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Effects of 2,4-Dichlorophenoxyacetic acid (2,4-D) treatment on the epididymal spermatozoa, blood serum transaminases and its accumulation in liver of rats

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Summary

Phenoxyacetic acid herbicides constitute one of the largest groups of herbicides used in the world. The study was carried out to investigate the effect of herbicide 2,4-D (2,4-Dichlorophenoxyacetic acid) on some blood parameters and toxicity of the male reproductive system of Sprague Dawley (CD) rats. The level of 2,4-D in the liver and its metabolite: 2,4-DCP was ascertained using the HPLC method and the organ weight of the livers was also determined. Three different concentrations of pesticide were used. The animals were treated orally 25 ppm and 50 ppm with water and 50 ppm and 100 ppm with food for 30 days. No significant difference was found in the blood parameters between the groups. The level of 2,4-D in the liver was found to be significantly higher in both feed and water groups compared to those of the control group ($p<0.01$). The level of 2,4-DCP in the liver also increased in all the experimental groups compared to control ($p<0.01$) groups, with the exception of the water group which had the lowest concentration. As far as the abnormal spermatozoa rates of rats were concerned, the values of the experimentally fed groups were higher than the control group and the difference between them was statistically significant ($p<0.01$).

Keywords: herbicide, rat, liver, sperma

2,4-Dichlorophenol (2,4-DCP) is used in the production of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), and it is one of the abundant chlorophenols in the aquatic environment. These pollutants, which are widely distributed in nature, have detrimental biological effects, including chronic toxicity, mutagenicity and carcinogenicity (18). 2,4-DCP is also utilized in the manufacture of methylated chlorophenols that are used for mothproofing, in antiseptics and disinfectants (1). Because of this great ubiquity, 2,4-DCP, together with other chlorophenols, was listed as early as 1978, among 129 priority pollutants in 65 classes by the US Environmental Protection Agency (10).

In recent years, there has been increasing interest in the toxicology of the male reproductive system. Controversial reports on reproductive disorders associated with the exposure of males to occupational toxicants has partly triggered this interest (2, 6, 13). Increasing reproductive adverse effect observed after the Vietnam War has been considered as a result of exposure to Agent White which is a herbicide having 2,4-D among the active ingredients (15). However, there is no evidence on the relation of the exposure to 2,4-D and reproductive system disorders.

The exact mechanisms related to the toxic effects of 2,4-D remain obscure (14). Although it's been claimed that this herbicide has not accumulated in environment, food chain and body (3) there is some evidence in relation to accumulation in animal tissues (12). Our previous study (4) in rat which treated with 2,4-D in drinking water and food at levels of 25, 50 and 100 ppm for a period of 30 days to determine accumulation with subchronic exposure showed that 2,4-D and 2,4-DCP has accumulated in kidneys at low levels.

In the present study, it was investigated the subchronic effects of orally administered 2,4-D on sperm morphology and serum AST and ALT levels (blood parameters) and accumulation levels of 2,4-D and its metabolite in liver.

Material and methods

Animals and treatments. Forty, eight week old Sprague Dawley (CD) rat were obtained from the Istanbul University, Cerrahpaşa Faculty of Medicine, Experimental Animals Production and Investigation Centre Rats (130-140 g) were acclimated for 1 week prior to treatment under usual management conditions (20 ± 2°C and $50 \pm$ % 5 humidity). Rats were randomly divided into 5 (five) groups consisting 8 animals each, 4 groups used as treatment groups and 1 group left as control.

Experimental design. Group I (n = 8): treated with 25 ppm 2,4-D in drinking water for 30 days, group II (n = 8): treated with 50 ppm 2,4-D in drinking water for 30 days, group III (n = 8): treated with 50 ppm 2,4-D in food for 30 days, group IV (n = 8): treated with 100 ppm 2,4-D in food for 30 days, control group (n = 8): fed on pelleted and drinking water for 30 days. Treated food and water were freshly prepared and given each morning. After the 30 days treatment period, the rats were sacrificed by anaesthetic (diethyl ether) overdose followed by cervical dislocation and tissue samples and blood were collected.

Spermatological morphology. The cauda epididymis of each animal was dissected from surrounding tissues and milked into 500 µl Sp-TALP medium. Abnormal spermatozoon rates were determined on a glass slide preparing and a thin smear staining by Spermac® stain (Stain Enterprise, Republic of South Africa). Morphological defect rate was evaluated by counting 200 cells under a light microscope immersion objective (× 1000).

Apparatus and reagents. Analysis were done using the Shimadzu LC-10A liquid chromatography equipment at a wavelength of 235 nm, which had a UV detector and 20 µl volume for all standards and final extracts of liver samples. The mobile phase was a mixture of 4% acetic acid/acetonitryl (60 : 40). The Hypersil ODS (125 × 4 mm, 5 µm) coloumn was used in the separation of the compounds. Flow rate was adjusted to 1 ml/min. (pressure about 2100 psi) and the temperature was ambient. Acetonitryl, methanol, diethyl ether (HPLC grade), hydrochloric acid, 2,4-dichlorophenoxyacetic acid and 2,4-dichlorophenol were purchased from Merck (Darmstadt, Germany), Spermac® colour (Stain Enterprise, Republic of South Africa). 2,4-D and 2,4-DCP stock solutions (0.1 mg/ml) were prepared by dissolving in bidistilled water.

Extraction of the 2,4-D and its metabolite 2,4-DCP for HPLC. Liver (1 g) homogenate was mixed 10 ml of distilled water and 1ml 1 N HCl. To this was added 30 ml diethyl ether followed by shaking for half an hour and centrifuged at 20°C and 4000 rpm for 15 min. The supernatant was removed. This procedure was repeated 3 times. The diethyl ether collected was completely evaporated to dryness at 40°C under a stream of nitrogen gas. The C-18 SPE (IST, Mid Glamorgan, UK) cartridge was drained with a vacuum manifold system, washed first with 3 ml methanol and then 10 ml 4% acetic acid. A 20 µl volume of acidic solution was injected into the HPLC.

Statistics. All calculations and statistical analyses were generated in SPSS for windows version 10,0 (SPSS Inc. Chicago, IL, USA). Statistical analyses were performed using analyses of variance (ANOVA) followed by Duncan test. Differences were considered to be significant at $p < 0.01$ and $p < 0.05$.

Results and discussion

Spermatological morphology. Abnormal spermatozoon rate in the control group was % 3.25 ± 0.50 (tab. 1). In 2,4-D treated groups this rate was increased at the end of treatment period. Also when the abnormal spermatozoa rates of rats were considered, the values of all groups were higher than the control group except treated with 25 ppm herbicide in water group and the difference between them was significant statistically.

Tab. 1. Abnormal spermatozoon rates in CD rats (mean ± S.E.M), (n = 8)

	Abnormal spermatozoon Rate (%)
Control group	3.25 ± 0.50^{a}
Group I (treated with 25 ppm 2,4-D in drinking water)	7.25 ± 1.90^{ab}
Group II (treated with 50 ppm 2,4-D in drinking water)	12.00 ± 0.40^{c}
Group III (treated with 50 ppm 2,4-D in food)	14.75 ± 2.80^{bc}
Group IV (treated with 100 ppm 2,4-D in food)	16.25 ± 3.60^{bc}

Explanation: means with different letters differ significantly at $p \leq 0.05$

Tab. 2. Serum activity of AST and ALT in CD rats (mean ± S.E.M), (n = 8)

	AST	ALT
Control group	192.57 ± 25.27	106.67 ± 7.28
Group I (treated with 25 ppm 2,4-D in drinking water)	162.00 ± 10.74	103.36 ± 5.17
Group II (treated with 50 ppm 2,4-D in drinking water)	164.37 ± 11.78	113.84 ± 7.98
Group III (treated with 50 ppm 2,4-D in food)	166.25 ± 7.38	95.67 ± 9.23
Group IV (treated with 100 ppm 2,4-D in food)	193.28 ± 13.57	113.42 ± 5.92

Tab. 3. The level of herbicide and its metabolite, 2,4-DCP, in the liver (mean ± S.E.M), (n = 8)

	2,4-D	2,4-DCP
Control group	Not found	Not found
Group I (treated with 25 ppm 2,4-D in drinking water)	2.83 ± 0.42*	1.03 ± 0.19*
Group II (treated with 50 ppm 2,4-D in drinking water)	4.19 ± 0.28*	1.73 ± 0.47*
Group III (treated with 50 ppm 2,4-D in food)	5.46 ± 0.35*	3.52 ± 0.37*
Group IV (treated with 100 ppm 2,4-D in food)	7.90 ± 0.54*	4.64 ± 0.42*

Explanation: * $p \leq 0.01$

Effects of 2,4-D on serum transaminase activities in rats. Hepatic toxicity was monitored by quantitative levels of the ALT and AST activities, which are used as the biochemical markers of liver injury. Blood serum AST levels were decreased in all groups treated with 2,4-D in drinking water and treated with 50 ppm herbicide in food in comparison with the control group, while AST levels were increased in group treated with 100 ppm in food. On the contrary, blood serum ALT levels were increased in groups treated with 50 ppm herbicide in food and 50 ppm in drinking water, while the group treated with 25 ppm 2,4-D in drinking water and the group treated with 100 ppm 2,4-D in food showed a decrease in serum ALT levels but, these differences were not statistically significant ($p < 0.01$) (tab. 2).

Liver analysis. Assessment of all 2,4-D treated groups revealed that herbicide and its metabolite concentrations found in the liver were high. Differences were statistically significant except the level of 2,4-DCP in group treated with 25 ppm herbicide in drinking water ($p < 0.01$). The level of herbicide was the highest in the group administered relatively high dose in food (100 ppm). Table 3 shows the level of herbicide and its metabolite, 2,4-DCP, in the liver.

Although there are few studies on the effects of pesticides on aquatic and agricultural ecosystems, the

toxic effects of 2,4-D on animals have not been clearly established. This study was concerned with both the evaluation of the toxic effects of 2,4-D on male reproductive system and with determination of its levels in rat liver.

Information on metabolism and mechanism of action of 2,4-D are limited but it is known that in rats, calves, pigs and human volunteers 2,4-D is absorbed rapidly and almost completely when administered orally as the free acid or as the sodium or amine salts (11, 17). This compound, essentially unaltered, is mainly excreted by the renal route in treated mammalian species (5). After low doses, tissue concentrations were highest in kidneys, liver, blood and lungs (11). Also Deregowski et al. (7) reported that high levels of the herbicide in the lungs, heart, liver, spleen and kidneys, and low levels in the adipose tissue and brain, with intermediate values in the adrenals and testicles. Regarding to the food and water contamination levels of 2,4-D, in the present study it was given to the rats at the dosage of 25, 50 and 100 ppm with food and drinking water for 30 days. It was observed that herbicide was the highest (7.90 ppm) in liver of treated with 100 ppm in food ($p < 0.01$). Concentration of 2,4-DCP was increased as depended to the treatment dose. It was reported that, for chloring substituted compounds the higher lipid peroxidation was observed for 2,4-D and 2,4-DCP when compared to three chlorine atoms substituted compounds. Also higher percentage of peroxidation of erythrocyte plasma membrane was observed for metabolites than for precursor herbicides. As a result, chlorophenols have been reported to have higher toxicity than the main compound (8). In our previous study, herbicide and its metabolite was determined in kidney and results showed that the level of metabolite was higher than main compound (4). The level of 2,4-DCP in the liver was lower than that herbicides in this study, but it was higher than those in kidney after same dose administered (100 ppm). Ferri et al. (9) reported that the tryptophan 2,3-dioxygenase (triptophan peroxidase) enzyme which is important in the regulation of triptophan levels, has been inhibitited in case of herbicide application to the rats at the same dosage (100 ppm). An increase in the activity of alanine transaminase (AST) in serum was reported (16) after administration of herbicide orally to rats at doses of 200 ppm for 30 days and this result is similar to results of group treated with 100 ppm in the current study.

When the effects of 2,4-D on male reproductive system were examined, conflicting findings are seen to the present results. Agent white called herbicide spray which has 2,4-D among its active components have been used during the Vietnam War was observed by a major study (2) not to cause lowered sperm numbers and increased abnormal spermatozoa in US Veterans. Conversely, another study made on US Veterans has revealed lower sperm concentrations and normal spermatozoa rates (6). Another parallel study carried out on 32 male farm workers has shown that exposure to 2,4-D caused 50% decrease in sperm motility, lowered sperm number and increased abnormal spermatozoa compared to control (13). Oakes et al. (15) has reported, 2,4-D and Picloram including preparate called Tordon 75 D caused testicular toxicity in rats but its specific cell type and mechanism was unknown.

In the present study, 2,4-D was given to rats orally at 25, 50 and 100 ppm doses for 30 days. Especially the 50 and 100 ppm dose caused increased number of abnormal spermatozoa. Our results suggested that 2,4-D and its metabolites could accumulated in the liver and disorders of the male reproductive system when taken orally even at lower doses. As the present information is uncertain it is necessary to conduct more organ-specific and chemical-specific studies.

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