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USE OF ZINC NANOPARTICLES AND/OR PRODIGIOSIN TO MITIGATE HEAT STRESS IN RABBITS

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Abstract

Heat stress (HS) is an environmental challenge affecting animals' health, productivity and welfare. This work aimed to inspect the protective effect of zinc oxide nanoparticles (ZnNPs) and/or prodigiosin (PRG) against inflammation, immune dysfunction, oxidative stress and endoplasmic reticulum (ER) stress triggered by HS in growing rabbits. Growing weaned rabbits (one hundred males, 35 days of age) were randomly assigned into four groups. The first group was fed a basal diet without supplementation and the 2nd, 3rd, and 4th groups were fed diets containing zinc oxide nanoparticles (ZnNPs, 50 mg/kg diet), prodigiosin (PRG, 100 mg/kg diet) or their mixture (ZnPRG) under HS conditions for eight successive weeks. The dietary inclusion with ZnNPs and/or PRG significantly boosted FBW (final body weight), CBWG (cumulative body weight gain), and FCR (feed conversion ratio) and had no substantial impacts on the CFI (cumulative feed intake) as compared with those in HS one. All supplemented treatments significantly unveiled an increase in the values of RBCs, hemoglobin, and platelets and significant decreases in WBCs, basophils and monocytes with non-statistical effects on hematocrit, MCV, MCHC, neutrophils and eosinophils. Compared with the HS group, all supplemental groups showed a significant reduction in TNFα, IL4, IFN-γ, TLR-4 and amyloid A levels and DNA damage markers (OHdG) and significant increases in the levels of NO and lysosome activity. Rabbits in the ZnPRG group had significantly higher IgG and IgM levels compared to other groups. The highest value of CAT and GSH levels were found in rabbits that received the mixture of ZnNPs (50 mg) and PRG (100 mg) in their diets under elevated temperatures. Additionally, both treatments, PRG and ZnNPS, significantly (P<0.001) reduced the values of MDA and MYO, while all treated groups had significantly reduced PC contents compared with the HS group. Co-supplement with ZnPRG showed a considerable restoration in the higher immune expression of reticulum oxidative stress such as GRP78 and IRE1 in hepatic tissues induced by HS conditions. The mixture of ZnNPs and PRG presented more robust effects in mitigating the adverse impacts of HS in rabbits compared with the individual treatments. Collectively, ZnNPs and/or PRG alleviated oxidative stress and DNA damage. In addition, it enhanced the antioxidant capacity and immune function, and downregulated ER stress such as GRP78 and IRE1 signaling in the hepatic tissues of stressed rabbits.

Key words: heat stress, rabbit, redox status, immunity, inflammatory indices, reticulum oxidative stress

Heat stress (HS) negatively affects immune organs and cellular organelles, diminishing immune-antioxidant capabilities (Wu et al., 2019; Hirakawa et al., 2020). Moreover, animals exposed to HS exhibited hepatic injury induced by excess amounts of oxidative stress (OS), causing lipid peroxidation, protein oxidation, and DNA damage, which are involved in hepatic dysfunction (Xiong et al., 2020; Jmbabi et al., 2021).

Constantly, HS is an environmental challenge that has been described to be accompanied by augmented malondialdehyde (MDA) levels, weakened antioxidant defense, such as catalase (CAT), superoxidase dismutase (SOD), and glutathione (GSH) in the hepatocytes of animals (Xiong et al., 2020; Miao et al., 2022), demonstrating inflammation process. Also, a previous experiment has displayed that HS exposure leads to noticeably augmented secretions of inflammatory cytokines such as TNF-α (tumor necrosis factor-α), interleukin-1β (IL-1β), and IL-4, as well as greater DNA damages of heat-stressed chicken (Miao et al., 2022). Previous data show that high temperatures severely overstress animals, which accompany cellular dysfunction, including endoplasmic reticulum (ER) (Xiong et al., 2020; Yang et al., 2021).

The ER is the main place for synthesizing, folding, amendment, and developing secretory and membrane proteins, hence important functions in regulating many cellular developments (Oghbaei et al., 2021). Studies have informed that the environmental stressor could

interrupt ER homeostasis, thus resulting in ER stress (Oghbaei et al., 2021; Miao et al., 2022). At normal physiological state in the cell, GRP78 is the dominant of the unfolded protein response (UPR) in the ER, which strengthens it to degrade or refold via cellular degradation machinery (Ibrahim et al., 2019). While through the environmental stressors, the upregulation of GRP78 on the cell membrane intercedes the massive number of disturbed proteins. Regrettably, this compels it a way for pathogens to get into the cell and initiate diverse paths leading to cellular disturbances (Ibrahim et al., 2019). The IRE1 in another UPR activates by environmental stresses in chickens (Ma et al., 2022). Discovering a new natural molecule or using nanotechnology with powerful antioxidant and anti-inflammatory abilities to modulate inflammation and ER signaling might be possible therapeutic tactics to relieve HS-induced hepatic damage in stressed rabbits.

Recently, nutritionists have focused their research on using nutritional tactics to enhance immunity and productivity, sustain health and reduce inflammation and OS in animals exposed to HS (Yang et al., 2019; Li et al., 2021; Yang et al., 2021). The valuable actions of microbial metabolites for boosting animal health have been recognized in many previous works (Venil et al., 2013; Faehnrich et al., 2016; do Nascimento et al., 2020) due to its robust antioxidant, anti-inflammatory as well as eco-friendly mediators, higher biodegradability, and promising opportunities for several pharmacological uses (do Nascimento et al., 2020; Ellamie et al., 2020; Jin et al., 2020). In this sense, prodigiosin (PRG) is a natural red pigment produced by *Serratia marcescens* and has many biological properties that are immunomodulatory, anticancer, anti-inflammatory and anti-microbial (Venil et al., 2013; Faehnrich et al., 2016). Zinc is an essential microelement for health, reproduction and the physiological pathways in the cellular system. The usage of nano minerals, such as zinc oxide nanoparticles (ZnNPs), represents a fortunate and developing technology in the sustainability of the livestock sector and for environmental uses (Ramiah et al., 2019; Abdelnour et al., 2021). Because of its smaller size and higher bioavailability of nano-zinc, it can be integrated into the intestinal cavity, thus decreasing emission and environmental contamination. Many previously published studies showed that the dietary inclusion of ZnNPs significantly improved the growth, health and feed efficiency in stressed rabbits (Abdel-Wareth et al., 2022), broilers (Ramiah et al., 2019; Li et al., 2021), and fish (Sherif et al., 2022). Based on this data, it was hypothesized that the ZnNPs incorporated with PRG would more effectively reduce the negative impacts of HS due to the powerful anti-inflammatory and antioxidant actions.

Consequently, and after the extensive screening of published papers, no previous work used both molecules to mitigate the adverse effects in stressed rabbits via enhancing the immune antioxidant and reducing the ER stress and inflammation caused by HS. Hence, we theorized that the dietary mixture of ZnNPs and PRG in the stressed rabbit diets might enhance the health, redox regulation, immunity, and combating ER stress in stressed rabbits due to its anti-inflammatory, antioxidant and immunomodulatory effects. Consequently, this exertion is planned to scrutinize the ameliorative effect of ZnNPs and/or PRG in strengthening the growth, immunity, and redox regulation and weakening rabbits' inflammation and ES stress under HS conditions.

Material and methods

Materials preparation

In our previous investigation (Othman et al., 2019; Abdelnour et al., 2020), we isolated and identified the PRG from *Serratia marcescens* and lyophilized then the PRG was supplemented into diets conferring to the experiment protocol. For the zinc nanoparticles (ZnNPs), the *Lactobacillus plantarum* VITES07 was used for the biogenic ZnNPs, as described in our previous work (Aioub et al., 2022). Then, the TEM recognized the ZnNPs (transmission electron microscope, type JEOL-JEM 2100). The size of ZnNPs is arranged less than 100 nm, with well dispersed and spherical shape (Figure 1).

Animals and experimental design

One hundred New Zealand White weaned-growing rabbits (804±14.17 g, 35 days, males) were involved in this experiment. The weaned rabbits were acquired from the Rabbit Research Unit, Faculty of Agriculture, Zagazig University, Egypt. All the procedures that used the rabbits for this trial followed the Guidelines for the Use and Care of Laboratory Animals (8th Edition, 2011) and were approved by the scientific committee of the IACUC of Zagazig University. All rabbits were kept in the same environmental and management conditions. Animals were housed in galvanized wire battery cages (50 × 45 × 40 cm^3), providing conventional feeders and an automatic system of a nipple.

The animals were haphazardly distributed into four groups; HS group, rabbits received a basal diet without supplementation; the ZnNPs group, rabbits received a basal diet supplemented with 50 mg of zinc nanoparticles (ZnNPs)/kg diet; PRG group, rabbits received a basal diet supplemented with 100 mg of prodigiosin/kg; and the ZnPRG group, rabbits received a basal diet supplemented with 50 mg and 100 mg/kg diet of ZnNPs and PRG, respectively for eight weeks. Growing rabbits were fed basal diets formulated to cover the nutrient requirements as endorsed by NRC (1977). As depicted in Table 1, we mentioned the constituents and chemical analysis of the diet of growing rabbits used in this experiment. Under summer conditions in Sharkia Province, Egypt, it is important to assess the HS severity in growing rabbits during the experimental period (Marai et al., 2002; Abdelnour et al., 2020). For this aim, relative humidity (RH) and ambient temperature (AT) values were used to detect

the THI (temperature humidity index) values and thereby detect the severity of HS in rabbits.

Table 1. Ingredients and nutrient contents of the basal diet of growing rabbits

Items	Basal diet
Ingredient	%
Maize	20
Soybean meal	20
Wheat bran	16
Berseem hay	30
Barley grain	10
Molasses	2
Limestone	1
NaCl	0.5
Premix*	0.5
Calculated composition (%)**	
Metabolizable energy (MJ/kg)	7.95
Crude protein	17.50
Calcium	0.88
Available phosphorus	0.20

*Each 1 kg of premix (minerals and vitamin mixture) contains vit. A, 20,000 IU; vit. D_3, 15,000 IU; vit. E, 8.33 g; vit. K, 0.33 g; vit. B_1, 0.33 g; vit. B_2, 1.0 g; vit. B_6, 0.33 g; vit. B_5, 8.33 g; vit. B_{12}, 1.7 mg; pantothenic acid, 3.33 g; biotin, 33 mg; folic acid, 0.83 g; choline chloride, 200 g.
**Calculated according to NRC (1977).

The THI was calculated using the following equation:

$$THI = db - [(0.31 - 0.31(RH)] \times [(db^{\circ}C - 14.4)]$$

Where *db* is the dry bulb temperature in Celsius and *RH* is the percentage of relative humidity. The calculated *THI* values were subsequently classified as follows: <27.8 = absence of heat stress; 27.8 to 28.9 = moderate heat stress; 28.9 to 30.0 = severe heat stress, and >30.0 = extremely severe heat stress (Marai et al., 2002). RH and AT were assessed using a hygrothermograph (ST-50A, SEKONIC, Tokyo, Japan) in the animal facility.

Growth performance

After the end of the trial, the growth indices were assessed weekly. The final body weight (FBW), cumulative body weight gain (CBWG), cumulative feed intake (CFI), and finally, the feed conversion ratio (FCR; g feed/g gain) were recorded.

Hematological valuation

The blood samples were picked up from (6 rabbits/group) the marginal ear vein, using heparinized tubes and scattered into two subsamples at the end of the trial. The heparinized tubes were used to evaluate the erythrocytes and leucocyte counts by an automated hematology analyzer (Hospitex Hema Screen 18, Sesto Fiorentino, Italy). For obtaining the plasma samples, the second subsamples were exposed to centrifugation (T32c; Janetzki, Wallhausen, Germany) at 2100 g for 15 min, and plasma samples were detached and preserved at −20°C for assessing the immunity, redox status, inflammatory and DNA oxidative markers.

Assay of redox status and immunity

For redox regulation valuations, the antioxidant indices in the plasma of rabbits of experimental groups such as SOD (Superoxide dismutase; MBS8807589), GSH (Glutathione) and CAT (Catalase; MBS8807700) activities were determined using a specialized quantitative sandwich ELISA kit (My BioSource, San Diego, USA) following the guidelines instructions. The oxidative stress in the rabbit plasma, including MDA (Malondialdehyde, MBS8806802), PC (Protein carbonyl, MBS2601439) and MYO (Myeloperoxidase, MBS724170), was determined using a specialized ELISA kit purchased from My BioSource company (San Diego, USA) following the guidelines instructions. According to the manufacturing procedure, the blood immunoglobulins (IgA and IgM) were determined using commercial kits purchased from Bio-diagnostic Company (Giza, Egypt).

Inflammatory assessment

The levels of IFN-γ (Interferon-gamma; MBS2601171) and TNF-α (Tumor necrosis factor α; MBS7612133) in rabbit plasma were estimated via ELISA kits examination conferring to the manufacturer's directions (MyBioSource, San Diego, USA). The nitric oxide (NO) and lysosome activity were detected in the rabbit plasma as described previously by Rajaraman et al. (1998) and Ellis (1990), respectively. Toll-like receptor 4 (TLR-4) levels were assessed in the rabbit plasma via ELISA kits (MBS2005383; MyBioSource, San Diego, USA), according to the manufacturer's instructions. Interleukin 4 (IL-4) was determined using the calorimetric method (My BioSource, San Diego, USA, MBS733925). The concentration of amyloid A (ng/ml) was assessed with commercially available sandwich ELISA kits (Biosource, Camarillo, CA), conferring to the procedures endorsed by the manufacturer.

Oxidative DNA marker

An 8-hydroxy-2′-deoxyguanosine (8-OHdG; ng/ml) level indicates oxidative DNA impairment (Kondkar et al., 2020). Assessment of plasma 8-OHdG levels was achieved via a competitive commercial sandwich ELISA kit (Trevigen, Gaithersburg, MD, USA) following the manufacturer's instructions. The plasma levels of 8-OHdG were assessed from a standard curve and expressed in ng/mL.

Immunohistochemical investigation

At the end of the experimental period, six animals were sacrificed for hepatic immunobiological study. Hepatic tissues were collected from the sacrificed rabbits in experimental groups. Then, the liver samples were washed several times with PBS, and fixed in neutral buffered formalin (10%), and managed via the routine

paraffin embedding method. After that, five thick segments were acquired from paraffin blocks and exposed to gradual ethyl alcohol and then stained for hepatic endoplasmic reticulum markers using mouse monoclonal anti-IRE1 antibody (ab96481) with dilution rate 1: 200, rabbit polyclonal anti-GRP78 antibody (ab21685) overnight at 4°C and 3,30-Diaminobenzidine chromogen (Abcam, UK), according to the avidin-biotin-peroxidase complex protocol of Hsu et al. (1981). The area percentage of GRP78 and IRE1 immunostaining in hepatic tissues were evaluated from five selected files/animals (40X) using the Image J software (v1.46r, NIH, USA) (Schneider et al., 2012).

Statistical analysis

Data collected from the study were checked via the SPSS program (version 16.0, SPSS Inc., Chicago, IL, USA). A Shapiro-Wilk test was conducted in order to check for normality. All data were evaluated with a one-way ANOVA (with the diet as the fixed factor) using the post hoc Tukey's test. The statistical significance was considered at $P<0.05$.

Results

Temperature humidity index (THI) values

As shown in Table 2, the THI values were 31.3 at starting of the experiment, while at the end of the experiment were 31.6. Those values indicate that rabbits suffer from heat stress between severe HS and very severe HS. However, the lowest values of THI were observed at the 7 and 2 weeks with values of 29.22 and 29.77, respectively, during the experiment. So, it is critical to use some nutritional mitigations strategy to attenuate the undesirable impacts of HS as presented in the current work.

Growth performance

Rabbits fed diets with ZnNPs and/or PRG exhibited a significant increase in the FBW, CBWG (cumulative body weight gain), and FCR (feed conversion ratio) as compared with those in the untreated group (Table 3). The mixture of PRG and/or ZnNPs boosted stressed rabbits' growth productivity. No significant changes were noticed among all investigational groups concerning CFI (cumulative feed intake).

Hematological assessment

The RBCs, hemoglobin, and platelets were increased significantly in all supplemented groups, while the best results of MCH were detected only in the ZnPRG group (Table 4). The dietary supplementations with ZnNPs and/or PRG did not affect the rabbit blood's hematocrit, MCV, or MCHC. Moreover, all treated groups significantly reduced the WBCs, basophils and monocytes compared with the control one. No statistical differences were detected for neutrophils and eosinophils among the groups. The lowest values of monocytes and basophils were recorded in PRG with a statistical difference from other groups. Results in Table 4 demonstrated the non-significant difference between the control group and the ZnNPs group for basophils. Also, non-significant differences were detected between the control group and both ZnNPs and ZnPRG for monocytes.

Table 2. Average values of air temperature (T), relative humidity (RH) and temperature humidity index (THI) at the site of experimental

Items	Periods (weeks)							
	W1	W2	W3	W4	W5	W6	W7	W8
AT[1] (db°C)	33.5	31.5	33.5	32	32.5	33	31	32
RH[2] (%)	63	67.5	98.5	72	72	70	65.5	69
THI[3]	31.30	29.77	31.63	30.47	30.92	31.27	29.22	30.31

[1]Air temperature.
[2]Relative humidity.
[3]Temperature humidity index.

Table 3. Effect of zinc nanoparticles (ZnNPs), prodigiosin (PRG) or their mixture (ZnPRG) on growth performance of growing rabbits exposed to heat stress conditions

Items	Experimental groups[1]				Pooled SEM	P-value
	HS	ZnNPs	PRG	ZnPRG		
Initial body weight (IBW; g)	795.00	803.75	808.75	811.25	14.35	0.984
Final body weight (FBW; g)	1707.5 b	1905 a	1917.5 a	1872.5 a	30.51	0.031
Cumulative body weight gain (CBWG; g)	912.5 b	1101.25 a	1108.75 a	1061.25 a	28.97	0.036
Cumulative feed intake (CFI; g)	2940.00	2802.50	2595.00	2582.50	71.98	0.238
Feed conversion ratio (FCR; g feed/g gain)	3.22 a	2.5373 b	2.3409 c	2.43 bc	0.093	0.001

[1]Rabbit received basic diet without supplementation (HS), zinc oxide nanoparticle (ZnNPs) 50 mg/kg diet, prodigiosin (PRG) 100 mg/kg diet and the mixture (ZnPRG), respectively.

a, b, c – means within a row without common letters differ at $P<0.05$.

Table 4. Effect of zinc nanoparticles (ZnNPs), prodigiosin (PRG) or their mixture (ZnPRG) on blood hematology of growing rabbits exposed to heat stress conditions

Items[1]	Experimental groups[2]				Pooled SEM
	HS	ZnNPs	PRG	ZnPRG	
Erythrocytes					
RBCs	3.36 b	4.8 a	4.82 a	4.64 a	0.223
hemoglobin	9.7433 b	12.193 a	11.676 a	11.91 a	0.324
platelet	823.33 b	1041.6 a	1057.66 a	1044 a	32.28
hematocrit	37.09	37.25	37.49	37.17	0.153
MCV	94.26	95.69	99.34	95.81	1.33
MCH	28.88 b	28.74 b	30.87 ab	32.01 a	0.511
MCHC	30.85	32.87	31.57	28.67	0.797
Leucocytes					
WBCs	8.20 a	5.33 b	5.49 b	4.47 b	0.443
basophils	0.27 a	0.24 ab	0.21 bc	0.19 c	0.011
eosinophils	0.38	0.37	0.33	0.41	0.016
monocytes	0.82 a	0.783 ab	0.653 b	0.776 ab	0.026
lymphocytes	82.96 ab	81.7 ab	80.88 b	84.92 ab	0.664
neutrophils	12.33	11.66	11.33	12.00	0.207

[1]RBCs: red blood cells (10^6); MCV: mean corpuscular volume (%); MCH: mean corpuscular hemoglobin (%); MCHC: mean corpuscular hemoglobin concentration; WBCs: white blood cells (10^3).

[2]Rabbit received basic diet without supplementation (HS), zinc oxide nanoparticle (ZnNPs) 50 mg/kg diet, prodigiosin (PRG) 100 mg/kg diet and the in combination (ZnPRG), respectively

a, b, c – means within a row without common letters differ at $P<0.05$.

Table 5. Effect of zinc nanoparticles (ZnNPs), prodigiosin (PRG) or their mixture (ZnPRG) on inflammatory response of growing rabbits exposed to heat stress conditions

Items[1]	Experimental groups[2]				Pooled SME
	HS	ZnNPs	PRG	ZnPRG	
TNFα	116 a	64.33 b	50.66 c	46.33 c	8.51
IL4	99.33 a	91.00 b	80.66 c	80.33 c	2.51
IFN-γ	78.00 a	58.00 b	59.67 b	60.33 b	2.56
NO	33 b	50.33 a	56.66 a	57.66 a	3.12
Lysosome	1.343 c	1.77 b	2.17 a	2.0733 a	0.11
TLR-4	6.97 c	4.47 b	2.793 a	2.96 a	0.52
Amyloid A	4.20 a	3.10 b	3.13 b	3.35 b	0.14
Ohdg	3.72 a	1.21 b	1.03 b	1.15 b	0.34

[1]Tumor necrosis factor α = TNFα; IL4 = Interleukin-4; IFN-γ = Interferon gamma; NO = Nitric acid; LYZ = Lysosome activity; Toll-like receptor 4 = TLR-4; 8-hydroxy-2'-deoxyguanosine = 8-OHdG.

[2]Rabbit received basic diet without supplementation (HS), zinc oxide nanoparticle (ZnNPs) 50 mg/kg diet, prodigiosin (PRG) 100 mg/kg diet and the in combination (ZnPRG), respectively.

a, b, c – means within a row without a common letter differ at $P<0.05$.

Inflammatory responses

As presented in Table 5, all supplemental groups exhibited a significant reduction in inflammatory responses such as TNF-α, IL4, IFN-γ, TLR-4, amyloid A and DNA damage markers (OHdG) accompanied by a significant increase in the levels of NO and lysosome activity as compared with the control group. Both treatments, PRG and ZnPRG, reduced TNF-α, IL4, and TLR-4 levels in the plasma of heat-stressed rabbits compared with those in other groups. All treated groups significantly decreased IFN-γ ($P<0.0001$) and amyloid A ($P<0.0007$) levels when compared with the HS group. The same trend was detected in the plasma OHdG. Where the treated groups showed the lowest OHdG levels ($P<0.0001$) compared with the control group. The nitric oxide level was significantly improved by dietary feed supplement inclusion. PRG and ZnPRG showed significantly higher lysosome activity levels ($P<0.01$) compared to other groups. In addition, ZnNPs also exhibited higher levels ($P<0.01$) for lysosomes compared with the control one. Generally, supplementing 100 mg of PRG with 50 mg of ZnNPs to the diets of heat-stressed rabbits reduced the inflammation markers and DNA damage compared with the HS group.

Table 6. Effect of zinc nanoparticles (ZnNPs), prodigiosin (PRG) or their mixture (ZnPRG) on redox regulation and immune response of growing rabbits exposed to heat stress conditions

Items[1]	Experimental groups[2]				Pooled SME
	HS	ZnNPs	PRG	ZnPRG	
Oxidative stress					
MDA	0.333 a	0.266 b	0.232 c	0.22 c	0.0121
PC	3.15 a	2.32 b	2.06 b	2.16 b	0.1375
MYO	8.55 a	3.06 b	1.35 c	1.08 c	0.9145
Antioxidant indices					
SOD	0.257	0.242	0.243	0.242	0.0053
GSH	0.19 b	0.16 c	0.23 b	0.32 a	0.0181
CAT	1.59 c	2.09 b	2.06 b	3.01 a	0.1573
Immune response					
IgM	60.33 c	7 1.00 b	86.00 a	88.00 a	3.5512
IgG	45.3 c	50.00 b	54.33 b	70.00 a	2.9037

[1]MDA = Malondialdehyde; PC = Protein carbonyl; MYO = Myeloperoxidase; Immunoglobins M = IgM; Immunoglobins G = IgG; SOD = Superoxide dismutase; GSH = Glutathione; CAT = Catalase.

[2]Rabbit received basic diet without supplementation (HS), zinc oxide nanoparticle (ZnNPs) 50 mg/kg diet, prodigiosin (PRG) 100 mg/kg diet and the in combination (ZnPRG), respectively.

a, b, c – means within a row without a common letter differ at $P<0.05$.

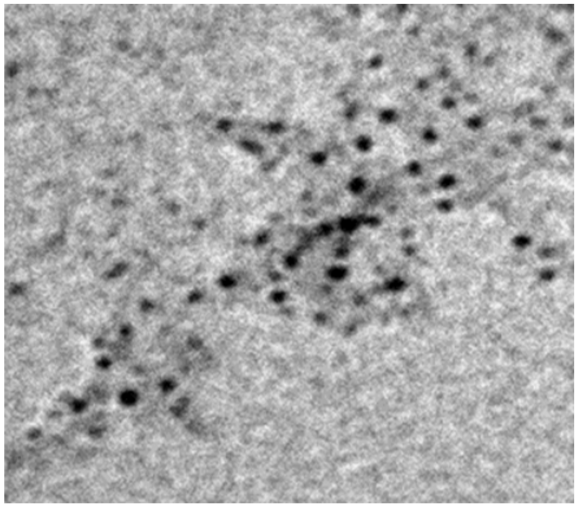

Figure 1. The synthesized ZnNPs characterized by TEM (transmission electron microscope, type JEOL-JEM 2100)

Redox status and immune responses

Rabbits in the ZnPRG group had significantly higher IgG and IgM levels than in other groups (Table 6). The highest values of CAT and GSH levels were found in the rabbits that received 50 mg of ZnNPs and 100 mg of PRG in their diets under elevated temperature (Table 6). No significant difference between ZnNPs and PRG groups was observed for CAT and IgG. Additionally, both treatments, PRG and ZnNPs, reduced the MDA and MYO values significantly ($P<0.001$), while all treatments effectively reduced the PC contents compared with the HS group. Rabbits supplemented with ZnNPs and/or PRG showed no statistical differences in SOD activity ($P=0.77$).

Immunohistochemical findings

Immunostaining revealed a highly positive expression of IRE1 in the hepatic tissue of heat-stressed rabbits, as represented in Figure 2 A. PRG (Figure 2 B) or ZnNPs (Figure 2 C) individual administration resulted in a slight down-expression of IRE1 in hepatic tissues of stressed rabbits compared with HS (Figure 2 A). In contrast, treatment with PRG incorporated with ZnNPs significantly lowered its expression in hepatic tissues (Figure 2 D). The same trend in the expression of GRP78 was noticed in Figure 3 A–D. The expression of GRP78 revealed a significant increase in the hepatic tissues of stressed rabbits (Figure 3 A). The supplementation of PRG and/or ZnNPs resulted in a significant reduction in the expression of GRP78, with the effective lower expression detected in the ZnPRG group.

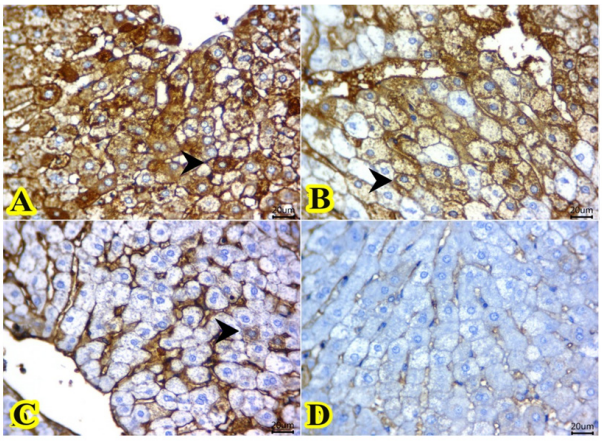

Figure 2. Representative photomicrograph of inositol-requiring enzyme 1 (IRE1) in hepatic tissues of heat-stressed rabbits treated with 0 (**A**), ZnNPs (**B**), PRG (**C**) and mixture of ZnPRG (**D**). **A.** Hepatic tissues showed higher and extensive expression of IRE1 in rabbits exposed to thermal stress. **B**. Rabbits that received ZnNPs showed moderate expression of IRE1 in their hepatic cells. **C**. Rabbits fed with PRG showed a slight expression of IRE1 in hepatic cells. **D**. Rabbits fed diets containing ZnNPs and PRG exhibited a weakening of stained cell in hepatic tissues (scale bar 20 µm)

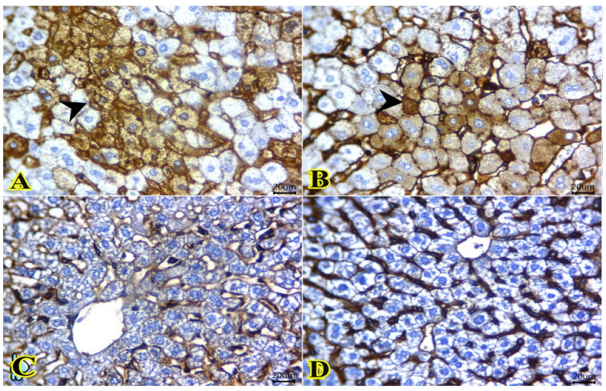

Figure 3. Photomicrograph of glucose-regulated protein 78 (GRP78) in hepatic tissues of heat-stressed rabbits treated with 0 (**A**), ZnNPs (**B**), PRG (**C**) and mixture of ZnPRG (**D**). **A.** Hepatic tissues of heat-stressed rabbits showing an increase in the expression of GRP78. **B**. Hepatic tissues of heat-stressed rabbit fed with ZnNPs showing moderate expression of GRP78. **C**. Sections of hepatic tissues in heat-stressed rabbits fed with PRG demonstrated a slight expression of GRP78. **D**. Sections of hepatic tissues of rabbits fed with ZnNPs and PRG demonstrate no immune staining of GRP78 (scale bar 20 µm)

Discussion

The current investigation revealed that the dietary inclusion of ZnNPs and/or PRG attenuated the adverse impacts of HS in growing rabbits by reducing oxidative stress, boosting the immuno-antioxidants mediators, and decreasing inflammation. Moreover, we also demonstrated that HS-persuaded stimulation of GRP78 and IRE1 signaling in hepatic tissues was noticeably diminished by PRG or/and ZnNPs, demonstrating a considerable function on the ER stress signaling in the hepatic damages of stressed rabbits. Globally, HS is an environmental challenge disturbing the productivity, health, and redox regulation of animals in the agriculture sector. Rabbits are very sensitive to HS (thermoneutral zone 25ºC±3) and find it difficult to diminish their heat load due to the smaller number of sweat glands. A nutritional strategy such as natural compounds or trace elements could play a critical role in reducing those negative influences. Recently, applying nanomaterials has been expanded as a promising method for sustainable agriculture to maintain the suitable quantity of existing forms of trace elements.

Also, natural pigments such as PRG were found to be powerful antioxidants and anti-inflammatory activities via boosting the antioxidant defense SOD and GSH and reducing the inflammation mediators such as TNF-α, IL-4, and IFN-γ as described previously (Venil et al., 2013; Rodriguez-Amaya, 2019). So, no previous work used the combination of PRG and ZnNPs to attenuate the HS-induced oxidative stress, inflammation and ER stress in rabbits. Hence, the strength of immune responses and reducing inflammation and ER stress in stressed rabbits substantially affect rabbit productivity for boosting health and homeostasis. THI is considered one of the most common tools for assessing the severity of HS in animals (Sejian et al., 2018). In the present work, rabbits suffered from severe HS depending on the values of THI obtained from the indoor farm (Marai et al., 2002).

As reported in our results, the dietary inclusion of ZnNPs and/or PRG effectively boosted the growth productivity of stressed rabbits. In the same trend as our results, Abdel-Wareth et al. (2022) found that the ZnNPs (up to 80 mg/kg diet) significantly enhanced the growth indices in growing rabbits exposed to HS. Authors suggested that the nano form of Zn has increased bioavailability and better absorption than organic or metallic forms. The improvement of growth might be revealed to the vital role of Zn in growth, synthesis, and cell division through enhancing the metabolism of the nutrients and improving protein and lipid synthesis (Chrastinová et al., 2015) via its imperative function in enzyme pathways as a constituent of numerous Zn metalloenzymes which are associated with several biological functions such as nutrient digestion, absorption and enzymatic structure (Li et al., 2021). Our data are identical to several previous works (El-Katcha et al., 2017; Abdel-Wareth et al., 2022), who reported that the ZnNPs enhanced the growth performance in several livestock species (broiler and rabbits) kept under high environmental temperatures (Hassan et al., 2021). For the effects of PRG in enhancing the growth performance in stressed rabbits, similar previous works are identical with our data obtained from the current study. The dietary inclusion of fucoxanthin (200 mg/kg) improved significantly the growth performance in broiler and had no effects on the feed intakes (Gumus et al., 2018). Studies have found that the addition of natural pigments has moderate influences on the growth productivity in animals (Sasaki et al., 2010; Wang et al., 2017). In an opposite trend, astaxanthin-rich yeast added

to broiler diets showed no significant impacts on growth indices. This may be related to the different sources and doses of pigments, animal strain, or experimental conditions.

The positive influence of PRG alone or in combination with ZnNPS on the growth performance was found in this study. Nevertheless, the protective effects of those molecules in stressed rabbits are unclear. Additional inquiry is desirable to clarify the roles of these natural molecules alone or in blends with other molecules in the intestinal integrity and structure or microbiota changes of animals. Recently, nanoparticles or microbial pigments have been generally hired as constructive supplements in the pharmaceuticals, food industry, and, lately, in aquaculture and livestock nutrition. This nano form of minerals might introduce a favorable method for the sustainable livestock sector to sustain the acceptable quantity of available trace elements (Abdelnour et al., 2021). Many nanoparticles or natural pigments have presented several biological activities in controlling infection and their significant role in medicinal uses. Hence, the attention to using natural pigments that can exchange synthetic dyes has expanded over the last decades. Of late, the tendency to employ nanotechnology or natural pigments in animal nutrition to boost productivity, immune-antioxidant capacity, and reduce inflammation and ER stress have been increasingly prolonged under adverse environments (Rodriguez-Amaya, 2019).

The HS triggers excessive amounts of oxidative stress, resulting in an imbalance between oxidant and antioxidant systems, thereby supporting cellular and organelles damages in animals (Oghbaei et al., 2021; Ma et al., 2022; Miao et al., 2022). Using natural molecules with robust antioxidants is a vital strategy for manipulating the antioxidant defense in stressed rabbits. The lowest values of MDA, PC and MYO were noticed in the Zn-PRG group, showing the robust antioxidant capacity of ZnNPs and/or PRG (Abdelnour et al., 2020) in removing oxidative stress in the plasma. As is well known, HS promotes oxidative stress that is associated with inflammation causing impairment to proteins, DNA, and cellular phospholipid membranes triggered by lipid peroxidation (Abdelnour et al., 2020; Sheiha et al., 2020; Abdelnour et al., 2021).

In line with our data, Hassan et al. (2021) and Abdel-Wareth et al. (2022) clarified that the ZnNPs could alleviate the adverse impacts of HS on the rabbit's health via their vital functions in cellular defense from OS by depressing free radicals and preventing lipids peroxidation as well as boosting the antioxidant protection. Catalase (CAT), sodium dismutase (SOD), and glutathione (GSH) are the main enzymes in the defensive system through the ability to neutralize the OS synthesis from the body. Our findings are identical to Wang et al. (2017), who elucidated that the diet fortified with lutein (up to 120 mg/kg diet) in broiler chicken significantly boosted the antioxidative defense and reduced lipid peroxidation. Moreover, it was found that fucoxanthin or PRG added to the broiler (Gumus et al., 2018) or rabbits (Abdelnour et al., 2020) diets, respectively, significantly improved the levels of CAT, GSH, and SOD and diminished MDA levels. Feeding stressed animals with several types of nanoparticles, or microbial pigments displayed a promising impression in declining the menace of severe health complications in respect of their antioxidant action (Ramiah et al., 2019; Abdel-Wareth et al., 2022; Sherif et al., 2022)

TNFα, IL-4, IFN-γ and TLR-4 were significantly lessened in all supplemented groups compared to the HS group. Many published works indicated that the HS-induced release of inflammatory cytokines represents a decline in the immune system (Wu et al., 2019; Xiong et al., 2020). The feeding strategy represented in feed additives supplements during HS, targeting the inflammatory cytokines, could be a potential approaches tool for mitigating HS in animals. Yang et al. (2019) showed the ability of PRG to alleviate intestinal inflammation in rats caused by HS. HS triggered superior expression of IL-4 in rabbits exposed to HS, indicating immunity suppression (Sheiha et al., 2020). Ramiah et al. (2019) found that the ZnNPs significantly reduced the inflammatory cytokines in stressed broilers. The mixture of ZnNPs and PRG showed a robust anti-inflammatory mediator in rabbit diets. Consuming anti-inflammatory molecules enhances the immunological IgG and IgM, thus boosting health and supporting ducking disease.

Hence, the existing investigation displays that the mixture of ZnNPs and PRG shows synergistic biological impacts by positively affecting immunity and health and reducing inflammatory marks and ER stress. This effect was not explored previously in animals. After that, the anti-inflammatory mechanisms of those molecules are desired in diverse animal species. Nutritional strategy for targeting inflammation, OS and ER stress induced by HS is critical those days with global warming affecting animal productivity (Sejian et al., 2018; Ellamie et al., 2020; Hassan et al., 2021; Li et al., 2021; Yang et al., 2021). The lysosome activity is a part of the innate immunity of animals that keeps against pathogenesis. Thus, it has been mentioned that HS can robustly induce autophagy and weaken lysosome function (Yi et al., 2017).

Consequently, dietary supplementation with ZnNPs and/or PRG showed higher levels of NO and lysozyme activity in stressed rabbits. We proposed that this increment in NO levels in treated groups might be interrelated with the rise of blood flow to the skin. That way, it helps the rabbits dissipate heat loss via skin. The presence of ZnNPs and/or PRG may allow the rabbits to sustain their blood homeostasis by aggravating endogenous cellular defense machinery to manage OS, inflammation and ER stress persuaded by HS (Cheng et al., 2019). Further investigations on various animal species should be explored with an increasing interest concerning nanoparticles or other pigments as a reliable substitute for feed supplements in animal nutrition.

HS increases pro-inflammatory signals through its action on the innate immune receptors comprising TLR-

4, which is vital in the thermo-tolerance of an animal. Moreover, Cheng et al. (2019) clarified that HS augmented the expression of TLR-4 cytokines and diminished the antioxidant enzymes and tight junction proteins in stressed rats. 8-OHdG is considered a DNA oxidation marker. Some studies found a positive relationship between plasma 8-OHdG levels and cardiovascular disease (Graille et al., 2020). Moreover, higher levels of 8-OHdG were detected after exposure to environmental pollution, heavy metal, or stress (Xiao et al., 2018). In our data, feed supplementing with ZnNPs and/or PRG significantly decreased the 8-OHdG levels in the plasma of stressed rabbits. This could reflect the ability of ZnNPs and PRG to sustain the DNA integrity in the cellular system.

As reported previously, HS triggered an excess of OS, resulting in ER stress, where GRP78 and IRE1 are activated. Moreover, ER stress might increase inflammation and apoptosis (Ibrahim et al., 2019; Tang et al., 2022). Evidence displays that ER stress shows an important role in pathological and physiological processes, such as oxidative stress and inflammation of an organ or cellular parts (Tang et al., 2022). To discover the machinery underlying the valuable impacts of PRG and/or ZnNPs in stressed rabbits, we assessed fluctuations in the hepatic tissues' immunoexpressing of GRP78 and IRE1. The results ascertained the downregulation of both GRP78 and IRE1 in hepatic tissues of stressed rabbit fed diets fortified with ZnNPs and PRG. It has been reported that IRE1 is activated in response to an aggregation of UPR in the ER (Tang et al., 2022).

GRP78 is indispensable in cellular signaling, immunity, and inflammation pathways (Ibrahim et al., 2019). The upregulation of GRP78 was induced by SH, which plays a critical role in the cellular membrane and intercedes the vast amount of unfolding proteins (Oghbaei et al., 2021). Chronic HS-persuaded ER stress also augmented the generations of misfolded proteins, leading to an increase in misfolded protein synthesis and degradation, as well as vesicle transport ailment (Tang et al., 2022). Similar to our data, Ma et al. (2022) found that HS-activated ER stress persuaded apoptosis and triggered hepatic injury in broilers. Recent studies reported the dietary inclusion of some molecules, such as alpha lipoic acid (Xiong et al., 2020) and ursolic acid (Yang et al., 2021) in animals. Dietary supplementation with the mixture of ZnNPs and PRG significantly resorted the upregulation of ER stress, such as GRP78 and IRE1, in the hepatic tissue of stressed rabbits. The protective role of PRG and ZnNPs in hepatic injury, through constraining ER stress (Xiong et al., 2020) disclosed that HS persuaded superior protein levels of GRP78 as an ER stress marker, indicating that ER stress was activated in testicular tissues of chicken after exposure to HS. The mixture of ZnNPs and PRG displays antioxidant and anti-inflammatory activities and immune-stimulatory effects that have been associated with many health revenues. Based on the above conclusions, it could be proposed that ZnNPs incorporated with PRG could be a valuable nutritional strategy in improving the immune-antioxidant features, and reducing oxidative stress, inflammation and ER stress of rabbits kept under elevated temperatures.

Conclusion

This study presented novel evidence on the beneficial effects of ZnNPs and/or PRG against HS, pinpointing the possible involvement of oxidative stress, DNA damage, inflammation and ER stress signaling. Dietary ZnNPs, PRG and their mixture inclusion prevented oxidative stress, DNA damage, immune weakening, inflammatory response, and ER stress signaling in stressed rabbits. Hence, ZnNPs and/or PRG could be promising candidates to avoid the adverse effects of HS in rabbits, pending further explorations to clarify the precise mechanism(s) underlying their positive effects.

Conflicts of interest

The researchers declare no conflict of interest.

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