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Toxic Effects of Xylazine on Endothelial Cells in Combination with Cocaine and 6-monoacetylmorphine

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Abstract

The use of xylazine as a drug of abuse has emerged worldwide in the last 7 years, including Puerto Rico. Clinical findings reported that xylazine users present greater physiological deterioration, than heroin users. The aim of this study was to assess the xylazine toxicity on endothelial cells, as this is one of the first tissues impact upon administration. Human umbilical vein endothelial cells in culture were treated with xylazine, cocaine, 6-monoacetylmorphine (heroin metabolite) and its combinations, at concentrations of 0.10 μ M to 400 μ M, for periods of 24, 48 and 72 hours. IC₅₀ were calculated and the Annexin V assay implemented to determine the cell death mechanism. Results indicated IC₅₀ values at 24 hours as follow: xylazine 62 μ M, cocaine 210 μ M, 6-monoacetylmorphine 300 μ M. When these drugs were combined the IC₅₀ value was 57 μ M. Annexin V results indicated cell death by an apoptosis mechanism in cells treated with xylazine or in combination. Results demonstrated that xylazine use inhibits the endothelial cell proliferation, at lower concentrations than cocaine and 6-monoacetylmorphine. These findings contribute to the understanding of the toxicity mechanisms induced by xylazine on endothelial cells.

Keywords

xylazine; cocaine; 6-monoacetylmorphine; apoptosis; drug abuse

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Conflict of interest

The authors have declared that no conflict of interest exists.

Introduction

Xylazine is an emerging drug of abuse, synthesized in 1962 [1], used in veterinary applications and not FDA approved for human use. This drug is an α -2-receptor (α -2) agonist [2]. The α -2 receptor is Gi protein coupled (GPCR), located in the pre and postsynaptic neuron cleft cell. The peripheral localization of α -2 receptors includes platelets, pancreatic β cells (inhibiting insulin release), adipocytes and vascular endothelium [3]. Their mechanism of action in the presynaptic cleft is related to the adenylyl cyclase inhibition, by the alpha-GTP complex [2], [4], [5]. This α -2 receptor agonist also inhibits the calcium channel activation and stops neurotransmitter vesicle release. Moreover in the postsynaptic cleft the mechanism of action activates the inward rectifier K⁺ channel, causing subsequent membrane depolarization and neurotransmission diminishment [6]. This mechanism of action allows xylazine to lower blood pressure, by reducing sympathetic peripheral outflow and thus reduces peripheral tone.

The use of xylazine as a drug of abuse has increased in the last decade, as reported worldwide [4, 7–9]. Puerto Rico has a particular high incidence among addicts population [10–12]. The modality of this drug use is known as “speedball”, which means the mixture of cocaine and heroin in the same syringe [12]. As xylazine is substituting heroin as a drug of abuse and also been the main adulterant of heroin, the current mixture modality is now among cocaine and xylazine or a mixture of cocaine, xylazine and heroin. This information was obtained from the chemical analysis of seized drug, performed by the Controlled Substance Section at the Puerto Rico Institute of Forensic Science (PRIFS) [13].

Xylazine is mainly administered through the intravenous route (IV), and therefore the vascular endothelium cells primarily receives the drug insult [12, 14]. When referring to the speedball modality, it must be considered that the drug mixture present is xylazine and 6-monoacetylmorphine, since heroin is immediately metabolized in 6-monoacetylmorphine when it enters the human body [15]. The vascular endothelium functions are important; these include synthesis and secretion of active biological substances. Its integrity is indispensable, because it acts as a selective barrier against the passage of blood constituents through the vessel wall. Furthermore, vascular endothelium cells participate in the control of blood fluidity, vascular tone, chemotaxis and leucocyte adhesion [16].

Little is known about the chronic and acute toxic effects of xylazine, or its combination with other drugs in humans. This study was focused on elucidating the potential cytotoxic effects and death mechanism of xylazine and its combination with cocaine and/or 6-monoacetylmorphine in human vascular endothelial cells. Jointly, we expect the outcomes of these experiments will add to the understanding of potential vascular injury and consequently other health deteriorating effects, which could be associated to xylazine abuse.

Materials and methods

1. Stock solutions and reagents

Experimental compounds stock solutions of all drugs were prepared at concentrations of 3 mM in ethanol 70%, obtained from Sigma Aldrich, (St. Louis, MO). Those stock solutions

were kept in sterile glass vials and stored at 4 °C. The positive control (camptothecin), cocaine, xylazine and yohimbine were obtained from Sigma Aldrich, and heroin metabolite (6-monoacetylmorphine) obtained from Cerilliant Corporation (Round Rock, Texas). Presto blue (PB) reagent obtained from Life Technologies (Grand Island, NY). Annexin V-FITC apoptosis detection kit, Propidium Iodide and Hoechst 33342 were obtained from Biotium, Hayward, CA.

2. Cell culture

The cell line used in this study was human umbilical vein endothelial cell line EA.hy926, kindly provided by Dr. Cora-Jean S. Edgell, from the University of North Carolina at Chapel Hill (UNC). Cells were cultured to confluence on DMEM culture media (ATCC, Manassas, Virginia) with 10% fetal bovine serum (ATCC, Manassas, Virginia) [17, 18]. These cultures were maintained at 37 °C and 5% CO₂ [19]. Cell viability was determined to be consistently over 90% by trypan blue exclusion method prior to seeding. The monolayers were treated with the tested compounds within 24 hours after confluence.

3. Determination of IC₅₀

3.1 Cell treatment—Prior to treatment, EA.hy926 cells were sub-cultured and kept at a density of 5.0×10^5 cells per 3.5 mL of culture media plus additives in 25 cm² flasks to assure stable metabolic state and exponential growth. Cells were seeded in 96 well plate and treatment solutions of cocaine, 6-monoacetylmorphine (6-MAM), xylazine, their combinations, camptothecin and yohimbine were prepared for each experiment by dilution in medium, and added. EA.hy926 cells were exposed for 24, 48 and 72 hours in experimental doses ranging from 0.10 µM to 400 µM. Cell viability and the IC₅₀ of each drug were determined by the presto blue (PB) reagent and analyzed using fluorostar Optima (BMG) fluorescence reader.

3.2 Presto-Blue assay—EA.hy926 viability assay with PB reagent was performed according to the manufacturer's protocol [20, 21]. Cells in suspension were seeded at 50,000 cells/well in a 96-well plate, 100µL final volume per well. After treatment period cells were washed and incubated with 10 µL of PB reagent and 90 µL of medium. Changes in cell viability were detected using fluorescence read at excitation 570 nm; emission 610 nm [22]. The cell viability was expressed as a percentage relative to untreated cells.

3.3 Statistical analysis—For the assessment of cell viability in EA.hy926 exposed to the tested drugs, the dose– response curves were generated and plotted using a non-linear regression analysis and the IC₅₀ values were calculated with Graphpad Prism software (v. 5.03).

4. Annexin V Apoptosis assay

Apoptosis was evaluated using the Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions [19, 23, 24]. Briefly, approximately 5×10^5 cells were treated for 24 hours with xylazine (60 µM), Cocaine (160 µM), 6-MAM (160 µM), camptothecin (50 µM), xylazine/cocaine (50 µM), xylazine/6-MAM (50 µM) and xylazine/cocaine/6-MAM (40 µM) and vehicle. After exposure, cells were stained with Annexin V-FITC

conjugate and Hoechst 33342 and incubated at 37 °C for 15 minutes [23, 25]. Supernatant was then removed, and re-suspended in 100 µl Annexin V binding buffer supplemented with 10 µg/ml PI (Propidium Iodide) and immediately analyzed. Analysis was performed using image cytometry with the Nucleo Counter NC3000 (Chemometec, Allerød, Denmark).

5. Statistical analysis

For the assessment of cell viability in EA.hy926 exposed to the tested drugs, dose–response curves were generated using a non-linear regression analysis and the IC₅₀ values were calculated with Graphpad Prism software (v. 5.03). Statistical analysis was completed by one-way and two way ANOVA, with Tukey post hoc test, where $p < 0.05$ was considered significant.

Results

Drug response and IC₅₀ Determination

The effect of the tested drugs on cell viability was consistent with a dose-response effect for all treatments at the different time periods. No significant difference was observed on cells treated with xylazine and its combination with yohimbine, cocaine and/or 6-MAM at 24 hours period, when compared with the positive control (camptothecin). Cocaine and xylazine in combination with 6-MAM shows similar effect as positive control at 48 hours treatment period. Lastly, at 72 hours treatment period combination of xylazine with 6-MAM and xylazine with cocaine and 6-MAM, are comparable to camptothecin toxicity, as shown in Figure 1.

The capacity of xylazine, cocaine, 6-MAM and their combinations to inhibit cell viability after 24, 48 and 72 hours of exposure were evaluated on EA.hy926 cells culture treated with doses in the range of 0.1 µM to 400 µM. Results showed a clear dose response with all drugs and the combinations. The highest IC₅₀ values obtained at 24 hours were 210 µM for cocaine, 300 µM for 6-MAM. The lowest were 62 µM for xylazine, 75 µM for xylazine/cocaine, 67 µM for xylazine/6-MAM and 57 µM for xylazine/cocaine/6-MAM. The lowest IC₅₀ values at 48 hours treatment were 88 µM for cocaine, 61 µM for the combinations of xylazine/cocaine and 64 µM for xylazine/6-MAM. After 72 hours of treatment the IC₅₀s were as follow: Xylazine (58 µM), 6-MAM (67 µM), the combinations of xylazine/6-MAM (68 µM) and xylazine/cocaine/6-MAM (27 µM). Meanwhile cocaine (91 µM) and xylazine/cocaine combination (90 µM) presented the higher values. The IC₅₀ values including positive and negative controls, at all periods are presented in Table 1.

Annexin V staining

As an indication of apoptosis, the Annexin V assay implies the translocation of phosphatidylserine (PS) to the external cell membrane. The Annexin V staining has been performed as a screening method for apoptotic activity; this methodology has been used to detect cells undergoing apoptosis after exposure to different substances [26]. Apoptosis cells present a typical migration of PS to the outer leaflet of the cell membrane; Figure 2 shows a representation of the annexin V assay. All tested drugs and their combinations presented significant ($P < 0.05$) apoptosis induction. Figure 3A presents the Annexin values for

xylazine, cocaine and 6-MAM. Xylazine presented 63% of apoptotic cells, cocaine 56%, and 6-MAM 54%. While Figure 3B presents the values for the combination of xylazine with cocaine (50%), 6-MAM (30%) and both drugs (47%). All compounds are comparable to the positive control camptothecin (64%), clearly showing apoptotic activity.

Discussion

In this study we assessed the biological activities and cell death mechanism of xylazine, cocaine, heroin metabolite (6-monoacetylmorphine) and their combinations. These drugs were assayed for toxicity effect and were determined to be capable of growth inhibition upon human venous umbilical endothelial cells (EA.hy926). The IC₅₀ value of the positive control (camptothecin), presents a significant toxic effect stable along the treatment periods. The results of xylazine, cocaine and 6-MAM present different IC₅₀ values on the EA.hy926 cell line. Xylazine presents higher toxic effects at 24 hours treatment, the two way ANOVA analysis presented no significant difference ($P>0.05$) when is compared to camptothecin, a recognized cytotoxic compound. This evidence suggests that xylazine toxic effect is comparable to camptothecin. Xylazine shows an increase in the IC₅₀ value at 48 hours treatment, but at 24 and 72 hours those values remain similar. The higher IC₅₀ value at 48 hours treatment could be related to an initial survival of cells in response of a high toxicity insult [27, 28] followed by a cell death after longer period of time of drug exposure. Moreover, yohimbine (alpha 2 receptor antagonist) has no toxic effect into the vascular endothelium cells at the tested doses.

Cells treated with the combination of xylazine and yohimbine presented IC₅₀ value comparable to those obtained from cells treated with xylazine alone; yohimbine mechanism of action has no ability to block xylazine toxic effect in endothelial cells. Implying that the observed toxicity effect of xylazine is independent from its mechanism of action, as an alpha 2-receptor agonist.

The results obtained from Annexin V assay showed clear apoptotic activity in cells treated for 24 hours with xylazine, cocaine, 6-MAM and their combinations. These treatments presented phosphatidylserine migration to the exterior of the cell membrane as evidenced by the annexin V staining. This phosphatidylserine migration is indicative of an apoptotic cell death mechanism. The apoptotic cell death process is an ubiquitous occurrence that is observed in a diversity of cell types, such as endothelial cells [29]. This finding is in agreement with previous studies showing that cocaine induces apoptosis in bovine coronary artery endothelial cells (BCAECs) [30]. These studies demonstrated that cocaine has induced Bcl-2 cleavage and activation of caspase-3 and caspase-9 in BCAECs. Their speculations suggest that cocaine exerts its effects on endothelial cells through three mechanisms: cleavage of Bcl-2, induction of Bax translocation, and attenuation of nitric oxide (NO) production [30]. Implying cocaine could be inducing the activation of caspases by denitrosylation of pro-caspases caused by the attenuation of NO, at this moment this remains unclear. Further evidence supports that cocaine apoptosis induction is related to increase levels of reactive oxygen species (ROS) and mitochondrial release of cytochrome c in human myocytes and fetal muscle cells [31, 32]. Lastly this cocaine apoptosis induction was also demonstrated in human coronary artery endothelial cells. In addition to several

animal studies involving cocaine to apoptosis induction, in cultured neurons, thymocytes, testes, and hepatocytes cells [33–35].

Furthermore results obtained from cells treated with heroin concur with previous studies reporting apoptosis induction in different rat tissues, such as primary cultured cerebellar granule cells (CGC) [36], hepatocytes [37] and neuronal cells [38]. Moreover it is important to point out that heroin, has another metabolite in addition to 6-MAM, which is the active metabolite, morphine, responsible for the pharmacological effects in humans. Morphine also is used in clinical applications for severe pain treatment [37, 39]. Heroin and morphine were shown to affect mitochondrial function [40], in rat cortical neuron's treated with heroin has showed decreased mitochondrial potential, triggering cell death through mitochondria-dependent apoptotic pathway [41, 42]. In these studies, morphine presented an inhibitory effect in ATPase activity [37]. The capability of heroin, 6-MAM, morphine and cocaine to induce apoptosis has been showed in a variety of cells types, but some concerns could be raised about the drug concentrations used in this study (from 0.1 to 400 μM). Nonetheless, the reported range concentrations of cocaine in plasma for human drug abusers are among 0.3 μM and 1 mM [37, 43]. The morphine concentrations of the blood levels detected in fatalities, implicating heroin abuse, are in range from 0.35 μM to 3.82 mM [44, 45], morphine concentration could be considered proportional to 6-monoacetylmorphine because it is metabolized to morphine as show in Figure 4. Significantly, morphine (including metabolites) and cocaine are accumulated in intracellular compartments. Consequently, blood levels determined in reported studies obviously miscalculate intracellular concentrations [37], because these concentrations are not contemplated in the final drug levels. The concentrations used in this study however, are comparable to doses to which the vascular endothelium, in drug users are exposed.

Cocaine and heroin are well known harmful drugs of abuse and their combination (speedball) being very common in drug users in many countries [42, 46]. Their toxic effect has been studied considerably, mainly in relation to neuronal and vascular damage [30, 40, 41, 46, 47]. There are no significant studies however, in regard to the impact upon the health of xylazine users, neither the combination of speedball and xylazine. Xylazine structurally belong to the phenothiazine drug family [4, 48]; these drugs are a class of compounds which interact with dopamine receptor as antagonist in the central nervous system (CNS) and other receptor depending on the chemical structure substitutions [49]. The relation among chemical structures is illustrated in the Figure 5. Phenothiazines are commonly used in combination with other drugs in antipsychotic treatment, showing also various biological activities involving antibacterial, antiplasmodic, antitumor and antihelmintic properties [49, 50]. These compounds have been shown to possess cytostatic and cytotoxic effects in a variety of non-CNS cells [51].

Phenothiazines interact with diverse biological macromolecules such as DNA, RNA, proteins, and human erythrocytes (releasing oxygen by interacting with hemoglobin (Hb) [49].

Recent studies results show phenothiazine enters the inner mitochondrial membranes and triggers the mitochondrial permeability transition associated to cytochrome c release [51].

This cellular death is associated to the apoptosis process, which concurs with our results, obtained from the cells treated with xylazine and its combination with cocaine and 6-MAM. These results indicated that xylazine and its combination induce cellular death by apoptosis. Cases related to xylazine toxicity reported human plasma concentrations in a range among 1.0 μM and 20 μM [4, 52], these cases weren't consider as drug users. The xylazine concentration on plasma users could be higher than these reported cases, considering that an epidemiologic study reported an increase in the frequency of injection since beginning to use xylazine (4–7/daily) [11, 12]. Which means, that xylazine concentrations used in this study are comparable, to the doses used by addicts. These concentrations account only for the parent drug, metabolites concentration were not determined. These metabolites are partially unknown, recently some were identified in human urine, but at this moment forensic toxicology laboratories are not screening for these metabolites [7, 8].

Understanding the effect of xylazine on endothelial cells is relevant since the endothelium plays an important role in regulating vascular tone by releasing nitric oxide (NO), participates in hemostasis, cellular proliferation, inflammation, and immunity [30]. Endothelial cell toxicity may result from chemical agents or drug interaction with the molecular targets expressed on membranes. Those chemical agents or drug interaction could initiate a signaling cascade inducing subsequent lesions [53]. Therefore, an imbalance could results in loss of vasoconstriction or vasodilation, which may lead to vascular damage. In general, drug-induced vascular injury is characterized by inflammation, neutrophil infiltrates, internal elastic lamina breaks and apoptosis [14, 16, 30]. Thus, arterial lesions induced may be a result of altered function and compromised endothelial cells [54, 55]. Drug-induced vascular injury remains to be a toxicological worry; hence, it is significant to identify the molecular targets of toxicity leading to the induction and progress of vascular injury, such as apoptosis death.

Apoptosis is a functional process in normal cell death, development and maintenance of tissue homeostasis. Diverse pathologic processes could be triggered by dysregulation of apoptosis [16, 56, 57]. Disproportionate apoptosis harms the integrity of the endothelial wall and is correlated with infection, inflammatory disease and atherosclerosis [58]. For example, disproportionate apoptosis of vascular endothelial cells in angiogenesis is imperative for ischemic disease [14, 59–61].

Conclusion

This study reports the toxic effects of xylazine, cocaine, 6-monoacetylmorphine and their combinations on the EA.hy926 cell line, human umbilical vein endothelial cells. The IC_{50} values demonstrated that xylazine and its combination with cocaine and/or 6-monoacetylmorphine show higher toxicity, comparable to camptothecin (positive control). Meanwhile cocaine and 6-monoacetylmorphine have exhibited less toxicity, when compared with xylazine and their combination with cocaine and/or 6-monoacetylmorphine. Moreover, drug-drug interaction between xylazine and their combination with cocaine and 6-monoacetylmorphine was not observed, given the similarities between IC_{50} . No synergistic effects among drug combination, was significant, meaning that xylazine has exhibited higher toxicity than cocaine and 6-monoacetylmorphine. Cells treated with xylazine and

yohimbines have shown that the death mechanism is independent of its agonist effect through the alpha-2 receptor.

Our results clearly indicate that the mechanism of death on human umbilical vein endothelial cells exposed to xylazine, cocaine, 6-MAM and their combinations involve an apoptotic pathway. This mechanism of cell death needs to be further characterized to determine whether an intrinsic or extrinsic pathway is activated. These findings contribute to the understanding of the toxicity mechanisms induced by xylazine on endothelial cells. Furthermore its potential health impact among addicts, when used alone and its combination with cocaine and/or heroin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- This study addresses toxics xylazine effect and its speedball combination, in humans.
- Assesses xylazine toxicity on human endothelial cells and its speedball combination.
- Xylazine and speedball combination IC₅₀ values have similar toxicity as camptothecin.
- The Annexin V assay demonstrated apoptosis as the cell death mechanism.
- These findings support that xylazine has higher toxicity than cocaine and 6-MAM.

A:

B:

C:

Figure 1.

Dose response comparisons of EA.hy926 cells; 1A: treated with camptothecin (positive control) and yohimbine, 1B: treated with xylazine and its combinations with yohimbine, 1C: treated with cocaine, 6-monoacetylmorphine (6-MAM) and its combinations with xylazine. Dose range from 0.10 μM to 400 μM , at treatment periods of 24, 48 and 72 hours.

Figure 2. Annexin V assay representation

The characteristic migration of PS to the exterior (extracellular) side of the cell membrane is typical of apoptosis. The translocation of PS from the cytoplasmic face to the external face of the plasma membrane can be detected using Annexin V during early apoptosis. Once on the cell surface, PS can be easily detected by staining with the fluorescent conjugate of Annexin V, a protein that has a high affinity for PS, detection could be analyzed by fluorescence microscopy. As apoptosis progresses the plasma membrane becomes compromised and loses membrane integrity later. Necrotic cells expose PS and lose membrane integrity concurrently after process initiation. Propidium Iodine (PI), a DNA binding dye, can be used to discriminate necrotic cells from apoptotic. Necrotic cells that have lost of membrane integrity will show red staining (PI) throughout the nucleus and green (annexin v) in the membrane. While apoptotic cells will show only green staining (annexin v) in the membrane.

Figure 3. Annexin V staining assay results

Data is shown in percentage of apoptotic cells, treated with all drugs, their combinations, vehicle as negative control and camptothecin as positive control, exhibiting significant apoptotic activity. A; The percentages of apoptotic cells treated with the drugs were camptothecin (Positive Control) 64%, xylazine 63%, cocaine 56%, and 6-MAM 54%. B; Drugs combinations treatments were xylazine/cocaine 50%, xylazine/6-MAM 30% and 47% for xylazine/cocaine/6-MAM. Approximately 5×10^5 cells were treated for 24 hours with xylazine (60 μ M), Cocaine (160 μ M), 6-MAM (160 μ M), camptothecin (50 μ M), xylazine/cocaine (50 μ M), xylazine/6-MAM (50 μ M) and xylazine/cocaine/6-MAM (40 μ M); and vehicle. One-way ANOVA statistical analysis with Tukey post hoc test was performed. All values were compared with positive control to determine the significant difference among drugs, if $P < 0.05$. P value Summary: $P < 0.001 = ***$, $P < 0.01 = **$.

NC = Negative Control, PC = Positive Control, XYL = Xylazine, COC = Cocaine, 6-MAM = 6-Monoacetylmorphine (Heroin metabolite).

Figure 4.

Illustration of structures involved in the metabolization of heroin molecule (A-Diacetylmorphine) to 6-Monoacetylmorphine (B) and finally to Morphine (C), once it enters the human body.

Phenothiazine

Xylazine

Figure 5.
Chemical structure illustration of phenothiazine drugs group (A) and xylazine (B).

Table 1

The half maximal inhibitory concentration (IC₅₀/μM) values of xylazine, cocaine, 6-monoacetylmorphine (6-MAM), camptothecin, yohimbine (negative control) and their combinations, obtained from a 10 doses study is showed below. The doses range was from 0.1 to 400 μM, and the IC₅₀ values were calculated with Graphpad Prism software (v. 5.03), from the dose response curves plotted using a non-linear regression analysis. Two way ANOVA statistical analysis was performed, all values were compared with the positive control (camptothecin) to determine the significant difference among drugs and treatment periods, if P<0.05. n/a = not applicative. Values are presented as mean ± SD (n = 9). P value summary: P > 0.05 = ns. * P value summary: P < 0.01. ** P value summary: P < 0.001. *** P value summary: P < 0.0001.

Drug	IC50 (μM) Treatment Period					
	24 Hours	P value	48 Hours	P value	72 Hours	P value
Xylazine	62	Ns	110	***	58	**
Cocaine	210	***	88	ns	91	***
6-MAM	300	***	138	***	51	*
Camptothecin	81	n/a	56	n/a	12	n/a
Yohimbine	6887	***	5717	***	593	***
Xylazine/Cocaine	75	Ns	61	ns	90	***
Xylazine/6-MAM	67	Ns	64	ns	38	ns
Xylazine/Cocaine/6-MAM	57	Ns	100	**	27	ns
Xylazine/Yohimbine	51	Ns	101	**	57	**