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Scientific Paper

THE EFFECT OF LAUREL LEAF EXTRACT AGAINST TOXICITY INDUCED BY 2,3,7,8-TETRACHLORODIBENZO-*P*-DIOXIN IN CULTURED RAT HEPATOCYTES

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2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a very toxic environmental pollutant that raises great public concern about its impact on human health. Recent studies indicate that laurel leaf extract exhibits antioxidant properties that can counter the toxic effects of certain compounds in the liver. The aim of this study was to assess how effective LE is against the toxicity of TCDD in a primary culture of rat hepatocytes. The extract (50 mg L^{-1}, 100 mg L^{-1}, and 200 mg L^{-1}) was added to cultures alone or with TCDD (1.61 mg L^{-1} and 3.22 mg L^{-1}) for 48 hours. Cell viability was measured using the [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide] (MTT) assay and the lactate dehydrogenase (LDH) cytotoxicity assay, while oxidative damage was assessed by measuring total antioxidant capacity (TAC) and total oxidative stress (TOS). DNA damage was also analysed using the micronucleus (MN) assay of the cultured hepatocytes. TCDD alone lowered, and laurel extract had no effect on cell viability. TCDD also increased TOS and significantly decreased TAC. It significantly increased the frequency of micronucleated hepatocytes in a dose-dependent manner. In cultures exposed to LE alone, TOS did not change and TAC significantly increased in a dose-dependent manner. Added to TCDD, laurel countered its toxic effects and showed protective effects against TCDD-mediated DNA damage. This points to the therapeutic potential of laurel against TCDD toxicity in the liver.

KEY WORDS: *antioxidant capacity, cell viability, genotoxicity, hepatoprotectivity*

Dioxins and dioxin-like aromatic hydrocarbons are industrial by-products, and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is one of the most toxic among them (1, 2). Its effects include dermal toxicity, immunotoxicity, hepatotoxicity, carcinogenicity, teratogenicity, and neurobehavioral, endocrine, and metabolic changes (3). Moreover, animal studies suggest increased incidence of several cancer types associated with TCDD treatment (4).

TCDD induces degeneration of hepatocytes (5) and promotes liver cancer (6). All these disorders involve a strong oxidative stress after TCDD exposure. Given their nature, research to find an agent to protect hepatocytes against oxidative stress is focused on antioxidants (7).

Laurel (*Laurus nobilis* L.) has long been used for its astringent, healing, and diuretic properties (8). This plant is used in cooking and folk medicine for stomach ache, flatulence, and gastric diseases in many parts of the world (9). Moreover, recent experimental results indicated that the laurel leaf extract (LE) exhibited

antibacterial properties (10). In addition, laurel bark extract inhibited lipid peroxidation (LPO) (11).

However, hepatoprotective effects of LE against TCDD - including the DNA damage - have not yet been investigated. In our present study, we examined the effects of LE on the viability, antioxidative capacity, and genome damage in rat liver cells exposed to TCDD.

MATERIALS AND METHODS

Test materials

TCDD (CAS No. 1746-01-6) was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). *L. nobilis* leaf samples for various extractions and processing experiments were collected from the Mersin-Silifke region in Turkey in May 2010. They were dried in the shade and crushed. After crushing, 100 g of air dried leaf powder was extracted with ethanol (95 %; 40 °C to 60 °C) in a Soxhlet extractor for 18 h to 20 h, and the solution was evaporated to dryness under reduced pressure and controlled temperature using a rotary evaporator. The extract (6.85 g) was stored in a refrigerator at 4 °C in air-tight bottles until further use.

Animals

For the study we used male Sprague-Dawley rats 200 g to 300 g of body weight, bred at the Medical Experimental Research Centre, Ataturk University, Turkey. All experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (12). The study protocol was approved by the local ethics committee.

Hepatocyte isolation and cultivation

Rats (n=3) were sacrificed by CO_2 overdose, and the livers were removed immediately. Hepatocytes were isolated using collagenase perfusion (13). The liver was perfused through the portal vein with calcium-free Hanks balanced salt solution to remove blood for about 10 min at a flow rate of 2.5 mL min^{-1}. As soon as the liver became greyish brown in colour, a second buffer solution containing Hank's balanced salt supplemented with 4 mmol L^{-1} calcium chloride and 0.5 mg mL^{-1} collagenase (Sigma-Aldrich) was run at the same rate until the liver appeared to have broken up. It was then minced into 3-mm to 4-mm pieces with a sterile scalpel. Following mechanical dissociation, the cells were filtered through gauze and centrifuged at 200x*g* for 5 min. Then the hepatocytes were collected in a medium containing bovine serum albumin and bovine insulin (Sigma-Aldrich). The cell suspension was filtered through gauze again and allowed to sediment for 20 min to eliminate cell debris, blood, and sinusoidal cells. The cells were then washed three times by centrifugation at 50x*g* and tested by Trypan blue for viability (always in the range of 82 % to 93 %). The hepatocytes were then suspended in a mixture of 75 % Eagle's minimum essential medium and 25 % medium 199, supplemented with 10 % foetal calf serum containing streptomycin, penicillin, bovine insulin, bovine serum albumin, and $NaHCO_3$ (2.2 mg) (Fluka AG, Germany). For the experimental procedure, hepatocytes were plated in multi-well tissue culture plates. The medium was changed 3 h to 4 h later. The effect of TCDD and LE was studied after 48 h of exposure in cultures maintained with a medium deprived of foetal calf serum, but supplemented with hydrocortisone hemisuccinate (14). Hepatocytes were cultured for additional 8 h before treated with TCDD and/or LE.

Cell treatment

After eight hours of plating, when primary hepatocytes adhered to the plate, culture media was aspirated and replaced with an equal volume of media supplemented with 1.61 mg L^{-1} or 3.22 mg L^{-1} TCDD and/or with 50 mg L^{-1}, 100 mg L^{-1}, or 200 mg L^{-1} LE and incubated in a CO_2 incubator for 48 hours.

MTT assay

Cell viability was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) test (15, 16). After the treatment with TCDD and/or LE, the hepatocytes were incubated with 0.7 mg mL^{-1} MTT at 37 °C for 30 min and washed with PBS. Blue formazan was extracted from the cells with isopropanol/formic acid (95:5) and spectrophotometrically determined at 560 nm.

LDH assay

Lactate dehydrogenase (LDH) activity was measured as an index of cytotoxicity, using an LDH kit (Bayer Diagnostics, France) adapted to the auto analyzer ADVIA 1650 (Bayer Corp., Tarrytown, NY, USA) and expressed as the percentage of extracellular LDH activity in the total activity on the plates (17).

TAC AND TOS ASSAYS

After 48 h, supernatants were collected to measure the total antioxidant capacity (TAC) and total oxidant status (TOS) levels using commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey) (18). The major advantage of the TAC test is that it measures the antioxidant capacity of all antioxidants in a biological sample and not just the antioxidant capacity of a single compound. In this test, antioxidants in the sample reduce dark blue-green coloured ABTS radical to its colourless reduced ABTS form. The change in absorbance at 660 nm is related to total antioxidant level in the sample. The assay is calibrated with a stable antioxidant standard solution called Trolox Equivalent, that is a vitamin E analogue.

In the TOS, oxidants in the sample oxidise the ferrous ion-chelator complex to ferric ion. The oxidation reaction is prolonged by enhancer molecules, which are abundant in the reaction medium. The ferric ion forms a coloured complex with chromogen in an acidic medium. Colour intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules in the sample. The assay is calibrated with hydrogen peroxide, and the results are expressed in terms of micromolar hydrogen peroxide equivalent per litre (H_2O_2 Equiv / μmol L^{-1}).

Micronucleus assay

Rat hepatocyte micronucleated cell frequency was measured using the method of Suzuki et al. (19). Immediately before the measurement, 10 μL to 20 μL of hepatocyte suspension was mixed with an equal volume of acridine orange (0.5 mg mL^{-1}) and 4',6-diamidino-2-phenylindole dihydrochloride (10 μg mL^{-1}) solution for fluorescent staining. Micronucleated cells were counted in 2000 well-isolated hepatocytes per animal using a fluorescence microscope (BX-70, Olympus Co. Ltd., Tokyo, Japan). A single observer (F. Geyikoglu) counted and confirmed hepatocytes with round or distinct micronuclei that stained like the nucleus, but whose diameter was one quarter or less than that of the nucleus.

Statistical analysis

The experimental data were analysed using one-way analysis of variance (ANOVA) and Fischer's least significant difference (LSD) tests to determine whether any treatment significantly differed from control or between each other. Results are presented as mean ±SD, and the level of $p<0.05$ is regarded as statistically significant.

RESULTS

Figure 1 shows cell viability measured by the MTT assay. Cells treated with TCDD concentrations of 1.61 mg L^{-1} and 3.22 mg L^{-1} showed 20 % and 60 % lower viability than the control cell line, respectively.

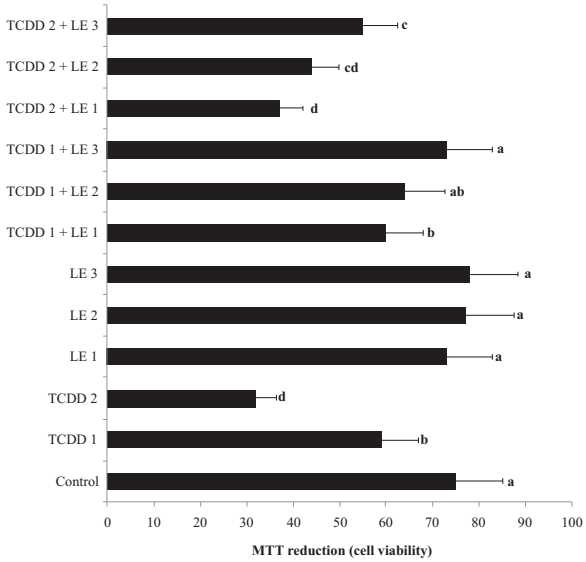

Figure 1 *Cell viability in rat hepatocyte cultures maintained 48 h in the presence of TCDD, LE and their combinations.*
TCDD 1: 1.61 mg L^{-1} TCDD; TCDD 2: 3.22 mg L^{-1} TCDD; LE 1: 50 mg L^{-1} of laurel leaf extract; LE 2: 100 mg L^{-1} of laurel leaf extract; LE 3: 200 mg L^{-1} of laurel leaf extract; means of five measurements marked by different letters differ significantly ($P<0.05$).

TCDD-induced hepatocellular damage was clearly evidenced by increases in LDH levels compared to controls. LE alone did not affect LDH activity (Figure 2). Moreover, at the concentrations of 100 mg L^{-1} and 200 mg L^{-1}, LE significantly reduced TCDD toxicity.

Table 1 shows the effects of LE on biochemical parameters in all experimental groups. Hepatic TAC levels decreased ($p<0.05$) in TCDD-treated groups while TOS increased. In contrast, cells treated with LE alone at 100 mg L^{-1} and 200 mg L^{-1} showed higher antioxidant capacity than other groups but TOS levels were similar to control. Moreover, LE at 100 mg L^{-1} and 200 mg L^{-1} significantly ($p<0.05$) increased the

Table 1 *Extracellular TAC and TOS levels in cultured rat hepatocytes incubated with TCDD and/or LE and in control culture. TCDD 1: 1.61 mg L^{-1} TCDD; TCDD 2: 3.22 mg L^{-1} TCDD; LE 1: 50 mg L^{-1} of laurel leaf extract; LE 2: 100 mg L^{-1} of laurel leaf extract; LE 3: 200 mg L^{-1} of laurel leaf extract; means of five measurements marked by different letters differ significantly (P<0.05).*

Treatments	TAC Trolox Equiv. / mmol L^{-1}	TOS H_2O_2 Equiv. / µmol L^{-1}
Control	5.1±0.5^{b}	8.3±2.1^{a}
TCDD 1	4.1±0.6^{c}	11.4±2.6^{c}
TCDD 2	3.4±0.4^{d}	16.4±2.9^{e}
LE 1	5.0±0.5^{b}	8.0±2.8^{a}
LE 2	5.6±0.7^{a}	8.4±2.7^{a}
LE 3	5.8±0.6^{a}	8.6±2.1^{a}
TCDD 1 + LE 1	4.2±0.5^{c}	11.0±2.9^{c}
TCDD 1 + LE 2	4.6±0.7^{c}	10.1±3.2^{b}
TCDD 1 + LE 3	5.1±0.4^{b}	9.4±2.5^{a}
TCDD 2 + LE 1	3.5±0.4^{d}	15.9±3.1^{e}
TCDD 2 + LE 2	3.9±0.5^{cd}	13.7±3.0^{d}
TCDD 2 + LE 3	4.2±0.5^{c}	13.0±2.6^{d}

TAC level compared to hepatocytes treated with TCDD alone.

Table 2 shows the frequency of micronucleated rat hepatocytes. Compared to control, TCDD statistically increased their frequency, while LE did not affect it. Moreover, it countered the TCDD effects in a clear dose-dependent manner.

Table 2 *Percentage of micronucleated rat hepatocytes in cultures incubated with TCDD and/or LE and in control culture.*
HEP = hepatocyte; MN cells = percentage of micronucleated hepatocytes per 2000 cells. TCDD 1: 1.61 mg L^{-1} TCDD; TCDD 2: 3.22 mg L^{-1} TCDD; LE 1: 50 mg L^{-1} of laurel leaf extract; LE 2: 100 mg L^{-1} of laurel leaf extract; LE 3: 200 mg L^{-1} of laurel leaf extract; means marked by different letters differ significantly (P<0.05).

Treatments	MN cells /%	Range / %
Control	0.3±0.1^{a}	0.2 to 0.4
TCDD 1	0.9±0.3^{c}	0.7 to 1.3
TCDD 2	1.7±0.4^{d}	1.5 to 2.3
LE 1	0.2±0.1^{a}	0.1 to 0.3
LE 2	0.3±0.1^{a}	0.2 to 0.4
LE 3	0.3±0.1^{a}	0.2 to 0.4
TCDD 1 + LE 1	0.9±0.2^{c}	0.7 to 1.4
TCDD 1 + LE 2	0.6±0.3^{b}	0.4 to 0.8
TCDD 1 + LE 3	0.5±0.2^{b}	0.4 to 0.7
TCDD 2 + LE 1	1.6±0.4^{d}	1.3 to 2.0
TCDD 2 + LE 2	1.3±0.4^{cd}	1.0 to 1.7
TCDD 2 + LE 3	1.1±0.2^{c}	0.8 to 1.4

DISCUSSION

According to Hassoun et al. (20) and Jin et al. (21), TCDD increases the production of reactive oxygen species (ROS) and LPO (20-21) in various tissues. This might be due to the inhibition of antioxidant enzyme activity (21). In contrast, Speroni et al. (8) have reported that LE exhibits high anti-radical activity. In fact it can restore the oxidant/antioxidant balance and lessen the destruction of cell membranes, proteins, and DNA, as has been confirmed for other plants such as duckweed or dandelion (22, 23). Our results corroborate this antioxidant activity of laurel, also observed by Kaurinović et al. (24) and Conforti et al. (25); LE alone (at the concentrations of 100 mg L^{-1} and 200 mg L^{-1}) significantly increased TAC. In addition, supplemented to TCDD (1.61 mg L^{-1}), LE restored TAC to control

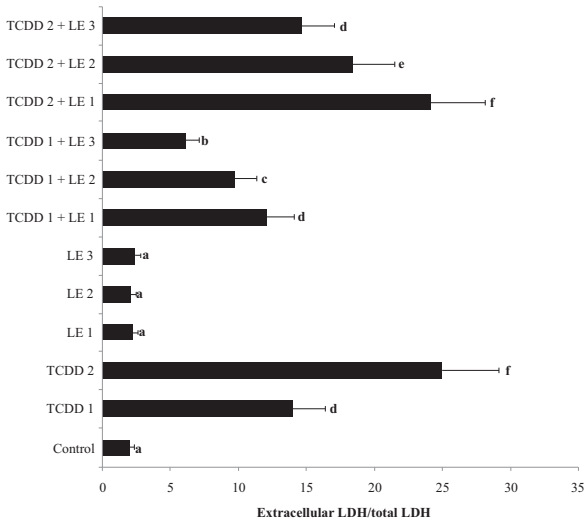

Figure 2 *Extracellular level of LDH (as mU mL^{-1}) in rat hepatocyte cultures maintained 48 h in the presence of TCDD, LE and their combinations. For abbreviations see legend in Figure 1.*

levels. With higher LE doses the TAC increased by 2.9 % to 24.3 %. Total antioxidant capacity combines the activities of non-enzymes such as glutathione (GSH) and enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) (26). The most abundant intracellular antioxidant, GSH, lowers endogenous ROS and/or exogenous oxidative damage in animal cells (27). GSH-Px enzyme metabolises peroxides such as H_2O_2 and protects cell membranes from LPO (28-29). According to Ralph et al. (30), amino acids may protect against toxicity by serving as precursors for GSH. GSH becomes depleted if cells are deprived of energy substrates. By providing an energy source and by acting as a precursor to GSH, LE may increase GSH content of hepatocytes and protect them from toxic substances. Recent reports also confirmed the neuroprotective property of LE (31).

Anti-oxidative therapy has an important role in preventing hepatic damages (32-34). Dall'Acqua et al. (35) found ten different flavonoid O-glycosides, one flavonoid C-glycoside, catechin and cinnamtannin B_1 in laurel leaf. All these compounds have antioxidant properties (35-37).

TCDD exposure is associated with increased production of ROS, lipid peroxidation, and DNA and membrane damage (38). ROS can alter vital cell components like polyunsaturated fatty acids, proteins, and nucleic acids (39). In our study, TCDD exposure increased the frequency of micronucleated rat hepatocytes. MN cell frequency lowered by LE in TCDD-treated cells may point to antioxidant activity in oxidative DNA damage (40). In a study by Ganguly (41), LE was a potent oxygen radical scavenger that improved carcinoma prognosis. Al-Kalaldeh et al. (42) evidenced antiproliferative activity of laurel extracts against human breast adenocarcinoma cells. Loizzo et al. (43) reported anticancer properties of LE *in vitro* against human amelanotic melanoma C32, renal cell adenocarcinoma ACHN, hormone-dependent prostate carcinoma LNCaP, and MCF-7 breast cancer. In addition, Hibasami et al. (44) isolated costunolide and zaluzanin D from laurel that proved to be effective against leukaemia HL-60 cells.

In conclusion, our findings encourage the use of LE in diet as prevention of TCDD toxicity.

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Sažetak

BLAGOTVORNO DJELOVANJE EKSTRAKTA LOVOROVA LISTA NA HEPATOTOKSIČNOST U ŠTAKORA UZROKOVANU 2,3,7,8-TETRAKLORDIBENZO-*P*-DIOKSINOM *IN VITRO*

2,3,7,8-tetraklordibenzo-p-dioksin (TCDD) vrlo je toksičan onečišćivač okoliša koji izaziva veliku zabrinutost zbog mogućih posljedica za zdravlje ljudi. Nedavna su ispitivanja pokazala da ekstrakt lovorova lista (*Laurus nobilis* L.) ima antioksidacijska svojstva koja ublažavaju hepatotoksičnost. Cilj je ovog ispitivanja bio istražiti djelotvornost ekstrakta lovorova lista protiv toksičnoga djelovanja TCDD-a na primarnoj kulturi hepatocita. Kulturama je ekstrakt dodan u dozama od 50 mg L^{-1}, 100 mg L^{-1} odnosno 200 mg L^{-1} sam ili zajedno s TCDD-om u dozama od 1,61 mg L^{-1} odnosno 3,22 mg L^{-1}. Izloženost je u oba slučaja trajala 48 h. Zatim je ispitana vijabilnost hepatocita s pomoću MTT-testa i testa na laktat dehidrogenazu (LDH). Oksidativno oštećenje mjerili smo s pomoću ukupnog antioksidativnoga kapaciteta i ukupnoga oksidativnog stresa. Oštećenje DNA hepatocita procijenjeno je s pomoću mikronukleus testa. MTT i LDH pokazali su da TCDD smanjuje vijabilnost hepatocita, ali ne i ekstrakt lovorova lista. TCDD je također povisio razinu ukupnoga oksidativnog stresa te smanjio ukupni antioksidativni kapacitet. Broj mikronukleusa rastao je s dozom TCDD-a. Nasuprot tomu, u hepatocitima izloženim samo ekstraktu lovorova lista nisu izmijenjene razine ukupnoga oksidativnog stresa, dok je ukupni antioksidativni kapacitet značajno rastao s dozom. Ekstrakt lovorova lista usto je ublažio toksično djelovanje TCDD-a na hepatocite u primarnoj kulturi. Stoga lovorov list štiti protiv oštećenja DNA uzrokovanoga TCDD-om. To ukazuje na mogućnost terapijske primjene ekstrakta lovorova lista protiv toksičnoga djelovanja TCDD-a u jetri.

KLJUČNE RIJEČI: *antioksidativni kapacitet, genotoksičnost, hepatoprotektivnost*, *vijabilnost stanica*

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