N-Acetylcysteine Promotes DNA Repair after Genotoxic Damage Induced by Copper Sulphate in Human Lymphocytes

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ABSTRACT

DNA damage may be induced by high reactive oxygen species levels and is related to cancer, amongst many diseases. Previous studies have shown that the excess of copper in cells increases the reactive oxygen species. Moreover, oxidative stress also diminishes the DNA repair enzymes, activity, hence, increasing cancer risk. There are existing reports about the role of N-acetylcysteine in DNA protection related to its antioxidant activity; however, its role in DNA repair has not been studied. In the present work, the DNA repair by N-acetylcysteine was analyzed through the comet assay in human lymphocytes after inducing genotoxicity by means of copper sulphate. The gathered data indicates that N-acetylcysteine is innocuous at 1000 µM and significantly reduced the DNA damage in human lymphocytes, which were previously treated with CuSO₄, while N-acetylcysteine exerted an adjuvant effect on the repair of DNA in human lymphocytes.

Abbreviations: ROS: Reactive Oxygen Species; DSBs: Double-Strand Breaks; NAC: N-Acetylcysteine

Introduction

The excess of many chemical substances in humans, such as copper has been reported to induce DNA damage and trigger cancer development [1]. The main reason for copper involvement in DNA damage has been associated to the increase of reactive oxygen species (ROS) [2]. ROS can induce DNA damage by means of two mechanisms: generation of DNA mutations, usually base pair substitutions, and DNA cleavage, where single strands breaks induce DNA instability [3]. The comet assay enables detection of the DNA double-strand breaks (DSBs) and it has been used to evaluate copper genotoxicity caused by oxidative damage in several plants, animals [4,5] and human cells as lymphocytes [6,7]. The DNA damage induced by ROS, can be decreased by the antioxidant action of N-acetylcysteine (NAC). Previous studies have reported the effect of NAC on DNA protection from ROS damage [8]. Xie, et al. evidenced the DNA protection effect of NAC against radiation harm on mice lymphocytes [9]. On the other hand, Ansari, et al. reported that NAC protects DNA from oxidative damage in rat intestine [10]. Additionally, NAC has been well established, such as a cytoprotective drug with an antioxidant effect as well as anti-inflammatory functions [11]. Therefore, NAC exerts a fundamental role in DNA protection, which may act as a chemoprotective agent to some diseases [12]. However, if DNA damage already exists, it needs to be repaired because the cell repair system is diminished due to oxidative lesions and this may increase the risk of cancer [13]. Due to the fact that DNA damage can lead towards the initiation of cancer progression, DNA damage repair proteins are present in cells in order to maintain the genome integrity. DNA damage is repaired in two manners: first, lesions are recognized for a restricted activity or inactivated enzymes involved in the repair process [14]. Certain studies associate oxidative stress condition with a restricted activity or inactivated enzymes involved in DNA damage repair [15]. Oxidative stress has been reported as the most frequent cause of DNA damage, such being the reason for the NAC to have a protective effect; yet could it be possible that...
NAC exerts a repairing effect on DNA? The comet assay procedure was followed in order to study the DNA repair effect of the NAC, it was used blood (5ml) from a healthy subject for four times to obtain lymphocytes, afterwards the cells were treated with copper sulphate and NAC.

**Objectives**

In the present work, we performed the following objectives:

1) To determine the DNA damage caused by copper sulphate.
2) To determine the N-acetylcysteine innocuity/damage to DNA.
3) To evaluate the effect of N-acetylcysteine on DNA repair.

**Material and Methods**

**Peripheral Blood Extraction**

Peripheral blood was obtained four times from the same healthy subject by venipuncture (5 ml) in a blood sample tube containing EDTA as anticoagulant and it was diluted 1:1 with PBS 1X. We isolated the lymphocytes from the same subject to test the effect of Cu\(\text{SO}_4\) and NAC. This procedure was done according to the Mexican bioethics NOM-012-55A3-2012 and following the Helsinki declarations protocol.

**Lymphocyte Isolation**

Two parts of blood-PBS (1:1) mixture were added to three parts of Ficoll paquepremium. The tube was centrifuged for 30 min at 3500 rpm, and then the white coat was separated and resuspended in PBS at 5 ml final volume. The lymphocytes were centrifuged for 5 min at 2500 rpm and the supernatant was removed. The pellet was resuspended into 1 mL RPMI-1640 medium containing 10% of FBS. Lymphocytes were incubated at 37°C, 5% CO\(_2\).

**Test Material Treatment**

Immediately, after the lymphocyte’s isolation, 2 x 10\(^4\) cells per 100 µL of PBS were used to test the effect of Cu\(\text{SO}_4\) and NAC. Each tested material was added to the lymphocyte’s samples (Table 1) and were incubated at 37°C for 2 hours. After the treatment, the cells were centrifuged for 5 minutes at 3000 rpm, afterwards, the lymphocytes pellet was resuspended in 100 µL of PBS accordingly, and 10 µL were removed for a trypan blue viability test, additionally, a comet assay was conducted on the samples.

| Groups          | PBS 1X | H\(_2\)O\(_2\) 200 µM | Cu\(\text{SO}_4\) 50 µM | Cu\(\text{SO}_4\) 100 µM | Cu\(\text{SO}_4\) 200 µM | NAC 500 µM | NAC 1000 µM | NAC 1500 µM |
|-----------------|--------|-----------------------|------------------------|------------------------|------------------------|------------|------------|------------|
| Negative Control| +      | -                     | -                      | -                      | -                      | -          | -          | -          |
| Positive Control| -      | +                     | -                      | -                      | -                      | -          | -          | -          |
| Cu\(\text{SO}_4\)_1 | -      | -                     | +                      | -                      | -                      | -          | -          | -          |
| Cu\(\text{SO}_4\)_2 | -      | -                     | -                      | +                      | -                      | -          | -          | -          |
| Cu\(\text{SO}_4\)_3 | -      | -                     | -                      | -                      | +                      | -          | -          | -          |
| NAC 1           | -      | -                     | -                      | -                      | -                      | +          | -          | -          |
| NAC 2           | -      | -                     | -                      | -                      | -                      | -          | +          | -          |
| NAC 3           | -      | -                     | -                      | -                      | -                      | -          | -          | +          |
| *NAC/ Cu\(\text{SO}_4\)* | -      | -                     | -                      | -                      | +                      | -          | -          | -          |

Note: *Group NAC/ Cu\(\text{SO}_4\) was incubated 2h with Cu\(\text{SO}_4\) µM and then 2h with NAC 1000 µM.

**Comet Assay Alkaline Version**

The agarose microgels were prepared as follows: the first layer was prepared with 50 mL of agarose normal melting point at 1.0 percent and distributed along a slide previously degreased with ethanol. For the distribution of the agarose, a rectangular glass cover was used to obtain a homogeneous layer and removed after the agarose solidified. The second layer consisted of 90 mL mixture of low melting agarose (ABPF) 0.5% and lymphocyte suspension (mix of 100 µL of the lymphocytes suspension and 100 µL of ABPF), the agarose-cell mixture was distributed on the first layer with a coverslip. Afterwards, it was allowed to solidify at 4 °C for 5 min, and then the coverslip was removed. The third layer was prepared with 90 mL of ABPF, distributed on the second agarose layer with the aid of a coverslip, and it was allowed to solidify at 4 °C for 5min. From this instance on, the work was conducted in a darkroom to prevent light damaged on the DNA after the cell lysis. The agarose microgels were immersed in 50 mL of lysis solution (4 °C) for 1.5 h allowing cell lysis.

The microgels were washed with 1X PBS and placed horizontally in the electrophoresis chamber; the chamber was filled with running buffer (10N NaOH, 300 mM EDTA), pH of 13.0, and allowed to stand for 20 min to allow the unwinding of the DNA. Electrophoresis was performed according to the Mexican bioethics NOM-012-55A3-2012 and following the Helsinki declarations protocol.
conducted under the following conditions: 25 V, 300 mA, 20 min. After the electrophoresis time, the microgels were neutralized by placing them twice in a Petri dish with Tris-base (pH 7.0) 5 mL for 2 min. The microgels dried at room temperature and stained with 50 mL of 25 mM ethidium bromide [16]. The genotoxic damage was evaluated by observing the microgels on a fluorescence microscope. All the experiments were tested in triplicate.

Statistical Analysis

Significant differences between groups were evaluated by two-way ANOVA and a tukey pos hoc test [17].

Results

Evaluation of N-Acetylcysteine Innocuity

In order to evaluate NAC innocuity, three different NAC doses in human lymphocytes were tested (Figure 1). The results did not indicate significant difference compared against the negative control (PBS 1X) at 500 µM and 1000 µM. Even though NAC at 1500 µM had significant differences in regard to the negative control, such dose did not generate the same DNA damage as the positive control (H_2O_2) (p<0.05). It was decided to use 1000 µM for the experiments that followed.

Copper Sulphate Genotoxicity

Different CuSO_4 doses were tested, and it was observed a significant difference in 50 µM, 100 µM and 200 µM compared to negative control. These results indicate the DNA damage in a dose dependent manner (Figure 2). Despite the fact that CuSO_4 at 50 µM and 100 µM were significantly different from the negative control, these doses do not generate the same damage (p<0.05) as the positive control. Only copper sulphate at 200 µM caused the same damage (p > 0.05) as positive control and for that reason CuSO_4 200 µM was used to test the effect of NAC in the DNA repair.
N-Acetylcysteine Effect on DNA Repair

In order to study the effect on DNA repair, N-acetylcysteine 1000 µM and CuSO₄ 200 µM were used. Lymphocytes treated with CuSO₄ 200 µM induced 57% damage to DNA. On the other hand, lymphocytes that were treated for 2h with CuSO₄ 200 µM and then 2h with NAC 1000 µM, showed a 25.5% decrease on DNA damage (Figure 3). It is interesting to notice that although the DNA damage had a significant difference compared to the negative control (p<0.05), subsequent treatment with NAC after the DNA damage, significantly decreased in comparison to the positive control and CuSO₄ 200 µM by itself (p<0.05). The genotoxic effect and DNA repair analyzed by comet assay is shown in Figure 4. Figure 4A demonstrates a negative control; Figure 4B exhibits positive control; Figure 4C shows NAC 1000 µM; Figure 4D indicates CuSO₄ 200 µM genotoxicity; and Figure 4E proved the NAC effect on the DNA repair.

Figure 3: N-acetylcysteine effect on DNA repair. The genotoxic effect of CuSO₄ is shown both, alone and together with N-acetylcysteine. N-acetylcysteine was added after 2h of lymphocyte treatment with CuSO₄ and the effect on DNA repair is shown. Positive and negative control are shown too. Different letters denote values statistically different by one-way ANOVA analysis followed by a Tukey’s multiple comparison test. P < 0.05 was considered significant. Bars represent SD (standard deviation).

Figure 4: Evaluation of DNA damage by comet assay. The presence and length or absence of the comet tail can be observed in each figure.
A. PBS 1X.
B. H₂O₂ 200 µM.
C. N-acetylcysteine 1000 µM.
D. CuSO₄ 200 µM.
E. CuSO₄ 200 µM / NAC 1000 µM.
Discussion

CuSO$_4$ has been reported to cause DNA damage, which is referred to as genotoxicity. It has been documented that copper concentrations are significantly elevated in several types of malignancies, and in turn, it leads to ROS increase and redox imbalance. The DNA damage caused by ROS was accepted as the beginning of cancer development [18]. In this study, the genotoxic effect of CuSO$_4$ was evaluated by comet assay. Three CuSO$_4$ concentrations (50 µM, 100 µM, 200 µM) induced DNA damage with significant difference compared to negative control, being the concentration 200 µM the most genotoxic. According to previous reports, the main genotoxic effect by CuSO$_4$ is due to the increase of reactive oxygen species (ROS) [19] which promotes that endogenous antioxidants and DNA repair systems get overwhelmed by redox imbalance as indicates previous study by Kermanizadeh, et al. [20]. Cells contain a variety of DNA repair enzymes to repair oxidant-induced DNA damage but, according to Mesquita, et al. those enzymes do not function properly in the presence of high amounts of ROS increased by CuSO$_4$ [21].

Our results are consistent with previous reports about copper genotoxicity in marine animals [22] and mice [23] in which copper induced ROS increase and oxidative DNA damage in vivo. The next goal was to determine if NAC is innocuous and if it mediates the DNA repair and the NAC 500 µM and 1000 µM did not show significant differences on the DNA damage compared to the negative control, which indicates cellular innocuousness. Given the fact that lymphocytes treated first with CuSO$_4$ 200 µM and then with NAC 1000 µM showed lower DNA damage compared to those treated only with CuSO$_4$, such results suggest that the NAC 1000 µM exerts an adjuvant effect on DNA repair as well a DNA protection as indicated Xie, et al. [8]. A possible explanation for DNA repair by NAC could be the restoration of redox balance due to its antioxidant effect as indicated by previous reports [11,24]. In this case, NAC regulates the redox balance, for the correct function of the enzymatic defense mechanisms, which contains superoxide dismutase, catalase and glutathione-peroxidase as the most important antioxidant enzymes [25].

At the other extreme, antioxidant protection can be non-enzymatic [26]. Glutathione is a non-enzymatic antioxidant composed of L-glutamate, L-cysteine and L-glycine, and is the predominant intracellular non-protein sulphydryl in a wide range of cells [27]. NAC can promote Glutathione synthesis, and both play a critical role in cellular defense and helps maintain structural and functional viability of proteins, same as DNA repair enzymes, by means of reducing the amounts of ROS by their reducing property through its thiol-disulfide exchange activity [28]. Furthermore, the reduction of ROS leads to the decrease of oxidative DNA damage and enables the enzyme to mediate the DNA repair [29]. It is possible to determine the mechanism by which NAC exerts its action as DNA repair compound by measuring ROS levels and enzymatic activity on DNA repair after treatment with CuSO$_4$/NAC.

Conclusion

The conclusion of our collected data indicates that NAC is innocuous to 500 µM and 1000 µM, and CuSO$_4$ induces DNA damage in a concentration dependent manner in human lymphocytes. The DNA damage caused by CuSO$_4$ can be repaired by NAC. Based upon these findings, NAC should be evaluated for its innocuity in further studies as well as a source of Geno protective and auxiliary compounds in DNA repair in several other types of cells.

Recommendations

We recommend testing a large range of NAC and CuSO$_4$ concentrations in different cancer and normal cell lines. However, the measure of ROS levels and the evaluation of enzymatic activity of the DNA repair system could provide additional evidence about the NAC and CuSO$_4$ Geno protective and genotoxic mechanisms, respectively.

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