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ULTRA HIGH MOLECULAR WEIGHT POLYETHYLENE IS CYTOTOXIC AND CAUSES OXIDATIVE STRESS, EVEN WHEN MODIFIED.

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Ultra High Molecular Weight (UHMW) Polyethylene (PE) is the most used biomaterial for articulating surface of total joint replacements (TJR). Since its introduction decades ago, many efforts have been made to improve its properties and to understand its behaviour with the final goal to provide a longer duration of the implants. Mechanical abrasion with particles production is an unavoidable process, but catastrophic PE wear with a huge quantity of PE reactive debris production was observed in the last years, with consequent severe periprosthetic osteolysis and aseptic loosenings. Now it is well known that the severe PE particle disease is due to UHMWPE oxidative degradation caused by a γ-sterilization in air (1-4). In order to solve this problem, the evolution of the conventional UHMWPE drove to alternative sterilization methods (ethylene oxide and gas plasma) and to the development of new PE derivatives with a supposed increased resistance to abrasion in vivo, such as the crosslinked PE (X-PE) and the vitamin E-stabilized PE (E-PE), Unfortunately, particle disease and periprosthetic osteolysis still remain an unsolved problem (5-9). The osteolysis is an inflammatory process caused by a foreign body (10), wherein the particles trigger monocyte recruitment and macrophage activation, leading to chronic inflammation, with release of cytokines and proteases, and bone reabsorption (8). It is well recognized that PE particles induce different reactions according to dimension (fragments larger than 10 μm produce foreign body-induced giant cell granuloma, whereas smaller fragments are phagocytized), number, shape, superficial hydrophobicity and oxidation (11-14). The majority of the studies have been focused on how conventional and new PEs develop debris particles different in sizes and behaviours, reacting in different ways with human cells and conducting to unpredictable clinical results (7, 15-17). But beside the shape and the dimension of a PE particle, the chemico-physical properties of its surface might play a fundamental role in stimulating the inflammatory reaction, the cytotoxicity and the apoptosis. In particular, it has been suggested that PE particles *per se* do not cause any response although macrophages are activated, probably due to the superficial characteristics of PE and macrophage binding receptors.

Different approaches have shown that some markers of inflammation and oxidative stress were induced after exposure to UHMWPE, in particular to their debris: among them, lactate dehydrogenase (LDH) release and lipid peroxidation (19) and cytokines production (20) have been observed. The mechanisms by which these events occur are not still well elucidated. Some authors demonstrated that in particular the oxidized UHMWPE is toxic in different cellular models: Renò et al. (21) showed that oxidized UHMWPE reduced proliferation in human fibroblasts and induced a strong release of gelatinase B (matrix metalloproteinase 2, MMP-2), and the surface oxidation of UHMWPE has been correlated to increased apoptosis and necrosis in human granulocytes (22). Moreover, the oxidation of UHMWPE has been strongly related to the development of inflammation around the implants (23) and Fiorito et al. (24) demonstrated that, in this inflammatory status, the production of reactive oxygen species (ROS) by inflamed synovial cells plays a very crucial role.

The supplementation of X-PE and E-PE has been shown

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to improve significantly the UHMWPE bioreactivity (25). Nevertheless, until now, a direct cytotoxic effect of UHMWPE has not been clearly demonstrated and the few cellular tests used have shown controversial results. On the other hand, it is very important to be sure that the new proposed types of UHMWPE are safe and do not induce any toxic or inflammatory reaction. For this reason, Bhatia et al. (26) have emphasized the importance to develop in vitro cytotoxicity assay(s) sufficiently rapid to screen large numbers of potential biomaterials.

Aim of this study has been to investigate whether UHMWPE with different chemico-physical properties at their surface, namely conventional (PE), oxidized (Ox-PE), crosslinked (X-PE) and Vitamin E-added (E-PE) UHMWPE, have or not different biological effects on the cells. In this work we show that in a human osteoblast cell line all these types of PE exerted similar and significant toxic effects versus non-stimulated control cells, by using means of investigation very easy to perform, i.e. release of LDH in the extracellular medium, generation of ROS and production of nitrite, a stable derivative of nitric oxide (NO), which is frequently released during inflammatory reactions. A similar effect of UHMWPE was observed in two monocyte/macrophage lines, used as control cells similar to the osteoclast lineage.

MATERIALS AND METHODS.

Biomaterials

UHMWPE films (approx. 200 μm thick) were microtomed from compression molded sheets of standard GUR 1020 UHMWPE (PE), highly cross-linked UHMWPE (X-PE) (75 kGy , Ebeam irradiation followed by remelting) and of UHMWPE blended with 0.5% w/w vitamin E (E-PE) (MediTECH/ Quadrant, Fort Wayne, IN, USA). Half of the standard PE samples were exposed to accelerated ageing in a ventilated oven at 95°C for 210 h, in order to obtain a third group of samples: oxidised UHMWPE (Ox-PE). The same manufacturing steps were used for all specimens, with the aim to obtain comparable surface roughnesses. In order to preserve the chemical and physical properties of the biomaterials, sterilization was achieved through 70% ethanol immersion followed by washing in sterile demineralised water.

Water contact angle measurements.

Static contact angle (CA) measurements were carried out by the sessile drop method using a KRÜSS DSA 100 (KRÜSS, Hamburg, Germany) apparatus and distilled water. The volume of the individual water droplet used for the static CA measurements was 5 μl. The water contact angle has been measured at four different locations on each sample to ensure homogeneity.

FTIR spectroscopy

Attenuated Total Reflectance (ATR)-Fourier-transform infrared (FTIR) spectra of the sample surfaces before the experiments were collected by using a FTIR Microscope (Spectrum Spotlight, Perkin-Elmer) equipped with an ATR objective (Germanium, incidence angle of the IR beam 45°, 100x100 μm^2 nominal surface area). The average penetration of the IR beam in the present conditions is in the order of 1 μm. Each spectrum was collected using 32 scans at a 4 cm^{-1} resolution. The ATR spectra were corrected for the wavelength dependence of the beam penetration by the Atrcorr algorithm of Grams AI/8.0 (Thermo Electron corporation).

CELLS AND REAGENTS

MG-63 human osteoblast cells, murine alveolar macrophages (MH-S) and human monocytes (THP-1) (provided by Istituto Zooprofilattico Sperimentale "B. Ubertini", Brescia, Italy). MG-63 and MH-S cells were cultured up to confluence in 35 mm-diameter Petri dishes, while 1.000.000 of THP-1 were used for each experiment, with M199 or RPMI medium supplemented with 10% fetal bovine serum (FBS), penicillin, streptomycin and L-glutamine in a humidified atmosphere containing 5% CO_2 at 37°C. The confluent cells were incubated for 72 h in the absence or presence of slides (1 cm^2), attached at the bottom of the dish, of PE, Ox-PE, X-PE and E-PE, on and around which the cells were allowed to grow and then checked for the data described in the Results. The protein contents of cell lysates were assessed with the BCA kit from Pierce (Rockford, IL). Plasticware was from Falcon (Becton Dickinson, Franklin Lakes, NJ). Unless otherwise specified, other reagents were purchased from Sigma Aldrich (Milan, Italy).

LACTATE DEHYDROGENASE (LDH) LEAKAGE

To check the cytotoxic effect under the different experimental conditions described in Results, LDH activity was measured in both culture supernatant and cell lysate at the end of the incubation time, as described previously (27). Both intracellular and extracellular enzyme activities, measured spectrophotometrically as absorbance variation at 340 nm (37°C), were expressed as μmoles of NADH oxidized/min/dish, then extracellular LDH activity was calculated as a percentage of the total

Table I. *Water contact angle*

Sample	Water contact angle (degrees)
PE	93
Ox-PE	84
E-PE	91
X-PE	92

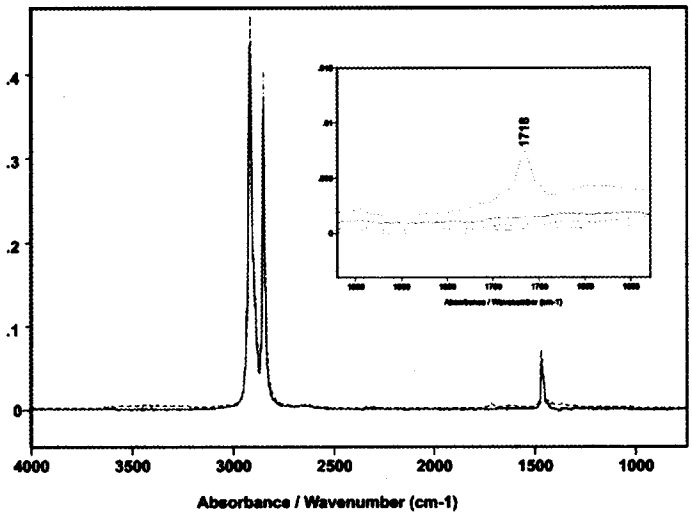

Fig. 1. *FTIR-ATR spectra of: (·····)PE; (-----) OX-PE; (-·-·-)E-PE; (____)X-PE.*

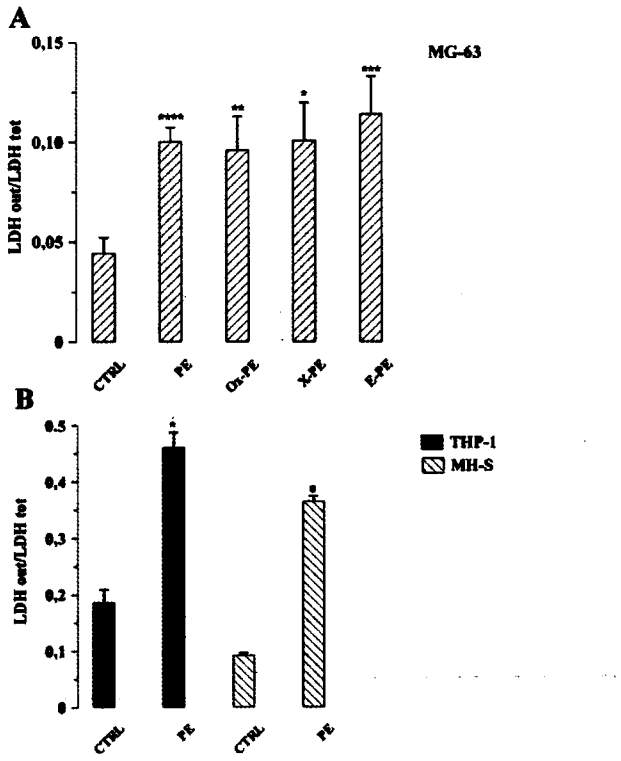

Fig. 2. *Effects of UHMWPE on cytotoxicity, measured as leakage of intracellular LDH.*
*A. MG-63 cells were incubated for 72 h, in the absence (CTRL) or presence of slides (1 cm^2), attached at the bottom of the dish of standard UHMWPE (PE), oxidised UHMWPE (Ox-PE), highly cross-linked UHMWPE (X-PE) and UHMWPE blended with 0.5% w/w vitamin E (E-PE), on and around which the cells were allowed to grow and then checked for the data (n = 8), which are represented as mean + SEM. Significance vs. CTRL: ****p<0.001, ***p<0.01, **p<0.02, *p<0.05.*
*B. THP-1 and MH-S cells were incubated under the same experimental conditions in the absence (CTRL) or presence of standard UHMWPE (PE) (n = 6), represented as mean + SEM. Significance vs. CTRL: *p<0.05, °p<0.005.*
At the end of each incubation the LDH activity in the extracellular medium and cell lysate was measured, and the cytotoxicity was expressed as percentage of extracellular LDH on the total (extracellular + intracellular) LDH activity, as described under Materials and Methods.

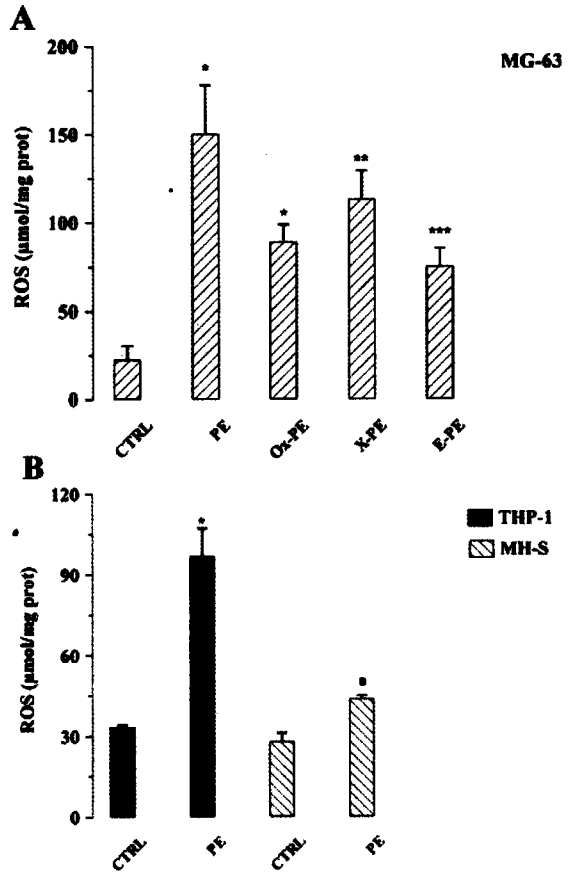

Fig. 3. *Effects of UHMWPE on oxidative stress, measured as ROS generation.*
*A. MG-63 cells were incubated for 72 h, in the absence (CTRL) or presence of slides (1 cm^2), attached at the bottom of the dish of standard UHMWPE (PE), oxidised UHMWPE (Ox-PE), highly cross-linked UHMWPE (X-PE) and UHMWPE blended with 0.5% w/w vitamin E (E-PE), on and around which the cells were allowed to grow and then checked for the data (n = 8), which are represented as mean + SEM. Significance vs. CTRL: ***p<0.002, **p<0.02, *p<0.05.*
*B. THP-1 and MH-S cells were incubated under the same experimental conditions in the absence (CTRL) or presence of standard UHMWPE (PE) (n = 6). Data are represented as mean + SEM. Significance vs. THP-1 CTRL: *p<0.05, vs. MH-S CTRL •p<0.05.*
At the end of each incubation the ROS generation was measured with DCHF-DA, as described under Materials and Methods.

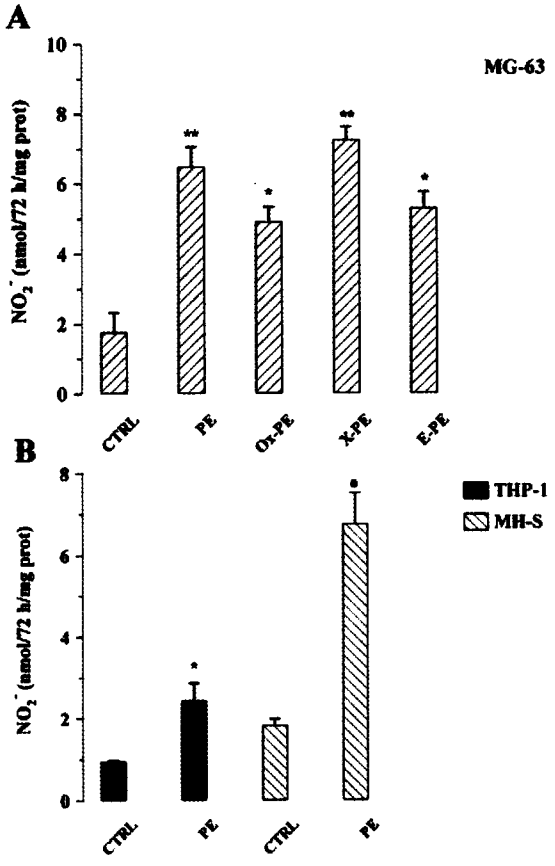

Fig. 4. *Effects of UHMWPE on the nitrite production.*
*A. MG-63 cells were incubated for 72 h, in the absence (CTRL) or presence of slides (1 cm^2), attached at the bottom of the dish of standard UHMWPE (PE), oxidised UHMWPE (Ox-PE), highly cross-linked UHMWPE (X-PE) and UHMWPE blended with 0.5% w/w vitamin E (E-PE), on and around which the cells were allowed to grow and then checked for the data (n = 8), represented as mean + SEM. Significance vs. CTRL: ***p<0.001, **p<0.002, *p<0.02.*
*B. THP-1 and MH-S cells were incubated under the same experimental conditions in the absence (CTRL) or presence of standard UHMWPE (PE) (n = 6). Data are represented as mean + SEM. Significance vs. THP-1 CTRL: *p<0.05, vs. MH-S CTRL •p<0.05.*
At the end of each incubation the nitrite accumulation in the extracellular medium was measured with the Griess method, as described under Materials and Methods.

(intracellular + extracellular) LDH activity in the dish.

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS)

After each experimental incubation, the cells were loaded for 30 min with 10 μM 2',7'-dichlorodihydr ofluorescein diacetate (DCFH-DA). DCFH-DA is a cell-permeable probe that is cleaved intracellularly by non-specific esterases to form DCFH, which is further oxidized by ROS to form the fluorescent compound dichlorofluorescein (DCF). Then, cells were washed twice

with PBS to remove excess probe and DCF fluorescence was determined at excitation wavelength of 504 nm and emission wavelength of 529 nm, using a Synergy HT microplate reader (Biotek Instruments, Winooski, VT). The fluorescence value was corrected for the content of cell proteins and expressed as μmol/mg cellular protein.

Measurement of nitrite production. After each experimental incubation, the culture supernatant was removed and tested for nitrite, which is a stable derivative of NO, using the Griess method as previously described (28). Nitrite amount was corrected for the content of cell proteins and expressed as nmol/mg cellular protein.

Statistical analysis

All data in text and figures are provided as means ± SE. The results were analyzed by a one-way Analysis of Variance (ANOVA) and Tukey's test. $p < 0.05$ was considered significant.

RESULTS

ATR-FTIR spectra of the different UHMWPEs

The ATR-FTIR spectra representative of the different polyethylene groups (PE, X-PE, E-PE and Ox-PE) before the tests are shown in Fig. 1. The only significant difference was observed in the spectrum of the aged group, Ox-PE, where the multiple absorption centered at 1718 cm^{-1} confirmed the presence of abundant oxidised products. In particular, the concentration of ketones can be estimated to be in the order of 1 mol/l. Oxidation of polyethylene is known to increase the surface hydrophilicity through the incorporation of polar, oxygen-containing, functional groups. Accordingly, a decrease in contact angle was observed in our Ox-PE group (Table 1).

Different types of UHMWPE caused similar cytotoxicity and oxidative stress

We incubated the MG-63 human osteoblast cells with PE, Ox-PE, X-PE and E- PE. THP-1 human monocytes and MH-S murine alveolar macrophages were incubated with PE only, to give a confirmation of its oxidative effects on osteoclast-like cell types. After a 72 h incubation with MG-63 cells, a significantly increased release of LDH, used as sensitive index of cytotoxicity, was observed (Fig. 2A). Moreover, MG-63-cells exhibited a significant increase of ROS production, used as sensitive index of oxidative stress (Fig. 3A). The toxic effects of PE were confirmed also in both THP-1 and MH-S cells (Fig. 2B-3B).

Different types of UHMWPE induced a similar nitric oxide (NO) production

A significant increase of NO production (Fig. 4A), a free radical marker of inflammation, was also detected in MG-63 cells incubated for 72 h with all types of UHMWPE. The NO production induced by PE was confirmed also in THP-1 and MH-S cells (Fig. 4B).

DISCUSSION

UHMWPE is a material widely used for knee and hip implantations, but, until now, no clear association has been established between UHMWPE, its oxidation state and the toxic effects exerted by this material. For the first time, we demonstrate a strong toxic effect exerted not only by conventional and oxidized UHMWPE, but also in the presence of X-linked and vitamin E-treated UHMWPE, until now considered inert materials. This effect could be observed both in osteoblast cells and osteoclast-like macrophage lines.

Our data suggest that both conventional UHMWPE and its derivatives evoke oxidative stress, measured as generation of ROS, accompanied by signs of cytotoxicity, expressed as LDH leakage in the extracellular medium, and by an increased generation of nitrite, a stable derivative of NO, a free radical released during inflammatory reactions, often in response to an oxidative stress evoked by cytokines. This result suggests that the different UHMWPE materials have similar toxic effects and that the present modifications used to improve its bioreactivity are not yet inert. Despite different superficial characteristics, such as the presence or not of an oxidised status, the films of PE seem to induce similar toxic effects on osteoblasts; it is not yet clear if a different physical shape, for instance a conversion into small particles, could cause a different cellular response. Furthermore, it is not clear how these results may have clinical relevance in vivo and if they should induce a review of all structured definition of biocompatibility. Further investigations are needed.

To investigate the mechanism by which this oxidative stress occurs, we are now performing experiments to elucidate whether the UHMWPEs trigger the expression of some enzymes (inducible NO synthase, heme oxygenase-1) and transcription factors (NF-kB, AP-1) activated by oxidative stress. Moreover, our future efforts are aimed to "mimic" the effects of wear debris of such biomaterials, by incubating the cells with particles generated by mechanical disintegration of the UHMWPEs, to clarify the mechanism by which these materials influence the cellular redox metabolism and how oxidative stress leads to the pathogenesis of prosthetic loosening.

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