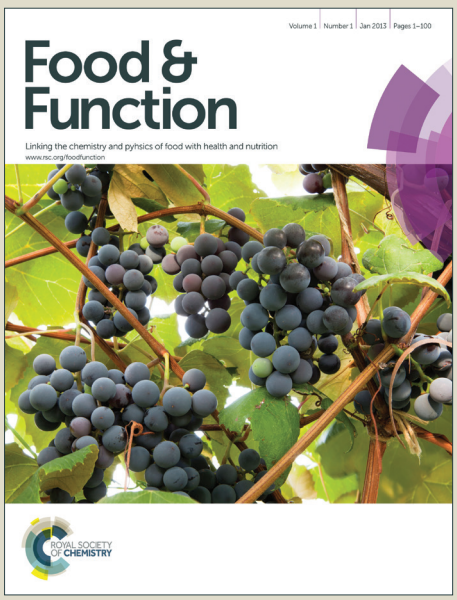
# Food & Function

Accepted Manuscript




This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

*Accepted Manuscripts* are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

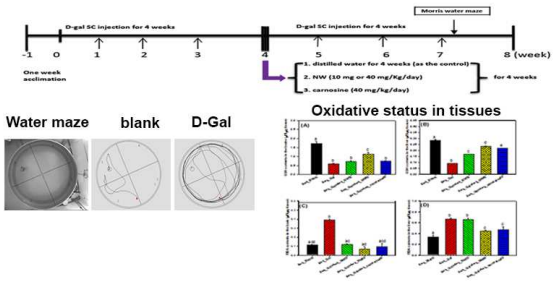
www.rsc.org/foodfunction

# Asn-Trp dipeptides improve the oxidative stress and learning dysfunctions in D-galactose-induced BALB/c mice

Chuan-Hsiao Han, Yin-Shiou Lin, Tai-Lin Lee, Hong-Jen Liang, Wen-Chi Hou

## Table of Content Entry

Asn-Trp (NW) interventions for 4 weeks can attenuate the oxidative stress and learning dysfunctions induced by daily D-galactose subcutaneous injection for 8 weeks



# Asn-Trp dipeptides improve the oxidative stress and learning dysfunctions in D-galactose-induced BALB/c mice

Chuan-Hsiao Han,[a,b,#] Yin-Shiou Lin,[c,#] Tai-Lin Lee,[a] Hong-Jen Liang,[d,e,*] and Wen-Chi Hou[c,e,*]

[a]School of Pharmacy, Taipei Medical University, Taipei, Taiwan

[b]Department of Health and Creative Vegetarian Science, Fo Guang University, Yilan, Taiwan

[c]Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan

[d]Department of Food Science, Yuanpei University, Hsinchu, Taiwan

[e]Traditional Herbal Medicine Research Center, Taipei Medical University Hospital, Taipei, Taiwan

[#]These two authors contributed equally to this study.

***To whom correspondence should be addressed:**

Prof. Hou, Wen-Chi (Fax: 886 (2) 2378-0134; E-mail: wchou@tmu.edu.tw)

Or

Prof. Liang, Hong-Jen (Fax: 886(3)610-2379; Email: jenliang@mail.ypu.edu.tw)

## ABSTRACT

The oral administrations of Asn-Trp (NW) or carnosine (β-alanyl-L-histidine) dipeptides to D-galactose (Gal)-induced BALB/c mice were used to evaluate antioxidant activities *in vivo*. The D-galactose (Gal) was subcutaneously injected into the dorsal necks of mice daily for eight weeks to induce oxidative stress (Gal group). From the beginning of the fifth week, groups of NW10, NW40 (10 or 40 mg NW $kg^{-1}$) or carnosine40 (40 mg carnosine $kg^{-1}$) were administered orally concurrent Gal injection to the end of studies. It was found that the malondialdehyde (MDA) contents in these intervention groups were much lower than the Gal group. The mice in NW40 group showed significantly improvements compared to the Gal group in reference memory task and probe trial test evaluated by Morris water maze. Mice in the intervention groups showed higher GSH levels and oxygen radical antioxidant capacity activities and lower MDA levels in the brain or liver tissues compared to the Gal group. The levels of advanced glycation end-products, including $N^{\varepsilon}$-(carboxymethyl)lysine (CML) and argpyrimidine, in the brain tissues of the NW40 interventions showed significantly lower compared to the Gal group. These results suggest that NW may be useful in developing functional foods for antioxidant and anti-aging purposes.

## 1. Introduction

Proteins or peptides are reported to be bioactive *in vitro*, as seen in antioxidant activity[1–4], antihypertensive activity[5–7], immunomodulatory activity[8–10] and anti-atherosclerotic activity[11], which may have regulatory functions in humans beyond involvement in nutrition alone.[12–15] The well-known peptides, such as Val-Pro-Pro and Ile-Pro-Pro, are obtained from milk fermentation by *Lactobacillus helveticus* with antihypertensive activities[16]; the reduced glutathione is a tripeptide that has critical, protective, and physiological functions in cells[17], with angiotensin converting enzyme inhibitory activity[18], as well as antioxidant activities.[3,4] Carnosine, a dipeptide of β-alanyl-L-histidine, is also known to exhibit antioxidant activities[19] and angiotensin converting enzyme inhibitory activity[18], and to delay aging in cultured cells.[20]

Reactive oxygen species (ROS) are related to various chronic diseases, such as diabetes mellitus, cancer, aging, cardiovascular diseases, and neurodegenerative diseases.[21] Several theories have been proposed with respect to the aging process, one of which is the "Free Radical Theory of Aging."[22] The respiratory chain reactions and glycation-related cell responses may be major ROS sources in cells. Glycation, also known as the Maillard reaction, involves nonenzymatic Schiff-based modifications of a specific amino acid side chain in proteins *in vitro* and *in vivo* via the reduction of sugars or sugars' metabolized intermediates or lipid peroxidation products, such as glyoxal or methylglyoxal, to form irreversibly advanced glycation end-products (AGEs).[23] AGEs can interact

with the receptor for AGE (RAGE) to promote ROS production or via NF-κB signaling pathways to express proinflammatory cytokines, such as TNF-α and IL-6.[24,25] AGEs, including $N^{\varepsilon}$-(carboxymethyl)lysine (CML) or argpyrimidine, etc., have been chemically characterized.[23,26] Increasing evidence suggests that AGE formation and accumulation during normal aging are all highly correlated with cardiovascular disease and diabetes complications.[27] We have previously found that Asn-Trp (NW) dipeptides performed much better in antioxidant activities than carnosine *in vitro* and had a similar anti-AGE formation capacity using galactose/bovine serum albumin (BSA) model systems.[4] Therefore, in this study, D-galactose (Gal) was used to induce oxidative stress in BALB/c mice. Meanwhile, NW or carnosine intervention was employed to evaluate *in vivo* the improvements on antioxidant activities, anti-AGE formations and anti-aging performance using Morris water maze evaluations.

## 2. Materials and methods

### 2.1. Materials

Carnosine and D-galactose were obtained from Sigma Chemical Co. (St. Louis, MO, USA). NW dipeptide was synthesized by Mocell Biotech Co., Ltd (Shanghai, China) with purity greater than 95% by HPLC chromatography and mass spectroscopy. Anti-argpyrimidine antibody was obtained from Cosmo Bio Co. Ltd. (Tokyo, Japan), anti-CML antibody (ab27684) was obtained from Abcam Inc. (Cambridge, MA), and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and

HRP-conjugated goat anti-rabbit IgG were from Sigma Chemical Co. (St. Louis, MO, USA). Glutathione (GSH) was measured using glutathione assay kits (No. 703002; Cayman Chemical Co., MI, USA), malondialdehyde (MDA) was measured using BIOXYTECH® MDA-586™ assay kits (*OXIS* Research™, OR, USA), and oxygen radical absorbance capacity (ORAC) was measured using the OxiSelect™ assay kit (STA-345, Cell Biolabs Inc., San Diego, CA).

**2.2. Animals and experimental protocols**

Male 10-week-old BALB/c mice (N=50) were purchased from National Laboratory Animal Center (Taipei, Taiwan). Each mouse was individually housed in wire-bottomed stainless steel cages in a temperature- and humidity-controlled room (at 22°C) with a 12-h light/dark cycle. The mice had free access to standard mouse/rat chow (Prolab® RMH2500, 5P14 Diet; PMI Nutrition International, Brentwood, MO, USA) and water. All animal experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Taipei Medical University (LAC-99-0142). After acclimation for one week, mice were randomly divided into 5 groups (N=10 for each group), including a blank group and 4 Gal-induced groups (the induced and 3 peptide-intervened groups). Oxidative damage was induced in the Gal-induced group by using Gal, following a modification of a previously described procedure.[28-30] Gal (12 g dissolved in 100 mL of normal saline) was subcutaneously injected daily (injection titer, 0.1 mL per 10 g mouse weight) into the dorsal necks of BALB/c mice for 4 weeks; the mice were weighed and plasma MDA levels were determined and

112 then randomly divided into 4 Gal-induced groups which were subcutaneously injected each day for
113 another 4 weeks with concurrent daily single oral administration through a feeding gauge for the
114 induced group (the control, distilled water) and 3 peptide-intervened groups [NW10 and NW40 (10
115 or 40 mg NW $kg^{-1}$ per day) or carnosine40 (40 mg carnosine $kg^{-1}$ per day)] in distilled water. For the
116 blank group, the mice were subcutaneously injected with normal saline for the first 4 weeks,
117 followed by subcutaneous injection of normal saline concurrent with oral administration of distilled
118 water daily for 4 weeks. The mice in these 5 groups were trained and evaluated for learning
119 dysfunction by using the Morris water maze before the end of the experiments.
120

121 **2.3. Learning behavior evaluations by Morris water maze**

122 Learning behaviors of reference memory task and probe trial test following Gal-induced oxidative
123 damage with or without NW or carnosine intervention was evaluated by the Morris water maze[31-33]
124 with modifications. During the last week of the experiment, the mice (N=5) in each group were
125 trained three times per day with an inter-trial interval around 30 min. The water tank (150 cm in
126 diameter and 60 cm in height) contained a movable escape platform (10 cm in diameter and 30 cm
127 in height) located at the center of the 3rd quadrant, and learning behavior was recorded using a
128 video behavioral (Diagnostic & Research Instruments Co., Ltd., Taiwan) and computerized
129 tracking system (Framgrabber II-33/34, HasoTech Co., Germany). For reference memory task, the
130 trainings in the first and second day were as followings. Each mouse was first placed on the

platform, where it remained for 30 s for the first training. The mouse was then placed on the starting quadrant and allowed to attempt to locate the platform for 2 min. If the mouse did not locate the platform within 2 min, it was guided to find the platform, where it remained for 30 s until the end of training. The maximal latency was assigned 2 min. On the third day, it was recorded for the latency (sec) and the swimming speed (cm/sec). For probe trial, after the 9$^{th}$ training in reference memory task, the platform was removed from the water tank and mice in each group were test in a spatial probe trial for 2 min. The numbers of crossing in the target quadrant was recorded where the platform had been located during reference memory task.

**2.4. Blood and tissue treatments**

During experimental periods, blood samples of mice were drawn from facial veins at the 4$^{th}$, 6$^{th}$, and 8$^{th}$ week by using 5-mm animal lancets. After the samples were centrifuged at 367 ×*g*, the plasma obtained was saved and stored at -80°C for MDA determination. At the ends of the experiments, the mice that had been subjected to passive avoidance test, but not been subjected to the Morris water maze test, were weighed and sacrificed, and blood samples were collected by cardiac puncture; the brains and livers were isolated, and all samples were immediately stored at -80°C for further measurements. Several oxidative status parameters were measured, including the AGE content in the brain tissues, MDA content and total GSH content in the brain and liver tissues, and ORAC in the brain and liver tissues. Whole brains and livers were immediately ground into a fine powder in liquid

nitrogen by using a mortar and pestle. The fine powders were suspended in 1 mL of 1× phosphate-buffered saline (PBS) for protein extraction. After the suspensions were centrifuged at 12,500 ×*g* at 0°C for 60 min, the supernatants were stored at -80°C for further investigation. The proteins were quantified using the BCA protein assay kit by using bovine serum albumin as a standard (Pierce Biotechnology, Inc., Rockford, IL, USA).

**2.5. Oxidative status parameters**

The MDA content in plasma (μM) or in the organ extracts (μM μg tissue$^{-1}$) was determined by BIOXYTECH® MDA-586™ assay kits according to the manufacturer's instructions (Portland, OR, USA) bases on the reaction of *N*-methyl-2-phenylindole with MDA to generate a chromogenic product with a maximum absorption at 586 nm, and expressed as using 1,1,3,3-tetramethoxypropane as the standard. The total GSH content in organ extracts (μM μg tissue$^{-1}$) was determined using glutathione assay kits (no. 703002; Cayman Chemical Co., Ann Arbor, MI, USA). ORAC activities in organ extracts were determined using the OxiSelect™ assay kit (STA-345; Cell Biolabs Inc., San Diego, CA, USA) according to the manufacturer's instructions, in which a peroxyl radical was acted to quench fluorescein over time. Antioxidant potentials present in the assay system block peroxyl radical-mediated fluorescein oxidation until the antioxidants in the sample are depleted. The area under the curve of Trolox was used to plot a standard curve of ORAC activity. The ORAC activities in organ extracts were expressed as μM Trolox equivalents (TEs) μg tissue$^{-1}$.[30]

169

170 **2.6. AGEs immune stainings**

171 Equal amounts of proteins from mouse brain extracts that had undergone different treatments were
172 subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis,
173 the gels were cut for protein stains and immune stains. For protein stains, the gel was fixed with
174 12.5% trichloroacetic acid for 30 min and then stained with Coomassie brilliant blue R-250.[34] For
175 immune stains, the gel was equilibrated in Tris-glycine buffer (pH 8.3) and transferred onto
176 immobile polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). PVDF
177 membranes were blocked with 1% gelatin in solution containing NaCl, EDTA, and Tris (NET) for
178 one hour at room temperature and incubated overnight at 4°C. An anti-argpyrimidine antibody or an
179 anti-CML antibody was used at a 5000-fold dilution. The PVDF membrane was washed 3 times with
180 phosphate buffered saline and Tween-20 (PBST) for 10 min. Next, HRP-conjugated secondary
181 antibody solution (5000-fold dilution in 0.25% gelatin in NET solution) was added, and the
182 membrane was washed with PBST. Immunoblots were detected using the Western chemiluminescent
183 HRP substrate kit containing luminol reagents and peroxide solutions (no. WBKL S0050;
184 Immobilon™, Millipore). Each blot was imaged using the Syngene GeneGnome5 imaging system
185 (Syngene, Cambridge, UK) and the major bands were quantified by GeneSys/GeneTools software
186 (Syngene).

187

### 2.7. Statistical analyses

General data were calculated as mean ± SD, and for water maze evaluations, data were expressed as mean ± SEM. Multiple group comparisons were performed using one-way analysis of variance (ANOVA) followed by the post-hoc Tukey's test, and values that have not been indicated with the same alphabet were significantly different ($P < 0.05$) or using one-way ANOVA followed by the post-hoc Dunnett's test for MDA changes in plasma, and a difference compare to Gal induction was considered statistically significant when $P < 0.05$ (*), or $P < 0.01$ (**), or $P < 0.001$ (***). Statistical analysis was performed using the GraphPad Prism 5.0 software (San Diego, CA, USA).

## 3. Results

### 3.1. Plasma MDA contents

The experimental protocol is shown in Fig. 1(A), including the 1-week acclimation and 8-week experimental period (4-week Gal subcutaneous injection followed by 4-week Gal subcutaneously injection together with NW or carnosine interventions). The learning behavior was evaluated during the last week. The animal weights among the groups were not significantly different during the course of the experiments (data not shown); however, the plasma MDA content of the groups changed over the experimental period. Compared to the Gal group by using one-way ANOVA analyses and the post-hoc Dunnett's test, the blank group showed a significantly lower MDA content ($P < 0.001$, ***; $P < 0.05$, *) compared to the control (Gal group) at the 4-, 6- and 8-week time

intervals. However, with only 4-week peptide interventions concurrent with 8-week Gal induction, the NW10, NW40 or carnosine40 showed a significantly lower MDA content ($P < 0.001$, ***; $P < 0.01$, **) compared to the Gal group (Fig. 1B). It was also found that the plasma MDA content in 4-week peptide interventions showed no significant difference ($P > 0.05$) among the intervention groups. Thus, NW or carnosine intervention for 4 weeks may reduce the increased plasma MDA content by 8-week D-galactose subcutaneous injection.

### 3.2. Learning behavior evaluations

During the last week of NW or carnosine intervention, the mice in each group were evaluated using a Morris water maze for a reference memory test and the probe test as an index of spatial memory and learning capacity.[32,33] After the mice were trained 3 times per day for 2 successive days, a reference memory test was administered on the third day and results were recorded to evaluate latency (time required to reach the platform, Fig. 2A) and swimming speed (Fig. 2B). Using one-way ANOVA followed by the post-hoc Tukey's test to evaluate the results of the reference memory test, the 8-week_Gal (control) required a longer time to reach the platform (latency) and showed a significant difference ($P < 0.05$) compared to the 8-week_Blank (Fig. 2A). However, the 40 mg NW $kg^{-1}$ interventions concurrent with Gal induction (8-week_Gal+4-week_NW40), but not NW10 or carnosine40 interventions, showed a significantly reduced latency (Fig. 2A) compared to the 8-week_Gal (control) in which the latency was comparable to that of the blank. There was also no

226 significant difference ($P > 0.05$) found among the groups with respect to the swimming speeds (Fig.
227 2B). This meant that the different latencies among the groups of Fig. 2A were raised from different
228 capacities of spatial memory and learning. After the platform was removed, a spatial probe trial was
229 performed to record the number of crossings in the target quadrant in which the platform had been
230 located (Fig. 2C). Using one-way ANOVA followed by the post-hoc Tukey's test to evaluate the
231 results of the probe trial, the 8-week_Gal (control) had fewer crossing numbers in the target quadrant
232 and showed a significant difference compared to the 8-week_Blank (Fig. 2C). However, the 40 mg
233 NW $kg^{-1}$ interventions concurrent with Gal induction (8-week_Gal+4-week_NW40), but not NW10
234 or carnosine40 interventions, were shown to significantly elevate the crossing numbers (Fig. 2C)
235 compared to the 8week_Gal (control) in which the crossing number was comparable to that of the
236 blank. The present data suggested that NW40 interventions could improve the impaired spatial
237 memory and learning induced by D-galactose.
238
239 **3.3. GSH and MDA content in tissue extracts of the brain and liver**
240 NW or carnosine interventions concurrent with Gal-injection can reduce mouse plasma MDA content.
241 It was of interest to determine whether Gal injection could induce the systematic elevation of
242 oxidative stress in the mouse body. Therefore, brain and liver tissue extracts were selected for GSH
243 and MDA determinations. The levels of GSH (Figs. 3A and 3B) and MDA (Figs. 3C and 3D) were
244 determined in the tissue extracts of the brain and liver after sacrifice. It was found that Gal injection

(8-week_Gal) could significantly decrease the GSH content ($P < 0.05$) and significantly elevate the MDA content ($P < 0.05$) compared to the blank (8-week_Blank) in both tissue extracts of the brain and liver. It was clear that the oxidative stress induced by Gal injection was systematically raised in the mouse body. When one-way ANOVA followed by the post-hoc Tukey's test was used to evaluate the GSH content in both tissues, only 40 mg NW $kg^{-1}$ intervention (8-week_Gal+4-week_NW40) could significantly elevate the GSH content ($P < 0.05$) in brain tissues compared to the control (Fig. 3A). The intervened groups (10 mg or 40 mg NW $kg^{-1}$ or 40 mg carnosine $kg^{-1}$) could significantly increase the GSH content in liver tissues ($P < 0.05$, Fig. 3B). From the present results, the NW40 showed greater effects than carnosine40 in elevating the GSH content in brain and liver tissues (Figs. 3A and 3B). On the other hand, the intervened groups (10 mg or 40 mg NW $kg^{-1}$ or 40 mg carnosine $kg^{-1}$) could significantly reduce the MDA content in brain tissues ($P < 0.05$, Fig. 3C) and comparable to that of the blank; the 40 mg NW or carnosine $kg^{-1}$ showed a significantly lower MDA content compared to the control (8-week_Gal) in liver tissues ($P < 0.05$, Fig. 3D). From the present results, the NW40 showed similar effects to carnosine40 in reducing the MDA content in brain and liver tissues (Figs. 3C and 3D). Thus, NW40 or carnosine40 intervention could increase the GSH content and decrease the MDA content in brain and liver tissues of model mice that had undergone long-term D-galactose induction.

### 3.4. ORAC activity in the brain and liver tissues

ORAC activity, another parameter used to estimate overall oxidative stress in the brain and liver tissues, is expressed in terms of μM TE μg tissue.$^{-1}$ Using one-way ANOVA followed by the post-hoc Tukey's test to evaluate ORAC activity in the tissues, the 8-week_Gal group (the control) showed the lowest ORAC activity in brain or liver tissues; however, the intervened groups (10 mg or 40 mg NW kg$^{-1}$ or 40 mg carnosine kg$^{-1}$) could significantly elevate ORAC activity in brain tissues ($P < 0.05$, Fig. 4A) or liver tissues ($P < 0.05$, Fig. 4B). The NW40 showed better than carnosine40 in elevating ORAC activity in brain tissues, but not in liver tissues. Thus, NW10, NW40 or carnosine40 intervention could elevate ORAC activity in the brain and liver tissues of model mice that had undergone long-term D-galactose induction.

### 3.5. AGE formation in the brain tissues

After sacrifice, AGE formations in mouse brain tissues were detected using an anti-argpyrimidine antibody (Fig. 5B) or an anti-CML antibody (Fig. 5C) in the PVDF membrane. These were compared to the equal amounts of extracted proteins in the PAGE gel by protein stains (Fig. 5A). Lanes B, C, 1, 2 and 3 correspond to the groups of 8-week_Blank, 8-week_Gal, 8-week_Gal+4-week_NW10, 8-week_Gal+4-week_NW40, and 8-week_Gal+4week_carnosine40, respectively. The protein staining results showed no clear changes among the groups (lanes in Fig. 5A); however, the immune stained bands around the 55-kDa detected by the anti-argpyrimidine antibody (the selected square frame in Fig. 5B) or between 43- and 55-kDa detected by the anti-CML antibody (the selected square

283 frame in Fig. 5C) showed a decreased intensity with NW or carnosine intervention compared to
284 bands for the control of the 8week_Gal group (lane C, Figs. 5B and 5C). The calculation intensities
285 of the selected square frame in Fig. 5B and Fig. 5C are shown in Fig. 5D and Fig. 5E, respectively.
286 When one-way ANOVA followed by the post-hoc Tukey's test was used to evaluate AGE formations
287 in the brain tissues, the NW40 or carnosine40 intervention showed a reduction and a significant
288 difference in AGE formations compared to 8-week_Gal ($P < 0.05$, the control) detected by
289 anti-argpyrimidine antibody, and the NW40 showed better anti-AGE formations than carnosine40 ($P$
290 $< 0.05$, Fig. 5D). On the other hand, the intervened groups (10 mg or 40 mg NW $kg^{-1}$ or 40 mg
291 carnosine $kg^{-1}$) could significantly reduce AGE formations ($P < 0.05$) compared to 8-week_Gal (the
292 control) detected by anti-CML antibody (Fig. 5E), which was comparable to that of the blank.
293

294 ## 4. Discussion

295 The present results of the animal experiments show that dipeptides of NW or carnosine
296 intervention, especially with daily single oral administration of 40 mg $kg^{-1}$ for 4 weeks, can improve
297 the oxidative stress and anti-AGE formations in Gal-induced BALB/c mice for 8 weeks *in vivo*. The
298 accumulated Gal may react with proteins and peptides to form AGEs *in vivo*; the increased AGEs
299 can accelerate the aging process.[28] Memory decline, an aging characteristic, is thought to generate
300 gradually a spatial memory loss. The oxidative stress and ROS have been proposed as being
301 important causes of aging.[22,31] The model of chronic administration of Gal in rodents could result in

symptoms of brain aging, including memory deficits, in which the spatial memory-dependent hippocampal functions were decreasing.[28,35] It was reported that age-related impairment in spatial learning and memory can be attenuated by antioxidant treatments.[36] It was reported that purple sweet potato colors attenuate Gal-induced aging in mice by elevating the survival of neurons via the PI3K pathway and inhibiting cytochrome C-mediated apoptosis.[37] In the present results, the oxidative stress induced by Gal injection was systematically elevated in brain and liver tissues (Figs. 3 and 4) by decreasing the GSH content and ORAC activity, and increasing the MDA content. MDA, a lipid peroxidation product, is reported to be a marker of oxidative stress in serum and the liver[38], and the AGE diet could cause oxidative stress in livers showing non-alcoholic steatohepatitis.[39,40] The NW40 and carnosine40 interventions showed similar effects in reducing the MDA content in plasma (Fig. 1B) and brain and liver tissues (Figs. 3C and 3D), and ORAC activity in liver tissues (Fig. 4B); the NW40 showed better elevating effects than carnosine40 in the GSH content in brain and liver tissues (Figs. 3A and 3B) and ORAC activity in brain tissues (Fig. 4A). However, only NW40 intervention showed improvements on spatial memory and learning (Figs 2A and 2C). This meant that the oxidative status, especially in the brain tissues, of the GSH content, as well as ORAC activity greater than the MDA content of NW40 intervention might pass threshold levels (but not NW10 or carnosine40 intervention) to reflect in part the correlations of improvement in Gal-induced aging and learning dysfunctions. Our previous results showed that NW exhibited 12- to 23-fold higher than carnosine in ORAC activities *in vitro*.[4] Carnosine is found in long-lived mammalian tissues at

relatively high concentrations (up to 20 mM).[41] The real concentrations of carnosine in experimental animals during Gal induction and concurrent 40 mg $kg^{-1}$ carnosine intervention were not clear. Nakashima *et al*.[42] reported the use of stable isotope labeling liquid chromatography-MRM-tandem mass spectrometry to determine the intact absorption of bioactive dipeptides in rats. Hong *et al*.[43] reported that small peptides could be seen after absorption into the tissues by MALDI-imaging mass spectrometry. It might be possible to use the above-mentioned techniques to check the absorption and distribution of NW and carnosine *in vivo*. This might explain in part the spatial memory and learning improvements seen with NW40; it will also use the neuron cells to investigate the protective roles and possible mechanisms of NW or carnosine against AGEs or glyoxal, all of which need further investigation.

The responses to glycated products may be one of the ROS sources in cells in which the ROS formations are closely related to neurodegenerative diseases.[21] AGE can bind to RAGE on the membrane to elevate ROS production through activation of NAD(P)H oxidase.[24] In the present results, 40 mg $kg^{-1}$ of NW or carnosine interventions can reduce the AGE formations (Figs. 5D and 5E). It was reported that NW and carnosine at 100 μM showed similar preventive CML formations in BSA molecules *in vitro*.[4] These similar effects were also found in the present NW40 and carnosine40 interventions *in vivo* (Fig. 5E). It is proposed that the reduced AGE formations in the brain tissues of intervention groups detected by anti-argpyrimidine antibodies rather than anti-CML antibodies might reflect in part the correlations of NW40 interventions in improvements of

340 Gal-induced aging and learning dysfunctions. Therefore, it is proposed that anti-glycation activity of
341 NW or carnosine may reduce AGE formations in cells and then diminish ROS productions together
342 with peptides' antioxidant properties in essence to improve oxidative stresses in Gal-induced mice.
343 Argpyrimidine was derived from the interactions of the arginine residue on proteins with
344 methylglyoxal. Increased amounts of argpyrimidine have been correlated with diabetic nephropathy
345 and neurodegenerative diseases.[44] The CML was reported to be the most thoroughly studied with
346 respect to the chemical and biological properties of AGE.[45] However, the immune-stained detection
347 used in the present research cannot cover all forms of chemically identified AGE. Hashimoto *et al.*[46]
348 developed a highly sensitive detection method for free AGEs by using LC-MS/MS-MRM, which
349 might make it possible to view the changes of the whole AGE profile in tissues and need further
350 investigation.

351 The NW dipeptide can be obtained from computer-aided pepsin simulated hydrolysis of
352 dioscorin, the yam (*Dioscorea* spp.) tuber storage protein.[4] The NW peptide has not yet been isolated
353 from dioscorin hydrolysates; however, we detected the NW signal in peptic hydrolysates of dioscorin
354 by TOF-MS/MS (M/Z 319.1, data not shown) after reverse-phase HPLC fractionations. We had
355 reported that yam dioscorin and its protease hydrolysates,[1,47] and the synthesized peptides derived
356 from computer-aided dioscorin-simulated hydrolysis by pepsin,[3,4] exhibited antioxidant activities *in*
357 *vitro.* Dioscorin intervention (80 mg $kg^{-1}$ of mouse body weight) was also shown to increase
358 antioxidant activities and improve oxidative stresses in galactose-induced mice *in vivo*.[30] Though the

359 dioscorin intervention was performed daily for 6 weeks, it was found that 4-week intervention could
360 already reduce plasma MDA and showed a significant difference ($P < 0.01$) compared to the
361 Gal-induced only, which had the same time intervals as NW interventions in the present experiment.
362 As suggested by Reagan-Shaw *et al*.[48] for dose translation from animal to human studies based on
363 body surface area normalization, it was calculated that the human equivalent dose was 3.24 mg $kg^{-1}$
364 of human body weight from NW or carnosine of 40 mg $kg^{-1}$ of mouse body weight in the present
365 experiment, or the human equivalent dose was 6.49 mg $kg^{-1}$ of human body weight from 80 mg
366 dioscorin $kg^{-1}$ of mouse body weight.[30] An adult weighing 60 kg may have to consume 195 mg NW
367 or carnosine or 390 mg dioscorin per $day^{-1}$ to achieve the similar antioxidant benefits. This should be
368 investigated further. Therefore, it is suggested that NW dipeptide may play a vital role and/or
369 coordinate with other peptides from dioscorin after being ingested to increase antioxidant activities
370 and improve oxidative stresses in Gal-induced mice *in vivo*.[30]
371

372 ## 5. Conclusion

373 The present results showed that 40 mg $kg^{-1}$ NW or carnosine intervention for 4 weeks can attenuate
374 the oxidative stress induced by daily D-galactose subcutaneous injection for 8 weeks. However, only
375 40 mg NW $kg^{-1}$ (NW40) intervention could improve spatial memory and learning dysfunctions in
376 which the GSH content and ORAC activity in the brain tissues of NW40 intervention might pass
377 threshold levels to reflect in part the correlations of improvement in Gal-induced aging and learning

dysfunctions. The possible mechanisms still need further investigation. It is suggested that NW may be useful in developing functional foods for antioxidant and anti-aging purposes.

## Acknowledgements

The authors would like to express thanks to National Science Council, Republic of China (NSC 102-2313-B-038 -004 -MY3) for financial supports.

## Notes and references

1 W. C. Hou, M. H. Lee, H. J. Chen, W. L. Liang, C. H. Han, Y. W. Liu and Y. H. Lin, J. Agric. Food Chem. 2001, 49, 4956-4960.

2 Y. W. Liu, C. H. Han, M. H. Lee, F. L. Hsu and W. C. Hou, *J. Agric. Food Chem.*, 2003, **51**, 4389-4393.

3 C. H. Han, J. C. Liu, S. U. Fang and W. C. Hou, *Food Chem.*, 2013, **138**, 923-930.

4 C. H. Han, Y. S. Lin, S. Y. Lin and W. C. Hou, *Food Chem.*, 2014, **147**, 195-202.

5 F. L. Hsu, Y. H. Lin, M. H. Lee, C. L. Lin and W. C. Hou, *J. Agric. Food Chem.*, 2002, **50**, 6109-6113.

6 C. L. Lin, S. Y. Lin, Y. H. Lin and W. C. Hou, *J. Sci. Food Agric.*, 2006, **86**, 1489-1494.

7 D. Z. Liu, H. J. Liang, C. H. Han, S. Y. Lin, C. T. Chen, M. Fan and W. C. Hou, *J. Sci. Food Agric.*, 2009, **89**, 138-143.

8 S. L. Fu, Y. H. Hsu, P. Y. Lee, W. C. Hou, L. C. Hung, C. H. Lin, C. M. Chen and Y. J. Huang, *Biochem. Biophys. Res. Communic.*, 2006, **339**, 137-144.

9 Y. W. Liu, H. F. Shang, C. K. Wang, F. L. Hsu and W. C. Hou, *Food Chem. Toxicol.*, 2007, **45**, 2312-2318.

10 Y. W. Liu, J. C. Liu, C. Y. Huang, C. K. Wang, H. F. Shang and W. C. Hou, *J. Agric. Food Chem.*, 2009, **57**, 9274-9279.

11 T. Matsui, M. Sato, M. Tanaka, Y. Yamada, S. Watanabe, Y. Fujimoto, K. Imaizumi and K.

Matsumoto, *Brit. J. Nutri.*, 2010, **103**, 309-313.

12 R. Hartmann and H. Meisel, *Cur. Opin. Biotechnol.*, 2007, **18**, 163–169.

13 K. Erdmann, B. W. Y.; Cheung and H. Schroder, *J. Nutr. Biochem.*, 2008, **19**, 643–654.

14 C. Fitzgerald, E. Gallagher, D. Tasdemir and M. Hayes, *J. Agric. Food Chem.*, 2011, **59**, 6829–6836.

15 A. B. Nongonierma and R. J. FitzGerald, *Food Funct.*, 2013, **4**, 1843-1849.

16 N. Yamaguchi, K. Kawaguchi and N. Yamamoto, *Eur. J. Pharmacol.*, 2009, **620**, 71-77.

17 H. Sies, *Free Rad. Biol. Med.*, 1999, **27**, 916–921.

18 W. C. Hou, H. J. Chen and Y. H. Lin, *J. Agric. Food Chem.*, 2003, **51**, 1706-1709.

19 R. Kohen, Y. Yamamoto, K. C. Cundy and B. N. Ames, *Proc. Nat. Acad. Sci. USA*, 1988, **95**, 2175–2179.

20 G. A. McFarland and R. Holliday, *Exp. Cell Res.*, 1994, **212**, 167–175.

21 K. B. Beckman and B. N. Ames, *Physiol. Rev*., 1998, **78**, 547–581.

22 F. L. Muller, M. S. Lustgarten, Y. Jang, A. Richardson and H. V. Remmen, *Free Rad. Biol. Med.*, 2007, **43**, 477–503.

23 S. Kikuchia, K. Shinpoa, M. Takeuchi, S. Yamagishi, Z. Makita, N. Sasaki and K. Tashiroa, *Brain Res. Rev.*, 2003, **41**, 306–323.

24 N. A. Calcutt, M. E. Cooper, T. S. Kern and A. M. Schmidt, *Nat. Rev. Drug Discover.*, 2009, **8**, 417-429.

424 25 G. Müch, B. Westcott, T. Menini and A. Gugliucci, *Amino Acids*, 2012, **42**, 1221-1236.

425 26 A. Lapolla, D. Fedele, R. Seraglia and P. Traldi, *Mass Spectrom. Rev.*, 2006, **25**, 775-797.

426 27 H. Unoki and S. I. Yamagishi, *Curr. Pharmaceut. Design*, 2008, **14**, 987-989.

427 28 X. Song, M. Bao, D. Li and Y. M. Li, *Mech. Ageing Dev.*, 1999, **108**, 239-251.

428 29 K. Parameshwaran, M. H. Irwin, K. Steliou and C. A. Pinkert, *Rejuvent. Res.*, 2010, **13**, 729-735.

429 30 C. H. Han, Y. F. Lin, Y. S. Lin, T. L. Lee, W. J. Huang, S. Y. Lin and W. C. Hou, *Food Chem.*

430 *Toxicol.*, 2014, **65**, 356-363.

431 31 C. S. Chiu, Y. J. Chiu, L. Y. Wu, T. C. Lu, T. H. Huang, M. T. Hsieh, C. Y. Lu and W. H. Peng,

432 *Amer. J. Chin. Med.*, 2011, **39**, 551-563.

433 32 Y. Yamaguchi, H. Miyashita, H. Tsunekawa, A. Mouri, H. C. Kim, K. Saito, T. Matsuno, S.

434 Kawashima and T. Nabeshima, *J. Pharmacol. Exp. Ther.*, 2006, **317**, 1079-1087.

435 33 C. L. Lee, T. F. Kuo, J. J. Wang and T. M. Pan, *J. Neurosci. Res.*, 2007, **85**, 3171-3182.

436 34 V. Neuhoff, R. Stamm and H. Eibl, *Electrophoresis*, 1985, **6**, 427-448.

437 35 Y. X. Shen, S. Y. Xu, W. Wei, X. X. Sun, J. Yang, L. H. Liu and C. Dong, *J. Pineal. Res.*, 2002,

438 **32**, 173–178.

439 36 D. J. Socci, B. M. Crandall and G. W. Arendash, *Brain Res.*, 1995, **693**, 88–94.

440 37 J. Lu, D. M. Wu, Y. L. Zheng, B. Hu and Z. F. Zhang, *Brain Pathol.*, 2010, **20**, 598–612.

441 38 R. Mateos, E. Lecumberri, S. Ramos, L. Goya and L. Bravo, *J. Chromatogr. B*, 2005, **827**, 76-82.

442 39 H. Hyogo and S. Yamagishi, *Curr. Pharm. Des.*, 2008, **14**, 969-972.

443 40 R. Patel, S. S. Baker, W. Liu, S. Desai, R. Alkhouri, R. Kozielski, L. Mastrandrea, A. Sarfraz, W.

Cai, H. Vlassara, M. S. Patel, R. D. Baker and L. Zhu, *PLoS ONE*, 2012, **7**, e35143. Doi:10.1371/journal.pone.0035143.

41 C. Brownson and A. R. Hipkiss, *Free Rad. Biol. Med.*, 2000, **28**, 1564-1570.

42 E. M. Nakashima, A. Kudo, Y. Iwaihara, M. Tanaka, K. Matsumoto and T. Matsui, *Anal. Biochem.*, 2011, **414**, 109-116.

43 S. M. Hong, M. Tanaka, S. Yoshii, Y. Mine and T. Matsui, *Anal. Chem.*, 2013, **85**, 10033-10039.

44 P. J. Thornalley, *Clin. Lab.*, 1999, **45**, 263–273.

45 P. Nagai, J. I. Shirakawa, R. I. Ohno, N. Moroishi and M. Nagai, *Amino Acids*, 2014, **46**, 261-266.

46 C. Hashimoto, Y. Iwaihara, S. J. Chen, M. Tanaka, T. Watanabe and T. Matsui, *Anal. Chem.*, 2013, **85**, 4289-4295.

47 Y. H. Liu, H. J. Liang, H. C. Cheng, Y. W. Liu and W. C. Hou, *Bot. Stud.*, 2006, **47**, 231-237.

48 S. Reagan-Shaw, M. Nihal and N. Ahmad, *FASEB J.*, 2007, **22**, 659–661.

458 ## Figure Legends

459 **Fig. 1**. (A) The protocols of animal experiments. (B) The changes of plasma MDA contents after
460 NW or carnosine oral administrations (groups of Gal+NW10, Gal+NW40, and Gal+carnosine40)
461 every two weeks. Data were expressed as mean ± SD. Multiple group comparisons were
462 performed using One-wayANOVA followed by the post-hoc Dunnett's test for MDA changes in
463 plasma, and a difference compare to Gal induction was considered statistically significant when $P$
464 < 0.05 (*), or $P$ < 0.01 (**), or $P$ < 0.001 (***).

465

466 **Fig. 2**. The mice learning behaviors were evaluated by Morris water maze. For reference memory
467 task, (A) the latency (time to reach the platform, sec) and (B) swimming speed (cm/sec) of the
468 third day was recorded; for probe trial, (C) the number of crossing in the target quadrant for 2 min
469 was recorded where the platform had been located during reference memory task. Groups
470 included the D-galactose-induced mice for 8 weeks (8week_Gal group) together with 4-weeks NW
471 or carnosine oral administrations (10 or 40 mg $kg^{-1}$) (groups of 8week_Gal+4week_NW10,
472 8week_Gal+4week_NW40, and 8week_Gal+4week_carnosine40) and the blank (8week_Blank).
473 Data were expressed as mean ± SEM. Multiple group comparisons were performed using one-way
474 ANOVA followed by the post-hoc Tukey's test, and values that have not been indicated with the
475 same alphabet were significantly different ($P$ < 0.05).

476

**Fig. 3**. After being sacrificed, the mouse brain tissue extracts (A, C) and mouse liver tissue extracts (B, D) were used to determine the GSH contents (A, B) and MDA contents (C, D) in groups of D-galactose-induced mice for 8 weeks (8week_Gal group) together with 4-weeks NW or carnosine oral administrations (10 or 40 mg $kg^{-1}$) (groups of 8week_Gal+4week_NW10, 8week_Gal+4week_NW40, and 8week_Gal+4week_carnosine40) and the blank group (8week_Blank). Data were expressed as mean ± SD. Multiple group comparisons were performed using one-way ANOVA followed by the post-hoc Tukey's test, and values that have not been indicated with the same alphabet were significantly different ($P < 0.05$).

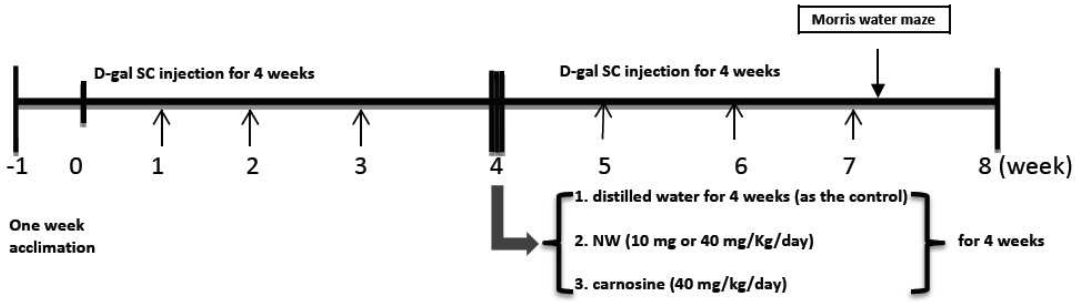
**Fig. 4.** ORAC activity in the (A) brain tissues and (B) liver tissues in groups of D-galactose-induced mice for 8 weeks (8week_Gal group) together with 4-weeks NW or carnosine oral administrations (10 or 40 mg $kg^{-1}$) (groups of 8week_Gal+4week_NW10, 8week_Gal+4week_NW40, and 8week_Gal+4week_carnosine40) and the blank group (8week_Blank). Data were expressed as mean ± SD. Multiple group comparisons were performed using one-way ANOVA followed by the post-hoc Tukey's test, and values that have not been indicated with the same alphabet were significantly different ($P < 0.05$).

**Fig. 5.** After electrophoresis, mouse brain tissue extracts were performed for protein stains in 10% SDS-PAGE gels by Coomassie brilliant blue R-250 (A) or electroblotted onto PVDF membranes

for advanced glycation endproducts (AGE) detection either by (B) an anti-argpyrimidine antibody or (C) an anti- $N^{\varepsilon}$-(carboxymethyl)lysine (anti-CML) antibody. The AGE formations closed to the 55 kDa, the selected square frame in the (B) and between 43 kDa and 55 kDa, the selected square frame in the (C), respectively, were quantified and showed in the (D) and (E). The AGE formations were stained by chemiluminescent HRP substrate kits containing luminol reagents and peroxide solutions. Lanes B, C, 1, 2, and 3 were groups of 8week_Blank, 8week_Gal, 8week_Gal+4week_NW10, 8week_Gal+4week_NW40, and 8week_Gal+4week_carnosine40, respectively. The 10 μg proteins were loaded onto each well. M was protein prestained markers. Multiple group comparisons were performed using one-way ANOVA followed by the post-hoc Tukey's test, and values that have not been indicated with the same alphabet were significantly different ($P < 0.05$).

**Figure 1**

**(A)**



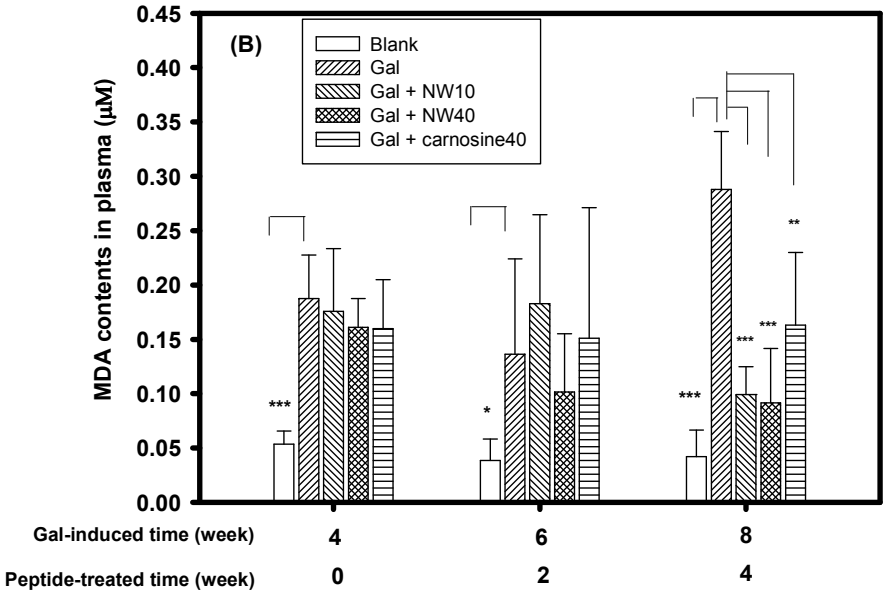



**Figure 2**

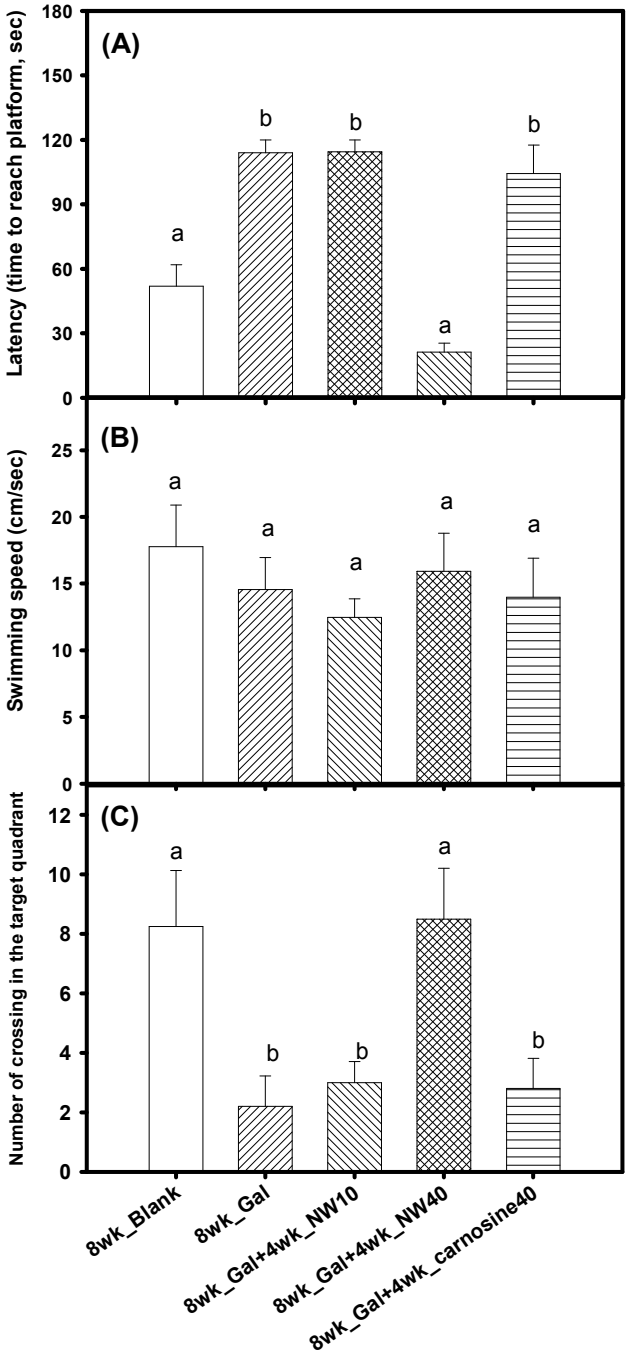


**Figure 3**

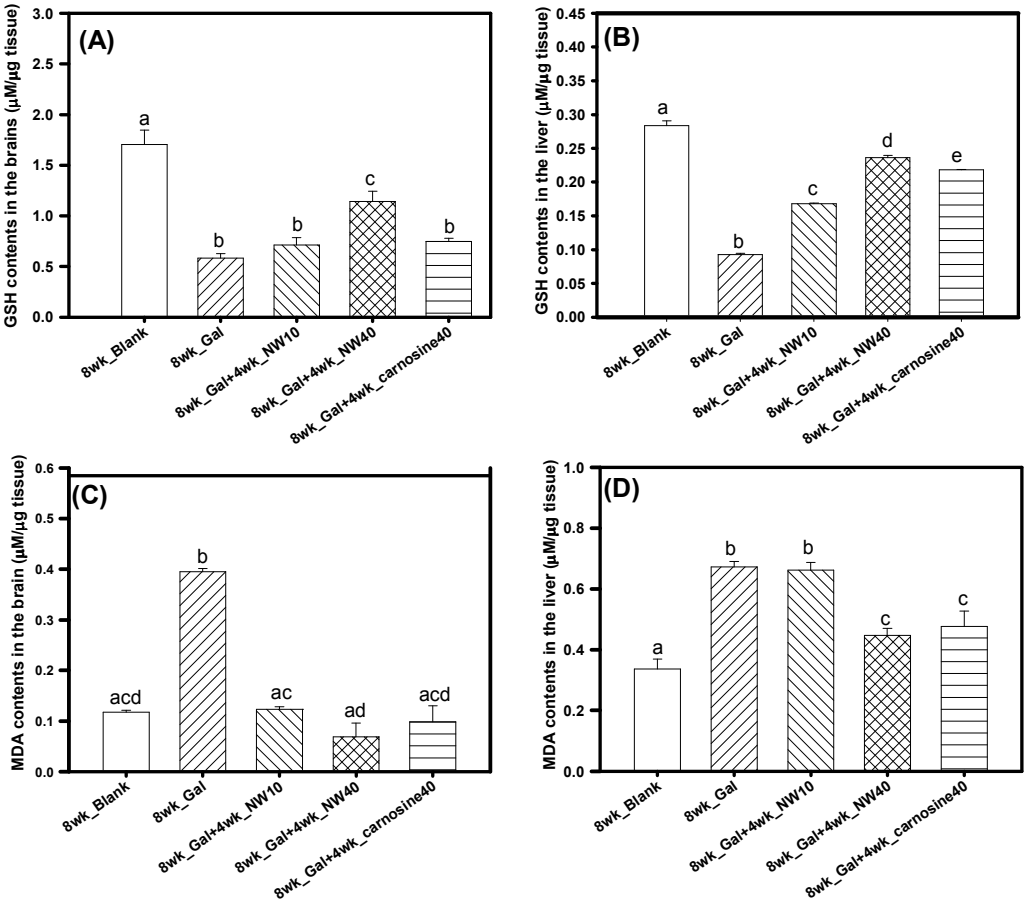


Figure 4

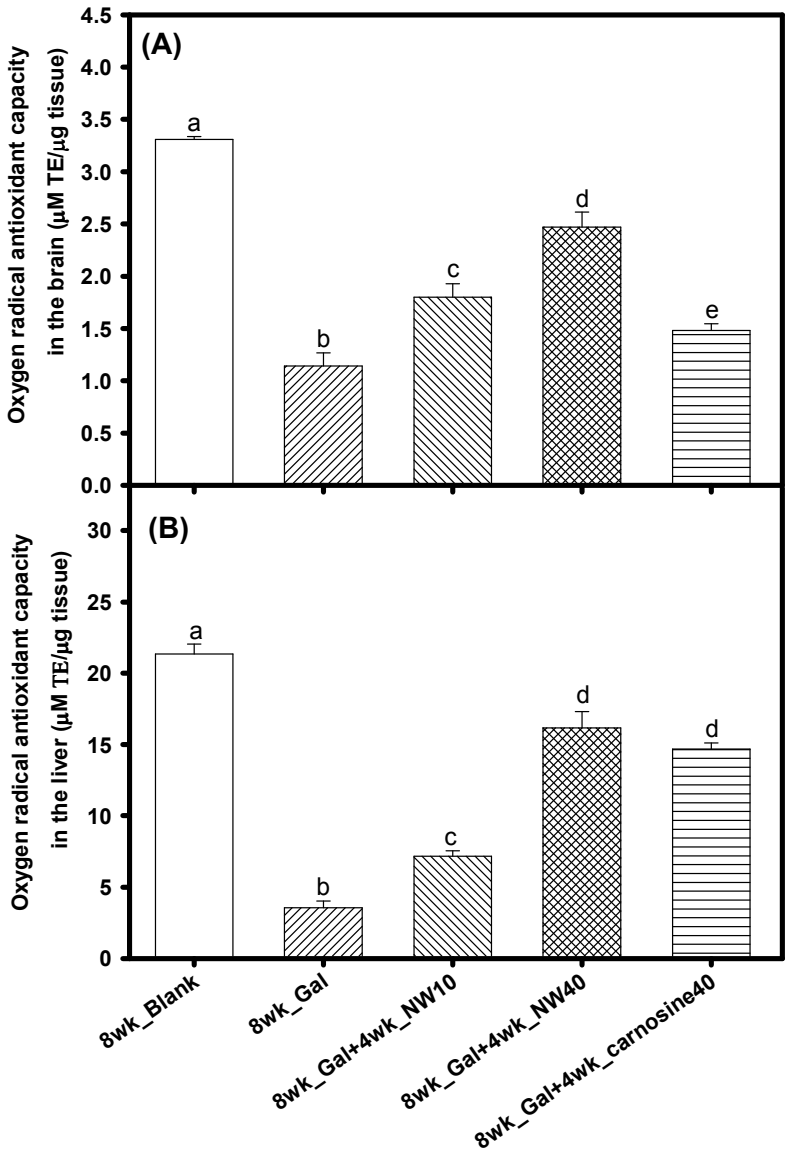


Figure 5