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Nigella Sativa Oil as an Immunostimulant Adjuvant in H5 Based DNA Vaccine of H5N1 Avian Influenza Virus

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Abstract: Nigella sativa oil was found to exhibit non specific immunostimulant effect and has the ability to induce cellular immune response. Most of the available vaccines of avian influenza H5N1 had variable efficacy to protect chickens against allviruses. Since DNA vaccine is considered to be a promising novel approach for vaccination against influenza A virus, the goal of this study was to prepare HA1 based DNA vaccine with nigella sativa oil as an adjuvant and study its immunostimulant effect.The HA1 gene from Egyptian virus A/chicken/Egypt/1055/2010(H5N1) was extracted and subcloned intoplasmid containing Cytomegalovirus (CMV) immediate-early enhancer/promoter region, a â-globin/IgG chimeric Intron and neomycinePCIneo mammalian expression vector. The immunological response was investigated by intramuscular immunization ofspecific pathogens free(SPF) chickens with PCIneo-H5 nigella sativa adjuvant vaccine. The H5-DNA vaccine with nigella sativa oil adjuvant induced potent cell mediated immune response in chickens reached up to 86% phagocytic percent and 0.5 lymphocyte proliferation at 14th day post vaccination.

Key words: H5 Based DNA Vaccine · Highly Pathogenic Avian Influenza Virus H5N1 · Nigella Sativa Oil Adjuvant · Cell Mediated Immune Response · Protective Efficacy Of H5 DNA Vaccine

INTRODUCTION

Nigella sativa commonly known as black seed belongs to botanical family of Ranunucleae. The general chemical composition of the nigella sativa seeds is fats (31-35.5%w/w), proteins (16-19.9%w/w), Carbohydrates (33.9%), fibers (4.5-6.5%) and moisture (5-7%) as described byAnsari and Sadiy[1] and Salem [2] (Fig. 1). There were 2 kinds of nigella sativa oil which present in the expressed or solvent-extracted oil namely the fixed oil (>30% of the seeds weight) and the volatile oil (0.5% of the seeds weight). The essential oil of nigella sativa seeds was found to contain a crystalline compound called nigellone or thymoquinone that possessed a protective action against disease. The active constituent of the volatile oil, nigellone was first isolated by Mahfouz and El Dakhakhny [3] and thymoquinone which was then isolated. When thymoquinone exposed to air, dimerization occurs with the formation of dithymoquinone

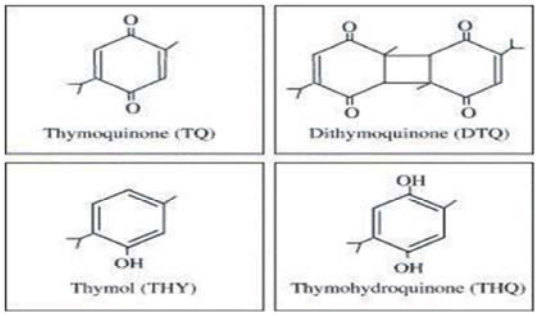

Fig 1: The Chemical structure of the active ingredients: TQ, DTQ, THY, and THQ, in the oil of *N. sativa* L seed as described by Salem [2].

[4]. El Alfy*et al.*[5]isolated a white crystalline compound identified as thymohydroquinone, which is probably a reduction product of thymoquinone. literature survey revealed only the ability of the total oil to induce immunity effect [6].

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Nigella sativa oil was found to exhibit nonspecific immunostimulant effect [7] and has the ability to induce cellular immune response as increasing lymphocytes pathogenesis and macrophage activities percent and macrophage index [8]. It was found that it enhance T cells, natural killer cell activity and both interleukine-IB (IL-IB) and interleukine-3 (IL-3) [9, 10]. These facts trigerred us to evaluate the effect of nigella sativa oil on the influenza DNA vaccine.

Based on animal study, treatment with nigella sativa oil increased the phagocytic activity and phagocytic index of peritoneal macrophages and lymphocyte count in peripheral blood in diabetic hamsters [11]. Nigella sativa also reversed the immunosuppression induced by lead and calcium in animal study [12]. Based on laboratory study, a melanin extract from nigella sativa may induce the expression of three cytokines (TNF-alpha " Tumor necrosis factor alpha", IL-6 and VEGF "Vascular endothelial growth factor") that enhance immunogenicity and promote tumor regression [13]. Black seed has also been shown to modulate the immune system by altering levels of inflammatory mediators [14].

The current H5 vaccines had insufficient efficacy in protecting chickens and turkeys after experimental infection with the newly emerging variant HPAI H5N1 strains in Egypt has been recently demonstrated [15-17]. Therefore, a vaccine that could protect chickens from a lethal infection and prevent the spread of the virus is urgently needed and the development of cost-effective avian influenza (AI) vaccine is a priority to prevent pandemic flu outbreaks. The DNA-based immunization is a promising strategy to prevent persistent viral infections and diseases. This approach can induce a broad range of immune responses and has been successfully used to provide protective immunity against influenza [18, 19].

Because hemagglutinin (HA) protein is a major viral surface antigen against neutralizing antibodies elicited, recombinant HA was a target as a candidate avian influenza vaccine. Since most antigenic and neutralization sites are in the HA1 domain of HA [20], using HA1 domain of influenza virus as antigen is of great importance in vaccine development [21] specially in countries facing vaccination failure inforce endemic viruses to be adapted like in Egypt so the aim of this study was to prepare H5 AI DNA vaccine against the current circulating H5N1 avian influenza viruses in Egypt adjuvanted with nigella sativa oil for its non-specific immunostimulant effect.

MATERIALS AND METHODS

Preparation of H5 Ai DNA Vaccine with Nigella Sativa Oil as an Adjuvant: The selected DNA vaccine strain was A/chicken/ Egypt/ 1055/2010 (H5N1), Gene bank Accession no. HQ 198268. The virus was propagated on Confluent monolayer of Madin-Darby Canine Kidney cells (MDCK) and titrated by calculating the TCID50 according to Reed and Muench[22]. Plaque assay was used to measure plaque forming unit (PFU) on MDCK. The coding sequence of HA1 gene was amplified by RT-PCR and cloned in pGEM® T Easy Vector System I (Promega, USA). The HA1 was then cut with NheI and XhoI restriction enzymes 10µ/µl (promega) by double digestion and directionally subcloned into PCI-neo mammalian expression vector (promega, USA) which contains the cytomegalovirus (CMV) early promoter. The proper ligation at both forward and reverse junctions of the constructed PCIneo-HA1 recombinant plasmid was confirmed by sequencing. The expression of the HA1 gene were tested invitro by chemical transfection of vero (African green monkey kidney) cell line by using Trans Fast™ Transfection Reagent (promega, USA) and the expression was detected by SDS-PAGE (SDS-polyacrylamide gel electrophoresis) [23] and theability of inducing mRNA expression for HA1 using oligo $(dT)_{18}$ primer and RT-PCR [24].

The HA1/PCI-neo recombinant plasmid DNA constructwas used as aqueous phase for preparing the vaccine according to Madbouly *et al*.[25, 26] as follow:

Aqueous Phase: 100 µg plasmid DNA diluted in PBS was mixed with tween 80 in ratio 96% plasmid DNA to 4% tween 80 with thouroughly mixing

Oil Phase: Nigella sativa oil was mixed with span 80 in ratio of 9 parts oil to one part span 80 with thouroughly mixing.

Then thouroughly mixing of aqueous and oil phase in ratio 1:4 where 1 part of aqueous phase was mixed with 3 parts of prepared nigella sativa adjuvant (oil phase) with continuous mixing till preparing a stable oil emulsion vaccine with low viscosity of 1:4

Evaluation of the Prepared Vaccine: Ten SPF chickens of 3 weeks old were immunized with single dose of 10 µg plasmid DNA mixed with nigella sativa oil, 0.5 ml by direct intramuscular injection in thigh muscle, another group of 10 SPF chickens of 3 weeks old was used as negative control.

Phagocytic activity and percentage of chicken peripheral monocyte using C. albicans was done according to Richardson and Smith[27] and Barry and Gilsson[28]. The phagocytic activity was calculated according to the following equations:

- Percentage of phagocytosis = Number of ingesting phagocytes X 100 / Total No. phagocytes including non ingesting cells
- Phagocytic index = Total number of phagocytes with or more than 3 blastospores / Total No. phagocytes ingesting blastospores.

The lymphocyte proliferation assay was done by isolation of peripheral blood lymphocytes by FicollHypaque, the mononuclear cell layer was collected and washed and resuspended in RPMI-1640 (Lonza, German y) then the lymphocyte proliferation was measured by using XTT cell proliferation assay kit (ATCC) cat. no. 30-1011K according to the instruction manual and measuring the absorbance of the assay by ELISA reader.

Statistical Analysis: The analysis of variance was outlined as completely randomized design in factorial arrangement using *MSTAT-Cstatisticalpackagesoftware*, there are more significance at 0.05 probability

RESULTS

HA1 gene was amplified by RT-PCR showing the expected size of HA1 which is 1016 bp, the HA1 gene was subcloned in PCI-neo expression vector and the sequence was confirmed and the PCI-neo HA1 construct had strong expression in vero cell line as detected by SDS-PAGE and mRNA detection.

Evaluation of phagocytic activity and percentage of chicken peripheral monocyte (Table 1) showed that there was a highly significant increase in the Phagocytic activity (phagocytosis percent and phagocytic index) to the HA1 DNA vaccine compared to control group ($P<0.05$) ($P=0$). The phagocytic activity increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine mixed with nigella sativa.

Evaluation of lymphocyte proliferation assay (Table 2) showed that there was a highly significant increase in Lymphocyte proliferation to H5 DNA vaccine compared to the control group ($P<0.05$) ($P=0$) and there was no significant difference between lymphocyte proliferation in vaccinated group and positive control ($P>0.05$). The lymphocyte proliferation increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine

DISCUSSION

Previous studies showed that the nigella sativa oil had the ability as a good adjuvant for viral vaccines due to its non specific immunostimulant effect which was represented in significant increase of phagocytic activity, phagocytic index and lymphocyte pathogenesis [7].

The advantages of nigella sativa oil as adjuvant for its non specific immunostimulating effect, anti-inflammatory and antioxidant activity, antimicrobial effect

Table 1: Phagocytic activity and percentage of chicken peripheral monocyte

	Phagocytic %		Phagocytic index	
Days post vaccination	Vaccinated group	Control group	Vaccinated group	Control group
3	75%	62%	0.6	0.2
7	80%	63%	0.7	0.4
14	86%	72%	0.7	0.2
21	83%	65%	0.8	0.5
28	83%	62%	0.7	0.2
35	81%	63%	0.6	0.4
42	78%	72%	0.8	0.2

Table 2: Evaluation of lymphocyte proliferation assay

Days post vaccination	Vaccinated group	Positive control	Negative control
3	0.4	0.6	0.2
7	0.6	0.6	0.2
10	0.5	0.6	0.2
14	0.5	0.6	0.2
21	0.5	0.6	0.2

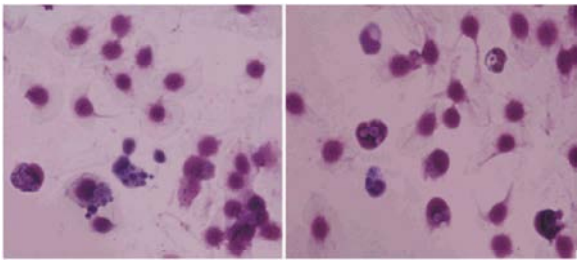

Fig 2: The phagocytic activity in vaccinated group (left) and in control group (right)

and growth promoting effect triggered us to evaluate it as djuvant for preparation of H5 AI DNA vaccine.

Highly pathogenic avian influenza (HPAI) subtype H5N1 is currently endemic inEgypt, with the first outbreaks reported in February 2006 [29], the current H5 vaccines had insufficient efficacy to protect chickens against the newly emerging 2.2.1 variant HPAI H5N1 strains in Egypt [15]. Efficient vaccination against influenza A virus is a difficult task that has not yet been accomplished by immunologists. DNA vaccine seemed to be a promising novel approach for vaccination against influenza A virus [18, 19]and the HA is no doubt the major target for protective immunity against AIV [30], so the goal of this study was to prepare HA DNA vaccine adjuvant with nigella sativa oil.

In our study, an H5 HA1 based DNA vaccine adjuvanted with nigella sativa was produced, expression of HA1 protein was measured and the type of immune response induced by intramuscular injection of a DNA vaccine encoding the HA1 gene of avian influenza H5N1 2010 variant strain was investigated.

Initially, HA1 gene was amplified by RT-PCR showing the expected size of HA1 which is 1016 bp, the HA1 gene was subcloned in PCI-neo expression vector and the sequence was confirmed and the PCI-neo HA1 construct had strong expression in vero cell line as detected by SDS-PAGE and mRNA detection.We evaluated the cell mediated immune response and our results showed strong potent induction of cell-mediated immunity influenza virus response in the chickens vaccinated with DNA vaccine 2 weeks following a single dose of HA1 DNA vaccine. The potent induction of cell-mediated immunity reflects endogenous expression of the antigenic protein either in muscle cells or professional APC after I/M immunization [31].

Our results revealed that there was a highly significant increase in the Phagocytic activity (phagocytosis percent and phagocytic index) to the HA1 DNA vaccine compared to control group ($P<0.05$) ($P=0$). The phagocytic activity increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine mixed with nigella sativa (Table 1 and Fig. 2).

Macrophages play an important role in innate and adaptive immunity as professional phagocytes by internalizing and degrading pathogens [32]. Macrophages are known to function as APC, providing cytokines for the activation of T cells. Macrophages also express the co-stimulatory molecules CD80 and CD86, which play a dominant role in T cell activation. Furthermore, these cells may play an important role in T lymphocyte activation through Ag presentation and coligation of the TCR complex [33].

Our results revealed that there was a highly significant increase in Lymphocyte proliferation to H5 DNA vaccine compared to the control group ($P<0.05$) ($P=0$) and there was no significant difference between lymphocyte proliferation in vaccinated group and positive control ($P>0.05$). The lymphocyte proliferation increased post vaccination up to a peak value reached 2 weeks following the single dose of HA1 DNA vaccine (Table 2). Cellular proliferation is an essential feature of the adaptive immune response, T lymphocytes help both humoral and cellular responses, when a cognate antigen is encountered, lymphocytes become activated; undergo clonal proliferation [33].

Nigella sativa possesses non specific immunostimulant effect [10] and this was revealed in our study showed that using of nigella sativa oil as adjuvant had the ability to increase the phagocytic percent, phagocytic index and lymphocyte proliferation and this results were agreed with Madbouly *et al*. [7] who found that nigella sativa oil ehibit non specific immunostimulant effect as evidenced by increased values of bursal and splenic indices and lymphocyte immune response. Also these results were agreed with Tarek [8] who showed that the nigella sativa oil has the ability to induce cellular immune response as increasing lymphocyte pathogenesis.

Previous studies reported that nigella sativa increased both IL-1B and IL-3 [9] and suggested that nigella sativa had a prominent effect on a subset of CD4 positive T-cells i.e. Th2 subset that secretes IL-3, the enhanced IL-1B production indicates a stimulatory effect on macrophages, either through a direct effect or via IL-3 which had to be a potent inducer of monocyte functional activities of various haemopoietic cell types [34]. Nigella sativa oil is reported to exhibit

immune-potentiating, immune-modulating and interferon-like activities and increase interferon gamma, macrophages and CD4+ T cells [35].

In this study immunization with HA DNA generated potent cell-mediated immunity and it is worth noting that protection against a lethal challenge in the absence of HI titers can be facilitated by cell-mediated immunity, which is consistent with previous reports [36].

Alternatively, the protection observed with the HA DNA vaccine may be mediated solely through the induction of cell-mediated immunity. This conclusion is consistent with reports which have demonstrated that mice that lack mature B cells and do not secrete immunoglobulin can clear an influenza virus infection from the respiratory tract [37] In general, the sufficient and differential cytotoxic T lymphocyte (CTL) immune responses restrict the replication of infected virus and probably eliminate viral infection quickly [38].

CONCLUSION

The results presented in this study showed that HA1 based DNA vaccine with nigella sativa oil adjuvant is a promising novel approach for vaccination against avian influenza virus as it induced potent cell mediated immunity which is sufficient for protection against lethal challenge.

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