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Prevention of cadmium-induced toxicity in liver-derived cells by the combination preparation Hepeel®

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ABSTRACT

Cadmium is a heavy metal of considerable environmental concern that causes liver damage. This study examined the possible prevention of cadmium toxicity in human HepG2 cells and primary rat hepatocytes by Hepeel®, a combined preparation of tinctures from seven different plants. Hepeel® prevented cadmium chloride ($CdCl_2$)-induced cell death in both HepG2 cells and hepatocytes, and also reduced the loss of glutathione, lipid peroxidation, nuclear fragmentation, caspase activation and release of mitochondrial cytochrome C. To compare their relative efficacy, the seven constituent plant tinctures of Hepeel® were also separately tested. The tinctures China and Nux moschata, which exert solely anti-oxidative effects, failed to reduce cytotoxicity, and only protected against loss of glutathione and lipid peroxidation. In contrast, the tinctures Carduus marianus and Chelidonium, demonstrated anti-apoptotic effects, and protected HepG2 cells and primary hepatocytes against $CdCl_2$-induced cell death. These results demonstrate how the effectiveness of Hepeel® is determined by the synergistic features of its constituent tinctures. Furthermore, we conclude that cadmium toxicity in the liver is mainly due to stimulation of the intrinsic apoptotic pathway, but may be intensified by increased oxidative stress.

1. Introduction

Environmental exposure to fluctuating concentrations of heavy metals poses an enormous challenge for biological organisms. Toxic metals cause a vast array of adverse effects, including neurotoxicity, hepatotoxicity, immunotoxicity and carcinogenicity (Waalkes et al., 2000; Godt et al., 2006). Due to the global dispersion of heavy metals and their extensive use in modern society, some human exposure to toxic metals is inevitable. This ongoing prevalence of metal exposure necessitates protective measures at the environmental, social and individual level.

Cadmium is one of the most common toxic heavy metals, due to its primary accumulation in the liver and kidney (Godt et al., 2006). Cadmium causes hepatic, renal, skeletal, respiratory, and vascular disorders in humans (Nordberg, 1992; Waalkes et al., 2000), and it may also affect Leydig cells of the testes and hepatocytes and stellate cells of the liver (Koizumi et al., 1992; Dudley and Klaassen, 1984; Fariss, 1991; Souza et al., 2004a,b). Furthermore, cadmium is a potent carcinogen (Godt et al., 2006).

There is growing evidence that oxidative stress (Sarkar et al., 1995) via reactive oxygen species (ROS) generation and mitochondrial damage are among the basic mechanisms of cadmium toxicity (Sarkar et al., 1995; Koizumi et al., 1994; Rikans and Yamano, 2000). Recently, apoptotic mechanisms involving caspase-dependent and caspase-independent pathways were described for cultured hepatocytes and livers exposed to cadmium *in situ* (Habeebu et al., 1998; Aydin et al., 2003; Pham et al., 2006; Oh and Lim, 2006; Li and Lim, 2007; Lasfer et al., 2008). However, despite much progress in research, the relative contribution of oxidative stress and apoptotic mechanisms to cadmium toxicity is still unclear.

The combination preparation Hepeel® is frequently used to stimulate liver function and improve antioxidant function in acute and chronic diseases, such as cholangitis and cholecystitis (Gebhardt, 2003). Hepeel® also demonstrates several other protective features, such as induction of glutathione-S-transferase activity (Gebhardt, 2003). These findings prompted the present investigation of the hepatoprotective potential of Hepeel®, and its seven constituent plant tinctures, against cadmium-induced hepatocellular damage. To thoroughly examine this, and to provide comparative experimental data for two different cell types, we used the human hepatoblastoma cell line HepG2 and primary rat hepatocytes. Exposure to Hepeel® largely prevented cell death, and oxidative and apoptotic pathomechanisms were differentially affected by the constituent tinctures. The combined anti-oxidative and anti-apoptotic properties of Hepeel® and its constituent tinctures support its overall protective effect against cadmium-induced toxicity in liver cells.

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2. Materials and methods

2.1. Materials

Hepeel® tinctures were prepared from seven different plants, according to procedures 3a and 4a of the German Homeopathic Pharmacopoea (HAB, 2000), and were provided by the Biologische Heilmittel Heel GmbH (Baden-Baden, Germany). The following seven constituent tinctures were used: (1) Chelidonium, prepared from *Chelidonium majus* L. (Ch-B 007009, 10^{-2} dilution), (2) Carduus marianus, prepared from *Silybum marianum* L. (Ch-B 007034, 10^{-2} dilution), (3) Veratrum, prepared from *Veratrum album* L. (Ch-B 007050, 10^{-3} dilution), (4) Colocynthis, prepared from *Citrullus colocynthis* L. (Ch-B 007058, 10^{-3} dilution), (5) Lycopodium, prepared from *Lycopodium clavatum* L. (Ch-B 007001, 10^{-3} dilution), (6) Nux moschata, prepared from *Myristica fragrans*, Houtt (Ch-B 007026, 10^{-3} dilution), and (7) China prepared from *Cinchona pubescens*, Vahl (Ch-B 007018, 10^{-3} dilution).

The commercially available formulation of Hepeel® is a combination of all tinctures at the dilutions given above, with the addition of Phosphorus, a 10^{-4} dilution of yellow phosphor. Hepeel® was supplied in sterile ampoules by Biologische Heilmittel Heel GmbH. The relative volume composition of 1.1 ml Hepeel® injection solution is: Chelidonium (*Chelidonium majus*, 10^{-3} dilution) 1.1 μl, Carduus marianus (*Silybum marianum*, 10^{-1} dilution) 0.55 μl, Veratrum (*Veratrum album*, 10^{-5} dilution) 2.2 μl, Colocynthis (*Citrullus colocynthis*, 10^{-5} dilution) 3.3 μl, Lycopodium (*Lycopodium clavatum*, 10^{-2} dilution)) 1.1 μl, Nux moschata (*Myristica fragrans*, 10^{-3} dilution) 1.1 μl, China (*Cinchona pubescens*, 10^{-2} dilution) 1.1 μl and Phosphorus (10^{-2} dilution) 0.55 μl.

Dichlorodihydrofluorescein diacetate (DCFH) was purchased from Sigma (Daisenhofen, Germany). All other chemicals were from Roche Diagnostics (Mannheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) or Sigma (Daisenhofen, Germany). Cell culture plates with tissue culture quality were from Techno Plastic Products AG (Trasadingen, Switzerland).

2.2. Culture of HepG2 cells

HepG2 hepatoblastoma cells were cultured in 1× Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Eggenstein, Germany) supplemented with 2 mM glutamine, 10% foetal calf serum, 40 U/ml streptomycin and 50 U/ml penicillin, as previously described (Gebhardt, 2003). Cells were passaged weekly, when confluent. Cell stocks (passages 31 till 40) were kept frozen in liquid nitrogen. Frozen cells were thawed, cultured for one week, and passaged at least once before use. Confluent HepG2 cell cultures were used for all experiments.

2.3. Preparation and culture of rat hepatocytes

Sprague–Dawley rats were bred and maintained at the Medizinisch-Experimentelles Zentrum at the University of Leipzig, according to local ethical rules for animal care. They were kept on normal maintenance diet V1534 (Sniff, Soest, Germany) and tap water, ad libitum. Primary hepatocyte cultures were prepared from the livers of male rats (260–310 g) with collagenase perfusion, as previously described (Gebhardt, 1997). Cells were cultured in Williams medium E (Lonza, Verviers, Belgium) on collagen-coated plastic plates, at a uniform cell density of 125.000 cells/cm^2. During the first 2 h, culture medium was supplemented with 10% calf serum, and serum-free medium was used thereafter. The medium volume was maintained at 100 μl/cm^2 of plating area. Additional details of cell culture have been reported elsewhere (Gebhardt, 1997; Gebhardt et al., 1994). For toxicity experiments, incubation in various agents usually started 2 h after plating.

2.4. Induced toxicity with cadmium chloride

The optimal concentration range of $CdCl_2$-induced cytotoxic effects was different for each cell type. For HepG2 cells, culture medium was supplemented with concentrations ranging from 3 to 8 μM. For primary rat hepatocytes, optimal concentrations ranged from 2 to 6 μM. The highest $CdCl_2$ concentrations caused the greatest cell death in each cell type. In HepG2 cells, 8 μM $CdCl_2$ caused about 95% cell death, within 30 h of incubation. In hepatocytes, 6 μM $CdCl_2$ caused 72% cell death within 24 h of incubation.

2.5. Preparation of Hepeel® and tinctures

To prepare a working dilution of each tested compound, one part Hepeel® or tincture was mixed with 9 parts (v/v) of serum-free Williams Medium E, and gently shaken for 20 min at room temperature. This working solution of effective 0.1 dilution was used for further dilutions with Williams Medium E as specified in figure legends. Appropriate controls replaced each tincture or Hepeel® with equal volumes of ethanol.

2.6. Determination of cytotoxicity

Cytotoxicity of the tested compounds was determined using the colorimetric MTT-assay (MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide), as previously described (Gebhardt, 1997).

2.7. Determination of lipid peroxidation and ROS production

Malondialdehyde (MDA) measurements were used to quantify lipid peroxidation (Gebhardt, 1997). Briefly, HepG2 cells or rat hepatocytes seeded on 60 mm Petri dishes were incubated with or without $CdCl_2$ (3 or 4 μM) for 60 min after 30 h and 24 h of cultivation, respectively. In order to enhance oxidative stress, some plates were simultaneously exposed to *t*-butyl hydroperoxide (*t*-BHP; final concentration 1.5 mM). Thereafter, cells were washed with 0.9% NaCl, resuspended, and scraped into 1 ml of 50 mM potassium phosphate buffer (pH 7.4), then homogenised by sonication for 10 s (15% of maximum power, Sonopuls HD 2200, Bandelin electronic, Berlin, Germany). MDA was determined by thiobarbituric acid (TBA) assay (Esterbauer and Cheeseman, 1990; Gebhardt, 1997). The protein content of homogenates was measured following the procedure of Lowry et al. (1951).

Measurement of intracellular ROS was accomplished by using the DCFH assay (Wang and Joseph, 1999). HepG2 cells or rat hepatocytes cultured overnight in collagen-coated 96-well black flat bottom plates were washed 3 times with Krebs-Ringer-HEPES (KRH) solution pH 7.2 (Pavlica and Gebhardt, 2005). Cells were preloaded with 0.1 mM DCFH in either DMEM (HepG2 cells) or Williams Medium E (rat hepatocytes) for 30 min, then washed 3 times with KRH buffer. Cells were then treated simultaneously with $CdCl_2$ (3 μM) and the test compound diluted 1:10 with different starting dilutions indicated in Table 3 for an additional 30 min. Fluorescence (485/520 nm, Spectrofluor, TECAN) was recorded every min for up to 30 min, while temperature was maintained at 37 °C. Percentage increase in fluorescence units/well was calculated by the formula: $Ft_{30} - Ft_0/Ft_0 \times 100$, where Ft_0 = fluorescence at time 0 min, and Ft_{30} = fluorescence at time 30 min (Pavlica and Gebhardt, 2005).

2.8. Determination of cellular glutathione content

To measure glutathione (GSH) content, cells were cultured in 6-well plates for 30 h (HepG2 cells) or 24 h (primary hepatocytes). Test compounds were added 2 h after plating, along with the first change of medium. At the end of the incubation period, cells were washed and scraped into HEPES buffered isolation medium as previously described (Pavlica and Gebhardt, 2005). Determination of total GSH content was performed according to method of Gebhardt and Fausel (1997).

2.9. Detection of apoptotic nuclei with DAPI

The blue nucleic acid dye DAPI (4′,6-Diamidino-2-phenylindole) was dissolved in methanol at 5 mg/ml, and stored as stock solution. Cells were washed twice with potassium phosphate buffer (PBS) and fixed with ice-cold methanol. Thereafter, a working solution of DAPI (1 μg/ml) in methanol was added, and cell nuclei were stained for 15 min at 37 °C. Destaining was achieved by replacing methanolic DAPI with pure methanol, followed by two rounds of washing with PBS.

2.10. Determination of caspase activity

Measurement of caspase-3 activity was based on the cleavage of a colorimetric substrate determined by the increase in absorbance at 405 nm. The assay was performed according to the instructions of the manufacturer (caspase-3 activity assay kit; Oncogene, Bad Soden, Germany) and adapted for HepG2 cells as described by Ohuchida et al. (2004). Recombinant caspase-3 was used for assay calibration.

Caspase-Glo™ 3/7 assay (Promega, Mannheim, Germany) is based on the cleavage of the proluminescent substrate by caspase 3/7 activity in the sample. A luminescent signal is generated via luciferase, and the assay was performed as previously described (Pavlica and Gebhardt, 2005). Briefly, 24 h (hepatocytes) or 30 h (HepG2 cells) after exposing the cells to medium containing Hepeel® or each tincture, the medium was removed. Then 0.1 ml of fresh medium was added to each well, together with 0.1 ml of the Caspase-Glo™ 3/7 reagent solution reconstituted according to the recommendations of the supplier. The well volume was shaked for 30 s, then incubated for 1 h at room temperature. After additional shaking for 20 s, luminescence was measured using a Multilabel-Reader Mithras LB 940.

2.11. Preparation of cellular fractions and Western blot analysis

To measure cytochrome C release, cellular extracts were prepared by lysing the cells in 10 mM Tris–HCl buffer (pH 7.4) containing 2 mM EDTA, 1 μM β-mercaptoethanol, 1 μM leupeptin, 100 μM PMSF (phenylmethylsulfonyl fluoride), and 250 mM sucrose. Cells were homogenised by repeated passaging through a 26-gauge needle, and were centrifuged at 14,000 × g × 20 min at 4 °C. Cytosolic supernatants and pellets containing mitochondria were diluted in order maintain equal concentrations of mitochondrial protein, then used for Western blot analysis as previously described (Haupt et al., 2000). Cytochrome C was detected with anti-cytochrome C (A-8) antibody (sc-13156, Santa Cruz Biotechnology Inc., Heidelberg, Germany) followed by alkaline phosphatase-conjugated secondary antibody.

2.12. Statistical evaluation

Data were analysed for significance with a Student's *t*-test for comparisons between two groups. Data are presented as mean ± standard deviation (SD) of three to four measures, except when stated otherwise.