Hypoxia-simulating agents and selective stimulation of arsenic trioxide-induced growth arrest and cell differentiation in acute promyelocytic leukemic cells

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Background and Objectives. We recently reported that hypoxia-mimetic agents cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) could induce differentiation of acute myeloid leukemic (AML) cells. Here, we investigate whether these two agents influence the *in vitro* differentiation-inducing effect of arsenic trioxide (As_2O_3) on AML cells, an effective drug for the treatment of acute promyelocytic leukemia (APL) that is a unique subtype of AML with a specific fusion protein, PML-RARα.

Design and Methods. The APL cell line NB4 and non-APL promonocytic leukemic cell line U937 were treated with As_2O_3 (0.5 μM) combined with $CoCl_2$ (50 μM) or DFO (10 μM). The U937/PR9 subclone, whose expression of PML-RARα protein can be induced by Zn^{2+}, was also investigated. Cellular differentiation was evaluated by morphological criteria and myeloid differentiation-related antigens and marker gene expression. The hypoxia-inducible factor-1α (HIF-1α) mRNA and protein were detected, respectively, by semi-quantitative/real-time quantitative reverse transcription polymerase chain reaction and immunoblots. PML-RARα protein was also analyzed.

Results. $CoCl_2$ and DFO potentiated the growth-inhibiting and differentiation-inducing effects of low-dose As_2O_3, the latter enhancing $CoCl_2$ and DFO-induced accumulation of HIF-1α protein in NB4 cells but not in U937 cells. These two hypoxia-mimetic agents also accelerated As_2O_3-induced modulation and degradation of PML-RARα protein in NB4 cells. Furthermore, inducible expression of the fusion gene restored the co-operative effects of As_2O_3 and $CoCl_2$/DFO on U937/PR9 cells in terms of growth arrest, differentiation induction and HIF-1α protein accumulation.

Interpretation and Conclusions. Mimicked hypoxia enhanced As_2O_3-induced differentiation, in which HIF-1α and PML/RARα proteins played an important role. These data provide new insights into the understanding of the mechanisms of the action of As_2O_3 in the treatment of patients with APL.

Key words: arsenic trioxide, hypoxia, hypoxia-inducible factor-1, differentiation

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Acute promyelocytic leukemia (APL), a unique subtype of acute myeloid leukemia (AML), is characterized by blockage of granulocytic differentiation at the promyelocytic stage and a specific reciprocal chromosomal translocation t(15;17)(q22;q21).[1,2] This genetic alteration leads to the production of the abnormal fusion protein PML-RARα (for promyelocytic leukemia-retinoic acid receptor-α),[2-4] which causes leukemia by incompletely understood mechanisms.[5-7] Inspired by the important and exciting discoveries in the 1970s and early 1980s that leukemic cells can undergo phenotypic reversion *in vitro* under the influence of some agents, all-trans retinoic acid (ATRA) was identified as a strong differentiation-inducing agent for APL cells in the late 1980s.[8] Subsequently, the clinical efficacy and safety of ATRA in the treatment of APL was well established.[9,10] Following ATRA, intravenous administration of arsenic trioxide (As_2O_3) was found to have a better clinical efficacy for patients with relapsed APL.[11-17]

In spite of these clinical progresses, the mechanism of the action of As_2O_3 in the treatment of APL still remains largely unknown. Using cytological assays of bone marrow samples, we demonstrated that daily intravenous infusion of As_2O_3 induced *in vivo* differentiation of APL cells.[17,18] Soignet *et al.*[12] also reported that As_2O_3 induced a progressive decrease in the proportion of cells expressing CD33, an antigen typically associated with primitive myeloid cells, along with an increase in the proportion of cells expressing CD11, a mature myeloid element-associated antigen. Similar results were observed in an APL model using syngeneic grafts of leukemic blasts from PML-RARα trans-

genic mice[19] and in a xenograft tumor model with ATRA-resistant UF-1 APL cells in transgenic SCID mice producing human granulocyte-macrophage -stimulating factor.[20] However, *in vitro* studies showed that high concentrations (1-2 μM) of As_2O_3 induced apoptosis of APL cells.[18,21] As_2O_3-induced apoptosis was subsequently observed in many other cancer cells as reviewed by Chen *et al.*[22] Although low concentrations (0.1-0.5 μM) of As_2O_3 were shown to induce an atypical differentiation of APL cells, after prolonged incubation,[23,24] the *in vitro* differentiation action of As_2O_3 appeared to be less significant than its *in vivo* action. Thus, it is reasonable to deduce that some factors in the bone marrow microenvironment modulate the *in vivo* action of As_2O_3. We recently reported that mild hypoxia and non-toxic concentrations of hypoxia-mimetic agents, cobalt chloride ($CoCl_2$) and desferrioxamine (DFO), induced the differentiation of human AML cells with an increase in hypoxia-inducible factor-1α (HIF-1α) protein.[25-27] Based on these findings, in the current study we investigated whether hypoxia-mimetic agents potentiate the differentiation of leukemic cells induced by low-dose As_2O_3.

Design and Methods

Drugs and chemicals

As_2O_3, ATRA, $CoCl_2$ and DFO powders were purchased from Sigma (St. Louis, MI, USA). As_2O_3 was first dissolved in 1.0N NaOH and then diluted to 5mM with phosphate-buffered saline (PBS) as a stock solution. $CoCl_2$ and DFO were dissolved in ultrapure water to form 50 mM and 10 mM stock solutions, respectively, and ATRA was dissolved in ethanol as a 10mM stock solution.

Cell lines and treatment

In addition to the APL cell line NB4 (kindly provided by Dr. Lanotte from Saint-Louis Hospital, Paris, France) and the promonocytic leukemia cell line U937 (from the Cell Bank of Shanghai Institutes for Biological Sciences, Shanghai, China), the U937/PR9 cell line was also investigated. This cell line stably expresses PML-RARα in a Zn^{2+}-inducible fashion as described previously[28] and was kindly provided by Dr. Pelicci (Istituto di Clinica Medica I, Policlinico Monteluce, Perugia University, Italy). All cells were cultured in RPMI-1640 medium (Sigma, St Louis, MI, USA) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL, Gaithersburg, ML, USA) in 5% CO_2/95% air humidified atmosphere at 37°C. For experiments, all cells were seeded at 2-5×10^5 cells/mL and incubated with the indicated concentrations of As_2O_3, DFO or $CoCl_2$. Due to the short half-life of DFO,[29] one half of the medium was replaced daily with fresh medium plus the corresponding concentrations of compounds. For induction of PML-RARα in U937/PR9 cells, 0.1 mmol/L $ZnCl_2$ was added to the culture medium and PML-RARα expression was confirmed by western blotting. Cell viability was determined by the trypan-blue exclusion assay.

Table 1. Primers to amplify HIF-1α, NCF-1, ORM-1 and β-actin cDNA for semi-quantitative RT-PCR.

Genes		*Primers*
HIF-1α	forward	5'-TCATCAGTTGCCACTTCCAC-3'
	reverse	5'-TTCACAAATCAGCACCAAGC-3'
NCF-1	forward	5'-CACCTTCATCCGTCACATCGC-3'
	reverse	5'-TAGTTGGGCTCAGGGTCTTCC-3'
ORM-1	forward	5'-TGCCTCCTGGTCTCAGTATGG-3'
	reverse	5'-GCTGGACATTCAGGTAGGTGG-3'
β-actin	forward	5'-CATCCTCACCCTGAAGTACCC-3'
	reverse	5'-AGCCTGGATAGCAACGTACATG-3'

Differentiation assay

Leukemic cell differentiation was evaluated by morphology with Wright's staining, the nitroblue tetrazolium (NBT) reduction test and differentiation antigens (CD11 and CD14). For the NBT reduction test, which was performed as previously described,[30] 500 cells were counted under a light microscope and the percentage of NBT-positive cells was calculated. The differentiation antigens were measured by flow cytometry (Beckman-Coulter, Miami, FL, USA) with Becton Dickinson Simultest Control r1/r2α as a negative control, as described previously.[23]

Semi-quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted by TRIzol reagent (Gibco BRL, Gaithersburg, ML, USA) and reverse transcription (RT) was performed using the TaKaRa RNA PCR kit following the manufacturer's instructions (Takara, Dalian, China). HIF-1α, neutrophil cytosolic factor-1 (NCF-1) and orosomcoid-1 (ORM-1) cDNA were amplified together with β-actin cDNA in the same tube using the primers described in Table 1. Polymerase chain reaction (PCR) amplification was performed for 28-30 cycles with denaturing at 94°C for 30s, annealing at 58°C for 40s and extension at 72°C for 45s, in a GeneAmp PCR System 9600 (Perkin-Elmer, Norwalk, CT, USA). The signal intensities of amplified HIF-1α fragments were normalized to β-actin using a densitometer.

Real-time quantitative RT-PCR for HIF-1α mRNA

Total RNA was extracted by TRIzol reagent (Gibco BRL, Gaithersburg, ML, USA) and was treated with RNase-free DNase (Promega, Madison, WI, USA). cDNA was then synthesized using the cDNA synthesis kit according to the manufacturer's instruction (Applied Biosystem, Forster City, CA, USA). For real-time quantitative RT-PCR, specific oligonucleotide primers were used for HIF-1α (forward: TGATTGCATCTCCATCTC CTACC and reverse: GACTCAAAGCGACAGATAACACG) and β-actin as the internal control (forward: CATCCTCACCCTGAAGTACCC and reverse: AGCCTGGATAGC AACGTACATG). The primers listed in Table 1 were used for NCF-1 and ORM-1. Real-time quantitative RT-PCR was performed with the double-stranded DNA dye SYBR Green PCR Master Mixture Reagents (PE Biosystems, Warrington, UK) using the ABI PRISM 7900 system (Perkin-Elmer, Torrance, CA, USA), as described previously.[31] PCR were done in triplicate. Using the ΔCt method, β-actin was co-amplified to normalize the amount of RNA added to the reaction and the data were subjected to cycling threshold analysis.

Immunofluorescent analysis

Indirect immunofluorescent analysis for PML/PML-RARα proteins was performed as described previously.[18,23] Briefly, cells were collected onto slides by cytospin (Shandon, Runcorn, UK), and then fixed sequentially by 4% paraformaldehyde (5 min, 4°C) and cold methanol (10 min, 4°C). After washing with PBS, cells were incubated with the mouse anti-PML monoclonal antibody (Santa Cruz, CA, USA) for 1 hour at room temperature, followed by incubation with a fluoroscein thiocyanate-conjugated goat anti-mouse IgG (Santa Cruz, CA, USA) for one additional hour at room temperature in the dark and visualized under fluorescent microscope (Olympus BX-51, Olympus Optical, Japan).

Nuclear extract and western blot

To determine the level of nuclear PML-RARα protein, nuclear extracts were prepared from the harvested NB4 cells. Briefly, cells (1-2×10^7) were lysed in buffer A (10 mM HEPES, pH 7.9, 1.5m M $MgCl_2$, 10 mM KCl, 0.5mM dithiothreitol) on ice for 10 min, and then incubated with buffer A containing 0.2% Nonidet P-40 on ice for 20 min. The samples were centrifuged and the pellets were resuspended in buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 0.5mM dithiothreitol) containing 0.5 μg/mL leupeptin, 0.2 mM phenlymethlysulfonyl flouride, 0.5 μg/mL aprotinin, and 0.5 μg/mL pepstatin, and were then gently mixed on ice for 20 min. Following centrifugation, the supernatant was used as a nuclear extract. Total cellular protein extracts or nuclear extracts were loaded onto an 8-10% sodium dodecyl sulfate polyacrylamide gel for electrophoresis and immunoblots were performed according to commonly-used methods with a mouse anti-human HIF-1α monoclonal antibody, a mouse anti-human HIF-1β monoclonal antibody (BD Biosciences, Palo Alto, CA, USA), or an anti-RARα antibody (a kind gift from Dr. P. Chambon from the Institute of Genetics and Molecular and Cellular Biology, CNRS/INSERM/ULP, France). As necessary, blots were stripped and re-probed with a mouse anti-β-actin monoclonal antibody (Oncogene, San Diego, CA, USA) as an internal loading control.

Statistical analysis

Student's t-test was used to evaluate the difference between two different treatments. A p value less than 0.05 was considered statistically significant.

Results

$CoCl_2$ and DFO each potentiates the growth-arresting effect of As_2O_3 in NB4 cells

We first determined whether $CoCl_2$ or DFO could influence the growth inhibition induced by low-dose As_2O_3. For this set of experiments, NB4 cells were treated with low-dose (0.5 μM) As_2O_3 with and without 50 μM of $CoCl_2$ or 10 μM of DFO for the time course. As shown in Table 2, the treatment of As_2O_3 combined with $CoCl_2$ or DFO induced significantly more growth inhibition than their treatment with As_2O_3 alone, although treatment with As_2O_3, $CoCl_2$ or DFO individually also produced a time-dependent growth arrest of NB4 cells with $CoCl_2$ and DFO being slightly more effect than As_2O_3. Moreover, treatment with As_2O_3, $CoCl_2$ or DFO individually, at the indicated concentration, failed to reduce cell viability, and their combination also kept cell viability over 75% (Table 2).

As_2O_3-induced differentiation is enhanced by $CoCl_2$ and DFO in NB4 cells

Next, we investigated possible effects of the combined treatment of As_2O_3 with $CoCl_2$ or DFO on the differentiation of NB4 cells. In agreement with previous reports,[18,23] treatment of NB4 cells with 0.5 μM As_2O_3 for 4 to 6 days produced an atypical maturity-related alteration in morphology (Figure 1). Following a single administration of 50 μM $CoCl_2$ or 10 μM DFO, NB4 cells also exhibited maturity-related morphological changes, such as condensed chromatin and a decreased nuclei/cytoplasm ratio with smaller nuclei (Figure 1), consistent with our previous reports.[25-27] Notably, treatment with either As_2O_3, $CoCl_2$ or DFO also increased $CD11b^+$ cells (Figure 2A) but failed to

Table 2. Effects of As_2O_3, $CoCl_2$ or DFO and their combined treatment on cell growth inhibition and survival in NB4 cells.

Treatment	*Day 4*	*Day 6*
	Growth inhibition (mean±SD %)	
0.5 μM As_2O_3	11.4±8.5	41.8±9.9
50 μM $CoCl_2$	26.1±3.9	56.4±3.1
0.5 μM As_2O_3+50 μM $CoCl_2$	58.6±2.9 (*0.0003;#0.011)	81.0±3.4 (*0. 0008;#0.023)
10 μM DFO	42.4±2.8	60.2±3.1
0.5 μM As_2O_3+10 μM DFO	61.2±2.1 (*0.0007;#0.010)	84.1±1.2 (*0.001;#0.018)
	Cell viability (mean±SD %)	
Control	96.0±1.0	95.7±0.6
0.5 μM As_2O_3	94.0±2.0	92.0±1.5
50 μM $CoCl_2$	90.3±1.5	86.0±1.0
0.5 μM As_2O_3+50 μM $CoCl_2$	90.3±1.5	82.0±1.5
10 μM DFO	90.7±3.1	88.7±1.2
0.5 μM As_2O_3+10 μM DFO	88.7±2.3	78.0±2.0

*NB4 cells were treated with the indicated regimes for 4 days and 6 days. The percentages of cell growth inhibition and cell viability were calculated as described in the Design and Methods. Each value represents the mean±S.D of triplicate samples in an independent experiment. All experiments were repeated three times and similar results were obtained. *p values for combined treatment v.s. DFO or $CoCl_2$ alone; and #p values for combined treatment v.s As_2O_3 treatment alone.*

increase NBT-positive cells (Figure 2B). More excitingly, cells treated with As_2O_3 plus $CoCl_2$ and even more so As_2O_3 plus DFO, produced morphologically more mature myeloid cells (Figure 1) with significantly increased $CD11b^+$ expression (Figure 2A), compared to those treated with the single agent. Of note, we did not find the positive NBT reaction (Figure 2B), one of the markers of mature granulocytes. We also measured mRNA levels of NCF-1 and ORM-1 genes by semi-quantitative and real-time quantitative RT-PCR, since these have been used as leukemic cell differentiation signatures.[32] As shown in Figure 3, the NCF-1/ORM-1 mRNA levels were significantly higher when NB4 cells were treated with As_2O_3 plus $CoCl_2$ or DFO than when treated with a single agent. Overall, these results indicate that $CoCl_2$ and DFO enhance As_2O_3-induced differentiation of NB4 cells.

As_2O_3 enhances $CoCl_2$ and DFO-induced HIF-1α protein in NB4 cells

The treatment of NB4 cells with As_2O_3 (0.5 μM), $CoCl_2$ (50 μM), and DFO (10 μM), either alone or in combination for 24 hours, failed to alter the HIF-1α mRNA level as assessed by both semi-quantitative (Figure 4A) and real-time quantitative RT-PCR (Figure

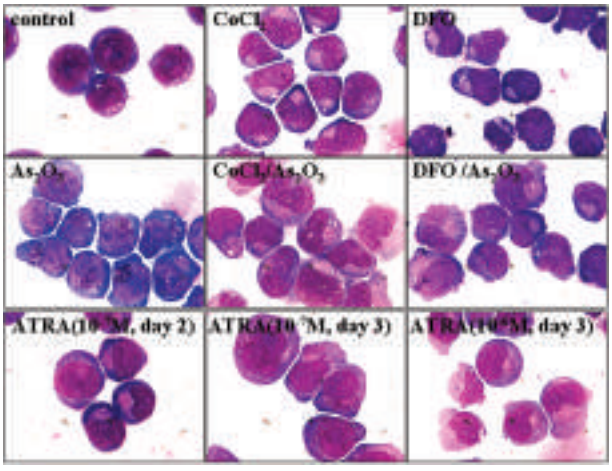

Figure 1. Morphologic features of NB4 cells after the treatment with As_2O_3, $CoCl_2$, or DFO alone and in combination. NB4 cells were treated with or without 0.5 μM As_2O_3, 50 μM $CoCl_2$, 10 μM DFO and their combination for 6 days. Cell morphology was examined under a microscope following Wright's staining of cells that were collected onto slides by cytospin (magnification ×1000). NB4 cells treated with the indicated concentrations of ATRA for 2 and/or 3 days were used as controls.

4B). Unlike $CoCl_2$ and DFO, treatment with As_2O_3 alone for 24 hours did not influence HIF-1α protein expression, but it did significantly potentiate $CoCl_2$ and DFO-induced increases in HIF-1α protein (Figure 4C). Of note, HIF-1β protein levels remained stable in NB4 cells regardless of the treatment regimens used (Figure 4C).

As_2O_3 fails to enhance $CoCl_2$/DFO-induced differentiation in U937 cells

In order to determine whether the combined effects of As_2O_3 with $CoCl_2$ or DFO are present in other AML subtypes, we examined the effects of As_2O_3 plus $CoCl_2$ or DFO on promonocytic leukemic U937 cells. Consistent with previous reports,[25-27] treatment with $CoCl_2$ at 50 μM or DFO at 10 μM produced significant growth arrest in U937 cells (Figure 5A). More surprisingly, and unlike what occurred with NB4 cells, As_2O_3 failed to enhance $CoCl_2$ or DFO-induced growth arrest (Figure 5A) and differentiation (Figure 5B) in U937 cells, as determined by cell morphology (*data not shown*) and the percentages of $CD11b^+$ cells (Figure 5B, left). Neither As_2O_3, $CoCl_2$ or DFO individually nor their combination increased $CD14^+$ cells (Figure 5B, right), an indication of more mature monocytic cells.[30,31] In parallel, As_2O_3 did not enhance $CoCl_2$ or DFO-induced HIF-1α protein accumulation in U937 cells (Figure 5C).

$CoCl_2$ and DFO enhance As_2O_3-induced modulation and degradation of PML-RARα fusion protein in NB4 cells

The differential effects of As_2O_3 plus $CoCl_2$ or DFO on NB4 and U937 cells led us to analyze the possible impacts of their combination on the APL cell-specific

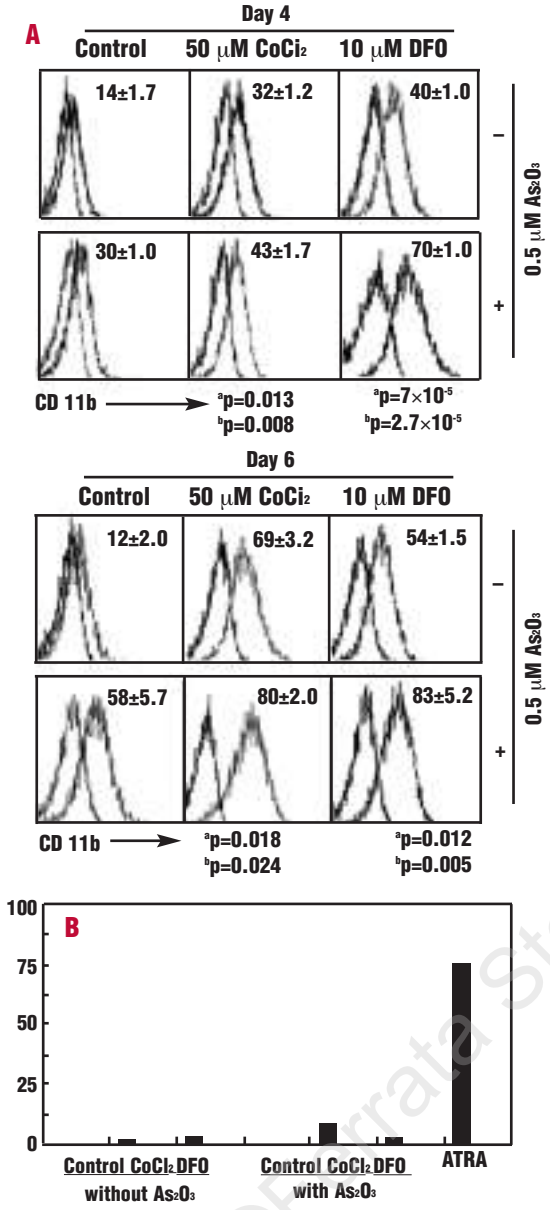

Figure 2. Effects of treatment with As_2O_3, $CoCl_2$, or DFO alone and in combination on the percentages of CD11b-positive and NBT-positive cells in NB4 cells. A. NB4 cells were treated with or without 0.5 μM As_2O_3, 50 μM $CoCl_2$ or 10 μM DFO and their combination for the indicated days. $CD11b^+$ cells were estimated by flow cytometry as described in the Design and Methods. For each panel, left and right lines represent a negative control (non-specific) and CD11b-positive cells, respectively. The percentage of $CD11b^+$ cells is shown as the mean±S.D of a separate triplicate experiment. All experiments were repeated at least three times with similar results. *p* values for combined treatment *v.s.* DFO/$CoCl_2$ treatment alone[a] and *v.s.* As_2O_3 treatment alone[b] are also indicated. B. NB4 cells were treated by the indicated agents for 6 days and the percentage of NBT^+ cells was detected. NB4 cells treated with 1 μM ATRA for 3 days were used as the positive control.

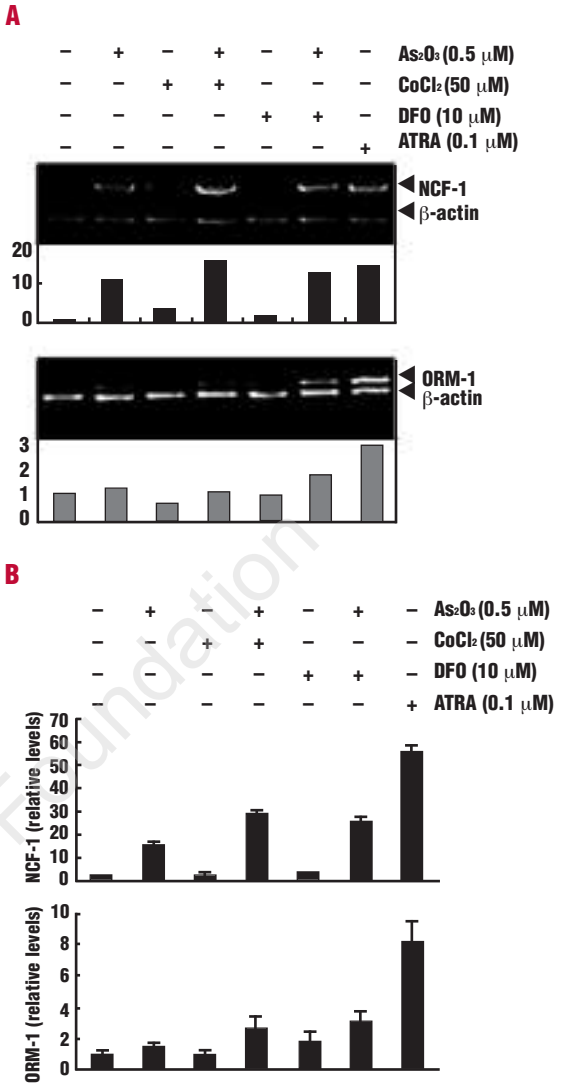

Figure 3. Effects of treatment with As_2O_3, $CoCl_2$, or DFO alone and in combination on NCF-1 and ORM-1 expression in NB4 cells. NB4 cells were treated with or without 0.5 μM As_2O_3, 50 μM $CoCl_2$ and/or 10 μM DFO and the levels of NCF-1 (top) and ORM-1 mRNA (bottom) were determined by semi-quantitative RT-PCR (A) and real-time quantitative RT-PCR (B) with β-actin mRNA as an internal control. NB4 cells treated with 0.1 μM ATRA were used as the positive control. For semi-quantitative RT-PCR, the ratios of NCF-1/ORM-1 mRNA against β-actin mRNA are given, and for real-time quantitative RT-PCR, ΔCt values of NCF-1/ORM-1 against β-actin were calculated and the ratios relative to untreated cells are given. All experiments were repeated three times with similar results.

fusion protein PML-RARα. We and others previously reported that As_2O_3 induced a remarkable modulation and degradation of PML/PML-RARα staining pattern in NB4 cells,[18,23,33] which involved their progressive sumoylation, followed by the proteosomal degradation of PML/RARα.[34,35] Consistently with these observations, we also showed in the present study that after incubation with As_2O_3 at 0.5 μM for 12 hours, the normal pattern of PML speckles (less than 10 so-called PODs or nuclear bodies[33,34] in each nucleus) was restored and the speckles further aggregated to form two to three large granules before they disappeared (Figure 6A).

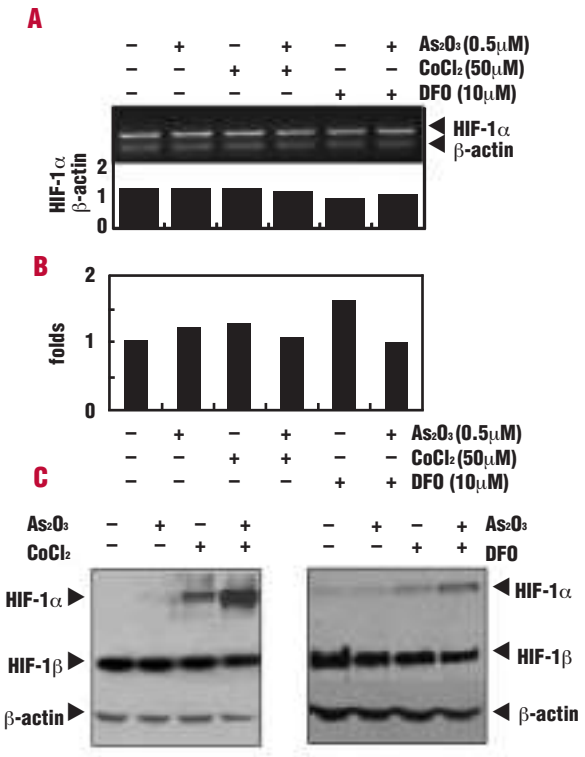

Figure 4. Effects of As_2O_3, $CoCl_2$, or DFO treatment alone and in combination on HIF-1 expression in NB4 cells. NB4 cells were treated with or without 0.5 μM As_2O_3 and/or 50 μM $CoCl_2$, 10 μM DFO for 24 hours. A and B. The levels of HIF-1α mRNA were determined by, respectively, semi-quantitative RT-PCR(A) and real-time quantitative RT-PCR (B). For semi-quantitative RT-PCR, the signal intensities of amplified HIF-1α fragments were normalized against β-actin using a densitometer. Each point represents the mean from triplicate samples. C. HIF-1α and HIF-1, proteins were analyzed by western blots with β-actin as a loading control. All experiments were repeated three times with similar results.

Treatment with $CoCl_2$ or DFO alone did not significantly change the PML staining pattern. However, the combination of As_2O_3 with $CoCl_2$/DFO greatly accelerated As_2O_3-induced disappearance of PML staining (Figure 6A). These changes were further confirmed by western blot analysis of PML-RARα protein. As shown in Figure 6B, treatment with the addition of $CoCl_2$ or DFO potentiated the reduction of PML-RARα protein induced by 0.5 μM As_2O_3, although $CoCl_2$ or DFO individually did not cause any changes in the fusion protein level.

Inducible expression of PML-RARα enhances the sensitivity of U937 cells to differentiation induced by As_2O_3 plus $CoCl_2$ or DFO

To investigate the possible involvement of PML-RARα in combined effects of As_2O_3 plus $CoCl_2$ or DFO on NB4 cells, the U937/PR9 and U937 cells were pretreated for 24 hours without and with 100 μM Zn^{2+}, the latter inducing PML-RARα expression in U937/PR9 cells (Figure 7A). These cells were then treated with 0.5 μM As_2O_3 and/or 50 μM $CoCl_2$/10 μM DFO for 6 days in the presence and absence of 100 μM Zn^{2+}. In the presence of 100 μM Zn^{2+}, As_2O_3 enhanced the growth-inhibitory effect of $CoCl_2$ and DFO in U937/PR9 but not in U937 cells (*data not shown*). Considering that untreated U937/PR9 cells had higher (up to 90%) CD11b-positive rates for unknown reasons (*data not shown*), CD11c was selected to evaluate their differentiation effects. As depicted in Figure 7B, As_2O_3 failed to enhance $CoCl_2$ or DFO-induced differentiation in U937 cells regardless of the presence of

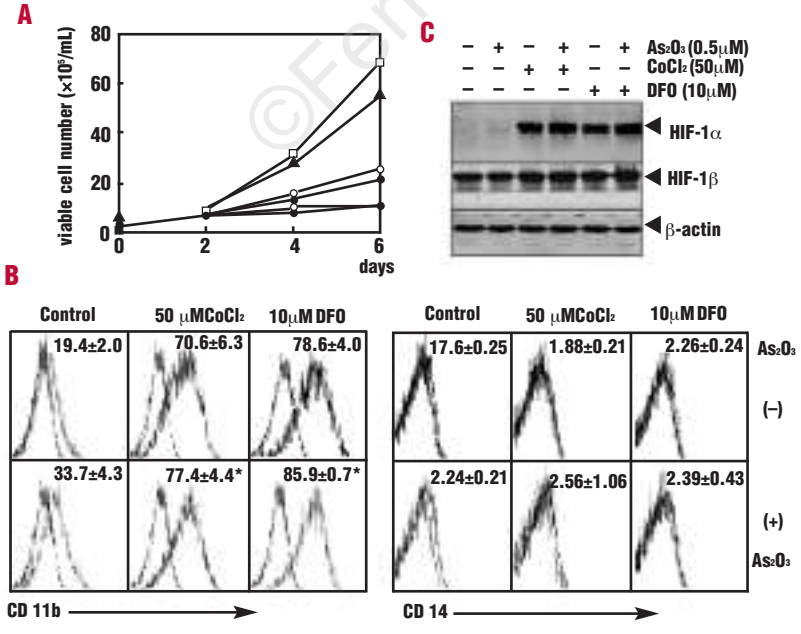

Figure 5. Effects of As_2O_3, $CoCl_2$, or DFO treatment alone and in combination on cell growth (A), cell differentiation-related antigens (B) and cellular HIF-1 protein level (C) in U937 cells. In panel A, U937 cells were treated with vehicle (□), 50 μM $CoCl_2$ (△) or 10 μM DFO (○) alone and in combination with 0.5 μM As_2O_3 (corresponding solid symbols) for various times as indicated and the number of viable cells was determined. Each point represents the mean+S.D. of triplicates in a separate experiment. In panel B, U937 cells were treated with or without 0.5 μM As_2O_3 and/or 50 μM $CoCl_2$/10μM DFO for 6 days. CD11b and CD14 cells were detected by flow cytometry. For each panel, left and right lines represent negative control (non-specific) and CD-positive cells, respectively. The percentages of $CD11b^+$ and $CD14^+$ cells are shown as the mean±S.D. of triplicates in a separate experiment. *$p>0.05$ compared with individual treatment with $CoCl_2$ or DFO. In panel C, U937 cells were treated with or without 0.5 μM As_2O_3, 50 μM $CoCl_2$ or 10 μM DFO and their combination for 24 hours. The levels of HIF-1α and HIF-1β proteins were analyzed by using β-actin as a loading control via immunoblots. All experiments were repeated three times with similar results.

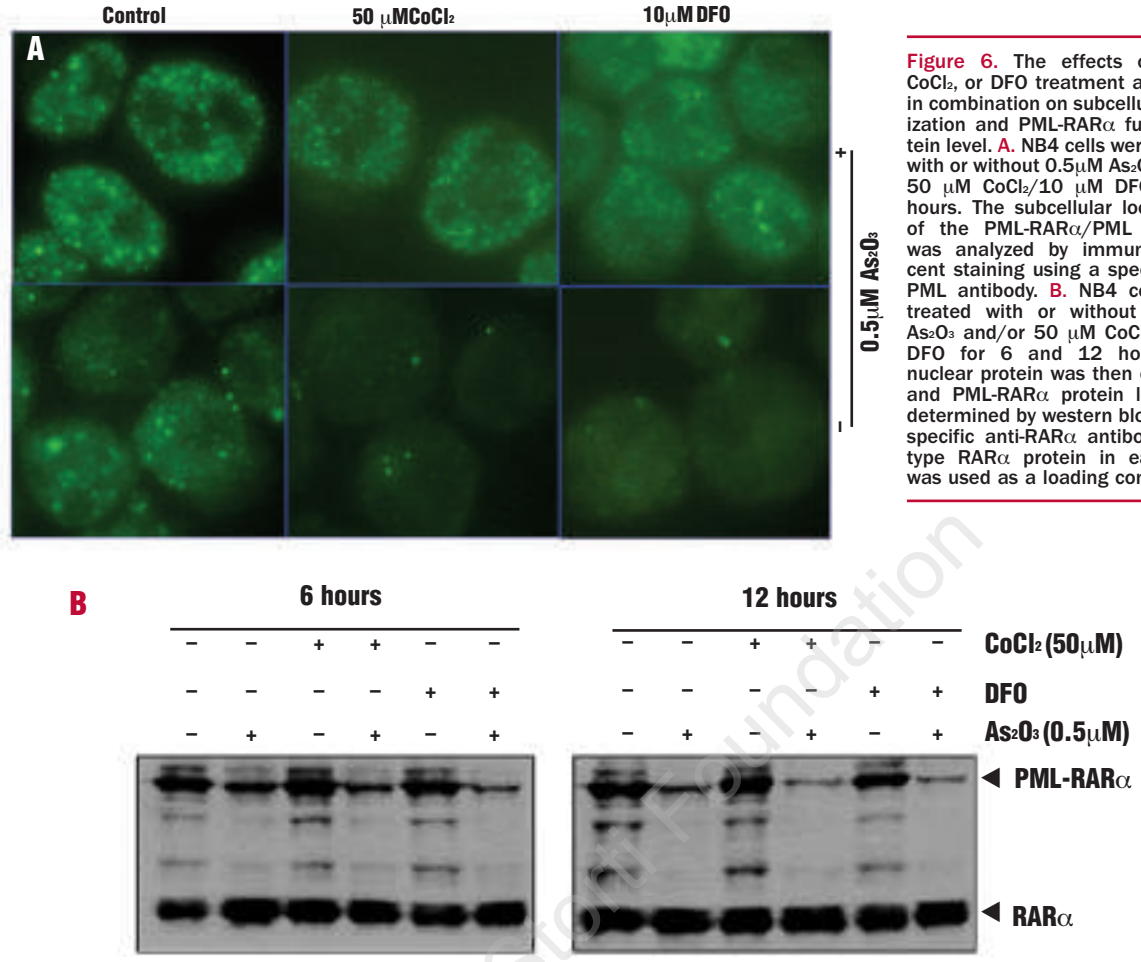

Figure 6. The effects of As_2O_3, $CoCl_2$, or DFO treatment alone and in combination on subcellular localization and PML-RARα fusion protein level. A. NB4 cells were treated with or without 0.5μM As_2O_3 and/or 50 μM $CoCl_2$/10 μM DFO for 12 hours. The subcellular localization of the PML-RARα/PML complex was analyzed by immunofluorescent staining using a specific anti-PML antibody. B. NB4 cells were treated with or without 0.5 μM As_2O_3 and/or 50 μM $CoCl_2$/10 μM DFO for 6 and 12 hours. The nuclear protein was then extracted and PML-RARα protein level was determined by western blot using a specific anti-RARα antibody. Wild-type RARα protein in each lane was used as a loading control.

Zn^{2+}. In contrast, As_2O_3 significantly increased $CoCl_2$/DFO-induced differentiation in the Zn^{2+}-incubated U937/PR9 cells. Of note, U937/PR9 cells appeared to be less sensitive to $CoCl_2$-, and even more so, especially DFO-induced differentiation than their parental U937 cells regardless of the presence or absence of Zn^{2+}. These results indicate that the inducible expression of PML-RARα protein enhances the sensitivity of U937 cells to growth inhibition and differentiation induced by the combined treatment of As_2O_3 and $CoCl_2$ or DFO. In agreement with this notion, As_2O_3 also significantly increased $CoCl_2$/DFO-induced HIF-1α protein accumulation in Zn^{2+}-treated U937/PR9 cells (Figure 7C) but not in Zn^{2+}-treated and untreated U937 cells (*data not shown*).

Discussion

It is well known that the physiological oxygen level in most peripheral organs of the body, including bone marrow, is 5% or less, which is much lower than the 95% in the air used in *in vitro* cell cultures. Although leukemia is traditionally regarded as a *liquid* tumor because leukemic cells floating in the peripheral circulation do not form a well-circumscribed *mass* as do solid tumors, on the other hand, the level of oxygen in the bone marrow of AML patients is low and might be further reduced as a result of the fast growth of leukemic cells.[36] Furthermore, the low oxygen level in bone marrow is possibly aggravated by the anemia which is often present in newly diagnosed AML patients. In this study we show for the first time that non-toxic concentrations of hypoxia-mimetic agents ($CoCl_2$ and DFO) enhanced low-dose As_2O_3-induced growth arrest and cell differentiation of an APL cell line, NB4, but not of a non-APL leukemic cell line, U937, as evidenced by morphological criteria, myeloid differentiation-related antigens and the marker genes NCF-1 and ORM-1. These results suggest that a low oxygen concentration *in vivo* potentiates the clinical effect of As_2O_3 used in the treatment of APL. In other words, the induction of APL cell differentiation by As_2O_3, which has been clearly demonstrated in clinical studies,[12,17,18] might be in some way related to a low oxygen concentration *in vivo*.

HIF-1, a critical transcription factor in the mammalian oxygen-sensing pathway, consists of two basic

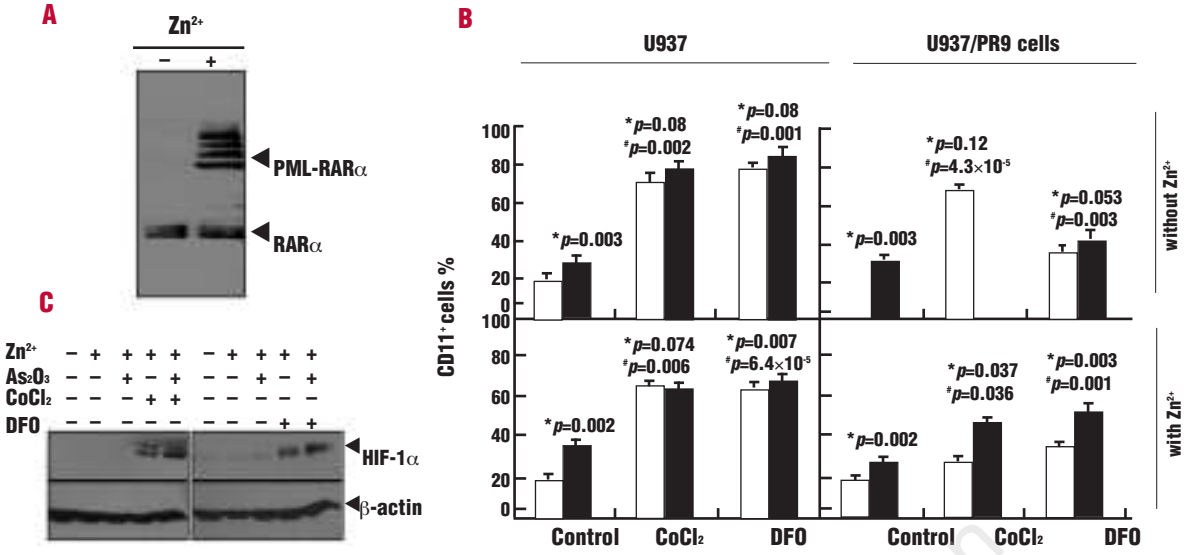

Figure 7. The effects of inducible expression of PML-RARα on actions of As_2O_3 and/or $CoCl_2$/DFO in U937/PR9 cells. A. U937/PR9 cells were treated with 100 μM $ZnCl_2$ for 24 hours and PML-RARα protein level was determined by western blot using a specific anti-RARα antibody. B. U937/PR9 cells (right) and their parental U937 cells (left) were pretreated with and without 100 μM $ZnCl_2$ for 24 hours, followed by 50 μM $CoCl_2$/10 μM DFO with (bold) and without (empty) 0.5 μM As_2O_3 in the presence (bottom) and absence (top) of 100 μM $ZnCl_2$ for 6 days. CD11c positive cells were detected by flow cytometry. *p* values for combined treatment v.s. DFO/$CoCl_2$ treatment alone and v.s. As_2O_3 treatment alone are indicated by * and # symbols, respectively. C. U937/PR9 cells were pretreated with and without 100 μM $ZnCl_2$ for 24 hours, followed by 50 μM $CoCl_2$/10 μM DFO with and without 0.5 μM As_2O_3 for 24 hours. HIF-1α proteins were then analyzed by western blots with β-actin as a loading control. All experiments were repeated three times with similar results.

helix-loop-helix-PAS domain-containing subunits, HIF-1α and HIF-1β/aryl hydrocarbon receptor nuclear translocator (ARNT).[37] HIF-1β is constitutively expressed, while the degradation of HIF-1α protein is regulated by oxygen concentration. Under normal oxygen tension, HIF-1α protein is rapidly degraded by ubiquitylation and the proteasome system through the hydroxylation of the conserved proline residues of HIF-1α by oxygen- and iron-dependent HIF-1α-specific prolyl hydroxylase.[37] In contrast, under hypoxic conditions or following administration of hypoxia-mimetic agents, such as $CoCl_2$ and DFO, the degradation of HIF-1α protein is blocked. The protein then transfers into the nucleus, forms a heterodimer with the HIF-1β subunit, and regulates gene expression by binding to the hypoxia responsive element (HRE) in the target genes.[37,38] Our previous studies suggested that $CoCl_2$/DFO-induced cell differentiation in leukemic cells was possibly mediated via the HIF-1α protein, which interacted physically with and enhanced the transcriptional activity of CCAAT-enhancer binding protein-alpha (C/EBPα).[25,26] It is worth pointing out that our recent preliminary experiments showed that the inducible stable expression of HIF-1α protein could directly induce U937 cells to undergo differentiation (*Song LP et al., unpublished data*). Here, we showed that As_2O_3 enhanced $CoCl_2$ and DFO-induced accumulation of HIF-1α protein, as well as $CoCl_2$ and DFO-induced cell differentiation in NB4 cells. In parallel to the failure of As_2O_3 to alter $CoCl_2$ or DFO-induced accumulation of HIF-1α protein, $CoCl_2$ or DFO-induced differentiation of U937 cells was not influenced by As_2O_3. These results further supported the contention that HIF-1α protein plays a role in leukemic cell differentiation.

It is well known that the APL-specific fusion protein PML-RARα can block cell differentiation and apoptosis through dominant negative inhibition of biological functions of wild-type PML, RARα and other proteins as well. In APL cells the heterodimerization of PML-RARα with wild-type PML (a tumor suppressor involved in many complex functions through gene transcription regulation such as growth arrest and apoptosis) disrupts the normal subcellular localization of PML protein producing a nuclear micro-speckled pattern that results in the loss of functions of PML and/or nuclear bodies.[33,34] Modulation and/or degradation of the chimeric protein have been widely accepted as important molecular mechanisms by which ATRA induces APL cell differentiation.[2] Although As_2O_3 exerts *in vitro* effects on many cell types, to date APL seems to be the most striking clinical target for As_2O_3. In this work, we showed the differential effects of As_2O_3 plus hypoxia-mimetic agents on the growth and differentiation of NB4 and U937 cells, which might partly explain why As_2O_3 is only clinically effective in the treatment of patients with APL but not in those with other subtypes of leukemia. As document-

ed, U937 cells became sensitive to ATRA[39] and to As_2O_3 in the presence of PML/RARα. Sternsdorf *et al.*[40] reported that As_2O_3 induced apoptosis only in U937 cells expressing the PML-RARα. Puccetti *et al.*[41] also demonstrated that sensitivity to As_2O_3-induced apoptosis in U937 cells can be increased either by overexpression of PML or by conditional expression of activated RAS, which upregulates PML. Here we showed that, unlike As_2O_3 that rapidly modulated the subcellular localization of PML-RARα fusion protein and induced its degradation in NB4 cells, $CoCl_2$ and DFO treatment alone had no obvious effects on PML-RARα protein. However, $CoCl_2$ and DFO each significantly enhanced the action of As_2O_3 on the PML-RARα fusion protein. These results possibly indicate that, like other leukemia-related fusion genes, such as AML1-ETO[26] and bcr-abl,[42] PML-RARα fusion protein might modulate the synthesis and/or degradation of HIF-1α protein, although this remains to be confirmed. Given that As_2O_3 enhanced DFO and $CoCl_2$-induced growth inhibition, differentiation induction and HIF-1α protein accumulation in NB4 cells but not in U937 cells, we hypothesized that the PML-RARα fusion protein might play a role in the action of As_2O_3 plus hypoxia-mimetic agents.

We, therefore, treated U937/PR9 cells with As_2O_3 plus $CoCl_2$ or DFO in the presence and absence of PML-RARα induction by Zn^{2+}. The results showed that inducible expression of PML-RARα protein restored the combined effects of growth inhibition and differentiation induction produced in U937 cells by As_2O_3 with $CoCl_2$ or DFO. More intriguingly, As_2O_3 also enhanced $CoCl_2$ or DFO-induced HIF-1α protein accumulation in the presence of PML-RARα expression in U937/PR9 cells. These results indicate that PML-RARα might contribute to enhanced As_2O_3-induced differentiation and HIF-1α protein accumulation under the hypoxia-simulating circumstance in NB4 cells. In summary, our discoveries shed new light on the mechanisms of action of As_2O_3 and may help to improve the efficacy of As_2O_3 in the treatment of APL.

HY, Y-LW: performed most experiments and drafted and revised the article; Z-GP: contributed to PML-RAR-related work; YJ: contributed to HIF-1-related work; YY: contributed to FACS analysis; YH, Y-SZ, QZ: analysis of data; G-QC: conception and design, of the study and critically revising the paper for important intellectual content and final approval of the version to be published. The authors reported no potential conflicts of interest.

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