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# The effects of erdosteine, *N*-acetylcysteine and vitamin E on nicotine-induced apoptosis of cardiac cells

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**ABSTRACT: This study was conducted to investigate the frequency of apoptosis in rat cardiomyocytes after intratraperitoneal nicotine injection, in order to examine the roles of inflammatory markers [myeloperoxidase (MPO) and tumor necrosis factor alpha (TNF-$\alpha$)] in nicotine-induced cardiac damage and to determine the protective effects of three known antioxidant agents (*N*-acetylcysteine (NAC), erdosteine and vitamin E) on nicotine toxicity in the heart. Female Wistar rats were divided into seven groups, each composed of nine rats: two negative control groups, two positive control groups, one erdosteine-treated group (500 mg kg$^{-1}$), one NAC-treated group (500 mg kg$^{-1}$) and one vitamin E-treated group (500 mg kg$^{-1}$). Nicotine was intraperitoneally injected at a dosage of 0.6 mg kg$^{-1}$ for 21 days. Following nicotine injection, the antioxidants were administered orally; treatment was continued until the rats were killed. Heart tissue samples were stained with hematoxylin-eosin for histopathological assessments. Apoptosis level in cardiomyocytes was determined by using TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick endlabelling) method. Staining of cytoplasmic TNF-$\alpha$ in cardiomyocytes and heart MPO activity were evaluated by immunohistochemistry. The treatments with erdosteine, NAC and vitamin E significantly reduced the rate of nicotine-induced cardiomyocyte apoptosis. The effect of vitamin E on apoptosis regulation was weaker than the effects of erdosteine and NAC. Erdosteine, NAC and vitamin E significantly reduced the increases in the local production of TNF-$\alpha$ and heart MPO activity. This findings suggest that the effects of erdosteine and NAC on apoptosis regulation are stronger than that of vitamin E. Copyright © 2007 John Wiley & Sons, Ltd.**

KEY WORDS: cardiomyocyte; apoptosis; nicotine; erdosteine; N-acetylcysteine; vitamin E

## Introduction

Cardiovascular disease is a leading cause of morbidity and mortality worldwide. Cigarette smoke is an important factor in the development of oxidative stress and cardiovascular disease, and nicotine is a major component of cigarette smoke that promotes these effects (Villablanca, 1998).

Cardiovascular disease can be initiated by multiple factors; a major contributing factor to the initiation and progression of cardiovascular disease is the loss of cardiomyocytes. Adult cardiomyocytes are thought to be terminally differentiated cells because of their inability to regenerate and replace damaged myocardium (Gill *et al*., 2002; Clerk *et al*., 2003). The lack of proliferation in cardiac myocytes is incompatible with neoplastic evolution in the heart, but suggests a possible association of DNA adducts with non-proliferative degenerative diseases, particularly smoking-related cardiomyopathy. In fact, several studies in humans and animals provide evidence that the heart is a preferential target for the localization of both spontaneous and smoking-related DNA adducts (De Flora *et al*., 1996; Izzotti *et al*., 1998).

Myocardial cell death can occur in a destructive, uncontrolled manner via necrosis or by a highly regulated programmed cell suicide mechanism termed apoptosis (Gill *et al*., 2002). Apoptosis is a highly organized, energy-dependent mechanism whereby a cell neatly commits suicide without causing damage to surrounding tissue. Apoptosis occurs normally during development, in tissue turnover, and in the immune system; it plays a physiological role in controlling cell mass and architecture in many tissues, including the myocardium (Neuss *et al*., 2001). The inappropriate onset or defects in sensitivity to an apoptotic stimulus can give rise to a number of clinical conditions including cancers, autoimmune diseases and degenerative disorders. Heart disease of ischemic and non-ischemic origin is characterized by abnormal changes in myocardial loading; these changes activate a variety of cellular responses, and ventricular function deteriorates as a consequence of oxidative stress and increased reactive oxygen species (ROS) formation, which stimulates cardiomyocyte apoptosis (Dhalla *et al*., 2000).

Nicotine is a major physiological modulator at the level of immune cell apoptosis or programmed cell death.

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Nicotine activates an inflammatory cascade resulting in the production of several cytokines such as tumor necrosis factor-alpha (TNF-$\alpha$), and potentiates chemotactic responses and oxygen radical generation by neutrophils (Yildiz *et al*., 1999). Nicotine contributes a major proportion of the net oxidative stress imposed by cigarette smoke, and at the same time, depletes antioxidant defense mechanisms (Villablanca, 1998). Excessive levels of oxygen radicals alter cell signaling and damage structural proteins, lipids and DNA, and thus triggering apoptosis (Chandra *et al*., 2000). Therefore, the regulation of apoptosis by antioxidant agents seems to attenuate the impact of heart damage on ventricular anatomy and performance.

*N*-acetylcysteine (NAC) is a membrane-permeable precursor of GSH and interacts directly with intracellular oxidants. In addition to its antioxidant properties, NAC has the capacity to inhibit several inflammatory elements related to oxidant stress and is involved in the pathophysiology of inflammation (Blackwell *et al*., 1996; Blesa *et al*., 2003). Erdosteine [*N*-(carboxymethylthioacetyl)-homocysteine thiolactone], a novel mucoactive agent, has antioxidant and antiinflammatory properties. Erdosteine contains two blocked sulphydryl groups which, after hepatic metabolization, become available for free radical scavenging and antioxidant activity (Dechant and Noble, 1996; Braga *et al*., 2000). Vitamin E, a lipid-soluble vitamin and potent free radical scavenging antioxidant, prevents lipid peroxidation and helps maintain the integrity of cellular organelles (Minko *et al*., 2002).

This study was conducted to investigate the frequency of apoptosis in rat cardiomyocytes after intraperitoneal nicotine injection, in order to examine the roles of the inflammatory markers myeloperoxidase (MPO) and TNF-$\alpha$ in nicotine-induced cardiac damage and to determine the protective effects of three known antioxidant agents, NAC, erdosteine and vitamin E, on nicotine toxicity in the heart.

## Materials and Methods

### Animals

This study was conducted at the experimental research center, University of Akdeniz (Antalya, Turkey). Sixty-three Wistar rats (200–250 g) were used in the study. The animals were fed a commercial balanced diet and tap water *ad libitum*. The rats were housed in cages and kept at a controlled temperature (22 ± 2 °C) and humidity (55–60%) with a 12 h light/dark cycle. The investigation followed the National Research Council guidelines (NIH publication no. 85-23, revised 1996) and was approved by the Animal Care and Use Committee of the University of Akdeniz.

### Drugs and Reagents

Erdosteine (Ilsan-Iltas Drug Industries, Istanbul, Turkey), *N*-acetylcysteine (Bılım Drug Industries, Istanbul, Turkey), vitamin E (Bayer Drug Industries, Istanbul, Turkey), nicotine hydrogen bitartrate (Sigma, St Louis, MO, USA) were used. The *in situ* cell death detection kit (Roche, Germany), anti-TNF alpha kit (Histopathology Ltd., Akác, Hungary) and anti-MPO kit (NeoMarkers Inc., Portsmouth, NH, USA) were employed.

### Experimental Groups

The rats were divided into seven groups, each composed of nine rats: two negative control groups (intraperitoneal saline plus oral distilled water or sodium bicarbonate), two positive control groups (intraperitoneal nicotine plus oral distilled water or sodium bicarbonate), one erdosteine-treated group (nicotine plus erdosteine at a dose of 500 mg $kg^{-1}$), one NAC-treated group (nicotine plus NAC at a dose of 500 mg $kg^{-1}$) and one vitamin E-treated group (nicotine plus vitamin E at a dose of 500 mg $kg^{-1}$).

### Experimental Procedure

Nicotine hydrogen bitartrate (Sigma, St Louis, MO, USA) was dissolved in 1 ml of sterile saline solution and injected intraperitoneally at a dosage of 0.6 mg $kg^{-1}$ for 21 days, as previously described (Helen *et al*., 2003). Erdosteine was dissolved with an equivalent molar quantity of sodium bicarbonate in distilled water, and NAC and vitamin E were each dissolved in distilled water. Following nicotine injection, the antioxidants were administered orally once a day via a syringe with a gavage needle; treatment was continued until the rats were killed. Control rats were intraperitoneally administered isotonic saline solution at a volume equal to that of the nicotine injection, and distilled water at a volume equal to that of the NAC and vitamin E treatments or a molar quantity of sodium bicarbonate equivalent to that of the erdosteine treatment dissolved in distilled water was given orally according to the drug administration protocol. The rats were killed with an overdose of urethane anesthesia at 21 days after the nicotine injection, a thoracotomy was performed, and the heart was explored. The heart tissue was prepared for histopathological examination and analysis of apoptosis, TNF-$\alpha$, and MPO.

### Histological Examination

The heart tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Sections 5 μm thick



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sections were placed on polylysine-coated slides and stained with hematoxylin and eosin (H & E). The slides were evaluated under light microscopy (Olympus BX51; Olympus Corp., Tokyo, Japan) at 40× magnification. The histopathological evaluation of heart injury was performed based on the following parameters: degeneration, necrosis and inflammation. The severity of the heart injury was judged by using a blind semiquantitative scoring system according to the previously defined criteria: no injury = 0, mild injury = 1, moderate injury = 2, and severe injury = 3.

### Analysis of Apoptosis

The apoptosis level in cardiomyocytes was determined by using a terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) kit according to the manufacturer's protocol. Briefly, the sections were deparaffinized and rehydrated. Then the sections were incubated with proteinase K, rinsed, incubated in 3% $H_2O_2$, permeabilized with 0.1% Triton X-100, rinsed again, and incubated in the TUNEL reaction mixture. Following incubation, the sections were rinsed and visualized using Converter-POD with DAB. The sections were counterstained with hematoxylin and eosin (H & E). Apoptotic cells containing DNA fragmentation at a single cell level were identified by the TUNEL method. The cardiomyocytes per heart section were counted under a selected 400× microscopic field by two pathologists blind to the experimental protocol. The apoptosis index was expressed as a percentage of TUNEL-positive cells in 1000 cells counted in the same section (D'Agostini *et al*., 2001).

### Analysis of TNF-$\alpha$

The local production of TNF-$\alpha$ was evaluated immunohistochemically using an anti-TNF-$\alpha$ kit according to the manufacturer's protocol. Briefly, the heart tissue samples on polylysine-coated slides were deparaffinized and rehydrated. Then, the microwave antigen retrieval procedure was performed, and the samples were incubated in a 3% $H_2O_2$ solution to inhibit endogenous peroxidase. To block nonspecific background staining, the sections were incubated with a blocking solution. Then the sections were incubated with primary anti-TNF-$\alpha$ antibody, followed by incubation with biotinylated goat anti-mouse antibody. After incubating with the chromogenic substrate (DAB), the sections were counterstained with hematoxylin and eosin (H & E). The slides were examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at 400×, and all analyses were performed by two pathologists blind to the group assignments. The staining of cytoplasmic TNF-$\alpha$ in cardiomyocytes was evaluated, and the results were expressed as the percentage of cardiomyocytes cytoplasmically stained positive for TNF-$\alpha$ in 1000 cells counted in the same section (Yang *et al*., 2004).

### Analysis of MPO

Heart MPO activity was evaluated immunohistochemically using an anti-MPO kit according to the manufacturer's protocol. Briefly, the heart tissue samples on polylysine-coated slides were deparaffinized and rehydrated. Then, the microwave antigen retrieval procedure was performed, and the samples were incubated in a 3% $H_2O_2$ solution to inhibit endogenous peroxidase. To block nonspecific background staining, the sections were incubated with a blocking solution. Then the sections were incubated with primary anti-MPO antibody, followed by incubation with biotinylated goat anti-mouse antibody. After incubating with the chromogenic substrate (DAB), the sections were counterstained with hematoxylin and eosin (H & E). The slides were examined under a light microscope (Olympus BX51; Olympus Corp., Tokyo, Japan) at 400×, and all analyses were performed by two pathologists blind to the group assignments. The staining of cytoplasmic MPO in the neutrophils was evaluated, and the results were expressed as the percentage of neutrophils cytoplasmically stained positive for MPO in 1000 cells counted in the same section (Genovese *et al*., 2005).

### Statistical Analysis

Statistical analyses were conducted using the SPSS statistical package (SPSS 9. for Windows, Chicago, IL, USA). The results were expressed as mean values ± standard deviation. Differences in quantitative variables between the groups were analysed using one-way analysis of variance (ANOVA) followed by *post-hoc* multiple comparison tests and Student's *t*-test. The Mann–Whitney *U*-test was used to compare the mean scores of histological parameters between groups. All statistical tests were two-tailed, and a vaule of $P < 0.05$ indicated significance.

## Results

### Histological Analysis

There were no significant histological differences between the distilled water- and sodium bicarbonate-treated groups (data not shown). The effects of drug treatment on heart histology are given in Table 1. Statistically significant differences were observed in the histopathological examination of heart tissue sections in the nicotine-treated



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**Table 1.** Effects of treatment on heart histology

| Histopathological findings | Negative control ($n = 9$) mean ± SD | Positive control ($n = 9$) mean ± SD | Erdosteine 500 mg kg$^{-1}$ ($n = 9$) mean ± SD | NAC 500 mg kg$^{-1}$ ($n = 9$) mean ± SD | Vitamin E 500 mg kg$^{-1}$ ($n = 9$) mean ± SD |
|---|---|---|---|---|---|
| Degeneration | 0.8 ± 0.4 | 2.4 ± 0.7[a] | 0.7 ± 0.5[c] | 1.1 ± 0.4[c] | 1.2 ± 0.8[c] |
| Necrosis | 0.2 ± 0.4 | 1.6 ± 1.1[b] | 0.4 ± 0.5[c] | 0.5 ± 0.8[c] | 0.2 ± 0.4[c] |
| Inflammation | 0.4 ± 0.5 | 0.8 ± 0.4[a] | 0.7 ± 0.5[c] | 0.4 ± 0.5[c] | 0.6 ± 0.5[c] |

Significantly higher compared with the negative control group ([a] $P < 0.05$, [b] $P < 0.0001$).
Significantly lower compared with the positive control group ([c] $P < 0.05$).

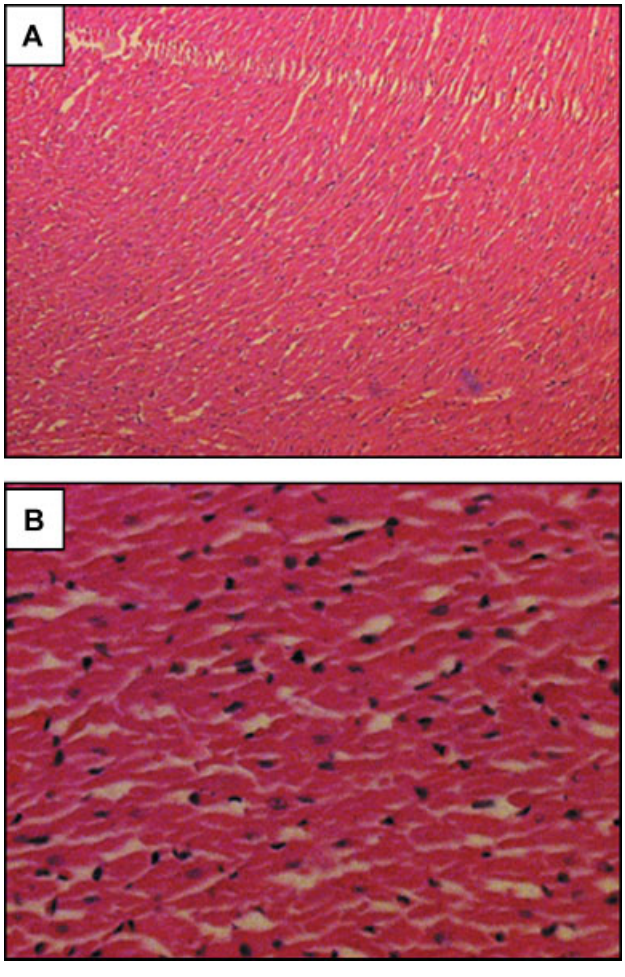


**Figure 1.** Photomicrographs of rat heart tissue sections (H & E, ×40 magnification). (A) negative control group; (B) nicotine-treated group. This figure is available in colour online at www.interscience.wiley.com/journal/jat

and control groups. In the nicotine-treated group, the following histopathological differences were found (Fig. 1): degeneration ($P = 0.001$), necrosis ($P = 0.009$) and inflammation ($P = 0.005$).

Degeneration, necrosis and inflammation were significantly reduced by erdosteine treatment ($P = 0.001$, $P = 0.029$ and $P = 0.04$, respectively), NAC treatment ($P = 0.002$, $P = 0.049$ and $P = 0.027$, respectively), and vitamin E treatment ($P = 0.001$, $P = 0.009$ and $P = 0.04$, respectively).

**Table 2.** Effects of treatment on apoptosis in cardiomyocytes

| Treated group | Apoptosis index (%) mean ± SD |
|---|---|
| Negative control ($n = 9$) | 6.0 ± 4.9 |
| Positive control ($n = 9$) | 84.9 ± 11.4[a] |
| Erdosteine (500 mg kg$^{-1}$) ($n = 9$) | 9.8 ± 7.8[b] |
| NAC (500 mg kg$^{-1}$) ($n = 9$) | 7.9 ± 6.2[b] |
| Vitamin E (500 mg kg$^{-1}$) ($n = 9$) | 27.2 ± 10.4[b] |

Significantly higher compared with the negative control group ([a] $P < 0.0001$).
Significantly lower compared with the positive control group ([b] $P < 0.0001$).

## Analysis of Apoptosis

There were no significant differences in apoptosis between the distilled water- and sodium bicarbonate-treated groups (data not shown). The effects of nicotine and antioxidants on apoptosis in cardiomyocytes are given in Table 2. The number of TUNEL-positive cardiomyocytes was significantly higher in the nicotine-treated group than in the control group ($P = 0$). Nicotine administration resulted in the induction of DNA fragmentation ladders, which are characteristic of apoptotic cell death (Fig. 2). Treatment with erdosteine ($P = 0$), NAC ($P = 0$) and vitamin E ($P = 0$) significantly reduced the rate of nicotine-induced cardiomyocyte apoptosis. The effects of erdosteine ($P = 0.001$) and NAC ($P = 0$) on apoptosis regulation were stronger than those of vitamin E.

## Analysis of TNF-$\alpha$

There were no significant differences in TNF-$\alpha$ between the distilled water- and sodium bicarbonate-treated groups (data not shown). The effects of nicotine and antioxidants on the local production of TNF-$\alpha$ in cardiomyocytes are given in Table 3. The percentage of cardiomyocytes with positive cytoplasmic staining for TNF-$\alpha$ was significantly



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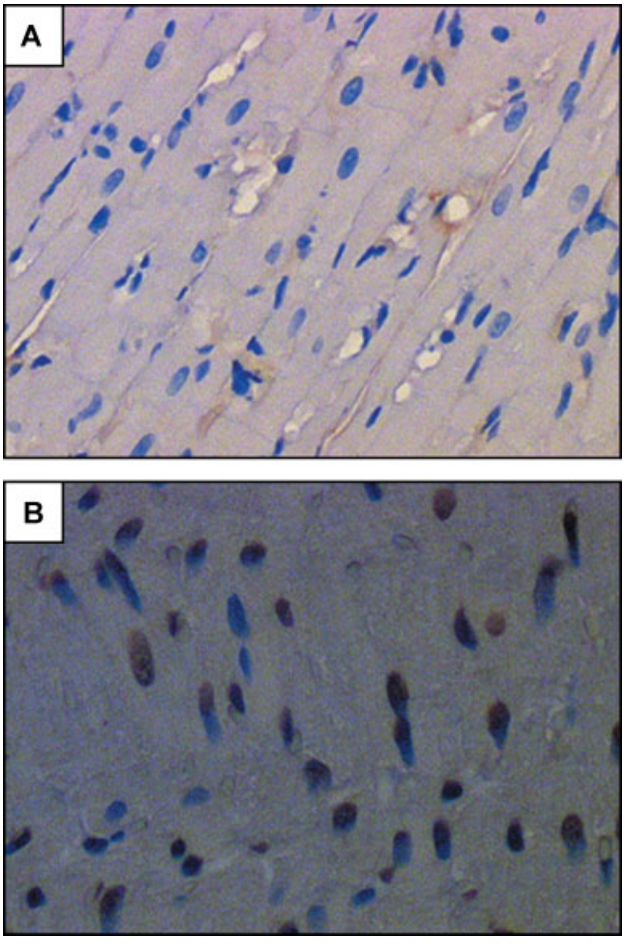


**Figure 2.** Photomicrographs of apoptotic cells (brown-stained nuclei) in cardiomyocytes as detected by the TUNEL method (original magnification, 200×). (A) negative control group; (B) nicotine-treated group. This figure is available in colour online at www.interscience.wiley.com/journal/jat

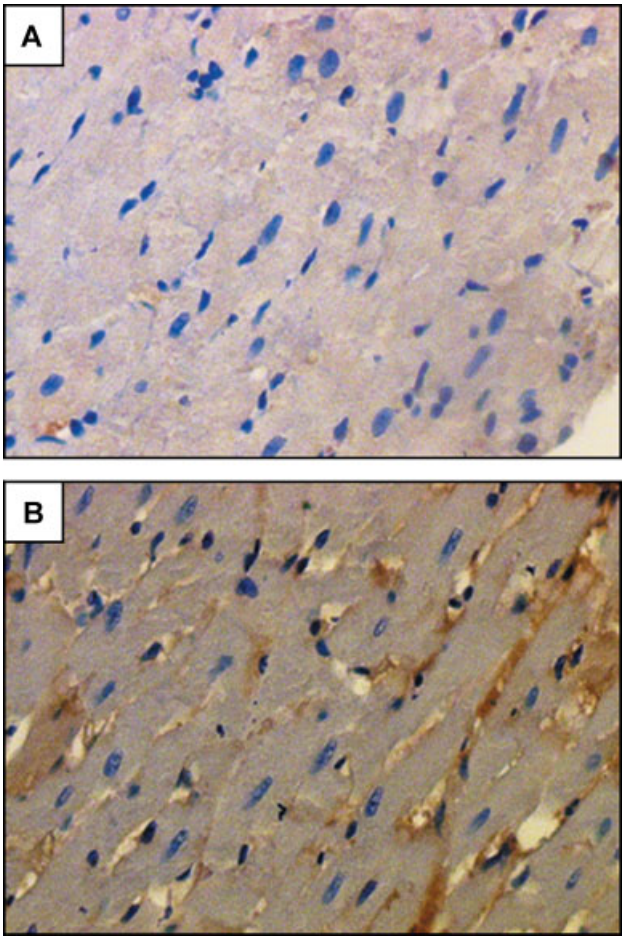


**Figure 3.** Photomicrographs of TNF-$\alpha$ analysis in cardiomyocytes as detected by immunohistochemical staining (original magnification, 200×). (A) negative control group; (B) nicotine-treated group. This figure is available in colour online at www.interscience.wiley.com/journal/jat

**Table 3.** Effects of treatment on local production of TNF-$\alpha$ in cardiomyocytes

| Treated group | Local production level of TNF-$\alpha$ (%) mean ± SD |
|---|---|
| Negative control ($n$ = 9) | 2.2 ± 3.3 |
| Positive control ($n$ = 9) | 71.7 ± 13.6[a] |
| Erdosteine (500 mg kg$^{-1}$) ($n$ = 9) | 5.8 ± 1.9[b] |
| NAC (500 mg kg$^{-1}$) ($n$ = 9) | 3.5 ± 2.0[b] |
| Vitamin E (500 mg kg$^{-1}$) ($n$ = 9) | 6.8 ± 10.5[b] |

Significantly higher compared with the negative control group ([a] $P < 0.0001$).
Significantly lower compared with the positive control group ([b] $P < 0.0001$).

**Table 4.** Effects of treatment on heart MPO activity

| Treated group | Heart MPO activity (%) mean ± SD |
|---|---|
| Negative control ($n$ = 9) | 2.2 ± 2.9 |
| Positive control ($n$ = 9) | 17.8 ± 5.9[a] |
| Erdosteine (500 mg kg$^{-1}$) ($n$ = 9) | 5.7 ± 5.3[b] |
| NAC (500 mg kg$^{-1}$) ($n$ = 9) | 3.3 ± 1.9[b] |
| Vitamin E (500 mg kg$^{-1}$) ($n$ = 9) | 6.7 ± 4.2[b] |

Significantly higher compared with the negative control group ([a] $P < 0.0001$).
Significantly lower compared with the positive control group ([b] $P < 0.0001$).

higher in the nicotine-treated group than in the controls ($P$ = 0; Fig. 3). Erdosteine ($P$ = 0), NAC ($P$ = 0) and vitamin E ($P$ = 0) significantly reduced the increase in TNF-$\alpha$ staining, and there were no significant differences in TNF-$\alpha$ staining among the three antioxidant groups. The increase in TNF-$\alpha$ staining correlated with the increase in TUNEL-positive cells ($r$ = 0.952, $P$ = 0).

## Analysis of MPO

There were no significant differences in MPO between the distilled water- and sodium bicarbonate-treated groups (data not shown). Table 4 shows the effects of nicotine and antioxidants on heart MPO activity. The percentage of neutrophils with cytoplasm that stained positive for MPO was significantly higher in the nicotine-treated



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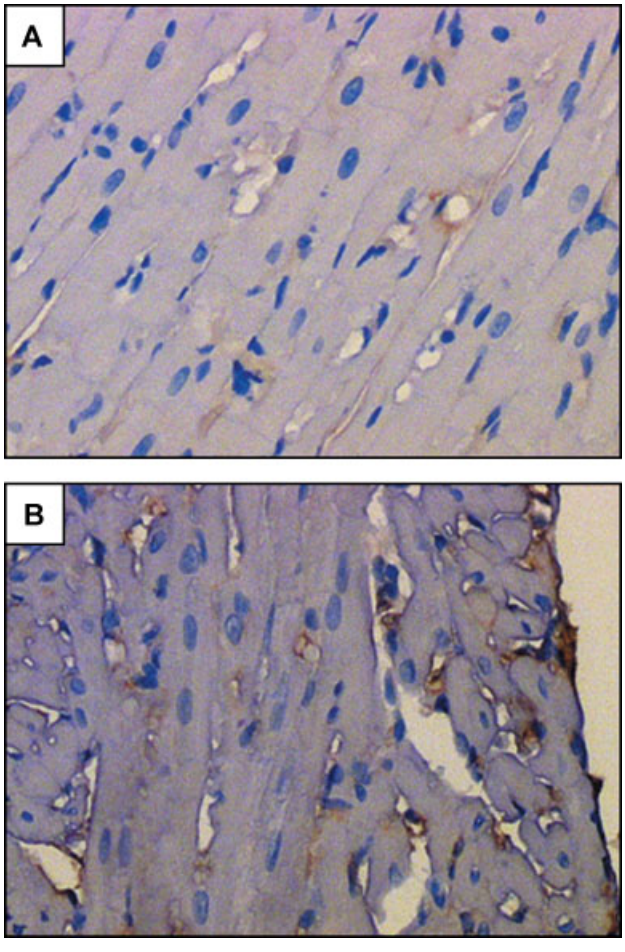


**Figure 4.** Photomicrographs of MPO analysis in heart tissue of nicotine-treated rats, as detected by immunohistochemical staining (original magnification, 200×). (A) negative control group; (B) nicotine-treated group. This figure is available in colour online at www.interscience.wiley.com/journal/jat

group than in the controls ($P = 0$; Fig. 4). Erdosteine ($P = 0$), NAC ($P = 0$) and vitamin E ($P = 0$) significantly reduced the increase in heart MPO activity, and there were no significant differences in MPO activity among the three antioxidant groups. The increase in heart MPO activity correlated with the increase in TUNEL-positive cells ($r = 0.841$, $P = 0$).

## Discussion

Nicotine has electrophilic properties, or can be metabolically converted into an electrophile, and attacks the nucleophilic sites in nucleic acids and proteins, resulting in the formation of covalent DNA adducts. Nicotine not only induces DNA damage, which may be the first step in the neoplastic process, but also allows the proliferation of aberrant cells, thus altering a crucial mechanism in the balance between cell survival and death (McPhail *et al*., 1998; Heeschen *et al*., 2001). Nicotine is generally not suspected as the culprit because the parasympathetic function, which is activated in part by nicotinic acetylcholine receptors, is generally cardioprotective, whereas sympathetic function is increasingly being implicated in cardiovascular dysfunction and inflammation (Yun *et al*., 2005).

In this study, the heart injury pattern in nicotine-treated rats was characterized by extensive degeneration, necrosis and inflammation. With regard to the route of administration and dose of nicotine, an intraperitoneal injection was chosen at a dose of 0.6 mg $kg^{-1}$ $day^{-1}$, that allows plasma levels similar to those found in the light smoker (Gomes *et al*., 2004). The dose of nicotine used and the duration of nicotine exposure was previously confirmed to show induction of oxidative stress and tissue damage (Helen *et al*., 2003). Erdosteine, NAC and vitamin E significantly reduced degeneration, necrosis and inflammation.

Nicotine administration in the present study resulted in the induction of DNA fragmentation ladders in the cardiomyocytes, indicating apoptosis of these cells. The induction of apoptosis in myocardial cells probably represents a defense mechanism in the cardiovascular system against the toxic and genotoxic effects of nicotine in cigarette smoke and may reflect molecular events in the pathogenesis of heart diseases. The treatments with erdosteine, NAC and vitamin E significantly reduced the rate of nicotine-induced cardiomyocyte apoptosis. These results illustrate that nicotine genotoxicity may be mediated, at least in part, by oxidative stress and may be attenuated by preloading cells with antioxidants (Fiordaliso *et al*., 2004; Qin *et al*., 2003). Indeed, it has been reported that supplementation with NAC and vitamin E reduced cell death caused by oxidative mechanisms in different cellular structures and blocked apoptosis induced by changes in mitochondrial membrane permeability and subsequent release of cytochrome c (Tsuda *et al*., 2000; De Flora *et al*., 2001). However, the effects of erdosteine on the regulation of apoptosis had not been previously examined. Furthermore, antioxidants have signal transduction regulatory properties independent of their ability to scavenge free radicals or repair oxidative damage, and these may prevent the incidence of cell death (Allen and Tresini, 2000).

The effect of vitamin E on apoptosis regulation was weaker than the effects of erdosteine and NAC, suggesting that vitamin E may be less effective against nicotine-induced cardiomyocyte apoptosis. Endogenous vitamin E is of great importance in the antioxidant defense system. Vitamin E deficiency exacerbates myocardial injury owing to oxidative stress, and prolonged myocardial injury is associated with a decrease in tissue vitamin E concentration. Unfortunately, the chronic administration of vitamin E *in vivo* resulted in a high plasma concentration but did not exert protection against myocardial injury (Campo *et al*., 1998).



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Cigarette smoke may trigger a generalized vascular inflammatory process which contributes to cardiovascular disease. This process may involve increased expression of proinflammatory cytokines such as TNF-$\alpha$ (Sasayama *et al*., 2000; Torre-Amione *et al*., 1995). TNF-$\alpha$ is a non-specific inflammatory cytokine that is increased early in the response to cellular stress and mediates the migration of neutrophils into injured or inflamed tissues. Studies have shown that the spectrum of biological activities for TNF-$\alpha$ is not limited to cytotoxic effects, but rather TNF-$\alpha$ exerts pleiotropic effects in a wide variety of mammalian cell types, including adult cardiomyocytes. TNF-$\alpha$ plays a pathogenetic role in cardiac disease in which left ventricular dysfunction develops with a strong negative inotropic effect (Krown *et al*., 1996). It is thought that at least part of the pathogenic effect of TNF-$\alpha$ in the heart is attributable to the induction of cell death (Torre-Amione *et al*., 1995; Song *et al*., 2000). TNF-$\alpha$ treatment has been reported to increase ROS production from mitochondria, and these mitochondrial ROS are critical modulators of TNF-$\alpha$-induced apoptosis which is mediated, at least in part, by a delay in the activation of nuclear factor-kappa B (NF-$\kappa$B) (Hughes *et al*., 2005).

In this study, the administration of nicotine increased the rate of TNF-$\alpha$ staining in the cytoplasm of cardiomyocytes, suggesting that cardiomyocytes secrete TNF-$\alpha$ in response to nicotine and that TNF-$\alpha$ plays an important role in nicotine-induced heart injury. Indeed, cardiomyocytes have been shown to be an important source of TNF-$\alpha$ production in the pathogenesis of cardiovascular diseases (Sasayama *et al*., 2000). Erdosteine, NAC and vitamin E significantly reduced the increases in the local production of TNF-$\alpha$, suggesting their antiinflammatory properties. NAC abrogated TNF-induced oxidative stress and negative effects on contraction in isolated cardiomyocytes, and also inhibited the NF-$\kappa$B-dependent cardiac hypertrophic response to TNF-$\alpha$ (Cailleret *et al*., 2004). Vitamin E, which decreases the level of TNF-$\alpha$ in human monocytes, modulated TNF-$\alpha$ activity and further decreased DNA fragmentation (Devaraj and Jialal, 2005). The positive correlation between local TNF-$\alpha$ production and TUNEL-positive cells suggests that TNF may be associated with the development of cardiomyocyte apoptosis. Antioxidants reduced the rate of cardiomyocyte apoptosis and inhibited the increase in local TNF-$\alpha$ levels caused by nicotine.

Neutrophils are important in host defense and may inflict oxidative damage during inflammation. Upon activation, neutrophils not only generate oxygen radicals but also secrete MPO as part of their oxidative repertoire. MPO, a heme enzyme secreted by activated neutrophils, generates a number of oxidants proposed to play a critical role in host defense and local tissue damage (Arnhold *et al*., 2001). The proinflammatory effects of MPO are typically viewed to be a result of its capacity to oxidize halides, lipids, proteins and DNA (Tousoulis *et al*., 2003).

In the present study, nicotine increased the ratio of neutrophils with cytoplasmic staining for MPO, indicating that neutrophils play an important role in nicotine-induced heart injury. Erdosteine, NAC and vitamin E significantly reduced the increase in heart MPO activity attributable to nicotine administration. These results suggest that the antioxidants reached concentrations in the heart tissue sufficient to exert antiinflammatory and antioxidant effects and to reduce the migration of neutrophils into the inflamed region, thereby mitigating tissue damage in the heart. Thus, in addition to their direct antioxidant effects, antioxidants may indirectly protect heart tissue from free radical damage by decreasing neutrophil recruitment into the heart (Blackwell *et al*., 1996; Hughes *et al*., 2005).

The increase of heart MPO activity correlated with the increase in TUNEL-positive cell, and the inhibition of DNA alterations in the presence of antioxidants paralleled the reduction in heart MPO activity, suggesting a possible role for neutrophils in the pathogenesis of nicotine-triggered cardiomyocyte apoptosis. Recent studies have shown that accumulated neutrophils may be involved in the pathogenesis of cardiomyocyte apoptosis by releasing various cytokines and oxidant species (Nakamura *et al*., 2000).

In conclusion, the results of this study suggest that long-term exposure to nicotine can lead to continual DNA damage and that antioxidant supplementation may be of therapeutic benefit in protecting the cardiovascular system from the damaging effects of nicotine contained in cigarette smoke. Further studies are needed to elucidate the mechanisms of nicotine-induced apoptosis and to investigate the direct and specific effects of these antioxidants on apoptosis regulation. Apoptosis represents a potentially preventable form of cell death because of its active nature, and the understanding of this genetic cell death pathway may lead to therapeutic strategies for altering myocardial injury by inhibiting apoptosis.

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