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Protective effects of N-acetylcysteine on homocysteine induced injury in chick embryos

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1. ABSTRACT

Protective effects of N-acetylcysteine (NAC) on homocysteine (Hcy)-induced injury have been reported *in vitro*. However, it is not known whether NAC has a similar effect in Hcy-induced injury during embryonic development. In this study, we tested the hypothesis that exogenous NAC can attenuate Hcy-induced injury in chick embryos. Hcy-induced apoptosis and reduced embryo viability were effectively attenuated by application of exogenous NAC. NAC could also rescue Hcy-induced inhibition of extra-embryonic vascular development. 2',7'-Dichlorofluorescein diacetate, an indicator of reactive oxygen species, was detected in H9C2 cells after treatment with Hcy. The results of this study provide the first evidence that NAC can protect against the adverse effect of Hcy during chick embryo development, and suggest that these effects are at least partly mediated by oxidative stress.

2. INTRODUCTION

Homocysteine (Hcy) is a nonessential sulfur-containing amino acid and an intermediate metabolic product of the demethylated essential amino acid methionine. Hcy is usually converted to cysteine via transsulfuration, or to methionine by the addition of a methyl group donated by folate (1, 2). However, the serum concentration of Hcy increases if these pathways are disrupted due to genetic mutations of the enzymes involved, or deficiencies of folate, vitamin B6 or vitamin B12 (3-6). During the past decade, Hcy has become widely recognized as an independent and graded risk factor related to a variety of debilitating and potentially fatal diseases in humans. Elevated levels of Hcy (hyperhomocysteinemia, HHcy) have been associated with atherosclerotic cardiovascular disease in the coronary, cerebral, and peripheral vascular beds, Alzheimer's disease, dementia, and loss of cognitive function (7-12). HHcy is also a

significant risk factor associated with pregnancy loss, and congenital defects of the heart and neural tube (13, 14). However, the causative role of Hcy remains controversial and needs to be more fully elucidated.

Oxidation of two Hcy molecules yields the oxidized disulfide (homocystine), two protons, and two electrons, while facilitating the formation of reactive oxygen species (ROS). The formation of mixed disulfides also contributes to the formation of ROS. ROS are toxic to cells and tissues as a result of the damage caused to lipids, nucleic acids and proteins (15). Under physiological conditions, cells protect themselves from ROS damage through the actions of antioxidants that remove free radical intermediates and suppress oxidation. Disturbance of the balance between endogenous oxidants and antioxidants leads to oxidative stress. Oxidative stress has been shown to be an important mechanism behind Hcy toxicity in neuronal and vascular cells (16, 17), and Hcy has been found to induce vascular dysfunction, atherogenesis and neurological dysfunction via oxidative stress (18, 19). This effect is mediated by increased production of ROS and inactivation of nitric oxide. Meanwhile, Hcy was shown to cause brain lipid peroxidation by blocking NMDA receptors (20) and to directly enhance the neurotoxicity of amyloid beta by inducing oxidative stress (20).

High Hcy levels *in vitro* induce obvious changes in cytokine expression in vascular cells (21-23). For example, mice with genetic HHcy demonstrate 6-fold increases in plasma tumor necrosis factor α (TNF-α) levels (24). TNF-α can induce cell death via reactive oxidative intermediates (25, 26). Ungvari *et al* (27) showed that HHcy raised TNF-α expression in coronary arteries of Wistar rats, with the subsequent induction of a proinflammatory vascular phenotype through oxidative stress.

Both intracellular and extracellular antioxidants play critical roles in neutralizing ROS. Antioxidants either prevent ROS from being formed, or remove them before they can damage vital cellular components (28). Glutathione (GSH) acts as an antioxidant because the thiol group in its cysteinyl moiety can be reversibly oxidized. GSH acts as a substrate for glutathione peroxidases to remove lipid hydroperoxides and hydrogen peroxide from endothelial cells (29). During hypercholesterolemia and under ischemia-reperfusion conditions, exhaustion of endogenous GSH may also change the vascular wall's ability to detoxify peroxynitrite (30). NAC is a thiol-containing compound that is known to effectively increase intracellular GSH levels, and for which extensive clinical experience exists. NAC is an antioxidant/free radical scavenger or reducing agent that protects against cell death, and has been shown to prevent TNF-α-induced death of oligodendrocytes and L929 cells (31, 32).

Rosenquist *et al* (14) administered Hcy to chick embryos to test the hypothesis that elevated plasma Hcy levels *per se* may be teratogenic during embryo development. They demonstrated that Hcy caused dysmorphogenesis of the heart and neural tube, as well as of the ventral wall. Twenty-seven percent of embryos showed neural tube defects and 23% of embryos showed ventricular septal defects. Epeldegin *et al* (33) showed that Hcy induced spina bifida in chick embryos, and Miller *et al* (34) demonstrated Hcy-induced changes in brain membrane composition. Overall, these studies have confirmed that Hcy is teratogenic in chick embryos.

Exogenous NAC may attenuate Hcy-induced injury. The protective effects of NAC on Hcy-induced cell injury (necrosis and apoptosis) have previously been demonstrated *in vitro* (35, 36), but not *in vivo*. Our present study therefore aimed to test the hypothesis that exogenous NAC could attenuate Hcy-induced injury in chick embryos. We investigated the effects of NAC supplementation on Hcy-induced reductions in embryo viability and Hcy-induced apoptosis. We also studied the effects of NAC supplementation on Hcy-induced inhibition of extra-embryonic vascular development.

3. MATERIALS AND METHODS

3.1. Cell culture and treatment

The H9C2 rat ventricular cell line used in this study was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's Modified Eagle Medium (DMEM). Cultures were supplemented with 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin. Cells were exposed to 1.5 mM D,L-homocysteine (Sigma-Aldrich, St. Louis, MO, USA) for 12 h.

3.2. Embryo treatments

White Leghorn chick eggs (Bovan strain) were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd. The compounds were directly injected into the center of the egg yolk via a small hole at the blunt end of the egg, using an established protocol. The normal stages of chick development were based on the experimental procedures of Rosenquist *et al.* (14). Chick embryos were treated with 50 μL of various treatment agents [0.1 μm, 1 μm, 10 μm D,L-Hcy, 10 μm NAC (Sigma-Aldrich, St. Louis, MO, USA), and 10 μm D,L-Hcy plus 10 μm NAC] or 0.9% saline vehicle alone at Hamburger-Hamilton (HH) stages 6, 8 and 12 (37). Embryos were harvested for analysis at 72 h of incubation (HH stage 19–20).

3.3. Embryo viability

Embryo viability was indirectly monitored by measuring embryo mass and the percent of live embryos at 3 days of development (theoretical stage 19–20). Live embryos were defined as those possessing a beating heart. Injections were performed on three different occasions (N=3), and 20–23 fertile eggs were injected per treatment group per set of injections.

3.4. Whole-mount terminal deoxynucleotidyl transferase-mediated biotinylated dUTP nick end labeling (TUNEL)

TUNEL staining was performed as described previously, with minor modifications. Briefly, chicken

Table 1. The effects of homocysteine on chick embryo viability

Exogenous treatment	% Living embryos	Embryo mass (g)
Saline (control)	95.8±3.6 (N=3)	0.0176±0.0019 (N=24)
Hcy (10 μm)	60.0±6.2 (N=3) [1]	0.0117±0.0021 (N=20) [2]
Hcy (1 μm)	85.5±1.9.0 (N=3)	0.0149±0.0028 (N=23) [1]
Hcy (0.1 μm)	85.8±2.2 (N=3)	0.0159±0.0030 (N=23) [1]

Hcy: homocysteine, Data presented as mean±standard deviation, [1]P<0.01, [2]P<0.001

Table 2. The effects of homocysteine and/or N-acetylcysteine on chick embryo viability

Exogenous treatment	% Living embryos	Embryo mass (g)
Saline (control)	98.0±3.3 (N=3)	0.0181±0.0018 (N=24)
Hcy (10 μm)	63.9±6.6 (N=3) [1]	0.0112±0.0021 (N=20) [2]
Hcy (10 μm) and NAC (10 μm)	80.0±6.7 (N=3)	0.0147±0.0025 (N=22) [1]
NAC (10 μm)	91.4±4.0 (N=3)	0.0166±0.0017 (N=23)

Hcy: homocysteine; NAC: N-acetylcysteine, Data presented as mean±standard deviation, [1]P<0.01, [2]P<0.001

embryos were fixed, dehydrated, and rehydrated through graded methanol concentrations into PBS containing 0.1% Tween-20 (PBST). The Apoptosis Detection System, DeadEnd Colorimetric TUNEL System (Promega, Madison, WI, USA) was used for *in situ* visualization of DNA fragmentation in whole mount embryos. After washing in PBST, embryos were treated with proteinase-K, refixed in methanol-free 4% formaldehyde, incubated in equilibration buffer for 10 min at room temperature, and transferred to a solution consisting of equilibration buffer, nucleotide mix and TdT enzyme (all supplied with the kit) for 12 h at 4°C, in the dark. The reaction was terminated by immersing the slides in 2×SSC for 30 min at room temperature. Embryos were then immersed in 0.3% hydrogen peroxide for 1 h at room temperature to block endogenous peroxidases. After three washes with PBT, the embryos were incubated with streptavidin-horseradish peroxidase for 2 h at room temperature and then stained with diaminobenzidine for 15 min. Finally, the embryos were examined and recorded using a 20× objective on a Nikon TE 2000-U microscope (Nikon Instruments, Inc., Melville, NY).

3.5. Extra-embryonic vascular development

Each surviving chick embryo (determined by the presence of a heartbeat) was photographed using a SONY Cyber-shot camera. The images were scaled to 1 cm in the plane of the embryo, and scanned into Adobe Photoshop (Microtek, Scanmaker III, Redondo Beach, CA) for quantification of relative extra-embryonic vascular area, using ImagePro software, as described by Hayek *et al* (38).

3.6. ROS detection

For ROS detection, treated cells were incubated in 10 μM 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA) for 20 min at 37°C and washed three times for 5 min with DMEM. Fluorescence was observed at 488 nm wavelength excitation

3.7. Data analysis

Group data are expressed as means ± SEM. Statistical comparisons (performed using ANOVA followed by Dunnett's method) were carried out using Microsoft Excel. Two-tailed P<0.01 values indicated statistically significant differences.

4. RESULTS

4.1. Embryo viability

The optimal exposure dose of Hcy was determined by treating embryos at HH stages 6, 8 and 12 with increasing Hcy concentrations (0.1, 1, and 10 μm). The results showed that exogenous Hcy reduced embryo viability in a dose-dependent manner, as indicated by Hcy-induced reductions in the percentages of live embryos and in embryo masses (Table 1). Hcy (10 μm) gave the highest rate of (% surviving) * (% dead. Therefore, 10 μm of Hcy is the best exposure dose which is equal to the daily dosage as used by Rosenquist *et al* (14), and a single dose of 10 μm Hcy was therefore used in the following experiment.

Exogenous NAC had no significant effect on embryo survival or embryo mass, compared with saline controls. However, when embryos were co-treated with Hcy and NAC, embryo survival and embryo masses were significantly higher than when embryos were treated with Hcy alone (Table 2).

4.2. Whole-mount TUNEL

Exogenous Hcy increased apoptosis, as measured by whole-mount TUNEL assay. Obvious TUNEL-positive staining was observed in Hcy (10 μm) treated embryos (Figure 1A), compared with saline-treated embryos (Figure 1B). There was no obvious TUNEL-positive staining in NAC-treated (10 μm) embryos (Figure 1C). However, when mixed with Hcy, exogenous NAC resulted in a significant decrease in TUNEL-positive staining (Figure 1D).

4.3. Extra-embryonic vascular development

The first endothelial cells start to differentiate at HH stage 8. Circulation begins around HH stage 12, and the vitelline vessels begin to branch in response to tissue growth and the resulting hypoxic stimulation (37). Embryos in this study were analyzed at 72 h (HH stage 20), by which time most of the main early developmental patterning events have already taken place. Chick embryos demonstrated significantly impaired extra-embryonic vascular development following exposure to Hcy (10 μm) (Figure 2A). Hcy (10 μm) also strikingly reduced vascular density, defined as the relative vascular area measured

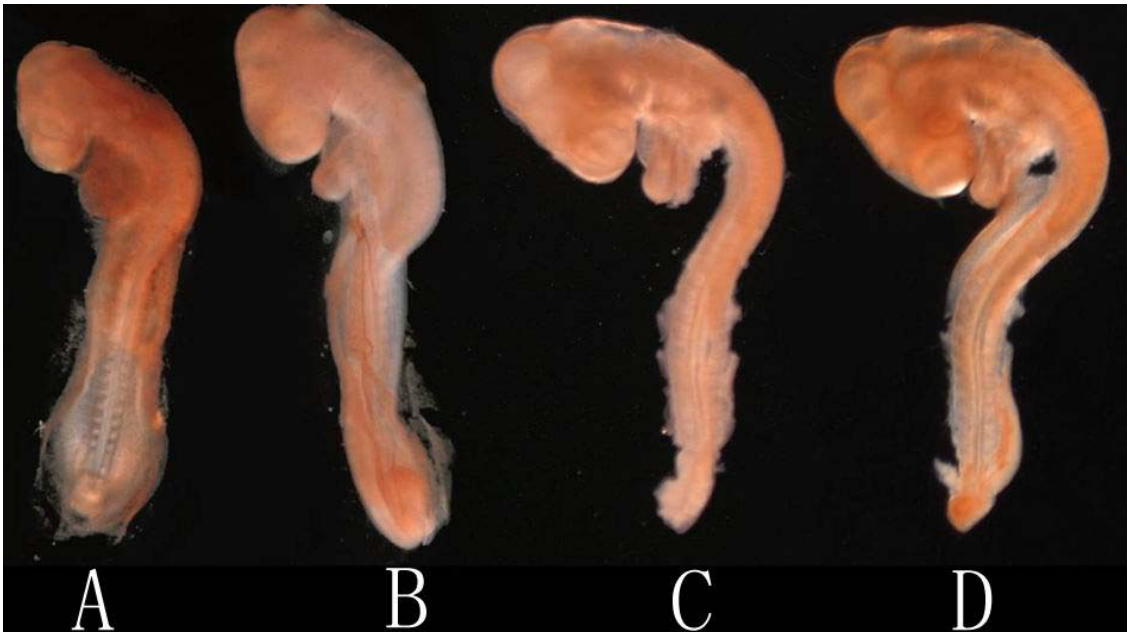

Figure 1. Whole-mount TUNEL assay for apoptosis. Obvious TUNEL-positive staining was observed in Hcy (10 μm)-treated embryos (A) compared with saline-treated controls (B).There was no significant TUNEL-positive staining in NAC (10 μm)-treated embryos (C). When co-administered with Hcy, exogenous NAC resulted in a significant decrease in TUNEL-positive staining (D).

using ImagePro software. The average relative vascular area for a normal chick embryo at day 3 was 3.41. Treatment with 10 μM Hcy significantly decreased the relative vascular area to 1.38, $P<0.001$ (Figure 2B). Exogenous NAC did not alter the relative vascular area compared with the saline control, but resulted in a 1.58-fold increase in relative vascular area (from 1.38 to 2.18, $p<0.001$) when mixed with Hcy. These results showed that exogenous NAC could partially rescue Hcy-induced inhibition of extra-embryonic vascular development.

4.4. ROS detection

To further confirm the effects of Hcy, H9C2 cells (rat ventricular cell line) were treated with Hcy (1.5 mM) for 12 h. DCFH-DA, an indicator of ROS, was detected by fluorescence at 488 nm wavelength excitation. The result showed that DCFH-DA staining was increased after Hcy treatment (Figure 3A), compared with control (Figure 3B), indicating Hcy-induced ROS generation.

5. DISCUSSION

Hcy reduced embryo viability in a dose-dependent manner, as indicated by reduced embryo survival and decreased embryo mass. TUNEL-positive staining was also observed in Hcy-treated embryos. Two Hcy molecules can undergo auto-oxidation to form a dimer (Hcy-oxidized disulfide), so releasing two hydrogen ions and two electrons. Hydrogen peroxide and hydroxyl radicals can thus be generated (15), and these ROS can in turn promote membrane lipid peroxidation and subsequent necrosis and apoptosis. It was therefore not surprising that Hcy promoted apoptosis, as measured by whole-mount TUNEL assay. DCFH-DA staining was increased compared with controls when H9C2 cells were treated with Hcy for 12 h, demonstrating Hcy-induced ROS generation.

Extra-embryonic vascular development was significantly impaired in chick embryos following exposure to Hcy (10 μM), and relative vascular area was strikingly reduced. Hcy is known to inhibit endothelial cell proliferation and migration, resulting in reduced angiogenesis (39, 40), and Hcy has been shown to induce cardiac outflow malformations and vessel wall thickening (41, 42). Latacha and Rosenquist (43) also demonstrated that Hcy inhibited extra-embryonic vascular development in the avian embryo. Hcy may facilitate ischemia, necrosis, apoptosis, and membrane lipid peroxidation via impaired angiogenesis and cardiovascular circulation. The failure of the Hcy-treated embryos to thrive may thus be due to disruption of the vasculature.

Exogenous NAC effectively attenuated Hcy-induced reduction in chick embryo viability and Hcy-induced apoptosis. Exogenous NAC was also able to rescue the Hcy-induced inhibition of extra-embryonic vascular development. Our results provide the first demonstration of the protective effects of NAC against Hcy-induced adverse effects in chick embryos. NAC acts via two main mechanisms: (a) as a scavenger of free radicals and (b) by increasing intracellular levels of GSH (31). Both of these antioxidative activities may help to protect against cell death induced by oxidative stress. The auto-oxidation of Hcy can disrupt redox homeostasis and thus cause

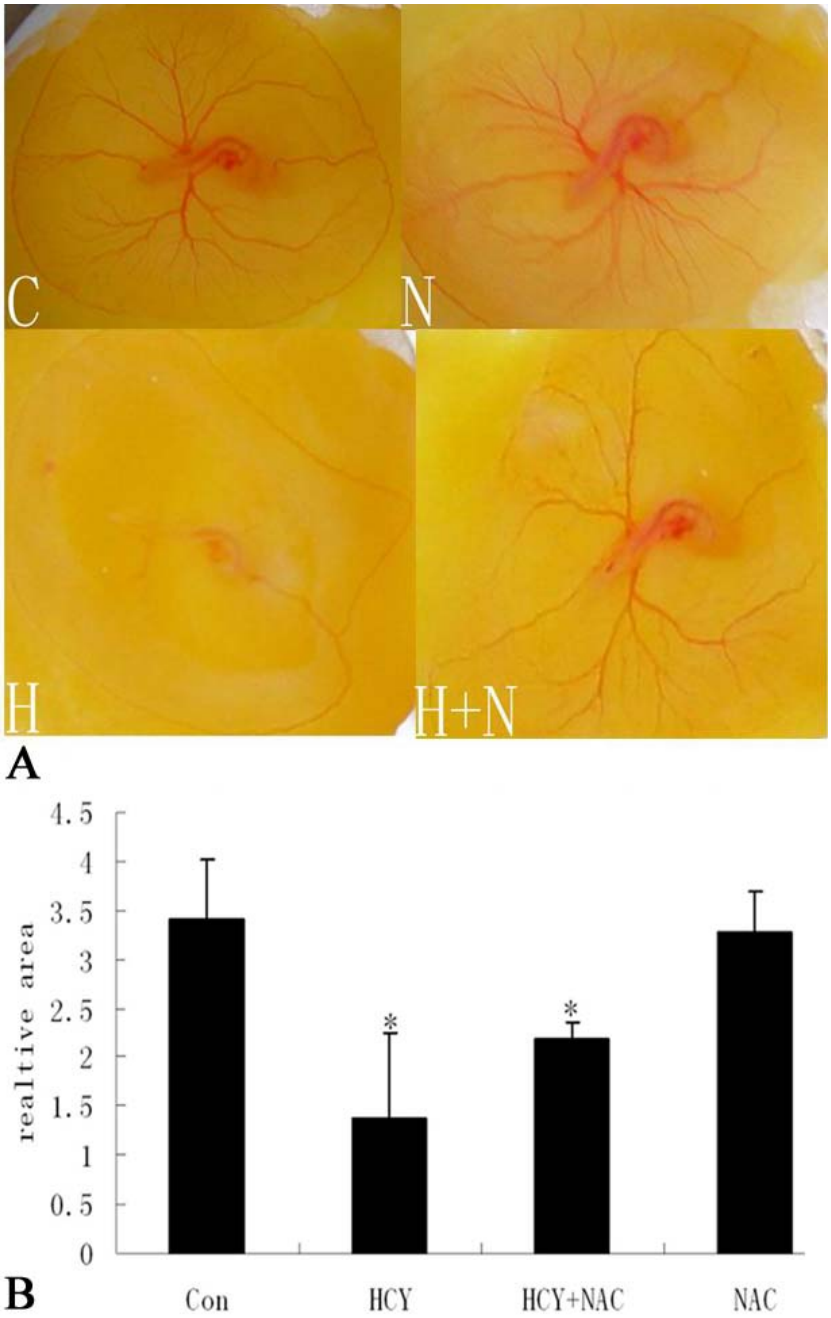

Figure 2. Hcy inhibited the development of the extra-embryonic vasculature. NAC partially rescued the development of extra-embryonic vasculature (A). C: saline (control); H: Hcy (10 μm); N: NAC (10 μm); H+N: Hcy (10 μm) + NAC (10 μm). Hcy significantly reduced the relative area of extra-embryonic vasculature (B). The average relative vascular area for a normal chick embryo at day 3 was 3.41. Treatment with 10 μM Hcy significantly decreased the relative vascular area to 1.38. NAC did not alter the relative vascular area compared with the saline control, whereas exogenous NAC mixed with Hcy resulted in a 1.58-fold increase in relative vascular area from 1.38 to 2.18. The error bars indicate one standard error of the mean (SEM) between repeated experiments. Each experiment was repeated three times with 13 embryos per group. **P*<0.001.

oxidative stress, and Hcy can also reduce the expression of the cellular isoform of glutathione peroxidase, as demonstrated both *in vitro* and *in vivo* (44, 45). Glutathione peroxidase utilizes GSH to reduce hydrogen peroxide and lipid peroxides to their respective alcohols, and may also act as a peroxynitrite reductase (46). Weiss *et al* (47) demonstrated that overexpression of cellular glutathione peroxidase could rescue Hcy-induced endothelial

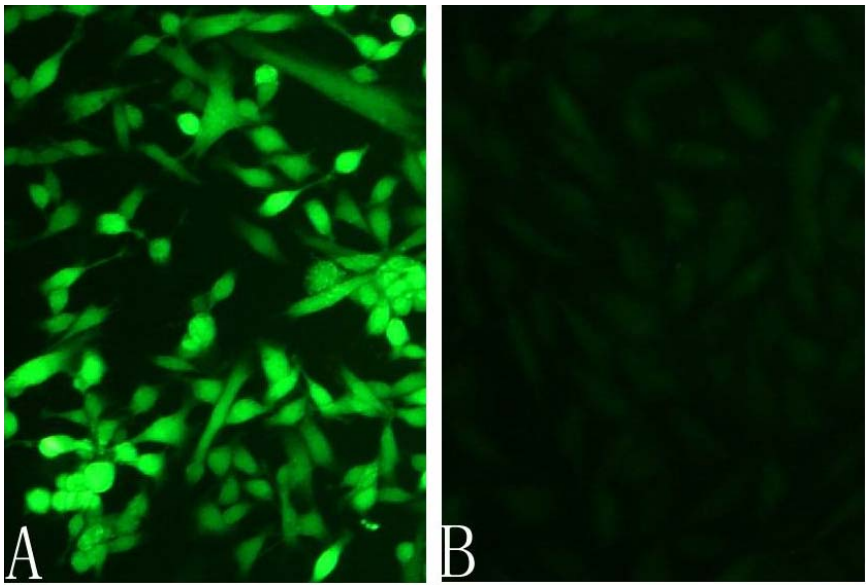

Figure 3. ROS detection in H9C2 cells. DCFH-DA staining was obviously increased in H9C2 cells after treatment with Hcy (1.5 mM) for 12 h (A), compared with control (B), indicating Hcy-induced ROS generation.

dysfunction. Thus, NAC may protect chick embryos from Hcy-induced adverse effects either by acting as a scavenger of free radicals or as a precursor of GSH.

In conclusion, this is the first study to demonstrate that exogenous NAC can attenuate Hcy-induced apoptosis and rescue Hcy-induced inhibition of extra-embryonic vascular development *in vivo*. However, the mechanism by which Hcy damages the chick embryo is complicated and multifactorial, and further studies are needed to illuminate the mechanisms whereby NAC attenuates Hcy-induced impairment of embryo development.

6. ACKNOWLEDGMENTS

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Abbreviations: NAC, N-acetylcysteine; Hcy, homocysteine; ROS, reactive oxygen species; GSH, glutathione; DCHFDA, 2′,7′-dichlorodihydrofluorescein diacetate

Key Words: homocysteine; N-acetylcysteine; reactive oxygen species; oxidative stress

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