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Effect of Chitosan on Physical status of Mammals and *in vivo* Antimicrobial Effect on Pathogenic Bacteria

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Abstract: *The shells as raw materials were extracted from shrimp (Metapenous Monoceros) to extract chitosan in this experiment. Demineralizations process was carried out by 4% HCl at room temperature in the ratio of 1:14(w/v). The deproteinization process was initiated by 5% NaOH at 90°C for 24 hours with a solvent to solid ratio of 12:1(v/w). Removal of acetyl groups from the chitin was achieved by using 70% NaOH solution with a solid to solvent ratio of 1:14 (w/v) at room temperature for 72 hours. Extracted chitosan was soluble in 1% acetic acid. Rabbit were treated with extracted chitosan to determine in vivo effects. 2.74g of chitosan was up taken by each rabbits per day. 80gm of standard food was given to each rabbit during experiments. Physical status was observed and stool samples were collected to observe antimicrobial before chitosan treatment and after chitosan treatment. Presence of pathogenic bacteria was detected by streaking loop-full sample on three specific media. Coliform detection was initiated by MacConkey Agar. Salmonella-Shigella detection was initiated by S-S Agar. Pseudomonas detection was initiated by Cetrimide Agar. Before chitosan treatment 2300g body weight was found, stool status was rigid and physical movement was normal. After chitosan treatment a continuous bacterial growth in the 7^{th}, 10^{th}, 15^{th} day but none of Coliform and Salmonella-Shigella growth occurred that indicates a ratio of growth of normal microbial flora. The physical status of the rabbit was changed during chitosan treatment. Continuous reduction in weight and looseness of stool was observed, but physical movement was normal.*

Keywords: *Bangladesh, Shrimp, Chitosan, Lipid Reduction, Antimicrobial Effect*

I. Introduction

Chitosan is a ß-1, 4-linked polymer of glucosamine (2-amino-2-deoxy-ß-D-glucose) and lesser amounts of N-acetylglucosamine. Chitosan is a natural, cationic, hydrophilic, nontoxic, biocompatible and biodegradable polysaccharide suitable for application in pharmaceutical technology. It is formed by deacetylation of chitin which is present in the exoskeleton of crustaceans like crab, shrimp lobster, krill, and squid etc is a (poly-N-acetylglucosamine) (Allan, et al. 1978). The degree of deacetylation (%DA) can be determined by NMR spectroscopy, and the %DA in commercial chitosan is in the range 60-100 % (Ashford, et al. 1977). It is one of the most abundant bio-degradable materials in the world. The distinction between chitin and chitosan is somewhat blurred. Some mention, when chitin is more than 50 percent deacetylated is chitosan, whereas others define chitosan as soluble in 1 percent acetic acid, chitin being insoluble. Most commercially available chitosan preparations are more than 85 percent deacetylated, and have molecular weights between 100kDa and 1000kDa. Chitosan is nontoxic and non-allergenic, so the body does not reject these compounds as foreign invaders. Biocompatibility, biodegradability and absorption properties of chitosan and its derivatives are much higher than synthetically substituted cellulose. (Peter, 1995)

An important property of chitosan is its positive charge in acidic solution. This is due to the presence of primary amines on the molecule that bind protons. The amino group in chitosan has a pKa value of approximately ~6.5; thus, chitosan is positively charged and is soluble in acidic pH to neutral solution with a charge density depending on pH and the %DA value (Rinaudo, 1999). This makes chitosan a bio-adhesive which readily binds to any negatively charged surfaces. The bio-adhesive nature of chitosan can be attributed to the same type of ionic interactions with mucosal membrane components. As chitosan is soluble in acidic pH, hydrogels are also formed in the presence of negative charged drugs or polyanions and represent a sustained drug release form. The maximum soluble concentration varies with different chitosan concentration, but is usually around 10-20g /L of chitosan. Chitosan solutions have good film-forming property and are therefore potentially useful in gels and coatings (Vårum, 1994). As chitosan is the second most abundant dietary fiber after cellulose and of low cost, chitosan and its derivatives have been used at least at the experimental level in a

diverse range of applications. In this study we will observe the physical status of mammals after chitosan treatment and antimicrobial activity of chitosan against pathogenic bacteria in mammals (rabbit).

II. Materials and Methods

2.1 Raw Materials Collection

The shrimps (*Metapeuous monoceros*) were collected from Khulna shrimp industry, Khulna, Bangladesh (Lat. 22.8167° N, Long. 89.5500° E) and the shell and operculum are removed from the animal. The shrimps exoskeletons collected are placed in Ziploc bags and refrigerated overnight.

2.2 Raw Materials Processing

Approximately 1500 grams of crushed shrimp's exoskeletons wet samples were placed on foil paper and measured using a balance. The shrimp exoskeletons were crushed into smaller pieces using a meat tenderizer. The samples were oven-dried for 4 consecutive days at 65°C until constant weight. The dry weights of the samples were determined to be 1269 grams. The obtained shrimp is made into 4 equal parts for efficient material handling. (Toan, 2009).

2.3 Extraction of Chitin and Chitosan

The chitin and chitosan sequence involves washing of crushed exoskeletons. Crushed shrimps exoskeletons were placed in 1000 ml beakers and soaked in boiling sodium hydroxide (2 and 4% w/v) for one hour in order to dissolve the proteins and sugars thus isolating the crude chitin. 4% NaOH is used for chitin preparation, concentration used by the scientists at the Sonat Corporation (Lertsutthiwong et al., 2002).
After the samples are boiled in the sodium hydroxide, the beakers containing the shrimp shell samples are removed from the hot plate, and allowed to cool for 30 minutes at room temperature. The exoskeletons are then further crushed to pieces of 0.5-5.0 mm using a meat tenderizer.

2.4 Demineralization

The shells were suspended in 4% HCl at room temperature in the ratio of 1:14(w/v). After 36 hours, the shells were quite squashy and were rinsed with water to remove acid and calcium chloride.

2.5 Deproteinization

The demineralized shells were then treated with 5% NaOH at 90°C for 24 hours with a solvent to solid ratio of 12:1(v/w). The residue was then collected and washed to neutrality in running tap water. Then it was dried in sun and the product is chitin.

2.6 Deacetylation

Removal of acetyl groups from the chitin was achieved by using 70% NaOH solution with a solid to solvent ratio of 1:14 (w/v) at room temperature for 72 hours. The mixture was stirred after some times for homogenous reaction. The resulting chitosan were washed to neutrality in running tap water and rinsed with distilled water. Then filtered and dried in sun.

2.7 Purification of Chitosan

We know that chitosan dissolve in 1% acetic acid but chitin cannot. Now the test tube containing the most white colored 5g chitosan mixture was dissolved in 2.5% solution of acetic acid to make a 2% solution of chitosan. The solution was stirred properly and rotated thoroughly in a hand mortar to dissolve all the chitosan in it. The remaining precipitate that was observed in it was the chitin that could not dissolve in acetic acid.
The obtained chitosan has to be purified to make it suitable for the pharmaceutical use. The purification process was designed in two steps:

1. Removal of insoluble with filtration
2. Re-precipitation of chitosan with 1 N NaOH

2.8 Removal of insoluble with filtration

1 mg/ml chitosan acetic acid 1% (v/v) solution is prepared by a magnetic stirrer until a homogenous solution is obtained. The insoluble particles were removed by filtration through Whatman filter paper 22μm.

2.9 Reprecipitation of Chitosan with 1N NaOH

Chitosan was precipitated from filtered chitosan solution by titration with 1 N NaOH until pH value of 8.5. The chitosan obtained is washed several times with distilled water by centrifuging at 8,000 to 10,000 xg.

2.10 Chitosan Treatment on Mammals (Rabbit)

Three healthy, mature, susceptible rabbits were taken under treatment during this experiment. These animals were examined under three observations:

a) Antimicrobial activity.
b) Condition of normal microbial flora.
c) Physical condition.

These observations were initiated in two phases: Before chitosan treatment and after chitosan treatment (2^{nd}, 5^{th}, 7^{th}, 10^{th}, 15^{th} day of treatment).

2.11 Chitosan Introduction in Mammals

2.74g of chitosan was up taken by each rabbits per day. 80gm of standard food was given to each rabbit during experiments.

2.12 Stool Sample Collection

Stool sample were collected to observe antimicrobial activity and condition of normal microbial flora before chitosan treatment and after chitosan treatment (2^{nd}, 5^{th}, 7^{th}, 10^{th}, 15^{th} day of treatment).

2.13 Stool Sample Preparation

Stool sample were prepared by serial dilution method. 10gm of sample was dissolved with vortex in 90ml of saline water and labeled as 10^{-1}. Others test tubes were labeled as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}. 1ml of sample from 10^{-1} dilution was transferred into the 10^{-2} test tube with 9ml saline solution by using a sterile 1ml pipette.

Each bottle contains 90ml of sterile water. This first bottle now has a 1:100 dilution. 1ml of sample from 10^{-2} test tube was transferred into the 10^{-3} test tube by using a new sterile 1ml pipette and shacked with vortex. Then 1ml of sample from 10^{-3} test tube was transferred into the 10^{-4} test tube by using a new sterile 1ml pipette and shacked with vortex. This process was continued for test tube labeled as 10^{-5}, 10^{-6} and 10^{-7}. Then 1ml sample from each tube was used for pour plate method with TSA.

2.14 Determination of the Presence of Pathogenic Bacteria

Presence of pathogenic bacteria was detected by streaking loop-full sample on three specific media.

a) Coliform detection was initiated by MacConkey Agar,
b) *Salmonella-Shigella* detection was initiated by S-S Agar and
c) *Pseudomonas* detection was initiated by Cetrimide Agar.

2.15 Media Preparation

The required amount of dehydrated medium, as stated by the manufacturer, was carefully weighed into clean conical flasks and appropriate volumes of distilled water were added. The flasks were shaken and gently heated to dissolve the medium completely, and then autoclaved at 121°C under 15lb pressure for 15 min. They were cooled to about 45°C and approximately 15 ml of medium was poured into each sterile Petri dish used in the experiment, to solidify.

All plates were checked for any contamination before use by overnight incubation; plates with any visible colonies were then discarded and another such plate was used.

III. Results

3.1 Physical Observation before Chitosan Treatment

Table 1: Physical observation (before chitosan treatment)

Parameters	Rabbit-1	Rabbit-2	Rabbit-3
Body Weight	2300gm	2100gm	2500gm
Stool Status	Rigid	Rigid	Rigid
Physical Movement	Normal	Normal	Normal

3.2 Microbiological Analysis before Chitosan Treatment

Table 2: Growth observation

Media	Growth	Remark
MacConkey Agar	+	Coliform Detected
S-S Agar	+	*Salmonella-Shigella* Detected
Cetrimide Agar	-	N/A

Table 3: Growth on specific media

Pathogens	Rabbit-1	Rabbit-2	Rabbit-3
Coliform	Present	Present	Present
Salmonella-Shigella	Present	Present	Present
Pseudomonas	Absent	Absent	Absent

3.3 Physical Observation after Chitosan Treatment

Table 4: Body weight after chitosan treatment

Day	Rabbit-1		Rabbit-2		Rabbit-3	
	Weight	**Reduction (%)**	**Weight**	**Reduction (%)**	**Weight**	**Reduction (%)**
2nd	2300gm	0%	2100gm	0%	2500gm	0%
5th	~2300gm	0%	~2100gm	0%	~2500gm	0%
7th	~2200gm	4.35%	~2000gm	4.76%	~2400gm	4%
10th	2200gm	4.35%	~1900gm	9.5%	2400gm	4%
15th	2100gm	8.7%	1800gm	14%	2300gm	8%

Table 5: Stool status after chitosan treatment

Day	Rabbit-1	Rabbit-2	Rabbit-3
2nd	Rigid	Rigid	Rigid
5th	Loose	Loose	Loose
7th	Loose	Loose	Loose
10th	Loose	Loose	Loose
15th	Loose	Loose	Loose

Table 6: Physical movement after chitosan treatment

Day	Rabbit-1	Rabbit-2	Rabbit-3
2nd	Normal	Normal	Normal
5th	Normal	Normal	Normal
7th	Normal	Normal	Normal
10th	Normal	Normal	Normal
15th	Normal	Normal	Normal

3.3 Microbiological Analysis after Chitosan Treatment

Table 7: Coliform detection after chitosan treatment

Day	Rabbit-1	Rabbit-2	Rabbit-3
2nd	Present	Present	Present
5th	Present	Present	Present
7th	Absent	Absent	Absent
10th	Absent	Absent	Absent
15th	Absent	Absent	Absent

Table 8: *Salmonella-Shigella* detection after chitosan treatment

Day	Rabbit-1	Rabbit-2	Rabbit-3
2nd	Present	Present	Present
5th	Present	Present	Present
7th	Absent	Absent	Absent
10th	Absent	Absent	Absent
15th	Absent	Absent	Absent

Table 9: *Pseudomonas* detection after chitosan treatment

Day	Rabbit-1	Rabbit-2	Rabbit-3
2nd	Absent	Absent	Absent
5th	Absent	Absent	Absent
7th	Absent	Absent	Absent
10th	Absent	Absent	Absent
15th	Absent	Absent	Absent

Figure 1: Coliform, *Salmonella-Shigella, Pseudomonas* detection in Rabbit-1

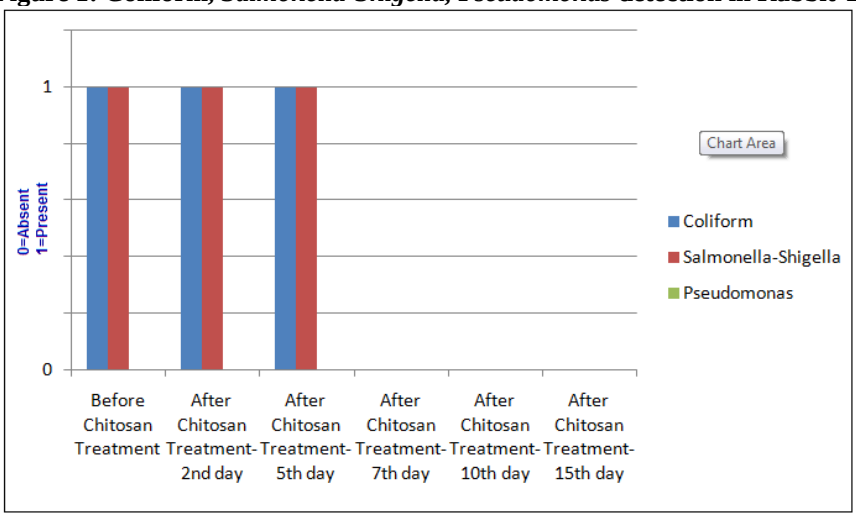


Figure 2: Coliform, *Salmonella-Shigella, Pseudomonas* detection in Rabbit-2

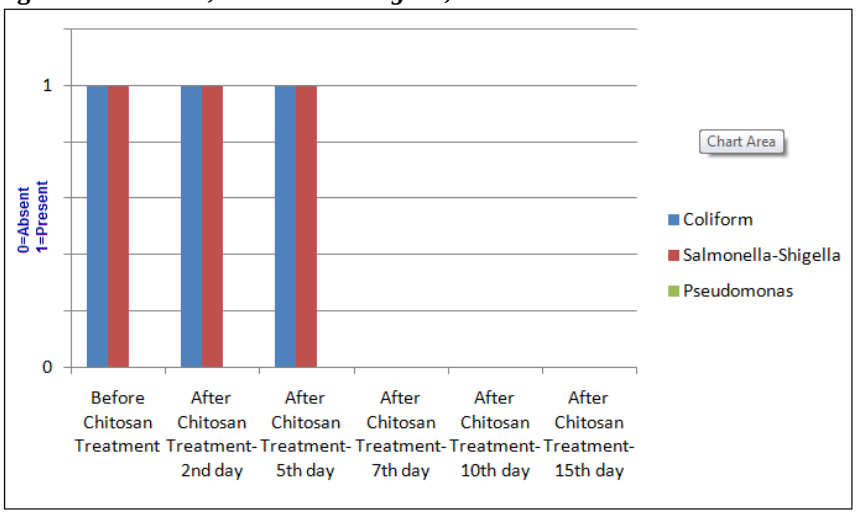


Figure 3: Coliform, *Salmonella-Shigella, Pseudomonas* detection in Rabbit-3

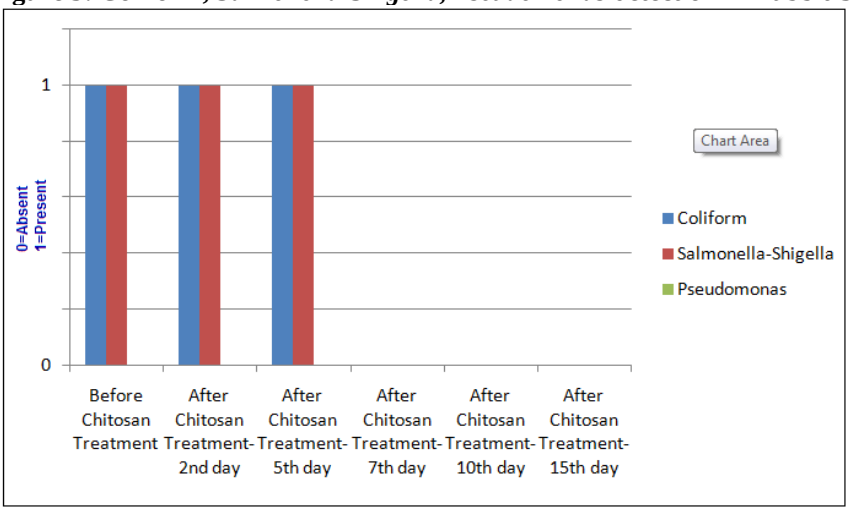
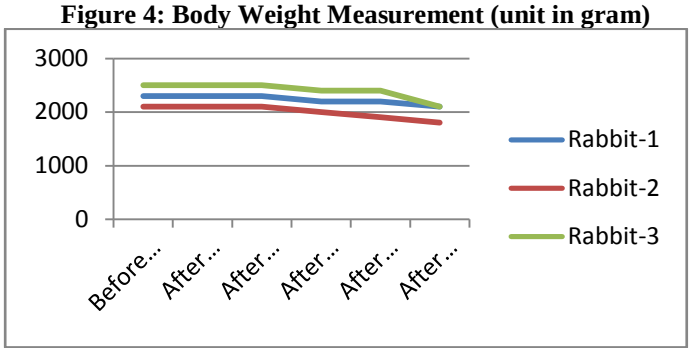


Figure 4: Body Weight Measurement (unit in gram)



IV. Discussion

The physical status of the rabbit was changed during chitosan treatment. Continuous reduction in weight and looseness of stool was observed, but physical movement was normal. After 15 days of chitosan treatment weight loss (%) observed 8.69, 14.28 and 8.0 in the rabbit 1, 2 and 3 respectively. This ensures lipid reduction property of chitosan on mammals which vary upon daily energy uptake and body weight.

A continuous bacterial growth was found in the 7^{th}, 10^{th}, 15^{th} day but none of pathogenic bacterial growth was observed. This refers to the safe antimicrobial of chitosan on mammals.

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