Combined Effects of Ephedrine-Containing Dietary Supplements, Caffeine, and Nicotine on Morphology and Ultrastructure of Rat Hearts

Christopher E. Brown,1 Stanley E. Trauth,1 Richard S. Grippo,1 Bill J. Gurley,2 and Anne A. Grippo1

Cigarette smokers have an increased risk for coronary artery disease. Nicotine present in cigarettes can adversely affect the cardiovascular system via stimulation of both sympathetic and parasympathetic neurons. Caffeine, another cardiovascular and central nervous system (CNS) stimulant, is commonly found in Ephedra and Ephedra-free dietary supplements. These caffeine-containing supplements also have been linked to cardiovascular toxicities. Although no longer on the U.S. market, Ephedra-containing supplements are another source of cardiovascular and CNS stimulants, namely the ephedrine alkaloids. Together caffeine, nicotine, and ephedrine can individually stress the cardiovascular system, and an overlap of these agents is predicted in smokers and dieters. To understand the collective effects of these stimulants on the heart morphology and ultrastructure, rats were exposed to synthetic combinations of nicotine (0.2 mg/kg/day), ephedrine (0–30 mg/kg/day), and/or caffeine (0–24 mg/kg/day) as well as an extract from a caffeine-containing Ephedra supplement (Metabolife 356). After exposure for 3 days, the hearts were removed and examined for hypersensitivity myocarditis and myocardial necrosis. None of the drugs tested alone affected heart tissue morphology, nor were atypical cardiac cells observed. However, in combination, significant interactions were found between caffeine and ephedrine; the interventricular septum was most susceptible, with a significant increase in atypical cardiac cells observed. Nicotine pretreatment caused greater susceptibility to cardiotoxicity associated with combinations of caffeine + ephedrine or Metabolife, particularly in the left ventricle wall. These results indicate that sympathomimetic combinations present in Ephedra supplements may have produced cardiotoxicity reported in consumers of these products. Moreover, the presence of nicotine exacerbates these toxic effects.

Introduction

From 1994 until 2004, Ephedra-containing dietary supplements were marketed as natural diet aids and exercise performance enhancers. The popularity of these products stemmed, in part, from the effects of ephedrine alkaloids (e.g., ephedrine, pseudoephedrine, methylephedrine, nor-ephedrine, and norpseudoephedrine) present in many species of the plant Ephedra (Ma Huang).1 When taken orally, ephedrine alkaloids elicit sympathomimetic effects by stimulating the release of endogenous catecholamines (e.g., norepinephrine) that act as agonists for α- and β-adrenergic receptors (ARs).2 This indirect action on ARs accounts for many of the pharmacologic effects of ephedrine alkaloids.3 In addition, ephedrine can act directly upon the β1-AR subtypes leading to increased heart rate and cardiac output that together result in increased blood pressure. Such direct actions may also promote cardiac arrhythmias via stimulation of the sinoatrial node. Ephedrine’s action as a direct β2-AR agonist accounts for its bronchodilatory effects. As a result of their sympathomimetic activity, Ephedra-containing supplements have been linked to a host of serious adverse health effects, including heart attack, stroke, seizures, psychosis, hepatotoxicity, and death.2,4–12 In 2004, due to an increased risk of adverse health effects, the U.S. Food and Drug Administration (FDA) declared Ephedra-containing supplements adulterated.13 Accordingly, these products were removed from the market.

At their zenith, most Ephedra-containing dietary supplements were formulated as multi-ingredient products, often containing botanical sources of caffeine (e.g., guarana, green tea, and kola nut) and other phytochemicals with sympathomimetic properties (e.g., synephrine, yohimbine, and...
phenylethylamine). One of the most widely consumed central nervous system (CNS) stimulants worldwide, caffeine, is generally perceived by the public to be safe and lacking long-term medical problems. However, caffeine has been shown to exacerbate the stimulant and pressor effects of ephedrine, phenylpropanolamine, and other adrenergic agents. Recent studies have also shown that ephedrine, particularly in the form of Ma Huang (Ephedra), in combination with caffeine can produce myocardial necrosis as well as other structural changes in the heart. Taken together, these findings suggest that ephedrine/caffeine combinations posed a greater risk for adverse health effects.

Tobacco usage has been prevalent for hundreds of years, with many detrimental health effects. The nicotine in tobacco has both central and peripheral nervous system activities, the latter resulting in harm to the cardiovascular system. Nicotine has variable effects on heart rate and its ability to increase blood pressure stems from complex cardiovascular pharmacodynamics, which involve both adrenergic and cholinergic mechanisms. For many Ephedra users that experienced adverse health effects, smoking was a common comorbidity, yet the contribution of nicotine to Ephedra-related cardiovascular side effects has not been studied. Thus, the goal of this research was to assess the effects of nicotine combined with multi-ingredient ephedrine-containing dietary supplements on the structure and function of the mammalian heart. The study’s objectives were to determine the effects of nicotine, in combination with ephedrine and caffeine, on rat heart morphology and ultrastructure. To accomplish this, hearts of rats exposed to combinations of nicotine, ephedrine, and caffeine, or a multi-ingredient Ephedra-containing dietary supplement were examined for tissue changes, including myocardial necrosis. This tissue was analyzed as part of a larger study utilizing implanted wireless transducers to test for cardiovascular effects of nicotine and these dietary supplements.

Materials and Methods
Pharmacological agents
Nicotine tartrate and caffeine were purchased from Sigma Chemical Co. (St. Louis, MO). EPH-833 (AST Sports Science Performance Nutrition, Evergreen, CO), a single-ingredient product containing only Ephedra, was purchased from local retailers and was used for ephedrine administration after extraction per concentrations determined by high-performance liquid chromatography (HPLC) in our laboratory (Table 1). Single lots of Metabolife 356 (Metabolife International, Inc., San Diego, CA), a multi-ingredient product containing Ephedra, natural caffeine sources, and other botanical extracts, were purchased from local retailers. The ephedrine and caffeine content of Metabolife 356 were determined by HPLC after extraction, based on earlier methods used in our laboratory (Table 1). Dosages of all agents were standardized to the amounts of ephedrine and caffeine determined in extracts of each agent, as determined by our HPLC method.

Animal treatment
Male Sprague-Dawley rats (250–330 g) were used for all experiments. All animal protocols were accepted through the Arkansas State University Institutional Animal Care and Use Committee (IACUC). Animals were offered standard Purina rat chow and water ad lib, and were maintained on a 12 hour light/12 hour dark cycle and were unrestrained throughout the experiment. A total of 14 days was chosen for the entire experiment to minimize stress and death of the animals. For purposes of a parallel study (data not shown) and based on procedures and treatments from earlier experiments in our laboratory, rats were implanted on day 0 with CS50-PXT radiowave transmitters (Data Sciences Inc., St. Paul, MN). After anesthesia by intraperitoneal pentobarbital administration (50 mg/kg), the abdominal cavity was opened, and the pressure catheter of the transmitter was inserted into the aorta, and two ECG leads attached to the transmitter were placed at about shoulder level under the skin. Because of the extensive analyses of these data, radiotelemetry findings will be presented in a forthcoming report. However, preliminary studies showed that blood pressure and heart rate stabilized within 4 days of transmitter implantation. Therefore, on day 4, rats in each study group were anesthetized by minimal ether inhalation and implanted subcutaneously between the scapulae with slow-release osmotic pumps (Alzet, Cupertino, CA; volume 212 μL, 14-day release) filled with either nicotine (0.2 mg/kg/day) or saline, which were delivered throughout the duration of the experiment. Beginning on day 8, rats were briefly anesthetized by volatile ether and dosed by oral gavage for three successive days (doses 1, 2, and 3 on days 8, 9, and 10 of the experiment, respectively) with 1.5 mL extracts of each treatment, as summarized in Table 1. In previous experiments in our laboratory, three successive doses of EPH-833 (100 mg/kg/dose) caused cardiac physiological changes in male Sprague-Dawley rats. In this study, we used the same 100 mg/kg/dose of EPH 833 to compare to our earlier data. Remaining doses of individual agents (i.e., ephedrine and caffeine) were chosen based on the levels of ephedrine and caffeine found in the extract of Metabolife 356 (full = that found in one Metabolife 356 tablet = ~23 mg ephedrine/kg/day + 160 mg caffeine/kg/day; half = that found in half a tablet = ~12 mg ephedrine/kg/day + ~80 mg caffeine/kg/day). This approach allowed statistical comparisons of the same amount of ephedrine (as EPH 833 extract) and/or pure caffeine found in Metabolife 356, vs. administration of the multi-ingredient Metabolife 356 supplement itself.

Cardiovascular tissue harvesting and analysis
On day 11, rats were decapitated; the hearts and lungs were excised immediately and placed into 10% neutral buffered formalin for at least 48 hours. After fixation, the formalin was extracted and replaced by 70% ethanol solution. The heart with major blood vessels was dissected from other tissues, and sectioned to allow measurement of the base-to-apex distance (BAD). The heart was then cut cross-sectionally at the equidistant point of the BAD, and the anterior section was further cut cross-sectionally at the pulmonary trunk, revealing the semilunar valve (Fig. 1). A third cross-sectional cut was made to separate the anterior portion into equal halves, which allowed viewing of three surfaces by scanning electron microscopy (SEM). A fourth cross-sectional cut was made posterior to the third cut, which allowed for histological analysis of the posterior portion of the heart, and an apical (anterior) portion.
SEM procedures

Tissue samples were dehydrated in a graded series of ethanol solutions (70%–100%), followed by fluid exchange to 100% amyl acetate. A Samdri-780 critical point drier (Tousimis Research Corporation, Rockville, MD) was used (31°C, 1072 psi, ventilation rate 100 psi/min) to remove amyl acetate, and then tissue samples were mounted on 12.7-mm aluminum stubs and coated with gold/palladium (Cressington sputter coater; Cressington 108 Sputter Coater; Cressington Scientific Instruments Ltd., Watford, United Kingdom). Tissues were analyzed both qualitatively and quantitatively with a Vega TS 5136XM scanning electron microscope (Tescan USA, Inc., Cranberry Township, PA). Five measurements, including left ventricle (LV), right ventricle (RV), and interventricular septum (IVS), were taken at the midpoint of the BAD to ensure consistent analyses between specimens. These measurements were representative of an average of values ($n=5$) from each structure, since the ventricular walls and the IVS were not of uniform thickness. The diameter of the LV was also measured, as well as a composite measurement, consisting of LV, its lumen, and the IVS; these were not averaged (Fig. 2). The aortic semilunar valve was observed for abnormal cells or damage to the valvular tissue.

Histological procedures

Specimens were prepared according to standard techniques: After infiltration and embedding in paraffin, tissue blocks were sectioned (Reichert-Jung 820 rotary microtome; Leica Biosystems Nussloch GmbH, Nussloch, Germany) into 8-μm serial ribbons, with sections affixed to microscope slides with Haupt’s gelatin fixative. Fifteen slides were generated to produce a total count of 60 sections for each tissue block. Stains included hematoxylin and eosin (H&E) to show general cytology and to perform differential cell counts based on nuclei, and a modified Barbeito-Lopez trichrome staining technique (BLTS), which was used to detect myocardial degeneration. Coverslips were then affixed to each stained slide with Permount.

### Table 1. Concentrations of Ephedrine and Caffeine in Solutions Administered by Gavage to Rats on Days 8–10

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ephedra mg/1.5 mL dose</th>
<th>Approx. mg/kg/dose</th>
<th>Caffeine mg/1.5 mL dose</th>
<th>Approx. mg/kg/dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water control</td>
<td>30</td>
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<td>0</td>
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<tr>
<td>Saline pretreated</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Nicotine pretreated</td>
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<td>100</td>
<td>0</td>
<td>0</td>
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<td>EPH 833 extract (EPH)</td>
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<td>12</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Saline pretreated</td>
<td>3</td>
<td>12</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Nicotine pretreated</td>
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<td>12</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Caffeine half dose (caff half)</td>
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<td>0</td>
<td>24</td>
<td>80</td>
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<tr>
<td>Saline pretreated</td>
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<td>0</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Nicotine pretreated</td>
<td>0</td>
<td>0</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>EPH extract + caffeine half dose (EPH + caff half)</td>
<td>3.5</td>
<td>12</td>
<td>24</td>
<td>80</td>
</tr>
<tr>
<td>Saline pretreated</td>
<td>3.5</td>
<td>12</td>
<td>24</td>
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<td>Nicotine pretreated</td>
<td>3.5</td>
<td>12</td>
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<tr>
<td>EPH extract + caffeine full dose (EPH + caff full)</td>
<td>7</td>
<td>24</td>
<td>48</td>
<td>160</td>
</tr>
<tr>
<td>Saline pretreated</td>
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<tr>
<td>Nicotine pretreated</td>
<td>7</td>
<td>24</td>
<td>48</td>
<td>160</td>
</tr>
<tr>
<td>Metabolife half dose (Metab half)</td>
<td>3.5</td>
<td>12</td>
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<td>80</td>
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<td>Saline pretreated</td>
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<td>Nicotine pretreated</td>
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<tr>
<td>Metabolife extract full dose (Metab full)</td>
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<td>24</td>
<td>48</td>
<td>160</td>
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<td>Nicotine pretreated</td>
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Histologically, the myocardium and IVS of the tissue samples were observed for any cellular changes or infiltration of mononuclear inflammatory cells. A Nikon Eclipse E600 light microscope (Nikon Instruments, Inc., Melville, NY) with an attached Nikon digital camera (DXM 1200) was used to observe slides and capture photomicrographs.

Stereological techniques

Stereological techniques were employed to determine numerical densities of both cardiac and noncardiac cell populations. Noncardiac cell populations, which are also referred to as atypical cardiac cell populations, consisted of mononuclear inflammatory cells and apoptotic cardiac myocyte nuclei. Cardiac cell nuclei were chosen by their ellipsoid shape and relatively weak staining profile, and noncardiac cell nuclei by their relatively intense staining and spherical shape (Fig. 3). These nuclear characteristics were used to identify each cell as cardiac or noncardiac (atypical) and to determine numerical densities of both cell populations: photomicrographs (400×; sampling area 0.0360 mm²) of the LV and RV free walls and the IVS were randomly sampled with a grid micrometer and a random-numbers table. A grid of evenly spaced points was randomly tossed on the photomicrograph, and points containing cardiac and noncardiac cell nuclei were differentially counted. The percent volume (V%) for both cardiac and noncardiac cell types was calculated using the equation

\[ V_V = \frac{\sum p}{\sum P} \]

where \( p \) = point counts for each nuclear profile; and \( P \) = total number of points within the test area. The test grid consisted of 475 points over a total area of 0.0297 mm². Stereological techniques were used on H&E-stained slide 9 in most cases, and the same regions were randomly sampled from posterior and anterior portions of the heart.

Statistical analyses

Analysis of covariance (ANCOVA) was used to test for significant differences between treatment groups for rat heart measurements obtained by SEM. Presurgical mass was used as a covariate to account for possible mass-dependent morphological differences between treatment groups. Kolmogorov–Smirnov tests were used to assess the normality of the distributions. After significant ANCOVA tests, Dunnett’s multiple comparisons were used to compare treatment groups with controls, and Tukey’s multiple comparisons were used to test for differences in the ventricular walls, IVS, LV diameter, and composite measurement of the heart between the treatment groups, as a result of combination dosing. In addition, the BAD measurements were also tested for significance by ANCOVA and Tukey’s and Dunnett’s multiple comparisons for a significant change in the total heart length as a result of combination dosing. Stereological data were analyzed by one-way ANOVA to detect any significant differences in cell population densities among treatment groups. Dunnett’s multiple comparisons and Tukey’s multiple comparisons were then used to determine significant changes in cell population densities. Paired t-tests were used to detect any significant differences in cell population densities between the LV wall, RV wall, and IVS within each treatment group. All statistical analyses were performed with Minitab Version 14 (Minitab, Inc., State College, PA). For all tests, an alpha-level <0.05 was considered significant, with family error adjusted to 0.05 for Dunnett’s and Tukey’s multiple comparisons. Data represent means for each group ± one standard deviation.

Results

Clinical observations

Of the 68 rats treated, six died unexpectedly after one or more exposures to Metabolife 356 extract: four died after saline pretreatment + half-dose Metabolife; one died after saline pretreatment + full-dose Metabolife 356; and one after nicotine pretreatment + full-dose Metabolife 356. Some animals were found dead within 24 hours of administration,
and others were euthanized when cardiovascular and temperature data recorded by radiotransmitters indicated irreversible morbidity.

**Tissue morphology**

As analyzed by SEM after pretreatment with nicotine or saline, plus water, ephedrine, caffeine, or combination dosing, no significant differences from controls administered water were observed in the rat heart morphology (BAD, left and right ventricular wall thicknesses, IVS, and left ventricular diameter) within pretreatment groups (data not shown).

**Stereological analysis**

Based on H&E and BLTS staining, the nuclei of noncardiac cells were pyknotic, thus suggesting myocardial degeneration. Areas containing these cells included infiltration mononuclear inflammatory cells and cells with apparent apoptotic cardiac myocyte nuclei.

In general, the LV wall appeared to be more sensitive to Metabolife 356 or Ephedra + caffeine in rats pretreated with nicotine, compared to those pretreated with saline. In the anterior region of the LV wall (Fig. 4), nicotine pretreatment followed by Ephedra + caffeine half dose resulted in significantly more noncardiac cells (1.435 ± 0.271; n = 4) than in animals given the same supplement, but pretreated with saline only (0.763 ± 0.199; n = 4) (p = 0.05).

In the posterior region of the LV wall (Fig. 5), among saline-pretreated rats, the administration of Ephedra + caffeine significantly increased the number of atypical cardiac cells (p < 0.001), compared to Ephedra alone. In animals administered low-dose Ephedra + caffeine, in which the concentration of Ephedra was 3.5 mg/day, the atypical cardiac cell percent was 1.025 ± 0.277 (n = 4), while in cardiac tissue of those receiving 30 mg/day Ephedra alone, percent atypical cells was 0.268 ± 0.201. In the same region, a significantly (p < 0.001) larger percentage of noncardiac cells was evident in nicotine-pretreated rats later administered full-dose Metabolife 356 (0.971 ± 0.298; n = 9), compared to full-dose Metabolife 356-treated rats pretreated with saline (0.598 ± 0.245; n = 6). Among the nicotine-pretreated rats only, both caffeine half dose (0.945 ± 0.445; n = 3) and Metabolife 356 full dose (0.971 ± 0.298; n = 9) resulted in significantly higher atypical cells counted than water treatment (0.254 ± 0.119; n = 5) (p < 0.001).

In the RV wall anterior (Fig. 6), saline pretreatment, followed by Ephedra + caffeine half dose (0.738 ± 0.377; n = 4), resulted in a higher percentage of atypical cells compared to saline and water controls only (0.267 ± 0.184; n = 8) (p = 0.001); no significant differences were noted in animals

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**FIG. 4.** Percent volume of atypical cardiac cells in rat heart anterior left ventricle (LV). Animals were pretreated with either saline or nicotine for 4 days, which continued during treatment for 3 days with sympathomimetics indicated or with water. *Significant differences between the pretreatment groups. Table 1 lists exact concentrations of ephedrine and caffeine for each treatment group. EPH, ephedra; Caff half, caffeine half dose; Caff full, caffeine full dose.

**FIG. 5.** Percent volume of atypical cardiac cells in rat heart posterior LV. Animals were pretreated with either saline or nicotine for 4 days, which continued during treatment for 3 days with sympathomimetics indicated or with water. *Significant differences between the pretreatment groups; % significant differences within the pretreatment group between the treatment group and water control; % indicates significant differences within the pretreatment group between Ephedra alone versus the same Ephedra concentration together with low-dose caffeine. Table 1 lists exact concentrations of ephedrine and caffeine for each treatment group.
pretreated with nicotine along with any supplement treatment in this heart region.

Abundant atypical cells in the RV wall posterior (Fig. 7) were evident with nicotine pretreatment along with either half-dose Ephedra + caffeine (1.297 ± 0.158; n = 4), full-dose Ephedra + caffeine (1.263 ± 0.105; n = 4), or full-dose Metabolife 356 (0.947 ± 0.319; n = 9) compared to nicotine and water (0.358 ± 0.203; n = 5), with each of these differences significant to p < 0.001. These supplements did not result in higher percentages of atypical cells in this region when nicotine was absent.

For the IVS anterior (Fig. 8), in both saline-pretreated and nicotine-pretreated rats, there was significantly a larger percentage of atypical cells after additional treatment of half-dose Ephedra + caffeine or full-dose Metabolife 356, compared to water treatment (p < 0.05 for each comparison among IVS anterior cells). Among saline-pretreated rats, Ephedra + caffeine half dose resulted in 1.053% ± 0.353% atypical cardiac cells (n = 4); Metabolife 356 full dose resulted in 0.858% ± 0.302% atypical cells (n = 6); water alone resulted in 0.303% ± 0.293% (n = 8) atypical cells. In nicotine-pretreated rats, Ephedra + caffeine half dose resulted in 1.435% ± 0.189% atypical cells (n = 4); Ephedra + caffeine full dose resulted in 1.462% ± 0.185% (n = 4); Metabolife 356 full dose resulted in 1.37% ± 0.467% atypical cardiac cells (n = 4); water alone resulted in 0.400% ± 0.321 atypical cardiac cells.

In the posterior IVS (Fig. 9), again only nicotine pretreatment, but not saline pretreatment, resulted in a higher number of atypical cells when administered with both full and half doses of Ephedra + caffeine (half dose = 1.133 ± 0.300; n = 4; full dose = 1.238 ± 0.231; n = 4), as well as Metabolife 356 at full dose (1.104 ± 0.427; n = 9), compared to nicotine and water (0.400 ± 0.261; n = 5) (p < 0.05 for each comparison among these cells). No significant differences were observed among supplement treatment groups of animals pretreated with saline.

Discussion

The results of this study suggest that a synergistic effect on the rat cardiac morphology occurred as a result of interactions between nicotine, caffeine, and Ephedra. The cardiotoxicity caused by combination dosing of Ephedra and caffeine has already been shown\textsuperscript{19,20,35}; however, the present study revealed an enhancement of cardiotoxicity when nicotine was administered in combination with Ephedra and caffeine.

Our findings suggest that smoking and prior or concurrent nicotine exposure may have been significant predisposing factors to many consumers who experienced cardiovascular toxicities with Ephedra-containing dietary supplements. Moreover, the cardiotoxic effects observed with
this combination of sympathomimetic agents lend credence to the toxicological reasoning behind the FDA's removal of Ephedra-containing dietary supplements.

Earlier investigations of cardiotoxicity due to sympathomimetic agents implicated β-AR stimulation with causing subsequent cardiovascular changes, including vasoconstriction and hypertension. These changes may lead to myocardial ischemia or possibly direct toxicity within cardiac myocytes. In addition, cardiac myocyte apoptosis may follow remodeling mechanisms demonstrated by the infarcted rat heart. Cardiac morphology did not change significantly with stimulant treatment, in either saline-pretreated or nicotine-pretreated rats. Morphological changes under similar combination dosing by Nyska et al. were chiefly observed in the LV free wall and IVS, where atypical cells were found more abundantly after combination stimulant administration. Further, more significant differences may have been seen with a longer period of dosing: In the study by Zhao et al., significant morphological changes in the myocardial infarcted (MI) rat heart were not seen until day 14 post-MI. From days 14 to 28 post-MI, myofibroblasts became the predominating cell type; therefore, myocardial morphological abnormalities may not be seen in the rat hearts until at least 1 week after exposure to nicotine, caffeine, and Ephedra. The dosing regimen used in this study was based on earlier reports of cardiac physiological changes in Sprague-Dawley rats with up to three successive doses of Ephedra-containing dietary supplement. Also, Nyska et al. and Howden et al. reported more exaggerated results for 14-week-old rats as compared to 7-week-old rats. (The age of rats used in the present study was 6.5–9 weeks.) This difference has been suggested by several authors to be due to a diminished ability of older rats to respond to myocardial ischemia compared to younger rats. Stereological techniques using random sampling and a grid micrometer permitted quantification and statistical analysis of atypical cardiac cell populations. Although specific cell types were not differentiated, noncardiac cell type volume fractions indicated significant cardiotoxicity among treatment groups due to pyknotic nuclei and the infiltration of inflammatory cells. These results support earlier findings of cardiotoxicity in rats exposed to caffeine and ephedrine, and facilitate more specific measurement of these changes.

Caffeine alone has not been shown to induce cardiotoxicity; this was substantiated in the present study. Here, in saline-pretreated animals, caffeine did not affect the number of atypical cardiac cells in rats. However, in animals pretreated with nicotine and administered caffeine alone, at the lower dose tested, the atypical cardiac cell volume was higher in the posterior LV.
Exposure to Ephedra-derived ephedrine alone, even at a relatively high concentration, did not cause significant increase in atypical cardiac cells, regardless of nicotine pretreatment. When much lower concentrations of the same Ephedra extract were combined with caffeine, however, either as a combination of the individual agents or as contained in Metabolife 356, a significant increase in atypical cardiac cells was observed, suggesting a cardiotoxic interaction when caffeine and ephedrine are coadministered. Nicotine apparently caused a predisposition to the cardiotoxic effects of caffeine and ephedrine in all heart regions studied, except the anterior RV free wall.

Dunnick et al.\textsuperscript{20} found significant cardiotoxicity and morbidity at 25 or 50 mg ephedrine/kg, in combination with 15 or 30 mg caffeine/kg after a single dose. Interestingly, ephedrine administered as Ephedra (Ma Huang) was found to be more toxic than purified ephedrine given in a similar combination dosage. In the present study, Ephedra-derived ephedrine was used, and higher concentrations of caffeine were administered (160 or 80 mg/kg), based on that present in Metabolife 356. These higher caffeine doses resulted in significantly greater numbers of atypical cells in the anterior RV and IVS, even in combination with a lower Ephedra dose (23 or 12 mg ephedrine/kg) in saline-pretreated rats. The use of ephedrine derived from the Ephedra extract may have intensified the cardiotoxicity seen, and again nicotine pretreatment seemed to exacerbate the cardiotoxic effects of these agents. An exacerbation of the cardiotoxic effects seen with Ephedra-containing supplements may be attributable to enhanced sympathomimetic activity stemming from inhibition of catechol-O-methyltransferase (COMT) by catechin polyphenols present in guarana and green tea. COMT, an enzyme widely distributed in tissues, functions to deactivate biologically active endogenous and exogenous catechols, including the catecholamine neurotransmitters dopamine, epinephrine, and norepinephrine.\textsuperscript{42} Catechin polyphenols, such as (−)-epigallocatechin-3-gallate and (−)-epicatechin-3-gallate, are found in high concentrations in green tea and guarana extracts.\textsuperscript{42,43} After oral administration, catechin polyphenols distribute into many peripheral tissues, including cardiac muscle.\textsuperscript{44}

The IVS was most susceptible to the effects of caffeine and ephedrine. In the anterior section of this region, both caffeine + ephedrine combination as well as the multicomponent supplement Metabolife 356 resulted in larger numbers of atypical cells compared to water controls, in both saline- and nicotine-pretreated rats. However, only rats pretreated with nicotine responded negatively to supplements in the posterior region of the IVS. Changes to the ventricular tissue could be particularly detrimental to overall cardiovascular health, considering that the high-pressure forces it must sustain for proper ejection volume to the arterial vasculature.

Metabolife 356, a dietary supplement consisting of Ephedra- and guarana-derived caffeine, showed significant signs of cardiotoxicity over control groups. Among the 68 animals tested in this study, all six premature deaths followed Metabolife 356 administration. Neither Ephedra (at higher concentration than was available in Metabolife 356) nor caffeine, either alone or in combination, was fatal. Rats pretreated with nicotine and administered high-dose Metabolife 356 demonstrated significantly higher volume percentages of atypical cardiac cells than nicotine + water in the posterior sections of all three cardiac regions, as well as the anterior IVS, suggesting a cardiotoxic effect of Metabolife 356 that is further enhanced by nicotine. Evidence from purified caffeine and Ephedra treatments after nicotine pretreatment may implicate other constituents of Metabolife 356 in affecting the noncardiac cell-type volume fraction. An alternate explanation to this effect may be differing concentrations between the Metabolife 356 and caffeine/Ephedra dosing regimens.

Scarabelli et al.\textsuperscript{38} reported that apoptotic factors are produced by endothelial cells during an ischemic response. These findings would suggest that cardiotoxicity occurs within a certain radius of coronary blood vessels. This type of multifocal cardiotoxicity, which was also reported by Nyska et al.,\textsuperscript{19} would not likely be seen with random sampling of the myocardium, as in this study. It remains unclear whether the multifocal nature of cardiotoxicity caused by combination dosing of nicotine, Ephedra, and caffeine can be described as diffuse cardiotoxicity or as radiating from multifocal coronary vessels.

Given that significant effects were seen under a relatively short time course, combination dosing of these agents implies potent and toxic effect. Therefore, we suggest that consumers of combination dietary supplements to be particularly cautious of simultaneous tobacco usage.

Conclusions

Nicotine, caffeine, and Ephedra interacted synergistically to increase atypical cardiac cells in rats, particularly in the IVS. Nicotine exposure therefore may have predisposed the consumers who experienced negative cardiovascular effects with caffeinated Ephedra-containing dietary supplements.

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