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Macrophage biocompatibility of CoCr wear particles produced under polarization in physiological hyaluronic acid solution

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ABSTRACT

Macrophages are cells involved in the primary response to debris derived from wear of implanted CoCr alloys. The biocompatibility of wear particles from a high carbon CoCr alloy produced under polarization in physiological hyaluronic acid (HA) solution was evaluated in J774A.1 mouse macrophages cultures. Polarization was applied to mimic the electrical interactions observed in living tissues. Wear tests were performed in a pin-on-disk tribometer integrating an electrochemical cell in phosphate buffer solution (PBS) and in PBS supplemented with 0.3% HA, physiological synovial fluid concentration, used as lubricant solution. Wear particles produced in 0.3% HA solution showed a higher biocompatibility in J774A.1 macrophages in comparison to those elicited by PBS. A considerable improvement in macrophages biocompatibility in the presence of 0.3 % of HA was further observed by the application of polarization at potentials having current densities typical of injured tissues suggesting that polarization produces an effect on the surface of the metallic material that leads to the production of wear particles that are macrophages biocompatible and less cytotoxic. The results showed the convenience to consider electric interactions together with other particles parameters, as are size and composition, to get a better understanding of the biological effects of the wear products.

Keywords: polarization; CoCr alloy; wear particles; hyaluronic acid; macrophages biocompatibility.

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1. INTRODUCTION

Macrophages are cells involved in inflammatory processes [1]. All orthopedic biomaterials may induce a biologic host response to generated wear debris, which is strictly dependent on the nature of the debris. Metal wear particles and metal ions from prosthetic devices may induce a cascade of adverse cellular reactions that may include inflammatory complications, macrophage activation, bone resorption, and, although rarely, neoplasia [2,3]. In this context, macrophages play a decisive role in the hostile inflammatory reactions that can lead to the loosening and implants failure.

Implanted metal surfaces in biological environments are exposed to cells and to physiological milieu interacting between them, an interaction that affects both, the cells and the metallic surface. Implanted metallic materials, such as CoCr alloys, undergo dissolution and formation of a passive film that is affected by factors such as pH, ions present in the physiological medium, temperature, and biopotentials. Biopotentials are natural electrical properties that control the normal growth and development of different types of cells and tissues [4,5]. When a tissue is injured its potentials undergo alterations to the normal potential of intact tissue [6,7]. Both, biopotentials and injury potentials are found in bone and these potentials induced between injured and intact tissues persist until the tissue heals. Potentials in injured tissue can span over hundreds of microns and are generated by current or ions flowing through the injured tissue [8,9] ranging over 10-100 mV/cm [10]. Assuming the resistivity of soft tissues to be 100 Ωcm [9,11] the resulting currents are in the 1-100 $\mu A/cm^2$ range [8,12]. Fukada and Yasuda have already described in 1957 the piezoelectric nature of the bone tissue [13]. Endogenous electrical properties of bone may play a role in the feedback mechanism of bone remodeling and development [14,15]. *In vivo*, these electrical signals work in collaboration to provide the correct environment for normal bone growth and development, but can be disrupted or altered by an injury after a trauma and during the healing process. Moreover, the

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resulting voltage gradients may induce modifications in the electrochemical potential of metallic implants and consequently may affect their surface properties.

Díaz *et al*. [16] recently characterized the CoCr alloy oxide films in a phosphate buffer solution containing 0.3% of hyaluronic acid, a concentration in the range reported for synovial fluid of healthy joints [17] and under potentials with current densities similar to those reported for injured tissues (1–100 μA/cm^2). Potentiostatic pulses applied during the growth of the CoCr oxide film produced a modification of the film that affected its chemical composition, thickness, and structure compared to the passive film formed in air [16]. These modifications induced surface heterogeneities at the atomic scale, geometric irregularities, such as nano-roughness, and a variation of the oxide composition [16]. Moreover, application of potentials of 0.7 V vs Ag/AgCl induced changes in the oxide layer with the formation of 10-50 nm diameter nanopores, uniformly distributed along the surface and an increase in Cr (VI) and Mo (VI) concentration [16].

Despite the presence of the passive film, metals are susceptible to corrosion, particularly in aqueous environments, which may affect the surrounding tissue. Corrosion events generate electrical currents due to electron transfer from ions in the solution to the metallic surface where reactions are occurring. Wear-corrosion phenomena and micromotion or fretting-corrosion mechanically removes material, including the passive film, causing continuous activation/repassivation cycles [18]. These continuous and dynamic processes not only weaken the surface performance but also lead to an increase in the debris around the implant. Wear debris is considered one of the main factors responsible for aseptic loosening of orthopedic endoprostheses [19,20]. Implant failure due to aseptic loosening, or osteolysis, may result from the release of wear debris or electrochemical ions generated during corrosion events [20-22].

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From the electrochemical point of view, the metallic surfaces of implants, the breakdown of the passive film under wear corrosion process causes a drastic decrease in the open circuit potential of the metal towards negative potentials, i.e., from the passive to active state. This situation can suppose a polarization of about 500-700 mV with respect to the original open circuit potential. The change from the passive to active state can be induced mechanically under wear and electrochemically applying anodic polarization on the tribological system. Several researchers have studied the wear corrosion processes by application of anodic potentiodynamic polarization under wear processes [23,24].

The object of this paper was to evaluate the biocompatibility of particles produced during wear-corrosion assays of a CoCr alloy at potentiodynamic range to cover a wide polarization window on the samples. The hyaluronic acid, one of the main lubricant molecules of the synovial liquid, was selected as the electrolyte for the generation of wear particles in conditions that represent more closely the prosthesis environment. Since macrophages are the main cells involved in the primary response to foreign bodies, cytotoxicity and biocompatibility of the wear particles were evaluated using these cells, measuring lactate dehydrogenase and mitochondrial activity respectively.

2. RESULTS AND DISCUSSION

2.1. WEAR-CORROSION TESTS

The interaction of physiological fluids with the bearing surfaces of hip implants is of great importance in the research of artificial joint lubrication, although this study has been so far little explored.

The effect of sliding of the alumina ball on the HCCoCr alloys is clearly shown in the drastic change in the open circuit potential. As an example, Figure 1 shows the change in the open circuit potential when the HCCoCr surfaces in PBS supplemented with 0.3 % HA (PBS-HA)

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are subjected to wear. The open circuit potential without wear was around -0.25 V vs Ag/AgCl, decreasing sharply when the alumina ball (pin) started the circular movement under 5 N load at 120 rpm. At this moment, the open circuit potential decreased until achieving values of about -0.55 V vs Ag/AgCl, i.e., about 300 mV, and remained constant until the end of the test. The reduction in the potential value towards more negative values indicates that the HCCoCr surface becomes electrochemically active. This variation is due to the breakdown of the passive film under sliding, promoting the release of metallic ions and particles.

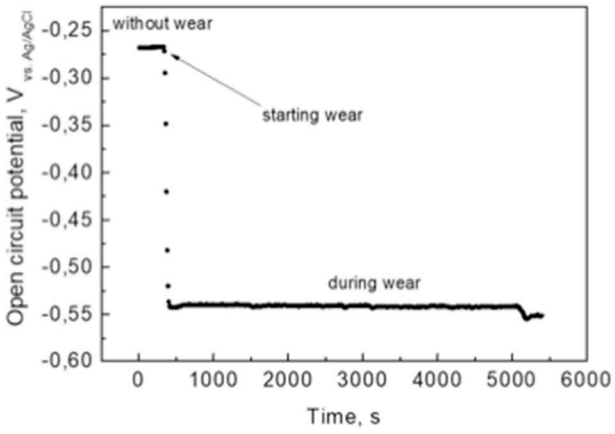

FIGURE 1

Figure 1. Open circuit potential of HCCoCr disks under wear. Open circuit potential was measured in PBS containing 0.3% HA (PBS-HA) during the performance of the wear corrosion test.

Figure 2 panel a and b shows the coefficient of friction (COF) for HCCoCr/alumina pair in PBS and PBS supplemented with 0.3 % HA (PBS-HA) during anodic potentiodynamic

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polarization and the anodic polarization curves drawn at 10 mV/min of HCCoCr in PBS and PBS containing 0.3 % HA under wear conditions (between point 1 and 2 in Figure 2a), respectively. The anodic polarization curve of HCCoCr in PBS-HA without wear has been also added in Figure 2b for comparative reasons. It can be seen that under sliding at the corrosion potential (before point 1 in Figure 2a), the COF was significantly higher in PBS than in PBS-HA. This result agrees with the hypothesis that the hyaluronic acid has a known lubricant role in the joint acting as a shock absorber [25] thus facilitating smooth joint movement by reducing friction between both surfaces. At the next stage (from point 1 to 2, in Figure 2a), the difference between both COF (in PBS and PBS-HA) is remained, but higher fluctuations were detected. The fluctuations could be related to the continuous formation of hard particulate matter that enhances friction between both counterparts and decrease the friction when is ejected from the track to surrounding areas where is accumulated (Figure 3). The deformation on the CoCr surfaces while sliding is applied activates mechano-chemical reactions causing not only the detachment of the passive film [26] but also bulk material resulting in an increase of COF. The hyaluronic acid in PBS maintains the lubricant effect during most of the wear corrosion tests.

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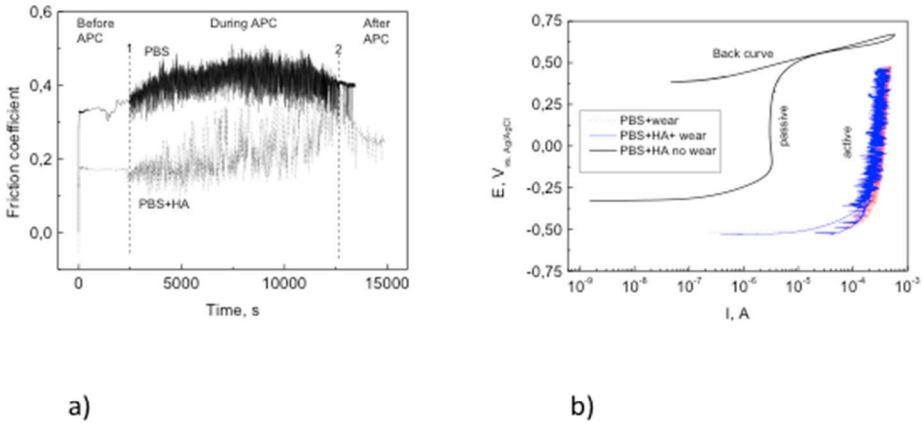

FIGURE 2

Figure 2. Friction coefficient of HCCoCr/alumina pair (a) and anodic polarization curves (APC) of HCCoCr disks (b) under wear corrosion tests. Wear corrosion tests were performed in the following solutions: PBS and PBS containing 0.3% HA (PBS-HA). Friction coefficient was measured before, during and after application of anodic polarization current (APC). Anodic polarization curve for HCCoCr alloy in PBS-HA without wear was also drawn for comparative analysis.

As a consequence of mechanically assisted corrosion, the passive film on the HCCoCr surface was rapidly broken in both media, PBS and PBS-HA producing an increase of approximately 3 orders of magnitude in current (Figure 2b) with respect to the anodic polarization curve without wear. Corrosion progresses on the wear track drawn by the sliding of alumina ball on the HCCoCr disks (Figure 3). Having in mind the wide passive region seen in the anodic polarization curve drawn without wear, (Figure 2) the potential applied could be employed in forming rapidly the new oxide film. However, the sliding rate is quick

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enough to avoid the repassivation and formation of new protective chromium oxides. All these results indicate that under these experimental conditions (5 N load and sliding rate of 120 rpm) the passive film is completely broken without possibility of reparation.

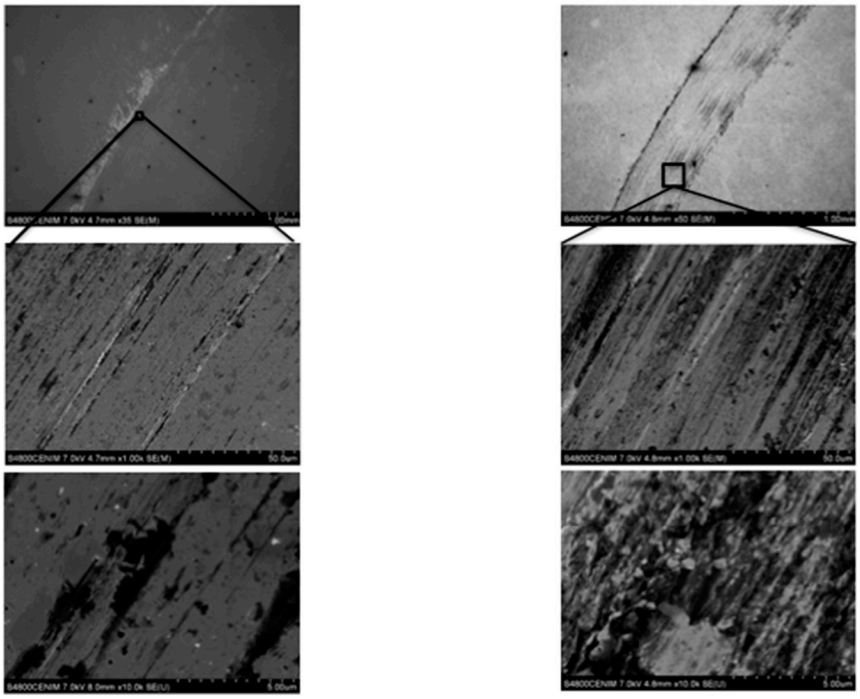


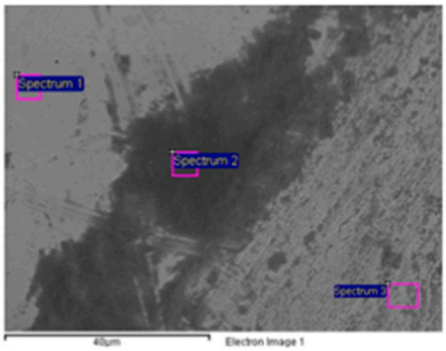
FIGURE 3

Figure 3. Secondary electron images of wear tracks on HCCoCr disks. Images by SEM of HCCoCr samples in PBS-HA under wear at the open circuit potential (PBS-HA) and applying anodic potentiodynamic polarization (PBS-HA+POL).

Figure 3 shows the secondary electron (SE) images of the tracks of HCCoCr in PBS-HA after wear corrosion tests, at the corrosion potential (PBS-HA) and under anodic potentiodynamic polarization (PBS-HA+POL). In both cases, debris is accumulated at the surrounding areas next to the wear tracks, but the surface inside the track is especially degraded when anodic potentiodynamic potential is applied. Figure 4 shows, as an example, the different spectra taken on the different area of interests in PBS-HA-POL outside and inside the track and on the debris accumulated around the track. There are some important facts that should be

highlighted. The first one is the high % C that contains the accumulated debris at the surrounding areas of the wear track in PBS-HA+POL. The second one is that Co/Cr ratio is mostly maintained outside the track and in the debris having a similar value of 1.85 and 1.71, respectively. It means that this deposit in the wear track is mainly composed of C and O, the greatest proportion probably coming from the hyaluronic acid. Table 1 shows the mean values of the chemical composition from EDX analysis inside and outside of the track in PBS-HA and PBS-HA+POL. It can be seen that in both cases, C content increases inside the track. In addition, the oxygen content is higher inside the track under polarization than inside the track at the corrosion potential.

PBS-HA+POL



Element	outside		Inside
	Spectrum 1	Spectrum 2	Spectrum 3
Co	58,15	14,22	45,74
Cr	31,45	7,69	26,79
Mo	3,76	1,00	3,32
C	6,95	61,94	13,60
O	-0,32	15,14	10,54

FIGURE 4

Figure 4. EDX analyses of wear tracks on HCCoCr disks. Secondary electron image and EDX analyses on different points outside and inside the track in HCCoCr disks in PBS-HA+POL sample.

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TABLE 1. Chemical composition by EDX analysis inside and outside of the track from the wear corrosion tests of CoCr samples in PBS+0.3 % HA at the open circuit potential and under anodic potentiodynamic polarization.

	Inside track		Outside track
Element	**PBS-HA at. % (mean ± σ)**	**PBS-HA+POL at.% (mean ± σ)**	**PBS-HA+POL at. % (mean ± σ)**
Co	43.40 ± 4.19	34.2 ± 4.12	56.26 ± 2.36
Cr	20.98 ± 5.68	20.50 ± 2.12	29.88 ± 1.34
Mo	3.63 ± 1.06	2.90 ± 0.84	3.69 ± 0.09
C	26.49 ± 2.77	25.85 ± 6.65	9.94 ± 2.62
O	3.84 ± 4.70	16.57 ± 5.18	0.22 ± 1.62

On the other hand, the morphology and chemical characterization of the wear particles revealed some interesting results. Figure 5 shows, as an example, the secondary electron image of wear particles collected from the tribocorrosion test in PBS containing 0.3% HA and the chemical composition of some of these particles. Metallic particles released from the HCCoCr surfaces during the wear test in PBS-HA were mainly composed of O, Cr with minor amount of Co (pt 1, 4 and 5, Figure 5). Usually, the smallest particles are enriched in chromium oxide, and therefore, are mainly produced from the removal of the protective oxide layer. However, Co percentage increases in the largest particles. All of them have phosphorous in their composition. Table 2 shows the chemical composition of wear particles collected from the wear corrosion tests in PBS, PBS-HA and PBS-HA+POL. The particles were mainly composed of chromium oxide in all cases. In previous assays, it has been proven by XPS (data not shown) that the immersion of the HCCoCr surfaces in PBS-HA causes a decrease in the Co species in the passive film and enrichment in chromium oxide. In addition, phosphorus is included in the oxide film of the HCCoCr surfaces immersed in PBS. The presence of P in the particles is explained by the adsorption of phosphate on the particle surface, which is provided by the physiological PBS. This means that most of the particles

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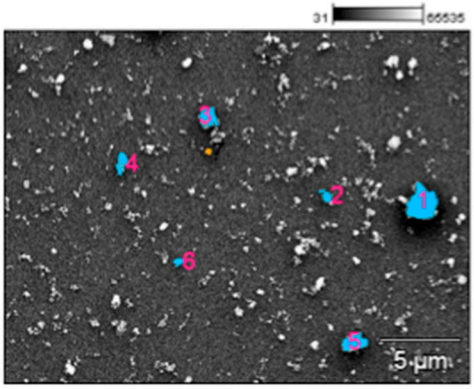
come from the native passive film (whose thickness is about 5-7 nm) and are mainly composed of chromium oxide and phosphate from the PBS.



	C Wt.%	O Wt.%	Al Wt.%	P Wt.%	Cr Wt.%	Co Wt.%
pt1	0	34,02	0	16,87	35,3	13,81
pt2	0	41,31	0	20,24	38,45	0
pt3	0	33,72	0	23,16	43,13	0
pt4	0	41,95	0	15,76	30,75	11,54
pt5	0	23,79	10,62	26,36	34,38	4,85
pt6	0	54,36	0	16,31	29,33	0

FIGURE 5

Figure 5. Secondary electron images and chemical composition of wear particles. Particles (pt) were collected from wear corrosion tests performed in PBS containing 0.3% HA (PBS-HA) and deposited on silicon wafer to analyze their chemical composition.

TABLE 2. Chemical composition of wear particles collected from wear corrosion tests in PBS, PBS containing 0.3% HA (PBS-HA) and PBS containing 0.3% HA under anodic polarization (PBS-HA+POL).

	O (mean ± σ)	Cr (mean ± σ)	Co (mean ± σ)	P (mean ± σ)
PBS	40.10±9.9	33.42±6.5	4.31±5.6	18.59±4.6
PBS-HA	38.19±9.4	35.22±4.6	5.03±5.7	19.78±3.9
PBS-HA+POL	10.91 ±6.5	38.52±9.8	36.46±16.7	9.409±6.8

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Nevertheless, the application of polarization during wear tests changed the chemical composition of the particles collected. In this case, the wear particles produced under anodic polarization increased the Co/Cr ratio. As wear particles obtained without polarization, these particles also contained P although in a lower proportion.

The potential applied on the HCCoCr also induced a change of the chemical composition of the passive film. Díaz *et al*. established that the increase in polarization (from 0.5 to 0.7V) induced the preferential dissolution of cobalt whereas chromium was concentrated in the surface oxide film [16]. The passive film grown at potential of 0.5 $V_{vs\ Ag/AgCl}$ (into the passive region of the anodic polarization curve) consisted predominantly of Cr_2O_3 and $Cr(OH)_3$. However, the oxidation at a potential of 0.7 $V_{vs\ Ag/AgCl}$ caused the appearance of Cr (VI) in the passive film.

The continuous sliding of the alumina ball on the HCCoCr surface under anodic potentiodynamic polarization, however, did not allow the regeneration of the oxide film creating an active state where base material was directly exposed to the electrolyte without enough time to build the new oxide film. Considering this situation, the results reveal that the anodic polarization on CoCr surfaces under wear processes accelerated and induced the release of larger metallic particles with higher Co content probably coming from the base material.

2.2. MACROPHAGE CELL RESPONSE

Since macrophage is a primary immune cell type resident in tissues and is the main cellular type involved inflammatory process [1] is necessary to evaluate the response of this cell line to wear particles generated from the implanted materials in order to know its biologic host response. Here macrophage response was evaluated by measuring the effect of wear particles in cell toxicity and in respiratory activity.

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Cytotoxicity induced by HCCoCr wear particles obtained in the different tribocorrosion tests was analyzed by measuring LDH activity released from cells (Figure 6), whose levels increase upon plasma membrane damaged being a sign of cell death [27]. As is shown in Figure 6, exposure of macrophages cultures to wear particles induced a degree of cytotoxicity that was mainly dependent of the conditions used during wear-corrosion assays and particle concentration. As shown in Figure 6 panel A, particles concentration of 0.5 mg/ml obtained in PBS produced almost 58 % cytotoxicity, a percentage that was highly reduced to almost 12 % when wear particles were generated from tribocorrosion tests in presence of 0.3 % of hyaluronic acid, an effect that could indicate a protective role of the hyaluronic acid on the metallic surface under wear stress conditions. Concentrations of 1 mg/ml of wear particles from PBS test produced an increase in the macrophage cytotoxicity to almost a 75 %, a value elevated in comparison with the cytotoxicity induced by the wear particles obtained in PBS containing 0.3 % of HA, where cytotoxicity reached a 14 % (data not shown). No additional increase in the cytotoxicity was observed at higher concentrations of wear particles (2 mg/ml) generated in PBS as macrophages cytotoxicity appeared comparable to the one elicited by exposure to lower concentrations of particles (0.5 and 1 mg/ml), where approximately a 64 % cytotoxicity was detected (data not shown). Concentrations of 2 mg/ml of particles produced in PBS containing 0.3 % of HA elicited an increase in the macrophages cytotoxicity that reached almost a 46 % (Figure 6, panel B). Although such increase was higher than the one produced by concentrations of 0.5 and 1 mg/ml, which was 12 and 14 % respectively, it was considerably reduced to a 24 % when polarization conditions characteristic of damage tissue were applied (Figure 6, panel B). These results indicate that the biopotential of an injured tissue, here applied as anodic polarization, in combination with concentration range of HA reported for synovial fluid [17], have a positive synergistic effect on the biocompatibility of wear particles.

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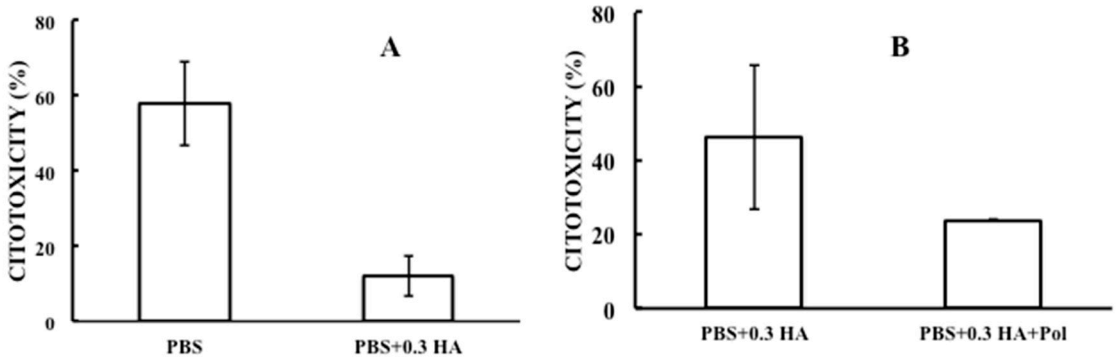

FIGURE 6

Figure 6. Macrophage cytotoxicity, measured as LDH activity, of cell cultures exposed for 72 hours to HCCoCr wear particles. Panel A: Exposure of macrophages culture to 0.5 mg/ml wear particles. Particles were obtained in PBS and in PBS containing 0.3% HA (PBS-HA). Panel B: Exposure of macrophages culture to 2 mg/ml wear particles obtained in PBS containing 0.3% HA with and without polarization application, PBS-HA+POL and PBS-HA respectively. Experimental data were done as independent triplicate.

Application of anodic polarization during tribocorrosion assays generated wear particles from CoCr alloy that affected macrophage mitochondrial activity response. It is well known that mitochondrial activity measurement is directly proportional to the number of metabolically active cells in culture that is for this reason a measure of cell viability and biocompatibility [27]. As shown in Figure 7 panel A, wear particles derived from HA solution tests produced a progressive reduction in the respiratory response of macrophages that is wear particle dose-dependent. This effect was dramatically reduced when polarization was applied during wear-

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corrosion tests as no significant effects in the respiratory activity were observed (Figure 7 panel B). These results suggest that polarization treatment in combination with presence of 0.3 % of hyaluronic acid produces changes in material behavior that seems to be beneficial to cell viability and biocompatibility.

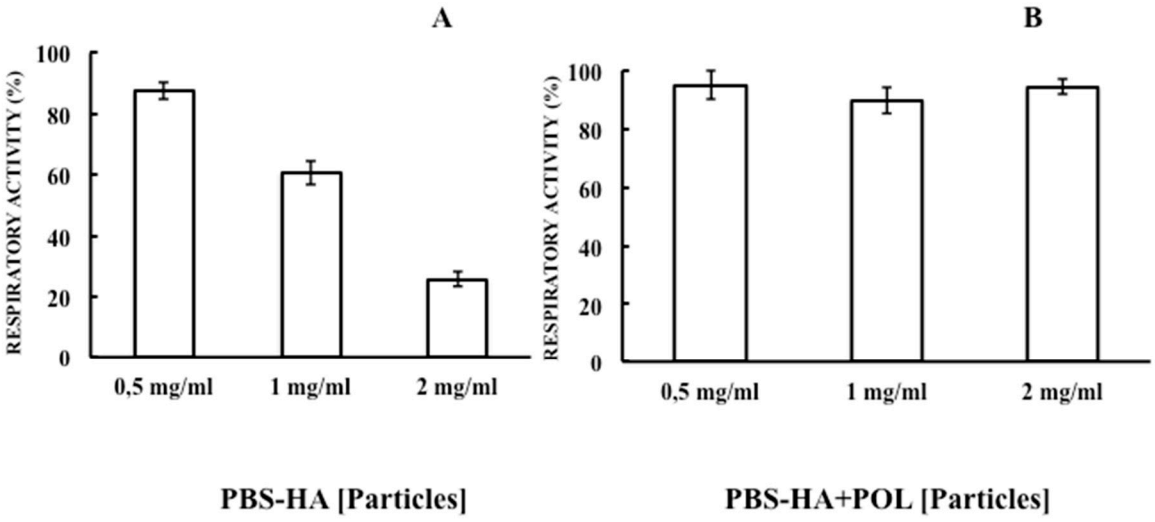

FIGURE 7

Figure 7. Mitochondrial activity of macrophages cell cultures exposed for 72 hours to different doses of HCCoCr wear particles. Wear particles were obtained in PBS containing 0.3% HA (PBS-HA) without (panel A) and with polarization (panel B; PBS-HA+POL). Cell cultures were exposed to the following wear particles concentrations: 0.5, 1 and 2 mg/ml. Experiments were done as independent triplicate.

In summary, the in vitro assays seem to indicate that the particles from wear corrosion in PBS supplemented with 0.3% hyaluronic acid under anodic polarization are more biocompatible and less cytotoxic. At this point, three different characteristics of the wear particles must be considered: size, number and chemical composition. It is known that particles size is

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inversely proportional to the cytotoxicity degree [28,29], a data that could explain why particles from wear corrosion tests in PBS and in PBS supplemented with 0.3% HA in the absence of anodic polarization elicited a lower macrophages cell biocompatibility, as in these samples a higher proportion of small particles were detected (data not shown). The particles produced in these solutions and in these experimental conditions proceed from the breakdown of the passive film formed in the material surface where an enriched in chromium oxide was observed, a compound with high toxicity [30]. It is also necessary to consider that macrophages assays here reported were done taking in consideration the concentration of particles, that means that for the same total mass the proportion of particles with small size probably is higher in the assays performed in absence of polarization, either in PBS or PBS+0.3% HA, that could also give explanation for the lower macrophage biocompatibility of these particles.

As it is here reported, the wear particles from corrosion tests under polarization in a solution of PBS containing a concentration of 0.3 % HA have larger size and a chemical composition with higher content in Co, suggesting that the application of anodic polarization during wear tests promotes the detachment of larger particles from the HCCoCr base material, particles size and composition that could account for the lower cytotoxicity observed.

3. Materials and methods

3.1. Material

A high carbon CoCr alloy (hereafter HCCoCr) that complies with ASTM F75 standard was used as material. HCCoCr composition is shown in Table 3. “Double heat-treated” disks, i.e. solution treatment (ST) followed by hot isostatically pressing (HIP), of 38 mm in diameter and 4 mm thickness, were obtained from BIOMET Spain Orthopaedic (Valencia, Spain). The

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sample preparation consisted of grinding on SiC paper, followed by mechanical polishing with 3 µm diamond paste.

TABLE 3: Chemical composition (wt %) of High Carbon CoCr alloy**.**

	C	Co	Cr	Mo	Ni	S	P	Al	W	Mn	Fe	Si	N	Ti	Cu
HC	0.22	62	29.4	6.4	0.1	0.004	0.001	0.01	0.03	0.7	0.16	0.7	0.16	-	-

3.2 Wear-corrosion tests under electrochemical control

Wear-corrosion experiments were carried out on a pin-on-disk tribometer. 6 mm diameter alumina ball pins were used as HCCoCr disk counterpart. The HCCoCr disks were 38 mm diameter and 4 mm thickness. Both disks and pins were previously washed with double distilled water and cleaned in an ultrasonic ethanol bath for 10 min. The alumina pins were placed in a pin plastic holder and fixed on the load cell. A low normal load of 5N was applied on the counterpart. The working electrode motion was provided by a rotating motor at a rotation rate of 120 rpm that produced, at the end of the alumina ball, a circular wear track (5 mm in diameter) on the HCCoCr disk surface.

The tribometer configuration consisted of an integrated electrochemical cell (3-electrode cell) including the HCCoCr disk as working electrode, a ring-shaped Pt wire counter electrode and a saturated Ag/AgCl reference electrode. All the potentials of HCCoCr disks during the wear corrosion tests were measured versus the reference electrode. Wear-corrosion tests were performed in two different aqueous media: Phosphate Buffer Solution (PBS) containing 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 8 g/L NaCl and 1.150g/L Na_2HPO_4 (anhydrous) and PBS

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supplemented with 0.3 % hyaluronic acid (HA), a concentration range reported for the synovial fluid of healthy joints [17].

The wear-corrosion behavior was studied simultaneously measuring the friction coefficient and electrochemical parameters. The wear corrosion procedure that appears in Figure 8, was the following:

- ***Before wear (no sliding):***
    - Measurement of the corrosion potential for 10 minutes.
- ***Under sliding (at 120 rpm, 5N):***
    - Simultaneous measurement of the corrosion potential and the coefficient of friction (COF) for 40 min.
    - Under anodic potentiodynamic polarization, simultaneous measurement of current and coefficient of friction (COF) for 200 minutes**.** The anodic potentiodynamic polarization was applied from the corrosion potential to a polarization of 1 V at a scanning rate of 10 mV/min, and back curve was drawn until reaching the corrosion potential. The back curve was also measured to analyze the repassivation ability of the HCCoCr alloy.

For comparative purposes, the anodic potentiodynamic polarization without wear in PBS and PBS-HA was also measured.

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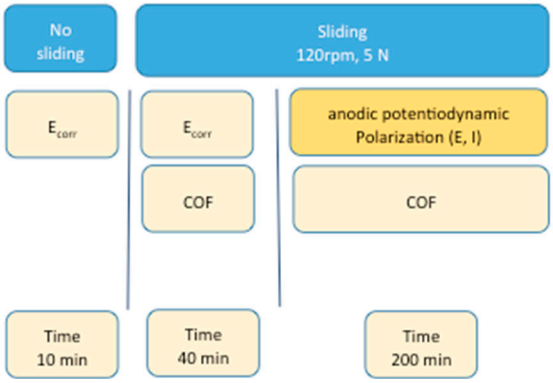

FIGURE 8

Figure 8. Schema of the experimental procedure of the wear corrosion tests.

Surface characterization of the worn surface after wear corrosion tests under polarization and without polarization was performed by profilometry and using a JEOL-6500F microscope equipped with a field Emission Gun (FEG) coupled to an Energy Dispersive X-ray (EDX) spectrometer. Secondary electron (SE) images were taken at 7,5 keV and EDX analysis were performed at 20 keV.

3.3. Characterization of particles

Debris from tribocorrosion tests performed in PBS, PBS containing 0.3 % hyaluronic acid (both without applying polarization) and in PBS supplemented with hyaluronic acid under anodic polarization was collected for the subsequent characterization. Metallic particles were isolated, purified and characterized following the protocol developed by Billi *et al.* for metal particles [31]. This procedure allows exhaustive removal of organic and inorganic impurities

from the metallic particles. To completely digest the hyaluronic acid, metal particles in PBS supplemented with 0.3% HA were digested adapting the protocol developed by Kavanaugh *et al*. [32]. Specifically, 150 µl 1M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid HEPES (pH 7.5), 6 µl 1M $MgCl_2$ and 75 µl 0,5M $CaCl_2$ were added in a centrifuge tube (Biologix) to a volume of metallic particles from 0.3 % (3 g/L) hyaluronic acid solution until complete a final volume of 3 ml. Then, 300 µl of a solution containing 0.05 % hyaluronidase enzyme in 0,1 M NaH_2PO_4 and 0,15 M NaCl were added to the mixture and incubated at 37 ºC for 24 hours in an orbital agitator at 250 rpm. After this time, the calcium excess in the mixture was eliminated by adding 1ml of 24 mM ethylenediaminetetracetic acid (EDTA) that was incubated at 37 ºC for 3 hours in an orbital agitator at 250 rpm.

Wear corrosion media (PBS and the digested hyaluronic acid solutions) containing metallic particles were rotated at 28 rpm for 24 h at room temperature to disperse the metal particles evenly before their isolation. The particles were then purified via density gradient centrifugation through multiple layers of denaturants and metal-selective high-density layer. This led to well-dispersed particles deposited onto a 5 mm x 5 mm featureless display silicon wafer (Ted Pella, Inc., Redding, CA, USA) coated with a monolayer of marine mussel glue (Cell-TakTM; BD Biosciences, San Jose, CA, USA). The silicon wafer was then coated with 10Å iridium.

The morphology of metallic particles was studied with a field emission scanning electron microscope (FE-SEM) (Supra VP-40; Zeiss, Peabody, MA) at a voltage of 15 kV and chemically analyzed by means of Energy-dispersive spectroscopy (EDS) analysis (Thermo Ultradry feature sizing system; Thermo Electron Scientific Instruments, Madison, WI).

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3.4. Macrophages cell cultures assays

The biocompatibility of wear-particles was tested in a mouse macrophage cell line (J774A.1), from DSMZ Human and Animal Cell Bank. Macrophages cell cultures were exposed to different concentrations of wear particles.

Wear-particles obtained from the wear-corrosion tests were centrifuged and the particle pellet was weighted, UV sterilized for 15 minutes and resuspended in sterile bi-distillated water and maintained in aliquots at -20 ºC until use. Wear particles, just before cell cultures assays, were thawed, resuspended by vigorously mixing with a vortex, and diluted at a concentration of 20 mg/ml 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 g/ml, Gibco, BRL), named as complete cell culture medium. A concentration of 20 mg/ml was used as stock solutions for the particles concentration tested in different cell assays. To assure a polydisperse distribution of the particles vigorous vortexing was applied in all experimental steps that required particles manipulation.

To evaluate the effect of HCCoCr particles on cell cultures, macrophages were seeded on a 96-well culture plates at 75,000 cells/ml cell density in complete cell culture medium. A final volume of 100 µl of cell suspension in complete cell culture medium was added to each well of 96-well plates. After 24 hours in culture, cell media were removed and replaced by 100 µl of fresh complete cell culture medium containing the following concentrations of HCCoCr particles: 0, 0.5, 1 and 2 mg/ml. Cell cultures were maintained for 72 hours in a cell culture chamber at 37 ºC and 5 % CO_2. Incubation time was selected based on the set-up of cell cultures assays for metallic particles studies carried out in the lab. Mitochondrial activity (WST-1 assay) and plasma membrane damage (LDH assay) were used to evaluate the biocompatibility and cytotoxicity respectively as described below [27].

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3.5. Mitochondrial Activity Measurement

Reduction of the WST-1 reagent (4-[3-4-iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (Roche Diagnostics GmbH, Mannheim, Germany)) was used to evaluate the effect of different concentrations of the HCCoCr wear particles on mitochondrial activity of macrophages cultures. The mitochondrial activity measurement is directly proportional to the number of metabolically active cells in culture. After 72 hours in culture, 10 µl of the cell proliferation kit reagent WST-1 was added to each well containing 100 µl of fresh complete cell culture medium, and the mixture was incubated inside the cell culture incubator for 30 minutes. After incubation, 100 µl of each reaction mixture were transferred to a 96-well cell plate, and the absorbance of the samples were measured as differential absorbance, 415 nm minus 655 nm, in an iMark microplate absorbance reader (Bio-Rad, CA, USA), using the absorbance given by complete cell culture medium as a blank. All experiments were carried out in triplicate.

3.6. Measurement of Lactate Dehydrogenase Activity

To measure and quantify the effect of HCCoCr wear particles on cell death and cell lysis, lactate dehydrogenase (LDH) activity was measured in the supernatants of cell cultures by an enzymatic assay using the Cytotoxicity Detection Kit^{plus} (Roche Diagnostics GmbH, Mannheim, Germany). Supernatants were collected from cell culture after being exposed for 72 hours to different HCCoCr particles concentrations and were centrifuged for 5 minutes at 1024g. The enzymatic assays were performed according to the LDH kit protocol provided by Roche Diagnostics. Complete cell culture medium was used as a control for absorbance base line. LDH activity was measured based on differential absorbance, 490 nm minus 655 nm, in an iMark microplate absorbance reader (Bio-Rad, CA, USA). LDH catalyzes the conversion of lactate to pyruvate, reducing NAD^+ to $NADH/H^+$, which is used by the catalyst to reduce a

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tetrazolium salt to a formazan salt, which is responsible for the change in absorbance at 490 nm. Quantification of LDH activity is used as an indicator of plasma membrane damage, as is a stable cytoplasmic enzyme present in all cells and rapidly release into the cell culture supernatant when the plasma membrane is damaged being a sign of cell death. The percentage of cytotoxicity is calculated taking as control a total cell lysate in the absence of any particles. The percentage cytotoxicity was calculated as follow: Cytotoxicity (%) = [(exp. value – low control) / (high control - low control)]× 100; where experimental value (exp. value) corresponds to the absorbance of the treated sample in study exposed to wear HCCoCr particles, low control is the absorbance from the untreated cell cultures with no particles, that corresponds to spontaneous LDH released and high control is the absorbance value obtained after total cell cultures lysis that corresponds to the maximum releasable LDH activity. The background absorbance corresponding to complete cell culture media was subtracted from the absorbance of all samples before cytotoxicity calculations. All experiments were carried out in triplicate.

4. CONCLUSIONS

- The cytotoxicity and the viability showed here suggest that the simultaneous use of anodic polarization conditions in an attempt to simulate currents associated with the biopotentials present in injured tissue in presence of 0.3 % of HA in the wear solution produces an effect in the metallic material that generates wear particles that are macrophages biocompatible and less cytotoxic.
- It is necessary to considerer different parameters characteristics of the prosthesis environment, not only particle size and composition and but the polarization, in order to have a closer view of the biological consequences of the wear products derived from implanted CoCr alloys.

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- As more parameters from the prosthesis environment are considered in *in vitro* assays to study cell-biomaterial interaction, as are the electric interactions, a better knowledge of the different processes that are taking place *in vivo* at the cell-biomaterial interface will be obtained.

CONFLICTS OF INTEREST

There are no conflicts of interest to declare. The authors will receive no benefit of any kind either directly or indirectly.

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