

Full Length Research Paper

# Kinetin protects against lipid peroxidation and improves antioxidant status in cultured astrocytes and mouse brain exposed to D-galactose

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The aim of this study was to evaluate the neuroprotective effects of kinetin (Kn) on oxidative damage induced by D-galactose (D-gal). *In vitro*, cultured astrocytes were distributed equally in different culture flasks to serve as control, model, and test groups. They were incubated respectively with 0  $\mu$ M (model group), 50, 100 or 200  $\mu$ M Kn along with 15 mM D-gal for 24 and 72 h, but cells in the control group received only normal medium. Activities of total-superoxide dismutase (T-SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) levels were measured by biochemical analysis. *In vivo*, mice were also randomly divided into control, model, and test groups. They were subcutaneously injected with D-gal (125 mg/kg), and administered with 0 (model group), and 10, 20 and 40 mg/kg Kn (test groups) per day simultaneously by gastric perfusion, but mice in the control group only received 0.4 ml physiologic saline solution per day by gastric perfusion. After 6 weeks, T-SOD and GSH-Px activities, and MDA levels in the brain tissue were assessed by biochemical analysis. Results show that both D-gal-treated astrocytes and mice brains displayed impaired antioxidant systems, an increase in MDA levels, and a decrease in T-SOD and GSH-Px activities. After Kn treatment, the changes of antioxidant enzymes activities and MDA levels were reversed both *in vitro* and *in vivo*. In conclusion, results of this study indicate that supplementation of Kn protects cultured astrocytes *in vitro* and mouse brain from D-gal-induced oxidative damage.

**Key words:** Kinetin, D-galactose, oxidative stress, astrocytes, brain aging.

## INTRODUCTION

Free radical, a major factor in aging and various diseases, are produced constantly by normal metabolic processes. Excessively high concentrations of free radical overwhelm the antioxidant defense system and induce oxidative damage within the body. The brain is particularly vulnerable to free radical than other tissues, since the brain utilizes one-fifth of the total oxygen demand of the body (Hall and Braughler, 1989) and it is

not particularly enriched in any of the antioxidant enzymes (Benzi et al., 1989). Accumulation of reactive oxygen species (ROS) as a result of imbalances in its production and the activities of cellular antioxidants may initiate neurotoxic events such as Alzheimer's and Parkinson's diseases (Olanow, 1992; Richardson, 1993; Chauhan and Chauhan, 2006). Astrocytes, the most abundant glial cell types in the brain, have been known to carry out a number of functions including the dynamic regulation of synaptic network formation, neural electrical activity, the blood-brain barrier integrity, and defense system of the brain against ROS (Takuma et al., 2004; Musalmah et al., 2006; López et al., 2007). The ability of astrocytes to maintain these functions may be a critical determinant of neuronal survival and brain function after damage. Thus, astrocytes could be one of the targets for neuroprotection

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**Abbreviations:** Kn, Kinetin; D-gal, D-galactose; T-SOD, total-superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde; ROS, reactive oxygen species.

(Li et al., 2008).

According to the theory which free radical induces aging (Harman, 1956), many believe that antioxidant intake, both from the diet or from supplementation, is beneficial to slowing the brain aging process. For example, antioxidants as vitamin C can directly and rapidly scavenge free radicals or inhibit their formation. Thus, sufficient dietary intake of vitamin C by individuals is beneficial in improving learning and memory of aged mice (Harrison et al., 2009). Kinetin (N<sup>6</sup>-furfuryladenine, Kn) was first isolated and identified in 1955 (Rattan and Clark, 1994; Barciszewski et al., 1999) from autoclaved herring sperm DNA. Since then this compound has been widely used as a cytokinin in various aspects of plant research, including plant cell division and differentiation, retardation of senescence and protection against abiotic oxidative stress. For a long time, Kn was recognized as a unnatural synthetic product. However it was found in 1996 that Kn occurs in commercially available DNA, in freshly extracted cellular DNA from human cells, in plant cell extracts and human urine (Barciszewski et al., 1996; Raman et al., 1996; Barciszewski et al., 2000). Recently, Kn was extracted in the endosperm liquid of fresh young coconut fruits (Ge et al., 2005). Most surprisingly, Kn also exerts anti-aging effects even in human skin fibroblasts (Rattan and Clark, 1994), dermal microvascular endothelial cells (Lee et al., 2006) and fruitflies (Sharma et al., 1995). Although the detailed mechanisms of Kn action has not yet been completely revealed, several lines of evidence indicate that Kn may act as an antioxidant or indirectly as a regulator of antioxidants. For example, Kn protects against Fenton reaction-mediated oxidative stress to DNA by acting as a radical scavenger or by binding iron in such a way that it is no longer a Fenton reductant or in a way that prevents iron from associating with the DNA (Olsen et al., 1999); makes a complex with superoxide dismutase (SOD) and can indirectly enhance catalase (CAT) and glutathione peroxidase (GSH-Px) activities in order to counteract hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) stress (Rattan and Sodagam, 2005); has free radical scavenging effect in activated platelets (Hsiao et al., 2003). These results indicate that Kn may be a potential preventor of lipid peroxidation in living animal cell. However, no published data are available shown the neuroprotective effect of Kn in mammals. Therefore, in this study, we examined the effect of Kn supplementation on antioxidant defense system and lipid peroxidation in cultured astrocytes and mouse brain on the basis of the potential health benefits of Kn.

## MATERIALS AND METHODS

Dulbecco's Modified Eagle's Medium (DMEM) used in the study was obtained from GIBCO (New York, USA), and fetal calf serum was from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (Hangzhou, China). D-gal was purchased from Shanghai Chemical-Regent Company (Shanghai, China) and dissolved in 0.9% NaCl (w/v) at the concentration of 5 mg/ml. Kn was supplied

by Xiamen Xinglongda Chemical-Regent Company (Xiamen, China). 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was obtained from GIBCO (New York, USA). Commercial kits used for determination of T-SOD, GSH-Px, and MDA were purchased from Jiancheng Institute of Biotechnology (Nanjing, China). All other reagents were of analytical grade.

## Preparation of Kn solutions

Since Kn is practically insoluble in water (<1.5 mg/L), all concentrated Kn solutions were prepared by dissolving in a minimum amount of 0.06 M HCl and stored at 4°C.

## Animals

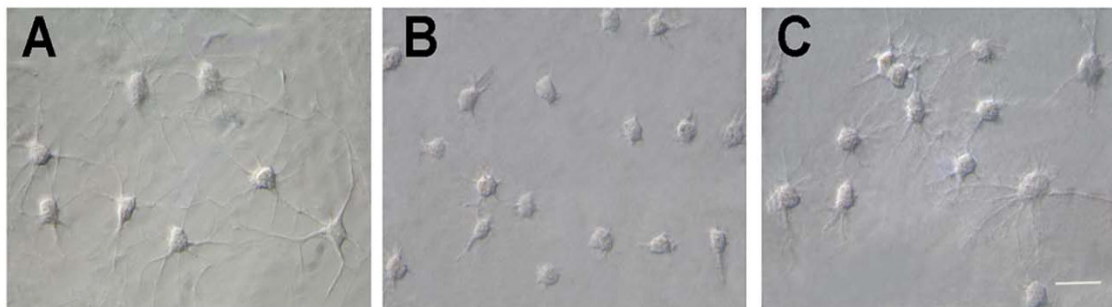
Newborn (1 day old) Sprague–Dawley rats and ICR mice (♂, 20 ± 2 g) were obtained from the Laboratory Animal Research Centre of the Fourth Military Medical University in China. All animal experiments were conducted according to institutional and ethical guidelines involving the use of animals (The Committee of Science and Technology of the People's Republic of China). Mice were group-housed (5 mice per cage) with free access to food and water, kept in a regulated environment at constant temperature (20 ± 2°C) and humidity (70 ± 4%) under a 12 h light-dark cycle.

## Primary culture of rat cerebrum cortical astrocytes

Primary cultured rat astrocytes were prepared from the cerebral cortices of newborn Sprague–Dawley rats as described previously (Reetz et al., 1997; Cristiano et al., 2001; Yu et al., 2002; Zaheer et al., 2004; Goursaud et al., 2009), with minor modification. Brains were aseptically removed from the skulls, cerebral cortices were dissected out, and meninges carefully peeled off. Trypsin-dissociated cells were sieved through sterile nylon mesh (100 µm pore diameter) with the plunger of a syringe. Then the cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C in a humidity of 5% CO<sub>2</sub> atmosphere. The medium was changed after 1 to 2 days and then twice a week. On reaching confluence, the cultures were shaken at 260 rpm overnight to remove microglia and oligodendrocyte.

## Oxidative stress model of astrocytes and Kn treatment

In the near-confluent state, cells were trypsinized. Following dissociation, the cells were stained with 0.4% trypan blue and the cell number was counted using a hemocytometer under a phase contrast microscope. Approximately 1×10<sup>5</sup> cells/ml medium were then transferred and distributed equally in different culture flasks to serve as control, model, and test groups. Before use, astrocytes were grown to confluence with normal medium. On the day of the experiment, the culture medium was removed and replaced with different medium. In the control group astrocytes were incubated in normal medium. In the model group, 15 mM D-gal was incorporated to the medium. The culture medium in the test groups was additionally supplemented with 50, 100 and 200 µM Kn respectively along with 15 mM D-gal. After been treated at different periods (24 and 72 h), cells from the control, model and test groups were observed under a phase contrast microscope, and processed for the estimation of viability and biochemical parameters as described below. Pilot tests with various amounts of HCl equivalent to those present in the Kn medium did not show any harmful effects on the survival and growth of astrocytes. Therefore no further controls with HCl were included in the experiments.



**Figure 1.** Effect of Kn on the cell morphology in cultured mouse astrocytes after 72 h. (A) control culture; (B) cultures after addition of 15 mM D-gal; (C) after addition 15 mM D-gal and 100  $\mu$ M Kn. White scale bar represents 25  $\mu$ m.

### Cell viability analysis

Cell viability was assessed with MTT assay. After each one of the treatment periods (24 and 72 h), the free-cell medium was removed and a 5 mg/ml MTT solution was added. After 4 h incubation at 37°C, the MTT solution was removed and formazan was dissolved in DMSO. MTT reduction in living cells was quantified at 490 nm wavelength using a microplate reader (Biotek, USA).

### Biochemical estimations of primary cultured astrocytes

After 24 and 72 h treatment, cells were scraped off in ice-cold PBS, centrifuged, and the pellets were stored at -80°C. Cells were disrupted by three cycles of freeze-thawing, followed by centrifugation at 18,000  $g$  for 15 min at 4°C. The supernatant was collected and used for protein determination and biochemical parameters assays. Antioxidant enzymes activities and MDA levels were determined by using commercially available kits supplied by Jiancheng Institute of Biotechnology (Nanjing, China). All procedures completely complied with the manufacturer's instructions. Protein concentrations were determined according to the method of Bradford (1976) using Coomassie protein assay dye and bovine serum albumin as a standard. Theoretically, the activity of T-SOD was based on its ability to inhibit the oxidation of hydroxylamine by the xanthine-xanthine oxidase system (Oyannui et al., 1984), and the activity of GSH-Px was determined by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione (Zhang et al., 2007). Thiobarbituric acid reaction (TBAR) method was used to determine the MDA levels which can be measured at the wavelength of 532 nm by reacting with thiobarbituric acid (TBA) to form a stable chromophoric production (Ohkawa et al., 1979).

### Animals grouping and treatment

After acclimatization to the laboratory for 1 week, mice were randomly divided into five groups and each group contained ten mice. For the control group and model group, mice received 0.4 ml physiologic saline solution (0.9%)/day by gastric perfusion and the test groups mice were administered Kn (dissolved in the 0.06 mol/L HCl) by gastric perfusion at doses of 10, 20 and 40 mg/kg body weight per day, respectively. Except the control group, each group was induced by a single hypodermic injection of D-gal (125 mg/kg body weight/day) simultaneously. After 6 weeks, all mice were sacrificed by decapitation. Brains were removed and washed thoroughly with ice-cold physiologic saline solution (0.9%). Before

detection, the brains were homogenized in cold physiologic saline. The homogenate (10%) was centrifuged at 4000  $g$  at 4°C for 10 min, and the supernatant was used for assay. Pilot experiments with various amounts of 0.06 mol/L HCl equivalent to those present in the Kn groups did not show any harmful effects on the growth, survival and biochemical systems of the mice. Therefore, no further controls with HCl were included in experiments.

### Biochemical estimations of the mouse brain

Antioxidant enzymes activities, MDA levels and protein concentration were determined by using commercially available kits supplied by Jiancheng Institute of Biotechnology (Nanjing, China). All procedures completely complied with the manufacturer's instructions.

### Statistical analysis

Data analyses were performed using the SPSS13.0 software, and all data were expressed as means  $\pm$  SEM. The level of statistical significance for differences between mean values was set at  $P < 0.05$ .

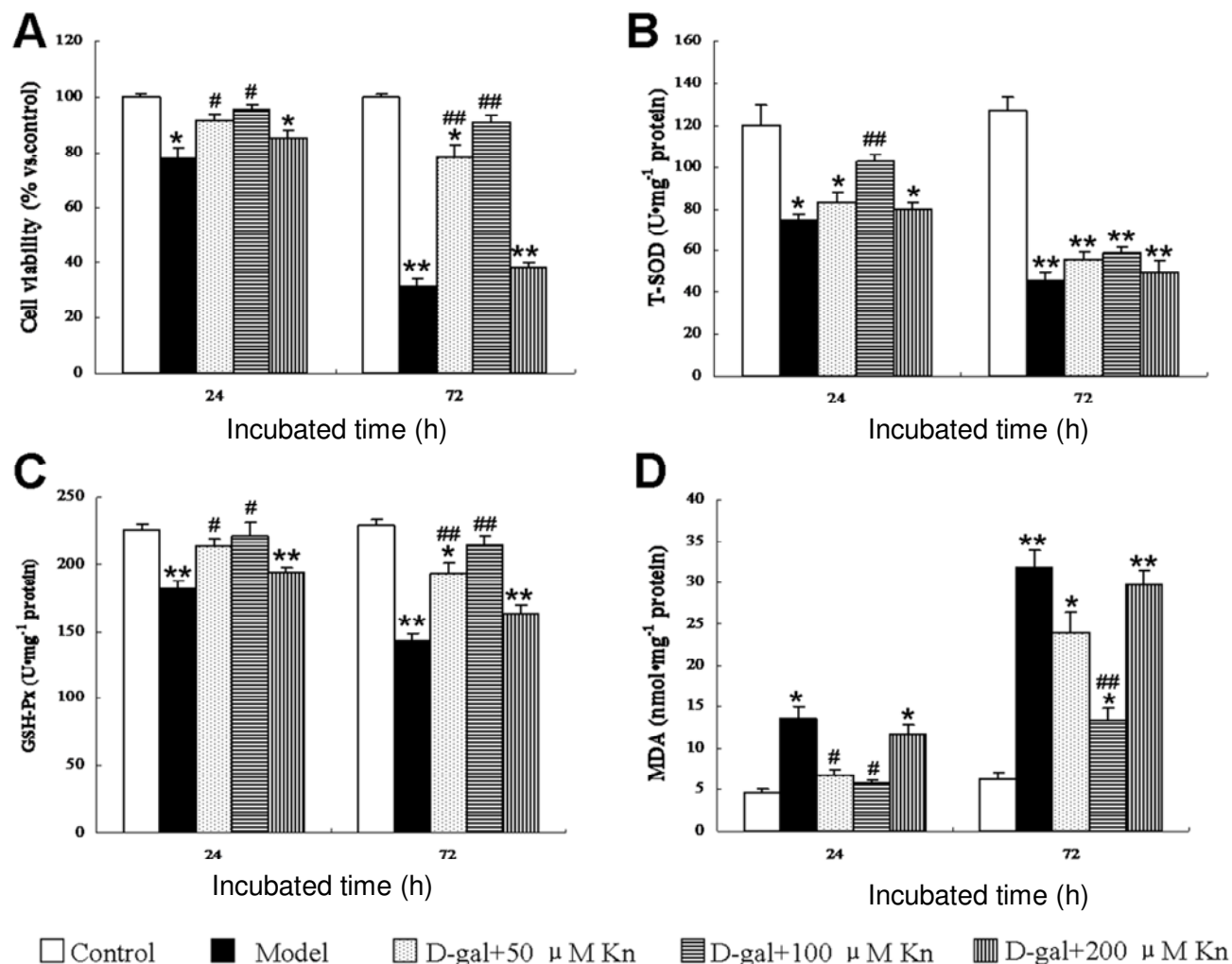
## RESULTS

### Effects of Kn on the morphological changes

Visual inspection by phase contrast microscopy demonstrated that those normal cultured astrocytes had a flat, polygonal shape with extended long, overlapping processes (Figure 1A). D-gal incubation for 72 h led to disappearance of multiple long processes from the cells (Figure 1B). The addition of Kn, however, dramatically prevented cell morphological deterioration. Most of the astrocytes showed normal cell morphology with overlapping processes (Figure 1C).

### Effect of Kn on cell viability

The effect of Kn on the viability of the cultured cells is illustrated in Figure 2A. There was a 22.28 and 68.43%



**Figure 2.** Protective effect of Kn against D-gal-induced oxidative stress to primary cultured rat astrocytes. (A) Cell viability; (B) T-SOD activities; (C) GSH-Px activities; (D) MDA levels. The data shown are mean  $\pm$  SEM of triplicate tests. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group; # $P < 0.05$ , ## $P < 0.01$ , compared with model group using paired T test.

reduction respectively in viable cell number when astrocytes were incubated with D-gal for 24 and 72 h. Treatment with 50 and 100  $\mu$ M Kn protected significantly astrocytes against D-gal-induced cell viability loss ( $P < 0.05$ ) at 24 and 72 h. This protection decreased when Kn was increased to 200  $\mu$ M. Figure 2A shows that cell viability decreased only 4.25 and 9.25% respectively after following 24 and 72 h incubation with 100  $\mu$ M Kn. However, 200  $\mu$ M Kn was found to provide a slight, but not a significant protection against D-gal-induced cytotoxicity (cell viability decreasing 61.70% at 72 h).

#### Effects of Kn on antioxidant enzymes activities and MDA levels in cultured astrocytes

The activities of the two antioxidant enzymes T-SOD and GSH-Px are shown in Figure 2B and C. Both T-SOD and

GSH-Px showed a significant decrease after 24 and 72 h incubation in the presence of D-gal. GSH-Px activities were increased with the 50 and 100  $\mu$ M Kn incubation for 24 and 72 h respectively which was statistically significant as compared with model group. T-SOD activities showed slight differences. Thus, only at 24 h was a statistically significant improvement with 100  $\mu$ M Kn treatment noted. MDA levels at 24 and 72 h in different experimental conditions are presented in Figure 2D. MDA levels in the control group were found to be 4.75 and 6.34 nmol/mg protein and increased to 13.65 and 31.81 nmol/mg protein in the presence of D-gal at 24 and 72 h respectively. Incorporation of 100  $\mu$ M Kn prevented significantly this rise in MDA levels; the level been 5.71 nmol/mg protein at 24 h and 13.43 nmol/mg at 72 h. Thus, an inhibition of 58.17% at 24 h and 57.78% at 72 h was observed as compared with the model group. MDA levels with the 50  $\mu$ M Kn incubation were

significantly reduced only in 24 h. However, 200  $\mu$ M Kn was found to provide a slight, but not a significant protection against MDA levels change in 24 and 72 h.

### Effects of Kn on T-SOD and GSH-Px activities and MDA levels in D-gal-induced senescent mouse brain

Figure 3 indicates that mice that received daily subcutaneous injection of D-gal at the 125 mg/kg doses significantly had reduced activities of T-SOD and GSH-Px in brains ( $P < 0.05$ ). On the contrary, remarkable increase in MDA levels was observed as compared with the injection of physiologic saline only. Treatment with 40 mg/kg Kn did not significantly attenuate MDA levels and antioxidant enzymes activities in D-gal-treated mice brains, while treatment with 10 mg/kg Kn could significantly restore antioxidant enzymes activities and attenuate MDA levels ( $P < 0.05$ ). There was no remarkable improvement on the T-SOD activities with 20 mg/kg Kn treatment. However, GSH-Px activities were significantly recovered ( $P < 0.05$ ).

## DISCUSSION

The brain is especially susceptible to oxidative stress than any other organ. One approach to protect against ROS in brain aging and neurodegenerative disorders is to enhance oxidative defense mechanism via antioxidants. Attention has been focused on a wide array of natural antioxidants that can scavenge free radicals and protect cells from oxidative damage, such as proanthocyanidins (Gong et al., 2008) and baicalein (Liu et al., 2007). Kn is a well known natural nutrient which retards senescence in plants, human skin cells and fruitflies for a long time. Since Kn was found to have antioxidant potential in living animal cell, it is important to find out whether the anti-aging and antioxidative effects of Kn can be observed for other animal organs also.

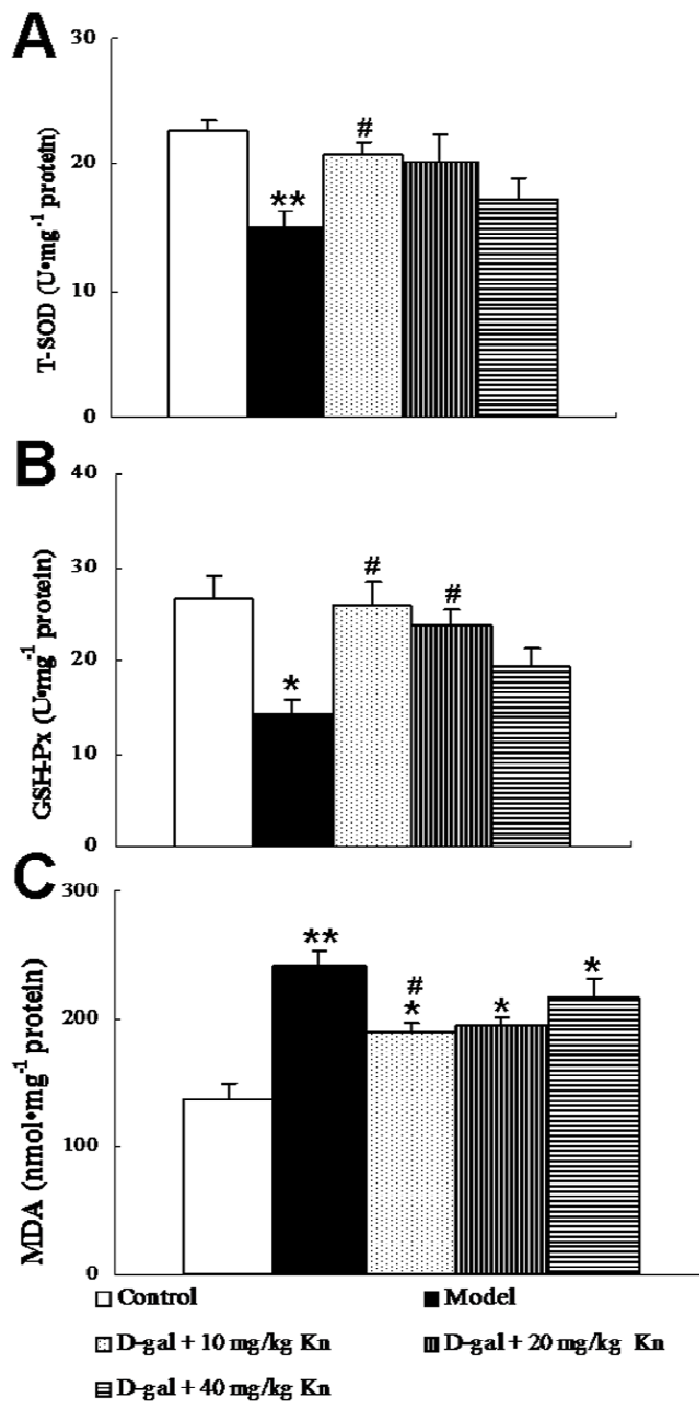
D-galactose (D-gal) is a normal reducing sugar in the body. At the normal level, it is usually converted into glucose by galactose-1-phosphate uridylyltransferase and galactokinase. However, at high levels, it can be oxidized into aldehydes and  $H_2O_2$  in the presence of galactose oxidase (Lei et al., 2008; Liu et al., 2010). Rodent chronically injected with D-gal has been widely used as an animal aging model for brain aging or anti-aging pharmacology research in china (Zhang et al., 2007). It was reported that D-gal could impair neurogenesis, a process similar to the natural aging in mice (Lu et al., 2006; Zhang et al., 2007; Zhong et al., 2009). The present data showed that the exogenous D-gal caused a great deal of astrocytes damage, degraded antioxidant enzymes activities and increased MDA levels both on astrocytes and mouse brain. Our results are in conformity to previous studies and further confirm that D-gal could

cause the brain aging, cytotoxicity and astrocytes damage (Mohanty et al., 2002; Lu et al., 2006; Lei et al., 2008).

The antioxidant potential of Kn has also been studied in other experimental models (Barciszewski et al., 1999; Hsiao et al., 2003). It is also known as free radicals scavenger (Hsiao et al., 2003). However, protective effects of Kn in D-gal-induced oxidative stress to astrocytes and mouse brain had never been established. This work indicate that reversion of T-SOD and GSH-Px activities, MDA levels, and improvement of cell viability were observed when astrocytes and mice were respectively treated with Kn of low concentrations compared with model group, which indicate a reduction of oxidative stress that turned out to be significant. However, there were no significant improvement on antioxidant enzymes activities and MDA levels after supplementation with Kn of high concentrations (200  $\mu$ M and 40 mg/kg). This is not a surprising observation since it is known that the anti-aging effects of Kn on human cells (Rattan and Sodagam, 1994) and fruitflies (Sharma et al., 1995) were best seen within a concentration range of 40 to 200  $\mu$ M and 25 ppm (equivalent to 125  $\mu$ M) respectively, above which Kn begins to inhibit cell growth and decrease fruitflies lifespan. Furthermore, Celik et al. (2006) reported that a remarkable increase in MDA levels and a significant decrease in SOD activities were observed in rat brain by 100 ppm Kn treatment (equivalent to 100 mg/kg). Although the highest concentrations (200  $\mu$ M and 40 mg/kg) of Kn in this study did not show clear toxicity, its protection effect decreased significantly. Our results are partly in agreement with those of previous studies. Thus, Kn of a low concentration was significantly more effective than high concentration in protecting D-gal induced damage to astrocytes and mouse brain.

Barciszewski et al. (2007) reported that the function of Kn seems to be different according to the cell types. Kn delays the aging of endothelial cells by enhancing cell proliferation capacity and metabolic capacity (Lee et al., 2006). However, Kn stimulates nuclear chromatin of the cultured *in vitro* human fibroblasts without stimulating proliferation. Those changes were manifested by an increase in sensitivity of nuclear chromatin to acid hydrolysis, which is accompanied by a higher activity of transcription (Rattan and Clark, 1994). The changes in the levels of enzymes and MDA presented in this study might indicate that both *in vivo* and *in vitro* supplement of Kn could affect antioxidant defense in the brain. Although the exact mechanism is not known at present, it is quite likely that Kn plays the role of antioxidant activities by interaction with rRNAs and tRNAs for the stimulation of SOD and GSH-Px synthesis (Sharma et al., 1995), and the increase in antioxidant enzymes activities inhibited the increase of MDA levels significantly.

In conclusion, our observations suggest that Kn of a low concentration was significantly more effective than



**Figure 3.** Protective effect of Kn against D-gal-induced oxidative stress to mouse brain. (A) T-SOD activities; (B) GSH-Px activities; (C) MDA levels. Data represent means  $\pm$  SEM of measurements from 10 mice per group. \* $P < 0.05$ , \*\* $P < 0.01$ , compared with control group; # $P < 0.05$ , ## $P < 0.01$ , compared with model group using paired T test.

high concentration in protecting D-gal induced damage to astrocytes and mouse brain, and it may be a potential nutrition for treatment of neurodegenerative diseases. However, the effect of higher concentrations of Kn on D-

gal-induced damage need to be further studied. At the same time, more studies needs to be done in order to elucidate the detailed way in which Kn protects astrocytes and mouse brain against D-gal-induced

oxidative stress.

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