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The adaptogenic anti-ageing potential of resveratrol against heat stress-mediated liver injury in aged rats: Role of HSP70 and NF-kB signalling

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ABSTRACT

Heat stress (HS) is a major international problem which has attracted a considerable attention due to its oxidative tissue effects and high morbidity and mortality rates, especially among elderly people. Discovering an effective antioxidant is pivotal for overcoming HS-induced injury. Therefore, the aim of this study was to estimate the hepatic protective effects of orally supplemented resveratrol (RES) against HS-mediated liver injury in young and old male Wistar albino rats. Compared to control rats, RES administered orally at a dose of 20 mg/kg BW for 21 successive days efficiently ameliorated HS-induced oxidative damage by significantly increasing ($P \leq 0.05$) the level of reduced glutathione and glutathione peroxidase, and decreasing the levels of malondialdehyde and TNF-α in hepatic tissue of both young and aged rats. However, level of NF-κB was downregulated significantly in aged rats rather than young rats. Moreover, RES significantly decreased ($P \leq 0.05$) the serum levels of aspartate transaminase and alkaline phosphatase in both ages of rats compared to their corresponding HS-stressed rats. Furthermore, RES upregulated the immunohistochemical expression of caspase 3 and heat shock protein 70 in young and aged rats, however it was more pronounced in young one. In addition, RES administration moderately normalized ($P \leq 0.0001$) the harmful effects of HS on the hepatic architecture of both young and aged rats. In conclusion, this study reveals for the first time that RES exerts promising hepatoameliorative effects against HS-induced oxidative stress in the young and aged rats via its antioxidant, anti-inflammatory, and anti-apoptotic effect, as well as via its inhibitory effect against the NF-κB signalling in a cellular system.

1. Introduction

Warming of the global climate has become an obvious and notable phenomenon, particularly after the recorded increase in the mean global temperature of 0.74 °C between 1906 and 2005 (Pachauri et al., 2014). Although humans and tropical animals are usually able to adapt to minimal changes in the temperature of their surrounding environment, the relative length and severity of heat waves have increased with the rise in the mean global temperature (Perkins et al., 2012). Prolonged exposure to environmental heat stress (HS) may lead to significant health problems, especially in elderly individuals, even after excluding the presence of overt cardiovascular disturbances; the majority of reported deaths during heat exposure may occur among the elderly (Schmeltz et al., 2016).

Along with advancements in therapeutic strategies for treating ageing-related diseases, the global elderly population continues to grow rapidly. As such, an increasing number of individuals will experience health problems or even death if the global climate continues to warm. Heat stroke is a common heat-related health problem in aged individuals, particularly during the summer. Severe hyperthermia is the main cause of heat stroke; heat stroke usually causes damage to several organs, such as the liver, kidneys, and brain (Margolis, 2014), which may show various lesions, such as thrombi and infarcts, and heat stroke can even cause death.

Both animals and humans possess many chemical and physiologic adaptive mechanisms against thermal exposure to protect the functions of different cells and allow efficient recovery from moderate hyperthermia (Belhadj Slimen et al., 2014; Bunker et al., 2016). However, the response and sensitivity of tissues to such heat-related injury differ among organs. Concerning the liver, thermal injury could activate

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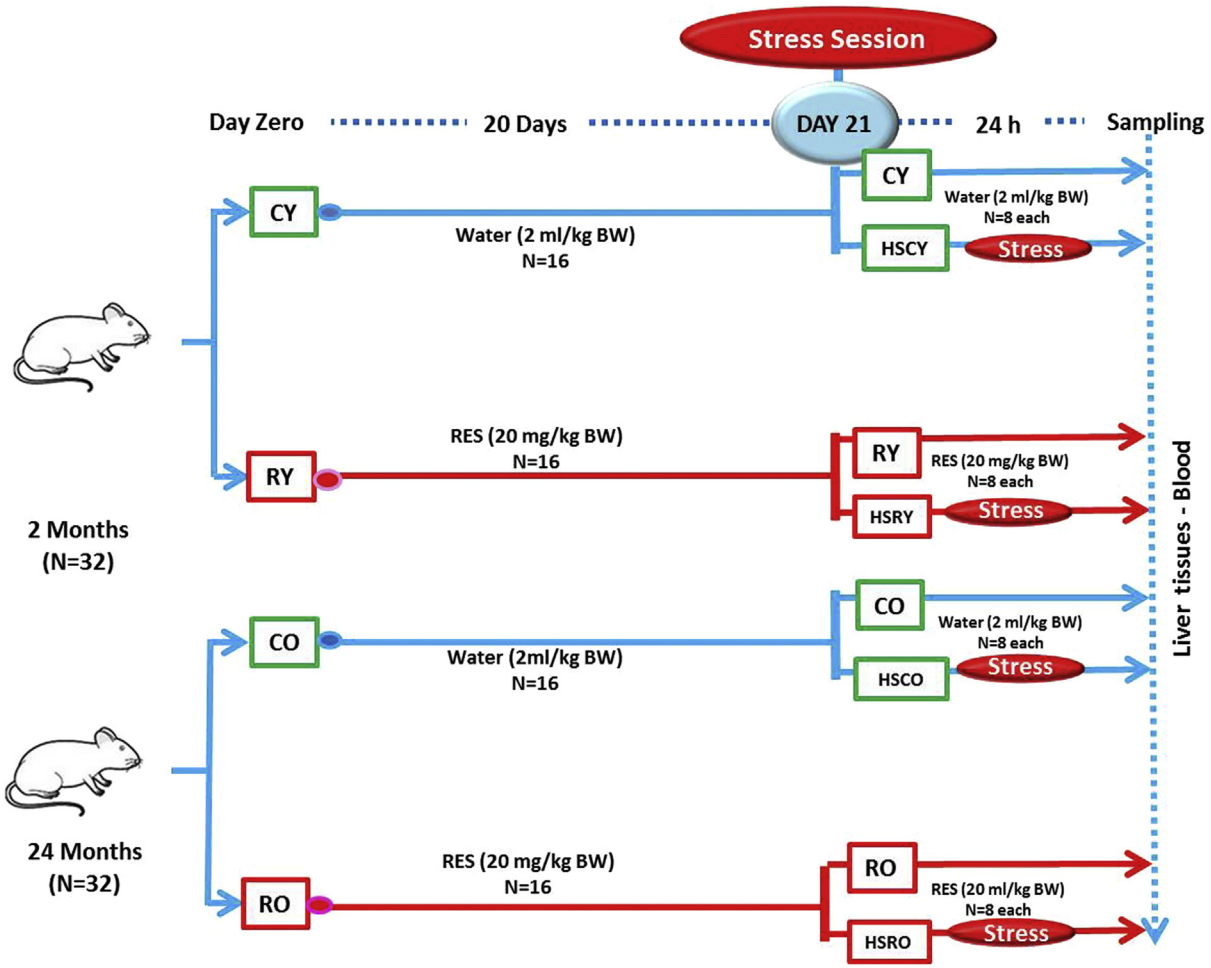

Fig. 1. Schematic summary of the study protocol. Young and old rats were allocated into four groups: control young (CY, receiving 2 ml water/kg BW daily for 21 days), resveratrol (RES)-supplemented young (RY, receiving 20 mg RES/kg BW daily for 21 days), control old (CO, receiving 2 ml water/kg BW daily for 21 days), and RES-supplemented old (RO, receiving 20 mg RES/kg BW daily for 21 days). On day 21, rats in each group were equally divided into another groups, one of which was exposed to heat stress (HSCY, HSRY, HSCO, HSRO) while the other was not (HCY, HRY, HCO, HRO). After 24 h, the rats were anesthetized and euthanized, and blood and liver samples were collected. RES was administered 1 h before the stress session.

nuclear factor-kappa B (NF-κB) with the subsequent production of proinflammatory cytokines, such as tumour necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) (Schwabe et al., 2001); this activation of NF-κB would lead to the inflammation and massive necrosis of hepatic tissues, as well as the increased activation of hepatic stellate cells (HSCs) and subsequent fibrosis (Shen et al., 2014). Therefore, thermal-induced injury may be ameliorated via the modulation of oxidative and inflammatory damage in the liver.

Thus, the identification of efficient antioxidants and anti-inflammatory agents for use against HS is essential. Resveratrol (RES; 3,4,5 trihydroxystilbene) is among the naturally occurring phytoalexins, which are polyphenolic compounds commonly present in various plants, such as peanuts, grapes, and berries. RES has been successfully used to treat cardiac, neural, diabetic, and asthmatic diseases (Saiko et al., 2008), additionally, RES plays a role in the amelioration of acetaminophen-induced hepatotoxicity (Şener et al., 2006). Recently, RES has become commonly used as a potent anti-ageing dietary supplement (Ramis et al., 2015). Interestingly, it has been found that RES can activate the production of heat shock promoters, such as heat shock protein 70 (HSP70), in several cell lines (Morimoto, 1998) and can induce mild to moderate HSP upregulation as a response to lethal thermal shock (Putics et al., 2008).

This study was designed to investigate the potential protective ability of orally supplemented RES against HS-mediated injury in terms of liver function and structure in young and aged rats, with special insight into the possible mechanism(s) of action.

2. Materials and methods

2.1. Chemicals

Pure RES (3, 4, 5 trihydroxystilbene; 100 mg/serving; Cat. NEL-1291, NeoCell Co., Irvine, California, United States) was suspended in water and shaken well before oral dosage. Kits for ALT and AST (Cat. AT 1034), ALP (Cat. AP 1020), MDA (Cat. MD 2529), SOD (EC 1.15.1.1, Cat. SD 2521), GSH (Cat. GR 25 11), and GSH-Px (EC 1.11.1.9, Cat. GP 2524) were purchased from Biodiagnostic, Egypt. NF-kB p50/65 EZ-TFA Transcription Factor (Cat. 70–510) was obtained from Billerica, USA. Kits for TNF-α (Cat. ab208348) and HSP70 mouse monoclonal antibody (Abcam, Cat. ab5442) were purchased from Abcam, Cambridge, UK). All reagents used in the experiments were of analytical grade and of the highest purity.

2.2. Animals

A total of 64 male Wistar albino rats (32 rats 2 months of age (average weight 150–180 mg) and 32 rats 24 months of age (average weight 520–570 mg)) were obtained from the Animal House, Medical

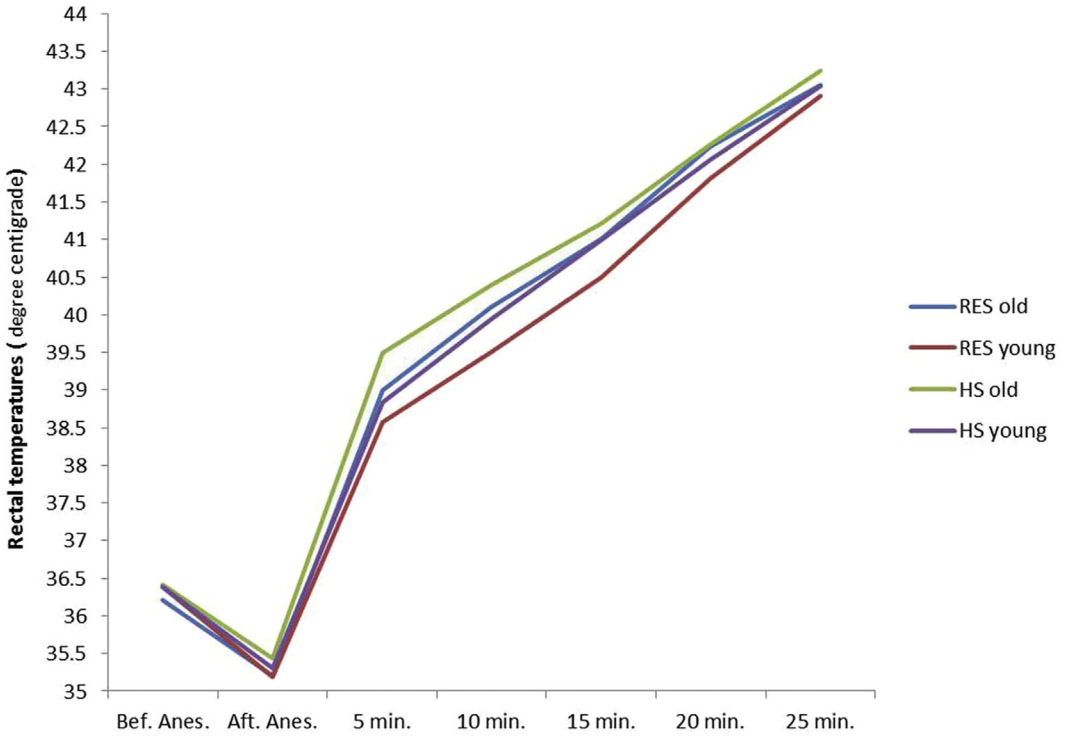

Fig. 2. The mean total time of heat stress (HS) and the mean rectal temperature of young and old rats in the HS and resveratrol (RES)-pretreated groups during and before the HS session. Bef. Anes: before anesthesia; Aft. Anes: after anesthesia.

Table 1
Effect of resveratrol (RES), heat stress (HS), and their combination on serum levels of hepatic enzymes of male Wistar albino rats.

Parameter	Age	CTR	RES	HS	RES + HS
ALT (U/L)	Young	38.13 ± 2.40b	37.00 ± 1.79b	173.25 ± 6.40a	170.25 ± 6.46a
	Old	40.56 ± 1.99b	38.63 ± 1.22b	177.50 ± 4.69a	175.13 ± 5.51a
AST (U/L)	Young	37.25 ± 2.94 cB	40.50 ± 1.75 cB	123.38 ± 5.51 aB	100.38 ± 3.40bB
	Old	59.50 ± 2.40 cA	60.69 ± 2.51 cA	188.13 ± 7.90 aA	171.63 ± 4.40bA
ALP (U/L)	Young	159.13 ± 7.60c	165.50 ± 7.12c	263.50 ± 6.90a	225.75 ± 6.83b
	Old	149.25 ± 5.74c	164.25 ± 4.22c	269.13 ± 5.15a	235.88 ± 9.31b

All values are expressed as the mean ± SEM. Mean values bearing different small letters within the same row are significantly different ($P < 0.05$). While mean values bearing different capital letters within the same column are significantly different at ($P < 0.05$) CTR: control; RES: resveratrol; HS: heat stress; ALT: alanine transaminase; AST: aspartate transaminase; ALP: alkaline phosphatase.

Table 2
Effect of resveratrol (RES), heat stress (HS), and their combination on the oxidative/antioxidative parameters of male Wistar albino rats.

Parameter	Age	CTR	RES	HS	RES + HS
MDA (ng/mL)	Young	1.40 ± 0.16b	1.49 ± 0.16b	2.91 ± 0.13 aB	1.73 ± 0.12b
	Old	1.62 ± 0.16b	1.84 ± 0.10b	3.34 ± 0.15 aA	1.95 ± 0.11b
SOD (u/mg protein)	Young	912.88 ± 46.47 aA	908.75 ± 42.53 aA	613.13 ± 14.73bA	686.25 ± 17.90bA
	Old	756.25 ± 24.85 aB	782.50 ± 29.99 aB	403.75 ± 20.78bB	410.00 ± 24.35bB
GSH nmol/L	Young	19.88 ± 1.49 aA	19.25 ± 1.67 aA	7.46 ± 0.74 cA	12.38 ± 1.29bA
	Old	15.31 ± 0.99 aB	14.13 ± 0.74 aB	3.25 ± 0.55 cB	5.83 ± 0.70bB
GSH–Px (nmol/mg protein)	Young	260.00 ± 18.59bA	309.63 ± 17.06 aA	157.75 ± 9.29d	212.88 ± 11.98c
	Old	192.13 ± 6.99 aB	191.88 ± 5.26 aB	136.88 ± 19.11b	181.75 ± 10.04a

All values are expressed as the mean ± SEM. Mean values bearing different small letters within the same row are significantly different ($P < 0.05$). While mean values bearing different capital letters within the same column are significantly different at ($P < 0.05$) CTR: control; RES: resveratrol; HS: heat stress; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: reduced glutathione; GSH-Px: glutathione peroxidase.

Research Institute, Alexandria University, and kept in the experimental laboratory of the Pathology Department, Faculty of Veterinary Medicine, Alexandria University, Egypt. Animals were kept in galvanized metal cages under controlled conditions (22 ± 1 °C, humidity 60 ± 2%, natural dark-light cycle) with free access to a standard rodent diet (crude Protein (23%), crude fat (3.0%), crude fiber (7.0%), acid insoluble ash (8%), calcium (1–2.5%), phosphorus (0.9%), sodium (0.5–1%), and moisture (12%) and water.

All experimental procedures were performed in accordance with the rules set by the Institutional Guidelines for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Alexandria University, Egypt (approval code: # 06) for year 2018. All efforts were made to minimize animal suffering during the experimental and sampling periods.

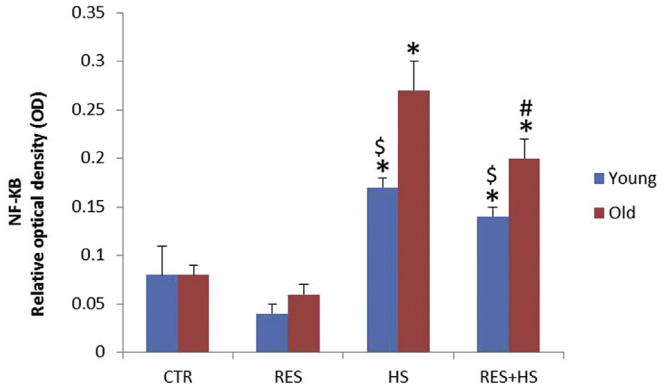

Fig. 3. Effect of resveratrol (RES), heat stress (HS), and their combination on nuclear factor-kappa B (NF-κB) in male Wistar albino rats; all values are expressed as the mean ± SEM, n = 8. The marked mean values are significantly different from those of the control ($^{*}P > 0.05$), HS ($^{\#}P > 0.05$), and old rats ($^{\$}P > 0.05$), as determined by a GLM using a 4 × 2 factorial design with Duncan's post hoc test for multiple comparisons.

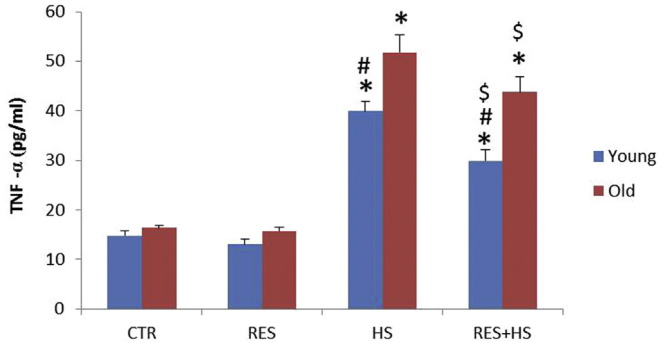

Fig. 4. Effect of resveratrol (RES), heat stress (HS), and their combination on tumour necrosis factor alpha (TNF-α) in male Wistar albino rats; all values are expressed as the mean ± SEM, n = 8. The marked mean values are significantly different from those of the control ($^{*}P > 0.05$), HS ($^{\#}P > 0.05$), and old rats ($^{\$}P > 0.05$), as determined by a GLM using a 4 × 2 factorial design with Duncan's post hoc test for multiple comparisons.

2.3. Induction of HS

Rats were randomly allocated into four groups: (1) control young (CY, 16 young rats received water 2 ml/kg BW/day); (2) control old (CO, 16 old rats received water 2 ml/kg BW/day); (3) RES-supplemented young (RY, 16 young rats supplemented with RES 20 mg/kg BW/day); and (4) RES-supplemented old (RO, 16 old rats supplemented with RES 20 mg/kg BW/day). RES was administered daily by oral gavage for 21 days (Sinha et al., 2002). The choice of RES dose was based on (Schmatz et al., 2012) study; in which treatment of rats with 20 mg/kg of RES provide efficient protection against oxidative damage in liver. Moreover, this dose was concluded as safe dose for all chemical, haematological, and biological functions of rats (Juan et al., 2002). On the 21st day, eight rats from each group were anesthetized via an intraperitoneal injection of sodium pentobarbital (50 mg/kg) 1 h after RES dosage and exposed to HS according to the procedure described by Meyer and Silva (1998). Simply, rats were secured to a supporting device and then placed in a warm water bath at 45 °C. The rectal temperature of each individual rat was monitored before and after anesthesia, and then monitored regularly every 5 min using a clinical glass thermometer. Thermometer probe was gently inserted intrarectal to a length of 3–4 cm until a stable reading was obtained or for up to 20 s; rats were manually restrained at the base of the tail and the thermometer probe was dipped into medical Vaseline before inserting. Monitoring of rectal temperature repeated each 5 min until the temperature reached around 42 °C for all rats, then, rats were kept within the water bath for an additional 5 min and the final temperature was monitored; the mean total time for the stress session was 25 min. After 24 h, all rats were lightly anesthetized with ether, and blood samples were collected from the medial eye canthus. Subsequently, the rats were humanely euthanized, and liver samples were collected (Fig. 1).

2.4. Evaluation of hepatic enzyme levels in serum

Serum samples were prepared from the collected blood samples by centrifugation for 10 min at 3000 rpm, and liver function was qualified via estimation of the serum levels of alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) using commercially available kits (Cat. AT 1034 (45) and AP 1020, respectively, Biodiagnostic, Egypt) according to the manufacturer's instructions.

2.5. Evaluation of oxidative damage in liver tissue

Lipid peroxidation was estimated as the malondialdehyde (MDA) level in the liver tissue homogenate determined using commercially available colorimetric assay kits (Cat. MD 2529, Biodiagnostic, Cairo, Egypt). The principle of this assay is based on the reaction between MDA and thiobarbituric acid, which yields a pink-coloured complex; the absorbance at 532 nm was determined (Mihara and Uchiyama, 1978). The activity of superoxide dismutase (SOD, EC 1.15.1.1) was estimated colorimetrically using commercially available kits (Cat. SD 2521, Biodiagnostic, Cairo, Egypt); SOD activity was evaluated by the inhibition of nitroblue tetrazolium dye reduction by phenazine methosulphate enzyme (Sun et al., 1988). Moreover, the hepatic content of reduced glutathione (GSH) was colorimetrically measured using an assay (Cat. GR 25 11, Biodiagnostic, Cairo, Egypt) that relies on the GSH-mediated reduction of 5,5′-dithiobis(2-nitrobenzoic acid), resulting in a yellow-coloured complex; the absorbance at 405 nm was estimated within 15 min (Adams et al., 1983). Glutathione peroxidase (GSH-Px, EC 1.11.1.9) activity was evaluated in accordance with previously described methods (Paglia and Valentine, 1967) using laboratory-supplied kits (Cat. GP 2524, Biodiagnostic, Cairo, Egypt).

2.6. Evaluation of NF-κB and TNF-α levels in hepatic tissue

To investigate whether HS-induced hepatic injuries are associated with activation of the NF-κB pathway, the binding activity of NF-κB to DNA was estimated in liver tissue using commercially available (NF-kB p50/65 EZ-TFA Transcription Factor Assay Colorimetric, Cat. 70–510, Millipore, Billerica, MA, USA) for electrophoretic mobility shift assay (EMSA) and ELISA. Liver samples were processed according to Fillebeen et al. (2014). Briefly, liver samples were dissected into 1–2 mm^3 and washed in 50 ml ice-cold PBS. Then, the liver pieces were homogenised in 0.5 ml of ice-cold cytoplasmic lysis buffer with a tissue homogenizer for 10 s then the homogenates were chilled on ice for 20 min. After that, samples spanned for 10 min at 13,000 rpm in a microcentrifuge at 4 °C. The protein concentration was estimated in the supernatant by using the Bradford assay.

In addition, TNF-α was estimated by ELISA according to the manufacturer's instructions (Cat. ab208348, Abcam, Cambridge, UK). The TNF-α concentrations (pg/ml) and the relative optical density (OD) values at 450 nm were obtained using an automated microplate ELISA reader (Sorin Biomedica SpA., Italy).

2.7. Histopathologic evaluation and semi-quantitative scoring of liver tissue

Following euthanasia, tissue specimens were collected from the liver of CY, CO, RY, RO and stressed rats and then immediately fixed in neutral-buffered formalin solution for 48 h. Fixed tissues were

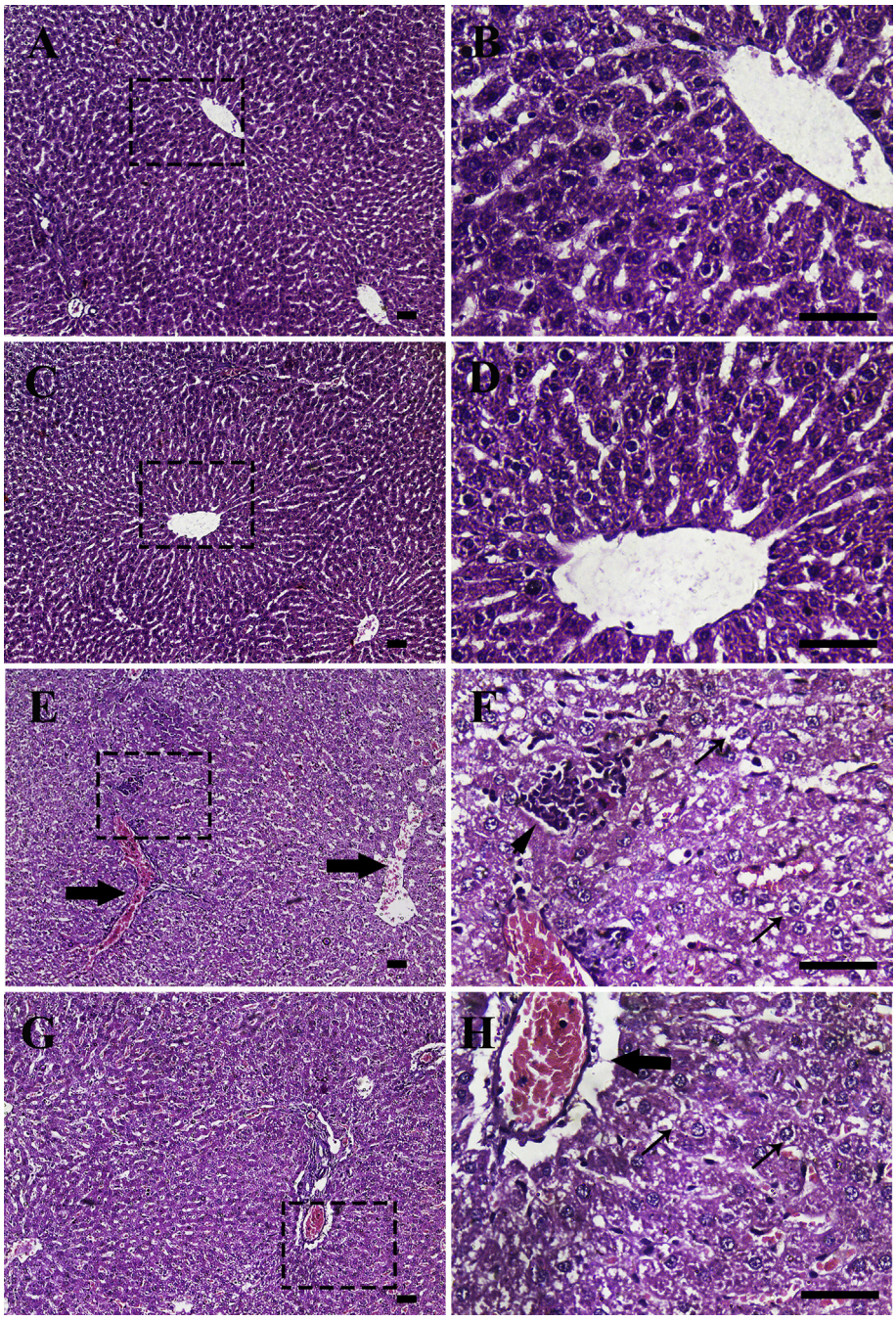

Fig. 5. Representative photomicrographs of H&E-stained liver tissue from young rats following heat stress (HS) exposure and/or resveratrol (RES) treatment. Light microscope examination of hepatic tissue from control (A, B) and RES (20 mg/kg BW, daily oral gavage for 21 days)-pre-treated rats (C, D) showing hepatic structures within normal histologic limits and normal hepatic cord, central vein, and portal triads. Examination of HS-exposed rats showing congestion of the central and portal vein (thick arrows) (E), with diffuse hydropic degeneration (thin arrows) and focal areas of coagulative necrosis infiltrated with mononuclear inflammatory cell (arrowhead) (F). Examination of HS-exposed rats pre-treated with RES showing hepatic vascular congestion (thick arrow) and mild vacuolization (arrows) (G, H). *Scale bar* = 50 μm.

processed by the paraffin-embedding technique according to previously described methods (Bancroft and Gamble, 2013). Several four-μm-thick sections were stained with haematoxylin and eosin (H and E) and the micrographs of the sections were captured with a digital camera (Leica EC3, Leica, Germany) connected to a microscope (Leica DM500). The extent of hepatic tissue injury was then evaluated via a semi-quantitative scoring system; five randomly selected fields were evaluated for each section. The scoring of hepatic lesion severity relied on the tissue involvement percentage, as described by Khafaga and El-Sayed (2018): none (0): no involvement of evaluated field; mild (1): involvement of 0–25% of evaluated field; moderate (2): involvement of 25–50% of evaluated field; and severe (3): involvement of 50–100% of evaluated field.

2.8. Immunohistochemical and histomorphometric evaluation

Four-micron-thick sections were obtained from each paraffin block, deparaffinized, and rehydrated in a descending grade of ethanol. Antigens were retrieved using 0.01 mol/L citrate-buffered saline (pH 6.0), and endogenous peroxidase activity was quenched using 0.3% (v/v) H_2O_2 in phosphate-buffered saline. Then, the non-specific binding of immunological reagents was blocked by incubating the samples with normal goat serum 10% (v/v) for 1 h. Liver sections were incubated overnight at 4 °C with HSP70 mouse monoclonal antibody (1:300; Abcam, Cat. ab5442, Cambridge, UK) and cleaved Caspase-3 rabbit polyclonal (1:100, BioCare Medical, Cat. CP229C, Concord, CA, USA). After washing with PBS, according to the primary antibody host, the sections were incubated with biotin-conjugated goat anti-mouse IgG antiserum (Histofine kit, Nichirei Corporation, Japan) or biotin-conjugated goat anti-rabbit IgG antiserum (Histofine kit, Nichirei

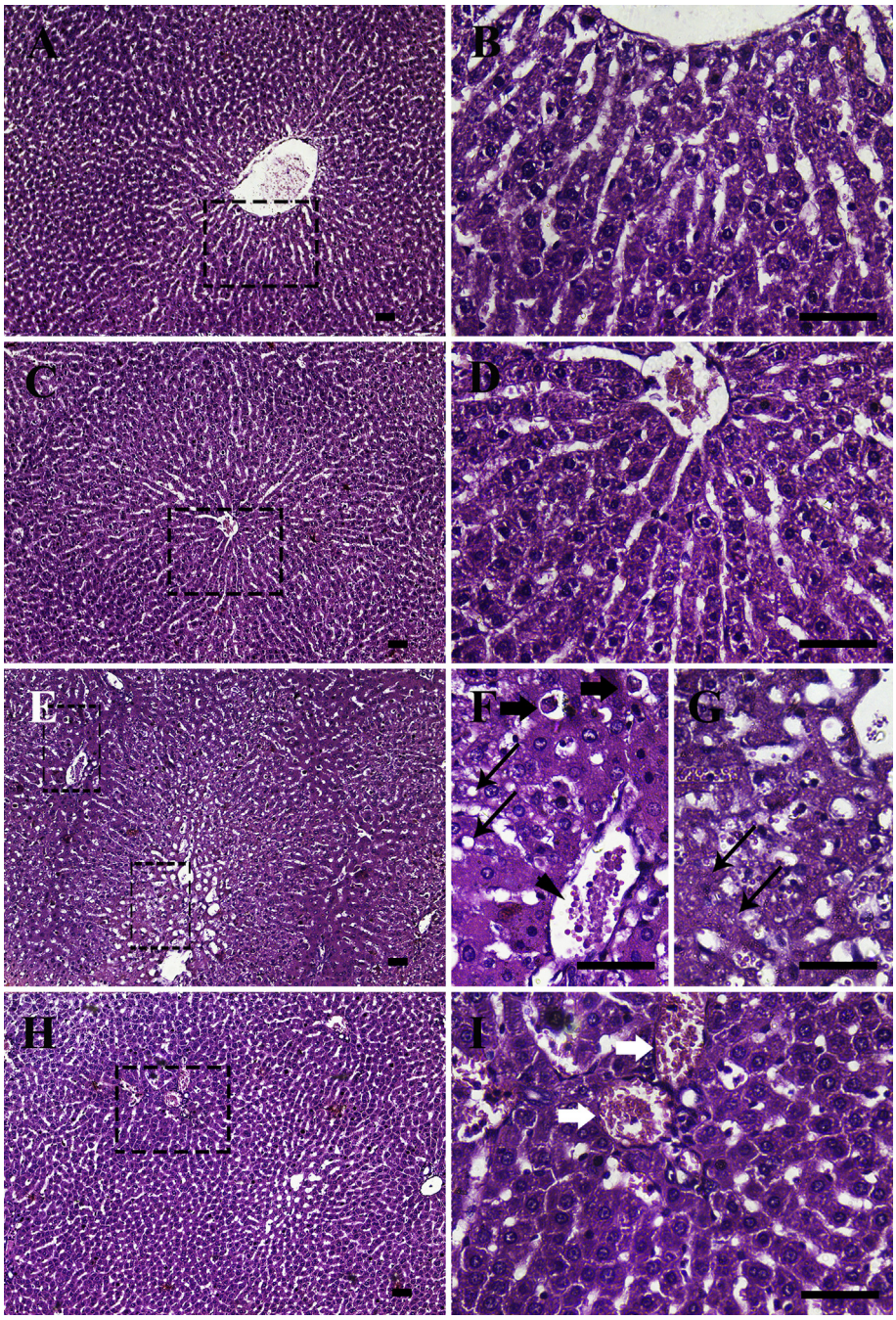

Fig. 6. Representative photomicrographs of H&E-stained liver tissue from old rats following heat stress (HS) exposure and/or resveratrol (RES) treatment. Light microscope examination of hepatic tissue from control (A, B) and RES (20 mg/kg BW, daily oral gavage for 21 days)-pre-treated rats (C, D) showing hepatocyte structures within normal histologic limits and normal hepatic cord, central vein, and portal triads. Examination of HS-exposed rats showing marked disarrangement of hepatic cords (E) with dilated congested portal vein (arrowhead), apoptotic cells (thick arrows) and sharp cytoplasmic fat vacuole with the characteristic signet ring appearance (thin arrows) (F). In addition, centrilobular coagulative necrosis was observed with dark eosinophilic cytoplasm, pyknotic or completely absent nucleus, and indistinct cell borders (thin arrows) (G). Examination of HS-exposed rats pre-treated with RES showing hepatic vascular congestion (arrows) (H, I). *Scale bar* = 50 µm.

Corporation, Japan) for 60 min. Then, the sections were washed with PBS, followed by incubation with streptavidin-peroxidase conjugate (Histofine kit, Nichirei Corporation, Japan) for 30 min. The streptavidin-biotin complex was visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB)-H_2O_2 solution, pH 7.0, for 3 min. Then, the sections were counterstained using Mayer's haematoxylin solution (Lebda et al., 2018; Noreldin et al., 2016, 2018). For the quantitative histomorphometric analysis, original micrographs were captured from the immunostained slides (5 random fields from each section, ×100 for HSP70 and ×400 for caspase 3) using a digital camera (Leica EC3, Leica, Germany) connected to a microscope (Leica DM500). The area and area percentage of HSP70 immune expression were measured in the hepatic tissue of control and treated rats. However, the caspase 3 positive hepatocytes nuclei were counted in each examined high power fields (HPF) by the aid of manual computer-assisted cell counting (Image J plug-in -cell_counter.jar) using ImageJ software (v1.46r, NIH, Bethesda, MD, USA) (Schneider et al., 2012).

2.9. Statistical analysis

Data were subjected to ANOVA by a general linear model (GLM) using a 4 × 2 factorial design according to the following model:

$$Yijk = \mu + Tj + Ai + TAij + eijk,$$

where Yijk = an observation, µ = overall mean, Tj = treatment effect (j = CTR, RES, HS, and HS + RES), Ai = rat age effect (i = young (2 months old) and old (24 months old)), TAij = interaction between treatment and age factor, and eijk = random error. Differences among the means of the same effect were estimated as previously described (Duncan, 1955). The semi-quantitative scoring of hepatic injury parameters and histomorphometric analysis of HSP and caspase 3 immune expressions were subjected to nonparametric analysis using

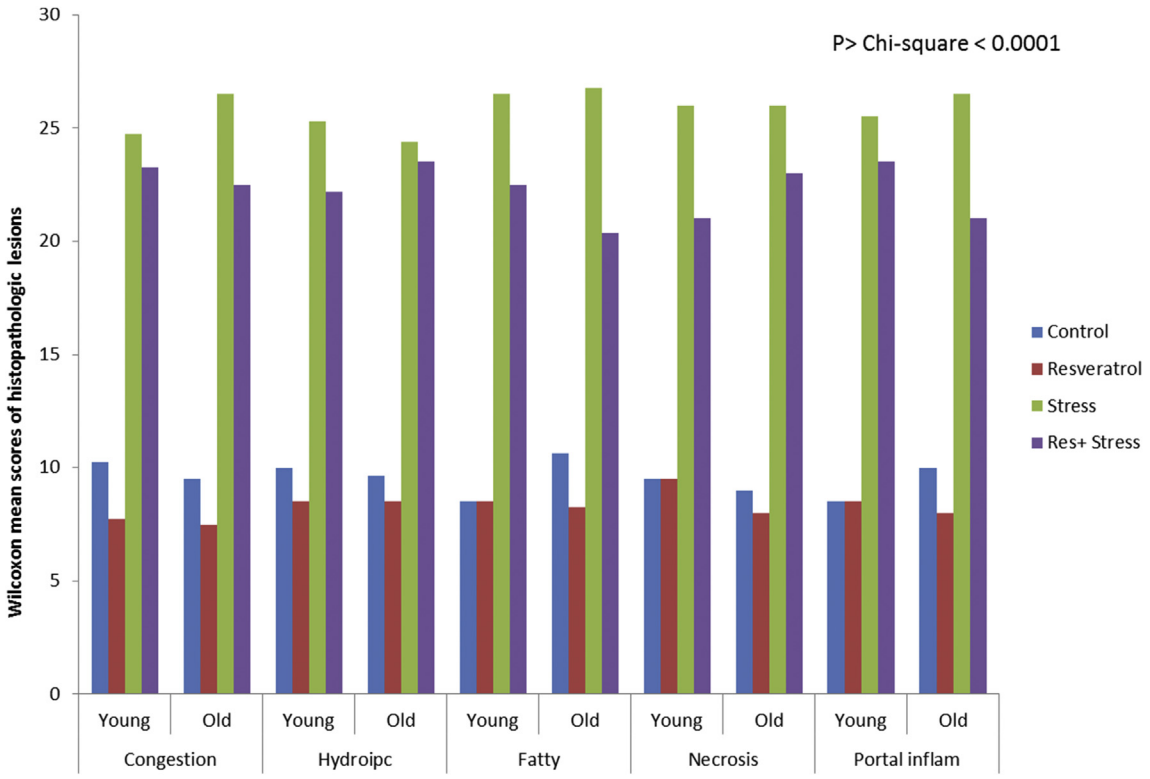

Fig. 7. Semi-quantitative scoring of hepatic injury parameters in male Wistar albino rats pre-treated with resveratrol (RES, 20 mg/kg BW) and exposed to heat stress (HS); all scores were subjected to nonparametric analysis using Kruskal–Wallis test to assess the significance between mean scores obtained from Wilcoxon rank sum test ($P >$ Chi-square < 0.0001).

Kruskal–Wallis test to assess the significance between mean scores obtained from Wilcoxon rank sum test.

3. Results

3.1. Rat rectal temperature

The mean total time of the stress session was 25 min. Before heat stress session, rectal temperatures of rats were obtained before and after anesthesia; their record was 36.38, 36.41, 36.39, and 36.21 for young HS, old HS, young RES + HS, and old RES + HS non-aesthetic rats; respectively. However, it dropped after anesthesia to 35.31, 35.44, 35.19, and 35.20 in young HS, old HS, young RES + HS, and old RES + HS rats; respectively. In addition, the mean final rectal temperatures of young and old HS-exposed rats were 43.04 and 43.24; respectively. Young and old RES + HS rats showed a mean final rectal temperature of 42.91 and 43.05; respectively. During stress session, the mean rectal temperatures of young HS, old HS, young RES + HS, and old RES + HS rats were 38.84, 39.50, 38.58, and 38.58, respectively, after 5 min; 39.94, 40.40, 39.51, and 39.51 after 10 min; 40.99, 41.30, 40.50, and 41.01 after 15 min; 42.06, 42.26, 41.81, and 42.24 after 20 min; and 43.04, 43.24, 42.91, and 43.05 after 25 min (Fig. 2).

3.2. Assessment of serum hepatic enzyme levels

As shown in Table 1, the serum levels of ALT, AST, and ALP were increased significantly ($P < 0.05$) in both young and old HS-exposed rats with and without RES pretreatment compared to the corresponding control rats. However, the serum levels of AST and ALP enzymes were significantly decreased ($P < 0.05$) by supplementation of RES in old and young stressed rats compared to stressed rats not given RES. Moreover, a significant difference ($P < 0.05$) in the AST level was found between young and old rats from all groups as compared to their counterparts.

3.3. Assessment of oxidative damage in liver tissue

To assess the antioxidant ameliorative role of RES against HS-induced oxidative liver injury, the level of lipid peroxidation was estimated in liver tissue as the MDA level; Table 2 illustrates that the MDA level was significantly increased ($P < 0.05$) only in young and old heat-stressed rats compared to the corresponding control rats. However, this increase was more pronounced in old stressed rats, which showed significant MDA upregulation ($P < 0.05$) compared to young stressed rats. In addition, the level of SOD was significantly increased ($P < 0.05$) in both young and old stressed rats with or without RES supplementation compared to control rats. Furthermore, the serum level of reduced glutathione (GSH) was significantly decreased ($P < 0.05$) in both young and old stressed rats followed by RES-supplemented stressed rats compared to control rats. Additionally, the serum level of GSH-Px was significantly reduced in young stressed rats followed by RES-supplemented stressed rats compared to control rats; however, stressed old rats exhibited significantly lower ($P < 0.05$) levels of such enzymes than RES-supplemented stressed old rats. Interestingly, a significant drop ($P < 0.05$) in the levels of antioxidative enzymes, including SOD and GSH, was observed in old rats from control, HS, and RES + HS groups compared with their counterparts in young rat groups; GSH-Px showed this significant drop only for control and RES-supplemented rats. The oral administration of RES did not induce a significant alteration in any analysed parameter compared to control rats.

3.4. Assessment of NF-κB and TNF-α in hepatic tissue

As illustrated in Figs. 3 and 4, the induction of NF-κB and TNF-α expression via HS exposure was assessed in liver tissue. In comparison

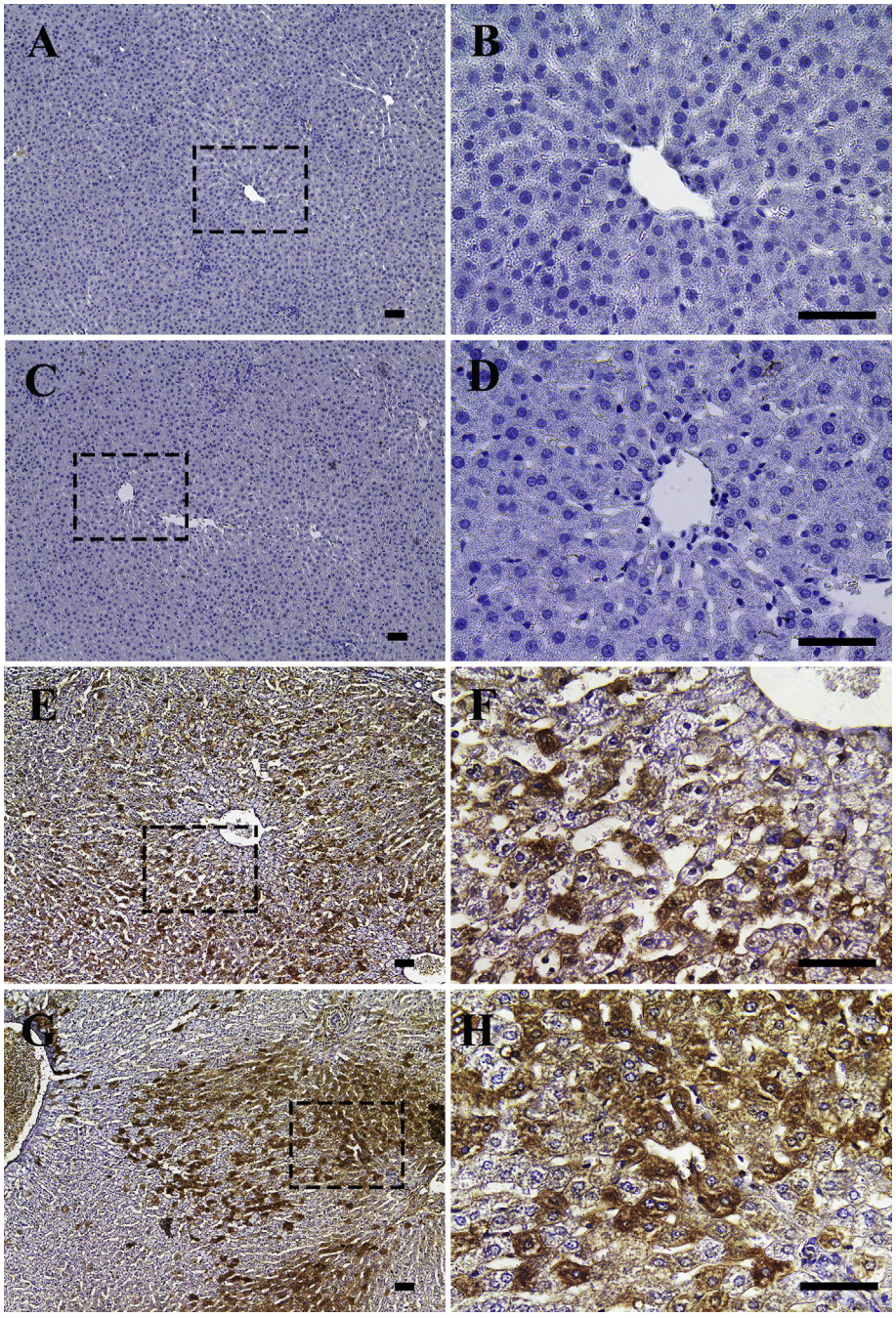

Fig. 8. Representative photomicrographs of HSP70 immunoreactivity in the liver tissue of young rats following heat stress (HS) exposure with and without resveratrol (RES) treatment. The liver tissue of control (CTR) (A, B) and RES (20 mg/kg BW, oral gavage daily for 21 days)-pre-treated (C, D) rats show negative HSP70 immune expression. HS-exposed rats exhibited moderate HSP70 immunoreactivity in the liver (brown colour) (E, F). HS-exposed rats pretreated with RES show strong HSP immunoreactivity in the liver (G, H). *Scale bar* = 50 μm.

with the control rats, RY and RO rats did not show a significant difference ($P \leq 0.05$) in the NF-κB or TNF-α level in the liver tissue. However, the production of NF-κB was increased significantly ($P < 0.05$) in rats of both ages compared to the corresponding control rats after exposure to HS; pretreatment with RES showed an improved effect in old rats compared to young rats. Concerning TNF-α, rats exposed to HS showed a significant upregulation ($P < 0.05$) of TNF-α compared to control rats; although the TNF-α level remained significantly greater than that in control rats, the HS-induced increase was significantly improved ($P < 0.05$) in young and old rats pretreated with RES. Additionally, significantly lower ($P < 0.05$) NF-κB and TNF-α levels were found in heat stressed old rats treated or not with RES than those in the corresponding young rats.

3.5. Histopathologic evaluation and semi-quantitative scoring of liver tissue

Liver tissue from both young and old euthermic control and RES-pretreated rats showed normal histological structures of hepatic lobules, hepatic cords, and portal tirades (*grade 0 injury*) (Fig. 5A, B, C, D; Fig. 6 A, B, C, D), except for mild occasional cytoplasmic vacuolization of both the hydropic and fatty type in old rats. After exposure to HS, the hepatic tissue from young rats showed moderate to severe vascular and sinusoidal dilatation and congestion, with diffuse cytoplasmic vacuolization mainly of the hydropic type. In addition, multifocal area of

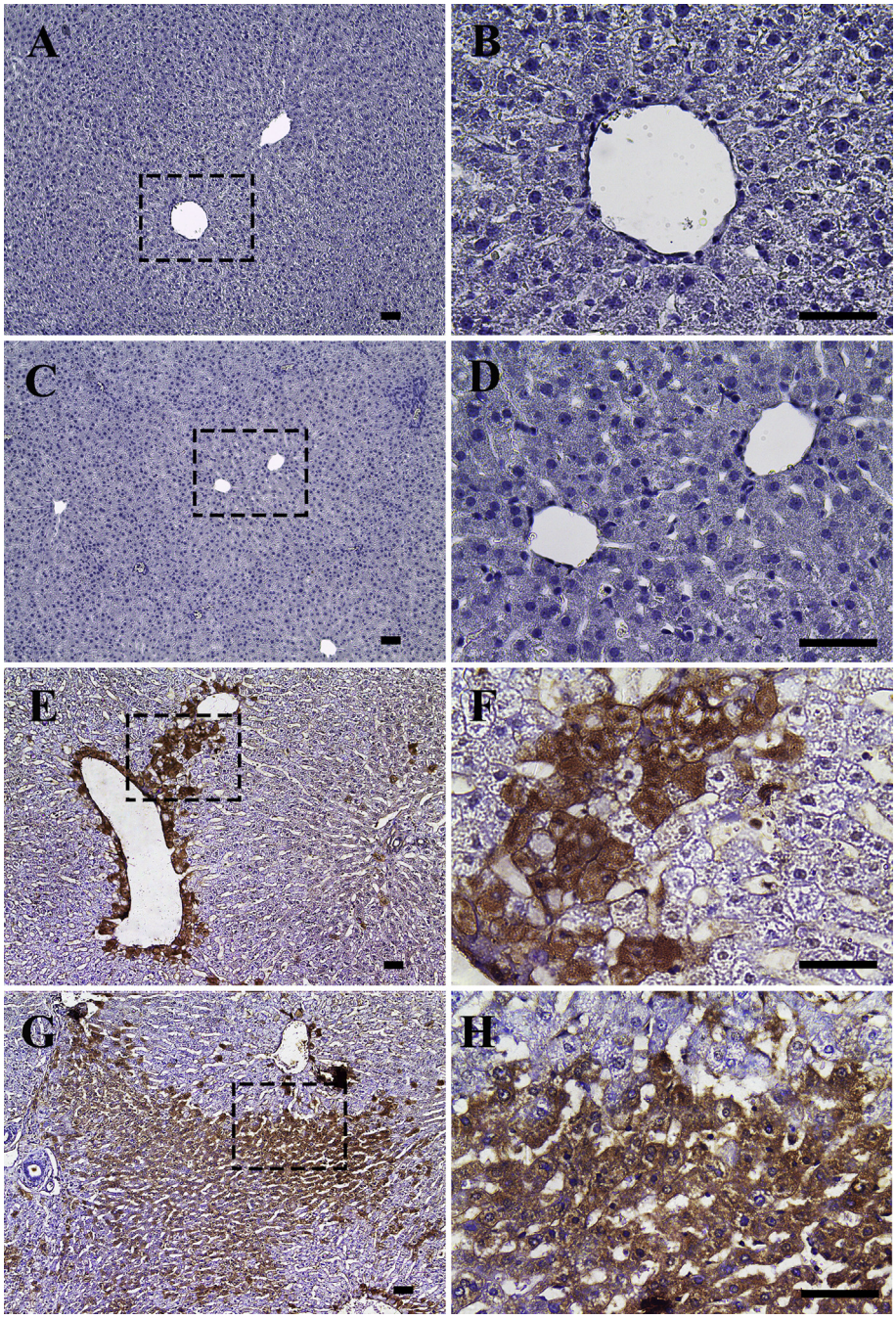

Fig. 9. Representative photomicrographs of HSP70 immunoreactivity in the liver tissue of old rats following heat stress (HS) exposure with and without resveratrol (RES) treatment. The liver tissue of control (CTR) (A, B) and RES (20 mg/kg BW, oral gavage daily for 21 days)-pre-treated (C, D) rats show negative HSP70 immune expression. HS-exposed rats exhibited moderate HSP70 immunoreactivity in the liver (brown colour) (E, F). HS-exposed rats pre-treated with RES show strong HSP immunoreactivity in the liver (G, H). *Scale bar* = 50 μm.

coagulative necrosis, with the characteristic hyper-eosinophilic cytoplasm and pyknotic or completely absent nuclei, was noted, along with the infiltration of mononuclear cells, predominantly lymphocytes (Fig. 5 E, F). Additionally, clustered mass of mononuclear inflammatory cells and neutrophils was observed within and around portal veins with mild to moderate vascular endothelial injury. However, microscopic examination of the liver tissue from old rats revealed characteristic disarrangement of hepatic cords with dilated congested or bloodless sinusoids (Fig. 6 E). Additionally, marked presence of centrilobular coagulative necrosis was observed, sometimes with lymphocyte infiltration or extravasation of red blood cells (Fig. 6 G), in addition to moderate distribution of apoptotic cells and cytoplasmic fat vacuole characterized by peripheral nuclei yielding the characteristic signet ring appearance (Fig. 6 F). Moreover, diffuse cytoplasmic vacuolization and thickening of portal triad with mononuclear cellular infiltration was evident in other examined tissues from the same group. Pretreatment of young and old rats with RES improved the HS-mediated tissue injury; the hepatic tissue showed a nearly normal morphology, except for vascular congestion in old rats (Fig. 6 H, I), and vascular congestion accompanied by mild vacuolization in young rats (Fig. 5 H, I). Non-parametric analysis for the semi-quantitative scores of hepatic injuries (including vascular and sinusoidal congestion, cytoplasmic vacuolization of the hydropic and fatty type, inflammatory infiltrate, and focal hepatic necrosis) showed a significant increase ($P < 0.0001$) in all HS-

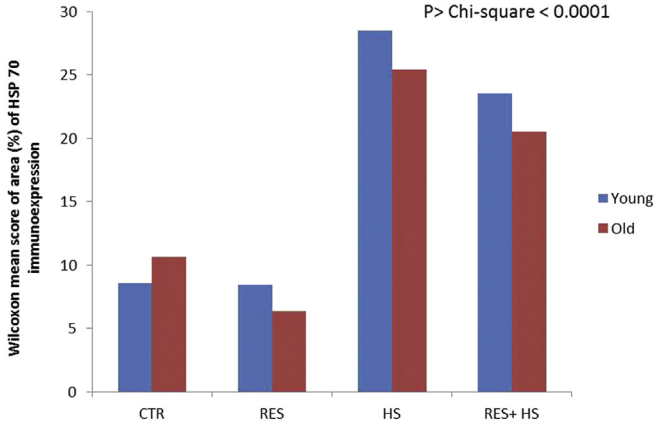

Fig. 10. Effect of resveratrol (RES), heat stress (HS), and their combination on the Wilcoxon mean score of area percent of HSP immune expression in male Wistar albino rats; all scores were subjected to nonparametric analysis using Kruskal–Wallis test to assess the significance between mean scores obtained from Wilcoxon rank sum test ($P >$ Chi-square < 0.0001).

exposed young and old rats compared to the control rats. However, pretreatment with RES resulted in a significant improvement ($P < 0.0001$) in the scores of most of these parameters compared to the scores of the HS-exposed rats (Fig. 7), although the scores remained significantly higher than the corresponding scores in the control group.

3.6. Immunohistochemical and histomorphometric evaluation

As shown in Figs. 8–10 under control conditions, the immune expression of HSP70 and the nonparametric analysis of such expression showed negative to mild expression in young and old control (Fig. 8 a,b; Fig. 9 a,b) and RES-pretreated (Fig. 8 c,d; Fig. 9 c,d) rats. In addition, rats exposed to HS expressed a significant increase ($P < 0.0001$) in HSP immunoreactivity compared to control non-stressed counterpart; however, such immunoreactivity was more pronounced ($P < 0.0001$) in HS young rats than in HS old rats (Fig. 8 e,f; Fig. 9 e,f, Fig. 10). Pretreatment with RES induced non-significant increase ($P < 0.0001$) in HSP70 immunoexpression in both old and young HS-exposed rats compared to HS-exposed rats not given RES (Fig. 8 g,h; Fig. 9 g,h, Fig. 10). Concerning the immune expression of caspase 3, as shown in Figs. 11–13, control and RES-treated rats of both age (young and old) showed negative immunostaining for caspase 3 (Fig. 11 a,b,c,d; Fig. 12 a,b,c,d). However, significant increase ($P < 0.0049$) in number of caspase 3 positive nucleus was reported in liver tissues from rats exposed to thermal injury (Fig. 11 e,f; Fig. 12 e,f, Fig. 13). In addition, prior-treatment with RES induced significant reduction ($P < 0.0049$) in number of positive stained nucleus compared to HS-exposed rats, albeit it not identical to control (Fig. 11 g,h; Fig. 12 g,h, Fig. 13).

4. Discussion

Currently, HS-induced morbidity and mortality in aged populations have gained great concern, particularly after the dramatic warming of the global climate. This increase in the awareness of heat stress has led to the emergence of many lifestyle precautions and dietary supplements, which are believed to play a role in combating the adverse effects of ageing on the biological functions of different organs. The primary purpose of the current study was to investigate the potential anti-ageing effect of oral RES supplementation on HS-induced alterations in old and young rats.

In this study, we focused on the liver because it is the sentinel organ for HS and plays a critical role in the pathophysiological response to thermal injury (Hall et al., 2000a, 2000b). Imbalances in the general antioxidant system and disturbances in the physiological and immunological statuses are common adverse effects of HS (Abdelnour et al., 2019; Mujahid et al., 2007b; Panda et al., 2008).

The results of the present study support this theory; both young and old rats exposed to HS showed a marked increase in lipid peroxidation, as indicated by a significant increase in the MDA level and a significant reduction in the levels of antioxidant enzymes, including GSH, SOD, and GSH-Px, in liver tissue. Similar results have previously been obtained by (Liu et al., 2013; Mujahid et al., 2007b; Panda et al., 2008; Ramnath et al., 2008). However, significant improvements in the levels of MDA, GSH and GSH-Px were found in rats pretreated with RES; such improvements may be attributed to the polyphenolic compounds included in RES (Yang et al., 2009), which could render it a potential anti-stress and anti-ageing agent able to alleviate HS-related (Sahin et al., 2012).

In addition, several investigators have observed the pleiotropic health properties of plant polyphenols, including antioxidant, immune-enhancing, anti-inflammatory, antiviral, antibacterial, and anti-tumourigenic properties (Duthie et al., 2000; Khafaga and Bayad, 2016a, b; Putics et al., 2008). A similar effect of RES against HS-induced oxidative effects has previously been reported in quails (Sahin et al., 2010, 2012). Additionally, it is well established that the over accumulation of MDA abrogates antioxidant enzyme activity, with subsequent oxidative injury (Mujahid et al., 2007a); accordingly, RES eliminates oxide precursors (Liu et al., 2011), activates antioxidant-associated proteins (Sgambato et al., 2001), and scavenges ROS (Das, 2011).

In this study, the striking pathology observed in old rats confirmed that the aged liver is more susceptible to HS-related oxidative injury; the old rats showed the most severe and widespread hepatic lesions, including inflammatory and degenerative changes and even hepatic necrosis. These findings may reflect the inability of aged animals to express appropriate adaptive mechanisms against heat-induced injury. Supporting this theory, animal studies (Bloomer et al., 2014; Hall et al., 2000b) have indicated that HS-related death may be due to liver dysfunction.

Similarly, the levels of cytosolic liver enzymes, such as AST, ALT, and ALP, were significantly increased following heat exposure, indicating increased cell membrane permeability associated with hepatic necrosis (Hafez et al., 2015; Mukherjee et al., 2013). However, RES-pretreated rats showed significant improvements in hepatic histopathologic structures and the serum levels of liver enzymes, including AST and ALP, which could also be attributed to the polyphenolic compounds contained in RES.

HSPs (HSP27, HSP70, and HSP90) are the first type of proteins that are generated in hepatic tissue in response to HS, and their generation is accompanied by haemorrhagic shock (Abdelnour et al., 2019; Kregel, 2002); they also play a major role in maintaining the structural and functional integrity of different organs against stress-induced tissue damage (Mahmoud et al., 2004; Yu et al., 2008). Importantly, the reduced ability of aged organisms to produce HSPs as an adaptive response to thermal injury has previously been reported (Avenatti et al., 2018; Hall et al., 2000b). In the current study, the immunohistochemical expression of HSP70 was significantly increased after HS exposure. However, it was more pronounced in young rats than in old rats; this finding may provide an acceptable explanation for the reduced thermotolerance of older populations (Semenza et al., 1996).

Indeed, the cellular ability to produce HSPs in response to environmental stress may be reduced but not eliminated by ageing, wherein ageing results in major changes in the intensity and type of stressor that can activate HSP accumulation (de Toda and De la Fuente, 2015; Hall et al., 2000b). Several mechanisms could be proposed regarding the modulation of the HSP70 response during stress, including dysfunctional transcriptional regulation (Butt et al., 2017). In this study, rats pretreated with RES showed a marked elevation in HSP70 expression. Oral pretreatment with RES has been shown to induce strong activation of the endogenous antioxidant defence system with a

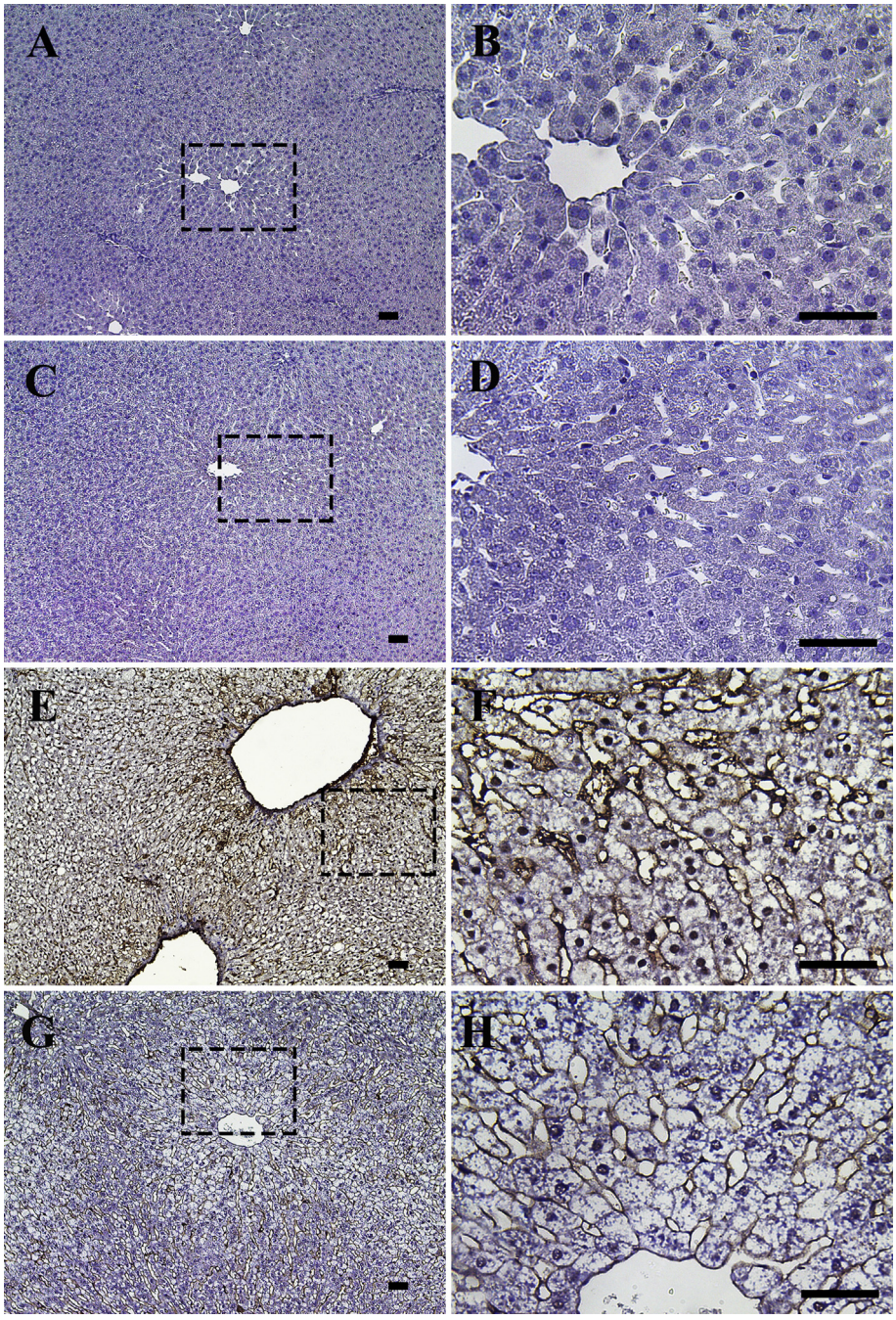

Fig. 11. Representative photomicrographs of caspase 3 immunoreactivity in the liver tissue of young rats following heat stress (HS) exposure with and without resveratrol (RES) treatment. The liver tissue of control (CTR) (A, B) and RES (20 mg/kg BW, oral gavage daily for 21 days)-pre-treated (C, D) rats show negative caspase 3 immune expression. HS-exposed rats exhibited strong caspase 3 immunoreactivity in the liver (brown colour) (E, F). HS-exposed rats pretreated with RES show mild to moderate caspase 3 immunoreactivity in the liver (G, H). *Scale bar* = 50 μm.

subsequent enhancement in the antioxidative status and, in turn, a great increase in HSP expression (Kanitkar and Bhonde, 2008). This result is consistent with the findings of Putics et al. (2008), who concluded that RES can induce the activation of HSPs, particularly the major chaperone, HSP70, in human peripheral lymphocytes and cell lines. In contrast, Sahin et al. (2012) and Liu et al. (2013) observed reduced HSP70 expression in the hepatic tissue of heat-stressed quail and black-boned chickens, respectively, which may be attributed to differences in the examined species, supplemented dose, or HS regimen.

HS is associated with NF-κB activation. In a normal neutral state, NF-κB is present intracellularly in an inactive form, and it is activated immediately after cell stimulation by different types of stressors, such as toxins, HS, or cytokines. Activated NF-κB translocates into the nucleus and subsequently binds to specific DNA binding sites. The binding of NF-κB is responsible for the regulation of the expression of different genes, with in turn regulate various biological processes, such as inflammation and apoptosis (Bettermann, 2017; El Okle et al., 2018; Nelson et al., 2004). Similarly, it was reported that HS induced increased NF-κB activation in quail liver and black-boned chicken jejunum, thereby activating multiple genes (Orhan et al., 2012; Sahin et al., 2010). Our present study also found a similar significant increase in the expression of NF-κB in liver tissue after HS exposure, suggesting that the reported post-stress pathologic lesions might be related to the high abundance of NF-κB, which may be involved in the stimulation of

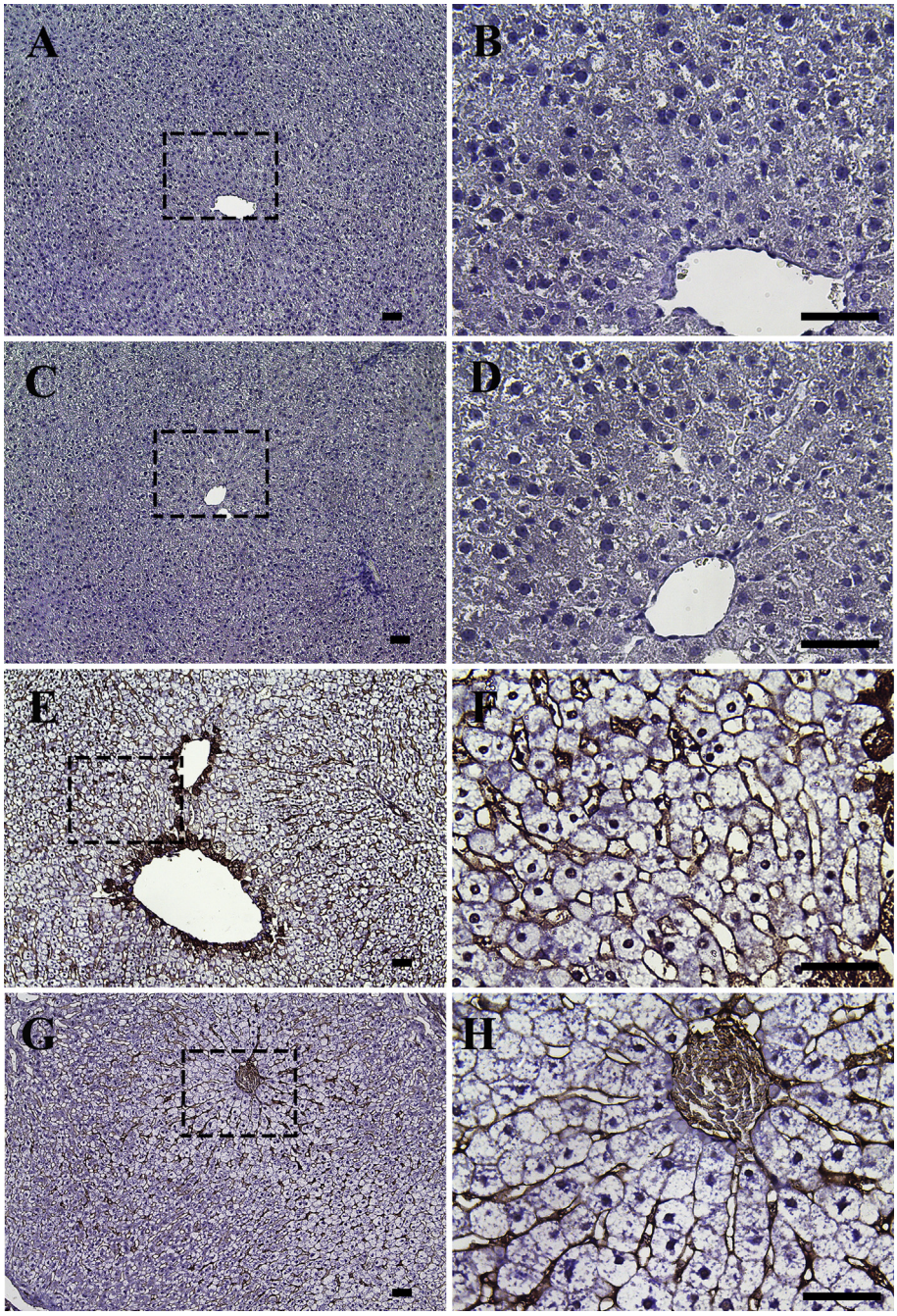

Fig. 12. Representative photomicrographs of caspase 3 immunoreactivity in the liver tissue of old rats following heat stress (HS) exposure with and without resveratrol (RES) treatment. The liver tissue of control (CTR) (A, B) and RES (20 mg/kg BW, oral gavage daily for 21 days)-pre-treated (C, D) rats show negative caspase 3 immune expression. HS-exposed rats exhibited strong caspase 3 immunoreactivity in the liver (brown colour) (E, F). HS-exposed rats pre-treated with RES show mild to moderate caspase 3 immunoreactivity in the liver (G, H). *Scale bar* = 50 μm.

various genes related to the induction of hepatic damage. Moreover, the current results indicate significant NF-κB inhibition in RES-pretreated rats with a concurrent upregulation of HSP expression. Therefore, it may be suggested that NF-κB regulates the transcription of HSP-related genes (Lanneau et al., 2010).

As mentioned above, binding of NF-κB to specific DNA binding sites is able to induce and regulate apoptosis (Bettermann, 2017). In addition, Gu et al. (2014) concluded that cells exposed to intense thermal stress can develop reduction in the mitochondrial membrane potential, which in turn resulted in increased release of mitochondrial cytochrome *c* leading to activation of caspase-9/caspase-3-induced early apoptosis through mitochondrial pathway. Constituently, in this study, the immune staining of caspase 3 - as an important apoptotic marker- was increased significantly in heat stressed rats of both age. However, it was significantly decreased in stressed rats pretreated with RES, reflecting the anti-apoptotic properties of RES.

Similarly, Liu et al. (2016) reported the transveratrol-mediated inhibition of NF-κB in black-boned chickens. Another theory regarding activation of the NF-κB pathway is the interaction of HSP90 and HSP70 with Toll-like receptor (TLR)2 and TLR4, followed by the expression of inflammatory cytokines, such as TNF-α (Colaco et al., 2013); this theory is supported by the findings of our study, in which the level of TNF-α was increased significantly in HS-exposed rats with concurrent increases in the levels of NF-κB, HSP70, and caspase 3. However, RES-

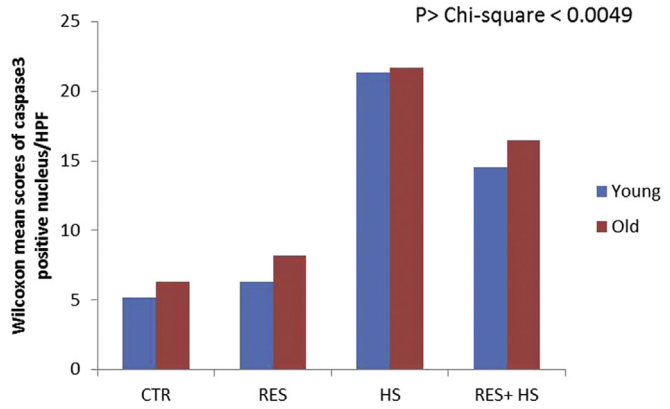

Fig. 13. Effect of resveratrol (RES), heat stress (HS), and their combination on the Wilcoxon mean score of caspase 3 immune positive hepayocytes in male Wistar albino rats; all scores were subjected to nonparametric analysis using Kruskal–Wallis test to assess the significance between mean scores obtained from Wilcoxon rank sum test ($P >$ Chi-square $<$ 0.0049).

pretreated rats showed significantly reduced levels of TNF-α, NF-κB, and apoptotic marker caspase 3, and upregulated HSP70 expression, along with the logical attenuation of oxidative stress and histopathologic lesions.

In conclusion, based on the current findings, it could be concluded that RES is a promising anti-ageing supplement, which may help in the attenuation of thermal-induced hepatic injury in aged individuals. RES exerted antioxidant, anti-inflammatory, and anti-apoptotic hepatoprotective effects via inhibition of the NF-κB and mitochondrial pathway in a cellular system. In addition, the immune expression of HSP70 was reduced in aged rats, indicating a reduced capacity for thermoregulation. Further investigations are needed to determine the effects of RES on complete physiologic profiles, molecular biology parameters, and deep proteomic analysis in aged animals exposed to heat stress. In addition, the clarification for dose dependent effect of RES and the link between aging and thermal stress via investigation of cytokines and ROS expression is still required.

Conflicts of interest

All authors declare that there is no conflict of interest.

Abbreviations

ALP	Alkaline phosphatase
ALT	Alanine transaminase
AST	Aspartate transaminase
GSH–Px	Glutathione peroxidase
GSH	Reduced glutathione
HSP70	Heat shock protein 70
IL-6	Interleukin 6
MDA	Malondialdehyde
NF-κB	Nuclear factor-kappa B
SOD	Superoxide dismutase
TNF-α	Tumour necrosis factor-alpha

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2019.04.012.

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