

Assesment the role of oxidative stress and efficacy of caffeic acid phenethyl ester (CAPE) on neurotoxicity induced by isoniazid and ethambutol in a rat model

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Abstract. – OBJECTIVE: The aim of this study were to investigate a role of oxidative stress and the therapeutic efficacy of caffeic acid phenethyl ester (CAPE) in the pathogenesis of neurotoxicity induced by isoniazid and etambutol in a rat model.

MATERIALS AND METHODS: Male Sprague-Dawley rats were randomly divided into eight experimental groups: control, INH, ETM, INH+ETM, INH+CAPE, ETM+CAPE, INH+ETM+CAPE, and CAPE treatment group, with ten animals in each group. INH and ETM doses were given orally within tap water for 30 days. CAPE was administered into relevant groups for 30 days. Brain tissue and sciatic nerve were removed for biochemical and histopathological investigation.

RESULTS: In the INH, ETM, and INH+ETM groups, malondialdehyde (MDA) and total oxidant status (TOS) levels were significantly higher than those of the control group ($p < 0.05$). Also, in these groups, brain total antioxidant capacity (TAC) levels, and superoxide dismutase (SOD) and PON-1 activities were decreased significantly with respect to control group ($p < 0.05$). By CAPE supplement within INH and ETM groups, there was a significant decrease in MDA and TOS ($p < 0.05$). In addition to a significant increase in TAC levels, and SOD and PON-1 activities both in brain and sciatic nerve tissues ($p < 0.05$).

CONCLUSIONS: CAPE may protect against INH- and ETM-induced neurotoxicity in rat brain and sciatic nerve.

Key Words:

Isoniazid, Ethambutol, Oxidative stress, CAPE, Neurotoxicity.

Introduction

Tuberculosis treatment is usually carried out by combination of various antituberculosis drugs such as isoniazid (INH), rifampicin, ethambutol (ETM) and pyrazinamide with the intention of less risk for development of drug resistance¹. Among the traditional forms of first-line antituberculosis therapy, INH is most often related with the side effects on central and peripheral nervous system as well as most prominently peripheral neuropathy, psychosis and seizures. Moreover, adverse reactions like optic neuropathy have been reported with other antituberculosis drugs like ETM². Although there are a few reports over a sensory peripheral neuropathy of ETM, it is not known yet whether ETM aggravates the neurotoxicity induced by INH in the combination therapy³. While these agents are effectively used in patients with tuberculosis, the treatment may have to be discontinued by a variety of neurotoxic side effects including restlessness, insomnia, headaches, muscle twitching, psychiatric symptoms, seizures, peripheral neuropathy, and optic neuropathy^{2,4}. However, several hypotheses were suggested, the exact mechanisms of INH-induced neurotoxicity have not been determined yet. Oxidative stress, of course, is one of the mechanisms proposed to be responsible for INH neurotoxicity⁵. Increased free radical production gives rise to toxic effects on membrane phospholipids, resulting in formation of toxic products, such as malondialdehyde (MDA). Both the decrease of total antioxidant capacity

(TAC) and the increase of total oxidant status (TOS) are closely related to oxidative stress. The normalization of those levels by any therapeutic agent is very important for the alleviating of oxidative tissue damage^{6,7}. Anti-oxidant compounds have long been proven to be remarkably beneficial to combat the oxidative injury of the central and peripheral nervous systems. Therefore, the brain and nerve damage generated by the combined therapy of INH and ETM can be reduced by adding an antioxidant substance like caffeic acid phenethyl ester (CAPE), which is extensively used in oxidative stress related studies^{8,9}. CAPE is one of the major components of honeybee propolis and its numerous effects such as anti-cancer, anti-inflammatory, potent antioxidant, immunomodulatory, anti-allergic, and neuroprotective properties have been announced⁸⁻¹⁰. In the previous studies, we reported that CAPE might protect spinal cord, brainstem and sciatic nerve from oxidative damage caused by methotrexate treatment in a rat model¹¹. Furthermore, ameliorating role of CAPE against isoniazid-induced oxidative damage on red blood cells was declared before⁹. In the present study, it is aimed to investigate neurotoxic side effects induced by INH and ETM of antituberculosis agents in brain and sciatic nerve, and to determine the presence of any positive effects of them by adding CAPE. To the best of our knowledge, this is a first study assessing the protective effects of CAPE on the neurotoxicity induced by INH and ETM via oxidative parameters and histopathological analyses.

Materials and Methods

This study was approved by Dicle University Animal Ethics Committee and was performed in accordance with the Animal Welfare Guidance for Laboratory Animals prepared by Animal Ethics Committee of Dicle University. Male Sprague-Dawley rats aged 8-12 weeks, weighing 230 ± 30 g were obtained from the Laboratory Animal Production Unit of Dicle University, and used in the experiment. The rats were placed in a temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50\% \pm 5\%$) controlled room in which 12 h light/dark cycles were maintained for 1 week before the start of the experiment. A standard diet and tap water were provided *ad libitum*. The rats were divided into eight experimental groups as in ten animals in each group: Control, INH treatment group, ETM treatment

group, INH+ETM treatment group, INH+CAPE treatment group, ETM+CAPE treatment group, INH+ETM+CAPE treatment group, and CAPE treatment group. INH and ETM were given orally at a dose of 50 mg/kg per day inside tap water for 30 days^{9,12} and CAPE was administered at dose of 10 mg/kg intraperitoneally for 30 days in relevant groups as described previously¹¹. The saline solution (an equal volume of INH and ETM) was given via the same way for 30 days in the control group. INH and ETM were obtained from Dicle University Tuberculosis Dispensary (Dicle University, Turkey), dissolved in tap water, and orally administered for 30 days at a dose of 50 mg/kg per day via plastic disposable syringe. After rats received the above treatments, they were fed *ad libitum* until midday. Brain and nerve tissue samples were obtained after rats were anaesthetized by ether. Half of these tissues were stored at -50°C until biochemical analysis. The other part of the brain and sciatic nerve tissues were fixed in 10% formaldehyde for histopathological examination.

Biochemical Analyses

The brain and sciatic nerve samples were homogenized and immediately stored at -50°C . Assays were performed on the supernatant of the homogenate that is prepared at 14,000 rpm for 30 min at $+4^\circ\text{C}$. The protein concentration of the tissues was measured by the Lowry et al¹³. Superoxide dismutase (SOD) activity was measured according to Fridovich¹⁴. Serum PON-1 levels were measured spectrophotometrically by a modified version of the Eckerson et al method¹⁵. Lipid peroxidation level was measured by Ohkawa et al and expressed as MDA¹⁶. Nitric oxide (NO) levels were determined by the Griess method¹⁷. The TAC of supernatant fractions was evaluated using a novel automated and colorimetric measurement method developed by Erel¹⁸ and its results are expressed as nmol Trolox equivalent/mg protein. The TOS of supernatant fractions was evaluated using a novel automated and colorimetric measurement method developed by Erel¹⁹. The assay was calibrated with hydrogen peroxide, and the results are expressed in terms of nmol H_2O_2 equivalent/mg protein^{20,21}.

Histopathological Analyses

For light microscopic investigation, brain tissue specimens were fixed in 10% formaldehyde, dehydrated in alcohol solution, embedded in paraffin, and used for histopathological examina-

tion. Four micrometer thick sections were cut, deparaffinized, hydrated, and stained with hematoxylin and eosin (H&E) under a light microscope (Nikon Eclipse 80i, Tokyo, Japan). An experienced histologist who was blind to the groups examined all tissue sections microscopically to characterize the histopathological changes.

Statistical Analysis

The Kolmogorov-Smirnov test assessed the normality of the distribution for all variables. Mann-Whitney U-test was used for variables that do not meet the normality assumption. The one-way analysis of variance (ANOVA) and post hoc multiple comparison tests (LSD) were performed on the data of biochemical variables to examine differences among groups. A p -value of < 0.05 was considered statistically significant. Data are expressed as mean $\pm$ SD.

Results

Biochemical

Biochemical results of the brain and sciatic nerve tissues are shown in Tables I and II. In the INH group, the level of MDA and TOS in both the brain and sciatic nerve tissues were higher than those of the control group ($p < 0.001$). SOD and PON-1 activities were also decreased in the INH group compared with the control group ($p < 0.001$). TAC levels of

brain and sciatic nerve tissues were significantly decreased in the INH group compared with the control group ($p = 0.007$). By CAPE adding to INH treatment caused a significant decrease in MDA and TOS generation in the same tissues compared to INH alone ($p < 0.001$). Furthermore, there was a significant increase in TAC levels, SOD and PON-1 activities in CAPE plus INH group compared to INH alone ($p < 0.001$). In the ETM group, similar to INH group, MDA and TOS levels were significantly higher than those of the control group ($p < 0.001$). However, with the addition of CAPE in ETM group, there was a significant decrease in MDA and TOS generation in brain and sciatic nerve tissues compared to ETM alone ($p < 0.05$). According to TAC levels and SOD and PON-1 activities, it was found a decrease in the ETM group compared with the control group ($p < 0.05$). Whereas, CAPE and ETM treatment caused a significant increase in PON-1 activity in brain and sciatic nerve tissues ($p < 0.05$) in addition to a significant increase in TAC levels compared to ETM alone ($p = 0.001$).

In the INH + ETM group, MDA and TOS levels were determined significantly higher than those of the control group ($p < 0.001$). Whereas CAPE adding within this group, seen a decrease in MDA and TOS generation in comparison to the INH + ETM group ($p < 0.05$) (CAPE plus INH + ETM group). In a similar way, TAC levels, and SOD and PON-1 activities were decreased in the INH + ETM group compared with

Table I. Biochemical results of brain tissues

Group	TAC (μ mol Trolox Eq/g protein)	TOS (mmol H ₂ O ₂ Eq./g protein)	MDA (nmol/g protein)	SOD (U/L)	PON-1 (U/L)
Control	0.50 $\pm$ 0.06	156.1 $\pm$ 39.6	238.0 $\pm$ 20.0	3.97 $\pm$ 0.58	15.9 $\pm$ 1.5
INH (II)	0.34 $\pm$ 0.10	233.2 $\pm$ 49.2	368.4 $\pm$ 36.8	2.09 $\pm$ 0.35	10.7 $\pm$ 1.8
CAPE (III)	0.52 $\pm$ 0.08	180.5 $\pm$ 22.1	221.5 $\pm$ 42.2	3.71 $\pm$ 1.51	15.5 $\pm$ 2.8
CAPE (I)	0.48 $\pm$ 0.20	123.7 $\pm$ 39.8	227.9 $\pm$ 46.0	3.03 $\pm$ 0.74	12.9 $\pm$ 2.8
ETM (V)	0.37 $\pm$ 0.08	235.5 $\pm$ 71.4	362.2 $\pm$ 58.5	2.21 $\pm$ 0.88	10.1 $\pm$ 2.1
ETM+CAPE (VI)	0.57 $\pm$ 0.15	161.1 $\pm$ 23.6	238.9 $\pm$ 41.5	3.07 $\pm$ 1.01	12.5 $\pm$ 1.8
ETM (IV)	0.30 $\pm$ 0.09	251.4 $\pm$ 60.2	411.4 $\pm$ 51.7	2.10 $\pm$ 0.33	8.4 $\pm$ 1.9
INH+INH+CAPE (VIII)	0.52 $\pm$ 0.13	184.6 $\pm$ 36.7	286.9 $\pm$ 41.5	3.40 $\pm$ 1.88	15.7 $\pm$ 2.7
Values					
I-II	0.007 (**)	0.002 (**)	0.001 (***)	0.001 (***)	0.001 (***)
II-IV	0.014 (*)	0.001 (***)	0.001 (***)	0.05 (*)	0.035 (*)
I-V	0.03 (*)	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
V-VI	0.001 (***)	0.001 (***)	0.001 (***)	N.S.	0.033 (*)
I-VII	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
VII-VIII	0.001 (***)	0.011 (*)	0.001 (***)	0.02 (*)	0.001 (***)
II-VII	N.S.	N.S.	N.S.	N.S.	0.039 (*)
V-VII	N.S.	N.S.	0.029 (*)	N.S.	N.S.
I-III	N.S.	N.S.	N.S.	N.S.	N.S.

Table II. Biochemical results of rat sciatic nerve tissue.

Groups	TAC (mmol Trolox Eq/g protein)	TOS (mmol H ₂ O ₂ Eq./g protein)	MDA (nmol/g protein)	SOD (U/L)	PON-1 (U/L)
Control (I)	0.30 ± 0.04	86.1 ± 9.1	22.18 ± 6.72	60.46 ± 12.33	1.08 ± 0.15
INH (II)	0.11 ± 0.03	167.8 ± 58.2	32.80 ± 7.61	37.35 ± 6.70	0.22 ± 0.28
CAPE (III)	0.29 ± 0.04	86.4 ± 6.6	20.44 ± 3.87	67.41 ± 12.62	1.13 ± 0.15
INH+CAPE (IV)	0.37 ± 0.20	65.2 ± 13.9	22.38 ± 7.14	59.41 ± 14.83	1.09 ± 0.15
ETM (V)	0.14 ± 0.03	200.6 ± 44.5	39.29 ± 11.28	37.67 ± 7.74	0.21 ± 0.15
ETM+CAPE (VI)	0.22 ± 0.35	58.9 ± 24.4	22.0 ± 3.26	53.10 ± 15.41	1.78 ± 0.82
ETM+INH (VII)	0.15 ± 0.04	178.1 ± 20.6	41.21 ± 3.85	32.97 ± 3.07	0.30 ± 0.15
ETM+INH+CAPE (VIII)	0.12±0.14	92.2 ± 70.1	23.69 ± 6.25	57.48 ± 22.15	0.26 ± 0.9
<i>p</i> values					
I-II	0.007 (**)	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
II-IV	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)	0.008 (**)
I-V	0.031 (*)	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
V-VI	N.S.	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
I-VII	0.04 (*)	0.001 (***)	0.001 (***)	0.001 (***)	0.001 (***)
VII-VIII	N.S.	0.039 (*)	0.001 (***)	0.001 (***)	N.S.
II-VII	N.S.	N.S.	0.001 (***)	N.S.	N.S.
V-VII	N.S.	N.S.	N.S.	N.S.	N.S.
I-III	N.S.	N.S.	N.S.	N.S.	N.S.

N.S.: Not significant $p > 0.05$. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$; Degrees of statistical significant among groups in each table.

the control group ($p < 0.05$). But, there was a significant increase in TAC levels, and SOD and PON-1 activities thanks to CAPE addition with INH + ETM group ($p < 0.05$). CAPE + INH + ETM treatment caused a significant increase in SOD activity of sciatic nerve tissues compared to the INH + ETM group ($p < 0.001$). INH plus ETM treatment caused a significant increase in MDA levels in brain tissues when compared to ETM alone ($p = 0.02$). Likewise, INH plus ETM treatment caused a significant increase in MDA levels in sciatic nerve tissues compared to INH alone ($p = 0.012$).

Histopathological Findings

In comparison to the control group (Figure 1A), distinct edema was observed in both ETM (Figure 1B) and INH + ETM groups (Figure 1C). Few edema was observed in the ETM+CAPE group compared to the ETM group (Figure 1D). Similarly, fewer edema was observed in the ETM+INH+CAPE group compared to the ETM+INH group (Figure 1E). A moderate level of edema and vascular congestion was observed in the INH group. Similar to the control group, no edema was observed in brain tissues in INH+CAPE and CAPE groups. Neurodegenerative changes, necrosis or hemorrhage were not observed in any of the groups.

Discussion

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates. It is thought to be involved in the development of many diseases or may exacerbate their symptoms. As a result of an increase in ROS that attacks cellular structures like lipids, proteins, and DNA, causes oxidative damage. Fortunately cells have appropriate defense mechanisms and enzymes that metabolize free radicals or their precursors. Some antituberculosis drugs have been reported to a cause of oxidative damage in various tissue⁵. Measurement of TOS and MDA provides a sensitive index of lipid peroxidation and oxidative stress^{5,18}. In a study, it was declared that an increase in lipid peroxidation was correlated with the degree of oxidative effects of INH in rat hippocampus⁵. Gokalp et al⁹ reported that INH caused a significant increase in MDA levels of rat red blood cells, and its amount were decreased by CAPE co-administration. Rana et al (22) reported that there was an increase in the lipid peroxidation hepatic damaged rats by isoniazid and rifampicin. In the current study, it was also determined a significant increase in TOS and MDA levels in brain and sciatic tissues of the

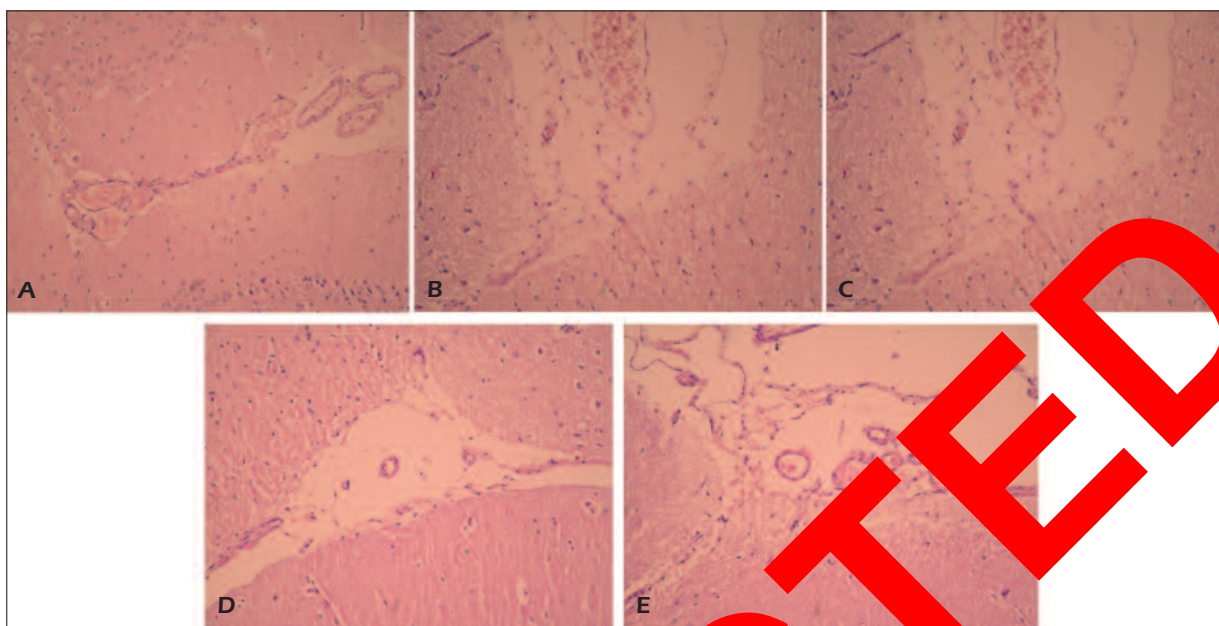


Figure 1. **A**, The brain tissue appear normal in control group (haematoxylin and eosin staining, magnification $\times 200$). **B**, The distinct edema was observed in ETM group (haematoxylin and eosin staining, magnification $\times 200$). **C**, The distinct edema was observed in INH + ETM group (haematoxylin and eosin staining, magnification $\times 200$). **D**, Fewer edema was observed in the ETM+CAPE group (Figure 1D) compared to the ETM group (haematoxylin and eosin staining, magnification $\times 200$). **E**, Fewer edema was observed in the ETM+INH+CAPE group compared to the ETM+INH group (haematoxylin and eosin staining, magnification $\times 200$).

both INH and ETM groups. In the INH and ETM were co-administered, lipid peroxidation reached the highest level, which may indicate that INH + ETM potentiate lipid peroxidation each other. Markedly increased malondialdehyde peroxidation and ROS suggest that the neurotoxic effect was created by oxidative damage. In other words, INH and ETM augment oxidative stress by either modulating the production of ROS and toxic cytokines leading to inflammation or direct tissue damage. In this study, CAPE, a powerful antioxidant and chemical scavenger, decreased lipid peroxidation and oxidants in an impressive way. Significantly reduction in MDA and TOS levels most likely indicate that CAPE might be a novel agent to protect the brain and sciatic nerve tissue from oxidative stress, resulting from INH and ETM toxicity. In addition, CAPE normalizes both MDA and TOS levels increased by anti-tuberculosis medication, which may also indicate that CAPE can be effective in both central and peripheral nervous systems.

Cells have antioxidant defense mechanisms against free radical production. This restricts or decrease their harmful effects²³. The overproduction of oxidants can be detoxified by enzymatic

and non-enzymatic endogenous antioxidants, such as glutathione, uric acid, SOD, catalase, and glutathione peroxidase, causing their cellular stores to be depleted^{6,23}.

Although the individual measurement of either oxidants or antioxidant components may give information about oxidative injury, but the determination of oxidants along with antioxidants is more useful in this context. Thereby, TOS and TAC levels may be measured simultaneously to assess oxidative stress more precisely^{6,18,19}. However, it also investigated MDA levels, and SOD and PON-1 activities in INH and/or ETM groups. In the study, it was displayed that TAC levels were significantly decreased in both INH and ETM administered rats compared to controls. The decrease in TAC may result from the overconsumption of all endogenous antioxidants during the neurotoxicity of these drugs. Additionally, there was a lower SOD in the same groups in comparison to controls. SOD catalyzes the conversion of superoxide radical into hydrogen peroxide. Thus, this enzyme protects the cell against toxic effects of superoxide radicals^{9,23}. PON-1 is an antioxidant enzyme that prevents oxidation of low-density

lipoprotein by hydrolyzing lipid peroxides^{5,24}. In addition to lipid peroxides, PON-1 has effects on hydrogen peroxide as well²⁵. The decreased in SOD and PON-1 activities are probably a result of inactivation of ROS that is produced by these drugs, or decreased production and/or increased catabolism of these enzymes due to INH and ETM toxicity. As a result, PON-1 activity is reduced in case of high oxidative stress^{5,25}. So, the decline in SOD and PON-1 activities of rat brain and sciatic tissues might be related to INH- and ETM-induced oxidative stress. In both INH- and ETM-treatment groups, supplementation of CAPE improved SOD and PON-1 activities in brain and sciatic nerve tissues. Our study indicates that CAPE significantly prevents the depletion of brain and sciatic tissue SOD and PON-1 activities by scavenging free radicals produced by both INH and ETM administration.

Deteriorations in cellular energy and metabolism balance may result in deteriorations in liquid balance of the cell. Hydrophobic degeneration is mostly the primary finding of cellular injury. While a significant toxic effect may cause death of cell by necrosis, a moderate toxic effect will cause the death of cell by vascular congestion, edema, apoptosis, or cellular degeneration^{26,27}. In the present study, both INH and ETM did not cause necrosis in the brain tissue; however, ETM caused a significant amount of edema and vascular congestion in the INH group; and, a moderate amount of edema and vascular congestion was observed in rats that received INH. CAPE treatment reduced the amount of edema and vascular congestion caused by these medications. Histopathological data in these groups were consistent with biochemical findings.

Conclusions

Our study suggests that INH and ETM are accompanied by increased lipid peroxidation and decreases in rat brain and sciatic nerve tissues when experimentally administered. So, it is considered that oxidative stress is a cause of INH- and ETM-induced neurotoxicity. Simultaneously, significant reduction in toxicity by the addition of CAPE also suggests that CAPE ameliorates neurotoxicity induced by INH and ETM, and can be combined as a potential neuroprotective drug for tuberculosis therapy.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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