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Cell viability evaluation of vero cells viability cultured on different chitosan films: development of functional biodressings possibilities

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Abstract: iomaterials used in regenerative medicine must have biocompatibility and be non-toxic, as they are used in contact with living tissues for repair or replacement. Therefore, they must provide a suitable microenvironment for ex vivo cell culture. Chitosan is a biomaterial highly used in cell culture research because it is non-toxic, biocompatible, biodegradable, exhibits high hydrophilicity, and has important antibacterial characteristics. This work has studied the interaction of VERO cells with chitosan films produced with different crosslinking agents, by evaluating the viability and cell morphology. Assays were followed for 96 hours for analysis of cell proliferation, viability, and morphology. The cells presented different developments in the different films, being the film with acetic acid the least suitable for cultivation showing cell viability of 58%, on the other hand, films made from chitosan solutions with lactic or citric acid had cell viability of around 70%. Therefore, chitosan films can be explored as biomaterials for the production of biodressings, for example, by exploiting the best crosslinking agent in their production (ie different acids).

Keywords: Chitosan. Films. Cell culture. Biomaterial.

Introduction

Cell culture in two dimensions

Cell culture refers to cultures derived from dispersed cells obtained from tissues, primary cultures, or from a cell line [14]. This technique allows the maintenance of living cells under controlled laboratory conditions and enables a better understanding of the molecular mechanisms of the cell, allowing important scientific advances in, for example, vaccine production and tumor cell biology [1].

The controlled conditions occur through the conditioning of the culture media, nutrient mixtures such as mineral salts [macro and micronutrients], carbohydrates, vitamins, and regulators necessary for cell growth, always maintained with pH, temperature, and oxygenation appropriated to the specific lineage keeping cells and tissues active for longer periods. Sometimes, the media also contain fetal bovine serum [FBS], whose main functions are to stimulate growth and other cellular activities through hormones and growth factors, increase cell adhesion by specific proteins, and provide proteins for hormone transport, minerals, and lipids [21].

Biopolymers are macromolecules produced by living organisms such as polysaccharides, proteins, nucleic acids, and lipids [11]. These biomaterials have the advantages of being abundant, affordable, similar to ECM, biodegradable and their degradation products are non-toxic and biocompatible. Due to this set of factors, the use of these biomaterials is abundant in numerous sectors of the biomedical industry [17]. Bioresorbable polymers are polymeric materials and solid devices that show degradation through size reduction and which are reabsorbed *in vivo*, materials that are eliminated by metabolic pathways in the body. Bioreaction is a concept that reflects the total elimination of low molar mass degradation by-products and by-products without residual side effects [22]. Bioresorbable devices have been used *in vitro* as a support for cell growth and proliferation of various cell types [6]. Among the several known bioresorbable biomolecules, chitosan occupies a prominent position in cell culture due to its particular characteristics.

Chitosan is a biopolymer obtained by the deacetylation reaction of chitin in alkaline medium. It is the second most abundant biopolymer in nature and can be extracted mainly from the crustacean and insect exoskeleton. It is insoluble in an aqueous medium and most organic solvents and has low chemical reactivity [9]. The main difference between chitosan and chitin is the average content of the 2-acetamido-2-deoxy-D-glucopyranose [GlcNAac] and 2-amino-2-deoxy-glucopyranose [GlcN] units present in the polymer chains, with effects on the solubility of these polymers. The degree of acetylation [GA] is defined as the average fraction of GlcNAc units present in the polymer chains. The product of chitin deacetylation is considered chitosan when soluble in dilute acid solution [3].

The main properties of chitosan that lead to its high use as a biomaterial in cell culture research are (1) high hydrophilicity, due to a large number of hydroxyl groups and amino groups present in the polymer chain, which allows its use as a biomaterial in the form of films, gels and membranes [9]; (2) non-toxicity, biocompatibility; and (3) biodegradability [8]. At neutral pH, chitosan acquires a positive global charge by protonating its amino groups. This property is of great importance for cell culture because it gives to chitosan the ability to electrostatically bind to glycosaminoglycans, proteoglycans, and other negatively-charged molecules [4].

Chitosan has been widely studied as a start material due to its antimicrobial properties and ability to manage the inflammatory response, besides promoting fibroblasts migration [10,15].

Analysis of the healing process from a macroscopic point of view reveals that chitosan plays an important role in the recovery of acute skin lesions on rats, accelerating the healing process and providing reduced lesion width, which reinforces its potential for medical application [5].

However, it has been shown that the presentation of the chitosan biomaterial, for example, gels, sponges, scaffolds, nanoparticles, films, or combined dressings influences its performances [16].

Preliminary results from our group indicate that cells do not reject collagen, hyaluronic acid, and chitosan-based support structures [18]. However, this work tested the interaction of cells in chitosan films made with different acids as reticulate agents, aiming to study the interaction of VERO cells with this biomaterial through morphological analysis and cell viability and point out a better starting presentation of a chitosan film for a biodressing development.

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Methodology

Production of chitosan films

The chitosan films used were kindly provided by Prof. Dr. Enio Nazaré de Oliveira Junior, from the Department of Chemistry, Biotechnology and Bioprocess Engineering of the Federal University of São João del–Rei. The production of chitosan–based films followed the procedure described by YOSHIDA et al. (2009). Different films were prepared by dissolving commercial chitosan samples, with the degree of acetylation from 5.8 to 6.3%, in 1% (v/v) of acetic acid (AA), lactic acid (AL), or citric acid (AC) solutions at 0.5% (w/v) concentration. The solutions were homogenized on a magnetic stirrer at room temperature for 24 hours until complete dissolution. They were then filtered using an 11 µm filter under vacuum and 100 mL of the filtrate was added to glass plates with a length of 29 cm and width of 21 cm. The films were dried at room temperature.

Preparation of the cell population

The tubes containing VERO cells were thawed in a water bath and the cells were resuspended in 8 mL RPMI culture medium (SIGMA), 20% fetal bovine serum (FBS) (SIGMA), and 1% of an antibiotic solution (10mg penicillin; 10mg streptomycin; 25µg amphotericin B per ml – SIGMA). The cell solution was centrifuged at 1150xg for 5 minutes and the supernatant was discarded. The pellet was resuspended in 5 mL of the same culture medium used for centrifugation and the cell suspension was transferred to a T–25 culture flask. Then the culture was incubated in a 5% CO_2 incubator at 37°C. After acclimatization of the cells, the FBS concentration was reduced to 10% and the cells were transferred to a T–75 flask, once a suitable confluence has been reached the experiments were performed. Thus, the medium used in all experiments was the 10% FBS and 1% antibiotics RPMI

Sterilization and preparation of chitosan films for cultivation

Chitosan films were cut into squares (1 cm^2) and deposited individually, in triplicate, under the bottom of the wells of a 24–well culture plate.

For sterilization, the films remained dipped in 70% alcohol at room temperature. After 3 days the alcohol solution was discarded and the wells containing the films were washed thoroughly with sterile phosphate–saline buffer (PSB). Then 450µL of penicillin/streptomycin antibiotic was added to each well allowing it to act for 30 minutes to eliminate any remaining contamination. The films were washed 3 times with PSB and acclimated in 500µL RPMI 10% FBS and 1% antibiotic culture medium.

Cell cultivation

The VERO culture at 80% of confluence was trypsinized and resuspended in RPMI culture medium, as described above.

To quantify cell concentration, the cell count was performed in a Haemocitometer. The culture plate containing the different films and the controls were seeded with 0.5 x 10^5 cells and the volume of each well was raised to 1 mL with the culture medium in use. The plates were incubated in a 37°C/ CO_2 incubator.

Morphological analysis by microscopy

For morphology and confluence analysis, VERO cell cultures were observed using the phase–contrast inverted microscope (BX41 Olympus) shortly after the beginning of cultivation, and at 24, 48, and 96 hours of cultivation at magnitudes of 100, 200, and 400x.

Viability test

The culture medium was discarded and the wells were washed with 2 mL of sterile PSB three times. The films were transferred to another identical plate so that the counting would only occur for cells that were adhered to the biomaterial. 250 µL of 0.25% trypsin (SIGMA) was added to each well and the plate was incubated for 3 minutes at 37 ° C. Then 250µL of culture medium was added to all wells. The suspensions were transferred to 2 mL Eppendorf® tubes. 10µL of each suspension was removed and 10µL of 0.4% Trypan blue was added. Thus, 20µL solutions were obtained from each well containing the dye. 10µL of each sample was transferred to Haemocitometer and counted under an optical microscope. After counting the four quadrants in each reticulum, Equation 1 was used to obtain cell concentration in cells / mL.Equation 1:

$$N = \frac{n \times D \times 10^4}{8} \quad (1)$$

Being:

N = number of cells/ml; "N" is the number of cells counted in the quadrants of the two Neubauer chamber reticles and "D" the dilution factor.

Cell viability was calculated by the percentage of viable unstained cells by Trypan blue dye during counting.

Results and Discussion

Cell growth in chitosan movies

The RPMI culture medium used had in its composition the phenol red pH indicator, therefore, it was possible to monitor qualitatively the pH of the medium during the tests, once it was possible to observe color changes according to the pH variation. Immediately after cell seeding on chitosan films, a sudden change in pH was observed, with a more prominent change in AL films, followed by AC and AA, as can be seen in Figure 1.

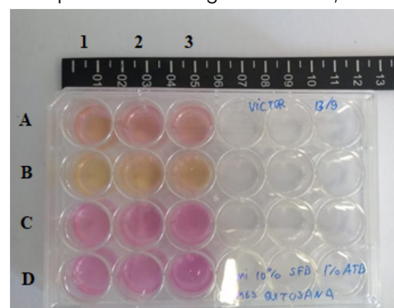


Figure 1 – 24–well plate containing chitosan films immediately after cell population passage. Chitosan 0.5% in lactic acid [A1 and A2 and A3]; Chitosan 0.5% in citric acid [B1, B2 and B3]; Chitosan 0.5% in acetic acid [C1, C2 and C3]; Negative control [D1, D2 and D3].

This pH variation can be explained by the different pKa values for these acids (Table 1). The lower the pKa value of an acid, the greater the acid dissociation constant (K_a).

Acids	*pK_aa 25°C*
Acetic acid	4.75
Citric acid	pK_{a1}=3.15 pK_{a2}=4.77 pK_{a3}=6.40
Lactic acid	3.85

Table 1 – pKa at 25 °C for acids used in film production.
Source: IUPAC *Gold Book*, 1997.

In the cell culture system, it is important to control the optimal pH (7.0–7.6) using a buffer and supplementing the culture medium to resists pH variations, especially in the lag phase of cell growth [14]. Therefore, the sudden variation in the pH of the culture medium can affect the culture deve–lopment, decreasing the cell viability, and increasing the lag phase duration.

Cell number count

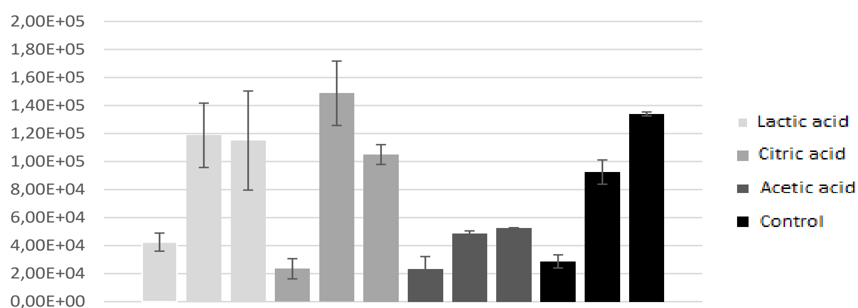
It was found that cells can interact well with chitosan films until they reach a high confluence especially the films which had AL and AC as reticulate agents. Chitosan films in AA were less favorable for cell multiplication (Graph 1).

At 24 hours there was initial cellularization in all films, but the AC and AA films exhibited a low number of cells when compared to the control. The acidification of the medium, generated after the contact of the films with the culture medium and the toxicity of these acids, especially the acetic and citric acids, may have interfered in the initial development of the cells. Utyama (2003), when studying the cytotoxicity of acetic acid on Artemia salina Leach, founds that the acid was cytotoxic at all concentrations studied.

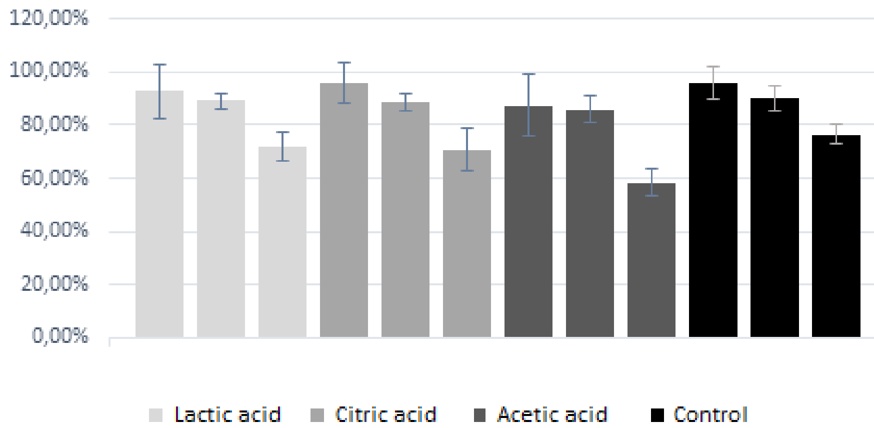
After 48h, it was observed that the cellularization of the films reached similar numbers to the control. In general, it is found around 1 x 10^5 cells/cm² when cells are cultured in monolayer [19]. Once the films are about 1cm², the cell concentration values for AL and AC films were compatible with those in the literature. The considerable improvement in cellularization within 48 hours can be explained by the pH control system in the CO_2 incubator. The dissolved CO_2 in equilibrium with bicarbonate ions generates a buffering system in the important medium in the lag phase of cell growth [13]. AA films, on the other hand, were not fit for cellularization and had about half cell numbers compared to other films and the control.

At 96 hours, the cellularization was kept stable for the AL and AC films. AA films maintained the same low cell concentration of 48h, proving to be less viable.

Data from cell viability analysis corroborate with the indication of the AA film as the least viable for cellularization, once at 24 hours of cultivation the viability of the cells cultured in it was 10% lower than the viability found in cells grown in the other films. AL (92.6%) and AC (96%) had results aligned with the control (96%). This decrease in cell viability in AA films becomes more evident at 96h when cell viability in AA films was only around 58%, while in the other films, including control, cell viability was up to 76%., This corroborates the toxicity of these films and the results found in cell count and light microscopy. Graph 2 presents the results for the observed cell viability.



Graph 1 – Cell concentration [cells / mL] of monolayer cultures on the different films tested and in the control comparing the same acids at different interaction times.



Graph 2 – Cell viability of monolayer growing cultures on different films tested and on control comparing different acids at different interaction times.

Analysis of VERO cells culture development in chitosan films

Figure 2 – shows the morphology of the VERO cell population in each film after 24, 48, and 96 hours after seeding.

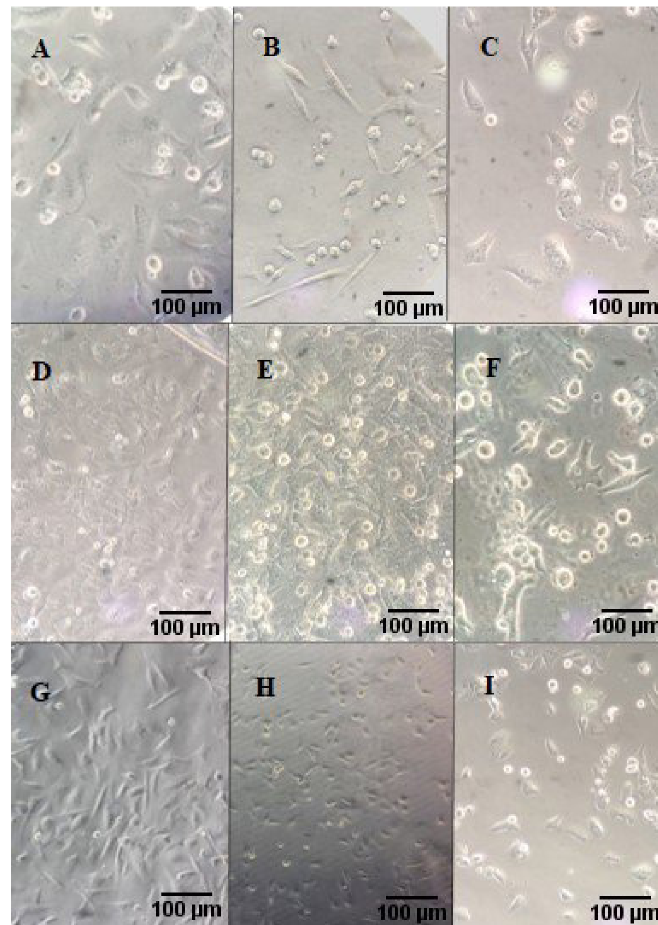


Figure 2 – Optical microscopy of triplicate wells containing 0.5% chitosan films after 24 hours [A, B, C], 48 hours [D, E, F], and 96 hours [G, H, I] of culture. A, D and G represent the lactic acid films, B, E, H the citric acid films, and C, F, I the acetic acid films. Source: Own author.

Spread cells exhibiting fibroblast conformation were observed within 24 hours of incubation, which corroborates the morphological characterization mentioned in the literature, where fibroblasts are described as regenerating cells with a tendency to grow rapidly [7].

Besides, it was possible to observe that immediately upon contact with the films, the culture medium acidifies rapidly due to the pKa of the acids used in the preparation of the films. This fact may have interfered in viability and cell number in the first 24h of culture.

One factor that should be considered is that the films were not produced in a sterile and controlled environment, therefore had impurities that could be observed by phase–contrast optical microscopy and according to PATRULEA et al. 2015 this may have an impact on the material performance

and work on this issue is still necessary on the field, as well as better characterization. This way, a more detailed analysis of the films and a better evaluation of the cellular interaction process with the film will require scanning electron microscopy (SEM) analysis.

It is valuable to note that, despite a bulk of data about chitosan as a successful biomaterial used on the wound healing process, there are few works on chitosan–based films and the available products based on different chitosan presentations don't include any film–based one [12].

Once films are stable and easy to handle this presentation turns out to be interesting on patronization and further studies.

Conclusion

VERO cells showed satisfactory late viability when grown on chitosan films. These were also biocompatible and capable of providing adhesiveness to VERO cells which achieved a population close to maximum confluence in AL and AC films. On the other hand, AA films maintained a low number of cells along with all the cultivation and showed low cell viability at the end of 96h, proving to be unviable and cytotoxic to the cells. This way we call attention to the fact that chitosan films are a good supply to manufacture biodressings once it is a cheap, easy, and safe biomaterial to be used in contact with cells, however, it is necessary to choose carefully the reticulate agent used in its fabrication.

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