Journal of Dental Sciences 20 (2025) 1782–1791

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.e-jds.com

Original Article

3,4-methylenedioxymethamphetamine induces reactive oxygen species-mediated autophagy and thioredoxin-interactive protein/nucleotide-binding domain, leucine-rich containing family, pyrin domain-containing-3 inflammasome activation in dental pulp stem cells

Shih-Yu Lee [a], Shang-Wen Chiu [b,c], I-Hsun Li [d], Wei-Cheng Tsai [a], Chung-Hsing Li [b,c*]

[a] *Graduate Institute of Aerospace and Undersea Medicine, National Defense Medical Center, Taipei, Taiwan*
[b] *Division of Orthodontics, Pediatric Dentistry, and Special Needs Dentistry, Department of Dentistry, Tri-Service General Hospital, Taipei, Taiwan*
[c] *School of Dentistry and Graduate Institute of Dental Science, National Defense Medical Center, Taipei, Taiwan*
[d] *School of Pharmacy, National Defense Medical Center, Taipei, Taiwan*

Received 2 April 2025; Final revision received 23 April 2025
Available online 6 May 2025

KEYWORDS
Dental pulp stem cells (DPSCs);
3,4-methylenedioxy methamphetamine (MDMA);
Oxidative stress;
Nucleotide-binding domain, leucine-

Abstract *Background/purpose:* 3,4-methylenedioxymethamphetamine (MDMA) is a synthetic substituted amphetamine. Research primarily focuses on its neurotoxicity and psychological effects, while studies examining its impact on mesenchymal stem cells (MSCs) and dental pulp stem cells (DPSCs) are relatively limited. This study investigated the cytotoxicity and molecular mechanisms of MDMA in DPSCs.
Materials and methods: Cell viability, apoptosis, autophagy, immunophenotype, reactive oxygen species (ROS), and the related signaling pathways were analyzed using a cell counting kit, Western blot, flow cytometry, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) dye after treating DPSCs with indicated concentrations of MDMA.

* Corresponding author. School of Dentistry and Graduate Institute of Dental Science, National Defense Medical Center, No. 161, Sec. 6, Minquan E. Rd., Neihu Dist., Taipei City 11490, Taiwan.
E-mail address: chiyenchli@yahoo.com.tw (C.-H. Li).

https://doi.org/10.1016/j.jds.2025.04.025

rich containing family, pyrin domain-containing-3 (NLRP3) inflammasome; Autophagy

Results: MDMA significantly decreased cell viability and increased cleaved poly adenosine diphosphate-ribose polymerase (PARP), cleaved caspase-8, cleaved caspase-3, and Bcl-2-associated X protein (Bax), while reducing B-cell lymphoma 2 (Bcl-2). Annexin V and 7-aminoactinomycin D (7-AAD) flow cytometry showed that these cells underwent early apoptosis without altering MSCs' immunophenotypic properties. MDMA also induced ROS accumulation and nucleotide-binding domain, leucine-rich–containing family, pyrin domain –containing-3 (NLRP3) inflammasome activation. Autophagy activation was observed with adenosine monophosphate-activated protein kinase (AMPK) activation, protein kinase B (AKT) inactivation, and mammalian target of rapamycin (mTOR) suppression. Autophagy inhibitor chloroquine (CQ) attenuated MDMA-induced cell death, suggesting that excessive autophagy contributes to cell damage.

Conclusion: MDMA induces ROS-mediated autophagic cell death and thioredoxin-interacting protein (TXNIP)/NLRP3 inflammasome activation, causing detrimental cell damage. The historical roots of drug addiction should be considered when modifying dental approaches for patients with a history of MDMA use, as well as in the screening of DPSCs donors and patients undergoing stem cell therapy.

Introduction

3,4-methylenedioxymethamphetamine (MDMA) is an amphetamine derivative, commonly known as Ecstasy, Molly, and Adam. It was first synthesized by German pharmaceutical company Merck as a by-product during the research of blood-clotting agents in 1912.[1] It remained largely unstudied until the 1970s Shulgin resynthesized it, reported that MDMA can produce psychotomimetic effects, and suggested that MDMA might be useful as a psychotherapeutic adjunct.[2] The biological mechanisms of MDMA primarily involve serotonin and dopamine neurotransmitter systems.[3] It stimulates the release of these neurotransmitters, leading to significant alterations in both mood and environmental perception. Users seek out MDMA for its euphoric, empathogenic, hallucinatory, and stimulant effects. Its usage is particularly prevalent among the young population.[4] Although direct epidemiological data on MDMA use among dental stem cell donors are limited, a 2022 report on substance use in dental patients highlighted a substantial discrepancy between self-reported drug use during dental visits and estimates from national survey data. Specifically, the reported prevalence of MDMA use in dental electronic health records was significantly lower than that reported in the general U.S. population. For example, lifetime use of MDMA was reported at 10.6 % in the National Survey on Drug Use and Health (NSDUH), compared to just 0.1 % in dental records from the New York University College of Dentistry (NYUCD). Similarly, current use was reported as 0.6 % in the NSDUH and 0.0 % in the NYUCD records. This marked discrepancy suggests substantial underreporting or underassessment during dental appointments.[5] These findings underscore the likelihood that MDMA exposure may be underestimated among dental patients, including potential donors of dental stem cells, highlighting the relevance of investigating MDMA's impact on DPSC.

Accumulating evidence suggests that MDMA can cause neurotoxicity, cognitive deficits, and brain damage as a consequence of long-term abuse.[6–8] Several mechanisms have been implicated in MDMA-induced neurotoxicity, including oxidative stress, excitotoxicity, mitochondrial dysfunction, and necrosis.[9] These findings underscore the potential risks associated with MDMA use, particularly in high doses or over extended periods. MDMA also causes various health issues, including oral and dental health problems such as MDMA-induced bruxism,[10] increased risk of developing dental erosion,[11] and oral ulcers.[12] Recent MDMA research has significantly focused on its psychological treatment effects, particularly in addressing post-traumatic stress disorder (PTSD) and anxiety, as well as its impact on neural cells.[13–15] However, the evidence of its effect on mesenchymal stem cells (MSCs) or dental-derived MSCs is relatively limited and unexplored.

Dental pulp is the soft, living tissue located in the center of the tooth, consisting of connective tissue, nerves, and blood vessels. It provides sensory function, which responds to thermal and mechanical stimuli, providing pain sensation as a defensive mechanism.[16] Additionally, it plays a crucial role in repairing and regenerating dental tissues by forming tertiary dentin in response to external stimuli such as trauma, restorative procedure, or dental caries.[17] This dentin-pulp complex formation ability prompted the investigation of the stem cell potential in dental pulp cells. In 2000, Gronthos et al. successfully isolated dental pulp cells from adult human dental pulp and identified them as stem cells due to their clonogenicity, high proliferative capacity, and ability to regenerate tissue.[18] These unique properties of dental pulp stem cells (DPSCs) have significant clinical implications. Clinical approaches, such as vital pulp therapy, utilize these regenerative capabilities to preserve the vitality and function of the dental pulp after injury or disease and to stimulate the regeneration of the dentin-pulp complex.[19]

Additionally, DPSCs exhibit multipotent differentiation potential,[20] immunomodulation properties,[21] and can maintain the expression of key MSC markers after prolonged culture expansion.[22] It can be obtained from deciduous and wisdom teeth through small-scale local surgery, providing a

less invasive method of extracting stem cells. Although individual procedures yield minimal tissue volume and cell numbers, stem cell isolation achieves high success rates and efficiency even following long-term cryopreservation, with the cells exhibiting robust growth and differentiation potential in suitable culture environments.[23] These characteristics make DPSCs a valuable source in tissue engineering and regenerative medicine. Although tooth banking is not currently a popular practice, there has been a growing establishment of dental stem cell banks in a few countries, such as Korea, USA, United Kingdom, etc.[24] This study investigated the effects of MDMA on DPSCs and its molecular mechanisms, which may contribute to modifying clinical approaches on dental pulp therapy for patients with a history of MDMA use and establishing more comprehensive screening protocols and standards for stem cell banking and donation.

Materials and methods

Cell culture

DPSCs were obtained from Lonza (Basel, Switzerland) (Catalog #PT-5025) and cultured in DPSC Basal Medium (Lonza) supplemented with DPSCGM SingleQuots Kit (Lonza) in a humidified environment at 37 °C with 5 % (vol/vol) CO_2. The medium was replaced every two or three days. Cells were passaged at 80 % confluence, and passages 4—10 were used in the present study.

Cell viability assay

Cell viability was assessed using a cell counting kit (CCK-8, Dojindo, Tokyo, Japan). Briefly, DPSCs (1×10^3 cells/well) were seeded into 96-well plates overnight and then treated with different concentrations of MDMA (0, 0.1, 1, 10, 30, 100, 300, 500, 1000 and 2000 μM) for 48 h. The medium was removed with subsequent 10 % of the CCK-8 in DPSC Basal Medium incubation for 90 min. The absorbance was measured at 450/650 nm using a spectrophotometer (SpectraMax 190, Molecular Devices, San Jose, CA, USA).

Immunophenotype analysis

DPSCs surface markers were evaluated by flow cytometric analysis. After MDMA stimulation, cells were harvested and incubated with various antibody solutions (Invitrogen, Waltham, MA, USA), including MSCs-associated cell surface cluster of differentiation (CD) markers (CD44-PE, CD73-PE, and CD90-PE) and hematopoietic markers (CD31-PE, CD34-PE, and CD45-PE) for 1 h at room temperature. Data was acquired using a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest software.

Intracellular reactive oxygen species measurement

Intracellular reactive oxygen species (ROS) levels in DPSCs were measured using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) dye (Cell Biolabs, Inc., San Diego, CA, USA). Briefly, DPSCs were seeded in a 24-well plate (1×10^5 cells/well) overnight and treated with indicated concentrations of MDMA for 24 h. The medium was removed and replaced with 100 μM DCFH-DA in culture medium for 30 min at 37 °C in the dark. Intracellular ROS images were detected by an inverted fluorescence microscope with a CCD camera. Fluorescence quantitation was measured using a fluorescence microtiter plate reader (POLARstar Galaxy, BMG Labtech, Ortenberg, Germany) at 485 and 530 nm wavelengths.

Western blotting

In brief, proteins were extracted by RIPA lysis buffer, and the concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The denatured proteins were then separated by SDS-PAGE. After electrophoresis, proteins were transferred to the PVDF membrane, then blocked in 5 % non-fat dry milk for 30 min and incubated overnight with apoptosis, thioredoxin-interacting protein (TXNIP)/nucleotide-binding domain, leucine-rich—containing family, pyrin domain—containing-3 (NLRP3), and autophagy-related primary antibodies (Cell Signaling Technology, Danvers, MA, USA). Corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies were used for 1 h incubation at room temperature. The immunoreactive proteins were visualized using an enhanced chemiluminescence kit (ECL, Amersham Biosciences, Buckinghamshire, UK) and detected using a luminescent image analyzer (LAS-3000, Fujifilm, Tokyo, Japan).

Annexin V/7-aminoactinomycin D staining

The Annexin V and 7-aminoactinomycin D (7-AAD) Apoptosis Kit were used to detect cell apoptosis following the manufacturer's instructions (Biotium, Inc., Fremont, CA, USA). Briefly, after MDMA stimulation for 72 h, the cells were harvested and then resuspended in 100 μL binding buffer (5×10^5 cells/sample). Subsequently, cells were stained with 5 μL Annexin V and 2 μL 7-AAD for 30 min on ice in the dark. Data was acquired using FACSCalibur (BD Biosciences) and analyzed using CellQuest software.

Acridine orange staining

The acridine orange (AO) staining was used for autophagy detection following the manufacturer's instructions (MedChemExpress, Monmouth Junction, NJ, USA). Briefly, DPSCs were treated with indicated concentrations of MDMA for 48 h. Wash the cells with phosphate-buffered saline (PBS, Gibco, Waltham, MA, USA), and discard the solution, then add the culture medium containing AO at a concentration of 1 μg/mL and incubate for 1 h at 37 °C in the dark. Cells were washed and harvested for flow cytometry analysis using FACSCalibur (BD Biosciences) and CellQuest software.

Statistical analysis

Results represent the mean ± SEM (standard error of the mean). The sample size is 3 in each group ($n = 3$).

Significant differences among group means were determined via one-way ANOVA followed by a Bonferroni post-hoc test using IBM SPSS Statistics Version 22 (IBMr SPSSr Statistics 22). Statistical significance was accepted when $P < 0.05$.

Results

Cell viability and immunophenotypic property analysis

CCK-8 results demonstrated that the cell viability was significantly decreased when DPSCs were treated with 100, 300, 500, 1000, and 2000 μM of MDMA for 48 h (Fig. 1A). We further evaluated the MSCs immunophenotypic properties of DPSCs after 500 μM MDMA stimulation. The results demonstrated no significant changes in the expression of MSCs-associated positive (CD44, CD73, and CD90) and negative markers (CD31, CD34, and CD45) respectively (Fig. 1B).

ROS and associated TXNIP/NLRP3 inflammasome axis analysis

The ROS intensity significantly increased when DPSCs were treated with 10, 100, 300, and 500 μM of MDMA for 24 h (Fig. 2A and B). Western blot was performed with TXNIP-NLRP3 associated proteins (TXNIP, NLRP3, apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain [ASC], pro-caspase-1, cleaved caspase-1, pro-interleukin (IL)-1β, and IL-1β) (Fig. 2C). The relative protein expression levels of TXNIP (Fig. 2D), NLRP3 (Fig. 2E), cleaved caspase-1 (Fig. 2F), and IL-1β (Fig. 2G) were significantly increased.

Cell apoptosis analysis

The annexin V and 7-AAD double-staining showed that the apoptotic cells significantly increased when treating DPSCs with 500 μM of MDMA for 72 h (Fig. 3A and B). Western blot

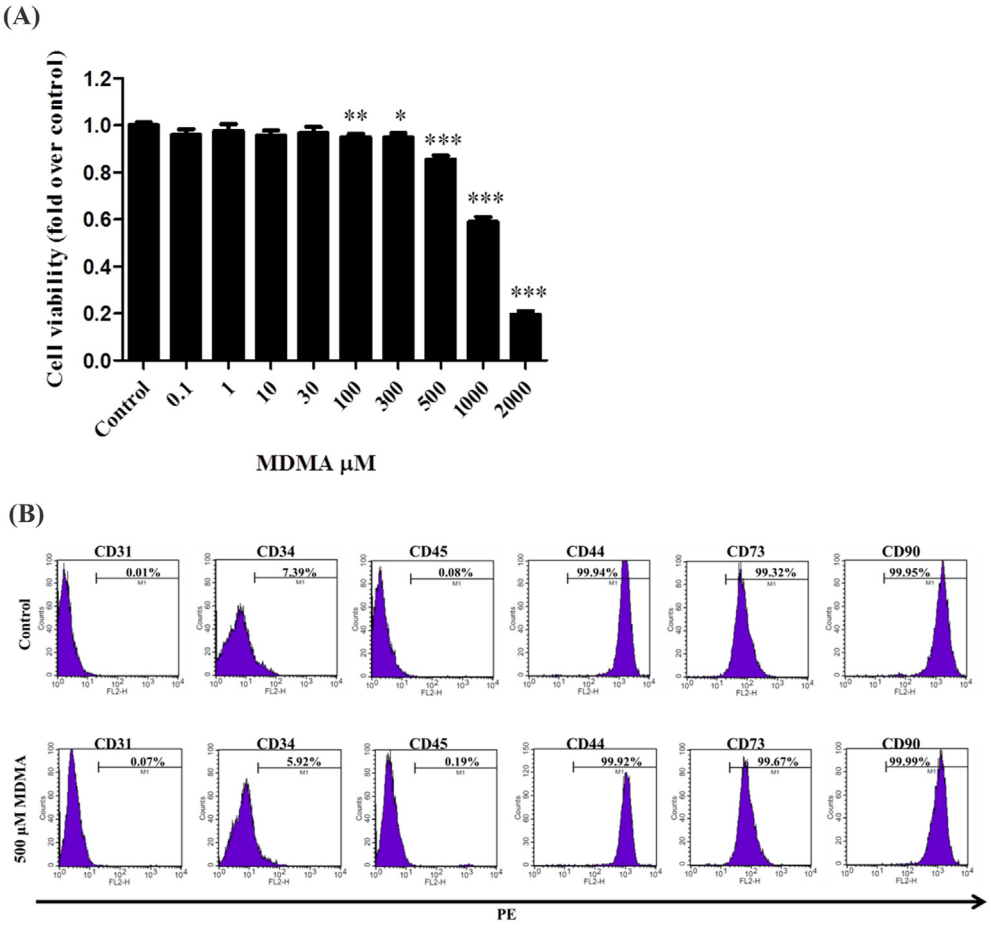

Figure 1 Effects of MDMA on cell viability and MSCs immunophenotypic property in DPSCs. DPSCs were treated with 0, 0.1, 1, 10, 30, 100, 300, 500, 1000 and 2000 μM of MDMA for 48 h. Cell viability was measured using a cell counting kit (CCK-8), and the results represent the mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control (A). Cells were treated with and without 500 μM of MDMA for 48 h, and the MSCs immunophenotypic property of DPSCs was assessed by flow cytometry. MSCs lineage markers (CD44, CD73, and CD90) and hematopoietic lineage markers (CD31, CD34, and CD45) were detected (B). Abbreviation: MDMA - 3,4-methylenedioxymethamphetamine, MSCs - mesenchymal stem cells, DPSCs - dental pulp stem cells, CD - cluster of differentiation.

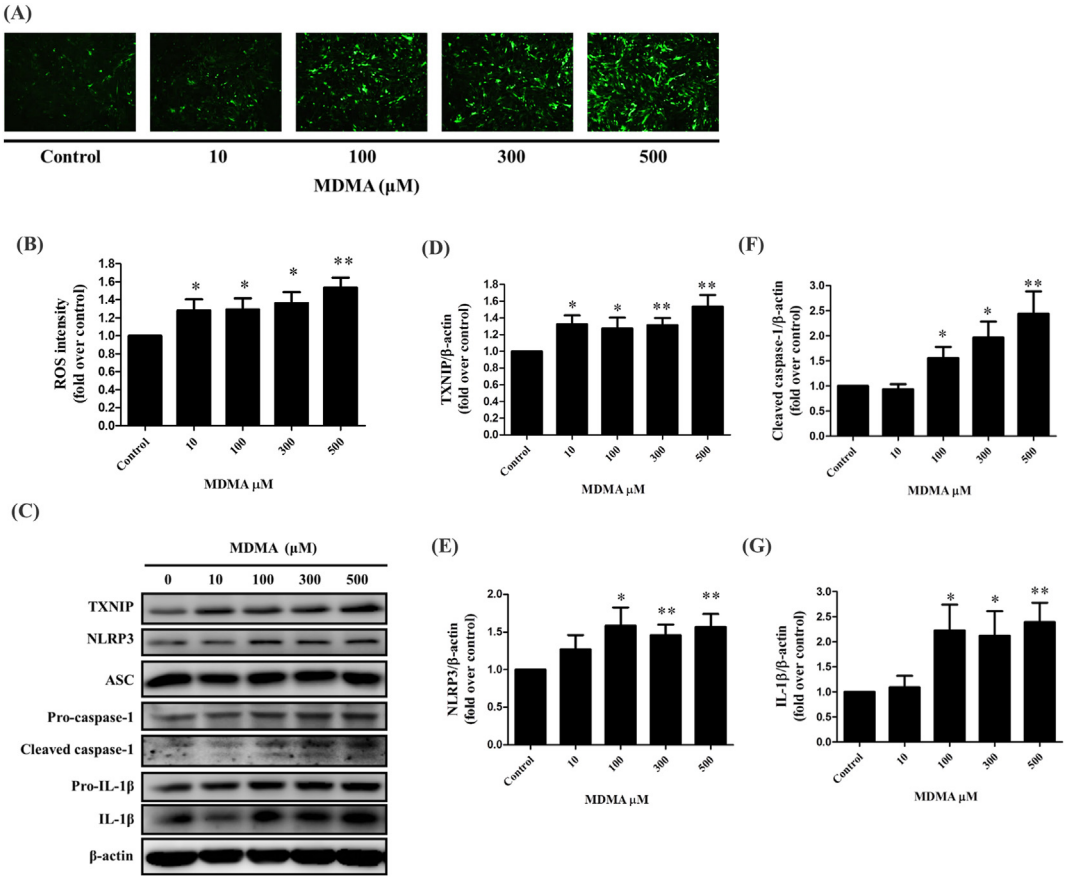

Figure 2 Effects of MDMA on ROS-TXNIP-NLRP3 inflammasome axis in DPSCs. DPSCs were treated with 0, 10, 100, 300, and 500 μM of MDMA for 24 h. The ROS formation was detected by DCFH-DA dye with an inverted fluorescence microscope (A), and the fluorescence intensity was measured by a microtiter plate reader (B). Protein expression of TXNIP, NLRP3, ASC, pro-caspase-1, cleaved caspase-1, pro-IL-1β, and IL-1β were analyzed by Western blot (C). Quantitative analysis of TXNIP (D), NLRP3 (E), cleaved caspase-1 (F), and IL-1β (G) were conducted. Results represent the mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. Abbreviation: MDMA - 3,4-methylenedioxymethamphetamine, ROS - reactive oxygen species, TXNIP - thioredoxin-interacting protein, NLRP3 - nucleotide-binding domain, leucine-rich—containing family, pyrin domain-containing 3, DPSCs - dental pulp stem cells, DCFH-DA - 2′,7′-dichlorofluorescein diacetate, ASC - apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain, IL - interleukin.

was performed with apoptosis-related proteins (poly adenosine diphosphate-ribose polymerase [PARP], cleaved PARP, pro-caspase-8, cleaved caspase-8, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, Bcl-2-associated X protein [Bax], and B-cell lymphoma 2 [Bcl-2]) (Fig. 3C and D). The levels of cleaved PARP (Fig. 3E), cleaved caspase-8 (Fig. 3F), and cleaved caspase-3 (Fig. 3G) significantly increased, whereas cleaved caspase-9 showed no significant difference (Fig. 3H). The pro-apoptotic protein Bax was significantly increased with decreasing anti-apoptotic protein Bcl-2 expression in high concentrations of MDMA stimulation (Fig. 3I and J).

Cell autophagy analysis

Western blot was further performed with autophagy-related proteins (microtubule-associated protein 1 light chain 3 beta [LC3B]-I and —II, sequestosome-1 [p62], autophagy related [ATG]12-ATG5, ATG7, and Beclin-1) (Fig. 4A). MDMA significantly increases the LC3B-II/LC3B—I ratio in a dose-dependent manner (Fig. 4B). Moreover, the results also revealed that the protein levels of p62 (Fig. 4C), ATG12-ATG5 (Fig. 4D), and ATG7 (Fig. 4E) were increased by MDMA stimulation for 48 h. However, the level of Beclin-1 showed no significant difference (Fig. 4F). Autophagy activation was consistently detected and analyzed by AO staining and flow cytometry (Fig. 4G and H). The population of autophagy cells significantly increases in a dose-dependent manner (Fig. 4I). We further found that MDMA regulated the upstream regulators of autophagy pathways (Fig. 4J) with significant decrease in phosphorylated protein kinase B [p-AKT]/AKT and phosphorylated mammalian target of rapamycin [p-mTOR]/mTOR (Fig. 4K and L), while the phosphorylated adenosine monophosphate-activated protein kinase [p-AMPK]/AMPK increased (Fig. 4M). To clarify the relationship between autophagy and apoptosis in MDMA-treated DPSCs, the autophagy inhibitor chloroquine

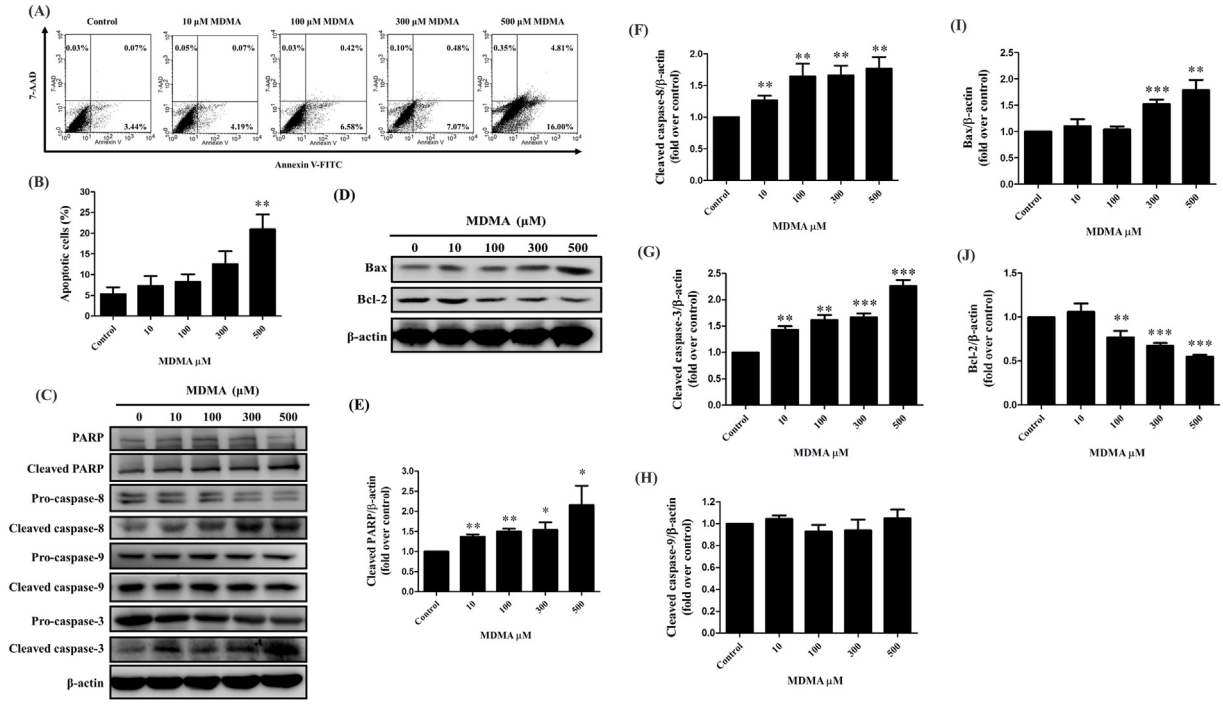

Figure 3 Effects of MDMA on apoptosis in DPSCs. DPSCs were treated with 0, 10, 100, 300, and 500 μM of MDMA for 72 h, and cell apoptosis was detected by annexin V and 7-AAD double staining (A). The apoptotic cell percentage is displayed (B). Protein expression of apoptosis-related proteins PARP, cleaved PARP, pro-caspase-8, cleaved caspase-8, pro-caspase-9, cleaved caspase-9, pro-caspase-3, cleaved caspase-3, Bax, and Bcl-2 were analyzed by Western blot (C and D). Quantitative analysis of cleaved PARP (E), cleaved caspase-8 (F), cleaved caspase-3 (G), cleaved caspase-9 (H), Bax (I), and Bcl-2 (J) was conducted. Results represent mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. Abbreviation: MDMA - 3,4-Methylenedioxymethamphetamine, DPSCs - dental pulp stem cells, 7-AAD - 7-aminoactinomycin D, PARP - poly adenosine diphosphate-ribose polymerase, Bax - Bcl-2-associated X protein, Bcl-2 - B-cell lymphoma 2.

(CQ) (MedChemExpress) was conducted. Cells were pre-treated with 2 μM of CQ for 1 h, followed by incubation with MDMA for 24 h. The effects of CQ intervention on cell viability and the expression of LC3B–I, LC3B -II, pro-caspase-3, cleaved caspase-3, PARP, and cleaved PARP in MDMA-treated cells were detected (Fig. 4N and O). The cell viability significantly increased with CQ intervention before 500 and 1000 μM of MDMA stimulation. Western blot results demonstrated that CQ intervention before MDMA did not alter the protein expression level of the LC3B-II/LC3B–I ratio (Fig. 4P). However, it significantly diminished the levels of cleaved caspase-3 and cleaved PARP compared to 500 μM MDMA-treated cells (Fig. 4Q and R).

Discussion

MDMA is an amphetamine derivative known for its neurotoxic effects, largely due to its ability to induce oxidative stress and trigger various intracellular pathways that lead to cell damage. Long-term use of amphetamines can cause serious oral health effects, including methamphetamine-induced caries, periodontal diseases, and oral ulcers.[12,25] Other structural analogues of amphetamine, such as MDA have also been reported to cause bruxism.[10] While its impacts on MSCs and dental-derived MSCs were relatively limited, the present study investigated the mechanisms by which MDMA affects DPSCs, with a particular focus on its role in cell viability, oxidative stress, NLRP3 inflammasome, autophagy, and apoptosis.

The concentration of MDMA used in in vitro studies varies widely depending on the research model, ranging from nanomolar (nM) to millimolar (mM). For example, studies on arginine vasopressin (AVP) release have utilized concentrations ranging from 0.1 to 1000 nM to examine the effects of MDMA on isolated rat hypothalamus,[26] while investigations into cardiac toxicity have employed concentrations of 1000 to 10,000 μM (1–10 mM) to assess mechanisms of cellular damage.[27] A previous study reported that, in a double-blind placebo controlled trial involving a 75 mg oral dose of MDMA administered to healthy recreational users, the average peak plasma concentration was 178 ng/mL,[28] corresponding to approximately 0.92 μM based on MDMA's molecular weight (193.25 g/mol). Although the concentrations used in our experiments (0.1–2000 μM) exceed typical human plasma levels, they are consistent with prior mechanistic studies aimed at elucidating cellular responses under heightened exposure and exploring potential dose-dependent cellular effects. In the present study, significant effects were observed starting from as low as 10 μM in some conditions, while in others, notable changes emerged at 100 μM or even

300 μM. These effects remained significant at higher concentrations, demonstrating a clear dose-dependent pattern. This aligns with previous studies suggesting that MDMA exerts concentration-dependent impacts on cell viability, oxidative stress, and other cellular processes.

The observed increase in cleaved-PARP, cleaved caspase-8, cleaved caspase-3, and Bax expression, alongside a decrease in Bcl-2 and positive Annexin V/7-AAD double-staining, confirmed the induction of early apoptosis in DPSCs. Despite this cytotoxicity, the immunophenotypic profile associated with MSCs remained unchanged, suggesting that MDMA may not compromise the core stem cell identity in the short term. However, studies on neural stem and progenitor cells have shown that chronic or repeated MDMA exposure can markedly reduce cell proliferation and impair neurogenesis, with long-term exposure in animal models leading to significant declines in neural progenitor cell populations and neuronal differentiation.[29] These findings raise the possibility that chronic MDMA exposure could exert more profound or cumulative effects on stem cell populations, which may not be captured by short-term assays.

The elevation of ROS levels is a key factor in the oxidative stress response, which contributes to cellular damage, triggering both autophagy and apoptosis. Our results demonstrated that MDMA significantly induced cellular ROS accumulation and apoptosis in DPSCs. This response can lead to cellular damage, impacting their therapeutic potential. Previous studies indicated that ROS can trigger NLRP3 inflammasome activation through TXNIP.[30] NLRP3 inflammasome activation can enhance host defense immunity such as releasing IL-1β and IL-18,[31] and it is also

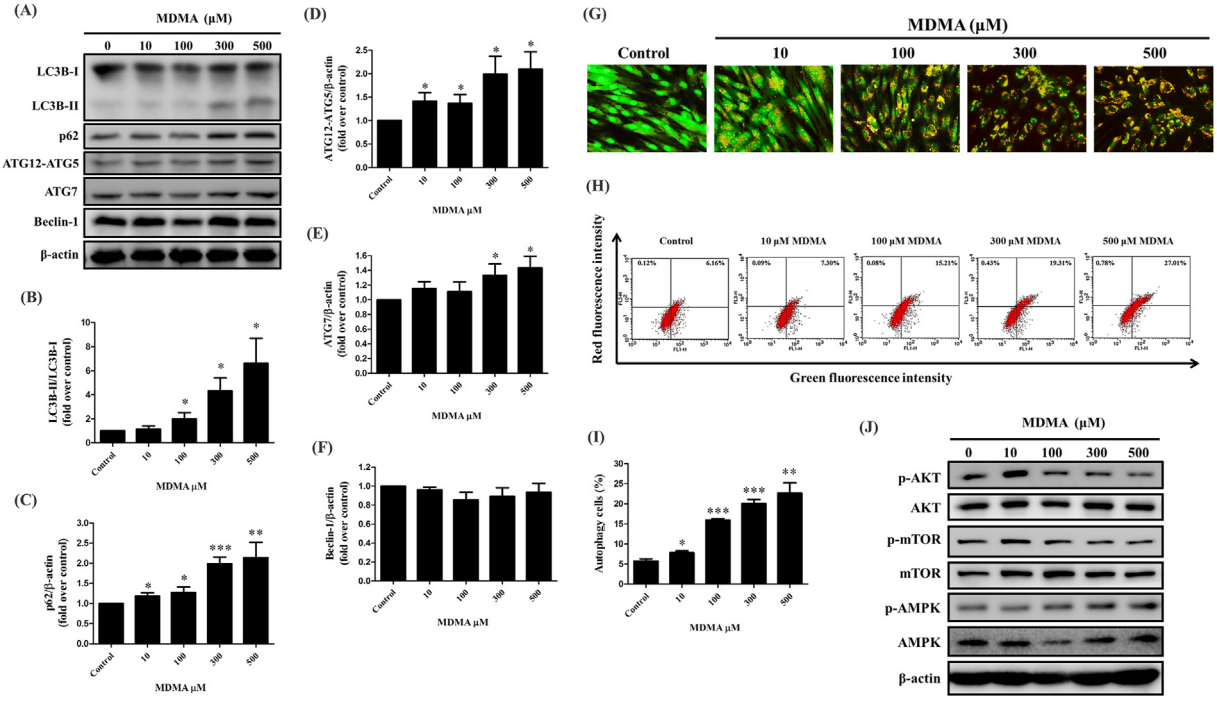

Figure 4 Effect of MDMA on autophagy signaling pathways in DPSCs. DPSCs were treated with 0, 10, 100, 300, and 500 μM of MDMA for 48 h, and the protein levels of LC3B—I, LC3B-II, p62, ATG12-ATG5, ATG7, and Beclin-1 were analyzed by Western blot (A). Quantitative analysis of LC3B-II/LC3B—I (B), p62 (C), ATG12-ATG5 (D), ATG7 (E), and Beclin-1(F) were conducted. Results represent mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. Cell autophagy was detected by acridine orange staining (G) and flow cytometry (H). The population of DPSCs underwent autophagy was conducted (I). Moreover, the upstream signal pathways of autophagy (AKT, mTOR, and AMPK) were assessed by Western blot (J). Quantitative analysis of the protein expression levels of p-AKT/AKT (K), p-mTOR/mTOR (L), and p-AMPK/AMPK (M) were also conducted. Results represent mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. DPSCs were pre-treated with 2 μM of CQ for 1 h, followed by incubation with MDMA for 24 h. Cell viability was evaluated (N), and the effects of CQ, 500 μM MDMA, and CQ intervention before MDMA on the protein expression levels of LC3B—I, LC3B-II, Pro-caspase-3, cleaved caspase-3, PARP, and cleaved PARP were detected by Western blot (O). Quantitative analysis of LC3B-II/LC3B—I (P), cleaved caspase-3 (Q), and cleaved PARP (R) were conducted. Results represent mean ± SEM (n = 3), analyzed via one-way ANOVA followed by a Bonferroni post-hoc test. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus control. #$P < 0.05$, ##$P < 0.01$, and ###$P < 0.001$ for CQ intervention before MDMA versus MDMA stimulation. Abbreviation: MDMA - 3,4-Methylenedioxymethamphetamine, DPSCs - dental pulp stem cells, LC3B-microtubule-associated protein 1 light chain 3 beta, p62-sequestosome-1, ATG-autophagy related, AKT - protein kinase B, p-AKT - phosphorylated protein kinase B, mTOR - mammalian target of rapamycin, p-mTOR - phosphorylated mammalian target of rapamycin, AMPK - adenosine monophosphate-activated protein kinase, p-AMPK - phosphorylated adenosine monophosphate-activated protein kinase, CQ - chloroquine, PARP - Poly adenosine diphosphate-ribose polymerase.

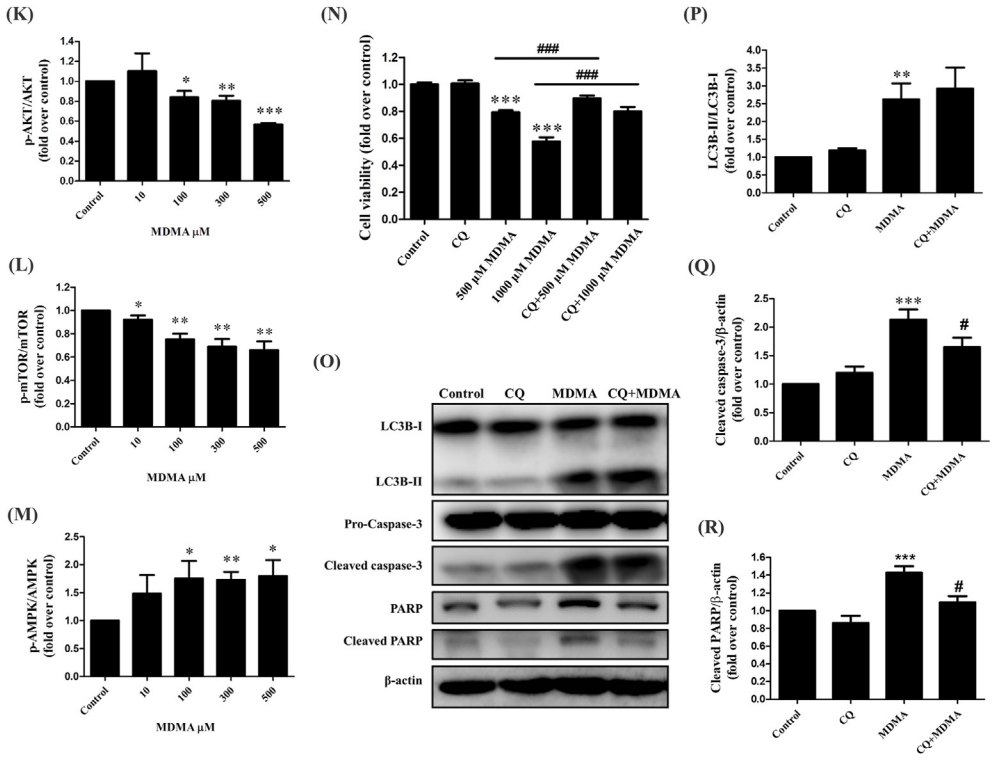

Figure 4 (*continued*).

related to periodontitis and pulpitis.[32,33] In our study, MDMA induces TXNIP-NLRP3 inflammasome activation with significantly increased expression of TXNIP, NLRP3, cleaved caspase-1, and IL-1β, indicating that MDMA may cause inflammatory reactions and damage to DPSCs. Apart from these, NLRP3 inflammasome activation has been reported to enhance adipogenic differentiation and inhibit osteogenic differentiation in MSCs derived from human umbilical cord.[34] The specific role and effect of NLRP3 inflammasome in DPSCs differentiation potential needs further investigation.

Oxidative stress is also closely related to autophagy signaling.[35] Autophagy is a cellular process involved in the recycling of organelles and maintaining cellular homeostasis, and it is closely related to the regulation of stem cell self-renewal and differentiation in hematopoietic stem cells (HSCs), bone marrow-derived MSCs, neural stem cells (NSCs), adipose-derived stem cells (ASCs), etc.[36] Autophagy activated by nanomaterials has also been reported to promote odontogenic differentiation of DPSCs.[37] The present study demonstrated that MDMA stimulation can lead to the activation of autophagy in DPSCs, as indicated by the increased expression of autophagy-related markers such as LC3B, p62, ATG12-5, ATG7, and Beclin-1. We further found that the AMPK pathway was activated with AKT and mTOR pathway suppression. However, the differentiation potential and clinical impacts of MDMA-induced autophagy on DPSCs require further exploration. Autophagy response is thought to play a biphasic role in cells, potentially contributing to supporting cell survival by recycling damaged components, while excessive autophagy activation can lead to autophagic cell death. For instance, autophagy can reduce periodontal inflammation and promote cell survival in periapical lesions or worsen periodontal diseases by promoting apoptosis and inflammation.[38] To clarify the relationship between autophagy and apoptosis in MDMA-treated DPSCs, the autophagy inhibitor CQ was conducted. CQ functions primarily by inhibiting lysosomal acidification and consequently blocking autophagy.[39] We investigated the effect of CQ on MDMA-treated DPSCs and found that the use of CQ can attenuate MDMA-induced cleaved caspase 3, cleaved PARP activation, and cell death. This indicates that excessive autophagy activation induced by MDMA contributes to cell damage, consistent with previous findings that MDMA can excessively activate autophagy, leading to damage in nerve cells.[40]

In conclusion, the present study demonstrated that the fundamental immunophenotypic characteristics of MSCs in DPSCs are not altered after MDMA stimulation. However, MDMA can induce ROS-mediated TXNIP/NLRP3 inflammasome and autophagy activation, contributing to detrimental effects in DPSCs. And the use of CQ can partially improve cell viability in MDMA-treated DPSCs. Understanding these complex interactions not only aids in preventing and treating MDMA-induced damage to DPSCs and other stem cell populations but also contributes to the establishment of comprehensive screening protocols for stem cell donation. Moreover, the historical roots of drug addiction should be considered in modifying dental approaches for patients with a history of MDMA use.

This study primarily investigated the short-term effects of high-dose MDMA exposure on DPSCs under in vitro conditions. While this approach enabled the assessment of immediate cellular responses with proposed mechanisms (Fig. 5), it does not account for potential alterations that may arise from chronic or repeated exposure, which may

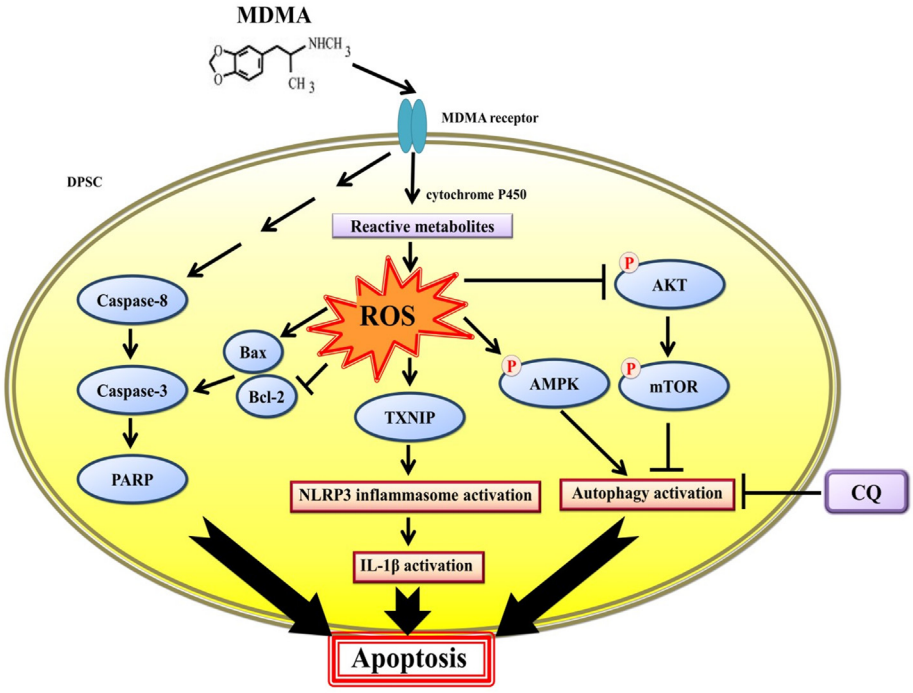

Figure 5 Proposed mechanisms of MDMA on DPSCs. MDMA induces ROS-TXNIP-NLRP3 inflammasome and autophagy activation, contributing to cytotoxicity in DPSCs. Abbreviation: MDMA - 3,4-Methylenedioxymethamphetamine, DPSCs - dental pulp stem cells, ROS - reactive oxygen species, TXNIP - thioredoxin-interacting protein, NLRP3 - nucleotide-binding domain, leucine-rich—containing family, pyrin domain-containing 3, PARP - poly adenosine diphosphate-ribose polymerase, Bax - Bcl-2-associated X protein, Bcl-2 - B-cell lymphoma 2, IL - interleukin, AMPK - adenosine monophosphate-activated protein kinase, AKT - protein kinase B, mTOR - mammalian target of rapamycin, CQ — chloroquine.

better reflect real-world substance use patterns. Therefore, the findings may not fully represent the in vivo consequences of long-term MDMA exposure on stem cell properties. Further studies incorporating chronic exposure models and donor-derived DPSCs from individuals with a history of MDMA use are warranted to evaluate the long-term effects on stem cell viability, differentiation potential, and therapeutic utility.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

Acknowledgment

I would like to sincerely thank Ms. Kuan-Ming Lin for her editorial contributions, revision, and support throughout the writing process.

References

1. Benzenhöfer U, Passie T. The early history of "ecstasy". *Nervenarzt* 2006;77:95—9.
2. Benzenhöfer U, Passie T. Rediscovering MDMA (ecstasy): the role of the American chemist alexander T. Shulgin. *Addiction* 2010;105:1355—61.
3. Schenk S, Highgate Q. Methylenedioxymethamphetamine (MDMA): serotonergic and dopaminergic mechanisms related to its use and misuse. *J Neurochem* 2021;157:1714—24.
4. Yang KH, Kepner W, Nijum A, Han BH, Palamar JJ. Prevalence and correlates of past year ecstasy/MDMA use in the United States. *J Addiction Med* 2023;17:592—7.
5. Le A, Palamar JJ. Underreported drug use among dental patients: a call for dental care professionals to improve drug use screening. *J Am Dent Assoc* 2023;154:191—3.
6. Sarkar S, Schmued L. Neurotoxicity of ecstasy (MDMA): an overview. *Curr Pharm Biotechnol* 2010;11:460—9.
7. Pantoni MM, Anagnostaras SG. Cognitive effects of MDMA in laboratory animals: a systematic review focusing on dose. *Pharmacol Rev* 2019;71:413—49.
8. Capela JP, Carmo H, Remião F, Bastos ML, Meisel A, Carvalho F. Molecular and cellular mechanisms of ecstasy-induced neurotoxicity: an overview. *Mol Neurobiol* 2009;39:210—71.
9. Halpin LE, Collins SA, Yamamoto BK. Neurotoxicity of methamphetamine and 3, 4-methylenedioxymethamphetamine. *Life Sci* 2014;97:37—44.
10. Dinis-Oliveira RJ, Caldas I, Carvalho F, Magalhães T. Bruxism after 3,4-methylenedioxymethamphetamine (ecstasy) abuse. *Clin Toxicol* 2010;48:863—4.
11. Brand HS, Dun SN, Nieuw Amerongen AV. Ecstasy (MDMA) and oral health. *Br Dent J* 2008;204:77—81.
12. Biancardi MR, Da Silveira HA, Fernandes D, et al. Ecstasy abuse and its effects on the oral mucosa. *J Craniofac Surg* 2019;30:e189—91.
13. Latimer D, Stocker MD, Sayers K, et al. MDMA to treat PTSD in adults. *Psychopharmacol Bull* 2021;51:125—49.
14. Wolfson PE, Andries J, Feduccia AA, et al. MDMA-assisted psychotherapy for treatment of anxiety and other

psychological distress related to life-threatening illnesses: a randomized pilot study. *Sci Rep* 2020;10:20442.
15. Hosseini A, Shetab Boushehri SMH, Shetab Boushehri SV. Evaluation of cytotoxic, necrotic, apoptotic, and autophagic effects of methamphetamine and 3, 4-methylenedioxymethamphetamine on U-87 MG (glial) and B104-1-1 (neuronal) cell lines. *Neurotox Res* 2022;40: 1499—515.
16. Yu C, Abbott PV. An overview of the dental pulp: its functions and responses to injury. *Aust Dent J* 2007;52:S4—16.
17. Farooq I, Ali S, Anderson P, eds. *Dentin. An illustrated guide to oral histology*, 1st ed. United States: Wiley, 2021:35—53.
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;97:13625—30.
19. Zhang W, Yelick PC. Vital pulp therapy—Current progress of dental pulp regeneration and revascularization. *Int J Dent* 2010;2010:856087.
20. Nuti N, Corallo C, Chan B, Ferrari M, Gerami Naini B. Multipotent differentiation of human dental pulp stem cells: a literature review. *Stem Cell Rev Rep* 2016;12:511—23.
21. Min Q, Yang L, Tian H, Tang L, Xiao Z, Shen J. Immunomodulatory mechanism and potential application of dental pulp-derived stem cells in immune-mediated diseases. *Int J Mol Sci* 2023;24:8068.
22. Bakopoulou A, Apatzidou D, Aggelidou E, et al. Isolation and prolonged expansion of oral mesenchymal stem cells under clinical-grade, GMP-compliant conditions differentially affects "stemness" properties. *Stem Cell Res Ther* 2017;8:247.
23. Papaccio G, Graziano A, d'Aquino R, et al. Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol* 2006;208:319—25.
24. Sivolella S, Scanu A, Xie Z, Vianello S, Stellini E. Biobanking in dentistry: a review. *Jpn Dent Sci Rev* 2022;58:31—40.
25. Ye T, Sun D, Dong G, et al. The effect of methamphetamine abuse on dental caries and periodontal diseases in an Eastern China city. *BMC Oral Health* 2018;18:8.
26. Fallon JK, Shah D, Kicman A, et al. Action of MDMA (ecstasy) and its metabolites on arginine vasopressin release. *Ann N Y Acad Sci* 2002;965:399—409.
27. Tiangco DA, Lattanzio FA, Osgood CJ, Beebe SJ, Kerry JA, Hargrave BY. 3, 4-Methylenedioxymethamphetamine activates nuclear factor-κB, increases intracellular calcium, and modulates gene transcription in rat heart cells. *Cardiovasc Toxicol* 2005;5:301—10.
28. Samyn N, Boeck GD, Wood M, et al. Plasma, oral fluid and sweat wipe ecstasy concentrations in controlled and real life conditions. *Forensic Sci Int* 2002;128:90—7.
29. Dutta RR, Taffe MA, Mandyam CD. Chronic administration of amphetamines disturbs development of neural progenitor cells in young adult nonhuman Primates. *Prog Neuropsychopharmacol Biol Psychiatry* 2018;85:46—53.
30. Lane T, Flam B, Lockey R, Kolliputi N. TXNIP shuttling: missing link between oxidative stress and inflammasome activation. *Front Physiol* 2013;4:50.
31. Laudisi F, Spreafico R, Evrard M, et al. Cutting edge: the NLRP3 inflammasome links complement-mediated inflammation and IL-1β release. *J Immunol* 2013;191:1006—10.
32. Marchesan JT, Girnary MS, Moss K, et al. Role of inflammasomes in the pathogenesis of periodontal disease and therapeutics. *Periodontol* 2000 2020;82:93—114.
33. Jiang W, Lv H, Wang H, et al. Activation of the NLRP3/caspase-1 inflammasome in human dental pulp tissue and human dental pulp fibroblasts. *Cell Tissue Res* 2015;361:541—55.
34. Wang L, Chen K, Wan X, Wang F, Guo Z, Mo Z. NLRP3 inflammasome activation in mesenchymal stem cells inhibits osteogenic differentiation and enhances adipogenic differentiation. *Biochem Biophys Res Commun* 2017;484:871—7.
35. Filomeni G, De Zio D, Cecconi F. Oxidative stress and autophagy: the clash between damage and metabolic needs. *Cell Death Differ* 2015;22:377—88.
36. Chen X, He Y, Lu F. Autophagy in stem cell biology: a perspective on stem cell self-renewal and differentiation. *Stem Cell Int* 2018;2018:9131397.
37. Lu J, Li R, Ni S, et al. Metformin carbon nanodots promote odontoblastic differentiation of dental pulp stem cells by pathway of autophagy. *Front Bioeng Biotechnol* 2022;10: 1002291.
38. Tan YQ, Zhang J, Zhou G. Autophagy and its implication in human oral diseases. *Autophagy* 2017;13:225—36.
39. Pasquier B. Autophagy inhibitors. *Cell Mol Life Sci* 2016;73: 985—1001.
40. Li IH, Ma KH, Weng SJ, Huang SS, Liang CM, Huang YS. Autophagy activation is involved in 3,4-methylenedioxymethamphetamine ('ecstasy')-induced neurotoxicity in cultured cortical neurons. *PLoS One* 2014;9: e116565.