Original Article

Effect of Heat Stress, Zinc, and Cholecalciferol (Vitamin D_3) on the Cortisol and Cytokine-mediated Responses of Laboratory Rats in Kirkuk City, Iraq

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ABSTRACT

Background: Both major and minor stressful events can have direct adverse effects on a variety of immunological mechanisms. Experimental data have shown that any improvement in vitamin D_3 status can result in a major change in the expression of genes that affect biological disorders linked to vitamin D_3 deficiency, such as cardiovascular diseases, autoimmune disorders, and cancer.

Objectives: This study aimed to measure the effects of heat stress and zinc and vitamin D_3 supplementation on serum cortisol, interleuken-6 (IL-6), and interferon-gamma (INF-g) levels in male rats.

Methods: Five equal groups of rats (n=10) were assigned to different treatment groups as follows: control, heat stress(exposed to 35 °C for 2 hours/day), zinc (50 mg/kg diet), vitamin D_3 (50 µg/kg of diet), and combined intervention (heat stress plus zinc and vitamin D_3 supplementation). The treatment regimen lasted for four weeks.

Results: Statistically significant differences were reported for all comparisons of cortisol levels between the five treatment groups ($P \leq 0.001$) except between the control and vitamin D groups and between heat stress and zinc groups ($P > 0.05$). Regarding the Il-6 levels, a statistically significant difference observed between all groups ($P \leq 0.001$) except between the control and zinc groups ($P > 0.05$). Finally, statistically significant differences were also noticed regarding the IFN-g levels between all study groups, except in the heat stress and zinc groups compared to the control group ($P > 0.05$).

Conclusion: Heat stress and zinc and vitamin D_3 supplementation can significantly affect the levels of stress-related hormones and the expression of immune response–stimulating cytokines. These findings suggest that physiological and nutritional factors are closely interlinked in modulating immune homeostasis.

Keywords: Heat stress, Interleuken-6 (IL-6), Interferon-gamma (INF-g), Vitamin D_3, Zinc

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Introduction

Elevated ambient temperature as a stress source triggers a sequence of stress responses in the body. It causes disruptions in internal metabolism, damage to organs and tissues, and exhaustion (Cramer et al., 2022). Differences in body temperature may lead to heat-stress consequences (Sakoi et al., 2024). The hypothalamus-pituitary-adrenal (HPA) axis is the principal neuroendocrine section involved in the stress response (Hinds & Sanchez, 2022). The HPA axis produces adrenocorticotropic hormone (ACTH) and cortisol. Changes in ACTH and cortisol adapt to the stressful conditions and serve as indices of stress level, playing a particularly important role in the body. Different types of stress may impact immune function, such as thermal, cold, and fear-related stress. Interleukin-2 (IL-2) is primarily secreted by activated T cells and has multiple functions in immunity. IL-2 also influences the HPA axis, and its concentration can serve as a valuable measure of cellular immune function (Mbiydzenyuy % Qulu, 2024).

Zinc is an important trace element necessary for normal growth, membrane stability, protein metabolism, and the function of over 200 metalloenzymes (Kaur et al., 2023; Palani et al., 2022). Additionally, during times of stress, trauma, or inflammation, zinc is redistributed internally, with zinc being mobilized from certain tissues such as the gut and pancreas and transferred to other organs, such as the liver (Ullah et al., 2023). This redistribution presumably serves to protect high-priority zinc-dependent processes, such as the liver's synthesis of acute-phase proteins during a stress reaction (Knez & Boy, 2023). This internal redistribution of zinc is mediated largely by stress hormones, such as cortisol, ACTH, catecholamines, and, to a greater extent, cytokines, such as IL-1, tumor necrosis factor, and IL-6 (Juszczyk et al., 2021).

Along with cortisol, retinoid, and sex hormones, cholecalciferol (vitamin D_3) is a component of steroid hormones. Vitamin D_3 exhibits pleiotropic properties, contributing significantly to immunological and metabolic functions, as well as mineral and bone homeostasis (Gotelli et al., 2024; Mohammed et al., 2020). Recent findings indicate that vitamin D_3 is necessary for human physiological purposes, minimizing inflammation and oxidative stress (Wimalawansa, 2023). Experimental data indicate that any improvement in vitamin D_3 status will result in major changes in the expression of genes that impact biological activities linked to vitamin D_3 deficit, such as cardiovascular disease, cancer, and autoimmune disorders (Sîrbe et al., 2022).

The main aims of the our study were to assess the serum levels of cortisol, IL-6, and interferon-gamma (INF-γ) in male rats subjected to heat stress and zinc and vitamin D_3 supplementation individually and collectively as measures of stress and immune response to these challenging conditions. This study is significant and innovative since it investigated the impact of heat stress, an escalating issue due to climate change, on stress and immunological indicators, while simultaneously assessing the potential protective effects of zinc and vitamin D_3 supplementation. The analysis of cortisol, IL-6, and IFN-γ responses, both separately and together, yields novel insights into the interaction between environmental stressors and nutritional therapies, presenting significant implications for the management of stress-induced immunological modifications.

Materials and Methods

Experimental design

Study duration

The study extended for six weeks, starting from the animals' adaptation to the exposure period (four weeks) and ending with the final week devoted to parameter measurements, to investigate the influences of heat stress and zinc and vitamin D_3 supplementation in Kirkuk City.

Ethical approval

All experimental procedures were approved by the Scientific Committee at the Veterinary Medicine College, Kirkuk University, Iraq (Approval No. 5, Date: 24/06/2025).

Animal model

Fifty males albino rat aged nearly 8 weeks, weighing 180-200 g, were used in the current study. The selected animals were healthy, adapted, and equally assigned to the experimental groups (10 rat each group).

Housing conditions

All rats were housed in stainless steel cages (60×60×60 cm) designed for laboratory animals. The cages were maintained under standardized laboratory conditions throughout the experiment, including proper ventilation, an ambient temperature of 20–25 °C, and a 14-hour light/10-hour dark cycle. Rats received a standard laboratory diet, supplemented with vitamin D_3 and zinc according to the experimental design.

The experimental groups

The rats were randomly assigned to five groups (10 rats per group) as follows:

a) Control group: This group was kept under standard laboratory conditions (ambient temperature and normal diet).

b) Heat stress group: This group was exposed to elevated ambient temperature (35 ˚C) for 2 hours/day using specially equipped chambers.

c) Zinc supplementation group: This group received a diet supplemented with zinc at a dose of 50 mg/kg.

d) Vitamin D_3 supplementation group: This group received a diet supplemented with cholecalciferol (vitamin D_3) at a dose of 50 µg/kg (equivalent to 2000 IU/kg, 1 IU=0.025 µg).

Combined intervention group (heat stress plus zinc plus vitamin D_3): This group was exposed to heat stress and received both zinc and vitamin D_3 supplementation.

6. The duration of the experiment

Week one involved body weight measurement and the adaptation phase

Serum zinc and vitamin D_3 levels were assessed prior to the initiation of the experiment by atomic absorption spectroscopy and the 25(OH) Vitamin D ELISA kit (Abcam® Cat. No. 213966), respectively.

Atomic absorption spectroscopy (AAS):

- Sample preparation: Samples were diluted to bring them within the detectable range of the AAS instrument.
- Calibration curve preparation: Multiple zinc standard solutions were prepared for this purpose.
- Instrument calibration: The instrument was adjusted according to the manufacturer's instructions.
- Wavelength setting: The zinc absorption line was set to a wavelength of 213.9 nm.
- Measurement: Absorbance was measured for each sample, and using the calibration curve the zinc concentration was determined.

The experiment continued for four weeks, according to the grouping design shown above.

Week five of the study involved the measurement of serum biomarkers, including cortisol (Cortisol ELISA Kit Abcam® Cat. No. 108665), IL-6 (Human IL-6 Simple Step ELISA Kit Abcam® Cat. No. 178013), and IFN-γ (Human IFN-γ Simple Step ELISA Kit Abcam® Cat. No. 174443). Blood samples were collected from the rats by orbital sinus puncture. The samples were left to clot and then centrifuged at 4000 rpm for 15 minutes to obtain a sufficient amount of serum.

Serum vitamin D_3 concentrations were measured using a commercial ELISA kit a expressed in ng/mL. Values were converted to SI units (nmol/L) using the factor 1 ng/mL=2.5 nmol/L. Serum zinc concentrations were measured using atomic absorption spectrophotometry and expressed in µg/mL. Values were converted to SI units (µmol/L) using the factor 1 µg/mL=15.3 µmol/L. All biochemical analyses were performed in duplicate.

All procedures related to the measurement of the study parameters strictly followed the manufacturing companies' instructions.

Statistical analysis

SPSS software, version 27 was used for data analysis. Data were presented as Mean±SD. One-way ANOVA was used to compare the mean serum concentrations of the studied parameters among the groups, and post hoc analyses were also applied to identify which groups differed. Statistical significance was set at $P \leq 0.05$.

Results

The mean serum concentrations of zinc and vitamin D_3 in all studied groups before and after the intervention are presented in Tables 1 and 2. In the control group, the mean serum zinc and vitamin D_3 concentrations were 14.84±4.13 µmol/L and that of was 91.65±14.4 nmol/L for studied groups before the intervention (Table 1). These values were 16.2±1.1 µmol/L and 80.4±4.5 nmol/L after the intervention (Table 2).

Table 3 shows the descriptive statistics of cortisol (ng/mL), IL-6 (pg/mL), and IFN-γ (ng/mL). The mean serum concentrations of cortisol, IL-6, and IFN-γ among the five studied groups revealed very highly statistically significant differences when the values were compared ($P \leq 0.001$) (Figures 1, 2, and 3).

Table 1. Serum zinc and vitamin D_3 levels in the experimental groups before treatment

Group	Measure	Mean	SE	SD	Range	Minimum	Maximum
Control	Zinc (µmol/L)	14.84	1.22	4.13	14.54	7.96	22.49
	Vit D_3 (nmol/L)	91.65	4.55	14.4	41.58	72.38	113.95
Heat stress*	Zinc (µmol/L)	15.1	0.45	1.1	3	13.8	16.8
	Vit D_3 (nmol/L)	74.5	1.7	4.2	11	68	80.2
Zinc Sup.**	Zinc (µmol/L)	14.6	0.32	0.87	2	13.6	15.8
	Vit D_3 (nmol/L)	71.8	1.6	4	11.6	66.4	78.36
Vit D sup.***	Zinc (µmol/L)	15.3	0.82	2.3	2.6	14.4	16.9
	Vit D_3 (nmol/L)	73.8	1.56	3.8	9.54	68.5	79.81
Combined****	Zinc (µmol/L)	14.7	0.34	1.2	2.7	13.5	18.1
	Vit D_3 (nmol/L)	72.6	2.73	3.69	9.2	67.84	80.7

*The group subjected to ambient temperature (35 °C) 2 hours/day, **The group supplemented with zinc at 50 mg/kg, ***The group supplemented with vitamin D_3 at 50 µg/kg (2000 IU/kg), ****The group exposed to the above three treatments simultaneously.

Serum cortisol levels changed significantly among the groups. The Bonferroni post hoc analysis (Table 3) showed a significantly higher increase in serum cortisol levels in the heat-stressed, zinc-supplemented, and combined exposure groups compared to the control group (P≤0.001), while its levels showed a decrease in the vitamin D_3 supplementation group (P>0.999), as shown in Figure 1. In the case of IL-6, all pairwise comparisons of the mean serum levels showed very highly statistically significant differences (P≤0.001); however, for the zinc supplementation group versus controls, there was no statistically significant difference attained (P>0.999) (Figure 2).

Table 2. Serum zinc and vitamin D_3 levels in the experimental groups after treatment

Group	Measure	Mean	SE	SD	Range	Minimum	Maximum
Control	Zinc (µmol/L)	16.2	0.43	1.1	2.8	15.3	17.8
	Vit D_3 (nmol/L)	80.4	1.8	4.5	12.3	74.6	88.2
Heat stress*	Zinc (µmol/L)	16.7	0.49	1.4	3.6	15.3	18.5
	Vit D_3 (nmol/L)	82.1	1.8	5.3	11.3	76.7	89.6
Zinc Sup.**	Zinc (µmol/L)	15.9	0.61	2.07	2.9	14.8	17.8
	Vit D_3 (nmol/L)	79.4	1.94	5.6	12.26	73.48	86.04
Vit D sup.***	Zinc (µmol/L)	16.9	1.21	0.42	3.1	14.5	18.65
	Vit D_3 (nmol/L)	81.7	1.76	4.72	10.52	77.3	87.8
Combined****	Zinc (µmol/L)	16.1	1.71	2.78	2.88	14.8	18.3
	Vit D_3 (nmol/L)	79.8	2.23	5.2	9.86	76.2	86.8

*The group subjected to ambient temperature (35 °C) 2 hours/day, **The group supplemented with zinc at 50 mg/kg, ***The group supplemented with vitamin D_3 at 50 µg/kg (2000 IU/kg), ****The group exposed to the above three treatments simultaneously.

Table 3. Serum cortisol, IL-6, and IFN-γ levels in the experimental groups

Measure	Groups	Mean	SE	SD	Range	Minimum	Maximum
Cortisol (ng/mL)		97.05	9.4	29.73	105.02	47.45	152.47
IL-6 (pg/mL)	Control	2.93	0.37	1.17	4.19	1.23	5.42
IFN-γ (ng/mL)		7.02	0.75	2.39	6.89	3.83	10.72
Cortisol ng/ml)		345.2	14.01	44.31	125.79	270.31	396.1
IL-6 (pg/mL)	Heat stress*	25.41	1.13	3.58	10.84	21.53	32.37
IFN-γ (ng/mL)		8.69	0.83	2.63	7.05	4.99	12.04
Cortisol (ng/mL)		357.67	7.38	23.34	64.59	316.56	381.16
IL-6 (pg/mL)	Zinc Sup.**	3.57	0.32	1.02	3.	2.28	5.28
IFN-γ (ng/mL)		5.84	0.68	2.16	7.18	0.79	7.96
Cortisol (ng/mL)		83.2	6.49	20.52	61.03	55.15	116.19
IL-6 (pg/mL)	Vit D sup.***	7.17	0.44	1.4	4.93	5.11	10.04
IFN-γ (ng/mL)		31.31	0.95	3.01	10.35	25.36	35.7
Cortisol (ng/mL)		259.87	17.8	56.29	187.55	150.46	338.01
IL-6 (pg/mL)	Combined****	12.78	0.78	2.47	8.36	7.98	16.34
IFN-γ (ng/mL)		36.37	1.58	4.98	14.9	29.74	44.64

*The group subjected to ambient temperature (35 °C) 2 hours/day, **The group supplemented with zinc at 50 mg/kg, ***The group supplemented with vitamin D_3 at 50 µg/kg (2000 IU/kg), ****The group exposed to the above three treatments simultaneously.

Moreover, when the multiple comparisons involved the mean serum IFN-γ levels across the groups, it appeared that the mean concentration of the controls did not differ statistically significantly when compared to these of the heat-stress group and the zinc supplementation group (P>0.999). However, a very highly statistically significant difference was observed when the comparison involved the control group one side and the vitamin D_3 supplementation and the combined groups on the other side (P≤0.001). Furthermore, no statistically significant difference was observed when comparing the heat-stress group versus the zinc-supplementation group (P=0.542). Finally, the mean serum level of IFN-γ in the vitamin D_3 supplementation group was highly statistically significant (P≤0.01) compared to the combined group; all other multiple comparisons across groups showed very highly statistically significant differences (P≤0.001) (Figure 3).

Discussion

The activation of the HPA axis is a major neuroendocrine mechanism in the stress response, leading to an abrupt increase in circulating corticotrophin (ACTH) and a subsequent rise in glucocorticoids, which are important for adaptive responses (Hinds & Sanchez, 2022). Therefore, plasma levels of glucocorticoids and ACTH are reliable measures of the stress response intensity, especially during the acute phase (Arfuso et al., 2022). The current study's findings were in line with previous sudies that found a considerable increase in serum corticosterone (CORT) and plasma corticotrophin (ACTH) concentrations, signaling that acute heat exposure has a severe stressful response (Li et al., 2022).

The immune effects of environmental stress are complex and unpredictable. Stress affects the immune system either through alterations in inflammatory reactivity at the level of innate immunity, or at the level of acquired immunity through the modulation of Th1 and Th2 cytokines (Alotiby, 2024). The findings of this research support previous findings that stress may also raise IL-6 levels (Roohi et al., 2021). Increased levels of plasma IL-6 have been observed in three models of stress in laboratory animals: immobilization, electric shock, and

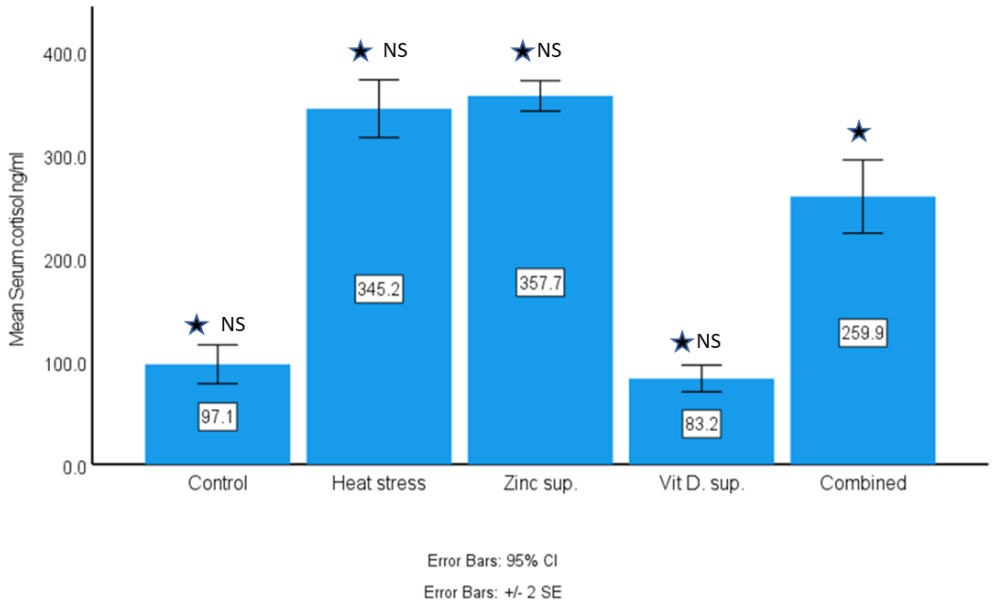

Figure 1. Post-hoc analysis of cortisol levels among the experimental groups

Note: Statistical significance for all comparisons except for the controls versus the vit D supplementation and heat stress groups versus the zinc supplementation group; these comparsons were not significant (NS).

exposure to a sound previously associated with the electric shock. Since it depends on the integrity of the adrenal glands, it is possible that this stress-induced IL-6 has a non-immune origin (Zhou et al., 1993). However, the origin of stress-induced IL-6 may also be immune, as suggested by a physiological study in which epinephrine perfusion in the liver causes IL-6 production by Kupffer cells (Reusswig et al., 2024). Stress significantly increases IFN-γ levels, a finding that aligns with our results (McLeod et al., 2022). However, in contrast to our data, both in vivo and in vitro exposure to catecholamines and glucocorticoids suppress the production of various pro-inflammatory cytokines, such as IFN-γ, which suppresses Th1-mediated cellular immunity and shifts the immune response to Th2- mediated humoral immunity (Balakin et al., 2025). However, in these studies, there

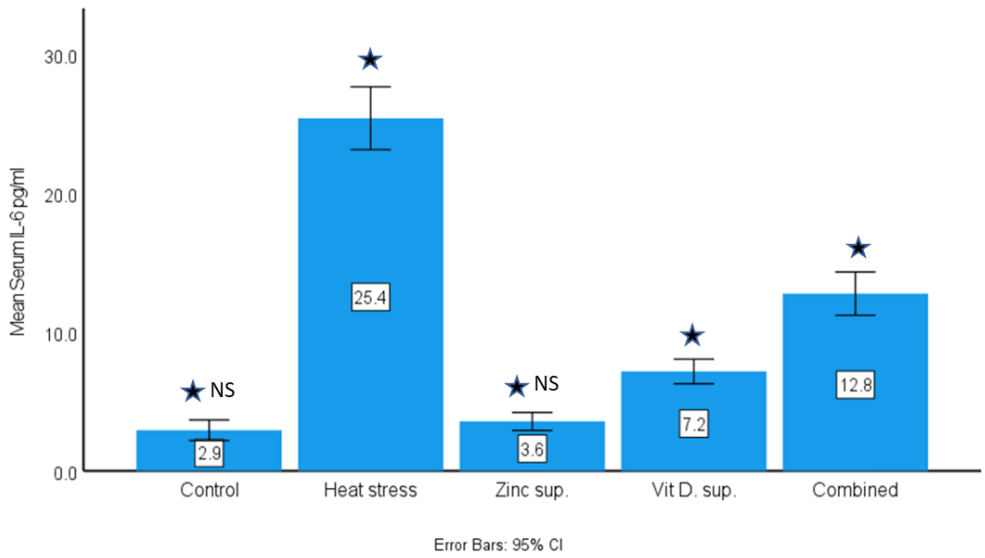

Figure 2. Post-hoc analysis of IL-6 levels among the experimental groups

Note: Statistical significance for all comparisons except for the controls versus the zinc supplementation group; these comparisons were not significant (NS).

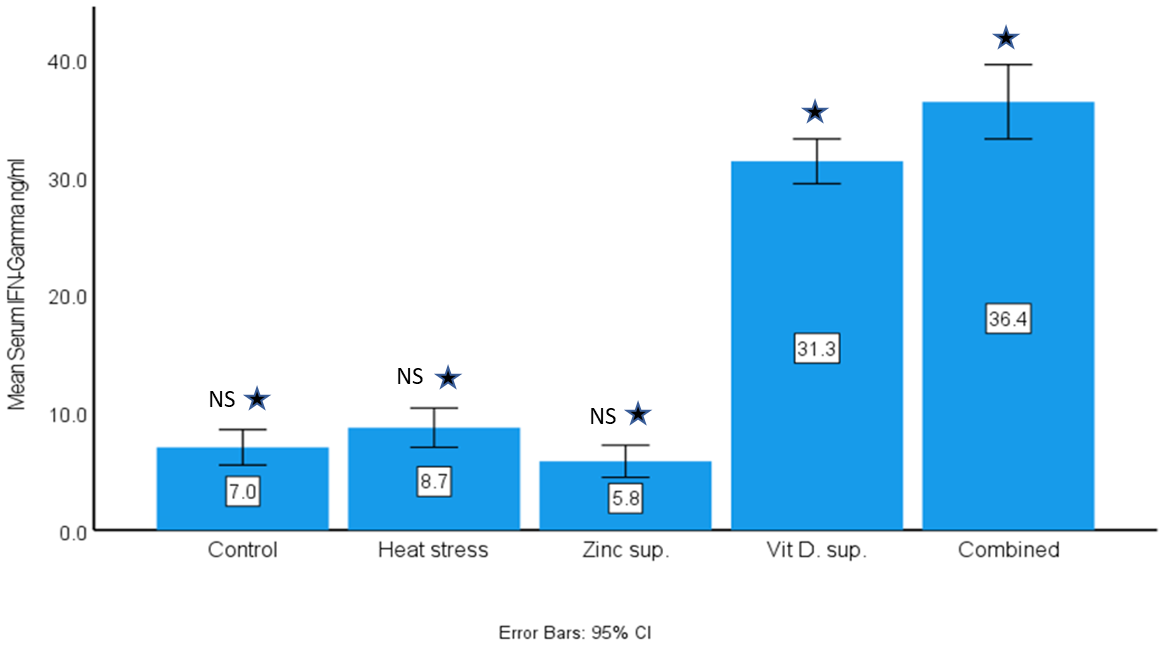

Figure 3. Post-hoc analysis of IFN-γ levels among the experimental groups

Note: Statistical significance for all comparisons except for the controls versus the heat stress and zinc supplementation groups; these comparisons were not significant (NS).

was either direct use of exogenous glucocorticoids with effects examined in vitro, or the duration of the stress exposure was neglected (Bassil et al., 2022).

Zinc has several important functions due to the fact that it is a cofactor for more than 200 enzymes. One of its most important functions is its role in the antioxidant defense system (Costa et al., 2023). Oxidative damage of the cell membrane by free radicals occurs during zinc deficiency, therby changing the status of antioxidant enzymes and substances (Jomova et al., 2025). The results of this study were generally in line with other studies on *Trypanosoma cruzi* and *Trypanosoma evans*i, where zinc as a mineral supplement displayed enhancement of the immune status, along with a delay in the onset of illness (Brazão et al., 2008; Dalla Rosa et al., 2012). Zinc acts in several mechanisms, including receptor signaling processes, metabolic pathways of various enzymes, and the improvement of the immune response (Kim & Lee, 2021). The latter notion was supported by the current study, which revealed an alteration in serum cytokine levels in response to zinc supplementation, which in turn can have a stimulatory effect on immune responses (Faghfouri et al., 2021). In another study to determine the effect of zinc supplementation on the induction of metallothione in rats (Brzóska et al., 2021), the findings were in agreement with our study, which found increased cortisol and IL-6 levels in the zinc-supplemented group compared to the control group (Ceylan et al., 2021).

Vitamin D_3 has recently been identified as a significant immunomodulator, improving the innate immune system and dampening the adaptive immune response, with a shift toward T helper 2 and regulatory T cell responses (Bishop et al., 2021). Our results showed decreased serum cortisol levels in the vitamin D-supplemented group compared with the control group (Pancar et al., 2021). Regarding the interaction of interest, the results are controversial, as some reports indicate that vitamin D supplementation does not affect cortisol levels (Yosaee et al., 2020); others report decreased glucocorticoid synthesis and hindering steroidogenesis (Muscogiuri et al., 2015) or augmented serum concentrations of glucocorticoid primarily because of relative and/or temporary glucocorticoid resistance (Mohamed & Abdel-Rehim, 2020). Expression of the vitamin D receptor in adrenal cortex cells (Muscogiuri et al., 2015) and alternative effects of vitamin D (Grudet et al., 2020) are perhaps key explanations for the discrepancies, as Vitamin D can activate neuroendocrine pathways (Slominski et al., 2018).

The evaluation of how stress influences the immune system involves a wide range of external stimuli that interfere with normal physiology, such as anxiety, metabolic stress, disruptive events, etc. This diversity of forms of stress has various effects on the immune system, some seemingly contradictory (Ovsiannikova et al., 2024). The notion that stress is solely immune-inhibitory should be reconsidered, since some types of stress may

lead to cytokine overexpression and increased immune responses in humans and laboratory animals (Dobrovinskaya et al., 2024). It has been postulated that this diversity of effects may depend on the duration of stress and the specific stress model applied (Koffer et al., 2016). Additional factors that could influence the immunological responses observed include the type of stress (metabolic, psychological, or parasitic), its intensity, frequency, novelty, degree of coping, and predictability (Al-Bayati et al., 2023; Alotiby et al., 2024; Azeez & Al-Hussary, 2012).

Conclusion

The present study concluded that stress and dietary alterations can influence levels of stress hormones and immune-stimulatory cytokines. Cortisol levels, as a measure of stress, were affected by heat, zinc supplementation, and the combined treatment, but not by vitamin D supplementation. The inflammatory marker IL-6 was affected by all treatments except zinc supplementation. Furthermore, heat stress and zinc supplementation did not alter IFN-γ levels, but vitamin D supplementation and the combined treatment did. Clinically, these results suggest that targeted micronutrient supplementation—especially vitamin D, alone or combined with zinc—may offer potential benefits in mitigating heat-induced immune dysregulation and enhancing stress resilience, thereby supporting better physiological adaptation in populations exposed to thermal stress. Finally, despite this study, further studies are required under different laboratories to examine heat stress, zinc, and cholecalciferol (vitamin D_3) supplementation, in order to improve and expand our knowledge of cortisol and cytokine-mediated responses as potential biochemical markers.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Scientific Committee of the Veterinary Medicine College, University of Kirkuk, Kirkuk, Iraq (No:. 5, Date: 24/06/2025).

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Authors' contributions

Conceptualization, methodology, investigation, and writing the original draft: Noor Faisal Noaman; Investigation and resources: Ryeam Sami Hameed; Formal analysis and data curation: Kasim Sakran Abass; Visualization, review, and editing: Damat Fikrat Saber; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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