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# Evaluation of in vitro toxic effects of cement dusts: A preliminary study

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## Abstract

Cement is widely used for construction and several reports have suggested a potential toxicity of cement dusts although it has never been definitively assessed. To determine the cytotoxic and bioactive effects of cement dusts, cultures of normal rat fibroblasts were exposed to different types of cements and cell growth parameters, apoptosis and the occurrence of DNA damage (both in terms of DNA breaks and oxidative damage) were analyzed. Cells were exposed to cement extracts or cultured in direct contact with cement dusts and the results obtained were compared to cells cultured in fresh medium. A dose-dependent decrease in viable cells was observed with all tested cements. Different results were obtained in the cell-cement direct contact tests compared to the indirect contact tests performed using extracts. Inhibition of cell growth was associated in most cases with an accumulation of cells in the S-phase of the cell-cycle and the appearance of an apoptotic peak. DNA strand breaks, assessed by comet test, and increase in the levels of 8-OHdG, an important marker of DNA oxidative damage, always occurred by incubating cells in the presence of cement extracts or dusts. However, after removal of cement, a rapid damage repair was generally observed with an almost complete recovery within 12 hours. In conclusion, all cements analyzed in this study displayed a limited toxicity in vitro without significant differences amongst them. Overall, the results obtained indicate that cements should be treated as hazardous materials but they do not allow to make accurate predictions regarding the in vivo effects. Further studies are warranted to reach a better understanding of the potential toxic effects of cements, to identify the responsible mechanisms and to evaluate the possibility of modulating and/or preventing them.

## Keywords

cement, toxicity, oxidative damage, rat fibroblasts

## Introduction

Cement is one of the most widely used construction materials. The most commonly used cement contains mainly calcium silicate with aluminium and iron compounds together with a small amount of gypsum. However, a variety of additives, such as alkaline hardeners, may be used to produce special purpose cements. For example, Portland cement is composed of lime, alumina, silica and iron oxide as tetra calcium alumino ferrate ($4CaO{\cdot}Al_{2O^3}.Fe^{2O}{}_3$), tricalcium aluminate ($3CaO{\cdot}Al_{2O^3}$), tricalcium silicate ($3CaO{\cdot}SiO_2$) and dicalcium silicate ($2CaO{\cdot}SiO_2$). Small amounts of magnesia (MgO), Na, K and S are also present

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(Neghab and Choobineh, 2007). The other cements generally present an even more complex composition. Cement plant workers are exposed to fine cement dust (0.05 to 5.0 μm aerodynamic diameter; Kalacić, 1973), which is produced by heating ground cement rock or other limestone-bearing materials into a fused clinker that is then ground into a fine powder (Rom, 1998). The harmful effects of cement dust upon living organisms are due to the irritating, sensitizing and pneumoconiotic properties of its components. In animal studies, it has been observed that cement dust induces atrophic and hypertrophic changes in nasal and pharyngeal mucosa and chronic exfoliative bronchitis (Maciejewska and Bielichowska-Cybula, 1991). The examination of workers exposed to cement dust has suggested a higher prevalence of chronic respiratory symptoms and the reduction of ventilatory capacity (Yang et al., 1996) as well as other disorders of the upper respiratory airways such as chronic rhinitis, laryngitis and pharynx catarrh (Maciejewska and Bielichowska-Cybula, 1991) and dyspepsia (Coppeta et al., 2008). It has been demonstrated that adverse respiratory health effects (increased frequency of respiratory symptoms and decreased ventilatory function) observed among cement workers cannot be explained by age, body mass index (BMI) and smoking (Al-Neaimi et al., 2001). Moreover, exposure to cement dust may increase the risk of lung and bladder cancer and a dose-related risk for stomach cancer has also been reported (Smailyte et al., 2004).

To date, most of the evidence about toxicity of cement dust derives from clinical studies on cement plant workers while limited information is available on the underlying mechanisms. In this study, the potential toxic effects of cements were assessed using a series of in vitro assays that are simple, reproducible, cost-effective, relevant and suitable for evaluation of basic biological aspects relating to biocompatibility (Chang et al., 1998). To determine the cytotoxic and bioactive effects of cement, we measured the viability, apoptotic and proliferation properties, fragmentation and oxidative damage of DNA in a culture system of normal fibroblasts exposed to cement dusts or their extracts.

## Materials and methods

All reagents were purchased from Sigma and were of the highest grade. All experiments were performed according to Good Laboratory Practice regulations and were repeated at least three times in triplicate. Data shown are mean ± standard deviationn (SD).

### Cell culture

The RAT-1 rat immortalized fibroblasts were obtained from American Type Tissue Culture Collection and were cultured in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

### Cement dusts and extracts preparation

Cement dusts were provided by the Italcementi SpA, Italy. Two different approaches were used to test cements toxicity: (i) direct contact, in which cells were cultured with cement dusts to mimic in vitro the close contact occurring in vivo; (ii) indirect contact, in which cells were exposed to cement extracts, in order to study the potential toxicity due to soluble components released by cement in the surrounding biological fluids. In the direct contact experiments, cement dusts were suspended directly in MEM and then sonicated for 5 min. Extracts were prepared following the international standard for medical device ISO 10993-4:2002 (ISO-10993-12:1996(E), 1996; ISO-10993-5:1999, 1999). Briefly, cement dusts were dissolved at a concentration of 0.2 g/ml in MEM and maintained at 37°C for 72 hours in agitation. At this time, culture media were centrifuged and filtered before adding them to cell cultures.

### Citoxicity and cell proliferation assays

Citotoxicity of cement dusts was evaluated using the MTT assay as an indicator of the metabolic competence of the cells. Briefly, $2 \times 10^4$ cells/well were seeded in 24-well culture plates, grown for a further 24 hours and then incubated in medium containing increasing amount of cement dusts (10, 25, 50, 100 and 1000 μg/cm$^2$) or extracts (0, 10, 20, 40, 60, 80 and 100%). At the end of the incubation (72 hours), the medium was removed and cultures were incubated with medium containing 1 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma) for 2 hours at 37°C. The medium was then discarded and 500 μL acid-isopropanol (0.04 N HCl in isopropanol) was added to each well to stop the cleavage of the tetrazolium ring by dehydrogenase enzymes that convert MTT to an insoluble purple formazan in living cells.

Plates were then kept in agitation at room temperature for about 15–20 min and the level of the coloured formazan derivative was determined on a multiscan reader at a wavelength of 540 nm (reference wavelength 630 nm). Data are expressed as the percentage of surviving cells in treated cultures compared to control ones incubated with medium exposed to extracting conditions and represent the mean ± SD of three independent experiments each one performed in triplicate.

For cell proliferation assays, cells were plated at a density of $1 \times 10^4$ cells/well in 6-well culture plates. After 24 hours, medium was changed and cultures were exposed to cement dusts or extracts. The number of cells per well was determined every day using a Coulter counter (Beckman Coulter, Fullerton, California, USA) and medium was changed every 2 days. Results of the cell proliferation assay are expressed as percentage of surviving cells in treated cultures compared to untreated controls. All experiments were performed in triplicate and were repeated three times.

## Flow cytometry and apoptosis detection

For cell cycle analysis, exponentially growing cells were treated as previously described and were then collected, washed with phosphate-buffered saline (PBS), fixed in 5 mL of 70% ethanol and stored at 4°C. For the analysis, cells were collected by centrifugation and the pellets were resuspended in 0.2 mg/mL of propidium iodide (PI) in Hank's balanced salt solution containing 0.6% NP-40 and RNase (1 mg/mL). The cell suspension was then filtered and analyzed for DNA content on a Coulter EPICS 753 flow cytometer, as previously described (Sgambato et al., 2001). The percentage of cells in different phases of the cell cycle and in the sub-G1 (subdiploid DNA content) peak were determined using a ModFit 5.2 computer program. The assays were repeated at least three times and gave similar results. The data reported are the results of a typical experiment.

Apoptosis was detected using the annexin V-FITC kit (Oncogene Research, Cambridge, Massachusetts, USA), following the manufacturer's instructions. Briefly, cell pellets were resuspended in ice-cold binding buffer and annexin V-FITC and PI solution were added. After 15 min incubation in the dark, the cell suspension was filtered and analyzed by flow cytometry. The results shown are the mean of three replicate experiments. SD was <20% for all tested conditions.

## Comet assay

Single cell gel electrophoresis or Comet assay was performed as previously reported (Sgambato et al., 2001). Briefly, after exposure to cements extracts or dusts, cells were collected, resuspended in low-melting agarose ($1.0 \times 10^4$ cells/100 μL of 0.5% low-melting agarose in PBS) and immediately pipetted onto agarose-coated slides (1.5% in PBS containing 5 mM ethylenediaminetetraacetic acid [EDTA]). Cells were then covered with a layer of agarose (0.5% in PBS) and allowed to solidify briefly. The slides were immersed in ice-cold lysing solution (2.5M NaCl, 100 mM EDTA, 10 mM Tris, 1% Sarkosyl, 10% dimethyl sulfoxide and 1% Triton X-100 (pH 10.0)) for 60 min at 4°C. They were then placed on an electrophoretic tray with an alkaline buffer (0.3 N NaOH, 1 mM EDTA) and allowed to equilibrate for 20 min at room temperature before the electrophoresis performed at 300 mA for 20 min in the same buffer. The slides were then washed, stained for 5 min with 2 mg/mL ethydium bromide (EB) and analyzed with a fluorescence microscope Eclipse E600 (Nikon Corporation, Tokyo, Japan). Images were acquired with a camera coupled with a computer and were analyzed using the software Image-Pro Plus 4.1 (Media Cybernetics, Silver Spring, Maryland, USA). This was also used to determine the relationship between migration of nuclear genetic material, or 'head' of the comet, and the resulting 'tail.' DNA damage is expressed as the 'tail moment,' which takes into consideration both the quantity of DNA present in the tail and the extent of migration of genetic material (length of the tail). In each experiment, at least 50 randomly selected cells were evaluated and results are expressed as mean ± SD of three independent experiments.

## Immunoperoxidase detection of 8-OHdG

Immunohistochemical detection of 8-OHdG was performed as previously described (Sgambato et al., 2004). Cells were grown directly on culture chamber-slides (Nalge Nunc International, Naperville, Illinois, USA) at a density of $1 \times 10^4$ cells/well and, after exposure to cements extracts or dusts, were processed as previously reported. Briefly, after fixation cells were treated with 100 μg/mL RNAase for 1 hour at 37°C followed by 20 μg/mL proteinase K for 10 min at room temperature in order to denaturate DNA and render it accessible to the antibodies. Slides were then treated with HCL 4N and 50 mM Trizma base for 5 min

at room temperature. After washing with PBS, slides were incubated with 0.3% $H_2O_2$ in methyl alcohol at room temperature for 30 min to quench endogenous peroxidase activity. Non-specific binding was blocked with 1.5% normal horse serum and incubation with monoclonal antibody 1F7 specific for 8-OHdG (kindly supplied by Prof. R Santella, Columbia School of Public Health, New York, USA) diluted 1:50 in horse serum was performed overnight at +4°C. Slides were then incubated with biotinylated horse anti-mouse secondary antiserum, and the reaction was visualized with the Avidin-Biotin (ABC Vectastain) enzymatic complex for 30 min at 37°C followed by diaminobenzidine (DAB). Semi-quantitative evaluation of the staining was carried out by an optical microscope (ECLIPSE E600, Nikon, at 400×) and evaluated using the Image Pro-Plus version 4.1 software (Media Cybernetics). Nuclear staining was evaluated in approximately 100 cells of randomly chosen images by operators who were blind to cell treatment. Negative and positive controls (untreated and 1.0 mM $H_2O_2$-treated cells, respectively) were included within each batch of slides. The image was obtained in black and white and the average optical density was recorded. The mean value for negative samples was 52.8 ± 22.5 (Mean ± SD) and positive samples always displayed a significantly higher staining (379 ± 147). Since we observed a significant variability in the values obtained for both negative and positive samples among each batch, the results obtained with cement-exposed cultures were standardized to controls and stratified into three categories in terms of staining intensity: 1 = weak, when staining was higher, but less than two times, than negative control; 2 = moderate, when staining was higher than 2× negative control but less than 50% of the value of the positive control; 3 = high, when staining was higher than 50% of the value of the positive control.

## Results

### *Cements inhibit cell growth and proliferation of rat fibroblasts*

In a first feasibility study, we aimed to determine the potential toxicity of cement dusts and to calculate the concentration inhibiting the growth of Rat-1 normal diploid rodent fibroblasts by 50% (IC50). Cytotoxicity assays were carried out by use of the MTT test. Exponentially growing cultures were exposed to increasing cement dust (10-1000 μg/cm$^2$) or extract (10%–100%) and cell viability was assessed after 72 hours. A dose-dependent decrease in viable cells was observed with all tested cements with an $IC_{50}$ ranging between 71 and 559 μg/cm$^2$ in the direct-contact tests and 21% and 54% of extract in the indirect contact tests.

**Table 1.** $IC_{50}$ of cement dusts or extracts in rat-1 cells

| Cement types | Direct contact[a] (μg/cm$^2$) | Indirect contact[a] (% of extract) |
|---|---|---|
| Clinker | 209.5 | 53.5 |
| Portland | 166.6 | 20.8 |
| M.C.12.5 Muracem | 151.0 | 39.0 |
| TX Active white | 103.8 | 33.4 |
| Plastocem grinded | 339.3 | 40.3 |
| I 52.5 R | 87.0 | 41.4 |
| II ALL 32.5 R | 107.5 | 45.9 |
| II ALL 42.5 R | 100.7 | 49.9 |
| II/ALL 32.5 Grinded | 186.7 | 54.4 |
| II/ALL 42.5 R Grinded | 192.3 | 34.2 |
| III/ALL 32.5 R Grinded | 285.4 | 53.8 |
| III/ALL 42.5 R Grinded | 559.3 | 41.8 |
| IV/A(P) 32.5 R | 71.2 | 40.1 |
| IV/A(P) 42.5 R | 274.2 | 39.8 |
| IV/B(P) 32.5 R | 264.7 | 42.0 |

[a] Concentration of cement dusts (direct contact) or extracts (indirect contact) inhibiting cell growth by 50% after 72 hours.

It is noteworthy that significant differences were observed between the direct and indirect contact. In fact, in the cell-cement direct contact tests, none of the cements appeared able to kill all the cells even at the highest concentration (1 mg/cm$^2$) tested and Portland cement (one of the most widely used cements) displayed an intermediate toxicity ($IC_{50}$ of 167 μg/cm$^2$) compared to other cements. On the other hand, in the indirect contact tests performed using extracts, Portland Cement appeared to be more toxic than all the other cements at the tested concentrations. Indeed, extracts of Portland Cement caused a striking inhibition of cell survival with an $IC_{50}$ of 21% and no cells surviving at concentrations ≥40%. All the other cements also caused a dramatic reduction of cell growth at concentrations ≥40% and death of the culture at the highest concentrations (80%–100%).

Interestingly, for the cements for which both the basal and grinded forms were available a different toxicity was observed between the two forms and, generally, a higher toxicity was observed with the grinded forms (Table 1).

To further investigate the effects of cements on cell proliferation, we then evaluated changes in cell

**Table 2.** Effects of cement dusts on cell cycle parameters

| Cement types | G0/G1 | S | G2/M | Apoptosis |
|---|---|---|---|---|
| None (control) | 70.1 | 16.3 | 13.6 | 1.9 |
| Clinker | 75.8 | 12.4 | 11.8 | 1.7 |
| Portland | 43.2 | 37.3 | 19.5 | 9.8 |
| M.C.12.5 Muracem | 67.1 | 16.9 | 16.0 | 8.9 |
| TX Active white | 56.5 | 23.4 | 20.1 | 5.1 |
| Plastocem Grinded | 42.2 | 41.4 | 16.4 | 9.3 |
| I 52.5 R | 49.8 | 32.3 | 17.9 | 15.4 |
| II ALL 32.5 R | 59.6 | 20.6 | 19.8 | 12.7 |
| II ALL 42.5 R | 42.9 | 35.7 | 21.4 | 16.6 |
| II/ALL 32.5 Grinded | 43.6 | 35.5 | 20.9 | 17.5 |
| II/ALL 42.5 R Grinded | 39.8 | 39.9 | 20.3 | 20.7 |
| III/ALL 32.5 R Grinded | 52.2 | 29.3 | 18.5 | 7.1 |
| III/ALL 42.5 R Grinded | 45.7 | 35.7 | 18.6 | 5.5 |
| IV/A(P) 32.5 R | 51.0 | 29.2 | 19.8 | 6.1 |
| IV/A(P) 42.5 R | 47.6 | 30.6 | 21.8 | 15.1 |
| IV/B(P) 32.5 R | 46.4 | 31.9 | 21.7 | 11.6 |

cycle distribution and in cell growth of cells exposed to cement extracts or directly to dusts. The concentration of 25 and 50 μg/cm$^2$ and 10% and 20% extracts were selected for the direct-contact and the indirect-contact tests, respectively, since no significant toxicity was observed for any of the cements at these concentrations.

Exponentially growing cultures of Rat-1 cells were cultured in the presence of the indicated concentrations and the number of cells per well was determined every day. In the cell-cement direct contact texts, all cements inhibited cell growth at the highest concentration. Only clinker did not display any significant effect on cell proliferation while Portland displayed an intermediate behaviour compared to all the other cements (data not shown).

In the indirect-contact tests, all the extracts displayed a substantial effect on cell growth with exception of the TX Active White. Portland appeared to be the most toxic, especially at the highest concentration of extracts tested (data not shown). For cell cycle analysis, exponentially growing cultures of Rat-1 fibroblasts were exposed to 20% of extracts or to 25 μg/cm$^2$ of cement dusts and the distribution of cells in the different phases of the cell cycle was determined after 72 hours in both treated and parallel untreated cultures by flow cytometry analysis analysis. As shown in Table 2, in the direct contact tests all the cements but Clinker and the M.C. 12.5 Muracem induced a marked increase in the percentage of cells in the S phase of the cell cycle, which increased from a value of about 16% in the control up to 41% with the Plastocem grinded. In parallel, the percentage of cells in the G0/G1 phase of the cell cycle decreased from about 70% in the control to about 42% with the Cem. Plastocem grinded and with II/ALL 32.5 grinded and III/ALL 42.5 (both basal and grinded).

Different results were obtained with cement extracts (Table 3), which induced a lower reduction (around 10%) in the percentage of cells in the G1/G0 phase of the cell cycle compared to control cultures. The reduction of cells in the G1/G0 phase was mainly associated with an increase of cells in the S phase of the cell cycle, with a slight increase of cells in the G2/M phase. However, three types of cements (Clinker, M.C.12.5 Muracem and TX Active White) did not induce any variation in the percentage of cells in the different phases of the cell cycle compared to control cells. The most striking effects were observed with Portland, Plastocem grinded and Cem IV/A(P) 42.5 R, which caused a striking reduction of the G0/G1 phase of the cell cycle with a parallel increase of cells in the S and G2/M phases.

To further evaluate the effects of cements on cell viability, the occurrence of apoptosis was evaluated by staining the cells with annexin which specifically binds to phosphatidylserine exposed on the surface of apoptotic cells (Martin et al., 1995). As shown in Tables 2 and 3, about 2.0% of apoptotic cells were detectable in control-untreated cells and all extracts and dusts induced an increase in the percentage of apoptotic cells whose highest value was detected in cultures exposed to Cem. II/ALL 42.5 R Grinded extract (12%) or dust (20%).

**Table 3.** Effects of cement extracts on cell cycle parameters

| Cement types | G0/G1 | S | G2/M | Apoptosis |
|---|---|---|---|---|
| None (control) | 70.1 | 16.3 | 13.6 | 1.9 |
| Clinker | 71.5 | 18.2 | 10.3 | 4.5 |
| Portland | 52.1 | 30.4 | 17.5 | 7.5 |
| M.C.12.5 Muracem | 69.8 | 15.6 | 14.6 | 7.7 |
| TX Active white | 70.7 | 15.0 | 14.3 | 5.4 |
| Plastocem Grinded | 51.2 | 22.2 | 26.6 | 7.7 |
| I 52.5 R | 57.1 | 27.8 | 15.1 | 7.3 |
| II ALL 32.5 R | 65.5 | 21.1 | 13.4 | 7.8 |
| II ALL 42.5 R | 62.1 | 22.5 | 15.4 | 9.2 |
| II/ALL 32.5 Grinded | 63.5 | 19.8 | 16.7 | 9.4 |
| II/ALL 42.5 R Grinded | 60.4 | 21.3 | 18.3 | 12.0 |
| III/ALL 32.5 R Grinded | 65.1 | 19.4 | 15.5 | 7.0 |
| III/ALL 42.5 R Grinded | 64.9 | 20.8 | 14.3 | 6.5 |
| IV/A(P) 32.5 R | 69.0 | 16.3 | 14.7 | 8.3 |
| IV/A(P) 42.5 R | 53.1 | 24.9 | 22.0 | 11.4 |
| IV/B(P) 32.5 R | 63.7 | 19.8 | 16.5 | 9.0 |

As for the extracts, all cement dusts induced an increase in the percentage of apoptotic cells and the strongest effect was observed with Cem. II/ALL 42.5 R Grinded.

## *Cements induce both DNA strand breaks and oxidative damage*

The occurrence of DNA single strand breaks was evaluated by Comet test in cell cultures treated with 20% of extracts or with 25 μg/cm$^2$ of cement dusts for 1 hour or 12 hours. Tail moment was always increased by incubating cells in the presence of cement extracts or dust. In the direct contact test, the highest damage was induced by cement Plastocem grinded, followed by II/A LL 42.5 R grinded, II/ ALL 32.5 R grinded, Cem III/A LL 42.5 R grinded and III/ ALL 32.5 R grinded. It is noteworthy that the highest mean value of tail moment obtained with Plastocem was 163, which is about 15% higher than the value obtained with Portland (mean value 142). No further increase in DNA damage was detected after 12 hours incubation when most of the values were reduced remaining higher than Portland only for Cem. Plastocem, II/A LL 42.5 R grinded, II/ ALL 32.5 R grinded, Cem III/A LL 42.5 R grinded and Cem III/ ALL 32.5 R grinded.

DNA damage also increased in the indirect contact tests. The strongest evidence for DNA damage was observed in cells exposed to Cem MC 12.5 Muracem, Plastocem, I 52.5R, II/ALL 32.5 R grinded and II/ALL 42.5 R grinded. However, the tail moment was always lower than in the direct contact test and after 12-hour treatment, the typical comet only remained evident in Cem MC 12.5 Muracem and I 52.5R (mean value 137), as shown in Table 4.

Immunohistochemical detection of 8-OHdG was performed as previously described (Sgambato et al., 2004) using a specific anti-8-OHdG monoclonal antibody. Staining intensity was highly heterogeneous in exposed cells

In the direct contact tests, only a slight oxidative DNA damage was observed after short term (1 hour) exposure, which increased in some of the cements after longer exposure (12 hours), but remaining always lower than the positive control. Thus, with Clinker, Cem II ALL 32.5 R, II ALL 42.5 R and Cem IV/A(P) 32.5 R DNA, a slight increase in staining was only observed at 12 hours but not after 1 hour exposure. Portland and Cem I 52.5 did not induce DNA damage at any of the time points tested. On the contrary, with other cements, such as TX Active white, M.C.12.5 Muracem, Plastocem and Cem IV/B(P) 32.5 R, a weak staining was observed after 1 hour but disappeared at later time point.

In the indirect contact tests, a positive staining was observed with all cements after 1 hour exposure, with the strongest staining being detected in cells exposed to TX Active white and M.C.12.5 Muracem. In no case an increase of the staining was observed at 12 hours compared to 1-hour exposure (Table 5).

**Table 4.** Evaluation of DNA strand breaks induced by cements on rat fibroblasts

| | Tail moment Mean ± SD | | | |
|---|---|---|---|---|
| Control | 100 | | | |
| Positive control | 590 ± 112 | | | |
| Cement Types | Direct contact | | Indirect contact | |
| | 1 hours | 12 hours | 1 hours | 12 hours |
| Clinker | 128 ± 47 | 102 ± 32 | 120 ± 25 | 120 ± 18 |
| Portland | 142 ± 29 | 127 ± 12 | 127 ± 25 | 117 ± 19 |
| M.C.12.5 Muracem | 139 ± 38 | 114 ± 19 | 144 ± 35 | 137 ± 29 |
| TX Active white | 152 ± 25 | 113 ± 18 | 120 ± 27 | 118 ± 22 |
| Plastocem Grinded | 163 ± 35 | 166 ± 39 | 166 ± 36 | 129 ± 49 |
| I 52.5 R | 115 ± 17 | 115 ± 19 | 151 ± 31 | 137 ± 32 |
| II ALL 32.5 R | 117 ± 12 | 116 ± 14 | 116 ± 14 | 117 ± 18 |
| II ALL 42.5 R | 128 ± 17 | 125 ± 22 | 115 ± 14 | 119 ± 22 |
| II/ALL 32.5 Grinded | 158 ± 37 | 156 ± 44 | 156 ± 37 | 116 ± 24 |
| II/ALL 42.5 R Grinded | 162 ± 38 | 158 ± 36 | 158 ± 33 | 126 ± 22 |
| III/ALL 32.5 R Grinded | 132 ± 12 | 146 ± 39 | 120 ± 15 | 116 ± 17 |
| III/ALL 42.5 R Grinded | 152 ± 22 | 154 ± 36 | 124 ± 23 | 126 ± 16 |
| IV/A(P) 32.5 R | 119 ± 21 | 119 ± 18 | 119 ± 17 | 118 ± 24 |
| IV/A(P) 42.5 R | 123 ± 19 | 117 ± 13 | 117 ± 21 | 118 ± 23 |
| IV/B(P) 32.5 R | 119 ± 17 | 118 ± 19 | 117 ± 24 | 118 ± 19 |

**Table 5.** Evaluation of oxidative DNA damage induced by cements on rat fibroblasts (nuclear staining intensity)[a]

| | | | | |
|---|---|---|---|---|
| Control | 0* | | | |
| Positive control | 3 | | | |
| Cement types | Direct contact | | Indirect contact | |
| | 1 hour | 12 hour | 1 hour | 12 hour |
| Clinker | 0 | 1 | 1 | 0 |
| Portland | 0 | 0 | 1 | 1 |
| M.C.12.5 Muracem | 1 | 0 | 3 | 1 |
| TX Active white | 1 | 0 | 3 | 1 |
| Plastocem Grinded | 1 | 0 | 1 | 1 |
| I 52.5 R | 0 | 0 | 2 | 2 |
| II ALL 32.5 R | 0 | 1 | 1 | 1 |
| II ALL 42.5 R | 0 | 1 | 1 | 1 |
| II/ALL 32.5 Grinded | 1 | 1 | 2 | 1 |
| II/ALL 42.5 R Grinded | 1 | 1 | 1 | 1 |
| III/ALL 32.5 R Grinded | 1 | 1 | 1 | 1 |
| III/ALL 42.5 R Grinded | 1 | 2 | 1 | 1 |
| IV/A(P) 32.5 R | 0 | 1 | 1 | 1 |
| IV/A(P) 42.5 R | 1 | 1 | 1 | 1 |
| IV/B(P) 32.5 R | 1 | 0 | 1 | 1 |

[a] Numbers indicate staining intensity: 0 = absent; 1 = weak; 2 = moderate; 3 = strong (see text for details).

## Discussion

In this study, the potential toxic effects of different types of cements were evaluated on rat fibroblasts. In fact, although several epidemiological evidence suggest a potential toxicity of cement dusts, no data are available on the underlying molecular mechanisms (Al-Neaimi et al., 2001; Coppeta et al., 2008; Kalacić, 1973; Neghab and Choobineh, 2007; Smailyte et al., 2004; Yang et al., 1996). Moreover, most of the available data mainly relate to respiratory

or gastrointestinal disorders (Al-Neaimi et al., 2001; Coppeta et al., 2008; Neghab and Choobineh, 2007; Yang et al., 1996) and limited data are available on the potential long-term effects of cement exposure, such as in terms of increased risk of cancer as well as other diseases (Maciejewska and Bielichowska-Cybula, 1991; Rom, 1998; Smailyte et al., 2004). In vitro cell systems are an excellent model for studying molecular mechanisms of toxicity since they allow to obtain rapid and reliable results that can be easily verified without using laboratory animals. Rat diploid immortalized fibroblasts are widely used as cell model for toxicological assays since rat and human cells show a good metabolic similarity and results obtained with rat cells are usually confirmed in humans. We found that cements can display a variety of effects both in terms of cells viability and proliferation and of DNA damage. However, these effects were limited and significantly lower than the ones observed with the positive control. Moreover, the results of the DNA damage assays demonstrate that the type(s) of DNA damage induced can be repaired by the cells, thus confirming the limited long-term toxicity of cement exposure, in accordance with epidemiological data.

Exposure to cements dusts or extracts inhibited cell growth in a time- and dose-dependent fashion, with a highly variable $IC_{50}$ (Table 1). Inhibition of cell growth was accompanied in most cases by reduction of the percentage of cells in the G0/G1 phase of the cell cycle and an accumulation of cells in the S-phase (Tables 1 and 2). These findings suggest a potential toxic effect probably linked to an inhibition of the DNA synthesis process or to an inhibition of the S-to-G2/M transition due to a block in cell division activities such as chromosome condensation or spindle formation. The appearance of an apoptotic peak confirms the presence of a toxic effect able to trigger cell death by apoptosis, even if this effect is not pronounced and the induction of apoptosis cannot fully account for the inhibitory effect observed on cell growth that likely involves a cytostatic-type mechanism accompanied by a block in cell proliferation (Tables 2 and 3).

To our knowledge, this is the first study investigating the potential toxic effects of a variety of cement types and comparing them to the effects of Portland, which is the most widely used cement. Our findings show that most cements have effects comparable to that obtained with Portland, which is widely used and well-known for its harmless effects on human health. However, an interesting finding of this study was that different results can be obtained by using the classical cell-cement direct contact tests or indirect-contact tests using extracts. For example, while Portland appeared to be one of the less toxic compounds in the direct contact tests, it was the most toxic in the indirect contact test. These findings might be relevant in vivo since toxic substances released by cement particles in biological fluids might in theory be adsorbed and exert their potential toxic effect at a distance from the sites of accumulation. Thus, the use of both types of approaches is warranted for a better definition of the potential toxic effects of cements.

The results of the present study do not provide any clue on the potential molecular targets of the observed toxic effects. DNA can definitely be considered a potential direct molecular target of cements. In fact, most of them caused a significant accumulation of cells in the S-phase of the cell cycle associated with an increase in the levels of 8-OHdG, an important marker of DNA oxidative damage and in the extent of DNA strand breaks as assessed by comet test (Table 2). However, damage was subsequently rapidly reduced and almost disappeared within 12 hours treatment with most cements.

It is difficult to interpret the biological significance of the DNA damage that can be detected by comet test. However, it is of interest that the extent of DNA fragmentation declined after the initial increase and almost disappeared within 12 hours for most cements, thus suggesting a promptly reversible damage. However, we cannot entirely rule out the possibility that the most damaged or sensitive cells die and/or are lost during preparation of the comet test although we tend to exclude this hypothesis since we did not observe a significant increase in the amount of dead cells in exposed compared to control cultures (data not shown). Further studies with time- and dose-dependent curves are needed, however, to investigate these issues. We hypothesize that the early appearance of these effects on the DNA might indicate that DNA is one of the first molecular targets of the toxic effect of cements and that DNA damage itself might trigger the observed effects in terms of cell-cycle arrest and induction of apoptosis.

In conclusion, this study demonstrates that cements can induce DNA damage, which might represent an important mediator of cement-associated toxic effects. However, it seems that cells can repair this type of damage, so that long-term effects, such as cell transformation, might be unlikely. Nonetheless, although our findings do not enable us to make

accurate predictions regarding the in vivo effects of the exposure, they indicate that prolonged exposure, even to low doses, could have biological effects whose in vivo significance remain to be defined. Further studies are warranted to reach a better understanding of the potential toxic effects of cement exposure and to identify the responsible mechanisms and to evaluate the possibility of modulating and/or preventing them.

## Conflict of Interest

Antonio Bergamaschi, is the Chief Occupational Physician for the Italcementi Group.

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