Full Length Research Paper

N-acetylcysteine an *Allium* plant compound protects against chromium (VI) induced oxidant stress and ultrastructural changes of pancreatic beta-cells in rats

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The present study was carried out to determine the effectiveness of N-acetylcysteine (NAC) in alleviating chromium (VI)-induced beta-cell damages in rats. Sixty male albino rats were randomly divided into four groups (n = 15/group). Group I remained untreated; Group II received single dose of potassium dichromate (50 mg/kg b.w., s.c.); Group III received NAC (200 mg/kg b.w., i.p.) before chromium (VI) injection (50 mg/kg b.w., s.c.); Group IV received NAC alone (200 mg/kg b.w., i.p.). Pancreatic tissue malondialdehyde (a marker of lipid peroxidation), pancreas antioxidant power, blood glucose level and ultrastructure of beta-cells were evaluated. Results showed that the administration of chromium (VI) resulted in a state of pancreatic injury and extensive oxidative damage in rats as manifested by the increase in lipid peroxidation and the decrease in activities of antioxidant enzymes such as glutathione peroxidase and catalase. In serum, there was significant increase in the level of glucose in these animals. Administration of NAC shortly prior to chromium (VI) significantly mitigated most of these changes to control values. Based on ultrastructural observations, the administration of NAC may effectively rescue beta-cells from oxidative damage without affecting their function and structural integrity. It can be concluded that NAC if administrated before chromium (VI) injection improves glycemic state by enhancing insulin secretion and antioxidant competence in pancreatic beta-cells.

Key words: N-acetylcysteine, hexavalent chromium, hyperglycemia, lipid peroxidation, pancreatic beta-cell.

INTRODUCTION

Widespread pollution by heavy metals has important consequences for human health and the quality of the environment (Abdel-Moneim and Abdel-Mohsen, 2010). Among the toxic metals, chromium (Cr) is widely used in industries such as electroplating, alloy and steel manufacturing, leather tanning, metal finishing, pigment Chromium commonly enters the environment in the effluents from these industries. Once released into soil and water, it is considered a major cause of environmental pollution. Chromium is listed among the 126 priority pollutants by the US EPA. It is also listed among the 25 most hazardous substances posing the greatest risk to human and ecosystem health at the priority superfund sites (USDHHS-USEPA, 1987). Chromium exists in several oxidation states, though Cr (III) and Cr (VI) species are the most stable, common forms. Cr (III) constitutes an essential nutrient, while Cr (VI) is highly toxic and a strong oxidizing agent (O'Brien et al., 2000). Occupational exposure to Cr (VI) compounds is associated with several adverse effects on health such as lung toxicity and bronchial asthma, and it also causes nephro- and hepatotoxicity (Costa, 1997; Bright et al., 1997; Dartsch et al., 1998). Squamous cell carcinoma is the most frequent type of lung cancer among Cr (VI)-exposed workers.

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Chromium is a Fenton metal and generates free radicals by itself. Fenton reactions are associated with membranous fractions including mitochondria, microsomes and peroxisomes. The generation of free radicals occurs when chromium undergoes redox-cycling reaction (Anand, 2005). Cr (VI) enters cells rapidly, and once inside the cell it is reduced by intracellular reductants to short-lived chromium intermediates such as Cr (V), Cr (IV) and ultimately kinetically stable form of Cr (III). This reduction process generates reactive oxygen species (ROS), such as superoxide (O\(^{-}\)), hydroxyl (OH\(^{-}\)) and (H\(^{2}\)O\(^{2-}\)), which serve as sources of hydroxyl radicals. Cr (VI) exposure generates oxidative stress in many systems (Harris and Shi, 2003; Pulido and Parrish, 2003). Oxidative stress results from an imbalance between the antioxidant defense systems and ROS generation. The most abundant antioxidant in the cell is glutathione, which is a strong antioxidant by acting directly in the detoxification of ROS (Schafer and Buettner, 2001; Wu et al., 2004). It is also important in maintaining the cellular redox balance and participates in one of the most important defense systems against oxidative stress (Schafer and Buettner, 2001; Wu et al., 2004). The enzymes involved in the glutathione anti-oxidant defense system are glutathione peroxidase (GPx) and glutathione reductase (GR) which detoxify hydrogen peroxide in a reaction couple to glutathione redox cycling. Among the enzymatic defense, catalase (CAT) also plays a fundamental role by reducing hydrogen peroxide to water and oxygen (Scandalios, 2005; Valko et al., 2006; Yang et al., 2006).

*Allium* plant, such as onion and garlic has attracted particular attention of modern medicine because of its widespread health use in warding off illnesses and due the association with fibrinolytic activity and cardiovascular protection (Anderson, 1998; Campos et al., 2003). Several lines of evidence suggested that the sulfur compounds of *Allium* plants due their potential antioxidant activities might be responsible, at least in part, for these health beneficial effects (Yin et al., 2002; Campos et al., 2003). N-Acetylcysteine (NAC) (C\(_5\)H\(_9\)NO\(_3\)S), an organosulfur from *Allium* plants, has been successfully used as adjuvant therapy in various bronchi-pulmonary disorders (Davreux et al., 1997), to improve hepatic function (Pastor et al., 1997), to reduce lung injury (Sprong et al., 1998) and metal toxicity (Gurer et al., 1998), but the mechanisms responsible for these activities are not yet completely understood. Recently, Diniz et al. (2006) have shown the preventive effects of NAC on dyslipidemic profile and alleviation of hyperglycemia in rats given standard chow and 30% sucrose in its drinking water. It was also demonstrated that NAC has beneficial effects on sucrose-induced insulin resistance and oxidative stress (Song et al., 2005; Blouet et al., 2007). On the other hand, previous investigation has shown that Cr (VI) is an important modulator of carbohydrate metabolism causing hyperglycemia (Das El-Saad et al., 2009), but whether NAC can reverse these adverse effects merits to be investigated. Here we evaluate the glycemic control and pancreatic beta-cells protective effects of NAC in rats after acute chromium (VI) exposure.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Potassium dichromate (K\(_2\)Cr\(_2\)O\(_7\)) and N-Acetylcysteine (NAC) were purchased from Sigma Chemical Company, St. Louis, USA and all other chemicals used in the experiment were of analytical grade and were purchased from Merk (Germany), BDH Chemical (Mumbai, India) or Sigma (USA).

**Animals and administration schedule of potassium dichromate and antioxidant N-acetylcysteine**

Sixty male albino rats weighting 120 to 160 g were obtained from the animal house of Medical Research Institute, Alexandria University, Egypt. Animals were kept on commercial diet and tap water provided ad libitum. The rats were housed at 25 ± 2°C and in daily dark/light cycle. All animals received human care and our study complies with the instruction’s guidelines. After 2 weeks of acclimation, animals were divided into four equal groups (n=15/group) as follow:

- **Group I** (Control group): Untreated animals.
- **Group II** (Cr VI): Rats were subcutaneously injected with single dose of K\(_2\)Cr\(_2\)O\(_7\) (50 mg/kg b.w.).
- **Group III** (NAC+Cr VI): Rats were first intraperitoneally injected with NAC (200 mg/kg b.w.) and then K\(_2\)Cr\(_2\)O\(_7\) (50 mg/kg b.w., s.c.) with the time difference of 2 h.
- **Group IV** (NAC): Rats were intraperitoneally injected with NAC (200 mg/kg b.w.).

Dose selection of K\(_2\)Cr\(_2\)O\(_7\) and NAC was based on the methods of Solis-Heredia et al. (2000) and Egrefoglu et al. (2006). The substances were administered in the morning (between 09:00 and 10:00 h) to non-fasted animals.

**Collection of samples**

Rats from each group were sacrificed 24 h after administration of the compounds and blood samples were collected. Serum was separated into plastic tubes and the pancreatic tissues were quickly excised and washed immediately with ice-cold physiological saline (0.9% NaCl) and one part immediately stored at -80°C until analysis of biochemical parameters. The other part of the tissue was taken from the animals for electron microscopic analysis.

**Biochemical assays**

Lipid peroxidation (LPO) was measured in pancreatic tissue homogenates according to the method of Ohkawa et al. (1979) based on the formation of thiobarbituric acid reactive substances (TBARs) and expressed as the extent of malondialdehyde (MDA) production. The activity of glutathione peroxidase (GPx) was expressed as unit/100 mg tissue using method of Rotruck et al. (1973). Catalase (CAT) was assayed according to the method of Sinha (1972) and expressed as unit/100 mg tissue. Total protein were assessed in serum using a commercially available spectrophotometric-enzymatic
results

Table 1. Efficiency of N-acetylcysteine on chromium (VI) induced alterations on pancreatic tissue MDA, GPx, CAT and serum glucose level.

<table>
<thead>
<tr>
<th>Biochemical parameters</th>
<th>Group I Control</th>
<th>Group II Cr (VI)</th>
<th>Group III NAC+Cr (VI)</th>
<th>Group IV NAC</th>
</tr>
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<tbody>
<tr>
<td>MDA (nmol released/100 mg tissue)</td>
<td>13.83±1.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.21±1.20&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.22±1.03&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.92±0.53&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPx (units/100 mg tissue)</td>
<td>25.12±2.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.23±1.54&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.02±2.81&lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.03±1.32&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAT (units/100 mg tissue)</td>
<td>51.72±5.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>42.82±2.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.12±4.63&lt;sup&gt;c&lt;/sup&gt;</td>
<td>52.34±4.23&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>92.20±7.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>120.90±6.37&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102.75±6.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>95.54±5.23&lt;sup&gt;c&lt;/sup&gt;</td>
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</table>

All values are expressed in mean ±SE from 6 rats in each group. Values within rows not sharing common superscript letters differ significantly at p< 0.05. MDA: malondialdehyde (nmol released/100 mg tissue), GPx: glutathione peroxidase (unit/100 mg tissue), CAT: catalase (unit/100 mg tissue).

Ultrastructural studies

For electron microscopic examinations of pancreatic tissues, primary fixation was done in 3% glutaraldehyde in sodium phosphate buffer (200 mM, pH 7.2) for 3 h at 4°C. Pancreatic tissues were washed with the same buffer and postfixed in 1% osmium tetroxide (Agar Sci. Ltd.) in sodium phosphate buffer, pH 7.2, for 1 h at 4°C. Tissue samples were washed with the same buffer for 3 h at 4°C and then embedded in Araldite (Agar Sci. Ltd.). Thin sections were cut with LKB ultramicrotome. Samples were stained with 2% uranyl acetate and lead citrate. The sections were viewed and photographed on a Jeol 100 CX transmission electron microscope (Jeol Ltd., Japan) at 80 kV.

Statistical analyses

Data were analyzed using SPSS 15.0 for windows. Significance was calculated using one-way analyses of variance (ANOVA) followed by least significance difference (LSD) for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

Biochemical results

Table 1 demonstrates the influence of toxicity of Cr (VI) and NAC administration on pancreatic LPO levels, activities of GPx and CAT, and serum glucose concentration. The acute administration of Cr (VI) resulted in a state of pancreatic injury and extensive oxidative damage in rats as manifested by the significant (p < 0.05) increase in LPO levels. In parallel, significant (p < 0.05) decreases in the activities of GPx and CAT were recorded. The level of LPO significantly increased by 38.90% whereas the level of GPx and CAT decreased by 23.44 and 17.20%, respectively as compared to control. The administration of NAC significantly (p < 0.05) reduced LPO and restored GPx and CAT activities towards near normalcy suggesting better ameliorative potential of this antioxidant in pancreatic tissues. Pre-treatment with NAC to Cr (VI) treated animals led to a decrease of LPO by 10.35% and increase in the activities of GPx and CAT enzymes by 14.50 and 7.70%, respectively in comparison to Cr (VI)-treated group. On the other hand, the level of blood glucose significantly (p < 0.05) increased (p < 0.05) in Cr (VI)-treated group by 31.13% compared with control.

Pretreatment with NAC before Cr (VI) reduced the level of blood glucose by 15.01% when compared to Cr (VI) alone.

Ultrastructural observations of pancreatic beta-cells

Figures 1 to 3 depicted the ultrastructure of β cells of control and experimental groups of rats. Transmission electron microscopic analysis of β cells in pancreatic islet showed no pathological alterations in control rats. The nucleus, nuclear envelope, mitochondria, endoplasmic reticulum and Golgi complex of β cells were normal. Beta cells contained a number of secretory granules, which had a space between the core and the membrane and were diffusely distributed in the cytoplasm. The granules composed of a central core, usually with moderate homogenous, or slightly heterogeneous electron density, and an external single-layered membrane (Figure 1). The electron micrographs of β cells in pancreatic islet of Cr (VI) treated rats displayed loss of nuclear envelope, mitochondrial vacuolization, swelling, and dilatation of the endoplasmic reticulum and a striking enlargement of the Golgi apparatus (Figure 2A-B).

The secretory granules of β cells of Cr (VI) treated rats were obviously fewer than that of control rats. Most nuclei of these cells contained the normal pattern of chromatin. The protective effect of NAC on Cr (VI) treated rats was evident with a moderate increase in secretory granules of β cells in islet (Figure 3), which elucidated the efficacy of NAC on β cells. These NAC+Cr (VI) supplemented animals showed intact mitochondria, Golgi apparatus, endoplasmic reticulum, and two types of secretory granules with a crystalline core or a homogeneous core surrounded by a membrane. It is worthy to mention that NAC had no deleterious effect on the ultrastructure of beta cells of pancreas.
Figure 1. Electron micrograph of β cell in Langerhans islet of control rat pancreas. N: Nucleus, M: mitochondria, RER: rough endoplasmic reticulum, G: Golgi complex, secretory granules (SG). Scale bar: 2 µm

Figure 2. Electron micrograph of β cell in Langerhans islet of rat pancreas after Cr (VI) treatment. (A) Large number of empty vesicles (EV), dilated nuclear envelope (NE), swelling and vacuolization in mitochondria (M) and less secretory granules (SG) can be noted. N: nucleus. Scale bar: 2 µm. (B) Vacuolization of cytoplasm (V), Swollen Golgi body (G), dilation of endoplasmic reticulum (RER) and mitochondria (M) displaying loss of cristae and limiting membranes can be observed. Note also distorted nucleus (N) containing condensed chromatin patches. SG: secretory granules. Scale bar: 2 µm.

DISCUSSION

Chromium induces a broad spectrum of toxicological effects and biochemical dysfunctions constituting serious hazards to health (Norseth, 1986). Recent studies have reported that oxygen-free radicals are considered to be important mediators of acute Cr (VI) induced toxicity (Boşgelmez et al., 2008). Accordingly, among the main approaches used to ameliorate Cr (VI) induced toxicity is the use of agents with powerful antioxidant properties. In
the present work, we have studied the relative cytoprotective efficiencies of NAC against Cr (VI) induced pancreatic beta-cell injury.

Redox disturbances are known to have a negative impact on the body system through ROS generation, which destroy proteins, lipids and DNA by oxidation (Halliwell and Gutteridge, 1990). Although chromium itself does not directly generate free radicals, it indirectly generates various radicals such as superoxide, nitrogen species like peroxynitrite, nitric oxide and hydroxyl causing damage consistent with oxidative stress (Pritchard et al., 2000). These radicals attack the cell membrane and lead to the destabilization and disintegration of the cell membrane as a result of lipid peroxidation (Susa et al., 1996). In the present study, we observed after Cr (VI) exposure a marked elevation in pancreatic MDA, an index of lipid peroxidation. Pre-treatment with NAC was very effective in the prevention of oxidative damage induced by Cr (VI), objectified by a significant decrease in pancreatic MDA. This could be explained according to two reasons. First, NAC possesses a cysteine with a free thiol group and thus can mimic the antioxidant action of GSH. Treatment with NAC may thereby increase the antioxidant available to sequester the damaging heavy metal, but it can also reduce other radical oxygen species generated by the metal. Second, NAC has been shown to be a biosynthetic precursor of GSH (Kelly, 1998). Reduced glutathione (GSH) acts as a ROS scavenger within the cell. Increasing the synthesis of GSH would improve the antioxidant/prooxidant ratio within the cell.

Antioxidant enzymes are considered to be the body’s primary defense, which prevents biological macromolecules from oxidative injury and removes peroxides, free radicals and superoxide anion generated inside the cell. These enzymes are cytosolic in nature and are encoded from the nucleus and subsequently targeted to the subcellular compartments. Catalases and peroxidases on the other hand convert H$_2$O$_2$ to harmless H$_2$O and molecular O$_2$. These enzymes work independently, cooperatively and synergistically to maintain the integrity of organ tissues. The level of such enzymes is well maintained under normal physiological conditions. However, increases or decreases in the amount of such enzymes are marked through their modification in gene expression, decreased uptake or when cells are overloaded with oxidants (Vuillaume, 1987; Barber and Harris, 1994). On the other hand, reactive oxygen radicals have the ability to sufficiently modify a protein, leading to altered enzyme activity (Bellomo et al., 1983). In the present study, statistically significant depletion in the activity of the antioxidative enzymes relates to the high ROS levels in the pancreatic tissues. Such type of correlation is already reported in a wide variety of tissues treated with heavy metals including Cr (VI) (Benov et al., 1990; El-Demerdash et al., 2006; Abdel-Moneim and Said, 2007; Abdel-Moneim et al., 2008; Boggelmez et al., 2008; Newairy et al., 2009; Chandra et al., 2010; Soudani et al., 2010). Most of the antioxidant enzymes become inactive after Cr (VI) exposure either due to the direct binding of heavy metal to enzyme active sites if it contains SH groups or to the displacement of metal cofactors from active sites (Soudani et al., 2010). In NAC+Cr (VI) treated rats, the activities of GPx and CAT

**Figure 3.** Electron micrograph of β cell in Langerhans islet of rat pancreas after NAC+Cr (VI) treatment. The restoration of the nuclear shape (N), mitochondrial configuration (M), rough endoplasmic reticulum (RER), Golgi body (G) and secretory granules (SG) are evident in β cells. Scale bar: 2 µm.
reached almost the control value levels, indicating that NAC eliminated the toxic effects of Cr (VI). Furthermore, NAC prevented the rise in blood glucose level/hyperglycemia in rats treated with acute dose of K$_2$Cr$_2$O$_7$. Oxidative stress is produced under diabetic conditions and it is likely involved in progression of pancreatic β-cell dysfunction (Kajimoto and Kaneto, 2004). Various mechanisms have been suggested to contribute to the formation of ROS-free radicals under diabetic condition. Glucose oxidation is believed to be the main source of free radicals. In its enediol form, glucose is oxidized in a transition-metal dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. The superoxide anion radicals undergo dismutation to hydrogen peroxide, which if not degraded by catalase or glutathione peroxidase, and in the presence of transition metals, can lead to production of extremely reactive hydroxyl radicals (Jiang et al., 1990). Superoxide anion radicals can also react with nitric oxide to form reactive peroxynitrite radicals (Halliwell and Gutteridge, 1990). Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals (Tsai et al., 1994). Another important source of free radicals in diabetes is the interaction of glucose with proteins leading to the formation of an Amadori product and then advanced glycation endproducts (AGEs) (Hori et al., 1996). These AGEs, via their receptors (RAGEs), inactivate enzymes and alter their structures and functions (McCarthy et al., 2001), promote free radical formation (Baynes and Thorpe, 1999), and quench and block antiproliferative effects of nitric oxide (Vlassara, 1997). By increasing intracellular oxidative stress, AGEs activate the transcription factor NF-$κ$B, thus promoting up-regulation of various NF-$κ$B controlled target genes (Mohamed et al., 1999). NF-$κ$B enhances production of nitric oxide, which is believed to be a mediator of islet beta cell damage. In the present study, electron microscopic examination of pancreatic β cells in rats treated with Cr (VI) revealed marked degranulation, nuclear membrane breakdown, cell membrane rupture, swelling of mitochondria and hypertrophied cytoplasmic organelles such as Golgi body and endoplasmic reticulum. These changes reflect an inhibition in insulin synthesis and secretion as reported in diabetic animals (Degirmenci et al., 2005; Aruselvan and Subramanian, 2007; Zhou et al., 2009). Normal architecture of β cells, as shown by electron microscopy, was due to the remained β cells mass protected by NAC that secrete insulin and alleviate Cr (VI) mediated complications such as degranulated secretory vesicles, swollen mitochondria and endoplasmic reticulum. The preservation of β cell mass and insulin secretory granules in NAC+Cr (VI) treated rats could be attributed to normoglycemic effect and even further potentiated efficacy of NAC. Antihyperglycemic effect of NAC is generally dependent upon the degree of β cell destruction. NAC brings about its anti-

hyperglycemic effect through insulin secretion from the remnant β cells and insulin sensitivity, as other studies showed (Kaneto et al., 1999; Tanaka et al., 1999). Antidiabetic effects of the antioxidant NAC were demonstrated in db/db mice (Kaneto et al., 1999). As these mice developed hyperglycemia, their insulin content and insulin gene expression decreased. NAC treatment was associated with preserved insulin content and insulin mRNA as well as increased amounts of PDX-1, an impont-tan insulin gene transcription factor. NAC also reversed the progressive worsening of hyperglycemia, the decrease in glucose tolerance and insulin secretion, the decrease in insulin gene expression in ZDF rats (Tanaka et al., 1999). A number of other antioxidants have also been observed to exert hypoglycemic activity through insulin release stimulatory effects (Kannappan and Anuradha, 2009).

Conclusions

The present study provided evidence that pretreatment with NAC leads to amelioration of diabetic complication in animal models. NAC preserves pancreatic beta cells in part by reducing Cr (VI)-induced oxidative stress and enhancing insulin secretion. Hence, the combined effects of NAC in eliciting normoglycemia at remarkably low insulin reserves and in preserving residual insulin stores suggest that it may prevent the progressive deterioration of beta cells in prediabetic and newly diagnosed insulin dependent diabetes mellitus.

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