Evaluation of liposomal carnosine in adjuvant arthritis

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Abstract

Liposomal carnosine could overcome the problems associated with direct application of this peptide. Anti-inflammatory and antioxidant effects of liposomal and non-liposomal carnosine in adjuvant arthritis were compared. The experiments were done on healthy animals, untreated arthritic animals, arthritic animals with oral administration of carnosine (CARN), and with subcutaneous administration of liposomal carnosine (CARN-L), both administered in the same daily dose of 150 mg/kg b.w. during 28 days. CARN reduced hind paw volume on day 28. Both forms markedly decreased interleukin-1β, matrix metalloproteinase-9 and monocyte chemoattractant protein-1(MCP-1) in plasma on day 14. Only CARN-L reduced significantly MCP-1. Malondialdehyde, 4-hydroxynonenal, resistance to Fe^{2+}induced oxidation and protein carbonyls were significantly corrected after administration of any form of carnosine. CARN-L corrected more effectively the oxidative stress in plasma than did CARN. In brain tissue, our results showed protective ability of both forms of carnosine against oxidation of proteins and lipids. They also corrected the resistance to Fe^{2+}induced oxidation in arthritic animals. We found that only CARN-L decreased the mRNA expression of inducible nitric oxide synthase in cartilage tissue. It can be concluded that the liposomal drug-delivery system is improving the pharmacological properties of carnosine administered in arthritis.

Key words

Liposomes • Adjuvant arthritis • Oxidative stress • Carnosine • Inflammation

Introduction

Rheumatoid arthritis (RA) is the most common autoimmune inflammatory arthritis in adults (Helmick et al. 2008). Although traditional treatment is reducing the pain without toxic pharmacological consequences, it is ineffective in reversing cartilage degradation and only slows it down. Moreover, its potential side effects limit the length of therapy (Cameron et al. 2009). There is thus an urgent need to find new methods and strategies for the treatment of RA. Several researchers reported that antioxidants are potential agents in RA therapeutic management (Mateen et al. 2016; Khojah et al. 2016). In the development of adjuvant arthritis (AA) not only immunological and inflammatory pathological changes are involved, but also the redox homeostasis is shifted towards increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Recent evidence from animal models of RA emphasized the importance of neutrophils in the initiation and progression of AA (Cross et al. 2006). We assessed ROS production in stimulated neutrophils of arthritic rats and it was found to be increased, with maximum on day 14 and 21 of AA. Neutrophils in whole blood of AA animals reacted excessively to stimulation and produced 6-9 times more ROS (Nosal et al. 2007). We also demonstrated oxidative damage to the tissues in AA: ROS levels in the joint and the spleen were significantly elevated (Drabikova et al. 2009). The control of inflammation in arthritic patients by natural as well as synthetic antioxidants could become a relevant component of antirheumatic prevention and therapy (Bauerová and Bezek 1999). Carnosine (β-alanyl-L-histidine) is a naturally occurring antioxidant and cell protector (Boldyrev and Johnson 2002). CARN is available as a dietary supplement with no known side effects or identified adverse drug interactions (Min et al. 2008). One of the reasons for the limited use of carnosine as a drug is considered to be its fast metabolism by means of serum and kidney carnosinases. These enzymes quickly decrease the level of carnosine in serum,

thus preventing its long-lasting action (Pegova et al. 2000). Our previous study showed a protective effect of CARN against oxidative damage and also its systemic anti-inflammatory effects (Ponist et al. 2016). Liposomes are nanosized artificial vesicles of spherical shape that can be produced from natural or synthetic phospholipids (Schuber et al. 1998). Encapsulation of antioxidants in liposomes has been shown to improve their therapeutic potential against oxidant-induced tissue injuries, as liposomes evidently facilitate intracellular delivery and extend the retention time of incorporated agents inside the cell. Thus antioxidant liposomes hold great promise in the treatment of many diseases in which oxidative stress plays a significant role (Zacharias 2011). Entrapment of carnosine into liposomes may represent an ameliorative approach to overcome the problems related with direct application of this antioxidant peptide by improving bioavailability in brain lipid tissues and providing protection against carnosinase.

The aim of this study was to examine anti-inflammatory and antioxidant effects of liposomal carnosine in plasma and brain in adjuvant arthritis (AA) and to compare these effects with its non-liposomal form.

Materials and Methods

Animals, experimental design and treatments

Male Lewis rats, weighing 160–180g, were purchased from the Breeding Farm Dobra Voda (Slovakia) and housed five per cage under standard conditions with food and water *ad libitum* and a 12-hour-light/12-hour-dark cycle. The experimental protocol was approved by the Ethics Committee of the Institute of Experimental Pharmacology and Toxicology and by the Slovak State Veterinary and Food Administration in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes and was found in accordance with Slovak legislation. AA was induced by

a single intradermal injection of 0.1ml suspension of heat-inactivated *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI, USA) in incomplete Freund's adjuvant at the base of the tail. The experiments included four groups of animals (8 animals in each group): *Group (1)* healthy animals as reference controls (CO), *Group (2)* arthritic animals without any drug administration (AA), *Group (3)* arthritic animals with the administration of carnosine (CARN) 150mg/kg b.w. in a daily oral dose during 28 days, and *Group (4)* liposomal carnosine (CARN-L) 150mg/kg b.w. in a daily subcutaneous dose during 28 days. Body weight of the rats was regularly measured to calculate the precise application doses before each administration of the given substance. On day 14, blood samples were taken from the retro-orbital plexus under Zoletil®/xylasine anesthesia. On day 28, the animals were sacrificed under the same anesthesia and blood for plasma preparations was withdrawn along with brain and cartilage tissue from each rat. All samples were stored at -80°C until biochemical analysis.

Preparation of liposomes by lipid film hydration

Liposomes were prepared by thin film hydration method. Briefly, sterol lipids and fatty acids were dissolved in an organic solvent to assure a homogeneous lipid mixture. The solution was prepared at 10-20 mg lipid/ml of organic solvent (ethanol 100%). Then the mixture was evaporated in a rotary evaporator. Carnosine was added to the obtained lipid film in buffer (PBS) solution and finally the heterogeneous system was thoroughly mixed using ultrasonic equipment to obtain liposomes (the ratio drug/lipids was 0.2:1). All solutions were sterile and the preparation of liposomes was performed in sterile conditions.

Clinical parameter: change of hind paw volume

The hind paw volume (HPV, %) increase was calculated as the percentage increase in the HPV on a given experimental day relative to the HPV at the beginning of the experiment. This parameter was recorded on days 1, 14, 21, and 28 with the use of an electronic water plethysmometer (UGO BASILE, Comerio-Varese, Italy).

Measurement of monocyte chemoattractant protein-1 (MCP-1) in plasma

For determination of plasmatic concentration of MCP-1 an ELISA kit from eBioscience® was used. The assay procedure was applied as described in the product manual. An anti-rat MCP-1 monoclonal coating antibody was adsorbed onto microwells. Rat MCP-1 was present in the sample or standard bound to antibodies adsorbed to the microwell; a biotin-conjugated monoclonal anti-rat MCP-1 antibody bound to rat MCP-1 was captured by the first antibody. Streptavidin-HRP was bound to the conjugated anti-rat MCP-1. Following incubation, unbound biotin conjugated anti-rat MCP-1 and Streptavidin-HRP were removed during a wash step and substrate solution reactive with HPR was added to the wells. A colored product was formed in proportion to the amount of soluble rat MCP-1 present in the sample. The reaction was terminated by addition of acid and absorbance was measured at 450 nm in comparison with reference wavelength 620 nm (microplate reader MRX II, Dynex, USA). The results were calculated from standard calibration curve on internal standards.

Measurement of interleukin 1β (IL-1β) and matrix metalloproteinase 9 (MMP-9) in plasma

For determination of plasmatic concentration of IL-1β and MMP-9, an ELISA kit from R&D Systems Quantikine® was used. The assay procedures were applied as described in the product manuals.

Chemiluminescence measurement of plasma and brain homogenate antioxidant capacity

The method used was based on the measurement of chemiluminescence (ChL) in brain homogenates and plasma of the experimental animals oxidized by Fe^{2+} ions was added in excess to the reaction medium (Fedorova et al. 1999). To initiate lipid peroxidation (LPO), 2.5 mM Fe^{2+} ions were added. The initial ChL burst (h, mV) represents the level of pre-existent LPO products (reflects the stationary level of lipid hydroperoxides), the lag period of Fe^{2+} -induced oxidation (τ, s) characterizes the resistance of the sample against oxidation, dependent on the intrinsic antioxidant capacity of a biological sample. ChL signal was monitored using LKB 1251Chemiluminometer (Sweden) and was expressed in mV (Dobrota et al. 2005).

Measurement of malondialdehyde in plasma and brain homogenates

Malondialdehyde (MDA) ELISA Kit (Cusabio cat № CSB-E08557h) for the quantitative determination of endogenic MDA concentrations in plasma and tissue homogenates was used. The assay employs an inhibition enzyme immunoassay technique. Antibody specific for MDA was pre-coated onto a microplate. Standards and samples were pipetted into the wells with horseradish-peroxidase (HRP) conjugated MDA. A competitive inhibition reaction between MDA (standards and samples) and HRP-conjugated MDA with the pre-coated antibody specific for MDA was launched - the higher the amount of MDA in samples, the less antibody bound by HRP-conjugated MDA will bind. Following a wash to remove any unbound reagent, a substrate solution was added to the wells and color was developed. The color development was stopped and the intensity was measured.

Measurement of 4-hydroxynonal and protein carbonyls in plasma and brain homogenates

Determination of oxidative stress parameters was done by using *protein carbonyl* ELISA Kit (Immundiagnostik AG cat№ K7822) and *4-hydroxynonenal* ELISA Kit (Cusabio

cat № CSB-E16214h). The assay procedures were applied as described in the product manuals.

Total RNA isolation and quantitative RT-PCR

Total RNA was isolated from the rat cartilage tissue. The cartilage was pulverized in liquid nitrogen followed by RNA extraction with the RNAzol RT (Sigma-Aldrich). RNA was converted into complementary DNA (cDNA) using the PrimeScript RT Reagent Kit (Takara) following the protocols of the manufacturers. Amplification and detection of cDNA of reference and target genes were performed on a 7300 Real-Time PCR System (Applied Biosystems) using HOT FIREPolEvaGreenR qPCR MixPlus (ROX) (Solis Biodyne). Relative mRNA expressions of iNOS were analyzed using the ΔΔCt value method (Winer et al. 1999). β-actin was used as a reference gene. PCR products were evaluated by melting curve analysis to confirm the specific amplification. The sequences of the primers were designed and checked using Primer 3 and Oligo Analyzer 1.0.3 (table 1).

Statistical analyses

Mean and S.E.M. values were calculated for each parameter in each group. Statistically significant differences among treated groups, untreated group and control group were tested using parametric Analysis of Variance (ANOVA). After *post hoc* (Tukey-Kramer) testing, significance designations were specified as follows: extremely significant ($^{***}p< 0.001$), highly significant ($^{**}p < 0.01$), significant ($^{*}p< 0.05$), and not significant ($p> 0.05$).

Results

Clinical parameter

The hind paw volume (HPV) of arthritic animals increased during the experiment in comparison to the control group on all days monitored. The highest increase was observed at

the end of the experiment – day 28. CARN and CARN-L reduced this parameter, but this effect was not statistically significant, except on day 28 when CARN significantly reduced HPV (fig. 1).

Inflammatory parameters in plasma evaluated on day 14

The level of IL-1β in untreated arthritic animals was increased approximately 12 times on day 14 when compared to healthy controls. CARN and CARN-L significantly decreased IL-1β concentration in plasma to the basal level analyzed in healthy animals (table 2). Further arthritis significantly increased MMP-9 and MCP-1 in plasma on day 14 (table 2 and fig.2). CARN and CARN-L were effective in reducing significantly the MMP-9 levels (table 2). Concerning MCP-1 in plasma, only CARN-L (fig.2) reduced the levels of this parameter significantly. Thus CARN-L was generally more effective in improving the inflammation analyzed in plasma than CARN.

Antioxidant capacity of CARN and CARN-L in plasma and brain tissue homogenate

Plasma: Neither AA nor CARN administered changed the content of lipid hydroperoxide (data not shown). However, AA significantly reduced the resistance to Fe^{2+}induced oxidation and both liposomal and non-liposomal CARN were able to effectively increase it. In comparison to CARN, CARN-L was much more effective (fig. 3). Oxidation of plasmatic proteins was assayed as protein carbonyls, which were increased in untreated arthritic animals. CARN and its liposomal form administered decreased effectively the protein carbonyls to control level. The arthritic process increased lipid peroxidation in plasma detected as levels of MDA and HNE. CARN and CARN-L reduced the levels of MDA and both forms of carnosine studied were also able to decrease HNE to control level (table 3).

Brain: The content of lipid hydroperoxides was increased in animals with AA without significance. CARN and CARN-L administered decreased the signal for damaged lipids (data not shown). Arthritis in experimental animals significantly reduced the resistance to Fe^{2+} induced oxidation and both forms of CARN administered were able to increase it effectively, with CARN-L being even more effective (fig. 3). Protein carbonyls were increased in the brain of untreated arthritic animals, though not significantly. CARN and CARN-L decreased brain protein carbonyls. Lipid peroxidation in the brain measured as content of MDA was increased 2-times in arthritic animals in comparison to healthy control. Both forms of carnosine administered reduced the content of MDA to control level. Moreover, HNE content was increased due to arthritis. CARN and CARN-L were able to decrease this parameter significantly (table 4).

Generally, the antioxidant effect of CARN-L in plasma was stronger than that of CARN. This trend was not observed in the brain.

Effect of CARN and CARN-L on mRNA expression of iNOS in rat cartilage tissue

In our study, the expression of iNOS mRNA was significantly increased in untreated arthritic animals (fig. 4). The administration of CARN did not lead to changes in mRNA expression of iNOS. CARN-L reduced this parameter without statistical significance.

Discussion

We intend to find new substances of natural origin with anti-inflammatory and antioxidant effect in AA. In an experimental study (Ponist et al. 2016) we investigated the effect of carnosine *in vitro* and *in vivo*, using two different animal models. The results indicated its potential systemic anti-inflammatory effects. It still remains unclear if the ability of CARN to restore redox balance is the only mechanism responsible for its anti-

inflammatory effects in AA. One goal of this research was to assess whether drug-delivery systems, such as liposomes, could improve the therapeutic potency and bioavailability of CARN.

Several researchers have focused on the application of liposomes as drug carriers in the treatment of RA (Kapoor et al. 2014). In this study, we monitored adjuvant arthritis (AA) progression by using the change of hind paw volume (HPV). L-CARN reduced HPV change on both experimental days similarly as did CARN. Moreover, CARN reduced hind paw edema significantly on day 28. Topical application of mannitol exerted a potent and fast anti-edema effect in a rat model of joint inflammation, suggesting a possible utilization in patients affected by RA (Cavone et al. 2012). Intravenous therapy with liposomal dexamethasone phosphate DxM-P suppressed joint swelling in a significant, dose-dependent, and long-lasting manner and showed superior therapeutic efficacy compared to matched doses of free DxM-P (Anderson et al. 2010). Thus liposomal encapsulation of current anti-rheumatic drugs, and of new potential substances for RA treatment, concerning also other drug-delivery systems, may clearly potentiate the clinical efficacy of the compounds evaluated.

Monitoring of cytokines, chemokines and other pro-inflammatory mediators which play essential roles in the progression of inflammatory diseases is very important for evaluation of the effectivity of the treatment. For determination of inflammation in our study, we assessed plasmatic levels of cytokine IL-1β, chemokine MCP-1 and protease MMP-9. IL-1 is the first molecule occurring at high levels in the synovial tissue in this T-cell-dependent arthritis. The early phases of the disease seem to be characterized by a systemic increase in IL-1β (Ferraccioli et al. 2010). Monocytes, macrophages and dendritic cells (DCs), as well as neutrophils, are among the immune cells capable of producing large amounts of IL-1β (Wilson et al. 1994; Thornberry et al. 1992). Arthritic animals demonstrated a significant increase in neutrophil numbers on day 14 in AA (Perecko et al. 2013). It seems, that

neutrophils are important producers of IL-1β in AA. We found in literature, that CARN decreased more than by 50% the production of ROS measured by luminol-dependent chemiluminescence of PMA-stimulated human neutrophils (Sharonov B.P., Govorova N.J. and Lyzlova S.N. 1990). Therefore, we assume, CARN and CARN-L inhibited the activity of neutrophils and their production IL-1β. From our results, we can conclude, liposomal form of CARN has no effect on the ability of CARN to reduce the level of IL-1β. CARN and CARN-L decreased the IL-1β to basal level. It is possible that both carnosine forms are affecting the function of macrophages, which produce IL-β. In one of our previous studies, both high- and low-molecular-weight non-animal chondroitin sulphate were effective in reducing plasmatic levels of proinflammatory cytokines in AA, including IL-β (Bauerova et al. 2014). CARN and its liposomal form might be good candidates for decrease of IL-1 levels in patients with RA.

MCP-1 may play an important role in the recruitment of peripheral blood leukocytes into the RA joints. Data from a study performed in RA patients provide evidence that the chemokine signalling pathway is involved in MCP-1 expression in the patients´ tissues, which may contribute to chronic inflammation associated with RA. Targeting this signalling pathway may provide a novel therapeutic direction in RA (Zhang et al. 2015). CARN-L significantly decreased the plasmatic level of MCP-1 on day 14, while CARN failed to affect it. It seems that incorporation of CARN into liposomes increases its ability to reduce MCP-1 levels in plasma during AA development. CARN-L may play some role in the pathway of MCP-1 signalling.

Matrix metalloproteinases (MMPs) produced by macrophages and synovial fibroblasts were shown to be involved in the destruction of articular tissues in RA (Cunnane et al. 2014). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 can regulate MMPs gene expression (Malemud and Schulte 2008). Since production of MMPs is controlled by TNF-α, the use anti-TNF agents has been suggested in the treatment of RA (Klimiuk et al. 2004). In

299 our study, both forms of carnosine were significantly effective in reducing MMP-9 in plasma.
300 This ability of liposomal and non-liposomal CARN might be also involved in the decrease of
301 IL-1β plasmatic levels to basal values. Unfortunately, the results from the hind paw volume of
302 animals for CARN and CARN-L do not correspond well with the intensive reduction of IL-1β
303 plasmatic levels on day 14. This might be explained by the fact that not only IL-1β but also
304 TNF-α and IL-6 regulate MMP-9 gene expression.

305 In animal models of RA, increased oxidative stress has been well documented by other
306 authors (Ahmed et al. 2015; Wruck et al. 2011). In our study, oxidative stress in AA was
307 analyzed by parameters in brain homogenates and in blood plasma. Lipid peroxidation is one
308 of the major consequences of oxidative stress and leads to the production of conjugated diene
309 hydroperoxides and unstable substances, which disintegrate into various bioactive aldehydes
310 such as MDA, HNE (Phaniendra et al. 2015). Another important consequence of oxidative
311 damage in RA is the deleterious effect on proteins, as free radicals can modify both their
312 structure and functions (Dalle-Donne et al. 2006). CARN and CARN-L significantly
313 corrected all parameters of oxidation stress measured in plasma, except the content of lipid
314 hydroperoxides. CARN-L was better in corrections of oxidative stress in plasma than CARN,
315 only in the resistance to Fe2+ induced oxidation. There was no difference in the ability of
316 CARN and CARN-L to protect the proteins and lipids in plasma against oxidation.

317 RA is not a disease typically involving the CNS, but brain dysfunctions occur in 20 to
318 30 % of rheumatic patients (Appenzeller et al. 2004). There are only few studies describing
319 oxidative stress and brain damage during experimental arthritis such as AA (Skurlova et al.
320 2010; Skurlova et al. 2011; de Almeida Gonçalves et al. 2015). Carnosine is accumulated in
321 excitable tissues (brain, heart and skeletal muscles) of vertebrates in large amounts (Abe
322 1995). In *in vitro* experiments carnosine protected brain neurons against oxidative injury
323 (Boldyrev et al. 1999). Liposomes are widely used as carriers or delivery vehicles for

therapeutic agents/drugs to transport them to specific sites inside the human body (e.g. brain). Liposomes could be the carrier system for the drugs, they do not cross the blood brain barrier (Spuch and Navarro 2011). We therefore evaluated the effect of liposomal and non-liposomal CARN on oxidative stress in brain homogenates. Our results with CARN and CARN-L showed protective ability of both forms of carnosine against protein and lipid oxidation. They corrected the resistance to Fe^{2+}induced oxidation in arthritic animals. CARN-L was more effective than CARN but the difference was without significance.

We also evaluated mRNA expression of inducible nitric oxide synthase (iNOS) in the cartilage tissue of rats with AA. The inducible isoform of NOS is found in the synovial tissue and cartilage and its expression is regulated by catabolic cytokines, such as IL-1β and TNF-α (Amin et al. 1999). A preclinical study indicated that NO generation by iNOS induced in chondrocytes was a key event in the induction of adjuvant arthritis (Yonekura et al. 2003). The results obtained in our experiment showed that only the liposomal form of carnosine decreased mRNA expression of iNOS in AA, and that not significantly. Liposomes can be assumed to improve bioavailability of carnosine in the rat cartilage tissue.

To the best of our knowledge, this is the first report in literature evaluating liposomal carnosine in AA. In an animal model of RA, the obtained results demonstrated that subcutaneously administered CARN-L ameliorated all parameters of inflammation and oxidative stress measured. Moreover, in comparison with its non-liposomal form, CARN-L was more effective in several parameters, especially MCP-1, the resistance to Fe^{2+}induced oxidation in plasma and brain homogenates, and the mRNA expression of iNOS in rat cartilage tissue. CARN-L showed a beneficial effect in AA, pointing to its potential use in the treatment of RA in humans.

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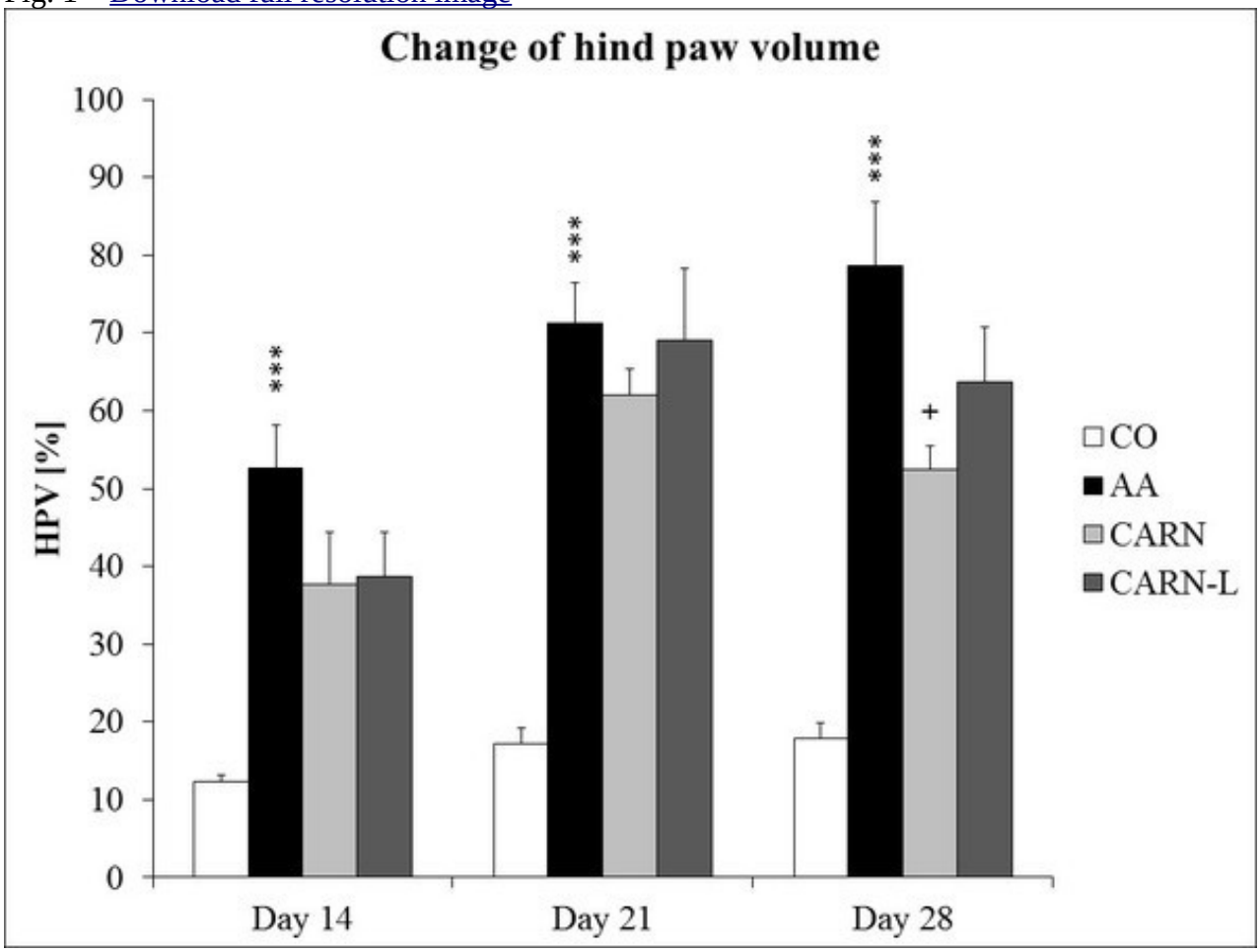

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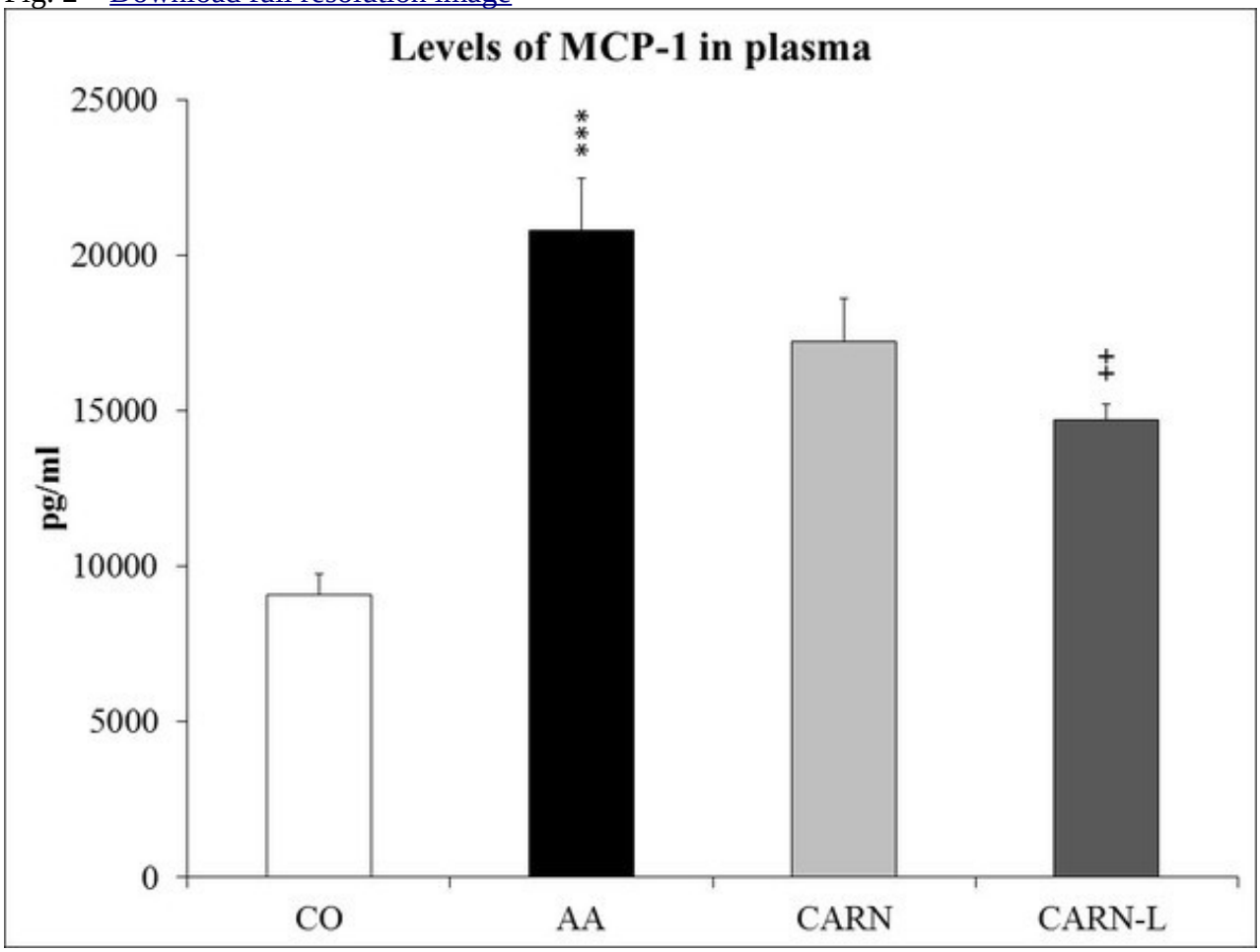

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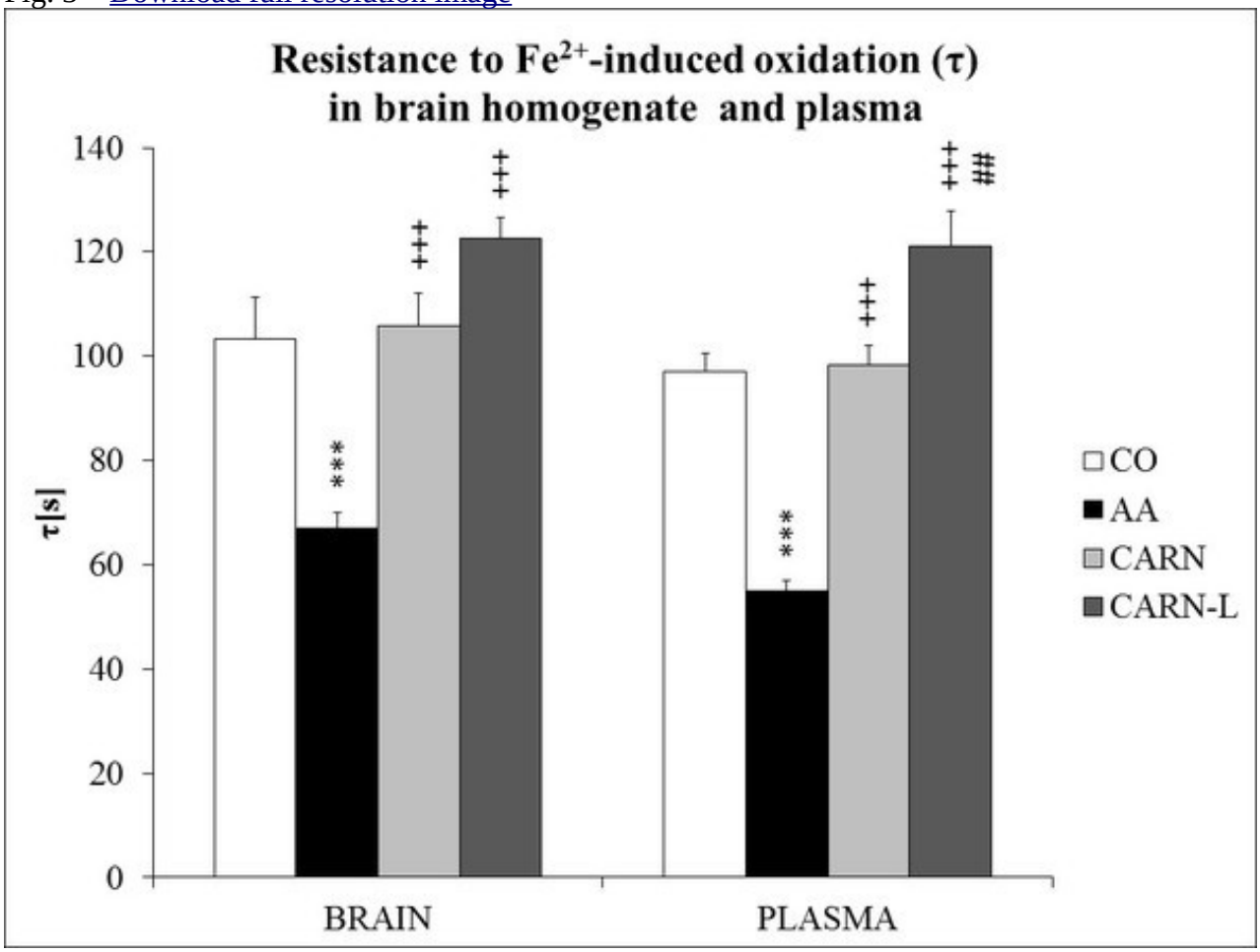

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