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FACULDADE DE ODONTOLOGIA DE ARAÇATUBA
Departamento de Odontologia Preventiva e Restauradora
Programa de Pós-graduação em Ciências

DEFESA DE TESE DE DOUTORADO

Síntese de trimetafosfato de cálcio e seu efeito contra a erosão inicial, energia livre da superfície do esmalte e desenvolvimento de nova membrana para engenharia de tecidos periodontais: estudo *in vitro*

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Araçatuba – SP
2024

Priscila Toninatto Alves de Toledo

SÍNTESE DE TRIMETAFOSFATO DE CÁLCIO E SEU EFEITO CONTRA A EROSÃO INICIAL, ENERGIA LIVRE DA SUPERFÍCIE DO ESMALTE E DESENVOLVIMENTO DE NOVA MEMBRANA PARA ENGENHARIA DE TECIDOS PERIODONTAIS: ESTUDO *IN VITRO*

Tese apresentada à Faculdade de Odontologia da Universidade Estadual Paulista "Júlio de Mesquita Filho", Campus de Araçatuba, para a defesa de tese para obtenção do título de Doutor em Ciência, área de concentração Saúde Bucal da Criança.

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Dedicatória

Dedico este trabalho,

Aos meus pais **Marilza** e **Luiz Carlos**, ao meu irmão **Guilherme, a minha vó Maria e** aos **meus tios (Osmair, Osvaldir, Marilde, Elaine e Isabel Cristina)** e **primos (Estela, Eitor, Eduardo, João Vitor e Gabriel).**

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"Nada na vida deve ser temido, somente compreendido. Agora é hora de compreender mais para temer menos."

- Marie Curie

TOLEDO P.T.A. Síntese de trimetafosfato de cálcio e seu efeito contra a erosão inicial, energia livre da superfície do esmalte e desenvolvimento de nova membrana para engenharia de tecidos periodontais: estudo *in vitro*. 136 f. Tese (Doutorado em Ciências, área de concentração Saúde Bucal da Criança) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2024.

Resumo Geral

O trabalho de tese foi dividido em 2 capítulos. O primeiro capítulo teve como objetivo sintetizar ciclotrifosfato (TMP) com substituição total do sódio pelo cálcio (Ca^{2+}), avaliar *in vitro* a energia livre de superfície (γ_s) e o efeito na inibição da lesão erosiva inicial do esmalte. O segundo capítulo visou criar e avaliar nanofibras biodegradáveis à base de polímeros, contendo distintas concentrações de trimetafosfato de cálcio (CaTMP) para engenharia de tecidos periodontais. No primeiro capítulo, ciclotrifosfatos com substituição total do sódio pelo cálcio foram sintetizados e este biomaterial foi caracterizado morfologicamente por espectroscopia de raios X por energia dispersiva (EDS) e microscopia eletrônica de varredura (MEV). Avaliou-se a energia livre de superfície através de um goniômetro utilizando três líquidos sonda: água deionizada, diiodometano e etilenoglicol. Blocos de esmalte bovino (4 x 4 mm) foram distribuídos aleatoriamente de acordo com a média de dureza de superfície (SH) do total de blocos e o intervalo de confiança entre 7 grupos de dentifrícios experimentais contendo 1% dos compostos: 100%NaTMP/0%CaTMP (NaTMP); 80%NaTMP/20%CaTMP (20CaTMP); 50%NaTMP/50%CaTMP (50CaTMP); 20%NaTMP/80%CaTMP (80CaTMP); 100%CaTMP/0%NaTMP (CaTMP). Como controle negativo, foi utilizado um dentifrício placebo e, como controle positivo, um dentifrício com 1100 ppm de F (NaF, Merck, CAS 7681-49-4, Alemanha). Para análise do efeito protetor, os blocos de esmalte hígidos foram imersos em solução de dentifrícios com saliva humana por 2 horas (sob agitação), seguidos por 4 desafios erosivos (ácido cítrico, 0,75%, pH 3,5, por 1 minuto, sob agitação). A porcentagem de variação da dureza superficial (%SH) foi calculada após o tratamento e os desafios ácidos de 1, 2, 3 e 4 minutos. Além disso, os precipitados foram analisados por MEV. As variáveis foram submetidas à análise de variância de

medidas repetidas a dois critérios, seguida pelo teste de Student-Newman-Keuls ($p<0,05$). No segundo capítulo, soluções de poli(éster ureia) (PEU) (5% p/v) contendo Ca-TMP (15%, 30%, 45% p/p) foram eletrofiadas em estruturas fibrosas. As fibras foram avaliadas por MEV, EDS, análise termogravimétrica (TGA), espectroscopia de infravermelho por transformada de Fourier (FTIR), difração de raios-X (DRX) e ensaios mecânicos. A taxa de degradação, a taxa de inchaço e a liberação de cálcio também foram avaliadas. A interação célula/CaTMP e célula/scaffold foi avaliada usando células-tronco de dentes decíduos esfoliados humanos (SHEDs) para viabilidade celular, adesão e atividade de fosfatase alcalina (ALP). Análise de variância (ANOVA) e testes post-hoc ($\alpha = 0,05$) foram utilizados. Assim, o primeiro capítulo concluiu que o dentifrício contendo CaTMP apresentou o maior efeito protetor em comparação aos outros grupos ($p < 0,001$). O efeito protetor foi 100%CaTMP/0%NaTMP > 20%NaTMP/80%CaTMP > 50%NaTMP/50%CaTMP > 80%NaTMP/20%CaTMP > 1100 ppm F > 100%NaTMP/0%CaTMP > placebo ($p < 0.05$). Todos os grupos formaram precipitados com camadas mais espessas ao utilizar qualquer concentração de CaTMP. Quanto maior a percentagem de CaTMP nos grupos, maior foi a adsorção de CaTMP e mais sítios doadores de elétrons na superfície do esmalte foram alcançados. No segundo capítulo, as membranas baseadas em PEU e PEU/CaTMP apresentaram diâmetros de fibra de 469 nm e 414–672 nm, respectivamente. A caracterização química atestou a incorporação de CaTMP nas fibras. A adição de CaTMP resultou em maior estabilidade de degradação e menor variação dimensional do que as fibras de PEU puras; no entanto, foram observadas características mecânicas semelhantes. O cálcio mínimo foi liberado após 21 dias de incubação em solução enriquecida com lipase. Os extratos de CaTMP aumentaram a viabilidade celular e a atividade de ALP, embora não tenham sido encontradas diferenças significativas entre os grupos de suporte. No geral, concluiu-se que os dentifrícios contendo CaTMP têm um efeito protetor superior na redução das lesões erosivas iniciais do esmalte e que o CaTMP foi efetivamente incorporado nas fibras de PEU, melhorando a estabilidade da membrana sem comprometer as propriedades morfológicas e sem promover função celular significativa.

Palavras-chaves: Esmalte dentário, Fosfatos, Desmineralização, Dentifrícios, Engenharia Tecidual, Nanofibras.

TOLEDO P.T.A. Synthesis of calcium trimetaphosphate and its effect against initial erosion, enamel surface free energy, and development of a new membrane for periodontal tissue engineering: an *in vitro* study. 136 f. Tese (Doutorado em Ciências, área de concentração Saúde Bucal da Criança) – Faculdade de Odontologia, Universidade Estadual Paulista, Araçatuba, 2024.

General Abstract

The thesis work was divided into 2 chapters. The first chapter aimed to synthesize cyclotriphosphate (TMP) with complete substitution of sodium by calcium (Ca^{2+}), to evaluate *in vitro* the surface free energy (γ_s) and the effect on inhibiting the initial erosive lesion of the enamel. The second chapter aimed to create and evaluate biodegradable nanofibers based on polymers containing different concentrations of calcium trimetaphosphate (CaTMP) for periodontal tissue engineering. In the first chapter, cyclotriphosphates with total substitution of sodium by calcium were synthesized, and this biomaterial was morphologically characterized by dispersive X-ray spectroscopy (EDS) and scanning electron microscopy (SEM). The surface free energy was evaluated using a goniometer with three probe liquids: deionized water, diiodomethane, and ethylene glycol. Bovine enamel blocks (4 x 4 mm) were randomly distributed according to the average surface hardness (SH) of all blocks and the confidence interval among 7 experimental toothpaste groups containing 1% of the compounds: 100%NaTMP/0%CaTMP (NaTMP); 80%NaTMP/20%CaTMP (20CaTMP); 50%NaTMP/50%CaTMP (50CaTMP); 20%NaTMP/80%CaTMP (80CaTMP); 100%CaTMP/0%NaTMP (CaTMP). As a negative control, a placebo toothpaste was used, and as a positive control, a toothpaste with 1100 ppm F (NaF, Merck, CAS 7681-49-4, Germany) was used. For the analysis of the protective effect, the sound enamel blocks were immersed in a solution of toothpastes with human saliva once for 2 hours (under agitation), followed by 4 erosive challenges (citric acid, 0.75%, pH 3.5, for 1 minute, under agitation). The percentage of surface hardness variation (%SH) was calculated after the treatment and the acid challenges of 1, 2, 3, and 4 minutes. Additionally, the precipitates were analyzed by SEM. The variables were subjected to two-way repeated measures analysis of variance, followed by the Student-Newman-Keuls test ($p<0.05$). In the second chapter, solutions of poly(ester

urea) (PEU) (5% w/v) containing Ca-TMP (15%, 30%, 45% w/w) were electrospun into fibrous structures. The fibers were evaluated by SEM, EDS, thermogravimetric analysis (TGA), Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), and mechanical tests. The degradation rate, swelling rate, and calcium release were also evaluated. The cell/CaTMP and cell/scaffold interaction were assessed using human exfoliated deciduous teeth stem cells (SHEDs) for cell viability, adhesion, and alkaline phosphatase activity (ALP). Analysis of variance (ANOVA) and post-hoc tests ($\alpha = 0.05$) were used. Thus, the first chapter concluded that the toothpaste containing CaTMP showed the highest protective effect compared to the other groups ($p < 0.001$). The protective effect was ranked as follows: 100%CaTMP/0%NaTMP > 20%NaTMP/80%CaTMP > 50%NaTMP/50%CaTMP > 80%NaTMP/20%CaTMP > 1100 ppm F > 100%NaTMP/0%CaTMP > placebo ($p < 0.05$). All groups formed precipitates with thicker layers when using any concentration of CaTMP. The higher the percentage of CaTMP in the groups, the greater the adsorption of CaTMP and more electron donor sites on the enamel surface were achieved. In the second chapter, the membranes based on PEU and PEU/CaTMP presented fiber diameters of 469 nm and 414–672 nm, respectively. Chemical characterization confirmed the incorporation of CaTMP into the fibers. The addition of CaTMP resulted in greater degradation stability and less dimensional variation than pure PEU fibers; however, similar mechanical properties were observed. Minimal calcium was released after 21 days of incubation in a lipase-enriched solution. The CaTMP extracts increased cell viability and ALP activity, although no significant differences were found between the support groups. Overall, it was concluded that toothpastes containing CaTMP have a superior protective effect in reducing initial erosive enamel lesions and that CaTMP was effectively incorporated into PEU fibers, improving the stability of the membrane without compromising morphological properties and without promoting significant cellular function.

Keywords: Tooth enamel, Phosphates, Demineralization, Dentifrices, Tissue Engineering, Nanofibers.

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Figure 1. Initial screening for Ca-TMP (10, 25, 50, 100, 150, and 200 μM) in contact with SHEDs. (A) Percentage of cell viability (AlamarBlue®) normalized by the negative control (NC) within each time point. Welch's ANOVA/Games–Howell; α = 5%. (B) Alkaline phosphatase (ALP) activity of SHEDs cells after 14 days. Data presented as mean ± SD. Two-way ANOVA/Tukey; α = 5%. (C) Representative fluorescence images of SHEDs cells at days 1 and 3 stained by F-actin (ActinRed™ 555).

Figure 2. Evaluation of chemical–morphological membranes. (A–D) Scanning electron microscope (SEM) images, energy dispersive X-ray spectroscopy (EDS—5000× magnification), and histogram of fiber diameter of PEU (control), 5% PEU + 15% Ca-TMP, 5% PEU + 30% Ca-TMP, and 5% PEU + 45% Ca-TMP, respectively. (E) Fiber diameter, (F) scaffold porosity, and (G) swelling ratio evaluations (mean ± SD, $p < 0.05$).

Figure 3. Evaluation of chemical–morphological membranes. (A) Fourier-transform infrared spectroscopy (FTIR) spectra, (B) X-ray diffraction (XRD), (C) thermogravimetric analysis (TGA) of electrospun fibers, and (D) contact angle data presented as mean ± SD, and (E) images formed be-tween water and the PEU- or PEU/Ca-TMP-based membranes containing different concentrations.

Figure 4. Mechanical characterization of membranes. Elongation at break (%), tensile strength (MPa), Young's modulus (MPa), yield stress (MPa), and yield strain (%) graphs of dry and wet PEU fibers loaded with Ca-TMP. Data presented as (mean ± SD). Two-way ANOVA/Tukey; α = 5%. Significant interaction between variables for yield stress (MPa) and yield strain (%), p-value = 0.0037 and 0.0004, respectively.

Figure 5. Membrane degradation and total calcium release quantification. (A) Degradation of electrospun membranes conditioned in PBS and lipase-enriched solutions over 91 days. Note the greater dimensional stability of the membranes even after 3 months of incubation. (B) Calcium ion release profile from the electrospun fibers immersed in a lipase-enriched solution derived over 21 days. Note the minimal calcium released from the fiber after 3 weeks of incubation. Data presented as (mean ± SD).

Figure 6. SHEDs cells and Ca-TMP electrospun membranes interaction. (A) Percentage of cell viability (AlamarBlue®) normalized by the PEU without Ca-TMP at day 1 value. Data presented as mean ± SD, $p < 0.05$. (B) Alkaline phosphatase (ALP) activity of SHEDs cells after 7 and 14 days. Repeated measures ANOVA/Sidak post-hoc; $\alpha = 5\%$. (C) Representative fluorescence images of SHED cells spread over the membranes at 1, 3, and 7 days stained by F-actin (ActinGreen™ 488).

Sumário

INTRODUÇÃO GERAL

A erosão dentária é caracterizada pela perda química da substância mineral do dente, causada pela exposição a ácidos não derivados de bactérias orais. O desgaste dentário erosivo, por sua vez, abrange a perda gradual da superfície mineralizada do dente devido a vários fatores físicos ou quimiofísicos, incluindo erosão dentária, atrito e abrasão (Jadeja et al., 2023; Schlueter et al., 2020). O desgaste erosivo dos dentes é cada vez mais reconhecido como uma condição comum na clínica dentária, com prevalência relatada variando entre 30 a 50% nos dentes decíduos e 20 a 45% nos dentes permanentes (Lussi et al., 2019). Portanto, é necessário estabelecer abordagens preventivas e terapêuticas efetivas, focadas na etiopatogenicidade da lesão. As medidas preventivas devem começar precocemente e envolver medidas causais, como orientações dietéticas, para reduzir a frequência e a severidade dos desafios erosivos (Magalhães et al., 2009; Buzalaf et al., 2012).

Estudos recentes mostraram que o trimetafosfato de sódio (NaTMP) reduz a erosão das estruturas dentárias quando associado ao fluoreto (200 a 1100 ppm F) (de Toledo PTA et al., 2022; Cruz et al., 2015; Danelon et al., 2018). A literatura indica que grupos aniônicos, a partir de fosfatos lineares, têm a capacidade de atrair íons cálcio, atuando como agentes nucleadores de cristais de apatita (Li e Chang, 2008; Leone et al., 2008). Os fosfatos são geralmente classificados como ciclofosfatos, polifosfatos ou fosfatos inorgânicos ramificados. Os polifosfatos têm a fórmula geral $M_{(n+2)}P_nO_{(3n+1)}$, com os íons fósforo dispostos em cadeias lineares. Esses fosfatos são nomeados de acordo com a quantidade de fósforos, como por exemplo, tripolifosfatos e tetrapolifosfatos, embora o mono- e difosfatos ainda sejam chamados de ortofosfato e pirofosfato, respectivamente. Os ciclofosfatos (chamados de metafosfatos) têm a composição representada pela fórmula $(MPO_3)^n$ (Greenfield & Clift, 1975; Kulaev et al., 2005). Até recentemente, apenas o ciclotrifosfato (trimetafosfato de sódio – TMP) e o ciclohexafosfato (hexametafosfato de sódio - HMP) foram empregados em processos de mineralização (Schröder & Müller, 1999; Kulaev et al., 2005).

A adição de fosfato cíclico a produtos fluoretados, como o NaTMP, demonstra que é possível aumentar o potencial anti-erosivo (de Toledo PTA et al., 2022; Danelon et al., 2018), pois o NaTMP atuaria bloqueando a difusão de ácido, reduzindo assim o amolecimento do esmalte em profundidade (Danelon et al., 2018). Entretanto, nenhum estudo analisou se a substituição do sódio pelo cálcio na molécula do TMP

resultaria em um composto com maior atividade contra a erosão dentária e uma maior energia livre de superfície, sem a necessidade de associação com fluoreto. Além disso, o TMP é um fosfato inorgânico cíclico e está presente nos fosfolipídios, o que pode resultar em interações diferenciadas com a superfície do esmalte. Considerando que o NaTMP e o CaTMP podem apresentar diferentes interações com a superfície do esmalte, é essencial avaliar suas interações em um ambiente rico em Ca^{2+} e PO_4^{3-} (simulando o ambiente salivar) para corroborar as evidências científicas que sustentam as hipóteses levantadas. Ademais, sabe-se que as propriedades superficiais de um substrato e sua interação com o meio podem ser analisadas utilizando a energia livre de superfície (γ_s) (Neves et al., 2018).

Ao contrário dos produtos exclusivamente fluoretados, que apresentam um efeito restrito à superfície, os ciclofosfatos também reduzem a perda mineral no interior do esmalte (Takeshita et al., 2011). A ação do TMP na proteção da hidroxiapatita (HA) ainda não está completamente compreendida, mas os resultados sugerem que o mecanismo está relacionado à adsorção do TMP na superfície do esmalte (Gonzalez et al., 1973; van Dijk et al., 1980), o que resulta em uma redução da troca iônica entre o esmalte e o meio bucal. O TMP se liga aos sítios de grupos hidroxila da HA (Delbem et al., 2014; Amaral et al., 2018), diminuindo a perda de cálcio e promovendo a cristalinidade da HA. A adsorção de TMP é influenciada pela presença de fluoreto, já que o aumento de sua concentração no meio reduz a adsorção do TMP na HA, sugerindo uma competição entre o fluoreto e o TMP pelo mesmo sítio de ligação na HA (Amaral et al., 2018). Portanto, a proporção molar TMP:F é crucial tanto na perda mineral do esmalte (Takeshita et al., 2009; Takeshita et al., 2011; Manarelli et al., 2011; Favretto et al., 2013; Souza et al., 2013; Amaral et al., 2018), quanto na formação de fluoreto de cálcio no esmalte. Esse efeito é importante para o controle do processo de cárie dentária, pois a ação anticárie dos produtos fluoretados está associada à deposição de fluoreto de cálcio no esmalte e sua dissolução durante os desafios cariogênicos (Magalhães et al., 2009; Delbem et al., 2010). A associação TMP/F aumenta a deposição de fluoreto fracamente ligado (fluoreto de cálcio) à HA e diminui o fluoreto fortemente ligado à HA (Delbem et al., 2014; Amaral et al., 2018).

De acordo com um estudo recente, o trimetafosfato de sódio (NaTMP) bloqueia a difusão de ácido, reduzindo o amolecimento do esmalte em profundidade (Danelon et al., 2018). Paralelamente, o NaTMP tem sido amplamente utilizado por sua

capacidade de melhorar as propriedades biológicas e mecânicas dos polímeros e pelos efeitos positivos do cálcio na mineralização óssea (Joglekar et al., 2020). A substituição dos íons sódio por íons cálcio poderia gerar um composto que combine as propriedades benéficas de ambos, TMP e cálcio, nos processos de mineralização. O NaTMP atua como um reticulador eficaz e não apresenta toxicidade em nenhuma concentração testada (Chaouat et al., 2008), além de potencializar locais de nucleação para a precipitação de apatita com fosfato de cálcio (Favretto et al., 2018), resultando em uma superfície mais aniônica (Oliveira et al., 2022; Nalin et al., 2021). Considerando a capacidade do NaTMP de melhorar as propriedades biológicas e mecânicas dos polímeros e os efeitos do cálcio na mineralização óssea, a substituição dos íons sódio por íons cálcio poderia criar um composto inovador para os processos de mineralização. Portanto, seria relevante sintetizar o trimetafosfato de cálcio para avaliar o impacto desta substituição no NaTMP em relação à erosão inicial do esmalte, à energia livre de superfície e nas estruturas nanofibrosas de PEU, permitindo investigar as propriedades mecânicas e biológicas dessas estruturas para a engenharia de tecidos periodontais.

Os resultados dos dois subprojetos estão apresentados na forma de dois capítulos:

Capítulo 1. Protective Potential of Calcium Cyclotriphosphate-Based Toothpaste Against Dental Enamel Erosion and Its Impact on Surface Free Energy. Artigo sendo preparado para publicação no periódico Journal of the Mechanical Behavior of Biomedical Materials.

Capítulo 2. Calcium Trimetaphosphate-Loaded Electrospun Poly(Ester Urea) Nanofibers for Periodontal Tissue Engineering. Artigo publicado na revista Journal Functional Biomaterials. 2023 Jun 30;14(7):350. doi: 10.3390/jfb14070350.

Capítulo 1

Protective Potential of Calcium Cyclotriphosphate-Based Toothpaste Against Dental Enamel Erosion and Its Impact on Surface Free Energy

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Abstract

This *in vitro* study aimed the synthesize and characterize cyclotriphosphate (NaTMP) with a total replacement of sodium by calcium (CaTMP) and to assess its effect on initial erosion and surface free energy of dental enamel. CaTMP was synthesized and morphologically characterized using energy-dispersive X-ray spectroscopy (EDS) and scanning electron microscopy (SEM). Enamel blocks (n = 182) were randomly allocated to seven groups of experimental toothpastes containing 1% of the compounds: 100%NaTMP/0%CaTMP; 80%NaTMP/20%CaTMP; 50%NaTMP/50%CaTMP; 20%NaTMP/80%CaTMP; 100%CaTMP/0%NaTMP; Placebo; and 1100 ppm F. The blocks were immersed in human saliva for 2 hours and then in a toothpaste slurry for 2 minutes. Subsequently, they underwent four erosive challenges in citric acid (0.75%, pH 3.5), each challenge lasting 1 minute. The surface hardness of the blocks was determined after treatment and after the four erosive challenges. The surface free energy (mN/m) was calculated after toothpaste treatment. In addition, the precipitates were analyzed by SEM. The percentage of surface hardness (%SH) was assessed with a two-way ANOVA and Tukey test ($p<0.05$). Surface and interaction free energy were analyzed with a one-way ANOVA and Student-Newman-Keuls test ($p<0.05$). Toothpastes containing CaTMP showed the greatest protective effect compared to the other groups ($p<0.05$). The higher the percentage of CaTMP in the solutions, the greater the CaTMP adsorption and more electron-donor sites were observed. All groups formed precipitates with a thicker layer when using CaTMP concentrations. It was concluded that toothpastes containing CaTMP demonstrated a superior protective effect on initial erosive lesions and promoted more electron-donor sites on the enamel surface.

Keywords: Dental Enamel, Phosphates, Tooth Erosion, Toothpastes

1. Introduction

Dental erosion is characterized by the chemical loss of the tooth's mineral substance due to exposure to acids not derived from oral bacteria, whereas erosive tooth wear encompasses the gradual loss of the mineralized tooth surface due to various physical or chemo physical factors, including dental erosion, attrition, and abrasion (Jadeja et al., 2023; Schlueter et al., 2020). Erosive tooth wear is increasingly recognized as a common condition in dental clinics, with reported prevalence ranging from 30 to 50% in primary teeth and 20 to 45% in permanent teeth (Lussi et al., 2019), particularly exacerbated when associated with gastroesophageal reflux disease (Chatzidimitriou et al., 2023). Advanced stages of erosive tooth wear can compromise both aesthetics and function, significantly impacting the patient's quality of life (Srivastava, 2023). Dental erosion remains a significant concern, necessitating thorough investigation into its causes, effective prevention strategies, and appropriate treatment protocols.

Fluoride (F) has been utilized as both a preventive and therapeutic measure in managing dental erosion (Inchingolo et al., 2023). Although F may have a protective effect in conditions where erosive factors are not excessive (Né et al., 2022), some studies have shown a limited effect of toothpastes containing at least 1000 ppm F on eroded dental substrates (West et al., 2017; Ganss et al., 2011; Magalhães et al., 2007). In this context, various treatment protocols involving fluoride compounds have been implemented to encourage the formation of a mineral precipitation layer in eroded lesions (Né et al., 2022; West et al., 2021). Among potential therapeutic options, the efficacy of other remineralizing agents, such as phosphate salts, in managing dental erosion has been investigated (Luka et al., 2023; de Toledo et al., 2022; Danelon et al., 2018; Carvalho et al., 2015).

Among the various phosphate salts, sodium trimetaphosphate (NaTMP), a cyclic polyphosphate, has exhibited promising properties, including strong adsorption onto dental substrates (Nunes et al., 2022; Emerenciano et al., 2018), the capability to reduce H^+ diffusion and enhance calcium and phosphate diffusion (Emerenciano et al., 2018; Amaral et al., 2018), and the ability to bind to OH^- groups (Nalin et al., 2021; Amaral et al., 2018). Furthermore, studies have demonstrated that NaTMP reduces the erosion of dental structures when associated with fluoride (200 to 1100 ppm F) (Paiva et al., 2023; de Toledo et al., 2022; Danelon et al., 2018; Cruz et al., 2015).

Research indicates that anionic groups from linear phosphates can attract calcium ions, serving as nucleating agents for apatite crystals (Nalin et al., 2021; Amaral et al., 2018).

It is understood that the efficacy of NaTMP relies on its combination with fluoride (Nunes et al., 2022; Danelon et al., 2018). While recent studies have explored the remineralizing effects and anti-erosive potential of NaTMP in fluoride products, none have analyzed the chemical substitution of sodium ions with calcium ions in the TMP molecule (CaTMP). Such an electrostatic alteration could potentially yield a compound that combines the characteristics of both ions, potentially enhancing its anti-erosive capacity and enabling its use without the need for co-administration with fluoride. Moreover, studying the interactions and chemical bonds between CaTMP and the enamel surface through surface free energy can help to better understand the mechanisms that involve the effects of CaTMP in the initial erosion of enamel.

Meanwhile, NaTMP has been widely used due to its ability to enhance the reduction of enamel solubility, indicating an affinity for the enamel surface and/or hydroxyapatite (Souza et al., 2013). Substituting sodium ions with calcium ions could result in a compound that combines the properties of both NaTMP and calcium in mineralization processes. Therefore, this study aimed to synthesize cyclotriphosphates (NaTMP) with a complete replacement of sodium by calcium (CaTMP) and to produce compounds with different proportions (weight/weight) of CaTMP/NaTMP, assessing their effect on initial dental enamel erosion and surface free energy. The null hypothesis of the study was that toothpaste containing CaTMP would have a similar effect to a 1100 ppm F toothpaste against initial dental enamel erosion and surface free energy.

2. Materials and Methods

2.1 Synthesis of cyclotriphosphates (Trimetaphosphate: TMP) with total sodium for calcium ($3Ca_2O_9P_3$)

The calcium trimetaphosphate (CaTMP) used had a molecular weight of 594.07 and a particle size of 236 ± 50 nm. It was synthesized in the Laboratory of Pediatric Dentistry of the Department of Preventive and Restorative Dentistry at São Paulo State University (FOA/UNESP) (Toledo, et al., 2023). TMP compounds with total replacement of sodium by calcium were prepared by dissolving 30 g of commercial

sodium TMP (NaTMP) ($Na_3P_3O_9$, CAS 7785-84-4, Sigma-Aldrich Co., St. Louis, MO, USA) in 40 mL of deionized water. The solution was treated in a cationic ion exchange column to replace sodium ions with calcium ions (Watanabe et al., 1998). Composition adjustment was achieved by rigorously controlling the initial amount of sodium TMP, pH, and ion exchange resin fraction. Precipitation and incorporation of calcium were conducted using a calcium hydroxide solution ($Ca(OH)_2$, Code HC09743RA, Exôdo, Brazil), until the initial pH of the solution was reached before the ion exchange. The solutions were incubated in an oven at 37 °C for 24 hours (Griffith 1962), then filtered, and the resulting powder was subjected to drying in an oven at 70 °C for 24 hours. Subsequently, the powder was homogenized in a ball mill (Planetary Micro Mill PULVERISETTE 7 classic line, Fritsch GmbH, Idar-Oberstein, Germany) for 2 hours. The compound was named calcium TMP: CaTMP. Compounds containing 20%/80%, 50%/50%, and 80%/20% CaTMP/NaTMP (w/w) were mixed in a ball mill as previously described.

2.2 Characterization of CaTMP compounds

The morphology and overall structure of the CaTMP powder were assessed using a field-emission scanning electron microscope (SEM/ZEISS DSM-940A model, Oberkochen, Germany). Sample of the powder mounted onto aluminum stubs, coated with a thin layer of gold–palladium through sputter-coating, and examined at an acceleration voltage of 5 kV. In addition, energy dispersive X-ray spectroscopy (EDS) analysis was performed under SEM to acquire semi-quantitative data regarding the chemical composition of the compound.

2.3 Experimental design

To conduct the study, enamel blocks (4 mm × 4 mm, n = 182) were obtained from bovine incisor teeth that had been immersed in a 2% formaldehyde solution at pH 7.0 for 30 days prior to any experimental procedures (de Toledo et al., 2022). These blocks underwent enamel surface polishing using 600, 800, and 1200-grade water-cooled silicon carbide paper disks (Buehler, Lake Bluff, IL, USA), followed by a final polish using a felt disk (Polishing Cloth 40–7618, Buehler) moistened with a 1-μm diamond polishing suspension (Extec Corp., Enfield, CT, USA). This sequential polishing process facilitated selection by determining the initial surface hardness (SHi).

Surface hardness (SH) was determined as follows: a reference impression was made 1000 µm (Knoop, 500 g, 10 seconds, Shimadzu HMV-2000, Shimadzu Corporation, Kyoto, Japan) from the central region of the enamel block. At a distance of 200 µm from the right vertex of the largest impression, five impressions were made (initial SH - SHi) separated by a distance of 100 µm (Knoop, 25 g, 10 seconds, Shimadzu HMV-2000) (de Toledo et al., 2022). The enamel blocks were randomly distributed according to the mean SH of the total blocks and the confidence interval between 7 experimental groups containing 1% of the compounds: 100%NaTMP/0%CaTMP (NaTMP), 80%NaTMP/20%CaTMP (20%CaTMP), 50% NaTMP/50%CaTMP (50%CaTMP), 20%NaTMP/80%CaTMP (80%CaTMP) and 100%CaTMP/0%NaTMP (CaTMP). A placebo toothpaste was used as a control and, as a positive control, a toothpaste containing 1100 ppm F (NaF, Merck, CAS 7681-49-4, Germany). Sample size calculation (n = 12 per group) was based on a previous study (de Toledo PTA et al., 2022), considering mean values of surface hardness (SH) and percentage loss in surface hardness (%SH) as the primary endpoint. Additionally, expected standard deviations, an α error of 5%, and a β error of 15% were considered during the calculation. After treatment and erosive challenges, the surface hardness was measured and the percentage loss in surface hardness (%SH) was determined. Additionally, analysis of enamel surface free energy, scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) were also performed. (Fig. 1).

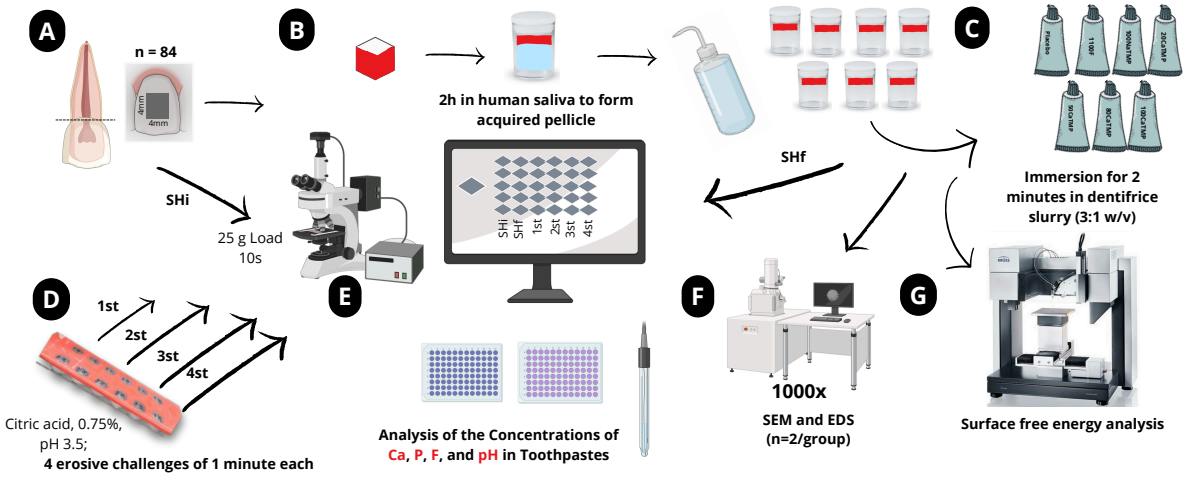

Fig. 1. (A) Enamel Block Preparation (B) Acquired pellicle formation (C) Treatment (D) Analysis of the percentage of surface hardness after erosion challenges (E) Analysis

of the F, Ca and P Concentration Present in Toothpastes (F) SEM and EDS after treatment on enamel surface (G) Surface free energy analysis.

2.4 Toothpaste formulation and fluoride (F), calcium (Ca), phosphate (P), and pH assessment

Toothpastes were formulated using the following ingredients: titanium dioxide, carboxymethyl cellulose, methyl p-hydroxybenzoate sodium, saccharin, mint oil, glycerin, abrasive silica, sodium lauryl sulfate, and deionized water. Each ingredient was carefully mixed to achieve a total weight of 100 g for each toothpaste. Toothpastes containing calcium trimetaphosphate and sodium trimetaphosphate were prepared at their specified concentrations for their respective groups. Additionally, a toothpaste containing sodium fluoride (NaF, Merck, CAS 7681–49–4, and Germany) at a concentration of 1100 ppm F was formulated. Furthermore, a placebo toothpaste formulation without calcium trimetaphosphate, sodium trimetaphosphate, and fluoride (F) was also prepared. All toothpaste formulations were stored at room temperature and hermetically sealed to maintain sample integrity.

Fluoride levels in the experimental toothpastes were measured following the method outlined by Delbem et al. (2009). Initially, a sample from the suspension was treated with 2 mol L^{-1} HCl for total fluoride assessment and then buffered with 1 mol L^{-1} NaOH. Supernatants obtained by centrifugation (906 × *g*; 20 min) were utilized for ionic fluoride assessment. The same volume of TISAB II (total ionic strength adjustment buffer, Orion, Thermo Scientific Inc., Beverly, MA, USA) was added to the solutions. Fluoride measurements were performed using an ion-selective electrode (Orion 9609-BN; Orion Research Inc., Beverly, USA) connected to an ion analyzer (Orion 720 A^{+}; Orion Research Inc.), calibrated with standards ranging from 0.125 to 4.0 µg F/mL.

Additionally, total phosphorus (TP) and calcium (TCa) levels in the toothpastes were determined (Delbem et al., 2009). Samples were subjected to acid hydrolysis by adding 2 mol L^{-1} HCl at a temperature just below the boiling point. After a 20-minute centrifugation (906 ×*g*) of the suspensions, ionic phosphorus and calcium were measured. Ionic calcium (ICa) and total calcium (TCa) were determined using the Arsenazo III colorimetric method as described by Vogel et al. (1983), with calibration standards ranging from 40 to 200 µg Ca/mL. Ionic phosphorus (IP) and total phosphorus (TP) were measured using a colorimetric method described by Fiske &

Subbarow (1925) with a spectrophotometer (Hitachi U-1100 UV/Vis spectrophotometer - Hitachi High Technologies, Tokyo, Japan) at a wavelength of 660 nm. Results were expressed as µg/g.

2.5 Collection of human saliva

Human saliva was collected from six healthy volunteers one hour after breakfast, with approval from the Research Ethics Committee (CAAE: 52188021.2.0000.5420). These volunteers reported good oral health and were not using any medication (Wetton et al., 2007). Human saliva was stimulated with the aid of paraffin (Parafilm® M, Sigma-Aldrich, St. Louis, MO, USA), and was expectorated into ice-containing vials. Subsequently, the collected saliva was centrifuged (Hanil-Combi-514 R, Incheon, South Korea) for 10 min at 4 °C, at a force of *2000* × *g*. The supernatants were collected, pooled, and stored in 50 mL vials at - 72 °C (Schipper et al., 2007). This temperature was maintained to preserve the properties of human saliva until its use at room temperature.

2.6 Treatment and erosive challenges

Prior to the acid challenges, the enamel blocks were individually immersed in human saliva (4 mL/block) for 2 hours to allow for the formation of an acquired pellicle. Subsequently, the blocks were washed in deionized water for 30 seconds. Following this, the blocks were individually immersed in suspensions/slurry of toothpaste in deionized water (1:3 - weight: weight) corresponding to their respective groups (4 mL/block) and agitated at 100 rpm for 2 minutes. Surface hardness (SHf) measurements were then recorded after treatment for each group. The surface hardness (SHf) measurements were taken after treatments of each group. After washing with deionized water, the blocks were individually immersed in 4 mL of citric acid solution (0.75%, pH 3.5; Synth, Brazil) under agitation (100 rpm) for 1 minute at room temperature, followed by another 30-second wash with deionized water. This process was repeated four times to complete four erosive challenges after treatment with the toothpaste slurry. Final surface hardness (SHf) measurements were taken after each erosive challenge to calculate the percentage of surface hardness change (%SHC=[(SHf-SHi)/(SHi)]×100) at 1, 2, 3, and 4 minutes into the challenge (de Toledo et al., 2022).

2.7 Surface free energy analysis

To determine the contact angle, the treated blocks were kept at 22 ºC for 45 minutes to allow for the stabilization of the formed film (van der Mei et al., 2002). The parameters of γ_s^{LW} and γ_s^{AB} and the acid (γ_s^+, acceptor component) and base (γ_s^-, donor component) components of the surface free energy (mN/m) were calculated according to the van Oss model, Chaudhery and Good to determine the free energy of interaction of substrates (Harnett et al., 2007; van der Mei et al., 2002; van Oss et al., 1995). Measurements were conducted using an automatic goniometer (DSA 100S, Krüss, Hamburg, Germany), employing three probe liquids with known surface energy parameters: water (polar), diiodomethane (nonpolar) and ethylene glycol (polar with acidic and base).

To determine the contact angle, a volume of 0.5 µL of each liquid was automatically dispensed into a different quadrant of the enamel surface of each block using a glass syringe (500 µL) and a 0.5 mm needle. After 1 second of dripping, the contact angles (right and left) were measured using a CCD camera to capture images and the tangent method (Drop Shape Analysis DSA4 Software, version 2.0-01, Krüss). Each drop was measured five times for 5 seconds at a temperature of 22 ºC and relative humidity of 44% ± 6 (Harnett et al., 2007, van der Mei et al., 2002).

The interaction free energy (ΔG_{iwi}) was also calculated to determine the hydrophobicity/hydrophilicity of the enamel surface: $\Delta G_{iwi} > 0$ indicates a hydrophilic surface, and $\Delta G_{iwi} < 0$ indicates a hydrophobic surface (Harnett et al., 2007). $\Delta G_{iwi} = -2(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}})^2 - 4(\sqrt{\gamma_s^+\gamma_s^-} + \sqrt{\gamma_w^+\gamma_w^-} - \sqrt{\gamma_s^+\gamma_w^-} - \sqrt{\gamma_s^-\gamma_w^+})$ (Harnett et al., 2007).

2.8 Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX)

To assess the deposition ability of toothpastes on enamel surfaces, enamel blocks were prepared as previously described and polished (2/per group). Each block was divided into two halves, with one half covered with nail polish. Subsequently, the blocks were exposed to human saliva for 2 hours to form an acquired pellicle. Following this, a mixture of toothpaste and human saliva was applied to each block for two minutes, followed by rinsing in deionized water for 30 seconds. Afterward, the nail polish was carefully removed using a scalpel blade and acetone, and the blocks were washed once more and dried with paper towels. The enamel blocks were then affixed to stubs with carbon conductive tape and coated with a thin gold layer (~5 nm thick) for 90 seconds in a sputter coater (Quorum - Q150T E, West Sussex, UK). SEM

images of each sample were captured using a scanning electron microscope (SEM) (Carl Zeiss, EVO LS15, Carl Zeiss NTS LTD, Germany) at a magnification of 1000x and an acceleration voltage of 20 kV to assess the presence of deposits on the enamel surfaces. Additionally, the composition of these deposits was analyzed using energy-dispersive X-ray spectroscopy (EDX) (Oxford Instruments, INCAx-act, 133 eV, England) operating at 20 keV with a spatial resolution of approximately 2 μm and a counting time of 210 seconds (de Toledo et al., 2022).

2.9 Statistical analysis

Experimental groups and phases (baseline, posttreatment, 1^{st} , 2^{nd} , 3^{rd} , and 4^{th} acid challenges) were considered as factors of variation. All variables exhibited normal distributions (Shapiro–Wilk test) and homogeneous variances (Cochran test). The percentage of surface hardness (%SH) was analyzed using a two-way repeated-measures analysis of variance (ANOVA), followed by the Tukey test. Surface and interaction free energy variables were analyzed using one-way ANOVA, followed by the Student-Newman-Keuls test. All statistical analyses were conducted using SigmaPlot software (version 12.0, SigmaPlot, Systat Software Incorporation, San Jose, CA, USA) with a significance level set at 5%.

3. Results

The SEM images revealed irregular grain crystals for NaTMP (Fig. 2.A) and regular spherical grains for CaTMP (Fig. 2.B). EDS analysis identified peaks corresponding to the chemical elements Na at 1.04 keV, O at 0.52 keV, and P at 2.02 keV, common to both NaTMP and CaTMP samples (Fig. 2.C), as well as peaks at 0.26 and 3.69 keV characteristic of the Ca element in CaTMP (Fig. 2.D).

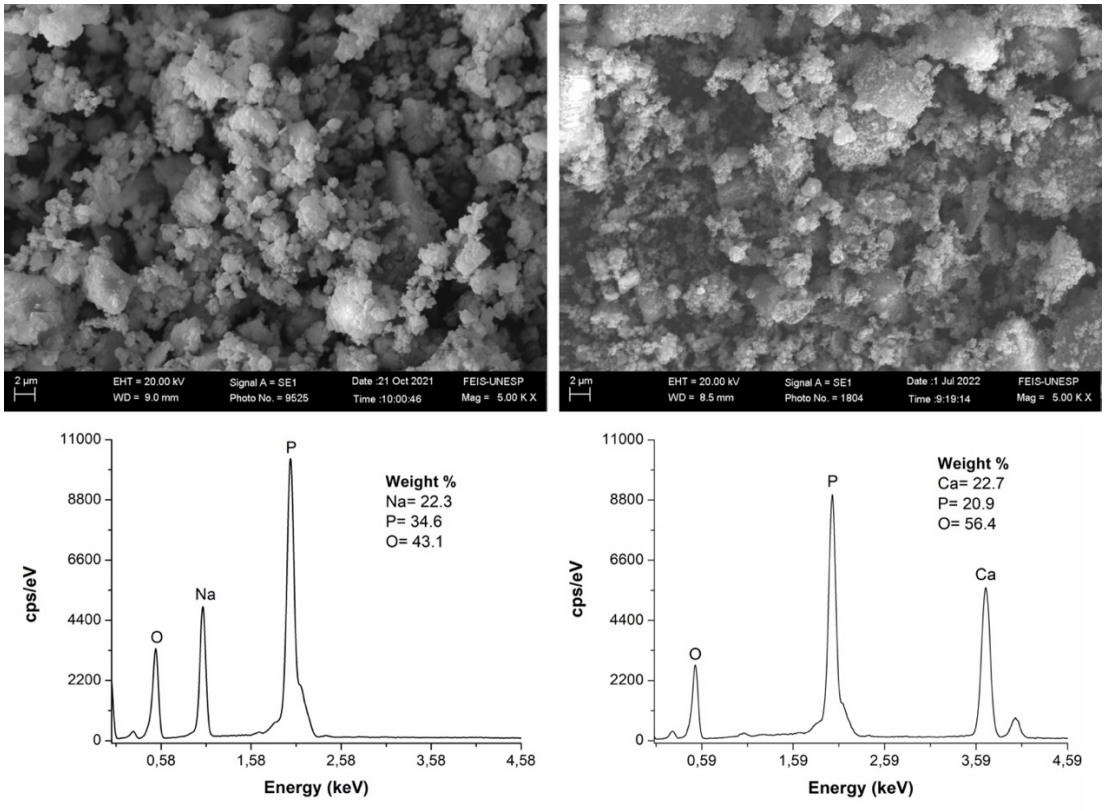

Figure 2 – Scanning Electron Microscopy images of NaTMP (A) and CaTMP (B) samples, (C) Energy-dispersive X-ray spectroscopy of NaTMP (C) and CaTMP (D) powder.

The mean (±SD) concentrations of total fluoride (TF) and ionic fluoride (IF) in the experimental toothpastes, were, respectively: 1) Placebo (16.5 ± 1.6) and (14.4 ±1.6); 2) 1100 ppm F (1134.1 ± 43.6) and (1107 ± 59.3); 3) 100%NaTMP/0%CaTMP (10.2 ± 0.2) and (11.4 ± 0.4); 4) 80%NaTMP/20%CaTMP (13.3 ± 1.7) and (12.5 ± 0.6); 5) 50%NaTMP/50%CaTMP (10.5 ± 0.64) and (12.9 ± 0.7); 6) 20%NaTMP/80%CaTMP (12.0 ± 0.8) and (11.3 ± 0.9) and 7) 100%CaTMP/0%NaTMP (21.6 ± 16.6) and (17.3 ± 2.4). The fluoride concentration was within the range of 10% proposed for toothpastes. All experimental toothpastes had a pH ranging from 6.83 to 7.19. The ionic concentrations of calcium/phosphorus (ICa and IP) were lower than the concentrations of total calcium/phosphorus (TCa and TP), influenced by the CaTMP concentration. The higher the concentration of CaTMP in the groups, the higher the concentrations of ionic and total calcium/phosphorus.

Table 1 – Mean values (µg/g) of calcium (Ca), phosphorus (P), fluoride (F), ionic (I) and total (T), and pH in the toothpastes

Groups	Fluoride		Calcium		Phosphorus		pH
	Ionic	Total	Ionic	Total	Ionic	Total	
Placebo	14.4 (1.6)*	16.5 (2.1)	–	–	255.3 (22.2)	348.5 (27.6)	6.89 (0.06)
1100 ppm F	1107.3 (59.3)	1134.1 (43.6)	–	–	295.8 (3.6)	606.4 (66.1)	7.05 (0.06)
100%NaTMP/ 0%CaTMP	11.4 (0.4)	10.2 (0.2)	–	–	419.4 (2.6)	2933.5 [47.9]	6,83 (0.04)
80% NaTMP/ 20% CaTMP	12.5 (0.6)	13.3 (1.7)	–	3419.8 (269.3)	588.5 (28.5)	5122.2 (147.2)	7.15 (0.03)
50% NaTMP/ 50% CaTMP	12.9 (0.7)	10.5 (0.64)	–	2837.1 (255.6)	616.5 (44.3)	3646.0 (60.8)	6.90 (0.04)
20% NaTMP/ 80% CaTMP	11.3 (0.9)	12.0 (0.8)	–	2190.2 (16.9)	448.5 (13.6)	3551.7 (5.0)	7.05 (0.06)
100%CaTMP/ 0%NaTMP	17.3 (2.4)	21.6 (16.6)	–	5675.2 (172.4)	769.0 (38.0)	6534.5 (33.2)	7.19 (0.05)

(−) Not detectable. (*) Standard deviation from the average

Regarding post-treatment hardness gain, the toothpaste containing 100%CaTMP/0%NaTMP exhibited the highest values, significantly surpassing those of the other groups ($p<0.05$; Table 2). Furthermore, this experimental toothpaste also demonstrated the most effective anti-erosion protective effect on enamel after erosive challenges ($p<0.05$; Table 2).

Table 2 – Mean values (SD) of the percentage of change in surface hardness (%SH) in enamel according to the groups and experimental conditions (n=12)

Groups	Experimental conditions				
	After treatment	**1st Challenge**	**2nd Challenge**	**3rd Challenge**	**4th Challenge**
Placebo	$0.2^{a,A}$ (0.1)	$-33.9^{b,A}$ (1.2)	$-51.4^{c,A}$ (1.1)	$-60.3^{d,A}$ (0.8)	$-66.8^{e,A}$ (0.4)
1100 ppm F	$2.2^{a,B}$ (0.3)	$-17.6^{b,B}$ (0.2)	$-27.4^{c,B}$ (0.7)	$-52.4^{d,B,D}$ (1.8)	$-58.8^{e,B,D}$ (0.3)
100%NaTMP /0%CaTMP	$0.7^{a,A}$ (0.1)	$-39.0^{b,C}$ (1.2)	$-47.3^{c,C}$ (1.3)	$-51.1^{d,B}$ (0.4)	$-59.4^{e,B}$ (0.2)
80% NaTMP/ 20% CaTMP	$2.5^{a,B}$ (0.3)	$-35.9^{b,D}$ (1.3)	$-43.8^{c,D}$ (0.8)	$-57.1^{d,C}$ (1.7)	$-62.0^{e,C}$ (0.9)
50% NaTMP/ 50% CaTMP	$3.5^{a,B}$ (0.3)	$-30.5^{b,E}$ (1.4)	$-38.4^{c,E}$ (1.0)	$-56.6^{d,C}$ (0.6)	$-59.2^{e,B}$ (1.9)
20% NaTMP/ 80% CaTMP	$5.7^{a,C}$ (0.9)	$-20.8^{b,F}$ (1.3)	$-27.5^{c,B}$ (0.9)	$-53.0^{d,D}$ (1.8)	$-57.2^{e,D,E}$ (1.2)
100%CaTMP /0%NaTMP	$7.4^{a,D}$ (1.3)	$-7.1^{b,G}$ (0.8)	$-17.1^{c,F}$ (0.6)	$-43.4^{d,E}$ (0.6)	$-48.1^{e,F}$ (0.8)

Distinct lowercase letters indicate a significant difference between experimental conditions for each agent. Different capital letters indicate a significant difference between agents for each experimental condition (two-way repeated measures ANOVA, Tukey, $p<0.05$).

The addition of calcium trimetaphosphate in various concentrations to sodium trimetaphosphate in toothpastes resulted in the formation of a thin, homogeneous precipitate layer containing approximate amounts of Ca, P, and O (Fig. 3.C−G).

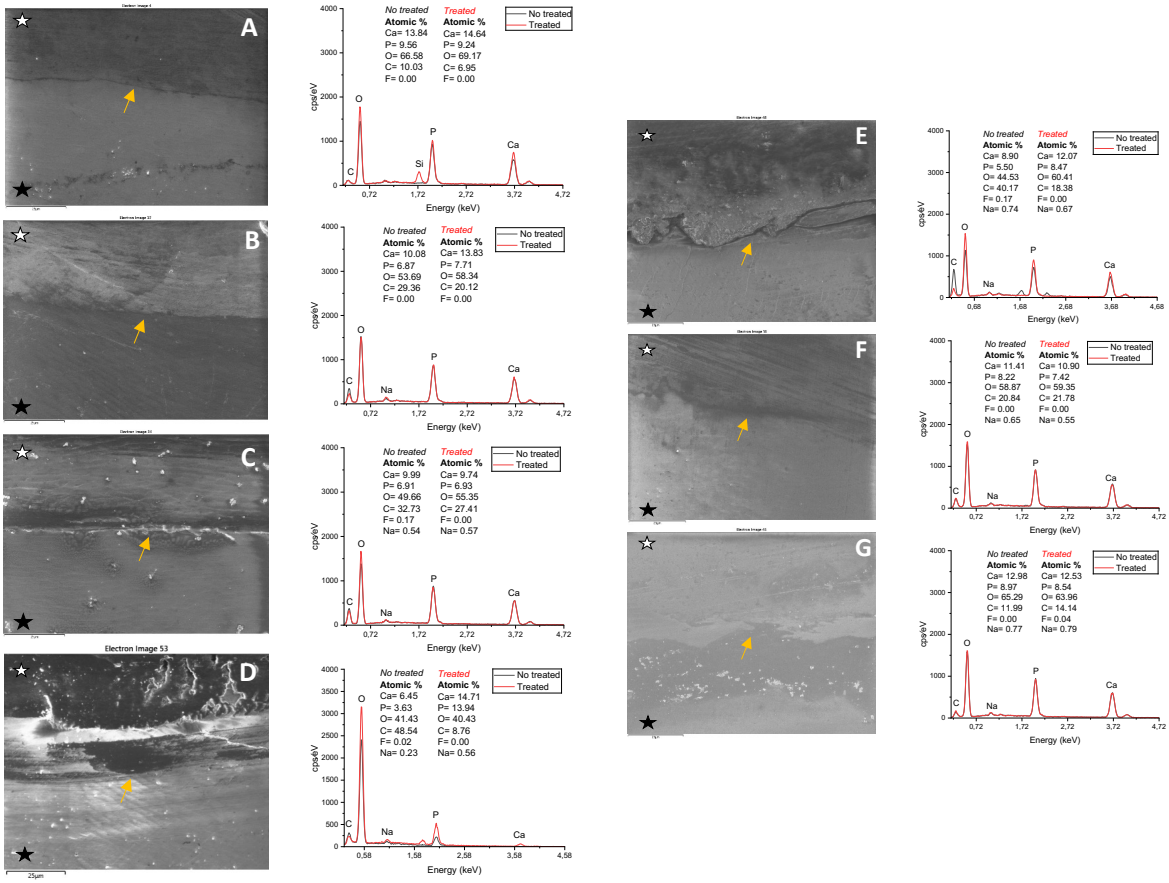

Figure 3 – SEM images of a representative specimen in different groups and respective spectrum from EDX analysis: (A) Placebo, (B) 1100 ppm F, (C) 100%NaaTMP/0%CaTMP, (D) 80%NaTMP/20%CaTMP/, (E) 50%NaTMP/50%CaTMP (F) 20%NaTMP/80%CaTMP and (G) 100%CaTMP/0%NaTMP. White asterisks indicate sound enamel. Black asterisks indicate precipitated enamel formed after the treatments. Arrows indicate the limit between sound enamel and precipitated enamel formed after treatments.

All CaTMP formulations tested on enamel resulted in a significant increase in γ_s and a decrease in ΔG_{iwi} ($p<0.05$) compared to the group containing 100%NaTMP/0%CaTMP (Table 3). When CaTMP treatments were compared with 1100 ppm F, there was a decrease in γ_s and an increase in ΔG_{iwi} ($p<0.05$), indicating a dose-response relationship (Table 3). Surface free energy (γ_s) was only significantly higher with Placebo, ($p<0.05$; Table 3). The values of polar component (γ_s^{AB} = Lewis acid-base) became more negative with increasing % of CaTMP ($p<0.05$; Fig. 4.A). Among the parameters from γ_s^+ = eletron-acceptor (Lewis acid) and γ_s^- = eletron-donor

(Lewis base), lower γ_s^- values were observed with increasing % of CaTMP ($p<0.05$; Fig. 4.B).

Table 3 – Means of the surface (γ_s) and interaction (ΔG_{iwi}) free energy after treatment of enamel surface (n = 12)

Treatments	Variables	
	γ_s, mN/m	ΔG_{iwi}, mN/m
Placebo	24.2[a] (3.4)	-49.0[a] (8.1)
1100 ppm F	-14.2[b] (6.1)	33.9[b] (7.9)
0%CaTMP/100%NaTMP	-44.7[c] (8.1)	44.6[c] (7.4)
80% NaTMP/20% CaTMP	-25.1[d] (6.2)	36.2[bc] (5.0)
50% NaTMP/50% CaTMP	-31.2[e] (8.6)	34.1[b] (9.4)
20% NaTMP/80% CaTMP	-33.7[e] (7.2)	37.6[bc] (6.8)
100%CaTMP/0%NaTMP	-33.7[e] (4.0)	40.2[bc] (8.2)

Distinct superscript letters indicate statistical significance among the groups in each variables (one-way ANOVA, Student-Newman-Keuls test; $p<0.05$). Values between parentheses indicate the standard deviation of the mean. NaTMP, sodium trimetaphosphate; CaTMP, calcium trimetaphosphate;

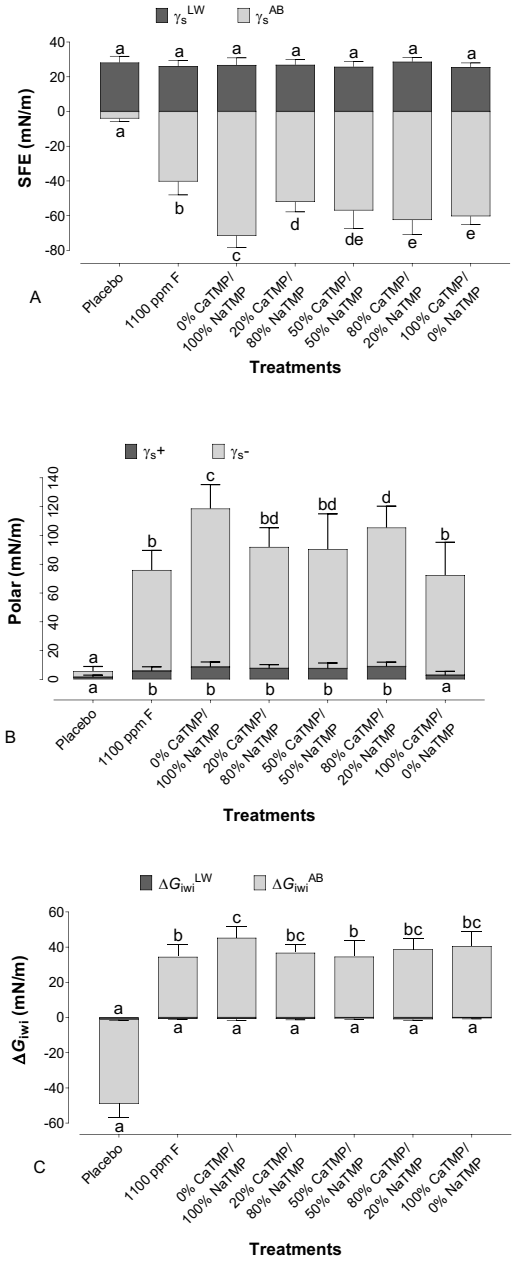

Figure 4. (A) Surface free-energies and their components (γ_s^{LW}: Lifshitz-van der Waals surface tension component; γ_s^{AB}: Lewis acid-base interaction) with different enamel-surface treatments. (B) Influence of the treatments on the component polar of surface free energy on enamel surface: Lewis-acid (γ_s^+) and Lewis-base (γ_s^-). Values denote mean and standard deviation (n = 12). (C) The mean values of the apolar (ΔG_{iwi}^{LW}) and polar (ΔG_{iwi}^{AB}) components of the interaction free energy. Distinct letters show significant differences among the groups (Student-Newman-Keuls, $p<0.05$). Asterisk means there is no significant difference between groups.

4. Discussion

In *in vitro* erosion models aimed at reducing enamel loss, the combination of fluoride and phosphates has emerged as a promising alternative (Paiva et al., 2023; de Toledo et al., 2022; Viana et al., 2019). Previous studies have demonstrated that the inclusion of NaTMP enhances the anti-erosion efficacy of fluoride toothpastes (Paiva et al., 2023; de Toledo et al., 2022; Danelon et al., 2018), even in formulations with lower fluoride concentrations (250 ppm F) (Cruz et al., 2015; de Toledo et al., 2022). The increased precipitation of calcium phosphate may further augment the anti-erosion effects of TMP. Therefore, the present *in vitro* study assessed toothpastes containing F, NaTMP, and cyclotriphosphates with a complete replacement of sodium by calcium (CaTMP) in their ability to inhibit initial enamel erosion lesions and alter surface free energy. The findings of this study indicated that toothpastes containing Na/CaTMP resulted in less enamel softening compared to 1100 ppm F.

The findings of the present study demonstrated that the addition of CaTMP significantly enhanced the protective effect against enamel erosion and promoted additional electron-donor sites on the enamel surface. Consequently, the null hypothesis of the study was rejected based on the data obtained. In a previous study (Cruz et al., 2015), formulations consisting solely of NaTMP without fluoride supplementation were not investigated. This decision was informed by prior evidence indicating that the efficacy of NaTMP alone is inferior to that observed with fluoride-containing toothpastes. These findings are consistent with our results, indicating that toothpastes containing only TMP exhibit either similar or diminished effects, depending on the concentration and challenge conditions (Table 2).

The presence of fluoride (F) in toothpaste is known to induce the formation of CaF_2 on eroded enamel surfaces, thereby partially mitigating enamel erosion following erosive cycles, as evidenced in a previous study (Magalhães et al., 2007). These CaF_2 globules serve as a physical barrier, hindering acid contact with the enamel and contributing to enamel hardening (Magalhães et al., 2007). This layer readily dissolves in acid, but it acts as an additional physical barrier, impeding the dissolution of the underlying enamel mineral. These precipitates not only directly protect the underlying enamel surface but also release fluoride ions in response to erosive challenges (Huysmans et al., 2014). Regarding trimetaphosphate (TMP), its adsorption onto enamel can impede acid diffusion (de Toledo et al., 2022; Amaral et al., 2018), thereby

reducing enamel softening (Danelon et al., 2018). The complete substitution of sodium with calcium has shown promising outcomes in terms of protection against erosive challenges, likely attributed to the precipitation of calcium phosphate, which offers defense during erosive episodes. Consequently, the increased precipitation of calcium phosphate resulting from the sodium-to-calcium replacement obstructs acid diffusion and minimizes enamel softening across all conducted challenges, even in the absence of fluoride.

Moreover, other phosphate salts have also been noted for their capacity to adsorb onto enamel, including calcium (Ca^{2+}) and phosphorus (PO_4^{3-}) (Neves et al., 2018; Emerenciano et al., 2023). Our study demonstrated that increasing the concentration of CaTMP led to higher levels of ionic and total calcium/phosphorus, facilitating faster and deeper subsurface protection. Alternatively, toothpastes containing 100%CaTMP/0%NaTMP enhanced saliva's efficacy, showcasing superior anti-erosion properties. They exhibited a 28% and 18% improvement in repair within 2 minutes, respectively, and demonstrated enhanced resilience against subsequent erosive tests compared to placebo and toothpastes with 1100 ppm F, as depicted in Table 2.

Given that enamel primarily comprises calcium (Ca), phosphorus (P), and oxygen (O), the application of agents containing phosphorus and calcium leads to minimal quantitative changes, attributed to the limited sensitivity of the Energy-Dispersive X-ray Spectroscopy (EDX) technique (Fig. 2). A marginal variation in Ca, P, and O percentages between sound enamel samples may suggest minimal mineral loss. While EDX does not specify whether the deposited calcium phosphate is crystalline or amorphous, the enamel hardness induced by each toothpaste formulation varied. This observation is supported by the increase in surface hardness (%SH) of sound enamel following a single application of the treatments, as detailed in Table 2. Moreover, examination of Scanning Electron Microscopy (SEM) images of sound enamel (Fig. 3) revealed qualitative disparities in the surface layer among groups, potentially influencing surface hardness.

Additionally, this study investigated the impact of CaTMP at various concentrations on enamel surface free energy. Consistent with prior reports (Nalin et al., 2021; Neves et al., 2018), the balance between (γ_s^+) and base (γ_s^-) forces indicates the hydrophobic or hydrophilic nature of a surface, as dispersive forces (non-polar energy) are always present (van Oss, 1995; Harnett et al., 2007). Treatment with

sodium TMP was found to facilitate the adsorption of Ca^{2+} and PO_4^{3-}, resulting in reduced γ_s, with a dose-response relationship observed between TMP concentrations in solutions, γ_s^{AB} and γ_s^-. Furthermore, in a previous study assessing enamel treated with calcium polyphosphate (CAGP), it was demonstrated that CAGP adsorbed on the enamel, leading to reduced γs attributed to lower γ_s^{LW} values (Nalin et al., 2021). In the current study, CaTMP was also adsorbed onto the enamel, thereby reducing γ_s due to lower γ_s^{AB} values.

A recent study that demonstrated that incorporating CaTMP into a membrane for periodontal tissue engineering resulted in hydrophobicity (Toledo et al., 2023). Nevertheless, the methodological variations and differences in the composition of the biomaterial does not allow a direct comparison with present data. In our study, treatment of dental enamel with CaTMP yielded a hydrophilic surface, evidenced by a decrease in γ_s due to reduced γ_s^{AB} values and no significant alteration in γ_s^{LW} values (Fig. 4.A). The results of γ_s^- further support these findings, indicating higher values (>28.5 mN/m) and ΔG_{iwi} values that confirm the hydrophilic nature of the enamel surface after CaTMP treatment, irrespective of the concentration used (van Oss, 1995). Van der Waals interactions occur between all atoms and molecules (non-polar energy). This study suggests that CaTMP treatment rendered the enamel surface hydrophilic based on $\theta_w < 65°$ and $\gamma_s^- > 28.5$ mN/m criteria.

Considering that calcium and phosphate have the potential to form ionic species with different charges and activities, enamel reactivity depends on the electrical charge created on the enamel surface. In this context, these elements are strongly associated with CaTMP (Table 3), exhibiting higher total calcium and phosphorus levels compared to NaTMP. With the enamel surface presenting more electron-donor sites after CaTMP treatment, the reactivity with Ca^{2+} is enhanced, leading to increased adsorption of PO_4^{3-} and Ca^{2+} and resulting in lower γ_s^{AB} values and a reduction in the γ_s^- parameter (Fig. 4). Nucleation of Ca^{2+} and PO_4^{3-} occurs within the adsorbed CaTMP layer, resulting in oversaturation of these ions near the enamel surface. Furthermore, calcium and phosphate ions are retained within the CaTMP layer rather than being deposited directly onto the enamel surface, keeping the pores open and facilitating ion diffusion into the enamel (Nalin et al., 2021). Consequently, cationic acid-resistant proteins from saliva could explain the lower hardness loss following erosion challenges. The findings of this study offer new insights into elucidating the mechanism of CaTMP in reducing erosion and enhancing

enamel protection (de Toledo et al., 2022), thereby supporting the hypotheses of previous findings. The study clearly demonstrates the impact of CaTMP concentration on tissue loss, emphasizing the importance of considering this factor in the development of oral care products.

It's crucial to note that human saliva was utilized in this study to better mimic clinical conditions, as it has been proposed that the presence of the acquired salivary pellicle can mitigate enamel loss and softening. Saliva's diluting action, buffering capacity, and supersaturation with respect to tooth mineral content contribute to this protective effect (Buzalaf et al., 2012). This early erosion experimental model provides valuable insights into the action of newly synthesized compounds on the enamel surface following exposure to acid challenges. Surface hardness is well-suited for analyzing acid challenges in enamel without quantifying substance loss (Stenhagen et al., 2010), allowing determination of enamel softening and susceptibility to repair and substance loss as the erosive process progresses. Absolutely, it's important to interpret the results of this study with caution. Further studies are warranted, particularly long-term experiments, to evaluate the product deposited on the surface and its contribution to the protective effect against erosion. Additionally, it's important to acknowledge potential limitations of the results, such as the absence of the abrasive effect of brushing and the use of bovine enamel, which is known to be more susceptible to acid attacks. In addition, *in vivo* or *in situ* studies would provide additional insights into this issue.

5. Conclusion

Under the conditions of this *in vitro* methodology, it was concluded that toothpastes containing CaTMP demonstrated superior protective effects against initial enamel dental erosion when compared to the 1100 ppm F toothpaste. Additionally, they promoted an increase in electron-donor sites on the enamel surface, resulting in a more hydrophilic surface.

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Data availability Data: will be made available on request.

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Calcium Trimetaphosphate-Loaded Electrospun Poly(Ester Urea) Nanofibers for Periodontal Tissue Engineering

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Calcium Trimetaphosphate-Loaded Electrospun Poly(Ester Urea) Nanofibers for Periodontal Tissue Engineering

Abstract

The objective of this research was to create and appraise biodegradable polymer-based nanofibers containing distinct concentrations of calcium trimetaphosphate (Ca-TMP) for periodontal tissue engineering. Poly(ester urea) (PEU) (5% w/v) solutions containing Ca-TMP (15%, 30%, 45% w/w) were electrospun into fibrous scaffolds. The fibers were evaluated using SEM, EDS, TGA, FTIR, XRD, and mechanical tests. Degradation rate, swelling ratio, and calcium release were also evaluated. Cell/Ca-TMP and cell/scaffold interaction were assessed using stem cells from human exfoliated deciduous teeth (SHEDs) for cell viability, adhesion, and alkaline phosphatase (ALP) activity. Analysis of variance (ANOVA) and post-hoc tests were used ($\alpha = 0.05$). The PEU and PEU/Ca-TMP-based membranes presented fiber diameters at 469 nm and 414–672 nm, respective-ly. Chemical characterization attested to the Ca-TMP incorporation into the fibers. Adding Ca-TMP led to higher degradation stability and lower dimensional variation than the pure PEU fibers; however, similar mechanical characteristics were observed. Minimal calcium was released after 21 days of incubation in a lipase-enriched solution. Ca-TMP extracts enhanced cell viability and ALP activity, although no differences were found between the scaffold groups. Overall, Ca-TMP was effectively incorporated into the PEU fibers without compromising the morphological properties but did not promote significant cell function.

Keywords: nanofibers; tissue engineering; calcium; phosphates; poly(ester urea)

2. 1. Introduction

Periodontal disease, a multifactorial inflammatory disorder affecting the supporting tissues of the teeth, poses a significant health burden worldwide. Over half of the global population is estimated to suffer from some form of periodontal disease, making it one of the most prevalent oral health conditions. This chronic condition leads to tooth loss and has profound implications for overall systemic health [1,2]. Conventional treatment modalities such as scaling, root planing, and surgical interventions have shown limited success in achieving complete regeneration of periodontal tissues. In recent years, tissue engineering has emerged as a promising approach to address the limitations of conventional therapies by leveraging the principles of stem cell biology, biomaterial science, and molecular biology [3].

Numerous techniques have emerged to regenerate compromised periodontal tissues, especially using membranes as scaffolds [4]. For clinical applications, an optimal membrane should fulfill specific criteria. These criteria encompass biocompatibility and biodegradability, as well as providing a suitable surface and a porous three-dimensional framework that facilitates cell migration, attachment, infiltration, proliferation, and differentiation. Additionally, the membrane should possess sufficient mechanical strength to support tissue regeneration by maintaining the space [4]. This way, various biomaterials can be chosen to manufacture barrier membranes, including synthetic and natural materials. Along with selecting the right biomaterial, it is crucial to use appropriate manufacturing methods when developing a membrane [5]. Electrospinning is a versatile and user-friendly technique. It is widely used to produce nanofibrous scaffolds with a high surface area-to-volume ratio, facilitating cell attachment and proliferation [6]. They can also be made with or without incorporating therapeutic agents for treating infections, controlling inflammation, modulating cell differentiation, and promoting tissue regeneration [6,7].

PEU, which stands for poly(ester urea), is a promising polymer in the biomedical field due to its biodegradability, biocompatibility, and tunability. These materials have found extensive application in tissue engineering and various medical fields. Notably, their versatility makes them particularly suitable for repairing periodontal defects, as they exhibit excellent compatibility with soft and hard tissues [8–11]. PEU is a copolymer comprising three building blocks: an aliphatic diol or diamine, a diisocyanate, and a dicarboxylic acid or its anhydride. Combining these building blocks forms ester and urea linkages in the polymer backbone. By varying its

composition, the mechanical properties of PEU can be tuned broadly. For example, using longer or shorter chain diols or diamines can change the flexibility and stiffness of the polymer [12]. Similarly, using different amino acids can also influence the mechanical properties of the polymer [13]. Additionally, by adjusting the length of the diol or the amino acid's hydrophilicity, the degradation rate can be easily controlled [12].

The addition of molecules as antibiotics and cations (Sr^{2+}, Mg^{2+}, Si^{4+}, and/or Zn^{2+}) to improve scaffolds' biological or antimicrobial properties has been widely tested [14,15]. Due to their remarkable resemblance to the mineral components of bone and their exceptional osteoconductive and osteoinductive properties, calcium phosphates (CaP) have been widely employed in bone tissue engineering applications [16–19]. The crucial role of low calcium (Ca^{2+}) ion concentrations in supporting cell proliferation, migration, and differentiation has been reported [17–21]. Meanwhile, sodium trimetaphosphate (STMP, $Na_3P_3O_9$) is a cyclic polyphosphate working as an effective crosslinker, not showing toxicity at any concentration, and has been used in the food industry since being FDA-approved for humans [20]. Studies investigating the potential of STMP found that it provides nucleation sites for calcium phosphate apatite precipitation [21] since it leads to a more anionic surface [22,23]. When added to a polyamide-based scaffold, STMP produced higher elastic modulus, elongation at break, and tensile strength, in addition to generating a hydrophilic surface that facilitates cell adhesion, which is essential for bone repair [24].

Based on the ability of STMP to improve the biological and mechanical properties of polymers and the effects of calcium on bone mineralization, the replacement of sodium ions by calcium ions may generate a compound that combines the properties of both (TMP and calcium) on mineralization processes. Thus, to determine the effect of sodium replacement for calcium in the STMP on cell adhesion, proliferation, and differentiation, the present study incorporates different concentrations of Ca-TMP into PEU nanofibrous scaffolds and investigates the mechanical and biological properties of those scaffolds for periodontal tissue engineering.

2. 2. Materials and Methods

2. 2. 1. Materials

We obtained 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) from Sigma-Aldrich in St. Louis, MO, USA. The calcium trimetaphosphate (Ca-TMP) used had a molecular weight of 594.07 and a particle size of 236 ± 50 nm. It was synthesized in the Laboratory of Pediatric Dentistry of the Department of Preventive and Restorative Dentistry at São Paulo State University (FOA/UNESP). The alpha-minimum essential medium (α-MEM), fetal bovine serum (FBS), and penicillin/streptomycin were procured from Gibco in Grand Island, NY, USA. Invitrogen in Waltham, MA, USA, provided AlamarBlue® (DAL1100), ActinRed™ 555, and ActinGreen™ 488. Additionally, we used the SensoLyte p-nitrophenyl phosphate (pNPP) ALP kit from AnaSpec, Inc. in Fremont, CA, USA.

2. 2. 2. PEU (Poly Ester Urea) Synthesis

PEU (poly ester urea) 50% PHE6 P(1-VAL-10) or 50% P6V10: M(1-PHE-6) (50.0 g, 0.07 mol, 0.5 eq.), M(1-VAL-10) (47.4 g, 0.07 mol, 0.5 eq.), sodium carbonate (43.4 g, 0.41 mol, 3.1 eq), triphosgene (15.7 g, 0.05 mol, 0.4 eq.) (Mn = 69.9 kDa, Mw = 134.4 kDa, ĐM = 1.9, Tg = 38.4 °C, Td = 235.2 °C) was produced via an interfacial step growth polymerization as described previously [9,25]. In summary, an interfacial polymerization process was carried out by combining chloroform and water. In a 2 L reactor equipped with an overhead mechanical stirrer, the desired molar equivalents (1 eq., in total) of di-ptoluenesulfonic acid monomer salts and sodium carbonate (3.15 eq.) were introduced. Around 500 mL of hot distilled water (100 °C) was added to the flask, and the solution was stirred at a rate of 200 RPM until complete dissolution. Subsequently, the flask was cooled in an ice bath to achieve a temperature of 10 °C. Once cooled, triphosgene (0.43 eq.) was dissolved in 500 mL of chloroform and added to the solution. Immediate whitening of the solution occurred upon adding the organic solution, which was stirred for 48 h at 400 RPM. The resulting solution was transferred to a separatory funnel and washed with 500 mL of deionized water. The chloroform layer was precipitated dropwise into 4 L of hot water. In the case where residual salt peaks were detected in the H-NMR analysis, the crude product was dissolved in a volume of acetone measuring 2 L and subsequently precipitated into hot water with a volume of 4 L. This process was carried out to effectively eliminate any remaining salts present. 1H NMR (500 MHz, 303 K, DMSO-d6, from X = 6 from valine monomer component, Y = 2 from phenylalanine monomer component) δ = 0.81–0.85 (m, 12H, -$CH(CH_3)_2$), 1.16–1.19 (b, 2YH, -COO $CH_2CH_2(CH_2)YCH_2CH_2OOC$-), 1.18–1.26 (br,

2XH, -$COOCH_2CH_2(CH_2)X$ CH_2CH_2OOC-) 1.43–1.44 (b, 4H,-$COOCH_2CH_2(CH_2)YCH_2CH_2OOC$-), 1.53–1.54 (br, 4H, -$COOCH_2CH_2$ $(CH_2)XCH_2CH_2OOC$-), 1.95–1.98 (m, 2H, -$CH(CH_3)_2$), 2.50 (s, DMSO), 2.90–2.92 (m, 2H, -$NHCH(CH_2Ph)COO$-), 3.29–3.33(H_2O), 3.95 (t, 2H, -$COOCH_2CH_2(CH_2)YCH_2CH_2OOC$-),4.03–4.06 (m, 6H, -$CHCOOCH_2(CH_2)XCH_2OOCCH$-) 4.36–4.39 (m, 2H, -$NHCH(CH_2Ph)COO$-), 6.36–6.38 (d, 2H, -NH-), 6.48–6.52 (d, 2H, -NH-), 7.13–7.25 (m, 10H, -C_6H_5) ppm (Figure S1).

2. 2. 3. Cell/Ca-TMP Interaction

Previously isolated and characterized SHEDs were generously donated by Dr. Jacques Nör (University of Michigan, School of Dentistry, Ann Arbor, MI, USA) and were used to assess cell function in response to Ca-TMP powder. SHEDs were cultured in an α-MEM. The medium was supplemented with 15% FBS and 1% penicillin/streptomycin. SHEDs at passages 4–6 were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and utilized for the experimental procedures. To determine a non-cytotoxic concentration to be incorporated into the PEU fibers, solutions containing different concentrations of Ca-TMP (10, 25, 50, 100, 150, and 200 μM) were placed in contact with SHEDs for 1, 3, and 5 days. The cells were seeded in a density of 1×10^4 cells/well in 96-well plates and incubated at 37 °C and 5% CO_2 for 24 h to the initial adhesion. After that, the Ca-TMP-containing media was added, and this media was refreshed every two days. Cell viability was assessed at each time point using 10% alamarBlue®. After 3 h of incubation, the fluorescence was measured at 560 nm excitation and 590 nm emission (Spectra Max Id3). The fluorescence values were transformed in percentages using the average of the negative control group (α-MEM without FBS) as 100%. For the qualitative analysis of cell adhesion and spread, cells were fixed using 4% paraformaldehyde, and the actin filaments were stained with ActinRed™ 555.

2. 2. 4. Fabrication of PEU and PEU Matrices Loaded with Ca-TMP

To prepare four different polymer solutions, PEU was dissolved in hexafluoropropylene (HFP) at a concentration of 5% (w/v). These solutions were stirred overnight. Subsequently, the PEU solutions were loaded with different concentrations (0, 15, 30, 45 wt%, relative to the total polymer weight) of Ca-TMP

particles. To ensure that the particles were evenly spread within the polymer solution, the mixtures were stirred for 24 h and sonicated for 30 min. Next, the solutions were individually loaded into plastic syringes (Becton, Dickson and Company, Franklin Lakes, NJ, USA) coupled with 27 G metallic blunt-tip needles (CML Supply, Lexington, KY, USA). The process of electrospinning was carried out at room temperature with specific parameters. These included a fixed spinning distance of 20 cm, a rotating collector (120 RPM) with a diameter of 2.5 cm and a peripheral speed of 0.15 m/s, a flow rate of 1.5 mL/h, and electric voltages of 16 kV. The approximate time of the electrospinning session was 2.6 h for each group. The electrospinning system consisted of a high-voltage source (ES50P-10W/DAM, Gamma High Voltage Research Inc., Ormond Beach, FL, USA), a syringe pump (Legato 200, KD Scientific Inc., Holliston, MA, USA), and a grounded stainless steel collecting drum connected to a high-speed mechanical stirrer (BDC6015, Caframo Limited, Georgian Bluffs, ON, Canada). After the electrospinning process, the mats produced, known as matrices, underwent a minimum of 48 h of vacuum drying to eliminate any remaining solvent [26,27].

2. 2. 5. Characterization of the Morphology and Composition of the Electrospun Fibers

The morphology and general structure of the electrospun fibers were assessed using a field-emission scanning electron microscope (TESCAN MIRA3, Kohoutovice, Czech Republic). Samples (n = 3) from each group were affixed onto an aluminum stub, coated with a thin layer of gold–palladium through sputter-coating, and imaged at an acceleration voltage of 5 kV. Fiber diameter distribution was subsequently determined using Image J software (version 1.53, National Institutes of Health, Bethesda, MD, USA). Measurements were conducted on 50 individual fibers per image acquired (three images per group) at a consistent magnification of 5000×. The percentage of porosity of the scaffolds was calculated from three images of each scaffold at a 5000× magnification using the same software. In addition, energy dispersive X-ray spectroscopy (EDS) analysis was carried out under SEM (n = 3) to obtain semi-quantitative data regarding the chemical composition of the fibers.

The chemical properties and Ca-TMP incorporation in all matrices were assessed (n = 2) using Fourier-transform infrared spectroscopy (FTIR) in attenuated total reflection mode (Nicolet iS50, Thermo Fisher Scientific, Waltham, MA, USA). The

FTIR measurements were performed over a range of 700–4000 cm^{-1} at a resolution of 4 cm^{-1}, with 64 scans conducted in random regions. X-ray diffraction (XRD) was used to analyze the crystalline phase composition (n = 2) using a Rigaku Ultima IV diffractometer (Rigaku Americas Corporation, Woodlands, TX, USA) in the Bragg–Brentano geometry. Cu Kα radiation with a wavelength (λ) of 1.54 Å was utilized. Scans were conducted in the 2θ range from 5° to 45°, with a step size of 0.05° and a scanning speed of 1°/min. Rigaku's data analysis software (PDXL Version 2.6.1.2) and the Inorganic Crystal Structure Database (ICSD) were employed for phase identification. Thermogravimetric analysis (TGA) of the fibers was performed (n = 2) using a simultaneous thermal analyzer (SDT Q600, TA Instruments, Dallas, TA, USA). The heating rate was set at 10 °C/min. Three fibers from each group were subjected to a step-by-step program under a pure nitrogen (N2) environment. This program involved holding the temperature at 50 °C for 1 min, heating it from 50 to 650 °C at a rate of 10 °C/min, and finally holding it at 650 °C for 2 min. Data were recorded after the final step.

2. 2. 6. Contact Angle

Fibers produced by electrospinning from pure PEU and Ca-TMP-loaded solutions were then affixed onto microscope glass coverslips (n = 4) sourced from Fisherbrand (Fisher Scientific UK Ltd., Loughborough, UK). A goniometer (M200 Rame-Hart Goniometer, Succasunna, NJ, USA) was employed to determine the surface contact angle of the fibrous matrices. Three consecutive drops of distilled water, each approximately 1 μL in volume, were placed on each sample. The contact angles of the drops were measured and subsequently averaged to obtain a representative value.

2. 2. 7. Mechanical Properties, Swelling, and Degradation Profile

The mechanical performance (e.g., elongation at break, tensile strength, Young's modulus, yield stress, and yield strain) of all the matrices was evaluated using uniaxial tensile testing on an eXpert 5601 machine from ADMET (Norwood, MA, USA) using a load cell from the same company (SM-250-961—250 lbf) of 1 kN. Rectangular samples with dimensions of 25 × 3 mm^2 were utilized for the tests, with a gauge length of 19 mm and eight samples per group examined. The testing was conducted in two

ways: dry, which means the samples were tested immediately without any storage, and wet, which means the samples were stored in a PBS solution for 24 h before being tested. The crosshead speed during testing was set at 1 mm/min. To determine the thickness of each sample, a digital caliper (Mitutoyo Corp., Kanagawa, Japan) was used to take measurements at three separate locations. We collected mechanical data from the stress-strain curves of each sample and presented the results as the average value along with the standard deviation (SD).

2. 2. 8. Swelling and Degradation Profile

To determine the water retention capability of the membranes, the fibers (1 cm × 1 cm; n = 4/group) were incubated in PBS at 37 °C for 24 h. The wet and dry weights of the specimens were determined before and after incubation at 37 °C. The changes in the fiber's weight between the wet (Ww) and dry (Wd) states were used to determine the volumetric swelling through the following equation:

$$\text{Swelling ratio} = ((Ww - Wd))/Wd \times 100$$

The in vitro degradation of the manufactured fibers was assessed by immersing 10 × 10 mm electrospun fibers (n = 4) in solutions containing PBS and lipase (150 U/L) at 37 °C while continuously monitoring changes in their weight over a period of time (91 days) [28]. At predetermined time intervals, the samples were taken out of the incubation medium, lightly dried using low-lint wipes manufactured by Kimberly Clark Corporation (Kimwipes), rinsed twice with deionized water, and vacuum-dried for 24 h before recording their weights. The degradation profile was determined using the following formula: Wt represents the remaining weight at a given time, and W0 denotes the initial dried weight.

$$\text{Degradation ratio (\%)} = Wt/W0 \times 100$$

2. 2. 9. Calcium Release Assay

Calcium release from particles was studied in duplicates by immersing 1 cm^2 of the membranes from PEU + 45% Ca-TMP and pure PEU groups (n = 3) in 1 mL of PBS and lipase at 37°C. During the 21-day period, we used the supernatant to measure calcium levels using the calcium colorimetric assay kit (MAK022, Sigma

Aldrich) according to the manufacturer's instructions. This kit detects the chromogenic complex formed between calcium ions and o-Cresolphthalein to determine the concentration of calcium ions. The absorbance, which is directly proportional to the concentration of calcium ions present in the sample of this complex, was read at 575 nm on a plate reader (Spectra Max Id3, Molecular Devices LLC, San Jose, CA, USA), and it was calculated from a standard calcium solution prepared in parallel. The values obtained by reading the PEU group were used as background and subtracted from the PEU + 45% Ca-TMP group to obtain the actual reading.

2. 2.10. Cell/Scaffold Interaction

SHEDs were also used to assess cell function in response to the formulated PEU/Ca-TMP fibers using the same cell culture conditions described in 2.2. To evaluate the cell response in contact with PEU/Ca-TMP fibers, all electrospun matrices were cut into 15 × 15 mm^2 samples and disinfected in a laminar flow hood by UV light (1 h on each side), then adapted into 24-well plates with metal rings. SHEDs were seeded at a density of 30,000 cells/matrix and incubated for 1 h to allow initial cell adhesion on the samples. As mentioned, cell viability was assessed using alamarBlue® after 1, 3, and 7 days. Fibers of PEU without Ca-TMP on day 1 were used as the control [29,30]. As previously mentioned, the adhesion and spreading of SHEDs on scaffold surfaces were assessed at 1, 3, and 7 days with ActinGreen™ 488. Matrix surfaces (n = 4) were analyzed under direct fluorescence microscopy (Echo Revolve, BICO Company, San Diego, CA, USA) at a 10 × magnification.

2. 2. 11. Alkaline Phosphatase (ALP) Activity

To induce osteoblastic differentiation in SHEDs treated with 50 μM of Ca-TMP (Section 2.3) and cells seeded on the fibers (Section 2.10), two types of media were employed: osteogenic differentiation medium (OM) and basal medium. OM consisted of alpha-MEM media supplemented with 100 nM dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbate phosphate, and 10% FBS. ALP (alkaline phosphatase) activity measurements were obtained by collecting cell lysates at 7 and 14 days for cells seeded on membranes and at 14 days only for cells/Ca-TMP interaction. The SensoLyte pNPP ALP kit was utilized according to the manufacturer's instructions for ALP activity detection. Briefly, the cells were suspended in 500 μL of Triton X-100, and the resulting cell homogenates were centrifuged at 2500× g for 10

min at 4 °C. The supernatants obtained were used in the ALP assay, where pNPP served as the phosphatase substrate, and the kit-provided ALP acted as the standard. Controls consisted of media or PEU fibers lacking Ca-TMP. Absorbance at 405 nm was measured using a microplate reader (SpectraMax iD3) to quantify the ALP activity.

2. 2. 12. Statistical Analysis

Data were submitted to normality (Shapiro–Wilk) and homogeneity of variance (Levene) tests. Cell viability, fiber diameter, contact angle, and mechanical test data were analyzed using one-way ANOVA followed by Tukey or Games–Howell post-hoc. Cell viability on scaffolds and ALP activity data were analyzed using two-way ANOVA followed by Sidak post-hoc test. Fluorescence images were analyzed qualitatively. All the experiments were performed, on at least two different occasions, to verify their reproducibility. Statistical inferences were based on a 5% significance level.

2. 3. Results

2. 3.1. Cell/Ca-TMP Interaction

To determine the cytocompatibility of pure Ca-TMP, we first evaluated the viability of several increasing concentrations on SHEDs cells before including it in membrane manufacturing. Initially, all concentrations decreased cell viability in a dose-dependent way for 50 μM to 200 μM amounts. However, cell viability increased from day 1 to day 3 for all tested concentrations, with 50 μM showing the significantly highest viability. From day 3 to day 5, there was a minor decrease in cell viability for all groups, but the 50 μM kept showing as the best concentration (Figure 1A). Based on this, we used this amount of Ca-TMP to evaluate the alkaline phosphatase activity. The results of ALP activity are shown in Figure 1B. After 14 days of culture, the Ca-TMP applied to the osteogenic media promoted a significantly higher activity when compared to osteogenic media only. The qualitative cell adhesion and spread staining results are consistent with the quantitative findings, indicating no hindrance in cell spreading caused by the concentration of Ca-TMP (Figure 1C). To determine the particle size, a SEM analysis was performed. As depicted in Figure S1B, the particles ranged from 171 nm to 320 nm with an average size of 236 ± 50 nm.

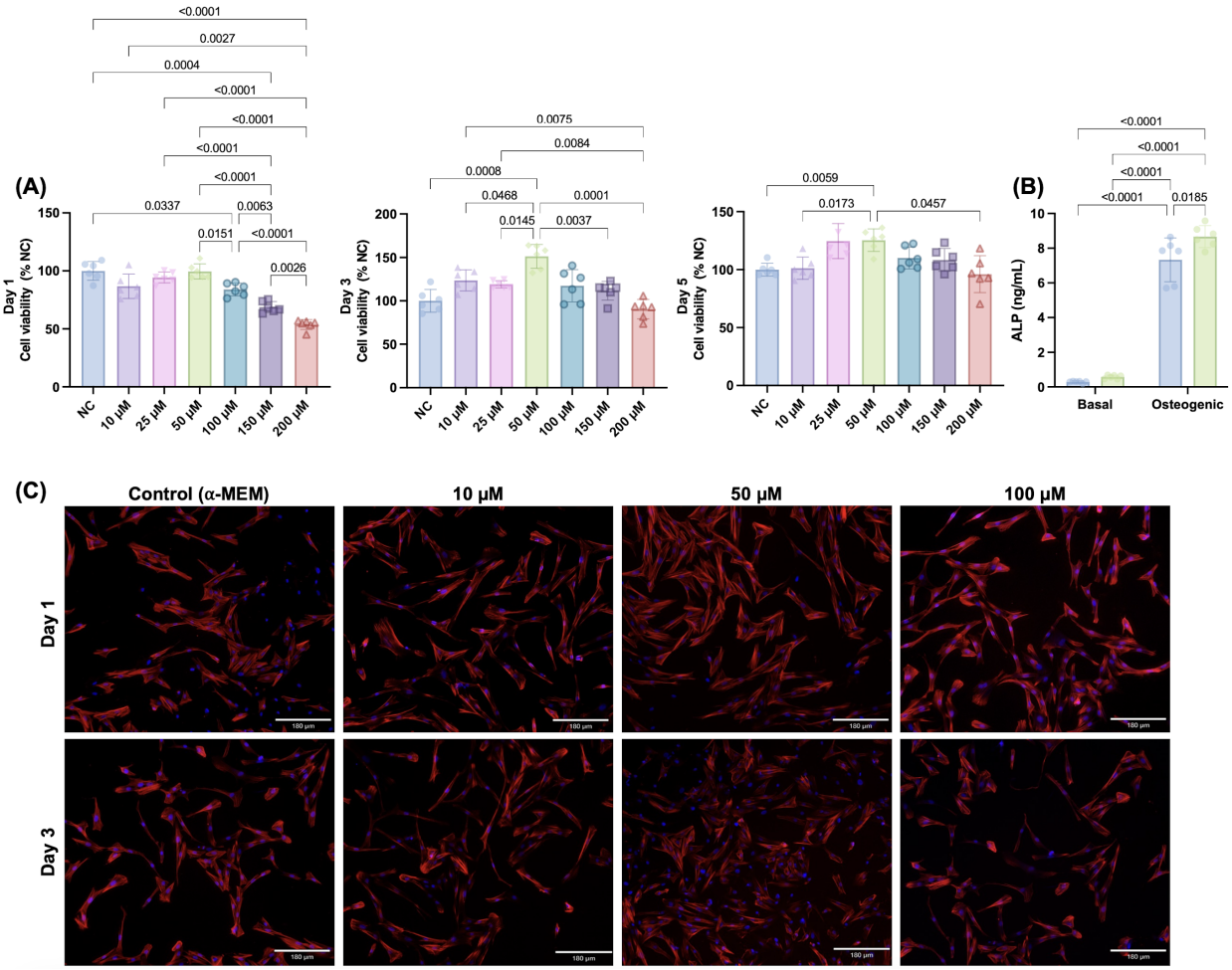

Figure 1. Initial screening for Ca-TMP (10, 25, 50, 100, 150, and 200 µM) in contact with SHEDs. (A) Percentage of cell viability (AlamarBlue®) normalized by the negative control (NC) within each time point. Welch's ANOVA/Games–Howell; α = 5%. (B) Alkaline phosphatase (ALP) activity of SHEDs cells after 14 days. Data presented as mean ± SD. Two-way ANOVA/Tukey; α = 5%. (C) Representative fluorescence images of SHEDs cells at days 1 and 3 stained by F-actin (ActinRed™ 555).

2. 3. 2. Morphological and Chemical Characterizations of the Fibers

The addition of Ca-TMP produced thicker fibers than pure PEU, except for the PEU + 30% Ca-TMP group, which presented a thinner fiber diameter. Also, while the pure fibers were visually smooth (Figure 2A), the SEM revealed Ca-TMP nanoparticle precipitates present in the fibers, differing in shape and amount, within a nano-sized scale, which was confirmed by the EDS analysis (Figure 2B–D). The PEU and PEU/Ca-TMP-based membranes presented fiber diameters at 469 nm and 414–672 nm, respectively (Figure 2E). There were no significant differences ($p > 0.05$) in the

percentage of porosity regardless of the presence and concentration of Ca-TMP (Figure 2F). Lastly, the FTIR analysis also revealed Ca-TMP incorporation into the membranes and PEU into the PEU-based membranes (Figure 3A). The broad stretch at 3350–3500 cm^{-1} is for the N-H (urea), and the stretch at 1735 cm^{-1} is characteristic of the C=O (ester) bond stretching and confirms the presence of an ester bond. The stretch at 1650–1690 cm^{-1} is typical of the C=O (urea) bond stretching from the urea and confirms the presence of the urea bond. The stretch around 1000–1300 cm^{-1} represents the C-O (ester) bond and the C-H (alkyl) bending. All these characteristic signals confirm that we had successfully synthesized the poly(PHE6XX-VAL-10XX) PEU [31].

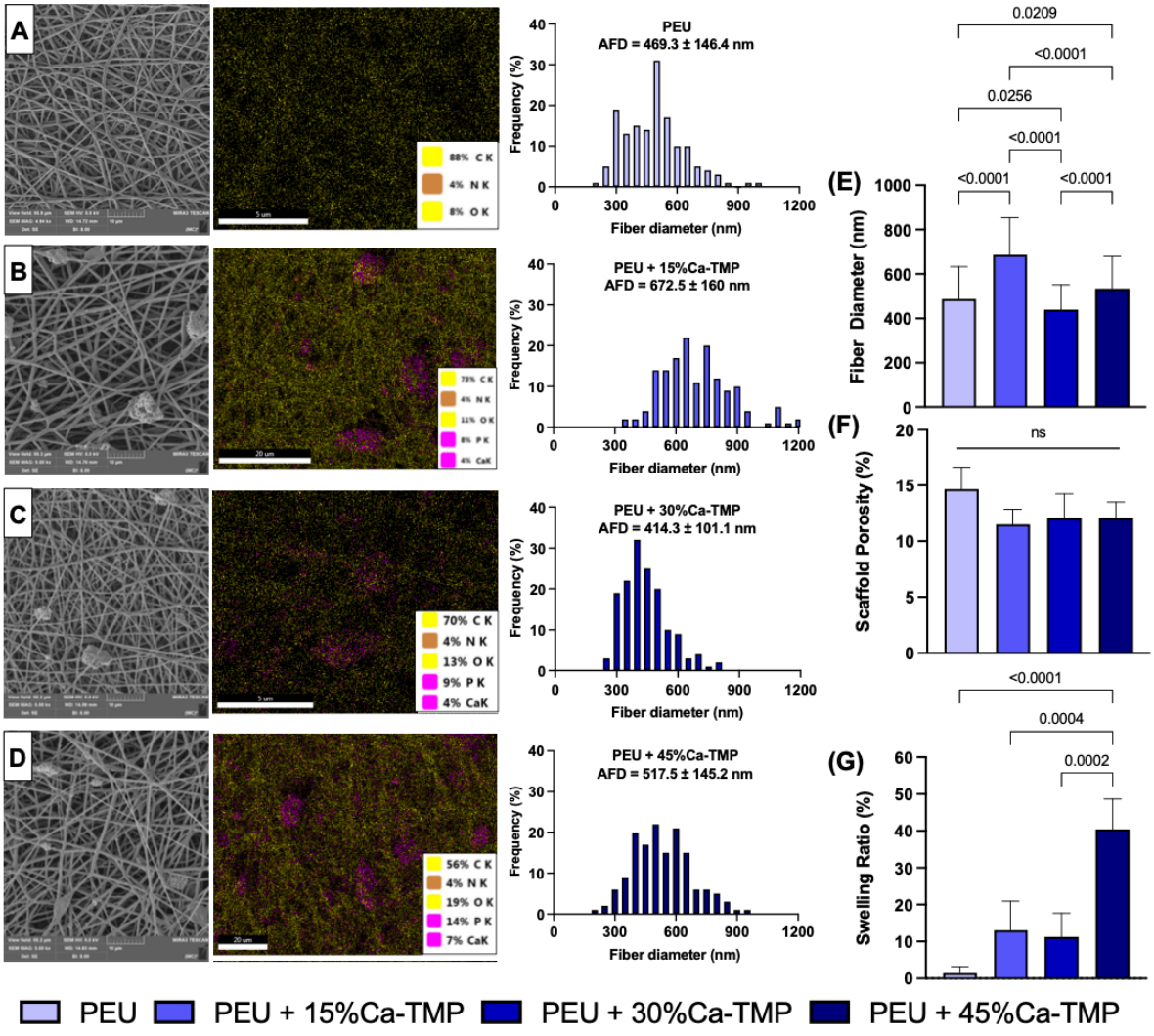

Figure 2. Evaluation of chemical–morphological membranes. (A–D) Scanning electron micro-scope (SEM) images, energy dispersive X-ray spectroscopy (EDS—

5000× magnification), and histogram of fiber diameter of PEU (control), 5% PEU + 15% Ca-TMP, 5% PEU + 30% Ca-TMP, and 5% PEU + 45% Ca-TMP, respectively. (E) Fiber diameter, (F) scaffold porosity, and (G) swelling ratio evaluations (mean ± SD, $p < 0.05$).

The X-ray diffraction pattern (Figure 3B) of Ca-TMP can be described as a reflection at 29.45 degrees with FWHM of 0.35 degrees sitting on an amorphous halo, which is the major portion of Ca-TMP that is amorphous with an estimated content of 93 wt%. The d-spacing of the crystalline phase is 3.03 angstrom based on Bragg's law, and the grain size is estimated to be around 23.5 nm from the peak width according to the Scherrer equation. The as-synthesized PEU shows an amorphous halo in the range from 15 to 37 degrees, which overlaps several diffractions, with the major ones at 19.6 and 23.9 degrees. The PEU-Ca-TMP profiles can be roughly viewed as the linear combination of PEU and Ca-TMP. The diffraction around 29.45 degrees is significantly weakened in these composites, suggesting the interaction between PEU and Ca-TMP disrupted the crystallization of Ca-TMP.

The thermal gravimetric analysis (TGA) of the nanometer fibers also evidenced the efficient incorporation of Ca-TMP, showing the thermal stability of the polymer up to 250 °C. Above that temperature, a similar weight-loss pattern was observed for all samples (Figure 3C). The degradation temperatures were detected around 250 °C, 260 °C, 285 °C, and 285 °C for PEU, PEU + 15% Ca-TMP, PEU + 30% Ca-TMP, and PEU + 45% Ca-TMP fibers, respectively. When PEU and Ca-TMP interact, it can alter the molecule's structure. This can lead to stronger connections between scaffold polymeric chains, resulting in improved thermal stability and higher degradation temperatures.

2. 3. 3. Contact Angle

Regardless of the Ca-TMP content, all PEU-based membranes had a hydrophobic surface with a contact angle greater than 90°, indicating low wettability ($p < 0.05$). The PEU/Ca-TMP membranes containing 15, 30, and 45% of Ca-TMP nanoparticles revealed a less hydrophobic surface than the pure PEU membrane, with just the 15% Ca-TMP one being statistically different ($p < 0.05$) (Figure 3D,E).

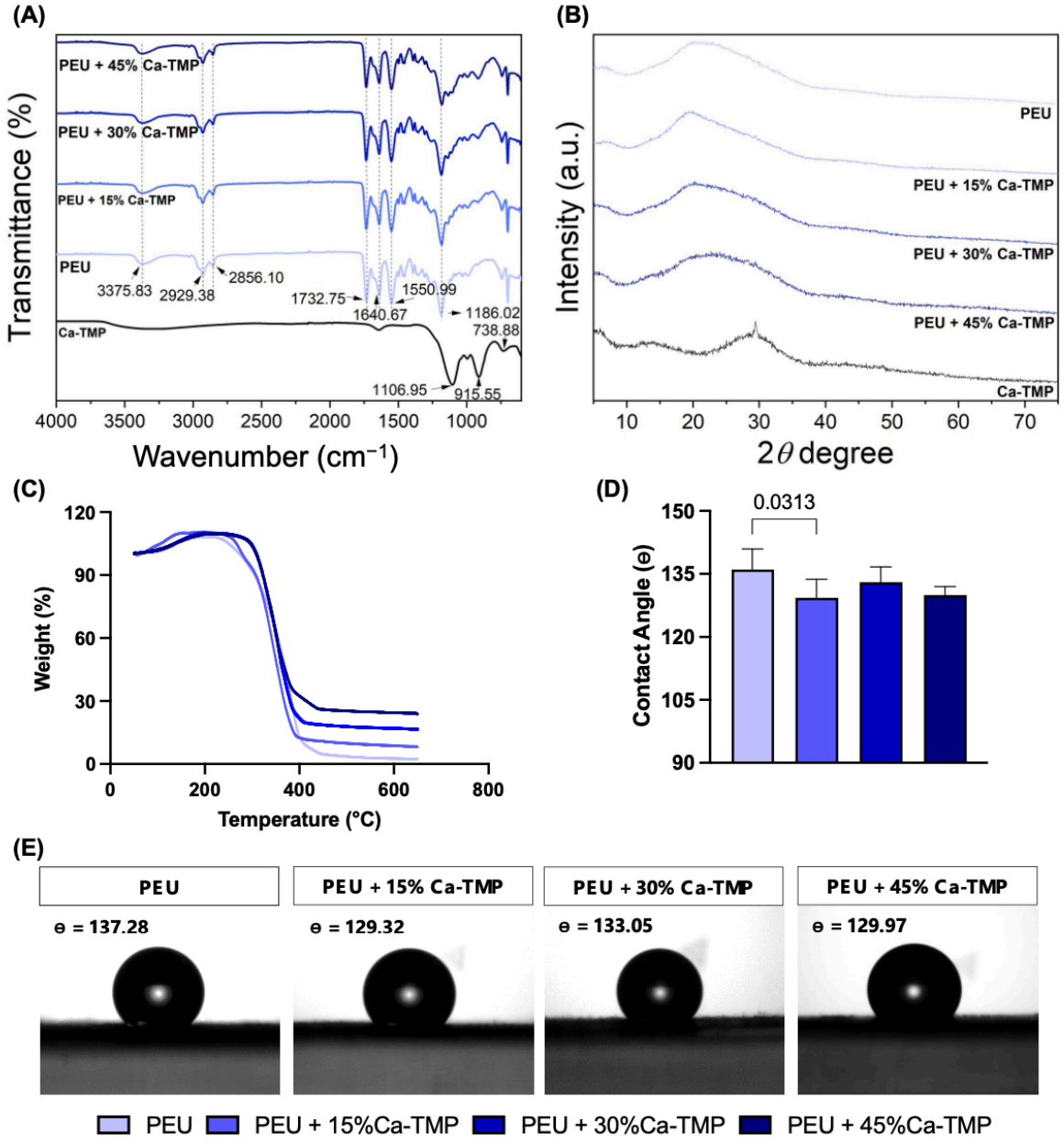

Figure 3. Evaluation of chemical–morphological membranes. (A) Fourier-transform infrared spectroscopy (FTIR) spectra, (B) X-ray diffraction (XRD), (C) thermogravimetric analysis (TGA) of electrospun fibers, and (D) contact angle data presented as mean ± SD, and (E) images formed be-tween water and the PEU- or PEU/Ca-TMP-based membranes containing different concentrations.

2. 3. 4. Mechanical Properties, Swelling, and Degradation Profile

The average specimen thickness for each group was as follows: PEU = 0.25 mm, PEU + 15%Ca-TMP = 0.27 mm, PEU + 30%Ca-TMP = 0.28 mm, and PEU +

45%Ca-TMP = 0.30 mm. The mechanical performance (e.g., elongation at break, tensile strength, Young's modulus, yield stress, and yield strain) of the fabricated nanofiber membranes in wet and dry conditions are presented in Figure 4. The PEU membranes showed a soft nature with moderate flexibility regardless of the storage condition. There were no notable differences in elongation at break between the fibers made from PEU, PEU + 15%Ca-TMP, and PEU + 45%Ca-TMP, in both dry and wet conditions ($p > 0.05$). However, the PEU + 30%Ca-TMP group displayed an increase in elongation compared to the PEU and PEU + 15%Ca-TMP groups in wet conditions and compared to the PEU and PEU + 45%Ca-TMP groups in dry conditions ($p < 0.05$). The groups observed a significant difference ($p < 0.05$) in the tensile strength between wet and dry conditions, increasing tensile strength in the wet state. In terms of Young's modulus, all the groups demonstrated an increase in modulus and a significant difference in the wet versus dry conditions ($p < 0.05$), except for the comparison between the PEU + 45%Ca-TMP group in the wet state and the PEU + 15%Ca-TMP group in the dry state ($p > 0.05$).

The strain–stress curve showed that the yield stress was 1.05 ± 0.16 MPa for mats made of pure PEU in the wet condition. However, mats loaded with 15/30/45% Ca-TMP and PEU had higher yield stress values of 1.99 ± 0.46, 2.01 ± 0.38, and 2.06 ± 0.59 MPa, respectively. This indicates that adding Ca-TMP significantly increased the yield stress compared to the dry condition and even to PEU alone ($p < 0.05$). In the dry state, the yield stress of pure PEU was found to be 0.70 ± 0.05. For PEU mixed with 15%, 30%, and 45% Ca-TMP, the yield stress was 0.67 ± 0.21, 1.37 ± 0.27, and 0.69 ± 0.14 MPa, respectively. There was no significant difference between the groups, except for the PEU mixed with 30% Ca-TMP. The yield strain graph for PEU and PEU-loaded with 15/30/45% Ca-TMP electrospun mats showed that in wet conditions, the percentages were 13.79 ± 2.33, 17.38 ± 0.95, 19.43 ± 3.59, and 17.20 ± 2.66%, respectively. In dry conditions, the percentages were 32.82 ± 6.04, 27.40 ± 3.64, 22.77 ± 5.57, and 21.97 ± 4.98% for the PEU and PEU-loaded with 15/30/45% Ca-TMP, respectively. As a result, the Ca-TMP groups had a lower yield strain in wet conditions compared to dry conditions, with a significant difference in the PEU +15%Ca-TMP ($p < 0.05$).

Based on the swelling profile results, it was observed that the introduction of Ca-TMP particles had an impact on the swelling properties of the fibers. The swelling percentage increased for PEU + 15%Ca-TMP and PEU + 30%Ca-TMP compared to

pure PEU. Additionally, PEU + 45%Ca-TMP had the highest swelling ratio among all groups, which was statistically significant (Figure 2G). Regarding the degradation profile of the various PEU-based membranes, all groups demonstrated high stability through the degradation process (Figure 5A). Pure PEU presented ~96% and ~100% remaining mass by day 91, the PEU + 15% Ca-TMP fibers had about ~94% and ~95% of the remaining mass, followed by ~88% and ~92% for PEU + 30% Ca-TMP and ~85% and 87% for PEU + 45%Ca-TMP fibers, incubated in PBS and lipase, respectively. In this way, we demonstrated that adding Ca-TMP changed the degradation profile of the fibers in a concentration-dependent manner; however, it is important to mention that all groups maintained their stability from day 1 to day 91, with minimal variation in mass loss once they reached a plateau.

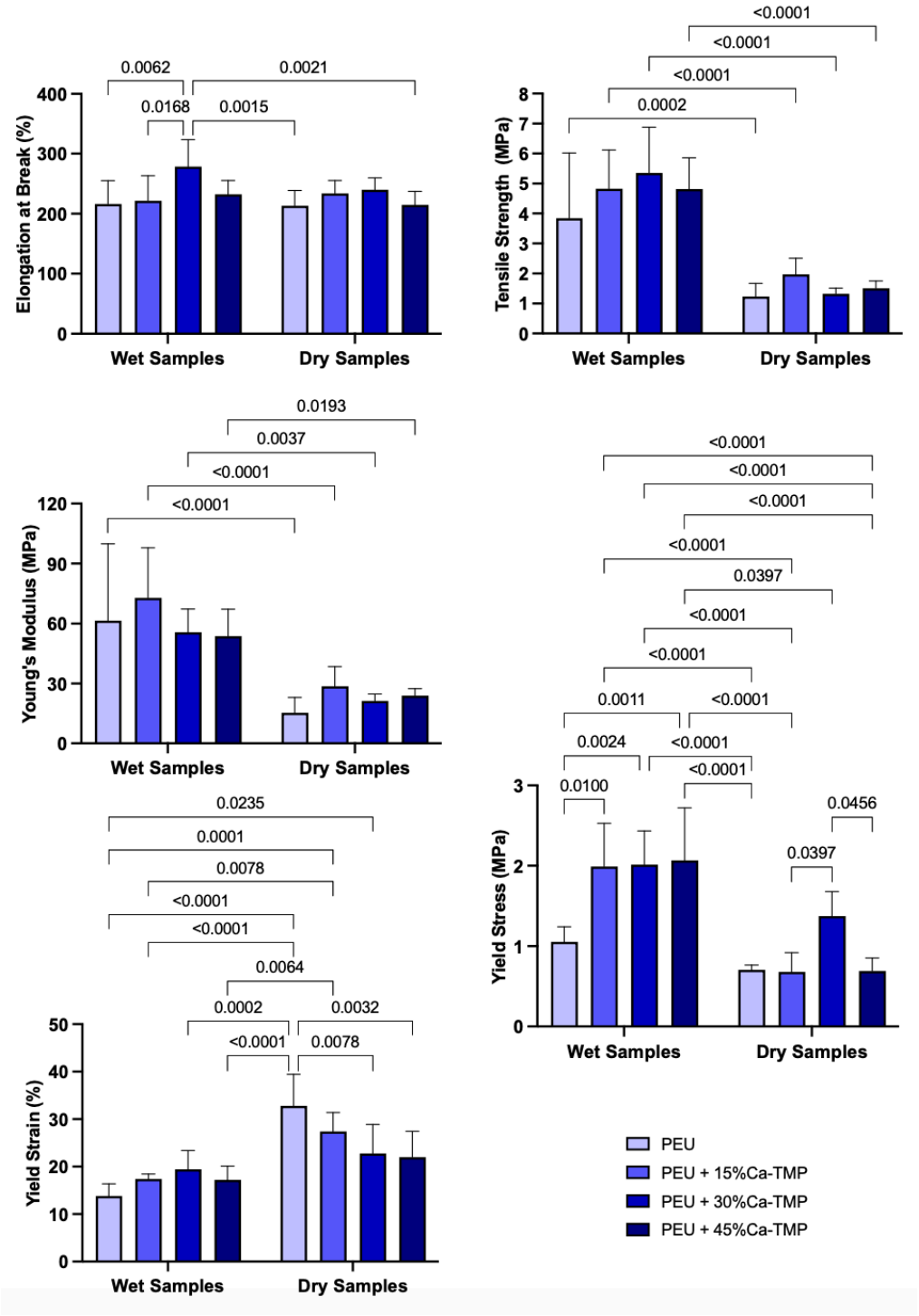

Figure 4. Mechanical characterization of membranes. Elongation at break (%), tensile strength (MPa), Young's modulus (MPa), yield stress (MPa), and yield strain (%) graphs of dry and wet PEU fibers loaded with Ca-TMP. Data presented as (mean ± SD). Two-way ANOVA/Tukey; α = 5%. Significant interaction between variables for yield stress (MPa) and yield strain (%), p-value = 0.0037 and 0.0004, respectively.

2. 3. 5. Calcium Release

The cumulative amount of calcium deposition was measured over 21 days and showed no calcium release for samples incubated in PBS. However, conditioning in lipase solution, the PEU + 45% Ca-TMP group showed a burst release of 0.793 ± 0.031 µg/µL after one day of incubation. For day 3, the values rose slightly to 0.821 ± 0.098 µg/µL, meaning that little new calcium was released, possibly due to the short time between readings (2 days). From day 7 to 21, the release of calcium increased in a higher fashion, reaching 0.882 ± 0.040 and 1.014 ± 0.184 µg/µL for days 7 and 14, respectively, peaking at day 21, with 1.069 ± 0.215 µg/µL (Figure 5B).

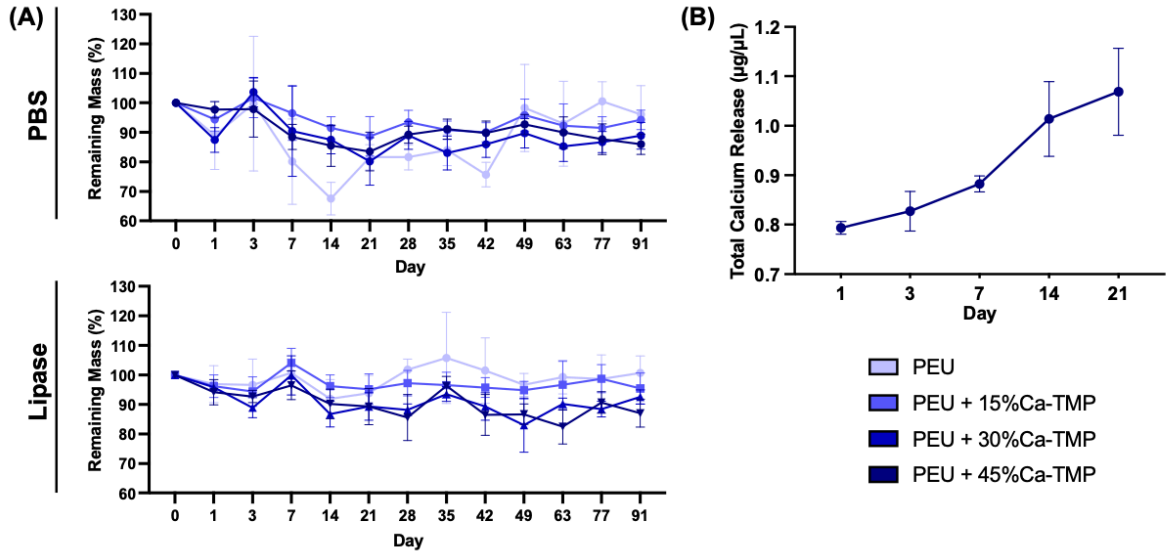

Figure 5. Membrane degradation and total calcium release quantification. (A) Degradation of electrospun membranes conditioned in PBS and lipase-enriched solutions over 91 days. Note the greater dimensional stability of the membranes even after 3 months of incubation. (B) Calcium ion release profile from the electrospun fibers immersed in a lipase-enriched solution derived over 21 days. Note the minimal calcium released from the fiber after 3 weeks of incubation. Data presented as (mean ± SD).

2. 3. 6. Cell/Scaffold Interaction

Throughout the time points, all scaffolds' formulations significantly increased intragroup cell viability (lowercase letters, $p < 0.0001$). However, when analyzing within the same time point, no differences were found regardless of the composition of the scaffold (uppercase letters, $p > 0.05$). Overall, all groups presented cell viability of ~100%, ~700%, and 1170% on days 1, 3, and 7, respectively (Figure 6A). All scaffolds containing Ca-TMP showed better cell adhesion when compared with pure

PEU fibers, with intensified cell spreading observed for Ca-TMP-incorporated fibers at 7 days (Figure 6C). At day 7, the pure PEU and PEU + 45% Ca-TMP fibers cultivated in basal (−0.09 ± 0.06 and 1.02 ± 0.62 ng/mL) and osteogenic media (2.15 ± 0.05 and 4.46 ± 1.73 ng/mL) were different when comparing over media (lowercase letters, $p < 0.05$), but similar values within the same treatment (uppercase letters, $p > 0.05$). In a late analysis (day 14), pure PEU and PEU + 45% Ca-TMP fibers cultivated in basal (7.65 ± 0.80 and 3.44 ± 0.34 ng/mL) and osteogenic media (11.00 ± 2.20 and 8.41 ± 1.64 ng/mL) PEU + 45% Ca-MP resulted in a significant decrease compared to the pure PEU group regardless of the median conditioned ($p < 0.0001$) (Figure 6B).

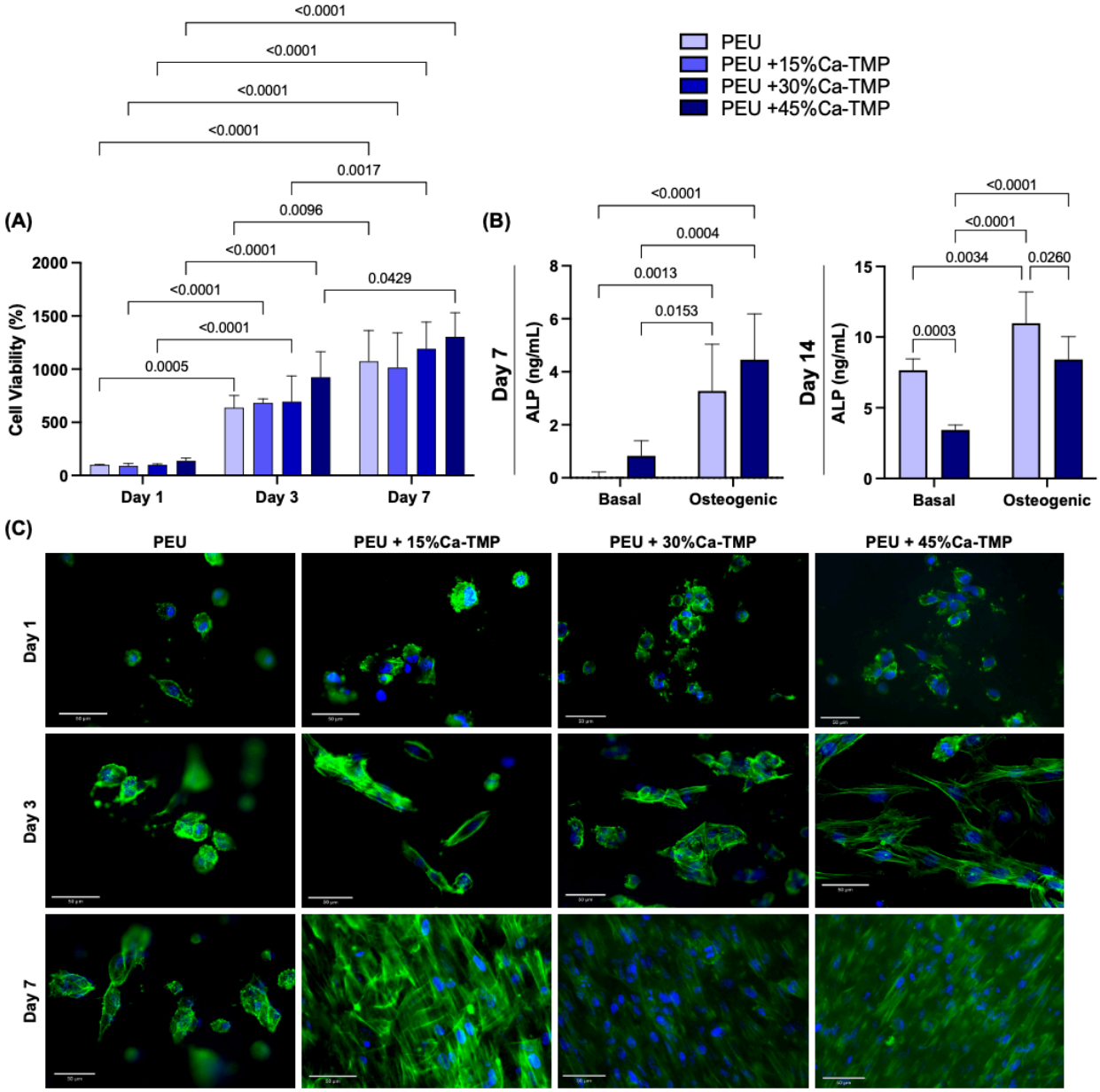

Figure 6. SHEDs cells and Ca-TMP electrospun membranes interaction. (A) Percentage of cell viability (AlamarBlue®) normalized by the PEU without Ca-TMP at day 1 value. Data presented as mean ± SD, $p < 0.05$. (B) Alkaline phosphatase (ALP) activity of SHEDs cells after 7 and 14 days. Repeated measures ANOVA/Sidak post-hoc; $\alpha = 5\%$. (C) Representative fluorescence images of SHED cells spread over the membranes at 1, 3, and 7 days stained by F-actin (ActinGreen™ 488).

2. 4. Discussion

In this study, we extensively investigated the morphology and chemical composition of PEU membranes loaded with Ca-TMP, a calcium phosphate compound that has been explored for its potential use in biomedical applications due to its biocompatibility and ability to promote mineralization. In our study, we conducted an initial concentration screening of Ca-TMP to evaluate its cytocompatibility and determine the optimal concentrations to be incorporated into membranes. Our experiments found that the cells reacted positively to the material in a controlled environment. We noticed that specific concentrations of Ca-TMP were especially beneficial for the growth and multiplication of cells. Based on these results, we decided to include three concentrations of Ca-TMP in the PEU-based membranes.

According to the SEM micrographs, the membranes showed notable differences in fiber morphology, influenced by the polymer system and the incorporated Ca-TMP content. Membranes from the pure PEU group had more homogeneous fibers, while membranes based on PEU/Ca-TMP showed fibers with a larger diameter than PEU. Therefore, increasing Ca-TMP concentration resulted in an increased diameter, except for the group containing 30% Ca-TMP. This may be due to viscosity changes leading to stronger chain entanglement leading to a greater fiber formation [32]. Furthermore, the SEM images showed that the percentage of porosity did not change with incorporating Ca-TMP into the PEU scaffold formulation. Overall, different morphological characteristics may play a role in structural properties, such as degradation and mechanical properties. These two factors are critical aspects when considering the modification of the PEU materials [26].

Ca-TMP integration was confirmed through visual observation using SEM and identification using EDS. FTIR and XRD were subsequently used to verify the successful integration. Together, these results confirmed the chemical characteristics of the membranes synthesized in the study. When creating scaffolds, it is crucial to

consider wettability. This can affect how cells attach, multiply, differentiate, and ultimately re-generate tissue once the scaffolds come into contact with moisture (i.e., body fluids present at the site of periodontal surgery). Many studies have shown that hydrophilic scaffolds have a higher affinity for cells, improving cell proliferation and wound healing [33,34]. According to our findings, all PEU-based membranes had a hydrophobic surface (i.e., contact angle greater than 90°), showing poor wettability [35]. However, when Ca-TMP was added to the PEU membrane, the contact angle was reduced, but not enough to transform the membrane from hydrophobic to hydrophilic, possibly due to the high hydrophobicity of sodium trimetaphosphate [22].

Despite the unfavorable hydrophobicity of the membranes, the scaffolds showed adequate morphology and chemical composition. For a membrane to be clinically useful, it needs to have satisfactory mechanical properties to prevent the collapse in periodontal defects as a result of compressive forces from soft tissues and mastication [6,36] and to resist cell adhesion and proliferation [6,36,37]. Considering these important characteristics, PEU was used due to its ability to be adjustable and controlled from the moment there is a change in the chemical composition of the compound. Our results showed that the mechanical properties of the PEU/Ca-TMP fibers were comparable to those of pure PEU in terms of elongation at break, except for the PEU +30%Ca-TMP. For the tensile strength and modulus of elasticity, all the groups exhibited high values in wet compared to dry conditions. Furthermore, in general, we observed significant differences in mechanical behavior between the dry and wet conditions for the scaffolds, even due to the high hydrophobicity of the material, which hindered the promotion of wettability on the surface of the membranes [6].

Although PEU is a material that offers great flexibility in modifying its amino acid chains to tune its mechanical properties, our work has shown that the PEU membranes we tested had a relatively low tensile strength, measuring around 1 MPa. To achieve optimal performance in periodontal regeneration, studies have suggested that a material with a tensile strength of 2 to 3 MPa, similar to the extracellular matrix (ECM), would be ideal [38]. The increase in stiffness and decrease in yield strain of electrospun membranes loaded with Ca-TMP in the wet condition compared to dry is probably because the fibers suffer from shrinkage after being kept at 37 °C for 24 h, promoting high stiffness and less elastic movement. Therefore, further modifications to the amino acid chains or other techniques may be necessary to enhance the

mechanical properties of PEU fibers and optimize their performance for periodontal regeneration applications. Incorporating Ca-TMP into the material resulted in some improvement in its mechanical properties, but the observed differences were not significant enough to alter the overall mechanical behavior of the material.

A critical factor for scaffolds is their biodegradability. Typically, after implantation, the mechanical strength of membranes diminishes, and they lose their ability to serve as a physical barrier between tissues [27,39–45]. The degradation rate of a membrane is a critical factor in tissue regeneration, as these membranes need to remain functional for a minimum of 4 to 6 weeks to facilitate successful regeneration of the periodontal system and surface coverings. Moreover, the fibers' degradation rate needs to match the tissue neoformation [3,40,46,47]. Consequently, having control over the degradation process of fibrous membranes becomes crucial in ensuring their efficacy. The present article shows that PEU and PEU/Ca-TMP fibers show high stability, with a low degradation rate of approximately > 80% for all groups after 3 months. This may have happened because, again, changing the PEU's chemical composition alters its mechanical properties. However, it was not enough to promote greater degradability of the material [13].

Calcium is widely recognized as a crucial factor in osteoinduction, as it plays a significant role in affecting the growth and specialization of cells [48,49]. Successful incorporation of substances into the manufacture of membranes must also be accompanied by the ability to release them at an appropriate rate. Although we confirmed the successful incorporation of Ca-TMP into the fibers via several methods, our study revealed that the Ca-TMP-incorporated membranes exhibited insufficient degradability to effectively re-lease calcium since, even for the PEU membrane loaded with the higher Ca-TMP tested (45%), minimal calcium was released after 21 days of incubation. This is related to the fact that the degradation rate was low, as previously described, showing high stability at day 91 and not being sufficient to promote high calcium release and stimulate the differentiation of cells [50]. Even though calcium (Ca^{2+}) was not released in large amounts from the membranes, it is still important to evaluate its effectiveness, as it is an essential signal transduction element involved in regulating various cellular activities and is required at key stages of the cell cycle to ensure orderly progression and proliferation [51]. Whit this in mind, we investigated cell compatibility using human dental pulp stem cells (SHEDs), knowing their significant potential for osteogenic differentiation [52], key in periodontal bone

regeneration. The results demonstrated no differences in cell viability for the four tested membranes over the three time points, mainly due to insufficient calcium released to stimulate cell proliferation [53,54]. The same happened with the alkaline phosphatase data; after seven days, the Ca-TMP group showed higher mineral differentiation activity but not enough to promote a significant difference between them and the osteogenic medium. However, although the scaffold did not promote osteogenic differentiation, it was clear that the membranes were not cytotoxic and even promoted better cell distribution and filopodia development.

In addition to PEU hindering degradation and drug release, the use of Ca-TMP seems to be another key factor in their unfavorable findings, as its synthesis is derived from STMP, which is used as a traditional scaffold crosslinker, responsible for the addition of intra- and intermolecular bonds at random locations in the polymer through an esterification reaction [55–57]. Although we worked with calcium trimetaphosphate, in the material synthesis process, sodium was removed, and calcium was added, maintaining its morphology [trimetaphosphate (P3O9)-3] [58]. Because of this, adding calcium to the trimetaphosphate could have promoted a crosslinking process in the PEU membrane, further increasing the material's mechanical properties and not being suitable for promoting periodontal regeneration. However, the findings of this study must be considered despite the unexpected results since the polymer itself is subject to changes in its structure to improve its response, and the biomaterial has never had its cytotoxicity evaluated. Also, further investigations are essential to discover whether another polymer is favorable for Ca-TMP release and promotes periodontal regeneration.

2. 5. Conclusions

The present study found that pure Ca-TMP powder at 50 μM concentration improved stem cells' cell viability and ALP activity from human exfoliated deciduous teeth when they came in direct contact with it. Additionally, Ca-TMP-loaded PEU membranes were successfully made with suitable surface topography and mechanical properties. While the PEU membranes containing Ca-TMP did not negatively impact cell viability and proliferation, they did not affect osteogenic differentiation.

Supplementary Materials: The following supporting information can be downloaded at www.mdpi.com/xxx/s1, Figure S1: (A) 1H NMR spectra of 50% PHE6 P(1-VAL-10) with integration values and corresponding peak assignments. (B) Scanning electron microscope (SEM) image of the pure Ca-TMP powder and histogram showing the particle size distribution; Figure S2: force-strain and stress-strain curves for all groups in dry and wet conditions.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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ANEXOS

ANEXO A
REFERÊNCIAS INTRODUÇÃO GERAL

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ANEXO B

CERTIFICADO DE APROVAÇÃO DO COMITÊ DE ÉTICA EM PESQUISA

UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: Síntese de ciclotrifosfato de cálcio e seu efeito contra a erosão inicial do esmalte: estudo in vitro

Pesquisador: Alberto Carlos Botazzo Delbem

Área Temática:

Versão: 1

CAAE: 52188021.2.0000.5420

Instituição Proponente: Faculdade de Odontologia do Campus de Araçatuba - UNESP

Patrocinador Principal: Financiamento Próprio

DADOS DO PARECER

Número do Parecer: 5.051.427

Apresentação do Projeto:

Para este estudo in vitro, será sintetizado e caracterizado ciclotrifosfatos com substituição total do sódio pelo cálcio e preparado compostos contendo diferentes proporções de Ca-TMP e Na-TMP (peso/peso) e analisar o efeito de soluções contendo 1% destes compostos contra a erosão inicial do esmalte. Para determinar o efeito na erosão do esmalte, blocos de esmalte bovino sadios (n=84) serão selecionados por dureza de superfície inicial e submetidos a 5 grupos de soluções experimentais contendo 1% dos compostos: 0% de Ca-TMP e 100% de Na-TMP (Na-TMP), 20% de Ca-TMP e 80% de Na-TMP (20% Ca-TMP), 50% de Ca-TMP e 50% de Na-TMP (50% Ca-TMP), 80% de Ca-TMP e 20% de Na-TMP (80% Ca-TMP), e 100% de Ca-TMP e 0% de Na-TMP (Ca-TMP). Como Controle será utilizada a água deionizada e, como controle positivo, uma solução contendo 1100 ppm F. Para a análise do efeito erosivo, os blocos de esmalte serão imersos em solução de dentifrícios com saliva humana uma vez por 2 minutos, seguidos por 4 desafios erosivos (ácido cítrico, 0,75%, pH 3,5, por 1 minuto, sob agitação). A percentagem de variação da dureza superficial (%SH) será calculada após os desafios ácidos de 1, 2, 3 e 4 minutos. As variáveis serão submetidas à análise de variância de medidas repetidas a dois critérios seguidas pelo teste de Student-Newman-Keuls ($p<0,05$).

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UNESP - FACULDADE DE ODONTOLOGIA-CAMPUS DE ARAÇATUBA/ UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

Objetivo da Pesquisa:

O objetivo do estudo in vitro será sintetizar ciclotrifosfatos com substituição total do sódio pelo cálcio (Ca-TMP; Ca(n+1)P3O9) e preparar compostos contendo diferentes proporções de Ca-TMP e Na-TMP (peso/peso) e analisar o efeito de soluções contendo 1% destes compostos contra a erosão inicial do esmalte.

Avaliação dos Riscos e Benefícios:

Riscos:

Haverá risco mínimo, pois haverá coleta de saliva que será estimulada com auxílio de parafina, sendo expectorada em frasco estéril.

Benefícios:

O benefício será entender melhor o mecanismo de ação de compostos de TMP com substituição total do sódio pelo cálcio no processo da erosão do esmalte.

Comentários e Considerações sobre a Pesquisa:

Pesquisa apresenta-se apta para a sua realização.

Considerações sobre os Termos de apresentação obrigatória:

Todos os termos foram adicionados de acordo com a resolução 466/12 do CNS.

Recomendações:

Nao Há.

Conclusões ou Pendências e Lista de Inadequações:

Pesquisa apresenta-se apta para a sua realização.

Considerações Finais a critério do CEP:

Salientamos que, de acordo com a Resolução 466 CNS, de 12/12/2012 (título X, seção X.1., art. 3, item b, e, título XI, seção XI.2., item d), há necessidade de apresentação de relatórios semestrais, devendo o primeiro relatório ser enviado até 01/04/2022.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_1830372.pdf	28/09/2021 15:43:34		Aceito

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Continuação do Parecer: 5.051.427

Projeto Detalhado / Brochura Investigador	projetodepesquisa.pdf	28/09/2021 15:41:54	Alberto Carlos Botazzo Delbem	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	tcle.pdf	28/09/2021 15:41:18	Alberto Carlos Botazzo Delbem	Aceito
Folha de Rosto	folhaderosto.pdf	28/09/2021 15:40:58	Alberto Carlos Botazzo Delbem	Aceito

Situação do Parecer:
Aprovado

Necessita Apreciação da CONEP:
Não

ARACATUBA, 21 de Outubro de 2021

Assinado por:
Aldiéris Alves Pesqueira
(Coordenador(a))

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ANEXO C
NORMAS DE APRESENTAÇÃO DO PERIÓDICO JOURNAL OF THE MECHANICAL BEHAVIOR OF BIOMEDICAL MATERIALS

We now differentiate between the requirements for new and revised submissions. You may choose to submit your manuscript as a single Word or PDF file to be used in the refereeing process. Only when your paper is at the revision stage, will you be requested to put your paper in to a 'correct format' for acceptance and provide the items required for the publication of your article.
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Introduction
Authors are requested to submit a cover letter that clearly states the novelty of the work presented in their manuscript.
Types of Contributions
Research Paper: A full-length article describing original research. There is no limit on the number of words, figures etc but authors should be as succinct as possible.
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Technical Note: A short article describing a new experimental technique or analytical approach.
Short Communication: An article presenting new work in reduced form, which for some reason is not suitable for a full research paper. For example a case study.
Opinion Piece: A short article presenting the author's opinion on a particular question. Normally shorter and less comprehensive than a review article, making use of published and/or unpublished results.
Tutorial: An article of an educational nature, explaining how to use a particular experimental technique or analytical method. Normally written by invitation only but the editor welcomes suggestions.
Please ensure that you select the appropriate article type from the list of options when making your submission. Authors contributing to special issues should ensure that they select the special issue article type from this list.
The journal also accepts letters, which should be sent directly to the editor in chief for consideration.

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• Referee suggestions and contact details provided, based on journal requirements

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Declaration of competing interest

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Declaration of generative AI in scientific writing

The below guidance only refers to the writing process, and not to the use of AI tools to analyse and draw insights from data as part of the research process.

Where authors use generative artificial intelligence (AI) and AI-assisted technologies in the writing process, authors should only use these technologies to improve readability and language. Applying the technology should be done with human oversight and control, and authors should carefully review and edit the result, as AI can generate authoritative-sounding output that can be incorrect, incomplete or biased. AI and AI-assisted technologies should not be listed as an author or co-author, or be cited as an author. Authorship implies responsibilities and tasks that can only be attributed to and performed by humans, as outlined in Elsevier's AI policy for authors.

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ANEXO D

NORMAS DE APRESENTAÇÃO DO PERIÓDICO JOURNAL OF FUNCTIONAL BIOMATERIALS

Manuscript Submission Overview

Types of Publications

JFB has no restrictions on the maximum length of manuscripts, provided that the text is concise and comprehensive. Full experimental details must be provided so that the results can be reproduced. *JFB* requires that authors publish all experimental controls and make full datasets available where possible (see the guidelines on **Supplementary Materials** and references to unpublished data).

Manuscripts submitted to *JFB* should neither be published previously nor be under consideration for publication in another journal. The main article types are as follows:

- *Article:* Original research manuscripts. The journal considers all original research manuscripts provided that the work reports scientifically sound experiments and provides a substantial amount of new information. Authors should not unnecessarily divide their work into several related manuscripts, although short *Communications* of preliminary, but significant, results will be considered. The quality and impact of the study will be considered during peer review.
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Manuscripts for *JFB* should be submitted online at **susy.mdpi.com**. The submitting author, who is generally the corresponding author, is responsible for the manuscript during the submission and peer-review process. The submitting author must ensure that all eligible co-authors have been included in the author list (read the **criteria to qualify for authorship**) and that they have all read and approved the submitted version of the manuscript. To submit your manuscript, register and log in to the **submission website**. Once you have registered, **click here to go to the submission form for *JFB***. All co-authors can see the manuscript details in the submission system, if they register and log in using the e-mail address provided during manuscript submission.

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Authors are encouraged to use the **Microsoft Word template** or **LaTeX template** to prepare their manuscript. Using the template file will substantially shorten the time to complete copy-editing and publication of accepted manuscripts. The total amount of data for all files must not exceed 120 MB. If this is a problem, please contact the Editorial Office **jfb@mdpi.com**. Accepted file formats are:

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Manuscript Preparation

General Considerations

- **Research manuscripts** should comprise:
    - **Front matter**: Title, Author list, Affiliations, Abstract, Keywords.

- **Research manuscript sections**: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
- **Back matter**: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, **References**.

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  The minimum required size for the GA is 560 × 1100 pixels (height × width). The size should be of high quality in order to reproduce well.
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  The data presented in this study are openly available in [repository name e.g., FigShare] at [**doi**], reference number [reference number].
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Additional data and files can be uploaded as "Supplementary Files" during the manuscript submission process. The supplementary files will also be available to the referees as part of the peer-review process. Any file format is acceptable; however, we recommend that common, non-proprietary formats are used where possible. For more information on supplementary materials, please refer to **https://www.mdpi.com/authors/layout#_bookmark83**.

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Citations and References in Supplementary files are permitted provided that they also appear in the reference list of the main text.

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Research and Publication Ethics

Research Ethics

Research Involving Human Subjects

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required, authors must either provide an exemption from the ethics committee or are encouraged to cite the local or national legislation that indicates ethics approval is not required for this type of study. Where a study has been granted exemption, the name of the ethics committee which provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation regarding why ethical approval was not required.

A written informed consent for publication must be obtained from participating patients. Data relating to individual participants must be described in detail, but private information identifying participants need not be included unless the identifiable materials are of relevance to the research (for example, photographs of participants' faces that show a particular symptom). Patients' initials or other personal identifiers must not appear in any images. For manuscripts that include any case details, personal information, and/or images of patients, authors must obtain signed informed consent for publication from patients (or their relatives/guardians) before submitting to an MDPI journal. Patient details must be anonymized as far as possible, e.g., do not mention specific age, ethnicity, or occupation where they are not relevant to the conclusions. A **template permission form** is available to download. A blank version of the form used to obtain permission (without the patient names or signature) must be uploaded with your submission. Editors reserve the right to reject any submission that does not meet these requirements.

You may refer to our sample form and provide an appropriate form after consulting with your affiliated institution. For the purposes of publishing in MDPI journals, a consent, permission, or release form should include unlimited permission for publication in all formats (including print, electronic, and online), in sublicensed and reprinted versions (including translations and derived works), and in other works and products under open access license. To respect patients' and any other individual's privacy, please do not send signed forms. The journal reserves the right to ask authors to provide signed forms if necessary.

If the study reports research involving vulnerable groups, an additional check may be performed. The submitted manuscript will be scrutinized by the editorial office and upon request, documentary evidence (blank consent forms and any related discussion documents from the ethics board) must be supplied. Additionally, when studies describe groups by race, ethnicity, gender, disability, disease, etc., explanation regarding why such categorization was needed must be clearly stated in the article.

Ethical Guidelines for the Use of Animals in Research

The editors will require that the benefits potentially derived from any research causing harm to animals are significant in relation to any cost endured by animals, and that procedures followed are unlikely to cause offense to the majority of readers. Authors should particularly ensure that their research complies with the commonly-accepted '3Rs [1]':

- Replacement of animals by alternatives wherever possible,
- Reduction in number of animals used, and
- Refinement of experimental conditions and procedures to minimize the harm to animals.

Authors must include details on housing, husbandry and pain management in their manuscript.

For further guidance authors should refer to the Code of Practice for the Housing and Care of Animals Used in Scientific Procedures [2], American Association for Laboratory Animal Science [3] or European Animal Research Association [4].

If national legislation requires it, studies involving vertebrates or higher invertebrates must only be carried out after obtaining approval from the appropriate ethics committee. As a minimum, the project identification code, date of approval and name of the ethics committee or institutional review board should be stated in Section 'Institutional Review Board Statement'. Research procedures must be carried out in accordance with national and institutional regulations. Statements on animal welfare should confirm that the study complied with all relevant legislation. Clinical studies involving animals and interventions outside of routine care require ethics committee oversight as per the American Veterinary Medical Association. If the study involved client-owned animals, informed client consent must be obtained and certified in the manuscript report of the research. Owners must be fully informed if there are any risks associated with the procedures and that the research will be published. If available, a high standard of veterinary care must be provided. Authors are responsible for correctness of the statements provided in the manuscript.

If ethical approval is not required by national laws, authors must provide an exemption from the ethics committee, if one is available. Where a study has been granted exemption, the name of the ethics committee that provided this should be stated in Section 'Institutional Review Board Statement' with a full explanation on why the ethical approval was not required.

If no animal ethics committee is available to review applications, authors should be aware that the ethics of their research will be evaluated by reviewers and editors. Authors should provide a statement justifying the work from an ethical perspective, using the same utilitarian framework that is used by ethics committees. Authors may be asked to provide this even if they have received ethical approval.

MDPI endorses the ARRIVE guidelines (**arriveguidelines.org/**) for reporting experiments using live animals. Authors and reviewers must use the ARRIVE guidelines as a checklist, which can be found at **https://arriveguidelines.org/sites/arrive/files/documents/ARRIVE%20Compliance%20Questionnaire.pdf**. Editors reserve the right to ask for the checklist and to reject submissions that do not adhere to these guidelines, to reject submissions based on ethical or animal welfare concerns or if the procedure described does not appear to be justified by the value of the work presented.

1. NSW Department of Primary Industries and Animal Research Review Panel. Three Rs. Available online: **https://www.animalethics.org.au/three-rs**
2. Home Office. Animals (Scientific Procedures) Act 1986. Code of Practice for the Housing and Care of Animals Bred, Supplied or Used for Scientific Purposes. Available online: **https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/388535/CoPanimalsWeb.pdf**
3. American Association for Laboratory Animal Science. The Scientific Basis for Regulation of Animal Care and Use. Available

online: **https://www.aalas.org/about-aalas/position-papers/scientific-basis-for-regulation-of-animal-care-and-use**

4. European Animal Research Association. EU regulations on animal research. Available online: **https://www.eara.eu/animal-research-law**

Research Involving Cell Lines

Methods sections for submissions reporting on research with cell lines should state the origin of any cell lines. For established cell lines the provenance should be stated and references must also be given to either a published paper or to a commercial source. If previously unpublished *de novo* cell lines were used, including those gifted from another laboratory, details of institutional review board or ethics committee approval must be given, and confirmation of written informed consent must be provided if the line is of human origin.

An example of Ethical Statements:

The HCT116 cell line was obtained from XXXX. The $MLH1^{+}$ cell line was provided by XXXXX, Ltd. The DLD-1 cell line was obtained from Dr. XXXX. The DR-GFP and SA-GFP reporter plasmids were obtained from Dr. XXX and the Rad51K133A expression vector was obtained from Dr. XXXX.

Research Involving Plants

Experimental research on plants (either cultivated or wild) including collection of plant material, must comply with institutional, national, or international guidelines. We recommend that authors comply with the **Convention on Biological Diversity** and the **Convention on the Trade in Endangered Species of Wild Fauna and Flora**.

For each submitted manuscript supporting genetic information and origin must be provided. For research manuscripts involving rare and non-model plants (other than, e.g., *Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa*, or many other typical model plants), voucher specimens must be deposited in an accessible herbarium or museum. Vouchers may be requested for review by future investigators to verify the identity of the material used in the study (especially if taxonomic rearrangements occur in the future). They should include details of the populations sampled on the site of collection (GPS coordinates), date of collection, and document the part(s) used in the study where appropriate. For rare, threatened or endangered species this can be waived but it is necessary for the author to describe this in the cover letter.

Editors reserve the rights to reject any submission that does not meet these requirements.

An example of Ethical Statements:

Torenia fournieri plants were used in this study. White-flowered Crown White (CrW) and violet-flowered Crown Violet (CrV) cultivars selected from ‘Crown Mix’ (XXX Company, City, Country) were kindly provided by Dr. XXX (XXX Institute, City, Country).

Arabidopis mutant lines (SALKxxxx, SAILxxxx,…) were kindly provided by Dr. XXX, institute, city, country).

Clinical Trials Registration

Registration

MDPI follows the International Committee of Medical Journal Editors (ICMJE) **guidelines** which require and recommend registration of clinical trials in a public trials registry at or before the time of first patient enrollment as a condition of consideration for publication.

Purely observational studies do not require registration. A clinical trial not only refers to studies that take place in a hospital or involve pharmaceuticals, but also refer to all studies which involve participant randomization and group classification in the context of the intervention under assessment.

Authors are strongly encouraged to pre-register clinical trials with an international clinical trials register and cite a reference to the registration in the Methods section. Suitable databases include **clinicaltrials.gov**, **the EU Clinical Trials Register** and those listed by the World Health Organisation **International Clinical Trials Registry Platform**.

Approval to conduct a study from an independent local, regional, or national review body is not equivalent to prospective clinical trial registration. MDPI reserves the right to decline any paper without trial registration for further peer-review. However, if the study protocol has been published before the enrolment, the registration can be waived with correct citation of the published protocol.

CONSORT Statement

MDPI requires a completed CONSORT 2010 **checklist** and **flow diagram** as a condition of submission when reporting the results of a randomized trial. Templates for these can be found here or on the CONSORT website (**http://www.consort-statement.org**) which also describes several CONSORT checklist extensions for different designs and types of data beyond two group parallel trials. At minimum, your article should report the content addressed by each item of the checklist.

Dual Use Research of Concern

MDPI follows the practical framework defined in **Guidance for Editors: Research, Audit and Service Evaluations** and introduced by the Committee on Publication Ethics (COPE). Research that could pose a significant threat, with broad potential consequences to public health or national security, should be clearly indicated in the manuscript, and potential dual-use research of concern should be explained in the cover letter upon submission. Potential areas of concern include but are not limited to biosecurity, nuclear and chemical threats, and research with a military purpose or application, etc. For these manuscripts to be considered for peer review, the benefits to the general public or public health must outweigh the risks. The authors have a responsibility to comply with relevant national and international laws.

Sex and Gender in Research

We encourage our authors to follow the **'Sex and Gender Equity in Research – SAGER – guidelines'** and to include sex and gender considerations where relevant. Authors should use the terms sex (biological attribute) and gender (shaped by social

and cultural circumstances) carefully in order to avoid confusing both terms. Article titles and/or abstracts should indicate clearly what sex(es) the study applies to. Authors should also describe in the background, whether sex and/or gender differences may be expected; report how sex and/or gender were accounted for in the design of the study; provide disaggregated data by sex and/or gender, where appropriate; and discuss respective results. If a sex and/or gender analysis was not conducted, the rationale should be given in the Discussion. We suggest that our authors consult the full **guidelines** before submission.

Borders and Territories

Potential disputes over borders and territories may have particular relevance for authors in describing their research or in an author or editor correspondence address, and should be respected. Content decisions are an editorial matter and where there is a potential or perceived dispute or complaint, the editorial team will attempt to find a resolution that satisfies parties involved.

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JFB is a member of the Committee on Publication Ethics (**COPE**). We fully adhere to its **Code of Conduct** and to its **Best Practice Guidelines**.

The editors of this journal enforce a rigorous peer-review process together with strict ethical policies and standards to ensure to add high quality scientific works to the field of scholarly publication. Unfortunately, cases of plagiarism, data falsification, image manipulation, inappropriate authorship credit, and the like, do arise. The editors of *JFB* take such publishing ethics issues very seriously and are trained to proceed in such cases with a zero tolerance policy.

Authors wishing to publish their papers in *JFB* must abide to the following:

- Any facts that might be perceived as a possible conflict of interest of the author(s) must be disclosed in the paper prior to submission.
- Authors should accurately present their research findings and include an objective discussion of the significance of their findings.
- Data and methods used in the research need to be presented in sufficient detail in the paper, so that other researchers can replicate the work.
- Raw data should preferably be publicly deposited by the authors before submission of their manuscript. Authors need to at least have the raw data readily available for presentation to the referees and the editors of the journal, if requested. Authors need to ensure appropriate measures are taken so that raw data is retained in full for a reasonable time after publication.
- Simultaneous submission of manuscripts to more than one journal is not tolerated.
- The journal accepts exact translations of previously published work. All submissions of translations must conform with our **policies on translations**.

- If errors and inaccuracies are found by the authors after publication of their paper, they need to be promptly communicated to the editors of this journal so that appropriate actions can be taken. Please refer to our **policy regarding Updating Published Papers**.
- Your manuscript should not contain any information that has already been published. If you include already published figures or images, please obtain the necessary permission from the copyright holder to publish under the CC-BY license. For further information, see the **Rights and Permissions** page.
- Plagiarism, data fabrication and image manipulation are not tolerated.
    - **Plagiarism is not acceptable** in *JFB* submissions.

      Plagiarism includes copying text, ideas, images, or data from another source, even from your own publications, without giving any credit to the original source.

      Reuse of text that is copied from another source must be between quotes and the original source must be cited. If a study's design or the manuscript's structure or language has been inspired by previous works, these works must be explicitly cited.

      All MDPI submissions are checked for plagiarism using the industry standard software iThenticate. If plagiarism is detected during the peer review process, the manuscript may be rejected. If plagiarism is detected after publication, an investigation will take place and action taken in accordance with our policies.
    - **Image files must not be manipulated or adjusted in any way** that could lead to misinterpretation of the information provided by the original image.

      Irregular manipulation includes: 1) introduction, enhancement, moving, or removing features from the original image; 2) grouping of images that should obviously be presented separately (e.g., from different parts of the same gel, or from different gels); or 3) modifying the contrast, brightness or color balance to obscure, eliminate or enhance some information.

      If irregular image manipulation is identified and confirmed during the peer review process, we may reject the manuscript. If irregular image manipulation is identified and confirmed after publication, we may correct or retract the paper.

  Our in-house editors will investigate any allegations of publication misconduct and may contact the authors' institutions or funders if necessary. If evidence of misconduct is found, appropriate action will be taken to correct or retract the publication. Authors are expected to comply with the best ethical publication practices when publishing with MDPI.

Citation Policy

Authors should ensure that where material is taken from other sources (including their own published writing) the source is clearly cited and that where appropriate permission is obtained.

Authors should not engage in excessive self-citation of their own work.

Authors should not copy references from other publications if they have not read the cited work.

Authors should not preferentially cite their own or their friends', peers', or institution's publications.

Authors should not cite advertisements or advertorial material.

In accordance with COPE guidelines, we expect that "original wording taken directly from publications by other researchers should appear in quotation marks with the appropriate citations." This condition also applies to an author's own work. COPE have produced a discussion document on **citation manipulation** with recommendations for best practice.

Reviewer Suggestions

During the submission process, please suggest three potential reviewers with the appropriate expertise to review the manuscript. The editors will not necessarily approach these referees. Please provide detailed contact information (address, homepage, phone, e-mail address). The proposed referees should neither be current collaborators of the co-authors nor have published with any of the co-authors of the manuscript within the last three years. Proposed reviewers should be from different institutions to the authors. You may identify appropriate Editorial Board members of the journal as potential reviewers. You may suggest reviewers from among the authors that you frequently cite in your paper.

Extensive English Editing

It is the authors' responsibility to submit their work in correct English. The APC includes only minor English editing, conducted by native English speakers. The APC does not include extensive English editing. If extensive editing is required, your paper could be returned to you at the English editing stage of the publication process. This could delay the publication of your work. You may have your work reviewed by an experienced English-speaking colleague or use a paid language-editing service before submitting your paper for publication. We offer rapid English editing, completed in 1 business day, here: **Author Services**.

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JFB accepts submissions that have previously been made available as preprints provided that they have not undergone peer review. A preprint is a draft version of a paper made available online before submission to a journal.

MDPI operates ***Preprints***, a preprint server to which submitted papers can be uploaded directly after completing journal submission. Note that *Preprints* operates independently of the journal and posting a preprint does not affect the peer review process. Check the *Preprints* **instructions for authors** for further information.

Expanded and high-quality conference papers can be considered as articles if they fulfill the following requirements: (1) the paper should be expanded to the size of a research article; (2) the conference paper should be cited and noted on the first page of the paper; (3) if the authors do not hold the copyright of the published conference paper, authors should seek the appropriate permission from the copyright holder; (4) authors are asked to disclose that it is conference paper in their cover letter and include a statement on what has been changed compared to the original conference paper. *JFB* does not publish pilot studies or studies with inadequate statistical power.

Unpublished conference papers that do not meet the above conditions are recommended to be submitted to the **Proceedings Series journals**.

Authorship

MDPI follows the International Committee of Medical Journal Editors (**ICMJE**) guidelines which state that, in order to qualify for authorship of a manuscript, the following criteria should be observed:

- Substantial contributions to the conception or design of the work; or the acquisition, analysis, or interpretation of data for the work; AND
- Drafting the work or reviewing it critically for important intellectual content; AND
- Final approval of the version to be published; AND
- Agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Those who contributed to the work but do not qualify for authorship should be listed in the acknowledgments. More detailed guidance on authorship is given by the **International Council of Medical Journal Editors (ICMJE)**.

Any change to the author list should be approved by all authors including any who have been removed from the list. The corresponding author should act as a point of contact between the editor and the other authors and should keep co-authors informed and involve them in major decisions about the publication. We reserve the right to request confirmation that all authors meet the authorship conditions.

For more details about authorship please check **MDPI ethics website**.

Reviewers Recommendation

Authors can recommend potential reviewers. Journal editors will check to make sure there are no conflicts of interest before contacting those reviewers, and will not consider those with competing interests. Reviewers are asked to declare any conflicts of interest. Authors can also enter the names of potential peer reviewers they wish to exclude from consideration in the peer review of their manuscript, during the initial submission progress. The editorial team will respect these requests so long as this does not interfere with the objective and thorough assessment of the submission.

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- Adequacy of reviewer comments and author response;
- Overall scientific quality of the paper.

In all of our journals, in every aspect of operation, MDPI policies are informed by the mission to make science and research findings open and accessible as widely and rapidly as possible.

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Editorial staff or editors shall not be involved in processing their own academic work. Submissions authored by editorial staff/editors will be assigned to at least two independent outside reviewers. Decisions will be made by other Editorial Board Members who do not have a conflict of interest with the author. Journal staff are not involved in the processing of their own work submitted to any MDPI journals.

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According to The International Committee of Medical Journal Editors, “Authors should avoid entering into agreements with study sponsors, both for-profit and non-profit, that interfere with authors’ access to all of the study’s data or that interfere with their ability to analyze and interpret the data and to prepare and publish manuscripts independently when and where they choose.”

All authors must disclose all relationships or interests that could inappropriately influence or bias their work. Examples of potential conflicts of interest include but are not limited to financial interests (such as membership, employment, consultancies, stocks/shares ownership, honoraria, grants or other funding, paid expert testimonies and patent-licensing arrangements) and non-financial interests (such as personal or professional relationships, affiliations, personal beliefs).

Authors can disclose potential conflicts of interest via the online submission system during the submission process. Declarations regarding conflicts of interest can also be collected via the **MDPI disclosure form**. The corresponding author must include a summary statement in the manuscript in a separate section “Conflicts of Interest” placed just before the reference list. The statement should reflect all the collected potential conflicts of interest disclosures in the form.

See below for examples of disclosures:

Conflicts of Interest: Author A has received research grants from Company A. Author B has received a speaker honorarium from Company X and owns stocks in Company Y. Author C has been involved as a consultant and expert witness in Company Z. Author D is the inventor of patent X.

If no conflicts exist, the authors should state:

Conflicts of Interest: The authors declare no conflicts of interest.

Editorial Procedures and Peer-Review

Pre-check

Immediately after submission, the journal's Managing Editor will perform the technical pre-check to assess:

- Overall suitability of the manuscript to the journal/section/Special Issue;
- Manuscript adherence to high-quality research and ethical standards;
- Standards of rigor to qualify for further review.

The academic editor (i.e., the Editor-in-Chief in the case of regular submissions, the Guest Editor in the case of Special Issue submissions, or an Editorial Board member in the case of a conflict of interest and of regular submissions if the Editor-in-Chief allows) will be notified of the submission and invited to perform an editorial pre-check. During the editorial pre-check phase, the academic editor will assess the suitability of the submission with respect to the scope of the journal, as well as the overall scientific soundness of the manuscript, including the relevance of the references and the correctness of the applied methodology. Academic editors can decide to reject the manuscript, request revisions before peer-review, or continue with the peer-review process and recommend suitable reviewers.

Peer-Review

Once a manuscript passes the initial checks, it will be assigned to at least two independent experts for peer-review. A single-blind review is applied, where authors' identities are known to reviewers. Peer review comments are confidential and will only be disclosed with the express agreement of the reviewer.

In the case of regular submissions, in-house assistant editors will invite experts, including recommendations by an academic editor. These experts may also include *Editorial Board Members* and Guest Editors of the journal. Potential reviewers suggested by the authors may also be considered. Reviewers should not have published with any of the co-authors during the past three years and should not currently work or collaborate with any of the institutions of the co-authors of the submitted manuscript. For more details about potential conflicts of interest, please check here, **https://www.mdpi.com/reviewers#_bookmark9**.

Optional Open Peer-Review

The journal operates optional open peer-review: *Authors are given the option for all review reports and editorial decisions to be published alongside their manuscript. In addition, reviewers can sign their review, i.e., identify themselves in the published review reports.* Authors can alter their choice for open review at any time before publication, but once the paper has been published changes will only be made at the discretion of the *Publisher* and *Editor-in-Chief*. We encourage authors to take advantage of this opportunity as proof of the rigorous process employed in publishing their research. To guarantee impartial refereeing, the names of referees will be revealed only if the referees agree to do so, and after a paper has been accepted for publication.

Editorial Decision and Revision

All the articles, reviews and communications published in MDPI journals go through the peer-review process and receive at least two reviews. The in-house editor will communicate the decision of the academic editor, which will be one of the following:

- *Accept after Minor Revisions*:
  The paper is in principle accepted after revision based on the reviewer's comments. Authors are given five days for minor revisions.
- *Reconsider after Major Revisions*:
  The acceptance of the manuscript would depend on the revisions. The author needs to provide a point by point response or provide a rebuttal if some of the reviewer's comments cannot be revised. A maximum of two rounds of major revision per manuscript is normally provided. Authors will be asked to resubmit the revised paper within a suitable time frame, and the revised version will be returned to the reviewer for further comments. If the required revision time is estimated to be longer than 2 months, we will recommend that authors withdraw their manuscript before resubmitting so as to avoid unnecessary time pressure and to ensure that all manuscripts are sufficiently revised.
- *Reject and Encourage Resubmission*:
  If additional experiments are needed to support the conclusions, the manuscript will be rejected and the authors will be encouraged to re-submit the paper once further experiments have been conducted.
- *Reject*:
  The article has serious flaws, and/or makes no original significant contribution. No offer of resubmission to the journal is provided.

All reviewer comments should be responded to in a point-by-point fashion. Where the authors disagree with a reviewer, they must provide a clear response.

Author Appeals

Authors may appeal a rejection by sending an e-mail to the Editorial Office of the journal. The appeal must provide a detailed justification, including point-by-point responses to the reviewers' and/or Editor's comments using an **appeal form**. Appeals can only be submitted following a "reject and decline resubmission" decision and should be submitted within three months from the decision date. Failure to meet these criteria will result in the appeal not being considered further. The *Managing Editor* will forward the manuscript and related information (including the identities of the referees) to a designated *Editorial Board Member*. The Academic Editor being consulted will be asked to provide an advisory recommendation on the manuscript and may recommend acceptance, further peer-review, or uphold the original rejection decision. This decision will then be validated by the *Editor-in-Chief*. A reject decision at this stage is final and cannot be reversed.

Production and Publication

Once accepted, the manuscript will undergo professional copy-editing, English editing, proofreading by the authors, final corrections, pagination, and, publication on the **www.mdpi.com** website.

Promoting Equity, Diversity and Inclusiveness within MDPI Journals

Our Managing Editors encourage the Editors-in-Chief and Associate Editors to appoint diverse expert Editorial Boards. This is also reflective in our multi-national and inclusive workplace. We are proud to create equal opportunities without regard to gender, ethnicity, sexual orientation, age, religion, or socio-economic status. There is no place for discrimination in our workplace and editors of MDPI journals are to uphold these principles in high regard.

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To improve the reproducibility of scientific research, the **Resource Identification Initiative** aims to provide unique persistent identifiers for key biological resources, including antibodies, cell lines, model organisms and tools.

We encourage authors to include unique identifiers - RRIDs- provided by the **Resource Identification Portal** in the dedicated section of the manuscript.

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