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Proliferative and Antimicrobial Evaluation of the Benzalkonium Chloride Loaded Walnut Shell-Rich Chitosan Gels

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ABSTRACT

Tissue engineering studies combine cells, biomaterials, and biomolecules to mimic native tissue. The selection of appropriate materials for tissue engineering applications encourages best practices from the lab to clinical trials, and natural biomaterials have the potential to offer desired features for these applications. Material abundance, ease of the process, and biocompatibility are the first milestones to choosing a suitable material. Lignocellulose is one of the most promising biomaterials for its biocompatible, antioxidant, and biodegradable features and is the most abundant material in nature. A walnut shell-added chitosan gel was developed in this study by exploiting chitosan's desired properties, such as biocompatibility, biodegradability, and mechanical capabilities, which boosted cell proliferation. Furthermore, the gel system was reinforced with benzalkonium chloride (BAC), a well-known eye drop sterilizing agent. The hydrogels were subjected to Fourier-transform infrared spectroscopy (FTIR) analyses, and BAC-related signals were observed. The results of BAC-loaded hydrogels revealed that the viability of the primary fibroblasts was enhanced on the BAC-loaded gels compared to tissue culture polystyrene, but the difference was not found statistically significant. Yet, antibacterial activity results demonstrated that only BAC-loaded gel systems have solid antibacterial activity. Additionally, the fibroblasts had the strongest proliferation profile on the walnut shell-added chitosan hydrogels compared to other test groups, but the films' bactericidal activity of the hydrogels was not apparent. After revising the BAC and walnut shell concentrations in the hydrogels, the findings demonstrated that the injectable gel system could be used for cell transplantation *in vitro* and *in vivo*.

Keywords: Chitosan, Injectable gel, Benzalkonium chloride, Toxicology, Bactericidal.

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Introduction

Because of major developments in 3D microtissue manufacturing and biomaterials, the recent decade has been dubbed the "golden era of tissue engineering." [1]. The development of materials that mimic the natural tissue environment or directly from nature has aided in advancing tissue engineering. The use of walnut shells, an industrial waste, in powder is one of the best examples. Thanks to its cellulose-containing structure, studies in which walnut shell powder has been added to scaffolds as a material that increases mechanical durability have been encountered more frequently in recent years. For example, in the study by Sharahi et al., electrospun fibers containing poly(caprolactone) and gelatin were enriched with walnut shell powder. It was shown that they did not have a toxic effect on mesenchymal stem cells in addition to the improved mechanical properties [2].

Another waste product is chitosan obtained from shellfish and is one of the biomaterial groups supporting cellular development with active amine and hydroxyl groups [3, 4]. Chitosan is employed in tissue engineering applications as nanoparticles, fiber mats, films, or directly as a hydrogel due to similarities of chitosan with the extracellular matrix [5-8]. Furthermore, hydrogel forms that may release drugs and biomolecules have shown promise in skin or bone patch construction trials; however, the mechanical properties of chitosan-based biomaterials are limited and necessitate the addition of other additives such as gelatin, silk, or others [8]. Walnut powder, known as a thickener, can offer a solution with its nature and desirable mechanical properties [2].

The antibacterial capabilities of chitosan are affected by a variety of factors, including pH, molecular weight, concentration, degree of deacetylation, and the kind of bacterium utilized in the study. [9]. Due to their multi-parameter fabrication techniques, before employing the resultant chitosan-based materials as biomaterials, their antibacterial activity should be evaluated, and other antibacterial agent cues should be included in the materials, if necessary. Benzalkonium chloride (BAC) is an antibacterial agent that can be used as an alternative to alcohol in the growing usage of hand disinfectants in recent years. It is also utilized in skin care products, eye

drops, cosmetics, and medicinal purposes [10]. BAC also has virus inactivation potential against several viruses [11]. BAC, on the other hand, possesses antibacterial characteristics due to its ionic structure but is hazardous to mammalian cells at high doses, including skin and air track cells. Hence, the American College of Toxicology (ACT) recommends that the concentration of BAC in contact with the skin should be between 0.1% and 0.13% [12].

In the study, injectable chitosan-walnut shell gels enriched with BAC were created, and FTIR studies and primary fibroblast viability assessments of the gels were performed in addition to bactericidal testing. According to our findings, the chitosan: walnut shell: BAC has the potential for viable cell transplantation *in vitro* and *in vivo* analysis.

Materials and Methods

Walnut Shell Activation

The walnut shell powder was a generous gift from EcoShell, LP, Canada, with a 325-micrometer powder size. Following the deionized water washing and drying stages, 10 g of the powder was immersed in 64% (w/w) sulfuric acid (Sigma Aldrich, 258105) for four days; the powder was carried into a dialysis bag to remove excessive sulfuric acid for ten days. Each day, the dialysis bag's water was refreshed, and the final sample was centrifuged at 5000 rpm for 10 min. Then the sample was lyophilized for 24 hours to remove excess water and stabilize the active cellulosic part. The dried sample was stored at 4 °C for ten days.

Gel Preparation

Chitosan (Sigma Aldrich, 448877) gels were prepared in 1% (v/v) acetic acid (Sigma Aldrich, A6283) solution. The solution was kept at room temperature, and the 1% (w/v) processed walnut powder was added to the chitosan gel. The chitosan: walnut shell gel was mixed for 4 hours at 1000 rpm. To prepare chitosan: walnut shell: BAC gel, chitosan: walnut shell gel was mixed with 0.1% (v/v) BAC (Thor, Acticide BAC50), and the gel was mixed under the previous condition.

FT-IR Analysis

One ml of the three variations was frozen at -80 °C freezer for one night, and the samples were lyophilized overnight. The lyophilized samples were measured at the Thermo Fisher Nicolet ATR FTIR module between 400-4000 nm. The findings of each sample were recorded three times, and the average value is depicted in Figure 1.

MTT Analyses

Human dermal fibroblasts were grown in the human dermal fibroblast medium (Promocell, C-23020) until reaching %80-90 confluency in the humidified incubator at 37 °C. After the trypsinization step, 10^4 cells/well was counted and applied to the 20 µl of the gel groups and TCPS. Following one day of incubation, one mM MTT solution was added to the samples, and formazan salts were dissolved in isopropanol. The plate readout was completed using the Biotek Neo system at 590 nm (n>3). Tissue cultured polystyrene (TCPS) was used as a control group for the analysis. Additionally, bright field microscope images of the cells on the gels were recorded using an inverted microscope (AE2000, Motic).

Antimicrobial Analysis

An agar-well diffusion test was employed for the evaluation of the gel samples. The tested microorganisms are *Escherichia coli*, *Staphylococcus aureus*, Shigella, *Bacillus pumilis*, and *Pantoea aggelomerans*. Each microorganism was grown in a Lurian Bertani broth at 35°C until reaching the optical density of 1.0. Following overnight incubation, the suspension of each examined microorganism was adjusted to OD 0.6 and uniformly spread on agar plates using a sterile cotton bud. To load 20 uL of the gel samples, a hole was punched in the bacteria spread agar with a sterile 100 uL volume tip. After the incubation at 35°C, the inhibition zones were measured by using a digital caliper. The 6 µg/mL concentration of ampicillin was used as a positive control. Each sample was done in triplicate.

Results and Discussion

BAC-loaded gels have a proper proliferative profile and bactericidal activity.

The cell viability analysis indicated that chitosan provides a more suitable environment for dermal fibroblasts than the control group (TCPS) since it presents many active $–NH_2$ and –OH groups at FTIR spectra. (Figure 1) The peak around 1589 cm^{-1} (the N-H bending of the primary amine) in the spectra of the test groups indicates the functional amine group sourced from chitosan. Additionally, at 2917 and 2856 cm^{-1}, two new intensive peaks correspond to asymmetric and symmetric vibration stretching bands of the $– CH_2$ group, respectively, demonstrating chitosan-BAC interaction [10].

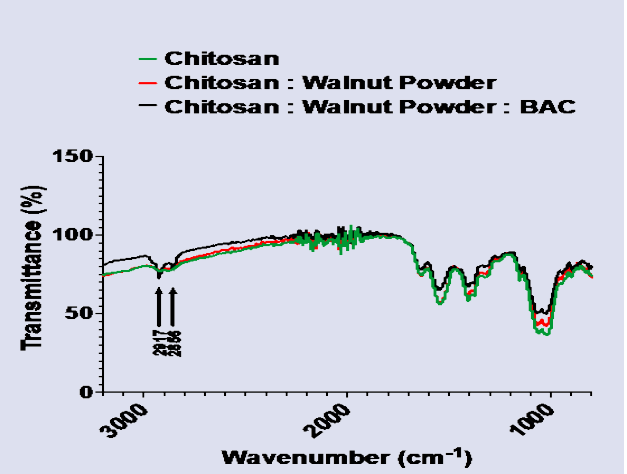

Figure 1. FTIR results of the chitosan, chitosan: walnut powder, and chitosan: walnut powder: BAC gel combinations, (n=3).

It is well-known that amine groups can increase protein concentration per surface area in a short time, and therefore cells show a tendency to attach to the surface [8]. There are different assumptions for protein adsorption on the hydroxyl group decorated surfaces, but it seems that the existence of the groups does not affect cell viability results [13, 14]. The cell viability results indicate that chitosan can provide the desired environment for fibroblast growth, but the difference is insignificant compared to a control group (Figure 2). Activated walnut shell powder-enriched gel form has the highest cell viability results (191.30±8.60), and the difference is statistically significant compared to a control group (100±16.09). Sharahi et al. reported that activated walnut powder supports cellular viability due to carrying many active groups, such as hydroxyl and carboxylic acid [2].

Interestingly, the BAC-loaded gel combination did not cause substantial toxicity even though it has been classified as a product type 1 disinfectant for personal hygiene in biocidal product regulation (BPR) [15]. The direct treatment of BAC solutions on the cells leads to membrane abnormalities because of the mode of action of quaternary ammonium compounds of BAC molecules. In a chitosan environment, the quaternary ammonium groups have the potential to reach different active groups, which may lead to controlling the cytotoxic effect of the BAC.

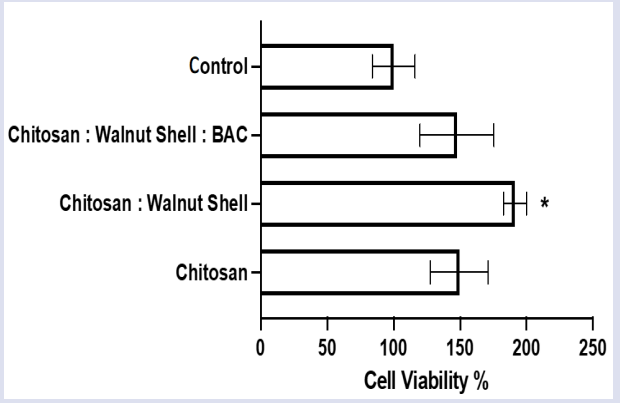

Figure 2. Cell viability results of the human dermal fibroblast on the gel formulations and TCPS control group. * represents p<0.05 compared to TCPS. (n>3 for each group)

In Figure 3, the cellular attachment on the gel was presented. The fibroblast on the gel formulations has shown a tendency to attach to the gel formulation surface, but the cells demonstrated a strong migration profile into the gel formulation. Therefore, for instance, the chitosan cell viability results on the chitosan gels showed around 50% proliferation, but the effect was not observed in figure 3A since the bright-field images could provide information on the top side of the gel formulations. The migration profile can be seen more clearly in Figure 3C. But, the cells that interacted with the formulation showed wide cell body presentation, unlike normal fibroblast morphology compared to other test groups, and it may be caused BAC toxicological effect (Figure 3C). The cell viability analysis also confirmed this finding since BAC-added formulation caused a decrement in the proliferation of the cells.4

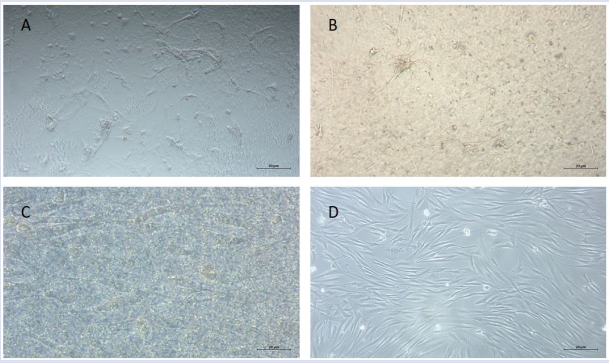

Figure 3. Optical microscope images of the human dermal fibroblast on the A) chitosan, B) Chitosan: Walnut shell, C) Chitosan: Walnut shell: BAC gel combinations, and D) TCPS control group. * represents p<0.05 compared to TCPS. (n>3 for each group)

Based on the well diffusion method, the antibacterial effectiveness of gel solutions on the microbes is shown in Table 1. According to the results, BAC-containing gel has a broad antibacterial activity on the microorganisms. Only chitosan and chitosan: walnut combination did not show any antibacterial effect. The experimental results showed that the BAC-containing gel had demonstrated bactericidal activity against gram-positive and gram-negative bacterial strains.

Table 1. Antibacterial effect of the gels on different microorganisms

	Diameter of Zone of Inhibition (mm)*									
Microorganisms	***Escherichia coli***		***Staphylococcus aureus***		**Shigella**		***Bacillus pumilis***		***Pantoea aggelomerans***	
Samples	**Mean**	**SD****	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
Chitosan:Walnut:BAC	17,19	0,82	24,83	1,08	19,22	0,82	24,18	0,67	29,19	0,55
Chitosan:Walnut	0	-	0	-	0	-	0	-	0	-
Chitosan	0	-	0	-	0	-	0	-	0	-
Positive Control (Ampicilin 6ug/mL)	34,04	1,04	56,87	1,08	36,19	0,96	38,39	1,18	29,19	0,55

* The zone of inhibition rate contains the 6mm diameter of well.
** SD: standard deviation

As a result, an injectable gel system has a critical potential for tissue engineering research and further reconstructing tissues in clinical applications [16]. In biomedical applications, in situ-forming gel systems have some troubleshooting which are defect geometries, polymerization problems, and contamination risk [17, 18]. The injectable gel development studies mainly focus on antibacterial solutions, whether innate or doping exogenous materials [19, 20]. Our antibacterial properties of BAC-containing gel have provided good stability for the

different microorganisms in both clinical and laboratory research applications. However, additional research is needed to determine how concentration screening of BAC, grain size, and quantity of walnut shell powder in the gel system affects cellular activities.

Conclusion

In the study, a low-cost, biodegradable, and biocompatible antibacterial gel based on quaternary ammonium and benzalkonium chloride (BAC) was developed for tissue engineering studies. The gel promises the potential for a wide range of industrial applications. It has a resistant and broad antibacterial effect on gram-positive (Staphylococcus aureus, Bacillus pumilis) and gram-negative (E.coli, Shigella, Pantoea agglomerans). This study is the first research using walnut shell powder as a supporter material in injectable gel system technologies with a combination the chitosan and BAC. In the future, the injectable nature of the gels can be used as filling material in dentistry and orthopedic surgery, and the mechanical and physicochemical properties of the gels will be studied for further studies within the scope of our studies.

Conflicts of Interest

The author declares no conflicts of interest. No competing financial interests exist.

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