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Research Article

Biochemical, Genetic, Pathological and Histochemical Alterations as Forensic Markers of Hypothermia in Rats

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Abstract

Human and animals have many adaptive mechanisms against extreme thermal conditions. Failure of these mechanisms induces various alterations in many organs especially brain. This study was performed on rats to investigate the effect of exposure to extreme cold till loss of consciousness or until death. Serum levels of glucose, Triglyceride (TG), Cholesterol (CHO), Total Protein (TP) and cortisol were determined. Malondialdehyde (MDA) and Total Antioxidant Capacity (TAC) were estimated in brain tissue. The genetic alteration of hypothermia was studied by measuring DNA damage using comet assay. Additionally, histopathological and immunohistochemical alterations in brain tissue were recorded. Hypothermia, significantly decreased serum glucose and CHO while increased TG and cortisol levels but had no effect on TP level. Also, marked increase in MDA level and DNA damage in brain tissue concurrently with reduction of TAC were recorded. Histopathological alterations including vasogenic perivascular edema, necrosis/ loss of Purkinje cells of cerebellum, neuronal degeneration/ necrosis and perineuronal edema in cerebrum and hippocampus were observed. Moreover, hypothermia decreased the immunopositivity of synaptophysin and neurofilaments but temporary increased the Glial Fibrillary Acidic Protein (GFAP) in brain tissue. In conclusion, hypothermia, despite several adaptive mechanisms, induced lethal harmful biochemical, genetic, pathological and immunohistochemical alterations which can be used as useful markers for hypothermia.

Introduction

In forensic pathology, diagnosis of death from hypothermia represents to some extent a problem due to the absence of specific features [1,2]. Hypothermia is defined as a decrease in body temperature below its normal physiological level [2,3]. Based on body temperature and clinical signs, hypothermia can be classified into mild, moderate, severe or profound hypothermia [4]. Prolonged exposure to subfreezing temperature increases the incidence of death [2].

The body can adapt to hypothermia or cold through vasoconstriction of blood vessels in the skin and muscle to conserve body heat. In addition, there is an increase in heat production through shivering and elevation of metabolic rate [5-8]. In case of inability of the body to adapt to hypothermia, the body experiences severe-cold related illness and permanent tissue damage such as cardiac arrhythmia, severe respiratory and central nervous system depression with reduction in hypothalamic function and metabolic rate that may end by death [4-9].

Postmortem biochemistry, light microscopy and Immunohistochemistry (IHC) are helpful tools in diagnosis of fatal hypothermia [2]. Exposure to cold is a type of stress that accelerates metabolic rate and helps in generation of Reactive Oxygen Species (ROS) that may overwhelm the cellular antioxidant defensive mechanisms and consequently induce oxidative stress [10-12].

It has been demonstrated that cold stress induces changes in biochemical parameters with significant increase of the lipid peroxidation product and reduction of antioxidant enzyme activities in rats and mice tissues [13-15].

Exposure of brain to short periods of hypothermia is helpful in cases of trauma; on the other hand, long exposure to hypothermia can harm the brain [4]. Pyramidal and glial cell degeneration, hemorrhage and vacuoles with varying degrees were reported previously in brain tissues during moderate (2-8°C) and severe (0 to -2°C) hypothermia [16].

Immunohistochemical expression of Glial Fibrillary Acidic Protein (GFAP) on hypothalamus sand synaptophysinon hippocampus has been performed previously during hypothermia [17,18]. To the best of our knowledge, immune-expression of neurofilament, GFAP and synaptophysin have not been investigated previously on cerebrum and cerebellum of brain suffering from hypothermia. Neurofilament and GFAP are indicators for neuron integrity, while synaptophysin plays an important role in neurotransmission which is present in all neurons in the brain and spinal cord that participate in synaptic transmission [19].

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This experiment was assigned to elucidate the effect of hypothermia on some biochemical, genetic, pathological and immunohistochemical markers in rats that may be useful as forensic markers.

Materials and methods

Animals

Thirty male Sprague-Dawley rats, weighing 150 ± 25 g, were purchased from Al-Zyade Experimental Animals Production Center (Giza, Egypt). All animals were kept in polypropylene cages with wood chip bedding. Rats were kept in a well-ventilated animal house of normal light–dark cycle (12 hrs light/dark) and temperature (26 ± 2°C). Food and water were provided *ad libitum*. Experimental design and all animal-handling procedures were approved by the Research Ethical Committee of the Faculty of Veterinary Medicine, University of Sadat City, Egypt.

Experimental design

After a week of acclimatization, rats were randomly divided into three equal groups, 10 rats each. Control group (G1) was kept at room temperature (26 ± 2°C). Animals of G2 and G3 were exposed to extreme cold and kept individually in small polypropylene cage with mesh wire cover (20×20×18 cm) at freezer of the refrigerator (-10 to -12°C) until loss of consciousness (G2) or death (G3). The rats of all groups were deprived totally from water and food along the experimental period.

Rats of G1 were anesthetized and blood samples were collected by heart puncture. Blood samples were collected by direct heart puncture from rats of G2 after being in the comatose state and from rats of G3 directly after sure death. Sera were separated and stored at -20°C as aliquots for further biochemical analysis.

After blood collection, rats were decapitated for brain tissues collection. Brains of five rats from each group were rapidly excised. The right half of each brain was washed with 0.9 % NaCl solution and kept at -20°C for further tissue biochemical analysis while the left halves were fixed in 10% Neutral Buffered Formalin (NBF) and prepared for histopathological and immunohistochemical investigations. Brains of the other five rats of each group were preserved in Phosphate Buffered Saline (PBS) at -20°C for comet assay.

Serum biochemical analysis

The sera were used for estimation of serum glucose, Triglyceride (TG), Cholesterol (CHO) and Total Protein (TP) colormetrically according to manufacture instructions of commercial kits [20-22]. Serum cortisol concentration (ng/ml) was determined by ELISA method [23].

Brain lipid peroxidation and antioxidant capacity

Brain tissue were homogenized for estimation of Malondialdehyde (MDA) level and total antioxidant capacity (TAC) following kits' instructions [24,25].

Comet assay

Comet assay or alkaline single-cell gel electrophoresis was performed using the modified method of Ellahueneet al. [26]. After the steps of cell lysis and electrophoresis, the slides were stained with 50 µL of ethidium bromide (2 mg/ml), cover slipped and examined using Zeiss Axioscope fluorescence microscope at 400X magnification. Comet image analysis was performed using Comet 5 image analysis software (Kinetic Imaging Ltd. Liverpool, UK) as the following: for each sample 100 randomly cells were selected, photographed and scanned. Cells with small heads and large fan-like tails which indicated apoptotic cells were excluded from the analysis [27].This image analysis system calculated the integrated intensity profiles for each cell, the comet cell components, and the range of derived parameters. DNA damage in brain cells was estimated by measuring percentage of DNA damage, intensity of DNA migration length (tail length) and percentage of DNA in tail. The tail moment was expressed as tail length multiplied by percentage of DNA in tail / 100.

Histopathology and Immunohistochemistry (IHC)

After 72 hrs of fixation in NBF, brain samples were dehydrated, embedded in paraffin wax, and sectioned, 3-µm thick sections, for Haematoxylin and Eosin (HE) staining, and 5-µm thick sections on positively charged slides, for immunohistochemical analysis.

To detect the synaptophysin and neurofilaments, the following protocol was used: After deparaffinization, tissue sections were treated with 3% H_2O_2 for 15 min at Room Temperature (RT) for endogenous peroxidase inactivation and then subjected to antigen retrieval by microwaving for 20 min at full power in citrate buffer (pH 5.4), followed by blocking with 10% Normal Goat Serum (NGS) for 5 min in the microwave. Tissues were incubated with the primary antibody (synaptophysin 1:100 dilution, Dako; Neurofilament 1:800, Novus biological; not to the negative control). After washing, sections were incubated with a secondary polymer reagent (Dako ChemMate INVISION Kit/HRP [DAP], Dako, Carpinteria, California, USA) for 30 min at RT. After further washing, the substrate was added (3,3'diaminobenzidine; Dako) and the sections were finally counter stained with haematoxylin and covered slipped under DPX mounting medium (Sigma Life Scienc, Steinheim, Germany).

To detect Glial Fibrillary Acidic Protein (GFAP) by IHC, the following protocol was used: deparaffinization, hydration with tap water for 20 min, followed by treatment with 5% H_2O_2 for 15 min and washing with Distilled Water (DW). Antigen retrieval was done using proteinase K for 5 min/ RT. Washing by DW then by Phosphate Buffer Saline (PBS). Blocking was done in microwave/ 5 min at power 3 (250 W) by using 5% bovine serum albumin. Tissues were incubated with the primary antibody (Polyclonal Rabbit Anti-Glial Fibrillary Acidic Protein, Dako, USA) (1 in 500 dilution) for 30 min/ RT. Labeling was done as mentioned above.

Statistical analysis

Data are presented as mean ± SE. Statistical significance of the data was analyzed using SPSS program version 16. For comparison, One-Way analysis of variance (ANOVA) test followed by Duncan's multiple range test for post-hoc analysis. Statistical significance was acceptable to a level of $p \leq 0.05$.

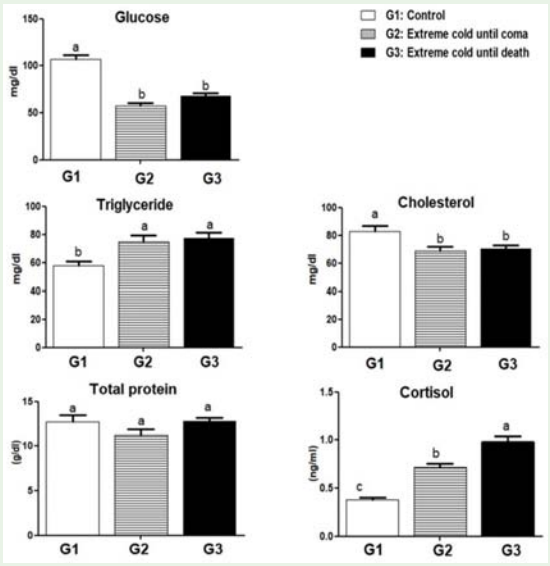

Figure 1: Serum glucose, triglyceride, cholesterol, total protein and cortisol levels of control and cold- exposed rats.

Values are presented as mean ± S.E. (n=5 animals/ group).

Different superscript means significant change at (p≤0.05) according to one-way ANOVA followed by Duncan's multiple range test for post hoc analysis.

Table 1: Mean values of comet assay parameters in brain of different groups.

	Damaged DNA %	Tail length (µm)	Tail DNA %	Tail moment
Control group (G1)	15.3±1.7^{b}	4.76±0.45^{b}	15.46±0.38^{c}	0.73±0.05^{c}
Extreme cold until coma group (G2)	28.0±4.1^{a}	6.03±0.23^{b}	22.96±1.4^{b}	1.38±0.03^{b}
Extreme cold until death group (G3)	34.3±2.3^{a}	9.30±0.47^{a}	30.80±2.2^{a}	2.85±0.14^{a}

Values are presented as mean ± S.E. (n=5 animals/ group).

Different superscript means significant change at (p≤0.05) according to one-way ANOVA followed by Duncan's multiple range test for post hoc analysis.

Results

Clinical signs and Post-Mortem (P.M.) lesions

Rats of group 2 (exposed to cold until coma) showed weak heart beats, decreased respiratory rate and muscle stiffness. On the other hand, rats of group 3 that died from cold showed complete stoppage of respiration and heart beats, absence of corneal reflex and whole-body stiffness. At necropsy, cherry red color of organs and mucosae was observed.

Serum biochemistry

Serum glucose, TG, CHO, TP and cortisol levels are shown in figure 1. Comparing to normal control group, cold- exposed rats (G2 and G3) showed significant decreased (p≤0.05) of serum

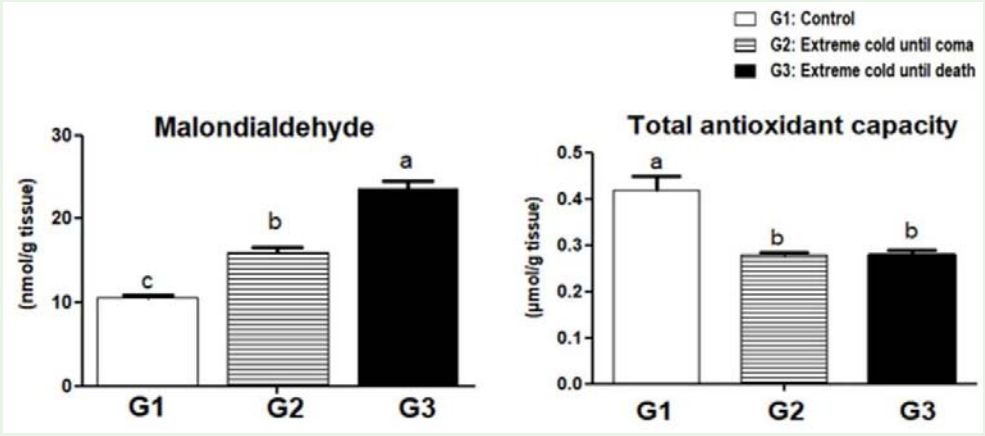

Figure 2: Levels of malondialdehyde and total antioxidant capacity in brain of control and cold-exposed rats.

Values are presented as mean ± S.E. (n=5 animals/ group).

Different letters means significant change at (p≤0.05) according to one-way ANOVA followed by Duncan's multiple range test for post hoc analysis.

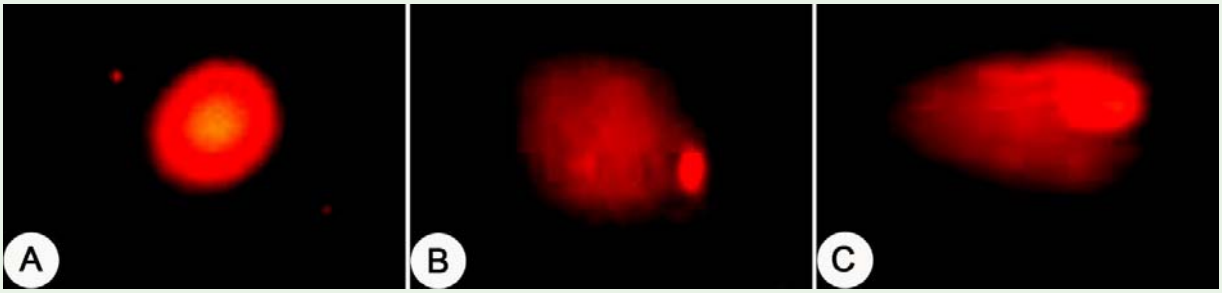

Figure 3: Comet study of brain tissue. Ethidium bromide stain A) Intact nucleus of control group; B & C) Tailed nucleus of cold- exposed rats of groups 2 and 3, respectively.

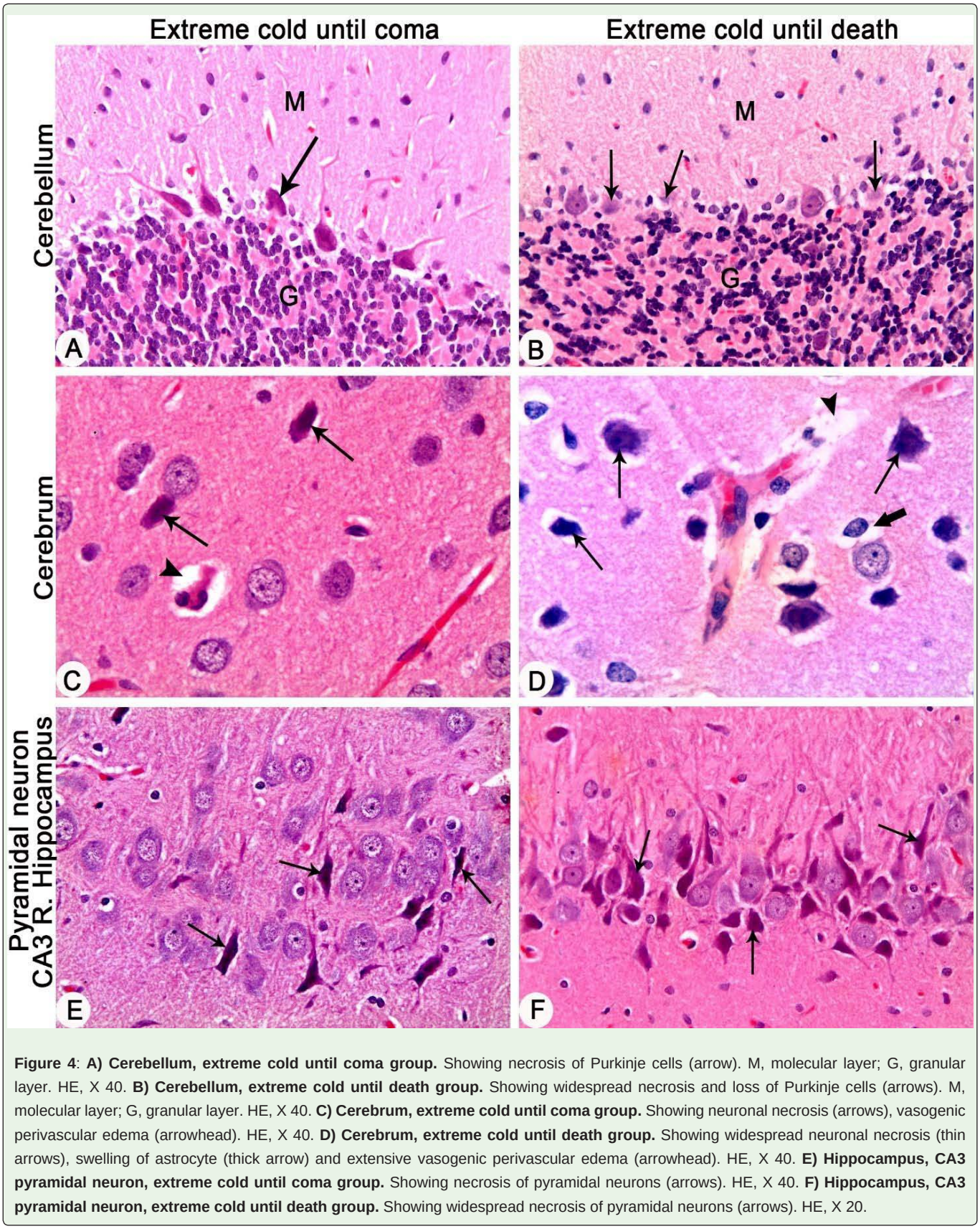

Figure 4: **A) Cerebellum, extreme cold until coma group.** Showing necrosis of Purkinje cells (arrow). M, molecular layer; G, granular layer. HE, X 40. **B) Cerebellum, extreme cold until death group.** Showing widespread necrosis and loss of Purkinje cells (arrows). M, molecular layer; G, granular layer. HE, X 40. **C) Cerebrum, extreme cold until coma group.** Showing neuronal necrosis (arrows), vasogenic perivascular edema (arrowhead). HE, X 40. **D) Cerebrum, extreme cold until death group.** Showing widespread neuronal necrosis (thin arrows), swelling of astrocyte (thick arrow) and extensive vasogenic perivascular edema (arrowhead). HE, X 40. **E) Hippocampus, CA3 pyramidal neuron, extreme cold until coma group.** Showing necrosis of pyramidal neurons (arrows). HE, X 40. **F) Hippocampus, CA3 pyramidal neuron, extreme cold until death group.** Showing widespread necrosis of pyramidal neurons (arrows). HE, X 20.

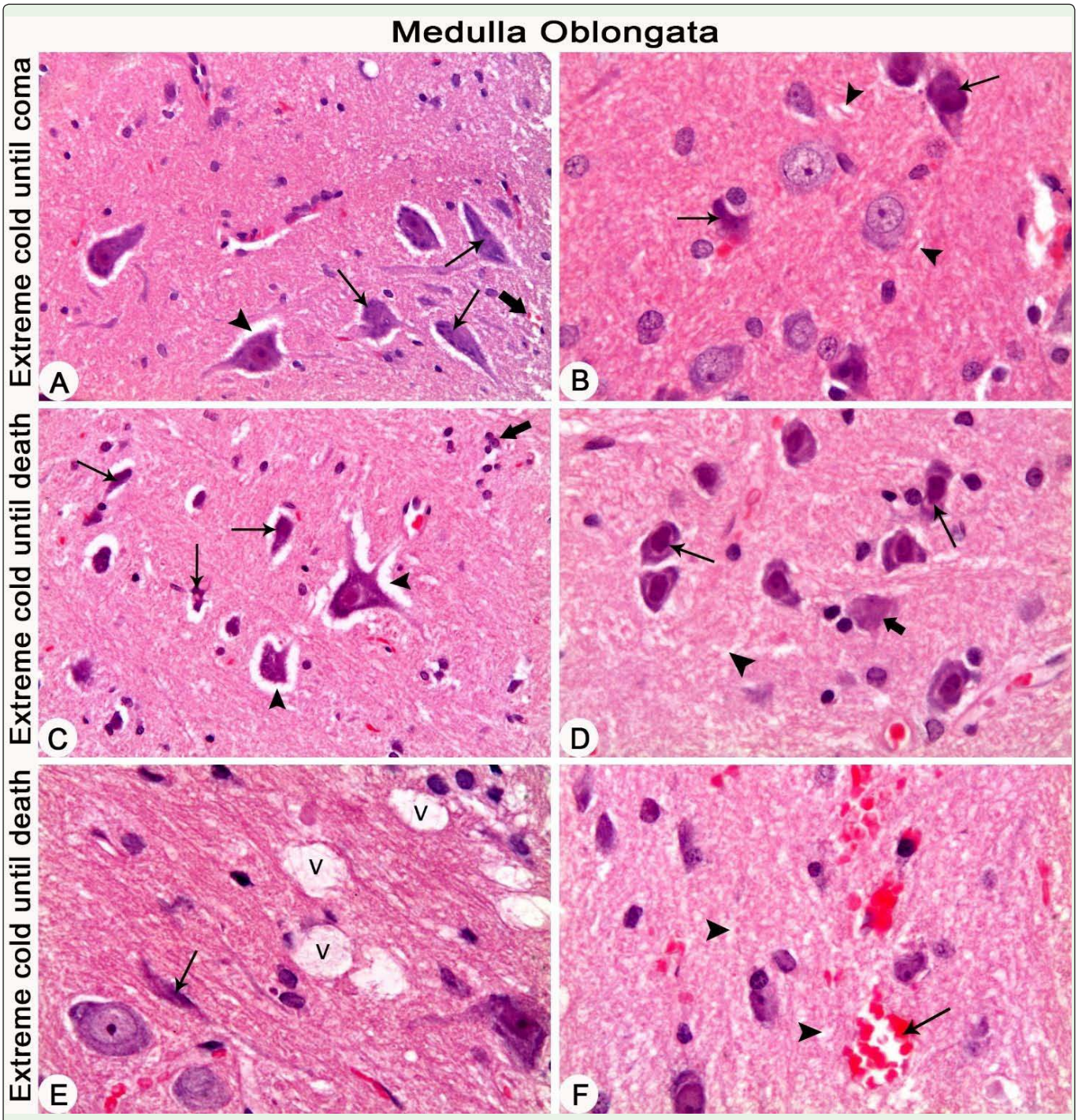

Figure 5: Medulla oblongata, rat.(A&B) Extreme cold until coma group. A) Showing neuronal necrosis (thin arrows), perineuronal edema (arrowhead), and hemorrhage (thick arrow). HE, X 40. **B)** Showing neuronal necrosis (arrows), edema in neuropil (arrowhead). HE, X 40. **C, D, E & F) Extreme cold until death group. C)** Showing widespread neuronal necrosis (thin arrows), perineuronal edema (arrowheads), and proliferation of oligodendroglia *'satellitosis'* (thick arrow). HE, X 40. **D)** Showing central chromatolysis (thin arrows), edema in neuropil (arrowheads), and neuronal necrosis (thick arrow). HE, X 40. **E)** Showing neuronal necrosis (arrow), accumulation of fluid vesicles within the white matter (V). HE, X 40. **F)** Showing hemorrhage (arrow), edema in neuropil (arrowheads). HE, X 40.

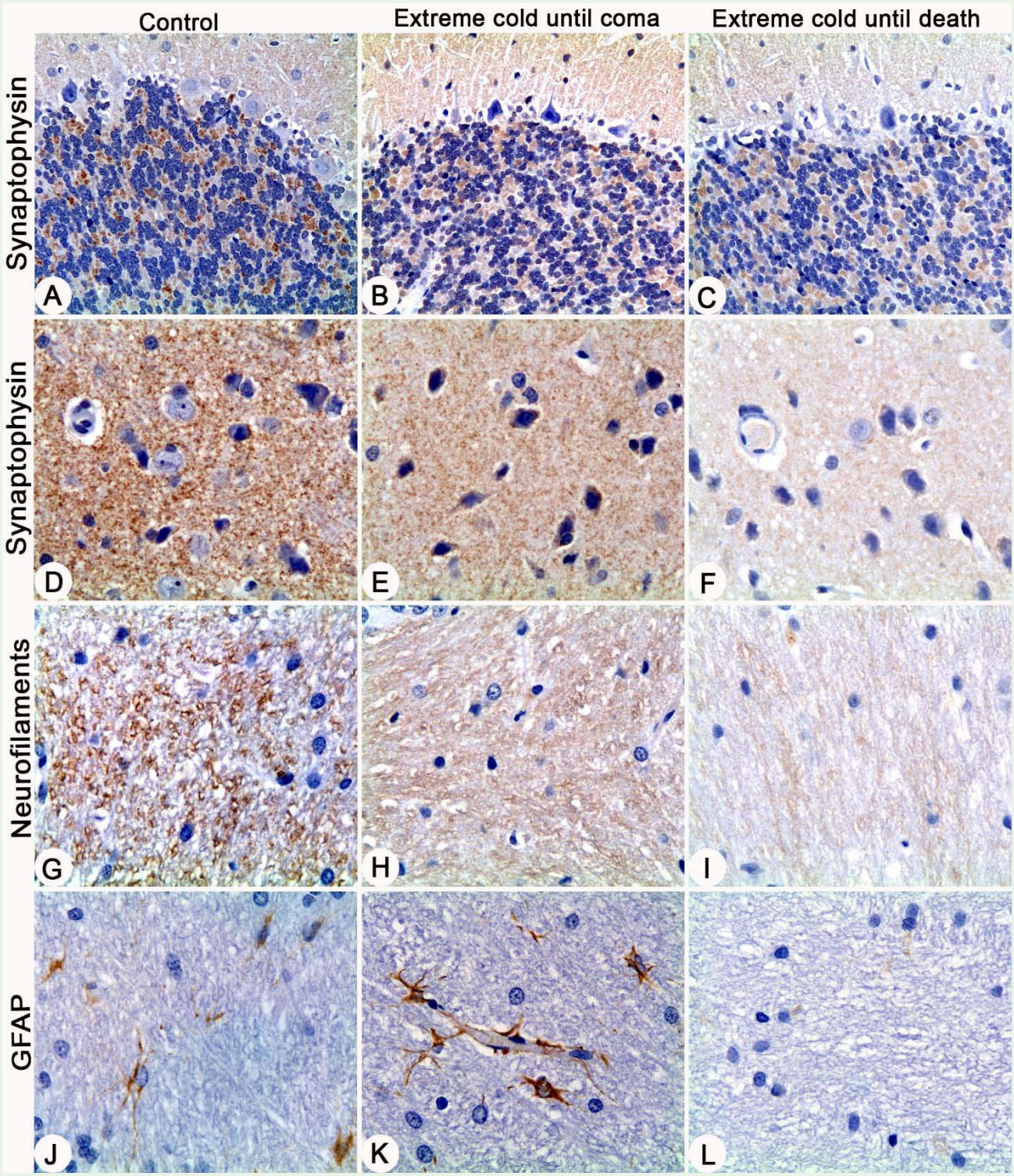

Figure 6: Brain, rat, IHC, X 40. A, B & C) Cerebellum, synaptophysin IHC. Showing intensive, mild, and weak immunolabeling in control, extreme cold until coma, and extreme cold until death groups, respectively. **D, E & F) Cerebrum, synaptophysin IHC.** Showing intensive, mild, and weak immunolabeling in control, extreme cold until coma, and extreme cold until death groups, respectively. **G, H & I) Cerebellar white matter, neurofilaments IHC.** Showing intensive, mild, and weak immunolabeling in control, extreme cold until coma, and extreme cold until death groups, respectively. **J, K & L) Cerebellar white matter, GFAP IHC.** Showing mild, moderate, and no immunolabeling in control, extreme cold until coma, and extreme cold until death groups, respectively.

glucose (-46.3% and -36.44%) and CHO (-16.9% and -14.9%) levels and significant increase of serum TG levels (29.7% and 34.55%), respectively. There were no significant changes of serum TP levels between different groups. Moreover, serum cortisol levels showed significant changes between different groups ($p \leq 0.05$), cold exposed rats (G2 and G3) showed significant increase of serum cortisol levels by about 90.47% and 159.26%, respectively, compared to normal rats.

Brain oxidative/ antioxidant status

As shown in figure 2, there were significant changes of MDA level in brain tissue between rats of the three groups at $p \leq 0.05$. MDA levels were significantly increased in G2 and G3 compared to normal rats by about 51% and 123.6%, respectively. Total antioxidant capacity in brain tissue showed significant reduction in cold exposed groups (G2 and G3) compared with that of G1 by about -33.3% for each.

Comet assay of brain tissue

From the obtained results (Table 1 and Figure 3), the damaged DNA percentage was significantly lower in control group in relation to cold- exposed groups and concomitantly the tail length showed gradual elevation in group 2 and group 3 compared with that of control group. Also Tail DNA% and tail moment showed gradual and significant elevation between different groups.

Histopathology and immunohistochemistry

Histopathologically, necrosis of Purkinje cells was detected in the cerebellum of extreme cold until coma group (Figure 4A). Widespread necrosis and loss of Purkinje cells were detected in extreme cold until death group (Figure 4B). Neuronal necrosis and vasogenic perivascular edema were detected in the cerebrum of extreme cold until coma group (Figure 4C). In extreme cold until death group, extensive and widespread neuronal necrosis, swelling of astrocytes and extensive vasogenic perivascular edema were detected in the cerebrum (Figure 4D). In hippocampus, some of CA3 pyramidal neurons were degenerated and necrosed in extreme cold until coma group (Figure 4E). However, in extreme cold until death group, the necrosis of these cells was intensive and widespread (Figure 4F).

In Medulla oblongata, neuronal degeneration/ necrosis, perineuronal edema, edema in neuropil, and hemorrhage were detected in extreme cold until coma group (Figure 5A-B). In extreme cold until death group, the medulla showing widespread neuronal necrosis, extensive perineuronal edema, proliferation of oligodendroglia '*satellitosis*', central chromatolysis, edema in neuropil, accumulation of fluid vesicles within the white matter, and hemorrhage (Figure 5C-F).

Immunohistochemically, synaptophysin positive signals were intensive, mild, and weak in control, extreme cold until coma, and extreme cold until death groups respectively in both cerebellum (Figure 6A-C), and cerebrum (Figure 6D-F). In cerebellar white matter, neurofilaments immunopositivity were intensive, mild, and weak in control, extreme cold until coma, and extreme cold until death groups, respectively (Figure 6G-I). GFAP immunopositive signals were mild, moderate, and absent in control, extreme cold until coma, and extreme cold until death groups, respectively, in cerebellar white matter (Figure 6J-L).

Discussion

The line between life and death in hypothermia is somewhat difficult to be determined. Postmortem biochemical and histopathological investigations are valuable in detecting the adaptive responses and metabolic changes that occur following cold exposure. In this study, we assessed some serum and brain biochemical parameters, brain DNA degradation, in addition to brain histopathological and immunohistochemical alterations in adult rats exposed to subfreezing temperature as a trial to determine useful markers for hypothermia.

It has been reported that exposure of rats to cold stress for few hours stimulates both the Hypothalamic-Pituitary-Adrenal Axis (HPA) and The Sympathetic Nervous System (SNS) resulting in a series of neural and endocrine adaptations with some changes in the metabolic pathways [28-30].

In current investigation, exposure of rats to cold stress significantly reduced serum glucose and cholesterol levels, but increased serum triglyceride levels. However, no significant changes in serum total protein were recorded. These results partially agree with those previously recorded in rats in which cold stress at 8°C reduced protein, cholesterol and triglyceride levels [31]. In addition, acute cold stress (-10°C for 3 hours) produced a significant increase in adrenomedullin levels in plasma, heart and kidney tissues of rats with reduction of cholesterol and plasma protein levels; however, triglyceride and plasma glucose levels were elevated [30].

Contrary to our findings, rats stressed in cold water at 15°C for 5 consecutive days showed increased serum protein levels [32]. The variation between our findings and those reported earlier may be attributed to difference in animal's strains and to different experimental conditions as we restrained rats under subfreezing temperature for about 7-8 hours until loss of consciousness or death.

In adult humans, the decrease in core temperature during environmental cold exposure is compensated by increasing heat production via shivering and vasoconstriction. Involuntary muscle contractions during shivering are mainly fueled by carbohydrates and lipids [33-35].

Besides shivering thermogenesis, cold stress induces elevation in non-shivering thermogenesis by enhancing substrate combustion from lipid and carbohydrates with increased oxygen consumption resulting in accelerated the release of reactive oxygen species [36,37].

Moreover, it was reported that upon cold exposure, norepinephrine is released from sympathetic nerves and binds to adrenergic receptors on brown adipocytes to induce non-shivering thermogenesis. Stimulation of adrenergic receptors induces Cyclic Adenosine Monophosphate (cAMP) production and subsequent induction of lipolysis, beta oxidation, and uncoupling of oxidative phosphorylation in the mitochondria [38]. Adrenergic stimulation due to cold exposure enhances glucose uptake and glycolysis in brown adipose tissue with lowering in glucose level [33,39,40].

Exposure of wild mice to cold environment (4°C) for 4 hrs with or without fasting for 20 hrs led to increase in glucose uptake, lipolysis and increase in serum non-esterified fatty acids [41]. In acute adaptation to cold stress, oxidation of adipose tissue fatty acids and

utilization of glucose is critical for providing the energy required to fuel heat generation and thermogenesis [42]. Therefore, reduction in serum glucose and cholesterol levels and increase of serum triglyceride level in our study is logical findings.

Furthermore, thyroid hormones, in the presence of norepinephrine, are major determinants of thermogenic activity in muscle and liver of cold-acclimated rats [43]. Brown adipose tissue is the main site for adaptive thermogenesis due to presence of the mitochondrial Uncoupling Protein-1 (UCP-1), which uncouples electron transport from the phosphorylation of ADP with loss of energy as a heat. UCP-1 expression is stimulated by Norepinephrine (NE) but requires increase secretion of thyroid hormones [44,45]. In hyperthyroid state, synthesis of endogenous plasma triglycerides and free fatty acids was increased due to adipose tissue lipolysis [46]. On the other hand, hyperthyroidism can be associated with hypocholesterolemia due to increase in cholesterol turnover [47].

Increased resting metabolism and fat oxidation and even extreme shivering were recorded in human exposed to acute cold environment (-15°C). Increased psychological stress activities represented in a significant increase the levels of s-amylase activity and cortisol [48].

Cortisol, Adrenocorticotrophic Hormone (ACTH) and adrenaline can be used as fatal hypothermia markers as hypothermia stimulates the hypothalamic–pituitary–adrenal axis [16,49-52]. In our study, serum cortisol was significantly elevated in rats exposed to cold stress. Additionally, MDA level was significantly elevated in brain of rats exposed to cold compared to control animals, while total antioxidant capacity showed significant reduction. The oxidative insult on brain tissue led to increased rate of DNA damage in rats exposed to cold stress compared to control animals.

At exposure of rats to extreme cold, they suffer from severe bradycardia and reduction in cardiac output with blood coagulopathy that help in reduction of blood supply to all organs. Brain requires around 25% of constant cardiac output for its metabolic needs. Therefore, any deficiency in cerebral blood flow may cause cerebral ischemic degeneration and neurological dysfunctions [53]. Also, the high rate of oxidative metabolic activity, as a first adaptive mechanism against cold stress, leading to increase in production of Reactive Oxygen Species (ROS) with low concentrations of endogenous antioxidants causing exceptional vulnerability of the brain to oxidative stress [54].

In this study, hypothermia induced necrosis and loss of Purkinje cells in cerebellum, neuronal degeneration/ necrosis and vasogenic perivascular edema in cerebrum and medulla oblongata. These pathologic changes are increased in severity with time by hypothermia as result coincides with data reported previously [16]. Cold stress is associated with increasing ROS production which causing lipid peroxidation, membrane injury and disturbance in tissues integrity [13,14,55].

In comparing with control group, immuno-expression of synaptophysin in the cerebrum and cerebellum of brain decreased in the extreme cold until coma and more decreased in and extreme cold until death groups. This result agrees with the previous reports demonstrating that immuno-expression of synaptophysin has been decreased during hypothermia in brain hippocampus [17]. Synaptophysin is a pre-synaptic vesicles protein indicator for synaptic efficacy [56]. Its reduction during hypothermia indicates a decrease in synaptic efficacy [17].

Also, immuno-expression of neurofilament in the cerebrum and cerebellum of brain decreased in both groups in comparing with the control group. Neurofilaments are abundant in axons and are essential for the radial growth of axons during development and transmission of electrical impulses along axons [57]. Decrease of neurofilament during hypothermia may indicate axonal degeneration.

Immuno-expression of GFAP increased in the extreme cold until coma when compared with control group and completely decreased or no staining in the extreme cold until death group was observed. GFAP is an intermediate filament protein or soluble protein present in cytoplasm of astrocytes [58,59]. GFAP expression increases as a specific marker of astrocyte proliferation during severe hypothermia and decrease by the death of the animal.

Conclusion

The presented investigations demonstrated that hypothermia induces biochemical changes in blood and brain of rats, enhanced brain DNA degradation and induced many histopathological and histochemical alterations in the brain making it possible to use these changes as useful markers for hypothermia in other mammals such as *Homo sapiens* with some expected variations across different species. If adaptive mechanisms to hypothermia fail to overcome these alterations, death will be the end result.

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