Supplementation File

Materials and methods

Testicular index

The testicular index or the relative testicular weight index was calculated as a measure of testicular weight loss due to thermal stress induced degenerative changes [1].

The body weight (W) of the experimental animal was recorded just before the sacrifice. Testes were dissected out of the experimental animal after sacrifice and weighed(T). Testicular index (I) of the individual animals was calculated using the following formula.

Testicular index (I) = T / W × 100

Preparation of the sperm sample

Cauda epididymis was dissected out of the experimental animals immediately after sacrifice and spermatozoa sample was prepared using the diffusion technique of sperm collection described by Slott VL et al with some modifications [2]. Briefly, cauda epididymis was immersed into 1 ml of preheated (37°C) 5% sucrose solution in phosphate buffer on a watch glass, and few incisions were made on the epididymal wall to make way for the matures sperms to diffuse out into the medium. It was then incubated in a CO_2 incubator at 37°C, 5% CO_2 for 30 minutes. The epididymis was taken out of the fluid, and the fluid with diffused out spermatozoa was used for the analysis.

Sperm count

Sperm count was done using an improved Neubauer Haemocytometer counting chamber [3]. Briefly, 10µl of the sperm sample solution was taken using a micropipette and poured between the space of the cover glass and the Haemocytometer, to fill the chamber altogether. The Haemocytometer was kept at the room temperature for 15-20 min to allow the sperms to settle down. The counting was done using the central 25 squares three times with the same sample, and the mean was used for the calculation. The calculation was done using the following formula. Where N is the mean sperm count in the central 25 chambers of the Haemocytometer and V is the total volume of the 25 Haemocytometer chambers in ml.

Sperm count per ml of diluted sperm sample = N/V

Histological assessment

Method of this part was discussed in the main manuscript. Eosine-Hematoxylin stained tissue sections of the testis were represented here. The images were captured using Dewinter, DGI510CCD microscope (magnification: 20X).

Determination of testicular lipid peroxidation

This method has discussed in the main manuscript.

Results

Testicular index

The result of the testicular index showed that the exposure to 41°C for 1 hr reduced the value to 24.39% as compared to the control animals. On the other hand, the values were reduced to 5.94% and 7.1%, respectively when exposed to 39°C for 1 hr and 39°C for 2 hr, indicating that the exposure of 41°C for 1 hr was more lethal in terms of testicular dysfunction than the other experimental hyperthermic conditions (**Fig. SC**).

Sperm count

In case of sperm count, the number was significantly reduced (51.97%) when the animals were exposed to 41°C for 1 hr. However, upon the exposure of 39°C for 1 hr and 39°C for 2 hr, the reduction in the sperm counts was nonsignificant (**Fig. SA**).

Histological assessment

Under light microscopic observation, a significant degenerative change in testicles was evident when the animals exposed to 41°C for 1 hr. Necrotic changes in the cells around the seminiferous tubule were also seen as well as the number of spermatozoa in the lumen of the tubule was reduced markedly when exposed to 41°C for 1 hr. (**Fig. SD**)

Lipid peroxidation assessment

The Lipid peroxidation (LPO) also supported the observation obtained from the sperm count, testicular index and histological studies, as the TBARS levels were much higher in case of the animals exposed to 41°C for 1 hr as compared to the control and the other two groups. (**Fig. SB**)

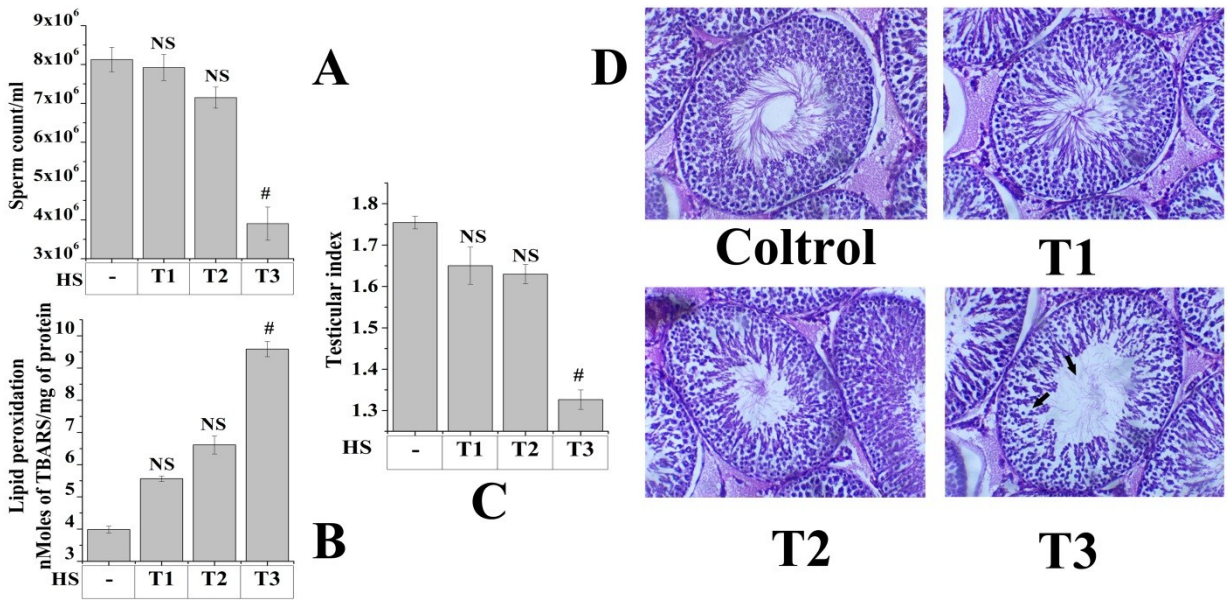

Fig: S. Evaluation of the testicular dysfunction by different heat exposed conditions (both degree and duration). Representative bar graph showing (A) Sperm count, (B) LPO, (C) testicular index. Tissue architectural changes were determined by H&E staining (Magnification20×). (D) A representative micrograph is showing H&E stained testis for the experimental groups. T1: 39°C heat exposure for 1 hr, T2: 39°C heat exposure for 2 hr, T3: 41°C heat exposure for 1 hr. Values are represented as mean ± SEM (n=6). $p<0.05$ was considered as significant. Statistical comparison: #Control vs. T3; NS= Non-significant.

Discussion

The modulation of the testicular index, the degree of lipid peroxidation in testis of rat exposed to different degree and duration of heat exposure, indicated a significant degenerative change which was developed at the ambient temperature of 41°C for 1 hr. The rectal temperature of the animals attended 39°C, suggesting a possible involvement in altering testicular function. Exposure to a higher temperature above 41°C for 1 hour or higher exposure time at 41°C were proved to be fatal for the experimental animals. The result also demonstrated that heat exposure with 41°C for 1 hr could be useful for the development of experimental heat stress model for testicular dysfunction.

Reference

1. D. S. Das, N. Wadhwa, N. Kunj, K. Sarda, B. S. Pradhan and S. S. Majumdar, Dickkopf Homolog 3 (DKK3) Plays a Crucial Role Upstream of WNT/β-CATENIN Signaling for Sertoli Cell Mediated Regulation of Spermatogenesis, *PLoS ONE*, 2013, 8, e63603.
2. V. L. Slott, J. D. Suarez and S. D. Perreault, Rat sperm motility analysis: methodologic considerations, *Reprod. Toxicol.*, 1991, 5, 449-458.
3. Y. Wang, Epididymal Sperm Count, *Current Protocols in Toxicology*, 2002, 14, 16.16.11-16.16.15.