Cardiotoxicity and Apoptotic Activity in Subacute Endosulfan Toxicity and the Protective Effect of Vitamin C in Rabbits: A Pathological Study

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ABSTRACT: Cardiovascular disease is one of the most significant causes of mortality in humans and animals, and its etiology is usually unknown. The aim of this study was to investigate the cardiac pathology of endosulfan toxicity and the protective effect of vitamin C in rabbits. Twenty-four rabbits were divided into 4 groups: (1) the END group was given a daily sublethal dose of endosulfan in corn oil by oral gavage for 6 weeks; (2) the END + C group received the endosulfan as well as vitamin C over the same 6-week period; (3) the OIL + C group received corn oil daily and vitamin C every other day; and (4) the OIL group received only corn oil daily. We observed microscopic hemorrhages, single-cell necrosis, inflammatory reactions, and fibrotic changes in the myocardium in the END group. Small hemorrhages and single-cell necrosis also were seen in some hearts in the END + C group, but no inflammation was observed. Caspase-3 immunoreactivity was more significant in myocardial cells in the END group compared with the others. A protective effect of vitamin C on lesions was observed in the END + C group. These results showed that endosulfan resulted in toxic changes in the hearts of rabbits, but this toxicity could be decreased with vitamin C treatment.

KEY WORDS: endosulfan, toxication, pathology, rabbit, heart, immunohistochemistry

I. INTRODUCTION

Cardiovascular disease is one of the most significant global causes of death, and it is estimated to be the leading cause of the death in developing countries.\(^1\) A critical event in heart failure is myocyte loss, which usually occurs by apoptosis. Programmed cell death, or apoptosis, in the myocardium has been linked to ischemia, reperfusion injury, and excessive mechanical forces associated with increases in ventricular loading.\(^2\) Apoptosis is an intrinsic biochemical pathway that plays an essential role in normal development and tissue homeostasis. A growing family of aspartate-specific cysteine proteases, termed caspases, functions as the central effector molecules of mammalian apoptosis.\(^3\) Caspases are present in the cytosol as inactive proenzymes but they become activated when apoptosis is initiated, and they play an essential role at various stages of apoptosis.\(^4\)

Endosulfan is an organochlorine insecticide belonging to the cyclodine group and is used all over the world to control various insect pests on a variety of food and noncrop products.\(^5-7\) Despite judicious use, both humans and animals commonly are exposed to endosulfan via different routes.\(^8,9\) Because of its toxicity, the World Health Organization has classified endosulfan as a moderately hazardous class II pesticide.\(^9\) Its breakdown products are persistent in the environment, with an estimated half-life of 9 months to 6 years.\(^7,10\)

Vitamin C (ascorbic acid) is a low-molecular-weight antioxidant that defends the cellular compartment against water-soluble oxygen and nitrogen radicals. It is an effective antioxidant of the hydrophilic phase.\(^11\) Vitamin C is capable of scavenging oxygen-derived free radicals (reactive oxygen species [ROS]), thus preventing tissue damage, and can act to overcome oxidative stress.\(^12\)

Endosulfan is still in use in many countries including Turkey. It has low volatility and a slow rate of biotransformation, and it is highly lipid soluble and resistant to degradation.\(^13-15\) Endosulfan is reported to
have numerous degenerative effects on different organs. Not much is known about heart pathology following endosulfan toxicity and, in general, lesions are examined histochemically and ultrastructurally. Oxidative stress and its correlation with heart lesions from endosulfan toxicity is well known, but there are currently no reports on histopathological findings and apoptotic activity in the heart. The aim of this study was to evaluate the pathological effects of subacute endosulfan toxicity and the potential protective effect of vitamin C. To achieve this, caspase-3 expression following endosulfan toxicity in myocardial cells from New Zealand white rabbits was assessed immunohistochemically.

II. METHODS

This study was approved by the Institutional Animal Use and Care Committee of Akdeniz University and was performed in accordance with the National Institute of Health Guidelines for the Care and Handling of Animals.

A. Animals

Twenty-four male, 6- to 8-month-old New Zealand white rabbits were used in this study. For the duration of the study the rabbits were fed standard rabbit chow and tap water ad libitum and were housed in cages with a controlled temperature (22°C) and 12-hour light/dark cycles. The physical condition of each rabbit was assessed daily for any obvious signs of illness.

B. Experimental Design

The 24 rabbits were allocated randomly to 4 equal groups of 6. The rabbit groups were treated as follows: (1) the END group was exposed to sublethal concentrations of endosulfan (1 mg/kg body weight/day) in corn oil for 6 weeks; (2) the END + C group received endosulfan (1 mg/kg body weight/day) and vitamin C (20 mg/kg body weight ascorbic acid) every other day over this period; (3) the OIL + C group received corn oil daily via oral gavage and vitamin C (20 mg/kg body weight ascorbic acid) every other day for the 6-week period; 4) the OIL group, the control group, received only corn oil via oral gavage.

C. Histopathology

The rabbits were killed 1 week after their last treatment, and necropsy was performed on all the animals. Hearts samples were fixed in 10% buffered formalin, routinely processed, embedded in paraffin, and stained with hematoxylin and eosin (HE) to be examined by light microscopy. Histopathological and immunohistochemical changes were examined.

D. Immunohistochemistry

Selected tissue sections were stained immunohistochemically to demonstrate caspase reactivity using anti-caspase 3 (CPP32 Ab4, 1:100; Neomarker, Fremont, CA) and a routine streptavidin-biotin peroxidase technique, according to the manufacturer’s instructions.

To evaluate the percentage of immunopositive cells, 100 cells from each slide were assessed in 10 different microscopic high-powered fields examined under the 40× objective of a trinocular microscope (Nikon E600) and microphotography apparatus.

E. Statistical Analysis

A one-way analysis of variance was used to detect any differences between the groups with regard to caspase immunopositive cell count. To determine differences, the nonparametric Duncan multiple comparison method was used. Calculations were made using the SPSS 13.0 program pack (SPSS Inc./IBM, Chicago, IL). P < 0.05 was accepted as statistically significant.

III. RESULTS

Depression and inappetence were the most commonly observed clinical symptoms in the END group. No clinical signs were evident in the other groups. No mortality was recorded in any of the groups throughout the study. No heart lesions were observed during necropsy.

Histopathological examination of the hearts of the rabbits in the END group revealed hemorrhages, inflammatory cell infiltrations in 5 rabbits, and fibrotic changes in 2 rabbits (Fig. 1A–C). Normal heart histology was observed in the OIL and OIL groups.
+ C groups. Slight hemorrhages and single-cell necrosis were observed in the myocardium of one rabbit in the END + C group, but no inflammation was observed in any hearts in this group.

Numerous myocardial cells with strong caspase-3 immunoreactivity were observed in the END group (Fig. 1D). A small number of apoptotic myocardial cells were seen in the END + C group (Fig. 1E). There were a few scattered caspase-3 positive cells seen in the OIL group, and these were only scantily seen in the OIL + C group (Fig. 1F). Data from statistical analyses of the percentage of caspase-3–positive cells are shown in Table 1. Apoptotic activity was markedly higher in papillary muscle than in the other areas of the heart.

IV. DISCUSSION

Heart failure is the final common pathway of many diverse etiologies that are characterized by impaired function with high morbidity and mortality. Apoptosis of myocardial cells has been documented in many cardiovascular pathologies, including myocardial infection, ischemia, and end-stage heart failure. ROS are the primary stimulus for apoptosis in the heart. Caspases generally are accepted as the executioners of apoptosis and are significant in implementing death of cardiac myocytes. Endosulfan is a pesticide that can cause toxic effects in many organs including the heart. Although the toxic effects of endosulfan are well known in the liver and genital system, very little is known about its cardiotoxicity. Thus, this study examined the histopathological and immunohistochemical findings in rabbit hearts with experimentally induced endosulfan toxicity. It also looked at the effect of vitamin C on this endosulfan toxicity because of the known antioxidant properties of ascorbic acid. First, the results indicate that endosulfan can cause heart lesions and induce apoptotic activity in myocardial cells and, second, that vitamin C can down-regulate this toxic affect.

Pesticides are economically important chemicals. Endosulfan is widely used and has been known to cause poisoning in humans and animals in Turkey. However, little is known about the heart pathology and apoptotic activity in the heart following endosulfan toxicity. The results of this study show the possible toxic histopathological effects of endosulfan on myocardial cells and demonstrate increased apoptotic activity in rabbit heart cells. The results indicate that endosulfan is toxic for rabbits, and even subacute toxicity can manifest as severe lesions in the heart. The most severe heart lesions were observed in the END group, and an amelioration of this effect was seen in the group treated with vitamin C (END + C).

Vitamin C is an essential micronutrient required for the normal metabolic functioning of the body. It prevents genetic damage caused by toxicants by several mechanisms and is known as an antimutagen, acting mainly by interfering with the generation of free radicals and the formation of toxic metabolites. Although significant caspase-3 activity, lesions, and inflammation were observed in the hearts from the END group, the group treated with vitamin C showed decreased caspase-3 activity and no inflammation. Hemorrhaging was also milder in the END + C group compared with the END group. Thus, the endosulfan toxicity observed on the rabbit hearts appeared to be down-regulated with vitamin C treatment.

Apoptosis is a highly regulated and conserved process of cell suicide that is involved in several cellular activities in eukaryotes. The caspases, a family of cysteine proteases, play a central role in the initiation and execution phases of apoptosis. Insecticides are capable of inducing apoptosis by multifunctional pathways. Oxidative stress affects a number of different cell functions, and when cells are exposed to oxidative stress, they often die by apoptosis or necrosis. This study showed

<table>
<thead>
<tr>
<th>Group</th>
<th>Caspase-3-positive myocardial cells (mean)</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>END</td>
<td>15.85 ± 5.85a</td>
<td></td>
</tr>
<tr>
<td>END + C</td>
<td>8.83 ± 1.14b</td>
<td></td>
</tr>
<tr>
<td>OIL + C</td>
<td>5.50 ± 1.64b</td>
<td></td>
</tr>
<tr>
<td>OIL</td>
<td>7.16 ± 2.13b</td>
<td>&lt;0.001</td>
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The superscript letters (a and b) indicate a significant difference between the means of the groups (P < 0.001).
that endosulfan toxicity in rabbit hearts resulted in increased apoptotic activity, inflammation, lesions (hemorrhage or necrosis), and fibrosis.

Myocyte cell death by apoptosis occurs in 2.8 million cells at 2 hours after coronary artery occlusion, and necrosis occurs in only 90,000 cells. Since mechanical forces produced by pathological loads may activate apoptosis, papillary muscles are exposed to high levels of resting tension. Apoptotic myocyte cell death plays a major role in ventricular remodeling after infarction, but whether physical forces, oxidant stress, architectural rearrangement of myocytes, and impaired force development of the myocardium is well known in endosulfan toxicity.

**FIGURE 1.**

A: Histopathological appearance of the myocardial hemorrhage (arrows) in a rabbit heart from the END group (hematoxylin & eosin [HE] stain; bar = 400 µm).

B: Myocardial inflammatory reaction in a rabbit from the END group (HE stain; bar = 100 µm).

C: Fibrosis at the myocardium (arrows) in a rabbit in the END group (HE stain; bar = 200 µm).

D: Significant caspase-3 immunoreactivity in myocardial cells of the papillary muscle in a rabbit in the END group (avidine biotin peroxidase method with 3,3-diaminobenzidine [DAB]; Harris hematoxylin counter stain; bar = 100 µm).

E: Decreased caspase-3 immunoreactivity in myocardial cells of the papillary muscle in a rabbit heart in the END + C group (Avidine Biotin Peroxidase method with DAB; Harris hematoxylin counter stain; bar = 100 µm).

F: Slight caspase-3 immunoreactivity in myocardial cells in a rabbit from the OIL group (aminobiphenyl method with DAB; Harris hematoxylin counter stain; bar = 100 µm).
Apoptotic activity was markedly higher in papillary muscle than in the other areas of the heart.

Although cardiac failure has been reported from accidental toxication with endosulfan, pathological lesions within the heart have not been demonstrated. The recognition that cell death in the myocardium is not only necrotic in nature but is also mediated by activation of the apoptotic suicide program raises several questions concerning the magnitude of this phenomenon, and whether these 2 distinct forms of cell death are disease-dependent or coexist in the pathologic heart is unknown. The toxicity induced in this study was subchronic and, as such, these 2 mechanisms for cell death may have been possible with higher apoptotic activity.

V. CONCLUSION

This study has shown that subacute endosulfan toxicity can cause hemorrhages, inflammation, necrosis, and fibrosis in the hearts of rabbits. The endosulfan-induced apoptotic activity in myocardial cells, especially papillary muscle cells, was increased in the hearts in the END group. In addition, a protective effect of vitamin C on endosulfan toxicity in the rabbit heart was observed. These results suggest that endosulfan is a cardiototoxic chemical as well as a nephrotoxic, hepatotoxic, and neurotoxic one, and vitamin C has an ameliorating effect on subacute endosulfan toxicity on pathological lesions in the rabbit heart. Endosulfan may be a possible cause of heart failure with unknown etiology and needs to be considered by practitioners, especially in cases of idiopathic heart failure.

REFERENCES


