VERSITA

Cent. Eur. J. Med. • 6(1) • 2010 • 58-63
DOI: 10.2478/s11536-010-0050-5

Central European Journal of **Medicine**

The effect of vitamin C on amiodarone-induced toxicity in rat thymocytes

Research Article

Cekic S[1], Pavlovic D[2],Sarac M[3], Kamenov B[4], Dimic A[5], Pavlovic V[1]

[1] *Institute of Physiology, Medical Faculty University in Nis, 18000 Nis, Serbia*

[2] *Institute of Biochemistry, Medical Faculty University in Nis, 18000 Nis, Serbia*

[3] *Medical Faculty University in Nis, 18000 Nis, Serbia*

[4] *Clinic of Pediatrics, Medical Faculty University in Nis, 18000 Nis, Serbia*

[5] *Institute for Prevention, Treatment and Rehabilitation of Rheumatic and Cardiovascular Diseases, Medical Faculty University in Nis, 18000 Nis, Serbia*

Received 19 April 2010; Accepted 5 August 2010

Abstract: Although, the antiarrhythmic effect of amiodarone (AMD) is well characterized, the mechanism of its toxicity on extracardiac tissues is still poorly understood. Several antioxidants have been shown to prevent AMD-induced toxicity by antioxidant and/or non-antioxidant mechanisms. In the current study, we evaluated the possible protective effect, in vitro, of vitamin C on AMD-induced toxicity in rat thymocytes. Rat thymocytes were cultured with increasing AMD concentrations (1–20 μM) with or without vitamin C (1000 μg/ml), for 24 hours. Cells treatment with AMD resulted in a concentration-dependent increase of hypodiploid cells and a significant decrease in cellular glutathione content. Vitamin C combined with AMD significantly decreased the proportion of hypodiploid cells and markedly increased the cellular glutathione content, compared with AMD treatment alone. These results suggest that treatment with vitamin C may prevent AMD-induced toxicity in rat thymocytes by restoring cellular glutathione content.

Keywords: *Amiodarone • Vitamin C • Thymocytes • Apoptosis • Glutathione*

1. Introduction

Amiodarone (AMD) is a strong class III antiarrhythmic agent frequently used in clinical practices. The antiarrhythmic effect of AMD is derived from its multichannel blocking effects, including blocking of potassium and sodium channels and beta receptor blocking [1]. On the other hand, administration is often limited by its toxic side effects (pulmonary, liver, thyroid and cardiomyocytes toxicity), and drug withdrawal is often necessary [2-6]. When administered at higher concentrations, AMD induces mitochondrial swelling, collapse of the mitochondrial membrane potential [7], and apoptosis [5]. AMD-induced cytotoxicity on the cells of the immune system has been also documented in thymocytes [8] and monocytes [9], and also a cellular immunity-decreasing effect has been demonstrated in splenocytes [10]. The ability of AMD to generate the free radicals that may be involved in the pathogenesis of its toxicity has been controversial recently: Some studies have proposed this to be the source of its toxicity [11,12] and other authors have argued that oxidative stress is not involved in the pathogenesis of AMD toxicity [13,14]. Further, despite some reports suggesting that the potential mechanisms of AMD-induced toxicity include direct cytotoxicity, the development of lysosomal phospholipidosis,

* *E-mail: vojapav@yahoo.com*

and membrane destabilization [15,12], the precise mechanism of AMD toxicity remains poorly understood.

Vitamin C (ascorbic acid) is an essential water soluble nutrient that primarily exerts its effect on host–defense mechanisms and immune homeostasis [16]. Under physiological conditions, it functions as a potent reducing agent that efficiently quenches potentially damaging reactive oxygen species (ROS) produced by normal metabolic respiration [17]. Vitamin C enhances antioxidant defenses of T cells, T cells' responsiveness to antigens, and inhibits T cell apoptosis-signaling pathways, indicating a significant role in regulating the immune function [18,19]. Currently, a protective effect of different antioxidants on AMD-induced oxidative stress has been documented in liver [3] and splenocytes [12], but has not been studied in the thymus. Therefore, the present study was designed to evaluate the effect of vitamin C on AMD-induced cytotoxicity in the rat thymus, and to assess whether this effect includes the changes in cellular glutathione content.

2. Material and Methods

2.1. Animals

Experiments were performed on adult male Wistar rats (140–170 g), 8–10 weeks old, bred at the Vivarium of the Institute of Biomedical Research, Medical Faculty, Nis, under conventional laboratory conditions. The experimental animals were treated in accordance with national animal protection guidelines.

2.2. Materials

The culture medium (CM) was prepared using RPMI 1640 (Sigma, St Louis, MO, USA), according to the manufacturer's instructions. The CM contained 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 µg/mL), and 10% fetal calf serum (FCS).

Propidium iodide (PI) was purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Vitamin C (L-ascorbic acid) was obtained from Galenika a.d., Belgrade, Serbia, and amiodarone from EBEWE Pharma Ges, Unterach, Austria. 5-chloromethylfluorescein diacetate (5CMF-DA) was purchased from Molecular Probes, Eugene, OR, USA.

2.3. Preparation of thymocytes

Thymocytes were isolated as described in a previous study (20). Briefly, thymus was aseptically removed and placed in cold CM containing 10% FCS. The thymocytes were released by sliding the thymus along the steel mesh. Cell suspensions were filtered through a sterile nylon filter to remove stroma; the cells were then washed twice with cold CM containing 10% FCS. The viability of the freshly isolated cells was always above 94% (trypan blue exclusion test).

2.4. Cell culture

Isolated thymocytes were seeded into 96-well round bottomed plates (NUNC, Aarhus, Denmark) at a density of $1x10^6$ cells/well. Cells were treated with increasing concentrations of AMD (1–20 µM), vitamin C (1000 µg/ml), AMD and vitamin C, or in CM only (controls). All cell cultures were done in triplicate and cultivated for 24 hours in an incubator (Galaxy, Wolf laboratories, USA) with 5% CO_2 at 37°C. Concentrations of the AMD and vitamin C used in this study were adopted from previous reports [8,18] regarding the effect of these compounds on thymocyte toxicity.

2.5. Apoptotic DNA analysis

Thymocytes undergoing apoptosis were identified by their reduced relative nuclear DNA content using PI staining as originally described [21]. Apoptotic single cells were detected using a flow cytometer (Coulter XL-MCL) as a reduction in the fluorescence of the DNA-binding dye PI in the apoptotic nuclei. The percentage of apoptotic cells (the subdiploid DNA peak in the DNA fluorescence histogram) was determined.

2.6. Measurement the cellular content of glutathione (GSH)

The cellular content of GSH in thymocytes was estimated by 5-chloromethylfluorescein diacetate (5CMF-DA) as described previously [22]. A direct relationship between the intensity of 5CMF-DA fluorescence and the cellular content of glutathione, with correlation coefficient of 0.965 between them, has been documented earlier [22]. GSH levels were detected with a Perkin-Elmer fluorimeter (Wallac Victor^{2}V, Turku, Finland), using an excitation wavelength of 480 nm, while fluorescence emission was collected at 525 nm. For each sample, basal fluorescence intensity values were subtracted from those obtained after different treatments and results are presented as ratio of mean fluorescence intensity (MFI).

2.7. Statistical analysis

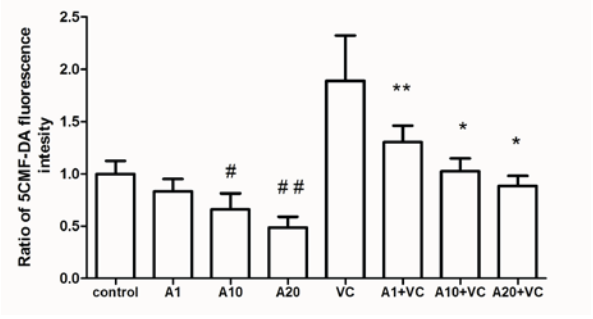
All values are expressed as mean ± SD. Comparisons among groups were carried out using the analysis of variance (ANOVA) coupled to the Dunnett's and Tukey's post hoc tests. A p value < 0.05 was considered significant.

Table 1. In vitro effect of amiodarone and vitamin C on rat thymocyte apoptosis.

Culture conditions	Apoptosis (%)
Control	27.06 ± 3.27
A1	30.03 ± 3.42
A10	40.13 ± 3.15**
A20	44.83 ± 4.79**
VC	25.3 ± 4.10
A1+VC	26.8 ± 3.45
A10+VC	28.26 ± 3.29#
A20+VC	31.4 ± 4.73#

*Rat thymocytes (1x10^6 cells/well) were cultured for 24 hours with increasing concentrations of AMD, vitamin C, and AMD, vitamin C alone or in medium (control), and assayed for apoptosis by flow cytometry, as described in Material and Methods. Results are presented as mean percentage ± SD. Abbreviations: A1 – cells treated with 1 μM AMD, A10 – cells treated with 10 μM AMD, A20 – cells treated with 20 μM AMD, VC – cells treated with vitamin C, A1+VC – cells treated with 1 μM AMD and vitamin C, A10+VC – cells treated with 10 μM AMD and vitamin C, A20+VC – cells treated with 20 μM AMD and vitamin C. **p < 0.01 compared to medium treated (control) cells, #p < 0.05 compared to corresponding AMD treated cells.*

Figure 1. In vitro effect of amiodarone and vitamin C on 5CMF-DA fluorescence intensity (cellular glutathione content) in rat thymocytes.



*Cells (1x10^6 cells/well) were treated with increasing concentrations of amiodarone, vitamin C and amiodarone, vitamin C and in culture medium (control) only. 5CMF-DA fluorescence intensity (cellular glutathione content) was measured using a fluorimeter, as described in Material and Methods. Results are given as ratio of MFI ± SD of triplicate samples. Abbreviations: A1 – cells treated with 1 μM AMD, A10 – cells treated with 10 μM AMD, A20 – cells treated with 20 μM AMD, VC – cells treated with vitamin C, A1+VC – cells treated with 1 μM AMD and vitamin C, A10+VC – cells treated with 10 μM AMD and vitamin C, A20+VC – cells treated with 20 μM AMD and vitamin C. #p < 0.05, ##p < 0.01 compared to control (non-treated cells), *p <0 .05, **p < 0.01 compared to corresponding AMD-treated cells.*

3. Results

AMD-induced thymocyte apoptosis was evaluated by flow cytometric analysis in which the appearance of the subdiploid peak indicated the hypodiploid DNA of apoptotic cells. The percentage of hypodiploid cell concentration dependently increased following a 24-hour exposure to AMD. The effect of AMD reached statistical significance at 10- and 20-μM of the drug (Table 1); the addition of vitamin C significantly decreased ($p < 0.05$) the percentage of hypodiploid cells after AMD treatment.

Since a decrease in cellular glutathione content is a common feature in cytotoxic actions induced by some drugs, to further evaluate the effect of AMD on rat thymocytes, cellular glutathione content of rat thymocytes was examined. We found that AMD treatment resulted in a significant dose-dependent decrease in 5CMF-DA fluorescence intensity, indicating the decrease in cellular content of glutathione (Figure 1). These results correlated with the increased percentage of hypodiploid cells. On the other hand, simultaneous treatment with AMD and vitamin C markedly ameliorated the 5CMF-DA fluorescence intensity ($p < 0.05$, $p < 0.01$), suggesting a significant increase in the cellular content of glutathione (Figure 1).

4. Discussion

Apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of immune homeostatic mechanisms leads to a variety disorders, including infections, allergy, immunodeficiency, and cancer [23]. The thymus, an important primary lymphoid organ where successive stages of cell development generate functionally competent T cells, is a target for various stimuli that may modulate thymic cell differentiation and apoptosis [24].

The results of this study showed that treatment with AMD was able to induce thymocyte apoptosis in vitro. These findings correlated with a marked decrease in the cellular content of glutathione, as determined by 5CMF-DA fluorescence intensity. AMD-induced apoptosis has been shown in rat thymocytes [8], human monocytes [9], and also in different nonimmune cells, including cardiomyocytes [6], alveolar epithelial cells [2], thyroid cells [5], and hepatocytes [4]. Adverse effects of AMD on cell immune responses have been reported to be mediated by phospholipase inhibition or drug-induced oxidative stress and membrane destabilization [15,10]. Our results showing evidence of glutathione involvement in AMD-induced thymocyte apoptosis are in line with the findings that AMD induced pulmonary and liver toxicity with glutathione depletion and the resulting oxidative stress [3]. Glutathione is the major low molecular weight

antioxidant in cells, and its levels often decrease during oxidative stress. Robinson et al. [25] demonstrated impaired T cell and macrophage immune function in rats upon glutathione depletion. Taken together with our results, it seems that exposure of thymocytes to AMD leads to reduction of intracellular glutathione content, which may have an important role in development of oxidative stress and resulting cytotoxicity. Since the oxidant–antioxidant balance is an independent regulator of immune response, and prooxidant predominance results in decreased immune functions, an alternative mechanism for the unfavorable immunological effects of AMD may be its free radical-generating effect [10].

Antioxidant vitamins have a number of biological activities, including immune stimulation and alteration of the metabolic activities of carcinogens. These vitamins can also prevent cell toxicity by inhibiting the DNA damage induced by reactive oxygen metabolites [26]. The present results showed that vitamin C treatment ameliorated the reduced cellular glutathione content and markedly decreased the apoptosis rate of thymocytes, suggesting a protective role for vitamin C on AMD-induced cytotoxicity. A preventive role of antioxidants against AMD-induced toxicity has been demonstrated in liver [12], lung [3], and splenocytes [10]; in this study we have demonstrated a protective role for vitamin C in thymocytes. Vitamin C is a low molecular weight antioxidant that defends the cellular compartment against water-soluble oxygen and nitrogen radicals. It has been demonstrated that vitamin C could modulate the immune system by enhancing the antioxidants properties of T cells and inhibiting T cell apoptosis-signaling pathways [18,16]. A direct redox relationship between vitamin C and glutathione is well documented [27], and it is based on the glutathione-sparing effect of ascorbic acid under oxidative stress. However, another study showed that vitamin C can elevate intracellular glutathione levels through the pentose phosphate pathway [28], although it may not be the main protective mechanism of vitamin C during oxidative stress. Indeed, other studies have proposed that reactive oxygen species sensitize T cells to apoptosis by decreasing Bcl-2 protein expression [29,30]. Such observations are consistent with findings that ascorbic acid prevents apoptosis by upregulating the Bcl-2 protein expression in various cell types [31] and also those of our recent report in rat thymocytes [32]. By detoxifying ROS, antioxidants may thereby reverse the ROS-induced decline in Bcl-2 and prevent cellular death [29,33,34,35].

5. Conclusions

To summarize, in vitro application of vitamin C exerted a protective role against AMD-induced apoptosis and cellular glutathione depletion in rat thymocytes. Thus, sufficient intake of vitamin C by individuals might have an important role in the preventive effect of antioxidants against AMD-induced toxicity and immunosuppression.

Acknowledgements

This work was supported by the Ministry of Science and Environmental Protection of Serbia (Project 145081).

References

[1] Kodama I, Kamiya K, Toyama J. (1999). Amiodarone: ionic and cellular mechanisms of action of the most promising class III agent. Am J Cardiol, 84, 20-8

[2] Bargout R, Jankov A, Dincer E, Wang R, Komodromos T, Ibarra-Sunga, Filippatos G, Uhal BD. (2000). Amiodarone induces apoptosis of human and rat alveolar epithelial cells in vitro. Am J Physiol, 278, 1039-44

[3] Jamshidzadeh A, Baghban M, Azarpira N, Bardbori AM, Niknahad H. (2008). Effects of tomato extract on oxidative stress induced toxicity in different organs of rats. Food Chem Toxicol, 46, 3612-15

[4] Kaufmann P, Torok M, Hanni A, Roberts P, Gasser R, Krahenbuhl S. (2005). Mechanisms of benzarone and benzbromarone induced hepatic toxicity. Hepatology, 41, 925-35

[5] Di Matola T, D'Ascoli F, Fenzi G, Rossi G, Martino E, Bogazzi F, Vitale M. (2000). Amiodarone induces cytochrome c release and apoptosis through an iodine-independent mechanism. J Clin Endocrinol Metab, 85, 4323-30

[6] Isomoto S, Kawakami A, Arakaki T, Yamashita S, Yano K, Ono K. (2006). Effects of antiarrhythmic drugs on apoptotic pathways in H9c2 cardiac cells. J Pharmacol Sci, 101, 318-24

[7] Varbiro G, Toth A, Tapodi A, Veres B, Sumegi B, Gallyas F. (2003). Concentration dependent mitochondrial effect of amiodarone. Biochem Pharmacol, 65, 1115-28

[8] Enomoto R, Komai T, Yoshida Y, Sugahara C, Kawaguchi E, Okazaki K, Kinoshita H. Komatsu H, Konishi Y, Lee E. (2004). Terfenadine induces

thymocyte apoptosis via mitochondrial pathway. Eur J Pharmacol, 496, 11-21

[9] Piccotti JR, LaGattuta MS, Knight SA, Gonzales AJ, Bleavins MR. (2005). Induction of apoptosis by cationic amphiphilic drugs amiodarone and imipramine. Drug Chem Toxicol, 28, 117-33

[10] Agoston M, Cabello RG, Blazovics A, Fehr J, Vereckei A. (2001). The effect of amiodarone and/or antioxidant treatment on splenocyte blast transformation. Clin Chim Acta, 303, 87-94

[11] Ray SD, Patel D, Wong U, Bagchi D. (2000). In vivo protection of DNA damage associated apoptotic and necrotic cell deaths during acetaminophen-induced nephrotoxicity, amiodarone-induced lung toxicity and doxorubicin- induced cardiotoxicity by a novel IH 636 grape seed proanthocyanidin extract. Res Commun Mol Pathol Pharmacol, 107, 137-66

[12] Agoston M, Orsi F, Feher E, Hagymasi K, Orosz Z, Blazovics A, Feher J, Vereckei A. (2003). Silymarin and vitamin E reduce amiodarone-induced lysosomal phospholipidosis in rats. Toxicology, 190, 231-41

[13] Sarma JS, Pei H, Venkataraman K. (1997). Role of oxidative stress in amiodarone-induced toxicity. J Cardiovasc Pharmacol Ther, 2, 53-60

[14] Bolt MW, Racz WJ, Brien JF, Massey TE. (2001). Effects of vitamin E on cytotoxicity of amiodarone and desethylamiodarone in isolated hamster lung cells. Toxicology, 166, 109-18

[15] Massey TE, Leeder RG, Rafeiro E, Brien JF. (1995). Mechanisms in the pathogenesis of amiodarone-induced pulmonary toxicity. Can J Physiol Pharmacol, 73, 1675-85

[16] Hartel C, Strunk T, Bucsky P, Schultz C. (2004). Effects of vitamin C on intracytoplasmic cytokine production in human whole blood monocytes and lymphocytes. Cytokine, 27, 101-6

[17] Arrigoni O, De Tullio MC. (2002). Ascorbic acid: much more than just an antioxidant. Biochim Biophys Acta, 1569, 1-9

[18] Campbell JD, Cole M, Bunditrutavorn B, Vella AT. (1999). Ascorbic acid is a potent inhibitor of various forms of T cell apoptosis. Cell Immunol, 194, 1-5

[19] Wu CC, Doriarajan T, Lin TL. (2000). Effect of ascorbic acid supplementation on the immune response of chickens vaccinated and challenged with infectious bursal disease virus. Vet Immuno Immunopathol, 74, 145-52

[20] Pavlovic V, Cekic S, Kocic G, Sokolovic D, Zivkovic V. (2007a). Effect of monosodium glutamate on apoptosis and Bcl-2/Bax protein level in rat thymocyte culture. Physiol Res, 56, 619-26

[21] Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods, 139, 271-9

[22] Chikahisa L, Oyama Y, Okazaki E, Noda K. (1996). Fluorescent estimation of H2O2-induced changes in cell viability and cellular non-protein thiol level of dissociated rat thymocytes. Jpn J Pharmacol, 71, 299-305

[23] Zhang N, Hartig H, Dzhagalov I, Draper D, He YW. (2005). The role of apoptosis in the development and function of T lymphocytes. Cell Res, 15, 749-69

[24] Savino W, Dardenne M. (2000). Neuroendocrine control of thymus physiology. Endocr Rev, 21, 412-43

[25] Robinson MK, Rodrick ML, Jacobs DO, Rounds JD, Collins KH, Saporoschetz IB, Mannick JA, Wilmore DW. (1993). Glutathione depletion in rats impairs T-cell and macrophage immune function. Arch Surg, 128, 29-34

[26] Verma RS, Mehta A, Srivastava N. (2007). In vivo chlorpyrifos induced oxidative stres: attenuation by antioxidant vitamins. Pestic Biochem Phys, 88, 191-6

[27] Meister A. (1994). Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem, 269, 9397-400

[28] Puskas F, Gergely P, Banki K, Perl A. (2000). Stimulation of the pentose phosphate pathway and glutathione levels by dehydroascorbate, the oxidized form of vitamin C. Faseb J, 14, 1352-61

[29] Hildeman DA, Mitchell T, Aronow B, Wojciechowski S, Kappler J, Marrack P. (2003). Control of Bcl-2 expression by reactive oxygen species. Proc Natl Acad Sci, 100, 15035-40

[30] Pavlovic V, Pavlovic D, Kocic G, Sokolovic D, Jevtovic-Stoimenov T, Cekic S, Velickovic D. (2007b). Effect of monosodium glutamate on oxidative stress and apoptosis in rat thymus. Mol Cell Biochem, 303, 161-6

[31] Saitoh Y, Ouchida R, Kayasuga A, Miwa N. (2003). Anti-Apoptotic Defense of bcl-2 Gene Against Hydroperoxide-Induced Cytotoxicity Together With Suppressed Lipid Peroxidation, Enhanced Ascorbate Uptake, and Upregulated Bcl-2 Protein. J Cell Biochem, 89, 321-34

[32] Pavlovic V, Pavlovic D, Kocic G, Sokolovic D, Sarac M, Jovic Z. (2009). Ascorbic acid modulates monosodium glutamate induced cytotoxicity in rat thymus. Bratisl Lek Listy, 110, 205-9

[33] Prakova G, Gidikova P, Slavov E, Sandeva G, Stanilova S. (2009). Serum neopterin in workers

exposed to inorganic dust containing free crystalline silicom dioxide. Cent Eur J Med, 4, 104-109

[34] Babusyte A, Jeroch J, Stakauskas R, Salakuskas R. (2009). The production of reactive oxygen species in peripheral blood neutrophilis modulated by airway mucous. Cent Eur J Med, 4, 245-252

[35] Cayir K, Kardeniz A, Yildirim A, Kalkan Y, Karakoc A, Keles M, Tekin SB. (2009). Protective effect of L-karnitine against cisplatin-induced liver and kidney oxidant injury in rats. Cent Eur J Med, 4, 184-191