Cell Stress and Chaperones (2024) 29:769–776
https://doi.org/10.1016/j.cstres.2024.11.002

Protective role of short-chain fatty acids on intestinal oxidative stress induced by TNF-α

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Received: 9 September 2024 / Revised: 31 October 2024 / Accepted: 9 November 2024
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Abstract
Inflammatory bowel diseases (IBDs) are driven by an exaggerated inflammatory response, which leads to a marked increase in oxidative stress. This, in turn, exacerbates the inflammatory process and causes significant cellular and tissue damage. Intestinal dysbiosis, a common observation in IBD patients, alters the production of bacterial metabolites, including short-chain fatty acids (SCFAs), which are key by-products of dietary fiber fermentation. While the role of SCFAs in intestinal physiology is still being elucidated, this study aimed to investigate their effects on intestinal oxidative stress, particularly under inflammatory conditions induced by the proinflammatory mediator tumor necrosis factor alpha (TNF-α). The Caco-2/TC7 cell line was employed as an in vitro model of the intestinal epithelium, and the cells were treated with a range of SCFAs, including acetate, propionate, and butyrate. The levels of protein and lipid oxidation were quantified, as well as the activity of antioxidant enzymes. Our findings demonstrate that microbiota-derived SCFAs can effectively mitigate TNF-α-induced oxidative stress by modulating antioxidant enzyme activity. The proinflammatory mediator TNF-α induces lipid peroxidation by inhibiting catalase and glutathione peroxidase activities. SCFAs are able to upregulate antioxidant enzyme activity to restore lipid oxidative levels. These results underscore the critical role of the gut microbiota in maintaining intestinal homeostasis and highlight the therapeutic potential of SCFAs in managing oxidative stress-related pathologies.

Keywords Inflammatory bowel disease · Gut · Inflammation · Colitis · SCFA

Introduction

Dysregulation of homeostatic mechanisms and maintenance of inflammation are frequently associated with a number of digestive diseases, including inflammatory bowel diseases (IBD).[1] IBD is a group of chronic and progressive inflammatory disorders affecting the gastrointestinal tract, whose prevalence has been increasing over the past decade.[2] The etiology of IBD is multifactorial, encompassing a complex interplay between host genetics, gut microbiome, and environment. These interactions result in the disruption of normal gut homeostasis and immune function, leading to increased activity of T-effector lymphocytes and the production of reactive oxygen species (ROS) and proinflammatory cytokines, ultimately causing tissue damage.[3] The production and secretion of inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, or IL-6, and the imbalance between these and anti-inflammatory cytokines (IL-5, IL-10, and IL-11), are directly related to chronic inflammation of the gut, tissue destruction, and cellular and molecular damage.[4] Given the high metabolic activity of the gut, the basal production of ROS, mainly superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), is elevated. However, this production is hugely increased in bowel diseases such as IBD. A number of

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Published online: 14 November 2024

studies have demonstrated a correlation between oxidative stress and IBD, with elevated intestinal ROS production and diminished antioxidant defense.[5]

In recent years, research on IBD has focused on the gut microbiota with significant findings indicating substantial alterations. A reduction in microbial diversity, particularly among bacterial species belonging to the phyla Bacteroidetes and Firmicutes, is observed in IBD patients, accompanied by an increase in Proteobacteria.[6] Moreover, there is strong evidence that the immune system responds abnormally to the microbiota. Therefore, the gut microbiota and its composition could play a decisive role in the pathogenesis of IBD.[7]

A crucial element within the complex relationship between the intestine and the microbiota is the synthesis of by-products by microbial communities. Among these metabolites (such as B group vitamins, K vitamin, essential amino acids, etc.), short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, have been identified as significant regulators of intestinal homeostasis, including the promotion of intestinal epithelial barrier integrity,[8] the provision of primary energy substrates for colonocytes (particularly butyrate),[9] or the mediation of anti-inflammatory effects.[10,11] Several studies have documented a reduction in SCFA levels in patients with IBD in comparison to control subjects. This is accompanied by a state of intestinal dysbiosis, characterized by a decline in the abundance of SCFA-producing bacterial species and an overall reduction in microbial diversity.[12]

Despite the known benefits of SCFAs, the precise mechanisms through which they regulate gut homeostasis and modulate inflammatory processes remain poorly understood. The objective of this study is to examine the influence of SCFAs on intestinal oxidative stress induced by the proinflammatory cytokine TNF-α, which plays a pivotal role in the pathogenesis of IBD. Elevated levels of TNF-α are commonly found in the serum of patients with IBD. Biologics targeting TNF-α have significantly advanced the treatment of IBD, becoming the most effective agents for inducing and maintaining remission.[13] Our findings indicate that SCFAs can counteract TNF-α-induced oxidative stress, which may have potential therapeutic benefits in reducing gut inflammation.

Results

SCFAs effects on oxidative status

Firstly, we analyzed the impact of the three SCFAs (acetate, propionate, and butyrate) on basal oxidative cellular status. Furthermore, a mixture condition was included to simulate a physiological scenario, in which the three SCFAs are produced in a ratio of 60:25:15 (acetate/butyrate/propionate).[16] Following a 24-hour treatment with 1 mM SCFAs, the levels of protein oxidation and lipid peroxidation were quantified. As illustrated in Figure 1(a) and (b), the administration of SCFAs did not result in any notable changes in oxidation levels, whether in protein or lipid compounds. Similarly, the enzymatic antioxidant defense, as measured by catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities, was not altered by the 24-hour treatment with SCFAs.

SCFAs can revert oxidative stress induced by TNF-α

In order to model an inflammatory condition, TNF-α was employed as the main proinflammatory mediator. The cells were pretreated for one hour with 5 ng/mL of TNF-α, and subsequently, SCFAs were added at a concentration of 1 mM or in combination (mixture), and incubated for 24 hours. The results are presented in Figure 2. TNF-α was found to significantly elevate the level of lipid oxidative damage, with a nearly doubling of the control condition, while exhibiting no impact on protein oxidation. This increase in lipid peroxidation is associated with a notable decline in the antioxidant capacity of CAT and GPx (Figure 2(a), (c), and (e)). SCFA treatment was able to reverse the TNF-α-induced oxidative damage to levels approaching those of the control group. The levels of lipid peroxidation were significantly reduced by acetate, propionate, butyrate, and the combination of the three, with no synergistic effect observed between the SCFAs.

With respect to CAT (Figure 2(a)), all SCFAs and the combination exhibited the capacity to enhance CAT activity. Nevertheless, only propionate demonstrated a considerable increase in activity in comparison to TNF-α treatment. Similarly, SCFAs and the combination enhanced GPx activity, though the effect was not statistically significant (Figure 2(e)). TNF-α did not affect protein oxidation or SOD activity, and neither did treatment with SCFAs (Figure 2(b) and (d)).

Discussion

Gut microbiota performs a multitude of functions that are highly relevant to the organism, including the production of essential metabolites, such as vitamins (K, B2, B12), or SCFAs.[17] The present study is concerned with the effects of SCFAs on the intestinal oxidative status. Our findings indicate that SCFAs can mitigate

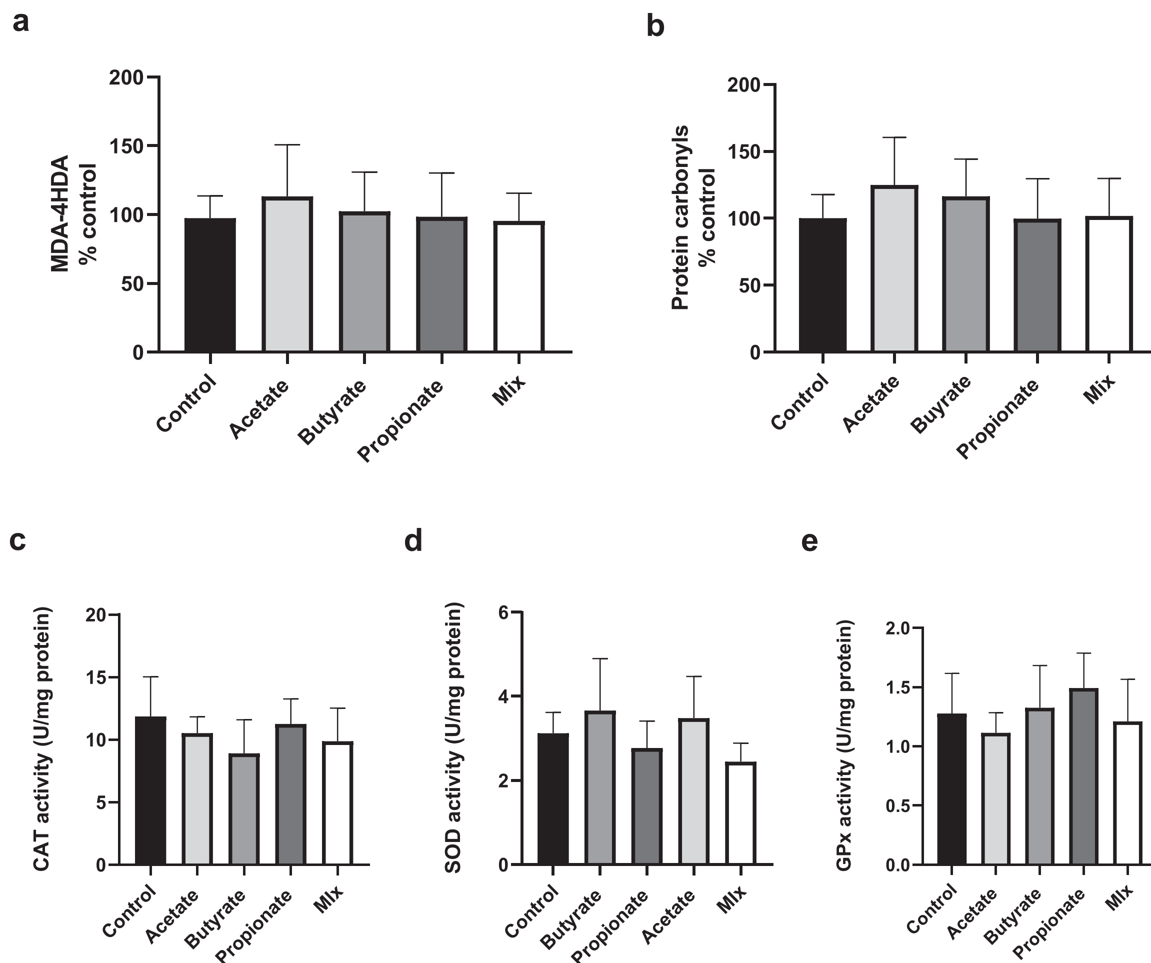

Fig. 1 Effects of SCFAs on oxidative status. Lipid peroxidation (a), protein oxidation (b), catalase (CAT) activity (c), superoxide dismutase (SOD) activity (d), and glutathione peroxidase (GPx) (e) activity were measured in Caco-2 cells treated throughout the course of one day with either acetate, butyrate, propionate at 1 mM or mixture (mix) 60:25:15 ratio (acetate/butyrate/propionate). Results are shown as the percentage of the control (100%) and are the mean ± standard deviation of four independent experiments (n = 8). Abbreviations used: 4-HDA, 4-hydroxyalkenals; MDA, malondialdehyde.

the oxidative stress induced by TNF-α, modulating the antioxidant enzyme activity.

It has been demonstrated that oxidative stress is associated with a number of pathological conditions, including IBD, where oxidative stress is closely linked to alterations in intestinal motility and patients' defecation habits.[18] Indeed, oxidative stress and inflammation play a pivotal role in the pathogenesis of IBD, where heightened inflammation and augmented oxidative stress at the intestinal level are present. Actually, IBD patients display a lower presence of antioxidant agents concomitant with an elevated production of ROS relative to healthy individuals.[19] Furthermore, there is compelling evidence indicating that a significant intestinal dysbiosis may play a role in the pathogenesis of IBD.[20]

Our findings show a protective role of SCFAs that can counteract intestinal oxidation in a TNF-α-induced inflammatory cell model. In agreement, SCFAs have been demonstrated to exert a protective effect on the intestinal epithelial barrier and improve intestinal immunity. Acetate and propionate regulate the expression of epithelial tight junctions and protein synthesis in LPS-stimulated cells.[21] Similarly, acetate has been liked to renoprotective effects, with studies indicating that it can ameliorate dysmorphic mitochondria in the proximal tubules, and reduce ROS production in chronic kidney disease.[22]

It appears that SCFAs do not exert any discernible influence on the intestinal oxidative status or antioxidant enzyme activity in physiological conditions. Nevertheless, it is well established that SCFAs can improve the gut health through a range of local effects, including the maintenance of intestinal barrier integrity, mucus production, and the protection against inflammation.[23]

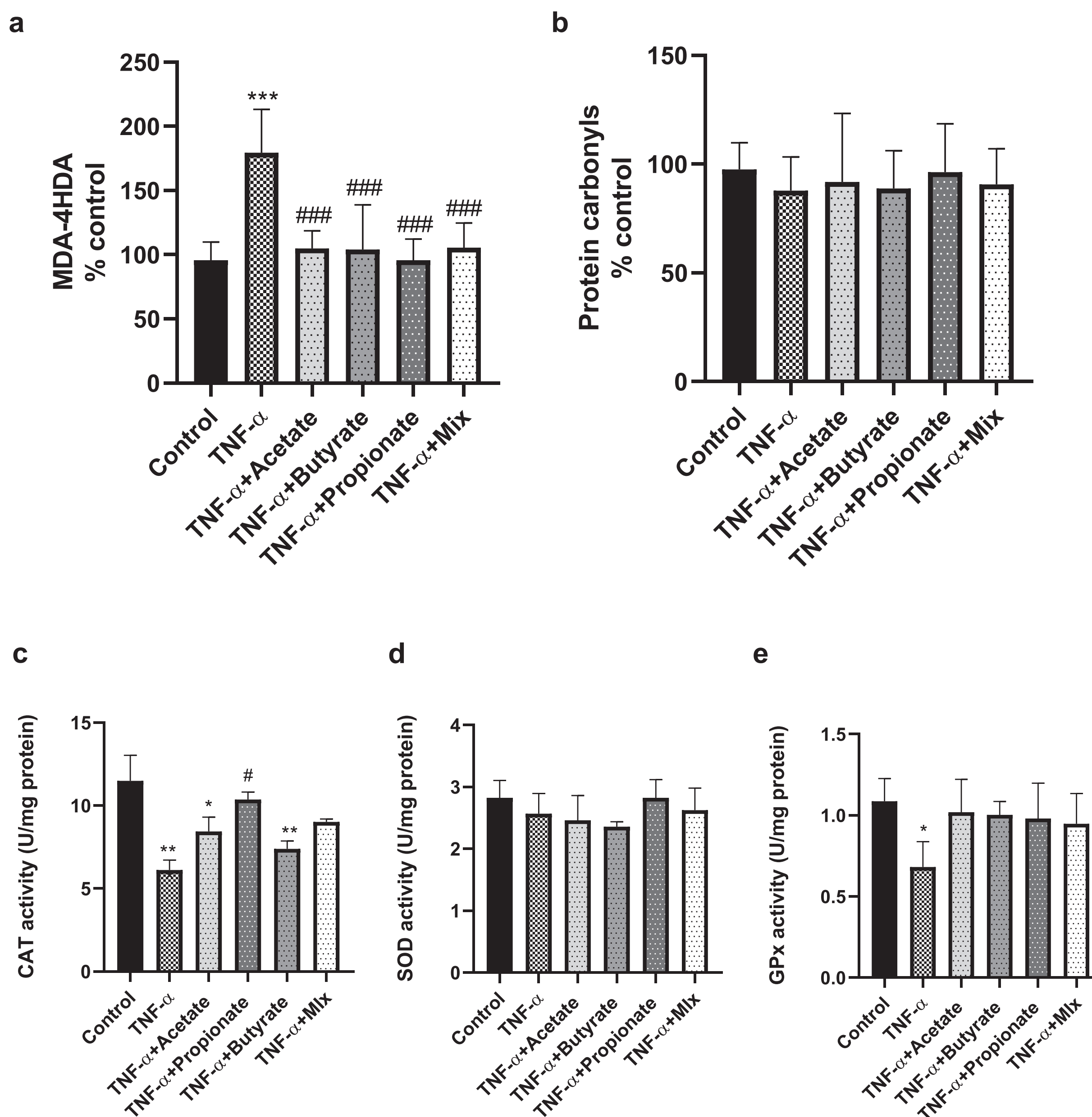

Fig. 2 SCFAs can revert oxidative stress induced by TNF-α. Lipid peroxidation (a), protein oxidation (b), catalase (CAT) activity (c), superoxide dismutase (SOD) activity (d), and glutathione peroxidase (GPx) (e) activity were measured in Caco-2 cells treated throughout the course of 1 day with either acetate, butyrate, propionate at 1 mM or mixture (mix) 60:25:15 ratio (acetate/butyrate/propionate). The cells were 1 hour pretreated with 5 ng/mL of tumor necrosis factor alpha (TNF-α). Results are shown as the percentage of the control (100%) and are the mean ± standard deviation of four independent experiments (n = 8). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, compared with control (C); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with TNF-α treatment. Abbreviations used: 4-HDA, 4-hydroxyalkenals; MDA, malondialdehyde.

The present study has demonstrated that SCFAs can reverse the oxidative damage induced by TNF-α. SCFAs reduced the TNF-α-induced lipoperoxidation increasing CAT and GPx activities. Our findings are corroborated by other studies that have also observed reversal of oxidative damage induced by inflammatory agents such as LPS.[24] Indeed, butyrate and acetate have been demonstrated to reduce the induction of DNA damage by the oxidant H_2O_2.[25] Our results show that SCFAs enhanced the activity of CAT and GPx, thereby counteracting the lipid oxidative damage induced by TNF-α. In agreement, other studies have also demonstrated the enzymatic modulation of SCFAs. *In vivo* models of bacterial infection have demonstrated that SCFAs increase the mRNA expression of GPx[26] and the CAT activity in primary human colon cells.[27] Butyrate

producer *S. boulardii* can inhibit lipid peroxidation and prevent the reduction of antioxidant levels, including GPx and CAT, in a model of oxidative stress induced by LPS in rats.[28] In an in vivo intestinal inflammation model, the production of SCFAs has been linked to increased antioxidant markers and a reduction in serum malondialdehyde (MDA) and ROS concentrations.[29] Furthermore, other microbial metabolites, specifically bioactive polysaccharide components derived from Huaier, have also shown to mitigate DSS-induced colitis by suppressing oxidative stress and inflammation, maintaining the intestinal barrier, and modulating the intestinal microbiota.[30]

The combination of SCFAs in physiological proportions reduces oxidative damage to a similar extent as that observed in control samples, while increasing CAT activity to a significant degree (and potentially elevating GPx activity). These findings highlight the crucial antioxidant role of SCFAs. In an inflammatory context, the impact of SCFAs may prove pivotal in counteracting the oxidative damage. It is important to note that intestinal inflammation frequently occurs alongside dysbiosis. Consequently, in addition to the oxidative damage induced by inflammation, aberrant production of SCFAs may result from intestinal dysbiosis.[31] In fact, the control of intestinal dysbiosis may represent a potential strategy for the reduction of inflammation and oxidative stress. Regulating the intestinal microbiome, through the inhibition of the abnormal expansion of Enterobacteriaceae during colitis, has demonstrated to alleviate the oxidative damage and restore intestinal barrier function.[32] Similarly, Bifidobacterium longum subsp. longum YS108R, a SCFA-producing bacterium, suppresses inflammation via NF-κB signaling pathway and attenuates oxidative stress via the Nrf2 signaling pathway. These effects contribute to the amelioration of colonic pathological damage in a colitis mouse model.[33]

Prebiotic supplementation, that increases SCFA production, has demonstrated to confer significant benefits on gut health. The bacterial fermentation of β-glucan produces a plethora of metabolites including vanillin, dihydroxyphenylacetic acid, caffeic acid, acetic acid, butyric acid, and lactic acid. These metabolites have been demonstrated to induce anti-inflammatory effects through the inhibition of intracellular ROS production and suppression of the NF-κB and JNK pathways.[34] In agreement, it has been demonstrated that β-glucans supplementation increases ATP levels, mitochondrial membrane potential, SCFA production, and Nrf2 DNA binding activity, while reducing ROS production and trimethylamine N-oxide levels in a rat model of ulcerative colitis.[35] Similarly, oligosaccharides, which induce SCFA production, can decrease LPS-induced intestinal inflammation increasing the antioxidant activities of SOD, GPx, and CAT.[36] The administration of non-digestible isomaltooligosaccharide and *Lactiplantibacillus ZDY2013* to DSS-induced colitis mice increases the level of intestinal tight junctions and mucins, which in turn have been shown to result in the alleviation inflammatory responses and the level of oxidative stress.[32]

Conclusion

Our study demonstrates the significant function of SCFAs in the modulation of intestinal oxidative status. The presence of SCFAs is indispensable for the optimal intestinal homeostasis. This suggests that an imbalance in their production due to dysbiosis may alter the antioxidant defense and contribute to oxidative damage. The anti-inflammatory potential of SCFAs lends support to the notion that SCFAs may exert a protective influence on intestinal inflammation. The findings of this study indicate that strategies aimed at increasing the abundance of specific SCFA-producing bacteria, prebiotic supplementation, and high-fiber diets may prove beneficial in the management of oxidative stress, particularly in the context of intestinal inflammatory conditions.

Materials and methods

Caco-2 cells: intestinal epithelial model

In this study, the Caco-2/TC7 cell line was used as an in vitro model for intestinal epithelium. The cell line was established in 1994 by Zweibaum/Rousset laboratory.[14] The cells were maintained at 37 °C and 5% CO_2 and fed by a high-glucose Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, antibiotics (100 U/mL penicillin and 100 g/mL streptomycin), 1% nonessential amino acids and 20% heat-inactivated fetal bovine serum from Life Technologies (Carlsbad, CA, USA). The cells were passaged weekly using an enzymatic method with a combination of 0.25% trypsin and 1 mM ethylene diamine tetra-acetic acid (EDTA), and subsequently sub-cultured in flasks from Sarstedt (Nuembrecht, Germany) at a density of 10^4 cells/cm². Subsequently, the medium was replaced every 48 hours, beginning at the time of seeding. The experiments were conducted on cells that had been cultured for 14 days, at which point full enterocyte-like differentiation had been achieved. For the experiments, the cells were seeded in 6-well cell culture plates (Sarstedt, Nuembrecht, Germany) at a density of 2×10^5 cells/well. Oxidative stress experiments were carried out

after treatment with different SCFAs at a concentration of 1 mM for 24 hours in medium without fetal bovine serum. Each condition was performed in duplicate. In order to simulate an in vitro inflammatory situation, cells were pretreated with 5 ng/mL of TNF-α for 1 hour. Following a 24-hour period of SCFAs treatment, the medium was removed from the wells, washed twice with PBS and stored at −80 °C until further analysis. To determine antioxidant enzyme activity, the same procedure was followed, with the exception that the samples were not frozen but rather analyzed on fresh cells.

Following a wash with PBS, samples were obtained by scraping the wells with a cell scraper in Tris-mannitol buffer (2 mM Tris, 50 mM mannitol, pH 7.1) with 100 mM phenylmethylsulfonyl fluoride, 25 g benzamidine, a protease inhibitor cocktail (Complete Mini, EDTA-free; Roche, Barcelona, Spain), and 0.02% sodium azide. The samples were disrupted using a Potter–Elvehjem homogenizer with a PTFE pestle and the resulting suspension was disrupted by sonication (15, 1-bursts, 60 W). Following sonication, the tubes were centrifugated at 3000 rpm for 10 minutes at 4 °C, after which the supernatants were collected for subsequent experimentation. The entire procedure was conducted at 4 °C to avoid degradation.

Determination of oxidative damage

To assess the oxidative damage produced in the cells, protein and lipid oxidation of the cells were measured. The Bradford method (Bio-Rad, Hercules, CA, USA) was employed to quantify total protein in the cell homogenate by spectrometry at 595 nm. This quantification served as the normalization factor for the results obtained in the subsequent assays.

Protein oxidation was analyzed by measuring carbonylated species concentration derivates of oxidative damage in proteins as previously described.[15] Cell homogenates were incubated with 2,4-dinitrophenylhydrazine at a final concentration of 1.5 mM and the resulting protein carbonylation was quantified spectrophotometrically at 375 nm. The results were expressed in nmol carbonyl groups per mg protein and presented as a percentage relative to the control value (100%).

Lipid peroxidation was evaluated by quantifying the levels of MDA and 4-hydroxyalkenals (4-HDA), as previously outlined.[15] In summary, the reaction between MDA and 4-HDA with N-methyl-2-phenyl-indole resulted in the formation of a stable chromophore, which was quantified by spectrophotometry at 586 nm, using 1,1,3,3-tetramethoxypropane as the standard. The results were expressed as nanomoles of MDA+4-HDA per milligram of protein and were normalized to the control value (100%).

The assays for protein quantification, protein oxidation and lipid oxidation, were performed in 96-well plates using a SPECTROstar Nano Microplate Reader (BMG LABTECH, Germany). In instances where required, quartz bottom microplates were utilized.

Antioxidant Enzymes Activity

CAT activity was determined using the classic protocol described by H. Aebi[37] with some modifications as previously described.[15] The method is based on the spectrophotometric monitoring at 240 nm of hydrogen peroxide (substrate) to water and oxygen (products) at a wavelength of 240 nm. The rate of production of water and oxygen per millimole of protein per minute was used as an indicator of CAT activity.

SOD (cytosolic superoxide dismutase CuZnSOD form in this study) is a metalloprotein that catalyzes the dismutation of superoxide anion (O^{2-}) into hydrogen peroxide (H_2O_2). The xanthine/xanthine oxidase system was used as the generator of the superoxide anion, which resulted in the reduction of cytochrome C. SOD activity was quantified in a carbonate-bicarbonate buffer (0.1 M, pH 10) containing 1 mM EDTA and 10 μM sodium azide. Xanthine oxidase (0.015625 U), cytochrome C (5 mM), and xanthine (70 μM) were added to the samples. Blank samples were prepared by replacing xanthine oxidase with buffer. The reduction was spectroscopically measured at 550 nm and the SOD activity calculated as U/mg prot × min.

GPx activity assay is based on the oxidation of glutathione during the GPx-catalyzed reaction. This reduction is continuously facilitated by an excess of glutathione reductase and NADPH. The oxidation of NADPH to $NADP^+$ serves as an indicator of GPx activity and can be monitored by spectrophotometry. The GPx activity was determined in a potassium phosphate buffer (0.1 M, pH 7.0), containing 1 mM EDTA, 1 mM glutathione, 0.25 U glutathione reductase, and 1.5 mM NADPH (previously solubilised in $NaHCO_3$ buffer 12 mM). A solution of hydrogen peroxide at a final concentration of 2 mM was added to the reaction mixture. Blank samples were prepared by replacing H_2O_2 with buffer. The reduction in NADPH absorbance during the oxidation of NADPH to $NADP^+$ is indicative of GPx activity. This was measured spectrophotometrically at 340 nm and calculated as NADPH nmol/mg prot × min.

Statistical Analysis

The results are presented as the mean ± standard deviation. The statistical analysis of the differences

between groups was performed using GraphPad Prism software (version 8.01, GraphPad Software, San Diego, CA, USA). A one-way analysis of variance was conducted, followed by Tukey's multiple comparisons test, with the objective of identifying significant differences between the groups. A *P*-value of less than 0.05 was considered statistically significant.

Funding and support Fundación Ibercaja-Unizar (JIUZ-2018-BIO-04).

Author contributions **Eva Latorre:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Jose Emilio Mesonero:** Visualization, Validation, Methodology. **Laura Grasa:** Validation, Methodology. **Berta Buey:** Writing – review & editing, Investigation. **Miguel Ferrer:** Writing – original draft, Investigation.

Data availability statement The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations of interest The authors declare that there is no conflict of interest regarding the publication of this article.

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