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Research Paper

Adrenomedullin and Protecting Spinal Motor Neurons Against Doxorubicin-induced Toxicity

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Spinal motor neuron (SMN), Doxorubicin (DOX), Adrenomedullin (AM), Oxidative stress, Inflammation

ABSTRACT

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

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 OptiPrep gradient and 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 flow cytometric 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 decreased following DOX treatment dose-dependently (IC_{50}=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 gene expression (P<0.01).

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

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Highlights

- Adrenomedullin (AM) and its receptors are present in spinal motor neurons (SMNs).
- AM reduced the oxidative stress induced by doxorubicin (DOX) in SMNs.
- AM attenuated the apoptotic effects of doxorubicin in spinal motor neurons.
- AM down regulated the expression of various inflammatory mediators.

Plain Language Summary

DOX is a highly effective chemotherapeutic agent used for the treatment of many cancers. Despite therapeutic benefits, it has side effects, including motor dysfunction. AM is a peptide expressed within the brain, spinal cord, and other organs. It has protective properties against diseases such as heart failure, pulmonary and renal diseases, sepsis, and inflammatory diseases. In the current study, we assessed the effects of AM on neurotoxicity induced by doxorubicin in cultured SMNs. The findings indicated that AM has the capacity to ameliorate DOX-induced cell death through its anti-inflammatory and antioxidant properties. Therefore, it may serve as a protective agent to alleviate the adverse effects of chemotherapy in cancer patients.

1. Introduction

Spinal motor neurons (SMNs) are essential to the spinal cord neural circuit and play crucial roles in spinal reflexes. SMNs also transmit signals from the brain to the muscles (Bäumer et al., 2014). Progressive loss of SMNs that occurs in amyotrophic lateral sclerosis and the use of chemotherapeutic drugs leads to muscle atrophy and disability (Drechsel et al., 2012; Starobova & Vetter, 2017; Verma et al., 2017). Doxorubicin (DOX) is among the potent chemotherapeutic drugs used to treat several cancers (Khan et al., 2014; McGowan et al., 2017). DOX exerts its therapeutic effects through the inhibition of replication and an increase in the production of inflammatory mediators and reactive oxygen species (ROS) (Capelôa et al., 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 et al., 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 the brain and the spinal cord (Hong et al., 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 et al., 2013), sepsis (Geven et sl., 2018), and ulcerative colitis (Martinez-Herrero et al., 2017). An in vitro study on mesenchymal stem cells showed that AM overexpression could protect the cells from serum deprivation and hypoxia-induced apoptosis (Si et al., 2018). Moreover, AM 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 AM's role in transmitting nociceptive impulses (Ma et al., 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 the neurotoxic effects of DOX in DRG neurons (Mahmoodazdeh et al., 2020). The present study aims to determine *AM*, *CLR*, and *RAMPs* expressions in rat embryonic SMNs and assess AM's protective and anti-oxidant effects against DOX-induced neurotoxicity.

2. Materials and Methods

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. (The USA). ROS detection kit was provided by Molecular Probes (The USA), and an 8-iso prostaglandin F2α (iPF2α) ELISA kit was obtained from Cayman Chemical Co. (The 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).

Isolation and culture of SMNs

SMNs were cultured according to Wang's 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 ˚C 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 ˚C) to separate SMNs from debris (F1), SMNs(F2), and non-neural cells (Figure 1). The SMNs (F2 layer) were carefully separated, re-suspended in an 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 (2 mM), streptomycin (100 mg), and penicillin (100 units) at 37 ˚C, 5% CO_2, 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 et al., 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.

Cell viability assay

To evaluate DOX toxicity, the SMNs were treated with DOX (0-100 µM), and their viability was measured 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 (Mahmoodazdeh et al., 2020). MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5- diphenyl-2H-tetrazolium bromide) was used to assess the effects of treatment on cell survival (Mahmoodazdeh et al., 2020). IC_{50} value of DOX was calculated using Prism 6 software. For further evaluations, the SMNs were cultured in 48-well plates (2×10^5 cells/well) and treated with AM (50 nM), DOX (10.54 µM; IC_{50}), and AM + DOX (50 nM+10.54 µM). SMNs were pretreated with AM for two h, DOX was added to the wells, and cell viability was measured after 24 h.

Apoptosis assay

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

ROS assay

Intracellular ROS were measured using 2',7'-dichloro fluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR, USA) probe and flowcytometry (Chen et al., 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 a Multimode Plate Reader.

Oxidative markers assay

NO levels in the cell lysate were assayed using the Griess-reduction method and nitrite standard curve (1−100 nmol/mL) (Hare et al., 2008). The Griess reagent (5% H_3PO_4 containing 1% sulfanilamide and 0.1% naphthyl ethylenediamine) 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. The 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) and normalized based on each sample's total protein concentration.

Table 1. The sequences of the primers used for qPCR

Gene	Accession Number	Sequence	Size (bp)
CRL	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
IL1β	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
SOX9	AB073720.1	F: AGTCGGTGAAGAATGGGCAA R: ACCCTGAGATTGCCCGGAG	161
MMP-13	M60616.1	F: CAAGCAGCTCCAAAGGCTAC R: TGGCTTTTGCCAGTGTAGGT	130

Mitochondrial membrane potential (MMP) assay

Staining with Rhodamine 123 (Rh123) was used to MMP (Sakamuru et al., 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 the fluorescence intensity of Rh123 was measured (excitation 488 and emission 525 nm).

Gene expression analysis by qPCR

The relative expressions of *AM*, *CRL*, *RAMPs*, *MMP-3*, *MMP-13*, *iNOS*, *TNF-α*, *IL-1β*, and *SOX9* 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.

Statistical analysis

SPSS software, version 16 was used to analyze the data. According to the Shapiro-Wilk test, the data were 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 a significant difference.

3. Results

AM protected SMNs against DOX-induced cell death

The MTT assay results (Figure 2) revealed a dose- and time-dependent increase in SMN cell death following DOX treatment (IC_{50} 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 was chosen for the subsequent experiments.

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).

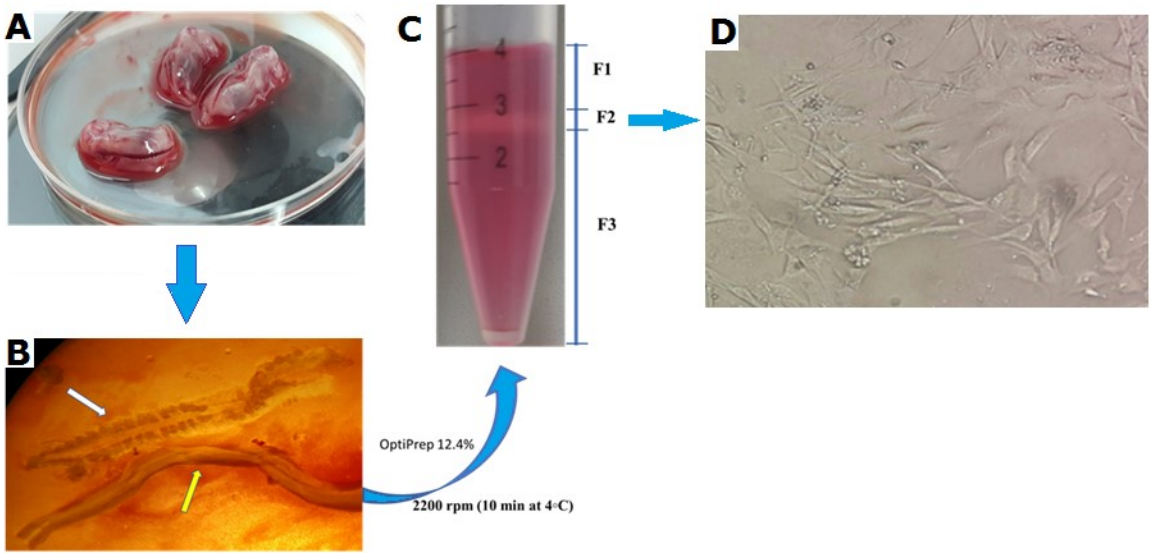

Figure 1. Isolation of SMNs from rat embryo

A) Spinal cord was removed from rat embryos E14-E15

B) DRGs and spinal cord membrane (white arrow) were separated from the spinal cord (yellow arrow) (spinal cords was digested, mechanicaly triturated and resulting cell suspension was centrifuged at OptiPreb 12.4%)

C) Three fractions(F1, F2, F3) were obtained (SMNs were enriched at F2 fraction)

D) Phase contrast image of SMNs culture 72 h after FUDR treatment

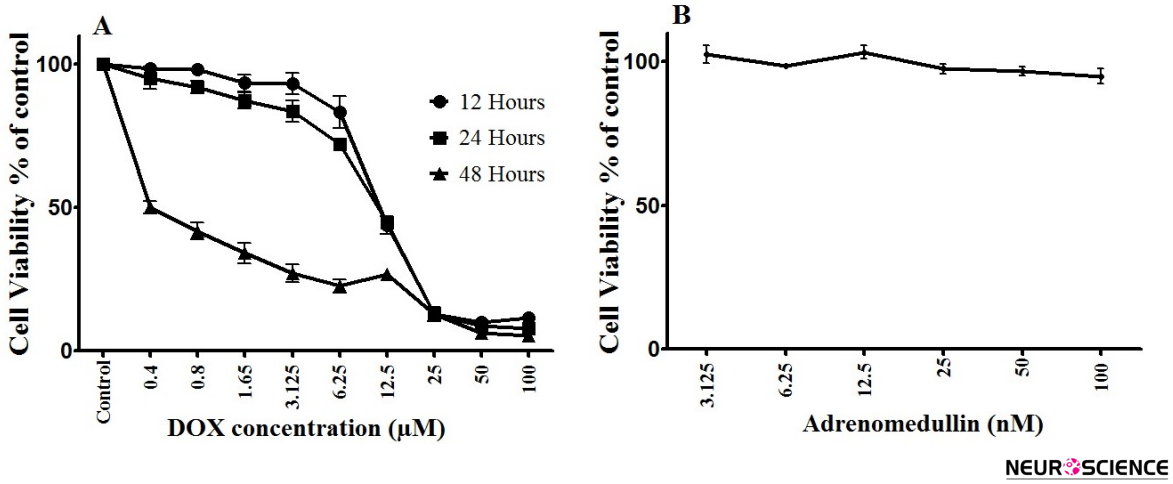

Figure 2. The effects of DOX and AM on the viability of SMNs

A) SMNs were treated with various concentrations of DOX (0.0-100 µM) for 12, 24, and 48 hours and cell viability were measured using MTT assay

B) The effects of AM treatment (3.125-100 nM for 24 h) on the cell viability of SMNs

Notes: The presented data are Mean±SEM of at least six independent experiments.

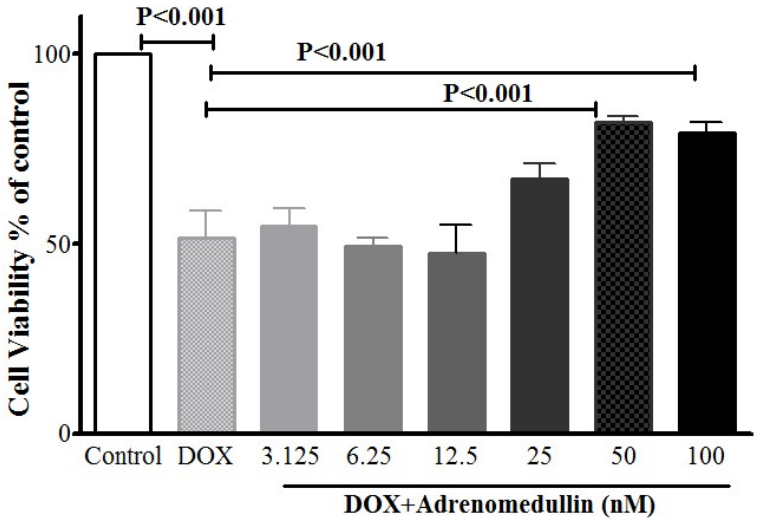

Figure 3. Protective effect of AM against DOX-induced cell death of SMNs

Notes: SMNs were treated with DOX (IC_{50}=10.54 µM) in the absence and presence of various concentrations of AM (3.125-100 nM) for 24 h and cell viability was evaluated using MTT assay. The presented data are Mean±SEM of at least six independent experiments. ANOVA followed by the LSD post hoc test was used to analyze the data. AM (50 and 100 nM) increased the cell viability of DOX-treated cells (P<0.001).

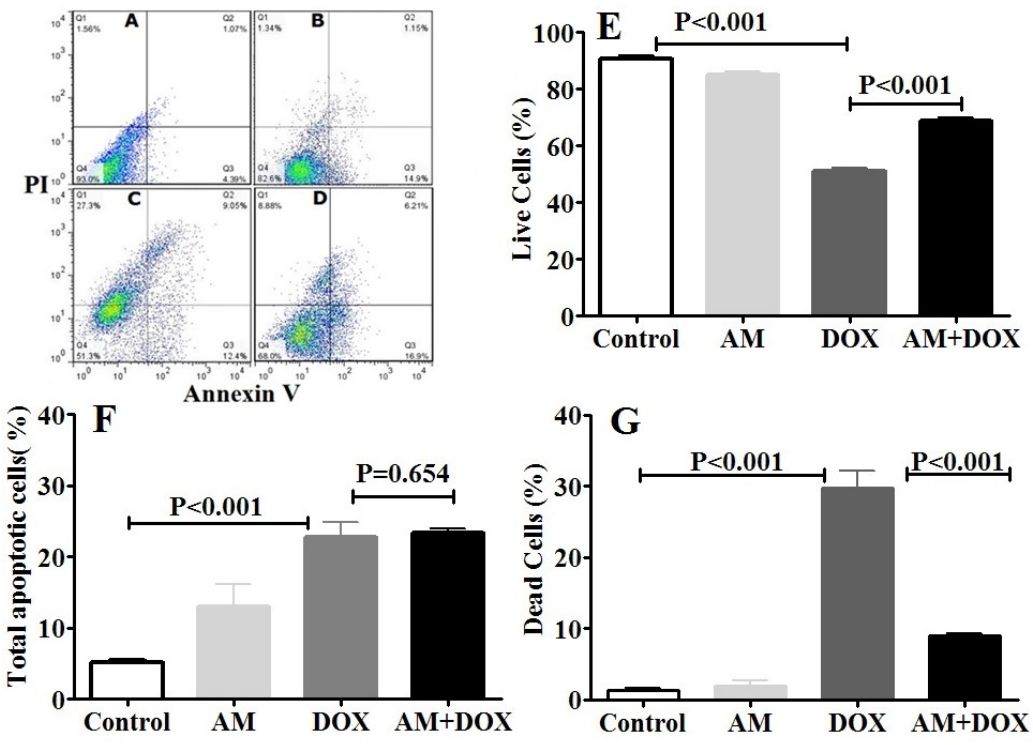

Figure 4. The effects of AM on DOX-induced cell death and apoptosis of SMNs

Notes: SMNs were treated with DOX (10.54 µM) in the absence or presence of AM (50 nM) for 24 h, and cell viability was measured using annexin V/PI flow cytometry. A, B, C, and D representing flow cytometric scatter diagram for the control, AM, DOX and AM+DOX treated groups, respectively. E, F, and G figures represent the percentages of live, total apoptotic (early +late), and necrotic cells in various groups. The presented data are Mean±SEM from at least three independent experiments. ANOVA followed by the LSD post hoc test was used to analyze the data. P<0.05 was considered as significant difference between the groups.

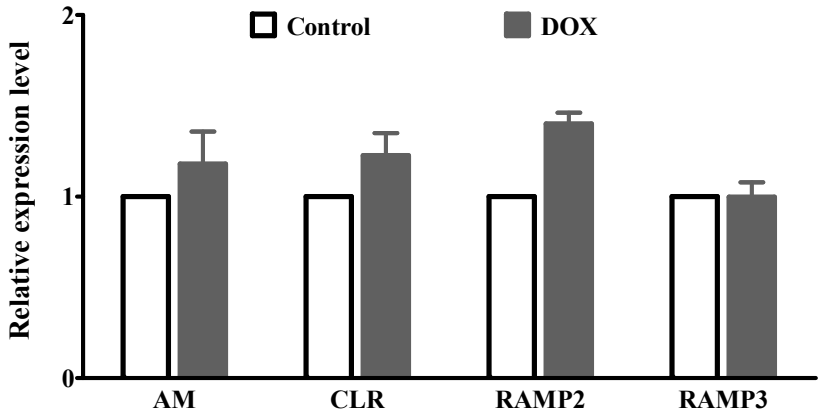

Figure 5. Relative expression of *AM, CLR, RAMP2, RAMP3* in control and DOX-treated motor neurons

Notes: The cells were treated with DOX (10.54 µM) for 24-hours. The level of AM and its receptor components was determined using real time PCR. Relative expression of each mRNA compared to β-actin mRNA was calculated in the control and DOX-treated groups. The presented data are Mean±SEM from three independent experiments. ANOVA followed by the LSD post hoc test was used to analyze the data.

AM also reduced the DOX-induced necrosis of SMNs (P<0.0001) (Figure 4).

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*.

DOX-Induced oxidative stress 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 6A, DOX elevated intracellular ROS

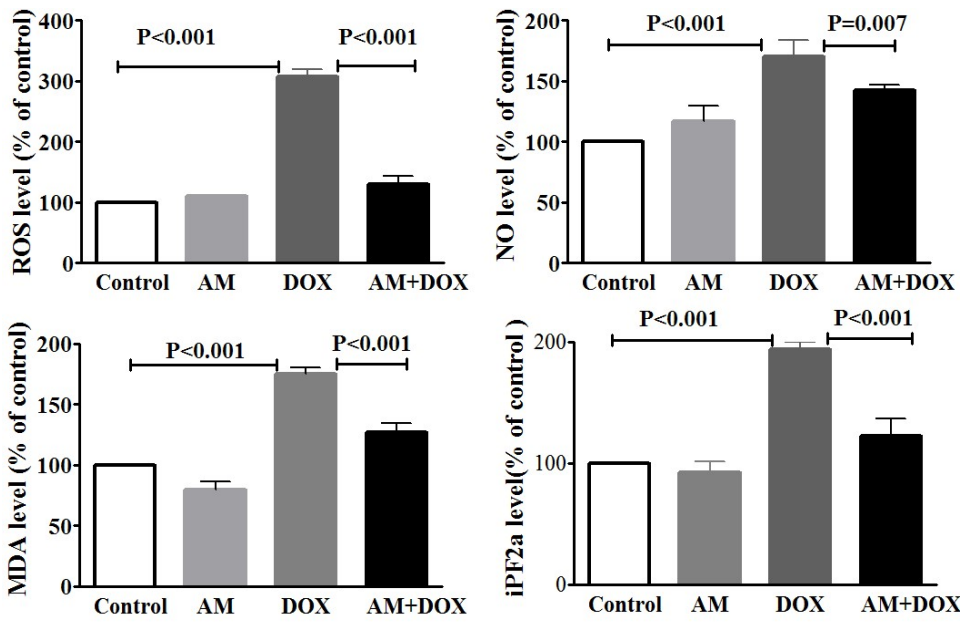

Figure 6. Effect of AM on the levels of ROS, NO, MDA, and iPF2α in DOX-treated motor neurons

Notes: The neurons were treated with DOX (10.54 µM) with and without AM (50 nM) pretreatment. ROS, NO, MDA, and iPF2α levels were determined as described in the methods. The presented data are Mean±SEM from three independent experiments. ANOVA followed by the LSD post hoc test was used to analyze the data. P<0.05 was considered as significant difference between the groups.

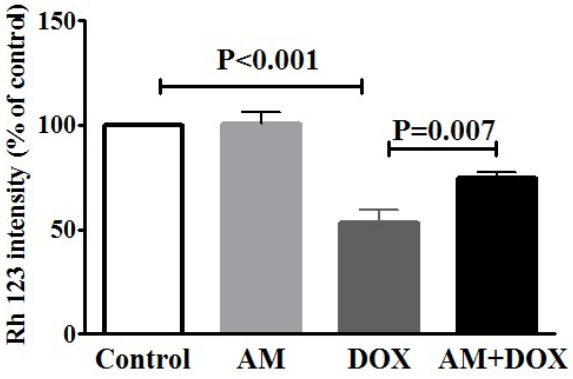

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Figure 7. Effects of AM on DOX-induced MMP dissipation in motor neurons

Notes: The cells were treated with DOX (10.54 µM) in the absence or presence of AM (50 nM) for 24 h. MMP was measured using rhodamine 123 staining. The presented data are Mean±SEM. ANOVA followed by the LSD post hoc test was used to analyze the data. P<0.05 was considered as significant difference between the groups.

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 6B). 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) (Figure 6).

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-

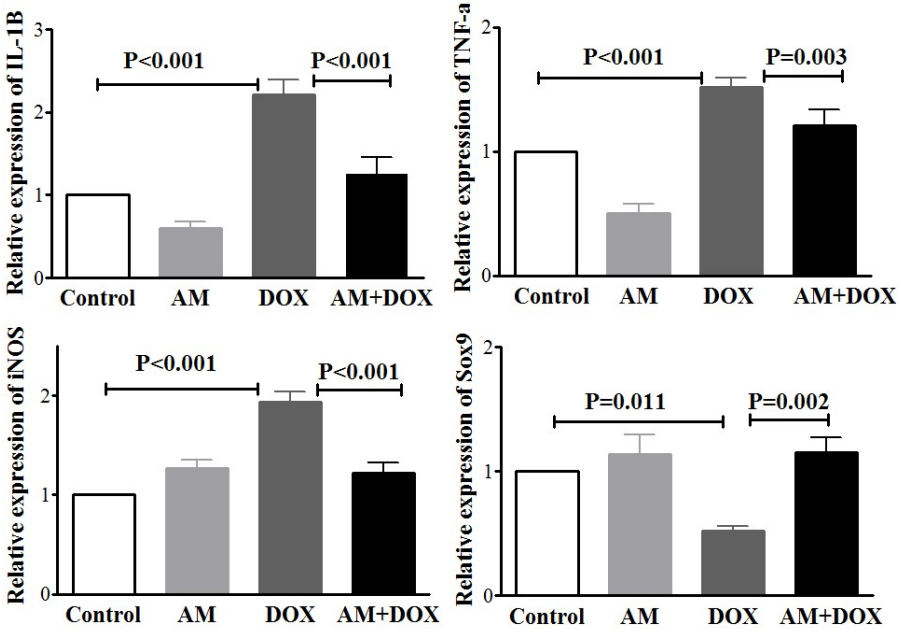

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Figure 8. The effect of AM on DOX-induced gene expressions of *IL-1β*, *TNF-a*, *iNOS* and *SOX9*

Notes: SMNs were treated with DOX (10.54 µM for 24 h) in the absence or presence of AM (50 nM). The expression of *IL-1β*, *TNF-a*, *iNOS*, and *SOX9* were determined using real-time PCR. Relative expression of each mRNA compared to β-actin mRNA was calculated in the control and treated groups. Fold changes are shown relative to untreated cells. The presented data are Mean±SEM. ANOVA followed by LSD post hoc test was used to analyze the data. P<0.05 was considered as significant difference between the groups.

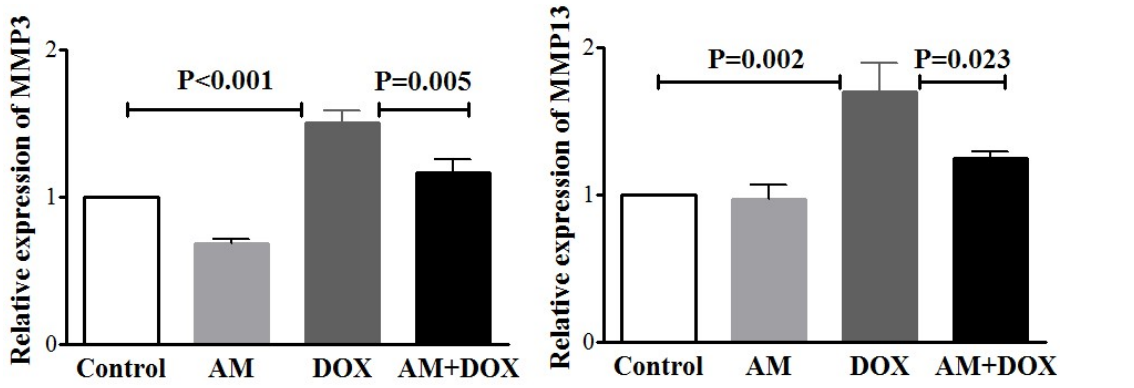

Figure 9. The effect of DOX and AM on *MMP-3* and *MMP-13* mRNA levels

Notes: The isolated SMNs were treated with DOX (10.54 µM for 24 h) in the absence and presence of AM (50 nM). The expression of *MMP-3* (A) and *MMP-13* (B) were determined using real time PCR. Relative expression of each mRNAs compared to β-actin mRNA was calculated in the control and treated groups. The data were reported as the percentage of the control group. The presented data are Mean±SEM. ANOVA followed by LSD post hoc test was used to analyze the data. P<0.05 was considered as significant difference between the groups.

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).

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) increased compared to the control SMNs (Figure 8). AM blocked the effects of DOX on *iNOS*, *TNF-α*, *SOX9*, and *IL-1β* expressions. As shown in Figure 9, DOX also increased the expression levels of *MMP-3* (P<0.001) and *MMP-13* (P=0.007). AM pretreatment revealed no significant effects on *MMP-13* mRNA while decreasing 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 et al., 2018). This research utilized this model to determine AM's possible neuroprotective role. 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 (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. (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 the flow cytometric 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 block the IL-1β-induced inflammation by decreasing the expressions of *iNOS* and cyclooxygenase-2 as well as the concentrations of NO and prostaglandin E2 (Hu et al., 2015).

In line with other studies, the findings showed that DOX significantly induced MMP dissipation. AM ameliorated DOX-induced mitochondrial damage in cardiac muscle cells (Yoshizawa et al., 2016). Therefore, prevention of mitochondria damage may play a role in the observed AM effects. Some reports regarding the anti-inflammatory properties of AM are also available. Hu et al. showed that AM could attenuate inflammation-induced apoptosis of rats' Leydig cells (Hu et al., 2015). This finding agreed with the present study results, which demonstrated a significant reduction in the expressions of inflammatory mediators following AM pretreatment in the SMNs, suggesting that AM may exert anti-inflammatory action against toxicity induced by DOX. Several mechanisms have been proposed for the anti-inflammatory properties of AM. For example, AM may inhibit the release of cytokine-induced neutrophil chemoattractants, 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 regulating the 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 critical in activating *MMPs*. Thus, antioxidant and anti-inflammatory agents could act as 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 cell survival (Scott et al., 2010). Proteasome-induced degradation of *SOX9* was revealed following DNA damage by several genotoxic agents (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, leading 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, the *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, a decrease in cell viability 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 indicated that pretreatment 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.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of Shiraz University of Medical Sciences approved this study (Code: IR.SUMS.REC.1396.582).

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Conflict of interest

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References

Bäumer, D., Talbot, K., & Turner, M. R. (2014). Advances in motor neurone disease. *Journal of the Royal Society of Medicine, 107*(1), 14-21. [DOI:10.1177/0141076813511451] [PMID] [PMCID]

Bucchia, M., Merwin, S. J., Re, D. B., & Kariya, S. (2018). Limitations and challenges in modeling diseases involving spinal motor neuron degeneration in vitro. *Frontiers in Cellular Neuroscience, 12,* 61. [DOI:10.3389/fncel.2018.00061] [PMID] [PMCID]

Capelôa, T., Caramelo, F., Fontes-Ribeiro, C., Gomes, C., & Silva, A. P. (2014). Role of methamphetamine on glioblastoma cytotoxicity induced by doxorubicin and methotrexate. *Neurotoxicity Research, 26*(3), 216-227. [DOI:10.1007/s12640-014-9464-1] [PMID]

Chen, X., Zhong, Z., Xu, Z., Chen, L., & Wang, Y. (2010). 2′,7′-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy. *Free Radical Research, 44*(6), 587–604. [DOI:10.3109/10715761003709802] [PMID]

Cirrincione, A. M., Pellegrini, A. D., Dominy, J. R., Benjamin, M. E., Utkina-Sosunova, I., & Lotti, F., et al (2019). Paclitaxel-induced peripheral neuropathy is caused by epidermal ROS and mitochondrial damage through conserved MMP-13 activation. bioRxiv. [DOI:10.1101/743419]

Demir, H., Onur, O. E., Denizbasi, A., Akoglu, H., Eroglu, S. E., & Ozpolat, C., et al. (2013). The effects of adrenomedullin in traumatic brain injury. *Peptides, 43,* 27-31. [DOI:10.1016/j.peptides.2013.02.018] [PMID]

Drechsel, D. A., Estévez, A. G., Barbeito, L., & Beckman, J. S. (2012). Nitric oxide-mediated oxidative damage and the progressive demise of motor neurons in ALS. *Neurotoxicity Research, 22*(4), 251-264. [DOI:10.1007/s12640-012-9322-y] [PMID] [PMCID]

Fingleton, B. (2017). Matrix metalloproteinases as regulators of inflammatory processes. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1864*(11), 2036-2042. [DOI:10.1016/j.bbamcr.2017.05.010] [PMID]

Geven, C., Kox, M., & Pickkers, P. (2018). Adrenomedullin and adrenomedullin-targeted therapy as treatment strategies relevant for sepsis. *Frontiers in Immunology, 9,* 292. [DOI:10.3389/fimmu.2018.00292] [PMID] [PMCID]

Haorah, J., Ramirez, S. H., Schall, K., Smith, D., Pandya, R., & Persidsky, Y. (2007). Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *Journal of Neurochemistry, 101*(2), 566-576. [DOI:10.1111/j.1471-4159.2006.04393.x] [PMID]

Hare, J. M., Beigi, F., & Tziomalos, K. (2008). Nitric oxide and cardiobiology-methods for intact hearts and isolated myocytes. *Methods in Enzymology, 441,* 369–392. [DOI:10.1016/S0076-6879(08)01221-4] [PMID]

Holmes, D., Campbell, M., Harbinson, M., & Bell, D. (2013). Protective effects of intermedin on cardiovascular, pulmonary and renal diseases: Comparison with adrenomedullin and CGRP. *Current Protein & Peptide Science, 14*(4), 294–329. [DOI:10.2174/1389203713149990049] [PMID]

Hong, X., Liu, W., Song, R., Shah, J. J., Feng, X., & Tsang, C. K., et al. (2016). SOX9 is targeted for proteasomal degradation by the E3 ligase FBW7 in response to DNA damage. *Nucleic Acids Research, 44*(18), 8855–8869. [DOI:10.1093/nar/gkw748] [PMID] [PMCID]

Hong, Y., Liu, Y., Chabot, J. G., Fournier, A., & Quirion, R. (2009). Upregulation of adrenomedullin in the spinal cord and dorsal root ganglia in the early phase of CFA-induced inflammation in rats. *Pain, 146*(1-2), 105-113. [DOI:10.1016/j.pain.2009.07.015] [PMID]

Hu, W., Zhou, P. H., Rao, T., Zhang, X. B., Wang, W., & Zhang, L. J. (2015). Adrenomedullin attenuates interleukin-1β-induced inflammation and apoptosis in rat Leydig cells via inhibition of NF-κB signaling pathway. *Experimental Cell Research, 339*(2), 220-230. [DOI:10.1016/j.yexcr.2015.10.024] [PMID]

Khan, M. S., Singh, M., Khan, M. A., Arya, D., & Ahmad, S. (2014). Scientific validation of cardioprotective attribute by standardized extract of Bombyx mori against doxorubicin-induced cardiotoxicity in murine model. *EXCLI Journal, 13,* 1043–1054. [PMID]

Li, M. Y., Zhu, X. L., Zhao, B. X., Shi, L., Wang, W., & Hu, W., et al. (2019). Adrenomedullin alleviates the pyroptosis of Leydig cells by promoting autophagy via the ROS-AMPK-mTOR axis. *Cell Death & Disease, 10*(7), 489. [DOI:10.1038/s41419-019-1728-5] [PMID] [PMCID]

Liu, R., Lin, G., & Xu, H. (2013). An efficient method for dorsal root ganglia neurons purification with a one-time anti-mitotic reagent treatment. *Plos One, 8*(4), e60558. [DOI:10.1371/journal.pone.0060558] [PMID] [PMCID]

Ma, W., Chabot, J. G., & Quirion, R. (2006). A role for adrenomedullin as a pain-related peptide in the rat. *Proceedings of the National Academy of Sciences of the United States of America, 103*(43), 16027–16032. [DOI:10.1073/pnas.0602488103] [PMID] [PMCID]

Mahmoodazdeh, A., Shafiee, S. M., Sisakht, M., Khoshdel, Z., & Takhshid, M. A. (2020). Adrenomedullin protects rat dorsal root ganglion neurons against doxorubicin-induced toxicity by ameliorating oxidative stress. *Iranian Journal of Basic Medical Sciences, 23*(9), 1197–1206. [PMID]

Martinez-Herrero, S., Larrayoz, I. M., Narro-Iniguez, J., Rubio-Mediavilla, S., & Martinez, A. (2017). Lack of adrenomedullin aggravates acute TNBS-induced colitis symptoms in mice, especially in females. *Frontiers in Physiology, 8,* 1058. [DOI:10.3389/fphys.2017.01058] [PMID] [PMCID]

McGowan, J. V., Chung, R., Maulik, A., Piotrowska, I., Walker, J. M., & Yellon, D. M. (2017). Anthracycline chemotherapy and cardiotoxicity. *Cardiovascular Drugs and Therapy, 31*(1), 63-75. [DOI:10.1007/s10557-016-6711-0] [PMID] [PMCID]

Montuenga, L. M., Martínez, A., Miller, M. J., Unsworth, E. J., & Cuttitta, F. (1997). Expression of adrenomedullin and its receptor during embryogenesis suggests autocrine or paracrine modes of action. *Endocrinology, 138*(1), 440-451. [DOI:10.1210/endo.138.1.4881] [PMID]

Moruno-Manchon, J. F., Uzor, N. E., Kesler, S. R., Wefel, J. S., Townley, D. M., & Nagaraja, A. S., et al. (2016). TFEB ameliorates the impairment of the autophagy-lysosome pathway in neurons induced by doxorubicin. *Aging, 8*(12), 3507-3519. [DOI:10.18632/aging.101144] [PMID] [PMCID]

Nishida, K., Kuchiiwa, S., Oiso, S., Futagawa, T., Masuda, S., & Takeda, Y., et al. (2008). Up-regulation of matrix metalloproteinase-3 in the dorsal root ganglion of rats with paclitaxel-induced neuropathy. *Cancer Science, 99*(8), 1618–1625. [DOI:10.1111/j.1349-7006.2008.00877.x] [PMID] [PMCID]

Nissinen, L., & Kähäri, V. M. (2014). Matrix metalloproteinases in inflammation. *Biochimica et Biophysica Acta, 1840*(8), 2571–2580. [DOI:10.1016/j.bbagen.2014.03.007] [PMID]

Pevny, L., & Placzek, M. (2005). SOX genes and neural progenitor identity. *Current Opinion in Neurobiology, 15*(1), 7–13. [DOI:10.1016/j.conb.2005.01.016] [PMID]

Piera-Velazquez, S., Hawkins, D. F., Whitecavage, M. K., Colter, D. C., Stokes, D. G., & Jimenez, S. A. (2007). Regulation of the human SOX9 promoter by Sp1 and CREB. *Experimental cell Research, 313*(6), 1069–1079. [DOI:10.1016/j.yexcr.2007.01.001] [PMID] [PMCID]

Roche, K. C., Gracz, A. D., Liu, X. F., Newton, V., Akiyama, H., & Magness, S. T. (2015). SOX9 maintains reserve stem cells and preserves radioresistance in mouse small intestine. *Gastroenterology, 149*(6), 1553-1563.e1510. [DOI:10.1053/j.gastro.2015.07.004] [PMID] [PMCID]

Sabri, M. I. (2018). Chemical neurotoxins and disruption of the axonal transport system. In Z. Iqbal (Ed.), *Axoplasmic Transport* (pp. 185-207)., Florida: CRC press. [DOI:10.1201/9781351070003-15]

Saito, T., Okamura, A., Inoue, J., Makiura, D., Doi, H., & Yakushijin, K., et al. (2019). Anemia is a novel predictive factor for the onset of severe chemotherapy-induced peripheral neuropathy in lymphoma patients receiving rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisolone therapy. *Oncology Research, 27*(4), 469–474. [DOI:10.3727/096504018X15267574931782] [PMID] [PMCID]

Sakamuru, S., Attene-Ramos, M. S., & Xia, M. (2016). Mitochondrial membrane potential assay. *Methods in Molecular Biology (Clifton, N.J.), 1473,* 17–22. [DOI:10.1007/978-1-4939-6346-1_2] [PMID] [PMCID]

Scott, C. E., Wynn, S. L., Sesay, A., Cruz, C., Cheung, M., & Gomez Gaviro, M. V., et al. (2010). SOX9 induces and maintains neural stem cells. *Nature Neuroscience, 13*(10), 1181–1189. [DOI:10.1038/nn.2646] [PMID]

Si, H., Zhang, Y., Song, Y., & Li, L. (2018). Overexpression of adrenomedullin protects mesenchymal stem cells against hypoxia and serum deprivationinduced apoptosis via the Akt/GSK3β and Bcl2 signaling pathways. *International Journal of Molecular Medicine, 41*(6), 3342-3352. [DOI:10.3892/ijmm.2018.3533] [PMID] [PMCID]

Starobova, H., & Vetter, I. (2017). Pathophysiology of chemotherapy-induced peripheral neuropathy. *Frontiers in Molecular Neuroscience, 10,* 174. [DOI:10.3389/fnmol.2017.00174] [PMID] [PMCID]

Takhshid, M. A., Owji, A. A., Vasei, M., Panjehshahin, M. R., Tabei, S. M., & Tabatabaee, H. R., et al. (2004). Expression of spinal cord Fos protein in response to intrathecal adrenomedullin and CGRP in conscious rats. *Brain Research, 1020*(1-2), 30–36. [DOI:10.1016/j.brainres.2004.05.112] [PMID]

Tsikas, D. (2017). Assessment of lipid peroxidation by measuring malondialdehyde (MDA) and relatives in biological samples: Analytical and biological challenges. *Analytical Biochemistry, 524,* 13–30. [DOI:10.1016/j.ab.2016.10.021] [PMID]

Uetake, R., Sakurai, T., Kamiyoshi, A., Ichikawa-Shindo, Y., Kawate, H., & Iesato, Y., et al. (2014). Adrenomedullin-RAMP2 system suppresses ER stress-induced tubule cell death and is involved in kidney protection. *Plos One, 9*(2), e87667. [DOI:10.1371/journal.pone.0087667] [PMID] [PMCID]

Verma, T., Mallik, S. B., Ramalingayya, G. V., Nayak, P. G., Kishore, A., & Pai, K. S. R., et al. (2017). Sodium valproate enhances doxorubicin-induced cognitive dysfunction in Wistar rats. *Biomedicine & Pharmacotherapy, 96,* 736-741. [DOI:10.1016/j.biopha.2017.09.150] [PMID]

Voors, A. A., Kremer, D., Geven, C., Ter Maaten, J. M., Struck, J., & Bergmann, A., et al. (2019). Adrenomedullin in heart failure: Pathophysiology and therapeutic application. *European Journal of Heart Failure, 21*(2), 163–171. [DOI:10.1002/ejhf.1366] [PMID] [PMCID]

Wang, W., Qi, B., Lv, H., Wu, F., Liu, L., & Wang, W., et al. (2017). A new method of isolating spinal motor neurons from fetal mouse. *Journal of Neuroscience Methods, 288,* 57-61. [DOI:10.1016/j.jneumeth.2017.06.014] [PMID]

Webster, K. A. (2012). Mitochondrial membrane permeabilization and cell death during myocardial infarction: Roles of calcium and reactive oxygen species. *Future Cardiology, 8*(6), 863-884. [DOI:10.2217/fca.12.58] [PMID] [PMCID]

Wu, S. N., So, E. C., Liao, Y. K., & Huang, Y. M. (2015). Reversal by ranolazine of doxorubicin-induced prolongation in the inactivation of late sodium current in rat dorsal root ganglion neurons. *Pain Medicine, 16*(5), 1032-1034. [DOI:10.1111/pme.12681] [PMID]

Yoshimoto, T., Fukai, N., Sato, R., Sugiyama, T., Ozawa, N., & Shichiri, M., et al. (2004). Antioxidant effect of adrenomedullin on angiotensin II-induced reactive oxygen species generation in vascular smooth muscle cells. *Endocrinology, 145*(7), 3331-3337. [DOI:10.1210/en.2003-1583] [PMID]

Yoshizawa, T., Takizawa, S., Shimada, S., Tokudome, T., Shindo, T., & Matsumoto, K. (2016). Effects of adrenomedullin on doxorubicin-induced cardiac damage in mice. *Biological & Pharmaceutical Bulletin, 39*(5), 737–746. [DOI:10.1248/bpb.b15-00832] [PMID]

Yoshizawa, T., Takizawa, S., Shimada, S., Tokudome, T., Shindo, T., & Matsumoto, K. (2016). Effects of adrenomedullin on doxorubicin-induced cardiac damage in mice. *Biological & Pharmaceutical Bulletin, 39*(5), 737–746. [DOI:10.1248/bpb.b15-00832] [PMID]

Yu, Z. G., Xu, N., Wang, W. B., Pan, S. H., Li, K. S., & Liu, J. K. (2009). Interleukin-1 inhibits Sox9 and collagen type II expression via nuclear factor-κB in the cultured human intervertebral disc cells. *Chinese Medical Journal, 122*(20), 2483-2488. [PMID]

Zhao, L., & Zhang, B. (2017). Doxorubicin induces cardiotoxicity through upregulation of death receptors mediated apoptosis in cardiomyocytes. *Scientific Reports, 7,* 44735. [DOI:10.1038/srep44735] [PMID] [PMCID]

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