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Study on the Role of Ganoderma lucidum-derived Recombinant LZ-8 Protein in Neutrophil Recovery and Bone Marrow Mobilisation

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ABSTRACT

Background: *Ganoderma lucidum* is a fungus from the *Polyporaceae* family of Agaricomycetes class, from which recombinant LZ-8 (rLZ-8) protein can be obtained that has pharmacoeffect on the neutrophil counts and bone marrow mobilization. This protein has the potential to mobilize hematopoietic stem cells (HSCs) through signaling pathways like CXCR4-SDF1. In addition, it has fewer detrimental treatments for neutropenia in comparison to existing therapies.

Methods: This prospective experimental study (November 2023–October 2024) evaluated the effect of recombinant Lingzhi-8 (rLZ-8) on white blood cell recovery and HSC mobilization in BALB/c and C57BL/6J mice. Neutropenia was induced by intraperitoneal cyclophosphamide, followed by saline, G-CSF, or rLZ-8 treatment. rLZ-8 was synthesized and purified using expression vector technology with high purity and low endotoxin. HSC markers were analyzed by flow cytometry, functional activity by CFU assay, SDF-1 by Western blotting, and localization by immunofluorescence. Data analysis was performed using SPSS with the Mann-Whitney U test.

Results: Significant mobilization of WBCs and neutrophils was observed in saline, cyclophosphamide, G-CSF, and rLZ-8-treated groups. The cyclophosphamide-only model group showed marked WBC and neutrophil reduction with mild recovery ($p<0.05$). In contrast, G-CSF and rLZ-8 groups showed progressive increases, with near-normal levels on Day 9 ($p<0.05$). Increasing rLZ-8 doses enhanced SDF-1 expression up to 300 AU and significantly restored bone marrow activity compared to the model group ($p=0.01$).

Conclusion: The study has concluded that Ganoderma lucidum-derived recombinant LZ-8 protein can promote neutrophil and recovery from neutropenia by leading to bone marrow mobilization.

Key-words: LZ-8 protein, G-CSF, BALB/c, C57BL/6J, Ganoderma lucidum, LZ-8 protein, Recombinant immunomodulatory protein, Bone marrow mobilisation

INTRODUCTION

Ganoderma lucidum (Gl), also known as Lingzhi/Reishi, is a fungus sourced from China and belongs to the *Ganoderma* genus and *Polyporaceae* family of *Agaricomycetes* class.

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This fungus has been used as a Chinese medication since ancient times, including as an immune booster, improving liver health, respiratory health, and managing anxiety [1]. It has a fan-shaped cap, a long stem, septate hyphae, and ellipsoidal spores. It involves long-chain polysaccharides, triterpenoids (antioxidants), and peptides like Lingzhi-8 (LZ-8). Sterols and alkaloids may also be present in this mushroom [2].

G. lucidum contains more than 400 bioactive compounds, out of which polysaccharides extracted from it are found to have an immunomodulatory effect. The fungal protein LZ-8 has a hundred-ten amino acids

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responsible for immunomodulation and ceasing tumor formation [3]. These non-covalent homodimers originate from the fruiting body of the fungus. The structure of LZ-8 is almost like the Fip-fve of *F. velutipes*. It has N and C-terminal FNIII domains. N-terminal domain forms a dumbbell-shaped dimer and consists of alpha-helix and beta-strands. The C-terminal domain comprises two beta-sheets sandwiched between beta-strands A-B-E and G-F-C-D (Fig. 2) [4]. Scientific reports indicated that LZ-8 activates and elicits differentiation of dendritic cells by activating specific pathways (for example, MAPK and NF-κB) [5]. It also stimulated dendritic cells, thus eliciting it as a potential adjuvant for the manufacture of DNA vaccines targeting human cancers [6]. The recombinant LZ-8 (rLZ-8) further induced apoptosis and suppressed the growth of lung cancer cells by degrading the epidermal growth factor receptor in a process that was dependent on c-Cbl ubiquitination, endocytosis, and clathrin [7].

A study depicted that FIP-gts facilitated WBC recovery. The effects of chemotherapy-induced myelosuppression and mucosal damage within the intestine were markedly reduced by both FIP-fve and FIP-gts, thus lowering the threat of bone metastasis and changing the bone microenvironment [9]. In a leukopenia mouse model, rLZ-8 had markedly raised the numbers of WBC [10].

Neutrophils in circulation are considered the first line of defense for humans against pathogens. They activate and regulate innate and adaptive immunity [11]. Reduced neutrophils can be seen as a side effect of myelosuppressive anti-cancer therapy. Because of this, the hematopoietic procedure is suppressed, thus weakening the body's defense mechanism and restricting the dosage of chemotherapy. These patients have a high chance of bacterial or fungal infections [12].

The major HSCs environment in mammals is found in the bone marrow, where it consists of osteoblasts lining [3-6], stromal cells, and cellular matrix [2–6]. HSCs are believed to be attached to those cells by an array of adhesion molecule interactions. If the HSC environment is disrupted, HSCs are released in the peripheral circulation, causing bone marrow mobilization [13]. The bone marrow is needed to form neutrophils, a highly controlled and energy-intensive process, to sustain immunological activities and its relatively short survival span [14]. Granulocyte colony-stimulating factor (G-CSF) is an essential regulator of this process [15]. The upregulation of transcription factors C/EBPβ and PU.1 encourages differentiation into the granulocyte lineage of myeloid progenitor cells [16].

In contrast, it favored the differentiation of neutrophils, which mature in the bone marrow to become part of the blood, and activated stem cell mobilization by regulating the CXCR4-SDF1 pathway, used as a short-term compensatory response to infection and suppression of hematopoiesis. On the contrary, the count of neutrophils increased sharply during the treatment period with G-CSF, but began to fall, and that is not a stable situation. Furthermore, there are not many therapeutic drugs available for the treatment of neutropenia.

Our study evaluates how recombinant LZ-8 protein derived from *G. lucidum* can help in hematological recovery and induce neutrophil counts by discussing its effects on neutrophil counts and bone marrow mobilizations. The study's outcome may lead to the development of novel therapeutic strategies directed at improving neutropenia with fewer side effects than the currently employed ones.

The study aims to analyze the effect of rLZ-8 on the recovery of WBC counts and neutrophil counts in mice models with induced neutropenia. The study has compared the recovery of the WBC count and neutrophil count in mice models inoculated with rLZ-8 with mice models inoculated with either saline or given no inoculation (positive control).

MATERIALS AND METHODS

Research Design- This is prospective experimental research conducted from November 2023 to October 2024, involving BALB/c and C57BL/6J mice models. Figure 3 shows the steps of this study. The rLZ-8 and G-CSF dosages and administration timings were selected based on prior pharmacokinetic studies and preliminary data demonstrating their efficacy in stimulating neutrophil recovery. This study used established dosage ranges for G-CSF to mirror standard clinical applications and investigated a range of rLZ-8 doses to identify the most effective concentration.

Preparation of rLZ-8- An established expression and purification technique was used to produce the rLZ-8 protein, ensuring high purity and low endotoxin levels

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suitable for animal administration. After cloning into an expression vector, the LZ-8 gene was expressed in Pichia pastoris and cultivated in a regulated bioreactor. The rLZ-8 will then go through purification procedures employing several chromatography steps after the protein expression is tracked and adjusted to maintain a high yield. To ensure the proper composition and concentration for animal administration, the quality of the purified protein was confirmed using high-performance liquid chromatography (HPLC).

Animal model- A neutropenia model was developed using BALB/c and C57BL/6J mice. To cause neutropenia, cyclophosphamide was injected intraperitoneally. Different treatment regimens (normal, model, rLZ-8, and granulocyte-colony stimulating factor (G-CSF) were administered to mice in groups. Using flow cytometry and other assays, samples of peripheral blood and bone marrow were obtained to determine the numbers of white blood cells and neutrophils.

Animal study design- The study considered 150 (BALB/c and C57BL/6J) mice. To ensure sufficient statistical power, *this* sample size on preliminary studies and a power analysis indicated that 150 mice would yield a power of at least 80%. It also detects a statistically significant difference ($p<0.05$) between groups. This sample size provides confidence in capturing variations in neutrophil counts and bone marrow mobilisation. This study aims to examine, using an in vivo model, how the recombinant Lingzhi-8 (rLZ-8) protein affects the mobilization and proliferation of HSCs. The study aims to evaluate rLZ-8's impact on several hematological parameters and clarify its possible pharmacodynamic qualities in promoting WBC recovery after induced leukopenia. The study aims to comprehend how rLZ-8 affects immunological response, HSC viability, and differentiation to investigate its potential therapeutic use in diseases marked by immune deficiency and bone marrow suppression.

To ascertain the most efficient way to administer rLZ-8, the animals were divided into normal, model, and different treatment groups. Each group will receive injections via subcutaneous and intravenous routes. To quantitatively examine the hematological response, samples of blood and bone marrow were taken at pre-arranged intervals, and automated hematology analyzers were used to measure WBC counts.

Blood cell count- The mice's blood samples were collected, and a hematology analyzer was used to count the white blood cells. Using flow cytometry, bone marrow cells were gathered to identify neutrophils and hematopoietic stem cells. The study implemented randomisation and blinding in group assignments, treatment administrations, and data collection processes to reduce potential biases. Each animal was randomly assigned to a treatment group, and researchers conducting the experiments were blinded to group allocations.

Flow cytometry analysis- Flow cytometry was used to investigate HSC populations and their distinct cell markers in bone marrow and peripheral blood for a more thorough cellular investigation. To enable a thorough assessment of cell differentiation and mobilization patterns, progenitor cell markers, neutrophils, CXCR4+ cells, and other pertinent cell types were labeled with specific antibodies. To isolate single-cell suspensions for colony-forming unit (CFU) experiments, bone marrow samples were also processed. The functional ability of HSCs to generate several hematological colonies, including CFU-GEMM, CFU-G, and CFU-GM, under rLZ-8 therapy was shown by these tests.

Western blot analysis- The amount of stromal cell-derived factor 1 (SDF-1), a crucial regulator in HSC trafficking and homing, was measured using Western blot analysis on bone marrow supernatants to better understand the molecular basis of rLZ-8's impact on hematopoiesis. The samples will undergo protein analysis based on capillary electrophoresis to guarantee precise SDF-1 identification and quantification, standardized against GAPDH. By identifying signaling pathways that are impacted by rLZ-8, these findings may help uncover possible mechanisms for how it affects HSC mobilization. Future studies will include complementary in vitro analyses to elucidate the molecular mechanisms underlying rLZ-8 effects on hematopoietic stem cell mobilisation and neutrophil proliferation. Such data would provide a more comprehensive understanding of rLZ-8's impact at a cellular level.

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Immunofluorescence imaging- Immunofluorescence imaging was used to see how rLZ-8 interacts with hematopoietic cells, including HSCs. Fluorescently tagged rLZ-8 and primary antibodies that target important receptors involved in cell signaling pathways, including G-CSFR, CSF1R, and GM-CSFR, were added to the cells during incubation. To stop non-specific antibody binding, the cells were frozen, permeabilized, and blocked after incubation. Further imaging using a structured illumination microscope (SIM) would improve comprehension of rLZ-8's cellular mechanism of action by enabling high-resolution visualization of protein localization and receptor interactions. In addition to saline-treated controls, this study incorporated G-CSF treatment as a positive control to measure neutrophil recovery and bone marrow mobilisation. This approach allows a comparative analysis between the novel rLZ-8 and established G-CSF treatments, strengthening the validity of this finding.

Statistical Analysis- The study has used SPSS 27 for effective analysis. The mean±standard deviation is used to express the continuous data. The Mann-Whitney U test was used to compare the means. MS Excel was used for calculating the percentage of the frequencies. The Mann-Whitney U test was chosen due to the non-normal distribution of some dataset parameters. This non-parametric test provides a robust method for comparing group differences without assuming normality. However, if normality can be established in future studies, parametric tests may offer additional insights. Non-significant differences observed in certain comparisons, such as between the low-dose rLZ-8 and saline groups, highlight potential limitations in treatment effects at lower concentrations. Further research could examine whether alternative dosing regimens yield more consistent results across groups.

Ethical Consideration- All animal protocols and procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), ensuring compliance with animal welfare standards and ethical research practices. The study adhered to the guidelines stipulated in the Guide for the Care and Use of Laboratory Animals.

RESULTS

The essential baseline parameters were studied for several groups of mice, comprising the normal group, model group, G-CSF group, and rLZ-8 therapy groups at low, medium, and high dosages. The study found that the group with a medium dosage of rLZ-8 had shown to have the most promising results in terms of neutrophil mobilization. The mean body mass of the mice is similar in all the tested groups, with a slight range of differences between 20.2 and 20.5 grams.

The similarity in weights indicates that differences in body mass would not influence the results. All groups have mice aged 8 – 10 weeks, hence there is consistency in age at which baseline characteristics are assessed. The dosage of cyclophosphamide administered to the Normal group is what sets that group apart from all the other groups. The Normal group is not treated with any cyclophosphamide, unlike the other five groups, which are administered 50 mg/kg for three days.

It seems reasonable to assume that this may be one of the factors causing a major neutrophil decline in the Model group, where neutrophils are very low, 0.75±0.05% as compared to the Normal group at 60.0±2.0% where no cyclophosphamide was given. The G-CSF group, also administered 50 mg/kg of cyclophosphamide, recorded a greater neutrophil count of 28.66±1% implying that neutrophil levels were partially restored because of G-CSF. With the rLZ-8 dose groups, there is also a dose-wise effect on the neutrophil counts.

Neutrophil counts from the Low Dose group (0.5 mg/kg) are 22.96±2.18%, lower than those of the G-CSF group but higher than the Model group. Neutrophil levels within the Medium Dose group (1.0 mg/kg) are increased even further at 45.36±3.63% coming close to the width of the Normal group. The high-dose group (1.5 mg/kg) records neutrophils at a proportion of 33.22±9.60%. Although this significant amount, it is still lower than the Medium Dose group (Table 1).

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Table 1: Baseline characteristics of the mice models in each group

Characteristic	Normal Group (n=25)	Model Group (n=25)	G-CSF Group (n=25)	rLZ-8 Low Dose Group (n=25)	rLZ-8 Medium Dose Group (n=25)	rLZ-8 High Dose Group (n=25)
Number of Mice (n)	25	25	25	25	25	25
Weight (g)	20.5±1.2	20.2±1.3	20.4±1.4	20.3±1.2	20.4±1.1	20.2±1.3
Age (weeks)	08-Oct	08-Oct	08-Oct	08-Oct	08-Oct	08-Oct
Cyclophosphamide Dosage	0 mg/kg	50 mg/kg (3 days)	50 mg/kg (3 days)	50 mg/kg (3 days)	50 mg/kg (3 days)	50 mg/kg (3 days)
Neutrophil Count (%)	60.0±2.0	0.75±0.05	28.66±1.00	22.96±2.18	45.36±3.63	33.22±9.60
Treatment Administered	Saline (placebo)	None	G-CSF (31.25 mg/kg)	rLZ-8 (0.5 mg/kg)	rLZ-8 (1.0 mg/kg)	rLZ-8 (1.5 mg/kg)

Using Electrophoresis, a protein supernatant was obtained, and its expression was found to increase from 40 to 120 kDA. The only protein that was detected is rLZ-8, whose mass was very similar to that of the native LZ-8 protein. The protein purity reached 99.87% at precisely 11.289 minutes, which indicates that the purification procedures were successful. The purity dropped to 0 after this peak and was maintained there for 22.5 minutes. This shows an exact chromatography purification event, while the LZ-8 protein eluted with excellent purity at 11.289 minutes **(**Fig. 1). The medium-dose rLZ-8 group showed a statistically significant increase in neutrophil mobilisation compared to the model group, approaching levels like G-CSF-treated mice.

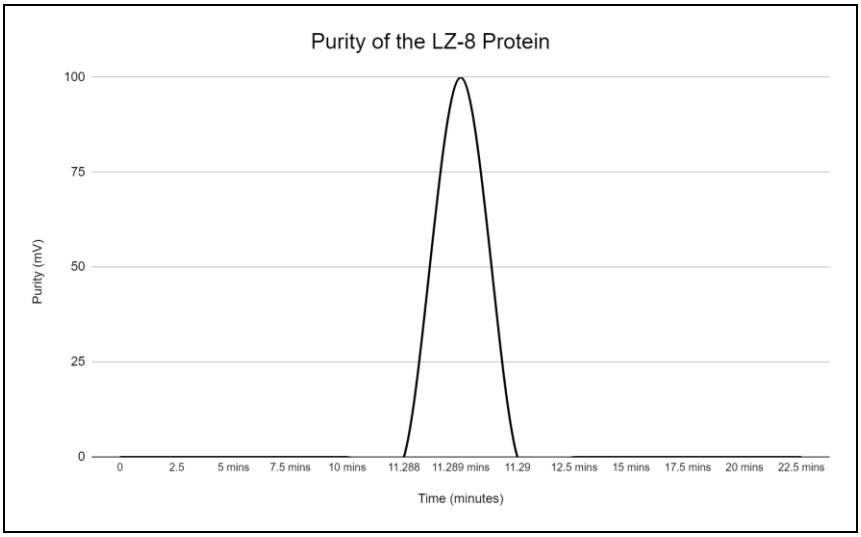

Fig. 1: Purity of LZ-8 Protein as obtained in this study

The change in white blood cell (WBC) estimates over 13 days in three groups: saline-treated, three-day cyclophosphamide-treated, and one-day treatment. The saline group had a WBC count of 7.5 on Day 1. In contrast, the 3-day cyclophosphamide group had a significantly lower count of 3.5. Even the 1-day administration group had an even lower count of 1.8 ($p<0.032$). On Day 3, the 1-day group's WBC count declined significantly to 0.07 ($p<0.01$). In the 3-day group, the count dropped to 3.3, while the saline group maintained 7.3.

The cyclophosphamide-treated groups showed significant improvement in WBC counts on Day 5. With the 1-day group reaching 2.1 and the 3-day group reaching 3.9 ($p<0.05$). Whenever the saline group maintained a count of 8. On Day 7, the 1-day group's WBC count rose to 3.2, while the 3-day group recovered to 5.9, below the saline group's 7.6 ($p<0.05$). The 1-day and 3-day cyclophosphamide groups had WBC counts of 6.7 and 7 on Day 9 ($p>0.05$, which indicates no significant difference between the groups). WBC counts on Days 11 and 13 were similar in all groups: saline 8.9 and 8, 3-day

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7.8 and 7.9, and 1-day 7.8 and 8.5 (p>0.09). In both cyclophosphamide-treated groups, WBC counts recovered completely by the conclusion of the monitoring period **(**Fig. 2).

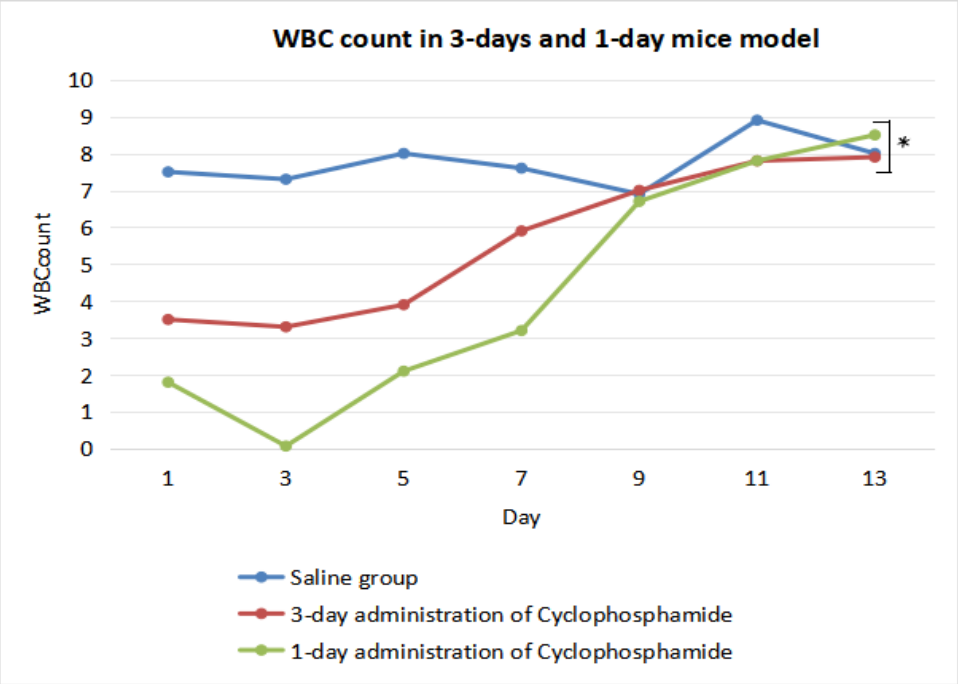

Fig. 2: Variation of WBC counts in animal models inoculated with cyclophosphamide for 1 day (230 mg/kg) and 3 days (50 mg/kg)

The study documented variation in WBC count among groups administered via different routes, including subcutaneous and intravenous, over 11 days. On Day 3, the saline group showed a WBC count of 9.1, while the subcutaneous group exhibited a significantly lower count of 0.09, and the intravenous group had a count of 1.1. The model group, which received no treatment, had an even lower WBC count of 0.01, indicating severe depletion. In contrast, the non-model-based group maintained a relatively higher count of 8.3. By Day 5, recovery started in the subcutaneous group, with the WBC count increasing to 3.2, and the intravenous group remained at a low count of 1. However, the non-model-based group showed a slight increase in WBC count to 9.6, while the model group showed a slight recovery, with a count of 0.04. The saline group continued to stabilize at 8.9. On Day 7, the subcutaneous group presented further development, with the WBC count growing to 5.6. The intravenous group also noticed that it rose to 1.9, while the non-model-based group reached 9.8. The model group's count increased slightly to 1.1, and the saline group declined slightly to 8.1. By Day 9, the subcutaneous group showed a notable recovery, with the WBC count accomplishment of 8, while the intravenous group improved to 3.9. The non-model-based group presented a slight reduction to 8.9, and the model group saw substantial improvement, with the WBC count increasing to 5.6. On the other hand, the following figure shows that the saline group remained stable at 9.2. On Day 11, the subcutaneous group showed the highest retrieval, with a WBC count of 10.1, while the intravenous group reached 6. The non-model-based group presented a small decline to 8.5, and the model group sustained recovery, with a WBC count of 6.1. The saline group continued to stabilize at 8.5. There is no substantial difference between the groups (p>0.05). The study showed variation in peripheral WBC counts in animal models inoculated with rLZ-8 proteins and found that the subcutaneous route of administration (Fig. 3) resulted in the most significant recovery in WBC counts, particularly from Day 7 onwards, followed by the intravenous route. Both groups outperformed the model group, which showed only gradual recovery, and the non-model-based group maintained high WBC counts throughout the study.

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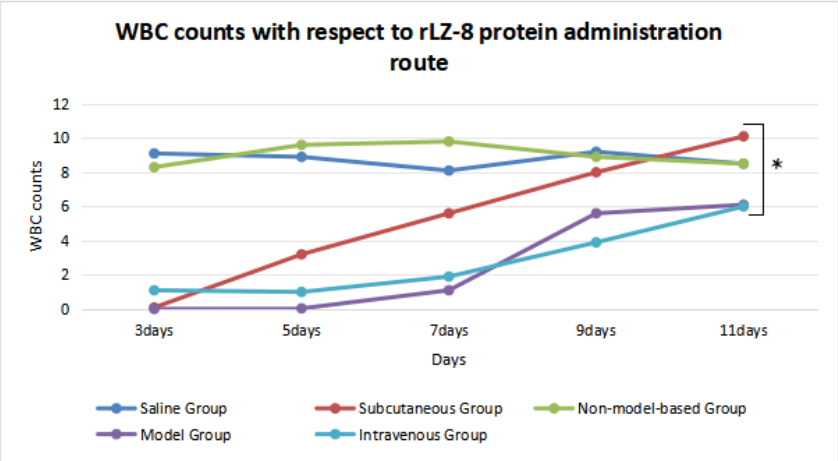

Fig. 3: Variation of Peripheral WBC counts in animal models inoculated with rLZ-8 proteins via different routes

The saline group had a neutrophil ratio of 6.2 on Day 4, while the model and G-CSF groups had much lower ratios. With the model group having no neutrophils and the G-CSF group having 5.2. The rLZ-8 group demonstrated a ratio comparable to G-CSF, at 5.1. By Day 5, the saline group exhibited a modest increase to 6.3, whereas the model group continued to demonstrate no recovery (0). The G-CSF group exhibited an enhancement to 9.3, while the rLZ-8 group showed a rise to 6.2. For Day 6, the saline group's neutrophil ratio stayed at 6.4, whereas the model groups did not change. The G-CSF group exhibited a significant increase to 16.2, while the rLZ-8 group ascended to 6.5. By Day 7, the G-CSF group demonstrated a notable increase in the neutrophil ratio, reaching 29.5, whereas the rLZ-8 group showed considerable improvement to 20.1. The saline group showed a minor decrease to 6.1, whereas the model group showed a modest gain to 1.9.

On Day 8, the rLZ-8 group exceeded the G-CSF group, achieving a neutrophil ratio of 31.5, whereas the G-CSF group decreased to 16.5. The saline group decreased somewhat to 5.9, while the model group showed negligible improvement to 2. On Day 9, the rLZ-8 group demonstrated the greatest neutrophil ratio at 45.2, closely followed by the G-CSF group at 50.1. In contrast, the saline group maintained a steady 6.2, whereas the model group showed a notable increase to 8.9. On Day 9, the p-value comparing the (rLZ-8 and G-CSF) groups to the (saline and model) groups were 0.025, signifying a statistically significant difference in neutrophil ratios. This indicates that both rLZ-8 and G-CSF therapies were markedly more effective at enhancing neutrophil ratios than the saline and model groups. The data suggest that rLZ-8 and G-CSF significantly enhanced neutrophil recovery, with rLZ-8 exhibiting a comparable recovery rate to G-CSF from Day 7 onwards. Fig. 4 shows fluctuations in peripheral neutrophil ratios over 9 days across four groups: the saline group, model group, G-CSF group, and rLZ-8 group.

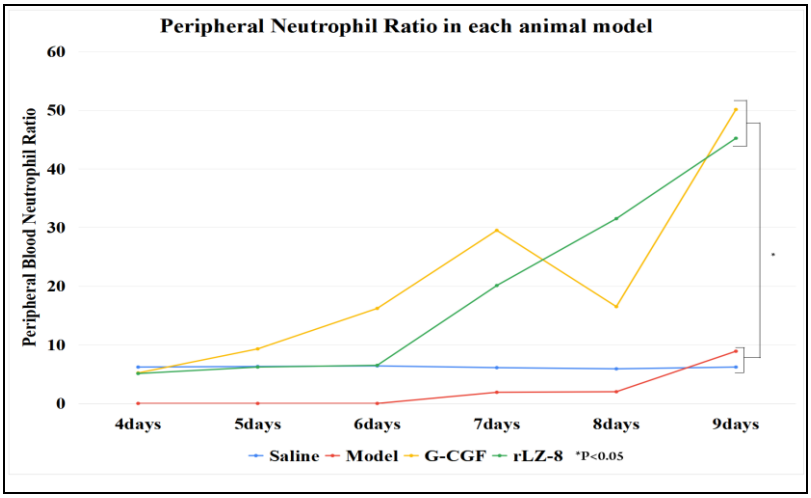

Fig. 4: Variation of Peripheral Neutrophil Ratio in animal models in each group

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The study also noted the fluctuation in bone marrow neutrophil ratios throughout a 9-day duration across four groups (saline, model, G-CSF, and rLZ-8 groups). On Day 4, the saline group exhibited a high neutrophil ratio of 39.9, but the model group demonstrated negligible recovery, with a ratio of 0.01. The G-CSF group had a ratio of 5.2, whereas the rLZ-8 group demonstrated a marginally elevated ratio of 5.6. On Day 5, the saline group experienced a small reduction to 38.2, but the model group exhibited a minor improvement to 2.1. The G-CSF group rose to 9.6, but the rLZ-8 group stayed constant at 5.6. On Day 6, the saline group exhibited an increase in neutrophil ratio to 42, whereas the model group improved to 9.6. The G-CSF group exhibited considerable growth, attaining 39.5, whereas the rLZ-8 group experienced a notable rise to 35.2. On Day 7, the saline group maintained a consistent value of 40.9, whereas the model group exhibited an improvement to 20. The rLZ-8 group rose to 62.5 after the G-CSF group rose to 78.6. The p-value of 0.0485 between the rLZ-8 group and the model group on Day 7 signifies a statistically significant difference.

By Day 8, the model group further improved, achieving a neutrophil ratio of 35.6. The G-CSF group had a minor decrease to 55.3, whereas the rLZ-8 group displayed more enhancement, attaining 78.9. The saline group stayed constant at 40.5. On Day 8, the rLZ-8 and model groups had a significant difference in neutrophil ratios, as indicated by the p-value of 0.0369. On Day 9, the rLZ-8 group sustained a neutrophil ratio of 78.9, like the G-CSF group, which attained 82.6. The model group demonstrated ongoing improvement, attaining a score of 55.2, whereas the saline group maintained a consistent score of 40.8. The p-value of 0.0299 between the rLZ-8 and model groups on Day 9 indicates a statistically significant difference.

The study found that both rLZ-8 and G-CSF treatments markedly enhanced bone marrow neutrophil ratios relative to the model group (Fig. 5). The rLZ-8 therapy demonstrated consistent enhancement, nearly paralleling the efficacy of G-CSF by the conclusion of the research period.

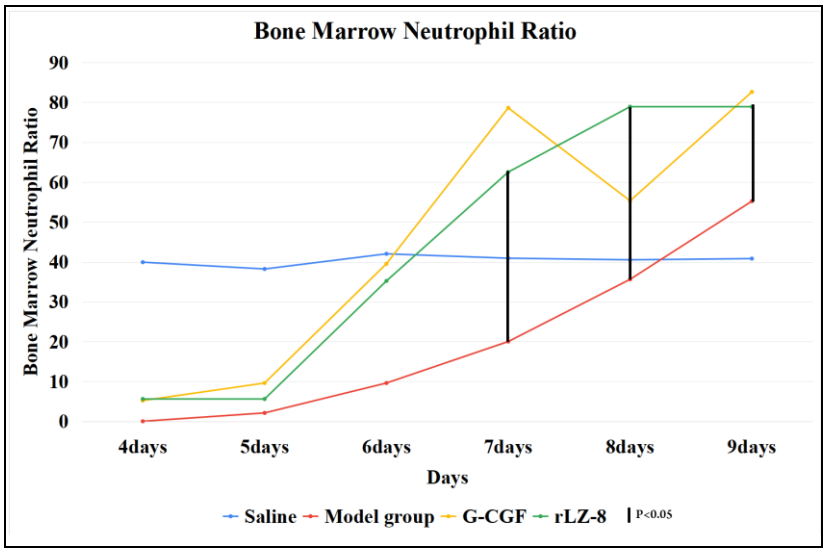

Fig. 5: Variation of Bone Marrow Neutrophil Ratio in animal models in each group

The model group administered cyclophosphamide demonstrated the lowest SDF-1 expression at 100 AU. High dosages of rLZ-8 boosted SDF-1 levels by 300 AU and had a p-value of 0.0156, showing considerable statistical significance compared to the model group. G-CSF similarly increased SDF-1 expression to 250 AU, with a p-value of 0.011. The findings indicate that rLZ-8, particularly at elevated doses, significantly increases SDF-1 expression, which may facilitate hematopoietic stem cell mobilization. Table 2 shows that the SDF-1 expression levels across several treatment groups, adjusted to GAPDH.

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Table 2: SDF expression of the mice models in each group

Group	SDF-1 Expression (AU)	Normalised to GAPDH (AU)	p-values (compared to Model Group)
Control (No treatment)	150	1.2	0.85
Model (Cyclophosphamide)	100	0.8	-
rLZ-8 (Low Dose)	200	1.5	0.04
rLZ-8 (High Dose)	300	2	0.01
G-CSF	250	1.8	0.01

DISCUSSION

Fungal immunomodulatory protein, or FIP, has been utilized to discover and develop new pharmaceutical drugs. The first FIP isolated was LZ-8 in 1989; ten FIPs have been identified so far. [17] including FIP (gts, gja gmi, fve and vvo). The genetic characteristics of FIPs originated from numerous fungi exhibit a remarkable similarity to the immunoglobulin superfamily. This fact significantly contributed to the anti-tumor, anti-allergic, lymphocyte proliferation, cytokine expression induction, and anti-graft rejection properties [18]. Although FIP members share sequential homology, their function has prominent differences. The structures of FIPs are modified because of the variations in the surface hydrophobicity of proteins. The interaction between the two homologous monomers is influenced by the unique structure formed due to a differential electrostatic potential. Because of their relatively low molecular weight (13kDa with 110 to 114 amino acids), FIPs were suitable for in vitro production and structural modifications. Genetically engineered strains are often used to produce highly effective pharmacological molecules and continue to be used in further in-depth studies of FIP proteins [19].

Recombinant LZ-8 stimulates HSC self-renewal and differentiates into the granulocyte-derived precursor cells. The ratio of the neutrophil population rises continuously, implying that there is always a process of replenishment of cells. LZ-8's immunomodulatory activities activate HSC precursor cells that, in turn, raise cytokine expressions, elevating neutrophil production and development. After G-CSF treatment, the neutrophil ratio inclines, then falls and increases again due to HSC differentiation. G-CSF stimulates granulocyte progenitor cells that express G-CSFR but do not cause all HSCs to proliferate. Both G-CSF and rLZ-8 promote neutrophil mobilization through the CXCR4-SDF1 axis. G-CSF mediates its effects on granulocyte progenitor cells expressing G-CSFR but does not stimulate the proliferation of all HSCs. Moreover, the repopulating potential per cell is decreased in G-CSF-mobilized HSCs compared to the untreated ones, which may eventually reduce cell numbers [20].

Moreover, as the CFU result was confirmed, achieving directional differences depends on the concentration of rLZ-8. In addition, cytokines of the microenvironment play a critical role in differentiation [21]. According to Mossadegh-Keller *et al.*, HSCs respond to environmental stressors and link the stress-induced cytokines. Lineage-specific cytokines could directly affect the HSCs in vitro and *in vivo*, causing a change in cell identity [22]. For instance, CLPs isolated from transgenic mice differentiate into macrophages and granulocytes in response to IL-2 stimulation [23], whereas rLZ-8 induces the transcription of IL-2 gene expression [5]. The observed effects of rLZ-8 on neutrophil recovery may be partially mediated through the CXCR4-SDF1 pathway, a critical axis for hematopoietic stem cell mobilisation. Additional studies on this signalling mechanism could elucidate how rLZ-8 uniquely influences neutrophil counts compared to traditional therapies.

All the cells of HSCs express CSF1R, a class III receptor tyrosine kinase containing an intracellular transmembrane and five heavily glycosylated immunoglobulin domains in the extracellular space [24]. It is also an M-CSF receptor, another critical CSF family member. CSF-1 and IL-34 can activate this receptor kinase by interacting with the extracellular domain. Induction of intracellular autophosphorylation and receptor dimerization leads to the activation of intracellular signal transduction. Numerous receptor activation results from various ligand recognition patterns and binding locations [25,26].

Recombinant LZ-8 is a candidate ligand of CSF1R that boasts multiple sites for the binding of CSF-1 and IL-34,

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which explains the divergence in its dimerization. This diversity affects HSCs in various ways in the case of self-renewal and differentiation and helps activate downstream metabolism. Therefore, binding sites of rLZ-8 and CSF1R are crucial to understanding cellular pathways responsible for FIP immunoregulation [24].

G-CSF and chemokines such as CXCR4 are the common mobilizers of bone marrow precursors, but they may cause splenomegaly or severe bone pain. LZ-8 may reduce SDF-1 expression or alter the expression of adhesion molecules such as VCAM-1, thereby impairing HSC adhesion to the bone marrow stroma. The protein may be much safer for patients who need mobilization of the bone marrow because of its ability to enhance the migration of stem cells [27,28].

CONCLUSIONS

The study concludes that Ganoderma lucidum–derived recombinant LZ-8 (rLZ-8) protein effectively promotes neutrophil recovery from neutropenia by stimulating bone marrow mobilization. This is supported by the significant increase in neutrophil counts and enhanced mobilization of hematopoietic stem cells observed in the study. The findings indicate that rLZ-8 has strong therapeutic potential for neutropenia management. Among the tested doses, the medium dose of rLZ-8 was the most effective at increasing neutrophil counts, even more so than G-CSF. The study makes an important clinical contribution by demonstrating that rLZ-8 may serve as a safer and effective alternative to existing neutropenia treatments through enhanced neutrophil production and bone marrow activation. However, additional animal studies are required before proceeding to clinical trials. Overall, rLZ-8 appears to be a promising alternative to G-CSF for neutropenia management, with potential future application in humans after further mechanistic and clinical investigations.

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