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RESEARCH ARTICLE

Evaluation of Nigella sativa's cold press oil as vaccine adjuvant

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Nigella sativa soğuk bası yağının aşı adjuvantı olarak değerlendirilmesi

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Öz

Amaç: Organik ve bitkisel kökenli, düşük düzeyde toksik olan, daha ucuz ve etkili bir adjuvant geliştirilmesi amacıyla soğuk bası Nigella sativa yağı (SBNSY) ve bu yağın temel bileşenlerinden timokiononun adjuvant potansiyeli incelendi.

Gereç ve Yöntem: SBNSY veya timokionon ile ovalbumin verilerek oluşturulan spesifik antikorların ölçümü değerlendirildi. Swiss albino fareler (n=36) A0 (kontrol), A1 (timokionon), A2 (SBNSY), A3 (içeriği; timokionon miktarında % 10 oranında arttırım sureti ile değiştirilmiş olan SBNSY) ve A4 (ticari adjuvant, Al(OH)3) olmak üzere farklı şekillerde verildi. Etkinin değerlendirilmesi, immünizasyondan sonra ovalbumine karşı oluşan spesifik antikoların I-ELISA ile ölçümü yolu ile ve laboratuvarımızda yapıldı.

Bulgular: Serum ve konjugatın optimal sulandırmaları sırası ile 1/200 ve 1/40.000 olarak bulundu. 450 nm'de okunan ortalama OD değerleri kontrol (A0), A1, A2, A3 ve A4 grupları için sırası ile 0,043 (± 0,002), 0,668 (± 0,074), 0,644 (± 0,018), 0,675 (± 0,066), ve 0,745 (± 0,09) idi.

Öneri: Her üç formulasyon da (A1, A2, A3), kontrol grubuna göre ovalbumine karşı sıvısal immün yanıtın oluşturulmasında ticari adjuvant (A4) formulasyonu kadar etkili bulundu ($p > 0,05$). Farede, bu çalışmada denenen her bir Nigella sativa temelli adjuvant adayının potansiyel olarak Al(OH)3'e alternatif olabileceği kanaatine varıldı.

Anahtar kelimeler: Timokionon, adjuvant, ovalbümin, fare

Abstract

Aim: In order to identify an organic and plant based, less toxic, cheaper and more effective adjuvant, a cold pressed oil of Nigella sativa (CPNSO) and one of the essential components of Nigella sativa oil (NSO), thymoquinone (Thymoquinone) were investigated as adjuvant candidates.

Materials and Methods: Adjuvant potentials of the both were measured by examining specific antiibody titers to ovalbumin given in presence of either of CPNSO or thymoquinone, in vivo. Such potentials of thymoquinone alone (A1), CPNSO (A2), mCPNSO (experimentally formed by addition of thymoquinone to CPNSO making up mCPNSO that was contained 10% more thymoquinone than original CPNSO did; A3) and Al(OH)3 (A4) in presence of ovalbumin in Swiss albino mice (n=36, female). The effects were determined by measuring anti-ovalbumin antibodies after immunization to ovalbumin by home-made ELISA in mice.

Results: Sera and conjugate were optimally diluted 1/200 and 1/40.000, respectively. Mean OD values at 450 nm of the groups control (A0), A1, A2, A3 and A4 were 0,043 (± 0,002), 0,668 (± 0,074), 0,644 (± 0,018), 0,675 (± 0,066), and 0,745 (± 0,09), respectively.

Conclusion: By comparison control, all three (A1, A2, A3) of the test formulations were found to be as effective as commercial (A4) formulation in triggering humoral response to ovalbumin ($p > 0,05$). Therefore, each of Nigella sativa based adjuvant candidates has an alternative potential to Al(OH)3 in the mouse.

Keywords: Thymoquinone, adjuvant, ovalbumin, mouse

Introduction

The most effecient, practical, economical and modern approach to struggle against infections is to optimaly maintain all the measures of primary prevention. Vaccination is one of the crucial applications of this type of prevention (Thrusfield 2013). The purpose of vaccination is to maintain a long-term protection from an infection by triggering a strong immune response. Unlike formula of attenuated (or live) vaccines, antigens in killed or subunit vaccines are needed to be combined with adjuvants such as aluminum hydroxide to produce sufficient immune responses. One of the major advantages of these type of vaccines is that they never cause any vaccine infections, making bacterin vaccines superior some times. In veterinary medicine both types of (either killed/subunit or live) vaccines are used widely, anyway.

Most adjuvants likely act by one or more of the following mechanisms; a) enhancing antigen presentation, b) improving antigen stability, c) and immunomodulating anyhow. However, complete mechanism of action is remains uncertain (Heegaard et al 2011, Awate et al 2013). Currently, there is a number of adjuvants other than oil emulsions and alum compounds that are either in clinical trials or already available for commercial vaccine production. Theoretically, selecting an adjuvant depends on many criteria such as types of animal, pathogen or antigen of interests etc. Apparently, there has not been just one adjuvant available that fitting for all the requirements of any kinds of vaccines so far (Spickler and Roth 2003, Awate et al 2013, Sander et al 2019).

Recent research showed that immunomodulatory properties of Nigella sativa Oil (NSO) and its major active ingredient, thymoquinone are remarkable. Both NSO and thymoquinone have been reviewed on how to modulate humoral and cellular immune responses and Th1/Th2 ratio elsewhere (Majdalawieh and Fayyad 2015).

Thymoquinone is apparently the most critical ingredient of NSO. It is responsible for implementing some of the bioactivities as documented by in vitro or in vivo trials conducted so far (although thymoquinone shows some degree of hydrophobicity in the body that limits its bioactivities). Therefore, we now hypothesize that NSO containing higher amounts of thymoquinone than natural NSO is resulted in forming more adjuvant effect, in vivo. Thus, the aim of this study was to compare the adjuvant abilities of thymoquinone (A1), CPNSO (A2), mCPNSO (rich in thymoquinone ingredient, A3) and $Al(OH)_3$ (A4) to generate anti-ovalbumin antibody response in mice.

Material and Methods

Thirty 6-8 week-old clinically healthy swiss-albino female mice were divided into 5 groups; thymoquinone alone (A1), Cold Pressed Nigella sativa oil (CPNSO; A2), modified CPNSO; (mCPNSO; experimentally formed by addition of thymoquinone to CPNSO making up mCPNSO that was contained 10% more thymoquinone than original CPNSO did; A3) and Al(OH)3 (A4). A further six mice were used only once for sampling at the beginning of the study. All cages contained a layer of bedding material. Mice were socially housed during the day and night by grouping. Mice were allowed access to water and food ad libitum. Throughout the experiment mice were examined daily for clinical signs of diseases. A commercial Cold Pressed Nigella sativa oil (CPNSO) (Zade®; 250 mL) was used. NSO contains thymoquinone about 1,56 mg/mL (Khairulla et al. 2016). After filtrations by several times CPNS was then diluted using Dimethyl sulfoxide (DMSO) and distilled water. Endotoxin level was determined by using a commercial kit (Zhanjiang A and C Biological, Zhanjiang, China) and used if it was ≤0.5 EU/mL.

A modified CPNSO (mCPNSO) was obtained by addition of thymoquinone (Santa CruzBiotechnology, sc 215986) to CPNSO. mCPNSO approximately contained 10% more thymoquinone than original CPNSO. Both types of NSO was prepared by under sterile conditions and kept at -8 °C until use.
Ovalbumin (OVA) was prepared according to Garulli et al (2008) with some minor modifications. Briefly; OVA (Ovalbumin 257-264 chicken S7951 Sigma-Aldrich) was diluted using saline solution to concentration of 1 mg/mL (stock solution) and sterilized by filtration using 0,22 µm filters and kept at -20 °C.

OVA mixed with thymoquinone solution or homoginezed in CPNSO or mCPNSO or adsorbed to aluminum hydroxyde were all used for immunizations at doses of 200 µg/mouse (Table 1). OVA concentration in each of these mixtures (OVA-vaccines) was 1mg/mL. Thus, 0,2 mL of each OVA-vaccines (200 µg/ OVA/mouse) were injected to each mouse from trial groups. for immunization. First injections were made by IM route and 14 day after first injections, second administrations of same quatities of OVA-vaccines were made by the same route. Before first injections, blood sampling to collect serum was made from six mice. Two weeks after second injections, all the mice from the groups were blood-sampled.

Tests for safety and sterility of OVA-vaccines

No further test was made for safety since the mouse was the final species. To check sterility of the OVA-vaccines 1ml samples from each was cultured onto the media such as Blood Agar, MacConkey Agar and Sabouraud Dextrose Agar, incubated optimally and examined daily for a week in terms observations for growths.

Table 1. Groups, samplings and immunizations

Group	1st injection (IM) (0.2 ml) of immunization	2d injection (IM) (0.2 ml) of immunization	Blood sampling (Once)
A0	Control (Saline)†	Control (Saline)	2 weeks after 2nd injection of immunization
A1	Tq (0.1 µl) +OVA (200µg)	Tq (0.1 µl) +OVA (200µg)	2 weeks after 2nd injection of immunization
A2	CPNSO (0.1 µl) +OVA* (200µg)	CPNSO (0.1 µl) +OVA* (200µg)	2 weeks after 2nd injection of immunization
A3	mCPNSO (0.1 µl) +OVA (200µg)	m CPNSO (0.1 µl) +OVA (200µg)	2 weeks after 2nd injection of immunization
A4	$Al(OH)_3$ (0.1 µl) +OVA (200µg)	$Al(OH)_3$ (0.1 µl) +OVA (200µg)	2 weeks after 2nd injection of immunization

**OVA: Ovalbumin. Saline: †A salt solution that contais 0.9 percent sodium chloride*

I-ELISA

Anti-OVA antibodies from serum samples that were collected afer two weeks of the second OVA injections were measured by a home-made I-ELISA in the Department's Microbiology Laboratory as described before with some modifications (Li et al 2012). Optimal concentrations for serum and conjugate were found to be 1/200 and 1/40.000, respectively. Adsorbance was read at 450 nm using a microplate reader (Biotek ELX 800, USA).

Statistics analysis

In order to determine whether differences exist among the means of four groups, analysis of variance (ANOVA) and Dunnett t test were used with a $p < 0.05$ test of significance.

Results

In this study, neither local advers effects nor overt signs of distress were observed in any of the mice after immunization injections. OVA antigens in three different experimental and one commercial adjuvants were used as vaccines to immunize mice and production of anti-OVA antibodies from all mice were then measured by I-ELISA. Although the higest figure was determined from the A4, no statistically significant difference was seen between the A4 and any of the A1, A2, or A3 (Table 2).

Mice in all the groups that received OVA-vaccines produced higher titers of antibodies against ovalbumin that were comparable to the control ($p < 0.05$).

Discussion

Vaccine production has a long history in Turkey, going back to the second half of the 19th century. Development of novel adjuvants that would fulfill all the requirements of different types of vaccines is key element in protection animals from the infectious diseases.

OVA is known to be a good choice of antigen for humoral immunity experiments. The toxic dose of OVA in mice 450 µg/mouse/injection (female mouse IP dose) (AbuKhader 2012). In our study the dose and route were 200 µg and IM, respectively. No advers effect was observed.

Table 2. Adjuvant potentials of different substances by I-ELISA (Mean ±SD)

	Gruplar				
	A0	A1	A2	A3	A4
OD	0,043 ± 0,002[a]	0,668 ± 0,074[b]	0,644 ± 0,018[b]	0,675 ± 0,066[b]	0,745 ± 0,09[b]

A0: control, A1: Tq (0.1 µl) +OVA, A2: CPNSO (0.1 µl) +OVA* (200µg), A3: mCPNSO (0.1 µl) +OVA (200µg), *A4:* $Al(OH)_3$ (0.1 µl) +OVA (200µg). *There were six mice per group. ODs were measured at 450nm. Dilutions for serum and conjugate were 1/200 and 1/40000, respectively. Values with different letter differ at p < 0.05.*

By comparison with human vaccine production, veterinary vaccine sector in the world represents lower financial figures although larger number of pathogens and hosts are the reality of veterinary medicine practice (Meeusen et al 2007). Thus, its smaller market share limits the finances allocated to veterinary vaccine research. For example, a commercial papillomavirus vaccine against cervical cancer in human has been estimated to have a market share of more than $ 1 billion (Meeusen et al 2007).

In the other or veterinary side, combined market shares of two vaccines which are considered among the best-selling animal products ever, the FMD vaccine and the *Mycoplasma hyopneumoniae* vaccine is reported to have only 10-20 % of the papillomavirus vaccine in human vaccine market (Meeusen et al 2007). Therefore, veterinary vaccinology has in part a pioneering role and led to new research involving recombinant proteins and plasmid DNAs (Rankin et al 2002). However, there is still a need for new discoveries on producing vaccines that are cost-effective and rich in immunogenicity (Spickler and Roth 2003). This also means that new generation of vaccines still requires more effective adjuvants.

Most adjuvants are either chemicals or substances or components obtained from infectious agents. However, molecules from plants having immunomodulatory properties are also proposed as adjuvants (Hue et al 2003, EL-Mady 2011, Awate et al 2013, Sander et al 2019). Historically, the adjuvant effect of ginseng has been reported earlier than the discovery of a similar effect as shown by NSO. Hu et al (2003) have immunized cattle by OVA with or without ginseng extract and reported that OVA caused significantly higher anti-OVA antibody production when administered in ginseng extract suggesting primarily that there might be some plant based substances, generating some solutions to adjuvant requirement.

The Nigella sativa is a well-known herb in many parts of the world including Mediterranean Basin. NSO by itself or through its bioactive constituents such as thymoquinone or other ingredients exhibit many pharmacological properties including anti-oxidant, analgesic, anti-inflammatory, anti-astmatic, antipyretic, antimicrobial, anticarcinogenic, antihypertensive and immunostimulant (El-Mehdy 2011, Mady et al 2013, Imran et al 2018). Specifically thymoquinone has been reported to have positive effects on cytokine synthesis and maturation process of dendritic type of professional antigen presenting cells (Xuan et al 2010). In accordance with this, thymoquinone modulates mouse CD8+ cells by increasing capability for IFN-γ synthesis (Salem et al 2011). Additionally, it was experimented by Mady et al (2013) that the H5-DNA antigen was first adjuvanted to NSO to prime in chickens and then was observed that NSO has induced a potent cell mediated immunity. Lastly, thymoquinone triggered bovine immune cell blastogenesis, in vitro (Ucan et al 2018). All these data simply recommended us that more thymoquinone should cause higher positive influence on immune system. To our best knowledge, no adjuvant effect of NSO artifically made rich in its thymoquinone content has been experimented before (Xuan et al 2010, El-Mady 2011, Ucan et al 2018). By comparison with an ordinary cold pressed NSO, no statistically significance on adjuvant effect of NSO, fortified by addition of extra thymoquinone was observed by this study ($p \leq 0.05$). The reason for this might be due to the fact that the amount for thymoquinone enrichment should be more than the quantity that was used in this study to get any more outcome. Another explanation might be the natural composition of NSO itself that has an equilibrium. In any case, our hypothesis is rejected since 10% thymoquinone enrichment does not caused any increase in adjuvant effect (higher specific antibody titers).

On the other hand, based on the results of this study, thymoquinone can potentially be added to the list of new adjuvant candidates since it showed similar adjuvant potential to NSO (Table 2). By this research, all adjuvanted OVA trials (groups of thymoquinone, CPNSO, mCPNSO and aluminium hydroxide) have significantly higher anti-OVA antibody responses than the non-adjuvanted trial (control). Additionally, there was no statisticaly significant difference between the responses of mCPNSO and ordinary/natural CPNSO ($p \leq 0.05$).

Conclusion

No significant differences were detected between adjuvant effects of either of thymoquinone, CPNSO, mCPNSO or Al(OH3) in response to OVA in mice. We suppose that more studies on thymoquinone alone or not might help us to understand their bioactive mechanisms that would provide an oppurtunity to develop non-toxic, abundantly produced and well-characterized adjuvants in future.

Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Ethical Approval

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