1   s-Allyl Cysteine, s-Ethyl Cysteine and s-Propyl Cysteine Alleviate

2   beta-Amyloid, Glycative and Oxidative Injury in Brain of Mice Treated by

3   D-galactose

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14   Running title: Neuro-protection of SAC and SEC

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1 **ABSTRACT**

2 The neuro-protective effects of s-allyl cysteine, s-ethyl cysteine and s-propyl cysteine in

3 D-galactose (DG) treated mice were examined.   DG treatment increased the formation of

4 $A\beta_{1-40}$ and $A\beta_{1-42}$, and enhanced mRNA expression of β-amyloid precursor protein (APP)

5 and beta-site APP cleavage enzyme 1 (BACE1), and reduced neprilysin expression in

6 brain ($P<0.05$); however, the intake of three test compounds significantly decreased the

7 production of $A\beta_{1-40}$ and $A\beta_{1-42}$, and suppressed expression of APP and BACE1 ($P<0.05$).

8 DG treatments declined brain protein kinase C (PKC) activity and mRNA expression

9 ($P<0.05$).   Intake of test compounds significantly retained PKC activity and the

10 expression of PKC-alpha and PKC-gamma ($P<0.05$).   DG treatments elevated brain

11 activity and mRNA expression of aldose reductase (AR) and sorbitol dehydrogenase, as

12 well as increased brain level of carboxymethyllysine (CML), pentosidine, sorbitol and

13 fructose ($P<0.05$).   Test compounds significantly lowered AR activity, AR expression,

14 and CML and pentosidine levels ($P<0.05$).   DG treatments also significantly increased

15 the formation of reactive oxygen species (ROS) and protein carbonyl; and decreased the

16 activity of glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase

17 ($P<0.05$); however, the intake of test compounds in DG-treated mice significantly

18 decreased ROS and protein carbonyl levels, and restored brain GPX, SOD and catalase

19 activities ($P<0.05$).   These findings support that these compounds via their anti-Aβ,

20 anti-glycative and anti-oxidative effects were potent agents against the progression of

21 neurodegenerative disorders such as Alzheimer's disease.

22

23 KEYWORDS: β-amyloid; s-allyl cysteine; s-ethyl cysteine; s-propyl cysteine; glycation;

24 protein kinase C

INTRODUCTION

Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common form of dementia in many countries. The 'amyloid cascade hypothesis' considers the amyloid-beta peptide (Aβ) a central role in the pathogenesis of AD (*1, 2*). Aβ is derived from the β-amyloid precursor protein (APP), a large transmembrane protein, in which a transmembrane aspartyl protease termed as beta-site APP cleavage enzyme 1 (BACE1) is responsible for the cleavage (*3, 4*). On the other hand, Aβ could be degraded by a variety of proteases such as neprilysin (NEP) (*5*). Thus, any agents with ability to down-regulate APP or BACE1, or up-regulate NEP, may potentially protect neurons against Aβ-induced neurotoxicity via decreasing Aβ formation and/or increasing Aβ clearance. In addition, it has been documented that Aβ deposition in brain tissue causes generation of free radicals and advanced glycation endproducts (AGEs) such as carboxymethyllysine (CML) and pentosidine, which in turn enhances oxidative and glycative damage, and consequently leads to neuronal cells apoptosis (*6, 7*). Thus, any agents with anti-glycative and anti-oxidative effects may also attenuate AD development.

s-Allyl cysteine (SAC), s-ethyl cysteine (SEC) and s-propyl cysteine (SPC) are hydrophilic cysteine-containing compounds naturally formed in *Allium* plants such as garlic and onion (*8*). Our previous study has reported that pre-intake of SEC and SPC markedly protected mice brain against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine induced Parkinson-like oxidative and inflammatory injury via increasing retention of dopamine and glutathione, elevating glutathione peroxidase (GPX) mRNA expression and diminishing tumor necrosis factor-α mRNA expression (*9*). Our another study further observed that SEC and SPC protected PC12 cells against Aβ-caused oxidative and apoptotic damage via retaining mitochondrial membrane potential and decreasing DNA

1 fragmentation (*10*). These findings support that these cysteine-containing compounds

2 via acting as anti-oxidative and anti-inflammatory agents are able to prevent or mitigate

3 cytotoxicity and oxidative stress occurred in neurodegenerative disorders. Therefore, an

4 animal study was conducted to further examine the *in vivo* protective effects and possible

5 action modes of these agents against AD progression.

6   It has been documented that D-galactose (DG) induced aging-related and/or AD-like

7 pathological changes including the increase of reactive oxygen species (ROS) and the

8 decrease of antioxidant enzyme activity in brain (*11, 12*). The animal study of Hsieh et

9 al. (*12*) reported that DG treatment increased AGEs level in circulation and enhanced Aβ

10 expression in brain. In our present study, DG injected mouse was used as an AD model

11 to examine the anti-Aβ, anti-glycative and anti-oxidative effects of SAC, SEC and SPC.

12

13 **MATERIALS AND METHODS**

14   **Chemicals.** SEC (99.5%) was purchased from Aldrich Chemical Co. (Milwaukee,

15 WI, USA). SAC (99.5%) and SPC (99%) were supplied by Wakunaga Pharmaceutical

16 Co. (Hiroshima, Japan).

17   **Animals.** Three- to four-week-old male C57BL/6 mice were obtained from National

18 Laboratory Animal Center (National Science Council, Taipei City, Taiwan). Mice were

19 housed on a 12-h light-12-h dark schedule, and fed with water and mouse standard diet for

20 16 wk. Use of the mice was reviewed and approved by both Chung Shan Medical

21 University and China Medical University animal care committees.

22   **Experimental Design.** Mice at 19-20 wk old were used for experiments. Mice

23 were divided into two groups, in which one group was treated with DG (100 mg/kg body

24 weight) via i.p. daily injection and the other group was treated with saline injection.

25 Both DG and non-DG treated mice were further divided into four sub-groups, in which

water, SAC, SEC or SPC was supplied.   SAC, SEC or SPC, each compound at 1 g/L, was directly added into the drinking water.   Consumed water volume and body weight were recorded weekly.   After 7-wk DG treatment and cysteine-containing compounds supplementation, mice were sacrificed by decapitation.   Brain was quickly removed and collected.   Brain tissue at 0.1 g was homogenized on ice in 2 mL of phosphate buffer saline (PBS, pH 7.2) and the filtrate was collected.   Protein concentration of filtrate was determined by a commercial assay kit (Pierce Biotechnology Inc., Rockford, IL, USA) with bovine serum albumin as standard.   In all experiments, the sample was diluted to a final concentration of 1 mg protein/mL.

Measurement of Aβ Levels. $A\beta_{1-40}$ and $A\beta_{1-42}$ were measured using commercial colorimetric ELISA kits (The Genetics Company, Schlieren, Switzerland) according to manufacturer's instructions.   All values were standardized to the protein concentration of sample.

Determination of BACE1 and NEP Activities. For BACE1 activity assay, brain tissue was homogenized and lysed in 100 μL buffer (20mM MES pH 6.0, 150mM NaCl, 2mM EDTA, 5 μg/mL leupeptin, 0.2mM PMSF, 1 μg/mL pepstatin A, 2 μg/mL aprotinin, and 0.5% Triton X-100).   After centrifuged at 16,000 xg for 20 min at 4 °C, 50 μL of lysate was incubated with reaction buffer containing 10 μM of fluorogenic substrate, MOCAc-Ser-Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe-Arg-Lys-Arg-Arg-NH2   (Peptide Institute, Inc., Osaka, Japan), at 37 °C for 1 h.   Absorbance at 405 nm was measured on a fluorescence plate reader.   NEP activity was determined using the NEP-specific fluorogenic peptide substrate, *N*-dansyl-D-Ala-Gly-p-(nitro)-Phe-Gly (DAGPNG; Sigma Chem. Co., St Louis, MO, USA).   Briefly, 50 μg brain membrane protein was incubated with 50 μM DAGPNG and 20 μM elanapril dissolved in 0.5 mL Tris-HCl, pH 7.4.   Then,

0.8 mU leucine aminopeptidase was added to the reaction mixture and further incubated for 20 min at 37 °C. Following centrifugation at 3000 xg for 5 min, the supernatant was removed and fluorescence (340 nm excitation, 520 nm emission) was read on a fluorescence plate reader.

Protein Kinase C (PKC) Activity Assay. Sample (100 µL) was incubated with a salt solution for 15 min in the presence or absence of 100 µM PKC-specific substrate, and followed by adding 5 mg/mL digitonin and 1 mM ATP mixed with γ-[^{32}P]ATP (<1500 cpm/pmol). The reaction was stopped by 5% trichloroacetic acid, and then spotted onto P81 phosphocellulose paper and washed with phosphoric acid and acetone. The amount of incorporated radioactivity into the substrate was determined by scintillation counting. PKC activity was normalized by the corresponding protein content.

Measurement of CML, Pentosidine, Sorbitol and Fructose Content. CML was immunochemically determined with ELISA technique using the CML-specific monoclonal antibody 4G9 and calibration with 6-(N-carboxymethylamino)caproic acid (Roche Diagnostics, Penzberg, Germany). Intra- and interassay variability were 5.3 and 6.2%, respectively. Pentosidine was analyzed by a HPLC equipped with a C18 reverse-phase column and a fluorescence detector according to the method described in Miyata et al. (*13*). Briefly, sample was lyophilized and acid hydrolyzed in 500 µL 6 N HCl for 16 h at 110 °C in screw-cap tubes purged with nitrogen. After neutralization with NaOH and diluted with PBS, sample was used for HPLC measurement. In addition, 100 mg brain was homogenized with PBS (pH 7.4) containing U-[^{13}C]-sorbitol as an internal standard. After precipitating protein by ethanol, the supernatant was lyophilized. The content of sorbitol and fructose in each lyophilized sample was determined by liquid chromatography with tandem mass spectrometry, according to the method of Guerrant and Moss (*14*).

Activity of Aldose Reductase (AR) and Sorbitol Dehydrogenase (SDH). The method of Nishinaka and Yabe-Nishimura (*15*) was used to measure AR activity in brain by monitoring the decrease in absorbance at 340 nm due to NADPH oxidation. SDH activity was assayed according to the method of Ulrich (*16*) by mixing 100 μL homogenate, 200 μL NADH (12 mM) and 1.6 mL triethanolamine buffer (0.2 M, pH 7.4), and monitoring the absorbance change at 365 nm.

Determination of Malonyldialdehyde (MDA), ROS, Protein Carbonyl and Glutathione (GSH) Levels. MDA, an index of lipid peroxidation, was measured by using a commercial assay kit (OxisResearch, Portland, OR, USA). The method described in Gupta et al. (*17*) was used to measure ROS level. Briefly, 10 mg tissue was homogenized in 1 mL of ice cold 40 mM Tris–HCl buffer (pH 7.4), and further diluted to 0.25% with the same buffer. Then, samples were divided into two equal fractions. In one fraction, 40 μL 1.25 mM 2', 7'-dichlorofluorescin diacetate in methanol was added for ROS estimation. Another fraction, in which 40 μL methanol was added, served as a control for auto fluorescence, which was determined at 488 nm excitation and 525 nm emission using a fluorescence plate reader. Protein carbonyls were determined with the Zentech PC kit (BioCell, Auckland, New Zealand). Briefly, 50 μL sample was mixed with a 200 μL dinitrophenylhydrazine (DNP) solution. The adsorbed DNP–protein was reacted with an anti-DNP-biotin antibody, and followed by reacting with streptavidin-linked horseradish peroxidase probe and chromatin reagent. The absorbance at 450 nm was measured. GSH concentration was determined by a commercial colorimetric GSH assay kit according to the manufacturer's instruction (OxisResearch, Portland, OR, USA). Reduced GSH was determined in this study.

Catalase, Superoxide Dismutase (SOD) and GPX Activity Assay. The activities of catalase, SOD, and GPX were determined by catalase, SOD, and GPX assay kits

1 (Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA).   The enzyme activity was

2 expressed in U/mg protein.

3   **Real-time Polymerase Chain Reaction (RT-PCR) for mRNA Expression.** Brain

4 tissue was homogenized in guanidinethiocyanate, and total RNA was isolated using Trizol

5 reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA).   One µg RNA was used to

6 generate cDNA, which was amplified using Taq DNA polymerase.   PCR was carried out

7 in 50 µL of reaction mixture containing Taq DNA polymerase buffer (20 mM Tris-HCl,

8 pH 8.4, 50 mM KCl, 200 mM dNTP, 2.5 mM $MgCl_2$, 0.5 mM of each primer) and 2.5 U

9 Taq DNA polymerase.   The specific oligonucleotide primers of targets are shown in

10 Table 1.   The cDNA was amplified under the following reaction conditions: 95 ºC for 1

11 min, 55 ºC for 1 min, and 72 ºC for 1 min.   28 cycles were performed for

12 glyceraldehyde-3-phosphate dehydrogenase (GAPDH, the housekeeping gene) and 35

13 cycles were performed for others.   Generated fluorescence from each cycle was

14 quantitatively analyzed by using the Taqman system based on real-time sequence

15 detection system (ABI Prism 7700, Perkin-Elmer Inc., Foster City, CA, USA).   In this

16 study, mRNA level was calculated as percentage of the control group.

17   **Statistical Analysis.** The effect of each treatment was analyzed from ten different

18 preparations (n = 10).   Data were reported as means ± standard deviation (SD), and

19 subjected to analysis of variance (ANOVA).   Differences among means were determined

20 by the Least Significance Difference Test with significance defined at $P<0.05$.

21

22 **RESULTS**

23   As shown in Table 2, DG treatment and intake of SAC, SEC and SPC did not

24 significantly affected water intake, body weight and brain weight ($P>0.05$).   DG

25 treatment significantly increased the formation of $A\beta_{1-40}$ and $A\beta_{1-42}$ in brain (Table 3,

$P<0.05$); however, the intake of three test compounds significantly decreased the brain level of $A\beta_{1-40}$ and $A\beta_{1-42}$ ($P<0.05$). DG treatment enhanced mRNA expression of APP and BACE1, and reduced NEP expression in brain (Figure 1, $P<0.05$). The intake of test compounds lowered APP and BACE1 expression ($P<0.05$); but failed to affect NEP expression ($P>0.05$). As shown in Table 4, DG enhanced BACE1 activity and decreased NEP activity ($P<0.05$); the intake of test compound significantly reduced BACE1 activity only ($P<0.05$). DG treatments also declined brain PKC activity and mRNA expression of PKC-alpha, PKC-beta and PKC-gamma (Figure 2, $P<0.05$). Intake of test compounds significantly retained PKC activity and the expression of PKC-alpha and PKC-gamma ($P<0.05$).

Brain level of CML, pentosidine, sorbitol and fructose was significantly increased in DG-treated mice (Table 5, $P<0.05$). Three test compounds significantly reduced the levels of these parameters ($P<0.05$). As shown in Table 6 and Figure 3, DG treatments elevated brain activity and mRNA expression of AR and SDH ($P<0.05$); however, three test compounds significantly lowered AR activity and expression ($P<0.05$), but did not affect SDH activity and expression ($P>0.05$). As shown in Table 7, intake of test compounds significantly raised GSH content in brain from mice without DG treatment ($P<0.05$). DG treatments led to a significant increase in the formation of MDA, ROS and protein carbonyl; and a decrease in GSH level ($P<0.05$); however, the intake of test compounds in DG-treated mice significantly reduced MDA, ROS and protein carbonyl levels, and restored GSH level in brain ($P<0.05$). DG treatments lowered brain activity of GPX, SOD and catalase (Table 8, $P<0.05$); however, the intake of test compounds significantly retained brain GPX, SOD and catalase activities ($P<0.05$).

DISCUSSION

9

1   As reported by others (*11, 12*) and our present study, DG treatments caused AD-like

2   pathological characteristics such as increased Aβ deposition, AGEs generation and

3   oxidative stress.   Our present study further found that the intake of SAC, SEC and SPC

4   down-regulated APP mRNA expression, diminished activity and expression of BACE1,

5   retained PKC activity and expression, and decreased Aβ formation.   In addition, we

6   notified that these compounds markedly reduced the production of CML, pentosidine,

7   ROS, protein carbonyl, and declined AR activity, as well as maintained activity of GPX

8   and SOD, which contributed to attenuate glycative and oxidative stress in brain of

9   DG-treated mice.   These novel findings suggested that these cysteine-containing

10   compounds were able to penetrate blood brain barrier and exhibited anti-Aβ,

11   anti-glycative and anti-oxidative neuro-protection to alleviate AD progression.

12   Furthermore, these compounds might exert their functions at the level of transcription

13   because they mediated mRNA expression of several factors.

14   Both APP level and BACE1 activity are crucial factors responsible for Aβ

15   accumulation and AD progression (*18*).   So far, decreasing APP and/or inhibiting

16   BACE1 have been considered as targets for developing therapeutic strategy for AD (*18,*

17   *19*). $A\beta_{1-40}$ and $A\beta_{1-42}$ are major Aβ forms occurred in brain tissue and circulation of AD

18   patients, they also play clinical significance for AD deterioration because both are

19   insoluble and able to cause aggregation of amyloid plaques (*20, 21*).   In our present study,

20   the intake of test compounds lowered BACE1 activity, and repressed the mRNA

21   expression of APP and BACE1, which subsequently decreased available Aβ precursors

22   and lowered Aβ formation.   Our data regarding brain levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ also

23   agreed that the supplementation of test compounds reduced Aβ production.   These

24   results indicated that these compounds exhibited anti-Aβ action via mediating APP and

1 BACE1. On the other hand, Aβ could be degraded by a variety of proteases including

2 NEP, which favors Aβ catabolism and clearance (*22*). In our present study, treatments

3 from cysteine-containing compounds failed to affect NEP expression and activity. Thus,

4 the anti-Aβ effects of these agents was not due to they regulate this protease. It is

5 reported that PKC activation lowered Aβ accumulation via modulating

6 non-amyloidogenic pathway of APP cleavage and led to the generation of soluble αAPP,

7 which was released into the extracellular media, and precluded the deposition of Aβ (*23,*

8 *24*). Our results revealed that SAC, SEC and SPC restored PKC activity, and improved

9 DG-caused down-regulation in PKC-alpha and PKC-gamma expression in mice brain

10 under AD-like condition, which further benefited APP cleavage and decreased the

11 available APP for Aβ formation. These findings suggested that the anti-Aβ action of

12 these test compounds might be partially due to they enhance PKC activation.

13 Enhanced glycative stress is involved in AD progression. CML and pentosidine,

14 two AGEs, have been implied in AD associated pathological development (*6, 25*). The

15 lower generation of CML and pentosidine in brain tissue of test compound-treated mice as

16 we observed indicated that glycative injury in those mice has been mitigated. Aldose

17 reductase and sorbitol dehydrogenase are two key enzymes responsible for AGEs

18 generation in polyol pathway. Increased activity and expression of these enzymes

19 facilitate the production of sorbitol and fructose, which in turn promote AGEs formation

20 and glycative stress (*26, 27*). Our present study found that three test compounds

21 declined both activity and mRNA expression of aldose reductase, which subsequently

22 decreased the production of sorbitol. Although these test compounds failed to affect

23 SDH activity and expression, it is highly possible that the lower available sorbitol further

24 decreased fructose production. Since fructose and sorbitol levels had been reduced, the

observed lower formation of CML and pentosidine could be partially explained. These findings indicated that these compounds could alleviate glycative stress in brain via suppressing polyol pathway and inhibiting AGEs formation, which consequently contributed to attenuate AD progression.

Oxidative damage is another hallmark of AD (*28*). It is reported that AD patients had increased carbonyl compounds and/or decreased GSH level in circulation (*29, 30*). In our present study, the intake of test compounds increased GSH content in brain from mice without DG treatment. This finding implied that these agents might be able to spare GSH and favor GSH homeostasis, which definitely contributed to enhance anti-oxidative protection for this tissue. In addition, we found the supplement of these compounds not only maintained GSH level but also effectively restored GPX, catalase and SOD activities in brain from DG-treated mice. These results explained the lower formation of MDA, ROS and protein carbonyl, and indicated that SAC, SEC and SPC could abate oxidative injury in brain of DG-treated mice via both non-enzymatic and enzymatic antioxidant protective actions. It is reported that Aβ deposition favors free radicals generation and exacerbates oxidative damage (*31*). In our present study, test compounds markedly reduced Aβ production in brain, which might in turn benefit lowering free radicals formation and mitigating oxidative stress. On the other hand, it is known that free radicals could promote AGEs formation (*32*). Thus, it is highly possible that the lower AGEs production in brain from test compound-treated mice as we observed was partially due to these compounds diminish free radicals generation via their anti-oxidative activities.

It has been documented that AD could be considered as type 3 diabetes mellitus (DM) because both DM and AD shared common pathological features including oxidative and glycative stress (*33, 34*). The results of our present study revealed that AD progression

not only increased the production of AGEs but also enhanced the activity and expression of glycation associated enzyme such as AR in brain. These findings supported that there was a closed link between AD and DM, and agreed that AD was a form of DM. Thus, the agents with anti-AD activity might also benefit the prevention or treatment of DM. It is interesting to find that the neuro-protective effects from three test agents in DG-treated mice was different because SAC was more effective in maintaining GPX activity, SEC was marked in restoring PKC-gamma expression and SPC was greater than other agents in lowering $A\beta_{1-40}$ generation and suppressing BACE1 expression and activity. Obviously, the allyl group of SAC, ethyl group of SEC and propyl group of SPC played important roles in determining their bioactivities. These compounds are hydrophilic peptide derivates and naturally formed in *Allium* foods such as garlic. Further studies are necessary to ensure the safety of these agents before they are applied for human. It is known that the content of these cysteine-containing compounds in *Allium* plants is dependent on the species or vegetation period (*35*). Thus, it may not be always possible to obtain these compounds by supplementing the diet with garlic or other *Allium* plants.

In conclusion, the treatments from s-allyl cysteine, s-ethyl cysteine or s-propyl cysteine effectively decreased $A\beta$ production, and alleviated glycative and oxidative stress in DG-treated mice. These agents exhibited anti-$A\beta$, anti-glycative and anti-oxidative effects via suppressing APP and BACE1 expression, retaining activity and expression of PKC, declining activity and expression of AR, as well as enhancing activity of GPX, SOD and catalase. These findings support that these compounds were potent agents against the progression of neurodegenerative disorders such as Alzheimer's disease.

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1   **Table 1**. Forward and reverse primers for real time PCR analysis.

Target	forward	reverse
APP	5′-GAC TGA CCA CTC GAC CAG GTT CTG-3'	5'-CTT GAA GTT GGA TTC TCA TAC CG-3′
BACE1	5'-TTG CCC AAG AAA GTA TTT GA-3'	5'-TGA TGC GGA AGG ACT GAT T-3'
NEP	5'-GAC CTA CCG GCC AGA GTA-3'	5'-AAA CCC GAC ATT TCC TTT-3'
PKC-alpha	5'-GAA CCA TGG CTG ACG TTT AC-3'	5'-GCA AGA TTG GGT GCA CAA AC-3'
PKC-beta	5'-TTC AAG CAG CCC ACC TTC TG-3'	5'-AAG GTG GCT GAA TCT CCT TG-3'
PKC-gamma	5'-GAC CCC TGT TTT GCA GAA AG-3'	5'-GTA AAG CCC TGG AAA TCA GC-3'
AR	5'-CCC AGG TGT ACC AGA ATG AGA-3'	5'-TGG CTG CAA TTG CTT TGA TCC-3'
SDH	5'-TGG GAG CTG CTC AAG TTG TG-3′	5′-GGT CTC TTT GCC AAC CTG GAT-3′
GAPDH	5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3'	5'-CCT TGG AGG CCA TGT AGG CCA T-3'

1 **Table 2.** Water intake, body weight and brain weight of mice with or without

2 d-galactose (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC or SPC.

3 Data are mean±SD (n = 10).

	Water intake mL/day/mouse	Body weight g	Brain weight g
Control	2.9 ± 0.4^a	29.5 ± 2.0^a	0.56 ± 0.08^a
SAC	2.5 ± 0.6^a	30.3 ± 1.4^a	0.58 ± 0.11^a
SEC	3.0 ± 0.5^a	29.0 ± 1.8^a	0.52 ± 0.06^a
SPC	2.7 ± 0.6^a	28.9 ± 2.2^a	0.60 ± 0.09^a
DG	3.1 ± 0.3^a	28.8 ± 1.7^a	0.53 ± 0.05^a
DG+SAC	2.4 ± 0.7^a	29.4 ± 1.9^a	0.57 ± 0.09^a
DG+SEC	2.8 ± 0.5^a	30.1 ± 1.3^a	0.59 ± 0.10^a
DG+SPC	2.9 ± 0.4^a	29.0 ± 2.1^a	0.56 ± 0.07^a

4 [a]Means in a column without a common letter differ, $P<0.05$.

5

1 **Table 3.** Level (pg/mg protein) of $A\beta_{1-40}$ and $A\beta_{1-42}$ in brain from mice with or without

2 d-galactose (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC or SPC.

3 Data are mean±SD (n = 10).

	$A\beta_{1-40}$	$A\beta_{1-42}$
Control	0.14±0.03[a]	0.10±0.04[a]
SAC	0.11±0.04[a]	0.12±0.05[a]
SEC	0.13±0.05[a]	0.11±0.03[a]
SPC	0.10±0.02[a]	0.09±0.04[a]
DG	2.43±0.19[d]	2.51±0.21[c]
DG+SAC	1.65±0.13[c]	1.42±0.17[b]
DG+SEC	1.57±0.10[c]	1.45±0.10[b]
DG+SPC	1.22±0.11[b]	1.32±0.08[b]

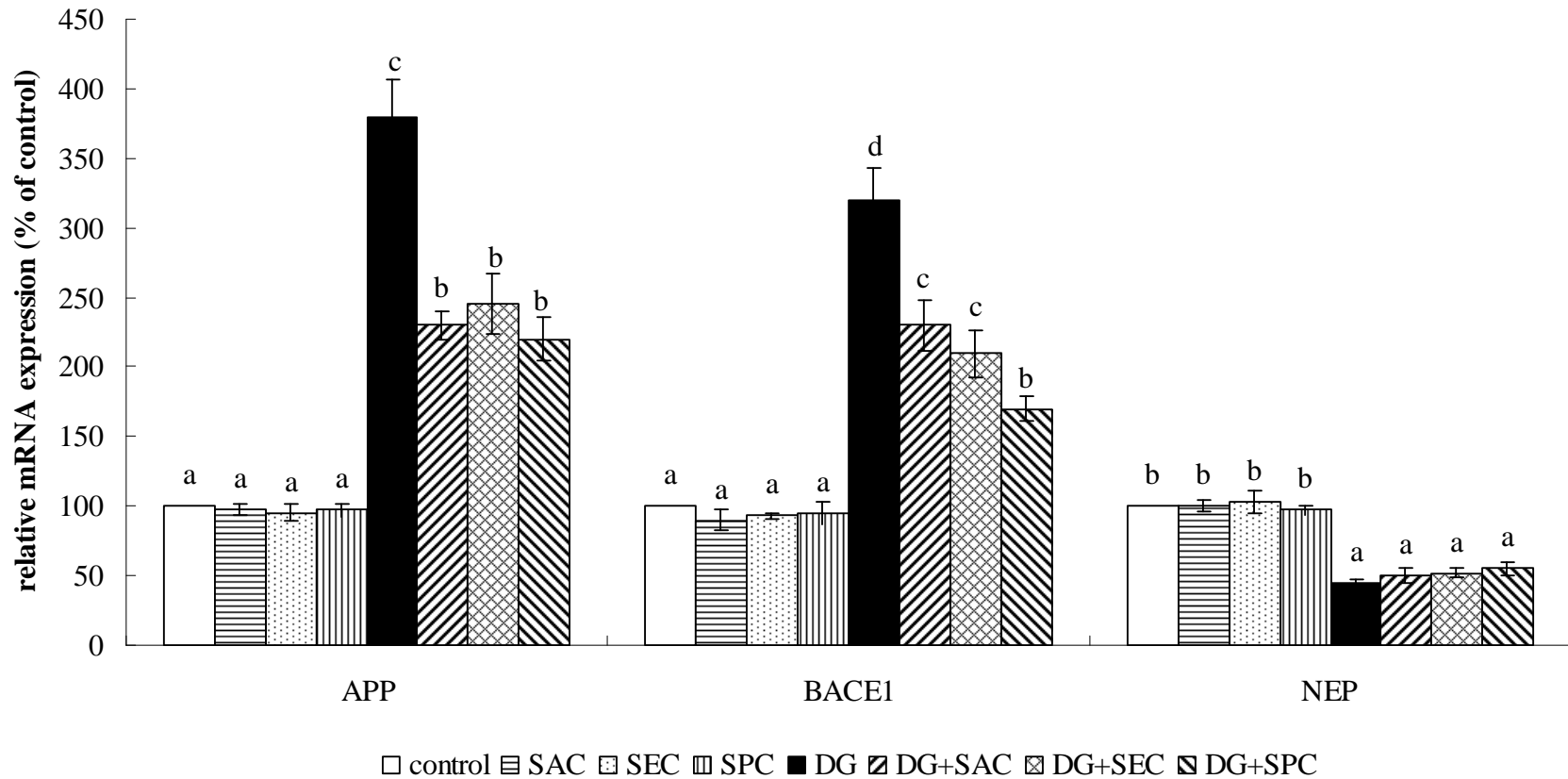4 [a-d]Means in a column without a common letter differ, $P<0.05$.

1 **Table 4.** Activity (nmol/mg protein) of BACE1 and NEP in brain from mice with or

2 without d-galactose (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC or

3 SPC.   Data are mean±SD (n = 10).

	BACE1	NEP
Control	7.62 ± 0.61^{a}	2.50 ± 0.24^{a}
SAC	7.34 ± 0.45^{a}	2.61 ± 0.19^{a}
SEC	7.28 ± 0.52^{a}	2.48 ± 0.22^{a}
SPC	7.53 ± 0.38^{a}	2.53 ± 0.27^{a}
DG	13.70 ± 0.89^{d}	1.03 ± 0.11^{b}
DG+SAC	11.43 ± 0.72^{c}	1.17 ± 0.09^{b}
DG+SEC	11.21 ± 0.68^{c}	1.14 ± 0.15^{b}
DG+SPC	9.36 ± 0.70^{b}	1.12 ± 0.16^{b}

4 [a-d]Means in a column without a common letter differ, $P<0.05$.
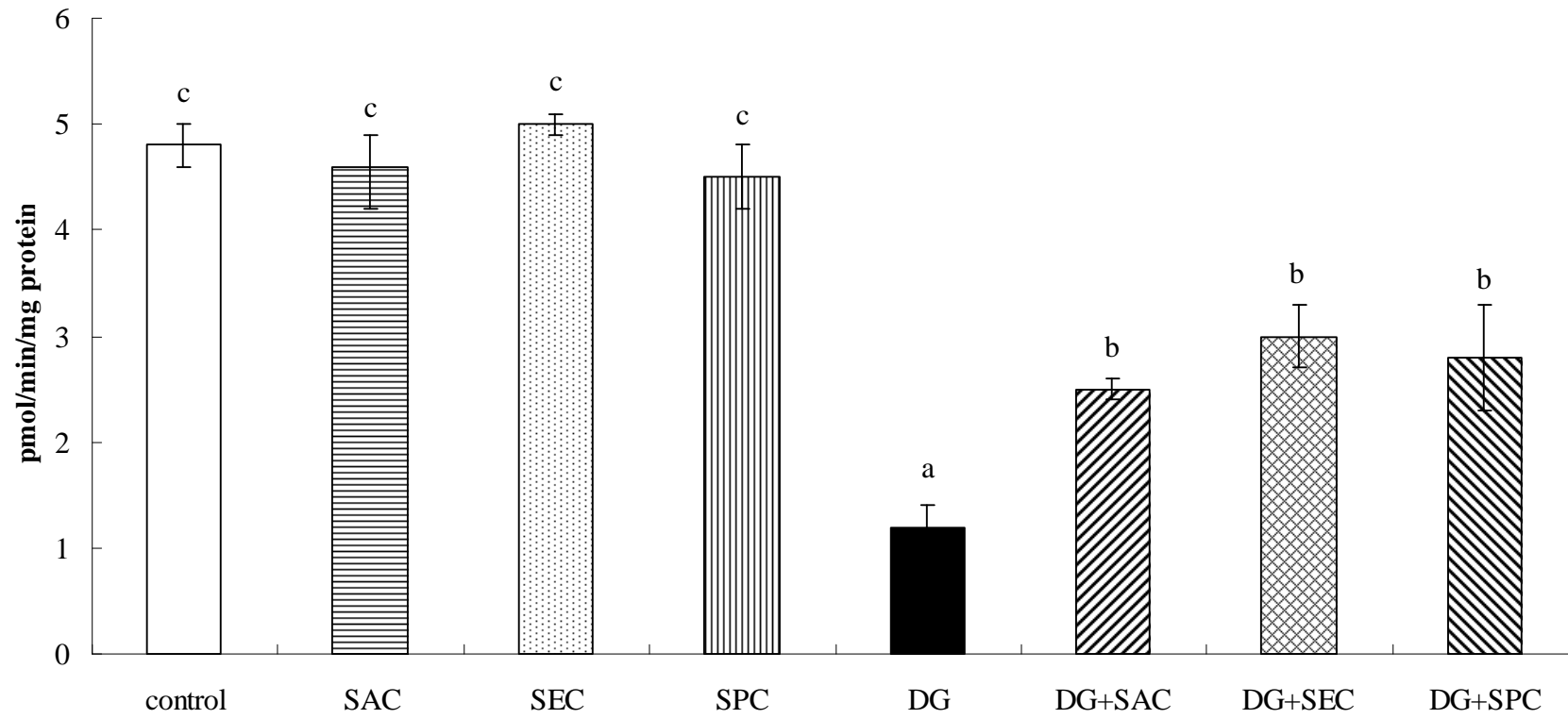
5

1   **Figure 1.** mRNA expression of APP, BACE1 and NEP in brain from mice with or

2   without d-galactose (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC or

3   SPC.   Data are mean±SD (n = 10).   [a-d]Means among bars without a common letter
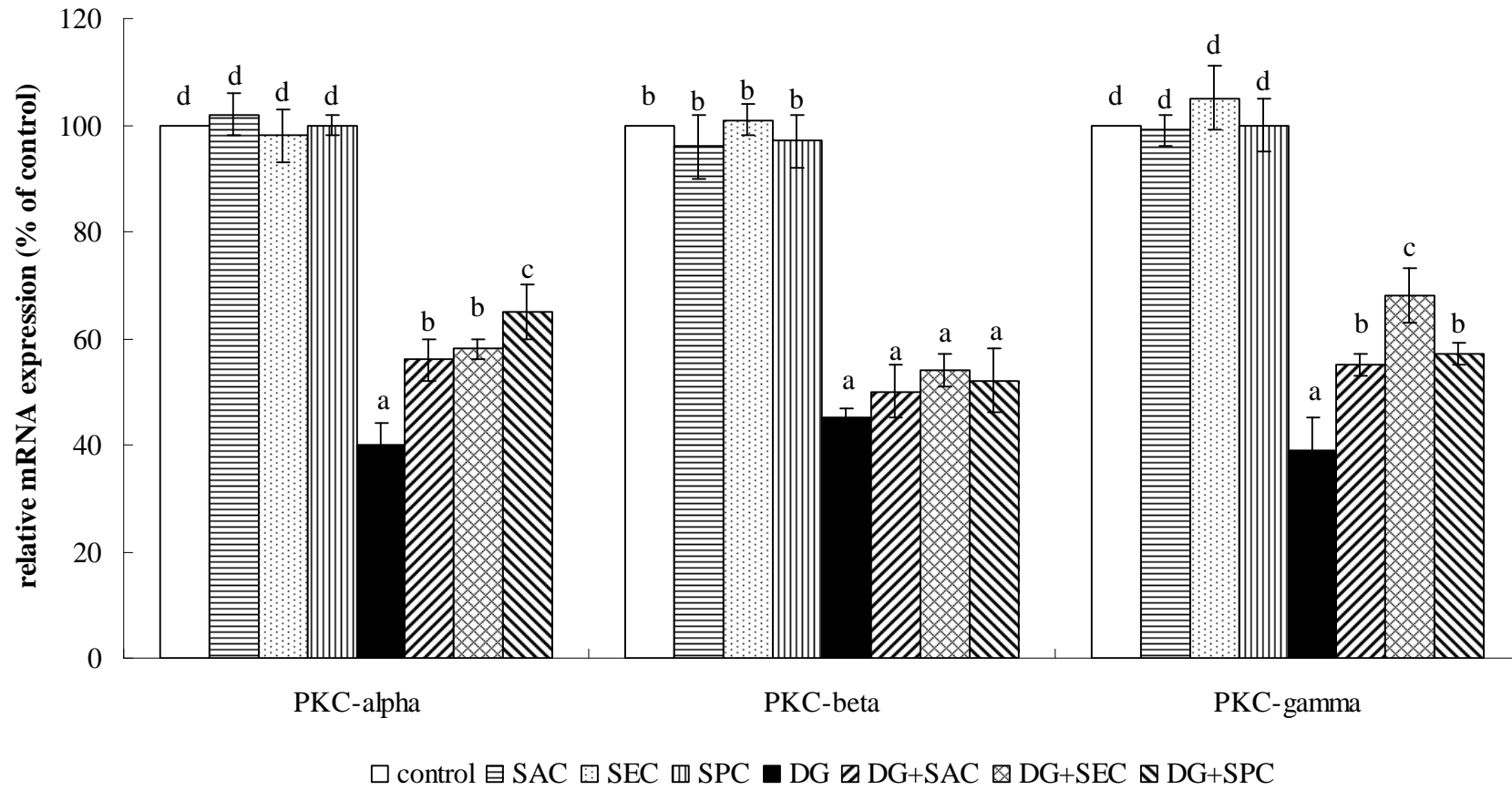
4   differ, $P<0.05$.

1   **Figure 2.** Activity (pmol/min/mg protein, upper part) and expression (lower part) of

2   PKC in brain from mice with or without d-galactose (DG) treatment and consumed 0

3   (control) or 1 g/L SAC, SEC or SPC. Data are mean±SD (n = 10). [a-d]Means

4   among bars without a common letter differ, $P<0.05$.

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1

1   **Table 5.** Level of CML, pentosidine, sorbitol and fructose in brain from mice with or without d-galactose (DG) treatment and consumed 0

2   (control) or 1 g/L SAC, SEC or SPC.   Data are mean±SD (n = 10).

	CML	Pentosidine	Sorbitol	Fructose
	pmol/mg protein	pmol/mg protein	nmol/mg protein	nmol/mg protein
Control	6 ± 3^a	0.20 ± 0.07^a	3.21 ± 0.31^a	13.0 ± 1.4^a
SAC	4 ± 2^a	0.17 ± 0.04^a	3.06 ± 0.24^a	12.8 ± 0.9^a
SEC	5 ± 3^a	0.19 ± 0.05^a	2.89 ± 0.19^a	12.7 ± 1.0^a
SPC	5 ± 4^a	0.18 ± 0.06^a	3.20 ± 0.26^a	12.8 ± 0.6^a
DG	64 ± 9^c	1.39 ± 0.12^c	8.75 ± 0.67^d	72.9 ± 5.8^c
DG+SAC	32 ± 5^b	0.67 ± 0.05^b	5.13 ± 0.21^b	30.1 ± 2.7^b
DG+SEC	30 ± 6^b	0.56 ± 0.07^b	6.09 ± 0.33^c	29.7 ± 2.2^b
DG+SPC	34 ± 7^b	0.71 ± 0.08^b	5.16 ± 0.38^b	31.0 ± 3.0^b

3   [a-d]Means in a column without a common letter differ, $P<0.05$.

1 **Table 6.** Activity of AR (nmol/min/mg protein) and SDH (U/g protein) in brain from

2 mice with or without d-galactose (DG) treatment and consumed 0 (control) or 1 g/L

3 SAC, SEC or SPC.   Data are mean±SD (n = 10).

	AR	SDH
Control	1.07 ± 0.25^a	4.11 ± 0.61^a
SAC	0.98 ± 0.16^a	4.14 ± 0.57^a
SEC	1.03 ± 0.19^a	3.96 ± 0.45^a
SPC	1.05 ± 0.08^a	4.02 ± 0.64^a
DG	3.82 ± 0.30^d	7.81 ± 1.02^b
DG+SAC	2.40 ± 0.24^c	7.12 ± 0.84^b
DG+SEC	1.68 ± 0.16^b	7.20 ± 0.69^b
DG+SPC	2.29 ± 0.19^c	7.27 ± 0.93^b

4 [a-d]Means in a column without a common letter differ, *P*<0.05.

5

1   **Figure 3.** Expression of AR and SDH in brain from mice with or without d-galactose

2   (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC or SPC. Data are

3   mean±SD (n = 10). [a-d]Means among bars without a common letter differ, $P<0.05$.
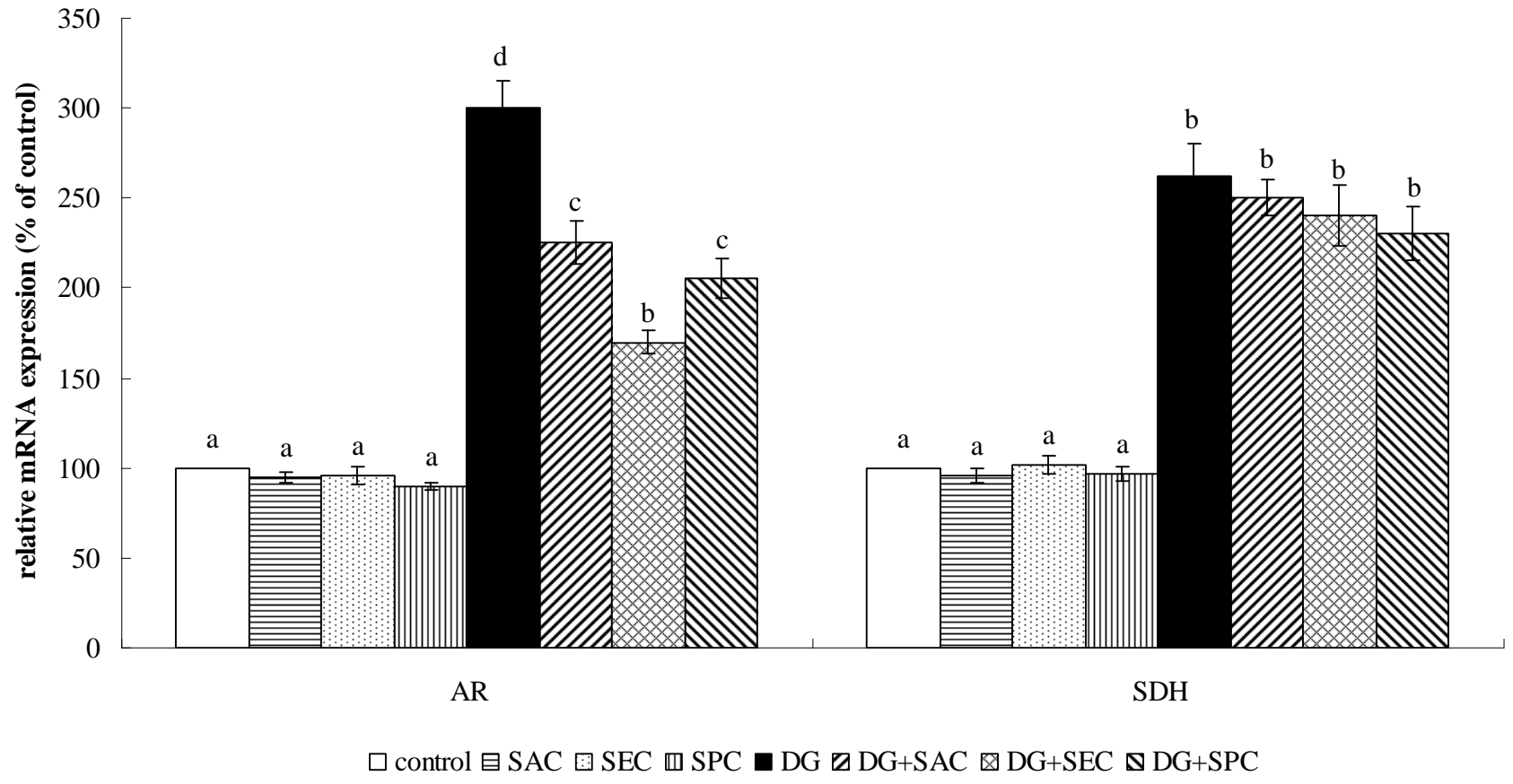
1   **Table 7.** Level of MDA, ROS, protein carbonyl and GSH in brain from mice with or without d-galactose (DG) treatment and consumed 0

2   (control) or 1 g/L SAC, SEC or SPC.   Data are mean±SD (n = 10).

	MDA	ROS	protein carbonyl	GSH
	μmol/mg protein	nmol/mg protein	pmol/mg protein	ng/mg protein
Control	0.26 ± 0.10^a	0.24 ± 0.07^a	16.8 ± 1.2^a	94 ± 8^c
SAC	0.21 ± 0.08^a	0.17 ± 0.09^a	14.1 ± 1.3^a	130 ± 12^d
SEC	0.20 ± 0.06^a	0.18 ± 0.05^a	15.2 ± 0.9^a	126 ± 15^d
SPC	0.18 ± 0.05^a	0.17 ± 0.04^a	14.5 ± 1.0^a	125 ± 10^d
DG	1.51 ± 0.33^c	1.37 ± 0.23^c	141.9 ± 7.3^c	46 ± 5^a
DG+SAC	1.02 ± 0.25^b	0.94 ± 0.16^b	80.1 ± 4.7^b	97 ± 7^c
DG+SEC	1.05 ± 0.17^b	1.03 ± 0.13^b	84.2 ± 5.0^b	79 ± 6^b
DG+SPC	0.92 ± 0.06^b	0.87 ± 0.10^b	82.1 ± 5.2^b	77 ± 6^b

3   [a-d]Means in a column without a common letter differ, $P<0.05$.

1 **Table 8.** Activity (U/mg protein) of GPX, SOD and catalase in brain from mice with

2 or without d-galactose (DG) treatment and consumed 0 (control) or 1 g/L SAC, SEC

3 or SPC.   Data are mean±SD (n = 10).

	GPX	SOD	catalase
Control	22.0 ± 2.3^d	7.3 ± 1.5^c	2.7 ± 0.6^c
SAC	24.1 ± 2.6^d	7.5 ± 1.7^c	2.6 ± 0.8^c
SEC	23.5 ± 2.0^d	7.7 ± 1.2^c	2.9 ± 0.4^c
SPC	22.9 ± 2.2^d	7.8 ± 1.4^c	2.3 ± 0.7^c
DG	8.8 ± 1.0^a	1.9 ± 0.7^a	0.6 ± 0.2^a
DG+SAC	16.2 ± 1.6^c	3.7 ± 1.0^b	1.2 ± 0.5^b
DG+SEC	12.9 ± 1.4^b	4.0 ± 0.9^b	1.4 ± 0.4^b
DG+SPC	13.3 ± 1.5^b	4.2 ± 1.1^b	1.5 ± 0.3^b

4 [a-d]Means in a column without a common letter differ, $P<0.05$.