PROTECTIVE ROLE OF SELENIUM AGAINST OVER-EXPRESSION OF CANCER-RELATED APOPTOTIC GENES INDUCED BY *O*-CRESOL IN RATS

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Cresols are monomethyl derivatives of phenol frequently used as solvents and intermediates in the production of disinfectants, fragrances, pesticides, dyes, and explosives, which is probably why they are widely distributed in the environment. General population may be exposed to cresols mainly through inhalation of contaminated air. In this study we evaluated the toxicological effects of *o*-cresol on differential gene expression profile of rat liver and prostate. Experiments were conducted on 80 male rats, 60 of which were exposed to *o*-cresol (1.5 g kg^{-1}, 5 g kg^{-1}, or 15 g kg^{-1}) through feed for 8 weeks. Three groups of rats were supplemented with 0.1 mg kg^{-1} selenium (Se, in the form of, sodium selenite) in addition to *o*-cresol to evaluate its effectiveness against *o*-cresol toxicity. Control group received neither o-cresol nor Se, while one group received Se alone. Survival was similar between the exposed and control animals. Rats exposed to 15 g kg^{-1} of *o*-cresol showed a 16 % loss in body weight by the end of the study, which may have been related to *o*-cresol making feed unpalatable at this concentration. Liver and prostate tissue samples were collected at the end of the treatment. mRNA analysis revealed that apoptotic genes (CYP3A, COX-2, PPARγ, BAX, BCL2 AKT-1, and PKCα) related to cancer were up-regulated in liver and prostate tissues isolated from groups exposed to 5 g kg^{-1} and 15 g kg^{-1} *o*-cresol in comparison to control. Changes in gene expression profile were prevented when rats were supplemented with Se. The exact mechanisms underlying its protective effect remain to be clarified by future studies.

KEY WORDS: *apoptotic genes, phenol derivatives, RT-PCR, trace element*

Cresols are monomethyl derivatives of phenol that come in three isomers: ortho, meta, and para. In nature, cresols occur in some plant oils and are by-products of burning natural materials such as cigarettes, petroleum fuel, wood and coal (1). Commercially, cresols are either synthesised or distilled from petroleum or coal tar (2). They are used in a wide variety of products such as disinfectants, preservatives, dyes, fragrances, herbicides, insecticides, explosives, resin, rubber, polymers, elastomers, and food.

High production and distribution of cresols in the environment increase the risk of widespread exposure for humans. They have been found in food and drink (3, 4), air, sediment, soil, and water (5-9). According to the US EPA, air cresol levels are about 32 ng m^{-3}, rising to 1.5 µg m^{-3} for *o*-cresol in the vicinity of intense traffic and gas stations. In soil and groundwater these levels can be as high as 55 mg kg^{-1} and 16 mg L^{-1}, respectively (10).

Several regions in Egypt such as El-Mahalla El-Kubra and Kafr El-Dawar cities have seen an industrial boom over the last decade (11). El-Mahalla El-Kubra is famous for textile dyeing and printing, but is also known for a water pollution problem with cresols and other contaminants, which threaten to enter the food chain and affect a larger fish population.

Routes of cresol exposure (including metabolites and phenolic compounds such as toluene) for humans include inhalation, skin, or mouth (12-14). Cresols can irritate the respiratory tract (1) and affect the skin of rabbits (15) and humans exposed to cresol-containing products (1, 16).

The carcinogenicity of cresols has yet to be determined. Boutwell and Bosch (17) observed increased incidence of skin papillomas in mice. Similarly, Yanysheva et al. (18) observed increased incidence of forestomach papillomas and carcinomas in mice. In contrast, Sanders et al. (19) found no clear evidence of cresol carcinogenicity in male rats and female mice.

Long and intense exposure to *o*-cresol may alter gene expression either directly or indirectly and lead to cancer. Only a few studies have investigated the genotoxicity of cresol, and none has investigated its effects on the expression of apoptotic genes associated with cancer development. Our study was therefore the first to investigate how exposure to *o*-cresol affected the expression of CYP3A, COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα, using reverse transcription polymerase chain reaction *(*RT-PCR). These have been established as reliable markers of carcinogenicity (20-24). In addition, we evaluated the protective effect of selenium against *o*-cresol-induced genetic alterations, whose antioxidative role has been well documented (25-33).

MATERIALS AND METHODS

Chemicals

Reagents and solvents used in this study were of the highest purity. *o*-cresol was purchased from Sigma-Aldrich (St. Louis, USA). Selenium was purchased from Merck-Schuchardt Chemical Co. (Darmstadt, Germany). Reagents for RT-PCR were purchased from Invitrogen (Paisley, UK) and Fermentas (Leon-Rot, Germany).

Experimental Animals

Eighty adult male albino rats weighing 120 g to 140 g were obtained from the Animal House Colony, National Research Centre, Giza, Egypt. The animals were kept individually in wire-bottom cages at room temperature (25±2) °C under the 12-hour dark-light cycle. They were receiving standard laboratory diet and water *ad libitum*. The animals were allowed to adjust to new conditions for one week before the experiment started. All received care in compliance with the guidelines of the Ethics Committee of Medical Research, National Research Centre, Giza, Egypt.

Experimental design

After one-week adjustment to new conditions, the animals were divided in eight groups with ten rats in each, as follows: group 1 - untreated control ; groups 2, 3, and 4 - animals receiving basic diet (34) with 1.5 g kg^{-1}, 5 g kg^{-1}, or 15 g kg^{-1} of *o*-cresol, respectively; group 5 - animals receiving basic diet without *o*-cresol but supplemented intragastrically with 0.1 mg kg^{-1} Se as sodium selenite; and groups 6 to 8 - animals receiving the same as groups 2, 3, and 4 plus 0.1 mg kg^{-1} Se. The experiment lasted eight weeks. The highest exposure concentrations of *o*-cresol were based on observations of minimal toxicity at these levels in previous cresol studies (35).

During the experiment, the animals were checked for morbidity and mortality twice a day. Body weights were recorded at baseline, every week, and at the end of the experiment, when the animals were euthanised and liver samples dissected and thoroughly washed with isotonic saline. Prostate tissues (seminal vesicles, prostate lobes, and bladder) were also removed and placed in a Petri dish containing PBS buffer (137 mmol L^{-1} NaCl, 3 mmol L^{-1} KCl, 4 mmol L^{-1} Na$_2$HPO$_4$, and 1.5 mmol L^{-1} KH$_2$PO$_4$, pH 7.4) to prevent desiccation. The bladder was emptied, and the tissues gently blotted to remove excess PBS. Tissues were then snap-frozen in liquid nitrogen and stored at -80 °C until analysis.

Semi-quantitative RT-PCR

First-strand cDNA synthesis from extracted rat RNA

Total RNA (Poly(A)$^+$ RNA) was extracted from 50 mg of liver and prostate tissues using the standard TRIzol extraction method (Invitrogen, Paisley, UK) and recovered in 100 µL diethylpyrocarbonate (DEPC)-treated water by passing the solution a few times through a pipette tip.

Total RNA was treated with one unit of RQ1 RNAse-free DNAse (Invitrogen, Karlsruhe, Germany) to digest DNA residues, re-suspended in DEPC-treated water, and quantified photospectrometrically at 260 nm. Total RNA was assessed for purity from the ratio between quantifications at 260 nm and 280 nm,

and was between 1.8 and 2.1. Integrity was verified with the ethidium bromide-stain analysis of 28S and 18S bands using formaldehyde-containing agarose gel electrophoresis. Aliquots were either used immediately for reverse transcription (RT) or stored at -80 °C.

To synthesise first-strand cDNA, 5 µg of complete Poly(A)$^+$ RNA was reverse transcribed into cDNA in a total volume of 20 µL using 1 µL oligo [poly(deoxythymidine)$_{18}$] primer (36). The composition of the reaction mixture was 50 mmol L^{-1} MgCl$_2$, 10x RT buffer (50 mmol L^{-1} KCl; 10 mmol L^{-1} Tris-HCl; pH 8.3), 200 U µL^{-1} reverse transcriptase (RNase H free, Fermentas, Leon-Rot, Germany), 10 mmol L^{-1} of each dNTP, and 50 µmol L^{-1} of oligo(dT) primer. RT reaction was carried out at 25 °C for 10 min, followed by 1 h at 42 °C, and completed with denaturation at 99 °C for 5 min. Reaction tubes containing RT preparations were then flash-cooled in an ice chamber until used for DNA amplification through polymerase chain reaction (PCR) (37).

RT-PCR assay

First-strand cDNA was used as a template for semi-quantitative RT-PCR with a pair of specific primers in a 25-µL reaction volume. The sequences of specific primer and product sizes are listed in Table 1.

β-Actin was used to normalise mRNA levels of the target genes. The reaction mixture for RT-PCR consisted of 10 mmol L^{-1} dNTP's, 50 mmol L^{-1} MgCl$_2$, 10x PCR buffer (50 mmol L^{-1} KCl; 20 mmol L^{-1} Tris-HCl; pH 8.3), 1 U µL^{-1} taq polymerase, and autoclaved water. Table 1 lists the specific gene primer sequences and PCR cycling conditions. PCR products derived from β-actin were then loaded onto 2.0 % agarose gel. Each RT-PCR was repeated for each rat, generating at least ten new cDNA products per group.

Statistical Analysis

All data were analysed with one-way analysis of variance using SAS general linear models procedure (38) followed by Scheffé's test to assess differences between groups. The values are expressed as mean ± SEM. The level of significance was set at $P<0.05$.

RESULTS

Rat survival and body weight

There were no significant differences in the survival rate between the control and *o*-cresol-exposed

Table 1 *Primers and PCR thermocycling parameters*

| Gene	Primer sequence (5´-3´)	Conditions of the PCR assay	PCR amplicons (bp)
CYP3A	GAA GCA TTG AGG AGG ATC AC GGG TTG TTG AGG GAA TCC AC	25 cycles: 94 °C, 40 s; 54 °C, 40 s; 72 °C, 45 s Final extension: 72 °C, 5 min	376
COX2	CTG TAT CCC GCC CTG CTG GTG ACT TGC GTT GAT GGT GGC TGT CTT	25 cycles: 94 °C, 30 s; 65 °C, 30 s; 68 °C, 1 min Final extension: 68 °C, 2 min	279
PPARγ	TGT GTG ACA GGA AAC AGC TAT GAC CAT G ATG CAG TTT CGT AAA ACG ACG GCC AGT	30 cycles: 94 °C, 30 s; 65 °C, 30 s; 72 °C, 1 min Final extension: 72 °C, 2 min	384
BAX	ACA AAG ATG GTC ACG GTC TGC C GGT TCA TCC AGG ATC GAG ACG g	25 cycles: 94 °C, 30 s; 65 °C, 30 s; 68 °C, 1 min Final extension: 68 °C, 2 min	429
BCL2	CTC AGT CAT CCA CAG GGC GA AGA GGG GCT ACG AGT GGG AT	25 cycles: 94 °C, 30 s; 65 °C, 30 s; 68 °C, 1 min Final extension: 68 °C, 2 min	450
AKT-1	TAC CTG AAG CTA CTG GGC AAG GAG AAG AAG GTG GAC AGA GCA	35 cycles: 93 °C, 30 s; 56 °C, 45 s; 74 °C, 45 s Final extension: 74 °C, 10 min	270
PKCα	TGA ACC CTC AGT GGA ATG AGT GGC TGC TTC CTG TCT TCT GAA	30 cycles: 93 °C, 30 s; 60 °C, 45 s; 74 °C, 45 s Final extension: 74 °C, 10 min	325
β-Actin	GTG GGC CGC TCT AGG CAC CAA CTC TTT GAT GTC ACG CAC GAT TTC	25 cycles: 94 °C, 30 s; 65 °C, 30 s; 68 °C, 1 min Final extension: 68 °C, 2 min	540
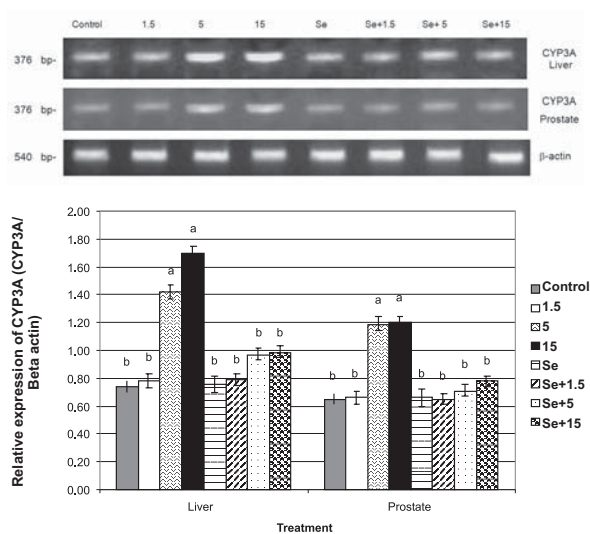
Figure 1 *Semi-quantitative RT-PCR confirmation of CYP3A gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05).*

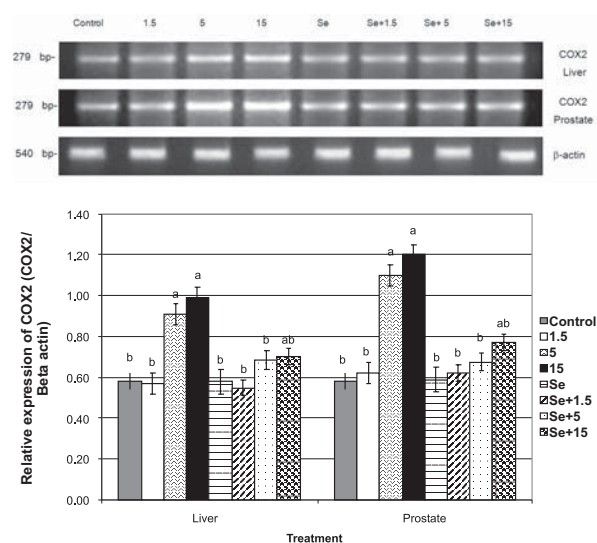**Figure 2** *Semi-quantitative RT-PCR confirmation of COX-2 gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05), but neither "a" or "b" are significantly different from "ab".*
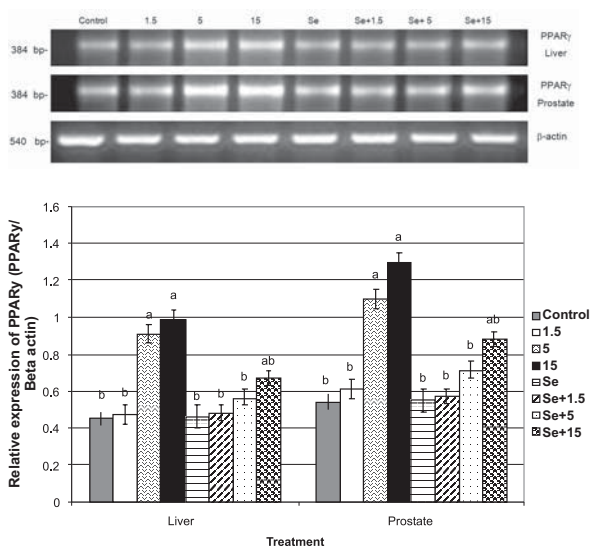

Figure 3 *Semi-quantitative RT-PCR confirmation of PPARγ gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05), but neither "a" or "b" are significantly different from "ab".*

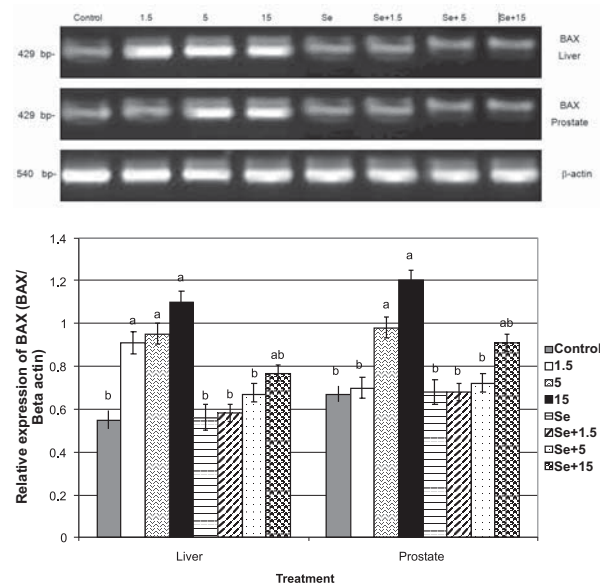**Figure 4** *Semi-quantitative RT-PCR confirmation of BAX gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05), but neither "a" or "b" are significantly different from "ab".*

groups. Approximately 91 % of the animals (range 87 % to 97 %) survived to the end of the study.

Mean body weights of rats receiving 1.5 g kg⁻¹ (group 2) and 5 g kg⁻¹ *o*-cresol (group 3) did not significantly differ from control over time. However, mean body weight of rats exposed to 15 g kg⁻¹ *o*-cresol (group 4) was only 84 % of control by the end of the

study (446 g *vs.* 498 g; P<0.05). The difference in mean weight gain was apparent within one week of the study (131 g *vs.* 142 g). Feed consumption in group 4 was lower than in controls in the first week (10.4 g day⁻¹ *vs.* 17.4 g day⁻¹), but recovered to near control levels by the second week (16.1 g day⁻¹ *vs.* 17.2 g day⁻¹).
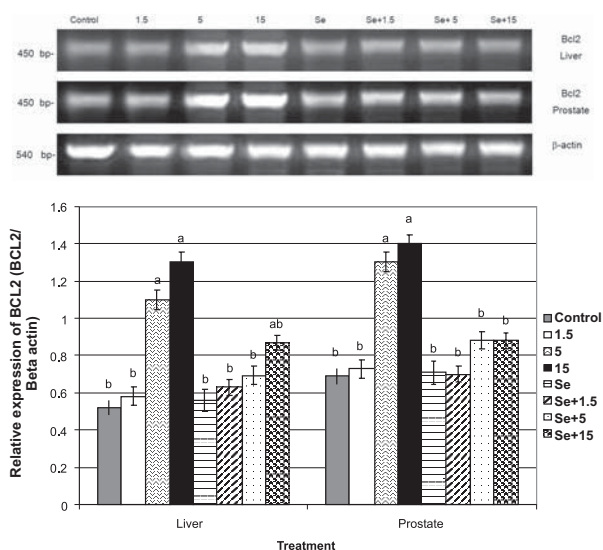
Figure 5 *Semi-quantitative RT-PCR confirmation of BCL2 gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05), but neither "a" or "b" are significantly different from "ab".*

Figure 6 *Semi-quantitative RT-PCR confirmation of AKT-1 gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05), but neither "a" or "b" are significantly different from "ab".*

Gene expression

CYP3A gene was more expressed in the liver than in prostate tissues. In contrast, COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα genes were more expressed in prostate tissues than in the liver.

In groups 3 and 4, liver CYP3A was significantly higher than in control and other treated groups, while exposure to low-dose *o*-cresol (group 2) did not increase CYP3A expression in comparison to the control group (Figure 1). Treatment with Se (group 5) did not increase CYP3A expression in the liver and prostate tissues and it remained similar to control group (Figure 1). Moreover, Se lowered CYP3A over-expression induced by 5 g kg⁻¹ (group 7) and 15 g kg⁻¹ *o*-cresol (group 8) in the liver and prostate tissue (Figure 1).

Groups 3 and 4 also showed increased expression of COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα genes (Figures 2-7), while group 2 remained within the control range, save for BAX, whose expression in the liver was significantly higher than in control (Figure 4).

In group 5 on Se alone, COX-2, PPARγ, BAX, BCL2, AKT-1 and PKCα expression in the liver and prostate tissues was similar to control (Figures 2-7). Se significantly to lowered COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα over-expression in the liver and prostate tissues of group 7 (Figures 2-7) and significantly lowered BCL2, AKT-1, and PKCα in
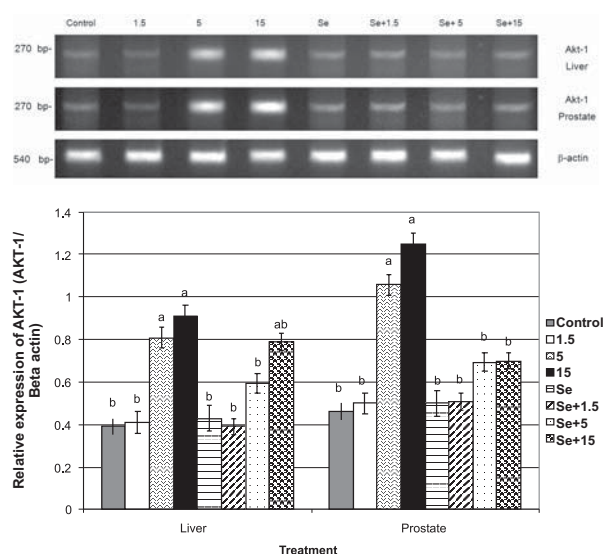
prostate tissues of group 8 (Figures 5-7). It also significantly lowered over-expression of PKCα in the liver of group 8 (Figure 7). However, it could not significantly lower the over-expression of COX-2, PPARγ, and BAX in the liver of the most exposed group 8 (Figures 2-4).

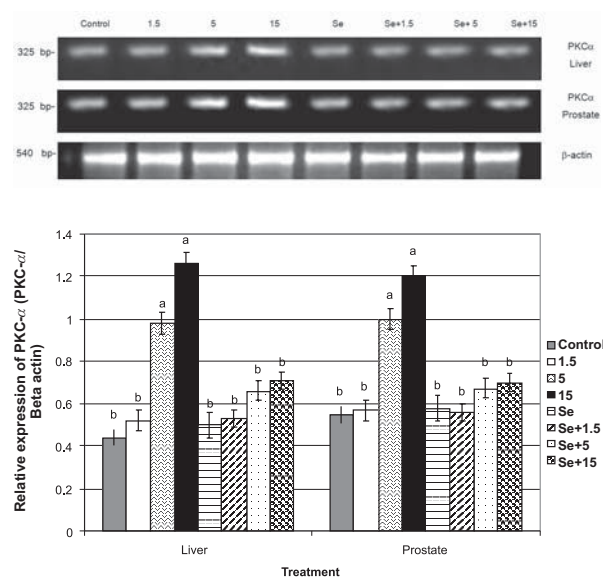
Figure 7 *Semi-quantitative RT-PCR confirmation of PKCa gene in male rats treated with o-cresol (1.5 g kg⁻¹, 5 g kg⁻¹, or 15 g kg⁻¹) with or without selenium (0.1 mg kg⁻¹) for 8 weeks. "a" is significantly different from "b" (P<0.05).*

DISCUSSION

This study was conducted to determine the expression of cancer-related apoptotic genes in male rats exposed to o-cresol in order to fill the gap in data on chronic cresol toxicity that may be relevant for human exposure.

While *o*-cresol did not affect survival, animals with the highest exposure (15 g kg^{-1}) ate less in the first week, which eventually reflected on their body weight, even though they returned to normal feeding by the second week. This is in agreement with the findings by Sanders et al. (19), whose rats exposed to a 15 g kg^{-1} mixture of *m*- and *p*-cresols had a significantly lower body weight than controls in the first week of exposure. These results were attributed to unpalatability of the feed containing high amounts of cresols.

Cresols are known respiratory irritants in humans and animals (2) and concentrations higher than 15 g kg^{-1} would probably have irritated the respiratory and other biological membranes of our rats.

Our study confirmed that *o*-cresol affects the expression of apoptotic genes that are indicators of cancer development. CYP3A was higher in the liver than in prostate tissues, but COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα were higher in prostate tissues than in the liver. Similarly, Lehman-McKeeman et al. (39) found that exposure of mice to environmental xylene increased CYP3A mRNA level in the liver. In addition, Badawi et al. (40) reported that over-expression of COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα genes was responsible for prostate cancer in aged rats.

Only a few studies have investigated the carcinogenic potential of cresols. Boutwell and Bosch (17) and Yanyscheva et al. (18) have suggested that cresols promote tumours in rodents. Villard et al. (41) found that cytochrome P450 isozymes CYP1A, CYP2B, CYP2E, and CYP3A were involved in liver and lung carcinogenesis in mice exposed to cigarette smoke.

Some believe that prostate cancer is associated with altered hormonal milieu (42, 43), lowered apoptotic potential (44-46), lowered antioxidant enzyme activity (47), and accumulation of DNA damage (48). Although these changes are all implicated in carcinogenesis, the precise mechanism underlying prostate cancer development remains unclear. According to Badawi et al. (20), one of key players in prostate cancer is cyclooxygenase-2 (COX-2), which can serve as an early predictor of cancer risk.

Selenium, on the other hand, was able to inhibit over-expression of CYP3A, COX-2, PPARγ, BAX, BCL2, AKT-1, and PKCα genes in rats exposed to 5 g kg^{-1} and 15 g kg^{-1} *o*-cresol. Selenium is involved in antioxidant defence (25) and is associated with anti-cancer activity (28, 29). Legg et al. (49) reported that high dietary intake of Se reduces prostate cancer risk.

Cellular glutathione peroxidase (GPx) is the first identified selenoprotein and there is a strong correlation between blood Se and GPx activity (50). GPx acts as an antioxidant by directly reducing H$_2$O$_2$, and lipid hydroperoxides cleaved by phosphor-lipase A2 (50). Therefore, inhibited over-expression of CYP3A, COX2, PPARγ, BAX, BCL2, AKT-1, and PKCα in our study may be related to higher activity of GPx and/or other antioxidant enzymes in male rats treated with Se. This remains to be verified or rejected by future studies, which should look deeper into the mechanisms underlying the protective effect of Se.

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Sažetak

ZAŠTITNO DJELOVANJE SELENIJA PROTIV PREKOMJERNE EKSPRESIJE APOPTOTSKIH GENA POVEZANIH S KARCINOMOM U ŠTAKORA IZLOŽENIH *O*-KREZOLU

Krezoli su monometilni derivati fenola koji se često rabe kao otapala te kao posrednici u proizvodnji dezinfekcijskih sredstava, mirisa, pesticida, boja i eksploziva. Otuda i njihova rasprostranjenost u okolišu. Opća je populacija izložena krezolima uglavnom putem zraka.

U ovome se toksikološkom istraživanju ocijenilo djelovanje *o*-krezola, jednoga od tri krezolova izomera, na ekspresiju gena u tkivima jetre i prostate mužjaka štakora. Istraživanje je provedeno na 80 mužjaka, od kojih je 60 tijekom osam tjedana bilo izloženo *o*-krezolu (1,5 g kg^{-1}, 5 g kg^{-1}, odnosno 15 g kg^{-1}) preko krmiva. Tri skupine štakora primale su uz o-krezol nadomjestak selenija u dozi od 0.1 mg kg^{-1} (Se, u obliku natrijeva selenita) radi ocjene njegove djelotvornosti protiv toksičnosti *o*-krezola. Kontrolna skupina nije primala ni *o*-krezol ni Se, dok je jedna skupina primala samo Se. Preživljenje je bilo podjednako u svih skupina životinja. Štakori izloženi najvišoj dozi *o*-krezola (15 g kg^{-1}) imali su 16 % manju tjelesnu masu od kontrolne skupine na kraju ispitivanja, što može biti povezano s lošim okusom krmiva zbog primjese visoke doze *o*-krezola.

S istekom osmotjednoga izlaganja o-krezolu životinje su eutanazirane te su prikupljeni uzorci tkiva jetre i prostate. Analiza m-RNA pokazala je značajno povišenu ekspresiju apoptotskih gena CYP3A, COX-2, PPARγ, BAX, BCL2, AKT-1 i PKCα, koji su povezani s nastankom karcinoma u skupinama štakora izloženim *o*-krezolu (5 g kg^{-1} i 15 g kg^{-1} u odnosu na kontrolu. Ova je prekomjerna ekspresija poništena u štakora koji su primali selenij. Još nisu jasni mehanizmi iza ovoga zaštitnog djelovanja, na što će odgovoriti buduća istraživanja.

KLJUČNE RIJEČI: *apoptotski geni, derivati fenola, RT-PCR, elementi u tragovima*

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