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Effects of combined toxicity of methamphetamine and ketamine on apoptosis, oxidative stress and genotoxicity in HepG2 cells

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ABSTRACT

Methamphetamine (MA) and ketamine (KET) are widely abused drugs individually. Previous surveys have revealed that the combined consumption of MA and KET were prevalent in illicit drugs abusers. However, few studies on the toxic effects induced by the combination of MA and KET have been reported. In this study, combined treatments were carried out using 3 × 3 full factorial design to determine the combined effects of MA and KET on apoptosis, oxidative stress and genotoxicity in HepG2 cells. Higher apoptosis and oxidative damage were observed in the MA treatments groups. Compared with control groups, the maximum apoptotic rate and level of malondialdehyde were ~7.7 fold and ~5.5 fold respectively. The mechanism that excessive oxidative stress resulted in cell apoptosis and DNA damage was inferred. For the joint effects, synergistic or additive interactions were found at different biological endpoints for various combinations, likely due to the mechanism in which MA promotes the metabolism of KET, which together provokes even greater oxidative stress. In conclusion, synergistic or additive interactions between MA and KET enhance cytotoxicity, oxidative damage and genotoxicity in HepG2 cells more than either of the drugs alone, which implies higher risk for abusers when exposed to the polydrug situation.

1. Introduction

Methamphetamine (MA) and ketamine (KET) are widely abused drugs currently, especially in Asian countries (Hser et al., 2016). MA is a member of the group of amphetamine-type stimulants (ATS), and abuse of MA has increased constantly in recent years, triggering severe social problems and arousing extensive public concern (Chomchai and Chomchai, 2015; Sommers and Baskin, 2006; Sommers et al., 2006). Between 2009 and 2013, the production and consumption of MA increased by 158%, and the MA-related mortality rates increased dramatically (Luikinga et al., 2018). MA is often used as the main ingredient in many illegally marketed recreational tablets, which may contain several psychoactive drugs such as ketamine, amphetamine and 3,4-methylenedioxymethamphetamine (MDMA) (Kuwayama et al., 2007; Moradi and Ziarati, 2018; Senn et al., 2007). KET, a derivative of phencyclidine (PCP), has also been abused increasingly for recreational use (Sassano-Higgins et al., 2016), and made available at a relatively low price (Gahlinger, 2004). The abuse-frequency of KET has increased from < 1% in 1999 to becoming the third most commonly abused drugs in 2015 in Hong Kong (Siu et al., 2018).

At present, the research hotspot for MA and KET focuses on the effects of the drugs on the nervous system. Both MA and KET have been shown to result in neurotoxicity and nervous system damage (Cartágenes et al., 2017; Moszczynska and Callan, 2017). However, results of earlier studies indicate that these two drugs also have toxic effects on peripheral organ tissues, including the hepatotoxicity (Darke et al., 2008; Katsumata et al., 1993). The types of toxic effects include, but are not limited to, oxidative stress, cell apoptosis and genotoxicity. MA is able to trigger oxidative stress, lipid peroxidation and mitochondria dysfunction in liver (Eskandari et al., 2014; Koriem and Soliman, 2014; Mashayekhi et al., 2014). *In vivo* and in vitro studies have demonstrated that MA-induced hepatotoxicity is associated with

Abbreviations: ANOVA, analysis of variation; ATS, amphetamine-type stimulants; CAT, catalase; CYP, cytochrome P450 proteins; GSH, glutathione; KET, ketamine; MA, methamphetamine; MDA, malondialdehyde; MDMA, 3,4-methylenedioxymethamphetamine; PCP, phencyclidine; ROS, reactive oxygen species; SCGE, single gel electrophoresis; SOD, superoxide dismutase

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cell cycle arrest and apoptosis (Dias da Silva et al., 2013; Sharikova et al., 2018; Wang et al., 2017). In addition, MA was reported to produce genotoxicity effects such as DNA damage and chromosomal aberration (Jeng et al., 2005; Li et al., 2003; McCallum et al., 2011; Ropek et al., 2019). Previous studies also suggested that KET caused liver injury not only by inducing generations of free radicals and lipid peroxidation, but also by inhibiting mitochondrial ATP synthesis and inducing apoptosis (Abdel-Salam et al., 2015; Chang et al., 2009; Lee et al., 2009). Furthermore, earlier studies showed that KET and its metabolite produced genetic toxicity in vitro assays (Adhvaryu et al., 1986; Toyama et al., 2006).

Psychoactive illicit drugs are commonly adulterated with drugs from different categories in order to gain revenue for the distributers by obtaining more doses (Solimini et al., 2017), as well as to enhance the drugs' effects (Kelly and Parsons, 2009). The consumptions of ATS, KET and ATS + KET are prevalent in illicit drugs abusers (Solimini et al., 2017; Zhang et al., 2013). Previous surveys have revealed that young people who frequently nightclubs and street-wise adolescents are more likely to actively abuse the combination of MA and KET (Kelly and Parsons, 2009; Martin et al., 2006). Although the use of MA or KET alone rarely leads to fatal intoxication (Gill and Stajíc, 2000; Sribanditmongkol et al., 2000), the situation may change in the case of polydrug abuse. Various components in the mixture might interact with each other in the body, leading to serious toxic effects. Previous studies have demonstrated that the combined treatments of MA and KET produced mutual enhancement of neurotoxicity (Ke et al., 2008; Xu et al., 2006). However, few studies on other types of toxic effects induced by the combination of MA and KET have been reported. Therefore, it is necessary to evaluate the combined toxic effects of MA and KET. The results of such investigation may have certain significance in deepening our understanding of these commonly abused drugs and searching for treatment methods.

Factorial design, an efficient method for evaluating the interaction of two or more mixed compounds, is in wide use because it can easily analyze and interpret interaction effects without calculating toxicity indexes such as LD50, EC50, etc. (Feron and Groten, 2002; Groten et al. 1996, 2001). HepG2 cells is commonly used, human-derived hepatoma cell line with high differentiation level that maintains certain enzymes involved in phase I and II metabolism (Westerink and Schoonen, 2007a, b). This cell line is often used in toxicological experiments, because the two important mechanisms of drug-induced hepatotoxicity, namely reactive oxygen species (ROS) formation and glutathione depletion, are observable, which suggests similarities to human primary hepatocytes to some extent (Schoonen et al., 2005; Van den Hof et al., 2014). In addition, the 5th International Workshop on Genotoxicity Testing also reported human p53-competent cells like HepG2 may be a good model to evaluate genotoxicity (Pfuhler et al., 2011). Therefore, we selected factorial design as the evaluation method and HepG2 cells as the experimental object.

The objective of the present study was to evaluate the putative combined effects of MA and KET on apoptosis, oxidative stress and genotoxicity in HepG2 cells. In this paper, a 3 × 3 full factorial design was used to investigate the interactive effects of binary mixtures. Cell apoptosis was measured by flow cytometry, oxidative stress was measured by four biomarkers (MDA: Malondialdehyde; SOD: Superoxide Dismutase; CAT: Catalase; GSH: Glutathione), and DNA damage was measured by Comet assay.

2. Material and methods

2.1. Chemicals

Methamphetamine (Cerilliant, USA) and ketamine (Cerilliant, USA) were obtained from the Forensic Center of Public Security of Yun Fu City (Guangdong, China) and were dissolved in sterile deionized water to prepare 200 mM stock solutions. The solutions were filtered through 0.22-μm filters (Millipore, USA) and then diluted with cell culture media.

2.2. Cell culture

HepG2 cells were obtained from the biochemistry laboratory (First Affiliated Hospital of Jinan University, Guangzhou, China) and were used in experiments that investigated the toxic effects of MA, KET, and MA + KET. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco) in 25-cm^2 flasks (Corning, USA) that were maintained in a humidified incubator with 5% CO_2 at 37 °C. When the cultures had been confluent, the cells were detached with trypsin/EDTA (Gibco) and then sub-cultured. The 5th to 9th passage from the stock cultures of HepG2 cells were used for the experiments.

2.3. Experimental design

HepG2 cells were exposed to MA or KET at concentrations of 0.1, 0.5, 1.0, 1.5, and 2.0 mM which were referred to the previous studies (Dias da Silva et al., 2013; Lee et al., 2009; Shen et al., 2015; Wang et al., 2017). Based on the results of single treatment groups in cytotoxicity assay, two concentrations of MA and KET (0.1 mM for low level and 1.0 mM for high level) were used to estimate the cytotoxic interaction of these two drugs. Cells were exposed to the MA + KET mixtures using 3 × 3 full factorial design, which contains the equal proportion (0.1 + 0.1 and 1.0 + 1.0 mM) and unequal proportion (0.1 + 1.0 and 1.0 + 0.1 mM) mixtures. All the selected concentrations in single and combined treatment groups ensured above 50% viability to match the request of comet assay (Garaj-Vrhovac et al., 2013).

2.4. Cytotoxicity assays

HepG2 cells were seeded into wells of a 96-well plates (8000 cells per well), in a volume of 100 μL of complete culture medium. On the day of the experiment, the media was carefully aspirated and the cells were exposed to MA, KET or MA + KET diluted in media for 24 h. Each 96-well plate also included three wells where the cells were not exposed to test agents; these wells served as the negative controls. At the indicated time point, 10 μL of MTT (5 mg/mL) was added into each well and the plate was incubated in a humidified incubator with 5% CO_2 at 37 °C for 4 h. The formazan crystals were subsequently dissolved in 100 μL dimethyl sulfoxide and the absorbance measured at 490 nm.

2.5. Apoptosis assays

HepG2 cells were seeded into 6-well plates (1.5×10^5 cells per well) in 2 mL medium and cultured for 24 h. The cells were exposed to the selected concentration of MA, KET or MA + KET and cultured for an additional 24 h. Thereafter, the cells were rinsed with phosphate buffer saline (PBS, Gibco) and then trypsinized to detach. Before the tests, cells were washed with PBS and then set aside until testing.

The Annexin V-FITC/PI Apoptosis Detection kit (BD Biosciences, USA) was used to distinguish viable, early apoptotic and late apoptotic/necrotic cells. Briefly, the cells were resuspended in 100 μL binding buffer, followed by the addiction of 2.5 μL Annexin V-FITC and 2.5 μL propidium iodide. The cells were gently mixed and incubated for 15 min at 25 °C. Thereafter, 200 μL binding buffer was added and the cells were gently mixed. The cell suspension was transferred into the Falcon tube and analyzed with the Gallios flow cytometer (Beckman-Coulter, Brea, CA, USA).

2.6. Oxidative stress biomarkers response assays

HepG2 cells were seeded and collected as above. Meanwhile,

Table 1
Cytotoxicity of MA and KET alone or in combination in HepG2 cells after 24 h exposure.

MA (mM)	Viability (%control)	KET (mM)	Viability (%control)	MA + KET (mM)	Viability (%control)	
					Measured value	Theoretical value
0.1	98.2 ± 1.1	0.1	98.3 ± 1.5	0.1 + 0.1	93.5 ± 2.1	96.7 ± 1.8
0.5	96.0 ± 2.1*	0.5	97.4 ± 1.5	0.1 + 1.0	77.0 ± 1.8##	84.5 ± 1.9
1.0	85.5 ± 1.8**	1.0	86.1 ± 1.6**	1.0 + 0.1	76.1 ± 2.1##	84.0 ± 2.3
1.5	76.1 ± 1.6**	1.5	80.8 ± 1.2**	1.0 + 1.0	61.0 ± 1.4##	71.8 ± 2.4
2.0	65.7 ± 1.4**	2.0	75.8 ± 1.4**			

Data are expressed as cytotoxic effect in treatment groups compared to control and represent the mean of three independent experiments.
*, ** in single exposures represent differences between control group and treatment groups at $p < 0.05$, $p < 0.01$, respectively; #, ## in combined exposures represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$, respectively.

solvent control and positive control (treated with 2 mM H_2O_2 for 30 min) followed the treatments applied to all experimental groups. The above collected cells were broken by ultrasonic processor and the supernatant was used to determine the activity of superoxide dismutase (SOD) and catalase (CAT) and the content of malondialdehyde (MDA) and glutathione (GSH). The detection kits were used for determination of SOD (Beyotime Institute of Biotechnology, Jiangsu, China), MDA (Leagene Institute of Biotechnology, Beijing, China) and GSH (Nanjing Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's instructions. The determination of intracellular CAT activity was conducted using the enzyme dynamic method as previously described (Aebi, 1984) and detected by UV spectrophotometer (SHIMADZU, UV-3600 Plus). The protein concentrations of these supernatants were determined by performing the bicinchoninic acid (BCA) protein assay kit. (Leagene Institute of Biotechnology, Beijing, China).

2.7. Comet assays

HepG2 cells were seeded and collected as above. DNA damage was evaluated using the alkaline single gel electrophoresis (SCGE) method (Pfuhler et al., 2011) with slight modification. Briefly, 70 μL 0.5% low melting-point agarose (LMP) mixed with 10 μL cell suspensions was added onto slides that were pre-coated with 1.5% normal melting-point agarose (NMP) and solidified at 4 °C for 20 min. Then the slides were slowly lowered into cold lysing buffer at 4 °C for 1 h in the dark and immersed in electrophoretic buffer to unwind DNA for 40 min successively. The electrophoresis was carried out at 25 V for 25 min and then neutralized in 0.4 M Tris-HCl buffer (pH 7.5) three times for 10 min. The slides were stained with 30 μL propidium iodide (PI) and observed with a fluorescence microscope (Leica, Germany). Tail DNA percentages (%) were obtained from the comet assay software project (CASP) to evaluate DNA damage.

2.8. Statistical analysis

All above experimental data were presented as mean values ± standard deviations of three replicated experiments. For the individual treatment assay, statistical significance of parameters between the control group and test group were analyzed using SPSS 24.0 with one-way analysis of variation (ANOVA), for which $p < 0.05$ was considered statistically significant. Three types of combined effects, additive, synergism and antagonism effect, can be observed in the combined action of chemicals (Groten, 2000). For the combined treatment assay, the interactive effects were evaluated by comparing the "theoretical value", which is defined as the summation of toxic effects induced by each chemical in the individual treatment assay, to the "measured value" from the endpoint detection in the combined treatment assay (Klarić et al., 2010; Li et al., 2017; Weber et al., 2005). The theoretical values (T) are calculated using the following formula:

$$T = C_{mix} + (M_A - C_A) + (M_B - C_B)$$

Heretofore, C_{mix} represents the results of control groups in combined treatments. C_A, C_B represents the results of control groups in A/B individual treatments. M_A, M_B represent measured values in the A/B individual treatments.

The significance of differences between theoretical and measured values was determined using one-way ANOVA. The results were considered to be synergic or antagonist when the p values were less than 0.05, while the additive effects were considered to exist if $p > 0.05$.

3. Results

3.1. Cell viability of HepG2 cells exposed to MA or KET alone and their binary mixtures

The viability of HepG2 cells after a 24-h exposure to single or combined MA and KET was measured using MTT cytotoxicity assay and results are displayed in Table 1. Results are expressed as a percentage compared with the corresponding negative control. Single exposure to MA and KET caused a dose-dependent decrease of cell viability. MA concentrations ≥0.5 mM were cytotoxic, while KET concentration ≥1.0 mM showed cytotoxicity. MA was more effective than KET in decreasing cell viability when compared at a concentration of higher than 1.0 mM.

After a co-exposure experiment, stronger cytotoxicity was found with the combined MA + KET than with MA or KET mono-exposure (0.1 mM, 1.0 mM). Almost all combined treatment presented significant differences when compared with theoretical values, excluding the combination of MA + KET (0.1 + 0.1 mM). The strongest cytotoxicity was observed in the 1.0 + 1.0 group.

3.2. Apoptosis of HepG2 cells exposed to MA or KET alone and their binary mixtures

Fig. 1 and Fig. 2 depict the relative number (% of cell population) of viable and total apoptotic cells after 24 h of treatment with MA, KET or their mixtures. After exposure to MA and KET alone, the viable fraction showed significant downward trends at concentrations ≥1.0 mM. Total apoptosis was calculated by the sum of early and late apoptotic cells. Similarly, in the single treatments, the total apoptotic fraction showed remarkable increases from 1.0 mM to 2.0 mM.

The majority of the combination treatment group had lower viable fraction and higher apoptotic fraction, except for combinations in which both MA and KET had low concentration (0.1 + 0.1 mM). The measured viable and apoptotic fraction was almost equal to the theoretical value at 0.1 + 0.1 but highly significant differences were shown in other combination groups. The combination of MA + KET (1.0 + 1.0 mM) displayed the strongest cytotoxicity as demonstrated by the lowest viable fraction (64.3 ± 2.02%) and highest apoptotic fraction (32.6 ± 2.02%).

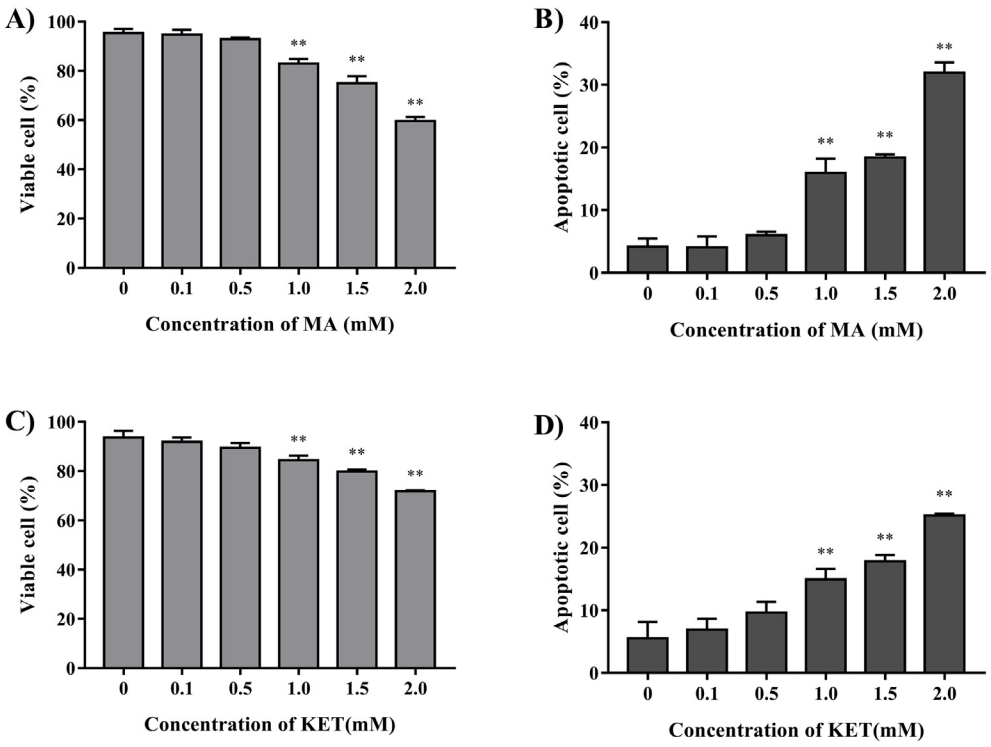

Fig. 1. The population of (A) living cell after MA treatments, (B) apoptotic cell after MA treatments, (C) living cell after KET treatments and (D) apoptotic cell after KET treatments in HepG2 cells for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between control group and treatments groups at $p < 0.05$, $p < 0.01$, respectively.

3.3. Lipid peroxidation (LPO) levels in cells exposed to single or combined groups

The results in Table 2 show significant increases of the MDA level in cells treated with MA or KET alone whose concentrations were higher than 1.0 mM compared to results of the negative control group. The MA-treated group showed a higher level of oxidative damage, with the maximum MDA level reaching 19.6 ± 1.29 nmol/mgprot, while the maximum MDA level for the KET-treated group was 10.6 ± 0.47 nmol/mgprot.

The combination-treated group showed different variations in trends of MDA level, which are displayed in Table 2 and Fig. 3. The most obvious difference was observed in the MA + KET (0.1 + 0.1 mM) treated group where the measured MDA level (17.0 ± 0.76 nmol/mgprot) increased significantly when compared with the theoretical value (11.6 ± 1.38 nmol/mgprot). However, the measured values showed no significant differences in the 0.1 + 0.1 and 0.1 + 1.0 groups.

3.4. Antioxidant system in cells exposed to single or combined groups

The results of SOD, CAT and GSH in HepG2 cells after exposed to MA, KET or their mixtures were shown in Tables 3–5. For single treatments, SOD activity decreased significantly only in the highest concentrations of MA treatment and KET treatment (Table 3). However, CAT activity showed a maximum of 140% increase after MA treatment and 130% increase after KET treatment (Table 4). Significantly decreased GSH levels were observed in all MA treatment and partial KET treatment (1.0–2.0 mM) as shown in Table 5.

In combined treatments (MA + KET), compared with the theoretical values, the combination of 0.1 + 1.0, 1.0 + 0.1 and 1.0 + 1.0 showed significantly decreased SOD activity while the 0.1 + 0.1 group showed no significant difference in SOD activity when compared with theoretical values (Fig. 4.). CAT activity in 1.0 + 0.1 and 1.0 + 1.0 groups was lower compared with the control group and theoretical values. But for the 0.1 + 1.0 groups, the CAT activity showed significant increases when compared with the control and the theoretical activity. For the 0.1 + 0.1 group, no significances in CAT activity were observed in comparison with control or theoretical value. (Fig. 5.). As for the intracellular GSH levels (Fig. 6.), the combined treatments had lower GSH levels than the single treatment groups. Compared with the theoretical values, the measured GSH content in all combined-treatment groups decreased remarkably.

3.5. Effects of MA or KET and their binary mixtures on DNA strand breaks (comet assay)

Results of comet assays for single-exposure and combined-exposure groups are shown in Fig. 7. Low concentrations of both MA and KET did not induce DNA damage when compared with the negative control group. However, in the high concentration group (1.0 mM), these two drugs resulted in a significant increase of tail DNA percentage values compared with the negative control group and the low concentration treatment (Fig. 7A.). Also, the genotoxicity of MA was stronger than that of KET in the same high-concentration group due to the %DNA in the tail.

The combined exposures resulted in elevated DNA damage, as demonstrated by higher %DNA in the tail than the control group (Fig. 7B.). The theoretical values of combined treatments were shown in Table 6. Although the mixture containing low level of MA and KET (0.1 + 0.1) resulted in stronger DNA damage, no significance differences were observed between the measured value and theoretical value. However, for other binary mixture groups, remarkable differences were observed, indicating enhancement of genotoxicity.

4. Discussion

Recent studies of MA and KET toxicity have focused on their single effects. However, in practice, abusers of these drugs are more likely to abuse multiple psychotropic substances to achieve greater pleasure. Therefore, our attention must shift to the combined toxicity of the drugs being abused, which is likely to be higher when used as a mixture. The present study demonstrated that MA and KET alone caused oxidative damage, DNA damage, and cell apoptosis in HepG2 cells, and their

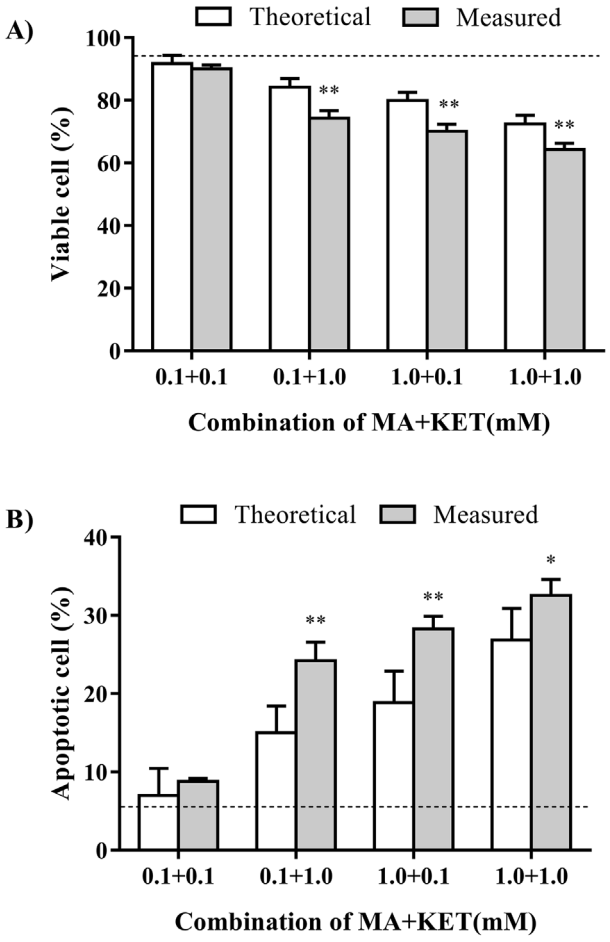

Fig. 2. The population of (A) living and (B) apoptotic cell in HepG2 cells after exposure to MA + KET for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between control group and treatments groups at $p < 0.05$, $p < 0.01$, respectively. Dashed line represents living or apoptotic of negative control.

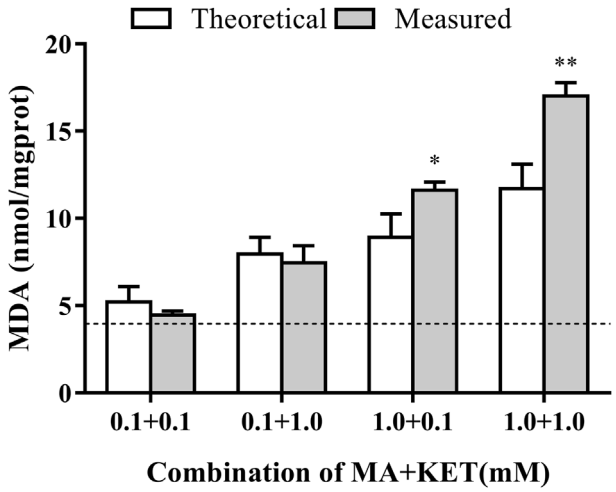

Fig. 3. MDA contents of lipid peroxidation in HepG2 cells after exposure to MA + KET for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$, respectively. Dashed line represents MDA content of negative control.

binary mixture produced even stronger toxic effects, indicating that the mixture may have additive or combined effects.

Cytotoxicity is a basic index for evaluating the toxicity of chemicals. In this study, MA was found to have higher toxicity to HepG2 cells when compared with KET at the same concentration. Results of the 24-h combined exposure (MA + KET) groups showed greater cytotoxicity than in the single exposure groups. In the combined exposure, except for the 0.1 + 0.1 group, the cell survival rate measured in the other three groups was lower than the theoretical survival rate, suggesting a synergistic effect, while the 0.1 + 0.1 group showed additive effects. In the apoptosis experiment, the percentage of living cells after individual and combined treatments showed the similar trend as indicated by the viability obtained by MTT assays. Previous studies have reported that MA activated hepatocyte apoptosis through oxidative stress and cell cycle arrest (Rahmati et al., 2014; Wang et al., 2017), while KET induces HepG2 cells apoptosis through the Bax-mitochondria-caspase pathway (Lee et al., 2009). Results of this work also illustrate that MA and KET can cause evident apoptosis, which showed a dose-dependent effect. For the co-exposures, the apoptosis rates of all combined treatment groups were higher than those of the single drug groups. All the experimental groups, except the 0.1 + 0.1 group, showed synergistic effects.

Previous reports indicated that the oxidative stress induced by MA or KET is closely related to apoptosis (Abdel-Salam et al., 2015; Pavlovic et al., 2018; Shah et al., 2013). In order to investigate the underlying mechanism of cell death and DNA damage, MDA, GSH, SOD and CAT were determined. MDA is the final product of lipid-peroxidation, whose content can reflect the degree of oxidative stress indirectly. In the single exposures, the significant accumulation of MDA after MA or KET treatment indicated that the cells had suffered severe

Table 2
Effects of MA and KET alone on MDA levels in HepG2 cells after 24 h exposure.

MA (mM)	MDA content (nmol/mgprot)	KET (mM)	MDA content (nmol/mgprot)	MA + KET (mM)	MDA content (nmol/mgprot)
0	3.56 ± 0.67	0	4.40 ± 0.32	0.1 + 0.1	4.45 ± 0.24
0.1	4.62 ± 0.48	0.1	4.60 ± 0.75		
0.5	5.03 ± 0.70	0.5	5.72 ± 1.18	0.1 + 1.0	7.46 ± 0.96[*,a]
1.0	8.33 ± 1.11[*]	1.0	7.35 ± 0.82[*]		
1.5	13.9 ± 0.6[*]	1.5	8.82 ± 0.90[*]	1.0 + 0.1	11.6 ± 0.5[*,b,c]
2.0	19.6 ± 1.3[*]	2.0	10.6 ± 0.5[*]		
2 mM H_2O_2	16.1 ± 2.1[*]	2 mM H_2O_2	16.3 ± 1.4[*]	1.0 + 1.0	17.0 ± 0.8[*,b,d]

Data were presented as mean ± SD of three replicates.
*Treatment-groups versus control-group ($p < 0.05$).
[a] MA 0.1 mM versus MA + KET (0.1 + 0.1 mM; 0.1 + 1.0 mM) ($p < 0.05$).
[b] MA 1.0 mM versus MA + KET (1.0 + 0.1 mM; 1.0 + 1.0 mM) ($p < 0.05$).
[c] KET 0.1 mM versus MA + KET (0.1 + 0.1 mM; 1.0 + 0.1 mM) ($p < 0.05$).
[d] KET 1.0 mM versus MA + KET (0.1 + 1.0 mM; 1.0 + 1.0 mM) ($p < 0.05$).

Table 3
Effects of MA and KET alone on SOD activity in HepG2 cells after 24 h exposure.

MA (mM)	SOD activity (U/mgprot)	KET (mM)	SOD activity (U/mgprot)	MA + KET (mM)	SOD activity (U/mgprot)
0	16.5 ± 0.7	0	16.7 ± 0.8	0.1 + 0.1	14.6 ± 0.6[c]
0.1	16.3 ± 0.6	0.1	16.8 ± 0.8		
0.5	16.3 ± 1.3	0.5	16.2 ± 0.6	0.1 + 1.0	11.3 ± 0.7*[a,d]
1.0	15.3 ± 0.4	1.0	16.5 ± 1.0		
1.5	14.0 ± 0.9*	1.5	15.6 ± 0.8	1.0 + 0.1	10.3 ± 0.5*[b,c]
2.0	10.2 ± 0.4*	2.0	12.7 ± 0.9*		
2 mM H_2O_2	6.24 ± 1.04*	2 mM H_2O_2	6.52 ± 1.12*	1.0 + 1.0	9.54 ± 0.81*[b,d]

Data were presented as mean ± SD of three replicates.
*Treatment-groups versus control-group ($p < 0.05$).
[a] MA 0.1 mM versus MA + KET (0.1 + 0.1 mM; 0.1 + 1.0 mM) ($p < 0.05$).
[b] MA 1.0 mM versus MA + KET (1.0 + 0.1 mM; 1.0 + 1.0 mM) ($p < 0.05$).
[c] KET 0.1 mM versus MA + KET (0.1 + 0.1 mM; 1.0 + 0.1 mM) ($p < 0.05$).
[d] KET 1.0 mM versus MA + KET (0.1 + 1.0 mM; 1.0 + 1.0 mM) ($p < 0.05$).

Table 4
Effects of MA and KET alone on CAT activity in HepG2 cells after 24 h exposure.

MA (mM)	CAT activity (U/mgprot)	KET (mM)	CAT activity (U/mgprot)	MA + KET (mM)	CAT activity (U/mgprot)
0	2.03 ± 0.06	0	2.20 ± 0.04	0.1 + 0.1	2.24 ± 0.05*
0.1	2.18 ± 0.08*	0.1	2.26 ± 0.04*		
0.5	2.33 ± 0.04*	0.5	2.63 ± 0.12*	0.1 + 1.0	2.89 ± 0.07*[a]
1.0	2.93 ± 0.04*	1.0	2.71 ± 0.07*		
1.5	2.86 ± 0.05*	1.5	2.40 ± 0.12	1.0 + 0.1	2.00 ± 0.05*[b,c]
2.0	2.02 ± 0.08	2.0	2.13 ± 0.08		
2 mM H_2O_2	2.98 ± 0.01*	2 mM H_2O_2	2.87 ± 0.02*	1.0 + 1.0	1.62 ± 0.19*[b,d]

Data were presented as mean ± SD of three replicates.
*Treatment-groups versus control-group ($p < 0.05$).
[a] MA 0.1 mM versus MA + KET (0.1 + 0.1 mM; 0.1 + 1.0 mM) ($p < 0.05$).
[b] MA 1.0 mM versus MA + KET (1.0 + 0.1 mM; 1.0 + 1.0 mM) ($p < 0.05$).
[c] KET 0.1 mM versus MA + KET (0.1 + 0.1 mM; 1.0 + 0.1 mM) ($p < 0.05$).
[d] KET 1.0 mM versus MA + KET (0.1 + 1.0 mM; 1.0 + 1.0 mM) ($p < 0.05$).

Table 5
Effects of MA and KET alone on GSH levels in HepG2 cells after 24 h exposure.

MA (mM)	GSH content (nmol/mgprot)	KET (mM)	GSH content (nmol/mgprot)	MA + KET (mM)	GSH content (nmol/mgprot)
0	54.4 ± 4.6	0	50.9 ± 1.3	0.1 + 0.1	35.0 ± 1.8*[a,c]
0.1	45.6 ± 0.7*	0.1	50.9 ± 1.1		
0.5	42.6 ± 1.7*	0.5	49.0 ± 0.4	0.1 + 1.0	29.1 ± 0.4*[a,d]
1.0	42.0 ± 1.4*	1.0	41.6 ± 1.6*		
1.5	44.9 ± 1.9*	1.5	36.8 ± 0.8*	1.0 + 0.1	33.5 ± 0.7*[b,c]
2.0	38.6 ± 0.4*	2.0	34.4 ± 1.5*		
2 mM H_2O_2	35.9 ± 1.1*	2 mM H_2O_2	37.1 ± 1.8*	1.0 + 1.0	18.6 ± 0.8*[b,d]

Data were presented as mean ± SD of three replicates.
*Treatment-groups versus control-group ($p < 0.05$).
[a] MA 0.1 mM versus MA + KET (0.1 + 0.1 mM; 0.1 + 1.0 mM) ($p < 0.05$).
[b] MA 1.0 mM versus MA + KET (1.0 + 0.1 mM; 1.0 + 1.0 mM) ($p < 0.05$).
[c] KET 0.1 mM versus MA + KET (0.1 + 0.1 mM; 1.0 + 0.1 mM) ($p < 0.05$).
[d] KET 1.0 mM versus MA + KET (0.1 + 1.0 mM; 1.0 + 1.0 mM) ($p < 0.05$).

oxidative stress. GSH, SOD and CAT play important role in antioxidant system, and their changes in content or activity reflect the oxidative damage to cells. In this study, the decline in GSH level and SOD activity as well as the increase in CAT activity after MA or KET treatments indicated that antioxidative ability was impaired in cells. The increase of CAT activity in cells is one of the responses to excessive hydrogen peroxide, which agree with the phenomenon that an increase of CAT levels in rats' liver was observed after MA exposure (Koriem et al., 2013). Moreover, it could be observed that MA was potent to induce oxidative damage when compared with KET, which may be related to the ability of MA to reduce Cu/Zn-SOD activity (Deng and Cadet, 2000; Jayanthi et al., 1998). Excessive ROS in cells will cause depletion of GSH and reduce the antioxidant activity of SOD and CAT (Venkatesan et al., 2007; Yu et al., 2015), resulting in blocked ROS-clearance and massive ROS accumulation, which will cause oxidative damage to membrane lipids, proteins, and DNA (Hildeman et al., 2003), and ultimately cell apoptosis. The data of four oxidative stress biomarkers were consistent with the results of cytotoxicity and apoptosis assay.

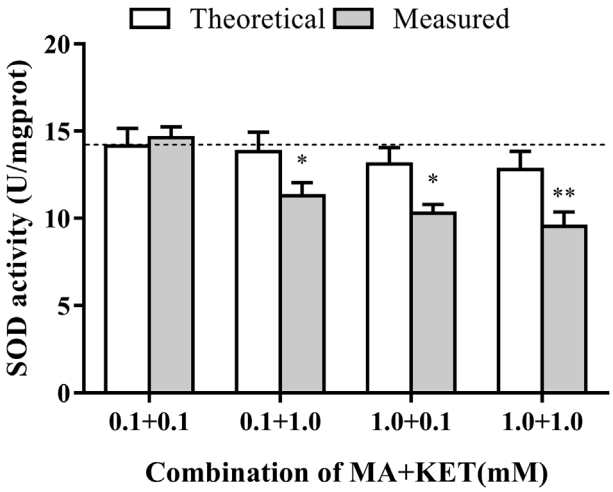

Fig. 4. SOD activity of HepG2 cells after exposure to MA + KET for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$, respectively. Dashed line represents SOD activity of negative control.

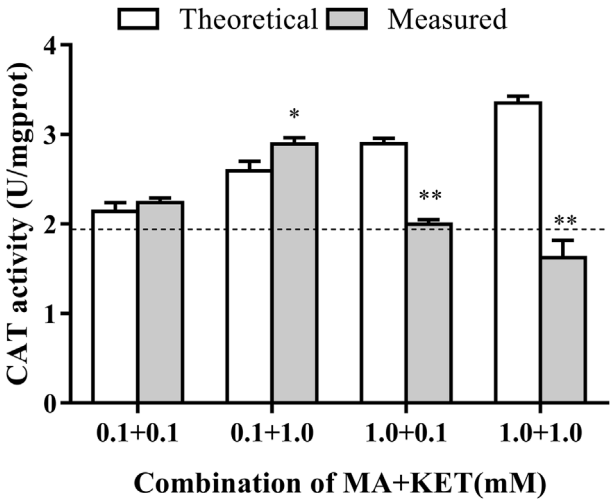

Fig. 5. CAT activity of HepG2 cells after exposure to MA + KET for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$, respectively. Dashed line represents CAT activity of negative control.

In toxicity studies of combined drugs, different interactions between different biomarkers can be observed due to different mechanisms. The co-exposures of MA and KET appear to have synergistic effects on oxidative damage, suggesting that the two drugs have different toxic effects. Strangely, in the 0.1 + 0.1 group, almost all the interactions observed in the four oxidative stress biomarkers were additive, while almost all the interactions in the other combinations were synergistic. A possible explanation is that MA could induce the activity of CYP3A (Cytochrome P450 proteins, CYP) (Dostalek et al., 2005), which was mainly involved in KET metabolism (Dinis-Oliveira, 2017). Therefore, KET metabolism could be enhanced with the presence of MA. In addition, during the metabolism of KET, a variety of free radicals were produced (Reinke et al., 1998), which played a role in oxidative stress and ROS-induced toxicity (Kovacic, 2005; Kovacic and Somanathan, 2011). Thus, for the 1.0 + 0.1 and 1.0 + 1.0 groups, the generation of ROS could be aggravated by the enhancement of KET metabolism with the presence of MA, resulting in synergistic effects on oxidative damage. In the 0.1 + 1.0 group, 0.1 mM MA did not cause significant

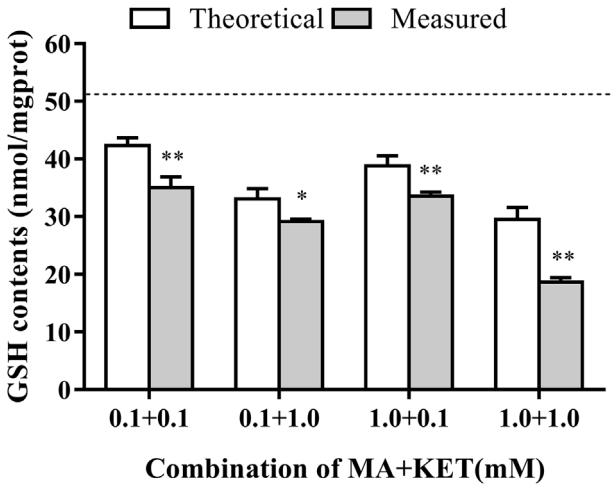

Fig. 6. GSH contents in HepG2 cells after exposure to MA + KET for 24 h. Data are expressed as means ± S.D. from three independent experiments. *, ** represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$, respectively. Dashed line represents GSH levels of negative control.

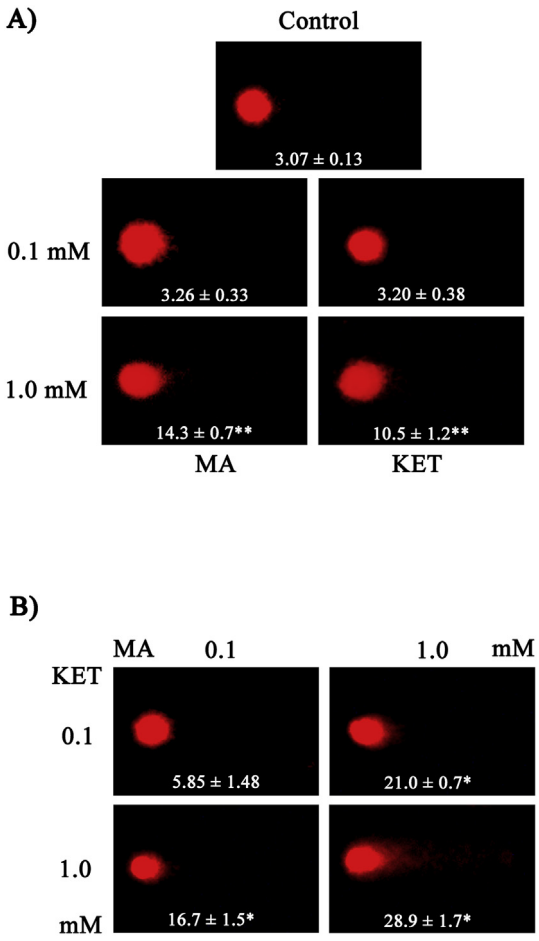

Fig. 7. Representative images of comet assay of HepG2 cells after 24 h (A)single exposures and (B) combined exposures. Tail DNA percentage (%) were presented as the numbers in the images. *, ** in single exposure represent difference between control group and treatments groups, while in combined exposures represent difference between theoretical values and measured values at $p < 0.05$, $p < 0.01$ respectively.

Table 6
The theoretical values of %DNA in tail in combined exposure treatments.

MA + KET (mM)	Theoretical value of %DNA in tail (%)
0.1 + 0.1	3.39 ± 0.50
0.1 + 1.0	10.7 ± 1.2
1.0 + 0.1	14.4 ± 0.8
1.0 + 1.0	21.7 ± 1.4

oxidative damage, but its stimulating effect on KET metabolism made it more toxic than being used individually, and the oxidative damage, DNA damage, and apoptosis were mainly caused by KET. For the 0.1 + 0.1 group, low concentrations of MA and KET were insufficient to produce significant oxidative damage and metabolic enhancement, showing additive effect as result.

Comet assay is a powerful method to evaluate the genetic toxicity of chemical substances. In our study, MA and KET alone caused DNA damage in HepG2 cells at 1.0 mM, providing new evidence for the previously reported genetic toxicity of MA and KET (Adhvaryu et al., 1986; Jeng et al., 2005; Li et al., 2003; Toyama et al., 2006). After co-exposure, the combined groups all showed more severe DNA damage than that caused by single exposures. Additive effect was observed in 0.1 + 0.1 group while synergistic effects were observed in the other three groups. A possible explanation is that MA not only caused DNA damage through oxidative stress, but also prevented DNA replication and repair (Wang et al., 2017). Therefore, the synergistic effects of combined-treatments on DNA damage can be attributed to both enhanced oxidative stress and inhibited DNA repair when MA was at high concentration. At low dosage of MA, DNA damage depended on KET concentration and the synergistic effect was observed only at higher KET concentrations.

Taken together, results of our study suggest that the cytotoxicity and genotoxicity of combined MA and KET on HepG2 cells cannot be ignored. As two widely abused drugs at present, MA and KET are jointly abused in many instances, leading to enhancement of toxicity through synergistic and additive interactions.

5. Conclusion

MA and KET both induce cell apoptosis, oxidative damage and DNA damage in HepG2 cells, and MA appears to be more toxic than KET, with a higher apoptosis rate, greater oxidative stress and severe DNA damage. Enhanced toxic effects, both synergistic and additive, are demonstrated with combined MA and KET, suggesting that coadministration of MA and KET may result in greater risk of abuse. Results of this study are important for improving our knowledge on the molecular mechanisms underlying MA and KET toxicity, which may aid in implementing new therapies to prevent or mitigate the acute consequences of MA and KET abuse as single or combined stimulants. Further research is needed to perfect risk assessment and design novel therapeutic approaches.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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