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SIRT1: A likely key for future therapeutic strategies for pain management

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ABSTRACT

Sirtuin 1 (SIRT1), a NAD+ -dependent histone deacetylase, plays a crucial role in mitigating oxidative stress, regulating inflammation, and maintaining mitochondrial function. Reduced SIRT1 activity has been linked to elevated pro-inflammatory cytokines, mitochondrial dysfunction, and chronic pain, all of which are observed in long COVID pathology. Emerging evidence identifies mitochondrial dysfunction and oxidative stress as central contributors to these symptoms. Increases reactive oxygen species (ROS) such as superoxide, nitric oxide, and peroxynitrite, leading to oxidative damage, chronic inflammation, and central/peripheral sensitization. Nutraceuticals, particularly the polyphenolic fraction of bergamot (BPF), have demonstrated potent antioxidant, anti-inflammatory, and antiviral properties. This study highlights BPF's ability to modulate SIRT1 activity in a rat model of inflammation and hyperalgesia. It provides novel evidence of SIRT1 nitration within the nucleus as a key event in inflammatory pain pathogenesis. BPF administration preserved SIRT1 activity, reduced oxidative stress markers such as malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8-OHdG), and minimized post-translational modifications of nuclear proteins, including nitration, acetylation, and carbonylation. Additionally, it alleviated hyperalgesia and allodynia. These findings underscore the therapeutic potential of polyphenols like BPF in reducing oxidative stress and inflammation-driven pain. By activating SIRT1, BPF may provide relief for pain conditions. Further research on SIRT1-targeted therapies is essential to combat inflammation and oxidative stress, preventing chronic conditions and enhancing treatment options.

1. Introduction

Many people experience pain as a health issue with serious social repercussions. The COVID-19 pandemic, caused by the SARS-CoV-2 virus, has highlighted the condition known as Long COVID, which is characterized by persistent symptoms that continue even after the acute phase of the infection [1]. Among these, pain is one of the most reported symptoms.

Long COVID's symptoms show that the virus' effects extend beyond the respiratory system, affecting multiple organs and systems [2]. The most common reported symptoms among Long COVID patients are chronic fatigue, difficulty with physical exercise, cognitive disturbances

Abbreviations: COVID, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; ROS, reactive oxygen species; RNS, reactive nitrogen species; SIRT1, Sirtuin 1; BPF, Polyphenolic fraction of Bergamot; MDA, malondialdehyde; 8-OHdG, 8-hydroxydeoxyguanosine; SO, superoxide; NO, nitric oxide; PN, peroxynitrite; NSAIDs, non-steroidal anti-inflammatory drugs; IL-1β, interleukin 1 β; IL-6, interleukin 6; TNF-α, Tumor Necrosis Factor-alpha; PARP, poly-ADP-ribose polymerase; FOXO1/3/4, forkhead-box transcription factor 1/3/4; HSF1, heat shock factor 1; HIF-1a, hypoxia-inducible factor 1 alpha; NF-kB, nuclear factor kappa B; Nef2, nuclear factor E2-related factor 2; PPAR, proliferator-activated receptor-gamma; MnTBAP, manganeseIII beta-octabromo-meso-tetrakis4-carboxyphenylporphyrin; HPLC, High-Performance Liquid Chromatography; BCA, Bicinchoninic Acid; TBARS, thiobarbituric acid reactive substances; DNPH, 2,4-dinitrophenylhydrazine; ANOVA, analysis of variance; PTM, post-translational modification; 4-HNE, 4-hydroxynonenal.

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and pain conditions suggesting possible systemic alterations beyond the initial viral infection [1]. Recent research suggests that oxidative stress could be a key factor underlying the persistence and variety of symptoms associated with Long COVID [3].

Redox balance is crucial for maintaining cellular homeostasis, apoptosis, modulation of immune responses, cellular aging, and pain response [4,5]. Pain and inflammation are associated with a reduction in the cellular redox balance, leading to increased oxidative damage induced by reactive oxygen species (ROS) such as superoxide (SO), nitric oxide (NO) and peroxynitrite (PN). This oxidative damage contributes to the development and maintenance of both central and peripheral sensitization [4,6–8].

Furthermore, it has been demonstrated that SO, NO and PN contribute to these signaling pathways through the post-translational modification of key proteins [6–10]. PN is a potent pro-nociceptive nitroxidative species, formed when superoxide combines with nitric oxide. It can nitrate protein bound tyrosine altering the structure and inhibiting or increasing enzyme activities [7,11]. Tyrosine nitration is one of the modifications occurring under conditions of PN over-production, it consists of a covalent addition of a nitro group (-NO2) to one of the two equivalent orthocarbons of the aromatic ring in tyrosine residues [12].

Despite the availability of pharmacological and non-pharmacological pain relief options, their use is questionable due to their low efficacy and adverse effects [13].

For instance, traditional non-steroidal anti-inflammatory drugs (NSAIDs) remain a cornerstone of inflammatory pain management. However, their use is limited by debilitating side effects including increased risks of heart attack and stroke which led to a decline in their use [7,9]. This highlights the urgent need for the development of new and effective strategies to manage pain and inflammation while preventing the transition to chronic pain.

Therapeutic strategies targeting SO, NO and PN can both prevent and reverse the pathologies associated with pain of various etiologies [14–16]. Modulation of protein kinases or ion channels, alterations in glutamatergic neurotransmission, and neuroinflammation are specific of the development of central sensitization and can occur in the periphery, spinal cord, or supraspinal region [9,17].

Moreover, our research documented that inflammation induced by intraplantar injection of carrageenan in rats led to significant increases in paw edema, with release of pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in paw exudates, nitrotyrosine formation, poly-ADP-ribose polymerase (PARP) activation, and hyperalgesia. Interestingly, the administration of synthetic antioxidants was able to reverse this condition, reducing signs of inflammation and pain. These findings are particularly relevant as they mirror the pathological mechanisms observed in COVID-19, where similar inflammatory cascades, oxidative stress markers, and hyperactivation of inflammatory mediators play a crucial role in disease progression [15].

In this scenario, sirtuins, NAD+ -dependent histone deacetylase enzymes, play a key role in several crucial cellular biological processes, including oxidative stress, cellular inflammation, and cellular functional homeostasis [4,6,10,18]. The most well-characterized sirtuin is the nuclear protein Sirtuin 1 (SIRT1) which is involved in diseases caused by inflammation, including cancer, metabolic disorders, neurodegenerative diseases, cardiovascular diseases, chronic obstructive pulmonary disease, COVID-19 induced respiratory distress. SIRT1 could also play a central role in pain management [19–21].

SIRT-1 has been identified as one potential biomarker associated with inflammatory processes in long COVID patients confirming that these patients develop an inflammatory state that damages many tissues ending in chronic pain condition [21,22].

Many non-histone proteins are deacetylated by SIRT1, which has been proven to affect pathophysiological processes like cell differentiation, apoptosis, autophagy, metabolism, and inflammation [23].

SIRT1 can directly deacetylate a variety of transcription factors or co-factors, such as P53, forkhead-box transcription factor 1/3/4 (FOXO1/3/4), heat shock factor 1 (HSF1), hypoxia-inducible factor 1 alpha (HIF-1a), nuclear factor kappa B (NF-kB), nuclear factor E2-related factor 2 (Nef2) [23,24].

Specifically, SIRT1 down-regulates p53 activity, increasing lifespan, cell survival and neuroprotection; it also deacetylates peroxisome proliferator-activated receptor-gamma (PPAR) and its co-activator 1 alpha, promoting fat transit, increasing the size and number of mitochondria, and positively regulating insulin secretion [25,26].

Hence, SIRT1's deacetylase activity has an effect on a variety of biological procedures [23].

Recent evidence emphasizes the significant role of SIRT1 in the context of long COVID [3,27,28]. In pain suffering Long COVID patients, dysregulation of SIRT1 activity has been linked to increased levels of pro-inflammatory cytokines, mitochondrial dysfunction, and oxidative stress [3,29–31]. It is believed that these alterations may contribute to the development of the main symptoms of long COVID, such as chronic fatigue, cognitive difficulties, inflammation, and pain [32,33]. Through the deacetylation activity, SIRT1 emerges as a key target for therapeutic strategies in these diseases.

Recently, it was discovered that substances of natural origin, such as the polyphenolic compounds present in fruits, vegetables, and plants (e.g., resveratrol, fisetin, quercetin, and curcumin), can increase SIRT1 activity [34,35].

The use of nutraceuticals rich in polyphenols for inflammation and pain management has significantly increased in recent years. Polyphenols, such as those found in bergamot, demonstrated potent anti-inflammatory and antioxidant activities and anti-viral effects [36–38].

Natural products such as bergamot derivates can reverse hyperalgesia and allodynia induced by oxidative stress in animal models of inflammatory pain [10,39,40]. Based on these findings, we now suggest that alterations in nuclear SIRT1 in a model of inflammatory pain contribute to the emergence and persistence of pain, and that polyphenolic fraction of bergamot treatment can improve the pain condition. The objectives of this study are to explore the therapeutic potential of SIRT1 in modulating inflammation, analyzing its role in the molecular mechanisms underlying inflammatory pain, using a well-characterized animal model of inflammatory pain, and evaluating the efficacy of natural compounds with antioxidant properties in reducing oxidative damage caused by free radicals, with potential applications for studying pain and symptoms related to Long COVID.

2. Materials and methods

2.1. Animals

Male Wistar rats (175–200 g, Envigo) were utilized in accordance with European Economic Community regulations (2010/63/EU) under authorization number 32659.27 and 577–2016-PR, the NIH Guidelines on Laboratory Animal Welfare, and Italian regulations for the protection of animals in experimental and scientific research (D.L. 26/2014), approved on 9/05/2023 and 8/06/2016, respectively. The number of animals selected was adequate to achieve statistical significance ($p < 0.05$), as recommended by the International Society for the Study of Pain guidelines.

Each cage housed two rats, and they were kept in a controlled environment with stable temperature (21 ± 1 °C) and humidity (60 ± 5 %), maintaining a 12-hour light/dark cycle. Food and water were provided ad libitum. Each cage was equipped with enrichments to enhance animal welfare. The experiments were conducted between 7:00 AM and 10:00 AM in a quiet setting. Unless otherwise noted, all drugs were procured from Sigma Aldrich (Milan, Italy) and were dissolved in saline solution (0.9 % sodium chloride).

2.2. Experimental group

Animals were randomly divided into the following groups:

Vehicle group: Rats (n = 10) received an intraperitoneal (i.p.) injection of saline 15 min before intraplantar (i.pl.) injection of saline into the right hindpaw.

Carrageenan group: Rats (n = 10) received an intraperitoneal (i.p.) injection of saline 15 min before intraplantar (i.pl.) injection of carrageenan (1 % suspension in 0.85 % NaCl; Calbiochem, cat. 22049) into the right hindpaw.

Drug groups: Rats (n = 10 for each group) treated with polyphenol fraction of bergamot (BPF, 25 mg/Kg) or manganese (III) beta-octabromo-meso-tetrakis (4-carboxyphenyl) porphyrin (MnTBAP, 10 mg/Kg) 15 min before intraplantar (i.pl.) injection of carrageenan (1 % suspension in 0.85 % NaCl; Calbiochem, cat. 22049) into the right hindpaw.

High-Performance Liquid Chromatography (HPLC) was employed to assess the polyphenol content in the BPF powder. Furthermore, toxicological evaluations indicated that no harmful compounds were present. Microbiological standard tests also confirmed the absence of bacteria and mycotoxins [41]. MnTBAP (Calbiochem, Darmstadt, Germany; code 475870) was sourced from Sigma (Milan, Italy) and was prepared in a saline solution (0.9 % sodium chloride).

The dose and the timing of compounds has been chosen according to the bibliography [10,42–44]. For all groups, after 6 hours rats were sacrificed and spinal cord (L4–L6) were explanted and the urine was collected. The tissue was immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analyses.

2.3. Behavioral test

Rats were acclimatized in a Plexiglas cage for about 20 minutes before each measurement.

Hargreaves's protocol [45] was used to evaluate damage in non-responsive animals' tissues. The thermal stimulus was directed to a single hind paw (cut off latency of 20 s) through mobile unit, consisting of a high intensity projector bulb. The withdrawal latency period of injected and contralateral paws was determined. Each successful test point represented the difference (sec) in withdrawal latency between paws [withdrawal latency of contralateral (left paw) minus withdrawal latency of injected paw (right paw)] at each time point. The test ended when the animal failed to respond by 20 s. Results were expressed as Paw-withdrawal latency changes (sec).

Paw volume was measured with a plethysmometer, as previously described [46] before and at 6 hours carrageenan injection. Edema was expressed as the increase in paw volume (mL) after carrageenan injection, relative to the paw's pre-injection volume. All experimenters were blinded to treatment conditions. Data were unblinded during data analysis.

2.4. Tissue preparation for nuclear protein extraction

Spinal cord (L4-L6) was homogenized with lysis buffer (10 mM Hepes pH8; 50 mM NaCl; 1 mM EDTA pH8; 0.2 % Triton; 500 mM Sucrose; 250 μM PMFS; 1 % protease inhibitor cocktail (v/v; Sigma, cat. 8340)) at 1:3 w/v ratio. Therefore, 14.000 rpm centrifuge (4 °C, 10 min) was performed to obtain supernatants from solubilized extracts (cytoplasmatic fraction). Pellet obtained was washed with buffer (10 mM Hepes pH8; 50 mM NaCl; 0.1 mM EDTA pH8; 25 % Glycerol), centrifuged at 14.000 rpm (10 minutes, 4°C) and then incubated for 10 minutes with lysis buffer (10 mM Hepes pH8; 350 mM NaCl; 0.1 mM EDTA pH8; 25 % Glycerol; 250 μM PMFS; 1 % protease inhibitor cocktail (v/v; Sigma, cat. 8340)). After incubation, samples obtained were sonicated for 15 seconds on ice and centrifuged at 14.000 rpm centrifuge (4°C, 10 min) to obtain supernatants from solubilized extracts (nuclear fraction). Then, the obtained samples were stored at −80 °C until usage. Bicinchoninic Acid (BCA) protein assay (Thermo Scientific, cat. 23225, Milan, Italy) was used to determine protein concentration.

2.5. Immunoprecipitation and western blotting analyses

Immunoprecipitation and western blot analyses were performed with the previous obtained proteins, using specific antibodies.

For the immunoprecipitation of nitrated proteins, nuclear proteins (250 μg) were incubated with agarose-conjugated anti-nitrotyrosine monoclonal antibody (10 μg; Upstate Biotechnology) and then washed in PBS (pH 7.4). The mix of bead-antibody and binding proteins was resuspended in 50 μl of sample buffer [2X, 0.5 M Tris-HCL (pH 6.8), 2.5 % glycerol / 0.5 % SDS / 200 mM 2- mercaptoethanol / 0.001 % bromophenol blue], and heated to 95 °C (8 min). Western blot analyzes of the immunoprecipitated protein complex and total lysates were performed using specific antibodies. Briefly, 10 % or 7.5 % SDS-PAGE minigels were used to resolve nuclear proteins, and then transferred to nitrocellulose membranes. Membranes were blocked at room temperature in 1 % BSA/0.1 % thimerosal in 50 mM Tris−HCl (pH 7.4)/150 mM NaCl/0.01 % Tween-20 (TBS/T), for 2 h. Then, membranes were incubated at 4 °C, O/N with: anti-SIRT1 (1:1000; Santa Cruz Biotechnology, cat. sc-15404); anti-acetylated lysine (1:1000, Cell Signaling, cat. 9441); anti-nitrotyrosine (O/N, 4 °C, 1:1000; Millipore, cat. AB5411), washed in TBS-Tween20 and incubated for 1 h at room temperature with anti-mouse (1:10,000; GE Healthcare, cat. NA931) or anti-rabbit (1:15,000; GE Healthcare, cat. NA934) secondary antibodies conjugated to horseradish peroxidase. Enhanced chemiluminescence (ECL; GE Healthcare, cat. RPN2232) was used to visualize the protein, after washing. Among the lanes, no difference for laminin (1:1000; Bd Transduction Laboratories, 612163) was detected. Thus, for each lane densitometry data were normalized against laminin. ImageQuant 5.2 software (Molecular Dynamics) was used to determine the quantitation of protein bands by densitometry.

2.6. Malondialdehyde assay

Malondialdehyde (MDA) detection was performed by measuring thiobarbituric acid reactive substances (TBARS). Lysate, after interaction with 10 % NaOH, 20 % Acetic Acid and TBA, were boiled at 95 °C for 1 h and placed on ice for 10 min. Then, samples were centrifuged at 1600 × g (10 min at 4 °C), loaded into a black 96-well microtiter plate, and fluorometrically measured at an excitation of 530 nm and emission of 550 nm, using Infinite 200 microplate fluorometer (Tecan, Männedorf, Switzerland).

2.7. 8-hydroxy-2′-deoxyguanosine (8OHdG)

8-hydroxy-2′-deoxyguanosine (8OHdG) analyses was conducted using a competitive immunoassay with the 8-hydroxydeoxyguanosine (8-OHdG) ELISA kit (Biomatik, Wilmington, DE, USA), following the manufacturer's protocol. Absorbance was measured at 450 nm using a Tecan Sunrise microplate reader (Tecan). The results were expressed in pg/mL.

2.8. SIRT1 deacetylase activity assay

SIRT1 deacetylase activity was determined using the SIRT1 Fluorimetric Activity Assay/Drug Discovery Kit (EnzoLife, cat. BML-AK555–0001) following the manufacturer protocol. The nuclear extract (5 μg) was incubated with the Fluor de Lys substrate buffer at 37 °C for 1 h, and then by Fluor de Lys Developer at 37 °C for 40 min. samples were fluorometrically measured at an excitation of 360 nm and emission of 460 nm, using an Infinite 200 microplate fluorometer (Tecan).

2.9. Detection of protein carbonylation

Protein carbonyl groups was determined using the Oxyblot protein oxidation kit (Millipore, cat. 57150) following the manufacturer protocol. Briefly, protein carbonyl derivatives were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH). The level of carbonyl protein groups was analyzed by Western Blot. SIRT1 and laminin levels were detected after stripping the membrane, using stripping buffer solution (Thermo scientific, cat. 21059) following the manufacturer protocol.

2.10. Statistical analysis

The Kolmogorov–Smirnov test was used for analysis of the data distribution. After confirmation of normal data, differences between groups were compared by analysis of variance (ANOVA). Two-way repeated measures ANOVA with Bonferroni comparisons were used for data obtained from each time point. Other data were analyzed via one-way ANOVA followed by the Newman–Keuls test. Statistical significance was fixed at $p < 0.05$. The results are expressed as mean ± SEM. Analyses were carried out using GraphPad Prism software (v8.00; GraphPad Software, Inc., San Diego, CA, USA).

3. Results

3.1. Carrageenan-induced thermal hyperalgesia and edema are associated with increased malondialdehyde (MDA) and 8-hydroxydeoxyguanosine (8OHdG) levels

Intraplantar injection of carrageenan in rats produced a time-dependent development of thermal hyperalgesia and edema, peaking between 2 and 6 hours (plateau phase; Fig. 1).

Our recent studies have extensively documented that during inflammation induced by carrageenan administration in rats, there was also an increase in pro-inflammatory cytokine levels, which appeared to decrease following the administration of antioxidants [15]. Here we observed that this response was associated to an increase in Malondialdehyde (MDA) (Fig. 2), a significant byproducts of lipid peroxidation, and 8-hydroxydeoxyguanosine (8OHdG) (Fig. 3), a key indicator endogenous oxidative damage to DNA. These compounds can modify essential proteins through cross-linking, leading to their inactivation [8,47].

We observed elevated levels of MDA in the lumbar tract of the spinal cord (Fig. 2) and increased levels of 8OHdG in the urine of carrageenan-treated rats (Fig. 3). Intraperitoneal injections of BPF (25 mg/kg) and MnTBAP (10 mg/kg), administered 15 minutes prior to carrageenan injection, ameliorated the development of thermal hyperalgesia and edema (Fig. 1), supporting previous studies [4,8]. Additionally, these

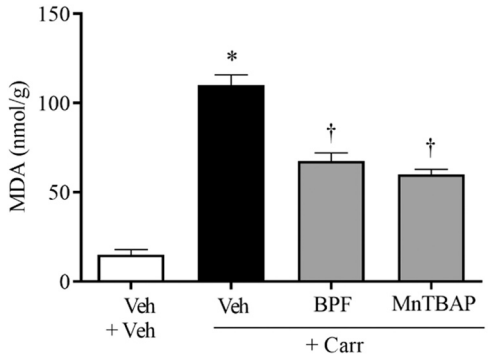

Fig. 2. Inflammatory pain is associated with oxidative stress damage. Intraplantar (i.pl.) injection of carrageenan increased MDA levels in the spinal cord (L4-L5) of rats. Intraperitoneal (i.p.) administration of MnTBAP (10 mg/kg; 15 min prior to each carrageenan injection) or BPF (25 mg/kg; 15 min prior to each carrageenan injection) attenuated MDA expression. Results are expressed as mean ± SEM for 3 different experiments. * $p < 0.05$ compared to veh; † $p < 0.05$ compared to veh + carr.

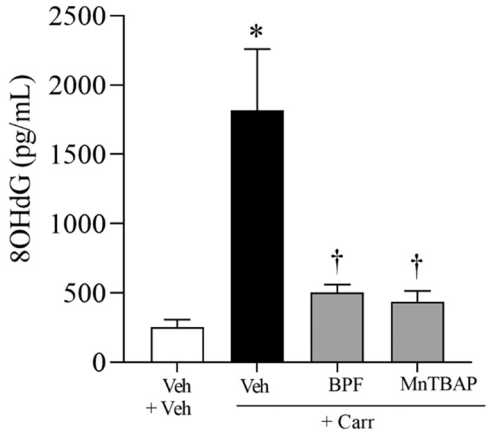

Fig. 3. Intraplantar (i.pl.) injection of carrageenan increased 8OHdG levels observed in the urine of rats. Intraperitoneal (i.p.) administration of MnTBAP (10 mg/kg; 15 min prior to each carrageenan injection) or BPF (25 mg/kg; 15 min prior to each carrageenan injection) attenuated 8OHdG expression. Results are expressed as mean ± SEM for 3 different experiments. * $p < 0.05$ compared to veh; † $p < 0.05$ compared to veh + carr.

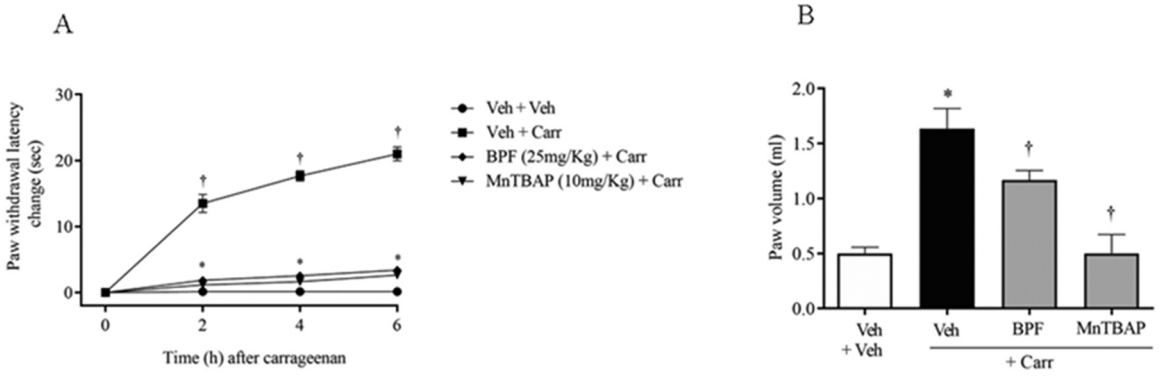

Fig. 1. Thermal hyperalgesia and edema induced by intraplantar carrageenan injection. (A–D) Intraperitoneal injections of MnTBAP (10 mg/kg) or BPF (25 mg/kg) 15 min prior to each carrageenan injection inhibit thermal hyperalgesia (A) and edema formation, indicative of inflammatory response (B). Results are expressed as means ± SEM. *$p < 0.05$ compared to Veh + carr; †$p < 0.05$ compared to Veh + Veh.

treatments effectively prevented the increases in MDA and 8OHdG levels (Figs. 2 and 3).

3.2. Oxidative stress is linked to increased post-translational modification of nuclear proteins in carrageenan-treated rats

ROS/RNS are mainly produced in the mitochondria. Although low levels of ROS/RNS are critical to the physiological mechanism in cells, large amounts of ROS/RNS are harmful to cells [48].

Redox modification or oxidation of protein by ROS/RNS can contribute to the formation of different mechanisms, such as carbonylation of specific aminoacidic residue, a crucial marker of protein oxidation, responsible for neurodegenerative and age-related disorders [49,50].

In this contest, to evaluate protein damage induced by oxidative stress, we evaluated acetylation, carbonylation and nitration levels of nuclear proteins.

We observed that carrageenan-treated rats revealed significant

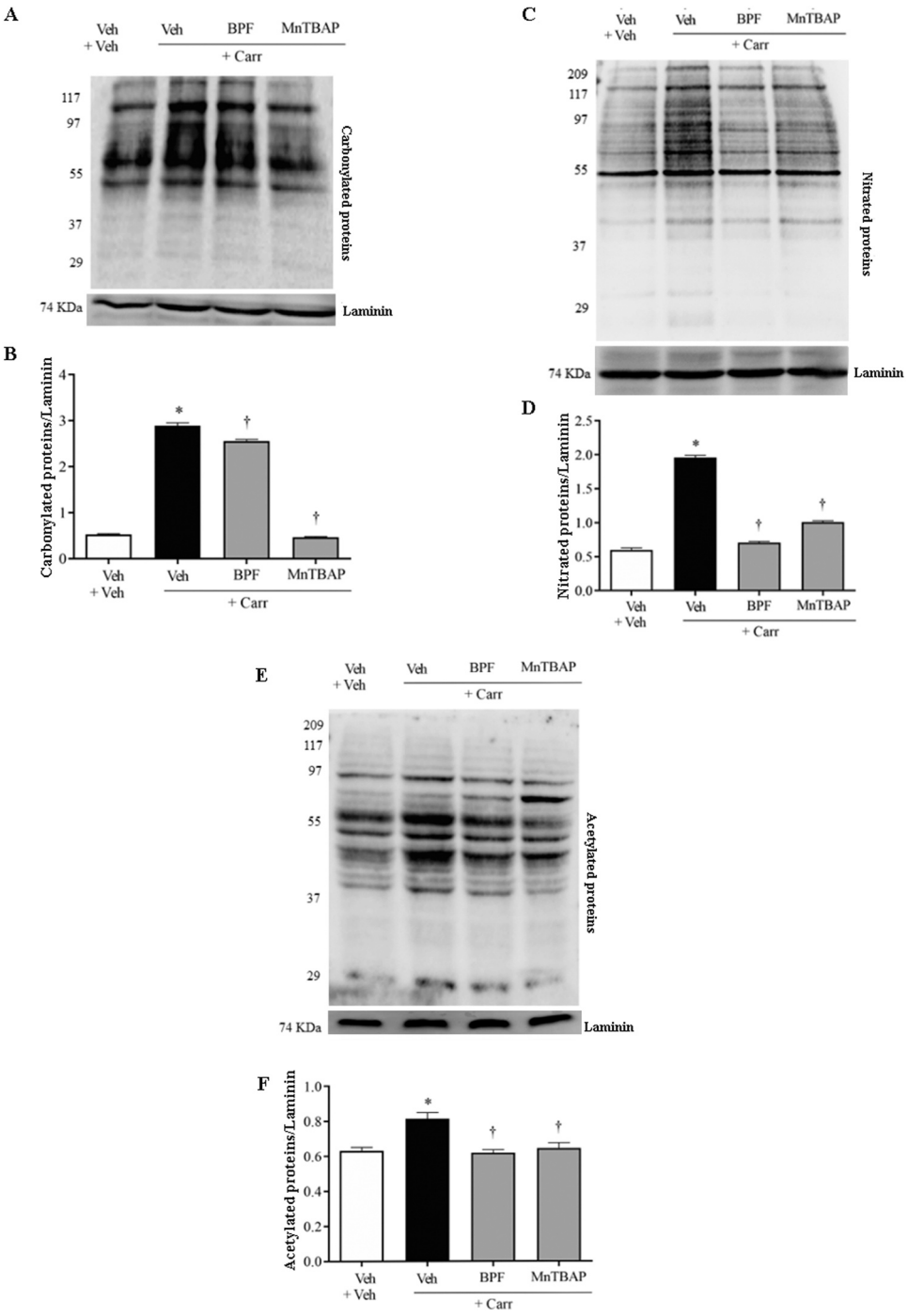

Fig. 4. Thermal hyperalgesia is associated with post-translational modification of nuclear proteins. Western blot analyses showed significant carbonylation (A), nitration (C) and acetylation (E) of nuclear proteins in the spinal cord of rats that received intraplantar injection of carrageenan. Pre-treatment with MnTBAP (10 mg/kg) or BPF (25 mg/kg), 15 min prior to each carrageenan injection, prevents this post-translational modification (A, C, E). No differences for laminin expression were detected among the lanes in these conditions (A, C, E). Gels are representative of results from 3 different experiments. Densitometric analyses of all experiments are reported (B, D, F). Results are expressed as mean ± SEM for 3 different experiments. * $p < 0.05$ compared to veh; † $p < 0.05$ compared to veh + carr.

increases in these post-translational modifications. Intraperitoneal injection of BPF (25 mg/kg) and MnTBAP (10 mg/kg) both 15 minutes before carrageenan administration, inhibiting the development of hyperalgesia prevents the post-translational modification of nuclear proteins (Fig. 4). Some effects may be attributed to bergamot's activation of SIRT1, which counteracts hyperacetylation of transcription factors like NF-κB, thus maintaining a balanced inflammatory response [51]. This mechanism is further supported by the ability of SIRT1 inhibitors to reverse the protective effects of bergamot on post-translational modifications (PTMs).

Indeed, it has been showed that flavonoid fraction of bergamot exhibits potent anti-inflammatory properties by modulating the SIRT1/NF-κB pathway [51]. In particular, it has been observed that bergamot reduces the expression and secretion of pro-inflammatory cytokines (IL-6, IL-1β, TNF-α) through inhibition of NF-κB activation, preventing its nuclear translocation and DNA binding [51].

3.3. *Oxidative stress and the role of nuclear SIRT1 in inflammatory pain induced by carrageenan in rats*

Sirtuin 1 (SIRT1) protein was the first SIRT to be studied in mammals; it is particularly known for its antioxidant and anti-inflammatory properties. SIRT1 mediates the deacetylation of proteins in an NAD^+-dependent manner [30]. Indeed, SIRT1 deacetylation function is involved in numerous biological processes, including apoptosis, lipid metabolism, oxidative stress, and inflammation. For this reason, changes in SIRT1 expression and function are crucial in cellular metabolism, and exert protective effects in the context of viral infections, including SARS-CoV-2 [52].

In Fig. 5, an increase in SIRT1 nitration is shown in spinal cord nuclear extracts of rats during inflammation induced by carrageenan administration. This mechanism was associated with the inhibition of its biological activity.

Administration of BPF (25 mg/Kg) and MnTBAP (10 mg/Kg) in rats, 15 min before injection of carrageenan, led to a decrease in SIRT1 nuclear nitration levels and as a consequence, an increase of its activity (Fig. 5). The treatments do not alter SIRT1 protein levels but only SIRT1 activity.

4. Discussion

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them [52,53]. This imbalance can lead to cellular damage, which exacerbates pain and inflammation [53].

Chronic inflammation, as in Long COVID, is the condition significantly increase oxidative stress levels [54,55]. As ROS and reactive nitrogen species (RNS) accumulate, they damage cellular components, such as lipids, proteins, and DNA. For instance, oxidative damage to lipids leads to the formation of lipid peroxidation products like 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which in turn generate nitrating species like peroxynitrite and nitrosonium cation [6,10,44]. These reactive molecules can cause tyrosine nitration, leading to protein dysfunction and further cell inactivation [56]. Additionally, the resulting DNA damage, as indicated by elevated levels of the biomarker 8-hydroxydeoxyguanosine (8-OHdG), is associated with ongoing cell turnover and an increased risk of genetic errors [7,57].

Oxidative stress also influences gene expression by altering DNA methylation patterns, leading to either hypermethylation or hypomethylation of specific genes [14]. Such modifications can disrupt normal cellular functions, further exacerbating pathogenic processes.

Our findings demonstrate that oxidative stress plays a crucial role in hyperalgesia (increased pain sensitivity). In experimental models of inflammatory pain, the accumulation of MDA and 8-OHdG was observed (Figs. 1–3), with significant attenuation of these markers upon antioxidant treatment with MnTBAP (10 mg/kg), and BPF (25 mg/kg) (Figs. 1–3). These data suggest that antioxidant interventions may offer a promising therapeutic approach for managing pain. By targeting this oxidative imbalance, antioxidants may alleviate the inflammatory and nerve-related pain, offering a potential avenue for symptom

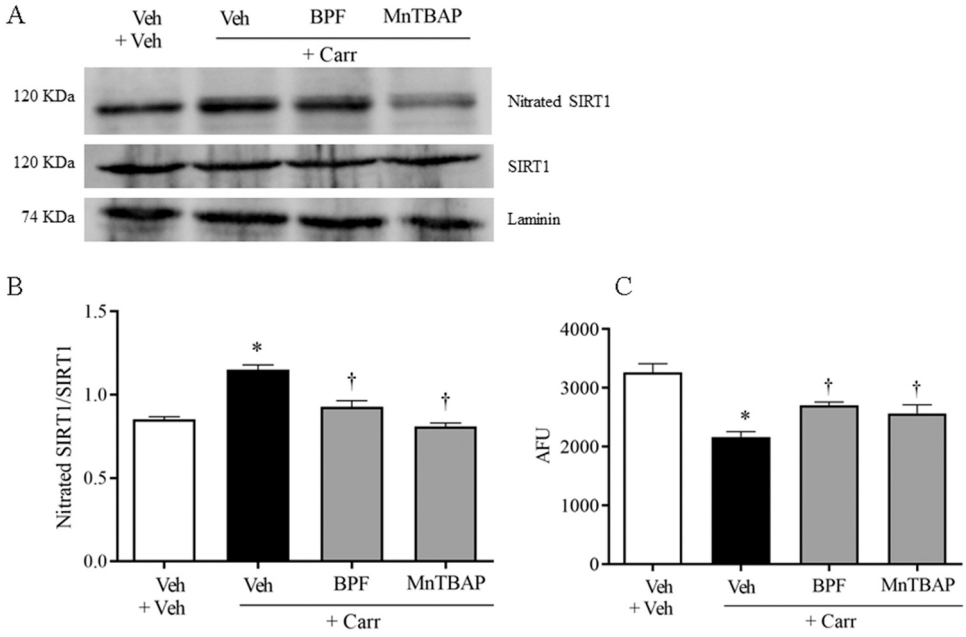

Fig. 5. Increased MDA, 8OHdG, and post-traslational modification of nuclear proteins correspond to nitration (A) and inactivation (C) of SIRT1 proteins in spinal cord tissues as measured by immunoprecipitation. Pre-treatment with MnTBAP (10 mg/kg) or BPF (25 mg/kg), 15 min prior to each carrageenan injection, prevented SIRT1 nitration (A) and restored its biological activity (C). No differences for laminin expression were detected among the lanes in these conditions (A, C, E). Gels are representative of results from 3 different experiments. Densitometric analyses of all experiments are reported (B, D, F). SIRT1 and nitrated SIRT1 were first normalized with laminin and then these values were used to obtain nitrated SIRT1/SIRT1 ratio. WB: SIRT1; IP: nitrated SIRT1. Results are expressed as mean ± SEM for 3 different experiments. * $p < 0.05$ compared to veh; † $p < 0.05$ compared to veh + carr.

management. In addition to direct oxidative damage, post-translational modifications (PTMs) of proteins are crucial in signal transduction during oxidative stress. These modifications can alter protein function, particularly in key inflammatory proteins that regulate the immune response. In animal models, carbonylation, nitration and acetylation of nuclear proteins have been observed in response to oxidative stress during carrageenan-induced inflammatory pain (Fig. 4). Antioxidant treatments inhibited these PTMs (Fig. 4), suggesting that modulating oxidative stress pathways may provide a therapeutic strategy for alleviating pain and inflammation in patients. SIRT1, a critical enzyme involved in regulating inflammation, oxidative stress, and immune system is often dysregulated in conditions associated with chronic inflammation and pain [58].

SIRT1 is known to regulate inflammatory pathways and oxidative stress responses, both of which are major contributors to the cytokine storm and multi-organ damage seen in severe cases.

Our previous findings indicate that in a rat model of inflammation, paw edema and elevated pro-inflammatory cytokines in the exudates were observed [15]. Administration of synthetic antioxidants reduced paw edema and pro-inflammatory cytokines [15].

SIRT1 exerts protective effects during viral infections, including SARS-CoV-2, by modulating immune cell responses and enhancing the body's antioxidant defense mechanisms. Specifically, SIRT1 inhibits the activation of pro-inflammatory cytokines and promotes the resolution of inflammation, thus mitigating the risk of hyper-inflammatory states. Additionally, SIRT1 activity is associated with the regulation of endothelial cell function and mitochondrial health [32].

Therapeutic strategies targeting SIRT1 are gaining attention for their potential to treat inflammatory disorders, where hyper-inflammation plays a central role in symptom persistence [3,27]. Evidence suggests that SIRT1 activity is often suppressed in various organ damage scenarios associated with hyper-inflammation [28,30]. This suppression exacerbates the inflammatory process and may contribute to the chronic pain and fatigue seen also in long COVID patients.

SIRT1 activity is regulated by several factors, including the NAD+ / NADH ratio, SIRT1-binding proteins, and PTMs, which can fine-tune its function and impact its role in cellular processes [59].

PTMs play a pivotal role in regulating SIRT1 function. Since SUMOylation was discovered as the first PTM to modulate its deacetylase activity, a variety of other chemical modifications have been identified, highlighting the complexity of SIRT1 regulation. These modifications, occurring at various protein residues, can affect SIRT1's activity, subcellular localization, and interactions with other proteins. Interestingly, in models of inflammation and pain, such as carrageenan-induced edema in rats, SIRT1 undergoes nitration and inactivation in the presence of hyperalgesia (Fig. 5).

This suggests that SIRT1's protective roles may be compromised during inflammatory pain, further exacerbating the pain response.

Polyphenols, such as bergamot derivates, have been shown to inhibit these PTM's and may offer a potential treatment strategy for managing inflammatory pain and modulating hyperalgesia while available pharmacological therapies have limited efficacy. Thus, novel therapeutic targets are urgently required for the development of more effective analgesics. By the employment of natural products patients can immediately benefit from their analgesic action due to the significantly shorten time required to bring these medications to market compared to synthetic drugs.

Polyphenols are recognized for their antioxidant and anti-inflammatory properties, and several studies have shown that they can activate sirtuins in various disease models [60,61]. It is well established that antioxidants neutralize excess free radicals produced during pain, thereby preventing oxidation and offering protection for biological molecules [60,61]. Antioxidants function through different mechanisms to inhibit radical formation and form stable end-products. Due to their diverse mechanisms of action, antioxidants are increasingly viewed as cost-effective therapeutic agents for a range of disorders, including chronic pain [62].

The anti-inflammatory, antioxidant, and antiviral effects of bergamot derivatives are particularly relevant in the management of long COVID, where persistent inflammation and oxidative damage are major contributors to the ongoing symptoms, including fatigue and pain [63].

Further research into molecular mechanisms underlying bergamot's biological effects is critical for advancing its therapeutic applications. Studies have shown that administering resveratrol, BPF, or oleuropein in animal models of pain effectively reduces allodynia and hyperalgesia by neutralizing ROS/RNS [7,14,42].

Alterations in SIRT1 activity are crucial not only for the onset and persistence of inflammatory pain but also for the chronic inflammatory profile like the one described in long COVID patients. Polyphenolic compounds, particularly those derived from bergamot, could represent a promising innovative therapeutic approach by modulating oxidative stress addressing inflammatory pain with the added potential of providing protection against the chronicization of pain response, thus potentially aiding in the prevention of chronic inflammatory conditions. This dual benefit underscores the importance of further research into targeted treatments for SIRT1 in various conditions involving inflammation and oxidative damage.

5. Conclusion

Our findings demonstrated that protecting SIRT1 activity by antioxidant drugs, could be beneficial during oxidative stress-induced inflammation and pain. Besides, re-establishing the activity of SIRT1 by antioxidants would be a new target in therapeutic intervention for the management and rehabilitation of pain suffering patients.

Thus, oxidative stress, and pain forms a complex web, where dysregulated oxidative stress and inflammatory responses contribute to the persistence of symptoms.

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CRediT authorship contribution statement

Oppedisano Francesca: Visualization. **Palma Ernesto:** Writing – review & editing, Visualization. **Malafoglia Valentina:** Writing – review & editing. **Scarano Federica:** Data curation. **Serra Maria:** Visualization, Data curation. **Macrì Roberta:** Visualization, Data curation. **Mazza Valeria:** Visualization, Data curation. **Muscoli Carolina:** Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Caminiti Rosamaria:** Visualization, Data curation. **Mollace Vincenzo:** Writing – review & editing, Visualization. **Passacatini Lucia Carmela:** Visualization, Data curation, Fundind acquisition. **Tomino Carlo:** Writing – review & editing, Visualization. **Nucera Saverio:** Writing – original draft, Data curation. **Maiuolo Jessica:** Visualization. **Ilari Sara:** Writing – original draft, Data curation, Conceptualization.

Declaration of Competing Interest

The authors declare no conflict of interest.

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During the preparation of this work, the author(s) used ChatGPT to

enhance language fluency and check for potential instances of phrasing that could be misconstrued as plagiarism. After utilizing this tool, the author(s) reviewed and edited the content as necessary and take full responsibility for the final version of the publication.

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