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Surface oxidation of UHMWPE for orthopedic use increases apoptosis and necrosis in human granulocytes

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Ultra high molecular weight polyethylene (UHMWPE) used in orthopedic prosthesis is often sterilized with γ-rays and the subsequent oxidation was suggested to favor the *in vivo* wear. UHMWPE debries produced by wearing trigger an inflammatory response that can led to the implant failure. To explore direct effects of UHMWPE oxidation on immunocompetent cells and their possible role in the prosthesis failure, peripheral blood cells (PBCs) have been grown for 24 and 48 h onto plastic (Ct), UHMWPE (PE) and heat oxidized UHMWPE (PEOx) discs. PBCs necrosis and apoptosis were assessed in flow cytometry using propidium iodide staining. After 24 h, no statistically significant differences were observed in the amount of apoptotic and necrotic cells between Ct, PE and PEOx samples while, after 48 h, both necrotic and apoptotic cells were strongly increased in PEOx samples where also the granulocytes population appeared strongly reduced ($6.3 \pm 1.1\%$) compared to PE ($10.5 \pm 1.5\%$) and Ct ($15.1 \pm 0.9\%$) samples. We conclude that surface oxidation may interfere with prosthetic failure and/or integration via granulocytes modulation.
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1. Introduction

Ultra high molecular weight polyethylene (UHMWPE) is widely used in orthopaedic prosthetic implants [1]. It has been observed that UHMWPE sterilization using high energy radiation in air atmosphere induces a strong oxidation [2] resulting in a decreased polymer molecular mass [3]. Oxidation alters therefore UHMWPE mechanical properties increasing wearing and causing a strong inflammatory response and a consequent prosthesis failure [4]. No reports about cell and tissue direct interaction with oxidized UHMWPE before the wearing phenomenon are available and the role of immunocompetent blood cells in the ''first contact'' phase with the oxidized surface is a matter of discussion. The interaction of cells and tissue with biomaterials following the prosthesis implantation has been widely studied and it has been observed that specific leukocytes inflammatory response is a key step in the healing process [5]. Usually the chemotactic recruitment of leukocytes around an implant promotes tissue repair and peripheral blood cells (PBCs) interaction with polymeric artificial surfaces through the protein layer could play an important role in the regulation of the inflammatory response [5]. Polymorphonuclear neutrophilic leukocytes (neutrophils), that represents almost the 85–90% of granulocytes, accumulate in the extravascular spaces during the early stages of inflammation following a prosthesis implant [6] and neutrophil apoptosis has been proposed to play a role in the control of inflammation [7]. Apoptosis is the normal non-inflammatory pathway for clearence of defective or aged cells in the organism without causing damage to the surrounding tissue [8], while necrosis is characterized by loss of membrane integrity and release of potentially toxic intracellular contents. Apoptotic cells has to be efficiently removed by phagocytes such as macrophages in order to avoid a ''secondary necrosis'' that can amplify inflammation and lead to a cronic inflammatory condition [9]. The aim of this work was to quantify the effects of orthopedic UHMWPE oxidation on PBCs viability, necrosis and apoptosis in order to investigate the effects of oxidised polymeric biomaterial on the ''first contact'' inflammatory response.

2. Materials and methods

2.1. UHMWPE oxidation and surface analysis

A compression molded plate of prosthetic UHMWPE (Gur 1020, Poly Hi Solidur, Germany) was used. The materials, in compliance with ASTM F 648-98 was without additives. The plate was initially sawn to a size suitable for microtomy ($5 \times 3 \times 7$ cm). A PolyCuts Microtome (Reichert-Jung) was used. Microtomy was performed at cutting speed 20 mm/s in air at room temperature. Slices of about 200 μm were recovered.

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Some of the slices were oxidized in air in oven at 120 °C for 80 h. The behavior of polyethylene heat oxidized is very similar to the γ-ray when a quantitative comparison of oxidation products was perform [10]. Moreover thermal treatment has been chosen to obtain oxidation levels easily measurable and reproducible. The original (PE) and oxidized (PEOx) samples were analyzed with infrared spectroscopy in attenuate total reflection (ATR). An FTIR Microscope (Perkin Elmer System 2000, Autoimage) equipped with an ATR objective (Germanium, incidence angle of the IR beam 45°, $100 \times 100\,\mu m^2$ nominal surface area) was used. ATR spectra were collected using 64 scans. The ATR spectra were corrected for wavelength dependence of the beam penetration by a computer program (Atrcorr program, Grams 32, Galactic) assuming 1.5 to be the refractive index of UHMWPE.

2.2. Peripheral blood cells isolation and treatment

EDTA collected human peripheral venous blood (10 ml) from 6 healthy donors (2 men and 4 women, average age ± S.E. = 25 ± 3) was lysed using an ammonium chloride lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4) for 20 min at 4 °C. Pellet was centrifuged twice in sterile phosphate buffer (PBS), cells were counted and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Gibco) containing penicillin (100 u/ml), streptomycin (100 mg/ml) and L-glutamine (2 mM) (Sigma). PBCs were seeded at a concentration of 1×10^5 cell/cm^2 onto polystyrene plates, UHMWPE discs (PE, 2 cm^2) or UHMWPE oxidized discs (PEOx, 2 cm^2). After 24 and 48 h exposure cells were collected and centrifuged at 1200 rpm for 5 min. Cell were resuspended in 2 ml PBS at pH 7.4, stained with a supravital concentration of propidium iodide (PI, 40 μg/ml) [11] and incubated at 37 °C for 45 min.

2.3. Flow cytometry analysis

PBCs viability, necrosis and apoptosis was analyzed using flow cytometry. All experiments were performed using a FACScan cytometer (Becton-Dickinson) equipped with a 15-mW air-cooled argon ion laser operating at 488 nm. Propidium iodide (PI) red fluorescence was measured through a 620 nm BP filter and displayed on a four-decade log scale. A minimum of 10 000 events was collected per sample at a low sample flow rate setting (12 μl/min). Cellular populations still present after incubation (mainly lymphocytes and granulocytes) were observed in a countour plot displaying physical parameters (forward scatter and side scatter) (Fig. 2). The granulocytes percentage was calculated on the basis of physical parameters drawing a gate around the granulocyte population (Fig. 2).

Apoptotic and necrotic cells were counted in dot plot displaying PI fluorescence and forward scatter values using the R1 gate for necrotic (''bright'' PI fluorescence) and R2 gate for apoptotic cells (''dim'' PI fluorescence). Data analysis was performed with WinMDI software version 2.8 and the Student's *t*-test was used for data coupled with a significance of $p < 0.05$.

3. Results

3.1. Surface oxidation

The ATR spectra for PE and PEOX are reported in Fig. 1 (courtesy of Prof. L. Costa, Chemistry Department I.F.M., University of Turin). The ATR spectrum of PEOx showed the presence of OH groups (3450–3350 cm^{-1}) due to alchohol and acid compounds, ester groups (1740 cm^{-1}), ketons (1718 cm^{-1}) and acid (1710 cm^{-1}) [12]. The esters groups (1740 cm^{-1}) were also present on the surface of the PE samples and that small level of oxidation was dependent on the microtoming process [13].

3.2. Apoptosis and necrosis analysis

Cells were analyzed in flow cytometry using both physical parameters (forward and side scatter) and PI fluorescence. As shown in Fig. 2, PBCs cultured onto polystirene discs (Ct) in the presence of 10% FCS and collected after 24 h incubation were mainly lymphocytes (Lymph, 61.5 ± 3%, mean ± S.E.M.) and granulocytes (Gran, 16.3 ± 1%) as identified on the basis of physical

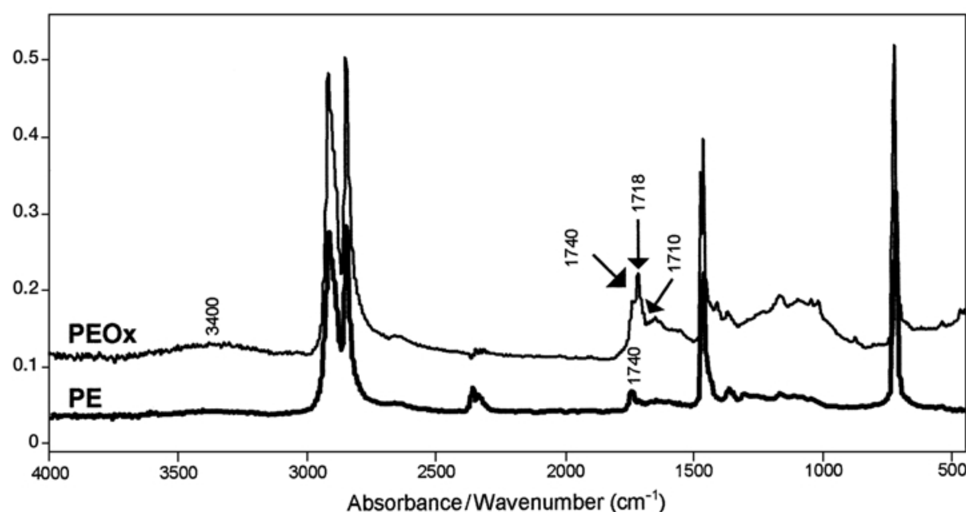

Figure 1 ATR spectrum of UHMWPE (PE) and UHMWPE thermally oxidized (PEOx).

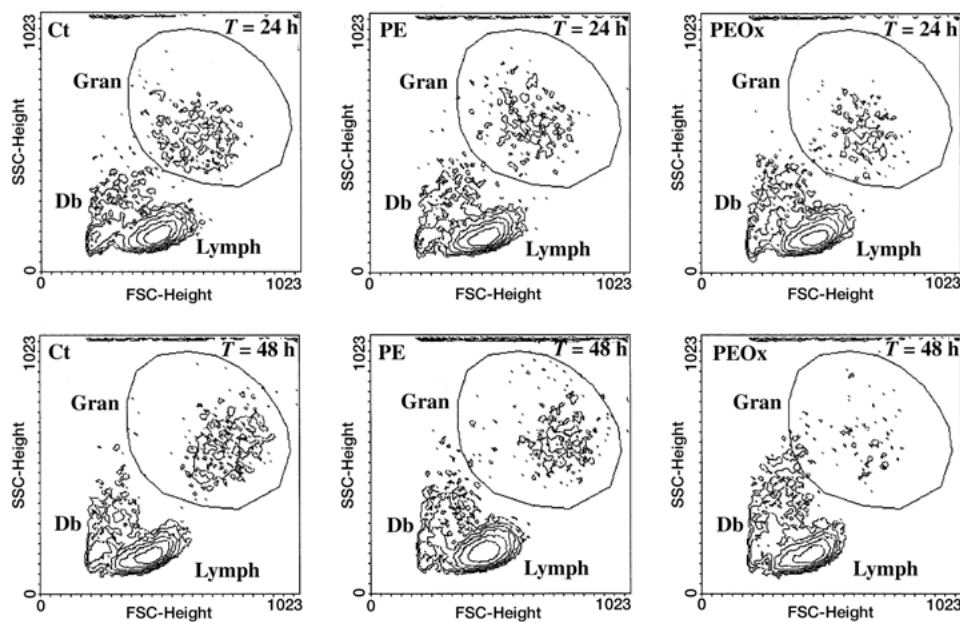

Figure 2 Representative conturn plots of forward scatter versus side scatter for PBCs seeded onto polystyrene plates (Ct), UHMWPE (PE) and UHMWPE oxidized (PEOx) for 24 (upper row) and 48 h (lower row). Granulocyte have been identified on the basis of physical parameters.

parameters. The initial percentage of granulocytes present in the blood samples used was $61 \pm 5\%$ and the drop in the granulocytes cell number was due to a ''costitutive'' apoptosis occurring during *in vitro* culture and involving 50–70% of neutrophils in the first 20 h [14]. In fact, a large amount of debris (Db) was present in control (Ct, $15.2 \pm 2\%$) as well as in the PE ($13.3 \pm 2\%$) and PEOx ($17.2 \pm 1.9.\%$) samples after 24 h. Debries represented cellular components released by dead cells in the growth medium at the end of the ''secondary necrosis'' process. Granulocyte percentage in PE ($14.4 \pm 1\%$) and PEOx ($13.4 \pm 1.2\%$) samples was similar to the control levels at 24 h, while an evident and statistically significant reduction was observed after 48 h in PE and PEOX samples compared to Ct ones (Fig. 1 lower row, Fig. 4A). No significant differences were observed in the lymphocytes percentages for any sample (data not shown). Staining with PI supravital concentration showed the presence in every samples of three main population (Fig. 3): necrotic cells PI positive (''bright'' population gated in R1), apoptotic cells PI positive (''dim'' population gated in R2) and live cells PI negative. Viable granulocytes showed a light orange-red autofluorescence due to the granules present in their cytoplasm. The value of granulocytes autofluorescence slightly increased with the incubation time (Fig. 3). In order to avoid an overstimation of apoptotic cells (gate R1), granulocytes have been identified on the basis of

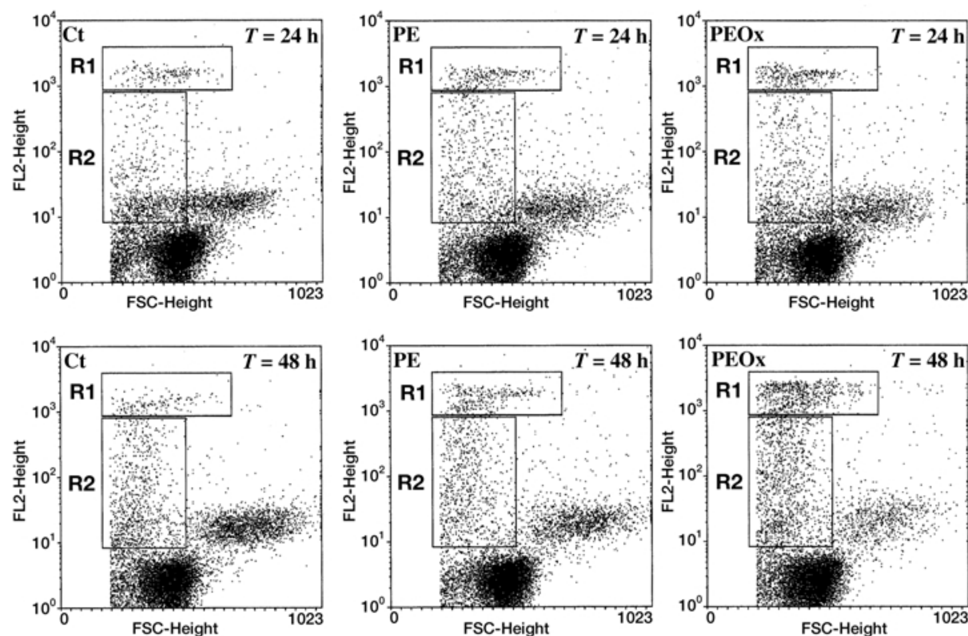

Figure 3 Representative plots of forward scatter versus PI fluorescence for PBCs seeded onto polystyrene plates (Ct), UHMWPE (PE) and UHMWPE oxidized (PEOx) for 24 (upper row) 48 h (lower row). Necrotic cells (PI positive, bright fluorescence) and apoptotic cells (PI positive, dim fluorescence) have been gated respectively in the region R1 and R2.

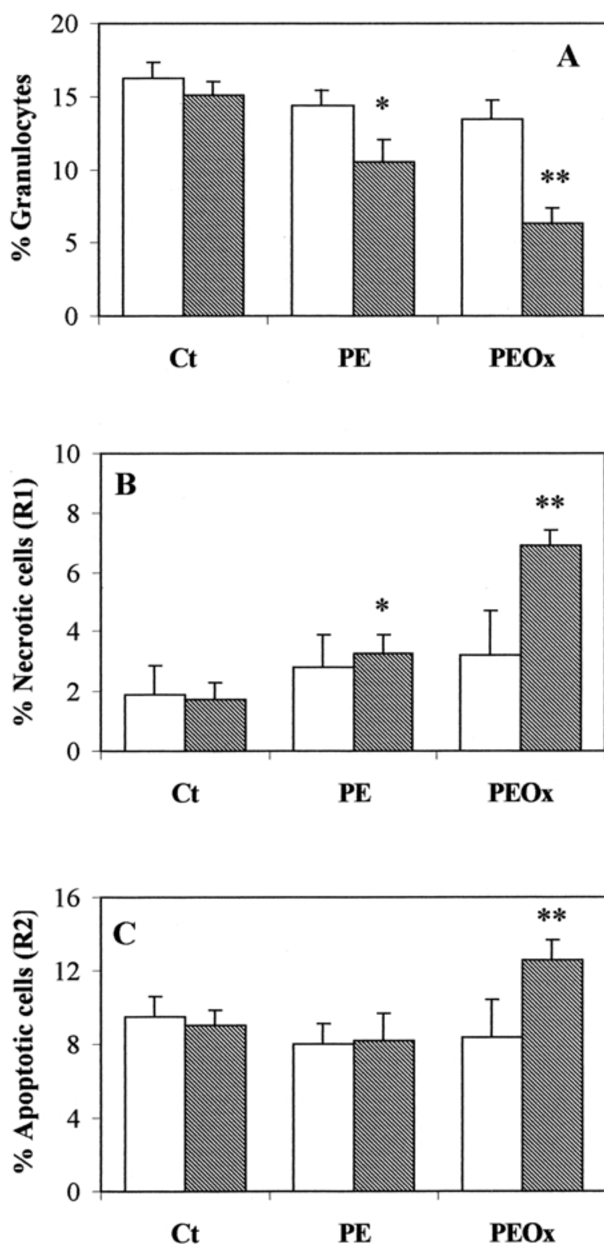

Figure 4 Effects of 24 (white bars) and 48 h (dotted bars) incubation onto plastic (Ct), UHMWPE (PE) and UHMWPE oxidized (PEOx) discs on granulocyte percentage (A), necrosis (B) and apoptosis (C) in PBCs. Results were obtained from 6 different experiments and displayed as the average value $\pm$ S.E.M: $^{*}p < 0.05$, $^{**}p < 0.001$.

FSC values and gated out from R1. After 24 h there was no significant difference in the number of both apoptotic and necrotic cells present (Fig. 4B and C) in Ct, PE and PEOx samples. Necrotic cells increased in PE ($3.3 \pm 0.6\%$) and PEOx ($6.9 \pm 0.5\%$) samples after 48 h (Fig. 3 lower row, Fig. 4B) compared to Ct ($1.7 \pm 0.6\%$). Furthermore PEOx samples showed also a significant increase in the apoptotic cell percentage ($12.6 \pm 1.1\%$) compared to both PE e Ct samples (Fig. 3 lower row, Fig. 4(C)).

4. Discussion

UHMWPE oxidation has been indicated as the responsible for increased *in vivo* biodegradation of prosthesis [15], but few reports are available about oxidative phenomenon direct effect/s on the cellular environment surrounding the prosthesis surface. In fact, the only data available concerned UHMWPE oxidized particles derived by implants wearing and these particles have been demonstrated to induce apoptosis in macrophages [16]. Apoptosis is a physiologic mode of cell death under genetic control and characterized by the selective degradation of DNA, chromatin condensation, mitochondrial swelling with preservation of the integrity of plasma membrane [17]. Late apoptotic cells *in vivo* are phagocited by macrophages or by surrounding cells [7], while *in vitro* they can come across a secondary necrosis characterized by the loss of plasma membrane integrity and releasing damaging intracellular contents [17]. Secondary necrosis can occur also *in vivo* when apoptotic cell load and phagocytic clearance mechanisms are mismatched, leading to the development of a chronic inflammatory conditions [17]. In order to mimic the ''oxidized scenario'' identical to that occurring after prosthesis implantation of X-ray sterilized prosthesis, UHMWPE discs treated with a thermal oxidative process [18] have been used to test PBCs viability after 24 and 48 h culture using a flow cytometric technique. Flow cytometry has been extensively used to discriminate both apoptosis and necrosis [17] occurring in small peripheral blood populations such as eosinophils [19] or induced by different polymers in polymorphonuclear cells [20]. Necrotic and late apoptotic cells can be easily stained by PI (1–5 μg/ml) that crosses the damaged plasma membrane and interacts with the DNA (and also double-stranded RNA) emitting an orange-red fluorescence [21]. It has been observed that using PI at a concentration of 40 μg/ml, it can also stain the unfragmented DNA of early and middle unfixed apoptotic cells after 30–45 min incubation [11, 22]. Using PI staining, we observed that after 48 h PEOx increased both apoptosis and secondary necrosis in PBCs and decreased at the same time the granulocytic population compared to the PE and Ct samples (Figs. 1 and 2). These data suggest that granulocytes could be the main cellular population affected by the oxidative state of UHMWPE. Granulocytes are recruited to inflammatory sites in response to tissue injury or infections and their apoptosis occurs at the end of inflammation. In our experimental model PEOx samples induced a stronger granulocytic activation compared to PE and plastic samples, thus an higher percentage of granulocytes become apoptotic. Moreover we observed an increase in the releasing of a matrix metalloproteasis (MMP-9, Gelatinase B) from PBCs after incubation with PEOx (Renò F., unpublished result). This observation could be linked to an activation of neutrophiles granulocytes [23]. On the PEOx surface decomposition of secondary hydroperoxides produced by γ-ray sterilization process in air produced large amounts of alcohols, ketons and acids (Fig. 1) [10]. It has been reported that hydroxyl radical are potential intracelluair mediators of apoptosis in neutrophil granulocytes [24] and the presence of hydroperoxides slowly decomposing in PEOx [25] suggests that these chemical species could be involved in the observed apoptosis. Moreover, the first oxidative effect on UHMWPE was that the apolar polymer surface has been changed in a polar surface in PEOx and it has been reported that surfaces displaying hydrophilic and anionic chemistriers (polar) induced apoptosis in adherent

macrophages at a higher magnitude than hydrophobic or cationic (apolar) surfaces reducing the cellular adhesion [25]. The key role of oxidized species in the induction of apoptosis and necrosis in PBCs is also suggested by the observation that UHMWPE not oxidized (PE) induced a light increase in necrosis after 48 h (Fig. 4B). This phenomenon can be addressed to the presence of a small quantity of oxidized species on the polymer surface due to the microtoming (Fig. 1) [13]. Than PEOx could induce granulocytes apoptosis activating them or through its chemo-physical characteristics or both mechanisms. The new apoptotic PEOx ability could have different roles in the implant integration as apoptosis might regulate the extinction of the inflammation, the increased apoptotic rate observed in PEOx sample could stop the initial tissue inflammatory response inhibiting the normal healing process. On the other hand, if the increase of apoptosis is not followed by an increase in the removal of late apoptotic cells, a secondary necrosis process could trigger a chronic inflammation.

In conclusion, the present study has shown that oxidation induced in the UHMWPE surface by sterilization procedures, as well as by thermal treatment, is potentially able to modulate the ''first contact'' inflammatory state increasing the apoptotic rate occurring in human granulocytes. Further studies are called to understand how and if this modulatory effect could affect the prosthesis integration.

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References

1. S. LI and A. H. BURNSTEIN, *J. Bone Joint Surg. Am.* **76** (1994) 1080.
2. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREVER, M. CROVA and P. GALLINARO, *Biomaterials* **19** (1998) 659.
3. V. PREMNATH, W. H. HARRIS, M. JASTY and E. W. MERRILL, *Biomaterials* **17** (1996) 1741.
4. M. JASTY, C. BRAGDON, K. LEE, A. HANSON and W. H. HARRIS, in ''Biological, material and mechanical consideration of joint replacement'', edited by B. F. Morrey (Raven Press, New York, 1993) p. 103.
5. L. TANG and J. W. EATON, *Am. J. Clin. Pathol.* **103** (1995) 466.
6. D. F. WILLIAMS, *J. Mat. Sci.* **22** (1987) 3421.
7. J. SAVILL and C. HASLETT, *Semin. Cell Biol.* **6** (1995) 385.
8. J. SAVILL, *J. Leukocyte Biol.* **61** (1997) 375.
9. C. WARD, I. DRANSFIELD, E. R. CHILVERS, C. HASLETT and A. G. ROSSI, *TiPS* **20** (1999) 503.
10. J. LACOSTE and D. J. CARLSOON, *J. Polymer. Sci.* **30** (1992) 493.
11. L. ZAMAI, E. FALCIERI, G. MARHEFKA and M. VITALE, *Cytometry* **23** (1997) 303.
12. K. JACOBSON, L. COSTA, M. P. LUDA and L. TROSSARELLI, *Polymer Degradation and Stability* **55** (1997) 329.
13. K. JACOBSON, L. COSTA, P. BRACCO, N. AUGUSTSSON and B. STENBERG, *Polymer Degradation and Stability* **73** (2001) 141.
14. L. C. MEAGHER, J. M. COUSIN, J. R. SECKL and C. HASLETT, *J. Immunol.* **156** (1996) 4422.
15. E. M. BRACH DEL PREVER, M. CROVA, L. COSTA, A. DELLERA, G. CAMINO and P. GALLINARO, *Biomaterials* **17** (1996) 873.
16. I. CATELAS, A. PETIT, D. J. ZUKOR, R. MARCHAND, L. YAHIA and O. L. HUK, *Biomaterials* **20** (1999) 625.
17. Z. DARZINKIEWICZ, G. JUAN, X. LI, W. GORCZYCA, T. MURAKAMI and F. TRAGANOS, *Cytometry* **27** (1997) 1.
18. L. COSTA, M. P. LUDA and L. TROSSARELLI, *Polymer Degradation and Stability* **58** (1997) 41.
19. K. SANDSTROM, L. HAKANSSON, A. LUKINIUS and P. VENGE, *J. Immunol. Meth.* **240** (2000) 55.
20. T. FABRE, F. BELLOC, B. DUPUY, M. SCHAPPACHER, A. SOUM, J. BERTRAND-VARAT, C. BAQUEY and A. DURANDEAU, *J. Biomed. Mater. Res.* **44** (1999) 429.
21. M. VITALE, L. ZAMAI, G. MAZZOTTI, A. CATALDI and E. FALCIERI, *Histochem.* **100** (1993) 223.
22. L. ZAMAI, B. CANONICO, F. LUCHETTI, P. FERRI, E. MELLONI, L. GUIDOTTI, A. CAPPELLINI, G. CUTRONEO, M. VITALE and S. PAPA, *Cytometry* **44** (2001), 57.
23. G. OPDENAKKER, P. E. VAN DEN STEEN, B. DUBOIS, I. NELISSEN, E. VAN COILLIE, S. MASURE, P. PROOST and J. VAN DAMME, *J. Leukoc. Biol.* **69** (2001) 851.
24. E. ROLLET-LABELLE, M. J. GRANGE, C. ELBIM, C. MARQUETTY, M. A. GOUGEROT-POCIDALO and C. PASQUIER, *Free Rad. Biol. Med.* **24** (1998) 563.
25. L. COSTA, M. P. LUDA, L. TROSSARELLI, E. M. BRACH DEL PREVER, M. CROVA and P. GALLINARO, *Biomaterials* **19** (1998) 1371.
26. W. G. BRODBECK, M. S. SHIVE, E. COLTON, Y. NAKAYAMA, T. MATSUDA, and J. M. ANDERSON, *J. Biomed. Mater. Res.* **55** (2001) 661.

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