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Inhibition of histone deacetylases class I improves adipogenic differentiation of human periodontal ligament cells

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Abstract

Periodontal ligament stem cells (PDLSCs) show plasticity towards the adipogenic lineage; however, little has been done on the participation of epigenetic mechanisms. Histone acetylation is a dynamic process, though balanced by histone acetyltransferases (HATs) and histone deacetylases (HDACs) activities. This process can be halted by HDACs inhibitors, such as trichostatin A (TSA) and valproic acid (VPA). This study aimed to determine the role of HDACs class I in adipogenic differentiation of PDL cells. PDLSCs were treated with TSA at concentrations of 100, 200, and 250 nM, or VPA at 1, 4 and 8 mM. Cell viability was assessed using MTT assays. Gene expression of pluripotency markers (*NANOG, OCT4, SOX2*), HAT genes (*p300, GCN5*), and HDACs genes (*HDAC1-3*) was analyzed by RT-qPCR. Adipogenic differentiation was evaluated via oil red O staining, and acetylation of histone H3 lysine 9 (H3K9ac) was examined by Western blot. VPA treatment resulted in a 60% reduction in cell proliferation, compared to a 50% when using TSA. Cell viability was not affected by either inhibitor. Furthermore, both TSA and VPA induced adipogenic differentiation, through an increase in the deposition of lipid droplets and in *GCN5* and *p300* expression were observed. Western blot analysis showed that TSA increased H3K9ac levels on adipogenic differentiation of PDLSCs. These findings highlight the potential of HDAC inhibitors as a tool for modulating H3K9 acetylation status and thus influencing adipogenic differentiation of PDLCs.

Keywords: Adipogenic differentiation, Dental Stem cells, Histone acetylation, Histone H3 lysine 9, Periodontal ligament Stem Cells

1. Introduction

Dental stem cells (DSCs) have emerged as promising tools for cell-based therapy given their accessibility, self-renewal potential, low immunogenicity, and multilineage differentiation ability [1]. Among DSCs, the periodontal ligament (PDL) stands out, a red fibrous membrane that connects the tooth root to the alveolar bone cement. It has been widely demonstrated that PDL perivascular wall contains an interesting cell population denominated periodontal ligament stem cells (PDLSCs), which display similar features to other mesenchymal stem cells (MSCs).

Since PDLSCs can express stemness-related genes, which facilitate their multilineage capacity, these cells can give rise to muscle, bone, nerve, cartilage, and fat cells [2–4]. Researchers have focused on unraveling the underlying molecular mechanisms in order to understand adipogenic differentiation of PDLCs. This process is regulated by several signaling pathways and transcription factors that control gene expression and cellular fate [5]. For instance, the peroxisome proliferator-activated receptor gamma (*PPARγ*) pathway is a key regulator of adipocyte differentiation and is expressed in PDLSCs.

Other transcriptional factors, such as CCAAT/enhancer-binding proteins (*C/EBPs*) and adiponectin (*ADIPOQ*), also play crucial roles in adipogenesis within PDLSCs [6]. It has been reported that both changes in transcriptional activity and cell differentiation involve chromatin reconfiguration mediated by epigenetic mechanisms, including DNA methylation, microRNAs, and post-translational modifications (PTMs) of histones [7–10].

PTMs of histones are associated with chromatin structures related to gene expression. For example, histone methylation can target genes for turn-on or off, while histone acetylation relaxes chromatin structure and thus faci-

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litates transcriptional factors to bind to DNA to regulate it positively [11]. In this context, histone acetylation is dynamically regulated by the concerted activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) [7, 12]. These enzymes perform acetylation and deacetylation at the N-terminal lysine residues [13]. Specifically, in nucleosomal histone H3 there are epigenetic signatures indicative of transcriptional activation such as histone H3 lysine 9 (H3K9ac), histone H3 lysine 14 (H3K14ac), and histone H3 lysine 27 (H3K27ac) [14, 15].

HAT and HDACs are essential for maintaining stem cell proliferation and self-renewal, genome integrity, and transcriptional regulation, among other process [16]. Acetylation of histones has been correlated to PDLSCs osteogenic ability [17, 18], but its contribution to adipogenesis in DSCs has been little explored. On the other hand, histone deacetylase inhibitors (HDACis) have emerged as influential modulators of chromatin acetylation. For instance, HDACis are chemical compounds that selectively impede HDACs enzymatic activities, leading to maintaining higher histone acetylation levels and thus, establishing a permissive chromatin state for gene expression [19]. HDACis, such as trichostatin A (TSA) and valproic acid (VPA), have been known to influence cellular commitment to alternative cell lineages, including neural, hepatic, myogenic, and osteogenic ones [20–23].

However, the specific impact of TSA and VPA on adipogenic differentiation of PDLCs remains to be fully understood. Additionally, the contribution of H3K9ac, an epigenetic modification that is intricately associated with the adipogenic response, in orchestrating a more permissive chromatin state merits investigation. Such modifications facilitate the recruitment of transcription factors and coactivators, thereby potentiating gene expression pertinent to adipogenesis, including the master regulator peroxisome proliferator-activated receptor gamma (PPARγ). Consequently, a comprehensive exploration of these aspects holds substantial promise in advancing our understanding of adipogenic differentiation in PDLCs. This study aimed to investigate the effect of inhibitors of HDACs class I on adipogenic induction and to examine the role of acetylated H3K9 in the adipogenic response of PDLCs.

2. Materials and methods

2.1. Isolation and culture of primary human PDL cells

Human third molars were collected from healthy volunteers (n=3; aged 13–27 years old) at the Pediatric Dentistry and Oral Surgery Clinics of the Universidad Autónoma de Yucatán, following a protocol approved by the Ethics Research Committee of Dr. Hideyo Noguchi Regional Research Center, Universidad Autónoma de Yucatán (CIE-06-2017). All isolation and cell culture procedures have been detailed in a previous report [4]. PDL tissue was separated from the root surface, minced into small pieces (1–2 mm), and placed in 35-mm culture dishes containing alpha-modified Eagle's medium (α-MEM, Gibco, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (FBS, Gibco, USA) and 1% antibiotic (penicillin/streptomycin, Gibco, USA). Primary cells from PDL tissue were incubated at 37 °C in 5% CO_2 under saturated humidity and medium changes twice a week. At 80-90% confluence, primary cells were seeded into subcultures. Morphological characterization of PDLCs was performed using an inverted phase contrast microscope (LABOMED, TCM 400 Model). Primary cells from passages 4-6 were used in this study.

2.2. Trilineage differentiation of the isolated cells *in vitro*

In order to investigate the osteogenic, adipogenic, and chondrogenic differentiation, PDLCs at passage 6 were seeded at a density of 3×10^4 cells/well in 12-well plates and incubated in osteogenic medium (a-MEM, Gibco, Grand Island, NY, USA) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µg/ml streptomycin) containing 10 nM dexamethasone (Sigma-Aldrich; Merck KGaA), 5 mM β-glycerophosphate (Sigma-Aldrich; Merck KGaA,) and 500 µM ascorbic acid (Sigma-Aldrich; Merck KGaA) for 14 days to induce mineralization. The cells were then stained with 1% Alizarin Red (Sigma-Aldrich; Merck KGaA) for 1 h at 37°C. In order to determine their adipogenic ability, PDLCs at passage 6 were seeded at a density of 3×10^4 cells/well in 12-well plates and incubated in basal medium (Gibco; Thermo Fisher Scientific, Inc.) [α-MEM containing insulin (1.7µM), dexamethasone (1µM), 3-isobutyl-1methylxanthine (500 µM), and indomethacin (60 µM)] for 21 days and then stained with Oil Red O (5 mg/ml; Sigma-Aldrich; Merck KGaA) for 30 min. For chondrogenesis induction, PDLCs at passage 6 were seeded at a density of 3×10^4 cells/well in 12-well plates and incubated in chondrogenic medium StemPro® (Gibco; Thermo Fisher Scientific, Inc.) and stained with Alcian blue (Sigma). All cell images were observed using an inverted contrast-phase light microscope (Nikon Corporation).

2.3. MTT assay of cell viability

The effect of different concentrations of TSA (0, 100, 250, nM) or VPA (0, 4 y 8 mM) on PDLCs viability was examined using a 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (n=6) (MTT; #M6494, Invitrogen, USA) at 0 and 72 h after treatment. Briefly, PDLCs were seeded at 2×10^4 cells/cm^2 in 12-well plates containing a growth medium for 24 h at 37°C before being exposed to increasing concentrations of TSA or VPA (Sigma-Aldrich, USA). Control groups were treated with growth medium inhibitors. The optical density of formazan crystals dissolved in dimethyl sulfoxide solution was determined at 570 nm. Viability percentage was calculated by dividing the optical density of the cells under treatment by the optical density of the cells under normal growth without treatment. The morphological changes in TSA or VPA-treated cells were observed with an inverted phase contrast microscope (LABOMED). The experimental scheme is illustrated in Fig. 1.

2.4. Western blotting

Cells treated with or without TSA or VPA were lysed by the addition of RIPA® buffer (Invitrogen) supplemented with a protease inhibitor cocktail (Sigma-Aldrich) to the cultures. Cells were detached with a cell scraper, and protein content was quantified by BCA assay (Bioscience). Samples containing 25–50 µg of total protein lysates were used in each experiment. Western blot analysis was performed with primary antibodies against H3 (1:10000; Millipore, cat. No. 07-690) and H3K9ac (1:2000; Millipore, cat. No. 07-352). Proteins were visualized using the ECL

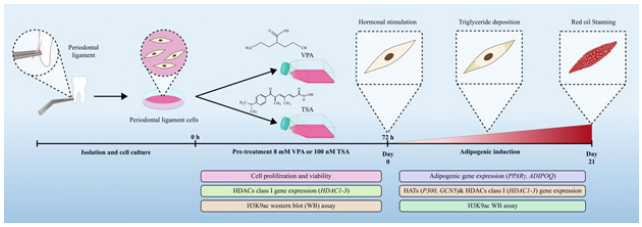

Fig. 1. Timeline of the experimental scheme.

system, according to the manufacturer's instructions, and exposed to X-ray films (Amersham Hyperfilm™ ECL). ImageJ software was used for the quantitative analyses.

2.5. Total RNA Extraction and Quantitative RT-PCR

RT-PCR was performed to measure the levels of pluripotency-associated gene expression (*NANOG, OCT4, KLF4, c-MYC*, and *SOX2*), HDACs (*HDAC1-3*), HAT (*p300* and *GCN5*) and adipogenic markers (*PPARγ* and *ADIPOQ*). Total RNA was isolated from PDLCs (passages 4 and 6) using a Direct-zol RNA kit (Zymo Research), according to the manufacturer's instructions. For cDNA synthesis, reverse transcription reactions were performed with 1 µg of total RNA using the PrimeScript RT–PCR Kit (Takara Biotechnology, Dalian, China) following the manufacturer's instructions. Platinum™ Taq DNA Polymerase (Invitrogen™), 1 µM of each primer, and 150 ng/µL of cDNA in a 25 µL volume were used during PCR in C1000 Touch Thermal cycler (BIO-RAD, Foster City, CA, USA). PCR products were separated through electrophoresis in 1.2 % agarose gels, stained with ethidium bromide (Sigma-Aldrich, USA), and visualized under UV light. Images were acquired using Gel Doc Xr+ System (BIO-RAD). 18S RNA was used as an internal control. Quantitative RT-PCR was performed in triplicate using iTaq Universal SYBR Green Supermix (BIO-RAD, CA, USA) in an Eco Realtime PCR System (Illumina, San Diego, CA, USA) and analyzed using EcoStudy software (Illumina, San Diego, CA, USA). Changes in gene expression were calculated relative to 18s RNA using the 2−ΔΔCT method (22) to measure the expression of pluripotency markers (*NANOG*, *OCT4*, *SOX2*, *KFL4*, *cMYC*) and adipogenic markers (*PPARγ* and *ADIPOQ*) using the primers listed in Table 1.

2.6. Statistical analysis

All experiments were repeated three times. Data were analyzed and expressed as the mean ± standard deviation for each set. For statistical analysis, independent samples comparison Student's t-test, one-way ANOVA were used between groups using SIGMA Statistics software. Statistical significance was set at *P <0.05.

3. Results

3.1. *In vitro* multipotent abilities of PDLCs and expression of pluripotent mesenchymal markers

The multipotency ability of PDLCs was verified by induction in the osteogenic, adipogenic, and chondrogenic media *in vitro*. Alizarin Red staining (Fig. S1) revealed several calcified nodules in the cultures after 14 days of osteogenic induction. After 4 weeks of culture in the adipogenic medium, intracellular lipid vacuoles appeared in PDLCs, whose presence was confirmed by Oil Red O staining (Fig. S1). Alcian blue-stained cells indicated the presence of acidic proteoglycan in chondrogenic differentiation (Fig. S1).

3.2. Effects of TSA and VPA on morphology and viability of PDLCs

In order to determine the effect of HDACs class I inhibitors on human PDLCs, different concentrations of TSA (100, 200, and 250 nM) or VPA (1, 4, and 8 mM) were tested by analyzing their morphology and viability. Untreated cells exhibited a spindle-like shape as normal

Table 1. The primer sequences of genes employes in this study.

Gene	Sequence	TM (°C)
KFL4	F: 5′-TAC CAA GAG CTC ATG CCA CC-3' R: 5′-CGC CTA ATC ACA AGT GTG GG-3'	60
c-MYC	F: 5′-GGA CCC GCT TCT CTG AAA GG-3' R: 5′-TAA CGT TGA GGG GCA TCG TC-3'	60
NANOG	F: 5'-TGC TGA GAT GCC TCA CAC GGA-3' R: 5'-TGA CCG GGA CCT TGT CTT CCT T-3'	60
SOX2	F: 5'-GAA AGG GAC CGA GGA GTA-3' R: 5'-CCG AGT GTG GTT CTG TAA C -3'	62
OCT4	F: 5'-GAA AGG GAC CGA GGA GTA-3' R: 5'-CCG AGT GTG GTT CTG TAA C -3'	62
HDAC 1	F: 5'-TCG ATC TGC TCC TCT GAC AA-3' R: 5'-GCT TCT GGC TTC TCC TCC TT-3'	60
HDAC 2	F: 5'-TGT GCC TCA GTT GCT TCA TC-3' R: 5'-GAT GCA GTG AGC CAA GAT CA-3'	60
HDAC 3	F: 5'-GGA GCT GGA CAC CCT ATG AA-3' R: 5'-GAC TCT TGG TGA AGC CTT GC-3'	60
PPARγ	F: 5′-CAG TGG GGATGC TCATAA-3′ R: 5′-CTT TTG GCA TAC TCT GTG AT-3′	58
ADIPOQ	F: 5′-ATG GTC CTG TGA TGC TTT GA-3′ R: 5′-GTT GAG TGC GTA TGT TAT TTT T-3′	50
18S	F: 5'-GGA CAG GAT TGA CAG ATT GAT-3' R: 5'-AGT CTC GTT CGT TAT CGG AAT-3'	60

mesenchymal cells. None of the tested concentrations of TSA or VPA induced detectable changes in cell morphology (Fig. 2A). Interestingly, it should be noted that cell viability remained unaffected when using the different concentrations of TSA or VPA (Fig. 2B). Results suggest that PDLCs did not show a concentration-dependent cellular sensitivity to HDACis. Since 100 nM TSA and 8 mM VPA treatments did not lower PDLCs viability compared to the control, these concentrations were used in subsequent experiments.

3.3. Effect of TSA and VPA on HDACs class I gene expression

A comparison of *HDACs 1-3* gene expression in cells treated with TSA or VPA inhibitors was performed to analyze the involvement of HDACs class I in the modulation towards adipogenic commitment of PDLCs. Screening of HDAC family showed changes in mRNA levels induced by HDAC inhibitors (Fig. 2C). For instance, *HDAC1* was downregulated by 40%, while *HDAC2* was lowered by 20% with 100 nM TSA. Contrastingly, with 250 nM TSA, there was an increase in mRNA levels of 2 and 8 times for *HDAC1* and *HDAC2*, respectively. These results suggest a differential response in regulating *HDAC* gene expression by TSA, depending on the specific HDAC isoform. Based on these data, the 100 nM concentration was selected for adipogenic differentiation assays. Regarding the expression of *HDACs* in VPA-treated cells, results point to a dose-dependent response. For *HDAC2* and *HDAC3*, 8 mM VPA reduced their expression by 50% and 80%, respectively, while for *HDAC1*, it increased by 60%. These findings highlight the importance of considering the inhibitor´s dose in the transcriptional activity of HDACs and its implication for cellular responses. On the other hand, we cannot discard an effect at the enzymatic level.

3.4. Effect of HDACs inhibitors on adipogenic differentiation of PDL stem cells

Given that no reduction in cell viability mediated by HDACis was observed, a pre-treatment strategy involving 100 nM TSA or 8 mM VPA for 72 h prior to initiating adipogenic induction was included (Fig. 1A). Based on our previously published adipogenic protocol [4], the possibility of an enhancement of the adipogenic response of PDLCs by pre-treating them with these inhibitors was explored [24, 25]. PDLCs presented a higher differentiation capacity towards the adipogenic lineage when cells were pre-treatment with these HDACis (Fig. 3A, image e-f). Cells treated with the inhibitors presented changes in their fibroblastoid-like morphology to more rounded cells (Fig. 3A, image e-f). This effect is stronger on cells treated with 8 mM VPA, showing a round cell morphology and an apparent larger size (Fig. 3A, image f). On the other hand, the increased adipogenic response concurred with *PPARγ* and *ADIPOQ* expression, genes that are important markers of adipogenic commitment [26]. This points out that TSA and VPA treatments enhanced *PPARγ* expression, compared to control cells (Fig. 3B). Furthermore, *ADIPOQ,* which encodes a cytokine that is secreted by adipocytes and accumulates gradually during adipogenesis [27], showed higher expression in TSA or VPA-treated cells than in uninduced cells (Fig. 3B). Our findings suggest that TSA and VPA treatments could promote the synthesis and accumulation of adipokines.

There was a reduction in the expression levels of *HDACs 1-3* when the inhibitors were present during adipogenic differentiation. Notably, *HDAC3* expression exhibited a more pronounced decrease, particularly in PDLCs treated with TSA, as depicted in Fig. 3C. These findings concur with the observations made in the microphotographs, where an augmented formation of droplets was evident in the presence of TSA. Therefore, the effect of HDAC inhibitors on the expression levels of histone acetyltransferases (HATs) was explored, with a particular focus on *p300* and *GCN5* during adipogenic differentiation. A significant upregulation of *HAT* expression in PDLCs during adipogenic differentiation was noted; specifically, *p300* expression levels showed a two-fold increase in induced PDLCs compared to non-induced cells (Fig. 3D). Additionally, *GCN5* exhi-

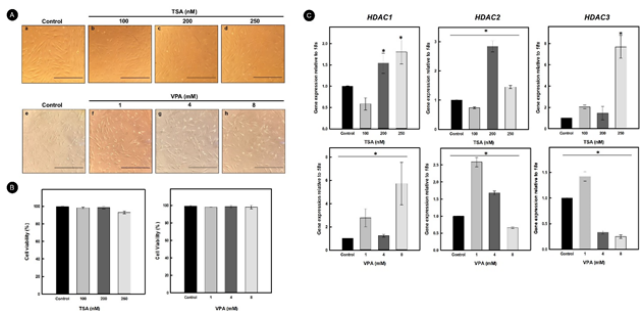

Fig. 2. Effect of TSA and VPA on the viability, and gene expression of *HDACs 1-3* in PDLCs. A: Photomicrograph of PDLCs with TSA (100, 200, and 250 nM) or VPA (1, 4, and 8 mM. B: Viability with different concentrations of TSA or VPA. C: Relative gene expression levels HDACS class I (*HDAC1-3*) of PDLCs treatment with different concentrations of HDACis. The expression of each gene was normalized to the average expression of the endogenous reference gene *18S*. Values are means ± ES, n = 3, ANOVA test with Tukey's post-test showed *P< 0.05.

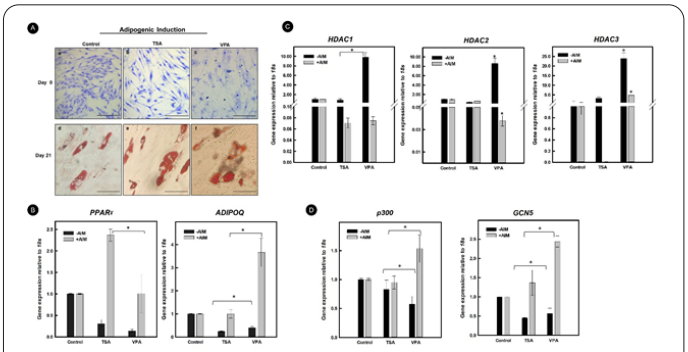

Fig. 3. Effect of TSA and VPA on lipid droplet deposition and gene expression levels of adipogenic markers, HAT, HDACs on adipogenic differentiation. A: Microphotographs of PDLC cultures in adipogenic medium in the presence or not of the inhibitors at 0 and 21 days of adipogenic induction. B: Relative gene expression levels HDACS class I (*HDAC1-3*) of PDLCs treatment with different concentrations of HDACis before and after adipogenic differentiation. C: Relative expression levels adipogenic gene markers (*PPARγ* and *ADIPOQ*) before and after adipogenic induction in PDLCs treatment with different concentrations of HDACis. D: Relative expression levels HATs (*p300, GCN5*) in PDLSCs treatment with different concentrations of HDACis before and after adipogenic differentiation. The expression of each gene was normalized to the average expression of the endogenous reference gene *18S*. Values are means ± ES, n = 3, ANOVA test with Tukey's post-test showed *P< 0.05.

bited a prominent increase in expression when exposed to TSA and VPA during adipogenic induction, compared to non-induced cells. These findings suggest a potential link between increased *p300* and *GCN5* expression levels and the observed upregulation of *PPARγ* during adipogenesis (Fig. 3B).

3.5. Histone acetylation during adipogenesis

Since H3K9ac is an epigenetic mechanism involved in SCs differentiation [28, 29], the effect of TSA or VPA on acetylation status on the adipogenic differentiation of PDLCs was assessed. Western blot assays revealed that TSA-treated PDLCs prior to differentiation showed a decrease of 70% in H3K9ac, while in VPA-treated PDLCs, it was only of 30%, compared to the control (Fig. 4A). On the other hand, H3K9ac levels after adipogenesis (Fig. 4B) increased by approximately 30% in the treatment with TSA, while VPA decreased H3K9ac by approximately 70% compared to the control. Western blot assays showed that in cells undergoing differentiation, TSA treatment increased H3K9ac levels (Fig. 4B).

4. Discussion

TSA and VPA can efficiently suppress the activity of multiple HDACs class 1, leading to an increase in histone acetylation and other non-histone proteins. These inhibitors influence various processes, such as proliferation, arrest of cell cycle progression, gene expression, and induction of cell differentiation. and apoptosis [30, 31]. In dental stem cells, TSA and VPA have been reported to affect proliferation in a dose-dependent manner [32–35]. For example, Jin et al [35] reported that 50 nM increased growth, while 100 nM had a cytotoxic effect on DPSCs. On the contrary, Sulistyowati et al [36] reported that 200 nM TSA is an appropriate dose to treat DPCs *in vitr*o without affecting their cell viability. Regarding VPA, a wide range of concentrations (0.01-100 mM) have been reported in human dental pulp cells [33, 34, 37] pointing out that higher VPA concentrations have an antiproliferative effect. In the present study, similar results were recorded. The application of a high concentration of HDACis decreased the number of cells, which implied an antiproliferative effect *in vitro*. However, these conflicting results may be caused by differences in cell type (primary cells vs. transformed cells), their epigenetic state, and their microenvironment. Additionally, it has been reported that the modulatory mechanism of HDACis in the proliferation of MSCs occurs by arresting the cell cycle in the G2/M phase [38]. Future studies are required to analyze whether the molecular components of the cell cycle are affected by HDACs class I inhibition in PDLCs.

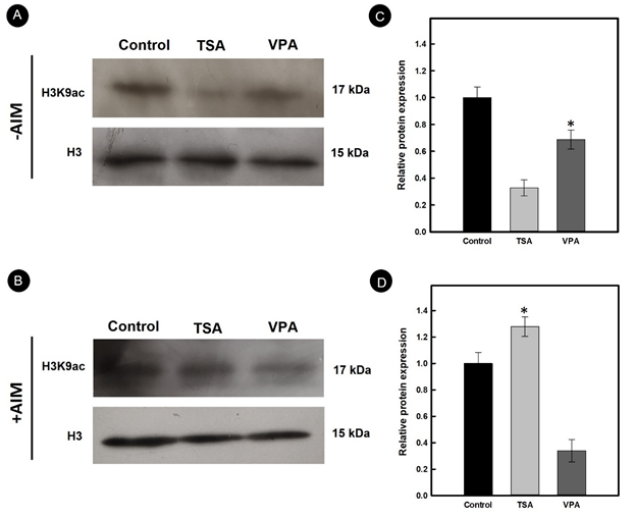

Fig. 4. H3K9ac levels in response to TSA or VPA treatments in PDLCs. A, B: Immunodetection of histone H3K9ac and H3 in PDLSCs exposed to TSA or VPA for 72h before and after adipogenic differentiation (representative western blot images), Ponceau S staining served as loading control. C, D: Quantification of H3K9ac levels was calculated from the intensity of each band. H3K9ac levels were normalized with H3 levels by using ImageJ. software. +MIA: Adipogenic induction medium. -MIA: Without adipogenic induction medium.

Adipogenesis is the process by which undifferentiated precursor cells differentiate into adipocytes. HDACs and HATs are components in epigenetic regulation; however, there are few reports of HDACs or HAT gene expression in adipocyte differentiation [39]. RT-qPCR assays revealed that *HDAC1* and *3* were dynamically downregulated in response to TSA or VPA (Fig. 3B). Inversely, *p300* and *GCN5* transcript levels increased with both inhibitors (Fig. 3D). Our results demonstrate that inhibition of HDACs class 1 during differentiation stimulated adipogenesis. This fact suggests that in PDLCs, there is a malleability of the epigenome, which allows HDACis to stimulate the formation of droplets compared to untreated cells (Fig. 3A). It also reinforced the idea that an increase in acetylation levels could be favoring the expression of *PPARγ*, a master adipogenic transcription factor, and *ADIPOQ*. Hence, an increase in *PPARγ* and *ADIPOQ* mRNA levels was observed in both inhibitor treatments. These findings suggest a potential link between increased *p300* and *GCN5* expression levels and the observed upregulation of *PPARγ* during adipogenesis (Fig. 3C). Previous studies have reported on the elevated expression of these histone HATs in the context of adipogenesis, wherein they play crucial roles in the activation of *PPARγ* and *C/EBPα* pathways [40, 41]. In brown preadipocytes, the loss of *GCN5/PCAF* hinders adipogenesis by suppressing the expression of *PPARγ* and *PRDM16*, indicating the probable involvement of H3K9 acetylation in white adipose tissue browning [42]. Studies involving ribozyme-mediated depletion of *CBP/p300* have demonstrated repression in the expression of genes targeting *PPARγ*; consequently, attenuating the process of adipogenic differentiation in preadipocytes [43]. Furthermore, some investigations into the molecular mechanisms underlying p300-mediated histone modifications have revealed its binding to the promoter and enhancer regions of *PPARγ2*. Specifically, p300 interacts with H3/H4ac and H3K27ac, respectively, at these regulatory regions, suggesting its role in regulating the transcriptional activity of *PPARγ* [44, 45]. It has been reported in PDLSCs that a gradual loss of repressive histone mark, H3K9me3 and DNA methylation on the *PPARγ* promoter occur to facilitate adipocyte differentiation [47]. Although the exact mechanism by which the inhibitors decreased the levels of *HDAC1* and *3* in PDLCs has not yet been determined, it seems that the decrease in their expression could be generating chromatin decondensation and facilitating transcription factors to bind in genomic regions of adipogenic genes. In our future work, this hypothesis is already being addressed by ChIP-seq.

Adipogenesis is also controlled by changes that occur in the nucleus, including a chromatin reorganization [46]. In this context, it has been reported that there are euchromatin histone marks (H3K9ac, H3K4me3, H3K27ac, and H3K36me3) that increase their expression in MSCs differentiated cells[47–49]. This would be the reason to evaluate if TSA or VPA could generate changes in the levels of H3K9ac in the adipogenesis of PDLCs. An increased level of H3K9ac on day 21 of adipogenesis with TSA and a reduction with VPA-treated cells were observed (Fig. 4B). Steger et al [50] reported that there is an increase in H3K9 acetylation on day one of adipogenesis in the 3T3-L1 line [46, 50]. Unfortunately, the participation of H3K9 acetylation through HAT or HDACs during differentiation of dental stem cells has generally been focused on osteogenesis, while adipogenesis has been little explored. For example, Li et al [51] reported that histone acetyltransferases GCN5 participate in the osteogenic differentiation of PDLSCs by regulating H3K9 acetylation in the *DKK1* promoter region [51]. Another research group has shown that overexpression of *p300* increases H3K9 acetylation in specific regions of genes associated with the odontogenic potential of DPSCs [1]. Additionally, Luo et al [52], observed that TSA and VPA increased histone H3 acetylation in DPCs. In the case of HDACs, it has been observed that HDACs 1 and 3 generate the up-regulation of the gene expression of odontogenic differentiation-related proteins in DPSCs [52].

Reports are indicating that VPA generates chromatin decondensation, promoting histone acetylation [53] and influencing cell fate choice [54]; however, these also focused only on osteogenesis. For example, Um et al [55] reported that VPA enhanced osteogenic differentiation in PDLSCs after constant treatment. Paino et al [34], reported that pretreatment of DPSCs with VPA improves mineralized matrix formation, and determined that HDAC1 and HDAC2 are enzymes that contribute to osteoblastic differentiation. There is disagreement about the effect of HDAC inhibitors on adipogenesis. Some reports have described a negative effect on adipogenesis after inhibition of HDACs. For example, Lv et al [56] reported that TSA suppresses adipogenesis in 3T3-L1 preadipocytes, while Ferrari et al [57], reported that MS-275 (class I HDAC inhibitor) promotes adipogenesis in C3H/10T1/2. These divergent results could be explained in terms of epigenomic differences among MSCs and therefore, in their response to cellular commitment. Another factor to consider is that HDACs deacetylate non-histone proteins, including those with a regulatory function in key transcription factors of adipogenic differentiation. Therefore, the treatment of cells with agents, such as TSA or VPA, could be affecting regulatory elements other than histones, resulting in epigenetic and non-epigenetic effects.

5. Conclusion

Our results suggest that perturbation in H3K9 acetylation dynamics contributes to improving the response to adipogenesis in PDLCs. These findings highlight the potential of inhibitors of class I HDACs as a tool to modulate the acetylation status of H3K9. The challenge for future studies is to generate knowledge about the epigenetic mechanisms in white to brown adipogenesis, which could help generate autologous anti-obesity therapeutic strategies with a scope in oral diseases.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and proved the final manuscript for publication.

Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Institutional Research Ethics Committee Dr. Hideyo Noguchi, Regional Research Center, Universidad Autónoma de Yucatán (Approval number CIE-06-2017).

Informed Consent

Informed consent was obtained from all subjects involved in the study authorization to be published.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors' contributions

Angelica Serralta-Interian: Investigation, methodology, data curation, writing-original draft. Julio Montero Del Toro: Methodology, data curation. Geovanny Nic-Can: Investigation, Writing – review & editing. Rafael Rojas-Herrera: Data curation, writing-review & edition. Fernando Aguilar-Ayala: Data curation, writing-review & edition. Beatriz A. Rodas-Junco: Conceptualization, Supervision, writing-original draft. All authors approved the final version submitted. Writing-review and editing, resources.

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