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·Original Article·

Oxidative damage increased in *presenilin1*/*presenilin2* conditional double knockout mice

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Abstract: Objective This report aims to describe the oxidative damage profile in brain of *presenilin1* and *presenilin2* conditional double knockout mice (dKO) at both early and late age stages, and to discuss the correlation between oxidative stress and the Alzheimer-like phenotypes of dKO mice. **Methods** The protein level of $A\beta_{42}$ in dKO cortex and free 8-OHdG level in urine were measured by ELISA. Thiobarbituric acid method and spectrophotometric DNPH assay were used to determine the lipid peroxidation and protein oxidation in cortex, respectively. SOD and GSH-PX activities were assessed by SOD Assay Kit-WST and GSH-PX assay kit, separately. **Results** Significant decrease of $A\beta_{42}$ was verified in dKO cortex at 6 months as compared to control mice. Although lipid peroxidation (assessed by MDA) was increased only in dKO cortex at 3 months and protein oxidation (assessed by carbonyl groups) was basically unchanged in dKO cortex, ELISA analysis revealed that free 8-OHdG, which was an indicator of DNA lesion, was significantly decreased in urine of dKO mice from 3 months to 12 months. Activities of SOD and GSH-PX in dKO and control cortices showed no statistical difference except a significant increase of GSH-PX activity in dKO mice at 9 months. **Conclusion** Oxidative damage, especially DNA lesion, was correlated with the neurodegenerative symptoms that appeared in dKO mice without the deposition of $A\beta_{42}$. Triggers of oxidative damage could be the inflammatory mediators released by activated microglia and astrocytes.

Keywords: *presenilins*; Alzheimer's disease; oxidative damage; 8-OHdG

1 Introduction

Alzheimer's disease (AD), which is characterized by progressive loss of neurons, accumulations of senile plaques and neurofibrillary tangles, is the most common cause of dementia[1-4]. It has been reported that mutations in *presenilin* genes are associated with the early onset of AD[5]. *Presenilin1* (PS1) and *presenilin2* (PS2), two homologous genes that share ~60% amino acids, are considered to be the active sites of γ-secretase complex, which is responsible for the proteolytic processing of amyloid precursor protein (APP), Notch and several other functional proteins[6,7]. The proteolysis of APP by γ-secretase produces β-amyloid peptide (Aβ), which is the main component of senile plaques and a crucial trigger of pathological process of AD[1,4]. It has been confirmed that PS1 knockout mice are embryonic lethal, whereas forebrain-specific PS1 knockout mice based on Cre/loxP system are normal but deficient in enrichment-induced neurogenesis[8-10]. However, PS2 knockout mice had no obviously abnormal phenotypes[10,11]. It is supposed that knockout of both PS1 and PS2 may reduce the production of Aβ, thus delay or prevent the onset of AD. Interestingly, while the forebrain-specific conditional PS1 and PS2 double knockout mice (dKO mice) indeed showed decreased Aβ content,

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they also exhibited several Alzheimer-like neurodegenerative symptoms, including impairments in both synaptic plasticity and hippocampus-dependent memory, severe shrinkage of the cortex, neuronal atrophy, tau phosphorylation and enlargement of the lateral and third ventricles[2,3,12].

Oxidative stress refers to the imbalance between the antioxidative defense system and the oxidative system[13-15]. Among various tissues, brain is very prone to attack, since it possesses high rate of oxygen consumption, plentiful polyunsaturated fatty acids and abundant transition metals, but contains relative paucity of antioxidants[16,17]. In recent years, multiple lines of evidence have suggested that oxidative stress is a causal, or at least an ancillary factor in the neuropathology of AD[13,18,19]. Furthermore, increasing amount of experiments indicated that aggregated Aβ was capable of generating free radicals, and thus induced the oxidative damage in AD-affected brains[13,20,21]; vice versa, Aβ was also subject to attack by free radicals, which then lead to the alterations of Aβ properties and accelerated Aβ fibrils formation[13,20]. Then here come the questions: what is the oxidative stress profile in dKO mice brain that has no Aβ deposition? What is the real correlation between oxidative stress and Alzheimer-like symptoms induced by PS1/PS2 double knockout? A group in our lab had found that oxidative stress enhancement appeared at early stages (≤ 7 months) and lipid peroxidation was enhanced in a gender- and age-related manner in dKO mice[22,23]. The present study therefore aims to thoroughly investigate oxidative stress status in dKO mice not only at early stages but also at late stages, by examining the oxidative products in multi-levels—lipid, protein, DNA and antioxidase levels in brain. Based on this study, we will discuss the relationship between oxidative stress and Alzheimer-like neurodegenerative phenotypes.

2 Materials and methods

2.1 Mouse breeding and genotyping Breeding and genotyping of *presenilins* (PSs) dKO mice have been described previously[2,3,9]. Briefly, dKO mice were obtained by crossing the forebrain-specific PS1 knockout heterozygous mice with PS2 knockout heterozygous mice on B6/CBA F1 genetic background. Mice with the *Cre* transgene, f*PS1*/f*PS1* and *PS2*-/- served as dKO, their littermates (no Cre, fPS1/+ and *PS2*+/+ or *fPS1*/+ and *PS2*+/-) served as controls. All mice were housed under 20-26 ºC temperature and 40-70% humidity with 12 h light/dark cycles, and all the experiments were approved by the Animal Ethics Committee in ECNU, China.

2.2 Enzyme-linked immunosorbent assay for $A\beta_{42}$ ELISA kit for $A\beta_{42}$ was purchased from R&D Systems (USA) and assays were performed according to the manufacturer's instructions. Briefly, 96-well plates were precoated with the anti-mouse $A\beta_{42}$ antibody and blocked. 50 μL tissue lysate of whole cortex at 6 months was loaded into the wells and then incubated at 37 ºC for 30 min. After maintaining the washing solution in the well for 0.5 min 5 times, HRP-conjugated goat anti-human antibody (50 μL) was added into each well and incubated at 37 ºC for 30 min. After washing, 100 μL of chromogenic agent (TMB) was added for the color reaction at 37 ºC for 15 min in darkness. 50 μL of reaction terminating solution was added to develop the final color. Absorbance at 450 nm was measured using GENios microplate reader (Tecan GENios, Austria). Concentrations were normalized to the loading amount of wet tissue.

2.3 Lipid peroxidation products determined by thiobarbituric acid method Malondialdehyde (MDA), which is used to assess the level of lipid peroxidation, was determined by thiobarbituric acid method using the MDA Assay Kit (Nanjing Jiancheng Corp., China). Mice of dKO and control aged 3, 6, 9 and 12-month were decapitated rapidly, and the whole cerebral cortex was separated quickly and stored at -80 ºC for later use. Tissues were weighed and then 9 times volume of the phosphate buffer saline (PBS, 0.137 mol/L sodium chloride, 2.7 mmol/L potassium chloride, 10 mmol/L dibasic sodium phosphate, 2 mmol/L potassium dihydrogen phosphate) based on the tissue weight was added. Tissues were homogenized and centrifugated at 3 000 r/min for 15 min at 4 ºC. 25 μL of the supernatant and the same volume of thiobarbituric acid were added to 1 000 μL chromogenic agent mixture. Reaction way undergone at 94 ºC for 40 min, and then the absorbance at 532 nm was measured by ultraviolet-visible spectrophotometer (Biochrom, England). 10 nmol/L of 1,1,3,3-tetraethoxypropane and ethyl alcohol served as standard and blank control, respectively. BCA Protein Assay Kit (Beyotime Ins.Bio, China) was used to measure the protein concentration. Data were corrected by the protein

concentration to obtain the final MDA content.

2.4 Spectrophotometric DNPH assay for carbonyl groups Carbonyl groups were measured as described previously[24]. Briefly, each tissue of whole cortex was homogenized separately in lysis buffer (Beyotime Ins.Bio, China) containing 1mmol/L PMSF on ice and centrifugated at 12 000 r/min for 5 min at 4 ºC. Protein concentration was assessed as described above. Protein samples (80 μL) were mixed with 10 mmol/L 2, 4-dinitrophenylhydrazine (DNPH) (80 μL) that dissolved in 2 mol/L HCl, and then they were incubated at room temperature for 1h with vortex every 10 min. 30% TCA (80 μL) was added to precipitate the protein, followed by centrifugation at 12 000 r/min for 15 min at 4 ºC. After the pellets were washed three times with 1mL ethanol-ethyl acetate (1:1, v:v), 6 mol/L guanidine HCl (160 μL) was added to dissolve the pellets. Tubes were kept at 37 ºC for 30 min with shaking, then were centrifugated at 5 000 r/min, 4 ºC for 5 min to remove the insoluble substance. Absorbance at 366 nm was measured by ultraviolet-visible spectrophotometer and concentration of the carbonyl groups was obtained by a molar absorption coefficient of 22 000 L/(mol•cm). The final carbonyl content was calculated in nmol/mg protein.

2.5 Enzyme-linked immunosorbent assay for free 8-hydroxy-2-deoxyguanosine (8-OHdG) Mice urine samples were collected by metabolic cage and stored at -80 ºC until analysis. A competitive ELISA kit (New 8-OHdG check, Japan Institute for the Control of Aging, Japan) was used to determine the concentrations of 8-OHdG in the urine samples according to the manufacturer's instructions. Briefly, equal volumes (50 μL) of urine samples and anti 8-OHdG monoclonal antibody were loaded into 96-well plates that were precoated with 8-OHdG, and were incubated at 37 ºC for 60 min. After washing the plates three times, HRP-conjugated anti-mouse antibody (100 μL) was added to each well followed by incubation for 60 min at 37 ºC. After washing again, substrate (100 μL) was added into each well and incubated at room temperature for 15 min in the darkness. Then reaction terminating solution (100 μL) was added to develop the final color. Absorbance at 450 nm was measured using GENios microplate reader. Data were corrected by urinary creatinine (LabAssay™ Creatinine, Wako, Japan) to get the final results.

2.6 Antioxidase examination To measure the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-PX), SOD Assay Kit-WST (Dojindo Inc., Japan) and GSH-PX assay kit (Nanjing Jiancheng Corp., China) were employed respectively, according to the manufacturer's protocol. Protein concentration was measured as described above. SOD activity was assessed by adding 20 μL cortex tissue solution or double distilled water (ddH_2O, as the blank control) to 96-well microplate. After adding WST working solution (200 μL), 20 μL of enzyme working solution was added to each sample and control well, then the plate was incubated at 37 ºC for 20 min. The absorbance at 450 nm was detected by GENios microplate reader. SOD activity was calculated in U/mg protein.

For GSH-PX activity measurement, 40 μL of 1mol/L glutathione (GSH) was added to both enzymatic reaction and non-enzymatic reaction tubes. The cortex tissue solution was then added to the enzymatic reaction tube. After adding H_2O_2 (20 μL) into each tube, reaction was strictly controlled for 5 min by adding reaction terminating solution (400 μL). All the operations above should be maintained in 37 ºC water bath. At this time, 40 μL of cortex tissue solution was added to non-enzymatic reaction tube. All reaction tubes were centrifugated at 4000 r/min for 10 min, then 250 μL of chromogenic agent mixture was added to each tube that contained 200 μL of the supernatant and incubated at room temperature for 15 min. Here, instead of the supernatant, the same volume of 20 μmol/L GSH or GSH standard working solution served as the standard or blank control, respectively. Absorbance at 412 nm was measured by ultraviolet-visible spectrophotometer for the final calculation with the correction by protein concentration.

2.7 Statistical analysis All data were expressed as mean ± SEM. Statistical significance was determined by Student's *t*-test using SigmaPlot Software (Systat Software Inc.). $P < 0.05$ was considered as statistically significant.

3 Results

3.1 Decreased protein level of $A\beta_{42}$ in dKO mice cortex ELISA was employed to determine the protein levels of $A\beta_{42}$ in the cortex of dKO and control mice at 6 months age. Data showed a significant decrease of $A\beta_{42}$ in dKO cortex as compared with that in controls (Fig.1), which confirmed that conditional PS1 and PS2 double knockout reduced the production of

toxic Aβ.

3.2 Increased lipid peroxidation at the very early age of dKO mice Cortical homogenate of dKO and control mice aged 3, 6, 9 and 12 months was used to determine the level of lipid peroxidative product MDA. Data showed that MDA level was increased in the cortex of dKO at 3, 6 and 9 months when compared with that of control mice, however, only the increase at 3 months was statistically significant (Fig. 2A).

3.3 Protein oxidation profile in dKO mice Since it is well accepted that protein carbonyl represents another oxidative stress biomarker, we next applied spectrophotometric DNPH assay to estimate the protein oxidation level in dKO mice. Results showed a decrease of protein carbonyl, level in 3 months dKO cortex and an increase beginning at 6 months. However, there appeared no statistical difference (Fig. 2B).

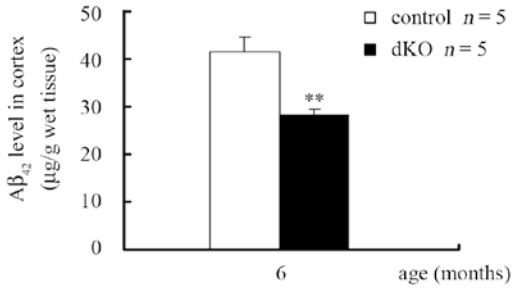

Fig. 1 Confirmation of dccreased $A\beta_{42}$ content in dKO mice. ELISA analysis demonstrated the reduced production of $A\beta_{42}$ (μg/g wet tissue) in whole cortex of dKO mice at 6 months age when compared with controls.

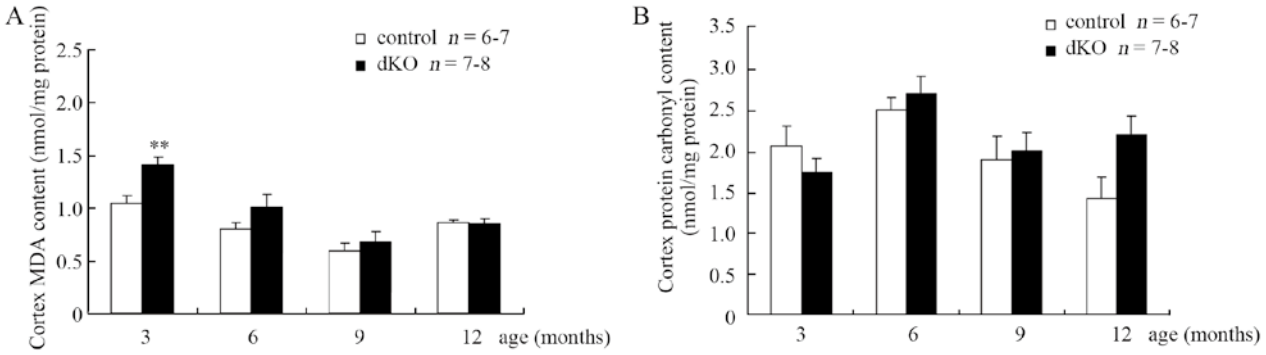

Fig. 2 Alterations of lipid peroxidation and protein oxidation profile in dKO mice. A: The MDA content (nmol/mg protein) at 3, 6, 9 and 12 months was used to assess the lipid peroxidation. Significantly increased lipid peroxidation in whole cortex existed at the 3-month age in dKO mice, whereas MDA level gradually reverted to the control level as dKO mice were getting older. B: Spectrophotometric DNPH assay results showed the trend of increase of carbonyl groups content in dKO as compared with control mice, but with no statistical difference (*P*=0.07). Here *n* indicates the number of individual mouse in each tested group.

3.4 Decreased free 8-OHdG level in urine of dKO mice at all age points Previous studies elucidated that DNA was the primary attack target of ROS, especially the hydroxyl radicals, producing 8-OHdG[25]. Here ELISA was employed to assess the concentration of free 8-OHdG in urine, thus to detect whether the repair system of oxidized DNA was functional or not. Data demonstrated that free 8-OHdG was significantly reduced in dKO mice at 3, 6, 9 and 12 months as compared with that in the age-matched controls, which suggested that dKO mice might have a lower ability to repair the oxidized DNA (Fig. 3).

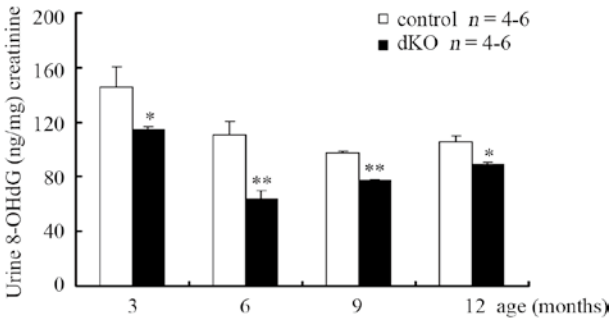

Fig. 3 Free 8-OHdG was greatly decreased in dKO urine at four age points. ELISA was employed for measurement of free 8-OHdG (ng/mg creatinine) in urine in dKO and control mice. Data indicated a significant decrease of free 8-OHdG in dKO mice at the age of 3, 6, 9 and 12 months. Here *n* indicates the number of individual mouse in each tested group.

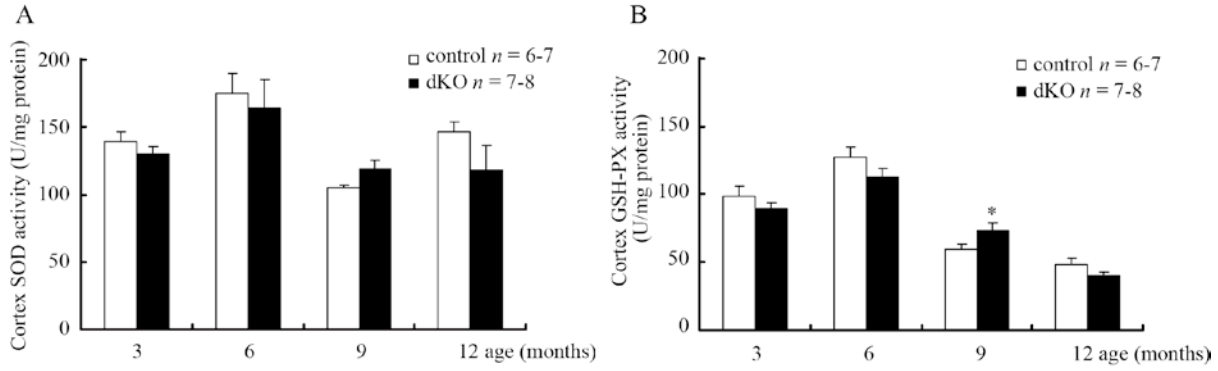

Fig. 4 Detection of SOD and GSH-PX activities in whole cortex of dKO and control mice. Data showed some alterations of (A) SOD activity (U/mg protein) and (B) GSH-PX activity (U/mg protein) in whole cortex of dKO mice at 3, 6, 9 and 12 months. Here *n* indicates the number of individual mouse in each tested group.

3.5 Alterations of antioxidant enzymes in dKO mice

Interestingly, activities of antioxidant enzymes SOD and GSH-PX in dKO mice showed a similar change tendency, both decreased at 3, 6 and 12 months and increased at 9 months in dKO cortex. However, there appeared no significant difference in most groups except a marked increase of GSH-PX activity in dKO cortex at 9 months (Fig. 4).

4 Discussion

It has been reported that Aβ production was reduced in PS1/PS2 conditional double knockout mice (cDKO)[12]. Although our dKO mice are also forebrain knockout of PS1/PS2, there are two differences between dKO and cDKO. One is the different loxP insert site on PS1 allele, and the other is that these two strains of PS1/PS2 conditional knockout mice are generated by two separate research groups and are maintained in different conditions[2,9,12,26]. To examine the Aβ level in our dKO mice, we evaluated protein level of $A\beta_{42}$ using ELISA assay for the first time. Our data demonstrated that the $A\beta_{42}$ protein level was exactly reduced in dKO mice cortex, which confirmed that loss-of-function of *presenilins* lead to the reduction of $A\beta_{42}$ toxicity.

Lipid peroxidation occurs when free radicals attack polyunsaturated fatty acid in membrane phospholipids, and its primary or secondary products will decompose into several small molecules, such as MDA, 4-hydroxy-2,3-trans-hexenal (4-HNE) and isoprostanes[27]. Using MDA as an indicator of lipid peroxidation, we found that lipid oxidative damage was an early event (before 6 months age) in dKO mice, which was consistent with the previous study[22]. However, using isoprostanes as a marker, Zhu *et al.* found that lipid peroxidation was enhanced in an age dependent manner[23], which suggested that there were different lipid peroxidative products at various age points induced by PS1/PS2 double knockout, and isoprostanes could be a more sensitive indicator of lipid peroxidation.

Protein carbonyls are generated when protein side chains are attacked by reactive oxygen species (especially at Pro, Arg, Lys and Thr)[28,29]. Most of the studies demonstrated that protein oxidation was increased in AD patients cortex or hippocampus[29-31]. However, we found that although protein carbonyls showed the trend of increase in dKO, there was no significant difference between dKO and control mice. This result suggested that free radicals had not yet induced the visible protein oxidation in dKO mice, or perhaps spectrophotometric DNPH assay used in this study could not detect the low level of protein oxidation. More sensitive techniques are needed for further analysis.

Although both lipid peroxidation and protein oxidation induced by loss function of *presenilins* are not so significant in this study, DNA oxidation in dKO mice is obvious. Free radicals such as O_2^-, H_2O_2, ·OH and $OONO^-$ can attack the DNA in tissues and cause a lot of damage, including strand breaks, sister chromatid exchange, DNA-DNA and DNA-protein cross linking, and base modifications[30]. Among various biomarkers of oxidative DNA damage, 8-OHdG is the one most commonly used[25,30]. Free 8-OHdG is generated via specific DNA repair enzyme, cutting the oxidative guanine in

intact DNA, which is then discarded from the organism through the urinary system. Our data showed a significant decrease of free 8-OHdG level in dKO mice as compared to that in controls at all tested age points (from 3 to 12 months), reflecting a functional deficiency of oxidative DNA repair enzyme, which suggested a dramatic increase in DNA oxidative level induced by PS1/PS2 knockout. This result gives us a hint that DNA oxidative damage could be more correlated with AD-like neurodegenerative symptoms induced by loss function of *presenilins*, and that DNA damage could result in the alterations of other targets (mitochondria for example) in neurodegeneration, which needs to be further verified.

In general, our results and previous findings[22,23] suggest that activities of antioxidant enzymes, such as SOD and GSH-PX, had no significant alterations in dKO cortex, which indicated the imbalance between the unchanged defense system and the increased oxidative system, finally resulting in oxidative damage, especially DNA damage, in dKO mice brain.

As mentioned above, our results confirmed that the protein level of $A\beta_{42}$ was significantly decreased. So, what is the trigger of oxidative stress in dKO mice? Reasons that have been reported for oxidative stress may include: (a) damaged mitochondria; (b) loss of homeostasis of iron and copper in brain; (c) lesions of the brain; (d) glycation, glycoxydation and advanced glycation end products; (e) activation of microglia or astrocytes; (f) proteolysis dysfunction[32]. From our data in dKO, we found that there existed robust activation of microglia or astrocytes and enhanced release of inflammatory mediators in dKO cortex as early as 3 months age (data is being prepared for another paper). Thus we speculate that robust inflammatory response could be one of the reasons for oxidative damage, especially DNA damage, in dKO mice that exhibited several AD-like neurodegenerative phenotypes induced by the loss-of-function of the *presenilins*. Next we will apply interference in dKO mice using anti-inflammation drugs or antioxidants to further investigate the relationships among inflammation, oxidative damage and AD-like symptoms.

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Presenilin1/*Presenilin2* 双基因敲除小鼠中氧化损伤增加

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摘要：目的 观察3，6，9，12月龄*presenilin1*和*presenilin2*双敲除小鼠(dKO)脑组织氧化损伤情况,探讨氧化损伤与*presenilins*功能丧失所引起的阿尔茨海默病样症状之间的关系。**方法** 酶联免疫反应(ELISA)检测dKO小鼠大脑皮层中$A\beta_{42}$水平和尿液中8-羟基脱氧鸟苷（8-hydroxy-2-deoxyguanosine, 8-OHdG）水平；硫代巴比妥酸法检测脂质过氧化水平，分光光度计测定DNPH法检测蛋白质损伤水平；试剂盒法检测超氧化物歧化酶(SOD)和谷胱甘肽过氧化物酶(GSH-PX)活性。**结果** 与对照组相比，dKO小鼠大脑皮层中$A\beta_{42}$水平显著降低。同时，除了在3月龄dKO小鼠大脑皮层中发现脂质过氧化水平有显著增加外，蛋白质损伤在各年龄段dKO小鼠中均没有明显变化。ELISA检测结果显示尿液中游离的8-OHdG水平在各年龄段的dKO小鼠中均显著降低。SOD和GSH-PX活性均无明显变化，只有9月龄dKO小鼠中的GSH-PX活性有显著增高。**结论** 氧化损伤特别是DNA损伤参与dKO小鼠神经退行性症状的发生过程；由于dKO小鼠大脑皮层中没有$A\beta_{42}$的沉积，推测造成氧化损伤的原因可能是由活化的小胶质细胞和星形胶质细胞所释放的免疫因子介导的。

关键词：早老素基因；阿尔茨海默病；氧化损伤；8-OHdG