

Material Science and Engineering with Advanced Research

Cytotoxicity of Low, Medium and High Molecular weight Chitosan's on Balb/c 3T3 Mouse Fibroblast Cells at a 75-85% De-acetylation Degree

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Article Type: Research, **Submission Date:** 08 February 2018, **Accepted Date:** 26 February 2018, **Published Date:** 20 March 2018.

Citation: Sias R Grobler and Perchyonok VT (2018) Cytotoxicity of Low, Medium and High Molecular weight Chitosan's on Balb/c 3T3 Mouse Fibroblast Cells at a 75-85% De-acetylation Degree. Mater. Sci. Eng. Adv. Res 2(2): 27-30. doi: <https://doi.org/10.24218/msear.2018.26>.

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Abstract

Chitosan is nowadays in the limelight as carrier system for drugs, proteins, hormones, enzymes and genes. Therefore, it is important to also investigate the cytotoxicity of different facets of it against cells. In this study the cytotoxicity towards three different molecular weights (a low molecular weight 50-90kDa, a medium molecular weight 190-310kDa and a high molecular weight 310-375kDa) on mouse Balb/c 3T3 fibroblast cells were investigated. The degree of de-acetylation was between 75-85%. The cell survival rate was determined according to the standard MTT assay. The presence of all three chitosan's showed a positive effect on the survival rate (above the control value) which increased with increasing molecular weight (from 108% through 118% to 120%). It was concluded that not only improved the presence of chitosan the cell survival rate to a higher level than that of the control but the higher the molecular weight of chitosan the better the survival rate while a molecular weight of about 375kDa seems to near the highest possible positive effect.

Introduction

Chitin is one of the most common natural polysaccharides and was first identified long time ago in 1884. This biopolymer is synthesized by a number of living organisms in plants and animals (for instance in the exoskeleton of arthropods or the cell walls of fungi and yeast) and is the most abundant polymer after cellulose.

Chitin is the second most abundant naturally occurred homo polysaccharide composed of β -(1,4)-linked-D-N-acetyl glucosamine (GlcNAc) after cellulose. Chitosan is a linear heteropolysaccharide composed of β -(1,4)-linked-D-glucosamine (GlcN) and N-acetyl glucosamine (GlcNAc), which is derived from chitin.

Chitosan (Figure 1) is the most important derivative of de-acetylated chitin and is a versatile hydrophilic, linear amino-polysaccharide and a natural polymer with a structure based on repetitive de-acetylated and acetylated units randomly distributed [1]. Chitosan has been proposed as a bio-adhesive polymer and became more and more important in the pharmaceutical field as a carrier system for drugs, hormones, proteins, enzymes and genes [2-6].

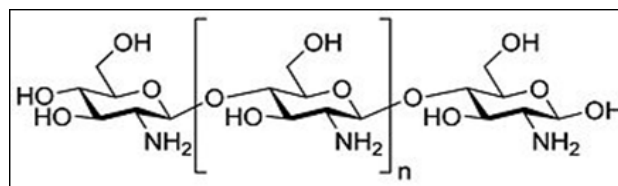


Figure 1: The chitosan molecule

Chitosan being soluble in acidic aqueous media is used in food stuff, cosmetic, biomedical and pharmaceutical applications. It is becoming a more and more popular therapeutic agent and its use is constantly extended. The cationic nature allows it to form electrostatic complexes or multilayer structures with other negatively charged molecules. Furthermore, antioxidant-chitosan hydrogels (that of resveratrol, propolis and β -carotene) were found to significantly improve the bond strength to dentine with or without phosphoric acid pre-treatment [7,8] as many other hydrogels do [9].

Further more, it is found to be hypoallergenic and anti-bacterial which support its use in the army as field bandages. However chitosan has only been approved by the FDA as a drug carrier in some combinations, for example, for wound healing [10]. Chitin is manufactured industrially by crushing shrimp shells, then washing the solids with acids to remove inorganic and protein

aceous material. The purified chitin is de-acetylated to chitosan by treatment with a strong base such as sodium hydroxide. The reaction occurs in two stages under first-order kinetic control. This reaction pathway, when allowed to go to completion (complete de-acetylation) yields up to 98% produce. Chitosan may be further purified by preparation of solutions in acid followed by neutralization and precipitation [11,12].

The purpose of this paper was to evaluate the possible cytotoxic effect of a low molecular weight chitosan, a medium molecular weight chitosan and a high molecular weight chitosan on Balb/c mouse 3T3 fibroblasts. Even though chitosan as such might prove to be important in many specialities, there are many facets of it which need further attention like cytotoxicity studies.

Materials and Methods

The three different chitosan's (Aldrich Ltd, Australia) were used as received. The de-acetylation degree for all three was between 75-85%. They differed in their molecular weight: a low molecular weight 50-90kDa, a medium molecular weight 190-310kDa and a high molecular weight 310-375kDa.

Mouse Balb/c 3T3 fibroblast cells (National Repository for Biological Materials, Sandringham, USA) were maintained under standard tissue culture conditions and allowed to reach a strong growth phase near confluence [4,5,7]. Cells were then trypsinised and plated out in 96 well plates. The 3 different molecular weights of chitosan's were dissolved in DMEM medium to a concentration of 1mg/ml. As the control no chitosan was added. There after the pH was set at 8.0. One hundred μ l of the chitosan medium (or medium without chitosan for the control) was added to cells in the 96 well plates and left in the incubator

for 24hours. Thereafter 10 μ l of MTT previously prepared and filtered was added to all wells. After 3 hours all medium was discarded and 100 μ l of DMSO added. The MTT stained only viable cells and an indication of the degree of stain is determined by absorbance values measured at a wavelength of 540nm on a spectrophotometer. Each of the 3 different molecular weights with their controls were repeated 3 times. Each level consisted of a control measurement paired with an experimental one (Figure 2).

Results

The difference between the pairs (control versus treatment) for all three molecular conditions were analysed using the Friedman's Two Way Analysis of Variance by Ranks (a non-parametric test). They all differed significantly at a 1% level (Figure 2). The differences between the three weight levels as well as between the 3 control groups (Figure 2) were analysed using a univariate ANOVA. No significant differences were found between the 3 control groups on a 5% significant level (Figure 1). Pairwise comparisons between the weight levels reveal that the difference between the lowest weight level (w1) and two higher weight levels were significant on a 5% level but the difference between medium molecular weight (w2) and the high molecular weight (w3) was not significant ($p > 0.05$, Figure 2).

Discussion

The cell survival rate was tested at a concentration of 1mg/ml growth medium with an exposure period of 24 hours. These settings are in line with the literature as an upper level for various cytotoxic tests [13,14].

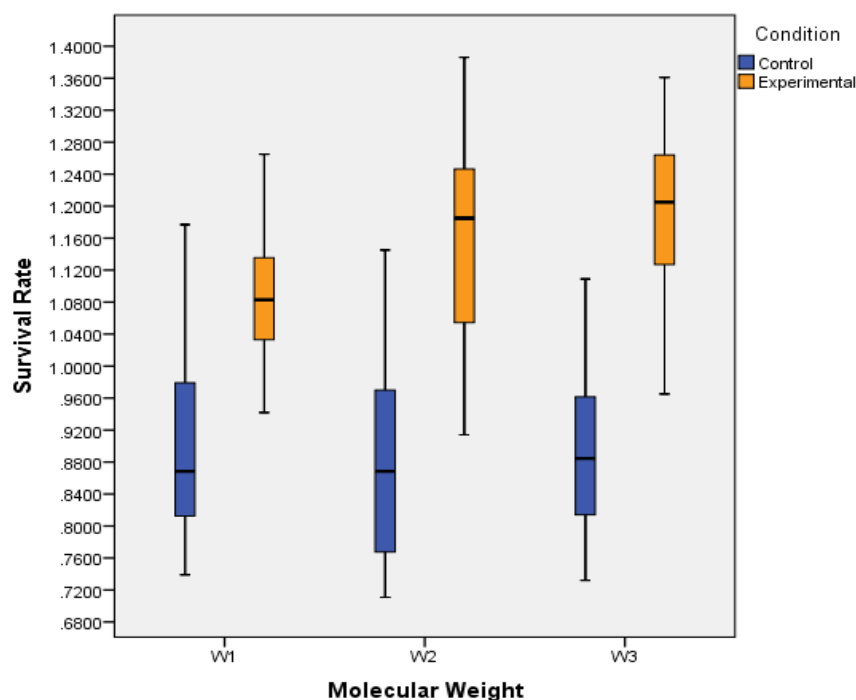


Figure 2: This figure shows the effect of different chitosan molecular weights on the survival rate of the 3T3 fibroblast cells. It shows the survival rate of each molecular weight (where: w1 = 50-90kDa, w2 = 190-310kDa, w3 = 310-375kDa) with their control. The survival rates were 108%, 118% and 120% respectively

The standard Balb/C mouse 3T3 fibroblast cells has extensively been used to give one a good indication of what could be expected, in general, as different cell-lines could differ in their cell survival rates [15-19].

Different degrees of de-acetylation (DDA) of chitosan is normally obtained by the treatment of chitin with alkali and a higher DDA is obtained by increasing the time and temperature, while the molecular weight (mw) of chitosan is dependent on the initial source of material which could be crab, fungi, shrimp etc. In this study chitosan with different molecular weights but the same degree of de-acetylation (75-85%) was investigated for their cytotoxicity on standard mouse 3T3 fibroblast cells. It was previously reported [20] that the higher the degree of de-acetylation of chitosan the better for the survival rate of the fibroblast cells and that the presence of chitosan improved the survival rate to levels higher than that of the control. In this study it was found that the higher the molecular weight of chitosan the better it is for the survival rates of the cells (Figure 2). From Figure 2 it can also be seen that the positive effect of the survival rate of the increasing molecular weights became less when the value for the medium molecular weight is compared to that of the high molecular weight (118% vs 120%, Figure 2). Therefore, it seems that the positive effect reaches a ceiling value when weight levels increased to the high molecular weight (310-275kDa). It was also reported [21] that molecular weight (mw) and DDA played an important role in the physicochemical and biological properties of chitosans. On the other hand Nunthanid [22] reported that chitosans with high mw have higher moisture adsorption and tensile strength than those with the same DDA but lower mw. Hidaka [23] reported that 94% DDA chitosan membranes gave minimal film degradation, mild inflammatory reaction and minimal osteogenesis while between 65 to 80% gave the opposite response.

In a previous study where the cytotoxicity of different degrees of de-acetylation degrees (40%, 70% and 87%) of chitosan on the 3T3 cell line was investigated it was found that from about a 70% de-acetylation degree towards the higher 87% an improvement on the cell survival rate was found [20]. For their high molecular weight (for the 87% DDA) a survival rate of 115% was reported which is close to the 120% (Figure 2) found in this study.

However, Hamilton [24] reported no relationship between the mw, DDA of chitosan, or growth of cells. While a similar report by the growth of keratinocytes was noted [25]. From the above it is concluded that there are controversial findings as far as the effect of molecular weights are concerned.

Conclusion

Not only improved the presence of chitosan the cell survival rate to a higher level than that of the control but the higher the molecular weight of chitosan the better the survival rate while a molecular weight of about 375kDa seems to near the highest possible positive effect.

Acknowledgement

Financial support was obtained from The University of the Western Cape and the SA Dental Association.

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