

RESEARCH ARTICLE

# Low-Concentration Arsenic Trioxide Inhibits Skeletal Myoblast Cell Proliferation via a Reactive Oxygen Species-Independent Pathway

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## Abstract

Myoblast proliferation and differentiation are essential for skeletal muscle regeneration. Myoblast proliferation is a critical step in the growth and maintenance of skeletal muscle. The precise action of inorganic arsenic on myoblast growth has not been investigated. Here, we investigated the *in vitro* effect of inorganic arsenic trioxide (As<sub>2</sub>O<sub>3</sub>) on the growth of C2C12 myoblasts. As<sub>2</sub>O<sub>3</sub> decreased myoblast growth at submicromolar concentrations (0.25–1 μM) after 72 h of treatment. Submicromolar concentrations of As<sub>2</sub>O<sub>3</sub> did not induce the myoblast apoptosis. Low-concentration As<sub>2</sub>O<sub>3</sub> (0.5 and 1 μM) significantly suppressed the myoblast cell proliferative activity, which was accompanied by a small proportion of bromodeoxyuridine (BrdU) incorporation and decreased proliferating cell nuclear antigen (PCNA) protein expression. As<sub>2</sub>O<sub>3</sub> (0.5 and 1 μM) increased the intracellular arsenic content but did not affect the reactive oxygen species (ROS) levels in the myoblasts. Cell cycle analysis indicated that low-concentrations of As<sub>2</sub>O<sub>3</sub> inhibited cell proliferation via cell cycle arrest in the G1 and G2/M phases. As<sub>2</sub>O<sub>3</sub> also decreased the protein expressions of cyclin D1, cyclin E, cyclin B1, cyclin-dependent kinase (CDK) 2, and CDK4, but did not affect the protein expressions of p21 and p27. Furthermore, As<sub>2</sub>O<sub>3</sub> inhibited the phosphorylation of Akt. Insulin-like growth factor-1 significantly reversed the inhibitory effect of As<sub>2</sub>O<sub>3</sub> on Akt phosphorylation and cell proliferation in the myoblasts. These results suggest that submicromolar concentrations of As<sub>2</sub>O<sub>3</sub> alter cell cycle progression and reduce myoblast proliferation, at least in part, through a ROS-independent Akt inhibition pathway.

## Introduction

Inorganic arsenic is well-known toxicant and a potent human carcinogen [1,2]. Chronic exposure to arsenic through consumption of polluted water is a major risk to the world population. Nevertheless, arsenic has also been used for many centuries as medicinal agents for the treatment of syphilis, ulcers, and psoriasis [3,4]. In addition, the U.S. Food and Drug Administration approved arsenic trioxide as a chemotherapeutic agent for treating acute promyelocytic leukemia (APL) [5]. Arsenic is also a transplacental carcinogen in rodents and probably in human [6,7]. Furthermore, the evidence demonstrates that arsenite induces human neuroblastoma cell proliferation via a vascular endothelial growth factor (VEGF) signaling pathway [8].

Arsenic is capable of producing reactive oxygen species (ROS), eliciting DNA damage and slowing cell-cycle progression [9]. Several studies have indicated that arsenic trioxide induces cell cycle arrest and apoptosis in various cells. These cells include lymphoid neoplasms [10] and the head and neck cancer cell line PCI-1 [11], human fibroblasts [12], human bronchial epithelial cell line BEAS-2B [13], and human colonic, breast, and pancreatic cancer cells [14]. The effect of inorganic arsenic on the cell cycle progression in myoblasts is still unclear.

In postnatal skeletal muscle, satellite cells (quiescent muscle precursor cells) reside beneath the basal lamina. They are the primary cellular source of muscle growth and regeneration [15,16]. In response to muscle injury, satellite cells activate, proliferate, and differentiate to form a pool of myoblasts, and then fuse together to repair or replace damaged muscle fibers [17]. In skeletal muscles undergoing hypertrophy, an increase in myonuclear numbers, which produced by the satellite cells, precedes the enlargement of myofiber size [18]. Evidence shows that maternal undernutrition during gestation results in a decrease in myoblast proliferation coupled with an earlier onset of differentiation to fibers. This alters the development of muscle fibers and reduces the birth weight of newborn lambs [19,20]. Therefore, myoblast proliferation is an early and critical cellular event essential for skeletal muscle growth.

Previous studies indicate that arsenic suppresses the myoblast differentiation (myogenesis) [21,22]. Arsenic also inhibits the osteoblast differentiation from bone marrow stromal cells [23]. Moreover, arsenic displays striking suppression of muscle regeneration in a mouse skeletal muscle injury model [22]. In addition, arsenic at micromolar concentrations induces apoptosis in the myoblasts [24]. However, the precise action of inorganic arsenic on myoblast proliferation remains to be clarified. In this study, we focused on the investigation of dose-related arsenic effects and possible mechanisms of action of inorganic arsenic ( $\text{As}_2\text{O}_3$ ) on myoblast proliferation.

## Materials and Methods

### Cell culture

C2C12 mouse myoblasts were obtained from American Type Culture Collection (CRL-1772; Manassas, VA, USA) and cultured in growth medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin). The cells were incubated in a 5%  $\text{CO}_2$  environment at 37°C.

$\text{As}_2\text{O}_3$  (Sigma-Aldrich, St. Louis, MO, USA) 0.1 M (stock solution) was prepared in 1 M NaOH, diluted to  $10^{-3}$  M in PBS, and adjusted to pH 7.2 using HCl. It was diluted further with phosphate-buffered saline (PBS), and the solutions were kept at 4°C until use.

### Cell proliferation assay

Cell proliferation assays were conducted in six-well culture dishes. C2C12 myoblast cultures were seeded at  $1 \times 10^4$  cells per well in DMEM. After a 16-h attachment period, arsenic test

media was added, which consisted of 10% fetal bovine serum media with 0–1  $\mu\text{M}$   $\text{As}_2\text{O}_3$ . Plates were then incubated in an atmosphere of 37°C and 5%  $\text{CO}_2$  for a further 24, 48 and 72 h. After the appropriate treatment period, cells were trypsinized, re-suspended in 0.4% trypan blue solution and counted using a hemocytometer. The positions of the samples on the plate were randomly assigned, and all samples were run in triplicate. Results are representative of at least three independent experiments.

### Bromodeoxyuridine incorporation assay

C2C12 myoblasts were cultured in growth medium with or without  $\text{As}_2\text{O}_3$  (0.25, 0.5, and 1  $\mu\text{M}$ ) in 96-well microplates for 48 h. Subsequently, bromodeoxyuridine (BrdU) (Roche Diagnostics, Indianapolis, IN, USA) was added and the cells were incubated for an additional 4 h. After the culture supernatant was removed, the cells were fixed, and then incubated with an anti-BrdU antibody conjugated to peroxidase (anti-BrdU-POD). Bound anti-BrdU-POD was detected by a substrate reaction, and then quantified in an enzyme-linked immunosorbent assay (ELISA) plate reader.

### Annexin V-FITC apoptosis detection

Apoptosis was detected using an annexin V-fluorescein isothiocyanate (FITC) kit purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). The assay was performed following the manufacturer's instructions. Briefly, C2C12 cells were seeded in 60 mm plates and cultured under the same condition as in the cell proliferation assay. Next, the cells were treated with  $\text{As}_2\text{O}_3$  (0, 0.25, 0.5, and 1  $\mu\text{M}$ ) for 72 h. Both floating and attached cells were harvested and wash twice with ice-cold PBS. The cells were then re-suspended in 100  $\mu\text{l}$  of binding buffer and incubated with annexin V-FITC and propidium iodide. After incubation for 30 min in the dark, 400  $\mu\text{l}$  binding buffer was added to each tube and the samples were immediately analyzed using a FACScan flow cytometer (Becton-Dickinson).

### Cell cycle analysis

C2C12 cells were plated  $2 \times 10^4$  cells/100-mm-diameter tissue culture dishes without or with the indicated concentrations of  $\text{As}_2\text{O}_3$  for 72 h. The cells were washed with PBS, detached with trypsin and fixed with 75% ethanol overnight. Samples were washed and re-suspended in 0.5 ml of PBS containing with 100  $\mu\text{g}/\text{ml}$  RNase A and 5  $\mu\text{g}/\text{ml}$  propidium iodide for 30 min. The DNA contents were measured by a FACScan flow cytometer (Becton-Dickinson). Ten thousand individual cells in each group were sampled.

### Protein extraction and immunoblotting

Cell lysates were prepared using radio-Immunoprecipitation assay (RIPA) buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid, 0.1% sodium dodecyl sulfate, 1 mM NaF, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{mL}$  aprotinin, and 1  $\mu\text{g}/\text{mL}$  leupeptin]. The cell suspension was left on ice for 20 min, and then centrifuged at  $10,000 \times g$  for 20 min at 4°C. In some experiments, the nuclear proteins were extracted using a nuclear extraction kit (Affymetrix, Fremont, CA, USA). Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Following electrophoresis, the proteins were electrotransferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were then blocked with 5% nonfat powdered milk for 1 h and subsequently incubated overnight at 4°C with primary antibodies [anti-p21, anti-p27, anti-cyclin-dependent kinase (CDK)2, anti-CDK4, anti-cyclin

B1, anti-cyclin D1, anti-cell division cycle 2 (CDC2), anti-proliferating cell nuclear antigen (PCNA), anti-histone H1, anti-Akt1/2/3, anti-pERK, anti-pERK1, anti- $\alpha$ -tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-pAkt (Ser473) (Epitomics, Burlingame, CA)]. The membranes were next washed three times in Tris buffered saline and tween 20 (TBST) and incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Millipore). The blots were developed using an enhanced chemiluminescence reagent and exposed to and X-ray film.

### Detection of arsenic contents

C2C12 myoblasts were treated with  $\text{As}_2\text{O}_3$  (0.5–10  $\mu\text{M}$ ) for 24 h. After incubation, cells were harvested and washed with PBS three times followed by the addition of 0.1% nitric acid. The mixture was then vortexed and frozen at  $-20^\circ\text{C}$  overnight. The arsenic levels were determined in the supernatant containing intracellular arsenic by inductively coupled plasma mass spectrometry (ICP-MS). The detection limit for arsenic was approximately 0.1 ppb ( $\mu\text{g/L}$ ).

### Measurement of intracellular ROS formation

A fluorescein-labeled dye, 2',7'-dichlorofluorescein diacetate (DCFH-DA) was used to determine the generation of intracellular ROS. The non-fluorescent dye in cells was hydrolyzed to 2',7'-dichlorofluorescein (DCF) upon interaction with intracellular ROS. Cells were incubated with 20  $\mu\text{M}$  DCFH-DA for 30 min at  $37^\circ\text{C}$ , and then cells were washed twice with ice-cold PBS and harvested. The cells were immediately analyzed using a FACScan flow cytometer (Becton Dickinson) to determine the ROS generation.

### Statistical analysis

Results were expressed as means  $\pm$  SEM. The significant differences from the respective controls for each experimental test condition were assessed by analysis of variance (ANOVA) and the Bonferroni t-test with  $P < 0.05$  considered significant.

## Results

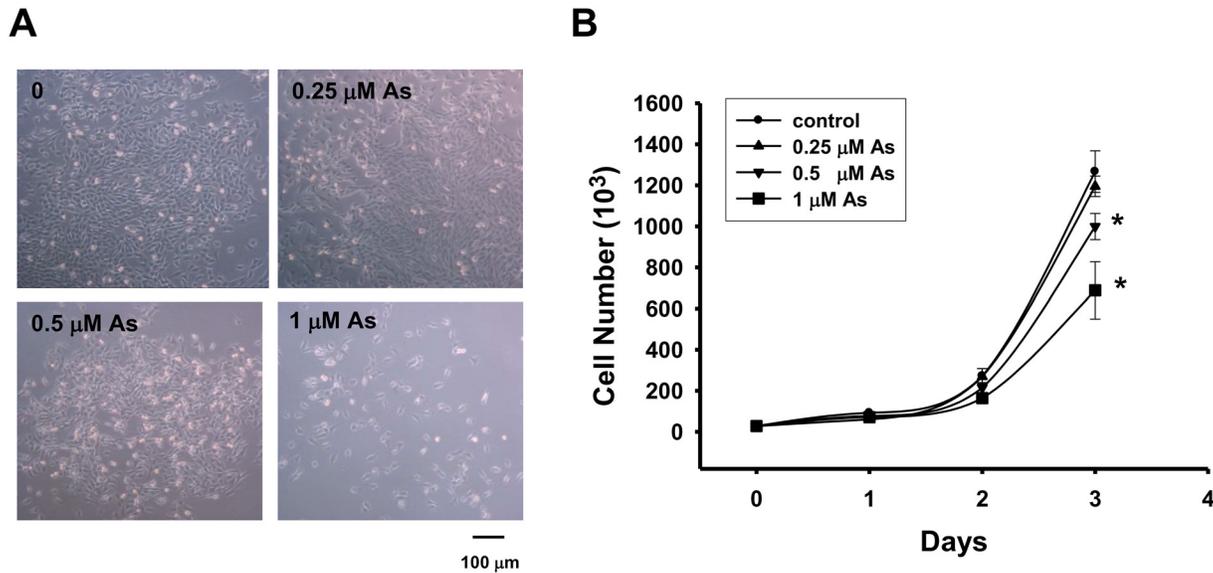
### Effects of submicromolar $\text{As}_2\text{O}_3$ on myoblast growth and apoptosis

To determine the effect of  $\text{As}_2\text{O}_3$  on the cell growth of myoblasts, the C2C12 cells were cultured in growth media with or without the treatment of  $\text{As}_2\text{O}_3$  (0.25–1  $\mu\text{M}$ ) for 24–72 h. As shown in [Fig 1](#),  $\text{As}_2\text{O}_3$  reduced the C2C12 myoblast growth in a dose and time-dependent manner. Treatment with 0.5 and 1  $\mu\text{M}$   $\text{As}_2\text{O}_3$  for 72 h significantly decreased C2C12 myoblast growth.

We next investigated whether or not apoptosis was involved in the myoblast growth inhibition by  $\text{As}_2\text{O}_3$ . As shown in [Fig 2](#),  $\text{As}_2\text{O}_3$  (0.25–1  $\mu\text{M}$ ) did not induce the myoblast apoptosis determined by annexin-V staining after 72 h treatment.

### Effects of low-concentration $\text{As}_2\text{O}_3$ on PCNA protein expression and BrdU incorporation

Next, we confirmed the effect of low-concentration  $\text{As}_2\text{O}_3$  (0.25–1  $\mu\text{M}$ ) on myoblast proliferation. As shown in [Fig 3A](#), the nuclear protein expression of PCNA (a marker for cell proliferation) [25] in C2C12 myoblasts was significantly decreased in a dose-dependent manner after  $\text{As}_2\text{O}_3$  treatment. Moreover, BrdU incorporation into the myoblasts, which reflects the rate of DNA synthesis, also significantly reduced in a dose-dependent manner after  $\text{As}_2\text{O}_3$  treatment ([Fig 3B](#)). These results indicate that  $\text{As}_2\text{O}_3$  inhibits the myoblast proliferation at submicromolar concentrations.



**Fig 1. As<sub>2</sub>O<sub>3</sub> decreases myoblast growth.** C2C12 myoblasts were cultured with or without As<sub>2</sub>O<sub>3</sub> (0.25, 0.5 and 1 μM) for 24–72 h. (A) Cell morphology in culture was assessed by light microscopy after incubation for 72 h in the presence or absence of As<sub>2</sub>O<sub>3</sub>. (B) The cell numbers were determined using trypan blue assay. Data are presented as means ± SEM of three independent experiments. \**P* < 0.05 vs control.

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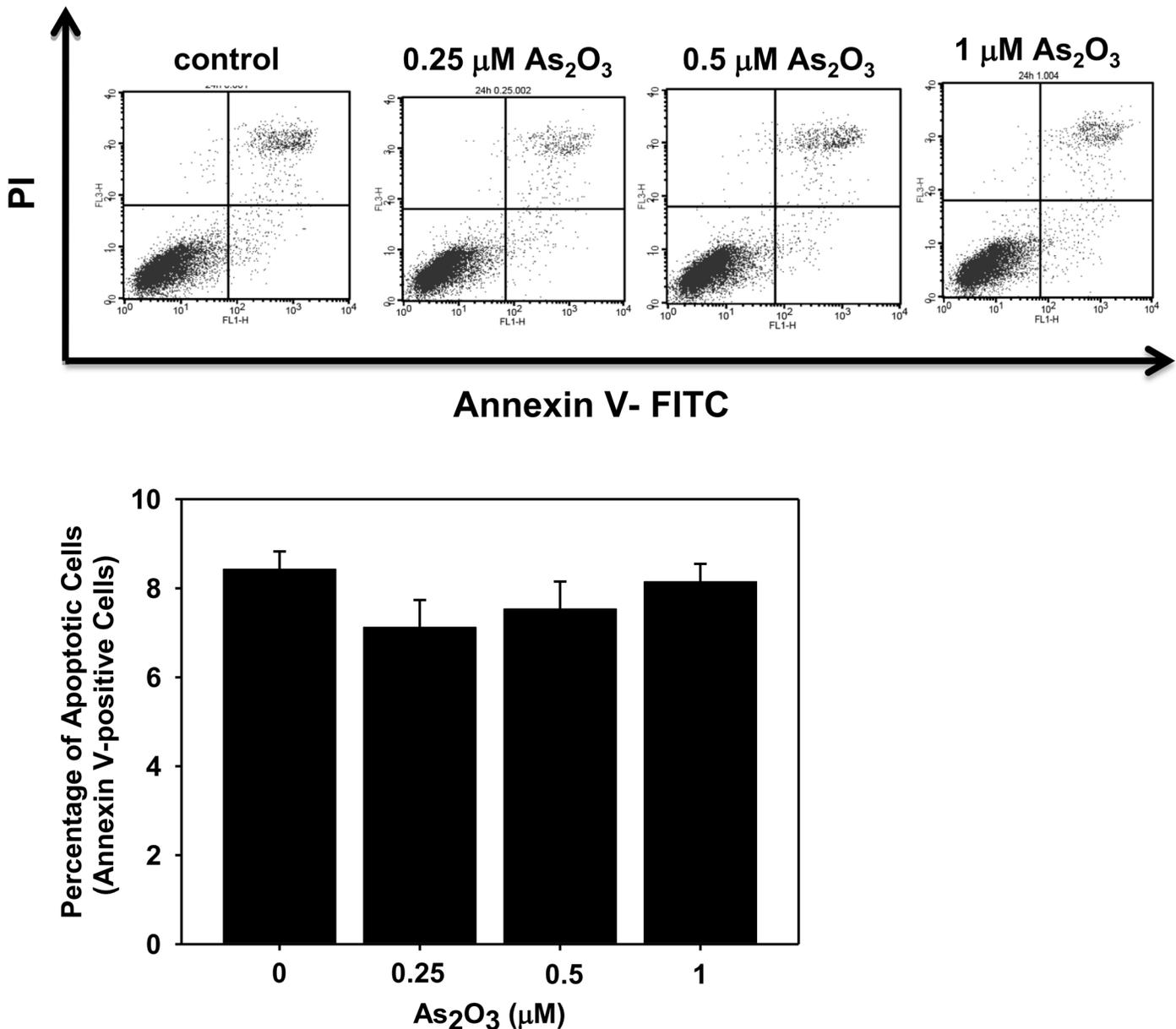
### Effects of low-concentration As<sub>2</sub>O<sub>3</sub> on intracellular arsenic contents and ROS levels

ROS regulates high-concentration As<sub>2</sub>O<sub>3</sub> (10–80 μM)-induced cell apoptosis [24,26]. Thus, we tested the arsenic contents and ROS levels in As<sub>2</sub>O<sub>3</sub>-treated myoblasts. As shown in Fig 4A, the arsenic contents of C2C12 myoblasts increased in a dose-dependent manner 24 h after treatment with As<sub>2</sub>O<sub>3</sub> (0.5–10 μM). The generation of ROS in the myoblasts was determined by DCF fluorescence. As shown in Fig 4B, As<sub>2</sub>O<sub>3</sub> 0.25–1 μM did not affect ROS generation in the myoblasts, but 10 μM of As<sub>2</sub>O<sub>3</sub> markedly increased the intracellular ROS level.

### Effects of low-concentration As<sub>2</sub>O<sub>3</sub> on G1 and G2-M phase arrest and protein expression of cell cycle-regulatory proteins

We investigated whether or not low-concentrations of As<sub>2</sub>O<sub>3</sub> affected the cell cycle distribution by flow cytometry. As shown in Fig 5, As<sub>2</sub>O<sub>3</sub> (0.5 and 1 μM) significantly increased the percentages of C2C12 myoblasts in the G1 and G2/M phases after 72 h of exposure. This was accompanied by a significant decrease in the proportion of cells in the S phase. These results indicate that low-concentration inorganic arsenic inhibits the proliferation of C2C12 myoblasts by inducing G1 and G2/M phase cell cycle arrest.

The cell cycle regulatory proteins were evaluated to determine the possible mechanism of low-concentration As<sub>2</sub>O<sub>3</sub>-altered cell cycle progression. As shown in Fig 6, there were no changes in the protein levels of the CDK inhibitors p21 and p27 in C2C12 myoblasts treated with As<sub>2</sub>O<sub>3</sub> (0.25–1 μM) for 48 h. Moreover, G1 progression and the G1/S transition are regulated by cyclin D1, which activates CDK 4 and CDK6, and cyclin E, which activates CDK2. Treatment of C2C12 myoblasts with low-concentrations of As<sub>2</sub>O<sub>3</sub> for 48 h decreased the protein levels of CDK2, CDK4, cyclin E, and cyclin D1 in a dose-dependent manner. Moreover, the cyclin B1 and CDC-2 proteins are related to the progression of G2 phase [27,28]. We further found that As<sub>2</sub>O<sub>3</sub> treatment down-regulated cyclin B1, but not cdc 2, protein expressions



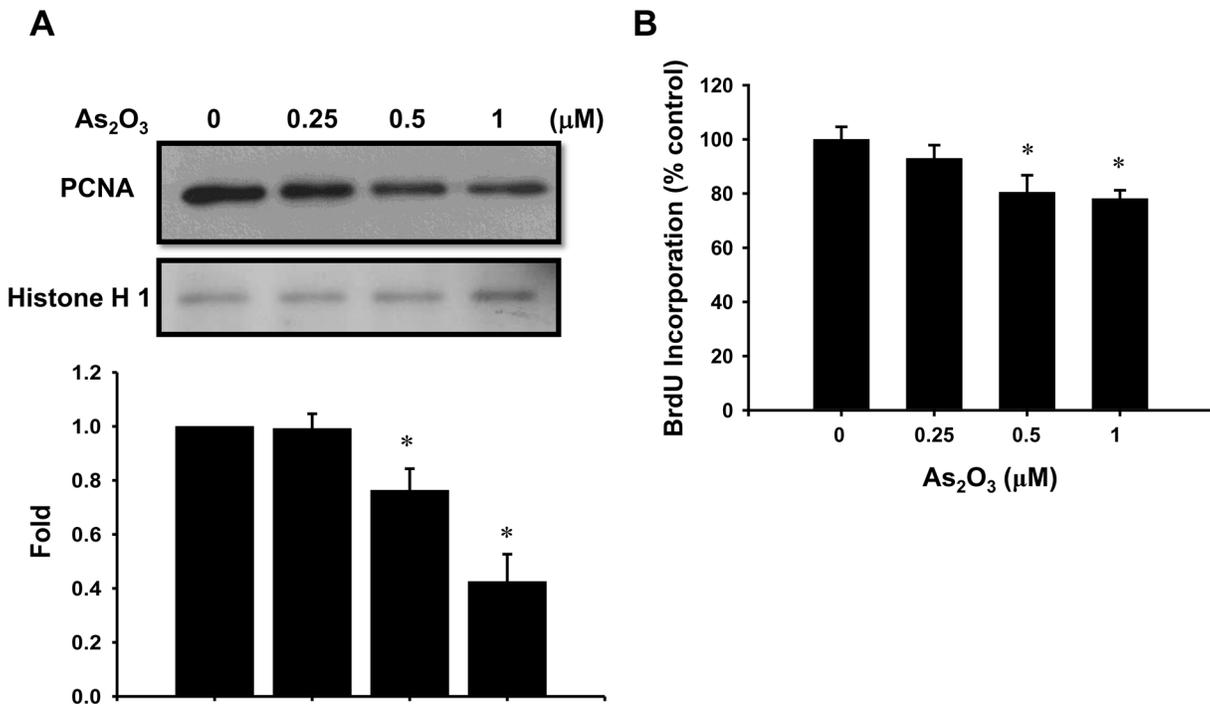
**Fig 2. Low-concentration As<sub>2</sub>O<sub>3</sub> does not induce myoblast apoptosis.** (A) C2C12 myoblasts were cultured with or without As<sub>2</sub>O<sub>3</sub> (0.25, 0.5 and 1 μM) for 72 h. Apoptotic cells were identified using an annexin-V assay. The percentage of annexin-V positive cells was calculated. Data are presented as means ± SEM of three independent experiments. No significant differentiations were observed between the groups ( $P > 0.05$ ).

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(Fig 6). These results indicate that low-concentrations of As<sub>2</sub>O<sub>3</sub> inhibit the CDK and cyclin expression, which are involved in the transition from G1/to S and G2 to M phase.

### Effect of low-concentration As<sub>2</sub>O<sub>3</sub> on the phosphorylation of Akt

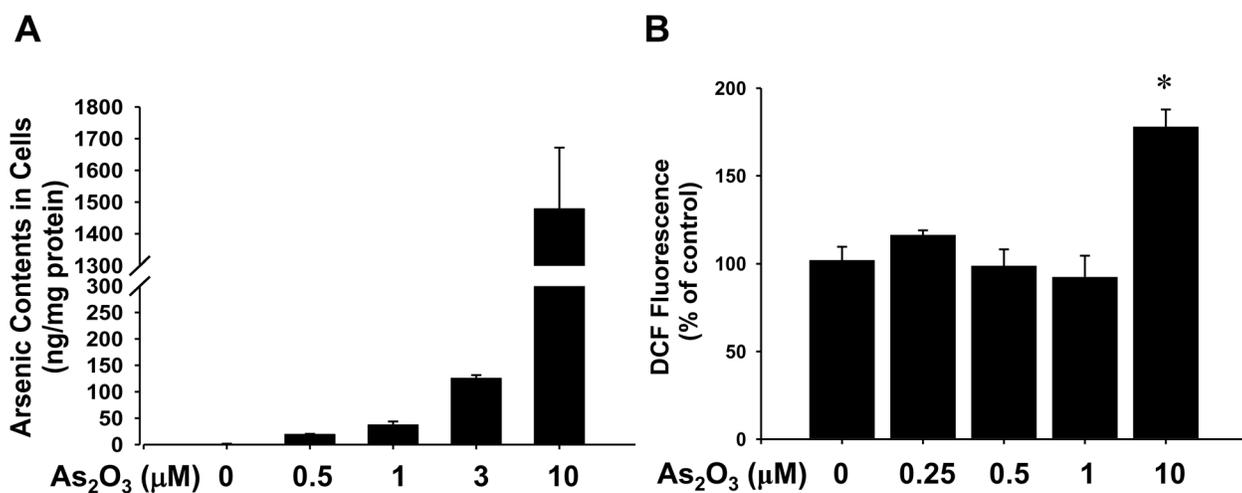
Akt (also known as protein kinase B) signaling pathway coordinates or synergistically promotes cell growth and progression throughout the cell cycle [29]. We investigated whether or not the As<sub>2</sub>O<sub>3</sub>-induced myoblast proliferation inhibition was associated with Akt signaling pathway. As shown in Fig 7, As<sub>2</sub>O<sub>3</sub> (2.5–1 μM) suppressed the phosphorylation of Akt (at



**Fig 3. As<sub>2</sub>O<sub>3</sub> decreases the nuclear levels of proliferating cell nuclear antigen (PCNA) protein expression and bromodeoxyuridine (BrdU) incorporation into the myoblasts.** (A) C2C12 myoblasts were treated with As<sub>2</sub>O<sub>3</sub> (0.25–1 μM) for 72 h. After incubation, the nuclear extracts were prepared from the cells and analyzed by Western blotting. Histone-1 expression was used as the internal control. PCNA protein expression was quantified by densitometry. (B) C2C12 myoblasts were treated with As<sub>2</sub>O<sub>3</sub> (0.25–1 μM) for 48 h. BrdU incorporation was measured using an ELISA kit. Data are presented as means ± SEM of three independent experiments. \**P* < 0.05 vs control.

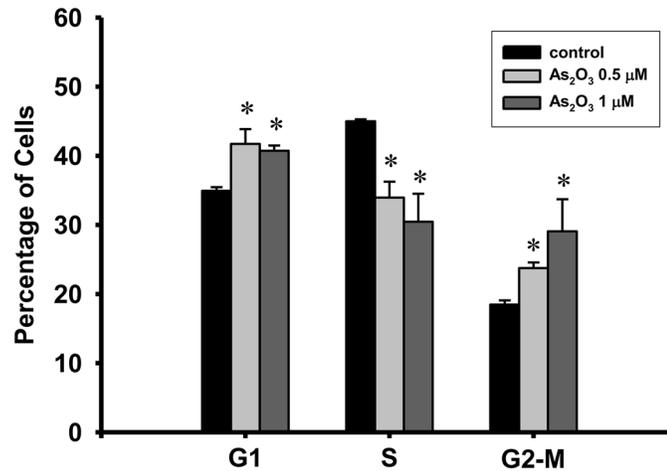
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Ser473) in a dose-dependent manner, whereas total form of Akt protein expression was not altered. Moreover, the insulin-like growth factor-1 (IGF-1)/Akt signaling pathway is involved in the positive regulation of skeletal muscle mass [30]. IGF-1 (50 and 100 ng/ml) markedly increased the Akt phosphorylation in myoblasts and reversed the Akt phosphorylation



**Fig 4. Detection of intracellular arsenic and reactive oxygen species (ROS) in myoblasts.** C2C12 myoblasts were treated with As<sub>2</sub>O<sub>3</sub> (0.5–10 μM) for 24 h (A) or 4 h (B). Arsenic contents and ROS levels in the cells were determined as described in Materials and Methods. Data are presented as means ± SEM for at least triplicate determinations. \**P* < 0.05 vs control.

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**Fig 5. As<sub>2</sub>O<sub>3</sub> alters the cell cycle phase distribution in myoblasts.** C2C12 myoblasts were treated with As<sub>2</sub>O<sub>3</sub> (0.5 and 1 μM) for 72 h. Cells were washed, fixed, and stained with PI, and then the DNA histogram was analyzed by flow cytometry. Data are presented as means ± SEM of three independent experiments. \**P* < 0.05 vs control.

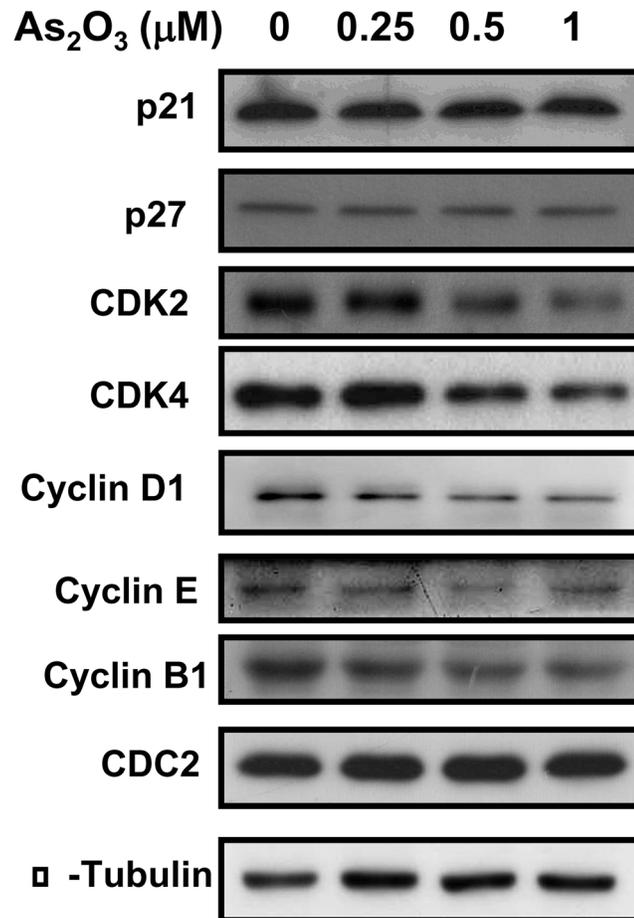
doi:10.1371/journal.pone.0137907.g005

inhibition by As<sub>2</sub>O<sub>3</sub> (1 μM) (Fig 8A). IGF-1 also significantly reversed the inhibition of myoblast cell proliferation by As<sub>2</sub>O<sub>3</sub> (1 μM) (Fig 8B).

## Discussion

As<sub>2</sub>O<sub>3</sub> is an active agent for leukemia (APL) therapy [5]. As<sub>2</sub>O<sub>3</sub> has also been considered to be a novel therapeutic agent for lymphoproliferative and autoimmune syndromes [31]. The plasma arsenic levels performed a peak level of 5.54 μM to 7.30 μM in patients for which As<sub>2</sub>O<sub>3</sub> was administered intravenously at the dose of 10 mg/d for APL treatment [32]. As<sub>2</sub>O<sub>3</sub> was reported to induce acute promyelocytic leukemia cell differentiation at lower concentrations (0.1–0.5 μM), but induce cell apoptosis at higher concentrations (0.5–2 μM) [33]. It has been shown that arsenic level of > 0.64 mg/L in drinking water is associated with an increase in liver cancer mortality in both sexes [34]. In addition, high concentrations of As<sub>2</sub>O<sub>3</sub> (30, 60, and 90 μM) for various periods (24, 48, and 72 h) caused apoptosis in primary cardiomyocytes [35]. As<sub>2</sub>O<sub>3</sub> (5–7 μM) also increased oxidative stress, mitochondrial dysfunctions, or apoptosis in H9c2 cardiomyoblasts [36–38]. On the other hand, our previous studies showed that C2C12 myoblasts and primary mouse and human myoblasts cultured in differentiation media with As<sub>2</sub>O<sub>3</sub> (0.1–0.5 μM) for 4 days significantly inhibited the myoblast differentiation [22]. Moreover, C2C12 myoblasts underwent apoptosis in response to As<sub>2</sub>O<sub>3</sub> (3–10 μM) for 24 h of treatment [24]. In the present study, the myoblast growth (proliferation) was decreased by As<sub>2</sub>O<sub>3</sub> at submicromolar concentrations (0.25–1 μM) after 72 h of exposure. These results indicated that As<sub>2</sub>O<sub>3</sub> at the concentrations of 0.5–1 μM may not only affect the myoblast differentiation but also inhibit myoblast cell growth without apoptosis induction. The findings also suggest that exposure to As<sub>2</sub>O<sub>3</sub> at doses relevant to human exposure may alter the myoblast proliferation and may interfere with the skeletal muscle cell development/growth.

Muscle growth, maintenance, and repair of injured muscle fibers require myogenesis [39]. Decreased proliferation of myoblasts could reduce the number of muscle fibers. C2C12 myoblasts are a good *in vitro* model for myoblast proliferation and differentiation and are easily reproducible in cell cultures [40,41]. We found that treatment with 0.25–1 μM As<sub>2</sub>O<sub>3</sub> resulted in the inhibition of C2C12 myoblast growth in a dose-dependent manner. Moreover, PCNA is

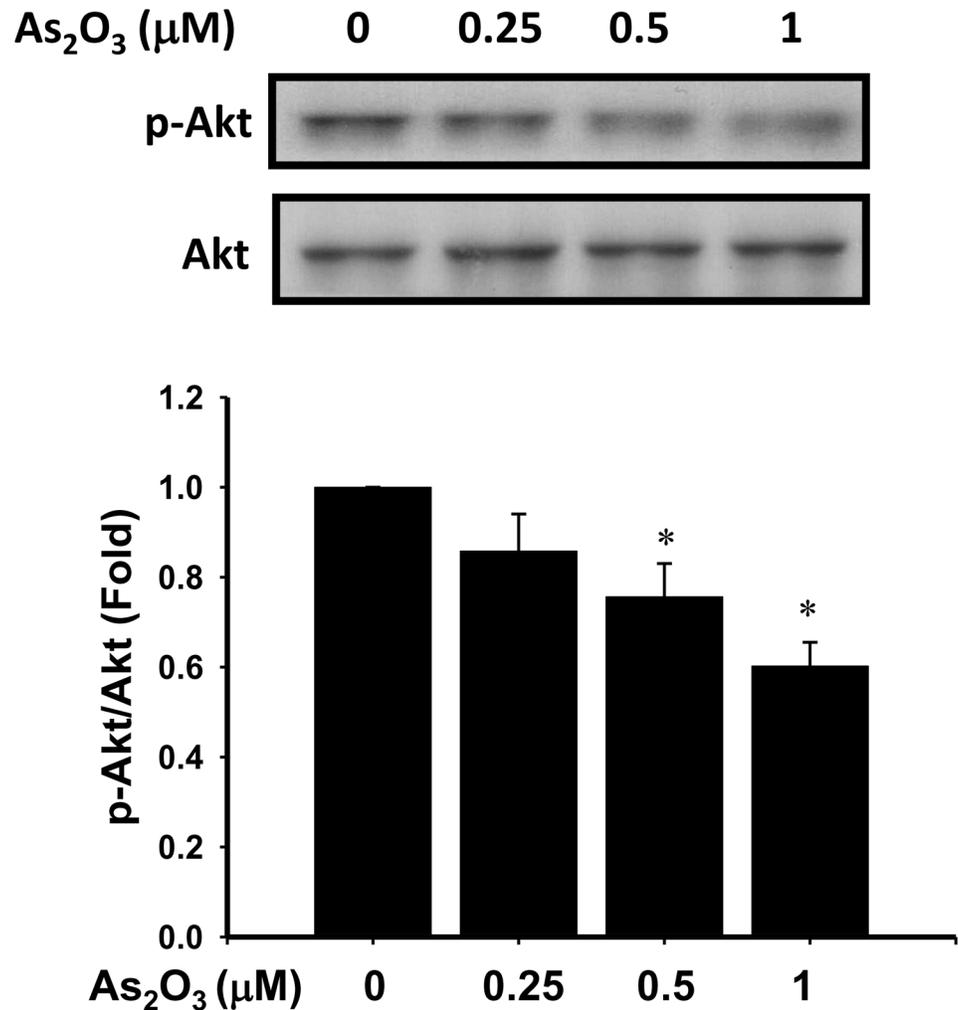


**Fig 6. As<sub>2</sub>O<sub>3</sub> affects the expressions of cell cycle-related proteins in myoblasts.** C2C12 myoblasts were harvested at 48 h after incubation with As<sub>2</sub>O<sub>3</sub> (0.25–1 μM). The protein expressions of p21, p27, cyclin-dependent kinase (CDK)2, CDK4, cyclin D1, cyclin E, cyclin B, and cell division cycle (CDC)2 were analyzed by Western blotting. Alpha-tubulin served as the sample equal loading control. Results are representative at least three independent experiments.

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an auxiliary protein of DNA polymerase δ, the level of which correlates with DNA synthesis during the cell cycle. The PCNA level is maximal during the S-phase of the cell cycle [42]. PCNA is also a marker for evaluating the proliferation activity of cells [25]. Our data showed that low-concentration As<sub>2</sub>O<sub>3</sub> induced decreases in nuclear PCNA protein expression and BrdU incorporation in the myoblasts, but did not induce cell apoptosis. We also found that arsenic contents are increased in myoblasts after the treatment with low-concentration As<sub>2</sub>O<sub>3</sub>, indicating that As<sub>2</sub>O<sub>3</sub> enters into myoblasts. Unexpectedly, As<sub>2</sub>O<sub>3</sub> (0.5 and 1 μM) did not affect the generation of ROS in myoblasts. These findings indicate that As<sub>2</sub>O<sub>3</sub> at submicromolar concentrations inhibits skeletal myoblast proliferation without cytotoxicity and its mechanism of action does not depend on ROS.

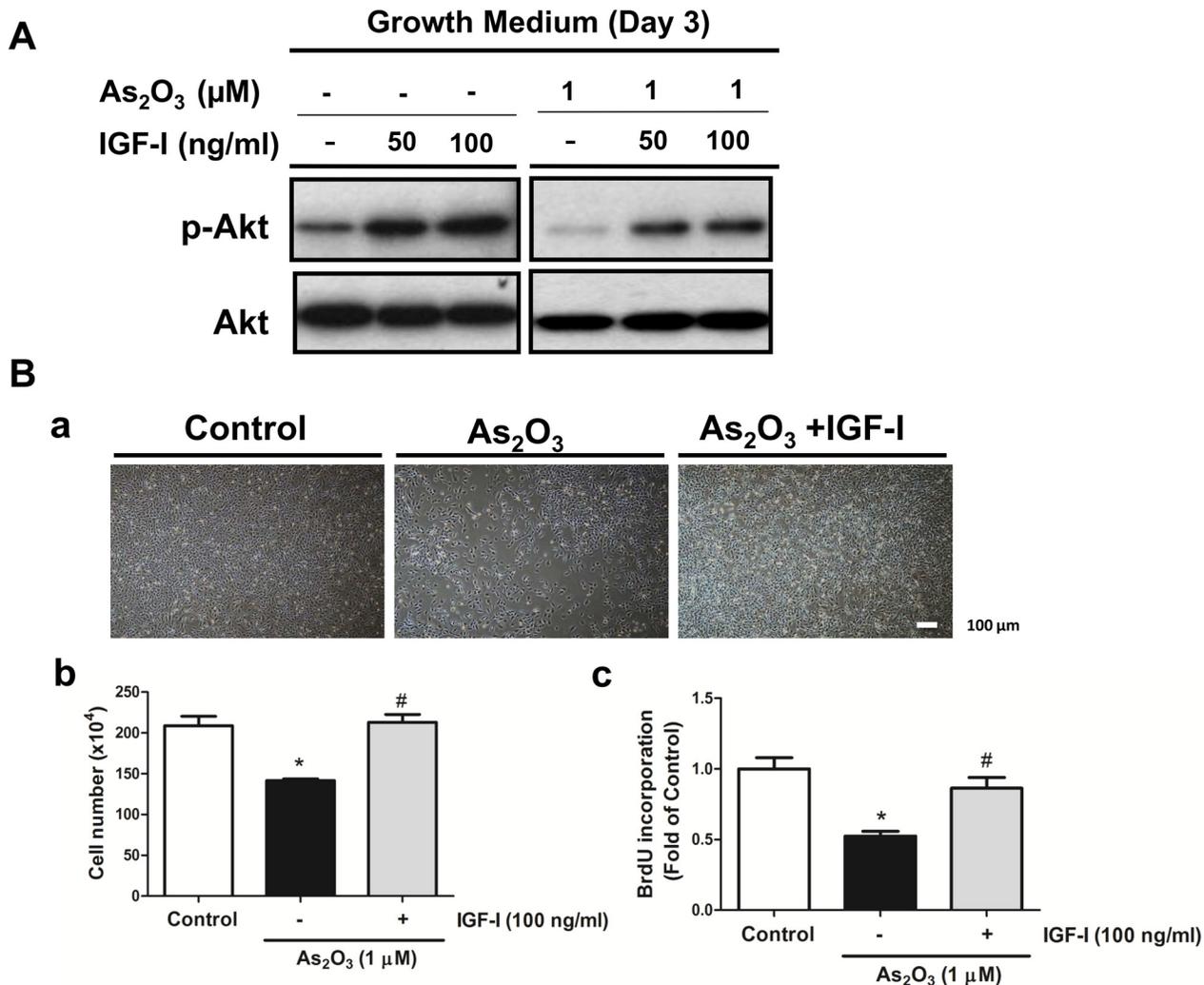
The cell cycle is regulated by complexes of cyclins and cyclin-dependent kinases (CDKs), whose formation and activation promote the cell cycle progression [43]. The main targets for this regulation are the cyclin-CDK complexes, cyclin-D1/CDK4 and cyclin-E/CDK2 [43,44]. These complexes control the G1 to S transition through phosphorylation and inactivation of the retinoblastoma protein and PCNA expression [44], while Cdc2 protein and cyclin B1 regulate the progression of G2/M phase [27]. As<sub>2</sub>O<sub>3</sub> (1–2 μM) inhibits the proliferation of human



**Fig 7. As<sub>2</sub>O<sub>3</sub> decreases the phosphorylation of Akt in myoblasts.** C2C12 myoblasts were harvested at 48 h after incubation with As<sub>2</sub>O<sub>3</sub> (0.25–1 μM). The phosphorylation of Akt and total Akt protein were determined by Western blotting. Protein expression was quantified by densitometry. Data are presented as means ± SEM of three independent experiments. \**P* < 0.05 vs control.

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endothelial cells by preventing the cell cycle progression from G1 to S and by causing G2/M phase arrest of the cell cycle [45]. Yih and Lee (2000) also reported that arsenic (5 μM) induced G2/M arrest with no apoptosis in human fibroblasts [12]. Renal cell carcinoma A498 cells treated with 2.5 μM As<sub>2</sub>O<sub>3</sub> for 72 h resulted in a down-regulation of cyclin D1 [46]. In addition, As<sub>2</sub>O<sub>3</sub> down-regulates cyclin D1 transcription via a reduction of Sp1 transcription factor in gallbladder carcinoma cells [47]. In the present study, the results of myoblast cell cycle analysis showed that 0.5 and 1 μM As<sub>2</sub>O<sub>3</sub> induced G1 phase cell cycle arrest. These cells therefore could not make the transition to the S-phase of the cell cycle, altering the progression of G2 to M phase. Moreover, 0.5 and 1 μM As<sub>2</sub>O<sub>3</sub> significantly decreased the cyclin D1 and CDK4 protein levels in a dose-dependent manner. This indicates that arsenic-mediated G1 cell cycle arrest and down-regulation of cyclin E protein in myoblasts are related to suppression of cyclin D1 and CDK4. Low-concentration As<sub>2</sub>O<sub>3</sub> also led to a marked dose-dependent decrease in cyclin B1 expression, suggesting As<sub>2</sub>O<sub>3</sub> induced G2/M phase arrest. On the other hand, the up-regulation of CDK inhibitors can inhibit the cyclin-CDK complexes and causes cell cycle arrest [48].



**Fig 8. Inhibitory effects of As<sub>2</sub>O<sub>3</sub> on Akt phosphorylation and cell proliferation are reversed by insulin-like growth factor 1 (IGF-I).** C2C12 myoblasts were pretreated with IGF-I (50 and 100 ng/ml) for 1 h and then incubated with 1 μM As<sub>2</sub>O<sub>3</sub> for 72 h. (A) The phosphorylation of Akt and total Akt protein were determined by Western blotting. (B) Cell morphology was observed under an inverted phase contrast microscope (a). Cells were counted after staining with 0.4% trypan blue (b) and bromodeoxyuridine (BrdU) incorporation assay (c). All data are presented as means ± SEM for at least three independent experiments. Scale bar = 100 μm. \**P* < 0.05 vs control; #*P* < 0.05 vs As<sub>2</sub>O<sub>3</sub>.

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However, the present work showed that neither p21 nor p27 protein expressions are affected by the treatment of low-concentration As<sub>2</sub>O<sub>3</sub> in myoblasts. Thus, it is likely that the CDK inhibitors p21 and p27 may not play a significant role in the cell cycle arrest in As<sub>2</sub>O<sub>3</sub>-treated myoblasts.

An accumulation of evidence supports a crucial role for Akt activation in regulating muscle cell survival, proliferation, and differentiation [22,24,49–51]. The proliferation of C2C12 myoblasts induced by insulin depends on Akt activity [52]. Using a specific RNA interference, the Akt1 isoform is required for C2 myoblast cell proliferation, while the Akt2 isoform plays a negative effect on cell cycle progression [53]. Over-expression of constitutive Akt (myrAkt) activity extended the half-life of cyclin D1 protein levels, whereas treatment with wortmannin (a PI3K inhibitor) accelerated cyclin D1 degradation [54]. Yang et al. (2007) reported that the PI3K/Akt/GSK-3β signaling cascade participates in myostatin-stimulated cyclin D1 degradation and

C2C12 myoblast proliferation inhibition [51]. Moreover, IGF-1-activated Akt signaling increases muscle mass through the induction of protein synthesis [30]. IGF-1 has also markedly increased the cyclin D1 expression in proliferating C2C12 myoblasts [55]. In the present study, we observed that As<sub>2</sub>O<sub>3</sub> at submicromolar concentrations inhibited the cell proliferation and phosphorylation of Akt and protein expression of cyclin D1 in C2C12 myoblasts in a dose-dependent manner. IGF-1 significantly reversed the inhibitory effect of As<sub>2</sub>O<sub>3</sub> on Akt phosphorylation and cell proliferation in myoblasts. These results indicate that the decreased phosphorylation of Akt may be partially responsible for the suppression of cyclin D1 protein levels and proliferation of myoblasts exposed to low-concentration As<sub>2</sub>O<sub>3</sub>.

In conclusion, the results of this study provide evidence that As<sub>2</sub>O<sub>3</sub> at submicromolar concentrations inhibits skeletal myoblast proliferation and induces cell cycle arrest. ROS are most likely not involved in the inhibitory effect of As<sub>2</sub>O<sub>3</sub> on myoblast proliferation. Low-concentration As<sub>2</sub>O<sub>3</sub> also suppressed CDK and cyclin expressions, which may be, at least in part, associated with the inhibition of Akt signaling. However, the more detailed mechanisms still need to be clarified in the future. Based on these results we suggest that As<sub>2</sub>O<sub>3</sub> interferes with myoblast growth and may be an environmental risk factor for skeletal muscle cell development and growth.

## Author Contributions

Conceived and designed the experiments: SHL KCL. Performed the experiments: SHL RSY YPY CYC KST. Analyzed the data: SHL RSY YPY KCL. Wrote the paper: KCL SHL.

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