CHAPTER III

MATERIALS AND METHODS

3.1 Cell culture

A549 cells (human alveolar epithelial cells), purchased from the American Tissue Culture Collection (ATCC), were cultured in DMEM (Sigma, Zwijndrecht, the Netherlands) supplemented with 10% heat inactivated fetal calf serum (FCS, Gibco Invitrogen, Breda, the Netherlands) and 1% penicillin/streptomycin (Sigma, Zwijndrecht, the Netherlands). Cells were maintained at 37°C in a 5% CO_2 atmosphere. Cells were routinely grown in 75 cm^2 cell-culture flasks and were passaged twice a week.

3.2 Cell Exposure

Confluent A549 cells were washed with HBSS, followed by exposure to HOCl (final concentrations ranging from 0 to 100 µM) in HBSS (37 °C). The negative control group was treated with a vehicle control (HBSS) and the positive control group was treated with H_2O_2. After incubating at 37°C for 15 minutes, the cells were harvested by trypsination for analyses of toxicity and DNA damage. Each individual exposure was repeated twice to ensure reproducibility. To examine the effects of HOCl exposure on DNA damage response, cells were exposed to HOCl in serum-free medium for 15 minutes, followed by washing and further incubation for up to 4 hours.

3.3 Cytotoxicity assay

Cytotoxicity of HOCl or H_2O_2 in A549 cells was determined using trypan blue (0,04%, Sigma) and cells were counted in an Burker counting chamber (Kosmider, Zyner et al. 2004). Viable cells will maintain membrane integrity and will not take up trypan blue.

Among the transcriptional targets of p53, the cyclin-dependent kinase (CDK) inhibitor $p21^{Cip1}$ and growth arrest and DNA damage (Gadd) genes play a key role in mediating G1 arrest. p21 inhibits growth of proliferating cells by inhibiting G1 cyclin-dependent kinases and proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase activity at DNA replication forks (Colman, Afshari et al. 2000). Like p53, the Gadd genes are induced in cells exposed to genotoxic stress. *Gadds* were originally identified in a genetic screen for early response genes induced by ultraviolet radiation (Fornace, Nebert et al. 1989). Each gene encodes a distinct gene product that participates in the cellular response to stress. In the lung, Gadd45 is selectively expressed in alveolar cells during hyperoxia. Gadd45 binds DNA replication and repair proteins cdc2, p21, and PCNA, suggesting a potential role in cell cycle control and DNA repair. Altogether, it suggested that p21 and Gadd45 may serve to coordinate DNA repair and replication in damaged cells (Roper, Gehen et al. 2005), and are considered as a sensitive indicator of genotoxic stress.

Cells with compromised cell membranes take up trypan blue, and were counted as dead. At least 100 cells were counted and the number of viable cells was calculated as percentage of the total cell population.

3.4 Comet Assay

DNA strand breakage in A549 cells was determined by single cell gel electrophoresis/alkaline comet assay, as described by Knaapen *et al.* (Knaapen, Schins et al. 2005). Microscope slides were coated with a layer of 1.5% agarose. A549 cells were harvested and suspended in HBSS. Subsequently, 25 µl of the cell suspension (2-10^6 cells/ml) was mixed with 75 µl 0.65% low melting point agarose. This mixture was added to the precoated slides and covered with a cover glass. Following solidification (45 minutes, 4°C), cover glasses were removed and slides were immersed in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 250 mM NaOH, pH 10; 10% DMSO and 1% Triton X-100) and stored at 4°C. After 18 h, slides were placed in an electrophoresis tank filled with buffer (300 mM NaOH, 1 mM EDTA, pH 13, 4°C) for 20 minutes. Electrophoresis was conducted at 300 mA and 25 V for 20 minutes. Subsequently, slides were neutralized by repeated washing (3-10 minutes) with neutralization buffer (90 mM Tris, 90 mM Borate, 2 mM EDTA, pH 7.5). Finally, slides were immersed in ethanol and allowed to dry under air. All steps described were performed in the dark/dimmed light to prevent additional DNA damage. Dried slides were stained with ethidium bromide (10 mg/ml) and comets were visualized using a Zeiss Axioskop fluorescence microscope. Samples were tested in two independent incubations within each single experiment. On every single slide, 50 cells were analyzed randomly and the comet tail moment (a product of the DNA fraction in the tail and the tail length) was measured. The mean value of the tail moment in a particular sample was taken as an index of DNA damage in this sample.

3.5 Isolation of DNA and measurement of 8-oxodG by HPLC-ECD

After incubation, cells were harvested by trypsination and lysed for 2 hours at 37°C in SET-SDS-Prot K solution (75 mM NaCl, 25 mM EDTA, 0,5 mg/ml proteinase K, 1% SDS), followed by incubation with 0,1 mg/ml RNAse-A and 100 U/ml RNAse T for 1 hour at 37°C. DNA was extracted using a standard phenol-chloroform method. The DNA was then dissolved in 2 mM Tris-HCl (pH 7.4) at a final concentration of 0.5 mg/ml. Oxidative DNA damage was measured using HPLC-ECD, as described previously (Knaapen, Seiler et al. 1999), and was expressed as the ratio of 8-OHdG to deoxyguanosine (dG).

3.6 Quantitative real time PCR

Quantitative real time PCR was applied to assess the effects of HOCl on the expression of DNA damage response genes. A549 cells were seeded in 28 cm^2 dishes and treated with HOCl. After 15 minutes, HOCl was removed and cells were further incubated in DMEM for 0 to 4 hours. After incubation, A549 cells were lysed in 1 ml Trizol® (Gibco Invitrogen, Breda, the Netherlands). Total RNA was isolated using phenol-chloroform method. The RNA yield was quantitated by UV spectrometry and then first strand cDNA was generated from 0,5 µg of total RNA using iScript™ cDNA synthesis kit (BIO-RAD, Veenendaal, the Netherlands). cDNA was used as the template for real time PCR to analysis mRNA expression of following genes: p21 and Gadd45. Real time PCR was performed with a MyiQ Single Colour real time PCR detection system (BIO-RAD, Veenendaal, the Netherlands) using SYBR© Green Supermix (BIO-RAD), 5 µl diluted cDNA and 0,3 µM primers (Table 1) in a total volume of 25 µl. PCR was conducted in the following condition: denaturation at 95°C for 3 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60°C for 45 seconds. After PCR, a melt curve temperature (60-95°C) was produced for product identification and purity. PCR efficiency of all primer sets, as assessed by the use of cDNA

dilution curves, was approximately 100%. Data were analyzed using the MyiQ Software System (BIO-RAD) and were expressed as relative gene expression (fold increase), using the $2^{-\Delta\Delta Ct}$ method and employing β-actin as house keeping genes (Livak and Schmittgen 2001).

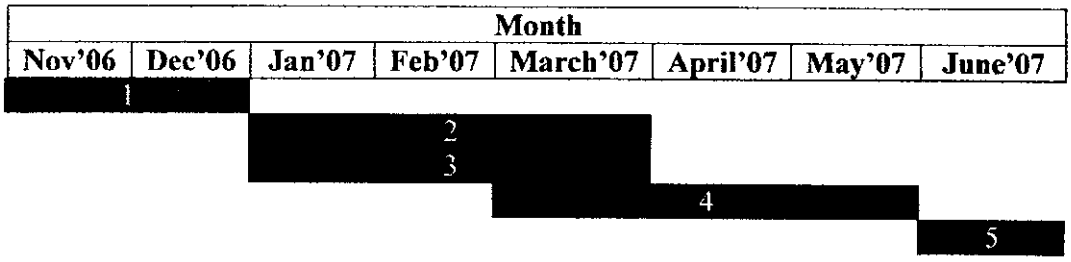
Table 1. Primer sequences for quantitative real time PCR

Gene	Sequence 5'→3'
β -actin	Forward primer: CCTGGCACCCAGCACAAT
	Reverse primer: GCCGATCCACACGGAGTACT
p21	Forward primer: GCAGACCAGCATGACAGATTTC
	Reverse primer: GCGGATTAGGGCTTCCTCTT
Gadd45	Forward primer: CGACCTGCAGTTTGCAATATGA
	Reverse primer: CCCCCACCTTATCCATCCTT

3.7 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM) from 2 independent experiment, unless stated otherwise. Statistical analysis was performed using SPSS version 11.5 for Windows. Differences between experimental groups were analyzed using one-way ANOVA followed by multiple comparison evaluations using Dunnett's method to test differences between treatments and control. Differences were considered statistically significant at $p < 0.05$.

TIME LINE



1. Culture A549 human alveolar epithelial cells
2. Measuring both DNA single strand breaks
3. Measuring 8-OHdG DNA lesions
4. Measuring the expression p21 and Gadd45 using quantitative real time PCR
5. Writting report