Effect of zinc on the T cells reconstitution after autologous hematopoietic stem cell transplantation: a study protocol

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Study protocol

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Abstract

Background: Post-transplant immune reconstitution has a significantly effect on "hematopoietic stem cell transplantation (HSCT)" outcomes. Delay in immune reconstitution increases the risk of infections and disease relapse after transplantation. Recovery of T cells is mainly thymus-dependent. Thymic atrophy is associated with various clinical conditions that lead to a reduced thymic output. Therefore, thymus rejuvenation can improve immune reconstitution after transplantation.

Zn plays a pivotal role in thymus rejuvenation. Zinc deficiency can lead to thymic atrophy, which increases susceptibility to infections. Zinc supplementation restores the immune system by boosting thymus output and T cell repertoire production.

This protocol was designed to investigate the effect of oral zinc supplementation on T cell recovery in patients undergoing HSCT.

Methods: Forty eligible candidates for autologous-HSCT will be selected. They will be randomly divided into "zinc" and placebo groups. Subsequently, they will receive three zinc or placebo tablets for the first 30 days post HSCT (+1 to +30), followed by one tablet or placebo for 60 days (+31 to +90). The copy numbers of "recent thymic emigrants (RTEs)" T cells and "T cell Receptor Excision Circles (TREC)" will be assessed before and after the intervention. All patients will be followed up for 365 days post HSCT for relapse and infection.

Discussion: This clinical trial is the first to determine the efficiency of "zinc" in T cell recovery post HSCT.

If successful, an available and inexpensive drug will improve immune system reconstruction after HSCT, reduce the risk of infection, particularly viral infections, and increase patient survival.

Trial registration number: IRCT20191211045701N1

Introduction

Post-transplant immune deficiency affects "hematopoietic stem cell transplantation (HSCT)" outcomes is significantly associated with mortality and morbidity (1, 2). .The innate immune

cells are rapidly recovered after stem cell transplantation but lymphocytes regeneration slowly occurs during one year post-HSCT (3). Delay in T cell recovery is attributed to a combination of several factors; including age-associated thymic atrophy, thymic damage due to chemotherapy, the source of stem cells, MHC histocompatibility antigen, the use of post-transplant immune suppression drugs, and "graft-versus-host disease (GVHD)" (4–7).

Long-term T cell immune reconstitution is mainly thymus-dependent in HSCT patients (6, 8, 9). A strong correlation between low thymopoiesis and development of opportunistic infections after HSCT reflects the critical function of thymus in post-transplantation T-cell recovery (2, 10, 11). Thymic function is

essential to recover recent thymic emigrant (RTEs) T cells, which provides a more diverse T cell repertoire (12). However, T cells are regenerated even with reduce activity in the atrophic thymus (13, 14).

Opportunistic infections and disease recurrence are major causes of HSCT failure. Preclinical studies have investigated strategies such as growth factors and cytokines to improve immune reconstitution following HSCT, although only a few have progressed to clinical protocols so far (15–21).

Zinc is a co-factor of more than 200 enzymes involved in many cellular functions, including proliferation, apoptosis, oxidative stress, immune responses and inflammation (22–24). Zinc is essential for regulating intracellular signaling pathways in innate and adaptive immune cells, so zinc homeostasis is crucial for adequate immune system function (25, 26). Zinc deficiency elevates the inflammatory response resulting in damage to the host tissue (27). Zinc modulates the pro-inflammatory response by targeting "Nuclear Factor Kappa B (NF-k B)", a master regulator of pro-inflammatory responses (28). It is also involved in controlling oxidative stress and regulating inflammatory cytokines (29). Mild zinc deficiency significantly decreases thymulin activity and T-cell number in humans (30, 31). Some studies confirmed the effect of zinc supplementation in thymic output and its ability to reduce the risk of infection in the elderly (27, 32, 33). Zinc supplementation can reduce the incidence and severity of mucositis in patients receiving chemotherapy by improving the effectiveness of immune system; however, the exact mechanism of this effect is not completely clear (34–37). A clinical study reported high-dose zinc supplementation improved thymic output and T cells reconstitution post HSCT in patients with multiple myeloma compared to placebo group. This study reported zinc-related adverse effects in five patients (55.5%) following zinc supplementation intake that it was likely multifactorial (four patients had nausea, and one had diarrhea). However, the small sample size and high-dose zinc supplementation limited this study. (38). Preclinical studies in murine models have also revealed that oral zinc supplementation improves thymus regeneration post-HSCT (39, 40).

Regarding the lack of evidence for zinc supplementation in improve T cell reconstruction post-HSCT and limitations of previous studies, this clinical study was has been designed to determine the efficacy of zinc supplementation on T cell reconstitution in patients undergoing autologous-HSCT. We will recruit 40 patients who are candidates for autologous HSCT. Eligible patients will be randomly divided into "zinc" and placebo groups. After HSCT, zinc supplementation will start on day + 1. The patient will receive three zinc tablets daily (each contains 30 mg of elemental zinc) for 30 days, followed by one zinc tablet or placebo for 60 days (+ 31 to + 90). Copy numbers of RTEs T cells and "T-Cell Receptor Excision Circles (TREC)" will be assessed before and after intervention. All patients will be followed up for 365 days post HSCT for recurrence and reactivation of CMV and EBV viral infection to evaluate T cell function.

This research will also provide valuable information that can be used in allogeneic transplantation.

Methods

Study Design:

This study will be a double-blind, placebo-controlled randomized clinical trial. In this parallel study, the candidates for autologous HSCT will randomly divide into two groups: "zinc" and placebo. The study design flow diagram is shown in Fig. 1.

This study will be performed in the bone marrow transplant department of Taleghani Hospital, Shahid Beheshti University of Medical Sciences.

Primary Objectives

The main purpose of this study is to evaluate the effects of "zinc supplementation" on improving T-cell reconstitution and thymic output post HSCT. Thus, the copy numbers of RTEs T cells and TRECs in "peripheral blood mononuclear cells (PBMCs)" as thymic output within each group and between the two groups before and after intervention will be determined and compared. Furthermore, we will evaluate relapse and non-relapse mortality (NRM) rate within 365 days post-HSCT to assess T cell function.

Secondary objectives:

1. Determination and comparison of Absolute Lymphocyte Count (ALC) post HSCT between two groups.

2. Determination and comparison of naïve T cells post HSCT between two groups.

Sample Size

Ready-to-use sampling will be available based on study entry criteria. The sample size was calculated using the following formula, which is recommended for parallel clinical trials:

$$n = [(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot s^2] / d^2$$

A total of 40 patients who are HSCT candidates will be divided into two equal groups by using block randomization method with a 95% confidence level, 80% power, and an additional drop-out rate of 20%.

Subjects:

The subjects will be selected from the patients with "Multiple Myeloma (M.M)" who are candidates for autologous HSCT and referred to the bone marrow transplant ward of Taleghani Hospital in Tehran. The patients will be candidates for autologous HSCT based on their medical records and the European Society for Blood and Marrow Transplantation protocols (EBMT). Eligible patients would be enrolled on the basis of the inclusion and exclusion criteria. The objectives and procedures of the study will be clearly explained to patients, and they will be informed about the possible side effects of zinc supplementation. All patients will provide informed consent before enrollment and a numerical code will be dedicated to ensuring patient anonymity. Additionally, the interviewer will be filled out a general questionnaire. The

participants have the right to withdraw from the study at any time even after obtaining informed consent. They will not receive fee to participate in this study and not pay extra for the drug and medical laboratory tests.

Inclusion And Exclusion Criteria:

The inclusion criteria are age between 40 to 60 years old, being able to swallow tablets, history of M.M, complete response to treatment, and being a candidate for autologous HSCT without comorbidity.

Exclusion criteria will include a history of allergic reactions to oral zinc supplementations, zinc serum levels above 200µg/dl, and taking oral zinc supplementations three months before intervention.

Blinding And Randomization:

In this double-blind study, the researcher and participants will be blind to "zinc" and placebo groups. To achieve this goal, the research assistant will offer researcher a zinc and placebo in the A and B packages. A research assistant collects and provides information to the outcome assessor.

Eligible patients will be divided into two equal groups using block randomization. Randomization will be conducted by a research assistant; however, the investigator, patients, staff and outcome assessors will be blinded to the type of intervention during the study.

Intervention:

The dosage of zinc was based on another clinical trial that used a high-dose oral zinc supplementation to improve immune reconstitution after HSCT(38). "Zinc Gluconate 30mg" tablets will be purchased from Dineh Iran Industries Complex (Tehran, Iran) and placebo tablets will be prepared in similar color, shape, and weight by a pharmacologist who is a faculty member of Shahid Beheshti University of medical sciences. The placebo used in this study will be based on microcrystalline cellulose (avicel) including propylene glycol, hydroxyl propyl methyl cellulose, titanium dioxide, alcohol and edible color. Zinc supplementations and placebos will be packed with A and B codes by other research assistant and will be delivered monthly to the participants. The consumption of tablets will be reminded and monitored by a text message and call phone.

All participants will be completely informed of the study and they will be asked to contact the researchers or their assistants in case of any adverse events (nausea, vomiting, diarrhea, rash, and so on)(41).

Assessments:

A demographic questionnaire including age, sex, education level, marital status, diagnosis, previous chemotherapy, height, and weight will be filled out for all participants before entering the study.

Two 3-day feed registration questionnaires will be filled out to ensure non-difference in food intake between the intervention and placebo groups. All people will be trained on how to complete these food records. Each patient will complete two "3-day feed registration" questionnaires before and after transplantation during the intervention at home. Each patient will have 6 questionnaires until the end of study. Based on the six completed food records, we will determine the number of calorie intakes by counting both groups' average macronutrients and micronutrients. The nutrient calculation will be performed by Nutritionist 4 software (First Data Bank, San Bruno, CA, USA) during the intervention. Patients will be monitored during intervention every week and any occurrence of adverse events will be recorded. They will also be asked to contact the researchers or their assistants in case of any adverse events (nausea, vomiting, diarrhea, rash, etc.) (42).

Due to the interference of "zinc" and "copper" absorption, these two micronutrients were measured before and every two weeks during the intervention using atomic absorption spectroscopy.

Blood samples will collect in the 2ml EDTA-coated tubes four times, including admission time, 30th, 90th, and 180th day following HSCT. "Peripheral Blood Mononuclear Cell (PBMCs)" will be isolated by density gradient centrifugation (Ficoll-Hypaque, Lymphodex, Inno-Train, Germany) and counted in the Neubauer chamber. After rinsing with PBS, the 10^6 mononuclear cells will be stained by fluorochrome-conjugated anti-human monoclonal antibodies panel including: CD4 /CD31 /CD45RA /CD45RO for 20 minutes at 4°C and will be analyzed by flow cytometry (Attune NxT Flow Cytometer).

Results of two populations of RTEs are expressed as percentages of CD4 CD45RA CD31 within CD4 T-cells and afterward as the absolute numbers of cells per microliter of blood. CD45RA CD4 naive T-cells and CD45RO CD4 memory T-cells will be quantified in the same sample (43).

For sjTREC quantification, DNA will be purified from 2 to 5 × 10^6 PBMCs using a DNA isolation kit. Specific primers and Taqman probes will be used for sjTREC and TRAC as a housekeeping gene for absolute quantification of sjTREC numbers: sjTRECs forward primer (5’-CAC ATC CCT TTC AAC CAT GCT-3’) and probe (5’-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-TAMRA-3’) and reverse primer for sjTRECs (5’-GCC AGC TGC AGG GTT TAG G-3’)(44).

Primers for the TRAC gene (forward 50-TGG CCT AAC CCT GAT CCTCTT-30, reverse 50-GGA TTT AGA GTC TCT CAG CTG GTA CAC-30 and probe 50-FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCCTAMRA-30) (45).

Quantitative PCR will be carried out in a total volume of 25 µl containing 500 to 1000 ng DNA, 10 to 15 µl TaqMan universal PCR master mix, 900 nM each primer, and 200 nM probe for sjTRECs and TRAC genes, respectively, under the following conditions: 95^C for 10 minutes for polymerase activation, followed by 45 cycles of amplification (95^C for 15 seconds’ denaturation, 60^C for 1 minute annealing/elongation).

sjTREC and TRAC reactions will be conducted in separate wells of the same plate. PCR reaction was performed on the 7500 Fast Real-Time PCR (Applied Biosystems). The number of sjTREC copies will be determined using a dilution series of plasmid TRECs-KRECs TCRAC, the pCR2.1-TOPO vector containing both sjTREC and TRAC fragment in T-A and Spe I acceptor sites, respectively (46). Standard dilutions of the vector from 10^6 to 10^1 copies prepare and run in each set of PCR to obtain a 6-point standard curve. Basically, sjTREC copy numbers will be calculated per 10^6 PBMCs using mean values of sjTREC and TRAC obtained in PCR:

Mean sjTRECs $\times 10^6$/mean TRAC×2

The absolute sjTREC concentration in peripheral blood (number of copies/mL) will be calculated using the following formula: (sjTREC copies/ 10^6 PBMCs) × (PBMCs/ml)/10^6

Follow-up

Relapse, cytomegalovirus (CMV) reactivation and Epstein-Barr virus (EBV) occurrence will be monitored within 365 days post-HSCT. Bone marrow aspirate and serum/urine protein electrophoresis in patients with multiple myeloma (MM) employ to detect relapse during follow-up.

Intervention and assessment schedule of the trial summarized in Fig. 2.

Statistical Analysis

SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) will be employed for data analysis. The data entry, coding, security and saving will be checked. Kolmogorov- Smirnov test will be used for checking normality. Wilcoxon, paired and independent t-tests and spearman and Pearson's correlation coefficients will be utilized. The significant level is considered 5%. Finally, Lawrence's method will be used to evaluate the effect of intervention groups on "Non-relapse mortality (NRM)" and relapse.

Patient And Public Involvement:

The research question and outcome measures were developed based on the oncologists’ experience following patients undergoing HSCT and the desire to find better immune reconstitution after HSCT. Patients and advisers were not involved in this study’s design, recruitment, or conduct. The patients or their families will be notified of the study results in writing and verbally.

Ethical Considerations

The Ethics Committee of Tarbiat Modares University and Shahid Beheshti University of Medical Sciences approved the study. We obtained permission for the survey from SBMU, Taleghani hospital, and bone marrow transplant wards.

Discussion

Post-transplant immune reconstitution is crucial for long-term survival after HSCT (47). Innate immunity recover following HSCT, but adaptive immunity reconstitution is a lengthy process that in the case of T-cell take several months to years (48, 49). Although, memory T-cells expand in peripheral blood and temporarily defend the host against previously encountered pathogens, reconstitution of naïve T-cell repertoire provides immune protection against a broad range of pathogens in the long term (45). The thymic function play a central role in T-cell repertoire generation (50, 51). Thymus atrophy results from aging, malnutrition, stress, and some clinical situations that decrease thymic output and naïve T-cell diversity(52). Therefore, therapeutic interventions are necessary for thymus regeneration, reducing the extent of thymic atrophy and boosting the immune system (9, 10, 21).

Studies have revealed that the improvement strategies of post-transplant T-cell reconstitution are related to improved survival in patients undergoing HSCT (53, 54), but more clinical studies are required for a specific protocol to be applied in clinical treatment (55, 56).

Since the role of many micronutrients, such as zinc, in immune system activities has been proven, dietary supplementations can be investigated as safe and inexpensive drugs to boost T-cell immune reconstitution following HSCT(57, 58).

Zn is pivotal in many metabolic, growing pathways and immune responses(59). Immune cells require sufficient zinc to achieve a high proliferation rate, differentiation, and function. Mild zinc deficiency has been detected in immune cells. Zinc has no specialized storage system in the body; however, a daily dose is required to maintain a steady state.

This double-blind clinical trial is a novel study that will be proposed for evaluating the efficacy of "zinc gluconate" on T-cell reconstitution and thymic output by assessing the number of RTEs and quantification of TRECs in patients undergoing autologous HSCT. TREC and RTE T-cells are used to produce naïve T-cells and Thymic output (60).

The strengths of the trial are using a randomized, double-blind design and protocol publication, employing a safe and available strategy to improve immune reconstitution, well-absorbed and low adverse effects of zinc supplementation, and determining thymic output in autologous HSCT. Additionally, this research will provide valuable information about Immune reconstitution following HSCT that can be used in allogeneic HSCT.

This trial has some limitations including slow patients recruitment and long-term follow-up. In addition, the study period will increase owing to the multiple eligibility criteria, selection of a single polyclinic

center, participants' self-reporting of their consumption, dietary intakes, and lack of cooperation in some participants for completing the intervention, which leads to replacement with other patients if the percentage loss will be more than expected.

Abbreviations

Hematopoietic Stem Cell Transplantation (HSCT), Graft Versus Host Disease (GVHD), Multiple Myeloma (M.M), Peripheral Blood Mononuclear Cell (PBMC), Absolute Lymphocyte Count (ALC), Recent Thymic Emigrant T-cells (RTEs), Signal joint T-cell receptor excision circles (sjTRECs), cytomegalovirus (CMV), Epstein-Barr virus (EBV), Non-relapse mortality (NRM), European Society for Blood and Marrow Transplantation protocols (EBMT), Nuclear Factor Kappa B (NF-kb).

Declarations

Ethics Approval and Consent to Participate: This study was approved on 14th January 2020 by the Ethical Committees of Tarbiat Modares University (IR.MODARES.REC.1398.195) and Shahid Beheshti University of Medical Sciences (IR.SBUM.REC.1399.010)

Patients who sign the consent form enter the study, a numerical code will be used for patients' anonymity, and the interviewer will fill out a general questionnaire.

Study Status: Sampling for this study will begin in April 2021.

Consent for Publication: Not Applicable

Availability of Data and Materials: Data and materials will be published in research articles.

Competing Interests: The authors declare that they have no competing interests**.**

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Authors' Contributions: MN has proposed this study. AZH, SP, and AH helped with the final study design. MS and HZ contributed to the nutrition aspects of the study.

SP, AH will help with clinical trial implementation. YS will help with part of the laboratory experiments.

All authors contributed to the refinement of the study protocol and approved the final manuscript

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Figures

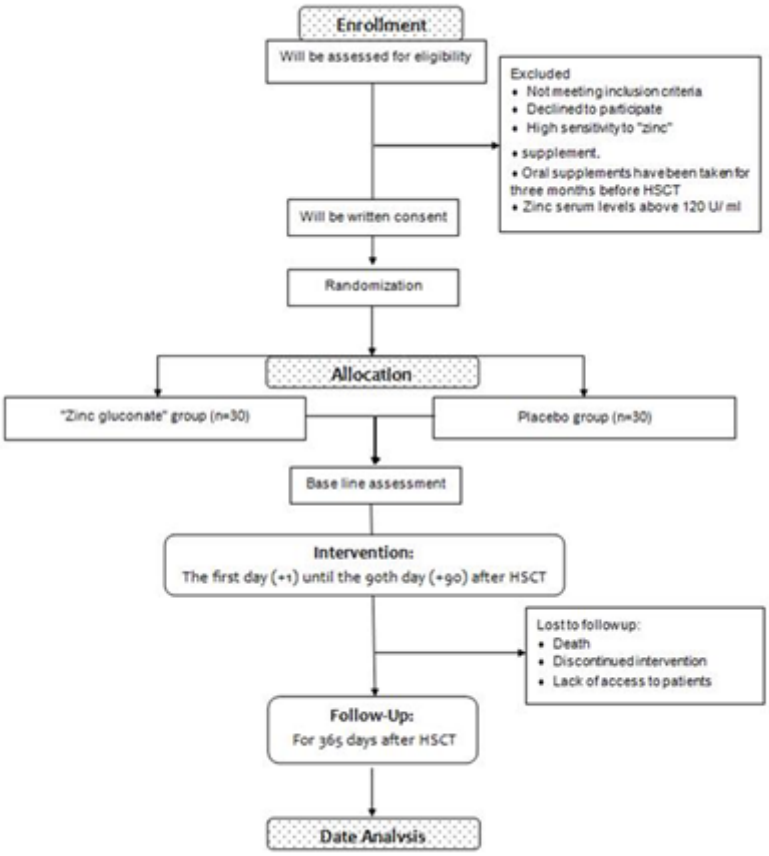

Figure 1

Flow diagram of trial

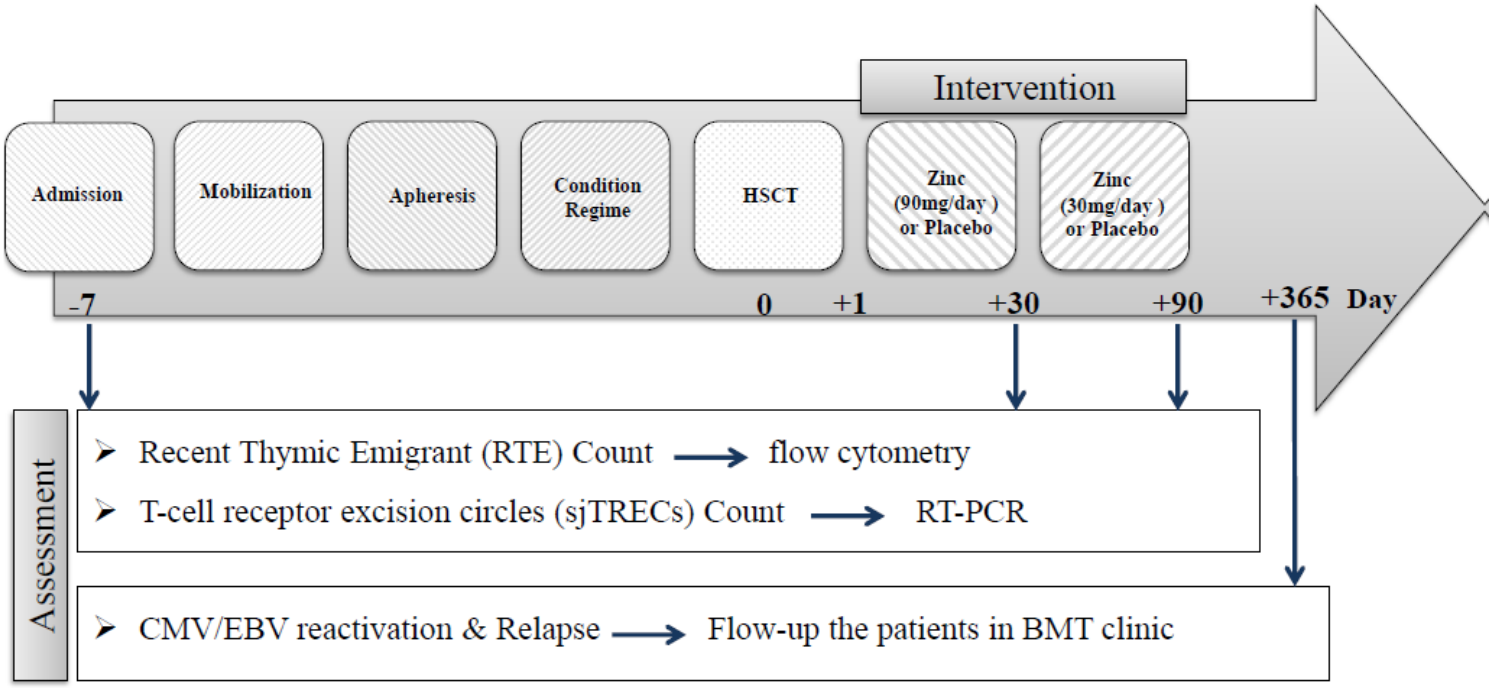

Figure 2

Intervention and assessment schedule of the trial

Supplementary Files

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- SPIRITChecklist.doc