MEDICAL SCIENCE MONITOR

LAB/IN VITRO RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2016; 22: 4836-4843
DOI: 10.12659/MSM.901772

Received: 2016.09.30
Accepted: 2016.11.04
Published: 2016.12.10

Chloral Hydrate Treatment Induced Apoptosis of Macrophages via Fas Signaling Pathway

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Statistical Analysis C
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Manuscript Preparation E
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Source of support: This work was supported by the Medical Scientific Research Foundation of Guangdong Province, China (no. A2014481)

Background: There are recent reports on several anesthetics that have anti-inflammatory and anti-infective effects apart from their uses for pain relief and muscle relaxation. Chloral hydrate is a clinical anesthetic drug and sedative that has also been reported to attenuate inflammatory response, but the mechanisms are not clearly understood.

Material/Methods: This study investigated the effect of chloral hydrate treatment on the apoptosis of macrophages and explored the underlying mechanisms. RAW264.7 macrophages were treated with various concentrations of chloral hydrate for various lengths of time. Morphological changes were observed under a light microscope and apoptosis was detected with annexin-V-FITC/PI double-staining assay, Hochest 33258 and DNA ladder assay, the expression of Fas/FasL was detected with a flow cytometer, and the Fas signaling pathway was assessed by Western blotting.

Results: The results showed that chloral hydrate treatment induced the morphology of RAW264.7 macrophages to change shape from typical fusiform to round in a concentration- and time-dependent manner, and was finally suspended in the supernatant. For the induction of apoptosis, chloral hydrate treatment induced the apoptosis of RAW264.7 macrophages from early-to-late stage apoptosis in a concentration- and time-dependent manner. For the mechanism, chloral hydrate treatment induced higher expression of Fas on RAW264.7 macrophages, and was also associated with changes in the expression of proteins involved in Fas signaling pathways.

Conclusions: Chloral hydrate treatment can induce the apoptosis of RAW264.7 macrophages through the Fas signaling pathway, which may provide new options for adjunctive treatment of acute inflammation.

MeSH Keywords: Apoptosis • Chloral Hydrate • Fas Ligand Protein • Macrophages

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/901772

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Background

In recent years, several anesthetics that have anti-inflammatory and anti-infective effects apart from their uses for pain relief and muscle relaxation been found, which may yield new options for adjunctive treatment of acute inflammation. For example, lidocaine, ketamine, isoflurane, and pentobarbital can prevent death in animals suffering from endotoxic shock [1,2] and protect them from septic peritonitis-induced liver and renal injury [3,4].

Chloral hydrate, as an long-established and safe sedative and assistant anesthetic drug applied clinically, is still commonly used in the adjunctive therapy of hyperpyretic convulsion in pediatric patients and auxiliary operation of MRI and ultrasound cardiogram [5–9] and is used for anesthesia of laboratory animals in many countries [10–13]. The time from oral administration of chloral hydrate to onset of sedation averages 15–60 min [14–16]. Hypnotic doses range from 25 to 50 mg/kg/day and sedation doses range from 25 to 100 mg/kg/day, up to a maximum individual dose of 500 mg [14,17,18]. The gastrointestinal tract rapidly absorbs chloral hydrate after oral or rectal use. It is promptly converted into trichloroethanol (TCE), the main active metabolite that prolongs the sedative effect for up to 12 h [14,16,19].

In our previous studies, we found that chloral hydrate (320 mg/Kg) treatment could significantly attenuate acute inflammation in mice treated with LPS/D-GalN and zymosan A *in vivo* [20]. We also found that chloral hydrate (0.5–1.0 mg/ml) and its major active metabolite, 2,2,2-trichloroethanol, treatment can significantly inhibit the levels of IL-6 and TNF-α secreted by peritoneal macrophages induced by LPS and peptidoglycan (PGN), as well as the activity of NF-κB in RAW264.7 macrophages *in vitro* [20–22]. Chloral hydrate treatment can also decrease PGN-induced higher expression of Toll-like receptor (TLR) 2 by RAW264.7 macrophages [22]. However, the mechanisms are largely unknown.

Apoptosis is programmed cell death induced by several molecular mechanisms and is of great important in the occurrence and progression of embryonic development, morphogenesis, cell stability in tissue, defense and immune response, cell injury, aging, and tumor development [23,24]. The Fas-FasL system plays an important role in apoptosis induction. After the cross-linking of Fas-FasL, the apoptosis signal will be transferred to the intracellular parts, inducing the apoptosis of cells expressing Fas antigen [25]. Many kinds of cells express Fas and FasL, and some can express both.

This study was designed to investigate the effect of chloral hydrate treatment on the apoptosis of RAW264.7 macrophages and to explore the underlying mechanisms, which may yield new options for adjunctive treatment of acute inflammation.

Material and Methods

Instruments and reagents

Chloral hydrate was from YongDa Chemical Reagent Development Center (TianJin, China). Annexin-V-FITC/PI double-staining cell apoptosis testing kit and DNA ladder were from KeyGEN Biological Technical Development Company (NanJing, China). Hochest 33258 was from Beyotime Biological Technical Institute (JiangSu, China). Fetal bovine serum (FBS) was from GIBCO company (Gran Island, NY, USA.). FITC-anti-mouse Fas (isotype control FITC-Armenian Hamster IgG2, λ2) and PE-anti-mouse FasL (isotype control PE-IgG2, λ2) were from BD Pharmingen (San Diego, CA, USA). Antibodies, including anti-FADD, anti-Phospho-FADD, anti-Cleaved caspase-8, anti-Cleaved caspase-3, and anti-Cleaved PARP and anti-β-actin were from Cell Signaling Technology Inc., (Beverly, MA, USA). HRP-anti-rabbit IgG or anti-mouse IgG were also from Cell Signaling Technology Inc. (Beverly, MA, USA). Proteinase inhibitors (PMSF, EDTA, aprotinin, leupeptin, prostatin A) were from Intron Biotechnology (Gyeonggi, Korea). A Protein Quantification kit (CBB solution®) was from Dojindo Molecular Technologies (Rockville, MD, USA). Nitrocellulose membrane was from PALL Life Sciences (MI, USA). Enhanced electrochemiluminescent (ECL) detection solution was from Pierce (Rockford, IL, USA). The murine macrophage RAW264.7 cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). The flow cytometer (FACSCanto II) was from Becton Dickinson (BD Biosciences, San Jose, USA).

Treatment of RAW264.7 macrophages with different concentrations of chloral hydrate at different times

RAW264.7 macrophages were cultured with IMDM medium containing 10% fetal bovine serum, and then treated with different concentrations of chloral hydrate with final concentration of 0, 0.25, 0.5, 1.0, and 2.0 mg/ml for 3 h, and we observed the morphologic changes under the light microscope. RAW264.7 macrophages were treated with chloral hydrate at 0.5 mg/ml for different times (1, 3, 5, and 7 h), and we observed the morphologic changes.

Detection of apoptosis of RAW264.7 macrophages treated with chloral hydrate

According to the detection of early and late apoptosis of RAW264.7 macrophages treated with chloral hydrate, RAW264.7 macrophages were divided into groups. First, RAW264.7 macrophages cultured in IMDM medium containing 10% fetal bovine serum were treated with chloral hydrate at 0.5 mg/ml for 1, 3, 5, 7, 12, and 24 h and then divided into 3 groups. In Group 1, RAW264.7 macrophages were treated with 0.25% trypsin (containing 0.02% EDTA) after 1, 3, 5, and 7 h treatment, and then

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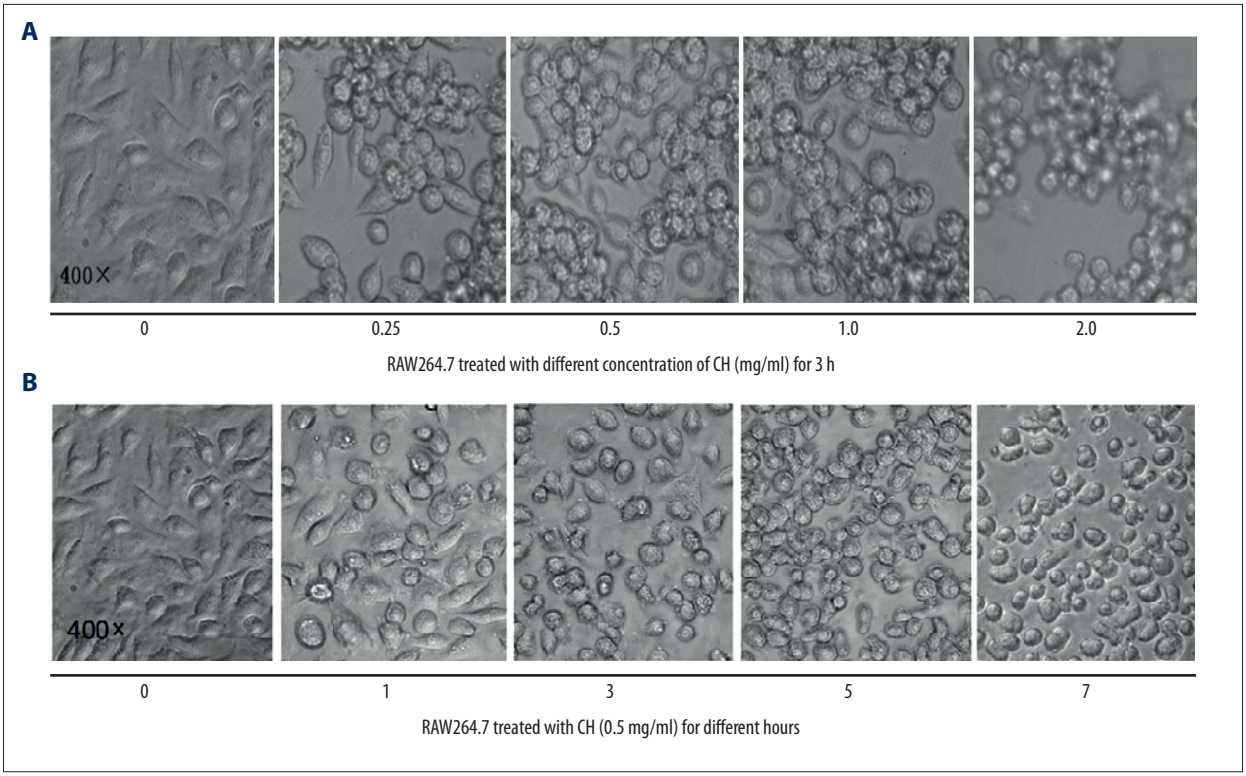

Figure 1. The effects of chloral hydrate treatment on the morphology of RAW264.7 macrophages. (**A**) RAW264.7 macrophages cultured with IMDM medium containing 10% fetal bovine serum were treated with chloral hydrate at different concentration for 3 h, and (**B**) chloral hydrate at 0.5 mg/ml for various lengths of time. The morphology is from an experiment that was repeated 3 times with similar results.

collected and stained with Annexin-V-FITC/PI according to the manufacturer's instructions. Flow cytometry was used to analyze the early and late apoptosis. In Group 2, RAW264.7 macrophages were treated with chloral hydrate at 0.5 mg/ml for 12 h, and then stained with Hochest33258 according to the manufacturer's instructions and observed under the fluorescence microscope. In Group 3, RAW264.7 macrophages were treated with chloral hydrate at 0.5 and 1.0 mg/ml for 12 h, and then digested with 0.25% trypsin (containing 0.02% EDTA) and tested for apoptosis by agarose gel electrophoresis with DNA ladder.

Detection of expression of Fas and FasL on RAW264.7 macrophages treated with chloral hydrate

RAW264.7 macrophages were treated with chloral hydrate at 0.5 and 1.0 mg/ml for 12 h and 24 h, respectively, and then digested with 0.25% trypsin (containing 0.02% EDTA) and tested for the expression of Fas and FasL with flow cytometry.

Detection of Fas signaling pathway in RAW264.7 macrophages treated with chloral hydrate

The cultured RAW264.7 cells in a 150-mm dish were treated with chloral hydrate for 12 h or 24 h and then washed with ice-cold PBS (phosphate-buffered saline) and harvested by scraping. The harvested cells were collected by centrifugation, lysed in ice-cold lysis buffer (50 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, and proteinase inhibitors [PMSF, EDTA, aprotinin, leupeptin, prostatin A]). After incubating on ice for 30 min, the insoluble materials were removed by centrifugation at 14 000 rpm for 20 min at 4°C. The protein contents of the cell lysates were determined by a Protein Quantification Kit with bovine serum albumin (BSA) as standard. An aliquot from each sample was boiled for 4 min, and then resolved by 12% SDS-PAGE. The proteins were electrotransferred to a nitrocellulose membrane and then blocked in PBS-T buffer (135 mM NaCl, 2.7 mM KCl, 4.3 mM NaPO4, 1.4 mM KH2PO4, 0.5% Tween-20) containing 5% skim milk overnight at 4°C. The blots were probed with the primary antibody and then washed 3 times in PBS-T, followed by incubation for 1 h with HRP-anti-rabbit IgG or anti-mouse IgG as secondary antibodies. The blots were then washed in PBS-T and visualized by an enhanced chemiluminescent (ECL) detection solution.

Statistical analysis

All data were analyzed with SPSS 13.0 software, and are expressed as mean ± standard deviation (SD). The

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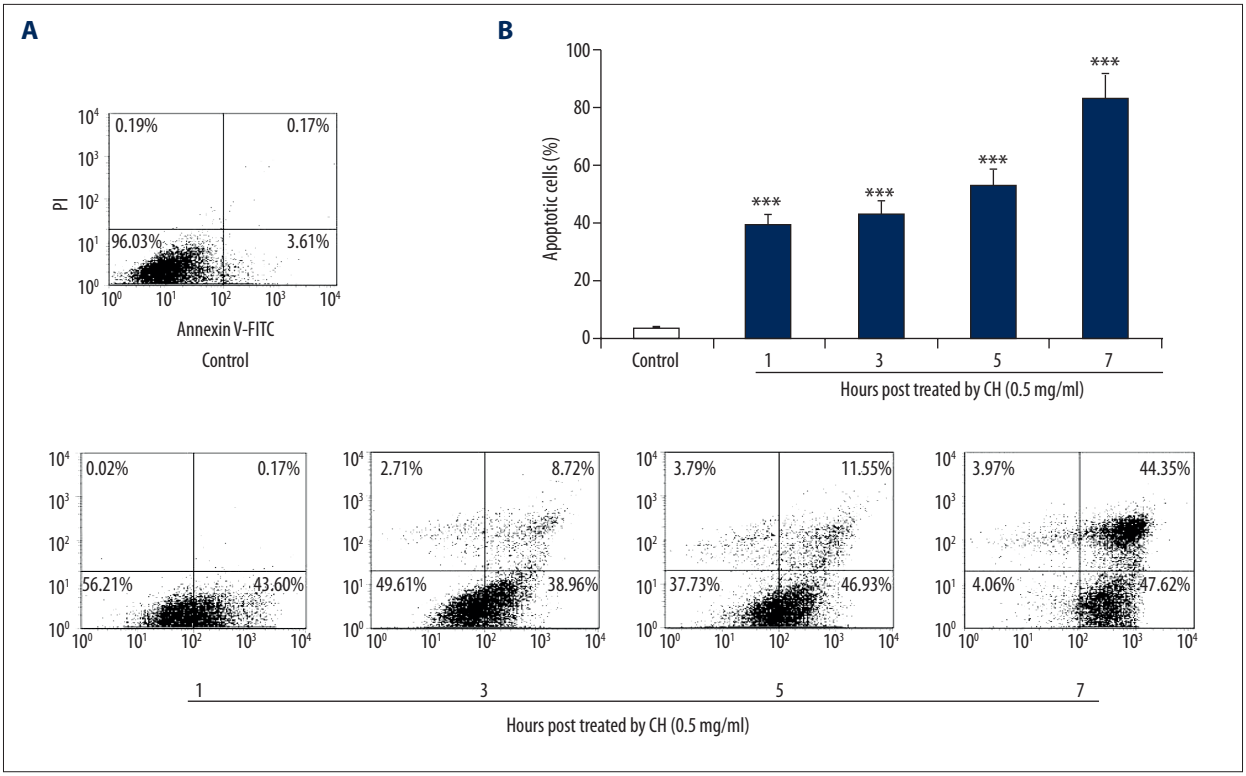

Figure 2. The effects of chloral hydrate (0.5 mg/ml) treatment for different times on the apoptosis of RAW264.7 macrophages. (**A**) RAW264.7 macrophages cultured with IMDM medium containing 10% fetal bovine serum were treated with chloral hydrate (0.5 mg/ml) for different times, and then detected with annexin-V-FITC/PI. (**B**) Statistical data are the mean ±S.D. of triplicates from an experiment that was repeated 3 times. Compared with control, *** $P<0.001$.

independent-samples *t* test was used to compare various factors in the chloral hydrate treatment group and the control group at the same time point. $P<0.05$ was considered significant.

Results

The effect of chloral hydrate on the morphology of RAW264.7 macrophages

The morphology of RAW264.7 macrophages changed from typical fusiform to round, and finally exfoliation and suspension, in a concentration-dependent manner of chloral hydrate treatment for 3 h (Figure 1A), especially for chloral hydrate at 0.5 mg/ml, which significantly induced the morphologic alterations (Figure 1A).

Also, the morphology of RAW264.7 macrophages changed from typical fusiform to round, and finally exfoliation and suspension, in a time-dependent manner with chloral hydrate treatment at 0.5 mg/ml (Figure 1B).

The effects of chloral hydrate treatment on apoptosis of RAW264.7 macrophages

First, annexin-V-FITC/PI staining showed that, compared with the control, chloral hydrate (0.5 mg/ml) treatment significantly induced the transformation from early apoptosis to late apoptosis after 1, 3, 5, and 7 h of treatment (F=39.433, P=0.000) (Figure 2). At 7 h, the early apoptosis rate was 47.62%, the late apoptosis rate was 44.35%, and the total apoptosis rate was 91.97% (Figure 2).

Second, Hochest33258 staining showed that, compared with the control, chloral hydrate (0.5 mg/ml) treatment significantly induced the apoptotic features of nuclei stained with Hochest33258, including obvious nucleus shrinking with increased density, condensation, and fracture (Figure 3A).

Third, the results showed that, compared with the control, chloral hydrate treatment for 12 h at 0.5 and 1.0 mg/ml significantly induced a typical DNA ladder, which is the typical feature of late apoptosis (Figure 3B).

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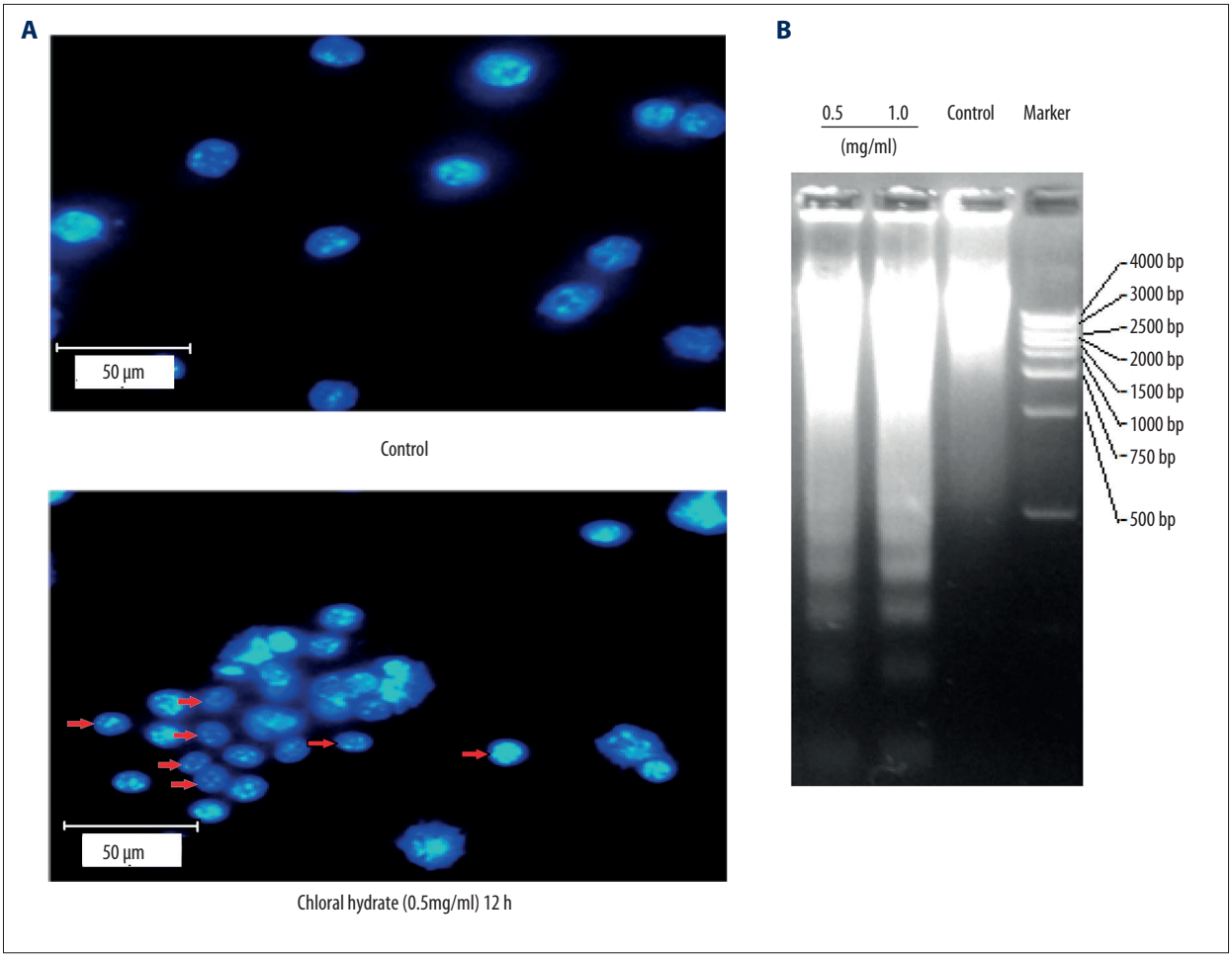

Figure 3. The effects of chloral hydrate treatment for 12 h on the apoptosis of RAW264.7 macrophages. (**A**) RAW264.7 macrophages cultured with IMDM medium containing 10% fetal bovine serum were treated with chloral hydrate (0.5 mg/ml) for 12 h, and then stained with Hochest 33258, or (**B**) digested with 0.25% trypsin (containing 0.02% EDTA) and tested for apoptosis by agarose gel electrophoresis with DNA ladder. Red arrow head: apoptotic nuclei. The apoptosis is from an experiment that was repeated 3 times with similar results.

The effects of chloral hydrate treatment on the expression of Fas and FasL on RAW264.7 macrophages

The expressions of Fas on RAW264.7 macrophages treated with chloral hydrate (0.5 and 1.0 mg/ml) for 12 and 24 h were all significantly up-regulated in a time-and dose-dependent manner (all $P<0.05$) (Figure 4). The expression of FasL in all RAW264.7 macrophages was not detected (Figure 4).

The effects of chloral hydrate treatment on Fas signaling pathway in RAW264.7 macrophages

The death receptor-mediated apoptotic pathway has been proposed as a therapeutic target in cancer treatment. Since the Fas/FasL system is also a key signal transduction pathway of apoptosis, we examined the involvement of the Fas/FasL system in RAW264.7 cells after being treated with chloral hydrate.

The cell surface receptor Fas has a Fas-associated protein with death domain (FADD) in the cytoplasmic domain. After the death signal factors bind to Fas, the FADD is phosphorylated. To determine the pathways of chloral hydrate-induced apoptosis, the levels of FADD and phospho-FADD were examined using Western blot analysis (Figure 5). The levels of FADD and phospho-FADD were increased after the treatment. These data suggest that chloral hydrate-induced apoptosis following the death receptor-mediated extrinsic pathway. Increased phospho-FADD stimulates binding of caspase-8 to FADD, and this leads to the activation of caspase-8, which can then cleave and activate downstream effector caspases such as caspase-3 or -7, leading to the apoptosis of RAW264.7 cells (Figure 5).

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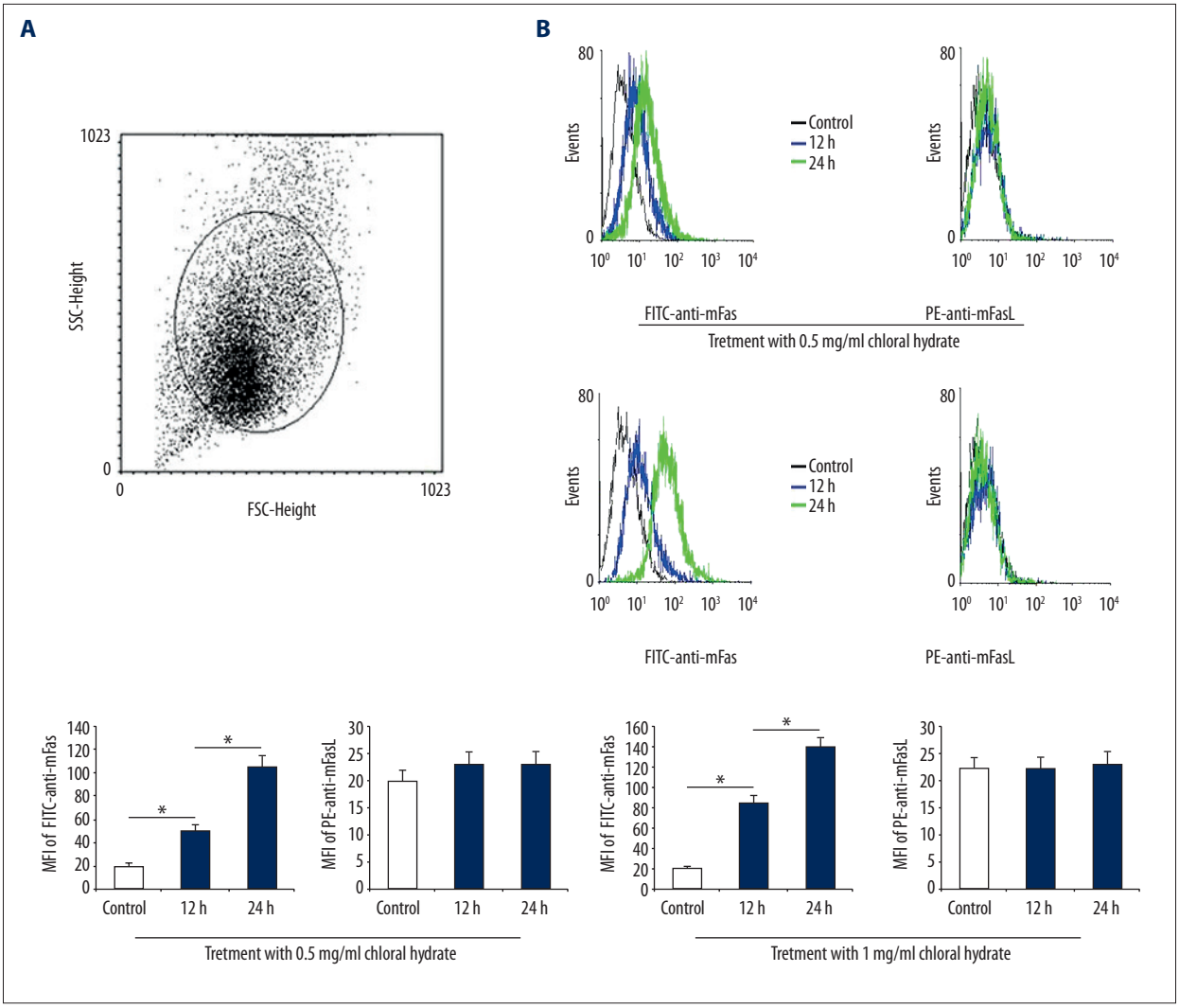

Figure 4. The effects of chloral hydrate (0.5 and 1mg/ml) treatment for 12 and 24 h on the expression of Fas and FasL on RAW264.7 macrophages. (**A**) RAW264.7 macrophages cultured with IMDM medium containing 10% fetal bovine serum were treated with chloral hydrate (0.5 and 1 mg/ml) for 12 and 24 h, and then detected with flow cytometry, and (**B**) the statistical data are the mean±S.D. of triplicates from an experiment that was repeated 3 times. Compared with control (Con), * $P<0.05$. (Gray: isotype control; blue and green 12 h and 24 h FITC-anti-mFas/FasL), respectively.

Discussion

We have reported that chloral hydrate treatment can attenuate the inflammatory reaction of RAW264.7 cells induced by several stimulators. In this study, we explored the underlying mechanisms. First, we investigated whether chloral hydrate treatment directly kills the targeted cells to necrose or triggers the apoptosis of the targeted cells. If chloral hydrate can directly kill the targeted cells *in vivo*, the new synthesis and secretion of inflammatory cytokines can be inhibited temporarily. However, necrotic cells will induce the cascaded or serious inflammatory response, even resulting in death because of inflammatory cytokine storm. In the contrast, apoptosis will not induce the inflammatory responses, because cell apoptosis happens in the process of body development or is induced by the body when the cell number is unbalanced, or in some disease conditions it provides the possibility of self-protection [26].

Effects of various concentrations of chloral hydrate have been reported in the literature [14,17,18,27], and the dose used in our previous study has been reported [20,21]. Here, RAW264.7 macrophages were treated with different concentrations of chloral hydrate (0.25 to 2 mg/ml) *in vitro*. This study demonstrated that chloral hydrate treatment can induce the apoptosis of RAW264.7 macrophages by active Fas signaling pathway. We focussed on the progress of early-to-late apoptosis with several apoptosis detection methods, including annexin-V FITC/PI double-stain assay, Hochest 33258 staining method,

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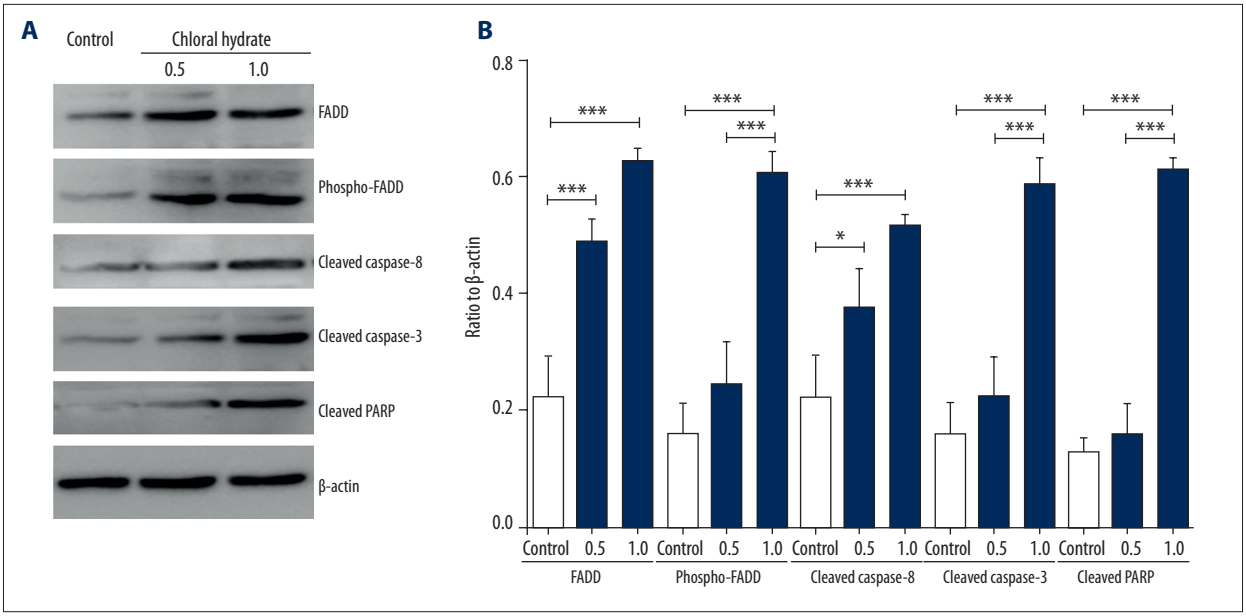

Figure 5. Western blot analyses of the proteins involved in apoptosis on RAW264.7 cells. (**A**) The RAW264.7 cells were treated with 0.5 and 1.0 mg/ml for 24 h, and the expression of proteins involved in Fas signaling pathways were examined using Western blot. β-actin was used as the internal control, and (**B**) the statistical data are the mean ±S.D. of triplicates from an experiment that was repeated 3 times. Compared with control (Con), * $P<0.05$. ** $P<0.01$, *** $P<0.001$.

and DNA ladder [28]. The results showed that chloral hydrate treatment could induce the apoptosis of RAW264.7 macrophages in a dose- and time-dependent manner. The results showed chloral hydrate at the concentration of 0.5 mg/ml for different times could significantly induce the transformation from early apoptosis to late apoptosis of RAW264.7 macrophages. Also, chloral hydrate treatment at the concentrations of 0.5 and 1 mg/ml for 12 h to RAW264.7 macrophages can induce typical DNA ladder, which is the typical feature of late apoptosis. Further, chloral hydrate treatment at a concentration of 0.5 mg/ml for 12 h to RAW264.7 macrophages can induce apoptotic feature of nuclei, including obvious nucleus shrinking with increased density, condensation, and fracture.

For the apoptosis pathway, the Fas and FasL family is one of the key apoptotic gene-related families, and the activation of Fas by its physiological ligand, FasL, has been proven to trigger apoptosis [29–31]. The main functions of Fas and FasL in physiological apoptosis are the killing of pathogen-infected target cells and the death of cells that are no longer needed and may be deleterious, as well as autoreactive lymphocytes [32]. As is known, the Fas and FasL pathway plays an important part in regulating apoptosis of T and B cells, including immune tolerance and maintaining the balance of lymphocyte number [33,34]. FasL stimulation can induce apoptosis of macrophages and cytokines release [35,36]. Li et al. reported that oxidative low-density lipoprotein induces the apoptosis of RAW264.7 macrophages through the Fas and FasL pathway [37]. LPS can also induce the apoptosis of RAW264.7 macrophages through the Fas and FasL pathway [38]. In the present study, the results showed that chloral hydrate treatment can induce RAW264.7 macrophages to express Fas in a dose- and time-dependent manner, and we speculate that chloral hydrate could induce apoptosis of RAW264.7 macrophages through the Fas and FasL pathway.

Next, we investigated the Fas signaling pathway. FasL-Fas signaling triggers apoptosis through FADD (Fas-associated protein with death domain, also called MORT1) adaptor protein-mediated recruitment and activation of the aspartate-specific cysteine protease, caspase-8 [32]. Our results show the clustering of the death domains (DDs) of receptors, which then recruit the cytosolic adapter protein FADD by binding to the death domains of FADD. FADD not only contains a DD but also a Death Effector Domain (DED) that binds to an analogous domain repeated in tandem within the zymogen form of Caspase-8. The complex of Fas receptor (trimer), FADD, and Caspase-8 is called the Death Inducing Signaling Complex (DISC). Upon recruitment by FADD, caspase-8 oligomerization drives its activation through self-cleavage [39]. Active caspase-8 then activates downstream caspases, committing the cell to apoptosis. Our study results show that chloral hydrate treatment can induce the apoptosis of RAW264.7 macrophages by activating the Fas signaling pathway.

Previously, we discovered a new anti-inflammation function of chloral hydrate, and the present study provides data on the underlying mechanisms, which we expect will provide new options for adjunctive treatment of acute inflammation.

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Conclusions

Chloral hydrate treatment can induce the apoptosis of RAW264.7 macrophages through the Fas signaling pathway, which may provide new options for adjunctive treatment of acute inflammation.

Disclosure of conflict of interest

The authors declare no competing financial or commercial interests.

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