Accepted Manuscript

Title: Cardiovascular effects of low versus high-dose beta-carotene in a rat model

Author: Evelin Csepanyi Attila Czompa David Haines Istvan Lekli Edina Bakondi Gyorgy Balla Arpad Tosaki Istvan Bak

PII: S1043-6618(15)00154-1
DOI: http://dx.doi.org/doi:10.1016/j.phrs.2015.07.021
Reference: YPHRS 2883

To appear in: *Pharmacological Research*

Received date: 10-4-2015
Revised date: 26-6-2015
Accepted date: 21-7-2015

Please cite this article as: Csepanyi Evelin, Czompa Attila, Haines David, Lekli Istvan, Bakondi Edina, Balla Gyorgy, Tosaki Arpad, Bak Istvan.Cardiovascular effects of low versus high-dose beta-carotene in a rat model.*Pharmacological Research* http://dx.doi.org/10.1016/j.phrs.2015.07.021

Cardiovascular effects of low versus high-dose beta-carotene in a rat model

Evelin Csepanyi[1], Attila Czompa[1], David Haines[1], Istvan Lekli[1], Edina Bakondi[2], Gyorgy Balla[3], Arpad Tosaki[1], Istvan Bak[1]*

[1]University of Debrecen, Faculty of Pharmacy, Department of Pharmacology, Debrecen, Hungary

[2]University of Debrecen, Faculty of Medicine, Department of Medical Chemistry, Debrecen, Hungary

[3]Hemostasis, Thrombosis and Vascular Biology Research Group, Hungarian of Academy of Sciences, Debrecen, Hungary

*Correspondence:

Dr. Istvan Bak

University of Debrecen, Faculty of Pharmacy, Department of Pharmacology, Bioanalytical Division, H-4032 Debrecen, Nagyerdei krt. 98., Hungary

Telephone, Fax: 00-36-52-255-586

E-mail: bak.istvan@pharm.unideb.hu

Graphical abstract

ABSTRACT

β-carotene (BC), a lipid-soluble tetraterpene precursor to vitamin A, widely distributed in plants, including many used in human diet, has well-known health-enhancing properties, including reducing risk of and treatment for certain diseases. Nevertheless, BC may also act to promote disease through the activity of BC derivatives that form in the presence of external toxicants such as cigarette smoke and endogenously-produced reactive oxygen species. The present investigation evaluates the dose-dependent cardioprotective and possibly harmful properties of BC in a rat model. Adult male rats, were gavage-fed BC for 4 weeks, at dosages of either 0, 30 or 150 mg/kg/day. Then hearts excised from the animals were mounted in a "working heart" apparatus and subjected to 30 minutes of global ischemia, followed by 120 minutes of reperfusion. A panel of cardiac functional evaluations was conducted on each heart. Infarct size and total antioxidant capacity of the myocardium were assessed. Heart tissue content of heme oxygenase-1 (HO-1) by Western blot analysis; and potential direct cytotoxic effects of BC by MTT assay were evaluated. Hearts taken from rats receiving 30 mg/kg/day BC exhibited significantly improved heart function at lower reperfusion times, but lost this protection at higher BC dosage and longer reperfusion times. Myocardial HO-1 content was significantly elevated dose-responsively to both BC dosage. Finally, *in vitro* evaluation of BC on H9c2 cells showed that the agent significantly improved vitality of these cells in a dose range of 2.5-10 μM.

Although data presented here do not allow for a comprehensive mechanistic explanation for reduced cardioprotection at high dose BC, it is speculated that since Fe^{2+} produced as a metabolite of HO-1 activity, may determine whether BC acts as an antioxidant or prooxidant agent, the strong induction of this enzyme in response to ischemia/reperfusion-induced oxidative stress may account for the high-dose BC loss of cardioprotection.

Keywords: beta-carotene, heart, ischemia/reperfusion, heme-oxygenase-1

Chemical compound studied in this article: CID: 5280489

1. INTRODUCTION

1.1 "Functional foods" in prevention of and management of chronic illness

Increasingly intensive efforts are currently being made to characterize properties of plant materials forming regular components of human diet and expand the range of their use in healthcare. Substantial ongoing increases in serious chronic diseases, particularly obesity- and lifestyle-related cardiovascular disorders and related co-morbidities, provide compelling incentive for use of natural products in healthcare – particularly in the form of "functional foods", which are items in normal diet configured to prevent and/or mitigate disease. Such substances are increasingly attractive to healthcare providers, since they are reasonably priced compared to prescription drugs and other potentially traumatic interventions. Moreover, health-enhancing properties for many of these materials have evolved in tandem with vertebrates as mutual adaptive strategies, a process called "xenohormesis" [1]. Hence there is compelling incentive to encourage adoption of diets rich in fruits, seeds, vegetables, legumes, fish oil and other materials rich in compounds that strengthen and stabilize healthy homeostatic processes and reduce risk of cancers, cardiovascular diseases and many other chronic illnesses.

1.2 β-carotene: chemical and biological properties

The present investigation explores the capacity of the vitamin A precursor β-carotene (BC) to influence aspects of myocardial cell, tissue, and organ function that may will allow development of novel strategies for use of this compound in prevention and management of heart disease. This tetraterpene carotenoid is a 536 Da lipid-soluble plant

pigment, widely distributed in nature, including many plants regularly consumed by animals and humans [2]. It is intensely red-orange and is responsible for multi-hued coloration in many plant species; and is also used as a commercial food colorant. Many human foods contain the compound, notably yams, pumpkins and carrots. Some of its medical benefits are well established. For example, the compound has been demonstrated moderately effective as adjuvant treatment in erythropoietic protoporphyria and has additionally shown some promise also been used to reducing risk of age-related macular degeneration; and susceptibility to breast cancer risk in pre-menopausal women [3-5]. A significant cautionary note on clinical use of BC, is evidence that lung cancer risk is increased in smokers by high-dose consumption of the agent [6]. This effect is hypothesized to occur due to instability of the compound in the presence of tobacco smoke – causing its degradation to carcinogenic metabolites [7].

1.3 Ischemia-reperfusion injury to cardiovascular tissue and antioxidant defense

The present investigation, which evaluates cardiovascular effects of BC, is focused on processes underlying ischemia and reperfusion (I/R) injury to the myocardium. Heart disease and cardiac surgery frequently involve procedures that deprive heart tissue of oxygenated blood, resulting in ischemia, a disruption of normal tissue homeostasis. Further derangement of tissue function may occur as a result of re-oxygenation by restoration of blood flow (reperfusion), a process that triggers a burst expression in physiologic production of highly reactive oxygen-containing species during the first 5 minutes of blood reflow [8]. These compounds greatly increase oxidative stress on reperfused tissues and effects that typically include oxidation of myocardial membrane lipids resulting in impairment of cell membrane function. This oxidative stress increase is a primary cause of reperfusion-induced damage [9]. Adverse effects on cardiovascular function caused by

these processes may be counteracted by antioxidant compounds capable of scavenging oxygen-containing free radicals that are the primary agents of oxidative stress-mediated damage. Such cytoprotective agents of (mainly) plant origin produce carotenoids, notably BC, along with polyphenolic compounds and other phytochemicals such as flavonoids, anthocyanidins, proanthocyanidins and other carotenoids which have well documented benefits to cardiovascular health [10-14].

1.4 β-carotene: a double-edged sword in health maintenance

The effects of BC have been intensively studied and both beneficial and potentially harmful effects of the compound have been noted [6,7]. In addition to its ability to affect risk and pathogenesis of cancer, BC has been considered for use in management of heart disease based on its free radical scavenging capacities, with the cautionary note that it may also act as a tissue-damaging prooxidant – depending on the physiologic environment [15,16]. Several clinical studies can be found having investigated the cardiovascular effects of BC, but all these results are rather controversial. Most of these studies found that BC had no any benefit and may have had adverse effects on the risk of death from cardiovascular diseases mainly among smokers [17-19]. The cardioprotective value of beta carotene also appears to vary among individuals based on genetic factors. This effect is illustrated by an intriguing April 2015 report demonstrating that healthy Korean women bearing single nucleotide polymorphisms that correlate with arterial stiffening, were differentially protected by dietary supplementation with BC, along with folate and vitamin E [20]. A primary mechanism by which BC counteracts pathogenesis of cardiovascular disease, has recently been shown to result from the compound`s ability to abate atherogenic processes by inhibition of peroxidation of cardiac-associated lipids [21].

125 The investigation described in the present report was undertaken to evaluate the effects
126 of BC administration in a rat model, segregated into groups administered two different BC
127 dosages during a 4-week period. One test group, defined as “low dose” (LD) was fed 30
128 mg/kg/day; and a second, “high dose” (HD) group received 150 mg/kg/day, with a cohort
129 of control (C) rats given hydroxyethyl cellulose-water vehicle. Following sacrifice at the
130 end of the 4-week dosing period, hearts surgically excised and mounted in a “working-
131 heart” apparatus were evaluated for cardiac function parameters and tissue biomarker
132 correlates of physiological regulation of heart activity. Potentially toxic effects of BC on
133 cardiomyocyte function was conducted by *in vitro* studies of BC dosage effect on H9c2
134 cells. The comparison of outcomes in hearts from animals treated with low-dose, high-
135 dose BC and vehicle, provided a clear perspective into how this compound affected
136 features of heart function relevant to maintenance of healthy heart activity and treatment
137 of disease.

138

139 **2. MATERIALS AND METHODS**

140 *2.1 Animals*

141 The experiments were accomplished using adult male rats (Charles River
142 Laboratories), with a body weight range of 350-400 grams. All animals received humane
143 care in compliance with the “Principles of Laboratory Animal Care” (formulated by the
144 U.S. National Society for Medical Research, as described in U.S. National Institutes of
145 Health publication No. 86-23, revised 1996) and the “Guide for the Care and Use of
146 Laboratory Animals”. Maintenance and treatment of animals used in the present study
147 was additionally approved by the Institutional Animal Care and Use Committee of the
148 University of Debrecen, Debrecen, Hungary. The animals were housed in wire-bottomed

cages (three rats per cage) throughout the study and were maintained on a 12:12-h light-dark cycle; and provided with laboratory rodent chow pellets, and water *ad libitum*.

2.2 Groups and administration of β-carotene

Rats used in the present study were segregated into 3 groups and gavage-administered the following agents: hydroxyethyl cellulose-water (1:4) vehicle control (C); LD-BC (30 mg/kg/day) and HD-BC (150 mg/kg/day) suspended in hydroxyethyl cellulose-water, respectively. BC was obtained from Sigma-Aldrich Kft. (Budapest, Hungary).

2.3 Ischemia-reperfusion and isolated working hearts

Following 4-week treatment with vehicle or BC, the rats were anesthetized with intraperitoneal injections of ketamine-xylazine (75/10 mg/kg), with heparin anticoagulant administered intraperitoneally (1000 IU/kg). After thoracotomy, the hearts were excised and placed in ice-cold modified Krebs-Henseleit bicarbonate buffer (118.5 NaCl, 4.7 KCl, 2.5 $CaCl_2$ x H_2O, 25 $NaHCO_3$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, and 10.0 glucose (in mM)), then cannulated through the aorta and perfused in a Langendorff apparatus in “non-working” mode (100 cm of water) for 5 minutes to flush blood out from the hearts. Subsequently, Langendorff perfusion was conducted using a cannulated pulmonary vein, during which the isolated heart preparatum was switched to working mode (at a filling pressure of 17 cm of the buffer). After 10 minutes of working perfusion, 30 minutes of global ischemia (ISA) was initiated for each procedure, by clamping the pulmonary inflow and the aortic outflow. At the end of the ischemic period, 120 minutes of reperfusion (REP) was initiated by unclamping the inflow and outflow lines. The first 10

minutes of REP was conducted in Langendorff mode to avoid the fatal ventricular arrhythmias as described [22].

2.4 Cardiac function measurements

Baseline assessment of cardiac function for each isolated heart was made following 10 minutes of working perfusion. To examine the recovery of the left ventricle, these parameters were measured after 30, 60, and 120 minutes of REP. Cardiac function evaluation for each experiment was conducted as previously described [22]. Briefly, heart rate (HR) was measured using a computer acquisition system (ADInstruments, PowerLab, Castle Hill, Australia); coronary flow (CF) values were obtained by timed collection of effluent draining from each heart; aortic flow (AF) measurements were made using a calibrated flow meter; cardiac output (CO) was generated as the sum of AF and CF. Stroke volume (SV) was calculated as the quotient of CO/HR [23]; and alteration in SV values, as a function of treatments, were calculated as the ratio of SV at reperfusion divided by baseline SV and multiplied by 100.

2.5 Infarct size determination

Estimations of infarct size (IS) were conducted using the triphenyl tetrazolium chloride (TTC) staining method as previously described [22]. Briefly, following each 30-minute ISA/120-minute REP period, hearts were perfused with 50 ml 1 % (w/v) solution of TTC in phosphate buffer (pH 7.4), and the samples were stored at -70 °C for subsequent analysis. The frozen samples were sectioned, weighted, and blotted dry. The dried sections were scanned on an Epson J232D flat-bed scanner. The infarcted area (white coloration) and the risk area (entire scanned section) were measured using

196 planimetry software (Image J, National Institute of Health, Bethesda, Maryland, USA).
197 Estimates of infarcted zone magnitude were subsequently obtained by multiplying
198 infarcted areas by weight of each slice. The resulting numbers represent weight of the risk
199 zone and the infarcted zone. Infarct size was expressed as percentage of the weight of
200 infarcted tissue and the weight of risk zone (whole heart) [13].
201
202 *2.6 Western blot analysis of heart tissue*

203 Content of HO-1 protein in the myocardium were obtained by Western blot as
204 previously described [22]. Briefly, approximately 300 mg of left ventricular myocardial
205 tissue were homogenized on ice using a tissue homogenizer (IKA T10 basic ULTRA-
206 TURRAX®) in isolating buffer (25 mM Tris-HCl, 25 mM NaCl, 1 mM orthovanadate, 10
207 mM NaF, 10 mM pyrophosphate, 10 mM okadaic acid, 0.5 mM EDTA, 1 mM PMSF,
208 and 1x protease inhibitor cocktail) and centrifuged at 2000 rpm at 4 °C for 10 minutes.
209 The supernatants were transferred to fresh tubes and centrifuged at 10,000 rpm at 4 °C for
210 20 minutes, after which the resulting supernatant was used as cytosolic fraction. The
211 protein concentration was measured by ND-1000 Nano drop spectrophotometer with
212 BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Thirty µg of protein in each
213 sample were loaded in 10 % polyacrylamide gel and resolved using SDS-PAGE
214 electrophoresis and then transferred to 0.45 µm pore size nitrocellulose membrane to
215 concentrate the samples. After blocking the membranes with 7 % nonfat dry milk in
216 TBST, membranes were incubated overnight with primary antibody solution in 1 % of
217 nonfat dry milk in TBST (GAPDH 1/40000, antibody was obtained from Cell Signaling
218 Technology, Boston, MA; and HO-1 1/50 was ordered from Sigma-Aldrich Kft.
219 Budapest, Hungary) at 4 °C. Then, the membranes were washed 3 times, each for 10
220 minutes, in TBST and incubated with horseradish peroxidase-conjugated secondary

antibody solution (Cell Signaling Technology) containing 1 % of nonfat dry milk in TBST, for two hours at room temperature. The membranes were treated with Western blot Enhanced Chemiluminescent HRP substrate (Millipore, Billerica, MA) to visualize the bands. After the Enhanced Chemiluminescent treatment, the membranes were exposed on x-ray films (Agfa, Mortsel, Belgium). The films were then digitalized by flat-bed scanner (Epson J232D) and analyzed using ImageJ program and normalized the HO-1 band intensities to GAPDH.

2.7 MTT cell viability assays for β-carotene cytotoxicity

Evaluation of BC cytotoxicity on cellular survival was accomplished using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [24]. Briefly, H9c2 cells (ATCC, CRL-1446, LGC Standards GmbH, Wesel Germany) dissociated by trituration in medium (Dulbecco's modified eagle's medium from Sigma with 10% FBS, 1% penicillin-streptomycin), were seeded into 96 well plates at a density of 3000 cells/well and cultured for 1 day to establish adhesion of the wells. BC containing medium was prepared as described by Wertz et al. for keratinocyte cultures with some modifications [25]. Cells were treated with 0, 2.5, 5, 10 and 20 μM BC for 4 hours, respectively. Next, following a 30-minute incubation period, half of the wells were treated with 125 μM H_2O_2. Four hours later addition of 20 μl MTT solution (5 mg/ml in PBS) to each well and an additional 3 hours incubation at 37 °C to allow mitochondrial uptake, the medium were removed and cells were lysed by addition of 150 μl of isopropanol, incubated for 15 minutes followed by measurement of absorbance at 570 and 690 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech). Within each experiment, absorbance values were averaged across 4 replicate wells and repeated 3 times. BC cytotoxic effect assessments were estimated based on linear correlation of

absorbance values with MTT-associated H9c2 viability and reported as percentage of cells surviving 4 hours of BC exposure relative to control cells not exposed to BC.

2.8 Tissue antioxidant capacity (TAC)

TAC of heart tissue was measured using the CS0790-1KT antioxidant assay kit (Sigma-Aldrich Kft., BP., Hungary). Briefly, at the end of reperfusion, the hearts were frozen and stored at -70 °C until analysis. Approximately 100 mg of left ventricular myocardial tissue from each heart were homogenized in 0.5 ml of 1x Assay buffer, centrifuged at 12000 rpm, for 15 min at 4 °C and the supernatants collected for assay. Following sample preparation according to manufacturer's instructions, the absorbance was measured at 405 nm using a plate reader (FLUOstar OPTIMA, BMG Labtech). TAC values for each heart was derived from absorbance values and expressed as Trolox equivalent (μM).

2.9 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5 software. The data are expressed as mean +/- SEM. One-way ANOVA followed by Bonferroni post-test was carried out for heart function data analysis. MTT data were compared by t-test. For Western blot and TAC data outcomes, repeated measures of one-way ANOVA followed by Tukey's post-test was conducted. Differences were considered significant at values of $P<0.05$.

3. RESULTS

3.1 β-carotene dose effects on cardiac function in isolated hearts subjected to I/R

Figure 1 shows the dose-responsive influence of 0, 30 or 150 mg/kg/day BC gavage-administered to rats during a 4-week period on cardiac function in hearts isolated from the animals and subjected to 30 minutes of global ISA and 120 minutes of REP in a "working-heart" apparatus. No BC-mediated effects on AF were observed in hearts not subjected to REP, however, relative to hearts from vehicle-treated control animals, hearts from animals subjected to 30 min of ISA followed by 30 and 60 min of REP exhibited significantly increased AF when taken from rats treated with LD-BC; but interestingly, not which were subjected to 120 min of REP or from those fed with HD-BC (Figure 1A). No significant BC dosage effects on CF were observed in non-reperfused hearts, or in hearts from animals subjected to 30 min of I/R (Figure 1B). Furthermore, HD-BC treatment resulted in a significant increase on CF values after 60 and 120 min of REP relative to organs from non-BC-treated controls ($P<0.05$). Likewise CO in non-ISA/REP hearts and those subjected to 120 minutes of REP did not vary significantly as a result of BC treatment, whereas significant CO increases in these hearts relative to organs from non-BC-treated controls were observed in those sustaining reperfusion periods of 30 minutes ($P<0.05$) and 60 minutes ($P<0.05$) from rats receiving 30 mg/kg/day BC, but not 150 mg/kg/day of the agent (Figure 1C). Treatment of animals with 150 mg/kg/day BC resulted in significant increase in HR for I/R hearts ($P<0.05$) (Figure 1D). Evaluation of the effects of BC on heart SV, revealed no significant BC effect on this variable in non-I/R hearts, or organs subjected to I/R. It was further noted that SV values in hearts receiving 30 and 60 minutes of REP from animals treated with 150 mg/kg/day BC were reduced, however it was not significant, relative to those from rats receiving low BC dose (Figure 1E). This apparent loss of cardioprotection at elevated BC dosage was further confirmed by measurement of stroke volume decline (dSV). These results demonstrated that relative to non-I/R hearts, hearts subjected to 30 and 60, but not 120 minutes of REP

exhibited lesser declines in stroke volume when taken from animals treated with 30 mg/kg/day BC than from rats receiving 0 or 150 mg/kg/day BC, however it did not reach the significant level. (Figure 1F).

Hearts were isolated from 3 groups of rats (n = 6 per group). Rats in each test group received hydroxyethyl cellulose-water (1:4) vehicle control (white bars); BC 30 mg/kg/day (black-shaded bars); and BC 150 mg/kg/day (gray-shaded bars) and sacrificed following a 4-week time period of BC treatment. Hearts isolated from each rat were subjected to 30 minutes of global ISA followed by either 120 minutes of REP in an isolated "working-heart" apparatus to induce I/R-induced injury and evaluated for selected cardiac functions. Results are shown as average values from each group of animals ± SEM of AF (1A); CF (1B); CO (1C); HR (1D); SV (1E); and dSV (1F). *****$P<0.05$ for comparison of the magnitude of each cardiac function measured in each test group receiving BC, relative to hearts from vehicle-treated control animals. $^{\#}P<0.05$ for comparison of the magnitude of cardiac function measured in LD-BC relative to hearts from HD-BC treated animals.

3.2 β-carotene dose effects on I/R-induced infarct zone extent and tissue antioxidant capacity

The influence of BC on the extent of I/R injury-associated infarct zones is shown in Figure 2A. Macroscopic analysis of TTC solution-perfused heart sections from rats subjected to 30 minutes of global ISA followed by 120 minutes of REP in a "working-heart" apparatus revealed that LD-BC treatment, correlated with significant reduction of the extent of infarcted myocardium relative to the control values (*$P<0.05$). This protective effect is abolished in hearts taken from animals treated with high-dose BC

319 ($^{\#}P<0.05$), moreover, the average extent of I/R-induced infarcted zones of hearts of these
320 animals was non-significantly increased relative to hearts from rats administered vehicle
321 only.

322 The phenomenon of diminished BC-mediated protective effect on cardiac physiology was
323 also dramatically illustrated in outcomes of experiments to assess contribution of the
324 agent to tissue antioxidant capacity (TAC), a process that has evolved as a homeostatic
325 countermeasure to oxidative stress. As shown in Figure 2B, isolated, I/R-injured working
326 hearts taken from rats administered LD-BC, exhibited TAC values significantly in excess
327 of vehicle-treated controls ($*P<0.05$) and hearts isolated from HD-BC treated animals
328 ($^{\#}P<0.05$). Nevertheless, TAC values measured in hearts from animals received HD-BC
329 were not significantly higher that hearts from control rats received vehicle.

330 Infarct size was measured in hearts (n=4) following 120 min of REP by perfusion with
331 triphenyl tetrazolium (TTC) solution, followed by macroscopic analysis of transverse
332 sections of each heart. Average sizes of infarct zone for hearts in each group ±SEM are
333 shown for each treatment group. TAC values for each tissue sample is expressed as
334 Trolox equivalent (μM) ±SEM are shown for each treatment group. *****$P<0.05$ for
335 comparison relative to values for hearts from vehicle-treated control (C) animals. $^{\#}P<0.05$
336 for comparison relative to values for hearts from HD-BC treated animals.
337

338 *3.3 β-carotene effects on heme oxygenase-1 (HO-1) protein expression*

339 Myocardial tissue levels of HO-1, which is a major mediator of cardiac homeostasis,
340 were determined by Western blot analysis. As shown in Figure 3., the presence of HO-1
341 protein was evaluated in isolated working hearts taken from control rats administered
342 hydroxyethyl cellulose-water vehicle (C), over 4 weeks; a second test group given LD-

343 BC; and a third group, fed with HD-BC. Hearts excised from these animals were either
344 sham-treated (BL-baseline) (Figure 3. panel A), or I/R-injured (I/R) (Figure 3. panel B)
345 by 30 minutes of global ISA and 120 minutes of REP. The results show that in control
346 hearts which were not subjected to I/R (C-BL) HO-1 expression was not significantly
347 different from non-injured hearts removed from rats receiving LD-BC (LD-BL), while
348 high dose treatment (HD-BL) resulted in significantly increased HO-1 level (*$P<0.05$)
349 (Figure 3.A). Furthermore, in hearts from non-treated animals and subjected to I/R (C-
350 I/R) we detected mild but non-significant increase of HO-1 compared to non-ischemic
351 baseline hearts (C-BL) (Figure 3.B) while, production of HO-1 protein in I/R injured
352 hearts from rats fed with either low-dose (LD-I/R) or high-dose BC (HD-I/R), was
353 significantly higher compared to non-treated non-injured (C-BL) group (*$P<0.05$) (Figure
354 3.B). Moreover, HO-1 expression in hearts removed from vehicle treated animals and
355 subjected to I/R (C-I/R) was not significantly different from hearts excised from rats
356 receiving LD-BC and subjected to I/R (LD-I/R), however, hearts from high-dose treated
357 animals expressed significantly elevated levels of HO-1 protein relative to corresponding
358 levels measured in vehicle-treated controls (C-I/R) ($^{\#}P<0.05$) (Figure 3.B).

359 Expression of HO-1 protein in rat myocardium was measured in homogenized
360 cardiac tissue samples from vehicle or BC treated hearts, with or without I/R injury.
361 Western blot analyses were conducted on each tissue homogenate in triplicate and the
362 signal intensity of resulting bands corresponding to HO-1 protein was measured using the
363 Scion for Windows Densitometry Image program. Tissue content of each protein is
364 shown as a ratio of arbitrary units for HO-1 protein to GAPDH signal. Data are expressed
365 as mean ± SEM of 6 different blots. *$P<0.05$ for comparison of average levels of HO-1 in
366 ventricular myocardium from BC-treated animals, versus non-ischemic control (C-BL)

hearts. # P<0.05 for comparison of average levels of HO-1 in ventricular myocardium from BC-treated animals subjected to I/R, versus I/R-injured control (C-I/R) hearts.

3.4 MTT assay-based evaluation of β-carotene cytotoxicity

To determine if the significant loss of cardioprotective capacity of BC when administered at the levels defined as "high-dose" in the present study, experiments were carried out to evaluate cytotoxicity of the agent in rat H9c2 cardiomyoblasts, an *in vitro* model, which along with the MTT cell viability assay, has proven to be a highly versatile tool for use in cardiovascular pharmacology [26]. As shown in Figure 4., significant increases in H9c2 cell viability was observed in cultures of these cells grown 24 hours in Dulbecco's medium supplemented with 2.5, 5 and 10 μM BC, relative to cells grown with no BC added (*P<0.05). Furthermore, this protective effect was not observed in cells treated with 20 μM of BC.

Evaluation of BC cytotoxicity on cellular survival was accomplished using the MTT assay. H9c2 cells grown 24 hours in Dulbecco's modified eagle's medium and treated with 0, 2.5, 5, 10 and 20 μM BC, were followed by 4-hour treatments with 125 μM H_2O_2 and 5 mg/ml MTT reagent, lysis of cells and measurement of absorbance was at 570- and 690 nm. Absorbance values were averaged across 4 replicate wells, and repeated 3 times. Outcomes are shown as average percentage of cell viability for each BC dosage level, relative to control cultures not BC-treated. *****P<0.05 for comparison of average % viability versus non BC-treated cultures.

4. DISCUSSION

Outcomes of the present study contribute to emerging insight into how widely available plant materials may be used to enhance strategies for maintenance of cardiovascular health and develop novel therapeutic approaches. Here, the capacity of β-carotene is evaluated for its ability to alter processes resulting in reduced tissue oxygenation, leading to ischemic injury to the heart and with strong relevance to the kidneys, brain and many other organs [27]. A major finding reported here, is that increasing BC dosage of may not add to any cardiovascular benefits. Moreover, the agent may mediate – and indeed, may exacerbate existing pathological mechanisms.

The efficiency and vigor with which a heart is able to maintain healthy circulation may be quantitatively described by cardiac functional measurements, in particular, AF, CF, CO, SV and dSV [28] and the ability of agents that enhance antioxidant signaling to improve these functions in isolated ischemic/reperfused rat hearts has previously been demonstrated [29]. Accordingly, the significant LD-BC-mediated improvement in AF (Figure 1A), CO (Figure 1C), were not unexpected. Nevertheless, the apparent abolition or mitigation of these protective effects against I/R injury in rats treated with BC at the higher dose is intriguing.

The rat model was selected for the present investigation based on the well-known features of this animal`s physiology its widespread use in cardiovascular research [30] , and extensive use of these animals in previous cardiac-related drug discovery and basic scientific work by authors of this report [10,11,13,22,31,32]. The experiments described here, were nevertheless undertaken in the context of known limitations on the translational value of carotenoid research using rat data. A significant consideration in studies such as the present one, is that although rats and mice readily convert BC to vitamin A, bioavailability of carotenoids through gut absorption is very low, requiring administration of supraphysiologic BC dosages – greatly in excess of levels from any

natural source [33]. The experiments were designed to partly offset this limitation by administration of BC in a high dose range for all conditions. Accordingly, use of these outcomes and findings in design of improved human healthcare strategies must be made conservatively, taking into account experimental versus normal dietary or therapeutic dose ranges. In the present study, even the lowest dose of BC administered to rats is very high, equivalent to more than 2 g/day/70 kg man. On a daily basis, a normal human diet may contain 3 orders of magnitude less BC [34]. The degree of translational value that these findings have for managing cardiovascular disease, will require human nutritional studies – for which the present study provides a guide to major outcomes that might be evaluated. Here it is important to emphasize the value of human, versus extended animal studies. No single animal model completely replicates BC absorption and metabolism of humans [33]. Moreover, since the present study was designed to obtain mostly qualitative evaluations of low-, versus high-dose BC effects on major cardiac parameters, no attempt was made to determine precise rat-to-human dose equivalents. Thus, human clinical evaluations of the effects described here, constitute appropriate and easily accomplished follow-on studies.

Although data presented here do not allow a comprehensive mechanistic analysis, the observation that low-dose-mediated improvement of AF and CO observed at 30 and 60, but not 120 minutes of post-ischemic REP suggest that BC at the lower dosage is effectively quenching oxidative stress-promoting prooxidant compounds produced by cardiac tissue as a consequence of inflammatory reactions triggered by I/R-induced damage and/or otherwise counteracting adverse effect of these metabolites. If this is the case, then it is reasonable to predict that the higher levels of oxidative stress present with longer reperfusion periods, might overwhelm the quenching ability of BC, significantly diminishing the beneficial effects of the 150 mg/kg/day treatments. The relative ability of

BC to quench prooxidant molecules may also account for differences in magnitudes of myocardial infarct zones and tissue antioxidant capacity shown in Figure 2. The significantly reduced average infarct size observed in I/R-injured hearts of animals treated with LD-BC, relative those not administered the agent, is consistent with previous work by authors of the present report, demonstrating that the extent of myocardial infarct zones in I/R-injured hearts may be diminished with agents that reduce oxidative stress [13,22,35]. Accordingly, the significant LD-BC-mediated reduction of infarct size (Figure 2A) and increased TAC (Figure 2B), were not surprising. Moreover, abolition of these effects in hearts of animals treated with 150 mg/kg/day BC, would be expected if the quenching ability of the molecule were suppressed at a higher dose, allowing a more prooxidant cardiac tissue environment at the higher dosage. The dose-responsively increased expression of HO-1 by myocardial tissue observed for both BC and duration of REP shown in Figure 3., further supports the possibility that BC loses quenching potential and may become prooxidant as dosage increases. HO-1 is an ubiqutous antioxidant response to a wide variety of oxidative stressors, hence it is expected that its expression would increase as a result of I/R injury [27].

It is further expected that BC acting as a protective antioxidant would not be diminished by BC also acting as a quenching agent and contributor to TAC, as shown in Figure 2B. However, if this model is accurate, BC at the elevated dose, acting to promote prooxidant tissue environments is expected to stimulate HO-1 production – which is what was observed (Figure 3.). The possibility of such a mechanism is additionally validated, by the observation that HO-1 expression in I/R-injured hearts of rats treated with high-dose BC (HD-I/R) was elevated relative to production of the enzyme by hearts from rats treated with high dose BC, but not subjected to I/R injury (HD-BL) (Figure 3.), since

464 elevated HO-1 expression would be an expected consequence of significantly higher
465 levels of oxidative stress present in I/R-injured tissue.

466 This analysis is supported, albeit to a minor extent by outcomes of the *in vitro* assays
467 for BC cytotoxicity on H9c2 rat myocardial cells. Outcomes of these experiments (Fig.
468 4.) show that viability of these cells is significantly enhanced in comparison to
469 unstimulated control cultures, by addition of BC at 2.5, 5 and 10 μM, with a loss of this
470 effect at 20 μM. Although these data are insufficient on which to base definitive
471 conclusions, the results suggest that as BC dosage is increased, the protective effect of the
472 compound is diminished and may include deleterious properties.

473 An explanation for apparent cardioprotective effect of BC at one selected dosage and
474 mitigation or elimination of that protection at a higher dose is speculative based on the
475 outcomes presented here. This notwithstanding, previous studies provide some clues as to
476 the mechanisms that may underlie the observations in this report. It is known that free
477 radical species of the kind responsible for oxidative stress in vertebrate tissues, react with
478 BC to produce prooxidant degradation products, such as apo-8'-beta-carotenal, that are
479 particularly damaging to subcellular organelles, particularly mitochondria, resulting in
480 further disruption of the redox balance of myocardial cells, oxidation of critical proteins
481 and exacerbation of damage caused by ischemia-reperfusion injury [15,16]. Ironically,
482 the HO-1 expression response to oxidative stressors may also contribute to loss of
483 cardioprotection at elevated BC dosage. This is a possibility because heme oxygenases,
484 including HO-1, reduce oxidative stress by degrading heme produced in the tissue by red
485 blood cell turnover to produce metabolites with cytoprotective qualities, principally
486 biliverdin (converted rapidly into bilirubin) and subtoxic levels of carbon monoxide,
487 which is promotes antioxidant signaling. Interestingly, although ferrous iron (Fe^{2+}) also
488 produced by normal HO-1 activity is a benign metabolite and readily cleared, its presence

489 under some physical settings determines whether BC acts as an antioxidant or prooxidant
490 compound [36]. This property has potential consequences that are paradoxical in the
491 context of the putative role of HO-1. Normally, increased activity of this enzyme reduces
492 oxidative stress and protects tissues from toxic insult. In the present study, I/R injury to
493 cardiac tissue, strongly activated HO-1, resulting in elevated levels of its metabolic
494 products, including Fe^{2+}. Since, as described by Andersen et al., Fe^{2+} enhances the
495 prooxidant properties of BC, influences that elevate HO-1 in the presence of high BC
496 levels, potentially overwhelm the cytoprotective effects of HO-1 and increase oxidative
497 stress on a tissue. This conclusion is speculative and will require further analysis of BC
498 cardiovascular effects. Such a mechanism is nevertheless consistent with the known
499 behavior of BC in the presence of Fe^{2+} - and if valid, accounts for the results shown in
500 Figure 4. Potential relevance of this finding to management of cardiovascular disease is
501 provided by an observation that an inducer of HO-1 administered orally to human
502 patients significantly altered serum levels of transferrin, which is the physiologic
503 transport molecule for Fe^{2+} [37] indicating that aspects of iron metabolism that may also
504 impact BC utilization may be modulated by drugs that affect HO-1 activity and possibly
505 selective chelators of Fe^{2+}.

506 **CONCLUSIONS:** The findings described above, suggest that the level of HO-1 activity
507 in response to I/R injury may be a critical determinant for the role of BC as either a
508 protective agent against I/R-induced heart damage, or as a contributor to I/R-related
509 syndromes through activation of its prooxidant properties. Validity of this model depends
510 on the degree to which redox activities of BC may be influenced in either direction by
511 presence of Fe^{2+} - which has been identified as a primary factor. Ongoing characterization
512 of these processes is continuing by this laboratory. A particularly valuable outcome
513 anticipated for this ongoing research initiative, is the potential for combining use of BC

514 with HO-1 inducers, such as the sour cherry seed extracts described by Csiki et al [37], in
515 modulation of the clinical effects of BC.

516 **FUTURE DIRECTIONS:** Current efforts by authors of this report to mitigate the
517 cardiotoxic effects of β-carotene, while retaining the cardioprotective value of this
518 compound and also HO-1 activity, are focused on development of a novel functional food
519 class containing whole leaf extract of *Tamarindus indica L.* (tamarind), which in March
520 2015 was reported to chelate ferrous iron, with a binding constant of 1.085 mol L^{-1} [38].
521 It is predicted that Fe^{2+} chelation in this potency range will augment transferrin activity to
522 the extent that the capacity of endogenous HO-1 to reduce oxidative stress will be
523 retained, while simultaneously counteracting BC toxicity by removal of Fe^{2+}. This
524 conclusion is currently speculative and await outcome of ongoing studies.

525

526 **ACKNOWLEDGEMENTS**

527 This study was supported by grants from OTKA K-104017 (A. T.) and PD-111794 (I.
528 L.), Grant of UD (I. B.), DEOEC BMC Korea-1/2011 (I. L.) the TÁMOP- 4.2.2.A-
529 11/1/KONV-2012-0045 and TÁMOP-4.2.6.-15/1-2015-0001 (E. Cs., A. Cz., D. H., I. L.,
530 G. B., A. T., I. B.) projects co-financed by the European Union and the European Social
531 Fund. This study was, in part, supported by the Hungarian Academy of Sciences MTA-
532 DE Vasc. Biol. 11003 (G. B.). E. Cs., A. Cz., I. L., and A.T. assisted in fundamental
533 research in the frame of TÁMOP-4.2.4. A/2-11-1-2012-0001 National Excellence
534 Program-elaborating and operating an inland student and researcher personal support
535 system, was realized with personal support. The project was subsidized by the European
536 Union and co-financed by the European Social Fund.

The authors confirm that there are no conflicts of interest with respect to any of the topic material presented herein.

5. REFERENCES

1 Hooper PL, Hooper PL, Tytell M, Vigh L. Xenohormesis: Health benefits from an eon of plant stress response evolution. Cell Stress Chaperones 15 (2010) 761-770.

2 Arnum SDV. Vitamin a. Wiley Online Library 2000.

3 Gandini S, Merzenich H, Robertson C, Boyle P. Meta-analysis of studies on breast cancer risk and diet: The role of fruit and vegetable consumption and the intake of associated micronutrients. Eur J Cancer 36 (2000) 636-646.

4 Seddon JM, Ajani UA, Sperduto RD, Hiller R, Blair N, Burton TC, Farber MD, Gragoudas ES, Haller J, Miller DT, et al. Dietary carotenoids, vitamins a, c, and e, and advanced age-related macular degeneration. Eye disease case-control study group. JAMA 272 (1994) 1413-1420.

5 Thomsen K, Schmidt H, Fischer A. Beta-carotene in erythropoietic protoporphyria: 5 years' experience. Dermatologica 159 (1979) 82-86.

6 Misotti AM, Gnagnarella P. Vitamin supplement consumption and breast cancer risk: A review. Ecancermedicalscience 7 (2013) 365.

7 Russell RM. Beta-carotene and lung cancer. Pure and Applied Chemistry 74 (2002) 1461–1467.

8 Hearse DJ, Tosaki A. Free radicals and reperfusion-induced arrhythmias: Protection by spin trap agent pbn in the rat heart. Circ Res 60 (1987) 375-383.

9 Tosaki A, Das DK. Reperfusion induced arrhythmias are caused by generation of free radicals. Cardiovasc Res 28 (1994) 422; discussion 424-432.

10 Haines DD, Bak I, Ferdinandy P, Mahmoud FF, Al-Harbi SA, Blasig IE, Tosaki A. Cardioprotective effects of the calcineurin inhibitor fk506 and the paf receptor antagonist and free radical scavenger, egb 761, in isolated ischemic/reperfused rat hearts. J Cardiovasc Pharmacol 35 (2000) 37-44.

11 Pataki T, Bak I, Kovacs P, Bagchi D, Das DK, Tosaki A. Grape seed proanthocyanidins improved cardiac recovery during reperfusion after ischemia in isolated rat hearts. Am J Clin Nutr 75 (2002) 894-899.

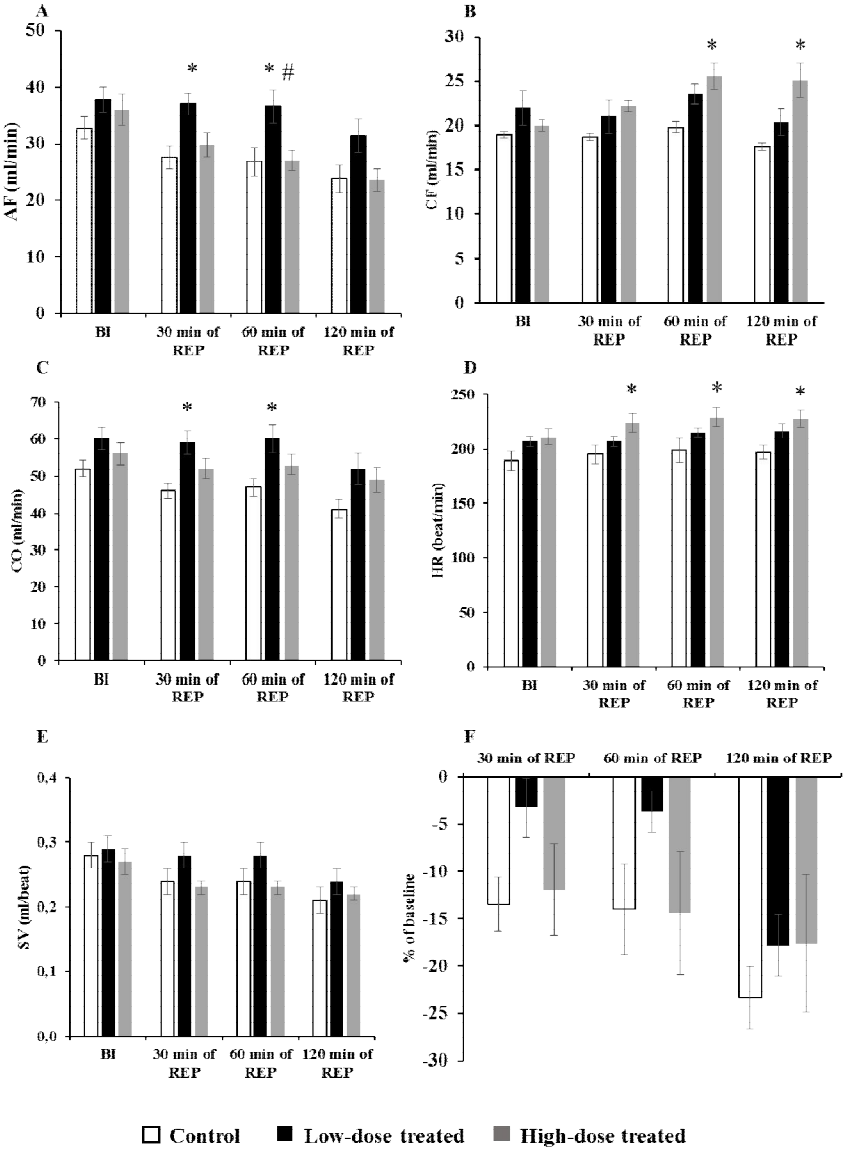
12 Szabo ME, Gallyas E, Bak I, Rakotovao A, Boucher F, de Leiris J, Nagy N, Varga E, Tosaki A. Heme oxygenase-1-related carbon monoxide and flavonoids in ischemic/reperfused rat retina. Invest Ophthalmol Vis Sci 45 (2004) 3727-3732.

13 Bak I, Lekli I, Juhasz B, Nagy N, Varga E, Varadi J, Gesztelyi R, Szabo G, Szendrei L, Bacskay I, Vecsernyes M, Antal M, Fesus L, Boucher F, de Leiris J, Tosaki A. Cardioprotective mechanisms of prunus cerasus (sour cherry) seed extract against ischemia-reperfusion-induced damage in isolated rat hearts. Am J Physiol Heart Circ Physiol 291 (2006) H1329-1336.

14 Haines DD, Varga B, Bak I, Juhasz B, Mahmoud FF, Kalantari H, Gesztelyi R, Lekli I, Czompa A, Tosaki A. Summative interaction between astaxanthin, ginkgo biloba extract (egb761) and vitamin c in suppression of respiratory inflammation: A comparison with ibuprofen. Phytother Res 25 (2011) 128-136.

15 Alija AJ, Bresgen N, Sommerburg O, Langhans CD, Siems W, Eckl PM. Cyto- and genotoxic potential of beta-carotene and cleavage products under oxidative stress. Biofactors 24 (2005) 159-163.
16 Siems W, Salerno C, Crifo C, Sommerburg O, Wiswedel I. Beta-carotene degradation products - formation, toxicity and prevention of toxicity. Forum Nutr 61 (2009) 75-86.
17 Omenn GS, Goodman GE, Thornquist MD, Balmes J, Cullen MR, Glass A, Keogh JP, Meyskens FL, Valanis B, Williams JH, Barnhart S, Hammar S. Effects of a combination of beta carotene and vitamin a on lung cancer and cardiovascular disease. N Engl J Med 334 (1996) 1150-1155.
18 Goodman GE, Thornquist MD, Balmes J, Cullen MR, Meyskens FL, Jr., Omenn GS, Valanis B, Williams JH, Jr. The beta-carotene and retinol efficacy trial: Incidence of lung cancer and cardiovascular disease mortality during 6-year follow-up after stopping beta-carotene and retinol supplements. J Natl Cancer Inst 96 (2004) 1743-1750.
19 Biesalski HK, Grune T, Tinz J, Zollner I, Blumberg JB. Reexamination of a meta-analysis of the effect of antioxidant supplementation on mortality and health in randomized trials. Nutrients 2 (2010) 929-949.
20 Park CY, Jung S, Kim MK, Choi BY, Shin MH, Shin DH, Lee YH, Chun BY, Hong KW, Hwang JY. Habitual dietary intake of beta-carotene, vitamin c, folate, or vitamin e may interact with single nucleotide polymorphisms on brachial-ankle pulse wave velocity in healthy adults. Eur J Nutr (2015)
21 Zhang PY, Xu X, Li XC. Cardiovascular diseases: Oxidative damage and antioxidant protection. Eur Rev Med Pharmacol Sci 18 (2014) 3091-3096.
22 Czompa A, Gyongyosi A, Czegledi A, Csepanyi E, Bak I, Haines DD, Tosaki A, Lekli I. Cardioprotection afforded by sour cherry seed kernel: The role of heme oxygenase-1. J Cardiovasc Pharmacol 64 (2014) 412-419.
23 Szilagyi S, Pollesello P, Levijoki J, Haikala H, Bak I, Tosaki A, Borbely A, Edes I, Papp Z. Two inotropes with different mechanisms of action: Contractile, pde-inhibitory and direct myofibrillar effects of levosimendan and enoximone. J Cardiovasc Pharmacol 46 (2005) 369-376.
24 Arunachalam S, Kim SY, Lee SH, Lee YH, Kim MS, Yun BS, Yi HK, Hwang PH. Davallialactone protects against adriamycin-induced cardiotoxicity in vitro and in vivo. J Nat Med 66 (2012) 149-157.
25 Wertz K, Seifert N, Hunziker PB, Riss G, Wyss A, Lankin C, Goralczyk R. Beta-carotene inhibits uva-induced matrix metalloprotease 1 and 10 expression in keratinocytes by a singlet oxygen-dependent mechanism. Free Radic Biol Med 37 (2004) 654-670.
26 Zordoky BN, El-Kadi AO. H9c2 cell line is a valuable in vitro model to study the drug metabolizing enzymes in the heart. J Pharmacol Toxicol Methods 56 (2007) 317-322.
27 Haines DD, Lekli I, Teissier P, Bak I, Tosaki A. Role of haeme oxygenase-1 in resolution of oxidative stress-related pathologies: Focus on cardiovascular, lung, neurological and kidney disorders. Acta Physiol (Oxf) 204 (2012) 487-501.
28 Gohean JR, George MJ, Chang KW, Larson ER, Pate TD, Kurusz M, Longoria RG, Smalling RW. Preservation of native aortic valve flow and full hemodynamic support with the torvad using a computational model of the cardiovascular system. ASAIO J (2014)
29 Chiba Y, Muraoka R, Noguchi H, Hiramatsu Y, Kimura T, Ihaya A, Morioka K. [significance of selenium deficiency on myocardial protection of the mature and immature rat hearts]. Nihon Kyobu Geka Gakkai Zasshi 39 (1991) 1882-1887.
30 Rice KM, Fannin JC, Gillette C, Blough ER. Efficacy of female rat models in translational cardiovascular aging research. J Aging Res 2014 (2014) 153127.
31 Bak I, Varadi J, Nagy N, Vecsernyes M, Tosaki A. The role of exogenous carbon monoxide in the recovery of post-ischemic cardiac function in buffer perfused isolated rat hearts. Cell Mol Biol (Noisy-le-grand) 51 (2005) 453-459.
32 Varga E, Nagy N, Lazar J, Czifra G, Bak I, Biro T, Tosaki A. Inhibition of ischemia/reperfusion-induced damage by dexamethasone in isolated working rat hearts: The role of cytochrome c release. Life Sci 75 (2004) 2411-2423.
33 Lee CM, Boileau AC, Boileau TW, Williams AW, Swanson KS, Heintz KA, Erdman JW, Jr. Review of animal models in carotenoid research. J Nutr 129 (1999) 2271-2277.

631 34 Schwingshackl L, Hoffmann G, Buijsse B, Mittag T, Stelmach-Mardas M, Boeing H, Gottschald
632 M, Dietrich S, Arregui M, Dias S. Dietary supplements and risk of cause-specific death, cardiovascular
633 disease, and cancer: A protocol for a systematic review and network meta-analysis of primary
634 prevention trials. Syst Rev 4 (2015) 34.
635 35 Juhasz B, Kertesz A, Balla J, Balla G, Szabo Z, Bombicz M, Priksz D, Gesztelyi R, Varga B, Haines
636 DD, Tosaki A. Cardioprotective effects of sour cherry seed extract (scse) on the hypercholesterolemic
637 rabbit heart. Curr Pharm Des 19 (2013) 6896-6905.
638 36 Andersen HJ, Chen H, Pellett LJ, Tappel AL. Ferrous-iron-induced oxidation in chicken liver
639 slices as measured by hemichrome formation and thiobarbituric acid-reactive substances: Effects of
640 dietary vitamin e and beta-carotene. Free Radic Biol Med 15 (1993) 37-48.
641 37 Csiki Z, Papp-Bata A, Czompa A, Nagy A, Bak I, Lekli I, Javor A, Haines DD, Balla G, Tosaki A.
642 Orally delivered sour cherry seed extract (scse) affects cardiovascular and hematological parameters
643 in humans. Phytother Res 29 (2015) 444-449.
644 38 Escalona-Arranz JC, Perez-Roses R, Rodriguez-Amado J, Morris-Quevedo HJ, Mwasi LB,
645 Cabrera-Sotomayor O, Machado-Garcia R, Fong-Lorez O, Alfonso-Castillo A, Puente-Zapata E.
646 Antioxidant and toxicological evaluation of a tamarindus indica l. Leaf fluid extract. Nat Prod Res
647 (2015) 1-4.
648



649

650 **Figure 1.: β-carotene effects on cardiac function in isolated working hearts**.

651

652

653

654

655

656

657

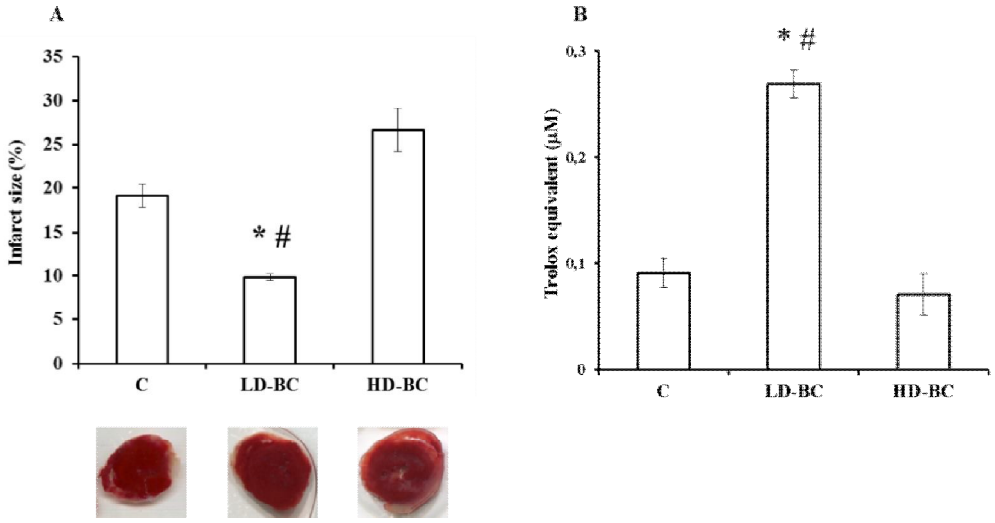


Figure 2.: Effect of β-carotene on infarcted zone magnitude (A) and tissue antioxidant capacity (B).

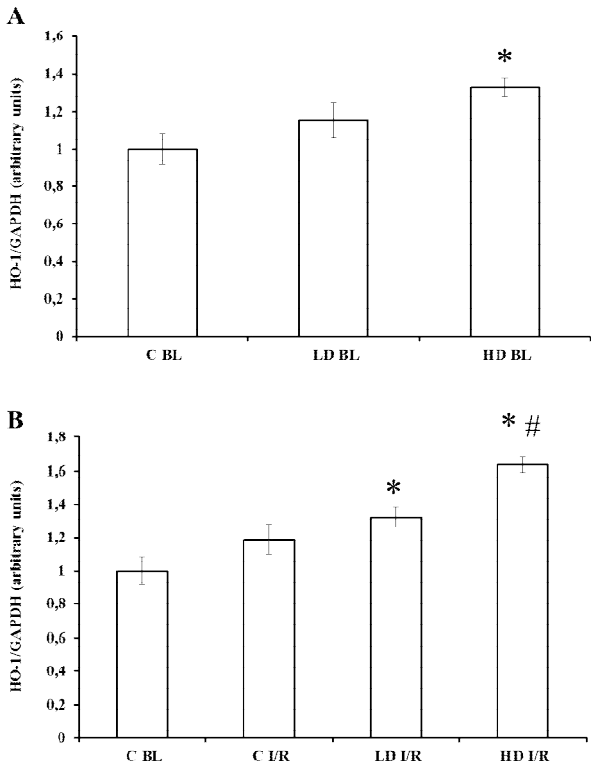
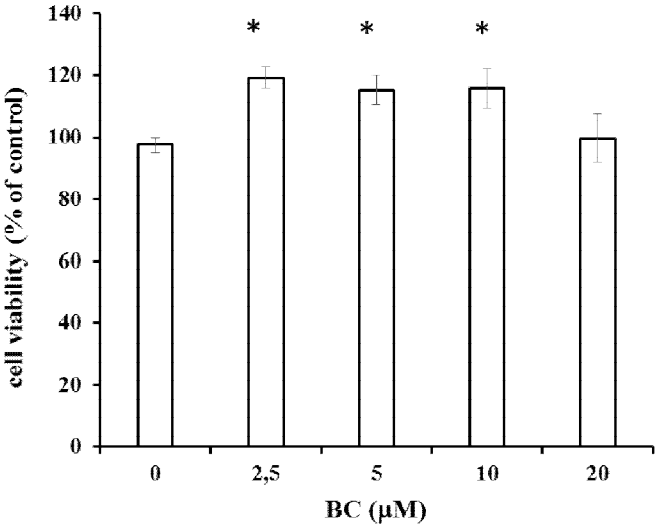


Figure 3.: Western blot analysis for cardiac expression of heme oxygenase-1 (HO-1) protein.



665

666 **Figure 4.: *In vitro* (MTT) assays for β-carotene cytotoxicity on H9c2 cells**.

667