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Toxicology of anhydroecgonine methyl ester: A systematic review of a cocaine pyrolysis product

Andrea Romero-Noreña [a,*], Heidy Johanna Contreras-Martinez [b], Miguel Mendivil-Perez [c]

[a] *Pharmaceutical Sciences Research Group, PhD student in Health Sciences, Universidad CES, Medellín, Colombia*
[b] *Research Group in Pharmaceutical Sciences, associate professor, Doctorate in Health Sciences, Universidad CES, Medellín, Colombia*
[c] *Neurosciences Group of Antioquia, associate professor, Universidad de Antioquia. (UdeA), Medellín, Colombia*

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ABSTRACT

Anhydroecgonine Methyl Ester (AEME), also known as methylecgonidine, is the main pyrolysis product of smoking cocaine (cocaine base paste or basuco, crack, or freebase). This review aims to synthesize the available scientific evidence on the toxicokinetic and toxicodynamic effects of AEME. A search of scientific articles published in Science Direct, SCOPUS, and MEDLINE up to May 2024 was conducted. Twenty-four articles, including 13 experimental animal studies, 2 clinical trials, and 3 observational studies, were reviewed. AEME is readily deposited in the alveoli; its absorption improves in combination with cocaine and has a broad tissue distribution. It is metabolized primarily in the liver, with a half-life of approximately one hour, and is mainly excreted through urine. Moreover, AEME acts as a partial agonist of M1 and M3 muscarinic cholinergic receptors, influences dopaminergic system neuroadaptation, increases the production of reactive oxygen species, imbalances the activity of glutathione-associated enzymes, and reduces melatonin levels, affecting its antioxidant regulatory properties. When combined with cocaine, AEME activates the non-apoptotic pathway of caspase-9 and then, the apoptotic pathway via caspase-8, reducing neuronal viability in half the time of cocaine. AEME plays a significant role in cocaine toxicity and AEME itself.

1. Introduction

Anhydroecgonine Methyl Ester (AEME), also known as methylecgonidine, is the main volatile product generated from the pyrolysis of cocaine. It was first detected in 1985 as a biomarker of smoking cocaine use [22].

Since then, research has focused mainly on this toxicological line as a biomarker in blood, urine, hair, and sweat [18,19,3]. However, research on the toxicodynamic effects of AEME did not begin until 1995. It was found that AEME acts on the cardiorespiratory system and produces hemodynamic effects in addition to those of cocaine [17,27,28,30,4,5].

Since 2012, Brazilian researchers have performed *in vivo* and *in vitro* studies of AEME toxicodynamic properties in the central nervous system (CNS), mainly in the areas related to the reward system (i.e., hippocampus, prefrontal cortex, basal nuclei). As a result, AEME was identified as a neurotoxic able to induce apoptosis of cholinergic neurons [1,11–15,23,26].

Currently, there are no existing syntheses on the properties and effects of this xenobiotic on the CNS and other systems. Therefore, this systematic review aimed to gather the available scientific evidence on

Abbreviations: AEME, anhydroecgonine Methyl Ester; AE, anhydroecgonine; ANEME, anhydronorecgonine methyl ester; AEMENO, anhydroecgonine methyl ester non-oxidized; AEEE, anhydroecgonine ethylester; CNS, central nervous system; PFC, prefrontal cortex; HPC, hippocampal; Nacc, nucleus accumbens; CPu, caudate nucleus; STR, striatal nucleus; BP, blood pressure; HR, heart rate; MMDA, aerodynamic diameter measurement; RF, respiratory frequency; SGaw, specific airway conductance; Bmax, binding capacity; Kd, dissociation constant; P-F-HHSiD, p-fluoro-hexahidrosila-difenidol; GC/MS, Gas Chromatography/Mass Spectrometry; LDH, lactate dehydrogenase; PLC, phospholipase C; AOPP, protein oxidation; GPx, glutathione peroxidase; GR, glutathione reductase; ROS, reactive oxygen species; TBA-RS, lipid peroxidation; CAT, catalase; SOD, superoxide dismutase; Ca++, calcium; KCl, potassium chloride; Ach, acetylcholine; MAChR, muscarinic cholinergic receptor; D1R, dopaminergic receptor 1; D2R, dopaminergic receptor 2; D3R, dopaminergic receptor 3; DOPAC, 3,4-Dihydroxyphenylacetic acid; HVA, homovanillic acid; CB1R, endocannabinoid receptor 1; FAAH, fatty acid amide hydrolase; MAGL, monoacylglycerol lipase; AEA, anandamide; NE, norepinephrine; TMPH, 2,2,6,6-Tetramethylpiperidin-4-yl heptanoate; MK-801, Dizocilpine Maleate; NMDA, N-Methyl-D-aspartic acid; NBQX, 2,3-dioxo-6-nitro-7-sulfamoilbenzofquinoxalina; AMPA, ácido α-amino-3-hidroxi-5-metilo-4-isoxazolpropiónico.

* Correspondence to: Calle 10A #22-04, Medellín, Colombia.
E-mail address: norena.andrea@uces.edu.co (A. Romero-Noreña).

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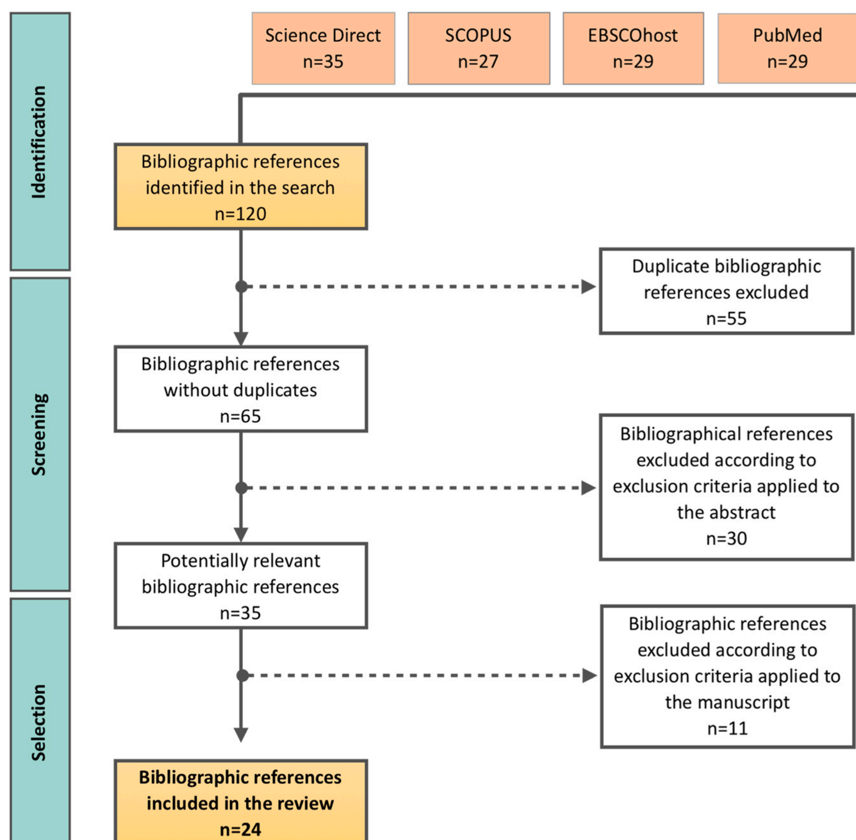

Fig. 1. *Item selection flowchart. Note:* This flow chart shows the stages of the method of this systematic review. First, the identification of the articles; second, the screening; and third, the final choice. The boxes on the right show the causes for exclusion. The first box in yellow shows the articles found, and the last yellow box shows the selected articles.

the toxicokinetic and toxicodynamic roles of AEME.

2. Methods

In May 2024, a search was conducted for scientific articles published in the literature addressing the toxicokinetic and toxicodynamic aspects of AEME. The following databases were utilized: ScienceDirect, SCOPUS, and MEDLINE, accessed through PubMed and EBSCOhost. The search was limited to titles, abstracts, and full texts, in English or Spanish, including all publication dates, types of articles, disciplines, and species. Keywords for the search were identified using the MESH Database, and Boolean connectors AND and OR were employed to refine it [9].

The search strategy was formulated as follows: (((Anhydroecgonine methyl ester [Title/Abstract]) OR (pyrolysis product[Title/Abstract])) AND ((Cocaine[Title/Abstract]) OR (Cocaine smoking[Title/Abstract]))); this search identified 120 articles, of which 55 references were duplicates across the 4 databases used, resulting in the identification of 65 unique references.

Selection criteria for articles were established as follows: (1) research articles, (2) short communications, (3) identification of AEME among diverse samples, (4) identification of AEME metabolites. Exclusion criteria were: (1) articles without available abstracts, (2) book chapters, (3) development and validation of instruments or techniques for AEME detection, (4) studies aimed at detecting and evaluating other cocaine metabolites or substances, (5) evaluation of use as a radioligand. Based on these criteria, 30 references out of the 65 identified were excluded due to the content of their abstracts.

Initially, references were selected based on title relevance and article type, followed by abstract review to verify suitability, and finally, full-text evaluation to assess adequacy [2,9]. A total of 24 articles were obtained and included in this review (Fig. 1). The selected articles were distributed among researchers who extracted information from each article using a template in Google Docs [2,25].

3. Results

The results were analyzed by categorizing them according to the toxicokinetic or toxicodynamic approach of the respective studies.

Of the 24 articles included, 13 reported findings from *in vitro* experimental studies, of which eight addressed toxicodynamic processes. In addition, six articles reported the results of *in vivo* studies, addressing toxicodynamic processes exclusively. Finally, two articles from clinical trials and three from observational studies addressed toxicokinetic processes.

Studies addressing a toxicokinetic approach were mainly conducted at the end of the last century and the beginning of the present century. Conversely, toxicodynamic studies were published during two periods: 1995–1997, when studies focused mainly on cardiorespiratory effects, and 2012–2022, when CNS effects were studied.

Resume Table (complementary documentation), presents the main results collected from each of the articles.

4. Discussion

4.1. Toxicokinetic of AEME

4.1.1. Physicochemical properties

Smoking cocaine (cocaine base paste or basuco, crack, freebase) corresponds to forms of cocaine base self-administered by smoking or inhalation. It requires an increase in temperature to change from solid to liquid and finally to vapor. Cocaine base has a melting point of 98 °C and

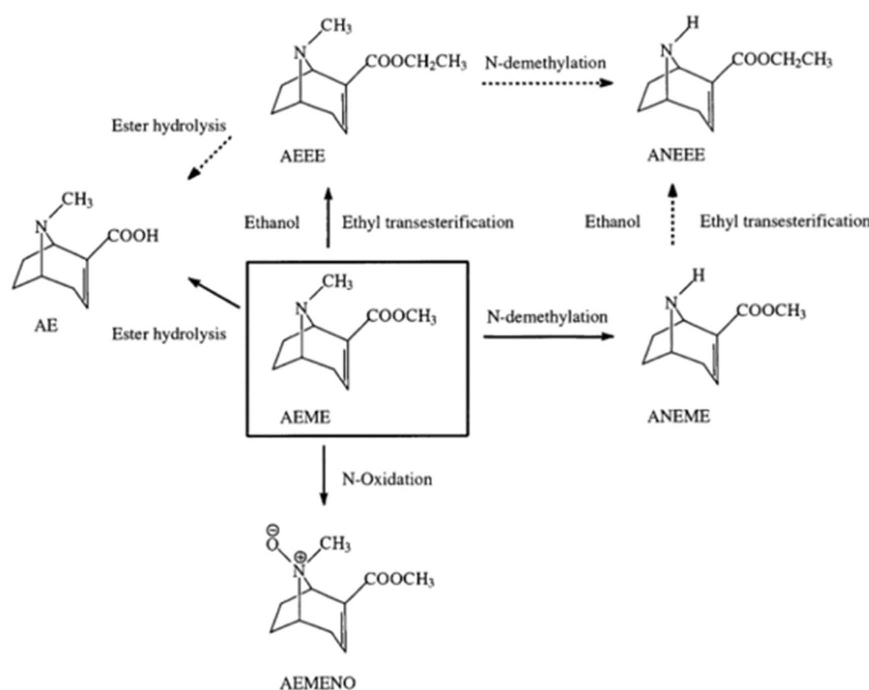

Fig. 2. *Metabolic pathways of the anhydroecgonine methyl ester. Note:* The figure shows the chemical composition of AEME (Anhydroecgonine methyl ester) and its metabolites: AE (Anhydroecgonine), AEEE (anhydroecgonine ethylester), AEMENO (anhydroecgonine methyl ester non-oxidized), ANEME (Anhydronorecgonine methyl ester) and ANEEE (anhydronorecgonine ethyl ester;). Solid arrows show proven pathways, and dashed arrows show postulated pathways. This image was adapted with permission from Fandiño AS, Toennes SW, and Kauert GF. Studies on hydrolytic and oxidative metabolic pathways of anhydroecgonine methyl ester (methylecgonidine) using microsomal preparations from rat organs. Chem Res Toxicol. 2002 Dec;15(12):1543–8. doi: 10.1021/tx0255828. PMID: 12482236. Copyright 2022 American Chemical Society.

exhibits a vapor pressure suitable for delivering cocaine above 160 ºC [20]. In contrast, the pyrolytic degradation of cocaine starts at around 170 °C [29].

In 1996, Wood et al. evaluated the vapor pressure and aerodynamic diameter measurement of cocaine and AEME using a simulated inhalation test of 30 mg of crack per pipe at different flow rates. A flow rate of 10 L/min recovered 21.3 % of the cocaine and 1.33 +/- 0.25 % of the AEME on the filter. Higher flow rates at 15 L/min produced greater distribution with smaller particulates [29].

It was also found that AEME disperses and disappears in 2 stages: a fast one, similar to cocaine, and a slow one. When air flows are slower, AEME particles are slightly larger, lasting longer in the environment than other volatile substances. Consequently, albeit at low levels, AEME may be present in the atmosphere for a more extended period than cocaine particles [29].

The AEME showed an elevated vapor pressure above the cocaine and a more gradual decrease in vapor pressure as the temperature decreased. Therefore, cocaine condenses first, forming a viscous droplet at a higher temperature than the AEME and providing the most significant surface area nearby as the AEME begins to condense. Accordingly, AEME droplets coat the cocaine droplet during aerosol formation [29].

4.1.2. Absorption

In relation to the absorption of AEME, there are few studies that refer to this process. Wood and collaborators propose, from their study in a crack pipe model, that a human who inhales or smokes cocaine would present a deposition fraction of approximately 0.3 with an aerodynamic diameter measurement (MMAD) of 1 mm; sg: 2.4. The aerosol generated by the typical crack pipe has an MMAD of <1 mm and is therefore easily deposited in the alveolar region [29].

Because temperature influences the size of condensed particles, low temperatures produce larger particles that are easily deposited in the upper airway [29], thus, temperature, inhalation depth, the instruments used, will influence the delivery and absorption of AEME in subjects who consume smokable cocaine. (Fig. 3).

4.1.3. Distribution

Regarding distribution, Garcia et al. compared the plasma concentrations of AEME and cocaine 15 minutes after administration alone or in combination in Wistar rats. The plasma concentration of cocaine was similar between the cocaine (480.7 +/- 64.3 ng/mL) and cocaine-AEME (426.9 +/- 48.7 ng/mL) groups. However, the AEME concentration was 2.5 times higher in the AEME-cocaine group (144.1 +/- 13.1 ng/mL) than in the AEME alone group (57.6 +/- 4.7 ng/mL; $p < 0.001$). This higher concentration of AEME in the presence of cocaine may be attributed to a toxicokinetic phenomenon suggesting that cocaine competes for the same metabolic pathways as AEME [13].

The volume of distribution of AEME (6–10 l/kg) is about three times greater than that of cocaine (1–3 l/kg), which implies that the tissue distribution of AEME is greater than that of blood. Therefore, the plasma concentration of AEME does not reflect the maximum concentration in brain tissue ([8]. Additionally, AEME crosses the placental barrier without further knowledge of its effects on the embryo or fetus (Kintz et al., 2002b), [19].

4.1.4. Metabolism

Regarding AEME metabolism, Fandiño, A.S., Toennes, S.W., and Kauert, G.F. conducted an experimental (in vitro) study using microsomes from rat liver, lung, kidney, and brain of three-month-old Sprague-Dawley rats, evaluating the resulting metabolites after incubation with Anhydroecgonine (AE), AEME, and Anhydronorecgonine Methyl Ester (ANEME). It was observed that in the incubation mixtures of rat liver and lung microsomes, the metabolites AEME, AE, ANEME, and Unoxidized Anhydroecgonine Methyl Ester (AEMENO) were identified. Conversely, in the incubation mixtures of rat kidney and brain microsomes, only AE was detected, suggesting an insignificant microsomal oxidation capacity for AEME in these organs. A higher yield of the hydrolysis product AE was found in incubated liver microsomes. Taking

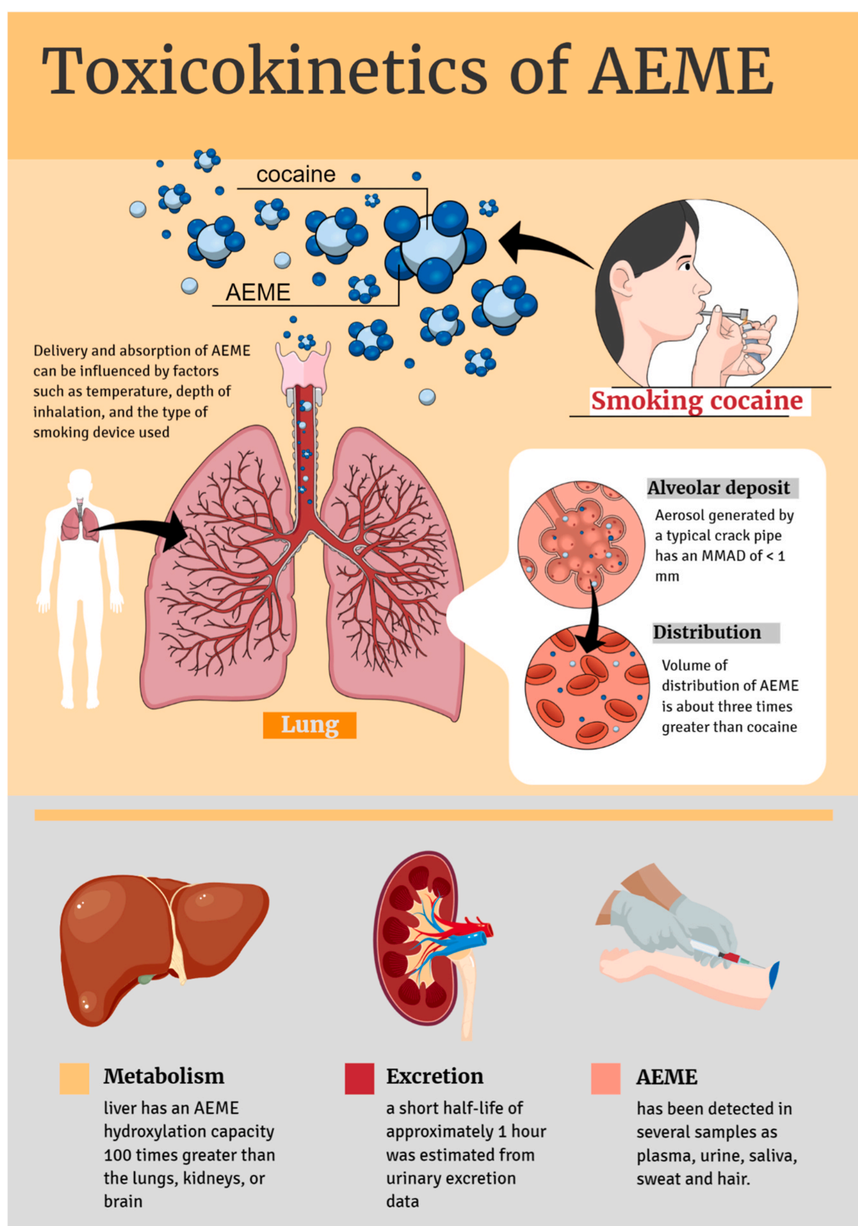

Fig. 3. *Schematization of AEME* toxicokinetics. *Note:* The figure shows an infographic resume of the toxicokinetics of AEME.

into account the yield of microsomal protein, the weight of the organs, and the activity of esterase, it is suggested that the liver has a hydrolytic capacity more than 100 times greater than that of the lung, kidney, or brain. Consequently, it is concluded that among the four tissues studied, the liver plays the most significant role in the in vivo hydrolytic metabolism of AEME [7].

Subsequently, this same study group evaluated the stability of AEME in human plasma. The effect of pH on AEME stability was examined through incubation studies of AEME with increasing pH phosphate buffers, revealing that AEME hydrolyzes much more rapidly at higher pH levels. These results are consistent with previous findings on the hydrolysis of cocaine in buffers. Additionally, it was found that AEME degrades in human plasma in vitro through spontaneous chemical hydrolysis and enzymatic cleavage by butyrylcholinesterase. The rate of hydrolysis depends on both pH and storage temperature. Based on these studies, optimal storage of samples containing AEME is recommended at pH 5, along with refrigeration and the addition of butyrylcholinesterase inhibitors such as sodium fluoride or echothiopate iodide [7].

Additionally, they identified the main metabolites of AEME: AE, ANEME, and AEMENO, this latest metabolite was identified after incubation of AEME with rat liver microsomes, and it was also detected in human samples. The chemical structure of AEMENO was confirmed through synthesis and in-depth study of its nanoESI-MSn fragmentation pathways. The use of ESI-MS was crucial for AEMENO detection due to its thermal lability during GC/MS analysis [8].

Similar to cocaine metabolizing into cocaethylene in the presence of ethanol, AEME also undergoes ethyl transesterification to form AEEE [6, 7]. Whether these metabolites play an active or toxic role remains unknown. Fig. 2 schematically illustrates the mentioned pathways and metabolites.

Studies by this group of researchers published in three articles in 2002 allow us to understand the metabolism of this cocaine pyrolysis product. These studies demonstrated that AEME undergoes N-oxidative metabolism in both rats and humans [8].

4.1.5. *Excretion*

In 1990, Jacob et al. determined the half-life elimination of AEME through controlled administration of crack in smoking cocaine users. A

short half-life of approximately 1 hour was estimated from urinary excretion data [18]. Subsequently, Cone et al. measured peak urinary AEME concentrations of 23 +/- 15 ng/mL, peaking at approximately 2.25 +/- 0.44 hours, representing 0.020 +/-0.1 % of the administered dose of cocaine base [3].

4.1.6. AEME detection

Different techniques have been used to detect AEME as a biomarker of smoking cocaine consumption. Kintz et al. evaluated distinct types of samples in active cocaine users. However, detecting AEME in plasma was not achieved in any samples. Conversely, 89 of 489 urine samples were positive (5–1477 ng/mL), even up to 54 hours later. Although there were few saliva samples (n = 6), all were positive (5–18 ng/mL). For sweat samples, only one case was positive (53 ng/patch), corresponding to a heavy crack user (1 g in 48 hours). Finally, 32 of 158 hair samples were positive (0.21–21.56 ng/mg), and in a binomial case of a mother-child, the detection of AEME was reported in the hair of the 35-week-old newborn baby [19]. While negative plasma sample findings may suggest the possibility of false negatives due to potential storage and handling issues, it is important to note that plasma samples can be sensitive to changes in pH and temperature, as has been documented in earlier studies.

In 2003, Liberty et al. evaluated the efficacy of a rapid patch in detecting cocaine analytes in sweat. The prevalence rate of cocaine detected was 92.2 % (180 patches), comparable to the urine drug detection rate of 91.1 %. However, 98 were positive for pyrolytic analytes, regardless of the concentrations of cocaine consumed. These data showed the success of the rapid patch in detecting smoking cocaine [21].

However, the identification of AEME in hair is not related to the consumption of smoking cocaine. Recently, Rubio et al. reported the detection of AEME in the hair of three individuals who consumed coca leaf, one by daily chewing and two in tea. The authors suggest that AEME may be formed in earlier processes of coca leaf storage, chewing, or drinking infusion. Therefore, other findings are recommended to support hair analyses, and differentiate between coca leaves and smoking cocaine [24]. Similarly, Gambier et al. found that AEME can also be produced "artificially." Cocaine-positive hair can occur after heat treatment by straightening, i.e. Therefore, considering the thermal treatment of hair during the analysis is recommended [10].

4.2. Toxicodynamic of AEME

4.2.1. Cardiorespiratory system

In 1995, Eurzouki and colleagues conducted the first AEME toxicodynamic study, investigating the cardiovascular effects of cocaine, pyrolytic products, and cocaine metabolites on rabbits. After administering cocaine intra-arterially at a dose of 1 mg, the rabbits experienced a decrease in both blood pressure (BP) and heart rate (HR). Similar effects were observed after administering AEME, ANEME, and AEEE intra-arterially at a dose of 3 mg. However, intravenous administration of AEME and ANEME increased respiratory frequency (RF), unlike intravenous cocaine. These findings suggest that the effects of cocaine mainly occur in the cardiorespiratory centers, whereas the effects of pyrolysis products mainly occur in the central and peripheral routes [5].

Considering the effects of AEME on RF and its potential peripheral effects, Willetts evaluated the effects of subcutaneously administered AEME on specific airway conductance (SGaw) in adult guinea pigs using plethysmography. The guinea pigs were evaluated with bronchoconstrictors such as acetylcholine (Ach) before nebulization. No differences were found between the control and intervention groups, preventing the demonstration of the antispasmodic effect of Ach. However, the authors also evaluated the effects of AEME inhalation by nebulization and found that a concentration of 16.12 mg/L (+/- 0.433) increases proteins and lactate dehydrogenase (LDH) in pulmonary lavage ($p < 0.01$). There were also traces of blood, but no significant changes in neutrophils were observed 24 hours after the intervention. In addition, the authors found systemic effects such as defecation, salivation, tremors, and startle upon AEME exposure. These findings suggested that AEME has cholinergic-type effects and can cause local irritation in the airway [27].

However, the researchers conducted an *in vivo* assay in squirrel monkeys to evaluate the effects of inhalation of AEME by chamber on SGaw, HR, and rectal temperature. A reduction in SGaw was reported after 5 minutes of exposure, with recovery after 25 minutes. Additionally, a reduction in HR was observed in two monkeys, indicating that AEME can produce bronchoconstriction and affect the cardiovascular system [28].

Similarly, this group performed an *in vitro* study in guinea pig tracheal rings. A dose-dependent relaxation was found upon administration of AEME to samples previously impregnated with Ach; tissue washing did not affect these effects. Finally, no change was observed upon exposure of the biventer cervical muscle-nerve of Leghorn hens to AEME, indicating no effect on striated muscle (el-Fawal & Wood, 2007), [4].

These findings were contradictory since the cholinergic bronchoconstrictor effects were only in two in vivo models associated with systemic effects such as increased salivation, defecation, and tremor. Accordingly, the expected effect of AEME on smooth muscle M3 muscarinic receptors would be a contraction, with no effect on striated muscle. Furthermore, it is believed that the relaxation effects on this muscle may be related to a paradoxical effect of parasympathomimetic, which stimulates nitric oxide synthesis by circumscribed endothelial cells [16].

Woolf et al. conducted two studies to evaluate the effects of AEME on cardiac function. In the first study, the effects of AEME were evaluated on ferret papillary muscle and human ventricular trabeculae and identified that AEME inhibits papillary muscle contractility. In the second study, the effects of AEME were evaluated on ferret cardiomyocytes and reported a depressed contractile effect caused by a combination of decreased Ca2++ availability and decreased myofilament Ca++ responsiveness [30]. The results showed that myocytes impregnated with an M2 antagonist, methoctramine, exhibited regular shortening despite exposure to AEME, indicating that AEME interacts with specific M2 receptors in myocytes to produce its negative inotropic effect. Compared to cocaine, AEME demonstrated a more potent effect as a cardiac depressant by altering the relationship between shortening and extracellular calcium concentration, particularly at higher extracellular calcium concentrations. Huang et al. also reported that at high concentrations, AEME has irreversible negative contractile actions that are not mediated by the muscarinic pathway, which suggests structural damage to myocytes [17].

4.2.2. Central nervous system

In Brazil, Garcia and collaborators published the first study regarding the toxicodynamic effects of AEME on the CNS, followed by different studies until 2021. In 2012, an *in vitro* study was conducted on hippocampal neurons (HPC) of Wistar rats exposed to AEME, AEME+Cocaine, and KCl (control) to assess neuronal viability. AEME was identified as neurotoxic with a more significant effect than cocaine. After 24 hours of exposure to concentrations greater than 10^1 mM AEME [F(10,154) = 41.17, $p < 0.001$] and 2 mM cocaine [F(9136) = 40.80, $p < 0.001$], the percentage of viable cells was 64.6 ± 8.2 % and 67.3 ± 7.1 %, respectively, with an additive neurotoxic effect to the cocaine-AEME combination, clearly reducing cell viability by 78.5 % [F (4,70) = 192.1, $p < 0.001$] [11].

Garcia et al. evaluated the effects of AEME on the cholinergic system by incubating HCP neurons with atropine (cholinergic antagonist) before exposure to AEME and cocaine. All concentrations of atropine prevented neurotoxicity by AEME, connecting its toxic effect with its cholinergic properties. In contrast, HCP neurons exposed to cocaine or the cocaine-AEME combination did not have the same effect, suggesting the involvement of other pathways influenced by cocaine [11]. The authors also evaluated the binding capacity (Bmax) and dissociation

constant (Kd) of AEME to muscarinic receptors, identifying AEME as a partial agonist of the muscarinic cholinergic receptor (mAChR) subtype M1 and M3 but not M2, generating an increase of intracellular Ca++. This effect was present even with low Ach concentrations. Likewise, their findings showed weak antagonistic effects of M2 and M4 and an orthosteric antagonistic effect of M5. Additionally, the effects of selective antagonists of M1 (pirenzepine) and M3 (p-F-HHSiD) on AEME-induced neurotoxicity were evaluated. Pirenzepine prevented neurotoxicity by 98.4 % ($p < 0.001$ compared to AEME) and with p-F-HHSiD by 85.4 % ($p < 0.05$ compared to AEME). In addition, the phospholipase C (PLC) inhibitor, U73122, was used to evaluate the involvement of PLC; U73122 showed neurotoxicity prevention by 102.6 % ($p < 0.01$ compared to AEME), indicating that the neurotoxic effects were mediated by PLC activation [12].

Areal et al. evaluated the effects of crack inhalation by chamber twice a day for 11 days in C57B1/6 mice. Blood analysis by Gas Chromatography/Mass Spectrometry (GC/MS) revealed that AEME concentrations were higher than those of cocaine. Mice exhibited hyperlocomotion behavior after inhalation sessions, represented in significantly longer runs inside the chamber during the first 5 minutes [F (1207) =48.85; $p < 0.0001$] and from 5 to 10 minutes later [F (1207) =20.39; $p = 0.0002$]. In addition, the mice exhibited a particular behavior described as "escape jumps" [F (1207) =11.31, $p = 0.0027$ for the 0 – 5 min segment and (1207) = 13.45, $p = 0.0013$ for the 5 –10 min segment]. Post-euthanasia, the prefrontal cortex (PFC) was assessed since it is an area of interest in the reward system altered in cocaine use disorders. Crack exposure promoted an increase in FosB expression ($p < 0.05$) and down-regulation of CREB ($p < 0.05$), genes related to cocaine seeking and reward processing. Conversely, crack increased the expression levels of dopamine pathway components ($p < 0.05$ for D1R and D3R, $p < 0.005$ for D2R and Tyrosine Hydroxylase) and decreased dopamine ($p = 0.0377$), DOPAC ($p = 0.0009$) and HVA ($p = 0.0021$) [1]. Additionally, alterations of the endocannabinoid system and its interaction with the dopaminergic system were evaluated; crack produced a down-regulation of CB1R, FAAH (fatty acid amide hydrolase, $p < 0.001$), and MAGL (monoacylglycerol lipase; $p < 0.05$) gene expression was found. Accordingly, D2R activation inhibits FAAH and stimulates MAGL, increasing anandamide (AEA) levels and possibly inducing CB1R sensitization as a dopamine regulatory mechanism [1].

Similarly, García et al. reported complementary results in Wistar rats exposed to AEME, AEME+cocaine, and cocaine via intraperitoneal route during nine days with a re-exposure at day 16 of intervention. In this study, the post hoc test showed that AEME was not related to increased rat running and movement alone ($p = 0.735$). However, the cocaine-AEME combination ($p < 0.001$) potentiated cocaine behavioral sensitization ($p = 0.003$). Additionally, changes in the dopaminergic system were evaluated in areas in charge of substance seeking within the reward system (nucleus accumbens Nacc and caudate nucleus CPu). The most relevant findings showed that treatment with all drugs increased dopamine levels in CPu compared to the control group (F3.44=12.25; $p < 0.001$), and the cocaine-AEME group showed the most significant magnitude of change. Likewise, cocaine and cocaine-AEME increased dopamine levels in NAcc. The increase in dopamine was related to the downregulation of D1R in the CPu for the cocaine and cocaine-AEME groups (F3,23 = 6.946; $p = 0.002$), with no change in D2R levels (F3, 23 = 0.742; $p = 0.539$) [13]. These changes are characteristic of dopaminergic neuroadaptation, reducing stimulatory D1R and increasing or not affecting inhibitory ones such as D2R.

In addition, the effects on the working memory of Wistar rats were studied after exposition to AEME intraventricularly (iv) at different doses (10, 32, and 100 μg). The researchers reported that the number of errors in working memory was significantly elevated at doses of 32 μg ($p < 0.05$) or 100 μg ($p < 0.05$) compared to the control group, suggesting that AEME impairs long-term spatial working memory. These effects may be related to its cholinergic activity, possibly involving M1, M2, and M3 receptors, which may have influenced dopaminergic activity, which was affected in previous studies. Subsequently, the findings were linked to alterations in antioxidant enzyme activity in the PFC, HPC, and striatal nucleus (STR). There was an increase in protein oxidation (AOPP) levels [$p < 0.05$], as well as in glutathione peroxidase (GPx) enzyme activity [$p < 0.05$] in the STR of the group receiving 100 μg of AEME compared to lower doses. Moreover, no significant differences were found in PFC or HPC. Notably, GPx activity increases in the STR as a compensatory mechanism to the increased amount of reactive oxygen species (ROS) produced by AOPPs. Conversely, no significant differences in the levels of lipid peroxidation (TBA-RS), CAT (catalase), or SOD (superoxide dismutase) were found in any of the structures [15].

Medeiros de Mesquita and collaborators administered AEME to Wistar rats at the end of the 12-hour light period for ten consecutive days to evaluate the effect of AEME on melatonin production, reporting a reduction of melatonin content in the pineal glands 5 h after turning off the lights and 2 h before turning them on (Two-way ANOVA: treatment: $p < 0.01$; time: $p < 0.001$; interaction: $p < 0.05$). Melatonin depletion by AEME affects the actions of melatonin as a photo-neuroendocrine transducer and as an antioxidant agent. Subsequently, the antioxidant enzymatic activity was evaluated in the PFC, HPC, and pineal gland, showing that AEME reduced nocturnal SOD activity in the PFC and HPC ($p < 0.05$), with no change in GPx or glutathione reductase activity (GR; $p > 0.05$). In addition, the pinealocytes were stimulated to produce melatonin by incubation with norepinephrine (NE). However, the production was not achieved, so inhibitors of potential AEME binding sites were used (TMPH, selective α3β4 nicotinic receptor antagonist; MK-801, NMDA receptor antagonist; NBQX, AMPA receptor antagonist; and atropine, muscarinic cholinergic receptor antagonist). Furthermore, all atropine concentrations reversed the inhibitory effect of AEME on melatonin synthesis, pointing to a cholinergic muscarinic effect on pinealocytes (one-way ANOVA: $p < 0.001$; Bonferroni: $p < 0.001$, AEME + NE compared to NE; $p < 0.05$, NE + AEME + atropine compared to NE + AEME) [23]. Also, a statistically significant reduction in the viability of HPC neurons upon exposure to AEME was reported (one-way ANOVA: $p < 0.001$; Bonferroni: $p < 0.001$, AEME 1 and 10 mM compared to control). Notably, incubating these cells with melatonin prior to AEME exposure protected neurons against its toxicity, increasing cell viability (Bonferroni: $p < 0.01$, AEME 0.1 mM + melatonin 1 nM compared to AEME 0.1 mM; $p < 0.001$, 1 mM AEME + 1 nM melatonin compared to 1 mM AEME) [23].

Later, Garcia et al. incubated HCP cells with AEME-cocaine combinations (1 and 2 mM, respectively) to continue the study of the imbalance in the activity of glutathione-related enzymes. As in previous studies, there was a reduction in cell viability, 59.5 ± 2.5 % for AEME ($p < 0.001$), 61.4 ± 4.6 % for cocaine ($p < 0.001$), and 32.6 ± 1.8 % for the AEME-cocaine combination ($p < 0.0001$). In addition, AEME reduced GPx and GR activity after 3 h of exposure (GPx [F (3, 8) = 9.796, p = 0.0047]; GR [F (3,8) = 19.43, $p < 0.001$]). Furthermore, the AEME-cocaine combination group showed more pronounced lipid peroxidation than the cocaine group after 48 h of exposure, 29.9 % for AEME ($p < 0.01$), 40.0 % for cocaine ($p < 0.01$), and 95.7 % for the AEME-cocaine combination ($p < 0.0001$), supporting an additive neurotoxic effect after exposure [14]. Remarkably, the reduction of GPx activity was reported in the *in vitro* study of an acute pattern, in contrast to what Gomes and collaborators reported in a chronic pattern with re-exposure, suggesting that these differences may be related to the period of exposure, route of administration, and dose.

Finally, in 2021 Udo MSB et al. described the autophagic and apoptotic processes of Wistar rat HPC neurons exposed to AEME, AEME-Cocaine, and cocaine. The combination of AEME and Cocaine resulted in membrane rupture within 3 hours of exposure ($p < 0.01$), which increased significantly after 6 hours ($p < 0.0001$). This effect was significantly faster than when either AEME or Cocaine was administered alone. Additionally, after 6 hours of exposure to the AEME-Cocaine combination, a higher percentage of cells were found to be in an early apoptotic stage, as evidenced by externalization of phosphatidylserine,

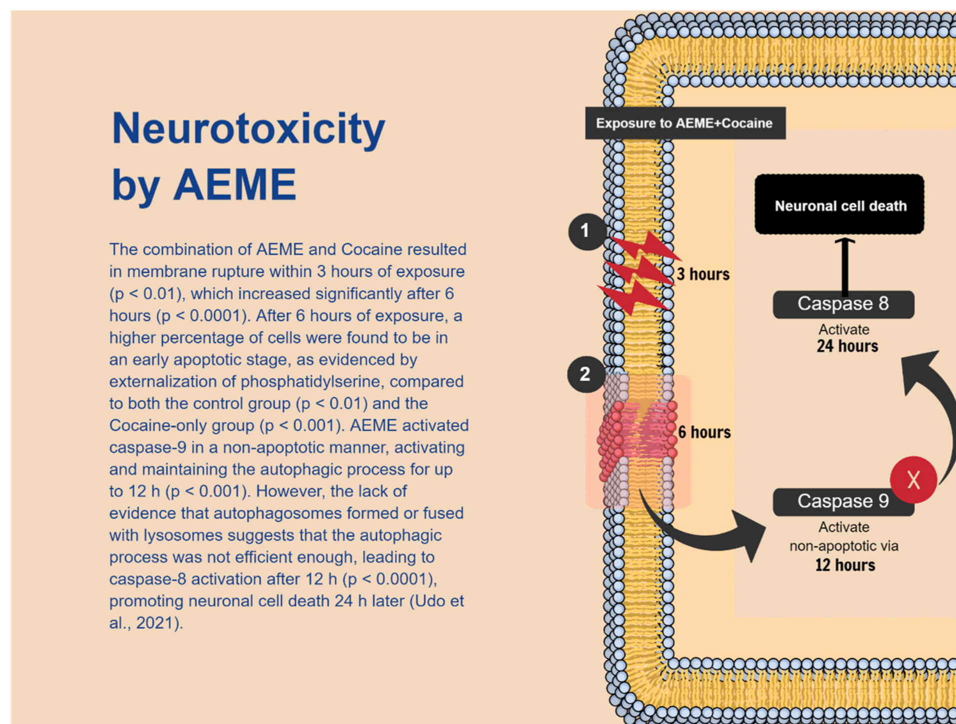

Fig. 4. *Schematization of AEME neurotoxicity findings. Note:* The figure schematizes the findings in neurotoxicity of hippocampal neurons.

compared to both the control group ($p < 0.01$) and the Cocaine-only group ($p < 0.001$). AEME activated caspase-9 in a non-apoptotic manner, activating and maintaining the autophagic process for up to 12 h ($p < 0.001$). However, the lack of evidence that autophagosomes formed or fused with lysosomes suggests that the autophagic process was not efficient enough, leading to caspase-8 activation after 12 h ($p < 0.0001$), promoting neuronal cell death 24 h later [26].

Several reports suggest that the partial cholinergic agonist effect of M1 and M3 may be involved in the neurotoxicity of primary HPC cells [23], participating in membrane rupture induced by increasing the number of dysfunctional proteins and organelles and [26], and the intracellular Ca concentration $^{++}$[12]. These changes lead to the activation of caspase-9 in a non-apoptotic manner, activating and maintaining the autophagic process until 12 h, then leading to caspase-8 activation after 12 h and promoting neuronal cell death 24 h later [11, 15]. Also, the AEME-Cocaine combination potentiates the changes, shortening the time of caspase-8 activation to 6 h, approximately half the time. The authors suggest that this potentiated effect involves dopaminergic effects from cocaine exposure and muscarinic effects from AEME exposure, favoring an even more neurotoxic effect [26] (Fig. 4).

Overall, AEME has been shown to significantly impact the addiction to smoking cocaine, with its effects being enhanced when combined with cocaine. The synergistic toxicity of AEME and cocaine manifests in respiratory absorption and distribution, leading to enhanced neurotoxic effects of cocaine, and reduced melatonin secretion through partial cholinergic agonist effects. Additionally, AEME potentiates alterations in GPx and Gr activity, generating an increase in ROS, hippocampal neuron membrane damage, and activation of apoptotic pathways, leading to rapid reduction of hippocampal neurons. Although the peripheral effects of AEME as a cholinergic agonist bronchoconstrictor and its adverse inotropic effects have been studied, its cardiotoxic effects and influence on other systems and neuronal processes are still not well understood. Further research on the effects of AEME and its interaction with cocaine will be crucial in improving our understanding of the pathophysiology of smoking cocaine addiction.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.101690.

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