

Effects of β -cryptoxanthin alone and in combination with methotrexate on inflammation, cardiac mitochondrial respiration and antioxidant status in experimental arthritis

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Summary

Rheumatoid arthritis (RA) is usually associated with systemic inflammation and cardiovascular disease. The effect of beta-cryptoxanthin (CRY) alone and in conjunction with a subtherapeutic dose of methotrexate (MTX) on inflammation, heart mitochondrial respiration and antioxidant status were studied in rats with adjuvant arthritis. CRY monotherapy did not reduce paw oedema and further impaired several mitochondrial respiratory parameters. Conversely, combination CRY+MTX therapy reduced early paw oedema and plasma IL-17A levels and enhanced lipophilic antioxidant capacity in cardiac mitochondria. These findings suggest varied CRY effects depending on the treatment environment and support the concept that its association with MTX could promote beneficial anti-inflammatory and mitochondrial antioxidant effects in experimental arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic systemic autoimmune disease with persistent inflammation of the synovial tissue resulting in destructive changes of joints and extra-articular manifestations [1]. Dysregulated immune responses including

increased production of pro-inflammatory cytokines, such as interleukin-17A (IL-17A) are associated with systemic inflammation and disease progression. Systemic inflammation is associated with cardiovascular complications, which increase mortality in RA patients [2]. The cardiac manifestations are pericarditis, myocarditis, cardiomyopathy, increased left ventricular mass, congestive heart failure, and ischemic heart disease [2-4]. Methotrexate (MTX) is a cornerstone medicine for RA, however efficacy and toxicity of the treatment varied between patients [2]. Therefore, natural substances with antioxidant and immunomodulatory activity are studied as potential adjuvants to the traditional antirheumatic therapy. β -Cryptoxanthin (CRY) is a xanthophyll carotenoid provitamin A, prevalent in citrus fruits with antioxidant, anti-inflammatory and osteoprotective characteristics [4]. CRY has been reported to protect against the articular cartilage degradation [3]. In experimental adjuvant-induced arthritis (AA) studies, carotenoids have shown their potential as adjunct therapy to MTX treatment [5].

The present study aimed to explore the effects of CRY alone or in conjunction with subtherapeutic dose of MTX on hind paw swelling, plasma IL-17A, blood cells count, and cardiac mitochondrial respiration and antioxidant content in rats suffering from AA.

2. Materials and Methods

2.1. Animals and experimental design

Adult male Lewis rats (6 weeks old, 160-180 g) were randomly divided into five experimental groups (n = 6-8): healthy control (HC), adjuvant arthritis (AA), AA with MTX treatment (0.3 mg/kg, orally 2 times per week), AA with CRY treatment (10 μ g/kg, orally daily) or AA with CRY+MTX treatment at the respective doses of monotherapy. Arthritis was generated by a single intradermal injection of 0.1 mL heat-killed *Mycobacterium butyricum* (12 mg/mL) in incomplete Freund's adjuvant near the base of the tail. Treatments were carried out for 28 days. All experimental techniques were approved by the State Veterinary and Food Administration of the Slovak Republic (Permit No. 3144/16-221/3).

2.2. Assessment of arthritis and inflammatory parameters

The volume of the hind paw was measured by water plethysmometer (UGO BASILE, Italy) on days 14, 21 and 28. The percent increase over baseline was reported as the change of hind paw volume (CHPV). On day 14 and 28 blood samples were taken and plasma concentrations of IL-17A were measured using commercial ELISA kits (R&D Systems, USA). On day 28, platelet, monocyte and neutrophil counts were analyzed using a Balio OV-560 RET hematology analyzer.

2.3. Respiration and antioxidant content in cardiac mitochondria

On day 28, cardiac mitochondria were isolated by differential centrifugation [6] and the respiration was assessed by high-resolution respirometry using an O2k respirometer following the SUIT-008 protocol [7,8]. Isolated heart mitochondria were

added to O2k chambers with MiR05 respiration medium containing 20 mM creatine at 37 °C. Respiratory states were evaluated by sequential addition of pyruvate and malate (5 and 2 mM), ADP (2.5 mM), cytochrome c (10 mM), glutamate (10 mM), succinate (10 mM), FCCP (1 μ M titration steps), rotenone (0.5 μ M) and antimycin A (2.5 μ M). Complex IV activity was assessed following TMPD/ascorbate (0.5+2 mM) addition and subsequent inhibition with sodium azide (200 mM). Oxygen flux was corrected for residual oxygen consumption and expressed as nmol O₂/s/mg mitochondrial protein. The concentration of antioxidants coenzyme Q10 (CoQ10), CoQ9 and α -tocopherol in heart mitochondria was determined by HPLC method [9].

2.4. Statistical analysis

Data are presented as mean $\pm$ SEM. Statistical differences were evaluated using one-way ANOVA followed by Tukey-Kramer post hoc testing. Values of $p < 0.05$ were considered statistically significant.

3. Results

3.1. Changes in hind-paw volume

Untreated arthritic rats exhibited significantly increased hind paw volume compared with healthy controls on days 21 (** $p < 0.01$) and 28 (* $p < 0.05$). CRY monotherapy was associated with pronounced paw swelling on days 14 and 21. In contrast, CRY+MTX significantly reduced hind-paw swelling compared with CRY monotherapy on days 14 (\$\$\$ $p < 0.001$) and 21 (\$\$ $p < 0.01$). By day 28, paw swelling was lower in both MTX and CRY+MTX-treated animals than in untreated AA rats, but without statistical significance (Table.1).

Table 1. Changes in hind paw volume and plasma IL-17A levels in experimental arthritis

Parameter	Day	HC	AA	AA+MTX	AA+CRY	AA+CRY+MTX
		Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM	Mean $\pm$ SEM
Change in hind paw volume (%)	Day 14	35.57 $\pm$ 3.17	30.14 $\pm$ 2.44	36.54 $\pm$ 2.88	84.77 $\pm$ 7.92	38.29 $\pm$ 4.07^{\$\$\$}
	Day 21	41.44 $\pm$ 2.15	90.39 $\pm$ 10.40^{**}	70.91 $\pm$ 8.45	108.21 $\pm$ 8.72[#]	60.32 $\pm$ 5.16^{\$\$}
	Day 28	57.05 $\pm$ 2.57	104.38 $\pm$ 6.63[*]	79.45 $\pm$ 5.99	111.58 $\pm$ 21.11	72.08 $\pm$ 5.33
Plasma IL-17A	Day 14	7.94 $\pm$ 0.19	87.38 $\pm$ 21.98^{***}	53.76 $\pm$ 16.29	76.88 $\pm$ 4.21	37.98 $\pm$ 4.11⁺
	Day 28	7.61 $\pm$ 0.23	24.12 $\pm$ 4.43[*]	33.22 $\pm$ 5.00	27.26 $\pm$ 4.05	22.93 $\pm$ 1.75

Values are presented as mean $\pm$ SEM. HC, healthy control; AA, adjuvant arthritis; MTX, methotrexate; CRY, β -cryptoxanthin; CRY+MTX, β -cryptoxanthin + methotrexate.

3.2. Plasma IL-17A concentrations

Plasma IL-17A was significantly elevated in untreated AA rats compared with healthy controls on days 14 (*** $p < 0.001$) and 28 (* $p < 0.05$). By day 14, MTX produced moderate, non-significant reductions relative to AA. CRY+MTX significantly reduced IL-17A compared with untreated AA rats on day 14 (+ $p < 0.05$). On day 28, CRY+MTX produced slight non-significant reductions relative to AA (Table.1).

3.3. Hematological parameters

Untreated AA rats exhibited substantially higher numbers of monocytes, neutrophils and platelets than the healthy controls ($p < 0.001$). Monotherapy with MTX and CRY caused minor, but not statistically significant, reductions in the numbers of monocytes and neutrophils. CRY alone also lowered platelet counts slightly, while MTX had no obvious effect. Compared with MTX monotherapy, CRY+MTX was associated with reduced monocyte, neutrophil and platelet counts although these did not achieve statistical significance.

3.4. Cardiac mitochondrial respiration and antioxidant status

Cardiac mitochondria from untreated AA rats exhibited reduced complex I (CI)-linked and combined CI+II-linked oxidative phosphorylation (OXPHOS) capacity compared with healthy controls. Maximum electron transfer (ET) capacity supported by CI+II and CII substrates, CIV activity, and the content of CoQ9-OX were decreased vs HC. CRY monotherapy further reduced CI-linked OXPHOS capacity and combined CI+II-linked OXPHOS and ET capacities compared with untreated AA rats. CRY also significantly increased the respiratory response to cytochrome c, consistent with compromised integrity of the outer mitochondrial membrane. The antioxidant status was not affected by CRY monotherapy. In contrast, CRY+MTX did not significantly alter respiratory parameters compared with untreated AA rats and increased mitochondrial contents of CoQ10-OX, CoQ9-OX, CoQ9-RED and α -tocopherol, together with a higher CoQ9-RED/CoQ9-TOTAL ratio.

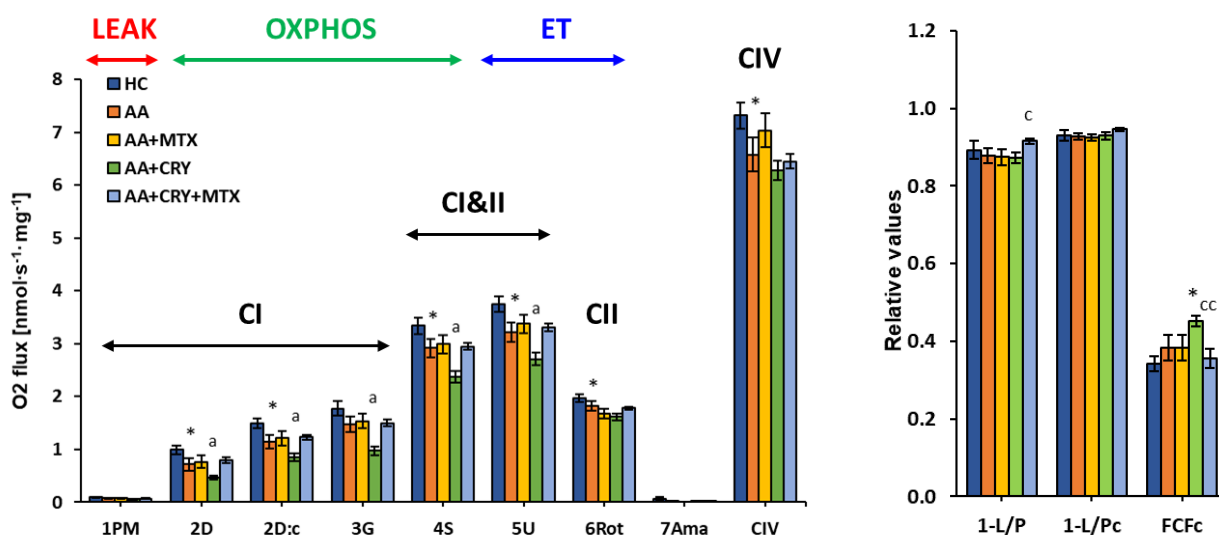


Figure 1. A) Respiration of heart mitochondria (nmol/s/mg protein) following SUIT-008 protocol (1). Mitochondria were added to respiration medium MiR05 (2) with 20 mM creatine at 37°C in the O₂k chamber. The titration steps were: 1PM – pyruvate+malate (5+2 mM), 2D – ADP (2 mM), 2D;c – cytochrome c (10 mM), 3G – glutamate (10 mM), 4S – succinate (10 mM), 5U – FCCP titration until maximum (1 μ M steps), 6Rot – rotenone (0.5 μ M), 7Ama – antimycin A (2.5 μ M). CIV was evaluated from the difference of respiration after addition of TMPD+ascorbate (0.5+2 mM) and subsequent addition of sodium azide (200 mM). The columns show mean $\pm$ sem of ROX-corrected respiration at the titration step shown on x-axis. B) *L-P* coupling efficiency, *L-P* coupling efficiency after cytochrome c and

flux control factor after addition of cytochrome c. CI, CI&II, CII, CIV – mitochondrial complexes. LEAK, OXPHOS, ET – respiratory states.

Experimental groups: HC – healthy control; AA – adjuvant arthritis control (untreated); MTX – AA rats treated with methotrexate (0.3 mg/kg, twice weekly); CRY – AA rats treated with β -cryptoxanthin (10 μ g/kg, daily); and CRY+M – AA rats treated with a combination of methotrexate and β -cryptoxanthin (0.3 mg/kg, twice weekly and 10 μ g/kg, daily). * p <0.05 vs HC, ^a p <0.05 vs AA, ^c p <0.05, ^{cc} p <0.01 vs AA+CRY.

4. Discussion and Conclusion

The effects of CRY differed between monotherapy and combination treatment. CRY alone did not attenuate paw swelling and further impaired selected mitochondrial respiratory parameters, whereas CRY+MTX reduced early paw swelling and plasma IL-17A, and induced an increase of lipophilic antioxidants content in heart mitochondria. Increased monocyte, neutrophil and platelet counts in untreated AA rats supported systemic inflammatory activation, although treatment-associated hematological changes were limited. Further work is needed to clarify the mechanisms and translational significance of these treatment-context-dependent effects.

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Author contributions

W.A. and Z.S. performed the experimental work and contributed to writing the original draft of the manuscript. Z.B., E.H., S.K., K.S., S.P., F.D. and J.M. contributed to the experimental work. K.B. conceptualization, funding acquisition, reviewed and revised the manuscript.

Conflicts of interest

None

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