



DIETARY GRAPESEED OIL NANO-EMULSION IMPROVES BLOOD HEALTH, ORGAN HISTOLOGY, ANTIOXIDANT STATUS, IMMUNE ABILITY, AND REDUCES INFLAMMATION IN YOUNG FATTENING RABBITS EXPOSED TO THERMAL STRESS

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Abstract

Heat stress (HS) represents a major environmental impact on rabbits' health, welfare, and production. Grapeseed oil (GSO) has improved health and growth. However, the mechanism by which it mitigates the negative effects of HS in growing rabbits is still under debate. This study explored the protective role of dietary grapeseed oil nano-emulsion (GON) against blood changes, immune dysfunction, organ histological damage, oxidative stress, and inflammation triggered by HS in growing rabbits. A total of 120 growing rabbits (5 weeks of age and average weight 657.32 g) were divided into 4 groups. The rabbits were administered with different doses of GON: 0 (HS, basal diet), 0.5 (GON0.5), 1 (GON1.0), and 1.5 (GON1.5) mL/kg of diet for 8 weeks under natural heat stress conditions. The results indicated that the GON1.0 and GON1.5 groups exhibited higher body weight values and gains than the other groups ($P < 0.05$), showing a linear relationship with GON dietary levels. A quadratic relationship was observed between FCR and GON, with the optimal dose being 0.7 mL/kg of diet. The GON1.5 group had higher red blood cell values than the other groups ($P < 0.05$), while all GON groups had significantly reduced leukocyte counts compared to the HS group ($P < 0.001$) in a quadratic manner. GON1.5 and GON1.0 significantly enhanced protein fractions in linear regression analysis. Additionally, ALT, AST, lipid profile, uric acid, LDH, GGT, and total and direct bilirubin were significantly decreased in all rabbits fed with GON ($P < 0.05$) in a quadratic manner. Enriching diets with GON (1 or 1.5 mL) significantly improved antioxidant status (SOD and GPx) and immunity (IgG and IgM) in a quadratic manner. It also resulted in a quadratic decrease in oxidative stress (MDA and PC) and inflammation (IL-6, lysosome activity, and IFN- γ) levels compared to the HS group ($P < 0.001$). There was a quadratic increase in IL-10 and nitric oxide with increasing the GON, and the optimal doses were 0.86 and 0.9 mL/kg of diet. The GON administration maintained organ architecture (liver, spleen, and intestines) and reduced the immune expression of iNOS in liver tissues induced by HS. Collectively, GON (1 mL/kg diet) is an effective feed additive for improving the health and growth of stressed rabbits by supporting antioxidant and immunity functions while reducing oxidative stress and inflammation pathways induced by HS.

Key words: growing rabbit, heat stress, grapeseed oil, nano-emulsion oil

Climate change poses a significant challenge for organisms worldwide. Rising temperatures can increase disease occurrence, mortality, food scarcity, and reduce productivity in animals. Heat stress (HS) induced by climate change significantly impacts the livestock sector. Rabbits play a significant role as meat suppliers globally, especially in European countries, China, and Egypt (Wu, 2022). Due to their lack of sweat glands, rabbits cannot dissipate heat (Marai et al., 2002). Scientists have long recognized that HS can decrease feed consumption and nutrient digestibility, impair growth performance, and result in economic losses on rabbit farms (Bashar et al., 2024; El-Raghi et al., 2023; Ismail et al., 2023). Additionally, HS damaged hepatocytes and renal tissues (Bashar et al., 2024), leading to renal and liver function failure and intestinal damage by promoting pathogenic bacteria growth. Elevated ambient temperature can increase oxi-

dative stress, impair immune function, and trigger inflammation in growing rabbits (Liang et al., 2022). Therefore, there is an urgent need to find effective, safe, sustainable, and eco-friendly methods to enhance immunity and reduce inflammation, helping animals withstand heat stress (Rashwan et al., 2023). Implementing a dietary strategy to mitigate the negative effects of HS is crucial.

The agricultural byproduct poses an additional environmental challenge. Valuing the waste from a food processing line that would otherwise be disposed of provides additional motivation for researching its anti-heat stress agent, as it contributes to the decline of food waste (Pérez-Aguilar et al., 2023). Vegetable and fruit by-products are valuable sources of natural bioactive compounds, which can help enhance sustainability in the food industry (Rashwan et al., 2023). Grapes are a valuable crop globally, with an annual production of 25 million tons.

By-products from grape processing, such as skins, seeds, and stems, account for about 20% of the total weight of the grape (Chu et al., 2024; Terra et al., 2007; Urkmez and Biricik, 2022). This poses a disposal challenge for wineries and the grape juice industry. Grape seeds contain 13 to 15% oil on a dry basis (Gitea et al., 2023). The main bioactive compounds of grapeseed oil (GSO) are vitamins, phenolic compounds, and fatty acids (Gitea et al., 2023; Mahanna et al., 2019). GSO has high amount of bioactive polyphenols frequently used as food preservatives or nutritional supplements due to their antimicrobial, antioxidant, and anti-inflammatory abilities (Charradi et al., 2018). The polyphenol extracted from grape seeds includes compounds like catechin, procyanidin, gallic acid, and epicatechin (Choi et al., 2023; Rashwan et al., 2023), with various health-promoting purposes such as vasodilatory and anti-allergic effects (Gitea et al., 2023; Mahanna et al., 2019). However, the major hurdles of dietary grapeseed oil include its weak water solubility and oral bioavailability. Therefore, a novel nanotechnology method is needed to address these obstacles. This is a highly innovative application of using grapeseed oil in nano-emulsion form (GON) as an anti-heat stress agent through its antioxidant, immunomodulatory, and anti-inflammatory actions in the diet of growing rabbits. It is commonly used as a nutritional supplement and has received the “Generally Recognized as Safe” certification from the US FDA. GSO has shown promise in animal models as a potential treatment for oxidation-interrelated disorders. Studies have shown that dietary supplementation of grapeseed extract (GSE) in broiler (El-Damrawy, 2014; Hajati et al., 2018), quails (Abdel-Wahab et al., 2018; Erişir et al., 2018), rabbits (Shamiah et al., 2024) and ruminants (Ma et al., 2023; Urkmez and Biricik, 2022), improved growth performance and blood health, enhanced antioxidant ability, feed efficiency, and regulation of inflammatory responses in various animal models (Ma et al., 2023; Urkmez and Biricik, 2022). Studies have shown that GSE can improve energy metabolism, microbial content and promote healthy growth in pre-weaning dairy calves under HS (Urkmez and Biricik, 2022) and thermoneutral zone (Ma et al., 2023). However, the impacts of GON dietary administration on growth, blood health, immunity, organ histology, and redox homeostasis in inflammatory responses of growing stressed rabbits remain to be fully explored. It is a multi-targeted and multi-faceted mixture of bioactive compounds. Based on the strong biological activities of GON, we hypothesize that GON can improve blood health, immunity, organ function, reduce inflammation, and oxidative stress in stressed rabbits. This could be an effective strategy for abating the negative causes of HS. Thereafter, the existing research was conducted to explore the protective effect of GON in mitigating the negative effects of HS in rabbits, specifically in terms of enhancing the blood health, organ histology, immune biomarkers, and reducing the inflammation-oxidative pathways.

Material and methods

Ethical statement and preparation of nano-emulsion

The animal handling and experimental protocol were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Zagazig (Code ID: ZU-IACUC/2/F/371/2023). The grapeseed oil (GOS) was purchased from the Ab Chem company for pharmaceutical materials, Mansoura, Egypt. The nano-emulsion of grapeseed oil (GON) was done according to the method described by Leisyah (2019), see Figure 1.

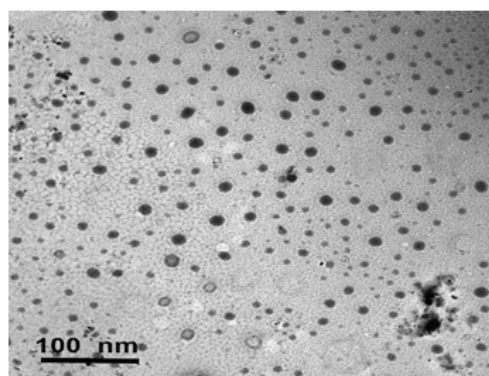


Figure 1. The nano-emulsion of grapeseed oil (GON) has a size of less than 100 nm and shows excellent distribution in the hydrophobic liquid. It also has a spherical shape

Animals and experimental design

A total of 120 growing male rabbits with an average weight of 657.32 g were registered in this investigation. The rabbits were independently accommodated in galvanized wire cages (35 × 35 × 60 cm³). All animals were divided into 4 experimental groups. Each group consisted of 30 animals; each of the two rabbits was considered a replicate. The trial was performed from July to August 2022 at the Agricultural Rabbit Farm, Zagazig University. Animals were fed a control basal diet (as a control group and serve as natural HS group) or supplemented with various levels of grapeseed oil nano-emulsion (GON) at levels 0.5 (GON0.5), 1 (GON1.0) and 1.5 (GON1.5) mL/kg diet. The dose of GON was added according to the previously published work (Nasr et al., 2022). According to the study protocol, the GON was mixed with premix and supplements into the diet. The cages were equipped with feeders that provided *ad libitum* access to food, and a fresh water supply was offered through an automatic nipple system. The growing rabbits were housed in a building with artificial lighting and natural environmental condition. All growing rabbits were maintained under similar hygienic situations and subjected to consistent environmental and managerial factors. Daily morning cleaning of the rabbit trays was performed to remove feces and urine. The diet was formulated to meet all the necessary nutrient requirements for fattening rabbits, as outlined by de Blas and Wiseman (2020). The chemical components of the diet on a dry matter basis re-

vealed the following composition (Table S1; supplementary data): ether extract (2.23%), crude protein (17.36%), nitrogen-free extract (59.46%), organic matter (91.42%), ash (8.58%), crude fiber (12.37%), and a digestible energy content of 2700 kcal/kg.

Climatic components

To assess the THI (temperature humidity index), the daily ambient temperatures (AT, °C) and relative humidity (RH, %) were documented throughout the investigational time using an automatic thermo-hygrometer (Wertheim, Germany). This device was installed in the rabbitry to collect the necessary data. The THI was calculated based on the collected humidity and temperature data, following the method outlined by Marai et al. (2002). $THI = db\ ^\circ C - [(0.31 - 0.31 (RH/100)) \times (db\ ^\circ C - 14.4)]$, Where db °C, is the dry bulb temperature in Celsius. Values of THI were categorized as follows: >30.0 (very severe HS), 29.0 to 30.0 (severe HS), 27.8 to 28.9 (moderate HS) and <27.8 (absence of HS).

Assessing growth indices and carcass traits

Each rabbit's feed intake (FI) and live body weight (LBW) were recorded every two weeks during the study. This data was used to determine the average daily weight gain (ADWG) and feed conversion ratio (FCR), which indicates the amount of feed consumed per unit increase in body weight for each specific period. After 8 weeks of feeding experiment, a random selection of ten rabbits from each group underwent a 12-hour fasting period before slaughtering according to the Islamic method. The non-edible parts, such as the tail, pelt, and viscera, were discarded. The carcass includes heart, liver, kidney, spleen, lungs, intestinal, and cecum weight evaluated as a proportion of the LBW. The length of the cecum in centimeters was calculated. The dressing proportion was calculated as $[(\text{weight of hot-dressed carcass/pre-slaughter weight}) \times 100]$.

Measurement of hematology and serum biochemistry

Six blood samples (from each group) were obtained from slaughtered rabbits. The samples were collected in sterile tubes and separated into two subsamples. The first subsample was utilized to assess hematological parameters following the method described by Siegel and Walton (2020) using an automated hematology analyzer (Hospitex Hema Screen 18, Sesto Fiorentino, Italy). The second subsample was allowed at room temperature for one hour and then centrifuged at $700 \times g$ for 20 min (Sigma 2-16KL, Germany). After centrifugation, the serum samples were removed and gathered at -20°C pending assessment. Serum levels of total protein (TP), albumin (ALB), alanine aminotransferase (ALT), uric acid, aspartate aminotransferase (AST), lactate dehydrogenase (LDH), gamma-glutamyl transferase (GGT), total bilirubin (TB), direct bilirubin (DB), were assessed using commercial kits purchased from CliniChem Ltd. (Budapest,

Hungary). Globulins (GLO) were assessed using the following equation $GLO = TP - ALB$, while ALB/GLO assessed the A/G.

Lipid profile assessment

Plasma levels of triglycerides (TG), total cholesterol (TC), and high-density lipoprotein (HDL) were measured using colorimetric assay kits from Biosino Biotechnology and Science, Beijing, China, following the manufacturer's instructions. The low-density lipoprotein (LDL) level was calculated based on TC and TG, as previously described by McNamara et al. (1990).

Antioxidant capacity assays

The total antioxidant status (TAC), superoxide dismutase (SOD), glutathione peroxidase (GPX), catalase (CAT), and malondialdehyde (MDA) were assessed using commercial ELISA kits: TAC-2513, SOD-2521, GPX-2524, CA2517, and MDA-2529, respectively. All commercial ELISA kits for antioxidant status were acquired from BioDiagnostic company in Giza, Egypt. We followed the methods of Young (2001), Nishikimi et al. (1972), Koracevic et al. (2001), Huang et al. (2016) and Kei (1978) for assessing the TAC, SOD, GPX, CAT, and MDA, respectively. The oxidized and reduced BSA's protein carbonyl (PC) content was determined using a modified colorimetric method (Buss and Winterbourn, 2002).

Immune and pro-inflammatory cytokines

The levels of IgG (ab187400) and IgM (ab190539) in rabbit serum were assessed using the quantitative sandwich method according to Jeklova et al. (2010). Lysozyme activity was measured using a lysozyme activity assay kit (ID: LS-K52-100) obtained from LifeSpan BioSciences, Inc. (USA), following the protocol outlined by Toro et al. (2015). Nitric oxide (NO, ab65328) was evaluated in the serum using the colorimetric method Nitric Oxide Assay Kit at a wavelength of 540 nm (Csonka et al., 2015). Interleukin 6 (IL-6; Cat. No.: E-EL-RB0014), Interferon Gamma (IFN- γ ; Cat. No.: E-EL-RB0679), and Interleukin 10 (IL-10; Cat. No.: E-CL-R0016) were assessed using ELISA kits provided by Elabscience (USA). Table S2 (Supplementary data) shows the sensitivity, ranges, and intra- and inter-assays of cytokines and immunoglobulins used in this study.

Histology of organ tissues

After the animals were slaughtered, liver, spleen, and intestinal samples were obtained from five rabbits in each treatment group. These samples were then fixed in a neutral formalin buffer (10%), dehydrated using increasing concentrations of ethyl alcohol, and cleared in two xylol changes. Afterwards, the samples were embedded in paraffin blocks and cut into 4- μm sections using a microtome (Leica RM 2155). To visualize the tissue, all sections were stained with eosin and hematoxylin, following the protocol described by Suvana et al. (2018). High-resolution

photomicrographs were obtained with a Leica DM 500 digital camera microscope equipped with an EC3 camera.

Immunohistopathology of iNOS in liver tissues

Liver samples were stained by immunohistochemistry (IHC) following the protocol described by Chetty et al. (2016) using antibodies for inducible nitric oxide synthase (iNOS) (Anti-iNOS antibody, ab3523, diluted 1:20). The tissue sections from all experimental groups were dewaxed and hydrated. Staining was then performed using the DAB chromogenic agent (Expose mouse and rabbit-specific HRP/DAB detection kit, Abcam; Cat.: ab80436). High-resolution photomicrographs were obtained with a Leica DM 500 digital camera microscope equipped with an EC3 camera.

Statistical analysis

To assess the normality of the obtained data, a Shapiro-Wilk test was performed following the methodology outlined by Razali and Wah (2011). The growth variables, feed utilization, serum metabolites, carcass traits, hematology, redox status, and immunity factors were evaluated using the MIXED procedure (PROC MIXED) provided by SAS Institute (2012). In the statistical model, individual rabbits were treated as a random effect, while the GON doses were considered fixed. Multiple comparisons between means were conducted using Duncan's multiple-range tests. For determining a possible dose-response curve, the response of each dependent variable for various levels of GON (0, 0.5, 1, and 1.5-ml GON/kg diet) was assessed using orthogonal contrast statements for linear and quadratic responses. Dose-response curves for significant relationships were fitted using the appropriate regression equation to determine the optimum dose of dietary GON by the GraphPad Program version 8. A P-value of <0.05 was considered statistically significant.

Results

THI values

As shown in Figure 2 C, the values were 29.38 and 30.96 in the study's first and last weeks, indicating severe heat stress in growing rabbits. The data on THI revealed that the ambient temperature and relative humidity were 31.5°C and 60% at the beginning of the study. By the end of the study, the ambient temperature and relative humidity were 31.5°C (Figure 2 A), and 71% (Figure 2 B), respectively.

Effects of dietary GON on growth performance

Table 1 summarizes growing rabbits' FI, BW, weight gain (BWG), and FCR under HS conditions. Throughout the feeding trial, the highest feed consumption was observed, with the highest level of GON (1.5 mL/kg). Diets containing 1 and 1.5 ml GON per kg significantly improved the final body weight of the rabbits at the end of the trial compared to other diets. Significant improvements in weight gain were noted in the 4th and 8th weeks of the growth period with the 1 and 1.5 mL GON/kg of diet compared to the HS group. All levels of GON inclusion resulted in better FCR values than HS throughout the feeding experiment ($P=0.001$, $P<0.001$, $P=0.001$, and $P=0.030$ at 1–4, 4–8, and 1–8 weeks, respectively). The highest performance of growing rabbits under HS conditions was achieved with the high dose of GON (1.5 mL/kg of diet). Linear polynomial regression analysis was observed between the dietary inclusion of GON and feed intake (Figure 3 A, 4–8 weeks), body weight (Figure 3 B, at 8 weeks), body weight gain (Figure 3 C, BWG 18 weeks). Otherwise, a quadratic relationship between GON and FCR (Figure 3 D, 4–8 weeks) with the optimum doses was established at 0.7 mL/kg diet for FCR (Figure 3 D, 4–8 weeks).



Figure 2 A–C. The ambient temperature (temp, °C, 2A), relative humidity (RH, %, 2B), THI values (C) throughout the experimental period in the natural environment

Table 1. Effects of dietary grapeseed oil nano-emulsion (GON; 0, 0.5, 1.0 and 1.5 mL/kg diet) on growth performance of growing rabbits under heat stress conditions

Weeks	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1.0	1.5		TRTs	Lin.	Quad.
Feed intake (g)								
1–4	2465.42	2374.16	2195.83	2491.25	54.60	0.215	0.335	0.084
4–8	1715 bc	1593.33 c	1905.83 a	1812.08 ab	29.97	0.001	<0.001	<0.001
1–8	4180.42 ab	3967.5 b	4101.66 ab	4303.33 a	49.24	0.101	0.335	0.084
Body weight (g)								
1–4	656.66	657.92	659.58	658.33	1.83	0.958	0.694	0.741
4–8	1475.42	1509.17	1467.50	1524.58	9.88	0.126	0.223	0.545
1–8	2018.75 b	2050.42 b	2107.50 a	2126.66 a	9.45	<0.001	0.000	0.669
Body weight gain (g)								
1–4	818.75	851.25	807.92	866.25	9.91	0.125	0.255	0.505
4–8	543.07 b	541.25 b	640.00 a	602.08 a	10.09	<0.001	0.001	0.288
1–8	1362.08 b	1392.50 b	1447.92 a	1468.34 a	9.50	<0.001	<0.001	0.739
Feed conversion ratio (g/g)								
1–4	3.01 a	2.78 b	2.87 b	2.87 b	0.02	0.001	0.335	0.084
4–8	3.15 a	2.94 c	2.97 bc	3.01 b	0.02	<0.001	<0.001	<0.001
1–8	3.07 a	2.85 b	2.83 b	2.93 b	0.03	0.030	0.115	0.011

GON: grapeseed oil nano-emulsion; HS: the basal diet, without GON supplementation. Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 (GON1.5) mL/kg of diet for 8 weeks. Data presented as mean±total SE. a, b, c – means in the same column bearing different letters are significantly different at $P<0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

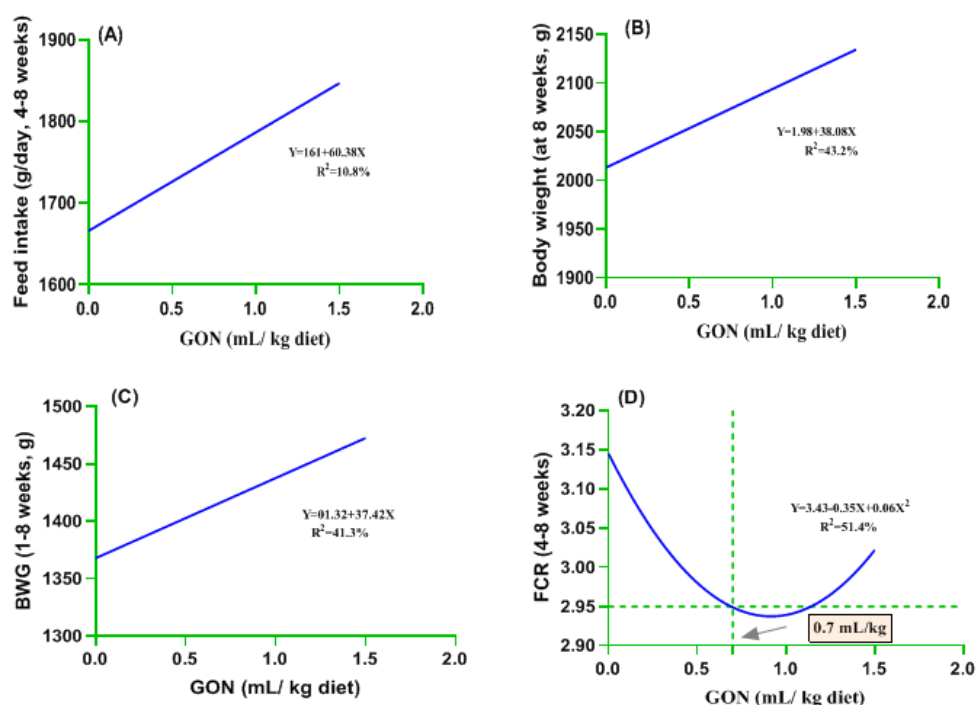


Figure 3 (A–D). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and feed intake (A, 4–8 weeks), body weight (B, at 8 weeks), body weight gain (C, BWG 18 weeks), and feed conversion ratio (D, 4–8 weeks)

Table 2. Effects of dietary grapeseed oil nano-emulsion supplementation (GON, 0, 0.5, 1.0 and 1.5 mL/kg of diet) on blood hematology of growing rabbit under HS condition

Attributes	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1	1.5		TRTs	Lin.	Quad.
RBC ($\times 10^6/\text{mm}^3$)	4.07 b	4.47 b	4.34 b	5.151 a	0.14	0.014	0.004	0.327
Hb (g/dL)	10.42 b	12.69 a	12.75 a	12.88 a	0.28	<0.001	<0.001	0.001
Ht (%)	32.48 c	37.21 b	37.76 b	39.07 a	0.67	<0.001	<0.001	0.002
MCV (fL)	86.14	85.54	87.42	87.35	0.41	0.297	0.143	0.745
MCH (%)	31.09 a	29.57 b	30.7 ab	30.85 ab	0.24	0.093	0.810	0.073
MCHC (%)	34.44	34.32	34.72	34.66	0.20	0.907	0.595	0.944
PLAT ($\times 10^3/\text{mm}^3$)	89.00 d	113.50 c	126.25 b	133.25 a	4.38	<0.001	<0.001	<0.001
WBC ($\times 10^3/\text{mm}^3$)	12.38 a	8.03 b	8.07 b	8.14 b	0.49	<0.001	<0.001	<0.001
LYM (%)	60.68 a	59.14 a	50.01 b	46.45 b	1.67	<0.001	0.211	0.010
MON (%)	3.31 a	2.22 b	2.17 b	2.14 b	0.14	<0.001	0.000	0.481

Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 mL (GON1.5) mL/kg diet for 8 weeks. Data presented as mean $\pm$ total SE. HS: the basal diet without GON supplementation. RBC: red blood cells; Hb: hemoglobin; Ht: hematocrit; PLAT: platelet count; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; WBC: white blood cells; LYM: lymphocytes and MON: monocytes. a, b, c – means in the same column bearing different letters are significantly different at $P < 0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

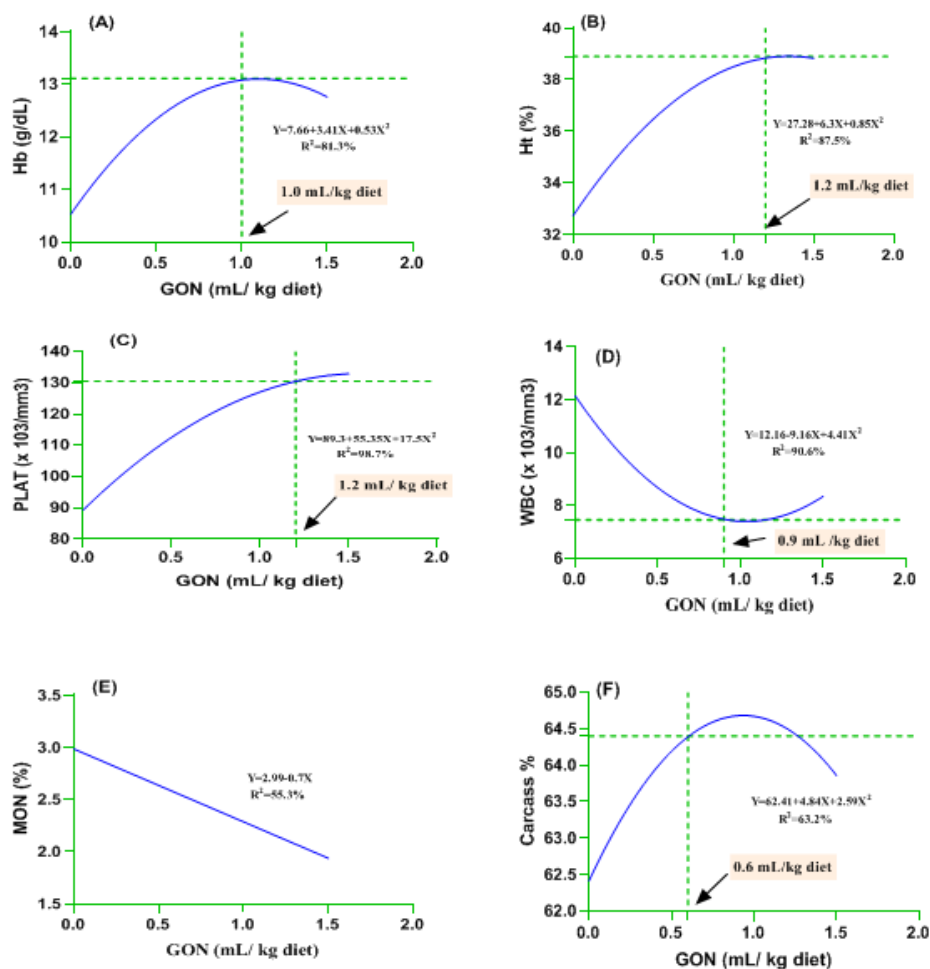


Figure 4 (A–F). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and hemoglobin (A, Hb), hematocrit (B, Ht), platelet count (C, PLAT), white blood cells (D), monocytes (E, MON), and carcass percentage (F)

Effects of dietary GON on blood hematology

The results in Table 2 demonstrate the impact of dietary GON on the blood hematology parameters of growing rabbits exposed to HS. All the tested levels of GON

led to significant increases ($P < 0.001$) in hematocrit (Ht %), platelet count (PLAT), and hemoglobin concentration (Hb) compared to the HS group. The highest increase in red blood cell (RBC) count ($P = 0.014$) was observed with

the highest supplementation level of GON (1.5 mL/kg). The group receiving 1.5 mL GON/kg showed the highest values for Ht, RBC, PLAT, and Hb among all groups. On the other hand, incorporating graded levels of GON in the diets of growing rabbits did not significantly affect mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) values. There was a significant increase ($P<0.001$) in the white blood cell (WBC) count for the HS group compared to those receiving dietary GON. The dietary levels of 1 and 1.5 mL of GON significantly decreased lymphocytes (LYM) and monocytes (MON). The polynomial regression analysis indicated that the optimal concentrations were 1 mL/kg diet for Hb (Figure 4 A), 1.2 mL for Ht (Figure 4 B), 1.2 mL for PLAT (Figure 4 C), and 0.9 mL for WBCs (Figure 4 D). A linear polynomial regression analysis was observed between the dietary inclusion of GON and MON (Figure 4 E).

Effects of dietary GON on carcass traits

Supplementing doses of GON significantly affected the carcass, liver, kidney, spleen, lungs, heart percentages, and cecum length ($P<0.05$). At the same time, they had no notable impacts ($P>0.05$) on the body, carcass, intestine, and cecum weights as shown in Table 3. Including GON in the experimental diets improved carcass percentages ($P=0.001$) but reduced the percentages of liver, heart, kidney, spleen, and lung weights, as well as the length of the cecum ($P=0.002$, $P=0.048$, $P=0.029$, $P=0.009$, $P<0.001$, and $P<0.001$, respectively) compared to the basal diet. The carcass percentage showed a quadratic increase in response to the treatment (Figure 4 F). The optimal doses for carcass percentage were deter-

mined to be 0.6 mL of GON per kg of diet. However, significant increases in the weight percentage of lungs and cecum length were observed with the basal diet, while the same items recorded the lowest values with the high dose of GON.

Effects of dietary GON on blood biochemistry

The effects of GON supplementation on the blood biochemistry of heat-stressed growing rabbits are shown in Table 4. Both levels of supplementation, 1 and 1.5 mL of GON, led to significant increases ($P<0.001$, $P=0.001$, and $P=0.003$, respectively) in serum total protein, albumin, and globulin concentrations, with no effect ($P>0.05$) on the albumin/globulin ratio. Feeding stressed rabbits with various levels of GON exhibited a linear increase in TP (Figure 5 A), ALB (Figure 5 B) and GLO (Figure 5 C). Regression analysis data displayed a quadratic relationship ($P<0.001$) between various levels of GON and AST (Figure 5D), ALT (Figure 5 E), LDH (Figure 5 F), TB (Figure 5 G), GGT (Figure 5 H), DB (Figure 6 A), and uric acid (Figure 6 B). The optimal GON levels were 0.87, 1.0, 1.13, 1.2, 0.96, 0.9, and 0.88-mL/kg of diet for AST, ALT, LDH, TB, GGT, DB, and uric acid, respectively. The average values of AST, ALT, LDH, GGT, total bilirubin, direct bilirubin, and uric acid were significantly reduced ($P<0.001$) due to GON supplementation. A quadratic regression analysis indicates that rabbits fed diets with GON had lower TC, TG, and LDL levels (Table 4), and higher HDL levels, especially at 1 and 1.5 mL/kg of the diet. The optimum doses for TC (Figure 6 C), TG (Figure 6 D), HDL (Figure 6 E), and LDL (Figure 6 F) were 0.7, 0.7, 1.1, and 0.8 mL/kg of GON, respectively ($P<0.001$).

Table 3. Effects of dietary grapeseed oil nano-emulsion (GON; 0, 0.5, 1.0 and 1.5 mL/kg of diet) supplementation on carcass traits of growing rabbit under HS condition

Items	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1	1.5		TRTs	Lin.	Quad.
Body weight (g)	2018.75	2035.00	2042.50	2090.00	18.56	0.610	0.224	0.693
Carcass weight (g)	1260.00	1292.50	1327.50	1328.75	13.36	0.217	0.054	0.513
Carcass (%)	62.57 c	63.70 b	65.16 a	63.69 b	0.27	0.001	0.004	0.001
Liver (%)	3.29 a	2.76 b	2.50 b	2.65 b	0.09	0.002	0.001	0.013
Heart (%)	0.37 a	0.35 b	0.35 b	0.36 b	0.01	0.048	0.024	0.269
Kidney (%)	0.70 a	0.64 b	0.66 ab	0.64 b	0.01	0.029	0.090	0.031
Spleen (%)	0.053 a	0.043 b	0.048 b	0.046 b	0.01	0.009	0.000	0.340
Lungs (%)	0.77 a	0.69 b	0.62 c	0.56 d	0.02	<0.001	0.395	0.613
Intestine (%)	220.50	221.00	220.75	223.75	1.13	0.759	0.784	0.481
Cecum weight (%)	0.46	0.46	0.48	0.46	0.05	0.489	0.000	0.327
Cecum length (cm)	11.55 a	10.67 b	9.68 c	9.27 c	0.25	<0.001	0.420	0.045

Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 ml (GON1.5) mL/kg diet for 8 weeks. HS: the basal diet without GON supplementation. Data presented as mean±total SE. a, b, c – means in the same column bearing different letters are significantly different at $P<0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

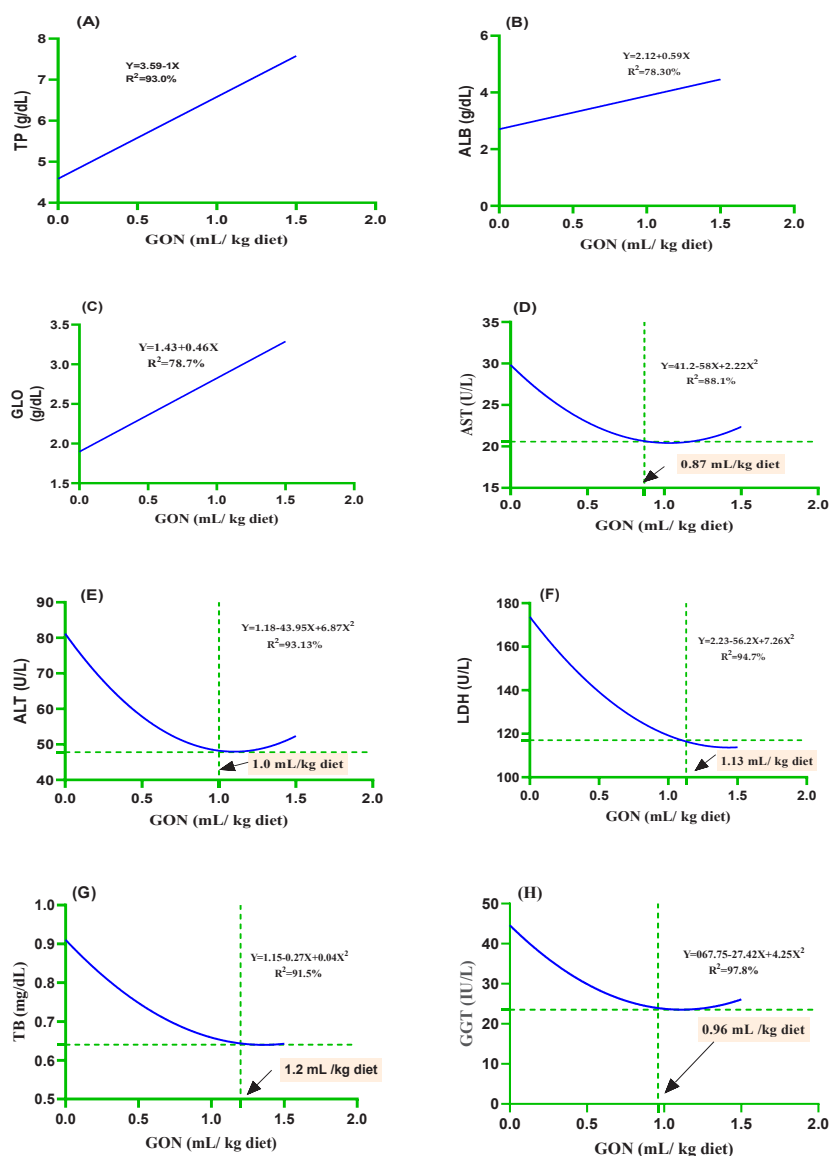


Figure 5 (A–J). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and total protein (A, TP), albumin (B, ALB), globulin (C, GLO), aspartate aminotransferase (D, AST), alanine aminotransferase (E, ALT), lactate dehydrogenase (F, LHD), total bilirubin (G, TB), and gamma-glutamyl transferase (H, GGT)

Table 4. Effects of dietary grapeseed oil nano-emulsion (GON; 0, 0.5, 1.0 and 1.5 mL/kg diet) supplementation on some blood biochemical of growing rabbit under HS condition

Attributes	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1	1.5		TRTs	Lin.	Quad.
1	2	3	4	5	6	7	8	9
TP (g/dL)	4.59 b	5.47 b	6.77 a	7.48 a	0.35	<0.001	<0.001	0.693
ALB (g/dL)	2.61 b	3.26 b	4.20 a	4.25 a	0.22	0.001	<0.001	0.170
GLO, g/dL)	1.91 c	2.27 bc	2.95 b	3.23 a	0.18	0.003	<0.001	0.838
A/G ratio (g/dL)	1.41	1.45	1.47	1.32	0.05	0.860	0.595	0.528
AST (IU/L)	30.20 a	21.81 b	21.51 b	22.00 b	1.14	<0.001	<0.001	<0.001
ALT (IU/L)	82.48 a	52.13 b	53.99 b	51.07 b	3.99	<0.001	<0.001	<0.001
LDH (IU/L)	172.40 a	143.13 b	115.33 c	115.07 c	7.28	<0.001	<0.001	0.003
GGT (IU/L)	44.66 a	29.66 b	26.00 b	24.00 c	2.47	<0.001	<0.001	<0.001
TB (mg/dL)	0.92 a	0.71 b	0.69 b	0.63 c	0.04	<0.001	<0.001	<0.001
DB (mg/dL)	0.44 a	0.316 b	0.34 b	0.32 b	0.02	<0.001	<0.001	0.003

Table 4 – contd.

1	2	3	4	5	6	7	8	9
Uric acid (mg/dL)	2.78 a	2.43 b	2.44 b	2.38 b	0.05	<0.001	<0.001	0.006
TC (mg/dL)	104.16 a	75.53 b	75.32 b	74.54 b	3.810	<0.001	<0.001	<0.001
TG (mg/dL)	112.76 a	81.01 b	81.40 b	81.17 b	4.171	<0.001	<0.001	<0.001
HDL (mg/dL)	26.04 c	35.00 b	40.15 a	40.56 a	1.820	<0.001	<0.001	0.004
LDL (mg/dL)	40.62 a	33.94 b	31.82 b	32.49 b	1.122	<0.001	<0.001	0.004

GON: grapeseed oil nano-emulsion; HS: the basal diet without GON supplementation. TP: total protein; ALB: albumin; GLO: globulin; A/G: albumin/globulin ratio; ALT: alanine aminotransferase; AST: aspartate aminotransferase; LDH: lactate dehydrogenase; GGT: gamma-glutamyl transferase; TB: total bilirubin; DB: direct bilirubin; TC: total cholesterol; TG: triglycerides; LDL: low-density lipoprotein; HDL: high-density lipoprotein. Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 (GON1.5) mL/kg of diet for 8 weeks. Data presented as mean±total SE. a, b, c – means in the same column bearing different letters are significantly different at $P<0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

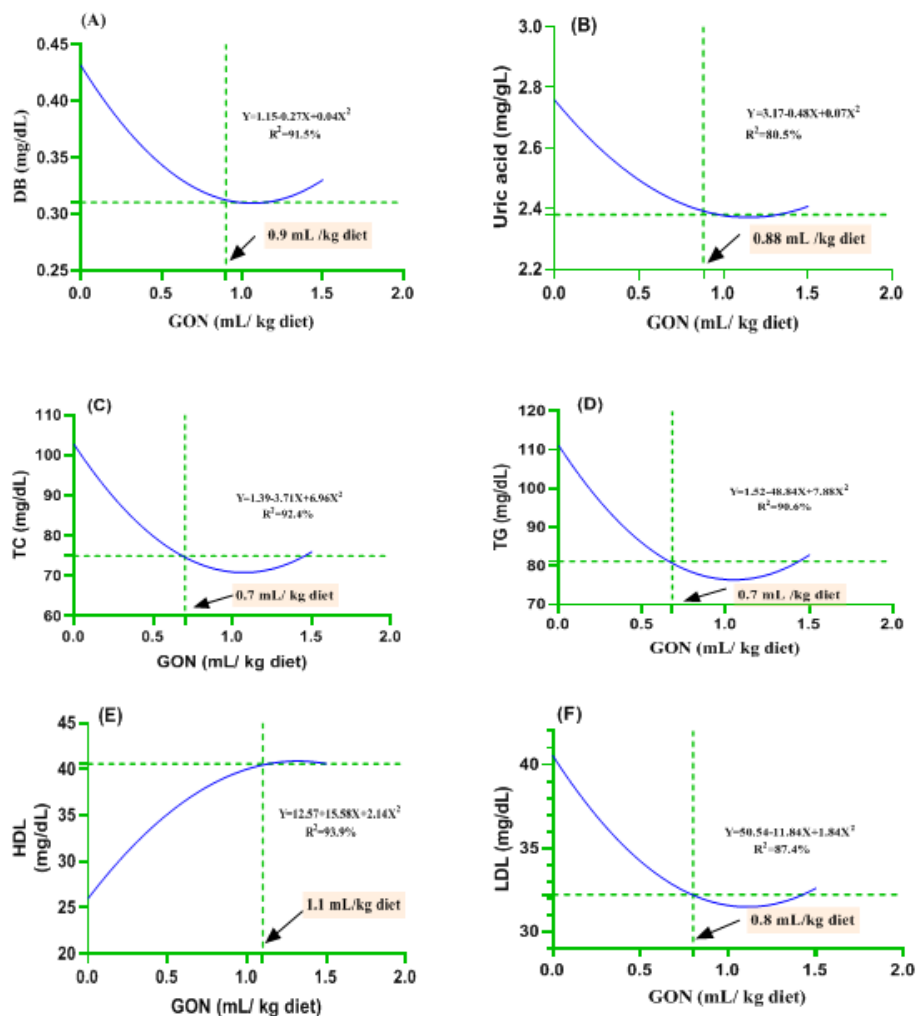


Figure 6 (A–F). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and direct bilirubin (A, DB), uric acid (B), total cholesterol (C, TC), triglycerides (D, TG), low-density lipoprotein (E, LDL), and high-density lipoprotein (F, HDL).

Effects of dietary GON on redox status

The oxidative biomarkers in growing rabbits exposed to heat stress are presented in Table 5. The GON supplements had a significant quadratic impact (except for TAC, linear effect) on all oxidative biomarkers in serum ($P<0.001$). Compared to the control group (HS), the TAC values exhibited a significant linear improvement

at levels of 1 and 1.5 mL/kg in the diet (Figure 7 A). SOD, CAT and GPx levels were significantly increased in a quadratic manner with the supplementation of GON ($P<0.001$). At the same time, PC and MDA were significantly decreased in a quadratic manner ($P<0.001$). The catalase enzyme was also boosted with the lower dose of dietary GON (0.5 mL/kg). On the other hand, all tested

levels of GON resulted in a significant decrease in MDA and PC levels ($P<0.001$ and $P=0.001$, respectively). The optimal GON levels were 0.78, 1.1, 0.3, 1.13 and 0.9 mL/

kg of diet for SOD (Figure 7 B), GPx (Figure 7 C), CAT (Figure 7 D), MDA (Figure 7 E), and PC (Figure 7 F), respectively.

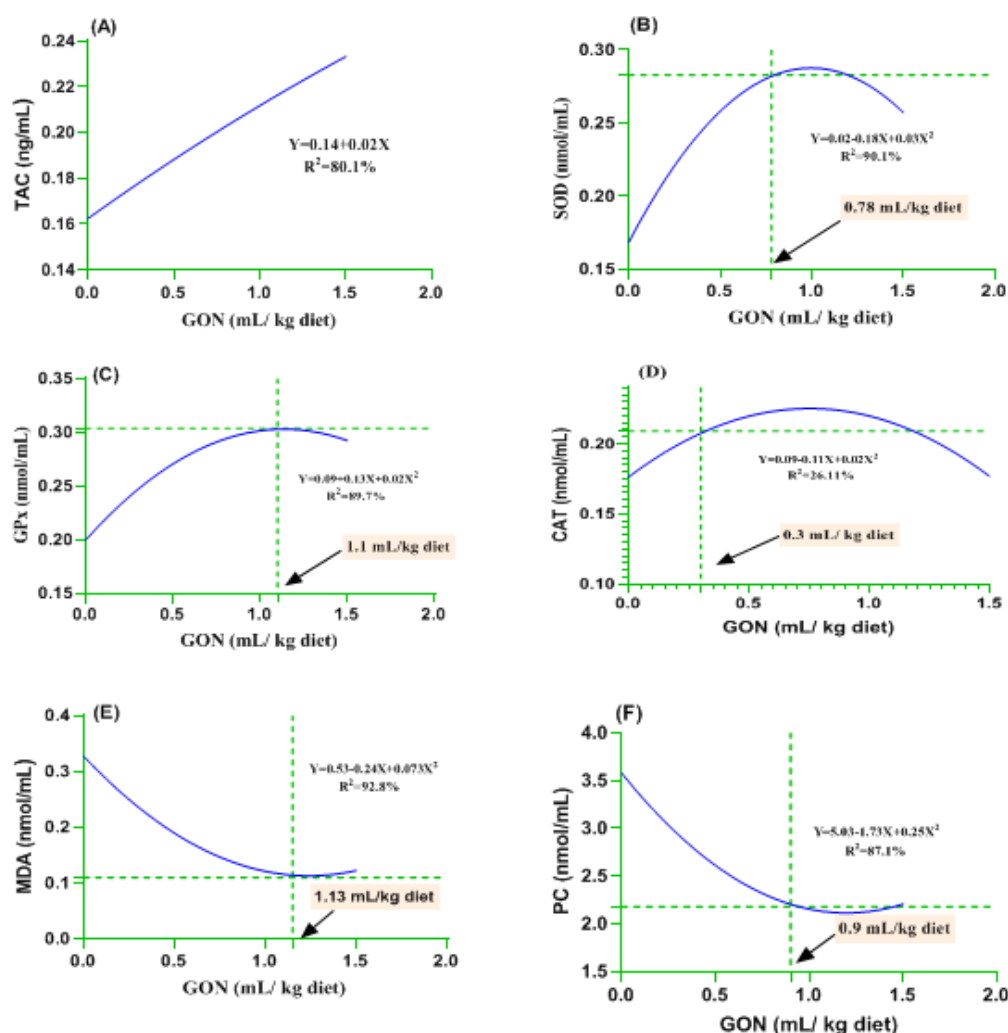


Figure 7 (A–F). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and total antioxidant capacity (A, TAC), superoxide dismutase (B, SOD), glutathione peroxidase (C, GPx), catalase (D, CAT), malondialdehyde (E, MDA), and protein carbonyl (F, PC)

Table 5. Effects of dietary grapeseed oil nano-emulsion (GON; 0, 0.5, 1.0 and 1.5 mL/kg diet) supplementation on redox status of growing rabbit under HS condition

Attributes	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1	1.5		TRTs	Lin.	Quad.
TAC (ng/mL)	0.17 b	0.18 b	0.22 a	0.23 a	0.01	<0.001	<0.001	0.788
SOD (nmol/mL)	0.16 b	0.270 a	0.270 a	0.26 a	0.01	<0.001	<0.001	<0.001
GPx (nmol/mL)	0.20 b	0.29 a	0.29 a	0.30 a	0.01	<0.001	<0.001	<0.001
CAT (nmol/mL)	0.16 b	0.26 a	0.18 b	0.19 b	0.01	<0.001	0.981	0.008
MDA (nmol/mL)	0.33 a	0.17 b	0.14 b	0.12 b	0.03	<0.001	<0.001	0.001
PC (nmol/mL)	3.59 a	2.58 b	2.19 b	2.20 b	0.18	0.001	<0.001	0.011

GON: grapeseed oil nano-emulsion; HS: the basal diet without GON supplementation. TAC: total antioxidant capacity; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; PC: protein carbonyl; MDA: malondialdehyde. Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 (GON1.5) mL/kg of diet for 8 weeks. Data presented as mean±total SE. a, b, c – means in the same column bearing different letters are significantly different at $P<0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

Table 6. Effects of dietary grapeseed oil nano-emulsion (GON; 0, 0.5, 1.0 and 1.5 mL/kg diet) supplementation on immunity status of growing rabbit under HS condition

Attributes	GON (mL/kg diet)				SEM	P-values		
	HS	0.5	1	1.5		TRTs	Lin.	Quad.
IgG (ng/ml)	28.35 b	54.48 a	54.01 a	53.64 a	3.40	<0.001	<0.001	<0.001
IgM (ng/ml)	41.09 b	77.38 a	79.23 a	80.36 a	4.99	<0.001	<0.001	<0.001
IL-6 (ng/mL)	0.29 a	0.13 b	0.12 b	0.12 b	0.03	<0.001	<0.001	<0.001
IFN- γ (ng/ml)	0.31 a	0.18 b	0.15 c	0.15 c	0.02	<0.001	<0.001	<0.001
IL-10 (ng/ml)	0.18 b	0.28 a	0.29 a	0.29 a	0.01	<0.001	<0.001	<0.001
NO (μ g/ml)	0.23 b	0.49 a	0.50 a	0.50 a	0.04	<0.001	<0.001	<0.001
Lysosome activity (μ U/ml)	0.29 a	0.18 b	0.18 b	0.18 b	0.02	<0.001	<0.001	0.001

IFN- γ : interferon-gamma; IgG: immunoglobulins G; IgM: immunoglobulins M; NO: nitric oxide; IL-6: interleukin 6; IL-10: interleukin 10. Growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (HS group), 0.5 (GON0.5), 1.0 (GON1.0) and 1.5 (GON1.5) mL/kg diet for 8 weeks. HS: the basal diet without GON supplementation. Data presented as mean $\pm$ total SE. a, b, c – means in the same column bearing different letters are significantly different at $P<0.05$. TRTs, treatment; Lin, linear regression; Quad, quadratic regression.

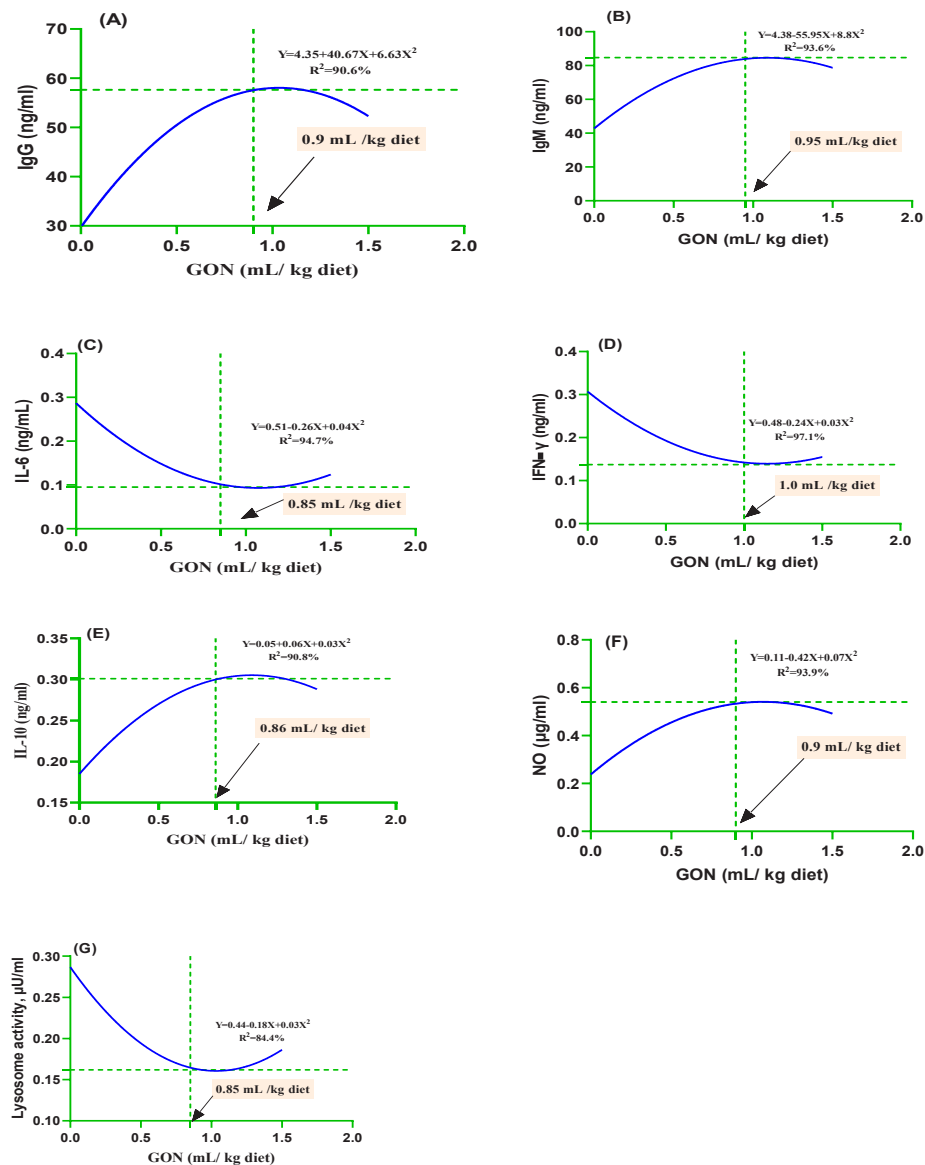


Figure 8 (A–G). A polynomial regression analysis between various levels of grapeseed oil nano-emulsion (GON) and IgG (A), IgM (B), interleukin-6 (C, IL-6), interferon-gamma (D, IFN- γ), interleukin-10 (E, IL-10), nitric oxide (F, NO), and lysosome activity (G)

Effects of dietary GON on immunity and inflammatory responses

Table 6 displays the immune indicators in the serum of rabbits supplemented with GON under heat stress conditions. Incorporating GON in rabbits' diets significantly affected all the measured immune indicators in serum ($P < 0.001$). The levels of IgG, IgM, IL-10, and NO were significantly increased ($P < 0.001$) with all supplemental levels of GON in a quadratic manner. In contrast, the levels of IL-6, IFN- γ , and lysozyme activity (LZM) were significantly suppressed ($P < 0.001$) by adding GON to rabbits' diets. All supplemented groups exhibited improvement in IgM and IgG in a quadratic manner, with the optimum dose of GON being 0.9 mL/kg of diet (Figure 8 A) and 0.96 mL/kg of diet (Figure 8 B), respectively. The same trend was observed in IL-10 (Figure 8 E) and NO (Figure 8 F). The values of IL-6 (Figure 8 C), IFN- γ (Figure 8 D), and lysozyme activity (Figure 8 G) were significantly decreased in a quadratic manner with optimum doses of 0.85, 1.0, and 0.85 mL of GON/kg of diet, respectively.

Effects of dietary GON on organ histology

The liver tissues of stressed rabbits (Figure 9 A) showed vacuolation in most hepatocytes, perivascular necrotic areas formed from necrotic debris, leukocyte infiltrates (including prominent giant cells), and hemorrhage. The other groups, where rabbits were fed diets with 0.5 mL/kg (Figure 9 B), 1 mL/kg (Figure 9 C), or 1.5 mL/kg (Figure 9 D) of GON, exhibited centrilobular

hydropic degenerated cells within some hepatic lobules. They showed a preserved majority of hepatic parenchyma with a few hydropic degenerated cells and normal histology of hepatic cords, central veins, and portal triads. The changes in spleen tissues were also reported in this study (Figure 10 A–D). Rabbits in the HS groups (Figure 10 A) showed atrophied white pulp, filled splenic sinusoids with numerous mature and immature lymphocytes, proliferated macrophages, megakaryocytes, and depleted white pulp lymphoid populations with interstitial edema. Additionally, dilated splenic sinusoids with various inflammatory cell types were observed. Rabbits fed diets with various levels of GON (0.5 in Figure 10 B, 1.0 in Figure 10 C, and 1.5 in Figure 10 D) exhibited normal cytostructures of white pulp lymphoid nodules and preserved red pulp, which included sinusoids, reticular fibers, many macrophages, lymphocytes, and megakaryocytes.

The histological changes in the intestines of growing rabbits are presented in Figure 11 (A–D). The rabbits fed the control diet (Figure 11 A) showed signs of necrotic enteritis in some examined sections, characterized by necrotic villus tips, inflammatory cell infiltration within the lamina propria, and dilated submucosal blood vessels. However, preserved muscular layers were detected. In rabbits from the GON0.5 group (Figure 11 B), metaplastic changes were observed in the lamina epithelialis, with numerous goblet cells and intraluminal mucus. Some intestinal glands were destroyed, but the muscular layer appeared normal.

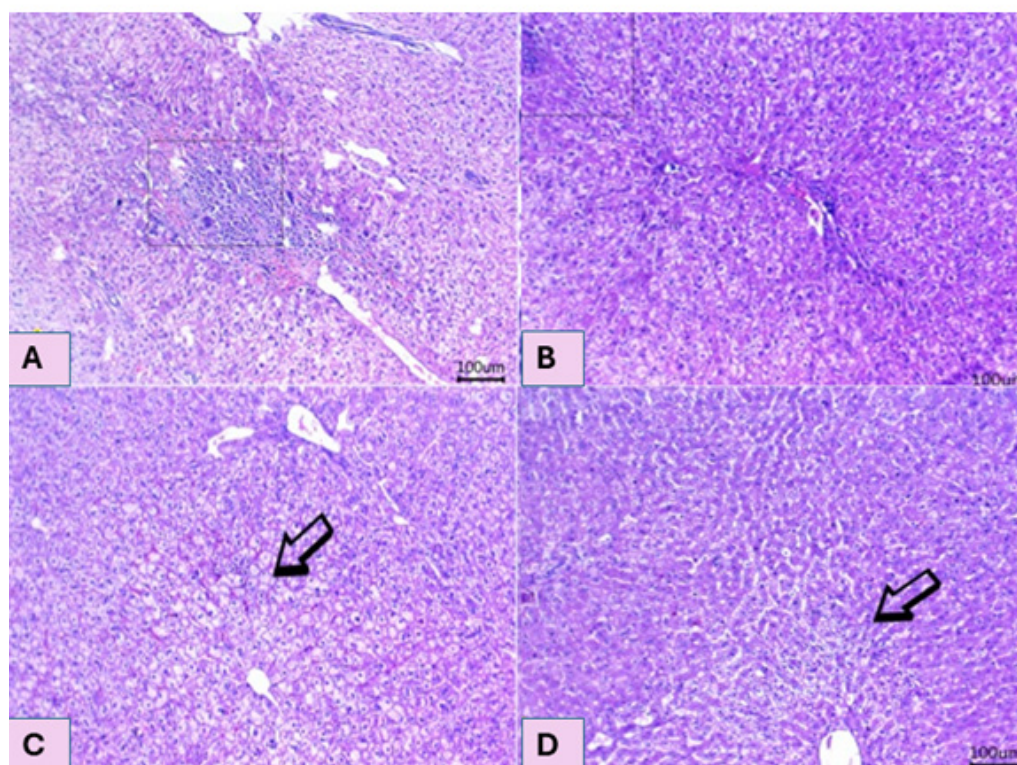


Figure 9 (A–D). The histological changes in the liver samples of growing rabbits exposed to heat stress and fed diets with various levels of grape-seed oil nano-emulsion (GON) 0 (A), 0.5 (B), 1 (C) and 1.5 (D) mL/kg of diet for 8 weeks

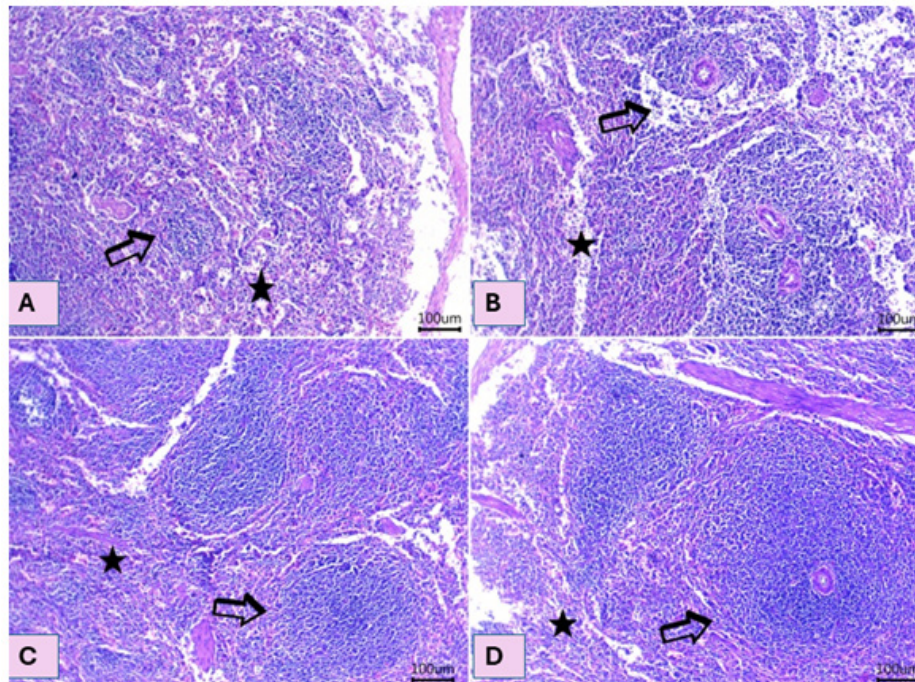


Figure 10 (A–D). The histological changes in the spleen samples of growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (A), 0.5 (B), 1.0 (C) and 1.5 (D) mL/kg of diet for 8 weeks

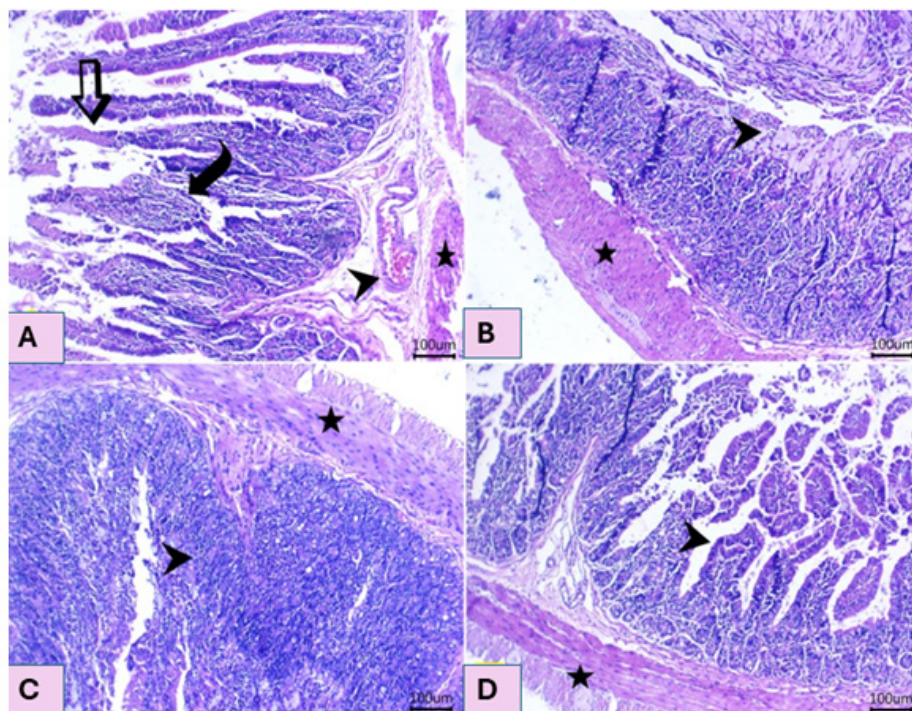


Figure 11 (A–D). The histological changes in the intestinal samples of growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (A), 0.5 (B), 1.0 (C) and 1.5 (D) mL/kg of diet for 8 weeks

In contrast, rabbits from the GON1 (Figure 11 C) and GON1.5 (Figure 11 D) groups also showed metaplastic changes in the lamina epithelialis with numerous goblet cells and intraluminal mucus, as well as destructed intestinal glands and normal musculosa. Additionally, they

exhibited a normal epithelial lining of villi, leukocytic aggregates within the lamina propria, and normal submucosa and musculosa. The histological structures of mucosa, submucosa, inner circular, and outer longitudinal smooth muscle layers were also normal in these groups.

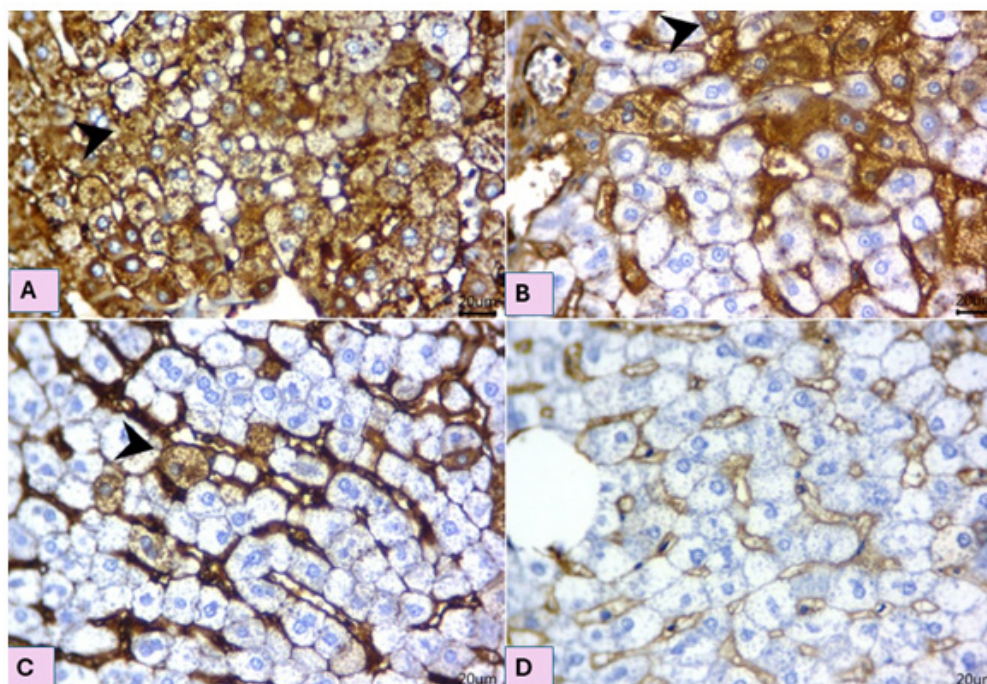


Figure 12 (A–D). Immunohistopathologic of iNOS in hepatic tissues of growing rabbits exposed to heat stress and fed diets with various levels of grapeseed oil nano-emulsion (GON) 0 (A), 0.5 (B), 1.0 (C) and 1.5 (D) mL/kg of diet for 8 weeks

Immune-histological study

The immune expression of iNOS in liver tissues of growing rabbits is shown in Figure 12 (A–D). Rabbits fed the control diet and exposed to HS (Figure 12 A) exhibited an intense expression of iNOS in hepatic tissues. At the same time, the GON0.5 group showed moderate expression of iNOS in rabbit liver (Figure 12 B). The GON1 (Figure 12 C) and GON1.5 (Figure 12 D) groups displayed lower expression of iNOS in the hepatic tissues.

Discussion

The present findings indicate the capability of using grapeseed oil nano-emulsion (GON) as a byproduct to enhance the growing performance, feed competence, blood health, and organ histology of growing rabbits exposed to environmental stress. Eco-friendly, applicable, safe, and efficient natural byproducts are an alternative to synthetic constituents to reduce environmental pollution. This study's findings clarify that adding GON can serve as a novel anti-heat stress agent for growing rabbits, providing a safer, more environmentally friendly, and sustainable approach. GON (1 or 1.5 mL/kg of diet) significantly improved growth and feed efficiency by promoting immunity (IgM and IgG) and antioxidant (CAT, GPx, SOD, and TAC) responses in stressed rabbits.

Additionally, GON suppressed oxidative markers (MDA and PC) and inflammation induced by high envi-

ronmental temperatures. This positive effect was also evidenced by maintaining organ architecture (liver, spleen, and intestines) and reducing the immune expression of iNOS in liver tissues. In stressed poultry, growth indices of quails (Erişir et al., 2018) and broilers (El-Damrawy, 2014) significantly increased after dietary supplementation with GSE. Recently, feeding calves with 4 g/d of GSE per animal significantly improved body weights, nutrient digestibility, and feed intake (Ma et al., 2023). Grape seeds have been used as a growth promoter in poultry (Abdel-Wahab et al., 2018), due to their antioxidant and antimicrobial effects. In rabbits, Hassan et al. (2016) indicated that dietary supplementation of GSE (200 or 300 mg/kg diet) significantly improved the growth performance during heat stress conditions. The improvement in growth shown in this experiment may be attributed to the ability of GON to enhance appetite, increase digestive enzymes (Ma et al., 2023), and boost nutrient digestibility (Urkmez and Biricik, 2022). Another reason is that GON has antimicrobial action by reducing pathogenic bacteria and increasing beneficial bacteria (Kitsiou et al., 2023) in the intestinal cavity of rabbits. GSE is rich in phenolic components and can be an efficient anti-heat stress, immunomodulatory and antioxidant mediator (Choi et al., 2023). The antioxidant activity of GSE is attributed to various mechanisms, such as the ability of polyphenols to scavenge free radicals.

Environmental stress, such as HS, can lead to changes in the hematology profile, potentially causing anemia (Imbabi et al., 2023). The synthesis of RBCs in the red

bone marrow is crucial for providing tissue oxygen. However, the elevated cellular temperature can reduce RBC synthesis, leading to oxygen deficiency and anemia. Numerous studies have supported this hypothesis. In our study, the GON significantly improved the counts of erythrogram parameters (except for MCV and MCHC) in growing rabbits and significantly reduced the leukocytes and their differentials. Calves fed with GSE (25–100 mg/kg BW) had better levels of erythrogram parameters such as hematocrit ($P<0.001$), RBC count ($P<0.001$), and hemoglobin concentration. However, the WBC, lymphocyte, and monocyte counts did not change significantly (Urkmez and Biricik, 2022). Moreover, calves fed GSE had a lower neutrophil count compared to CON. The improvement in the erythrogram may be attributed to the anticoagulant effect of grapeseed extract via increasing the prothrombin time (Bijak et al., 2011). In broiler, Hajati et al. (2018) found that adding GSE (300 and 450 mg/kg diet) significantly improves the RBCs and hemoglobin while decreasing lymphocyte in broiler exposed to chronic HS. This anticoagulant effect (Bijak et al., 2011) may enhance blood flow in the veins, thereby increasing heat loss to the environment through evaporative cooling in growing rabbits (El-Raghi et al., 2023; Imbabi et al., 2024). GON can mitigate the negative effects of HS on blood hematology by increasing the count of RBCs, Hb, PLT, and Ht. According to several studies (Bashar et al., 2024; El-Raghi et al., 2023; Ismail et al., 2023), the use of natural essential oils significantly improved erythrogram variables and decreased the counts of WBC in growing rabbits. Dietary GSE (200 or 300 mg/kg diet) increased the percentage of hot carcass, carcass weight, intestine and edible giblets, while total non-edible parts of stressed rabbits were diminished compared to the standard group (Hassan et al., 2016). Lipid parameters (TG, LDL, HDL) in the serum of stressed broiler fed diets with GSE (200 or 300 mg/kg) were significantly diminished compared to the control group (El-Damrawy, 2014). TG levels were significantly improved in calves fed diets with GSE (4 g/day/animal) (Ma et al., 2023). Administration of GSE significantly reduced the plasma concentrations of TC, total lipids, TG and LDL in stressed rabbits (Hassan et al., 2016).

Serum biochemistry is an important factor associated with the health of animals, as it reflects the physiological metabolism of the body and changes in various organ functions. Feeding stressed rabbits with 200 or 300 mg of GSE significantly increased the TP, ALB and GLU compared to the control group (Hassan et al., 2016), as reported in our current research. Thermal stress can change biological blood variables, with enzymes like AST and ALT being released into the bloodstream due to tissue damage. In this study, high temperatures caused a significant rise in serum ALT and AST levels in growing rabbits, indicating oxidative injury in renal tissues and hepatocytes. In this study, plasma glucose, TC, triglyceride, AST, and ALT levels in quail under heat stress decreased with GS supplementation. Abu Hafsa and

Ibrahim (2018) observed that GS extract reduced plasma glucose, total lipids, triglyceride, and cholesterol levels without affecting ALT and AST levels. Previous research demonstrated GSO's hepatoprotective and nephroprotective effects (Kumar et al., 2024; Ulusoy et al., 2012).

Additionally, plasma TG, TC, and glucose levels in quail increased in response to high ambient temperatures (Abdel-Wahab et al., 2018; Erişir et al., 2018). Elevated TC and glucose levels were linked to high plasma corticosterone levels induced by HS. However, plasma cholesterol levels were substantially boosted in this study, while glucose levels remained normal under heat stress. Chronic stress in rabbits has been associated with stress-induced hypoglycemia (Miinalainen et al., 2009), attributed to the depletion of hepatic glycogen stores due to increased glucose demand and glycolysis. Studies have revealed that heat stress can trigger homeostasis dysfunction by increasing lipid and protein oxidations and decreasing antioxidant enzymes such as SOD, GPx, and CAT in cellular systems. Enhancing diets with natural antioxidants can help combat free radicals, neutralize oxidants, and boost antioxidant capacity. In our study, dietary supplementation with GON led to a significant increase in the levels of CAT, SOD, GPx, and TAC in growing rabbits. Like our data, Hassan et al. (2016) observed that stressed rabbits fed diets with GSE (200 or 300 mg/kg diet) had higher levels of GPx, SOD, CAT and lower values of MDA.

Additionally, GON supplementation significantly reduced PC and MDA levels. These conclusions are reliable with research by Urkmez and Biricik (2022), which showed that stressed calves given varying doses of grapeseed extract (25–100 mg/kg BW) had greater levels of TAC, SOD, as well as lower MDA levels compared to the control group. In contrast, the levels of GSH-Px, CAT, and IL-1 α in the serum of calves fed with GSE were not affected. Supplementing diets with GSE (200 mg/kg) significantly improves liver SOD and GSH levels, while decreasing MDA in stressed broiler chickens (El-Damrawy, 2014). Additionally, Erişir et al. (2018) found that 10 g of GSE significantly improved GSH and CAT levels, while significantly reducing MDA in kidney and liver tissues of stressed quails (16 h at 34°C, 8 h at 22°C). Moreover, calves fed diets with 4 g/animal/day of GSE significantly improved the antioxidant enzymes such as CAT and SOD (Ma et al., 2023). Likewise, the serum concentrations of TG, CAT, SOD, Ig G and IgM were remarkably greater in the GSE group than those in the CON group in calves as shown in the previous study.

Reactive oxygen species (ROS) are essential in cell death pathways. Excessive ROS production can damage organelles and biomolecules, leading to an inflammatory response contributing to heat stress development. Plasma IFN- γ concentration was diminished in calves fed GSE (25 mg/kg BW) compared to other groups (Urkmez and Biricik, 2022). Additionally, significant improvements were observed in IgM and IgG levels in the serum of calves following GSE supplementation, and there was

also a considerable reduction in the levels of MDA (Ma et al., 2023). The same authors found that the levels of IL-6 and TNF- α were significantly decreased in the calves fed 4 g of GSE/day. Rabbits are susceptible to various health issues that can impact their well-being, such as immune dysfunction and HS (Bashar et al., 2024). Weakened immune systems can make rabbits more vulnerable to infections, diseases, and parasites, reducing their ability to fight off threats effectively. Additionally, rabbits are particularly sensitive to high temperatures, and heat stress can lead to dehydration, hyperthermia, and even organ failure (El-Raghi et al., 2023; Ismail et al., 2023).

Addressing environmental immune issues and providing a comfortable, temperature-regulated environment are crucial for maintaining the health and welfare of pet rabbits. GON has several compounds, including epicatechin gallate, catechin, procyanidins, and gallic acid (Shamiah et al., 2024). Flavonoids have recently been considered because of their frequent therapeutic effects, such as anti-inflammatory, antibiotic, antidiarrheal, and high antioxidant role (Xie et al., 2015). GSO is known to act as a powerful natural antioxidant and metal chelator (Choi et al., 2023), scavenging free radicals initiated by OS, due to the rich polyphenols and flavonoids (de Oliveira et al., 2020). Reports above have shown that GS polyphenols encourage the actions of antioxidant enzymes and reduce the effects of OS in lambs (Hassan et al., 2016). Further to its antioxidant actions, GSE have been reported to reduce neutrophil infiltration and standardize the relief of inflammatory intermediaries (Wang et al., 2023).

Environmental HS can damage the tight junction proteins in the gastrointestinal epithelium of animals, leading to strengthened gastrointestinal permeability and the entry of lipopolysaccharides into the bloodstream. This can trigger the release of pro-inflammatory cytokines, causing local and systemic inflammation. Fundamental pro-inflammatory components such as IFN- γ , IL-1 β , and IL-6 regulate inflammatory reactions in the body. Nallathambi et al. (2020) stated that GSE has an anti-inflammatory consequence by lowering intestinal permeability and transcripts of pro-inflammatory cytokines. In this study, dietary supplementation with GON reduced the levels of IFN- γ , lysosome activity, and IL-6 while slightly increasing IL-10 content in the serum of growing rabbits. This indicates that GON helped alleviate inflammatory responses in stressed rabbits.

Previous research on stressed rabbits showed that it could lower IFN- γ and IL-6 levels and mitigate inflammatory responses under HS conditions, which aligns with our results (Bashar et al., 2024; El-Raghi et al., 2023; Ma et al., 2023). Another study suggested that grapeseed products could reduce pro-inflammatory cytokine levels by preventing the NF- κ B signaling pathway (Gessner et al., 2012). The positive effects of GON on regulating inflammatory factors may be attributed to its ability to inhibit the NF- κ B signaling pathway. Future studies should explore the potential mechanisms

through which GON influences calf immunity. IL-10, on the other hand, can inhibit the production of pro-inflammatory factors and their binding to receptors, thereby reducing the damage caused by inflammatory reactions (Terra et al., 2007). The mechanism of its action may be related to the regulation of the JAK/STAT3 signaling pathway (Chu et al., 2024). In our experiment, GON significantly improved the levels of IL-10, thus reducing inflammation. The anti-inflammatory effect of GON may be attributed to the high content of procyanidins (Terra et al., 2007). IFN- γ is a cytokine that is primarily produced by cells of the immune system. Higher levels of IFN- γ in the serum may reflect the occurrence of inflammation, or exposure of the host to adverse environment. In this sense, HS can trigger an increase of IFN- γ and IL-4, as shown in this study and other previous works (Bashar et al., 2024; El-Raghi et al., 2023). Targeting the inflammation process via anti-inflammatory molecules could represent an effective method for reducing the HS effects in animals. After that, GON has a considerable amount of anti-inflammatory molecules such as proanthocyanidin (Chu et al., 2024), procyanidins (Terra et al., 2007), and resveratrol (Mahanna et al., 2019).

To gain insight into how GON can protect organs from HS-induced injuries, we conducted histological analyses of growing rabbits' liver, spleen, and intestines after feeding them with GON. The present study indicates that HS can damage the histology of the liver via vacuolation in most hepatocytes, perivascular necrotic and debris, leukocyte infiltrates (including prominent giant cells), and hemorrhage. Meanwhile, the liver tissues of rabbits fed with GON exhibited normal histological architecture. In the studies by El-Raghi et al. (2023) and Ismail et al. (2023), nano-essential oil protected liver cells in rabbits exposed to heat stress. Moreover, the use of nanomaterials (50 mg of zinc nanoparticles) and algae (1 g/kg diet) can improve the structure of intestinal, and liver samples during HS conditions in rabbits (Bashar et al., 2024). HS can induce spleen and intestinal damage in growing rabbits, while GON improves their structures. The iNOS isoform is typically activated by inflammatory stimuli and produces NO during stressful conditions. A study found that supplementing L-citrulline under thermoneutral conditions increased mice's iNOS expression and NO production. This suggests that natural molecules may have a beneficial effect on NO production in certain conditions. It has been observed that GON supplementation, especially at 1 or 1.5 mL/kg diet, resulted in lower iNOS expression than the stressed group. This indicates that GON can reduce the iNOS expression in hepatic tissues, revealing the mitigating impacts of HS in rabbits. Uyanga et al. (2021) found that the addition of L-citrulline (1% of the basal diet) significantly downregulated the mRNA expression of iNOS in the hypothalamus of broilers exposed to HS (35°C for 5 h). Also, it has been shown that the procyanidin isolated from GSE diminished the expression of iNOS and NF- κ B translocation

(Terra et al., 2007). In supporting our data, Pistol et al. (2023) found that byproducts of GS can alleviate the intestinal syndromes in post-weaning piglets via antimicrobial action.

Conclusion

The study findings suggest that adding grapeseed oil nano-emulsion (GON) can be a new anti-heat stress agent for growing rabbits, offering a safer, more eco-friendly, and sustainable solution. GON (0.8 or 1.2 mL/kg diet) significantly enhanced growth and feed efficiency by boosting immunity (IgM and IgG) and antioxidant responses (CAT, GPx, SOD, and TAC) in stressed rabbits. Moreover, GON reduced oxidative markers (MDA and PC) and inflammation caused by high temperatures. This positive impact was also seen in maintaining organ health (liver, spleen, and intestines) and decreasing iNOS immune expression in liver tissues. The study indicates that GON is a beneficial additive for enhancing growth, blood health, and productivity in stressed rabbits. Further research is needed to explore GON's potential in mitigating animal heat stress effects.

Ethics statement

The animal handling and experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Zagazig (Code ID: ZU-IACUC/2/F/371/2023).

Acknowledgements

The authors extend their appreciation to the Dean-ship of Research and Graduate Studies at King Khalid University for funding this work through Large Research Project under grant number RGP2/342/45.

Funding

The authors extend their appreciation to the Dean-ship of Research and Graduate Studies at King Khalid University for funding this work through Large Research Project under grant number RGP2/342/45.

CRedit authorship contribution statement

Nada Attia and Sameh A. Abdelnour: data curation, formal analysis, methodology, software. Usama M. Abdel Monem, Fatima S. Alaryani, Kamlah A. Majrashi and Bakry Khalil: visualization, writing – original draft. Fatima S. Alaryani, Kamlah A. Majrashi and Sameh A. Abdelnour: writing – review & editing, writing – original draft. Revision and editing Mohammed O. Alshaharni and Mohannad Abuajamieh. All authors revised this version and accepted it for publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests.

Data availability

Data will be made available on request.

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Received: 3 VII 2024

Accepted: 4 XI 2024