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Original Article

Rat Hepatocellular Primary Cells: A Cellular and Genetic Assessment of the Chitosan Nanoparticles-Induced Damage and Cytotoxicity

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Abstract

Chitosan (CH) is a non-toxic vital polymer that is derived naturally from chitin. Due to its anti-bacterial and anti-fungal properties, it has attracted researchers' attention. The anti-bacterial activity of 1-3 CH is ideal in an acidic medium due to its weak solubility at pH levels higher than 6.5. The type of CH and the degree of its polymerization affect its anti-microbial activity, as well as some of its other chemical and physical properties. The present study was conducted to investigate the damage induced by chitosan nanoparticles (CHNPs) at various concentrations on the cultured rat hepatic cells. The CHNPs were synthesized by the ionotropic gelation of CH with sodium tripolyphosphate anions. Hepatic cells were cultured from tissues freshly isolated from the liver of normal laboratory rats. Cells were allowed to reach a confluence level before the treatment with CHNPs. In total, five different concentrations of CHNPs were used, and cell cytotoxicity was evaluated using the MTT assay. The genetic expression of P2Y1, P2Y2, and P2Y4 purinergic receptors was evaluated on the cellular level using Qualitative Reverse Transcription Polymerase Chain Reaction technique. The primary culture of rat hepatic cells was thoroughly exposed to a range of CHNPs. Under normal conditions, the cells showed normal cellular morphology with clearly defined borders and normal nuclear structure. Apoptotic cellular damage was observed in the cultured hepatic cells when exposed to CHNPs. Moreover, irregular cellular morphology and heavy pigmentation were noticed in the hepatic cells when exposed to a high concentration of CHNPs. Purinergic receptor gene expression indicated an inflammatory response by an increased gene fold change post-exposure to CHNPs. This study concludes that CHNPs have a strong cytotoxic effect on the cultured rat hepatic cells. Overall, CHNPs showed an inhibitory response to hepatic cells evoking a purine receptor-mediated inflammatory response.

Keywords: Cell culture, Chitosan nanoparticles, Hepatic

1. Introduction

Chitosan (CH) is a polysaccharide derivative that is naturally derived from chitin (1). It has some immunoenhancing, antitumor, and antimicrobial activities. Additionally, it can be used as a long-term stabilizing agent for preventing the agglomeration of nanoparticles (NPs) (2). There is also a newly-developed strategy to utilize CH as a reducing agent for gold nanoparticles (AuNPs) production. Interestingly, CH-AuNPs were bactericidal against the antibiotic-resistant strains of *Staphylococcus aureus* and *Pseudomonas aeruginosa* (3). The *in vivo* and *in vitro* studies that deal with nanoparticle cytotoxicity have paradoxical findings and do not present adequate information about its effects. Alternative medicine carriers, such as polymeric NPs, have been offered as a solution to such issues due to their superior repeatability and stability characteristics than those of

liposomes. Solid and colloidal particles the dimensions of which range from 1 to 1,000 nm are known as NPs. They are syntheses of macromolecular components and can be employed as adjuvants in vaccinations or medicine transporters when the active agent is dissolved, adsorbed, entrapped, encapsulated, or chemically bonded. It is possible to inject NPs intravenously, as the smallest diameter of the capillaries is about 4 µm (4). Particles with a size of more than 100 nm are absorbed faster by the reticuloendothelial system (in the bone marrow, liver, spleen, and lung), while smaller particles have a longer circulation time. The particles with a negative charge are removed rapidly, compared to particles that have a positive or neutral charge (1, 5). Previous research findings proposed that syntheses hydrophilic NPs with a neutral charge can be applied to avoid phagocytosis by the phagocytic cells of the immune system, such as macrophages. Therefore, they can improve the therapeutic efficiency of laden medicine particles (5).

Chitosan NPs (CHNPs) are the most widely studied material and the least water-soluble polymers. They are non-toxic, biodegradable, biocompatible, and inexpensive. Moreover, they have a positive charge and show a promoting impact of absorption. These features make CH a very appealing substance as a carrier for medicine transmission (6).

Recently, there has been extensive research on estimating molecular signals that control adult stem cell proliferation. A study by Pearson et al. investigated several genes associated with the development of the retina in low vertebrates and how they respond to diverse molecular signals (7, 8). Extrinsic signaling actively controls cell fate and progenitor cell determination. Moreover, extrinsic cues, such as neurotransmitters, considerably influence progenitor cell development and fate (8). They also play an important role in cellular proliferation pathways, as well as internal regulators. Purinergic receptors can be identified in the germinal layer of embryogenesis at an early stage of development. Purinergic signaling, which is mediated by nucleotides, such as adenosine diphosphate and adenosine triphosphate (ATP), is important for embryogenesis (9). Purinergic receptors are spliced into two classes, namely P2Y and P2X receptors. P2Y are membrane-bound G protein receptors. (8). The activation of purinergic receptors leads to enhanced proliferation and additional neuronal development in olfactory epithelium cells (10). Previous studies investigated the role of purinergic signaling in cell cycle regulation in the ventricular zone of chicks. The researchers discovered an increase in $[Ca^{2+}]$ in the ventricular zone of the chick retina caused by the activation of purinergic receptors, which had a significant impact on the mitotic rate of the ventricular zone cells (6-8). Purinergic signaling is also involved in the regulation of different types of regional brain development. The ATP signaling has been found to influence developmental patterns in a variety of tissues. As previously stated by Neary, Kang (11), retinal development is mostly governed by the calcium ion waves elicited by the ATP receptor activity, increasing calcium ions that influence the rate of progenitor cell division. During the early stages of cochlea development, a small structure known as the kolliker' organ is present (5). Purinergic signaling is essential in the development of a variety of tissues. Purinergic receptors are found in a variety of embryonic mammalian organs, indicating their significant role in cell proliferation and development. Adult stem cell populations, including those present in the olfactory bulb, have been reported to be regulated and maintained by these receptors (10, 12). They were also found to be involved in the human epidermis at various stages of development.

This study was designed to investigate the damage induced by CHNPs at various concentrations on cultured rat hepatic cells.

2. Materials and Methods

2.1. Preparation of the Chitosan Nanoparticles

Chitosan (CAS Number: 9012-76-4) was obtained from Sigma Aldrich. The CHNPs were synthesized via the ionotropic gelation of CH with sodium

tripolyphosphate (STPP) anions. The solution of STPP has been prepared by the ddH2O at a 1 mg/mL concentration. The CHNPs were prepared with the drop-precise addition of STPP solution (CH/TPP=4:1) with magnetic stirring at 1,000 rpm at normal room temperature. The suspension was formed in similar conditions. The NPs were detached at 20,000 rpm by centrifugation at 14˚C for 30 min. Afterward, supernatants were discarded. Consequently, the particles were frozen, dried, and stored at 4˚C. Finally, frozen-dried NPs weight was calculated.

2.2. Scanning Electron Microscope Analysis

Field emission scanning was done with a scanning electron microscope (SEM) to observe the morphological features of the NPs. The spraying of the samples was conducted by 5-nm thick coated gold (magnification 25000×).

2.3. Culture and Maintenance of Primary Hepatic Cells

Rat liver tissue fragments were extracted under anesthesia. Tissues were microscopically dissected and incubated in 100 µl trypsin (0.25%) at 37˚C for 20 min. They were vigorously shaken and mechanically pipetted every 10 min. The dissociated tissue was layered over 500 µl Roswell Park Memorial Institute (RPMI) 1,640 with 10% fetal bovine serum solution and 1 µ of Penicillin/streptomycin (Capricorn, Germany) and was centrifuged at 3,000 rpm for 5 min. The supernatant was removed, and the pellet was resuspended in 500 µl RPMI 1,640, 10% FBS, as well as 1 µ of Penicillin/streptomycin (Capricorn, Germany), and 0.5 µl of Penicillin/streptomycin (Capricorn, Germany) was also added to the culture. Cells were put in 24 non-TC treated plates and cultured for 7 days.

2.4. MTT Assay

To determine cell viability by the MTT assay, the cells were cultured at 1×105 cells/mL in 96 well micro-titer plates in the RPMI medium. The cells were brooded overnight for attachment. Different concentrations of CHNP (2.5, 5.0, 10.0, 20.0, 40.0, and 80.0 µg/ mL) extracts were dissolved in the medium, added to the three replicates, and incubated for 48 h. Afterward, the cells were handled with MTT at 2 µg/mL concentration for 3 h. After the incubation period (37˚C, 5% CO_2), all the components were well aspirated, DMSO 1% was added to each well, and the absorbance was measured at 492 nm by the utilized microplate reader.

2.5. Quantitative Real-Time Polymerase Chain Reaction

This reaction was conducted by P2Y1, P2Y2, and P2Y4 receptor gene primers (purchased from the Integrated DNA Technologies IDT, U.S.), as listed in table 1.

The total Ribonucleic Acid was extracted from all organs and skin sites using the ExCellenCT Lysis Kit commercial purification system (abm-Applied Biological Materials, Canada) following the manufacturer's protocol. The Qualitative Reverse Transcription Polymerase Chain Reaction was also used. Gene fold changes were calculated using $-2^{\Delta\Delta CT}$, each reaction was repeated three times, and the mean threshold cycle value was taken to work out the $-2^{\Delta\Delta CT}$ (6, 10).

Table 1. P2Y1, P2Y2, and P2Y4 receptor gene primers

Primers	Left	Right	Product length (bp)
P2Y1	Ctgtgtggaccccattcttt	Tcgggacagtctccttctga	439
P2Y2	Gagcatcctcaccacctca	gctattccagggttccaggt	634
P2Y4	Gaagaagcagcagaacacca	caaggagtctgcactggtca	319

3. Results and Discussion

SEM examination of CHNPs shows small round particles, measuring between 40 and 50 nm (magnification 130,000×) (Figure 1).

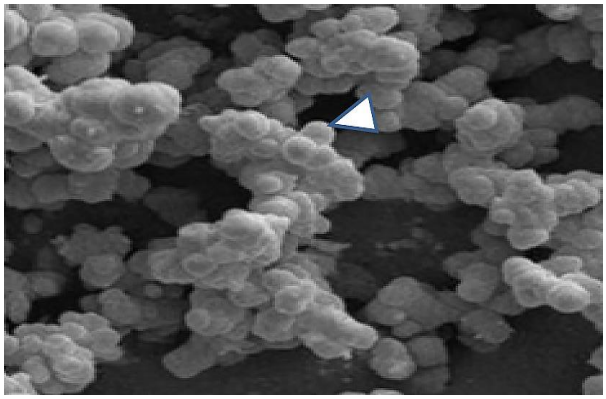

Figure 1. SEM examination of CHNPs

The recorded data showed that the control cells had minimal cell cytotoxicity in comparison with other concentrations. A sharp increase in rat hepatic cell cytotoxicity was noted after initial exposure to the lowest dose of CHNPs. However, the continuous rise in cell cytotoxicity was minimal among other concentrations.

The CHNPs were synthesized from chitin and were produced as NPs per SEM examination. The measurement of NPs ranging from 40-50 nm, as shown by many studies that followed the same procedure, was also confirmed in this study. The primary culture of rat hepatic cells was thoroughly exposed to a range of CHNPs, as shown in figure 2. Under normal conditions, the cells showed normal cellular morphology with clearly defined borders and normal nuclear structure. Apoptotic cellular damage was observed in cultured hepatic cells when exposed to CHNPs at a concentration of 2.5 10 µg/mL, as illustrated in figure 3. Irregular cellular morphology and heavy pigmentation were also observed in hepatic cells when exposed to a 5.0 µg/mL of CHNPs.

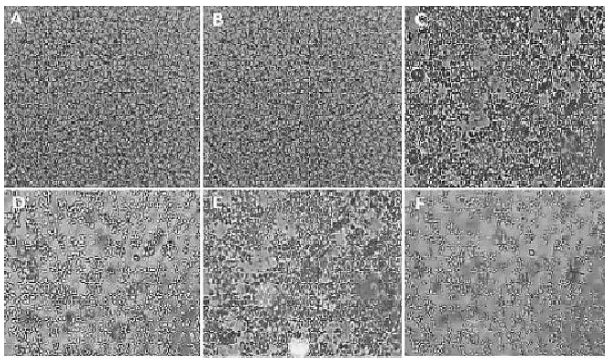

Figure 2. The primary culture of rat hepatic cells

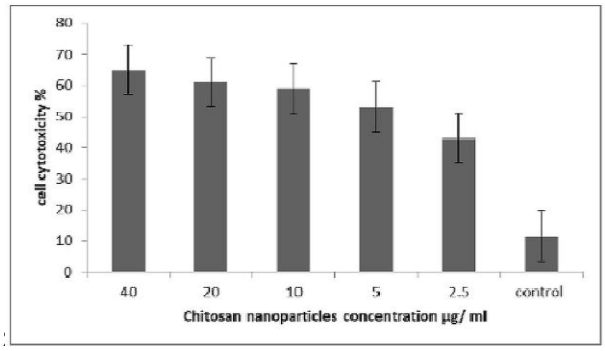

Figure 3. Cell death percentage is observed to increase with chitosan nanoparticle concentration

Purinergic receptors are highly involved in many cellular responses. In this study, it was demonstrated that the genetic expression of P2Y1, P2Y2, and P2Y4 purinergic receptors gradually increased as a result of induced cytotoxic effect due to exposure to CHNPs (Figure 4). The findings of the present study are similar to those demonstrated by Kandra and Kalangi (5), showing the role of purinergic signaling in the healthy and diseased liver. Many studies have revealed that purinergic signaling is activated during inflammatory response as a means of the restoration process to reduce inflammatory responses. Quantitative genetic examination of purine receptors in this study demonstrated the gradual increase in genetic overexpression as a means of correction mechanisms in hepatic cells (Figure 4). This is similar to the findings of a study by Li, Wang (6), which demonstrated the

reduction in cell proliferation in P2Y knockout animal models. Genetic overexpression of P2Y1 is mediated by ATP, which is shown to increase after cellular damage as a response to cellular restoration. In this study, P2Y1, P2Y2, and P2Y4 receptors were all observed to gradually increase due to cellular and histological damage. The concept demonstrated in the present study is consistent with several previous research findings (13).

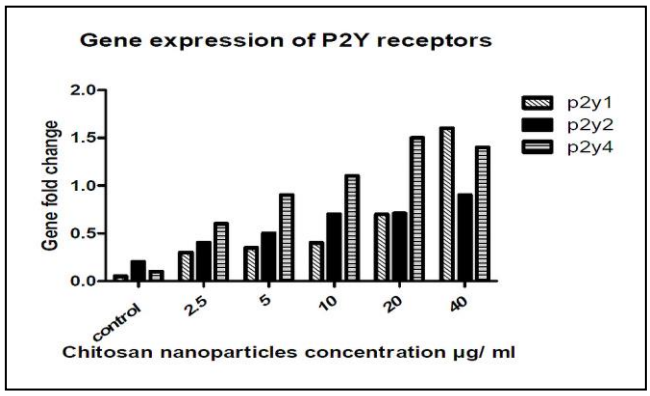

Figure 4. Difference in gene fold change of P2Y1, P2Y2, and P2Y4 receptors genes in primary hepatic cell suspension exposed to various chitosan nanoparticles concentrations, compared to normal hepatic cell suspension under normal conditions

This study concludes that CHNPs have a strong cytotoxic effect on cultured rat hepatic cells. Morphological appearance shows variable cell changes, including apoptotic changes, as well as nuclear damage. The cytotoxic effect is confirmed by the gradual increase in purinergic receptors gene expression indicating an inflammatory response by the increased gene fold change post-exposure to CHNPs. Overall, CHNPs showed an inhibitory response on hepatic cells evoking a purine receptor-mediated inflammatory response.

Authors' Contribution

All researchers involved in this study had an equal contribution.

Conflict of Interest

The authors declare that this research project was managed in the absence of any financial or commercial relations that could be interpreted as a likely discrepancy of interest.

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