Dietary supplementation with Biobran/MGN-3 increases innate resistance against viral infections that cause influenza-like illnesses in elderly subjects: a randomized, double-blind, placebo-controlled clinical trial

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Introduction

Immunodeficiency of aging is manifested in terms of increased infections, autoimmune disorders, and cancers in geriatric population. Immunodeficiency is apparently a multi-factorial problem, where the loss of immunologic vigor in geriatric population plays a major role in the above-mentioned diseases. This has been substantiated with the reported age-dependent reduction of leukocyte qualitative functions in many studies (Fulop et al., 1985; Tortorella et al, 2002; Tortorella et al., 2006; Sansoni et al., 2008). Of particular interest was the reported age-dependent reduction of natural killer (NK) cells, which constitutes the primary defense line against malignant and virally-infected cells (Lotzova 1984; Moretta et al., 2002; Chretien et al., 2014; Ivarsson et al, 2014). Enhancing NK cell activity is therefore an essential therapeutic target in geriatric population.

Biobran/MGN-3 is defined by the Medicines and Healthcare products Regulatory Agency (MHRA) as a food supplement (McDermott, 2006). Biobran/MGN-3 is manufactured by hydrolyzing rice bran with the enzymatic extract of medicinal Shiitake mushrooms (Ghoneum and Jewett, 2000; McDermott, 2006). Enzyme hydrolysis of rice bran produces arabinoxylane, a hemi-cellulose compound, which constitutes the active ingredient of Biobran/MGN-3. Several studies demonstrated the potent immunomodulator effect of Biobran/MGN-3 (Ghoneum and Jewett, 2000; Ghoneum and Gollapudi, 2003; Badr El-Din et al., 2008; Ghoneum and Agrawal, 2011; Cholujova et al., 2013; Ghoneum et al., 2014). For example, earlier studies by Ghoneum have shown the superb capability of MGN-3 to enhance NK cell activity in human subjects aged 20-46 years (Ghoneum 1998). The induction of NK cell activity reached 2-3 folds of the baseline level after one month of supplementation. Biobran/MGN-3-induced upregulation of NK cell activity was associated with an increase in the granular content of NK cells. Other studies demonstrated that Biobran/MGN-3-induced NK cell activity was associated with increased Tumor Necrosis Factor (TNF-α, a cytokine involved in activating the immune system) and increased number of NK cells, T-cells and B-cells (Ghoneum and Jewett, 2000).

Influenza is a seasonally-epidemic viral infection causing 3-5 million severe illnesses and up to approximately 500,000 annual deaths around the world (Stohr 2002; Lee 2011). The elderly are more susceptible to influenza infection and complications, which was attributed to the phenomenon of immunosenescence or age-associated decline of immune system activity (Pop-Vicas and Gravenstein 2011; Talbot 2017). Diagnosis of influenza can't be made without virological confirmation, which is costly and rarely performed in public health surveillance systems. The World Health Organization (WHO) has recently revised the definition of ILI to be "acute respiratory illness with a measured temperature of ⩾ 38 ° C and cough, with onset within the past 10 days" (Fitzner et al., 2017). Mounting effective innate immune response against influenza and other viral infections involve detection of viral infection by the intracellular foreign nucleic sensors Retinoic acid-inducible gene 1 (RIG-1) and Melanoma Differentiation-Associated protein 5 (MDA5) and induction of downstream antiviral proteins such as interferon-stimulated genes 15 (ISG15) and Myxoma Resistance Protein 1 (MX1) (Barral et al., 2009, Baum et al., 2010, Takeuchi and Akira 2010, Schoggins and Rice 2011).

In the current study, we test the hypothesis that Biobran/MGN-3 could reverse the decline of NK cell activity and improve resistance to influenza-like illnesses in older adults.

Rationale of the study

Aging is associated with decline of NK cell activity, which renders geriatric population more susceptible to viral infection and malignancies. Biobran/MGN-3 dietary supplement was shown to increase NK cell activity in young adults and is expected to produce the same effect in geriatric population. Increase NK activity would increase resistance to viral infections and cancers in older adults.

Research questions

1. Could Biobran/MGN-3 supplementation reduce the incidence of influenza-like illnesses in older adults?

2. Could Biobran/MGN-3 supplementation reduce the incidence density of influenza-like illnesses in older adults?
3. Does Biobran/MGN-3 supplementation increase NK cell activity in the geriatric population?
4. Does Biobran/MGN-3 supplementation upregulate expression of RIG-1, MDA5, ISG15, MX1?

Hypothesis

Biobran/MGN-3 dietary supplements increases NK cell activity, upregulates RIG-1, MDA5, ISG15, MX1, and improves resistance to influenza-like illnesses in geriatric population.

Aim of the study

To improve the quality of life together with reduction of health care costs among geriatric population via increasing immune system activity against infections and malignancy.

Specific objectives

1. To assess the effect of daily dietary supplementation with Biobran/MGN-3 (500 mg/d for 3 months) on the incidence rate of influenza-like illnesses in older adults. The incidence rate will be calculated by dividing the number of incident ILI cases by the total number of the group participants.
2. To assess the effect of daily dietary supplementation with Biobran/MGN-3 (500 mg/d for 3 months) on the incidence density of influenza-like illnesses in older adults. The incidence density will be calculated by dividing the number of incident cases by the total person-time at risk.
3. To assess the effect of daily dietary supplementation with Biobran/MGN-3 (500 mg/d for 3 months) on NK number and activity in geriatric population. Activity of NK cells will be assessed using the degranulation assay.
4. To assess the effect of Biobran/MGN-3 (100 μg/ml for 72 h) on the pulmonary epithelial BEAS-2B cell lines expression levels of viral nucleic acid sensors Retinoic Acid-Inducible Gene 1 (RIG-1) and Melanoma Differentiation-Associated protein 5 (MDA5) as well as some of their downstream antiviral proteins Interferon-stimulated Genes 15 (ISG15), and Myxoma Resistance Gene 1 (MX1) using flow cytometry.

Subject and Methods

A) Technical design

1. study design:

Double-blind, placebo-controlled clinical trial. The study will span the time period from November 2018 till the end of February 2019, a period with known peak incidence of ILI attacks (Reafy et al., 2016).

2. Setting:

Outpatient clinics, Zagazig University Hospitals, and follow-up at home with phone calls and visits.

3. Subjects:

Ninety apparently healthy older adults ≥ 56 years were originally recruited from the residents of Zagazig city visiting outpatient clinics at Zagazig University Hospitals. Only local residents were recruited so as to facilitate follow-up home visits and to reduce the dropout rate. Old age is associated with NK cell senescence. We recruited subjects ≥ 56 years of age in order to meet the WHO definition of old age in African nations (32). In addition, this age is close to public service retirement in Egyptian society, which is associated with significant social, mental, and psychological stress and therefore could be associated with significant decline in NK activity.

Males and females were randomly assigned into two groups (n=40/group) that received either placebo or Biobran/MGN-3 (500 mg/day for 3 months). Both the health care giver and the participants were blinded to the ingested supplement. Participants' health were monitored via weekly home visits and they were instructed to report any complaint or side effect by phone to the health care giver.

Diagnosis of ILI was made by documenting the incidence of acute respiratory illness with a measured temperature of ≥ 38 °C with cough (Fitzner et al., 2017). After diagnosis, the subject was helped to follow the proper health management

plan by the health care giver. During the study, all participants were required not to take any vitamins or medications during the study without consultation.

Inclusion criteria:

1. Ages of 56+ years
2. Both sexes will be included.
3. Willing to participate in the study and give a written consent.

Exclusion criteria:

1. Subjects who took influenza vaccine, cortisone, or any other immunosuppressive agents such as radiation or chemotherapy.
2. Diagnosed with infections or malignancies.
3. Presence of auto-immune disorders.
4. Pregnant and lactating ladies.
5. Marked portal hypertension, pancytopenia, renal, or kidney failure.
6. Presence of major psychological insult or under medication for psychological insult.
7. Presence of chronic infections such as TB or HBV.
8. Inability to give a written consent.

Risk to subjects:

Biobran/MGN-3 is a food supplement that is produced from rice bran. It is available over-the-counter in the United States, Europe, and Japan. Clinical studies with Biobran/MGN-3 at larger doses and longer durations than what will be used in the current study were shown to be completely safe (Ghoneum, 1998; McDermott, 2006). Therefore, the risk to the participating subjects is negligible.

Sample size:

Previous clinical trials demonstrated that daily intake of Biobran/MGN-3 in young adults at doses of 15 mg/kg/d (~1000 mg/d), which is around 2 folds of the dose (500 mg/d) used in the current study, and 30 mg/kg/d (~2000 mg/d) increased NK cells activity after one month by 2 folds and 5 folds, respectively (Ghoneum,

1998). Therefore, it is reasonable to propose that doses of 500, 1000, and 2000 mg/d are within the linear portion of the Biobran/MGN-3 dose response curve. Thus, it is expected that Biobran/MGN-3 at a dose of 500 mg/kg will yield an effect size of 0.8. A power analysis using this effect size for a repeated measure analysis of variance with two measurements was performed using G*Power software package (version 3.1.9.2, Franz Faul, Germany). With the criterion of significance (alpha) set at 0.05, a sample size of 13 subjects per group (total of 39 subjects) results in a power of 0.80 to yield a statistically significant result regarding NK cell activity.

With regards to the preventive efficacy on influenza-like illnesses, we propose to enroll 90 subjects as a pilot study.

B) OPERATIONAL DESIGN:

1. Questionnaire:

Detailed clinical history and thorough clinical examination were conducted to assess subject eligibility to participate in the study.

2. Laboratory investigation:

Blood sampling, plasma and PBMCs isolation: Study participants will be asked to donate blood samples (10 ml) at the start (basal) and at the termination of the study (4 weeks). Blood will be collected into heparinized tubes, diluted with an equal volume of DPBS (no Ca^{++}/Mg^{++}), and peripheral mononuclear cells (PMBC) will be isolated by a standard Ficoll-Hypaque density gradient centrifugation method within 2h. Part of the plasma will be aspirated and freezed for analysis of liver and kidney functions. The lymphocyte band will be aspirated from the gradient interface, washed once with PBS supplanted with 1 mM EDTA and 0.1% BSA to remove platelets and once with a complete medium (CM; RPMI medium supplemented with 10% fetal bovine serum, 100-U⁄ml penicillin, 100 mg⁄ml streptomycin and 50 mg/ml gentamicin.), counted and assessed for viability in a trypan blue dye (0.2% (v/v) in PBS), and prepared as subsequently described for the different assays.

Liver and kidney functions: AST (serum glutamic oxaloacetic transaminase or SGOT), ALT (serum glutamic-pyruvic transaminase or SGPT), uric acid, creatinine will be assessed in plasma.

Degranulation assay for NK cell activity: NK cell activity will be measured with degranulation assay as previously described by Elsaid et al. (23). Briefly, 100 µl of heparinated blood will be used to examine either unstimulated control (RPMI 1640 medium alone), stimulated (combination ofPhorbol-12-myristate-13-acetate (PMA, 50 ng/ml, Sigma, St. Louis, MO, USA) and Ca^{2+} ionophore (Ionomycin, 250 ng/ml, Sigma, St. Louis, MO, USA), positive control (PMA/Ionomycin plus cytochalasin (5 µg/mL, Sigma, St. Louis, MO, USA), IgG isotypic staining negative control. For stimulation, samples will be incubated for 5 hours at 37°C under 5% (v/v) CO2 with FITC-labeled mouse anti-human CD-107a (clone H4A3, BD Bioscience, San Jose, CA, USA) plus monensin (GolgiStop, BD Bioscience, San Jose, CA, USA), which will be added to a final concentration of 6 µg/mL 1 hour after incubation. In the IgG isotypic staining negative control sample, incubation will be performed with mouse anti-human FITC-labeled IgG AB (clone G18-145, BD Bioscience, San Jose, CA, USA) instead of FITC-labeled mouse anti-human CD-107a (34). At the end of incubation, BD PharmLyse (BD Bioscience) will be used to lyse RBCs. White blood cells will be collected by centrifugation (300x g, 5 min), washed once, re-suspended in 100 µl staining buffer, and then stained by incubation with 5 µl of PE-labeled mouse anti-human CD56 AB clone R19-760 (NCAM-1, BD Bioscience) and 5 µl of PerCP-labeled mouse anti-human CD3 clone SK7 (BD Bioscience) for 15 minutes at 37°C. After staining, cells will be centrifuged (300x g, 5 min), washed once, and re-suspended in FACS buffer. BD FACS Calibur with CellQuest software will be used for analysis (BD Bioscience, San Jose, CA,USA).

Cell culture and flow cytometry: BEAS-2B cells (ATCC) will be grown on collagen-coated (5 g/cm2) plates in bronchial epithelial growth medium using the BEGM Bullet kit (Lonza, Walkersville, MD), maintained at 37°C, 5% CO2, seeded in 6-well plates and grown until 90% confluent. Cells will then be incubated with either PBS (control) or Biobran/MGN-3 100 µg/ml (Daiwa Technologies, Japan) for

72h. After harvesting, cells were washed with PBS, permeablized/fixed by BD cytofix/cytoperm buffer as per manufacture instruction, washed, collected, re-suspended in 100 µl of the first block solution with 10% rabbit serum for 30 min at room temperature. Cells will then incubated for 15 min at 37° C with 5 µl of either fluorescent-labeled monoclonal anti retinoic acid-inducible gene-1 (RIG-1) PE (Biorbyt, San Francisco, USA), monoclonal rabbit anti humans melanoma differentiation-associated protein-5 (MDA5) AB, clone # 33H12L34 (catalog # 700360, ThermoFisher Scientific, Rockford, IL, USA), monoclonal rabbit Anti-humans Myxoma Resistance Protein-1 (MX1) AB (catalogue # orb228747) (Biorybt, San Francisco, USA), monoclonal rabbit anti-humans anti-interferon-stimulated genes-15 (ISG15) AB (catalog # ab133346) (Abcam, Cambridge, MA, USA), or the IgG isotype matched control AB (negative control). For RIG-1, cells will be washed once in FACS buffer, collected by spinning, re-suspended in FACS buffer and analyzed. For MDA5, MX1, and ISG15, cells will be washed in permeablized/fixed BD cytofix/cytoperm buffer, spun down, re-suspended in staining buffer, and exposed to the second block in 10% goat serum for 30 min at room temperature. Cells were finally stained by incubating with phycoerythrin-conjugated goat anti-rabbit IgG secondary antibody (catalog # F0110) at 10 µL/10^6cells (R&D Systems, Minneapolis, MN, USA). FACS Calibur (BD Biosciences) and BD Cell Quest Software (BD Biosciences) will be used for the acquisition and analysis of the data. All cell culture and flow experiments were performed in triplicates.

3. Statistical analysis:

Fisher's Exact test and OpenEpi, version 3.01, was used for comparing the incidence rates and the incidence density rates between groups. Mean ± standard deviation is used to express continuous variables. Shapiro-Wilks test was used to determine the normality of data distribution. Two-tailed Student's t-test was used to compare Biobran/MGN-3 and placebo groups. Paired t-test was used to examine treatment effects within groups (i.e., comparisons of post-treatment versus pre-treatment). $P \leq 0.05$ was considered statistically significant. SPSS version 20 was used for computing statistical analyses values.

C-ADMINISTRATIVE AND ETHICAL DESIGN:

1. Ethical approval:

The study protocol will be subjected for reviewing by the IRB at Zagazig University.

2. Consent:

Informed consent will be obtained from all subject after explaining all study details, assuring confidentiality, and assuring their right of withdrawal at any time.

3. Confidentiality:

After IRB approval, the original data will be maintained by the PI. For other researchers, data will be de-identified, such that the data does not include any identifying markers such as names, initials, telephone, address or other demographic information.

Results and discussion

The collected data will be analyzed as previously explained, interpreted, and published in a peer-reviewed journal.

Significance of the study

1. Benefits to subjects:

Aging is associated with reduction of NK cell activity. Biobran/ MGN-3 dietary supplementation in the geriatric population would increase NK cell activity and improve resistance to viral infections and malignancies.

2. Benefits to the Community:

Geriatric population is rapidly increasing in Egypt and worldwide. Biobran/ MGN-3 dietary supplementation is expected to improve the quality of life and to reduce health care costs in this susceptible age group.

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