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Original article

N-acetylcysteine relieves oxidative stress and protects hippocampus of rat from radiation-induced apoptosis by inhibiting caspase-3

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ABSTRACT

It has been recognized that radiation-induced effects remain a significant risk. An accumulation of reactive oxygen species (ROS) is considered to be one factor that contributes to neurodegenerative changes. The aim of our study was to investigate the potential radioprotective effects of NAC. Male Sprague-Dawley rats underwent radiation. Irradiation was performed at room temperature with a 4-Gy dose of radiation. A dose of N-acetylcysteine (NAC) was performed 15 min prior to irradiation intraperitoneally. The methods of immunohistochemistry, TUNEL staining, Nissl staining, qRT-PCR, analysis of reactive oxygen species and Western blot were performed. In conclusion, our results demonstrate that NAC inhibits apoptosis induced by irradiation via the inhibition of caspase-3. We demonstrated a decrease in caspase-3 mRNA that was present at 24 h of NAC treatment. Such mRNA decrease was accompanied by a decrease of protein. In the present study, NAC effectively antagonized oxidation induced by irradiation. These results provide evidence that the neural protective effect and the antioxidant effect of NAC contribute to metabolic activity.

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1. Introduction

Neuronal cell apoptosis is associated with various neurological damaging factors, including radiation exposure [1]. It has been recognized that radiation-induced effects remain a significant risk. Protection of biological systems from ionizing radiation is of paramount importance in planned as well as unplanned exposures to radiation [2,3], and the development of novel and effective agents to combat radiation damages using nontoxic radioprotectors is of considerable interest in defense, nuclear industry, space travels, and health care, particularly in radiodiagnostics and therapy. Reactive oxygen species (ROS), in particular the hydroxyl radical, can lead to functional alterations in lipids, proteins, and nucleic acids, and an accumulation of ROS is considered to be one factor that contributes to neurodegenerative changes, for example in Parkinson's disease [4] and Alzheimer's disease [5]. An increase in ROS production occurs following irradiation [6], and therefore, it is not surprising to find that at least some effects of exposure of cells to ROS are mimicked by exposure of cells to ionizing radiation. Neuronal tissue is susceptible to oxidative stress because of its high oxygen consumption and modest antioxidant defenses [7].

N-acetylcysteine (NAC) is an amino acid derived from cysteine with anti-inflammatory and antioxidant properties already used in the clinical setting, for example in acetaminophen intoxication, idiopathic pulmonary fibrosis, bronchitis, ischemia-reperfusion injury, cardiac injury and doxorubicin cardiotoxicity [8]. It is both an analog and a precursor of intracellular glutathione synthesis leading to restoration of the cell redox status. NAC has been reported to provide cardiac protection in animal models through its antioxidant and anti-inflammatory properties [9,10]. Acting on oxidative stress and inflammation, NAC has therefore immunomodulatory properties associated with cardioprotective effects.

Abbreviations: NAC, N-acetylcysteine; Caspase, cysteinyl aspartate specific proteinase; ROS, reactive oxygen species; IR, irradiation; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labeling; GSH, glutathione; CT, cycle threshold; DCF, dichlorofluorescein.

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Reduced glutathione (GSH) is a multifunctional intracellular non-enzymatic antioxidant. GSH is highly abundant in the cytosol, nuclei, and mitochondria and is the major soluble antioxidant in these cell compartments. It is considered to be the major thiol-disulphide redox buffer of the cell [11]. The evidence from both in vitro and in vivo studies suggests that NAC is capable of replenishing intracellular GSH by reducing extracellular cystine to cysteine [12], or by supplying sulfhydryl (–SH) groups that can stimulate GSH synthesis and enhance glutathione-S-transferase activity [13,14]. NAC is a potent free radical scavenger in consequence of its nucleophilic reactions with ROS [15]. Thus, NAC treatment may be beneficial for conditions of GSH depletion and free radical formations during oxidative stress [16]. It is suggested that NAC may protect cell membranes against lipid peroxidation and protein oxidation, and helps maintain the integrity of cellular organelles [17,18]. However, it remains to be determined whether and how NAC influences basic cellular processes such as apoptosis.

It is unknown whether NAC inhibits the caspase cell death pathway to anti-apoptosis induced by radiation in hippocampus, and it is also unknown which intracellular signaling pathways contribute to this phenomenon. The aim of our study was to investigate the potential radioprotective effects of NAC, representing a clinically used radioprotector, in the prevention of oxidative damage caused by irradiation in normal rat tissue after whole body irradiation.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats weighing 200–220 g were randomly divided into three groups (12 rats/group): the irradiation group (IR group), the irradiation with NAC (IR + NAC group) and control group (con group). All rats were housed in groups of 4–5/cage in a temperature (24 ± 1 °C) and light controlled room (12 h light/dark cycle with lights on at 07:00 h). Food and water were provided ad libitum before and after treatment. The animals were allowed free access to tap water and a standard diet for the duration of the study. All animal procedures were performed in a facility accredited by the Radiation Hazard Evaluation Laboratory of the Institute of Radiation Medicine of Chinese Academy of Medical Science and Peking Union Medical College (Tianjin, China). All experimental procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

2.2. Drug treatments

The drug was intraperitoneally (i.p.) administered to rats at a dose of 1000 mg/kg NAC (containing 300 mg of N-acetylcysteine, Asist ampul, Hüsnü Arsan, Ilaç, Istanbul) in IR + NAC group. Rats in control group and IR group did not receive NAC but received 2.2 ml isotonic NaCl solution (i.p.). Saline and NAC injections in study groups were performed 15 min prior to irradiation.

2.3. Radiation model

The irradiation of the rats in IR and IR + NAC groups was performed at room temperature using a Cs-137 γ-ray instrument (Atomic Energy of Canadian Inc., Mississauga, Canada) to administer a 4-Gy dose of radiation at a dose rate of 0.71116 Gy/min. The animals in the control group did not receive any radiation. The study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Institute of Radiation Medicine of Chinese Academy of Medical Science and Peking union Medical College (Tianjin, China). Twenty-four hours subsequent to irradiation, the rats from each group were anesthetized with 10% chloral hydrate (30 mg/kg body weight) by intraperitoneal anesthesia.

2.4. Terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining, Immunohistology

Rat brains were harvested and immediately frozen in 2-methylbutane at −30 °C. Coronal sections were cut into 12-μm thick sections with a cryostat (CM 3000; Leica, Manheim, Germany) at the level of the CA3 subfield of hippocampus and then stored at −80 °C until required for further experiments. Coronal sections were air dried for 15 min, post-fixed in 10% formalin for 15 min, washed twice in PBS and then processed for immunohistology with rabbit anti-caspase-3 (1:1000 dilution; Abcam, Cambridge, MA, USA). The avidin-biotin-peroxidase complex method was conducted as previously described [19]. For detection of DNA fragmentation, the fluorescein-based TUNEL assay (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used. TUNEL staining was conducted according to the manufacturer's instructions. Briefly, sections were incubated for 90 min at 37 °C with TUNEL reaction mixture. Positive control sections were incubated with 200 U/ml DNase I (Gibco-BRL, Carlsbad, CA, USA) for 5 min prior to fixation. Negative control sections underwent the same procedure but terminal deoxynucleotidyl transferase was omitted from the reaction buffer to evaluate nonspecific labeling. TUNEL cell counts were performed on brain sections ($n = 6$) from the hippocampi. TUNEL-positive cells were averaged from counts on three adjacent brain sections of a rat. Images were visualized using a Leica microscope under an excitation/emission wavelength of 500/550 nm (green), captured using an Optronics DEI-750 3-chip camera equipped with a BQ 8000 sVGA frame grabber and analyzed with Bioquant software (Bioquant Image Analysis Corporation, Nashville, TN, USA) [20].

2.5. Nissl staining

This method is used for the detection of Nissl body in the cytoplasm of neurons, to identify the basic neuronal structure from necrotic neurons in brain and spinal cord. The following steps were followed: rinsed in tap water and then in distilled water; stained in 0.1% cresyl violet solution for 3–10 min; rinsed quickly in distilled water and then differentiated and dehydrated in alcohol then clearing and finally mounted with permanent mounting medium. The Nissl body was stained purple-blue [20].

2.6. Western blot analysis

Animals were euthanized at 24 h following irradiation, and hippocampi ($n = 6$ each group) were obtained. The total protein and nuclear protein were isolated from hippocampi using RIPA buffer (Beyotime, Jiangsu, China) according to the manufacturer's instructions. The protein concentration from the cytosol (supernatant) was determined spectrophotometrically from the absorbance at 595 nm (A595 nm) using the method as Riedl had done [21]. Samples containing equal amounts of protein were mixed with loading buffer with 5% 2-mercaptoethanol, heated for 5 min at 95 °C, loaded onto a 10% SDS-PAGE gel, and transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). After blocking with 5% milk and 0.1% Tween-20 in Tris-buffered saline (TBS), membranes were incubated overnight at 4 °C with the following primary antibodies: rabbit anti-caspase-3 antibody which was obtained from Cell Signaling Technology (1:500 dilution; Beverly, MA). Rabbit anti-β-actin (1:1500 dilution;

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