Universidade Federal De Uberlândia
Faculdade De Odontologia
Programa De Pós-Graduação Em Odontologia

Geovana Pires Da Silva

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Orientadora: Profª. Drª. Ana Paula Turrioni

Banca examinadora:
Profª. Drª. Ana Paula Turrioni
Profª. Drª. Fabíola de Carvalho Carlo
Profª. Drª. Fernanda Gonçalves Basso Lombardi

UBERLÂNDIA
2026

UNIVERSIDADE FEDERAL DE UBERLÂNDIA
Coordenação do Programa de Pós-Graduação em Odontologia
Av. Pará, 1720, Bloco 4L, Anexo B, Sala 35 - Bairro Umuarama, Uberlândia-MG, CEP 38400-902
Telefone: (34) 3225-8115/8108 - www.ppgoufu.com - copod@umuarama.ufu.br

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RESUMO

Células-tronco da polpa dentária humana (DPSCs) apresentam potencial para diferenciação neuronal, porém, a inflamação pode comprometer esse processo. Este estudo avaliou o impacto do TNF-α na neurodiferenciação das DPSCs e o papel modulador da lipoxina A_4 (LXA_4) e da resolvina D_1 (RVD_1). DPSCs foram isoladas e submetidas à diferenciação neuronal por 21 dias. A concentração ideal de TNF-α foi definida em estudo piloto, avaliado por PCR em tempo real. A produção de OCT3/4, Doublecortina e β-III-tubulina foi analisada por ensaio MTT, citometria de fluxo e imunocitoquímica. A ação da LXA_4 (10 ou 100 nM) e RvD_1 (10 ou 100 nM) foi avaliada por citometria de fluxo. Os dados foram analisados pelos testes de Kruskal-Wallis, complementado por Mann-Whitney ($p<0,05$) e One-Way ANOVA com pós-teste de Tukey. Foi demonstrado que 25 ng/ml de TNF-α induz inflamação sem causar citotoxicidade. A alta produção de doublecortina indicou o potencial de diferenciação neuronal das DPSCs. A baixa produção de β-III-tubulina indicou que o TNF-α inibe a progressão da diferenciação neuronal. O aumento da produção de β-III-tubulina na presença de LXA_4 10 e 100 nM ($p=0,02$; $p=0,03$) e RvD_1 100 nM ($p=0.02$), indica que esses mediadores atenuam os efeitos inibitórios do TNF-α. A terapia anti-inflamatória combinada às DPSCs pode potencializar a recuperação motora após neurotrauma.

Palavras-chave: célula-tronco; lesão medular; inflamação; medicina de reabilitação

ABSTRACT

Human dental pulp stem cells (DPSCs) exhibit neurogenic differentiation potential; however, inflammatory conditions may impair this process. This study investigated the impact of tumor necrosis factor-alpha (TNF-α) on DPSC neurodifferentiation and the modulatory role of the pro-resolving lipid mediators lipoxin A_4 (LXA_4) and resolvin D_1 (RvD1). DPSCs were isolated and subjected to neuronal differentiation for 21 days. The optimal TNF-α concentration was determined in a pilot study using real-time PCR. The expression of OCT3/4, doublecortin, and β-III-tubulin was analyzed by MTT assay, flow cytometry, and immunofluorescence. The effects of LXA_4 (10 or 100 nM) and RvD_1 (10 or 100 nM) were assessed by flow cytometry. Data were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney test ($p<0.05$) and one-way ANOVA with Tukey's post hoc test. TNF-α at a concentration of 25 ng/mL was shown to induce inflammation without causing cytotoxicity. The high production of doublecortin indicated the neuronal differentiation potential of DPSCs, whereas reduced β-III-tubulin expression demonstrated that TNF-α inhibits the progression of neuronal differentiation. Conversely, increased β-III-tubulin expression in the presence of LXA_4 (10 and 100 nM; $p=0.02$ and $p=0.03$, respectively) and RvD_1 (100 nM; $p=0.02$) indicate that these mediators attenuate the inhibitory effects of TNF-α. These findings suggest that anti-inflammatory therapy combined with DPSCs may enhance motor recovery following neurotrauma.

Keywords: stem cell; spinal cord injury; inflammation; rehabilitation medicine

SUMÁRIO

INTRODUÇÃO E REFERENCIAL TEÓRICO

A regeneração do sistema nervoso central (SNC) representa um grande desafio para a medicina regenerativa em função do estabelecimento de um microambiente desfavorável após lesões traumáticas ou isquêmicas e da baixa plasticidade dos neurônios maduros (Kvistad; Krakenes; Gavasso, 2024). Em condições fisiológicas, a inflamação constitui um processo essencial e protetor, atuando na contenção dos danos e na promoção do reparo tecidual. Por outro lado, quando prolongada, a resposta inflamatória compromete o processo de reparo, agravando o dano tecidual e limitando a recuperação funcional (Cavalcanti *et al*., 2025).

A resposta inflamatória que se segue após lesões do SNC é mediada por uma série de citocinas pró-inflamatórias, dentre elas o fator de necrose tumoral alfa (TNF-α). O TNF-α, sintetizado por macrófagos ativados e células imunes, é conhecido por coordenar a ativação de vias de sinalização pró-inflamatórias, estimulando a apoptose programada e a necrose (Gonzalez Caldito, 2023; He *et al*., 2025; Li *et al*., 2023b). Além disso estudos em modelos de células-tronco mesenquimais indicam que a exposição crônica às citocinas inflamatórias, em especial ao TNF-α, pode comprometer o potencial de diferenciação dessas células e promover alterações celulares que dificultem a regeneração tecidual (He *et al*., 2025; Li *et al*., 2023b).

No contexto das lesões medulares, além da liberação prolongada de citocinas pró-inflamatórias que mantém o dano tecidual, a recuperação funcional é adicionalmente limitada pela formação de cicatriz glial, que atua como barreira física e molecular à regeneração axonal (Cavalcanti *et al*., 2025; Kvistad; Krakenes; Gavasso, 2024). Em resposta a esses desafios, abordagens terapêuticas baseadas na utilização de células-tronco surgem como uma estratégia promissora, haja vista que são capazes de mediar a secreção de citocinas e fatores neurotróficos, além de estimular o potencial regenerativo de células-tronco neurais endógenas, favorecendo o reparo funcional nos casos de lesão medular (Kvistad; Krakenes; Gavasso, 2024).

Nesse cenário, as células-tronco originárias da polpa dentária humana (DPSCs) têm recebido grande destaque. Essas células podem ser facilmente obtidas, a partir de terceiros molares impactados ou dentes extraídos por motivos ortodônticos, e sua origem a partir da crista neural craniana, faz com que elas apresentem características biológicas semelhantes às progenitoras neurais (Li *et al*., 2023a; Staniowski; Zawadzka-Knefel;

Skośkiewicz-Malinowska, 2021). Além disso, as DPSCs apresentam grande capacidade proliferativa e multipotência, sob condições específicas, se diferenciando em diferentes linhagens celulares como odontoblastos, osteoblastos, células endoteliais e células neurais (Li *et al*., 2023a; Staniowski; Zawadzka-Knefel; Skośkiewicz-Malinowska, 2021). Por último, pesquisas emergentes indicam que as DPSCs possuem propriedades imunomoduladoras que lhes permite interagir com o sistema imunológico, promovendo processos anti-inflamatórios que podem ser benéficos em microambientes de lesão (Li *et al*., 2023a).

Ademais, a busca por mecanismos de resolução da resposta inflamatória tem ganhado destaque, especialmente com a descoberta de mediadores lipídicos pró-resolutivos especializados. Esses mediadores incluem as lipoxinas e resolvinas, formadas a partir do metabolismo de ácidos graxos poli-insaturados como o ômega-6 e ômega-3 (Derada Troletti *et al*., 2021; Julliard et al., 2022). A lipoxina A_4 (LXA_4) e a resolvina D_1 (RvD_1) exemplificam essa classe de mediadores lipídicos que facilitam a restauração da homeostase tecidual por meio da inibição do recrutamento de neutrófilos, redução da produção e secreção de citocinas pró-inflamatórias e promoção da fagocitose de debris celulares (Derada Troletti *et al*., 2021; Julliard *et al*., 2022).

A integração desses conhecimentos estabelece uma base teórica sólida para investigar como o microambiente inflamatório influencia a capacidade de diferenciação das DPSCs em linhagens neurais. Além disso, é importante investigar também estratégias que modulam a resposta inflamatória, como o uso de mediadores lipídicos pró-resolução, os quais reduzem os efeitos desfavoráveis da liberação prolongada de citocinas pró-inflamação, favorecendo o reparo neural. A compreensão desses mecanismos é essencial para orientar o desenvolvimento de abordagens terapêuticas inovadoras que facilitem o tratamento de doenças e lesões do SNC.

CAPÍTULO 1

Artigo:

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Artigo adequado às normas e diretrizes a ser submetido na revista Cell Biology International.

Lipoxin A_4 and Resolvin D_1 preserve neural inductive capacity of dental pulp stem cells cultured under inflammatory conditions

Geovana Pires da Silva, DDS[1], Yan Xu, MD[2], Alpdogan Kantarci, DDS, PhD[2,3], Hatice Hasturk, DDS, PhD[2], Ricardo A. Battaglino, PhD[4], Leslie R. Morse, DO[4], Ana Paula Turrioni, DDS, PhD[1]

[1] Department of Pediatric Dentistry and Orthodontics, School of Dentistry, Federal University of Uberlandia, Uberlandia, Minas Gerais, Brazil.

[2] ADA Forsyth Institute, Somerville, MA

[3] University of Minnesota, Minneapolis, MN

[4] Department of Physical Medicine and Rehabilitation, University of Miami, Miller School of Medicine, Florida, USA.

Corresponding author

Dr. Ana Paula Turrioni DDS, MSc, PhD, Cell Biology Research Group, Departament of Pediatric Dentistry, School of Dentistry, Federal University of Uberlândia, Uberlândia, MG, Brazil.

Avenida Pará, 1720, Bloco 2G sala 02 - Campus Umuarama.

CEP 38405-320

Tel: +55 (34) 3225-8146

e-mail: apturrioni@ufu.br

Abstract

Human dental pulp stem cells (DPSCs) exhibit neurogenic differentiation potential; however, inflammatory conditions may impair this process. This study investigated the impact of tumor necrosis factor-alpha (TNF-α) on DPSC neurodifferentiation and the modulatory role of the pro-resolving lipid mediators lipoxin A_4 (LXA_4) and resolvin D_1 (RvD_1). DPSCs were isolated and subjected to neuronal differentiation for 21 days. The optimal TNF-α concentration was determined in a pilot study using real-time PCR. The expression of OCT3/4, doublecortin, and β-III-tubulin was analyzed by MTT assay, flow cytometry, and immunofluorescence. The effects of LXA_4 (10 or 100 nM) and RvD_1 (10 or 100 nM) were assessed by flow cytometry. Data were analyzed using the Kruskal–Wallis test followed by the Mann–Whitney test ($p<0.05$) and one-way ANOVA with Tukey's post hoc test. TNF-α at a concentration of 25 ng/mL was shown to induce inflammation without causing cytotoxicity. The high production of doublecortin indicated the neuronal differentiation potential of DPSCs, whereas reduced β-III-tubulin expression demonstrated that TNF-α inhibits the progression of neuronal differentiation. Conversely, increased β-III-tubulin expression in the presence of LXA_4 (10 and 100 nM; $p=0.02$ and $p=0.03$, respectively) and RvD_1 (100 nM; $p=0.02$) indicate that these mediators attenuate the inhibitory effects of TNF-α. These findings suggest that anti-inflammatory therapy combined with DPSCs may enhance motor recovery following neurotrauma.

Keywords: stem cell; spinal cord injury; inflammation; rehabilitation medicine

1. Introduction

Neuroregenerative stem cell therapy faces several challenges, including the lack of standardized protocols, the selection of cell types that yield the best therapeutic results, limited access to neural stem cells, and the difficulty of cell differentiation in the absence of an inflammatory environment (Carvalho *et al*., 2024; Khan *et al*., 2023). Since 2000, dental pulp has been identified as a rich source of neural crest stem cells that are easily obtainable, highly proliferative in culture, and can differentiate into functionally active neurons in vitro (Gan *et al*., 2020). Tooth-derived stem cells can be obtained from both extracted third molars (human adult dental pulp stem cells or DPCSs) and from exfoliated deciduous teeth, which allows their harvesting and expansion with relatively limited ethical concerns (Fu *et al*., 2022; Gan *et al*., 2020; Jenkner *et al*., 2024; Kabatas *et al*., 2018; Luo *et al*., 2018). They originate from the cranial neural crest and express early markers of both mesenchymal and neuroectodermal stem cells (Luo *et al*., 2018). DPSCs are pluripotent and can differentiate into osteoblasts, chondrocytes, adipocytes, endothelial cells, and neural-like cells (Gan *et al*., 2020; Jenkner *et al*., 2024; Luo *et al*., 2018). They express trophic factors that promote neuronal proliferation and survival (Kabatas *et al*., 2018; Luo *et al*., 2018). Transplantation of human DPSCs into the damaged spinal cord enhances neuro-recovery in a rodent model of spinal cord injury by inhibiting apoptosis and preserving neural fibers/myelin sheaths, inhibiting axonal growth inhibitors, and differentiating into mature oligodendrocytes (Fu *et al*., 2024; Kabatas *et al*., 2018). These neuro-regenerative properties have not been demonstrated in embryonic stem cells, adult bone marrow stromal cells, or other stem cell populations (*Lukomska et al.*, 2019). Therefore, DPSCs have tremendous potential to advance treatment of neurotrauma due to stroke, traumatic brain injury, or spinal cord injury (SCI) (Fu *et al*., 2022; Kabatas *et al*., 2018).

SCI is associated with secondary inflammation, which may limit *in vivo* neuronal differentiation of transplanted dental pulp stem cells. With disruption of the blood-spinal cord barrier after SCI, neutrophils infiltrate the damaged cord, triggering inflammatory cytokine production, cell death, and the subsequent release of toxic metabolites, furthering tissue damage (Albashari *et al*., 2020; Zivkovic *et al*., 2021). Furthermore, inflammatory scar formation has been shown to inhibit spinal cord regeneration (Albashari *et al*., 2020; Zivkovic *et al*., 2021). Little is known about the impact of inflammation on the neural differentiation potential of dental pulp stem cells. Therefore, we sought to determine the effect of TNF-α treatment on *in vitro* neuronal differentiation of dental pulp stem cells.

Resolvins and lipoxins are endogenous anti-inflammatory agents derived from omega-3 and omega-6 fatty acids, respectively (Abdelmoaty et al., 2013). These lipid mediators have demonstrated pro-resolving potential in animal models ranging from asthma to colitis, in addition to promoting regeneration and wound healing, thereby reversing tissue damage (Abdelmoaty *et al*., 2013; Albuquerque-Souza *et al*., 2020; Martini *et al*., 2016; Park *et al*., 2023). Intrathecal injection of lipoxin A_4 (LXA_4) and resolvin D_1 (RvD_1) has been shown to attenuate inflammatory hypersensitivity in mice (Abdelmoaty *et al*., 2013; Martini *et al*., 2016; Park *et al*., 2023). However, neither mediator has been studied in experimental SCI. We therefore tested the hypothesis that

LXA_4 and RvD_1 will mitigate the inflammation-induced suppression of neural differentiation in dental pulp stem cells.

2. Methods

2.1 Experimental Groups

a. Time point 1 – Pilot study
Group 1: Undifferentiated cells treated with 1 ng/mL TNF-α
Group 2: Undifferentiated cells treated with 10 ng/mL TNF-α
Group 3: Undifferentiated cells treated with 25 ng/mL TNF-α
Group 4: Undifferentiated cells treated with 100 ng/mL TNF-α

b. Time point 2 - Stem Cells, and Neuronal Markers
Group 1: Undifferentiated cells
Group 2: Undifferentiated cells treated with 25 ng/mL TNF-α
Group 3: Differentiated cells
Group 4: Differentiated cells treated with 25 ng/mL TNF-α

c. Time point 3 – TNF-α, Lipoxin A_4, and Resolvin D_1 Treatment
Group 1: Undifferentiated cells
Group 2: Differentiated cells
Group 3: Differentiated cells treated with 25 ng/mL TNF-α
Group 4: Differentiated cells treated with 25 ng/mL TNF-α and 10 nM RvD_1
Group 5: Differentiated cells treated with 25 ng/mL TNF-α and 100 nM RvD_1
Group 6: Differentiated cells treated with 25 ng/mL TNF-α and 10 nM LXA_4
Group 7: Differentiated cells treated with 25 ng/mL TNF-α and 100 nM LXA_4

2.2 Isolation and culture of DPSC

DPSCs were isolated from impacted third molars, premolars, or deciduous teeth of 7 healthy donors who underwent routine dental care at the ADA Forsyth Institute. All donors provided a signed informed consent or assent form prior to the use of their teeth, as per the study protocol approved by Harvard University (IRB 037). Samples were selected from our tooth biorepository based on the following donor characteristics: absence of medical comorbidities, no active medication use (except for vitamin supplements), and sound teeth. The extracted pulp was plated and cultured in basal medium, DMEM/F12 supplemented with 15% FBS, 100 U/mL non-essential amino acids (Gibco Life Technologies), 100 U/mL penicillin and streptomycin, and glutamine (Gibco Life Technologies). Outgrowth of fibroblast-like cells occurred after 3-4 days, and at that point, the pulp was replated. The outgrowing cells were passaged after reaching 80% confluence, and passage 4 was used for all experiments. Cells were seeded in duplicate at a density of 2×10^4 cells/well. Neuronal differentiation was induced by replacing DMEM/F12+15% FBS culture media with neuronal differentiation media (NeuroCult

NS-A Differentiation Kit-Human, StemCell Technologies Inc, Tukwila, WA, USA) 48 hours after seeding. Cells were cultured under neuronal induction conditions for up to 21 days with culture media changed twice a week (Al-Maswary *et al.*, 2022; Király *et al.*, 2009; Liu *et al*., 2024).

2.3 Time Point 1 – Pilot Study

Experimental Group
Group 1: Undifferentiated cells treated with 1 ng/mL TNF-α
Group 2: Undifferentiated cells treated with 10 ng/mL TNF-α
Group 3: Undifferentiated cells treated with 25 ng/mL TNF-α
Group 4: Undifferentiated cells treated with 100 ng/mL TNF-α

Twenty-four hours after cell seeding, TNF-α at concentrations of 1, 10, 25, and 100 ng/mL (PeproTech, Rocky Hill, NJ, USA) was added to cultures of undifferentiated cells. After an additional 24 hours, the expression of inflammatory cytokines (IL-6, IL-8, and IL-1β) was analyzed by real-time PCR to determine the TNF-α concentration capable of inducing an inflammatory response while minimizing cytotoxicity.

Total RNA was extracted from cells using Trizol (Life Technologies). 10 µL total RNA was reverse-transcribed to cDNA using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). 1 µL of cDNA was used as a template for real-time PCR amplification reactions, performed using the iQ SYBR Green Supermix (Bio-Rad) in combination with 19 µL of each gene-specific primer, according to the manufacturer's instructions. The relative quantification of each target gene was normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control gene and calculated using the $2^{-\Delta\Delta CT}$ method. PCR reactions were conducted in duplicate using the following conditions: initial denaturation for 3 min at 95˚C, followed by 40 cycles of 15 sec denaturation at 95˚C and 60 sec annealing at 60˚C. Relative quantification was used for statistical analyses (Al-Maswary *et al.*, 2022; Király *et al.*, 2009; Liu *et al*., 2024).

2.4 Time Point 2 – Steams Cells, and Neuronal Markers

2.4.1 Cellular Metabolic Activity

The metabolic activity of cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay at 2 and 10 days of the experiment to assess TNF-α–induced toxicity in dental pulp stem cells. Cells were incubated for 4 hours in 5 mg/mL thiazolyl blue tetrazolium bromide (Sigma Aldrich, St. Louis, MO, USA). The precipitate was then suspended in 2-propanol (Sigma Aldrich, St. Louis, MO, USA), and absorbance was determined in triplicate (Al-Maswary *et al.*, 2022; Király *et al.*, 2009; Liu *et al*., 2024).

2.4.2 Flow Cytometry

Expression of stem cell and neuronal cell markers was determined by flow cytometry, assay at 21 days, using a twenty-four well plate per marker. Cells were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.1% Triton X-100 for 10 minutes and subsequently blocked with TBST in 5% BSA for 20 minutes. Cells were incubated for 1 hour at room temperature with the following primary antibodies against stem cells markers: rabbit anti-OCT3/4 (Abcam, 1:200), CD 44 (Abcam, 1:200) and neuronal markers, rabbit anti-doublecortin (Abcam, 1:50) and mouse anti-β III-tubulin (Abcam, 1:100). Fluorescence was achieved by incubating with secondary antibodies (Life Tech, Carlsbad, CA, goat anti-rabbit IgG or goat anti-mouse IgG, dilution 1:500) for 1 hour at room temperature in a dark room. Cells were then subjected to flow cytometry, and the percentage of cells expressing each marker was calculated (Al-Maswary *et al.*, 2022; Király *et al.*, 2009; Liu *et al*., 2024).

2.4.3 Immunofluorescence

Immunofluorescence was performed on cells assay at 21 days, according to standard protocols. Briefly, cells were cultured on glass cover slips under varying conditions, washed 3X10 minutes in 1XPBS+0.1% (vol/vol) Tween-20, fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 minutes, permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 minutes, and washed 3X10 minutes in ice-cold PBS. The cells were then incubated with the following primary antibodies (OCT 1:100, Doublecourtin 1:200, and β-III Tubulin 1:100, Abcam, Cambridge, MA, USA) in PBS with 3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour in a humidified chamber at 4°C. The cells were then washed 3X10 minutes in 1XPBS+0.1% (vol/vol) Tween-20 and incubated for 1 hour at room temperature with the biotinylated secondary antibodies (100 µl/coverslip, diluted 1:250 to 1:750 in blocking buffer, Life Tech, Carlsbad, CA, goat anti-rabbit IgG or goat anti-mouse IgG) in a humidified chamber. Finally, cells were washed 3X5 minutes in 1XPBS, incubated for 1 min with 1mg/mL 4'6' -Diamidin-2-phenylindol (DAPI, Vector Laboratories, Burlingame, CA), rinsed in 1XPBS, and the coverslips were mounted with Vectashield mounting medium and sealed. Images were acquired using a Carl Zeiss Axioplan fluorescence microscope at 20× magnification (LSM 410, Zeiss, Jena, Germany) (Al-Maswary *et al.*, 2022; Király *et al.*, 2009; Liu *et al*., 2024).

2.5 Time Point 3 – TNF-α, Lipoxin A_4, and Resolvin D_1 Treatment

TNF-α (25 ng/mL; PeproTech, Rocky Hill, NJ, USA), LXA_4 (10 and 100 nM; Cayman Chemical, Ann Arbor, MI, USA), or RvD_1 (10 and 100 nM; Cayman Chemical, Ann Arbor, MI, USA) were added to cultures of both differentiated and undifferentiated cells starting 48 hours after seeding. Gene expression was assessed by flow cytometry, assay at 21 days.

2.6 Statistical Analysis

Statistical analyses were performed using SPSS software version 22.0 (IBM Corp., Armonk, NY, USA). For analyses of cell metabolic activity and cytokine expression, data are presented as median and interquartile range for each condition. The Kruskal–Wallis test followed by the Mann–Whitney test was applied ($p<0.05$). For flow cytometry markers, data are expressed as mean ± standard deviation, and one-way ANOVA followed by Tukey's post hoc test was used.

3. Results

3.1 Donor Characteristics

Donor characteristics at the time of extraction are presented in Table 1. The age range was from 10 to 38 years. The majority was white (57,14%). One donor was an active smoker at the time of extraction. One donor reported vitamin D supplementation. No other medication use was reported. No donor reported an active medical problem at the time of extraction. All extracted teeth were in good condition, and third molars were the most frequently obtained teeth.

3.2 Time Point 1 – Pilot Study

All TNF-α doses induced significant increases in DPSC expression of the inflammatory cytokines IL-6, IL-8, and IL-1β after 24 hours of treatment (Figure 1). Among the tested concentrations, 25 ng/mL induced increased cytokine expression without exhibiting cytotoxic effects and was therefore selected for all subsequent experiments.

3.3 Time Point 2 – Steams Cells, and Neuronal Markers

3.3.1 Cellular Metabolic Activity

The MTT assay demonstrated that induction of differentiation resulted in a significant reduction in the metabolic activity of DPSCs at 2 and 10 days, both in the absence and presence of TNF-α (Figure 2). However, at 10 days, neural induction in the presence of 25 ng/mL TNF-α resulted in significantly higher metabolic activity when compared with cells induced in the absence of TNF-α ($p = 0.017$).

3.3.2 Flow Cytometry and Immunofluorescence

Flow cytometry analysis at 21 days of neuronal induction demonstrated a significant reduction in the percentage of differentiated cells expressing the stem cell

marker OCT3/4 (Figure 3A; $p < 0.001$). In addition, differentiated cells exhibited increased expression of the early neuronal marker doublecortin (Figure 3A; $p < 0.002$) and the intermediate neuronal marker β-III tubulin (Figure 3A; $p < 0.01$) when compared with undifferentiated cells. No differences were observed in the expression of the stem cell marker OCT3/4 or the early neuronal marker doublecortin when comparing differentiated cells cultured in the presence or absence of TNF-α. However, a marked reduction was observed in the percentage of differentiated cells expressing the intermediate neuronal marker β-III tubulin (Figure 3A; $p < 0.004$). These findings are consistent with impaired progression toward later stages of neuronal differentiation in the presence of 25 ng/mL TNF-α.

These findings were further confirmed by immunofluorescence analysis (Figures 3B–D). Reduced expression of the stem cell marker OCT3/4 was observed under differentiation conditions, as evidenced by discrete red labeling in a limited subset of cells (Figure 3B). As OCT3/4 is a nuclear marker, its visualization by immunostaining is inherently challenging. An increase in the expression of the early neuronal marker doublecortin was also observed in differentiated cells, characterized by intense cytoplasmic labeling and a filamentous pattern associated with morphological changes compatible with the onset of neuronal differentiation (Figure 3C). In contrast, the expression of the intermediate neuronal marker β-III tubulin, visualized as an intense green signal well distributed throughout the cytoplasm and along cellular processes, was significantly reduced in the presence of 25 ng/mL TNF-α when compared with differentiated cells cultured in the absence of the inflammatory stimulus, which exhibited an elongated morphology and filamentous organization consistent with more advanced stages of neuronal differentiation (Figure 3D).

3.4 Time Point 3 – TNF-α, Lipoxin A_4, and Resolvin D_1 Treatment

We tested the effects of LXA_4 and RvD_1 on mitigating TNF-α-induced suppression of DPSC neuronal differentiation. Flow cytometry analysis at 21 days confirmed that treatment with 25 ng/mL TNF-α significantly reduced β-III tubulin production (Figure 4; $p = 0.0002$). This reduction was partially blocked by treatment with RvD_1 and LXA_4 at 10 nM. A significant 78% increase in β-III tubulin expression was observed in cells subjected to neural induction in the concomitant presence of TNF-α and 100 nM RvD_1 ($p = 0.02$). Similarly, a significant increase in β-III tubulin expression was observed in cells subjected to neural induction in the concomitant presence of TNF-α and 10 nM LXA_4 ($p = 0.02$). Comparable results were obtained for LXA_4 at the 100 nM dose ($p = 0.03$), whereas a trend toward significance was observed for RvD_1 at 10 nM ($p = 0.09$). No reduction or improvement in neuronal differentiation was observed when DPSCs were induced to differentiate into neurons in the presence of RvD_1 or LXA_4 alone, at any of the concentrations tested (data not shown).

4. Discussion

The global incidence of spinal cord injuries is estimated to be 40 to 80 million cases per year, with more than 18,000 occurring in the United States (Zawadzka et al., 2021). Such injuries are characterized by an initial phase in which damage results directly from the trauma itself, followed by a secondary phase in which damage is caused by local inflammation (Anjum *et al*., 2020; Bonosi *et al*., 2022). During inflammation, interleukins and TNF-α are released, which can trigger neuronal apoptosis, glial scar formation, and axonal demyelination (Anjum *et al*., 2020; Bonosi *et al*., 2022). In this study, we demonstrated that the neuronal differentiation capacity of DPSCs is impaired under inflammatory conditions, particularly in the presence of TNF-α. Moreover, we showed that the endogenous pro-resolving lipid mediators LXA_4 and RvD_1 are able to attenuate TNF-α induced inhibition of neuronal differentiation, thereby mitigating the deleterious effects of inflammation.

The selection of the 25 ng/mL TNF-α concentration was based on a pilot study demonstrating, by PCR, that this dose increases the expression of inflammatory cytokines without inducing acute cytotoxicity after 24 hours. Similarly, Li *et al*. (2023) treated human adipose tissue–derived stem cells with TNF-α at concentrations ranging from 10 to 40 ng/mL for 48 hours and found that cellular proliferative capacity was altered in a dose-dependent manner. The authors therefore concluded that high concentrations of TNF-α induce autophagy and apoptosis, thereby affecting regeneration, immune regulation, and differentiation functions. Cheng *et al*. (2019) investigated the effects of TNF-α at concentrations ranging from 0 to 200 ng/mL on nucleus pulposus–derived mesenchymal stem cells (NPMSCs). Their results demonstrated that treatment with high concentrations of TNF-α (50–200 ng/mL) induced apoptosis in NPMSCs, whereas lower concentrations (0.1–10 ng/mL) promoted proliferation but inhibited differentiation at more advanced stages of maturation. There is currently no consensus in the literature regarding the optimal TNF-α concentration, as this choice depends on the cell type and experimental objective. Nevertheless, available evidence indicates that moderate concentrations are sufficient to induce relevant inflammatory signaling without triggering excessive cytotoxicity, supporting the selection of the intermediate dose used in the present study.

In the literature, the OCT3/4 marker is used to identify the maintenance of an undifferentiated phenotype, as its expression decreases with the onset of cellular differentiation (Patel & Parchem, 2022; Sucha *et al.,* 2021). In contrast, doublecortin is used to identify the potential of cells to exit the undifferentiated state and initiate neuronal differentiation (Dema *et al.*, 2024; Sucha *et al.*, 2021). Finally, β-III-tubulin is a cytoskeletal component of newly formed neurons and is widely used as a marker of more advanced stages of neuronal differentiation (Sucha *et al.,* 2021). Although TNF-α did not exhibit cytotoxic effects on DPSCs, the progression of neuronal differentiation was impaired in the presence of this cytokine, as evidenced by immunofluorescence and flow cytometry analyses at 21 days of induction. In this context, DPSCs preserved their neuronal differentiation potential, as evidenced by the expression of the early marker doublecortin. However, these cells failed to progress to later stages of neuronal maturation, as indicated by the reduced levels of the marker β-III tubulin.

Bueno *et al*. (2019) reported significant levels of β-III-tubulin during the neuronal differentiation of human periodontal ligament–derived stem cells, confirming the ability of these cells to acquire neuronal characteristics in vitro. However, this study did not simulate an inflammatory environment, which limits the extrapolation of its findings to clinical conditions. In contrast, Jung *et al*. (2016) demonstrated that culture media enriched with inflammatory cytokines, including interleukins and TNF-α, negatively regulate β-III-tubulin expression in stem cells undergoing neuronal differentiation, whereas environments with a lower inflammatory burden favor higher levels of this marker. Consistent with our findings, the results reported by Jung *et al.* (2016) support the hypothesis that an inflammatory microenvironment directly interferes with the progression of neuronal differentiation toward more advanced stages. Therefore, considering that inflammation is an inherent response to spinal cord injury, future stem cell–based therapeutic strategies should take into account the inflammatory milieu in which transplanted cells must proliferate and differentiate.

The mechanisms by which TNF-α inhibits stem cell differentiation have not yet been fully elucidated, although several hypotheses have been proposed in the literature (Sonmez Kaplan *et al*., 2023). The most widely accepted hypothesis suggests that TNF-α, in conjunction with other inflammatory cytokines, activates the transcription factor NF-κB, which in turn inhibits SOX2 expression (Wehling *et al.,* 2009). Evidence indicates that SOX2 is a key protein involved in the maintenance of pluripotency and in the regulation of the early stages of the cellular differentiation process. In addition, SOX2 expression has been described in neurogenic regions of the central nervous system, such as the hippocampus and cerebellum, reinforcing its role in neurogenesis (Pereira *et al*., 2013). Although no studies have directly linked NF-κB activation to the regulation of β-III-tubulin, given that β-III-tubulin is also a protein, it is plausible to hypothesize that the reduced expression of this neuronal marker may be associated with NF-κB-mediated inhibitory signaling.

Given that TNF-α inhibits the neural differentiation of DPSCs, it becomes relevant to discuss strategies capable of modulating this inflammatory response and, consequently, promoting neuroregeneration. In this context, Abdelmoaty *et al*. (2013) conducted an in vivo study to investigate whether spinal administration of LXA_4 and 17(R)-RvD_1, a more stable analog of RvD1, reduces the release of TNF-α and interleukins, thereby promoting pain relief. The authors observed that lipoxins and resolvins inhibit spinal nociceptive processing, indicating their potential as therapeutic agents. Complementarily, Albuquerque-Souza *et al*. (2020) demonstrated that the pro-resolving lipid mediators Resolvin E_1 (RvE_1) and Maresin-1 (MaR_1) are capable of restoring the regenerative properties of human periodontal ligament stem cells exposed to an inflammatory environment containing TNF-α and IL-1β. The authors reported that these mediators attenuate the deleterious effects of inflammation while promoting tissue repair under persistent inflammatory conditions (Albuquerque-Souza *et al*., 2020).

In our study, we observed that both LXA_4 and RvD_1 mitigated the negative effects induced by TNF-α on DPSC differentiation, with no significant differences between the mediators or the concentrations tested. This finding may be explained by the fact that both mediators exert their pro-resolving actions through binding to specific G protein–coupled receptors (GPCRs), particularly the ALX/FPR2 and GPR32 receptors (Park, Langmead,

& Riddy, 2020; Pirault & Bäck, 2018). By binding to these receptors with high affinity, these mediators are able to elicit effective biological responses even at relatively low concentrations, such as 10 nM.

Although the mechanisms by which pro-resolving lipid mediators attenuate TNF-α–mediated effects are not yet fully elucidated, several hypotheses have been proposed in the literature. One possibility is that both LXA_4 and RvD_1 inhibit NF-κB activation (Isopi *et al*., 2020; Jaén *et al*., 2021; Wang *et al.,* 2011). Considering that pro-inflammatory cytokines activate this signaling pathway, which regulates the transcription of genes involved in TNF-α synthesis, partial inhibition of NF-κB by lipid mediators could explain the observed reduction in inflammatory cytokine production (Albashari *et al*., 2020; Yu *et al*., 2020).

Despite the relevance of the findings, some limitations should be considered. First, this is an *in vitro* study that employed isolated cytokines to simulate the inflammatory stimulus. Although this model allows a controlled assessment of the effects of inflammation on the neuronal differentiation of DPSCs, it does not fully reproduce the complexity of the inflammatory microenvironment present *in vivo* spinal cord injury, which involves multiple cytokines and dynamic signaling interactions. Therefore, extrapolation of these results to the clinical context should be approached with caution.

In addition, the assessment of neuronal differentiation was predominantly based on the expression of molecular markers representative of maturation stages, which does not confirm the acquisition of functional neuronal properties. Furthermore, the protective effects of LXA4 and RvD1 were investigated only at a specific TNF-α concentration of 25 ng/mL, which does not allow conclusions to be drawn regarding whether these mediators maintain their efficacy under more intense inflammatory conditions. Future studies incorporating functional analyses and more complex inflammatory models will be essential to further elucidate the therapeutic potential of DPSCs in neuroinflammatory conditions.

Therefore, even in the presence of TNF-α induced inflammation, human DPSCs maintained neurotrophic factor production and the ability to proliferate. However, because the inflammatory environment was detrimental to the continuation of DPSC differentiation, they failed to reach full maturation and became postmitotic neuronal cells. Thus, we conclude that administering anti-inflammatory agents as LXA_4 and RvD_1 in conjunction with human DPSC transplantation may offer future therapeutic benefits in the treatment of spinal cord injury.

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Table 1. Donor Characteristics

Study ID	Age	Gender	Race	Active Smoker	Medications	Tooth
P1	29	F	White	No	None	Lower 3rd molar (#17)
P5	38	M	Black	Yes	None	Upper 3rd molar (#1)
P6	10	F	White	No	None	Primary upper canine (H)
P8	35	F	Asian	No	Vitamin D supplements	Upper 3rd molar (#1)
P10	14	F	Black	No	None	Upper 1st premolar (#5)
P17	30	F	White	No	None	Lower 3rd molar (#17)
P18	36	M	White	No	None	Upper 3rd molar (#1)

Supplementary table: Primers used in PCR methodology

	Following primers
IL-6	Forward: 5’AAATTCGGTACATCCTCGACGG3’ Reverse: 5’GGAAGGTTCAGGTTGTTTTCTGC3’
IL-8	Forward: 5’ACTGAGAGTGATTGAGAGTGGAC3’ Reverse: 5’AACCCTCTGCACCCAGTTTTC3’
IL-β	Forward: AAGGCGGCCAGGATATAACT-3' Reverse: 5'-TACGGCCTAAGGCAGGCAGTTG-3'
GAPDH	Forward: 5' AGAAAAACCTGCCAAATATGATGAC 3' Reverse: 5’ TGGGTGTCGCTGTTGAAGTC 3’

Figures

Figure 1. Cytokine gene expression in DPSCs stimulated with different TNF-α concentrations

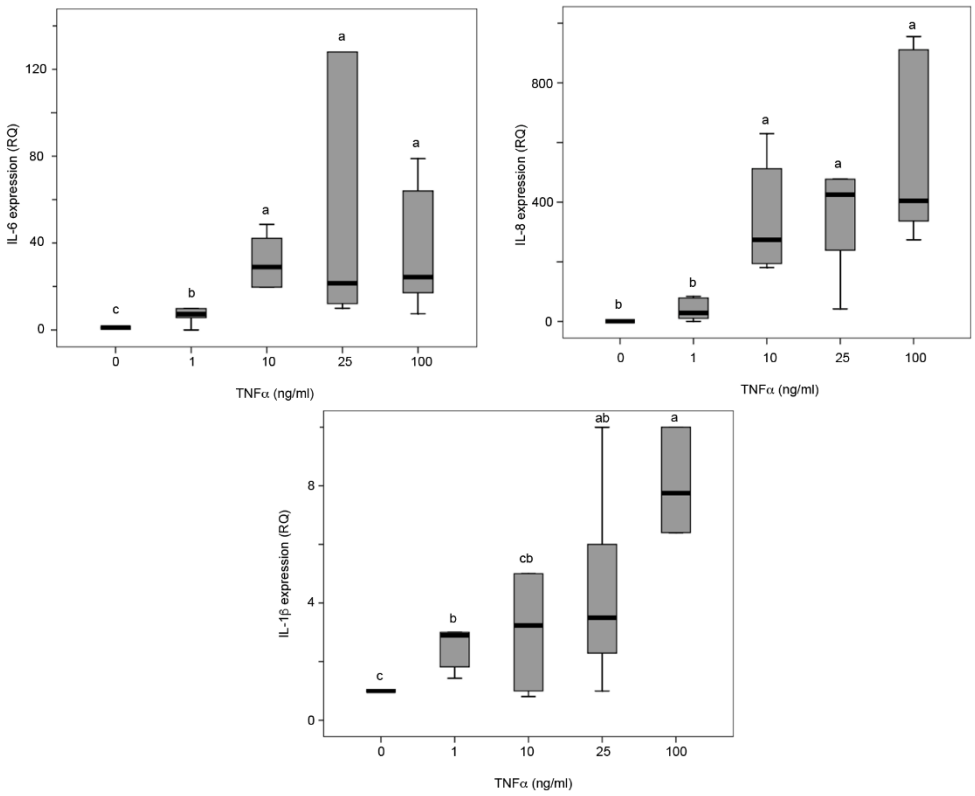

Figure 2. Cell viability detected by MTT in DPSCs submitted to neuronal differentiation

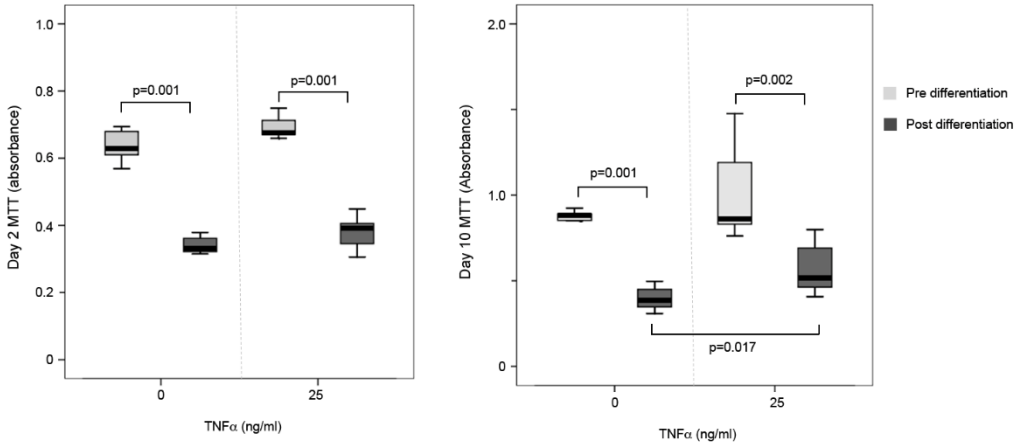

Figure 3A. Quantification of neuronal markers in DPSCs

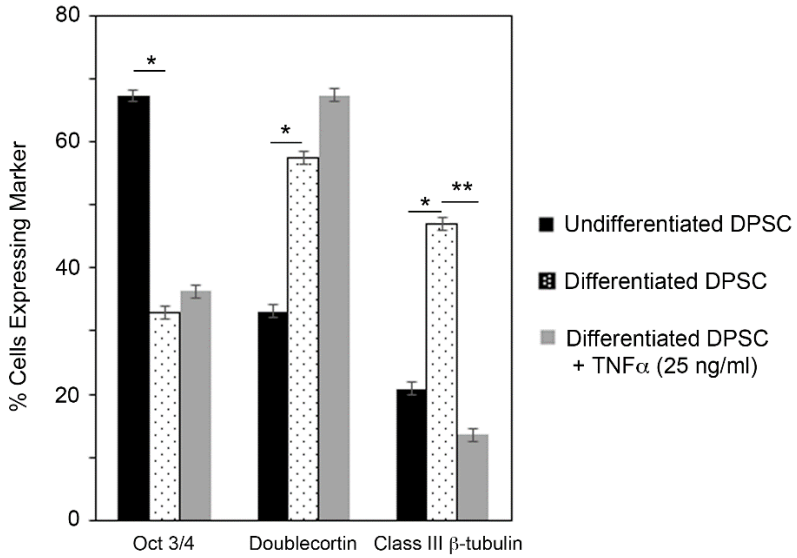

Figure 3B. OCT3/4 immunofluorescence in DPSCs

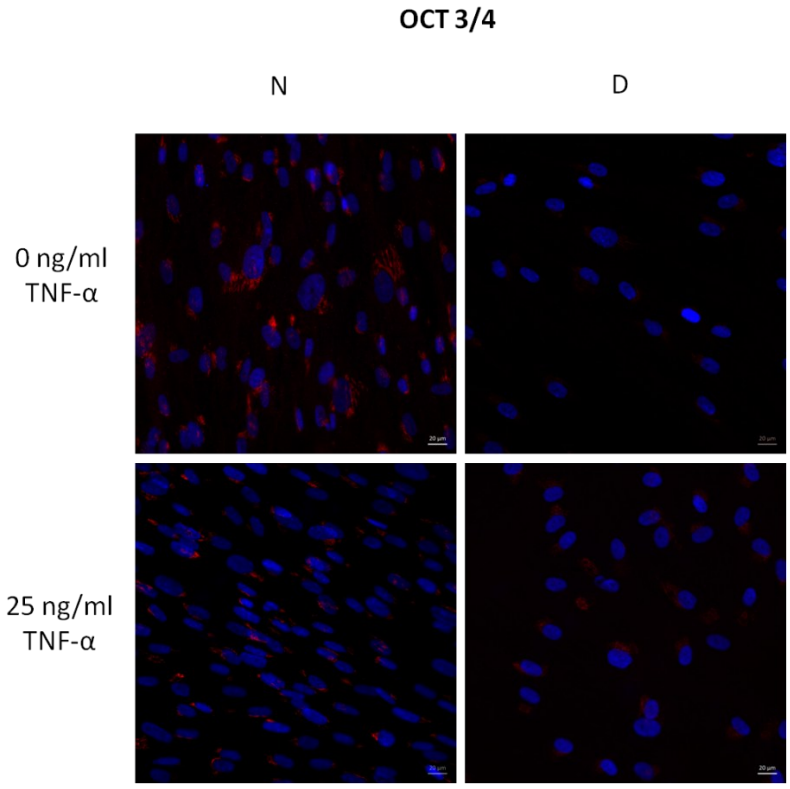

Figure 3C. Doublecortin immunofluorescence in DPSCs

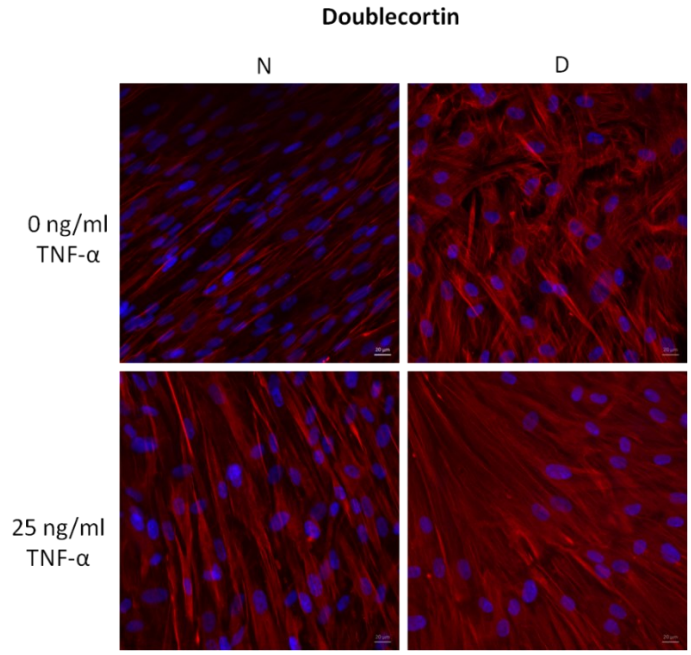

Figure 3D. β-III tubulin immunofluorescence in DPSCs

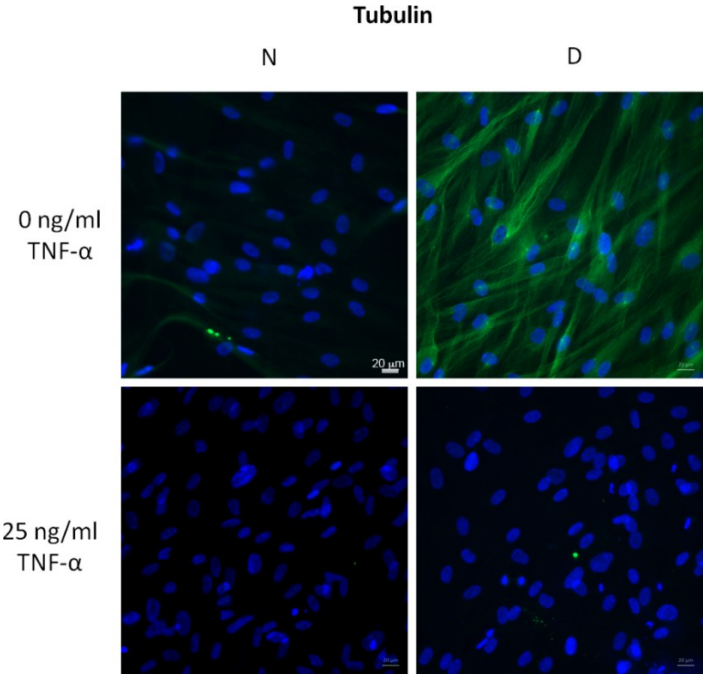

Figure 4. βIII-tubulin production in DPSCs after treatment with LXA4, RvD1 and TNF-α

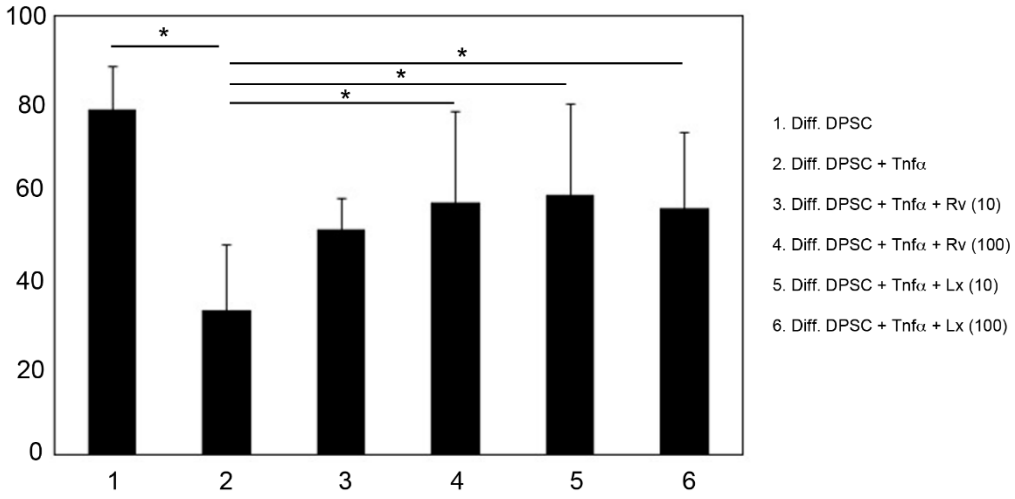

Figure Legends:

Figure 1. Cell viability detected by MTT Assay (absorbance values) by HDPCs submitted to neuronal differentiation or kept in DMEM/F12 for 2 days (a) or 10 days (b) in the presence (0 ng/mL) or absence (25 ng/mL) of TNF-α. The box contains 50% of the data points, and the middle line of the box is the median. The tips of the projecting bars show minimum and maximum values, n = 7. Mann–Whitney, $p > .05$

Figure 2. IL-6 (a), IL-8 (b), and IL-1β (c) gene expression (RQ values) by HDPCs submitted to different concentrations of TNF-α (0, 1, 10, 25, and 100 ng/mL), for 24 hours. The box contains 50% of the data points, and the middle line of the box is the median. The tips of the projecting bars show minimum and maximum values. n = 7. Mann–Whitney, $p > .05$

Figure 3. (A) OCT 3/4, Doublecortin and β-III tubulin production by HDPCs subjected to neuronal differentiation or kept in DMEM/F12 for 21 days in the presence (25 ng/mL) or absence (0 ng/mL) of TNF-α. Bar graphs indicating the mean values and standard deviation, n = 7. One-way ANOVA complemented by Tukey, $p > .05$. (B) OCT 3/4 (C) Doublecortin and (D) β-III tubulin immunofluorescence images representative of HDPCs subjected to neuronal differentiation or kept in DMEM/F12 for 21 days in the presence (25 ng/mL) or absence (0 ng/mL) of TNF-α.

Figure 4. β III-tubulin production by flow cytometry, after LXA_4, RvD1, or TNF-α (25 ng/mL). Bar graphs display the mean values and standard deviations, with n = 7. One-way ANOVA complemented by Tukey, $p > .05$

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APÊNDICE

HARVARD
Human Research Protection Program

Harvard University Faculty of Medicine
Office of Human Research Administration
90 Smith Street, 3rd Floor
Boston, MA 02115

Notification of Exemption Determination

Ricardo Battaglino
rbattaglino@forsyth.org

Protocol Title:	Neurogenerative properties of human dental pulp stem cells: a pilot study
Protocol #:	IRB14-1841
Funding Source:	Spaulding Rehabilitation Hospital Corporation
IRB Review Action:	Exempt

Dear Dr. Battaglino:

After review of your submission, the Institutional Review Board (IRB) of the Harvard University Faculty of Medicine determined that the above-referenced protocol meets the criteria for exemption per the regulations found at 45 CFR 46.101(b)(4).

Additional review by the IRB is not required. However, any changes to the protocol that may alter this determination must be submitted for review via a modification (by selecting the Create Modification activity in the ESTR system) to determine whether the research activity continues to meet the criteria for exemption.

The IRB made the following determination:

- Research Information Security Level: The research is classified, using Harvard's Data Security Policy, as Level 1 Data.

If you have any questions, please contact me at 617-432-7434 or kserpico@hsph.harvard.edu.

Sincerely,

Kimberley Serpico

Kimberley Serpico, MEd, CIP
IRB Review Specialist

University Area IRB http://cuhs.harvard.edu
Longwood Medical Area IRB http://www.hsph.harvard.edu/ohra/