Original article DOI: 10.2478/aiht-2023-74-3727

The effects of ketamine on viability, primary DNA damage, and oxidative stress parameters in HepG2 and SH-SY5Y cells

Andreja Jurič[1], Blanka Tariba Lovaković[1], Antonio Zandona[1], Dubravka Rašić[1], Martin Češi[2], Alica Pizent[1], Marijana Neuberg[3], Irena Canjuga[3], Maja Katalinić[1], Ana Lucić Vrdoljak[1], Arnes Rešić[4], and Irena Brčić Karačonji[1,5]

[1] Institute for Medical Research and Occupational Health, Zagreb, Croatia
[2] University of Zagreb Faculty of Food Technology and Biotechnology, Zagreb, Croatia
[3] University North, University Centre Varaždin, Varaždin, Croatia
[4] Children's Hospital Zagreb Department of Paediatrics, Zagreb, Croatia
[5] University of Rijeka Faculty of Health Studies, Rijeka, Croatia

[Received in March 2023; Similarity Check in March 2023; Accepted in June 2023]

Ketamine is a dissociative anaesthetic used to induce general anaesthesia in humans and laboratory animals. Due to its hallucinogenic and dissociative effects, it is also used as a recreational drug. Anaesthetic agents can cause toxic effects at the cellular level and affect cell survival, induce DNA damage, and cause oxidant/antioxidant imbalance. The aim of this study was to explore these possible adverse effects of ketamine on hepatocellular HepG2 and neuroblastoma SH-SY5Y cells after 24-hour exposure to a concentration range covering concentrations used in analgesia, drug abuse, and anaesthesia (0.39, 1.56, and 6.25 μmol/L, respectively). At these concentrations ketamine had relatively low toxic outcomes, as it lowered HepG2 and SH-SY5Y cell viability up to 30 %, and low, potentially repairable DNA damage. Interestingly, the levels of reactive oxygen species (ROS), malondialdehyde (MDA), and glutathione (GSH) remained unchanged in both cell lines. On the other hand, oxidative stress markers [superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT)] pointed to ketamine-induced oxidant/antioxidant imbalance.

KEY WORDS: anaesthetic; antioxidant enzymes; drug abuse; ROS; toxicity

Ketamine (2-(2-chlorophenyl)-2-(methylamino)-cyclohexanone), a derivative of phencyclidine, is a dissociative anaesthetic widely used in clinical practice, while its sub-anaesthetic administration has potential for the treatment of depression and chronic pain (1). However, ketamine is also the main ingredient in the so-called "date-rape" pills, whose abuse has been on increase (2). Owing to its hallucinogenic effects, it is also often used as a recreational drug (1).

Regarding its biological activity, ketamine (aka "K", "vitamin K" or "special K") is a non-competitive inhibitor of *N*-methyl-*D*-aspartate (NMDA), a sub-type of glutamate receptors, but at relatively high concentrations it can also interact with opioid receptors and type A gamma-aminobutyric acid receptors (1, 3).

It can cause a wide range of dose-dependent effects, from analgesia at lower doses (peak plasma concentrations 0.29–0.67 μmol/L) (4) to anaesthesia at higher doses (peak plasma concentrations 5–10 μmol/L) (4–7).

A wide range of effects are also attributed to two optical stereoisomers of ketamine: *S*(+) with four times greater affinity for the NMDA receptor than *R*(-), a less potent ketamine. *S*(+)-ketamine possess twice higher analgesic potency and fewer psychotomimetic effects (1, 7).

Corresponding author: Antonio Zandona, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, HR-10000 Zagreb, Croatia
E-mail: *azandona@imi.hr*

In spite of widespread use for various purposes, many aspects of ketamine toxicity are still poorly described. This study is based on the hypothesis that ketamine as a cell-receptor binding compound can cause diverse effects at the cellular level as well, that is, affect cell viability and survival, induce DNA damage, and disturb the oxidant/antioxidant balance. In order to verify this, we chose two cell models: hepatocellular HepG2 and neuroblastoma SH-SY5Y, each expressing ketamine-binding receptors of interest or metabolic enzymes responsible for ketamine metabolism (8–12). In a battery of cell-based assays we investigated the extent of cytotoxic and genotoxic effects and various factors associated with oxidative stress following 24 h exposure to ketamine.

MATERIALS AND METHODS

Chemicals and reagents

The analytical standard ketamine, consisting of a mixture of both isomers (1 mg/mL in methanol) was purchased from Lipomed (Arlesheim, Switzerland). Potassium dihydrogen phosphate (KH_2PO_4) was obtained from Merck (Darmstadt, Germany).

Ultrapure water (18 MΩ) was obtained from a Barnstead™ Smart2Pure™ 3 UV/UF Water Purification System (Thermo Fisher Scientific, Waltham, MA USA). Potassium hydroxide (KOH) and phosphoric acid (H_3PO_4) were purchased from Kemika (Zagreb, Croatia).

Other reagents and chemicals were bought from Sigma-Aldrich (Steinheim, Germany). Staurosporine, well-known inductor of apoptosis, was used as positive control for cytotoxicity experiments. Hydrogen peroxide (H_2O_2), oxidising agent, was used as positive control for other assays.

Cells

Cell lines used in the experiments were obtained from the European Collection of Authenticated Cell Cultures (ECACC) (Sigma-Aldrich). Human Caucasian hepatocyte carcinoma HepG2 cells (ECACC 85011430) were grown in Eagle's Minimum Essential (EMEM) medium containing 10 % (v/v) foetal bovine serum, 1 % (v/v) penicillin/streptomycin, and 1 % (v/v) non-essential amino acids. Human neuroblastoma SH-SY5Y cells (ECACC 94030304) were grown in Dulbecco's Modified Eagle's Medium F12 (DMEM F12) containing 15 % (v/v) foetal bovine serum, 2 mmol/L glutamine, 1 % (v/v) penicillin/streptomycin, and 1 % (v/v) non-essential amino acids. All media and supplements were purchased from Sigma-Aldrich. All cells were cultured at 37 °C in a 5 % CO_2 atmosphere, and the medium was changed every few days and passaged according to the manufacturer's protocol.

Cells for the viability assay were seeded in transparent 96-well plates at a density of 20,000 cells/well one day before the assay. Cells for reactive oxygen species and glutathione measurements were seeded in black 96-well plates at a density of 10,000 cells/well. For other assays they were seeded in transparent 24-well plates at a density of 50,000 cells/well. For the viability assay both cell lines were treated with ketamine concentrations from 0.39 to 100 μmol/L. For DNA damage and cell oxidative status determination ketamine concentrations corresponded to those found in human plasma for analgesia (0.09 mg/L i.e. 0.39 μmol/L), anaesthesia (1.49 mg/L i.e. 6.25 μmol/L), and drug abuse (0.37 mg/L i.e. 1.56 μmol/L) (6). The highest tested concentration of 100 μmol/L was selected based on reports that this ketamine concentration affects calcium signalling, which leads to apoptosis in undifferentiated neural PC12 cells (13).

All treated samples were compared to untreated control cells, based on results obtained from at least three independent experiments (each treatment performed in duplicate or triplicate).

Measurement of cell viability – MTS assay

Cytotoxic properties of ketamine were determined by measuring mitochondrial succinate dehydrogenase activity of viable cells following a protocol described elsewhere (14). After treatment with ketamine and incubation with the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI, USA), the absorbance was read at 492 nm on an Infinite M200PRO (Tecan Group Ltd., Switzerland) plate reader. Results are given as percentages (mean ± standard deviation) of viable cells compared to control (untreated) cells. Considering that a reliable genotoxicity assessment requires that cell viability is over 70 % (15), for further toxicity assessments we selected three concentrations which meet this requirement and which correspond to ketamine concentrations found in human plasma after analgesia (0.39 μmol/L), anaesthesia (6.25 μmol/L), and drug abuse (1.56 μmol/L).

Alkaline comet assay

The alkaline comet assay was performed to determine the level of primary DNA damage. Its effectiveness in genotoxicity assessments has been well established (16–18). A detailed procedure for microgel preparation has been described in our previous publication (18). Briefly, randomly selected comets were measured on duplicate slides under an epifluorescence microscope (200× magnification) using the Comet Assay IV™ software (Instem-Perceptive Instruments Ltd., UK). As the experiment was carried out in triplicate, altogether 600 comets per sample were assessed for tail intensity (DNA% in the comet tail) as an indicator of DNA damage.

Malondialdehyde determination

Malondialdehyde (MDA) analysis in cell lysates was based on the method reported by Khoschsorur et al. (19). Briefly, after the treatment, cells were rinsed twice in Krebs-Heneseleit buffer, centrifuged at 248 *g* for 5 min and sonicated on ice. After lysis, cell lysate was resuspended in Krebs-Heneseleit buffer (250 mL). The reaction mixture was prepared by mixing 25 μL cell lysates (or standard, i.e. 2.5 μmol/L 1,1,3,3-tetraethoxy propane) with water (225 μL), phosphoric acid (375 μL; 0.44 mol/L), and thiobarbituric acid (125 μL; 42 mmol/L) and incubating it in boiling water bath at 100 °C for 30 min.

Samples were analysed with a high-performance liquid chromatograph (HPLC) with a UV detector (Shimadzu Corporation, Kyoto, Japan) using the guard and analytical reverse-phase C-18 column (4.0 × 4.0 and 4.0 × 125.0 mm, respectively) with 5 μm particle size (LiChrospher, Merck) as described earlier in detail (18).

Reactive oxygen species detection

Reactive oxygen species (ROS) were determined using a fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) obtained from Sigma-Aldrich. HepG2 and SH-SY5Y were seeded into black 96-well plates at 10^4 cells/mL. On the day of the experiment, the medium was removed, cells rinsed with the PBS buffer (100 μL) and incubated with DCFH-DA (200 μL; 20 μmol/L) at 37 °C for 30 min (20). The dye was then washed off, and the cells

rinsed again with PBS (100 μL) and treated with either of the three concentrations of ketamine mentioned above.

Fluorescence was recorded (λ_{Ex} 485 nm, λ_{Em} 520 nm) on a multilabel plate reader (Victor3™, Perkin Elmer, Waltham, MA, USA). Fluorescence arbitrary units are given as percentages of control cell values.

Measurement of glutathione levels

Intracellular glutathione (GSH) was measured using fluorogenic monochlorobimane (mBCl), as it reacts with GSH to form a fluorescent product in an amount proportional to GSH content (21).

Cell cultures were placed in black-sided, clear-bottom 96-well plates in the same medium and conditions as for other assays. Cells were treated with the same ketamine concentrations as in other assays, followed by a 4-hour incubation at 37 °C. Then, the cells were rinsed with PBS and incubated with mBCl in PBS (200 μL; 20 μmol/L) at 37 °C for 30 min. The fluorescence (corresponding to GSH concentration) in cell samples was measured with a Victor3™ (Perkin Elmer) multilabel plate reader (λ_{Ex} 355 nm, λ_{Em} 460 nm). The obtained fluorescence arbitrary units are given as percentages of control values. The measurements were done in triplicate.

Measurement of glutathione peroxidase activity

Glutathione peroxidase (GPx) activity was measured with the commercially available assay kit No. 703102 (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions (22). Briefly, treated cells were separated by centrifugation (1000 *g* at 4 °C for 10 min), sonicated in cold buffer (50 mmol/L tris-HCl, pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid (EDTA), and 1 mmol/L dithiothreitol (DTT), and centrifuged again at 10,000 *g* and 4 °C for 15 min to obtain the supernatant. After a sequence of reactions, the decrease in absorbance due to nicotinamide adenine dinucleotide phosphate (NADPH) oxidation into $NADP^+$ directly proportional to GPx activity in the sample was measured at 340 nm using the SpectraMax i3x microplate reader (Molecular Devices, San Jose, CA, USA). One unit of activity corresponds to the amount of GPx needed to catalyse oxidation of 1 nmol of NADPH per minute and per milligram of protein. GPx activity is expressed as percentage of control values.

Measurement of superoxide dismutase activity

Superoxide dismutase (SOD) activity was measured with the commercially available assay kit No. 706002 (Cayman Chemical Company) according to the manufacturer's instructions (23). Briefly, treated cells were separated by centrifugation (1000 *g* and at 4 °C for 10 min), sonicated in cold buffer [20 mmol/L N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.2, containing 1 mmol/L ethylene glycol tetraacetic acid (EGTA), 210 mmol/L mannitol, and 70 mmol/L sucrose], and centrifuged again at 1500 *g* and 4 °C for 5 min to obtain the supernatant. Tetrazolium salt is used for the detection of superoxide radicals produced by xanthine oxidase and hypoxanthine. The inhibition of this reaction was measured at 505 nm using the SpectraMax i3x microplate reader (Molecular Devices). One unit of activity corresponds to the amount of SOD needed to produce 50 % dismutation of the superoxide radical. SOD activity is given as percentage of control values.

Measurement of catalase activity

Catalase (CAT) activity was measured with the commercially available assay kit No. 707002 (Cayman Chemical Company) according to the manufacturer's instructions (24). Cells collected after treatment and centrifugation at 1000 *g* and at 4 °C for 10 min were sonicated in cold buffer (50 mmol/L potassium phosphate, pH 7.0, containing 1 mmol/L EDTA) and centrifuged again at 10,000 *g* at 4 °C for 15 min to obtain the supernatant. The method relies on enzyme reaction with methanol in the presence of H_2O_2 at an optimal concentration, which yields formaldehyde. As the chromogen 4-amino-3-hydrazino-5-mercapto-1,2,3-triazole (Purpald®, Sigma-Aldrich) was used, the absorbance was measured at 540 nm using the SpectraMax i3x microplate reader (Molecular Devices). One unit of activity corresponds to 1 nmol of formaldehyde per minute and per milligram of protein. CAT activity is given as percentage of control values.

Statistical analysis

Cell viability data were analysed with the parametric one-way analysis of variance (ANOVA) and Dunnett's test using the Prism software (GraphPad Software, San Diego, CA, USA). The comet assay data, which had non-parametric distribution (and whose medians more accurately represented the centre of distribution), were analysed with the Mann-Whitney *U*-test on Statistica version 14 (TIBCO Software, Inc., Palo Alto, CA, USA). The same software was used for the analysis of biochemical parameters with the Kruskal-Wallis one-way analysis of variance. Statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the viability of HepG2 and SH-SY5Y cells after 24 h of exposure to ketamine at concentrations ranging from 0.39 to 100 μmol/L. Even though we tested low micromolar concentrations of the drug, almost all caused significant concentration-dependent cytotoxicity. SH-SY5Y cells were up to 1.2 times more sensitive to exposure than HepG2 cells. However, no concentration reduced viability below 70 %. These cytotoxic effects are noteworthy considering that they were caused by ketamine at plasma concentrations corresponding to those in human analgesia, anaesthesia, and drug abuse (4, 5).

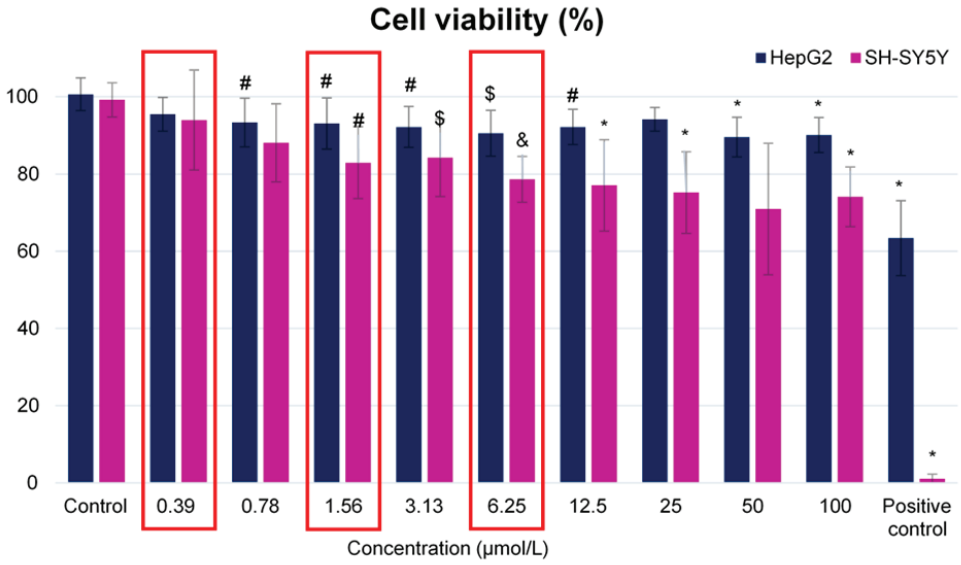

Figure 1 Mean (± SD) changes in the viability of HepG2 and SH-SY5Y cells exposed to ketamine (0.39–100 µmol/L) for 24 h relative to control (untreated cells). Concentrations found in human plasma upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L) are framed in red. Positive control – staurosporine (2 µmol/L); $^{\&}P<0.05$; $^{\#}P<0.01$; $^{\$}P<0.001$; $^{*}P<0.0001$ vs control (untreated cells)

Cytotoxic effects of ketamine have already been reported for HepG2 cells by Liang et al. (25) but at much higher concentrations (≥1.0 mol/L). On the other hand, Slikker et al. (26) reported no significant drop in the viability of neural stem cells at concentrations below 500 µmol/L. This is comparable to our data, since neural cells are more sensitive to ketamine than liver cells. Barr et al. (27) reported that a 20-hour exposure of canine peripheral blood lymphocytes to 12.62 µmol/L ketamine reduced viability up to 30 %.

Figure 2 shows primary DNA damage following exposure to the three ketamine concentrations corresponding to those found in human plasma upon analgesia, anaesthesia, and drug abuse. Compared to respective controls, all concentrations significantly increased tail intensity in both cell types. A slightly higher DNA damage was observed in samples with lower cell viability. However, the median tail intensities at all tested concentrations in SH-SY5Y cells and at the two higher concentrations in HepG2 cells were below 10 %. Such DNA damage level can be considered low, as the background levels reported in untreated cells are usually about 5 % (28–31).

Not many studies have investigated ketamine effects at the DNA level. Asghary et al. (32) have reported that ketamine has an affinity toward the guanine bases of DNA and a groove-binding interaction with double-stranded DNA. This can explain the level of DNA damage we observed in our study. Intercalation events caused by groove binding induced by ketamine and/or its metabolites may disturb DNA conformation, which may, in turn, affect the denaturation and electrophoresis stages of the comet assay (18). As we know, intercalation generally activates DNA repair, which may increase the amount of strand breaks (33, 34), and the alkaline comet assay is quite sensitive in detecting strand breaks (29, 35). Since simple DNA strand breaks are completely repairable, exposure to low concentrations of ketamine like ours seems to allow complete DNA repair and more complex chromosomal damage is unlikely. Along these lines, Cavalcanti et al. (36) reported complete repair of DNA strand breaks and no chromosomal aberrations in human peripheral blood leukocytes even at significantly (>300 times) higher ketamine concentration than ours.

In terms of oxidative stress, ketamine treatment did not cause membrane damage sufficient to increase lipid peroxidation in either cell type (Figure 3) nor did it affect ROS levels significantly compared to control in either cell line, regardless of concentration (Figure 4). These findings are similar to an earlier report (37), which also suggests that ketamine may have a prominent role in preventing oxidative stress. It is, therefore possible that suppressed oxidation by ketamine also resulted in its lower cytotoxicity.

Furthermore, ketamine did not affect GSH levels in either cell line (Figure 5), but did cause a slight disturbance of GPx activity (Figure 6). In HepG2 cells the lowering trend was concentration-dependent. Changes in the enzyme's activity could be related to differences in metabolic features between these cell types and call for further investigation. Nevertheless, lower GPx activity after ketamine treatment is in line with one previous report (38).

Superoxide dismutase enzyme constitutes the first line of defence against ROS. Since ketamine did not affect ROS levels significantly in our experiment, we expected that SOD activity would also not significantly change compared to control, yet it was greatly reduced in both cell lines (Figure 7). Such finding indicates an unbalanced antioxidant defence, assuming that antioxidant defence, which relies on CAT, GPx, and GSH besides SOD (39, 40) is under homeostatic control, and that variations in the level of one can be compensated by a change in another. Our results suggest that this is what happened with our cell lines. There may be other reasons for lower SOD activity, such as reduced metabolic rate in ketamine-treated cells, which could result with lower demand for SOD. These are yet to be identified by future research. As both cell lines used in our study are of tumour origin, we should also consider the

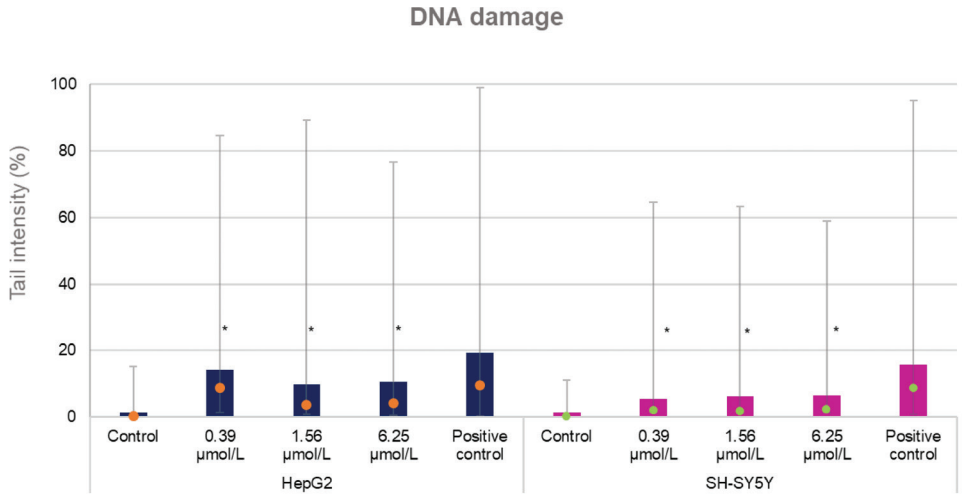

Figure 2 Changes in comet tail intensity of HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control (alkaline comet assay). Ketamine concentrations correspond to concentrations found in human plasma upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L). Results are expressed as mean, median and range. Positive control – H_2O_2 treated cells (100 µmol/L). *Statistically significant differences ($P<0.05$, ANOVA with *post-hoc* Tukey HSD test) vs control

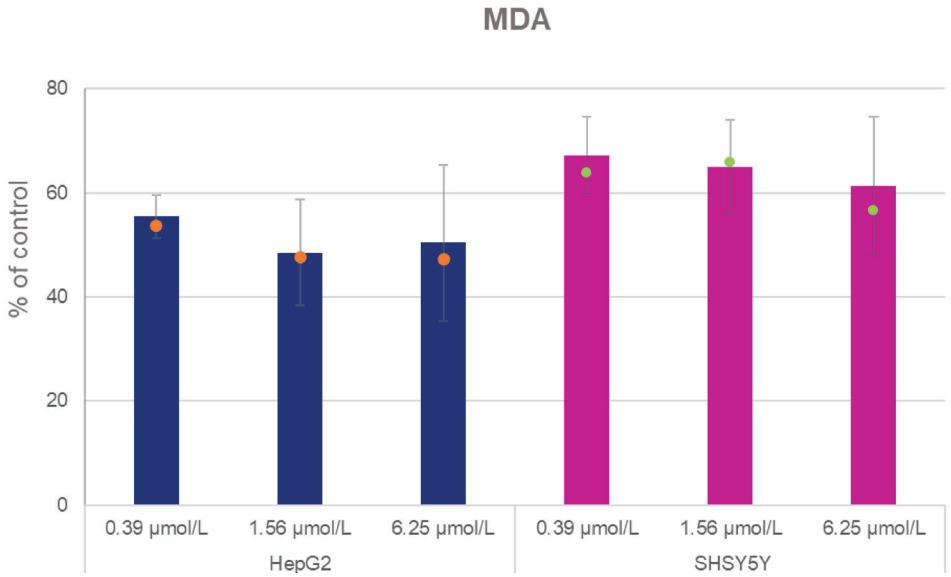

Figure 3 Changes in malondialdehyde (MDA) concentrations in HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug- abuse (1.56 µmol/L)]. Results are expressed as means ± SD and medians

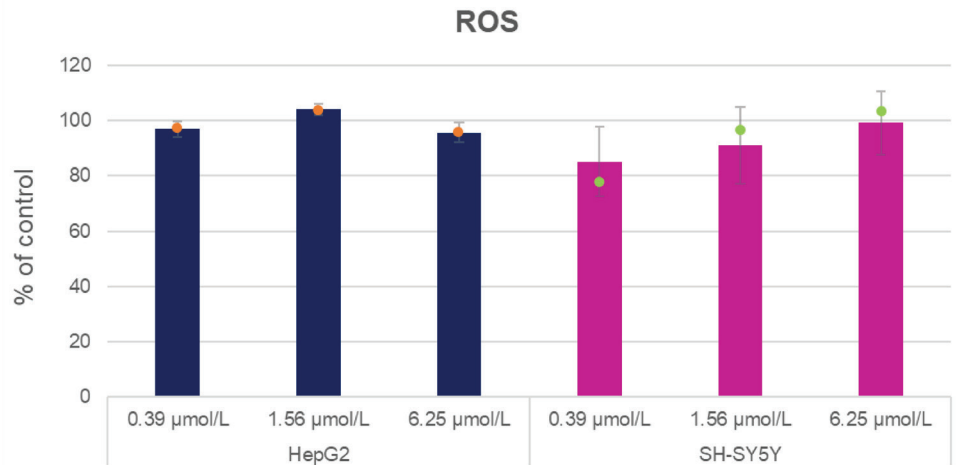

Figure 4 Changes in ROS levels in HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L)]. Results are expressed as mean ± SD and medians. ROS – reactive oxygen species determined using a fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA)

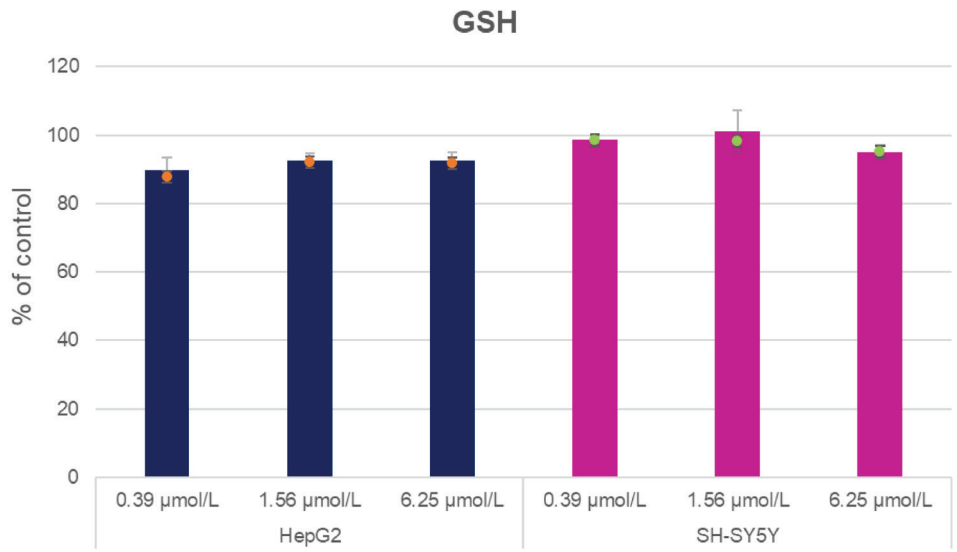

Figure 5 Changes in glutathione (GSH) levels in HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma relevant upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L)]. Results are expressed as means ± SD and medians

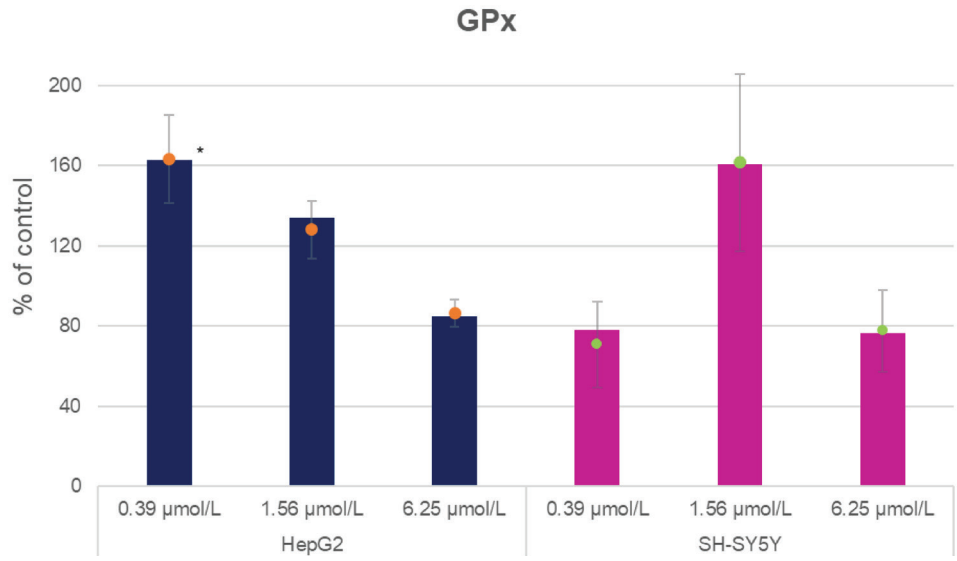

Figure 6 Changes in glutathione peroxidase (GPx) activity in HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma relevant upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L)]. Results are expressed as means ± SD and medians. * P<0.05 vs control (ANOVA with *post-hoc* Tukey HSD test)

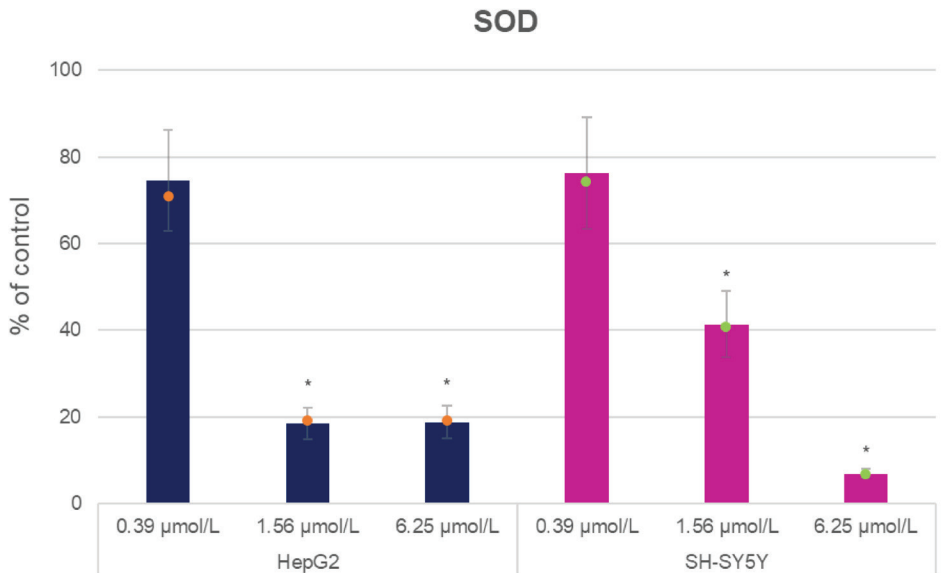

Figure 7 Changes in superoxide dismutase (SOD) levels in HepG2 and SH-SY5Y cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma relevant upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L)]. Results are expressed as means ± SD and medians. *P<0.05 vs control (Kruskal-Wallis one-way analysis)

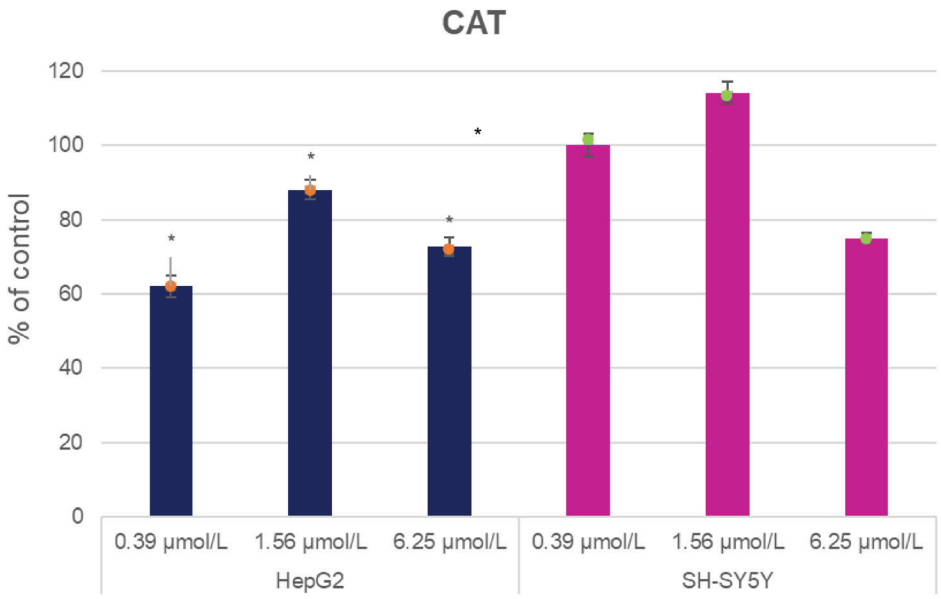

Figure 8 Changes in catalase (CAT) levels in HepG2 and SH-SYY cells exposed to ketamine for 24 h relative to control [ketamine concentrations correspond to concentrations found in human plasma relevant upon analgesia (0.39 µmol/L), anaesthesia (6.25 µmol/L), and drug abuse (1.56 µmol/L)]. Results are expressed as means ± SD and medians. *P<0.05 vs control (ANOVA with *post-hoc* Tukey HSD test)

possibility that their antioxidant defence is different than that of normal cells. In fact, an extensive review by Oberley and Oberley (41) suggests that lower SOD activity in tumour cells of human and animal origin constitutes a survival advantage over their normal counterparts. Besides, an interesting study (42) suggests that the SOD1 form of the enzyme, which is predominantly cytoplasmic, can move to the nucleus to counteract DNA damage. This seems to hold true for our findings of increased DNA damage and lower SOD activity in the cytoplasm (which was used for our measurements).

As for other antioxidative defences, the pattern of treatment-related changes in the CAT activity was similar in both cell types (Figure 8), but HepG2 cells showed a greater decline in CAT activity than SH-SY5Y cells, which may point to differences in their origin and overall metabolic competence.

These results are interesting if we consider the fact that ketamine is used to anaesthetise laboratory animals used for toxicity testing of various compounds, and that these tests use the same methods we have in this study. With this in mind, our results suggest that ketamine has a low potential to affect the level of primary DNA damage, and that its effects on antioxidative enzyme activities should be taken with greater reserve.

One of the limitations of our study is its *in vitro* design, which means that the obtained results cannot be directly extrapolated to *in vivo* effects in animals or humans. Another limitation is that the methods used here cannot give definitive answers about the mechanisms underlying the observed effects. There are still a number of issues that require further research, most notably of the mechanisms behind cell death induced by ketamine. For instance, we already know that treatment with 1–8 µmol/L ketamine induces concentration- and time-dependent apoptosis via the mitochondrial pathway and necrosis in Jurkat and SHEP neuroblastoma cells (43), in HepG2 cells (44), and SH-SY5Y cells (45). These pathways are probably mediated by ketamine's target receptor NMDA (11, 46) or by inhibition of nicotinic receptors.

CONCLUSION

Our results show that ketamine at concentrations relevant for analgesia, anaesthesia, or drug abuse does not produce harmful cytotoxic, genotoxic, and oxidative stress effects *in vitro*. However, the same range of concentrations may be harmful for the nervous system, which cannot be assessed using the *in vitro* design, and therefore calls for further research.

Even so, research on cell models such as ours certainly helps to better understand the impact of drugs on cell signalling and may contribute to research needed to advance more effective ketamine use in anaesthesia without any side effects.

Acknowledgments

This research was supported by the programme of cooperation between the Institute for Medical Research and Occupational Health (Zagreb, Croatia), University North (Varaždin, Croatia) and the Croatian Science Foundation (grant No. HrZZ-UIP-2017-05-7260).

REFERENCES

1. Li J-H, Vicknasingam B, Cheung Y, Zhou W, Nurhidayat AW, Jarlais DC Des, Schottenfeld R. To use or not to use: an update on licit and illicit ketamine use. Subst Abuse Rehabil 2011;2:11–20. doi: 10.2147/sar.s15458
2. Negrusz A, Gaensslen RE. Analytical developments in toxicological investigation of drug-facilitated sexual assault. Anal Bioanal Chem 2003;376:1192–7. doi: 10.1007/s00216-003-1896-z

3. Liu F, Patterson TA, Sadovova N, Zhang X, Liu S, Zou X, Hanig JP, Paule MG, Slikker W, Wang C. Ketamine-induced neuronal damage and altered N-methyl-d-aspartate receptor function in rat primary forebrain culture. Toxicol Sci 2013;131:548–57. doi: 10.1093/toxsci/kfs296
4. Flood P, Krasowski MD. Intravenous anesthetics differentially modulate ligand-gated ion channels. Anesthesiology 2000;92:1418–25. doi: 10.1097/00000542-200005000-00033
5. Little B, Chang T, Chucot L, Dill WA, Enrile LL, Glazko AJ, Jassani M, Kretchmer H, Sweet AY. Study of ketamine as an obstetric anesthetic agent. Am J Obstet Gynecol 1972;113:247–60. doi: 10.1016/0002-9378(72)90774-0
6. Zanos P, Moaddel R, Morris PJ, Riggs LM, Highland JN, Georgiou P, Pereira EFR, Albuquerque EX, Thomas CJ, Zarate CA, Gould TD. Ketamine and ketamine metabolite pharmacology: Insights into therapeutic mechanisms. Pharmacol Rev 2018;70:621–60. doi: 10.1124/pr.117.015198
7. Mion G, Villevieille T. Ketamine pharmacology: An update (pharmacodynamics and molecular aspects, recent findings). CNS Neurosci Ther 2013;19:370–80. doi: 10.1111/cns.12099
8. Hirota K, Sikand KS, Lambert DG. Interaction of ketamine with μ2 opioid receptors in SH-SY5Y human neuroblastoma cells. J Anesth 1999;13:107–9. doi: 10.1007/s005400050035
9. Friederich P, Dybek A, Urban BW. Stereospecific interaction of ketamine with nicotinic acetylcholine receptors in human sympathetic ganglion-like SH-SY5Y cells. Anesthesiology 2000;93:818–24. doi: 10.1097/00000542-200009000-00032
10. Choi JM, Oh SJ, Lee SY, Im JH, Oh JM, Ryu CS, Kwak HC, Lee JY, Kang KW, Kim SK. HepG2 cells as an *in vitro* model for evaluation of cytochrome P450 induction by xenobiotics. Arch Pharm Res 2015;38:691–704. doi: 10.1007/s12272-014-0502-6
11. Mak YT, Lam WP, Lü L, Wong YW, Yew DT. The toxic effect of ketamine on SH-SY5Y neuroblastoma cell line and human neuron. Microsc Res Tech 2010;73:195–201. doi: 10.1002/jemt.20774
12. Nagarajan SR, Paul-Heng M, Krycer JR, Fazakerley DJ, Sharland AF, Hoy XAJ. Lipid and glucose metabolism in hepatocyte cell lines and primary mouse hepatocytes: A comprehensive resource for *in vitro* studies of hepatic metabolism. Am J Physiol - Endocrinol Metab 2019;316:E578–89. doi: 10.1152/ajpendo.00365.2018
13. Bustamante J, Acosta L, Karadayian AG, Lores-Arnaiz S. Ketamine induced cell death can be mediated by voltage dependent calcium channels in PC12 cells. Exp Mol Pathol 2019;111:104318. doi: 10.1016/j.yexmp.2019.104318
14. Zandona A, Katalinić M, Šinko G, Radman Kastelic A, Primožič I, Kovarik Z. Targeting organophosphorus compounds poisoning by novel quinuclidine-3 oximes: development of butyrylcholinesterase-based bioscavengers. Arch Toxicol 2020;94:3157–71. doi: 10.1007/s00204-020-02811-5
15. Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206–21. doi: 10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J
16. Singh NP, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988;175:184–91. doi: 10.1016/0014-4827(88)90265-0
17. Kruszewski M, Sikorska K, Meczynska-Wielgosz S, Grzelak A, Sramkova M, Gabelova A, Kapka-Skrzypczak L. Comet assay in neural cells as a tool to monitor DNA damage induced by chemical or physical factors relevant to environmental and occupational exposure. Mutat Res Genet Toxicol Environ Mutagen 2019;845:402990. doi: 10.1016/j.mrgentox.2018.11.014
18. Jurič A, Zandona A, Lovaković BT, Rašić D, Pizent A, Kozina G, Katalinić M, Lucić Vrdoljak A, Karačonji IB. Cytotoxic, genotoxic, and oxidative stress-related effects of lysergic acid diethylamide (LSD) and phencyclidine (PCP) in the human neuroblastoma SH-SY5Y cell line. Arh Hig Rada Toksikol 2021;72:333–42. doi: 10.2478/aiht-2021-72-3604
19. Khoschsorur GA, Winklhofer-Roob BM, Rabl H, Auer T, Peng Z, Schaur R. Evaluation of a sensitive HPLC method for the determination of malondialdehyde, and application of the method to different biological materials. Chromatographia 2000;52:181–4. doi: 10.1007/BF02490453
20. Griffiths SM, Singh N, Jenkins GJS, Williams PM, Orbaek AW, Barron AR, Wright CJ, Doak SH. Dextran coated ultrafine superparamagnetic iron oxide nanoparticles: compatibility with common fluorometric and colorimetric dyes. Anal Chem 2011;83:3778–85. doi: 10.1021/ac200103x
21. Kamencic H, Lyon A, Paterson PG, Juurlink BHJ. Monochlorobimane fluorometric method to measure tissue glutathione. Anal Biochem 2000;286:35–7. doi: 10.1006/abio.2000.4765
22. Cayman Chemical Company. Glutathione Peroxidase Assay Kit [displayed 8 February 2023]. Available at https://www.caymanchem.com/product/703102/glutathione-peroxidase-assay-kit
23. Cayman Chemical Company. Superoxide Dismutase Assay Kit [displayed 8 February 2023]. Available at https://www.caymanchem.com/product/706002/superoxide-dismutase-assay-kit
24. Cayman Chemical Company. Catalase Assay Kit [displayed 8 February 2023]. Available at https://www.caymanchem.com/product/707002/catalase-assay-kit
25. Liang Z, Yin P, Zhao L. Effects of combined toxicity of methamphetamine and ketamine on apoptosis, oxidative stress and genotoxicity in HepG2 cells. Food Chem Toxicol 2019;132:110653. doi: 10.1016/j.fct.2019.110653
26. Slikker W Jr, Liu F, Rainosek SW, Patterson TA, Sadovova N, Hanig JP, Paule MG, Wang C. Ketamine-induced toxicity in neurons differentiated from neural stem cells. Mol Neurobiol 2015;52:959–69. doi: 10.1007/s12035-015-9248-5
27. Barr CA, Alvarado F, Chang YM, Luo J, Garden OA. The impact of alfaxalone, propofol and ketamine on canine peripheral blood lymphocyte cytotoxicity *in vitro*. Res Vet Sci 2021;136:182–4. doi: 10.1016/j.rvsc.2021.02.019
28. Koppen G, Azqueta A, Pourrut B, Brunborg G, Collins AR, Langie SAS. The next three decades of the comet assay: A report of the 11th International Comet Assay Workshop. Mutagenesis 2017;32:397–408. doi: 10.1093/mutage/gex002
29. Collins A, Møller P, Gajski G, Vodenková S, Abdulwahed A, Anderson D, Bankoglu EE, Bonassi S, Boutet-Robinet E, Brunborg G, Chao C, Cooke MS, Costa C, Costa S, Dhawan A, de Lapuente J, Bo' C Del, Dubus J, Dusinska M, Duthie SJ, Yamani NE, Engelward B, Gaivão I, Giovannelli L, Godschalk R, Guilherme S, Gutzkow KB, Habas K, Hernández A, Herrero O, Isidori M, Jha AN, Knasmüller S, Kooter IM, Koppen G, Kruszewski M, Ladeira C, Laffon B, Larramendy M, Hégarat LL, Lewies A, Lewinska A, Liwszyc GE, de Cerain AL, Manjanatha M, Marcos R, Milić M, de Andrade VM, Moretti M, Muruzabal D, Novak M, Oliveira R, Olsen AK, Owiti N, Pacheco M,

Pandey AK, Pfuhler S, Pourrut B, Reisinger K, Rojas E, Rundén-Pran E, Sanz-Serrano J, Shaposhnikov S, Sipinen V, Smeets K, Stopper H, Teixeira JP, Valdiglesias V, Valverde M, van Acker F, van Schooten FJ, Vasquez M, Wentzel JF, Wnuk M, Wouters A, Žegura B, Zikmund T, Langie SAS, Azqueta A. Measuring DNA modifications with the comet assay: a compendium of protocols. Nat Protoc 2023;18:929–89. doi: 10.1038/s41596-022-00754-y

30. Gajski G, Gerić M, Živković Semren T, Tariba Lovaković B, Oreščanin V, Pizent A. Application of the comet assay for the evaluation of DNA damage from frozen human whole blood samples: Implications for human biomonitoring. Toxicol Lett 2020;319:58–65. doi: 10.1016/j.toxlet.2019.11.010
31. Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004;26:249–61. doi: 10.1385/MB:26:3:249
32. Asghary M, Raoof JB, Ojani R, Hamidi-Asl E. A genosensor based on CPE for study the interaction between ketamine as an anesthesia drug with DNA. Int J Biol Macromol 2015;80:512–9. doi: 10.1016/j.ijbiomac.2015.07.019
33. Lenglet G, David-Cordonnier MH. DNA-destabilizing agents as an alternative approach for targeting DNA: mechanisms of action and cellular consequences. J Nucleic Acids 2010;2010:290935. doi: 10.4061/2010/290935
34. Mišković K, Bujak M, Baus Lončar M, Glavaš-Obrovac L. Antineoplastic DNA-binding compounds: intercalating and minor groove binding drugs. Arh Hig Rada Toksikol 2013;64:593–602. doi: 10.2478/10004-1254-64-2013-2371
35. Azqueta A, Collins AR. The essential comet assay: a comprehensive guide to measuring DNA damage and repair. Arch Toxicol 2013;87:949–68. doi: 10.1007/s00204-013-1070-0
36. Cavalcanti BC, de Andrade Neto JB, de Sousa Silva AA, Barreto FS, de Oliveira Ferreira JR, da Silva CR, Aires do Nascimento FBS, do Amaral Valente Sá LG, Magalhães HIF, Nobre Júnior HV, de Moraes MO. Evaluation of genotoxicity and mutagenicity of ketamine on human peripheral blood leukocytes and in *Salmonella typhimurium*. Toxicol in Vitro 2020;62:104718. doi: 10.1016/j.tiv.2019.104718
37. Ergün Y, Darendeli S, Imrek S, Kilinç M, Öksüz H. The comparison of the effects of anesthetic doses of ketamine, propofol, and etomidate on ischemia-reperfusion injury in skeletal muscle. Fundam Clin Pharmacol 2010;24:215–22. doi: 10.1111/j.1472-8206.2009.00748.x
38. Kamiloglu NN, Kamiloglu A, Beytut E. Changes in antioxidant system, lipid peroxidation, heart and respiratory rate and rectal temperature with ketamine and ketamine-xylazine anaestesia in tuj rams. Kafkas Univ Vet Fak Derg 2009;15:205–10. doi: 10.9775/kvfd.2008.101-a
39. Traverso N, Ricciarelli R, Nitti M, Marengo B, Furfaro AL, Pronzato MA, Marinari UM, Domenicotti C. Role of glutathione in cancer progression and chemoresistance. Oxid Med Cell Longev 2013;2013:972913. doi: 10.1155/2013/972913
40. Ighodaro OM, Akinloye OA. First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): their fundamental role in the entire antioxidant defence grid. Alexandria J Med 2018;54:287–93. doi: 10.1016/j.ajme.2017.09.001
41. Oberley TO, Oberley W. Histology and histopathology from cell biology to tissue engineering antioxidant enzyme levels in cancer. Histol Histopathol 1997;12:525–35. PMID: 9151141
42. Tsang CK, Liu Y, Thomas J, Zhang Y, Zheng XFS. Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. Nat Commun 2014;5:3446. doi: 10.1038/ncomms4446
43. Braun S, Werdehausen R, Gaza N, Hermanns H, Kremer D, Küry P, Hollmann MW, Stevens MF. Benzethonium increases the cytotoxicity of S(+)-ketamine in lymphoma, neuronal, and glial cells. Anesth Analg 2010;111:1389–93. doi: 10.1213/ANE.0b013e3181f690e4
44. Lee ST, Wu TT, Yu PY, Chen RM. Apoptotic insults to human HepG2 cells induced by S-(+)-ketamine occurs through activation of a Bax-mitochondria-caspase protease pathway. Br J Anaesth 2009;102:80–9. doi: 10.1093/bja/aen322
45. Chen Y, Yang Z, Wei L, Wang J, Xuan W, Wang Y, Li J, Ke Z, Li Y. Yes-associated protein protects and rescues SH-SY5Y cells from ketamine-induced apoptosis. Mol Med Rep 2020;22:2342–50. doi: 10.3892/mmr.2020.11328
46. Zhang Y, Ye F, Zhang T, Lv S, Zhou L, Du D, Lin H, Guo F, Luo C, Zhu S. Structural basis of ketamine action on human NMDA receptors. Nature 2021;598(7882):E3. doi: 10.1038/s41586-021-04038-5

Učinak ketamina na vijabilnost, primarna oštećenja DNA i parametre oksidacijskog stresa u stanicama HepG2 i SH-SY5Y

Ketamin je disocijativni anestetik koji se koristi za izazivanje opće anestezije u određenim medicinskim postupcima kod ljudi, kao i u anesteziji laboratorijskih životinja. Zbog svojih halucinogenih i disocijativnih učinaka koristi se i kao rekreacijska droga. Anestetici također mogu prouzročiti toksične učinke na staničnoj razini i, utječući na preživljavanje stanica, izazvati oštećenje DNA te neravnotežu oksidansa i antioksidansa. Cilj ove studije bio je istražiti moguće štetne učinke ketamina na hepatocelularne HepG2 i neuroblastoma SH-SY5Y stanice nakon 24-satne izloženosti širokom rasponu koncentracija, uključujući koncentracije relevantne u slučajevima korištenja u analgeziji, zlouporabi droga i anesteziji (0,39, 1,56 odnosno 6,25 µmol/L). Naši rezultati pokazali su da je ketamin u ovim ispitivanim koncentracijama izazvao relativno nisku citotoksičnost, budući da je do 30 % smanjio preživljenje stanica HepG2 i SH-SY5Y, ali je uočen neznatan porast razine primarnih oštećenja DNA. Zanimljivo je da su razine reaktivnih kisikovih vrsta (ROS), malondialdehida (MDA) i glutationa (GSH) ostale nepromijenjene u objema staničnim linijama. S druge strane, markeri oksidacijskog stresa [superoksid dismutaza (SOD), glutation peroksidaza (GPx), katalaza (CAT)] upućivali su na oksidacijsko-redukcijsku neravnotežu izazvanu ketaminom.

KLJUČNE RIJEČI: anestezija; antioksidacijski enzimi; ROS; toksičnost; zlouporaba droga