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Impact of N-acetylcysteine and sesame oil on lipid metabolism and hypothalamic-pituitary-adrenal axis homeostasis in middle-aged hypercholesterolemic mice

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Hyperlipidemia and stress are important factors affecting cardiovascular health in middle-aged individuals. We investigated the effects of N-acetylcysteine (NAC) and sesame oil on the lipidemic status, liver architecture and the hypothalamic-pituitary-adrenal (HPA) axis of middle-aged mice fed a cholesterol-enriched diet. We randomized 36 middle-aged C57bl/6 mice into 6 groups: a control group, a cholesterol/cholic acid diet group, a cholesterol/cholic acid diet group with NAC supplementation, a cholesterol/cholic acid diet enriched with 10% sesame oil and two groups receiving a control diet enriched with NAC or sesame oil. NAC administration prevented the onset of the disturbed lipid profile, exhibiting decreased lipid peroxidation and alkaline phosphatase (ALP) levels, restored nitric oxide bioavailability and reduced hepatic damage, compared to non-supplemented groups. High-cholesterol feeding resulted in increased hypothalamic glucocorticoid receptors (GR) levels, while NAC supplementation prevented this effect. NAC supplementation presented significant antioxidant capacity by means of preventing serum lipid status alterations, hepatic damage, and HPA axis disturbance due to high-cholesterol feeding in middle-aged mice. These findings suggest a beneficial preventive action of plant-derived antioxidants, such as NAC, on lipid metabolism and on the HPA axis.

Hyperlipidemia is considered to be one of the most important Cardiovascular Disease (CVD) risk factors, while exercise, pharmacotherapy, diet and dietary supplements are often employed to regulate blood lipid levels[1]. It is a common place that CVD risk factors exhibit complex interplays or synergistic actions. For instance, oxidative stress is involved in the pathogenesis of hypercholesterolemia[2], while biological stress also plays a significant role in the susceptibility, progress, and outcome of CVD[3]. On the other hand, there is also a connection between biological stressors and increased production of free radicals and oxidative damage[4].

A "stressor" can be defined as an actual or perceived threat to an organism, while the response to a stressor is known as a "stress response"[5]. Malnutrition during pregnancy, separation of pups from their mothers[5], and modifications of dietary fat content are all well-established biological stressors commonly investigated in experimental studies related to the hypothalamic-pituitary-adrenal (HPA) axis function[6].

The activation of the HPA axis that occurs in response to disruptions of homeostasis or stressors, seems to result in an increase of circulating glucocorticoids[7]. Glucocorticoid receptors (GR) mediate the effects of adrenal steroids both in peripheral organs and the brain. High-fat diets are known to increase basal HPA axis activity and

might influence the central stress response[5,8,9]. Apart from regulating stress response, glucocorticoids also control metabolic resources and are causatively linked to the metabolic syndrome[10].

Literature data indicate numerous natural agents that could be potentially useful in the treatment of hyperlipidemia. In the last decade, nutraceuticals and functional foods have attracted great scientific interest, with a number of clinical trials and animal studies addressing their potential role in blood lipid control and/or cardioprotection[11]. For instance, plant and fish-derived oils or fatty acids (e.g., olive oil, canola oil, n3 fatty acids, plant stanols/sterols) as well as antioxidants (e.g., polyphenols) are rigorously researched for their beneficial effects on blood lipid levels and cardiovascular health[12–16].

The beneficial hypocholesterolemic and/or antioxidant effects of two plant-derived substances: N-acetylcysteine (NAC) [a cysteine-containing compound of Allium plants such as garlic and onion] and sesame oil in young mice (12 weeks old) have been reported in a previous study from our group[17]. Cardiovascular and metabolic deregulation is linked to advancing age in humans[18], while the impact of age on different blood parameters and metabolic pathways was examined by our group using a murine model of diet-induced hypercholesterolaemia and metabolic syndrome[19]. Older primates and rodents fed an atherogenic diet develop more extensive atherosclerotic damages compared to younger animals receiving the same diet[20]. Older animals appear to model more accurately age-related conditions. However, there is a paucity concerning studies assessing the effect of these functional substances in older animals, which are more prone to metabolic deregulation and CVD.

The aim of the present study was to assess the possible beneficial metabolic and antioxidant properties of these two plant-derived agents when administered to middle-aged mice, and to study their effect on disturbed hepatic function resulting from a high-cholesterol diet. Additionally, we investigated the consequences of high-cholesterol feeding on the HPA axis, as well as the impact of NAC and sesame oil administration on this system.

Results

Serum lipid parameters. No significant differences could be recorded between the groups at baseline measurements. Significant main effects of diet and NAC administration on total cholesterol (diet: $p < 0.001$, and NAC administration: $p = 0.006$), as well as on LDL cholesterol levels (diet: $p < 0.001$, and NAC administration: $p = 0.016$) were observed at T_1. The interaction between diet × NAC was also significant ($p = 0.01$ for total cholesterol and $p = 0.002$ for LDL cholesterol levels at T_1).

LDL cholesterol levels were significantly elevated in the high-cholesterol fed groups compared to their respective chow-fed groups: High-cholesterol group (HC) versus Normal chow group (NC) ($p < 0.001$); High-cholesterol group with NAC supplementation (HCN) versus Normal chow group with NAC supplementation (NN) ($p < 0.001$); and High-cholesterol group with sesame oil supplementation versus Normal chow group with sesame oil supplementation (NS) ($p = 0.022$) (Table 1). Total and LDL cholesterol levels were significantly decreased in the HCN group in comparison to HC group ($p < 0.05$).

HDL cholesterol levels were significantly lower in HC and HCN groups compared to NC and NN groups, respectively (Table 1). No significant differences were observed between HCS group with HC and NS groups ($p > 0.05$ in all cases); whereas triglyceride levels were reduced in the three cholesterol-fed groups when compared to their respective controls ($p < 0.05$).

The observed differences between the study groups remained significant after controlling for baseline measurements.

Serum lipid peroxidation – parameters of the nitric-oxide pathway. At the end of the experiment, serum lipid peroxidation was increased in the HC group compared to the control group ($p = 0.041$), while serum peroxides were decreased in the HCN group when compared to the HC group ($p = 0.015$). No significant differences were detected between HCS and HC groups ($p = 0.089$).

A significant effect of diet ($p = 0.002$), sesame oil supplementation ($p = 0.001$), NAC administration ($p = 0.003$), and diet × NAC interaction ($p = 0.001$) was observed concerning serum Nitrate ($NO3-$) levels. Animals belonging to the HCN group had increased Nitrate levels as compared to animals belonging to NN ($p < 0.001$), HC ($p < 0.001$) and NC groups ($p < 0.001$).

No significant differences could be observed between groups regarding serum levels of Nitrite ($NO2-$) and total Nitrate/Nitrite (NO) (Figure 1).

Liver and aorta histopathology. Hematoxylin-eosin-stained liver samples obtained from groups fed a high-cholesterol diet showed signs of hepatic steatosis and ballooning (Figure 2) with a significant increase in accumulation of fat droplets as compared to the chow-fed groups [HC vs NC ($p = 0.008$), HCN vs NN ($p = 0.002$) and HCS vs NS ($p = 0.002$)] (Table 2). However, the grade of steatosis in HCN mice was significantly reduced compared to mice in the HC group ($p = 0.042$). The architecture of hepatocytes in the HCN group was found to be similar to that in the NC group, exhibiting widely different morphological characteristics when compared to hepatocytes derived from HC or HCS groups (Figure 2).

Neither NAC nor sesame oil administration significantly reduced ballooning, hepatic inflammation or lobular activity. Fatty streaks or plaques were not observed in the aortic tissues of any of the experimental mice (data not shown).

Hepatic enzymes. Significantly elevated alkaline phosphatase (ALP) and serum glutamic oxaloacetic transaminase (SGOT) serum levels were observed in the HC group compared to the NC group ($p < 0.001$ in both cases). In the HCN group, ALP and SGOT serum concentrations were significantly increased as compared to the NC group ($p < 0.001$ in both cases). ALP levels were found significantly higher in the HCN group compared to the NN group ($p = 0.003$) and significantly lower than in the HC group ($p = 0.011$). HCS mice exhibited increased SGOT levels in comparison to the NS group ($p < 0.001$) (Figure 3).

Plasma corticosterone levels and GR levels in the hypothalamus. No significant differences were observed in plasma corticosterone levels between the study groups.

Notably, a significant increase was observed in GR-protein level in the hypothalamus of HC-fed animals as compared to the NC group ($p = 0.033$). This increase was reversed in the HCN group whose GR level was lower than in the HC group ($p = 0.033$) (Figure 4).

Discussion

Epidemiological data indicate that ageing increases the risk of various pathological conditions related to cholesterol[21], with cardiovascular-event rates increasing in a curvilinear fashion after age 65 in men and 75 in women[18]. The identification of safe, effective and cost-effective protective agents has gained significant scientific attention. Functional foods/nutraceuticals such as fish, chocolate, green tea and also some vegetables and spices are known to exhibit beneficial effects on cardiovascular health. Garlic, oily seeds and fish oils are known to have lipid-lowering effects in humans, inhibiting fat absorption and/or suppressing hepatic cholesterol synthesis[22].

The present study focused on the potential hypolipidemic and hepatoprotective activity of NAC and sesame oil in middle-aged mice. Although adverse reactions to NAC are described in the available literature, these are in most cases dose-dependent and rarely serious[23], while sesame oil can be safely consumed.

We observed that the cholesterol/cholic acid diet increased total and LDL cholesterol levels in mice. NAC supplementation resulted in a significant decrease of serum lipid levels, with a simultaneous

Table 1 | Body weight, food consumption and serum lipid levels. Body weight (g) at the beginning and at the end of the experimental study, food consumption levels (g/day), total cholesterol (mg/dL), LDL-cholesterol (mg/dL), HDL-cholesterol (mg/dL) and triglycerides levels (mg/dL) in serum before (T_0) and after the 8-week experimental period (T_1).

Body weight, food consumption and serum lipid levels													
		NC		HC		HCN		HCS		NN		NS	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body Weight (g)	T_0	27	1.15	27.66	1.50	28.67	1.03	29	1.15	27.66	1.96	27.67	0.81
	T_1	27.5	1.00	27	1.67	27	1.09	28.5	1.00	27.66	0.82	27.33	1.63
Food Consumption (g/day)		3.80	0.30	3.99	0.41	4.13	0.52	3.76	0.43	4.66	0.30	3.96	0.41
Total cholesterol (mg/dL)	T_0	75.00	6.28	75.33	3.50	72.50	3.50	72.66	15.99	75.66	13.06	61.00	21.34
	T_1	73.62	11.35	171.00	33.94	121.33	17.70	167.40	72.72	71.83	19.97	80.80	13.53
LDL-cholesterol (mg/dL)	T_0	29.52	11.96	37.27	2.22	36.00	2.47	36.03	9.39	39.77	8.84	22.60	13.79
	T_1	21.95	8.05	145.40	33.84	97.03	18.50	145.04	71.01	31.63	16.80	41.28	7.06
HDL-cholesterol (mg/dL)	T_0	36.50	4.75	31.00	2.37	29.33	1.50	29.17	6.43	29.00	4.09	28.20	9.36
	T_1	39.37	12.62	18.00	2.00	17.67	1.21	18.20	5.63	29.17	7.73	28.80	9.44
Triglycerides (mg/dL)	T_0	44.87	18.52	35.33	5.28	35.83	3.06	37.33	7.39	34.50	5.54	51.00	14.00
	T_1	61.50	8.99	38.00	8.41	33.17	11.88	20.80	3.27	55.17	7.03	53.60	17.12

NC, Control mice; HC, mice fed high cholesterol diet; HCN, mice fed high cholesterol diet and treated with NAC; HCS, mice fed high cholesterol diet enriched with sesame oil; NN, mice fed control diet and treated with NAC; NS, mice fed control diet enriched with sesame oil. Values are presented as means ± 1 standard deviation (SD).

decrease of their peroxidation. The hypolipidemic effects of NAC are also documented in studies utilizing young rodents on high-fat diets[24,25].

The present study shows that NAC can be used to improve serum lipid levels, with its beneficial effects exhibiting possible connections to its anti-oxidant properties[26]. The lipid-lowering action of NAC in experimental animal models of hyperlipidemia can be partially attributed to the suppression of mRNA expression of lipogenic-related enzymes[24]. In addition, in an experimental mouse model of high-sucrose diet feeding, NAC prevented the metabolic shifting in cardiac tissue, enhancing fatty acid oxidation[27]. Furthermore, the maintenance of the normal structure of lipoprotein receptors is indispensable for their function, facilitating the cellular uptake of serum lipids from the blood. On the other hand, reactive oxygen species oxidize lipoproteins and inhibit lipid intracellular uptake[28–30]. It is possible that the decreased serum cholesterol levels in mice fed a NAC-supplemented diet are due to the antioxidative effects of NAC. However, there are no mechanistic studies available that show clearly whether these effects are incidental or causal to the lipid-lowering effects of NAC.

NAC supplementation exhibited differential effects on serum peroxides in control groups in comparison to the high-cholesterol group. This result could be observed only in our study comprising older animals, rather than in our previous reports focusing on younger mice. Notably, age seems to affect NAC's selective antioxidant action, which is restored in the metabolically-challenging environment of the high-cholesterol fed mice. This might be a new aspect of NAC's function and further studies are required to investigate the involved mechanisms in this dual action.

According to the results of our study, NAC supplementation also prevents the anticipated extensive hepatic damage produced by high-fat, high-cholesterol diets. These results are consistent with the decreased levels of ALP activity in cholesterol-fed mice receiving NAC supplementation. In our previous study involving young mice[17], we observed a clear improvement of liver steatosis and ballooning in the high-cholesterol fed group treated with NAC that resulted in levels comparable to controls. However, in the present study, we did not observe a similar degree of improvement in middle-aged mice. In addition, NAC failed to restore ALP levels towards normalcy. Despite the fact that the liver has a remarkable ability to regenerate and respond to stress, these capabilities are gradually reduced with age[31,32]. Liver volume, blood flow and number of hepatocytes decrease as the liver ages[33]. More importantly, the reduced activity of multiple hepatic enzymic systems[32], in addition to the aforementioned changes, modifies the capacity of hepatic tissue to resist any potential injury as it ages[34].

Furthermore, younger mice seem to adapt better to metabolic alterations caused by high-cholesterol feeding. The fact that NAC did not totally prevent liver damage can be attributed to the influence of age on the severity of hepatic injury caused by the hypercholesterolemic diet or to the higher dose of NAC needed to be administered to older animals in order to exhibit a comparable protective effect. Quantitative intrahepatic lipid measurements and/or qPCR measurements of relevant gene markers, including IL1B, IL6, TNFalpha, CD68, Col1A and Emr1 could provide a more detailed understanding of pathophysiological differences between the studied groups.

In our study, NAC-treated animals exhibited a significantly reduced degree of steatosis and ameliorated hepatocyte architecture, compared to high-cholesterol fed mice. However, NAC administration did not return liver function markers to normal levels. Even though a discrepancy between liver function markers and histopathology findings is commonly reported in the available literature, we cannot exclude that liver function might be still impaired. Similar to our findings, the co-administration of NAC and metformin in patients with non-alcoholic steatohepatitis did not alter aspartate aminotransferase, gamma-glutamyl transferase and alkaline phosphatase levels regardless of a significant decrease in hepatic steatosis and fibrosis[35]. In addition, despite the amelioration in serum ALT levels, no significant alterations in ALP and SGOT levels could be recorded after a three-month period administration of N-acetylcysteine in patients with non-alcoholic fatty liver steatosis[36].

Only a limited number of relevant studies have been performed in female mice. Ivanovski reported that NAC was capable of reducing atheroma progression in female apoE (−/−) mice with uremia-enhanced atherosclerosis, probably via a decrease in oxidative stress[37]. In addition, sesamin exhibited significant hypolipidemic effects in LDL-receptor deficient female mice[38].

In our study, sesame oil failed to exert an analogous effect to that of NAC among high-fat fed animals. Although previous studies suggest that sesame oil can exhibit hypolipidemic properties in experimental animal models fed high-fat diets; this was not the case in our study[39].

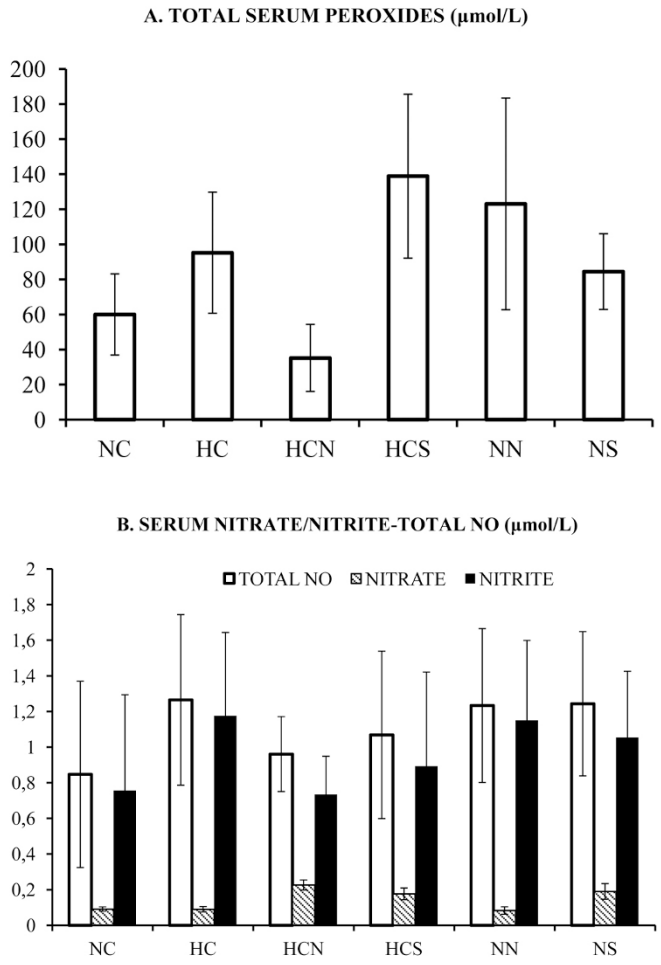

Figure 1 | Total serum Peroxides (µmol/L) (A) and Nitrate, Nitrite (endogenous) and Total Nitrate/Nitrite (NO) levels (µmol/L) (B) at the end of the 8-week experimental period. (NC), Control mice; (HC) mice fed high cholesterol diet; (HCN) mice fed high cholesterol diet and treated with NAC; (HCS) mice fed high cholesterol diet enriched with sesame oil; (NN) mice fed control diet and treated with NAC and (NS) mice fed control diet enriched with sesame oil.

In this context, we can possibly assume that the dietary cholesterol abolished the ability of sesame oil to improve the lipidemic status of high-fat fed animals. This finding was also observed in our previous study using younger animals[17].

The failure of sesame oil to exert a hypolipidemic activity might be related to the increased total caloric intake of animals fed with sesame oil-supplemented high-cholesterol diets[17]. On the other hand, a previous study of albino rats fed a hypercholesterolemic diet showed improved antioxidant status and significant decrease of plasma lipid concentration following non-defatted sesame powder administration[40]. The study concluded that these beneficial effects can be attributed to fiber, sterol, polyphenol and flavonoid contents of sesame seeds, which were identified as enhancing fecal-cholesterol excretion and bile-acid production, and increased antioxidant enzyme activities. However, the wide range of different substances present in sesame powder compared to sesame oil does not permit the derivation of the possible mechanisms involved in these differences.

The release of nitric oxide (NO) by healthy vascular endothelium prevents the adherence of platelets and leukocytes to the arterial wall, thus limiting the risk for athero-thrombotic processes[41]. In our study, we observed significantly higher levels of NO-end products (nitrate) in cholesterol-fed mice treated with NAC, compared to the non-treated cholesterol-fed group. In a previous study, researchers suggested that NAC intake increased serum nitrite and nitrate levels in fenthion treated mice[42]. The authors attributed this effect to either an activation of the NO synthase or to the inhibition of NO degradation and concluded that the protective effect of NAC against oxidative stress might be related to restoration of NO availability.

Modifications of the administered diet may lead to changes in neuroendocrine and metabolic responses. High-fat feeding may influence the organism's response to stress[7,43]. In our study, high-cholesterol feeding induced an increase in hypothalamic GR levels, which are considered to be the main mediators of the negative feedback of corticosteroids on HPA axis activation; thus implying an enhanced capacity for terminating a stress response[44]. Given that GRs in the brain are also implicated in the central energy expenditure and feeding control, this alteration might result in a consequent potentiation of orexigenic signals within the hypothalamus of these mice[45]. In this context, high-energy and high-fat diets have been reported to increase the expression of GR in mouse hippocampus[46,47].

High-fat feeding increases circulating free fatty acids (FFA), presenting a stimulatory effect on the HPA axis[45,48]. It is probable that

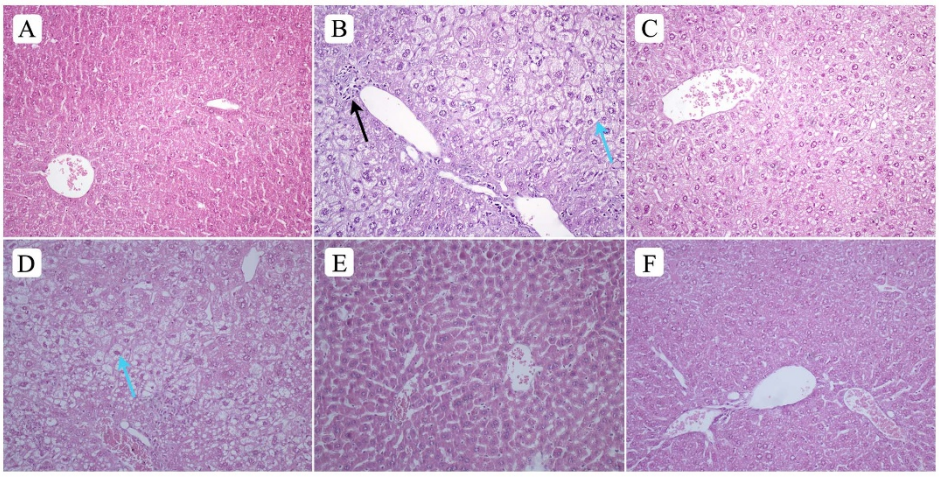

Figure 2 | **Haematoxylin and eosin staining of hepatic tissue (400×).** Control mice (A); mice fed high cholesterol diet (B); mice fed high cholesterol diet and treated with NAC (C).; mice fed high cholesterol diet enriched with sesame oil (D); mice fed control diet and treated with NAC (E) and mice fed control diet enriched with sesame oil (F). Turquoise arrows in B and D indicate hepatocyte lipid inclusion (steatosis) and ballooning. Black arrow in B indicates lobular activity.

Table 2 | Histological observations of hepatic tissue. 3, severe; 2, moderate; 1, mild; 0, no changes in histology (scores)

Histopathological evaluation of hepatic tissue

Group	Steatosis -Median (interquartile range)	Ballooning –Median (interquartile range)	Portal inflammation-Median (interquartile range)	Lobular activity –Median (interquartile range)
NC	0.5 (1)	0 (0)	0 (0)	0 (0)
HC	2.5 (1)	2 (1.25)	0 (1)	1 (0.5)
HCN	1.5 (2)	1 (0.25)	0.5 (1)	1 (1.25)
HCS	3 (0.25)	2 (1.25)	1 (2.25)	2 (1.25)
NN	0 (0)	0 (0)	0 (0)	0 (0)
NS	0 (0)	0 (0)	0 (0)	0 (0.5)

NC, Control mice; HC, mice fed high cholesterol diet; HCN, mice fed high cholesterol diet and treated with NAC; HCS, mice fed high cholesterol diet enriched with sesame oil; NN, mice fed control diet and treated with NAC; NS, mice fed control diet enriched with sesame oil.

the alterations observed in the lipidemic status of cholesterol-fed mice or among mice consuming cholesterol plus sesame oil, might be responsible for the increased hypothalamic GR levels through a similar mechanism. Subsequently, the observed preventive action of NAC on hypothalamic GR levels might be explained by the lipid-lowering properties of this agent. Although there are no available studies investigating the impact of NAC or sesame oil on the axis of stress, other antioxidants are known to have protective properties against brain damage induced by mental or physical stress[49].

In humans, NAC is considered to be a safe plant-derived pharmaceutical agent. A recent experimental study suggested that combination therapy of NAC with statin and angiotensin II (AngII) type 1 (AT1) receptor blocker (ARB) should be clinically evaluated as a pharmaceutical alternative in order to prevent oxidative stress, hypertension, cardiac hypertrophy, heart failure, morbidity and mortality arising from cardiac pathology[50]. Nutraceutical supplements such as NAC, in combination with a lipid-lowering diet, may eventually provide a safe, inexpensive, and well-tolerated alternative to patients who are intolerant to statins[51–53]. However, further clinical trials are required in order to solidify generalize our findings. In this context, we assume that our study provides a useful tool for such future studies by supplying evidence for its preventative action in middle-aged animal models following hypercholesterolemic diets.

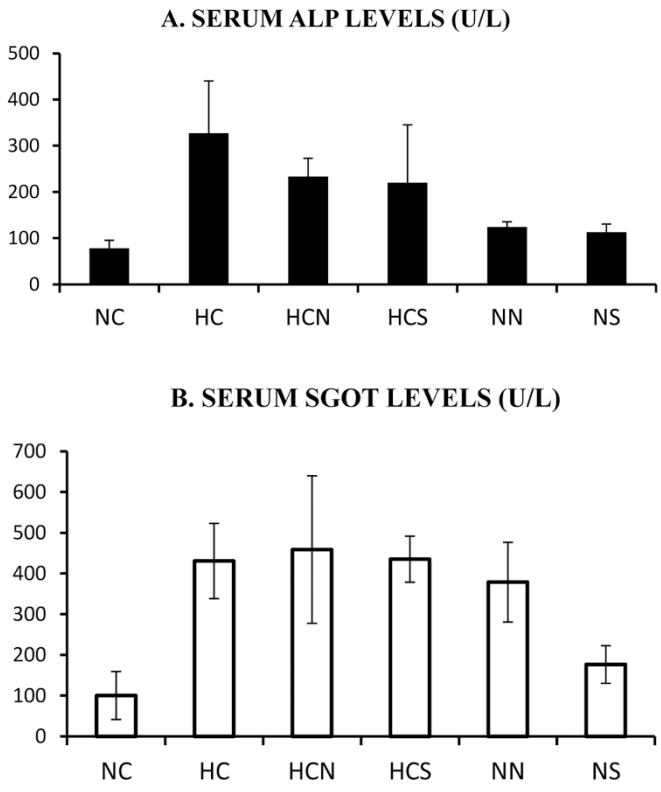

Figure 3 | Serum ALP (U/L) (A) and serum SGOT levels (U/L) (B) at the end of the 8-week experimental period. (NC), Control mice; (HC) mice fed high cholesterol diet; (HCN) mice fed high cholesterol diet and treated with NAC; (HCS) mice fed high cholesterol diet enriched with sesame oil; (NN) mice fed control diet and treated with NAC and (NS) mice fed control diet enriched with sesame oil.

Conclusion

In conclusion, NAC but not sesame oil supplementation prevented the onset of hyperlipidemia and showed hepatoprotective activity against fatty liver disease related to diet-induced hyperlipidemia in middle-aged mice. It is possible that this effect is mediated through an antioxidant action. However, further studies are required to examine if the antioxidant action NAC is causal or parallel to its protective effects. Furthermore, we observed that the changes in hypothalamic GR protein levels which occurred in response to high-cholesterol feeding were prevented by NAC co-administration, suggesting a potential impact of NAC on the HPA axis function.

Methods

Animals – study design. Thirty-four-week-old C57BL/6 male mice were obtained from BSRC "Al. Fleming" (Vari, Greece) and left to be acclimatised for one week before the experiment start. The number of animals was selected by taking into account our previous study results and the directives of the relevant Ethics Committee. The animals were housed under conditions of controlled temperature (23 ± 2°C) and humidity (60%) with 12 h light/dark cycles. All possible precautions were taken to avoid animal suffering at each stage of the experiment. The experimental protocol was approved by the Veterinary Directorate of Athens Prefecture and by the Ethics Committee of the Medicine School of the National and Kapodistrian University of Athens according to the EU legislation regarding the use of laboratory animals in biomedical science.

Mice had free access to food and water throughout the study (8 weeks). During the study, food and water consumption was recorded daily, while body weight was measured on a weekly basis.

Thirty six mice (n = 36) were randomized into six experimental groups (n = 6/group) as follows: Normal Control (**NC**): animals received normal chow diet (4RF25, Mucedola, Milan, Italy, 3.48% fat, Energy value: 2.789 Kcal/g); High Cholesterol diet (**HC**): animals received normal chow diet supplemented with 2% cholesterol and 0.5% cholic acid (Energy value: 2.789 Kcal/g) for 8 weeks; High Cholesterol and NAC supplementation (**HCN**): animals received the high cholesterol diet and 230 mg/kg NAC daily diluted into their drinking water (Flumil Antidoto, IFET, Greece). The dosage of NAC administration was determined according to previous reports on rodents[54]. High Cholesterol and Sesame oil (**HCS**): mice received the high-cholesterol diet enriched with 10% sesame oil (Energy value: 3.353 Kcal/g) for the same period. Normal diet and NAC (**NN**): animals fed normal chow with NAC supplementation. Normal diet and Sesame oil (**NS**): mice received normal chow enriched with 10% sesame oil.

The sesame-oil-enriched diet was prepared as previously described[17] in order to achieve a final concentration of sesame oil at a level of 10% per gram of chow. Cholesterol and cholic acid (approximately 95% and 99% purity, respectively) were obtained from Sigma-Aldrich (Germany).

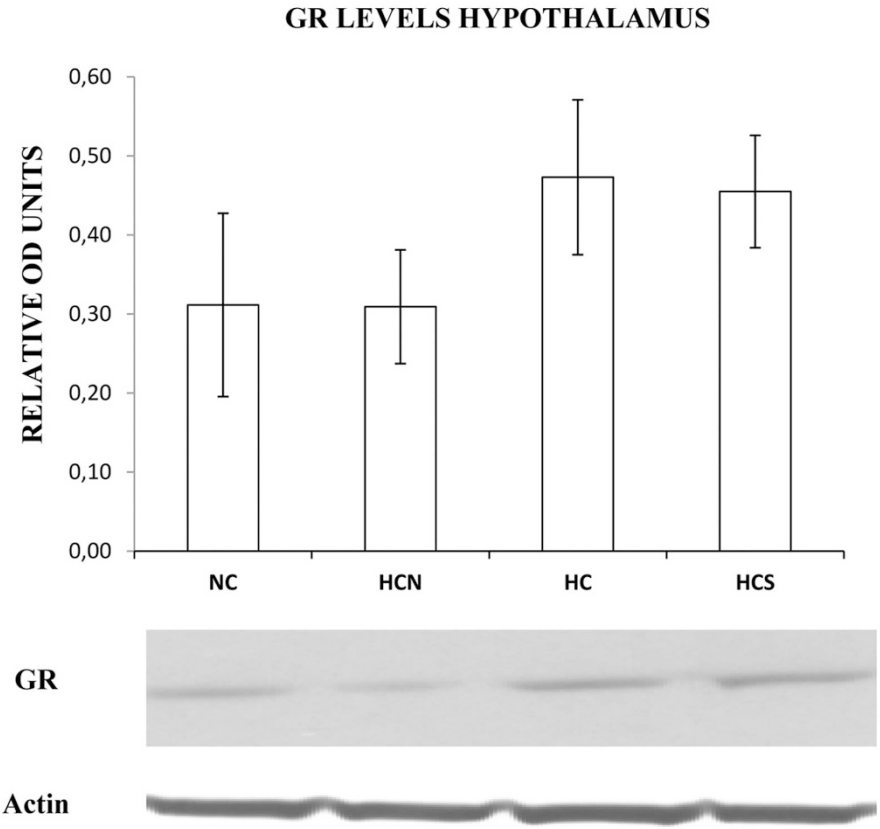

Figure 4 | Glucocorticoid Receptor (GR) protein expression levels in the hypothalamus of control mice (NC); mice fed high cholesterol diet and treated with NAC (HCN); mice fed high cholesterol diet (HC) and mice fed high cholesterol diet enriched with sesame oil (HCS), as depicted by Western blot analysis. (Bars represent the means ± standard deviation (SD) of the optical density (OD) of receptors' band divided by that of the respective actin band in each sample).

Blood collection – serum & plasma measurements. Blood samples of mice were collected at the beginning (T_0) and at the end of the experimental period (T_1) (at 9:00 AM, after a 12-hour fast) using capillary tubes introduced into the medial retro-orbital venous plexus under light ether anaesthesia. Serum concentrations of total, LDL, and HDL cholesterol, as well as triglycerides and total peroxides were determined as described in our previous study[17].

Nitrate, endogenous Nitrite and total Nitrate/Nitrite (NO) levels were assessed using a commercially available kit (R&D SYSTEMS, MN, U.S.).

Serum samples for determination of serum ALP and SGOT levels were measured in the same batch in an automated analyzer (TECHNICON RA-XT/Technicon, Dublin, Ireland) using commercially available kits.

Corticosterone levels in plasma of NC, HC, HCN and HCS groups were tested using an RIA kit for small rodents (MP Biomedicals, Orangeburg, NY). The inter- and intra-assay coefficients of variation were both 8%. The values were expressed as ng of corticosterone/mL plasma.

Tissue collection and histopathological staining. At the end of the 8-week period, animals were euthanized under ether anaesthesia. Liver and aortas were dissected immediately for further histopathological analysis as described in our previous work[17]. In brief, liver sections were stained with hematoxylin-eosin and examined blindly by two independent researchers using light microscopy. Liver evaluation was conducted as previously described by the Pathology Committee of Non-Alcoholic Steatohepatitis Clinical Research Network[55]. The histological features were grouped into 4 broad categories: steatosis, ballooning, portal inflammation and lobular activity. A score from 0 (absence) to 3 (severe) was assigned to each parameter. The animals' hypothalami belonging to NC, HC, HCN and HCS groups were individually dissected on ice, frozen in liquid nitrogen and stored at −80°C until use.

Western blot analysis. Frozen hypothalami were homogenized in an ice-cold buffer (50 mM Tris HCl pH 7.6, 150 mM sodium chloride, 0.5 mM dithiothreitol, 0.1% w/v sodium dodecyl sulfate and 1 mM phenylmethylsufonylfluoride) and the homogenates were centrifuged at 14,000 RPM for 20 minutes at 4°C. The supernatants were then used for western blot analysis as previously described[6]. The anti-GR monoclonal antibody used (clone 2F8; 1 : 10) was kindly donated by Dr. M. N. Alexis[56] and anti-b-actin (1 : 10,000) was purchased from Chemicon International Inc; Temecula, CA. The signal was visualized on Kodak X-Omat AR films, by chemiluminescence (Amersham, UK). To measure the relative density of immunoreactive bands, the films were scanned and analyzed by using the Image Pro Plus Software (Media Cybernetics). The bands were normalized by dividing the band intensity of GR antibody by the corresponding band intensity of beta-actin antibody (housekeeping protein) for each sample. Beta-actin is considered a prevalent housekeeping protein which remains stable among experimental groups.

Statistical analysis. The results, apart from histological scores, were expressed as means ± 1 standard deviation (SD). Body weight, food consumption, serum lipid, total peroxides, NO and hepatic enzymes levels were analysed for statistical significance by Two-Way ANOVA with diet and NAC intake or diet and sesame oil supplementation as the between-subjects factors. Analysis of Covariance (ANCOVA) was performed in order to compare T1 measurements after controlling for baseline levels. Hypothalamic GR protein levels and plasma corticosterone levels were evaluated by One-Way ANOVA. Histological scores were expressed as median (interquartile range) and were compared using the Kruskall-Wallis test, followed by the Mann-Whitney's U test when appropriate. Benjamini & Hochberg's False Discovery Rate (FDR) was utilized in order to assess differences between multiple groups, as well as to control family-wise error to a < 0.05. All tests were two-sided. Statistical significance was set at $p < 0.05$.

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Author contributions

L.M.K. has been involved in experimental design, animal experiments, laboratory analysis, data collection and statistical analysis. G.A. carried out the histopathological examination. C.K. performed the Western Blot Analysis and corticosterone measurements and E.K. also participated in Western Blot Analysis and in experimental design and manuscript drafting, I.S.V. participated in experimental design and performed part of the statistics, D.D. and T.K. have been involved in experimental design. V.P. participated in drafting the manuscript and in evaluating the experimental results. I.T. participated in the experimental design and also in manuscript drafting. D.N.P. participated in experimental design, animal experiments, laboratory analysis and data collection. All of the above helped in drafting the manuscript. All authors read and approved the final manuscript.

Additional information

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