CYTOTOXIC EFFECT AND ANTIMICROBIAL ACTIVITY OF CHITOSAN NANOPARTICLES AND HAFNIUM METAL BASED COMPOSITE: TWO SIDES OF THE SAME COIN — AN IN VITRO STUDY

1.ABSTRACT

In recent years, progress has been made to treat the loss or failure of an organ or bone tissue in form of organ transplantation, surgical reconstruction and the use of artificial prostheses.Chitosan (CTS) is a biocompatible polymer that has been widely researched for tissue engineering purposes. It has demonstrated a significant role in bone tissue engineering in the last two decades. Being a natural polymer obtained from chitin, a major component of crustacean exoskeleton, it has varied uses. Lately, attention has been given to chitosan composite materials due to its minimal foreign body reactions, antibacterial nature, biocompatibility, biodegradability, and the ability to be moulded into various shapes and forms .It can be used as porous structures, suitable for cell ingrowth and osteoconduction.The aim of this research was to assess biocompatibility of a chitosan nanoparticle and hafnium metal based composite and project its use for bone tissue engineering.In the present study we have prepared chitosan nanoparticles and its based hafnium composite and it was analysed for its cytotoxic effect using brine shrimp lethality assay and antimicrobial activity using disc diffusion method.

Keywords: Chitosan, hafnium , composite, cytotoxic effect, antimicrobial activity, brine shrimp lethality assay

2.INTRODUCTION

Research on biomaterials for dental implants and bone substitutes have expanded considerably over the last few decades [1–3] . The establishment of a load bearing biomaterial must be integrated with natural bone. Biocompatibility, osteoconductivity, high porosity and biomechanical compatibility, are essential criteria the implanted biomaterial should possess [3–5]. The best bioactive biomaterials in bone tissue engineering, renowned for their excellent biocompatibility with the human body environment include Chitosan (CTS) and hydroxyapatite (HAp) [6–8].

Chitosan is a biocompatible polymer that has been researched upon for tissue engineering objectives [9–12]. The first discovery of chitosan dates back to the middle of the eighteenth century, but the compound did not reach its fame until the 1930s, when its crystalline structure was discovered [13–15]. Though the compound was originally used only in limited applications, chitosan and chitosan-based composites are currently used more diversely in different fields including water treatment, cosmetics, food, paper and textile industries, agriculture, photography products, fuel cells and batteries, detergents, gene therapy, cancer therapy, drug and vaccine delivery and biotechnology [11,16–20].

Hafnium is a passive metal with a number of interesting properties, such as high ductility and strength, as well as resistance to corrosion and mechanical damage [21–23]. However, the behaviour of hafnium in the biological environment has not been studied in great depth. Thus, further studies of hafnium coating under biological conditions are needed in order to determine the suitability of this material, for biomedical applications. On the other hand, composites demonstrate tailored physical, biological and mechanical properties as well as expectable degradation behaviour [24–26]. The apt selection of a particular composite for a given application demands a thorough understanding of pertinent cells and/or biocompatible response.

Previously our department has published extensive research on various aspects of prosthetic dentistry [27–37], this vast research experience has inspired us for the present investigation. We have prepared chitosan nanoparticles and its based hafnium composite to evaluate the benefits of the new combination. This novel composite was prepared and analysed for its cytotoxic effect using brine shrimp lethality assay and antimicrobial activity using disc diffusion method.

3.MATERIALS

3.1.Synthesis of Chitosan Nanoparticles

Raw materials for the chitosan preparation were chitosan nanoparticle, distilled water, glacial acetic acid and hafnium metal particles (Figure 1). 500 mg of chitosan dissolved in 49.5 mL of double distilled water. 0.5 mL of glacial acetic acid was added to this solution (Fig 2a). A 500 mg of hafnium metal was added to this preparation and kept in a magnetic stirrer for 24 hours for the preparation of composite (Figure 2 a, b and c).

Figure 1: Chitosan preparation

Figure 2 : CTS- hafnium composite preparation;

a : preparation 500 mg of chitosan dissolved in 49.5 mL of double distilled water and 0.5 mL of glacial acetic acid added to this solution

b: 500 mg of hafnium metal

c: 500 mg of hafnium metal added to this preparation and kept in magnetic stirrer for 24 hours for the preparation of composite

3.2.Preparation of test organism

Brine shrimp eggs were collected from sla®, India were used as the test organism [38–42]. Artificial seawater was prepared by Filtering and dissolving 36 g of sea salt in 1 l of distilled water for hatching the shrimp eggs . The seawater was put in a small plastic container (hatching chamber) with a partition for dark (covered) and light areas. Shrimp eggs were added into the dark side of the chamber while the lamp above the other side (light) will attract the hatched shrimp. Two days were allowed for the shrimp to hatch and mature as nauplii (larva). After 2 days, when the shrimp larvae are ready, 5 mL of the artificial seawater and different concentrations of composite viz. 5, 10, 20, 30, and 50 µg/mL.

3.3. Brine Shrimp Lethality Assay (BSLA) for the studied CTS-hafnium composite

Ten nauplii were used in each test. Three replications were used for each concentration and the blank control was always included. Control groups were used in cytotoxicity study to validate the test method and ensure that the results obtained were only due to the activity of the test agent and the effects of the other possible factors were nullified. After 24 hours, using a dissection microscope, the number of surviving shrimps was counted and recorded [38–42].

3.4. Antimicrobial activity for the studied CTS-hafnium composite

The antimicrobial activity was tested using the agar disc diffusion method [43–46]. If the test sample possesses antimicrobial activity, the bacteria is killed or growth is hampered and there will be a clear area around the wafer where the bacteria have not grown enough to be visible This is referred to as zone of inhibition and in this study 3 concentrations of the CTS-hafnium composite (50 ,100 and 150µg/mL)were used in a single disc for 2 most common oral microbiota viz, Streptococcus mutans and lactobacillus. The distance from the centre of each concentration of CTS-hafnium composite is measured to determine its antimicrobial potential. Inhibition produced by the test sample is compared with that produced by known concentration of a reference antibiotic compound.

4.RESULTS AND DISCUSSION

4.1.Cytotoxicity

Cytotoxic Effect from the brine shrimp lethality test done it is noted that on the 1st day five of nauplius survived, while on day 2 it got decreased to three nauplius, and on day 3 only one nauplii remained to survive (Figure 3).

Figure 3: Image showing the petri dishes with brine shrimp nauplii in various concentrations (5, 10, 20, 30, 50 µg/mL)

The mean of three replications used for each concentration viz, 5 µg/mL (3.67 ± 0.58), 10 µg/mL (1.33 ± 0.58), 20 µg/mL (1 ± 0.00), 30 µg/mL (1 ± 0.00), 50 µg/mL (0 ± 0.00) and the blank control (10 ± 0.00)was tabulated (Table 1).

Table 1: Table showing the Mean ± S.D of number of surviving shrimps for each concentration (5, 10, 20, 30, 50 µg/mL) and the blank control

GROUPS	CONCENTRATIONS OF CTS-HAFNIUM AND NEUTRAL	MEAN ± S.D
CONTROL	Neutral	10 ± 0.00
	5 µg/mL	3.67 ± 0.58
TEST	10 µg/mL	1.33 ± 0.58
	20 µg/mL	1 ± 0.00
	30 µg/mL	1 ± 0.00
	50 µg/mL	0 ± 0.00

As the concentration of the nanoparticles increased, the toxicity got decreased and nauplius survived. When the concentration of nanoparticles decreased, the toxicity increased, and nauplius died.. Hence, from the current study, it is noted that, as we used less concentration it caused only half the amount of toxicity (Figure 4). There was a significant difference between and within the concentrations used ($p<0.01$), when One way ANOVA statistical analysis (IBM SPSS Statistics 20®) was performed (Table 2). Hence, if the concentration is above 50%, it can be used for biomedical applications.

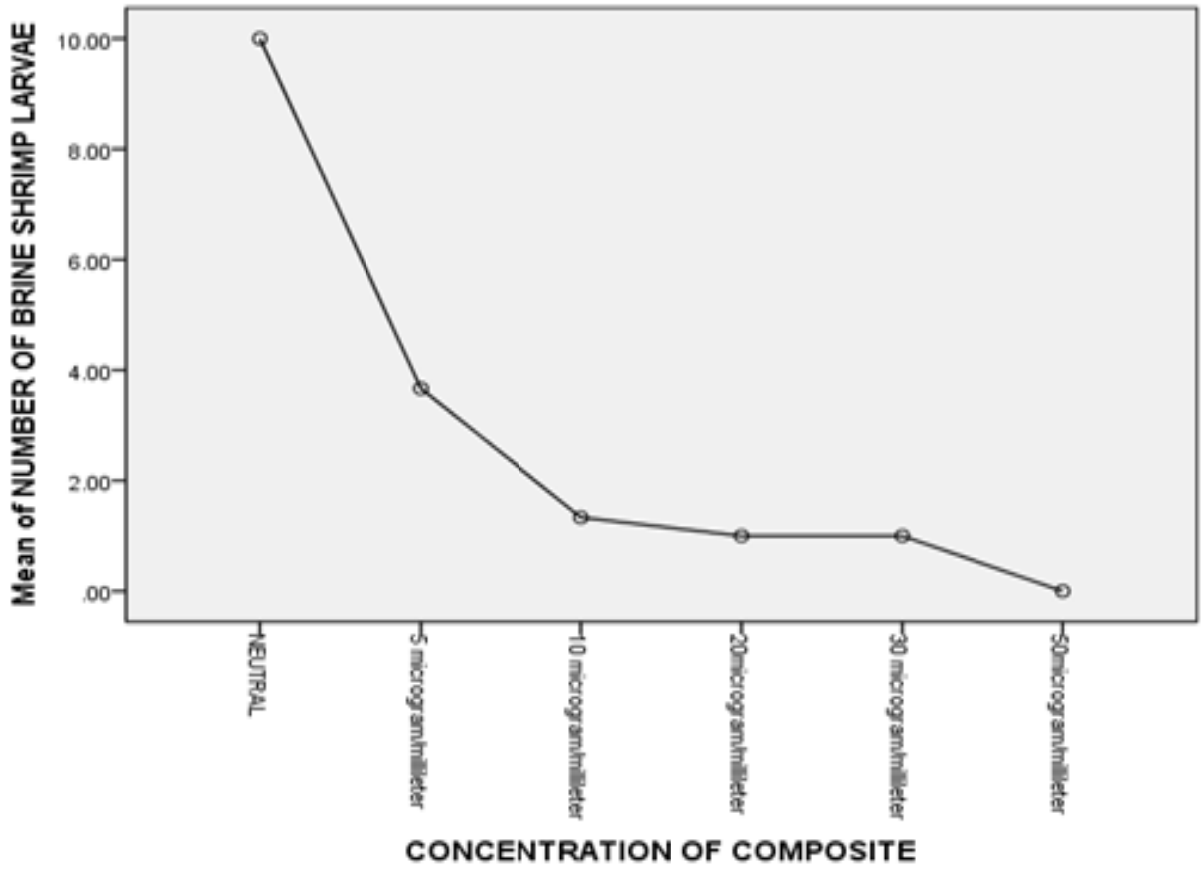

Figure 4: Graph showing number of brine shrimp larvae present at different concentrations of the composite solution viz. 5, 10, 20, 30, and 50 µg/mL

Table 2: Table showing the significant difference between and within the concentrations used (One way ANOVA, p<0.01)

NUMBER OF BRINE SHRIMP LARVAE

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	207.167	5	41.433	372.900	.000
Within Groups	1.333	12	.111		
Total	208.500	17			

4.2.Antimicrobial Activity

The mean measured distance from the centre for each concentration of CTS-hanium composite for S.mutans bacteria (Figure 5) and lactobacillus bacteria were noted (Figure 6). The zone of inhibition values measured for S.mutans for each concentration were 31mm, 13mm, 14mm, 16mm and for Lactobacillus the values were 28mm , 13mm, 16mm, 17mm for reference antibiotics, 50,100 and 150 µg/mL respectively.

Figure 5: Figure showing the zone of inhibition of the different concentrations (50 ,100 and 150µg/mL) of CTS-hafnium composite and the reference antibiotic for S.mutans bacteria

Figure 6: Figure showing the zone of inhibition of the different concentrations (50 ,100 and 150µg/mL) of CTS-hafnium composite and the reference antibiotic for lactobacillus bacteria

Table 3: Table showing the Mean of distance of zone of inhibition from the centre of each concentration (50 ,100 and 150µg/mL) of CTS-hafnium composite and the reference antibiotic control

		DISTANCE OF ZONE OF INHIBITION (in mm)	
GROUPS	**CONCENTRATIONS OF CTS-HAFNIUM AND REFERENCE ANTIBIOTIC**	***Streptococcus mutans***	***Lactobacillus* sp**
CONTROL	ANTIBIOTIC	13	13
TEST	50 µg/mL	14	16
	100 µg/mL	16	17
	150 µg/mL	31	28

The current study we have prepared chitosan nanoparticles and its based hafnium composite and it was analysed for its cytotoxic effect using brine shrimp lethality assay. The studied composite had antimicrobial activity but was cytotoxic at higher concentrations.CTS with various composites can be potential bone implant materials with good osteoconductivity, osteoinductive and osteogenic properties. The structural, mechanical, chemical interaction and in vitro study of CTS, with various composite preparations have been carried out for other industrial purposes [47–50]. Although many CTS composite materials have been developed, questions persist with their biocompatible properties. Hence, much research is in progress to address the gap in the development of these properties.

The brine shrimp lethality assay (BLSA) is proven as a useful tool for preliminary assessment of toxicity [51–54]. It is a comprehensive bioassay for the bioactive compounds of natural and synthetic origin [55–57] . It has advantages of being rapid (24 hours), inexpensive, and simple (e.g., no aseptic techniques are required). It easily employs a large number of organisms for statistical validation and requires no special equipment and a relatively small amount of sample (2-20 mg or less). Moreover it does not require animal serum as is needed for other cytotoxicity tests.

5.CONCLUSIONS

Composite Chitosan-based materials have been found to have a predominant role in bone tissue engineering in recent years. Limited evidence exists with substantial research work to address the cytotoxicity of CTS-metal composites. The current study substantiates the antimicrobial activity and highlights the possible cytotoxicity of the CTS and hafnium composite. Though challenges still exist, the addition of hafnium metal to improve the properties of CTS would surely support and stimulate the function of natural bone. The development of research on the efficacy of CTS-hafnium composite may open great possibilities for future bone tissue engineering and hence should be explored for osteoblastic activity

6.REFERENCES

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