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Arsenic trioxide blocked proliferation and cardiomyocyte differentiation of human induced pluripotent stem cells: Implication in cardiac developmental toxicity

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ABSTRACT

Arsenic trioxide (ATO) has been recommended as the first-line agent for the treatment of acute promyelocytic leukaemia (APL), due to its substantial anticancer effect. Numerous clinical reports have indicated that ATO is a developmental toxicant which can result in birth defects of human beings. But whether arsenic trioxide can lead to human cardiac developmental toxicity remains largely unknown. So the present study aims to explore the influence and mechanisms of ATO on human cardiac development by using a vitro cardiac differentiation model of human induced pluripotent stem cells (hiPSCs). Here we found that clinically achievable concentrations (0.1, 0.5 and 1 μM) of ATO resulted in a significant inhibition of proliferation during the whole process of cardiac differentiation of hiPSCs. Meanwhile, TUNEL assay revealed that ATO could cause cell apoptosis during cardiac differentiation in a concentration-dependent manner. Consistently, we found that ATO reduced the expressions of mesoderm markers Brachyury and EOMES, cardiac progenitor cell markers GATA-4, MESP-1 and TBX-5, and cardiac specific marker α-actinin in differentiated hiPSCs. Furthermore, ATO treatment had caused DNA damage which was shown in the upregulation of γH2AX, a sensitive marker for DNA double-strand breaks. Taken together, ATO blocked cardiomyocyte differentiation, induced apoptosis and cell growth arrest during cardiac differentiation of hiPSCs, which might be associated with DNA damage.

1. Introduction

Arsenic is a common and natural substance which exists in organic and inorganic forms (Dong et al., 2015). However, arsenic trioxide (ATO) has been used as a medicinal agent for a long time (Emadi and Gore, 2010). For example, ATO was used for the clinical treatment of acute promyelocytic leukemia (APL) (Ma et al., 2015). In addition, ATO also appears to have the ability to inhibit different kinds of solid tumors such as breast (Chow et al., 2004; Ye et al., 2005; Wang et al., 2011), lung (Chien et al., 2011), liver (Li et al., 2013) and colon (Nakagawa et al., 2002) cancers. Meanwhile, ATO has the ability to trigger the apoptosis and cell cycle arrest in many kinds of cancer cells (Cheung et al., 2007). However, the effect of ATO on cardiomyocyte differentiation of hiPSCs remains incompletely unknown.

Abbreviations: ATO, arsenic trioxide; hiPSCs, human induced pluripotent stem cells; APL, acute promyelocytic leukemia; hPSCs, human pluripotent stem cells; hESCs, Human embryonic stem cells; PBS, phosphate-buffered saline; EdU, 5-Ethynyl-20-deoxyuridine; CVPCs, cardiovascular progenitor cells; CM, cardiomyocyte; GAPDH, glyceraldehyde phosphate dehydrogenase; EOMES, eomesodermin; MESP-1, mesoderm posterior BHLH transcription factor 1; TBX-5, T-box transcription factor 5; GATA-4, GATA binding protein 4

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Human pluripotent stem cells (hPSCs) including embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) (Lin et al., 2017) have been shown to differentiate into multiple cell types in vitro and in vivo (Minami et al., 2012). In 2006, Takahashi and Yamanaka found that reprogramming fibroblasts into pluripotent stem cells (iPSCs) by ectopic retroviral expression of 4 transcription factors (Oct-4, Sox-2, c-Myc, Klf-4), and the iPSCs showed the similar ability to mouse ESCs (Takahashi and Yamanaka, 2006). This finding provided a new solution in drug discovery and cellular therapy (Yu et al., 2007; Cherry and Daley, 2013). After that, iPSCs had been shown to have the ability to differentiate into cardiomyocytes (Zwi et al., 2009). Cardiomyocyte differentiation from human induced pluripotent stem cells provided a useful platform to explore cardiac developmental toxicity in vitro.

In this study, we aimed to evaluate the toxic effects of ATO on cardiac development by a vitro cardiac differentiation model of hiPSCs (Jha et al., 2017). Our study reveals the inhibitory role of ATO in cardiomyocyte differentiation by triggering cellular DNA damage, which provides new insights in molecular mechanism of ATO induce cardiomyocyte differentiation toxicity, and gives the safety guidance for the clinical application of ATO in pregnant patients.

2. Materials and methods

2.1. Culture of hiPSCs and cardiomyocyte differentiation

hiPS cells were cultured in PGM_1 medium (Cellapy, China) at the temperature of 37 °C in 5% CO_2. When the density reached to 60–70%, hiPSCs were washed with phosphate-buffered saline (PBS) once and then incubated with 0.5 mM EDTA for 2 min in 37 °C. And then the cells were washed gently for 4 times with PGM_1 medium and passaged to Matrigel-coated plates. When cells were 70–80% confluence at 3–4 days after plating, the medium was changed from PGM_1 to differentiation basal medium I (CELLAPY, CA2004500, CHINA) to induce mesoderm cell differentiation. After culturing for 2 days, the medium was changed from differentiation basal medium I to differentiation basal medium II (CELLAPY, CA2004500, CHINA) to induce cardiovascular progenitor cell (CVPC) differentiation. Finally, the medium was changed from differentiation basal medium II to differentiation basal medium III (CELLAPY, CA2004500, CHINA) at day 4 to induce cardiomyocyte differentiation. Different concentrations of ATO (0, 0.1, 0.5 and 1 μM) were separately added to the medium.

2.2. TUNEL assay

TUNEL assay was performed as described previously (Han et al., 2019) with the In Situ Cell Death Detection Kit (TUNEL fluorescence FITC kit, Roche, Indianapolis, IN, USA). Then washing the cells that grew on Glass Bottom Cell Culture Dish with PBS and fixed in 500 μL 4% paraformaldehyde solution for 15 min at 37 °C. Then the cells were permeabilized in a solution containing 0.1% Triton X-100 for 2 min, followed by incubation in 500 μl freshly prepared TUNEL reaction mixture for 1 h at 37 °C in the dark. The cells were then washed with PBS followed by incubating with DAPI for 15 min. Then the stained cells were examined under a confocal fluorescence microscope (FV10C-W3, Olympus, Japan).

2.3. EdU assay

The EdU assay was used to measure the proliferation of the cells under the influence of ATO. Cells that incubated on Glass Bottom Cell Culture Dish were treated as experiment design. After appropriate treatment, cells were fixed with 4% paraformaldehyde followed by penetrating with 0.4% Triton X-100. Cells' proliferation was determined by 5-Ethynyl-20-deoxyuridine (EdU) assay kit (Ribobio Co., Ltd. Guangzhou, China). The cells were observed by a confocal fluorescence microscope (FV10C-W3, Olympus, Japan). Nuclei that double labeled with EdU and DAPI were considered to be positive cells.

2.4. Real-time polymerase chain reaction

Real-time quantitative reverse transcriptase PCR were performed as described previously (Cai et al., 2015). Total RNA was extracted by TRIzol reagent (Ambion, Life Technologies, USA) according to manufacturer's instructions. The FastStart Universal SYBR Green Master (Rox) was used in real-time PCR for relative quantification of RNAs. Then using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) to analyze. GAPDH and 18S were used as an internal control. All primer sequences are listed in Table 1.

2.5. Immunofluorescence assays

Cells were fixed with 4% paraformaldehyde for 15 min at 37 °C. Then cells were washed by PBS for 3 times followed by penetrating with 0.3% Triton X-100 for 15 min at room temperature. After culturing with normal goat serum for 1 h, the cell were stained with γH2AX (ab26350, Abcam) overnight. Before staining with secondary antibody (EarthOx, San Francisco, CA, USA) cells were washed by PBS for 3 times. The images were captured by confocal fluorescence microscope (FV10C-W3, Olympus, Japan).

2.6. Statistical analysis

Group data was described as the mean ± S.E.M. One-way ANOVA accompanied by Bonferroni's Multiple Comparison. Tests were used to analyze comparisons. $p < 0.05$ was considered to indicate a significant difference. Data were analyzed by using the GraphPad Prism 5.0 software.

Table 1
Oligonucleotide primers and PCR conditions for quantitative real-time PCR.

Species	Genes	Forward primer	Reverse primer	Annealing (℃)
Human	GAPDH	GGTGAAGCAGGCGTCGGAGG	GAGGGCAATGCCAGCCCCAG	51
	18S	CCTGGATACCGCAGCTAGGA	GCGGCGCAATACGAATGCCC	51
	MESP-1	GCCAGAGCCTGACCAAGATC	GACTCTCCTCGCTGAGGCCTA	51
	Brachyury	CTATTCTGACAACTCACCTGCAT	ACAGGCTGGGGTACTGACT	51
	GATA-4	TCCCTCTTCCCTCCTCAAAT	TCAGCGTGTAAAGGCATCTG	51
	TBX-5	AAATGAAACCCAGCATAGGAGCTGGC	ACACTCAGCCTCACATCTTACCCT	51
	EOMES	GTCTGAAGGGGCAAGGATTTA	CAACCTGGGACCAACAAACTA	51
	α-actinin	GTGAACACCCCTAAACCCGA	ATCCTGTTAGCCGCTGTCTC	51

3. Results

3.1. Effects of ATO on proliferation and differentiation of hiPSCs into mesoderm

To investigate the effect of ATO on mesoderm differentiation of hiPSCs, we firstly treated cells with clinically achievable concentrations of ATO (0.1, 0.5 and 1 μM). After culturing for 24 h, we observed ATO could cause the increase of cell death in hiPSCs during mesodermal differentiation (Fig. 1a). EdU incorporation assay showed that ATO significantly inhibited the proliferation of differentiating hiPSCs in a concentration-dependent manner (Fig. 1b). In particular, almost no EdU-positive (red) cells were observed when the concentration of ATO up to 1 μM (Fig. 1b). Then, we investigated whether treatment with ATO could lead to the apoptosis of hiPSCs during mesoderm differentiation. As shown in Fig. 1c, there were more TUNEL-positive (green) cells in ATO group than Control group. These results suggested that ATO triggers apoptosis of differentiating hiPSCs. Furthermore, we explored the influence of ATO treatment on mesodermal differentiation of hiPSCs. qRT-PCR assay showed that exposure to ATO for 24 h and 48 h resulted in the downregulation of mesodermal markers Brachyury and EOMES (Fig. 1d), indicating that ATO inhibits mesodermal differentiation of hiPSCs. Taken together, the above data suggests that ATO not only blocks mesoderm differentiation of hiPSCs but also causes apoptosis.

3.2. Effects of ATO on differentiation of mesoderm cells into cardiovascular progenitor cells

Next, we sought to determine the effects of ATO on the differentiation of hiPSCs from mesodermal cells to cardiovascular progenitor cells (CVPCs). As shown in Fig. 2a, exposing to ATO resulted in a concentration-dependent cell death in this stage. Meanwhile, EdU incorporation assay showed that the number of EdU positive cells were gradually reduced after ATO treatment (Fig. 2b). Consistently, the percentage of TUNEL-positive cells were markedly increased after treating with ATO (Fig. 2c). To further study the effects of ATO on differentiation of hiPSCs from mesodermal cells to CVPCs, we detected the expressions of cardiovascular progenitor cell markers GATA-4, MESP-1 and TBX-5 by qRT-PCR. As shown in Fig. 2d, the expressions of GATA-4, MESP-1 and TBX-5 were significantly downregulated after the treatment with ATO for 24 h. These results indicate that ATO at clinical relevant concentration has the potential to induce cell apoptosis and inhibit the differentiation of hiPSCs from mesodermal cells to CVPCs.

3.3. Effects of ATO on cardiomyocyte differentiation of CVPCs

We further explored the impacts of ATO on cardiomyocyte (CM) differentiation of CVPCs. Firstly, we found that the proliferation of differentiating cells wre significantly decreased after treating with ATO, by calculating the percentage of EdU positive cells comparing with control group. (Fig. 3b). TUNEL staining revealed that the apoptosis of cells were substantially increased after ATO treatment, indicating that ATO induces the apoptosis of hiPSCs during the differentiation from CVPCs into CMs (Fig. 3c). Furthermore, we evaluated the influence of ATO treatment on cardiomyocyte differentiation of hiPSCs from CVPCs by detecting the expression of cardiac specific marker α-actinin. The result showed that α-actinin expression was downregulated after treating with ATO for 24 and 48 h (Fig. 3d). These results suggest that ATO inhibits proliferation and differentiation of hiPSCs from CVPCs into cardiomyocytes.

3.4. ATO induced DNA damage in differentiated hiPSCs

Finally, we investigated the potential mechanism underlying ATO-induced the inhibition of proliferation and cardiac differentiation in hiPSCs. It has been reported that ATO is able to increase the expression of γH2AX, a novel marker of DNA damage. Thus, in this study, we investigated whether ATO caused DNA damage in differentiating hiPSCs. The result showed that the number of γH2AX positive cells was increased by ATO during the differentiation of hiPSCs into mesodermal cells (Fig. 4a). During mesodermal differentiation of hiPSCs, the number of γH2AX positive cells were also increased after exposing to ATO (Fig. 4b). Similarly, ATO induced a larger than 2-fold increase in the number of γH2AX during cardiomyocyte differentiation of hiPSCs (Fig. 4c). All these results suggest that ATO treatment is able to trigger DNA damage in cardiac differentiation.

4. Discussion

During the past decades, ATO has been widely used for treating many hematological malignancies (Takahashi, 2010). Many studies have showed that ATO could cause cell death and induce apoptosis in various kinds of cancer cells (Hoffman and Mielicki, 2013). For example, ATO has been shown to kill APL cells by inhibiting cell proliferation and inducing differentiation (Wang, 2003). Although ATO has a good therapeutic effect on tumor cells, its clinical application is limited due to severe side effects (Unnikrishnan et al., 2004) such as cavity effusion, cardiotoxicity and peripheral nervous infection (Nouri et al., 2006; Liu et al., 2007). In particular, it was reported that ATO was capable to cause developmental toxicity in animals (Rebuzzini et al., 2015). However, the evidence and mechanisms of human cardiac developmental toxicity induced by ATO have not been clarified. Using in vitro cardiomyocyte differentiation model of induced pluripotent stem cells, we systematically explored the cardiac developmental toxicity of ATO in this study.

It has been reported that ATO led to the growth arrest and cell apoptosis in breast cancer cells (Kasukabe et al., 2015). Also, ATO inhibited the growth of myoblast but did not cause apoptosis at clinical relevant concentrations of 0.5–1 μM (Liu et al., 2015). In our study, we found that ATO at the concentrations of 0.1, 0.5 and 1 μM inhibited cell growth in the whole process of cardiomyocyte differentiation. Particularly, we observed a significant reduction of cell proliferation by ATO at the final stage (CVPCs to CM). Many studies have demonstrated that ATO can induce apoptosis in K562 cells (Song et al., 2014), Raji cells (Li et al., 2014), human melanoma cells (Pastorek et al., 2014) and human gastric cancer SGC-7901 cells (Gao et al., 2014). It also has been reported that high concentrations of ATO (30, 60 and 90 μM) can cause apoptosis in primary cardiomyocytes (Raghu and Cherian, 2009). Nevertheless, in this study, we found that low concentrations of ATO were able to induce apoptosis of differentiated hiPSCs. It indicates that differentiated cells from hiPSCs are more susceptible to ATO damage. In addition, it has been suggested that ATO at the high concentrations can induce apoptosis, while ATO at the low concentrations can induce partial differentiation (Ouyang et al., 2008). For example, ATO can induce differentiation of CD133+ hepatocellular carcinoma cells at a low dose (Zhang et al., 2014). In this study, ATO at both low (0.1 μM) and high (1 μM) concentrations will suppress the proliferation and induce the apoptosis during hiPSCs differentiation into cardiomyocytes.

Human iPSCs have the ability to differentiate into all the three germ layers (Tao et al., 2014). Under certain conditions, cardiomyocytes can be derived from human induced pluripotent stem cells (Cao et al., 2012). It has been well documented that cardiac differentiation of hiPSCs includes three stages: mesoderm cells, cardiovascular progenitor cells (CVPCs) and cardiomyocytes (Hu et al., 2015). The abnormal changes of any step during hiPSCs differentiation will lead to the dysfuction of hiPSCs-CMs. In this study, we further observed the effects of ATO on cardiac differentiation of hiPSCs. Our findings showed that the expressions of Brachyury and EOMES were remarkably reduced by ATO during mesodermal development. We also observed that ATO decreased the expression of GATA-4, MESP-1 and TBX-5 mRNA during CVPCs development, and the downregulation of α-actinin during its

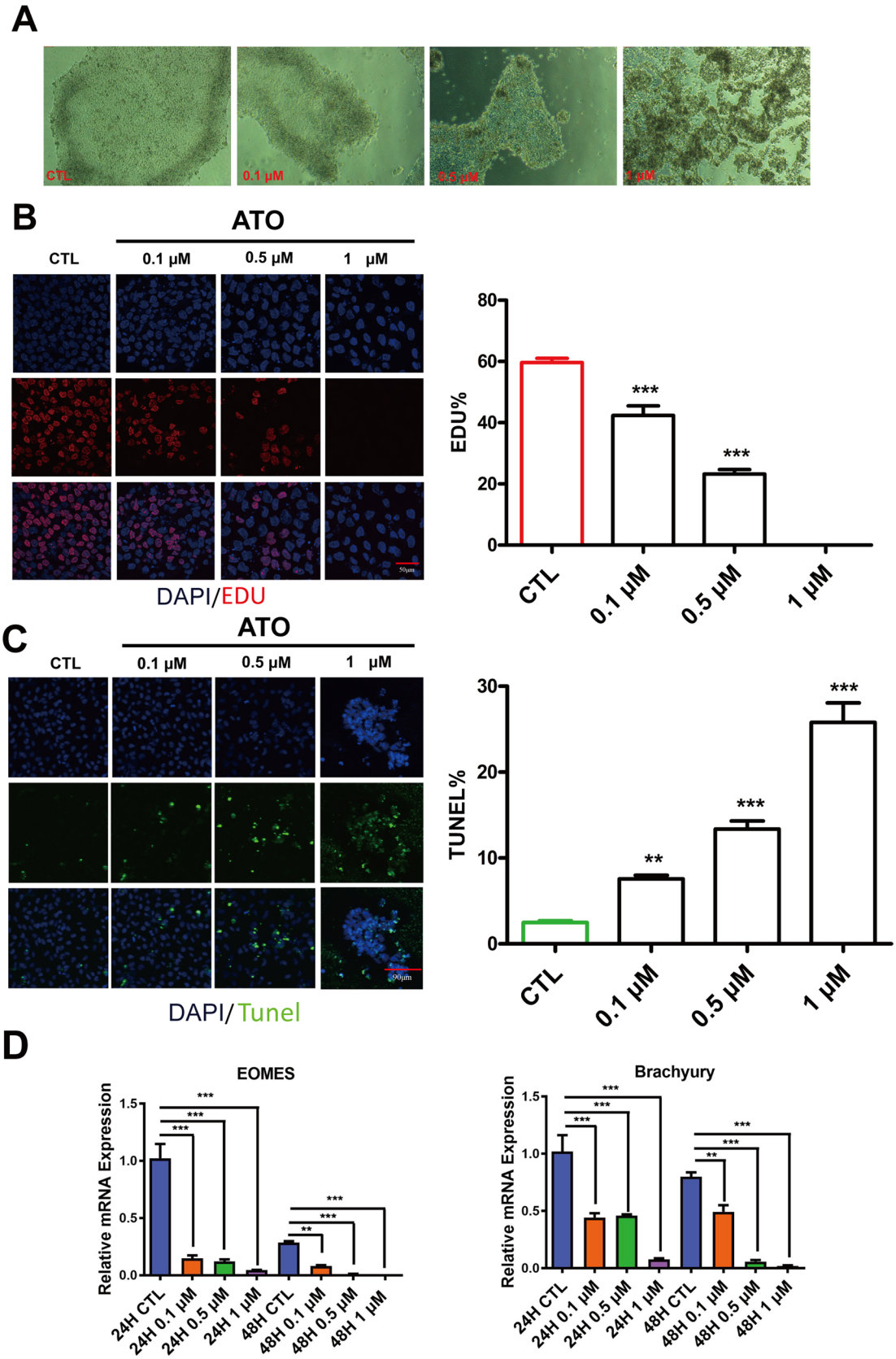

Fig. 1. ATO inhibited the proliferation and mesoderm differentiation of hiPSCs. (A). The effects of 0.1, 0.5 and 1 μM ATO on the growth of hiPSCs. (B). EdU assay was used to detect the proliferation of hiPSCs, assessing by fluorescence microscopy (200×). Representative images of EdU staining showed the proliferated cells (stained in red). Nuclei were stained in blue with DAPI. (C). Tunel assay showed that the apoptosis of hiPSCs was increased by ATO. Tunel-positive staining indicated apoptotic cells (stained in green). Nuclei were stained in blue with DAPI. Scale bar, 100 μm. (D). Expression levels of Brachyury and EOMES after the treatment of ATO for 24 h and 48 h were determined by qRT-PCR. The data are represented as mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. 24H CTL.

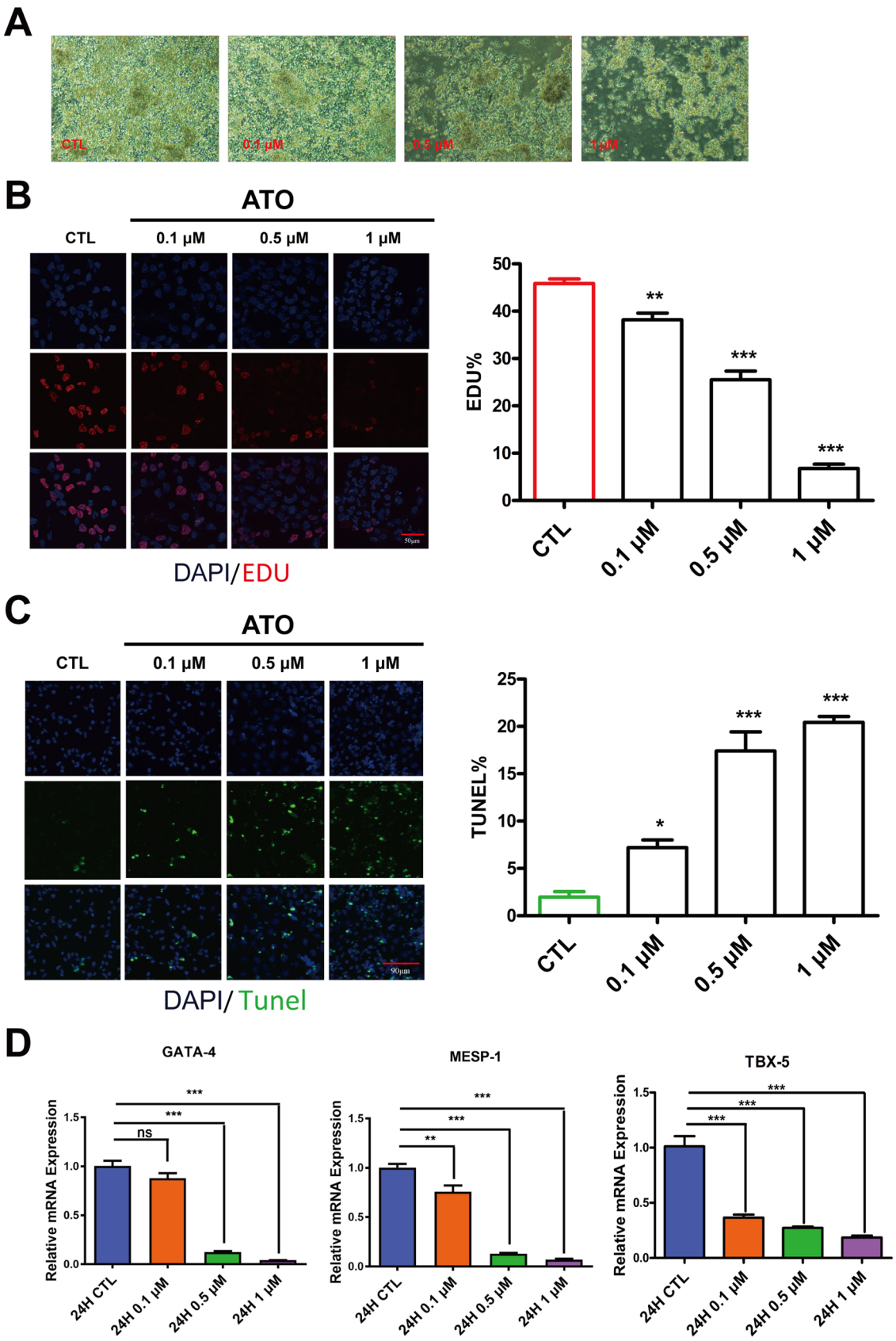

Fig. 2. ATO inhibited the proliferation and cardiovascular progenitor cell diferentiation of hiPSCs. (A). The effects of 0.1, 0.5 and 1 μM ATO on the growth of hiPSCs were observed by microscrope. (B). EdU assay was used to detect proliferation of hiPSCs, assessing by fluorescence microscopy (200×). Representative images of EdU staining showing proliferation cells (stained in red). Nuclei were stained in blue with DAPI. (C). ATO treatment increased the apoptosis of hiPSCs in a concentration-dependent manner. Representative images of Tunel staining showing apoptotic cells (stained in green). Nuclei were stained in blue with DAPI. Scale bar, 100 μm. (D). Treatment of ATO for 24 h reduced the expression levels of GATA-4 and MESP-1mRNA. The data are represented as mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control.

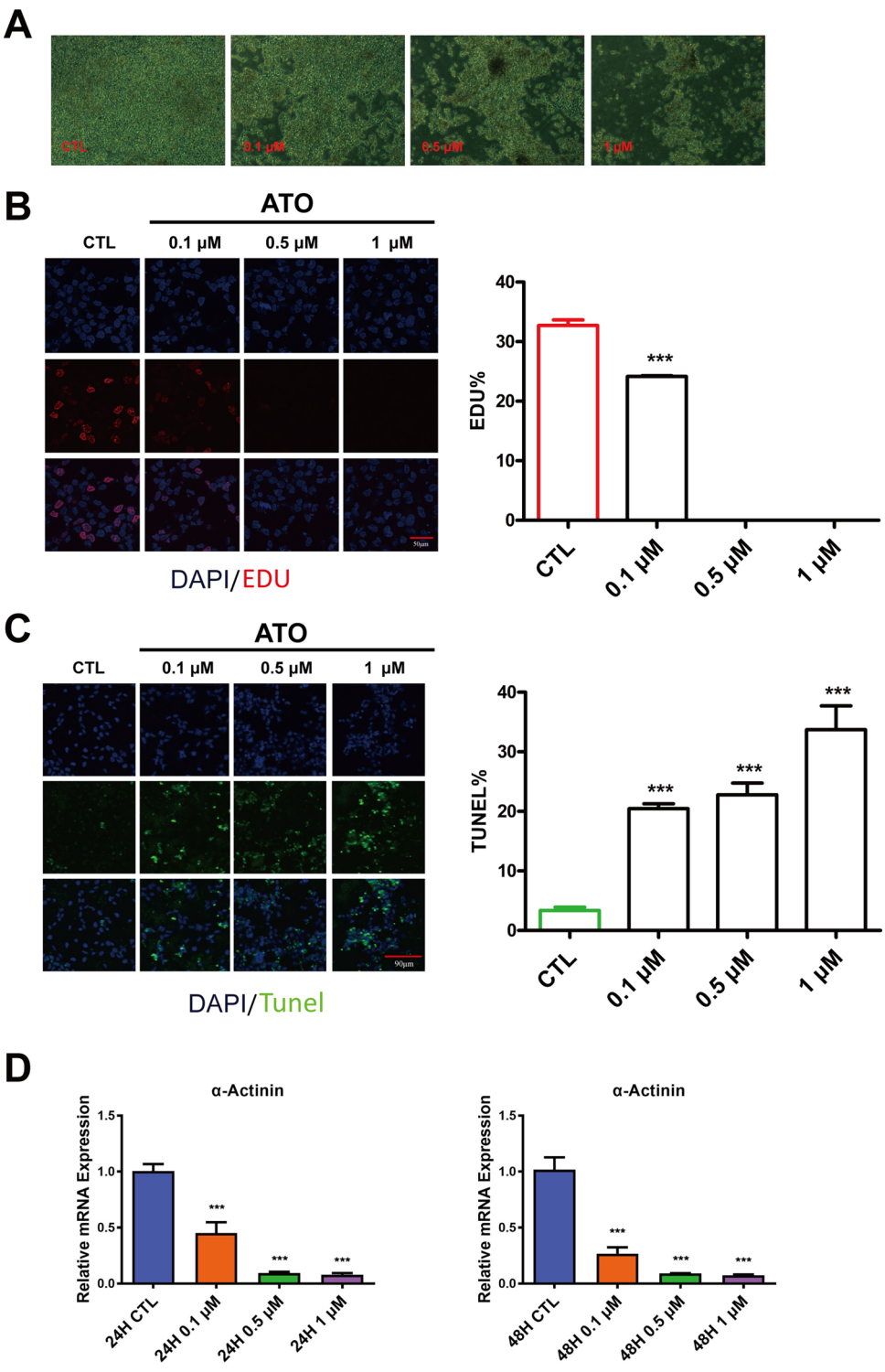

Fig. 3. ATO inhibited the proliferation and cardiomyocyte differentiation of hiPSCs. (A). The effects of different concentrations of ATO on the growth of hiPSCs were obtained from microscrope. (B). The proliferation of hiPSCs was assessed by EdU assay (200×). The representative images of EdU-positive cells (stained in red) after ATO treatment. Nuclei were stained in blue with DAPI. (C). The number of apoptosis of differentiated hiPSCs was increased by ATO. Tunel staining showed apoptotic cells (stained in green). Nuclei were stained in blue with DAPI. Scale bar, 100 μm. The data are represented as mean ± SEM of three independent experiments. $^{***}p < 0.001$ vs. Control. (D). Expression levels of α-actinin after the treatment of ATO for 24 h and 48 h were determined by qRT-PCR. The data are represented as mean ± SEM of three independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. Control.

differentiation from CVPCs into CM. These data indicate that different concentrations of ATO (0.1, 0.5 and 1 μM) inhibit the differentiation of hiPSCs into mesoderm cells, cardiovascular progenitor cells and cardiomyocytes.

It has been reported that ATO induced DNA damage in HL-60 cells (Yedjou and Tchounwou, 2007). And it also has been shown that ATO can induce DNA damage in human colon cancer cells (Stevens et al., 2010) and human lymphoblastoid cells (Hornhardt et al., 2006). In order to determine the molecular mechanism by which ATO inhibited the proliferation and cardiac differentiation in hiPSCs, we detected the expression of γH2AX, a DNA damage marker (Natale et al., 2017). The upregulation of γH2AX expression indicated that ATO induced DNA damage in a concentration-dependent manner.

Thus, our results showed that clinically relevant concentrations of ATO (0.1, 0.5 and 1 μM) could induce apoptosis, inhibit proliferation of differentiated hiPSCs and inhibit cardiomyocyte differentiation of hiPSCs. We further uncovered that DNA damage might be the major cause of the inhibitory role of ATO in cardiomyocyte differentiation of

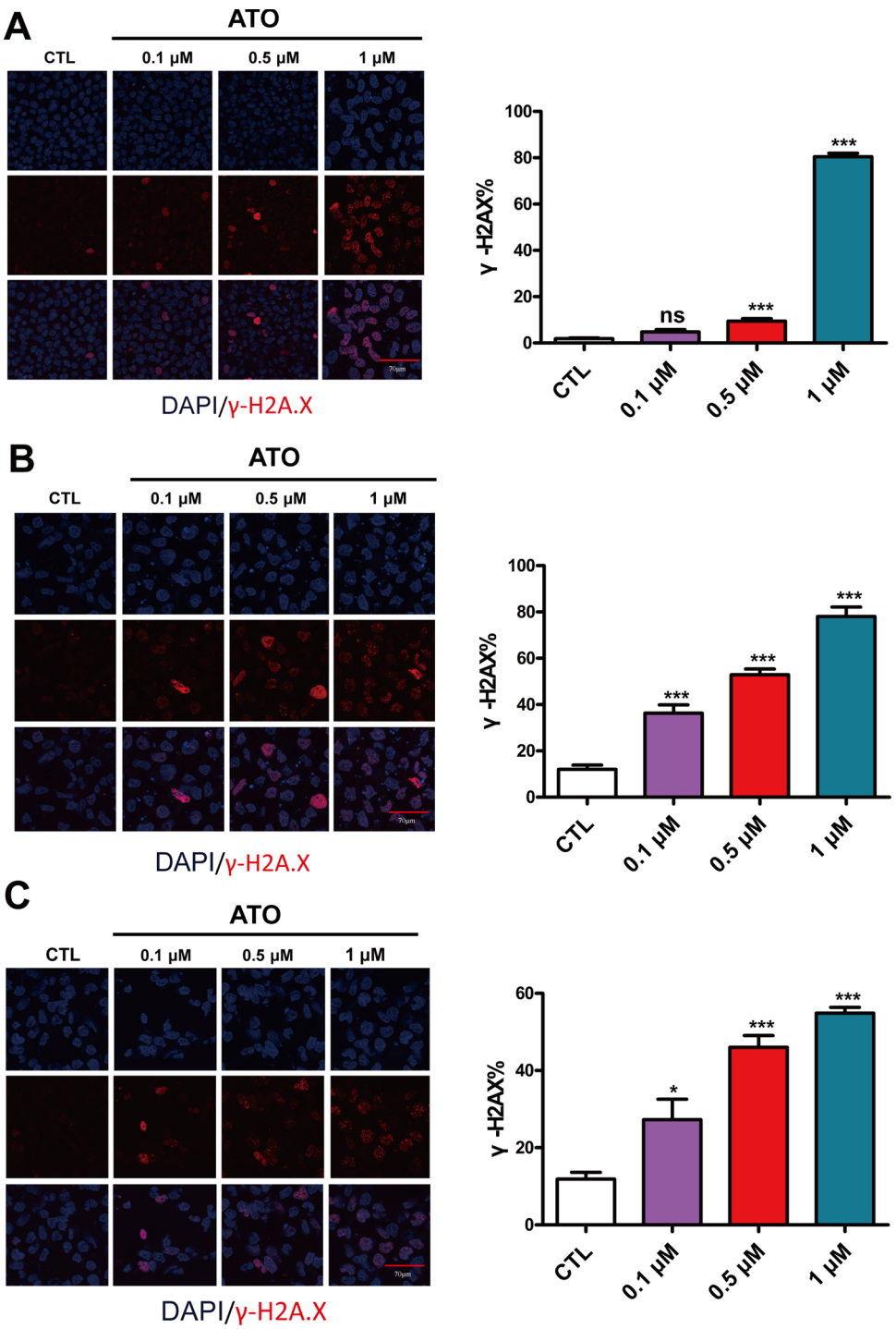

Fig. 4. ATO caused DNA damage in hiPSCs during cardiac differentiation. (A). Immunofluorescence analysis of DNA damage in hiPSCs during the stage of mesoderm differentiation by staining with anti-γH2AX (red) and DAPI (blue). Data are shown as mean ± SEM (n = 3 independent experiments). ***$p < 0.001$ vs. Control. (B). The hiPSCs during its CVPCs differentiation were stained with anti-γH2AX (red) and DAPI (blue). Data are shown as mean ± SEM (n = 3). ***$p < 0.001$ vs. Control. (C). Immunofluorescence staining of anti-γH2AX (red) and DAPI (blue) in hiPSCs during CM differentiation. Data are shown as mean ± SEM (n = 3). *$p < 0.05$, ***$p < 0.001$ vs. Control.

hiPSCs. Our results will provide novel insights into ATO toxicity in cardiac development, and give clinical safety guideline for ATO application in pregnant women.

5. Conclusions

Exposing to ATO at the clinical relevant concentrations resulted in the inhibition of cell growth and cardiomyocyte differentiation, and induction of apoptosis in differentiating hiPSCs by DNA damage.

Conflict of interest

No conflicts of interest are declared by the authors.

Acknowledgements

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