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Antifungal properties of silver coating on tumour endoprostheses: an *in vitro* study

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Abstract. – OBJECTIVE: We tested and quantified the *in vitro* effect of silver coating on preventing development of fungal biofilm over titanium, as found in some megaprosthesis used for musculoskeletal oncological reconstruction, to evaluate the antiseptic effect of this additional feature on this class of pathogens.

MATERIALS AND METHODS: Different strains and species of *Candida* (*C. albicans*, *C. tropicalis*, *C. parapsilosis*) were cultured over 6 silver-coated and 6 non silver-coated titanium (Ti-6Al-4V) samples following a standardized procedure. Then spectrophotometrical analysis and viability assay were conducted after 5 days of incubation to quantify the different extension of biofilm produced by pathogens

RESULTS: Significant differences between groups ($p<0.05$) were found in terms of biofilm extension and pathogens viability over the different materials for any single experiment reported, with silver-coating group showing substantially lower values in terms of fungal development in all conducted assays.

CONCLUSIONS: The results suggest that silver coating is a reliable and effective implementation for antifungal purpose, in addition to its widely known and demonstrated antibacterial potential. Therefore, the use of silver-coated implants may be an even wiser choice in an oncological surgical procedure where patients are particularly at risk for this infective complication due to immunosuppression caused by pharmacological treatments, although the relevant antifungal potential here shown needs to be confirmed *in vivo*.

Key Words
Silver, Megaprosthesis, Bone tumours, Candida, Biofilm, Antifungal, Xtt assay.

Introduction

Prosthetic joint infections (PJI) in reconstructive surgery following oncological bone resections are a dramatic yet frequent complication after these procedures[1,2]. Chemotherapies, radiotherapy, invasive catheters and ordinary pharmacological treatments for these conditions can induce alterations in immune functions leading to significantly higher incidence of infectious diseases[3,4].

Bacteria cause most of the infectious processes diagnosed among these patients; nevertheless, they have higher fungal infections rates as well, which significantly increase the risks of perioperative complications due to their peculiar resistance to conventional pharmacological therapies. This is mainly caused by the ability of fungal pathogens to create a superficial "biofilm" over host surfaces. Biofilm consists of concentrated microbial communities with limited drug reception and susceptibility, making device-associated infection of this kind extremely difficult to treat[5]. Specifically, *Candida* species are emerging as important nosocomial pathogens, and an implanted device with a detectable biofilm is frequently associated with their isolation[6].

Research in the biomedical field has long been analyzing prosthetic materials to minimize the chance of implant failures due to infections. Titanium alloys (mostly in 6Al-4V composition) have been widely adopted in the biomedical field for prosthetic and fixation device fabrication,

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showing higher resistance to bacterial colonization compared to other standard biomaterials. But the incidence of local infections following the implantation of these devices isn't optimal yet[7].

The antibacterial activity of silver has long been known and has lately found a variety of applications in production of biomedical devices because of its antiseptic properties while its toxicity to human cells has been demonstrated to be reasonably low[8]. Silver coating of prosthetic implants has been proposed and widely studied over the years, especially for reconstructive surgery in musculoskeletal oncology with the aim of reducing the peculiar very high incidence of postoperative infections in this field[9,10]. *In vitro* and *in vivo* studies of the antibacterial efficacy of the use of silver coating over megaprosthesis have been publicated showing very promising results[11,12]. However, there are currently very few studies testing the actual efficacy of silver in preventing fungal spreading over the implants.

Our intention in this study was to quantify and compare the adhesion and production of biofilm by different *Candida* species *in vitro* to simulate the likeliness of fungal infection over biomedical titanium and to assess how silver coating may limit this process.

Materials and methods

12 circular disks (diameter: 2 cm; height: 2 mm) were fabricated using standard titanium alloy (Ti) equivalent in composition to commonly used modular endoprosthesis (MUTARS® Implantcast, Germany) already involved for a similar experimental study on cell growing properties[13]. However, given the purpose of the analysis to compare the antifungal properties of this material when provided with a silver coating on the surface (Ti-Ag), half of the produced disks featured this additional property. These metallic slides were intended to be on the bottom of culture wells where different fungal strains would be inoculated in order to evaluate their growing and adhesion potential on the two different surfaces. Two experimental study groups were created and the disks allocated to them based on their composition: the "titanium group" (Ti, 6 plates) *vs.* the "silver-coated titanium" group (Ti-Ag, 6 plates).

Fungal Strain Selection

Three different *Candida* species were selected for our analysis in order to evaluate and compare the production of biofilm by some of the most commonly isolated and treated variants. Two strains of clinical isolates for namely *Candida albicans* (CA), *Candida tropicalis* (CT) and *Candida parapsilosis* (CP) were obtained from the Department of Microbiology at our institution. We decided to experimentally test two isolates for each species to average the respective growth potential of different strains reducing the chance of bias in collected results.

Fungal Culture

Procedures were as follows for each of the multiwell culture plates: we put each of the strain samples into yeast nitrogen base supplemented with glucose at controlled temperature (37°C) for 12 hours to enhance proliferation. The yeast cells were then harvested, washed with phosphate-buffered saline (PBS; pH 7.2) and quantitatively adjusted to measure 1 X 10^6 cells/mL. Each plate containing the different experimental disks was then inoculated with cell suspension (100 µL) of *Candida* strains following the group division previously illustrated. Foetal calf serum (FCS) was then added to cover the plate which rested for 24 hours at controlled temperature (37°C) to promote adhesion. A new wash with PBS was later performed to eliminate non adherent fungal cells, and finally the disks inside the culture plates were covered in brain hearth infusion (BHI) agar and incubated for 5 days at controlled temperature (37°C) to simulate and enhance biofilm development over the surfaces. We substituted the medium daily during this phase.

XTT Cell Growth Assay

At the end of the culturing phase, we quantitatively measured the biofilm size and its viability using validated colorimetric tetrazolium salt XTT reduction assay[14]. This method is based on the cleavage of 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT) to form an orange formazan dye by metabolic active cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multiwell spectrophotometer (ELISA reader). Therefore, the experimental disks were then transfered to a new 12-wells tissue culture plate containing 2 ml of PBS per well. 25 µL XTT (Sigma) and its activating solution (acetone added with 1 Mm of menadione) were added to each plate for 6 hours of incubation at controlled temperature (37°C).

Table I. Complete data collection from spectrophotometrical assay of fungal biofilm growth on the different materials for each strains of fungal species involved. (CA: Candida albicans; CT: Candida tropicalis; CP: Candida parapsilosis; SD: Standard deviation).

Strain	Experimental groups		Mean difference	*p*-value
	Titanium	Silver-coated titanium		
CA1	0.65	0.39	0.26	< 0.0001
CA2	0.65	0.34	0.31	< 0.0001
CT1	1.31	0.82	0.49	0.0034
CT2	0.82	0.58	0.24	0.0005
CP1	0.69	0.35	0.34	0.0008
CP2	0.65	0.43	0.22	0.0017

After incubation, the content of the well was quantified spectrophotometrically measuring optical absorbance at fixed wavelength of 492 nm by an ELISA reader. Each experiment was performed in triplicate and mean values were calculated from the readouts.

Statystical Analysis

Statistical significance was tested for two-tailed paired or unpaired *t*-test as appropriate. Statistical analysis of data was performed using the Statistical Package for the Social Sciences (SPSS) software version 20.0 (SPSS Inc., Chicago, IL, USA). A p-value < 0.05 was considered statistically significant.

Results

Data collected from spectrophotometrical analysis of the different experimental samples showed a lower fungal biofilm growth over silver-coated titanium disk in all assays when compared to standard titanium alloy disk (Table I). *Candida tropicalis* demonstrated to be the most aggressive strain of those tested with the highest values registered in terms of colony size, regardless the material inside the growth plates. The statistical analysis showed significant antifungal effects of silver-coated titanium against any of the different species and strains involved (Table II), with an average intergroup difference of about 30% in all tests (Figure 1).

Table II. Statistical analysis of the data collected from the from spectrophotometrical assays. Silver-coated titanium group showed significant difference in reducing development of biofilm development when compared to standard titanium alloy.

Titanium Group					
Strain	Optical density relevation for each experiment			Mean values	SD
CA1	0.64	0.65	0.66	0.65	0.01
CA2	0.67	0.64	0.63	0.65	0.02
CT1	1.23	1.27	1.43	1.31	0.11
CT2	0.82	0.83	0.82	0.82	0.00
CP1	0.63	0.72	0.72	0.69	0.05
CP2	0.71	0.61	0.64	0.65	0.05
Silver-Coated Titanium Group					
Strain	Optical density relevation for each experiment			Mean values	SD
CA1	0.39	0.41	0.37	0.39	0.02
CA2	0.37	0.31	0.33	0.34	0.03
CT1	0.89	0.73	0.84	0.82	0.08
CT2	0.61	0.60	0.53	0.58	0.04
CP1	0.32	0.34	0.40	0.35	0.04
CP2	0.42	0.44	0.44	0.43	0.01

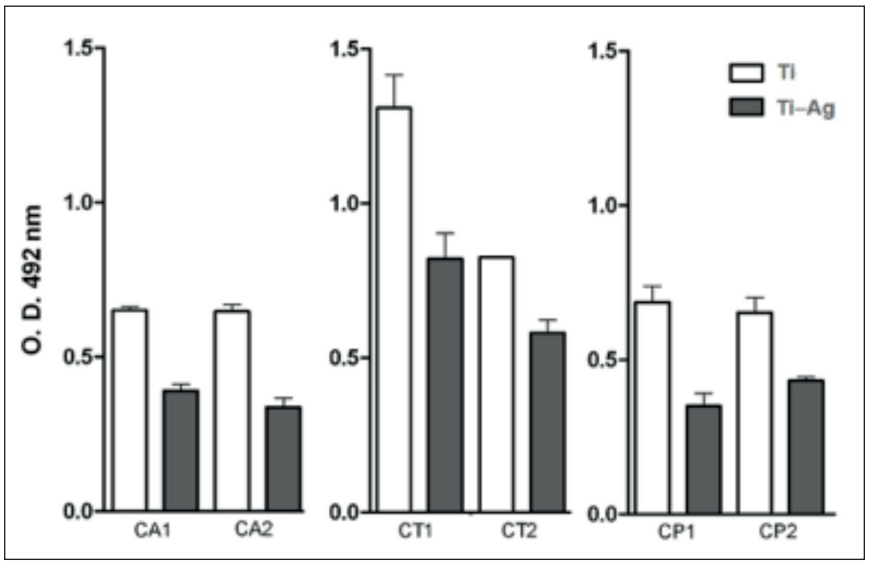

Figure 1. Histogram comparing the mean values of 492nm optical densities measured through XTT cell proliferation assay. Values are about 30% lower in the silver-coated titanium group (Ti-Ag) compared to standard titanium alloy group (Ti). (O.D: optical density measurement).

Discussion

Over the last few decades, megaendoprosthesis gradually became a reliable surgical option for the purpose of extensive bone reconstruction mostly in the field of musculoskeletal oncology, even though very complex cases of prosthetic revision surgery and selected trauma surgery cases may also benefit from the use of it[15-18]. On the other hand, high susceptibility to implant-related infections has been reported, with incidence ranging 3% to 30% in first-time surgeries, increasing up to 45% in patients undergoing revision surgery for previously failed megaprosthetic implant[19].

The most important risk factors for infection of orthopaedic devices are prolonged surgical time, age > 65 years, previous local or systemic infections, diabetes, malnutrition, chemotherapies, multiple surgeries needed and bad local soft tissue condition[20]. Many of these are commonly found in patients affected by bone sarcomas or other malignant tumours eligible for bone resection and reconstruction of the affected limb using these devices.

Treatment of orthopaedic prosthetic infections can be variable and depends on timing of diagnosis[21]; early infection can be treated with surgical debridement and irrigation followed by a long period of antibiotic therapy; late infections are very difficult to eradicate and usually require removal of the implant.

Among the different pathogens involved in prosthetic colonization, rare but still troubling entities are represented by fungi which are related to about 1% of orthopedics metalware infections, increasing to 3% in surgical revisions, especially in immunocompromised patients[22]. *Candida* species are the most frequently isolated fungi in implant-related infections, but there are cases reported in literature caused by *Aspergillus* as well[23]. Pathogenetic mechanisms are numerous, with haematogenous route being the most commonly involved after a primary cutaneous, urinary or digestive infection. Direct inoculation may also cause acute local symptoms after surgery and/or after percutaneous procedures to the joint (i.e., arthrocentesis, local infiltration)[24-28].

Formation of biofilm over orthopaedic implant surfaces has been observed and most of the studies on this issue focus on infections by strains of *C. Albicans*. Three phases of this process were identified: an early phase, when yeast cell adhere to the device surface; an intermediate phase, featuring formation of a matrix with dimorphic switching from yeast to hyphal forms; a maturation phase, when matrix extension increase and a three-dimensional structure is created. Mature *Candida* biofilm is therefore made of yeasts, hyphae and pseudohypee within a matrix of polysaccharides, carbohydrate, protein and other unknown cells[6]. Khun et al[29] demonstrated *in vitro* that *C. albicans* produces significantly more biofilm than the other *Candida* species (as *C. parapsilosis*, *C. glabrata* e *C. tropicalis*), thus representing a serious threat to prosthtetic implant survival.

In this regard, researchers demonstrated how chemical nature of surfaces could influence the size of biofilm production, and recently intro-

duced silver-coated megaprosthesis showed promising higher antiseptic efficacy when compared to standard alloys in different studies, particularly in the early postoperative phases[11,30]. Nevertheless, silver is highly biocompatible and has proven to be a safe additional component in implantable device[31,32].

To our knowledge, no extensive research to date tested the efficacy of silver coating over biomedical titanium devices in the prevention of fungal colonization and biofilm production. The results of our *in vitro* comprehensive analysis seem to confirm that antiseptic properties of silver, already demonstrated for bacteria, are able to inhibit colonization of implants by fungi as well. If this feature will be confirmed *in vivo* as well, the indication for use of silver coated orthopaedic modular megaprostheses could dramatically increase, particularly in the field of muscoloskeletal oncology where the treatment of postoperative complications, such as infections, are definitely harder and harmful to patients. The results were similar for any strains of *Candida* tested, suggesting similar effects on all fungal species commonly involved in infectious disease.

It must be noted that concerns have been raised by researchers on different metabolic pathways of various fungal species and strains when exposed to the reagents used in the viability assay we adopted for this analysis (XTT test) which could result in analytic biases when comparing the different pathogens viability[33]. However, this assay has been validated over the years as reliable and its one of the most widely used in literature[34]. Moreover, our intention was to carry out a comparative analysis of the effect of the different materials tested on fungal biofilm production rather than a precise and reliable quantification of this product, and we believe that the experiment we designed was suitable for this purpose.

Conclusions

Silver-coated titanium used for the fabrication of megaendoprostheses showed higher resistance to fungal colonization *in vitro* as demonstrated by a smaller production of biofilm on the surface of this material when compared to samples of titanium alloy. This evidence is in accordance with previous similar analysis regarding bacteria, but still needs to be tested and extensively analyzed *in vivo* in order to have a better knowledge and reliable data to strongly suggest a wider adoption of this devices especially for the treatment of muscoloskeletal malignant tumour where perioperative implant-related complications rates still remain unacceptably high. On the other side this potential antiseptic properties of the materials does not affect periprosthetic soft tissues, which would still be at risk of colonization from fungal and other pathogenic microrganisms.

Conflict of Interests

The authors of this manuscript certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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