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An In-Vitro Study of the Cytotoxicity of Chitosan Nanoparticles-Containing Orthodontic Primer

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Abstract
Background: Demineralization is an anticipated side effect of fixed orthodontic treatment. Incorporating antimicrobial compounds into orthodontic adhesive material may be an effective method for averting white spots. The objective of this study was to evaluate the cytotoxicity of an orthodontic primer modified with varying quantities of chitosan nanoparticles. Materials and methods: 0%, 1%, 5%, and 10% concentrations of Chitosan nanoparticles have been combined with Transbond TM XT primer. Three discs of eight millimeters in diameter and two millimeters in thickness were constructed for each group, for a total of twelve discs. Results: included the application of scanning electron microscopy (SEM) and X-ray diffraction (XRD) to confirm the Nano powder's properties. The data was statistically examined using one-way analysis of variance (ANOVA), and the results indicated statistically non-significant differences between groups ($p>0.05$).
In conclusion, the addition of chitosan nanoparticles to the orthodontic adhesive system's primer exhibited an acceptable level of cytotoxicity and was within the appropriate ISO limits (70% cell viability).

Introduction:

Most patients are unable to maintain optimal hygiene throughout orthodontic treatment, and enamel demineralization or white spot lesions form around the brackets, affecting up to 96% of orthodontic patients, with a higher incidence after 12 months of orthodontic appliance use (1). Many materials and medications handle these problems, but some have negative side effects that limit their use (2-3). Numerous researchers have postulated that antibacterial chemicals are likely to be utilized in the formulation of adhesives. Nevertheless, similar to the case of bioactive glasses, the bactericidal effect of these compounds might have a limited duration of a few weeks, hence potentially leading to increased rates of debonding [(4)]. In addition, using metal nanoparticles resulted in increased cytotoxicity [(5)]. Chitosan nanoparticles (CNPs), a cationic polymer derived from natural sources such as crab and shrimp shells [(6)], appear to be effective against S. mutants and S. sanguinis at various doses [(7)]. Nonetheless, their potential toxicity for human use has not been well investigated. Regardless of the high cytotoxicity of orthodontic adhesive, particularly in the first hour of bonding, integrating nanoparticles into composite materials could create additional health risks due to nanoparticles' unique structural and chemical features [(8)].

Given the limitations of other available materials, adding chitosan nanoparticles may positively reduce the formation of (WSL) during orthodontic therapy. Consequently, the research aimed to prepare an orthodontic primer that includes different chitosan nanoparticle percentages and investigates its cytotoxic properties. The current study is the first to investigate the cytotoxicity of chitosan nanoparticles in combination with an orthodontic primer. The null hypothesis is that “there are no significant differences in biocompatibility between orthodontic primer with and without chitosan nanoparticles.

Materials and Method

1. Characterization of nanoparticles

2.1.1 X- Ray Diffraction Analysis:

The investigation of the crystal structure of chitosan nanoparticles was conducted utilizing an X-ray diffraction instrument, specifically the PHILIPS_PW1730 from the Netherlands. The experiments were conducted using an operational voltage of 40 kilovolts and a current of 30 mill amperes. The samples were measured using X-rays with a single wavelength of 1.5406 A, at angles ranging from 5 to 80 degrees. The measurements were taken at a constant speed of 0.2 degrees per second. The X-ray diffraction (XRD) pattern of chitosan nanoparticles in their pure form is depicted in Figure 1[(23).]

Scanning Electron Microscope Analysis:

The researchers employed scanning electron microscopy (SEM) to examine the morphological features of chitosan nanoparticles and confirm the geometric arrangement and average size of the constituent atoms. The nanoparticle underwent investigation using field-emission scanning electron microscopy (FE-SEM) with the TESCAN MIRA (Mira3-XMU model) apparatus situated in Brisbane, Queensland, Australia.

The process of preparing a freshly formulated primer and the subsequent preparation of specimens.

This study utilized a digital electronic scale developed by the Satorius Company in Göttingen, Germany, with a precision of 0.001 gm, to quantify the weight of chitosan nanoparticles and Transbond TM XT orthodontic primer manufactured by 3M-Unitek in Monrovia, USA. Chitosan nanoparticles were introduced into the Transbond TM XT primer at varying concentrations of 0%, 1%, 5%, and 10%. The weight of each individual drop of the primer was measured to be 0.05 g. Consequently, 20 drops were utilized to produce 1 g of primer for every experimental group. The chitosan nanoparticles were combined with the primer at doses of 0.01 g, 0.05 g, and 0.1 g, respectively. The control group was not

administered any supplementary chitosan nanoparticles. Following this, a vortex mixer produced by Stuart Scientific in England, United Kingdom, was utilized for a period of 2 minutes to attain the necessary degree of uniformity. The standardization of droplets is achieved through the employment of a sampler with a size range of 10-100 µm, which is produced by Cypress Diagnostics, a company based in Hulshout, Belgium. The addition and mixing procedures were conducted within a controlled laboratory setting with little ambient lighting (7). Initially, a transparent polyurethane tube was employed to fabricate a total of 12 molds, each possessing a diameter of eight millimeters and a thickness of two millimeters. A total of 12 prepared starter discs, with each group containing three discs, were designed. These discs had dimensions of eight mm in length and two mm in thickness (9).

The preservation and care of cell cultures

The REF cells were grown in RPMI-1640 media (Capricorn, Germany) supplemented with 10% fetal bovine serum (Capricorn, Germany), 100 units/mL penicillin, and 100 µg/mL streptomycin. The cells underwent passaging utilizing Trypsin-EDTA (Capricorn, Germany). They were reseeded twice a week when they reached 80% confluence. The incubation process was carried out at a temperature of 37 °C using a CO2 incubator (Cypress Diagnostics, Belgium)[10].

Cytotoxicity Estimation

In order to assess the cytotoxic impact of various concentrations (control group, 1% group, 5% group, and 10% group), an MTT assay was conducted utilizing 96-well plates obtained from Santa Cruz Biotechnology, USA [11]. Each well was injected with a cell line containing 10,000 cells. The REF cells were subjected to different treatment conditions, including a control group, a 1% group, a 5% group, and a 10% group, either after 24 hours or upon reaching a confluent monolayer. Cell viability was assessed at two time points, 24 and 48 hours post-treatment. This was accomplished by removing the culture medium, introducing 28 µL of a 2 mg/mL solution of MTT, and thereafter incubating the cells for a duration of 2.5 hours at a temperature of 37 °C. Following the removal of the MTT solution (Bio-World, USA), the crystals present in the wells were dissolved by the addition of 130 L of DMSO (Dimethyl Sulphur Oxide). The mixture was then incubated for a duration of 15 minutes at a temperature of 37 °C, with oscillation [12] . The absorbance was measured using a microplate reader at 492 nm, and the experiment was conducted in triplicate. The inhibition rate of cell proliferation (the proportion of cytotoxicity) was calculated using the following formula [13]:-

Inhibition rate = A- B/A*100

In this context, A represents the optical density of the control, while B represents the optical density of the samples [14] .

The cells were placed into 24-well micro-titration plates at a concentration of 1×105 cells mL−1 and incubated for 24 hours at a temperature of 37 °C. Following this, the cells had exposure to several concentrations, encompassing a control group, a 1% group, a 5% group, and a 10% group, over a period of 24 hours. After being exposed, the plates were subjected to staining with crystal violet dye and then placed in an incubator at a temperature of 37 °C for a period of 10 to 15 minutes (15-13). Thoroughly removing the color by rinsing it with water from the faucet until complete elimination was achieved. The cells were examined using an inverted microscope with a magnification of 100, and the resultant images were recorded using a digital camera that was linked to the microscope [16].

Sample Grouping:

Twelve sample (three-disc per group) of formulated primer and grouping as follows:

• Control group: Transbond™ XT primers without nanoparticle.

• 1% group: the primers were prepared with 1% chitosan nanoparticles.

• 5% group: the primers were prepared with 5% chitosan nanoparticles.

• 10% group: the primers were prepared with 10% chitosan nanoparticles.

Statistical Analysis
The Shapiro-Wilk test was used to evaluate the normality of the data, whereas Levene's test was used to assess homogeneity. IBM Company, situated in New York, United States, analyzed the collected data statistically using SPSS Statistics ™ version 26 (Statistical Package of Social Sciences) software. The investigation employed ANOVA with an acceptable threshold of significance of (p 0.05).

Result:

A) X- Ray Diffraction Analysis:
The X-ray diffractograms obtained from the chitosan nanopowder reveal the presence of six well-defined diffraction peaks at certain 2θ values. These peaks are observed at 5.900, 9.000, 10.320, 16.970, 20.100, and 40.730. The Scherrer equation was employed to determine the average size of the crystallite in the prepared sample, yielding a value of approximately 21.48 nm[23] .

B)Analysis by Scanning Electron Microscope:
To determine the atomic shape and average size of chitosan nanoparticles, the researchers analyzed their morphological properties using scanning electron microscopy (SEM). According to the data depicted in Figure 2, a significant proportion of nanoparticles exhibit a predominantly spherical morphology with diameters ranging from 6 to 33 nm.

C) The Normality of Data Distribution
Using the Shapiro-Wilk test, it was determined if the data followed a normal distribution. All p-values are greater than 0.05, indicating that the data are not statistically significant. If this is the case, accept H0, which indicates the data follow a normal distribution (P> 0.05).

D)**The variance homogeneity test**
The test called Levene's was conducted in order to evaluate the homogeneity of variance in the data. The results indicated that there was no statistically significant distinction observed among the groups, as evidenced by a p-value greater than 0.05.

D)Descriptive statistics of Cytotoxicity
Standard deviation (S.D.), minimum (Min.), maximum (Max.) and the mean value are illustrated in Figure 1. The control group exhibited the greatest mean value (A)=(96.000±2.646), followed by that of the 1% group (B)= (92.333±1.528), then the 5% group(C)=(90.000±4.359), and lastly, the 10% group (D) =(88.333±5.508). Table 1 presents a comparison of the mean differences among several groups using an analysis of variance (ANOVA). there were non-statistically significant differences among all groups(**P value> 0.05**) . The result revealed that the experimented materials had no cytotoxic impact against REF cell lines, as exhibited in Figure (3). In addition, the results demonstrated that all groups did not make apparent morphological changes in REF cell lines after treatment, as shown in Figures 4–8.

Discussion
Previous studies confirmed that the penetration depth of primer in etched enamel prisms was around 40.55–53.97 micrometers, which is the PowerPoint to infiltrate nanoparticles included in primer [17]. From this point on in the current study, nanoparticles were included with the primer rather than the adhesive, and this direct contact and penetration of nanoparticle-containing resin into the enamel made the enamel more resistant to white spot lesions, which occur during orthodontic treatment, have a detrimental effect on enamel, and are difficult to restore via natural remineralization [18].
The toxicity of mineral nanoparticles prevents their use in high concentrations for a long time, so many researchers have attempted to study another material safer than metal nanoparticles. One nanomaterial is chitosan, a polymeric material with antibacterial, antifungal and antiviral action recently used in many medical branches and has given satisfactory results [19-20]. However, prior investigations still need to examine the cytotoxicity of chitosan nanoparticles in

orthodontic primers. Consequently, a chitosan-containing primer was developed in this study, and its cytotoxic effects were evaluated.

Incorporating Chitosan nanoparticles offers several further advantages, such as their notable chemical stability, convenient accessibility, and effective antibacterial properties (21-22). Although Chitosan nanoparticles were synthesized by a dedicated manufacturer, X-ray diffraction (XRD) analysis indicates the presence of a distinct and well-defined crystalline peak at an angle position of 2θ = 20.21. The observed crystalline peak of pure chitosan at an angle of 20.21 degrees can be attributed to the presence of the Miller plate on the (220) plane, as indicated by the notation [23]. Consistent with prior studies [24], the structural study using SEM indicates that the formation of the chitosan's crystalline structure can be attributed to the presence of hydrogen bonds within the molecular framework. The presence of a wide peak spanning from 35 to 55 degrees signifies the amorphous phase of chitosan [25] and suggests the existence of its crystalline structure. According to the data, it has been shown that a significant proportion of nanoparticles have a near-spherical shape, with their diameters falling within the range of 6 to 33 nm.

The average atomic dimensions and histogram of atomic distribution were determined using the contemporary programming tools Image J and Origin Pro. The average size of atoms, as estimated by study of electron microscope images, is approximately 18.5 nanometers. The widely employed technique of ionic creation in the synthesis of chitosan is accountable for the reduced size of particles. The technological advancements in chitosan preparation have facilitated the efficient and expeditious manufacture of nanoparticles. Furthermore, prior studies have indicated that the characteristics of nanoparticles, such as their surface properties and size, can be altered through the manipulation of the chitosan and stabilizer proportions [26].

Nanotechnology provides patients and orthodontists with improved access to new physicochemical, mechanical, and antibacterial properties; however, even though nanoparticles can easily penetrate tissues and affect biological behaviors at various levels, they must pass specific biocompatibility tests to meet safety requirements [27].

This is the first study to investigate the application of the smaller nanoparticle chitosan. Various concentrations (from 0 to 10%) were investigated to pick the optimal concentration(s) to increase antibacterial performance attributes while avoiding cytotoxicity.

Cell viability measurements following exposure to adhesive system components allow the possible detrimental effects of such compounds to be determined [28]. The MTT technique has the advantages of simplicity, precision, dependability, and time savings. In addition, the MTT technique was effective for estimating cell densities in tiny culture volumes. We also employed an MTT test technique for similar reasons.

According to our findings, All the groups were non-statistical significant. This result is in agreement with a previous study [29], and cell viability was acceptable according to ISO standards (70%) [30]. However, cell viability lowered as nanoparticle concentration increased, with the lowest rate of cell death in the 10% at 88.33%, exhibiting that nanoparticle cytotoxicity is concentration-dependent[31]. This biocompatibility in 1% and 5% may be interrupted using appropriate disc dimensions that guarantee complete polymerization and low concentration[32]. In comparison, the slight increase in cytotoxicity in 10% may be explained by particle size, which was used in his study and this particle's ability to damage mitochondria membrane that causes loss of plasma membrane integrity.

Chitosan nanoparticles have been reported to be biocompatible with fibroblasts in different uses in dentistry. Chitosan nanoparticles can be mixed with the root canal sealant in primary teeth and used as a mouthwash in a dose-dependent manner [33]. These previous studies support the current research.

Limitations

The inclusion of a combination of chitosan nanoparticles and other agents in a novel formulation to achieve a synergistic effect on remineralization and antibacterial activities was not considered in this study.

Clinical implications

Given the nature of chitosan nanoparticles' antibacterial action and the flowability of the primer, it resolves to come into direct touch with enamel, and the chitosan nanoparticles may be used as a substitute for other harmful metal nanoparticles. Moreover, the antibacterial activity of chitosan nanoparticles increased with the percentage increase, such that adhesives containing 1% NPs significantly reduced S. mutans and S. sanguis biofilm inhibition; however, this concentration did not affect L. acidophilus biofilm inhibition until the 10% nanoparticle L. acidophilus aided in caries formation and was present in more advanced lesions. The current study provides the foundation for using a 10% concentration with acceptable biocompatibility.

Conclusion

The present study utilized chitosan nanoparticles measuring around 50 nm in size. The cytotoxicity of orthodontic primers, which were formulated with different concentrations of chitosan nanoparticles (1%, 5%, and 10%), was shown to be within tolerable limits. Nevertheless, it is essential to establish the practicality of utilizing these materials in vivo.

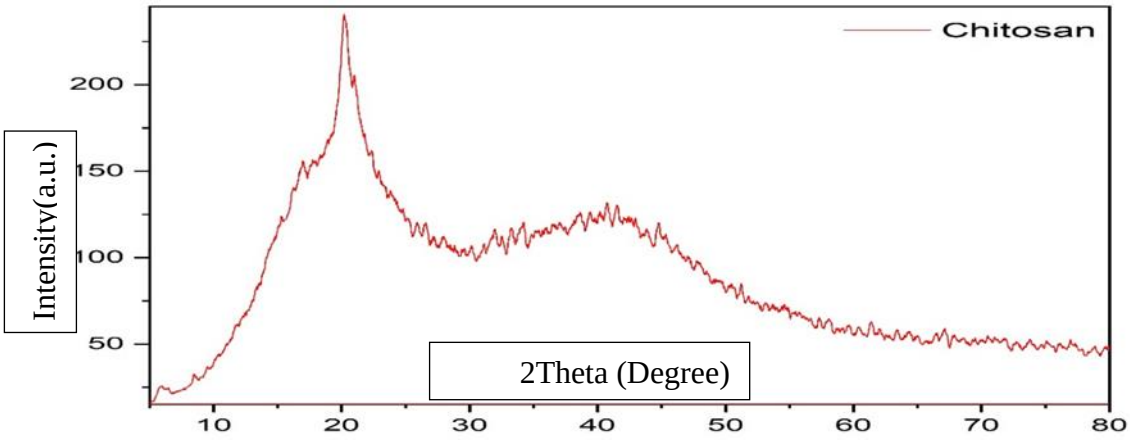

Figure 1: XRD patterns of chitosan nanoparticles

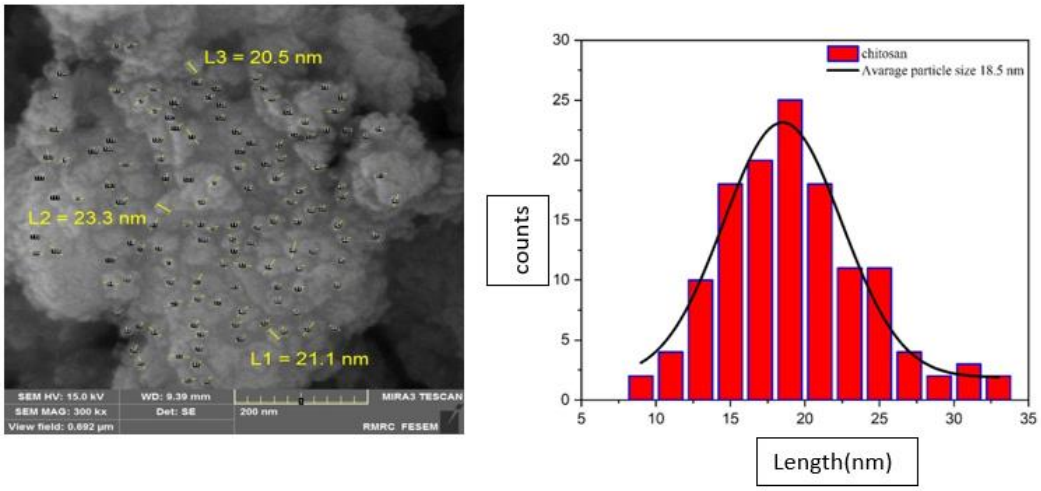

Figure 2: SEM image and histogram distribution of chitosan nano particles.

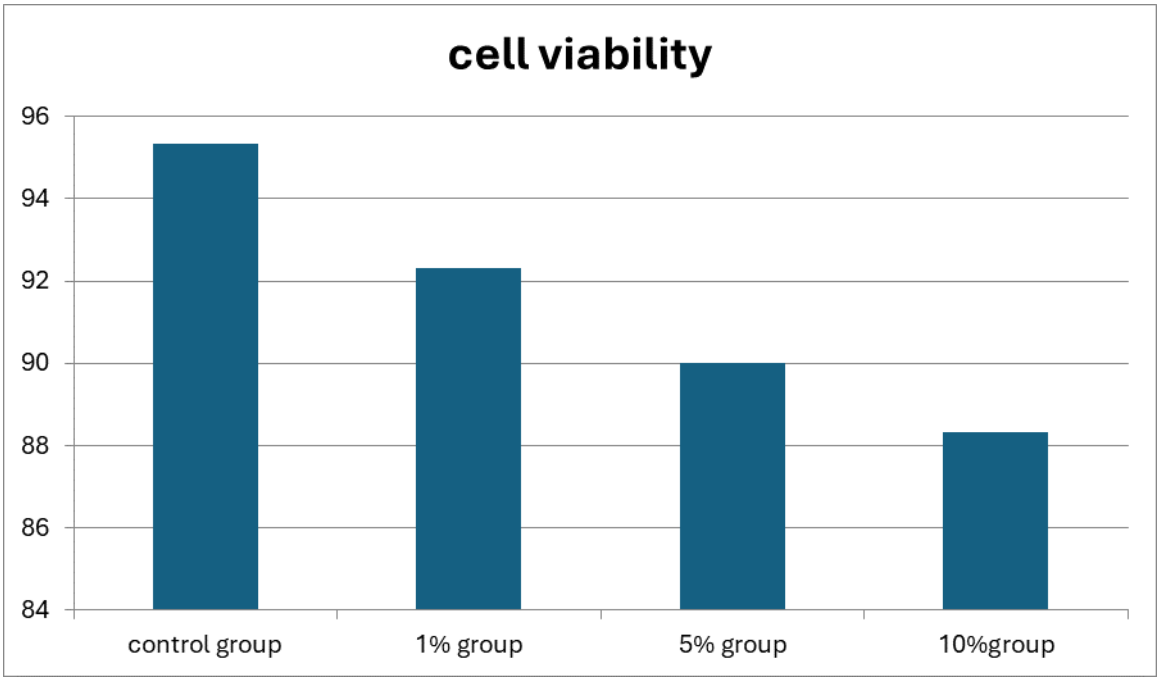

Figure 3: Bar chart for cell viability for all groups.

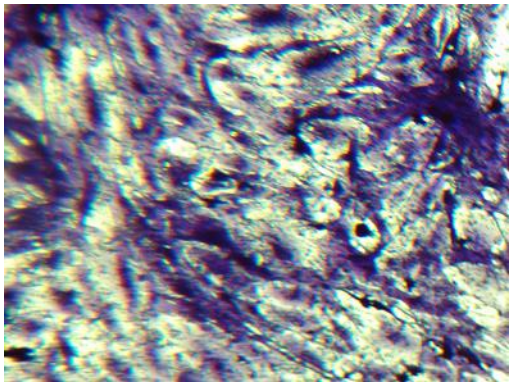

Figure 4: Control untreated REF cells (violet). Magnification power 40x.

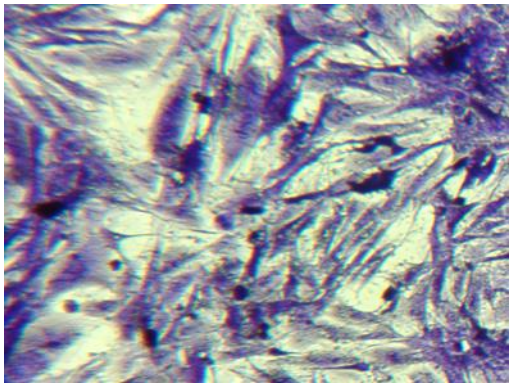

Figure 5: Morphological changes of REF cells after treated with control primer. Magnification power 40x

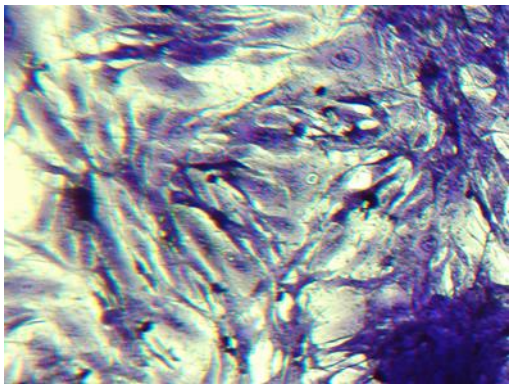

Figure 6: The morphology changes of REF cells after being treated with 1% chitosan-containing primer. Magnification power 40x

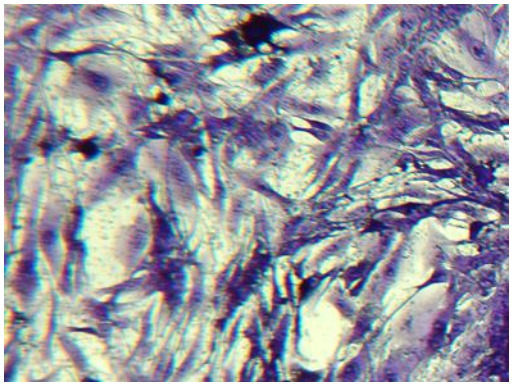

Figure 7: Morphological changes of REF cells after being treated with 5% chitosan-containing primer. Magnification power 40x.

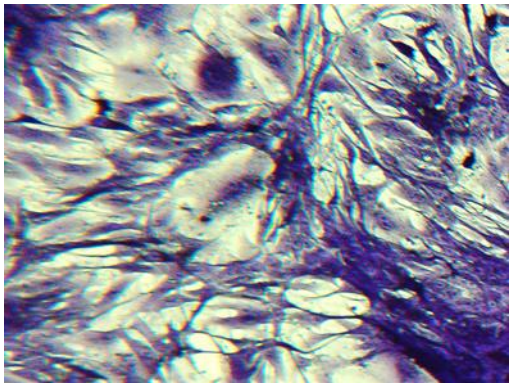

Figure 8: Morphological changes of REF cells after 10% chitosan- containing primer. Magnification power 40x

Table 1: Descriptive and statistical test Cell viability among groups (one way Analysis Of variance; ANOVA).

Groups	Minimum	Maximum	Mean	±SD	F	p value
Control	93.000	98.000	96.000	2.646	2.258	0.159 NS
1% group	91.000	94.000	92.333	1.528		
5% group	85.000	93.000	90.000	4.359		
10% group	83.000	94.000	88.333	5.508		

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