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Research Article

RESVERATROL PROTECTS WHOLE BODY HEAT STRESS-INDUCED TESTICULAR DAMAGE IN RAT MODEL

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ABSTRACT

Objective: The local thermoregulation in testis is important for optimum spermatozoa development. Excessive heat hampers this regulation resulting in alteration of normal testicular function. The present investigation confirms the role of free radicals in hyperthermia induced oxidative damage in testis and elucidates the dose-dependent ameliorating effect of resveratrol (RSV) against testicular oxidative damage. The aim of the present investigation is also to observe the role of selective concentration of RSV on heat induced oxidative changes in the damaged tissue.

Methods: 48 male Wister rats were exposed to hyperthermic condition for the past 7 days of the total 21 days of experiment. RSV was pre- and co-treated with heat stress daily in a dose-dependent manner (1 mg, 5 mg, and 10 mg/kg body weight) for 21days.

Results: Reactive oxygen species level was estimated using flow cytometry. Enhancement of hepatotoxicity markers in serum, lipid peroxidation and decreasing antioxidant status in the testis homogenate demonstrated that the oxidative damage in heat exposed tissue.

Conclusion: Histological study along with biochemical and molecular assessment of the redox balance of testicular tissue in the present study revealed that RSV significantly ameliorated the heat induced damage in testis. The findings suggest that RSV is an effective antioxidant polyphenolic compound that can protect testis against hyperthermia induced oxidative damage.

Keywords: Hyperthermia, Resveratrol, Reactive oxygen species, Antioxidant.

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INTRODUCTION

Hyperthermic stress is a major environmental and occupational heat hazard in our daily life. Male reproductive organ, testis is highly heat susceptible. In a healthy body, testicular temperature is 2–8°c lower than the abdominal and core body temperature [1]. This lower temperature within testes is maintained by counter current heat exchange process between the pampiniform plexus and testicular artery [2] and is required for normal functioning of testis like spermatogenesis and steroidogenesis. Heat stress (HS) leads to oxidative stress in testis as demonstrated by excessive reactive oxygen species (ROS) production and impaired antioxidant capacity [3].

Like many other natural antioxidant, Resveratrol (RSV) also can resist the stress induced degenerative changes. It is a member of a group of phytoalexin, which is majorly stored in grape skin [4]. Trans-isomer of RSV is more biologically active and has been used in the experiment [5].

It is already reported that RSV has potent ROS scavenging activity [6]. It has been observed that the concentration of RSV in blood reaches its maximum level in about 10–15 min after oral gavage [7,8] and can prevent liver diseases induced by free radical in different drug-induced hepatotoxicity [9]. However, it is not definitely known whether RSV can alleviate the HS mediated oxidative stress in testis of HS animals. Therefore, the study of the protective effect of RSV on HS induced testicular oxidative damage in rat model would be beneficial for designing new therapeutic approaches in the future.

The aim of present investigation is to compare the dose dependent ameliorative effect of RSV on whole body heat induced testicular damage in male rats and to find out the optimum dose of RSV pre-treatment against hyperthermic stress. The results may help to find, the current and future risk of infertility in male individuals, especially those who are working in areas vulnerable to excessive whole body heat exposure, and also how far RSV can protect testis from heat induced degenerative changes.

METHODS

48 healthy adult male Wistar rats, weighing about 100–120 g each, were used in the study. Eight experimental animal groups were made by keeping six animals in each of the polypropylene cages sized 18 inches × 12 inches × 9 inches. The rats were acclimatized in the animal house condition for 7 days. A constant temperature (24±2°C) and humidity (50±5%) was maintained along with a 12 h light/12 h dark cycle. Animals were fed on standard pellet food and water *ad libitum*. The animal ethical clearance was obtained from Institutional Animal Ethics Committee (IAEC) approved by CPCSEA (Reg. no. 1946/PO/Re/S/17/CPCSEA).

Drug and chemical

RSV (Tokyo chemical industry, Lot- W3ZZH-RA) was suspended in 1% carboxy methyl cellulose (LOBA CHEMIE PVT.LTD.) [10] and the suspension was used in this experiment.

Preparation of HS animal model

Animals to be stressed were placed in a (4 feet × 4 feet × 9 feet) room where a room heater was attached to increase the room temperature, and the temperature was monitored continuously with a thermometer placed inside the room. The temperature of the room was maintained at 41±1°C with relative humidity 30–40%. A thermostat was placed strategically that cuts off to prevent overheating of the room. A blower and air ventilator placed at the top of the chamber along with the room heater kept the temperature of the room within the desired range. The air flow was recorded using KATA thermometer. Animals were kept within a meshwork cage (1.5 ft × 1 ft × 9 inch) in the stress room

daily for 1 h at 41°C ambient temperature. Immediately after HS, rectal temperature of the experimental animals was measured using digital thermometer and the animals were placed at normal room temperature for 20 min. Rats were then returned to the animal house and provided with free access to drinking water.

Selection of effective dose of RSV

Three different doses of RSV were chosen for the present study as 1 mg/kg, 5 mg/kg, and 10 mg/kg body weight. These doses were administered orally into three different groups of rats. The effects of different doses were compared to examine the optimum dose of RSV, at which the amelioration was maximum against whole body HS induced testicular damage.

Experimental design

The animals were randomly divided into eight groups (n=6).

- Group 1: Served as control where rats were not exposed to HS and were kept untreated
- Group 2: Where rats were exposed to heat at temperature (41°C) and humidity (30–40%) for 1 h daily for last consecutive 7 days of experiment
- Group 3: Where rats were treated with RSV at a dose of 1 mg/kg body weight for 21 days
- Group 4: Where rats were treated with RSV at a dose of 5 mg/kg body weight for 21 days
- Group 5: Where rats were treated with RSV at a dose of 10 mg/kg body weight for 21 days
- Group 6: Where rats were exposed to heat for last consecutive 7 days along with oral administration of RSV at a dose of 1 mg/kg body weight once daily for 21 days (14 days prior to and 7 days along with the heat exposure)
- Group 7: Where rats were exposed to heat for last consecutive 7 days along with oral administration of RSV at a dose of 5 mg/kg body weight once daily for 21 days (14 days prior to and 7 days along with the heat exposure)
- Group 8: Where rats were exposed to heat for last consecutive 7 days along with oral administration of RSV at a dose of 10 mg/kg body weight once daily for 21 days (14 days before and 7 days along with the heat exposure).

All the animals were euthanized soon after hyperthermic exposure following protocol and ethical guideline. Serum and tissue samples were used for subsequent experiments (Fig. 1).

Blood sample

Blood was collected from each animal by the cardiac puncture technique under light either anesthesia. The sample was taken in a plain vial and the serum was separated by centrifugation and stored at 4°C for biochemical assay.

Tissue sample

Left testis of each rat was dissected out, rinsed with phosphate buffer saline (PBS) (pH7.4) and soaked with filter paper. Tissues were weighed and homogenized in chilled PBS. The homogenized samples were collected in a centrifuge tube and stored in –20°C for biochemical assays. The right testis of each animal was used for histological studies and other biochemical estimation.

Body weight

Body weight was recorded at an interval of 7 days throughout the experimental period, and last recording was taken just before the sacrifice.

Testicular weight and calculation of testicular index

After dissection, both the testes of all animals were weighed and used for the calculation of the testicular index using the following formula:

Testicular index = (Testicular weight/Body weight) × 100

Rectal temperature

During HS, rectal temperature was regularly recorded before and after HS by digital thermometer.

Histopathological study

Immediately after sacrifice the right testis of all the animals were taken out from the body and fixed in Bouin's fluid, and embedded in paraffin for making tissue section. The tissue sections were stained with eosin-hematoxylin and examined under light microscope (Victory plus- FL LED).

Analysis of hepatotoxicity markers

Important liver function enzymes, such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), and alkaline phosphatase (ALP), were estimated as the markers of hepatotoxicity. SGOT and SGPT were estimated using commercially available kit (Beacon Diagnostic PVT LTD, India) according to manufacturer's protocol; ALP activity was measured using enzymatic assay kit manufactured by Coral Clinical System, India (Mod. Kind and King's method).

Super oxide dismutase (SOD) activity

The activity of SOD in the testicular tissue homogenate was estimated using auto oxidation of hematoxylin, adjusted with ethylenediaminetetraacetic acid (EDTA). Briefly, 25 µl of the freshly prepared hematoxylin solution was mixed with 1.450 ml phosphate buffer and 25 µl tissue homogenate supernatant was added. Auto-oxidation was monitored for 2 min and recorded as an increase in the absorbance at 560 nm. In case of blank, distilled water was used instead of supernatant [11].

Catalase activity

Catalase activity was assayed according to the method of Takahara *et al.* [12] with minor modifications. Briefly 2.45 ml of the phosphate buffer and 50 µl of the tissue sample were mixed and the reaction mixture was initiated by adding 1 ml of H_2O_2 solution (30 mM). The decreasing absorbance was measured at 240 nm at 30 s intervals for 2 min. The enzyme blank was run simultaneously with 1.0 ml of distilled water instead of H_2O_2. The enzyme activity was expressed as n moles of H_2O_2 decomposed/minutes/mg of protein.

Total reduced glutathione (GSH) level

The GSH level was assessed using standard protocol [13]. 4 ml of PBS, 0.2 ml of EDTA, 0.1 ml sodium azide, 0.1 ml H_2O_2, 0.2 ml GSH, and 0.2 ml tissue supernatant were mixed and incubated at 37°C for 10 min. After incubation 0.5 ml, TCA was added to the mixture, mixed vigorously and centrifuged at 3000 rpm for 10 min. Then 0.25 ml supernatant was taken and 1 ml of Na_2HPO_4 and 0.25 ml DTNB were added to it. Absorbance was measured at 412 nm. In case of blank, distilled water was added to the reagent mixture instead of supernatant. Different known concentrations of GSH were used to prepare a standard curve. The amount of GSH was expressed as µg/mg of protein.

Lipid peroxidation (LPO) level

The LPO level was measured by following the method described by [14]. Briefly, 1 ml of tissue homogenate was mixed with equal volume of TBA-TCA-HCl mixture and was allowed to boil in boiling water bath for 15–20 min till the appearance of pink color. The reaction mixture was allowed to cool at room temperature and the absorbance was measured at 535 nm. PBS was added to the reaction mixture instead of tissue sample for blank.

Protein estimation

Protein was estimated following the method of [15]. 25 µl tissue homogenate, 0.975 µl NaCl, and 5 ml alkaline copper reagent were mixed and kept for 15 min. To it was then added 0.5 ml of Folin–Ciocalteu reagent and was kept for 30 min. Absorbance was taken at 660 nm. NaCl was added to the reaction mixture instead of tissue sample for blank. For standard curve preparation, BSA solution (0.1%) was added to the reaction mixture at different volumes.

Preparation of testicular cell suspension
The left testis was dissected out, washed, cut into small pieces in pre-warmed PBS (pH7.4, 37.5°C) with type I collagenase (0.25%) and incubated at 32.5°C for 15 min. After the incubation period, additional 15 min of digestion in PBS with trypsin (0.25%) was performed with gentle agitation. The isolated cells were then passed through a cell strainer (70 µm) and the collected sample was centrifuged at 3000 rpm for 15 min. The cells in the pellet were washed twice with PBS and suspended in DMEM F12 medium with fetal bovine serum (10%) for future use [16].

Fluorescence-activated cell sorting (FACS) analysis for assessment of intercellular ROS (iROS) of testis

The level of iROS was estimated using flow cytometric studies using 2′,7′-dichloro fluorescein di-acetate (H_2DCFDA) staining. Intensity of green fluorescence was compared to the untreated control to measure the increment of iROS. The isolated testicular cells (2×10^6) were incubated for 30 min at 37°C after re-suspension in complete medium containing H_2DCFDA following acquisition by a BD FACSAria™ III cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) using an argon laser at 488 nm [17].

Statistical analysis
The statistical analysis was performed by using the statistical program Origin Pro version 8.0. All the data in the figures are represented as mean values of their standard errors (mean±SEM), where n (n=6) represents the number of animals used. In histology and immunofluorescence experiments, at least three sections of the tissues collected from all the animals in each group were shown. Statistical significance was determined using one-way analysis of variance according to the Tukey method with the *post hoc* test. The significance level was $p<0.05$ in all cases.

RESULTS

Measurement of body weight
Body weight was reduced significantly in HS group compared to the control group and no significant changes were observed between control and protection (RSV treated) groups. RSV pre-treatment resisted this reduction of body weight in heat-induced animals significantly (Table 1).

Calculation of testicular index
Reduced level of testicular index was seen in hyperthermic exposed animals. In case of RSV pre-treated (5 mg and 10 mg/kg body weight) HS rats, improved levels of testicular index compared to stress group was seen while a dose of 1 mg was found ineffective (Fig. 3a).

Rectal temperature
It was observed that rectal temperature was raised over 39°C after heat exposure (when ambient temperature was 41°C) compared to control. RSV failed to prevent the rise of rectal temperature due to high ambient heat exposure (Table 1).

Heat-induced histological changes in testis
Histopathological study is the primary tool for observation of the nature and extent of damage in the tissue. The histopathological investigations of the testis sections have shown that heat exposure could not disorganize and change the architecture of testis but germinal epithelium showed significant degenerative changes. These include disrupted and atrophied seminiferous tubule, loss in number of germ cells, arrested spermatogenic cells in various stages of division, cell debris in the lumen of lobule, cytoplasmic vacuolization in many cells. In RSV treated stressed testis, thickness of basement membrane was reduced compared with that of stress group. The germ cells in tubules were arranged in an order like control group (Fig. 2).

Hepatic indices
Redox imbalance in the body has its primary effect on the hepatic tissues altering the activity of hepatic enzymes resulting altered metabolic activity. The levels of hepatotoxicity markers such as SGOT, SGPT, and ALP in HS groups were significantly increased and treatment with RSV significantly improved the status in a dose-dependent manner (1 mg, 5 mg, and 10 mg/kg body weight) (Fig. 3b-d).

SOD activity
SOD has a major role in maintenance of the redox balance of the body. It converts more active superoxide to comparatively less reactive H_2O_2.

Table 1: Comparison of the effect of whole body heat exposure on the body weight and rectal temperature of the experimental animal groups which were heat stressed, both without and with different dose treatment of resveratrol

Group of experimental animals	Body weight (g)*		Rectal temperature (°C)#	
	Before stress	After stress	Before stress	After stress
HS	121.67±4.41	118.33±3.33	37.38±0.08	40.12±0.07
HS+RSV (1 mg /kg)	125.67±0.67	123.33±1.67	37.52±0.06	39.94±0.05
HS+RSV (5 mg /kg)	115±2.89	115±2.89	37.37±0.09	39.97±0.09
HS+RSV (10 mg /kg)	125±2.89	125.67±3.48	37.27±0.1	39.93±0.12

*Body weights of the animals were recorded just before the start of the heat exposure and were recorded after the end of the HS period of 7 days, just before sacrifice, #Rectal temperature was recorded daily during the HS period of 7 days, just before and immediately after the heat exposure. Values are expressed as mean±SEM. (n=6). $p<0.05$ was considered as significant. SEM: Standard error of mean, HS: Heat stress, RSV: Resveratrol

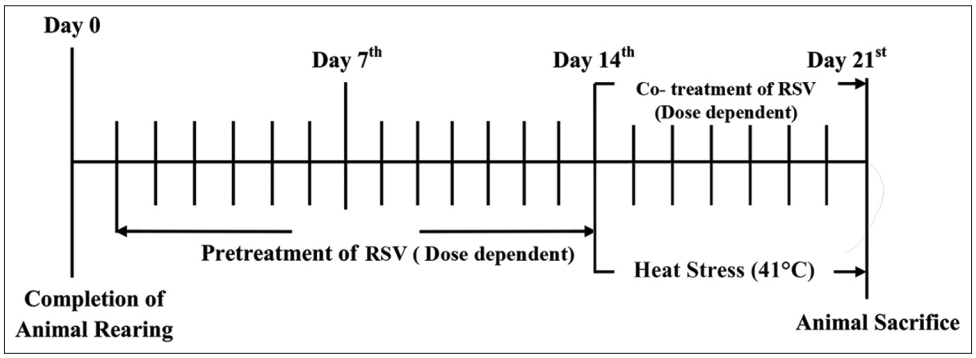

Fig. 1: Experimental design: Schedule and the intervals, illustrating the experimental design of induction of heat stress, and application of resveratrol as a protector

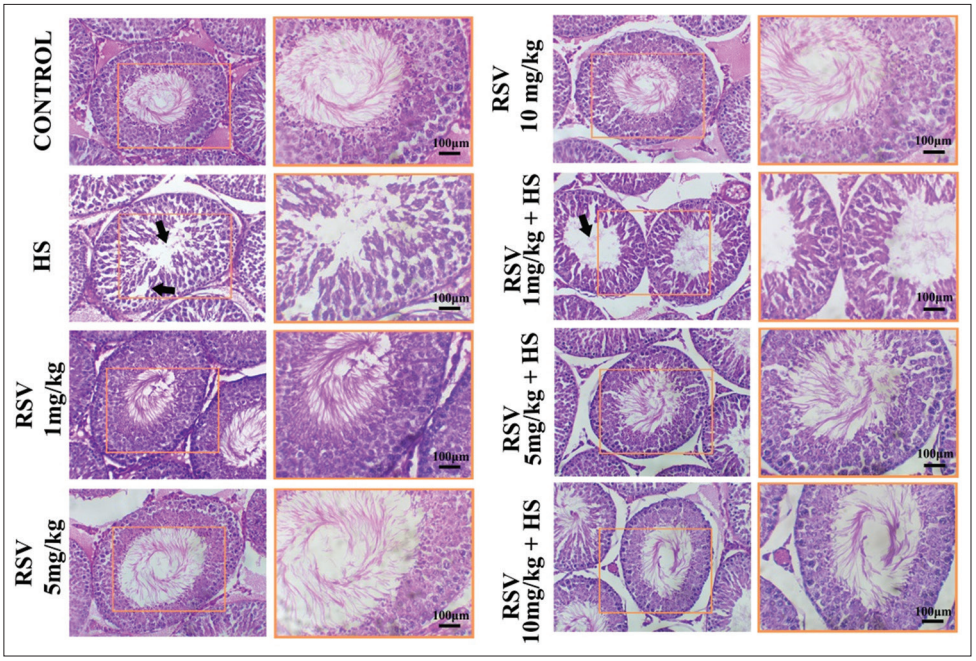

Fig. 2: Histological changes of heat stress induced testicular tissue and modulation by resveratrol: Tissue architectural morphology was determined by hematoxylin and eosin staining (Magnification 20×, 40×). A representative micrograph is showing H&E stained testis for the experimental groups

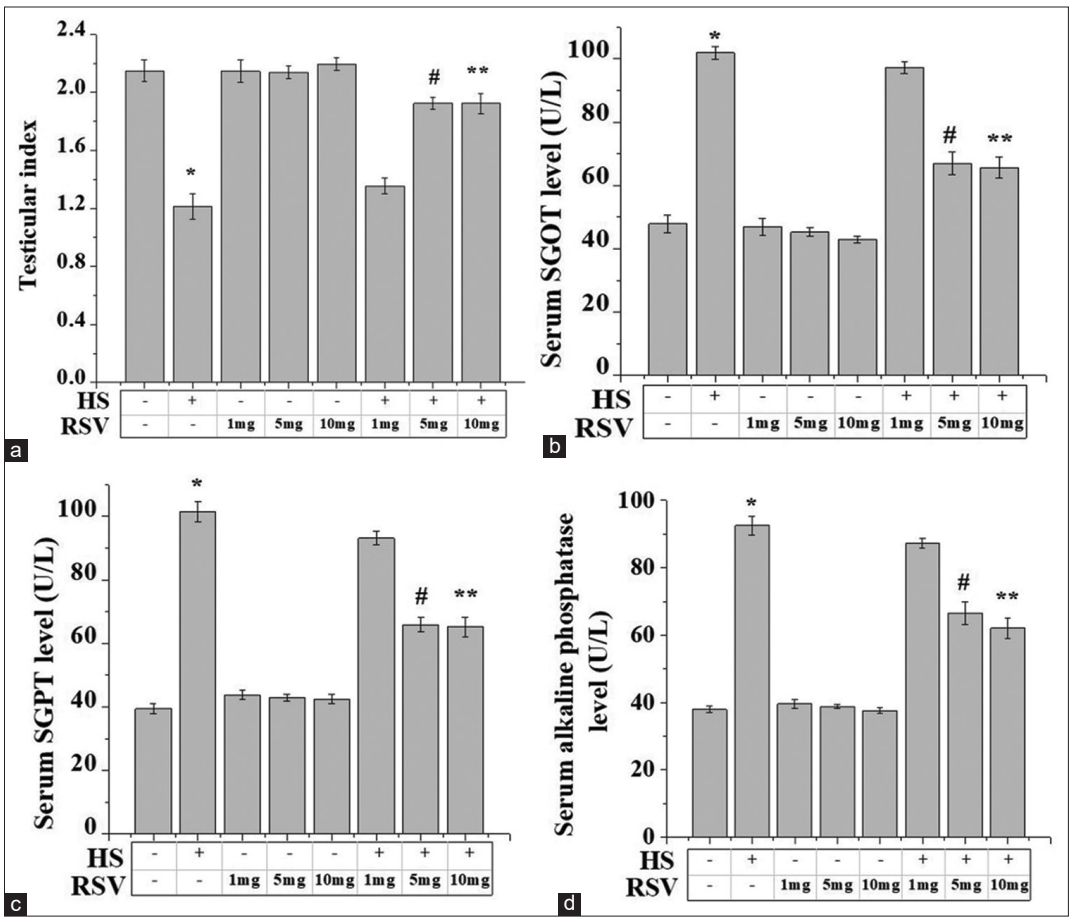

Fig. 3: Ameliorative effect of resveratrol (RSV) on the heat exposed animals: (a) Graph showing the testicular index Bar graph representing the activities of (b) serum aspartate amino transferase, (c) serum alanine aminotransferase, and (d) serum alkaline phosphatase of the different experimental group. Values are presented as mean±SEM (n=6) p<0.05 was considered as significant. Statistical comparison: *Control versus heat stress (HS); #HS versus RSV (5 mg/kg)+HS; **HS versus RSV (10 mg/kg)+HS

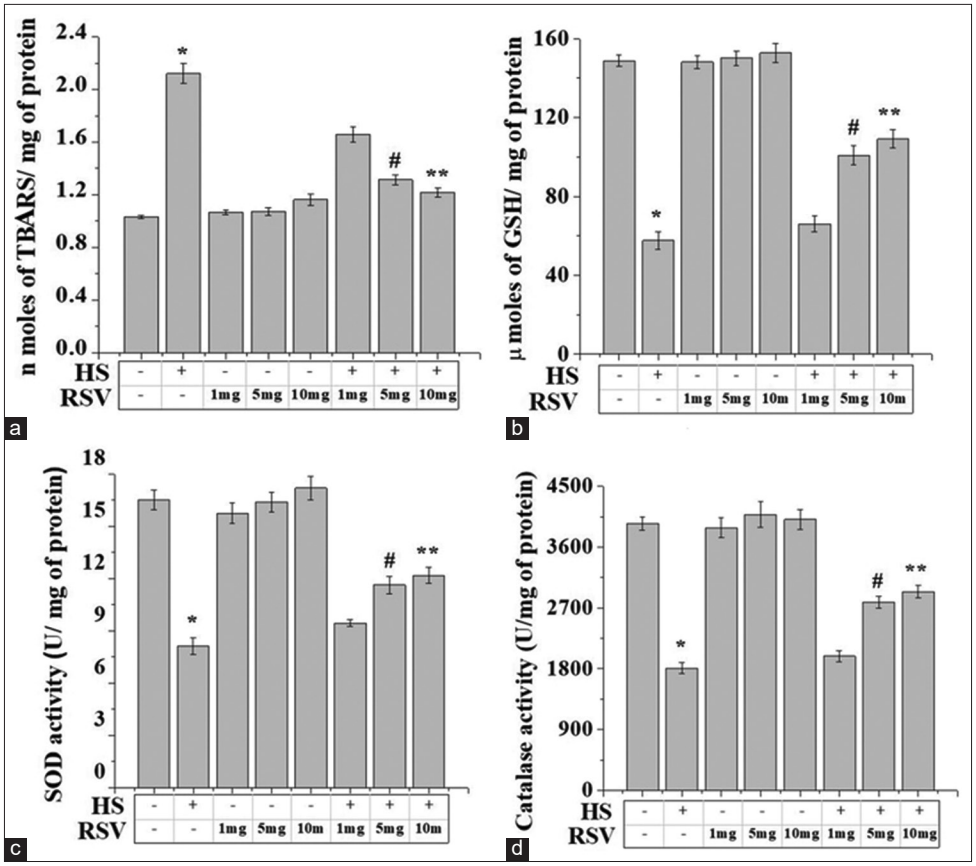

Fig. 4: Modulatory role of resveratrol (RSV) on the alteration of heat stress (HS)-induced oxidative stress indices: (a) Lipid peroxidation, (b) reduced glutathione, (c) Super oxide dismutase (SOD) activity, (d) catalase activity. Values are represented as mean±SEM (n=6). p<0.05 was considered as significant. Statistical comparison: *Control versus HS; #HS versus RSV (5 mg/kg)+HS; **HS versus RSV (10 mg/kg)+HS

SOD activity was significantly reduced in HS group. RSV significantly ameliorated this hyperthermia induced change and activity levels were gradually increased in the animals treated with dose of 1 mg, 5 mg, and 10 mg/kg body weight. RSV at a dose of 5 mg/kg showed higher efficiency than other two doses (Fig. 4c).

Catalase activity

Catalase is a peroxisomal enzyme which actively converts highly reactive H_2O_2 to non-reactive water and oxygen. Catalase activity was significantly decreased after heat exposure. The activity of catalase was significantly regained by RSV treatment (dose of 1 mg, 5 mg, and 10 mg/kg body weight). No significant changes between control and protection groups were found. RSV at the dose of 5 mg showed higher antioxidant activity than other two doses (Fig. 4d).

GSH level

GSH is a non-enzymatic endogenous antioxidant. Higher cellular level of GSH indicates a less stressed healthy cell having better capacity to scavenge ROS. GSH level was significantly decreased in HS group compared to control group. Dose-dependent studies of RSV pre-treatment on HS animals have shown that most effective increase of GSH level was at a dose of 5 mg/kg body weight (Fig. 4b).

LPO

The level of LPO is the primary marker of ROS induced damage. It is expressed as n moles of thiobarbituric acid reactive substances (TBARS) per mg of protein. In HS group, the TBARS level was significantly increased in comparison to control group. RSV significantly ameliorated this effect after heat exposure in a dose dependent manner (1 mg, 5 mg, and 10 mg/kg body weight) (Fig. 4a).

RSV ameliorated HS-induced testicular ROS

To evaluate the increment in the level of iROS and its role in triggering oxidative damage in testis, and also to determine the specific action of RSV in ameliorating HS induced iROS, testicular iROS was estimated using FACS. The level of iROS generation within the testicular cells is directly proportional to the rise in relative data capture format (DCF) fluorescence. The relative DCF fluorescence intensity was elevated in the stress exposed group compared to that of the untreated group. Whereas, it was markedly decreased by the RSV pre-treatment in the heat exposed group, compared to the HS group. Pre-treatment of RSV alone does not alter ROS generation compared to the control, indicating that RSV may inhibit iROS generation by scavenging free radicals in stressed condition (Fig. 5).

DISCUSSION

Exposure of animals to hot environment have large effects on reproductive functions such as disruption in spermatogenesis, oocyte development, oocyte maturation, and early embryonic development since male gonads are vulnerable to oxidative stress due its complex structure and presence of high concentration of unsaturated fatty acids, the present investigation was designed to confirm the role of free radicals in HS induced oxidative damage in testis of rat and to evaluate the protective role of RSV in acute whole body heat induced testicular damage in a murine system.

The hyper thermic stress in this study was enough to elevate rectal temperature from 37±1°C to 39±1°C after acute heat exposure for 1 h daily. The reduction in body weight caused by stress was probably due to reduced food intake and water consumption or exhaustion of body reserves as a result of increased metabolic activity [18] during stress.

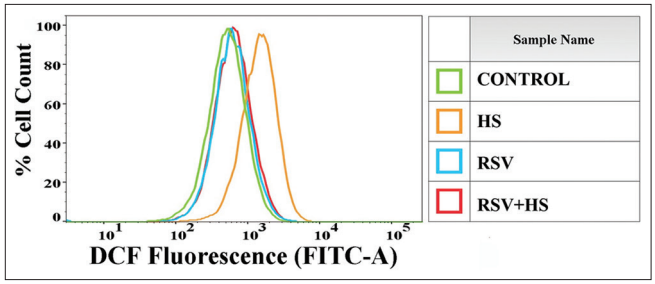

Fig. 5: Representative fluorescence-activated cell sorting histogram showing intercellular data capture format fluorescence in isolated testicular cells: Cells were isolated from respective rat testicles after completion of the heat stress and resveratrol treatments

RSV is a phytoalexin found in skin of grapes mulberries, peanuts. It plays an important role in anti-aging therapy as it is an important free radical scavenger [19].

RSV has also shown to inhibit cellular events associated with tumor initiation, promotion and progression [20]. Pre and co-treatment of RSV prevented this reduction of body weight significantly. This may be due to the fact that RSV being antioxidant reduced the excessive metabolic activity caused by stress [21]. The significant reduction of testicular weight and cytological changes in HS animals clearly suggest that elevated ambient temperature can alter the testicular structure. To get the effective dose of RSV as testiculo protector, dose-dependent responses were studied using 1 mg, 5 mg, and 10 mg/kg body weight, and significant result was obtained using RSV at a dose of 5 mg/kg body weight of rat.

Elevation of serum SGPT, SGOT, and ALP in HS animals indicates the disturbances in transamination reaction in liver cells. Decrease in GSH contents in testicular cells of hyperthermic rats suggests the disturbed testicular redox pool in HS rats [22] leading to tissue damage and LPO [23]. Several studies have reported that heat exposure could result in oxidative stress, which in turn could lead to cytotoxicity [24]. Rise of LPO in testis due to acute hyperthermia indicates the pathological consequences in rats due to heat induced oxidative stress. Malondialdehyde (MDA) is considered as an index of LPO, and this level reflects the degree of damage of testicular tissue. However, the result in RSV pre-treatment group showed decreased level of MDA as compared to stressed group. This indicates that RSV could scavenge free radicals and suppress the LPO as shown by lower TBARS level, and ameliorate the heat induced testicular damage.

This work provides enough evidence that RSV has a protective effect against heat induced oxidative stress in rat's testis. The protective action is possibly by elevating the GSH concentration and decreasing TBARS levels and by attenuating free radical scavenging enzymes. The adverse effect of whole body heat exposure may be due to enzymatic and non-enzymatic anti-oxidant defense dysregulation or the consequences of the oxidative stress due to poor ROS scavenging phenomenon that results in damage of testicular cells. From the results of intracellular antioxidant levels and oxidative damage marker, 5 mg/kg body weight of RSV dose was chosen for further molecular studies.

Numerous studies have reported that hyperthermic stress leads to excessive ROS generation, along with DNA damage [25]. This may cause male infertility [26]. Testis is one of the most heat sensitive parts of the mammalian system and even a single mild hyperthermic shock may result in an irreversible testicular damage [27]. Elevated level of ROS can hamper the cellular function. The result of the present study demonstrated a marked decrease in HS induced ROS generation with RSV pre-treatment, indicating the role of RSV in scavenging heat induced ROS generation by boosting the intracellular antioxidant system.

CONCLUSION

This study proved that RSV at a dose of 5 mg/kg of body weight protects rat testis against whole body heat induced oxidative damage most efficiently among the three doses. This work provides evidences that RSV has a protective effect in heat induced oxidative stress in testicular cells of rats by controlling stress induced ROS generation. This in mainly achieved by antioxidative action of RSV and increased levels of endogenous antioxidants such as SOD, CAT, and GSH. This, in turn, reduces tissue LPO levels to protect the tissue further. Thus, supplementation of RSV may improve the fertility in male residing or working in hot environment.

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AUTHORS CONTRIBUTION

Study designed: Puja Roy, Asima Das; Performed Research: Puja Roy, Soumya Sundar Kumar; Data Analyzed: Puja Roy, Krishnendu Manna, Soumya Sundar Kumar; Contributed new methods or models: Soumya Sundar Kumar, Asima Das; Wrote the paper: Puja Roy, Soumya Sundar Kumar; Drafting the paper or revising it critically: Asima Das; Approval of the submitted and final versions: Asima Das

CONFLICTS OF INTERESTS

The authors have declared no conflict of interest.

AUTHORS FUNDING

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ETHICS APPROVAL

The animal ethical clearance was obtained from IAEC approved by CPCSEA (Reg. no. 1946/PO/Re/S/17/CPCSEA).

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