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ORIGINAL ARTICLE

Benzydamine induces apoptosis in astrocytes in vitro

Benzadamina induz apoptose em astrócitos in vitro

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Abstract

Objective: the present work aims to evaluate the Benzydamine (BZD) effect on cell viability in astrocyte culture and investigated the death mechanism involved with its cytotoxic effect. **Methods**: in order to evaluate cell viability, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used, while flow cytometry was used to verify cell damage. The immunofluorescence assay was used to verify the expression of the marker's caspase-8, caspase9 and p65 subunit of Factor nuclear kappa B (NFκB). A statistical analysis for MTT assay and Flow Cytometry were made using ANOVA with Dunett's post-test; Student's t-test was made for the Immunofluorescence. Significance was set at $p < 0.05$. **Results**: the MTT reduction assay showed that BZD (3.1 to 100 µg/mL) caused a decrease in astrocytes viability. The flow cytometry showed that the cytotoxic effect of BZD was caused by the activation of the apoptotic death pathway, evidenced by the externalization of phosphatidylserine. The immunofluorescence revealed an increase in caspase-8 expression and no alteration in caspase-9 expression, demonstrating that there was an activation of the extrinsic pathway of apoptosis. The mean inhibitory concentration (IC50) of BZD (26.13 µg/mL) also caused an increase in NFκB p65 expression. **Conclusion**: taken together, the results of the present study suggest that BZD has a cytotoxic effect on astrocyte cells, and this effect comes from its ability to activate the extrinsic apoptotic pathway.

Keywords: benzydamine; astrocyte; caspase; factor nuclear kappa b.

Resumo

Objetivo: o presente trabalho tem como objetivo avaliar o efeito da Benzidamina (BZD) na viabilidade celular em cultura de astrócitos e investigar o mecanismo de morte envolvido com seu efeito citotóxico. **Métodos**: para avaliar a viabilidade celular foi utilizado o ensaio de redução do brometo de 3-(4,5-Dimetiltiazol-2-il)-2,5-difeniltetrazólio (MTT), enquanto a citometria de fluxo foi utilizada para verificar o dano celular. O ensaio de imunofluorescência foi utilizado para verificar a expressão do marcador caspase-8, caspase9 e subunidade p65 do Fator nuclear kappa B (NFκB). A análise estatística para ensaio MTT e Citometria de Fluxo foi feita utilizando ANOVA com pós-teste de Dunett; Foi feito o teste t de Student para Imunofluorescência. A significância foi estabelecida em $p < 0,05$. **Resultados**: o ensaio de redução do MTT mostrou que o BZD (3,1 a 100 µg/mL) causou diminuição na viabilidade dos astrócitos. A citometria de fluxo mostrou que o efeito citotóxico do BZD foi causado pela ativação da via de morte apoptótica, evidenciada pela externalização da fosfatidilserina. A imunofluorescência revelou aumento na expressão de caspase-8 e nenhuma alteração na expressão de caspase-9, demonstrando que houve ativação da via extrínseca de apoptose. A concentração inibitória média (CI50) de BZD (26,13 µg/mL) também causou aumento na expressão de NFκB p65. **Conclusão**: em conjunto, os resultados do presente estudo sugerem que o BZD tem efeito citotóxico nas células astrocitárias, e esse efeito advém de sua capacidade de ativar a via apoptótica extrínseca.

Palavras-Chave: benzidamin;, astrócito; caspase; fator nuclear kappa b.

INTRODUCTION

Benzydamine hydrochloride (BZD) is a non-steroidal anti-inflammatory drug used in the treatment of many diseases such as pharyngitis, painful and inflammatory processes, laryngitis, gingivitis, vulvovaginitis, stomatitis, among others. This drug has as one of its mechanisms of action the inhibition of prostaglandin synthesis and induction of apoptosis in many cell lines[1].

Shortly before being withdrawn from the market, BZD was common for its non-therapeutic use, especially among street children, because of its psychoactive effects[2]. There are numerous preparations for BZD in many countries, and misuse reports are increasingly rising 3–5. High BZD doses acutely can cause delirium, abnormal behavior, hallucinations, headache, diplopia, nystagmus, tinnitus, blurred vision, dizziness, seizures, lethargy, dyskinesia, loss of consciousness, and coma; these effects can be associated with even in part, with the astrocyte's dysfunction, but the mechanisms involved have not yet been fully elucidated[2,6].

Astrocytes are important cells in the homeostasis of the Central Nervous System (CNS). It has been involved in a variety of

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molecular mechanisms (exocytosis, membrane transporters, or diffusion through channels) by secreting numerous neurotransmitters, neurohormones, and trophic factors, which are directly related to the neuroprotective ability of these cells. Insults to the CNS, regardless of etiology, disrupt the body's homeostasis, and the astrocytes act through specific molecular cascades, protecting neurons against glutamatergic excitotoxicity, extracellular overload potassium ion, and reactive oxygen species (ROS). Astrocytes also provide energy substrates to stressed neurons[7,8] and govern the formation and maintenance of synaptic transmission, the synchronization of neuronal networks and signals to other cells (microglia, oligodendrocytes, endothelial cells, etc.)[9]. In this sense, the present study aimed to evaluate the BZD effect on astrocytes and investigate the death mechanism involved with its cytotoxic effect.

MATERIAL AND METHODS

Chemical, antibodies and reagents

BZD was obtained from Sigma-Aldrich. Anti-rabbit caspase 8 (sc-7890), anti-rabbit caspase 9 (sc-7885), and anti-rabbit NFκB p65 (sc-372) were from Santa Cruz Biotechnology. Alexa fluor 488 donkey anti-rabbit (A21206) and DAPI (4',6-diamidino-2-phenylindole, D1306) were obtained from Invitrogen (Carlsbad, CA). Fluoromalt (S3023) was from DAKO (Denmark).

Cell culture

The immortalized cortical astrocytes kindly provided by the Department of Pharmacology of the Federal University of São Paulo were seeded in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin (100,000 U/mL) and streptomycin (10 mg/mL) at 37°C in an atmosphere of 95% humidity and 5% CO2. Before each experimental analysis, cells were kept in a medium without FBS for 24 hours to synchronize them in the G0 phase of the cell cycle. For each experiment, cells were plated in a 96-well plate at a density of 1 × 105 cells/mL.

Cell Viability by MTT Assay

Cell viability was measured using a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. The activity of BZD was evaluated in different concentrations (3,1 µg/mL, 6,2 µg/mL, 12,5 µg/mL, 25 µg/mL, 50 µg/mL e 100 µg/mL) for a period of 12 hours or 24 hours of exposition. After treatment, the MTT reagent was added (2.5 mg/mL) for 4 hours. The solubilization of the formazan crystals was then carried out using 10% sodium dodecyl sulfate (SDS) in 0.01 N HCl. The results were obtained with a microplate reader at an absorbance of 570 nm. Cell viability was determined by comparison with the control group treated only with phosphate-buffered saline (PBS), whose mean absorbance was weighted as 100% viability. Mean inhibitory concentration (IC50) was calculated by non-linear regression.

Flow cytometry

After incubation with the IC50, IC50/2, and IC50×2 for 24 hours with BZD, astrocytes were submitted to flow cytometry assays to determine cell death mechanisms involved in the BZD cytotoxic effect. Cells were dislocated, centrifuged, and washed twice with binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM CaCl2, pH 7.4). Treated and untreated cells were labeled with annexin V-PE and 7-aminoactinomycin D (7-AAD for 15 minutes in the dark). Annexin has a strong binding affinity for phosphatidylserine, a membrane phospholipid that, during apoptosis, is translocated from the inner side of the cell membrane to its outer side. 7-aminoactinomycin D (7-AAD) binds to DNA, indicating cytoplasmic and nuclear cell damage, indicative of necrosis. Finally, the experimental groups were analyzed in FACSCalibur flow cytometry device (BD Biosciences, New Jersey, USA) using the CellQuest Pro1 software.

Immunofluorescence

For immunofluorescence assay, astrocytes cultured (1 × 105 cells/mL) were washed (5 min) with PBS and fixed in ice cold methanol for 10 min. After fixation, cells were permeabilized and blocked by incubating cells in a permeabilizing/blocking buffer (0.25% Triton X-100 and 1% BSA in PBS) for 1 hour at room temperature. Cells were then washed three times with washing buffer (0.025% Triton X-100, 0.2% BSA in PBS) and incubated with primary caspase-8 (1:100), caspase-9 (1:100) or NFκB p65 (1:100) antibody diluted in washing buffer overnight at 4ºC. Afterward, cells were rinsed in washing buffer and stained with secondary anti-rabbit antibodies conjugated to Alexa488 (1:500) at 37ºC for 1 hour. Following protocol, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). At the end of the staining protocol, the cells were mounted onto glass slides using fluoromalt (DAKO). The images were acquired through a confocal microscope (LM 710, Zeiss, Germany) using an objective 40×/NA 1.4. The immunofluorescence intensity (IF) was obtained through ImageJ (NIH, Bethesda, USA) and was calculated as the integrated density per cell corrected for background.

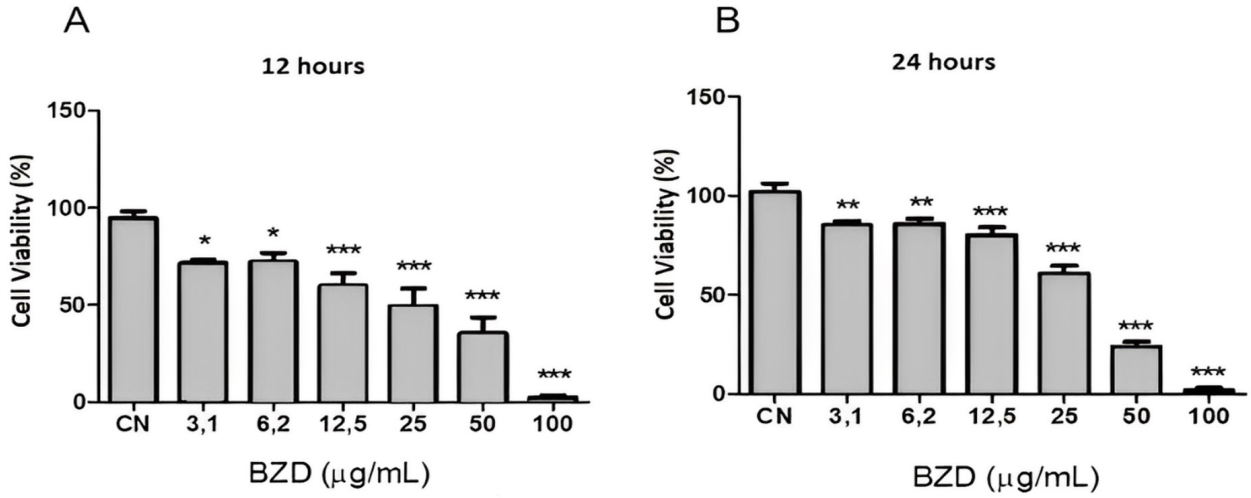
Statistical analysis

Experiments were performed in triplicate, and data were expressed as mean ± Standard Error Media (SEM). Data were analyzed using ANOVA with Dunett post-test for MMT assay and Flow Cytometry and Student's t-test for the Immunofluorescence. Significance was set at $p < 0.05$.

RESULTS

To study the effect of BZD on astrocytes, it was performed the MTT reduction assay. After 12 hours of incubation, all tested BZD concentrations were able to reduce cell viability compared

Figure 1. Assessment of BZD cytotoxicity in astrocytes at 12h (A) and 24h (B) of incubation. (A) BZD effects on astrocyte viability after 12 hours of incubation by MTT assay. (B) BZD effects on astrocyte viability after 24 hours of incubation. The experiments were performed in triplicate, and data are expressed as mean ± SEM. Statistical analysis was performed using ANOVA followed by post-test Dunnett. *p<0.05; **p<0.01; ***p<0,001 compared to the control group.



To identify indicative cellular changes suggestive of necrosis and/or apoptosis induced by BZD, cells incubated for 24 hours with the IC50 (26.13 mg/mL), 2×IC50 (52.26 mg/mL) and IC50/2 (13.06 mg/mL) of BZD were subjected to the labeling protocol for 7-AAD and annexin-V-PE (Figure 2 and 3). There was an increase of approximately 30% of events marked with annexin (Ax+/7AAd-), indicating the externalization of phosphatidylserine from the membrane, a phenomenon indicative of the occurrence of apoptosis. These events are in the lower right quadrant of the dot-plot graphic

Figure 2. Assessment of the cell death mechanism using flow cytometry. Dot plot graphics representative of evaluation of the death type involved in BZD cytotoxicity after 24-hours incubation with IC50/2 (13.06 mg/mL), IC50 (26.13 ug/ml), and IC50x2 (52.26 mg/mL) of the study drug. Lower left quadrant: Viable cells (non-marked). Lower right quadrant: cells stained only with Annexin V-PE (apoptosis). Upper left quadrant: marked cells only with 7-AAD (necrosis). Upper right quadrant: doubly labeled cells with 7-AAD and Annexin V-PE.

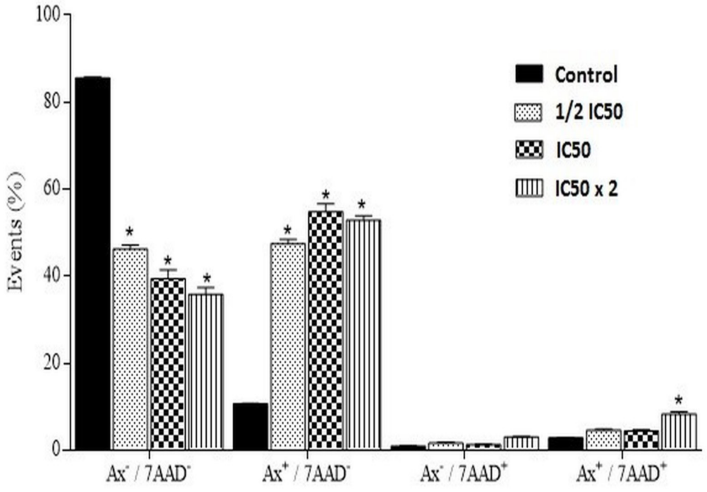
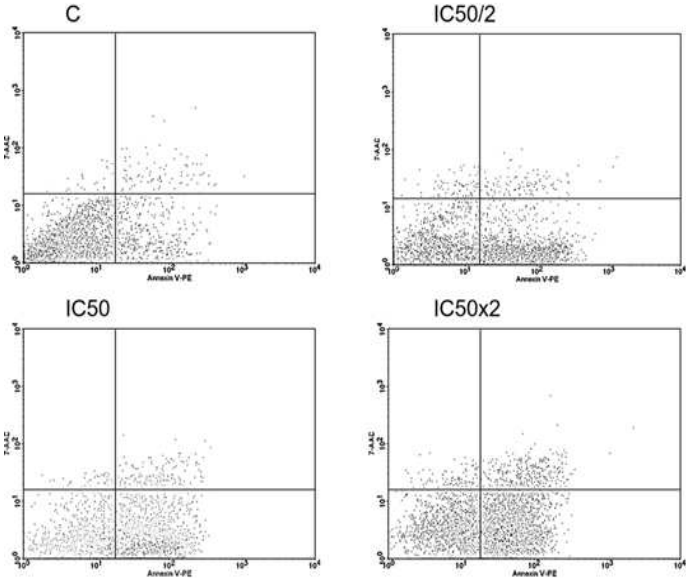


Figure 3. Quantification of the death type involved in BZD cytotoxicity after 24 hours of incubation with IC50/2 (13.06 mg/mL), IC50 (26.13 ug/ml), and IC50x2 (52.26 mg/mL) of the study drug. The experiments were performed in triplicate, and the data were expressed as a percentage of events ± SEM. Statistical analysis was performed using ANOVA followed by post-test Dunnett * p <0.05 compared to the control group.



The results of immunofluorescence showed that there was an increase in IF for caspase-8 after the treatment with BZD [14.13 ± 0.77] when compared to the control group [3.51 ± 0.13] (Figures 4 and 7A). There was no difference in IF for caspase-9 after the treatment with BZD [7.08 ± 0.48] when compared to the control group [6.32 ± 0.33] (Figures 5 and 7B). NFκB, a transcription factor activated mainly by the extrinsic pathway of apoptosis, showed a significant increase in the IF after the treatment with BZD [26.27 ± 2.26] when compared to the control group [4.45 ± 0.47] (Figures 6 and 7C).

Figure 4. Double-immunofluorescence staining of cortical astrocyte cultures for caspase-8 and DAPI. Representative images showing caspase-8 (green) and DAPI (blue) double staining from cortical astrocyte cells with no treatment (control group) and after IC50 treatment with BZD. The control culture showed only scant DAPI immunoreactivity and almost no caspase-8 staining. In contrast, culture treated with IC50 of BZD showed marked caspase-8 staining.

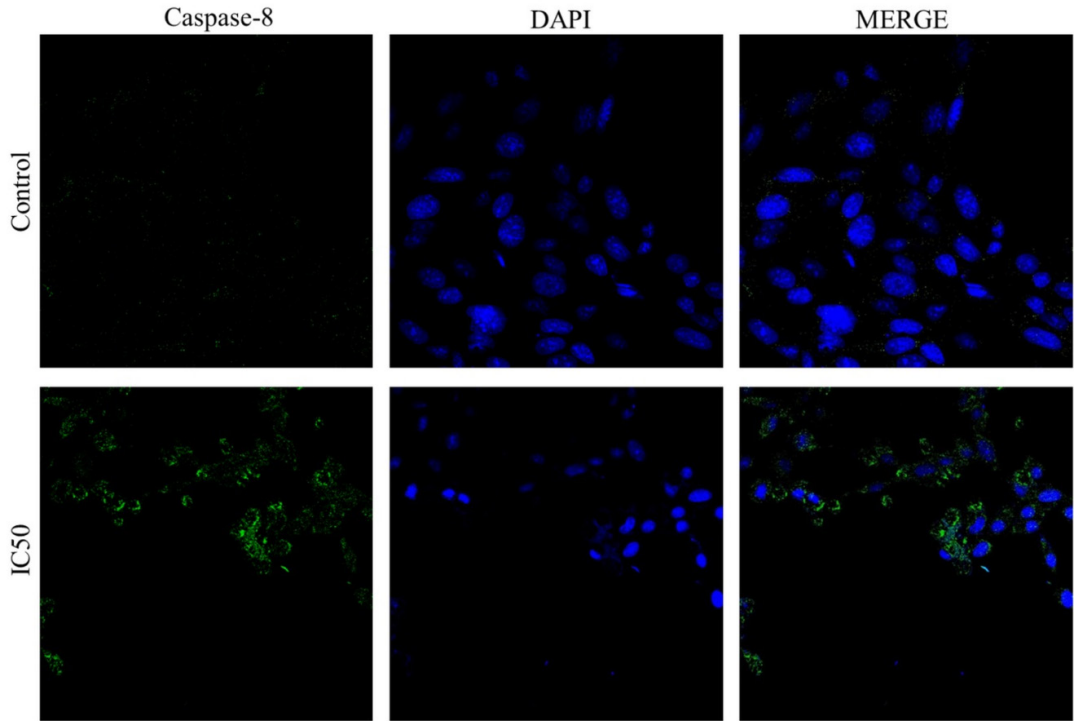


Figure 5. Double-immunofluorescence staining of cortical astrocyte cultures for caspase-9 and DAPI. Representative images showing caspase-9 (green) and DAPI (blue) double staining from cortical astrocyte cells with no treatment (control group) and after IC50 treatment with BZD. Control culture and BZD-treated culture showed only scant DAPI immunoreactivity and almost no caspase-9 staining.

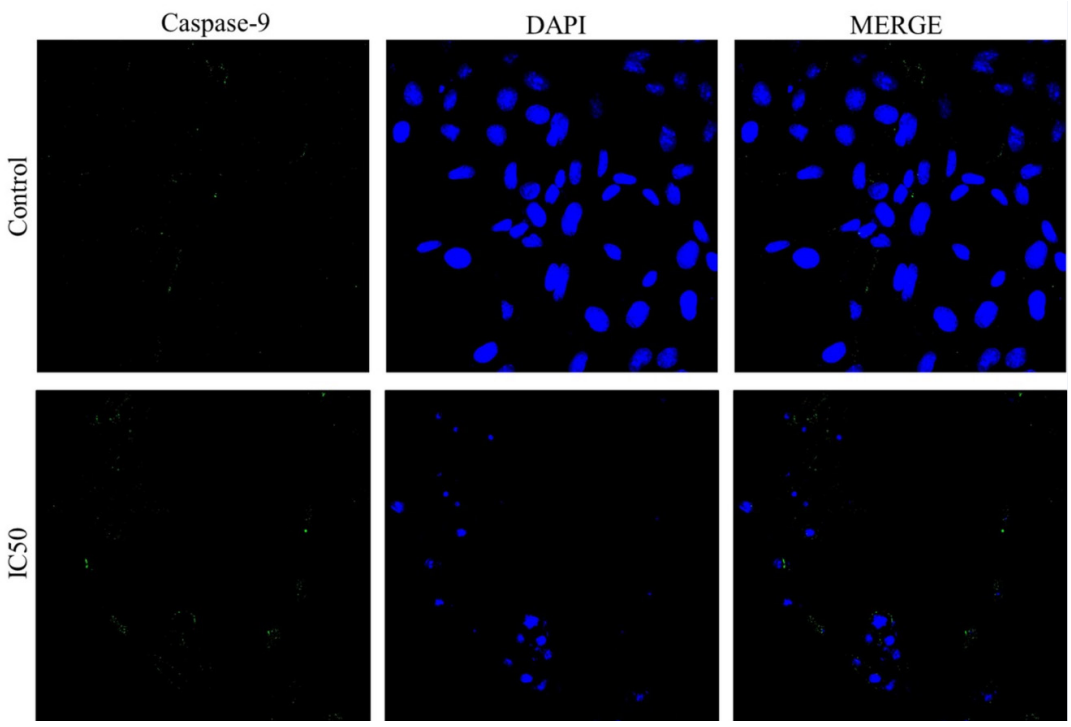


Figure 6. Double-immunofluorescence staining of cortical astrocyte cultures for NFκB p65 and DAPI. Representative images showing NFκB p65 (green) and DAPI (blue) double staining from cortical astrocyte cells with no treatment (control group) and after IC50 treatment with BZD. Control culture showed only scant DAPI immunoreactivity and almost none of NFκB p65 staining. In contrast, culture treated with IC50 of BZD showed marked NFκB p65 staining.

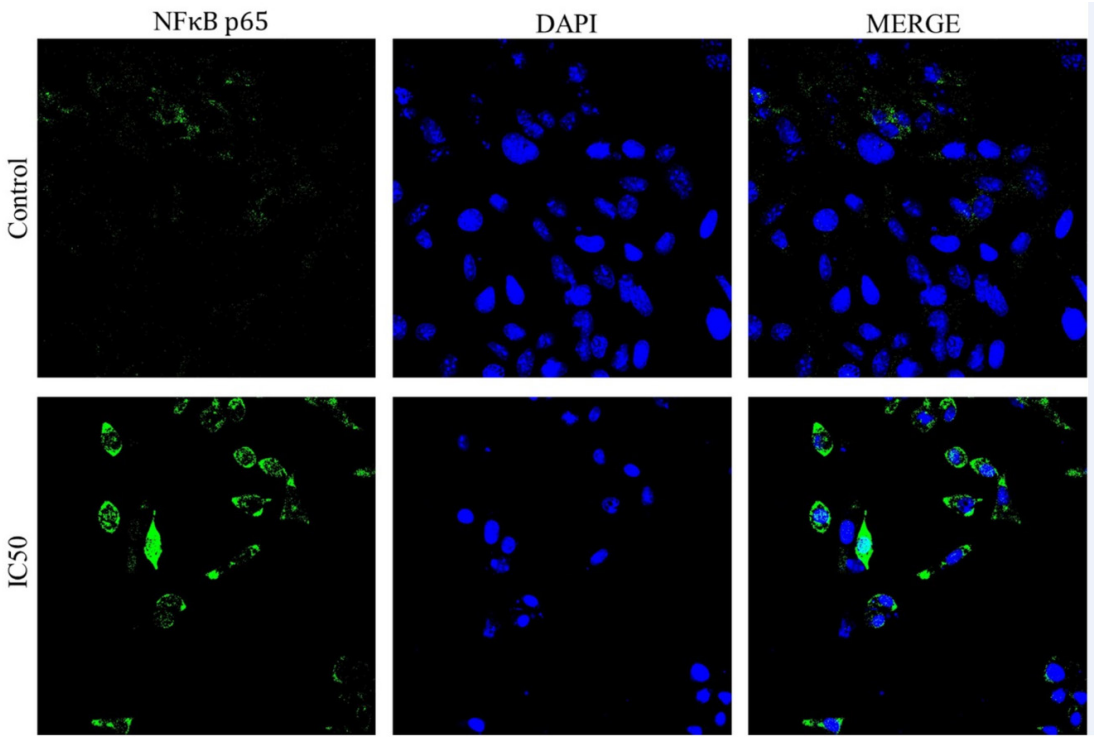
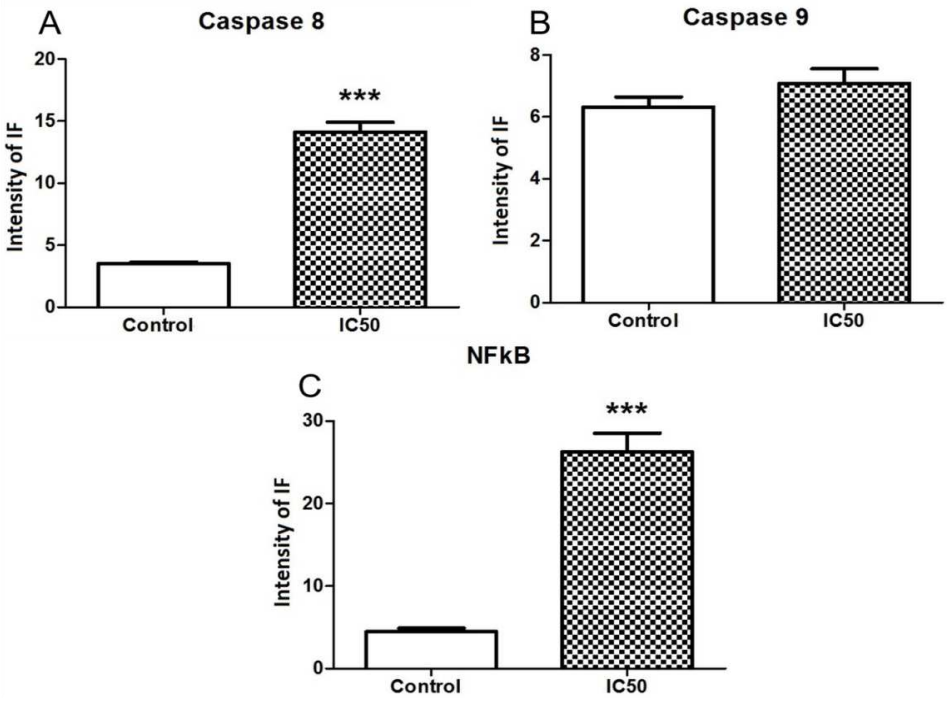


Figure 7. Quantification of the intensity of immunofluorescence for (A) caspase-8, (B) caspase-9, and (C) NFκB p65 in cortical astrocytes cultures. The experiments were performed in triplicate, and the data were expressed as IF ± SEM. Statistical analysis was performed using Student's t-test. ***p <0.001 compared to control group.



DISCUSSION

In the present work, BDZ decreased cell viability by activating apoptotic pathways. Studies showed the same cytotoxic profile in other cell lines like macrophages, leading to the overexpression of IL-1β, IL-6, and TNF-α caused by this drug[10].

The present work used the IC50, IC50/2, and IC50x2 from the 24-hour incubation period to investigate whether astrocytic cell death induced by BZD was caused by apoptosis or necrosis. The results showed that BZD induces predominantly cell death via apoptosis. Apoptosis is known to be activated by drugs of abuse as part of their cytotoxic mechanisms. The neurotoxicity of cocaine, for example, has been associated with the induction of apoptosis as well as the activation of caspases, the loss of mitochondrial potential, and the release of cytochrome c into the cytosol, among others[11]. It has also been known that the abuse of alcohol activates the apoptotic pathway, and this pathway is linked to its antagonist properties on glutamatergic N-methyl-D-aspartate (NMDA) receptors and to its Gamma-Aminobutyric Acid (GABA) agonists. Other abuse drugs, such as phencyclidine, ketamine, barbiturates, methamphetamines, and benzodiazepines, also activate apoptosis. BDZ also showed the ability to delay the cell cycle of carcinogenic cells[12].

There are two major apoptotic signaling pathways. The road of death receptors (extrinsic) and the pathway mediated by mitochondria (intrinsic). The extrinsic pathway is initiated by death receptors expressed on the cell surface belonging to the superfamily of tumor necrosis factor. One of these central pathways of extrinsic apoptosis is initiated by cytokines such as tumor necrosis factor-α (TNF-α), Fas ligand (FasL), and apoptosis-inducing ligand related to TNF-α (TRAIL). Once the receptor is activated, it oligomerizes, recruits intracellular adapter proteins, and forms complexes, while protein containing the death domain (FADD) is recruited by Fas signaling. The complexes recruit one or more members of the protease family of cell death called caspases, caspase-8 classically. The activated caspase-8 cleaves the effector caspase, particularly caspase-3. Caspase-3, then, is joined to several intracellular substrates, which leads to morphological changes of apoptosis[13,14].

The intrinsic pathway can be initiated by anti-cancer drugs, growth factor withdrawal, hypoxia, or inducing oncogenes. These stimuli induce permeabilization of the external mitochondrial membrane and activate the mitochondrial pathway. The mitochondrial pathway is involved by releasing apoptogenic factors, such as cytochrome c, from the mitochondrial intermembrane space to the cytosol. This release of cytochrome c into the cytosol triggers the activation of caspase-3 through the formation of the apoptosome complex with cytochrome c / Apaf-1 / caspase-9[15,16].

Thus, in this study, it was performed immunofluorescence markings for caspase-8 and caspase-9 in the culture of astrocytic cells after incubation with BZD to investigate 111the extrinsic and intrinsic pathways, respectively. Our results showed that the apoptotic route by which BZD causes its cytotoxic effect is the extrinsic one. Many drugs of abuse, which are known to induce programmed cell death, 17 found that methamphetamine, in PC12 neuron culture, activates the intrinsic pathway of apoptosis and increases levels of the p53 transcription factor, a factor that is primarily activated as a result of DNA damage.

Then, immunofluorescence was performed on the NFκB p65 portion. NFκB is a transcription factor that consists of five members (p50, p52, p65/RelA, c-Rel, and Rel B), which can be combined in various ways to form active transcriptional dimers, and the p50-p65 dimer is the most common of the many possible combinations[18,19]. This transcription factor is activated in response to cellular stresses (pro-inflammatory stimuli) involved in apoptosis regulation. Depending on the cell type and apoptotic agent, NFκB may mediate or prevent apoptosis[20–22]. This transcription factor can be found in a wide variety of cell types, including neurons and microglia[23,24]. NFκB, the committed member of the Rel/NFκB proteins family, form homo- or heterodimers by combining the p65 (or RelA), p50, p52, c-Rel, or RelB subunits. It is constitutively expressed in the cytoplasm, bound to IκB, a protein that masks the nuclear localization signal of NFκB by retaining it in the cytoplasm. Inductors of NFkB act via intracellular signaling cascades that activate IkB kinases (IKKS), which phosphorylates two N-terminal specific serines in IkBα, resulting in polyubiquitination of IkBα and degradation of the 26S proteasome. When IκBα is degraded, NFκB (mainly the portion p65) migrates to the nucleus and modulates transcription of target genes involved in cell death. Phosphorylation of serine at position 536 of the p65 NFκB portion makes this portion active and responsible for gene transcription[23–25].

One of the main ways to activate NFκB is the activation of caspase-8 enzyme 25. Because of this, to corroborate with the activation of the extrinsic pathway of apoptosis by BZD, it was held the marking of astrocytic cells by immunofluorescence for NFκB p65 phosphorylated. Our results showed that incubation of the cells with BZD led to a significant increase in phosphorylated NFκB p65 expression, reinforcing the hypothesis that BZD causes cytotoxicity through activation of the extrinsic pathway of apoptosis. The chronic administration of cocaine also induced NFκB activation in the nucleus accumbens, leading to toxicity to astrocytes and microglia[26,27]. High expression of NFκB may indicate autophagy or apoptosis in astrocyte cells mediated by endogenous or exogenous neuroinflammatory stimuli. The neuroinflammatory response can be maintained even if the cell death pathway has been ceased by the residual ROS and NFκB levels on the tissue[28,29].

In summary, BZD and other stimulants are common club drugs abused by young people. We showed in this study that BZD, like many other drugs of abuse, has deleterious effects on CNS, and its effect is caused by activation of extrinsic apoptotic pathway.

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