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Article

Study of the potential toxicity of adrenaline to neurons, using the SH-SY5Y human cellular model

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Prolonged overexposure to catecholamines causes toxicity, usually credited to continuous adrenoceptor stimulation, autoxidation, and the formation of reactive pro-oxidant species. Non-differentiated SH-SY5Y cells were used to study the possible contribution of oxidative stress in adrenaline (ADR)-induced neurotoxicity, as a model to predict the toxicity of this catecholamine to peripheral nerves. Cells were exposed to several concentrations of ADR (0.1, 0.25, 0.5 and 1mM) and two cytotoxicity assays [lactate dehydrogenase (LDH) release and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction] were performed at several time-points (24, 48, and 96h). The cytotoxicity of ADR was concentration- and time-dependent in both assays, since the lowest concentration tested (0.1mM) also caused significant cytotoxicity at 96h. N-acetyl-cysteine (1mM), a precursor of glutathione synthesis, prevented ADR-induced toxicity elicited by 0.5mM and 0.25mM ADR following a 96-h exposure, while the antioxidant Tiron (100μM) was non-protective. In conclusion, ADR led to mitochondrial distress and ultimately cell death in non-differentiated SH-SY5Y cells, possibly because of ADR oxidation products. The involvement of such processes in the catecholamine-induced peripheral neuropathy requires further analysis.

Keywords: Neurotoxicity. Adrenaline. N-acetyl-cysteine. Tiron. Reactive species.

Abbreviations list

ADR, adrenaline
DA, dopamine
DMEM, Dulbecco's Modified Eagle Medium
FBS, fetal bovine serum
LDH, lactate dehydrogenase
MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide
NAC, N-acetyl-cysteine
NA, noradrenaline
PBS, phosphate buffered saline
ROS, reactive oxygen species
Tiron, 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt

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INTRODUCTION

Physical challenges, emotional arousal, increased physical activity, or changes in the environment may activate the 'fight-or-flight response', causing stress and altered activity in several organs and tissues. These

changes occur through the release of several endogenous substances (Tank, Lee Wong, 2015). During the 'fight-or-flight response', activation of the sympathetic nervous system causes the release of adrenaline (ADR) and noradrenaline (NA) from the adrenal medulla and NA from sympathetic nerve terminals (as well as ADR in small amounts). This allows the increase of blood pressure and cardiac output, to cope with the challenging circumstances (Tank, Lee Wong, 2015). ADR is recognized as an important metabolic hormone that helps mobilize energy stores in the form of glucose and free fatty acids in preparation for physical activity or for recovery from hypoglycemia (Verberne *et al.*, 2016). Moreover, in *Diabetes mellitus*, the catecholaminergic system is functionally altered in many ways depending on the diabetes stage or tissue selected. *Diabetes mellitus* may lead to peripheral neuropathy, in which sustained exposure to catecholamines, namely ADR, can be a contributor to the damage observed in peripheral neurons (Cryer *et al.*, 1978; Gallego *et al.*, 2003). In fact, early studies have demonstrated that some diabetic patients had higher ADR excretion (Del Rio *et al.*, 1990; Del Rio, Marrama, Della Casa, 1992). This disruption of the adrenergic system, especially through higher ADR release from the adrenal medulla leads to the high exposure of several organs and peripheral neurons to this catecholamine. Unlike neurons in the central nervous system, peripheral neurons are not protected by the blood brain barrier. It is well known that adrenoceptor overstimulation causes toxicity. In addition, oxidative stress has also been described as being involved in catecholamine toxicity in several systems. In fact, the accumulation of dopamine (DA) and its degradation products produces oxidative stress and neuronal degeneration (Asanuma, Miyazaki, Ogawa, 2003; Kumar *et al.*, 2012; Offen *et al.*, 1996); and ADR (as other catecholamines) potentiates amyloid β-peptide toxicity in hippocampal neurons, contributing to Alzheimer's disease (Fu *et al.*, 1998). Despite these reports, the toxic effects of ADR on peripheral neurons have been poorly explored.

We have previously reported the toxic effects of catecholamines in the heart, namely those involving catecholamine oxidation products (Costa *et al.*, 2009a; Costa *et al.*, 2007; Costa *et al.*, 2009b; Costa *et al.*, 2009c). When the enzymes responsible for the catabolism of catecholamines are unable to cope efficiently with their sustained release, catecholamines can undergo oxidation. The oxidation rate is faster under enzymatic or metal catalysis, in the presence of superoxide radicals or higher pH. In general, catecholamines may be oxidized to unstable o-semiquinones that, after deprotonation and loss of a second electron, form the analogous o-quinones (Costa *et al.*, 2011). Partial deprotonation of the amine group of the side chain may lead to an irreversible 1,4-intramolecular cyclization, a reaction that occurs through nucleophilic attack of the nitrogen atom at position 6 of the quinone ring, to give "leucoaminochrome", which is subsequently oxidized to form aminochromes (Costa *et al.*, 2011). The formation of aminochromes is very fast in ADR, while for DA, the quinone intermediary is more stable, but in both cases reactive oxygen species (ROS) can be formed by oxygen reduction. Once formed, the aminochromes can be transformed into melanins, which are reactive compounds that easily undergo a series of reactions among them. *In vivo*, these oxidation pathways may be more complex, since other factors, such as nucleophilic groups or metal ions can be involved, and both aminochromes and quinones can react with cellular nucleophilic groups to cause cytotoxicity (Costa *et al.*, 2011) (Figure 1).

Due to its reactivity and the large amounts of ADR released by the adrenal medulla in stressful situations, diabetes or in drug abuse situations, the potential neurotoxic actions of ADR must be evaluated. Therefore, in this study we aimed to assess the toxicity of ADR to the SH-SY5Y human neuroblastoma cell line (a catecholaminergic cellular model) and determine whether antioxidants could prevent ADR-induced cytotoxicity.

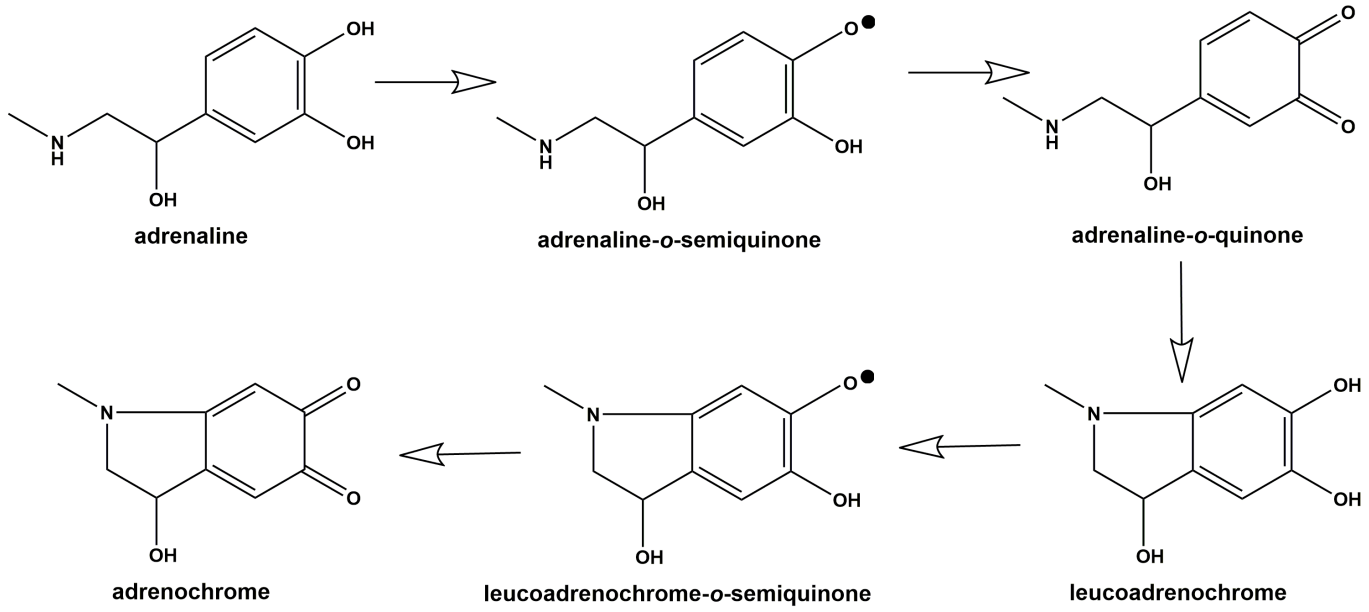

FIGURE 1 - Adrenaline (ADR) oxidation processes and related production of reactive species.

MATERIAL AND METHODS

Material

Materials for cell culture experiments were obtained from the following sources: ADR, trypsin/EDTA, *N*-acetyl-cysteine (NAC), dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), nicotinamide adenine dinucleotide reduced (NADH), pyruvate sodium, dodecyl sulfate (SDS), and 4,5-dihydroxy-1,3-benzenedisulfonic acid disodium salt (tiron) from Sigma-Aldrich (St. Louis, MO, USA); phosphate buffered saline (PBS), penicillin/streptomycin from Biochrom (Berlin, Germany); Dulbecco's Modified Eagle Medium (DMEM) high glucose, trypsin-EDTA 0.05% and fetal bovine serum (FBS) from Gibco–Invitrogen, Alfagene (Carcavelos, Portugal). Culture multi-well plates, petri dishes and cell flasks, as well as sterile pipettes and filters, were obtained from Corning Costar (Corning, NY, USA). All chemicals were of analytical grade.

Cell culture

SH-SY5Y neurons were acquired from Sigma-Aldrich (European Collection of Authenticated Cell Cultures-ECACC). Regarding cell maintenance, they were maintained in DMEM high glucose medium supplemented with 10% FBS, 100units/mL penicillin, 100mg/mL streptomycin. Cells were maintained and grown at 37ºC in 5% CO_2 and 95% air. For experiments, cells were seeded in a density of 25,000 cells/cm^2 in complete DMEM high glucose medium. Cells were allowed to adhere and reach confluence for 3-4 days. Before the beginning of each experiment, the medium was changed to new complete DMEM medium. Cells were then incubated with different ADR concentrations (up to 1mM) for 24, 48 and 96h. ADR, NAC and tiron solutions were made in PBS and control cells were incubated with PBS. When NAC or tiron was used, they were added to the cells 30 minutes before ADR (0.25 and 0.5mM), and the 96h time-point was used for these experiments, to evaluate the long-term neurotoxic effect of the catecholamine.

Lactate dehydrogenase (LDH) leakage assay

Cellular membrane damage was assessed quantitatively by measuring LDH release into the medium using a kinetic photometric assay, as described previously (Capela *et al.*, 2006b). Cell death was assessed

by calculating the ratio between LDH leakage into the medium with the respective total cellular LDH. Total cellular LDH was obtained after achieving the full cellular death by adding 0.5 % triton X-100 per well for 30 min at 37ºC (Capela *et al.*, 2006b).

MTT Assay

The MTT assay is based on the reduction of tetrazolium salts and was performed as an indirect measure of mitochondrial function at 24, 48, or 96h (Costa *et al.*, 2009c). To avoid any interference of ADR or its byproducts on the assay, the incubation medium was removed at the desired time-point and new complete warm medium was added with 500µg MTT/ml, followed by an incubation at 37°C for 3h (Almeida *et al.*, 2018). The reaction was stopped by adding 10% SDS in 0.01M HCl. This was followed by an over-night incubation at 37°C and photometric detection of formazan at 550 nm, as described previously (Rossato *et al.*, 2013). Data obtained are presented as percentage of control cells, whose value was set to 100 %.

Statistical analysis

Results are presented as mean ± standard deviation (SD). Data were pooled from 4 to 7 independent experiments, and normal distribution was confirmed using the following tests, D'Agostino & Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests. Since data passed at least one of these normality tests, statistical comparisons were performed using ANOVA tests. Comparisons between groups were performed with one-way ANOVA, and Bonferroni's *post-hoc* test was used once a significant *p* was achieved. In particular, for the LDH and MTT data included in Figures 2 and 3, an additional analysis was performed using a two-way ANOVA followed by Tukey's multiple comparisons test, to evaluate the effect of time in the cytotoxicity observed. All statistical analyses were performed using GraphPad Prism 8 software (GraphPad software, Inc, La Jolla, CA). Significance was accepted at *p* values < 0.05. Details of the statistical analyses are found in figure legends.

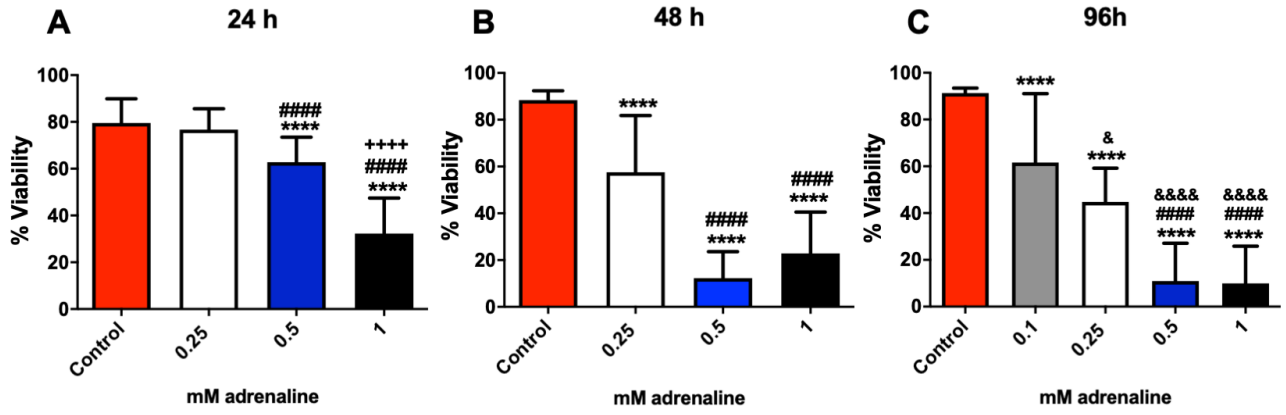

FIGURE 2 - Adrenaline (ADR) killed human SH-SY5Y neuronal cells in a concentration- and time-dependent manner. Cultures of human SH-SY5Y cells were exposed to ADR (0.1, 0.25, 0.5, and 1mM) for different time-points. ***A***, ***B*** and ***C***, Percentage of cell viability evaluated by the LDH release assay after ADR exposure for 24, 48 and 96h, respectively. Results were obtained from 23 - 42 wells from 4 - 7 independent experiments, each experiment having 6 different culture wells *per* condition. Statistical analysis conducted by one-way ANOVA, (ANOVA summary, Figure 2A F=148.3 and p<0.0001; Figure 2B F=114.8 and p<0.0001; Figure 2C F=92.8 and p<0.0001) followed by the Bonferroni's *post-hoc* test (****p<0.0001 vs control; $^{\&}$p<0.05 versus 0.1mM; $^{\&\&\&\&}$p<0.0001 versus 0.1mM; $^{\#\#\#\#}$p<0.0001 vs 0.25mM; $^{++++}$p<0.0001 vs 0.5mM).

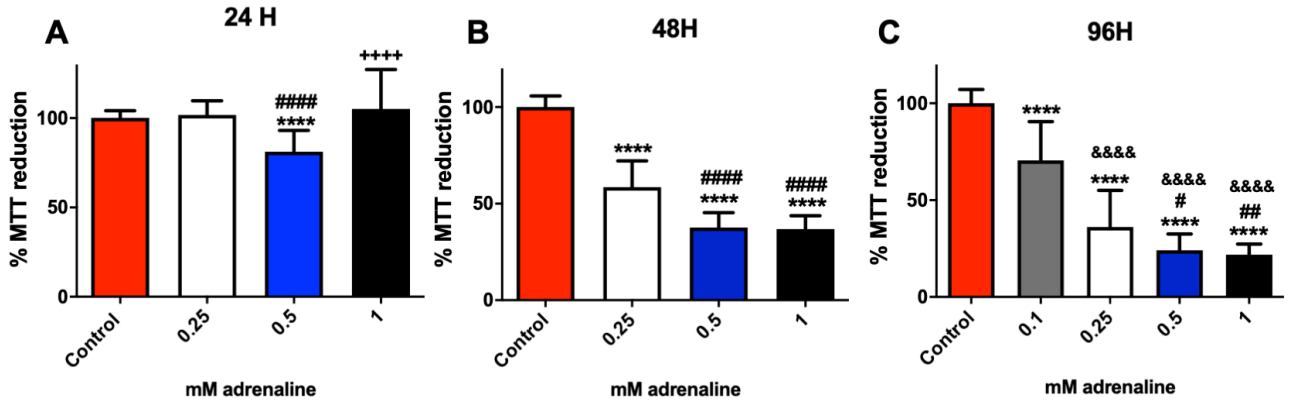

FIGURE 3 - Adrenaline (ADR) induced mitochondrial dysfunction in human SH-SY5Y neuronal cells in a concentration- and time-dependent manner. Cultures of human SH-SY5Y cells were exposed to ADR (0.1, 0.25, 0.5, and 1mM) for 24 (A), 48 (B) or 96h (C) and toxicity was evaluated by the MTT reduction assay. Results were obtained from 24 - 30 wells from 4 - 5 independent experiments, each experiment having 6 different culture wells *per* condition. Statistical analysis conducted by one-way ANOVA, (ANOVA summary, Figure 3A F=15.6 and p<0.0001; Figure 3B F=323.8 and p<0.0001; Figure 3C F=150.5 and p<0.0001) followed by the Bonferroni's *post-hoc* test (****p<0.0001 vs control; $^{\&\&\&\&}$p<0.0001 versus 0.1mM; $^{\#}$p<0.05, $^{\#\#}$p<0.01 and $^{\#\#\#\#}$p<0.0001 vs 0.25mM; $^{++++}$p<0.0001 vs 0.5mM).

RESULTS

Adrenaline causes time- and concentration-dependent loss of viability in SH-SY5Y neurons

The LDH release assay was performed in control cells and in cells exposed for 24, 48 or 96h to ADR. Viability for 0.5mM (62.80 ± 10.68%) and 1mM (32.32 ± 15.17%) ADR-incubated cells was significantly different from control cells (79.54 ± 10.35%) (Figure 2A) at 24h. This effect was also time-dependent. At the 48-h time-point, the viability of ADR 0.25mM-incubated cells (57.55% ± 24.27%) was significantly different from control cells (88.39 ± 4.01%) (Figure 2B). According to the LDH release assay, at 96h, 0.1mM ADR reduced cellular viability to 61.62 ± 29.41%, 0.25mM to 44.79 ± 14.45 %, 0.5mM to 10.93 ± 16.18% and 1mM to 9.98 ± 15.85%, when compared to control values (91.31 ± 2.14%) (Figure 2C). We also performed a two-way ANOVA of the data obtained from the LDH assay at the three time-points to confirm a significant time-dependent cytotoxicity. The increase in ADR toxicity with time of exposure was significant among all time-points for the 0.25mM concentration. Meanwhile for the 0.5mM and 1mM concentrations, it was only significantly different at the 24h and 48h time-points (data not shown).

Adrenaline produces time- and concentration-dependent mitochondrial dysfunction in human SH-SY5Y cells

The MTT reduction assay showed that ADR induced a concentration- and time-dependent cytotoxicity in the SH-SY5Y cells. At 24h, only the ADR 0.5mM-incubated cells showed significant differences on MTT reduction (81.17 ± 11.99%) when compared to control (100.00 ± 4.09 %) (Figure 3A). At 48h, all the ADR-exposed cells (0.25, 0.5 and 1mM) had significantly different MTT reduction compared to control cells (Figure 3B). At 96h, the cells exposed to 0.1mM (70.50 ± 20.07%), 0.25mM (36.15 ± 18.90%), 0.5mM (24.18 ± 8.33 %) and 1mM (21.87 ± 5.44%) had significant cytotoxicity when compared to control cells (100.00 ± 7.18%) (Figure 3C). We performed a two-way ANOVA of the data obtained from the MTT reduction assay at the three time-points to confirm that there was a time-dependent cytotoxicity. The increase in ADR toxicity with time of exposure was significantly different among all time-points for all tested concentrations (data not shown).

N-acetyl-cysteine prevents adrenaline-induced cytotoxicity in SH-SY5Y cells

NAC 1mM completely prevented the cytotoxicity evoked by ADR 0.5mM and 0.25mM following a 96-h cellular exposure, as assessed by the LDH release assay (Figures 4A and 4C). The MTT reduction assay showed that NAC 1mM partially prevented ADR cytotoxicity and the indirect mitochondrial toxicity observed after ADR exposure (Figures 4B and 4D). However, the ADR+NAC conditions did not match the control cells MTT reduction levels at 96h. Conversely, Tiron 100µM did not prevent any of the cytotoxicity-induced by ADR in the LDH release (Figure 4A and 4C) and he MTT reduction assays (Figures 3B and 3D).

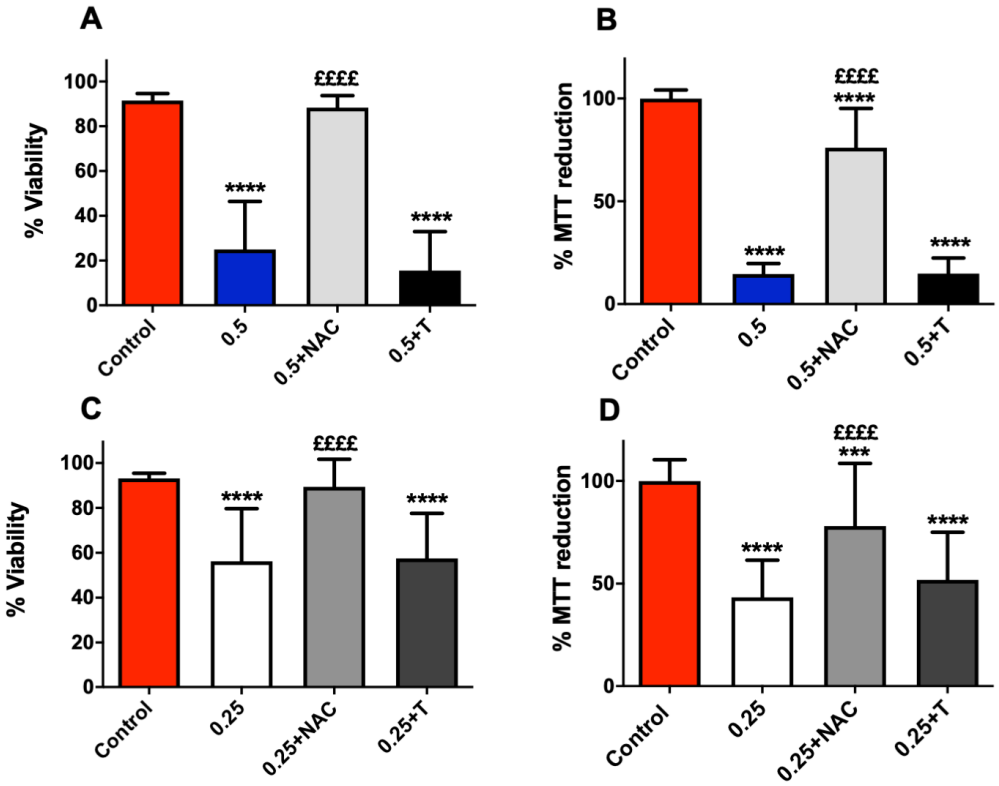

FIGURE 4 - N-acetyl cysteine (NAC) 1mM prevented the toxicity caused by adrenaline (ADR) after a 96-h exposure. The effect of the pre-incubation of NAC 1mM and tiron (T) 100µM on ADR 0.5mM (A) and ADR 0.25mM (C) cytotoxicity was assessed by the LDH release assay. The effect of the pre-incubation of NAC 1mM and tiron (T) 100µM on ADR 0.5mM (B) and ADR 0.25mM (D)-induced mitochondrial dysfunction in human SH-SY5Y cells was determined by the MTT reduction assay. Results were obtained from 24-30 wells from 4 - 5 different and independent experiments, each experiment having 6 different culture wells *per* condition. Statistical analysis conducted by one-way ANOVA, (ANOVA summary, Figure 4A $F=195.0$ and $p<0.0001$; Figure 4B $F=485.5$ and $p<0.0001$; Figure 4C $F= 42.76$ and $p<0.0001$; Figure 4D $F= 42.0$ and $p<0.0001$) followed by the Bonferroni's *post-hoc* test ($^{****}p<0.0001$ vs control; $^{£££}p<0.001$ and $^{££££}p<0.0001$ vs respective ADR concentration).

DISCUSSION

The SH-SY5Y human neuroblastoma cell line has long been used as an *in vitro* model in neurotoxicity studies, due to its human origin and catecholaminergic properties (Xicoy, Wieringa, Martens, 2017). SH-SY5Y cells are human-derived, and, in their non-differentiated state, they express immature neuronal markers and are considered immature catecholaminergic neurons (Kovalevich, Langford, 2013). Despite their immature

state, these cells are a good model system to study the neurotoxicity of catecholamines. The present studies revealed that ADR induced a time- and concentration-dependent cytotoxicity in SH-SY5Y cells. Cellular LDH release usually represents features of necrosis or late apoptosis in tested cells (Capela *et al*., 2013; Soares *et al*., 2013). Unlike what was seen in the LDH leakage assay, the MTT reduction assay did not reveal cytotoxicity caused by ADR at the higher concentration tested at the 24h incubation point. The reduction of MTT is mediated by cellular dehydrogenases, namely NADH dehydrogenase (complex I) and/or succinate dehydrogenase (complex II) activities (Dong *et al*., 1997), although the reduction can also occur via direct chemical reaction or other cellular dehydrogenases. We believe that, in this cellular paradigm, the MTT reduction can be an indirect measure of mitochondrial homeostasis. In fact, Fu and colleagues have shown in neuronal cells that ADR is the most toxic of all biogenic amines tested (Fu *et al*., 1998). They also demonstrated that the MTT reduction assay had similar response patterns to the mitochondrial membrane depolarization assay when cells were exposed to ADR (10 or 200 μM), showing a strong link between MTT reduction and mitochondrial dysfunction (Fu *et al*., 1998). Furthermore, as the byproducts of ADR can react directly with the MTT forming a colored formazan (unpublished results), and to avoid that bias, we removed the medium containing ADR and its oxidation products and replaced it with new media prior to performing the MTT assay.

Oxidative stress is involved in the etiology and pathogenesis of neurodegenerative disorders and the neurotoxicity of xenobiotic agents (Capela *et al*., 2009; Capela *et al*., 2006a; Carvalho *et al*., 2012; Cassagnes *et al*., 2018; Kumar *et al*., 2012). Moreover, it has been demonstrated that peripheral catecholamine levels may be dysregulated in diabetic patients and animal models of the disease (Adeghate*,* Ponery, Sheen, 2001; Del Rio *et al*., 1990; Del Rio, Marrama, Della Casa, 1992; Stewart *et al*., 1994) and may contribute to the peripheral neuropathy seen in those reports. Truthfully, diabetic neuropathies are a frequent late occurring complication and they affect the autonomic, sensory, and motor peripheral nervous system. Although the etiology of diabetic polyneuropathies and the underlying mechanisms are multifaceted, interesting work performed by Lelkes *et al*. in PC12 cells, showed that catecholamines are probably involved. These authors observed that continuous exposure of undifferentiated PC12 cells to elevated glucose enhances both basal and secretagogue-stimulated catecholamine release (Lelkes*,* Unsworth, Lelkes, 2001). Moreover, ROS formation is increased under high glucose conditions (Lelkes*,* Unsworth, Lelkes, 2001). The overproduction of ROS in situations of high catecholamine surge can reflect the intrinsic reactivity of catecholamines (DA, ADR, and NA) or the overstimulation of adrenoceptors (Smythies, Galzigna, 1998). Catecholamines are rapidly converted, chemically or enzymatically, into catechol ortho-quinones and then into highly deleterious semiquinone radicals after 1-electron reduction in cells. These semiquinones can then form aminochromes by reactions that are favored in the presence of ROS (Figure 1) (Costa *et al*., 2007). Catecholamine oxidation byproducts are highly reactive and electrophilic and can bind to cellular nucleophiles, namely glutathione, DNA, proteins or lipids (Cassagnes *et al*., 2018; Costa *et al*., 2011). These catecholamine byproducts have been found *in vivo*, and therefore may be major players in neurotoxicity (Smythies, Galzigna, 1998). Cassagnes *el al*. showed that exposure of SH-SY5Y to 100μM adrenochrome led to ROS formation, which was further increased when quinone reductase 2 expression was genetically overexpressed (Cassagnes *et al*., 2018). These data suggested that quinone reductase 2, an enzyme that reduces adrenochrome, when overexpressed in neuronal cells might cause ROS-induced cell death via the dismutation of superoxide radicals into hydrogen peroxide which then, through Fenton reactions, leads to the formation of the highly reactive hydroxyl radical (Cassagnes *et al*., 2018). Furthermore, NA, ADR, and DA were also shown to increase the vulnerability of cultured hippocampal neurons to amyloid β-peptide-induced toxicity. The catecholamines potentiated amyloid β-peptide toxicity at concentrations of 10-200 μM (Fu *et al*., 1998), while antioxidants (vitamin E, glutathione, and propyl gallate) protected cultured hippocampal neurons against their effects, while the beta-adrenergic receptor antagonist propanolol was ineffective (Fu *et al*., 1998). These data ruled for a major role of oxidative stress on the neurotoxicity found, which was unrelated to the adrenoceptor-stimuli.

Considering the potential role of oxidative stress in the cytotoxicity observed in cells exposed to ADR in our experimental paradigm, we tested the putative protection provided by two antioxidants. We observed that NAC fully counteracted the cytotoxic effect of ADR in the LDH leakage assay, while promoting strong protection in the MTT reduction assay. NAC is a precursor of reduced glutathione, the most important intracellular antioxidant. Glutathione is a tripeptide (glutamate-cysteine-glycine) that is synthesized and maintained at high (mM) concentrations in the cells (Rushworth, Megson, 2014). Although, there is a general belief that all thiols share the antioxidant power of glutathione, NAC is unable to scavenge one of the most abundant radical species, the superoxide radical anion, but it is a powerful scavenger of hypochlorous acid, reacts with hydroxyl radical and slowly reacts with hydrogen peroxide (Aruoma *et al*., 1989). Recent evidence on the antioxidant actions of NAC suggests that it is, in fact, a relatively weak direct antioxidant. It is a belief that NAC provides sulfhydryl moieties and acts as a glutathione precursor (Rushworth, Megson, 2014). In fact, NAC is frequently used as "antioxidant" *in vitro,* but direct experiments to assess its antioxidant potential suggest that ~10-fold more NAC is required as compared to glutathione to facilitate equivalent oxygen-centered radical scavenging in platelets (Gibson *et al*., 2009). One could argue that NAC provides strong protection against the ADR-induced neurotoxicity acting as glutathione precursor and by providing sulfhydryl moieties, but further research is needed to prove this hypothesis.

We also tested the effect of tiron on ADR-induced cytotoxicity in non-differentiated SH-SY5Y cells. Tiron (1,2- dihydroxybenzene-3,5-disulfonate) is a vitamin E analog, metal chelator, and a direct scavenger of hydroxyl, superoxide, alkyl, and alkoxyl radicals (Krishna *et al*., 1992). In our cellular model, tiron failed to protect against ADR-cytotoxicity at any concentration or time-point, thus further supporting the suggestion that the effect of NAC may not be related to its direct antioxidant activity. NAC, unlike tiron, has a poor scavenging ability, but it can deliver sulfhydryl moieties and directly react with reactive intermediates (Aldini *et al*., 2018; Zhitkovich, 2019). The outstanding protection of thiol containing compounds was already described for DA in a neuronal model. The thiol containing compounds, glutathione, NAC, and dithiothreitol (DTT) were markedly protective against the DA-induced cytotoxicity in PC12 cells, while vitamins C and E had lesser or no effect (Offen *et al*., 1996). The thiol antioxidants and vitamin C, but not vitamin E, prevented DA autoxidation and the production of DA-melanin. In the same study, thiol antioxidants protected cells by inhibiting DA-induced apoptosis. NAC (0.1mM) inhibited DA-induced toxicity and prevented the covalent binding of DA quinones to proteins in cultured rat forebrain neurons (Hoyt*,* Reynolds, Hastings, 1997) showing that the observed cytotoxicity is linked directly to the reactive intermediaries of the catecholamine. Additionally, depletion of cellular glutathione by buthionine sulfoximine, an inhibitor of gamma-glutamyl transpeptidase, significantly enhanced DA toxicity; however, co-treatment with NAC rescued the cells from the toxic effect of buthionine sulfoximine plus DA (Offen *et al*., 1996). Moreover, both DA and ADR induce nuclear factor erythroid 2-related factor 2 (Nrf2) activation, which is involved in the inducible expression of multiple antioxidant/detoxification genes, namely those involved in glutathione *de novo* synthesis. The effect of DA on Nrf2 activation is blocked by co-application of antioxidants (Shih*,* Erb, Murphy, 2007). In another study, the toxicity of the catechol metabolites of the recreational drug of abuse 'ecstasy' (α-methyldopamine and N-methyl-α-methyldopamine) on differentiated SH-SY5Y cells was prevented by NAC, but not by tiron, providing further evidence that the toxicity reflects the actions of the drugs' oxidation products rather than ROS formation (Ferreira *et al*., 2013). Therefore, we conclude that ADR oxidation products are major effectors of toxicity, given that NAC protection might be related to it's ability to reduce or prevent ADR oxidation, increase glutathione synthesis, or directly link to ADR products of oxidation.

A potential limitation of this study is the fact that the range of ADR concentrations we used would not be expected to occur under physiological or pathological conditions in humans. We investigated the cytotoxicity of ADR in a neuronal model using higher concentrations than those found *in vivo* to obtain concentration-toxicity curves and study underlying toxic mechanisms *in vitro*. Our study aims to assess the neurotoxicity profile of ADR that can steer future research to more sensitive endpoints, possibly more relevant to the human exposure scenario.

CONCLUSION

In conclusion, our work clearly demonstrates the potential of ADR to induce cytotoxicity in a neuronal model. ADR-mediated toxicity in this model appears to reflect the formation of reactive intermediates or oxidation products. Moreover, NAC was able to fully prevent the ADR-induced cytotoxicity on these human neuronal cells by mechanisms that may not involve scavenging of radicals. The involvement of such processes in potential catecholamine-induced peripheral neuropathy requires further analysis.

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