IN VolvEMENT OF MAIN OXIDATIVE STRESS MECHANISMS IN THE TOXICITY OF BENOMYL AND CARBENDAZIM IN RATS

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SUMMARY

This study aimed to evaluate the role of oxidative stress in toxicity induced by BNL (benomyl) and its metabolite CBZ (carbendazim), which are systemic broad-spectrum benzimidazole fungicides.

The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd), γ-glutamyl transpeptidase (GGT) and glutathione-S-transferase (GST) as well as the levels of malonyldialdehyde (MDA), being a lipid peroxidation marker and reduced glutathione (GSH) were measured in the liver, kidney, testis and brain tissues of the rats, after acute exposure of the two fungicides and their mixture.

In all tissues, it was observed that MDA levels were increased and changes in antioxidant defense system were occurred. The induction of oxidative stress manifested as changes in the activity and/or levels of antioxidative parameters has been suggested as the mechanism by which BNL and CBZ induces their toxic effects.

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ÖZET
Bu çalışmada, geniş spektrumlu sistemik benimidazol grubu fungüş-dlerden olan BNL (benomyl) ve metaboliti CBZ (carbendazim)’nin toksik etki mekanizmalarında oksidatif stresin rolü araştırıldı. BNL, CBZ ve karışımlarına akut maruziyet sonrası sıçanların karaciğer, böbrek, testis ve beyin dokularında oksidatif stres göstergesi olarak lipid peroksidasyon son ürünü malondialdehid (MDA) ve indirgenmiş glutatyon (GSH) seviyeleri, süperoksid dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GSH-Px), glutatyon redüktaz (GSH-Rd), gama glutamil transpeptidaz (GGT) ve glutatyon-S-transferaz (GST) antioksidan enzim aktivitelerinin tayini yapıldı. Tüm dokularda MDA düzeylerinin arttığı ve antioksidan savunma sistemlerinde değişiklik olduğu gözlandı. BNL ve CBZ’nin toksisitesinde, antioksidan parametrelerin aktivite ve/veya düzeylerinde oksidatif stresin indüksiyonu sonucu görülen değişikliklerin rol oynadığı sonucuna varıldı.

Key words: Oxidative Stress, Benomyl, Carbendazim, Pesticide toxicity

INTRODUCTION
Pesticides are used to control several types of damages caused by pests all over the world. Although the usage of pesticides is beneficial, they are also potential toxicant to humans and other animals. Therefore it is important to highlight the toxic mechanisms following pesticide exposures. BNL (benomyl, methyl-1-(butylcarbamoyl)-2-benzimidazole-carbamate) and its metabolite CBZ (carbendazim, methyl-2-benzimidazole carbamate) are systemic broad-spectrum benzimidazole fungicides controlling various fungal pathogens whose fungicidal activities are based on the ability to interfere with the assembly of fungal microtubules (1). In the environment, BNL rapidly degrades to its breakdown product, CBZ (2). Subsequently CBZ is metabolized into many compounds mainly 5-hydroxy-2-benzimidazole carbamate and 5,6-hydroxy-2-benzimidazole carbamate-N-oxides (3).

Administration of BNL and CBZ to rats is known to cause reproductive damage (4). They act as fungicides by binding to the colchicine binding site of fungal tubulin, resulting in inhibition of microtubule assembly in vitro (5). It has been proposed that BNL and CBZ cause testicular toxicity in mammals in a similar mechanism that disrupts microtubules (6). Nakai et al. (7) reported that while both BNL and CBZ caused adverse effects in
the testis, CBZ produced more severe long-term pathologic alterations of
the testis and efferent ductules than BNL.

According to the classification of EPA (Environmental Protection
Agency), BNL and CBZ are practically non-toxic and belong to toxicity
class IV, both have oral LD$_{50}$ levels higher than 10 g/kg in rats and both
are possible human carcinogen and belong to the group C carcinogens (8).

Oxidative stress is a significant factor in the toxicity of several xenobiotics
especially of pesticides (9-14). It is accepted to be a consequence of in-
creased production of free radicals and reactive oxygen species (ROS), and/
or decrease in antioxidant defense. ROS interact with several cellular con-
stituents such as DNA, proteins and lipids (15). Lipid peroxidation (LPO), a
frequent case in oxidative stress process gives several products and affects
enzyme activities, takes important place in the pesticide toxicity shown by
several groups (16-20). Besides LPO, protein oxidation is another important
case for the organism since proteins are the most abundant macromolecules
which have important roles in myriad of pathways (21). Antioxidant defense
system including GSH, SOD, CAT, GSH-Px, GSH-Rd, GGT and GST may
protect the organism from deleterious effects of ROS (22).

The present study was designed to evaluate the acute effects of
BNL and CBZ on oxidative stress parameters such as MDA, GSH and
enzymatic antioxidants SOD, CAT, GSH-Px, GSH-Rd, GST and GGT. For
this purpose, Wistar albino rats were fed with BNL, CBZ separately and
also together and mentioned parameters were tested in liver, kidney, brain
and testes tissues. This study was designed to highlight the toxicity mecha-
nisms of BNL and CBZ fungicides which are the mostly used ones.

**MATERIALS AND METHODS**

**Chemicals**

BNL (95% purity) and CBZ (98% purity) were obtained from Agrosan
and Teknik respectively. All chemicals were of analytical grade and pur-
chased from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

**Animals and experimental design**

Healthy male 12-13 weeks old Wistar albino rats weighing 160 ± 20
g were housed in clean polypropylene cages, maintained at 25 ± 2 °C, 50
± 15% relative humidity and constant 12h/12h dark and light cycle, and fed with standard free pellet diet and water ad libitum. The animals were obtained from the animal laboratory of Cerrahpasa Faculty of Medicine, Istanbul University and the experiments complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the Animal Ethical Committee of Cerrahpasa Faculty of Medicine, Turkey.

The rats were divided into four groups each containing 10 animals. Group I: rats were given corn oil as vehicle orally. Group II: rats were treated with BNL dissolved in corn oil at a concentration of 1 g/kg body weight (1/10 LD₅₀). Group III: rats were treated with CBZ dissolved in corn oil at a concentration of 0.64 g/kg body weight (1/10 LD₅₀). Group IV: rats were treated with BNL and CBZ dissolved in corn oil at concentrations of 0.5 and 0.32 g/kg body weight (1/20 LD₅₀) respectively.

24 hours after the treatments, rats were sacrificed by cervical dislocation. Liver, kidney, brain and testes were removed, washed immediately with ice-cold 0.9% NaCl and immediately stored at -80 °C until the time of analysis.

**Preparation of tissue homogenates**

The tissues were homogenized in 0.9% NaCl using an Ultra Turrax tissue homogenizer to make up the 10% homogenates (w/v) and then centrifuged at 10000 × g at 4 °C for 20 min to obtain cytosolic fractions. Tissue homogenates (10%) were used to determine the levels of MDA and GSH content. Cytosolic fractions of tissue homogenates (10%) were used to determine antioxidant enzyme activities.

**MDA measurement**

MDA, an end product of LPO, was determined according to the method described by Beuge and Aust (23). The principle of the method is the measurement of pink chromophore produced during the reaction of thiobarbituric acid (TBA) with MDA. The absorbance of the chromophore was measured using high performance liquid chromatography (HPLC) with UV diode-array detector at 532 nm according to the modification of the HPLC method of Draper and Hadley (24). 1,1,3,3-tetraethoxypropane (TEP) was used as the standard solution. MDA values were determined from the standard curve and presented as nmol/mg protein. HPLC was carried out on a Thermo Separation Products Liquid Chromatograph (Model
Spectra System®, TSP, CA, USA). The column elute was monitored with an UV 6000 LP diode-array detector. Analysis was performed at ambient temperature on a reversed phase C\textsubscript{18} column (Luna 5 μm, 25 cm x 4.6 mm ID, Phenomenex, CA, USA) fitted with guard columns (4 mm x 3 mm ID, Phenomenex) packed with same material. The mobile phase was methanol and phosphate buffer (pH 6.8) (60:40) at a flow rate of 1 ml/min.

**GSH measurement**

The levels of GSH in incubation solutions were determined according to the method described by Beutler (25). The chromophoric product 5-mercapto-2-nitrobenzoate, resulting from the reaction of the reagent 5,5’-dithiobis-2-nitrobenzoic acid (DTNB) and free sulfhydryl groups, were measured by spectrophotometry at 412 nm. Results were expressed as micromoles of GSH per milligram of protein using standard calibration curve.

**SOD activity measurement**

SOD (EC 1.15.1.1) activity was determined according to the method described by Sun et al. (26). The principle of the method is based on the inhibition of nitroblue tetrazolium (NBT) reduction by the xanthine/xanthine oxidase system as a superoxide generator. Activity was determined in the supernatants of 10000 × g of ethanol/chloroform (5/3, v/v) extracts of tissue homogenates (10%). SOD enzyme caused 50% inhibition in NBT reduction rate was accepted as one unit. Specific activity was expressed as unit SOD per milligram of protein using standard calibration curve.

**CAT activity measurement**

CAT (EC 1.11.1.6) activity was measured according to the method of Aebi (27) based on the determination of the rate constant of hydrogen peroxide decomposition by CAT enzyme. This reaction follows a first-order kinetic given by equation $k = (2.303/t) \log A_0/A_1$. Specific activity of CAT was expressed as $k$ per milligram of protein using standard calibration curve.

**GSH-Px activity measurement**

GSH-Px (EC 1.11.1.9) activity was measured according to the method of Pleban et al. (28) based on the decrease in the absorbance of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm. GSH-Px catalyses the oxidation of GSH by hydrogen peroxide. In
the presence of GSH-Rd and NADPH, oxidized glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to NADP+. Specific activity was expressed as unit of GSH-Px per milligram of protein using standard calibration curve.

**GSH-Rd activity measurement**

GSH-Rd (EC 1.6.4.2) activity was measured according to the method of Carlberg and Mannervik (29) by monitoring the oxidation of NADPH in the presence of GSSG. Specific activity was expressed as milliunit GSH-Rd per milligram of protein using standard calibration curve.

**GST activity measurement**

GST (EC 2.5.1.18) activity was measured according to the method of Habig et al. (30) The principle of the assay is based on the monitoring of the conjugate between glutathione and the substrate 1,2-dichloro-4-nitrobenzene at 345 nm. Specific activity was expressed as unit of GST per milligram of protein using standard calibration curve.

**GGT activity measurement**

GGT (EC 2.3.2.2) activity was measured according to the method of Tate and Meister (31). The principle of the assay is based on the reaction between L-γ-glutamyl-p-nitroanilide and glycylglycine and monitoring p-nitroaniline, the end product of the reaction, at 405 nm. Specific activity was expressed as unit of GGT per milligram of protein using standard calibration curve.

**Protein measurement**

Protein amounts of the tissue homogenates (10%) and cytosolic fractions were measured according to the method of Lowry et al. (32) using bovine serum albumin as standard.

**Statistical analyses**

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using Prism 4 (GraphPad) software. For determination of statistical significances of differences one-way ANOVA were performed, followed by multiple comparisons using the Bonferroni’s multiple comparison test. P values of less than 0.05, 0.01 and 0.001 were selected as the levels of significance.
RESULTS

Following the treatments, none of the rats showed morbidity and also no significant change was observed in the body weight of rats in all experimental groups (data not shown).

**MDA as an indicator of LPO**

As shown in Figure 1, in the liver and brain there was an increase in the levels of MDA but only the results in CBZ treated group were significant (p<0.01) when compared to control, in the kidney, the levels of MDA were significantly (p<0.001) elevated in all three groups compared to control and in the testis, there was an increase in the levels of MDA but only the results in BNL + CBZ treated group were significant (p<0.001) when compared to control.

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**Figure 1:** MDA formation in BNL, CBZ and BNL + CBZ treated rat tissues. Values are Mean ± SD (n=10); $^aP<0.01$, $^bP<0.001$ vs control group, ANOVA, Bonferroni’s Multiple Comparison Test)
**SOD and CAT**

In the liver, kidney, brain and testis the activities of SOD (Figure 2A) and CAT (Figure 2B) were significantly (p<0.001) decreased in all three groups compared to control.

![Figure 2: Changes in the activities of SOD (A) and CAT (B) enzymes in rat tissues following BNL, CBZ and BNL + CBZ treatments. Values are Mean ± SD (n=10); *P<0.001 vs control group, ANOVA, Bonferroni’s Multiple Comparison Test)](image)

**GSH and GSH-Rd**

In the liver and brain, the levels of GSH were significantly (p<0.001) decreased in all three groups compared to control. In the kidney and testis, there was a decrease in the levels of GSH but only the results in BNL and BNL + CBZ treated groups were significant (p<0.001) when compared to control (Figure 3A). In the liver and kidney the activities of GSH-Rd were significantly (p<0.001) decreased in all three groups compared to control and in the brain and testis, the decrease in the GSH-Rd activity was not significant when compared to control (Figure 3B).
**Other enzymatic antioxidants related to glutathione metabolism**

In the liver, kidney, brain and testis the activity of GSH-Px (Figure 4A) were significantly (p<0.001) decreased in all three groups compared to control. As shown in Figure 4B, GST activity results were different in all tissues. In the liver; BNL (p<0.05), CBZ (p<0.001) and BNL + CBZ (p<0.05) caused a decrease in the GST activity. In the kidney; there was a decrease in the activities of GST but only the results in BNL and CBZ treated groups were significant (p<0.001) when compared to control. In the brain, the decrease in the GST activity of all three groups was not significant when compared to control. In the testis; the decrease in the GST activity was significant in BNL (p<0.001) and BNL + CBZ (p<0.05) and also in CBZ (p<0.05) group when compared to control.
DISCUSSION

The present study clearly demonstrates the involvement of oxidative stress in the toxicity of BNL and CBZ. Due to low toxicity to nontarget species (8), rapid metabolism and excretion, BNL and CBZ are intensively used for fungal pathogenic infections. Therefore the identification of the role of oxidative stress in the toxicity may bring new aspect to decrease the toxicity in the organisms which are not target.

Induction of oxidative stress has been reported by many authors, as the main mechanism of toxicity and it has been a focus of toxicological research even today (33-41). There is evidence from animal and tissue culture studies that benzimidazole fungicides such as oxfendazole (42, 43), albendazole
and mebendazole (44), 2-furan-2-yl-1H-benzimidazole (45) induce oxidative stress. Several studies have detected elevated levels of MDA, decreased levels of GSH and decreased antioxidant activities in different tissue of rats exposed to BNL or CBZ (46-56).

Considering the previous studies on BNL and CBZ, this study was undertaken to highlight the full oxidative stress mechanism in the toxicity of these fungicides together. Dose of BNL, CBZ and BNL + CBZ were selected as derived from the LD$_{50}$ levels. The application of BNL and CBZ together based on the fact that in the agricultural applications they are used as a mixture to increase the efficiency. There are also other studies who tested the role of oxidative stress following the use of pesticide combinations (57-60).

BNL and CBZ has been reported to cause testicular damage in rats after their intraperitoneal and intratesticular administration (46, 56, 61). In the present study, we also planned to correlate the testicular levels of BNL and CBZ and the oxidative damage. But the amounts were very low (less then nanomol levels) and cannot be reliably detected (data not shown). Probably this was because of the difference in the administration route and metabolism rate following oral administration compared to the study of Lim and Miller (61).

In this study, the toxicity of xenobiotic-induced lipid peroxidation is considered to be a primary mechanism of cell membrane destruction and cell damage in liver, kidney, brain and testis. Liver, kidney, brain and testis were chosen for experiment with the reason that liver and kidney are known to play important role in the metabolism of the pesticides and this makes them sensitive to the toxicity (62). The brain tissue is highly susceptible to oxidative damage because of its high rate of ROS production, due to high rate of oxidative metabolism, an abundant supply of polyunsaturated fatty acids in cell membrane and a deficient antioxidant defense (22). Since BNL and CBZ are known to break microtubule assembly, similar situation exists for tubulin derived from microtubule-enriched organs like the brain and testis (61).

The most widely used marker of lipid peroxidation is MDA known as thiobarbituric acid reactive substance and product of peroxidized polyunsaturated fatty acids (15).

In this study; rats exposed to BNL had a significant increase renal content of MDA, rats exposed to CBZ had significant increase in the MDA lev-
Involvement of Main Oxidative Stress Mechanisms in the Toxicity

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els of liver, kidney and brain. BNL and CBZ, when administered together, caused significant increase in the MDA levels of kidney and testis. The results are compatible with the results of Ramírez-Mares and de Mejía (55) relevant to BNL, Rajeswary et al. (56) relevant to CBZ, Locatelli et al. (44), Karatas et al. (45), Saber and Shalaby (46) relevant to several benzimidazole fungicides although the treatments and the settings of the studies are different. The increase in MDA formation may be explained with the epoxide formation during the metabolism of BNL and CBZ in the organism (63). Epoxides may induce the reactive species formation such as hydroxyl radical and hydrogen peroxide as shown by Rajeswary et al. (56).

SOD catalyses the destruction of superoxide radical and converts it to peroxide that needs to be destroyed by other systems. Our data suggest that the decrease in the SOD activity may be due to the decreased dismutation of superoxide radicals to $\text{H}_2\text{O}_2$. Also SOD enzyme may be damaged directly with BNL and CBZ metabolism, due to oxidation of amino acid residues and aggregation (64). It was also suggested that the superoxide radicals cause an oxidation of the cysteine in the enzyme and decrease in the SOD activity (65). This result is in agreement with the results of Rajeswary et al. (56), Khan et al. (58), Tuzmen et al. (59), Khan (60), Yousef et al. (66) and Celik et al. (67).

CAT is an enzyme, that catalyses direct decomposition of $\text{H}_2\text{O}_2$ to ground state $\text{O}_2$. In basal conditions catalase provides protection against $\text{H}_2\text{O}_2$ generated by dismutation of superoxide radical (68). In this study, the significant reduction in CAT activity by BNL and CBZ which is in agreement with the results of Goel et al. (19), Rajeswary et al. (56), Khan et al. (58), Tuzmen et al. (59), Khan (60) and Celik et al. (67) may be also responsible for the accumulation of high concentrations of $\text{H}_2\text{O}_2$. Additionally $\text{H}_2\text{O}_2$ can participate in hydroxyl radical formation with Fenton reaction.

GSH, one of the most potent reducing biological molecules, affects scavenging of free radical reactions. The consequence of the oxidative stress in the organisms may be due to depletion of reduced glutathione and increase of its oxidized form (GSSG). In this study, the obtained results indicate that BNL, CBZ and their combination usage caused a decrease in the levels of GSH. These results are consistent with the results of Banks and Soliman (54), Ramírez-Mares and de Mejía (55), Rajeswary et al. (56), Prashantku-
Indeed the results supported the suggestion that a decrease in GSH-Rd activity induces the decrease in the GSSG conversion to its reduced form (70). In the present study, the decreased level in GSH induced by BNL and CBZ could seriously impair the optimum functioning of the various GSH dependent enzymes (22). As another explaining mechanism for the decrease of GSH, it has been reported that the butylcarbamoyl group of the BNL can be transferred to GSH in vivo through a direct nucleophilic displacement (71). The hepatic synthesis of GSH, limited by cysteine availability, is frequently slower than the conjugation with xenobiotics. GSH stores decreased during conjugation, leading to a loss of redox potential and the inability to quench peroxides via GSH-Px (55).

GSH-Rd enzyme plays an important role in maintaining glutathione redox state and high GSH/GSSG ratio. The decrease in GSH-Rd activity, in agreement with the results of Brocardo et al. (11), Rajeswary et al. (56), Khan et al. (58), Khan (60) and Celik et al. (67), explains the decrease in GSH in all tissues.

GSH-Px catalyzes the reduction of a variety of hydroperoxides (ROOH and $H_2O_2$) using GSH, thereby protecting mammalian cells against oxidative damage (72). The decreased activity of GSH-Px, in agreement with the results of Rajeswary et al. (56), Khan et al. (58), Khan (60) reveals that BNL and CBZ might have inhibited the enzyme directly by impairing the functional groups, or indirectly by rendering the supply of reduced glutathione (GSH) and NADPH insufficient needed for its action. Furthermore GSH-Px activity appears to be related to the activity of GSH-Rd which supplies reducing equivalent for its function.

GST provides protection to the tissues by catalyzing the conjugation of a variety of electrophilic xenobiotics to GSH (30). The decreased activity of GST, in agreement with the results of Brocardo et al. (11), Goel et al. (19), Rajeswary et al. (56), Hazarika et al. (57), Khan et al. (58), Khan (60), Yousef et al. (66) and Celik et al. (67), indicates the direct interaction of BNL and CBZ with this enzyme.

GGT couples the gamma-glutamyl moiety to a suitable amino acid acceptor for transport into the cell and makes it available for intracellular GSH synthesis, since most cells are unable to take up the intact form (73). The observed decrease in GGT activities of liver, brain and testis tissues,
in agreement with the results of Rajeswary et al. (56), indicates the suppressed synthesis of GSH. The increase in the GGT activities of kidney tissues explains the lower decrease in GSH level in CBZ treated group.

These enzymes act in coordination and the organisms may be pushed to oxidative stress state if any change occurs in the activities of enzymes (22). Another explanation for the decrease in the enzyme activities may be that BNL and CBZ lead to the inhibition of the enzymes synthesis in tissues as a result mRNA breakdown or cellular transcription mechanisms. Many by-products of oxygen metabolism initiate different outcomes at the subcellular level. The superoxide radical has been shown to inhibit the GSH-Px and CAT activities; (74) moreover, singlet oxygen and peroxyl radicals can inhibit SOD and CAT activities (75). These findings are in accordance with that of Khan et al. (58).

Considering the results, we can say that oxidative damage may differ according to the tissues. These observed variations among organs might be related to their respective enzymatic (22) and non-enzymatic (76) antioxidant potential. Whereas the SOD enzyme activity is high in testis, CAT and GST enzymes are high in liver compared to the other tissues. Kidney has higher amount of GSH according to the other tissues.

In conclusion, it can be demonstrated that the toxicity of acute high dose treatment of BNL and CBZ proceeds via derangement of the oxidative status as evidenced by enhancement in LPO and in antioxidative defense. Combination of BNL and CBZ were not more effective in causing toxicity via LPO mechanism compared to a single use of these fungicides.

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