

Review

TOXICITY OF CHITOSAN BASED PRODUCTS

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ABSTRACT

Chitosan is a natural polysaccharide with great biocompatibility and low toxicity. Most important factors that define its properties are the acetylation degree and molecular mass. This polysaccharide can be used in preventing or treating wound and burn infections due to its intrinsic antimicrobial properties, and also because of its ability to deliver extrinsic antimicrobial agents to wounds and burns. It can be used also as a slow-release drug-delivery vehicle for growth factors that help improving wound healing. Chitosan it is a natural polymer used in various application, from agriculture, cosmetic and food industry to medical and pharmaceutical fields. Over the past years, chitosan became an important tool in drug delivery therefore it is important to evaluate the safety profile of this biopolymer.

Keywords: chitosan toxicity, drug delivery, biocompatibility, cytotoxicity, natural polysaccharides

1. INTRODUCTION

Chitosan is one of the most used biopolymer in research fields and it is obtained from alkaline deacetylation of chitin [1, 2].

For many years now, chitosan represents a major scientific interest. This natural polysaccharide shows promise for safe use in biomedical applications. Being chemically versatile and also possessing many beneficial properties (biodegradability, biocompatibility, muco-adhesive and antimicrobial properties, antioxidant and hemostatic effect) it is considered a biologically compatible and possibly non-toxic material [3-5].

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Regarding hemostatic effect of chitosan, it is believed that it has the ability of activating macrophages and also causing cytokine stimulation, which has led to a great interest in wound healing applications [6-8].

Due to its high killing rate against microorganisms, particularly Gram-positive and Gram-negative bacteria and also because of the advantage of showing low toxicity towards mammalian cells, chitosan has attracted great attention from researchers [9].

2. SOURCE OF CHITOSAN

Marine organisms like shrimp, crabs, squid, and lobsters are the most important source of chitin. Present also in insects, mollusks and fungi, chitin is the most abundant biopolymer, after cellulose and lignin. Millions of tons of crustacean waste are generated every year from the seafood processing industry thus crustacean waste represents a renewable source of chitin [10].

Chitin is the precursor of chitosan and is a polysaccharide synthesized by numerous microorganisms and also higher plants. Chitinases from microorganisms, generally hydrolyze randomly N-acetyl- β -1,4-glucosaminide linkages, that means they are endochitinases [11, 12].

One of the biggest disadvantage of chitosan is the poor solubility at neutral pH, so in order to overcome this, scientist have developed various chitosan derivatives, by chemically modifying hydrophilic groups or by grafting on water soluble functional groups [13].

By improving water solubility of chitosan derivatives it was also enhanced the cationic nature of chitosan, which allowed a better interaction with antigens or cell membranes [14].

3. CHITOSAN TOXICITY

When it comes to chitosan toxicity, published studies reveal ambiguous information and various explanations that are not totally confirmed by the results. Some analysis of the toxicity of chitosan and chitosan derivatives show that regardless of molecular weight, toxicity of chitosan enhances with an increase in the acetylation degree. Another observations show that cytotoxicity is directly proportional to molecular mass [15].

Because of the already known beneficial properties, chitosan has become increasingly used as scaffold in drug delivery and tissue engineering applications [16].

Although there are many studies published, chitosan drug delivery products are not approved by the FDA, thereby very few biotech companies are using chitosan as raw material [17].

Interest in using chitosan as a pharmaceutical excipient is not new, however, this polysaccharide still does not appear to be present in any drug existing on the market. In drug delivery, excipients play a vital role, but developing new ones it is mostly a slow process which is related to cost and regulatory problems and also demonstrating that these kind of products are safe for human use [6, 17].

Still, there are reports that this polymer is under investigation for use in many pharmaceutical formulations including drug delivery [18]. Published studies have reported the use of chitosan as granulations, gels, coating agents [6].

For all that, chitosan reached human body not as a pharmaceutical excipient, but through its use as a dietary supplement. In addition, chitosan exists on the market as medical device on various forms and formats: from hemostatic dressing for the treatment of bleedings, to bandages and as a coating agent for contact lens [19]. In the food industry however, chitosan has been listed as a GRAS product (Generally Recognized As Safe) in the U.S. and furthermore, in Japan, Italy and Finland chitosan is recognized as food additive [20].

Most studies show that chitosan toxicity is related to degree of deacetylation and molecular weight [21].

Guangyuan et al showed that 50% acetylated chitosan was efficiently degraded by lysozyme. After 4 hours of incubation the polymeric solution has lost 66% of his viscosity. The degradation rate was dependent on the degree of acetylation, so the more acetylated form of chitosan was degraded faster [22].

3.1. *In vitro* toxicity

Chitosan nanoparticles may be an alternative that can facilitate drug delivery. Because they could pass from the gastrointestinal tract, alveolar sac or nasal cavity into the systemic circulation, they could cause some toxicity to the human body. There are some studies that reported the cytotoxic effect of chitosan nanoparticles [23].

It was observed that chitosan nanoparticles of 40 nm in size showed inhibitory effects on the proliferation of a tumor cell line and chitosan nanoparticles were more toxic than chitosan itself [24].

Other researchers have used live human cells in order to examine the cytotoxicity of chitosan nanoparticles. The results showed that they are able to penetrate the cell and thus reducing the cell viability, the proliferation and compromising the cell membrane [25, 26].

Beside lysozyme, which can degrade easily chitosan, there are three forms of human chitinase that were thoroughly studied and that showed hydrolytic activity against chitosan and its derivative: acidic mammalian chitinase (AMCase), di-N-acetylchitobiase, and chitotriosidase. They have been identified in plasma, liver and gastrointestinal tract [27].

Studies have revealed that degradation rate depend on MW and DD [28] thus a higher reaction rate was observed for degradation of high MW chitosan while low DD chitosan was degraded faster [12, 29].

Cytotoxicity of chitosan is dependent on the MW, DD, concentration of the polymer and some other unspecific factors. The half maximal inhibitory (IC₅₀) is approximately 0.2 – 2.0 mg/mL in most cell models [30].

It was reported that three commercialized types of chitosan with various DD and MW possessed different levels of adjuvant activity and these are caused not only by a single factor but rather a combination of multiple factors such as MW, DD, solubility and particle size [28].

Chitosan toxicity analysis have shown that toxicity enhances with the increase of the acetylation degree, regardless of molecular weight, but also it has been reported that chitosan toxicity depends directly on molecular mass [15, 31].

The results of experiments testing the cytotoxicity and genotoxicity of chitooligosaccharides upon lymphocytes revealed that even though these chitooligosaccharide

did not possess genetic toxicity, they have demonstrated a great cytotoxic effect which was dependent on molecular mass [32].

The information on cytotoxicity of chitosan derivatives differ dramatically and this may be explained by the fact that polysaccharide derivatives are very diverse and so are the cell lines on which the studies were performed. One hypothesis, presented by Ahmed [33] shows that low molecular weight chitosan derivatives with increased acetylation degree are more efficiently degraded.

3.2. *In vivo* toxicity

The most important aspect in the use of polymers, mainly chitosan, as drug delivery system, is the biodegradation or metabolic fate in the human body. It is important for chitosan to have a suitable molecular weight for renal clearance, or, if the polymer has a larger size, then it should undergo degradation, and so, it would become suitable for renal clearance [34].

When entering the vertebrate bodies, chitosan seems to accumulate mainly in the liver, but also kidney and stomach [35]. It is presumed that the liver is the most significant site of accumulation because this organ is the primary site of metabolism [17].

Some scientists believe that the rate of biodegradation of chitosan in living organisms is dependent on the degree of deacetylation (DD), the degradation rate decreases with the increasing of DD. It is possible that, if the studies are conducted given appropriate conditions and also adequate time, chitosan would be degraded enough to be excreted [5, 36, 37].

Chitosan may be hydrolyzed chemically by acids present in the stomach and when referring to enzymatic hydrolysis, chitosan can be degraded by breaking linkages like glucosamine–glucosamine, glucosamine–N-acetyl glucosamine and N-acetyl glucosamine–N-acetyl glucosamine [17].

There are enzymes existing in vertebrates that can degrade chitosan, most reported being lysozyme [38, 39] and also bacterial enzymes found in the colon. Moreover, scientists have identified eight human chitinases belonging to the glycoside hydrolase 18 family from which three of them have shown enzymatic activity on chitosan [40].

Strange is the fact that there were reported to be some proteases that can degrade chitosan films, but maybe more relevant to know is that scientists showed that chitosan can be digested by rat colonic and cecal bacterial enzymes and also porcine pancreatic enzymes [7].

It should be mentioned that the majority of enzymes used in assays that investigate the enzymatic activity are not from vertebrates. Therefore, taking into account that the specific activity of enzymes differ from species to species, it must be considered that there is a major variability between organisms and the way they interact with different substances [41].

Another study on determining chitosan nanoparticles toxicity was performed using the zebrafish embryo model. Researchers have exposed zebrafish embryos to chitosan nanoparticles for 96 hours and induced a dose-dependent inhibition of embryo by chitosan nanoparticles of different size. When comparing with the control group, they observed a significant decrease in hatching rate at 20 mg/L and 40 mg/L chitosan nanoparticles of 200 nm. In addition, they detected an increased mortality rate among zebrafish embryos, therefore at high concentrations (30 mg/L for 200 nm nanoparticles and 40 mg/L for 340 nm nanoparticles), chitosan nanoparticles had toxic effect, leading to the death of embryos within 96 hours of exposure. The surviving zebrafish embryos from the 5 mg/L group (200 nm

nanoparticles) showed signs of malformations. The rate of malformations seems to increase when increasing chitosan nanoparticles concentration. Interesting was that the embryos treated with chitosan nanoparticles of 340 nm size showed decreased malformations rate when compared with the 200 nm particles treated group. Therefore, smaller chitosan particles showed higher toxic effect on zebrafish embryos [42].

Lagarto performed acute toxicity studies in order to assess the safety of chitosan and chitosan acid salts from *Panurilus argus* lobster and observed no signs of toxicity during the experimental period and concluded that the approximate lethal doses of chitosan are higher than 2000 mg/kg in female rats. In addition, no major changes were observed regarding the body weight of control versus chitosan treatment groups. Macroscopic examinations of organs showed no abnormalities in treated rats [43].

Signs of toxicity or even mortality were not observed neither in the case of chitosan repeated oral dose toxicity. Instead, erythrocyte count was increased at a dose of chitosan of 300 mg/kg/day for males and at 1000 mg/kg/day for females. Apparently no other biochemical parameters were affected by chitosan doses [43].

Some studies reported similar cytotoxic effects of different chitosan derivatives [44, 45]. Others studies have shown good tolerance and safety performance of chitosan products administered orally [46]. Baldrick performed *in vivo* tests on rats and concluded that very few side effects appeared both in mouse and rat models after oral intake of chitosan (1 – 15 g/kg/day for 3 months) [6].

In addition, there were no relevant clinical signs found in human volunteers after taking oral doses of chitosan up to 6.75 g/day [44].

Villacis reported that people with shrimp allergies developed no allergic reaction after oral administration of shellfish derived glucosamine [47].

All these many studies and results suggest that chitosan may be a possible biocompatible and biodegradable polysaccharide that exhibits no, or minor toxicity, and can become a potential safe pharmaceutical material [6, 44].

3.3. Toxicity against microorganisms

Although *in vivo* tests have shown low toxicity of chitosan, this polymer and its derivatives seem to be toxic to bacteria [48-52], fungi [53-56] and parasites [57, 58].

Researchers have concluded that antimicrobial effects of chitosans depend mainly on molecular weight, small molecular weight chitosan being more toxic than larger chains against microorganisms [9, 59, 60]. The antimicrobial properties of chitosan represent a great advantage in treating infectious diseases [9, 61, 62].

Chitosan can be used in preventing or treating wound and burn infections because of its intrinsic antimicrobial properties, and due to its ability to deliver extrinsic antimicrobial agents to wounds and burns in order to protect the wounds from microorganisms infections [63].

Another useful advantage of chitosan is the ability of being a slow-release drug-delivery vehicle for growth factors that can help by accelerating wound healing processes. The large number of published papers in this area suggests that chitosan will continue to play an important role in the management of wounds and burns [9].

Interactions between different pathogenic microorganisms and chitosan were investigated by Andres [62]. Several bacterial strains as *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis* and *Staphylococcus saprophyticus* were used for mortality constant rate determination. A hypothesis regarding antibacterial mechanism was the influence of free amino groups present in chitosan on disruption of cell wall.

Comparative studies were realized on antimicrobial activity of chitosans and chitosan oligomers against Gram-negative and Gram-positive bacteria [64]. The oligomers of chitosan showed low antimicrobial activity while chitosans inhibited growth of most bacteria tested. Likewise 0.1% chitosan showed antimicrobial activity on Gram positive bacteria. 1% acetic acid used as a solvent for preparation of chitosan solution inhibits almost all tested bacteria growth, except lactic acid bacteria [65].

Muzzarelli [66] highlighted the antibacterial efficiency of N-carboxybutyl chitosan obtained from crustacean chitosan with 73% DD, against different strains of Gram positive and Gram negative pathogenic bacteria. In this study was showed that N-carboxybutyl chitosan was active against Gram positive bacteria and *Candida spp.* Electron microscopy experiments proved that N-carboxybutyl chitosan decomposed external part of the cell wall in *Staphylococci* and duplication has also been affected. An abnormally expanded periplasmic space was detected close to the N-carboxybutyl chitosan pads in Gram –negative microorganisms. N-carboxybutyl chitosan pads affected also cell wall of *Candida albicans* strains. Intracellular structures changed their distributions or characteristics [67].

In another study, Raafat [68] detected antibacterial activity of chitosan dose-dependent. However, for *Staphylococcus simulans*, chitosan treatment showed that the cells membrane and cell wall remained intact. For *Staphylococcus aureus* SG511 the treatment with chitosan leads to changes in expression profile of genes in charged with autolysis, regulation of stress and metabolism.

4. CONCLUSIONS

Considering those many factors that influence *in vivo* and *in vitro* toxicity (MW and DD) systematic studies, regarding various chitosans and chitosan based products, should be performed. Describing chitosan structure and correlating this with safety profile would provide data that will help regulatory scientists to understand the toxicity or non-toxicity of chitosan and chitosan derivatives.

Amongst MW and DD, there are many other factors to consider that influence the toxicity: salt form, source, purity, polydispersity.

Until now, studies have shown low toxicity of chitosan, ($\mu\text{g/mL}$), but when it comes to chitosan derivatives, the modification will produce new chemical properties, so the studies should be performed individually, taking into account each modification [17].

To ensure chitosan safety, scientist must eliminate protein, metal or other contaminants that could cause potential toxic effects. There are enzymes that show activity against chitosan, at least *in vitro*, but when it comes to derivatives it may be more difficult to generalize or to extrapolate the conclusions, because they could give indigestible molecules. It is necessary for these kind of compounds to be small enough to be clinically viable and to be renally excreted [17].

Amongst purity, MW and DD, researchers should be aware of the physiochemical and biological properties of chitosan, the properties of raw material, the biological source and manufacturing procedure.

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