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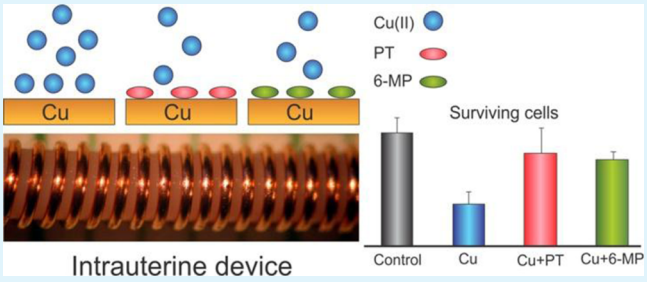
Decrease in Cytotoxicity of Copper-Based Intrauterine Devices (IUD) Pretreated with 6-Mercaptopurine and Pterin as Biocompatible Corrosion Inhibitors

Florencia Alvarez,[†] Claudia A.Grillo,[†] Patricia L.Schilardi,[†] Aldo Rubert,[†] Guillermo Benítez,[†] Carolina Lorente,[†] and Mónica Fernández Lorenzo de Mele*[,†,‡]

[†]Instituto de Investigaciones Fisicoquímicas Teóricas y Aplicadas (INIFTA, CCT La Plata −CONICET), Departamento de Química, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CC 16, Suc. 4 (1900) La Plata, Argentina.

[‡]Facultad de Ingeniería, Universidad Nacional de La Plata, Calle 47 y 1, (1900) La Plata, Argentina

ABSTRACT: The copper intrauterine device (IUD) based its contraceptive action on the release of cupric ions from a copper wire. Immediately after the insertion, a burst release of copper ions occurs, which may be associated to a variety of side effects. 6-Mercaptopurine (6-MP) and pterin (PT) have been proposed as corrosion inhibitors to reduce this harmful release. Pretreatments with 1×10^{-4} M 6-MP and 1×10^{-4} M PT solutions with 1h and 3h immersion times were tested. Conventional electrochemical techniques, EDX and XPS analysis, and cytotoxicity assays with HeLa cell line were employed to investigate the corrosion behavior and biocompatibility of copper with and without treatments. Results showed that copper samples treated with PT and 6-MP solutions for 3 and 1 h, respectively, are more biocompatible than those without treatment. Besides, the treatment reduces the burst release effect of copper in simulated uterine solutions during the first week after the insertion. It was concluded that PT and 6-MP treatments are promising strategies able to reduce the side effects related to the "burst release" of copper-based IUD without altering the contraceptive action.



KEYWORDS: *intrauterine device, copper, copper ions, pterin, 6-mercaptopurine, cytotoxicity*

1. INTRODUCTION

Copper-based intrauterine device (CuIUD) is a highly effective reversible contraception method used by more than150 million women (about 15% of the world's women in reproductive age). Apart from their high effectiveness they are a low-cost and long-lasting method. Their contraception action is mainly based on the uninterrupted release of cupric ions from this device. This release of ions induces inflammatory reactions in the endometrium in response to the foreign elements, creating an unfriendly environment that decreases the motility and viability of sperm and the receptivity of the endometrium to implantation of embryos.[1−4]

Two of the most popular T-shaped IUDs are TCu380A and TCu 220C. Cao et al.[5] and Gao et al.[6] informed that the highest corrosion rates of these devices in simulated uterine fluid (SUF) were obtained during the first three days after immersion. Afterward, a dramatic reduction in the copper dissolution was observed, confirming that the "burst release" effect mainly occurs during the first week after immersion in SUF. Notwithstanding this, the IUD effectiveness does not decrease after the initial period because copper amounts as low as 2 μg/day (measured for IUD with indometacin)[7] have proved to be enough to guarantee the contraception action.

Various side effects were frequently informed associated with the first week after implantation. During the follow up period, the most frequent complaints, mostly related to uterine bleeding, pelvic pain, intermenstrual spotting, and excessive bleeding, decrease markedly over time, mainly after the first month following insertion.[7] It has been demonstrated that copper ion burst release may affect the surrounding cells because they are exposed to cytotoxic levels of Cu ions.[8]

On the basis of previous arguments, the decrease in the initial high release of copper may be a way of reducing the side effects and cytotoxicity problems. Recently, different methods were proposed to improve the performance of IUDs, reducing the harmful effects.[9,10] The use of biocompatible organic corrosion inhibitors is proposed here as strategy for controlling copper dissolution within the initial period after the insertion of the IUD. Pterin (PT) (Figure 1a) and 6-mercaptopurine (6-MP) (Figure 1b) are proposed here as possible biocompatible inhibitors.

Pterin is one of the larger families of bicyclic N-heterocycles named pteridines. Pterin refers to a pteridine specifically substituted by an aminogroup at position 2 and a ketogroup at position 4 (Figure 1a).[11] Pterin derivatives have been proposed as antitumor agents.[12−14] The polarographic behavior of pterin

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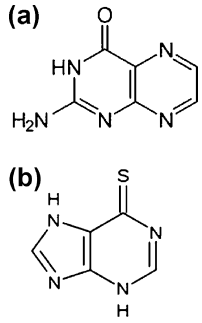

Figure 1. Molecular representation of (a) PT and (b) 6-MP.

in the presence of metallic ions has been studied by Kucharska (1997).[15] However, to the best of our knowledge, its behavior on copper surfaces is currently unknown.

6-MP is a plan molecule that can attach on metal surfaces with possible binding sites of N1, N3, N7, N9 and S10 (Figure 1b).[33] The thiol form is in equilibrium with its tautomer (Figure 1b). The adsorption of 6-MP on several metals has been extensively studied by electrochemical techniques, SERS, scanning tunnelling microscopy (STM) and X-ray photoelectron spectroscopy (XPS) and multiple-angle-of-incidence-plarization-and-infrared-reflection-adsoption-spectroscopy (MAI-PIRRAS) particularly in relation to its chemisorption on Au, Ag, Hg, and carbon nanotubes modified graphite electrodes to form self-assembled monolayers.[16−25] Conversely, no information in relation to 6-MP behavior on copper electrodes can be found.

6-MP has been utilized for many years as a chemotherapy drug and is increasingly employed in the treatment of a variety of diseases, including rheumatologic disorders, immuno diseases, post-transplant immonosuppression, inflammatory bowel disease, and lymphoblastic leukemia.[16] Additionally, it has been immobilized on gold nanoparticles to form bioanalytical and biomedical applications.[24]

In this article, the potential action of PT and 6-MP as weak corrosion inhibitors to reduce burst release effect is analyzed. In addition, their effect on lisosomal and mitochondrial activities in HeLa cells was investigated to test their biocompatibility in vitro.

2. MATERIALS AND METHODS

2.1. Electrochemical Tests. The electrochemical cell used in the measurements has double walls to allow the circulation of water at constant temperature. All measurements were made at 37.0 ± 0.1 °C. A platinum foil was used as counterelectrode and a saturated calomel electrode (SCE) as reference electrode. The potential values in the text are referred to the SCE. Copper electrodes (99.7% electrolytic metal copper, Merck, Darmstadt, Germany) were used as disks (1 cm diameter), whose lateral surface was covered with epoxy resin, leaving an exposed area of 0.785 cm^2. Each electrode was mechanically polished with emery paper of different grain sizes using water as lubricant and then washed with water and ethanol, and dried with nitrogen. The electrode surface was carefully observed under optical microscope (Olympus BX51, Olympus Corp., Tokyo, Japan), before and after the experiments, to evaluate possible changes of color and texture of copper. The electrodes were pretreated by immersion for 1 and 3 h in PT (1×10^{-4} M) and 6-MP (1×10^{-4} M) water solutions, respectively. Occasionally, ethanolic solutions were used for comparison. The tests were conducted in SUF whose composition is summarized in Table 1. This solution, formulated by Zhang et al.,[26] is widely accepted as a suitable medium to simulate the uterine fluid.[6,27−29] Considering that the pH of the uterine fluid is in the 6.0−8.0 range, tests were conducted in SUF at pH 6.0, which represents the condition with the higher copper release.[26] SUF was prepared with analytical grade chemicals and bidistilled water. Before each test, the electrolyte medium was deareated with pure nitrogen for 60 min. The polarization curves were carried out in duplicate and the results were repetitive. Polarization curves were performed at 1 mV s^{-1} from the open circuit potential to 0.0 V and potentiostatic current transients were measured at −0.1 V. In all cases a Radiometer potentiostat PGP201 was used.

2.2. Surface Analysis by EDX and XPS. XPS measurements were performed using a Al Kα source (XR50, Specs GmbH) and a hemispherical electron energy analyzer (PHOIBOS 100, Specs GmbH) operating at 40 eV pass energy. A two-point calibration of the energy scale was performed using sputtered cleaned gold (Au 4f7/2, binding energy (BE) = 84.00 eV) and copper (Cu 2p3/2, BE = 932.67 eV) samples. C 1s at 285 eV was used as charging reference.

EDX analysis was made using Apollo 40 Silicon Drift Detector.

2.3. Colorimetric Determination of Copper Concentration. Copper wires (0.3 mm diameter and 190 mm^2 area) similar to those used in copper-based IUD, with and without PT- or 6-MP treatments, were immersed in 3 mL of SUF (without glucose) and the concentration of cupric ions was colorimetrically determined by adding 1-(2 pyridylazo)-2-naphthol (PAN) to the samples. This dye forms colored complexes with a large number of metal ions, including Cu(II)[30,31] which are suitable for spectrophotometric analysis. Calibration curves were carried out by measuring the absorbance of the complex formed by the addition of 1 mL of appropriate dilutions of $CuSO_4.5H_2O$ (Baker) 1.7×10^{-2} M aqueous dissolution (concentration range: 1.7×10^{-6} M to 1.7×10^{-4} M, 6 points) to 1 mL of H_2SO_4 (Merck) 0.25 M and 1 mL of PAN (Fluka) 4×10^{-3} M ethanolic dissolution and brought to 10 mL with water. The absorbance was measured in a Perkin-Elmer Lambda 35 UV−visible spectrometer at 560 nm, maximum of the absorption spectra of the copper(II)−PAN solution. To quantify the amount of copper released, we repeated the procedure described above but replacing the $CuSO_4.5H_2O$ dissolutions by the same volume of the copper-containing SUF sample. Each measurement was performed in duplicate. For each set of measurements, the corresponding calibration curve was made by using fresh PAN dissolution. Exposure periods between 12 and 168 h (7 days) were assayed.

2.3. Cytotoxicity Evaluation. *2.3.1. Cell Cultures.* The human cervical cancer HeLa cell line was originally obtained from American Type Culture Collection (ATCC) (Rockville, MD, USA). Cells were grown as monolayer in Falcon T-25 flasks with D-MEM culture medium (GIBCO-BRL, Los Angeles, USA) supplemented with 10% inactivated fetal calf serum (Natocor, Carlos Paz, Córdoba, Argentina), 50 IU/mL penicillin and 50 mg/mL streptomycin sulfate (complete culture medium) at 37 °C in a 5% CO_2 humid atmosphere. Cells were counted in an improved Neubauer hemocytometer and viability was determined by the Trypan blue (Sigma, St. Louis, MO, USA) exclusion method; in all cases, viability was higher than 95%.

2.3.2. Evaluation of Cytotoxicity of PT and 6-MP. Cytotoxicity of corrosion inhibitors in HeLa cells was estimated using (1) metabolic competence by the colorimetric method of Mosmann (Mosmann 1983) as modified by Twentyman and Luscombe (Twentyman and Luscombe 1987), that measures the reduction of tetrazolium salt (MTT) (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to formazan by dehydrogenase enzymes of intact mitochondria

Table 1. Composition of the Simulated Uterine Fluid

	$NaHCO_3$	$NaH_2PO_4 \cdot 2H_2O$	$CaCl_2$	KCl	NaCl	glucose	urea
composition (g/L)	0.25	0.072	0.167	0.224	4.97	0.50	0.48

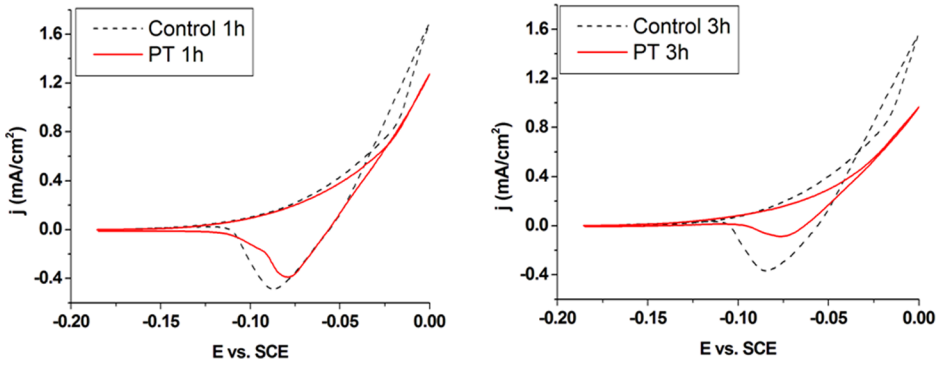

Figure 2. Polarization curves at 1 mV s^{-1} recorded in SUF with pretreated copper electrodes (PT 1 × 10^{-4} M) for 1 and 3 h.

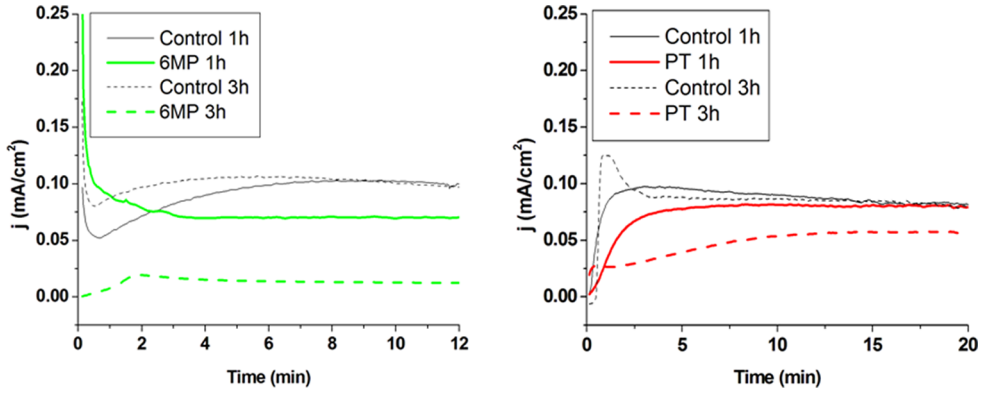

Figure 3. Current transients recorded in SUF with untreated and pretreated copper electrodes (PT 1 × 10^{-4} M and 6-MP 1 × 10^{-4} M) for 1 and 3 h.

in living cells and (2) Neutral Red (NR) (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) assay according to Borenfreud and Puerner.[32] This assay measures cellular transport based on the dye uptake by living cells.

For these analyses, 2.7 × 10^3 cells/well were cultured in a 96-multiwell plate and grown at 37 °C in 5% CO_2 humid atmosphere in complete culture medium for 24 h. This medium was then replaced with different corrosion inhibitors concentrations during 24 h. Dose range used in cytotoxic assays was 1 × 10^{-7} to 1 × 10^{-5} M both for PT and 6-MP. This concentration range was estimated theoretically considering a total released of the inhibitory monolayer-coating by the copper disk calculated according to previous reports.[21]

For MTT assays, after treatment with inhibitors, medium was removed, cells were washed with phosphate buffered solutions (PBS) and fresh medium containing MTT reagent (1 mg/mL final concentration) (Sigma, St. Louis, MO, USA) was added. After 3 h incubation, cells were washed again with PBS. Color was developed by the addition of 100 μL of dimethylsulfoxide (DMSO) (Merck, Química Argentina SAIC, Argentina) to each well for cell lysis and formazan crystals dissolution.

For NR assay, medium was removed after treatment with inhibitors, then cells were washed with PBS and fresh medium containing 40 μg/mL RN dye (Sigma, St. Louis, MO, USA) was added. After 3 h incubation, cells were washed with a PBS. Color was developed by the addition of 0.1 mL of 1% acetic acid in 50% ethanol.

In both cases, the plate was shaken for 10 min and the absorbance was measured at 540 nm using an automatic ELISA plate reader (BioTek μQuant). Absorbance change is assumed to be directly proportional to the number of viable cells. Cytotoxicity percentage was calculated as [(A-B)/Ax100], where A and B are the absorbance of control and treated cells, respectively. Each assay was repeated three times in independent experiments. Data were analyzed using one-way ANOVA test and multiple comparisons were made using p values corrected by the Bonferroni method.

2.3.3. Evaluation of the Effect of PT and 6-MP treatments of Copper Disks on the Viability of the Surrounding Cells by Acridine Orange Staining. Copper disks (99.7%, 0.1 mm thick, area = 0.223 cm^2) (Merck, Darmstadt, Germany), were first immersed in sulfuric acid 5.0% during 30 s in order to clean the metal surface. (Schwartz 2003) Afterward, they were washed with bidistilled water and dried with N_2.

Inhibitory coatings were generated by immersion of the copper disks in 1 × 10^{-2} M 6-MP and 1 × 10^{-4} M PT (solubility limit in water) solutions during 1 h at room temperature. After the immersion period, samples were washed twice with sterile water and subsequently added to cell cultures. The concentrations of the inhibitors employed were selected taking into account the dose range used for previous studies that showed an inhibitory effect.[21,22,25,33]

For this set of experiments 5 × 10^5 cells were seeded in Petri dish (10 cm diameter) and grown at 37 °C in 5% CO_2 humid atmosphere in complete culture medium for 24 h. The medium was then removed and a copper disk treated with the inhibitory coating was added in the center of each Petri dish. Subsequently, fresh medium was incorporated. Cells were grown under these conditions during 24 h. HeLa cell cultures without copper disk were used as negative controls. After exposure time, adherent cells were stained with Acridine Orange dye (Sigma, St Louis, MO, USA) and subsequently, they were examined by fluorescence microscopy (Olympus BX51, Olympus Corp., Tokyo, Japan) equipped with appropriated filter, connected to an Olympus DP71 (Olympus Corp., Tokyo, Japan) color video camera. The images were taken immediately after opening the microscope shutter to the computer monitor. Surface densities of cells were obtained from digital images using Image-Pro Plus program. Each assay was repeated two times in independent experiments.

For microscope analyses Petri dishes were divided into two regions (A and B) in order to evaluate the influence of the distance from the metal on the number of living cells. Thus, region A and B (outer radii = 2.0 and 4.0 cm, respectively) were determined by marking circles at the bottom of the dishes according to the inset of Figure 10.

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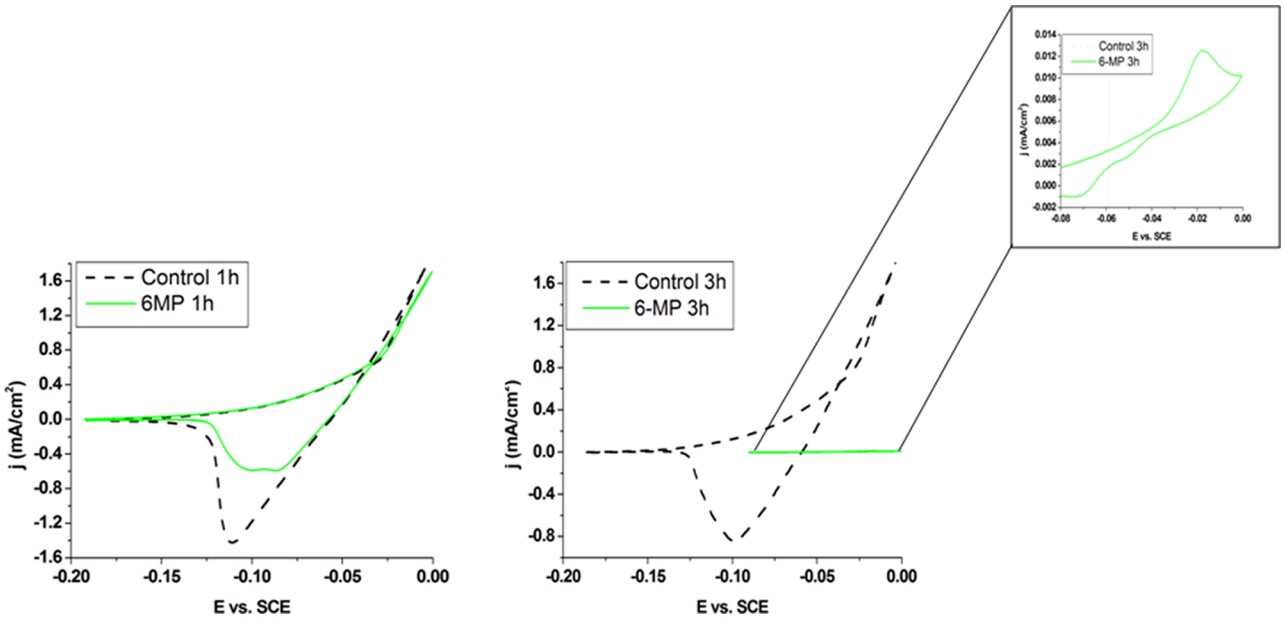

Figure 4. Polarization curves at 1 mV s^{-1} recorded in SUF with pretreated copper electrodes (6-MP 1 × 10^{-4} M) for 1 and 3 h.

3. RESULTS

3.1. Corrosion Tests. *3.1.1. Pretreatments with PT- and 6-MP-Containing Solutions.* Cyclic polarization curves made in SUF showed that pretreatment with PT-containing solution decreased the anodic current and the corresponding reduction process with respect to those of the control without PT (Figure 2). The effect was dependent on the immersion time of the pretreatment, with lower current densities and consequently, stronger effect when longer exposure times were assayed. In agreement, transient currents also showed a marked decrease of current density for 3 h immersion period but the effect was not so important for shorter (1h) exposures to the PT solution (Figures 3).

Similarly, current also decreased after 6-MP treatments (Figures 3 and 4). A dramatic decrease of current density was observed both during potentiodynamic (Figure 4) and potentiostatic (Figure 3) assays after pretreatments with 1 × 10^{-4} M 6-MP solutions for 3 h.

Results of the measurement of cupric ions liberated after 6-MP and PT treatments (Figure 5) are in agreement with electrochemical results. Thus, copper ions release from copper wires treated with 6-MP and PT for 3 h was lower than that of the control after all the periods assayed (between 12 h and 7 days). The longest exposure period (7 days) corresponds, according to Mora et al. (2002),[28] to the most harmful period of the copper ions release.

Both, XPS and EDX analysis of the samples before and after treatments were made. XPS showed (Figure 6) N1s signal that characterizes the adsorption of N-containing molecules like PT and 6-MP after both PT and 6-MP treatments, that was absent in the control without treatment. When both ethanolic and aqueous solutions of 6-MP were used to treat copper surface, S signal was also distinguishable. The N/S intensity ratio is close to 4, in good agreement with the molecular formula of 6-MP. Important O1s signals were also detected in all the samples, probably related to oxygen-containing copper corrosion products and adsorbed water. EDX analysis (Figure 7) also showed small S signals after 6-MP treatment in aqueous and ethanolic solutions.

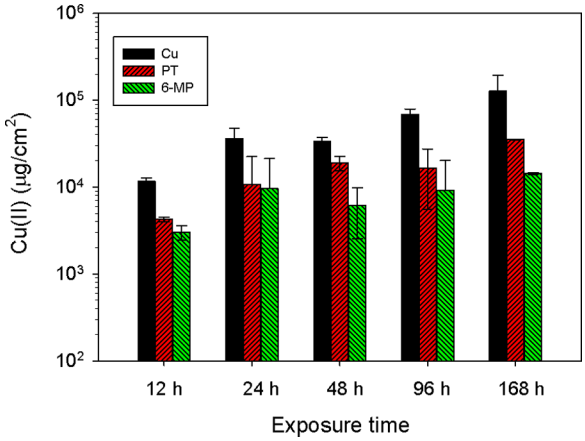

Figure 5. Concentration of copper ions released by copper wires without treatment (control) and treated with 6-MP or PT after different exposure periods.

3.2. Cytotoxicity of PT and 6-MP Evaluated by MTT and NR Assays. The mitochondrial activity observed using MTT test after cell exposure to culture medium with PT or 6-MP in the 1 × 10^{-7} and 1 × 10^{-5} M concentration range were similar to those of untreated control cultures (Figure 8).

Figure 9 shows that lisosomal activity tested by RN assay was not changed in the presence of PT in the 1 × 10^{-7} to 1 × 10^{-5} M concentration range but was significantly affected after exposures to 10^{-5} M 6-MP. .

3.3. Effect of Metal Ions Released by the Copper Disk with and without Treatment on the Number of Living Cells as a Function of the Distance from the Metal. Figure 10 shows results of cell viability by Acridine Orange staining in the presence of copper discs as a percentage of cell coverage of the control without copper discs. In Region A, after 24 h exposure to copper disks without treatment, the coverage of the samples with copper discs is markedly less than the corresponding control (Figure 10). This indicates that cells were severely affected by the metal ions released by the copper samples. However, less cytotoxicity was observed when the

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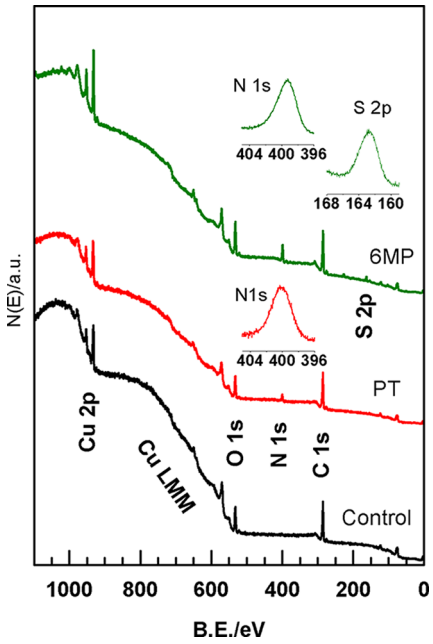

Figure 6. XPS spectra of Cu surface: untreated (control), and treated with PT and 6-MP aqueous solutions. Details of the S and N signals are also shown close to PT and 6-MP spectra.

discs were treated with PT and 6-MP indicating that the release of copper ions decreased under these conditions. On the other hand, at greater distances from the source of ions (Region B), the cytotoxic effect was less notorious (Figure 10)

4. DISCUSSION

We have hypothesized that significant reduction of cytotoxic effects caused by the burst release of copper ions during the first days after insertion may be achieved by using organic inhibitors. However, it should be guaranteed that these inhibitors are biocompatible and that the contraception action remains unaltered. In order to find a suitable method able to fulfill both requirements two organic compounds were proposed here as corrosion inhibitors for IUD: PT and 6-MP.

4.1. Copper Ions Release with and without PT and 6-MP Treatments. Copper release from 190 mm^2 copper wires was measured. The wire surface is similar to those of Copper T200 IUD (TCu 200, 200 mm^2) and Copper T220 IUD (220 mm^2) and half of the area of IUD T380 surface (380 mm^2). These devices are the most popular in developing countries.[6,34] Arancibia et al[1] reported that cuprous was undetectable in

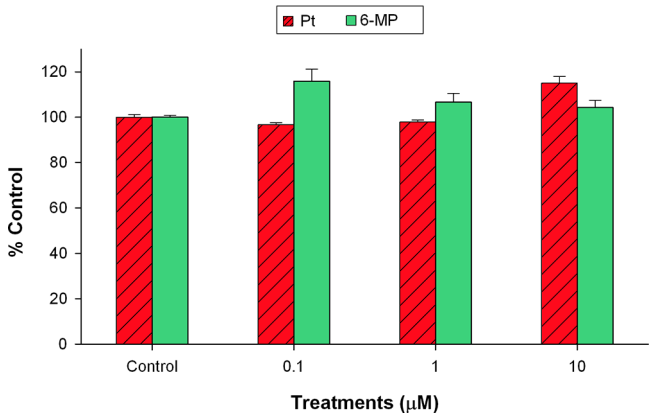

Figure 8. Cellular response to different concentrations of PT and 6-MP after 24 h of exposure evaluated by MTT assay.

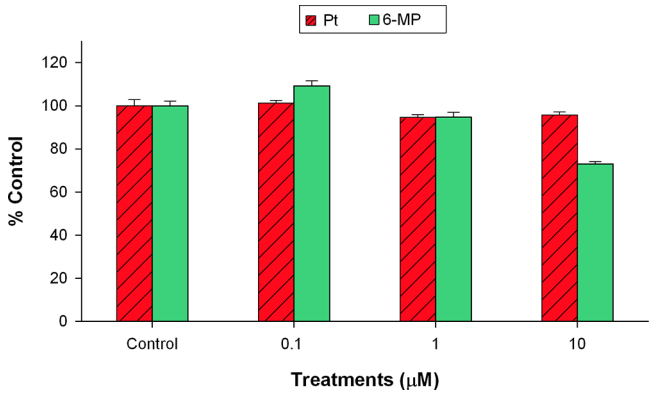

Figure 9. Cellular response to different concentrations of PT and 6-MP after 24 h of exposure evaluated by Neutral red assay.

uterine fluid samples. They confirmed that in aqueous solutions, like uterine solution, the life of these ions is very short because they quickly react to become cupric. Thus, analytical data correspond to total cupric ions (those originally in solution or resuspended after their precipitation).

Adsorption of organic substances, particularly those able to form self-assembled monolayers (SAMs), has proved to be an effective method to reduce corrosion of several metals.[16,25,35,36]

Our electrochemical, XPS, EDX, and copper ions concentration measurements as well as cytotoxicity assays suggest that dissolution of copper decreased after PT and 6-MP treatments. The adsorption process of 6-MP on different metallic substrates has been previously examined by several methods.[16−18,20−22,24]

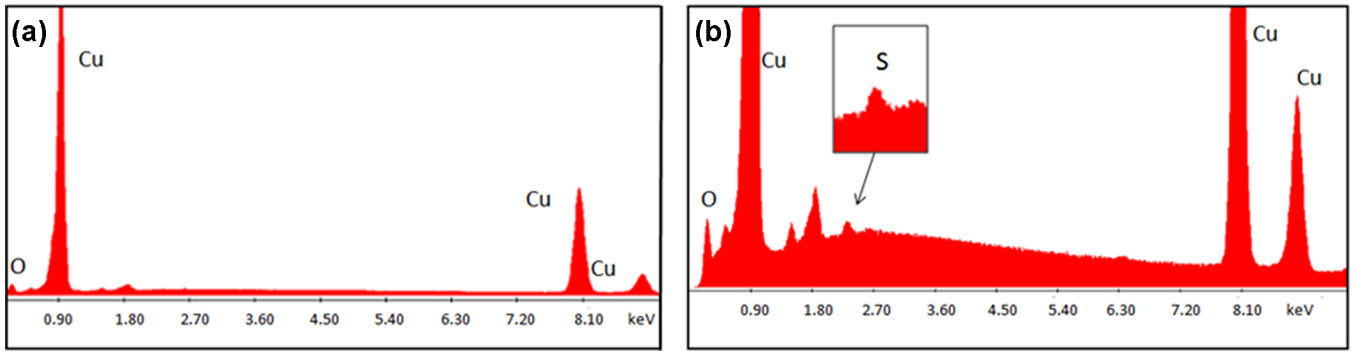

Figure 7. EDX analysis of Cu surface: (a) untreated and (b) treated with 6-MP aqueous solution.

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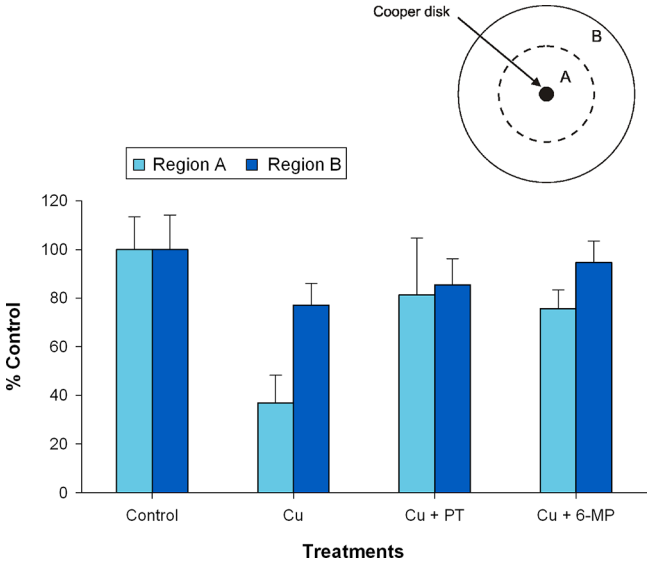

Figure 10. Cell viability by acridine orange staining evaluated as a function of the distance from the metal with and without treatment with PT and 6-MP, according to Petri dish regions (Region A and Region B, in the inset).

These results showed that 6-MP forms SAMs on various metals, that are stable in a wide potential range. Organic S-containing molecules such as 6-MP chemisorbed via S-Metal (thiolate) bonds and arrange in a parallel fashion. Accordingly, EDX and XPS analysis showed the presence of S signal on the surface. The relationship between N/S peak area ratio is close to 4, the ratio in the molecular formula ($C_5H_4N_4S$). Consequently, the important corrosion inhibition on copper surfaces by 6-MP treatment may be related to the formation of a partial or complete monolayer of 6-MP, with S−Cu bonds and N−Cu bonds. In the case of PT an important N1s signal was detected that may be associated to N-metal bonds.

To the best of our knowledge, there is no electrochemical study on the influence of PT on dissolution of metals. Our results suggest that, after immersion of copper in the PT-containing solution, amino groups characteristic of pterin may be present on the surface. However, it is worth mentioning that corrosion inhibition probably decreases if PT forms coordinated compounds with metal ions that could favor the desorption of this molecule from the surface.[11,15]

Consistent with potentiodynamic measurements in SUF, the adsorption of organic molecules induced the decrease in both anodic oxidation and reduction processes. In the case of 6-MP, the coverage of the adsorption layer seems to be almost completed after 3h-treatment and copper dissolution is highly inhibited after this period. However, it should be considered that PT and 6-MP are temporal corrosion inhibitors that reduces the initial burst release to more than a half the control value and guarantee the minimum level of cupric ions necessary for contraception (2 μg/day).[5] According to Mora et al.,[28] the former 7 days is the most risky period after IUD insertion because the concentration of copper ions decreased markedly after 5 days and remains nearly constant after 7 days. Consequently, when the inhibitors are used it is expected that after the 7 days exposure period reported here the situation should be similar or even less dangerous than in the absence of inhibitor.

Interestingly, the amount of the contraception agent (cupric ions) may be controlled by adjusting the treatment period of the wires within the solution with the inhibitor. Besides, the reduction of burst release effect by pretreatments with 6-MP or PT may be achieved by the IUD manufactures under sterile conditions before packing the device.

To interpret the copper dissolution behavior that guarantees contraception it must be considered that adsorbed layer may not be complete because of pinhole defects, collapsed sites, uncovered areas, etc. In these regions, the underlying metal may be in direct contact with the chloride-containing electrolyte solution being the electron transfer rate in these places greater than on the covered regions.[21] Besides, a complex competition of adsorbed derivatives ($Cu(PT)_{ad}$, $Cu(6\text{-}MP)_{ad}$) and chloride ions should occur on copper, resulting in the progressive removal of some organic molecules from the surface due to the chloride adsorption. This process can be interpreted according to the following equations:

In the case of PT adsorbed on copper:

$$Cu(PT)_{ad} + Cl^- \rightarrow Cu(Cl)_{ad} + PT + e^-$$

Similarly, in the case of 6-MP adsorbed on the metal:

$$Cu(6-MP)_{ad} + Cl^- \rightarrow Cu(Cl)_{ad} + 6-MP + e^-$$

On the bare surface Cl^- and OH^- ions also compete for the adsorption sites[37−39]

$$Cu + H_2O \leftrightarrow Cu(OH)_{ad} + H^+ + e^-$$

and

$$Cu + Cl^- \leftrightarrow Cu(Cl)_{ad} + e^-$$

The action of chlorides leads to copper oxidation followed by copper ions release, necessary for contraception.

4.2. Biocompatibility Tests. The second requirement for IUD application is biocompatibility of 6-MP and PT. Our RN and MTT results show only slight changes in lysosomal and mitochondrial activity in HeLa cell lines exposed to 1×10^{-7} M to 1×10^{-5} M PT for a 24 h period. This biocompatible response is in agreement with the fact that pterins are related to a variety of roles in biology including pigments, one-carbon transfer cofactors and redox cofactors.

In contrast, a reduction in lysosomal activity of cells measured by RN test was found in by RN test when cells were exposed to 1×10^{-5} M 6-MP solution. However, it should be considered that this concentration is much higher than the maximum concentration of 6-MP that can be reached after the total dissolution of a monolayer coating (ca. 6×10^{-10} mol cm^{-2}, assuming total dissolution of a 380 mm^2 (CuT380A area) monolayer in 0.1 mL of SUF).[21] Consequently, biocompatibility of the 6-MP treatment may be ensured because 1×10^{-5} M is beyond the concentration range of clinical relevance.

On the other hand, when cells were exposed to copper without treatment a significant decrease in the number of living cells in Region A was observed. These results are in agreement with our previous works[8,40] that reported cytotoxic and genotoxic effects of copper ions. These toxic effects may be associated to specific damage caused by copper ions. Particularly, cupric and cuprous ions can act in oxidation and reduction reactions with direct (changes in protein conformation) and indirect (metal-driven formation of ROS) damages. Copper-induced cellular toxicity may be explained considering that copper ions are prone to participate in ROS formation

through Fenton/Haber-Weiss reaction.[41] Actually, both treatments (PT and 6-MP) reduce the cytotoxic effect of copper-treated samples with respect to nontreated substrates. However, in Region A the beneficial effect of 6-MP was less remarkable than PT effect notwithstanding that lower release of the cytotoxic copper ions was found in case of copper treated with 6-MP. This result may be interpreted considering that during the dissolution process, together with copper ions, a high amount of 6-MP (probably higher than 1×10^{-6} M, according to NR assay) accumulates in the vicinity of the copper disc (region A) due to the low solubility of these organic molecules in water and the diffusion resistance of the biological layer.[42] Like others thiopurines, 6-MP may exert a concentration-dependent cytotoxic effect by non specific mechanisms such as block of replication by incorporation into DNA and of transcription by incorporation into RNA.[43] Reduction in the intracellular concentrations of ATP under supra-pharmacological concentration (2.5×10^{-5} M) has been reported. However, only slight ROS was measured after 24 h and a minor production after 72 h of treatment,[43] confirming the weak effect on this reaction.

5. CONCLUSIONS

Overall, our findings show that copper ion release is high ("burst release") during the first days after insertion and that these copper levels may cause cytotoxic effects in the surrounding cells. Experiments with HeLa cell line showed that PT and 6-MP pretreatments proposed here are suitable to reduce the cytotoxic effect of cupric ions released by IUD, without affecting the contraceptive action of IUD. Relevant advantage of 6-MP or PT treatments is that cupric ion release could be controlled by adjusting the immersion period of IUD within the inhibitor-containing solution. The reduction of burst release effect may be achieved by an easy method that can be implemented under sterile conditions before packing the device by the IUD manufactures. However, in vivo assays to evaluate the behavior of pretreated copper samples should be made to confirm that the treatments are also efficient in living organisms.

AUTHOR INFORMATION

Corresponding Author

*E-mail: mmele@inifta.unlp.edu.ar. Tel: 54 221 4257430, ext 148. Fax: 54 221 4254642.

Notes

The authors declare no competing financial interest.

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