MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (4): 774-778, 2009
ISSN: 1680-5593

Effects of Soybean Extract and L-Tryptophan on 2, 4-Dichlorophenol Induced Testicular Toxicity in Mice

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Abstract: Two, 4-Dichlorophenol (2, 4-DCP), an environmental pollutant has been in agriculture and synthetic chemical industry. The aim of present study was to analyse the testicular toxicity of 2, 4-DCP, which caused biochemical, spermatological and histological changes in male mice and to evaluate the possible ameliorative effect of soybean extract and L-Tryptophan (L-TRP). Soybean extract (25 mg/kg bw/day) and L-TRP (150 mg kg^{-2} bw/day) were given by intraperitoneal (ip) route for 14 days. 2, 4-DCP was administered to male mice with drinking water at dose of 1000 ppm for 14 days. Biochemical parameters in serum ((glucose, creatinine, Blood Urea Nitrogen (BUN), Aspartate Transaminase (AST), Alanine Transaminase (ALT), Lactate Dehydrogenase (LDH)), spermatological and histological changes were investigated at the end of the 14 days comparatively with control group. We conclude that soy extract and L-tryptophan alleviate 2, 4-DCP testicular toxicity.

Key words: Mice, L-tryptophan, soybean, 2, 4-dichlorophenol, abnormal spermatozoa, toxicity

INTRODUCTION

Soy and its derivates have gained an important value in human and animal (especially carnivores and farm and laboratory animals) diets due to their positive impact on health owing to isoflavons they include (Sharma and Sultana, 2004; Cassidy and Faughnan, 2000; Delclos *et al*., 2001). Recent knowledge emerging through epidemiologic and experimental studies have shown that soy is effective in preventing osteoporosis, hypercholesterolemia, cardiovascular diseases and hormone-dependent breast, prostate and endometrial cancers, in addition to having a strong antioxidant effect with phytochemicals it contains (Sharma and Sultana, 2004; Clarkson, 2002; Merz *et al*., 2000). Among the isoflavons in its structure, genistein is reported to be the strongest antioxidant in soy (Knight and Eden, 1996) while phenolic compounds such as syringic, vanilic, caffeic, ferulic, p-coumaric ve p-hydroxybenzoic acid are also antioxidant compounds isolated from soy (Packer *et al*., 1999). This information is supported by the another study (Vieira *et al*., 1999) in which they concluded that both caffeic acid and ferulic acid blocked human endothelial cell apoptosis and intracellular calcium increase caused by oxidized Low Density Lipoprotein (LDL). Furthermore, soybean contains significant amounts of all the essential amino acids (including L-Tryptophan) for human and animals.

L-tryptophan, has also been presented to be displayed on the market as a dietary supplement. Pathological conditions in which urinary excretion of some aromatic tryptophan metabolites are increased, have been determined as pyridoxine insufficiency, metabolic disorders, phenylketonuria, porphyria, metastatic malignant carcinoid, leukemia, lymphoma, mental disorders and collagen vascular diseases. Information regarding the relation between tryptophan dietary supplement or repeated doses on the reproductive system is still insufficient. Therefore, Tryptophan-Dioxygenase (TDO) is an enzyme responsible for the catabolism of tryptophan and plays a role in determination of plasma free-and brain tryptophan levels. Serotonin synthesis from tryptophan, as a serotonin (5-HT) precursor, depends on hepatic TDO activity (Walsh and Daya, 1998). Previous studies have revealed that a tryptophan derivative, N-acetylserotonin, is a free radical scavenger (Reiter *et al*., 1999).

On the other hand, gradually increasing use of herbicide for the fight against weeds during agricultural

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activities constitutes an important risk for environmental contamination. Herbicides and their degradation products are localized on the surface and underground water due to this contamination. Two, 4-D is the most commonly used herbicide in the world and is the third most frequently used herbicide in the US and Canada (Kaioumova *et al*., 2001). Two, 4-DCP is its main catabolic metabolite and is one of the most commonly found chlorophenols in the aquatic environment (Crespín *et al*., 2001). It is used in the manufacture of biocides, flame retardants and in the synthetic chemical industry and it may also be found as an impurity in the production of 2, 4-D for herbicidal formulation as a precursor (Jensen, 1996).

Two, 4-DCP is the most commonly detected phenolic compound in river water in the UK. On the other hand, chlorophenols have the ability to accumulate in living organisms. Accumulation of phenol compounds in living organisms damages the cell structure and impairs its functions. The toxic effects and biological degradation of chlorinated compounds depend on the number and position of chlorine groups in the aromatic ring and an increased level of toxicity and a decrease in biologic degradation is parallel to the increase in the number of chlorine groups (Zhang *et al*., 2004). Since water pollution is at high levels, the present study has investigated the toxic effects directed to 2, 4-DCP exposure of drinking water. Furthermore, the second aim of the present study was to demonstrate any ameliorative effect of L-Tryptophan (L-TRP) and soybean extract on 2, 4-DCP induced possible changes in the parameters evaluated.

MATERIALS AND METHODS

Animal treatments and drug administration: Eight-week-old male BALB-c mice (20-25 g) were used. Animals were kept under observation for 1 week before experimentation in the animal room and the water consumption was determined. Based on the water consumptions determined, the dose of 2, 4-DCP was established. Dose of L-TRP was calculated according to Brzozowski *et al*. (1997). Bidistilled water was used for 2, 4-DCP preparation and drinking water. L-TRP was dissolved in physiologic saline. The mice were divided into six groups, 6 male mice in each group: the group 1 (control group) was fed a normal diet and distillated water; group 2 received 1000 ppm 2, 4-DCP with drinking water, group 3 received 25 mg/kg/day soy extract by the intraperitoneal (ip) route; group 4 received 150 mg/kg/day L-TRP by ip route and group 5 received both 2, 4-DCP orally (at dose of 1000 ppm with drinking water) and soy extract (at dose of 25 mg/kg/day) by ip route; group 6 received both 2, 4-DCP (at dose of 1000 ppm with drinking water) orally, L-TRP (at dose of 150 mg/kg/day) by ip route for 21 days. The male mice were weighed after 2 weeks treatment and euthanasia was applied under ether anesthesia. Blood samples were collected from the heart shortly after death for biochemical analyses. For histological and spermatological analyses, the testes and the epididymes were harvested, in addition to the kidney and liver in order to determine the changes in organ weights and the weights of all organs were measured. The animals were housed under stable conditions, at 22-24°C and were exposed to a 12-h light/12-h dark cycle. The study is approved by local ethical committee.

Preparation of soybean extract: Soybean material was grinded in a mill and triturated and extracted with methanol (50 mL) under 15 min sonication in an ultrasonic bath at ambient temperature. The methanol was the brought to dryness using a rotating evaporator at 40°C. The dry matter obtained was dissolved in physiological saline solution and passed through sterile filter (0.45 μm) for intraperitoneal injection.

Biochemical analysis: The blood samples centrifuged and biochemical parameters ((glucose, creatinine, Blood Urea Nitrogen (BUN), Aspartate Transaminase (AST), Alanine Transaminase (ALT), Lactate Dehydrogenase (LDH)] were determined using commercially available kits (Bio-clinica).

Spermatological analysis: Abnormal spermatozoa rates were determined by Spermac® stain (Stain Enterprise, Republic of South Africa) on smear samples over microscopic slides. Morphological abnormalities were investigated by counting a total of 200 cells under the immersion objective of a light microscope (×1000 magnification). Sperm motility was evaluated microscopically within 1-2 min at 37°C and is expressed as percent progressive motility.

Histologic preparation of testes: Fixed testis tissue samples in 10% formalin were embedded in paraffin, sectioned at 5 μm and were stained with hematoxylin and eosin (H and E). Light microscopy was used for the evaluations.

Statistical analysis: All calculations and statistical analysis were performed using one-way Analysis of Variance (ANOVA). Differences were considered to be significant at $p<0.05$ against control group.

RESULTS

Body and organ weights: In groups 3 and 4, where only soy extract or TRP were given, despite the presence of an increase in body weight, there was no significant

Table 1: Necrotic cell counts (mean±SEM) (n = 6)

Control	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Necrotic cell	10.12±1.48	29.16±2.92*	16.40±1.69	17.00±2.025	18.20±2.577	18.80±1.158

Table 2: Spermatological changes in mice (mean±SEM) (n = 6)

		Abnormality (%)				
Treatment	Motility (%)	Acrosome	Head	Mid-piece	Tail	Total
Control (Groups)						
1	73.81±2.10	16.11±4.60	2.35±0.50	0	1.25±0.20	19.71±2.30
2	16.25±3.40*	49.35±9.30*	7.25±1.60*	0.75±0.10	5.25±0.60*	62.6±11.20*
3	70.83±3.60	15.16±3.70	4.0±0.600	1.33±0.30	6.66±0.80	27.16±5.50
4	59.16±7.30	24.5±4.700	8.16±2.40	2.66±0.80	6.16±1.20	41.5±6.70*
5	45.35±8.80*	22.7±1.800	9.0±1.800	1.0±0.300	4.50±3.60	37.2±3.600
6	38.25±4.60*	32.0±5.200	4.16±2.40	0	2.83±0.70	39.0±6.400

Table 3: Biochemical parameters in mice (mean±SEM) (n = 6)

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
Control						
AST (U mL^{-1})	95.6±11.30	135.2±13.1*	97.1±14.50	96.3±19.30	115.1±19.70	124.3±12.5
ALT (U mL^{-1})	44.2±4.600	73.2±10.7*	45.4±5.700	44.7±3.500	65.2±10.40	66.4±11.8
BUN (mg dL^{-1})	50.2±4.100	58.4±5.500	43.6±2.200	51.3±8.600	55.1±11.50	55.5±6.70
Creatinine (mg dL^{-1})	0.45±0.10	0.51±0.07	0.41±0.03	0.47±0.02	0.48±0.05	0.5±0.03
Glucose (mg dL^{-1})	120.3±12.50	135.8±16.20	116.7±9.200	132.0±11.70	128.2±14.20	131.3±12.5
LDH (U L^{-1})	1257.0±261.3	1856.0±369.5*	1013.0±196.1	1158.0±120.5	1335.0±331.2	1365.0±212.1

*$p<0.05$ by SPSS and ANOVA

difference. The increase in liver weight observed in group 3 compared to other groups was not statistically significant.

Histological findings: In the study performed, consequent to administration of substances, the necrotic cells in the seminiferous tubules were detected. The necrotic cells in group 2 were statistically different from control group ($p<0.05$). When the necrotic cell counts were compared, the values of all groups were higher than the control group but, these differences were not statistically significant. Treatment with soybean extract and L-tryptophan on 2, 4-DCP toxicity provided a reduction in the increased necrotic cell counts (Table 1).

Spermatological analyses: No statistically significant differences were observed between any of groups in sperm motility. However, 2, 4-DCP administration caused the significant decrease ($P<0.05$) in sperm motility and abnormality in comparison to the control group. A marked ($p<0.05$) increase in group 5 and group 6 was observed in sperm motility compared to group 2. Both L-TRP and soy extract prevented the motility decrease caused by 2, 4-DCP. Moreover, L-TRP was effective in improving the decreased motility by 2, 4-DCP and soy extract was effective in alleviating the increased abnormal spermatozoa ratio (Table 2).

Biochemical parameters: Blood parameters as markers of liver (ALT, AST) and kidney (BUN, Creatinine) damage and serum glucose and LDH levels have been demonstrated in Table 3. Blood serum AST, ALT and LDH levels were increased significantly in group 2 but there were no significant difference in BUN, creatinin and glucose values.

DISCUSSION

The studies carried out so far, 2, 4-D and its metabolite 2, 4-DCP toxicity, have shown their *in vivo* and *in vitro* effects on antioxidant enzyme systems (Zhang *et al*., 2004; Bukowska, 2003; Bukowska *et al*., 2000). 2, 4-DCP, which has stood out in water pollution, has led to investigations to be directed towards aquatic organisms. In a study (Zhang *et al*., 2004) on the intoxication mechanism in fish, 2, 4-DCP was reported to become biotransformed in the fish liver, then an oxidation and reduction cycle occurred and more reactive oxygen was formed, thereby being exposed to oxidative stress and the cell membrane system was damaged. In our study, in the group where only 2, 4-DCP was administered, the remarkable increase in serum ALT and AST levels as the most important biomarker of hepatocellular injury may have originated from oxidative liver injury during liver biotransformation. Also, the significantly increase of serum LDH level ($p<0.05$) in the same group is a marker for generalized tissue injury. Free radicals, called Reactive Oxygen Species (ROS) or Reactive Nitrogen Species (RNS), cause DNA damage, protein denaturation and cellular injury and necrosis by peroxidation of membran lipids. The free radicals play an important roles in 2, 4-DCP toxicity (Bukowska *et al*., 2000). TRP treatment that was

planned depending on the inhibitory effect of TRP 2, 3-dioxygenase enzyme, whose main compound 2, 4-D has an important role on TRP regulation (Ferri *et al*., 2003) constituted the basis of this study. L-TRP, as a melatonin precursor amino acid, is reported to have a scavenger effect on free oxygen radicals. Also melatonin and N-acetylserotonin, called to as indolamines, is reported to inhibit lipid peroxidation in brain, liver and kidney homogenates and to have antioxidant and free radical scavenger effects by preventing hemolysis in rat erythrocytes (Reiter *et al*., 1999; Leaden and Catala, 2007). Antioxidant effect of melatonin origin has been reported to occur by producing an indolyl cation radical reacting with H_2O_2 radical and the produced radical catching O_2 of present (Brzozowski *et al*., 1997; Moosmann and Behl, 2000). Bukowska *et al*. (2000) reported that 2, 4-D has little effect on the decrease of catalase enzyme, which plays an important role in scavenging free radical reactions in cells; on the contrary, its metabolite 2, 4-DCP (at a 500 ppm dose) decreased catalase activity by 84%. It has been shown that exposure to these herbicides *in vivo* and *in vitro* proliferated peroxisomes in rats and hamsters hepatocytes. Also, phenoxyherbicides produce their toxic effects interacting with lipids and proteins on erythrocyte membranes by impairing the phospholipid structure regulating the cellular membrane functions. A change in O_2 affinity of hemoglobin and an increase in methemoglobin levels have observed by the effects of 2, 4-D and its metabolite 2, 4-DCP (Bukowska *et al*., 2000). In the cellular region, where lipid density is high, tyrosine and TRP content is also high. Therefore, it is suggested that they protect the cells against oxidative disorder. Besides, long chain acylated tyrosine and TRP or short chain acylated derivates overcome oxidative cell death and prevent lipid peroxidation (Moosmann and Behl, 2000). In this study, groups receiving both TRP and 2, 4-DCP with highly antioxidant soy extract showed improvement in biochemical parameters and histological analysis, which support this information.

On the other hand, there are studies about the different effects of L-TRP treatment on spermatogenesis. Biswas *et al*. (1985) reported that L-TRP increased the brain 5-HT levels following 7 and 21 days' treatment and decreased the plasma testosterone levels and pointed to the relation between brain 5-HT level and testicular steroidogenesis inhibition. TRP dioxygenase responsible for TRP regulation and indoleamine dioxygenase, produce O_2 during their catalytic reactions. Enzymes requiring-NH and-SH groups for their activities are inhibited during lipid peroxidation. Amer and Aly (2001) reported that 2, 4-D and 2, 4-DCP increased the incidence of sperm head anomalies in male mice. This result supports our findings which is in the group only 2, 4-DCP was administered the remarkable increase in abnormal spermatozoa and sperm motility levels than controls and the other treated groups. However, abnormal spermatozoa rate in the L-TRP-treated group with repeated doses for 21 days, was determined relatively high. The probable reason for this is the high concentration of tryptophan in seminal plasma (Sarkar *et al*., 1947) and harmfull effect of free radicals produced by high O_2 as a result of catalytic reactions of indoleamindioxygenase which shows activity specially in mice epididymis tissue (Yoshida *et al*., 1980). Hence, this study cannot completely rule out the possibility that L-TRP supplementation unless it is used repeated doses.

CONCLUSION

We suggest that L-TRP and soy extract may prove beneficial in 2, 4-DCP toxicity.

ACKNOWLEDGEMENT

We would like to thank Assoc. Prof. Bulent Ekiz (Istanbul University, Faculty of Veterinary Medicine) for help in the statistical analysis.

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