**Abstract**: Nickel(II) is reported to be genotoxic, but the mechanisms underlying its genotoxicity are largely unknown. It can interfere with DNA repair and this may contribute to its genotoxicity. We studied the effect of nickel chloride on the repair of DNA damaged by UV radiation or N-methyl-N-nitro-N-nitrosoguanidine (MNNG) in human lymphocytes using the alkaline comet assay. Nickel(II) at 1 μM caused an accumulation of DNA breaks during repair incubation, which could follow from the inhibition of the polymerization/ligation step of UV-damaged DNA repair. On the other hand, nickel(II) inhibited the formation of transient DNA breaks brought by the repair process after incubation with MNNG at 5 μM, which might follow from interference with the recognition/incision step of excision repair. Additionally, nickel at 1 μM inhibited the activity of formamidopyrimidine-DNA glycosylase (Fpg) and 3-methyladenine-DNA glycosylase II (Alk A), enzymes involved in DNA excision repair. A decrease in endonuclease III (Endo III) activity was observed at 2 and 5 μM of nickel chloride. Our results suggest that nickel(II) at non-cytotoxic concentrations can inhibit various steps of DNA excision repair, and this may contribute to its genotoxicity.

**Key Words**: Nickel Chloride, DNA Repair, DNA Damage, MNNG, UV Light, Lymphocytes, Comet Assay
INTRODUCTION

Nickel compounds can be carcinogenic to humans and laboratory animals [1]. One possible mechanism involved in tumour formation by this metal is an increase in the production of reactive oxygen species, which can subsequently interact with DNA [2-6]. We have recently shown that free radicals may be involved in the formation of strand breaks and/or alkali labile sites as well as DNA-protein cross-links in human lymphocytes treated with nickel chloride [7]. Epigenetic changes exerted by nickel, such as the induction of cytosine methylation and histone deacetylation, which would lead to the inherited inactivation of the senescence and tumour suppressor gene(s), may also contribute to the carcinogenic process [8-10]. On the other hand, a risk of cancer promotion can result from a nickel-mediated decrease of an ability to remove endogenous DNA damage and DNA damage induced by environmental agents [11, 12]. Nickel has been shown to interfere with the repair mechanisms involved in removing UV-, platinum-, mitomycin C, \( \gamma \)-radiation- and benzo[a]pyrene-induced DNA damage [13-18]. Nickel(II) can impair DNA base excision repair (BER), thus diminishing the removal of oxidative DNA base modifications and abasic sites, as well as diminishing the closure of DNA strand breaks. Some compounds of nickel(II) have also been shown to inhibit the nucleotides excision DNA repair (NER). In consequence, the removal of (6-4)-photoproducts, cyclobutane pyrimidine dimers and bulky adducts may be incomplete. It is also possible that nickel(II) can interfere with recombination repair responsible for the removal of interstrand cross-links and DNA double-strand breaks induced by \( \gamma \)-radiation and some antitumor drugs (e.g., cisplatin and mitomycin C) [17, 18].

In this study, we investigated the effect of nickel chloride at very low concentrations (0.1 and 1 \( \mu \)M) on the repair of DNA damaged by UV radiation or \( N \)-methyl-\( N' \)-nitro-\( N \)-nitrosoguanidine (MNNG) in human lymphocytes using the alkaline (pH > 13) comet assay. The influence of nickel(II) on the activity of enzymes involved in DNA repair was also studied. We used enzymes, which have broad substrate specificity and recognize oxidative DNA damage: formamidopyrimidine-DNA glycosylase (Fpg) and endonuclease III (Endo III). Fpg is a glycosylase-initiating base excision repair enzyme in \( E. coli \). It recognizes and removes 7,8-dihydro-8-oxoguanine (8-oxoguanine), the imidazole ring-opened purines: 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua) and 4,6-diamino-5-formamidopyrimidine (Fapy-Ade), and small amounts of 7,8-dihydro-8-oxoadenine (8-oxoadenine) [19]. The removal of specific modified bases from DNA by this enzyme leads to the formation of apurinic or apyrimidinic sites, which are subsequently cleaved by its AP-lyase activity. This leaves a gap in the DNA strand, which can be detected by the comet assay [20]. Endo III coverts oxidized pyrimidines into strand breaks, which can be detected by the comet assay [21]. Hydrogen peroxide was used as an oxidized agent introducing DNA damage recognized by Fpg and Endo III.
We also studied another enzyme, 3-methyladenine-DNA glycosylase II (Alk A), which recognized 3-methyladenine, 7-methyladenine, 3-methylguanine, \(O^2\)-alkylcytosine and \(O^2\)-alkylthymine [19]. We used MNNG to introduce DNA damage detected by this enzyme.

**MATERIALS AND METHODS**

**Chemicals**

Nickel chloride (NiCl\(_2\)), RPMI 1640 medium without L-glutamine, low melting point (LMP) and normal melting point (NMP) agarose, phosphate buffered saline (PBS), 4’,6-diamidino-2-phenylindole (DAPI), \(N\)-methyl-\(N’\)-nitro-\(N\)-nitrosoguanidine (MNNG), hydrogen peroxide, hydroxyurea were purchased from Sigma. Fpg, Endo III and Alk A were donated by Dr. B. Tudek and Dr. J. T. Kusmierek of the Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland. All other chemicals were of the highest commercial grade available.

**Lymphocyte isolation**

Lymphocytes were isolated by centrifugation in a density gradient of Gradisol L (15 min, 280g, 4°C) from human peripheral blood obtained from young (23-25 years), non-smoking men. The viability of the cells was measured by the trypan blue exclusion assay and was found to be about 99%. The final concentration of the lymphocytes was adjusted to 1-3 \(\times\) 10\(^5\) cells/ml by adding RPMI 1640 to the single cell suspension.

**Cell treatment**

The lymphocytes in RPMI 1640 medium were pre-incubated with nickel chloride at 0.1 and 1 \(\mu\)M for 1 h at 37°C. Subsequently, MNNG was added to the suspension of the lymphocytes to give a final concentration of 5 \(\mu\)M, and an 1-h incubation at 37°C was carried out. In the experiment with UV radiation, the cells were irradiated at 2.5 J/m\(^2\) at 4°C. UV-irradiation was performed at room temperature with an EMITA VP-60 UV lamp (Famed, Łódź, Poland) emitting at 150-400 nm at a dose rate of 0.12 J/m\(^2\)s\(^{-1}\). Irradiation was carried out in RPMI 1640 medium. Control samples were also pre-incubated with nickel chloride at 0.1 and 1 \(\mu\)M for 1 h at 37°C. In order to increase the sensitivity of the DNA repair process in our experimental conditions, hydroxyurea at 10 mM, which is a DNA repair inhibitor, was used in the same manner as nickel chloride.

**DNA repair**

After treatment with MNNG or UV radiation, the lymphocytes and the control samples were washed and resuspended in fresh RPMI 1640 medium preheated to 37°C, containing nickel chloride at 0.1 and 1 \(\mu\)M or hydroxyurea at 10 mM. Aliquots of the suspension were taken immediately and 30, 60 and 120 min later. Further steps were as described in the section Comet assay.
DNA repair enzyme treatment
In experiments with the repair enzymes, the lymphocytes were treated with hydrogen peroxide at 20 µM (10 min, 4°C) in the case of Fpg and Endo III, or with MNNG at 5 µM (1 h, 37°C) in the case of Alk A. Fpg and Endo III were incubated with nickel chloride at concentrations in the range of 0.1-5 µM for 15 min at 37°C in a buffer containing 40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM EDTA and 0.2 mg/ml bovine serum albumin, pH 8.0. Alk A was incubated with nickel chloride for 15 min at 37°C in a buffer containing 70 HEPES-KOH, 1 mM EDTA and 1 mM β-mercaptoethanol, pH 7.6. Aliquots of 30 µl of Fpg, Endo III or Alk A at 1 µg/ml in buffer were applied to the cells in agarose on the slides after lysis, and incubated at 37°C for 30 min in the case of Fpg and Endo III [20], and 1 h in the case of Alk A. The control samples received appropriated enzyme buffer containing nickel chloride at the same concentration as the enzyme samples. Further steps were as described in the section Comet assay.

Comet assay
The comet assay was performed under alkaline conditions essentially as per the procedure of Singh et al. (1988) [22] with some modification [23] as described previously [24]. A freshly prepared suspension of the lymphocytes in 0.75% LMP agarose dissolved in PBS was spread onto microscope slides (Superior, Germany) pre-coated with 0.5% NMP agarose. The cells were then lysed for 1 h at 4°C in a buffer consisting of 2.5 M NaCl, 100 mM EDTA, 1% Triton X-100 and 10 mM Tris, pH 10. After lysis, the slides were placed in an electrophoresis unit, and DNA was allowed to unwind for 20 min in a solution consisting of 300 mM NaOH and 1 mM EDTA, pH > 13. Electrophoresis was conducted in an electrophoretic solution consisting of 30 mM NaOH and 1 mM EDTA, pH > 13 at an ambient temperature of 4°C (the temperature of the running buffer did not exceed 12°C) for 20 min at an electric field strength of 0.73 V/cm (28 mA). The slides were then washed in water, drained and stained with 2 µg/ml DAPI, and covered with cover slips. To prevent additional DNA damage, all the steps described above were conducted under dimmed light or in the dark. The comets were observed at 200× magnification in an Eclipse fluorescence microscope (Nikon, Japan) attached to a COHU 4910 video camera (Cohu, Inc., USA) equipped with a UV-1 A filter block and connected to a Lucia-Comet v. 4.51 personal computer-based image analysis system (Laboratory Imaging, Czech Republic). Fifty images were randomly selected from each sample and the tail DNA (%) was measured. The tail DNA is positively correlated with the level of DNA breakage in a cell [22]. The mean value of the tail DNA in a given sample was taken as an index of DNA damage in the sample.

Data analysis
The values of the comet assay in this study were expressed as the mean ± S.E.M. from three experiments, i.e. the data from three experiments was pooled and the
statistical parameters were calculated. All data was analyzed using the one-way ANOVA test. A P value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Nickel compounds are usually found to be weak mutagens, however they are well-known carcinogens [1]. These compounds may exert their carcinogenic activity through unusual genotoxic mechanisms. In combination with UV irradiation, nickel(II) was shown to enhance cytotoxicity, mutagenicity and sister chromatid exchange induction in V79 Chinese hamster cells [25]. Later experiments showed that these effects can be linked with the nickel(II)-mediated inhibition of DNA repair [11-18, 26-30]. The lowest reported concentration of nickel(II) that effectively inhibits DNA repair in cultured cells is 50 µM [11]. A concentration of as low as 10 µM, nickel(II) decreased the binding of XPA protein, which is essential in the formation of the pre-incision complex of the NER system in mammalian cells, to cisplatin-damaged oligonucleotide [26]. On the other hand, it was reported that a very high concentration of nickel(II) ~ 76 mM – was necessary to observe a repair inhibition of radiation-induced DNA double-strand breaks [17].

In this study, we found that nickel(II) at 1 µM can interfere with the repair of DNA damage induced by UV light and MNNG. At the lower concentration of nickel (0.1 µM), we did not observe this effect. In order to observe the maximal extent of DNA damage, we used broad-range UV (UVA, UVB, UVC), which remains also terrestrial UV (280-400 nm) solar spectrum.

In the UV spectrum range between 290 and 315 nm, the direct excitation of DNA is responsible for base modifications sensitive to Fpg protein (i.e. 8-hydroxyguanine and formamidopyrimidines) and T4 endonuclease V (i.e. cyclobutane pyrimidine dimers). While the yield of pyrimidine dimers per unit dose continues to decrease exponentially beyond 315 nm, the yield of Fpg-sensitive modifications increases to a second maximum between 400 and 450 nm. The DNA damage spectrum in this wavelength range consists of only a few other modifications (strand breaks, abasic sites and pyrimidine modifications sensitive to endonuclease III) [31].

UV photoproducts are removed by the nucleotide excision repair process, which involves five basic steps: the recognition of the DNA lesion; the cleavage of the damaged 3’ and 5’ strand of the lesion; the excision of the damaged strand, creating a gap; the synthesis of new DNA to fill the gap; and the ligation of the final nick [32]. UV-radiation at 2.5 J/m², which we used, caused an increase in the number of DNA lesions appearing during the repair incubation of the lymphocytes and revealed by the comet assay (Fig. 1). This lesion might come from the excision of the damaged fragment of DNA. Hydroxyurea at 10 mM inhibited the polymerization step of the excision repair of the UV-damaged DNA, which was seen between the 60 and 120 min stages of the repair incubation as an increase in the level of DNA damage. This metabolic inhibitor
causes a depletion of nucleotides by inhibiting ribonucleotide reductase; therefore, transient DNA breaks introduced in the repair process remained open [33]. The presence of nickel chloride at 1 μM caused a net increase in DNA damage resulting from the accumulation of DNA breaks (time 0 min P < 0.001; time 30 min P < 0.05; time 60 min P < 0.01; time 120 min P < 0.01; time 180 P < 0.01; time 240 P < 0.05), which could follow from the inhibition of the polymerization/ligation step of UV-damaged DNA repair (Fig. 1).

Fig. 1. The time course of DNA strand break formation in human lymphocytes irradiated with UV light at 2.5 J/m² (●) in the presence of nickel chloride at 0.1 μM (▼) and 1 μM (■) or hydroxyurea at 10 mM (▲) compared with the untreated control (○). The number of cells scored for each treatment was 50. Error bars denote S.E.M.

MNNG is an alkylating agent which can cause DNA base modifications, such as 3-methyladenine and 7-methylguanine. Alkylated bases can be repaired by a direct reversal, during which O⁶-MeGua-DNA methyltransferase transfers the methyl group from the O-alkylated base to its own cysteine residue, or by base excision repair, where glycosylases excise methylated bases leaving apurinic/apirimidinic sites for subsequent repair processes [34]. Previously, it was shown that O⁶-MeGua-DNA methyltransferase is inactivated by nickel(II) in HeLa cells [27]. During the repair incubation of the lymphocytes in the presence of nickel chloride, we observed a decrease in DNA damage in comparison with the cells incubated in the absence of this agent (time 30 min P < 0.001; time 60 min P < 0.01) (Fig. 2). The results obtained might suggest that nickel(II) inhibited the formation of transient DNA breaks brought by the repair process after incubation with MNNG, which might follow from the interfering with the recognition/incision step of the excision repair. We observed an
increase in DNA damage appearing during repair incubation in the presence of hydroxyurea at 10 mM. Therefore, hydroxyurea inhibited the polymerization step of the excision repair of the MNNG-damaged DNA.

Fig. 2. The time course of DNA strand-break formation in human lymphocytes incubated with MNNG at 5 µM for 1 h at 37°C (●) in the presence of nickel chloride at 0.1 µM (▼) and 1 µM (■) or hydroxyurea at 10 mM (▲) compared with the untreated control (○). The number of cells scored for each treatment was 50. Error bars denote S.E.M.

Fig. 3. DNA damage of human lymphocytes incubated for 10 min at 4°C with hydrogen peroxide at 20 µM (white bars) and treated with Fpg at 1 µg/ml (grey bars) in the presence or in the absence of nickel chloride. The number of cells analysed in each treatment was 50. Error bars denote S.E.M. *** P < 0.001.
The molecular mechanisms of repair inhibition by nickel(II) or other metals are still poorly known. It is possible that nickel may bind to DNA-repair enzymes and generate oxygen-free radicals to cause protein degradation \textit{in situ} [29]. On the other hand, a competition of toxic metals with essential ions may be considered as an important mechanism. Potentially sensitive targets for the toxic action of nickel(II) are zinc finger structures in DNA binding motifs present in several DNA repair enzymes, including the bacterial Fpg protein and the mammalian XPA protein, DNA ligase III and poly(ADP-ribose) polymerase (PARP). The effects of interference with DNA repair by nickel(II) were reported to be reversible by the addition of magnesium, indicating that the inactivation of DNA repair indeed occurred at the protein level by the displacement of the respective essential metal ions and by the distortion of the DNA structure \textit{per se}, nor by an interference with the expression of the repair proteins [11, 16].

We observed a decrease in Fpg activity (P < 0.001) towards DNA damaged by hydrogen peroxide after incubation with nickel chloride at 1 \(\mu\text{M}\) by comparison to the enzyme incubated in the absence of it (Fig. 3). Similarly, we observed a decrease in Alk A activity (P < 0.05) after the incubation of the enzyme with nickel chloride at 1 \(\mu\text{M}\) (Fig. 5). At the lower concentration of nickel, 0.1 \(\mu\text{M}\), we did not notice any decrease in the activity of these enzymes (P > 0.05). In the case of Fpg, our results are in contrast with those from the study of Asmuss \textit{et al.} [28]. Nickel(II) at concentrations of not greater then 1 mM was shown not to affect the activity of Fpg. In the same study, it was indicated that nickel(II) reduced the DNA-binding ability of XPA. XPA plays a central role in the first steps of nucleotide excision repair. It binds specifically to damaged DNA,

Fig. 4. DNA damage of human lymphocytes incubated for 10 min at 4°C with hydrogen peroxide at 20 \(\mu\text{M}\) (white bars) and treated with Endo III at 1 \(\mu\text{g/ml}\) (grey bars) in the presence or in the absence of nickel chloride. The number of cells analysed in each treatment was 50. Error bars denote S.E.M. *** P < 0.001.
including lesions induced by UVC or cisplatin [35]. We did not observe any change (P > 0.05) in the activity of Endo III after incubation with nickel(II) at 1 µM (Fig. 4). We detected a decrease in Endo III activity at higher concentrations of nickel chloride, 2 and 5 µM (P < 0.001). However, all the enzymes used in our studies are bacterial, so their inhibition does not fully explain the effect in mammalian cells.

Fig. 5. DNA damage of human lymphocytes incubated for 1 h at 37°C with MNNG at 5 µM (white bars) and treated with AlkA at 1 µg/ml (grey bars) in the presence or in the absence of nickel chloride. The number of cells analysed in each treatment was 50. Error bars denote S.E.M. * P < 0.05.

In our experiments, we detected the nickel(II)-mediated decrease of Alk A activity, the repair enzyme which does not have zinc finger structures. Hence, probably another mechanism than the substitution of essential ions is responsible for the observed nickel(II) action. One possibility is a reaction between the metal and the −SH groups of the proteins. Nickel(II) reacts easily with thiols and generates free radicals [36], which could inactivate the repair enzymes. On the other hand, nickel(II) could bind to proteins and by an influence on conformations, decrease its enzymatic activity. The results obtained suggest that nickel(II), at a very low, non-cytotoxic concentration (1 µM), might interfere with two different steps of DNA repair. It can inhibit the polymerization/ligation step of UV-damaged DNA repair and the recognition/incision step of MNNG-damage DNA repair. Nickel(II) decreases the activity of some repair enzymes. The mechanism of this action may include interactions with a specific structure containing zinc or the −SH groups of repair proteins. The effects of nickel(II) on repair processes contribute to nickel(II)-mediated genotoxicity.
Acknowledgements. This work was supported by grants 505/449 (K. W.) and 505/450 (J. B.) from the University of Łódź.

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