ARTIGO ORIGINAL

INFLUENCE OF DIFFERENT TITANIUM SURFACE TREATMENTS ON THE BIOLOGICAL BEHAVIOR OF OSTEOBLASTIC CELLS

Influência de diferentes tratamentos de superfície de titânio no comportamento biológico de células osteoblásticas

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ABSTRACT

Introduction: Different methods of treatment to dental titanium surfaces have been adopted to improve mechanical and chemical properties of the implant and to improve osseous integration. Objective:The objective of the present work was to compare the performance of three titanium surfaces, Porous® (obtained by double acid treatment), Vulcano Actives® (obtained by anodization), and PorousNano® (obtained by double acid treatment and fluoride incorporation) in regards to cell proliferation of osteoblast precursors and gene expression of extracellular matrix proteins type 1 collagen, osteocalcin, osteopontin and ostenectin. Materials and Methods: We utilized commercially pure grade 4 titanium discs to cultivate osteoblastic precursor cell line MC3T3-E1 (ATCC - USA). After 3, 7 and 10 days, we performed cell proliferation assay using Trypan blue exclusion testand gene expression analysis by Real-Time PCR. Results and Conclusion: Results were recorded and analyzed statistically using a level of significance of $P<0.05$. We observed higher cell proliferation and higher gene expression of proteins linked to synthesis and mineralization of the extracellular matrix on the Vulcano Actives® surface.

Keywords: Osteoblasts. Gene expression. Bone matrix. Dental implants.

RESUMO

Introdução: Diferentes métodos de tratamento de superfícies de implantes de titânio têm sido adotados para o controle da rugosidade e química de superfície no intuito de melhorar os resultados da osseointegração. Objetivo: O objetivo deste trabalho foi comparar o desempenho de três superfícies de titânio, Porous® (obtida pelo método de duplo tratamento ácido), Vulcano Actives® (obtida pelo método de anodização), e PorousNano® (obtida por duplo ataque ácido e incorporação do Flúor), quanto à proliferação de células ósseas pré-osteoblásticas e quanto à expressão gênica de proteínas da matriz óssea extracelular, a saber, colágeno tipo 1, osteocalcina, osteopontina e osteonectina. Metodologia: Foram utilizados discos de titânio grau 4 comercialmente puro e sobre estes discos foram cutivadas células pré-osteoblásticas MC3T3-E1 (ATCC - EUA). Após 3 dias, 7 dias e 10 dias foi realizado ensaio de proliferação celular através do método de exclusão vital por azul de Trypan e Análise de expressão gênica através de PCR em Tempo Real. Resultados e Conclusão: Os resultados foram tabulados e submetidos a análise estatística tendo sido adotado nível de significância de 5%. Os resultados do presente estudo in-vitro evidenciaram que dentre as superfícies estudadas, a Vulcano Actives® apresentou maior proliferação celular e maior expressão de proteínas associadas à síntese e mineralização de Matriz extra celular.

Palavras-chave: Implante Dentário; Conexão Implante Dentário; TiBase.

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INTRODUCTION

Defined as "the direct, structural, and functional connection between bone and the surface of a load-bearing implant," osseointegration revolutionized restorative dentistry, establishing itself as the primary alternative for replacing lost teeth. Among other factors, the surface of the implants and the quality of the bone bed have been highlighted as decisive in the incorporation of the implant into the bone. Alongside this, understanding the factors that regulate bone growth, continuous bone remodeling throughout life, and the regeneration of injured tissue is essential for comprehending osseointegration [1].

The formation of bone tissue involves multiple activities of the osteoblast. The combined application of molecular, biochemical, and ultrastructural approaches defines the stages of osteoblastic phenotype development, with each cell sub-population exhibiting unique morphological and functional properties. The peak levels of expressed genes reflect the maturation sequence of bone differentiation and growth, characterized by three main periods: proliferation, maturation, and extracellular matrix mineralization [4].

In an attempt to increase the success rate of implants, surface roughness control has been adopted to select the type of cell that will interact with the implant. It is known that osteoblastic cells adhere more rapidly to rough titanium surfaces, where there is a more pronounced production of extracellular matrix synthesis, compared to smooth surfaces. Controlling surface roughness has been the strategy adopted by the industry to reduce osseointegration time [7,13]. Research results indicate that in the future, new implant surfaces will have individualized morphology and chemical composition. It will be possible to control the interaction between proteins, tissues, and implant surfaces. These surfaces will be capable of stimulating bone formation, absorbing and releasing drugs to reduce the rate of implant loss, and controlling bone loss. [3.] It is already known that changes in the surface morphology of implants affect cellular response, adhesion, adsorption, shape change, differentiation, and proliferation during osseointegration [7,12]. Nevertheless, despite the advances in implant dentistry, the explanation of the influence of implant surfaces on osseointegration remains incomplete.

Several studies analyze morphology, topography, roughness, chemical composition, surface energy, chemical potential, level of work hardening, presence of impurities, thickness of the titanium oxide layer, and the presence of metallic and non-metallic compounds. The influence of all these factors on cell adhesion to implant surfaces determines the success of osseointegration [8,11].

Different implant surfaces have been obtained through particle blasting, acid etching, double acid etching, anodization, and

fluoride incorporation. The aim is to improve osseointegration outcomes, and the use of various research methodologies such as Resonance Frequency Analysis [10], Contratorque Testing [6], Gene Expression Analysis [9], among others, systematically contribute to the understanding of the regulatory mechanisms of osteoblastic activity and their consequences on implant dentistry practice.

Another research approach that has been widely used involves isolating bone cells that exhibit specific metabolic responses and biosynthesis of bone structural components in culture. From these studies, various classes of factors have been identified, allowing the definition of regulatory mechanisms that control the growth and differentiation of osteoblasts or osteoprogenitor cells. It is also recognized that a wide spectrum of physiological mediators of bone formation has differential and highly selective effects on the numerous cells involved in bone tissue formation and turnover. A clear understanding of the effects on osteoblasts is somewhat compromised by different species or experimental systems, although these variations are now being considered as related to the stage of cell maturation or differentiation, which has an impact on the osteoblast's responsiveness to physiological modulators of bone cell functions [5].

In this context, implant dentistry has deepened its investigations aiming for a greater understanding of the influence of implant surfaces on the speed and quality of osseointegration.

The present study investigated different surfaces of osseointegrable implants, considering cell proliferation and the gene expression of structural proteins related to the bone mineralization process.

METHODOLOGY

This research was conducted at the São Leopoldo Mandic Institute and Research Center, Campinas campus, in the Laboratory of Cellular and Molecular Biology. The present study was submitted for prior approval by the Ethics Committee in Research of the São Leopoldo Mandic Institute and Dental Research Center. Protocol 2010/0396.

CELL CULTURE

The MC3T3-E1 mouse osteoblast cell line, studied by Quarles et al. (1992) and Fratzl-Zelman et al. (1998), was obtained from the American Type Culture Collection (ATCC, VA, USA).

The osteoblastic cells were cultured in Minimum Essential Medium (MEM - Alpha) supplemented with 10% fetal bovine serum (Cultilab®, Campinas, SP, Brazil) and 1% antibiotic-antimycotic solution (Sigma, St. Louis, Missouri, USA).

All procedures were conducted in a laminar flow hood to maintain the sterility of materials and substances used for cell culture.

The cells were kept in a 37°C incubator in a humid atmosphere containing 95% air and 5% carbon dioxide. The culture medium was changed every 2-3 days, and the progression of the culture was evaluated by phase-contrast microscopy. After reaching confluence, the cells were detached from the culture flasks using 1 mM EDTA (Gibco, Invitrogen, Grand Island, NY, USA) and 0.25% trypsin (Gibco) and counted using a hemocytometer under a microscope (Fisher Scientific, Pittsburgh, PA, USA).

After reaching confluence, the cells were plated at a density of 110 cells/mm2 on titanium discs in 24-well plates for cell proliferation assays and analysis of extracellular matrix protein gene expression through real-time PCR experiments.

TITANIUM SURFACES

Three different titanium surfaces produced by Conexão Sistema de Prótese - São Paulo - Brazil, were used as substrates.

These surfaces were presented in the form of titanium discs with dimensions of 8.0 mm in diameter and 1.0 mm in thickness. The surface treatments were as follows: Porous® surface obtained through double acid conditioning, Vulcano Actives® surface obtained by anodization process, and PorousNano® surface obtained by double acid conditioning with fluoride deposition.

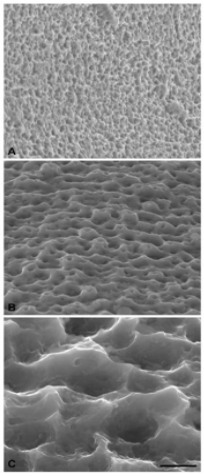


Figure 3 – Representative photomicrographs of the Porous® (A), Vulcano Actives® (B), and PorousNano® (C) surfaces. Scanning Electron Microscopy. Scale bar – 320 μm.
Source: Prof. Dr. Carlos Nelson Elias

The titanium discs were divided into groups as follows: 27 discs used as substrates for RT-PCR assay and 27 discs used as substrates for cell proliferation assay.

CELL PROLIFERATION ASSAY

For cell proliferation assessment, the trypan blue vital exclusion method was used at 3, 7, and 10 days of cell cultures plated on different titanium surfaces.

For this purpose, once the cells reached sub-confluency, they were enzymatically removed from the plates, and the resulting cell pellet from centrifugation was suspended in 1 mL of medium. Then, 10 µL of the cell suspension was mixed with 10 µL of trypan blue. One microliter of this solution was placed on a hemocytometer (Neubauer chamber - Fisher Scientific, Pittsburgh, PA, USA) and examined under a phase-contrast microscope (Nikon, Eclipse TS100) for cell counting and observation.

The total number of cells was obtained using the following mathematical equation:

$$\text{Total number of cells} = \frac{\text{Number of counted cells x Vol. initial x Dilution x } 10^4}{\text{Number of squares used for counting}}$$

EVALUATION OF GENE EXPRESSION OF PROTEINS THROUGH REAL-TIME PCR (RT-PCR).

After 3, 7, and 10 days, the transcription levels of the genes encoding type I collagen, osteopontin, osteocalcin, and osteonectin proteins were evaluated in osteoblastic cells plated on the different titanium substrates previously described.

RNA EXTRACTION

Total RNA was extracted from the cells using Trizol reagent (Life Technologies) according to the manufacturer's instructions. Briefly, cells were collected and homogenized with 0.5 ml of Trizol, and the separation of the aqueous and organic phases was performed by adding 0.1 ml of chloroform, followed by centrifugation (12,000 g, 15 minutes, 4°C). RNA was precipitated from the aqueous phase with 0.5 ml of isopropanol (12,000 g, 15 minutes, 4°C), washed with 75% ethanol, and resuspended in water.

COMPLEMENTARY DNA (cDNA) SYNTESIS

Samples of 1 µg RNA were treated with 1U of DNAse I, and cDNA synthesis by reverse transcription was performed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, reactions were conducted with 1 µg RNA, 0.5 µg oligo(dT)18, 1 mM of dNTP mix, 200 U RevertAid H Minus M-MuLV Transcriptase, and 20 U RiboLock RNAse Inhibitor, at 42°C for 60 minutes. Reactions were then terminated by heating at 70°C for 5 minutes.

PCR IN REAL TIME (RT-PCR)

Pairs of oligonucleotide primers were designed for amplification of the various genes of interest (COL1A1, SPP1, BGLAP2, SPARC, and GAPDH) using the Primer Express program (Life Technologies) (Table 1). Amplification reactions were performed using 40 ng of cDNA

and 0.3 μM of primer pairs, added to the Maxima SYBR Green qPCR Master Mix (Thermo Scientific). The reaction conditions were: 10 minutes at 95°C, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The 7500 Fast Real Time PCR System (Life Technologies) was used. Expression levels were quantified using the SDS System program (Life Technologies), and relative expression among samples was calculated according to the comparative Ct (threshold cycle) method, based on the 2^-ΔΔCt formula. GAPDH gene was used for expression normalization.

Table 1 - Representation of the proteins analyzed, corresponding genes, and sequences of the primers used

Protein	Gene	Gene Bank (NM)	Sequence
Collagen I	*COL1A1*	007742.3	F 5'-ACAAGGTGACAGAGGCATAAAGG-3' R 5'-GCCTGCAGGACCTGAAGCT-3
Osteopontin	*SPP1*	009263.2	F 5'-TGCTTGGGTTTGCAGTCTTCT-3' R 5'-CCAAACAGGCAAAAGCAAATC-3
Osteocalcin	BGLAP2	001032298.2	F 5'-ACAGACTCCGGCGCTACCTT-3' R 5'-ACTGGTCTGATAGCTCGTCACAAG-3'
Osteonectin	SPARC	009242.2	F 5'-TGAGACCTGTGACCTAGACAACGA-3' R 5'- CAGCAGGAGGCGTGAACTTAG -3'
GAPDH	*GAPDH*	008084	F 5'-TGGCAAAGTGGAGATTGTTGCC-3' R 5'-AAGATGGTGATGGGCTTCCCG-3'

F: *forward* ou *sense*

R: *reverse* ou *anti-sense*

ANALYSIS of qPCR DATA

The expression of the target genes was determined relatively, meaning that the expression values were normalized to endogenous control genes.

The Ct values of each gene were obtained in duplicate, and the arithmetic means of these were calculated for each sample. Then, the delta Ct (ΔCt) value was obtained, which consists of subtracting the mean Ct of a target gene from a particular sample from the Ct of the endogenous control gene of the same sample. The next calculation was ΔΔCt, corresponding to the subtraction of ΔCt of a target gene from a particular sample from the ΔCt of the gene of the calibrator sample (sample used as a basis for

comparison in relative quantification analyses). Finally, the values corresponding to 2^-ΔΔCt were calculated, representing the expression levels of the target genes in relative quantification assays. The obtained values were expressed in arbitrary units (AU).

ANALYSIS OF RESULTS

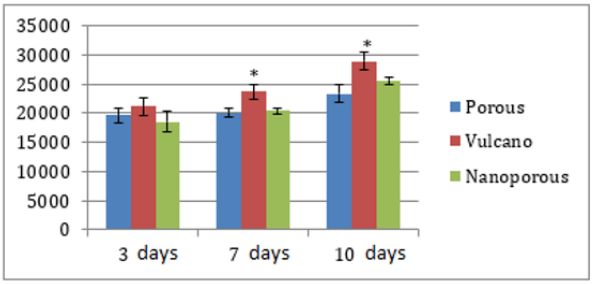
The data were tabulated and subjected to ANOVA followed by Tukey's test. In all analyses, the SAS program was used, and a significance level of 5% was considered (SAS Institute Inc., Cary, NC, USA, Release 9.2, 2010).

RESULTS

With the aim of investigating the influence of different titanium surfaces on pre-osteoblastic cells, cell proliferation was evaluated using the trypan blue vital exclusion method, as well as the expression of genes associated with osteoblast differentiation, encoding structural and mineralizing proteins of the bone matrix, represented by type I collagen (COL1A1), osteopontin (SPP1), osteocalcin (BGLAP2), osteonectin (SPARC), in addition to the endogenous control gene GAPDH (GAPDH), with all data subjected to descriptive statistical approaches.

CELL PROLIFERATION

As seen in Graph 1, regarding the number of osteoblastic cells, the greatest growth occurred at the 10-day time point, regardless of the surface ($p<0.05$). In the comparison between surfaces, there was no difference at the 3-day time point ($p>0.05$). However, for the 7 and 10-day time points, the Vulcano Actives® surface showed a higher number of cells ($p<0.05$).



Graph 1 - Cell proliferation assessment by the trypan blue vital exclusion method in pre-osteoblastic cells at 3, 7, and 10 days. Representative graph of biological triplicates. Values are expressed as mean (± SD).
Source: Author's own work.

GENE EXPRESSION

The mean values and standard deviations of gene expression for COL1A1, BGLAP2, SPP1, and SPARC when pre-osteoblastic cells were cultured on different titanium surfaces (Porous®, Vulcano®, and PorousNano®) after 3, 7, and 10 days of cell plating are shown in Graphs 2, 3, 4, and 5, respectively. For all experiments, the

gene expression relative to the Porous® surface at 3 days was adopted as control.

For the expression of the COL1A1 gene (Type I collagen) (Graph 2), it can be observed that for the Porous® surface, there was no significant difference between the time points ($p>0.05$). However, for the Vulcano Actives® and PorousNano® surfaces, the highest mean was observed at 3 days, and the 7 and 10-day time points did not differ from each other. Regarding the comparisons between surfaces, at 3 days, the highest mean occurred on the Vulcano® surface ($p<0.05$). At 7 days, there was no significant difference ($p>0.05$) between the three surfaces. However, at 10 days, a higher mean was observed for Vulcano Actives®, and a lower mean for PorousNano® ($p<0.05$), while Porous® showed an intermediate mean, not differing from the others ($p>0.05$).

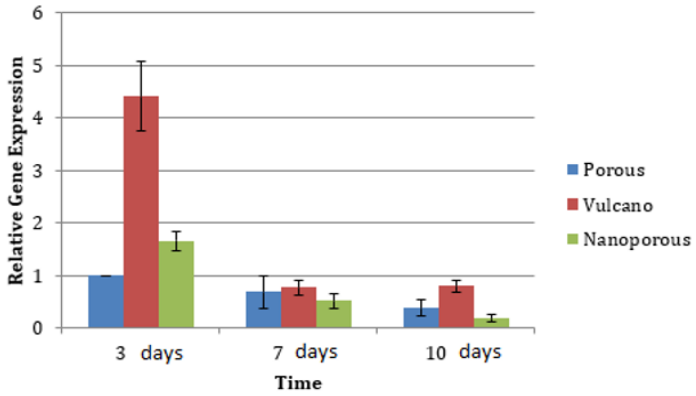
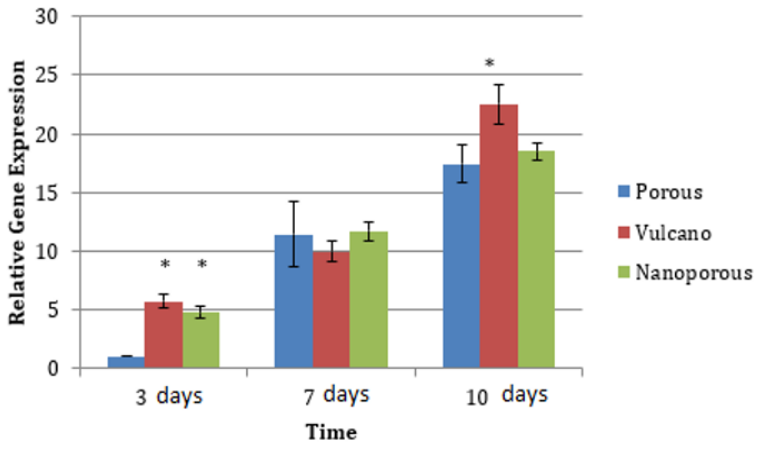


Gráfico 2: Relative gene expression of Col1 as a function of surface and time. Representative graph of the mean of biological triplicates.
Source: Author's own work.

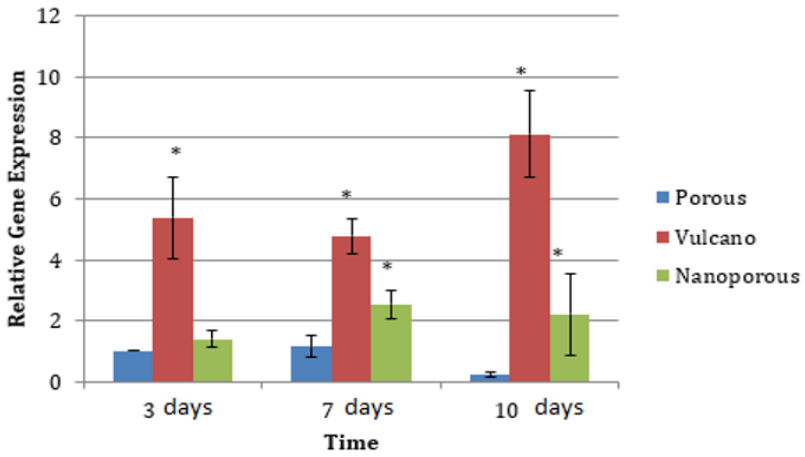
For the BGLAP2 (osteocalcin) gene expression (Graph 3), it can be observed that on the Porous®, Vulcano Actives®, and PorousNano® surfaces, the highest mean of relative gene expression occurred at 10 days, followed by 7 days and lastly 3 days ($p<0.05$). In the comparison between surfaces, at 3 days, no difference was observed between Vulcano® and PorousNano® surfaces ($p>0.05$), but both significantly differed from the control ($p<0.05$). There was no significant difference between surfaces at 7 days ($p>0.05$). At 10 days, the Vulcano Actives® surface showed a significantly higher mean than the others ($p<0.05$).



Graph 3: Mean and standard deviation of relative gene expression of OCC as a function of surface and time. Representative graph of the mean of biological triplicates.

Source: Author's own work.

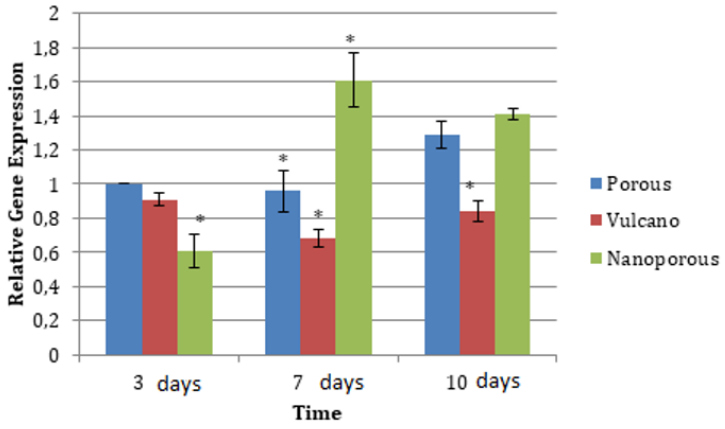
For the SPP1 (osteopontin) gene expression (Graph 4), the Porous® surface showed no significant difference between 3 and 7 days ($p>0.05$), however, there was a decrease in expression at 10 days ($p<0.05$). The Vulcano Actives® surface showed no difference between 3 and 7 days ($p>0.05$), but there was a significant increase in gene expression at 10 days ($p<0.05$). The PorousNano® surface showed no significant difference between the three time points ($p>0.05$). Regarding the comparison between surfaces, at 3, 7, and 10 days, a higher mean of relative gene expression was observed on the Vulcano Actives® surface ($p<0.05$). The PorousNano® surface showed no difference compared to the control surface at 3 days ($p>0.05$), but at 7 and 10 days, it showed higher expression ($p<0.05$).



Graph 4: Mean and standard deviation of relative gene expression of OPN as a function of surface and time. Representative graph of the mean of biological triplicates.

Source: Author's own work.

For the gene expression of SPARC (osteonectin) (Graph 5), the Porous® surface showed a higher mean at 10 days ($p<0.05$), with no statistically significant difference between the 3 and 7-day time points ($p>0.05$). For the Vulcano Actives® surface, there was no significant difference between the three time points ($p>0.05$). For the PorousNano® surface, there was an increase in gene expression at 7 and 10 days compared to 3 days ($p<0.05$). In comparing the surfaces, at 3 days, there was no difference between the Porous® and Vulcano Actives® surfaces ($p>0.05$), while the PorousNano® surface showed decreased gene expression ($p<0.05$) compared to the control. At 7 days, higher relative gene expression was observed on the PorousNano® surface, followed by the Porous® surface, with lower expression for the Vulcano® surface ($p<0.05$). At 10 days, there was no difference between the Porous® and PorousNano® surfaces ($p>0.05$), however, both showed significantly higher expression than the Vulcano Actives® surface ($p<0.05$).



Graph 5: Mean and standard deviation of relative ONC gene expression as a function of surface and time. Representative graph of the mean of biological triplicates.
Source: Author's own work.

CONCLUSION

Considering the proposed hypothesis and based on the employed methodology, it can be observed that:

- The Vulcano Actives® surface positively influenced cell proliferation.
- The expression of type 1 collagen, osteocalcin, and osteopontin was higher for the Vulcano Actives® surface compared to the other surfaces studied.
- The gene expression of osteonectin was higher for the PorousNano® surface compared to the other surfaces studied.

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Observação: os/(as) autores/(as) declaram não existir conflitos de interesses de qualquer natureza.