance of NETs and Cit-H3 during the course of hospitalization should be studied. If it can be shown clinically that Cit-H3 expression is followed by NET formation, it might be important to evaluate Cit-H3 expression in the blood upon admission to an ICU.

Table 2 shows that NETs and Cit-H3 were detected in patients with infection, resuscitation from CPA, acute poisoning, brain stroke, or heat stroke; surprisingly, we could not detect NETs or Cit-H3 in patients with trauma or heart disease. NETs are formed

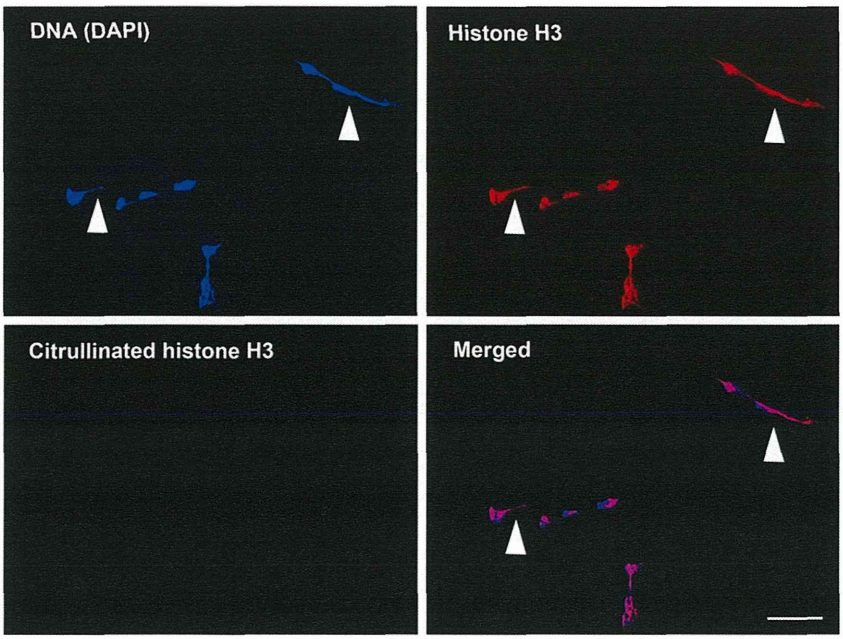


**Figure 2. Representative images of immunofluorescence staining to detect neutrophil extracellular traps (NETs).** NETs were visualized in the blood smear samples by immunocytochemistry and identified as extracellular string-like structures composed of chromatin (DNA and histone H3). NETs were present in the bloodstream of critically ill patients. Citrullination of histone H3 was not recognized in these images. In the blood smears surveyed in this study, we identified NETs in five patients (5/49, 10.2%). Blue, 4′,6-diamidino-2-phenylindole (DAPI); red, histone H3; green, citrullinated histone H3. Arrowheads indicate the double-stained areas containing NETs (Magnification ×400). Scale bar; 50 μm.

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