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Apoptotic insults to human HepG2 cells induced by *S*-(+)-ketamine occurs through activation of a Bax-mitochondria-caspase protease pathway

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Background. Ketamine is widely used as an i.v. anaesthetic agent and as a drug of abuse. Hepatocytes contribute to the metabolism of endogenous and exogenous substances. This study evaluated the toxic effects of *S*-(+)-ketamine and possible mechanisms using human hepatoma HepG2 cells as the experimental model.

Methods. HepG2 cells were exposed to *S*-(+)-ketamine. Cell viability and the release of lactate dehydrogenase (LDH) and γ-glutamyl transpeptidase (GPT) were measured to determine the toxicity of *S*-(+)-ketamine to HepG2 cells. Cell morphology, DNA fragmentation, and apoptotic cells were analysed to evaluate the mechanism of *S*-(+)-ketamine-induced cell death. Amounts of Bax, an apoptotic protein, and cytochrome *c* in the cytoplasm or mitochondria were quantified by immunoblotting. Cellular adenosine triphosphate levels were analysed using a bioluminescence assay. Caspases-3, -9, and -6 were measured fluorometrically.

Results. Exposure of HepG2 cells to *S*-(+)-ketamine increased the release of LDH and GPT, but decreased cell viability (all $P<0.01$). *S*-(+)-Ketamine time-dependently caused shrinkage of HepG2 cells. Exposure to *S*-(+)-ketamine led to significant DNA fragmentation and cell apoptosis ($P=0.003$ and 0.002). *S*-(+)-Ketamine increased translocation of Bax from the cytoplasm to mitochondria, but decreased the mitochondrial membrane potential and cellular adenosine triphosphate levels (all $P<0.01$). Sequentially, cytosolic cytochrome *c* levels and activities of caspases-9, -3, and -6 were augmented after *S*-(+)-ketamine administration (all $P<0.001$). Z-VEID-FMK, an inhibitor of caspase-6, alleviated the *S*-(+)-ketamine-induced augmentation of caspase-6 activity, DNA fragmentation, and cell apoptosis (all $P<0.001$).

Conclusions. This study shows that *S*-(+)-ketamine can induce apoptotic insults to human HepG2 cells via a Bax-mitochondria-caspase protease pathway. Thus, we suggest that *S*-(+)-ketamine at a clinically relevant or an abused concentration may induce liver dysfunction possibly due to its toxicity to hepatocytes.

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Ketamine, a widely used i.v. anaesthetic agent, has also emerged as an increasingly common drug of abuse among groups of young injected-drug users in the USA and other countries.[1 2] Ketamine has been reported to modulate the activities of neutrophils and leucocytes.[3 4] Our previous studies also showed that ketamine can induce dysfunction of macrophages and human umbilical vein endothelial cells.[5 6] Hepatocytes play critical roles in the metabolism of endogenous and exogenous substances.[7] A variety of drugs and toxins can damage hepatocytes, leading to cell

dysfunction or even death.[8 9] Ketamine modulates the expression of multiple forms of cytochrome P450-dependent mono-oxygenases in the rat liver and increases carbon tetrachloride-induced hepatic injuries and cocaine-mediated acute toxicity.[10] After operation, ketamine has been reported to possibly induce hepatotoxicity in patients given this i.v. anaesthetic agent.[11] Hepatocytes are the major tissues responsible for the biotransformation of ketamine.[12] However, the toxic effects of ketamine on hepatocytes, especially at higher dosages, are little known. The human hepatoma HepG2 cell line with the essential enzymes required for metabolism of drugs is a useful model for the study of drug metabolism and interactions.[13 14]

Apoptosis is an autonomously programmed cell death mechanism which participates in physiological and pathophysiological regulation of tissue homeostasis and cell activities.[15 16] Drug-induced hepatocyte stress can lead to activation of built-in death programmes for apoptosis. Increases in the translocation of the proapoptotic Bax protein from the cytoplasm to mitochondria can sequentially trigger depolarization of the mitochondrial membrane potential, enhancing the release of apoptotic factors such as cytochrome *c*, and activating caspases-9, -3, and -6, ultimately leading to DNA fragmentation and apoptosis.[17–19] In rats, ketamine was shown to induce apoptosis of cultured cortical or forebrain neurones.[20] Our previous study further showed that ketamine can induce DNA fragmentation and cell apoptosis in human umbilical vein endothelial cells.[6] However, studies investigating the effects of ketamine on hepatocyte activity are limited. Therefore, this study used HepG2 cells as the experimental model to further evaluate the toxic effects of *S*-(+)-ketamine and its possible signal-transducing mechanisms.

Methods

Cell culture and drug treatment

Human hepatoma HepG2 cells were purchased from American Type Culture Collection (Rockville, MD, USA). These cells were cultured as described previously.[21] *S*-(+)-Ketamine purchased from Sigma Company (St Louis, MO, USA) was dissolved in phosphate-buffered saline (PBS) (0.14 M NaCl, 2.6 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4).

Toxicity assay

Analyses of γ-glutamyl transpeptidase (GPT) and lactate dehydrogenase (LDH) release and cell viability were carried out to determine the toxicity of *S*-(+)-ketamine to HepG2 cells. Levels of GPT and LDH in the culture medium were quantified using a model 7450 automatic autoanalyzer system from Hitachi (Tokyo, Japan) as described previously.[21] A survival assay was carried out using a trypan blue exclusion method.[19]

DNA fragmentation and apoptotic cells

DNA fragmentation in HepG2 cells was quantified to evaluate if *S*-(+)-ketamine can damage nuclear DNA according to a previously described method.[22] The BrdU-labelled histone-associated DNA fragments in the cytoplasm of cell lysates were detected according to the instructions of the cellular DNA fragmentation enzyme immunoassay kit (Boehringer Mannheim, Indianapolis, IN, USA). Apoptotic cells were identified via detection of cells which were arrested at the sub-G1.[16] After drug treatment, the harvested HepG2 cells were fixed with cold 80% ethanol, incubated with 3.75 mM sodium citrate, 0.1% Triton X-100, and 30 $\mu g\ ml^{-1}$ RNase A, and resuspended in 20 $\mu g\ ml^{-1}$ propidium iodide. Stained nuclei were analysed by flow cytometry (FACS Calibur, Becton Dickinson, San Jose, CA, USA).

Cellular and mitochondrial Bax, cytochrome c, and β-actin

Levels of Bax, cytochrome *c*, and β-actin in mitochondria and the cytoplasm were immunodetected following a previously described method.[23] Bax protein was immunodetected using a mouse monoclonal antibody (mAb) against human Bax (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cytochrome *c* protein was immunodetected using a mouse mAb against pigeon cytochrome *c* protein (BioSource, Camarillo, CA, USA). β-Actin was immunodetected using a mouse mAb against mouse β-actin (Sigma) as an internal control. Intensities of the immunoreactive protein bands were determined using the UVIDOCMW version 99.03 digital imaging system (UVtec, Cambridge, UK).

Mitochondrial membrane potential and ATP levels

The membrane potential of mitochondria in HepG2 cells was determined according to a previously described method.[24] Briefly, cells were harvested and incubated with 3,3′-dihexyloxacarbocyanine ($DiOC_6$) at 37°C for 30 min in a humidified atmosphere of 5% CO_2 after drug administration. The fluorescent intensities of $DiOC_6$ in HepG2 cells were analysed by flow cytometry (Becton Dickinson). Levels of cellular ATP in HepG2 cells were determined by a bioluminescence assay as described previously,[25] which is based on the requirement of luciferase for ATP in producing a light emission, according to the protocol of Molecular Probes' (Eugene, OR, USA) ATP Determination kit.

Caspase activities

Activities of caspases-3, -6, and -9 in HepG2 cells were determined using fluorometric assay kits (R&D Systems,

Minneapolis, MN, USA) as described previously.[23] The peptide substrates for assays of caspase-3, -6, and -9 activities were DEVD, VEID, and LEHD, respectively. These peptides were conjugated to 7-amino-4-trifluoromethyl coumarin for fluorescence detection. In the inhibition study, HepG2 cells were pretreated with 50 μM Z-VEID-FMK, an inhibitor of caspase-6, for 1 h, and then exposed to *S*-(+)-ketamine. The data are expressed in terms of cell number.

Statistical analysis

The statistical significance of differences among the control and ketamine-treated macrophages was evaluated using the Kruskal–Wallis non-parametric ANOVA followed by Duncan's multiple-range test, and differences were considered statistically significant at *P*-values of <0.05.

Results

Toxicity of S-(+)-ketamine

Exposure of HepG2 cells to 10 and 50 μM *S*-(+)-ketamine for 24 h did not affect the release of GPT (Fig. 1A). Meanwhile, when treated with 100 and 200 μM *S*-(+)-ketamine for 24 h, the release of GST from HepG2 cells into the culture medium was augmented by 2.2- and 3.4-fold, respectively (Fig. 1A). Exposure of HepG2 cells to 200 μM for 6 and 24 h, respectively, increased the release of GPT by 2.2- and 3.2-fold (Fig. 1B). The release of LDH was enhanced by 2.4- and 3.5-fold after administration of 100 and 200 μM *S*-(+)-ketamine for 24 h (Fig. 1C). Treatment of HepG2 cells with 200 μM *S*-(+)-ketamine for 6 and 24 h caused significant 2.3- and 4.1-fold increases in the release of LDH (Fig. 1D). After exposure to 100 and 200 μM *S*-(+)-ketamine for 24 h, the viability of HepG2 cells decreased by 31% and 54%, respectively (Fig. 1E). Administration of 200 μM *S*-(+)-ketamine to HepG2 cells for 24 h significantly decreased cell viability by 31% and 60%, respectively (Fig. 1F).

Apoptotic insults

Exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 1 h did not affect cell morphology (Fig. 2A). When administered for 6 h, *S*-(+)-ketamine obviously caused shrinkage of HepG2 cells. Treatment with 200 μM *S*-(+)-ketamine for 24 h decreased cell numbers and caused much more serious shrinkage of HepG2 cells (Fig. 2A). Quantification of DNA fragmentation revealed that exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 1 h did not cause DNA damage (Fig. 2B). Meanwhile, after exposure for 6 and 24 h, *S*-(+)-ketamine significantly induced DNA fragmentation by 92% and 3.9-fold, respectively. Exposure to 200 μM *S*-(+)-ketamine for 6 and 24 h increased the percentages of HepG2 cells which underwent apoptosis by 22% and 45%, respectively (Fig. 2C).

Apoptotic mechanisms

Exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 6 and 24 h obviously enhanced the levels of Bax in mitochondria (Fig. 3A, *top panel*, lanes 3 and 4). Simultaneously, the amounts of cytosolic Bax in HepG2 cells decreased (Fig. 3A, *middle panel*, lanes 3 and 4). The levels of β-actin were immunodetected as the internal standards (Fig. 3A, *bottom panel*). These immunoreactive protein bands were quantified and analysed (Fig. 3B). Administration of 200 μM *S*-(+)-ketamine for 6 and 24 h significantly augmented the amounts of mitochondrial Bax by 2.2- and 2.5-fold, respectively. The levels of cytosolic Bax time-dependently decreased by 36% and 72% after *S*-(+)-ketamine administration for 6 and 24 h, respectively (Fig. 3B).

Exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 6 and 24 h significantly decreased the mitochondrial membrane potential by 33% and 55%, respectively (Fig. 3C). Consequently, the levels of cellular ATP in HepG2 cells time-dependently decreased by 28% and 56%, respectively (Fig. 3D). Administration of 200 μM *S*-(+)-ketamine to HepG2 cells for 6 and 24 h enhanced the amounts of cellular cytochrome *c* (Fig. 3E, *top panel*, lanes 3 and 4). The amounts of β-actin were immunodetected as the internal standards (Fig. 3E, *bottom panel*). These immunoreactive protein bands were quantified and analysed (Fig. 3F). Treatment of HepG2 cells with *S*-(+)-ketamine for 6 and 24 h significantly increased cellular cytochrome *c* levels by 2.4- and 2.76-fold, respectively (Fig. 3F).

Exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 1 h did not affect caspase-9 activity (Fig. 4A). After administration of *S*-(+)-ketamine for 6, 16, and 24 h, the activities of caspase-9 were augmented by 68%, 85%, and 2.6-fold, respectively. Treatment of HepG2 cells with 200 μM *S*-(+)-ketamine for 1 h did not influence caspase-3 activity (Fig. 4B). Meanwhile, when treated with *S*-(+)-ketamine for 6, 16, and 24 h, the activities of caspase-3 were significantly enhanced by 62%, 74%, and 2.4-fold, respectively. The activity of caspase-6 did not change under treatment with *S*-(+)-ketamine for 1 h (Fig. 4C). Exposure of HepG2 cells to 200 μM *S*-(+)-ketamine for 6, 16, and 24 h caused significant 76%, 2.1- and 3.7-fold, respectively, increases in caspase-6 activity.

Treatment of HepG2 cells with 200 μM *S*-(+)-ketamine significantly increased caspase-6 activity by 3.7-fold (Fig. 5A). Pretreatment with Z-VEID-FMK significantly decreased *S*-(+)-ketamine-induced caspase-6 activity by 53%. *S*-(+)-Ketamine caused a 3.1-fold increase in DNA fragmentation (Fig. 5B). Pretreatment of HepG2 cells with Z-VEID-FMK significantly alleviated *S*-(+)-ketamine-caused DNA fragmentation by 44%. Administration of 200 μM *S*-(+)-ketamine induced 46% greater apoptosis of

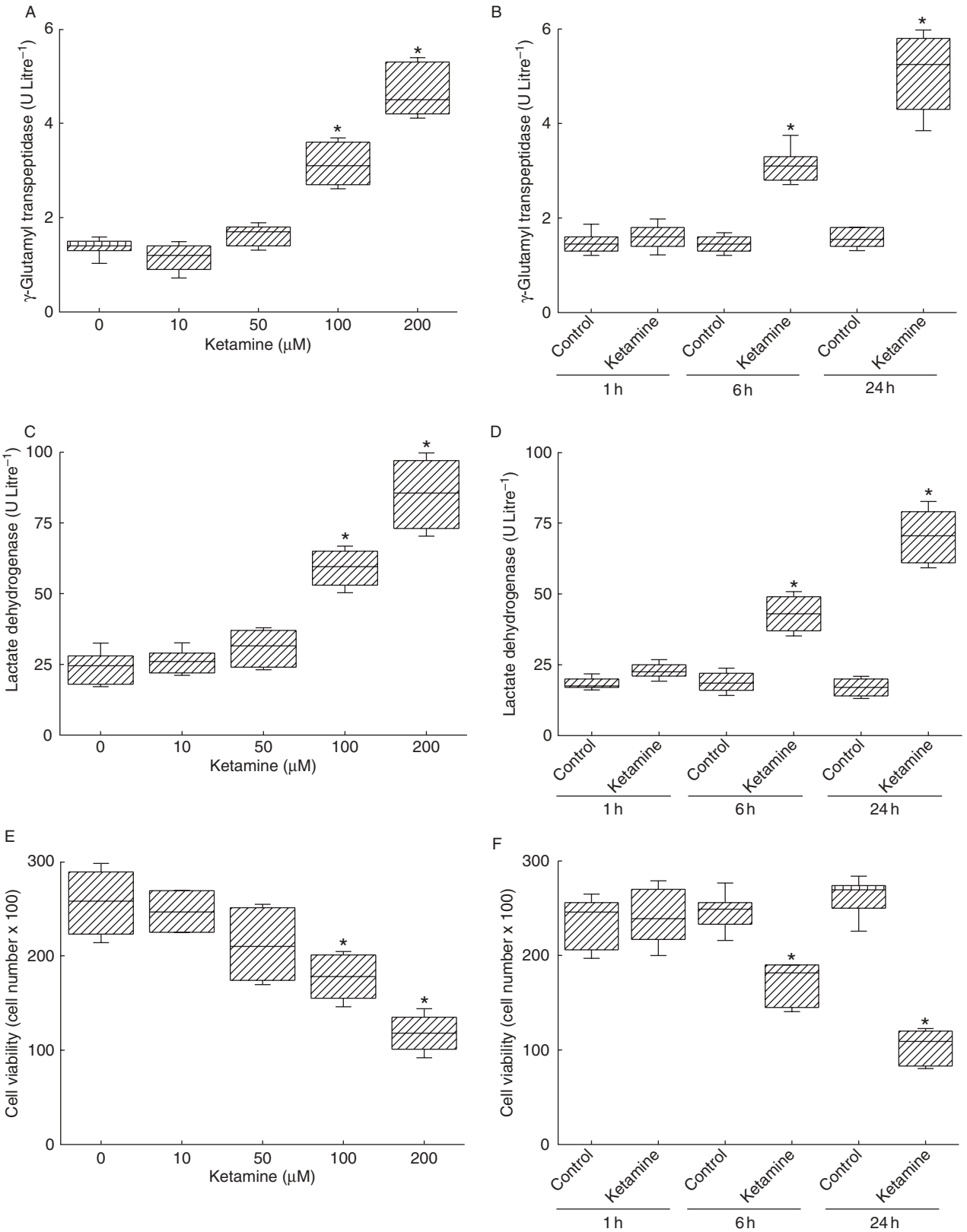

Fig 1 Effects of *S*-(+)-ketamine on the release of GPT and LDH, and cell viability. HepG2 cells were exposed to 10, 50, 100, and 200 μM ketamine for 24 h, or to 200 μM ketamine for 1, 6, and 24 h. The amounts of GPT (A and B) and LDH (C and D) released from human hepatocytes to the culture medium were quantified using an automatic autoanalyzer. Cell viability was determined by a trypan blue exclusion method (E and F). The figures are drawn as box and whisker plots showing median, inter-quartile, and full ranges. *Values significantly differ from the respective control, $P<0.05$, $n=6$.

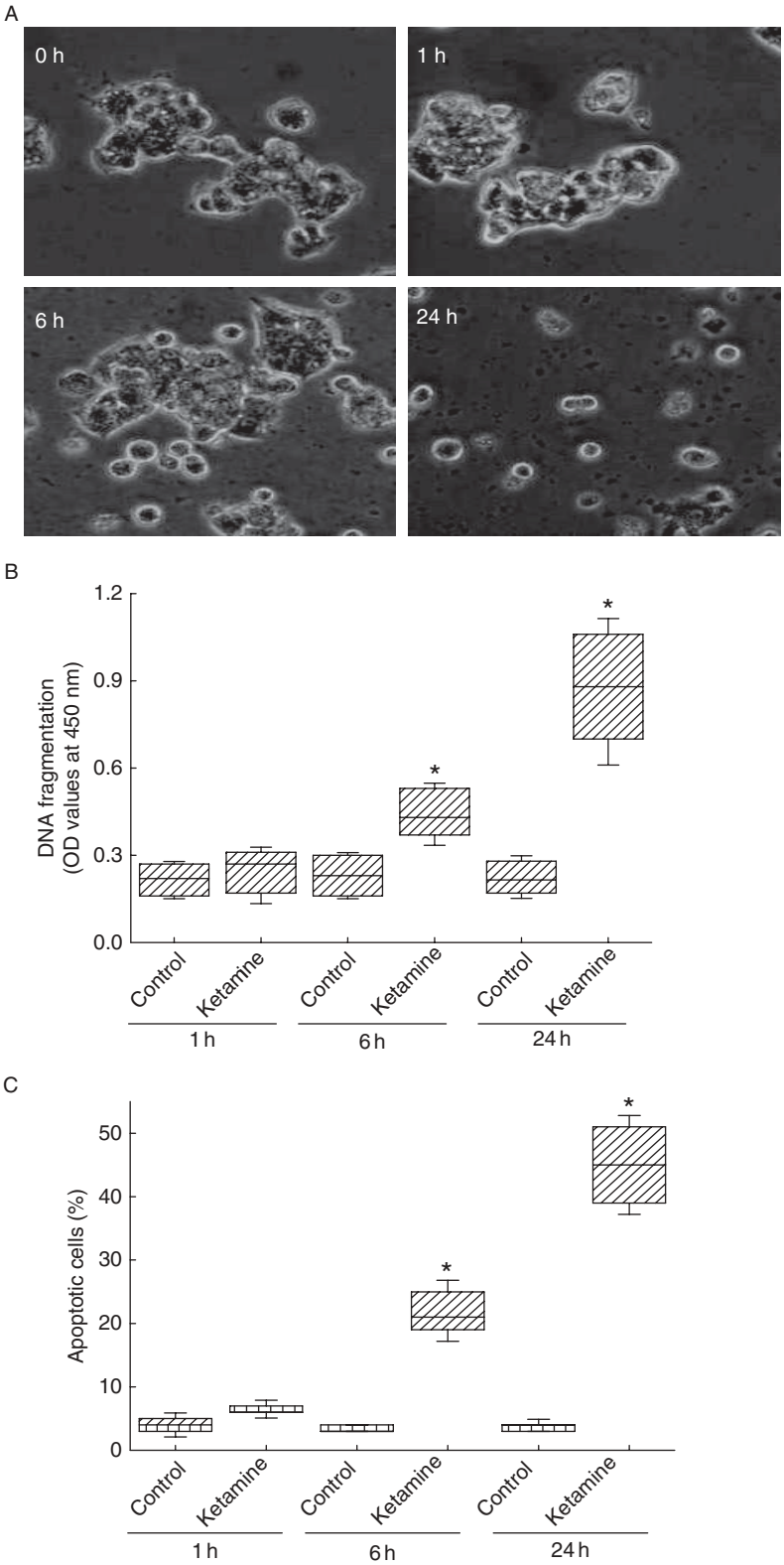

Fig 2 Effects of *S*-(+)-ketamine on cell morphologies, DNA fragmentation, and cell apoptosis. HepG2 cells were exposed to 200 μM ketamine for 1, 6, and 24 h. Cell morphologies were observed and photographed using a reverse-phase microscope (A). Fragmentation of genomic DNA was quantified using a BrdU-labelled histone-associated enzyme-linked immunosorbent assay (B). Apoptotic cells were analysed using flow cytometry (C). The figures are drawn as box and whisker plots showing median, inter-quartile, and full ranges. *Values significantly differ from the respective control, $P<0.05$, $n=6$.

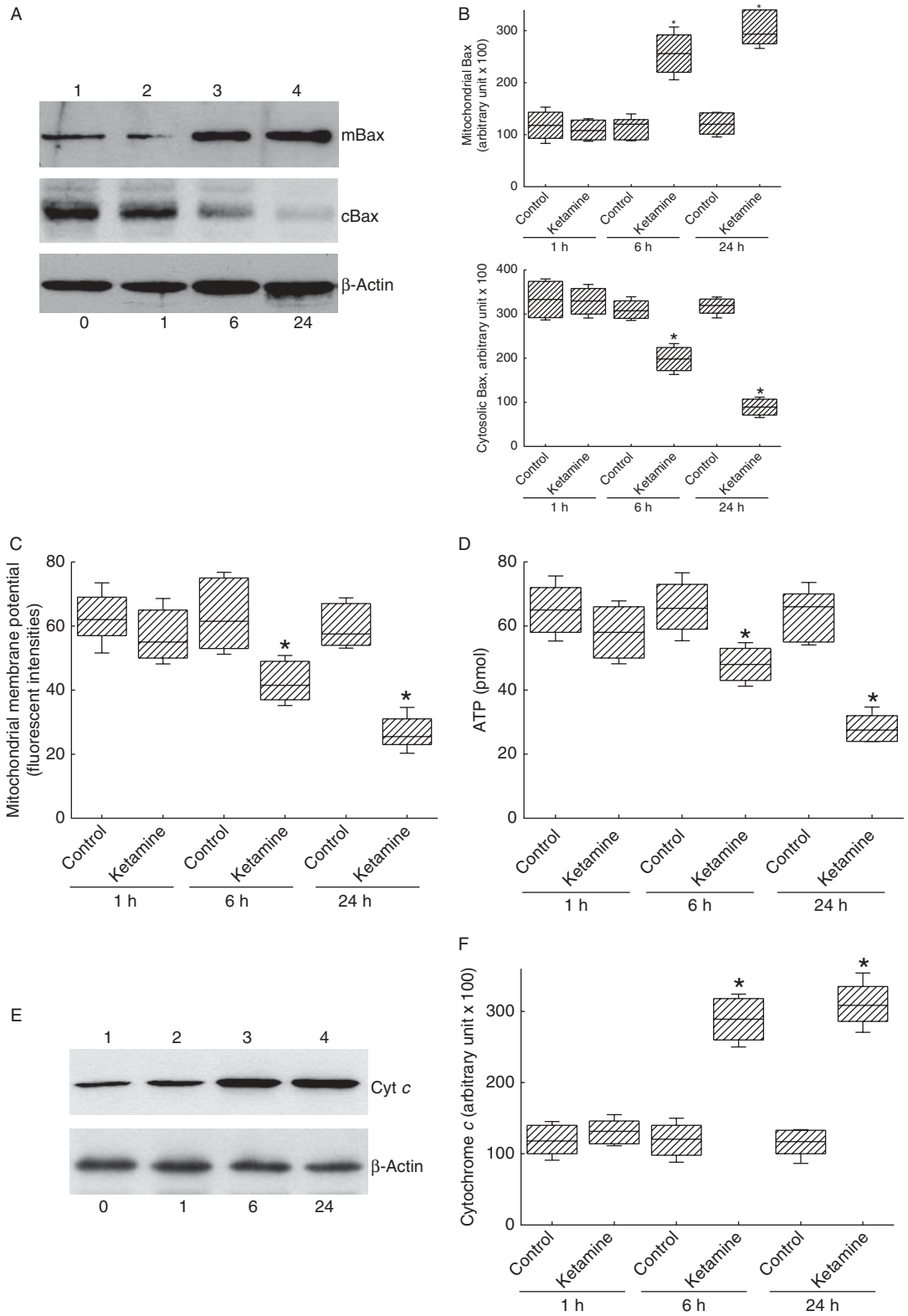

Fig 3 Effects of *S*-(+)-ketamine on Bax translocation, the mitochondrial membrane potential, cellular ATP levels, and release of cytochrome *c* (Cyt *c*). HepG2 cells were exposed to 200 μM ketamine for 1, 6, and 24 h. The amounts of mitochondrial and cytosolic Bax proteins were immunodetected (A, *top and middle panels*). β-Actin was immunodetected as the internal control (A, *bottom panel*). These immunoreactive protein bands were quantified and analysed (B). The mitochondrial membrane potential was detected by staining with $DiOC_6$ and quantified using flow cytometry (C). The levels of cellular ATP were analysed using a bioluminescence assay (D). Cyt *c* in the cytoplasm was immunodetected (E, *top panel*). β-Actin was analysed as the internal control (E, *bottom panel*). These protein bands were quantified and analysed (F). The figures are drawn as box and whisker plots showing median, inter-quartile, and full ranges. *Values significantly differ from the respective control, $P<0.05$, $n=6$.

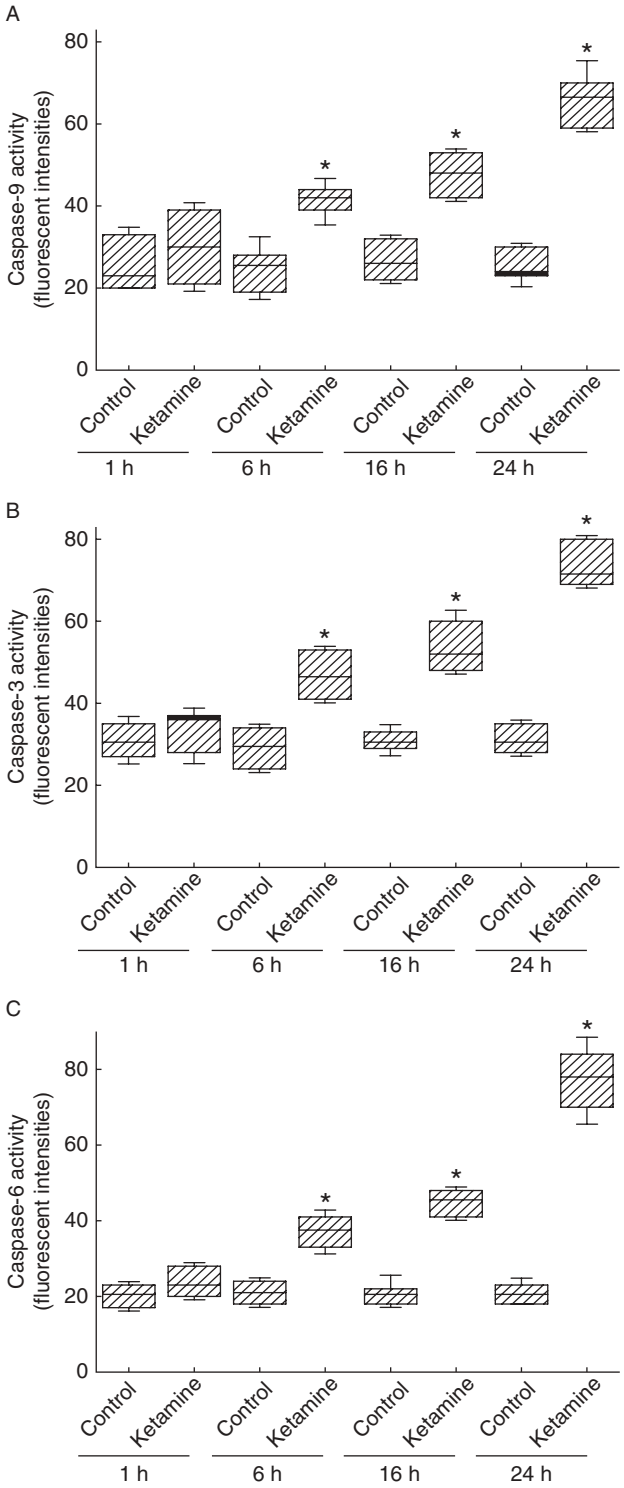

Fig 4 Effects of *S*-(+)-ketamine on the activities of caspases-9, -3, and -6. Human hepatocytes were exposed to 200 μM ketamine for 1, 6, 16, and 24 h. Activities of caspases-9, -3, and -6 were analysed by fluorogenic assays using LEHD, DEVD, and VEID as the respective substrates (A–C). The figures are drawn as box and whisker plots showing median, inter-quartile, and full ranges. *Values significantly differ from the respective control, $P<0.05$, $n=6$.

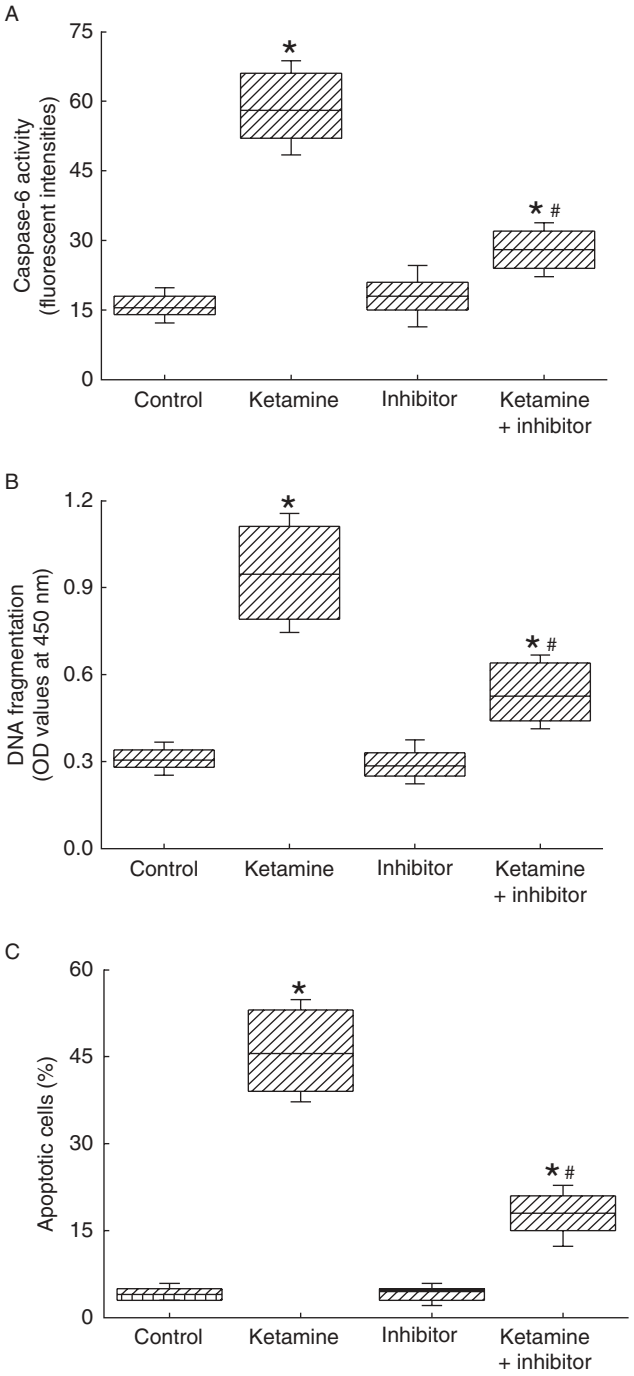

Fig 5 Effects of *S*-(+)-ketamine and Z-VEID-FMK on caspase-6 activity, DNA fragmentation, and cell apoptosis. Human hepatocytes were pretreated with 50 μM Z-VEID-FMK, an inhibitor of caspase-6, for 1 h, and then exposed to 200 μM ketamine for another 16 h. Caspase-6 activity was determined by a fluorogenic assay (A). DNA fragmentation was quantified using a BrdU-labelled histone-associated enzyme-linked immunosorbent assay kit (B). Apoptotic cells were quantified using flow cytometry (C). The figures are drawn as box and whisker plots showing median, inter-quartile, and full ranges. The symbols * and # indicate that a value significantly ($P<0.05$) differs from the respective control and ketamine-treated groups, respectively ($n=6$).

HepG2 cells (Fig. 5c). After pretreatment with Z-VEID-FMK, the *S*-(+)-ketamine-induced apoptotic insults to HepG2 cells significantly decreased by 58%.

Discussion

This study shows that *S*-(+)-ketamine can induce apoptotic insults to HepG2 cells, whereas exposure of HepG2 cells to 100 and 200 μM *S*-(+)-ketamine led to cell toxicity. Concentrations of ketamine of ≤100 μM are within the range of clinical relevance.[26] *S*-(+)-Ketamine was reported to possess different pharmacokinetic profile from racemic ketamine.[27] Previous studies showed that racemic ketamine at high concentrations could induce injuries of rat neurones and human umbilical vein endothelial cells.[6 20] Thus, *S*-(+)-ketamine in HepG2 cells appears to have more toxic effects than its racemic form. Our present results further reveal that exposure of HepG2 cells to 100 μM *S*-(+)-ketamine caused cell shrinkage, DNA fragmentation, and cell arrest at sub-G1 phase. The appearance of these characteristics indicates that cells are undergoing apoptosis.[28 29] Thus, *S*-(+)-ketamine induces insults to HepG2 cells via an apoptotic mechanism.

The apoptotic Bax protein participates in *S*-(+)-ketamine-induced apoptosis of HepG2 cells. Exposure of HepG2 cells to *S*-(+)-ketamine significantly increased mitochondrial Bax levels but simultaneously decreased cytosolic ones. Bax is an apoptotic protein and has been shown to regulate cell apoptosis.[16 17] Because mitochondria cannot synthesize the Bax protein, increased amounts of mitochondrial Bax are due to translocation from the cytoplasm. Our present data revealed that *S*-(+)-ketamine decreases the mitochondrial membrane potential. A previous study showed that Bax translocation to mitochondria can depolarize the mitochondrial membrane.[30] Thus, *S*-(+)-ketamine decreases the mitochondrial membrane potential possibly due to stimulation of Bax translocation. Blom and colleagues[18] reported that an inhibition in cellular ATP synthesis can induce cell apoptosis. In this study, we showed that administration of *S*-(+)-ketamine to HepG2 cells time-dependently decreased cellular ATP levels. Therefore, *S*-(+)-ketamine may enhance Bax translocation from the cytoplasm to mitochondrial membranes and then cause mitochondrial dysfunction through suppression of the mitochondrial membrane potential and cellular ATP synthesis in HepG2 cells, thus inducing cell apoptosis.

Cytochrome *c* mediates *S*-(+)-ketamine-induced apoptotic insults to HepG2 cells. Exposure of HepG2 cells to *S*-(+)-ketamine time-dependently increases the levels of cytosolic cytochrome *c*. Cytochrome *c* is one of the critical mitochondrion-related apoptotic factors.[28 31] Exposure of HepG2 cells to *S*-(+)-ketamine significantly increased caspase-9 activity. Cytochrome *c* released from mitochondria can interact with cytoplasmic apoptotic protease-activating factor-1 in forming apoptosomes and mediating caspase-9 activation.[32] Sequentially, the present data showed that activities of caspases-3 and -6 were enhanced by *S*-(+)-ketamine. Caspase-9 promotes digestion of procaspases-3 and -6 into activated subunits.[33] Caspase-3 has a cascade effect on the activation of caspase-6.[32] After activation, caspase-3 can cleave cellular key proteins such as lamin and nuclear mitotic apparatus proteins which affect cell functions.[34] Our present results further demonstrate that suppression of caspase-6 activation by its specific inhibitor, Z-VEID-FMK, significantly lowered *S*-(+)-ketamine-induced DNA fragmentation and cell apoptosis. Therefore, the *S*-(+)-ketamine-induced activation of caspases-9, -3, and -6 participates in cell apoptosis.

There are certain limitations in this study. HepG2 cells are a useful *in vitro* model, but overexpress cytochrome P450 enzymes.[13 14] In this study, we used HepG2 cells as our experimental model to show that *S*-(+)-ketamine can damage hepatocytes via an apoptotic mechanism. Future work should include investigation of the effect of *S*-(+)-ketamine on primary hepatocytes, and also *in vivo* using animals. Although ketamine has a short elimination half-life of <3 h in adults,[35] our study showed that *S*-(+)-ketamine can trigger apoptotic insults after exposure for 6 and 24 h. The *S*-(+)-ketamine-induced toxicity during prolonged exposure may be clinically relevant when used for longer periods such as for treatment of postoperative pain, prevention/treatment of neuropathic pain syndromes, and as a sedative in the intensive care unit. However, since our *in vitro* study uses a hepatoma cell line as the experimental model, clinical inference should not be made at this stage.

In summary, this study shows that *S*-(+)-ketamine can damage HepG2 cells via an apoptotic pathway. A schematic diagram is given in Figure 6. Administration of *S*-(+)-ketamine increases the translocation of the proapoptotic Bax protein from the cytoplasm to the mitochondrial outer membrane (Step 1). Simultaneously, *S*-(+)-ketamine

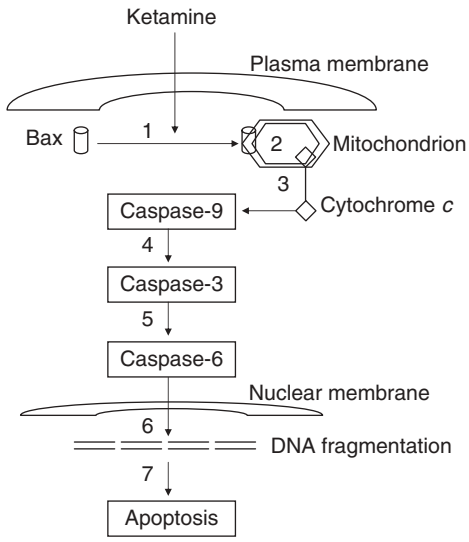

Fig 6 Signal-transducing mechanism of *S*-(+)-ketamine-induced apoptotic HepG2 cells.

induces mitochondrial dysfunction due to suppression of the mitochondrial membrane potential and cellular ATP synthesis (Step 2). The amounts of mitochondrion-related apoptotic factor cytochrome *c* increase after *S*-(+)-ketamine administration (Step 3). Sequentially, *S*-(+)-ketamine increases the activities of caspases-9, -3, and -6 (Steps 3–5), and consequently induces damage to genomic DNA (Step 6). Inhibition of caspase-6 activity significantly lowered *S*-(+)-ketamine-induced DNA fragmentation and cell apoptosis (Steps 6 and 7). Therefore, according to the present data, we suggest that *S*-(+)-ketamine induces apoptotic insults to HepG2 cells via a Bax-mitochondria-caspase protease pathway. Although racemic ketamine is more commonly used both clinically and by drug abusers, the more toxic effects of *S*-(+)-ketamine on hepatocytes provide important toxicological and pharmacological information about this anaesthetic agent when it is clinically applied.

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