Anti-oxidant and Anti-free Radical Activities of Natural Oil Blend Formulation: Implication for Immune Regulation and Potential Adjuvant Treatment in Infectious Diseases

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Highlights

a. The obtained result has great implication for immune regulation and adjuvant treatment in infectious diseases.

b. Natural Oil Blend is a combination of the following oils Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgarea, and Daucus carota with the purpose of viral inactivating properties.

c. Using quantitative flow cytometry, the influence of Natural Oil Blend on neutrophils phagocytosis and their respiratory burst induction were studied.

d. Natural Oil Blend enhances human neutrophils phagocytosis but dramatically decreases chemotactic peptide N-formyl-Met-Leu-Phe (fMLP)-, phorbol 12-myristate 13-acetate (PMA)-, and E. coli-induced oxidative burst.

e. Natural Oil Blend like alfa-tocopherol was able to promote the scavenging of free radicals, displaying a high dose–response representing a significant anti-oxidant activity.

f. Natural Oil Blend may serve as a potent pharmaceutical agent with combined anti-inflammatory, antimicrobial and anti-oxidant activity capable restoring impaired intracellular production of ROS and down-modulation of overwhelming inflammatory response of innate immune cells to chemotaxis- and phagocytosis-mediated respiratory burst induction.

Abstract

In the present work, the human peripheral blood polymorphonuclear neutrophils phagocytosis, neutrophils oxidative burst regulation, and anti-free radical activity of Natural Oil Blend (NOB) contained Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgarea and Daucus carota essential oils evaluated. Using quantitative flow cytometry in this study we investigated the influence of NOB on neutrophils phagocytosis, and also on their respiratory burst induction. We demonstrated that NOB enhances human neutrophils phagocytosis but dramatically decreases chemotactic peptide N-formyl-Met-Leu-Phe (fMLP)-, phorbol 12-myristate 13-acetate (PMA)-, and E. coli-induced oxidative burst. Furthermore, the antioxidant activity of NOB confirmed by in vitro studies the anti-free radical scavenging activity.
We observed that OB like αL-tocopherol was able to promote the scavenging of free radicals, displaying a high dose-response representing a significant anti-oxidant activity. Therefore, our results suggest that NOBF may serve as a potent pharmaceutical agent with combined anti-inflammatory, antimicrobial and anti-oxidant activity capable restoring impaired intracellular production of ROS and down-modulation of the overwhelming inflammatory response of innate immune cells to chemotaxis- and phagocytosis-mediated respiratory burst induction. This result has great implications for immune regulation and adjuvant treatment in infectious diseases.

Introduction

Natural oils are a mixture of volatile compounds (mainly mono- and sesquiterpenoids, benzenoids, amino acid-derivatives, phenylpropanoids, etc.). Natural oils also obtain a wide range of pharmacological actions, e.g. antimicrobial, antifungal, anti-inflammatory, antioxidant, hyperemic, anti-nociceptive, spasmyloytic, diuretic, choleretic, and carminative effects[1,2]. For centuries, natural oils used for combating infection in various cultures. Nowadays, the use of natural antimicrobial agents is noticeably growing for a wide variety of purposes including human and animal medication, food industry, and environmental protection[3].

Previously, we demonstrated that the natural oil blend formulation (NOBF) which prepared from a mixture of Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgare and Daucus carota, as well as other combination of natural oils[4], act as a potent antimicrobial and antifungal agent. The natural oil blend formulation (NOBF) inhibited the growth of several tested microorganisms, including gram-positive Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, gram-negative Pseudomonas aeruginosa, as well as the strains of fungi Candida albicans. The most prominent antimicrobial activity of NOBF observed against Staphylococcus epidermidis and Pseudomonas aeruginosa. Besides, NOBF demonstrated a prominent anti-inflammatory effect as reflected by the suppression of IL-1β, TNFα, IL-6, and IFNγ production in a dose-dependent manner by LPS-stimulated human mononuclear cells.

The phagocytic cells of the innate immune system such as neutrophils and macrophages are key components of antimicrobial host defense, which is because these cells are capable of generating large amounts of highly toxic molecules - reactive oxygen species (ROS)[5,6]. When phagocytes are activated, the production of ROS is initiated by NADPH oxidase because of the translocation of several cytosolic proteins (gp67phox, gp40phox, gp47phox, and the Rho-family GTPases, Rac2) to the membrane-bound complex carrying cytochrome b558 (gp91phox-gp22phox-Rap1a). The primary product of the reaction catalyzed by NADPH oxidase is superoxide (O2-), which can be converted to H2O2 by superoxide dismutase, to hydroxyl radicals (OH) and hydroxyl anion (OH-). ROS generated by phagocytes can damage DNA and several chemical moieties and are of central importance in host defense and inflammation[7,8].

Oxygen radicals generated in excess in a diverse array of microbial and viral infections. Emerging concepts in free radical biology are now shedding light on the pathogenesis of various diseases. Reactive oxygen species generated by infiltrating phagocytic cells and xanthine oxidase expressed in inflamed tissues and they believed to contribute to nonspecific (innate) and immunological host defense as well[9]. Free radical-induced pathogenicity in virus infections is of great importance, because evidence suggests that oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases including severe acute respiratory syndrome coronavirus (SARS-CoV) induced acute respiratory distress syndrome (ARDS)[10].

Therefore, the NOBF-induced production of ROS upon phagocytic activation of effector cells and oxidative burst may be natural for killing several micro-organisms, as shown by the resistance of NOBF-treated animals to bacterial and viral infection. Using quantitative flow cytometry in this study we investigated the influence of NOBF on human peripheral blood polymorphonuclear neutrophils (PMN) phagocytosis, and also on their respiratory burst induction. We demonstrated that NOBF enhances human PMN phagocytosis but dramatically decreases chemotactic peptide N-formyl-Met-Leu-Phe (fMLP)-, phorbol 12-myristate 13-acetate (PMA)-, and E. coli-induced oxidative burst. Furthermore, the anti-oxidant activity of NOBF confirmed by in vitro studies the anti-free radical scavenging activity.

Materials and Methods

Natural Oil Blend formulation (NOBF) preparation and composition

Each single oil Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgare and Daucus carota obtained from the vendors who comply with the strictest industry practices: Demeter Agro Research and Improvements Pty Ltd, New Directions Australia Pty Ltd and Australian Botanical Products Pty Ltd. Each natural oil is obtained through the steam distillation process and should undergo thorough checking for the quality and chemical compositions based on European Pharmacopeia. After the natural oils are declared to pass the quality checking, the mixture of the NOs conducted with the following sequence and percentage: Gardenia jasminoides, Commiphora myrrha, Boswellia serrata, Foeniculum vulgare and Daucus carota are added in equal quantities to form
the oil mixture they mixed with extra light olive oil. Hence, we get 1% concentration of the final product.

Peripheral blood sampling

After signed informed consent, 30 ml of venous blood samples obtained from 6 healthy subjects, 30 ml of venous blood samples 3 men (mean age of 25 ± 3.1 years) and another 30 ml from 3 women (mean age of 26 ± 3.4 years). The Ethical Committee approved the experiment of the Institute of Molecular Biology of the NAS RA (IRB 10RG0003427). Healthy volunteers selection was conducted based on reviews of medical history with the following exclusion criteria 1) studied subjects were not taking any medication at the time of the experiments; 2) none of the individuals suffered from acute/ chronic diseases or abnormalities; 3) smoking, consumption of alcohol, drugs and current or past use of any active substance; 4) pregnancy or lactating mothers.

Cytotoxicity of NOBF on human whole blood cells

Assessment of Natural oil blend formulation (NOBF) cytotoxicity performed with trypan blue dye exclusion and flow cytometry. Both techniques used to determine the number of viable cells present in a whole blood samples after 0-2 h incubation with NOBF and PMA. Cytotoxicity <30% was accepted as a criterion for valid positive result in this assay.

Neutrophil Phagocytosis

Assessment of natural oil blend formulation (NOBF) cytotoxicity performed with trypan blue dye exclusion and flow cytometry. Both techniques used to determine the number of viable cells present in whole blood samples after 0-2 h incubation with NOBF and PMA. Cytotoxicity <30% was accepted as a criterion for a valid positive result in this assay. Whole blood samples were incubated with 6×10-3 and 6×10-4 µg/µl NOBF were added to 100 µl of whole blood and incubated at 37°C for 10 min. DHR123 solution added and the tubes incubated for an additional 10 min at 37°C. After erythrocyte removal by lysing solution the DNA of the remaining cells was stained with propidium iodide and immediately subjected to flow cytometry analysis (10,000 events). The respiratory burst monitored by determining the percentage of fluorescing cells on a FACSCaliburTM instrument using CellQuestTM software (Becton Dickinson).

Antioxidant assay

The antioxidant activity of the NOBF was evaluated by DPPH radical scavenging assay using methodologies previously described [12]. A stock solution of DPPH reagent (0.5 mM) prepared in methanol. The solution was diluted with methanol (60 µM approx.) to yield an initial absorbance of 0.62 ± 0.02 at 517 nm. The reaction mixture was composed of 1950 µl of DPPH solution and 50 µl of the oil, diluted with different proportions of methanol. For each sample, a methanol blank also used. we used Alfà-tocopherol as a standard antioxidant. The absorbance measured after 10 and 60 min for the samples and standard, respectively. The DPPH-inhibition percentage calculated the radical-scavenging activity according to the equation: % = 100 (A-B)/A, where A and B are the blank and sample absorbance values, at the end of the reaction. Linear regression determined the concentration of antioxidant required for 50% scavenging of DPPH radicals (half-maximal effective concentration or EC50). The analysis performed in triplicate and the result presented as mean value ± standard deviation.

Statistics

Statistical analyses performed using the statistical software Graph Pad Prism 5.01 (Graph Pad Software, USA). Data compared using the One-way Repeated Measure ANOVA test. Normal distribution was checked visually from distributions and with Shapiro-Wilk’s W test. P values ≤ 0.05 considered as significant. Results expressed as the mean and standard error of the mean.

Results

The effect of natural oil blend formulation (NOBF) on PMN phagocytosis

First, we tested the cytotoxicity of different concentrations of NOBF on human whole blood cells by using both trypan blue dye exclusion and flow cytometry. natural oil blend formulation (NOBF) showed cytotoxic effects at the highest 6×10-2 µg/µl concentration and the absence of toxic effect of NOBF at 6×10-3 – 6×10-5 µg/µl.
concentrations. Next, we investigated the influence of NOBF on human peripheral blood PMN phagocytosis using quantitative flow cytometry. The obtained results of the flow cytometry study of PMN phagocytosis is provided in Figure 1. We observed that NOBF significantly increased (p<0.05) the PMN phagocytic function (i.e., percentage of cells able to undergo phagocytosis) comparing with the untreated control in the presence of both $6 \times 10^{-3}$ and $6 \times 10^{-4}$ µg/µl concentrations [Figure 1]. We did not observe significant differences between $6 \times 10^{-3}$ and $6 \times 10^{-4}$ µg/µl concentrations of NOBF on PMN phagocytic function in the whole blood model. Therefore, NOBF increased PMN phagocytic function which could be the innate immune basic mechanism underling antimicrobial activity of NOBF previously observed in vivo animal models [13-15].

![Figure 1: NOBF effect on normal donors whole blood PMN phagocytosis. Results are the means SD. NOBF 1 - $6 \times 10^{-3}$ µg/µl, NOBF 2 - ($6 \times 10^{-4}$ µg/µl concentrations. *p<0.05, comparing with untreated control.](image)

![Figure 2: Antioxidant activity of the NOBF in DPPH radical scavenging assay.](image)

**The effect of natural oil blend formulation (NOBF) on the respiratory burst of PMN.**

Although PMN cell population exerts antimicrobial activity against extracellular bacteria, not only through phagocytic function, there is also more antimicrobial activity through the utilization of ROS production for the control of intracellular microbes. The results of the flow cytometric determination of PMN respiratory burst, using the fluorescence probe DHR123, which is highly specific to superoxide anion, hydrogen peroxide, and hypochlorous acid [7,8], are presented in Table 1. According to the presented data, the NOBF in both used concentrations didn’t influence the number of PMN undergoing respiratory burst in the absence of ROS stimulators. However, we observed that NOBF dramatically decreases chemotactic peptide N-formyl-Met-Leu-Phe (fMLP)-, phorbol 12-myristate 13-acetate (PMA)-, and E. coli-induced oxidative burst comparing to the untreated whole blood control (p<0.05). Again, we did not observe significant differences between $6 \times 10^{-3}$ and $6 \times 10^{-4}$ µg/µl concentrations of NOBF on PMN respiratory burst in the whole blood model. Together, these results suggest that NOBF possesses a pronounced ability to down-regulate PMN oxidative burst induced by strong pro-inflammatory stimulators like fMLP, PMA, and bacterial cells.
Table 1: Oxidative burst induction of whole blood PMN and Natural oil blend formulation (NOBF) effects.

|                      | Spontaneous | fMLP | PMA | E. coli |
|----------------------|-------------|------|-----|---------|
| Whole blood control  | 11.2±2.5    | 57.1±13.9 | 98±0.8 | 99.6±0.1 |
| Whole blood + 6*10⁻⁴ µg/µl NOBF | 11.6±3.9 | 31.9±5.5 | 57.4±2 • | 56.5±3.2 • |
| Whole blood + 6*10⁻³ µg/µl NOBF | 14.5±5.2 | 37.1±13.6 | 46.6±12.1 • | 51.7±13.2 • |

Results are the means ± SD. •p ≤ 0.05 versus non-NOBF -treated control.

Antioxidant Activity

In the present work, the in vitro antioxidant activity of the NOBF evaluated by DPPH radical scavenging. The oil interacted with the DPPH by transfer of electron or hydrogen, neutralizing its free radical character [12]. The oil was able to promote the scavenging of DPPH radical, displaying a high dose-response (r² = 0.96), with inhibition varying from 5 to 65 %, at concentrations from 5 to 50 µg/ml. The half-maximal effective concentration (EC50) was 40.0 ± 3.5 µg/ml, calculated by linear regression (p < 0.05), representing a significant antioxidant activity and comparable to the standard alfa-tocopherol (38.8 ± 2.9 µg/ml), as can be seen in Figure 2.

Discussion

Natural oil blend formulation (NOBF) inhibited the growth of several tested microorganisms in vitro, including gram-positive Staphylococcus aureus, Staphylococcus epidermidis, Micrococcus luteus, gram-negative Pseudomonas aeruginosa, as well as the strains of fungi Candida albicans [4]. The most prominent antimicrobial activity of NOBF observed against Staphylococcus epidermidis and Pseudomonas aeruginosa.

While inflammatory response required for effective host defense during infections, exaggerated or uncontrolled inflammation is the main cause of healthy tissue destruction and chronic pathologies. Natural Oil Blend formulation (NOBF) was designed not solely as a formulation with an antimicrobial and antifungal potency but also as an anti-inflammatory agent, capable to limit pathogen-induced inflammation [4]. Natural oil blend formulation (NOBF) demonstrated a prominent anti-inflammatory effect as reflected by the suppression of IL-1β, TNFα, IL-6, and IFNγ production in a dose-dependent manner by LPS-stimulated human mononuclear cells. Production of anti-inflammatory IL-10 by LPS-primed cells was also suppressed by NOBF in a dose-dependent manner [4].

The ROS and reactive nitrogen species (RNS) as central components of the “respiratory burst” in activated leukocytes, play an natural role in the host immune defenses against pathogens [13,14]. However, based on their nonspecific and highly reactive nature, products of redox metabolism (e.g., O₂−, OH−, ONOO−, HClO, H₂O₂ and NO) may be potentially harmful to host cells [12, 20]. Apart from virulence factors expressed by pathogenic bacteria and viruses, the excessive production of ROS and RNS by activated immune cells creates a highly cytotoxic milieu that contributes to the direct damage of target organs in the context of unbalanced inflammatory responses [14-17]. Therefore, the modulation of the host’s redox system as an antimicrobial therapeutic strategy is challenging. The up regulation of ROS and RNS production may increase cytotoxicity and organ damage, while the downregulation of these molecules may favor the survival and spread of pathogenic microorganisms, leading to opposite scenarios with a similar endpoint, including higher mortality of the host [16,17].

Here we demonstrated that NOBF up-regulated human PMN phagocytic function but dramatically down-regulated fMLP- and PMA-as well as bacteria (opsonized E. coli)-induced, an oxidative burst of neutrophils. Increased level of ROS production in PMN during inflammatory conditions characterized by the impairment of PMA (protein kinase C)-dependent regulation of ROS production and also the over-regulation of chemotaxis/inflammation-mediated respiratory burst induction as well as phagocytosis-dependent activation of ROS production. In the present study we show that NOBF is a differentially regulated oxidative burst induction in PMN. In this study we demonstrated that NOBF does not affect human PMN spontaneous oxidative burst but dramatically decreases fMLP- and PMA-induced, an oxidative burst of cells [Figure 1 & Table 1]. Accordingly, NOBF could serve as a potent antioxidant capable of restoring impaired intracellular production of ROS in PMN and down-modulation of the overwhelming inflammatory response of PMN to chemotaxis- and phagocytosis-mediated respiratory burst induction.

The cells of living organisms generate free radicals as a result of pathophysiological and biochemical processes in response to factors such as, radiation, chemicals, toxins, and bacterial and viral infection. The NOBF creates an imbalance in the formation and neutralization of prooxidants that subsequently seek stability through electron pairing with biological macromolecules such as proteins, lipids, and DNA, leading to oxidative stress in the physiological system [18]. Furthermore, these effects lead to lipid peroxidation as well as protein or DNA damage in human cells, which consequently lead to aging and several chronic diseases such as cancer; diabetes, and atherosclerosis as well as cardiovascular; inflammatory, and other degenerative diseases in humans [18,19]. The ability of certain phytochemical extracts to inhibit or delay the oxidation of other molecules by suppressing the initiation or propagation of oxidizing chain reactions have made them active alternatives in complementary medicine [19-22]. These naturally
occurring antioxidants have reported to exhibit antioxidant and anticancer activities [21,22].

In the present study, the in vitro antioxidant activity of the NOBF -induced production of ROS upon phagocytosis activation of effector cells and oxidative burst may be essential for killing several microorganisms, as shown by the resistance of NOBF -treated animals to bacterial and viral infection. Using quantitative flow cytometry in this study we investigated the influence of NOBF on human peripheral blood polymorphonuclear neutrophils (PMN) phagocytosis, and also on their respiratory burst induction. We demonstrated that NOBF enhances human PMN phagocytosis but dramatically decreases chemotactic peptide N-formyl-Met-Leu-Phe (FMLP), phorbol 12-myristate 13-acetate (PMA), and E. coli-induced oxidative burst. Furthermore, the anti-oxidative activity of NOBF confirmed by in vitro studies the anti-free radical scavenging activity. It was evaluated by DPPH radical scavenging [Figure 2]. The NOBF like α-tocopherol was able to promote the scavenging of DPPH radical, displaying a high dose-response representing a significant antioxidant activity.

Antioxidant systems seem to play a crucial role in maintaining the morphological and functional integrity of all microorganisms and viral particles. Accordingly, disruptors of redox balance (e.g., inducers of oxidative stress or inhibitors of antioxidant molecules) proposed as candidates to new antimicrobial drugs [23]. Several drugs developed for different conditions (e.g., anti-inflammatory, antineoplastic, antidepressant, anxiolytic, and antipsychotic) also exhibit antimicrobial properties [23]. In many cases, these properties based on the disruption of microbial redox metabolism, a pharmacological effect that opens new avenues for drug repurposing and development of new strategies for the treatment of infectious diseases [24].

Cytokine storm is an excessive immune response to external stimuli. The pathogenesis of the cytokine storm is complex. The disease progresses rapidly, and mortality is high. Certain evidence shows that, during the coronavirus disease 2019 (COVID-19) epidemic, the severe deterioration of some patients has closely related to the cytokine storm in their bodies [24,25]. The pathogens then mediate the recruitment of immune cells, which eliminates the pathogens and ultimately leads to tissue repair and restoration of homeostasis. However, SARS-CoV-2 induces excessive and prolonged cytokine/chemokine responses in some infected individuals, known as the cytokine storm [26]. Cytokine storm causes ARDS or multiple-organ dysfunction, which leads to physiological deterioration and death. Timely control of the cytokine storm in its early stage through such means as immunomodulators and cytokine antagonists, as well as the reduction of lung inflammatory cell infiltration caused by ROS, would facilitate in improving the treatment success rate of patients with infectious diseases.

Conclusion

Natural oils used for our blend thought to have a strong biological potency associated with a suppression of unwanted inflammation and bacterial and viral infection. Disruption of the host excessive production of ROS and redox metabolism pronounced antioxidant activity and the ability to increase a key component of antimicrobial host defense.

The phagocytic activity of the innate immune system pronounced such as neutrophils, a pharmacological effect that opens new venues for Natural oil blend formulation (NOBF) development of new strategies for the adjuvant treatment of infectious diseases. Therefore, it has anticipated that overlapping action of different natural compounds will amplify antimicrobial potency, antioxidant activity and influence the magnitude of immune responses and restrict exaggerated production of ROS and cytokines, supporting the regulation of immune response to inflammatory stressors.

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Conflict of interest

The authors declare no conflict of interest.

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