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Oxidative stress and cell death induction by amitraz and its metabolite BTS-27271 mediated through cytochrome P450 and NRF2 pathway alteration in primary hippocampal cell

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ABSTRACT

Amitraz is a neurotoxic formamidine pesticide that induces cell death in hippocampal neurons, although its mechanisms are unknown. Amitraz produces reactive oxygen species (ROS), which could lead to cell death. Amitraz was shown to induce different cytochrome P450 (CYP) isoenzymes involved with ROS and apoptotic cell death induction. Finally, amitraz was described to decrease the activity of antioxidant enzymes regulated through KEAP1/NRF2 pathway, thus likely leading to a reduction of ROS elimination and to cell death induction. We evaluated the effect of amitraz or BTS-27271 co-treatment with or without the antioxidant N-acetylcysteine and/or the unspecific CYP inhibitor 1-aminobenzotriazole on cell viability and its related mechanisms in wild type and silenced primary hippocampal neurons after 24 h treatment. We observed that amitraz produced oxidative stress and CYPs induction leading to apoptotic cell death. ROS generation was partially mediated by CYPs induction and downregulation of NRF2-pathway through KEAP1 overexpression. These data could help explain the mechanism by which amitraz induces cell death and oxidative stress and provide a therapeutic strategy to protect against this effect in case of poisoning.

1. Introduction

Amitraz (1,5 di-(2,4-dimethylphenyl)-3-methyl-1,3,5-triaza-penta-1,4-diene) belongs to the class of formamidine pesticides and is used worldwide on both animals and crops to control pests as an insecticide and acaricide (Yilmaz and Yildizdas, 2003). Amitraz poisoning cases reported up to date generate an increasing general concern in health authorities (Demirel et al., 2006; Veale et al., 2011). Recently, a systematic review of human amitraz poisoning showed that most cases were accidental exposures and children, while a small portion were suicide attempts and adult cases. Ingestion was reported to be the most common route of exposure, and only a small fraction of cases were of percutaneous exposure (Dhooria and Agarwal, 2016).

Amitraz is rapidly absorbed, distributed, metabolized and eliminated, primarily via urine when administered orally to mammals (USEPA, 1996; JMPR, 1998). The spectrum of metabolites observed was similar in all species studied, being BTS-27271 the main metabolite formed and with higher distribution to the brain (Hu et al., 2019). BTS-27271 has been found to be more potent than amitraz with regard to its miticidal activity (Schuntner and Thompson, 1978) and mammalian toxicity (Pass, and Mogg, 1991; Chen and Hsu, 1994).

Amitraz is a potent neurotoxic compound that induces signs such as sedation, loss of righting reflex, motor incoordination, coma and seizures among others (Folz et al., 1984; Hsu and Schaffer, 1988).

Abbreviations: AMZ, Amitraz; 1-ABTZ, 1-aminobenzotriazole; BSA, bovine serum albumin; CAT, catalase; CYP, Cytochrome P450; CYB5R1, NADPH cytochrome P450 b5 reductase; DMSO, dimethylsulphoxide; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GCLC, glutamate cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; HO-1, heme oxygenase-1; KEAP1, Kelch-like ECH-associated protein 1; MTT, 3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide; NQO1, NADPH quinone oxidoreductase 1; NRF2, Nuclear factor erythroid 2-related factor 2; P450R, NADPH cytochrome P450 c reductase; PBS, phosphate-buffered saline; PX, peroxidase; SOD, superoxide dismutase

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Hippocampus is one of the most affected regions by amitraz. In this sense, Gilbert (1988) and Gilbert and Dyer (1988) reported that amitraz induced seizures in hippocampus of male rats. Recently, it has been reported that amitraz induces cell death, which was mediated partially through H1 and alpha-2 receptors in primary hippocampal cells, suggesting that other mechanism could be involved (Del Pino et al., 2015a, b).

Amitraz has been described to induce oxidative stress and cell death in human lymphocytes cell cultures (Radakovic et al., 2013). Recently, Kanbur et al., (2016) showed that amitraz induced oxidative stress after acute and repeated exposure in different tissue, being the most affected the brain in male rats. However, no studies have been conducted to elucidate the effect of BTS-27271 on oxidative stress generation and cell death induction. Reactive oxygen species (ROS) can directly modify cellular macromolecules leading to a variety of toxic effects including lipid peroxidation, protein dysfunction, nucleic acid oxidation, and finally cell death (Bensaad et al., 2009; Elmore et al., 2001). Therefore, the oxidative stress generation induced by amitraz or its metabolite could mediate the cell death observed in primary hippocampal cells.

The mechanism through which amitraz could induce oxidative stress is unknown. Oxidative stress can be the result of either an increase of ROS production and/or a reduction in the mechanisms of ROS elimination. Cytochrome P450 (CYP) isoenzymes induction are associated with different signaling pathways contributing to ROS (superoxide, H_2O_2, and OH^-) production and causing cell/tissue injury (Khatua and Bhattacharyya, 2001; Caro and Cederbaum, 2004; Sangar et al., 2010; Tian et al., 2010; Reed et al., 2011; Shah et al., 2013). In this sense, amitraz was reported to induce cytochrome P450 1A1/2, 2B1/2, 3A1/2 and increase their activity and that of nicotinamide adenine dinucleotide phosphate (NADPH) cytochrome P450 c reductase (P450R) and NADPH cytochrome P450 b5 (CYB5R1) enzymes in female rats' liver after 3 days exposure (Ueng et al., 2004). In addition, amitraz increased the activity of cytochrome P450 2E1, CYB5R1 and P450R after 40 days exposure in male rats' liver (Kanbur et al., 2016). Thus, these alterations could mediate the effects observed on oxidative stress generation and cell death after amitraz exposure. Moreover, amitraz has been reported to reduce peroxidase activity in uterus of female rats after 3 days exposure (Ueng et al., 2004) and the activity of superoxide dismutase (SOD), catalase (CAT), and peroxidase (PX) in different male rat tissues, mainly in the brain, after 40 days exposure (Kanbur et al., 2016), which could lead to a reduction of ROS elimination, contributing to the increase of ROS and induction of cell death.

Nuclear factor erythroid 2-related factor 2 (NRF2) is a key transcription factor that regulates the transcription of antioxidant protective genes such as, SOD, CAT, glutamate cysteine ligase catalytic subunit (GCLC), glutamate-cysteine ligase modifier subunit (GCLM), NAD(P)H quinone oxidoreductase 1 (NQO1), and heme oxygenase-1 (HO-1) (Li et al., 2004; Sha et al., 2015; Luo et al., 2019). Under normal conditions, not subjected to stress, Kelch-like ECH-associated protein 1 (KEAP1) binds to NRF2 in the cytoplasm and promotes its degradation. However, when subjected to oxidative stress, KEAP1 is inactivated, while NRF2 is accumulated and constantly activated, inducing the expression of downstream antioxidant enzymes (Sha et al., 2015; Luo et al., 2019). The antioxidant enzymes altered by amitraz are regulated through NRF2 pathway, so amitraz could mediate their alteration through disruption of this pathway. In addition, CYPs have been reported to participate in the regulation of NFR2 pathway (Gong and Cederbaum, 2006; Xu et al., 2018), so amitraz could mediate its effect by alteration of CYPs isoenzymes directly and indirectly through NRF2 pathway dysfunction.

According to all exposed above, we hypothesized that amitraz could induce cell death through disruption of CYPs isoenzymes and oxidative stress generation. The induction of ROS could be mediated by alteration of CYPs and NRF2 pathway. To prove this hypothesis, a study on primary hippocampal neurons after 24 h amitraz (1 μM–160 μM) or BTS-27271 (1 μM–160 μM) treatment with or without the antioxidant N-acetylcysteine (NAC; 1 mM) or unspecific CYPs inhibitor 1-aminobenzotriazole (1-ABTZ; 1 mM), was undertaken.

2. Methods

2.1. Chemicals

The compounds, amitraz (98%), BTS-27271, poly-L-lysine, dimethyl sulfoxide (DMSO), were obtained from Sigma (Madrid, Spain). Anti-microtubule associated protein-2 (MAP2) antibody and anti-glial fibrillary acidic protein (GFAP) antibody were obtained from Millipore (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

2.2. Primary hippocampal neuron culture

Dissociated primary cell cultures are a simplified model that, in a very pure and particular cell type, allows easy evaluation of features such as the vulnerability of different cell types to toxic stimuli (Silva et al., 2006). We used a hippocampal primary cell culture to evaluate whether amitraz and its metabolite BTS-27271 induce hippocampal neuronal cell death through oxidative stress generation and CYPs isoenzymes alteration.

All experiments were performed in accordance with European Union guidelines (2003/65/CE) and Spanish regulations (BOE 67/8509-12, 1988) regarding the use of laboratory animals. Pregnant Wistar rats on embryonic days 17–18 (Charles River, Barcelona, Spain) were anaesthetized with sodium pentobarbital, the embryos were removed and their brains dissected under a stereomicroscope (Olympus SZ51, Barcelona, Spain). Hippocampus was collected, processed and cells were cultured according to the procedure previously described by Del Pino et al. (2014). Cell viability was assessed by the trypan blue 0.4% (Sigma, Madrid, Spain) dye exclusion method and it was routinely higher than 95%. The culture cells were immune-histochemically characterized with MAP2 (neuronal marker) and GFAP (astrocytic marker) to check cell culture purity and a predominance of neurons (92%) was observed.

This primary neuronal culture, after a few days in culture (6–7 days *in vitro*), forms a network of functional synaptic contacts, acquiring the characteristics of mature neurons, and remains viable up to 3 weeks in culture. Amitraz was previously described to produce cell death in primary hippocampal cells from 100 μM concentration (Del Pino et al., 2015a, b) and to induce oxidative stress from oral doses of 25 mg/kg in male rats (Kanbur et al., 2016). In addition, amitraz is extensively metabolized, being BTS-27271 the main metabolite that reaches the brain at the higher concentration (326 μM) after 10 mg/kg oral dose treatment in male rats (Hu et al., 2019), thus we chose a range from 1 μM to 160 μM amitraz and BTS-27271 concentrations to test our hypothesis. On the seventh day, hippocampal cells were treated with amitraz or BTS-27271 with or without NAC (1 mM) or 1-ABTZ (1 mM) for 24 h, at least 3 replicate wells/treatment were used. A vehicle group was employed in parallel for each experiment as a control.

2.3. Real-time PCR analysis

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a spectrophotometer Nanodrop 2000 (ThermoFisher Scientific, Madrid, Spain) and the quality of total RNA samples was assessed using an Experion LabChip (Bio-Rad, Madrid, Spain) gel. First-strand cDNA was synthesized with 1000 ng of cRNA by using a PCR array first strand-synthesis kit (C-02; SuperArray Bioscience, Madrid, Spain) following the manufacturer's instructions and including a genomic DNA elimination step and external RNA controls. After reverse transcription, we performed QPCR using pre-validated primer sets (SuperArray Bioscience) for mRNAs encoding CYP1A1 (PPR57580A),

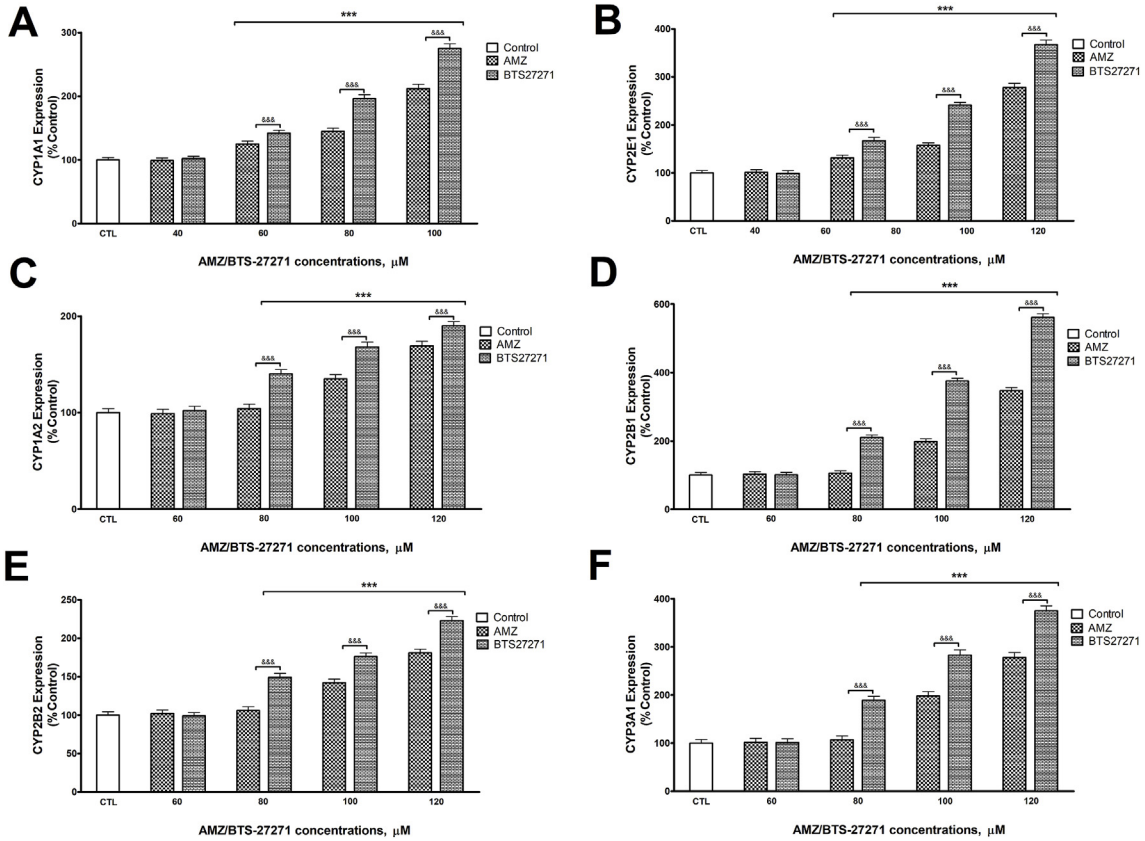

Fig. 1. Shows results from protein expression of (A) CYP1A1, (B) CYP2E1, (C) CYP1A2, (D) CYP2B1, (E) CYP2B2 and (F) CYP3A1 proteins after 24 h AMZ or BTS-27271 treatment. CYP1A1, CYP2E1, CYP1A2, CYP2B1, CYP2B2 and CYP3A1 expression was compared with controls [cells treated with vehicle were the negative control]. Each bar represents mean ± SD of 6 samples. Levels were measured using ELISA assays. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ.

CYP1A2 (PPR43442B), CYP1B1 (PPR71592A), CYP1B2 (PPR63384B), CYP2E1 (PPR42474B), CYP3A1 (PPR52864A), KEAP1 (PPR47610B), NRF2 (PPR45094A), CAT (PPR42937A), SOD1 (PPR43506A), NQO1 (PPR45314A), PX (PPR45366A) and ACTB (PPR06570C). We used ACTB as an internal control for normalization. Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SuperArray Bioscience). The thermocycler parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 seconds and 72 °C for 30 seconds. Relative changes in gene expression were calculated using the Ct (cycle threshold) method. The expression data are presented as actual change multiples (Livak and Schmittgen, 2001).

2.4. Protein determination

At the end of the treatments, primary hippocampal cells were washed with pre-chilled PBS, collected by scrapping, and lysed using RIPA buffer (Thermo Scientific, Madrid, Spain) with freshly added protease inhibitors cocktail (Thermo Fisher Scientific, Madrid, Spain). After centrifugation at 10,000 × *g* for 10 min at 4 °C, cell lysate supernatant was collected. Total protein concentration was assayed using a BCA kit (Thermo Fisher Scientific, Madrid, Spain).

Commercial ELISA kits (MBS092945, MBS2023157, MBS9310919, MBS531309, MBS2023931, MBS9428186, MBS7218529, MBS752046, MBS701908, MBS036924, MBS2024176 and MBS727547, MyBiosource, CA, USA) were used to determine CYP1A1, CYP1A2, CYP2B1, CYP2B2, CYP2E1, CYP3A4, KEAP1, NRF2, CAT, SOD1, NQO1 and PX, respectively, protein concentrations according to the manufacturer's instructions. A negative control was performed to determine interferences of amitraz and its metabolite with kits reagents. Concentrations were calculated as ng/mg of protein and values were expressed as the percentage of enzyme control content.

2.5. Gene knockdown

Primary hippocampal cells were transfected with siRNAs in 6-well plates (1×10^6 cells/well) using HiPerfect Transfection reagent according to the manufacturer's instructions (Qiagen, Barcelona, Spain). Two sets of siRNA duplexes (Qiagen, Barcelona, Spain) homologous to rat KEAP1 sequences for each one were designed using the HiPerformance Design Algorithm (Norvatis AG) and were purchased from Qiagen (catalog numbers SI01528443). As a transfection control, an All Stars Negative Control siRNA (Qiagen, Barcelona, Spain) was used. 48 h after transfection, the efficiency of siRNA-mediated KEAP1 knockdown was determined by RT-PCR using primers specific for rat KEAP1 mRNA (Qiagen, Barcelona, Spain). The effects of KEAP1 knockdown on cell injury were tested by MTT and caspases 3/7 assays. After 24 h of incubation with the siRNAs, the cells were washed with PBS and incubated for a further 24 h in culture medium with amitraz or BTS-27271 with or without NAC or 1-ABTZ.

2.6. Measurement of hydrogen peroxide levels

Hydrogen Peroxide (H_2O_2) is one of the reactive oxygen species produced under oxidative stress conditions. H_2O_2 content was measured using an using a hydrogen peroxide assay kit (Abcam, Cambridge, UK) according to the manufacturer's instruction. In brief, after 24 h treatment with amitraz or BTS-2727, primary hippocampal cells were harvested in H_2O_2 assay buffer and then centrifuged for 15 min at 1000 × *g*. A total of 50 μL of the supernatant was mixed with 50 μL of the reaction mix (assay buffer: 46 μL; OxiRed Probe: 2 μL; horse radish

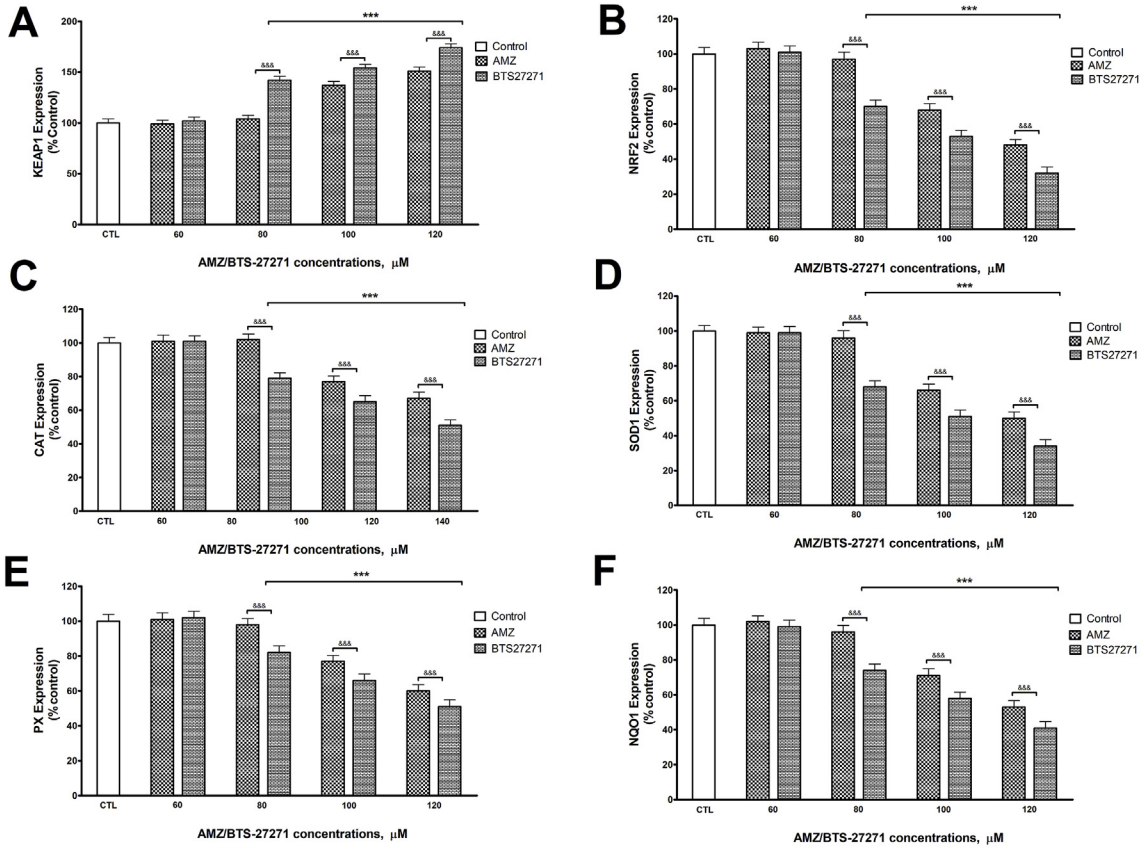

Fig. 2. Shows results from protein expression of (A) KEAP1, (B) NRF2, (C) CAT, (D) SOD1, (E) PX and (F) NQO1 proteins after 24 h AMZ or BTS-27271 treatment. KEAP1, NRF2, CAT, SOD1, PX and NQO1 expression was compared with controls [cells treated with vehicle were the negative control]. Each bar represents mean ± SD of 6 samples. Levels were measured using ELISA assays. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ.

peroxidase (HRP): 2 μL) and then incubated at room temperature for 10 min. The optical density was read at 570 nm with a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain), and the H_2O_2 concentration was calculated according to a standard concentration curve. A negative control was performed to determine interferences of amitraz and its metabolite with kit reagents. Hydrogen peroxide content in the samples was expressed in nanomole per milliliter and presented as percent untreated control.

2.7. Lipid peroxidation assay

Malondialdehyde (MDA) concentration was determined as an indicator of lipid peroxidation products. Intracellular MDA production was quantified after 24 h exposure to amitraz or BTS-2727, using a Lipid Peroxidation MDA Assay Kit (Abcam, Cambridge, UK) following the manufacturer's protocol. Briefly, after treatment, 1×10^6 cells were collected and homogenized on ice in MDA lysis buffer (300 μL) with 3 μL butylated hydroxytoluene (BHT) (100 ×), centrifuged for 10 min at 13,000 × *g* to remove insoluble material. Sample (200 μL) or standard (200 μL of MDA) was mixed with 600 μL of thiobarbituric acid (TBA) solution, incubated at 95 °C for 50 min and cooled to room temperature in an ice bath for 10 min. Each sample and standard (200 μL) was loaded (duplicate) into a clear 96-well plate and the absorbance was recorded at 532 nm using a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain). A negative control was performed to determine interferences of amitraz and its metabolite with kit reagents. Concentration of malondialdehyde determined as nmol/mg protein is presented as percent untreated control.

2.8. Protein oxidation assay

Carbonylation of protein was measured as an indicator of oxidative damage. The assessment of carbonyl formation was undertaken based on the derivatization of protein carbonyl groups with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of stable dinitrophenyl (DNP) hydrazone adducts. Protein carbonyl contents were quantified after 24 h exposure to amitraz or BTS-2727, using a Protein Carbonyl Content Assay Kit (Abcam, Cambridge, UK) following the manufacturer's procedures. Briefly, after treatment, 1×10^6 cells were collected and homogenized on ice in lysis buffer (100 μL), the lysates were derivatized with DNPH. Each sample and standard (100 μL) was loaded (duplicate) into a clear 96-well plate and the absorbance was recorded at 370 nm, using a microplate reader (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific, Madrid, Spain). A negative control was performed to determine interferences of amitraz and its metabolite with kit reagents. Protein carbonyl contents, determined as nmol/mg protein, are presented as percent untreated control.

2.9. Measurement of cell cytotoxicity (MTT assay)

Primary hippocampal cell cytotoxicity was measured by MTT assay after 24 h amitraz or BTS-27271 treatment. The MTT assay is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals by mitochondrial dehydrogenase. Cells were incubated for 4 h after amitraz or BTS-27271 treatment with 100 μL of yellow MTT solution (final concentration 0.5 mg/ml). After 4 h at 37 °C, the medium was removed and the formazan reaction product was dissolved in 250 μL DMSO. Absorbance was read at 562 nm (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, Thermo Fisher Scientific,

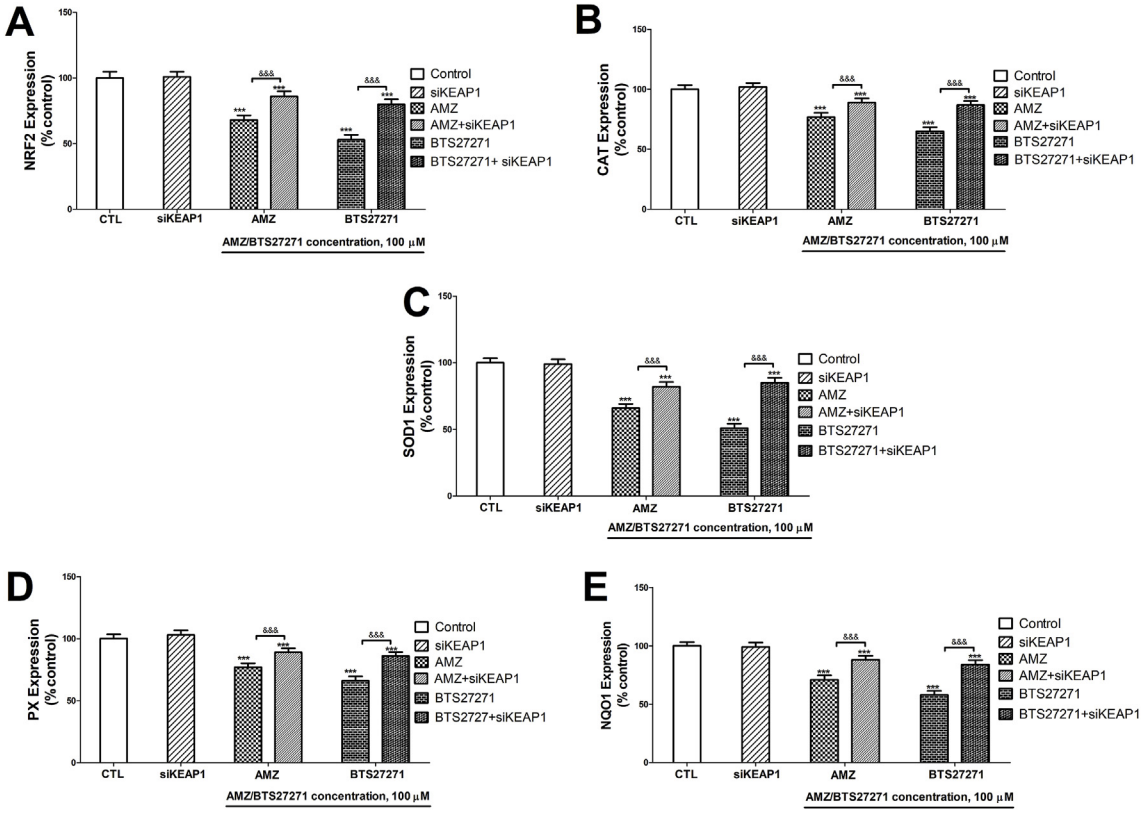

Fig. 3. Shows results from protein expression of (A) NRF2, (B) CAT, (C) SOD1, (D) PX, and (E) NQO1 proteins after 24 h AMZ or BTS-27271 treatment (100 μM). KEAP1, NRF2, CAT, SOD1, PX and NQO1 expression was compared with controls [cells treated with vehicle were the negative control]. Each bar represents mean ± SD of 6 samples. Levels were measured using ELISA assays. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ or BTS-27271.

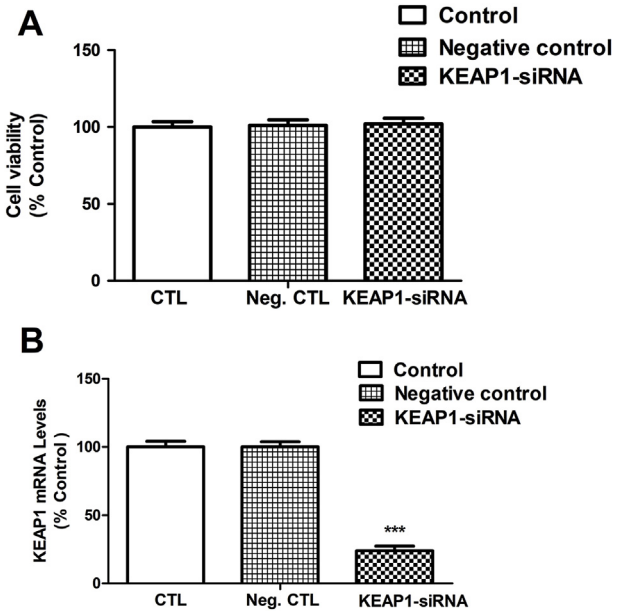

Fig. 4. Downregulation of KEAP1 in primary hippocampal cells and its impact on cell viability and gene expression was determined. Control: cells transfected without siRNA. Negative (Neg.) control: cells transfected with scrambled siRNA. KEAP1-siRNA: cells transfected with siRNA against KEAP1. (A) MTT test shows that KEAP1 downregulation did not significantly induce cell damage after 48 h. (B) KEAP1 downregulation could be detected by RT-PCR analysis 48 h after transfection. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. $^{***}p \leq 0.001$ compared to control.

Madrid, Spain) and results are expressed as the mean percentage of viable cells relative to untreated controls. A negative control was performed to determine interferences of amitraz and its metabolite with MTT. Control cells treated with DMEM were taken as 100% viability.

2.10. Caspase activity analysis

The presence of apoptotic cells was assessed by determination of caspase activation using Caspase-Glo 3/7 luminescence assay kits (Promega, Madrid, Spain) according to the manufacturer's protocol after treatment with indicated concentrations of amitraz or BTS-27271. In brief, at the end of treatment, culture cells were washed with PBS and the cells were scraped and collected in a microfuge tube in dark. Equal volumes of reagent and cell lysis buffer were added to a white-walled 96-well plate and incubated at room temperature in dark for 1 h and the resultant luminescence was read in a PerkinElmer LS50B plate-reading iluminometer (PerkinElmer, Madrid, Spain) and are presented as percent untreated control. A negative control was performed to determine interferences of amitraz and its metabolite with kit reagents. The luminescence of each sample was measured. The experiments were performed in triplicate.

2.11. Statistical analysis

For each experimental condition, the results represent data from at least three replicates. Data are represented as mean ± standard deviation (SD). Comparisons between experimental and control groups were carried out with the Student's *t*-test (data analyzed in Fig. 3) and either two-way ANOVA analyses (concentrations vs treatments) or one-way ANOVA analysis (analysis of different treatments), followed by the Tukey post-hoc test. Statistical difference was accepted when $p \leq 0.05$.

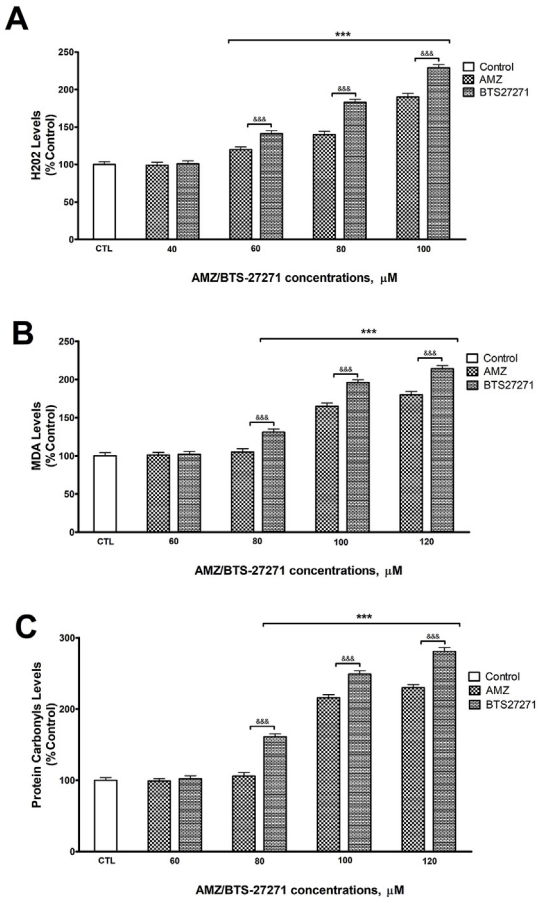

Fig. 5. Detection of ROS generation in primary hippocampal cells was measured by hydrogen peroxide (H_2O_2) assay. H_2O_2 concentration is presented as percent untreated control. The lipid peroxidation and protein oxidation level of primary hippocampal cells was measured by MDA and protein carbonyls assay. MDA and protein carbonyls concentrations are presented as percent untreated control. H_2O_2 (A), MDA (B) and protein carbonyl (C) content generated after 24 h AMZ or BTS-27271 (from 40 μM to 140 μM) treatment. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ.

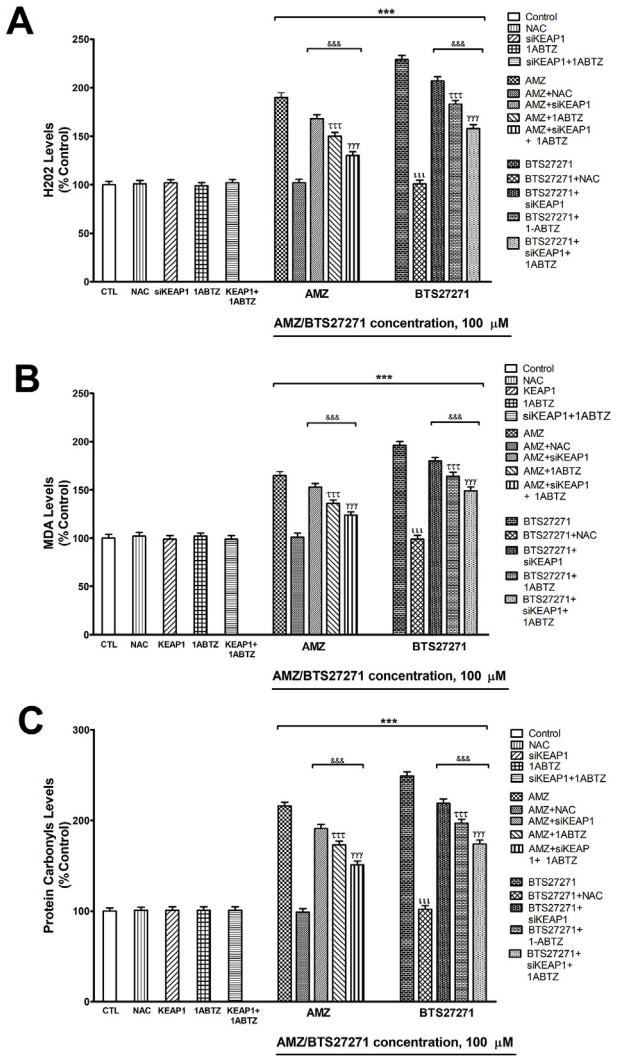

Fig. 6. Analysis of H_2O_2 (A), MDA (B) and protein carbonyl (C) contents generated after 24 h AMZ (100 μM) or BTS-27271 (100 μM) treatment. Data are presented as percent untreated control. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ or BTS-27271. $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with 1-ABTZ and AMZ or BTS-27271. $^{\gamma\gamma\gamma}p \leq 0.001$ compared to KEAP1 silenced cells treated with AMZ or BTS-27271.

Statistical analysis of data was carried out by computer using GraphPad software.

3. Results

3.1. Expression analysis

After incubation for 24 h with different concentrations of amitraz or BTS-27271 in hippocampal cells, the gene expression of KEAP1, CYP1A2, CYP2B1, CYP2B2, and CYP3A1, was significantly up-regulated, in a concentration-dependent way, from 100 μM to 80 μM concentrations, respectively, except for CYP1A1 and CYP2E1 that were up-regulated, in a concentration-dependent way, from 60 μM concentration (Fig. 1A–D and 2A). The increase of CYPs expression from higher to lower was CYP2B1, CYP2E1, CYP3A1, CYP2B2, CYP1A1 and CYP1A2 (Fig. 1A–F). The gene expression of NRF2, CAT, SOD1, NQO1 and PX was down-regulated, in a concentration-dependent way, after 24 h treatment with AMZ or BTS-27271 from 100 μM to 80 μM concentrations, respectively (Fig. 2B–F). The effect observed on the expression of these enzymes was bigger after BTS-27271 treatment than after amitraz treatment (Figs. 1 and 2). NAC co-treatment with AMZ or BTS-27271 had no effect on the KEAP1 expression (Data not shown). These results were corroborated with the analysis of these proteins expression. Moreover, KEAP1 silencing with amitraz reversed partially the disruption of NRF2, CAT, SOD1, NQO1 and PX expression induced by amitraz or BTS-27271 (Fig. 3).

3.2. Gene knockdown

Transfection of primary hippocampal neurons with control siRNA showed no effect on KEAP1 gene expression and cell viability. Transfection of KEAP1 siRNA caused large reductions in KEAP1 gene expression, although showed no effect on cell cytotoxicity (Fig. 4A and B).

3.3. Measurement of hydrogen peroxide levels

H_2O_2 is a reactive oxygen metabolic byproduct that serves as a key regulator of a number of oxidative stress-related states. After 24 h AMZ or BTS-27271 treatment, the hydrogen peroxide content increased, in a concentration-dependent manner, from 60 μM concentration compared

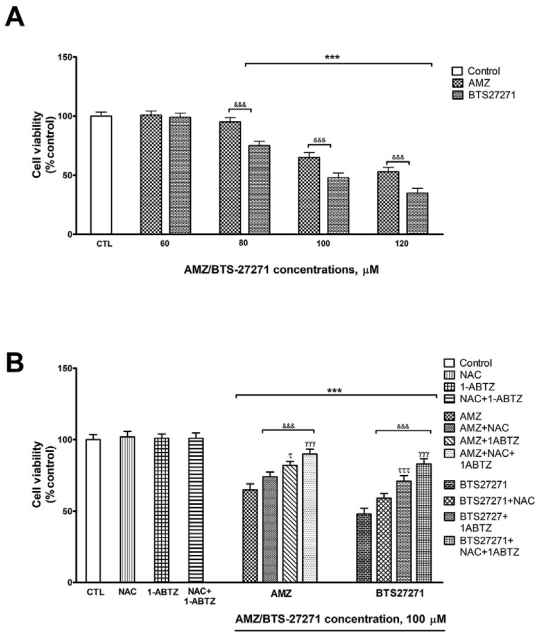

Fig. 7. AMZ and BTS-2721 effect on cell viability of primary hippocampal cells was determined by MTT assay. (A) AMZ and BTS-2721 (60–140 μM) effect on cell viability. (B) AMZ and BTS-2721 (100 μM) effect on cell viability of primary hippocampal cells co-treated with or without NAC (1 mM) or 1-ABTZ (1 mM). Cell viability was measured as MTT reduction (ordinate) and data were normalized as % control (CTL, white column). Data represents the mean ± SD of three independent experiments in triplicate. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ or BTS-27271. $^{\tau}p \leq 0.05$ and $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with NAC and AMZ or BTS-27271. $^{\gamma\gamma\gamma}p \leq 0.001$ compared cells co-treated with 1-ABTZ and AMZ or BTS-27271.

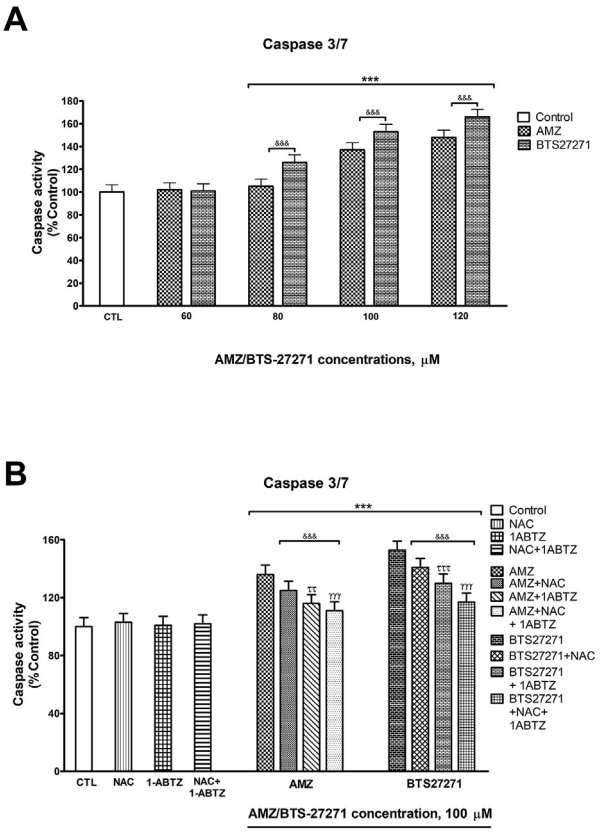

Fig. 8. Analysis of caspases 3/7 activity in primary hippocampal cells after AMZ or BTS-27271 treatment. (A) Analysis of caspases 3/7 activity after AMZ or BTS-27271 (60–140 μM) treatment. (B) Analysis of caspases 3/7 activity in primary hippocampal cells after AMZ or BTS-27271 (100 μM) treatment with or without NAC (1 mM) or 1-ABTZ (1 mM). Data represents the mean ± SD of three independent experiments in triplicate. $^{***}p \leq 0.001$ compared to control. $^{\&\&\&}p \leq 0.001$ compared to cells treated with AMZ or BTS-27271. $^{\tau\tau}p \leq 0.05$ and $^{\tau\tau\tau}p \leq 0.001$ compared to cells co-treated with NAC and AMZ or BTS-27271. $^{\gamma\gamma\gamma}p \leq 0.001$ compared cells co-treated with 1-ABTZ and AMZ or BTS-27271.

with control group (Fig. 5A). The increase in the content of H_20_2 was higher after BTS-27271 treatment than AMZ treatment. NAC treatment did not induce an increase in H_2O_2 content and co-treatment with AMZ or BTS-27271 completely attenuated the increase in H_2O_2 content observed after AMZ or BTS-27271 treatment alone (Fig. 6A). AMZ or BTS-27271 treatment of KEAP1 silenced cells or 1-ABTZ co-treatment with AMZ or BTS-27271 reversed partially the increase in the content of H_2O_2, being higher the reversion induced after 1-ABTZ co-treatment (Fig. 6A). AMZ or BTS-27271 co-treatment with 1-ABTZ of KEAP1 silenced cells induced a higher reversion of the increase in the content of H_2O_2 than that produced after co-treatment with 1-ABTZ of wild type cells (Fig. 6A).

3.4. Lipid peroxidation and protein oxidation assay

Lipid peroxidation and protein carbonylation was measured in primary hippocampal cells after 24 h exposure to AMZ or BTS-27271 at increasing concentrations. AMZ or BTS-27271 treatment induced an increase in MDA and protein carbonyl content, in a concentration-dependent manner, from 100 μM AMZ and 80 μM BTS-27271 concentrations compared with control group, indicating the enhancement of lipid peroxidation (Fig. 5B) and protein oxidation (Fig. 5C). BTS-27271 treatment induced bigger effects than AMZ treatment. Otherwise, NAC treatment had no effect in the MDA and protein carbonyl content (Fig. 6B and C). Co-treatment with AMZ or BTS-27271 and NAC produced a complete attenuation of the increased MDA and protein carbonyl content observed after AMZ or BTS-27271 treatment alone (Fig. 6B and C). AMZ or BTS-27271 treatment of KEAP1 silenced cells or 1-ABTZ co-treatment with AMZ or BTS-27271 reversed partially the increase in the content of MDA and protein carbonyls, being higher the reversion induced after 1-ABTZ co-treatment (Fig. 6B and C). After 24 h AMZ or BTS-27271 co-treatment with 1-ABTZ of KEAP1 silenced cells a higher reversion of the increase in the content of MDA and protein carbonyls produced after co-treatment with 1-ABTZ of wild type cells was observed (Fig. 6B and C).

3.5. Effect of AMZ and BTS-27271 on cell cytotoxicity

We used the MTT test to evaluate cell survival after 24 h exposure to AMZ or BTS-27271 at increasing concentrations. MTT test after 24 h cell culture incubation with AMZ or BTS-27271 showed, from 100 μM to 80 μM concentrations, respectively, a clear concentration-dependent reduction in cell viability compared with vehicle-treated cells (control negative) (Fig. 7A). NAC or 1-ABTZ treatment had no effect on cell viability (Fig. 8A). However, after AMZ or BTS-27271 co-treatment with NAC or 1-ABTZ, an amelioration in the reduction in cell viability was observed (Fig. 8A). This reduction of cell viability decrease was higher after unspecific CYPs inhibition than after antioxidant co-treatment. NAC and 1-ABTZ co-treatment with AMZ or BTS-27271 induced a higher reversion of cell death observed than after NAC or 1-ABTZ co-treatment with AMZ or BTS-27271 (Fig. 8A).

3.6. Effect on the activity of caspases 3/7

AMZ or BTS-27271 activated the caspases in primary hippocampal cells after 24 h treatment, in a concentration-dependent manner, from 100 μM to 80 μM concentrations, respectively, compared with control group (Fig. 7B). NAC or 1-ABTZ treatment had no effect on caspases

activation (Fig. 8B). However, after AMZ or BTS-27271 co-treatment with NAC or 1-ABTZ, a reduction in caspase 3/7 activation was observed (Fig. 8B). This reduction was higher after unspecific CYPs inhibition than after antioxidant co-treatment. NAC and 1-ABTZ co-treatment with AMZ or BTS-27271 induced a higher reversion than that observed after NAC or 1-ABTZ co-treatment with AMZ or BTS-27271 (Fig. 8B).

4. Discussion

In the present work, we show that amitraz and BTS-27271 induce, after 24 h exposure, an increase in the expression of CYP2E1 and CYP1A1 from 80 μM to 60 μM, respectively, and of KEAP1, CYP1A2, CYP2B1, CYP2B2 and CYP3A1 from 100 μM to 80 μM, respectively, in a concentration-dependent way, on primary hippocampal neurons. The increase of CYPs' expression from higher to lower was CYP2B1, CYP2E1, CYP3A1, CYP2B2, CYP1A1 and CYP1A2. In this regard, amitraz has been described to induce CYPs 1A1/2, 2B1/2 and 3A1/2 enzymes expression and activity, but not of CYP2E1 enzyme after 3 days exposure in liver from immatures female rats, ranking from highest to lowest CYP2B1, CYP3A1, CYP2B2, CYP1A1 and CYP1A2 (Ueng et al., 2004). These previous data support our results except for the expression of CYP2E1, which could be due to the difference of model used, tissue studied, the exposure period of time and the concentrations that reach the tissue. Ueng et al. (2004) also described that amitraz increased the activity of P450R and CYB5R1. In addition, amitraz was shown to increase the activity of CYP2E1, CYB5R1 and P450R after 40 days exposure in male rats' liver (Kanbur et al., 2016). The induction of these enzymes could increase their activity, although the exact mechanism through which amitraz increases their activity is unknown.

Otherwise, amitraz and BTS-27271 induce, after 24 h (from 100 μM to 80 μM, respectively) exposure, a decrease of NRF2, CAT, SOD1, NQO1 and PX expression, in a concentration-dependent way, on hippocampal neurons. In this sense, amitraz has been reported to decrease the activity of SOD, CAT and PX after acute and repeated exposure in brain and other tissues in rats (Ueng et al., 2004; Kanbur et al., 2016), supporting our findings. The silencing of KEAP1 partially reversed the amitraz and BTS-27271 effect on NRF2, SOD1, CAT, NQO1 and PX expression. KEAP1 has been reported to degrade NRF2, which regulates the expression of different antioxidant enzymes such as SOD1, CAT, NQO1, and PX among others (Li et al., 2004; Sha et al., 2015; Luo et al., 2019), so the overexpression of KEAP1 induced by amitraz and BTS-27271 mediated the downregulation of these enzymes. BTS-27271 effect on the expression of all the proteins commented above was bigger than that of amitraz. BTS-27271 has been reported to be more toxic than amitraz, as can be observed with LD_{50} and the induction of their mechanisms of action, which are produced at lower concentrations than amitraz (Del Pino et al., 2015a, b), supporting our results.

Amitraz and BTS-27271 treatment also increased ROS levels (from 80 μM to 60 μM, respectively), lipid peroxidation and protein oxidation (from 100 μM to 80 μM, respectively), in a concentration dependent-way, after 24 h exposure in hippocampal neurons. Amitraz, has been reported to induce ROS generation and lipid peroxidation in the brain and other tissues after acute and repeated exposure in male rats (Kanbur et al., 2016), which support our findings. Amitraz and BTS-27271 effect on oxidative stress was reversed partially after KEAP1 silencing or co-treatment with 1-ABTZ, producing the latter a higher reversion, suggesting that NRF2 pathway and CYPs alteration mediate, in part, the oxidative stress observed. KEAP1 overexpression has been reported to downregulate NRF2 pathway and the antioxidant enzymes expression regulated by it, thus reducing the mechanism of antioxidant defense that could leads to the increase of ROS levels (Luo et al., 2019), which support our findings. Besides, CYPs 1A1/2, 2B1/2, 2E1 and 3A1/2 have been described to induce oxidative stress through generation of ROS in their activity (Sapone et al., 2003; McLean et al., 2008; Liu et al., 2010; Liua et al., 2019; Luo et al., 2019), supporting or results. The induction of CYP2E1 and CYP1A1 took place at the same concentrations than the induction of ROS generation, while the downregulation of NRF2 pathway took place at the same concentrations than those of the induction of lipid and protein oxidation. These data suggest that although the production of ROS takes place at lower concentration induced by CYP2E1 and CYP1A1, it only produces cell damage when the antioxidant defense of the NRF2 pathway is overridden. ROS generation has been reported to stimulate the degradation of KEAP1 and the induction of NRF2, increasing the expression of the antioxidant enzymes (Nguyen et al., 2004; Gong and Cederbaum, 2006), which is a contradiction with the effect observed. NAC co-treatment with AMZ or BTS-27271 had no effect on the KEAP1 expression. Therefore, AMZ and BTS-27271 seems to regulate directly NRF2 pathway through KEAP1, blocking the effect of ROS generation induced by CYPSs on NRF2 pathway, weakening the mechanism of defense against ROS, leading to oxidative stress generation. Further studies are necessary to determine the specific contribution of the different CYPs to ROS generation. Otherwise, other mechanisms seem to contribute to ROS generation besides those commented above. In this sense, it was reported that amitraz (5 mg/kg intraperitoneally) decreases glutathione and non-protein sulfydryls due to its α_2-agonist action, causing weakness of the antioxidant defense mechanism (Costa el al., 1991), which could contribute to the oxidative stress observed.

Our results also show that amitraz and BTS-27271 induces, after 24 h treatment, in a concentration dependent-way, from 100 μM to 80 μM, respectively, cell death on hippocampal neurons. Amitraz and BTS-27271 exposure also activates caspase 3/7, indicating that apoptosis takes place. Multiple authors have described amitraz potential ability to induce cell death. Different studies have described that acute amitraz treatment decreases cell viability in mammalian cells *in vitro*, producing this from 100 μM concentration in MCF-7 human breast cancer (Ueng et al., 2004), from 1.19 mM in human lymphoblastoid WIL2NS cells (Young et al., 2005a,b), from 341 μM human luteinized granulosa cells (Young et al., 2005a,b), from 8.5 μM in CHO hamster cells (Padula et al., 2012) and from 11.9 μM in human lymphocytes (Radakovic et al., 2013), showing in the last two studies that amitraz induces apoptotic cell death, which support our results. According to all exposed above there is a discrepancy on the AMZ concentrations that induce cell death *in vitro*, which could be due to the use of different cell lines, solvents and cell culture protocols. Moreover, Oglesbya et al. (2006) reported that amitraz poisoning (78 mg/kg) induced necrosis in Scottish terrier's liver and kidney, which support that amitraz could induce cell death *in vivo*.

Amitraz and BTS-27271 induction of cell death was partially reversed after co-treatment with NAC or 1-ABTZ. NAC co-treatment with amitraz and BTS-27271 reversed completely the induction of oxidative stress. These results show the ROS generation mediates cell death. In addition, 1-ABTZ co-treatment with amitraz or BTS-27271 and NAC induced a higher reversion than NAC or 1-ABTZ co-treatment alone, which suggests that CYPs induced cell death, in part, independently from the induction of ROS, although other mechanisms could be involved on this effect. In this sense, it was reported that CYPs generate oxidative stress and apoptotic cell death (Chung et al., 2007; Tian et al., 2010; Leung et al., 2013; Shah et al., 2013). Previously, it was described that amitraz was able to reduce the cell viability through α_2 adrenergic receptor agonist and H_1 receptor antagonist effects (Del Pino et al., 2015a, b), which could contribute to the effect observed. Furthermore, amitraz could induce voltage-dependent Ca^{2+} channels activation, increasing Ca^{2+} cellular levels (Shin and Hsu, 1994), and it has been stated that abnormal Ca^{2+} elevation leads to apoptosis induction (Jiang et al., 2011; Chang et al., 2014). Amitraz also inhibits adenylyl cyclase, decreasing cyclic AMP levels (Chen and Hsu, 1994), which reduction may induce apoptotic cell death (Anway et al., 2005). On the other hand, no studies have been developed to research whether amitraz is able to induce the last type of cell death, autophagy, which may be induced by oxidative stress (Elmore et al., 2001; Bensaad et al., 2009).

To our knowledge, this is the first work to prove that amitraz and BTS-27271 induce CYP 1A1/2, 2B1/2, CYP2E1 and CYP3A1 and downregulate NRF2 pathway through overexpression of KEAP1, leading to oxidative stress generation in hippocampal cells. Moreover, we prove that ROS and CYPs induced by amitraz and BTS-27271 leads to cell death, in part, through them. Future studies should be developed to determine the other mechanisms implicated in the oxidative stress and cell death observed in hippocampal neurons and to corroborate this data, determining dose-response relationship for these effects *in vivo*. These results are of interest because they constitute one step forward to a better understanding of amitraz neurotoxicity mechanisms and may lead to a promising alternative treatment of the latter.

Conflict of interest

The authors declare that they have no conflict of interest.

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