STUDY THE EFFECT OF NANO-PREPARATIONS OF CHITOSAN ON THE PERFORMANCE OF THE CELLS AND THE VIRUS TITRE USED IN SHEEP POX VACCINE PRODUCTION

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Abstract

Sheep pox (SP) still represents a major threat to animal wealth. Vaccination is the only reliable remedy for such highly infectious and contagious diseases. Chitosan nanoparticles (CNP) could be successfully added to live as well as inactivated vaccines to enhance their immune responses as it exhibits a good delivery system. In this study, CNP was added to maintenance essential media (MEM) with ratios of 3, 15 and 30 µg/ml. Such preparations were overlayed on confluent vero cell sheets for 7 days. CNP augmented MEM was proved to be un-harmful for VERO cells. The prementioned concentrations of CNP-MEM were mounted the infected sheets of cells that had been inoculated with the Romanian vaccinal strain of sheep pox virus (SPV). Appearance of the cytopathic effect as well as SPV infectivity titers were slightly improved particularly with CNP-MEM that was augmented with 15µg/ml. The former infected culture was used to prepare pilot batch of CNP supplemented sheep pox vaccine. The immune response of the pilot batch was assessed comparing with the conventionally produced SP vaccine by inoculating them into separate groups of susceptible sheep. There was slight increase in the serum neutralizing antibody titers in the group inoculated with the CNP augmented vaccine over the traditional one. It has been deduced that CNP could be used in sheep pox vaccine preparation to enhance the vaccine quality.

Keywords: SPV, CNP, Vero cells, cytopathic effect (CPE) and serum neutralization test (SNT).

INTRODUCTION

Sheep pox virus (SPV) is a member of genus capripox, family Poxiviridae (Kitching, 2003), characterized by fever, nodules on the skin, mucous membranes and internal organs, emaciation, enlarged lymph nodes, edema of the skin and sometimes death (OIE 2010). Sheep pox is still problematic in the Middle East especially in Egypt where it causes severe catastrophic losses. Vaccination is the most effective and protective way to overcome the spreading of the disease and to counteract its drastic damage especially for farm animals. The major aim of vaccination is to generate sufficiently strong immune responses of the correct nature to protect against disease

(OIE, 2014). Some modulations on SP vaccine preparation recipe should be taken out so as to potentiate the vaccine immunogenicity (Paillot *et al*., 2008).

Chitosan is a cationic biopolymer widely distributed in nature. In recent years, chitosan nanoparticles has offered a wide choice of delivery systems such as aqueous dispension, gel and sponges which capable of carrying antigen and adivacate better performance for the viral vaccines (Arca *et al*., 2009). The unique character of Chitosan nanoparticles (CNP) could exhibit excellent characteristics as vaccine delivery system due to its bioadhesive, biocompatibility, biodegreability, low toxicity and penetration enhancement properties. It is mostly taken up by phagocytic cells inducing strong systemic and mucosal immune response. So, CNP has been previously tested as an adjuvant/ delivery system for different vaccines (Van der Lubben *et al.,* 2001 and Zhu *et al*., 2007, Deunne *et al*., 2008 and Volkova *et al.,* 2014).

Our herein study is devoted to assess the role of chitosan nanoparticles as vaccine penetration enhancers and its immunological effect for sheep pox vaccine to induce virus cell ultimate performance to get superior SP vaccine.

MATERIALS AND METHODS

1. Virus:

Sheep pox vaccinal Romanian strain was firstly described by Sabban (1960). The vaccinal strain was supplied from Pox virus vaccines Res. Dep., Veterinary Serum and Vaccine Research Institute (VSVRI), Abbasia, Cairo (with a titer of 10^6 $TCID_{50}$/ml).

2. Green monkey kidney cell line (Vero):

It was obtained from Pox virus vaccines Res. Dept., (VSVRI), Abbasia, Cairo. It was subcultured using Eagl's minimum essential medium (MEM) supplemented with 10% newly born calf serum and the maintenance medium was supplemented with 2% serum and adjusted at pH 7.8 (Ozawa and Hazrati, 1964 and Hosamani *et al*., 2004).

3 .Chitosan nanoparticles preparation:

Chitosan nanoparticles preparations were performed by dissolving chitosan at 3% (w/v) in 1% (w/v) acetic acid (CH_3COOH), then pH was adjusted to 4.6 – 4.8 with 10N NaOH. CNP were formed by the principle of ionic crosslinking between positively charged chitosan and negatively charged sodium tripolyphosphate (TPP) (0.25% w/v) according to Zhao *et al*. (2012). Chitosan nanoparticles were formed by dropping TPP to the chitosan solution with the ratio of (1:5) with magnetic stirring at room temperature for overnight. CNP was separated by centrifugation at 10,000 rpm for 30 minutes at 4°C and the supernatant was discarded while the sediment was reconstituted to the original volume with PBS. So, a stock CNP suspension with a concentration of 3 mg/ml was obtained.

Transmission electron microscopy (A Jeol JEM-1400 TEM) micrograph was used to measure the chitosan nanoparticles size, morphology and distribution of the chitosan nanoparticles at an acceleration voltage of 80 KV.

CNP suspension was added to MEM with ratios of 0.1% (3µg/ml), 0.5% (15µg/ml) and 1% (30µg/ml) (Qi *et al*., 2004).

4. Detection of Chitosan nanoparticles cytotoxicity on Vero cells:

Four groups of 75 ml prescription flasks were seeded with Vero cells. Each group was composed of five flasks. After 48 hours, the exhausted supernatant growth media were discarded and media change was carried out. For the first three groups the MEM used were augmented with 0.1%, 0.5% and 1% CNP for the first, second and third group respectively. The fourth group flasks were overlaid with blank MEM (without CNP). For 10 days, all the flasks were kept under daily microscopically inspection. The tested cell flasks were supcultured for three successive passages with adding CNP supplemented media in each group as the previous used concentration and observed in the same technique.

5. Detection the influence of CNP on sheep pox (SP) virus infectivity titer:

Five groups of 75 ml prescription flasks were seeded with Vero cells. The flasks were kept at 37°C for 48 hours. The first four groups were inoculated with SPV, each flask received 5 x 10^3 $TCID_{50}$ then the flasks were incubated at 37°C for 2 hours (for complete virus adsorption). The 1st three groups were overlaid with the above mentioned concentrations of CNP supplemented MEM (0.1%, 0.5% and 1% CNP respectively) while the 4th group flasks were proved with blank MEM. The fifth group flasks were kept as non-inoculated normal control. All the bottles were checked up daily for tracing of SPV cytopathic effect (CPE). Three successive passages of the cell inoculation were performed using MEM with each chitosan concentration.

On the 3rd passage of the tested inoculated cells, samples of the virus infected cells was collected daily and subjected to freezing and thawing for 3 times before tested by virus titration.

The infected fluids of the tested groups were collected separately for further investigation.

6. Virus titration:

The virus titration of the prepared virus fluids were performed using the 10 fold dilution inoculated onto VERO cells microtiter plate according to OIE (2010) and the virus titer was calculated following the rules given by Reed and Muench (1938).

7. Assessment of CNP-SP vaccine on the immune response of susceptible sheep comparing with the conventional one:

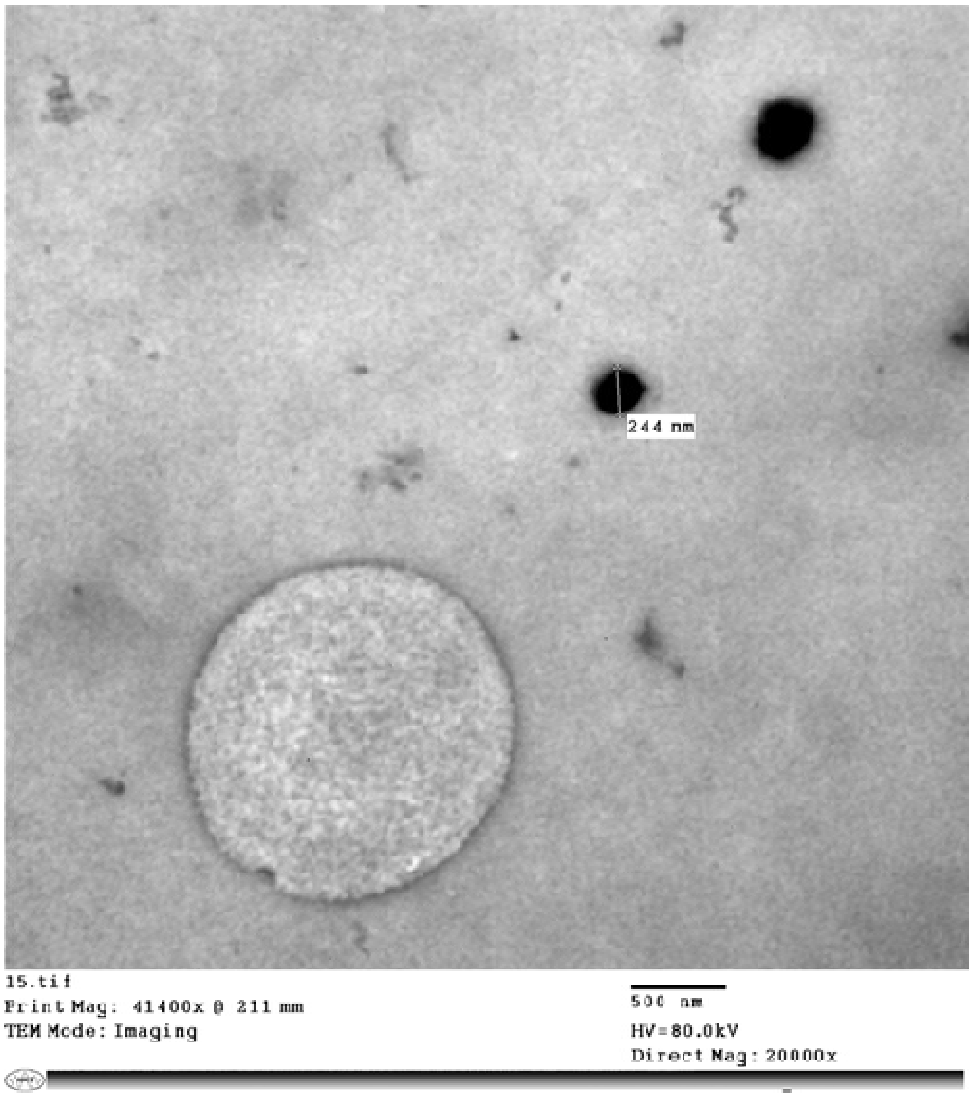
Nine sheep 6-8 months old were proved to be susceptible to sheep pox through screening their serum samples with serum neutralization test (SNT). The animals were categorized to three groups each made of 3 animals. The animals of the

first group were inoculated with SPV vaccine prepared with MEM augmented by CNP (15µg/ml). While the second group of animals were inoculated with conventional vaccine and the last group were kept as non-vaccinated controls. All the animals were checked up weekly by testing their serum samples with SNT to monitor the sheep-pox neutralizing antibody titers in vaccinated as well as non-vaccinated animals.

8. Serological technique:

Serum neutralization test (SNT) was carried out in accordance with the method described by OIE (2012). The result was expressed as neutralizing antibody titer.

RESULTS AND DISCUSSION



Dense particles refer to CNP with diameter 244 nm.

Fig. 1. Transmission electron microscopy micrograph of chitosan nanoparticles.

Table 1. The influence of CNP on the performance and infectivity titers of SPV

Cells' Bottle groups	Days Post Inoculation											
	3		4		5		6		7		8	
	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer	CPE	Titer
C 1	-	-	10%	100.9	30%	102.2	50%	103.9	80%	105.7	100%	103.1
C 2	10%	-	20%	101.3	40%	102.5	70%	104.2	90%	106.3	100%	103.7
C 3	-	-	20%	101.1	40%	102.2	70%	104.0	90%	106.1	100%	103.7
C 4	-	-	10%	101.1	30%	102.1	50%	104.0	70%	105.7	90%	104.2
C5	-	-	-	-	-	-	-	-	-	-	-	-

C1: Infected cell group covered with MEM supplemented with 0.1% chitosan
C2: Infected cell group covered with MEM supplemented with 0.5% chitosan
C3: Infected cell group covered with MEM supplemented with 1 % chitosan
C4: Infected cell group covered with blank MEM
C5: Control non infected cell group

Table 2. Assessment of the CNP-SPV vaccine on the immune response of susceptible sheep comparing with the conventional one expressed by neutralizing antibody titer.

Animals' Group		Weeks Post Vaccination							
		1	2	3	4	5	6	8	10
Gp (1)	1	8	32	256*	256	256	256	128	128
	2	8	32	128	256	256	128	128	128
	3	16	64	256*	256	256	256	256	128
Gp (2)	1	0	16	64	128*	128	64	64	32
	2	8	32	128	256*	128	128	128	64
	3	8	16	128	256*	128	128	64	64
Gp (3)	1	0	0	0	0	0	0	0	0
	2	0	0	0	0	0	0	0	0
	3	0	0	0	0	0	0	0	0

Gp (1): Animals vaccinated with SPV prepared from inoculated cell overlaid with MEM supplemented with 0.5% chitosan
Gp (2): Animals vaccinated with SPV prepared from inoculated cell overlaid with blank MEM
Gp (3): Unvaccinated control animals
* : the peak of antibody titer

Sheep pox is still rampant in Egypt causing severe drastic impacts on the national economy. Its high morbidity and mortality rates rendered it as one of the most dangerous viral disease of list A (OIE, 2014). Vaccination, so far, is the best cost-effective and prophylactic measures to control and even to eradicate such versus diseases (OIE, 2014).

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4) linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine. It was extracted from marine shrimp shells with a degree of deacetylation of 85% and

molecular weight of 220 kDa as determined by Qurashi *et al*. (1992). Several researches have studied the properties, biocompatibility and non-toxicity of the chitosan nanoparticles which makes it attractive as a natural agent for delivery of active agents. Chitosan nanoparticles could be considered as microporous polysaccharide biopolymers; therefore, pores are large enough and well suited to let virus particles diffuse through (Qi *et al*. 2004).

The size and distribution profile of the prepared chitosan nanoparticles when measured by transmission electron microscopy, as shown in Figure 1, represents a batch of nanoparticles with average diameter of 200-250 nm with regular shape and did not have adhesion or subsidence damage in morphology.

Chitosan nanoparticles cytotoxicity on Vero cells:

Direct examination using microscopic evaluation and observation the cell morphology changes has been widely used as indicators for cytotoxicity studies (Fischer *et al*., 2003; Janvikul *et al*., 2007). The data of this experiment referred to the innoxiously effect of CNP on Vero cells. The first three groups of cell flasks those received CNP supplemented MEM in different concentration (3µg, 15µg and 30µg/ml) showed neither morphological alterations nor detachment. Moreover, the cell vitality and activity of the all four groups were within the normal limit denoting that CNP had no deleterious effect on VERO cells. These findings were previously ascertained by Zhu *et al*. (2007) who stressed on the characteristic advantages of utilization of CNP as a supplement in preparing wide variety of vaccines on tissue culture. They added that the biodegrability of CNP suggested its incorporation in vaccine manufacturing.

The influence of CNP on sheep pox (SP) virus infectivity titer:

It has been recorded that SPV-CPE appeared in all the infected cell groups almost at the same time except the 2nd group flasks (tested with 15µg CNP/ml MEM) that showed CPE 24 hours earlier than the other groups. Also, the infectivity titers of all the four groups were very close except the 2nd group bottles that showed mild increase in SPV titer (Table 1) comparing with the virus titer of the other groups. The results obtained from this experiment referred to mild enhancement of the SP virus titer prepared with CNP suplemented MEM. Such finding is associated with the CNP penetration properties that enhance the capabilities of SPV to recognize and penetrate the cell receptors leading to improving cell-virus interaction. These nations come in curious coincidence with the proposals achieved by Gunbeyaz *et al*. (2010) who demonstrated that the particle size and the positive surface charge of chitosan make it more easily associated with the cell membranes and taken up better. Zhao *et al*. (2012) reported that the chitosan was found to effectively induce an increase in the infectivity of Newcastle live attenuated vaccine improving its efficacy and

subsequently its immunogenicity. On the other hand, Wang *et al*. (2011) reported that chitosan nanoparticles induced decrease in the infectivity titers of feline calici virus (FCV-F9) as well as Bacteriophage MS2 and Phix 174.

Assessment of CNP-SP vaccine on the immune response of susceptible sheep comparing with the conventional one:

It was found that sheep inoculated with SPV vaccine supplemented with CNP gave better immune response than the sheep vaccinated with the classic SPV vaccine (prepared without additive). As sheep vaccinated with SPV vaccine prepared with CNP augmented media (group 1) showed obvious increase in SN antibodies comparing with those vaccinated with the conventional SPV vaccine (group 2). Moreover, the onset of the antibodies was appeared in group 1 sheep earlier than group 2 ones. The peak of SN antibodies for the 1st group was achieved by the third week post vaccination while the peak of the SN antibodies occurred by the 4th week post vaccination in the 2nd group. The two antibody titer curves remained high till the 8th week post vaccination when the curve of the second group began to decline, whereas the 1st curve remain at the same level till the 10th week post vaccination. The serum samples collected from group 3 (control non-vaccinated sheep) did not exhibit neutralizing antibodies throughout the duration of the experiment (Table 2).

Those results denoted that the addition of the CNP to the MEM of the SPV infected cultures has led to improving the immune response of sheep to SP vaccine. These conclusions are in full agreement with the data obtained by Zheng *et al*. (2011) who stressed on the fact that low immunogenic protein like ovalbumin was improved when dissolved in chitosan nanoparticles. They discovered that CNP enhanced the serum OVA-specific IgG1, IgG2a and IgG2b antibody titers, moreover the nanoparticles induced spleenocyte proliferation which promoted the production of T-helper 1 (Th-1) and Th-2 and remarkably increased the activities of natural killer (NK) cells. Thus, the CNP coupled OVA had a strong potential to enhance both cellular and humoral immune responses and elicited a balanced Th1/Th2 response and is safe and efficacious to be conducted with prophylactic and therapeutic vaccine. Also Volkova *et al*. (2014) reported that CNP caused better immunogenic response when coupled with inactivated Newcastle vaccine and rendered it eligible to be used as oral vaccine. The new recipe of CNP-ND vaccine improved either the mucosal and systemic immune responses. The same dogma was also ascertained by Deunne *et al*. (2008) who managed to conspicuously elevate the immune response of sheep to FMDV specific synthetic peptides through their combination with nano-beads resulting in a significant increase in cellular and humoral immune responses.

From the obtained results it could be concluded that:
-Chitosan nanoparticles have no deleterious effect on Vero cells.

-CNP could be added to maintenance medium to improve the sheep pox virus vaccine (Romanian strain) infectivity on Vero cells.

-CNP supplemented SP vaccine proved to enhance the neutralizing antibodies in vaccinated sheep more than the traditional one.

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دراسة تأثير الجزيئات المصغرة (النانو)
لمادة الشيتوزان على كفاءة الخلايا وعيارية الفيروس
المستخدمين لانتاج لقاح جدرى الاغنام

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ما زال مرض جدرى الضأن يشكل تهديدا خطيرا للثروة الحيوانية والتحصين هو الحل الأمثل لمثل هذه الأمراض الوبائية. ثبت ان استخدام تقنيات النانو يحسن بدرجه كبيره من كفاءة اللقاحات وبخاصه جزئيات الشيتوزان المصغرة التى يمكن أن تضاف الى اللقاحات الحيه والمثبطه لتحسن من رد الفعل المناعي. عند دراسة اضافة جزئيات الشيتوزان النانو (CNP) بنسب 3 ،15 ، 30 ميكروجرام/مل الى الميديا المستخدمة على خلايا كلى القرد الأخضر الأفريقى (VERO cell) حيث وجد انه ليس له اى تأثير ضار على الخلايا لمدة سبعة أيام. وعند استخدام نفس التركيزات السابقه على الخلايا المحقونه بفيروس جدرى الضأن (العترة الرومانيه) وجد أنها قد أدت الى تحسن طفيف فى ظهور التأثير المرضى للفيروس على الخلايا (CPE) وكذلك على عيارية الفيروس وبخاصه التركيز 15 ميكروجرام/مل. تم تجربة فيروس لقاح جدرى الضأن المجهز باستخدام ميديا الحقن المضاف اليها (CNP 15µg/ml) فى عمل دفعه لقاح استرشاديه ومقارنتها بلقاح جدرى الضأن العادى (المحضر دون اضافات) حيث تم حقن الدفعتين فى حملان قابله للعدوى ووجد أن اللقاح المضاف اليه جزئيات الشيتوزان النانو قد أحدث أجساما مناعيه أعلى من اللقاح العادى وذلك باجراء اختبار السيرم المتعادل (SNT) وبذلك يمكن استخدام جزئيات الشيتوزان النانو لتحسين اداء لقاح جدرى الضأن.