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ORIGINAL ARTICLE

Improvement of Resveratrol Effects When Combined with Rice Oil in Rat Models of Inflammation

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Abstract— This study investigated the effects of systemic treatment with a new formulation of resveratrol (RSV) vehicled in rice oil (RSVO) in experimental rat models of inflammation. Male Wistar rats were evaluated in the following *in vivo* models: carrageenan-induced acute edema, complete Freund's adjuvant (CFA)-evoked sub-chronic edema, and CFA-induced polyarthritis. The animals were treated orally with RSVO (10–15 mg/kg) or RSV (100–200 mg/kg), depending on the experimental protocol. RSV was more effective than RSVO in carrageenan-elicited acute edema when dosed in either prophylactic or therapeutic schemes of administration. However, the repeated RSVO administration, at 10-fold lower doses, exhibited superior anti-inflammatory actions in either the sub-chronic edema or the chronic polyarthritis model elicited by CFA, when compared with RSV. The novel formulation RSVO displayed a lower plasma biotransformation when compared with the RSV-treated group—46% *versus* 88% of metabolites, respectively. RSVO also prevented polyarthritis-related cartilage destruction, an effect that might rely on the inhibition of the pro-inflammatory cytokine interleukin-6 (IL-6), associated with an increase of the anti-inflammatory cytokine interleukin-10 (IL-10). Noteworthy, the long-term administration of RSVO did not elicit any gastrointestinal harm. Our study revealed that RSVO was notably effective in the long-term inflammatory and degenerative responses triggered by CFA. This

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innovative formulation might well represent a promising alternative for treating chronic inflammatory diseases, such as arthritis.

KEY WORDS: acute and chronic inflammation; polyarthritis; complete Freund's adjuvant; resveratrol; resveratrol vehicled in rice oil.

INTRODUCTION

Chronic inflammation comprises physiological and structural tissue alterations underlying rheumatoid arthritis, systemic lupus erythematosus, and other autoimmune diseases [1]. Several nutritional compounds are the focus of great attention due to their potential beneficial effects. Thereby, some plant-derived compounds and bioactive peptides have been proven valuable in traditional medicine as alternatives for controlling inflammatory disorders [2]. Resveratrol (*trans*-3,5,4′-trihydroxystilbene; RSV) is a polyphenol compound that is produced in response to infection in plants such as *Vitis vinifera* [3]. The scientific research on RSV was greatly stimulated due to the "French paradox," which reports a reduction of mortality related to coronary heart disease by consuming RSV in red wine, even in a part of the French population with a high-fat diet [4]. Furthermore, numerous *in vitro* and *in vivo* studies have suggested anti-inflammatory, anti-tumor, and antioxidant properties for RSV [5, 6]. The anti-inflammatory effects of RSV have been described in several animal models including arthritis, acute pancreatitis, and experimental ulcerative colitis [7–9].

Previous studies demonstrated that RSV displays low bioavailability when administered orally (from 30 to 60 min), as this molecule is rapidly metabolized and consequently the peak plasma level decreases, forming various derivatives that are readily eliminated [10, 11]. Some strategies to increase its absorption include improving the bioavailability by optimized galenic preparations or by limiting the extensive metabolization of RSV into less active compounds, and to enhance the formation of the most active metabolites formed after oral intake [12]. Another approach might be the concomitant administration of other phenolic compounds that compete with RSV for phase-I/II metabolizing enzymes, resulting in higher systemic concentrations of the non-metabolized free form [13].

The rice bran oil contains high levels of tocopherols, tocotrienols, and phytosterols, and it is especially rich in γ-oryzanol. Noteworthy, γ-oryzanol has greater antioxidant activity in comparison to tocopherols, due to its superior temperature stability [14]. In recent literature, there are several examples of synergistic effects obtained by the combination of RSV with other bioactive compounds [15, 16]. For instance, an association of RSV, quercetin, and tocotrienol has been demonstrated to modulate cardiovascular lipid parameters in humans [17].

Rheumatoid arthritis causes enormous impacts on the patient's life quality and economy. Considering the serious adverse events allied to the chronic use of non-steroidal anti-inflammatory drugs and immunosuppressants, novel cost-effective anti-arthritis therapies are urgently needed [18]. Therefore, the development of new alternatives for managing chronic inflammatory and painful diseases continues to be an issue of high interest. It is well known that pharmacological responses to drugs are directly related to its concentration at required sites of action [19]. Following this rational, in the present study, we have assessed the effects of a new formulation containing RSV vehicled in rice oil (RSVO) in either acute or chronic models of inflammation in rats.

MATERIALS AND METHODS

Animals

Male Wistar rats (5–8 per group; total number: 140 animals; 180 to 200 g) were obtained from the Center of Experimental Biological Models (CeMBE/PUCRS). The animals were housed in groups of four per cage. Rats were maintained in controlled temperature (22 ± 2 °C) and humidity (60 to 70%), under a 12-h light-dark cycle, with food and water *ad libitum*. For the experiments, the animals were acclimatized to the laboratory for at least 1 h, and they were used only once in each test. All of the experimental procedures were carried out in accordance with the Guidelines for the Use and Care with Laboratory Animals from National Institute of Health and ethical guidelines for investigations of experimental pain in conscious animals. The Animal Ethics Committee of the Pontifícia Universidade Católica do Rio Grande do Sul approved the experimental protocols (CEUA-PUCRS, numbers 07/03611 and 18/017). The number of animals and the intensity of noxious stimuli were the minimum necessary

to demonstrate the consistent effects of the drug treatments. The distribution of experimental groups and the respective treatments are shown in Table 1.

Drugs and Reagents

The following drugs and reagents were used: *trans*-resveratrol (Sigma-Aldrich, MO, USA), rice bran oil (Helmut Tessmann, RS, Brazil) with the following composition: γ-oryzanol 1%, oleic acid 40%, linoleic acid 33.5%, vitamin E (tocopherol; 1.200 mg/mL). Inert oil 100% (Cristália, SP, Brazil). In addition, a surrogate standard (SS) of resveratrol-4,(4-hydroxyphenyl-13C6—with purity up to 98%—was obtained from Sigma-Aldrich (St. Louis, Missouri, EUA).

Preparation of *trans*-resveratrol Vehicled in Rice Oil

Solid RSV was dissolved in absolute ethanol and sonicated in an ultrasound bath for 30 min, at room temperature. This solution was slowly mixed with 250 mL of rice bran oil and placed in a horizontal shaker at 500 rpm, for 30 min. For complete homogenization, the mixture was sonicated for 1 h. The RSV content was determined by high-performance liquid chromatography with UV detection (HPLC-UV), as described elsewhere [20], using a calibration curve ranging from 0.10 to 200.0 mg/L. Sample extraction was carried out by mixing 10 mL of the final product with 400 mL methanol/acetone (7:3) and shaking the mixture (700 rpm) for 1 h, at room temperature. The extract was then cooled at − 20 °C for 15 h, in order to enable an efficient separation of the liquid phase containing RSV, from the frozen sample matrix by vacuum filtration. The liquid phase was evaporated to dryness under reduced pressure, and the residues were dissolved in 20 mL of methanol and analyzed by HPLC-UV at 306 nm. The calculated concentration of RSV vehicled in rice oil (RSVO) was 10.2 ± 0.1 mg/mL (Patent Number: WO 2011/097691 A1).

Carrageenan-Elicited Acute Paw Edema

The experiments were conducted according to the method described by Bernardi and colleagues, with some modifications [21]. Under light anesthesia with isoflurane (2%), the animals received an intradermal (i.d.) injection into the right hindpaw of saline (0.9% NaCl) containing carrageenan (300 μg per paw; 100 μL). As a control, the contralateral paw (left paw) received 100 μL of saline. Edema was measured by means of a plethysmometer (Ugo Basile, Varese, Italy) at several time points after carrageenan injection (30, 60, 120, 180, and 240 min). Edema was expressed in milliliters as the difference between the right and left hindpaw. In this model, two distinct schedules of treatment were adopted. In the prophylactic scheme, the animals were pre-treated orally (p.o.) with RSV (100 and 200 mg/kg) or RSVO (10 and 15 mg/kg), 30 min before carrageenan application. In the therapeutic scheme, the rats received RSV (100 mg/kg, p.o.) or RSVO (10 mg/kg, p.o.), 120 min after carrageenan. The control groups received the vehicle solutions: saline, inert oil or rice oil, at the same schedules of administration. The protocols of treatment for all of the tested compounds (doses, route of administration, and time of injection) were chosen in accordance with previous publications [22–24] or pilot experiments.

Sub-chronic Inflammation Evoked by Complete Freund's Adjuvant

The protocol used was similar to that described before [21]. Briefly, 2% isoflurane-anaesthetized animals received

Table 1. Distribution of Experimental Groups and Treatments

Experimental model: carrageenan-induced acute edema Prophylactic scheme: 30 min before carrageenan application				
Vehicle (0.9% NaCl)	RSV (100 or 200 mg/kg)	Vehicle (inert oil)	Rice oil	RSVO (15 or 10 mg/kg)
Experimental model: carrageenan-induced acute edema Therapeutic scheme: 120 min after carrageenan injection				
Vehicle (0.9% NaCl)	RSV (100 mg/kg)	Vehicle (inert oil)	Rice oil	RSVO (10 mg/kg)
Experimental model: sub-chronic edema induced by CFA				
Vehicle (0.9% NaCl)	RSV (100 mg/kg)	Vehicle (inert oil)	Rice oil	RSVO (10 mg/kg)
Experimental model: CFA-elicited polyarthritis				
Vehicle (0.9% NaCl)	RSV (100 mg/kg)	Vehicle (inert oil)	Rice oil	RSVO (10 mg/kg)

RSV, resveratrol; *RSVO*, resveratrol vehicled in rice oil; *CFA*, complete Freund's adjuvant

an i.d. injection into one hindpaw (right paw) of complete Freund's adjuvant (CFA) (mg/mL; 100 μL; heat-killed and dried *Mycobacterium tuberculosis*, each mL of vehicle containing 0.85 mL paraffin oil plus 0.15 mL mannide monooleate), which was suspended as a 1:1 oil/saline emulsion (in a total volume of 200 μL per paw). As a control, the contralateral paw (left paw) received 200 μL of saline. In this experiment, the animals were orally treated with RSV or RSVO (100 mg/kg and 10 mg/kg, respectively), 2-h post-CFA injection—once a day for 3 days. The control groups received the corresponding vehicle solutions at the same intervals of time. The edema was measured by using a plethysmometer at several time points following CFA injections (2, 4, 6, 8, 24, 48, and 72 h), and it was expressed in milliliters as the difference between the right and left hindpaw.

Polyarthritis Model

The polyarthritis model was induced by CFA injection, as indicated above, and it was assessed daily in a plethysmometer, between days 14 and 21 post-CFA application. The animals were treated orally with RSV or RSVO (100 mg/kg and 10 mg/kg, respectively), twice a day, for 8 days, starting at the 14th day after the CFA injection, until day 21. The control groups received the corresponding vehicle solutions at the same intervals of time. The edema was expressed in milliliters as the difference between the right and the left hindpaw. Rats were monitored daily and were weighed every 7 days (as a parameter of general health), for up to 21 days.

Quantification of RSV in Rat Plasma by LC-MS/MS

After the chronic treatment with the compounds, i.e., 21-day post-CFA injection, the rats were euthanized and the blood was collected (BD Vacutainer®, containing anticoagulant EDTA K2). Thereafter, blood samples were centrifuged (4500 rpm, 12 min, 4 °C) and plasma was collected, and stored at − 80 °C. Prior to quantification, samples were thawed, and RSV was extracted by liquid-liquid partitioning [25]. Briefly, 2.5 mL of ethyl acetate was added to 500 μL of plasma and vortexed-mixed for 30 s before centrifugation at 4000 rpm, for 10 min, at room temperature. The supernatant was transferred to a new tube, and the solvent was evaporated at room temperature under compressed air flow. The dried extract was reconstituted by the addition of 100 μL of 80% methanol in water and vortexed-mixed for 30 s prior to analysis.

RSV in rat plasma was determined by LC-MS/MS in order to reach the required selectivity and sensibility. The analysis was carried out on an Infinity 1290 liquid chromatograph system (Agilent, USA) equipped with a phenylhexyl C18 column (1.8 μm, 4.6 mm × 5.0 mm) and guard column of same material (Zorbax Eclipse, Agilent, USA), operated in gradient mode at 25 °C. The mobile phase consisted of 0.1% formic acid and 0.1% acetonitrile plus formic acid, with a flow rate of 0.5 mL/min, for 7 min. Liquid chromatograph was coupled to a triple-quadrupole mass spectrometer (6460 Triple-quad LC/MS; Agilent, USA), equipped with Jetstream electrospray source, operating in the negative mode. The parameters of the source were as follows: gas temperature 350 °C, gas flow 10 L/min, nebulizer 55 psi, sheath gas temperature 300 °C, sheath gas flow 10 L/min, capillary − 4500 V, nozzle voltage − 500 V and delta EMV 650. Spectrometric parameters were 140 V of fragmentation and collision energies between 12 and 25 V.

Trans-resveratrol identification was performed through the MRM transitions 227 > 143 and 227 > 185, at the retention time of 3.3 min, being the first transition used for quantification and the second for confirmatory analysis [26]. *Trans*-resveratrol concentrations were calculated by using the equation obtained from a matrix-matched calibration curve in triplicate. The curves were constructed using the surrogate analyte peak area ratio *versus* the concentration of analyte. The glucuronide, glycoside, and sulfate conjugates of resveratrol were identified through Neutral Loss scans, with the respective transitions: 403 > 227, 307 > 227, and 389 > 227 at retention times close to *trans*-resveratrol peak. The concentrations of these metabolites were estimated as RSV equivalents, by using the calibration curves constructed for *trans*-resveratrol.

Histological Assessment

After euthanasia, i.e., 21-day post-CFA administration, the right hindpaw tissue was removed with a scalpel and fixed in 10% buffered formalin for 24 h. Subsequently, the samples were dehydrated and embedded in paraffin. The evaluation of the inflammatory infiltrate and cartilaginous destruction was carried out using hematoxylin-eosin (HE)-stained slides. The histological sections were cut into 4-μm thickness. Each slide was scored in a blinded manner according to the inflammatory degree, from 0 to 4: 0, no inflammation; 1–2, slight thickening of the lining layer and/or some infiltrating cells; 3, thickening of lining layer and/or more pronounced influx of inflammatory cells; and 4, thickening of lining layer and numerous inflammatory cells. The destruction of cartilage was graded on a scale of 0 to 4, as follows: 0, no destruction of cartilage; 1–2,

localized cartilage erosions; 3, more extended erosions; and general cartilage destruction [27].

Determination of Tissue Cytokine Production

In the polyarthritis group, the animals were euthanized at the 21st day by deep isoflurane inhalation, and the paw subcutaneous tissues were collected. Samples were placed on phosphate-buffered saline (PBS) containing Tween-80 0.05%, phenylmethylsulphonyl fluoride 0.1 mM, benzethonium chloride 0.1 mM, EDTA 10 mM and aprotinin A 20 KIU, homogenized, centrifuged at 4500 rpm for 12 min. The supernatant was rapidly frozen and stored at − 70 °C for later measurement of interleukin (IL)-6 and IL-10 levels. Cytokine levels were evaluated using specific ELISA kits, according to the manufacturer's recommendations (R&D Systems).

Evaluation of Gastrointestinal Damage

The occurrence of gastrointestinal lesions was evaluated following the chronic administration of RSV (100 mg/kg), RSVO (10 mg/kg), or the positive control drug indomethacin (3 mg/kg). All of them were administered by the oral route, for 7 days, twice a day. At the eighth day following the treatment initiation, the rats were euthanized. Immediately after, the intestine (duodenum, jejunum, and ileum) was slit open opposite to the attached mesenteric tissue. The organs were washed with saline solution, and the mucosal surfaces were macroscopically examined according to an arbitrary scale [28]. The number and the gravity of erosions were scored by an examiner blinded to the experimental groups, on a scale of five grades: grade 0, no lesion; grade 0.5, hemorrhagic points; grade 1, ulcer length < 2 mm; grade 2, ulcer length > 2 mm; grade 3, lesion with perforation and hemorrhage. Experimental data was obtained by multiplying the score by the number of lesions. The mean scores for each group were calculated and expressed as lesion indexes.

Statistical Analysis

The results are expressed as the mean ± SEM of 5–8 animals per group. The statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Tukey post hoc test. Values of $P < 0.05$ were considered as indicative of significance. The inhibition rates were determined in percentage, based on the areas under the curve (AUC). All tests were performed using the GraphPad® 5.01 Software (San Diego, USA).

RESULTS

Effects of Prophylactic and Therapeutic Treatments with RSV or RSVO on Carrageenan-Evoked Acute Paw Edema

We firstly analyzed the effects of treatment with RSV or RSVO on the acute edema induced by carrageenan. The results showed that prophylactic administration of RSV (100 mg/kg and 200 mg/kg, p.o.), dosed 30 min before, significantly inhibited the edema induced by carrageenan, when compared to the respective control group, with inhibitions of 31 ± 7% and 22 ± 4%, respectively (Fig. 1a). The oral treatment of animals with RSVO failed to reduce the edema formation elicited by carrageenan, in either tested doses of 10 or 15 mg/kg (Fig. 1b).

Relating to the therapeutic schedule of treatment, the edema evoked by carrageenan was markedly reduced by RSV (100 mg/kg, p.o.), dosed 120 min after carrageenan application, with inhibitions of 35 ± 8% and 49 ± 9%, at 180 and 240 min, respectively. The therapeutic regimen using RSVO (10 mg/kg, p.o.) was also capable of significantly reducing the paw edema formation, with an inhibition percentage of 38 ± 3%, at 180 min (Fig. 1c).

Anti-edema Actions of RSV and RSVO in Sub-chronic Inflammation Elicited by CFA

In the sub-chronic protocol, we compared the effects of RSV and RSVO in a short period of evaluation following CFA application (until 3 days). The results depicted in Fig. 2 show that administration of RSV or RSVO (100 mg/kg and 10 mg/kg, p.o., respectively), given 2 h after the induction of paw edema, and once a day, for 3 days, significantly reduced the edema induced by CFA injection. For RSV treatment, the calculated inhibition was 11 ± 2%, until 8 h (Fig. 2b), and 23 ± 3% (Fig. 2c), until 72 h of assessment. Regarding the RSVO treatment, the percentages of inhibition were 23 ± 4% and 21 ± 2%, respectively (Fig. 2e–f). Notably, the inhibitions observed for RSVO were significantly higher than those obtained for RSV.

Superior Activity of RSVO in the Polyarthritis Model

The animals received either RSV or RSVO (100 mg/kg and 10 mg/kg, p.o., respectively, twice a day, for 8 days), between the 14th and the 21st days after CFA

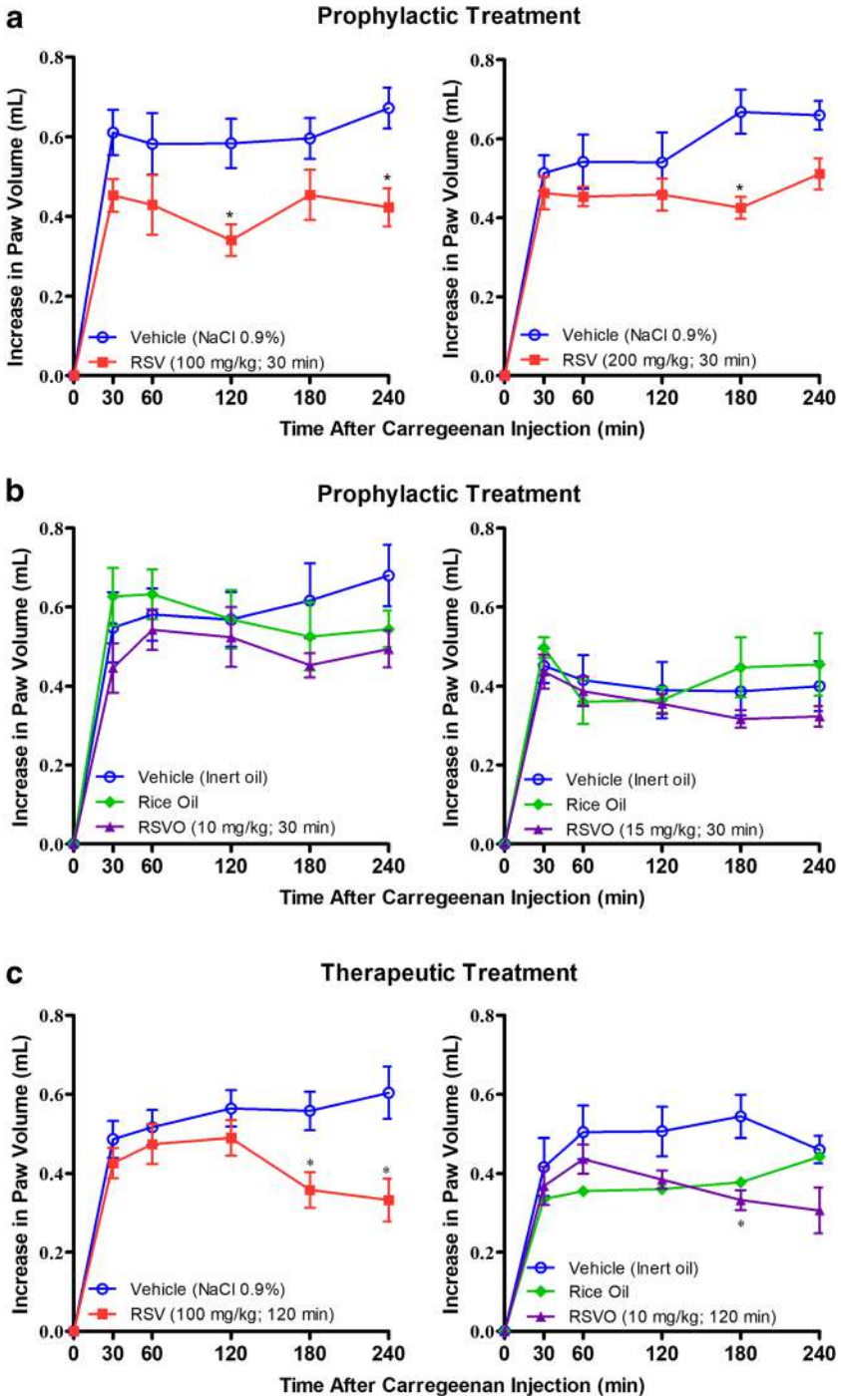

Fig. 1. Effect of resveratrol (RSV; 100 and 200 mg/kg, p.o.) and resveratrol vehicled in rice oil (RSVO; 10 and 15 mg/kg, p.o.), 30 min before (prophylactic treatment) and after 120 min (therapeutic treatment), on paw edema evoked by carrageenan. **a** RSV (100–200 mg/kg, p.o.) and saline 0.9% (vehicle); **b** RSVO (10–15 mg/kg, p.o.), rice oil and inert oil (vehicle); **c** RSV (100 mg/kg, p.o.) and RSVO (10 mg/kg, p.o.) Each point represents the mean of 6 animals and vertical lines show the SEM. *$P < 0.001$ significantly different from vehicle values.

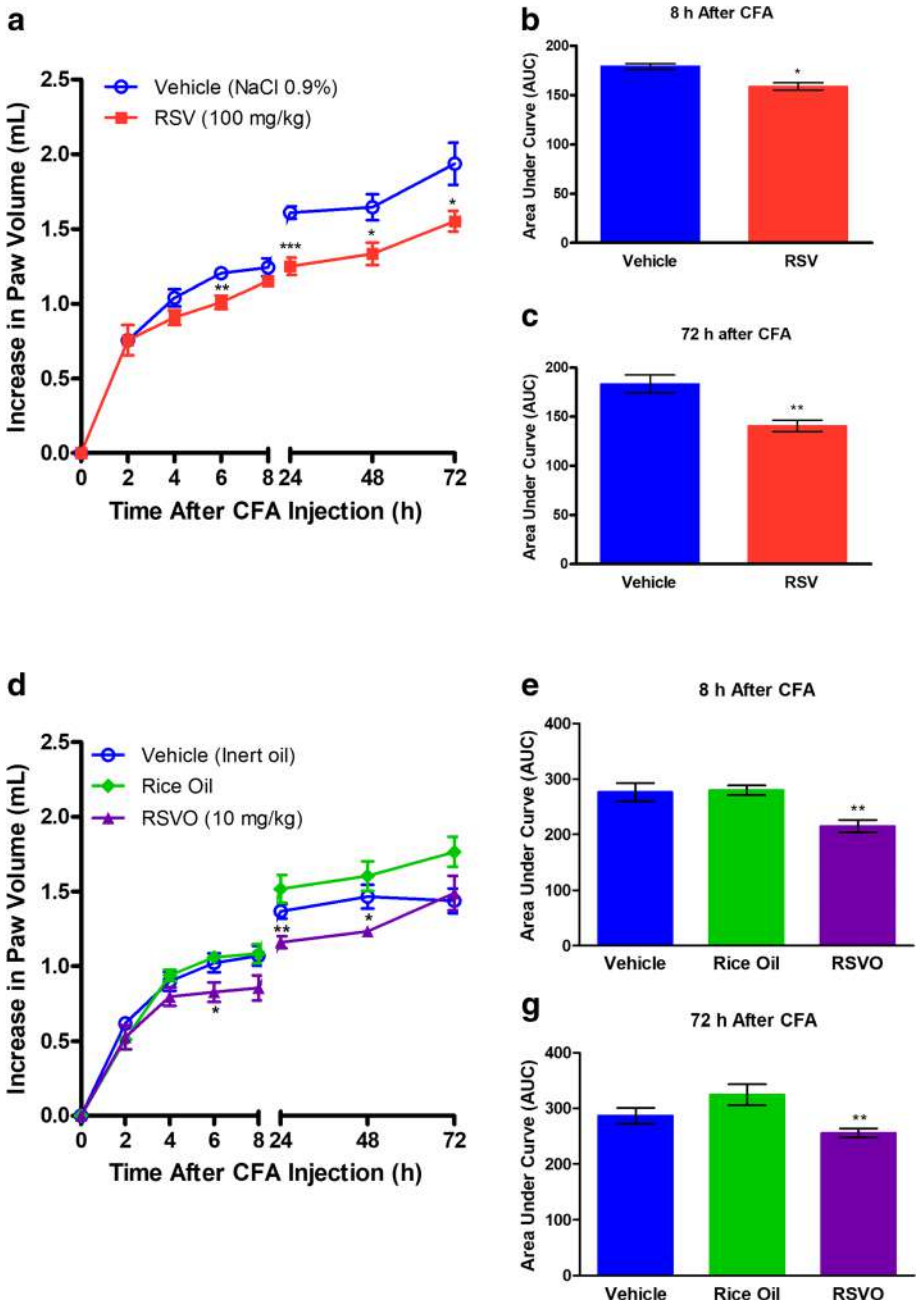

Fig. 2. Effect of resveratrol (RSV; 100 mg/kg, p.o.) and resveratrol vehicled in rice oil (RSVO; 10 mg/kg, p.o., 2-h post-CFA, daily, for 3 days), on paw edema generated by CFA. **a** RSV and saline 0.9% (vehicle); **d** RSVO, rice oil, and inert oil (vehicle). The percentages of inhibition were calculated based on the area under the curve (AUC), 8 h (**b**, **e**) and 72 h (**c**, **f**) after CFA injection. Each point represents the mean of 6–8 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective vehicle values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. CFA, complete Freund's adjuvant.

injection. Both formulations were capable of significantly reducing the long-term edema caused by CFA. Again, in this experimental set, RSVO exhibited a significantly greater inhibition (29 ± 4%; Fig. 3d), in comparison with RSV (17 ± 5%; Fig. 3b), according to the evaluation of the areas under the curve.

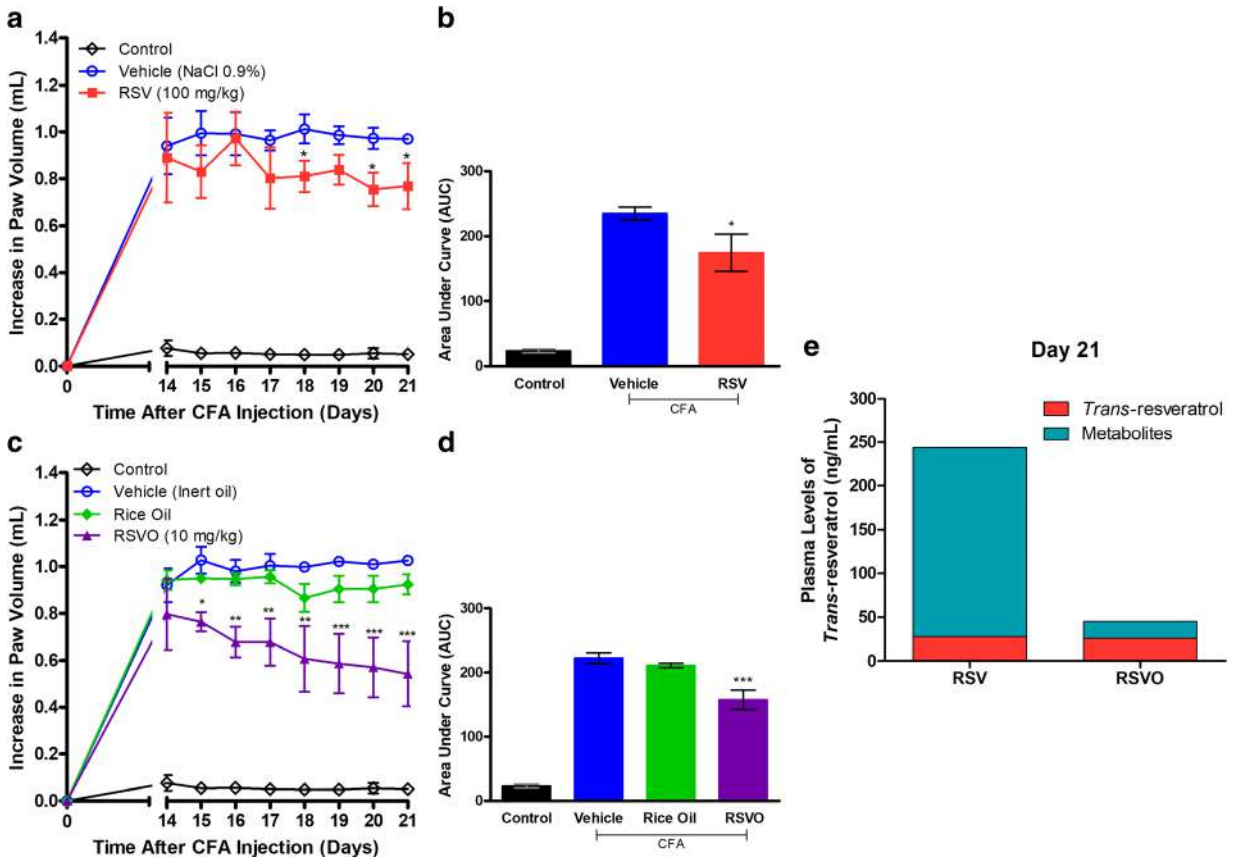

Fig. 3. Effect of resveratrol (RSV; 100 mg/kg, p.o.) and resveratrol vehicled in rice oil (RSVO; 10 mg/kg, p.o., 14-day post-CFA, twice a day, for 8 days), on paw edema evoked by CFA (polyarthritis model). **a** RSV and saline 0.9% (vehicle); **c** RSVO, rice oil and inert oil (vehicle). The percentages of inhibition were calculated based on the area under the curve (AUC), RSV (**b**) and RSVO (**d**) for 8 days. **e** Quantitative analysis of *trans*-resveratrol and estimation of metabolites concentration (*trans*-resveratrol equivalents)—in plasma rats, in the same schedule quoted above. Each point represents the mean of 5–10 animals and vertical lines show the SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different from vehicle values. CFA, complete Freund's adjuvant.

Biotransformation Parameters

Trans-resveratrol levels were evaluated in plasma of rats enrolled in the polyarthritis model, at 21 days. As depicted in Fig. 3e, animals that had been treated orally with RSVO (10 mg/kg) or RSV (100 mg/kg)—for 8 consecutive days, twice a day—had similar plasma levels of *trans*-resveratrol (28 ng/mL *vs* 30 ng/mL, respectively). As shown in Fig. 3e, approximately 88% of RSV—at the dose of 100 mg/kg—was biotransformed into metabolites of resveratrol. Alternatively, the treatment with the RSVO formulation (10 mg/kg) produced only 46% of resveratrol metabolites. The sum of *trans*-resveratrol and resveratrol metabolites is likely higher due to the 10-fold higher dosage of RSV *vs* RSVO.

RSVO Formulation Prevented Cartilage Destruction in the Polyarthritis Model

HE staining was performed to evaluate inflammatory infiltrates and cartilage integrity. Histopathological analysis showed a marked increase in inflammatory and cartilage destruction scores, according to the evaluation of hindpaw tissues in the polyarthritis CFA model (mean ± SEM; 3.0 ± 0.5 and 2.6 ± 0.2 respectively) (Figs. 4 and 5). The treatment with RSV (100 mg/kg) or RSVO (10 mg/kg) failed to significantly altering the inflammatory infiltration scores (Fig. 4b). Notably, the cartilage destruction scores were significantly decreased by the oral administration of RSVO, twice daily for 8 days, at the dose of 10 mg/kg (1.6 ± 0.2), whilst RSV (100

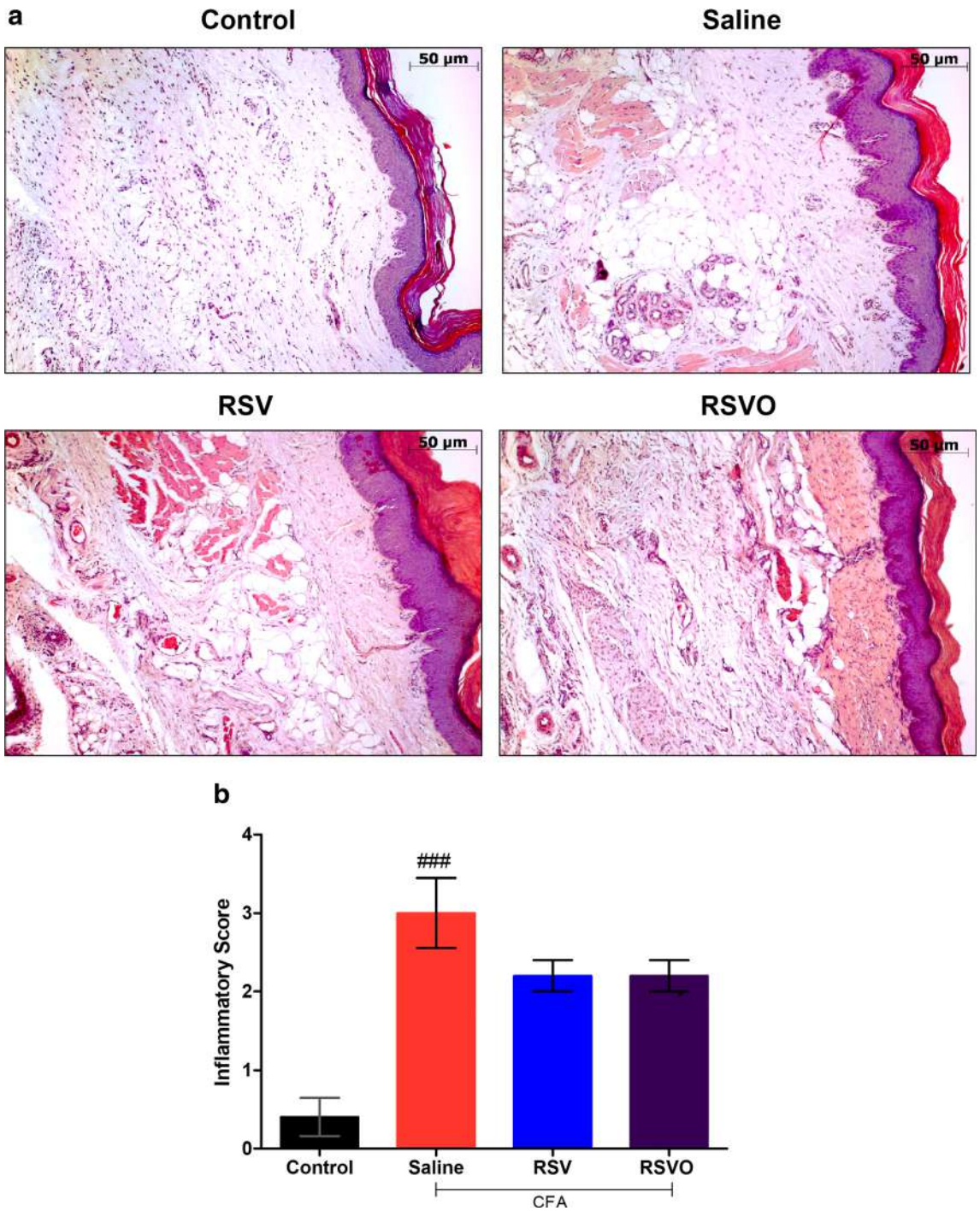

Fig. 4. Effect of treatment with RSV (100 mg/kg) and RSVO (10 mg/kg)—by oral route—for 8 consecutive days, i.e., from day 14th to 21st after CFA application, on the inflammatory infiltrate (**a**, **b**) (hematoxylin-eosin), in the animal model of polyarthritis. Each point represents the mean of 5 animals and vertical lines show the SEM. Asterisks denote the significance levels in comparison to respective control values: ###$P < 0.001$. The images were captured in magnification of × 100. Scale bar = 50 µm. CFA, complete Freund's adjuvant.

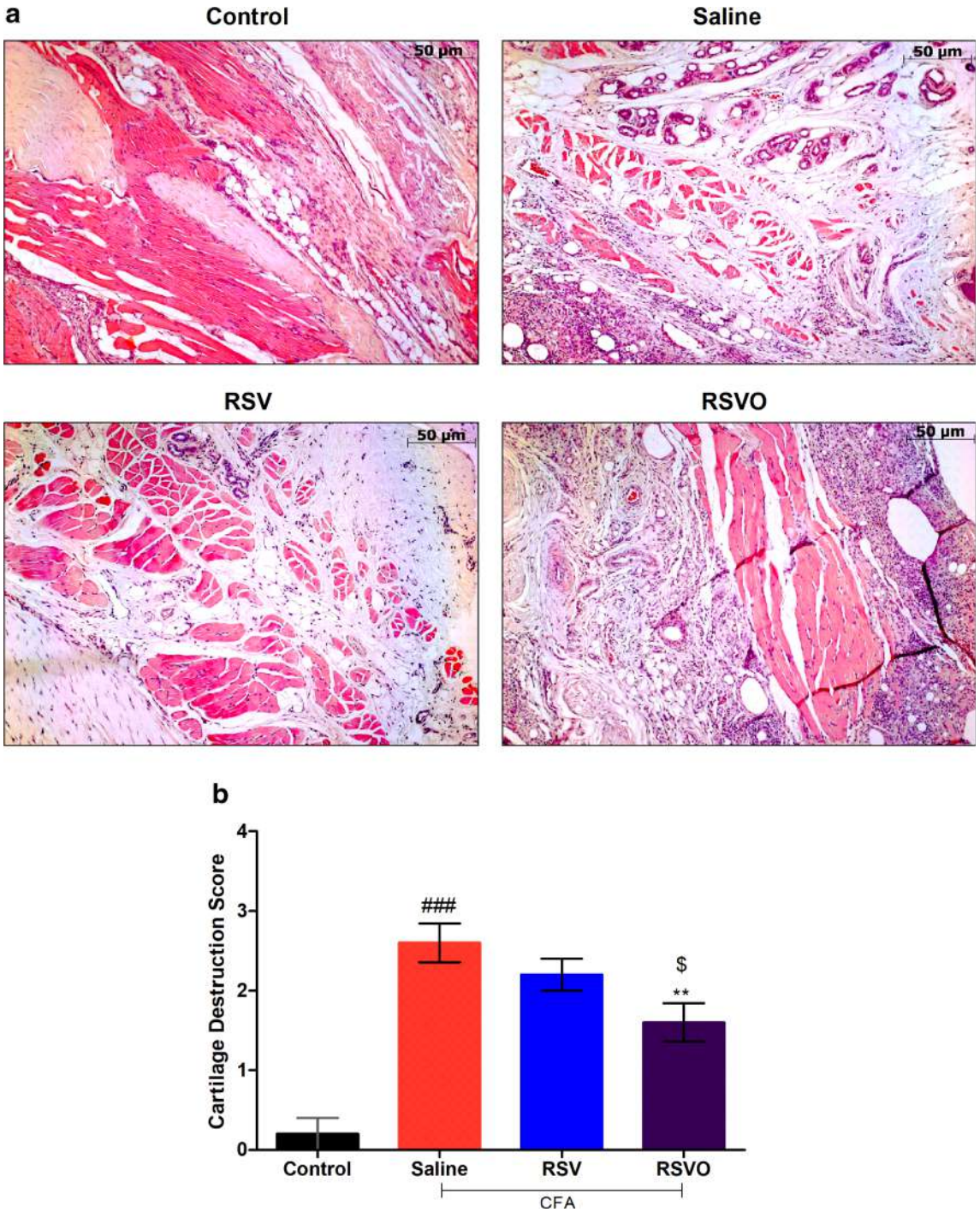

Fig. 5. Effect of resveratrol (RSV; 100 mg/kg, p.o.) and resveratrol vehicled in rice oil (RSVO; 10 mg/kg, p.o., twice a day, for 8 days) on cartilage destruction (**a** and **b**) in the CFA-elicited polyarthritis in rats. Each point represents the mean of 5 animals and vertical lines show the SEM. ###$P < 0.05$ significantly different from control values; **$P < 0.01$ significantly different from vehicle values; \$$P < 0.05$ significantly different from RSV values. CFA, complete Freund's adjuvant.

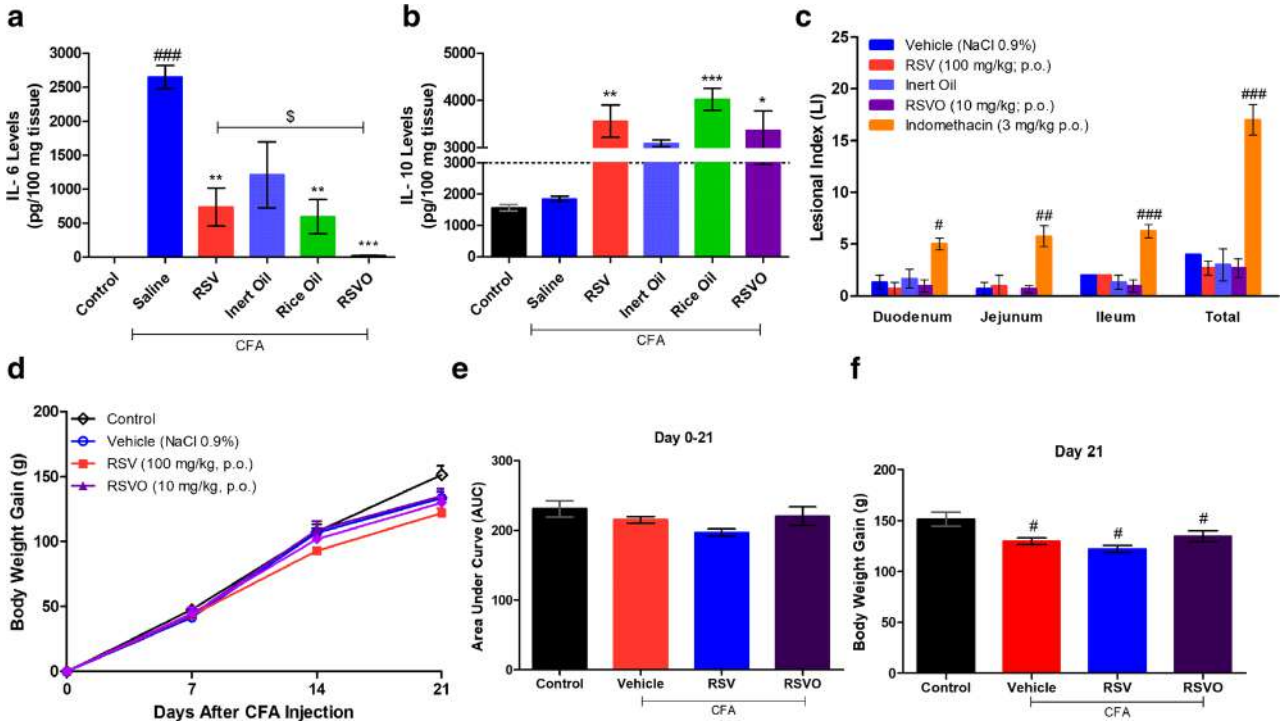

Fig. 6. Effect of resveratrol (RSV; 100 mg/kg, p.o.) and resveratrol vehicled in rice oil (RSVO; 10 mg/kg, p.o., twice a day, for 8 days) on IL-6 (**a**) and IL-10 (**b**) production, intestine lesion index (**c**) and body weight gain (**d**–**f**), in the CFA-caused polyarthritis in rats. Each point represents the mean of 5–6 animals and vertical lines show the SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ significantly different from vehicle values; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ significantly different from vehicle values—in the intestine lesion experiment, and significantly different from control values—in the body weight gain. CFA, complete Freund's adjuvant.

mg/kg) dosed at the same protocol of administration, failed to affect this parameter (Fig. 5b).

Evaluation of Cytokine Levels in the Rat Paw

IL-6 and IL-10 production was measured in the paw tissue after 21 days of CFA injection. CFA induced a marked increase of the pro-inflammatory cytokine IL-6. Interestingly, the treatment with RSV (100 mg/kg), rice oil, or RSVO (10 mg/kg) was capable of significantly reversing the increased production of IL-6 (Fig. 6a). In the RSV and rice oil-treated groups, the reduction percentages were 72 ± 11% and 77 ± 10%, correspondingly. Furthermore, the treatment with RSVO was able to abolish the production of IL-6, reaching the levels of the control group. Noteworthy, the treatment with RSV, rice oil, or RSVO produced a marked increase of the anti-inflammatory cytokine IL-10 (92 ± 19%, 117 ± 13% and 81 ± 22%, respectively) (Fig. 6b).

Gastrointestinal Safety After Chronic Administration of RSV and RSVO

This experimental set was designed to evaluate the gastrointestinal safety of RSV and RSVO, in comparison with the non-steroidal anti-inflammatory drug indomethacin, after the long-term administration of the compounds. As shown in Fig. 6c, the positive control drug indomethacin caused marked alterations of the gastrointestinal mucosa, displaying high lesional indexes. Neither RSV nor RSVO elicited any significant gastrointestinal damage, according to the evaluation of ulceration and hemorrhagic scores, presenting values near to the control groups.

Treatment with RSV and RSVO Does Not Restore the Body Weight Gain Over 21 Days

The body weight of animals was evaluated on days 0, 7, 14, and 21 after CFA application. As shown in Fig. 6d and e, a time-related analysis of body weight gain (from day 0 to 21) did not reveal any significant difference

among the experimental groups. However, all of the animals that received CFA had a significantly lower body weight, according to assessment at day 21, when compared with the control group. The respective values were control, 151 ± 7 g (mean ± SEM); vehicle, 130 ± 3 g; RSV, 122 ± 3 g; and RSVO, 135 ± 6 g (Fig. 6f).

DISCUSSION

The present study evaluated the effects of an innovative formulation containing the antioxidant agent RSV vehicled in rice oil (RSVO), in either acute or chronic models of inflammation in rats. Of note, our results show that RSVO formulation was greatly effective in reducing the long-term inflammatory responses elicited by CFA, displaying a favorable outcome when assessed for gastrointestinal toxicity. To our knowledge, this is the first study showing that low doses of RSV vehicled in rice oil displayed a marked anti-inflammatory effect when administered by oral route, especially in chronic protocols of administration.

As a first experimental approach, we have compared the effects of treatment with RSV and RSVO in the acute edema model induced by carrageenan. Previous literature data demonstrated that RSV significantly reduced carrageenan-induced rat paw edema, probably by interfering with prostaglandin production and COX-2 mRNA expression [29, 30]. More recently, it has been suggested that polydatin, a natural precursor of RSV, when associated with palmitoylethanolamide, reduced the development of edema and hyperalgesia through the modulation of pro-inflammatory and pro-nociceptive cytokines, namely IL-1β, TNF, and IL-6, in an animal model of carrageenan-evoked inflammation [31]. In our study, both prophylactic and therapeutic treatment with RSV, when given orally, at the dose of 100 mg/kg, significantly reduced the edema formation induced by carrageenan. However, the anti-inflammatory action of RSV in the carrageenan model was not superior at the 200 mg/kg dose, discarding a classic dose-response profile for RSV. Hence, we wondered whether lower doses of RSV vehicled in rice oil might interfere with carrageenan-elicited edema. Our results showed that oral prophylactic administration RSVO (10 or 15 mg/kg) was not able to significantly alter the edema formation caused by carrageenan, whereas it displayed a partial anti-edematogenic effect, when given therapeutically, 120 min after carrageenan injection. This experimental evidence allows us to suggest that the combination of RSVO resulted in a synergistic anti-inflammatory effect, according to evaluation in the therapeutic scheme of treatment.

When assessed in the sub-chronic edema model following CFA injection, the oral administration of RSVO (10 mg/kg) produced a sustained inhibition of edema formation, which was visibly greater than that seen for RSV (100 mg/kg), during the 3-day period of evaluation. The differences in the anti-edematogenic effects of RSV and RSVO might be explained by several reasons. Formerly, it has been largely described that RSV presents very low oral bioavailability in humans, as this molecule is rapidly metabolized [3]. It is reasonable to infer that the combination of RSV to rice oil resulted in an overall improvement of both the efficacy and the oral bioavailability of this compound, what likely accounts for a sustained drug release to the inflamed sites. Secondly, the rice oil itself holds a series of active compounds that might potentiate the anti-inflammatory effects of RSV. For instance, the rice bran oil is a rich source of γ-oryzanol, which contains a number of phytosteryl ferulates, such as 24-methylenecycloartanyl ferulate and cycloartenyl ferulate. In turn, these compounds have anti-inflammatory and antioxidant properties [14, 32, 33]. Rao and colleagues demonstrated that a diet supplemented with γ-oryzanol was able to decrease the secretion of inflammatory markers, such as thromboxane B_2, leukotriene B_4 and C_4, and IL-6 by rat peritoneal macrophages [34]. Furthermore, rice bran oil has been found to possess promising benefits in the prevention of cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease [14, 35].

An interesting study indicated that daily administration of RSV during 7 days reduced the severity of experimental chronic pulmonary fibrosis evoked by bleomycin in mice [36]. Considering the encouraging results obtained with RSVO in the sub-chronic CFA experimental paradigm, we have also compared the effects of oral administration of RSV (100 mg/kg) with RSVO (10 mg/kg), in the polyarthritis model elicited by CFA. Remarkably, the chronic schedule of treatment with RSVO, administered in a 10-fold smaller dose regimen for eight days, resulted in a marked inhibition of the inflammatory response caused by CFA, which was significantly higher in comparison to RSV. Again, we provide compelling evidence on the synergistic effects obtained by associating RSV to rice oil. When administered alone, the rice oil failed to significantly affecting the inflammatory effects evoked by CFA, in either the sub-chronic (until 8 h) or the polyarthritis experimental models. Nevertheless, the association of RSV to rice oil (RSVO) displayed clear beneficial advantages. Additionally, it was possible to observe that chronically

RSVO-treated group showed a lower biotransformation when compared to RSV (46% vs 88%, respectively), as indicated by the quantification of *trans*-resveratrol in the plasma. Supporting this data, Mamadou and colleagues have shown that nanoemulsions of RSV potentiated its absorption, as assessed by the jejunal absorptive transepithelial test [37]. Additionally, the solid dispersion system which consists of grape peel extract plus propylene glycol monocaprylate, poloxamer 188, polyoxyl 35 castor oil and polyethylene glycol 6000, demonstrated to enhance the oral bioavailability of RSV (0.9 to 10.5%) in rat plasma [38]. In the light of literature data, the present findings indicate that RSVO formulation bypassed the RSV degradation, promoting superior anti-inflammatory effects in the CFA-injected rats.

Elmali and colleagues showed that an intra-articular administration of RSV, once a day, for 2 weeks, starting at the onset of disease, protected against cartilage destruction and synovial inflammation, in a chronic inflammatory monoarthritis model in rabbits [39]. We showed that CFA evoked inflammatory cell infiltration and cartilage destruction in the hindpaw tissue—after 21 days of administration. In this study, the oral treatment with RSVO restored the cartilage integrity, whereas RSV administration failed to affect this parameter. Thus, the new formulation RSVO was capable of greatly reducing the edema formation, besides preventing the cartilage destruction secondary to polyarthritis induction. In spite of that, either RSVO or RSV did not significantly avoid the inflammatory cell infiltration in the polyarthritis model induced by CFA. This evidence contrasts somewhat with a previous study showing that RSV (20 mg/kg), given intraperitoneally in a long-term scheme of administration, inhibited the cell influx, synovial hyperplasia, and bone erosion in a mouse model of collagen-induced arthritis in mice [40]. The different experimental model or the tested rodent species might explain the discrepant data.

RSV has several mechanisms of action, such as the modulation of angiogenesis, inhibition of metastasis, modulation of DNA damage, regulation of sirtuins, and suppression of inflammation [41]. Concerning the inflammatory response, RSV has been shown to modulate the levels of the pro-inflammatory cytokines TNF, IL-1β, and IL-6 [5, 42]. An *in vitro* study conducted by Ferraresi and colleagues showed that RSV decreased the production of IL-6 in ovarian cancer cells [43]. Additionally, another study by Schwager indicated that RSV was able to reduced prostaglandin E2, chemokines (CCL5/RANTES, CXCL8/IL-8) and also increased IL-10, IL-6, and IL-1β production by chondrocytes, in acute and chronic inflammation [44]. Accordingly, in the experimental model of polyarthritis elicited by CFA, the treatment of rats with RSV markedly reduced the production of IL-6 and increased the levels of the anti-inflammatory cytokine IL-10. Additionally, the present data show that RSVO formulation displayed a potent anti-inflammatory activity, by abolishing the production of IL-6, and by increasing the generation of IL-10. Even the administration of rice oil alone caused a significant increase of IL-10 levels, what might help to explain the notable anti-inflammatory effects observed for RSVO in our study. Nevertheless, additional studies are still necessary to further clarifying the mechanisms of action of RSVO in inflammation.

It is tempting to suggest that RSVO might represent a potential alternative for treating chronic inflammatory diseases, such as arthritis. In fact, there is an urgent need for new alternatives for management of most inflammatory chronic diseases, what is of high interest to the medical practice today [45]. Regarding the treatment of arthritis, currently available drugs have been widely associated to acute and long-term toxicity. In the case of non-steroidal anti-inflammatory drugs (NSAID), one of the most relevant aspects concerns the gastrointestinal toxicity observed for this group of molecules, especially following long-term regimens of treatment [46]. We have compared the effects of the repeated oral administration of either RSV or RSVO (for 8 days), with those seen for the NSAID drug indomethacin, on the gastrointestinal tract. Strikingly, whereas the animals in the indomethacin group displayed high indexes of gastrointestinal damage, neither RSV nor RSVO elicited any evident signal of toxicity. RSV is able to inhibit both the activity and the expression of COX-2, without interfering with COX-1 [47]. Therefore, the low gastrointestinal toxicity of RSV and RSVO might be explained by its relative COX-2 selectivity. Noteworthy, it has been suggested that RSV can display gastroprotective effects in different experimental models via activation of sirtuins, especially, Sirt1 and Sirt3 [48]—a mechanism that deserves further investigation. In our experimental model, the induction of polyarthritis by CFA was associated with a slight body weight loss, according to the evaluation at 21 days. Nevertheless, nor RSV or RSVO was able to prevent this alteration in our experimental model. A pilot study demonstrated that a 4-month protocol of treatment with RSV prevented the body weight loss elicited by anti-HIV protease inhibitors in rats [49]. It is tempting to suggest that a long-term of

treatment with RSVO might prevent CFA-related body weight reduction.

Taken together, the present results provide evidence showing a superior profile for the new formulation named RSVO to prevent edema formation and cartilage destruction, lacking gastrointestinal toxicity, with a favorable bioavailability profile. It is tempting to suggest that RSVO might be used as an adjuvant to improve the life quality of individuals with arthritis.

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COMPLIANCE WITH ETHICAL STANDARDS

The Institutional Animal Ethics Committee approved all the experimental protocols used in the present study.

Conflict of Interest. The authors declare that they have no conflicts of interest.

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