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Evaluation of cellular viability in chitosan/L-arginine hydrogels

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Abstract

Background. There is a lack of studies evaluating the toxicity of nitric oxide (NO) precursors in chitosan/L-arginine hydrogels and their topical administration. However, clarifying the characteristics of these elements is essential for their possible use in non-surgical techniques of tooth movement acceleration. Such characteristics include interaction with different cell types, metabolism and drug safety.

Objectives. This in vitro study aimed to assess the cytotoxicity of chitosan hydrogels on human HeLa cells using different concentrations of L-arginine.

Materials and methods. The hydrogels were synthesized in a materials engineering laboratory, with a controlled environment, using 4 different L-arginine concentrations of 0%, 10%, 15%, and 20%. Once the hydrogels were prepared, their physical and chemical properties were characterized, and viability analysis was performed using 2 different methods, including a 48-h assay with *Artemia salina* nauplii and a 24-h cell culture with human HeLa cells followed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay. Data analysis was performed using a Mann–Whitney U test to evaluate positive and negative controls in the cell culture, with a significance level of 0.01. A Wilcoxon paired test contrasted the 24-h compared to 48-h *Artemia salina* assays, with a Kruskal–Wallis and post hoc Dunn test used to compare groups using a significance level of 0.05.

Results. In the more viscous hydrogels, *Artemia salina* nauplii decreased drastically in 24 h, while the 15% and 20% hydrogels had no statistical differences from the negative control. The 10% and 20% hydrogels were statistically different from the negative control when comparing cell culture data.

Conclusions. Our findings suggest that chitosan/L-arginine hydrogels could be used in humans without toxic effects. However, more trials and tests are needed to evaluate tooth movement rate during orthodontic treatment.

Key words: hydrogels, in vitro techniques, cell culture techniques

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Background

The current goal of orthodontic processes is to reduce treatment time and avoid the deleterious effects of controlled dental movement. As for the possibilities of movement acceleration, there are multiple approaches, including vibration stimulation,[1–3] low-intensity laser[4,5] and surgical techniques (piezosin, corticotomy or surgeries to generate regional acceleration (RAP)).[6–8] However, these approaches may negatively affect dental roots.[9] There is a non-surgical or radiation alternative involving drug stimulation with hydrogels containing nitric oxide (NO) precursors as mediators to eliminate possible adverse effects.

Given the physical and chemical characteristics of hydrogels, they can be modulated through structural modifications. Hydrogels composed of natural polymers are used in tissue engineering to control bioactive molecule delivery.[10–13] Also, collagen scaffolds are used for drug delivery in tissue engineering.[14]

Chitosan is an alkaline deacetylated co-polymer of chitin of the amino-polysaccharide type, consisting of D-glucosamine and N-acetyl-D-glucosamine.[10] Nitric oxide is an endogenously produced diatomic molecule that plays a crucial role in physiological processes such as angiogenesis, healing, neurotransmission, smooth muscle relaxation, inflammation, and bone metabolism.[15,16] The role of NO in osteogenesis depends on its concentration, with models using NO disruption demonstrating inhibition of osteoblast differentiation and a decrease in their activity.[17–19] Under normal physiological conditions, the effect of NO is biphasic, with the cytokines that promote osteoclastogenesis inhibited at high concentrations and their impact enhanced at low concentrations.[10,20–23]

Since there are no studies reporting NO toxicity precursors in chitosan/L-arginine hydrogels and their topical administration, it is essential to clarify their characteristics for use in non-surgical dental movement acceleration techniques, their interaction with different cell types, metabolism, and drug safety. This study aimed to evaluate the cytotoxicity of multiple chitosan hydrogels using different L-arginine concentrations in human HeLa cell cultures.

Materials and methods

Chitosan hydrogels

This in vitro study used a hybrid chitosan gel, obtained following the formulation and protocols developed by the research group of Faculty of Stomatology (Meritorious Autonomous University of Puebla, Mexico),[23,24] with a texture suitable for tipical intraoral use. Once the gel was synthesized, the amino acid (L-arginine) was adhered, and the link was made chemically.[25]

Hydrogels were obtained using L-arginine concentrations of 0%, 10%, 15%, and 20%, according to protocols standardized by the laboratory. A hydrogel with a percentage lower than 10% was not selected because it was important to ensure an adequate arginine concentration.

The hydrogel was prepared with a dilution of L-arginine (Santa Cruz Biotechnology, Santa Cruz, USA), according to the required concentration (1.244 mg for 10%, 1.97 mg for 15% and 2.8 mg for 20%), with 10 mL of distilled water until a homogeneous consistency was achieved. Then, 0.5 mL of acetic acid and 0.2 mL of glycerol were added and mixed with 0.5 mg of low molecular weight chitosan (Sigma-Aldrich, St. Louis, USA). The hydrogel was kept refrigerated and used for experimentation within a week of fabrication.

Physicochemical characterization

The physicochemical characterization was done in the Materials Engineering and Chemistry Laboratory of the University of Valle (Cali, Colombia) after the different hydrogels were dried and processed. Gel samples were dried at 60°C for 72 h, and their chemical composition was studied using Fourier-transform infrared spectroscopy in attenuated total internal reflectance mode (ATR-FTIR) (Perkin-Elmer, Waltham, USA) with Shimadzu equipment (Spectrophotometer FT-IR-8400; Shimadzu Corp., Kyoto, Japan) . A Fisherbrand AccumetTM AB150 pH (Thermo Fisher Scientific, Waltham, USA) meter measured pH using a pH/ATC electrode with a refillable epoxy body, previously calibrated with buffer solutions of pH at 4, 7 and 10. Density was determined with a 25 cm^3 pycnometer (Brand, Wertheim, Germany). The amount of each sample required to fill the pycnometer was weighed, and the density was calculated as mass/volume.

Cytotoxicity and cell viability tests

To perform the cytotoxicity tests, we used the HeLa cell line available at the in vitro cell culture laboratory of the Department of Pharmacology of the University of Valle. Six evaluation groups were determined according to the percentage of L-arginine:

- cells in medium alone (negative control);
- cells seeded in chitosan gel with 0% L-arginine;
- cells seeded in chitosan gel with 10% L-arginine;
- cells seeded in chitosan gel with 15% L-arginine;
- cells seeded in chitosan gel with 20% L-arginine;
- cells with cisplatin (positive control).

For the hydrogel treatment, each group was seeded in 96-mug plates under a humidified atmosphere of 5% CO_2 at 37°C for 24 h and allowed to proliferate. Thirty thousand (30,000) cells per mug were achieved following the protocol of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay manufacturer. Subsequently, the mugs were brought to a final volume of 200 µL using Hank's balanced salt solution (HBSS) without fetal bovine serum (FBS) since the formazan crystals of the MTT assay were not formed. We added 20 µL of hydrogel to each mug and incubated them for 24 h. Cell viability analysis was then carried out.

MTT feasibility assay

Cell viability was evaluated using the MTT Cell Proliferation Assay Kit (Abcam ab211091; Abcam, Cambridge, UK), which is based on the conversion of the water-soluble component MTT to an insoluble formazan product. Viable cells with active metabolism convert MTT to formazan, while dead cells lose their metabolic capacity and do not show any signal. A spectrophotometer was used to analyze the absorbance once the formazan crystallization process was completed to evaluate whether there was cell death. The absorbance measurement at optical density (OD) 590 nm is proportional to the number of viable cells.[27–29] To make the data more easily interpreted, the following equation was used[30]:

% viability = (OD treated cells/OD control cells) × 100

Artemia salina assay

Artemia salina culture was performed for 3 days within a sterile saline medium with a pH of 8.5 using 0.1 N NaOH under constant oxygenation and artificial light to simulate a natural and controlled saline environment. The specimens were harvested once the newly hatched nauplii were available. Samples in culture dishes were divided into 6 groups: 0%, 10%, 15%, and 20% L-arginine hydrogel, negative control with the same saline medium, and positive control with an enzymatic soap. Each group was analyzed in 5 dishes with 10 *Artemia salina* specimens in each mug to guarantee enough replicates for each treatment.

Once the number of samples per group was achieved, the hydrogels were introduced to the culture dishes for 24 h and 48 h, with 20 µL of hydrogel added to each mug. Statistical analyses were carried out according to the number of *Artemia salina* specimens not seen alive under an optical microscope (Primo Star; Carl Zeiss AG, Jena, Germany) at ×4 magnification.

HeLa cell culture assay

The viability data of each hydrogel concentration was recorded in Microsoft Excel 2019 sheets (Microsoft Corp., Redmond, USA) and later analyzed in GraphPad Prism v. 9.11 (GraphPad, San Diego, USA). The analysis consisted of calculating measures of central tendency and dispersion of the serial tests for each material. A summary table and graph of mean values with the corresponding standard errors (SEs) were constructed. Viability distributions were compared between pairs of the experimental samples (compared to the positive and negative controls) using Mann–Whitney U test.

Statistical analyses

Culture data were expressed as the mean ± SE of independent experiments for each condition. Differences between treatment groups were analyzed with the GraphPad software. Viability distributions were compared between pairs of the experimental samples (compared to the positive and negative controls) using Mann–Whitney U test. A significance level of less than 0.01 was used.

Artemia salina assay data were recorded in Excel and later analyzed in the Stata IC15 program (Stata Corp., College Station, USA). The analysis consisted of calculating measures of central tendency and dispersion of the presence of nauplii for each hydrogel concentration (0%, 10%, 15%, and 20%). A summary table and graph of means with the corresponding SE were constructed. The number of nauplii per pair was compared between 24 h and 48 h using a Wilcoxon paired test. The contrast between groups was performed using the Kruskal–Wallis test and post hoc in pairs with Dunn's test; this analysis excluded the negative control. We used a significance level of 0.05.

Results

Physicochemical characterization

The results showed absorption bands that characterize chitosan in the 3,200–3,600 cm^{-1} region, which are due to stretching of the –OH and –NH bonds. The absorption bands near the 2,870 cm^{-1} and 1,404 cm^{-1} region corresponded to the stretching and bending vibration of the C–H single bond in the polymer chain (Fig. 1). The peaks present in the 1,638 cm^{-1} and 1,545 cm^{-1} region corresponded to amide bands I and II, respectively.

For the pH results, there was a difference in acidity that corresponded to the percentage of the amino acid added. For instance, the hydrogel with 0% L-arginine component had a 3.32 score on the acidity scale, while the one with 20% had a more neutral score of 6.60.

Finally, the density presented by the different types of hydrogels was 1.14 g/cm^3 for the 0% hydrogel, 1.18 g/cm^3 for the 10% hydrogel, 1.09 g/cm^3 for the 15% hydrogel, and 1.07 g/cm^3 for the 20% hydrogel.

Artemia salina culture

Microscopic analyses demonstrated a significant decrease in specimens at 24 h in the 0% and 10% hydrogels, while there was no such mortality in the less viscous hydrogels (15% and 20%).

The mean number of nauplii in the hydrogel was higher at the 20% (9.0 ±2.24) and 15% (8.2 ±1.79) concentrations at 24 h. After 48 h, the number of nauplii decreased, but the same pattern continued, with more *Artemia salina* nauplii at higher hydrogel concentrations. For instance, an average of 7 (±1.73) nauplii were found in the 20% hydrogel, while 6.8 (±1.30) nauplii were found in the 15% hydrogel. The differences were

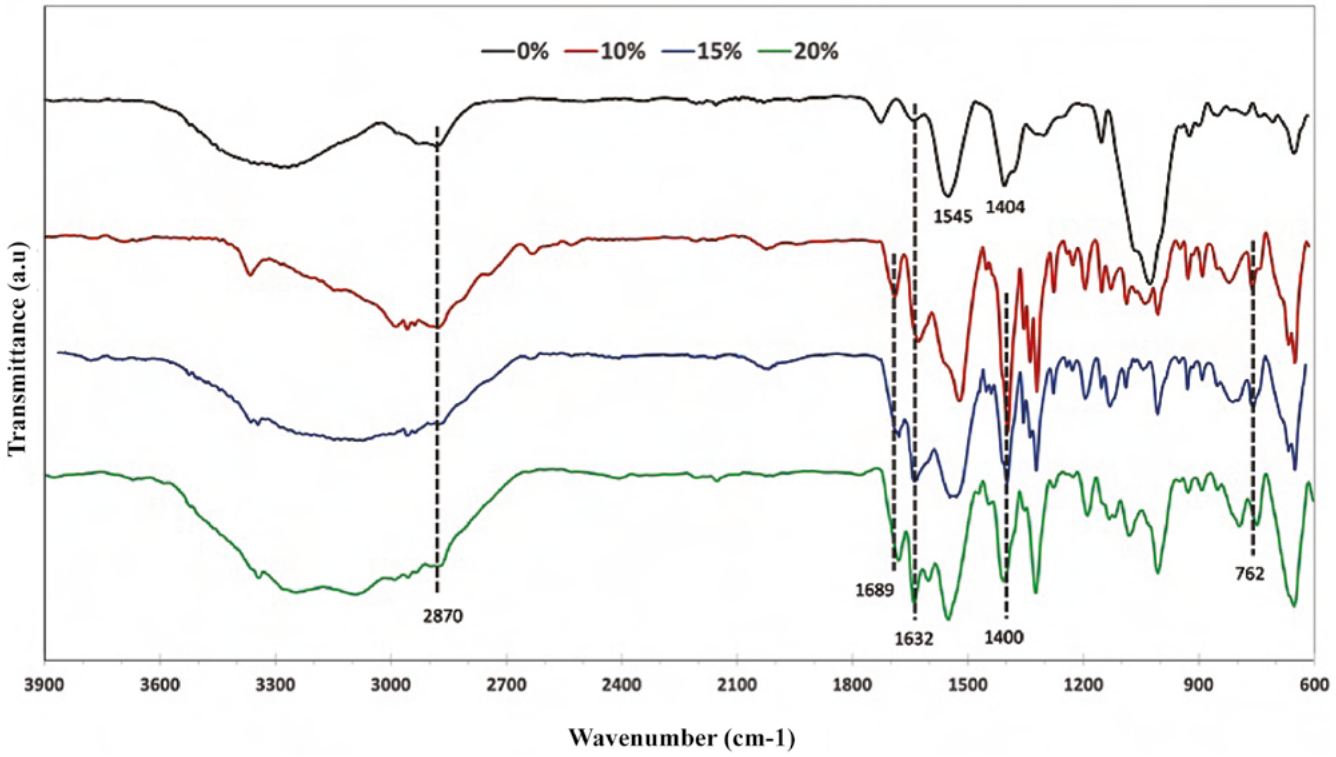

Fig. 1. Infrared Fourier-transform spectra of chitosan and arginine gels

Table 1. The average number of *Artemia salina* nauplii at 24 h and 48 h according to the concentration of the experimental hydrogel

Materials	n	24 h			48 h			p-value
		Mean	SD	SE	Mean	SD	SE	
H-0%	5	2.2	0.84	0.37	0.8	0.84	0.37	0.038
H-10%	5	2.8	0.45	0.2	1.6	0.55	0.24	0.034
H-15%	5	8.2	1.79	0.8	6.8	1.30	0.58	0.038
H-20%	5	9	2.24	1	7	1.73	0.77	0.039
C–	4	6.5	1.29	0.65	6	0.82	0.41	0.157
C+	4	0	0	0	0	0	0	–

SD – standard deviation; SE – standard error; H – hydrogel; C+ – positive control; C– – negative control.

Table 2. P-value of contrast tests by pairs (Dunn's test)

Materials	24 h				48 h			
	C–	H-0%	H-10%	H-15%	C–	H-0%	H-10%	H-15%
H-0%	0.007**	–	–	–	0.011*	–	–	–
H-10%	0.024*	0.309	–	–	0.045*	0.264	–	–
H-15%	0.207	0.000**	0.002**	–	0.349	0.002**	0.014*	–
H-20%	0.439	0.003**	0.012*	0.241	0.120	0.000**	0.001**	0.202

H – hydrogel; C+ – positive control; C– – negative control; *p < 0.05; **p < 0.01.

statistically significant between 24 h and 48 h at all concentrations (Table 1).

When comparing the groups with each other in pairs (Table 2), statistically significant differences were found at 24 h (Kruskal–Wallis p = 0.001) and 48 h (Kruskal–Wallis p = 0.001) (Fig. 2).

HeLa cell culture assay

Table 3 shows viability distributions between pairs of experimental samples compared to the controls (positive and negative). The bar graph of the mean and SE indicates higher viability in the 10% and 20% hydrogels, with 0.24 ±0.29 and

Table 3. Spectrophotometric viability analysis of absorbance level

Materials	Mean	SD	SE	H-ABS vs C+	p-value*	H-ABS vs C–	p-value**
C+	0.043	0.001	0.001	–	–	–	–
H-0%	0.065	0.013	0.008	0.021	0.100	0.064	0.100
H-10%	0.245	0.290	0.167	0.201	0.100	0.116	0.600
H-15%	0.114	0.054	0.031	0.070	0.100	0.015	0.600
H-20%	0.158	0.056	0.032	0.115	0.100	0.029	0.600
C–	0.129	0.013	0.008	–	–	–	–
0-ABS	0.042	0.001	0.001	–	–	–	–

SD – standard deviation; SE – standard error; H – hydrogel; ABS – absorbance; C+ – positive control; C– – negative control; *significance of correlation between each hydrogel absorbance and the positive control; **significance of correlation between each hydrogel absorbance and the negative control.

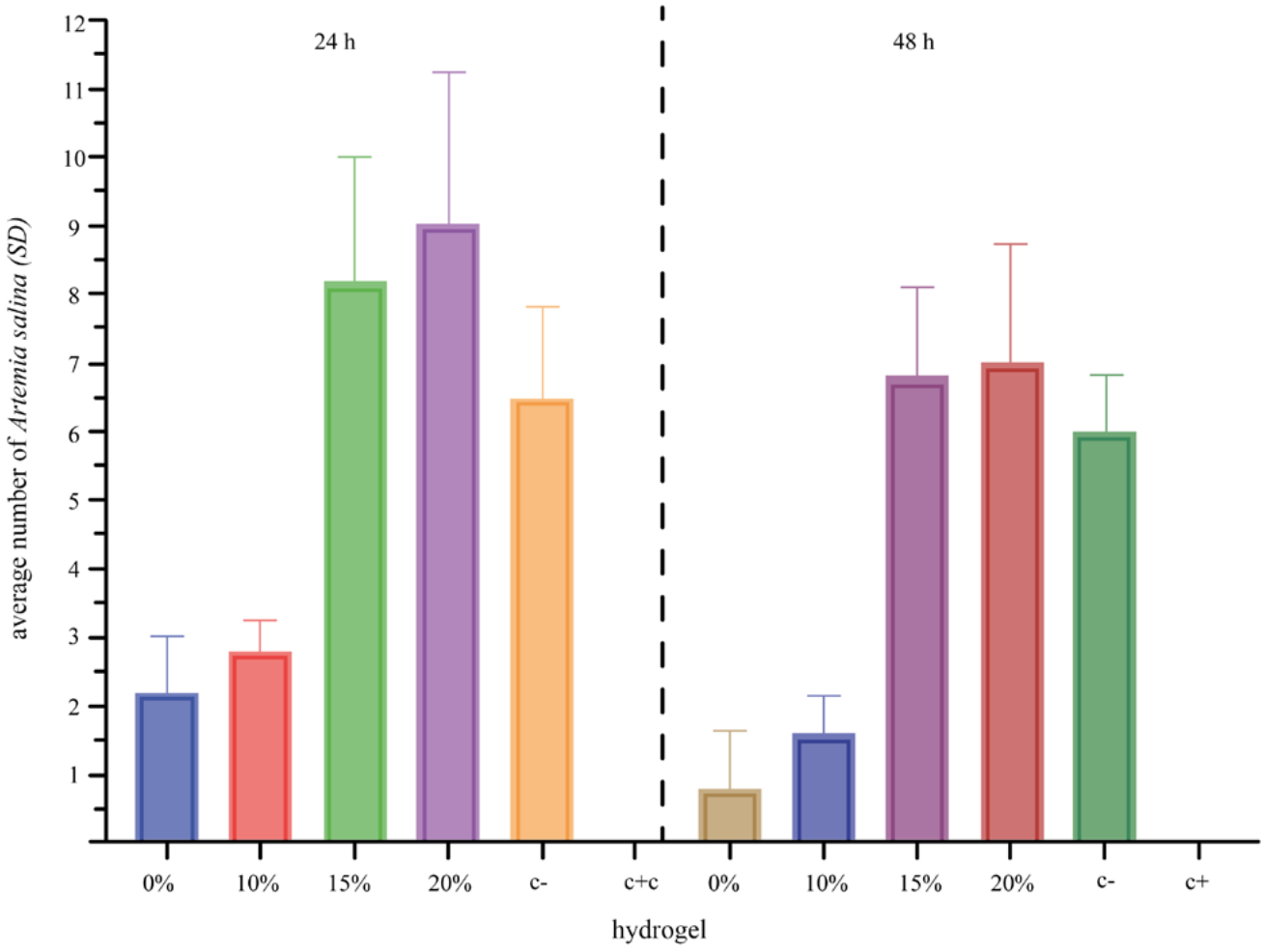

Fig. 2. Mean values of *Artemia salina* nauplii at 24 h and 48 h according to experimental hydrogel concentration

SD – standard deviation; C+ – positive control; C– – negative control.

0.16 ±0.06, respectively). Comparisons between the positive control and the hydrogel at different concentrations showed that the positive control values were lower than the material. The largest absolute difference was obtained by the 10% hydrogel (0.20, in favor of the material), and the same results were obtained with the negative control (0.12). Furthermore, the 0% and the 15% hydrogels had values lower than the negative control. The differences in the data reported in Fig. 3 were not statistically significant.

Discussion

This study aimed to assess the cytotoxicity of chitosan hydrogels on human HeLa cells using different concentrations of L-arginine. Based on our findings, we suggest that adding 10% of L-arginine to hydrogels generates an efficient product that achieves the desired effect, is easy to handle, and reaches optimal levels of cell viability.

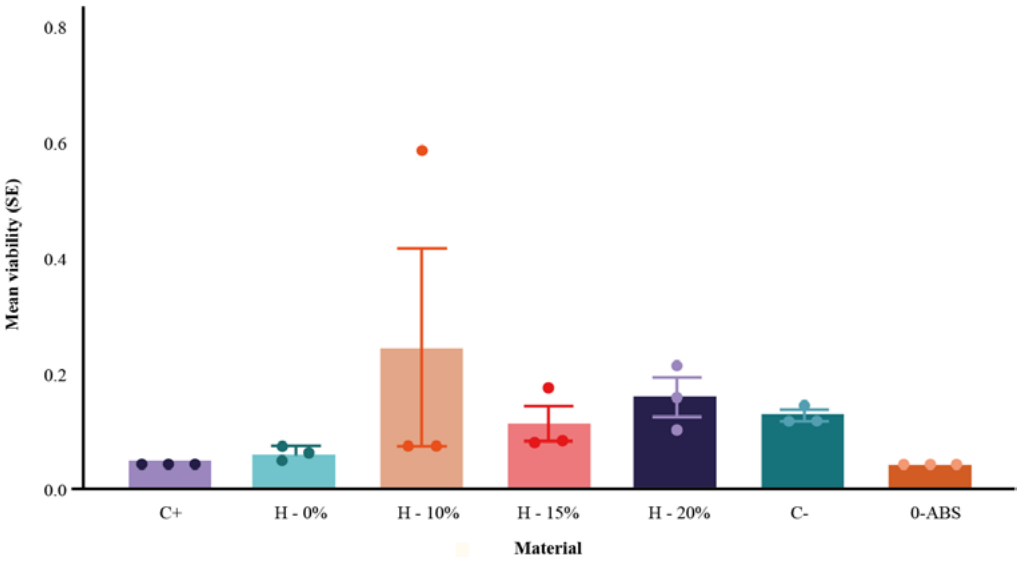

Fig. 3. Mean viability values according to the material used

SE – standard error; H – hydrogel; ABS – absorbance; C+ – positive control; C– – negative control

Artemia salina assay

The *Artemia salina* test is widely used for preliminary material compatibility studies.[31] The *Artemia salina* culture was done using a saline medium in culture dishes and was impregnated with different hydrogels to assess the viability of the specimens. The MTT and *Artemia salina* assays were performed to validate the method. The hydrogel in the wells of *Artemia salina* was too dense and reduced the movement of the nauplii, and for that reason, the MTT assay was carried out.

Different concentrations of L-arginine were used since it is a precursor of NO synthase and can be found endogenously at a cellular level. Raising the levels of the precursor (L-arginine) increases NO production or synthesis. Chitosan is a vehicle used to transport the amino acid and is stable over time.[32]

Pereira et al.[31] reported a hydrogel with chitosan cross-linked using ceftazidime and modified with acetyl-acetone and ethylenediamine (Cacen) or diethylenetriamine (Cacdien), and carried out cytotoxicity tests with *Artemia salina*, obtaining low mortality (less than 10%). These results showed the non-toxic nature of the chitosan derivate elements. Likewise, Parvez et al.[33] reported low cytotoxicity in an *Artemia salina* assay using a matrix of chitosan and gelatin for healing purposes.

Our results were likely influenced by the viscosity of the hydrogels, which reduced the movement of the specimens, unlike the lower density hydrogels. Similar results were reported by Pereira et al. and Parvez et al.,[31,33] where several samples died due to the high viscosity that limited the entry of oxygen. A layer of high concentration of the scaffold inhibited the permeability of oxygen, producing sequential death of several specimens.

HeLa cell culture assay

Cytotoxicity analyses ensure the biocompatibility of a material. Rakhshaei et al.[34] reported obtaining a gelled chitosan compound cross-linked with zinc nanoparticles and Arg-Gly-Asp. The viability of our analysis at various concentrations achieved a significance greater than 100% at 24 h and increased at 48 h without statistical significance.

Cell dispersion through the scaffold and its pores has been observed from the 2nd day. Meanwhile, Rodriguez et al.[35] highlighted the usefulness of low molecular weight chitosan hydrogels with polyvinyl alcohol (PVA) and evaluated the cytotoxicity of the hydrogel at 1, 3 and 7 days in a non-somatic cell line (colorectal adenocarcinoma), obtaining cell viability higher than 80%. However, as a result, there was a decrease in biocompatibility on most of the samples and their dilutions by the 7th day. In our study, cell viability achieved a higher percentage when arginine was applied, with 50% without L-arginine and 190%, 88% and 122% when 10%, 15% and 20% L-arginine were used, respectively. The characteristics of arginine explain this as a NO precursor that increases cellular metabolic capacity.[36,37]

Govindaraj and Raghavachari[38] demonstrated 120% viability in their fibroblast culture with a hydrogel partially cross-linked with succinic acid and urea with genipin. Two chitosan hydrogels have been reported by in vitro studies using human bone carcinoma cells and rodent fibroblast lines, where the hydrogel with polyethylene glycol dimethacrylate (PEDGMA) had the highest cytotoxicity compared with the negative control and the hydrogel in both cell lines.[39]

A chitosan hydrogel with dopamine-inulin was tested, with acceptable cytotoxicity values in the same fibroblast cell line.[12] In a study by Su et al.,[13] the same cell line was used to assess their 2 variants of carboxymethyl hydrogels with chitosan/polylactide (CMCS-PLA) and CMCS,

obtaining a viability of 97% after 72 h of evaluation. Other studies used different amino acids to improve physicochemical properties, mucoadhesion and solubility to develop safe and efficient products.[11,26]

Physicochemical characterization

Hydrogels can retain liquids in their macrostructure, but their stability and integrity can vary with the addition of other elements, such as amino acids or medications. For this reason, density is a relevant property when supplying hydrogels to patients. As reported by Sanchez et al.,[40] a chitosan hydrogel was obtained from purified technical shrimp, with this purified hydrogel displaying optimal physical characteristics. However, as described in the in vitro tests and the scanning electron microscopy (SEM) analysis, a better arrangement and distribution of the hydrogel pores was observed when they were cross-linked with external chemical elements such as glutaraldehyde or glyoxal. The purified hydrogel was also compared with non-cross-linked hydrogels, obtaining better water retention, temperature affectation and scaffolding structure. The hydrogels prepared with L-arginine in our research presented an absorption band around 1,632 cm^{-1}, that was assigned to the guanidine group, and a band at 1,689 cm^{-1}, that was associated with the stretching vibration of the carboxy carbonyl group. The bands at 1,400 cm^{-1} and 762 cm^{-1} were attributed to symmetric and asymmetric bending of the COO–, respectively.[41,42] The increase in the amide I peak in formulations with arginine suggests that arginine was effectively linked to the chitosan polymer chain.[42]

Mohandas and Rangasamy[43] found the same pH behavior of the hydrogel when they polymerized hydrogels with concentrations of L-arginine between 1.4 mM and 22.9 mM, obtaining a more neutral pH as the concentration of the amino acid increased given the alkaline nature of arginine.

Conclusions

The presence of L-arginine in our chitosan gels increased the viability of HeLa cells compared to the samples without this component, which was caused by an increase in the metabolic capacity of the NO precursors in the cultures.

The physical characteristics of the hydrogel with 10% L-arginine resulted in a better clinical application due to its density ratio and viability levels, with the density likely affecting the viability of *Artemia salina* nauplii. However, it is evident that hydrogels with lower densities were more viable. Based on our results, we suggest that chitosan/L-arginine hydrogels could be used in humans without toxic effects. However, more trials and tests are needed to evaluate tooth movement rate during orthodontic treatment.

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