

## Research Article

# Xylazine and Its Speedball Combination: Induction of Apoptosis by Intrinsic and Extrinsic Pathway in Human Umbilical Vein Endothelial Cells

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**Abstract**

Emerging drugs of abuse, such as xylazine (XYL), are receiving great interest due to increasing use and the potential health toxicity effects, in the addict population. XYL is an alpha two agonist without medical applications in humans. Our previous studies indicated XYL and its combination with cocaine (COC) and/or 6-monoacetylmorphine (6-MAM) induced apoptotic cell death and DNA fragmentation in endothelial cells. In addition XYL and 6-MAM trigger reactive oxygen species (ROS) production in these cells. This study aim is identify apoptosis path way in cell death and determine cell cycle effects of xylazine and its combination with COC and 6-MAM in Human umbilical vein endothelial cells (HUVEC, EA.hy926). HUVEC were treated with XYL (60  $\mu$ M), COC (160  $\mu$ M), 6-MAM (160  $\mu$ M), camptothecin (Positive control, 50  $\mu$ M), XYL/COC (50  $\mu$ M), XYL/6-MAM (50  $\mu$ M) and XYL/COC/6-MAM (40  $\mu$ M) for a period of 24 hours. Activation of caspases 8 and 9, and cell cycle assessment were analyzed using differential microscopy assays. Results reveal that all drugs tested in this study and their combinations activate caspases 8 and 9, involved in extrinsic and intrinsic pathways respectively. Also, these drugs in combination have presented cell cycle arrest in G2/M phase. While cells treated with COC and 6-MAM, show cell cycle arrest in G0/G1 phase. The findings suggest that these drugs trigger apoptotic process in human endothelial cells involving both extrinsic and intrinsic pathways, and furthermore induce cell cycle arrest in two of the major checkpoints.

**ABBREVIATIONS**

COC: Cocaine; DNA: Deoxyribonucleic Acid; 6-MAM: 6-Monoacetylmorphine; ROS: Reactive Oxygen Species; XYL: Xylazine.

**INTRODUCTION**

The combination of common drugs of abuse such as cocaine and heroin with new emerging substances have a high toxic potential [1,2]. Since the mechanisms of action for these combinations are partially undetermined and clinical laboratories are unable to detect them [3,4]. In the case of xylazine (XYL), an emerging drug of abuse, its alpha-2 receptor agonist mechanism of action was studied in animals, resulting on FDA approval for veterinary applications only [5,6]. Human intoxication cases have reported

bradycardia and transient hypertension followed by hypotension [7,8]. Also, sedation and reduced cardiac output were observed as an effect of norepinephrine release blocking by this alpha 2 agonist [9,10]. Its combination with heroin (opioid) is highly toxic because this opioid is a  $\mu$ (Mu) receptor agonist, that also causes sedation, hypotension and respiratory depression [1,2,4,11,12]. Concomitant use of these drugs may cause potentiation of physiological toxic effects, such as sedation, hypotension and respiratory depression by a different mechanism of action, which could cause death [13]. The use of XYL as heroin adulterant or substitute has increased in many countries, causing a great deal of concern among physicians and the National Institute on Drug Abuse (NIDA) [9,14,15]. XYL has unknown acute and chronic toxic effects, that may be important in clinical treatment and rehabilitation.

The main administration route by drug users is intravenous, gaining rapid access to the vascular system. Distribution thru the circulatory system, will deliver direct to organs such as the heart, lungs, liver and kidney, with full dose bioavailability [16,17]. Through this type of administration the vascular endothelium is continuously exposed to XYL, or its combination with COC and 6-MAM (heroin metabolite) and the effects are partially unknown. Cocaine and heroin effects in endothelial and other cells have been studied, due to the endothelium's extensive functions and implications of its damage in health, and disease development [18–28]. XYL toxic effects in the human endothelium have not been studied. Endothelial cells are involved in the exchange of nutrients, homeostasis and recovery of lesions [29,30]. Their role in mediating normal physiology is crucial for the human body [31]. Our previous studies has reported that XYL use and its combination with COC and 6-MAM triggers apoptotic death in EA.hy926[32]. These studies report that xylazine triggers ROS production as well as 6-MAM in these cells, and DNA fragmentation mainly in G0/G1 and G2/M phase [33].

Reactive species have a role in several signal transduction pathways, as signaling molecules and transcriptional regulation [34]. Significant amounts of superoxide anion are produced by the cells as part of normal functions [35–37]. Sustained high levels of ROS, implicating severe oxidative stress, could trigger tissue damage, subsequently inducing cellular death and disease [38]. In vitro studies in human neuronal progenitor cells exposed to cocaine demonstrated that high levels of oxidative stress lead to cell death. Programmed cell death, apoptosis, is used to remove selectively damaged cells [39, 40]. Dysregulated apoptosis process has been characterized in the pathophysiology of many diseases [41–43]. This uncontrolled process triggers diseases such as vasculitis and key events featured in renal disease [44]. Apoptotic cell death is regulated by either two pathways; extrinsic or intrinsic [45, 46].

In this study, we used EA.hy926 cells to identify the apoptotic pathway induced by xylazine and its combinations with COC and/or 6-MAM. These drugs and their combination were also investigated to elucidate their effect in cell cycle progression.

## MATERIALS AND METHODS

### Stock solutions and reagents

Experimental stock solutions of all drugs were prepared at concentrations of 3 mM in ethanol 70%, obtained from Sigma Aldrich, (St. Louis, MO) and were kept in sterile glass vials and stored at 4 °C. The positive control (camptothecin) cocaine and xylazine were obtained from Sigma Aldrich, and heroin metabolite (6-monoacetylmorphine) was from Cerilliant Corporation (Round Rock, Texas). DNA dye reagent (DAPI) was obtained from Chemometec (Allerød, Denmark). Green FAM FLICA k it was obtained from Immunochemistry Technologies, Bloomington, Minnesota.

### Instrumentation

Countess automated cell counter (Invitrogen, Carlsbad, California) was used for cell quantification. Analysis of cell cycle was achieved by cytometry using the Nucleo counter NC-3000 (Chemometec, Allerød, Denmark) instrument.

### Cell culture

The cell line used in this study was human umbilical vein endothelial cell (HUVEC) line Ea.hy926, kindly provided by Dr. Cora-Jean S. Edgell, from the University of North Carolina at Chapel Hill (UNC). Cells were cultured on DMEM culture media (ATCC, Manassas, Virginia) with 10% fetal bovine serum (ATCC, Manassas, Virginia) [47, 48]. These cultures were maintained at 37 °C and 5% CO<sub>2</sub>[49]. Cell viability was determined with trypan blue exclusion method, to be consistently over 90% previous to seeding cells. After monolayers reached confluence were used within 24 hours.

### Cell treatment

Cells were exposed for 24 hours to vehicle (negative control group), XYL (60 μM), COC (160 μM), 6-MAM (160 μM), and drugs at their approximated IC<sub>50</sub> (previously calculated [32]) camptothecin (positive control group, 50 μM), XYL/COC (50 μM), XYL/6-MAM (50 μM) and XYL/COC/6-MAM (40 μM). The EA.hy926 cells were cultured at a density of 5.0 x10<sup>5</sup> cells per 3.5 mL of culture media in 25 cm<sup>2</sup> flasks to assure steady metabolic state and exponential growth. Drugs treatment solutions and combinations were prepared freshly by dilution in medium, and then added to cultures.

### Caspase 8 and 9 activation assay

Caspase activation is a hallmark of apoptosis in response to drug toxicity. Activation of effector caspases is a key event in the apoptotic pathway; caspase 8 is activated by extrinsic stimuli and caspase 9 by intrinsic. These enzymes trigger many of the typical hallmarks of apoptotic cell death. This assay was performed using Fluorescent Labeled Inhibitors of Caspases (FLICA). These probes bind covalently with active specific caspase (8 or 9, individually). After treatment as described previously, cells were harvested and stained using the green FAM FLICA kit. This interaction allows cells with active Caspases 8 or 9 a green fluorescence emission, while cells with inactive caspases will present an absence of fluorescence. Camptothecin was employed as the positive control and the drug vehicle as the negative control. Following treatments, cells were rinsed with PBS, detached, centrifuged and quantified. These cells were stained for an hour following the manufacturer's instructions with slight modifications. The fluorescence was measured in fluorescence standard units (FSU) by image analysis with Nucleo counter NC3000 instrument; Nucleoview software interpreted data, as a percentage of cells.

### Cell Cycle Assessment

Determination of the effects on cell cycle progression is used as indicators of drug toxicity assessment. This assay measures stained cells, which indicate cellular DNA content related to cell cycle phase. Cells were harvested after treatment and implementing the previously defined conditions, fixed with 70% ethanol, incubated 24 hours at 4°C, stained with 500 μL of 1 μg/ml DAPI and incubated for 5 minutes, according to manufacturer's specifications and analyzed by image analysis with Nucleo counter NC3000 instrument.

### Statistical analysis

Significant changes in the cell exposed to the tested drugs

were assessed by statistical analysis, performed using one-way ANOVA, with Tukey post hoc test, where  $p < 0.05$  was considered significant. Calculated with Graphpad Prism software (v.5.03). Results were presented as mean + SD of replicates, from duplicates experiments and shown as negative control standardization (percent, %).

## RESULTS AND DISCUSSION

### Caspases 8 and 9 activation

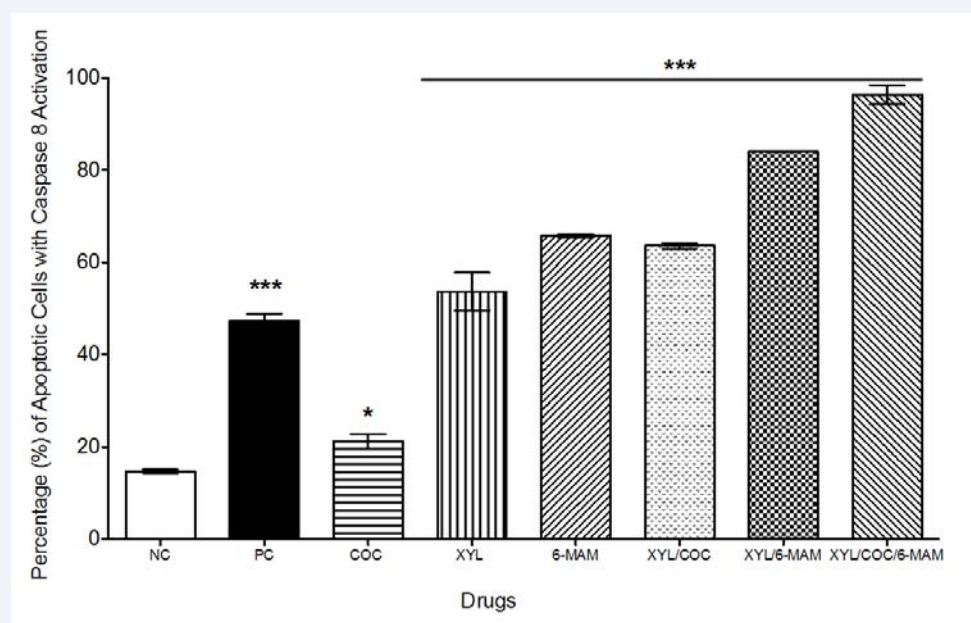
When EA.hy926 cells were exposed to all drugs and their combination at concentrations described previously, activation of caspases 8 and 9 was detected (Figure 1 and 2). Caspase 8 activation was detected at higher proportion in cells treated with 6-MAM, XYL and its combination with 6-MAM and COC, ( $P < 0.0001$ , Figure 1) when compared to the negative (NC) and positive control (PC, camptothecin), using ANOVA analysis. When comparing XYL to PC groups, a significant difference was observed ( $P < 0.001$ ), cells treated with XYL exhibited an increase in caspase 8 activation, more than PC, as well as cells treated with XYL in combination with 6-MAM and/or COC ( $P < 0.0001$ ). Meanwhile cells treated with COC presented lower caspase 8 activation than PC, but higher than NC ( $P < 0.01$ ).

Caspase 9 activation was detected in all treatments used in this study (Figure 2), showing higher activation in cells treated with 6-MAM and its combination with XYL and COC ( $P < 0.0001$ ) when compared with NC, PC, XYL, COC and XYL in combination with COC. No significant difference (ns,  $P > 0.05$ ) was observed between PC and COC treated groups. Meanwhile COC treated

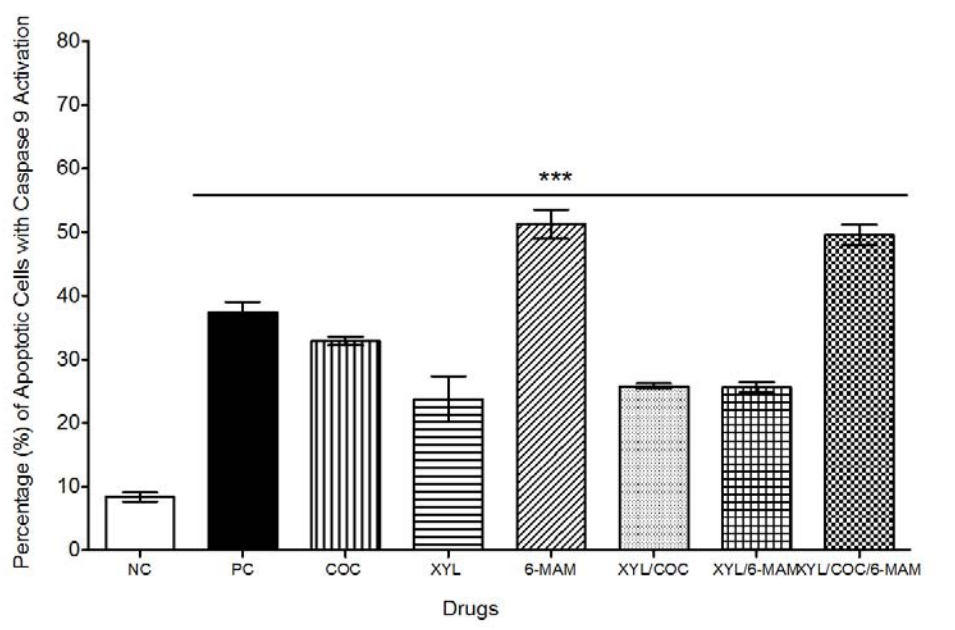
cells, presented significant higher caspase 9 activation than XYL ( $P < 0.001$ ). Cells treated with XYL and its combination with COC or 6-MAM present no significant difference in their response (ns,  $P > 0.05$ ), but higher than NC ( $P < 0.0001$ ). Cells treated with the three drug combinations present the higher caspase 8 and 9 activation, showing synergistic effect, higher than activation observed in cells treated with the drug individually, with exception of 6-MAM treated cells, which presented higher caspase 9 activation than the other drugs individually, but equal to the three drugs combination response.

### Results of Cell Cycle Assessment

When EA.hy926 cells were exposed to XYL, COC and 6-MAM and their combinations at concentrations describes previously, presents different cell cycle arrest among phases Sub-G0, G0/G1, S and G2/M, when were compared to NCgroup (Figure 3A and 3B). Sub-G0 and G0/G1 phases shows higher amount of cells arrested when were treated with COC and 6-MAM. In contrast, cells treated with XYL and its combination with COC and/or 6-MAM presented no significant ( $P > 0.05$ ) difference when compared to NC group in Sub-G0 and G0/G1 phases. Whereas cells treated with COC and 6-MAM show no significant cell cycle arrest in S and G2/M phase. However cells treated with XYL and its combination with COC and/or 6-MAM presented significant difference in cell cycle arrest ( $P < 0.0001$ ) in S and G2/M phases, when compared to NC group. No significant difference was observed in S phase among cells treated with XYL and its combination with COC or 6-MAM (ns,  $P = 0.05$ ), while cells treated with XYL in combination with COC and 6-MAM presented less cells arrested than XYL



**Figure 1** Effects of xylazine, cocaine, 6-monoacetylmorphine and their combination; on Caspase 8 activation in Human Umbilical Vein Endothelial Cells (EA.hy926). Caspases 8 activation in EA.hy926 cells after 24 h of exposure to vehicle (negative control group), xylazine (XYL, 60  $\mu$ M), cocaine (COC, 160  $\mu$ M), 6-monoacetylmorphine (6-MAM, 160  $\mu$ M), camptothecin (positive control group, 50  $\mu$ M), XYL/COC (50  $\mu$ M), XYL/6-MAM (50  $\mu$ M) and XYL/COC/6-MAM (40  $\mu$ M), were as follows: 1A; showed significant levels activation of caspases 8 in EA.hy926 cells, when exposed to XYL, COC, 6-MAM and their combinations. All values are expressed as mean  $\pm$  SD of 6–9 replicates, from 2 to 3 experiments. Statistical analysis performed was one-way ANOVA, with Tukey post hoc test, where  $p < 0.05$  was considered significant. P summary; \*\*\* significantly different from negative control group ( $P < 0.0001$ ), ns = no significant different from negative control group ( $P > 0.05$ ). The experiment was repeated for at least three times in replicate, expressed as change from negative control normalization (%).



**Figure 2** Effects of xylazine, cocaine, 6-monoacetylmorphine and their combination on caspase 9 activation in Human Umbilical Vein Endothelial Cells (EA.hy926). Caspase 9 activation in EA.hy926 cells after 24 h of exposure to vehicle (negative control group), xylazine (XYL, 60  $\mu$ M), cocaine (COC, 160  $\mu$ M), 6-monoacetylmorphine (6-MAM, 160  $\mu$ M), camptothecin (positive control group, 50  $\mu$ M), XYL/COC (50  $\mu$ M), XYL/6-MAM (50  $\mu$ M) and XYL/COC/6-MAM (40  $\mu$ M), were as follows: 2A presented activation levels of Caspases 9 in EA.hy926 cells when exposed to XYL, COC, 6-MAM and their combinations. 2B presented levels of caspases-9 activation in EA.hy926 cells when exposed to XYL, COC and 6-MAM. 2C presented levels of caspases-9 activation in cells when exposed to combination of XYL, COC and 6-MAM. All values are expressed as mean  $\pm$  SD of 6–9 replicates, from 2 to 3 experiments. Statistical analysis performed was one-way ANOVA, with Tukey post hoc test, where  $p < 0.05$  was considered significant. P summary; \*\*\* significantly different from negative control group ( $P < 0.0001$ ), ns = no significant different from negative control group ( $P > 0.05$ ). The experiment was repeated for at least three times in replicate, expressed as change from negative control normalization (%).

group ( $P < 0.001$ ), but higher than NC ( $P, 0.0001$ ). Cells treated with XYL and its combination with COC and/or 6-MAM exhibited no significant difference among their response, in G2/M phase.

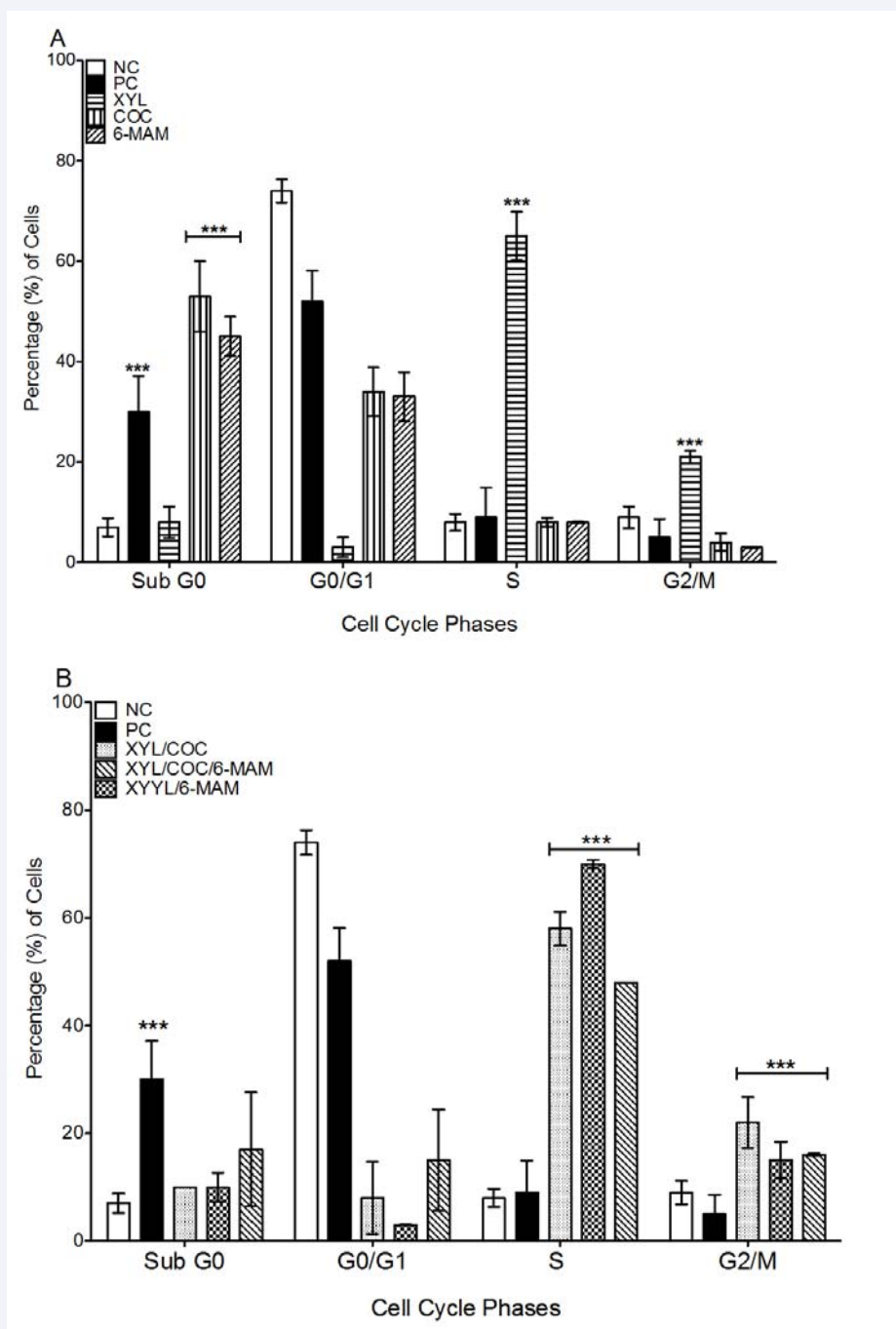
Cocaine and heroin are drugs of abuse well studied, with known harmful effects, their combination (known as speedball) is very common among addicts [50,51]. Their toxic effects in neuronal and vascular damage has been identified [21,23,24,26,51]. Meanwhile XYL has no significant studies of its toxic effects in humans. This drug belongs to a drug group known as phenothiazines, in reference to its chemical structure, [10, 52]. Phenothiazines act as antagonist of  $\alpha_2$ , dopamine and other receptors in the central nervous system (CNS), subject to chemical structure substitutions [53]. These drug groups exhibit cytotoxic effects in a diversity of non-CNS cells [54] and interact with macromolecules such as proteins, DNA and RNA [53].

Phenothiazines have been associated to cell death by apoptosis, by mitochondrial permeability induction [54]. This cellular death by apoptosis is in accordance with our previous results, obtained from cells treated with xylazine and its combination with cocaine and/or 6-MAM. Significant increase in the expression of activated caspase 8 and 9 has been detected in our study with EA.hy926 cells. This data suggests activation of both pathways in apoptotic cellular death process. Stress signals from inside the cells could activate intrinsic pathway, were caspase-9 is activated, and subsequently caspase-3 and poly ADP-ribose polymerase (PARP) activation. Moreover extrinsic

pathway induction activates caspase-8, which successively activates downstream effectors, that likewise include caspases-3 and PARP [46, 55].

Xylazine apoptotic cell death induction in endothelial cells is significant since the endothelium is the regulator of vascular tone, an active participant in hemostasis, cellular proliferation, inflammation, and immunity [24]. Drugs, xenobiotic compounds and chemical agents could interact with molecular targets expressed on membranes, inducing endothelial cell toxicity and initiating a signaling cascade. Consequently, lesions in vascular tissue, skin and vital organs could be developed [16]. Recently, disproportionate apoptotic death of endothelial cells have been related to vascular injury, in human studies, characterized by inflammation, neutrophil infiltration and lamina breaking [24, 31, 56]. Drug-induced vascular injury is a great concern in clinical toxicology; due to the extension in their function and potential systemic impact [16, 31, 44, 57].

Moreover cell cycle is regulated by entry checkpoints into each phase [58–60], cells entrance in G1 phase, followed by S phase (DNA replication) [61]. Subsequent to S phase, cells enter to G2 phase, where DNA repair and protein synthesis occurs and M phase is where separation of progenitor and daughter cells is achieved. DNA damage manifestation in these checkpoints mainly appears in late in G1 and G2 phase. This DNA damage could trigger [59, 61], cell cycle arrest in G0/G1 and/or G2/M phase and might be related to the apoptotic process, adduct formation



**Figure 3** Effects of xylazine, cocaine, 6-monoacetylmorphine and their combination on cell cycle in Human Umbilical Vein Endothelial Cells (EA.hy926). EA.hy926 cells after 24 h of exposure to vehicle (negative control group, NC), camptothecin (positive control group, PC, 50  $\mu$ M), xylazine (XYL, 60  $\mu$ M), cocaine (COC, 160  $\mu$ M), 6-monoacetylmorphine (6-MAM, 160  $\mu$ M), XYL/COC (50  $\mu$ M), XYL/6-MAM (50  $\mu$ M), and XYL/COC/6-MAM (40  $\mu$ M), were evaluated and compared to negative control group. Cell cycle assay was performed to measure cells containing less DNA dye with DAPI [65]. Cells were harvested after treatment, fixed with 70% ethanol, incubated 24 hours at 40C, stained with 500  $\mu$ L of 1  $\mu$ g/ml DAPI (5 min. incubation) and image analyzed with Nucleo counter NC3000 instrument. Results obtained when EA.hy926 cells were exposed to XYL, COC and 6-MAM and their combinations at concentrations described previously, presents different cell cycle arrestment among phases Sub-G0, G0/G1, S and G2/M, when compared to the negative control group (Figure 3A and 3B). Phase Sub-G0 shows higher amount of cells arrested when treated with COC and 6-MAM (Figure 3C). Phase G0/G1 shows no cell cycle arrest with significant difference ( $P > 0.05$ ) in cells treated with drugs combinations (Figure 3A and 3B). Cells exposed to XYL and its combination with COC and/or 6-MAM presented cycle arrest with significant difference ( $P < 0.0001$ ) in Phase S (Figure 3D and 3E). Similar results were observed in G2/M phase (Figure 3F and 3G). All values are expressed as mean  $\pm$  SD of 6–9 replicates, from 2 to 3 experiments.. Statistical analysis performed was one-way ANOVA, with Tukey post hoc test, where  $p < 0.05$  was considered significant. P summary; \*\*\*  $P < 0.0001$ , \*\*  $P < 0.001$ , \*  $P < 0.01$  significantly different, ns = no significant difference  $p > 0.05$ , when compared to negative control group.

The experiment was repeated for at least three times in replicate, expressed as change from negative control normalization (%)

and by reactive species interaction. The assay employed to evaluate cell cycle allows DNA content can be measured using fluorescent dye (DAPI); emission signals exhibited by DNA-selective stains are proportional to DNA mass. Our results indicate that EA.hy926 cells treated with XYL and its combination with COC and/or 6-MAM induce significant cell cycle arrest in G2/M and S phases; in contrast to cells treated with COC or 6-MAM that presented significant cell cycle arrest only at Sub-G0 Phase. Cell cycle arrest in S phase could be associated to mitochondrial membrane potential disruption, leading to cytochrome c release, and subsequently apoptotic cell death, which has been observed by previous research [50, 62–65]. This phase is highly regulated by cyclin dependent kinases and other proteins. Meanwhile cell cycle arrest in G2/M could be related to a secondary necrosis event, resulting from severe and acute mitochondrial dysfunction that initially activates apoptosis. Additionally, it could be associated to inhibition, activation or phosphorylation of proteins such as Cdc2, p35, Chk1 and Chk2 kinases, as well as other proteins [63, 66]. Molecular mechanism underlying XYL, and its combination with COC and/or 6-MAM, effects in the cell cycle still unknown, but is part of our future plans. Our previous work and this study are among the first research related to XYL molecular effects in human cells. Previous studies of COC and 6-MAM or heroin and their combination (speedball) concur with our findings, cell cycle arrest in Sub-G0 and G0/G1 phase presented in cells treated with COC or 6-MAM, a typical apoptotic response and has been related to mitochondrial disruption, Bcl-2 proteins cleavage and cytochrome c release, among other mechanisms, [21, 23, 27, 67, 68, 62, 69].

## CONCLUSION

This study reports primarily the pathways involved in the induction of apoptosis by xylazine and its combination with cocaine and/or 6-monoacetylmorphine on the EA.hy926, human umbilical vein endothelial cell line. Our results demonstrate that the effects of all drugs tested in this study are comparable to those induced by camptothecin (positive control) activating both pathways in apoptotic cell death. In addition this study identified caspase 8 and 9 activation as important intermediate steps involved in apoptotic cell death. Drug combination treatments effects are similar to those induced by drugs individually. Cell cycle assessment shows that effects of COC and 6-MAM treatments were observed in Sub-G0 and G0/G1 phase. Cells treated with XYL, and its combinations with COC and/or 6-MAM show effects mainly in S and G2/M phases.

Our study has contributed to understanding the mechanisms involved in xylazine toxicity alone and in combination with cocaine and/or 6-monoacetylmorphine on endothelial cells. Relationship among xylazine abuse and impairment of the endothelial barrier functions has been demonstrated in this study. This is an essential event in the pathological processes in a broad variety of diseases. Further, potential health risk in addict population could be suggested, once clinically monitored and documented, in users of xylazine and its combination with speedball modality.

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