Effect of chitosan supplementation on immune response in mice

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ABSTRACT

Ninety Swiss albino mice apparently healthy were divided into 3 groups (n=30). (G1) control group; (G2) supplemented with 200 µg of chitosan/mouse/day in water intake; (G3) supplemented with 500 µg of chitosan/mouse/day in water intake. The result reveled that administration of chitosan at dose of 500µg/mouse/day had significantly hyper responder compared to control group in all examined parameters. The phagocytic percentage was 80.33 ± 0.88 % and 72.00 ± 0.47 % in G3 and G2 respectively compared to 61.00 ± 0.52 %, in G1 after 21 days of supplementation. The phagocytic index was 2.12 ± 0.02 and 1.60 ± 0.03 in G3 and G2 respectively compared to 0.84± 0.01 in G1. The level of lysozyme showed significant increase and it was 403.04 ± 21.12 and 313.48 ± 21.07 in G3 and G2 respectively compared to 197.02± 15.85 in G1 after 21 days of supplementation. While tumor necrosis factor (TNF-α) showed significant decrease and it was 55.10 ± 0.05 and 55.70 ± 0.16 in G3 and G2 respectively compared to 59.30 ± 0.60 in G1 after 14 days of supplementation. The level of IgG showed significant increase and it was 966.66± 2.40 and 815.66 ± 1.20 in G3 and G2 respectively compared to 702.66 ± 3.71 in G1. The level of IgM showed significant increase and it was 231.33± 3.28 and 179.33 ± 2.60 in G3 and G2 respectively compared to 156.33 ± 1.20 in G1.

Key words: Chitosan, Phagocytosis, Immunostimulant.

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1. INTRODUCTION

Chitosan is the major component of arthropod exoskeletons (Alishahi and Aider, 2011; Rinaudo, 2006), tendons, and the linings of their excretory, respiratory and digestive systems, as well as insect's external structure and some fungi. Shell wastes of shrimp, lobsters, crabs and krill are the main commercial sources of chitin. Therefore, we can say that chitin is not only present in invertebrates but is also present in vertebrates (Shakeel et al., 2014). Shells of crustaceans such as crab, shrimp, and crawfish contain approximately 15-40 % chitin (Kurita, 2006). Chitosan is a natural non-toxic immunostimulant (Abu-Elala et al., 2015) derived from the process of de-acetylation of chitin, a major shell component of crustaceans such as crab, shrimp and crawfish (Harikrishnana et al., 2012). Chitosan have unique properties, including low-toxicity (Thanou et al., 2001 and Raafat and Sahl, 2009), biocompatibility (Kumar et al., 2004), low cost and good handling properties. It demonstrates marked anti-bacterial activities against wide range of bacteria (No et al., 2002). chitosan have received considerable attention for their commercial applications in biomedical, food and chemical industries (Xia et al., 2010).

Chitosan is insoluble in most of the solvents but is soluble in dilute organic acids such as acetic acid, formic acid, succinic acid, lactic acid, and malic acid below pH 6.0. The most commonly used solvent is 1% acetic acid at about pH 4.0. The solubility of chitosan depends on its biological origin, molecular weight and degree of acetylation (Goy et al., 2009). The various chemical and biological properties of chitosan are as follows: Natural polymer linear polyamine reactive amino groups' reactive hydroxyl groups available. Chelates many transitional metal ions (Wu et al., 2001), biocompatible (Ravi Kumar et al., 2004), biodegradable to normal body constituents, safe and non-toxic (Thanou et al., 2001 and Raafat and Sahl, 2009), binds to mammalian and microbial cells aggressively (Xu et al., 2004), accelerate the formation of osteoblast responsible for bone formation (Seol et al., 2004), haemostatic (Pusateri

et al., 2003), fungistatic (Liu et al., 2001), spermicidal, antitumor (Azab et al., 2006 and Azab et al., 2007), anticholesteremic (Varlamov et al., 2010), accelerate bone formation (Ardakani, 2012), immunoadjuvant and drug delivery agent (Huang et al., 2005). Researches on the possibility of developing chitosan as a natural immunostimmulant have increased especially in two decades. Previous research indicated that chitosan could stimulate the production of antibodies in the blood circulation because its amino groups could be recognized by the immune system (Tokura et al., 1999). Some studies suggested that chitosan possesses some characteristics such as immune enhancing anti-inflammatory effects and antimicrobial activities, and could be used as an immunostimulant for animals (Yoon et al., 2008 and Kong et al., 2014). Chitosan act as an adjuvant enhanced significantly serum IgG titers in mice (David et al., 2007). Chitosan improved the humoral and cellular immune functions in broilers (Liu et al., 2007).

So, the present study aimed to investigate the immunostimulant effect of chitosan on mice.

2. MATERIALS AND METHODS

2.1. Chitosan preparation

Chitosan was obtained from Petroleum Research Institute (Nasr city, Egypt) (degree of deacetylation, 85% as determined by elemental analysis, and the average molecular weight, 109 KDa as determined by viscometric methods) according to (Hussein et al., 2012). Stock chitosan solutions (1% [wt/vol]) were prepared in 1% aqueous acetic acid that was later diluted and administered to mice (Brodaczewska and Doligalska 2012).

2.2. Animals

This study was carried out on 90 Swiss albino mice apparently healthy of both sexes. The mice aged (4-6 weeks) weighed about 25-30 gram, were obtained from the Animal Health Research Institute (Dokki, Giza, Egypt). The mice were kept in polypropylene cages under proper environmental conditions at room temperature 22-24ºC and 12h light/dark cycle and fed with a commercial pellet diet (Wadi El Kabda Co., Cairo, Egypt). The animals were acclimatized to laboratory conditions for two weeks before beginning the experiment. The experiment continued for 3 weeks on which constant weight of diet was given for each mouse. All the experiment were designed and conducted according to the ethical norms approved by the Ethical Committee of Animal Health Research Institute. Mice were randomly grouped into 3 groups (30 mice in each group) G1 (control group), G2 (mice received an oral daily dose of chitosan (200 µg/mouse/day) (Mrukowiez et al., 2006) on water intake and group G3 mice received an oral daily dose of chitosan (500 µg/mouse/day) (Brodaczewska and Doligalska, 2012) for 3 weeks on water intake. There were no pregnant females were used in this study.

2.3. Blood sample collection (serum preparation)

For the collection of autologous serum (AS), 300 µl of blood was collected by retro-orbital puncture using heparinized blood capillary tubes under mild ether anesthesia. After collection, the blood samples were incubated at room temperature for 10 minutes and left to clot for 2 hrs at 4°C, the clotted blood was centrifuged; the serum was removed from the clot. The serum was centrifuged again for 30 min at 500xg at 4°C to remove any remaining insoluble material. It was stored in clean stopper plastic vial at 4°C for a week at maximum or stored at -20ºC until the analysis of serum parameters.

2.4. Phagocytic activity

The phagocytic activity was assessed according to Zhang et al. (2010) with some modification. Phagocytosis assay was done at 3, 7, 14 and 21 days after chitosan supplementation. The results are expressed as phagocytic percentage and phagocytic index.

2.5. lysozyme assay

Lysozyme activity was measured by agarose by gel lysis assay according to Schultz (1987).

2.6. TNF-α determination

The content of The TNF-α was evaluated in serum as described by Ogata et al., (1993) using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by Biosource, USA, according to the manufacturer's protocol. TNF-α activity were measured at day 3, 7 and 14 after supplementation of chitosan.

2.6. Immunoglubulin assay

IgG and IgM were measured in serum, using a commercially available enzyme-linked immunosorbent assay (ELISA) kit supplied by

GenWay Biotech, Inc. USA, according manufacturer's instructions.

2.7. Effect of chitosan on growth performance

Body weight after supplementation of mice with chitosan was individually recorded at 3, 7 and 14 days to evaluate the effect of chitosan on growth performance.

2.8. Statistical Analysis

All data were analyzed for mean and standard error of mean and were statistically analyzed by conducting analysis of variance (ANOVA) test for least significant Difference (LSD) for determination of the significance between means at $P > 0.05$ according to Petrie and Watson (1999).

3. RESULTS

The obtained results revealed that, administration of mice with chitosan in dose of 500 µg/mouse/day showed improvement in cellular and humeral immunity than 200 µg/mouse/day of chitosan in comparison with control group.

3.1. Phagocytic activity

Peritoneal macrophage phagocytic activity/ index was determined in mice supplemented with chitosan at dose of 200 µg/mouse/day (G2), chitosan at dose of 500 µg/mouse/day (G3) and untreated group (G1). After 3 days of supplementation the phagocytic percentage showed no significant increase in any of groups and the phagocytic index was nearly similar in all groups Figure (1).The phagocytic percentage showed significant increase in G3 and G2 respectively in comparison with G1 after 7, 14 and 21 days of supplementation (Table.1 and Figure.1). After 7, 14 and 21 days of supplementation the phagocytic index reveled significant increase in G3 and G2 respectively in comparison with G1 (Table.2 and Figure. 1).

3.2. Lysozyme activity

After 3 days of supplementation with chitosan lysozyme activity showed no significant decrease in any of treated groups compared to control group (G1). The highest serum lysozyme activity was seen in group G3 and G2 respectively compared to G1 after 7, 14 and 21 days of chitosan supplementation. (Table.3)

3.3. TNF-α activity

After 3 days of supplementation with chitosan TNF-α level showed no significant decrease in any of treated groups compared to control group (G1). While TNFα level revealed significant decrease at 7 days, 14 days in G3 compared to G1. The level of TNF-α in G3 was 56.84 ± 0.31 and 55.10 ± 0.05 after 7 days and 14 days respectively compared to 59.36± 0.54 and 59.30 ± 0.60 in control after 7 days and 14 days respectively. G2 showed significant decrease at 7 and 14 days compared to G1. The level of TNF-α in G2 was 57.57 ± 0.37 and 55.70 ± 0.16 after 7 days and 14 days respectively compared to 59.36 ± 0.54 and 59.30 ± 0.60 in G1 after 7 days and 14 days respectively. There were no significant increase between G2 and G3 till the end of experiment Table (4).

3.4. Immunoglobulins assay

The effect of chitosan supplementation on serum IgG and IgM are shown in Table (5). Supplementation of chitosan revealed significant increase in IgG level in chitosan treated groups in comparison with control. Serum IgG level was 966.66 ± 2.40 and 815.88 ± 1.20 in G3 and G2 respectively compared to 702.66 ± 3.71 in G1 (Table 5). Supplementation of chitosan showed significant increase in IgM level in chitosan treated groups in comparison with control. Serum IgM level was 231.33± 3.28, and 179.33 ± 2.60 in G3 and G2 respectively compared to 156.33 ± 1.20 in G1 (Table.5).

3.5. *Growth performance*

The supplementation of chitosan revealed no significant increase in body weight after 3 and 14 days in treated groups, while it showed significant increase in G3 in comparison with G1 after 7 days of supplementation. There weren't any significant increase between G2 and G3 till the end of experiment.

Table (1): Phagocytic percentage in mice supplemented with chitosan

Group	phagocytic percentage %			
	3 days	7 days	14 days	21 days
G1	61.00 ± 0.49[a]	61.00± 0.57[c]	60.33± 0.88[c]	61.00 ± 0.52[c]
G2	62.00 ± 0.57[a]	66.00 ± 0.50[b]	69.00 ± 0.53[b]	72.00 ± 0.47[b]
G3	63.00± 0.51[a]	69.00 ± 0.57[a]	74.33 ± 1.45[a]	80.33 ± 0.88[a]

Table (2): Phagocytic index in mice supplemented with chitosan

Group	phagocytic index			
	3 days	7 days	14 days	21 days
G1	0.87 ± 0.008^{a}	0.84 ± 0.022^{c}	0.85 ± 0.02^{c}	0.84 ± 0.01^{c}
G2	1.21 ± 0.08^{a}	1.37 ± 0.04^{ab}	1.62 ± 0.03^{b}	1.60 ± 0.03^{b}
G3	1.16 ± 0.06^{a}	1.49 ± 0.003^{a}	2.11 ± 0.02^{a}	2.12 ± 0.02^{a}

The mean difference within the same column and bearing different superscripts are significantly different at $p > 0.05$ levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (3): Serum Lysozyme activity (µg/ml) in mice supplemented with chitosan

Group	Serum Lysozyme (µg/ml)			
	3 days	7 days	14 days	21 days
G1	210.02 ± 16.95^{a}	182.41 ± 31.5^{c}	213.9 ± 14.56^{c}	197.02 ± 15.85^{c}
G2	232.2 ± 19.1^{a}	292.4 ± 20.12^{b}	313.4 ± 21.07^{b}	313.48 ± 21.07^{b}
G3	297.3 ± 22.82^{a}	380.2 ± 21.78^{a}	380.2 ± 22.8^{a}	403.04 ± 21.12^{a}

The mean difference within the same column and bearing different superscripts are significantly different at $p > 0.05$ levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (4): The effect of chitosan supplementation on TNFα (pg/ml) in mice

Group	TNFα (pg/ml)		
	3 days	7 days	14 days
G1	59.66 ± 0.86^{a}	59.36 ± 0.54^{a}	59.30 ± 0.60^{a}
G2	58.00 ± 0.02^{a}	57.57 ± 0.37^{b}	55.70 ± 0.16^{b}
G3	57.00 ± 0.03^{a}	56.84 ± 0.31^{b}	55.10 ± 0.05^{b}

The mean difference within the same column and bearing different superscripts are significantly different at $p > 0.05$ levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (5): The effect of oral supplementation of chitosan on IgG and IgM in mice

Group	IgG	IgM
G1	702.66 ± 3.71^{c}	156.33 ± 1.20^{c}
G2	815.66 ± 1.20^{b}	179.33 ± 2.60^{b}
G3	966.66 ± 2.40^{a}	231.33 ± 3.28^{a}

The mean difference within the same column and bearing different superscripts are significantly different at $p > 0.05$ levels. The values were given as means (±S.E.) of three replicates, ANOVA test.

Table (6): The effect of supplementation of chitosan on body weight

Group	Body weight (g)		
	3 days	7 days	14 days
G1	28.62 ± 0.23^{a}	29.00 ± 0.40^{c}	29.87 ± 0.21^{a}
G2	28.87 ± 0.12^{a}	29.50 ± 0.35^{bc}	29.87 ± 0.22^{a}
G3	29.02 ± 0.36^{a}	30.00 ± 0.20^{ab}	30.12 ± 0.23^{a}

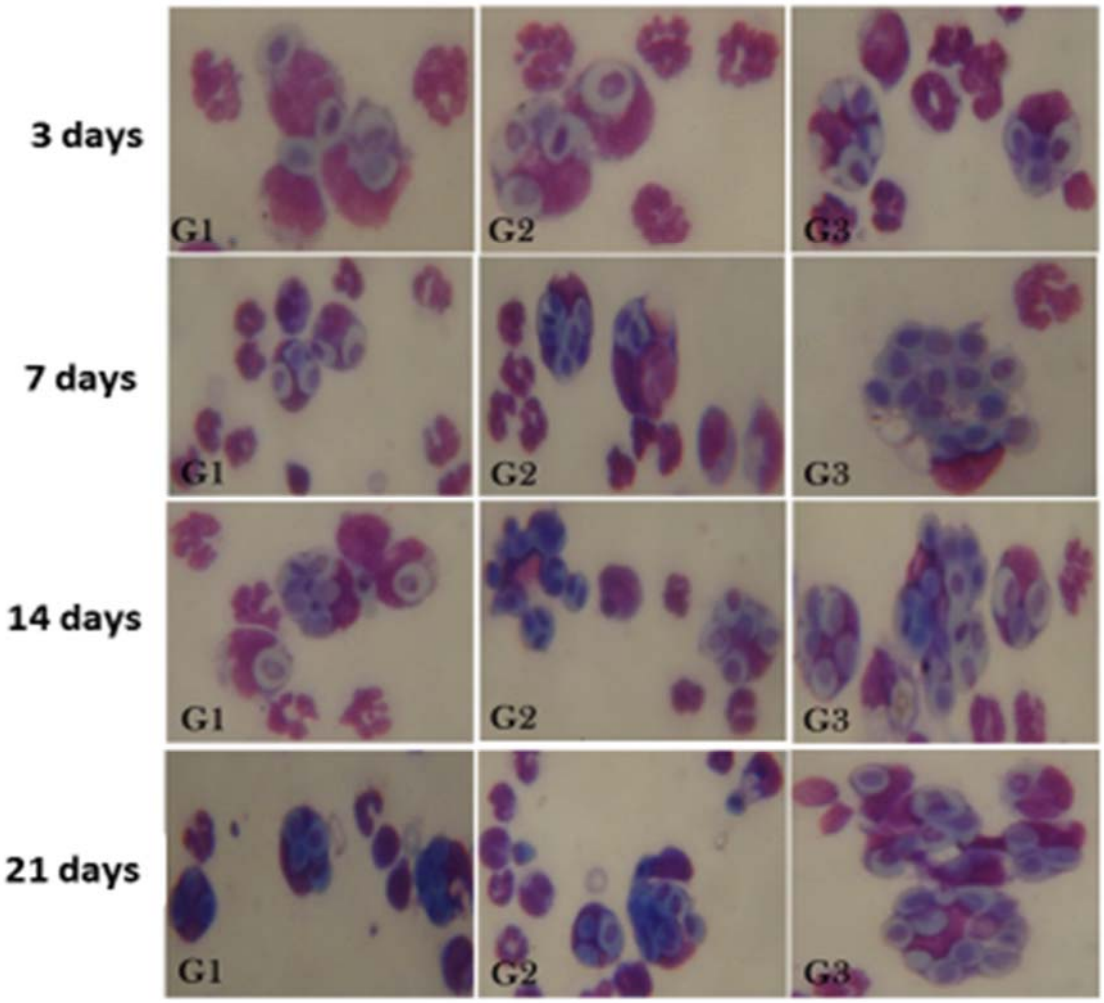

Figure (1): Effect of chitosan supplementation on mice peritoneal macrophage phagocytic activity engulfed chicken red blood cells. The phagocytic activity was examined under light microscopy at a total magnification power of 100x.

4. DISCUSSION

This study has been conducted to assess immuostimulant effect of chitosan. Mice were supplemented with two doses of chitosan (200µg/mouse/day and 500µg/mouse/day). The highest dose of chitosan revealed more effect on cellular and humeral immunity than the low dose, which means that chitosan is dose dependent.

The present study demonstrated that the dietary supplementation of chitosan had immune-modulating effects on some nonspecific immune functions of mice. In this study phagocytic activity of macrophage (phagocytic % and phagocytic index) was higher in mice supplemented with chitosan compared to control. The supplementation of chitosan at concentration of 500µg/mouse/day showed the best phagocytic % and phagocytic index of macrophage in this study. These results are in accordance with Harikrishnan *et al.,* (2012) and Abu-Elala *et al.,* (2015) who reported that phagocytic activity were significantly enhanced in fish fed with 1% chitin and chitosan diet in weeks 2 and 4 compared to control group.

The lysozyme activity is an important indicator of the immune defence in both invertebrates and vertebrates (Ellis, 1990). Lysozyme are proteins of low molecular weight found in polymorphonuclear leukocyte and mononuclear cells, lysozyme are considered as a member of innate humoral factors that elaborate from the body and showed dramatic increase in concentration in response to infection or tissue injury (Weir,1983). Lysozyme has an antibiotic ability and is released by leukocytes, it damage bacterial cell walls especially of Gram-positive and some Gram-negative bacteria (Grinde,1989) by hydrolysis of glycoside link between N-acetylmuramic and N-acetylglucosamine in peptidoglycan layer of bacterial cell wall and activate the complement

system and phagocytes by acting as an opsonin (Magnado, 2006).

The present study showed that the dietary supplementation of chitosan and increase lysozyme activity compared to control specially in G3 and G2. The supplementation of chitosan at concentration of 500 µg/mouse/day showed the best lysozyme activity followed by 200 µg/mouse/day. In the current study, the higher levels of serum lysozyme activity in the treated groups could have contributed to the noticeable enhancement of the non-spesific defense mechanisms (Engstad *et al.,* 1992). Similar results have been reported by (Geng *et al,* 2011; Harikrishnana *et al.,* 2012 and Abu-Elala *et al.,* 2015).

The production of pro-inflammatory cytokines such as TNF-α is increased in acute inflammatory responses associated with infection, injury, trauma or stress (Avitsur *et al.,* 2006). The present study demonstrated that the dietary supplementation of chitosan showed significant decrease in the level of TNF-α as compared to control. Daily administration of 500 µg/mouse/day chitosan for two weeks in mice (G3) showed a significant decrease of TNF-α as compared to control group. This result is in agreement with Chen *et al.,* (2008) who recorded that chitosan with low molecular weight (MW, 21–92 kDa) had specific immunomodulatory effects on MDM (monocyte-derived macrophages) including the shifting of Th2 cytokine polarization, decreasing the production of the inflammatory cytokines IL-6 and TNF-α. In contrast to the present results Wu *et al.,* (2015) who reported that demonstration of two important low molecular weight chitosan (3 kDa and 50 kDa) can induce activation of macrophages and enhanced significantly the pinocytic activity, and induced the production of tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), interferon-γ (IFN-γ) and nitric oxide (NO).

Dang *et al.,* (2011) and Li *et al.,* (2015) recorded that dietary supplementation of chitosan at dose of 500 mg/kg raised significantly the level of IL-1 compared with the control, whereas the IL-2 and TNF-α levels exhibited no significant difference in beef cattle.

Immunoglobulin is a protein produced by plasma B-cells that has the ability to recognize and neutralize foreign objects (Solem and Stenvik, 2006). Specific immunoglobulins in combination with lymphokines could indicate collectively the status of humoral immunity playing essential role in protecting against bacterial as well as viral infections (McKee *et al.,* 2007). This study investigated the effects of chitosan supplementation on immune functions. The results showed that chitosan at dose of 500 µg/mouse/day (G3) increased remarkably serum IgG and IgM however, the effects were weakened with decreasing the dose of chitosan to 200 µg/mouse/day (G2) in comparison with control (G1). This may be due to amino groups of chitosan that could be recognized by the immune system, then stimulate immune cells to proliferate and differentiate, and to release immunoglobulins (Tokura *et al.,* 1999). This result is in agreement with Lim *et al.,* (1997) who reported that concentrations of IgG, IgA, and IgM in mesenteric lymph node (MLN) lymphocytes were generally higher in rats fed chitosan than in those fed cellulose. Furthermore, dietary supplementation of chitosan in piglets increased serum immunoglobulins and recovered the loss which was caused by early weaning (Yin *et al.,* 2008; Li *et al.*, 2013).

Supplementation of chitosan with different doses showed no significant increase in body weight. Many researchers studied the effective utilization of chitosan as an animal feed supplement. This result is in agreement with Kobayashi *et al.,* (2002, 2006) who reported that chitosan has no effect on growth performance of broiler chickens fed on a 50 g/kg chitosan diet. Another study by Razdan and Pettersson (1994) showed that 30.00 g/ dietary chitosan decreased growth performance. In contrast to this study Shi *et al.,* (2005), Khambualai *et al.,* (2008, 2009) and Yuan and Hong (2012) recorded that a low concentration of 0.50 to 1.00 g/kg, dietary chitosan could gain superior performance than the control groups in broilers.

In conclusion, the current study showed that chitosan has powerful immunostimulant effect. Administration of mice with chitosan in dose of 500 µg/mouse/day showed improvement in cellular and humeral immunity than 200 µg/mouse/day of chitosan in comparison with control group, this mean that chitosan is dose dependent. chitosan had no effect on growth performance.

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