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Diego Garcia Miranda

BIOPHYSICALCHEMICAL EVALUATION OF DRUGS INCORPORATED INTO HYALURONIC ACID HYDROGELS FOR THE TREATMENT OF PAIN AND THE REGULATION OF THE INFLAMMATORY PROCESS IN TEMPOROMANDIBULAR DISORDER

The jury composed of:

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Tronci, Giuseppe	Full professor University of Leeds	Rapporteur
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Diego Garcia Miranda

ÉVALUATION BIOPHYSIQUECHIMIQUE DE MÉDICAMENTS INCORPORÉS DANS DES HYDROGELS D'ACIDE HYALURONIQUE POUR LE TRAITEMENT DE LA DOULEUR ET LA RÉGULATION DU PROCESSUS INFLAMMATOIRE DANS LE DYSFONCTIONNEMENT TEMPOROMANDIBULAIRE

Devant le jury composé de :

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BIOPHYSICALCHEMICAL EVALUATION OF DRUGS INCORPORATED INTO HYALURONIC ACID HYDROGELS FOR THE TREATMENT OF PAIN AND THE REGULATION OF THE INFLAMMATORY PROCESS IN TEMPOROMANDIBULAR DISORDER

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POTENTIAL IMPACT OF THIS RESEARCH

This study provides evidence regarding the technical feasibility of developing a hyaluronic acid-based neo-polymer conjugated with drugs for the treatment of temporomandibular disorder (TMD). The project aimed to develop the polymer by taking into account the three pillars of the disease's pathophysiology: mechanical friction, pain, and the inflammatory process. Further studies are needed for in vivo validation with a view toward human application.

IMPACT POTENTIEL DE CETTE RECHERCHE

Cette étude apporte des éléments de preuve quant à la faisabilité technique de la mise au point d'un néopolymère à base d'acide hyaluronique conjugué à des médicaments pour le traitement des troubles temporo-mandibulaires (TMD). Le projet visait à développer ce polymère en tenant compte des trois piliers de la physiopathologie de la maladie : le frottement mécanique, la douleur et le processus inflammatoire. Des études supplémentaires sont nécessaires pour une validation in vivo en vue d'une application chez l'homme.

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São José dos Campos, September 14, 2026.

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"C'est le temps que tu as perdu pour ta rose que fait ta rose si importante".

Antoine de Saint-Exupéry.

ABSTRACT

Miranda DG. Biophysicochemical evaluation of drugs incorporated into hyaluronic acid hydrogels for pain control and regulation of the inflammatory process in temporomandibular dysfunction [doctorate thesis]. São Paulo State University (UNESP), *Institute of Science and Technology*; Lyon: Université Claude Bernard Lyon 1 (UCBL); 2026.

Temporomandibular disorder (TMD) is a public health problem that affects 12% of the world's population. Its clinical presentation is diverse and may include myalgia, arthralgia, headache, malocclusion, clicking, crepitus, and reduced mouth opening, among other signs and symptoms. The pathophysiology of the condition is attributed to immuno-inflammatory dysregulation; therefore, treatments are based on regulating this process. Thus, in the search for an effective therapeutic option, this study aimed to develop and evaluate the biophysicochemical properties of hydrogels based on hyaluronic acid conjugated with bupivacaine, ketoprofen, or hydrocortisone for pain control and regulation of the inflammatory process in temporomandibular dysfunction. For the physicochemical analyses, field emission scanning electron microscopy (FEG-SEM), Fourier transform infrared spectroscopy (FTIR), thermogravimetry, and oscillatory rheology testing were performed. Biological assays were performed on mouse macrophages (RAW 264.7) to evaluate the cytocompatibility of the biomaterials using the alamar blue colorimetric assay, in accordance with OECD 129; in addition, genotoxicity was assessed using the micronucleus assay in accordance with OECD 487. Data with a normal distribution were analyzed using ANOVA supplemented by Tukey's test, with a significance level of 5% ($p \leq 0.05$); data that did not follow a normal distribution were analyzed using the Kruskal-Wallis test supplemented by Dunn's test, with a significance level of 5% ($p \leq 0.05$). As a result, physicochemical analysis by FTIR spectroscopy identified the characteristic bands of hyaluronic acid (HA), bupivacaine (BUP), ketoprofen (CET), and hydrocortisone (HID), with no significant chemical interactions between the drugs and the polymer. Morphological analysis by SEM-FEG revealed a porous, three-dimensional structure in all hydrogels, with no evident structural changes due to the incorporation of the drugs. Thermogravimetric analysis indicated that the combination with HA increased the thermal stability of the drugs, resulting in more gradual mass losses. Rheological analysis confirmed stable viscoelastic behavior ($G' > G''$), with autoclaving increasing the stiffness of pure AH, while the addition of drugs generally reduced the storage modulus (G'). Regarding biocompatibility, the conjugated hydrogels maintained or increased cell viability in RAW 264.7 macrophages, in contrast to the cytotoxic observed for the individual drugs at high concentrations. In the genotoxicity assessment, the bupivacaine and ketoprofen hydrogels did not exhibit genotoxic effects, whereas the hydrocortisone hydrogel induced micronuclei at concentrations of 0.5 and 1 mg, in proportion to the increase in drug concentration. In conclusion, hyaluronic acid hydrogels combined with bupivacaine, ketoprofen, and hydrocortisone exhibited viscoelastic properties and thermal stability suitable for the temporomandibular joint, in addition to demonstrating cytocompatibility and the absence of genotoxicity in in vitro tests.

Keywords: temporomandibular joint dysfunction syndrome; hyaluronic acid; immunoregulation; osteoarthritis.

RÉSUMÉ

Miranda DG. Évaluation biophysicochimique de médicaments incorporés dans des hydrogels d'acide hyaluronique pour le contrôle de la douleur et la régulation du processus inflammatoire dans les troubles temporo-mandibulaires [thèse]. Universidade Estadual Paulista (UNESP), Instituto de Ciência e Tecnologia; Lyon: Université Claude Bernard Lyon 1 (UCBL); 2026.

Les troubles temporo-mandibulaires (TTM) constituent un problème de santé publique touchant 12 % de la population mondiale. Leur présentation clinique est variée et peut inclure, entre autres signes et symptômes, des myalgies, des arthralgies, des céphalées, une malocclusion, des clics articulaires, des crépitements et une réduction de l'ouverture buccale. La physiopathologie de la maladie s'explique par une dérégulation immuno-inflammatoire ; par conséquent, les traitements visent à réguler ce processus. Ainsi, dans la recherche d'une option thérapeutique efficace, cette étude a visé à développer et à réaliser une évaluation biophysicochimique d'hydrogels à base d'acide hyaluronique conjugués à la bupivacaïne, au kétoprofène ou à l'hydrocortisone, pour le contrôle de la douleur et la régulation du processus inflammatoire lié aux TTM. Les analyses physico-chimiques ont inclus la microscopie électronique à balayage à émission de champ (FE-SEM), la spectroscopie infrarouge à transformée de Fourier avec réflexion totale atténuée (FTIR-ATR), la thermogravimétrie et des essais de rhéologie oscillatoire. Des analyses biologiques ont été effectuées sur des macrophages de souris (RAW 264.7) afin d'évaluer la cytocompatibilité des biomatériaux à l'aide du test colorimétrique Alamar Blue, conformément à la ligne directrice 129 de l'OCDE. La génotoxicité a été évaluée par le test des micronoyaux selon la ligne directrice 487 de l'OCDE. Les données suivant une distribution normale ont été analysées par ANOVA suivie du test de Tukey (seuil de signification $p \leq 0,05$), tandis que les données ne suivant pas une distribution normale ont été analysées par le test de Kruskal-Wallis suivi du test de Dunn (seuil de signification $p \leq 0,05$). L'analyse physico-chimique par spectroscopie FTIR-ATR a permis d'identifier les bandes caractéristiques de l'acide hyaluronique (HA), de la bupivacaïne (BUP), du kétoprofène (CET) et de l'hydrocortisone (HID), sans qu'aucune interaction chimique significative ne soit observée entre les médicaments et le polymère. L'analyse morphologique par microscopie électronique à balayage à émission de champ (FEG-SEM) a révélé une structure poreuse tridimensionnelle dans tous les hydrogels, sans modification structurelle apparente liée à l'incorporation des médicaments. L'analyse thermogravimétrique a indiqué que l'association avec l'acide hyaluronique (AH) augmentait la stabilité thermique des médicaments, entraînant une perte de masse plus progressive. L'analyse rhéologique a confirmé un comportement viscoélastique stable ($G' > G''$) ; l'autoclavage a accru la rigidité de l'AH pur, tandis que l'ajout de médicaments a généralement réduit le module de conservation (G'). En termes de biocompatibilité, les hydrogels chargés en médicaments ont maintenu ou augmenté la viabilité cellulaire des macrophages RAW 264.7, contrastant avec la cytotoxicité observée pour les médicaments seuls à fortes concentrations. Lors de l'évaluation de la génotoxicité, les hydrogels à la bupivacaïne et au kétoprofène n'ont présenté aucun effet génotoxique ; en revanche, l'hydrogel contenant de l'hydrocortisone a induit la formation de micronoyaux aux concentrations de 0,5 et 1 mg, de manière proportionnelle à l'augmentation de la concentration en médicament. En conclusion, les hydrogels d'acide hyaluronique combinés à la bupivacaïne, au kétoprofène et à l'hydrocortisone ont présenté des propriétés viscoélastiques et une stabilité thermique adaptées à l'articulation temporo-mandibulaire, tout en démontrant une cytocompatibilité et une absence de génotoxicité lors des tests in vitro.

Mots-clés: syndrome de l'articulation temporomandibulaire; acide hyaluronique; immunorégulation; arthrose.

RESUMO

Miranda DG. Avaliação Biofisicoquímica de fármacos incorporados a hidrogéis de ácido hialurônico para controle da dor e regulação do processo inflamatório na disfunção temporomandibular [tese]. Universidade Estadual Paulista (UNESP), Instituto de Ciência e Tecnologia; Lyon: Université Claude Bernard Lyon 1 (UCBL), 2026.

A disfunção temporomandibular (DTM) é um problema de saúde publica que afeta 12% da população mundial. Sua apresentação clínica é diversa, podendo ser composta de mialgia, artralgia, cefaleia, má oclusão, estalidos, crepitações, redução da abertura de boca, entre outros sinais e sintomas. A fisiopatologia da doença é explicada pela desregulação imuno-inflamatória, portanto os tratamentos são baseados na regulação desse processo. Assim, na busca por uma opção terapêutica eficaz, o presente trabalho visou o desenvolvimento e avaliação biofísicoquímica de hidrogéis a base de ácido hialurônico conjugado a bupvacaína; cetoprofeno ou hidrocortisona para controle da dor e regulação do processo inflamatório na disfunção temporomandibular. Para as análises físico-químicas foram realizados a microscopia eletrônica de varredura com emissão de campo (MEV-FEG), espectroscopia infravermelho por transformada de Fourier com reflectância total atenuada (FTIR-ATR), termogravitometria e ensaio de reologia em regime oscilatório. As análises biológicas foram realizadas sobre macrófagos de camundongos (RAW 264.7) avaliando a citotocompatibilidade dos biomateriais por meio do teste colorimétrico azul de alamar, conforme a OECD 129; Além da avaliação da genotoxicidade por micronúcleos seguindo a OECD 487. Dados com distribuição normal foram avaliados pelo método ANOVA complementado pelo Teste de Tukey, com nível de significância de 5% ($p \leq 0.05$); já os que não obtiveram distribuição normal foram analisados por Kruskal Wallis suplementado por Dunn's com nível de significância de 5% ($p \leq 0.05$). Como resultado, a análise físico-química por espectroscopia FTIR-ATR, identificou as bandas características do ácido hialurônico (AH), bupivacaína (BUP), cetoprofeno (CET) ou hidrocortisona (HID), sem interações químicas significativas entre os fármacos e o polímero. A análise morfológica por MEV-FEG revelou uma estrutura porosa e tridimensional em todos os hidrogéis, sem alterações estruturais evidentes pela incorporação dos fármacos. A termogravimetria indicou que a associação com o AH aumentou a estabilidade térmica dos fármacos, com perdas de massa mais graduais. A análise reológica confirmou um comportamento viscoelástico estável ($G' > G''$), com a autoclavagem aumentando a rigidez do AH puro, enquanto a adição de fármacos geralmente reduziu o módulo de armazenamento (G'). Quanto à biocompatibilidade, os hidrogéis conjugados mantiveram ou aumentaram a viabilidade celular em macrófagos RAW 264.7, contrastando com a citotoxicidade observada para os fármacos isolados em altas concentrações. Na avaliação de genotoxicidade os hidrogéis de Bupivacaína e Cetoprofeno não apresentaram efeitos genotóxicos, já o hidrogel com Hidrocortisona exibiu indução de micronúcleos, nas concentrações de 0,5 e 1 mg, proporcional ao aumento da concentração do fármaco. Em conclusão, os hidrogéis de ácido hialurônico associado a bupvacaína, cetoprofeno e hidrocortisona apresentaram propriedades viscoelasticas e termoestabilidade adequadas a articulação temporomandibular, além de exibirem citocompatibilidade e ausência de genotoxicidade nos testes *in vitro*.

Palavras-chave: síndrome da disfunção da articulação temporomandibular; ácido hialurônico; imunoregulação; osteoartrite.

LIST OF ABBREVIATIONS AND ACRONYMS

BUP	Bupivacaine
GeneQoL	Consortium for Research on Genetics and Quality of Life
DC/TMD	Diagnostic Criteria for Temporomandibular Joint Disorder
HA	Hyaluronic Acid
HYD	Hydrocortisone
ICOP	International Classification of Orofacial Pain
KET	Ketoprofen
OECD	Organization for Economic Cooperation and Development
DCR/ TMD	Research diagnostic criteria for temporomandibular dysfunction
TMD	Temporomandibular dysfunction
TMJ	Temporomandibular Joint

LISTE DES ABRÉVIATIONS ET DES ACRONYMES

AH	Acide hyaluronique
ATM	Articulation temporo-mandibulaire
BUP	Bupivacaïne
CD/DTM	Critères diagnostiques du dysfonctionnement temporo-mandibulaire
CDP/DTM	Critères diagnostiques de référence pour le dysfonctionnement temporo-mandibulaire
CET	Cétoprofène
CIDO	Classification internationale de la douleur orofaciale
DTM	Dysfonctionnement temporo-mandibulaire
GeneQoL	Consortium pour la recherche en génétique et qualité de vie
HYD	Hydrocortisone
OCDE	Organisation de coopération et de développement économiques

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1 INTRODUCTION

The American Academy of Orofacial Pain defines temporomandibular disorders (TMD) as an umbrella term for a group of pathophysiological conditions affecting the musculoskeletal and neuromuscular structures of the masticatory musculature and the temporomandibular joint (TMJ). It represents a major public health issue with variable distribution, affecting approximately 6% to 12% of the general population, and is most frequent in women aged 20 to 40 years. TMD is considered the most common cause of non-dental chronic pain in the orofacial region (Wu et al., 2021; Valesan et al., 2021).

The globally accepted classification tools for TMD diagnosis are the Research Diagnostic Criteria for Temporomandibular Disorders (RDC/TMD) and the Diagnostic Criteria for Temporomandibular Disorders (DC/TMD). Recently, the International Classification of Orofacial Pain (ICOP) was developed to increase consistency across studies, enabling standardization and reproducibility of results in both clinical and research settings (Gilheaney et al., 2020; Mnguni et al., 2021; Valesan et al., 2021).

In the DC/TMD, TMD diagnoses are subdivided into two main categories: painful disorders and joint disorders. Painful disorders include local myalgia, myofascial pain with spreading, myofascial pain with referral, arthralgia, and headache attributed to TMD. Joint disorders, on the other hand, are classified as disc displacement with reduction, disc displacement with reduction with intermittent locking, disc displacement without reduction with limited opening, disc displacement without reduction without limited opening, degenerative joint disease, and subluxation (Valesan et al., 2021; Wu et al., 2021).

Regarding clinical manifestations, the following may be observed: pain in masticatory structures; pain modified by jaw movement, function, or parafunction; headache of any type in the temporal region; headache influenced by jaw movement, function, or parafunction; clicking during jaw opening, closing, lateral movement, or protrusion; passive opening less than 40 mm; crepitus during jaw movement; and current or intermittent TMJ locking (Valesan et al., 2021; Wu et al., 2021).

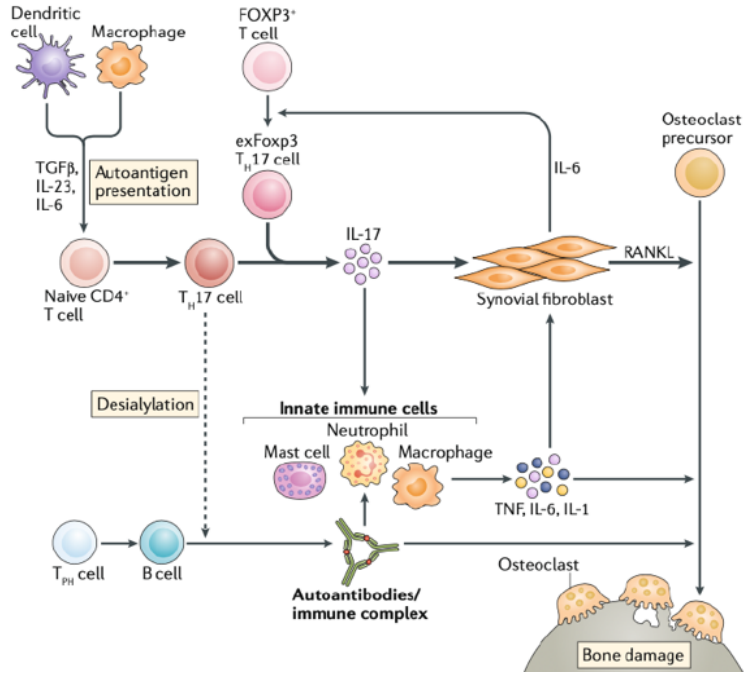
The pathophysiological process of TMD is complex because, although defined as a local inflammatory condition, its development can stem from both local and

systemic factors. Local factors prominently feature microtrauma secondary to bruxism, parafunctional habits, and improper disc displacement. Systemic factors include rheumatoid, psoriatic, and reactive arthritis. Thus, the host's immuno-inflammatory response in TMD is particularly noteworthy (Monasterio et al., 2018; Bouloux, 2018; Hunter and Bierma-Zeinstra, 2019).

Temporomandibular joint osteoarthritis is one of the most common forms of TMD and can be described as a low-grade inflammatory arthritic condition when compared to systemic arthritides (Monasterio et al., 2018).

Different cytokines, chemokines, chemokine receptors, enzymes, and bone resorption factors have been proposed as biomarkers for TMD. Elevated levels of interleukin (IL)-1β, IL-6, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and prostaglandin E2 (PGE2) are cited by several authors. To a lesser extent, factors such as matrix metalloproteinase (MMP)-2, MMP-9, aggrecanase-1, superoxide dismutase, and receptor activator of nuclear factor κB ligand (RANKL) have also been detected in the synovial fluid of patients with TMD (Monasterio et al., 2018; Bouloux, 2018; Hunter and Bierma-Zeinstra, 2019).

Figure 1 – Illustration of the immuno-inflammatory mechanisms of joint dysfunction



Source: Tsukasaki MT, 2019.

Regarding the pain profile of patients, Sebastiani et al. (2021) demonstrate that genetic polymorphism of IL-6 in certain individuals increases the expression of this cytokine, negatively contributing to pain sensitivity and duration. The Genetic Quality of Life Consortium (GeneQoL) suggested that the IL-6 gene is associated with overall quality of life, where the interaction of this cytokine with its receptors induces inflammation, activates vagal afferent nerves, and exacerbates pain severity and duration, in addition to triggering depression (Sprangers et al., 2014).

The disease has a well-established impact on social, economic, and healthcare structures. Studies indicate reduced levels of health-related quality of life, social interaction, and social networks, alongside an increased incidence of depression among TMD patients. Pain and dysphagia affect both the physical functioning and emotional well-being of patients, manifesting as a decreased ability to maintain a normal diet and reduced participation in social events (Gilheaney et al., 2020; Suomela et al., 2025).

TMD management aligns with the patient's clinical manifestations. Given the current understanding that the immuno-inflammatory process plays a key role in disease pathogenesis, interventions are based on the use of analgesics, non-steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and muscle relaxants. To reduce the concentration of pro-inflammatory cytokines in the synovial fluid and break down potential disc adhesions, arthrocentesis is recommended, which may or may not be combined with hyaluronic acid-based viscosupplementation. As a last resort, surgical interventions such as discopexy and arthroplasty, with or without joint replacement, are proposed.

INTRODUCTION (Français)

L'Académie Américaine de la Douleur Orofaciale définit les désordres temporo-mandibulaires (DTM) comme un terme générique regroupant un ensemble de conditions physiopathologiques qui affectent les structures musculo-squelettiques et neuromusculaires de la musculature masticatrice et de l'articulation temporo-mandibulaire (ATM). Il s'agit d'un problème majeur de santé publique à distribution variable, touchant environ 6 à 12 % de la population générale, plus fréquent chez les femmes âgées de 20 à 40 ans. Le DTM est considéré comme la cause la plus

fréquente de douleur chronique d'origine non dentaire dans la région orofaciale (Wu et al., 2021 ; Valesan et al., 2021).

Les outils de classification mondialement acceptés pour le diagnostic du DTM sont : les critères diagnostics de recherche pour les désordres temporo-mandibulaires (RDC/TMD) et les critères diagnostics des désordres temporo-mandibulaires (DC/TMD). Récemment, la Classification Internationale de la Douleur Orofaciale (ICOP) a été développée afin d'augmenter la cohérence entre les études, permettant la standardisation et la reproductibilité des résultats, tant sur le plan clinique que dans le domaine de la recherche (Gilheaney et al., 2020 ; Mnguni et al., 2021 ; Valesan et al., 2021).

Dans le DC/TMD, le diagnostic de DTM est subdivisé en deux catégories : les désordres douloureux et les désordres articulaires. Les désordres douloureux comprennent la myalgie locale, la douleur myofasciale avec propagation, la douleur myofasciale avec douleur référée, l'arthralgie et les céphalées attribuées au DTM. Quant aux désordres articulaires, ils sont classés en déplacement discal réduit, déplacement discal réduit avec blocage intermittent, déplacement discal non réduit avec limitation de l'ouverture, déplacement discal non réduit sans limitation de l'ouverture, maladie articulaire dégénérative et subluxation (Valesan et al., 2021 ; Wu et al., 2021).

Concernant les manifestations cliniques, on peut observer : une douleur au niveau des structures masticatrices ; une douleur modifiée par le mouvement, la fonction ou la parafonction mandibulaire ; des céphalées de tout type dans la région temporale ; des céphalées influencées par le mouvement, la fonction ou la parafonction mandibulaire ; des claquements lors de l'ouverture, de la fermeture, de la diduction ou de la propulsion de la mandibule ; une ouverture passive inférieure à 40 mm ; des crépitations pendant le mouvement mandibulaire ; ainsi qu'un blocage actuel ou intermittent de l'ATM (Valesan et al., 2021 ; Wu et al., 2021).

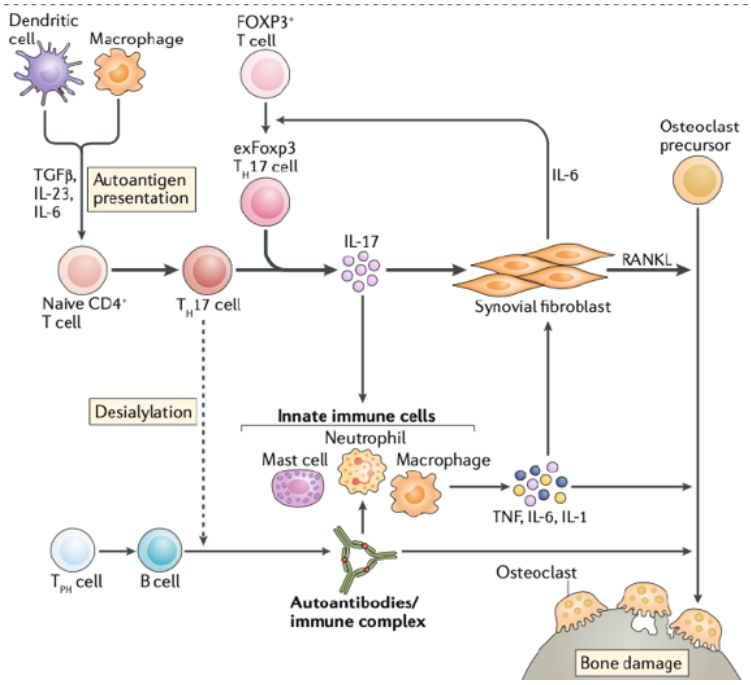
Le processus physiopathologique du DTM est complexe car, bien qu'il soit défini comme une affection inflammatoire locale, son développement peut découler de facteurs locaux et systémiques. Parmi les facteurs locaux, on met en évidence le microtraumatisme secondaire au bruxisme, les habitudes parafonctionnelles et le déplacement inadéquat du disque articulaire. Parmi les facteurs systémiques figurent la polyarthrite rhumatoïde, le rhumatisme psoriasique et l'arthrite réactive. Il convient

donc de souligner la réponse immuno-inflammatoire de l'hôte dans le DTM (Monasterio et al., 2018 ; Bouloux, 2018 ; Hunter et Bierma-Zeinstra, 2019).

L'ostéoarthrite de l'articulation temporo-mandibulaire est l'une des formes les plus fréquentes de désordre temporo-mandibulaire et peut être décrite como une affection arthritique à faible composante inflammatoire lorsqu'elle est comparée aux arthrites systémiques (Monasterio et al., 2018).

Différentes cytokines, chimiokines, récepteurs de chimiokines, enzymes et facteurs de résorption osseuse ont été proposés comme marqueurs du DTM. Certains auteurs citent des taux élevés d'interleukine (IL)-1β, d'IL-6, d'IL-17, d'interféron (IFN)-γ, de facteur de nécrose tumorale (TNF)-α et de prostaglandine E2 (PGE2). Dans un nombre plus restreint d'études, il est également possible de vérifier la présence de facteurs tels que la métalloprotéinase matricielle (MMP)-2, la MMP-9, l'aggrécanase-1, la superoxyde dismutase et le ligand du récepteur activateur du facteur nucléaire κB (RANKL), détectés dans le liquide synovial de patients atteints de DTM (Monasterio et al., 2018 ; Bouloux, 2018 ; Hunter et Bierma-Zeinstra, 2019).

Figure 1 – Illustration des mécanismes immuno-inflammatoires du dysfonctionnement articulaire



Source: Tsukasaki MT, 2019.

En ce qui concerne le profil algique des patients, Sebastiani et al. (2021) démontrent que le polymorphisme génétique de l'IL-6 chez certains individus entraîne une augmentation de l'expression de cette cytokine, contribuant de manière négative à la sensibilité et à la durée de la douleur. Le Consortium de Recherche sur la Génétique et la Qualité de Vie (GeneQoL) a suggéré que le gène de production de l'IL-6 est associé à la qualité de vie globale, où l'interaction de la cytokine avec ses récepteurs engendre l'inflammation, l'activation des nerfs afférents vagaux, et accentue la sévérité et la durée de la douleur, tout en déclenchant la dépression (Sprangers et al., 2014).

La pathologie a un impact bien établi sur les structures sociales, économiques et de santé. Des études mettent en évidence une diminution des niveaux de qualité de vie liée à la santé, des interactions sociales et des réseaux sociaux, associée à une augmentation de la dépression chez les patients atteints de DTM. La douleur et la dysphagie altèrent tant le fonctionnement physique que le bien-être émotionnel du patient, ce qui se traduit par une réduction de la capacité à maintenir une alimentation normale et par une participation moindre aux événements sociaux (Gilheaney et al., 2020 ; Suomela et al., 2025).

La prise en charge du DTM est adaptée aux manifestations cliniques du patient. Il étant aujourd'hui admis que le processus immuno-inflammatoire joue un rôle fondamental dans la pathogenèse de la maladie, les interventions reposent sur l'utilisation d'analgésiques, d'anti-inflammatoires non stéroïdiens (AINS), de corticoïdes et de relaxants musculaires. Dans le but de réduire la concentration de cytokines pro-inflammatoires dans le liquide synovial et de libérer d'éventuelles adhérences du disque articulaire, l'arthrocentèse est préconisée, pouvant être associée ou non à une viscosupplémentation à base d'acide hyaluronique. En dernier recours, des interventions chirurgicales telles que la discopexie et l'arthroplastie, avec ou sans prothèse articulaire, sont proposées.

2 ARTICLES

2.1 Article – Miranda DG, Ramos LP, Lopes NFDS, Silva NVH, Soares CP, Rodrigues FP, Morais VP, Sani-Taiariol T, Baldan MR, Vasconcellos LMR, Borges ALS, Grosgogeat B, Gritsch K. *Ketoprofen Associated with Hyaluronic Acid Hydrogel for Temporomandibular Disorder Treatment: An In Vitro Study. Le cétoprofène associé à un hydrogel d'acide hyaluronique pour le traitement des troubles de l'articulation temporo-mandibulaire: une étude in vitro.*

ABSTRACT

Temporomandibular disorders (TMD) are a public health problem that affects around 12% of the global population. The treatment is based on analgesics, non-steroidal anti-inflammatory, corticosteroids, anticonvulsants, or arthrocentesis associated with hyaluronic acid-based viscosupplementation. However, the use of hyaluronic acid alone in viscosupplementation does not seem to be enough to regulate the intra-articular inflammatory process. So, we propose to develop and evaluate the physicochemical and biological properties in vitro of hyaluronic acid hydrogels (HA) associated with ketoprofen (KET) as a new therapeutic treatment for TMD. The hydrogels were synthesized with 3% HA and 0.125, 0.250, 0.500, or 1% KET. Physicochemical analyses of Attenuated Total reflectance-Fourier transform infrared spectroscopy (FTIR), Thermogravimetry (TGA), Rheology by Frequency, Amplitude sweeps, temperature ramp, and scanning electron microscopy (SEM) were performed with or without sterilization and cycled. Cytocompatibility and genotoxicity (micronucleus assay) were performed in mouse macrophages (RAW 264-7) for 24 h. Results: FTIR spectrum showed characteristic absorptions of HA and KET. In the TGA, two mass loss peaks were observed, the first representing the water evaporation at 30 and 100 °C, and the second peaks between 200 and 300 °C, indicating the degradation of HA and KET. Rheology tests in the oscillatory regime classified the hydrogels as non-Newtonian fluids, time-dependent, and thixotropic. Mouse macrophages (RAW

264-7) presented viability of 83.6% for HA, 50.7% for KET, and 92.4%, 66.1%, 65.3%, and 87.7% for hydrogels, in addition to the absence of genotoxicity. Conclusions: Hyaluronic acid associated with ketoprofen shows satisfactory physicochemical and biological properties for use as viscosupplementation. As a limiting point of this study, further research is needed to evaluate the pharmacodynamic, toxicological, and pharmacokinetic characteristics of a complete organism.

Keywords: non-steroidal anti-inflammatory agentes; polysaccharides; osteoarthritis.

RÉSUMÉ

Les désordres temporo-mandibulaires (DTM) constituent un problème de santé publique affectant environ 12 % de la population mondiale. Le traitement repose sur l'utilisation d'analgésiques, d'anti-inflammatoires non stéroïdiens, de corticoïdes, d'anticonvulsivants ou sur l'arthrocentèse associée à la viscosupplémentation à base d'acide hyaluronique. Cependant, l'utilisation isolée de l'acide hyaluronique lors de la viscosupplémentation ne semble pas suffisante pour réguler le processus inflammatoire intra-articulaire. Ainsi, nous proposons de développer et d'évaluer in vitro les propriétés physico-chimiques et biologiques d'hydrogels d'acide hyaluronique (HA) associés au kétoprofène (KET) en tant que nouveau traitement thérapeutique pour les DTM. Les hydrogels ont été synthétisés avec 3 % de HA et 0,125 %, 0,250 %, 0,500 % ou 1 % de KET. Des analyses physico-chimiques par spectroscopie infrarouge à transformée de Fourier (FTIR) en réflectance totale atténuée, thermogravimétrie (TGA), rhéologie par balayage de fréquence et d'amplitude, rampe de température et microscopie électronique à balayage (MEB) ont été réalisées avec ou sans stérilisation et soumises à des cycles. La cytocompatibilité et la génotoxicité (test des micronoyaux) ont été évaluées sur des macrophages de souris (RAW 264.7) pendant 24 heures. Résultats : Le spectre FTIR a présenté des absorptions caractéristiques du HA et du KET. En TGA, deux pics de perte de masse ont été observés : le premier représentant l'évaporation de l'eau entre 30 et 100 °C, et le second entre 200 et 300 °C, indiquant la dégradation du HA et du KET. Les essais

rhéologiques en régime oscillatoire ont classé les hydrogels comme des fluides non newtoniens, dépendants du temps et thixotropes. Les macrophages de souris (RAW 264.7) ont montré une viabilité de 83,6 % pour l'AH, 50,7 % pour le KET et de 92,4 %, 66,1 %, 65,3 % et 87,7 % pour les hydrogels, en plus de l'absence de génotoxicité. Conclusions :L'acide hyaluronique associé au kétoprofène présente des propriétés physico-chimiques et biologiques satisfaisantes pour une utilisation en viscosupplémentation. Comme limite de cette étude, des recherches supplémentaires sont nécessaires pour évaluer les caractéristiques pharmacodynamiques, toxicologiques et pharmacocinétiques dans un organisme entier.

Mots-clés : anti-inflammatoires non stéroïdiens ; polysaccharides ; ostéoarthrite.

*Ketoprofen Associated with Hyaluronic Acid Hydrogel for Temporomandibular Disorder Treatment: An In Vitro Study**

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1. Introduction

The American Academy of Head, Neck and Facial Pain defines temporomandibular disorders (TMD) as a comprehensive term, being a set of pathophysiological conditions that affect the musculoskeletal and neuromuscular structures of the masticatory muscles and temporomandibular joint (TMJ) [1]. TMD is an important public health problem with a variant distribution, affecting around 6 to 12% of the global population, and is more frequent in women between the ages of 20 and 40 [2]. TMD is also considered the most common cause of chronic pain of non-dental origin in the orofacial area [3].

Osteoarthritis of the temporomandibular joint is one of the most common forms of temporomandibular dysfunction and can be described as a low-inflammation arthritic condition when compared to systemic arthritis [4]. The pathophysiological process of TMD is complex because although it is defined as a local inflammatory disease, its development can be due to local and systemic factors [5]. Local factors include microtrauma secondary to bruxism, parafunctional habits, and improper displacement of the articular disc. Systemic factors include rheumatoid, psoriatic, and reactive arthritis [6,7]. This highlights the importance of the host's immune-inflammatory response in the osteoarthritic process [8].

Regarding patients' pain, Sebastiani et al. [9] showed that the genetic polymorphism of IL-6 in some patients leads to an increase in the expression of this cytokine, contributing negatively to the sensitivity and duration of pain. The Consortium for Research on Genetics and Quality of Life suggested that the IL-6 production gene is associated with overall quality of life, where the interaction of the cytokine with its receptors generates inflammation, activation of the vagal afferent nerve, and promotes the severity and duration of pain, as well as triggering depression [10].

The condition has an established impact on social, economic, and health structures. Studies point to reduced levels of health-related quality of life, socialization, and social networks, together with increased depression among TMD patients [11,12,13]. Pain and dysphagia affect both the physical functioning and emotional well-being of the patient; this manifests as a reduction in the ability to consume typical diets and decreased participation in social events [14].

TMD treatment is based on the patient's clinical manifestations. Today, because it is understood that the immunoinflammatory process plays a fundamental role in the pathogenesis of the disease, interventions are based on the use of analgesics, non-steroidal anti-inflammatory drugs, corticosteroids, muscle relaxants, benzodiazepines, antidepressants, and anticonvulsants [15,16].

Arthrocentesis, a minimally invasive surgical procedure, aims to reduce the concentration of pro-inflammatory cytokines in the synovial fluid and cleave possible adhesions in the articular disc [17,18,19]. Arthrocentesis can also be associated with hyaluronic acid-based viscosupplementation to restore the biomechanical properties of the synovial fluid [20,21,22,23,24]. Viscosupplementation is a therapy that consists of an intra-articular injection of sodium hyaluronate (HA) into the joint compartments, applied to promote improvement in mandibular mobility, joint homeostasis, and friction decay, stimulating type B synoviocytes to produce additional synovial fluid, which results in improvements in the rheological properties of the synovial fluid, superior lubrication, absorption of functional loads and stimulation of chondrocyte proliferation in fibrocartilage (januzzi). However, the use of hyaluronic acid (HA) alone does not seem to be enough to regulate the intra-articular inflammatory process [16,25].

As for the principles of hyaluronic acid, it is a natural polysaccharide present in various tissues of the human body, including the skin, cartilage, and synovial fluid [26]. It consists of a linear molecule made up of repeated disaccharide units composed of

D-glucuronic acid and N-acetylglucosamine [27,28]. It has several biological functions, including regulating tissue hydration and lubrication, maintaining the structural integrity of tissues, and interacting with cells via specific receptors such as CD44 [29,30]. Hyaluronic acid is also an important component in the formulation of hydrogels since its ability to form bonds allows the crosslinking of polymers, resulting in materials with adjustable mechanical and drug-release properties [31,32,33].

Ketoprofen (KET) is a propionic acid derivative non-steroidal anti-inflammatory drug (NSAID) with analgesic and antipyretic properties [34,35]. In various branches of medicine and dentistry, KET is used to reduce pain associated with inflammatory processes and to treat chronic pain [36]. The indole methyl moiety in KET is involved in the enzyme-inhibiting properties. KET selectively inhibits cyclooxygenase 1 and 2 and blocks prostaglandin synthesis at the peripheral level [37]. Also, in recent studies, KET has been shown to be the therapy of choice for treating osteoarthritis [38,39]. We hypothesize that the combination of the biomechanical properties of the hyaluronic acid hydrogel and the immunomodulatory effect of KET could improve the success of treatment by temporomandibular dysfunction. Therefore, the aim of this study was to evaluate the physicochemical and biological properties in vitro of HA hydrogels associated with ketoprofen to propose a new therapeutic compound.

2. Results and Discussion

2.1. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of HA showed characteristic absorptions at 3300 cm^{-1} stretching of (OH) and (NH), 2882 cm^{-1} stretching (C-H) presented in lines 1 and 2; 1600 cm^{-1} asymmetric stretching of carboxyl groups (C = O) presented in line 4; 1560 cm^{-1} (amine II); 1402 cm^{-1} symmetric stretching of carboxyl groups (CO); 1148 cm^{-1}, the C-O-C group (O bridge); 1080 cm^{-1} exocyclic groups (C-O) and (C-C); and 1036 cm^{-1} (C-OH) [40,41,42,43].

The FTIR spectrum of KET showed characteristic absorptions at 1701 cm^{-1} acid carbonyl group (C=O), 1660 cm^{-1} ketone carbonyl group (C=O) [44], 1600 cm^{-1} aromatic stretching (C=C) presented in line 4, 1431 cm^{-1} CH-CH_3 deformation, 1080 cm^{-1} exocyclic groups (C-O) and (C-C), 1036 cm^{-1} (C-OH), 716 and 966 cm^{-1} out-of-plane deformation (C-H) [45,46,47,48].

The FTIR spectrum of the gel (3% HA + 1% KET) showed characteristic absorptions at 3300 cm^{-1} stretching of (OH) and (NH); 2882 cm^{-1} stretching (C-H) presented in lines 1 and 2 in Figure 1; 1660 cm^{-1} ketone carbonyl group (C=O); 1600 cm^{-1} aromatic stretching (C=C) or asymmetric stretching of carboxyl groups (C = O) presented in lines 3 and 4; 1148 cm^{-1}, the C-O-C group (O bridge); and 1080 cm^{-1} exocyclic groups (C-O), shown in lines 5 and 6; absorption in 716 cm^{-1} representative of out-of-plane deformation (C-H), shown in line 7. These results suggest the presence of both compounds in the hydrogel sample 3% HA + 1% KET without a spontaneous reaction between the compounds.

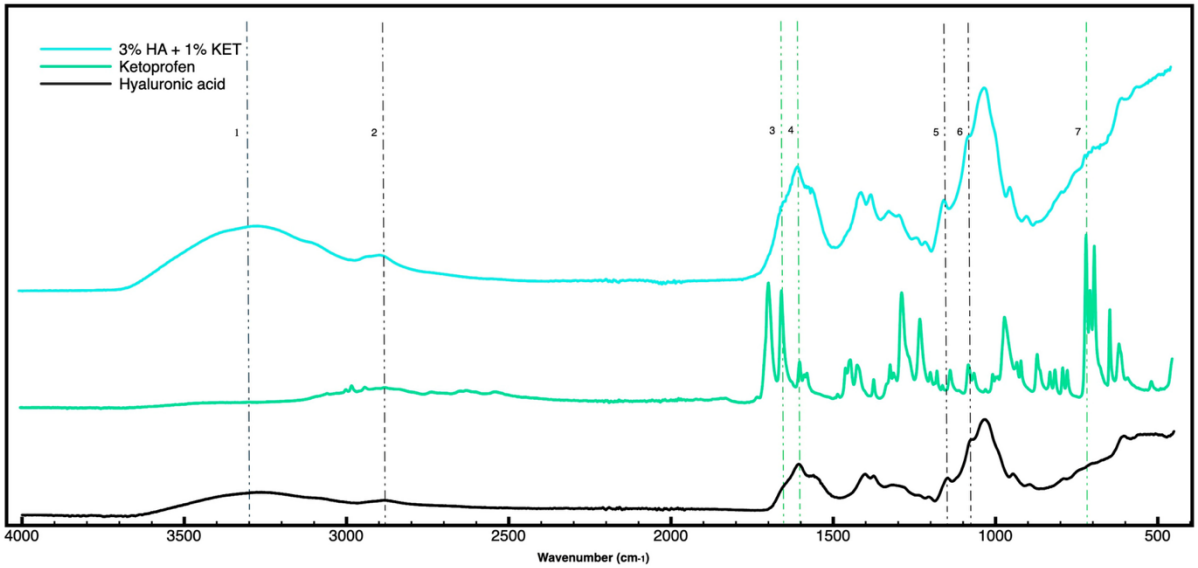


Figure 1. FTIR spectra of HA (line black), KET (line green), and hydrogel 3% HA associated with 1% KET (line blue). The vertically dotted black lines indicate the molecular signatures of HA present in the isolated molecule and in the gel combined with KET. The vertically dotted lines in green indicate the molecular signatures of KET present in the isolated molecule and in the gel combined with HA.

2.2. Thermogravimetric Analysis (TGA)

The thermogravimetric analysis graph of hyaluronic acid shows one peak of mass drop between 220 and 260 °C, representing 40% of the total mass [49]. The thermogravimetric analysis graph of ketoprofen shows one peak of mass drop between 200 and 290 °C, representing 100% of the mass [50]. The thermogravimetric analysis graph of 3% HA + 1% KET shows two regions of mass drop, the first between 30 and

100 °C, representing the evaporation of the water mass of the hydrogel, and the second between 200 and 300 °C, representing the degradation of hyaluronic acid and ketoprofen, expressed in Figure 2.

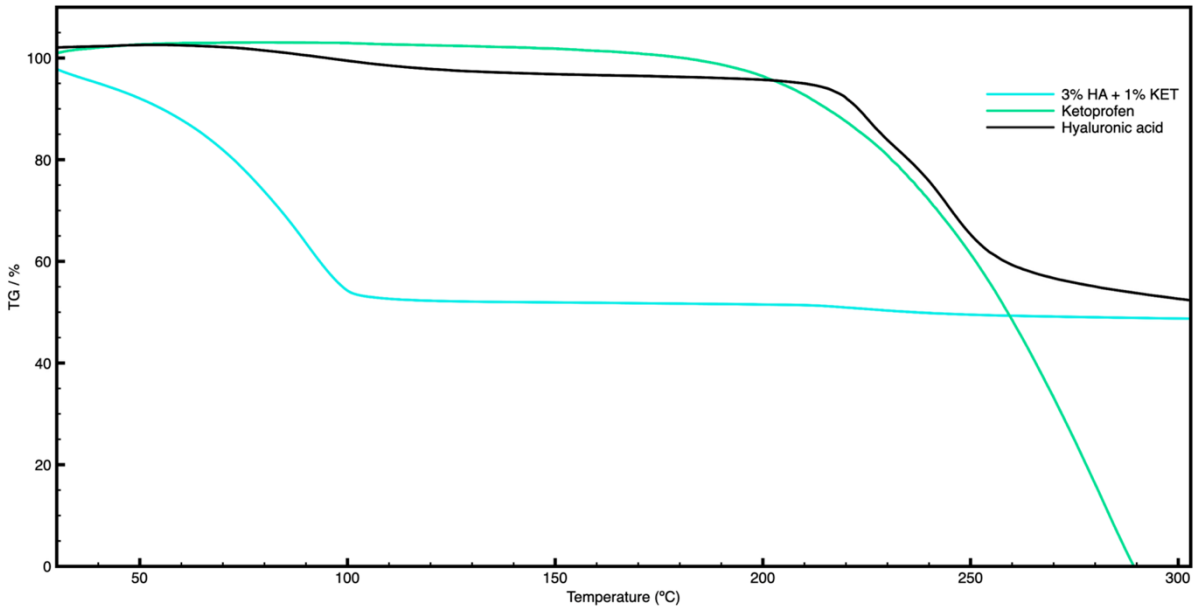


Figure 2. Analysis of the decomposition of masses in relation to temperature. TGA spectra of HA (line black), KET (line green), and hydrogel 3% HA associated with 1% KET (line blue).

It is known that the hyaluronic acid molecule at neutral pH attracts water molecules up to 10,000 times its mass. The water molecules interact with the hyaluronic acid through hydrogen bonds, which stabilize the molecular structure of the hydrogel. When the hydrogel is heated, the kinetic energy and, consequently, the thermal energy of the solution increases, leading to greater agitation of the molecules, breaking the hydrogen bonds, thus allowing the evaporation of the water.

Given that the human body temperature is around 36 to 37.5 °C, it is expected that the hydrogel will lose mass due to water loss. However, the hydrogel would be in a closed system (temporomandibular joint), which would reduce the dispersion of water molecules into the environment. In addition, the human body has physiological mechanisms for maintaining tissue hydration, which would contribute to maintaining the hydration of the hydrogel. Although hyaluronic acid is hydroscopic, the hydrogel

will have a volumetric limitation determined by the size of the joint capsule, thus limiting water absorption.

Regarding hyaluronic acid molecules and ketoprofen, there is no impact in the intracorporeal environment, given their high thermodegradation temperatures.

2.3. Rheology Assay

2.3.1. Frequency Sweep

The frequency analyses of non-autoclaved hydrogels (Figure 3A–D) indicate a higher elastic modulus for the hyaluronic acid alone sample (3% HA), while the addition of ketoprofen reduces the viscoelasticity (G')—a similar effect was observed in the study by Kurbasic et al. [51]. When we evaluated only the hydrogels associated with ketoprofen, it was possible to see that the viscoelastic modulus gradually increased with the concentration of the anti-inflammatory added. It was also possible to see that the addition of ketoprofen increases the gel's stability over time.

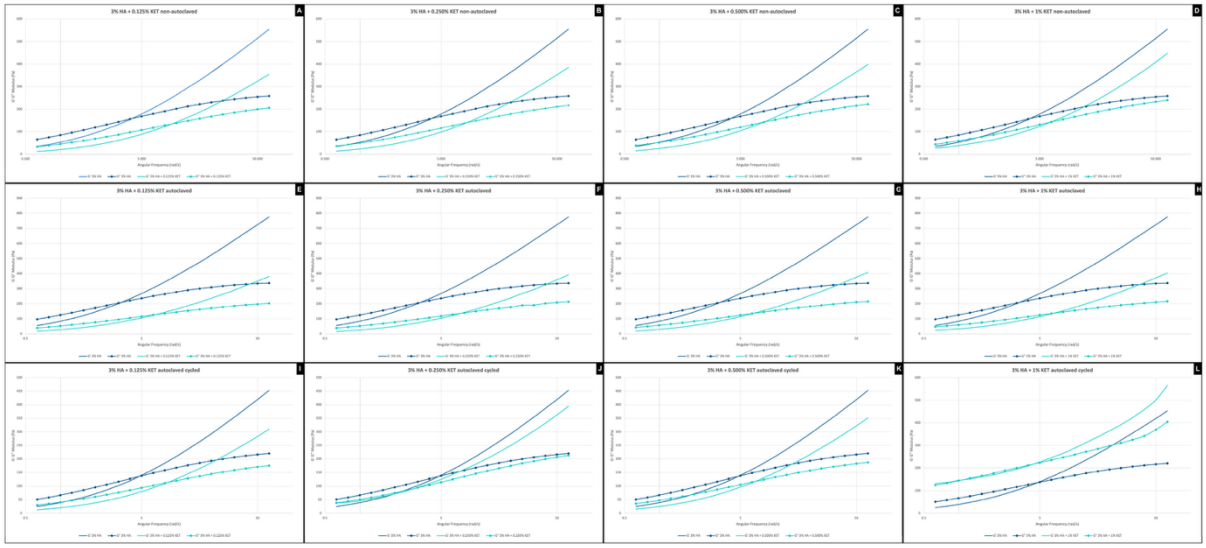


Figure 3. Frequency sweep of 3% HA + 0.125% KET hydrogel non-autoclaved (A), 3% hyaluronic acid HA + 0.250% KET hydrogel non-autoclaved (B), 3% HA + 0.500% KET hydrogel non-autoclaved (C), 3% HA + 1% KET hydrogel non-autoclaved (D), 3% HA + 0.125% KET hydrogel autoclaved (E), 3% HA + 0.250% KET hydrogel autoclaved (F), 3% HA + 0.500% KET hydrogel autoclaved (G), 3% HA + 1% KET hydrogel autoclaved (H), 3% HA + 0.125% KET hydrogel autoclaved cycled (I), 3% HA + 0.250% KET hydrogel autoclaved cycled (J), 3% HA + 0.500% KET hydrogel autoclaved cycled (K), 3% HA + 1% KET hydrogel autoclaved cycled (L).

The autoclaving process causes the hydrogels to stiffen, a phenomenon observed in the initial values of G' and G", making the material more viscoelastic. The comparison of stability between autoclaved and non-autoclaved materials shows no differences.

The cycling process applied to the autoclaved hyaluronic acid gel (3% HA) showed a reduction in the viscoelasticity modulus with G' starting at 450 Pa, compared to 800 Pa for the autoclaved material alone and 550 Pa for the synthesized material alone. It can also be seen that the 3% HA + 1% KET sample (Figure 3L) maintains the ratio between G' and G" but shows a gain in viscoelasticity, with an increase in the G' modulus, giving viscoelasticity and stability superior to the 3%HA group.

Fam et al., 2007 [52] evaluated the viscosity of knee synovial fluids, finding values from 1 to 175 Pa for healthy patients and 0.1 to 1 Pa for patients with diagnosed osteoarthritis. In the present work, it is possible to verify that the hydrogel associated with ketoprofen presents viscosity characteristics at zero shear rates of 150 Pa for the hydrogels containing 3% AH + 0.125% KET and 3% AH + 0.250 KET, and 170 Pa for the hydrogels 3% AH + 0.500% KET and 3% AH + 1% KET. These values show a similarity to the consistency of human joint fluids. It is important to highlight that studies that evaluate the viscosity of the synovial fluid of the temporomandibular joint are scarce in the literature, with no rheological data being found for discussion.

2.3.2. Amplitude Sweep

The non-autoclaved isolated hyaluronic acid gel (3%HA) has a higher stiffness than the samples associated with ketoprofen, as shown by the distance between the G' and G" moduli or even by the state of flow, in which the gel shows deformation near the 2% region. This result goes against that found in the study by Otto & Froelich [53], who observed no change in the amplitude curve after adding ketoprofen. This dissimilarity can be explained by the difference in the base polymer used to form the hydrogel, which, in our study, was HA, and in Otto & Froelich's study, was Carbopol ®.

The samples associated with KET show a decrease in elastic modulus. As an example, the 3% HA + 0.250% KET sample (Figure 4B) has a linear viscoelastic region, with a deformation of 1% to enter the flow state when G" is higher than G', i.e., fluidized. Among all the gel concentrations, the 3% HA + 0.500% KET (Figure 4C)

sample shows the greatest reduction in viscosity, with G' and G" modules very close to each other, almost becoming a viscoelastic liquid.

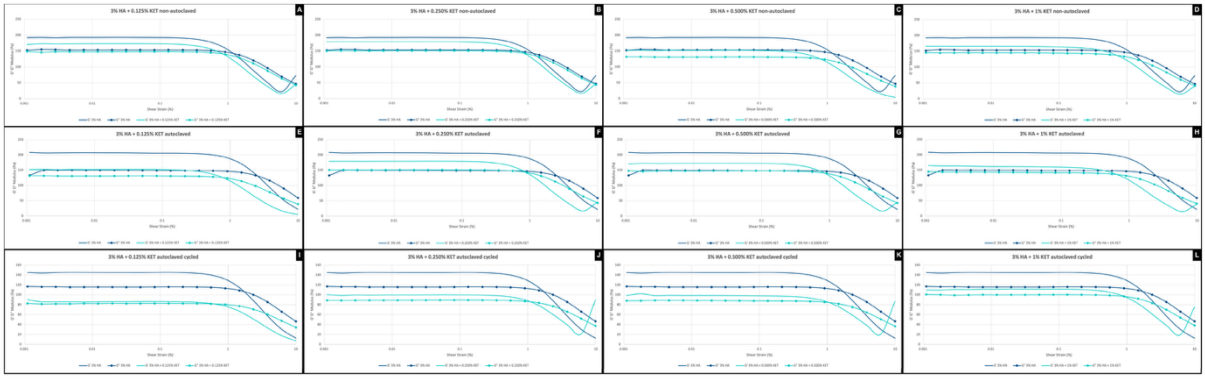


Figure 4. Amplitude sweep of 3% HA + 0.125% KET hydrogel non-autoclaved (A), 3% HA + 0.250% KET hydrogel non-autoclaved (B), 3% HA + 0.500% KET hydrogel non-autoclaved (C), 3% HA + 1% KET hydrogel non-autoclaved (D), 3% HA + 0.125% KET hydrogel autoclaved (E), 3% HA + 0.250% KET hydrogel autoclaved (F), 3% HA + 0.500% KET hydrogel autoclaved (G), 3% HA + 1% KET hydrogel autoclaved (H), 3% HA + 0.125% KET hydrogel autoclaved cycled (I), 3% HA + 0.250% KET hydrogel autoclaved cycled (J), 3% HA + 0.500% KET hydrogel autoclaved cycled (K), 3% HA + 1% KET hydrogel autoclaved cycled (L).

The autoclaving process did not cause any significant changes in the amplitude analysis of the gels associated with ketoprofen. The only point to note is that the sample isolated with HA (3% HA) has a G' modulus of more than 200 Pa, while the non-autoclaved sample has a G' modulus of less than 200 Pa, which indicates an increase in the modulus of elasticity.

When observing all the groups (Figure 4E–H) after the aging process (cycling), it is possible to see that the gels suffer a decrease in the modulus of elasticity (G'), showing polymer relaxation. The values measured in the G' modulus start at around 150 Pa, while the sample autoclaved without undergoing the cycling process has a G' modulus of over 200 Pa.

It was also possible to see that the addition of KET to the HA gel reduces the modulus of elasticity, where G' is around 150 Pa, while the 3% HA + 1% KET sample has a G' of around 110 Pa. The decrease in modulus of elasticity is proportional to the

decrease in KET concentration, in which the 3% HA + 0.125% KET (Figure 4E) group shows G' in the order of 90 Pa.

The amplitude analyses indicate that the stress process promoted by cycling (Figure 4I–L) has an impact on the elastic characteristics of the hydrogels, making them more fluid.

2.3.3. Temperature Ramp

The temperature ramp of the non-autoclaved gels indicates an intermediate elastic modulus characteristic at 210 Pa. The concentrations of 3% HA + 0.125% KET (Figure 5A) and 3% HA + 0.250% KET (Figure 5B) show low viscosity, while the concentrations of 3% HA + 0.500% KET (Figure 5C) and 3% HA + 1% KET (Figure 5D) show higher viscosity than the sample of hyaluronic acid alone (3% HA).

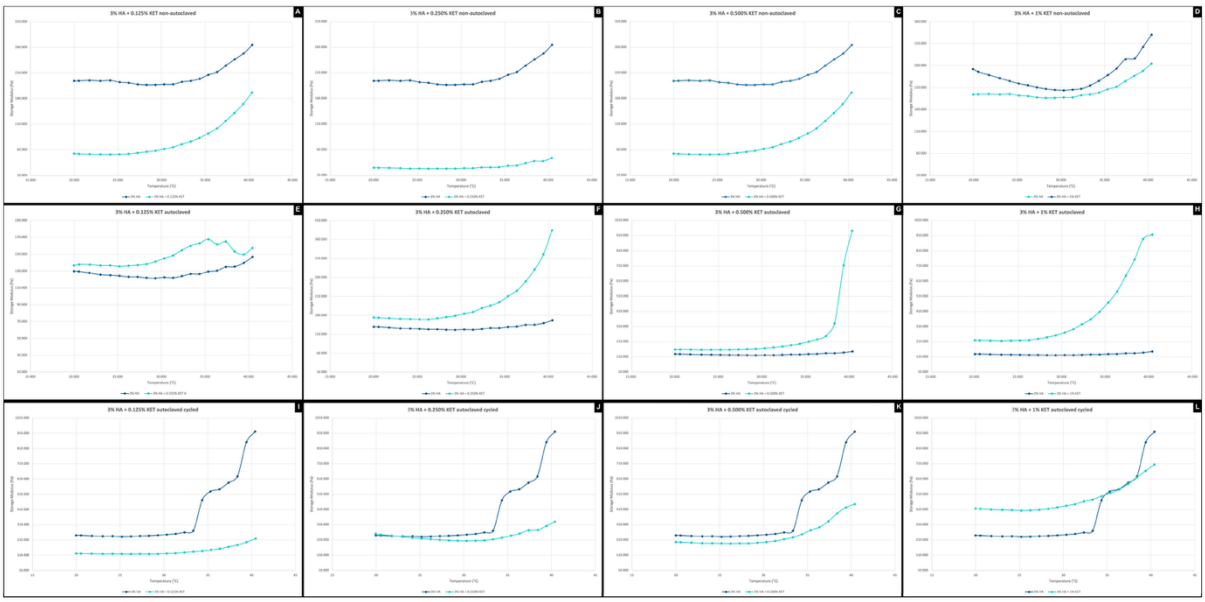


Figure 5. Temperature ramp of 3% HA + 0.125% KET hydrogel non-autoclaved (A), 3% HA + 0.250% KET hydrogel non-autoclaved (B), 3% HA + 0.500% KET hydrogel non-autoclaved (C), 3% HA + 1% KET hydrogel non-autoclaved (D), 3% HA + 0.125% KET hydrogel autoclaved (E), 3% HA + 0.250% KET hydrogel autoclaved (F), 3% HA + 0.500% KET hydrogel autoclaved (G), 3% HA + 1% KET hydrogel autoclaved (H), 3% HA + 0.125% KET hydrogel autoclaved cycled (I), 3% HA + 0.250% KET hydrogel autoclaved cycled (J), 3% HA + 0.500% KET hydrogel autoclaved cycled (K), 3% HA + 1% KET hydrogel autoclaved cycled (L).

The process of autoclaving the samples suggests a molecular rearrangement of the polymer, in which the gradual increase in the concentration of KET affects the increase in the viscosity profile. This can be seen in Figure 5, with emphasis on the 1% concentration (Figure 5E), which shows an upward viscosity ramp from a temperature of 25°C.

The groups that were autoclaved with subsequent cycling (Figure 5I–L) show the phenomenon of material aging, with a reduction in the rigidity and elastic memory of the material, when compared to the materials that were autoclaved only. García-Villegas et al. also observed the process that affected the viscosity values the most was temperature cycling due to the abrupt temperature changes [54]. The 3% HA + 1% KET (Figure 5I) sample shows stabilization in the elastic modulus, with no increase in stiffness as the temperature rises, i.e., an attenuation of the elastic modulus as the temperature varies.

2.4. Scanning Electron Microscopy with Field Emission Gun (SEM-FEG)

In scanning electron microscopy with a field emission gun, no structural difference could be observed between the isolated hyaluronic acid hydrogels when compared to those associated with ketoprofen (Figure 6). SEM micrographs revealed a sheet-like structure containing pores of various sizes and interconnected with a three-dimensional network, with spacing between the hydrogel layers. The micrograph in Figure 6E shows a structure composed of smaller pores and fewer spaces between the layers. Structures shown in Figure 6B–E have a rougher appearance than the ones shown in Figure 6A. The porosity of the gels was not evaluated metrically since the drying process influences the crosslinks and, therefore, the porosity of the material [55].

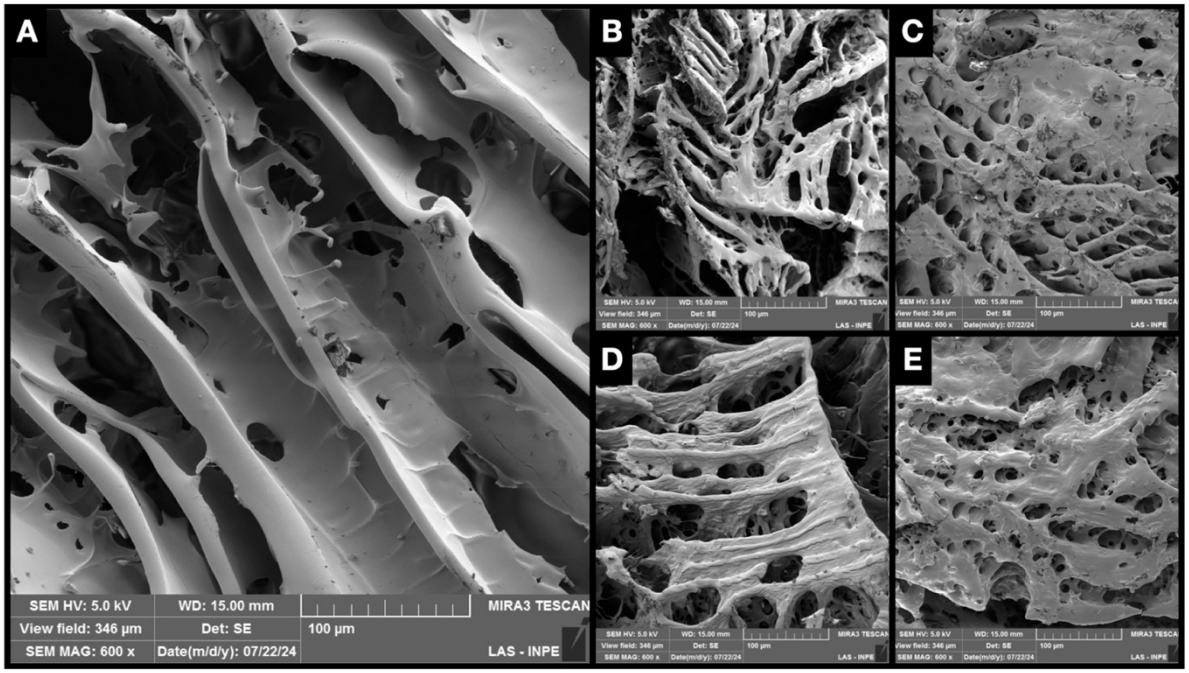


Figure 6. Micrography of 3% HA hydrogel (A), 3% HA + 0.125% KET hydrogel (B), 3% HA + 0.250% KET hydrogel (C), 3% HA + 0.500% KET hydrogel (D), 3% HA + 1% KET hydrogel (E).

2.5. Cell Viability by Resazurin Essay

After contact for 24 h with the 3% HA hydrogel, mouse macrophages (RAW 264.7) presented cell viability of 83.6%, while the HA hydrogels conjugated to concentrations of 0.125, 0.250, 0.500, and 1% KET presented viability of 92.4%, 66.1%, 65.3%, and 87,7%. The application of 1% ketoprofen resulted in 50.7% cell viability, as shown in Figure 7.

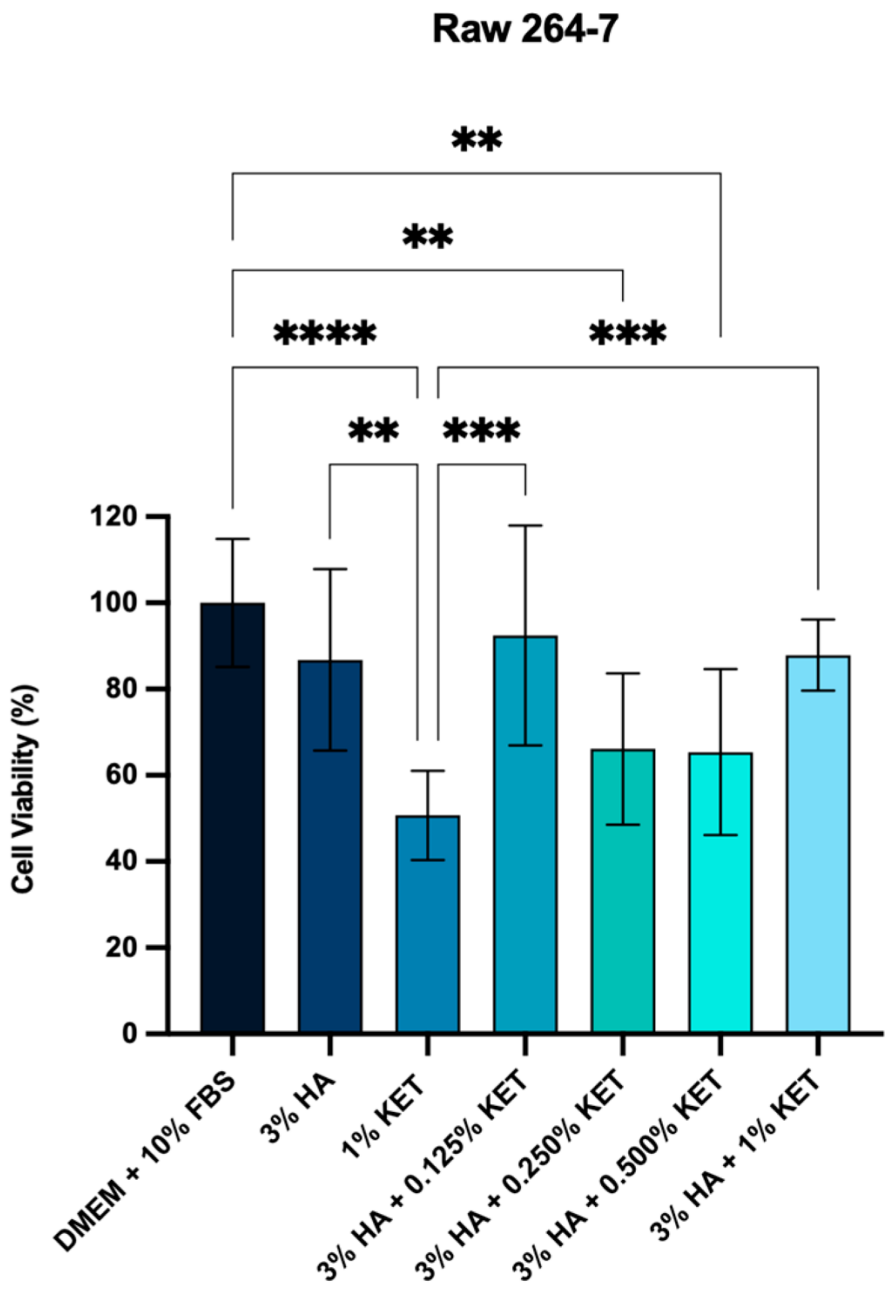


Figure 7. Cytocompatibility by hydrogels on mouse macrophages (RAW 264.7). p < 0.0021 (**), p < 0.0002 (***), p < 0.0001 (****).

Recent studies have sought to improve the availability, solubility, and compatibility of KET as an anti-inflammatory agent associated with materials. Chachlioutaki et al. evaluated the cytotoxic activity of zinc oxide (ZnO) nanofibers associated with KET at concentrations of 0.5 to 1 mg/mL. The polymer based on 3% hydroxypropylmethyl cellulose (HPMC) associated with 1 mg/mL of KET exhibited variations of 30 to 40% viability with the times of 24, 48, and 72 h of application on mouse macrophages (RAW

264-7). In contrast, the present study shows that the HA polymer associated with KET presents greater cytocompatibility with the macrophage lineage (RAW 264.7), exhibiting viability greater than 60%.

Criado-Gonzalez [56] evaluated the application of hybrid hydrogels based on phosphorylated tripeptides Fmoc-FFpY (F: phenylalanine, pY: phosphorylated tyrosine) associated with KET. The gel at a concentration of 0.5%, applied by direct contact, showed cell viability higher than 85% on mouse macrophage (RAW 264.7) and human dermal fibroblast (HDF) lines. The results corroborate the present study, indicating cell viability ranging from 83.6% to 60.3% on the macrophage line. It was possible to verify that concentrations of 0.125 and 1.0 demonstrate the best viability values, indicating the absence of toxicity to the mouse macrophage lineage.

Moses et al. [57] evaluated the application of KET in the synovial membranes of horses after stress by Lipolysaccharides (LPS). The tissues were cultured in the laboratory under aseptic conditions and incubated with 0.001 µg of LPS/mL for tissue inflammation. The evaluation demonstrated that the administration of 4 mg/mL of KET promoted a reduction of 1 pg/mL in prostaglandin production, in addition to not promoting significant changes in the production of hyaluronic acid and maintaining preserved tissue characteristics. Wyns et al. [57] reinforce the low cellular toxicity, as well as the anti-inflammatory activity of KET, showing the reduction in prostaglandins in pig monocytes, besides indicating the decrease in Interleukin 6. The findings of Moses et al. [57] and Wyns et al. [58] corroborate the present study, with a view to the clinical application of HA gels associated with KET, supporting cytocompatibility in cartilage tissue cells and mouse macrophages, additionally to highlighting the anti-inflammatory activity promoted by the non-steroidal drug.

2.6. Genotoxicity Assay by Micronucleus

The genotoxicity evaluation for the DMEM + 10% BFS, 3% HA, and 3% HA + 0.250% KET groups showed the formation of two micronuclei. The 1.0% KET, 3% HA + 0.500% KET and 3% HA + 0.125% KET groups showed three micronuclei. The 3% HA + 1.0% KET group showed the formation of five micronuclei, while the positive genotoxicity control caused the formation of 29 micronuclei.

The evaluation of the genotoxicity of KET and HA by micronuclei is scarce in the literature, making this work pioneering in this regard. Philipose et al. [59] evaluated the mutagenic potential of KET using the AMES test (in vitro) and the evaluation of sister

chromatid exchange (SCE) in cells extracted from the bone marrow of rats (in vivo). The results of the AMES test, using the Salmonella strains TA97a, TA100, and TA102, demonstrated the toxicity of the drug at a concentration of 5000 µg, at a threshold of concentrations of 1, 10, 100, 1000, and 5000 µg. The authors indicate the inconclusiveness of this evaluation because the same result was not obtained when the test was repeated.

The in vivo evaluation involved the administration of 270 mg/kg of KET to rat bone marrow cells, resulting in an average of 5.14 cells with chromatid exchange in a total of 150 cells evaluated, a value close to the control group (-), which presented 4.68 cells. In contrast, the group presented with the genotoxic drug (Cyclophosphamide 10 mg/kg) presented an average of 19.86 cells with chromatid exchange in a total of 150 cells evaluated. The authors conclude that ketoprofen has low genotoxic potential in in vivo tests [59]. These results corroborate the present study, which, despite using a different methodology, contributes to the literature, with results indicating the absence/low formation of micronuclei in mouse macrophages exposed to 1 mM/mL of KET.

Salústio et al. [60] reinforce the absence of genotoxicity of KET in relation to human metabolism. The researchers evaluated the activity of reactive acyl glucuronides, a molecule derived from the metabolism of UDP-glucuronosyltransferases (UGTs) that performs the hepatic excretion of xenobiotics on KET. The results conclude that KET presents cellular toxicity, obtaining less than 50% of predictions of mouse hepatocytes with a concentration of 0.12 mM of KET. However, the comet test shows that the drug does not present genotoxicity, presenting extraction of genetic material similar to the control group. These results corroborate the present study, where cellular observation of mouse macrophages was observed in 50.4% after the application for 24 h of 1 mM of KET and the absence of genotoxicity after the application of the same parameters, observed in Figure 8.

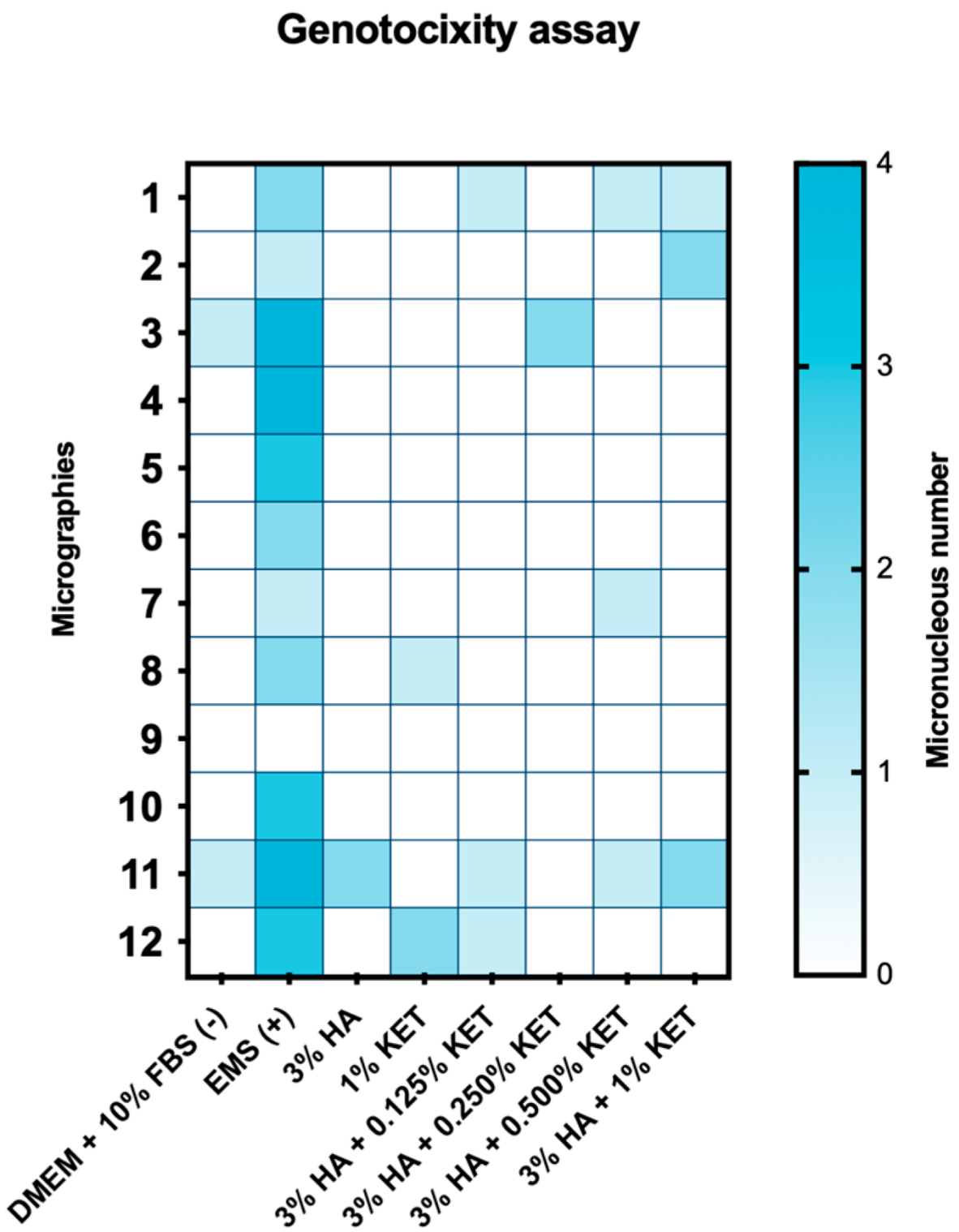


Figure 8. Genotocixity assay by micronucleus.

As a limiting point of this study, it is worth noting that the biological analyses were performed on a single type of cell in a monolayer, exclusively evaluating the metabolic behavior of an immune cell (macrophages). Further research is needed to evaluate the application of the drug in chondrocytes and synoviocytes, exhausting the pharmacodynamic profile of the drug in vitro. In addition, it is necessary to use in vivo tests to evaluate the toxicological and pharmacokinetic characteristics of a complete organism.

3. Conclusions

Hyaluronic HA associated with KET shows satisfactory physicochemical and biological properties for use as viscosupplementation. The 3% HA + 1.0% KET hydrogel seems to be the best choice since it has the highest concentration of KET and shows no significant biophysicochemical changes when compared to the controls. In vivo tests are necessary to determine the pharmacokinetics, pharmacodynamics, and lethal dose of the drug before clinical trials can begin to ascertain their therapeutic applicability.

4. Materials and Methods

4.1. Chemical Reagents

Hyaluronic acid (HA) (CAS nº: 9004-61-9, purity: 95.9%, molecular weight: 1.300.000 Dalton, Inlab Analitic®, code: HA2022042345X, São Paulo, Brazil), ketoprofen (CAS nº: 22071-15-4, purity: 99.08%, Lepuge®, code: KPO-2105014), phosphate buffer saline (PBS) (code: P2272, Sigma-Aldrich®, St. Louis, MO, USA), sodium hydroxide (NaOH) (CAS nº: 1320-73-2, code: S5881, purity: 98%, Sigma-Aldrich®, St. Louis, MO, USA), formic acid (CAS nº: 64-18-6, code: 27001, purity:98%, Sigma-Aldrich®, St. Louis, MO, USA), methanol (CAS nº: 67-56-1, purity: 99.8%, Synth®, Diadema, Brazil), Eagle's medium modified by Dulbecco—DMEM (LGC Biotechnology®, Cotia, Brazil), Fetal Bovine Serum—FBS (Invitrogen®, New York, NY, USA), 7-Hidróxi-3H-fenoxazin-3-ona-10-óxido (Resazurin) (CAS nº: 62758-13-8, code: R1017, Sigma-Aldrich®, St. Louis, MO, USA), Ethyl Methane sulfonate (EMS) (CAS nº: 62-50-0, code: M08080, Sigma-Aldrich®, St. Louis, MO, USA), Cytochalasin B (CAS nº: 14930-96-2, code: C2743, purity: 98%, Sigma-Aldrich®, St. Louis, MO, USA), 4',6-Diamidino-2-fenilindol, 2-(4-Amidinofenil)-6-indolecarbamidina with fluoroshield (DAPI) (CAS nº: 28718-90-3, code: F6057, Sigma-Aldrich®, St. Louis, MO, USA), Ethanol (CAS nº: 64-17-5, purity:99.5%, Synth®, Diadema, Brazil).

4.2. Equipments

Class II biological safety cabinet (Veco®, biosseg-06, Sumaré, São Paulo, Brazil), analytical balance (Mettler Toledo®, Balance XPR106DUH/A, Columbus, OH, USA), type I ultrapure water purification system (Allcron®, direct-Pure® Genie, São Paulo, Brazil), autoclave (Cristofoli Biossegurança®, Vitale21, Campo Mourão, Parana, Brazil), dual asymmetric centrifuge mixer (Hauschild®, Speed MixerDAC150. 1 FVZ, Waterkamp, Hamm, Germany), Ph meter (Digimed®, DM-20, São Paulo, Brazil), Spectrometer Matrix Assisted Laser Desorption Ionization-Time of Flight—MALDI-TOF-MS Ettan (Amersham Biosciences®, Amersham, UK), high-performance liquid chromatography with a photodiode detector instrument (HPLC) (Merck-Hitachi®, D-7000, Tokyo, Japan), freezer for cryogenesis (Freezer -80) (NuAire Laboratory Equipement®, Glacier polar edition -86 ultra-low temperature freezer, Plymouth, MA, USA), freeze dryer (Terroni®, LS3000, São Carlos, São Paulo, Brazil), Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer®, LR64912C, Waltham, MA, USA), thermogravimetric analyzer (TGA) (NETZSCH®, STA449F1 Jupiter, Selb, Bavaria, Germany), dynamic shear rheometer (Anton-paar®, SmartPave 92, Graz, Austria), Sputter Coater (Quorum Technologies®, Emitech—SC7620, Kent, UK); Scanning Electron Microscope with Field Emission Gun (FEG®, TESCAN MIRA 3®, Brno, Czech Republic), CO_2 Incubator (Sanyo®, MCO-19AIC(UV), Osaka, Japan), water bath precision (TermoFisher Scientific®, TSGP02, Waltham, MA, USA), refrigerated centrifuge (Labnet®, HEREMLE Z300®, Madrid, Spain), inverted microscope (Ziess®, Axiovert 40C, Jena, Thuringia, Germany), fluorescence microscope (Ziess®, Axio Observer A1, Jena, Thuringia, Germany), spectrophotometer (Lonza Biotek®, ELX808LBS, Winooski, VT, USA), thermal cycler (Nova Ética®, 521-D, São Paulo, Brazil).

4.3. Hydrogels Preparation

A 3% (w/v) solution of HA was prepared in ultra-pure type I water for physico-chemical analysis and biological analysis. Then 1%, 0.5%, 0.25% or 0.125% KET (w/w) was added. The hydrogel was homogenized in a mixer for 1 min and the pH was adjusted to 7.2 with NaOH.

4.4. Sterilization and Thermal Fatigue

The gels were sterilized in an autoclave for 5 min at 118 °C [61].

Sterilized hydrogels were subjected to thermal fatigue cycles in a controlled simulator. Simultaneously, the specimens were thermocycled between 5 °C and 55 °C, maintained at each temperature for 30 s, totaling 1 min per cycle. A total of 1000 thermal cycles were performed in a total of 2 days stored in the device [62].

4.5. Freeze-Drying

The hydrogel samples were frozen at −80 degrees for 24 h and then placed in a freeze-dryer for 3 days to sublimate the solvent.

4.6. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (FTIR)

The freeze-dried samples were analyzed by FT-IR, obtaining wave number spectra between 500 and 4000 cm^{-1}, with a nominal resolution of 4 cm^{-1} and 100 scans per sample.

4.7. Thermogravimetric Analysis (TGA)

The samples of the gels were analyzed by thermogravimetry, obtaining the thermal analysis curves by scanning the temperature range between 30 and 300 °C, with a rate change of 10 °C/min in an atmosphere of argon (Ar).

4.8. Rheology Assay

4.8.1. Frequency Sweep

In the frequency ramp, after the sample had settled, we waited 2 min for the hydrogels to recover and relax. Shear deformation was carried out with a 1% constant and frequency decreasing from 10 to 0.1 hertz.

4.8.2. Amplitude Sweep

In the amplitude ramp, after the sample had settled, we waited 2 min for the polymers to recover and relax. A controlled shear deformation ramp was applied from 0.001 to 100% with a frequency of 10 radians/second, with a fixed frequency.

4.8.3. Temperature Ramp

The sample was preheated to 20 degrees Celsius for 2 min, after which the temperature ramp test was applied with a controlled deformation ramp and frequency, with a deformation of 1%, a frequency of 10 radians/second, and a heating rate of 1 degree Celsius per minute.

4.9. Scanning Electron Microscopy with Field Emission Gun (SEM-FEG)

For SEM, the lyophilized hydrogels were transferred to aluminum stubs and covered with gold for 120 s at 40 mA. After the process, the discs were analyzed and photographed by the scanning electron microscope. Then, the metallized SEM

samples were used for surface elemental characterization by low energy dispersive X-ray cartography technique.

4.10. Biological Performance on In Vitro Cell Cultures

Cytocompatibility was carried out on mouse macrophages (RAW 264-7). Cell culture was carried out in DMEM supplemented with 10% FBS, incubated in an oven at 37 °C, with atmospheric humidity and 5% CO_2.

The tests were carried out in 24-well microplates using 4 × 10^4 cells, cultured in 500 µL of DMEM + 10% FBS medium, and incubated at 37 °C with 5% CO_2 for 24 h. After this period, the supernatant was discarded, and the cells were exposed to the treatments.

4.11. Treatment via Transwell

The treatments were carried out according to the groups: 500 µL of 3% HA; 500 µL of 1% KET; 500 µL of 3% HA + 1% KET; 500 µL of 3% HA + 0.500% KET; 500 µL of 3% HA + 0.250% KET; 500 µL of 3% HA + 0.125% KET; and 500 µL of DMEM + 10% FBS as a control. After 24 h of exposition, the treatments were discarded, and the wells were washed three times with PBS.

4.12. Cell Viability by Resazurin Essay

Metabolic activity was checked using resazurin at 440 µM. For this, 50 µL of the resazurin suspension was added to each well of the microplate, followed by the addition of 450 µL of DMEM + 10% FBS. Incubation in the dark was carried out for 16 h, after which the plate was read in a spectrophotometer at a wavelength of 570 nm. The optical densities (OD) obtained were converted into a percentage of cell viability using the following formula:

$$\% \text{ Metabolic Activity} = (\text{OD Treated Group} \times 100)/\text{Average OD Control Group}$$

4.13. Genotoxicity Assay by Micronucleus

Mouse macrophages at a concentration of 3 × 10^5 cells/mL were cultured in 96-well microplates with 1 mL of DMEM supplemented with 10% SFB for 24 h at 37 °C in a 5% CO_2 atmosphere. The cells were exposed to the experimental groups under the same conditions. The negative control group received only the culture medium, while the positive control group received EMS at a concentration of 5 mM; both treatments were applied for 24 h.

After the treatments, the cells were washed with PBS three times and incubated with cytochalasin B at a concentration of 6 µg/mL for 24 h at 37 °C in a 5%

CO_2 atmosphere. After the incubation, the cells were fixed in 100% methanol for 20 min, followed by staining with DAPI. The dye was removed after 5 min of contact with the cells, followed by three washes with PBS. Micronuclei were analyzed under a fluorescence microscope at 40X magnification with a total of 2000 cells evaluated per well.

4.14. Statistical Analysis

The data obtained was initially analyzed for normality using D'Agostino, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Those that showed normality were then analyzed by ANOVA complemented by Turkey. Those that did not show normality were evaluated by Kruskall Wallys, complemented by Dunn's test. All analyses utilized p value of 0.0001.

Author Contributions

Conceptualization, D.G.M., and L.d.P.R.; methodology, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., M.R.B., L.M.R.d.V., A.L.S.B., B.G., and K.G.; validation, D.G.M., L.d.P.R., F.P.R., V.d.P.M., T.S.-T., M.R.B., L.M.R.d.V., A.L.S.B., B.G., and K.G.; formal analysis, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., M.R.B., and K.G.; investigation, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., and M.R.B.; resources, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., and K.G.; data curation, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., and V.d.P.M.; writing—original draft preparation, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., M.R.B., L.M.R.d.V., A.L.S.B., B.G., and K.G.; writing—review and editing, D.G.M., L.d.P.R., N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., M.R.B., L.M.R.d.V., A.L.S.B., B.G., and K.G.; visualization, N.F.d.S.L., N.V.D.H.F.S., C.P.S., F.P.R., V.d.P.M., T.S.-T., M.R.B., L.M.R.d.V., A.L.S.B., B.G., and K.G.; supervision, L.d.P.R., L.M.R.d.V., A.L.S.B., B.G., and K.G.; project administration, D.G.M., and L.d.P.R.; funding acquisition, D.G.M., L.d.P.R., N.V.D.H.F.S., L.M.R.d.V., and K.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

Not applicable.

Informed Consent Statement

Not applicable.

Data Availability Statement

The raw data supporting the conclusions of this article will be made available by the authors upon request.

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Conflicts of Interest

The authors declare no conflicts of interest.

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2.2 Article – Miranda DG, Ramos LP, Camargo PC, Lopes NFS, Sani-Taiariol T, Baldan MR, Soares CP, Godoi BH, Gritsch K, Grosgogeat B, Borges ALS. *Biophysicochemical Design of a Dual-Function Hydrogel for Synergistic Shock-Absorption and Anti-Inflammatory Action for TMD Therapy. Conception biophysico-chimique d'un hydrogel à double fonction pour une action synergique d'absorption des chocs et anti-inflammatoire dans le traitement des troubles temporo-mandibulaires.*

ABSTRACT

Temporomandibular disorder (TMD) is recognized as a major public health problem, causing pain and physiological and psychosocial limitations. In this context, the present in vitro study investigated the synthesis of a hyaluronic acid (HA) hydrogel with hydrocortisone (Hyd), designed to enhance joint lubrication by reducing mechanical friction and delivering the anti-inflammatory drug. The hydrogels were prepared with 3% HA (30 mg/mL) and Hyd—0.125% (1.25 mg/mL), 0.250% (2.5 mg/mL), 0.500% (5 mg/mL), or 1% (10 mg/mL). Physicochemical analyses included Fourier transform infrared spectroscopy (FTIR), thermogravimetry (TGA), rheological tests (frequency, amplitude, and temperature ramp scans), and field emission scanning electron microscopy (FESEM), performed before and after sterilization and cycling. In addition, cytocompatibility was evaluated by protocol OECD 129 and confocal microscopy, as well as genotoxicity (OECD487) in mouse macrophages (RAW 264.7 strain) per 24 h of exposure. FTIR demonstrated the spectral signatures of the compounds with no covalent interactions between the drugs, as well thermal stability on TGA. Rheology demonstrated that Hyd protected the HA structure after autoclaving, maintaining viscoelastic properties. SEM confirmed homogeneous porous morphology. Biological assays showed cell viability > 70%, but with a dose-dependent increase in genotoxicity (4–17 micronuclei). Confocal analysis revealed increasing cytotoxicity at high Hyd concentrations, indicating a balance between biocompatibility and adverse effects at concentrations ≤ 0.5%. Among the tested formulations, the 3% HA + 0.250% Hyd hydrogel provided the best balance of viscoelastic stability, cytocompatibility, and low

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genotoxicity, supporting its potential as a dual-function intra-articular candidate for TMD therapy.

Keywords: steroidal anti-inflammatory agents; polysaccharides; osteoarthritis; hydrocortisone; hydrogels.

RÉSUMÉ

Les désordres temporo-mandibulaires (DTM) sont reconnus comme un problème majeur de santé publique, provoquant de la douleur ainsi que des limitations physiologiques et psychosociales. Dans ce contexte, la présente étude in vitro a étudié la synthèse d'un hydrogel d'acide hyaluronique (HA) associé à la hydrocortisone (Hyd), conçu pour améliorer la lubrification articulaire grâce à la réduction du frottement mécanique et à la libération du principe actif anti-inflammatoire. Les hydrogels ont été préparés avec 3 % de HA (30 mg/mL) et de la Hyd — 0,125 % (1,25 mg/mL), 0,250 % (2,5 mg/mL), 0,500 % (5 mg/mL) ou 1 % (10 mg/mL). Les analyses physico-chimiques ont compris la spectroscopie infrarouge à transformée de Fourier (FTIR), la thermogravimétrie (TGA), des essais rhéologiques (balayages de fréquence, d'amplitude et rampe de température) et la microscopie électronique à balayage à émission de champ (FE-MEB), réalisées avant et après la stérilisation et les cycles d'utilisation. De plus, la cytocompatibilité a été évaluée selon le protocole OCDE 129 et par microscopie confocale, ainsi que la génotoxicité (OCDE 487) sur des macrophages de souris (ligne cellulaire RAW 264.7) après 24 heures d'exposition. La FTIR a démontré les signatures spectrales des composés, sans interactions covalentes entre les médicaments, ainsi que la stabilité thermique en TGA. La rhéologie a démontré que la Hyd a protégé la structure du HA après l'autoclavage, maintenant les propriétés viscoélastiques. La MEB a confirmé une morphologie poreuse homogène. Les essais biologiques ont montré une viabilité cellulaire > 70 %, mais avec une augmentation dose-dépendante de la génotoxicité (4 à 17 micronoyaux). L'analyse confocale a révélé une augmentation de la cytotoxicité à des concentrations élevées de Hyd, indiquant un équilibre entre biocompatibilité et effets

indésirables à des concentrations ≤ 0,5 %. Parmi les formulations testées, l'hydrogel de 3 % de HA + 0,250 % de Hyd a offert le meilleur équilibre entre stabilité viscoélastique, cytocompatibilité et faible génotoxicité, renforçant son potentiel en tant que candidat intra-articulaire à double fonction pour le traitement du DTM.

Mots-clés : *anti-inflammatoires stéroïdiens ; polysaccharides ; ostéoarthrite ; hydrocortisone ; hydrogels.*

*Biophysicochemical Design of a Dual-Function Hydrogel for Synergistic Shock-Absorption and Anti-Inflammatory Action for TMD Therapy**

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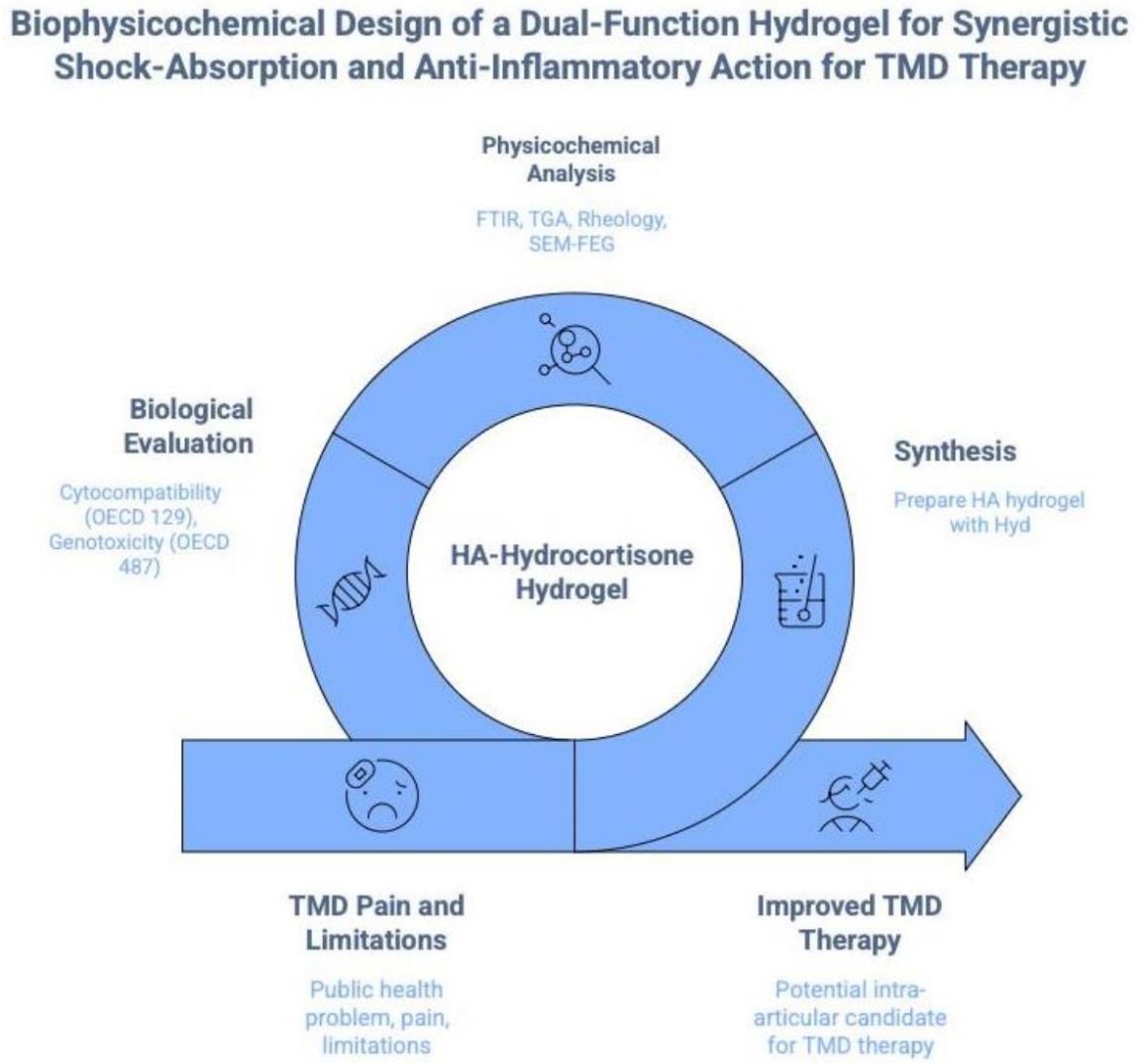
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Graphical abstract



1. Introduction

Temporomandibular dysfunction (TMD) is defined by the American Academy of Orofacial Pain as a collective term that covers various pathophysiological conditions involving the musculoskeletal and neuromuscular structures of the masticatory muscles and the temporomandibular joint (TMJ) [1]. Recognized as a major public health problem, its prevalence varies between 6% and 12% of the general population, with a higher incidence in women aged between 20 and 40. TMD stands out as the main cause of chronic orofacial pain of non-dental origin [2,3].

The Diagnostic Criteria for Temporomandibular Disorders divides TMD into three categories: the first is Muscle Pain (Muscular TMD), with disorders affecting the masticatory muscles; the second classification covers Joint Disorders (Articular TMD) involving displacement of the articular disk (a shock absorber structure) in relation to

the jaw bones or degenerative and inflammatory conditions of the joint such as osteoarthritis and osteoarthrosis; finally, the third classification, called Other Joint Disorders, involves conditions that mimic TMD but have different causes, such as rheumatoid arthritis, neoplasms, or fractures [1].

TMD patients often experience masticatory muscle pain aggravated by mandibular function, movement-related temporal headaches, joint noises (clicking or crackling), limited mouth opening (<40 mm) and episodes of joint locking, as demonstrated by Valesan et al. [3] and Wu et al. [2]. The pathophysiological process of TMD is complex, because although it is defined as a local inflammatory disease, its development can be due to local and systemic factors. Local factors include microtrauma secondary to bruxism, parafunctional habits and improper displacement of the articular disk. Systemic factors include rheumatoid, psoriatic and reactive arthritis. Therefore, the host's immune-inflammatory response stands out [4].

Osteoarthritis of the temporomandibular joint is one of the most common forms of temporomandibular dysfunction and can be described as a low-inflammatory arthritic condition when compared to systemic arthritis [4]. Different cytokines, chemokines, chemokine receptors, enzymes and bone resorption factors have been proposed as markers of TMD. Some authors cite high levels of interleukin (IL)-1β, IL-6, IL-17, interferon (IFN)-γ, tumor necrosis factor (TNF)-α and prostaglandin E2 (PGE2), while a smaller number of studies also show the presence of factors such as matrix metalloproteinase (MMP)-2, MMP-9, aggrecanase-1, superoxide dismutase and receptor-activating nuclear factor κB ligand (RANKL), detected in the synovial fluid of TMD patients [5,6,7].

The pathology has an established impact on social, economic and health structures. Studies point to reduced levels of health-related quality of life, socialization and social networks, together with increased depression among TMD patients. Pain and dysphagia affect both the physical functioning and emotional well-being of the patient, which manifests as a reduction in the ability to consume typical diets and decreased participation in social events [8].

The management of temporomandibular disorders (TMD) is tailored to the patient's specific clinical presentation. Given the established central role of the immunoinflammatory process in the disease's pathogenesis, current therapeutic protocols heavily rely on systemic pharmacotherapy, including analgesics, non-

steroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and muscle relaxants. For cases refractory to conservative medication, arthrocentesis is employed to lavage pro-inflammatory cytokines from the synovial space and release articular disk adhesions. This procedure is often combined with visco-supplementation using intra-articular hyaluronic acid, which aims to restore the viscoelastic properties of the synovial fluid, reducing mechanical friction and improving joint lubrication. In advanced stages, surgical interventions such as discopexy and arthroplasty remain the last resort [9,10,11,12].

Building upon the rationale of visco-supplementation, this study proposes a novel integrated therapeutic strategy. We hypothesize that a dual-action hydrogel, combining the mechanical benefits of hyaluronic acid for friction reduction with the sustained pharmacological anti-inflammatory activity of hydrocortisone, could synergistically address both the biomechanical and inflammatory components of TMD. Consequently, this work aims to develop and characterize a hyaluronic acid-based hydrogel incorporating hydrocortisone, elucidating its biophysicochemical properties to validate its potential as a new therapeutic for temporomandibular dysfunction.

2. Materials and Methods

2.1. Chemical Reagents

The following were acquired: hyaluronic acid (HA) (CAS nº: 9004-61-9, purity: 95.9%, molecular weight: 1,300,000 Dalton, Inlab Analitic®, code: HA2022042345X, São Paulo, Brazil), hydrocortisone (CAS nº: 125.29-1, purity: 98%), phosphate-buffered saline (PBS) (code: P2272, Sigma-Aldrich®, St. Louis, MO, USA), sodium hydroxide (NaOH) (CAS nº: 1320-73-2, code: S5881, purity: 98%, Sigma-Aldrich®, St. Louis, MO, USA), formic acid (CAS nº: 64-18-6, code: 27001, purity:98%, Sigma-Aldrich®, St. Louis, MO, USA), methanol (CAS nº: 67-56-1, purity: 99.8%, Synth®, Diadema, Brazil), Eagle's medium modified by Dulbecco—DMEM (LGC Biotechnology®, Cotia, Brazil), Fetal Bovine Serum—FBS (Invitrogen®, New York, NY, USA), 7-Hidróxi-3H-fenoxazin-3-ona-10-óxido (Resazurin) (CAS nº: 62758-13-8, code: R1017, Sigma-Aldrich®, St. Louis, MO, USA), Ethyl Methane sulfonate (EMS) (CAS nº: 62-50-0, code: M08080, Sigma-Aldrich®, St. Louis, MO, USA), Cytochalasin B (CAS nº: 14930-96-2, code: C2743, purity: 98%, Sigma-Aldrich®, St. Louis, MO, USA), 4',6-Diamidino-2-fenilindol, 2-(4-Amidinofenil)-6-indolecarbamidina with fluoroshield (DAPI) (CAS nº: 28718-90-3, code: F6057, Sigma-Aldrich®, St. Louis, MO,

USA), ethanol (CAS nº: 64-17-5, purity:99.5%, Synth®, Diadema, Brazil), and a Live/Dead® Baclight Viability and Counting kit (Molecular Probes, Eugene, OR, USA).

2.2. Equipment

The following were used: class II biological safety cabinet (Veco®, biosseg-06, Sumaré, São Paulo, Brazil), analytical balance (Mettler Toledo®, Balance XPR106DUH/A, Columbus, OH, USA), type I ultrapure water purification system (Allcron®, direct-Pure®Genie, São Paulo, Brazil), autoclave (Cristofoli Biossegurança®, Vitale21, Campo Mourão, Parana, Brazil) dual asymmetric centrifuge mixer (Hauschild®, Speed MixerDAC150. 1 FVZ, Water-kamp, Hamm, Germany), Phmeter (Digimed®, DM-20, São Paulo, Brazil), Spectrometer Matrix Assisted Laser Desorption Ionization-Time of Flight—MALDI-TOF-MS Ettan (Amersham Biosciences®, Amersham, UK), high-performance liquid chromatography with a photodiode detector instrument (HPLC) (Merck-Hitachi®, D-7000, Tokyo, Japan), freezer for cryogenesis (Freezer −80) (NuAire Laboratory Equipement®, Glacier polar edition −86 ultra-low-temperature freezer, Plymouth, MN, USA), freeze-dryer (Terroni®, LS3000, São Carlos, São Paulo, Brazil), Fourier transform infrared spectroscopy (FTIR) (Perkin Elmer®, LR64912C, Waltham, MA, USA), Thermogravimetric Analyzer (TGA) (NETZSCH®, STA449F1 Jupiter, Selb, Bavaria, Germany), Dynamic shear Rheometer (Anton-paar®, SmartPave 92, Graz, Austria), Sputter Coater (Quorum Technologies®, Emitech—SC7620, Kent, UK); scanning electron microscope with Field Emission Gun (FEG®, TESCAN MIRA 3®, Brno, Czech Republic), CO2 Incubator (Sanyo®, MCO-19AIC(UV), Osaka, Japan), water bath precision (TermoFisher Scientific®, TSGP02, Waltham, MA, USA), refrigerated centrifuge (Labnet®, HEREMLE Z300®, Madrid, Spain), inverted microscope (Ziess®, Axiovert 40C, Jena, Thuringia, Germany) fluorescence microscope (Ziess®, Axio Observer A1, Jena, Thuringia, Germany), spectrophotometer (Lonza Biotek ®, ELX808LBS, Winooski, VT, USA), thermal cycler (Nova Ética®, 521-D, São Paulo, Brazil), and laser scanning confocal microscope (LSM 700, Zeiss, Oberkochen, Germany).

2.3. Hydrogels Preparation and Sterilization

A 3% (30 mg/mL) solution of HA was prepared in ultra-pure type I water for physicochemical analysis and biological analysis. Then 1% (10 mg/mL), 0.500% (5 mg/mL), 0.250% (2.5 mg/mL) or 0.125% (1.25 mg/mL) Hyd was added. The hydrogel was homogenized in a mixer for 1 min and the pH was adjusted to 7.2 with NaOH.

The gels were sterilized in an autoclave for 5 min at 118 °C [13].

Sterilized hydrogels were subjected to thermal fatigue cycles in a controlled simulator. Simultaneously, the specimens were thermocycled between 5 °C and 55 °C, maintained at each temperature for 30 s, totaling 1 min per cycle. A total of 1000 thermal cycles were performed in a total of 2 days and specimens were stored in the device [14].

2.4. Freeze-Drying

The hydrogel samples were frozen at −80 degrees for 24 h and then placed in a freeze-dryer for 3 days to sublimate the solvent.

2.5. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (FTIR)

The freeze-dried samples were analyzed by FT-IR, to identify functional group retention, obtaining wave number spectra between 500 and 4000 cm^{-1}, with a nominal resolution of 4 cm^{-1} and 100 scans per sample.

2.6. Thermogravimetric Analysis (TGA)

The samples of the gels were analyzed by thermogravimetry, obtaining the thermal analysis curves by scanning the temperature range between 30 and 300 °C with a rate change of 10 °C/min in an atmosphere of argon (Ar), to evaluate the thermal degradation profile.

2.7. Rheology Assay in Cone Plate

2.7.1. Frequency Sweep

In the frequency ramp, after the sample had settled, we waited 2 min for the hydrogels to recover and relax. Shear deformation was carried out with a 1% constant and frequency decreasing from 10 to 0.1 hertz.

2.7.2. Amplitude Sweep

In the amplitude ramp, after the sample had settled, we waited 2 min for the polymers to recover and relax. A controlled shear deformation ramp was applied from 0.001 to 100% with a frequency of 10 radians/s, with a fixed frequency.

2.7.3. Temperature Ramp

The sample was preheated to 20 degrees Celsius for 2 min, after which the temperature ramp test was applied with a controlled deformation ramp and frequency; with a deformation of 1% and a frequency of 10 radians/second and a heating rate of 1 degree Celsius per minute.

2.8. Scanning Electron Microscopy with Field Emission Gun (SEM-FEG)

For SEM, the lyophilized hydrogels were transferred to aluminum stubs and covered with gold for 120 s at 40 mA. After the process, the disks were analyzed and photographed using the scanning electron microscope. Then, the metallized SEM samples were used for surface elemental characterization by a low-energy-dispersive X-ray cartography technique.

2.9. Biological Performance on in Vitro Cell Cultures

Cytocompatibility was carried out on mouse macrophages (RAW 264.7) at 11 passages which came from the Rio de Janeiro cell bank (BCRJ). Cell culture was carried out in DMEM supplemented with 10% FBS, incubated in an oven at 37 °C, with atmospheric humidity and 5% CO_2.

The tests were carried out in 24-well microplates using 4 × 10^4 cells, cultured in 500 µL of DMEM + 10% FBS medium, incubated at 37 °C with 5% CO_2 for 24 h. After this period, the supernatant was discarded, and the cells were exposed to the treatments.

Treatment via Transwell

The treatments were carried out according to the following groups: 500 µL of 3% HA; 500 µL of 1% HYD, 500 µL of 3% HA + 1% HYD, 500 µL of 3% HA + 0.500% HYD, 500 µL of 3% HA + 0.250% HYD, 500 µL of 3% HA + 0.125% HYD, and 500 µL of DMEM + 10% FBS as a control. After 24 h of exposition, the treatments were discarded, and the wells washed three times with PBS.

2.10. Cell Viability by Resazurin Assay

Metabolic activity was checked using resazurin at 440 µM. For this, 50 µL of the resazurin suspension was added to each well of the microplate, followed by the addition of 450 µL of DMEM + 10% FBS. Incubation in the dark was carried out for 16 h, after which the plate was read in a spectrophotometer at a wavelength of 570 nm (absorbance). The optical densities (ODs) obtained were converted into a percentage of cell viability using the following formula:

$$\% \text{ Metabolic Activity} = (\text{OD Treated Group} \times 100)/\text{Average OD Control Group}$$

2.11. Genotoxicity Assay by Micronuclei

Mouse macrophages at a concentration of 3 × 10^5 cells/mL were cultured in 96-well microplates with 1 mL of DMEM supplemented with 10% FBS for 24 h at 37 °C in a 5% CO_2 atmosphere. The cells were exposed to the experimental groups at same conditions. The negative control group received only the culture medium, while the

positive control group received EMS at a concentration of 5 mM, with both treatments applied for 24 h.

After the treatments, the cells were washed with PBS three times and incubated with cytochalasin B at a concentration of 6 µg/mL for 24 h at 37 °C in a 5% CO_2 atmosphere. After the incubation, the cells were fixed in 100% methanol for 20 min, followed by staining with DAPI. The dye was removed after 5 min of contact with the cells, followed by three washes with PBS. Micronuclei were analyzed under a fluorescence microscope at 40× magnification with a total of 2000 cells evaluated per well.

2.12. Confocal Analysis

After the treatments, the cells were stained using the Live/Dead® Baclight Viability and Counting kit, composed of SYTO-9 dye, colored green for the pigmentation of live cells (penetrates cells with an intact cell membrane) and propidium iodide dye, colored red for the pigmentation of dead cells (penetrates cells with an impaired cell membrane). The dyes, prepared in a ratio of 4 µL to 1 mL of sterile PBS, were dripped onto the coverslips and taken away for analysis after 20 min. A wavelength of 488 nm was used, with light emitted between 500 and 550 nm and light below 560 nm being collected by different filters. To form a 3D image, "stacks" were acquired made with z-sections at 1 µm intervals. Quantification of confocal microscopy images was performed using Imaris 10.0 software (Oxford Instruments®, Abingdon, UK). For each experimental condition, five random image fields were analyzed in three independent biological replicates.

2.13. Statistical Analysis

The data obtained was initially analyzed for normality using D'Agostino, Shapiro–Wilk, and Kolmogorov–Smirnov tests. Those that showed normality were then analyzed by ANOVA complemented by Tukey. Those that did not show normality were evaluated by Kruskal–Wallis, complemented by the Dunn's test. All analysis utilized a p value of 0.0001.

3. Results

3.1. Attenuated Total Reflectance-Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectrum of hyaluronic acid (Figure 1—blue line) displayed its characteristic functional groups. Key absorptions were identified as follows: a broad

band at 3600–3000 cm^{-1} (O-H and N-H stretches); peaks at 2950–2850 cm^{-1} (aliphatic C-H stretches); a doublet at 1650–1600 cm^{-1}, attributed to the amide I carbonyl stretch and asymmetric COO^- stretch; and a peak at 1560–1550 cm^{-1} for the amide II N-H deformation. The fingerprint region (1150–1000 cm^{-1}) showed C-O and C-O-C stretches, confirming the polysaccharide structure of HA [15,16,17,18].

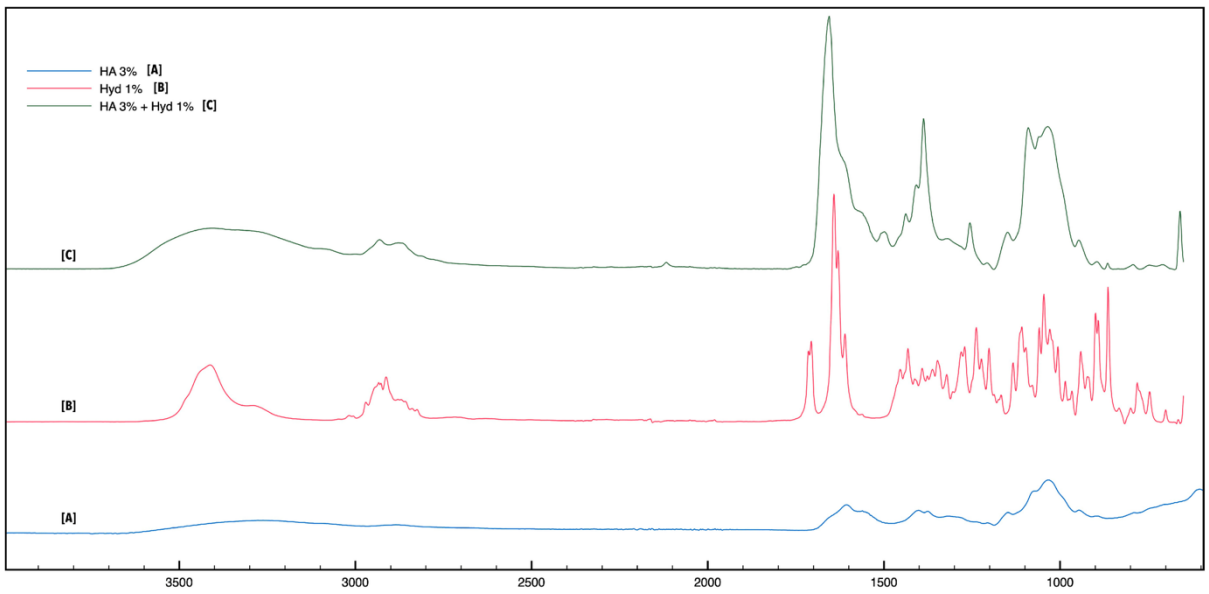


Figure 1. FTIR analysis by hyaluronic acid gels. Legend: FTIR spectra of HA [A] line, Hyd [B] line and hydrogel 3% HA associated with 1% Hyd [C] line.

The FTIR spectrum of hydrocortisone (Figure 1, red line) showed the functional groups in their steroid-based structure. A sharp O-H stretching band was observed at 3500–3300 cm^{-1}, characteristic of its hydroxyl groups and distinct from the broader equivalent in HA. Aliphatic C-H stretches from the steroid skeleton's methyl and methylene groups appeared at 2950–2850 cm^{-1}. The fingerprint region revealed a highly characteristic C=O stretch from ketone groups at 1750–1700 cm^{-1}, while peaks in the 1650–1600 cm^{-1} range were attributed to C=C stretches of the unsaturated ring system and/or conjugated ketones. Additional confirmatory peaks included C-H bending vibrations (1470–1370 cm^{-1}) and C-O stretches from alcohol and ether groups (1200–1000 cm^{-1}) [19,20].

The green line shows the spectrum of the 3% HA gel associated with Hyd. The intensity of the hydrocortisone peaks is noticeably higher in the mixture compared to

the pure HA spectrum, especially in the 1750–1600 cm^{-1}region (carbonyls and C=C) and in the 1200–1000 cm^{-1} region (C-O), where the Hyd peaks are strong. The broad O-H and N-H peaks in the 3600–3000 cm^{-1} region of HA are still prominent but may be slightly modulated by the presence of the O-H groups of hydrocortisone.

In the spectrum by % HA gel associated with Hyd, there is no drastic shift in peaks to indicate a strong covalent chemical interaction. However, small changes in the width or shoulders of the peaks in the O-H/N-H (3600–3000 cm^{-1}) or C=O (1650–1600 cm^{-1} for HA and 1750–1700 cm^{-1} for Hyd) region may suggest hydrogen bond interactions between HA and hydrocortisone. For example, a slight broadening or shift to lower wavenumbers in the O-H/N-H peaks may indicate the strengthening of hydrogen bonds.

3.2. Thermogravimetric Analysis (TGA)

The thermal degradation of hyaluronic acid shows a noticeable initial loss (approx. 30–100 °C), which corresponds to the loss of adsorbed water (moisture) in the sample. Subsequently, a significant and marked loss of mass begins in the 200–300 °C range. This stage is attributed to the thermal degradation of the polysaccharide, involving depolymerization and/or decomposition of the saccharide units, with the release of volatiles. The maximum degradation peak appears to occur around 250–260 °C [21]. Degradation continues at higher temperatures (above 300 °C), but at a slower rate, corresponding to the decomposition of carbonaceous residues. The curve shows that, at 400 °C, approximately 40–45% of the original mass still remains [20].

Hydrocortisone shows excellent initial thermal stability, with minimal (practically zero) mass loss up to around 200 °C. This indicates that hydrocortisone is a relatively anhydrous and thermally stable compound at low and medium temperatures. The main degradation of hydrocortisone begins at around 200 °C and proceeds more gradually compared to HA. The degradation is continuous and the mass decreases steadily up to 400 °C. At 400 °C, approximately 60–65% of the original mass is still present. Hydrocortisone is more thermally stable than HA in the 200–300 °C range, as shown in Figure 2 [21].

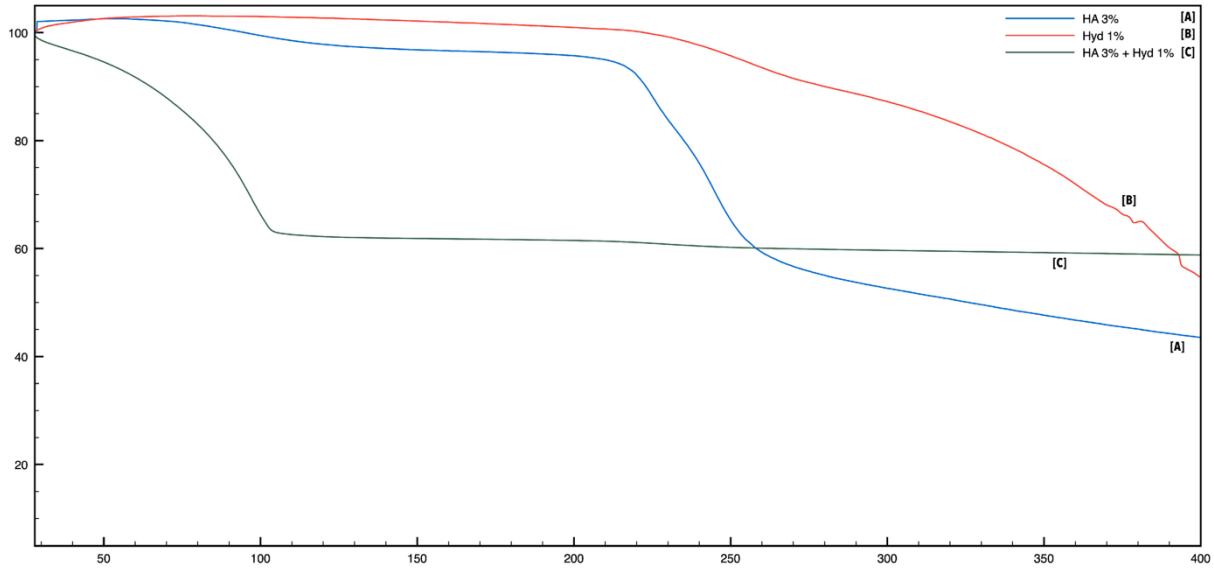


Figure 2. TGA. Legend: TGA spectra of HA 3% [A] line, Hyd 1% [B] line and hydrogel HA 3% associated with Hyd 1% [C] line.

The thermal behavior of the mixture of HA and hydrocortisone shows an initial loss of moisture (approx. 30–100 °C) which is more pronounced than that of pure HA. This may indicate a greater amount of water bound to the HA + Hyd gel structure. The mass loss is approximately 35–40% up to 100 °C. After the loss of moisture, the curve stabilizes for a short period (plateau) and then a more gradual degradation seems to occur before the main degradation of HA begins. The curve of the mixture follows a profile that is a combination of HA and hydrocortisone. Significant mass loss (similar to that of HA) begins at around 200–220 °C, indicating the degradation of the HA component in the mixture. However, the rate of degradation appears to be somewhat slower than that of pure HA in this range, and the residual mass at 400 °C (around 58–60%) is higher than that of pure HA (40–45%) but lower than that of pure hydrocortisone (60–65%).

3.3. Rheology Assay

3.3.1. Frequency Sweep

The angular frequency sweep graph shows that all hyaluronic acid gels (HA 3%), pure or with hydrocortisone (Hyd), exhibit predominantly solid viscoelastic behavior, with the storage modulus (G') remaining significantly higher than the loss modulus (G"), indicating a stable gel structure (approximately 0.5 to 80 rad/s), as shown in Figure 3.

It should be noted that G' increases moderately with frequency, a behavior typical of polymer networks. The addition of hydrocortisone, at any concentration tested (0.125%, 0.250%, 0.500%), did not drastically alter the dependence of G' on frequency, but caused a dose-dependent reduction in the absolute values of G' compared to pure HA, suggesting a slight plasticizing effect or interference in the HA network. The autoclave process promoted clear thermal degradation, resulting in a marked decrease in G' for all formulations, although the trend in behavior as a function of frequency remained unchanged.

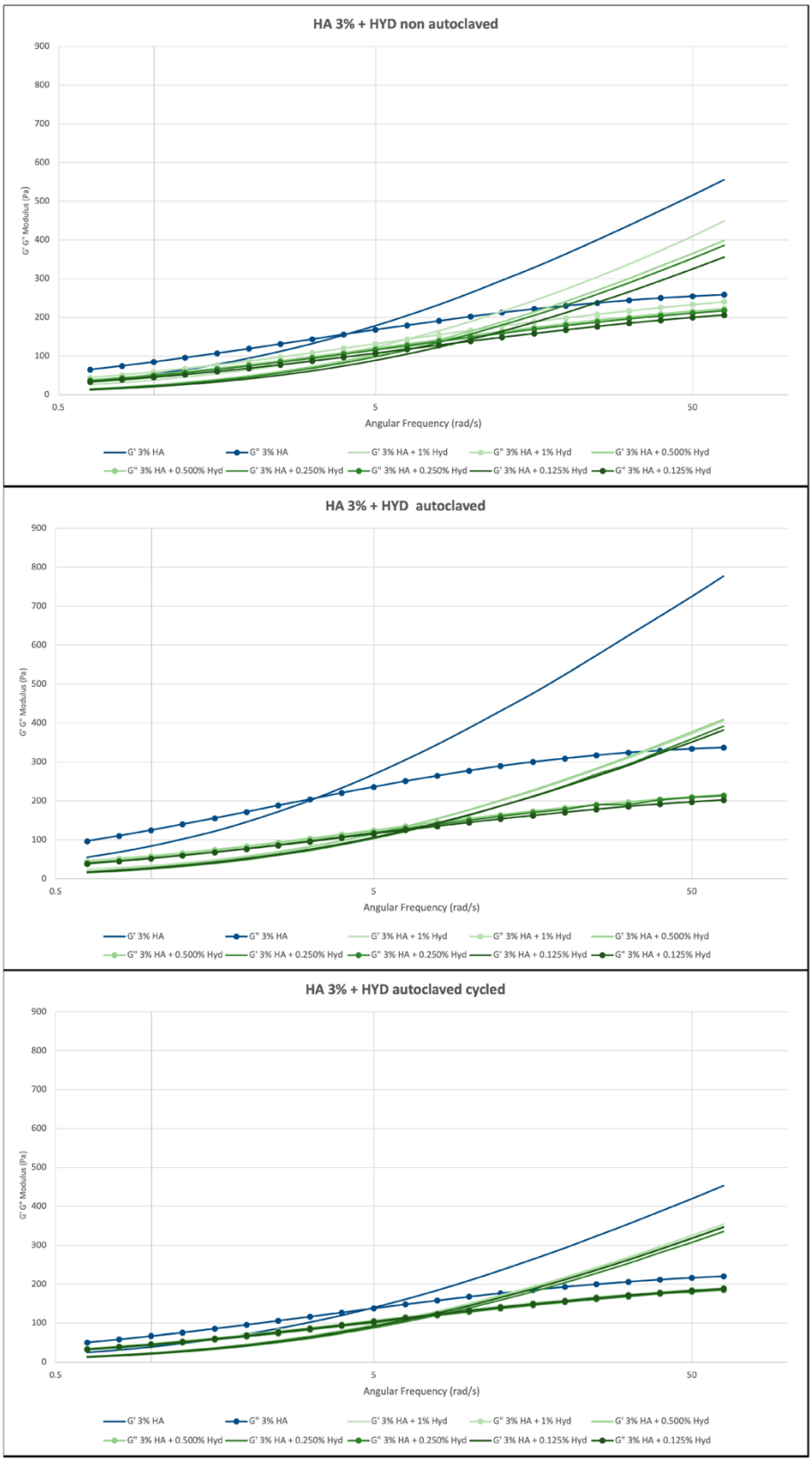


Figure 3. Angular frequency sweep. Legend: Graph of storage modulus (G') as a function of angular frequency for HA 3% gels and their combinations with different hydrogel concentrations (Hyd).

3.3.2. Amplitude Sweep

The results show that all gels exhibit predominantly solid viscoelastic behavior in the low deformation range (0.001% to 1%), with the storage modulus (G') significantly higher than the loss modulus (G") for all formulations, indicating a well-formed gel structure. It should be noted that the G' values for pure hyaluronic acid (HA 3%) are consistently the highest across the entire range tested, while the addition of hydrocortisone (Hyd) promotes a dose-dependent reduction in the elastic stiffness of the gel, evidenced by the progressive decrease in G' values as the drug concentration increases (from 0.125% to 1%). This effect, observed even at very low deformations, suggests that hydrocortisone acts as an interfering or plasticizing agent in the HA polymer network, reducing its initial elastic resistance without fundamentally altering the gel nature of the material within the linear viscoelastic region, as shown in Figure 4.

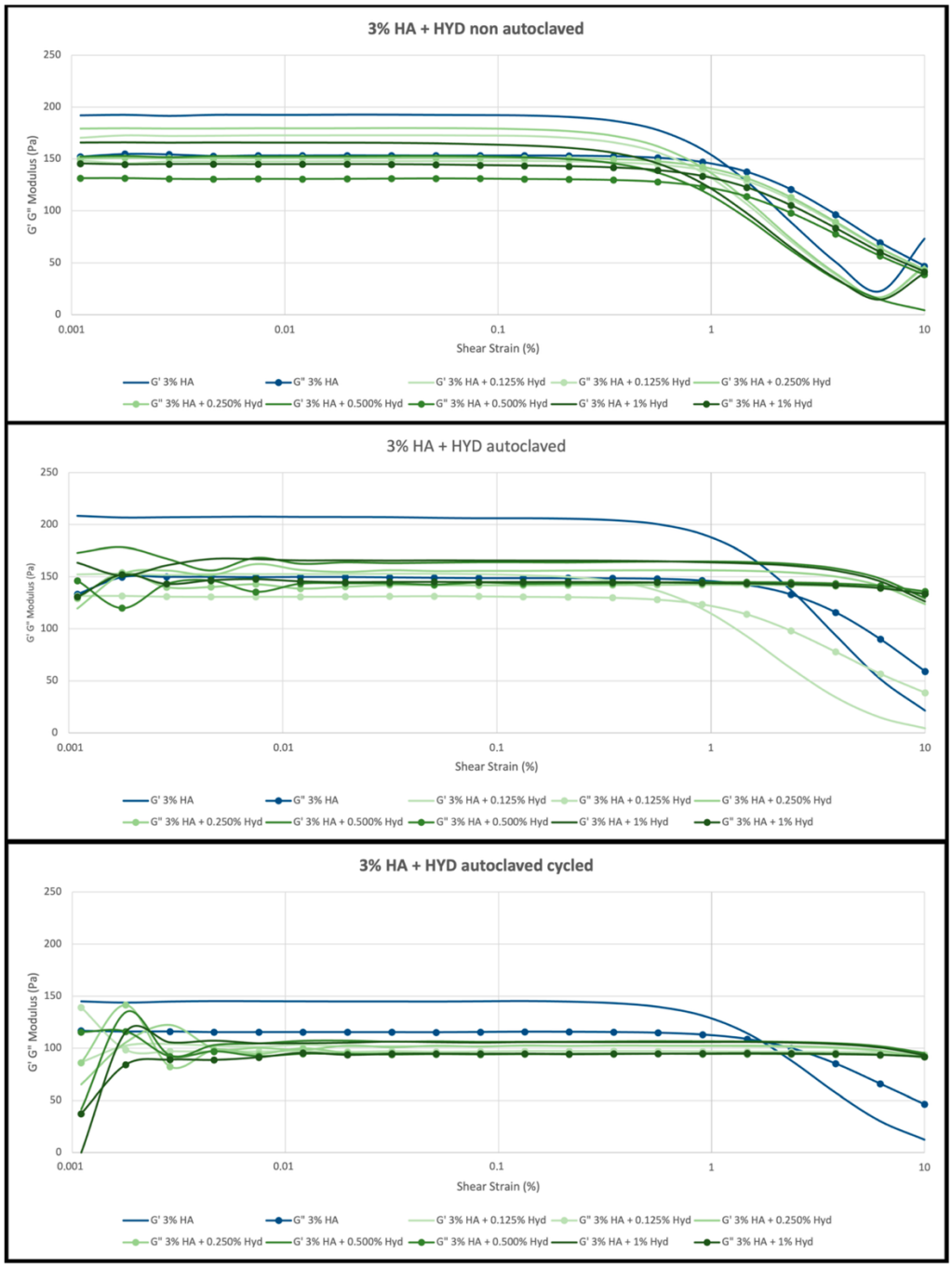


Figure 4. Amplitude sweep. Legend: Oscillatory amplitude curve (amplitude sweep). Storage modulus (G') and loss modulus (G") as a function of applied deformation (%) for 3% HA gels and their formulations supplemented with different concentrations of hydrocortisone (Hyd: 0.125%, 0.250%, 0.500%, 1%).

3.3.3. Temperature Ramp

According to the temperature range (15 °C to 40 °C), as shown in Figure 5, all hyaluronic acid gels (HA 3%), pure or with hydrocortisone (0.125% to 1%), showed a

linear and proportional increase in apparent density, both in non-autoclaved and autoclaved conditions. This behavior reflects the typical volumetric reduction in hydrophilic polymer networks under heating, resulting from thermal contraction or water expulsion. The pure HA formulation (non-autoclaved) served as a reference, with density ranging from 100 Pa to 500 Pa between 15 °C and 40 °C. The addition of hydrocortisone did not alter the overall thermodynamic trend but promoted a slight increase in absolute density values, especially at concentrations of 0.500% and 1%, suggesting greater compaction of the polymer network. The autoclaving process modified the thermal behavior of the gels: the autoclaved samples showed slightly higher density at low temperatures and a lower slope on the thermal ramp. This result indicates that prior thermal degradation altered the network structure, making it less susceptible to further contraction during heating.

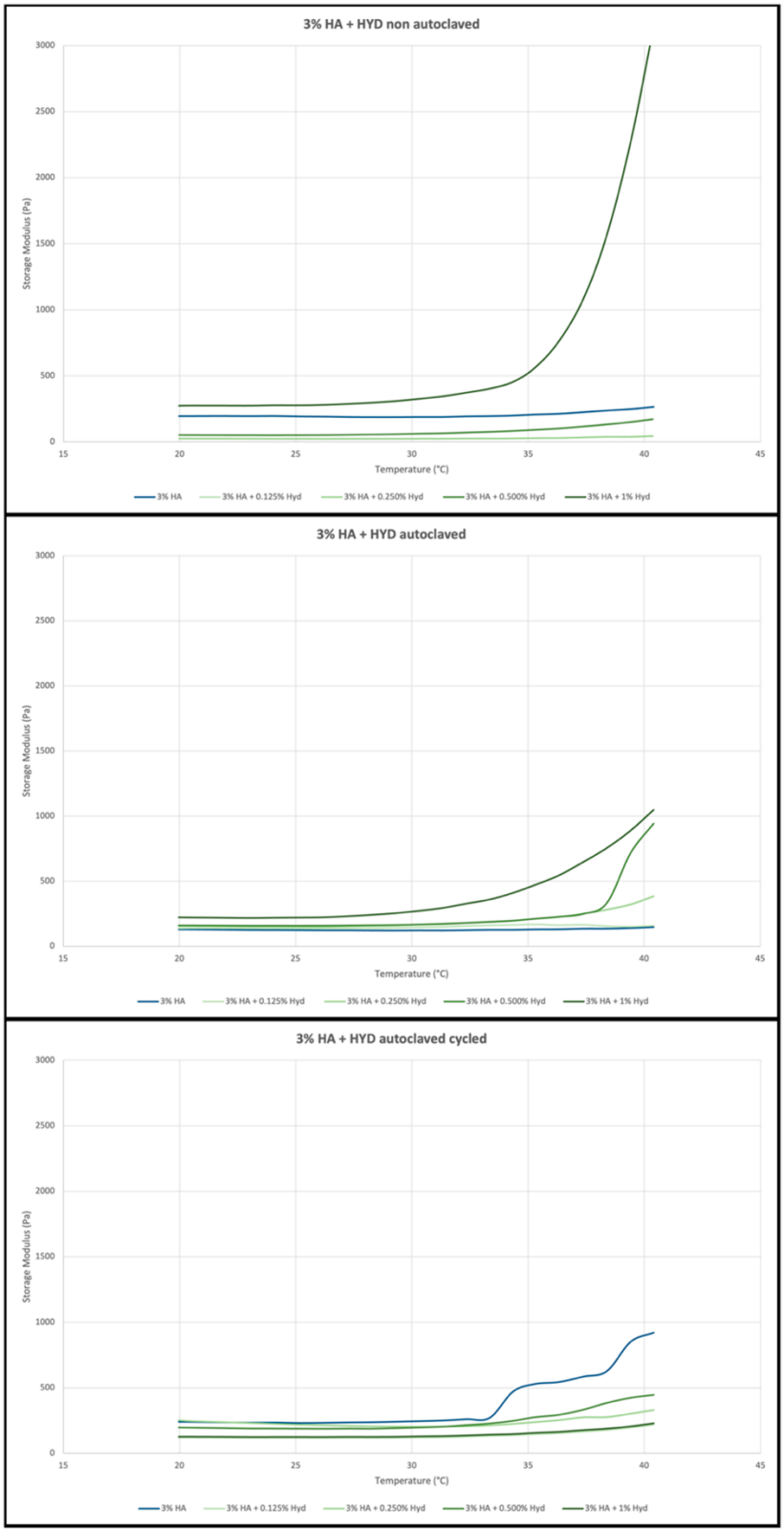


Figure 5. Temperature sweep. Legend: Storage modulus values (G', in Pa) measured for hyaluronic acid-based gels (HA3%) supplemented with different concentrations of hydrocortisone (Hyd: 0.125%, 0.250%, 0.500%, 1%), processed with and without autoclaving, in a temperature range of 15 to 40 °C.

3.4. Scanning Electron Microscopy with Field Emission Gun (SEM-FEG)

The photomicrograph reveals a spongy surface with interconnected pores, forming a dense fibrillar matrix due to the high molecular weight of hyaluronic acid, observed in Figure 6. The pore diameter was not assessed because the freeze-drying process used to prepare the sample directly influences the pore diameter.

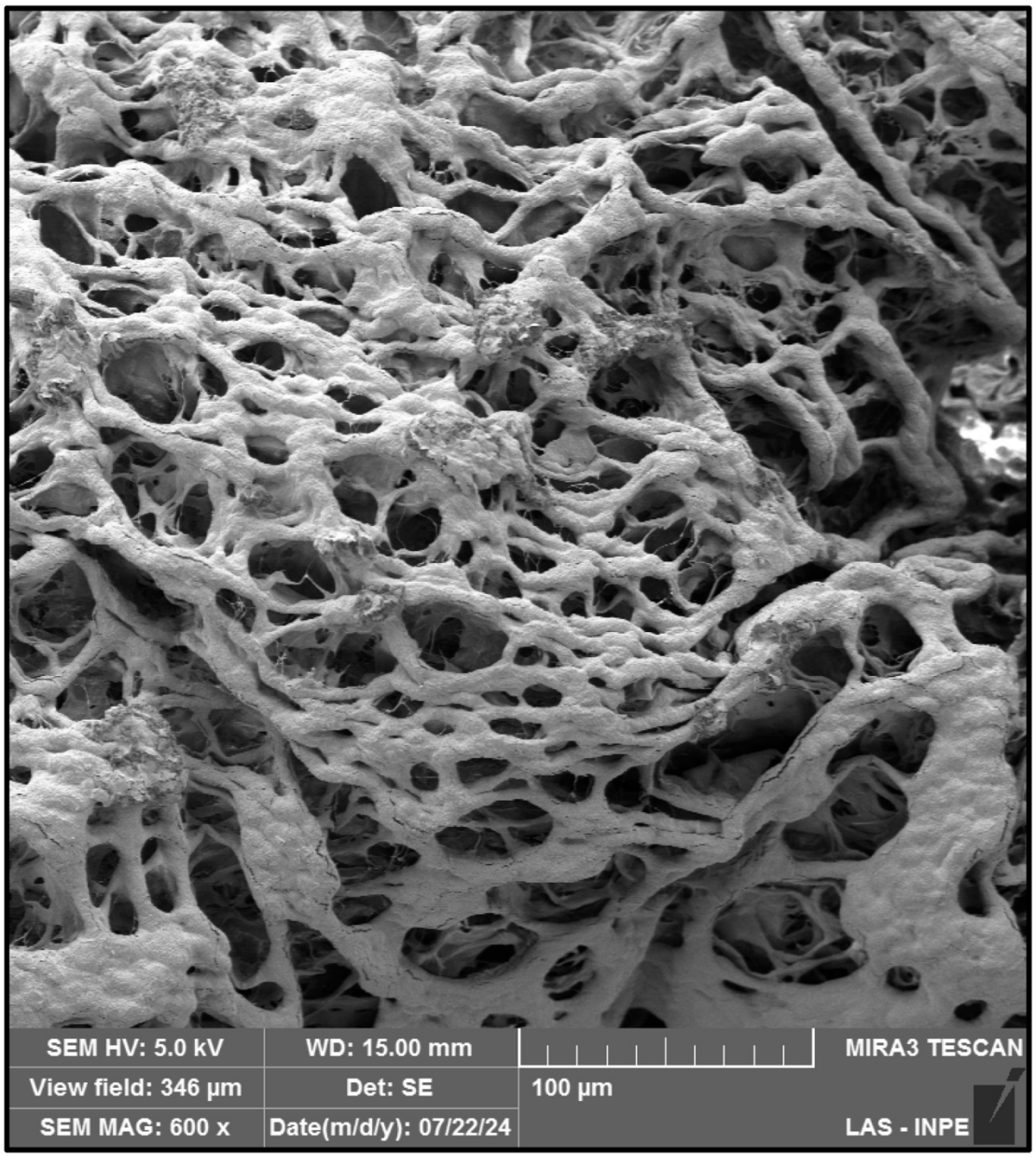


Figure 6. Morphological analysis of the surface of hyaluronic acid. Legend: Scanning electron micrograph (SEM) of hyaluronic acid (HA) gel operating at an acceleration voltage of 5.0 kV and secondary electron detectors (SE). The sample was analyzed under vacuum, with a working distance (WD) of 15.00 mm and a magnification of 600×.

No structural difference was observed between the isolated hyaluronic acid hydrogels when compared to those associated with hydrocortisone (Figure 7). SEMs revealed a spongy structure with several sheets containing pores of various sizes and interconnected by a three-dimensional network, with spacing between the hydrogel layers, indicating the complete dispersion of hydrocortisone in the hydrogel.

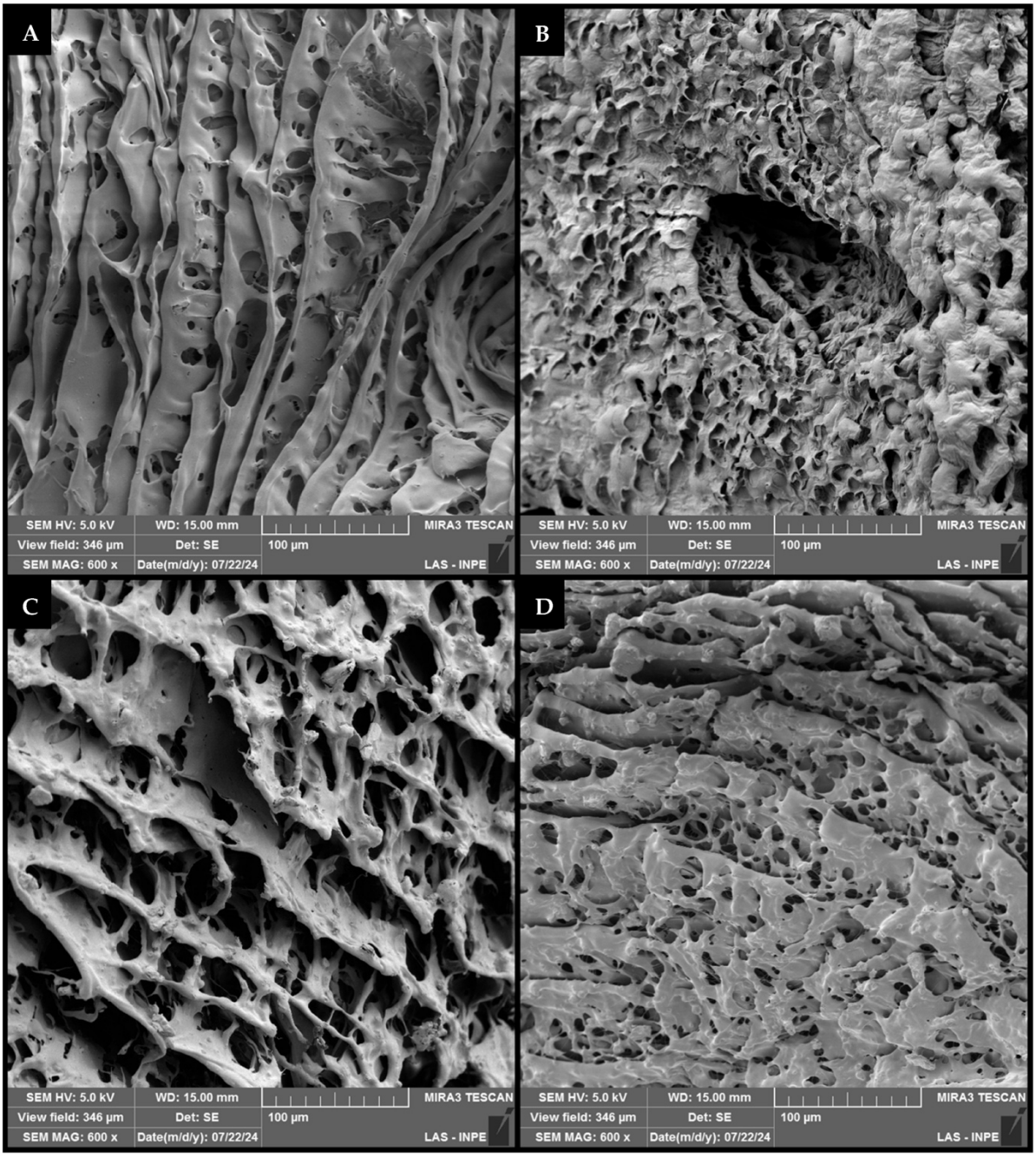


Figure 7. Morphological analysis of the surface of hyaluronic acid gels associated with hydrocortisone. Legend: Scanning electron micrograph (SEM) of hyaluronic acid (HA)

gels operating at an acceleration voltage of 5.0 kV and secondary electron detectors (SE). The sample was analyzed under vacuum, with a working distance (WD) of 15.00 mm and a magnification of 600×; HA 3% + Hyd 0.125% hydrogel (A); HA 3% + Hyd 0.250% hydrogel (B); HA 3% + Hyd 0.500% hydrogel (C); HA 3% + Hyd 1% hydrogel (D).

3.5. Cell Viability by Resazurin Essay

After contact for 24 h with the 3% hyaluronic acid hydrogel, mouse macrophages (RAW 264-7) obtained 86.7% cell viability. For the hydrogel groups associated with hydrocortisone, the viability percentages were 71.07%, 95.3%, 107.0% and 121.4% at concentrations of 0.125%, 0.250%, 0.500% and 1%. The group exposed only to 1% hydrocortisone had a viability percentage of 148.5%, as shown in Figure 8.

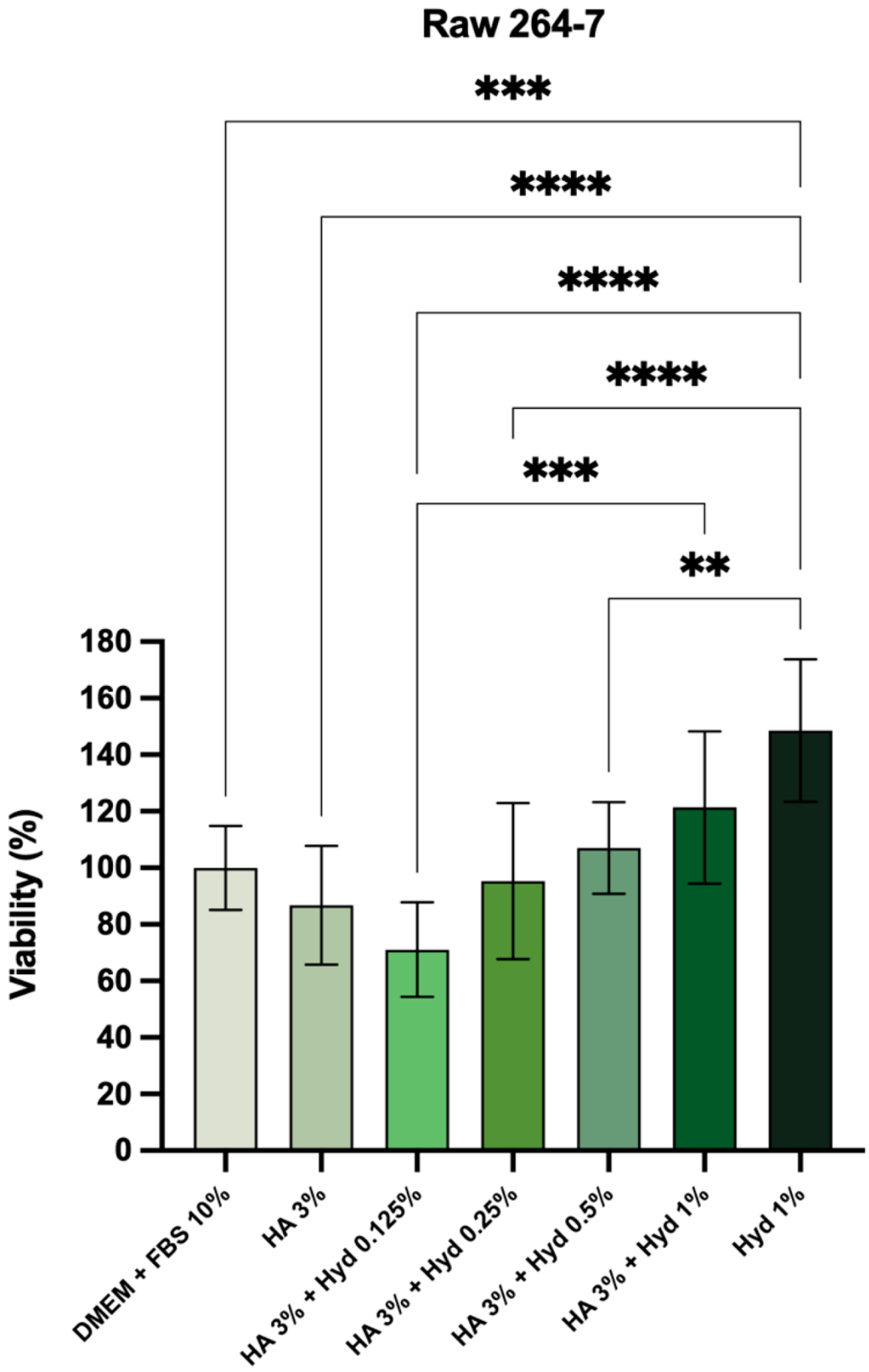


Figure 8. Cytocompatibility by hydrogels on mouse macrophage lineage. Legend: p < 0.001 (**), p < 0.0002 (***), p < 0.0001 (****).

3.6. Genotoxicity Assay by Micronucleus

The evaluation of genotoxicity indicated that the negative control group (DMEM + 10% SFB) and the application of the pure hyaluronic acid hydrogel promoted the formation of 2 micronuclei in the global count of 2000 cells. The group of hyaluronic acid hydrogels associated with hydrocortisone promoted the formation of 4, 11, 14 and 17 micronuclei at concentrations of 0.125%, 0.250%, 0.500% and 1%. The application of hydrocortisone alone promoted the formation of 22 micronuclei, and the positive control (EMS) yielded a total of 29 micronuclei, as shown in Figure 9.

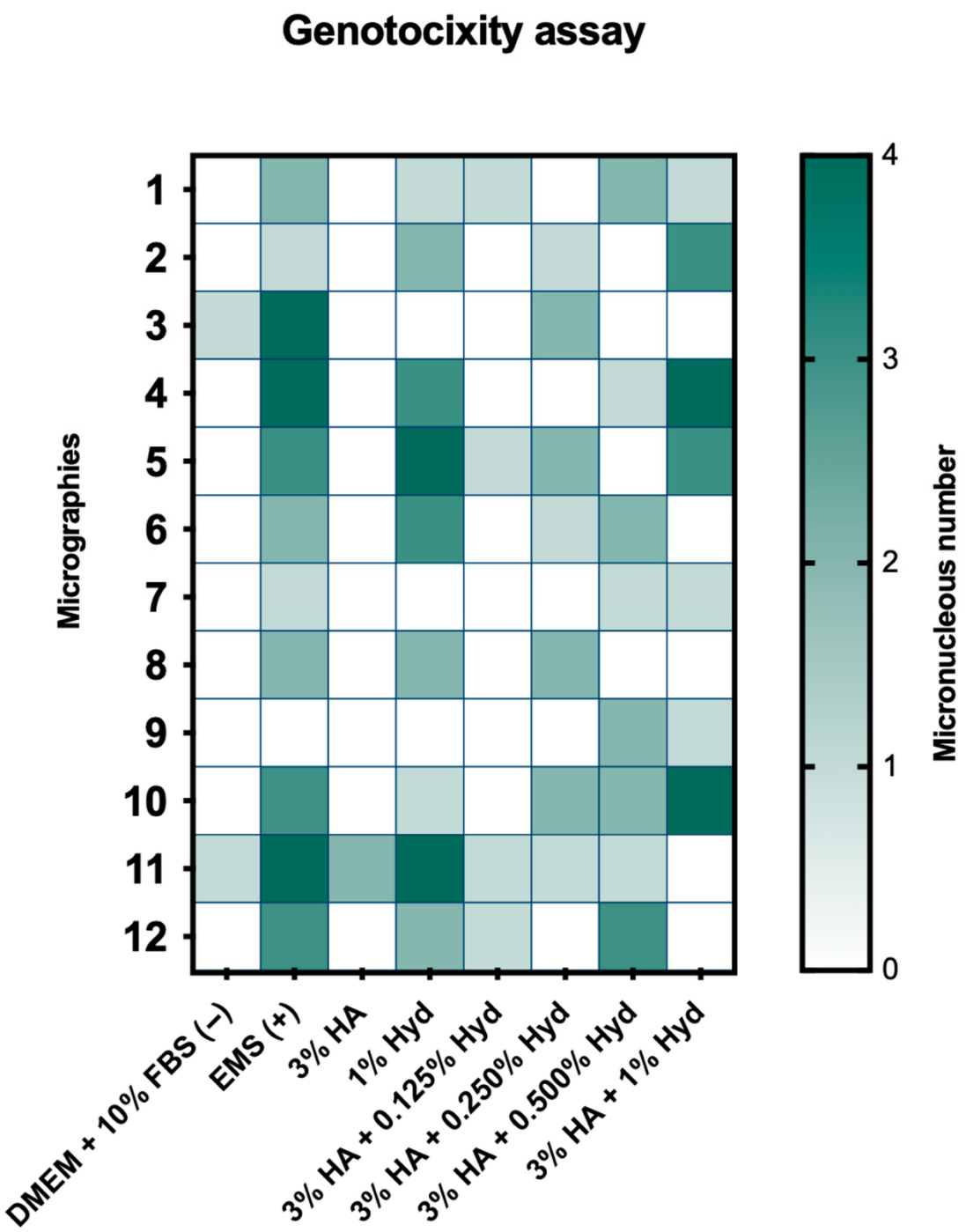


Figure 9. Micronucleus assay. Legend: Micrograph of cells stained with DAPI, showing the presence of micronuclei (indicated by color). The sidebar on the right quantifies the number of micronuclei per field of view (photomicrograph).

3.7. Confocal Analysis

The control group (A) and 3% hyaluronic acid hydrogel (HA3%, B) demonstrated excellent biocompatibility, with most macrophages showing predominantly green

staining, indicative of high viability. In contrast, 1% hydrocortisone (Hyd1%, C) induced notable cytotoxicity, evidenced by the significant presence of red/orange-stained cells. The evaluation of the combined HA and hydrocortisone formulations revealed that lower concentrations of hydrocortisone (0.125% in D and 0.250% in E) were well tolerated, maintaining cell viability. However, the progressive increase in hydrocortisone concentration in the hydrogels (0.500% in F, and 1% in G) correlated with a proportional increase in the proportion of cells with compromised membranes (red/orange staining), indicating a dose-dependent reduction in macrophage viability, as shown in Figure 10.

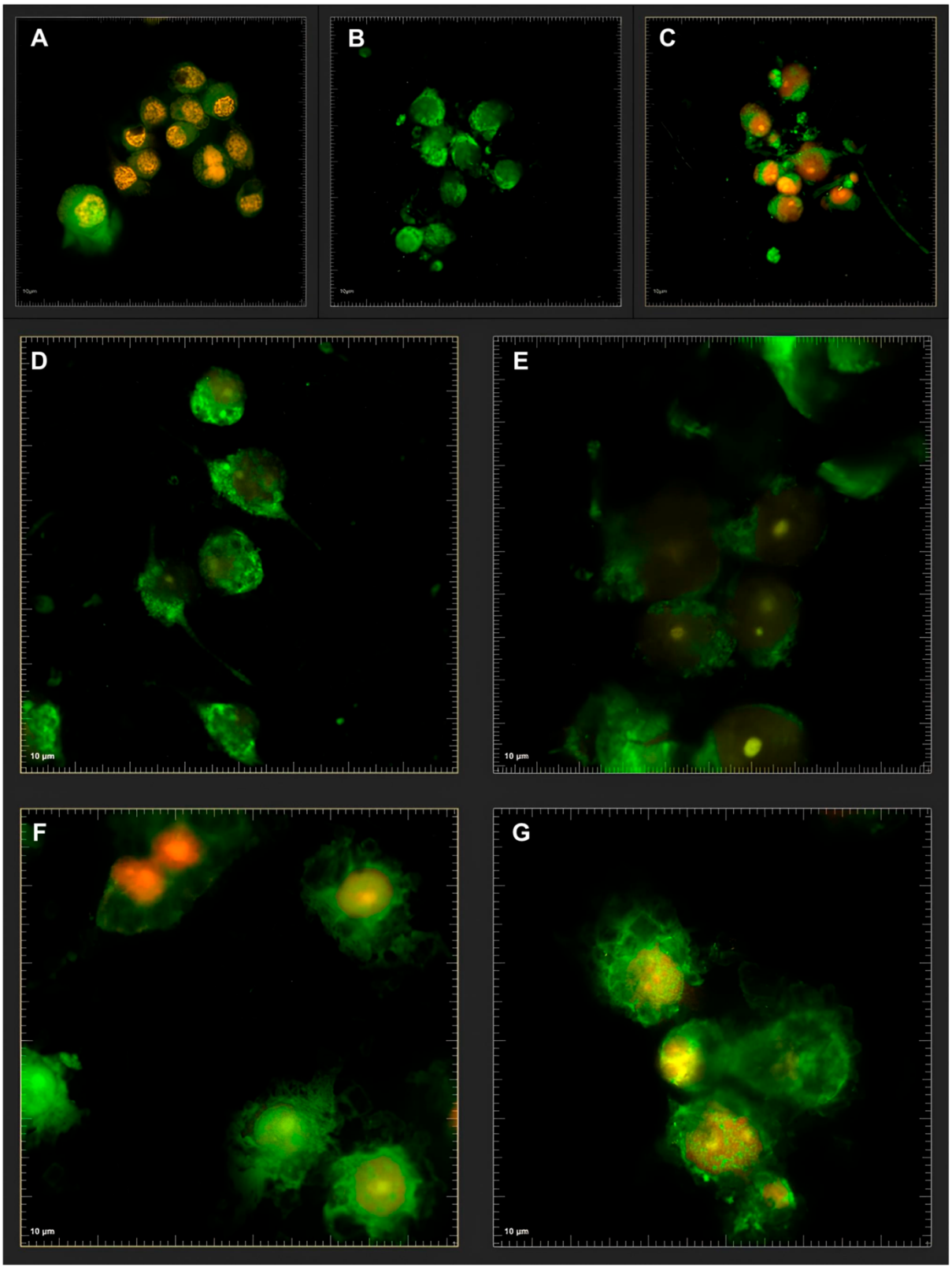


Figure 10. Confocal analysis by hydrogels on macrophage lineage (RAW 264.7). Legend: Control group—DMEM+SFB10% (A); HA3%—hyaluronic acid hydrogel (B);

Hyd1%—hydrocortisone (C); HA3% + Hyd 0.125% hydrogel (D); HA3% + Hyd 0.250% hydrogel (E); HA3% + Hyd 0.500% hydrogel (F); HA3% + Hyd 1% hydrogel (G). Cells stained green (Calcein AM) are functionally viable; cells stained red (Propidium Iodide) have structural damage and are not viable.

4. Discussion

FTIR analysis of the developed hydrogel confirmed the coexistence of hyaluronic acid [15] and hydrocortisone [16] without chemical degradation. The spectrum showed the characteristic peaks of both components, with no evidence of new covalent bond formation, as indicated by the absence of new absorption bands [15,16,17]. This molecular stability is critical, as it preserves the pharmacodynamic integrity of the drugs. Furthermore, these non-covalent interactions are anticipated to directly influence the release profile of hydrocortisone, potentially leading to a sustained release mechanism without compromising its therapeutic activity [15,16,17,18].

Thermogravimetric analysis showed different behaviors for hyaluronic acid (HA), hydrocortisone (Hyd) and their mixture. HA 3% showed an initial loss in 30–100 °C, attributed to desorption of water, followed by marked thermal degradation between 200 and 300 °C, corresponding to depolymerization and decomposition of the saccharide units, releasing volatiles. Above 300 °C, slower residual degradation was observed, with 40–45% mass remaining at 400 °C. In contrast, hydrocortisone showed high initial thermal stability, with minimal loss up to 200 °C, with gradual degradation, resulting in 60–65% residual mass at 400 °C. The hydrogel HA–hydrocortisone mixture exhibited more pronounced loss (35–40% up to 100 °C), suggesting greater water retention in the gel structure, followed by combined degradation of the components. Significant degradation began at 200–220 °C, with slower kinetics than pure HA, resulting in intermediate residual mass (58–60% at 400 °C), indicating an interaction between the components that moderates the decomposition of HA without affecting the stability of hydrocortisone alone [20,21,22].

Given the imperative for a sterile product, the rheological impact of autoclaving was a critical evaluation. The tests revealed that autoclaving degrades pure hyaluronic acid (HA) gels, significantly reducing their storage modulus (G') and viscoelastic integrity. Notably, the incorporation of hydrocortisone (Hyd) counteracted this effect. Formulations with higher Hyd concentrations (0.5% and 1%) demonstrated a

pronounced stabilizing effect, maintaining a higher G', lower temperature sensitivity, and greater deformation resistance after thermal stress [13,14,23]. This protective action positions the HA gel with 1% Hyd as the ideal candidate from a rheological point of view, as it uniquely fulfills the dual requirements of sterility and preservation of mechanical performance essential for long-lasting function in the temporomandibular joint [24,25].

The biological compatibility assessment of the hydrogels, based on the OECD 129 guideline (Acute Cytotoxicity Test), indicates biocompatibility with 86.7% viability for the hyaluronic acid group, as expected for a natural polysaccharide. The hydrogels associated with hydrocortisone meet the OECD 129 [26] criteria for non-cytotoxicity (viability > 70%), exhibiting a concentration-dependent effect. At lower concentrations (0.125%), viability is slightly reduced but remains acceptable. At higher concentrations (0.250–1%), viability increases, potentially due to anti-inflammatory and pro-survival effects. The 1% hydrocortisone group (148.5% viability) suggests overstimulation of cellular metabolism, likely due to glucocorticoid receptor activation, which can suppress apoptosis and increase metabolic activity in macrophages.

The genotoxicity results (OECD 487) [27] demonstrated that pure hyaluronic acid (HA) did not induce significant micronucleus formation (2 MN/2000 cells), remaining equal to the negative control (2 MN), confirming its safety. However, the addition of hydrocortisone (Hyd) showed a dose-dependent effect. The formulations with 0.125%, 0.250%, 0.500% and 1% Hyd resulted in 4, 11, 14 and 17 micronuclei, respectively, while Hyd alone (1%) generated 22 micronuclei, surpassing the positive control (EMS: 29). Concentrations ≥ 0.250% Hyd indicated genotoxic potential (≥5.5× the control), being clinically unacceptable above 0.500%. It is recommended to limit the Hyd concentration to ≤0.125% to ensure safety, in association with new in vivo tests for validation.

The confocal analysis corroborated these results, showing that pure hyaluronic acid (HA) (3%) presented high cell viability (predominance of green cells in the Live/Dead® assay), confirming its biocompatibility. In contrast, hydrocortisone alone (1%) marked cytotoxicity (red/orange cells), indicating a reduction in viability. In the combined formulations (HA + hydrocortisone), a dose-dependent increase in cell death was observed, with concentrations ≤ 0.250% exhibiting a better safety profile. These data suggest that HA partially attenuates the toxicity of hydrocortisone, but high

concentrations of the drug (>0.500%) compromise macrophage viability, reinforcing the need for dosage optimization for therapeutic applications.

Hyaluronic acid and hydrocortisone hydrogels with the concentrations evaluated in this study, applied to cartilage, are scarce in the literature. The study by Habib et al. [28] investigated the systemic effects of intra-articular betamethasone on the knee and assessed whether pre-injection of hyaluronic acid (HA) influenced these effects, concluding that HA did not alter the systemic response to corticosteroids. In contrast to the work of Castor and Prince [29] and Kongtawelert et al. [30], which demonstrated the negative effects of hydrocortisone on HA viscosity in vitro and cartilage degradation in vivo, respectively, the study by Habib et al. [28] did not explore the local impacts of betamethasone on HA homeostasis or the extracellular matrix. While previous studies highlight the deleterious action of corticosteroids on HA structure and dynamics, the article by Habib et al. [28] focused exclusively on systemic effects, without assessing possible degenerative changes or HA function in the joint. Thus, although all studies address the interaction between corticosteroids and HA, the approaches and outcomes evaluated differ significantly.

The study by Jahanbekam et al. [31] developed an ultrasound-responsive hyaluronic acid (HA) hydrogel for controlled release of hydrocortisone, aimed at treating osteoarthritis. The authors incorporated a second polymer (unspecified) to improve the mechanical properties and stability of the system but did not report the exact concentrations of hydrocortisone or HA used in the formulation. Despite this limitation, the hydrogel demonstrated biocompatibility, sustained drug release, and anti-inflammatory efficacy in vitro and in vivo, with ultrasound application optimizing tissue penetration. The results suggest that the strategy may reduce extracellular matrix degradation, although the lack of details on component concentrations hinders reproducibility and comparison with other formulations.

In the present paper, rheological tests have shown that hydrocortisone in concentrations ≥ 0.5% has a stabilizing effect on the HA polymer network, mitigating thermal degradation during autoclaving and cycling, with a storage modulus (G') higher than pure HA after thermal stress. However, higher concentrations of hydrocortisone (0.5–1%) reduce the intrinsic elasticity of the non-autoclaved gel, making it less like the properties of native human cartilage (G' ~0.1–2 MPa). Cell viability analysis revealed that hydrocortisone at 0.250% promoted 95.3% viability in macrophages,

while concentrations ≥ 0.5% induced dose-dependent cellular stress with a high cell reproduction rate (107–121.4%) and a significant increase in micronuclei (11–17 vs. 2 in the control), indicating genotoxic risk. Thus, the formulation with AH 3% + hydrocortisone 0.250% combines rheological stability after autoclaving, biocompatibility, absence of relevant genotoxicity and adequate viscoelastic properties. Higher concentrations of hydrocortisone (≥0.5%), although improving thermal resistance, compromise cell safety and genomic integrity, making its clinical application unfeasible. Therefore, a dose of 0.250% hydrocortisone in 3% HA is most appropriate for therapeutic applications in temporomandibular joint disfunction, balancing anti-inflammatory efficacy and preservation of the extracellular matrix. Additional in vivo studies are needed to validate durability and long-term tissue response.

This pioneering study on the biophysical and chemical characterization of hyaluronic acid hydrogels associated with hydrocortisone provides valuable data on the thermal stability and preliminary biocompatibility of the potential drug. However, its main limitations include restricted cell models, an absence of anti-inflammatory evaluation, limitations in the evaluation of hydrogel degradation by physiological characteristics such as the action of hyaluronidase or cyclic mechanical stress, and the absence of tests in animal models or controlled release in vivo, which prevents the evaluation of anti-inflammatory efficacy or hydrogel persistence in the joint.

5. Conclusions

Based on rheological, cytocompatibility, and genotoxicity results, the optimized formulation of 3% hyaluronic acid (HA) and 0.250% hydrocortisone in hydrogel presents the best balance between mechanical properties, biocompatibility, and safety, offering a promising dual-action hydrogel for TMD, combining the mechanical benefits of hyaluronic acid in reducing friction and increasing lubrication with the sustained anti-inflammatory pharmacological activity of hydrocortisone. These in vitro findings encourage the continuation of in vivo and subsequent clinical studies for the development of a new therapy for temporomandibular dysfunction.

Author Contributions

Conceptualization, D.G.M., L.d.P.R. and B.G.; Data curation, D.G.M., L.d.P.R., P.C.d.C., N.F.d.S.L., M.R.B., C.P.-S., B.H.G., K.G., B.G. and A.L.S.B.; Formal analysis, D.G.M., L.d.P.R., T.S.-T., M.R.B., C.P.-S., B.H.G., K.G., B.G. and A.L.S.B.; Investigation, D.G.M., L.d.P.R., P.C.d.C., N.F.d.S.L., T.S.-T., M.R.B., C.P.-S., B.H.G., K.G., B.G. and A.L.S.B.; Methodology, D.G.M., L.d.P.R., K.G., B.G. and A.L.S.B.; Project administration, D.G.M., B.G. and A.L.S.B.; Resources, D.G.M.; Supervision, D.G.M., B.G. and A.L.S.B.; Validation, D.G.M., L.d.P.R., C.P.-S., K.G., B.G. and A.L.S.B.; Writing—original draft, D.G.M., L.d.P.R., P.C.d.C., N.F.d.S.L., T.S.-T., M.R.B., C.P.-S., B.H.G., K.G., B.G. and A.L.S.B.; Writing—review and editing, D.G.M., L.d.P.R., K.G., B.G. and A.L.S.B. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest

The authors declare no conflicts of interest.

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2.3 Article – Miranda DG, Ramos LP, Camargo PC, Lopes NFS, Sani-Taiariol T, Baldan MR, Soares CP, Godoi BH, Gritsch K, Borges ALS, Grosgogeat B. ***Hyaluronic Acid-Bupivacaine Hydrogel System for Temporomandibular Joint disorders: Mechanical Lubrification and Local Anesthesia in a preclinical approach. Système d'hydrogel à base d'acide hyaluronique et de bupivacaïne pour le traitement des troubles de l'articulation temporo-mandibulaire: lubrification mécanique et anesthésie locale dans le cadre d'une approche préclinique.***

ABSTRACT

Temporomandibular disorders (TMD) are primarily characterized by pain and impaired mobility, affecting 5-12% of the adult population and significantly compromising quality of life. Current therapeutic management is multimodal but suffers from high failure rates, highlighting the need for improved interventions. We hypothesized the combination of hyaluronic acid (HA) with bupivacaine (BUP) in the search for a cytocompatibility hydrogel with improved physicochemical properties, suitable for intra-articular therapy of TMD, aiming at physical action, with the viscosity of HA and the prolonged analgesic effects of BUP in a single platform. FTIR analysis confirmed the presence of functional groups of both components. TGA revealed that the HA matrix significantly increased the thermal stability of BUP, delaying its decomposition. Rheological tests demonstrated predominant viscoelastic behavior (G' > G"), with adjustable stiffness depending on the concentration of BUP. Cytocompatibility assays on RAW 264.7 cells (OECD 129) showed that the HA-BUP formulation-maintained cell viability above 98%, in contrast to the use of the anesthetic (BUP) alone (53.6%), and did not exhibit genotoxicity (OECD 487). These findings conclude that the HA-BUP hydrogel has adequate biomechanical, thermal, and biocompatible properties in the laboratory setting. In vivo and clinical studies are needed to evaluate long-term efficacy and safety.

Keywords: Hydrogels; Hyaluronic acid; Anesthetics; Temporomandibular disorders.

RÉSUMÉ

Les désordres temporo-mandibulaires (DTM) se caractérisent principalement par la douleur et une mobilité réduite, affectant de 5 à 12 % de la population adulte et compromettant significativement la qualité de vie. Le traitement thérapeutique actuel est multimodal, mais présente des taux d'échec élevés, ce qui souligne le besoin d'interventions améliorées. Notre hypothèse a été de combiner l'acide hyaluronique (HA) avec la bupivacaïne (BUP) à la recherche d'un hydrogel cytocompatible possédant des propriétés physico-chimiques améliorées, adapté à la thérapie intra-articulaire des DTM, visant une action physique grâce à la viscosité du HA couplée aux effets analgésiques prolongés de la BUP au sein d'une même plateforme. L'analyse par FTIR a confirmé la présence des groupes fonctionnels des deux composants. La TGA a révélé que la matrice de HA a augmenté de manière significative la stabilité thermique de la BUP, retardant sa décomposition. Les tests rhéologiques ont démontré un comportement viscoélastique prédominant ($G' > G''$), avec une rigidité ajustable selon la concentration de BUP. Les essais de cytocompatibilité sur cellules RAW 264.7 (OCDE 129) ont montré que la formulation HA-BUP maintenait la viabilité cellulaire au-dessus de 98 %, contrairement à l'utilisation de l'anesthésique (BUP) seul (53,6 %), tout en ne présentant aucune génotoxicité (OCDE 487). Ces résultats permettent de conclure que l'hydrogel de HA-BUP possède des propriétés biomécaniques, thermiques et de biocompatibilité adéquates en environnement de laboratoire. Des études in vivo et cliniques sont nécessaires pour évaluer son efficacité et sa sécurité à long terme.

Mots-clés : hydrogels ; acide hyaluronique ; anesthésiques ; désordres temporo-mandibulaires.

*Hyaluronic Acid-Bupivacaine Hydrogel System for Temporomandibular Joint disorders: Mechanical Lubrification and Local Anesthesia in a preclinical approach**

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1. Introduction

Temporomandibular disorders (TMDs) encompass conditions affecting the temporomandibular joint (TMJ), masticatory muscles, and associated tissues. These disorders are typically characterized by pain and functional impairment (Maini & Dua, 2023). As a significant public health concern, TMDs rank as the second most prevalent source of musculoskeletal pain, after low back pain, with prevalence estimates ranging from 5% to 12% according to the National Institute of Dental and Craniofacial Research (NIDCR, 2020). While TMD symptoms are not age-specific, the most affected demographic is adults aged 20–40 years. Additionally, epidemiological studies indicate that women are significantly more likely than men to exhibit TMD symptoms (Makris et al., 2015; Manfredini et al., 2013).

Pain during mastication primarily results from abnormal TMJ or masticatory muscle function. Pain-induced restricted jaw mobility and subsequent difficulty in food intake significantly impair patients' quality of life (Makris et al., 2015; NIDCR, 2020). Wan et al. (2025) suggest that TMDs frequently coexist with mental health disorders, particularly depression and anxiety, affecting a substantial portion of the global population. The interplay between TMDs and mental health disorders contributes to complex comorbidity, perpetuating a cycle of mutual influence and reinforcement.

Peripheral and central sensitization are key mechanisms in the exacerbation and chronicity of painful TMDs. Peripheral sensitization increases nociceptive neuron responsiveness while lowering pain thresholds in areas of tissue damage (Sessle, 2011). Tissue injury releases inflammatory chemical mediators, activating immune cells such as neutrophils, mast cells, and macrophages. These activated cells subsequently produce inflammatory mediators, including serotonin (5-HT), prostaglandin E2 (PGE2), nerve growth factor (NGF), adenosine triphosphate (ATP), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β), which further stimulate nociceptive terminals, perpetuating the inflammatory process (Steinhoff et al., 2014).

Treating articular cartilage defects remains one of the most challenging clinical problems for maxillofacial surgeons. Articular cartilage is a highly organized tissue with substantial durability but limited intrinsic healing capacity. Trauma or degenerative pathology often leads to progressive tissue deterioration, resulting in debilitating joint pain, functional impairment, and degenerative arthritis. Chondral and osteochondral lesions due to injury or other pathologies commonly contribute to osteoarthritis

development, eventually leading to progressive joint destruction. Thus, the limited regenerative capacity of articular cartilage often makes joint arthroplasty an inevitable surgical intervention (Makris et al., 2015).

Currently, the standard surgical treatment for end-stage degenerative joint pathology is total joint replacement. Early surgical interventions for symptomatic cartilage lesions, including osteotomy and autologous osteochondral grafting have been proposed to restore joint congruency and minimize further joint deterioration. However, these techniques often fail to provide long-term clinical solutions, prompting the development of regenerative medicine and tissue engineering approaches for cartilage restoration.

In cases of severe joint pain with limited mouth opening, treatment is sometimes empirical (Makris et al., 2015; Wan et al., 2025). The range of methods for managing joint pain and restricted mandibular mobility is extensive, often involving combined therapies. Depending on the specific diagnosis and symptom severity, interventions may include psychotherapy, physical therapy, systemic pharmacotherapy, splint therapy, dry needling, intra-muscular injections, intra-articular injections, arthrocentesis, arthroscopy, open joint surgery, and joint replacement (Nowak et al., 2021; Pihut et al., 2016; Sessle, 2011; Sikora et al., 2020; Steinhoff et al., 2014).

Due to its minimally invasive application, intra-articular drug administration, such as hyaluronic acid (HA) and anesthetics, is a focus of current scientific research.

Hyaluronic acid (HA) is a natural, unbranched polymer belonging to the glycosaminoglycan (GAG) group, a major component of the extracellular matrix (ECM) (Maini & Dua, 2023; NIDCR, 2020). It is a polar (hydrophilic) molecule with a simple chemical structure, composed of repeating disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine linked by alternating β-1,3 and β-1,4 glycosidic bonds (Makris et a., 2015; Manfredini et al., 2013; Sessle, 2011; Sikora et al., 2020; Steinhoff et al., 2014; Wan et al., 2025). In its native form, HA exists as a very long polymer, termed high-molecular-weight HA (HMWHA) (NIDCR, 2020). However, under certain conditions, it can degrade into smaller fragments, referred to as low-molecular-weight HA (LMWHA) (NIDCR, 2020). HMWHA (>1000 kDa) is present in intact tissues and exhibits anti-angiogenic and immunosuppressive properties, whereas smaller polymers indicate tissue distress and act as potent inducers of inflammation and angiogenesis.

Discovered in 1957, bupivacaine (1-Butyl-N-(2,6-dimethylphenyl)-2-piperidinecarboxamide) is a widely utilized amino-amide local anesthetic (LA) and a World Health Organization Essential Drug (Shafiei et., 2020; WHO, 2017). Its mechanism of action involves binding to the intracellular domain of voltage-gated sodium channel alpha subunits, inhibiting sodium influx into axons and consequently blocking depolarization and pain signal transmission (Shafiei et., 2020). In musculoskeletal surgery, bupivacaine is applied via local infiltration anesthesia (LIA) following total hip arthroplasty (THA) and total knee arthroplasty (TKA). This method demonstrates lower systemic toxicity and fewer adverse effects compared to opioid-based analgesia (Andersen & Kehlet, 2014; Møiniche S et al., 1999; Mörwald et al., 2017; Rubin et al., 2018). Additionally, LA infiltrations serve diagnostic purposes in joint disorders such as hip and shoulder osteoarthritis.

Bhargava et al. (2023) compared the efficacy of prolotherapy—a regenerative therapy using bupivacaine-dextrose (HDP) versus autologous blood injection (ABI) in treating symptomatic TMJ hypermobility. In a preliminary randomized clinical trial, both methods significantly reduced pain and improved joint function, with HDP showing superior outcomes in joint stability and reduced recurrence compared to ABI. The study suggests HDP as a promising therapeutic option for TMJ hypermobility, though further research is needed to confirm these findings in larger cohorts with extended follow-up (Nagella et al., 2023).

Lubecka et al. (2023) included bupivacaine among intra-articular anesthetics evaluated for TMD pain relief. Their meta-analysis demonstrated that bupivacaine, like other LAs (e.g., lidocaine), provided significant pain reduction and functional improvement, though some studies noted differences in duration of effect. No serious adverse effects were reported with TMJ intra-articular administration, supporting its safety profile when properly applied. However, the authors emphasize the need for further research to directly compare efficacy and effect persistence among different anesthetics, including bupivacaine.

This study aimed to synthesize a novel hydrogel composed of hyaluronic acid (HA) combined with four different concentrations of bupivacaine (BUP). We sought to evaluate its physicochemical properties through rheology, thermogravimetric analysis (TGA), and Fourier-transform infrared spectroscopy (FTIR), as well as its in vitro cytocompatibility and genotoxicity. The overarching goal was to develop a dual-

action therapeutic platform for temporomandibular disorders (TMD), combining the viscosupplementation properties of HA with the potential for prolonged local analgesia from BUP.

2. Materials and methods

2.1. Hydrogels preparation and sterilization

This study was conducted using hyaluronic acid, composed of glucuronic acid and N-acetylglucosamine, with a molecular weight of 1.300.000 Daltons (CAS: 9004–61–9, purity: 95.9%, Inlab Analitic®, code: HA2022042345X, São Paulo, SP, Brazil).

In a Class II biological safety cabinet (Veco®, Biosseg-06, Sumaré, SP, Brazil), all reagents were weighed using an analytical balance (Mettler Toledo®, model XPR106DUH/A, Columbus, OH, USA). A 3% (w/v) solution of hyaluronic acid was prepared in ultrapure Type I water for physicochemical and biological analyses. Bupivacaine (BUP, CAS: 38396-39-3, purity: 98%, code: 23030098) was then incorporated at concentrations of 1%, 0.5%, 0.25%, or 0.125% (w/w). The resulting hydrogel was homogenized using Dual asymmetric centrifuge mixer (Hauschild®, Speed MixerDAC150. 1 FVZ, Water-kamp, Hamm, Germany), for 1 min, and the pH was adjusted to 7.2 with sodium hydroxide (NaOH, CAS: 1310–73–2, purity: 98%, code: S5881, Sigma-Aldrich®, St. Louis, MO, USA). Finally, the gels were sterilized by autoclaving (Cristofoli Biossegurança®, Vitale21, Campo Mourão, Parana, Brazil) at 118 °C for 5 min (Haridas et al., 2019).

2.1.1. Freeze-drying

The hydrogel samples were frozen at NuAire Laboratory Equipement® (Glacier polar edition -86 ultra-low temperature freezer, Plymouth, Minnesota, USA) for 24 h and then placed in a freeze-dryer (Terroni®, LS3000, São Carlos, São Paulo, Brazil) for 3 days to sublimate the solvent.

2.2. Chemical characterization by attenuated total reflectance-Fourier transform infrared spectroscopy (FTIR)

The freeze-dried samples were analyzed by FT-IR (Perkin Elmer®, LR64912C, Waltham, Massachusetts, USA), obtaining wave number spectra between 500 and 4000 cm-1, with a nominal resolution of 4 cm-1 and 100 scans per sample.

2.3. Thermal degradation profile by Thermogravimetric analysis (TGA)

The samples of the gels were analyzed by Thermogravimetric Analyzer (TGA) (NETZSCH®, STA449F1 Jupiter, Selb, Bavaria, Germany), obtaining the thermal

analysis curves by scanning the temperature range between 30 and 300 °C with a rate change of 10 °C/min in an atmosphere of argon (Ar).

2.4. Characterization of the viscoelastic profile in rheological analysis

The analysis was performed at Dynamic shear Rheometer (Anton-paar®, SmartPave 92, Graz, Austria).

2.4.1. Frequency sweep

In the frequency ramp, after the sample had settled, we waited 2 min for the hydrogels to recover and relax. Shear deformation was carried out with a 1% constant and frequency decreasing from 10 to 0.1 hertz.

2.4.2. Amplitude sweep

In the amplitude ramp, after the sample had settled, we waited 2 min for the polymers to recover and relax. A controlled shear deformation ramp was applied from 0.001 to 100% with a frequency of 10 radians/second, with a fixed frequency.

2.4.3. Evaluation of the thermal behavior of hydrogel on human physiological conditions by temperature ramp

The sample was preheated to 20 degrees Celsius for 2 min, after which the temperature ramp test was applied with a controlled deformation ramp and frequency; with a deformation of 1% and a frequency of 10 radians/second and a heating rate of 1° Celsius per minute.

2.5. Morphological characterization of the hydrogel surface scanning electron microscopy with field emission gun (SEM-FEG)

For SEM, the lyophilized hydrogels were transferred to aluminum stubs and covered with gold for 120 s at 40 mA (Sputter Coater - Quorum Technologies®, Emitech - SC7620, Kent, UK). After the process, the discs were analyzed and photographed by the scanning electron microscope with Field Emission Gun (FEG®, TESCAN MIRA 3®, Brno, Czech Republic).

2.6. Biological performance on in vitro cell cultures

Cytocompatibility was carried out on mouse macrophages (RAW 264–7), comes from the Rio de Janeiro cell bank (BCRJ). Cell culture was carried out in Eagle's medium modified by Dulbecco - DMEM (LGC Biotechnology®, Cotia, Brazil) supplemented with 10% Fetal Bovine Serum - FBS (Invitrogen®, New York, USA), incubated in an oven at 37 °C, with atmospheric humidity and 5% CO_2 (Sanyo®, MCO-19AIC(UV), Osaka, Japan).

The tests were carried out in 24-well microplates using 4 × 10^4 cells, cultured in 500 µL of DMEM + 10% FBS medium, incubated at 37 °C with 5% CO_2 for 24 h. After this period, the supernatant was discarded, and the cells were exposed to the treatments.

2.6.1. Treatment via transwell

The treatments were carried out according to the groups: 500 µl of 3% HA; 500 µl of 1% BUP, 500 µl of 3% HA + 1% BUP, 500 µl of 3% HA + 0.500% BUP, 500 µl of 3% HA + 0.250% BUP, 500 µl of 3% HA + 0.125% BUP and 500 µl of DMEM + 10% FBS as a control. After 24 h of exposition, the treatments were discarded, and the wells washed three times with Phosphate buffer saline (PBS code: P2272, Sigma-Aldrich®, St. Louis, USA).

2.7. Cell viability by resazurin essay (OECD 129)

Metabolic activity was checked using resazurin at 440 µM (7-Hidróxi-3H-fenoxazin-3-ona-10-óxido CAS n°: 62,758-13–8, code: R1017, Sigma-Aldrich®, St. Louis, USA). For this, 50 µl of the resazurin suspension was added to each well of the microplate, followed by the addition of 450 µl of DMEM + 10% FBS. Incubation in the dark was carried out for 16 h, after which the plate was read in a spectrophotometer (Lonza Biotek ®, ELX808LBS, Winooski, Vermont, USA) at a wavelength of 570 nm. The optical densities (OD) obtained were converted into a percentage of cell viability using the following formula:

% Metabolic Activity = (OD Treated Group x 100) / Average OD Control Group.

2.8. Genotoxicity assay by micronucleous (OECD 487)

Mouse macrophages at a concentration of 3 × 10^5 cells/mL were cultured in 96-well microplates with 1 mL of DMEM supplemented with 10% SFB for 24 h at 37 °C in a 5% CO2 atmosphere. The cells were exposed to the experimental groups at same conditions. The negative control group received only the culture medium, while the positive control group received Ethyl Methane sulfonate (EMS - CAS n°: 62–50–0, code: M08080, Sigma-Aldrich®, St. Louis, USA) at a concentration of 5 mM, both treatments applied for 24 h.

After the treatments, the cells were washed with PBS three times and incubated with cytochalasin B (CAS n°: 14930-96-2, code: C2743, purity: 98%, Sigma-Aldrich®, St. Louis, USA) at a concentration of 6 µg/mL for 24 h at 37 °C in a 5% CO_2 atmosphere. After the incubation, the cells were fixed in 100% methanol (CAS n°: 67-56-1, purity: 99,8%, Synth®, Diadema, Brazil) for 20 min, followed by staining with DAPI (4',6-

Diamidino-2-fenilindol, 2-(4-Amidinofenil)-6-indolecarbamidina with fluoroshield CAS n°: 28718-90-3, code: F6057, Sigma-Aldrich®, St. Louis, USA). The dye was removed after 5 min of contact with the cells, followed by three washes with PBS. Micronuclei were analyzed under a fluorescence microscope (Ziess®, Axio Observer A1, Jena, Thuringia, Ger-many) at 40X magnification with a total of 2.000 cells evaluated per well.

2.9. Statistical analysis

The data obtained was initially analyzed for normality using D'Agostino, Shapiro-Wilk, and Kolmogorov-Smirnov tests. Those that showed normality were then analyzed by ANOVA complemented by Tukey. Those that did not show normality were evaluated by Kruskal Wallys complemented by the Dunn's test. All analysis utilized p value of 0.0001.

3. Results

3.1. Chemical characterization by attenuated total reflectance-Fourier transform infrared spectroscopy (FTIR)

FTIR analysis, showed in Fig. 1, was used to characterize and validate the raw materials used, with isolated spectra of hyaluronic acid (HA) and bupivacaine (BUP), as well as to verify the molecular behavior of the compounds when combined in the hydrogel composition. The HA spectrum exhibited characteristic absorption bands corresponding to key functional groups: a broad band at 3300 cm^{-1} attributed to O-H and N-H stretching vibrations; a weak band at 2882 cm^{-1} associated with C-H stretching (lines 1 and 2); a strong absorption at 1600 cm^{-1} due to asymmetric C=O stretching of carboxyl groups (line 4); a peak at 1560 cm^{-1} assigned to amine II (N-H bending); a band at 1402 cm^{-1} corresponding to symmetric COO^- stretching; and absorptions at 1148 cm^{-1} (C-O-C bridging), 1080 cm^{-1} (exocyclic C-O and C-C stretching), and 1036 cm^{-1} (C-OH bending) (Alcântara et al., 2023; Elhiss et al., 2024; Kaya et al., 2024; Shao et al., 2024).

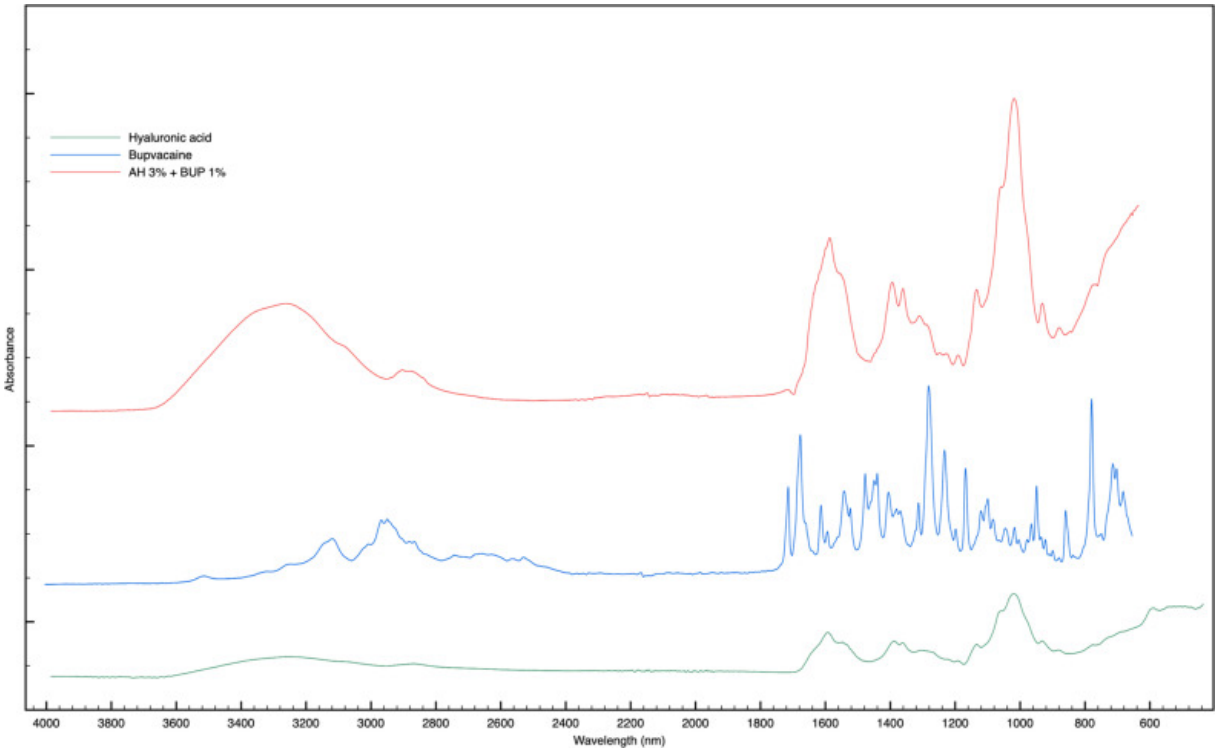


Fig. 1. FTIR spectra of HA (green line), BUP (blue line) and hydrogel 3% HA associated with 1% BUP (red line).

FTIR analysis of Bupivacaine showed the drug's signature bands. The O-H and N-H vibrations were identified at band 3105. Type I amide bonds presenting C=O carbonyl bonds were identified at 1676. Type II amide bonds in C-N-H bonds in the straight plane were identified at 1538. And type III amide bonds in the C-N bond were identified at 1281 (Nagella et al., 2023).

The FTIR spectrum of the gel (red line) displayed characteristic absorption bands corresponding to both drugs, confirming their presence in the hydrogel formulation (3% HA + 1% BUP). The absence of new peaks or significant shifts suggests no spontaneous chemical interaction between the compounds.

3.2. Thermal degradation profile by Thermogravimetric analysis (TGA)

Analysis reveals that pure bupivacaine is thermally less stable, undergoing rapid decomposition or volatilization around 100 °C. This volatilization of the pure drug is due to its solubilizer (water). After this main stage of mass loss, there is a small additional loss and a plateau of around 32% residual mass at the end of the analysis (600 °C), showed in Fig. 2.

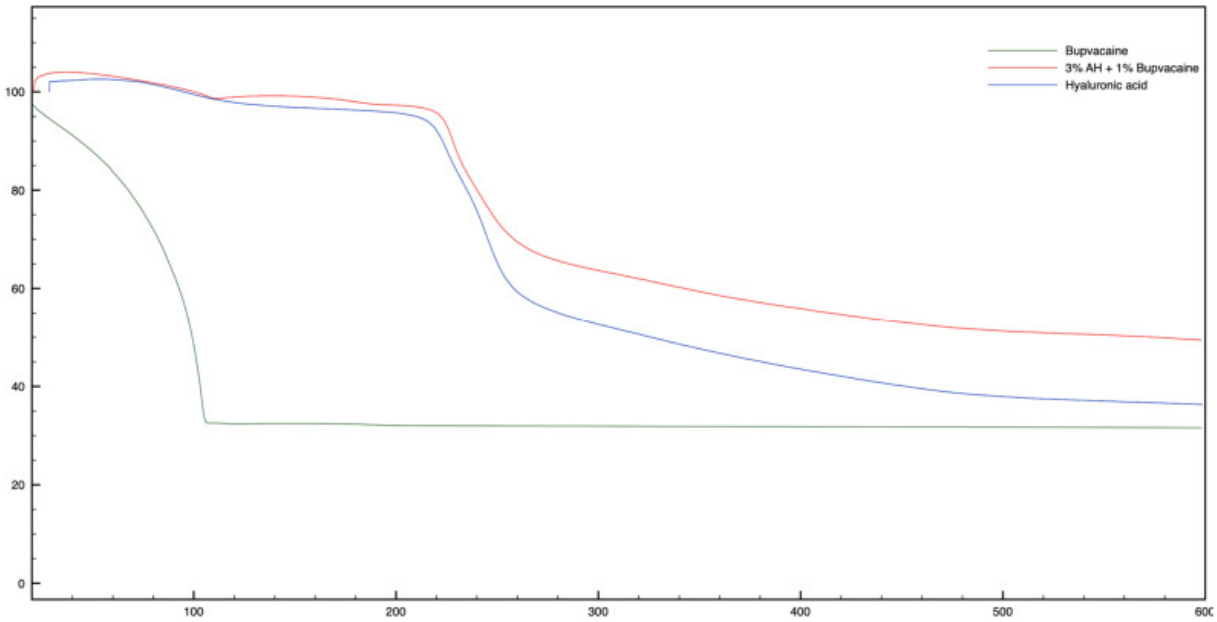


Fig. 2. Analysis of the decomposition of masses in relation to temperature. TGA spectra of HA (Blue line), BUP (Green line) and hydrogel 3% HA associated with 1% BUP (Red line).

Hyaluronic acid shows a more gradual decomposition over a wider temperature range, with a greater amount of residue at the end of the analysis. The main loss of mass occurs over a wider temperature range, starting at around 200 °C and continuing until the end of the analysis. At the end of the analysis (600 °C), hyaluronic acid retains a considerable amount of residual mass, around 45%. This suggests the formation of more thermally stable degradation products or the presence of non-volatile residues. The association of bupivacaine with hyaluronic acid gel influences the thermal behavior of bupivacaine. A small initial mass loss is observed, similar to hyaluronic acid, correlating with water loss. The main mass loss stage, associated with bupivacaine, occurs over a temperature range slightly shifted towards higher temperatures compared to pure bupivacaine (starting just before 200 °C). The slope of the curve at this stage is also less steep. After this stage, the mass loss continues gradually, following a trend closer to that of hyaluronic acid, although with a greater total mass loss. At the end of the analysis (600 °C), the hyaluronic acid gel with bupivacaine has an intermediate residual mass between the individual components, around 50%. This allows us to infer that there is a delay in the decomposition/volatilization of the 3%AH + 1% Bupvacaine gel.

3.3. Characterization of the viscoelastic profile in rheological analysis

3.3.1. Frequency sweep

Frequency scans, as shown in Fig. 3, reveal viscoelastic behavior typical of polymer networks structured by physical entanglements, where the storage (G') and loss (G") moduli show strong dependence on angular frequency. In non-autoclaved samples, a crossover point is observed at intermediate frequencies; below this point, the viscous character predominates (G" > G'), while at high frequencies the material exhibits a predominantly elastic response (G' > G'). Pure hyaluronic acid (HA) exhibits crossover at lower frequencies and reaches the highest G' values in the high-frequency range (550 Pa), suggesting a longer relaxation time. The incorporation of bupivacaine (BUP) shifts the intersection point to the right, indicating that the drug accelerates the relaxation dynamics of the chains, reducing the elastic resilience of the network under low deformation rates.

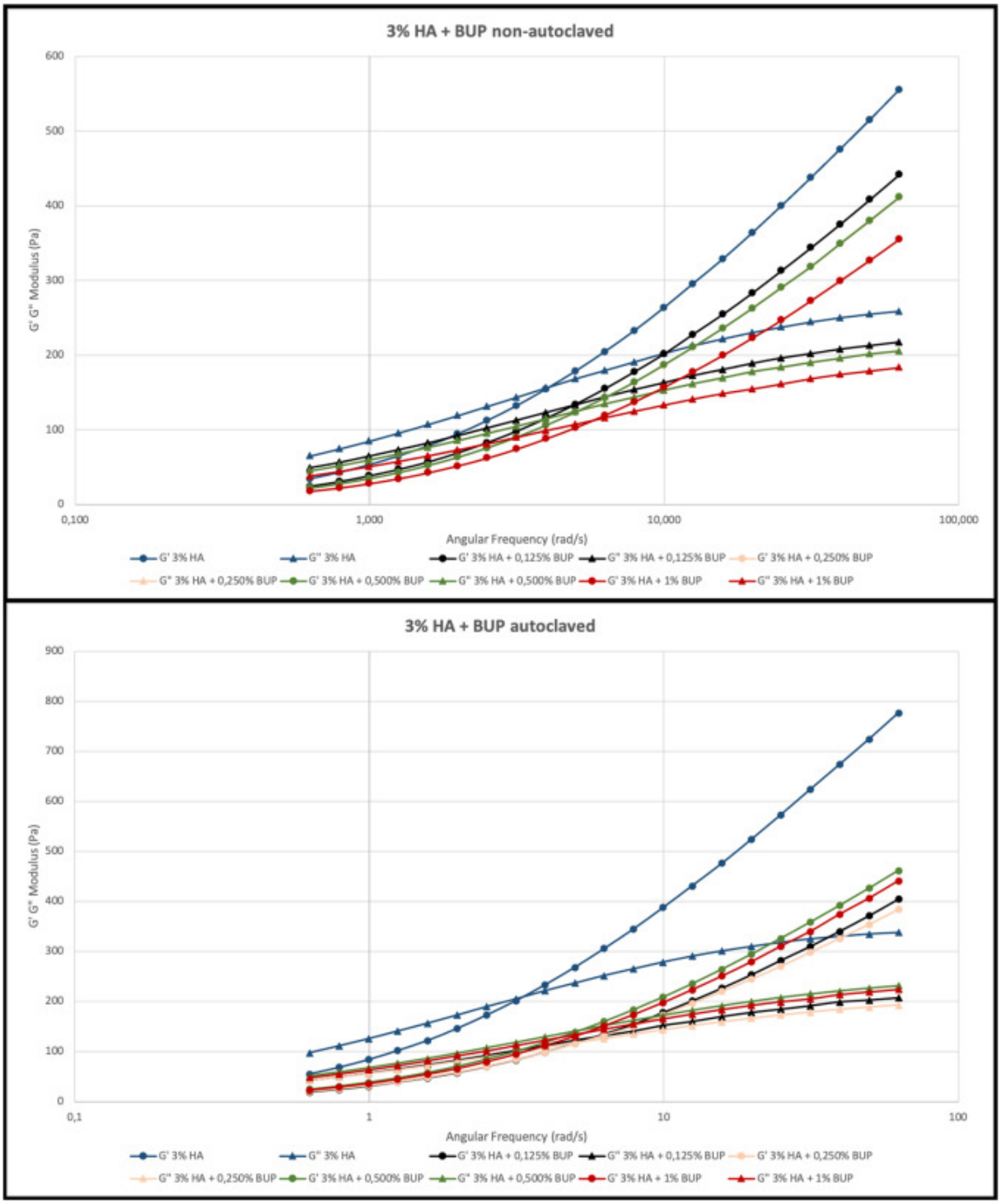


Fig. 3. Frequency sweep of 3% HA + BUP hydrogel non-autoclaved (A) and 3% hyaluronic acid HA + BUP hydrogels autoclaved (B).

After autoclaving, the rheological profile undergoes changes that point to a structural reorganization induced by thermal stress. Although pure HA shows a paradoxical increase in the magnitude of the elastic modulus at high frequencies (800 Pa),

formulations containing BUP exhibit an attenuation in the values of G' and G'' compared to the control, remaining grouped at lower levels. The sterilization process preserves frequency dependence, but the divergence between the G' and G'' curves becomes less pronounced for mixtures with bupivacaine compared to the original state. This phenomenon suggests that thermal degradation promoted a reduction in the molecular weight of the polymer, resulting in systems with lower stable entanglement density and an earlier transition to the viscous flow regime when compared to isolated hyaluronate after treatment.

3.3.2. Amplitude sweep

The amplitude sweep graphs, showed in Fig. 4, reveal that all formulations exhibit elastic gel behavior, characterized by a storage modulus (G') higher than the loss modulus (G'') in the linear viscoelasticity (LVE) region. In the non-autoclaved state, it is observed that the incorporation of bupivacaine (BUP) exerts a concentration-dependent structural reinforcement effect, with the 0.500% BUP sample reaching the highest level of stiffness (425 Pa). However, the 1% BUP concentration reverses this trend, suggesting that excess drug may destabilize interactions in the sodium hyaluronate network, reducing the magnitude of the elastic moduli.

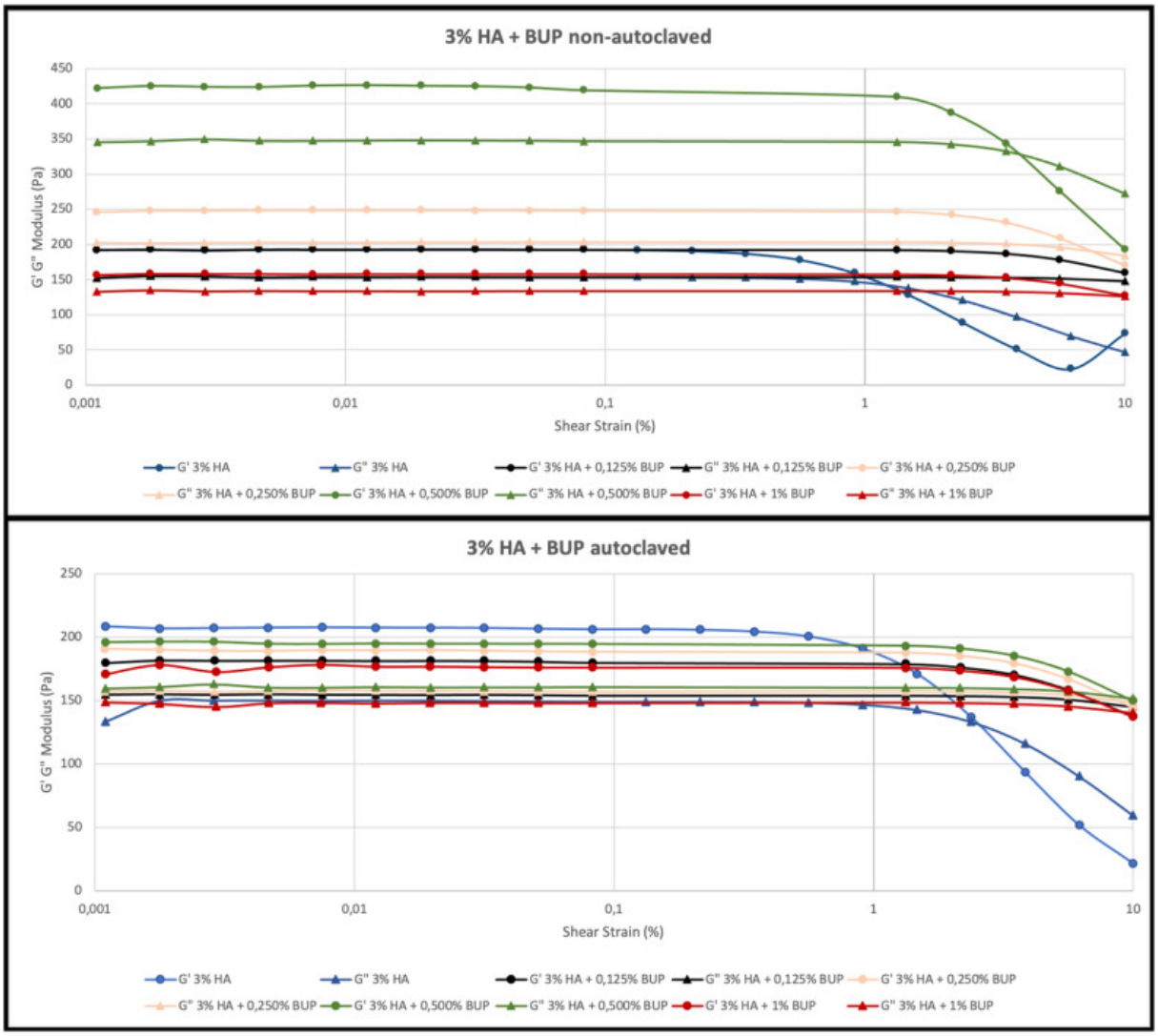


Fig. 4. Amplitude sweep of 3% HA + BUP hydrogel non-autoclaved (A) and 3% HA + BUP hydrogel autoclaved (B).

After the autoclaving process, significant thermal degradation of the rheological properties was observed, evidenced by the overall reduction in G' and G" values in all samples. Heat treatment promoted a standardization of mechanical behavior, where the distinction between different concentrations of BUP became less pronounced, with the modules stabilizing at lower levels (150−200 Pa). This phenomenon indicates that the thermal energy of sterilization caused the breakdown of hyaluronic acid polymer chains, resulting in less structured gels with lower resistance to deformation compared to the original samples.

3.3.3. Evaluation of the thermal behavior of hydrogel on human physiological conditions by temperature ramp

The results show that temperature, the autoclaving process, and the presence of the anesthetic interact in a complex manner, significantly altering the stiffness of the material (Fig. 5). In non-autoclaved samples, pure HA maintained a high storage modulus (G') up to 30 °C, followed by progressive softening. The addition of BUP drastically reduced the initial stiffness, but at higher concentrations (0.5% and 1%), it induced a marked increase in G' above 30–35 °C, suggesting a heat-induced structural reorganization. The autoclaved samples, on the other hand, exhibited radically different behavior: pure autoclaved HA showed much higher initial stiffness and a drastic phase transition above 30 °C, with an exponential increase in G'. The presence of BUP, however, attenuated or suppressed this transition, maintaining a much more moderate thermal response, although with G' values still higher than those of the corresponding non-autoclaved samples.

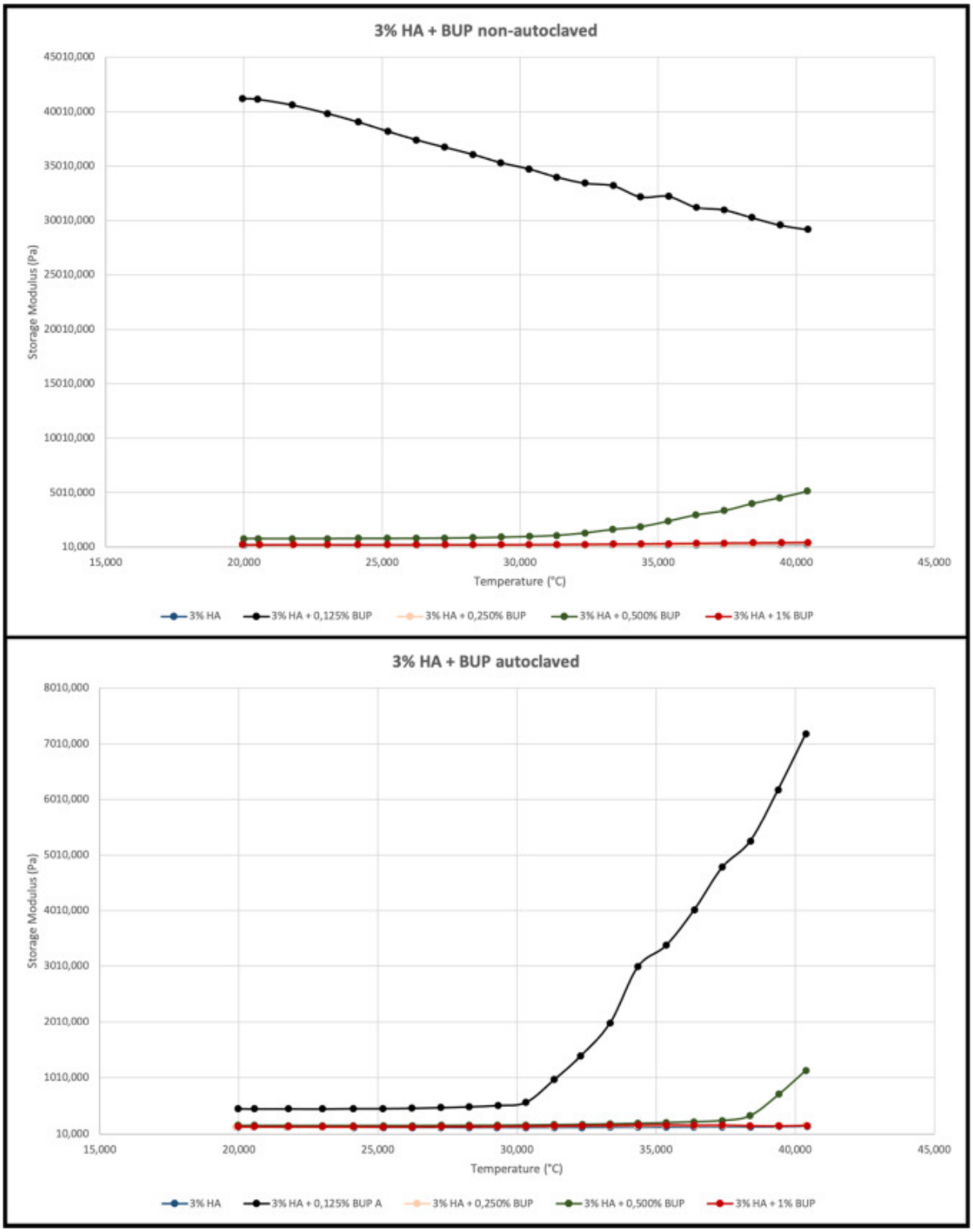


Fig. 5. Temperature ramp of 3% HA + BUP hydrogel non-autoclaved (A) and 3% HA + BUP hydrogel autoclaved (B).

3.4. Morphological characterization of the hydrogel surface scanning electron microscopy with field emission gun (SEM-FEG)

SEM-FEG analysis revealed no structural differences between isolated hyaluronic acid (HA) hydrogels and those combined with bupivacaine (Fig. 6). Both formulations exhibited a sheet-like morphology with a porous, three-dimensional network, featuring interconnected pores of varying sizes and distinct interlayer spacing. Notably, the drying process inherent to SEM preparation in-fluences crosslinking and alters porosity; thus, quantitative pore analysis was not per-formed.

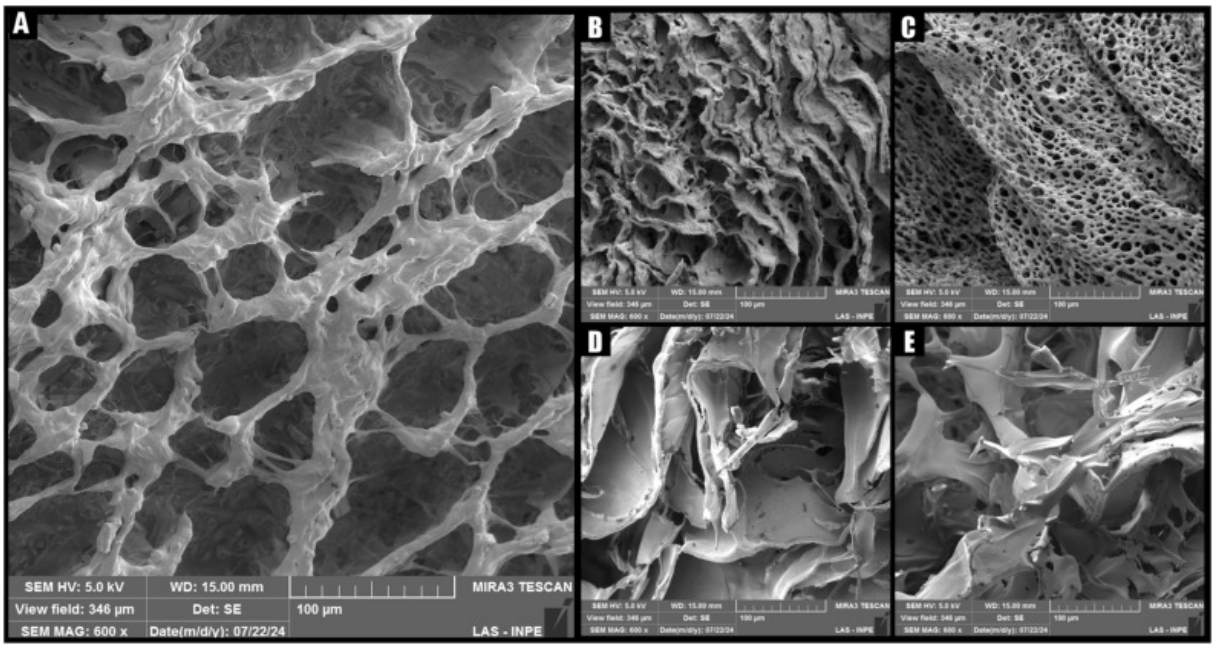


Fig. 6. Micrography of 3% HA hydrogel (A), 3% HA + 0.125% BUP hydrogel (B), 3% HA + 0.250% BUP hydrogel (C), 3% HA + 0.500% BUP hydrogel (D), 3% HA + 1% BUP hydrogel (E).

3.5. Cell compatibility by resazurin essay

After contact for 24 h with mouse macrophages (RAW 264-7) and application of the hyaluronic acid gel, the group showed 83.6% viability. The groups using the HA hydrogel conjugated to concentrations of 0.125, 0.250, 0.500 and 1% bupvacaine showed viability of 101.8%, 98.7%, 103.07% and 98.76%. The application of 1% bupvacaine alone promoted viability of 53.6%, as shown in Fig. 7.

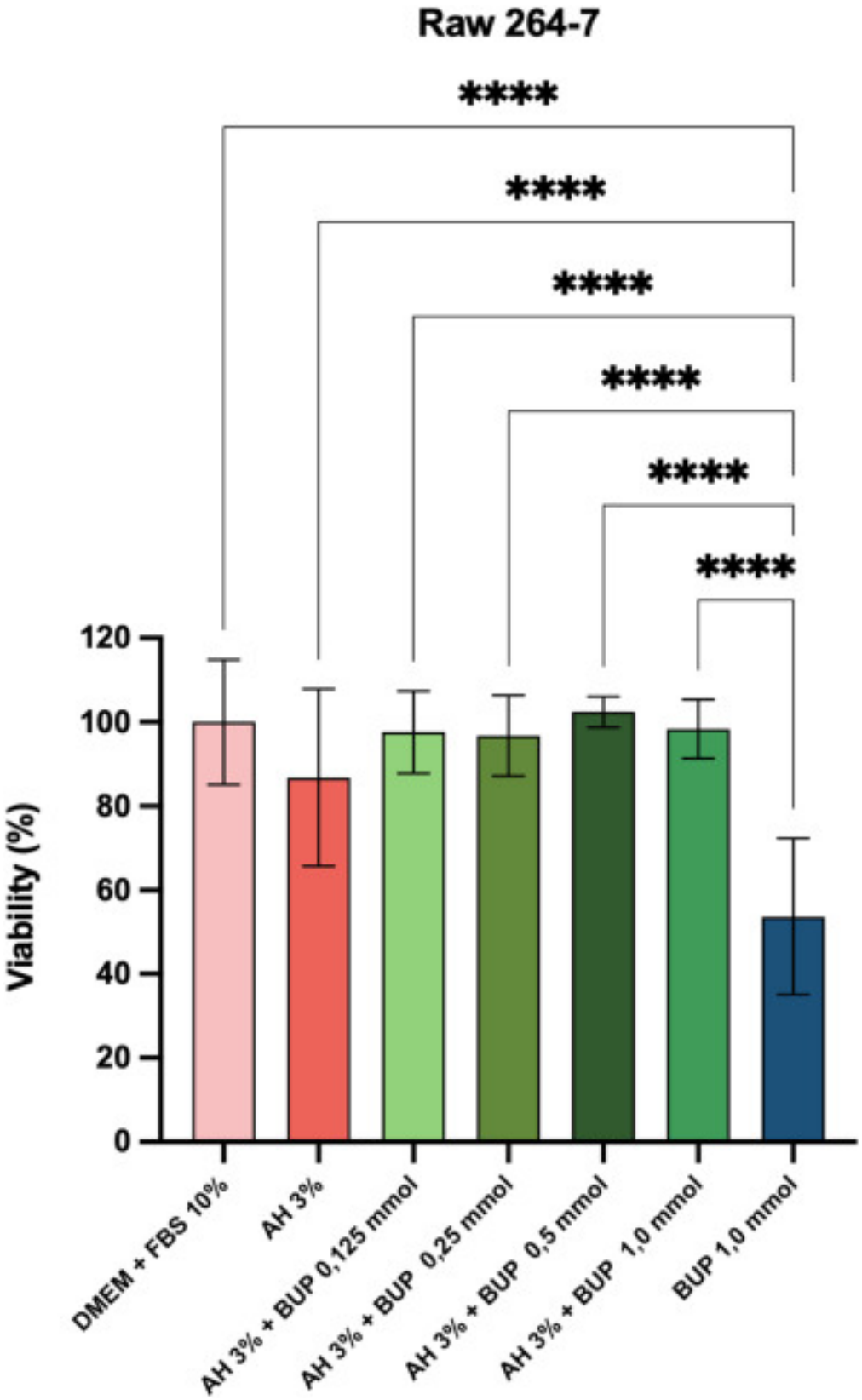


Fig. 7. Cytocompatibility by hydrogels on mouse macrophages (RAW 264.7). p < 0.0001 (****).

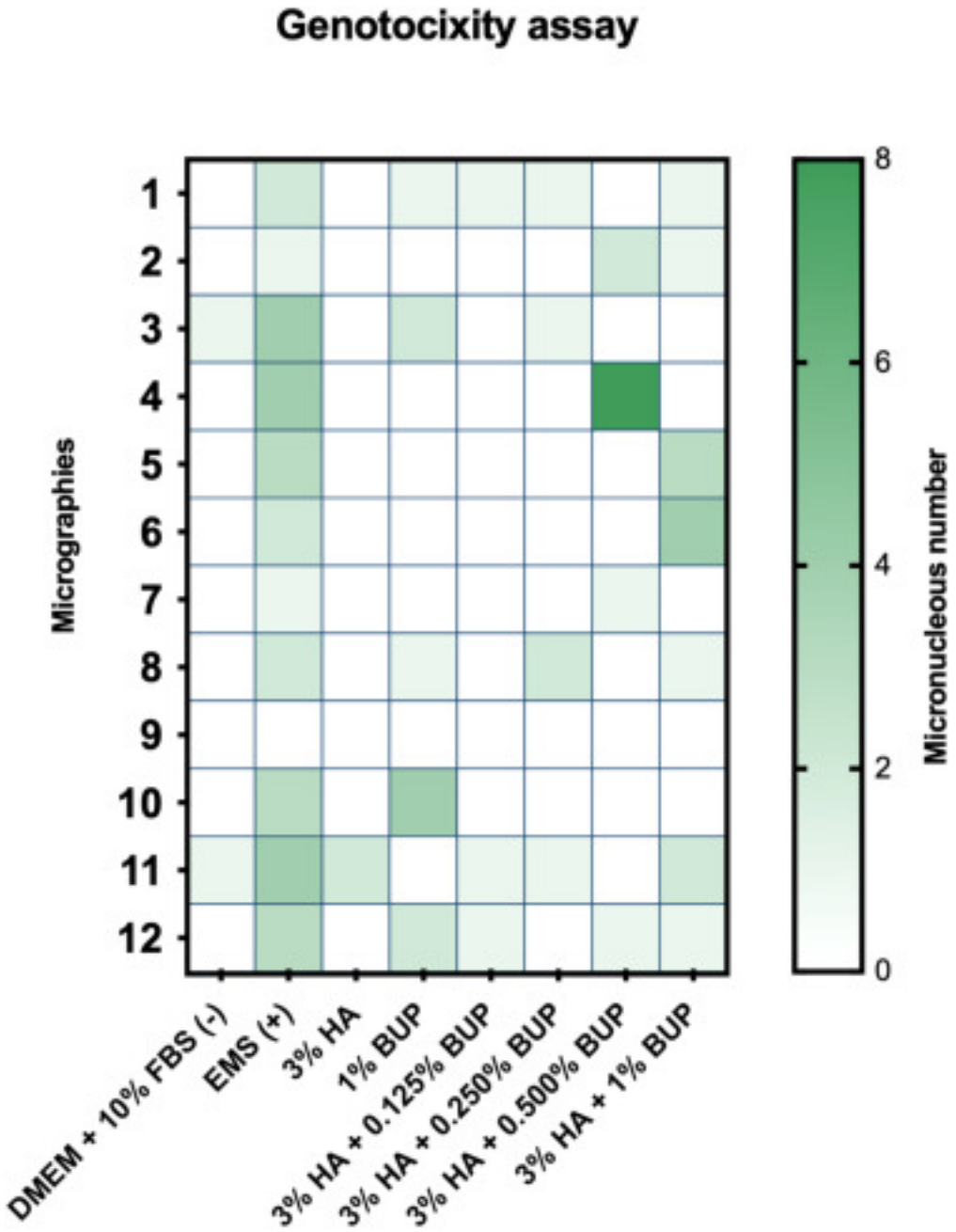


Fig. 8. Genotocixity assay by micronucleous.

3.6. Genotoxicity assay by micronucleous

Genotoxicity assessment revealed that DMEM + 10% FBS group and 3% HA group induced the formation of 2 micronuclei, while 1.0% BUP resulted in 10 micronuclei, as showed in Fig. 8. In the combination groups, 3% HA with increasing concentrations of BUP (0.125%, 0.250%, 0.500%, and 1.0%) produced 3, 5, 12, and 13 micronuclei, respectively. The positive genotoxicity control exhibited a significantly higher response, with 29 micronuclei formed.

4. Discussion

Hyaluronic acid viscosupplementation in the treatment of Temporomandibular Dysfunction is a minimally invasive technique, widely described in the literature and adopted clinically. Goiato et al. (2016) concluded in their systematic review that intra-articular injections of HA are beneficial for improving the pain and/or functional symptoms of TMD, however, other drug therapies such as corticosteroid injections, analgesics and non-steroidal anti-inflammatory drugs may be necessary for satisfactory results. For this reason, the present study developed a hyaluronic acid hydrogel associated with bupvacaine, evaluating its bio-physico-chemical characteristics for the treatment of TMD, to combine the mechanical properties of the natural polymer associated with the anesthetic effect of bupvacaine.

Chemical characterization of compounds mediated by FTIR revealed absorption bands characteristic of both compounds, Hyaluronic acid and Bupvacaine, confirming their presence in the formulation without evidence of significant chemical interactions (Al-Sibani et al., 2018). The absence of new peaks or significant shifts in the bands suggests that there were no spontaneous chemical interactions between HA and BUP during the preparation of the hydrogel. This is particularly important to ensure the stability of the formulation and the maintenance of the pharmacological properties of bupivacaine (Joshi & Sangamwar, 2022; Ström et al., 2015). The results obtained show that the combination of HA and BUP in the hydrogel does not induce significant chemical changes, indicating compatibility between the components. This lack of chemical interactions is favorable for therapeutic applications, as the individual properties of each compound are preserved. Further studies should be carried out to evaluate the controlled release of bupivacaine and the efficacy of the hydrogel under physiological conditions.

Starting the physical characterization. Thermogravimetric analysis (TGA) showed that pure bupivacaine has low thermal stability, volatilizing 68% of its mass at around 100 °C and leaving 32% residue at 600 °C. In contrast, hyaluronic acid (HA) showed gradual decomposition between 200 and 600 °C, with a final residue of 45%, indicating greater thermal stability due to its polymeric structure. The combined formulation of 3% HA + 1% bupivacaine showed intermediate behavior, with the decomposition of bupivacaine shifting to higher temperatures (starting around 200 °C) and a final residue of 50%, suggesting that HA acts as a thermal stabilizer, delaying the volatilization of

the drug without presenting irreversible chemical interactions. This valuable finding is relevant to the manufacturing and storage process of the future drug. The results corroborate the conclusions of Yang et al. (2025), who highlight the role of HA in increasing the physical stability of bupivacaine through thermal retardation or encapsulation. The main difference lies in the methodological approach, while the present work approaches these data by thermogravimetric analysis (high temperatures), the study by Yang et al. validates efficacy under physiological conditions. Together, they reinforce that HA-based formulations are promising for prolonged analgesia, whether in industrial (thermal processing) or clinical (intra-articular therapy) contexts.

In the analytical context of viscosity, the frequency sweep indicate that the HA hydrogel associated with bupivacaine, after the sterilization process, underwent a structural reorganization induced by thermal stress, thus decreasing its molecular weight. A similar phenomenon was reported by Haridas et al. (2019), who observed that heat-induced conformational changes in HA can increase its initial rigidity. The combined outcome demonstrates that while autoclaving improves the mechanical properties of the base HA gel, it does not fully counteract the softening effect induced by bupivacaine. The final gel's rigidity is therefore a balance between the reinforcing effect of sterilization and the concentration-dependent plasticizing effect of the anesthetic drug.

The observed increase in storage modulus (G') following autoclaving is consistent with a thermal strengthening of the HA polymer network. This can be mechanistically explained by the work of Li et al. (2026), who used molecular dynamics simulations to demonstrate that the viscoelastic response of HA is governed by the dynamics of its hydrogen bond network. The thermal energy from autoclaving likely promotes the reorganization and stabilization of this network, resulting in greater elastic solid behavior, as measured by the higher G'.

Conversely, the concentration-dependent reduction in G' caused by the incorporation of bupivacaine suggests the anesthetic disrupts the hydrogels microstructure. Objectively speaking, a plasticizing agent is a substance which, when added to a polymer, makes it more flexible, malleable, due to an increase in the mobility of the macromolecules and/or a decrease in the intensity of the chemical bonds. The plasticizing action of bupvacaine, highlighted in this study, can be confirmed by the

findings of Wójcik-Pastuszka et al. (2024) who point out that it is interesting to note that the incorporation of bupvacaine in formulations containing only the ionic polymer HA, decreased the viscosity compared to the viscosities of compositions without the anaesthetic. This must be related to the ionic interactions of Bupvacaine with the polymer and should be studied further to better characterize the molecular interactions. This data aligns with the molecular mechanism proposed by Li et al. (2026), wherein the mechanical properties of HA are highly sensitive to its hydrogen bonding integrity. It is plausible that bupivacaine molecules interfere with the inter-chain hydrogen bonds critical for network elasticity, leading to a softer, less rigid gel.

In the quest to extrapolate the data obtained from the rheological characterization of hydrogel, aiming at its applicability in replacing synovial fluid, there is a gap in the literature and a lack of guidelines that determine the shear viscosity in healthy joints (More et al., 2020). This factor is understandable, given the low volumes of synovial fluid in the human body, the absence of indications for removal of this fluid in healthy individuals, and rheology machines that require volumes in the milliliter range. The combination of these factors makes many of these studies unfeasible. However, Madkhali et al. (2016) observed that the shear viscosity of synovial fluid in healthy joints ranged from 1 to 175 Pa, while joints with osteoarthritis exhibited values from 0.05 to 14.05 Pa. Regarding the hydrogels developed in the present study, the results indicate values of 210 Pa for 3% HA and values of 180, 190, 200, and 175 Pa for the 3% HA groups associated with 0.125, 0.250, 0.500, and 1%. Notably, increasing the drug load to 1% BUP resulted in a loss of stiffness, with the modulus declining to 175 Pa, suggesting possible saturation or destructuring of the polymer network. Thus, the concentration of 0.500% BUP is established as the ideal equilibrium point, ensuring the greatest elastic stability and dynamic resistance among the pharmaceutical mixtures subjected to thermal stress. Given the results obtained compared to the data reported by Madkhali et al. (2016) the rheological behavior of the HA-BUP 0.125 and 1% hydrogels closely mimics the viscoelastic nature of healthy human synovial fluid (SF).

After completing the physical-chemical characterizations, we sought to verify the cellular compatibility of the material. The evaluation indicated that pure hyaluronic acid (HA) gel maintained 83.6% viability in RAW 264.7 macrophages after 24 h. The formulations of HA conjugated with bupivacaine (0.125%, 0.250%, 0.500%, and 1%)

showed high viability (98.7–103.1%), indicating biocompatibility based on OECD 129. In contrast, pure 1% BUP drastically reduced viability to 53.6%, evidencing its direct cytotoxic effect. The cytotoxicity of the anesthetic can be verified in the studies by Chen et al. (2022, 2024) on human chondrocytes (TC28a2) and breast carcinoma cells (MDA-MB231). For the TC28a2 line, the authors indicate an IC50 of 2.8 mM bupivacaine, while for the MDA-MB231 line, the IC50 was 1.8 mM. These data corroborate the findings of the present study and demonstrate that the association with HA neutralizes the toxicity of the anesthetic.

Systematic reviews, such as that by Lubecka et al. (2023), also report the use of bupivacaine alone in the temporomandibular joint. The authors conclude that the anesthetic remains effective at the site for only 24 h, does not improve jaw mobility, and has a statistically significant chondrotoxic potential. These findings are important given that the present study formulated a hyaluronic acid-based hydrogel with the anesthetic bupivacaine. The formulation aims to utilize the mechanical effect of hyaluronic acid's viscosity to reduce mandibular friction. Hyaluronic acid also exhibited a cytotoxicity-attenuating effect against mouse macrophages, demonstrating its potential as a drug delivery system.

It is also important to note that the review conducted by Lubecka et al. (2023), identified innovative approaches to the treatment of temporomandibular dysfunction. For instance, the study by Hegab et al. (2023), which evaluated the combination of platelet-rich plasma (PRP) and hyaluronic acid (HA) following arthrocentesis (AC), resulted in greater maximum voluntary mouth opening, reduced pain, and improved joint noises at 12 months, compared to AC+HA. These findings lead us to believe that arthrocentesis promoted the removal of synovial fluid filled with cytokines, chemokines, and residues from the inflammatory process, followed by the mechanical action of hyaluronic acid, reinforcing the proposition of this study, which aims to use hyaluronic acid as a mechanical agent to improve friction and, consequently, mandibular mobility.

*The anesthetic's genotoxicity was assessed by Önal et al. (2024) using the comet test on human colon fibroblast cell lines (CCD-18Co, CRL-1459™). The authors point out that the levels of DNA damage in the cells increased significantly at concentrations of 10 and 20 μM of bupivacaine ($p < 0.05$ * for 10 μM, $p < 0.01$ * for 20 μM) and increased severely at concentrations of 40 μM to 300 μM of bupivacaine. Despite different methodologies and cell types, the results of the present study differ from the findings*

of Onal et al. (2024) where mouse macrophages (RAW 264–7) submitted to the isolated application of bupvacaine and also to hydrogels did not exhibit the formation of micronuclei, thus concluding the absence of genotoxicity.

Gürbüzel et al. (2016) evaluated the anesthetic's genotoxicity using the somatic mutation and wing recombination test in Drosophila melanogaster. Three-day-old trans-heterozygous larvae were treated with bupivacaine. Analysis of standard crosses indicated that bupivacaine does not show mutagenic or recombinogenic activity up to high concentrations (500 µg/mL). Data in line with the results presented in this study.

As a limiting point of this study, it is worth noting that the biological analyses were carried out on a monolayer, evaluating exclusively the metabolic behavior of one immune cell (macrophages). Further research is needed to evaluate the application of the drug in chondrocytes and synoviocytes, exhausting the pharmacodynamic profile of the drug in vitro.

5. Conclusions

Biophysical-chemical analyses indicate that the hydrogel composed of 3% hyaluronic acid (HA) and bupivacaine (BUP) maintains the chemical integrity of the precursors, with no evidence of spontaneous covalent interactions, as demonstrated by the overlap of characteristic bands in infrared spectroscopy (FTIR). Thermogravimetrically, HA acts as a thermal stabilizer, delaying the volatilization of bupivacaine and raising its initial decomposition temperature to levels close to 200 °C. The rheological characterization of HA-BUP hydrogels revealed that HA 3% + 0.500% BUP demonstrated the best performance in terms of elastic stability and thermal stress resistance, although the formulations with 0.125% and 1% BUP stand out for their biomimetic properties, approaching the viscoelastic properties of native human synovial fluid. From a biological point of view, the association with the polymer neutralizes the intrinsic cytotoxicity of the anesthetic, increasing the viability of macrophages to levels above 100% and decreasing the potential genotoxic effects, which validates the biocompatibility of the system for intra-articular applications.

This study is a pioneer in evaluating the cytocompatibility and physicochemical characteristics of the 3% hyaluronic acid hydrogel associated with bupivacaine, proving to be suitable for viscosupplementation applications. However, further in vivo studies are needed to assess pharmacokinetics, pharmacodynamics and safety

parameters, including the determination of potential lethal doses, before moving on to clinical trials to confirm its therapeutic potential.

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Diego Garcia Miranda: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. Lucas de Paula Ramos: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. Gabriela Ferraz de Araujo: Writing – original draft, Investigation. Nicole Fernanda dos Santos Lopes: Writing – original draft, Investigation. Thalita Sani-Taiariol: Writing – original draft, Methodology, Investigation. Mauricio Ribeiro Baldan: Writing – original draft, Validation, Methodology, Investigation. Bruno Henrique Godoi: Writing – original draft, Visualization, Methodology, Investigation. Cristina Pacheco Soares: Writing – original draft, Validation, Methodology, Investigation. Kerstin Gritsch: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. Alexandre Luiz Souto Borges: Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. Brigitte Grosgogeat: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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3 GENERAL CONSIDERATIONS

This doctoral degree program, conducted under a Joint Doctorate (Cotutelle) agreement between Claude Bernard University Lyon 1 and São Paulo State University (UNESP), began with the completion of the required coursework. Following the consolidation of the theoretical framework and formal approval in these initial stages, the thesis development phase commenced. The line of research focuses on the design, biophysicochemical characterization, and biological validation of hyaluronic acid (HA)-based hydrogel systems functionalized with anti-inflammatory agents or a local anesthetic, aiming to propose a dual-action therapeutic platform for the management of Temporomandibular Disorders (TMD).

In endorsement of this thesis, the first published article is entitled: Ketoprofen Associated with Hyaluronic Acid Hydrogel for Temporomandibular Disorder Treatment: An In Vitro Study (Gels, 2024). The study aimed to develop and evaluate the in vitro physicochemical and biological properties of an injectable hydrogel platform composed of hyaluronic acid (3% HA) combined with the non-steroidal anti-inflammatory drug (NSAID) ketoprofen (at concentrations ranging from 0.125% to 1%), targeting a combined therapeutic approach for Temporomandibular Disorders (TMD). To this end, the chemical integrity, structural stability, and morphology of the polymeric matrix were mapped through Fourier-Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), and Scanning Electron Microscopy (SEM). Comprehensive rheological assays were conducted before and after sterilization processes to assess the impact of thermal stress on the HA network. Biological safety was determined by biocompatibility (cell viability) and genotoxicity assays using macrophage cell lines (RAW 264.7). The system displayed an ideal rheological behavior for viscosupplementation, characterized as a non-Newtonian fluid with a thixotropic profile (capable of recovering its original structure after shearing), indicating excellent stability for injectable application. Biologically, the formulation proved to be safe and highly biocompatible, maintaining cell viability rates up to 92.4% and showing no evidence of genotoxic potential in the tested cell line.

The second published article is entitled: Biophysicochemical Design of a Dual-Function Hydrogel for Synergistic Shock-Absorption and Anti-Inflammatory Action for

TMD Therapy. The study aimed to synthesize, develop, and evaluate the in vitro biophysicochemical and biological properties of an injectable hyaluronic acid (3% HA) hydrogel platform combined with the steroidal anti-inflammatory drug (corticosteroid) hydrocortisone (Hyd), aiming for a dual-action therapeutic approach (viscosupplementation/mechanical shock absorption and sustained drug release) for the treatment of Temporomandibular Disorders (TMD). The molecular integrity, chemical interactions, and thermal degradation profile of the system were mapped using Fourier-Transform Infrared Spectroscopy (FTIR) and Thermogravimetric Analysis (TGA). The internal architecture of the gel was evaluated by Scanning Electron Microscopy (SEM). Comprehensive rheological assays were conducted before and after autoclave sterilization to assess the impact of thermal stress on the viscoelasticity of the HA network. Biological safety was evaluated through cell cytotoxicity assays (OECD 129 guideline) and genotoxicity tests (micronucleus assay) using the RAW 264.7 macrophage line, supplemented by confocal microscopy analyses. FTIR analysis confirmed molecular stability and the coexistence of compounds through non-covalent interactions, preserving the pharmacodynamic potential of hydrocortisone. Rheological assays revealed that autoclaving degrades the pure HA matrix; however, the incorporation of hydrocortisone exerted a dose-dependent protective effect, preserving the storage modulus (G') and viscoelastic integrity after thermal stress. SEM confirmed a homogeneous porous morphology. Biologically, the system met the non-cytotoxicity criteria (cell viability >70%), but exhibited a dose-dependent increase in genotoxicity at higher concentrations. Among the tested formulations, the group consisting of 3% HA + 0.250% Hyd was identified as the optimal candidate, as it provided the best balance between viscoelastic stability, cytocompatibility, and low genotoxic potential.

The third article is entitled: Hyaluronic Acid-Bupivacaine Hydrogel System for Temporomandibular Joint disorders: Mechanical Lubrication and Local Anesthesia in a preclinical approach (Carbohydrate Polymer Technologies and Applications, 2026). The work focused on the development and preclinical validation of a hydrogel system composed of hyaluronic acid (3% w/v HA) conjugated with the local anesthetic bupivacaine (BUP, at concentrations from 0.125% to 1%). The main objective was to propose a bivalent platform for Temporomandibular Disorders (TMD), combining mechanical joint lubrication with prolonged local analgesia. Chemical interactions and

the molecular integrity of the system were evaluated by Fourier-Transform Infrared Spectroscopy (FTIR). The thermal stability profile and degradation patterns were determined by Thermogravimetric Analysis (TGA). The viscoelasticity window and biomimetic behavior were characterized by dynamic rheological assays before and after autoclave sterilization. In vitro biological safety was validated through cytotoxicity (cell viability) and genotoxicity assays (micronucleus assay - OECD 487) in RAW 264.7 macrophages. FTIR indicated the absence of deleterious spontaneous covalent bonding between the polymer and the drug, preserving the integrity of both. TGA revealed that the HA matrix acted as a protective thermal barrier, delaying bupivacaine degradation. Rheologically, the hydrogel exhibited dominant viscoelastic behavior ($G' > G''$), with the 0.125% and 1% formulations identified as perfectly biomimetic to healthy human synovial fluid. In the biological evaluation, the association with the polymer reduced the toxicity of the anesthetic, ensuring cell viability above 98% and a total absence of genotoxic potential.

The execution of this proposal allowed the consolidation of a robust set of in vitro data regarding the physicochemical, rheological, thermal, and biological properties of the formulations. These results not only underpin this thesis but also establish the necessary in vitro safety and efficacy criteria to guide subsequent in vivo and clinical research phases.

CONSIDÉRATIONS GÉNÉRALES

Le présent cycle de doctorat, réalisé en régie de Cotutelle entre l'Université Claude Bernard Lyon 1 et l'Université d'État de São Paulo (UNESP), a débuté par le suivi et la validation des modules d'enseignement des programmes respectifs. Après la consolidation du cadre théorique et la validation formelle de ces étapes, la phase de développement de la thèse a été initiée. La ligne de recherche est axée sur la conception, la caractérisation biophysico-chimique et la validation biologique de systèmes d'hydrogels à base d'acide hyaluronique (AH) fonctionnalisés par des agents anti-inflammatoires ou un anesthésique local, visant à proposer une plateforme thérapeutique à double action pour la prise en charge des Désordres Temporo-

Mandibulaires (DTM).

En soutien à cette thèse, le premier article publié s'intitule : Ketoprofen Associated with Hyaluronic Acid Hydrogel for Temporomandibular Disorder Treatment: An In Vitro Study (Gels, 2024). L'étude avait pour objectif de développer et d'évaluer les propriétés physico-chimiques et biologiques in vitro d'une plateforme injectable d'hydrogels d'acide hyaluronique (AH 3 %) associés à un anti-inflammatoire non stéroïdien (AINS), le kétoprofène (à des concentrations de 0,125 % à 1 %), visant une approche thérapeutique combinée pour les Désordres Temporo-Mandibulaires (DTM). Pour cela, l'intégrité chimique, la stabilité structurelle et la morphologie de la matrice polymérique ont été cartographiées par Spectroscopie Infrarouge à Transformée de Fourier (FTIR), Analyse Thermogravimétrique (TGA) et Microscopie Électronique à Balayage (MEB). Des essais rhéologiques complets ont été conduits avant et après les processus de stérilisation afin d'évaluer l'impact du stress thermique sur le réseau d'AH. La sécurité biologique a été déterminée par des essais de biocompatibilité (viabilité cellulaire) et de génotoxicité sur une ligne cellulaire de macrophages (RAW 264.7). Le système a présenté un comportement rhéologique idéal pour la viscosupplémentation, caractérisé comme un fluide non newtonien au profil thixotrope (capable de recouvrer sa structure originale après cisaillement), indiquant une excellente stabilité pour une application injectable. Sur le plan biologique, la formulation s'est avérée sûre et hautement biocompatible, maintenant des taux de viabilité cellulaire atteignant 92,4 % et montrant une absence d'indices de potentiel génotoxique sur la ligne cellulaire testée.

Le deuxième article publié s'intitule : Biophysicochemical Design of a Dual-Function Hydrogel for Synergistic Shock-Absorption and Anti-Inflammatory Action for TMD Therapy. L'étude avait pour objectif de synthétiser, développer et évaluer les propriétés biophysico-chimiques et biologiques in vitro d'une plateforme injectable d'hydrogels d'acide hyaluronique (AH 3 %) associés à un anti-inflammatoire stéroïdien (corticoïde), l'hydrocortisone (Hyd), visant une approche thérapeutique à double action (viscosupplémentation/absorption des chocs mécaniques et libération prolongée du principe actif) pour le traitement des Désordres Temporo-Mandibulaires (DTM). L'intégrité moléculaire, les interactions chimiques et le profil de dégradation thermique du système ont été cartographiés par Spectroscopie Infrarouge à Transformée de Fourier (FTIR) et Analyse Thermogravimétrique (TGA). L'architecture interne du gel a

été évaluée par Microscopie Électronique à Balayage (MEB). Des essais rhéologiques complets ont été menés avant et après le processus de stérilisation à l'autoclave pour évaluer l'impact du stress thermique sur la viscoélasticité du réseau d'AH. La sécurité biologique a été déterminée par des tests de cytotoxicité cellulaire (ligne directrice OCDE 129) et des essais de génotoxicité (test des micronoyaux) sur la ligne de macrophages RAW 264.7, complétés par des analyses en microscopie confocale. L'analyse FTIR a confirmé la stabilité moléculaire et la coexistence des composés par des interactions non covalentes, préservant ainsi le potentiel pharmacodynamique de l'hydrocortisone. Les essais rhéologiques ont révélé que l'autoclavage dégrade la matrice d'AH pure ; cependant, l'incorporation de l'hydrocortisone a exercé un effet protecteur dose-dépendant, préservant le module de conservation (G') et l'intégrité viscoélastique après le stress thermique. La MEB a confirmé une morphologie poreuse homogène. Sur le plan biologique, le système a répondu aux critères de non-cytotoxicité (viabilité cellulaire > 70 %), mais a montré une augmentation dose-dépendante de la génotoxicité aux concentrations les plus élevées. Parmi les formulations testées, le groupe composé de 3 % AH + 0,250 % Hyd a été identifié comme le candidat idéal, offrant le meilleur équilibre entre stabilité viscoélastique, cytocompatibilité et faible potentiel génotoxique.

Le troisième article s'intitule : Hyaluronic Acid-Bupivacaine Hydrogel System for Temporomandibular Joint disorders: Mechanical Lubrication and Local Anesthesia in a preclinical approach (Carbohydrate Polymer Technologies and Applications, 2026). Ce travail s'est concentré sur le développement et la validation préclinique d'un système d'hydrogel composé d'acide hyaluronique (AH 3 % m/v) conjugué à un anesthésique local, la bupivacaïne (BUP, à des concentrations de 0,125 % à 1 %). L'objectif principal était de proposer une plateforme bivalente pour les Désordres Temporo-Mandibulaires (DTM), combinant la lubrification mécanique articulaire à une analgésie locale prolongée. Les interactions chimiques et l'intégrité moléculaire du système ont été évaluées par Spectroscopie Infrarouge à Transformée de Fourier (FTIR). Le profil de stabilité thermique et les modes de dégradation ont été déterminés par Analyse Thermogravimétrique (TGA). La fenêtre de viscoélasticité et le comportement biomimétique ont été caractérisés par des essais rhéologiques dynamiques avant et après stérilisation à l'autoclave. La sécurité biologique in vitro a été validée par des essais de cytotoxicité (viabilité cellulaire) et de génotoxicité (test

des micronoyaux - OCDE 487) sur des macrophages de la ligne RAW 264.7. La FTIR a indiqué l'absence de liaisons covalentes spontanées délétères entre le polymère et le principe actif, préservant l'intégrité des deux composants. La TGA a révélé que la matrice d'AH agissait comme une barrière thermique protectrice, retardant la dégradation de la bupivacaïne. Du point de vue rhéologique, l'hydrogel a présenté un comportement viscoélastique dominant ($G' > G''$), les formulations à 0,125 % et 1 % ayant été identifiées comme parfaitement biomimétiques du liquide synovial humain sain. Lors de l'évaluation biologique, l'association au polymère a réduit la toxicité de l'anesthésique, garantissant une viabilité cellulaire supérieure à 98 % et une absence totale de potentiel génotoxique.

L'exécution de ce projet a permis de consolider un ensemble robuste de données in vitro concernant les propriétés physico-chimiques, rhéologiques, thermiques et biologiques des formulations. Ces résultats soutiennent non seulement cette thèse, mais établissent également les critères de sécurité et d'efficacité in vitro nécessaires pour guider les phases ultérieures de recherche in vivo et clinique.

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APPENDIX A – Article published during my doctoral studies in the jornal Scientific Reports (IF 3.9 JCR): Saliva culture as a predictive indicator for current blood infections and antimicrobial resistance in the ICU setting Ketoprofen Associated with Hyaluronic Acid Hydrogel for Temporomandibular Disorder Treatment: An *In Vitro* Study

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Saliva culture as a predictive indicator for current blood infections and antimicrobial resistance in the ICU setting

Leonardo Moura Brasil da Rocha Santos[1,2], Lucas de Paula Ramos[1,3,4], Carlos Eduardo Rocha Santos[1,2], Diego Garcia Miranda[1,4,5], Mariana Gadelho Gimenez[6], Vanessa Marques Meccatti[1], Amjad Abu Hasna[6]✉, Marcela dos Santos Oliveira[7], Morun Bernardino Neto[8] & Luciane Dias de Oliveira[1]

Antimicrobial resistance is a worldwide health problem and patients in intensive care are more vulnerable, requiring strict control measures and early identification. Currently, clinical culture materials are used to identify the bacterial agent, but saliva culture is not validated, which has great clinical relevance because it participates in several pathophysiological processes. The aim of this study was to validate saliva culture in an intensive care unit environment, determining its diagnostic value for infection. For this purpose, the results of the 39-month surveillance cultures, from the database of a private hospital were evaluated. A total of 323 cultures were paired between saliva, tracheal secretions, blood and urine from patients who were hospitalized for more than 5 days. The search for correlations between the results was performed using the Spearman correlation test. Severity and evolution data were also correlated. It was possible to correlate the presence of *Klebsiella* spp. between blood culture and saliva culture in 25% of the results (r = 0.01) and the correlation between saliva and tracheal secretion was 33% (r = 0.33447) with p < 0.0001. In conclusion, saliva can be an excellent discriminator of systemic infections, and can be considered a useful culture in clinical practice.

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APPENDIX B – Article published during my doctoral studies in the journal Antibiotics Basel (IF 4.6 JCR): New Insights into Cutaneous Asepsis: Synergism between *Pfaffia* and Rosemary Extracts.

Open Access Article

New Insights into Cutaneous Asepsis: Synergism between Pfaffia and Rosemary Extracts

by Diego Garcia Miranda [1,2,3,4,*,†], Florence Carrouel [2,†], Tatiane Cristina Alberti Silva [4,5], Mariana Cafalchio Rozzatto [4,5], Amjad Abu Hasna [4,6], Carlos Eduardo Rocha Santos [4], Flavia Villaça Morais [5,7], Luciane Dias de Oliveira [4,†] and Lucas de Paula Ramos [2,4,5,*,†]

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APPENDIX C– Article published during my doctoral studies in the journal Microorganisms (IF 4.6 JCR) *Pfaffia paniculata* Extract, a Potential Antimicrobial Agent against *Candida* spp., *Pseudomonas aeruginosa*, and *Streptococcus mutans* Biofilms.

Open Access Article

Pfaffia paniculata Extract, a Potential Antimicrobial Agent against *Candida* spp., *Pseudomonas aeruginosa*, and *Streptococcus mutans* Biofilms

by Diego Garcia Miranda [1,2,3,4,*,†], Lucas de Paula Ramos [3,4,5,†], Nina Attik [1,2], Thaís Cristine Pereira [4], Luciane Dias de Oliveira [4], Maria Cristina Marcucci [4], Flavia Pires Rodrigues [6], Graziella Nuernberg Back Brito [7] and Florence Carrouel [2,3,*]

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APPENDIX D – Article published during my doctoral studies in the jornal Plants Basel (IF 4.1 JCR) *Gymnema sylvestre* as a Potential Anti-Inflammatory and Anti-Biofilm Agent Against Anaerobic Infections: An *In Vitro* Study.

Open Access Article

Gymnema sylvestre as a Potential Anti-Inflammatory and Anti-Biofilm Agent Against Anaerobic Infections: An In Vitro Study

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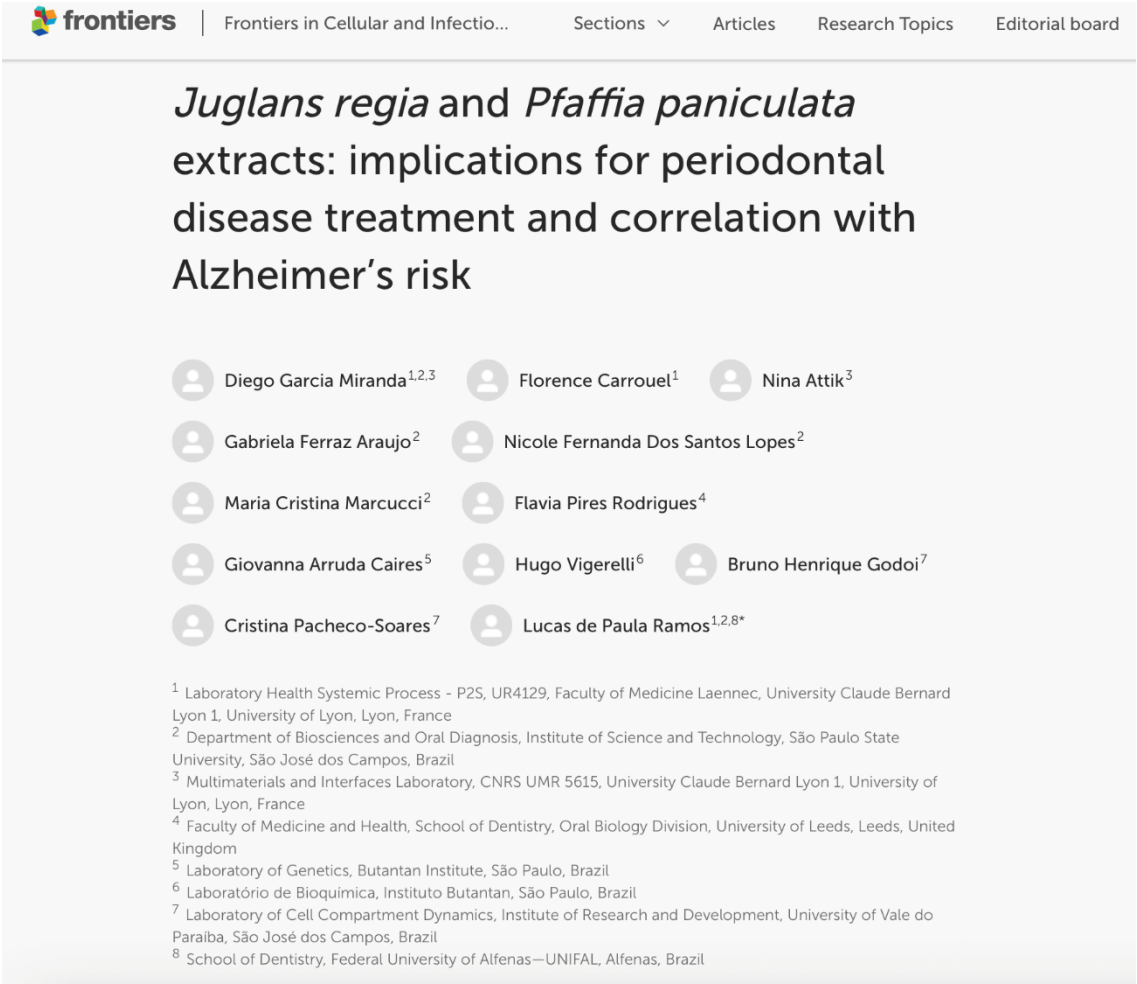
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APPENDIX E – Article published during my doctoral studies in the jornal Frontiers in Cellular and Infection Microbiol (IF 4.8 JCR): *Juglans regia* and *Pfaffia paniculata* extracts: implications for periodontal disease treatment and correlation with Alzheimer's risk.



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Juglans regia and *Pfaffia paniculata* extracts: implications for periodontal disease treatment and correlation with Alzheimer's risk

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APPENDIX F – Article published during my doctoral studies in the jornal Pharmaceutics (IF 5.5 JCR): Exploring Antioxidant, Antimicrobial and Anti-Inflammatory Effects of *Juglans regia* and *Pfaffia paniculata* Extracts: Implications for Intestinal Dysbiosis and Colorectal Cancer Risk Associated with Oral Pathogens.

Open Access Article

Exploring Antioxidant, Antimicrobial and Anti-Inflammatory Effects of *Juglans regia* and *Pfaffia paniculata* Extracts: Implications for Intestinal Dysbiosis and Colorectal Cancer Risk Associated with Oral Pathogens

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APPENDIX G – Article published during my doctoral studies in the jornal Applied Sciences (IF 2.5 JCR): Antifungal and Cytocompatible Properties of *Juglans regia* Extract for Dental Applications: A Novel Approach Against Oral *Candida* Infections.

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Antifungal and Cytocompatible Properties of *Juglans regia* Extract for Dental Applications: A Novel Approach Against Oral *Candida* Infections

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