Research in Veterinary Science 95 (2013) 437–443

Contents lists available at SciVerse ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Nigella sativa seed extract: 1. Enhancement of sheep macrophage immune functions *in vitro*

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ARTICLE INFO

Article history:
Received 5 May 2012
Accepted 22 February 2013

Keywords:
Monocytes-derived macrophages
N. sativa extract
Phagocytic capacity
Nitric oxide production
Microbicidal activity

ABSTRACT

Nigella sativa (*N. sativa*) seed, Black cumin, immunomodulatory activity has been investigated in human and mice. Little is known about the immunomodulatory effect of *Nigella sativa* (*N. sativa*) seed extract on animals' immune cells, specifically, antigen presenting cells such as macrophages. This study focused on the immunomodulatory effect of *N. sativa* seed extract on sheep macrophage functions *in vitro*. Sheep peripheral blood monocytes were isolated and derived to macrophages (MDM). The MDM were cultured with *N. sativa* seed extract and their morphological changes, phagocytic activity, nitric oxide production, and microbicidal activity were investigated. Marked morphological changes were observed in MDM cultured with *N. sativa* seed extract including cell size enlargement; increase in both cytoplasmic space and cytoplasmic granules. Significant increases in phagocytic activity to *Candida albicans* yeast and in number of yeast engulfed per individual MDM were observed in cells cultured with seed extract. MDM capacity to produce nitric oxide was higher in the culture media of the seed extract-cultured cells compared to the control. Interestingly, prominent enhancement in MDM microbicidal activity to yeast or bacteria was observed in MDM cultured with *N. sativa* seed extract confirming the potent immunostimulatory effect of the extract. From this study, it could be concluded that *N. sativa* seed extract can enhance macrophages' important innate immune functions that could control infectious diseases and regulate adaptive immunity.

1. Introduction

Botanical products exhibit a number of beneficial therapeutic properties, and it is thought that the mechanisms involved in these effects are due to the modulation of innate immunity. As reported by the WHO, plants and herbs are chemical factories that directly provide about 25% of currently used drugs as well as another 25% of drugs which are chemically altered natural products (reviewed by Salem, 2005). Many plants and herbs and their products have been used in treatment of numerous illnesses based on religious and cultural traditions in which plants are viewed as sources of health remedies (Huxtable, 1992). *Nigella sativa* (*N. sativa*), commonly known as black seed, belongs to the botanical family of Ranunculaceae, is one of the most useful medicinal herbs that has been traditionally in use in many Middle Eastern and Far Eastern countries as a natural folk remedy for over 2000 years. Nutritional supplementation with *N. sativa* seeds has been stated to enhance general health in both human and animal models. Essential oil (thymoquinone, thymol and dithymquinone) and active ingredients of *N. sativa* seeds (Kanter et al., 2003; Morikawa et al., 2004; Haq et al., 2009) have been reported to have therapeutic effects including: anti-oxidant (Kanter et al., 2003; Yu et al., 2008), anti-inflammatory (Huffman, 2003; Yang et al., 2012), anti-cancer (Huffman, 2003; Salem, 2005) and anti-microbial (Hanafy and Hatem, 1991; Salem and Hossain, 2000; Khan et al., 2003), anti-parasitic (Aboul-Ela, 2002) in addition to a variety of biological responses (Morikawa et al., 2004). In the last few years, some medicinal plants, such as *Panax ginseng*, *Asparagus racemosus*, *Viscum album*, *Ocimun santum*, and *Nigella sativa* (black cumin or black seed) were found to have immunomodulatory activity (EL-Kadi and Kandil, 1989; SaiRam et al., 1997; Mediratta et al., 2002). *N. sativa* aqueous extract was found to modulate BALB/c and C57/BL6 mice splenocytes proliferation, enhance peritoneal macrophages function and to increase natural killer cells anti-tumor activity (Majdalaweih et al., 2010). Thymoquinone, one of the *N. sativa* active ingredients, have been reported to inhibit the dendritic cells inflammatory functions (Yang et al., 2012); to enhance the survival and activity of antigen-specific CD8 T cells *in vitro* and to protect against tumor growth (Majdalaweih et al., 2010 and Xusan et al., 2010).

An invading pathogen must be held in check by the innate immune system until a specific immune response can be mounted. Macrophages are considered one of the most powerful innate lines

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0034-5288/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.rvsc.2013.02.015

of defense against microbial invasion. They have multifaceted functions in both innate and adaptive immunity. In innate immunity, macrophages participate in the inflammatory response via phagocytosis; generation of toxic oxygen radicals, reactive nitrogen intermediates; and secretion of diverse array of cytokines that regulate a protective inflammatory response. In cell-mediated immunity, macrophages first present microbial antigens to T cells, and then, activate lymphocytes by cytokines and through co-stimulatory signals (Roitt et al., 1998; Thomas et al., 2006; Janeway et al., 2008).

It is well documented that macrophages are target for many pathogens and have been shown to have a vital role in their pathogenesis (Santos et al., 2006). Ovine macrophages have been shown to play role in transmission of some pathogens including: Respiratory Syncytial Virus (Fach et al., 2007); Visna/Maedi virus (McNeilly et al., 2008); poxvirus (Embury-Hyatt et al., 2012); bluetongue virus (Maclachlan, 2011); *Brucella melitensis* (Fatma and Zabit, 2008) and many others. Modulating macrophages functions in sheep and in other animal species might be of importance in microbes pathogenesis and hence in protection against microbial invasion.

Although some studies have focused on examining the potential immune-modulatory and immunopharmacological effects of *N. sativa* and its active ingredients on humans and mice immune cells and have some conflicts in the obtained results. Such studies are also still relatively scarce, and hence, further investigation is needed to confirm earlier findings and explore the direct effect on immune cells of this promising medicinal herb. Also, previous studies did not explain if *N. sativa* immunomodulatory is species specific or not. However, little is known about the direct *in vitro* immunomodulatory effects of *N. sativa* products on animals' macrophages, in specific, sheep immune cells. The aim of the study was to explore the direct immunomodulatory activity of purified extract from *N. sativa* seeds on sheep macrophage functions *in vitro*.

2. Materials and methods

2.1. Animals

Fifty Balady sheep, about one year-old of either sex weighing 45–80 kg were usedas blood donor in this study for peripheral blood mononuclear cell separation and autologous sera collection. Sheep were housed at the farm of the Faculty of Veterinary Medicine, Zagazig University, Zagazig, Egypt. The study was performed in compliance with institutional guidelines for research on animals.

2.2. Microbial strains

Candida albicans (*C. albicans*) and multi-drug resistant *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) bacteria previously isolated from cows' mastitic milk and cows' vaginitis cases, respectively, were used to study the MDM microbicidal activity. These isolates were also resistant to *N. sativa* extract after testing on Muller-Hinton agar as confirmed before starting this study.

2.3. Nigella sativa (N. sativa) seed extract

Nigella sativa (*N. sativa*) seed purified extract was prepared by Medical Egyptian Plant and Pharmaceutical Company (MEP Co., Inshas, Sharkia Governorate, Egypt) according to the method found in the British pharmacopoeia book, 1984. *N. sativa* crude constituents' concentration was 10 mg/ml. The extract was filter-sterilized using 45 μm syringe filter, stored at −20 °C in dark container.

2.4. Generation of monocytes-derived macrophages

Sheep monocytes were isolated in 6-well or 24-well plates according to the method described by (Goddeeris et al., 1986) and then derived to functional monocytes-derived macrophages (MDM) according to the method described by (Elmowalid, 2002; Elmowalid, 2012). Briefly, 2 ml/well of complete, slightly acidic (pH 6.7), RPMI-1640 (Life Technologies, Grand Island, NY) containing 10% autologous serum and penicillin (100 IU/ml; streptomycin (100 μg/ml); and amphotericin B (25 μg/ml) was added to the adherent monocytes and the cells were incubated for 24 h at 37 °C in a humidified incubator under 5% CO_2 tension. After incubation, the conditioned culture media were removed and replenished by 1 ml/well of fresh pre-warmed complete RPMI-1640 (pH 6.7); 5% autologous serum and antimicrobials. The conditioned media were centrifuged at 800*g* for 20 min at room temperature to pellet the non-adherent cells and any cell debris. One milliliter of conditioned media was added to each well for a total of 2 ml/well. The adherent cells were cultured for an additional 48–72 h to allow monocytes to mature to functional macrophages with the media being changed every 24 h as described. The cells were examined each other day for any morphological, phenotypic, and functional changes.

3. Immunomodulatory activity of the *N. sativa* seed extract

Because most of the previous studies had focused only on humans and mice macrophages; because of the important role of macrophages in microbial pathogenesis; and because of the lack of data on the *N. sativa* direct immunomodulatory effect on sheep macrophages, our study was designed to investigate the direct effect of purified extract containing: nigellone, thymohydroquinone, and thymol (the main active ingredients) at total concentration of 2 mg/ml. In this study, blood was collected from at least eight randomly selected sheep in each experiment. Each experiment was repeated at least 3 times for reproducibility. All the concentrations of *N. sativa* seed purified extract mentioned in this study were predetermined in our laboratory through time and concentration-study curve.

3.1. N. sativa seed extract effect on monocytes-derived macrophages morphology

Functionally adherent MDM were cultured in 6-well plates at concentration of 1.5×10^6 cells/well in complete RPMI-1640 medium, *N. sativa* seed purified aqueous extract was added at final concentration of 40 μg/ml and the cells were incubated for 18 h at 37 °C. Wells without *N. sativa* purified seed extract served as control. After incubation, the medium was aspirated and the cells were washed with PBS. Subsequently, the cells were stained using Leishman's stain (Aljoumhoria Chemical Co., Cairo, Egypt) according to the vendor's protocol. MDM cells were examined for morphological changes under light microscopy at 900× total magnification. In some cases the cells were directly examined under inverted microscope at 400× total magnification and then photomicrographed.

3.2. MDM phagocytic capacity

MDM were treated in 24-well plate with 40 μg/ml aqueous extract of *N. sativa* seed for 18 h, then cell phagocytic capacity was determined (Frankenberger et al., 2000; Elmowalid, 2002; Elmowalid 2012). In brief, the culture media were removed and 300 μl/well of complete RPMI-1640 and FITC (Sigma, St. Louis, MO, U.S.A) – labeled yeast (ratio 50 labeled *C. albicans*/one MDM)