Dental Materials 40 (2024) 984–992

Contents lists available at ScienceDirect

Dental Materials

journal homepage: www.elsevier.com/locate/dental

Cytotoxicity effects and differentiation potential of ormocer-based and nanohybrid composite resins on human dental pulp stem cells

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ARTICLE INFO

Keywords:
Cell differentiation
Cytotoxicity
Dental pulp stem cells
Dental restorations
Nanohybrid composites
Ormocer-based composites

ABSTRACT

Objective: to compare conventional nanohybrid (Ceram.x Spectra) and ormocer-based (Admira fusion) dental composite resins effects on human dental pulp stem cells (hDPSCs) in terms of cytotoxicity, self-renewal, migration and osteogenic differentiation.
Methods: hDPSCs were cultured in presence of different dilutions (undiluted, form 1:2 to 1:100) of CeramX (CX) and Admira fusion (AD) eluates and viability assay in standard or osteogenic conditions were performed. Samples and eluates were prepared according to ISO 10993–12. In addition, apoptosis, self-renewal and migration activity evaluations were carried out. Osteogenic differentiation potential was tested by Alkaline Phosphatase Activity, alizarin red staining and gene expression of specific markers (ALP, RUNX2, OCN, OPN and COL1α1). Statistical analysis was performed by means of a One-way analysis of variance (One-way ANOVA) followed by a Tukey's test for multiple comparison; results were presented as mean ± standard error of mean (SEM).
Results: Admira Fusion demonstrated to be highly biocompatible and showed positive effects on hDPSCs proliferation and differentiation; on the contrary, conventional nanohybrid composite showed to be more cytotoxic and without any notable effect on stem cells differentiation. Moreover, the obtained results were further corroborated by a significant upregulation of osteogenic differentiation markers obtained in presence of ormocer-based composite resin eluate. Specifically, in AD 1:50 group expression levels of ALP, Runx2, Col1α1 were double than control (ALP, $p = 0.045$; Runx2, $p = 0.003$; Col1α1, $p = 0.001$) and CX 1:50 (ALP, $p = 0.006$; RUNX2, $p = 0.029$; Col1α1, $p = 0.005$). Moreover, in the same group, OPN and OCN resulted about 5 times more expressed as compared to control (OPN, $p = 0.009$; OCN, $p = 0.0005$) and CX 1:50 (OPN, $p = 0.012$; OCN, $p = 0.0006$).
Significance: The less cytotoxicity obtained by AD than conventional nanohybrid composite may be attributed to a reduced monomers release in the oral environment, supporting the hypothesis of limited adverse effect and enhanced healing potential, mainly when the material is positioned in close contact with pulp tissue.

1. Introduction

Composite resin materials are widely and successfully used in restorative dentistry due to their physio chemical features such as bond strength, mechanical properties, low solubility as well as optical and aesthetic qualities [1,2]. In addition, composite restorations require minimal preparations and allow to preserve hard dental structure providing excellent clinical results and high survival rates [3]. From a chemical point-of view, dental composite resins are composed of monomers, filler particles, as well as initiators, activators, stabilizers, and other additives [4].

Monomers and non-alloy additives are eluted in oral cavity within the first hours after placement, while leachable components are released due to degradation or erosion over time [5]. Specifically, composite resins can be degraded through several mechanisms as decomposition and dissolution in saliva, damaging during mastication, interactions with food and drugs, or bacterial activity [4]. Moreover, it has been reported that monomers may be released by polymerization of dental composites causing cracks at the tooth-material interface and resulting in increased sensitivity and microleakage [6,7]. The amount of

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https://doi.org/10.1016/j.dental.2024.05.011
Received 16 February 2024; Received in revised form 3 May 2024; Accepted 3 May 2024
Available online 9 May 2024

leachable monomers from composite resins is related to the degree of polymerization [8,9], that strongly depends on monomers' viscosity [10], concentration of used photoinitiator [11] and the environment in which the material is exposed [12]. In addition, the release of chemical substances may cause negative biological consequences demonstrating cytotoxic and genotoxic effects on cell cultures [13–15].

Interestingly, monomers seem to be able to spread through the dentine tubules and potentially interact with pulp cells even when dental composite is not in direct contact with pulp tissue [16]. Therefore, is paramount to understand whether these monomers might cause toxicity to dental pulp and have possible adverse effects on cells' viability and odontogenic differentiation [17,18]. To overcome this potential limitation of dental restorations, organically modified ceramic (ormocer) technology was recently developed with the aim to reduce unreacted monomers release [19]. Ormocer composites present an inorganic structure of polysiloxanes with pendants of conventional, organic and polymerizable molecules (methyl acrylate molecules) [20]. In these materials the methacrylate has been partially replaced by an inorganic component, such as ceramic nanoparticles, resulting in reduction of polymerization shrinkage than conventional composites resins [21]. Although recent studies reported on biocompatibility of ormocer composites [22–24], additional data regarding their effect on cell proliferation, differentiation and healing potential are still missing.

Therefore, the aim of the present in vitro study was to compare conventional nanohybrid (Ceram.x) and ormocer-based (Admira fusion) dental composites, evaluating the cytotoxicity against human dental pulp stem cells (hDPSCs) and how they influence hDPSCs self-renewal, migration and osteogenic differentiation. The null hypothesis was that different composite resins (nanohybrid vs ormocer-based) have same effects on hDPSCs cultures.

2. Materials and methods

2.1. Composite materials, sample preparation and generation of composite eluates

Ceram.x Spectra™ (Dentsply Sirona), a nanohybrid composite with pre-polymerized Sphere-Tec particles, and Admira® Fusion (Voco), a nanohybrid restorative material with the innovative ormocer technology were tested. According to the manufacturer, Admira® Fusion is a purely silicon oxide-based filling material and did not contain any classic monomer (Table 1).

The samples and the eluates were prepared according to ISO 10993–12 [25]. A stainless-steel mold was used for the preparation of standardized composite samples with a diameter of 6 mm and a thickness of 2 mm. The composite material was inserted into the holes of the mold and performed by a Heidemann spatula. The polymerization of the samples was carried out using a LED light-curing unit (3 M™ Elipar™ S10), placed in direct contact with the specimens with constant light intensity (1200 mW/cm^2) per 20 s, according to the manufacturers' instructions.

Table 1
Composite resins tested in the present study. Composition has been reported according to manufacturers.

	Ceram.x Spectra™ (CX)	Admira® Fusion (AD)
Manufacturer	Dentsply Sirona; Konstanz, Germany	VOCO; Cuxhaven, Germany
Batch number	2107000505	2139695
Colour	A3	A3
Ingredients	Ethoxylated Bisphenol A Dimethacrylate, Urethane modified Bis-GMA dimethacrylate resin, 2,2′-ethylenedioxydiethyl dimetharcylate, ytterbium trifluoride, 2,6-di-tert-butyl-p-cresol, pre-polymerized SphereTEC® fillers	Organically modified silicic acid, fumes silica, 2,6-di-tert-butyl-p-cresol
Filler	Barium glass, prepolymerized filler, ytterbium fluoride: HV: 78-80 wt% or 60-62 vol%.	Silicon oxide: 84 wt% or 60 vol%.

HV: High viscosity

The eluates were produced according to ISO 10993–12, disinfecting the composite samples for 60 s with ethanol 70% (Sigma-Aldrich), washing twice with sterile H_2O and then stored in 800 μL of Dental Pulp Stem Cell Growth Medium (DPSC-GM) (Lonza, PT-4516 containing human Dental Pulp stem cell Growth Supplement, PT-3927) per sample in an incubator, at 37 °C [26]. After 72 h, the composite samples were removed, and the extracts (at concentration 1:1) were recovered and stored at − 20 °C until use. The same preparation was conducted using osteogenic medium (DPSC-GM containing 10^{-8} M dexamethasone, and 8 mM β-glycerophosphate) and growth medium without serum. Since the 1:1 eluate concentration might affect outcomes of in vitro experiments, dilutions of the eluates, ranging from 1:1 to 1:100, were used to evaluate cellular effects on hDPSCs.

3. Human dental pulp stem cells culture

Human dental pulp stem cells (hDPSCs) were purchased from Lonza (PT-5025, Swiss, Basel, Switzerland), cultured and maintained following manufacturers' instruction in DPSC-GM, or induced to osteogenesis using the osteogenic medium described above, in an incubator at the temperature of 37 °C and 5% CO_2. In this study, hDPSCs from passages 4–6 were used. Cells were treated with Ceram.x (CX) or Admira (AD) eluates undiluted (1:1) or at different dilutions (form 1:2 to 1:100 according to the experimental settings).

3.1. Viability assay

The cytotoxicity of the eluates on hDPSCs was examined using the MTT [3-(4,5- dimethylthiazol-2-yl)− 2,5-diphenyltetrazolium bromide] assay, according to manufacturer's instructions (Sigma-Aldrich, M5655), as previously described [27]. The cells were placed into a 96-well at an initial density of 5 × 10^3 cells per well; after the cell adhered for 24 h, the appropriate extracts were added at different dilutions (1:1, 1:2, 1:4, 1:10, 1:50, 1:100) and the 96-wells were incubated for 96 h. The medium was eliminated by each well and MTT solution (final concentration 5 μg/ml) was added and incubated for 4 h at 37 °C and 5% CO_2. When the purple precipitates were formed, 100 μL acidic isopropanol (0.04 N HCl in absolute isopropanol) were added to create the formazan dye soluble. Absorbance was measured at 570 nm using a microplate reader (Tecan, Grödic, Austria). Cells cultured with hDPSCs medium were used as control. The assay was conducted also in osteogenic medium for 72 h starting the treatment with cells at confluence density. For both eluates, 1:1, 1:50 and 1:100 dilutions are tested, and the control was represented by cells in osteogenic medium. The results were obtained in triplicate from 3 separate experiments for each test.

3.2. Apoptosis assay

hDPSCs (80.000 cells/well in a 24 well-plate) were treated with the dilutions 1:1, 1:50 and 1:100 of CX and AD eluates in growth medium for 96 h. At the end of stimulation, caspase 3/7 enzyme activity (Elabscience, E-CK-A383) was calculated as enzymatic units/mg protein according to manufacturer's protocols to evaluate the apoptosis. The results were obtained in triplicate from 3 separate experiments for each assay.

3.3. Evaluation of clonogenic activity

To evaluate the hDPSCs clonogenic activity in the presence of the different eluates, a colony forming unit-fibroblast (CFU-F) assay was performed. Briefly, 2 × 10^2 cells/well were cultured into 6 well, in

growth medium at 37 °C and 5% of CO_2. The extracts at 1:1 and 1:50 dilutions were added after 24 h and cells were fixed 7 days after treatments and stained with crystal violet for 10 min at room temperature, then washed with sterile water until clear. The number of colonies in each well was obtained by manual counting with an optical microscope. Colonies with n ≥ 30 cells were considered. Cells without treatment were used as control. Data were presented as fold change as compared to control. The results were obtained in triplicate from 6 separate experiments for each test.

3.4. Wound healing assay

To assess the effect of different dental composites eluates on hDPSCs migration, a wound healing assay was performed. The cells were cultured into 6-well plates until confluency. The growth medium was replaced by a serum-free medium for 2 h, a wound was generated manually with a 1000 μL pipette tip and cells were treated with the extracts (1:1 and 1:50 dilutions in serum-free medium). The cell cultures were photographed with an optical microscope (LEICA DMi6000) at zero time point (T0) and after 72 h (T72) to quantify the wound closure area using ImageJ software (National Institutes of Health, Bethesda, MD, USA). At least 5 images were analysed for each sample. Cells without extracts served as control. Results were obtained in triplicate from 4 separate experiments for each test and shown as percentage of wound closure calculated as (area T0-area T72)/area T0 X 100.

3.5. Alkaline phosphatase activity

The cells were cultured in a 48-well plate at a density of 2.5×10^4 cells/well. After allowing to adhere overnight, the cells were cultured with osteogenic medium in the presence of dilutions 1:50 of the eluates for 10 days [28]. At the end of the treatment, Alkaline Phosphatase (ALP) activity was determined using an Alkaline Phosphatase Activity Assay Kit (Elabscience, E-BC-K091) according to the manufacturer's instructions and calculated as enzymatic units/mg protein. Moreover, cells were fixed for ALP staining with the Leukocyte Alkaline Phosphatase kit (Sigma-Aldrich, 85L2) following the manufacturer's instructions. The results were obtained in triplicate from 3 separate experiments for each assay.

3.6. Alizarin red staining assay

hDPSCs were plated in a 48-well plate at a density of 2.5×10^4 cells/well. Reached the confluence, the cells were cultured with osteogenic medium in the presence of dilutions 1:50 of the eluates for 10 days. The accumulated calcium deposition was analysed using alizarin red stain solution as described in a previous study [29]. Briefly, the cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 15 min at room temperature, then fixative was removed and wash with distilled water was conducted. Subsequently, 250 μL of alizarin red Staining solution (Merck, TMS-008-C) stain was added to cells for at least 30 min. After the cells were washed two times with deionized water, photographs were taken using an optical microscope equipped with a digital camera at 10X magnification (LEICA DMi6000). The quantification of alizarin red was obtained as previously described [30]. In brief, 10% acetic acid was added to cell samples for 30 min in shaking and then samples were evaluated at a wavelength of 405 nm using a using a microplate reader (Tecan, Grödic, Austria). The results were obtained in triplicate from 3 separate experiments for each test.

3.7. q-PCR analysis

Total RNA was extracted from cell cultures using the TriZOL^R Reagent (Invitrogen), following the manufacturer's instructions. Reverse transcription was carried out using 1.0 μg total RNA and High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, 4387406). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, 4367659) and gene-specific primers as detailed in Table 2. The amplification was performed using the StepOne™ Real-Time PCR System (Applied Biosystems, 4376357) with the following cycling conditions: cDNA denaturation and polymerase activation step at 95 °C for 10 min followed by 40 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min. The relative gene expression analysis of target genes was conducted in comparison with the GAPDH housekeeping control gene following the comparative $2^{-\Delta Ct}$ method. The normalized expression was calculated as fold change mRNA level versus control condition. The results were obtained in triplicate from 4 separate experiments for each assay.

3.8. Statistical analysis

The results were presented as mean ± standard error of mean (SEM) of at least three independent experiments, as indicated in the figure legends. Statistical analysis of the data was performed by means of a One-way analysis of variance (One-way ANOVA) followed by a Tukey's test for multiple comparison using GraphPad Prism 9.0 software (GraphPad Software, Inc., La Jolla, CA, USA). A p lower than 0.05 was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

4. Results

4.1. Cytotoxicity evaluation in growth medium and apoptosis

To evaluate possible cytotoxic effects of the composites on hDPSCs, cells were treated with CX and AD eluates. Ninety-six h after CX eluate treatment in growth medium, a cytotoxic effect on hDPSCs compared to the control was observed (Fig. 1A). The conventional composite causes a significant reduction of cell viability of 2.5 times ($p = 0.0008$) when not diluted (CX 1:1), and, to a lesser extent, at dilutions of 1:2 (1.86 fold, $p = 0.0111$) and of 1:4 (1.72 fold, $p = 0.0251$) than control. When cells were treated with CX 1:10, 1:50 and 1:100, no significant effects were reported on cell growing. On the other hand, the AD eluate did not display significant adverse effects on hDPSCs viability at all tested dilutions (Fig. 1B). To assess whether the observed reduction of cell viability in CX treated cells was due to apoptotic cell death, a caspase 3/7 activity was evaluated in cells after treatment with CX and AD at the more relevant dilutions, namely 1:1, 1:50 and 1:100 (Fig. 1C). The undiluted CX (1:1) eluate shows a high caspase 3/7 activity as compared to the control basal level (Control=0.084; CX 1:1 = 0.174; $p = 0.0010$). Moreover, CX 1:1 surpasses CX 1:50 and 1:100 dilutions by three-fold (CX 1:50 = 0.047; CX 1:100 = 0.055; $p < 0.0001$) in caspase 3/7 levels. Conversely, CX 1:50, CX 1:100 and all AD dilutions did not show significant changes in caspase activity as compared to untreated cells. Remarkably, it's been observed a statistically significant difference in cell apoptotic activity between conventional monomers and the innovative ormocer technology, particularly when comparing CX 1:1 to AD 1:1 (AD 1:1 =0.092; $p = 0.0026$), as well as between CX 1:1 and AD 1:50

Table 2
Primer sequences for qPCR analysis.

Primer	Forward sequence	Reverse sequence
RUNX2	F - ATGTGTGTTTGTTTCAGCAGCA	R - TCCCTAAAGTCACTCGGTATGTGTA
OCN	F - TGAGAGCCCTCACACTCCTC	R - ACCTTTGCTGGACTCTGCAC
OPN	F - CAGTTGTCCCCACAGTAGACAC	R - GTGATGTCCTCGTCTGTAGCATC
ALP	F - TCAGAAGCTCAACACCAACG	R - TTGTACGTCTTGGAGAGGGC
COL1α1	F - CCCGGGTTTCAGAGACAACTTC	R - TCCACATGCTTTATTCCAGCAATC
GAPDH	F - TCAGCAATGCCTCCTGCAC	R - TCTGGGTGGCAGTGATGGC

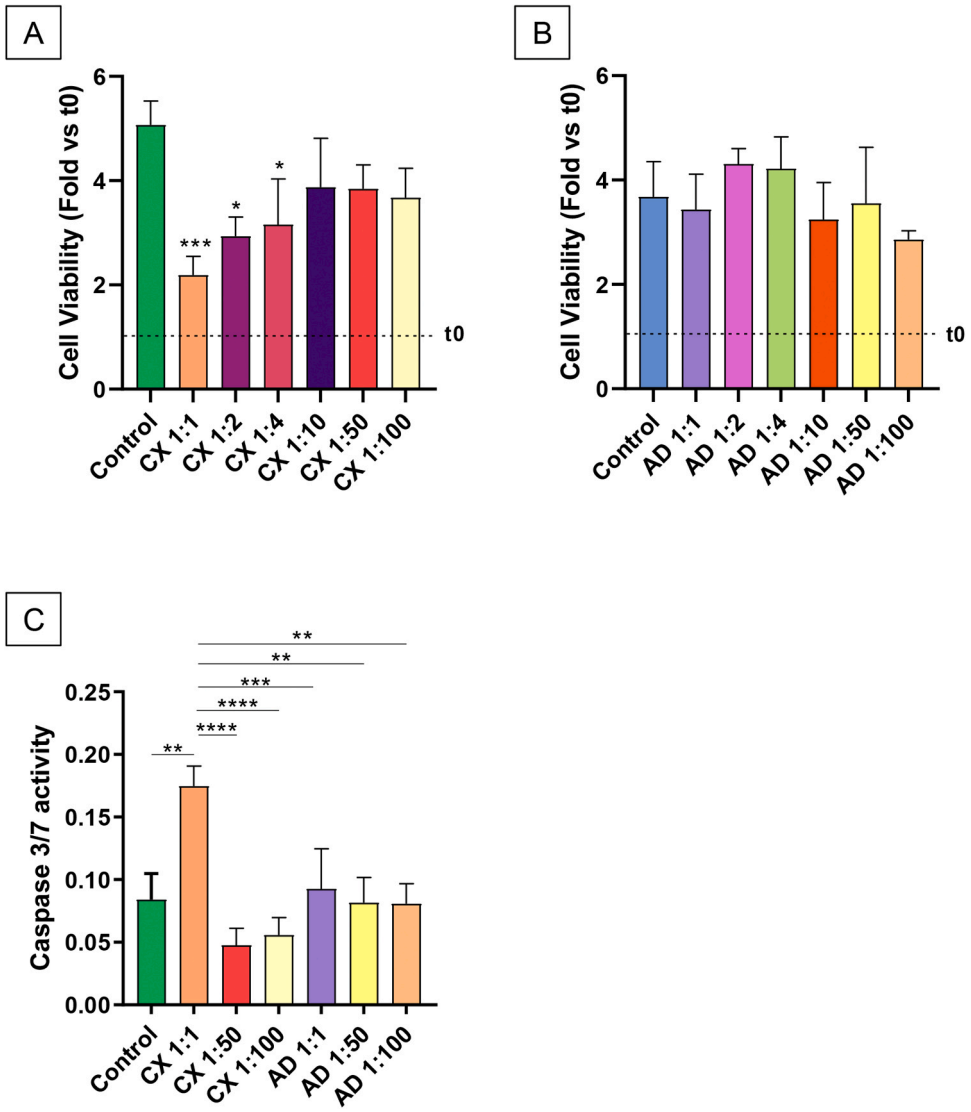

Fig. 1. Cell viability in growth medium of conventional composite Ceram.x (CX) (A) and the innovative technology Ormocer Admira® Fusion (AD) (B) evaluated with MTT assay after 96 h (n = 3); apoptosis evaluation tested with caspase 3/7 activity assay (n = 3) (C). * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

and 1:100 dilutions (AD 1:50 = 0.081; AD 1:100 = 0.080; p = 0.0007). Based on these observations, the subsequent experiments were performed with eluate dilutions of 1:1 and 1:50, that allowed the investigation of functional effects in cytotoxic conditions.

4.2. Self-renewal and migration activity evaluation

To investigate whether the composites might affect clonogenic ability of hDPSCs, a colony forming unit – fibroblast (CFU-F) assay was performed. Fig. 2A shows how the conventional undiluted eluate CX 1:1 reduced 4 times the clonogenic capacity of hDPSCs as compared to the control ($p < 0.0001$), while the CX 1:50 dilution did not result in significant difference with untreated cells, thus preserving clonogenic capacity. When treated with AD 1:1, hDPSCs exhibited a slight reduction in self-renewal as compared to the control group (fold: 0.638; p = 0.0337), while no differences with the untreated cells were observable in AD 1:50. Additionally, the conventional undiluted composite CX 1:1 seemed to limit the formation of colonies as compared to AD 1:1 and AD 1:50 dilutions (fold CX 1:1: 0.27, fold AD 1:1: 0.63, p = 0.0295; fold AD 1:50: 0.812, p = 0.0008).

To investigate the migration ability of hDPSCs in presence of different dental composites eluates, a wound healing after 72 h was assessed. As shown in Fig. 2B, non-diluted CX (1:1) eluate reduced the cell migration rates of 20.3% as compared to the control (p = 0.0486), while CX 1:50 dilution did not show any significant difference as compared to unstimulated cells. Conversely, in AD eluates no statistical differences were revealed at any dilutions as compared with the untreated cells. Consequently, the wound closure reduction observed after CX 1:1 treatment was also significantly lower than AD treatments. These results indicated that the two composites seemed to have different migration effects on hDPSCs. Particularly, significant difference among the conventional composite and the omocer technology were highlightable (percentage of closure CX 1:1 = 35.05%, AD 1:1 = 66.8%, p = 0.0011; AD 1:50 = 55.26%, p = 0.0497). These data demonstrated that CX and AD could differently affect the self-renewal and the migration ability of hDPSCs, also in relation to different dilutions.

4.3. Cells viability in osteogenic condition

To evaluate the effects of the composites on hDPSCs cell viability even in osteogenic condition, cells were treated with CX and AD eluates at more relevant dilutions in presence of osteogenic medium. Fig. 3A

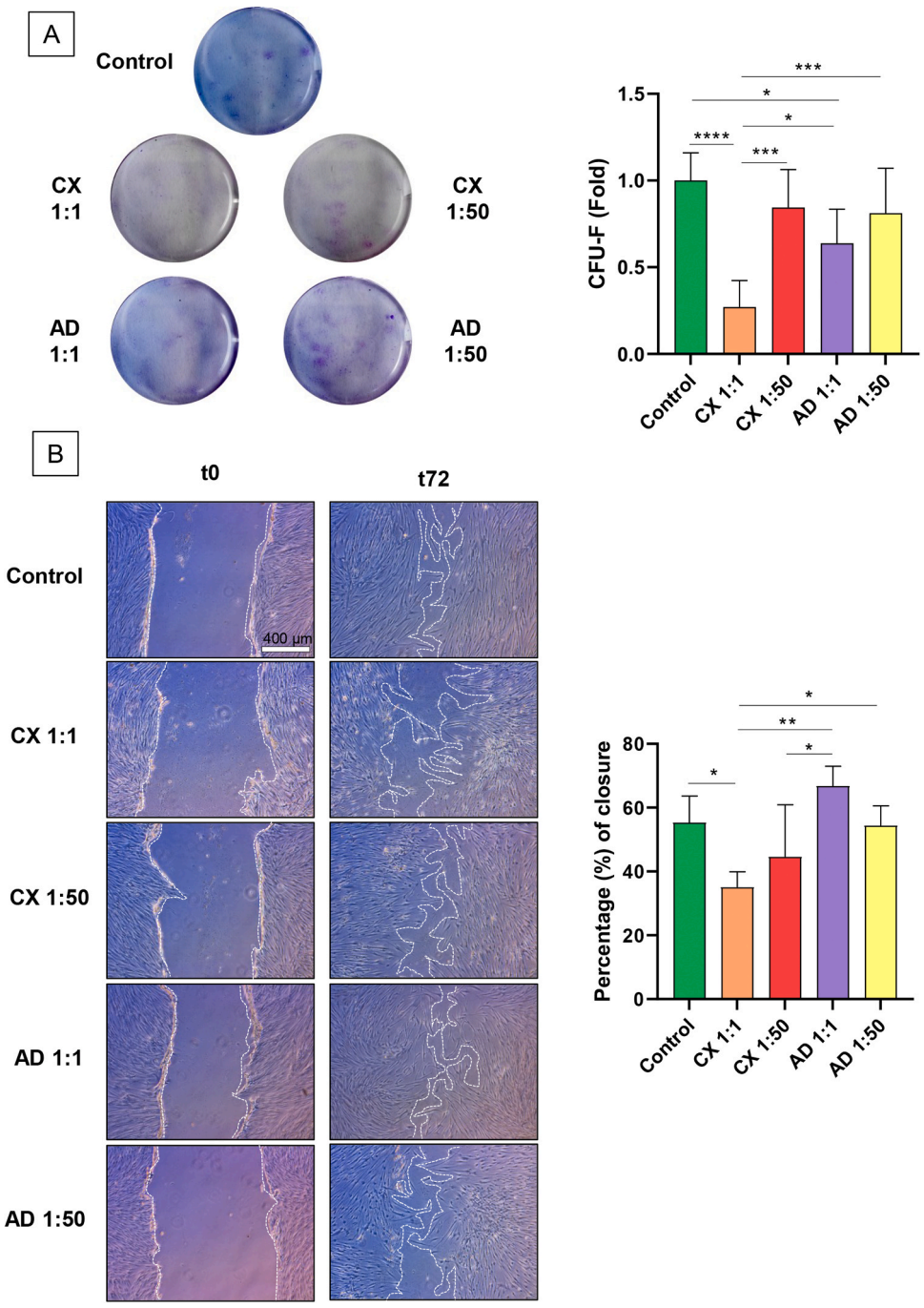

Fig. 2. Evaluation of the ability to form colonies assessed with crystal violet (n = 6) (A) and to migrate after 72 h of treatment tested with wound healing assay (n = 4) (B). scale bar= 400 µm. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$, * ** * $p < 0.0001$.

highlights how the undiluted CX (1:1) eluate caused a significant decrease (almost twice time) in the cell viability as compared to the control group ($p = 0.0065$). Instead, all dilutions of AD eluate showed no differences as compared to the untreated cells (Fig. 3B). These data supported the hypothesis that the two tested composites had different effect on cells viability also in osteogenic conditions.

4.4. Osteogenic differentiation of hDPSCs

To investigate the ability of the studied composites to influence osteogenesis, cells were induced towards osteogenic differentiation using only 1:50 dilutions of both CX and AD, to avoid cytotoxicity and to assess functional outcomes of the assays. As shown in Fig. 4A, ALP assays showed a significant decrease of ALP staining and activity in CX 1:50 eluate as compared to untreated cells (Control= 0.18, CX 1:50 = 0.089; $p = 0.0376$), while a significant increase in ALP activity occurred when cells were treated with AD extract 1:50 as compared to the control (AD 1:50 = 0.31; $p = 0.0044$), and to CX 1:50 (CX 1:50 = 0.089; $p < 0.0001$). These observations probably indicated that an adverse effect might be exerted by the CX eluate on the osteogenic differentiation and stressed a better behavior of AD 1:50. These results were partially confirmed by the ability of hDPSCs to generate mineralization nodules observable in Fig. 4B. The conventional composite seemed not to have a significant effect in terms of mineralization capacity as compared to control group, while AD 1:50 eluate promoted more calcium deposits than control (OD Control= 0.124, AD 1:50 = 0.2;

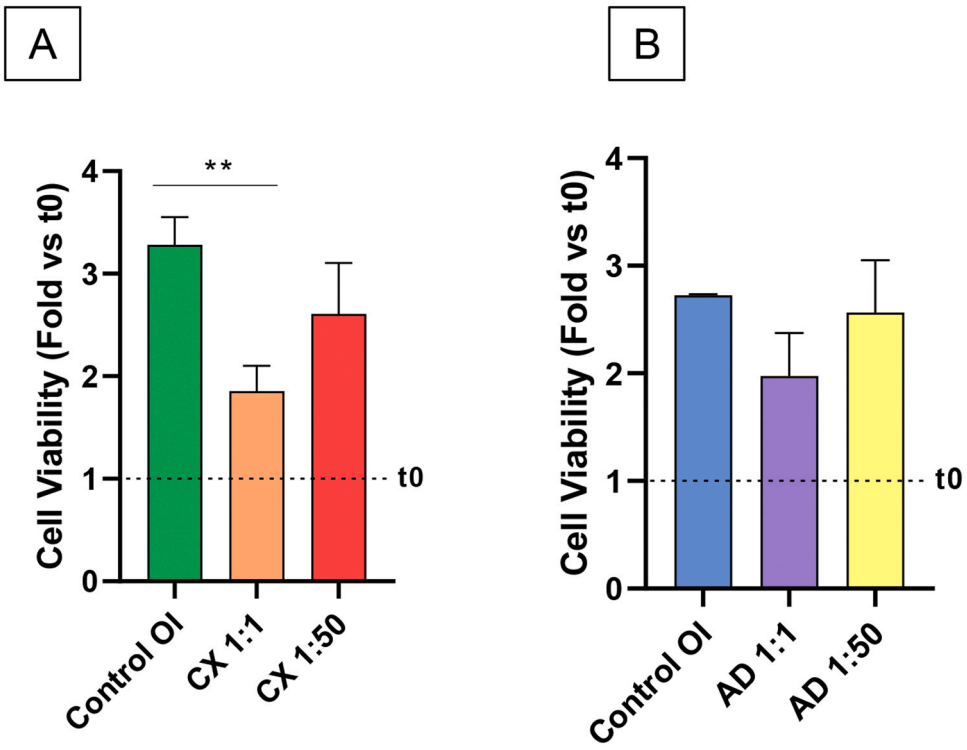

Fig. 3. Cell viability in osteogenic medium of conventional composite CX (n = 3) (A) and the innovative technology Ormocer AD (n = 3) (B) evaluated with MTT assay after 96 h. * * p < 0.01.

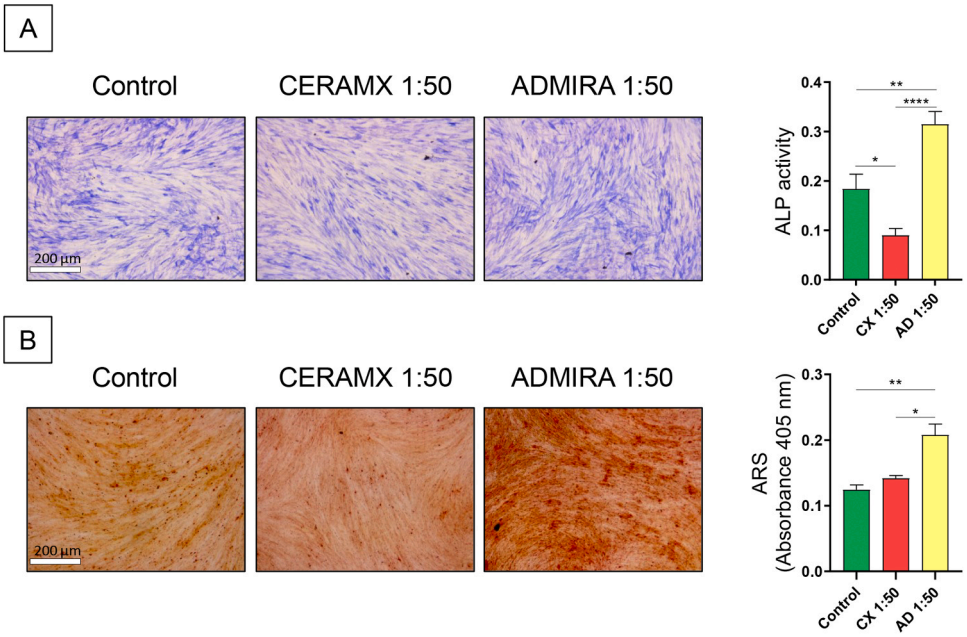

Fig. 4. Alkaline phosphatase activity shown as representative staining and enzymatic activity (n = 6) (A) and alizarin red representative staining and quantization (n = 3) (B) after 10 days of treatments (scale bar= XXX). * p < 0.05, * * p < 0.01, * ** * p < 0.0001.

p = 0.0041) and CX 1:50 (OD CX 1:50 = 0.141; p = 0.0127).

4.5. Gene expression

To corroborate osteogenic differentiation results, a gene expression analysis was conducted on specific markers 10 days after differentiation under CX 1:50 and AD 1:50 conditions (Fig. 5). The expression levels of ALP, RUNX2, OCN, OPN and COL1α1 did not differ among the conventional composite CX 1:50 and control group, while their mRNA levels were significantly higher in AD 1:50 than CX 1:50 and control. Particularly, mRNA levels in AD 1:50 group were double in ALP, Runx2, Col1α1 as compared to control (ALP, p = 0.045; Runx2, p = 0.003; Col1α1, p = 0.001) and CX 1:50 (ALP, p = 0.006; RUNX2, p = 0.029; Col1α1, p = 0.005). While OPN and OCN in AD group resulted about 5 times more expressed than control (OPN, p = 0.009; OCN, p = 0.0005) and CX 1:50 (OPN, p = 0.012; OCN, p = 0.0006). These results were in

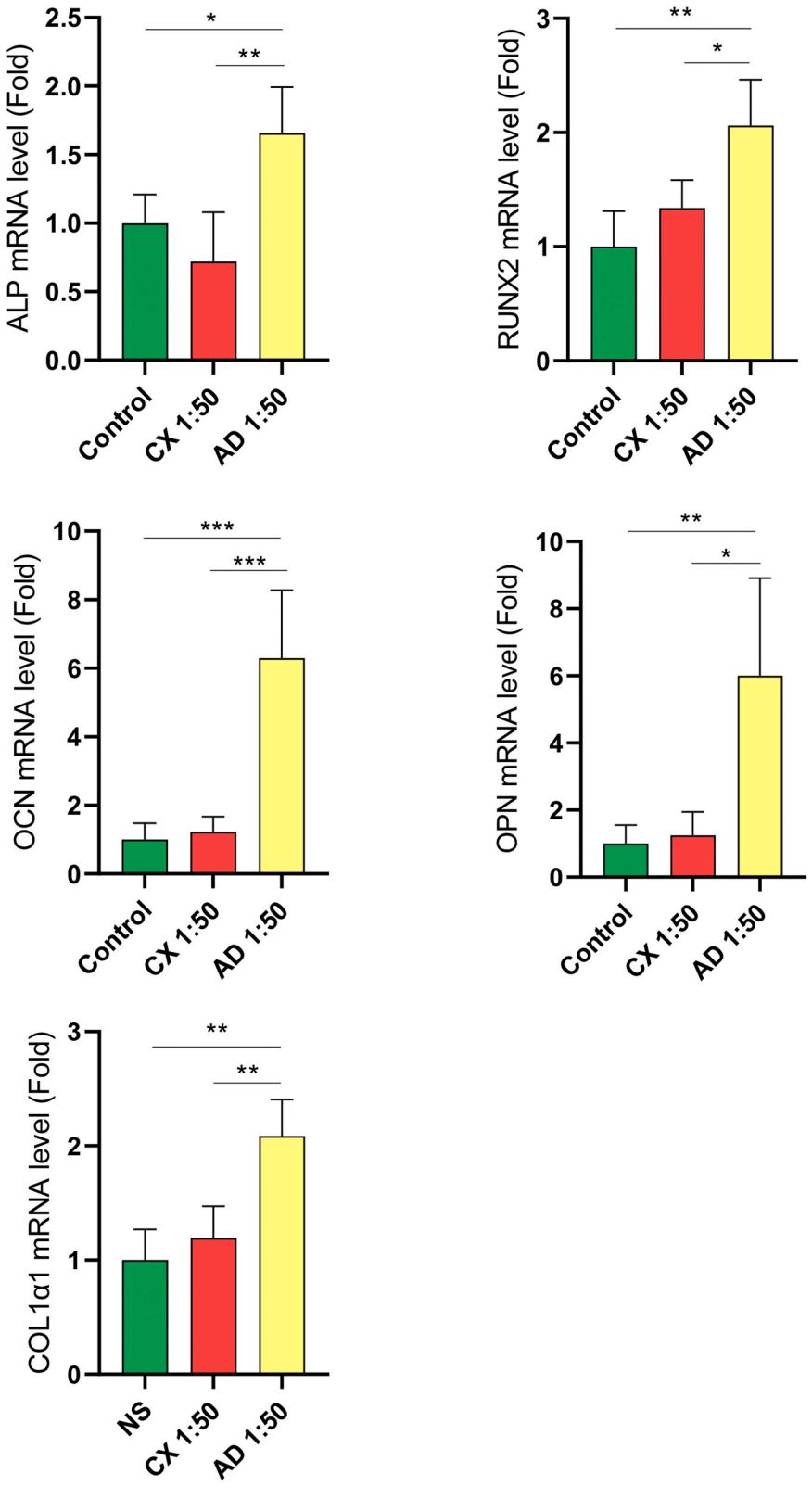

Fig. 5. qPCR for osteogenic marker genes (n = 4 per group). * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

line with the hypothesis that AD eluate at 1:50 dilution might have beneficial effects in inducing cell maturation and differentiation.

5. Discussion

The present study observed that conventional composite eluate might exert detrimental effect on hDPSCs functionality while new technology Ormocer mostly preserved cells properties, thus rejecting the

null hypothesis. Specifically, the obtained results demonstrated that the nanohybrid conventional composite eluate significantly impacted hDPSCs viability, inducing cytotoxic effects when undiluted or at lower dilutions of 1:2 and 1:4 than control, stressing the potential adverse effects of Ceram.x on cellular health. Intriguingly, further dilutions (1:10, 1:50, and 1:100) did not exhibit any noticeable impact on cell growth, indicating a threshold effect related to the concentration of Ceram.x that elicited cytotoxicity. For this reason, undiluted, 1:50 and 1:100 dilutions compared to 1:1 were used to evaluate apoptotic induction. Conversely, the Admira Fusion composite eluate demonstrated a starkly different profile, displaying no adverse effects on hDPSCs viability at all tested dilutions. These data were in line with previous studies that showed lower cytotoxic effect of ormocer composites than conventional ones (i.e. nanohybrid, nanofiller, etc) on human gingival fibroblasts [22,23,31] and hDPSCs [23]. Furthermore, the assessment of caspase 3/7 activity provided deeper insights into the underlying mechanisms of cytotoxicity. The elevated apoptotic activity observed in response to undiluted Ceram.x eluate, significantly surpassing both the control and its own dilutions, suggesting a potential induction of cell death. For this reason, the subsequent experiments were carried out with 1:50 dilution, omitting 1:10 dilution due to its proximity to cytotoxic levels and excluding 1:100 dilution as the lowest concentration. On the other hand, it was observed an absence of any significant difference in caspase 3/7 activity between Admira Fusion eluates and the control, supporting the hypothesis of its non-cytotoxic effect. The absence of cytotoxicity might be explained by the lack of conventional dimethacrylate monomers and unreacted c=c groups within the resin matrix [32,33], contributing to enhanced biocompatibility [34]. In fact, Ormocers exhibited reduced monomer release owing to their lower initial monomer content [19,35]. Additionally, their three-dimensional network structure effectively impedes the release of bonded monomers, contributing to minimized monomer release profile [36]. Therefore, the diverse behaviour of the two evaluated materials could be attributed to the minimal release of monomers from Ormocer-based composite, causing less toxicity than the methacrylate-based counterpart [37–39]. Accordingly, Ceram.x in its undiluted form, significantly impaired both clonogenic activity and cell migration rates, highlighting its potential adverse impact on essential cellular processes. On the other hand, Ormocer undiluted eluates showed a minor reduction in clonogenic potential, when cells were cultured at very low density in CFU-F experiment, that represents a more specific test for stem-like cell activity. Since a reduced clonogenic activity is not necessarily associated with a dysfunctional cell proliferation [40], the obtained results suggested a relatively milder impact of Admira 1:1 on stem-like features maintenance, while no adverse effects were exerted on cell proliferation and migration.

The evaluation of composites' cytotoxicity also plays a crucial role from a clinical point of view [41]. Indeed, in dental restorations the impact of monomers release and their potential toxicity on tissue healing strictly depend on several variables such as dentin permeability, dentin thickness, etching and depth of cavities [23]. The proximity to dental pulp tissue might be considered as a negative prognostic factor for undifferentiated cells recruitment and deposition of a mineralized matrix in response to an injury during reparative dentinogenesis [42,43]. Dental pulp stem cells are a potential source of multipotent mesenchymal stem cells able to proliferate and differentiate into osteoblast-like or odontoblast-like cells, namely cells responsible for mineralization [28,29,44]. Due to this reason, the effect of the studied composites on hDPSCs cell viability was appreciated even in osteogenic condition. Ceram.x eluates (1:50 and 1: 100) demonstrated a substantial reduction in cell viability than Admira Fusion ones, highlighting the superior biocompatibility of the latter. Moreover, a diverse impact on osteogenic differentiation and mineralization was appreciated between the tested materials. In particular, the decrease in ALP activity associated with Ceram.x eluate suggested a possible interference with early stages of osteogenesis. In contrast, the heightened enzymatic ALP activity observed in Ormocer extract implied a potential positive influence on osteogenic processes, that was further supported by deposition of mineralization nodules (particularly in AD 1:50); interestingly, CX 1:50 performed as the control in terms of mineralization process. These findings were even corroborated by the significantly higher expression levels of osteogenic differentiation markers in Ormocer group compared to both conventional composite and control groups. On the best of our knowledge, this is the first study that evaluate the hDPSCs differentiation in osteogenic pattern and pave the way for further clarifications on how Ormocers and its composition influenced osteogenic induction. The osteogenic differentiation was used as model of differentiation in cells able to provide mineralized matrix deposition [45], although the studied materials won't never be placed in direct contact with bone tissue. Moreover, analysis of the expression of genes common to odontoblasts and osteoblasts (as OCN, ALP) was performed. However, this could be considered as a potential limit of the present work and future studies should investigate in depth the specific effect of Ormocers on odontogenic differentiation.

Although the impact of Ormocer composite on undifferentiated mesenchymal stem cells should be further confirmed, based on the outcomes obtained by the present study, it may be speculated that Admira Fusion was highly biocompatible and showed positive effects on hDPSCs proliferation and differentiation in cells able to provide mineralization; on the contrary, conventional nanohybrid composite demonstrated to be more cytotoxic and without any notable effect on stem cells differentiation. These observations may be explained not only due to the reduced number of unreached monomers release, but probably on the less toxicity of delivered monomers. However, the differences in chemical composition of monomers released by ormocer-based composites vs conventional ones have not been yet elucidated [19,23], and this aspect may be considered as a limitation of the present in vitro study. Therefore, the qualitative effect of ormocer composites' monomers should be clarified in depth in future research to better elucidate the promising use as indirect pulp capping material [46–48] and to support the possible repair potential of dentin-pulp complex in case of deep cavitated lesions.

6. Conclusions

The impact of Ceram.x and Admira fusion composites on hDPSCs functionality diverged significantly; while Ceram.x, particularly at higher concentrations, displayed cytotoxic effects, Admira Fusion emerged as a promising alternative, demonstrating biocompatibility properties even in undiluted form and might present a more favourable choice for dental applications, mainly when deposition of mineralized tissue is required.

However, further studies focusing on long-term effects, in vivo assessments, and the specific cellular pathways involved would be instrumental in solidifying these observations, understanding how different composite materials influence cell behavior in osteogenic settings, and guiding clinical decision-making in restorative dentistry.

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