



Xanthohumol exerts protective effects in liver alterations associated with aging

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Received: 30 April 2017 / Accepted: 5 March 2018 / Published online: 13 March 2018
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Abstract

Background and aims Aging is associated with a deregulation of biological systems that lead to an increase in oxidative stress, inflammation, and apoptosis, among other effects. Xanthohumol is the main preylated chalcone present in hops (*Humulus lupulus* L.) whose antioxidant, anti-inflammatory and chemopreventive properties have been shown in recent years. In the present study, the possible protective effects of xanthohumol on liver alterations associated with aging were evaluated.

Methods Male young and old senescence-accelerated prone mice (SAMP8), aged 2 and 10 months, respectively, were divided into four groups: non-treated young, non-treated old, old treated with 1 mg/kg/day xanthohumol, and old treated with 5 mg/kg/day xanthohumol. Male senescence-accelerated resistant mice (SAMR1) were used as controls. After 30 days of treatment, animals were sacrificed and livers were collected. mRNA (AIF, BAD, BAX, Bcl-2, eNOS, HO-1, IL-1 β , NF- κ B2, PCNA, sirtuin 1 and TNF- α) and protein expressions (BAD, BAX, AIF, caspase-3, Bcl-2, eNOS, iNOS, TNF- α , IL1 β , NF- κ B2, and IL10) were measured by RT-PCR and Western blotting, respectively. Mean values were analyzed using ANOVA.

Results A significant increase in mRNA and protein levels of oxidative stress, pro-inflammatory and proliferative markers, as well as pro-apoptotic parameters was shown in old non-treated SAMP8 mice compared to the young SAMP8 group and SAMR1 mice. In general, age-related oxidative stress, inflammation and apoptosis were significantly decreased ($p < 0.05$) after XN treatment. In most cases, this effect was dose-dependent.

Conclusions XN was shown to modulate inflammation, apoptosis, and oxidative stress in aged livers, exerting a protective effect in hepatic alterations.

Keywords Liver · Aging · Inflammation · Senescence-accelerated mouse · Xanthohumol

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Introduction

Aging is a universal physiological condition in which a decrease in organic functions is clear along with an increase in aging-related diseases. Several studies have seen a link between aging and dysregulation of biological systems [1]. Aging has been reported as one of the main risk factors for chronic diseases. Liver is utterly involved in this process, since energy metabolism and detoxification pathways become damaged. Liver-related deaths also increase with age as disease susceptibility is enhanced.

Liver aging leads to both structural and functional alterations: liver undergoes a significant 30% reduction in volume with increasing age [2]. Similarly, hepatic blood flow is decreased by 35%, which may explain subsequent liver sinusoidal endothelial cell dysfunction [3]. In addition, studies have shown an outstanding decrease in the number of

mitochondria in the elderly subjects and moreover, a reduction in the expression of genes involved in oxidative stress such as cytochrome c [3]. It is also considered that a reduction in hepatocyte telomere length results in diminished cell mitosis and apoptosis, and therefore, in a decrease in cell proliferation [4, 5].

Cellular senescence is a mechanism which limits the proliferative potential damaged cells and, moreover, it also leads to inflammation, fibrosis and some other important disorders. It occurs as a result of oxidative stress, DNA damage or oncogene activation, and induces cell cycle arrest. Cellular senescence in hepatocytes has been demonstrated in several chronic parenchymal disorders including chronic viral hepatitis B and C, alcohol-related liver disease and non-alcohol-related fatty liver disease [6].

Xanthohumol (XN), a hop-derived prenylated flavonoid has been identified as a regulator of several pathways related to proliferation and apoptosis [7]. Moreover, several research works have revealed that XN possesses a handful of biological properties, including antioxidant, anti-inflammatory, and anticancer effects, as well as its capacity to inhibit NF- κ B and Akt activation in vascular endothelial cells [8, 9]. Recent studies have shown that XN contributes to the prevention of oxidative damage by reducing not only ROS but also nitrogen oxide production. It also exhibits anti-inflammatory properties by the inhibition of the cyclooxygenase activity [10].

The aim of the present study was to evaluate the possible effects of XN on the mRNA and protein expression of inflammation and apoptosis markers, oxidative stress intermediaries, and proliferative parameters in hepatic tissue of senescence-accelerated prone male mice (SAMP8) aged 2 and 10 months, with livers of SAMR1 male mice as controls.

Materials and methods

Animal model

Male senescence-accelerated prone (SAMP8) and senescence-accelerated resistant (SAMR1) mice of 2 months of age (young) and 10 months of age (old) were used in the study [11]. SAMP experience increases in pro-inflammatory cytokines and oxidative damage observed during aging, while SAMR is used as model of physiological, non-accelerated aging [12–15], therefore acting as controls of SAMP.

Animals were housed in cages under controlled environmental conditions (22 °C; 70% humidity), kept under a 12/12 h light/dark photoperiod, and fed ad libitum, i.e., food and water were available at all times with the quantity and frequency of consumption being the free choice of the animal.

This study was approved by the Research and Animal Experimentation Committee of the Complutense University of Madrid (Ref. 10/226347.9/16). All experiments were performed according to both European and Spanish laws regarding the handling and care of experimental animals (EU Directive 2010/63/EU for animal experiments).

Treatment

SAMP8 mice were divided into four experimental groups of five individuals each: (1) non-treated young mice, (2) non-treated old mice, (3) old mice treated with 1 mg/kg/day of XN and (4) old mice treated with 5 mg/kg/day of XN. SAMR1 mice aged 2 and 10 months were used as controls. Animals were purchased from Harlan Laboratories (Indiana, USA).

XN (98% purity, NIC Nookandeh Institute for Chemistry of Natural Substances, Homburg, Germany) was dissolved in ethanol and this solution was added to the animal drinking water. Non-treated animals were supplied only with ethanol 1%. This concentration of ethanol was not shown to have a toxicity effect on the liver, while in prior studies from our group concerning the effect of XN in the brain [16], the dose used was 0.1% of ethanol to avoid brain toxicity [17]. The solution was prepared daily and was available to animals during 24 h of the day. After a treatment period of 30 days, animals were sacrificed and plasma and livers were collected.

Plasma samples were flash frozen and stored at – 20 °C until liver functional tests were performed. Sample tissues were placed into cryotubes immediately after extraction, flash frozen in liquid nitrogen and stored at – 80 °C until mRNA and protein expression of inflammatory mediators (TNF- α —tumor necrosis factor-alpha-, IL-1 β —interleukin 1-beta-, NF κ B2—nuclear factor NF-kappa-B p100 subunit- and IL-10—interleukin 10-), apoptosis markers (Bcl-2—B-cell lymphoma-, BAD—Bcl-2-associated death promoter-, BAX—Bcl-2-associated X protein, AIF—apoptosis-inducing factor- and caspase 3), oxidative stress intermediaries (eNOS, iNOS, and HO-1), proliferating messenger PCNA and sirtuin 1 was measured in liver tissue.

Blood chemistry

Plasma samples were flash frozen and stored at – 20 °C until liver functional tests were performed. A non-hemolyzed sample was used by treating plasma with EDTA. Liver function tests (LFT) included the measurement of total bilirubin (TBIL), triglycerides (TG), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT) and lactate dehydrogenase (LDH).

RNA isolation and RT-PCR quantification

mRNA expression of TNF- α , IL-1 β , NF κ B2, BAD, BAX, AIF, Bcl-2, eNOS, HO-1, PCNA and sirtuin 1 was measured using real time (RT)-PCR. RNA was isolated from liver samples according to the method described by Chomczynsky and Sacchi [18] using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. The purity of the RNA was estimated using 1–1.5% agarose gel electrophoresis, and the RNA concentrations were determined using spectrophotometry (BioDrop™). Reverse transcription of 2 mg of RNA for cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison, WI, USA) and a pd(N)6 random hexamers. RT-PCR was performed using an Applied Biosystems 7300 apparatus with the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300 nM concentrations of specific primers (Table 1). Amplification of GAPDH mRNA was used as a loading control for each sample. Relative changes in mRNA expression were calculated using the 2- $\Delta\Delta$ CT method [19].

Table 1 Primers used in real-time PCR experiments

	Primers	Sequence (5'–3')
GAPDH	Forward	TGGTATCGTGCAAGGACTCATGAC
	Reverse	ATGCCAGTCAGCTTCCCGTTCAGC
IL-1 β	Forward	TGTGATGAAAGACGGCACAC
	Reverse	CTTCTTCTTTGGGTATTGTTTGG
TNF- α	Forward	ATGAGAAGTTCCCAAATGGC
	Reverse	CTCCACTTGGTGGTTTGCTA
Bcl-2	Forward	TGAGTACCTGAACCGGCATCT
	Reverse	GCATCCCAGCCTCCGTTAT
NF- κ B2	Forward	TGGAACAGCCCCAAACAGC
	Reverse	CACCTGGCAAACCTCCAT
BAX	Forward	GTGAGCGGCTGCTTGCT
	Reverse	GGTCCCGAAGTAGGAGAGGA
BAD	Forward	GCCCTAGGCTTGAGGAAGTC
	Reverse	CAAACCTCTGGGATCTGGAACA
eNOS	Forward	CCAGTGCCCTGCTTCATC
	Reverse	GCAGGGCAAGTTAGGATCAG
AIF	Forward	AGTCCTTATTGTGGGCTTATCAAC
	Reverse	TTGGTCTTCTTAATAGTCTTGTAGGC
Sirtuin 1	Forward	TCGTGGAGACATTTTAATCAGG
	Reverse	GCTTCATGATGGCAAGTGG
PCNA	Forward	GCCGTGAACCTACAGAGCAT
	Reverse	CACAGGAGATCACCACAGCA
HO-1	Forward	GTCAAGCACAGGGTGACAGA
	Reverse	ATCACCTGCAGCTCCTCAAA

GAPDH was used as a housekeeping gene to compare the samples

Protein extraction and Western blot analysis

Western blotting was used to measure levels of BAD, BAX, AIF, caspase-3, Bcl-2, eNOS, iNOS, TNF- α , IL-1 β , NF κ B2, and IL-10. Briefly, after homogenization with lysis buffer (100 mmol/l NaCl, 10 mmol/l Tris-Cl [pH 7.6]), (1 mmol/l EDTA [pH 8], 1 μ g/ml aprotinin, 100 μ g/ml PMSF), all samples were diluted (1:1) with 2 \times buffer (100 mmol/l TrisHCl [pH 6.8], 4% SDS, 20% glycerol, bromophenol blue 0.1, 200 mmol/l dithiothreitol) and were boiled for 10 min at 100 °C. To correct possible variations in the size of samples, protein concentration by the Bradford method was determined. Equivalent amounts of protein were subjected to electrophoresis on SDS polyacrilamide gel (10%) and transferred onto PVDF membranes. After blocking with 5% milk in TBS-Tween 20 (20 mmol/l TRIS, 150 mmol/l NaCl, 0.2% Nonidet P 40, 5% skim milk) for 60–90 min to prevent nonspecific binding and washing, the membranes were probed overnight at 4 °C with primary antibodies. The membrane was incubated with a rabbit polyclonal antibody (Table 2) (1:1000) for 12 h at 4 °C, followed by incubation with an anti-rabbit horseradish peroxidase conjugated antibody (#12–348 Millipore Billerica, Massachusetts, USA) (1:7000). Detection was performed using an ECL Plus assay kit (Amersham Life Science Inc., Buckinghamshire, UK). The blots were re-probed with antibodies against β -actin (#A5316 Sigma-Aldrich Co., St. Louis, MO, US) to ensure equal loading and transfer of proteins. After membrane washing, proteins were visualized by chemiluminescence (ECL system, Amersham, Oakville, Ontario, Canada). Densitometric measurement of protein level was performed using Quantity One 1-D Analysis Software (Bio-Rad GS 800). The optical density of the bands was normalized to β -actin levels, the loading control.

The same procedure was used to measure NF- κ B p52 and NF- κ B p65 in nuclear and in cytoplasmic fractionations previously separated through selective centrifugation.

Representative images of the Western blot results are shown in Table 3.

Statistical analysis

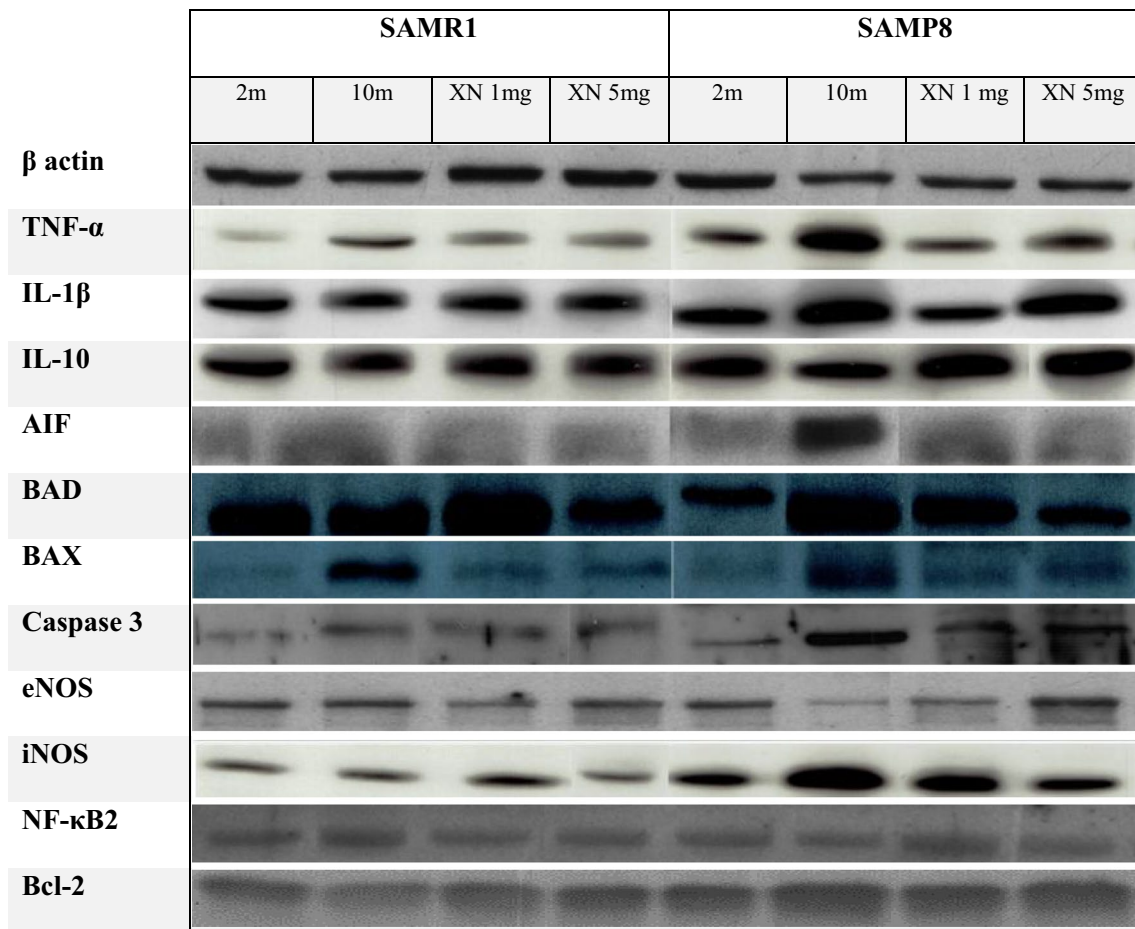
Data are expressed as mean \pm standard deviation of the number of determinations. Results were analyzed using ANOVA followed by a post hoc Tukey HSD test to find out which specific group's means were different. Pair of means were compared by Student's *t*-test. Statistical significance was set at $p < 0.05$.

Results

Liver functional tests showed overall stability with the exception of AST where a significant increase was observed in SAMP8 old mice in comparison with SAMP8 young

Table 2 Source of primary antibodies

Antibody	Catalog number	Company
TNF- α	500-P72	PeptoTech EC, Ltd. London, UK
IL-1 β	500-P80	
IL-10	500-P139	
BAD	21062	Signalway antibody, Maryland USA
iNOS	33261	
BAX	2772	Cell Signaling Technology Massachusetts, USA
Bcl-2	2870	
eNOS	32027	
AIF	5318	
Caspase 3	9665	
NF κ B p52-100	14-6733	eBioscience, California USA
NF κ B p65	14-6731	
HO-1	AB1284	Chemicon Intenrational, California USA

Table 3 Representative images of the Western blot results

mice. This rise was significantly reduced in the 5 mg XN-treated group (Table 4).

While no significant alteration was observed in TNF- α mRNA expression (Fig. 1a), increased levels of its protein expression were observed in both SAMR1 and SAMP8 old

mice compared to young animals. Both 1 mg and 5 mg XN-treated groups showed significantly lower levels than those observed in old animals in SAMR1 and SAMP8 groups. Also, the levels of 5 mg XN-treated animals were

Table 4 Liver functional tests

	SAMR 1				SAMP 8				
	Young	Old	XN 1 mg	XN 5 mg	Young	Old	XN 1 mg	XN 5 mg	
TBIL (mg/100)	0.936 (0.05)	1.013 (0.1)	0.903 (0.03)	0.863 (0.04)	1.00 (0.03)	1.063 (0.11)	0.87 (0.05)	0.855 (0.05)	NS
TG (mg/100)	117 (10.6)	114 (12.9)	91.3 (16.3)	135 (6.4)	143 (12.1)	186.6 (4)	149.6 (7.6)	119.3 (27.4)	NS
Cholesterol (mg/100)	73 (5.6)	81.3 (14.9)	89.3 (16.7)	81.6 (19.1)	78 (6.5)	88 (17.3)	103 (9.5)	87.2 (16)	NS
ALP (U/l)	78 (9.1)	81.3 (5.9)	76.6 (7.8)	71 (6.2)	82.3 (12)	96.3 (2.5)	80.3 (3.6)	73.5 (9.1)	NS
AST (U/l)	69.3 (11)	94.7 (4.8)	80.3 (6)	78 (8.9)	66 (9.1)	122.6 (10.9)*	78 (8.5)	64.8 (9) #	* $p < 0.05$ # $p = 0.015$ vs SAMP8 old
ALT (U/l)	16.6 (1.2)	16.1 (1.8)	15.9 (1)	16 (1.7)	15 (0.8)	18.6 (0.5)	16 (0.5)	16 (0.2)	NS
GGT (U/l)	7.6 (1.21)	5 (0)	5.3 (0.58)	4.6 (0.22)	4.6 (1.09)	6.6 (1.15)	5.6 (0.58)	5.2 (0.49)	NS
LDH (U/l)	58.9 (1.3)	75.7 (6.5)	57.3 (1.8)	55.8 (5)	60.1 (3.2)	77.6 (3.3)	70.6 (2.5)	63.8 (2.9)	NS

TBIL total bilirubin, TG triglyceride, ALP alkaline phosphatase, AST aspartate aminotransferase, ALT alanine aminotransferase, GGT gamma glutamyl transferase, LDH lactate dehydrogenase

significantly lower than those administered with 1 mg XN (Fig. 1b).

Both mRNA and protein expressions of IL-1 β were increased in old SAMP8 animals compared to young animals and to SAMR1 group. These increases were significantly counteracted in animals treated with XN. In addition, mRNA expression in 5 mg XN-treated group was significantly lower than that in 1 mg XN-treated mice (Fig. 1c, d).

NF- κ B2 mRNA expression was significantly lower in SAMR1 young mice compared to SAMP8 young animals. In SAMR1 old group, significantly higher levels were observed in comparison with their younger counterparts. Significantly lower levels were observed in both SAMR1 and SAMP8 5 mg XN-treated animals when compared to their respective non-treated old groups (Fig. 1e). However, NF- κ B2 protein levels did not show any significant changes when the total fraction was measured (Fig. 1f).

Likewise, age-related changes were not seen in the protein expression of anti-inflammatory marker IL-10 (Fig. 1g).

When the nuclear-cytoplasmic fractionation of liver homogenates was analyzed, no significant alterations were observed in NF- κ B p52 (Fig. 2a). However, NF- κ B p65 levels were significantly reduced when treating with 5 mg XN in comparison to the values observed in old SAMP8 group (Fig. 2b).

Regarding pro-apoptotic molecules, old SAMP8 mice showed elevated BAD mRNA expression in comparison with young SAMP8 mice and old SAMR1 mice (Fig. 3a). BAD mRNA expression of SAMP8 animals administered with 5 mg was significantly lower than that observed in old non-treated animals (Fig. 3a).

BAX protein expression of both SAMR1 and SAMP8 old non-treated mice was significantly higher than the one observed in young animals. Also, SAMP8 group showed significantly higher levels than SAMR1. While in SAMR1 animals only 5 mg XN-treated group showed significantly lower levels than old non-treated animals, in SAMP8 animals both doses of XN significantly reduced the expression of this marker compared to old non-treated SAMP8 mice (Fig. 3d).

Both mRNA and protein levels of apoptosis-inducing factor AIF were higher in old SAMP8 mice when compared to young SAMP8 and old SAMR1 mice. While for the mRNA expression, both 1 and 5 mg XN-treated SAMP8 groups showed significantly lower expressions than old non-treated animals, AIF protein expression of SAMP8 old animals was significantly reduced only when mice were given 5 mg XN (Fig. 3e, f).

Old SAMP8 non-treated animals showed significantly higher levels of caspase 3 than those in young animals and old SAMR1 mice. The treatment with both 1 and 5 mg XN significantly reduced caspase 3 levels (Fig. 3g).

Considering Bcl-2, age-related changes were not seen in the levels of this anti-apoptotic marker (Fig. 4a, b).

eNOS and iNOS protein levels experienced opposite changes with aging. While iNOS levels were enhanced in old SAMP8 mice in comparison with their younger counterparts and the control animals, eNOS levels were reduced with age significantly (Fig. 5b, c). eNOS protein expression of SAMP8 group treated with 5 mg XN was significantly higher than the one observed in old non-treated animals (Fig. 5b), whereas its mRNA levels did not show any significant change (Fig. 5a). Treatment with both 1 and 5 mg XN

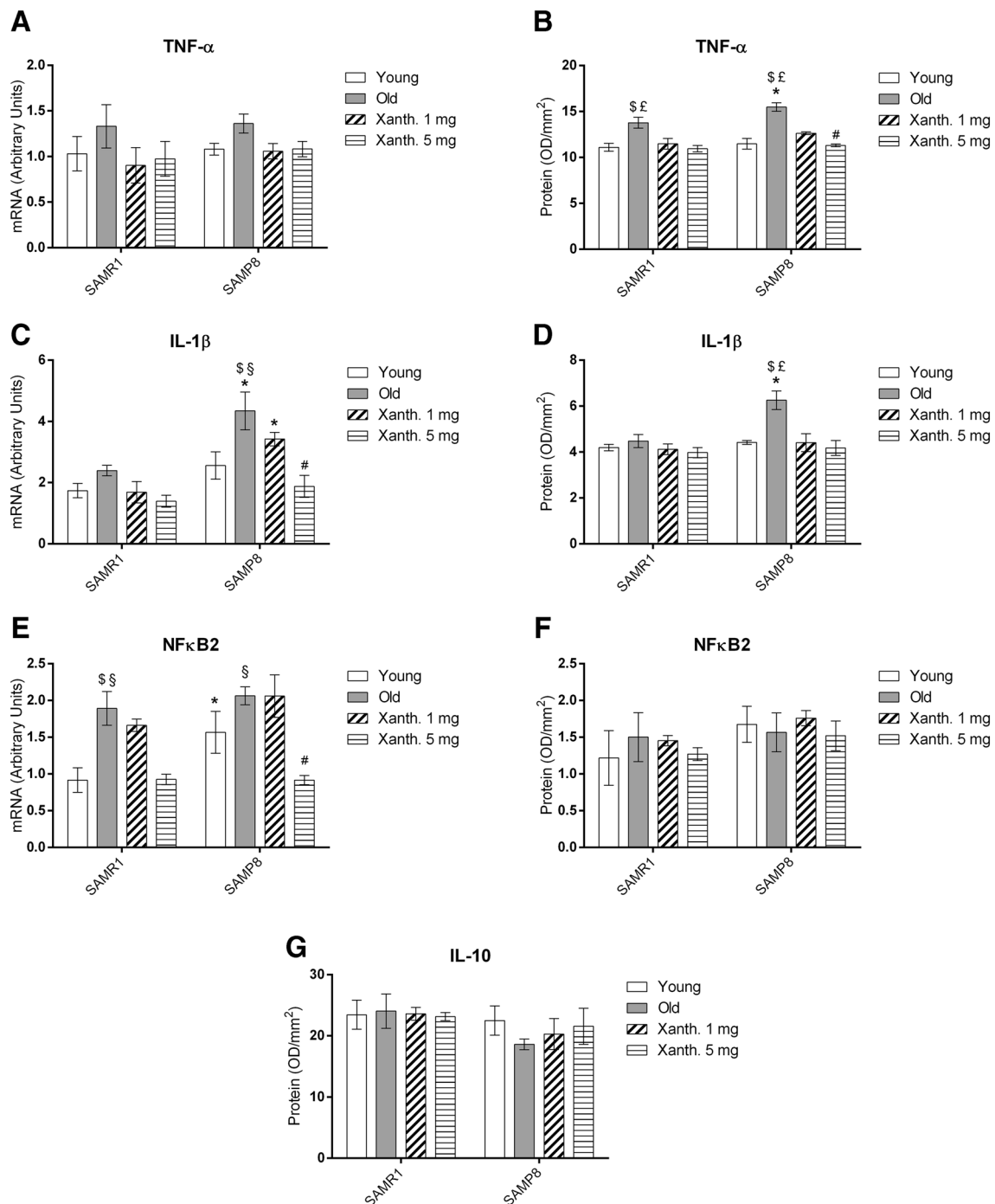


Fig. 1 mRNA and protein expressions of TNF- α (a, b), IL-1 β (c, d), NF κ B2 (e, f), and protein expression of IL-10 (g) in liver of young and old SAMR1 and SAMP8 mice non-treated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean \pm standard deviation of the number of determinations. * p < 0.05 with respect to values

obtained in their respective SAMR1 group; $^{\$}p$ < 0.05 with respect to values obtained in the young group; $^{\text{£}}p$ < 0.05 with respect to values obtained in the 1 and 5 mg XN-treated groups; $^{\#}p$ < 0.05 with respect to values obtained in the 1 mg XN-treated group; $^{\text{§}}p$ < 0.05 with respect to values obtained in the 5 mg XN-treated group

resulted in significantly lower levels of iNOS in SAMP8 animals, when compared to the old non-treated group (Fig. 5c).

Age-related changes were also found in the mRNA expression of HO-1 (Fig. 5d). Significantly higher levels

were found in the old SAMP8 group in comparison with SAMR1 animals. HO-1 mRNA expression of SAMP8 animals treated with 5 mg XN was significantly lower than the one observed in old non-treated animals (Fig. 5d).

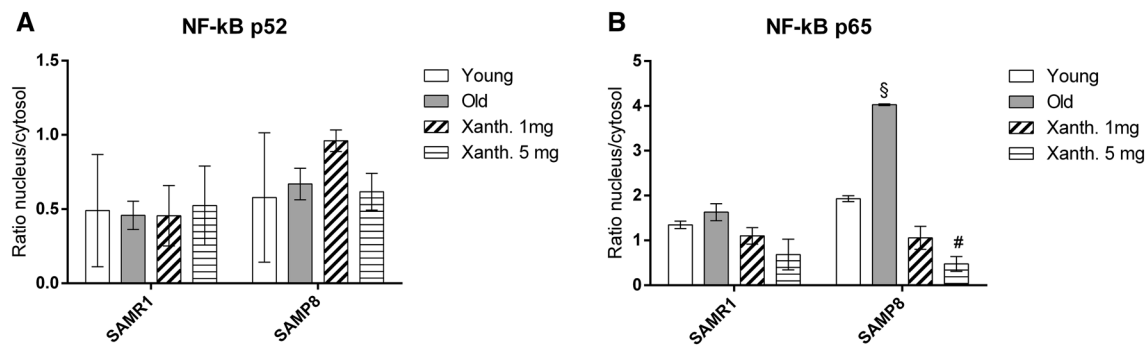


Fig. 2 Ratio of nucleus/cytoplasm fraction of NFκB p52 (a) and NFκB p65 (b) in liver of young and old SAMR1 and SAMP8 mice nontreated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean ± standard deviation of the number of determinations.

Finally, no age-related effect was found in the levels of the proliferation messenger PCNA and sirtuin 1 (Fig. 6a, b). XN did not provoke any change in the mRNA expression of both molecules.

Discussion

To determine whether XN exerted anti-inflammatory effects on age-related liver alterations, liver functional tests were performed. No significant alterations were observed suggesting that, in our study, the age-related hepatic alterations are not causing a hepatic dysfunction.

However, in our study, aging was responsible for a significant increase in TNF-α protein expression. TNF-α is a protein known to be critically involved in the regulation of infectious, inflammatory and autoimmune processes. Macrophages, such as the Kupffer cells, and T cells activate the production of TNF-α [20]. XN has been shown to inhibit inflammatory responses through the attenuation of nitric oxide (NO) release, or up-regulation of iNOS, IL-1β and TNF-α itself [21]. As stated earlier, XN was not able to diminish the transcription of the TNF-α gene, as no significant changes were shown after the mRNA amplification, but it did reduce the translation of the genetic material and, therefore a marked decrease was exhibited after the quantification of the protein expression by Western blotting, i.e., XN returned TNF-α levels to young mice values in both SAMP8 and SAMR1 groups. IL-1β, another pro-inflammatory mediator, is expressed in response to tissue damage. In our study, its protein and mRNA levels were increased in old SAMP8 animals. A well-known hypothesis establishes the capacity of IL-1β to induce liver fibrosis through the activation of HSCs [22].

The current study supports the idea of a decrease in TNF-α and IL-1β protein levels following XN treatment by inhibiting NF-κB protein expression. NF-κB survival

[#] $p < 0.05$ with respect to values obtained in the 1 mg XN-treated group; [§] $p < 0.05$ with respect to values obtained in the 5 mg XN-treated group

functions are accomplished through the activation of anti-apoptotic proteins and antioxidant genes. TNF-α and IL-1β act as negative regulators of NF-κB pathways.

While no significant differences were seen in NF-κB protein expression, it was observed that in SAMP8 old mice, the nucleus/cytoplasm ratio of NF-κB p65 was significantly reduced when treating with 5 mg XN [23]. Hence, an increase in pro-inflammatory mediators in aging may occur as a result of the increase in the two pro-inflammatory proteins analyzed (TNF-α and IL-1β) and of NF-κB translocation. Increased hepatic injury leads to stimulation of regenerative responses in progenitor cells and activation of Kupffer cells and the release of mediators such as IL-1β and TNF that intensify the hepatic injury. As it has been seen in previous studies with flavonones [16, 24], XN is thought to decrease levels of pro-inflammatory intermediaries and therefore, it could be helpful to reduce liver fibrosis and hepatic carcinoma due to aging.

While IL-1β and TNF-α are pro-inflammatory mediators, the function of IL-10 is predominantly anti-inflammatory. Reduced levels of IL-10 cytokine have been related to an increase in these other pro-inflammatory mediators. No significant changes in IL-10 were observed in this study, suggesting that the absence of IL-10 was associated with higher TNF-α and IL-1β levels, as it has been reported elsewhere [25–27].

Pro-apoptotic markers have been shown to increase with age. In this study, caspase 3 protein expression was seen to increase with aging showing an early activation of effector caspases. After XN treatment, its expression was diminished to young mice levels in SAMP8 group. This suggests an inhibition of aging-induced apoptosis by XN. Apoptosis plays an important role in the aging process. The rate of apoptosis is enhanced in most types of aging cell populations and organs as a protective mechanism against the accumulation of defective cells. However, the over-activation of the apoptosis pathway seems to be involved in most age-associated diseases [28]. Although apoptosis has

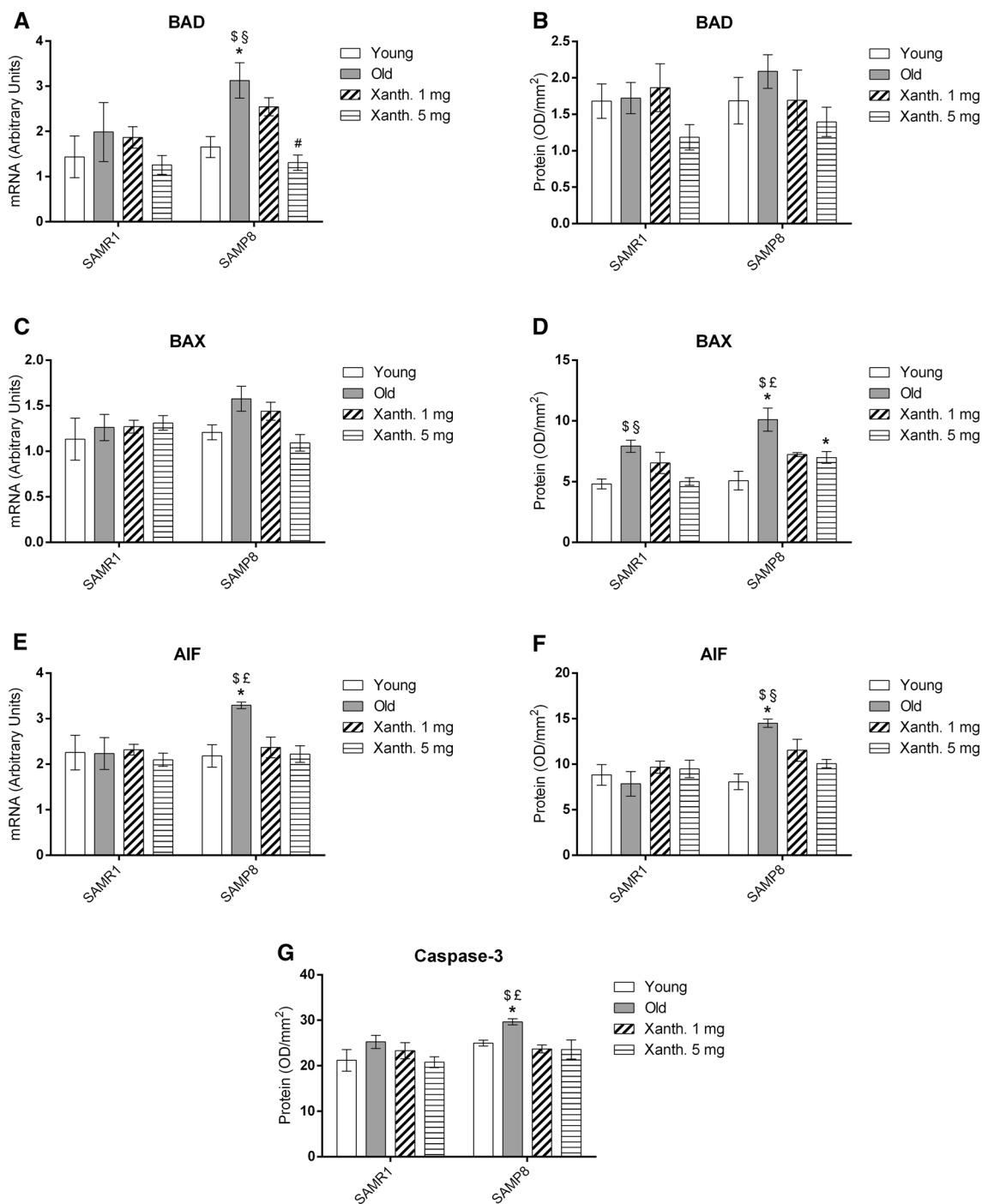


Fig. 3 mRNA and protein expressions of BAD (a, b), BAX (c, d), AIF (e, f), and protein expression of caspase-3 (g) in liver of young and old SAMR1 and SAMP8 mice non-treated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean \pm standard deviation of the number of determinations. * p < 0.05 with respect to values

obtained in their respective SAMR1 group; $^{\$}p$ < 0.05 with respect to values obtained in the young group; $^{\text{£}}p$ < 0.05 with respect to values obtained in the 1 and 5 mg XN-treated groups; $^{\#}p$ < 0.05 with respect to values obtained in the 1 mg XN-treated group; $^{\text{§}}p$ < 0.05 with respect to values obtained in the 5 mg XN-treated group

been recognized as one of the main processes to prevent cancer cells from growing in young subjects, its enhancement in aging is thought to be related with diseases such as cirrhosis or liver dysfunction [6].

BAX mRNA levels exhibited no remarkable change either with aging or after XN treatment. However, BAX protein expression was increased in old animals and XN exerted a protective effect. On the other hand, BAD mRNA levels

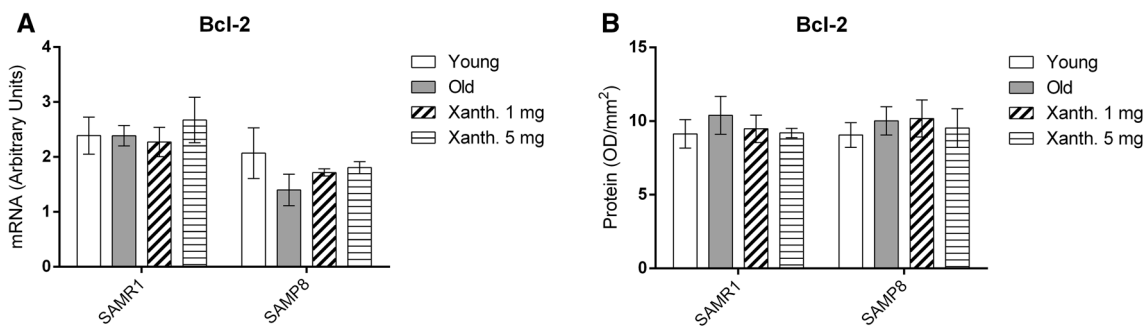


Fig. 4 mRNA (a) and protein (b) expressions of Bcl-2 in liver of young and old SAMR1 and SAMP8 mice non-treated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean ± standard deviation of the number of determinations

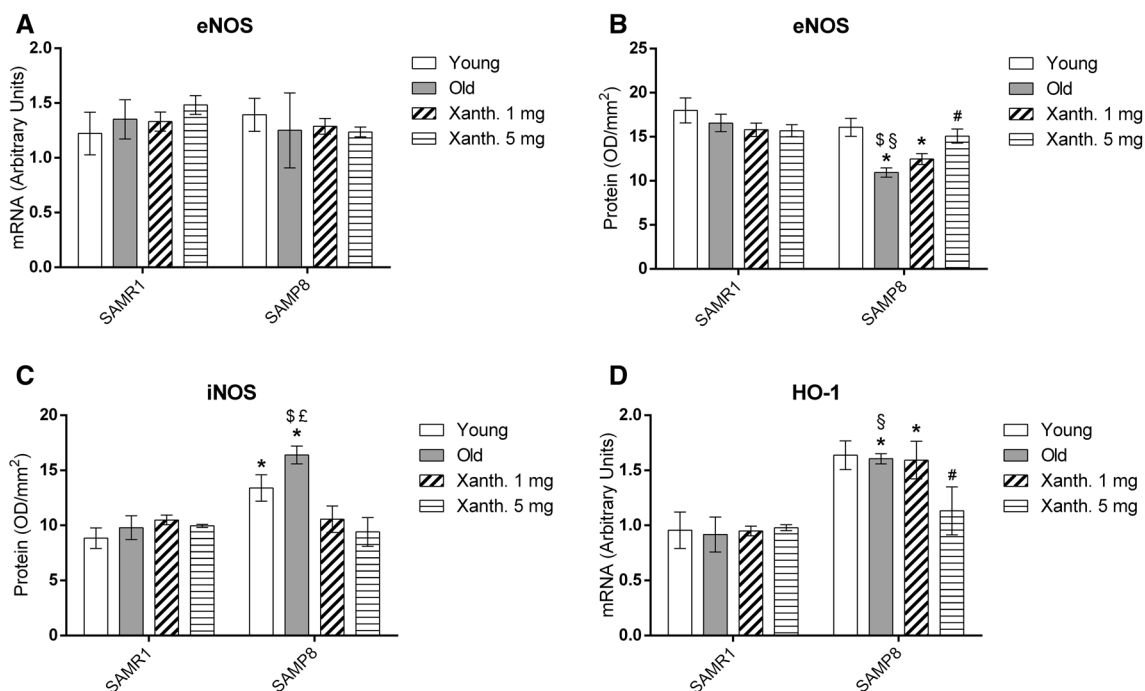


Fig. 5 mRNA (a) and protein (b) expressions of eNOS, protein expression of iNOS (c) and mRNA expression of HO-1 (d) in liver of young and old SAMR1 and SAMP8 mice non-treated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean ± standard deviation of the number of determinations. **p* < 0.05 with respect to values obtained in their respective SAMR1 group; \$*p* < 0.05 with

respect to values obtained in the young group; £*p* < 0.05 with respect to values obtained in the 1 mg and 5 mg XN-treated groups; #*p* < 0.05 with respect to values obtained in the 1 mg XN-treated group; §*p* < 0.05 with respect to values obtained in the 5 mg XN-treated group

did change with aging, i.e., its expression was increased but not its protein levels. This may be due to several processes including not only transcription and translation, but also RNA silencing, and the rate of RNA and protein degradation. XN, at a dose of 5 mg/kg/day, was able to reduce BAD mRNA levels. It is noticeable that while in old SAMP8 mice, the transcription of BAD became affected after XN treatment, only translation of BAX was reduced following the administration of the drug. Regarding, Bcl-2, and its closest relatives, Bcl-xL and Bcl-w, it has been reported that they induce survival instead of apoptosis. With age, a reduction

in its expression levels may be expected; however, this was not seen in this research work, pointing out the possibility of an imbalance between pro-apoptotic and anti-apoptotic Bcl-2 family members that leads either to apoptosis or survival. AIF is a flavoprotein that is normally located in the mitochondrion. After death stimuli, AIF translocates to the cytosol and to the nucleus where it participates in the induction of apoptosis. Nuclear chromatin condensation and DNA fragmentation are among its main functions [29]. Here, both AIF transcription and translation were up-regulated

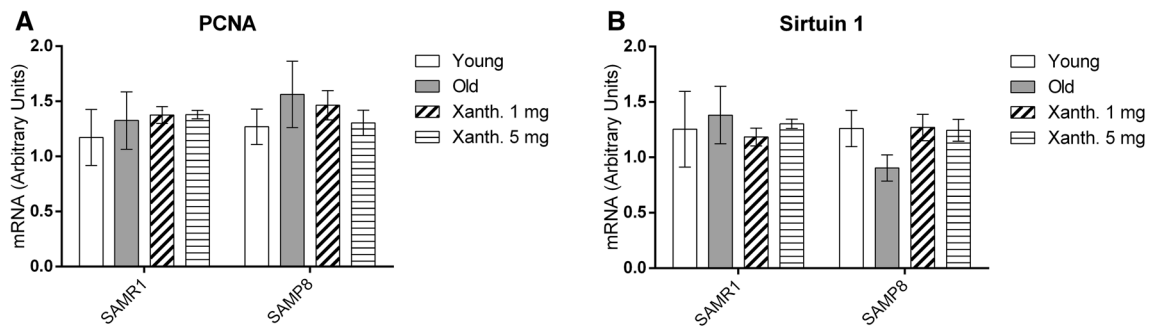


Fig. 6 mRNA expression of PCNA (**a**) and sirtuin 1 (**b**) in liver of young and old SAMR1 and SAMP8 mice non-treated and treated with 1 mg or 5 mg/kg/day XN. Data are expressed as mean \pm standard deviation of the number of determinations

with aging and showed a marked reduction following XN treatment.

To investigate the involvement of XN in cellular oxidative stress, eNOS, iNOS and HO-1 were tested as mediators of this process. Vascular function mainly depends on the balance between synthesis/degradation of NO, which is supported by the normal activity of eNOS. An increase in the synthesis of NO is due to an enhanced production by iNOS which causes vascular dysfunction. While NO produced by eNOS is a result of a normal metabolic response and regulates blood flow and blood cell interactions, iNOS activation has some detrimental effects for the liver function [30]. This background may explain why eNOS mRNA levels showed no significant change with increasing age despite the fact that protein expression was reduced with aging. Opposite to this, iNOS protein levels increased with aging, suggesting an induction in vascular dysfunction, and they diminished after XN treatment.

It has been demonstrated that HO-1, a stress protein whose aim is to degrade heme, is up-regulated by IL-1 and TNF- α as a consequence of hypoxia, oxidative stress, etc., and it is thought to contribute to the anti-proliferative and anti-apoptotic effects mediated by NO [31]. Hypoxia and oxidative stress become more important in liver aging, therefore, HO-1 increases to prevent progression of liver fibrosis. In our study, XN was able to decrease the HO-1 levels in old SAMP8 mice but only at the dose of 5 mg/kg/day.

Finally, the possible relationship between XN, proliferation and survival was addressed. PCNA plays an important role in nucleic acid metabolism being essential for DNA replication, DNA excision repair, as well as chromatin assembly [32]. PCNA analysis showed no significant differences between age groups either in SAMP8 or in SAMR1 animals suggesting that XN might not affect cell cycle pathways. Sirtuin 1 has been found to have a large number of functions apart from deacetylating histones that confers resistance to cellular stress: it acts as a life-extender by decreasing ROS levels as well [33]. In spite of this, in our study, sirtuin 1 showed no significant change.

In conclusion, liver from old SAMP8 mice showed a significant augmentation in its pro-oxidative, pro-inflammatory and pro-apoptotic state, in contrast with young SAMP8 mice. A similar effect was observed in old SAMR1 mice but to a lesser extent. XN was able to revert most of these effects, significantly reducing apoptosis, inflammatory and oxidative markers. This molecule may contribute to the counteraction of age-related changes in the liver and therefore may act as a protective compound in liver alterations.

Acknowledgements The authors would like to thank Medicine students Paula Corral, Bryan Hyacinthe, and Mario Calvo-Soto (School of Medicine, Complutense University of Madrid, Spain) for their continued interest and cooperation in our work. The skillful technical assistance of Rocío Campón (Dept. of Physiology, School of Medicine, Complutense University of Madrid, Spain) is also gratefully acknowledged.

Author contributions EV, JAFT designed the research; CFG, LR, SDP, CM conducted the research; EV, MF analyzed the data; CFG, LR, SDP, EV wrote the paper. All authors read and approved the final manuscript.

Funding This study was supported by Grants from Red de Fragilidad y Envejecimiento RETICEF (RD12/0043/0032) and GRUPOS UCM-BSCH (GR35/10-A).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

References

1. Sanz A, Stefanatos RK (2008) The mitochondrial free radical theory of aging: a critical view. *Curr Aging Sci* 1(1):10–21
2. Schmucker D (2005) Age-related changes in liver structure and function: implications for disease? *Exp Gerontol* 40(8–9):650–659
3. Preedy V (2014) Aging. Chapter 1. Oxidative stress and frailty: a closer look at the origin of a human aging phenotype
4. Aikata H, Takaishi H, Kawakami Y, Takahashi S, Kitamoto M, Nakanishi T et al (2000) Telomere reduction in human liver tissues with age and chronic inflammation. *Exp Cell Res* 256(2):578–582

5. Jaskelioff M, Muller F, Paik J, Thomas E, Jiang S, Adams A, Sahin E, Kost-Alimova M, Protopopov A, Cadiñanos J, Horner J, Maratos-Flier E, DePinho R (2010) Telomerase reactivation reverses tissue degeneration in aged telomerase-deficient mice. *Nature* 469(7328):102–106
6. Aravinthan A, Alexander G (2016) Senescence in chronic liver disease: is the future in ageing? *J Hepatol* 65:825–834
7. Pinto C, Cestero J, Rodríguez-Galdón B, Macías P (2014) Xanthohumol, a prenylated flavonoid from hops (*Humulus lupulus* L.), protects rat tissues against oxidative damage after acute ethanol administration. *Toxicol Rep* 1:726–733
8. Vanhoecke B, Derycke L, Van Marck V, Depypere H, De Keuleire D, Bracke M (2005) Antiinvasive effect of xanthohumol, a prenylated chalcone present in hops (*Humulus lupulus* L.) and beer. *Int J Cancer* 117(6):889–895
9. Zhang B, Chu W, Wei P, Liu Y, Wei T (2015) Xanthohumol induces generation of reactive oxygen species and triggers apoptosis through inhibition of mitochondrial electron transfer chain complex I. *Free Radical Biol Med* 89:486–497
10. Ungvari Z, Kaley G, de Cabo R, Sonntag WE, Csiszar A (2010) Mechanisms of vascular aging: new perspectives. *J Gerontol Ser A Biol Sci Med Sci* 65(10):1028–1041
11. Takeda T, Hosokawa M, Takeshita S, Irino M, Higuchi K, Matsushita T et al (1981) A new murine model of accelerated senescence. *Mech Ageing Dev* 17(2):183–194
12. Yagi H, Katoh S, Akiguchi I, Takeda T (1988) Age-related deterioration of ability of acquisition in memory and learning in senescence accelerated mouse: SAM-P/8 as an animal model of disturbances in recent memory. *Brain Res* 474(1):86–93
13. Tresguerres JA, Kireev R, Forman K, Cuesta S, Tresguerres AF, Vara E (2012) Effect of chronic melatonin administration on several physiological parameters from old wistar rats and SAMP8 mice. *Curr Aging Sci* 5(3):242–253
14. Paredes SD, Forman KA, García C, Vara E, Escames G, Tresguerres JA (2014) Protective actions of melatonin and growth hormone on the aged cardiovascular system. *Hormone Mol Biol Clin Investig* 18(2):79–88
15. Puig A, Rancan L, Paredes SD, Carrasco A, Escames G, Vara E et al (2016) Melatonin decreases the expression of inflammation and apoptosis markers in the lung of a senescence-accelerated mice model. *Exp Gerontol* 75:1–7
16. Rancan L, Paredes SD, García I, Muñoz P, García C, López de Hontanar G, de la Fuente M, Vara E, Tresguerres JAF (2017) Protective effect of xanthohumol against age-related brain damage. *J Nutr Biochem* 49:133–140
17. Montoliu C, Vallés S, Renau-Piqueras J, Guerri C (2002) Ethanol-induced oxygen radical formation and lipid peroxidation in rat brain: effect of chronic alcohol consumption. *J Neurochem* 63(5):1855–1862
18. Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty something years on. *Nat Protoc* 1:581–585
19. Shmittgen DT, Livak KJ (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta$ CT method. *Methods* 25:402–408
20. Pasparakis M, Alexopoulou L, Episkopou V, Kollias G (1996) Immune and inflammatory responses in TNF alpha-deficient mice: a critical requirement for TNF alpha in the formation of primary B cell follicles, follicular dendritic cell networks and germinal centers, and in the maturation of the humoral immune response. *J Exp Med* 184(4):1397–1411
21. Lee IS, Lim J, Gal J, Kang JC, Kim HJ, Kang BY et al (2011) Anti-inflammatory activity of xanthohumol involves heme oxygenase-1 induction via NRF2-ARE signaling in microglial BV2 cells. *Neurochem Int* 58(2):153–160
22. Gieling R, Wallace K, Han Y (2009) Interleukin-1 participates in the progression from liver injury to fibrosis. *Am J Physiol Gastrointest Liver Physiol* 296(6):G1324–G1331
23. Tak P, Firestein G (2001) NF- κ B: a key role in inflammatory diseases. *J Clin Investig* 107(1):7–11
24. Peluso I, Raguzzini A, Serafini M (2013) Effect of flavonoids on circulating levels of TNF-alpha and IL-6 in humans: a systematic review and meta-analysis. *Mol Nutr Food Res* 57(5):784–801
25. Garcia-Lafuente A, Guillamon E, Villares A, Rostagno MA, Martinez JA (2009) Flavonoids as anti-inflammatory agents: implications in cancer and cardiovascular disease. *Inflamm Res* 58(9):537–552
26. Paredes SD, Rancan L, Kireev R, González A, Louzao P, González P, Rodríguez-Bobada C, García C, Vara E, Tresguerres J (2015) Melatonin counteracts at a transcriptional level the inflammatory and apoptotic response secondary to ischemic brain injury induced by middle cerebral artery blockade in aging rats. *BioRes Open Access* 4(1):407–416
27. Meador BM, Krzysztoson CP, Johnson RW, Huey KA (2008) Effects of IL-10 and age on IL-6, IL-1beta, and TNF-alpha responses in mouse skeletal and cardiac muscle to an acute inflammatory insult. *J Appl Physiol* 104(4):991–997
28. Lu B, Chen H, Lu HG (2012) The relationship between apoptosis and aging. *Adv Biosci Biotechnol* 3:705–711
29. Dugas E, Nochy D, Ravagnan L, Loeffler M, Susin SA, Zamzami N et al (2000) Apoptosis-inducing factor (AIF): a ubiquitous mitochondrial oxidoreductase involved in apoptosis. *FEBS Lett* 476(3):118–123
30. Cau SB, Carneiro FS, Tostes RC (2012) Differential modulation of nitric oxide synthases in aging: therapeutic opportunities. *Front Physiol* 3:218
31. Schipper HM (2000) Heme oxygenase-1: role in brain aging and neurodegeneration. *Exp Gerontol* 35(6–7):821–830
32. Kelman Z (1997) PCNA: structure, functions and interactions. *Oncogene* 14(6):629–640
33. Guarente L (2007) Sirtuins in aging and disease. *Cold Spring Harb Symp Quant Biol*:483–488