Title: Adrenomedullin Protects Spinal Motor Neurons against Doxorubicin-Induced Toxicity

Authors: Mohammad Ali Takhshid[1,*], Amir Mahmoodazdeh[2], Mohammad Shafiee[3], Mohsen Sisakht[2], Zahra khosdel[2]

1. *Professor Division of Medical Biotechnology, Department of Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran.*
2. *Assistant Professor Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.*
3. *Associated Professor Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran.*

***Corresponding Author**: Mohammad Ali Takhshid, Professor Division of Medical Biotechnology, Department of Laboratory Sciences, School of Paramedical Sciences, Shiraz University of Medical Sciences, Shiraz, Iran. Email: takhshidma@sums.ac.ir

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Abstract

Purpose: In the present study, the culture of embryonic spinal motor neurons (SMNs) was used to assess the impacts of adrenomedullin (AM) on neurotoxic effects of doxorubicin (DOX).

Materials and methods: To prepare the culture of rat embryonic SMNs, spinal cords were isolated from the rat embryos, digested enzymatically, and triturated to obtain spinal cell suspension. Then, the SMNs were purified from the cell suspension using a single gradient of OptiPrep and were cultured. The SMNs were treated with DOX (0.0-100 µM) and AM (3.125-100 nM) and their viability and apoptosis were evaluated using MTT and annexin V flowcytometric assays. Oxidative stress was assessed through the measurement of cellular reactive oxygen species (ROS), nitric oxide (NO), malondialdehyde (MDA), and 8-iso-prostaglandin F2α (iPF2α) levels. Finally, qPCR was employed to determine the expressions of interleukin1-β (IL-1β), inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), SRY-related protein 9 (SOX9), matrix metalloproteinase (MMP)-3 and -13.

Results: The viability of SMNs was decreased following DOX treatment dose-dependently (IC50 = 10.54 µM). DOX increased the cellular ROS, MDA, NO, and iPF2α levels ($p<0.001$). Additionally, AM reduced DOX-induced cell death dose-dependently ($p<0.001$). AM (50 nM) pretreatment also reduced the DOX-induced oxidative stress ($p<0.01$) and -genes expression ($p<0.01$).

Conclusion: Based on the results, AM might be considered a protective factor against chemotherapy-induced toxicity in SMNs.

Keywords: Spinal motor neuron, Doxorubicin, Adrenomedullin, Oxidative stress, Inflammation

1. Introduction

Spinal Motor Neurons (SMNs) are an important part of the spinal cord neural circuit and play crucial roles in spinal reflexes. SMNs are also involved in the transmission of signals from the brain to the muscles (Bäumer, Talbot, & Turner, 2014). Progressive loss of SMNs that occurs in Amyotrophic Lateral Sclerosis (ALS) and the use of chemotherapeutic drugs leads to muscle atrophy and disability (Drechsel, Estévez, Barbeito, & Beckman, 2012; Starobova & Vetter, 2017; Verma et al., 2017). Doxorubicin (DOX) is among the potent chemotherapeutic drugs used in the treatment of a number of cancers (Khan, Singh, Khan, Arya, & Ahmad, 2014; McGowan et al., 2017). DOX exerts its therapeutic effects through the inhibition of replication and increase in the production of inflammatory mediators and Reactive Oxygen Species (ROS) (Capelôa, Caramelo, Fontes-Ribeiro, Gomes, & Silva, 2014; Zhao & Zhang, 2017). Despite its beneficial effects, irreversible toxic effects of DOX on the induction of cognitive and motor impairments such as learning disabilities and memory loss have been reported (Moruno-Manchon et al., 2016). DOX-mediated degeneration of the Dorsal Root Ganglion (DRG) and SMNs have also been shown in the spinal cord in both animal models (Wu, So, Liao, & Huang, 2015) and human studies (Saito et al., 2019).

Adrenomedullin (AM), a peptide belonging to the calcitonin family, is widely expressed in peripheral tissues and the Central Nervous System (CNS) in both the brain and the spinal cord (Y. Hong, Liu, Chabot, Fournier, & Quirion, 2009). Considering the peripheral tissues, AM has revealed protective effects against leydig cells pyroptosis (Li et al., 2019), heart failure (Voors et al., 2019), pulmonary and renal diseases (Holmes, Campbell, Harbinson, & Bell, 2013), sepsis (Geven, Kox, & Pickkers, 2018), and ulcerative colitis (Martinez-Herrero, Larrayoz, Narro-Iniguez, Rubio-Mediavilla, & Martinez, 2017). An in vitro study conducted on mesenchymal stem cells showed that AM overexpression could protect the cells from serum deprivation and hypoxia-induced apoptosis (Si, Zhang, Song, & Li, 2018). Moreover, AM strongly inhibited streptozotocin-induced cell death in cultured human renal tubule cells (Uetake et al., 2014). In the CNS, Calcitonin-Like Receptor (CLR), Receptor Activities Modifying Protein-2 (RAMP-2), and RAMP-3, as AM receptor components, are expressed in both glial and nerve cells. Animal studies have suggested the role of AM in the transmission of nociceptive impulses (Ma, Chabot, & Quirion, 2006; Takhshid et al., 2004). The neuroprotective effects of AM against ischemic brain

damage and acute brain injury were mediated through decreasing oxidative stress and pro-inflammatory cytokines production (Demir et al., 2013). A recent study also disclosed the protective effects of AM against neurotoxic effects of DOX in DRG neurons(Amir Mahmoodazdeh, Sayed Mohammad Shafiee, Mohsen Sisakht, Zahra Khoshdel, & Mohammad Ali Takhshid, 2020). The present study aims to determine AM, CLR, and RAMPs expressions in rat embryonic SMNs and to assess the protective and anti-oxidant effects of AM against DOX-induced neurotoxicity.

2. Materials and Methods

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences.

2.1 Reagents and chemicals

Rat AM was purchased from Bachem. Cell culture materials were bought from Gibco BRL (Life technologies, Renfrewshire, UK), except for poly- D-lysine, Trypsin-EDTA solution, penicillin/streptomycin solution, MTT assay kit, OptiPrep™ density gradient medium (D1556-250ML, Sigma, USA), and DOX that were the products of Sigma (USA). BCA protein assay kit and Nitric Oxide (NO) generation kit were purchased from Thermo Fisher Scientific, Inc. (USA). ROS detection kit was provided by Molecular Probes (USA) and 8-iso prostaglandin F2α (iPF2α) ELISA kit was obtained from Cyman Chemical Co. (USA). All primers were prepared by Metabion Co. (Germany). Apoptosis detection kits were purchased from Bio Vision (Switzerland) and Syber green Polymerase Chain Reaction (PCR) master mix was bought from Ampliqon A/S, Odense (Denmark).

2.2. Isolation and culture of SMNs

SMNs were cultured according to Wang method. (Wang et al., 2017). As illustrated in Figure 1, the spinal cord was separated from the DRGs, rinsed in L15 medium containing penicillin G (300 U/mL) and gentamycin (75 µg/mL), and digested in 0.025% trypsin (Invitrogen) at 37 ºC for 40 min. The spinal cells were then dissociated using trituration, and the suspended cells were put on a single gradient of OptiPrep™ and centrifuged for 10 min (4 ºC) to separate SMNs from debris (F1), SMNs(F2), and non-neural cells (Figure 1). The SMNs (F2 layer) were carefully separated, re-suspended in appropriate volume of culture medium, transferred to the poly-D-lysine coated culture plates, and cultured in Neurobasal-A medium supplemented with B27 (2%), GlutaMAX

(2mM), streptomycin (100 mg), and penicillin (100 units) at 37 °C, 5% CO2, and 80% relative humidity. After that, the cultured cells were treated with 5-fluorodeoxyuridine (20 mM ; 72 h) to prevent the growth of non-neural cells (Liu, Lin, & Xu, 2013). Finally, the purity of the SMNs in the culture was evaluated using phase contrast microscopy. Based on the results, SMNs comprised 87±5% of the cultured cells.

2.3. Cell viability assay

To evaluate DOX toxicity, the SMNs were treated with DOX (0-100 µM) and their viability was measured after for 12, 24, and 48 h. To evaluate the possible toxicity of AM, the SMNs were treated with AM (3.125-100 nM) for 24 h. The doses of AM were selected according to a previous study (A. Mahmoodazdeh, S. M. Shafiee, M. Sisakht, Z. Khoshdel, & M. A. Takhshid, 2020). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide) was use to assess the effects of treatment on cell survival. (A. Mahmoodazdeh et al., 2020). IC50 value of DOX was calculated using Prism 6 software. For further evaluations, the SMNs were cultured in 48-well plates (2×105 cells/well) and treated with AM (50 nM), DOX (10.54 µM; IC50), and AM + DOX (50 nM+10.54 µM). SMNs were pretreated with AM for 2 h and DOX was then added to the wells and cell viability was measured after 24 h.

2.4. Apoptosis assay

The effects of the treatments on SMNs apoptosis were determined evaluated using annexin V-FITC/ PI staining and flowcytometry. Briefly, the treated SMNs were harvested, stained, and analyzed using a flow cytometer (BD) at 488 nm.

2.5. ROS assay

Intracellular ROS were measured using 2',7'-dichloro fluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) probe and flowcytometry (Chen, Zhong, Xu, Chen, & Wang, 2010). Briefly, the SMNs were incubated with 10 µM of DCF-DA at 37 °C for 30 min. The cells were then washed three times with Phosphate-Buffered Saline (PBS), and the fluorescence intensity was measured (Excitation: 488 and emission:525 nm) using Multimode Plate Reader.

2.6. Oxidative markers assay

NO levels in the cell lysate were assayed using Griess-reduction method and nitrite standard curve (1−100 nmol/mL). (Hare, Beigi, & Tziomalos, 2008). Briefly, the Griess reagent (5% H3PO4 containing 1% sulfanilamide and0.1% naphthyl ethylene ediamine) was mixed with the cell lysates and incubated at room temperature in the dark. The absorbance of the samples was then measured at 540 nm. Thiobarbituric Acid method (Tsikas, 2017) was used to measure MDA levels in SMNs. To quantify iPF2α levels, the SMNs were lysed and treated with KOH (15% at 40 °C for 60 min) to release esterified forms of iPF2α. iPF2α was then measured using an ELISA kit (Cayman, item No. 51635) were normalized based on total protein concentration of each sample.

2.8. Mitochondrial membrane potential assay

Staining with Rhodamine 123 (Rh123) was used to measure the Mitochondrial Membrane Potential (MMP)(Sakamuru, Attene-Ramos, & Xia, 2016). Briefly, 24 h after the treatments, the SMNs were incubated with Rh 123 (1 μM) at 37 °C for 30 min. SMNs were then washed with PBS and fluorescence intensity of Rh123 was measured (excitation 488 and emission 525 nm).

2.9. Gene expression analysis by qPCR

The relative expressions of AM, CRLR, RAMPs, MMP-3, MMP-13, iNOS, TNF-α, IL-1β, and SOX-9 were measured using qPCR (ABI Biosystems), specific primers (Table 1), and β-actin as the housekeeping gene. Qiagen and Thermo Fisher kits(USA) were used for RNA extraction and cDNA synthesis.

2.11. Statistical analysis

SPSS software (version 16) was used to analyze the data. According to Shapiro-Wilk test, the data was normally distributed ($p<0.05$). Therefore, one-way Analysis of Variance (ANOVA) and Student-Newman-Keuls post-hoc test was used to analyze the data. The data were presented as mean ± SEM and $p<0.05$ was considered as significant difference.

3. Results

3.1. SMNs expressed AM, CLR, and RAMPs

The results of q-PCR revealed the expressions of CLR, RAMPs, and AM, in the SMNs (Figure 5.) DOX increased the expression of RAMP2 ($p=0.001$), while it had no significant effects on the expressions of AM, CLR, and RAMP3.

3.2. AM protected SMNs against DOX-induced cell death

The MTT assay results(Figure2) revealed a dose- and time-dependent increase in SMNs cell death following DOX treatment (IC50 values = 10.72 ± 3.42, 10.54 ± 2.59, and 0.95 ± 0.14 μM for 12, 24, and 48 h of treatment, respectively). AM exerted no significant toxic effects on the viability of SMNs (Figure 2B). AM reversed DOX (10.54 μM for 24 h)-induced cell death in a concentration-dependent manner ($p<0.0001$) (Figure 3). Based on the MTT results, one concentration of DOX (10.54 μM) and AM (50 nM) AM were chosen for the next experiments.

3.3. AM reversed DOX-induced SMNs cell death

Based on the apoptosis data, the SMNs viability decreased to 49.3% in the DOX-treated neurons (10.54 μM for 24 h). The decrease in the viable SMNs population in the DOX-treated cells was accompanied by a significant increase in the percentage of necrotic cells to 33.8%. However, no significant change was observed in the percentage of live cells in the AM group (50 nM). AM also reduced the DOX-induced necrosis of SMNs ($p<0.0001$) (Figure 4).

3.4. DOX-induced oxidative stress was ameliorated by AM

The levels of various markers were measured to assess the effects of DOX on oxidative stress (Figure 6). As illustrated in Figure 6 A, DOX elevated intracellular ROS in the SMNs compared to the controls ($p<0.001$). Pretreatment with AM (50 nM) significantly reduced DOX-induced ROS generation ($p<0.001$). Furthermore, AM pretreatment significantly ($p=0.004$) decreased DOX-induced NO production in the SMNs (Figure 6 B). Moreover, the levels of MDA ($p<0.001$) and iPF2α ($p=0.003$) were elevated in the DOX-treated SMNs. Similarly, AM decreased the DOX-elevated levels of iPF2α ($p=0.003$) and MDA ($p=0.007$) (Figures 6).

3.5. AM suppressed the effects of DOX on MMP

MMP dissipation can lead to mitochondrial dysfunction and cell death (Webster, 2012). The results of the MMP assay revealed a significant decline in MMP DOX-treated SMNs compared to the controls ($p<0.001$). AM treatment alone had no significant effects on the MMP level, but significantly reduced the effect of DOX (10.54 µM for 24 h) on the MMP loss (Figure 7).

3.6. The effects of AM and DOX-induced gene expression

Following treatment with DOX (10.54 µM for 24 h), the mRNA levels of TNF-α, iNOS, and IL-1β ($p<0.001$) were increased compared to the control SMNs (Figure 8). AM blocked the effects of DOX on iNOS, TNF-α, SOX-9, and IL-1β expressions. As shown in Figures 9, DOX also increased the expression levels of MMP-3 ($p<0.001$) and MMP-13 ($p=0.007$). AM pre-treatment revealed no significant effects on MMP-13 mRNA while decreased the DOX-induced MMP-3 expression.

4. Discussion

The cultures of isolated SMNs provide a valuable model for studying the mechanisms involved in motor neuron survival, degeneration, and regeneration (Bucchia, Merwin, Re, & Kariya, 2018). This model was utilized in this research to figure out the possible neuroprotective role of AM. The findings revealed the protective effects of AM against DOX-induced cell death in the SMNs. AM also ameliorated the DOX-induced oxidative stress and MMP dissipation. Finally, DOX-induced expressions of inflammatory mediators were reversed by AM. These findings suggested that AM might be considered a protective factor in the conditions associated with the degeneration of SMNs.

Although DOX poorly crosses the blood brain barrier, there is evidence that it can reach the motor neurons through retrograde axoplasmic transport (Sabri, 2018). In the current study, treatment of the isolated SMNs with DOX led to oxidative stress, increased expression of inflammatory

markers, and cell death. These findings were in line with the observed toxicity of DOX in DRG neurons (Amir Mahmoodazdeh et al., 2020) and cardiac cells (Yoshizawa et al., 2016).

Several studies have shown the protective effects of AM in different cells mediated through antioxidant and anti-inflammatory activities (Yoshimoto et al., 2004). The current study results revealed the expressions of AM, CLR, and RAMP-2 and -3 in the embryonic SMNs, which was in accordance with the findings of the research carried out by Montuenga et al. (Montuenga, Martínez, Miller, Unsworth, & Cuttitta, 1997) that indicated the expressions of AM and CLR in the ventral spinal cells from day 14 of the embryonic life. In the present investigation, the results of flowcytomtric assay demonstrated that AM (50 nM) reversed the DOX-induced necrosis of the SMNs, while it had no significant effects on DOX-induced apoptosis. AM also reduced the DOX-induced increase in the intracellular levels of oxidative stress markers (ROS, NO, MDA, and iPFα), suggesting the antioxidant effects of AM. Although the underlying mechanisms were not explored in our investigation, it has been revealed that AM could blocked the IL-1β-induced inflammation by decreasing the expressions of iNOS and cyclooxygenase-2 as well as the concentrations of NO and prostaglandin E2 (W. Hu et al., 2015).

In line with some other studies, the present study findings showed that MMP dissipation was significantly induced by DOX. AM ameliorated DOX-induced mitochondrial damage in cardiac muscle cells (30). Therefore, prevention of mitochondria damage may play a role in the observed AM effects. Some reports are also available regarding the anti-inflammatory properties of AM. Wei Hu et al. showed that AM was able to attenuate inflammation-induced apoptosis of rats' leydig cells (Wei Hu et al., 2015). This was in agreement with the present study results, which demonstrated a significant reduction in the expressions of inflammatory mediators following AM pre-treatment in the SMNs, suggesting that AM may exert anti-inflammatory action against

toxicity induced by DOX. Up to now, several mechanisms have been suggested for the anti-inflammatory properties of AM. For example, AM may be able to inhibit the cytokine-induced neutrophil chemoattractant release possibly via a cAMP-dependent pathway (Yu et al., 2009).

Matrix metalloproteinase enzymes are actively involved in the inflammatory process, mainly through their role in the regulation of availability and activity of inflammatory mediators (Nissinen & Kähäri, 2014). It has been shown that MMPs, especially MMP-3 and -13, have a role in cell toxicities induced by anti-cancer agents. The present study findings revealed a significant increase in the expression levels of these enzymes following DOX treatment. The same results were reported as the side effect of another chemotherapeutic agent; i.e., paclitaxel (Cirrincione et al., 2019; Nishida et al., 2008), suggesting that MMP-3 may play a role in chemotherapy-induced cell death. ROS (Haorah et al., 2007) and inflammatory mediators (Fingleton, 2017) were also found to play critical roles in the activation of MMPs. Thus, antioxidant and anti-inflammatory agents could act as the possible inhibitors of MMPs in abnormal states. Although the current study results proved the inhibitory effect of AM on the expression levels of MMPs, more studies are recommended to clarify the exact pathways.

SOX9 has been found to play a role in various physiological pathways such as CNS development (Pevny & Placzek, 2005) and neural stem cells survival (Scott et al., 2010). Proteasome-induced degradation of SOX9 was revealed following DNA damage by several genotoxic agents (X. Hong et al., 2016). However, SOX9 upregulation increased cell viability, suggesting its role in cell survival (Roche et al., 2015). In the present study, DOX decreased the SOX9 expression in the SMNs, while AM blocked the action of DOX, suggesting that SOX may play a role in AM's protective effects. CREB binds to its regulatory elements in SOX promoter leads to SOX upregulation (Piera-Velazquez et al., 2007), while its expression is downregulated by IL-1β and

TNF-α (Piera-Velazquez et al., 2007; Yu et al., 2009). Moreover, BMP-2 signaling pathway increases SOX9 gene expression. In DRG neurons, AM increases BMP-2 expression, suggesting the possible role of BMP-2 signaling in AM-induced SOX9 expression.

5. Conclusion:

In conclusion, the present study provided substantial evidence that AM ameliorated the DOX-induced toxicity in isolated motor neurons. Based on the results, decrease in cell viability that was mainly mediated through necrotic cell death, increased level of oxidative stress markers, loss of MMPs, and increased expressions of genes involved in inflammation were the toxic effects detected following the treatment of motor neurons with DOX. The findings clearly indicated that pre-treatment with a low dose of AM (50 nM) could protect the motor neurons from the DOX-induced cytotoxic effect, most probably through its anti-oxidant effects.

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Table 1. The sequences of the primers used for qPCR

Gene	Accession number	Sequence	Size (bp)
CRLR	NM_012717.1	F: CACACCAAGCAGAATCCAATC R: GTCATACACCTCCTCAGCAA	59.3
RAMP2	NM_031646.1	F: GAATCAATCTCATCCTACT R: TGTAATACCTGCTAATCAA	56.3
RAMP3	NM_020100	F: CTGACCTCTGCTACGCTTG R: TGACTCCTAACAACTCCATTCC	62.2
AM	NM_012715.1	F: GAACAACTCCAGCCTTTACC R: GAGCGAACCCAATAACATCAG	62
IL-1β	BC091141.1	F: GGAGAGACAAGCAACGACAA R: TTGTTTGGGATCCACACTCTC	123
iNOS	AABR07030077.1	F: GGATGTGGCTACCACTTTGA R: CATGATAACGTTTCTGGCTCTTG	107
TNF-α	AF269159.1	F: CCCACGTCGTAGCAAACCAC R: TAGGGCAAGGGCTCTTGATG	264
MMP-3	X02601.1	F: ATGATGAACGATGGACAGATGA R: CATTGGCTGAGTGAAAGAGACC	99
SOX-9	AB073720.1	F: AGTCGGTGAAGAATGGGCAA R: ACCCTGAGATTGCCCGGAG	161
MMP-13	M60616.1	F: CAAGCAGCTCCAAAGGCTAC R: TGGCTTTTGCCAGTGTAGGT	130

Figure Legends

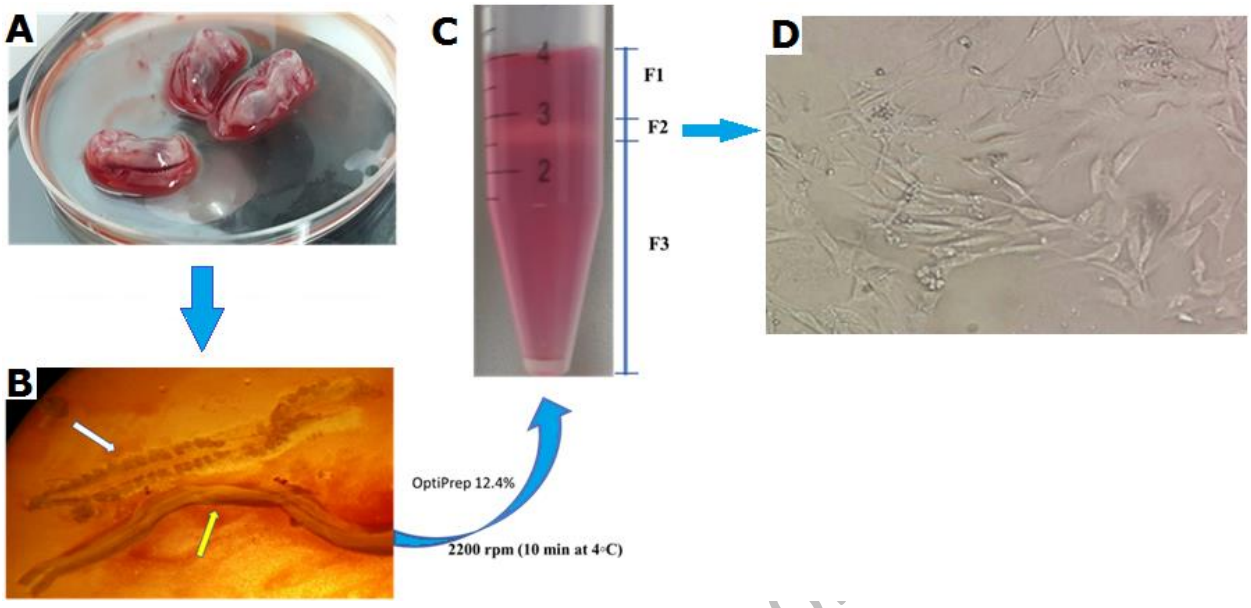

Figure 1. Isolation of the spinal motor neurons from the rat embryo. A) The spinal cord was removed from the rat embryos E14-E15. B) Dorsal root ganglions and spinal cord membrane (white arrow) were separated from the spinal cord (yellow arrow). The spinal cord was digested and mechanicaly triturated. The resulting cell suspension was centrifuged at OptiPreb 12.4%. C). Three fractions (F1, F2, F3) were obtained. The SMNs were enriched at F2 fraction. D) Phase contrast image of the SMN culture 72 h after FUDR treatment.

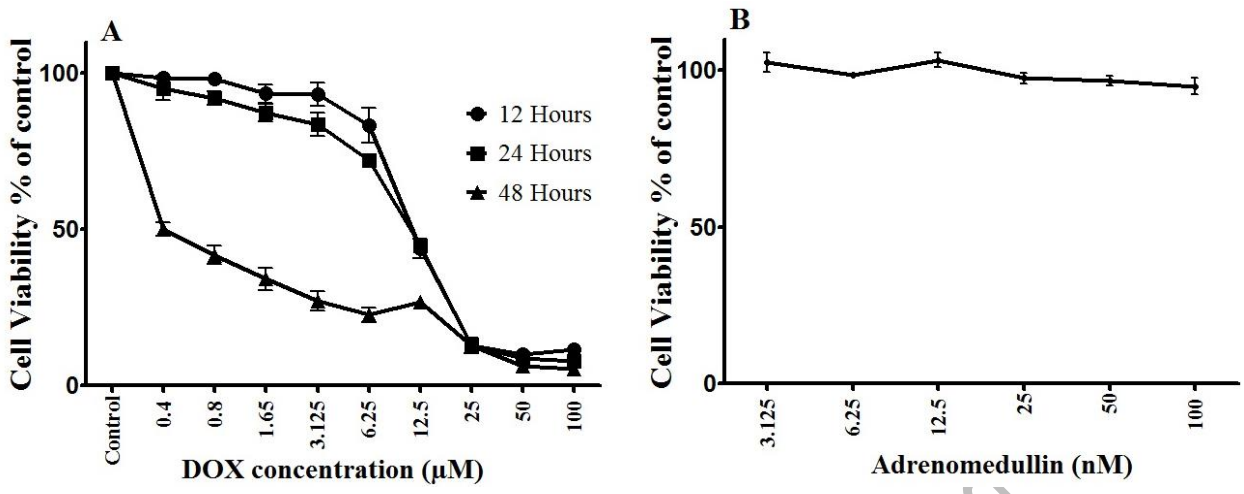

Figure 2. AM suppressed DOX-induced cell death in SMNs. The SMNs were treated with DOX (0.0-100 µM for 12, 24, and 48 hours) and AM (3.125-100 nM for 24 h), and cell viability was measured by the MTT assay ($p<0.05$ was considered significant).

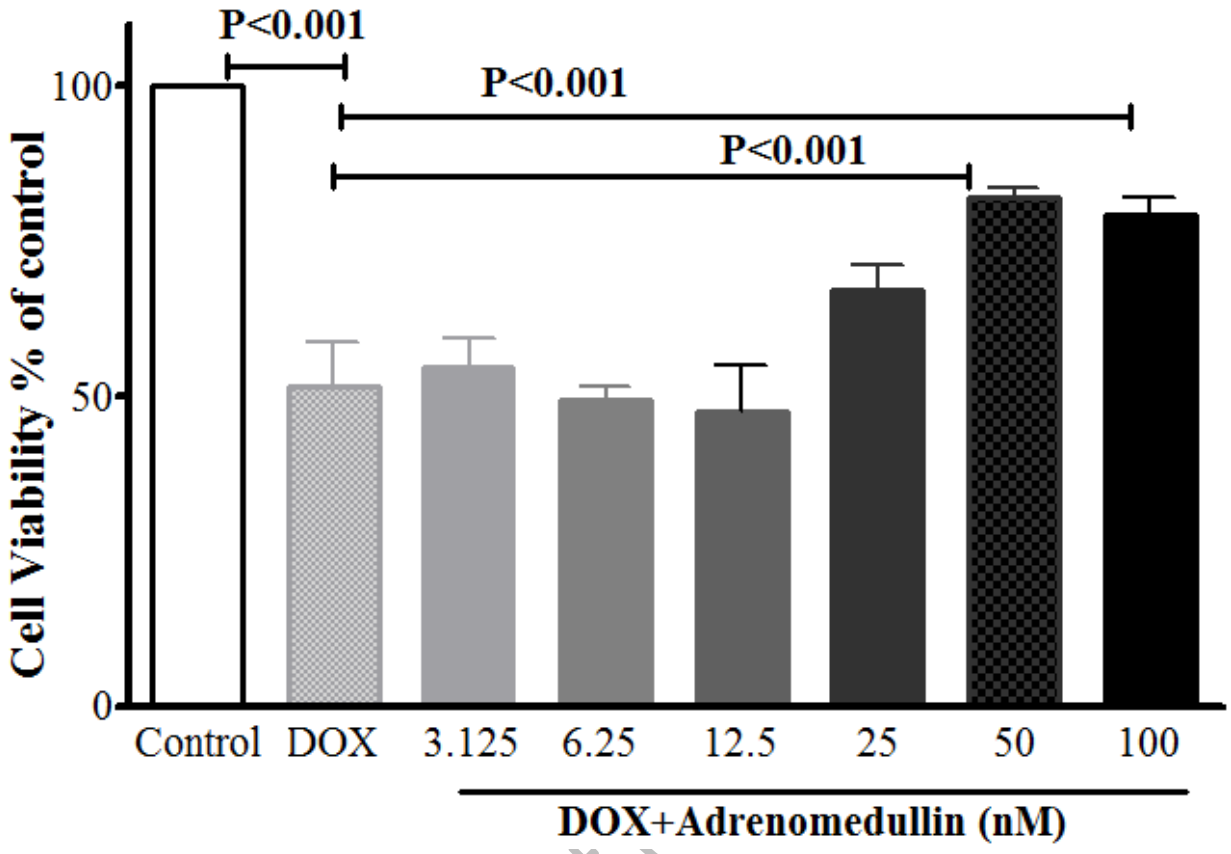

Figure 3. AM protected SMNs against DOX-induced cell death

The SMNs were treated with AM (50 nM) and DOX (10.54 μM) for 24 h and cell viability was measured using the MTT assay(n=6). ($p<0.05$ was considered significant).

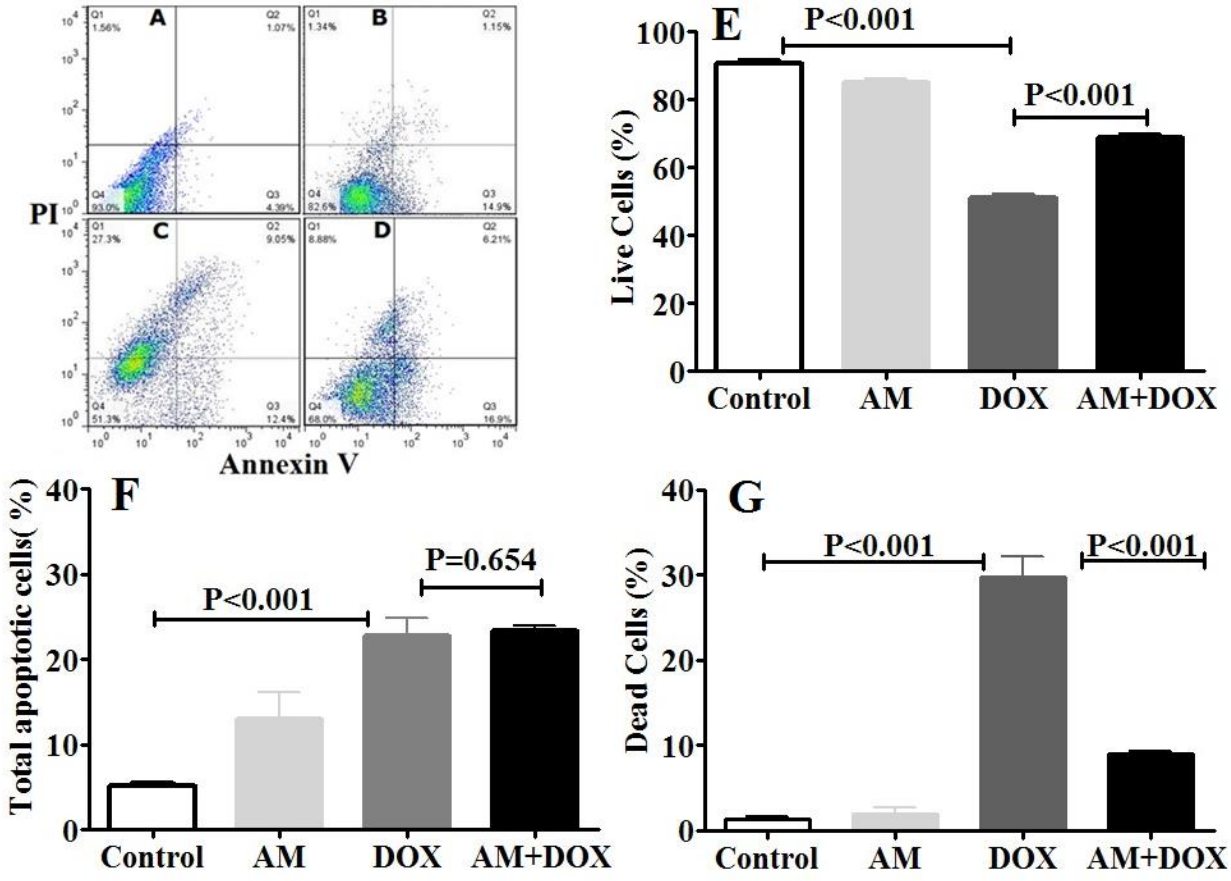

Figure 4. The effects of AM on DOX-induced cell death in the motor neurons. The SMNs were treated with AM (50 nM) and DOX (10.54 µM) for 24 h, and the level of apoptosis was measured using flow cytometry (A: control; B:AM; C: DOX; and D**:** AM-DOX treated groups). Bar charts indicate the percentages of live (E), apoptotic (F), and necrotic cells(G). ($p<0.05$ was considered significant).

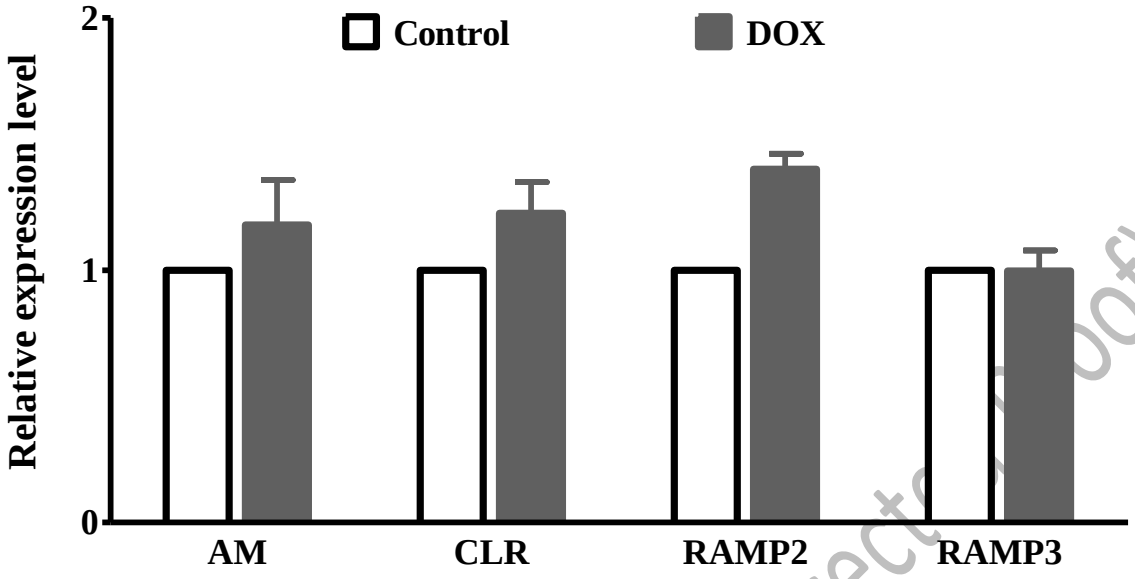

Figure 5. Relative expressions of AM, CLR, and RAMPs in the control and DOX-treated motor neurons. The SMNs were treated with AM (50 nM) and DOX (10.54 μM) for 24 h and the relative expressions of AM, CLR, and RAMPs were quantified using q- PCR and were reported as the percentage of the control group(n=6). ($p<0.05$ was considered significant).

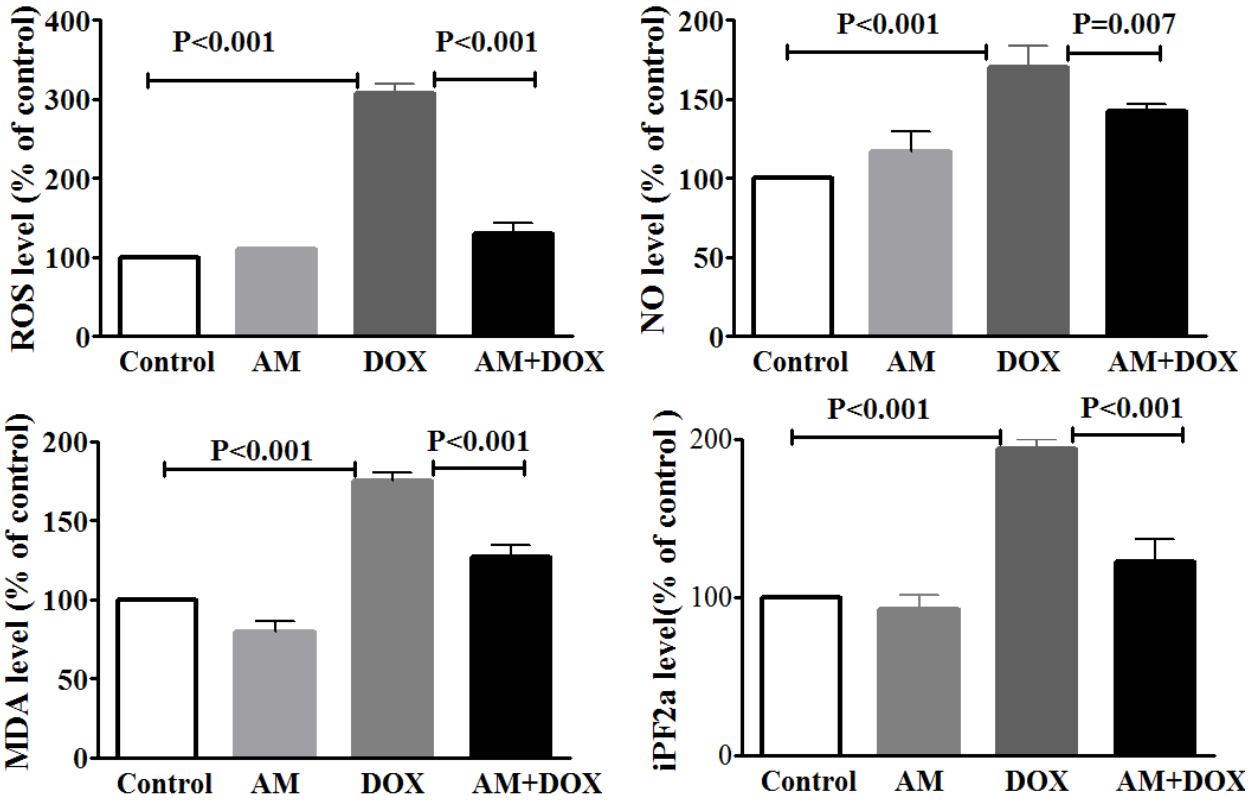

Figure 6. The effects of DOX and AM on the levels of oxidative stress markers. The SMNs were treated with AM (50 nM) and DOX (10.54 µM) for 24 h and the levels of oxidative stress markers were determined (n=6). ($p<0.05$ was considered significant).

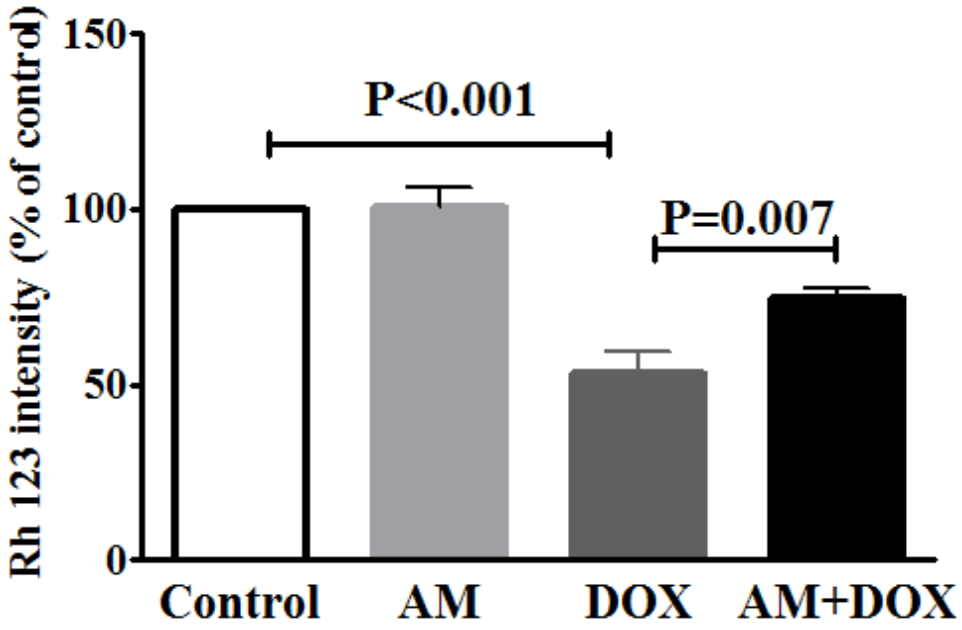

Figure 7. The effects of AM on DOX-induced MMP dissipation. The SMNs were treated with AM (50 nM) and DOX (10.54 µM) and for 24 h. MMP was determined using Rhodamine 123 staining. ($p<0.05$ was considered significant).

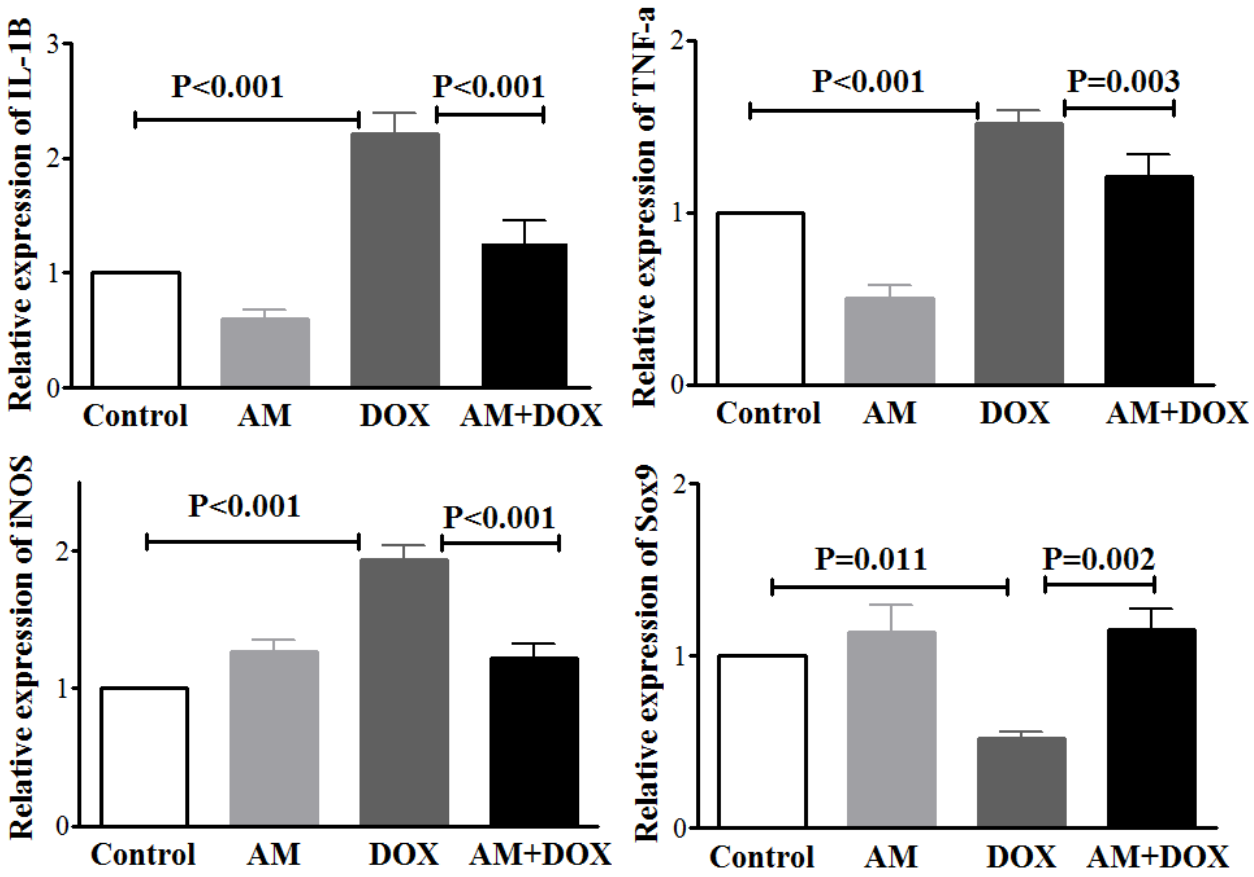

Figure 8. The effect of AM on the DOX-induced expressions of inflammatory mediators. SMNs were treated with AM (50 nM) and DOX (10.54 µM) for 24 h. The relative expressions of iNOS, SOX9 IL-1β, and TNF-α, were quantified using q-PCR and reported as the percentage of the control group(n=6). ($p<0.05$ was considered significant).

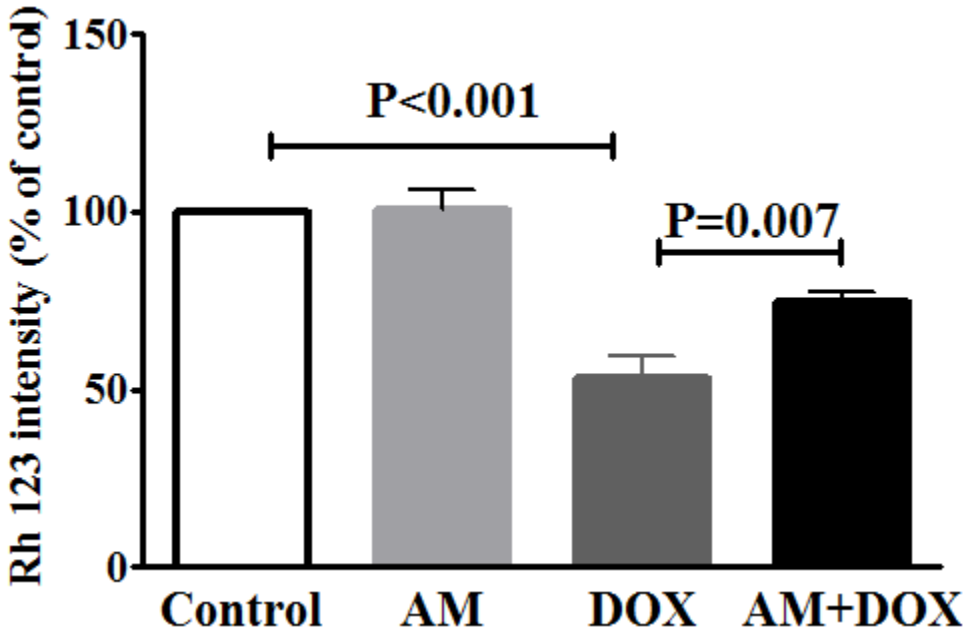

Figure 9. The effect of AM on DOX-induced MMPs expressions. SMNs were treated with AM (50 nM) and DOX (10.54 µM). The relative expressions of MMP-3 (**A**) and MMP-13 (**B**) were quantified q-PCR and reported as the percentage of the control group(n=6). ($p<0.05$ was considered significant).