

Research Article

Nanoformulation of Premixing Propofol Lipid Emulsion and Fentanyl Citrate and Their Effects on Acute Toxicity, Sedation, and Analgesia

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To illustrate the effects of premixing propofol lipid emulsion (PLE) and fentanyl citrate (FC) on acute toxicity, sedation, and analgesia, sixty female mice were randomly assigned to individual and premixed groups. The median lethal dose (LD_{50}), median effective dose (ED_{50}) of loss of righting reflex, and ED_{50} of tail flick test in both groups were determined using the up and down procedure (FC : PLE = 1 μ g : 2 mg). PLE was immediately administered to the mice from the individual group via the tail vein after injecting FC. By contrast, FC was mixed with PLE before injection from the premixed group. No significant differences in LD_{50} , histopathological examination, and ED_{50} sedation value were found between the groups. However, ED_{50} of analgesia in the premixed group decreased to half of that in the individual group. Transmission electron microscopic observation revealed ~10 nm fusiform particles at the juncture of 200–300 nm particles in the mixture of PLE and FC compared with the single PLE and its mixture with saline, which may be attributed to the structure of FC. In conclusion, premixing PLE and FC enhanced synergic analgesia twofold but did not influence toxicity and sedation compared with individual injections.

1. Introduction

Propofol (2,6-diisopropylphenol) is widely used to induce and maintain sedation during operation and intensive care. Propofol is an important clinical intravenous anesthetic that presents rapid action and short-term effects. Given its high hydrophobicity, propofol is injected in clinical settings as a lipid emulsion with a particle size of 200–259 nm [1]. Propofol lipid emulsion is commonly prepared as follows: 1% propofol, 10% soybean oil, 1.2% purified egg phospholipids (emulsifier), and 2.25% glycerol (tonicity-adjusting agent); sodium hydroxide is added to adjust the pH. However, the large amount of soybean oil long-chain triglyceride increases the serum triglyceride level and induces hypertriglyceridemia [2]. Thus, a new type of propofol medium/long-chain lipid emulsion has been developed, in which half of the soybean oil long-chain triglyceride is replaced with medium-chain triglycerides to reduce the abovementioned risks [3].

Given its weak analgesic effect [4], propofol has been considered to be combined with an opioid analgesic, such as fentanyl, in clinical medicine. As a strong agonist of μ -opioid receptors, fentanyl exhibits rapid action and short-term efficacy. The aqueous solubilities of fentanyl and fentanyl citrate are 0.2 and 25 mg/mL, respectively [5, 6]. Citrate modification reduces the hydrophobicity of fentanyl. Regardless of the formulation, the combination of fentanyl and propofol exhibits a natural synergy. Fentanyl reduces the first-pass pulmonary uptake of propofol, and propofol inhibits the oxidative metabolism of opioid through the cytochrome P450 enzyme [7–9]. Neither of these drugs can be injected without hydrophilic formulations. Trissel et al. [10] reported that hydrophilic formulations of fentanyl and propofol that are mixed in a polypropylene syringe are physically compatible and chemically stable. They are commonly premixed by Y-site administration in clinical settings. A similar premixture, the single-syringe combination of ketamine and propofol,

shows minimal adverse hemodynamic effects and favorable emergence characteristics for short procedures [11]. Premixing propofol with lidocaine is an effective measure to reduce injection pain [12, 13]. However, the effects of premixing propofol lipid emulsion and fentanyl citrate on acute toxicity, sedation, and analgesia remain unknown.

In this study, the median lethal dose (LD_{50}) and median effective dose (ED_{50}) of premixed and individual solutions of propofol lipid emulsion and fentanyl citrate were determined in mice. Hematoxylin and eosin- (HE-) stained pathological sections of important organs in the LD_{50} experiment were obtained. Transmission electron micrographs (TEM) of propofol lipid emulsion and/or fentanyl citrate were analyzed to elucidate the potential mechanism of the interaction between propofol lipid emulsion and fentanyl citrate.

2. Materials and Methods

2.1. Animals. A total of 60 female Kunming mice weighing 18–22 g were obtained from the Laboratory Animal Research Center of Xi'an Jiaotong University. All animal experiments were performed in accordance with the European Commission Directive 86/609/EEC (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes) and the Guiding Principles in the Use of Animals in Toxicology, and these experiments were approved by the Xi'an Jiaotong University Ethics Committee. Animal suffering was minimized. All mice were maintained in an animal house with a 12 h light/12 h dark cycle under $22 \pm 2^\circ\text{C}$ and $55\% \pm 5\%$ humidity. Sterilized food pellets and water were provided ad libitum.

2.2. Drug Administration. The propofol medium/long-chain lipid emulsion and fentanyl citrate were purchased from Fresenius Kabi Co., Ltd. (Beijing, China) and Yichang Humanwell Pharmaceutical Co., Ltd. (Yichang, China), respectively. The carbon support film on copper was purchased from Beijing Zhongjingkeyi Technology Co., Ltd. (Beijing, China).

All 60 mice were randomly assigned to individual and premixed groups for each test. Each group in each test was administered with different doses of fentanyl and propofol. The dosage ratio of fentanyl citrate and propofol lipid emulsion was 1:2000 in this study. Propofol lipid emulsion was immediately administered in the mice from the individual group via the tail vein after injecting fentanyl citrate. By contrast, fentanyl citrate was mixed with propofol lipid emulsion before tail vein injection in the mice from the premixed group. LD_{50} and ED_{50} were determined using the up and down procedure in accordance with the OECD Guidelines for Testing of Chemicals Number 425 (OECD, 2008) on female Kunming mice [14–16].

2.3. Determination of LD_{50} . In the limit test for each group, a single limit dose of 54.40 mg/kg propofol + 27.20 $\mu\text{g/kg}$ fentanyl was estimated and intravenously administered [17]. The electrocardiograms of all mice were monitored for 30 min using a BIOPAC system (BIOPAC Systems Inc., Goleta, CA, USA) to determine death case. A constant

straight line from the electrocardiograms was regarded as the death criterion. Histopathological examinations through HE staining were performed on the brain, lung, heart, liver, and kidney of a dead mouse within 1 h after death from the limit dose administered. In the main test for each group, one female mouse was randomly selected and intravenously administered with a preliminary dose of 17.84 mg/kg propofol + 8.92 $\mu\text{g/kg}$ fentanyl. Single animals were dosed in sequence at 1 h intervals for the rapid cardiovascular and respiratory depression of propofol [18, 19]. The subsequent ascending or descending doses were determined depending on the survival from the preceding dose of the animals with a dose progression factor of 1.25.

2.4. Righting Reflex Test. The up and down procedure was used in the loss of righting reflex (LRR) assay to determine ED_{50} of sedation [20]. The LRR for more than 10 s was regarded as positive for sedation; otherwise, the observation was regarded as negative [21]. For the limit test for each group, a single limit dose of 9.13 mg/kg propofol + 4.57 $\mu\text{g/kg}$ fentanyl was estimated and intravenously administered [17]. In the main test for each group, one female mouse was randomly selected and intravenously administered with a preliminary dose of 2.39 mg/kg propofol + 1.20 $\mu\text{g/kg}$ fentanyl. The subsequent ascending or descending doses were determined depending on the results of LRR from the preceding dose of the animals with a dose progression factor of 1.25.

2.5. Tail Flick Test. The tail flick test was used to estimate ED_{50} of analgesia. The tail of each mouse was immersed in hot water at $55 \pm 0.5^\circ\text{C}$. The tail flick latency was defined as the time interval taken by mice to flick their tails after heat exposure. Cutoff time was fixed at 30 s to avoid damage to the animal tails. The increase in tail flick latency was taken as antinociception and calculated as the percentage of maximal possible effect (MPE) by using the following formula: $\text{MPE}\% = (t_1 - t_c)/(t_{\text{cutoff}} - t_c) \times 100$, where t_1 is the withdrawal latency, t_c is the control latency, and t_{cutoff} is the cutoff time [22]. The up and down method is intended for quantal data; thus, the graded tail flick data were converted to a quantal scale with a response of at least 50% MPE defined as analgesic and a value less than 50% MPE defined as not analgesic [23]. In the limit test for each group, a single limit dose of 9.13 mg/kg propofol + 4.57 $\mu\text{g/kg}$ fentanyl was estimated and intravenously administered [22]. In the main test for each group, one female mouse was randomly selected and intravenously administered with a preliminary dose of 2.39 mg/kg propofol + 1.20 $\mu\text{g/kg}$ fentanyl. The following ascending or descending doses were determined depending on the results of MPE% from the preceding dose of the animals with a dose progression factor of 1.25.

2.6. Morphology of Propofol Lipid Emulsion and Fentanyl Citrate. The morphology of propofol lipid emulsion and fentanyl citrate was investigated via TEM (Hitachi, Japan) [24]. The samples included single fentanyl citrate (50 $\mu\text{g/mL}$), single propofol lipid emulsion (10 mg/mL), mixed propofol

TABLE 1: Acute toxicity, sedation, and analgesia test in the individual and premixed groups of propofol lipid emulsion and fentanyl citrate.

		Acute toxicity test		Sedation test		Analgesia test	
		LD ₅₀	CI 95%	ED ₅₀	CI 95%	ED ₅₀	CI 95%
Individual group	Propofol (mg/kg)	34.84	27.68, 43.30	7.31	5.98, 12.30	7.30	5.85, 7.31
	Fentanyl (μ g/kg)	17.42	13.84, 21.65	3.36	2.99, 6.15	3.65	2.93, 3.66
Premixed group	Propofol (mg/kg)	34.84	29.46, 47.00	5.85	4.78, 9.94	3.74*	2.95, 4.69
	Fentanyl (μ g/kg)	17.42	14.73, 23.50	2.93	2.39, 4.97	1.87*	1.48, 2.19

Median lethal dose (LD₅₀), median effective dose (ED₅₀), and 95% confidence interval (CI 95%). * $P < 0.05$ between groups.

lipid emulsion and fentanyl citrate (volume ratios of 20:1, 20:2, 20:3, 20:4, and 20:5), and mixed propofol and physiological saline (volume ratios of 20:2 and 20:5). The images were captured immediately after mixing. Samples were dropped on a copper grid, air-dried, and then stained with 3% phosphotungstic acid for TEM. To reduce the bias, TEM was operated by an experienced electron microscope technician who was blinded to the study design. The center area of copper grid was first located at low magnification (10000x), and the nearest nanoparticle gathered grids on four directions were visualized at low and high (50000x) magnifications under a relatively low electron current density. Eight TEM images were obtained for each sample to obtain the emulsion overview.

2.7. Statistical Analysis. The LD₅₀ and ED₅₀ values, as well as the 95% confidence interval (CI), were analyzed using the AOT 425 Statistical Program. ED₅₀ values with nonoverlapping 95% CIs were considered significantly different.

3. Results

3.1. LD₅₀ from the Up and Down Procedure. In the limit test, a female mouse in both groups died after intravenous administration of a single dose of 54.40 mg/kg propofol + 27.20 μ g/kg fentanyl. HE-stained examinations showed no significant abnormal changes in the brain, lung, heart, liver, and kidney in both groups after the limit dose (Figure 1). The results of the respective up and down test series in both groups in the main test were shown in Figure 2. The estimated LD₅₀ in the premixed group was 34.84 mg/kg propofol combined with 17.42 μ g/kg fentanyl, and LD₅₀ in the individual group was 34.84 mg/kg propofol + 17.42 μ g/kg fentanyl, which showed no significant difference between the two groups (Table 1).

3.2. Righting Reflex. The sequences of the up and down method for the righting reflex test in both groups are shown in Figure 3. The ED₅₀ value of sedation in the premixed groups was 5.85 mg/kg propofol combined with 2.93 μ g/kg fentanyl. The corresponding ED₅₀ in the individual groups was 7.31 mg/kg propofol combined with 3.66 μ g/kg fentanyl. No significant difference in ED₅₀ of LRR was detected between the individual and premixed groups (Table 1).

3.3. Tail Flick Test. The results of the respective up and down test series in both groups in the tail flick test of analgesia

were shown in Figure 4. ED₅₀ of analgesia in the individual groups was 7.30 mg/kg propofol combined with 3.65 μ g/kg fentanyl. The corresponding ED₅₀ in the premixed group was 3.74 mg/kg propofol combined with 1.87 μ g/kg fentanyl. The ED₅₀ value of the premixed group was significantly lower than that of the individual group (Table 1).

3.4. Morphology of Propofol Lipid Emulsion and Fentanyl Citrate. As shown in Figure 5, large particles ($>1\mu$ m) were absent in all TEM micrographs. The propofol lipid emulsion had a wide inhomogeneous particle diameter (200–300 nm) with a large-area solution field (Figure 5(a)). The particle size in all mixtures of propofol lipid emulsion and fentanyl citrate injection (Figures 5(b)–5(f)) and mixtures of propofol and physiological saline (volume ratio of 20:2) (Figure 5(g)) had no visible difference compared with that in the propofol lipid emulsion. However, the distribution area and numbers of particles in one visual field were greater in the mixtures than in the propofol lipid emulsion. Furthermore, ~10 nm fusiform particles were found at the juncture of the 200–300 nm particles in all propofol lipid emulsion and fentanyl citrate mixtures. However, these particles were absent in the single propofol lipid emulsion and in its mixture with physiological saline. In addition, the mixture of propofol and physiological saline at a volume ratio of 20:5 (Figure 5(h)) contained 40–60 nm particles. A TEM image of the black dried salt crystal of fentanyl citrate under vacuum was shown in Figure 5(i).

4. Discussion

Propofol and fentanyl are hydrophobic drugs. Their small molecular size and hydrophobic nature allow them to penetrate the blood cerebrospinal barrier and rapidly exert sedative and analgesic effects [25]. However, hydrophobic drugs cannot be directly injected. They must first be prepared into several types of hydrophilic formulations, such as molecular complexes, polymeric micellar systems, nanosuspensions, lipid formulations, and prodrugs, before they can be used [26]. In current clinical settings, propofol is prepared into propofol lipid emulsion, whereas fentanyl is prepared into fentanyl citrate. Propofol lipid emulsion and fentanyl citrate are hydrophilic formulations of hydrophobic drugs that are frequently injected separately and sequentially for anesthesia. Few reports focused on the premixture of fentanyl citrate and propofol lipid emulsion. The fentanyl citrate analog alfentanil hydrochloridum can be premixed with propofol

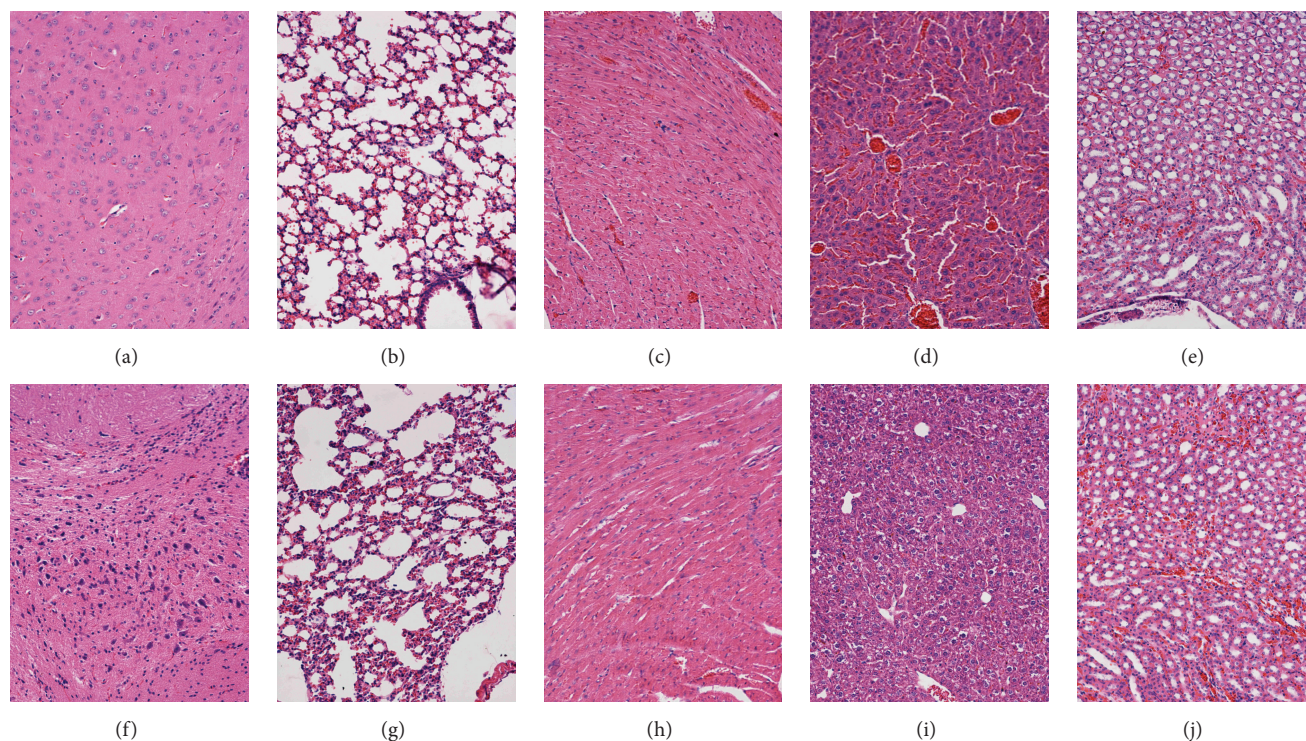


FIGURE 1: HE-stained pathological sections of organs at the highest administered dose (54.40 mg/kg propofol lipid emulsion combined with 27.20 µg/kg fentanyl citrate) (200x). (a–e) Pathological sections of the brain, lung, heart, liver, and kidney in the individual group. (f–j) Pathological sections of the brain, lung, heart, liver, and kidney in the premixed group.

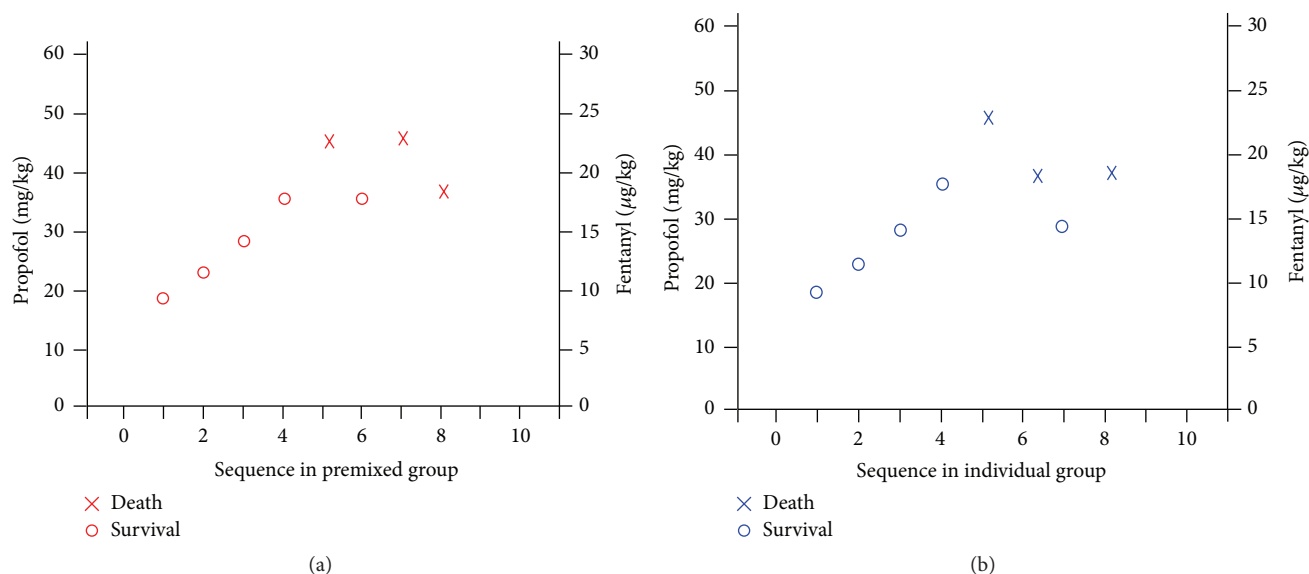


FIGURE 2: Sequence of the up and down procedure for LD₅₀. (a) Premixed group. (b) Individual group.

lipid emulsion at a ratio of 20:1 to 50:1 for use within 6 h of preparation, thereby implying the feasibility of preparing a premixture for fentanyl citrate and propofol lipid emulsion [13]. In consideration that such a premixing administration method is not contained in drug instructions, the premixed solution of propofol lipid emulsion and fentanyl citrate should be preliminarily investigated in animals.

In this study, no significant differences in LD₅₀ values and HE-stained histopathological examinations were found between the premixed and individual groups (Table 1, Figure 1). The premixture of fentanyl citrate and propofol lipid emulsion showed no increased toxicity. Similarly, the ED₅₀ value of sedation in the premixed group showed no significant difference compared with that in the individual group.

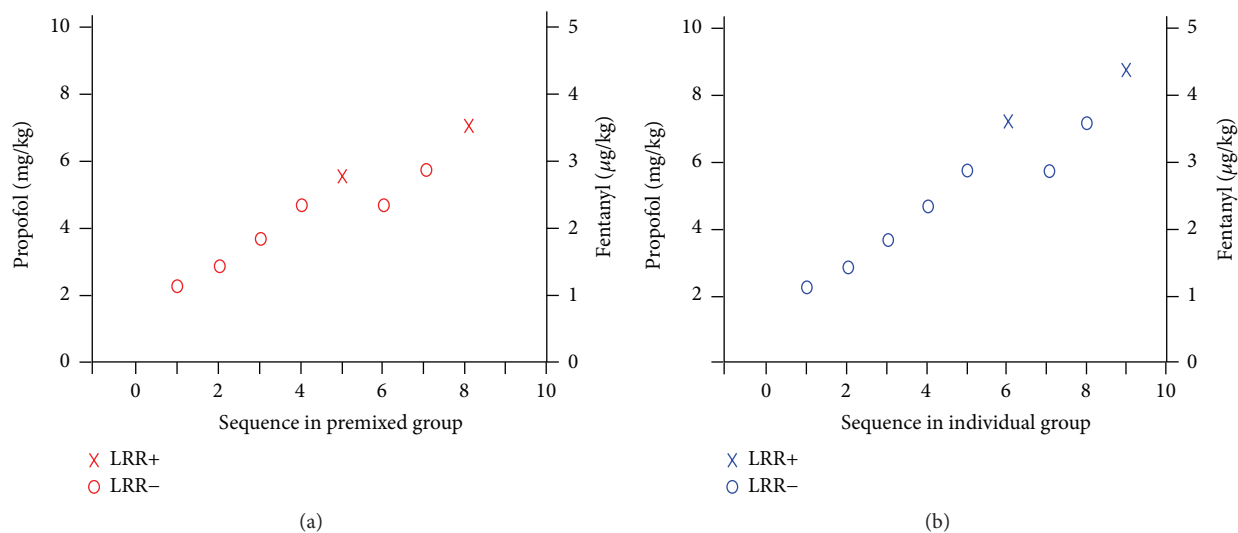


FIGURE 3: Sequences of the up and down procedure for ED_{50} of sedation. (a) Premixed group. (b) Individual group.

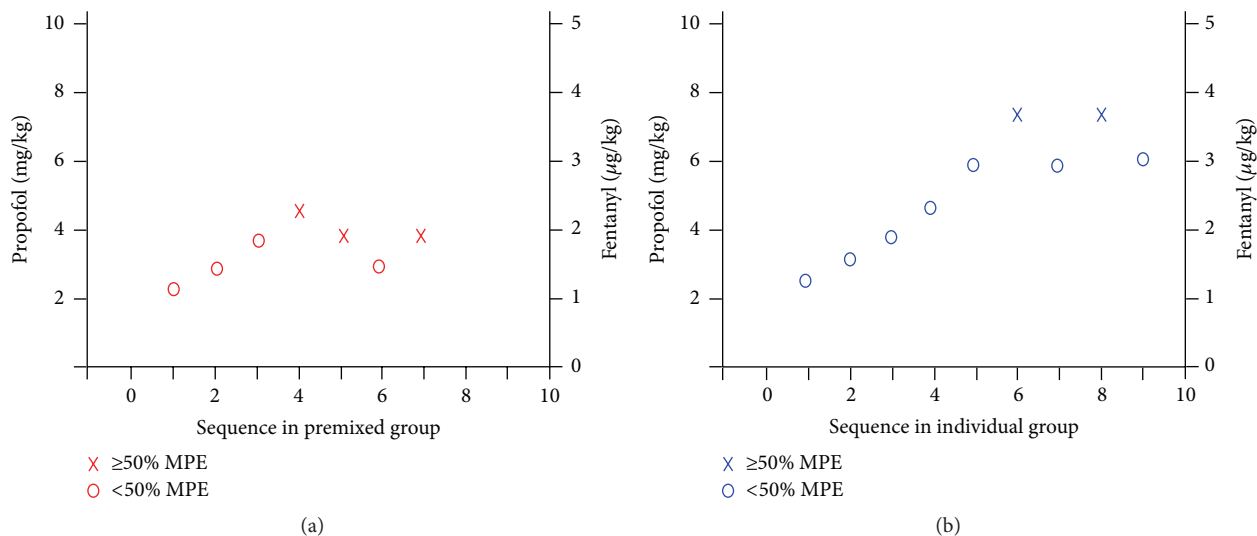


FIGURE 4: Sequences of the up and down procedure for ED_{50} of analgesia. (a) Premixed group. (b) Individual group.

This result indicates that the prior mixture did not change the synergistic effect of propofol on sedation. However, the ED_{50} value of analgesia in the premixed group was significantly lower than that in the individual group, demonstrating that the prior mixture enhanced the synergistic effect of fentanyl on analgesia. Thus, the animal experiment revealed that the synergistic analgesic effect was stronger in the premixed group than in the individual group. No significant differences in toxicity and synergic sedation were found between the two groups. Similar results have been reported in other literature. A single shot of propofol and rocuronium shows that the potency of rocuronium is significantly enhanced after propofol infusion for 30 min compared with 2 min of propofol; this finding indicates that propofol can increase the affinity of rocuronium for the receptor under steady-state conditions [27]. Meanwhile, a randomized double-blinded

trial found no statistically significant difference in the doses of propofol required for inducing anesthesia, regardless of whether the drug was administered as a freshly prepared propofol/lidocaine 10:1 mixture or as a separate injection after a dose of lidocaine [28].

The TEM micrographs of propofol lipid emulsion and fentanyl citrate or physiological saline with different volume ratios were examined to elucidate further the results of the animal experiment. Lipid emulsion is an unstable oil in water systems, and the intravenous administration of droplets ($>5\mu$ m in diameter) may pose the risk of pulmonary embolism [29]. For example, adding lidocaine to propofol emulsion in one or separated syringe is widely recommended to minimize injection pain [12, 30]. However, Masaki et al. [31] added 2 mL of 2% lidocaine to 20 mL of 1% propofol and observed particle sizes higher than 5μ m in scanning electron

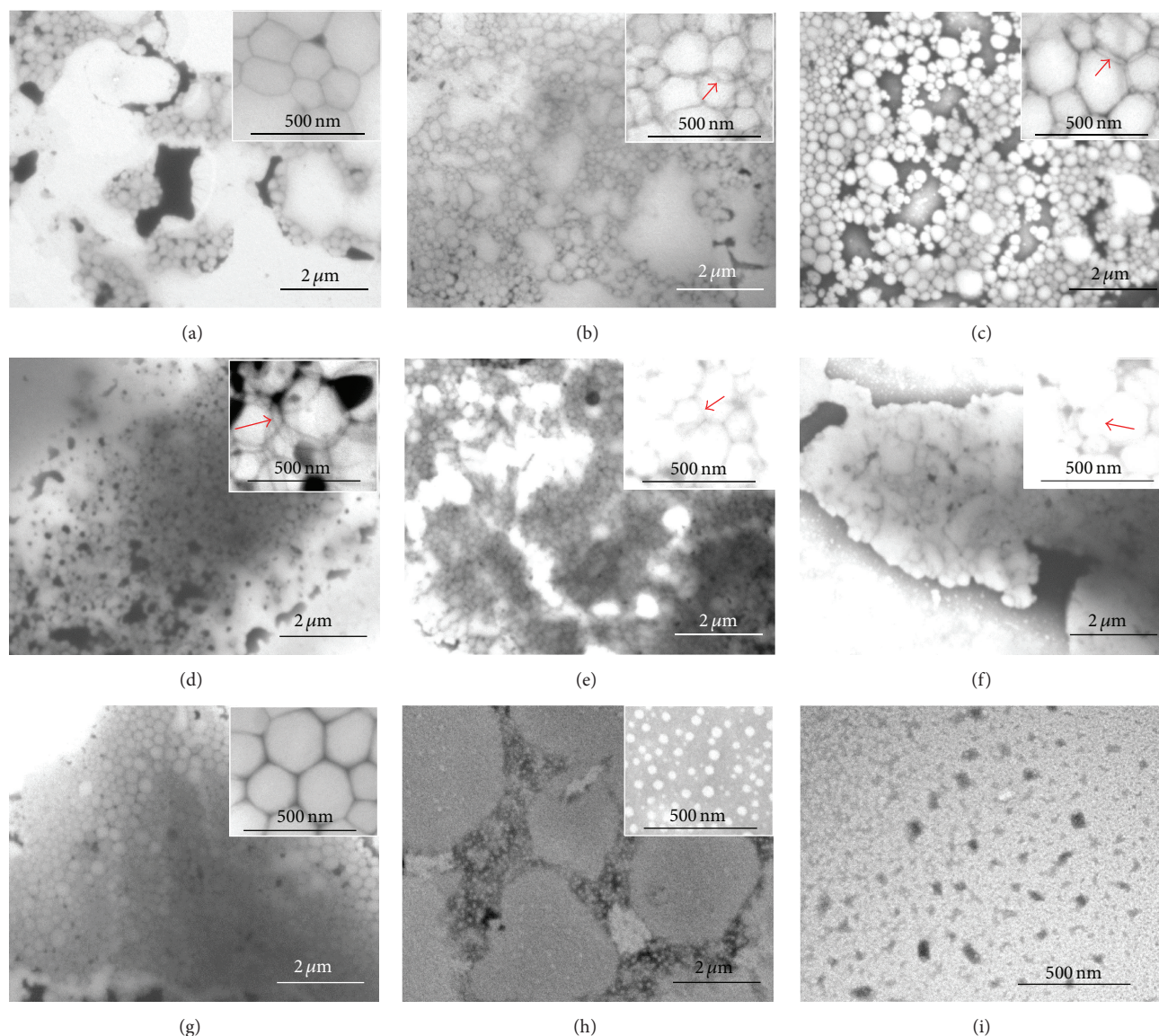


FIGURE 5: TEM micrographs of propofol lipid emulsion, fentanyl citrate, mixture of propofol lipid emulsion and fentanyl citrate, and mixture of propofol lipid emulsion and physiological saline. (a) Propofol lipid emulsion. (b–f) Mixed propofol lipid emulsion and fentanyl citrate (volume ratios of 20 : 1, 20 : 2, 20 : 3, 20 : 4, and 20 : 5). The arrow pointed to ~10 nm fusiform particles at the juncture of 200–300 nm particles. (g–h) Mixed propofol and physiological saline (volume ratios of 20 : 2 and 20 : 5). (i) Fentanyl citrate.

micrographs. Park et al. [32] reported that the particle size increases to $>5\ \mu\text{m}$ at 2 h after adding $>30\ \text{mg}$ of 4% lidocaine. Thus, the maximum volume dilution rate of propofol with 1% lidocaine is 20 : 1 in the drug instruction of propofol lipid emulsion and is injected immediately after premixing. In the present study, the particles were $<1\ \mu\text{m}$ after TEM analysis, indicating that the premixture did not significantly increase the size of propofol lipid emulsion and the risk of pulmonary embolism (Figure 5). In consideration that fentanyl and propofol are commonly combined and used during the induction period, the images were captured immediately after premixing. The absence of particles $>5\ \mu\text{m}$ might explain why the LD_{50} values and HE-stained histopathological test results did not significantly change in these two administration

methods. Furthermore, the cardiovascular or respiratory depression might be the main reason for death [18, 19]. The intravenous injection LD_{50} values of propofol and fentanyl were 53 and 11.2 mg/kg in mice according to the drug instructions, respectively. The dosage ratio of fentanyl citrate and propofol lipid emulsion was 1 : 2000 in this study. Thus, the death was mainly induced by propofol not fentanyl.

The large-area solution field in the propofol lipid emulsion was probably caused by the instability of the lipid emulsion system (Figure 5(a)). Lipid emulsion degrades because of Brownian motion or external agitation, which occurs in several sequential processes, including dispersed phase, flocculation, coalescence, and free oil [33, 34]. Although the morphology observed via TEM is not totally

equal to it in emulsion, TEM is an important method to observe nanosized lipid emulsions [24]. A large amount of free propofol leaked from the particles and dissolved in the oil phase of the propofol lipid emulsion. Particle size did not significantly change with increasing fentanyl citrate concentration in the mixture of propofol emulsion and fentanyl citrate injection (Figures 5(b)–5(f)). However, the particle number in these mixtures was greater than that in pure propofol lipid emulsion, and the solution field areas in these mixtures were smaller than those in the single propofol lipid emulsion. These data illustrate that fentanyl citrate injection increased the number of particles and reduced the free propofol from the propofol emulsion. Compared with the mixture of propofol and physiological saline at a 20:2 ratio (Figure 5(g)), the mixture of propofol lipid emulsion and fentanyl citrate at a 20:2 ratio showed ~10 nm fusiform particles at the juncture of large particles (200–300 nm) (Figure 5(c)). Compared with the mixture of propofol lipid emulsion and physiological saline at a 20:5 ratio (Figure 5(h)), the mixture of propofol lipid emulsion and fentanyl citrate at a 20:5 ratio produced significantly larger particles (200–300 nm) surrounded by ~10 nm fusiform particles (Figure 5(f)). These results suggest that the water/oil ratio played a negligible role in the change of the propofol lipid emulsion and fentanyl citrate mixture. Thus, the structure of fentanyl citrate should be considered. Fentanyl citrate is an organic basic compound with a pK_a value of 8.99 [35]. Fentanyl citrate has a hydrophobic part on one side and a hydrophilic part on the other side, with a positive charge at physiological pH 7.35–7.45 and a structure similar to that of a cationic surfactant [36]. Fentanyl citrate is a cationic surfactant located at the edge of the particle for the amphipathicity and electrostatic adsorption with the negative-charged emulsifier of egg phospholipid. These characteristics might explain the larger number of 200–300 nm particles that did not significantly change for water/oil ratio and the ~10 nm fusiform particles at the juncture of 200–300 nm particles in the mixture of propofol lipid emulsion and fentanyl citrate. Meanwhile, fentanyl might dissociate from citrate upon sodium hydroxide or dilution in the propofol lipid emulsion because of the instability of ionic bonds in fentanyl citrate and then dissolve in the soybean oil of lipid emulsion [37]. Such a high hydrophobicity allows fentanyl to penetrate the blood cerebrospinal barrier [38]. However, TEM only showed morphological changes, and the reasons for enhanced synergic analgesia were deduced by chemical theory, which needs further research to validate.

5. Conclusion

Compared with individual injections, the premixed solution of propofol lipid emulsion and fentanyl citrate at a volume ratio of 20:2 enhanced synergic analgesia twofold but did not influence acute toxicity and sedation. These results indicated that formulation interaction played an important role in the drug interaction. In clinical settings, not only the characteristics of drugs themselves but also their formulation should be considered. A rational administration would improve the effects of combined drugs.

Competing Interests

The authors declare no competing financial interests.

Authors' Contributions

Wei Gao and Baoyong Sha contributed equally to this work.

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