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Dimethoate-induced oxidative stress and DNA damage in rat blood cells: preventive effects of ferulic acid

Rat kan hücrelerinde dimetoat'ın neden olduğu oksidatif stres ve DNA hasarı: ferulik asit'in koruyucu rolü

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ABSTRACT

Objective: Chemicals called as insecticides are frequently used in agricultural control and cause toxic effects on non-target organisms, especially mammals. Exposure to insecticides may induce hematoxicity. This research is appertaining to the possible property of ferulic acid, utilized as nutritional supplement, to mitigate oxidative stress process caused via dimethoate.

Methods: Male Wistar rats were divided into six groups, randomly (six animal each). Animals were treated orally via gavage with ferulic acid (30 mg/kg body weight, everyday for 28 days) prior to treatment of low or high doses of dimethoate (3 mg/kg body weight, everyday for 28 days 1/100 LD50 and 30 mg/kg body weight, everyday for 4 weeks 1/10 LD50, respectively).

Results: Exposuring rats to dimethoate for 4 weeks contributed oxidative stress process with a rise in malondialdehyde levels of rats' erythrocytes. Also, treatment of dimethoate induced alterations in antioxidant defence system of erythrocytes

ÖZET

Amaç: İnsektisitler olarak adlandırılan kimyasallar, zirai mücadelede sıklıkla kullanılmakta ve başta memeliler olmak üzere hedef olmayan canlılarda toksik etkiye neden olmaktadır. İnsektisitlere maruz kalmak kan dokusunda meydana gelen toksisiteyi indükleyebilir. Bu araştırmanın amacı, dimetoat'ın neden olduğu oksidatif stres sürecini azaltmak için besin takviyesi olarak kullanılan ferulik asidin olası koruyucu özelliğini ortaya koymaktadır.

Yöntem: Erkek Wistar sıçanlar rastgele (her bir grupta altı hayvan olacak şekilde) olmak üzere altı gruba ayrıldı. Hayvanlar gavaj vasıtasıyla, düşük veya yüksek dozlarda dimetoat (sırasıyla; 3 mg/kg vücut ağırlığı 1/100 LD50, 28 gün boyunca her gün ve 30 mg/kg vücut ağırlığı 1/10 LD50, 4 hafta boyunca her gün) uygulamasından önce, ferulik asit (30 mg/kg vücut ağırlığı, 28 gün boyunca her gün) ile ağız yoluyla muamele edildi.

Bulgular: Sıçanların 4 hafta dimetoat'a maruz bırakılması, eritrosit malondialdehit seviyesinde oluşan artış ile gözlemlenen oksidatif stres sürecine katkıda bulunmuştur. Ayrıca dimetoat uygulaması, eritrositlerde

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as monitored by the increase in the activities of antioxidant enzymes such as glutathione peroxidase, glutathione-S-transferase, catalase and superoxide dismutase. Additionally, dimethoate caused DNA damage, too. Pretreatment with ferulic acid remediated the detriment caused via dimethoate, as ascertained by detention of lipid peroxidation, improvement of glutathione peroxidase, glutathione-S-transferase, superoxide dismutase and catalase activities and also DNA damage.

Conclusion: Dimethoate, an insecticide, has caused serious toxic effects on blood tissue of rats. Therefore, the conscious use of dimethoate should be ensured, its use should be minimized and development of different methods should be encouraged that replaced these chemicals in agricultural control. The present findings indicate that ferulic acid may amelierate dimethoate-caused oxidative stress process and DNA damage by altering antioxidant defense system and decreasing lipid peroxidation in blood.

Key Words: Dimethoate, ferulic acid, DNA damage, oxidative stress, pesticide, antioksidant, lipid peroxidation

glutatyon peroksidaz, glutatyon-S-transferaz, katalaz ve süperoksit dismutaz gibi antioksidan enzimlerin aktivitelerindeki artışla kendini gösteren antioksidan savunma sisteminde meydana gelen değişiklikleri indüklemiştir. Ek olarak dimetoat DNA hasarına da neden olmuştur. Ferulik asit ile yapılan ön muamele, lipid peroksidasyonunun azalması, glutatyon peroksidaz, glutatyon-S-transferaz, süperoksit dismutaz ve katalaz aktivitelerinin ve ayrıca DNA hasarının düzenlenmesi ile dimetoatın neden olduğu zararın boyutunu azaltmıştır.

Sonuç: Bir insektisit olan dimetoat ratlarda kan dokusu üzerinde ciddi toksik etkilere neden olmuştur. Bu nedenle, dimetoatın bilinçli olarak kullanılması sağlanmalı, kullanımı asgari seviyeye indirilmeli ve zirai mücadelede bu kimyasalların yerini alabilecek yöntemlerin geliştirilmesi teşvik edilmelidir. Mevcut bulgular, ferulik asidin dimetoat kaynaklı oksidatif stres sürecini ve DNA hasarını antioksidan savunma sistemini düzenleyerek ve kandaki lipid peroksidasyonunu azaltarak düzeltebileceğini göstermektedir.

Anahtar Kelimeler: Dimetoat, ferulik asit, DNA hasarı, oksidatif stres, pestisit, antioksidan, lipit peroksidasyonu

INTRODUCTION

Pesticides especially organophosphate (OP) compounds have been among the most widely ubiquitously and heavily used pesticides worldwide for over half a century. Regrettably, like other xenobiotics, OP compounds have adverse effects on people. Their extensive use is a main concern of public health in the World (1). Even so, given its ease of application, low cost, and effects against insects, its use is predicted to increase around the World by 2022 (2). The harmful impressions of OP insecticides cause severe different pathologies such as chronic renal diseases, testes atrophy, benign and malignant neoplasms of the endocrine organs, parathyroid hyperplasia, disorders in the liver and lymphatic system (3).

Dimethoate (DM) or O, O-dimethyl-S (N-methyl-carbomethyl)phosphorodithioate, an OP insecticide, is used in agriculture to protect crops against aphids and leaf miners (3). Besides, extensive use of DM can pose lots of negative impressions on the health of organisms thanks to its persistence in water, crops and soil (4). Annual DM production in the European Union

is estimated to be 6000–8000 tons (5) and DM values up to 39.9 mg/L have been determined in surface waters close to the Mediterranean Sea coast (6). The main risk groups for DM exposure are the general population (by household insecticide application or drinking water), farm owners, pesticide producers and workers (4, 7). Also, DM residue is found in foods such as cow's milk (8).

The generation of free radicals is expected to cause toxicity. For this reason, antioxidant supplementation may be used as an alternative method against pesticide toxicity (3). Thus, its relevance as a natural antioxidant has risen, such as its use as a method to protect oxidative stress in the pathophysiology of different health problems (9). Within many classes of antioxidant compounds, phenolics naturally found in nutrients have been given attention in recent studies (10). Ferulic acid (FA) or 4-hydroxy-3-methoxycinnamic acid is an important natural potent phyto-chemical that can be obtained from rice, wheat, apple, barley, orange, coffee, etc. It has an extensive range of biological activities like anti-inflammatory activity (11). FA has been established to be a powerful antioxidant, reported to end the chain reaction of free radicals and reduce the risk of heart problems such as coronary heart diseases. Also, FA has preventive effects in hepatic toxicity caused by drugs (12).

In this regard, studies on FA added to the diet is promising mainly because of its free radical scavenging efficiency, specifying that it could procured as a substantial antioxidant source in the diet. Since pesticides are frequently used in agriculture, non-target organisms are exposed to these chemicals even if they do not want to. Therefore, it is important to detect antioxidants that reduce or eliminate the effect of pesticides. There are few studies on the effectiveness of ferulic acid against pesticides. This research aimed to examine the preventive role of dietary FA adding on DM causing hematoxicity in Wistar rats, as it has not been investigated so far. We hypothesize that FA may reduce DM hematoxicity.

MATERIAL and METHOD

ANIMALS AND CHEMICALS

The procedure (G.U.ET—17.004) which we applied to the rats (200–250 g) in this research was approved by Gazi University Animal Experiments Local Ethics Committee. 36 male Wistar rats were provided from the Laboratory Animals Raising and Experimental Research Center, Gazi University, Ankara, Turkey. Water ad libitum and standard laboratory chow at 25±2 °C were applied to the rats. The investigation process of the research was carried out in accordance with international guidelines for care and use of laboratory animals. DM (≥99% purity), FA and other chemicals were obtained from Sigma Aldrich and dissolved in distilled water.

GROUPS AND APPLICATIONS

Rats were divided into 6 groups (6 animals in each gruop).

1. group: Control (1.0 ml/kg bw distilled water treated)

2. group: FA (30 mg/ kg bw daily) treated group

3. group: Low dose DM (3 mg/kg bw daily 1/100 LD50) treated group (13)

4. group: High dose DM (30 mg/kg bw daily 1/10 LD50) treated group

5. group: Low dose DM + FA treated group

6. group: High dose DM + FA treated group

DM and FA were given to rats daily by gavage during the experimental period (28 days).

OBTAINING OF ERYTHROCYTES FOR MEASURING OXIDATIVE STRESS PARAMETERS

At the end of the experimental period, blood samples of animals were taken into tubes containing heparin. Erythrocyte cells and plasma were separated via centrifugation (1600 rpm for 5 min) and then the erythrocytes were treated with %0.9 isotonic saline solution for washing. After this stage, phosphate buffer (pH 7.4) was added to erythrocytes for attaining 50% cell suspension. Erythrocyte cells were destroyed with

the help of osmotic pressure, they were centrifuged and supernatants were isolated, and data on the activities of antioxidant enzymes and MDA were assessed via Shimadzu UV-1700, Japan. Hemoglobin concentration (Hb) was measured using the method of Drabkin (14).

ASSAYS OF ANTIOXIDANT ENZYME ACTIVITY

Assay of catalase (CAT) activity

A mixture containing 2 ml erythrocyte dilution and 1ml Phosphate Buffered Saline at pH 7.00 (50 mM) was prepared Then it was incubated for 3 min (25 °C) and the reaction was started by adding 1 ml H_2O_2 (30 mM). The distribution of H_2O_2 was monitored directly with the Shimadzu UV-1700 spectrophotometer by the absorbance decreasing at 240 nm (25 °C) (15). The obtained data were represented as U/mgHb for erythrocytes.

Assay of glutathione peroxidase (GPx) activity

We measured the GPx activity using a substrate (H_2O_2) predicate in the procedure described in Paglia and Valentine's study (16). The reactions were observed indirectly as the NADPH oxidation rate for 3 minutes at 240 nm. The enzymatic activity of the samples were calculated as U of mg Hb.

Assay of glutathione-S-transferase (GST) activity

We assayed the GST activity via measuring Glutathione (GSH) and the 1-chloro 2, 4-dinitrobenzene (CDNB) conjugate generation (17). Enhancing absorbance was searched for 3 minutes at 340 nm. We expressed the GST activity as U of the CDNB - GSH conjugate formed/mg Hb.

Assay of superoxide dismutase (SOD) activity

SOD activity in fractions of cell cytoplasm was analysed by Marklund and Marklund's procedure (18). The procedure determined in Marklund and Marklund's method is based on measuring the pyrogallol illumination and autooxidation for 3 minutes at 440 nm. One unit activity of SOD was assessed as the amount of protein that induced detention of 50% pyrogallol autooxidation. The activity of enzyme was defined as U/mg Hb.

ASSAY OF MALONDIALDEHYDE (MDA) LEVELS

MDA data of erythrocytes, which are the major final product of lipid peroxidation (LPO) in membranes of cells, were investigated. For this assay, thiobarbituric acid was put in blood cells and the mixture was incubated for 60 min (95°C). Thiobarbituric acid reacts with MDA and the generation of coloured complex was sighted. MDA levels were analysed at 532 nm according to a procedure described previously (19). Data were expressed as nmol/mg Hb.

DETERMINING OF DNA DAMAGE

Blood samples (1,5 ml) taken from the control and experimental groups were taken to eppendorf tubes. After that, all the blood samples in eppendorf were added from ammonium sulfate solution prepared at 8 per thousand and all samples were centrifuged until the leukocytes collapsed clearly to the bottom of the ependorf tube with a 15-min interval at 8000 rpm (doi:10.1101/pdb.rec079152Cold Spring Harb Protoc2015). Then Enzyme-linked Immunosorbent Assay (ELISA) study was carried out for DNA damage and repair of leukocytes belonging to all the knitting of the experimental group (Roche, cat no: 11544 675 001).

STATISTICAL ANALYSIS

Statistical analysis of the obtained values was made with the help of SPSS program (Statistical Package for the Social Sciences), version 20.0 for Windows. One-way analysis of variance (anova) and Tukey tests were used to determine the differences of groups regarding oxidative stress parameters. $p < 0.05$ was considered statistically significant and the data were represented as the mean±SD (standard deviation). Statistical significance was assessed by the two-tailed Student's t-test with SPSS version 20.0 to determine DNA damage results.

The study was approved by the Gazi University Experimental Animal Local Ethics' Committee (Date: 03.01.2017 and Number: E.785).

RESULTS

Catalase activity in erythrocytes

Erythrocytes of animals treated with FA did not show significant changes compared to control (Fig. 1) ($p > 0.05$). Treatment with DM caused an increase in the erythrocyte CAT activity compared to the control group. FA treated rats administered with DM showed a decrease in erythrocyte CAT activity compared to DM administered groups without FA ($p < 0.05$).

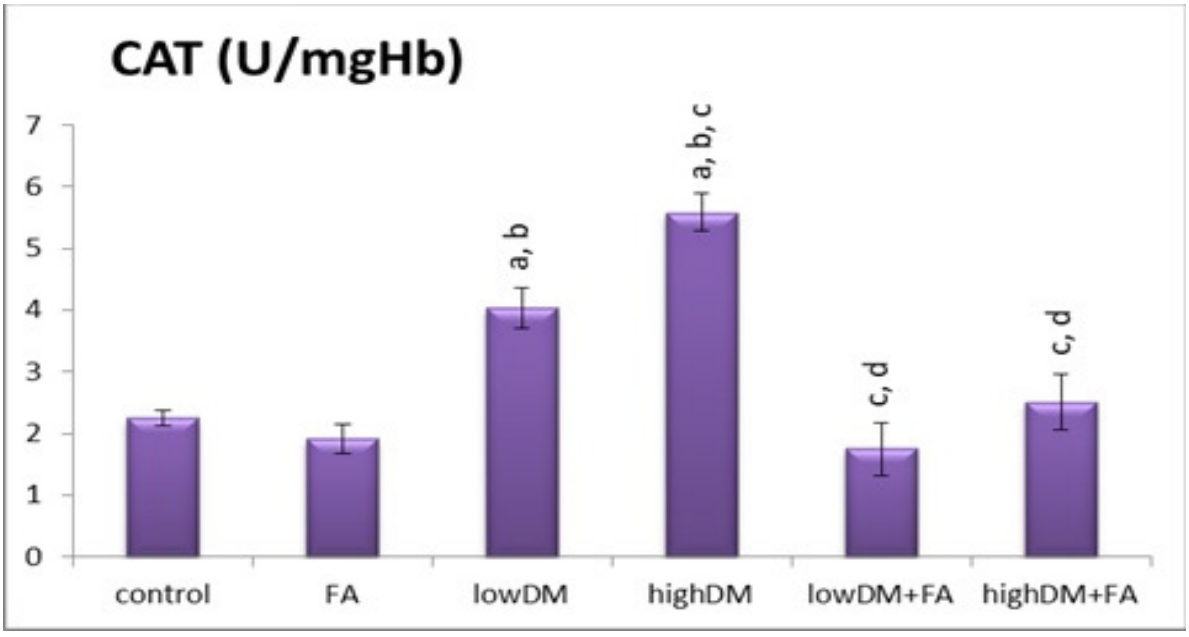

Figure 1. Effect of dimethoate (DM) treatment on catalase activity (U/mgHb) in experimental rats after subacute exposure of 28 days. Data of groups (6 rats in each group) are predicated as mean±SD. P values <0.05 were regarded significant.
[a]comparison of control group with other groups.
[b]comparison of Ferulic acid (FA) treated group with other groups.
[c]comparison of low dose DM treated group with other groups.
[d]comparison of high dose DM treated group with other groups

Glutathione peroxidase activity in erythrocytes

Erythrocyte GPx activity of FA treated rats was not significantly different from control values (Fig. 2) ($p > 0.05$). Treatment with DM (high dose only) caused an increase in the enzyme activity of erythrocytes compared to control. FA treated rats administered with high dose DM showed a decrease in erythrocyte GPx activity compared to the high dose DM administrated group ($p < 0.05$).

Glutathione-S-transferase activity in erythrocytes

GST activities of erythrocyte of FA treated rats showed no changes compared to control (Fig. 3) ($p > 0.05$). Treatment with DM caused a significant increase in erythrocyte GST activity compared with the control group. FA treated rats administered with DM showed amelioration in GST activity compared to DM treated groups ($p < 0.05$).

Superoxide dismutase activity in erythrocytes

SOD activity in FA administrated rats was not significantly different from control (Fig. 4) ($p > 0.05$). Treatment with DM caused an increase in SOD activity in erythrocytes compared to the control group. FA treated rats administered with DM showed a significant decrease in erythrocyte SOD activity compared to DM treated groups ($p < 0.05$).

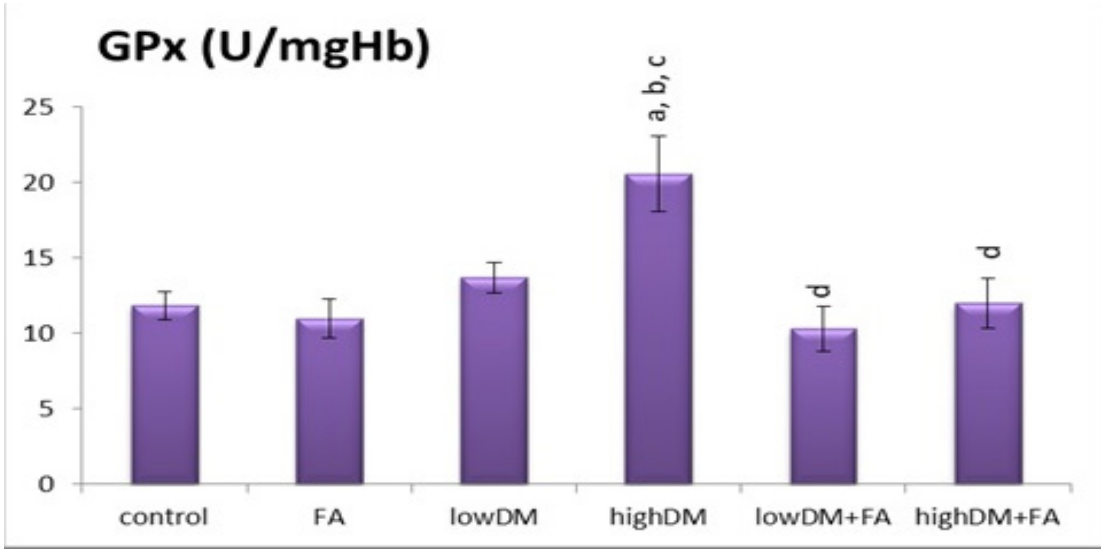

Figure 2. Effect of dimethoate (DM) treatment on glutathione peroxidase activity (U/mgHb) in experimental rats after subacute exposure of 28 days. Data of groups (6 rats in each group) are predicated as mean±SD. P values <0.05 were regarded significant.

[a]comparison of control group with other groups.

[b]comparison of Ferulic acid (FA) treated group with other groups.

[c]comparison of low dose DM treated group with other groups.

[d]comparison of high dose DM treated group with other groups

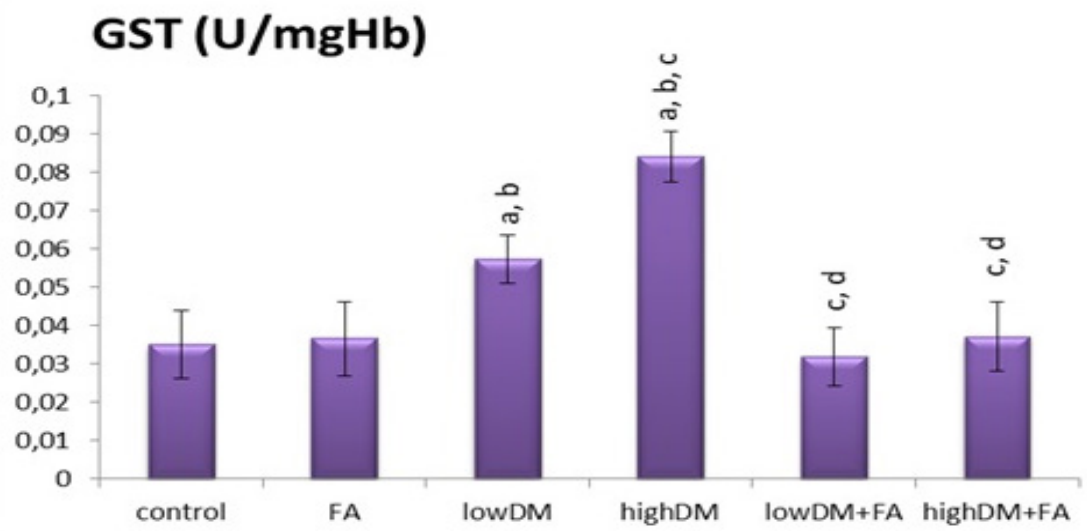

Figure 3. Effect of dimethoate (DM) treatment on glutathione-S-transferase activity (U/mgHb) in experimental rats after subacute exposure of 28 days. Data of groups (6 rats in each group) are predicated as mean±SD. P values <0.05 were regarded significant.

[a]comparison of control group with other groups.

[b]comparison of Ferulic acid (FA) treated group with other groups.

[c]comparison of low dose DM treated group with other groups.

[d]comparison of high dose DM treated group with other groups

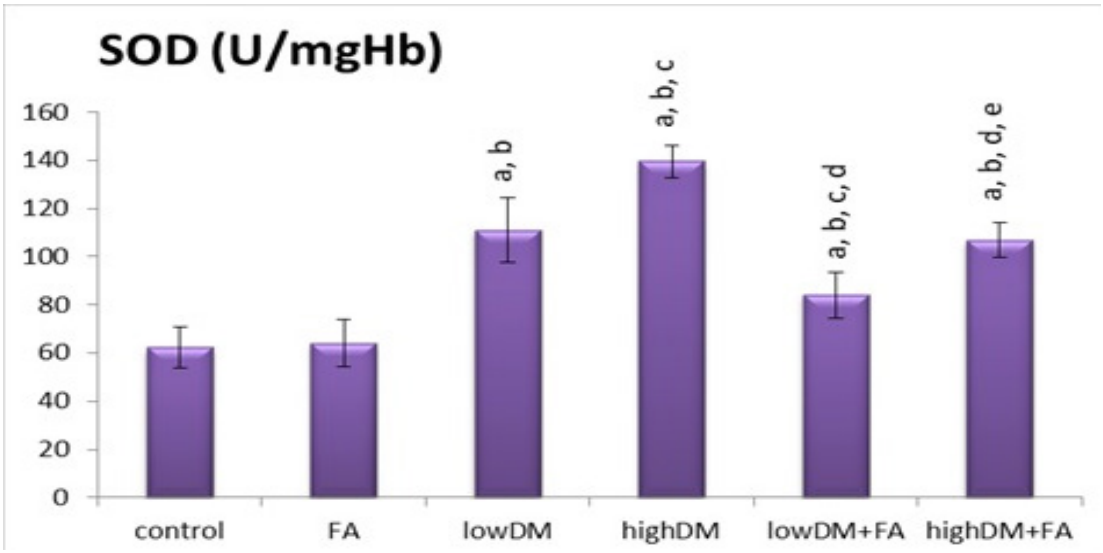

Figure 4. Effect of dimethoate (DM) treatment on superoxide dismutase activity (U/mgHb) in experimental rats after subacute exposure of 28 days. Data of groups (6 rats in each group) are predicated as mean±SD. P values <0.05 were regarded significant.
[a]comparison of control group with other groups.
[b]comparison of Ferulic acid (FA) treated group with other groups.
[c]comparison of low dose DM treated group with other groups.
[d]comparison of high dose DM treated group with other groups.
[e]comparison of low dose DM+ FA treated group with other groups

Lipid peroxidation in erythrocytes

Erythrocytes of FA treated rats showed no difference compared to control (Fig. 5) ($p > 0.05$). Treatment with DM (both low and high doses) caused increased MDA levels in erythrocytes compared to the control group. FA treated rats administered with DM showed a significant decrease in erythrocyte LPO compared to rats administered DM without FA treatment ($p < 0.05$).

DNA damage results

In this study, DNA damage levels of control and FA treated rats were very close to each other. Similar results were seen among rats treated with low dose DM and high dose DM, as well as between FA combined with low dose DM and high dose DM groups. According to the control and FA groups, DNA damage level significantly increased in the low dose DM and high dose DM treated groups. However, a significant decrese in DNA damage was observed in the groups treated with FA combined with low dose DM and high dose DM (Fig. 6).

DISCUSSION and CONCLUSION

These days people are increasingly exposed to chemical compounds such as OP pesticides. These chemicals have caused the generation of excessive amounts of free radicals that are responsible for various different alterations in cells of organisms. Recent examinations have shown the important effects of natural antioxidants found in nutritions to protect the damage induced by toxic chemicals (20). Thus, in this research, we investigated the potential effects of FA against DM toxicity.

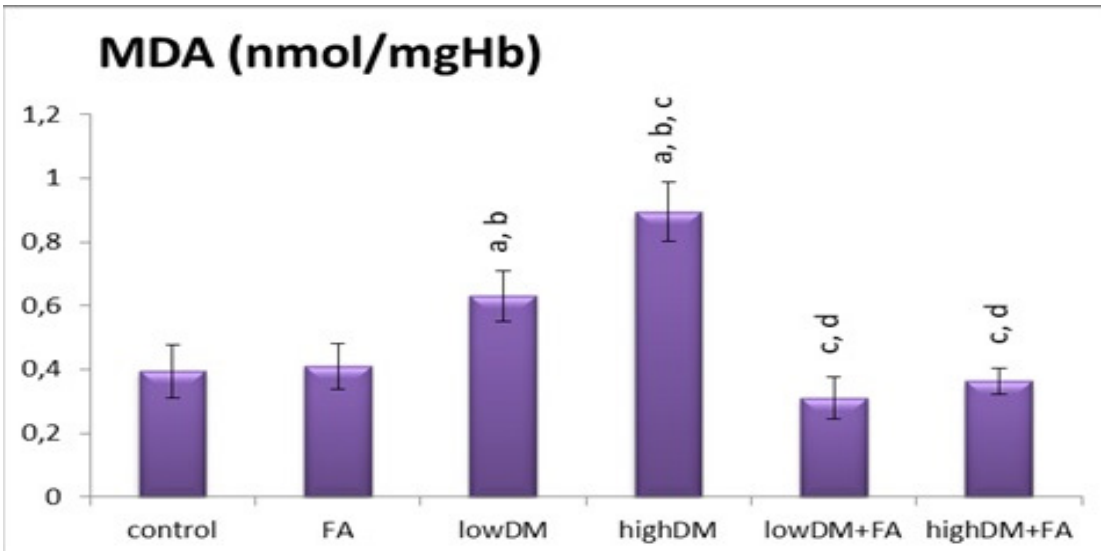

Figure 5. Effect of dimethoate (DM) treatment on malondialdehyde level (nmol/mgHb) in experimental rats after subacute exposure of 28 days. Data of groups (6 rats in each group) are predicated as mean±SD. P values <0.05 were regarded significant.

[a]comparison of control group with other groups.

[b]comparison of Ferulic acid (FA) treated group with other groups.

[c]comparison of low dose DM treated group with other groups.

[d]comparison of high dose DM treated group with other groups

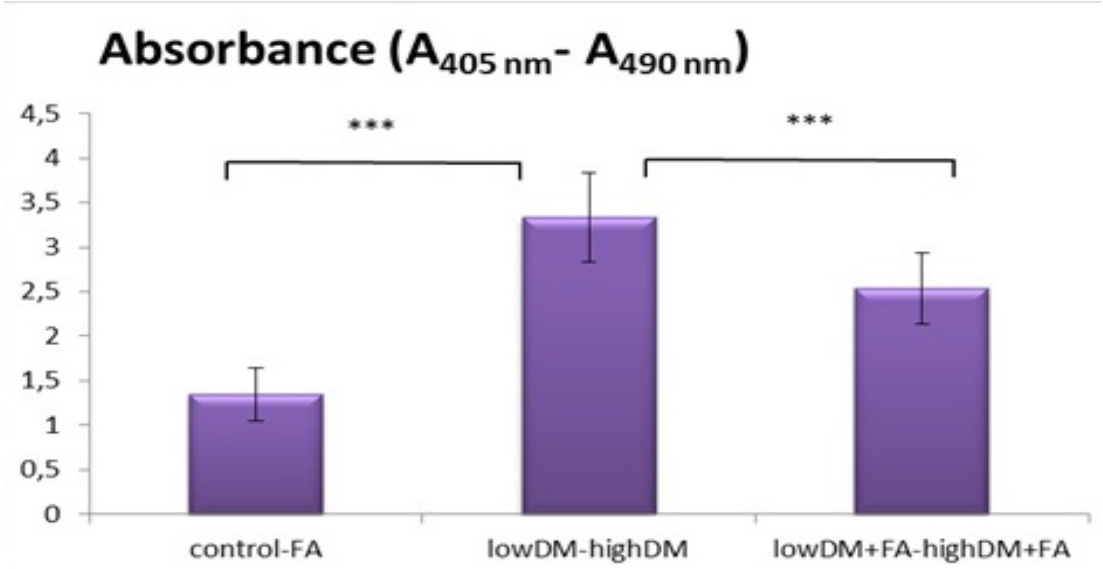

Figure 6. Effect of dimethoate (DM) treatment on DNA damage in experimental rats after subacute exposure of 28 days. Data are shown as means ± SD.

Statistical significance in two-tailed Student's t-test indicated by *P ≤ 0.05, **P ≤ 0.005, and ***P ≤ 0.0005

DM, the OP compound chosen for this research, induced human toxicity and has been widely used in many countries for the control of a wide variety of crop insects and indoor houseflies. It changes physiological parameters in subchronic and acute applications (21). The excessive use of DM induces toxic effects on human health and causes environmental pollution. The mechanisms of DM toxication are inhibiting the acetylcholinesterase activity and provoking oxidative stress through free radical formation and induction of LPO. DM exposure results in harmful effects on many organ systems in human and other mammals (22). In a previous study, dichlorvos, an OP pesticide, caused dose-response damages in rat erythrocytes (23). In another study, researchers investigated the effects of three OP pesticides, chlorpyrifos, malathion and diazinon in human erythrocytes. They observed the increase in LPO levels (24). DM intoxication induces oxidative stress resulting in LPO and free radical formation, which causes cellular injury (3). These effects may be related to the cytotoxic and genotoxic potential of DM in rat bone marrow and peripheral blood cells (25).

The mission of LPO markers and antioxidant enzymes have been ascertained in the pesticide pathogenesis in many studies (26, 27). For this reason, in this work, we examined the MDA contents as LPO marker and antioxidant enzyme activities. In this study, it was observed DM caused an increase in MDA levels both low and high doses. But high dose DM treatment caused more increment in MDA content. There are various studies showing that insecticides lead to an increase in MDA values (28, 29). Previous studies on insecticides indicated that MDA levels may increase because of detrimental impressions of OP insecticides on poly-unsaturated fatty acids (PUFA) found in cellular membranes (27, 28).

GST, SOD, CAT, and GPx are the antioxidant enzymes play important roles in the process of scavenging free radicals, especially reactive oxygen species (ROS), which potentiate lots of damage at the cellular level (30, 31). In previous studies, the enzymatic activities of these enzymes have been investigated to search oxidative stress (28, 32). Differences in enzymatic activities may be due to the formation of ROS (27, 33). In a former study, chlorpyrifos, malathion and diazinon induced changes in total antioxidant power in human erythrocytes (24). It is well known that DM changes some metabolic enzyme activities (21). DM intoxication has been shown to alter the antioxidant defense system in the liver of female adult rats (3). In this study, DM caused an increase in antioxidant enzyme activities both low and high doses except GPx activity. For GPx, low dose DM did not effect the activity. High dose DM treatment caused more increment in GST, CAT and SOD activities. The rise in the enzymatic activities of GST, SOD, CAT, and GPx in erythrocytes of DM administrated animals compared to control blood cells appears to be a response to the improved oxidative stress process (22).

DNA is known to be targeted by various chemicals whose activity results in genotoxic effects (34). Pesticides can cause DNA damage, too. In a previous study, it was showed that DM caused DNA damage in Oncorhynchus mykiss. The authors examined iwhether there is a relationship between lipid peroxidation, SOD, GPx, CAT activities and genotoxicity in vivo (35). They observed that DM caused oxdative stress and DNA damage, which are compatible with our results in blood cells of rats. As a result of this study, DNA damage was significantly increased in DM treated groups compared to the control group. However, a significant reduction in DNA damage was observed in the group treated with FA. These data can be evaluated depending on our experimental results as follows; exposure of rats to DM resulted in extensive acute further tissue inflammation including blood and blood injury reflected by the increased number of neutrophils and macrophages levels and DNA damage with cytoplasmic histone-associated DNA fragments (mono-and oligonucleosomes) after induced cell death in blood tissue. Thus, in DM treated groups, DNA fragmentation due to DM causing DNA damage was measured by ELISA method and the comparison of the

protective group was obtained. The high DNA damage observed in rat leucocytes after in vivo DM treatment shows parallelism with related investigations. In a previous study, it was determined that chlorpyrifos, an OP pesticide caused DNA damage in Mouse retina cells (28). Furthermore, Nazam et al. reported that DM induces DNA damage and mitochondrial dysfunction triggering apoptosis in rat bone-marrow and peripheral blood cells (25). In another study, it is reported that organophosphate compounds such as DM cause DNA damage by changing oxidative stress markers (34).

Actually, numerous studies indicated that the antioxidant activities of cells are ameliorated by the presence of antioxidant compounds in diet (3). Phenolic compounds have been proposed as natural antioxidant agents that protect cells against unwanted effects associated with oxidative stress. It has been proposed that they reduce ROS concentration directly (36). So, more attention has been paid to the preventive role of naturally occuring antioxidants against toxicity caused by chemicals, especially when ROS formations are involved (21). FA is known to scavenge both reactive nitrogen species and ROS (37). Because of its scavenge ROS activity, and enhancing the cell response, FA has very strong antioxidant properties due to its ROS activity and enhancing cell response (38, 39). In this study, FA protected changes in CAT, GPx, GST activities and MDA levels completely against low and high dose treatments of DM. These parameters were not different from the control group in lowDM+FA and highDM+FA groups, statistically. However, we did not see these complete preventive effects on SOD activity. For SOD, FA caused a decrease in activity but lowDM+FA and highDM+FA groups were statistically different from the control group. It has an antioxidant role referring to phenolic hydroxyl group in its structure. Studies have demonstrated that FA could hinder MDA formation from cell membranes, hinder lysis of erythrocytes induced by MDA and hydroxyl radical (HO. free radical) and so, inhibit LPO caused by H_2O_2 and O_2. Moreover, previous studies also indicated that the antioxidant effect of FA may be due to the capture of free radicals directly through the structural phenolic hydroxyl group.(10). Kohno et al. investigated the retinal protective effect of FA in a sodium iodate-induced model of retinal degeneration in mice and found that oral administration of FA attenuated the morphological and functional features of retinal degeneration (40). Mahmoud et al. indicated that FA prevents oxidative stress, inflammation, and liver injury via upregulation of Nrf2/HO-1 signaling in methotrexate-induced rats (12). As a result of this study, a significant reduction in DNA damage was observed in the groups treated with FA combined with low dose DM and high dose DM. In other words, FA has a protective effect against DM induced DNA damage.

The effects observed in the present research indicated that DM has toxic oxidant impacts on blood cells. This infomation may be based upon the probability of such a pesticide producing ROS and a state of oxidative stress and improving cell injury. FA, a naturally occuring antioxidant, could be beneficial in reducing the harmful effects of DM, maintaining the normal function of blood cells and actively inhibiting oxidative injury, suggesting its mission in ROS elimination, which is responsible for LPO process.

ETHICS COMITTEE APPROVAL

* The study was approved by the Gazi University Experimental Animal Local Ethics' Committee (Date: 03.01.2017 and Number: E.785).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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