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An Insight into the Histological Changes in Rodent Brain in Response to Graded Hyperthermia

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Abstract—*Environmental heat exposure is a natural hazard with large number of mortality across the globe. Exposure to Heat Stress is known to be associated with a plethora of physiological alterations to all the systems in the body. These complications are further exacerbated in case of Heat Stroke (HS). The intensity of HS induced death is rising with worldwide increase in the frequency and intensity of heat waves due to global warming. An individual undergoes various physiological, cellular and molecular modifications upon exposure to the dry and hot environment. This way the highly dynamic human system keeps a check to maintain its homeostasis. The extreme conditions have a tremendous impact on the Hypothalamic Pituitary Adrenal (HPA) Axis, resulting in thermoregulatory failure that can significantly alter the landscape of the organism giving rise to various Heat related maladies and Central Nervous System (CNS) Dysfunction. Suitable diagnostic and therapeutic markers need to be established to reduce mortality and morbidity associated with HS. So, our present study focuses on exploration of pathological changes in response to graded hyperthermia in different regions of rodent brain. Rats were exposed to Heat Stress (Ambient Temperature, T_a=45±0.5°C and Relative Humidity, RH=30±10%) in a heat simulation environmental chamber. Continuous measurement of Core Temperature, T_c and Skin Temperature, T_s was monitored. After completion of heat exposure, different regions of the brain were subjected to histological analysis. Concomitant increase of pyknotic neurons in hippocampus was observed with increase in T_c of heat exposed rats. Neuronal damage also occurred in the cortical region, with the widening of VRS (Virchow Robin Space). This is the first study correlating the core body temperature to changes in structural integrity of brain relating the severity of heat stress to extent of damage. Thus, HS culminates in perturbation of structural integrity of the brain.*

Keywords*: Heat Stroke; Hippocampus; HPA Axis; CNS Dysfunction; VRS*

1. INTRODUCTION

Heat stress is a common stressor that affects biological systems. Exposure to heat stress is known to be associated with a plethora of physiological alterations to all the systems in the body. Heat stress occurs when the thermoregulatory mechanism gets activated and when the system is unable to dissipate heat from the body to its surrounding ultimately resulting in hyperthermia. Heat stroke (HS), on the other hand is a pathological condition that is characterized by an elevated core temperature (>40°C), resulting in multi-organ dysfunction (MOD) and CNS dysfunction [1]. The intensity of HS induced death is rising with worldwide increase in the frequency and intensity of heat waves due to global warming [2,3]. That is why it becomes a matter of concern for all of us. When heat is combined with other stressors (viz. physical work, dehydration, fatigue, medical conditions, etc.) it leads to heat related illnesses, disability or even death. Depending on the magnitude, severity and duration of hyperthermia, CNS abnormality will result probably due to release of various neurochemicals and alteration in signal transduction pathways. Exposure to high temperatures induces cognitive impairment in experimental animals and humans [4,5]. Since, CNS is extremely sensitive to hyperthermia, therefore, brain, being the master regulator of all the physiological systems, needs to be investigated on a broader scale. So, we investigated the impact of graded hyperthermia on histology of rodent brain. Integrating these findings with genetic level studies will provide deeper insights into development of detection and treatment options for HS.

2. MATERIALS AND METHODS

2.1. Experimental Animals

Adult male *Sprague dawley* rats, (weight, 250-300 grams) were obtained from the Animal Resource Center of Defence Institute of Physiology and Allied Sciences (DIPAS), Defence Research and Development Organization (DRDO). The animals were housed at an ambient temperature of 25±1°C, with a 12-hour light/dark cycle. Pelleted rat chow and tap

water were made available *ad libitum*. All the protocols were approved by the Animal Ethical Committee of the Institute.

2.2. Heat stress protocol and experimental groups

All rats were handled daily and familiarized with the rectal temperature probe (Rectal Probe for Rats, RET-2, AD instruments) during the week preceding the heat stress protocols for each group of rats. On the day of the heat exposure, each rat was fitted with the rectal temperature probe inserted 6–7 cm into the rectum and then placed in a plastic cage, conscious and unrestrained. Rectal temperature was continuously monitored on a digital display using Lab chart 7, AD instruments. The experiments were terminated when the targeted core body temperature was attained. Animals were randomly assigned to 1 of the following 5 groups with 6 rats in each group (n=6): Group 1 was exposed to T_a=25±0.5°C and RH=30±10% in a heat simulation environmental chamber for 30 minutes to reach thermal equilibrium. This group served as the control. Group 2 was exposed to T_a=45±0.5°C and RH=30±10% till the rectal temperature reached 39°C. Similarly, animals in group 3 and group 4 were exposed to T_a=45±0.5°C and RH=30±10% till the rectal temperature reached 40 °C and 41°C respectively. For group 5, the exposure to T_a=45±0.5°C and RH=30±10% was continued till heat stress induced death of rats.

2.3. Sample collection

The heat exposed and control animals were anesthetized with an intraperitoneal dose of 80 mg/kg ketamine and 5 mg/kg xylazine. For histological analysis, rats were perfused transcardially with ice cold PBS, followed by 4% paraformaldehyde solution and whole brain was kept in 10% formalin.

2.4. Sectioning Paraffin-embedded Tissues

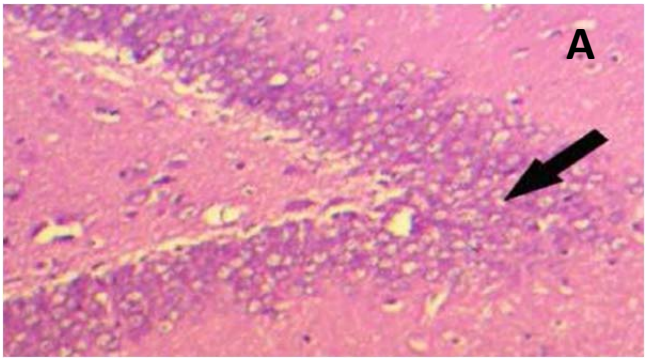
The tissue was immersed in 70% ethanol three times for 30 minutes each at room temperature (RT) followed by immersion in 90% ethanol two times for 30 minutes each. The tissue was then immersed in 100% ethanol three times for 30 minutes each at RT. Finally, the tissue was immersed in xylene (mixed isomers) three times for 20 minutes each at RT. Now, the tissue was embedded in paraffin at 58°C. 5-15 µm thick tissue sections were obtained using a rotary microtome. The sections were floated in a 56°C water bath. The sections were mounted onto gelatin-coated histological slides. The slides were dried overnight at RT and stored at RT till hematoxylin and eosin (HE) staining was performed.

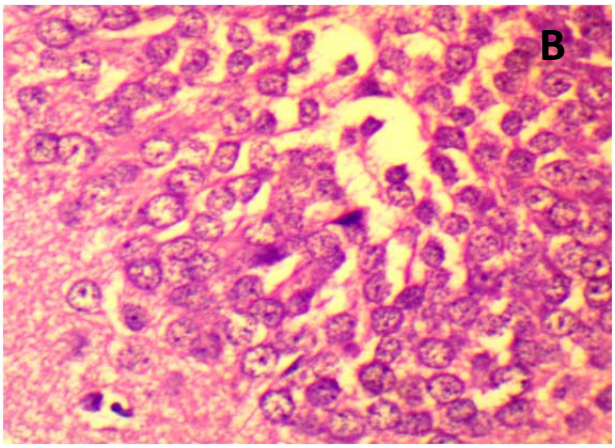
2.5. Hematoxylin and Eosin (HE) Staining

The samples were deparaffinised by 3 washes of 100% xylene, 3 minutes each. This was followed by 3 washes, 3 minutes each in decreasing concentrations of ethanol (95 and 90% respectively) and a final distilled water wash. The sample was then stained in hematoxylin for 6 minutes and rinsed in running tap water for 20 minutes followed by a 3 second wash in acid alcohol. The sample was rinsed well in tap water for 5 minutes followed by immersion in lithium carbonate for 3 seconds. The sample was then rinsed in tap water for 5 minutes, followed by counterstaining in eosin for 15 seconds. The sample was dehydrated by incubation in increasing concentration of ethanol (2 washes in 95% ethanol followed by 2 washed in 100% ethanol). The sample was cleared in Xylene and mounted [6]. Finally slides were subjected to microscopic evaluation.

3. RESULTS

Histological investigation was performed and representative photomicrographs of different regions of the brain were taken. When the core temperature of rats reached 39°C, only moderate edema occurred (Fig 3.2) as compared to control (Fig 3.1). Neuronal degeneration and necrosis were detected at >39° temperature (Fig 3.3). As the core body temperature progresses from 37°C to 41°C (temperature characterizing the onset of heat stroke), the lesions in the brain were further aggravated (Fig 3.4). VRS (Virchow Robin Space) widening in the cortical region and edema with 75% pyknotic neurons (Table 3.1, Fig 3.3,3.4,3.5) as compared to control (Fig 3.1) has been observed in heat stroked rats (Fig 3.4,3.5).





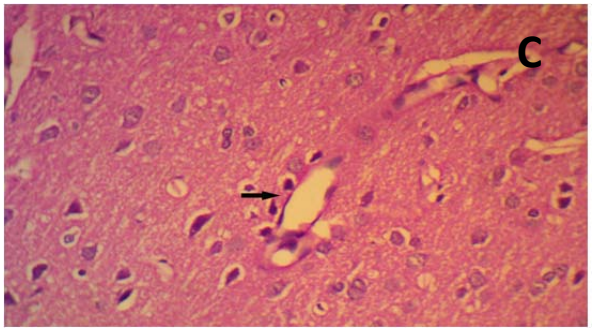


Figure 3.1. HE staining of Group 1 (Control) rat brain showing A. dentate gyrus (DG) region of Hippocampus (Arrow) with normal neurons (100X); B. normal neurons with large round vesicular nuclei in DG region (400X); C. Cerbral Cortex showing a cortical blood vessel with minimal peri-capillary (Virchow-Robin) space (Arrow) (400X)

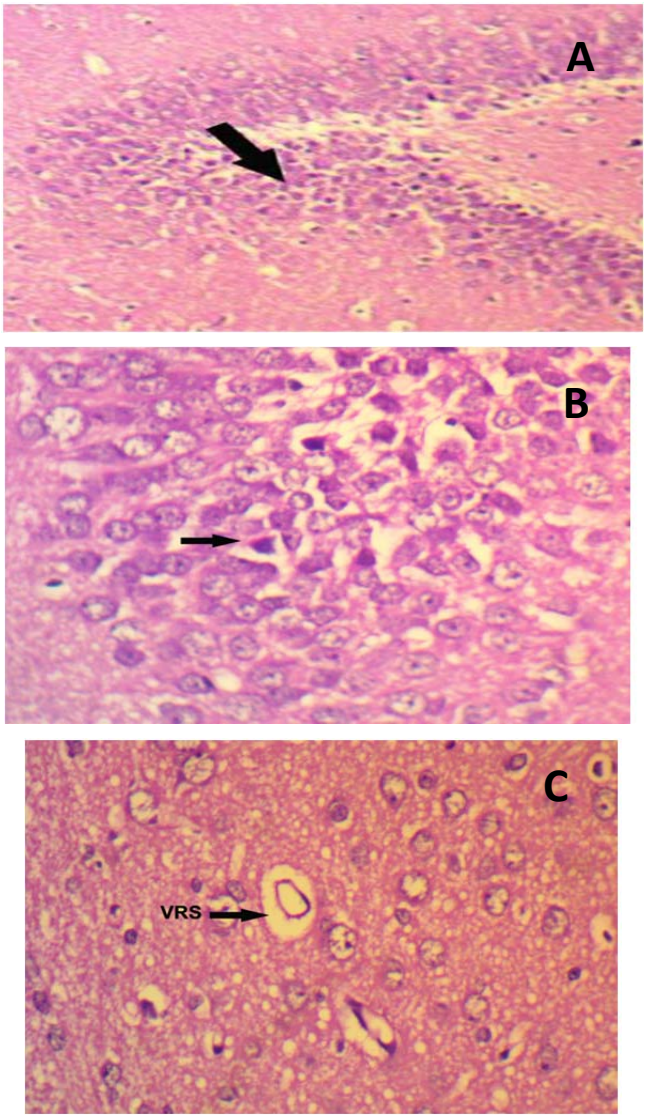


Figure 3.2. HE staining of Group 2 rat brain showing A. DG region (Arrow) with edema and occasional darkly staining neurons (100X); B. DG area showing mainly normal neurons with large round vesicular nuclei with some pyknotic neurons (Arrow) (400X); C. Cerbral Cortex showing a cortical blood vessel with widening of the peri-capillary (Virchow-Robin) space (Arrow) (400X)

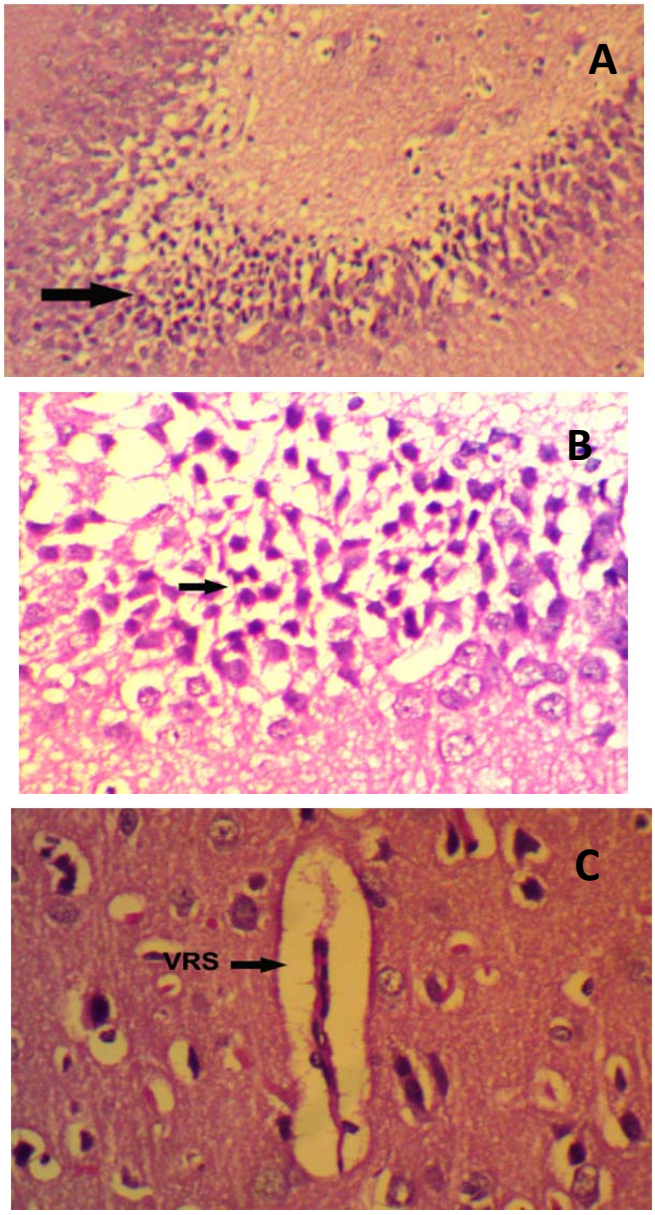


Figure 3.3. HE staining of Group 3 rat brain showing A. DG region (Arrow) with edema and several darkly staining neurons (100X); B. DG area showing edema and about 70 % of the neurons showing pyknosis with dense and darkly staining nuclei (Arrow). The surviving normal neurons are seen lying along the lower edge of the image (400X); C. Cerbral Cortex showing a cortical blood vessel with evident widening of the peri-capillary (Virchow-Robin) space (Arrow) (400X)

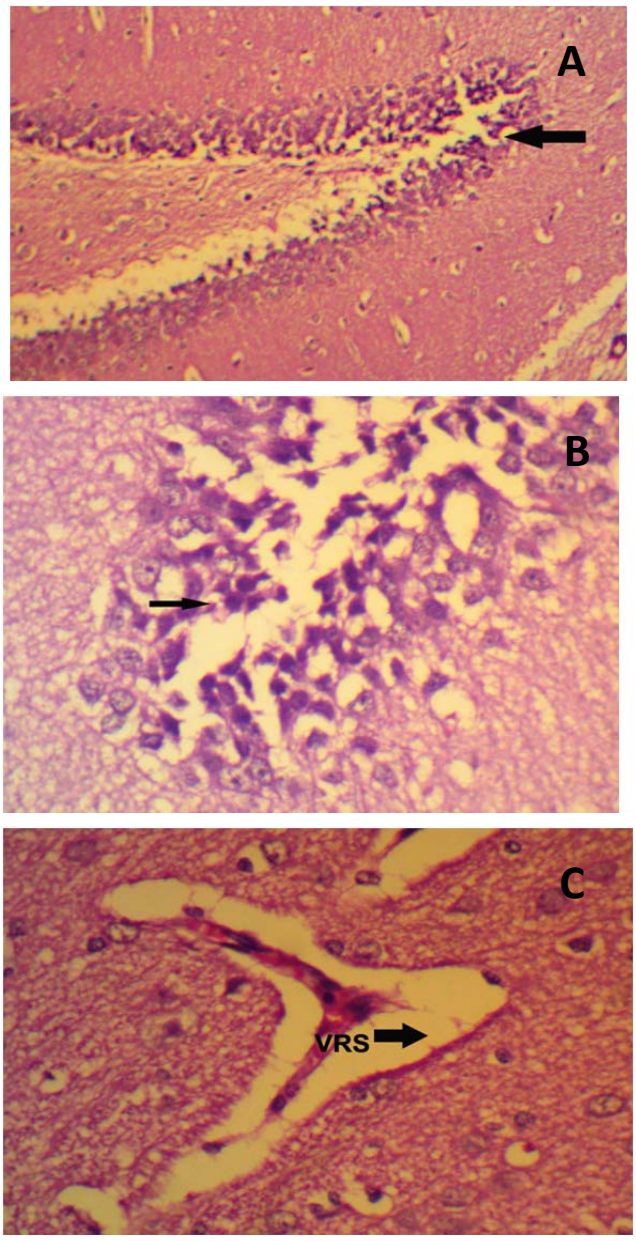


Figure 3.4. HE staining of Group 4 rat brain showing A. DG area (Arrow) with edema and several darkly staining neurons (100X); B. DG area showing about 50 % of the neurons showing pyknosis with dense and darkly staining nuclei (Arrow). The surviving normal neurons are seen lying along the outer margin of the DG (400X); C. Cerbral Cortex showing a cortical blood vessel with evident widening of the peri-capillary (Virchow-Robin) space (Arrow) (400X)

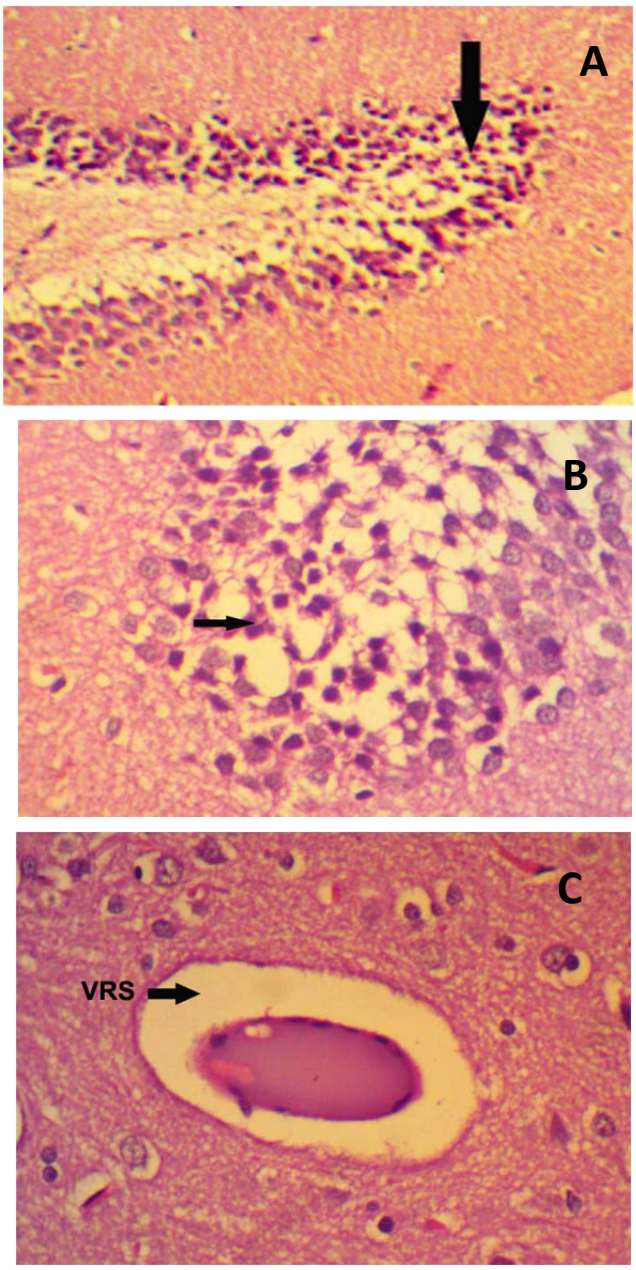


Figure 3.5. HE staining of Group 5 rat brain showing A. DG region (Arrow) with edema and several darkly staining neurons (100X); B. DG area showing edema and about 75 % of the neurons showing pyknosis with dense and darkly staining nuclei (Arrow). The surviving normal neurons are seen on the right edge of the image (400X); C. Cerbral Cortex showing a cortical blood vessel with evident widening of the peri-capillary (Virchow-Robin) space (Arrow) (400X)

Table 3.1: Showing the extent of damage to the Dentate Gyrus Neurons of the Hippocampal region and widening of the Virchow Robin Space of the Cortical region in Heat stressed Rat Brain compared to Control

Sl. No.	Experimental Groups	Hippocampus (Damage to DG Neurons)	Cortex (Widening of VRS)
1	1	Nil	Nil
2	2	+	++
3	3	+++	+++
4	4	++	+++
5	5	+++	+++

4. DISCUSSION

Heat Stroke is a form of hyperthermia, characterized by systemic inflammatory response resulting in MODS with delirium, convulsion and coma and other CNS disorders [1]. Suitable diagnostic and therapeutic signatures for HS need to be established to alleviate morbidity and mortality associated with HS. We investigated the effect of increasing magnitude of heat stress culminating in HS on histological aspects of brain. The histology was compared in all 5 groups with an objective of facilitating the understanding of structural changes with increase in degree of severity of hyperthermia and its correlation with alteration in functionality.

The blood–brain barrier (BBB) is a complex structure restricting passage of substances from the bloodstream much more than the endothelial cells in capillaries elsewhere in the body. The BBB acts very effectively to protect the brain from many toxins and common bacterial infections. It has been observed that acute heat stress causes increase in permeability of BBB [7,8] which has the most dangerous repercussions on the physiology of the individual as a whole.

In our present study, histological evaluation of heat exposed rodent brain revealed that rise in core body temperature was found to be associated with an increase in the level of brain damage, with the degree of hyperthermia and brain damage increasing proportionately. As the core temperature of rats increased more than 2°C than normal, moderate edema occurred (Fig 3.2) with respect to control (Fig 3.1) [9]. Normal neurons with large round vesicular nuclei with some pyknotic neurons were observed in Dentate Gyrus (DG) region of the hippocampus (Fig 3.2) (centre for learning and memory in the brain). The number of pyknotic neurons (about 70%) in hippocampal DG area was found to be increased at >39°C (Fig 3.3). As the core body temperature reached 41°C, the lesions in the DG region of brain were further aggravated, as evident with dense and darkly stained nuclei indicating higher extent of brain damage at higher temperature which is in consistent with the previous studies (Fig 3.4) [9,10]. These results clearly suggest that increased pyknotic neurons in hippocampal area due to heat stress are associated with memory impairment in rodent brain [10].

Virchow–Robin spaces (VRS), also known as perivascular spaces, are the immunological spaces between the arteries and veins (not capillaries) and pia mater that can be expanded by leukocytes. VRS may be enlarged to a diameter of five millimeters in healthy humans and are usually harmless. When enlarged, they can disrupt the function of the brain regions into which they project [11]. One of the most basic roles of the VRS is the regulation of CNS fluid movement and drainage. VRS ultimately drain fluid from neuronal cell bodies in the CNS to the cervical lymph nodes [11]. Another role of the VRS is as an integral part of the blood–brain barrier (BBB) [12]. Recent ongoing research has found associations between enlarged VRS and several neurodegenerative disorders like dementia, alzheimer's disease, stroke, multiple sclerosis, autism. In our study, VRS widening and edema with 75% pyknotic neurons (Table 3.1, Fig 3.3,3.4,3.5) as compared to control (Fig 3.1) has been observed in HS rats (Fig 3.4,3.5). Maximum VRS widening was observed in case of heat exposure till death indicating most severe damage to the cortical region ultimately leading to the alteration in the regulation of CNS fluid movement and drainage. These findings are consistent with the clinical manifestations of heat stroke patients with CNS dysfunction and neurological morbidity.

This study, combined with biochemical and genome-wide studies on heat stress and HS will provide insights into the mechanisms underlying the pathophysiology of organ damage upon progression from mild and moderate hyperthermia to HS with increasing core body temperature (in a graded pattern) providing cues for development of appropriate diagnostic and therapeutic measures for heat related illnesses including the potentially fatal HS. This is the first study correlating the core body temperature to changes in structural integrity of brain relating the severity of heat stress to extent of brain damage.

5. ACKNOWLEDGMENTS

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