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The protective role of curcumin on perfluorooctane sulfonate-induced genotoxicity: Single cell gel electrophoresis and micronucleus test

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ABSTRACT

Perfluorooctane sulfonate (PFOS) is a man-made fluorosurfactant and global pollutant. PFOS a persistent and bioaccumulative compound, is widely distributed in humans and wildlife. Therefore, it was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants in May 2009. Curcumin is a natural polyphenolic compound abundant in the rhizome of the perennial herb turmeric. It is commonly used as a dietary spice and coloring agent in cooking and anecdotally as an herb in traditional Asian medicine. In this study, male rats were treated with three different PFOS doses (0.6, 1.25 and 2.5 mg/kg) and one dose of curcumin, from *Curcuma longa* (80 mg/kg) and combined three doses of PFOS with 80 mg/kg dose of curcumin by gavage for 30 days at 48 h intervals. Here, we evaluated the DNA damage via single cell gel electrophoresis or comet assay and micronucleus test in bone marrow in vivo. PFOS induced micronucleus frequency and decreased the ratio of polychromatic erythrocyte to normochromatic erythrocyte in bone marrow. Using the alkaline comet assay, we showed that all doses of the PFOS strongly induced DNA damage in rat bone marrow and curcumin prevented the formation of DNA damage induced by PFOS.

1. Introduction

Perfluorinated organic compounds (PFOCs) have been widely used as lubricant paints cosmetic and fire-fighting foams (Kim et al., 2010). Among PFOCs, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonate (PFOS) have been detected in environment and variety of living organism worldwide (Kim et al., 2010; Giesy and Kannan, 2002). They are environmentally persistent because these compounds are resistant to hydrolysis, photolysis, microbial degradation and metabolism. Perfluorooctane sulfonate (PFOS), is a man-made fluorosurfactant and global pollutant. Therefore, it was added to Annex B of the Stockholm Convention on Persistent Organic Pollutants in May 2009 (http://chm.pops.int/Convention/Pressrelease/COP4Geneva8May2009/tabid/542/language/enUS/Default.aspx). PFOS can form from the degradation of precursors in addition to industrial production (Ye et al., 2008; EPA, 2002).

Curcumin, a natural polyphenolic compound abundant in the rhizome of the perennial herb turmeric, *Curcuma longa*, is known to possess comprehensive anti-inflammatory and anti-cancerous properties following topical or oral administration. It is commonly used as a dietary spice and coloring agent in cooking and anecdotally as an herb in traditional Indian and Chinese medicine (Miquel et al., 2002; Maheshwari et al., 2006). Curcumin has been shown to act as an antioxidant through modulation of glutathione (GSH) levels (Biswas et al., 2005). Curcumin is the most active component of turmeric, a botanical agent derived from the dried rhizome of the turmeric plant (*C. longa*), a perennial herb belonging to the ginger family that is cultivated extensively in south and southeast tropical Asia. Turmeric's pharmacological safety is accepted, considering that it has been consumed as a dietary spice, at doses up to 100 mg/d, for centuries (Ammon and Wahl, 1991).

The micronucleus assay is a mutagenic test system for the detection of chemicals which induce the formation of small membrane bound DNA fragments, i.e. micronuclei in the cytoplasm of interphase cells. Micronucleus test has been used to evaluate the exposure to chemicals for decades in many investigations in in vitro and in vivo studies in different organism (Çelik et al., 2003, 2005; Çelik and Kanık, 2006; Çavaş, 2011).

The single-cell gel electrophoresis assay (comet assay) is capable of detecting DNA damage with great sensitivity and has been used widely both in vitro and in vivo protocols to identify potentially environmental genotoxins (Tsuda et al., 2000). Comet assay has been widely used in different models from bacteria to man, employing diverse cell types to assess the DNA-damaging potential of chemicals and/or environmental conditions by many research groups and researchers (Narendra, 2000; Çavaş, 2011).

The main advantages of the comet assay include: (a) the collection of data at the level of the individual cell, allowing more robust statistical analyses (b) the need for a small number of cells per

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sample (<10,000) (c) sensitivity for detecting DNA damage and (d) use of any eukaryote single cell population both in vitro and in vivo, including cells obtained from exposed human populations and aquatic organisms for eco-genotoxicological studies and environmental monitoring (Collins et al., 1997).

In the present study, we aimed; (1) to evaluate the genotoxic and cytotoxic effects of PFOS at three different doses in rat bone marrow using micronucleus test system, single gel electrophoresis/comet assay and determining polychromatic erythrocyte (PCE)/normochromatic erythrocyte (NCE) ratio, respectively, (2) to evaluate the protective effects of curcumin against damages occurred by PFOS.

2. Materials and methods

2.1. Chemicals

Perfluorooctane sulfonate (PFOS; CAS No.: 1763-23-1) was supplied as grade form and purchased form Sigma. Curcumin was purchased from Sigma (St. Louis, MO, USA). AN (chemical purity >99%). Figs. 1 and 2 represent molecular structure of PFOS and curcumin, respectively.

2.2. Dose selection

Doses were selected according to LD_{50} dose of PFOS for rats. LD_{50} dose of PFOS is 251 mg/kg for rats (OECD, 2002). Therefore, in this study, the highest dose was set at 2.50 mg/kg body wt, i.e. 1% of the LD50 dose. The lowest dose was determined via pre study as 0.6 mg/kg body wt because this dose did not affect the animals neurologically and behaviorally. Curcumin dose was selected according to previous studies performed by other researchers (Balakrishnan et al., 2008; Sankar et al., 2010) and curcumin's pharmacological safety is accepted, considering that it has been consumed as a dietary spice, at doses up to 100 mg/d, for centuries (Ammon and Wahl, 1991).

Mitomycin C (MMC) (2 mg/kg), a single i.p. dose, was used as a positive control in this study. It is acceptable that a positive control is administered by a route different from or the same as the test substance and that it is given only a single time (Hayashi et al., 1994). The positive control and untreated control rats were treated identically with equal volumes of normal saline.

2.3. Animal and experimental design

Healthy adult female Swiss albino rats (Wistar rat) [6–8 weeks of age and average body weight (body wt) of 180–200 g] were used in this study. Rats were obtained from the Experimental Animal Center, University of Mersin, Turkey. The study was approved by the research and ethical committee at the University of Mersin. The rats were randomly selected and housed in polycarbonate boxes (six rats per box) with steel wire tops and rice husk bedding. They were maintained with 12 h dark/light cycle in a controlled atmosphere of 22 ± 2 °C temperature and 50–70% humidity with free access to pelleted feed and fresh tap water. The animals were allowed to acclimate for 14 days before treatment. In this study, rats were divided to nine groups including six rats. Therefore, the study was performed by nine different diet regimen one dose per 48 h for 4 weeks (~30 days).

The rats were treated by gavage with

(1) 0.6 mg/kg body wt only PFOS dissolved in saline.
(2) 1.25 mg/kg body wt only PFOS dissolved in saline.
(3) 2.50 mg/kg body wt only PFOS dissolved in saline.
(4) 0.6 mg/kg dose of PFOS (dissolved in saline) with curcumin (80 mg/kg body wt).
(5) 1.25 mg/kg dose of PFOS (dissolved in saline) with curcumin (80 mg/kg) body wt).
(6) 2.50 mg/kg dose of PFOS (dissolved in saline) with curcumin (80 mg/kg body wt).
(7) The rats were given only curcumin (80 mg/kg body wt).
(8) The untreated control rats were treated identically with equal volumes of normal saline only via gavage throughout the study.

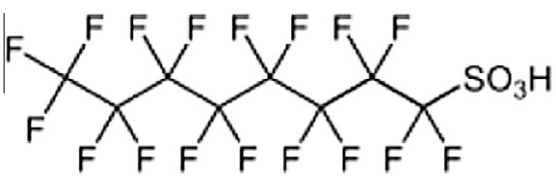
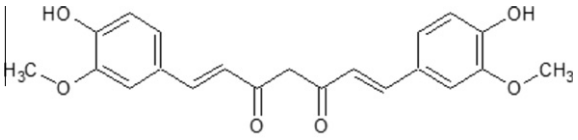


Fig. 1. Chemical structure of perfluorooctane sulfonate.



Curcumin

Fig. 2. Chemical structure of curcumin.

(9) Since positive controls may be administered by a different route and treatment schedule than the test agent (Hayashi et al., 1994) a single dose of MMC (2 mg/kg, i.p.) was administered at the 16th week dosing time.

2.4. Comet assay in bone marrow

The two femurs were removed and bone marrow cells were flushed from the femur into 1 ml of fetal bovine serum (FBS). Isolated bone marrow cell suspension was washed two times with RPMI supplemented with 10% FBS. Part of the isolated cells was used directly for the comet assay. Comet assay was performed under alkaline conditions according to the method of Singh et al. (1988) with slight modifications. Completely frosted microscopic slides were covered by a thin layer of 0.5% normal melting agarose (NMA) at about 50 °C (dissolved in $Ca^{2+}$and Mg^{2+} free phosphate buffer saline (PBS). Eppendorf tubes were placed in water bath at 40 °C. One hundred microliters of bone marrow suspension were diluted with 1 ml of PBS in eppendorf tube. Then 30 μl mixtures were mixed with 250 μl of LMA (0.5%). One hundred microliters of this mixture was spreaded on NMA-coated slides using micropipette and immediately was covered with coverslip. Slides were preserved in refrigerators at +4 °C for 15 min. The coverslips were slowly removed from top of slides. Then slides were placed in chalets including lysis solution and preserved for 1.5 h in refrigerator in dark. Slides were washed with chilled distilled water and placed on a horizontal gel electrophoresis unit filled with fresh electrophoretic buffer (300 mM NaOH + 1 mM EDTA) to allow DNA unwinding before electrophoresis for 20 min Electrophoresis was conducted at 20 °C using 25 V and 185 mA for 20 min. The above steps should be carried out in dark to avoid DNA damage. After electrophoresis, slides were washed with chilled distilled water and placed in neutralizing buffer (0.048 g/ml) (for 5 min).Then again slides were washed with chilled distilled water and placed in chilled ethanol for 10 min. DNA was stained with ethidium bromide (0.1 mg/ml, 1:4) and the slides were examined with a fluorescent microscope (BX51, Olympus, Japan). Each group should be six parallel samples.

2.5. Bone marrow micronucleus test

Rats were anaesthetized with Ketalar (Ketamine-HCl, Pfizer, Istanbul), 30 h after the last treatment and sacrificed. The frequency of micronucleated erythrocytes in femoral bone marrow was evaluated according to the procedure of Schmid (1973), with the slight modifications of Agarwal et al. (1994). The bone marrow was flushed out from both femora using 1 ml of fetal calf serum and centrifuged at 336 g for 10 min and the supernatant was discarded. Bone marrow smears were prepared on clean microscope slides, air dried, fixed in methanol and stained with acridine orange (125 μg/ml in pH 6.8 phosphate buffer) for 1 min just before the evaluation with a fluorescence microscope (Olympus BX51) using a 40× objective.

2.6. Scoring of micronucleus frequency

The frequency of PCEs per total erythrocytes was determined using a sample size of 200 erythrocytes per animal. The number of MNPCEs was determined using 2000 PCE per animal. Briefly, immature erythrocytes, i.e. PCEs were identified by their orange–red color, mature erythrocytes by their green color and micronuclei by their yellowish color.

2.7. Scoring of comet images

Comet images were analyzed according to Collins et al. (1995). One hundred comet images were scored for each treatment by two scorers (S.Y.E. and S.Y.) visually under fluorescence microscopy (BX51 Olympus). An intensity score from class 0 (undamaged) to class 4 (ultra high damage) (Sun et al., 2004) was assigned to each cell. The method of the observation was barred in a blind way during which the observer had no knowledge of the identity of the slide.

2.8. Statistical analysis

The "arbitrary units (AU)" was used to express the extent of DNA damage and calculated following formula.

$$AU = \sum_{i=0}^{4} i \times N_i$$