Objective: The objectives were to analyze the cardiac effects of exposure to tobacco smoke (ETS), for a period of 30 days, alone and in combination with beta-carotene supplementation (BC). Research methods and procedures: Rats were allocated into: Air (control, n = 13); Air + BC (n = 11); ETS (n = 11); and BC + ETS (n = 9). In Air + BC and BC + ETS, 500 mg of BC were added to the diet. After three months of randomization, cardiac structure and function were assessed by echocardiogram. After that, animals were euthanized and morphological data were analyzed. One-way and two-way ANOVA were used to assess the effects of ETS, BC and the interaction between ETS and BC on the variables. Results: ETS presented smaller cardiac output (0.087 ± 0.001 vs. 0.105 ± 0.004 l/min; p = 0.007), higher left ventricular diastolic diameter (19.6 ± 0.5 vs. 18.0 ± 0.5 mm/kg; p = 0.024), higher left ventricular (2.02 ± 0.05 vs. 1.70 ± 0.03 g/kg; p < 0.001) and atrium (0.24 ± 0.01 vs. 0.19 ± 0.01 g/kg; p = 0.003) weight, adjusted to body weight of animals, and higher values of hepatic lipid hydroperoxide (5.32 ± 0.1 vs. 4.84 ± 0.1 nmol/g tissue; p = 0.031) than Air. However, considering those variables, there were no differences between Air and BC + ETS (0.099 ± 0.004 l/min; 19.0 ± 0.5 mm/kg; 1.83 ± 0.04 g/kg; 0.19 ± 0.01 g/kg; 4.88 ± 0.1 nmol/g tissue, respectively; p > 0.05). Ultrastructural alterations were found in ETS: disorganization or loss of myofilaments, plasmatic membrane infolding, sarcoplasm reticulum dilatation, polymorphic mitochondria with swelling and decreased cristae. In BC + ETS, most fibers showed normal morphological aspects. Conclusion: One-month tobacco-smoke exposure induced functional and morphological cardiac alterations and BC supplementation attenuates this ventricular remodeling process.

Key Words: beta-carotene; smoking; cardiac function; hypertrophy; ventricular dilation.

Active and passive exposure to tobacco smoke (ETS) is an important cause of morbidity and mortality (Hays et al., 1998; NRC, 1986). Multiple studies have shown that chronic smokers are at increased risk of diseases related to atherosclerosis such as coronary obstruction, acute myocardial infarction, and sudden death. In this connection, chronic smoking causes endothelium dysfunction, increased oxidation of LDL-cholesterol, reduction of blood levels of HDL-cholesterol, and increased blood levels of adhesion molecules and fibrinogen, joint factors which may lead to platelet aggregation and eventually vascular spasm (Ridker et al., 2001).

Tobacco-smoke exposure has also been related to other cardiac effects. Greenspan et al. (1969) showed acute effects of nicotine on hemodynamic and functional cardiac variables. Rats exposed to chronic carbon monoxide, another component found in the vapor phase of mainstream cigarette smoke, showed increase in the endothelin-1 gene expression and induced myocardial hypertrophy (Loennechen et al., 1999). Impairment of the left ventricular function as evaluated by transthoracic echocardiography has also been shown in rats exposed to cigarette smoke for 30 days and 4 months (Castardeli et al., 2005; Paiva et al., 2003b). These findings suggest that exposure to nicotine/tobacco smoke may be associated with alterations of both functional and morphological cardiac variables.

The mechanism of the alterations induced by ETS is unknown. However, it is known that mainstream cigarette smoke is composed of several billion semi-liquid particles per cm$^3$ within a mixture of combustion gases with relatively high concentrations of free radicals, which are oxidants or pro-oxidants (Counts et al., 2004). Free radicals induce functional and structural damage of cardiac myocytes and may play an important role in acute coronary syndromes and heart failure (Ide et al., 1999; Siwik et al., 1999), where imbalance of oxidative stress status has been shown (Li et al., 2003). In this view, antioxidant nutrients such as carotenoids (Anand et al., 1997) are attractive agents that could protect against...
We hypothesized that ETS would adversely affect functional and structural cardiac variables and that this effect would be prevented by BC. Therefore, the objective of this study was to investigate the effects of tobacco-smoke exposure, isolated or in association with dietary BC, on ventricular remodeling in rats.

Materials and Methods

Experimental protocol. Six-week-old male Wistar rats (n = 44) were used in the study; the animals were housed and taken care of in compliance with all NIH guidelines. The experimental protocol was approved by the Animal Ethics Committee of the Faculdade de Medicina de Botucatu, UNESP, São Paulo, Brazil. The animals were randomly allocated into four groups and fed a cereal based diet for three months: (1) Group Air (control; n = 13), control rats neither exposed to tobacco smoke nor supplemented with BC; (2) Group Air + BC (n = 11) rats fed with the standard diet, supplemented with 500 mg of BC per kg of diet (Sigma/ St. Louis, MO) milled in feed; (3) Group ETS (n = 11), rats exposed to tobacco smoke; (4) Group BC + ETS (n = 9), rats exposed to cigarette smoke plus BC supplementation. The ETS and BC + ETS animals were exposed to cigarette smoke in a chamber (dimensions 95 × 80 × 65 cm) connected to a smoking device based on a model published by Wang et al. (1999) and modified by Paiva et al. (2003b, 2005). The smoke was drawn out of commercial cigarettes (composition per unit: 1.1 mg of nicotine; 14 mg of tar; and 15 mg of carbon monoxide) with a vacuum pump and was exhausted into the smoking chamber. All ETS and BC + ETS animals were exposed to tobacco smoke during the last 30 days of the study; in the first 14 days the number of cigarettes was gradually increased from 5 to 10 cigarettes over a 30-min period, twice in the morning and twice in the afternoon; for the remaining follow-up period, 10 cigarettes were used in each smoking trial. In a previous study, with similar tobacco exposure time, samples of arterial blood were collected from rats, and carboxyhemoglobin was measured. The carboxyhemoglobin levels in smoke-exposed rats were greater than in the control group (control rats: 0.9 ± 0.7% and ETS rats: 5.3 ± 2.8%, p < 0.008) (Castardeli et al., 2005). Thus, the carboxyhemoglobin values confirmed the efficacy of the exposure of smoking animals to cigarette smoke.

Biochemical determination. Rat blood was placed into a centrifuge tube and allowed to clot to obtain serum. Lipid hydroperoxide was measured with 0.1 ml of sample and 900 μl of a reaction mixture containing 100 μM of xylene orange, 250 μM of FeSO4, 25 mM of H2SO4 and 4 mM of butylated hydroxytoluene in 90% (v/v) methanol. Mixtures were incubated for 30 min at room temperature before measurement at 560 nm. Spectrophotometric determinations were performed in a Pharmacia Biotech spectrophotometer (model 974213, Amersham Pharmacia Biotech; Cambridge, U.K.) (Diniz et al., 2005).

Statistical analysis. Data are reported as means ± SEM. The study design used was a two-factor factorial (Montgomery, 1991, Norman and Streiner, 1993). A one-way ANOVA with three degrees of freedom (DF) was applied to observe the existence of difference between treatments and 40 DF for the residuals. In addition, to assess the main effects and the interaction between ETS and diet supplementation, the DF were decomposed in 1 DF to ETS, 1 DF to diet supplementation with BC and 1 DF to the interaction of ETS + BC with a two-way ANOVA. If an interaction of BC and ETS was observed a Tukey post-hoc analysis was performed.

RESULTS

Echocardiographic Study

Table 1 summarizes the echocardiographic data. There were differences only in the cardiac output (CO) among the groups...
assessed by one-way ANOVA; where the values from ETS group was smaller than Air group. Considering two-way ANOVA, no interaction was found between tobacco-smoke exposure and supplementation with BC. Values of left ventricular diastolic diameter (LVDD), LVDD/body weight (BW) and left atrium/BW were higher and cardiac index and CO were lower in ETS animals (groups ETS and BC + ETS) than in non-ETS animals (groups Air and Air + BC). In the BC-supplemented animals (groups Air + BC and BC + ETS), values for fractional shortening (FS) and ejection fraction (EF) were lower than in non-BC animals (groups Air and ETS). Tobacco exposure and BC supplementation did not have any effect on the other echocardiographic variables (Table 1).

Morphological and Oxidative Stress Data

The effects of ETS and BC supplementation on BW, atrium and ventricular weights are shown in Table 2. Analyzed by one-way ANOVA, the values of BW and atrium weight, RVW/BW, LVW/BW, and AW/BW ratio were higher in ETS group in comparison with Air group \((p < 0.05)\). In contrast, the BC supplementation attenuated these alterations in RVW/BW, LVW/BW, and AW/BW ratio, since there were no differences between BC + ETS and Air groups \((p > 0.05)\). Considering two-way ANOVA, no interaction was found between ETS and BC when BW, LVW, RVW and variables were considered. BW was similar when tested for the main effect ETS \((p = 0.987)\) and when tested for the main effect BC \((p = 0.226)\). Significantly higher values were found for LV weight and RV weight in the ETS animals (groups ETS and BC + ETS) when compared with non-ETS animals (groups Air and Air + BC). In addition, considering atrium weight, LV weight/BW, RV weight/BW, and atrium/BW, the results indicate that there was significant interaction of BC supplementation and ETS when those variables were considered. Values of LV weight/BW, RV weight/BW, and atrium/BW in group ETS were significantly higher than those found in groups Air and BC + ETS; values of atrium weight in group ETS were also higher than in group Air. No differences were observed in the comparison between groups Air and Air + BC.

The wet-to-dry-weight ratios of liver, lung and left ventricle and the oxidative status of the rats, assessed by the levels of lipid hydroperoxide in the liver, are shown in Table 3. Analyzed by one-way ANOVA, the values of wet-to-dry weight ratios of lung and LV were higher in ETS group than in Air group. The lipid hydroperoxide values in the liver where smaller in the Air + BC group than the Air group. Considering two-way ANOVA, wet-to-dry weight ratios in lung and LV tissues results indicate that there was significant interaction of BC supplementation and ETS when those variables were considered. Values of LV w/d and lung w/d in ETS group were significantly higher than those found in groups Air and BC + ETS. No differences were observed in the comparison between groups Air and Air + BC.

Ultra-structural Study

With regard to ultra-structural study of LV muscle, normal morphological aspects were found in animals from groups Air and Air + BC: sarcoplasm filled with myofibrils, well-defined...
sarcosomes, mitochondria presenting lamellar cristae, regular plasma membranes, nuclei with loose chromatin, and sarcoplasmic reticulum vesicles between myofibrils (Figs. 1A, 1B and 2A, 2B, respectively). In group ETS, focal changes were found in muscle fibers, consisting of disorganization or absence of myofilaments, infolding of plasma membrane, dilatation of sarcoplasmic reticulum, polymorphic and swelling mitochondria associated with decreased cristae (Figs. 1C, 1D).

In animals of group BC + ETS, some fibers showed focal alterations as disorganization or absence of myofilaments and infolding of the plasma membrane, but most of fibers had normal morphological aspects (Figs. 2C, 2D).

### DISCUSSION

Our results showed that ETS induced myocardial hypertrophy (both left and right ventricular weight), ventricular enlargement (increase in LVDD) with preservation of the ventricular geometry, as shown by the maintenance of values for LV wall thickness/LVDD in ETS. Further, the alterations of muscle fibers found in group ETS give support to a hypothesis of direct lesion from tobacco smoke on the heart. Alteration of ventricular weight, structure and alterations of ventricular geometry and volume in response to alteration of loading conditions or myocardial injury are viewed as examples of

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### TABLE 2

Morphological data

<table>
<thead>
<tr>
<th>Smoke</th>
<th>Diet</th>
<th>n</th>
<th>Final BW (g)</th>
<th>LVW (g)</th>
<th>RVW (g)</th>
<th>AW (g)</th>
<th>LVW/BW (g/kg)</th>
<th>RVW/BW (g/kg)</th>
<th>AW/BW (g/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>No BC</td>
<td>13</td>
<td>425 ± 15</td>
<td>0.722 ± 0.031</td>
<td>0.195 ± 0.009</td>
<td>0.079 ± 0.003</td>
<td>1.70 ± 0.03</td>
<td>0.46 ± 0.01</td>
<td>0.186 ± 0.007</td>
</tr>
<tr>
<td>Air</td>
<td>BC</td>
<td>11</td>
<td>424 ± 11</td>
<td>0.747 ± 0.022</td>
<td>0.191 ± 0.007</td>
<td>0.085 ± 0.005</td>
<td>1.77 ± 0.04</td>
<td>0.45 ± 0.01</td>
<td>0.198 ± 0.010</td>
</tr>
<tr>
<td>ETS</td>
<td>No BC</td>
<td>11</td>
<td>408 ± 8</td>
<td>0.821 ± 0.017</td>
<td>0.232 ± 0.008</td>
<td>0.097 ± 0.005</td>
<td>2.02 ± 0.05</td>
<td>0.57 ± 0.02</td>
<td>0.238 ± 0.014</td>
</tr>
<tr>
<td>ETS</td>
<td>BC</td>
<td>9</td>
<td>441 ± 16</td>
<td>0.808 ± 0.040</td>
<td>0.211 ± 0.011</td>
<td>0.085 ± 0.005</td>
<td>1.83 ± 0.04</td>
<td>0.48 ± 0.02</td>
<td>0.191 ± 0.007</td>
</tr>
<tr>
<td>P1</td>
<td>(3DF)</td>
<td></td>
<td>0.405</td>
<td>0.045</td>
<td>0.007</td>
<td>0.038</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.003</td>
</tr>
<tr>
<td>P2</td>
<td>ETS</td>
<td></td>
<td>0.987</td>
<td>0.008</td>
<td>0.002</td>
<td>0.057</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.036</td>
</tr>
<tr>
<td>P3</td>
<td>BC</td>
<td></td>
<td>0.226</td>
<td>0.824</td>
<td>0.178</td>
<td>0.523</td>
<td>0.148</td>
<td>0.004</td>
<td>0.103</td>
</tr>
<tr>
<td>P4</td>
<td>ETS × BC interaction (1 DF)</td>
<td></td>
<td>0.197</td>
<td>0.512</td>
<td>0.340</td>
<td>0.048</td>
<td>0.003</td>
<td>0.012</td>
<td>0.007</td>
</tr>
</tbody>
</table>

*Note.* Values are means ± SE; n = number of rats. BC = beta-carotene supplementation; ETS = exposed to cigarette smoking; BW = body weight; LVW = left ventricular weight; RVW = right ventricular weight; AW = atrium weight (left + right). P1 = p value of one-way ANOVA with 3 degree of freedom (DF); P2 = p value of two-way ANOVA with 1 DF to ETS effect; P3 = p value of two-way ANOVA with 1 DF to BC effect; P4 = p value of two-way ANOVA with 1 DF to BC × ETS interaction.

When interaction was observed * = significant differences were observed between ETS × no ETS groups; § = significant differences were observed between BC × no BC groups by Tukey test.

*Means with different superscript letters are significantly different in the multiple comparisons test in the one-way ANOVA (Tukey test).

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### TABLE 3

Wet-and-Dry Ratio of the Liver, Lung and Left Ventricle and Liver Lipid Hydroperoxide Concentration

<table>
<thead>
<tr>
<th>Smoke</th>
<th>Diet</th>
<th>n</th>
<th>Liver (w/d)</th>
<th>Lung (w/d)</th>
<th>LV (w/d)</th>
<th>LP (nmol/g tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>No BC</td>
<td>13</td>
<td>3.26 ± 0.02</td>
<td>4.68 ± 0.06</td>
<td>4.17 ± 0.02</td>
<td>4.84 ± 0.16</td>
</tr>
<tr>
<td>Air</td>
<td>BC</td>
<td>11</td>
<td>3.27 ± 0.04</td>
<td>4.67 ± 0.03</td>
<td>4.19 ± 0.09</td>
<td>3.85 ± 0.16</td>
</tr>
<tr>
<td>ETS</td>
<td>No BC</td>
<td>11</td>
<td>3.20 ± 0.03</td>
<td>5.24 ± 0.08</td>
<td>4.26 ± 0.02</td>
<td>5.32 ± 0.16</td>
</tr>
<tr>
<td>ETS</td>
<td>BC</td>
<td>9</td>
<td>3.29 ± 0.04</td>
<td>4.73 ± 0.06</td>
<td>4.17 ± 0.02</td>
<td>4.88 ± 0.16</td>
</tr>
<tr>
<td>P1</td>
<td>(3DF)</td>
<td></td>
<td>0.279</td>
<td>&lt;0.001</td>
<td>0.038</td>
<td>0.031</td>
</tr>
<tr>
<td>P2</td>
<td>ETS effect (1 DF)</td>
<td></td>
<td>0.265</td>
<td>&lt;0.001</td>
<td>0.114</td>
<td>0.031</td>
</tr>
<tr>
<td>P3</td>
<td>BC effect (1 DF)</td>
<td></td>
<td>0.181</td>
<td>&lt;0.001</td>
<td>0.161</td>
<td>0.042</td>
</tr>
<tr>
<td>P4</td>
<td>ETS × BC interaction (1 DF)</td>
<td></td>
<td>0.213</td>
<td>&lt;0.001</td>
<td>0.038</td>
<td>0.409</td>
</tr>
</tbody>
</table>

*Note.* Values are means ± SE; n = number of rats. BC = beta-carotene supplementation; ETS = exposed to cigarette smoking; w/d = wet-and-dry ratio; LV = left ventricular; LP = lipid hydroperoxide. P1 = p value of one-way ANOVA with 3 degree of freedom (DF); P2 = p value of two-way ANOVA with 1 DF to ETS effect; P3 = p value of two-way ANOVA with 1 DF to BC effect; P4 = p value of two-way ANOVA with 1 DF to BC × ETS interaction.

When interaction was observed * = significant differences were observed between ETS × no ETS groups; § = significant differences were observed between BC × no BC groups by Tukey test.

*Means with different superscript letters are significantly different in the multiple comparisons test in the one-way ANOVA (Tukey test).
ventricular remodeling (Cohn et al., 2000; Pfeffer et al., 1985; Pfeffer and Braunwald, 1990). It is well established that hypertrophy plays a key role in the remodeling process. The rearrangement of myocytes can vary, according to the mechanism of hypertrophy. Pressure-overload results in the development of concentric myocardial hypertrophy where parallel sarcomere replication leads to increased wall thickness; in contrast, volume-overload results in series sarcomere replication and eccentric hypertrophy (Carabello et al., 1992).

Our findings in the ETS group of rats suggest that exposure to smoke induced cell growth with maintenance of LV geometry. Therefore, at this point, the cell growth induced by ETS cannot be defined as concentric or eccentric hypertrophy.

FIG. 1. Electron micrographs of the myocardial cells. A, B: Group Air. Myofibrils (M) and mitochondria (m). Plasma membrane (arrow) and endothelial cell (E) in B. Sarcoplasmic reticulum (arrow head). Nucleus with nucleolus (N) in A ×13250. C and D: Group ETS. Loss of myofilaments (*). Polymorphic and swelling mitochondria associated with irregular or decreased cristae (m). Sarcoplasmic reticulum in D (arrow). ×13250.

A noteworthy finding in the present study is that ETS induced relevant alterations in cardiac functional variables. The decreased values for cardiac index and cardiac output indicate a decreased systolic myocardial function whereas the increased values for LA dimension and atrium weight suggest a diastolic dysfunction. It is well accepted that ventricular remodeling is initially a compensatory process influenced by hemodynamic overload or neurohormonal activation (Cohn et al., 2000). However, chronic ventricular remodeling is now recognized as a pathological process, which results in progressive ventricular dysfunction and clinical presentation of heart failure or sudden death (Cohn et al., 2000; Pfeffer and Braunwald, 1990). The results of our study are in agreement with this concept, since
the findings in the ETS animals (groups ETS and BC + ETS) were associated with evidence of ventricular dysfunction. In addition, the water content of the tissues was higher in ETS animals, in accordance with the presence of ventricular dysfunction.

Antioxidants have been studied for their capacity to prevent chronic disease. However, the use of antioxidants in cardiovascular diseases remains controversial. Discrepancy among results from their use may be related to patient characteristics or doses of antioxidants (Jialal and Devaraj, 2003). BC has been better characterized with regard to its antioxidant capabilities; observational and prospective epidemiologic studies have shown an inverse relationship between cardiovascular disease and dietary intake of carotenoids and/or blood levels (Albanes, 1999; Ford and Giles, 2000). Four large interventional trials were designed to test the hypothesis that BC protects against cancer and/or cardiovascular disease development in humans. In these studies, doses of BC were higher than those that could be achieved from the habitual diet, and the BC blood levels were 2–6 times higher than the 95th percentile level of BC in the Health and Nutrition Examination Survey of the United States (Albanes et al., 1997; Hennekens et al., 1996; Omenn et al., 1996; Vogel et al., 1997). Three out of these four intervention trials did not show any protective effect of BC against cancer or cardiovascular disease; actually, some adverse effects were found. In fact, the risk of fatal

**FIG. 2.** Electron micrographs of the myocardial cells. A, B: Group BC. Myofibrils (M) and mitochondria (m). Sarcoplasmic reticulum (arrow head). Plasma membrane (arrow) and endothelial cell (E) in A ×13250. C and D: Group BC + ETS. Mitochondria (m). Infolding of plasma membrane (arrow) in C and loss of myofilaments (*). Sarcoplasmic reticulum (arrow head) in D ×13250.
coronary heart disease was increased in the groups that received either beta-carotene or the combination of alphatocopherol and beta-carotene (Rapola et al., 1997); and the relative risk of death by lung cancer also increased by 17% in the beta-carotene-supplemented heavy smokers vs. placebo group (Omenn et al., 1996). Then, it can be speculated that BC is health promoting when taken at a physiologic level, but may take on circumstantially adverse properties when given at a high dose or in the presence of highly oxidative conditions (Paiva and Russell, 1999).

An important finding of the current investigation was that BC attenuated the remodeling process induced by ETS. In fact, compared with the echocardiographic, anatomical, and ultrastructural findings that occur in rats from group ETS, the combination of ETS and BC supplementation resulted in a reduction of the myocardial hypertrophy, as shown by decreased values of LVW/BW and RVW/BW and milder LV myocardial ultrastructural alterations, as shown by focal alterations of myofilaments, with preservation of the plasma membrane, sarcoplasm reticulum, and mitochondrias.

The mechanism of beta-carotene action in the cardiovascular system is unknown. However, antioxidant supplements may have beneficial cardiac effects after myocardial injuries (Palace et al., 1999; Qin et al., 2003; Sia et al., 2002; Siveski-Illskovic et al., 1995). For instance, in the adriamycin cardiomyopathy model, probucol has been shown to protect cardiac function by maintaining an adequate level of antioxidant status of the heart (Siveski-Illskovic et al., 1995). Following experimental myocardial infarct in rats, probucol increased survival and vitamin E preserved ventricular function (Palace et al., 1999; Sia et al., 2002). Many of the consequences of ETS have been associated with its oxidative characteristics. Indeed, we found increased levels of lipid hydroperoxide in ETS rats, in comparison with non-ETS animals. In contrast, the BC supplementation attenuated the lipid hydroperoxide levels induced by ETS. Therefore, a protective effect of BC might occur through its power of reducing myocardial oxidative stress and attenuating morphological alterations due to ETS.

An important issue is that most of the ingested BC is converted in the enterocytes, mainly to retinol, by the enzyme beta, beta-carotene 15,15′-monooxygenase (Wyss, 2004). This enzyme is highly active in rat intestine by hindering accumulation of beta-carotene in the body; accordingly, rats are classified as “nonaccumulators” (Krinsky et al., 1990). In this connection, it has been mentioned that a high dose of BC, higher than 20 mg/kg body weight per day (Russell, 2004) and a diet sufficient in vitamin A (van Vliet et al., 1996) can enhance BC accumulation in rat tissues. In our study, each animal ingested approximately 40 mg of beta-carotene/kg body weight/day, as the standard diet contained 12,000 IU of vitamin A/kg of diet. In addition, in a previous study (Zornoff et al., 2005), which used the same BC supplementation, the amount of BC given to the animals was absorbed and found in the systemic circulation and stored in the liver (1.18 ± 0.94 mmol/l × 100 and 26.9 ± 14.8 mmol/l × 100, respectively).

In conclusion, our findings suggest that one-month tobacco-smoke exposure induces functional and morphological cardiac alterations and that BC supplementation attenuates this ventricular remodeling process in rats.

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REFERENCES


