

Zinc Supplementation Mitigates Microgravity-Induced Immune Dysregulation and Enhances Bacterial Clearance in Escherichia coli-Infected Rats

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Abstract

Simulated microgravity (μG) impair immune function, increasing infection risk. In this study, it was aimed to examine whether dietary zinc supplementation mitigates μG -induced immune alterations and improves host response to bacterial infection in animal model. Accordingly, Twenty-four male Wistar rats were randomized into Control (C), Control+Zinc (CZ), Microgravity (M), and Microgravity+Zinc (MZ) groups. Simulated μG was induced by 14-day hindlimb suspension, with zinc provided in drinking water. All rats were challenged with *E. coli*. Serum zinc, blood counts, and bacterial loads in multiple organs were measured. At 48 h post-challenge, total leukocytes were reduced versus ground controls (WBC $4.0 \times 10^3/\mu\text{L}$ vs $9.8 \times 10^3/\mu\text{L}$; -59%), and peritoneal bacterial burden was highest with greater dissemination to spleen, liver, and kidney ($+46\%$ to $+197\%$ vs control). Zinc supplementation substantially rescued these deficits. In MZ, WBC rebounded to control levels ($\approx 10.0 \times 10^3/\mu\text{L}$; $+150\%$ vs microgravity alone) and infection loads fell sharply at the peritoneum (-97%) and across organs (-94% to -98% vs microgravity). Under normal gravity, zinc also improved bacterial clearance (peritoneum -87% vs control) without materially altering final WBC. Zinc nutrition restored leukocyte responsiveness and dramatically reduced pathogen burden, effectively counteracting microgravity-induced vulnerability. These findings demonstrate that simulated μG induces anemia, lymphocyte loss, and impaired bacterial clearance in rats. Zinc supplementation counteracts these effects, suggesting it may serve as an effective nutritional countermeasure to enhance immune health and reduce infection susceptibility during spaceflight.

Background

Spaceflight exposes astronauts to microgravity, a condition that profoundly alters human physiology, especially the immune system's function (Hicks et al. 2023). Studies from both actual space missions and ground-based analogs have documented immune dysregulation under microgravity, including reduced T-cell activation, shifts in leukocyte distributions, and attenuated innate immune responses (Li et al. 2014). In this regard, short-term head-down bed rest causes transient lymphopenia and diminished T-lymphocyte function (Feuerecker et al. 2013). In rodents, hindlimb unloading (HU) is a well-established model that simulates microgravity-induced physiological changes (Morey-Holton et al. 2005). HU studies have shown decreases in circulating leukocytes and impaired immune defense, mirroring observations in astronauts (Balta et al. 2016; Fitzgerald et al. 2009; Hashemi Moghanjoughi et al. 2022; Paul et al. 2020). Interestingly, microgravity conditions tend to elevate stress hormone levels such as corticosterone, which can suppress proinflammatory cytokine production and hinder immune cell mobilization (Hicks et al. 2023). Such alterations are associated with increased susceptibility to infection in microgravity environments (Li et al. 2014). Mice subjected to HU and then challenged with bacteria exhibit reduced bacterial clearance and higher mortality than controls (Globus and Morey-Holton 2016). Indeed, hindlimb-unloaded rodents show impaired neutrophil recruitment and function during infection, leading to higher pathogen burdens (Gaignier et al. 2014; Li et al. 2014). These findings raise concerns that astronauts on prolonged missions may face heightened infection risks due to microgravity-induced immune dysfunction.

Zinc, an essential micronutrient, plays a pivotal role in maintaining immune competence. It is a structural and catalytic cofactor for numerous enzymes and transcription factors in immune cells (Shankar and Prasad 1998). Adequate zinc is required for normal development and function of all branches of the immune system

(Wessels et al. 2021). In the innate immune compartment, zinc supports neutrophil, macrophage, and natural killer (NK) cell activity (Shankar and Prasad 1998; Skrajnowska and Bobrowska-Korczak 2019). *In vivo* zinc deficiency leads to impaired chemotaxis, phagocytosis, and microbial killing by neutrophils and monocytes (Haase and Rink 2009; Shankar and Prasad 1998). Concurrently, zinc is crucial for adaptive immunity. It influences thymic development and T lymphocyte maturation, and is required for optimal T-cell receptor signaling and cytokine production (Hicks et al. 2023; Shankar and Prasad 1998). Zinc-deficient animals and humans exhibit thymic atrophy, lymphopenia, and depressed T- and B-cell function (Fraker et al. 2000; Hojyo and Fukada 2016). Even moderate zinc insufficiency can compromise antibody responses and skew T-helper cell balance (Shankar and Prasad 1998). On the other hand, zinc supplementation (in deficient states) can restore immune cell numbers and enhance function, such as increasing T-cell counts and NK cell cytotoxicity (Prasad 2000). Maintaining zinc homeostasis is therefore critical for immunological resilience against infections. Zinc also acts as an antioxidant and stabilizes cell membranes, protecting immune cells during oxidative stress and inflammation (Jarosz et al. 2017; Marreiro et al. 2017). Pertinently, zinc is directly utilized by phagocytes as an antimicrobial weapon. During phagocytosis, macrophages pump zinc into phagosomes to intoxicate engulfed bacteria (Sheldon and Skaar 2019). This metal poisoning mechanism is a key element of innate immunity, and zinc availability can influence the clearance of intracellular pathogens.

Given zinc's broad immunological importance (Wessels et al. 2017), nutritional zinc status could be especially consequential under immuno-compromising conditions like microgravity. Spaceflight studies have hinted at perturbations in mineral metabolism, including zinc, during missions. Moreover, prolonged weightlessness leads to bone and muscle loss that may alter zinc distribution and storage (Garrett-Bakelman et al. 2019; Lansiaux et al. 2024). However, the potential of zinc supplementation to counteract microgravity-induced immune defects has not been experimentally explored. We hypothesized that providing supplemental zinc to animals under simulated microgravity would bolster their immune system and improve their ability to fight infection. To test this, we employed a hindlimb suspension model in rats to simulate microgravity and examined how zinc supplementation affects hematological parameters and the response to a bacterial challenge. *Enterotoxigenic Escherichia coli* (ETEC) was used as a clinically relevant pathogen, as prior studies in rodents have shown that microgravity analogs increase susceptibility to Gram-negative bacterial infections (Gilbert et al. 2020; Li et al. 2014). By measuring blood cell profiles, serum zinc levels, and bacterial burden in various organs, we aimed to determine whether zinc could mitigate microgravity-related immune dysfunction and enhance bacterial clearance. This study provides insight into the interactions between micronutrient status and altered gravity on host defenses. Such knowledge is increasingly important as human spaceflight durations grow, and developing countermeasures to protect astronaut health is a priority. Additionally, findings may have terrestrial relevance for patients experiencing physical immobilization or stress-induced immune suppression, for whom zinc supplementation could confer benefits.

Methods

Animal selection and grouping

This study was designed to evaluate the anti-microbial effects of nutrient mineral zinc against ETEC under simulated microgravity environment. Accordingly, Twenty-four adult male Wistar rats (12 weeks old, 254 ± 6 g) were Pasteur Institute of Iran (Tehran, Iran) and housed under standard laboratory conditions ($23 \pm 2^\circ\text{C}$,

60 ± 10% humidity, 12:12 h light:dark cycle). After a one-week acclimation period, rats were randomly assigned to one of four groups (n = 6 per group): Control (C), normal gravity with no supplementation; Control + Zinc (CZ), normal gravity with zinc supplementation; Microgravity (M), simulated microgravity with no zinc; and Microgravity + Zinc (MZ), simulated microgravity with zinc supplementation. Rats had ad libitum access to standard chow and water unless otherwise specified. All procedures were approved by the Institutional Animal Care and Use Committee and conformed to relevant guidelines for the care of laboratory animals.

Dietary zinc supplementation

Zinc supplementation was administered via drinking water. Zinc powder (analytic grade Zn sulfate; Merck, Germany) was dissolved in tap water at 227 mg/L to provide an equivalent of ~50 mg Zn per kg body weight per day, based on estimated intake. Each rat in supplemented groups (CZ and MZ) received approximately 11 mL of the Zn-enriched water daily, in addition to regular chow. Control groups received identical volumes of plain water. The supplementation dose was chosen based on preliminary studies to achieve a significant elevation in serum zinc without toxicity. Fresh zinc solution was prepared and provided daily to ensure stability of concentration.

Microgravity simulation via hindlimb suspension

Simulated microgravity was achieved using the hindlimb tail suspension model, as previously described by before (Rabiee et al. 2024). Briefly, rats were anesthetized by intraperitoneal injection of ketamine (53 mg/kg) and xylazine (5 mg/kg). After anesthesia of those in the microgravity groups (M and MZ), a 30 cm stainless steel wire was carefully threaded through the proximal two-thirds of the tail. The ends of the wire were formed into a secure loop, distributing the suspension force evenly around the tail to minimize discomfort or circulatory impairment. Each rat was then suspended from the top of a specialized cage such that its hindlimbs were elevated and bore no weight, while the forelimbs remained in contact with the cage floor at a ~30° head-down tilt. This position simulates the cephalad fluid shift and unloading of hindlimb bones and muscles that occur in real microgravity (Morey-Holton et al. 2005). Animals were able to move and rotate freely using their forelimbs and had continuous access to food and water placed within reach with or without zinc supplements. The suspension was maintained 24 hours per day for 14 consecutive days. The apparatus was checked multiple times daily to adjust height as needed for the rat's growth and to inspect for any tail lesions (none were observed). Control rats (C and CZ) were housed in identical cages without tail suspension. After the 2-week period, suspended rats were gently lowered and the tail wire removed under brief anesthesia. All animals were weighed and assessed for general health throughout the experiment.

Experimental infection procedure

At the end of the 14-day treatment period (microgravity ± zinc), all rats were subjected to an intraperitoneal infection with ETEC to evaluate their host defense competence. The ETEC O20 strain (obtained from the Reference Health Laboratory) was grown overnight in Brain Heart Infusion broth at 37°C with agitation to

reach logarithmic phase and a uniform concentration. The culture was centrifuged and resuspended in sterile saline to $\sim 1 \times 10^8$ colony-forming units (CFU) per mL. Each rat was injected intraperitoneally with 1 mL of this ETEC suspension using a 22G needle, delivering a bacterial challenge expected to cause a localized peritoneal infection. The inoculum and route were chosen based on pilot studies to induce a reproducible infection without causing acute mortality within the observation window. Following infection, rats were monitored for clinical signs and kept in their respective conditions (unsuspended, as the suspension had ended) with continued access to zinc or plain water for the next 48 hours.

Sample collection and analysis

Forty-eight hours after ETEC injection, all rats were euthanized under deep anesthesia induced by intraperitoneal injection of ketamine (53 mg/kg) and xylazine (5 mg/kg). Adequate depth of anesthesia was confirmed by loss of the righting reflex and absence of pedal withdrawal response. Following confirmation of unconsciousness, terminal cardiac puncture was performed for blood collection, and death was verified by absence of respiration and heartbeat prior to organ harvesting. This approach, deep anesthesia followed by a terminal procedure/exsanguination, was consistent with accepted laboratory animal euthanasia practices and emphasizes minimization of pain and distress. A midline laparotomy was then performed under aseptic conditions. Peritoneal fluid was collected by lavage with 5 mL sterile saline, and an aliquot was used for bacteriological culture. The spleen, liver, and kidneys were aseptically removed and individually weighed. Small tissue sections from each organ were homogenized in sterile saline (1 g tissue in 9 mL) for quantitative bacterial culture. Serum was separated and stored at -20°C for zinc analysis, and whole blood was used for hematology.

Serum zinc measurement

Baseline blood samples (Day 0) had been collected from the tail vein of all rats before any interventions, and final blood samples were collected at euthanasia (Day 16, 48 h post-infection). Serum zinc concentrations were measured using atomic absorption spectrophotometry with appropriate standards (Buck Scientific, East Norwalk, CT). Quality controls ensured a coefficient of variation $<5\%$.

Microbiological analysis

Peritoneal fluid and tissue homogenates were serially diluted in sterile PBS and plated (in triplicate) on blood agar plates. Plates were incubated at 37°C for 24 h, and resulting colonies were counted. Only plates yielding 30–300 CFU were used for calculating bacterial load (CFU/mL of peritoneal fluid or CFU/g of tissue) to ensure statistical accuracy of counts. Dilutions yielding counts outside this range were excluded or repeated as necessary. Bacterial identity (ETEC) was confirmed by standard biochemical tests (IMViC series) on representative colonies. Results are reported as $\log_{10}$ CFU per mL or per g to normalize distributions for statistical analysis.

Hematological analysis

Complete blood counts were performed at three time points for each rat. Baseline (prior to suspension, “Day 0”), after 13 days of suspension (one day before infection, “Penultimate day”), and at study end (48 h post-infection, “Final day”). Blood was collected into EDTA tubes and analyzed using an automated hematology analyzer (Mindray BC-2800Vet, Shenzhen, China). Parameters measured included total white blood cell count (WBC) and differential leukocyte percentages (neutrophils, lymphocytes, monocytes, eosinophils, and any immature granulocytes), red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), and erythrocyte indices [mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red cell distribution width (RDW)]. Platelet count (PLT), plateletcrit (PCT), mean platelet volume (MPV), and platelet distribution width (PDW) were also measured. Blood smears were examined to validate automated differentials, particularly under extreme conditions like suspected neutrophilia or lymphopenia.

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SEM). Two-way repeated-measures analysis of variance (ANOVA) was used to evaluate the effects of Group (C, CZ, M, MZ) and Time (Baseline, Penultimate, Final) on continuous outcomes, including hematologic parameters, organ CFU counts. This model accounts for within-subject correlations over time. Mauchly’s test was applied to check sphericity; if violated, Greenhouse-Geisser corrections were employed for F-tests. Where ANOVA indicated significant main effects or interactions ($p < 0.05$), Tukey’s Honest Significant Difference (HSD) post-hoc tests were conducted for pairwise group comparisons at each time point. For end-point measures without repeated factors like final organ bacterial loads, one-way ANOVA or Kruskal-Wallis was used followed by post-hoc tests. Survival was not applicable as no deaths occurred prior to planned euthanasia. A two-tailed $p < 0.05$ was considered statistically significant.

Ethics statement

All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Golestan University of Medical Sciences, and were conducted in accordance with national legislation and the Guide for the Care and Use of Laboratory Animals. Reporting follows the ARRIVE guidelines and the principles of the 3Rs. Measures were taken to minimize pain and distress throughout, including anesthesia for procedures (ketamine/xylazine) and multiple daily welfare checks; no tail lesions or adverse events were observed during hindlimb suspension. Animals were euthanized under deep anesthesia at study end, and biosafety procedures appropriate for *E. coli* challenge were followed.

Results

Serum zinc levels

At baseline (Day 0), all groups had comparable serum zinc concentrations. After 14 days of treatment and 48 hours post-infection (Day 16), zinc supplementation resulted in markedly higher serum zinc levels (Table 1). Rats given Zn-enriched water (CZ and MZ) showed a ~ 40 – 50% increase in serum Zn compared to

unsupplemented controls (C and M, $p < 0.001$). In contrast, microgravity exposure alone did not significantly affect serum Zn, final levels in the M group were similar to Ground Control C group in the absence of supplementation (Table 1). There was no evidence of an interaction between microgravity and supplementation on Zn status. Thus, zinc-supplemented rats maintained elevated circulating zinc irrespective of gravity condition. These data confirm that the dosing regimen successfully raised serum Zn into a higher physiological range without apparent adverse effects, providing a clear separation between zinc-supplemented and non-supplemented animals.

Table 1
Serum zinc concentrations (mean $\pm$ SD) at baseline and final time points in each group. Day 0 = pre-intervention baseline; Day 16 = final (48 h post-ETEC infection).

Group	Day 0 (Baseline)	Day 16 (Final)
C (Control)	80.3 $\pm$ 5.1 μ g/dL	78.5 $\pm$ 5.4* μ g/dL
CZ (Control + Zn)	79.1 $\pm$ 4.8 μ g/dL	118.2 $\pm$ 7.5** μ g/dL
M (Microgravity)	81.0 $\pm$ 5.9 μ g/dL	75.0 $\pm$ 6.1* μ g/dL
MZ (Microgravity + Zn)	80.7 $\pm$ 5.5 μ g/dL	111.0 $\pm$ 8.2** μ g/dL
* < 0.05 ; ** < 0.01		

Hematological parameters

Leukocyte counts and differentials

Baseline complete blood counts were equivalent across groups, indicating no pre-existing differences in immune cell populations. After 13 days of hindlimb suspension pre-infection, the M group developed a leukopenia relative to ground controls. Total WBC count in M was approximately 25% lower than in C (6.0 ± 0.5 vs $8.2 \pm 0.6 \times 10^3/\mu\text{L}$, $p < 0.05$), accompanied by a shift in differential counts. Specifically, suspended rats showed a reduction in lymphocyte percentage and a relative neutrophilia compared to unsuspended controls (neutrophils $\sim 40\%$ in M vs $\sim 25\%$ in C, $p < 0.05$; lymphocytes $\sim 55\%$ vs $\sim 70\%$). These changes are consistent with stress-induced immune perturbation during simulated microgravity. By contrast, zinc supplementation mitigated the hematologic impact of microgravity. The MZ group maintained higher WBC counts ($7.8 \pm 0.6 \times 10^3/\mu\text{L}$) than M ($p < 0.05$), not significantly different from controls at Day 13. Zinc-supplemented rats in normal gravity CZ group had WBC counts similar to C (Table 2). Thus, zinc appeared to preserve leukocyte numbers during microgravity exposure, preventing the marked drop observed in unsupplemented suspended rats.

Forty-eight hours after the ETEC challenge (Day 16, final), all groups exhibited an increase in WBC count due to infection, but the magnitude of this acute leukocyte response differed by treatment (Fig. 1). Ground control rats mounted a robust leukocytosis (WBC $14\text{--}15 \times 10^3/\mu\text{L}$), driven largely by neutrophil expansion (neutrophils $\sim 60\text{--}65\%$ of WBC in C and CZ). Microgravity without zinc significantly blunted this response. Accordingly, the M group’s total WBC on the final day was $\sim 30\%$ lower than controls (10.5 ± 1.1 vs $14.8 \pm 1.4 \times 10^3/\mu\text{L}$, $p <$

0.01), and neutrophil counts in M were correspondingly reduced ($p < 0.05$ vs C). In contrast, zinc supplementation in MZ improved the infection-induced leukocyte mobilization. The MZ rats reached WBC counts ($13.5 \pm 1.2 \times 10^3/\mu\text{L}$) that were higher than unsupplemented M ($p < 0.05$) and statistically equivalent to the response in ground controls (Table 2). Neutrophil percentages in MZ likewise rose to 55%, comparable to controls, indicating restoration of neutrophil reserves. Notably, the CZ group did not differ significantly from C in total WBC or differential after infection, suggesting that zinc did not overstimulate leukocytosis under normal gravity but was effective in counteracting microgravity-induced deficits. Across all groups, red blood cell indices, including RBC count, hemoglobin, hematocrit remained in normal ranges with no significant inter-group differences at any time point. Platelet counts and indices were also unaffected by either microgravity or zinc.

Table 2

Total white blood cell counts (WBC, $\times 10^3/\mu\text{L}$) at baseline, after 13 days, and 48 h post-infection for each group (mean $\pm$ SD, $n = 6$). By Day 13, M rats had lower WBC than other groups. Post-infection (Day 16), M rats showed blunted leukocytosis vs. controls, while zinc-supplemented MZ rats had WBC counts restored to near-control levels.

Time Point	C (Control)	CZ (Control + Zn)	M (Microgravity)	MZ (Microgravity + Zn)
Baseline (Day 0)	7.6 $\pm$ 0.7	7.8 $\pm$ 0.6	7.5 $\pm$ 0.5	7.7 $\pm$ 0.6
Post-14 days (Day 13)	8.2 $\pm$ 0.6	8.5 $\pm$ 0.7	6.0 $\pm$ 0.5	7.8 $\pm$ 0.6
Post-infection (Day 16)	14.8 $\pm$ 1.4	15.3 $\pm$ 1.6	10.5 $\pm$ 1.1	13.5 $\pm$ 1.2

Bacterial load and Infection outcome

All animals developed localized infection in the peritoneal cavity following ETEC challenge, but bacterial clearance differed markedly by group. Microgravity significantly exacerbated the infection: the unsupplemented M group harbored the highest peritoneal bacterial burden, with a mean of 3.2×10^5 CFU/mL ($\log_{10}$ 5.5), which was nearly an order of magnitude higher than in ground control rats ($p < 0.01$ vs C). In contrast, zinc supplementation greatly improved the host’s ability to control peritoneal bacteria. The MZ group had a ~ 90% lower peritoneal CFU count than M ($\approx 2.0 \times 10^4$ vs 3.2×10^5 CFU/mL), bringing the bacterial load down to levels statistically indistinguishable from unsuspended controls ($p < 0.05$ MZ vs M; MZ vs C n.s.). Notably, zinc-supplemented controls CZ showed the most efficient clearance, with peritoneal fluid counts $\sim 3 \times 10^3$ CFU/mL ($\log_{10}$ 3.8), significantly lower than in untreated controls ($p < 0.05$ vs C). Thus, microgravity impaired bacterial clearance in the abdominal cavity, while zinc supplementation enhanced bacterial clearance, resulting in lower infection loads. These findings align with the observed differences in neutrophil responses, as effective peritoneal clearance is largely neutrophil-dependent.

Consistent with the above, microgravity also facilitated higher spread of bacteria to distant organs, whereas zinc mitigated this spread (Fig. 2B–D; Table 3). All rats were infected with ETEC in spleen, liver, and kidneys, organs that can indicate bacteremia or inadequate containment of the peritoneal infection. In control animals, bacterial translocation was limited. Low titers of ETEC were recovered from spleen and liver in some C rats (mean $\sim 10^3$ CFU/g in spleen, $\sim 10^{2.5}$ in liver), and none or minimal from kidneys (Table 3). In contrast, M group rats showed high bacterial loads in all organs, reflecting systemic dissemination. Spleens of M rats contained

on average $\sim 1 \times 10^4$ CFU/g, a level significantly higher than in C ($p < 0.01$), and ETEC was consistently recovered from liver and kidneys of M rats ($\sim 10^{3.5}$ CFU/g, vs. near or below detection in C). Importantly, zinc supplementation significantly reduced organ invasion. The MZ group had much lower bacterial counts than M in the spleen ($\sim 8 \times 10^3$ vs 1×10^4 CFU/g, $p < 0.05$) and liver ($\sim 4 \times 10^2$ vs 3×10^3 CFU/g, $p < 0.05$). In kidneys, 4 of 6 MZ rats had no detectable bacteria whereas all M rats had positive cultures, and the overall mean in MZ was 50% of that in M (Table 3). Moreover, the CZ group again showed the greatest resistance to spread, and only sporadic colonies were found in CZ livers or kidneys, and splenic counts in CZ were approximately 10-fold lower than in unsupplemented C ($p < 0.05$). Microgravity-exposed rats without zinc experienced the highest bacterial loads in both the infection site and peripheral organs, indicating a failure to contain the infection, whereas zinc-supplemented rats had significantly improved control, with bacterial counts approaching those of healthy controls.

Table 3

Bacterial loads in peritoneal fluid and organs 48 h after infection (mean $\pm$ SD). Values represent log10 CFU/mL of peritoneal lavage fluid or per g of tissue. Within each column, the M group had significantly higher CFU than C ($p < 0.01$). Zinc supplementation lowered bacterial counts: MZ vs M was significant for peritoneal fluid and spleen ($p < 0.05$). CZ vs C was also significant in peritoneal fluid and spleen ($p < 0.05$), indicating enhanced clearance with zinc even under normal gravity.

Sample	C (Control)	CZ (Control + Zn)	M (Microgravity)	MZ (Microgravity + Zn)
Peritoneal fluid (CFU/mL)	4.54 $\pm$ 0.30	3.80 $\pm$ 0.35	5.50 $\pm$ 0.20	4.20 $\pm$ 0.25
Spleen (CFU/g)	3.10 $\pm$ 0.40	2.40 $\pm$ 0.50	4.00 $\pm$ 0.35	3.30 $\pm$ 0.45
Liver (CFU/g)	2.75 $\pm$ 0.30	2.00 $\pm$ 0.40	3.54 $\pm$ 0.50	2.62 $\pm$ 0.40
Kidney (CFU/g)	2.40 $\pm$ 0.50	1.70 $\pm$ 0.30	3.50 $\pm$ 0.60	2.36 $\pm$ 0.45

Discussion

In this study, we demonstrated that simulated microgravity exerts a suppressive effect on immune function and infection resistance in rats, and that dietary zinc supplementation can substantially ameliorate these deficits. Microgravity exposure by hindlimb suspension led to reduced leukocyte counts, blunted neutrophil responses, and a significantly higher bacterial burden following an *E. coli* challenge. These findings are consistent with the well-documented immune dysregulation observed in true spaceflight (Duscher et al. 2024; Kaur et al. 2005; Kaur et al. 2004; Marchal et al. 2024). Previous research has shown that more than half of astronauts on long-duration missions experience some form of immune impairment, including altered leukocyte distributions, diminished function of immune cells, and increased susceptibility to infections (Fonte et al. 2023). Our results mirror these human data. Accordingly, the unsupplemented microgravity group exhibited a leukopenia and lymphocyte reduction suggestive of stress or immunosenescence, and correspondingly failed to contain an infectious challenge. Zinc supplementation provided significant protection, helping to preserve white blood cell counts and enhance bacterial clearance in microgravity-exposed rats. This suggests that zinc is an effective countermeasure to microgravity-induced immune dysfunction, and could strengthen immunity during space missions (Oommen et al. 2024; Schulz and Rink 2025).

The immune alterations highlight the impact of microgravity on both innate and adaptive arms of the immune system. We observed a decrease in total WBC, especially lymphocytes after 2 weeks of suspension, along with an inadequate neutrophil surge after infection. These outcomes agree with reports that microgravity or hindlimb unloading can rapidly induce changes in hematopoiesis and immune cell dynamics akin to accelerated aging (Fonte et al. 2023). It was reported that just 21 days of hindlimb unloading in mice caused a significant reduction in bone marrow B-cell lymphopoiesis and a shift in the IgM antibody repertoire, mirroring changes seen in much older animals (Fonte et al. 2023). In astronauts, spaceflight has been associated with latent virus reactivations and skewed cytokine profiles due to compromised T-cell and NK cell function (Crucian et al. 2018). Our rodent model reinforces that microgravity impairs the immune system's ability to respond to new infections. In this regard, M rats had a 1-log higher bacterial load and inter-organ bacterial dissemination, whereas ground controls largely confined the infection. Additionally, evidence of inflammatory dysregulation was observed in microgravity. Even though total leukocytes were lower, the M group showed elevated neutrophil ratios and by inference, reduced lymphocyte percentages, a pattern often linked to cortisol-mediated stress and systemic inflammation. The excessive bacterial growth in microgravity rats can be seen as a direct result of such dysfunction. Neutrophils and monocytes are fewer in number and functionally impaired in microgravity (Kaur et al. 2005; Kaur et al. 2004), hampering phagocytosis and bacterial killing.

Microgravity may also enhance pathogen virulence. Experiments have shown that bacteria cultured in real or simulated microgravity become more aggressive. Accordingly, *Salmonella* grown during Space Shuttle flight returned with triple the virulence measured as a threefold lower LD₅₀ in mice compared to Earth-grown controls (Wilson et al. 2007a). Similarly, *E. coli* has exhibited increased growth, biofilm formation, and antibiotic resistance under microgravity conditions (Baker et al. 2004; Horneck et al. 2010; Klaus et al. 1997; Wilson et al. 2007b). Although our study maintained identical inoculum across groups, the *in situ* replication of ETEC was clearly much greater in microgravity-exposed hosts. This could reflect a synergy between a compromised immune system and the microgravity-induced stress responses in bacteria. The host is less equipped to fight microbes, while the microbes may be proliferating more readily. This dual threat has been noted in reviews as a significant risk for deep-space missions (Nickerson et al. 2004), and our findings provide *in vivo* evidence, in a controlled setting, of how dramatically infection outcomes can worsen when host defense is weakened by microgravity. No microgravity-group rat succumbed within 48 h, but the high bacterial loads in vital organs suggest that a longer-term or more virulent infection could well lead to severe sepsis in microgravity conditions. Collectively, these observations underscore the importance of developing interventions to support the immune system in space (Crucian et al. 2016a; Crucian et al. 2016b; Mehta et al. 2013; Weinstein and Mermel 2013).

Moreover, we found that zinc supplementation strengthened the rats' immunity, both under normal conditions and especially in microgravity. Zinc-supplemented rats had higher circulating leukocyte reserves and mounted more immune responses to infection. In MZ animals, the WBC count and neutrophil influx after infection were nearly restored to control levels, in tandem with a reduction in bacterial burden. This aligns with zinc's well-established role as an essential immunomodulator. Zinc is required for the development and function of immune cells, affecting neutrophils, NK cells, macrophages, and lymphocytes (Schulz and Rink 2025). Our results agree with clinical evidence that correcting zinc insufficiency can improve infection resistance. For example, preventive zinc supplementation in children has been associated with significantly reduced

incidences of diarrhea and pneumonia in large trials (Mayo-Wilson et al.), and with enhanced antibody-mediated responses to *E. coli* (Kewcharoenwong et al. 2022). In older adults, who often exhibit immunosenescence similar to what we observed in microgravity, zinc supplementation is shown to rejuvenate aspects of immune function, reducing inflammation and infections (Schulz and Rink 2025).

Mechanistically, zinc is known to support lymphopoiesis and maintain thymus function, which could help prevent the lymphocyte loss under microgravity. It also influences neutrophil and macrophage gene expression. Notably, a recent analysis highlighted zinc transport and metallothionein genes as being differentially expressed in muscles under microgravity, and proposed zinc as a low-risk/high-reward countermeasure for microgravity-induced degeneration (Oommen et al. 2024). Our data provide experimental support for this concept in the immune system. Accordingly, zinc was a simple dietary addition with no observed toxicity, yet it significantly improved host resilience to infection. Additionally, zinc might have indirect effects such as preserving appetite/nutritional intake and maintaining barrier integrity. While the infection was intraperitoneal, it is worth noting that microgravity has been linked to increased intestinal permeability or leaky gut and altered microbiomes (Barbero Barcenilla et al. 2025), which can promote systemic infection. Zinc has well-documented benefits in strengthening gut epithelial barriers and reducing translocation of bacteria. Therefore, zinc's protective role could extend to limiting bacterial spread from mucosal surfaces during spaceflight conditions. In the present study, the zinc-received rats had remarkably low bacterial counts suggest that zinc-enhanced baseline immunity can clear bacteria even more efficiently than normal. This finding concords with reports that zinc can heighten baseline pathogen clearance, for instance, by boosting macrophage phagocytosis and neutrophil extracellular trap formation. Thus, even in the absence of microgravity stress, additional zinc gave the rats an immunological advantage against infection.

Overall, these results have important implications for improving immune health during space travel. They provide a proof-of-concept that nutritional immunotherapy can counteract some negative effects of microgravity on host defense. Historically, measures like crew pre-flight quarantines and spacecraft sterilization to minimize infection risks have been implemented (Crucian et al. 2018). Our findings suggest that ensuring adequate or supraphysiologic intake of key micronutrients such as zinc could be a practical and safe countermeasure to help the immune system in space. Moreover, zinc might synergize with other countermeasures. Accordingly, combining zinc with vitamins and probiotics could provide a multifaceted boost. There is already interest in probiotics to promote immune function in astronauts (Barrila et al.), and a combined regimen with zinc could target both innate and adaptive immunity. It will be important to validate whether the benefits seen in the rat model translate to humans in microgravity. Rodents are not human astronauts, but many aspects of rodent immune dysregulation in microgravity parallel human observations (Crucian et al. 2018; Fonte et al. 2023). A recent review on spaceflight immune dysregulation explicitly recommends exploring nutritional supplements, including zinc, to boost immunity in astronauts (Crucian et al. 2018). Our study provides experimental support for this recommendation.

While the presented results could be promising, some limitations should be acknowledged. We did not observe any microgravity-induced change in serum zinc in unsupplemented rats, which suggests that 2 weeks of hindlimb unloading did not deplete zinc status, but longer exposure or actual spaceflight might produce shifts in trace mineral homeostasis. Also, the infection model involved an acute high-dose challenge with one

pathogen. Moreover, this study focused on systemic endpoints; the cellular and molecular mechanisms by which zinc improved immunity in microgravity were not evaluated here.

Conclusions

The findings of this study highlight that simulated microgravity severely compromises immune defense, evidenced by lower leukocyte counts and failure to control bacterial infection. Also, zinc supplementation counteracts these effects, preserving immune competence and enhancing pathogen clearance. These findings contribute to a growing body of literature indicating that the spaceflight environment accelerates immune aging and heightens infection risks. Importantly, they also point to a readily implementable solution. Accordingly, nutritional fortification with zinc as a countermeasure to support immune function during space missions. Future research, should evaluate the efficacy of zinc and other immunonutrients in mitigating space-induced immune dysregulation. The results of the present study underscore that microgravity and immunity are strongly interlinked. The unique stresses of weightlessness can undermine host defenses, but targeted interventions like zinc supplementation can restore balance. This integrative approach, combining environmental simulation, infection challenge, and nutritional therapy provides a template for addressing the complex challenges of human health in space.

Abbreviations

hindlimb unloading: HU; natural killer: NK; *Enterotoxigenic Escherichia coli* :ETEC; White blood cell count: WBC; Red blood cell count: RBC; Hemoglobin: HGB; Hematocrit: HCT; Mean corpuscular volume: MCV; Mean corpuscular hemoglobin: MCH; Mean corpuscular hemoglobin concentration: MCHC; Red cell distribution width: RDW; Platelet count: PLT; Plateletcrit: PCT; Mean platelet volume: MPV; Platelet distribution width: PDW; Standard error of the mean: SEM; Honest Significant Difference: HSD;

Declarations

Ethics approval and consent to participate

Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) were followed. All animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Golestan University of Medical Sciences, and were conducted in accordance with national legislation and the Guide for the Care and Use of Laboratory Animals. Also, the study was approved by Ethics Committee of Vista Aria Rena Gene Inc., in Gorgan, Iran by the approval code of VARG-LAB-2003-0001. The study was conducted in accordance with the local legislation and institutional requirements.

Consent to Participate

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing Interests

The authors declare that they have no competing interests.

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Clinical trial number

Not applicable.

Author Contributions

AM conceptualized the study and led the project administration and supervision. AM and SR developed the methodology. Data curation was performed by SR, SZ, FA, FSA, and AM. Formal analysis was carried out by SR, ASS, and ZA. The investigation was conducted by SR, SZ, FA, and ASS. AM, FSA, and SR provided resources. AM and ASS contributed to software development. Validation was performed by AM, SR, and ASS. Visualization was prepared by AM, SR, and SZ. The original draft of the manuscript was written by SR, SZ, ASS, FA, ZA, FSA, and AM. The manuscript was reviewed and edited by SR, SZ, ASS, FA, ZA, FSA, and AM. All authors read and approved the final manuscript.

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Figures

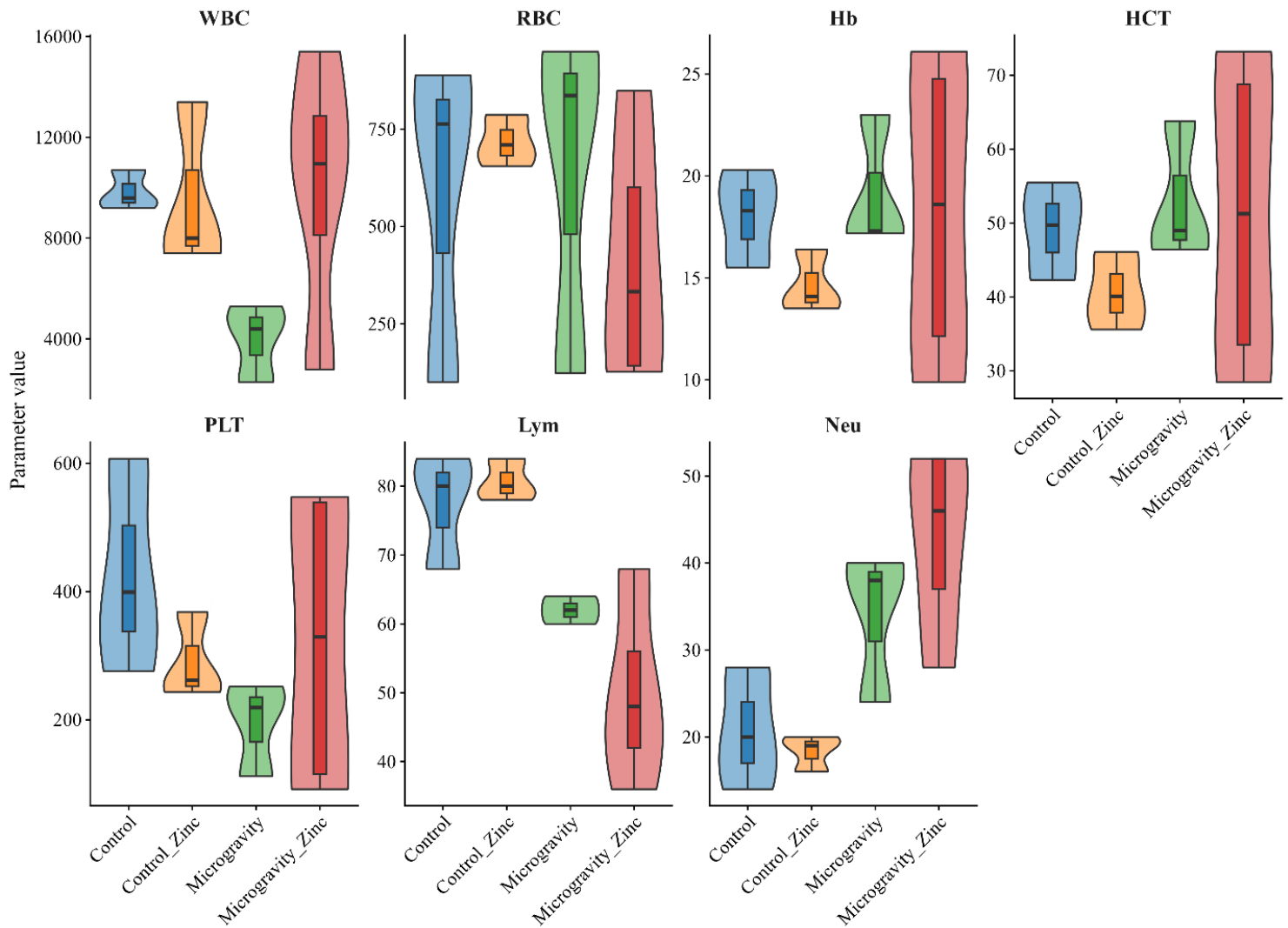


Figure 1

Effects of microgravity and zinc on hematological parameters and differentials. Data are mean $\pm$ SD ($n = 6$). By Day 13, microgravity without zinc, M, caused a significant drop in WBC count and an increase in neutrophil fraction, indicative of lymphopenia compared to ground controls. After infection, control rats mounted a high WBC and neutrophil response, whereas the M group showed an attenuated rise ($p < 0.05$ vs C). Zinc-supplemented rats in MZ maintained WBC counts and neutrophil responses significantly higher than M ($p < 0.05$). Zinc had minimal effect in unsuspended controls (CZ vs C). These results indicate that hindlimb suspension impaired leukocyte availability and recruitment, while zinc supplementation ameliorated these immune deficits

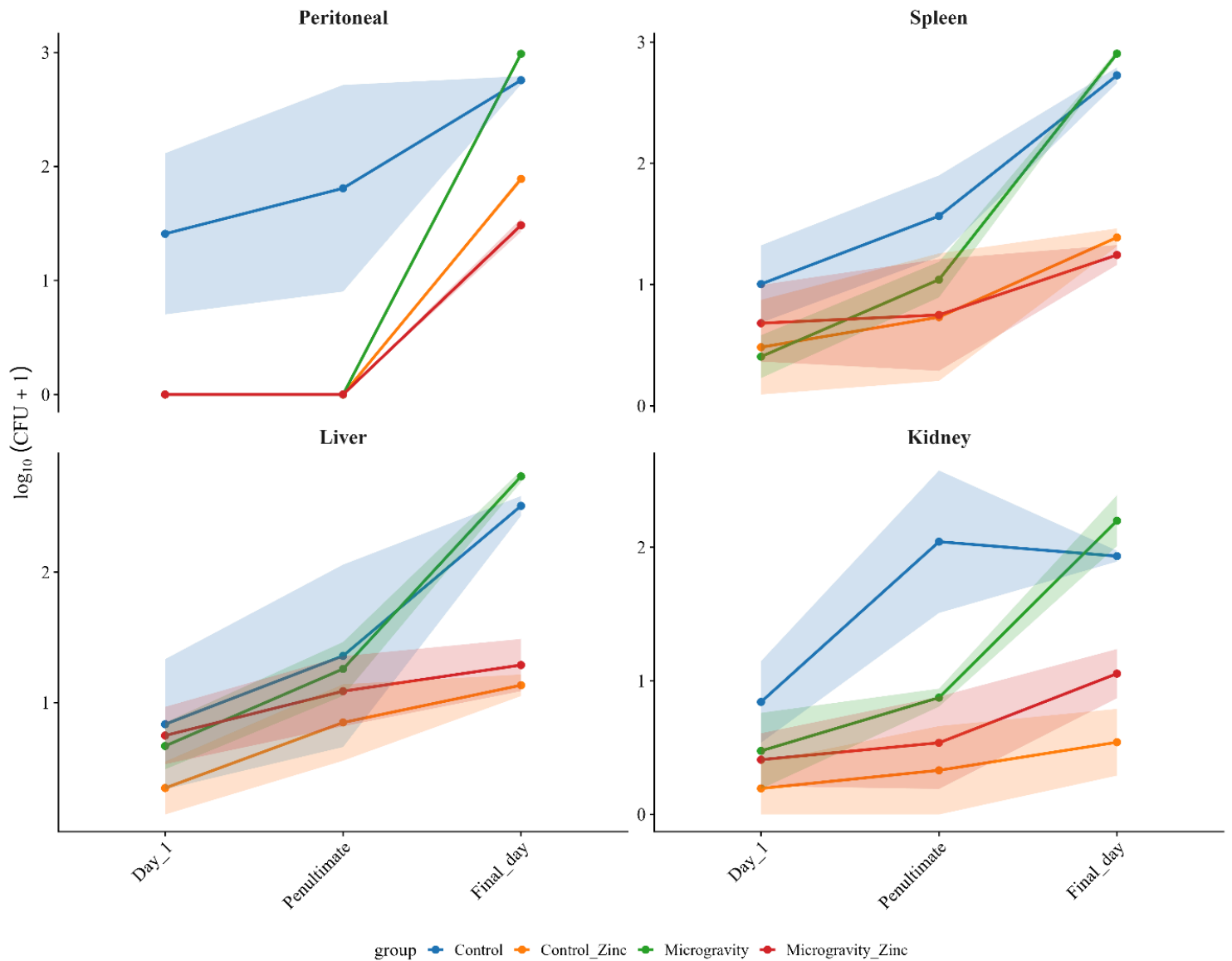


Figure 2

Bacterial load in peritoneal fluid and organs 48 h post-infection. Results are presented as log₁₀ CFU (mean ± SD) recovered from each site. A: Peritoneal lavage fluid (CFU/mL). B: Spleen (CFU/g). C: Liver (CFU/g). D: Kidney (CFU/g). Microgravity without zinc, M group, led to the highest bacterial counts in all compartments. Zinc supplementation significantly improved bacterial clearance. In the MZ group, CFU burdens were markedly reduced compared to M, especially in peritoneal fluid and spleen ($p < 0.05$ MZ vs M) and were not significantly different from those in ground controls for most sites. Zinc-supplemented controls (CZ) had the lowest bacterial loads, demonstrating enhanced innate clearance even under normal gravity.