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Prenatal high sucrose intake affected learning and memory of aged rat offspring with abnormal oxidative stress and NMDARs/Wnt signaling in the hippocampus

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Abstract

Maternal over-nutrition may predispose offspring to obesity, type 2 diabetes and other adult diseases. The present study investigated long-term impact of prenatal high sucrose (HS) diets on cognitive capabilities in aged rat offspring. The fasting plasma glucose concentration did not differ between the control and HS groups. However, the fasting plasma insulin and insulin resistance index values were significantly increased in HS offspring that showed abnormal glucose tolerance test. HS offspring exhibited increased escape latency and swimming path length to the platform, and reduced time in the target quadrant and the number of crossing the platform, as compared with the control group. The expression of *Grin2b*/NR2B, *Wnt2*, *Wnt3a* and active form of β -catenin protein were decreased, and Dickkopf-related protein 1 was increased in the HS group. In addition, the levels of lipid peroxidation biomarker thiobarbituric acid reactive substance, nicotinamide adenine dinucleotide phosphate oxidases 2 and superoxide dismutase 1 were significantly increased, and the activity of catalase was decreased in the hippocampus in the HS group. The results demonstrate that prenatal HS-induced metabolic changes cause cognitive deficits in aged rat offspring, probably due to altered N-methyl-D-aspartate receptors/Wnt signaling and oxidative stress in the hippocampus.

Keywords

Aged offspring; Prenatal high sucrose diets; Spatial cognition; Oxidative stress; NMDARs; Wnt/ β -catenin

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Author contributions

A.H., M.S., and Z.X. designed research; A.H., Y.Z., Y.Y., J.L., X.F. B. W., D.Z., L.W., Y.L. conducted research; A.H., L.Z., M.S., and X.Z. analyzed data or wrote the paper; A.H. had primary responsibility for final content. All authors read and approved the final manuscript.

Conflict of interest/disclosures

None.

1. Introduction

Age-related dementia such as Alzheimer's disease (AD) is one of the most common causes of disability among older people, which is expected to reach 106 million by 2050 (Wimo et al., 2013; Ogawa, 2014). Epidemiological and experimental studies have indicated that adverse nutritional status may increase the risk of cognitive loss (Solfrizzi et al., 2011). It has been demonstrated that maternal high calorie intake during pregnancy results in various chronic diseases linked to dementia (Barker, 1990; Lakhan and Kirchgessner, 2013). Recent studies showed that prenatal high sucrose (HS) diets impaired spatial learning associated with apoptosis in the hippocampus in adolescent offspring (Kuang et al., 2014). However, whether prenatal HS diets have a long-term influence on cognitive function in aged offspring is unknown.

Excess energy intake during pregnancy results in programming of adverse metabolic outcomes in offspring such as obesity and diabetes in adulthood (Sedova et al., 2007). Both chronic hyperglycemia and insulin resistance may trigger neuronal death through oxidative stress and affect cognitive processes (Trevino et al., 2015). Oxidative stress is widely accepted as a key player in neurodegeneration, especially in age-related dementia such as AD (Smith et al., 1996; Ansari et al., 2008). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (NOX) are a family of enzymes in generation of reactive oxygen species (ROS) (Bedard and Krause, 2007). In the central nervous system, NOX contributes to the development of AD, Parkinson disease, anxiety, and schizophrenia (Ma et al., 2017). High fat diets enhanced hippocampal and cerebral cortex oxidative stress via the activation of NOX2 (Zhang et al., 2005; Bruce-Keller et al., 2010). However, effects of prenatal HS diets on NOX in the hippocampus remain unclear.

A recent study suggested that obesity and hypertriglyceridemia induced cognitive impairment (Farr et al., 2008). In the hippocampus, both N-methyl-D-aspartate receptors (NMDARs) and Wnt signaling play important roles in regulating cognitive functions (Chen et al., 2006; Zhao et al., 2009). NMDARs are cation-passing channels in the maintenance of synaptic plasticity, learning and memory (Park et al., 2014). NMDARs contain NR1 and NR2 subunits, and NR2A and NR2B present in high density in the hippocampus (Erreger et al., 2005). Disruption of NMDARs in the hippocampus may lead to blockade of synaptic plasticity and memory decline (Morris et al., 1990). The Wnt pathway is activated when Wnts bind to the Frizzled (FZD) and low-density lipoprotein-related protein (LRP) receptors, followed by the recruitment of cytoplasmic protein Dishevelled (DVL) and inhibition of glycogen synthase kinase-3 (GSK-3) activity. Free β -catenin is ultimately translocated to the nucleus in the control of activation of Wnt-responsive target genes (Kishida et al., 2001). The Wnt signaling pathway was altered in patients with neurodegenerative diseases (Caricasole et al., 2003; Logan and Nusse, 2004). It is unknown whether NMDARs and Wnt signaling are involved in the long-term effects on hippocampal-dependent learning and memory in aged offspring exposed to prenatal HS. Furthermore, ROS may suppress both the Wnt pathway and the function of NMDARs (Buckner, 2004; Manolopoulos et al., 2010). Thus, we determined the effect of prenatal HS on hippocampal NMDARs/Wnt signaling in aged offspring in the present study.

2. Results

2.1. The effect of prenatal HS on food and fluid intake, body and brain weight, plasma glucose, insulin and glucose tolerance in aged offspring

No significant differences were observed in daily food intake (22.45 ± 0.583 g/day versus 24.56 ± 0.7627 g/day, $P > 0.05$) and fluid intake (33.68 ± 1.764 ml/day versus 37.5 ± 1.118 ml/day, $P > 0.05$) between the control and HS groups. The body weight of HS offspring was greater than that of the control ($P = 0.024$, Fig. 1A). The brain weight ($P = 0.006$, Fig. 1B) and the brain to body weight ratio ($P = 0.030$, Fig. 1C) were significantly decreased in HS offspring. No significant difference was found in fasting plasma glucose (FPG) levels between the two groups ($P = 0.903$, Fig. 2A). The fasting plasma insulin (FINS) ($P = 0.016$, Fig. 2B) and insulin resistance index (Homa-IR) values ($P = 0.002$, Fig. 2C) were significantly increased in HS offspring, as compared with the control. The difference in glucose tolerance test between the two groups was significant following an intraperitoneal glucose load ($F_{1, 14} = 4.756$, $P = 0.047$, Fig. 2D), and the area under the time curve (AUC) was significantly greater in the HS group than that in the control (13.57 ± 0.519 versus 15.87 ± 0.819 , $P = 0.033$).

2.2. The effect of prenatal HS on spatial memory performance in aged offspring

In the learning phase, aged HS offspring showed a significant increase in escape latency ($F_{1, 20} = 15.572$, $P = 0.001$, Fig. 3A), and the path length to the platform was prolonged ($F_{1, 20} = 6.297$, $P = 0.021$, Fig. 3B), as compared with the control. In the retention test, the time spent in the target quadrant ($P = 0.0138$, Fig. 3C) and the number of crossing the platform ($P = 0.049$, Fig. 3D) were significantly decreased in the HS group.

2.3. The effect of prenatal HS on expression of NMDARs and Wnt signaling related genes in aged offspring

NMDARs-dependent synaptic plasticity is suggested to mediate several forms of learning. The q-PCR showed that mRNA abundance of *Grin2b*, but not *Grin1* and *Grin2a*, was significantly decreased in the hippocampus of aged HS offspring ($P = 0.024$, Fig. 4A). Western blot analysis showed that NR2B protein abundance was decreased in aged HS offspring, as compared with the control ($P = 0.044$, Fig. 4B). Wnt signaling is necessary to maintain NMDARs transmission and synaptic plasticity. The q-PCR showed that mRNA abundance of *Wnt2* and *Wnt3a* were significantly decreased ($P = 0.03$; $P = 0.004$, respectively, Fig. 5A), whereas Dickkopf-related protein 1 (*Dkk1*) was increased ($P = 0.044$, Fig. 5C), as compared with the control. There were no significant differences in mRNA abundance of *Fzd4*, *Lrp5/6*, *Gsk3 β* and *β -catenin* in between the control and HS offspring ($P > 0.05$, Fig. 5B, C). However, there was a significant decrease in active form of β -catenin protein in HS offspring, as compared with the control ($P = 0.004$, Fig. 5E).

2.4. The effect of prenatal HS on lipid peroxidation and catalase in aged offspring

Thiobarbituric acid reactive substance (TBARS) is an index of lipid peroxidation in the hippocampus. Catalase is a metal protein that detoxifies H_2O_2 to H_2O . TBARS was increased in the hippocampus of HS offspring ($P = 0.011$, Fig. 6A). In contrast, prenatal HS

diets markedly decreased the activity of catalase in the hippocampus of aged offspring ($P = 0.002$, Fig. 6B).

2.5. The effect of prenatal HS on expression of Sod/SOD subtypes in aged offspring

Superoxide dismutase (SOD) is one of the important antioxidant enzymes with three distinct isoforms: SOD1, SOD2, and SOD3. The q-PCR showed that mRNA abundance of *Sod1*, but not *Sod2* and *Sod3*, was significantly increased in the hippocampus of HS offspring ($P = 0.029$, Fig. 7C). In addition, SOD1 protein abundance was increased as well in HS offspring, as compared with the control ($P = 0.048$, Fig. 7D).

2.6. The effect of prenatal HS on expression of Nox/NOX subtypes in aged offspring

NOX is a prominent source of ROS and is involved in various neurodegenerative disorders. The q-PCR showed that mRNA abundance of *Nox2*, but not *Nox1* and *Nox4*, was significantly increased in the hippocampus of HS offspring, as compared with the control ($P = 0.017$, Fig. 7A). Furthermore, Western blot analysis demonstrated that NOX2 protein abundance was also increased in HS offspring ($P = 0.015$, Fig. 7B).

3. Discussion

The present study investigated the long-term effect of prenatal HS diets on hippocampal-dependent behavior and the underlying mechanisms in aged rat offspring. Previous studies reported that prenatal HS diets increased the blood glucose in both pregnant dams and fetuses. Increased birth weight was also found in the HS group as compared with the control (Wu et al., 2014). During the adolescence, the body weight of HS offspring remained higher than the control, while the brain weight was decreased (Kuang et al., 2014). At the adult stage (5 months old), there was no difference in the body weight between the two groups (Wu et al., 2014). In the present study, we showed that aged HS offspring has an increase in body weight and a decrease in brain weight at 18 months of age. In addition, aged HS offspring presented significantly higher FINS and Homa-IR levels than those of the control group.

It has been demonstrated that impaired glucose tolerance, hyperinsulinemia, and insulin resistance are the characteristics of the pre-diabetic state (Tabak et al., 2012). Epidemiological and experimental studies have indicated that people with diabetes show an increased risk in developing cognitive deficits and dementia as getting old (Cukierman et al., 2005). Oxidative stress has been suggested as a key player in diabetic encephalopathy with cumulative effects over a longer period to manifest cognitive decline (Soares et al., 2013).

To determine if prenatal HS causes oxidative stress in the brain, we measured hippocampal levels of TBARS, a marker of lipid lipoxidation. We found a significant increase in TBARS levels in aged HS offspring. Since oxidative stress depends not only on ROS production, but also on the antioxidant capacity, we determined two important antioxidant enzymes, SOD and catalase. The SOD family includes three isoforms. Cu/Zn-SOD1 is distributed throughout the cytoplasm, nucleus and inner membrane space of mitochondria, Mn-SOD2 is restricted to the mitochondrial matrix, and Cu/Zn-SOD3 is located in the extracellular space (Fridovich, 1983). Catalase is an important enzyme responsible for the decomposition of

H₂O₂ to H₂O and O₂. We found that the expression of *Sod1*/SOD1 was increased, while the activity of catalase was decreased in the HS group. High concentration of SOD1 appears to lead to H₂O₂ formation and induces neuronal damage in the brain (Harris-Cerruti et al., 2004). It has been suggested that catalase may protect neurons from amyloid-induced cytotoxicity (Bruce et al., 1996). Our results indicated that prenatal HS intake induced excessive production of H₂O₂ in the hippocampus, which may induce impairment of neurons.

The present study further investigated whether prenatal HS diets might affect the expression of NOX, a family of ROS-producing enzymes. We found that the expression of *Nox2*/NOX2 in the hippocampus was significantly increased in aged HS offspring as compared to the control. Activation of NOX2 in neurons contributes to ROS production and apoptosis in the brain (Tammariello et al., 2000). Genetic deletion of *Nox2* attenuated oxidative stress and neuronal cell death in mice (Nair et al., 2011). Our results suggest that prenatal HS diets may aggravate oxidative damage in the hippocampus probably via the abnormal activation of NOX2.

Morris water maze was widely used to evaluate hippocampus-dependent spatial learning and memory in rodents. During adolescence, HS offspring exhibited prolonged escape latency and path length in a hidden platform-learning phase, but did not affect the memory retention (Kuang et al., 2014). In the present study, the performance in the spatial acquisition test became worse, and both the time spent in the target quadrant and the number of crossing the platform in retention test were decreased in aged HS offspring, as compared with the control, indicating that the prenatal insult plus aging may cause worse performance on learning and memory task.

NMDARs are a subtype of ionotropic glutamate receptors gated by the neurotransmitter glutamate (Mayer and Westbrook, 1987). We tested three important subunits of NMDARs (NR1, NR2A and NR2B) and found that only *Grin2b*/NR2B subtype was decreased in aged HS offspring. NR2B subunit is expressed at higher levels throughout the brain during early stages of development and declines after birth, while NR2A subunit-containing NMDARs increase through the life span (Laurie et al., 1997; Law et al., 2003), suggesting different expression patterns during various life periods. Previous study showed that maternal high fat diets decreased the expression of NR2B and the ratio of NR2B/NR2A in adult offspring (Page et al., 2014). NR2B subunit plays an important role in recruiting relevant molecules important for learning and memory (Foster et al., 2010). The finding of decreased *Grin2b*/NR2B in the present study suggests a possible involvement of NMDARs in the damaged brain function in aged HS offspring.

In addition to NMDARs, the expression levels of *Wnt2* and *Wnt3a*, as well as the active form of β -catenin protein were decreased, while *Dkk1* was increased in aged HS offspring. *Wnt2* and *Wnt3a* facilitate the activation of FZD and co-receptor LRP5/6 and ultimately lead to the activation of Wnt/ β -catenin signaling (Ai et al., 2007). Abnormal Wnt/ β -catenin signaling resulted in cognitive decline (Ivanova et al., 2016). Increased *Wnt2* or *Wnt3a* may enhance dendritic arbors and synaptic plasticity (Wayman et al., 2006), while the down-regulation of Wnt signaling in the hippocampus has been related to cognitive decline

associated with neuronal loss (Bayod et al., 2015). The present study showed unchanged *Wnt5a* and *Wnt7a* in aged offspring, suggesting a selective effect of Wnt signaling by prenatal HS. *Wnt5a* and *Wnt7a* mainly rely on non- β -catenin-dependent pathways and pertain to intracellular calcium release (Patapoutian and Reichardt, 2000). DKK1 is a secreted protein that acts as an inhibitor of Wnt signaling by binding to LRP5/6 (Semenov et al., 2001). The expression of DKK1 was found significantly increased in the hippocampus of AD mouse models, while reduced expression of DKK1 counteracted the age-related decrease in both neurogenesis and cognitive function (Caricasole et al., 2004; Seib et al., 2013). The finding of decreased *Wnt2*, *Wnt3a*, active β -catenin, and increased *Dkk1* in the present study suggests that prenatal HS may selectively influence the molecules of NMDARs/Wnt signaling pathway in the aged brain. However, to confirm impairment of cognitive functions, more motor and sensory tests of hippocampus function are needed in the future study.

In summary, the present study demonstrated that maternal HS diets during pregnancy is a risk factor for the development of age-related diseases in offspring. Cognitive impairments induced by prenatal HS diets may be correlated with oxidative stress and abnormal NMDARs/Wnt signaling during aging in the hippocampus. These results suggest that prenatal influence may affect health and disease during various life stages. Postnatal influence such as aging may be co-operated with prenatal influence to produce further damage to the brain function in old offspring. Thus, the findings provide new information for further understanding the causes and possible mechanisms of cognitive problems in the old age, as well as new insight in early prevention of the diseases initiated from early developmental stages.

4. Materials and methods

4.1. Experimental animals

Pregnant Sprague-Dawley rats (250–280 g) were obtained from Soochow University Animal Center. Rats were housed in a climate-controlled light-regulated facility with 12:12 h day-night cycles and allowed to free access to food and water. Pregnant rats were randomly divided into two groups (N = 11 each group). From gestational 1 to 21 day, one group was fed with 20% sucrose solution and the other was fed with fresh tap water (C). Both groups were provided with the same standard rat food containing 39.1% carbohydrates, 19.3% protein, 4.0% fat, 0.6% NaCl, 1.00% calcium, 0.70% phosphate and 0.68% potassium (Slaccas, Shanghai, China). During pregnancy, there was no difference in daily fluid intake (36.2 ± 1.4 ml/day versus 36.3 ± 1.5 ml/day). After delivery, all rats were provided with fresh tap water. An average litter size was 9–14. After weaning, male offspring from both groups (n = 1 from each dam) were randomly selected and raised with standard food until 18 month old. All procedures and protocols were approved by the Institutional Animal Care and Use Committee and conform to the Guidelines for the Care and Use of Laboratory Animals.

4.2. Task of Morris water maze

The water maze was modified from the standard version of the Morris test (Morris, 1984). It contained a circular pool (0.46 m in depth and 1.2 m in diameter) and a submerged platform

(26 cm in height and 10 cm in diameter). The pool was divided into four virtual quadrants, and each quadrant had different sign on the wall. During testing, the pool was filled with opaque water ($26\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$) by addition of nontoxic white paint. There were 4 acquisition trials each day with training lasting for 7 days and the position of the cues was not changed during testing. Each trial was started by placing a rat facing toward the wall. A trial was ended until the rat climbed on the hidden platform with all 4 paws or until 60 s elapsed. If the rats couldn't find the platform, they were guided to the platform and sat on it for 15 s before being removed and dried with a towel. In the end, the latency and path length for swimming to the platform were measured.

The navigational task was tested at the end of the day of acquisition training finished after the platform was removed. All rats were placed in the pool in a randomly determined quadrant only once and were allowed to swim within a limited time (60 s). The numbers of crossing the platform and the time spent in target quadrant were measured. All activities of rats in the testing were monitored, recorded, and analyzed using MT-200 water maze video tracking system (Taimeng, Chengdu, China).

4.3. Intraperitoneal glucose tolerance test and sample preparation

Glucose tolerance test was performed in fasted rats (16 h). Blood for glucose determination was drawn from the tail at intervals of 0, 30, 60, and 120 min after giving a glucose solution (2 g/kg body weight) by intraperitoneal injection.

Offspring rats were sacrificed using sodium pentobarbital (100 mg/kg; Jiangsu Henrui) intraperitoneally. Blood samples were collected from abdominal aorta and placed into anti-coagulation tubes. Rat brains were removed by cutting at the edge of vermis for measuring weight. Then, hippocampal tissues were collected and were frozen at $-80\text{ }^{\circ}\text{C}$ for analysis.

4.4. Blood sample assay

Plasma glucose was determined using Glucose Assay Kit (Shanghai Rongsheng Cat: 361500) according to manufacturer's instructions, and plasma insulin was assayed with radioimmunoassay (RIA) by Huaying Biotechnology Institute (Beijing, China). The sensitivity for insulin was 0.01 mIU/L. The intra-assay and inter-assay CV were about 3.0–6.7% and 7.5–9.9%, respectively. Insulin sensitivity of individual rats was evaluated using the homeostasis model assessment (Homa). The formula used as follows: $\text{FPG (mmol/L)} \times \text{FINS (mIU/L)} / 22.5$. Samples and data were handled in a blind manner.

4.5. TBARS and catalase assay

The measurement of TBARS is a well-established method for monitoring lipid peroxidation. Levels of TBARS in the hippocampus were measured using TBARS Assay Kit (Cayman Item No. 10009055). The reaction mixture contained 0.1 ml hippocampus homogenate (25 mg protein), 530 mg thiobarbituric acid, 2 ml acetic acid solution, (pH 3.5, 20%), 2 ml sodium hydroxide, and 0.1 ml SDS (0.1%). Samples were quantitatively analyzed by reading the absorbance at 532 nm (Dawn-Linsley et al., 2005). Catalase activity was assayed by calculating the rate decomposed H_2O_2 colorimetrically at a wavelength of 240 nm using Assay Kit (Nanjing Jiancheng, Cat: A007-1) (Sinha, 1972). The reaction mixture (1.5 ml)

contained 1 ml phosphate buffer (pH 7.0), 0.1 ml of hippocampus homogenate (supernatant), and 0.4 ml H₂O₂. The reaction was stopped by acetic acid reagent. The levels of TBARS and enzyme activities of catalase were expressed as units per milligrams of protein (U/mg protein). Total protein concentration was determined using the Bicinchoninic acid (BCA) assay (Beyotime).

4.6. Quantitative real-time PCR

Total RNA was isolated from the hippocampus tissues using RNA plus (RNA extraction reagent, TaKaRa). RNA was reverse transcribed using Revert Aid First-Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. Q-PCR was performed in 20 µL of the reaction mixture contained 10 µL SYBR Premix Ex Taq (TaKaRa), 5 µL cDNA (50 ng), and 5 µL primers (400 nM). The reaction was analyzed on iCycler Real-Time PCR Detection System (Bio-Rad) with the following conditions: 95 °C for 5 min and 45 cycles of 95 °C 5 s/62 °C 15 s/72 °C 15 s. The analysis was repeated three times for each sample and the expression of mRNA was normalized by each β-Actin mRNA and used 2^{-CT} method. The primer sequences are shown in Table 1.

4.7. Western blotting

Hippocampal tissues (50 mg) were lysed by 0.25 ml RIPA (Beyotime). Protein homogenates were separated on SDS-PAGE gels electrophoretically and transferred onto PVDF membranes. The membranes were incubated with the antibodies: NOX1 (Santa Cruz; sc-5821), NOX2 (Santa Cruz; sc74514), NOX4 (Santa Cruz; sc21860), SOD1 (Santa Cruz; sc-11407), SOD2 (Santa Cruz; sc- 30080), SOD3 (Santa Cruz; sc-67088), NR2B (Millipore; AB15362), β-catenin (1:2000, Millipore), active-β-catenin (1:2000, Millipore) and β-Actin (Beyotime; AA128) overnight at 4 °C. Then membranes were washed in Tris-buffered saline with Tween (TBS-T) and incubated with secondary antibodies (1:1000) for an hour. After washing with TBS-T; the bands were visualized after exposed to Hyperfilm (Amersham). Results were quantified using a UVP Bio-imaging system EC3 apparatus (UVP, Upland, CA, USA). The relative density of the bands was normalized to β-Actin.

4.8. Statistics analysis

Data were analyzed using Prism (GraphPad) and SPSS (version 17.0). Unpaired, two tailed Student's *t*-test or Mann-Whitney's test was used to determine differences between the control and HS groups for biochemical, hormonal, and physiological parameters, as well as for q-PCR and western blot data. Repeated measures ANOVA was conducted for analyzing the data from glucose tolerance and water maze tests, Pos-hoc comparisons were made using the LSD method. Difference among groups were considered statistically significant if *P* < 0.05.

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Abbreviations

AD	Alzheimer's disease
DKK1	Dickkopf-related protein 1
FINS	fasting plasma insulin
HS	high sucrose
FPG	fasting plasma glucose concentration
FZD	Frizzled
Homa-IR	insulin resistance index
LRP5/6	low-density lipoprotein-related protein receptors5/6
NMDARs	N-methyl-D-aspartate receptors
NADPH	nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidases
ROS	reactive oxygen species
SOD	superoxide dismutase
TBARS	thiobarbituricacid reactive substance

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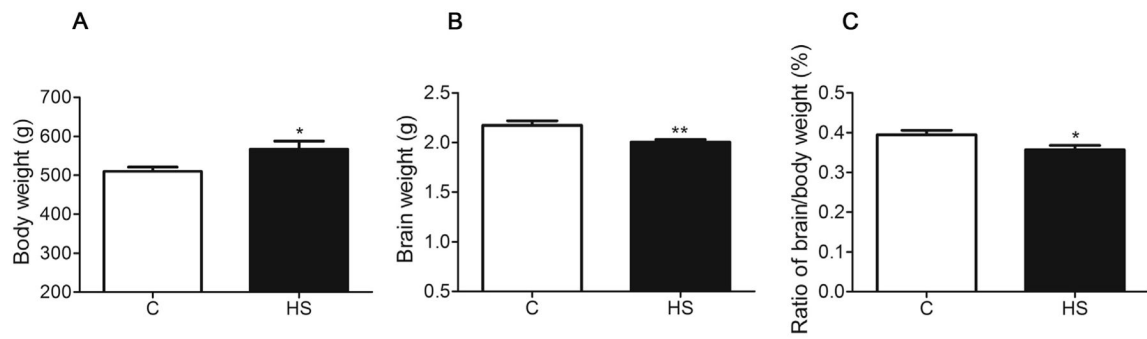


Fig. 1.

The effect of prenatal HS diets on body weight (A), brain weight (B), the ratio of brain/body weight (C) in aged offspring. C: control group (n = 11), HS: prenatal HS group (n = 10); Results are presented as mean \pm SEM; *, $P < 0.05$; **, $P < 0.01$ v.s. the control.

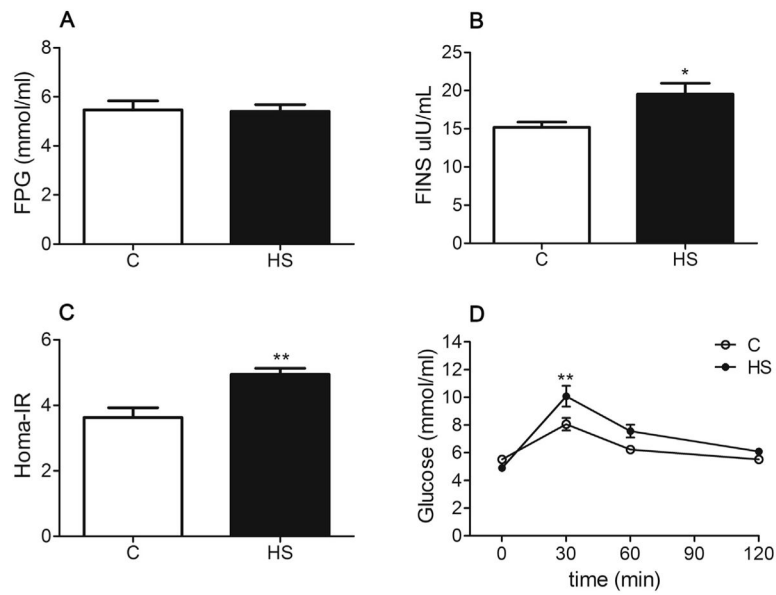


Fig. 2. The effect of prenatal HS diets on plasma glucose (A), plasma insulin (B), Homa-IR (C) and glucose tolerance test (D) in aged offspring. C: control group (n = 8), HS: prenatal HS group (n = 8); Results are presented as mean \pm SEM; *, P < 0.05; **, P < 0.01 vs. the control.

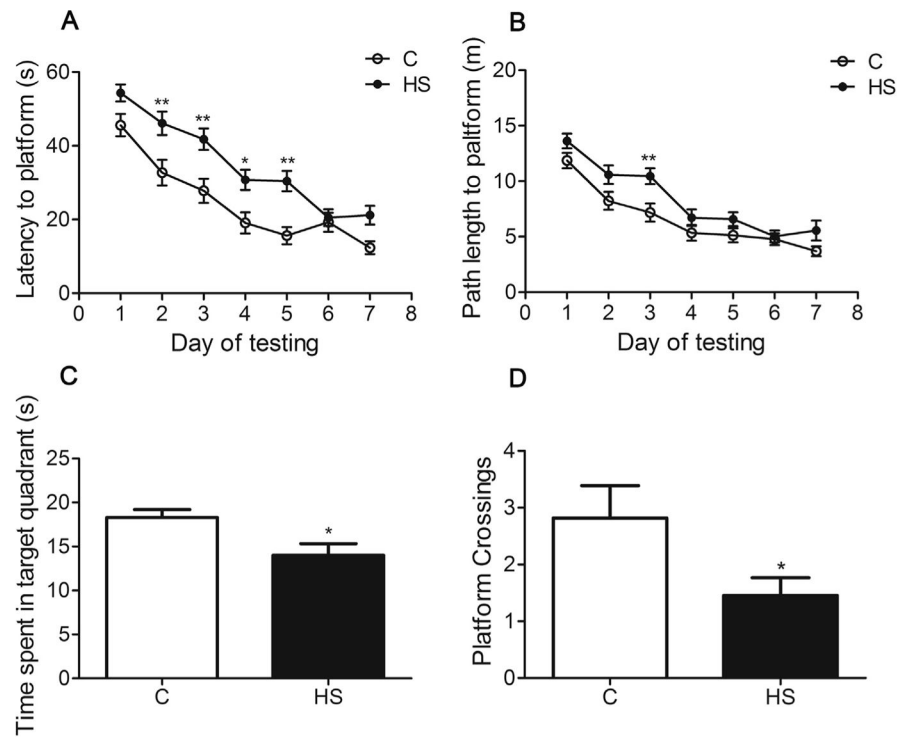
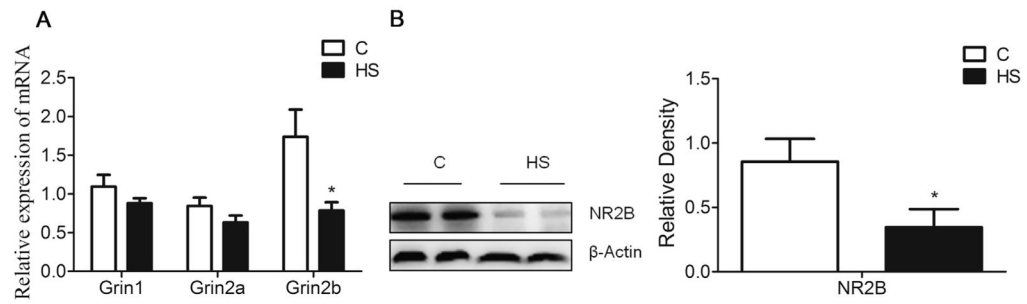
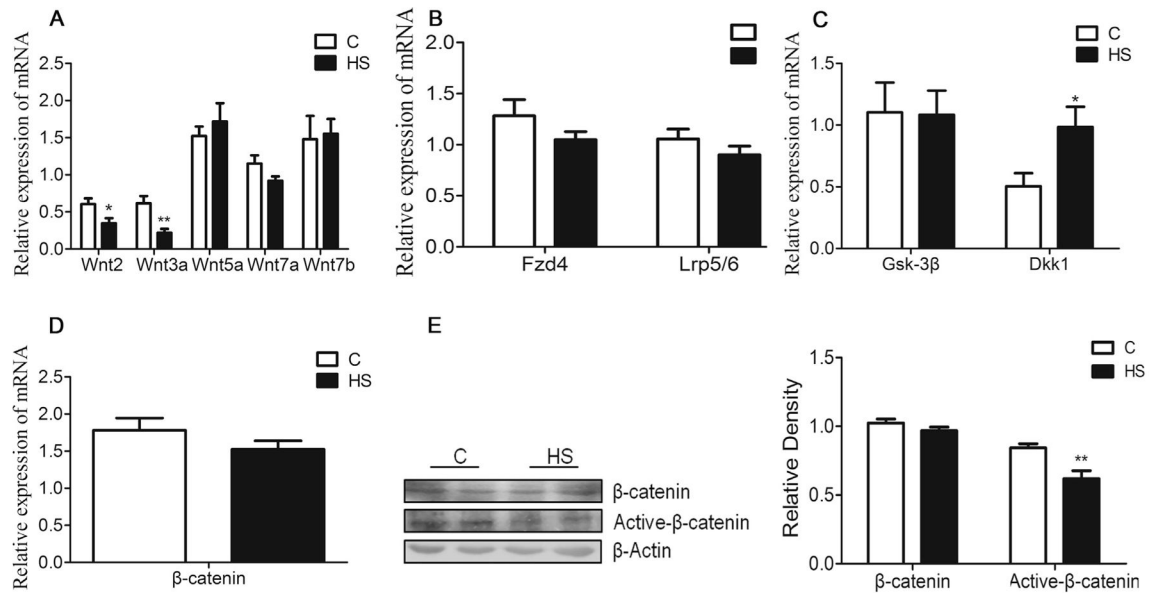


Fig. 3. The effect of prenatal HS diets on behavior performance in aged offspring. The HS group showed longer escape latency and path length to the platform (A, B). The time spent in target quadrant was decreased in HS group in navigation test (C). The number of crossing the platform was significantly decreased in the HS group (D). C: control group (n = 11), HS: prenatal HS group (n = 11); Results are presented as mean \pm SEM; *, $P < 0.05$; **, $P < 0.01$ vs. the control.

**Fig. 4.**

The effect of prenatal HS diets on the expression on NMDARs subunits in the hippocampus in aged offspring. The q-PCR results showed *Grin2b*, not *Grin1* and *Grin2a* expression level was decreased in the HS group compared with the control (A). Western blot analysis showed NR2B was decreased in HS group compared with the control group (B). C: control group (n = 7), HS: prenatal HS group (n = 7); Results are presented as mean \pm SEM; *, P < 0.05; **, P < 0.01 v.s. the control.

**Fig. 5.**

The effect of prenatal HS diets on the expression on Wnt/β-catenin signaling in the hippocampus in aged offspring. The mRNA expression of *Wnt2* and *Wnt3a* were decreased in the hippocampus compared with the control (A). There were no differences in mRNA expression of *Fzd4*, *Lrp5/6*, *Gsk3β* and *β-catenin*, while *Dkk1* was increased in HS group (B–D). The protein expression of β-catenin was no difference, but the form of Active-β-catenin protein was decreased compared with the control (E). C: control group (n = 7), HS: prenatal HS group (n = 7); Results are presented as mean ± SEM; *, P < 0.05; **, P < 0.01 v.s. the control.

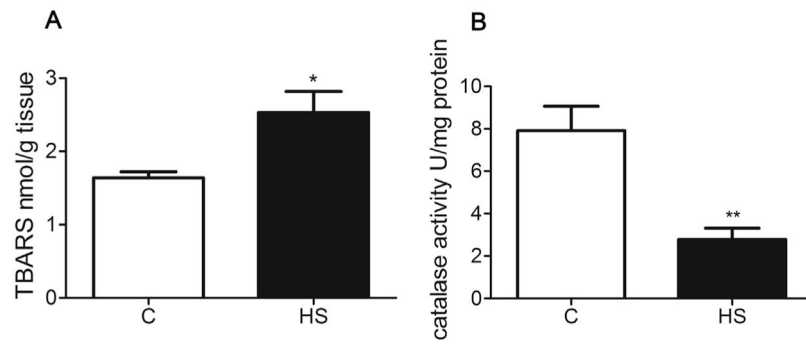
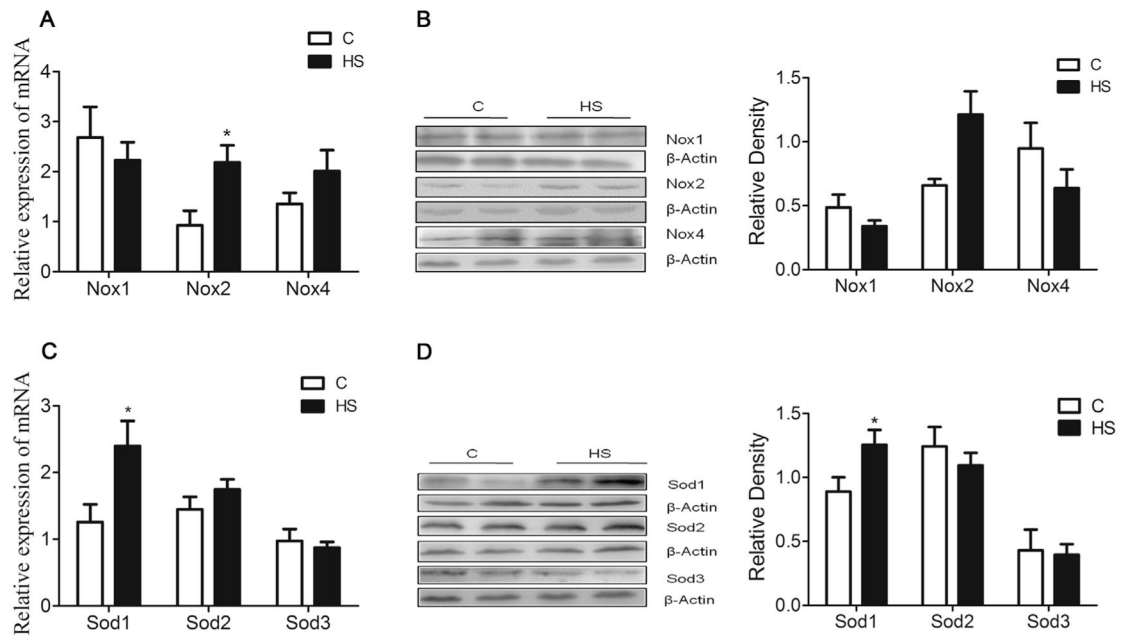


Fig. 6. The effect of prenatal HS diets on TBARS and catalase activity in aged offspring. C: control group (n = 7), HS: prenatal HS group (n = 7); Results are presented as mean \pm SEM; *, $P < 0.05$; **, $P < 0.01$, vs. the control.

**Fig. 7.**

The effect of prenatal HS diets on expression of *Nox/NOX* and *Sod/SOD* in the aged offspring. The mRNA expression and protein of NOX2 (A, B) and SOD1 (C, D) were significantly increased compared with the control. C: control group (n = 7), HS: prenatal HS group (n = 7); Results are presented as mean \pm SEM; *, P < 0.05; **, P < 0.01, vs. the control.

Table 1

Primer sequences.

Gene	Forward primer(5'-3')	Reverse primer(3'-5')	NCBI Ref. Seq.
β -actin	CCTAAGGCCAACCGTGAAAAG	GCTCGAAGTCTAGGGCAACATAG	NM_031144.3
Nox1	TTTTTCAGTTTGCCCTTTGCT	TCCAAGAGCTGAAGCAGGTT	NM_053683.1
Nox2	TCTTCAGCCATTCACACCAT	CAGAGAAGGGAGGCTCACC	NM_023965.1
Nox4	CGGGGTGGCTTGTGAAGTAT	CCTCCAGCAAAGATCCATG	NM_053524.1
Sod1	CGTCATTCACCTCGAGCAGA	AAAATGAGGTCTGCAGTGG	NM_017050.1
Sod2	CTGGACAAAACCTGAGCCCTA	GAACCTTGGACTCCCACAGA	NM_017051.2
Sod3	AGGCTCTTCTCAGGCCTCT	CACCAGTAGCAGGTTGCAGA	NM_012880.1
Grin1	ATACAGATGGCCCTGTCAGTGT	AGTGAAGTGGTCGTTGGGAGTA	NM_001270606.1
Grin2a	TGTCCAGAATCCTAAAGGCACA	CTGTACTTCCATTGGGTACCGTC	NM_012573.3
Grin2b	CACCTTCATCTGTGAGCATCTG	TATGGCTACCCCATGGATACAG	NM_012574.1
Wnt2	CAACATTGACTACGGGATCAAA	CATGACACTTGCATTCTTGTT	XM_575397.6
Wnt3a	AACCGTCACAACAATGAGGC	GCAGGTCTTCACTTCGCAAC	NM_001107005.2
Wnt5a	AAGCAGGTCGCAGGACAGTA	GAGTTGAAGCGGCTGTTGAC	NM_022631.2
Wnt7a	CCGAGAGATCAAGCAGAATGC	TCCAGTTTCATGTTCTCCTCCA	NM_001100473.1
Wnt7b	CAAGAGCTCCGAGTAGGGAGTC	ACAGCCACAGTTGCTCAGATTG	NM_001009695.1
Fzd4	GCTACAACGTGACCAAGATGC	ATGGGGATGTTGATCTTCTCTG	NM_022623.2
Lrp6	GTTGTAAGCTCGGTTCCAAATC	CAATTTTGGGAGATCTTTCCTG	NM_001107892.1
GSK-3 β	GTTTCATGTGCTTCTTTGTC	GGTACTTCCACAAGAACAGCC	NM_032080.1
Dkk1	CATTTTCATTTGATTCCTGGCG	CTTGAGTTGCTCACCAGTTCG	NM_001106350.1
β -catenin	GCTTTGCTCAACAAAACAAACG	ACAGACAGCACCTTCAGCACTC	NM_053357.2