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Abstract

Altered homocysteine metabolism defined as hyperhomocysteinemia is implicated as pathogenic factor in several cardiovascular diseases and atherosclerosis. The purpose of this study was to investigate the efficacy of prune extract, a good source of phenolic antioxidants, on lowering plasma homocysteine level in male hyperhomocysteinemic mice from average weight of 28g. The administration of lyophilized prune extract was carried out by intraperitoneal injection one day preceding and one hour before sacrifice of mice. Prune extract decreased significantly plasma homocysteine level, correlated with an increased activity of S-adenosylhomocysteine (SAH) hydrolase and NAD(P)H: quinone oxydoreductase-1 activities. Our results suggest a beneficial effect of prune extract on hyperhomocysteinemia with reduction of homocysteine level by its conversion on to SAH by S-adenosylhomocysteine hydrolase, which is activated by NAD^+, a by-product of NAD(P)H: quinone oxydo reductase-1.

Keywords: Homocysteine; Prune extract; Mice; S-adenosylhomocysteine hydrolase; NAD(P)H:quinone oxydo reductase-1.

Highlights: plasma homocysteine level is decreased after prune extract administration – hepatic S-adenosylhomocysteine hydrolase and NAD(P)H: quinone oxydoreductase-1 activities are increased after prune extract administration - hepatic S-adenosylhomocysteine hydrolase activity correlates with NAD(P)H: quinone oxydoreductase-1 activity.

1. Introduction

Homocysteine (Hcy), a metabolic by-product of methionine, can cause widespread hazards to human body if its level is increased in plasma, defined by hyperhomocysteinemia (Zhang et al., 2005). It can be remethylated into methionine by means of vitamin B12-dependent methionine synthase and 5-methyltetrahydrofolate as a methyl donor. Hcy can also be catabolized into cysteine by the transsulfuration pathway via cystathionine beta synthase (CBS) and cystathioninase, both enzymes being vitamin B6-dependent. A third way to remove Hcy is conversion to S-adenosylhomocysteine (SAH). The last reaction is mediated by SAH-hydrolase (SAHH) and favors the SAH formation in case of increased Hcy concentrations (Obeid et al., 2009)**.** Hcy is the main factor implicated or linked to several metabolic diseases like atherosclerosis, thrombosis, diabetes, Alzheimer, cerebral, and cardiovascular diseases (Jacobsen, 1998; Lawrence de Koning et al., 2003; Buysschaert et al., 2007; Van Dam & Van Gool, 2009). Some phenolic compounds and plant extracts such as catechin, quercetin, chlorogenic acid, coffee, wine phenolics have been demonstrated to be effective in decreasing plasma Hcy level (Nygård et al., 1997; Noll et al., 2009a; Noll et al., 2011; Kim et al., 2012). Prune, dried fruits of plum (*P*runus *domestica*) contain higher level of phenolic compounds. In according to the study of Donovan and colleagues (1998) the level of phenolic compounds in prunes (1840 mg/kg) surpasses the levels reported for many other popular fruits like as Red Flame seedless table grapes (<250 mg /kg of grape), white table varieties (<50 mg/kg of grape), apples (1200 mg/kg), oranges (830 mg/kg), pears (265 mg/kg), and cherries (850mg/kg of fruit) (Donovan et al., 1998). Phenolic compounds in prunes have been found to inhibit human low-density lipoprotein oxidation *in vitro*, and thus might serve as preventive agents against chronic diseases, such as heart disease and cancer (Stacewicz-Sapuntzakis et al., 2001). Hyperhomocysteinemia is well defined as risk factor for cardiovascular diseases, therefore, the present study investigated the effects of polyphenolic prune extract, which contains notably chlorogenic acid and its isomer neochlorogenic acid, on plasma Hcy level in hyperhomocysteinemic mice and its implication on SAHH activity, a third way of Hcy metabolism.

2. Materials and methods

2.1. Extraction of prune phenolic compounds

The extraction of phenolic compounds from prune was carried out by microwave assisted extraction under optimized conditions (Haddadi-Guemghar et al., 2014). The fruit samples were prepared for extraction by cutting into small pieces by a knife. Two grams of sample were placed in a round-bottom flask with 40 mL of

distilled water. The flask was placed in a microwave oven and connected to condenser. After irradiation (1mn at 500W), the flask was taken out and cooled in ice water bath (4°C). The solution was filtered through Wathman n°1 paper under vacuum. After concentrating using rotavapor at 40°C, the extract was lyophilized.

2.2. Total phenols and hydrogen peroxide determination

Total phenols (TP) content was determined by the Folin-Ciocalteu method. 1 ml of Folin-Ciocalteu reagent (diluted 10 times by water) was mixed with 100 µL of the prune extract. After 5min, 1 mL of aqueous solution of sodium carbonate (6%) was added. The mixture was kept for 60 min at room temperature. Absorbance was measured at 755 nm. Ethanol solution of gallic acid was used as standard (Velioglu et al., 1998). Hydrogen peroxide content was determined with the hydrogen peroxide assay kit (Abcam, France) as recommended by the supplier's instructions.

2.4. Mice, genotyping and experimental protocol

Mice were maintained in a controlled environment with unlimited access to food and water on a 12-h light/dark cycle. All procedures were carried out in accordance with internal guidelines of the French Agriculture Ministry for animal handing. Number of mice and suffering were minimized as possible. Mice heterozygous for targeted disruption of the Cbs gene (Cbs +/−) were generously donated by Dr. N. Maeda (Department of Pathology, University of North Carolina, Chapel Hill, NC, USA) (Watanabe et al., 1995). Cbs +/− mice, on a C57BL/6 background, were obtained by mating male Cbs +/− mice with female wild-type C57BL/6 (Cbs +/+) mice. DNA isolated from 4-week-aged mice tail biopsies was subjected to genotyping of the targeted CBS allele using polymerase chain reaction assay (Watanabe et al., 1995). The study was carried out on male 2-month-old Cbs +/- and Cbs +/+. The administration of prune extract was carried out by intraperitoneal injection of 100 µL of prune extract at concentration of 1mg/mL (1mg of lyophilized prune extract was resuspended in 1 mL of NaCl 0.9%) to Cbs +/- and Cbs +/+ mice. The mice control, both Cbs +/- and Cbs +/+, were treated by intraperitoneal injection of 100 µL of NaCl 0.9%. All mice were treated one day preceding and one hour before sacrifice. Two experimental protocols (one with five mice per group and the second with three mice per group) have been done, depending on the variability of the analysed biomarker.

2.5. Preparation of serum samples, tissue collection, and plasma tHcy assay

At the time of sacrifice, blood samples were collected into tubes containing an 1/10 volume of 3.8% sodium citrate, placed on ice immediately. Plasma was isolated by centrifugation at 2500*g* for 15 min at 4 ℃. Liver was harvested, snap-frozen and stored at – 80 ℃ until use. Plasma tHcy was assayed by using the fluorimetric high-performance liquid chromatography method described by Fortin and Genest (1995).

2.6. Determination of SAHH activity

SAHH activity assay was performed on 200 µg of total proteins obtained from liver samples following the protocol described previously (Villanueva & Halsted, 2004)**.**

2.7. Determination of NAD(P)H: quinone oxydo reductase-1 activity

Determination of NAD(P)H: quinone oxydo reductase-1 (NQO1) activity was assayed on 100 µg of total proteins obtained from liver samples following the protocol described by Ernster (Ernster, 1967), and modified by Benson and colleagues (Benson et al., 1980).

2.8. Data analysis

Statistical analysis was done with one-way ANOVA followed by Student's unpaired t test using Statview software. The results are expressed as medians with interquartile ranges. Data were considered significant when $p < 0.05$. A *p* value of 0.06–0.10 was considered to indicate a strong statistical tendency due to the small sample size. Correlations between tHcy level, SAHH activity and NQO1 activity were determined by using Spearman's rank correlation as data were not normally distributed according to Shapiro – Wilk test. Data were analyzed using R software (http://www.R-project.org) and considered significant when $p < 0.05$.

3. Results and discussion

3.1. Effects of prune extract on plasma tHcy level in wild type and hyperhomocysteinemic mice

Previous study demonstrated that administration of wine polyphenols such as catechin, epicatechin or wine extract modifies significantly Hcy metabolism, this effect depending on quantity of polyphenols administered

(Noll et al., 2009a). The concentration of prune exptract injected here to mice, in terms of total polyphenols, was measured by Folin ciochalteu method. It was 3mg GAE (gallic acid equivalents)/mL per injection. The volume of prune extract injected was 100 µL, thereby the quantity of total phenols expressed as equivalent of gallic acid was 300 µg. The effects of prune extract administration on plasma tHcy level in wild type mice (Cbs +/+) and hyperhomocysteinemic mice (Cbs +/-) were shown in Fig. 1. The prune extract administration decreased significantly plasma tHcy level in both Cbs +/+ and Cbs +/- mice, in comparison with mice on control diet respectively. Although the phenolic content of prune extract is different to phenolic content of red wine extract, the result was similar to those obtained previously (Noll et al., 2009a). The major polyphenols in wine extract are gallic acid, catechin, epicatechin and *p*-coumaric acid (Porgali et al., 2012), whereas neochlorogenic acid and chlorogenic acid are the predominant polyphenols in prune extract (Donovan et al., 1998).

3.2. Effect of prune extract on SAHH activity in wild type and hyperhomocysteinemic mice

The SAHH reaction, which forms Hcy from SAH, is reversible, with the equilibrium actually favoring the condensation of Hcy and adenosine to generate SAH (De La Haba et al., 1959). A third way to remove Hcy is its conversion to SAH. The last reaction is mediated by SAHH and favors the SAH formation in case of increased Hcy concentrations. The effect of prune extract administration on SAHH activity in wild type and hyperhomocysteinemic mice was shown in Fig. 2A. Prune extract administration increased significantly SAHH activity in both Cbs +/+ and Cbs +/- mice. We found a 1.6 fold increase in SAHH activity in liver of Cbs +/+ mice treated with prune extract in comparison with mice on control diet, and a two-fold increase in SAHH activity in liver of Cbs +/- mice treated with prune extract in comparison with mice on control diet. Using Spearman correlation test, we found a significant negative correlation between plasma tHcy level and SAHH activity (Fig. 2B, $r = -0.55$, $p < 0.02$). Noll and colleagues (Noll et al., 2009a) also found a decrease of plasma tHcy level, concomitant with an increase of hepatic SAHH activity, after wine polyphenolic extract administration.

3.3. Effect of prune extract on NQO1 activity in wild type mice and hyperhomocysteinemic mice

Previous study demonstrated that an increase in SAHH activity is not due to an increase in SAHH expression, but occurs concomitantly with an increase in NQO1 activity (Noll et al., 2009b). NQO1, also

referred to as DT-diaphorase, is a flavoprotein that catalyzes the two-electron reduction of quinones and quinonoid compounds to hydroquinones, using either NADH or NADPH as the electron donor (Chen et al., 2000). NQO1 co-produces NAD+, which is a cofactor of SAHH. The administration of prune extract to mice increases NQO1 activity in both Cbs +/+ and Cbs +/- mice (Fig. 3A). Moreover, NQO1 activity was negatively correlated with plasma tHcy level (Fig. 3B, r = - 0.65, $p < 0.01$). Several previous studies demonstrated that the induction of NQO1 is attributed to the NF-E2-related factor-2 (Nrf2), which binds to an upstream regulatory sequence, the antioxidant response element (ARE) and activates NQO1 gene expression (Krajka-Kuźniak et al., 2013)**.** Experimental studies in animal models and cell cultures have shown that several naturally occurring polyphenols or plant extract demonstrate the ability to activate the Nrf2/ARE pathway. Valerio and colleagues (Valerio et al., 2001) reported the induction of NQO1 expression by quercetin in human breast cancer cells. They show that treatment of human breast cancer cells for 24 h with 15 mM quercetin results in a two fold increase in NQO1 protein levels and enzyme activity, and a three- to four-fold increase in NQO1 mRNA expression (Valerio et al., 2001). Krajka-Kuźniak and colleagues (Krajka-Kuźniak et al., 2013) demonstrated the activation of Nrf2/ARE pathway by phloretamide, an apple phenolic compound in human hepatocytes. They have shown the translocation of Nrf2 from cytosol to nucleus and an increasing in the NQO1 transcript and protein after treatment of hepatocyte cells with phloretamide. Forman and colleagues proposed that the activation of the Nrf2/ARE pathway is the major mechanism of action for nutritional antioxidants *in vivo* (Forman et al., 2014). In prune, neochlorogenic acid is the principal phenolic compound (71%) followed by its isomer chlorogenic acid (24%). Kim and colleagues have shown the induction of NQO1 expression by chlorogenic acid in neuronal cultural cells, which suggested that prune phenolic compounds enhance NQO1 activity by inducing the expression of NQO1 (Kim et al., 2012). One other way to activate Nrf2 by polyphenols is the generation of hydrogen peroxide (Erlank et al., 2011). More importantly, polyphenols can undergo autoxidation with oxygen consumption and hydrogen peroxide production (Matsuura et al., 2014). We therefore analyzed hydrogen peroxide content in prune extract and found generation (figure 5), with a positive correlation with the volume of prune extract ($p < 0.0455$; $r= 1$). Therefore, even if we demonstrated that the use of microwaves in the extraction of phenolics compounds from prune not only gives the higher value but also increased the antioxidant activity (Haddadi-Guemghar et al., 2014), the activation of Nrf2 by prune extract may also be due to oxidized species generated by autoxidation of polyphenols. In this sense, hydrogen peroxide can act as a cell-survival signaling molecule (Groeger et al., 2009).

3.4. Correlation between SAHH activity and NQO1 activity

According to Spearman test, we showed a strong tendency to positive correlation between SAHH activity and NQO1 activity (figure 4, r = 0.42, $p< 0.1$). NQO1 activity and SAHH activity have an indirect relation since the SAHH enzyme is activated by NAD^+, which is produce in oxydo-reduction reactions catalyzed by NQO1. SAHH requires NAD+ to maintain its quaternary structure and catalytic activity, making the enzyme sensitive to the redox status of the cell that is reflected by the NAD^+/NADH ratio (Tehlivets et al., 2013).

4. Conclusion

This study demonstrates that prune extract is effective in reducing plasma tHcy level in both Cbs +/+ mice and Cbs +/- mice. The possible mechanism is associated with the conversion of Hcy to SAH by SAHH, which is activated by NAD^+, a by-product of NQO1 reaction. We suggest that prune extract enhances the NQO1 activity, which could increase the NAD^+/NADH ratio.

Conflict of interest

The authors declare there are no conflicts of interest.

Acknowledgements

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Figure legends

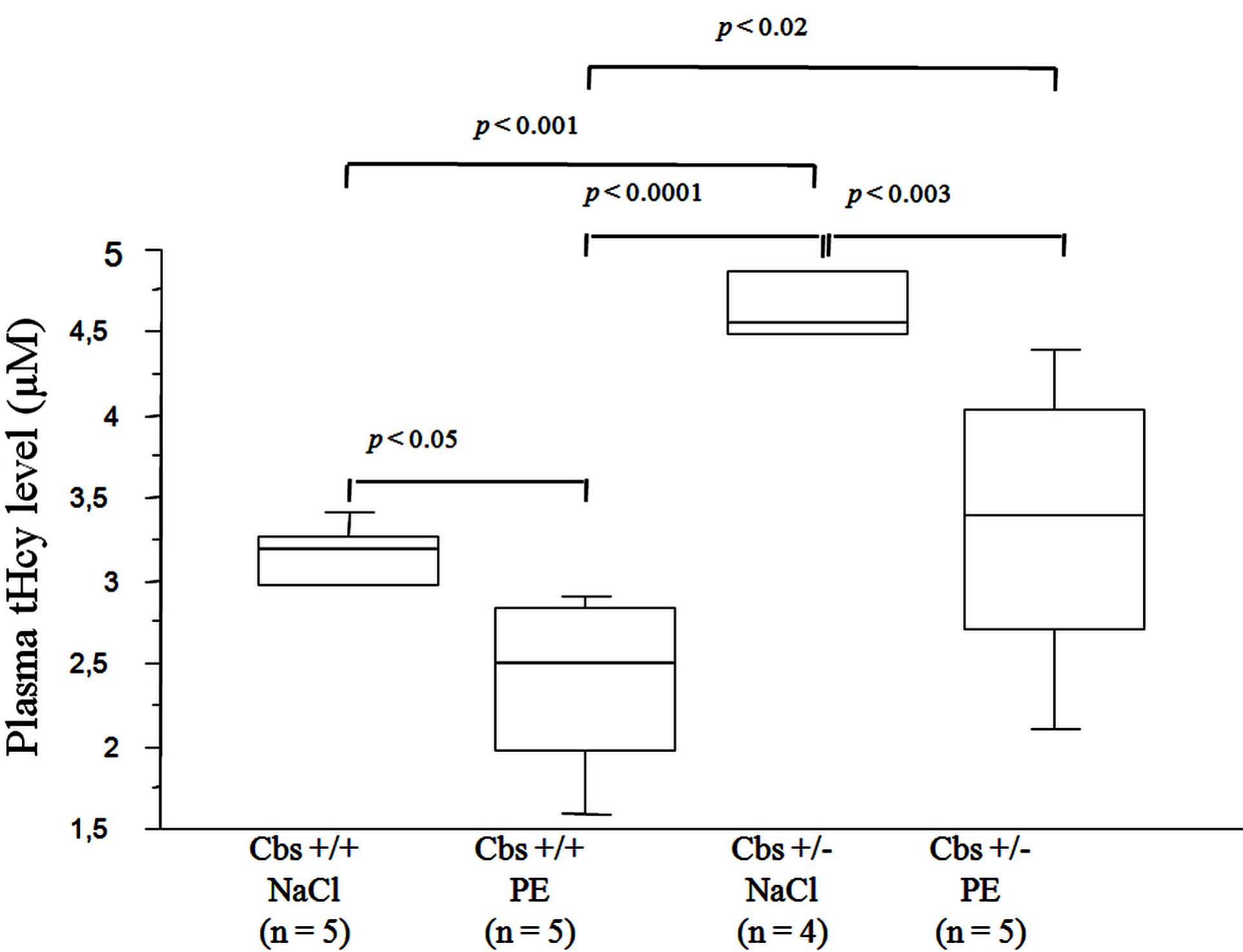
Fig. 1. Effects of prune extract on plasma tHcy levels in wild type (Cbs +/+) and hyperhomocysteinemic (Cbs +/-) mice fed the control diet supplemented (prune extract: PE) or not (NaCl). Data correspond to the medians with interquartile ranges. n = number of mice. Statistical analysis was done with one-way ANOVA followed by Student's unpaired t-tests.

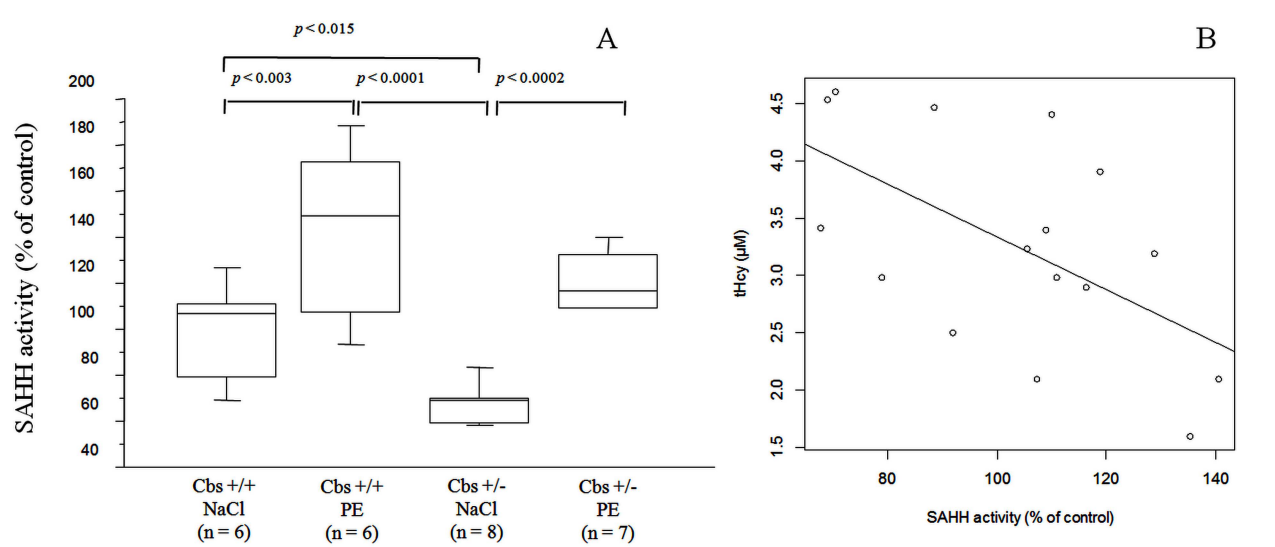
Fig. 2. Effects of prune extract on hepatic SAHH activity in wild type (Cbs +/+) and hyperhomocysteinemic (Cbs +/-) mice fed the control diet supplemented (prune extract: PE) or not (NaCl). Data of hepatic SAHH activity (A) correspond to the medians with interquartile ranges. Data were normalized to the mean of (Cbs +/+) mice on control diet not supplemented (NaCl). n = number of mice. Statistical analysis was done with one-way ANOVA followed by Student's unpaired t-tests. (B) Hepatic SAHH activity was negatively correlated with tHcy level ($p < 0.02$; r=- 0.55).

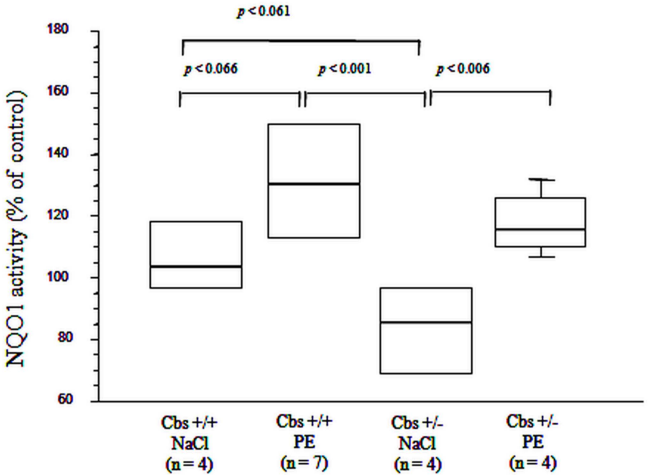
Fig. 3. Effects of prune extract on hepatic NQO1 activity in wild type (Cbs +/+) and hyperhomocysteinemic (Cbs +/-) mice fed the control diet supplemented (prune extract: PE) or not (NaCl). Data of hepatic SAHH activity (A) correspond to the medians with interquartile ranges. Data were normalized to the mean of (Cbs +/+) mice on control diet not supplemented (NaCl). n = number of mice. Statistical analysis was done with one-way ANOVA followed by Student's unpaired t-tests. (B) Hepatic NQO1 activity was negatively correlated with tHcy level ($p < 0.01$; r=- 0.65).

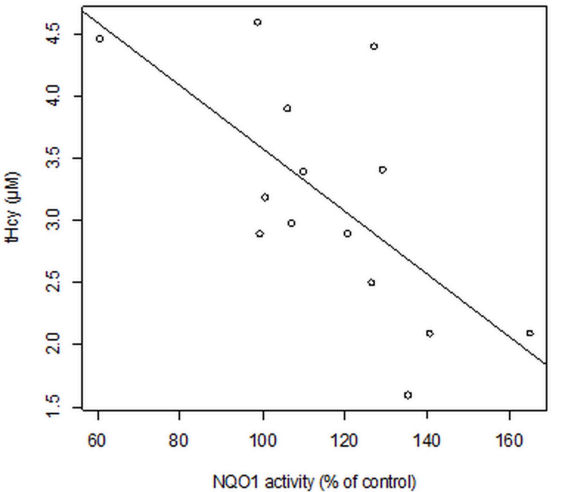
Fig. 4. Positive correlation between hepatic SAHH and NQO1 activity. Hepatic NQO1 activity was positively correlated with SAHH activity ($p < 0.01$; r= 0.42).

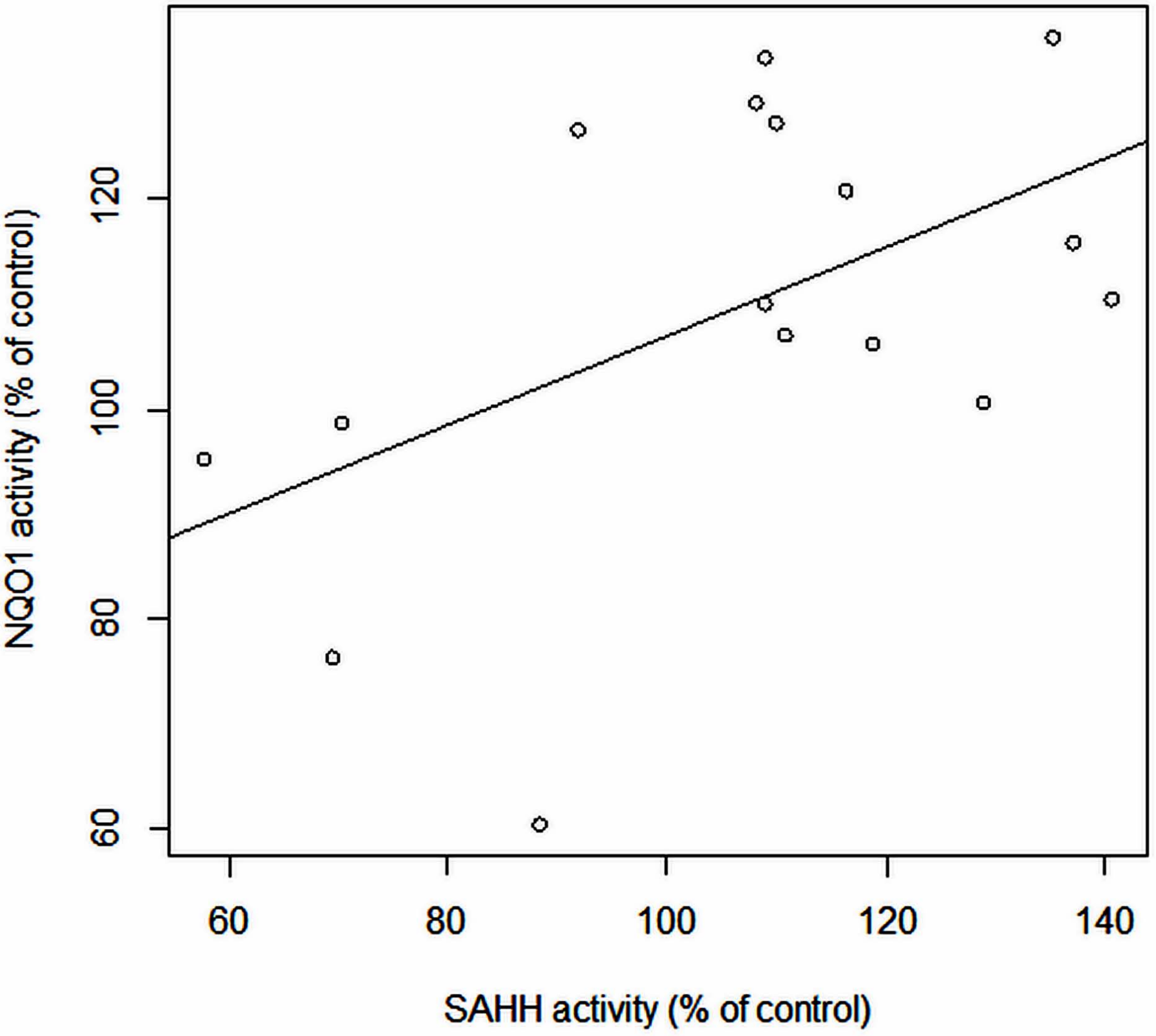
Fig. 5. Effects of prune extract on hydrogen peroxide generation. Hydrogen peroxide content was determined according to the volume of prune extract ($p < 0.0455$; r= 1).

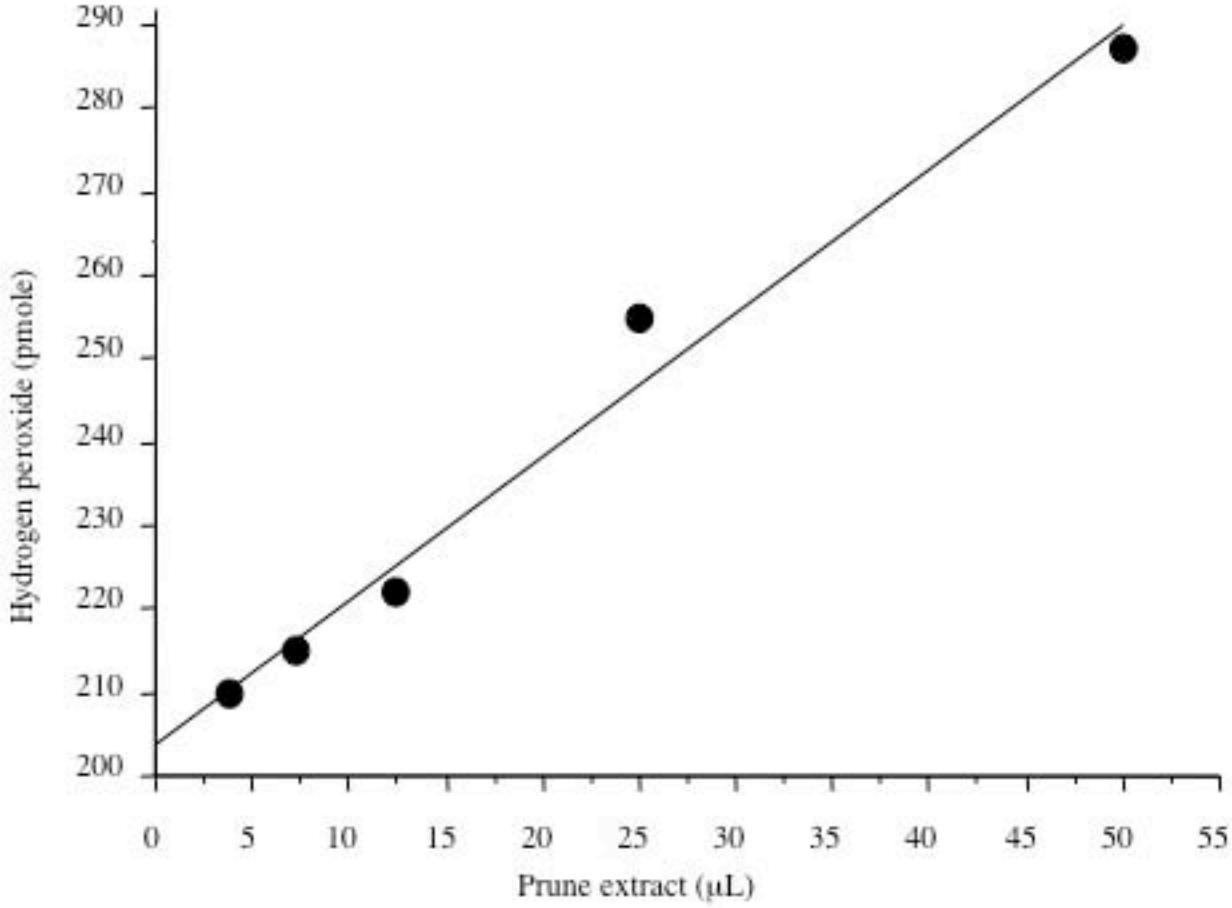

p < 0.02
p < 0.001
p < 0.0001
p < 0.003
p < 0.05
Plasma tHcy level (µM)
5
4,5
4
3,5
3
2,5
2
1,5
Cbs +/+ NaCl (n = 5)
Cbs +/+ PE (n = 5)
Cbs +/- NaCl (n = 4)
Cbs +/- PE (n = 5)


A
p < 0.015
p < 0.003
p < 0.0001
p < 0.0002
SAHH activity (% of control)
200
180
160
140
120
100
80
60
40
Cbs +/+ NaCl (n = 6)
Cbs +/+ PE (n = 6)
Cbs +/- NaCl (n = 8)
Cbs +/- PE (n = 7)
B
tHcy (μM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
80
100
120
140
SAHH activity (% of control)


p < 0.061
p < 0.066
p < 0.001
p < 0.006
NQO1 activity (% of control)
180
160
140
120
100
80
60
Cbs +/+
NaCl
(n = 4)
Cbs +/+
PE
(n = 7)
Cbs +/-
NaCl
(n = 4)
Cbs +/-
PE
(n = 4)


tHcy (µM)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
60
80
100
120
140
160
NQO1 activity (% of control)


NQO1 activity (% of control)
60
80
100
120
SAHH activity (% of control)
60
80
100
120
140


Hydrogen peroxide (pmole)
290
280
270
260
250
240
230
220
210
200
0
5
10
15
20
25
30
35
40
45
50
55
Prune extract (µL)