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Anhydroecgonine methyl ester (AEME), a cocaine pyrolysis product, impairs glutathione-related enzymes response and increases lipid peroxidation in the hippocampal cell culture

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ABSTRACT

Crack cocaine smokers inhale, alongside with cocaine, its pyrolysis product, anhydroecgonine methyl ester (AEME). We have previously described AEME neurotoxic effect and its additive effect when co-incubated with cocaine. Our aim was to evaluate, the effect of AEME, cocaine and AEME-cocaine combination on glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST) activities after 3 and 6 h of exposure, periods previous to neuronal death. Lipid peroxidation was evaluated through malonaldehyde (MDA) levels at 3, 6, 24 and 48 h of exposure. All treated groups reduced neuronal viability after 24 h of exposure. AEME and cocaine decreased GPx, GR and GST activities after 3 and 6 h, with an increase in MDA levels after 48 h. AEME-cocaine combination decreased the enzymes activities after 3 and 6 h, showing an additive effect in MDA levels after 48 h. These data show that the glutathione-related enzymes imbalance caused by AEME, cocaine or AEME-cocaine combination exposure preceded neuronal death and lipid peroxidation. Moreover, the additive effect on lipid peroxidation observed with AEME-cocaine exposure after 48 h, suggest a higher neurotoxic effect after crack cocaine use when compared to cocaine alone.

1. Introduction

Neurotoxicity can be defined as any permanent or reversible adverse effect caused by a biological, physical or chemical agent that affects the structure and/or the function of the nervous system [1]. The main mechanisms underlying drug-induced neurotoxicity include mitochondrial disruption, oxidative stress and intracellular calcium increase, leading to cell death by apoptosis or necrosis [2].

Oxidative stress is a pathophysiologic condition in which there is an imbalance between the generation of reactive oxygen species (ROS) and the antioxidant systems, increasing ROS production or availability [3]. There is an excessive production of superoxide anion radical ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2), which may produce the highly reactive hydroxyl radical ($^{\cdot}OH$) in the presence of transition metal ions. This condition can lead to cellular death due to the oxidation of important molecules, e.g. amino acids and polyunsaturated fatty acids (PUFA) [4]. The oxidation of PUFA, known as lipid peroxidation, leads to the production of some toxic metabolites, also called “oxidative stress second messengers”, such as malonaldehyde (MDA). Several of these breakdown products of lipid peroxides are reactive compounds and have higher half-life and diffusibility when compared to free radicals. They could also be used as a biomarker of lipid peroxidation [5,6].

The excess of ROS can be scavenged by antioxidant defenses, such as glutathione and its related enzymes, which are extremely important intracellular components [7]. Among them, glutathione peroxidase (GPx) catalyzes the reduction of H_2O_2 to H_2O with glutathione

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oxidation. In turn, glutathione reductase (GR) reduces the oxidized glutathione (GSSG) into glutathione (GSH) through the consumption of NADPH. Additionally, glutathione *S*-transferase (GST) is mainly involved in phase II xenobiotic biotransformation, with an important role in small electrophiles detoxification [8,9]. The central nervous system (CNS) is vulnerable to oxidative stress due to: 1) its aerobic metabolism, with high oxygen level consumption; 2) high amount of PUFA; 3) low levels of antioxidant defenses; and 4) the presence of transition metals ions, such as iron [2].

Oxidative stress and redox homeostasis disturbance are considered the hallmarks of several neurodegenerative diseases, such as Alzheimer's disease [10]. Some studies have also focused on this imbalanced redox state in the brain during drug addiction [9]. Cocaine is a highly addictive drug, leading to neurotoxic effects through several mechanisms, including mitochondrial dysfunction, oxidative stress, lactate dehydrogenase (LDH) leakage, caspase activation [11–14].

Crack cocaine, the smoked form of cocaine administration, has a greater addiction potential when compared to other routes e.g. intranasal cocaine [15]. Cocaine quickly volatizes when heating crack cocaine, reaching the CNS faster than any other route. Anhydroecgonine methyl ester (AEME) is produced during this heating process and inhaled alongside with cocaine [16]. The conversion of cocaine into AEME, which can reach up to 80 %, is mainly dependent on the heating temperature and cocaine purity [17]. There are few studies regarding the neurotoxicity of AEME. Previous studies of our group showed that AEME is more neurotoxic than cocaine and it may involve the activation of M_1 and M_3 muscarinic cholinergic receptors (mAChRs) [14, 37]. It also potentiated cocaine-induced behavioral sensitization, reinforcing the contribution of AEME to crack cocaine effects [18].

Thus, the purpose of this study was to investigate the effects of AEME and AEME-cocaine combination on glutathione-related enzymes activities, as well as on the lipid peroxidation through the quantification of MDA levels using rat primary hippocampal cell cultures.

2. Materials and methods

2.1. Animals

Pregnant Wistar rats (230–250 g) were obtained from Butantan Institute, São Paulo, Brazil. They were housed in plastic cages and maintained in a room with constant temperature (22 ± 1 °C) on a 12/12 h light/dark cycle, with the lights turned on at 7:00 a.m. Food and water were provided *ad libitum*. This study was performed according to the National Institutes of Health (NIH) guidelines and approved by the Animal Use Ethic Committee of Butantan Institute (# 372/07) and the Animal Experimentation Ethic Committee of the School of Pharmaceutical Sciences at the University of São Paulo (# 142/07).

2.2. Cocaine and anhydroecgonine methyl ester (AEME)

Cocaine was gently donated by the Criminal Institute of São Paulo, for research purposes, to the Laboratory of Toxicological Analyses (School of Pharmaceutical Sciences, University of São Paulo). Briefly, cocaine was purified (95 %) and converted into its salt form, cocaine hydrochloride, by bubbling hydrochloric acid into mixture of purified cocaine dissolved in diethyl ether. AEME was then synthesized using cocaine hydrochloride as start material. The synthesis and the purification processes are described by Garcia et al. [14]. The AEME product purity was > 98 % and its structure was confirmed by proton nuclear magnetic resonance (^{1}H-NMR) and electrospray ionization-mass spectrometry (ESI-MS) [14].

2.3. Rat primary hippocampal cell culture

Hippocampal neurons were dissociated from hippocampi of E18-E19 Wistar rat embryos. Pregnant rats were anesthetized with 55 mg/kg sodium pentobarbitone and the fetuses were rapidly decapitated in order to remove their hippocampi. The tissue was placed into a Petri dish containing 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) in a cooled Neurobasal medium (Gibco). Hippocampi tissues were previously washed with Hank's Balanced Salt Solution (HBSS) and then subjected to a mechanical fragmentation with appropriate scissors. After the mechanical fragmentation, the tissue fragments were transferred to a solution containing 0.25 % trypsin in Earl's Balanced Salt Solution (EBSS) with a pH adjusted to 7.2–7.4. After the incubation period (10 min at 37 °C), cells were washed with an EBSS solution containing 277.5 U/mL DNAse (Sigma) and 10 % fetal bovine serum (FBS) (Gibco) and centrifuged at 300 *g* (Eppendorf 5804R) for 2 min at 20 °C. Following the mechanical dissociation in an EBSS solution (with DNAse and fetal bovine serum) using Pasteur pipettes with different diameters sizes, the isolated neurons were centrifuged for 5 min at 300 *g*. Finally, the pellet containing the homogenized tissue was resuspended in Neurobasal medium (Gibco) supplemented with 0.5 mM L-glutamine, 25 µM L-glutamic acid, 100 U/mL penicillin, 100 µg/mL streptomycin and 2 % B27 supplement (Gibco) to reduce glial cell proliferation [19,20]. The cells were seeded onto 0.01 % poli-L-lysine-coated 24-well culture plate, at a density of 2×10^5 cells/cm^2, and maintained at 37 °C in a humidified atmosphere of 5 % CO_2, for 7–8 days, the time required for hippocampal neurons to develop a network of functional synaptic contacts [21]. On the second day, half of the old medium was replaced by the same volume of a fresh new medium with the same composition. The culture cells were immunohistochemically characterized with MAP2 (neuronal marker) and GFAP (astrocytic marker), showing a predominance of neurons (92 %) over astrocytes (8 %) [14].

2.4. Exposure conditions for cocaine and AEME

On the 7th day, the cell culture was incubated with 1 mM AEME, 2 mM cocaine or AEME-cocaine combination (1 and 2 mM, respectively), for different time periods (3, 6, 24 or 48 h). All solutions were daily prepared in culture medium. For GPx, GR and GST assays, hippocampal neurons were exposed for 3 and 6 h, periods in which there is no decrease in cell viability as already described by Garcia et al. [14]; for MDA levels, cells were exposed for 3, 6, 24 and 48 h.

2.5. Cell viability assay

The hippocampal cell viability was evaluated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma) reduction assay, as previously described by our group [14,37]. Briefly, after 3, 6 and 48 h of incubation with 1 mM AEME, 2 mM cocaine or AEME-cocaine combination, all the medium was removed and 100 µL of MTT solution (5 mg/mL MTT in PBS: neurobasal medium without phenol red, 1:9 v/v) was added. After 3 h of incubation at 37 °C in a humidified atmosphere (containing 5 % CO_2), the MTT solution was replaced by 200 µL of dimethyl sulfoxide. After 30 min of shaking, the absorbance was measured in a multiwell plate reader (BioTek Synergy H1 Hybrid Reader), using 250 mM KCl as a positive control of neuronal death. Results were expressed as a percentage of the control value and dimethyl sulfoxide was used as a blank solution.

2.6. Sample preparation for GPx, GR, GST and MDA assays

After each exposure period, hippocampal neurons were rapidly removed from 24-well culture plate using a solution containing 0.25 % trypsin in EBSS (pH 7.2–7.4). The maximum period of exposure to trypsin did not exceed 5 min. Cells were then inactivated with an equal volume of an EBSS solution containing 10 % FBS (Gibco) and centrifuged at 300 *g* (Eppendorf 5804R) for 2 min at 20 °C. This step was performed three times to guarantee complete inactivation of trypsin.

Cells homogenate were prepared in 0.1 M phosphate buffer, pH 7.3 (approximately 2.5 × 10^6 lysed cells/mL), and stored at −80 °C until analysis. After centrifugation at 1000 *g* for 30 s at 4 °C, the supernatant was collected and used in all procedures described as follow.

2.7. Glutathione peroxidase (GPx) activity

To evaluate GPx activity, *tert*-butyl-hydroperoxide was used as the reaction substrate [22]. Briefly, 120 µL of 0.1 M potassium phosphate buffer solution (pH 7.0), containing 1 mM EDTA, 2 mM glutathione, 0.048 U glutathione reductase and 40 µL of sample were added to a 96-well plate and incubated at 37 °C for 5 min. After, 30 µL of 1.2 mM NAPDH was added and the reaction was initiated by the addition of 10 µL of 0.46 % *tert*-butyl-hydroperoxide solution. The GPx activity was indirectly measured using a spectrophotometer by NAPDH consumption at 340 nm for 5 min (Power Wave x 340, Bio-Tek Instruments Inc., software KC4 v3.0, USA). GPx activity was calculated as U/mg of protein [23] and expressed as a percentage of control value, using 0.1 M phosphate buffer (pH 7.3) as a blank solution.

2.8. Glutathione reductase (GR) activity

GR activity was determined through NADPH consumption as described elsewhere [24]. Briefly, to 96-well plate 50 µL of sample were mixed with 150 µL of GR assay buffer (2 mL of 0.1 M potassium phosphate buffer, pH 7.0, containing 1 mM EDTA; 1.5 mL of Milli-Q water; 1.5 mL of 5 mM EDTA; 10 mg of oxidized glutathione and 2 mg of NAPDH). As previously mentioned for GPx activity, the absorbance decrease was measured at 340 nm for 10 min at 37 °C (Power Wave x 340, Bio-Tek Instruments Inc., software KC4 v3.0, USA). GR activity was calculated as U/mg of protein [23] and expressed as a percentage of control value, using 0.1 M phosphate buffer (pH 7.3) as a blank solution.

2.9. Glutathione S-transferase (GST) activity

GST activity was measured through the production of a complex formed by the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) [25]. Briefly, 40 µL of sample were added to 96-well plate and mixed with 140 µL of 0.1 M potassium phosphate buffer, pH 6.5, and 5 µL of 0.1 M CDNB. This mixture was pre-incubated for 2 min at room temperature. After, 15 µL of 0.1 M GSH was added and the increase of absorbance was monitored at 340 nm for 5 min at 25 °C (Power Wave x 340, Bio-Tek Instruments Inc., software KC4 v3.0, USA). GST activity was calculated as U/mg of protein [23] and expressed as a percentage of control value, using 0.1 M phosphate buffer (pH 7.3) as a blank solution.

2.10. Lipid peroxidation

Malonaldehyde (MDA) levels were measured as a parameter of lipid peroxidation [26,27]. Sample preparation and chromatographic conditions were similar to those described by Lobo Torres et al. [28]. Briefly, 200 µL of sample containing 0.2 % butylated hydroxytoluene (50 µL) was reacted with the same volume of 0.4 % thiobarbituric acid in an acidic medium (0.13 M HCl) at 90 °C for 1 h, centrifuged and filtered through a 0.2-µm Spin-X centrifuge tube filters (Corning®). Thus, the colored complex was quantified by reverse phase high performance liquid chromatography (HPLC/PDA, Shimadzu, Kyoto, Japan) using a C18 analytical column (Phenomenex 150 mm × 4.6 mm, 10 µm), eluted with a mobile phase containing 50 mM phosphate buffer (pH 7.0):methanol mixture (65:35, v/v) at 1 mL/minute and detected spectrophotometrically at 532 nm. A calibration curve was used to quantify MDA levels. The concentration ranged from 0.01–10 µM. MDA levels were calculated based on the analytical curve and results were corrected by the amount (in mg) of protein [23]. Results were expressed as a percentage of control value, using 0.1 M phosphate buffer (pH 7.3) as a blank solution.

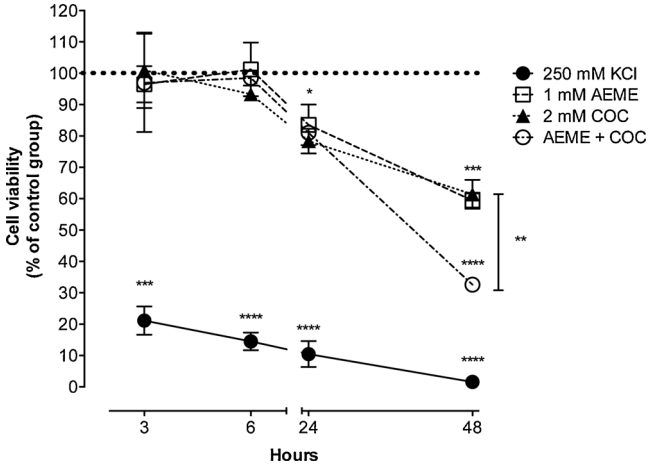

Fig. 1. Neuronal cell viability results (n = 3), expressed in percentage of control group, after 3, 6, 24 and 48 h of exposure to 1 mM AEME (□), 2 mM COC (▲) and AEME + COC (○). The positive control of neuronal death was 250 mM KCl (•). Dotted line represents the control group (error bar was omitted). AEME, anhydroecgonine methyl ester; COC, cocaine; and AEME + COC, AEME-cocaine combination. *$p < 0.05$, ***$p < 0.001$ and ****$p < 0.0001$ for all groups when compared with control group. After 48 h of exposure, **$p < 0.01$ for the AEME + COC when compared with either 1 mM AEME or 2 mM COC (ANOVA and Newman-Keuls multiple comparisons test). Results are presented as mean ± SEM.

2.11. Statistical analysis

Data represent three independent experiments performed in triplicate and are expressed as mean ± SEM. It was used an one-way analysis of variance (ANOVA) followed by Newman-Keuls multiple comparisons *post-hoc* test; $p < 0.05$ was considered statistically significant. Normality of the samples was checked by the Kolmogorov-Smirnov test. All data were plotted and analyzed by GraphPad Prism software version 5.0.

3. Results

3.1. Cell viability assay

The incubation of the hippocampal cells with either AEME, cocaine or AEME-cocaine combination did not decrease cell viability, except for the positive control (KCl), after 3 [$F(4, 10) = 12.69$, $p < 0.001$] and 6 h [$F(4, 10) = 28.06$, $p < 0.0001$] of exposure (Fig. 1). The results after 3 and 6 h of exposure, expressed in percentage of control group, were, respectively: 96.5 ± 5.8 % and 101.2 ± 8.6 % for AEME; 100.9 ± 12.0 % and 93.3 ± 2.1 % for cocaine; 97.0 ± 15.7 % and 98.5 ± 2.4 % for AEME-cocaine combination. After 24 h of exposure, there was a decrease in neuronal viability in all treated groups [$F(4, 10) = 72.30$, $p < 0.0001$]. The percentage of viable cells was: 83.5 ± 6.5 % for AEME ($p < 0.05$); 78.4 ± 3.9 % for cocaine ($p < 0.05$); and 81.0 ± 1.8 % for AEME-cocaine combination ($p < 0.05$) (Fig. 1). After 48 h of exposure, all treated groups showed a decrease in neuronal viability [$F(4, 10) = 51.09$, $p < 0.0001$], with the following percentage of viable cells: 59.5 ± 2.5 % for AEME ($p < 0.001$); 61.4 ± 4.6 % for cocaine ($p < 0.001$); and 32.6 ± 1.8 % for AEME-cocaine combination ($p < 0.0001$). Moreover, both AEME and cocaine treated groups are statistically different from AEME-cocaine combination group ($p < 0.01$ for each comparison) (Fig. 1).

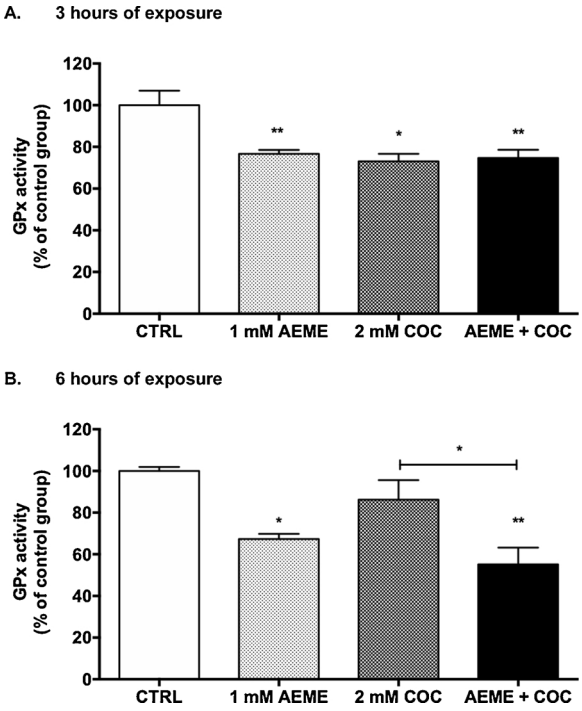

Fig. 2. Glutathione peroxidase (GPx) activity, expressed in percentage of control group, after 3 (**A**) and 6 h (**B**) of exposure to 1 mM AEME, 2 mM COC and AEME + COC (n = 3). CTRL, control; AEME, anhydroecgonine methyl ester; COC, cocaine; and AEME + COC, AEME-cocaine combination. *$p < 0.05$ and **$p < 0.01$ for all groups when compared with control group. After 6 h of exposure (**B**), *$p < 0.05$ for the AEME + COC when compared with 2 mM COC (ANOVA and Newman-Keuls multiple comparisons test). Results are presented as mean ± SEM.

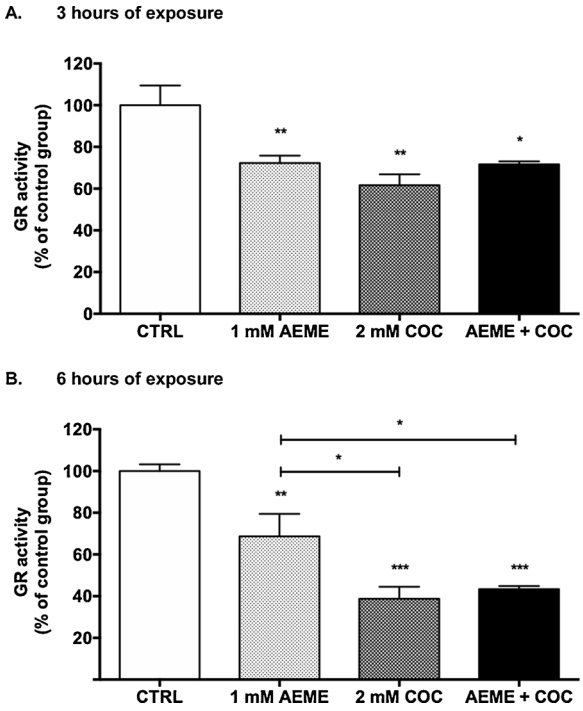

Fig. 3. Glutathione reductase (GR) activity, expressed in percentage of control group, after 3 (**A**) and 6 h (**B**) of exposure to 1 mM AEME, 2 mM COC and AEME + COC (n = 3). CTRL, control; AEME, anhydroecgonine methyl ester; COC, cocaine; and AEME + COC, AEME-cocaine combination. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ for all groups when compared with control group. After 6 h of exposure (**B**), *$p < 0.05$ for the 1 mM AEME when compared with either 2 mM COC or AEME + COC (ANOVA and Newman-Keuls multiple comparisons test). Results are presented as mean ± SEM.

3.2. Effect of AEME, cocaine and AEME-cocaine combination on GPx, GR and GST activities

After 3 h of exposure, there was a decrease in GPx activity in all treated groups [$F(3, 8) = 8.028$, $p < 0.01$; Fig. 2A]. When compared with control group, the reduction was, in percentage: 23.3 % for AEME ($p < 0.01$); 26.9 % for cocaine ($p < 0.05$); and 25.3 % for AEME-cocaine combination ($p < 0.01$). After 6 h of exposure, there was a decrease in GPx activity only for AEME and cocaine-combination group when compared with control group [$F(3, 8) = 9.796$, $p = 0.0047$; Fig. 2B], with a reduction of 32.7 % ($p < 0.05$) and 44.8 % ($p < 0.01$), respectively. There was also a significant decrease in GPx activity in AEME-cocaine combination compared with cocaine group ($p < 0.05$).

Regarding GR activity, after 3 h of exposure, it was observed a reduction in its activity in all treated groups [$F(3, 8) = 8.263$, $p < 0.01$; Fig. 3A]. The percentage of decrease was the following, compared with control group: 27.4 % for AEME ($p < 0.01$); 38.4 % for cocaine ($p < 0.01$); and 28.3 % for AEME-cocaine combination ($p < 0.05$). This effect remains after 6 h of exposure for all treated groups, with a more pronounced effect for cocaine and cocaine-combination groups [$F(3, 8) = 19.43$, $p < 0.001$; Fig. 3B]. The percentage of decrease was the following, compared with control group: 31.3 % for AEME ($p < 0.01$); 61.3 % for cocaine ($p < 0.001$); and 56.6 % for AEME-cocaine combination ($p < 0.001$). Moreover, there was significant decrease in GR activity in the AEME-cocaine combination group ($p < 0.05$), as well as in the cocaine group ($p < 0.05$), when compared to AEME group.

No significant differences were detected in GST activity after 3 h of exposure [$F(3, 8) = 3.688$, $p = 0.0621$; Fig. 4A]. However, after 6 h of exposure, there was a decrease in GST activity in all treated groups [$F(3, 8) = 7.554$, $p < 0.05$; Fig. 4B]. When compared with control group, the reduction was, in percentage: 43,2 % for AEME ($p < 0.05$); 37.9 % for cocaine ($p < 0.05$); and 59.3 % for AEME-cocaine combination ($p < 0.01$).

3.3. Effect of AEME, cocaine and AEME-cocaine combination on lipid peroxidation

No significant differences were observed in MDA levels either after 3 [$F(3, 8) = 3.085$, $p > 0.05$; Fig. 5A], 6 [$F(3, 8) = 2.517$, $p > 0.05$; Fig. 5B] or 24 h of exposure [$F(3, 8) = 0.241$, $p > 0.05$; Fig. 5C] for all tested groups. After 48 h of exposure, there was an increase in MDA levels in all treated groups [$F(3, 8) = 42.95$, $p < 0.0001$; Fig. 5C]. The percentage of increase in MDA levels was the following, compared with control group: 29.9 % for AEME ($p < 0.01$); 40.0 % for cocaine ($p < 0.01$); and 95.7 % for AEME-cocaine combination ($p < 0.0001$). Moreover, there was a significant increase in MDA levels in the AEME-cocaine combination group when compared to both AEME ($p < 0.001$) and cocaine groups ($p < 0.001$).

4. Discussion

Crack cocaine is the smoked form of cocaine with higher devastating effects when compared to other routes, such as intravenous or intranasal routes. These harmful effects can also be attributed, in part, to AEME, the main pyrolytic compound of crack cocaine, which is inhaled along with cocaine during smoking. This manuscript shows that one of the mechanisms involved in cocaine-, AEME- and AEME-cocaine combination-induced neurotoxicity might be oxidative stress through the impairment of glutathione-related enzymes and increase of lipid peroxidation.

Interestingly, we also found an additive effect regarding MDA levels, once AEME-cocaine combination group showed a more

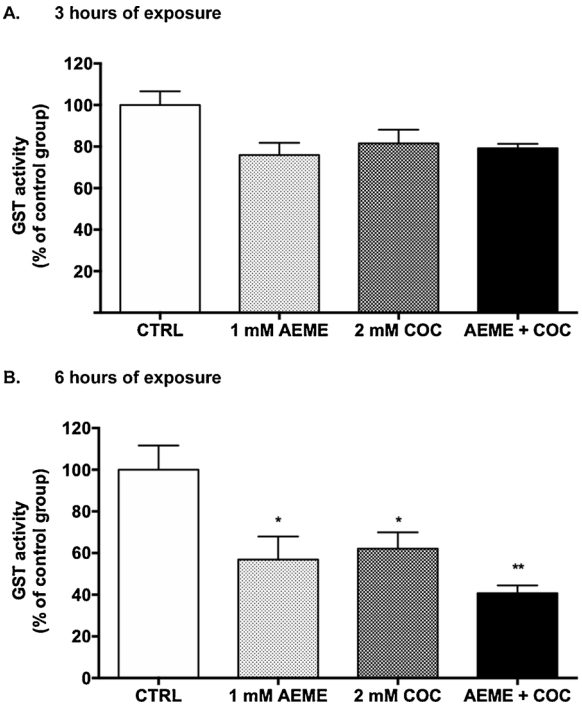

Fig. 4. Glutathione *S*-transferase (GST) activity, expressed in percentage of control group, after 3 (**A**) and 6 h (**B**) of exposure to 1 mM AEME, 2 mM COC and AEME + COC (n = 3). CTRL, control; AEME, anhydroecgonine methyl ester; COC, cocaine; and AEME + COC, AEME-cocaine combination. $*p < 0.05$ and $**p < 0.01$ for all groups when compared with control group (ANOVA and Newman-Keuls multiple comparisons test). Results are presented as mean ± SEM.

pronounced lipid peroxidation when compared to cocaine after 48 h of exposure. This result corroborates the MTT assay, which are in accordance with our previous study that observed an additive neurotoxic effect after 48 h of exposure to AEME-cocaine combination [14].

Cocaine is a tropane alkaloid found in *Erythroxylum coca* leaves that increases mesocorticolimbic dopamine activity throughout the blockade of the dopamine transporter, inhibiting its reuptake. The enhanced dopaminergic transmission, especially in the mesocorticolimbic region, results in cocaine reinforcement effects, which in turn explains its high addictive potential [29]. The massive increase in dopamine release could be also responsible for cocaine's neurotoxic effects due to dopamine auto-oxidation, which results in excessive ROS production (e.g. $O_2^{\bullet-}$ and H_2O_2), damaging macromolecules such as proteins and PUFA, leading to cellular death either by apoptosis or necrosis [30,31].

In vitro study with human neuronal progenitor cells demonstrated that even an acute exposure (30 min) to 1 μM cocaine was able to induce oxidative stress [32]. The authors showed an increase in total protein carbonyl levels, as well as in 4-hydroxinonenal levels, another breakdown product of lipid peroxides, with a decrease in reduced glutathione levels 48 h after a single exposure to 1 μM cocaine. Moreover, these changes occurred before neuronal apoptosis, showing that oxidative stress preceded cellular death [32]. Our data showed a decrease in the activity of all glutathione-related enzymes, with exception for GPx, after 6 h of exposure, and GST, after 3 h of exposure, in the hippocampal cells exposed to 2 mM cocaine in periods with no detection of neuronal death, i.e. 3 and 6 h. On the other hand, the MDA levels were increased only after 48 h of exposure, period in which neuronal death was identified.

Regarding oxidative stress *in vivo* studies with cocaine, male Wistar rats, intraperitoneally administered with 20 mg/kg cocaine, either acutely or repeatedly (10 days, once a day), showed higher H_2O_2 formation, intense lipid peroxidation and an increase in GPx activity, both

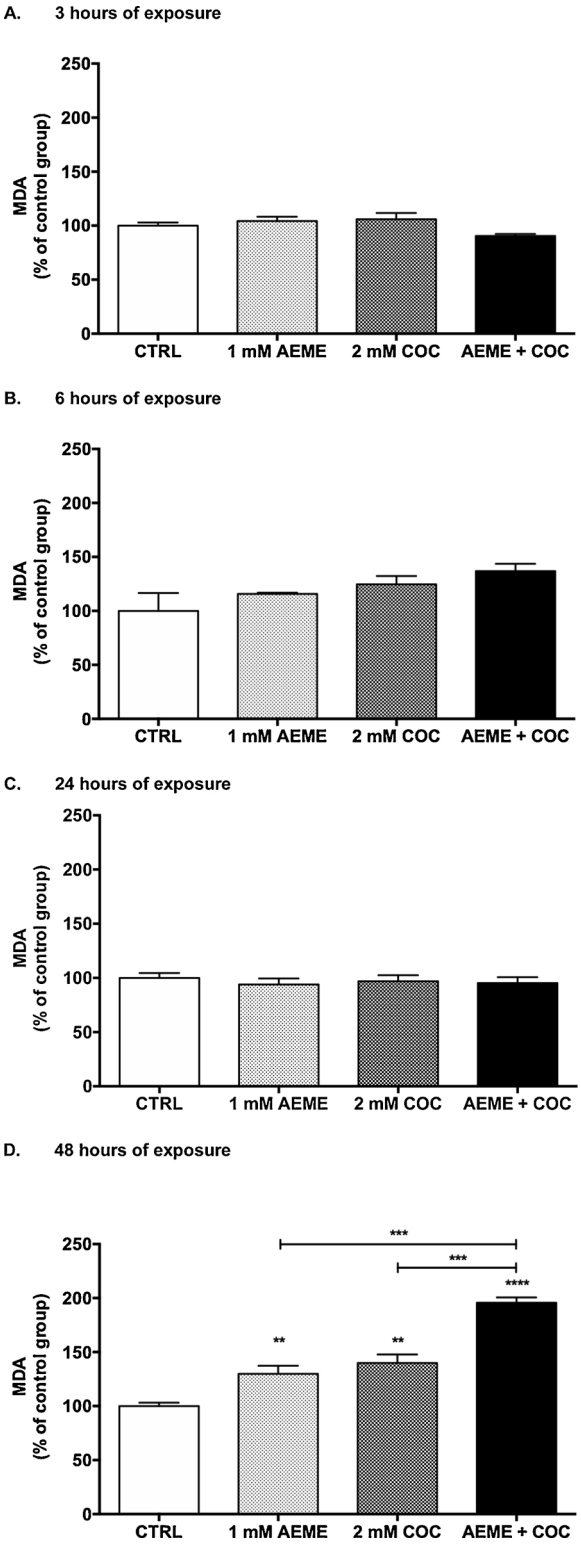

Fig. 5. Malondialdehyde (MDA) levels, expressed in percentage of control group, after 3 (**A**), 6 (**B**), 24 (**C**) and 48 h (**D**) of exposure to 1 mM AEME, 2 mM COC and AEME + COC (n = 3). CTRL, control; AEME, anhydroecgonine methyl ester; COC, cocaine; and AEME + COC, AEME-cocaine combination. $**p < 0.01$ and $****p < 0.0001$ for all groups when compared with control group. After 48 h of exposure (**C**), $***p < 0.001$ for the AEME + COC when compared with either 1 mM AEME or 2 mM COC (ANOVA and Newman-Keuls multiple comparisons test). Results are presented as mean ± SEM.

in the cortex and striatum [33]. The hippocampus of the offspring Sprague-Dawley rats, whose dams received intraperitoneal injection of 20 mg/kg cocaine from E17 to E20, presented an increase in TBARS, which was correlated with learning impairment in the water-maze test [34]. Conversely, male Wistar rats intraperitoneally administered with 15 mg/kg cocaine for 18 days showed a decrease in both cerebellar GPx activity and reduced glutathione/oxidized glutathione ratio [35].

It has been reported that cocaine relapse in rodents downregulates the cystine-glutamate exchanger, impairing glutamate homeostasis in the nucleus accumbens and also limiting the synthesis of glutathione. Uys et al. [36] showed that cocaine was able to increase the levels of protein S-glutathionylated and a decrease in GST expression. In fact, either the pharmacological blockade with ketoprofen or the genetic manipulation to deplete GST in rodents, increased the capacity of cocaine to induce locomotor sensitization or conditioned place preference. This reduction in GST may be responsible to neuroplasticity induced by cocaine administration [36]. Although we did not evaluate the expression of GST, we detected a decline of its activity after 6 h of exposure to 2 mM cocaine.

As we previously reported, AEME showed to be more neurotoxic than cocaine in a rat primary hippocampal culture, with an additive effect when co-incubated [14]. This effect seems to occur due to AEME partial agonist effect at M_1 and M_3 mAChRs. The activation of both mAChRs subtypes is involved in AEME-induced neurotoxicity [37]. The resulting intracellular cascade leads to calcium increase which, in turn, can culminate to apoptosis due to the activation of caspase signaling [38]. Arecoline, a mAChR agonist, induces neuronal apoptosis through the generation of ROS, the decrease in antioxidant defense system and the activation of caspase-3 [39]. In this study, we observed that AEME was able to reduce the activity of all glutathione-related enzymes, with an exception for GST after 3 h of exposure. We also demonstrated that this redox imbalance preceded both neuronal death and lipid peroxidation, i.e. after 48 h of exposure, similar to cocaine exposure.

Moreover, *in vivo* study with rats showed that AEME potentiated cocaine-induced behavioral sensitization in rats, which can contribute to the risk of crack cocaine abuse [18]. This drug-induced sensitization is influenced by melatonin, a pineal hormone with neuroprotective effects, which has its synthesis impaired by AEME exposure, both *in vivo* and *in vitro*. [40].

The injection of McN-A-343, a M_1 mAChR agonist, increased nitric oxide in striatal tissues which, if overproduced, can lead to neuronal death due to oxidative stress and lipid peroxidation [34,41]. Gomes et al. [42] evaluated the AEME potential to induce oxidative stress in the CNS and observed an increase in advanced oxidation protein products levels in the striatum followed by 100 μg AEME intracerebroventricular administration. Moreover, they also detected an increase in GPx activity in the same brain region, with no results in the hippocampus [42].

There are few reports regarding the *in vitro* neurotoxic mechanisms of AEME and its combination with cocaine. Indeed, our group first described the additive neurotoxic effect of AEME-cocaine combination *in vitro* after 48 h of exposure [14]. The combination of 1 mM AEME with 2 mM cocaine, showed a decrease in the activity of all glutathione-related enzymes, with an exception for GST after 3 h of exposure when compared to control group. Importantly, a more pronounced effect was observed for GPx activity after 6 h of exposure to AEME-cocaine combination when compared to cocaine. Likewise, the combination of both substances after 6 h of exposure showed a higher decrease in GR activity when compared to AEME effect alone. In fact, the higher levels of MDA formation in the AEME-cocaine combination group after 48 h of exposure could lead the additive neurotoxic effect previously described and confirmed here.

Male adult Wistar rats exposed to crack cocaine inhalation presented a decrease in TBARS, a lipid peroxidation marker, in the hippocampus and an increase in advanced oxidation protein products levels in the striatum [43]. The authors suggest that there is a compensatory mechanism involved in the decrease in TBARS levels in the hippocampus of rats exposed to crack cocaine smoke, attributing it to an adaptive response of the antioxidant defense system.

Oxidative stress was also evaluated in crack cocaine users. Male crack-abstinent patients submitted to a 14-days detoxification regime presented an increase in glutathione, total thiol content and nitric oxide levels, with a negative correlation between the frequency of crack cocaine use and nitric oxide levels on the first day of hospitalization [44]. The authors concluded that crack cocaine consumption increases oxidative stress in drug addicted subjects and the 14-days rehabilitation process was able to boost antioxidant defenses and improve oxidative parameters of the patients.

In summary, we showed that rat primary hippocampal cell culture exposed to 1 mM AEME or 2 mM cocaine disrupted the glutathione-related enzymes, with a more pronounced effect for AEME-cocaine combination, in periods which no neuronal death was observed. When neuronal death was confirmed after 48 h of exposure, an increase in MDA levels was detected, with an additive effect for AEME-cocaine combination, showing that crack cocaine users might be more prone to oxidative stress and lipid peroxidation, leading to a more harmful effects when compared to other routes of cocaine administration.

Declaration of Competing Interests

None.

Declaration of Competing Interest

The authors report no declarations of interest.

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