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RESEARCH ARTICLE

Long-term sulforaphane-treatment restores redox homeostasis and prevents cognitive decline in middleaged female and male rats, but cannot revert previous damage in old animals

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Abstract Aging is a complex and detrimental process, which disrupts most organs and systems within the organisms. The nervous system is morphologically and functionally affected during normal aging, and oxidative stress has been involved in age-related damage, leading to cognitive decline and neurodegenerative processes. Sulforaphane (SFN) is a hormetin that activates the antioxidant and anti-inflammatory responses. So, we aimed to evaluate if SFN long-term treatment was able to prevent age-associated cognitive decline in adult and old female and male rats. Memory was evaluated in adult (15-month-old), and old (21-month-old) female and male Wistar rats after three months of SFN treatment. Young rats (4-month-old) were used as age controls. The antioxidant response induction, the redox state (GSH/GSSG), and oxidative damage were determined in the brain cortex (Cx) and hippocampus (Hc). Our results showed that SFN restored redox homeostasis in the Cx and Hc of adult rats, thus preventing cognitive decline in both sexes; however, the redox responses were not the same in males and females. Old rats were not able to recover their redox state as adults did, but they had a mild improvement. These results suggest that SFN mainly prevents rather than reverts neural damage; though, there might also be a range of opportunities to use hormetins like SFN, to improve redox modulation in old animals.

Keywords Aging · Memory · GSH · Antioxidant enzymes · Brain · Cognition

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Introduction

Aging is a complex multifactorial biological process that is triggered by several intrinsic and extrinsic factors, contributing to the cellular and organismal physiological decline and impairment of stress-contending molecular pathways, which lead to frailty and increased risk of developing age-related diseases (Mc Auley et al. 2017; Titorenko 2018; Zhang et al. 2020).

The aging process is not homogeneous between species, and despite several conserved molecular pathways, slight variations cause a plethora of effects that influence how the organism ages (Cohen 2018). These differences are not only found between species; it has been widely studied that females and males from the same species age differently (Hernandez et al. 2020; Gurvich et al. 2021). In mammals, usually females live longer than males (Vina et al. 2005). Thus, sex differences during aging have been suggested to be related to the decrease in sex hormone levels, leading to a physiological decline (Horstman et al. 2012). However, since aging is a multifactorial phenomenon, there must be other factors that also contribute to the sex-differential aging process and the development of age-associated diseases.

Several years ago, various studies with transgenic animals reported that eliminating antioxidant enzymes (Pérez et al. 2009), or altering the mitochondrial respiratory chain to increase reactive oxygen species (ROS) generation (Lapointe and Hekimi 2008; Yang and Hekimi 2010), did not decrease animal's longevity. For this reason, the redox state was left out of the hallmarks of aging (López-Otin et al. 2013). Nevertheless, nowadays ROS are no longer viewed as just toxics by-products of redox metabolism that the cell must eliminate but are now appreciated for their role in regulating cellular signaling pathways and gene regulation in different species (Ursini et al. 2016; Mendoza-Martínez et al. 2020). Numerous functions of the organism depend on ROS production and redox homeostasis (Billingham et al. 2022; Ciesielska et al. 2021); so, changes in the redox state must be considered when studying aging (Jones 2015). For all the above, it is currently no longer intended to knock down ROS using large amounts of antioxidant molecules to improve health-span, instead, the use of low doses of molecules that are able to modulate the redox state has increased (Calabrese et al. 2020).

It is known that some phytochemicals and dietary components display a typical hormetic dose–response and generate an oxidative modification in the redox state activating adaptive stress response signaling pathways, and protecting the cells (Mattson and Cheng 2006; Rattan, 2008; Calabrese et al. 2019a, b). These molecules are known as "hormetins" since they act at the hormetic level (Ali and Rattan 2006; Calabrese et al. 2019a, b). In particular, sulforaphane (SFN) is a hormetin that has been shown to have excellent results (Calabrese and Kozumbo 2021).

SFN (1-isothiocyanate-(4R)-(methylsulfonyl) butane) is an organ-sulfured compound obtained from cruciferous vegetables, such as broccoli, cauliflower, cabbage, and Brussel sprouts (Lavich et al. 2015). Its precursor, the glucoraphanin can be found at higher concentrations in broccoli (0.8–21.7 μmol/g of dry weight) compared with other cruciferous, and it is hydrolyzed by the enzyme myrosinase when the vegetable is mechanically damaged, or attacked by pathogens, generating SFN as the main product (Guerrero-Beltrán et al. 2012). SFN administration in in vitro and in vivo models promotes the activation of nuclear factor erythroid 2-related factor 2 (Nrf2) (Hu et al. 2011), a master regulator of detoxification and antioxidant response, which also activates cell protection signaling pathways (Bai et al. 2013), and mediates mitochondrial protection (O'Mealey et al. 2017). Moreover, SFN can antagonize the nuclear factor ƙB (NFƙB), which is an important regulator of many pro-inflammatory genes (Jin et al. 2008), thus decreasing inflammation.

The nervous system is one of the most affected systems during aging; neural circuit neurodegeneration and glial cell dysfunction are contributing factors that promote mental and motor alterations in processes associated with cognitive decline, memory, and learning loss, as well as circadian rhythm alterations (Mattson and Arumugam 2018). These failures have been associated with the nervous system's high sensibility to impaired redox homeostasis (Friedman 2011), the induction of several neurodegenerative disorders, as well as age-dependent cognitive loss (Fan et al. 2019; Kandlur et al. 2020). Moreover, it has been established that cognitive and memory decline associated with an altered redox state is highly affected by sex and age (Hao et al. 2014).

In this regard, SFN has shown neuroprotective effects in cellular and animal models (Townsend and Johnson 2016). SFN administration to an Alzheimer's cellular model of murine neurons protected them from amyloid protein aggregates formation (Zhao et al. 2018). Canto et al. (2022) reported the neuroprotective effect of SFN in a mice model of retinitis pigmentosa; SFN reduced retinal neurodegeneration and modulated the inflammatory

profile. SFN also reduced ischemic injury and cognitive dysfunction in a rat model of vascular cognitive impairment, where it reduced neuronal and endothelial death and maintained the integrity of the blood–brain barrier after oxygen–glucose deprivation (Mao et al. 2019).

However, most of the studies that have been carried out with SFN in murine models used SFN for a short time and treated the animals with high doses. For example, Negrette-Guzmán et al. (2013) used SFN 1 mg/kg daily for 4 days before inducing kidney damage. While Silva-Palacios et al. (2019) injected 500 µg/Kg into the rat's heart before undergoing ischemia/reperfusion treatment. In general, the most common effective doses used in murine models range from 110 to 175 µmol/kg of body weight and are normally used for only a few days (Yagishita et al. 2019). Here we used the Cai group's scheme of low doses of SFN (0.5 mg/Kg, i.e. 2.82 µmol/Kg BW) for 3 months (Miao, et al. 2012; Li et al. 2020; Wang et al. 2021) to evaluate the SFN effect as a hormetin.

On the other hand, it was reported that no sex differences in SFN metabolism or tissue distribution were observed (Yagishita et al. 2019), however, we have recently shown that nerve conduction velocity, short-term and working memory, and redox state of the brain cortex and hippocampus, are differently affected in males and females during normal aging (Santín-Márquez et al. 2021). Moreover, redox metabolism is now recognized for its role in regulating cellular signaling pathways, so, although SFN metabolism might be similar in both sexes, by modulating the redox state, SFN might have different effects in males' and females' brains. Hence, this study aimed to evaluate if long-term SFN treatment was able to reduce age-related cognitive impairment via the restoration of redox homeostasis and if SFN differently affected adult and old male and female rats. In order to answer this question adult (12-month-old) and old (18-month-old) male and female Wistar rats were treated with SFN for 3 months, memory and learning were evaluated, as well as redox state (GSH/GSSG ratio), and some antioxidant defense system proteins such as Nrf2, KEAP1, CAT, SOD, and GPX.

Our results showed that SFN restored redox homeostasis in the brain cortex (Cx) and hippocampus (Hc) of adult rats, thus preventing cognitive decline in both sexes. However, the redox responses were not the same in males and females. Remarkably, although old rats were not able to recover their redox state as adults did, they certainly had a mild improvement. These results concur with an improvement in adult animals' redox homeostasis and antioxidant defense system, suggesting that SFN might prevent rather than revert neural damage. Yet, they also suggest that there might be a range of opportunities to use hormetins, like SFN, to improve redox modulation in old animals.

Materials and methods

Chemicals

All chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO). The reagents obtained from other sources are detailed throughout the text.

Animals

The Wistar rats (*Rattus norvegicus*) were provided by the closed breeding colony at the Universidad Autónoma Metropolitana-Iztapalapa (UAM-I). The assays were performed in young (4 months old), adult (12 months old), and old (18 months old), female and male rats. The animals were housed five-per-cage in polycarbonate cages in a 12 h light–dark cycle, with ad libitum standard chow diet (Harlan Laboratories Inc. Indianapolis) and water. The animals' health status was constantly evaluated. An acceptable state of health was considered when the animals did not have tumors, or skin or ear infections, and when they ate and drank properly. Rats with tumors and those that went blind were discarded from the study. All animals procedures were strictly carried out according to Mexican Official Ethics Standard NOM-062-ZOO-1999, and the Standard for the disposal of biological waste (NOM-087-ECOL-1995). The rats were euthanized by decapitation, according to the NOM-062-ZOO-1999, section 9.5.3.3.

SFN administration

Sulforaphane (SFN; LKT Laboratories, Cat. 4478-93-7) was administrated subcutaneously (0.5 mg/Kg, i.e. 2.82 µmol/Kg BW) 5 days per week for 3 months as reported by Miao et al. (2012). The SFN

was primarily dissolved in 1% DMSO and furtherly dissolved in saline solution. The control group was administrated with the vehicle (saline solution with proportional DMSO percentage per Kg of weight).

Experimental groups

Rats were divided into 6 groups (G) as follows: Young male (G1; n = 5) and female rats (G2 n = 5) (4 months old), that were only used as an age control and were therefore not treated with SFN.

Middle-aged adult male (G3; n = 5) and female (G4; n = 5) animals (12 months old), were treated with SFN for 3 months. These rats finished their treatments at 15 months of age and at that moment the determinations were made. These animals will be further referred to as the adult groups.

Old male (G5 n = 3) and female (G6 n = 3) rats (18 months old), that were treated with SFN for 3 months; they finished their treatments at 21 months of age and the determinations were performed.

Biochemical assays

The rats of each group were euthanized at the end of their respective treatment as mentioned above. The whole brain was carefully extracted and washed with saline solution. Brain cortex (Cx) and hippocampus (Hc) were dissected and stored in a − 70 °C ultra-freezer for the biochemical assays. The protein extraction and the Western blot assays were performed as previously reported (Hernández-Arciga et al. 2020) using the respective primary antibody, Nrf2 1:1000 (Santa Cruz Biotechnology sc-365949), Keap-1 1:500 (Santa Cruz Biotechnology sc-15246), SOD1 1:1000 (Santa Cruz Biotechnology sc-8637), CAT 1:1000 (Santa Cruz Biotechnology sc-271803), glutathione peroxidase 1:1000 (Santa Cruz Biotechnology sc-133160), and β-actin 1:1000 (Santa Cruz Biotechnology sc-47778).

Superoxide dismutase antioxidant activity (SOD)

The antioxidant enzyme activity was analyzed by spectrophotometry (Thermo Scientific™ GENESYS 10S UV–Vis; Madison, WI USA) through the xanthine/xanthine oxidase system based on Paoletti et al. (1986) protocols modified by Hernández-Arciga et al. (2018). One unit of enzyme was considered as the amount of SOD needed to inhibit 50% of the superoxide reaction with the nitro blue tetrazolium.

Catalase antioxidant activity (CAT)

CAT enzyme activity was spectrophotometry determined through the Aebi protocol (1984) modified by Hernández-Arciga et al. (2020), evaluating the decline in H_2O_2 levels at 240 nm every 15 s for 3 min. One catalase unit was considered as the amount of enzyme necessary to catalyze 1 µmol of H_2O_2 per min.

Glutathione peroxidase antioxidant activity (Gpx)

Gpx antioxidant activity was analyzed following the Ahmad protocol (1989) modified by Hernández-Arciga et al. (2018) evaluating GPx activity at 340 nm every 3 s per 40 s. One GPx unit was considered as the amount of enzyme necessary to neutralize H_2O_2 using NADPH.

Redox state determination

GSH/GSSG quotient was determined by high-performance liquid chromatography (HPLC) as described before (Hernández-Álvarez et al. 2019). The area under the curve for GSH and GSSG was determined.

Protein carbonyl groups

Carbonyls were determined to assess protein oxidative damage by using the DNPH alkaline method (Mesquita et al. 2014) modified by (Hernández-Arciga et al. 2018).

Memory test

The novel objects recognition test (NOR) was used because it takes advantage of the rat's innate behavior to explore novel objects, so it is commonly used to evaluate recognition memory. NOR test was performed as reported before (Santín-Márquez et al. 2021); the interactions number (IN) and interaction time (IT) for each object were recorded.

Statistical analysis

Univariate statistical analyses and graphs were carried out using the software Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data are presented as a mean ± standard deviation of each animal group. D'Agostino & Pearson's omnibus normality test and Levene's homoscedasticity test were performed. Not all groups presented normal distribution, and the n was limited, so the Kruskal–Wallis H test was performed. Pairwise, differences were analyzed by the post hoc test of Bonferroni. For all cases, $p < 0.05$ was taken as a significant difference between groups. The p values for all experimental groups in the analysis of Gpx, CAT, and SOD relative levels and antioxidant enzymes; Nrf2, KEAP1, and Nrf2/KEAP1 ratio; GSH/GSSG ratio; and carbonylated proteins content are presented in Supplementary tables. Statistically significant differences are marked with bold numbers.

Dimension reduction of antioxidant and memory parameters

Factor analysis

Owing to the high number of variables, a factor analysis (FA) was performed to reduce variables' dimensions into representative new variables. Missing data from old rat groups have been imputed. A FA was performed for each brain region, hippocampus, and cortex, considering the factoring method: Maximum likelihood and with rotation method: Varimax using the factanal function from the stats package in R.

We performed the Kaiser-Meyere-Olkin (KMO) test; variables that had individual MSA (measure of sampling adequacy) < 0.50 were excluded from the analysis. Data for Cx had an overall MSA of 0.61, and Hc data of 0.64. Afterward, a Barlett's test for sphericity was performed for Cx data ($X^2 = 137.2$, $p = 2.34e{-}16$, df = 28) and for Hc data ($X^2 = 119$, $p = 3.45e{-}13$, df = 28). Then we examined the scree plot (Supplementary Fig. 1A, B) of successive eigenvalues to identify the number of factors for the models, both models required three factors. After running the FA we got the significant test to corroborate that three factors were enough: Cx ($X^2 = 7.18$, $p = 0.42$, df = 7) and Hc ($X^2 = 7.18$, $p = 0.42$, df = 7).

Discriminant analysis

The variables generated with the FA, plus the ratio of the IN and the IT for each object (IT/IN ratio), were used for the discriminant analysis. Particularly, a regularized discriminant analysis (RDA), which accounts for small data sets, was selected. The RDA grid search was performed with Cross-Validation (fivefold) to determine optimal gamma and lambda values using the KlaR package in R. Accuracy was used to select the optimal model using the largest value. The final values used for the model were gamma: 0.00 and, lambda: 1.00 since these values correspond to a linear model, and considering that our data set did not meet homoscedasticity (Box'sM = 2445.3, $F_{252,1517} = 1.4E{-}0.06$), but it had multivariate normality (Skewness: b = 10 89.7, z = 89.67, df = 84, $p = 0.49$; Kurtosis: b = 60.58, z = − 0.76, $p = 0.45$), we decided to perform a Quadratic discriminant analysis (QDA) with a stepwise variable selection using JMP9.

Results

SFN effect on the Nrf2 transcription factor

SFN is known to induce the antioxidant response via the Nrf2-Keap1 pathway in vivo, therefore we evaluated Nrf2 and Keap1 in the Cx and the Hc from all groups. Young rats were used as age controls. Nrf2 relative levels significantly decreased with age in the Cx of non-treated adult females ($p = 0.028$) (Fig. 1A and Supplementary Fig. 2A). Moreover, SFN-treated adult females had higher Nrf2 levels in comparison with the same-age control group ($p = 0.032$), even obtaining higher Nrf2 levels than the young group ($p \leq 0.001$). Nrf2 levels were lower in both old groups than those observed in young and both adult groups, and no effect of SFN treatment was observed between old groups.

No differences in Keap1 relative levels were found between adult female groups, Adult SFN-treated group had higher Keap1 levels than the young group ($p = 0.009$). Nonetheless, significantly higher levels were observed in the SFN-treated old group when compared with the young, adult and, non-treated old group (Young: $p \leq 0.001$; Adult Ctl: $p = 0.014$; Adult

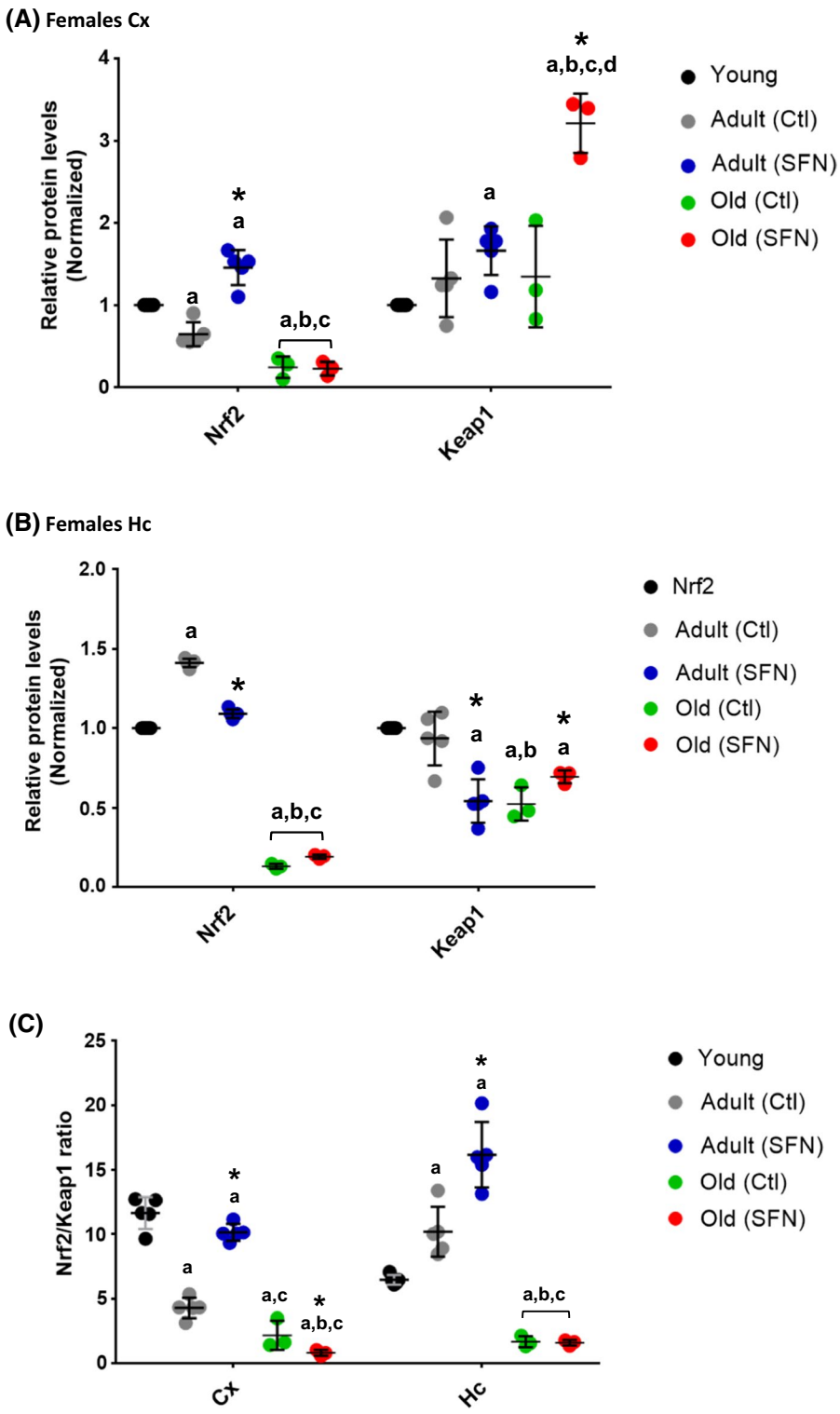


Fig. 1 Female Nrf2 and Keap1 relative levels. **A** Relative protein levels and densitometric analysis for the Cx blots. Data were normalized against actin. **B** Relative protein levels and densitometric analysis for the Hc blots. Data were normalized against actin. **C** Nrf2/Keap1 ratio for female Cx (left) and Hc (right). Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$. Nrf2 and Keap1 representative blots from young, adult, and, old female Cx and Hc are presented in Supplementary Fig. 2A and B

SFN: $p=0.028$; Old Ctl: $p=0.012$) (Fig. 1A and Supplementary Fig. 2A).

Interestingly, Nrf2 relative expression showed an opposite behavior in adult groups' Hc, where non-treated adult female rats' Nrf2 levels were higher in the older groups when compared with young males ($p=0.035$). Curiously, the SFN-treated adult group obtained lower Nrf2 relative levels compared to the non-treated female groups ($p=0.019$), and non-different Nrf2 relative levels were observed in the young group (Fig. 1B and Supplementary Fig. 2B). Similarly, SFN-treatment significantly reduced Keap1 levels in the Hc when compared with the young ($p=0.004$) and adult non-treated female groups ($p=0.016$). Likely, Nrf2 levels significantly decreased in both, treated (Young: $p=0.020$; Adult Ctl: $p\leq0.001$; Adult SFN: $p=0.003$) and non-treated (Young: $p=0.018$ Adult Ctl: $p=0.001$; Adult SFN: $p=0.048$), old female groups. No differences in Keap1 relative levels were found between the SFN-treated old group and the control old group.

The Nrf2/Keap1 quotient was calculated to determine the relationship between both protein levels as an indicator of the Nrf2 pathway activation. Figure 1C corresponds to the female's Cx and Hc Nrf2/Keap1 quotient. Higher Nrf2/Keap1 ratios were obtained in both studied brain regions from SFN-treated adults compared with the same-age control group (Cx: $p=0.018$ Hc: $p=0.001$). These data suggest a higher de novo Nrf2 synthesis or lower degradation rates. In old female groups, we observed lower Nrf2/Keap1 ratios than those observed in young females (Cx: Old Ctl: $p=0.002$, Old SFN: $p\leq0.001$; Hc: Old Ctl: $p=0.020$, Old SFN: $p\leq0.018$), non-treated adult (Cx: Old SFN: $p=0.035$; Hc: Old Ctl: $p\leq0.001$, Old SFN: $p\leq0.001$), and SFN-treated adult female groups (Cx: Old Ctl: $p=0.022$, Old SFN: $p\leq0.001$; Hc: Old Ctl: $p=0.003$, Old SFN: $p=0.004$). In summary, SFN treatment in female adult groups increased Nrf2 levels and lowered Keap1 relative levels, which in consequence, increased the Nrf2/Keap1 quotient in SFN-treated adult groups.

Concerning male groups, Nrf2 levels in Cx augmented with age (Fig. 2A and Supplementary Fig. 3A). Non-treated adults ($p=0.018$), non-treated old group ($p=0.011$), and SFN-treated old group ($p\leq0.001$) were, on average, fivefold higher when compared with the young group. Interestingly, SFN-treated male adults had significantly higher Nrf2 levels compared with all remaining groups (Young: $p\leq0.001$; Adult Ctl: $p\leq0.001$; Old Ctl: $p\leq0.001$; Old SFN: $p=0.005$).

Keap1 relative levels (Fig. 2A and Supplementary Fig. 3A) found in Cx were lower for SFN-treated adults when compared with same-age non-treated groups ($p=0.003$). Moreover, higher levels were observed in the old control group, as well as in the old SFN-treated group, than those observed in the young (Old Ctl: $p=0.016$; Old SFN: $p\leq0.001$) and SFN-treated adult males (Old SFN: $p=0.002$). No differences were noted in Keap1 levels from SFN-treated and non-treated old groups.

In male Hc (Fig. 2B and Supplementary Fig. 3B) no differences were observed in Nrf2 levels between young and adult rats, but significantly higher levels were determined in SFN-treated old males when compared with same-age non-treated males ($p=0.004$). The SFN-treated adult group obtained higher Nrf2 levels than both, the control ($p=0.018$) and treated ($p=0.003$) adult groups. Interestingly, lower Keap1 levels were found in both, young and non-treated adult males when compared with same-age females. As well as in Cx, SFN treatment reduced Keap1 levels in the Hc in comparison with the non-treated group.

Nrf2/Keap1 ratio (Fig. 2C) showed higher values in young males' Cx when compared with all other groups (Adult Ctl: $p=0.018$; Adult SFN: $p\leq0.001$; Old Ctl: $p=0.011$; Old SFN: $p=0.012$). Besides Nrf2/Keap1 ratio was significantly lower in the adult control group compared with the young group, and we found that the SFN-treated adult group obtained a similar Nrf2/Keap1 ratio to the young group, in consequence, higher than the non-treated adult Nrf2/Keap1 ratio ($p\leq0.001$). Both old groups, whether they were treated with SFN or not, obtained lower Nrf2/Keap1 ratios when compared with adult male groups (Old Ctl: Adult SFN: $p\leq0.001$; Old SFN: Adult SFN: 0.005). Nrf2/Keap1 ratios in males' Hc were homogenous and only were significantly lower in the SFN-treated group ($p=0.043$) and the old control group ($p=0.001$) compared with the young group. Interestingly, SFN-treated old group got a higher Nrf2/Keap1 ratio than the same-age control group ($p=0.022$).

These results suggest that SFN treatment induced the Nrf2-Keap1 pathway, probably by two distinct effects, by directly augmenting Nrf2 levels, or by

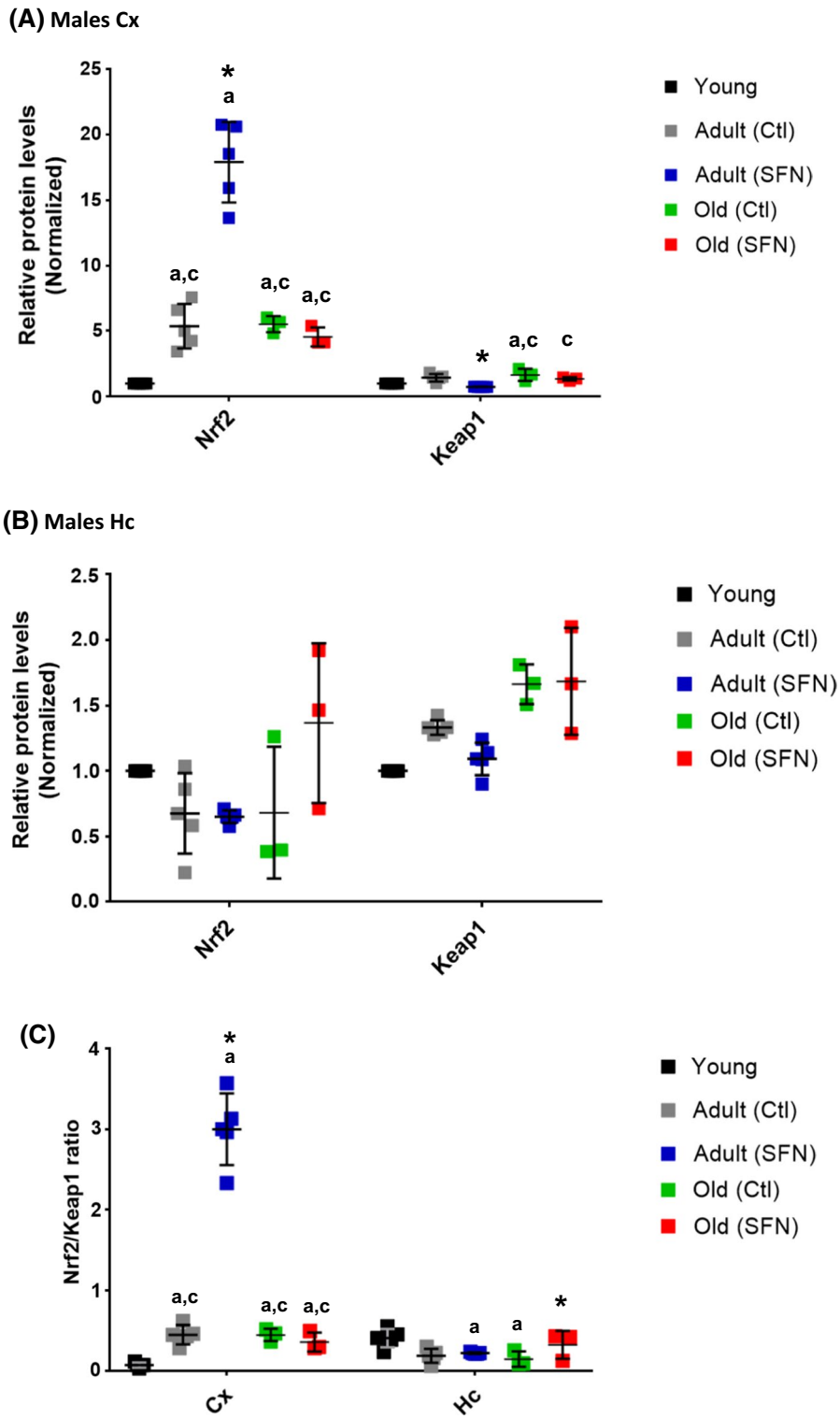


Fig. 2 Male Nrf2 and Keap1 relative levels. **A** Relative protein levels and densitometric analysis for the Cx blots. Data were normalized against actin. **B** Relative protein levels and densitometric analysis for the Hc blots. Data were normalized against actin. **C** Nrf2/Keap1 ratio for male Cx (left) and Hc (right). Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$. Nrf2 and Keap1 representative blots from young, adult, and old male Cx and Hc are presented in Supplementary Fig. 3A and B

reducing its cytosolic repressor, Keap1, in both female and male adult groups.

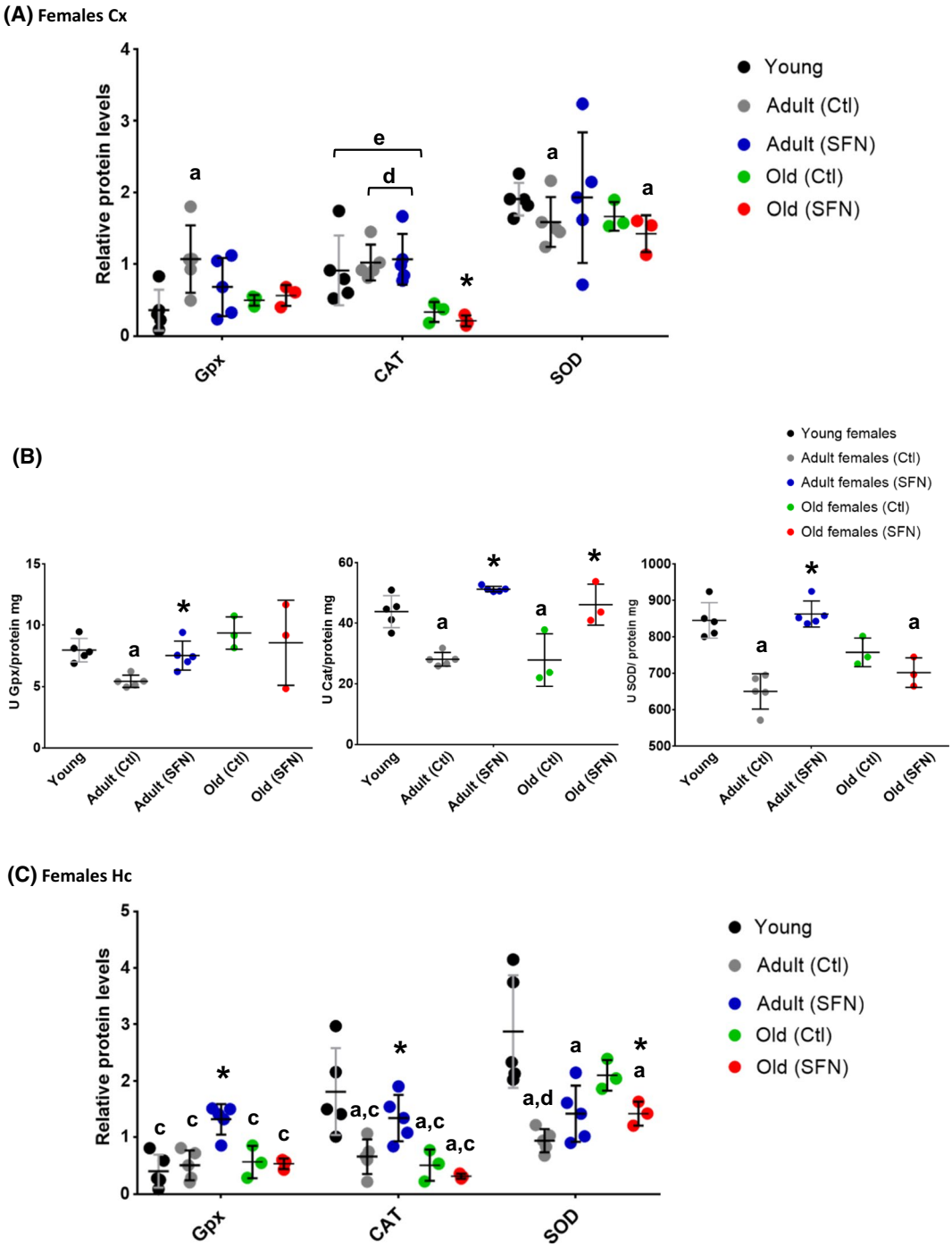
Enzymatic antioxidant response

The results obtained for the enzymatic content and activity in females' Cx indicated that Gpx levels were higher only in the non-treated adult females compared with the young group ($p=0.005$) (Fig. 3A, and Supplementary Fig. 4A), but no differences were found either in the SFN-treated group or in both old groups. No differences in CAT relative levels among young and adult groups were observed either, but a significant decrease was noted in both old groups compared with the younger groups [old (Ctl): vs adult (Ctl) $p=0.011$, vs adult (SFN) $p=0.011$; SFN-treated: vs young $p=0.008$, vs adult (Ctl) $p=0.001$, vs adult (SFN) $p=0.001$] (Fig. 3A, and Supplementary Fig. 4A). A statistically significant decrease was found in the old SFN-treated group in CAT relative levels when compared with the same-age control group ($p=0.046$). For SOD relative levels, the adult non-treated group and the old SFN-treated group obtained lower relative protein levels than the young group ($p=0.043$ and $p=0.12$, respectively) (Fig. 3A, and Supplementary Fig. 4A). Interestingly, despite the protein levels were similar in almost all groups, an increase in all three evaluated enzyme activities from the SFN-treated adult group compared with its same-age control group was observed (Gpx $p=0.041$; CAT $p\leq0.001$; SOD $p\leq0.001$) (Fig. 3B). It is important to mention that SFN treatment had no effects on Gpx and SOD activity in old groups, but it increased their CAT activity ($p=0.008$).

When the females' Hc was evaluated (Fig. 3C and Supplementary Fig. 4B), differences in antioxidant enzyme relative levels were found. For Gpx, higher relative levels were found in the adult SFN-treated group when compared with same-sex young ($p=0.001$) and old groups (Old Ctl: $p=0.016$; Old SFN: $p=0.014$), as well as with the same-age non-treated group ($p=0.006$) (Fig. 3C and Supplementary Fig. 4B). A similar effect was noted in CAT in which elevated relative levels were found in both, the young and adult SFN-treated groups, when compared with non-treated adults (young: $p=0.025$; adult SFN: $p=0.046$), with the non-treated old group (young: $p=0.006$; adult SFN: $p=0.020$) and with SFN-treated old group (young: $p\leq0.001$; adult SFN: $p=0.001$) (Fig. 3C and Supplementary Fig. 4B). For females' Hc SOD relative levels, we found a decrease with age, in consequence, the young group showed higher levels compared with adult groups (adult Ctl: $p\leq0.001$; adult SFN: $p=0.002$), as well as in the SFN-treated old group ($p=0.018$). No differences between adult or old groups were noted, no matter if they were treated with SFN or not. Old groups didn't respond to the SFN treatment, and we even noted a significant reduction in some enzyme levels in the old female treated group compared with its same-age control group.

Regarding the antioxidant activity, an increase in all enzymes' antioxidant activity in the adult treated female group was observed when compared with the same-age non-treated group (Gpx: $p=0.020$; CAT: $p=0.001$; SOD: $p\leq0.001$), but no effects were noted in the oldest groups (Fig. 3D), which showed no differences between treatments in any of evaluated the enzymes. In Gpx antioxidant activity, it was observed a higher activity in the young group compared with the rest of the groups (Adult Ctl: $p=0.001$; Old Ctl: $p=0.014$; Old SFN: $p=0.031$), except for the SFN-treated adult group, but similar antioxidant activity with adult SFN-treated group, showing a restoration of its activity. In contrast, CAT activity showed to keep similar scores in the non-treated adult female group, but an increase in both adult ($p=0.001$) and old ($p=0.004$) SFN-treated groups. Similarly, we observed an increase in CAT activity with age, showing higher activity in the old Ctl group when compared with the adult Ctl group ($p=0.035$).

In regard to males' Cx (Fig. 4A and Supplementary Fig. 5A), increased levels of Gpx were determined in both, the treated ($p=0.003$) and non-treated ($p=0.021$) adult groups, and the control old group ($p=0.039$) when compared with the young ones. SFN treatment did not increase Gpx levels in adults in comparison with its same-age control group. While in old groups a reduction in Gpx levels was noted in the SFN-treated group when compared with the same-age non-treated group ($p=0.047$) (Fig. 4A and Supplementary Fig. 5A). CAT displayed significantly higher levels in the young males, which decreased in adult rats (Adult Ctl: $p=0.028$; Adult SFN: $p=0.002$ Old Ctl: $p=0.001$; Old SFN: $p\leq0.001$), but no differences were found between the SFN-treated and non-treated adult groups. On the other hand, we noted a reduction in CAT relative levels in the old SFN-treated


(A) Females Cx
Relative protein levels
Young
Adult (Ctl)
Adult (SFN)
Old (Ctl)
Old (SFN)
Gpx
CAT
SOD
(B)
Young females
Adult females (Ctl)
Adult females (SFN)
Old females (Ctl)
Old females (SFN)
U Gpx/protein mg
U Cat/protein mg
U SOD/ protein mg
(C) Females Hc
Relative protein levels

◀ **Fig. 3** Female antioxidant enzymes levels and activity after SFN-treatment. **A** Gpx, CAT, and SOD densitometric analysis for the Cx blots. The data from Supplementary Fig. 4A were normalized against actin from young, adult, and old females' Cx respectively. **B** Gpx, CAT and, SOD antioxidant activity from young, adult and, old females' Cx. **C** Gpx, CAT, and, SOD densitometric analysis for the Hc blots. The data from Supplementary Fig. 4B were normalized against actin from young, adult, and old females' Hc respectively. **D** Gpx, CAT and, SOD antioxidant activity from young, adult and, old females' Hc. Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$. Gpx, CAT, and SOD representative blots were evaluated in young, adult, and old females' Cx and Hc as described in materials and methods and are presented in Supplementary Fig. 4A and B

group compared with the same age group ($p = 0.024$) (Fig. 4A, and Supplementary Fig. 5A).

As well as in Gpx, SOD levels increased in both adult groups, and again, SFN-treatment significantly increased the enzyme relative levels ($p = 0.028$). In the old groups, a similar behavior as in old females' groups was observed, in which SFN treatment reduced the levels of the antioxidant enzymes ($p = 0.019$). Again, a significant reduction was noted in old non-treated (Adult Ctl: $p = 0.028$; Adult SFN: $p = 0.031$) and SFN-treated (Adult Ctl: $p = 0.018$; Adult SFN: $p \leq 0.001$) when compared with old groups (Fig. 4A and Supplementary Fig. 5A). When the antioxidant enzymes' activities were determined, we confirmed that SFN-treatment increased all three-enzyme activity in the adult group when compared with the same-age non-treated group (Gpx: $p \leq 0.001$; CAT: $p = 0.044$; SOD: $p = 0.001$), but it didn't have an effect in the old group. Adult control groups had lower Gpx activity than young ($p = 0.011$) and adult SFN-treated ($p \leq 0.001$) groups (Fig. 4B). For CAT activity, young and non-treated adult male groups obtained similar values and were statistically lower than SFN-treated adult group (Young: $p = 0.014$; Adult Ctl: $p = 0.044$), old non-treated (Young: $p = 0.022$; Adult Ctl: $p = 0.043$) and SFN-treated groups (Young: $p = 0.045$; Adult Ctl: $p = 0.009$) (Fig. 4B). SOD antioxidant activity had similar behavior to CAT activity, in which the adult male control group had lower SOD antioxidant activity than the young group ($p \leq 0.013$), while the adult SFN-treated, old non-treated group, and SFN-treated group got similar SOD antioxidant activity. SFN treatment improved SOD antioxidant activity in the adult male group compared with the same-age control group ($p \leq 0.001$) (Fig. 4B).

Finally, no differences were noted in Gpx relative expression in adult males' Hc (Fig. 4C and Supplementary Fig. 5B), but higher relative levels were observed in the old SFN-treated group when compared with young ($p = 0.001$) and SFN-treated

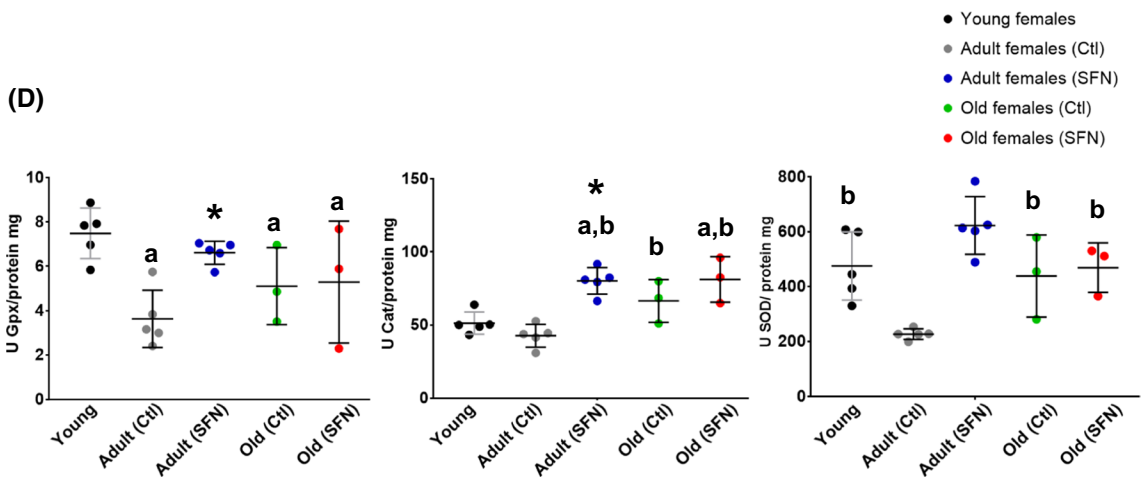
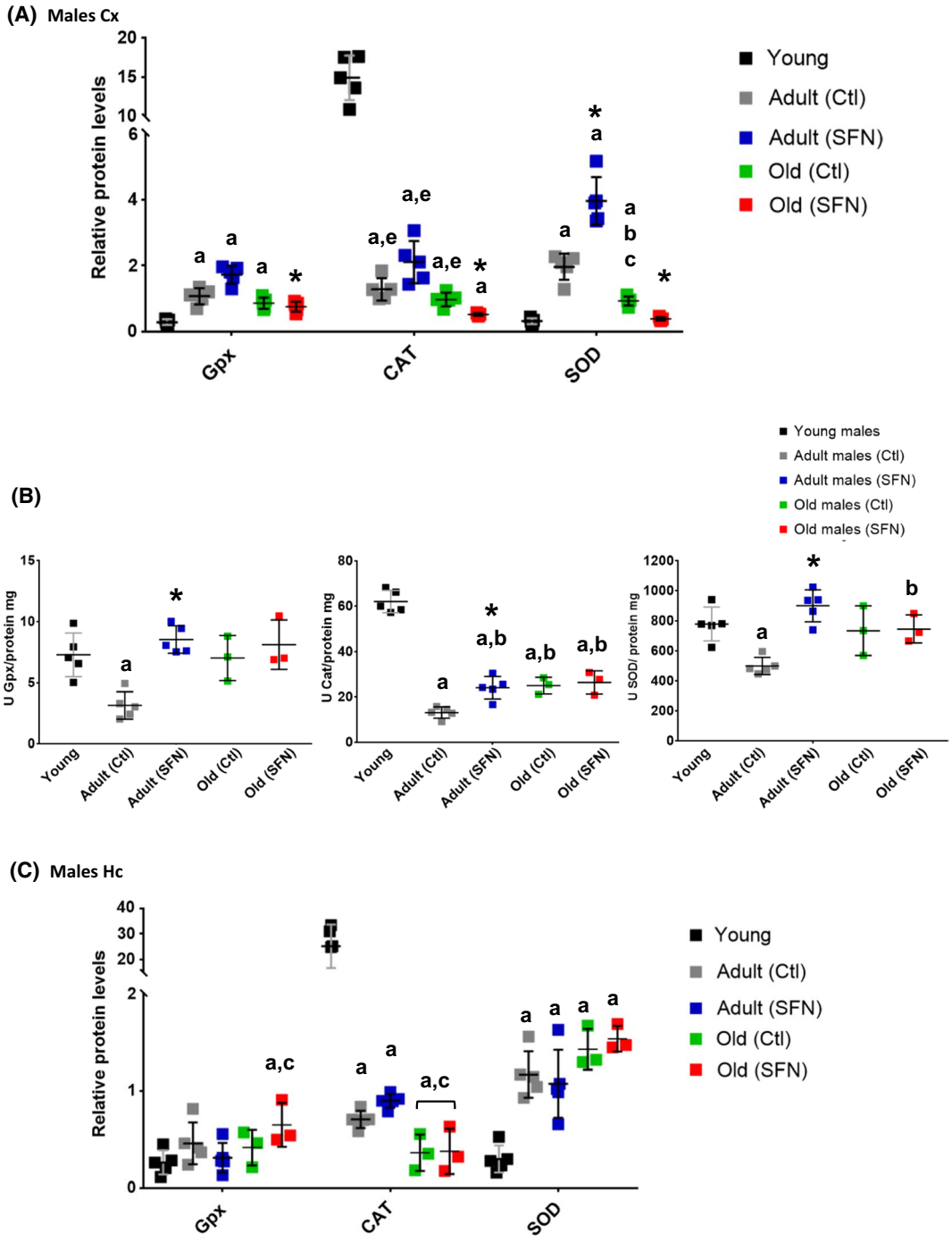


Fig. 3 (continued)


(A) Males Cx
Relative protein levels
Young
Adult (Ctl)
Adult (SFN)
Old (Ctl)
Old (SFN)
Gpx
CAT
SOD
(B)
Young males
Adult males (Ctl)
Adult males (SFN)
Old males (Ctl)
Old males (SFN)
U Gpx/protein mg
U Cat/protein mg
U SOD/ protein mg
(C) Males Hc
Relative protein levels
Young
Adult (Ctl)
Adult (SFN)
Old (Ctl)
Old (SFN)
Gpx
CAT
SOD

◀ **Fig. 4** Male antioxidant enzymes levels and activity after SFN-treatment. **A** Gpx, CAT, and SOD densitometric analysis for the Cx blots. The data from Supplementary Fig. 5A were normalized against actin from young, adult, and old males' Cx respectively. **B** Gpx, CAT and, SOD antioxidant activity from young, adult and, old males' Cx. **C** Gpx, CAT, and, SOD densitometric analysis for the Hc blots. The data from Supplementary Fig. 5B were normalized against actin from young, adult, and old males' Hc respectively. **D** Gpx, CAT and, SOD antioxidant activity from young, adult and, old males' Hc. Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$. Gpx, CAT, and SOD representative blots were evaluated in young, adult, and old males' Cx and Hc as described in materials and methods and are presented in Supplementary Fig. 5A and B

adults ($p = 0.009$). As in the Cx, we found, on average, tenfold higher CAT levels in the young males' Hc than those observed in the rest of the groups (Adult Ctl: $p = 0.003$; Adult SFN: $p = 0.002$; Old Ctl: $p \leq 0.001$; Old SFN: $p \leq 0.001$). No statistically significant differences between treated and control groups were noted neither in the adult nor in the old groups. SOD levels significantly augmented in the Hc with age (Adult Ctl: $p = 0.035$; Adult SFN: $p = 0.007$; Old Ctl: $p = 0.002$; Old SFN: $p \leq 0.001$) (Fig. 4C and Supplementary Fig. 5B). When the antioxidant activity was determined, Gpx antioxidant activity showed similar values to those observed in Cx, Gpx antioxidant activity in the non-treated adult group was significantly lower than in the young male group ($p = 0.005$) (Fig. 4D). Interestingly, SFN treated adult group showed higher Gpx antioxidant activity when compared with the same-age control group ($p \leq 0.001$), as well as with the old control group ($p = 0.039$). The adult male control group showed lower Gpx antioxidant activity than the non-treated ($p \leq 0.036$) and SFN-treated old groups ($p \leq 0.028$) (Fig. 4D). CAT antioxidant activity was lower in both, the adult (Adult Ctl: $p = 0.009$; Adult SFN: $p = 0.001$) and old (Old Ctl: $p = 0.001$; Old SFN: $p \leq 0.001$) groups compared with young males. No differences were observed between treated and control groups neither in adults nor in old males) (Fig. 4D).

So, SFN-treatment increased the antioxidant activity of the evaluated enzymes in both sexes' adult groups, but it had no effects in old-treated groups. Moreover, SFN-treatment decreased protein levels in old groups compared with their same-age control groups. Despite protein levels remaining unaltered, SFN-treatment increased the antioxidant activity of most of the evaluated enzymes.

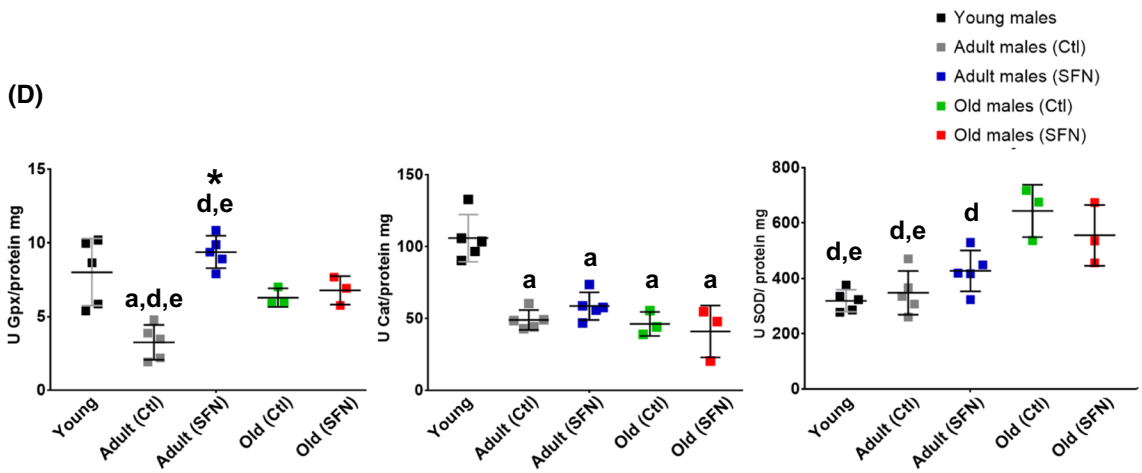


Fig. 4 (continued)

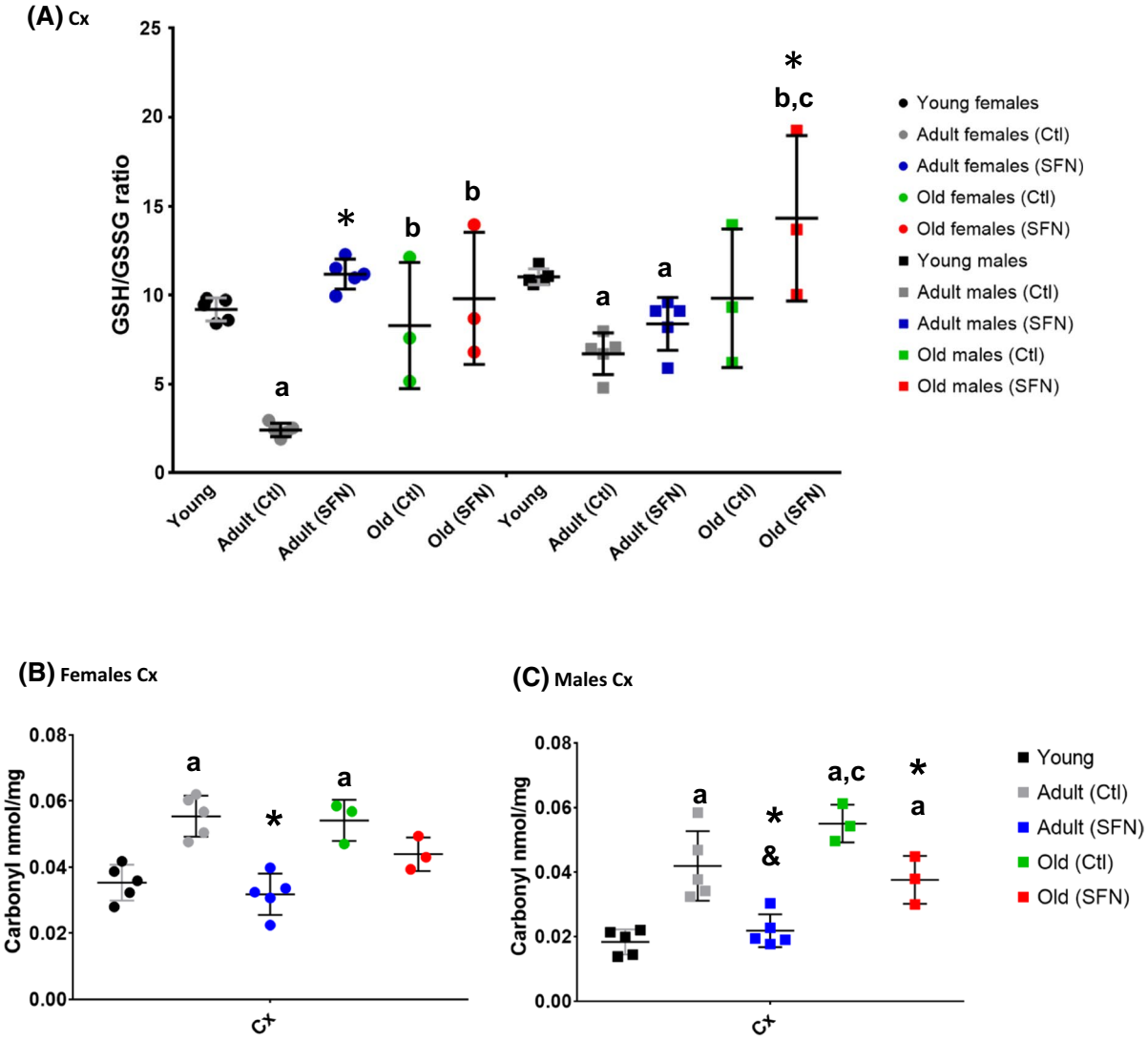


Fig. 5 Redox state and protein damage. The GSH/GSSG ratio was calculated for young, adult, and old females and males Cx (**A**) and Hc (**D**). Carbonylated proteins isolated from females (**B**) and males (**C**) Cx, as well as female (**E**) and male (**F**) Hc, were evaluated as described in materials and methods. Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$

SFN improves redox state

GSH/GSSG ratio was determined as a redox state indicator. In Cx (Fig. 5A), both sexes' adult groups obtained lower levels when compared with their respective same-sex young groups (females: $p = 0.022$; males: $p = 0.005$). Furthermore, SFN-treated adult females obtained a higher GSH/GSSG ratio when compared with the same-age non-treated group ($p \leq 0.001$), while no differences were observed against the young group. No differences were observed between the old groups, regardless of the SFN-treatment, but both groups were higher than the non-treated adult group (Old Ctl: $p = 0.005$; Old SFN: $p = 0.047$). Nevertheless, in males' old groups, SFN-treated old rats obtained a higher GSH/

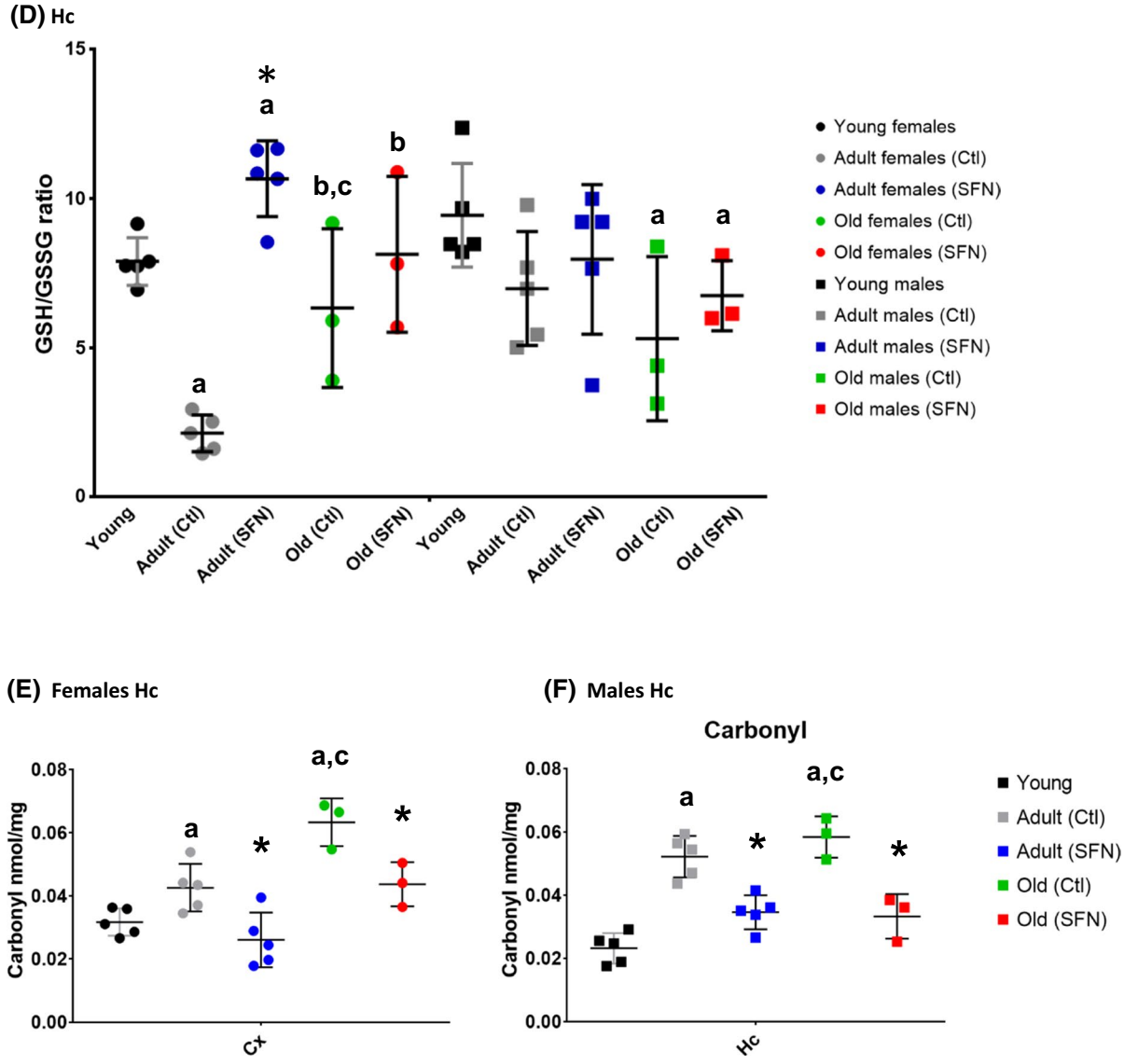
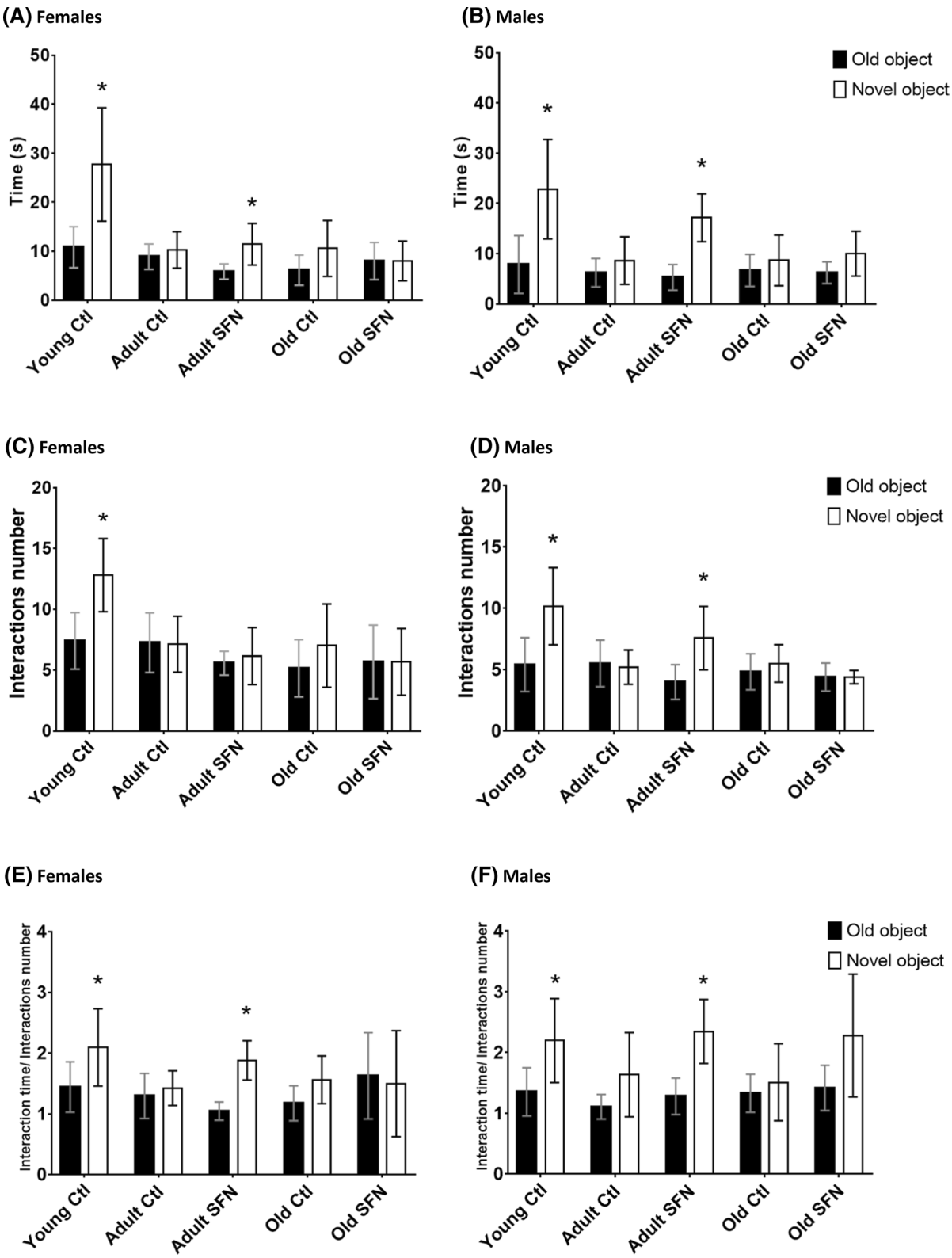


Fig. 5 (continued)

GSSG ratio ($p=0.028$) compared to the same-age non-treated group, Moreover, old males treated with SFN obtained an even higher GSH/GSSG ratio than both adult groups (Adult Ctl: $<p=0.001$; Adult SFN: $p=0.014$).

On the other hand, in the Hc, SFN-treatment only improved GSH/GSSG ratio in the adult females compared with the non-treated control group ($p\leq0.001$), and this GSH/GSSG ratio was higher when compared to the young group ($p\leq0.038$) (Fig. 5D). Both old groups obtained similar GSH/GSSG ratios to those observed in the same-sex young groups, and still were higher than non-treated adult groups (Old Ctl: $<p=0.046$; Old SFN: $p=0.009$). No differences were obtained for males' Hc GSH/GSSG ratios, among young and both adult groups, however, in old groups, lower GSH/GSSG ratios were observed compared with young groups (Old Ctl: $<p=0.003$; Old SFN: $p=0.043$).

In summary, SFN-treatment increased GSH/GSSG ratio in adult females in both evaluated brain regions. No effects were noted in both sexes' old groups' after


(A) Females
(B) Males
Old object
Novel object
Time (s)
(C) Females
(D) Males
Interactions number
(E) Females
(F) Males
Interaction time/ Interactions number
Young Ctl
Adult Ctl
Adult SFN
Old Ctl
Old SFN

◂**Fig. 6** Novel object recognition test. Old and novel object IT was determined in young, adult, and old females (**A**) and males (**B**) as described in materials and methods. IN was also counted in young, adult, and old females (**C**) and males (**D**). IT/IN quotient was calculated for females (**E**) and males (**F**). Each bar represents mean ± SD. Young: n = 5; Adult Ctl: n = 5; Adult SFN: n = 5; Old Ctl: n = 3; Old SFN: n = 3. Kruskal–Wallis non-parametric test was performed, followed by Bonferroni's multiple comparison test. Statistical significance as compared with same-sex young group (a) $p < 0.05$; against adult Ctl group (b) $p < 0.05$; against adult SFN group (c) $p < 0.05$; against old Ctl group (d) $p < 0.05$; against adult SFN group (e) $p < 0.05$; and against SFN-treated group (*) $p < 0.05$

SFN treatment. In males, no significant effects were observed in young and old groups, but, surprisingly, SFN-treated old group obtained a better Cx GSH/GSSG ratio in comparison to the same-age control group.

To determine if SFN treatment also reduced protein oxidation, we measured protein carbonylation. SFN-treatment successfully reduced carbonyl content in both sexes' Cx and Hc from adult groups when compared with their same-age control group. SFN treatment appeared to reduce carbonylated proteins in SFN-treated adult females' Cx ($p = 0.005$) and Hc ($p = 0.039$) (Fig. 5B and E), and in males' Cx ($p = 0.039$) and Hc ($p = 0.048$) (Fig. 5C and F).

In summary, the GSH/GSSG ratio increased in the Cx of both, females and males. Surprisingly, SFN treatment in adult females, not only restored the redox state in both evaluated regions, but it increased to significantly higher levels than those obtained in the young female group. SFN-treated groups also showed a decrease in protein carbonylation, showing an improvement in redox state and a protective effect not only in both sexes' adult groups but even in old groups.

Novel object recognition test

To evaluate the deterioration in recognition memory, young rats were used as a control to observe the discrimination between novel and old objects. The IT in seconds was evaluated, and the results are shown in Fig. 6, separated by sex, females (Fig. 6A) and males (Fig. 6B). As expected, young animals significantly explored the novel object for more time. Young females' IT was 2.4 times more with the novel than the old object, while males IT was 2.9 times more with the new object. Regarding the adult and old groups, none of these animals, regardless of their sex, was able to discriminate between the novel and the old object, thus, exploring them for a similar time. Interestingly, adult groups from both sexes treated with SFN, correctly discerned between the novel object and the old one, exploring it for a longer time. SFN-treated adult females explored 1.9 times more the novel object, while SFN-treated adult males explored it 3.6 times more. Curiously, none of the old groups discerned between the novel and the old object, even when treated with SFN.

As well as in IT, in females (Fig. 6C) and males (Fig. 6D), the number of interactions (IN) increased with the novel object than with the old ones (females 1.7 and males 2.0 times more). For the adult and old rats, there were no differences between the IN with the novel and old objects in the control groups for both sexes. Nevertheless, the IN in the adult SFN-treated male group was higher with the novel object compared with the old one (1.9) which was not observed in adult SFN-treated females. SFN-treatment did not affect IN in the old groups.

To determine how long each animal spent *per* interaction with the novel or the old object an IT/IN ratio was calculated (Fig. 6 E for females and Fig. 6 F for males). IT/IN ratio confirmed that SFN-treatment prevented the deficit in object recognition in the adult groups (15 months of age), but failed to do it in the old rats (21 months of age). No differences were found in the IT/IN ratio between males and females.

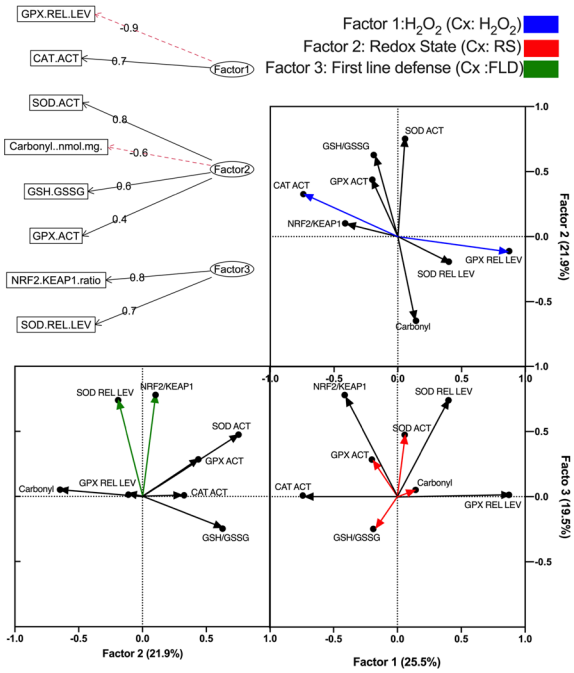
Factor analysis: relationship between redox parameters

To reduce the dimensions of all variables into a simpler system to analyze, we performed a FA as described in the methods section. All antioxidant enzymes' relative levels and antioxidant activity were included in the analysis, as well as the GSH/GSSG ratio, carbonylated protein content, and Nrf2/Keap1 ratio. The factor analysis reduced our variables to three, for both the Cx and the Hc.

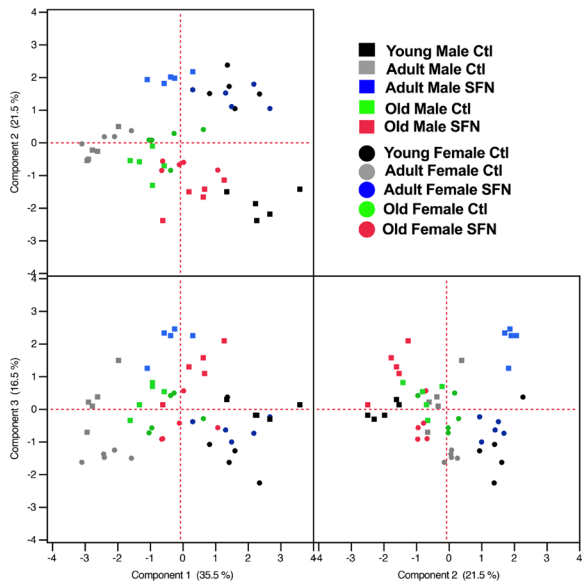
For the Cx, the three factors explained 67% of the variation. The first factor was named Cx H_2O_2 buffering ability (Cx: H_2O_2) and was strongly negatively related to GPx relative level, and positively related to CAT activity. Factor two, named Redox State (Cx: RS) was positively related to SOD activity, GSH/GSSG, and GPx activity, and negatively related to carbonyl content; finally factor three named as "First

FA Cortex

(A) Loading plot

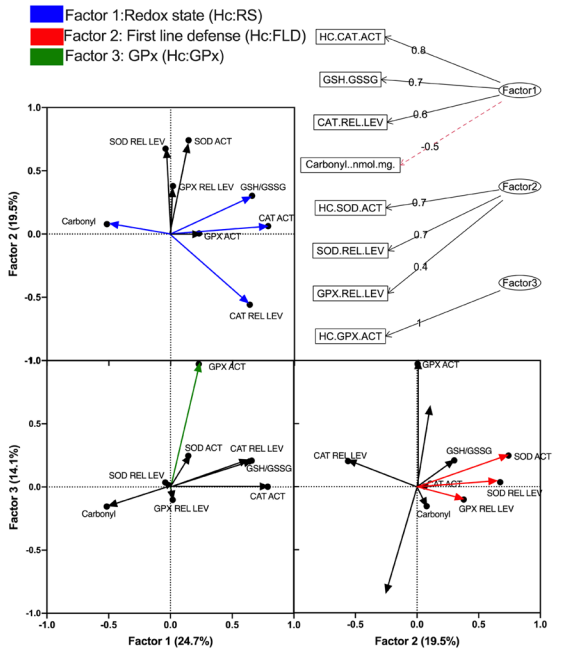


(B) Score Plot

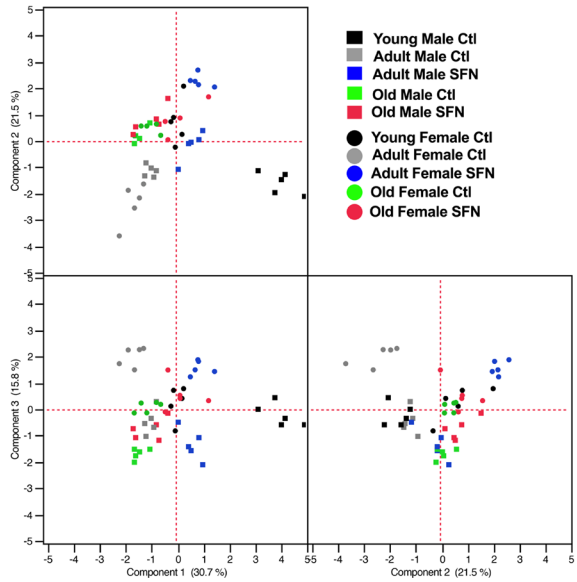


FA Hippocampus

(C) Loading plot



(D) Score Plot



◂**Fig. 7** Factor analysis of cortex data. All variables from both sexes were used to perform the factor analysis using the Kaiser-Meyere-Olkin (KMO) test; variables that had individual MSA (measure of sampling adequacy) < 0.50, were excluded from the analysis. **A** Loading Plot for the Cx. Loads which represent each treatment (black dots), were plotted for rotated factors 1, 2, and 3. This helped to identify which variables have the largest effect on the factors. Loadings can range from − 1 to 1. Loadings close to − 1 or 1 indicate that the variable strongly influences the factor. Loadings close to 0 indicate that the variable has a weak influence on the factor. Tree diagram of the factor model was added to facilitate interpretation. **B** Score Plot for the Cx. The plots were performed using factors 1, 2, and 3, to detect clusters and trends of the data. **C** Loading Plot for the Hc. Loads which represent each treatment (black dots), were plotted for rotated factors 1, 2, and 3. This helped to identify which variables have the largest effect on the factors. Loadings can range from − 1 to 1. Loadings close to − 1 or 1 indicate that the variable strongly influences the factor. Loadings close to 0 indicate that the variable has a weak influence on the factor. Tree diagram of the factor model was added to facilitate interpretation. **D** Score Plot for the Hc. The plots were performed using factors 1, 2, and 3, to detect clusters and trends of the data. Each point represents an individual. Circles represent females, while squares represent males

line of defense" (Cx: FLD), was positively related to Nrf2/Keap1 ratio and SOD relative levels (Fig. 7A). While for the Hc, the three factors explained a cumulative 59% of the variation. The three factors have similar names, but they consist of different components. The first factor was named Redox State (Hc: RS) and was mainly represented positively by CAT activity, GSH/GSSG ratio, CAT relative levels, and negatively by carbonyl content; factor two, named as "First line of defense" (Hc: FLD), was positively related to SOD activity, SOD relative levels, and GPx relative levels; and finally factor three, named as Hc: Gpx, which was only represented by GPx activity (Fig. 7C).

For the Cx score plot (Fig. 7B). We found that both sexes' young groups were placed mostly in the center and right panels, where factor 1 (Cx: H_2O_2) was also located. Non-treated adult groups from both sexes were placed mostly in the left panels, where not only did they have strong factor 1 but also factor 2 (Cx: RS) representation. Surprisingly, it was old-treated males who showed an improvement, gravitating towards factor 3 (Cx: FLD), compared to treated old females. The score plot of the Hc (Fig. 7D) shows that both sexes' young and SFN-treated adults are mainly located in the center of the panel and right panels (upper and down). Particularly young male group is mostly located in the right-down panel, where factor 1 (Hc: RS) is directed. In contrast to the young female group, which is mostly located in the center panel, where factor 2 (Hc: FDL) is directed. Interestingly, non-treated adults of both sexes and the male old group are mainly located in the left panels, suggesting greater carbonyl content. Remarkably, the female old group treated with SFN was found in the center and right panel, suggesting an improvement in their RS and FLD. This analysis suggests that SFN-treatment mildly recovers the redox homeostasis in old males' Cx, and the Hc of old females; and although the effect is not as evident as in adults, old animals do have an improvement.

Discriminant analysis: SFN effect

The discriminant model with the representative new variables and the IT/N as covariates was significant (Wilks' lambda = 3.6e-6, $F_{54,183} = 236.2$, $p < 0.0001$), and both canonical dimensions explained 81.12% of the total variance. The results obtained showed that SFN treatment greatly improved adult females' FLD in the Cx, being very similar to the one observed in young females. When untreated adult females were compared with untreated old females, it was found that old females similarly improved the Hc's FLD. Still, after the SFN-treatment their FLD slightly decreased compared to untreated old females. Something similar was observed for old males treated with SFN compared to the non-treated; not only did they decrease their Hc's FLD, but they switched to an increased Cx H_2O_2 buffering capacity. Interestingly, young males showed a better RS in the Hc and the Cx, while untreated male adults increased their FLD in Hc, similarly to old SFN-treated females, however, when adult males were treated with SFN, they increased their Cx's FLD, probably in an attempt to return to redox homeostasis. At the same time, they increased their memory (IT/IN) similarly to young females and adult-treated females.

Discussion

SFN is a natural compound that modifies the redox state and activates more than one protective response pathway promoting a beneficial effect on cells

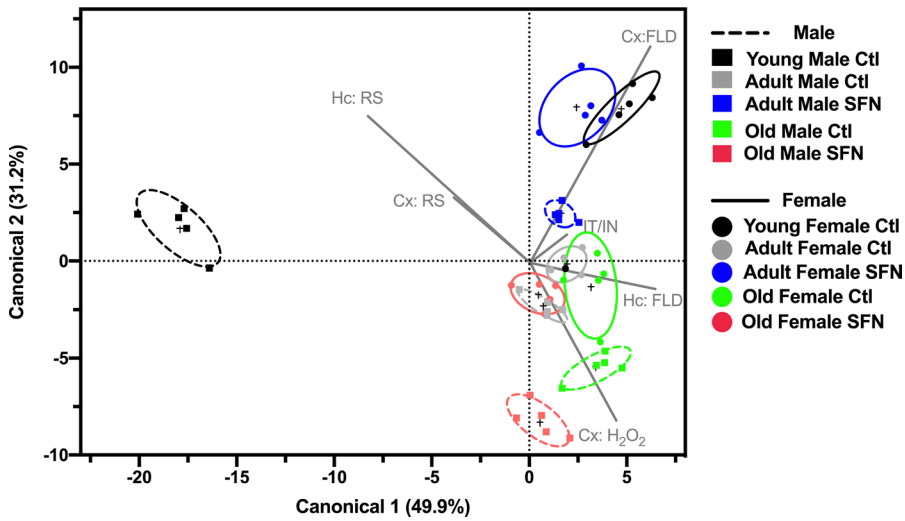


Fig. 8 Discriminant analysis. The first and second canonical dimensions that best separate the groups are represented in the X and Y axis, respectively. The percentage of variation explained by each dimension is indicated. The representative five new variables (Hc: RS, Hc: FLD, Cx: FLD, Cx: RS, Cx: H_2O_2) were used together with the IT/IN ratio. The variable Hc: GPx was excluded from the analysis as it didn't significantly contribute to the model. The length and direction of the biplot rays represent the level of association with each separated group. Each point represents an individual. Circles represent females, while squares represent males. Experimental groups are surrounded by 95% confidence level ellipses, dashed ellipses represent male groups and solid ellipses represent females. Group means are represented by a plus symbol (+). Statistically significant different groups' ellipses do not intersect

(Santín-Márquez et al. 2019a, b); hence, it can be considered a "nutritional hormetin" (Ratan et al. 2009). Neuroprotective effects have been attributed to SFN in in vitro (Liu et al. 2020; Maciel-Barón et al. 2018; Calabrese et al. 2020) and in vivo (Morroni et al. 2013; Zhou et al. 2016; Mao et al. 2019; Devi and Chamoli 2020) experiments. Therefore, in this study, we decided to administer SFN for long times and at low concentrations to evaluate its effectiveness as a hormetin to prevent aging deterioration and to compare the effects observed when treating female and male adult and old rats, to contrast the responses due to age and sex.

With the results obtained in our experiments, it was evident that adult animals of both sexes responded better to SFN-treatment than old animals, which is not surprising since it has been reported that the Nrf2 pathway is hardly activated in aging (Suh et al. 2004). Yet, we have reported that under certain Nrf2 inducer concentrations and times of exposure the Nrf2 response may be activated in aging (Alarcón-Aguilar et al. 2014). Accordingly, if we analyze each of the parameters studied, it can be observed that not all of them behave in the same way during aging. The same goes for differences by sex. The SFN-treated male adults obtained similar values to the ones determined in young groups, while the SFN-treated female adults showed better results. It is important to notice that young females are known to have a better antioxidant response and less ROS production (Bhatia et al. 2012; Kander et al. 2017), but with the age-associated hormonal decline, this protection also decreases (Viña et al. 2005; Wang et al. 2020a, b, c; Agarwal et al. 2012). Moreover, it has been reported that females are capable of responding better after an insult which affects the antioxidant response than males (Chamniansawat and Sawatdiyaphanon 2018). So, at first glance, it would seem that adult females respond better to SFN-treatment compared with same-age males. However, as was seen when adult and old animals were compared, regarding sex, not all the evaluated parameters behaved in the same way. So, in order to understand how the redox variables relate to each other, and to the behavioral test, we decided to perform a multivariate analysis. Specifically, the factor analysis (FA) was used to reduce variables' dimensions into representative new variables (called factors). Then a quadratic discriminant analysis (QDA)

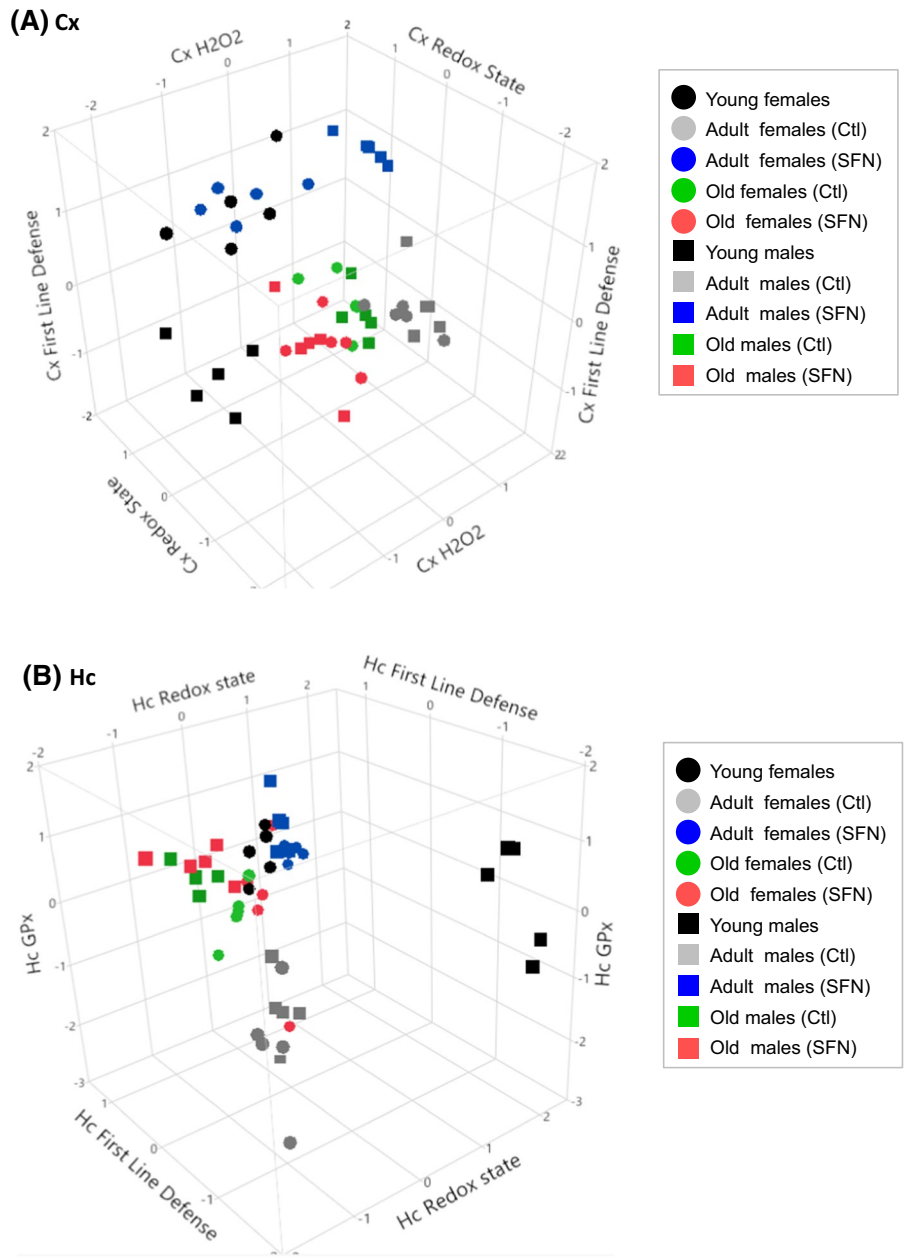


Fig. 9 3D graph of the FA factor in the cortex and hippocampus. The figure shows the spatial representation of the FA factors obtained from the data as described in the materials and methods section. Negative numbers indicate a negative correlation with the model, and positive numbers a positive one. The 3D plot was constructed using the JMP statistical discovery software

was performed to describe the relationships between the new factors generated using the FA, plus the IT/IN ratio. Interestingly, by grouping the data we found remarkable responses that were not evident when analyzing them individually. The results found in the FA (Fig. 7), and corroborated in the QDA (Fig. 8), showed that old rats treated with SFN in a hormetic manner modified their redox regulation, i.e. their global redox response and homeostasis were not the same as the old untreated rats; and although they were not able to recover their redox state as adults did, they certainly had an improvement.

To visually represent the relationships among the three new factors for the Cx (H_2O_2 buffering capacity, Redox State, and FLD) and for the Hc (Gpx, Redox State, and FLD), a 3D scatter plot for each brain region was performed (Fig. 9A, B). For the Cx (Fig. 9A) a distinct data distribution for most of the

groups was observed. In young females, the RS and FLD factors are positively correlated and negatively correlated with H_2O_2. Probably because the H_2O_2 levels are low and the antioxidant system does not require eliminating them. This suggests that SFN-treatment is modulating and buffering the redox state, so they don't have to "invest" in increasing their first line of defense parameters.

In the case of young males, there is a negative correlation with FLD and a positive one with RS, which confirms that the regulation of redox homeostasis in this case is carried out by a different mechanism. In adult and old animals of both sexes, the three factors turn to negative numbers, which indicates that they are losing their redox homeostasis. Interestingly, when treating the adults with SFN, they return to positive values, the males increase the three factors and the females only maintain two, like the young females, without activating H_2O_2. For Hc (Fig. 9B), we see a similar behavior, although it must be considered that the GPx factor only contains the activity of this enzyme.

In general, SFN enhanced adult female's first line of defense (NrF2/Keap1 and SOD relative levels) in the Cx, while in old females it slightly improved their Hc redox state (CAT activity and relative levels, GSH/GSSG, and decreased carbonyl content) and in consequence boosted their FLD (SOD activity and relative levels and GPx relative levels) slightly decreased compared to adults, but it continued to be their strongest characteristic. On the other hand, these results support that SFN-treatment improved males' Cx redox homeostasis, however, the age determined which parameters were being benefited. In adults, SFN greatly improved the first line of defense (NrF2/Keap1 and SOD relative levels); while in old treated males, the H_2O_2 buffering abilities were improved (GPx relative levels and CAT activity).

Nevertheless, even with these analyses, it is difficult to explain how a molecule like SFN can affect the redox response differently in males and females. Therefore, it is necessary to consider that SFN not only acts by activating Nrf2 when it modifies the redox state. In the same way that it can alter the oxidation state of the sulfhydryls in Keap1, it could also modify the cellular redox signaling by changing thiol/disulfide regulation in other molecules. It is known that the neurochemistry of the brain in males and females is different (Fowler et al. 2021), so changes in the redox state generated by the SFN could activate or inactivate different signaling pathways (Sun et al. 2019). Just to give an example, some neuropeptides, such as oxytocin and vasopressin, have cysteines whose sulfurs form disulfide bonds, making them susceptible to this type of redox modulation. Besides, SFN might be related to recuperating redox homeostasis by protecting the mitochondrial function (Greco et al. 2011; Greco and Fiskum 2010) and decreasing the inflammatory pathways Liang et al. 2018; Qi et al. 2016; Ruhee and Suzuki 2020). SFN might be acting simultaneously on those pathways to protect the redox state, thus more experiments in that direction should be performed shortly. Another hypothesis to understand these differences might be related to the known fact that the SFN molecule is largely conjugated with glutathione, cysteine, and N-acetylcysteine (NAC), these conjugates could also have different affinities and play an important role in differential sex regulation (Jardim et al. 2020).

Furthermore, not only is the redox effect difficult to explain, the role of sex differences during normal aging is also not fully understood. The hormonal background offers substantial differences between females and males in several morphological, cognitive, and molecular aspects (Hamson et al. 2016; de Souza et al. 2022). Besides hormonal implications in cognition, sex differences in the brain's antioxidant mechanisms have been described (Gaignard et al. 2015). Still, redox homeostasis deregulation is an important hallmark usually found in the central nervous system of aged organisms, regardless of the sex (Gaignard et al. 2015); additionally, sex hormone decrease with age has been related to an increase in oxidative stress in both sexes, having a severe effect in old females (Razmara et al. 2007; Sumien et al. 2021). It has been widely reported that the gradual decrease in sex hormones is correlated with cognitive impairment and with alterations in memory processes in both, female and male-aged rats (Bimonte-Nelson et al. 2003; Gurvich et al. 2018). Some reports establish that the typical decrease in sex hormone production begins during middle age, when the rats still have a reproductive capacity (8–10 months old) (Candeias et al. 2017), and progresses with age. Middle age is a crucial stage in which cognitive decline starts, and this correlates with a hormonal reduction (Morrison and Baxter 2012). Even so, sex-specific hormone levels can activate several stress-contending pathways, whose failure could

be involved in nervous system damage and cognitive impairment (Ferreira et al. 2012; Wang et al. 2020c).

Furthermore, better brain mitochondrial function and antioxidant defenses have been found in aged females when compared with males in rodent models (Guevara et al. 2009). GSH metabolism, which is increased in females during their youth, drastically decreases with aging, in contrast to males who show lower glutathione-related antioxidant defenses during adulthood (Wang et al. 2020a, b, c). Therefore, changes in the general redox state play an important role during the brain aging process. So, a pharmacological intervention able to induce the antioxidant response could mitigate aging-associated oxidative damage in the nervous system, preserving memory integrity.

Conclusion

The SFN-treatment improved memory and learning in female and male adult groups. Probably by protecting the nervous system from age-related oxidative stress. Our results suggested that SFN improves memory and learning, as well as redox state. Furthermore, it significantly reduced oxidative damage to proteins. Remarkably, SFN-treatment did not revert cognitive decline in old animals, so it could be used as a prevention treatment instead of a reverting one.

Nevertheless, it should be considered that the equivalence between the age of rodents and humans has been a controversial issue for a long time. There are different ways of comparing ages between species (Sengupta 2013; Dutta and Sengupta 2016; Agoston 2017; Wang et al. 2020a, b, c) and no consensus has been reached, but fundamental differences according to life stage have to be considered (Dutta and Sengupta 2016). Numerous clinical trials that showed successful results in preclinical studies in rodents were ineffective in humans. Interestingly, an important factor that was often neglected was the different biological timescales of rodents *versus* humans (Agoston 2017). So, before considering the SFN-treatment in humans, more studies must be performed.

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Declarations

Conflict of interest The authors have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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