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

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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.

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

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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 (Antonpaar®, 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).

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.

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.

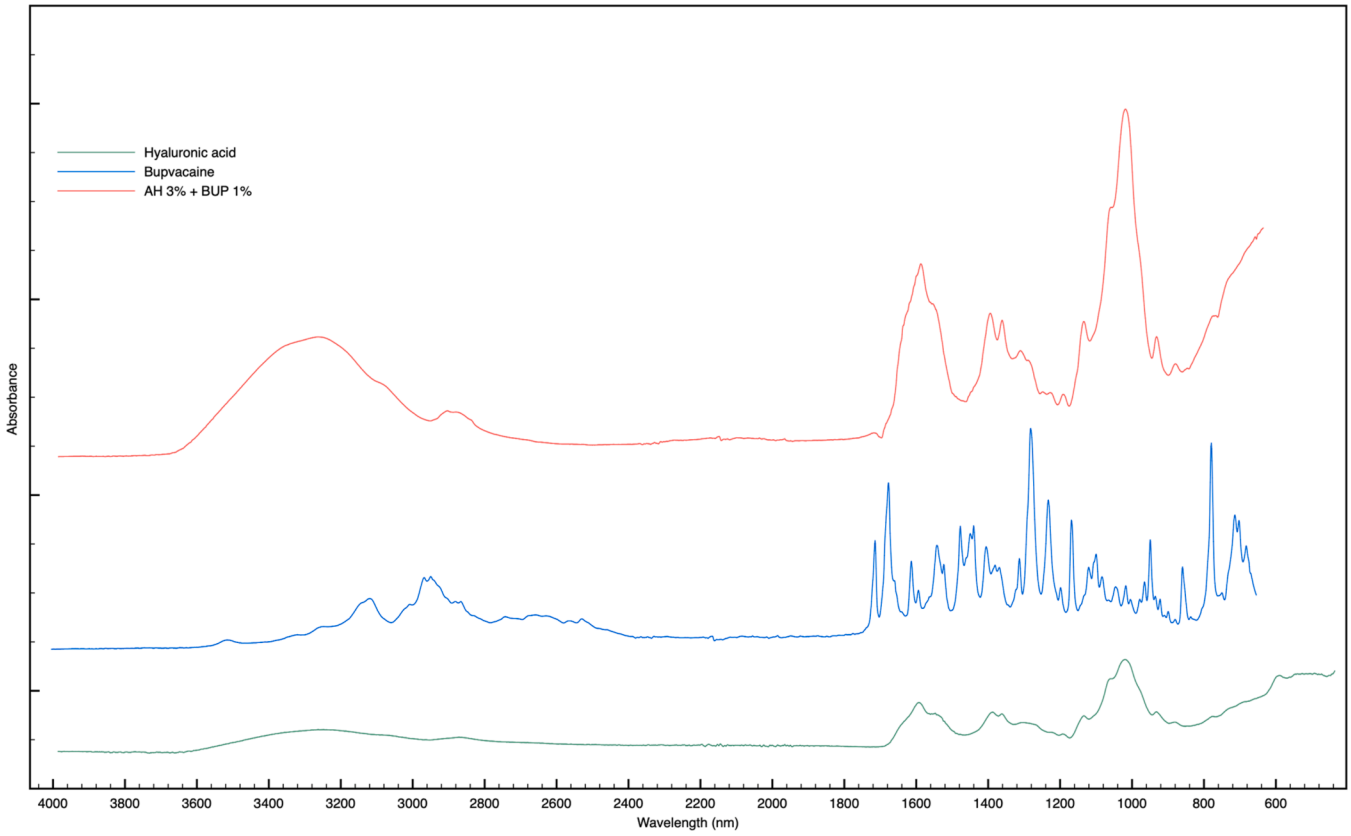

Fig. 1. FTIR spectra of HA (green line), BUP (blue line) and hydrogel 3% HA associated with 1% BUP (red line).

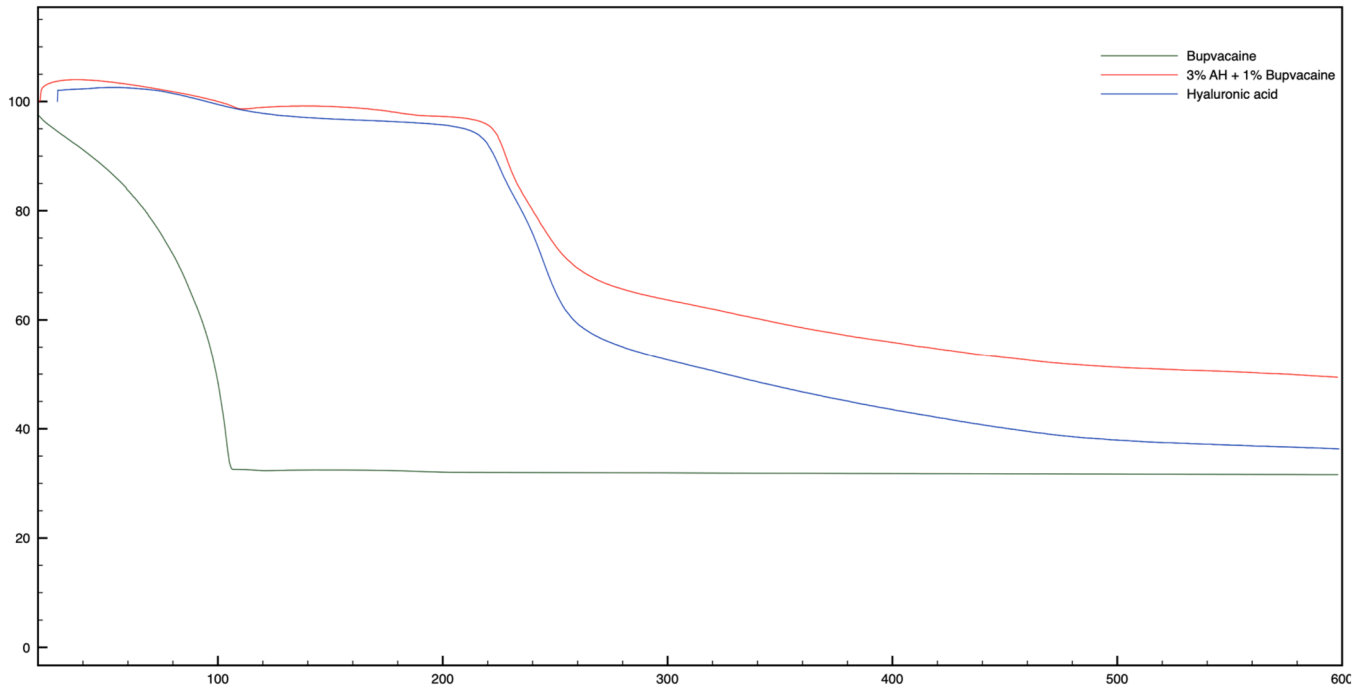

Fig. 2. Analysis of the decomposition of masses in relation to temperature. TGA spectra of HA (Blue line), BUP (Green line) and hydrogel 3% HA associated with 1% BUP (Red line).

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.

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.

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.

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

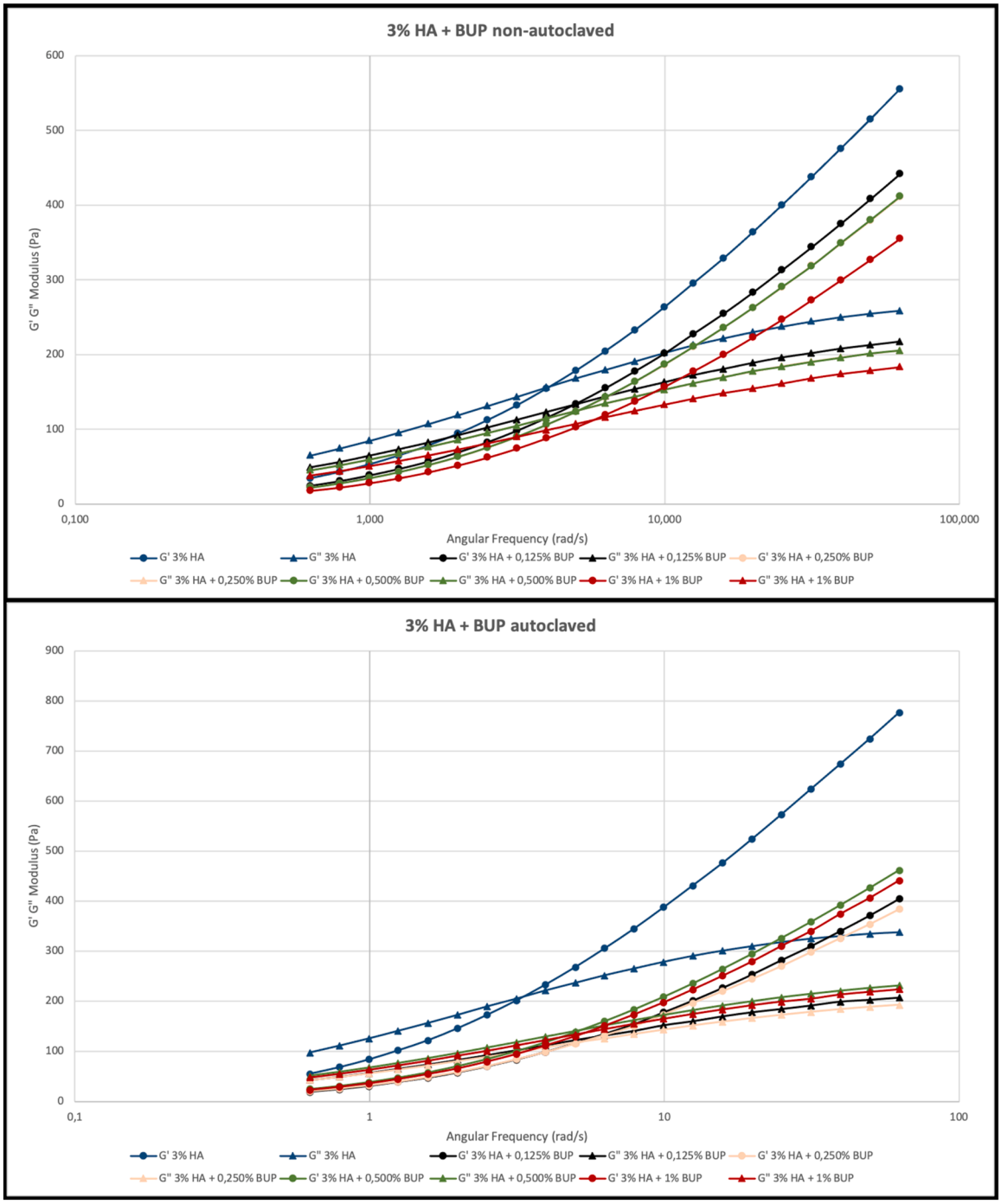

Fig. 3. Frequency sweep of 3% HA + BUP hydrogel non-autoclaved (A) and 3% hyaluronic acid HA + BUP hydrogels autoclaved (B).

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.

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.

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

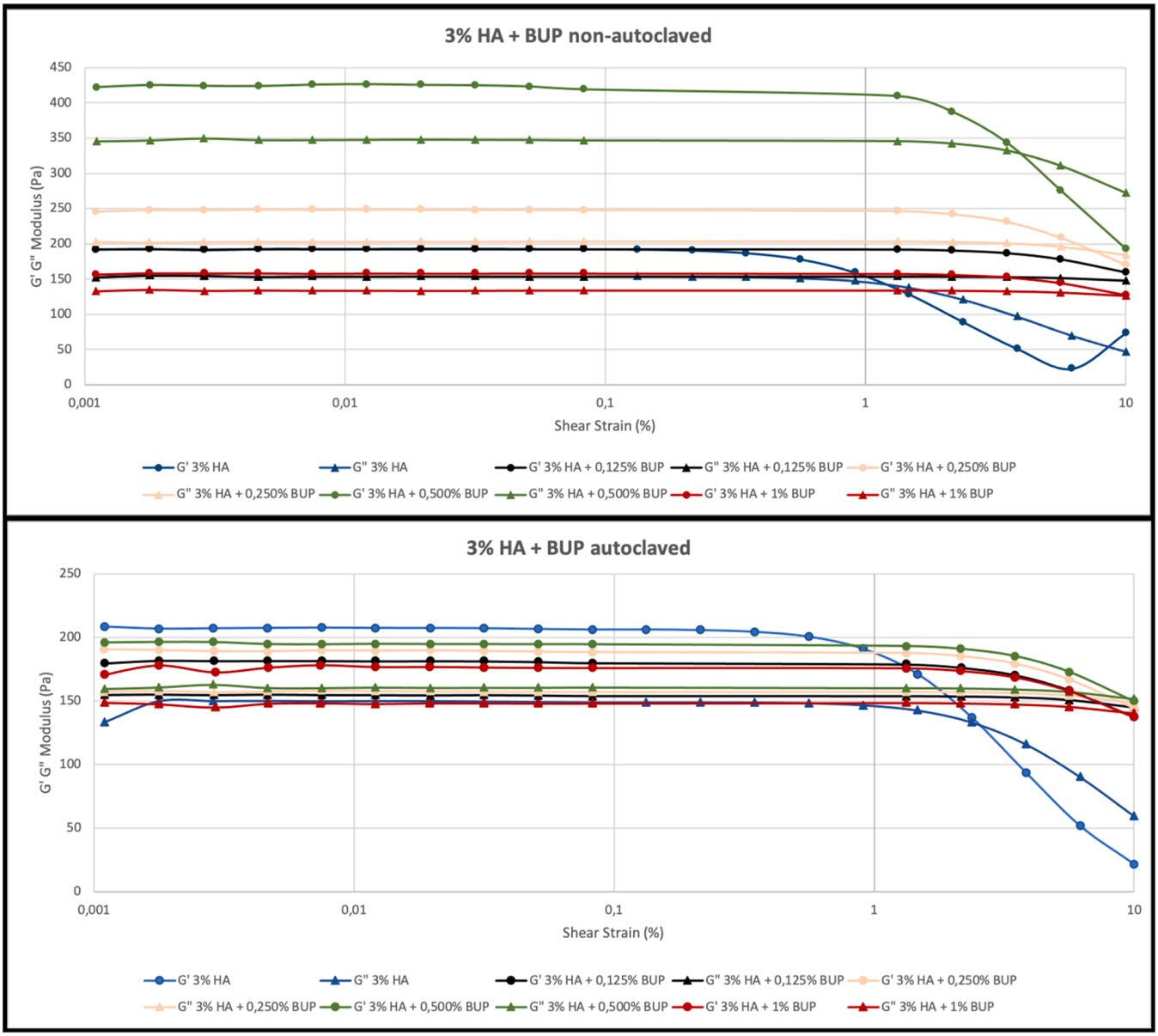

Fig. 4. Amplitude sweep of 3% HA + BUP hydrogel non-autoclaved (A) and 3% HA + BUP hydrogel autoclaved (B).

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

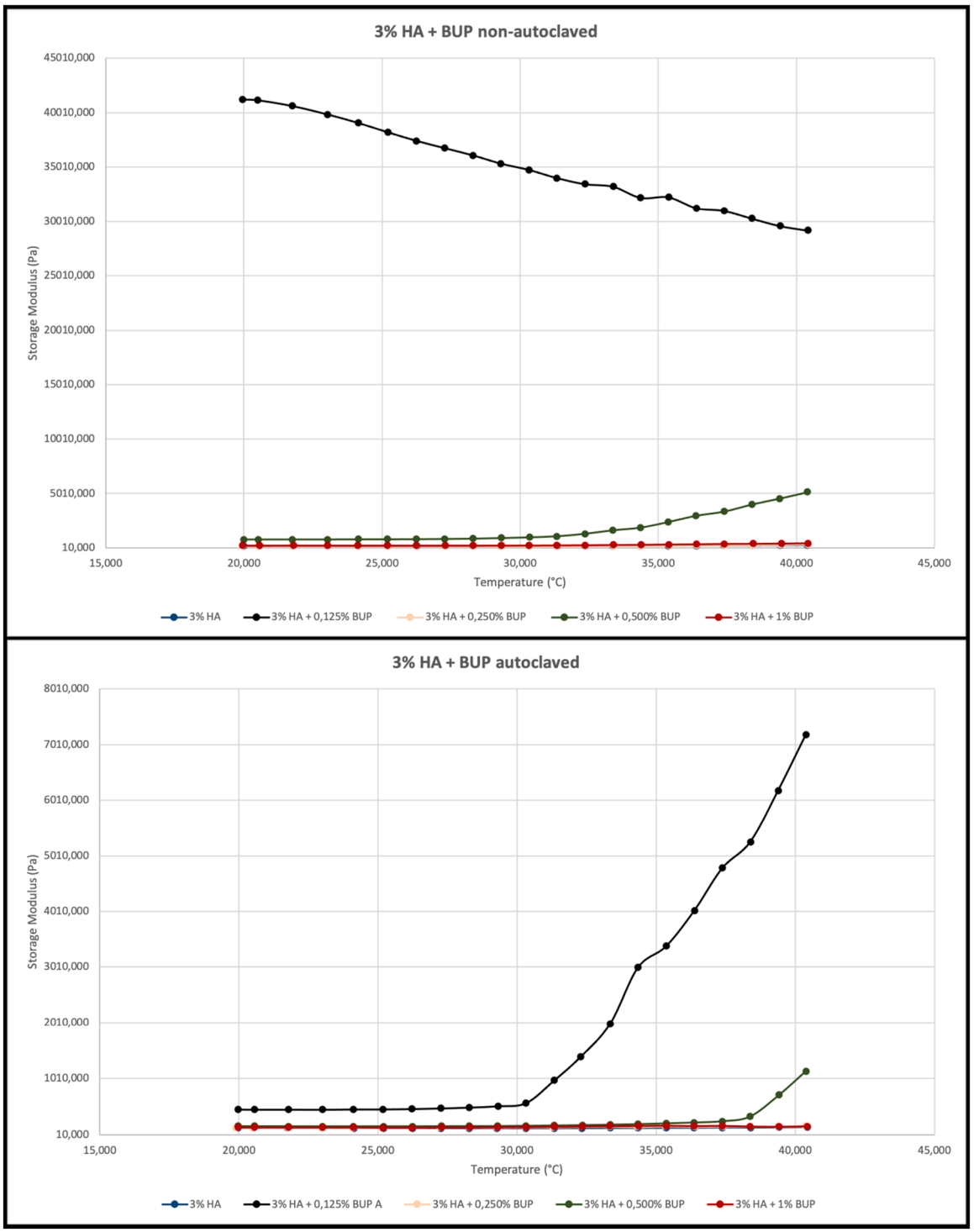

Fig. 5. Temperature ramp of 3% HA + BUP hydrogel non-autoclaved (A) and 3% HA + BUP hydrogel autoclaved (B).

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

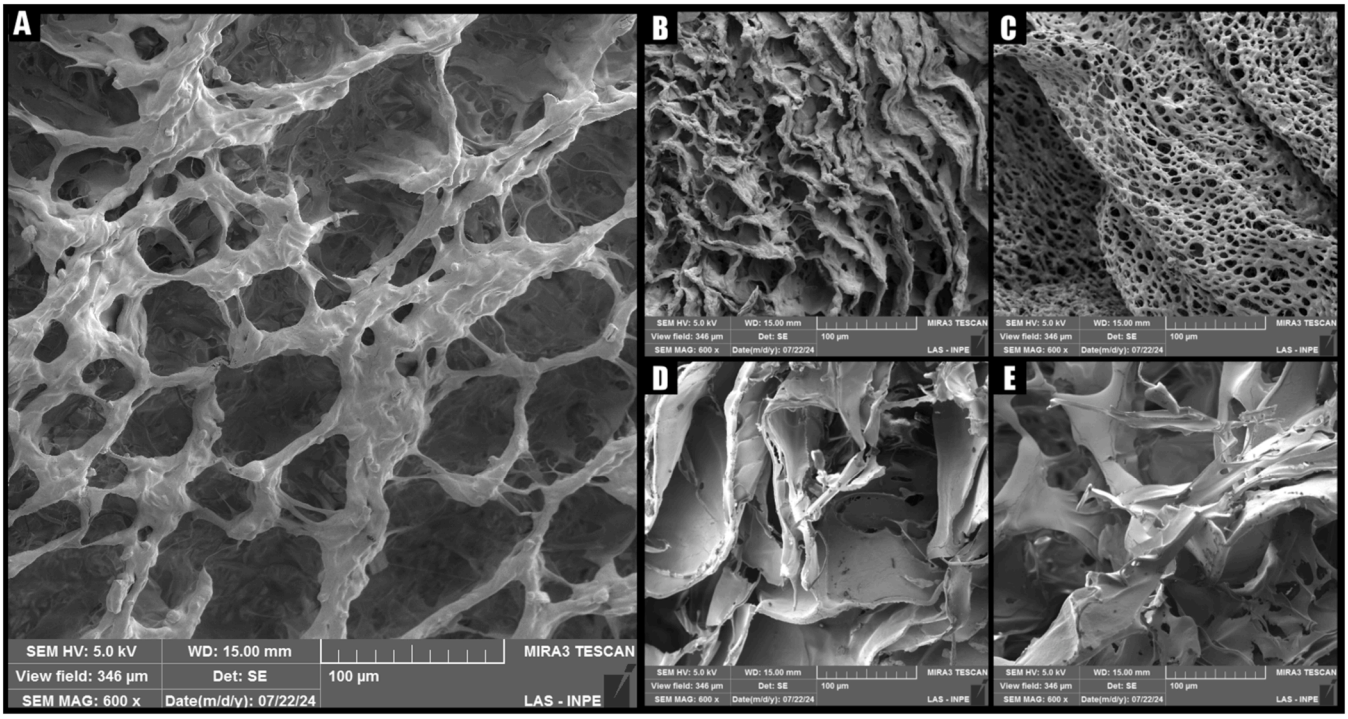

Fig. 6. Micrography of 3% HA hydrogel (A), 3% HA + 0.125% BUP hydrogel (B), 3% HA + 0.250% BUP hydrogel (C), 3% HA + 0.500% BUP hydrogel (D), 3% HA + 1% BUP hydrogel (E).

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

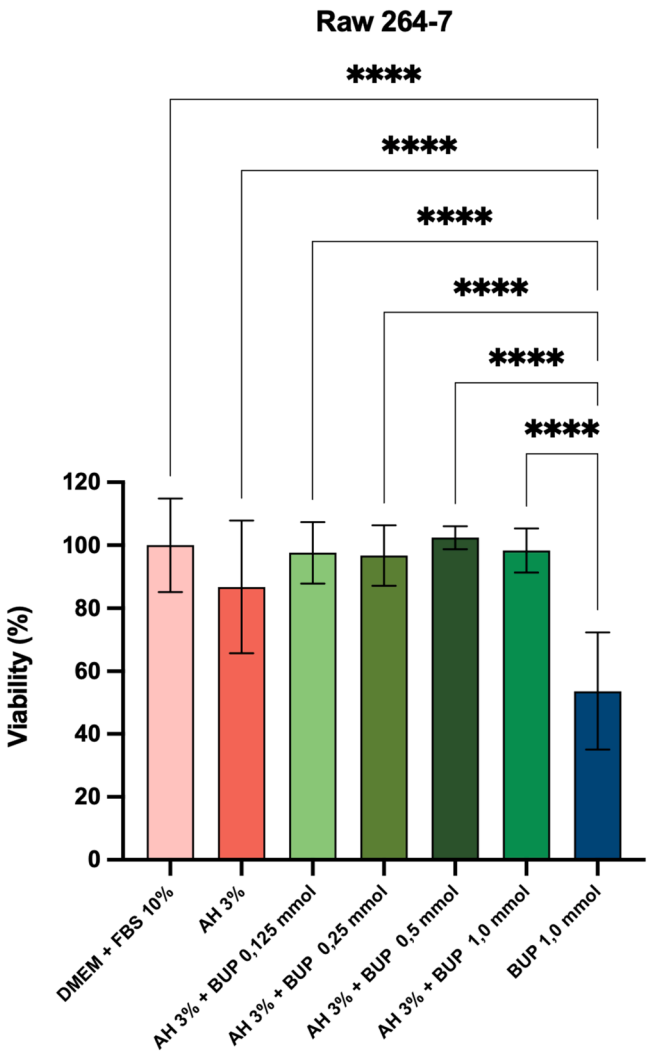

Fig. 7. Cytocompatibility by hydrogels on mouse macrophages (RAW 264.7). $p < 0.0001$ (****).

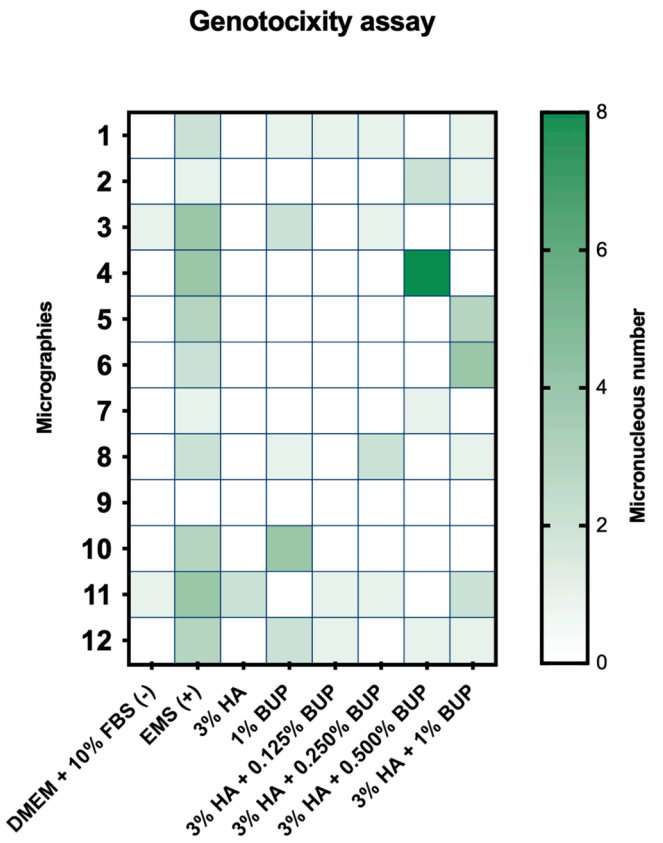

Fig. 8. Genotocixity assay by micronucleous.

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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