ORIGINAL ARTICLE

Carnosine inhibits degradation of hyaluronan induced by free radical processes *in vitro* and improves the redox imbalance in adjuvant arthritis *in vivo*

František Dráfi [1], **Katarína Bauerová** [1], **Katarína Valachová** [1], **Silvester Poništ** [1], **Danica Mihalová** [1], **Ivo Juránek** [1], **Alexander Boldyrev** [3], **Eva Hrabárová** [2], **Ladislav Šoltés** [1]

1 Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic
2 Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic
3 Research Center of Neurology, Russian Academy of Medical Sciences, Moscow, Russia

Correspondence to: František Dráfi, MSc.
Institute of Experimental Pharmacology and Toxicology,
Slovak Academy of Sciences,
Dúbravská cesta 9, 84104 Bratislava, Slovak Republic.
TEL: +421-2-59410666; FAX: +421-2-54775928; E-MAIL: frantisek.drafi@savba.sk

Abstract

OBJECTIVE: New ways of supplementary or combinatory therapy of rheumatoid arthritis (RA) are of great importance. The aim is to find an additive to classical RA therapy with natural molecules without side effects possessing anti-inflammatory and anti-oxidative properties. In this study we investigated the anti-oxidative and anti-inflammatory properties of the endogenous natural compound carnosine (CARN) *in vitro* and *in vivo*. Moreover, we tested also the inhibitory properties of the drug methotrexate (MTX) on dynamic viscosity of hyaluronan (HA) solutions in the same manner. **METHODS:** For *in vitro* testing of the inhibitory properties of CARN against degradation of HA solutions, we used the model of degradation of hyaluronan (HA) induced by free radicals. Both substances, CARN and MTX, were compared to glutathione (GSH). Rotational viscometry was used in evaluation of protective properties of compounds studied. The ability of CARN to restore the redox imbalance occurring in adjuvant arthritis (AA) of rats was also tested. We monitored the effect of CARN on hind paw volume (HPV) and on the levels of protein carbonyls, and thiobarbituric acid reacting substances (TBARS) in AA. **RESULTS:** In the reaction system with the prevalence of •OH and/or peroxy-type radicals, CARN in 200 µmol/L concentration tested was shown to exert a protective action on HA degradation. MTX was less effective than CARN in preventing HA degradation. Its ability to protect HA against radical degradation was evident only at the highest concentration of 400 µmol/L. In AA, carnosine significantly reduced TBARS and protein carbonyls in plasma, and also decreased the HPV of animals most effectively on the day 14. **CONCLUSIONS:** CARN proved its inhibitory properties against degradation of HA solutions at experimental conditions *in vitro* and showed its beneficial efficiency *in vivo*. Moreover, it reduced also HPV, the clinical marker of inflammation in AA.

INTRODUCTION

The pathogenesis of rheumatoid arthritis (RA) is associated predominantly with the formation of free radicals and pro-inflammatory cytokines at the site of inflammation. The inflammatory process develops in the tissue of the synovium; primary sources of reactive oxygen species (ROS) in RA are leukocytes (Firestein 2003; Bauerová & Bezek 1999). Hyaluronan (HA) is a major component of synovial fluid (SF) necessary for proper function of joints. Yet, due to the fact that SF does not contain any hyaluronidases, it is reasonable to assume that ROS might be involved also during HA catabolism in the inflamed joint. Any transition metal, *e.g.* iron or copper, can play an active role in oxidative HA catabolism. The polymer functions of HA are size-specific and its fragments constitute an information-rich system. While "space-filling" mega-dalton hyaluronans are immunosuppressive and anti-angiogenic, the inter-mediate-sized HA-polymer fragments are inflammatory, immunostimulatory and highly angiogenic (Šoltés & Kogan 2009). Thus, substances preventing HA from being degraded might have anti-inflammatory and anti-angiogenic effects. Methotrexate (MTX), a classic disease-modifying anti-rheumatic drug (DMARD), has become the predominant immunosuppressive agent used in the treatment of patients with RA. MTX induces metabolic changes leading to increased extracellular adenosine and suppresses the function of immune-competent cells (Chan & Cronstein 2002; Wessels *et al.* 2008). Carnosine (CARN), an essential endogenous molecule, has many physiological functions: radical scavenging, pH buffering, heavy metal chelating, anti-glycating, and neutralization of toxic aldehydes. CARN was found to have neuroprotective, hepatoprotective, cataract treating, and anti-aging abilities (Boldyrev 2005), but its anti-inflammatory potential in auto-immune systemic inflammatory diseases, as rheumatoid arthritis, has been scarcely investigated as yet.

MATERIALS AND METHODS

Biopolymer, chemicals and drugs

Hyaluronan sample (P9710-2A, M_w = 808.7 kDa) was kindly donated by Lifecore Biomedical Inc., Chaska, MN, U.S.A. NaCl and $CuCl_2 \cdot 2H_2O$ of analytical purity grade were from Slavus Ltd., Bratislava, Slovakia, L-ascorbic acid was the product of Merck KGaA, Darmstadt, Germany, L-glutathione (GSH) was from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Carnosine (CARN) was purchased from Hamary Chemicals Ltd., Japan, and methotrexate (MTX) from Pharmachemie B.V., Holland - Methotrexate-TEVA®.

Study of hyaluronan degradation

The volume of 7.90 mL of the HA solution (2.5 mg/mL) was stirred for 30 s. $CuCl_2$ solution (160 µmol/L) was added in the volume of 50 µL, stirred for 30 s and left to stand for 7 min and 30 s at room temperature. The volume of 50 µL of L-ascorbic acid solution (16 mmol/L) was added and stirred for 30 s. The mixture was immediately loaded into the viscometer tube (cup reservoir) (Valachova *et al.* 2009).

Study of inhibited hyaluronan degradation

First, the substance tested was added to the degradative system before •OH radicals were generated. Next, the substance tested was applied one hour after initiation of HA degradation, when peroxy-type radicals are being generated. The *in vitro* effect of CARN and MTX was studied at the concentrations of 100, 200, and 400 µmol/L. GSH as a standard was tested in the concentration of 100 µmol/L, which is equivalent to 100 µmol/L of •OH radicals generated by the above mentioned oxidative system.

Rotational viscometry

Degradation of HA was assessed by means of a rotational viscometer (Brookfield Engineering Labs., Inc., Middleboro, MA, U.S.A.). Recording of the viscometer output parameters started 2 min after the experiment onset. Dynamic viscosity of the solution was measured at 25.0 ± 0.1 °C in 3-min intervals for up to 5 h. The viscometer Teflon® spindle rotated at 180 r.p.m. (Šoltés *et al.* 2005).

Animals, experimental design and treatments

Male Lewis rats weighing 160–180 g were obtained from the Breeding Farm Dobra Voda, Slovakia. Adjuvant arthritis (AA) was induced in rats by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The injection was performed near to the base of the tail. The experiments included healthy animals (CO), arthritic animals not treated (AA) and arthritic animals treated with carnosine (AA-CARN) in the oral dose of 150 mg/b.w. daily. In each experimental group, 8–10 animals were used.

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The duration of the experiment was 28 days. After the animals were sacrificed under deep ketamin/xylasine anesthesia, blood for plasma preparation was taken on the day 28 and stored at minus 70 °C until biochemical analysis.

Clinical parameter evaluated: hind paw volume

We monitored the basic clinical parameter: change in the hind paw volume (HPV). The HPV increase was calculated as the percentage increase in HPV on a given experimental day, evaluated in comparison with the beginning of the experiment. Hind paw swelling measurements with the use of an electronic water plethysmometer (UGO BASILE, Comerio-Varese, Italy) were recorded on days 1, 14, 21, and 28 of the AA treatment.

Protein carbonyl assay

Enzyme-linked immunosorbent assay (ELISA) was used for quantitative determination of protein carbonyls in plasma. Protein samples were incubated with dinitrophenyl-hydrazine and adsorbed in multiwell-plates (Nunc Immunosorp plates, Roskilde, Denmark). A biotin-conjugated anti-dinitrophenyl-rabbit-IgG (Sigma, USA) was used as primary antibody and a monoclonal anti-rabbit-IgG-antibody peroxidase conjugate (Sigma, USA) as secondary antibody. Absorbance was determined at 492 nm. The method was calibrated using oxidized bovine serum albumin (Bauerová et al. 2010).

Thiobarbituric acid reactive substances (TBARS)

The reaction with thiobarbituric acid occurs by attack of the monoenolic form of malondialdehyde on the active methylene groups of thiobarbituric acid. Visible and ultraviolet spectrophotometry of the pigment confirms the primary maximum at 535 nm. TBARS were measured in heparinized blood plasma at 535 nm in a 0.5 cm cuvette (Poništ et al. 2010).

Statistical data analysis

The data for hind paw volume, TBARS, and protein carbonyls are expressed as arithmetic mean and S.E.M. Each group contained 8–10 animals. For significance calculations, unpaired Student´s t-test was used. p-values lower than 0.05 were considered a significant change, i.e. *$p<0.05$, **$p<0.01$, ***$p<0.001$. The arthritis group was compared with healthy control animals (*). The treated arthritis groups were compared with the untreated once (+).

RESULTS AND DISCUSSION

The HA free-radical degradation is involved in the pathogenesis of RA and osteoarthritis (OA). It belongs to the major causes of joint stiffness and worsened mobility of patients with RA or OA (Šoltés & Kogan 2009; Yamazaki et al. 2003). One of the most detrimental free radical species, responsible for the HA degradation in vivo, is the •OH radical induced by the Weissberger´s pro-oxidative system. Endogenous thiol antioxidant – GSH, was shown to be very effective in vitro at the HA protection against degradation comparable with a well-known DMARD compound – sodium aurothiomalate (Valachova et al. 2010). Thus, substances able to prevent HA degradation could be very helpful in the therapy of RA and OA. When testing CARN at the lowest concentration, it showed an accelerated effect on the decrease of the HA dynamic viscosity, followed by a moderate inhibitory action in the later phase in the system where •OH radicals were predominant (Figure 1, curve "b", "c", "d"). The dose of 100 µmol/L (Figure 2, curve "b") was more effective in the system of peroxy-type radicals than in the system of •OH radicals (Figure 1, curve "b"). A decreased rate of HA degradation was observed at the two higher concentrations of CARN, independently of the type of experimental arrangement (Figures 1, 2; curves "c", "d").
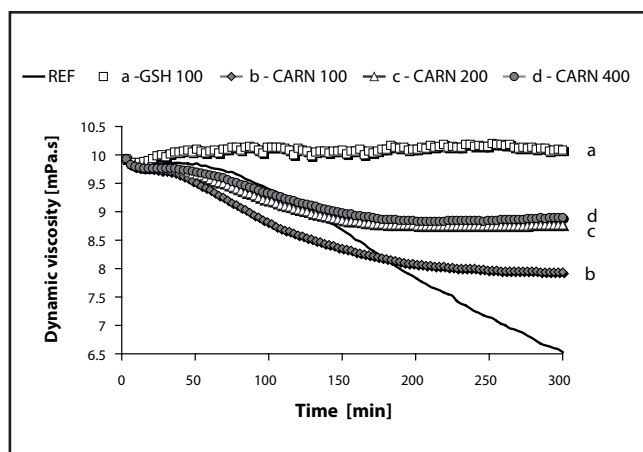

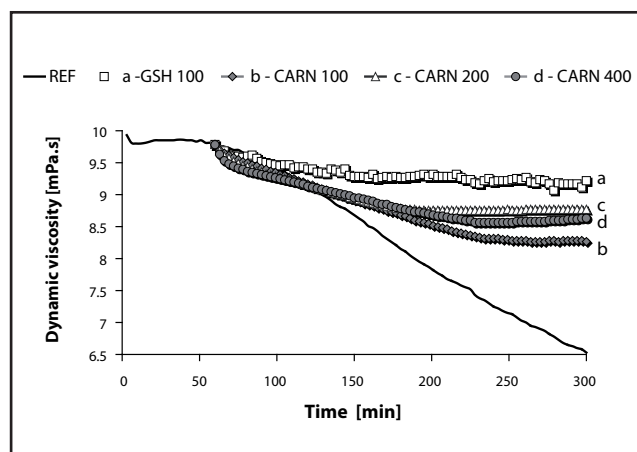**Fig. 1** Effect of CARN and GSH on HA degradation induced by Weissberger´s system on •OH radicals.

Fig. 2 Effect of CARN and GSH on HA degradation induced by Weissberger´s system on peroxy-type radicals.
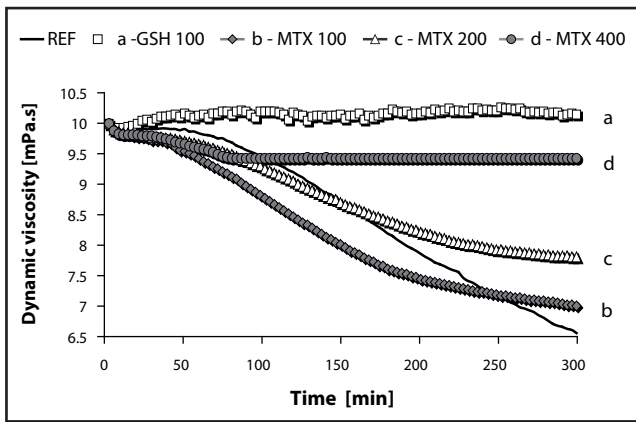
Fig. 3. Effect of MTX and GSH on HA degradation induced by Weissberger´s system on ·OH radicals.
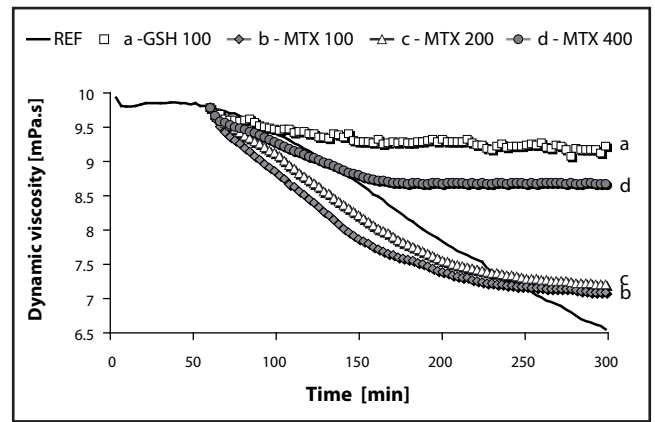

Fig. 4. Effect of MTX and GSH on HA degradation induced by Weissberger´s system on peroxy-type radicals

In contrast to CARN, MTX, at the two lower concentrations tested, increased the rate of HA degradation and partially acted on the levels of dynamic viscosity at the highest concentration tested (Figures 3, 4, curve "d"). In both experimental arrangements, CARN in comparison to MTX was more effective in the prevention of HA dynamic viscosity decrease.

In contrast to GSH (Figures 1–4 curve "a"), neither of the two substances tested had as notable and protective effect against HA degradation as had GSH. The dose-dependency was clearly shown when MTX was added at the beginning and also one hour after onset of HA degradation (Figures 3, 4 curves "b", "c", "d"). When CARN was tested, dose-dependency was not shown in the second phase, when peroxy-type radicals were formed. However in the same phase, MTX had the tendency to approximate partially the GSH curve (Figure 4, curves "a", "d").

Numerous studies have suggested a crucial role of oxidative stress in the pathogenesis of RA (Bauerová & Bezek 1999; Bohanec *et al.* 2009). The role of ROS in the etiology of RA is supported by numerous studies documenting that not only the damaging effects of ROS but also the role that ROS play in regulating various inflammatory processes contributes to the pathogenesis of the disease (Kunsch *et al.* 2005). Our previous results indicate that substances with anti-oxidative properties can have also beneficial effects in systemic chronic inflammatory diseases such as AA, not only in restoring redox imbalance but also by affecting clinical parameters of arthritis (Poništ *et al.* 2010, Bauerová *et al.* 2010; 2008 and 2009). CARN beneficially affected HPV measured in time profile (14, 21 and 28 days), significantly on day 14 when the clinical manifestation of the disease started (Figure 5). CARN was able to delay the disease onset, however it was not so effective later (days 21 and 28) when AA was fully developed. The markers of redox imbalance in plasma (TBARS and protein carbonyls) were significantly decreased, indicating the ability of CARN to restore redox imbalance *in vivo* (Figures 6, 7).
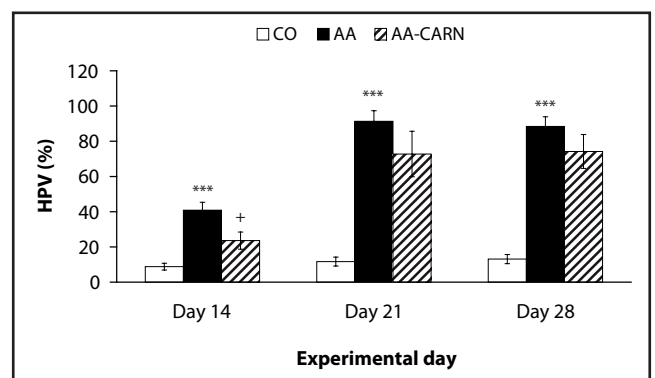

Fig. 5. Hind paw volume affected by CARN administration. The data are expressed as arithmetic mean ± S.E.M. Statistical significance was evaluated applying Student's t-test for independent variables: ***$p<0.001$ with respect to CO; +$p<0.05$ with respect to AA.

We suppose that the ability of CARN to restore redox imbalance may be also involved in the beneficial effect of CARN on HPV on day 14 (Figure 5). On the other hand, the poor effectivity of CARN later, in more severe stages of AA, indicates that the reduction of oxidative stress fails to be a sufficient therapeutic intervention in rheumatic diseases. However, the combination of antioxidants with immunosuppressive drugs (MTX) could be very effective, as we recently found for MTX administered together with coenzyme Q_{10} (Bauerová *et al.* 2010). Restoration of redox balance in chronic inflammatory diseases may be of significant importance in new therapeutic strategies.

CONCLUSION

In both *in vitro* experimental arrangements, CARN was more effective in the prevention of HA degradation in comparison to MTX. Moreover, its effect was close to the course of the GSH curve in the experimental *in vitro* setting where peroxy-type radicals were formed. CARN proved its anti-oxidative properties also *in*
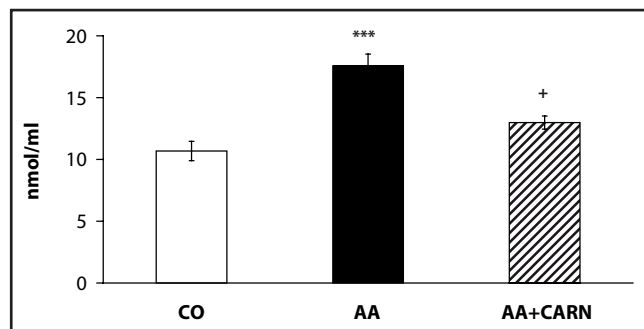
Fig. 6. TBARS plasmatic level on day 28 affected by CARN administration. The data are expressed as arithmetic mean ± S.E.M. Statistical significance was evaluated applying Student's t-test for independent variables: ***$p<0.001$ with respect to CO; +$p<0.05$ with respect to AA.
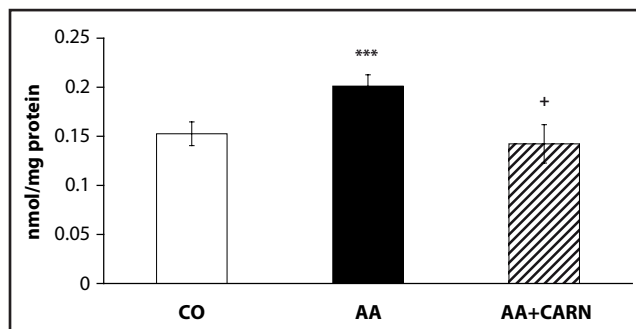

Fig. 7. Plasmatic level of protein carbonyls on day 28 affected by CARN administration. The data are expressed as arithmetic mean ± S.E.M. Statistical significance was evaluated applying Student's t-test for independent variables: **$p<0.01$ with respect to CO; +$p<0.05$ with respect to AA.

vivo by decreasing the systemic markers (TBARS and protein carbonyls) of redox imbalance occurring in adjuvant arthritis; furthermore, it exerted an anti-inflammatory action, demonstrated in the reduction of HPV of arthritic animals.

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