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MDMA and tramadol or tapentadol co-administration causes serotonin-independent neurological damage

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Abstract: The simultaneous consumption and misuse of different drugs, including 3,4-methylenedioxymethamphetamine (MDMA, "ecstasy") and opioids, raise concerns about fatal intoxications, as a result of increased serotonin levels. However, research on the simultaneous consumption of MDMA and opioids is limited. This study aimed to evaluate the toxic effect of the simultaneous exposure of the undifferentiated human SH-SY5Y neuroblastoma cell line to MDMA and tramadol or MDMA and tapentadol, focusing on serotonin toxicity. After a 48-hour exposure to the mixtures, there was a significant decrease in cell viability linked to an increase in oxidative stress, alterations in mitochondrial membrane potential, and an increase in caspase-3 activity. Additionally, increased intracellular glucose levels and changes in the expression of enzymes involved in energetic metabolism were observed. However, serotonin levels did not significantly increase compared with the exposure to MDMA alone. Tramadol or tapentadol exposure did not cause a significant increase in serotonin levels compared with non-treated cells. Then, serotonergic toxicity may not be associated with the damage observed. Further studies are needed to better understand the toxicity deriving from the simultaneous exposure to these drugs. The results additionally underlined the need for a careful prescription of tramadol and tapentadol.

Keywords: MDMA; tramadol; tapentadol; serotonin; *in vitro*; acute neurotoxicity

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Introduction

Serotonin syndrome (SS) is a consequence of an increase in intrasynaptic serotonin (5-HT) levels in the central nervous system (CNS). The increase in 5-HT levels leads to overactive central and peripheral 5-HT receptors, particularly 5-HT_{1A} and 5-HT_{2A} receptors [1]. The 5-HT increase is a result of the intake of prescription medication and psychoactive substances, such as tramadol, antidepressants, and 3,4-methylenedioxymethamphetamine (MDMA or "ecstasy") [1-4]. SS diagnosis is established by assessing the serotonergic drugs used by the patient and through the application of "The Hunter Serotonin Toxicity Criteria", concerning altered mental status, hypertonicity, and maximum temperature > 38 °C [1,5].
MDMA is a widely used recreational psychoactive substance, with several neurotoxic effects in humans, which may be associated with serotonergic and dopaminergic systems [6-12]. MDMA consumption,

when in combination with other serotonergic drugs, has been associated with fatal SS cases [2,11]. MDMA users often concomitantly consume several other drugs, such as ethanol, tobacco, lysergic acid diethylamide (LSD), *Cannabis*, and opioids [9,13-18]. There are few studies on the simultaneous consumption of opioids with MDMA, although some consumers report opioid use to "come down" from ecstasy or to minimize dysphoria following administration [13,19]. Pilgrim *et al.* studied fatalities involving the use of 5-HT-active licit drugs, with tramadol being the most frequently reported drug in this context, having often been detected in combination with antidepressants [3]. In addition, in another study by Pilgrim *et al.*, 1,123 fatalities were analyzed, and one or more 5-HT-active licit and illicit drugs such as tramadol, venlafaxine, fluoxetine, sertraline, citalopram, paroxetine, and MDMA, were detected [2]. In this study, of these seven drugs, tramadol was the most frequently consumed, and MDMA was also often detected [2]. In this context, co-administration of tramadol and MDMA may increase the risk of SS, given the increased risk of 5-HT accumulation in the CNS. Therefore, more studies should be performed to evaluate the effects of tramadol and MDMA combination. In turn, Franco *et al.* reported a fatal intoxication by tapentadol, suggesting respiratory depression, CNS depression, and serotonin syndrome as possible death-triggering events [20]. Additionally, although tapentadol serotonergic activity is much more limited than its norepinephrinergic action [4,21,22], Walczyk *et al.* also reported a case of probable SS after tapentadol overdose, in combination with other medications, in particular duloxetine and amitriptyline [23]. By telephone, these authors contacted Janssen Pharmaceuticals, a tapentadol manufacturer, to inquire if SS had been implied as a consequence of tapentadol intake [23]. The manufacturer's senior therapeutic specialist replied that no cases were reported in phase 2 and 3 clinical trials; nonetheless, in post-marketing, SS had been reported in patients using tapentadol combined with other serotonergic drugs [23]. In this sense, further studies are needed to assess whether tapentadol, combined with other drugs, may cause SS.

Therefore, the present work aimed to evaluate the toxicological effects of the exposure to tramadol or tapentadol, in combination with MDMA, in an *in vitro* human neuronal model, the SH-SY5Y cell line, to comparatively analyze cell viability, oxidative stress, mitochondrial alterations, as well as to determine if 5-HT release and serotonin toxicity are involved in cellular damage.

Materials and Methods

Cell culture

The human neuroblastoma cell line SH-SY5Y (ATCC) was cultured in complete growth medium containing Dulbecco's Modified Eagle's Medium (DMEM) with 1% (v/v) GlutaMAX™ and 4.5 g/L glucose (Lonza), supplemented with 10% (v/v) Fetal Bovine Serum (FBS, Gibco Invitrogen), 1% Non-Essential Amino Acids (NEAA, Gibco Invitrogen), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich).

SH-SY5Y cells were maintained in a humidified incubator (Heraeus Hera Cell) at 37 °C and a 5% CO_2 atmosphere. Cells were regularly subcultured through trypsinization [Trypsin EDTA 1× (ScienCell)] to new flasks. They were seeded into 6-, 24-, or 96-well plates and incubated for 24 hours in the humidified incubator before each experiment.

Drug preparation and exposure

Stock solutions of tramadol hydrochloride (Sigma-Aldrich), tapentadol hydrochloride (Deltaclon), and MDMA hydrochloride (Lipomed) were prepared in sterile distilled water and stored at -20 °C.

To induce toxic effects, high doses of tramadol, tapentadol, and MDMA were selected. Doses of 600 µM and 200 µM were selected for tramadol and tapentadol, respectively, considering previous results from our group [24]. MDMA dose was defined according to doses used by other authors [6,7,25-27]; concentrations up to 1 mM were tested in preliminary cytotoxicity assays, and then 1 mM MDMA was selected for all subsequent assays.

Cytotoxicity assays

The sulforhodamine B (SRB) assay was used as an indicator of cell density based on the measurement of cellular protein content, as previously described [24]. Cells were seeded into 24-well plates (6×10^4 cells/well) in a growth medium containing 5% FBS and lacking the antibiotic/antimycotic mixture, and exposed to tramadol, tapentadol, and MDMA. Wells without cells and with media containing the compounds were used as blanks, and non-exposed cells were used as controls. Before cell exposure, an additional plate (T0 plate) was prepared to determine the number of cells present when the compound(s) was(were) added. The mean T0 absorbance was subtracted from the absorbance value of each well upon exposure.

After incubation with 1 mM MDMA, 600 µM tramadol, 200 µM tapentadol, 1 mM MDMA + 600 µM tramadol or 1 mM MDMA + 200 µM tapentadol, for 48 hours, cells were fixed with 50 µl 50% (w/v) ice-cold trichloroacetic acid, by incubating 1 hour at 4 °C. Then, the plates were washed 5 times with deionized water and air-dried at room temperature. Cell proteins were then stained with 50 µl 0.4% (w/v) SRB (Sigma-Aldrich) in 1% (v/v) acetic acid, for 30 minutes; subsequently, the plates were washed 5 times with 1% (v/v) acetic acid. After drying at room temperature, protein/SRB complexes were

solubilized with 100 µl 10 mM Tris buffer/well. Absorbance was read at 515 nm in a plate reader (Biotek Synergy 2).
The results from 5 independent assays (each performed at least in triplicate) are expressed as the percentage of control cell viability.

Cell death analysis

Apoptosis detection through annexin V-FITC labeling

Cell death was analyzed through annexin V-FITC and propidium iodide (PI) labeling (annexin V-FITC Detection Kit, Biotool). Cells were plated in 6-well plates (2×10^5 cells/well), with coverslips, and exposed to tramadol, tapentadol, and MDMA. After exposure, cells were washed with cold PBS, and then the coverslips were incubated in the dark, at room temperature, for 15 minutes with a mixture of 5 µl annexin V-FITC, 5 µl PI and 90 µl binding buffer. Cells were then washed with PBS for 5 minutes. Finally, coverslips were mounted in 4',6-diamidino-2-phenylindole (DAPI). Non-treated cells were used as negative controls, and cells treated with 50 µM H_2O_2 were used as positive controls.
The coverslips were analysed under fluorescence microscopy in a Spinning Disc AxioObserver Z.1 SD microscope (Zeiss), equipped with an AxioCam MR3 camera. Photos were taken from representative fields using the AxioVision 4.8.2 software.

Caspase-3 activity determination

Cells were seeded into 6-well plates (2×10^5 cells/well), in complete growth medium, and then exposed to tramadol or tapentadol, alone or in combination with MDMA, for 48 hours. At the end of the exposure, cells were washed with PBS and then lysed with 150 µl Glo Lysis Buffer (Promega) for 5 minutes (at room temperature). The lysates were stored at -20 °C.
Caspase-3 activity was determined as described by Faria *et al.* [24] using caspase-3 peptide substrate Ac-DEVD-pNA (Sigma-Aldrich) (to a final concentration of 80 µM), followed by incubation at 37 °C for 24 hours, and measuring absorbance at 405 nm in a microplate reader (Biotek Synergy 2). The absorbance of blanks, used as non-enzymatic controls, was subtracted from each absorbance value. Results were normalized against protein content. All results were also normalized against the control group (non-exposed cells).

Measurement of reactive oxygen species production

The reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced intracellularly were quantified by a fluorescence method, using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), as described by Barbosa *et al.* [7]. DCFH-DA is a lipophilic probe that penetrates the cells, producing 2',7'-dichlorodihydrofluorescein (DCFH) after enzymatic hydrolysis. DCFH reacts with intracellular ROS and RNS, producing a fluorescent compound, 2',7'-dichlorodihydrofluorescein (DCF). A 4 mM DCFH-DA stock solution was prepared in DMSO (Sigma) and diluted in culture media before each assay (ensuring that the final concentration of DMSO did not exceed 0.5% (v/v)).
Cells were seeded into 96-well black plates (Greiner CELLSTAR®, Sigma) (1.5×10^4 cells/well). Prior to the exposure to tramadol, tapentadol, or MDMA, cells were rinsed twice with Hank's Balanced Salt Solution (HBSS) and then incubated with 10 µM DCFH-DA (Sigma) in culture medium for 30 minutes, at 37 °C, regarding intracellular accumulation. After DCFH-DA incubation, cells were washed again twice with HBSS and then incubated in 200 µL complete grow medium with tramadol, tapentadol, or MDMA, for 48 hours. Fluorescence intensities were determined using a microplate reader (Biotek Synergy 2), at 485 nm excitation and 530 nm emission. The absorbance of blanks (without cells) was subtracted from each absorbance value. A negative control (cells lacking compound) was also included in the plate.
Results were expressed as the DCF fluorescence percentage of the control (cells that had not been exposed to tramadol, tapentadol, or MDMA) from 5 independent assays, tested in triplicate within each assay.

Mitochondrial membrane potential ($\Delta\psi m$) assessment

Mitochondrial integrity was evaluated by measuring tetramethylrhodamine ethyl ester perchlorate (TMRE) inclusion, as previously reported [28]. Cells were seeded in complete growth medium in 96-well black plates (Greiner CELLSTAR®, Sigma) (1.5×10^4 cells/well) and exposed to the compounds for 48 hours. Following exposure, the medium was removed and fresh medium, containing 2 mM TMRE (Sigma-Aldrich), was added. Cells were incubated for 30 minutes at 37 °C in the dark. After incubation, the medium was removed and 0.2% bovine serum albumin (BSA) in HBSS was added to each well. Fluorescence intensity was measured using a microplate reader (Biotek Synergy 2), at 544 nm excitation and 590 nm emission. Cells lacking compound exposure were used as controls. The results were expressed as the percentage of the control from 5 independent assays, with each condition tested in triplicate.

Gene expression analysis through quantitative real-time polymerase chain reaction (qRT-PCR)

RNA extraction, cDNA synthesis, and qRT-PCR analysis were performed as described elsewhere [24]. Total RNA was extracted from cells growing in 6-well plates (2×10^5 cells/well), using 500 µl PureZol (Bio-Rad)/well, according to the manufacturer's directions. The High-Capacity cDNA Reverse

Transcriptase kit (Applied Biosystems) was used for cDNA synthesis, using 1 µg total RNA. qRT-PCR was performed with SYBR Green mix (Bio-Rad). The PCR amplification program involved an initial denaturation step (95 °C for 3 minutes), followed by 45 denaturation cycles at 95 °C for 10 seconds, primer annealing at 52 °C for 20 seconds, extension at 72 °C for 20 seconds, and plate reads. After the cycling steps, a melt curve was performed from 55 °C to 95 °C, with 0.5 °C increments for 5 seconds, followed by a plate read.
The results were normalized with 18S ribosomal RNA as a housekeeping gene. The effect of the compounds on the expression of different genes (Fe-S protein 1 of NADH dehydrogenase (NDUFS1), creatine kinase B (CKB), 5-HT_{1A} and 5-HT_{2A} 5-HT receptors) is presented in comparison with the expression in non-treated control cells. The nucleotide sequences of the primers used are provided in Table 1.

Table 1. Nucleotide sequences (5' → 3') of the primers used for qRT-PCR quantification of gene expression upon exposure of SH-SY5Y cells to tramadol, tapentadol, and MDMA.

Gene	Forward Primer	Reverse Primer	Reference
NDUFS1 (Fe-S protein 1 of NADH dehydrogenase)	TCGGATGACTAGTGGTGTTA	TTATAGCCAAGGTCCAAAGC	[29]
CKB (creatine kinase B)	CCTTCTCCAACAGCCACAAC	CAGCTCCGCGTACAGCTC	[30]
5-HT_{1A} (5-HT receptor)	TCATCGTGGCTCTTGTTCTG	CGGGGTTAAGCAGAGAGTTG	[31]
5-HT_{2A} (5-HT receptor)	GTTGCTTACTCGCCGAT	TGCCAAGATCACTTACACACAAA	[31]
18S rRNA (18S ribosomal RNA)	CAACATCGATGGGCGGCGGA	CCCGCCCTCTTGGTGAGGTC	[24]

Intracellular glucose and lactate quantification
Cell lysates were prepared as described in the "Caspase-3 activity determination" section. Glucose, lactate and protein intracellular levels were determined by using the Prestige 24i automated analyzer (Tokyo Boeki) as previously described [32], using two appropriate calibrators and a quality control. Glucose and lactate concentration results were normalized against the total protein content and the respective controls (non-exposed cells).

Serotonin release quantification
Cells were seeded into 24-well plates (6×10^4 cells/well) in a complete growth medium, and exposed to tramadol, tapentadol, and MDMA. After a 48-hour exposure, the medium was recovered regarding 5-HT quantification. Cell lysates were prepared as reported in the "Caspase-3 activity determination" section. The culture medium and the lysates were stored at -20 °C. Non-exposed cells were used as controls.
5-HT concentration in the medium was determined using an ELISA Kit (Serotonin ELISA kit, Enzo Life Sciences), as per the manufacturer's directions. Six different standards (with serotonin concentrations ranging from 0.49 to 500 ng/mL) were run in duplicate to plot a calibration curve. 5-HT levels were normalized against the protein content of each sample, as determined with the Prestige 24i automated analyzer (Tokyo Boeki), according to the manufacturer's instructions.

Statistical analysis
Results were expressed as means ± SD. All studies comprised at least 6 replicates performed in 3 independent assays. All analyses and graphics were plotted using GraphPad Prism® version 7.0a (GraphPad Software, LLC). Statistical comparisons between groups were performed as an Analysis of Variance (ANOVA), followed by post-hoc analysis through Dunnett's multiple comparisons test. Probability values of $p < 0.05$ were considered statistically significant.

Results

MDMA/tramadol and MDMA/tapentadol mixtures increase toxicity compared with each opioid alone
SH-SY5Y was used as a cellular model for tramadol, tapentadol, and MDMA exposure assays. The cells were exposed to high doses of these compounds to induce toxic effects. Therefore, we tested, in preliminary assays, different MDMA doses up to 1 mM (data not shown) and, because it was sufficient

to elicit toxic effects, the 1 mM dose was selected. Tramadol and tapentadol toxicity-inducing doses, previously determined as described in the "Drug preparation and exposure" section, were selected. The selection of doses was guided by data from reported cases of tramadol and tapentadol intoxication [33-35], doses commonly used in various *in vitro* studies on opioid exposure [36,37], and findings from our previous experiments with tramadol and tapentadol using the same cellular model [24]. SH-SY5Y cells were exposed to MDMA (1 mM), tramadol (600 μM), tapentadol (200 μM), or to the mixtures of MDMA (1 mM) with tramadol (600 μM) or MDMA (1 mM) with tapentadol (200 μM), for 48 h. Upon exposure, the toxic effects were evaluated by measuring cellular protein content, an indicator of cell density, through SRB assays. A single exposure to MDMA, tramadol or tapentadol, at the selected doses, causes a significant decrease in cell viability (Fig. 1).

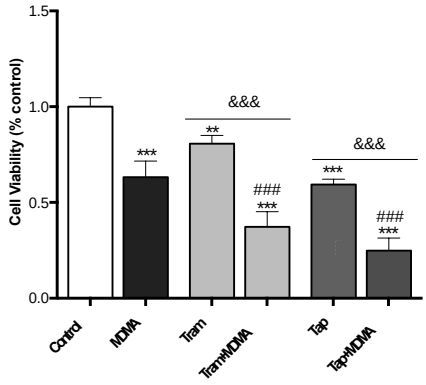

Figure 1. Cell viability, as assessed through SRB assays, upon exposure of SH-SY5Y cells to 1 mM MDMA, 600 μM tramadol (Tram), or 200 μM tapentadol (Tap) for 48 hours. Results were normalized against the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD from at least five independent assays, with each condition tested in triplicate within each experiment. ***$p < 0.001$, **$p < 0.01$ compound vs. control; $^{###}p < 0.001$ mixture effect vs. MDMA effect; $^{&&&}p < 0.001$ mixture effect vs. tramadol or tapentadol effects.

Exposure to MDMA/tramadol and MDMA/tapentadol mixtures increases necrosis and apoptosis

Given the toxic effects observed in SRB assays, and to evaluate the underlying mechanisms of toxicity and cell death, annexin V-FITC labeling was performed, and caspase-3 activity was determined. Annexin V-FITC labeling (Fig. 2A) evidenced cell death mainly through necrosis (red staining) in all conditions. In this assay, cells treated with H_2O_2 were used as a positive control for cell death (data not shown). Nonetheless, the exposure to the mixtures also led to an increase in caspase-3 activity (Fig. 2B).

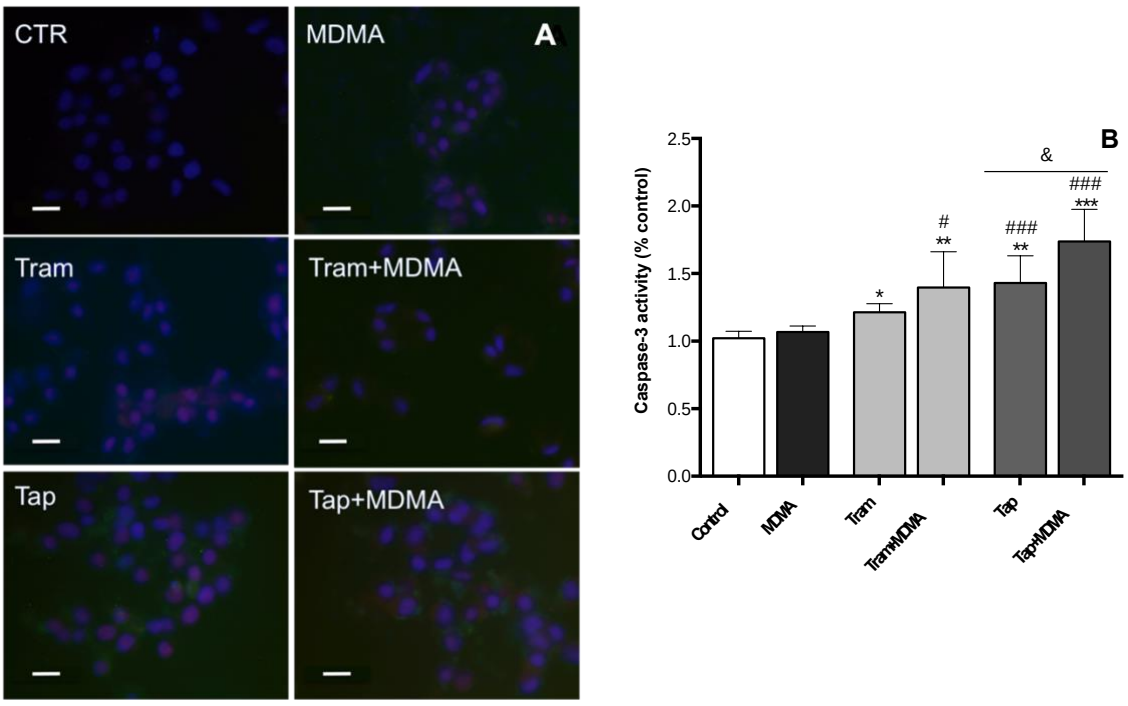

Figure 2. Annexin V-FITC (A) labeling and caspase-3 activity (B) in SH-SY5Y cells upon exposure to 1 mM MDMA, 600 μM tramadol (Tram), or 200 μM tapentadol (Tap) for 48 hours. (A) DNA was stained with DAPI,

annexin V-FITC was stained green, and necrotic cells were stained with propidium iodide. Scale bar, 20 μm. (B) Caspase-3 activity quantifications were normalized against the protein content and the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD, reflecting at least six replicates performed in three independent assays. ***$p < 0.001$, **$p < 0.01$, *$p < 0.1$ compound vs. control; $^{###}p < 0.001$, $^{#}p < 0.1$ mixture effect vs. MDMA effect; $^{&}p < 0.1$ mixture effect vs. tramadol or tapentadol effects.

To assess whether these drugs lead to an increase in oxidative stress, ROS/RNS levels were determined, and an increase in oxidative stress was observed when cells were exposed to the MDMA/tramadol or MDMA/tapentadol mixtures, compared with the exposure to each compound alone (Fig. 3A). As shown in Fig. 3B, the exposure to the mixtures also caused mitochondrial damage, resulting in a decrease in mitochondrial integrity. These results are in line with those of caspase-3 activity, suggesting that, upon exposure to the mixtures of MDMA and tramadol or tapentadol, apoptosis increases, while necrosis is the main mechanism of cell death in single exposures.

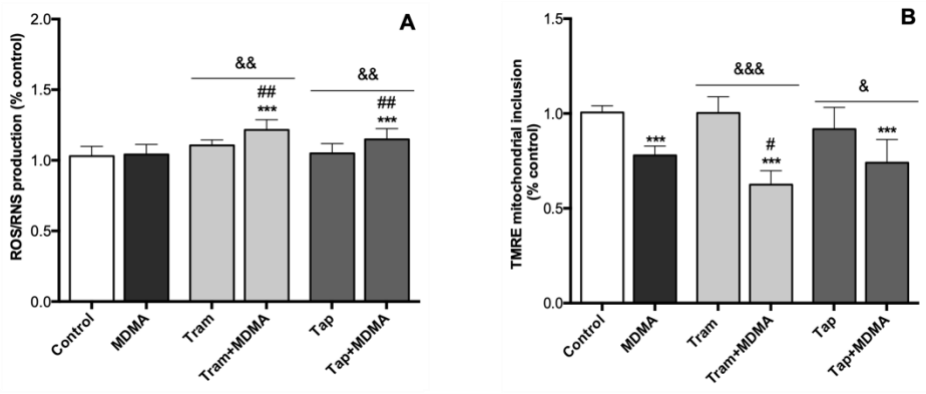

Figure 3. Effect of 1 mM MDMA, 600 μM tramadol (Tram) or 200 μM tapentadol (Tap) on reactive oxygen and nitrogen species (ROS and RNS) production (A) and mitochondrial membrane potential (B) in SH-SY5Y cells, after 48 hour-incubations. ROS/RNS were measured as the fluorescence emitted when 2',7'-dichlorodihydrofluorescein (DCFH) reacts with ROS and RNS. Mitochondrial membrane potential was measured as the tetramethylrhodamine ethyl ester perchlorate (TMRE) incorporation into mitochondria. The results were normalized against the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD, reflecting at least five independent assays run in triplicate. ***$p < 0.001$ compound vs. control; $^{###}p < 0.001$, $^{#}p < 0.1$ mixture effect vs. MDMA effect; $^{&&&}p < 0.001$, $^{&&}p < 0.01$, $^{&}p < 0.1$ mixture effect vs. tramadol or tapentadol effects.

MDMA/tramadol and MDMA/tapentadol mixtures cause alterations in gene expression and biochemical parameters

To assess the mechanisms implied in mitochondrial dysfunction, NDUFS1 and CKB expression levels were determined. After exposure to 1 mM MDMA, no significant alterations in NDUFS1 gene expression were observed (Fig. 4). However, the exposure to tramadol (600 μM) or tapentadol (200 μM) caused a significant decrease in NDUFS1 expression, with MDMA and tramadol or tapentadol mixtures further accentuating such decrease (Fig. 4). CKB expression levels decreased in all conditions (Fig. 4), with the highest decrease being observed in cells exposed to the MDMA/tramadol mixture.

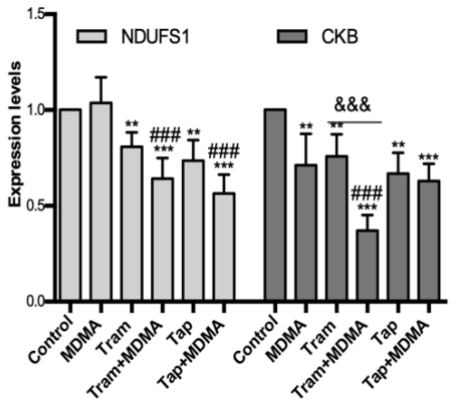

Figure 4. Relative NDUFS1 and CKB gene expression levels upon exposure of SH-SY5Y cells to 1 mM MDMA, 600 μM tramadol (Tram), or 200 μM tapentadol (Tap) for 48 hours. Gene expression levels were normalized against rRNA 18S gene expression and against the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD, reflecting at least three independent assays. ***$p < 0.001$, **$p < 0.01$ compound vs. control; $^{###}p <$

0.001 mixture effect vs. MDMA effect; $^{\&\&\&}p < 0.001$ mixture effect vs. tramadol or tapentadol effects. CKB: creatine kinase B; NDUFS1: Fe-S protein 1 of NADH dehydrogenase.

Intracellular glucose and lactate were also quantified to evaluate alterations in these metabolic parameters (Figs 5A, 5B). Glucose levels increased in all conditions, and the highest increase was observed after exposure to MDMA, alone or in combination with tramadol or tapentadol (Fig. 5A). No cumulative effects were observed, since there were no significant differences between the results from the exposure to MDMA alone and MDMA combined with each opioid. No significant differences in lactate levels were found after MDMA treatment (Fig. 5B). However, treatments with tramadol, tapentadol, or MDMA/tapentadol mixture caused a significant increase in intracellular lactate levels (Fig. 5B).

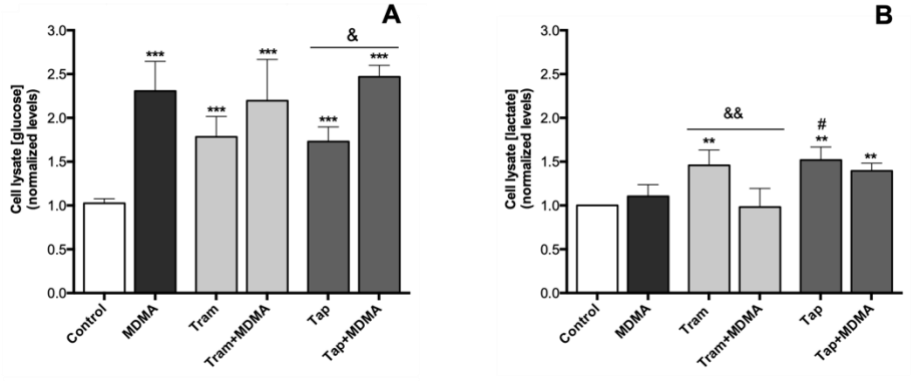

Figure 5. Glucose (A) and lactate (B) intracellular levels upon exposure of SH-SY5Y cells to 1 mM MDMA, 600 μM tramadol (Tram) or 200 μM tapentadol (Tap) for 48 hours. Results were normalized against protein content and against the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD, reflecting at least three independent assays. $^{***}p < 0.001$, $^{**}p < 0.01$ compound vs. control; $^{\#}p < 0.1$ mixture effect vs. MDMA effect; $^{\&\&}p < 0.01$, $^{\&}p < 0.1$ mixture effect vs. tramadol or tapentadol effects.

MDMA/tramadol and MDMA/tapentadol mixtures do not increase serotonin levels in SH-SY5Y culture medium when compared with MDMA alone

To evaluate the effects of MDMA, tramadol, and tapentadol on 5-HT levels, this neurotransmitter was quantified in culture medium samples (Fig. 6). In a single exposure, only MDMA caused a significant increase in 5-HT levels. After exposure to the mixtures, no differences were observed when compared with a single exposure to MDMA, with tramadol causing similar effects to tapentadol in combination with MDMA. In this context, the SH-SY5Y cell line was validated as an experimental model to study the 5-HT component since, besides releasing 5-HT, it expresses the 5-HT receptor genes 5-HT_{1A} and 5-HT_{2A}, with no changes in their expression levels upon exposure to the different drugs (data not shown).

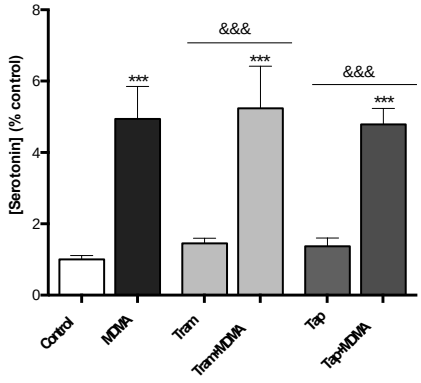

Figure 6. Serotonin levels in culture media samples after exposure of SH-SY5Y cells to 1 mM MDMA, 600 μM tramadol (Tram) or 200 μM tapentadol (Tap) for 48 hours. Serotonin levels were normalized against the protein content of the corresponding extract. They were also normalized against the value obtained in non-treated cells, set as 1. Results are expressed as means ± SD, reflecting at least six replicates performed in three independent assays. $^{***}p < 0.001$ compound vs. control; $^{\&\&\&}p < 0.001$ mixture effect vs. tramadol or tapentadol effects.

Discussion

The simultaneous consumption of opioids with other drugs is an increasing trend [18]. In a study performed with a sample of 2,000 Iranian adolescents, the adjusted odds ratio for the combined consumption of tramadol and MDMA, among the sample of tramadol misusers, was 8.9 (2.7–29.4) [38].

The authors concluded that tramadol could be a related factor or co-factor for alcohol, *Cannabis*, and MDMA abuse within the adolescent population [38]. Despite the higher incidence of simultaneous consumption of multiple drugs, the underlying toxic effects and corresponding cellular mechanisms are not yet completely understood. Therefore, in the present work, we evaluated the potential increase in MDMA toxicity when consumed in combination with tramadol or tapentadol. To this purpose, the undifferentiated neuronal cell line SH-SY5Y was used as an *in vitro* model. This cell line is commonly used in neurotoxicity studies since it presents several biochemical and functional features from neurons [24,39-42]. Moreover, when undifferentiated, SH-SY5Y cells produce different neurotransmitters, such as noradrenaline (NA), acetylcholine, glutamate, and 5-HT [43]. However, when SH-SY5Y cells are differentiated with retinoic acid, they develop a dopaminergic phenotype, characterized by an increase in dopamine levels and decreased levels of other neurotransmitters, such as 5-HT and NA [43]. Given this background, in the present work, we used undifferentiated cells, to study the potential serotonergic effect after drug exposure.

Under what we previously reported, SH-SY5Y exposure to tramadol and tapentadol caused a decrease in cell viability, with tapentadol causing the highest decrease [24]. When cells were exposed to the MDMA/tramadol or MDMA/tapentadol mixtures, a higher reduction in cell viability was observed, when compared with the exposure to each compound alone. To understand the higher toxicity observed after exposure to the mixtures, cell death mechanisms and oxidative stress were assessed. Our results showed that necrosis is the predominant mechanism of cell death in all conditions tested, although apoptosis increases for combined exposures. Treatment with tramadol or tapentadol alone was not expected to significantly increase apoptosis, as a previous study using the same cell model demonstrated weak caspase-3 activation [24]. Additionally, Annexin V-FITC labeling results suggested necrosis as the predominant mechanism of cell death, although the results in Fig. 2 suggest that apoptosis may also be involved. Concerning MDMA exposure, and in agreement with other studies [25], the exposure of differentiated SH-SY5Y cells did not cause statistically significant changes in the activity of caspase-3. Also, some authors suggest that, at higher concentrations of MDMA and MDMA metabolites, necrosis is the main mechanism of cell death, while apoptosis is the major event at lower concentrations [6]. Furthermore, undifferentiated SH-SY5Y cells, after treatment with *N*-methyl-α-methyldopamine (*N*-Me-α-MeDA) (0.1–1 mM), showed an increase in cell death through necrosis, with increased necrotic cell death having been suggested as a consequence of oxidative stress [44]. However, apoptosis is also a possible cause of MDMA-induced cell death. *In vitro* studies with MDMA reported apoptosis induction in rat cortical and cerebellar granule neurons [45]. In fact, caspase-3 activation, increased ROS and intracellular calcium levels, as well as MDMA metabolites, were associated with MDMA toxic effects [6,7,27,46]. Therefore, different concentrations, as well as different cell models, might be associated with different mechanisms of cell death [47,48]. In addition to cell death mechanisms, oxidative stress, a major contributor to cell death, was also assessed. After MDMA exposure, we did not observe an increase in ROS/RNS production, and the exposure to tramadol or tapentadol alone also did not cause an increase in oxidative stress damage, consistently with previous reports by our group [24]. Nonetheless, the MDMA and tramadol or tapentadol mixtures caused a significant increase in caspase-3 activity, ROS/RNS production, and necrotic cells, with ROS/RNS levels being higher under conditions for which caspase-3 activity was also higher. When 5-HT and dopamine are released after MDMA exposure, they are oxidatively deaminated by monoamine oxidase (MAO), leading to the production of hydrogen peroxide [6]. In this sense, MDMA exposure is associated with ROS and nitric oxide (NO) synthesis and consequent RNS production, which contributes to and explains MDMA neurotoxicity [45]. In parallel, Felim *et al.* [44] suggested that oxidative toxicity is a result of MDMA redox-active metabolites (*e.g.*, 3,4-dihydroxymethamphetamine, HHMA), which could cause cell death through necrosis. Increased ROS production was involved in the toxic effect induced by the mixture of MDMA and its metabolites [7]. Therefore, we suggest that the mixtures might accentuate the effects of each drug, resulting in a significant increase in ROS/RNS levels, and explaining the higher potential damage deriving from the misuse of these drugs. To further elucidate oxidative damage mechanisms, future experiments may include the measurement of oxidative damage to biomolecules and the quantification of antioxidant defenses. Accordingly, we also observed alterations in mitochondrial membrane integrity after exposure to MDMA alone and in combination with tramadol or tapentadol, although tramadol and tapentadol alone did not cause modifications in $\Delta\psi m$ [24]. The highest damage was observed in cells exposed to the MDMA/tramadol mixture. Mitochondrial damage was reported in the HepG2 cell line, after a 24-hour exposure to a higher MDMA concentration (1.8 mM) in normothermia; however, in hyperthermia conditions (40.5 °C), it increases at lower concentrations, such as 1.0 and 1.5 mM [28]. Nevertheless, Ferreira *et al.* [25] reported that MDMA promotes minor mitochondrial dysfunction, as analyzed through MTT assays. Similarly, Barbosa *et al.* [6] reported that MDMA, at concentrations up to 200 μM, did not promote significant mitochondrial dysfunction, as seen through MTT assays, nor did it promote an increase in cell death in SH-SY5Y differentiated cells. In contrast, an *in vivo* study suggested that MDMA affects spatial memory and brain mitochondrial function, with these effects significantly correlating with MDMA concentration [49]. Similarly to the loss of mitochondrial membrane integrity, ATP depletion is also a surrogate of mitochondrial dysfunction, as seen for MDMA-exposed HepG2 cells [28]. Although we did not observe alterations in the gene expression of NDUFS1, a respiratory chain enzyme, upon

MDMA treatment, its mixture with tramadol or tapentadol accentuated the decrease observed after treatment with each opioid alone. Tramadol and tapentadol are also associated with a decrease in ATP levels, leading to a bioenergetic crisis [24,37], while MDMA was associated with a transient energetic impairment [50]. Decreased expression of genes encoding for mitochondrial complexes, such as complexes I and IV of the respiratory chain, were also associated with MDMA exposure [12,51]. Nonetheless, the study of isolated skeletal mitochondria showed little evidence that MDMA could cause a bioenergetic crisis [52]. Therefore, their combination might exacerbate the effects described for both drugs alone.

On the other hand, our results showed alterations in CKB gene expression. The CKB gene encodes for an enzyme that catalyzes the interconversion of creatine and phosphocreatine, an important substrate in energy metabolism. Several studies showed an increase in CK serum levels because of rhabdomyolysis, after exposure to MDMA [45,52]. In addition, Rusyniak *et al.* [52] suggested that MDMA may have the ability to reversibly inhibit CK activity, while tramadol and tapentadol are associated with decreased CK expression [24,53]. Decreased CK activity and expression are associated with increased brain damage and neurodegeneration [54]. Thus, combined exposure to MDMA and tramadol or tapentadol might increase the likelihood of a bioenergetic crisis and consequent cell damage.

To further study the metabolic events underlying such bioenergetic crisis, glucose and lactate levels were measured. Intracellular glucose levels increased in the presence of MDMA, alone or in combination, while lactate levels did not change upon exposure to MDMA only. In the case of a 29-year-old woman with MDMA intoxication, severe hypoglycemia and hyperinsulinemia were reported, though the main adverse effects of MDMA did not include hypoglycemia [55]. Such hypoglycemia is consistent with higher glucose uptake and its intracellular increase. Likewise, *in vivo* studies with rats given a single or repeated MDMA dose showed that blood glucose levels decreased significantly after drug administration in both cases [56]. In animals receiving a single dose, a change in glucose metabolism was observed, with cerebellum hyperactivation and hippocampus, amygdala, and auditory cortex hypoactivation [56]. Moreover, a single MDMA dose (2, 10, and 20 mg/kg intravenous administration) induced a transient increase in interstitial glucose concentrations in the striatum [57], suggesting a subsequent increase in its cellular uptake. According to our results, tramadol consumption is also associated with hypoglycemic events [58,59], and *in vitro* studies with the same tramadol and tapentadol concentrations, cell model and exposure period show that both opioids promote increased glucose uptake [24]. Tramadol activates the insulin signaling cascade by inducing insulin receptor substrate-2 expression and enhancing glucose utilization in neuronal cells *in vitro* [59]. *In vivo*, tramadol was shown to decrease fasting serum glucose levels and ameliorate glucose metabolism [59,60]. In our study, a corresponding decrease in glucose levels in the culture medium samples would further support the hypothesis of increased glucose uptake by SH-SY5Y-treated cells. Accordingly, culture medium glucose quantification should be considered in future assays. Alternatively, the use of cytochalasin B, an inhibitor of glucose transporters 1 and 3, could also be an approach to this topic.

In turn, increased lactate levels have been associated with the bioenergetic crisis deriving from exposure to opioids, particularly to tapentadol and tramadol [24]. Consistently with this, the transient increase in glucose in rat striatum was associated with a concomitant increase in lactate levels [57]. Nonetheless, we did not find alterations in intracellular lactate levels after MDMA exposure, possibly reflecting their transient nature [57]. Interestingly, combined exposure to MDMA and tramadol or tapentadol did not increase lactate levels, although mitochondrial damage and increased glucose accumulation were observed. Oxidative glucose metabolism is affected by MDMA exposure through inhibition of the pyruvate dehydrogenase complex, subsequently limited Krebs cycle, and inhibition of the α-ketoglutarate dehydrogenase complex, an enzyme of the same metabolic pathway [50]. However, possible concomitant changes in the expression and activity of glycolytic enzymes may limit glycolysis, thus preventing the increase in lactate levels.

Given that MDMA neurotoxicity may be associated with the serotonergic system, that MDMA is a 5-HT releaser [45,61] and tramadol mechanism of action (and, on a minor scale, tapentadol) as 5-HT reuptake inhibitors [21,22,61-64], the highest toxicity of the mixtures might be a consequence of increased 5-HT levels. Moreover, MDMA exposure caused an increase in 5-HT levels, and this has also been observed with *in vivo* and *in vitro* approaches, given that MDMA leads to 5-HT efflux and inhibition of its transporter (5-HT transporter (5-HTT)) [45]. Nonetheless, our results showed that tramadol or tapentadol alone did not cause a significant 5-HT increase in the medium, as previously suggested by several authors [2,65]. Indeed, although tramadol is frequently associated with SS, some studies failed to detect 5-HT toxicity among 71 cases of tramadol overdose [65,66], which is in line with our *in vitro* data. Our results additionally suggest that the mixtures of MDMA and tramadol or tapentadol do not increase the risk of 5-HT toxicity. Consistently with this, MDMA effects in 5-HT release were reported to be decreased in the presence of 5-HT reuptake inhibitors in a review by Gillman, since they inhibit MDMA uptake by the presynaptic terminal [61]. The combination of changes detected after tramadol/tapentadol exposure, and after exposure to the mixture of MDMA and tramadol or tapentadol, is represented in Fig. 7. Regarding these results, while the possibility of the rabbit polyclonal antibody to serotonin used in this study reacting with MDMA is highly unlikely, an additional control containing culture medium with MDMA alone will help to rule out cross-reactivity in future assays.

Although we used an *in vitro* model, with limited metabolic activity, it is important to underline that there are *in vivo* toxicity results from MDMA exposure, but also from the exposure to its metabolites, such as HHMA and thioether conjugates [44]. *In vitro* methodologies have limitations because they do not study the simultaneous exposure to parent compounds and the metabolites produced by the exposed cells. This is particularly more difficult to reproduce in the context of multiple drug exposure. Barbosa *et al.* [6] compared the effects of MDMA and MDMA metabolites in SH-SY5Y differentiated cells and concluded that only MDMA metabolites decreased mitochondrial function and increased cell death. In this sense, it would be interesting to evaluate the toxicity of mixtures of MDMA metabolites and tramadol or tapentadol metabolites. In this context, future assays using differentiated SH-SY5Y cells could complement the mechanistic findings obtained in the present study. Additionally, the subsequent use of an *in vivo* model will enable a more integrated analysis, reflecting human physiological conditions. *In vivo* assays will be important to complement the results obtained *in vitro*. Similarly, MDMA and tramadol are metabolized by cytochrome P450 enzymes [44,67], for which pharmacogenetic variations may account for differences in their pharmacokinetics, explaining their toxicity [2,3,67]. In this context, Jamali *et al.* [68] concluded, in a rat model, that MDMA affects tramadol absorption, distribution, and metabolism. MDMA uses mechanism-based inhibition to inhibit the CYP2D6 isoenzyme, and tramadol undergoes CYP2D6 metabolism to form its main metabolite, *O*-desmethyltramadol (M1), also undergoing *N*-demethylation to *N*-desmethyltramadol (M2) via CYP2B6 and CYP3A4 [67,69,70]. Indeed, *in vitro* studies suggest that MDMA may inhibit the CYP2D6 isoenzyme through competitive interaction and/or the formation of a metabolic intermediate [19,45,71], thereby decreasing the metabolism of tramadol when taken together. A study with Wistar rats concluded that MDMA administration reduced M1 production and increased M2 production, which may lead to neurotoxic alterations [70]. It should also be emphasized that hyperthermia conditions are very important for the toxic effects of MDMA and its hepatic metabolites, making it relevant to simulate such conditions to better assess the resulting toxicological effects [6,28].

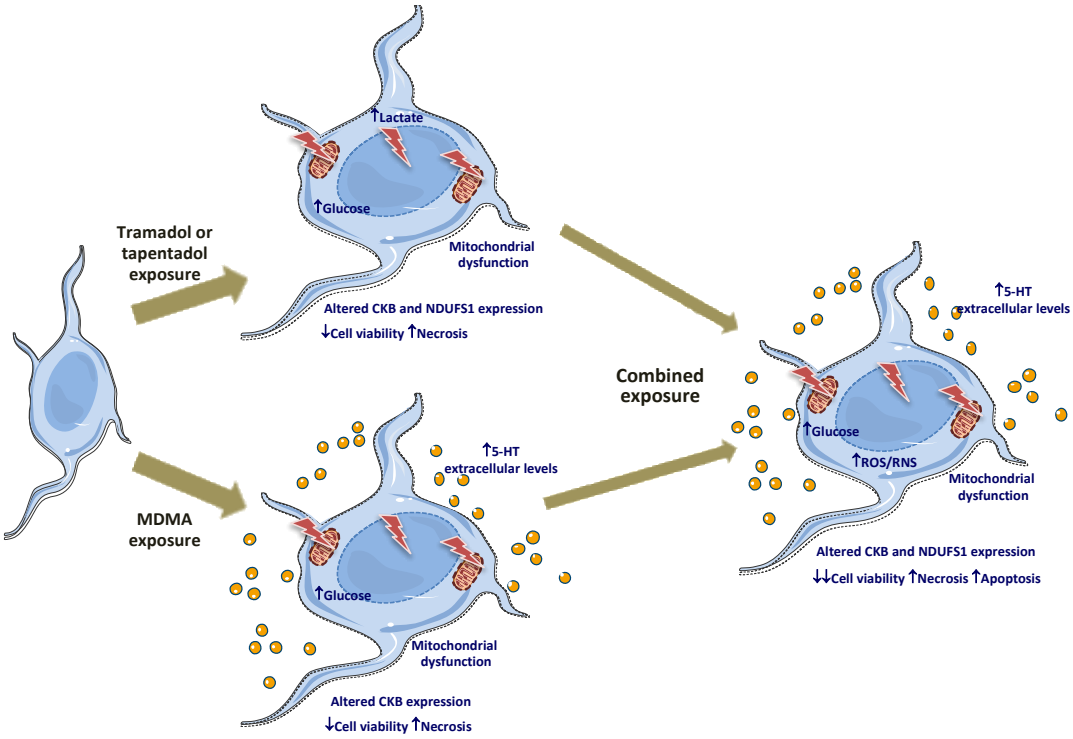

Figure 7. Schematic representation of the acute toxic effects induced by tramadol, tapentadol, MDMA, and MDMA/tramadol and MDMA/tapentadol combinations in undifferentiated SH-SY5Y cells. Exposure to both opioids leads to altered expression of cytosolic and mitochondrial enzymes (decreased expression of CKB and NDUFS1), increased intracellular glucose levels, and decreased lactate levels, resulting in increased cell death through necrosis. Exposure to MDMA leads to increased 5-HT release, decreased CKB expression, mitochondrial dysfunction, and cell death through necrosis. The combined exposure to MDMA and tramadol or tapentadol caused higher decreases in cell viability, with increased necrotic and apoptotic cell death, as well as an increase in ROS/RNS levels, in addition to the effects of single exposures. CKB: creatine kinase B; 5-HT: serotonin; NDUFS1: Fe-S protein 1 of NADH dehydrogenase; ROS/RNS: reactive oxygen species/reactive nitrogen species.

The results of the present study show that the combined exposure to MDMA and tramadol or tapentadol causes a higher decrease in cellular viability than the exposure to the individual drugs. Exposure to tramadol or tapentadol alone did not cause an increase in 5-HT levels when compared with the controls (untreated cells). However, the exposure to MDMA alone caused a significant 5-HT increase. In turn, no

additional increase in 5-HT levels was detected on exposure to mixtures, compared with the exposure to MDMA alone. Nevertheless, combined exposure caused increased caspase-3 activity and cell death through apoptosis, as well as increased oxidative stress and mitochondrial damage. Cells exposed to the mixtures also show more alterations in gene expression, and the MDMA/tramadol mixture led to the highest decrease in CKB expression and more pronounced mitochondrial damage.

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Author Contributions

JF and JB were involved in the study's conceptualization, methodology, validation, formal analysis, investigation, resources, data curation, original draft preparation, review and editing, visualization, supervision, project administration, and funding acquisition. OQ participated in the study's conceptualization and supervision, as well as in the draft's review and editing. RJD-O contributed to the study's conceptualization, methodology, validation, formal analysis, resources, supervision, and draft review and editing. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

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