EFFECT OF *ARONIA MELANOCARPA* FRUIT JUICE ON INDICES OF INFLAMMATION AND FIBROSIS IN A RAT MODEL OF AMIODARONE-INDUCED PNEUMOTOXICITY

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ABSTRACT

The effect of *Aronia melanocarpa* fruit juice (AMFJ) on indices of inflammation and fibrosis was studied in a model of amiodarone (AD)-induced pneumotoxicity in rats. AD was instilled intratracheally on days 0 and 2 (6,25 mg/kg as a 3,125 mg/mL water solution). AMFJ (10 mL/kg) was given orally to rats either from day 1 to day 10, or from day 11 to day 27. Thus, the animal groups were: control, AD, AD+AMFJ (day 1-10), and AD+AMFJ (day 11-27). The rats were sacrificed on day 28. The levels of IL-6 and IL-10 were measured in rat serum as markers of inflammation, and hydroxyproline (HP) level was determined in lung tissue as a marker of fibrosis. AD caused a tendency to elevate IL-6 and decrease IL-10. AMFJ counteracted these effects of AD. In rats from group AD+AMFJ (day 1-10), IL-6 level was significantly lower ($p<0,05$) than that of AD group, lower ($p<0,05$) even than the control value. AD significantly increased ($p<0,05$) HP content in lung homogenate. AMFJ antagonized that effect, and in AMFJ-treated rats HP levels did not differ significantly from the control value. Any AMFJ effects were more prominent in rats that were treated with the juice during the first 10 days after AD instillation. In conclusion, AMFJ reduced the signs of inflammation and could have a protective effect against AD-induced pulmonary fibrosis, especially if administered in the early phase after AD instillation.

Key words: *Aronia melanocarpa* fruit juice, amiodarone, lung, inflammation, fibrosis, rats

INTRODUCTION

Amiodarone (AD) is a very effective antiarrhythmic drug. It causes acute pneumonitis resulting in fatal pulmonary fibrosis (8). *In vivo* and *in vitro* studies have shown that AD is not only directly toxic to lung cells (16) but also could induce oxidative stress and increased production of reactive oxygen species (11,19), activation of alveolar macrophages and cytokine release (4,14,20).

Aronia melanocarpa (Michx.) Elliot (black chokeberry) fruits are extremely rich in phenolic compounds (12): procyanidins, flavonoids (mainly from the subclass of anthocyanins) and phenolic acids (chlorogenic and neochlorogenic). *Aronia* berries possess very high antioxidant capacity (9,12,18). Studies have demonstrated that constituents of *Aronia melanocarpa* fruits (flavonoids in-

cluding anthocyanins) possess anti-inflammatory activity due to the suppression of the release of proinflammatory cytokines such as tumor necrosis factor alpha and interleukins (IL-1beta, IL-6, IL-8) (7,17).

The aim of the study was to investigate the effect of *Aronia melanocarpa* fruit juice (AMFJ) on indices of inflammation and fibrosis in a rat model of AD-induced pulmonary toxicity.

MATERIALS AND METHODS

Experimental substances

Amiodarone hydrochloride (AD) and all other chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich Company (Germany). The Quantikine Rat IL-6 and IL-10 immunoassay kits were from R&D Systems (USA).

AMFJ was produced from *Aronia melanocarpa* Elliot fruits grown in the Balkan Mountains, Bulgaria. They were handpicked in September, crushed and squeezed. The juice

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was filtered, pasteurized at 80 °C for 10 min and stored at 0 °C till the experiment. The contents of phenolic substances in 100 mL AMFJ were: total phenolics, 709,3±28,1 mg as gallic acid equivalents; total flavonoids, 189,4±8,6 mg as catechin equivalents; total anthocyanins, 106,8±6,2 mg as cyanidin-3-glucoside equivalents, and quercetin, 11,8 mg. The values were the mean of duplicate determinations of three samples.

Animals and experimental treatments

The study was carried out on 24 male Wistar rats (weight 220-250 g, age 4 months). The animals were housed at a temperature of 22±2 °C and humidity of 50±10 %, given normal pelleted diet and water *ad libitum*.

All the procedures concerning animal treatment and experimentation were conducted in compliance with the national laws and policies, in conformity with the international guidelines (EEC Council Directive 86/609, IL 358, 1, December 12, 1987).

The animals were divided into four groups of 6 rats: control, AD, AD+AMFJ (day 1-10) and AD+AMFJ (day 11-27). The control group received two intratracheal (i.t.) instillations of sterile distilled water (2 mL/kg) on days 0 and 2, and distilled water (10 mL/kg) orally through an orogastric cannula from day 1 to day 10. The AD group received two i.t. instillations of AD (6,25 mg/kg, as a 3,125 mg/mL water solution) on days 0 and 2 (15), and distilled water (10 mL/kg) orally through an orogastric cannula from day 1 to day 10. The rats belonging to AD+AMFJ (day 1-10) group were treated with AD i.t. on days 0 and 2, and with AMFJ orally at a dose of 10 mL/kg from day 1 to day 10. Rats from AD+AMFJ (day 11-27) group were treated with AD i.t. on days 0 and 2, and with AMFJ orally at a dose of 10 mL/kg from day 11 to day 27. The animals were sacrificed on day 28 under thiopental anesthesia (50 mg/kg) by exsanguination through cutting *v. renalis*.

AD was dissolved in distilled water at 60 °C and allowed to cool to room temperature before the i.t. instillation.

Immunological assays of rat serum

The serum of the experimental animals was used for the measurement of IL-6 and IL-10 in pg/mL by the ELISA method in accordance with the immunoassay kits manufacturer's instructions.

Biochemical assays of lung homogenate

Lung homogenate was obtained from the right lung. The tissue was homogenized with KCl (1,15%) in 1:10 ratio. The homogenate was centrifuged (9000 x g, 30 min) and the supernatant was stored on ice. Hydroxyproline (HP) levels were measured in mg/mL as described by Bergman and Loxley (2). The method is based on the release of free HP from collagen by acid hydrolysis.

Statistical analysis

Results are presented as mean ± S.E.M. The data were tested by one-way ANOVA, followed by Dunnett's multiple comparison post test. A level of $p < 0,05$ was considered

significant. All analyses were performed using GraphPad Prism statistical software.

RESULTS

Immunological assays of rat serum

AD instillation resulted in a slight elevation of IL-6 in rat serum (104% of the control) (Fig. 1). AMFJ prevented AD-induced elevation of IL-6. IL-6 in rats from AD+AMFJ (day 1-10) group was 87% of the control value, and in rats from AD+AMFJ (day 11-27) group it was 91% of the control level. Thus, in animals belonging to AD+AMFJ (day 1-10) group, IL-6 was significantly lower than that of AD group ($p < 0,05$). It was significantly lower even than the control value ($p < 0,05$) (Fig. 1).

Fig. 1. Effect of AMFJ on IL-6 levels in rat serum 28 days after i.t. AD instillation; AMFJ applied with AD either from day 1 to day 10, or from day 11 to day 27
* $p < 0,05$ vs control; & $p < 0,05$ vs AD

In AD group, a tendency was observed for reduction of IL-10 to a level that was 91% of the control value (Fig. 2). AMFJ antagonized that effect of AD. In AMFJ-treated animals, IL-10 levels were higher (however, insignificantly) not only than the AD group level but also than the control one. Thus, IL-10 levels were 108% of the control value for AD+AMFJ (day 1-10) group and 106% of the control value for AD+AMFJ (day 11-27) group (Fig. 2).

Biochemical assays of lung homogenate

HP levels are presented on Fig. 3. In rats from AD group, there was a significant increase ($p < 0,05$ vs control) of HP levels in lung tissue (147% of the control value). In rats from AD+AMFJ (day 1-10) group, HP was 112% of the control value, and in rats from AD+AMFJ (day 11-27) group, HP was 129% of the control value. Thus, in rats with AD instillation and treatment with AMFJ, the HP levels did not differ significantly from the control value.

Fig. 2. Effect AMFJ on IL-10 levels in rat serum 28 days after i.t. AD instillation; AMFJ applied with AD either from day 1 to day 10, or from day 11 to day 27

Fig. 3. Effect of AMFJ on HP levels in rat lung tissue 28 days after i.t. AD instillation; AMFJ applied with AD either from day 1 to day 10, or from day 11 to day 27

* $p < 0,05$ vs control

DISCUSSION

Both indirect and direct toxic effects on target cells have been proposed for explanation of AD-induced pulmonary toxicity. There are findings that activation of macrophages and release of inflammatory and cytotoxic mediators drive AD-induced lung fibrosis (4,14). Up to date, there are no literature data on the effect of AD on the levels of the cytokines IL-6 and IL-10 available.

IL-6 is a potent, pleiotropic, inflammatory cytokine secreted by T cells and macrophages. It is produced at the site of inflammation and plays a key role in the acute phase re-

sponse as defined by a variety of clinical and biological features such as the production of acute phase proteins. In this experiment, serum IL-6 was significantly increased in rats in the early phase (on days 3 and 5) after i.t. AD instillation (unpublished data). This could be a result of IL-6 translocation from the lung tissue to the circulation (10). The present data demonstrated that 28 days after AD instillation, serum IL-6 remained still elevated to a certain extent. It is known that when IL-6 activity as a proinflammatory cytokine persists, acute inflammation turns into chronic one that includes immune responses (6). IL-6 seems to play an important role in fibrosis initiation and progression. Rats treated with AMFJ had significantly lower IL-6 levels in comparison both with the AD and the control groups. The effect of AMFJ was more pronounced in rats treated with the juice during the first 10 days after AD instillation. Thus, applied in the early phase of inflammation, AMFJ could, probably, prevent the shift from acute to chronic inflammation. This effect is consistent with the anti-inflammatory activity of the flavonoids which are essential juice constituents (7,17).

IL-10 is a potent anti-inflammatory cytokine. Nowadays it is known that the ability to synthesize that cytokine is not limited to certain T cell subsets, but is a characteristic of almost any leukocytes. Usually, IL-10 synthesis occurs as a consequence of acute and chronic inflammatory responses, and IL-10 neutralization often exacerbates inflammatory lesions (1). AD induced a tendency to decrease IL-10 in rat serum in the acute phase of inflammation (unpublished data), as well as in its chronic phase (current results). AMFJ antagonized that effect of AD, which, probably, contributed to its anti-inflammatory activity.

Since lung HP is almost exclusively derived from collagen (5), whole lung collagen content was estimated by measuring HP levels. The fibrotic response in AD group was confirmed by a significant increase in lung HP above the control values. AMFJ applied either during the first 10 days, or from day 11 to day 27, suppressed that effect of AD. HP levels in both rat groups treated with AMFJ did not differ significantly from the control value, however, the effect of AMFJ was more prominent in rats treated with the juice during the first 10 days after AD instillation.

Numerous data suggest the role of oxidative stress in amiodarone-induced lung fibrosis (11,13,19). Free radical reactions have been suggested to play a contributory role in the fibrogenesis either directly or through inflammatory stimuli. Lipid peroxidation and certain lipid peroxidation products induce genetic overexpression of fibrogenic cytokines, the key molecules in the mechanisms of fibrosis, as well as increased collagen transcription and synthesis. Both events can be downregulated, at least in experimental models, by the use of antioxidants. Consequently, both catalytic and scavenger antioxidants attenuate AD-induced lung injury and fibrosis in animals (3). The ability of AMFJ to scavenge reactive oxygen species has been demonstrated by many authors using different well-established assays (9,12,18). Therefore, AMFJ protective effect against AD-induced pneumotoxicity and lung fibrosis might be at

least partly due to the ability of AMFJ to act as a powerful antioxidant.

CONCLUSION

AMFJ administered to rats in a model of AD-induced pneumotoxicity reduces the signs of inflammation and fibrosis. Its protective effect is, probably, due to its antioxidant and anti-inflammatory properties.

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