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The molecular pathway triggered by zirconia in endothelial cells involves epigenetic control

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ABSTRACT

The requirement to achieve natural looking restorations is one of the most challenging aspects in dentistry. Although zirconia has provided new opportunities for achieving superior aesthetics and physicochemical outcomes, very little has been achieved for its cellular and molecular performance, especially considering angiogenesis and osteogenesis. As angiogenesis is a secondary event and concomitant to osteogenesis, an indirect effect of dental implant on endothelial cells could be the release of active molecules such as those already reported affecting osteoblasts. To better address this issue, we challenged human endothelial cells (HUVECs) with zirconia-conditioned medium up to 72 h to allow analysis specific gene expression and protein pattern of mediators of epigenetic machinery in full. Our data shows involvement of zirconia in triggering intracellular signaling through MAPK-ERK activation, leading the signal to activate histone deacetylase HDAC6 likely with concomitant well-modulated DNA methylation profile by DNMTs and TETs. These signaling pathways seem to culminate in cytoskeleton rearrangement of endothelial cells, an important prerequisite to cell migration expected in angiogenesis. Collectively, this study demonstrates for the first time epigenetic-related molecular mechanism involved in endothelial cells responding to zirconia, revealing a repertoire of signaling molecules capable of executing the reprogramming process of gene expression, which are necessary to drive cell proliferation, migration, and consequently angiogenesis. This set of data can further studies using gene editing approaches to better elucidate functional roles.

1. Introduction

Throughout the world, scientists are searching for alternative biomaterials with the ability to regenerate lost tissue while also recapitulating its original characteristics and functions (Henkel et al., 2013; Branemark et al., 1969; Selvaggi et al., 2015; Chen and Liu, 2016). Different materials and alloys have been evaluated along with changes in their topographic structure favoring cell adhesion and subsequent differentiation within osseointegration processes (Zambuzzi et al., 2011; Gemini-Piperni et al., 2014a; da Costa Fernandes et al., 2018a). Although bone tissue can recover from small injuries, for extensive lesions, bone requires surgical intervention to restore tissue normality and functionality (Fernandes et al., 2017; Chan et al., 2013; Aspenberg, 2013). Ideally, the biomaterial employed in tissue regeneration and successful osseointegration should have adequate physical-chemical composition and bioactivity (CJ da and Zambuzzi, 2020; Kang et al., 2018; Murphy et al., 2017).

Titanium alloys are widely used in the biomedical area, but there is a growing interest in understanding cellular responses to bioceramic materials, such as zirconia dioxide (ZrO_2) (Evans, 1994; Albrektsson and Wennerberg, 2019; Gautam et al., 2016; Cionca et al., 2000). Initially, ZrO_2 presented itself as an aesthetically attractive material, but studies have elucidated important physical-chemical properties to be considered in implantology. Zirconia has low thermal conductivity, as well as high support in effective compression ranging 1000 MPa to 2000 MPa, which is why zirconia is sometimes called "ceramic steel". In vitro models reveal that ZrO_2 does not cause cytotoxic effects and is capable of inducing the proliferation of pre-osteoblasts (da Costa Fernandes et al.,

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2018b).

Despite the growing number of studies that reveal the behavior of bone cells exposed to different alloys, including ZrO_2, little is known about the cells adjacent to the implant, such as those in the endothelial tissue. Angiogenesis is expected to carry regenerative cells and nutrients in the healing tissue surrounding implants in an osseointegration event. A coupled mechanism has been demonstrated between osteogenesis and angiogenesis (Ramasamy et al., 2014; Kusumbe et al., 2014; Ramasamy et al., 2016). Additionally, many studies in the area are focused on mechanisms that comprise cell adhesion, proliferation, and differentiation (da Silva et al., 2019; Sartoretto et al., 2019; de Souza Malaspina et al., 2009; da Silva Feltran et al., 2019; Zambuzzi et al., 2008, 2010), but the mechanisms prior to protein processing have been minimally explored in the area, including epigenetic marks, which can provide important knowledge about cell-to-biomaterial interaction.

Considering that the epigenetic mechanisms may drive behavior of cells in contact with implants, they are expected to have a pivotal role on modulating osseointegration. The possible changes that DNA undergoes in all cells are controlled by mechanisms of methylation (catalyzed by DNA methyltransferase - DNMT) and demethylation (catalyzed by Tet Methylcytosine Dioxygenase - TET) (Delcuve et al., 2009; Lappalainen and Greally, 2017; Prachayasittikul et al., 2017; Tsumagari et al., 2013). Mechanistically, epigenetic guarantees the turnover of methyl moiety to DNA basis, promoting the inhibition or propulsion of gene transcription. In somatic cells, this process occurs in CpG islands (Edwards et al., 2017; Liedtke et al., 2010). Another important mechanism to be considered is the condensation of chromatin that requires enzymes to modify its structures with acetylation of histones at lysine residues, in the N-terminal branch of the protein, guaranteed by enzymes histone acetyltransferase (HAT) and histone deacetylase (HDAC), and able to regulate gene transcription (Delcuve et al., 2009; von Knethen and Brune, 2019; Gong et al., 2018).

Taken epigenetics mechanisms into account, these mechanisms control the expression pattern of genes and later posttranscriptional mechanisms to regulate cell adhesion, proliferation, and differentiation that act on the osseointegration mechanism. Based on these statements, this study tests the hypothesis that epigenetic is involved in endothelial cells responding to zirconia and fills a gap in the comprehension about the performance of zirconia as a biomaterial in biomedical field.

2. Materials & methods

2.1. Materials and reagents

Zirconia (Alumina toughened Zirconia, composition: 76 % ZrO_2, 20 % Al_2O_3, 4%YO_3) discs were donated by SIN Implants System Company (SIN), São Paulo, Brazil. Antibodies: HDAC1 (10E2) Mouse mAb #5356; HDAC2 (3F3) Mouse mAb #5113; HDAC3 (7G6C5) Mouse mAb #3949; HDAC6 (D2E5) Rabbit mAb #7558; SirT1 (1F3) Mouse mAb #8469; SAPK/JNK antibody [phospho-Thr180/ Tyr182] #4511; and GAPDH (D16H11) Rabbit mAb #5174 were from Cell Signaling (Danvers, MA, USA). DNMT1 Antibody (60B1220.1) DNMT3A Antibody (64B1446) (Novus Biologicals, LLC, USA), TET3 Antibody; Anti-DNMT3B (orb372330), TET1 (orb228563), and TET2 (orb131790) were purchased from BiorByt (San Francisco, CA, USA); anti-ERK1/ERK2 antibody [ERK- 7D8] (ab54230) and anti-ERK1/2 (phospho-Thr202/ Tyr204) antibody (ab214362); anti-P38 (ab7952); anti-P38 [phospho-T180 1 Y182] (ab4822) from Abcam (Cambridge, MA, USA)

2.2. Zirconia-based conditioning medium

Zirconia discs were incubated individually up to 24 h in cell culture medium without Fetal Bovine Serum (0.01 mg/mL, with 0.6 cm diameter) as recommended by ISO 10993−5:2016. This conditioned medium was further used to challenge semiconfluent cultures of endothelial cells up to 72 h.

2.3. Cell culture

Human Umbilical Vein Endothelial Cells (HUVECs) were used for this study. Throughout the experiments, the cells were maintained in DMEM medium containing antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin). The medium was supplemented with 10 % Fetal Bovine Serum (Nutricell, Campinas, SP, Brazil). HUVECs were maintained at 37 °C, 5% CO_2, and 95 % humidity. After 72 h of treatment with conditioned medium, the cells were harvested to allow the biological analyzes.

2.4. Gene expression

By considering the same experimental protocol, the cells were harvested in TriZOL, exactly as recommended by the manufacturer. Total mRNA samples were quantitated using a microplate reader (SYNERGY-HTX multi-mode reader, Biotek, Tigan St, Winooski, VT, USA). The cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kits kit (Applied Biosystems, Foster City, CA, USA). For each gene evaluated here, we used 3 housekeeping genes to normalize the transcripts profile, as follows: *gapdh* gene. Thereafter, the pool of cDNA was used to evaluate the expression of the specific gene (Table 1).

2.5. Immunoblot

Semi confluent cultures of HUVECs were subjected to conditioned medium up to 72 h, when they were lysed [50 mM Tris−HCl, pH 7.4, 1% Tween 20, 0.25 % Sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM *O*-Vanadate, 1 mM NaF, and protease inhibitors (1 μg/mL aprotinin, 10 μg/mL leupeptin, and 1 mM aminoethylfluorosilicon 4-fluoride hydrochloride)]. The protein concentration was determined by the Lowry method (Hartree, 1972; Lowry et al., 1951a, b). Then, to cell extracts, we added an equal volume of Laemmli buff ;er [2X sodium dodecyl sulfate (SDS), 100 mM Tris−HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% SDS, 0.1 %bromophenol blue, and 20 % glycerol]. Protein extracts were resolved by SDS–PAGE (8% or 10 %) and transferred to PVDF membranes (Millipore). Membranes were blocked with either 1% bovine serum albumin (2.5 %) in Tris-buff ;ered saline (TBS)–Tween-20 (0.05 %) and incubated overnight with appropriate primary antibody at 1:1.000 dilutions. After washing in TBS–Tween-20 (0.05 %), membranes were incubated with horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse IgG antibodies, at 1:2.000 dilutions (in all immunoblotting assays), in blocking buff ;er for 1 h. Detection was per-formed by using enhanced chemiluminescence (ECL). For all runs, GAPDH was used as an internal control (housekeeping parameter).

2.6. Statistical analysis

Densitometry analysis of the immunoblots bands were done, and the arbitrary values were represented as mean ± standard deviation (SD). They were verified using student's *t*-test (2-tailed) with $p < 0.05$ considered statistically significant and $p < 0.001$ considered highly significant. In experiments with >2 groups, we used one-way ANOVA (non-parametric) with post-test of Bonferroni, to compare all pairs of groups. In this case, the significance level was considered when $\alpha = 0.05$ (95 % confidence interval). The software used was GraphPad Prism 6.

3. Results

The epigenetic mechanism guarantees cellular control of gene expression levels and modulates cell behavior responding to environmental changes. The mechanisms of epigenetic control are acetylation, methylation, and non-coding RNAs (Delcuve et al., 2009; Gupta et al., 2010; Razzouk and Sarkis, 2013; Wei et al., 2017). Specifically, the balance of methylation profile is guaranteed by enzymes responsible for catalyzing methyl groups to the DNA strands (DNMTs), while TETs

Table 1
Primers data sheet and qPCR technical details.

Gene (ID)	Primer	5′- 3′ Sequence	Condition of the Reaction	Product size
HDAC1 (3065)	Forward	CTGGCCATCATCTCCTTGAT	95 °C – 10 s; 58 °C – 30 s; 72 °C – 30 s	216 pb
	Reverse	ACCAGAGACGTGGAAACTGG		
HDAC2 (3066)	Forward	TTCTCAGTGCACCCAGTCAG	95 °C – 10 s; 59 °C – 30 s; 72 °C – 30 s	170 pb
	Reverse	CCAGTATCCTTGGGGGAAAT		
HDAC3 (8841)	Forward	ACGTGGGCAACTTCCACTAC	95 °C – 10 s; 58 °C – 30 s; 72 °C – 30 s	219 pb
	Reverse	GACTCTTGGTGAAGCCTTGC		
HDAC6 (10013)	Forward	AAGTAGGCAGAACCCCCAGT	95 °C – 10 s; 59 °C – 30 s; 72 °C – 30 s	416 pb
	Reverse	GTGCTTCAGCCTCAAGGTTC		
SIRT 1 (23411)	Forward	GCAGATTAGTAGGCGGCTTG	95 °C -15 s; 60 °C – 30 s; 72 °C – 30 s	152 pb
	Reverse	TCTGGCATGTCCCACTATCA		
DNMT1 (1786)	Forward	AGGACCCAGACAGAGAAGCA	95 °C – 15 s; 60 °C – 30 s; 72 °C - 30 s	201 pb
	Reverse	GTACGGGAATGCTGAGTGGT		
DNMT3A (1788)	Forward	AGGAAGCCCATCCGGGTGCTA	95 °C – 15 s; 60 °C – 30 s; 72 °C – 30 s	225 pb
	Reverse	AGCGGTCCACTTGGATGCCC		
DNMT3B (1789)	Forward	TCGACTTGGTGGTTATTGTCTG	95 °C – 15 s; 60 °C – 30 s; 72 °C – 30 s	129 pb
	Reverse	TCGAGCTACAAGACTGCTTGG		
TET1 (80312)	Forward	GCCCCTCTTCATTACCAAGTC	95 °C - 15 s; 60 °C – 30 s; 72 °C – 30 s	211 pb
	Reverse	CGCCAGTTGCTTATCAAAATC		
TET2 (54790)	Forward	GGTGCCTCTGGAGTGACTGT	95 °C -15 s; 60 °C – 30 s; 72 °C – 30 s	245 pb
	Reverse	GGAAAATGCAAGCCCTATGA		
TET3 (200424)	Forward	GGTCAGGCTGGTTTACAACG	95 °C -15 s; 60 °C – 30 s; 72 °C – 30 s	198 pb
	Reverse	GGCATAGACCCACACACATCT		
GAPDH (2597)	Forward	AAGGTGAAGGTCGGAGTCAA	95 °C – 10 s; 58 °C – 30 s; 72 °C – 30 s	345 pb
	Reverse	AATGAAGGGGTCATTGATGG		

promote their reverse process by hydrolyzing the methyl group by processing intermediate hydroxylmethylation status. Additionally, histone acetylation and deacetylation processes also are important epigenetic mechanisms, histones structures are acetylated and deacetylated at lysine residues in the N-terminal branch by typical histone acetyltransferase or histone deacetylase activities, respectively (Fig.1).

The set of data presented in Fig.2 reveals involvement of MAPKs in response to ZrO_2-enriched medium challenging. These data show that MAPK-ERK is significantly required, as the phosphorylated form of this MAPK was significantly up-modulated (Fig.2**a,b**). Conversely, the phosphorylations of both MAPK JNK (Fig.2**c,d**) and MAPK p38 (Fig.2**e, f**) were down-modulated in response to ZrO_2-enriched medium.

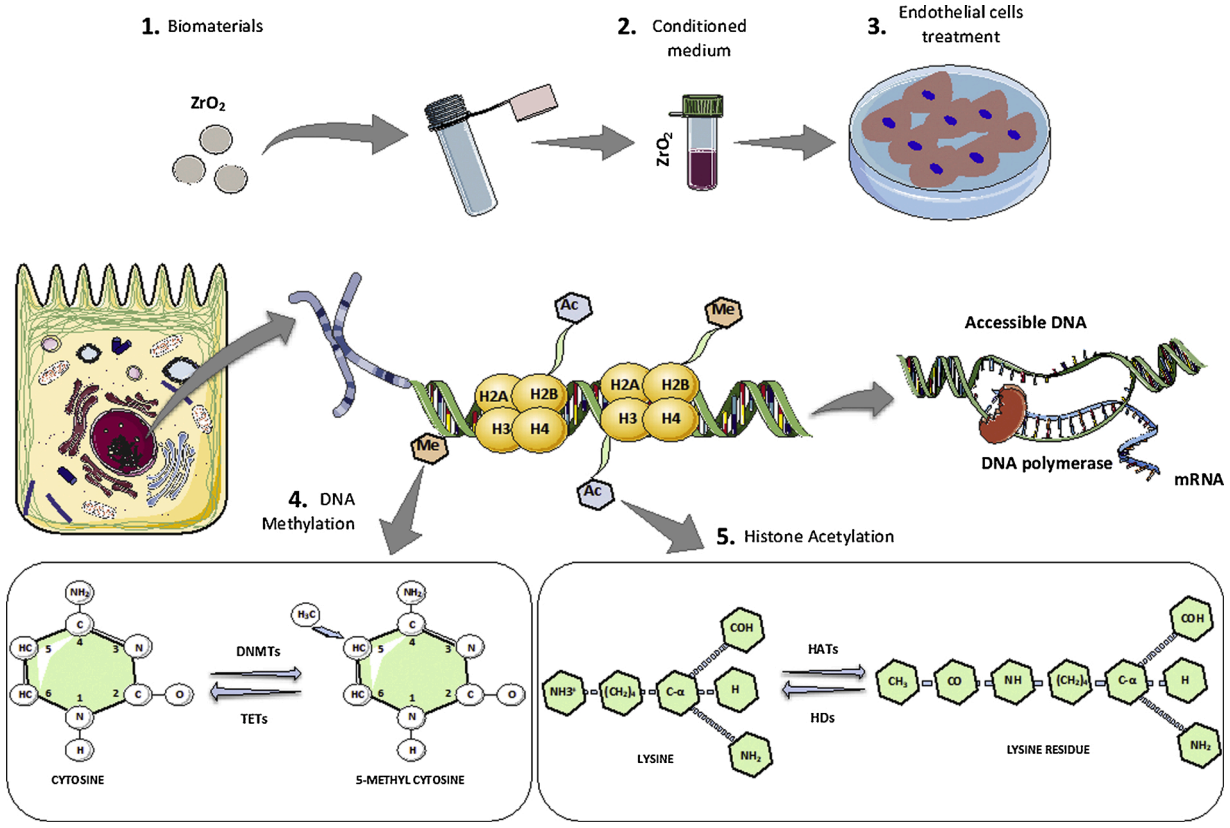

Fig. 1. Experimental design and epigenetic background. To further evaluate whether zirconia-enriched medium could modulate epigenetic machinery in endothelial cells, zirconia discs were incubated in cell culture medium up to 24 h (**1**) and later used to challenge endothelial cells up to 72 h (**2;3**). The epigenetic mechanism evaluated in this study was DNA methylation balance (**4**), and histone acetylation at lysine residue (**5**). Altogether, these molecular mechanisms have relevance in making the DNA accessible and guaranteeing gene expression.

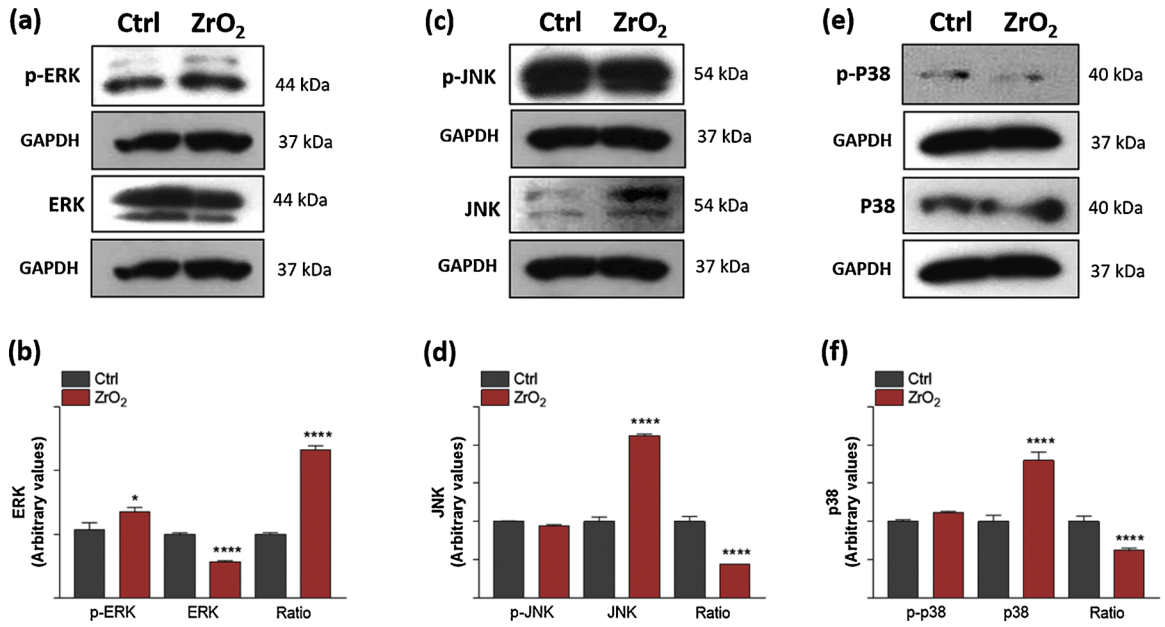

Fig. 2. Effect of ZrO_2-enriched medium on MAPKs activities. To evaluate MAPKs involvement, antibodies recognizing specific phosphorylations were explored by western blotting technology. One representative immunoblots of MAPK-ERK (**a**), MAPK-JNK (**c**), and MAPK-p38 (**e**) were used in this panel. Densitometric analysis of immunoblots bands were normalized to the total protein ratio of controls, and GAPDH was used as loading control (**b, d, f**). Differences were considered statistically significant when *p < 0.04, **p < 0.001, ***p < 0.0002, ****p < 0.0001 compared with the control (Ctrl) group. The results are represented as mean ± standard deviation of three independent experiments.

Altogether, these data demonstrated that MAPKs are important to maintain survival signaling active in response to the biomaterial.

Additionally, an overview was made about expression of genes related to epigenetic machinery (Fig.3). Among the histone deacetylases (HDACs) evaluated here, only the HDAC6 gene (Fig.3**d**) was significantly up-expressed in response to ZrO_2-enriched medium, while HDACs1, 3, 5 remained unchanged (Fig.3**a-c**). Importantly, SIRT1, which is SIRT1 is a member of the Sir2 family of NAD+-dependent histone deacetylases (Vaquero et al., 2004; Imai et al., 2000), was also up-expressed (Fig.3**e**). In addition, DNA methyltransferases (DNMTs) genes were also investigated; however, no significant changes occurred in their expressions in response to ZrO_2-enriched medium (Fig.3**f-h**). Finally, ten-eleven translocation (TET) proteins have been investigated in the experimental workflow proposed here (Fig.3**i,j**), and our data indicates up-expression only for TET1. Note that TETs are dioxygenases converting 5mC to 5-hydroxymethycytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) (He et al., 2011).

Later, the protein amounts of these enzymes were analyzed to estimate possible posttranscriptional processing and suggest the action of microRNAs, specifically epigenetically modifying mature mRNA. Thus, our data reveals that ZrO_2-enriched medium promotes an increase in protein content of HDAC1 (Fig.4**a,b**), HDAC3 (Fig.4**c,d**), and HDAC6 (Fig.4**g,h**), while HDAC2 and SIRT1 proteins had a significant reduction (Fig.4**e,f,i,j**, respectively).

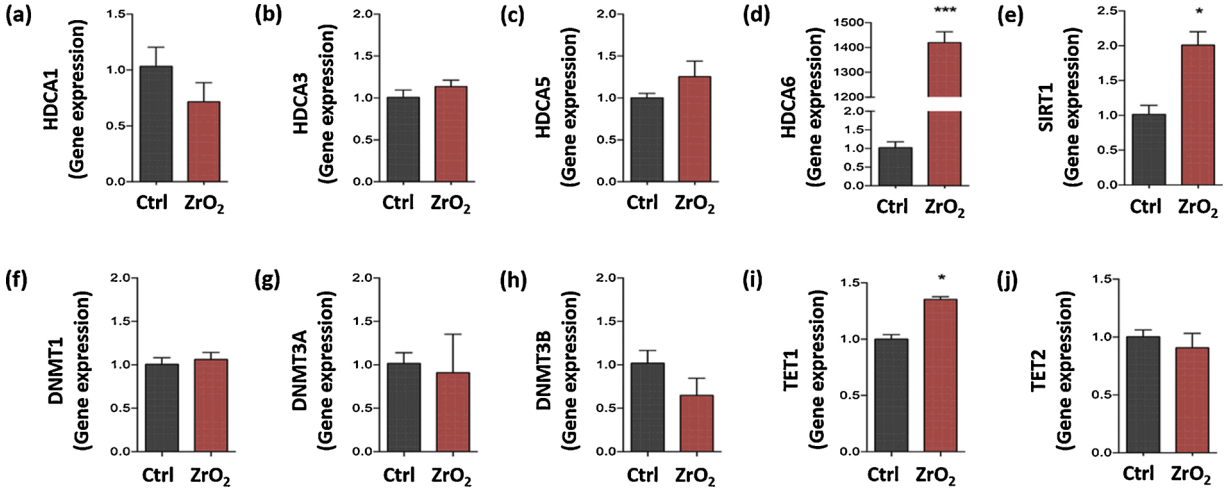

Fig. 3. Effect of ZrO_2-enriched medium on modifying expression of epigenetic related genes. To evaluate whether genes encode epigenetic related enzymes, qPCR technology was performed. For the expression of genes related to histone acetylation, the data shows no significances in HDACs1, 3, and 5 (**a-c**), while HDAC6 and SIRT1 genes were up-expressed in endothelial responding to ZrO_2 (**d,e**, respectively). Considering DNA methylation balance, although any changes were observed in DNMTs (**f-h**), TET1 gene was up-expressed (**i**), while TET2 remained unchanged (j). The Control group (Ctrl) values were normalized to 1 and the relative values obtained to ZrO_2 are shown in fold-changes. Differences were considered statistically significant when *p < 0.04, **p < 0.001, ***p < 0.0002, ****p < 0.0001 compared with the control (Ctrl) group. The results are represented as mean ± standard deviation of three independent experiments.

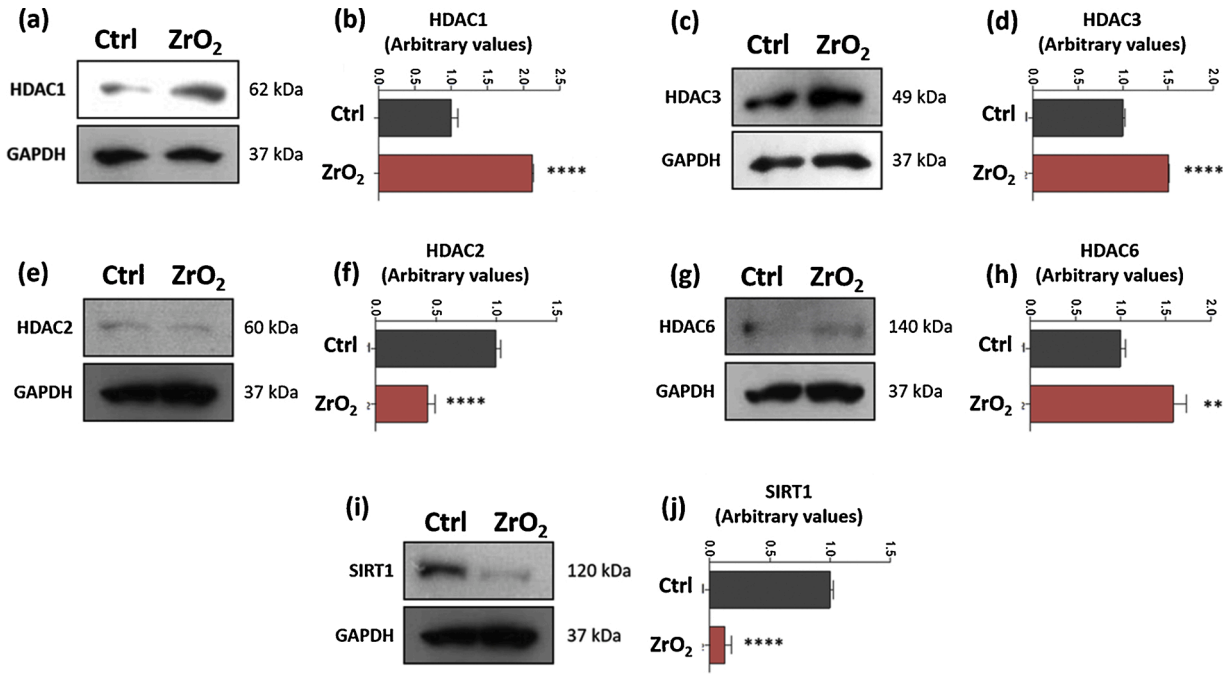

Fig. 4. Effect of ZrO_2-enriched medium on the protein content of histone deacetylase enzymes. To detect possible posttranscriptional mechanisms, we evaluated protein patterns of epigenetic related enzymes by performing western blotting. Firstly, histone behavior was better addressed to clarify potential involvement of chromatin compaction: one representative immunoblots of HDAC1 (**a**), HDAC3 (**c**), HDAC2 (**e**), HDAC6 (**g**), and SIT1 (**i**) are presented. Densitometric analysis of immunoblots was normalized to the protein ratio of controls (1) and GAPDH was used as loading control (b, d, f, h, j). Differences were considered statistically significant when *p < 0.04, **p < 0.001, ***p < 0.0002, ****p < 0.0001 when compared with the control (Ctrl) group. The results are represented as mean ± standard deviation of three independent experiments.

Additionally, the DNA methylation balance was considered in full. Here, the set of data shows a significant and dynamic requirement of DNMT1 (Fig.5**a,b**) and DNMT3A (Fig.5**c,d**), while DNMT3B decreased (Fig.5**e,f**); in addition, TETs 1 (Fig.5**g,h**) and 2 (Fig.5**i,j**) also increased. Collectively and comparing these findings with those displayed in Fig.3 evidences a dynamic posttranscriptional mechanism processing mRNA and rigorously driving the translational events in challenged endothelial cells, possibly requiring non-coding RNA as microRNAs and lncRNAs.

4. Discussion

In dentistry, the requirement to achieve natural looking restorations is one of the most challenging aspects. Although dental porcelain is considered the gold standard material for prosthetic rehabilitation, some characteristics, such as handling and aesthetic excellence, are quite difficult to obtain, which has resulted in the increased use of zirconia. The introduction of zirconia in this field has provided new opportunities for achieving superior aesthetic outcomes; however, as discussed by Vichi et al. (2011), further studies are required with this material (Vichi et al., 2011) to achieve better cellular and molecular performance to guide our comprehension for clinical uses.

Our group and others have shown that bone cells are modulated in contact with biomaterials by modulating intracellular cell signaling, and their surfaces properties such as topography/roughness trigger specific signaling pathways (da Costa Fernandes et al., 2018a; Bezerra et al., 2017a; Gomes et al., 2020; Bezerra et al., 2017b; Rossi et al., 2017; Zambuzzi et al., 2014; Fernandes et al., 2018); however, very little is known about the effect on endothelial cells relating to the phenomenon on angiogenesis, mainly considering the growth proposition for using zirconia in dental applications. Angiogenesis is a necessary event surrounding dental implanted devices as it provides the delivery of nutrients and oxygen to support the performance of healing mechanisms. Obice et al. (2019) discussed how angiogenic mediators, such as VEGF, play a key role in angiogenesis during the bone regeneration and remodeling process, but the mechanisms by which this regulation occurs are not yet known (Obice et al., 2019). Here, we highlighted possible epigenetic involvement in modulating endothelial cell performance by orchestrating methylation and acetylation of DNA and histones, respectively. As angiogenesis is a secondary event and concomitant to osteogenesis, it is possible to suggest an indirect effect of the dental implant on endothelial cells, maybe by releasing active molecules such as those already reported in affecting osteoblasts (da Costa Fernandes et al., 2018a; Rossi et al., 2017; Fernandes et al., 2018; Machado et al., 2019; Tokuhara et al., 2019). To better address this issue, we challenged human endothelial cells with zirconia-conditioned medium to allow molecular analysis focusing on epigenetics.

First, challenged HUVECs present an important increase of MAPK-ERK, a serine/threonine-protein kinase related to survival and proliferating mechanisms. (Czyz, 2019; Asati et al., 2016; Baroncelli et al., 2019). Upstream activated by a set of extracellular stimuli, it contributes with an important signaling cascade that can cause the remodeling of the cell cytoskeleton, maybe requiring HDAC6 (Seidel et al., 2016; Li and Yang, 2015). As cytoskeleton is a very important issue in cells responding indirectly to biomaterials (Kang et al., 2018; Gemini-Piperni et al., 2014b; Milani et al., 2010), and also considering to drive morphological changes of cells during proliferation and migration, this signaling pathway seems to be relevant in angiogenesis control surrounding zirconia. To prove this hypothesis, we investigated the relevance of histone deacetylases in endothelial cells responding to zirconia, with a better look at HDCA6. Surprisingly, both transcript and protein content of HDCA6 were significant involvement in zirconia-induced signaling pathways and this increase can be easily linked with the upstream involvement of MAPK-ERK, mainly because these coupled events are associated with cell migration (Williams et al., 2013). Altogether, a molecular landscape is now drawn, reinforcing the upstream signaling pathways related with endothelial migration, a prerequisite to angiogenesis in response to zirconia.

Additionally, to better investigate epigenetic marks in endothelial

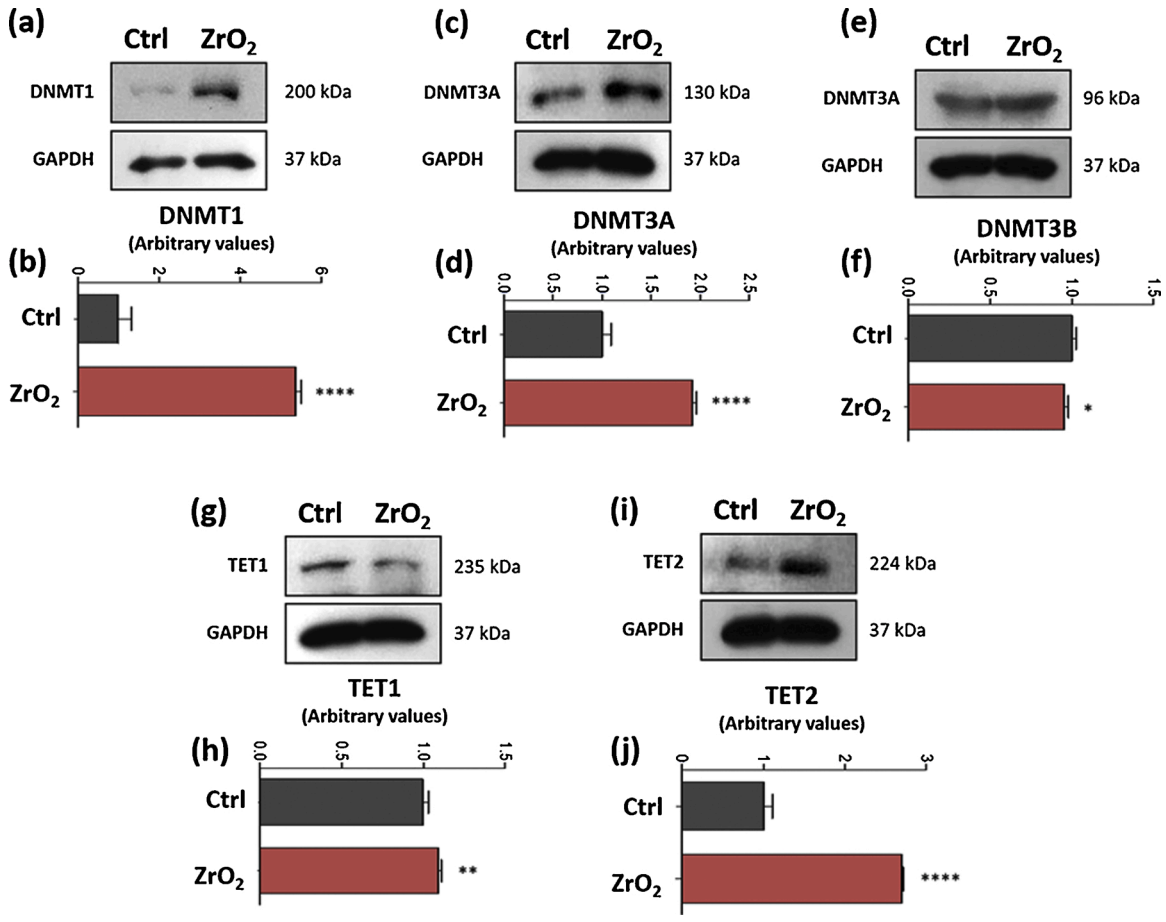

Fig. 5. Effect of ZrO_2-enriched medium on the protein content of DNA methylation modifying enzymes. To detect possible posttranscriptional mechanisms, we evaluated protein patterns of epigenetic related enzymes by performing western blotting. Secondly, DNA methylation modifying enzymes were evaluated by performing western blotting technology. One representative Immunoblot of DNMT1 (**a**), DNMT3A (**c**), DNMT3B (**e**), TET1 (**g**), and TET2 (**i**) are presented. Densitometry analysis of Immunoblot was normalized to the protein ratio of controls (1), and GAPDH was used as loading control (b, d, f, h, j). Differences were considered statistically significant when *$p < 0.04$, **$p < 0.001$, ***$p < 0.0002$, ****$p < 0.0001$ compared with the control (Ctrl) group. The results are represented as mean ± standard deviation of three independent experiments.

cells responding to zirconia, we also looked at enzymes involved with driving the balance of DNA methylation pattern on CpG sites, since it is the most common epigenetic modification. The methylation process is a well-regulated event governed by the antagonism of DNA methyltransferases (DNMTs) and ten-eleven translocation (TET) enzymes. While DNMTs are key enzymes in mediating the DNA methylation process, TETs are enzymes able to oxidize 5-methylcytosines (5mCs) and promote locus-specific reversal of DNA methylation. In turn, DNMT1 is an enzyme responsible for promoting maintenance and requiring methylation at the hemi-methylated sites generated in the DNA file chains and highly expressed during the S phase of the cell cycle, where duplication of genetic material occurs; therefore, it promotes the inhibition of the transcription gene (Loo et al., 2018; Svedruzic, 2011; Szyf, 2001). DNMT3A and DNMT3B are responsible for the new methylation of DNA strands and are included in chromosomes 2p23 and 20q11.2, respectively (Lyko, 2018; Yang et al., 2012). Mechanistically, the molecules released in the medium by zirconia discs affect endothelial cells by increasing the content of DNMTs; therefore, considering the increase in the enzymatic activity of DNMTs may indicate a rigorous mechanism in regulating transcription process (Baretti and Azad, 2018; Bogdanovic and Lister, 2017; Cui and Xu, 2018; Hervouet et al., 2018; Uysal et al., 2017), also considering TETs involvement in promoting the DNA demethylation. Taken into consideration, these data strongly suggest the dynamic requirement of epigenetic metabolism of signaling pathways for driving specific gene expression to respond to zirconia-induced environmental changes.

5. Conclusion

Collectively, this study demonstrates for the first time epigenetic-related molecular mechanism involved in endothelial cells responding to zirconia discs, revealing a repertoire of signaling molecules capable of executing the reprogramming process of gene expression, which are necessary to drive cell proliferation, migration, and consequently angiogenesis. This set of data furthers studies using gene editing such as CRISPR-Cas9 to better understand the functional roles of these mechanisms in endothelial cell phenotype and performance, as well as reinforcing DNA methylation and the acetylation of histories as a prerequisite for zirconia-related environmental responses to the signaling of the cell cycle progress and angiogenesis.

Author contribution statement

WFZ, CJCF, RAS, FJB: Conceived and designed the experiments.
CJCF, SAT: Performed the experiments.
WFZ, CJCF, PFW, SAT, RAS: Analyzed and interpreted the data.
WFZ, FJB: Contributed reagents, materials, analysis tools or data.
WFZ, CJCF, RAS, PFW, FJB: Wrote the paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.tice.2021.101627.

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