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**Electrochemically Reduced Graphene Oxide on CoCr biomedical alloy:
Characterization, Macrophage Biocompatibility and Hemocompatibility in rats
with graphene and graphene oxide**

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Abstract

Electrochemically reduced graphene oxide (ErGO) films on a biomedical grade CoCr alloy have been generated and characterized in order to study their possible application for use on joint prostheses. The electrodeposition process was performed by cyclic voltammetry. The characterization of the ErGO films on CoCr alloys by XPS revealed sp² bonding and the presence of C=O and C-O residual groups in the graphene network. Biocompatibility studies were performed with mouse macrophages J774A.1 cell cultures measured by the ratio between lactate dehydrogenase and mitochondrial activities. An enhancement in the biocompatibility of the CoCr with the ErGO films was obtained, a result that became more evident as exposure time increased. Macrophages on the CoCr with the ErGO were well-distributed and conserved the characteristic cell shape. In addition, vimentin expression was unaltered in comparison with the control, results that indicated an improvement in the CoCr biocompatibility with the ErGO on the material surface. The in vivo response of graphene and graphene oxide was assessed by intraperitoneal injection in wistar rats. Red blood cells are one of the primary interaction

sites so hemocompatibility tests were carried out. Rats inoculated with graphene and OG showed red blood cells of smaller size with a high content in haemoglobin.

Keywords: graphene, reduced graphene oxide, macrophages, biocompatibility, rats, blood red cell

1. Introduction

The Co-based and Ti-based alloys are the metallic biomaterials mainly used in the substitution or repair of hard tissues, either permanent or temporary applications, due to the excellent mechanical properties, high corrosion resistance and good biocompatibility. Among all the mechanical properties needed in permanent biomaterials, CoCr alloys are widely used in such cases where high wear resistance is required, such as joints. The implantation of metallic joint replacements into the human body involves the interaction with the physiological environment simultaneously to the action of wear that alter the operation success of implants and affect drastically the surface performance. The activation of the passive surface is enhanced by wear-corrosion phenomena that lead to a continuous activation/repassivation cycles and consequently, a strong metal ion accumulation that can induce aseptic loosening.

On the other hand, the characterization of the surface of retrieval Metal on Metal joint replacements has revealed tribological films composed of graphitic compounds in areas where sliding takes place [1,2]. Graphite, a well-known solid lubricant usually used in industrial applications where wear occurs under humid environments, seems to induce the decrease in friction/wear processes and low corrosion. These findings raise the question of integrating carbon-base compounds on the surface of biomedical implants prior to their implantation. Bearing this in mind, the discovery of the exceptional properties of graphene, makes it a suitable candidate to be deposited as a solid lubricant on the metallic surfaces. The importance of studying the surface modification with graphene-based structures on Cobalt-Chromium (CoCr) alloys, one of the most commonly used as joint replacements, is of great interest in order to decrease corrosion and enhance the wearability of implants. It is known that CoCr alloys undergo wear-corrosion phenomena causing debris and dissolution and the formation of a passive film. These phenomena are affected by factors such as: lubrication between surfaces, corrosive media, pH, temperature, biopotentials etc. Because of these processes, all biomaterials may induce a biological host response to generated wear debris, which is strictly

dependent on the nature of the debris. Wear particles and metal ions from prosthetic devices may induce a cascade of adverse cellular reactions that can include inflammatory complications, macrophage activation, bone resorption, and, although rarely, neoplasia [3,4,5]. In this context, macrophages play a decisive role in the hostile inflammatory reactions that can lead to the loosening and failure of the implants.

One of the most attractive routes to obtain graphene-enriched surfaces is the reduction of graphene oxide (GO) on metal substrates due to their low cost of synthesis and easy deposition. GO consists of a lattice of graphene which incorporates oxygenated functional groups, such as hydroxyl and epoxy groups on its basal plane and carboxyl groups on the edges [6].

In an attempt at restoration of the initial properties of graphene, the reduction of GO is promoted by chemical, thermal or electrochemical processes. The reduction of graphene oxide by thermal treatments results in a lower residual content of oxygen than the reduction performed by chemical reduction. However, thermal reduction in GO films on metal substrates can cause undesirable microstructural changes in the metal bulk. At this point, electrochemical methods appear as a good alternative to reducing the oxygen content incorporated in the carbon network without altering the bulk properties. Electrochemical reduction gives rise to voltammetric reduction waves at high negative potentials but complete removal of the oxygen content is hard to obtain. In fact, graphene produced from all these methods induces common structural defects and residual oxygen remains in the partially reduced graphene. To date, not many substrates have been used apart from glassy carbon [7], gold [8] aluminum [9] or zinc [10]. To the best of our knowledge, there has not been any study about the electrochemical reduction of graphene oxide on Co-based alloys.

The presence of oxygen in the carbon network opens future applications for the graphene films to improve specific properties of the surfaces. The oxygen can act as a link between the graphene and specific substances used in the treatment of diseases. However, the experimental results in *in vivo* applications are not conclusive to date.

Bearing in mind that toxicity is the crucial issue that must be addressed in order to exploit the use of GO in any interaction with living matter [11], cytotoxic and genotoxic effects should be considered. Both effects seem to be very closely related to the potential ability

to cross the cellular membrane. Several studies prove that GO can easily enter the cells and that this mechanism strongly depends on the size of the GO flakes [12].

The main damage caused by GO nanosheets in the cell is attributed to the interaction of the functional groups within the cell that leads to an increase in the production of reactive oxygen species (ROS) and consequently, cytotoxic effects. This effect has been observed in fibroblasts HDF cell lines and in mice after intravenous injection of GO at concentrations higher than 50 $\mu\text{g/mL}$ [13].

Genotoxicity of GO in cells of human MSCs has been verified by Akhavan et al in [14]. They correlate the ROS level in hMSCs with cytotoxic and genotoxic results. They also found that toxicity strongly depends on the size of the nanomaterial. De Marzi et al [15] studied the genotoxic and cytotoxic effects of pristine GO on different cell lines as a function of the average of lateral size of the GO flakes. From the literature found, the potential genotoxicity of GO has received little attention in nanobiomaterials applications. However, toxicity is the crucial issue that must be addressed and explained [16].

Scattered and contradictory results about the secondary effects produced by derivative graphene can be found in the literature [10,17,18,19]. This has promoted further *in vivo* research in order to clarify its effect.

In this work, electrochemically reduced graphene oxide films on a biomedical grade CoCr alloy are generated and characterized in order to study their possible application on the joint prostheses. Biocompatibility tests using a mouse macrophage cell line J774A.1 were performed as they are the principal cells involved in the primary response to foreign bodies and have a decisive role in the inflammatory reactions. Taking into account that the red blood cells are one of the primary interaction sites, hemocompatibility tests in wistar rats intraperitoneally inoculated with graphene and graphene oxide were carried out to assess the biomedical performance of graphene oxide compounds on the CoCr alloy.

2. Experimental procedure

2.1 Electrochemical reduction

The nominal composition of the CoCr alloy (wt.%), supplied by International Edge alloys given by the manufacturer is shown in Table 1.

Co	C	Mo	Cr	Ni	S	P	Al	W	Mn	Fe	Si	N	Ti	Cu	O
65.35	0.044	5.36	27.25	0.15	0.001	0.002	0.002	0.02	0.69	0.29	0.68	0.15	0.001	0.01	0.02

Table 1. Chemical composition in wt.% of CoCr alloy.

A graphene oxide (GO) aqueous suspension 4 mg/mL supplied by Grupo Antolin Holding (Burgos, Spain) was used in the electrochemical reduction, in normally aerated conditions. The corresponding pH (2.3) and conductivity (1.7 mScm^{-1}) values were measured with a Crison pH meter.

CoCr discs of 12 mm diameter and 2 mm thickness were immersed in the aqueous suspension of graphene oxide immediately after grinding, with SiC abrasive paper of increasing grain from 600 to 2000. Two-sided material was electrochemically covered with reduced graphene oxide films. Electrochemical experiments were performed at room temperature using a standard three-electrode electrochemical cell consisting of a graphite bar used as counter electrode, Ag/AgCl 3M KCl as reference electrode and CoCr samples as working electrodes.

An Autolab32 potentiostat/galvanostat was used for the electrochemical test. Cyclic voltammetry was carried out from -2.1 to -0.5 V (vs. Ag/AgCl) at a scan rate of 10 mVs^{-1} for five cycles to obtain Reduced Graphene Oxide (ErGO) films on the CoCr alloy. After electrochemical reduction, the ErGOCoCr samples were washed with deionized water and dried in the air.

2.2 Characterization of the ErGO films

ErGO films were observed with an optical and a JEOL-6500F microscope. X-Ray photoelectron spectra were performed with a Fisons MT500 spectrometer using a non-monochromatic Mg $K\alpha$ X-Ray radiation (1253.6 eV) operated at 300 W. Specimens were fixed on small flat discs supported on an XYZ manipulator placed in the analysis chamber. The residual pressure inside the analysis chamber was maintained in the 10^{-9} torr range during data acquisition. The spectra were recorded with a constant pass energy of 20 eV, which is typical of high-resolution conditions. The intensities were estimated by calculating the area under each peak after subtraction of the S-shaped background and

the fitting peaks of the experimental curve were defined by a combination of Lorentzian and Gaussian distributions of variable proportions. The calibration of binding scale was performed with the C1s sp² peak at 284.5 eV.

2.3 Corrosion behavior of ErGOCoCr

The corrosion behavior of the CoCr alloy in as-received and after electrochemical reduction of the graphene oxide was evaluated in a Phosphate Buffer Saline (PBS) solution: 0.2 g/L KCl, 0.2 g/L KH₂PO₄, 8 g/L NaCl and 1.150 g/L Na₂HPO₄ anhydrous. Electrochemical techniques such as the measurement of the corrosion potential, E_{corr} , and electrochemical impedance spectroscopy (EIS) were applied to assess the corrosion behavior. EIS experiments were performed at the corrosion potential by applying a sinusoidal wave of 10 mV in amplitude in a frequency range from 10⁵ Hz to 10⁻³ Hz spaced logarithmically (five per decade). All the electrochemical experiments were performed in triplicate.

The EIS results were analyzed by fitting the experimental impedance data with the proper equivalent circuit model. The equivalent circuit parameters were calculated by fitting the impedance function to the measured spectra by a Non-Linear Least-Squares program (NLLS program) for all the frequencies measured. The criteria used in estimating the quality of the fittings were the lowest chi-square value, and the lowest estimative errors (in %) for all the components.

2.4 Biocompatibility assays on CoCr and ErGOCoCr discs

Prior to being immersed in the cell culture, both sides of the metallic discs were sterilized for 5 minutes under UV in an active vertical flux cabin.

A mouse macrophages cell line (J774A.1) was obtained from DSMZ Human and Animal Cell Bank (ACC 170). Macrophages were seeded on UV-sterilized discs of CoCr and ErGO/CoCr of 12 mm diameter and in the absence of any discs (used as control) in a 24-wells culture plates in Dulbecco's Modified Eagle Medium (DMEM 41966; Gibco, BRL) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, BRL) and with a mixture of antibiotics (penicillin at 100 units/mL and streptomycin at 100 µg/mL, Gibco, BRL). Macrophages densities of 50000 cells/mL were used for cultures of 24 and 48 hours and 25000 cells/mL for 72 hours. Cell density was selected, based on the absence of any significant damage to cell culture at the exposure times studied. Cell

cultures were maintained in a chamber at 37 °C and 5 % CO₂. To evaluate the biocompatibility and the cytotoxicity of the materials under study, mitochondrial activity (WST-1 assay) and plasma membrane damage (LDH assay) were measured as previously described in [20]. The ratio LDH/WST-1 was employed to relate the plasma membrane damage to the number of metabolically active cells in the culture.

Briefly, cell supernatants were removed from each well at the end of the culture, saved for LDH activity measurement, and renewed by 1 mL of complete cell culture medium. A volume of 100 µL of the cell proliferation kit reagent WST-1 was added to each well containing 1 mL of cell culture medium and the reaction mixture was incubated inside the cell culture incubator for 60 minutes. After incubation, a volume of 100 µL of each sample was transferred to a 96-wells cell plate, and the absorbance of the samples was measured as differential absorbance, 415 nm minus 655 nm, in an iMark-microplate absorbance reader (Bio-Rad, CA, USA). The absorbance given by the complete cell culture medium was used as a control. Supernatants collected from cell cultures after 48 and 72 hours were centrifuged for 5 minutes at 1024g before the LDH enzymatic assays were performed as previously described in [20]. All data are the average of three independent experiments where each assay contained three samples.

2.5 Cell Fixation for optical microscopy and SEM observation

Morphological studies on the macrophages were performed after culture tests. CoCr and ErGOCO₂ discs were directly fixed on the culture plate with 1 mL of cold methanol and incubated for 10 minutes at -20 °C, followed by two 5 min wash-steps with Phosphate Buffer Saline (PBS) solution. The discs were maintained at 4 °C in 1 mL of PBS. Then, the discs were further fixed with a solution of 2.5 % of glutaraldehyde in PBS at 4 °C for 24 hours and washed with distilled water three times. After that, the cells were dehydrated by consecutively increasing the concentration in ethanol at 4 °C (10-minute wash-steps series) until 100 % ethanol concentration was achieved. Finally, a Trimethylsilane solution (TMS Sigma-Aldrich®) at 50% (0.5 mL of TMS in 0.5 mL of 100% ethanol) was added for 10 minutes. This solution was removed and 1mL of TMS at 100% was added for another 10 minutes. Lastly, TMS was removed and left to air-dry during 30 minutes. No staining by dye was carried out for optical observation.

Further observation by SEM in a JEOL-6500F microscope required the gold sputtering of the samples in order to get conductive surfaces.

2.6 *In vivo* tests

Grade H graphene nanoplatelets were acquired from XG Sciences (Lansing, USA). Graphene nanosheets consist of short stacks of graphene sheets of platelet shape. According to the manufacturer, Grade H nanoplatelets have an average thickness around 15 nm, a typical surface area from 50 to 80 m²/g and average particle diameter of 5 µm. In this research, the smallest size of the platelets was selected for the biomedical application, taking into account that the highest toxicity is produced by smaller sizes in the liver [21]. An aqueous suspension of 4 mg/mL graphene oxide was supplied by Grupo Antolin Holding (Burgos, Spain).

Adult Wistar male rats approximately 250±10 g body weight (bw) were maintained in the animal facilities of the Experimental Centre of the University of Alcalá de Henares. These animals were treated in accordance with the Ethical Committee of the University, reviewed and approved by the Madrid Community Ethics Committee for Regional Clinical Research (CEIC-R), Spanish regulations (RD 53/2013) and the European Union Guidelines for Ethical Care of Animals (86/609CEE).

Graphene and graphene oxide nanosheets at a concentration of 4 mg per kilogram of weight were suspended with probe sonication into an aqueous solution of 0.9% NaCl in a proportion of 1:1 (PBS:G or GO), i.e., 1 mg/mL. The rats were randomly divided into three different groups of five animals each: control, injection of 1 mL physiological solution without nanoplatelets (control), with graphene (G) and with graphene oxide (GO). The suspension of the derivate graphene/physiological saline solution was vigorously stirred just before use in order to avoid the agglomeration of the nanoplatelets. The nanoplatelets handling was performed taking into account the Prevention of Occupational Risks [22,23] under an extraction cabin and using masks. Sterilization of each aliquot was made under vapour at 134 bars for 5 minutes.

The corresponding physiological saline suspension (control, with graphene (G) and with graphene oxide (GO) nanoplatelets) was inoculated by intraperitoneal injection.

After 30 day's post-inoculation, the rats were anesthetised with isoflurane for 1,5 mL blood extraction and immediately were sacrificed in the CO₂ cabin. *In vivo* response of graphene and graphene oxide nanoparticles were studied by hematological analysis including red and white cells, proteins, GOT (AST), GPT (ALT), glucose, creatinine,

LDH, triglyceride, and clotting in UNILABS (Madrid, Spain). Organs were recovered from animals for histological analyses.

3. Results and discussion

3.1 Electrochemical reduction and characterization of ErGOCO_{Cr}

Figure 1 shows the cyclic voltammogram (CV) of CoCr surfaces for five cycles recorded from -2.1 V_{vs. Ag/AgCl} to -0.5 V_{vs. Ag/AgCl} at a scan rate of 10mV/s in the GO aqueous suspension of 4mg/mL. The CV exhibits a well-developed cathodic peak at -1.23 V_{vs. Ag/AgCl} that slightly shifts towards more positive potentials and lower current peaks in consecutive scans. The decrease in the corresponding cathodic currents in the successive scans demonstrates that the reduction of surface-oxygenated groups occurs quickly and irreversibly under oxygenated conditions [7,24,25]. The electrochemical reduction is believed to take place when the GO sheets adjacent to the CoCr surface accept electrons, yielding the insoluble ErGO that attaches directly on to the electrode surface.

As a result, the ErGO film progressively covers the CoCr substrate thus reducing the active points of the metallic surface.

Figure 1. Cyclic voltammogram, CV, carried out under oxygenated conditions and room temperature, on CoCr samples in an aqueous solution of graphene oxide (4 mg/mL).

The characterization of the ErGO film attached to the surface of the CoCr samples was revealed by XPS. Figure 2 shows the curve fitting of the C 1s spectra measured on ErGO films on CoCr after electrochemical reduction. GO fitting is shown for comparative purposes. The C 1s peak of ErGO film can be fitted with four contributions (see Table 2).

% peak area	Csp2 BE= 284.5	Csp3 BE= 285.4	C-O BE= 286.4	C=O BE= 288.4
ErGO	69.1	nd	20.9	10.0
GO	56.2	15.2	23.1	5.6

Table 2. Binding energy, BE, and percentage of peak area of C signals obtained from XPS fitting of graphene oxide, GO, and electrochemically reduced graphene oxide by CV, ErGO, on CoCr. nd-not detectable.

In general, the binding energy of 284.5 eV is attributable to the C-C sp² bonds and the C-C sp³ is usually seen at 285.4 eV. The last one is detected in the GO film but not in the ErGO samples. The other two contributions that appear at higher binding energies are associated with the oxygen groups retained from the GO dispersed in the aqueous suspension that have not been completely removed after reduction. The peak at 286.4 ±0.2eV corresponds to C-O groups and the peak at 288.4 ±0.2 eV is assigned to the carbonyl (C=O) functional group [6,7]. As can be seen in table 2, the main change produced after electrochemical reduction is an increase in the C sp² content, whereas the sp³ contribution disappears. This suggests that the electrochemical reduction is an efficient procedure to restore the sp² carbon network of graphene on the CoCr surfaces. These results agree with the Raman data previously published by the authors [26]. Nevertheless, the presence of different C-O contributions (Table 2) indicates that the reduction process is not complete, and that can induce a more hydrophilic character than graphene.

Figure 2. Fitting of high resolution spectra of C 1s of the graphene oxide, GO, and reduced graphene oxide, ErGO, films on CoCr obtained after cyclic voltammetry experiments.

Figure 3 shows the Nyquist diagrams of CoCr in as-received and after electrochemical reduction of the Graphene oxide (ErGOCr) in PBS for 4 days. In the initial hours of immersion, ErGOCr surfaces show a defined semicircle in the Nyquist diagrams. After 24 hours, the evolution of the impedance response with immersion time is very similar, independently of the surface condition until the end of the test.

Figure 3. Nyquist impedance diagrams of CoCr and ErGOCr surfaces immersed in PBS.

Figure 4 shows the equivalent circuit that simulates the experimental impedance diagrams whose fitted values are shown in Table 3. The electrical components used to fit the impedance data that appear in the Randles equivalent circuit (Figure 4), are: R_s , which is the solution resistance, CPE, is the constant phase element associated with the electrochemical response of the passive film, and R_p , the resistance offered by the passive film.

Figure 4. Model of equivalent circuit for fitting the experimental impedance data.

	Time, d	$R_s \pm \text{error}$ (%) Ω	$R_p \pm \text{error}$ (%) Ω	$CPE \pm \text{error}$ (%) $S s^n$	$n \pm \text{error}$ (%)	Chi2
CoCr	0	6.50±0.67	4.59 $10^5 \pm 6.40$	9.54 $10^{-5} \pm 0.54$	0.89±0.14	0.002
	1	6.20±0.73	2.97 $10^6 \pm 4.03$	7.13 $10^{-5} \pm 0.45$	0.91±0.11	0.003
	2	6.96±0.69	4.42 $10^6 \pm 6.36$	6.97 $10^{-5} \pm 0.43$	0.91±0.11	0.003
	4	5.46±1.13	4.74 $10^6 \pm 9.42$	7.41 $10^{-5} \pm 0.67$	0.92±0.16	0.007
ErGOCr	0	7.15±0.88	2.69 $10^5 \pm 1.88$	1.10 $10^{-4} \pm 0.69$	0.89±0.18	0.130
	1	6.91±0.86	1.95 $10^6 \pm 3.40$	9.99 $10^{-5} \pm 0.58$	0.89±0.15	0.120
	2	2.76±1.53	2.06 $10^6 \pm 7.69$	8.43 $10^{-5} \pm 0.94$	0.92±0.21	0.320
	3	5.83±1.32	3.93 $10^6 \pm 11.62$	8.72 $10^{-5} \pm 0.88$	0.91±0.22	0.068
	4	5.45±0.85	5.41 $10^6 \pm 12.72$	9.95 $10^{-5} \pm 0.55$	0.90±0.14	0.027

Table 3. Fitted impedance results by using equivalent circuit of Figure 3. R_s -solution resistance; R_p - passive film resistance; CPE- Constant Element Phase associated with the electrochemical response of the passive film; n- exponent of CPE and Chi2- error.

The passive film resistance increases with immersion time and the CPE data remain at the same value which is characteristic of the double layer capacitance without significant variation with immersion time. These results agree with those obtained for passive materials and the presence of ErGO films on the CoCr surfaces do not cause significant changes in the good corrosion behavior.

3.2 Biocompatibility assays on CoCr and ErGOCr films

Figure 5 shows the biocompatibility of mouse macrophages J774A.1 cultures of CoCr and ErGOCr surfaces after 24, 48 and 72-hour exposure. The ratio of LDH/WST-1 activities here reported are used as a biocompatibility indicator relating cell death and mitochondrial respiratory activities for macrophages culture for each material. The low value indicates the good viability of the culture. Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme that is rapidly released into the cell culture supernatant when the plasma is damaged. LDH activity was used as an indicator of plasma membrane damage and a sign of cell death [20]. The mitochondrial activity, measured by the reduction of the WST-1 reagent, is directly proportional to the number of metabolically active cells in the culture [20].

Figure 5. Effect of CoCr and CoCrErGO on the biocompatibility of macrophages J774A.1. Macrophages were cultured on CoCr and CoCrErGO discs and in the absence of any discs (control).

The comparative results obtained with CoCr and ErGOCr (Figure 5) showed a decrease in the LDH/WST-1 ratio upon exposure to ErGOCr that was observable after 48 hours and became more noticeable when the exposure time increased up to 72 hours. Data suggest there is less damage to the cell plasma membrane in presence of ErGO films and could indicate an improvement in the macrophage biocompatibility on ErGOCr discs.

Figure 6 shows the images obtained by optical microscope of macrophages J774A.1 on CoCr and ErGOCr after 48 hours in culture. The comparison of both panels reveals clear differences in the cell distribution pattern. Macrophages appear on the CoCr surface in discrete areas or patches as cells grouped in clusters or colonies, selecting some certain areas on the disc for the growth. The distribution of the cells was quite different on ErGOCr surfaces (Figure 6, right panel), as on this surface, cells seem regularly and quite uniformly distributed along the metal surface. This homogeneous distribution of

macrophages was observed on the ErGOCr discs even at exposure time of 48 hours where the incipient signs of an increase in the biocompatibility of the material was observed (Figure 5). This observation could be interpreted as the ErGO films on CoCr discs make a friendly surface for macrophages.

Figure 6. Optical microscopy images of macrophages J774A.1 on CoCr and CoCrErGO after 48 hours in culture. Macrophages were cultured for 48 hours on CoCr and CoCrErGO discs.

Figure 7 shows SE images of macrophages J774A.1 on CoCr and ErGOCr after 48 hours in culture. Differences in cell morphology were observed between CoCr and ErGOCr discs. On the CoCr surface, dark cells with a quite round shape and with a cotton-like appearance, as if the cells were fading, could be observed. Meanwhile, macrophages on ErGOCr surface maintained an observable cell volume, an elongated fusiform shape and showed cell extensions or projections that probably facilitate cell adhesion to the material. These results indicate the best behavior of macrophages on the ErGOCr surface. These features were also reinforced by vimentin analysis distribution, a protein responsible for the architecture of cytoplasm [27]. As is shown in Figure 8 panel CoCr, vimentin fluorescence (detected in green) almost completely disappeared on macrophages, after 48 h on CoCr discs surface. However, macrophages on ErGOCr discs show a vimentin fluorescence distribution that resembles the pattern showed by the macrophages in the absence of material (control panel).

Figure 7. SEM images of macrophages J774A.1 on CoCr and CoCrErGO discs. Macrophages were cultured for 48 hours on CoCr and CoCrErGO discs.

Figure 8. Vimentin expression in macrophages J774A.1 on CoCr and CoCrErGO discs. Macrophages were cultured for 48 hours in the absence (control) and on CoCr and CoCrErGO discs. Cell nuclei, in blue, were stained with Hoechst reagent and vimentin appears in green. Scales are in white in the right hand bottom corner of all figure panels and represent 150 μm .

3.3 *In vivo*

The *in vivo* response of graphene is mainly studied by intravenous administration in the literature [17,28,29]. However, our study is focused on the evaluation of the possible toxicity due to a slow leakage of graphene derivate coming from the prosthesis surface. Bearing this particular case in mind, the presence of graphene in the blood stream would be slow and non-uniform and this is why the methodology selected for graphene derivate administration has been intraperitoneal injection.

The haematological analysis showed significant results only in the red cells. Figure 9 shows the red blood cell volume after 30 days of intraperitoneal inoculation in rats of graphene (1) and GO (2) and with respect to control rats (0) ($p=0,006$).

Figure 9. Medium hematocrit volume after 30 days of intraperitoneal inoculation in rats of graphene (1) and GO (2) and with respect to control rats (0) ($p=0,006$).

Rats inoculated with graphene and OG showed red cells with smaller size ($p< 0,0005$), and with a high content in hemoglobin ($p< 0,0005$) (Figure 10).

Figure 10. Medium corpuscular hemoglobin concentration (CHCM) after 30 days of intraperitoneal inoculation in rats of graphene (1) and GO (2) and with respect to control rats (0) ($p<0,0005$).

The red cell volume is lower than in the control group (microcytosis), but this feature seems to be compensated for by a higher haemoglobin concentration. These results agree with the characteristic alterations in the red blood cells described in the literature. Kanakia et al. [28] used high doses (between 25 and 100 mg/kg weight) to analyse the toxicity,

finding no significant decrease in mass cell volume (MCV). Nevertheless, this feature has not been verified by us using a similar dose. Guo et al. [30] suggests that the different in vivo responses found by various authors can be due to the state of the functional groups on the particulate surface, and oxygen content/surface charges. Maybe the binding of Fe to the graphene network, especially in the graphene oxide (group 2), impairs the transport of oxygen in cells. The impaired transport could be compensated for by the increased amount of haemoglobin. Nevertheless, the microcytosis could be due to the membrane damage in the cell [31].

On the other hand, slight changes in coagulation and liver enzyme were found but without statistical significance (data not shown), in agreement with other authors [30,32,33].

Significant histological alterations were not found in the organs of the rats inoculated with graphene (group 1) or graphene oxide (group 2) with respect to the control group (group 0) (data not shown). On the other hand, a slight and not statistical significant increase in the hepatic enzymes in both treated groups (1 and 2) could be observed in accordance with references [21, 30]. In addition, a larger amount of prothrombin and thromboplastin in the treated group was observed but without statistical significance [18, 31]. Feng R, Yu et al [32] also found some alterations only in the thromboplastin.

4. Conclusions

- Films grown on CoCr by cyclic voltammetry resulted in graphene in which oxygen functional groups are partially reduced and deposited on the CoCr surfaces.
- The presence of ErGO films on the CoCr surfaces does not cause significant changes in the good corrosion behavior of CoCr alloys.
- Macrophage biocompatibility of CoCr surfaces improves with the deposition of the electrochemical reduced graphene oxide films, that increase with the exposure time to the material. The ErGO films on CoCr discs make a friendly surface for macrophages as their homogeneous distribution evidences.
- Graphene causes a decrease in the size of the red blood cells that is offset by an increase in the haemoglobin concentration. Further work must be done to verify these findings.

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