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# Hypothermia in Rat: Biochemical and Pathological Study

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## Abstract

Human and animals have many amazing protective mechanisms that enable them to adapt to extreme thermal conditions. Failure of these mechanism will induce a widespread alteration in many organs specially brain. This study will focus on some of the biochemical and histopathological characters in serum and brain of rats exposed to extreme cold till loss of consciousness or death. Serum glucose, triglyceride (TG), cholesterol (CHO) and total protein (TP) were estimated colormetrically, while, serum cortisol concentration was determined by ELISA. In brain tissues, malondialdehyde (MDA), total antioxidant capacity (TAC) and comet assay were evaluated. Additionally, histopathological and immunohistochemical investigation on brain tissues were conducted. Hypothermia, significantly decrease serum glucose and CHO levels and increase TG and cortisol levels but have no effect on TP level. During hypothermia, MDA and DNA lysis were increased in brain tissue while the TAC was decreased. Hypothermia induce, vasogenic perivascular edema, necrosis/ loss of Purkinje cells of cerebellum, neuronal degeneration/ necrosis and perineuronal edema in cerebrum and hippocampus. Hypothermia decrease the immunopositivity of synaptophysin and neurofilaments but temporary increase the glial fibrillary acidic protein (GFAP) in brain tissue. Hypothermia, despite the several adaptive mechanisms, can induce lethal harmful effects especially in brain tissues.

**Key Words:** Rat, Hypothermia, Biochemical, Immunohistopthological, Brain.(Hypothermia, Biochemical, Immunohistopthological, Met, Brain)

## Introduction

In forensic pathology, diagnosis of death from hypothermia represents to some extent a problem due to the absence of specific features (Turk et al. 2010; Palmiere et al. 2014). Hypothermia is defined as the decreasing in body temperature below its normal physiological level (Hart et al. 2011; Palmiere et al. 2014). Based on body temperature and clinical signs, hypothermia can be classified into mild, moderate, severe or profound hypothermia (Oncken et al. 2001). Prolonged exposure to subfreezing temperature increases the incidence of death (Palmiere et al. 2014).

The body can adapt to the 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 (Young 1981; Pilcher et al. 2002; Taylor 2014; Daanen & Van Marken 2016). 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 (Lishmanov et al. 1997; Oncken et al. 2001).

Postmortem biochemistry, light microscopy and immunohistochemistry (IHC) are helpful tools in diagnosis of fatal hypothermia (Palmiere et al. 2014). 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 induction of oxidative stress (Ji & Fu 1992; Selman et al. 2000; Heise et al. 2003).

It has been demonstrated that cold stress induces changes in biochemical parameters with significant increase in the lipid peroxidation level and reduction of antioxidant enzyme activities in rats and mice tissues (Kausik & Kaur 2003; Sahin & Gumuslu 2004; Yeap et al. 2014).

The previously recorded microscopic changes in hypothermia are focal pancreatic necrosis and haemorrhage, vacuolization in anterior pituitary gland cells, hepatocytes, adrenal and renal cells. Additionally, fatty degeneration of the renal tubular epithelium, cardiac myocytes and hepatocytes was recorded in hypothermia beside hypoxic cardiac changes (Palmiere et al. 2014). 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 (Oncken et al. 2001). A few or scarce data are present about the microscopic changes of cerebrum and cerebellum during hypo-

thermia. Pyramidal and glial cells degeneration, haemorrhage and vacuoles with varying degrees were detected previously in brain tissues during moderate (2 - 8ºC) and severe (0 to -2ºC) hypothermia (Elshama et al. 2016). Immunohistochemical expression of Glial fibrillary acidic protein (GFAP) on hypothalamus and synaptophysin on hippocampus have been performed previously during hypothermia (Strijkastra et al. 2003; Liu et al. 2015). To 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 indicator for neuron integrity, while synaptophysin play an important role in neurotransmission which is present in all neurons in the brain and spinal cord that participate in synaptic transmission (Misgeld et al. 2002).

This work was performed to elucidate some biochemical and histopathological changes in brain tissue exposed to extreme cold till loss of consciousness or death.

## Materials And Methods

### Animals

Fifteen 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±2oC). Food and water were provided ad libitum..

### Animal rights

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

All precautions were taken into consideration to avoid animal stress at acclimation period. Promotion of high standard care and animal well-being were practiced at all times. Painful procedures were performed under anesthesia to avoid any distress and pain that could be inflicted on the animals. Also we minimize the number of animals used in this study to avoid cold stress on large number of animals. In our country, there is no under zero cold stress so experimental work is only available to study cold stress from forensic point of view.

### Experimental design

After a week of acclimatization, rats were randomly divided to three equal groups, 5 rats each. Control group (G1) was kept at room temperature (26±2oC). Animals in extreme cold until coma group (G2) were kept individually in small polypropylene cage with mesh wire cover (20×20×18 cm) at deep freeze (-10 to -12 oC) till loss of consciousness. Animals in extreme cold until death group (G3) were exposed to cold as those of G2 but the exposure continued until death. The rats of all groups were deprived totally from water and food along the experimental period.

### Serum collection and tissue preparation

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. Samples were left to clot at room temperature and centrifuged at 3000 rpm for 15 min at 4oC. Sera were then, separated and stored at -20°C as aliquots for further biochemical analysis.

After blood collection, rats were sacrificed by cervical decapitation. Brain of six rats from each group were rapidly excised. The right half of each brain was washed with 0.9 % NaCl solution, then blotted over a piece of filter paper and perfused phosphate buffer saline (50 mM potassium phosphate pH 7.4) in an ice-containing medium. Subsequently, brain tissues were homogenized in 5 ml cold buffer per gram tissue using Dounce Tissue Grinder (Omni International, Kennesaw Georgia) and centrifuged at 4000 rpm for 15 min at 4°C using cooling centrifuge. The resulting supernatant was then transferred into Eppendorf tubes, and kept at -80°C until used for various biochemical assays. The left halves of brain tissues were fixed in 10% neutral buffer formalin (NBF) and prepared for histopathological and immunohistochemical investigations.

Brain tissues of the other four rats of each group were preserved in phosphate buffer 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 previously published reports using kits purchased from Biodiagnostic Company (Dokki, Egypt) (Lowry et al.1951; Allain et al. 1974; Fassati & Principle 1982). Serum cortisol concentration (ng/ml) was determined by ELISA method (Munro & Lasley 1988).

Evaluation of tissue lipid peroxidation and antioxidant capacity

Lipid peroxidation (malondialdehyde, MDA) and total antioxidant capacity (TAC) were evaluated in brain tissues using kits purchased from Biodiagnostic Company (Dokki, Egypt) (Ohkawa et al. 1979 & Koracevic et al. 2001).

### Comet assay

Comet assay or alkaline single-cell gel electrophoresis was performed using the modified method of Ellahuene et al. (2004). 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 (Tice et al. 2000). 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 the cells was estimated by measuring the degree of fluorescence or intensity of DNA migration length or tail length. The tail moment was expressed as tail length × percentage of migrated DNA / 100.

### Histopathology and Immunohistochemistry (IHC)

After 72 h 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% H2O2 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% H2O2 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 $p \leq 0.05$.

RESULTS

## Gross examination of the animals

Rats of group 2 (those 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. The rectal temperature ranged from 26-30 oC of rats exposed to cold.

## Serum biochemical analysis

Serum glucose, TG, CHO and TP concentrations are shown in Table 1. Serum glucose level showed significant changes between different groups at $p \leq 0.05$. It was significantly decreased in cold-exposed rats (G2 and G3) in relation to control rats (G1) by about -46.3% and -36.44%, respectively. Considering serum TG levels, there were significant increases in their levels in G2 and G3 compared with control group (G1) by about 29.7% and 34.55%, respectively. Serum CHO levels of G2 and G3 showed significant reduction than that of G1 about -16.9% and -14.9% respectively. Serum TP showed insignificant changes between different groups. Serum cortisol showed significant increase between different groups. It was increased in G2 and G3 by 90.47% and 159.26% respectively related to that of G1.

**Table (1):** Mean values of serum glucose, TG, CHO (mg/dl), TP (g/dl) AND cortisol (ng/ml) in control and cold- exposed rats. Values are presented as mean ± S.E. (n= 5 animals/ group)

| Parameters / Groups | Glucose | Triglyceride (TG) | Cholesterol (CHO) | Total protein (TP) | Cortisol |
|---|---|---|---|---|---|
| Control group (G1) | 107.3±4.05$^a$ | 57.6±3.2$^b$ | 82.7±3.9$^a$ | 12.7±0.8$^a$ | 0.378±0.025$^c$ |
| Extreme cold until coma group (G2) | 57.6±2.8$^b$ | 74.7±4.5$^a$ | 68.7±3.2$^b$ | 11.2±0.7$^a$ | 0.720±0.032$^b$ |
| Extreme cold until death group (G3) | 68.2±2.7$^b$ | 77.5±3.9$^a$ | 70.3±2.6$^b$ | 12.8±0.4$^a$ | 0.980±0.058$^a$ |

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

## Brain tissue lipid peroxidation and antioxidant capacity

As shown in Table 2, there were significant changes in lipid peroxide represented by MDA level in brain tissue between rats of the three groups. Lipid peroxide levels were significantly increased in G2 and G3 in relation to that of rats in G1 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%.

**Table (2):** Mean values of brain tissue lipid peroxide (MDA) (nmol/g tissue) and total antioxidant capacity (TAC) (µmol/g) levels of control and cold exposed rats.

| Parameters / Groups | Malondialdehyde (MDA) | Total antioxidant capacity (TAC) |
|---|---|---|
| Control group (G1) | 10.6±0.34$^c$ | 0.42±0.03$^a$ |
| Extreme cold until coma group (G2) | 16.0±0.58$^b$ | 0.28±0.005$^b$ |
| Extreme cold until death group (G3) | 23.7±0.88$^a$ | 0.28±0.01$^b$ |

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

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

## Comet assay of brain tissue

From the obtained results (Table 3 and Fig. 1), the intact DNA percentage was significantly higher 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 groups 1, 2 and 3.

**Table (3):** Mean values of comet assay parameters of control and cold exposed rats' brain cells.

| Parameters / 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 ($p \leq 0.05$) according to one-way ANOVA followed by Duncan's multiple range test for post hoc analysis

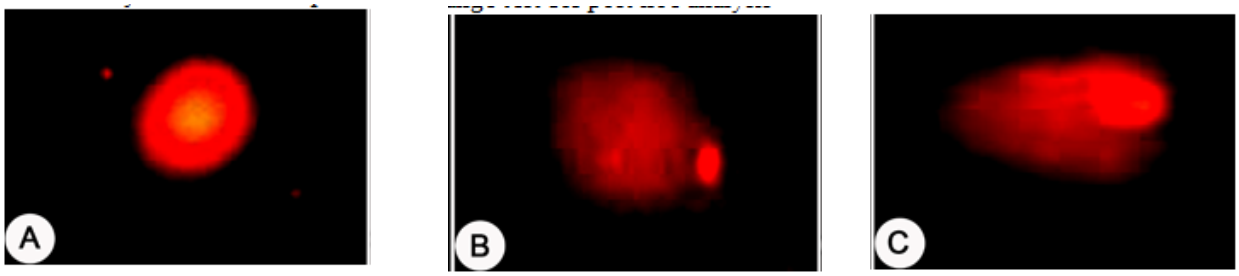


**Fig. I:** A) Intact nucleus of control group; B & C) Tailed nucleus of cold exposed rats of groups 2 and 3, respectively.

## Histopathology and immunohistochemistry

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

In Medulla oblongata, neuronal degeneration/ necrosis, perineuronal edema, edema in neuropil, and haemorrhage were detected in extreme cold until coma group (Fig. III-A & 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 haemorrhage (Fig. III-C, D, E & 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 (Fig. IV- A, B & C), and cerebrum (Fig. IV- D, E & 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 (Fig. IV- G, H & 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 (Fig. IV- J, K & L).

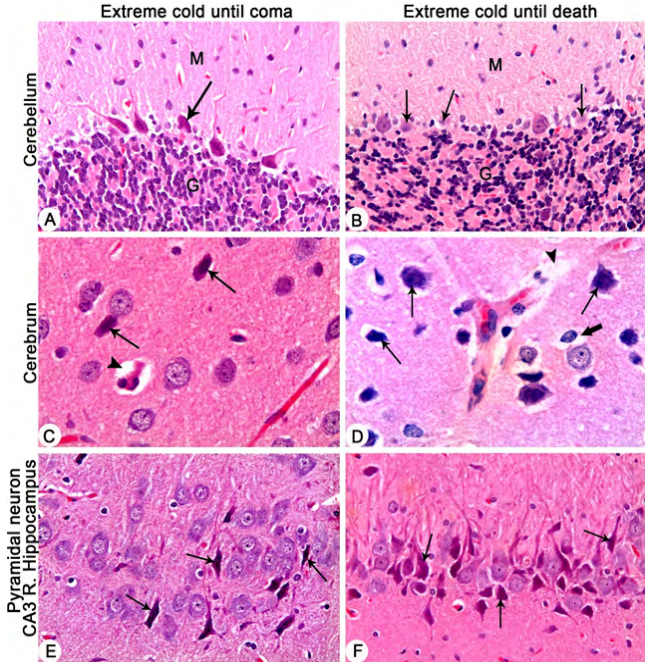


**Fig. II** (A-F). Brain, rat. A) Cerebellum, extreme cold until coma group. Showing necrosis of Purkinje cells (arrow). M, molecular layer; G, granular layer. HE, X 40. B) Cerebellum, extreme cold until death group. Showing widespread necrosis and loss of Purkinje cells (arrows). M, molecular layer; 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.

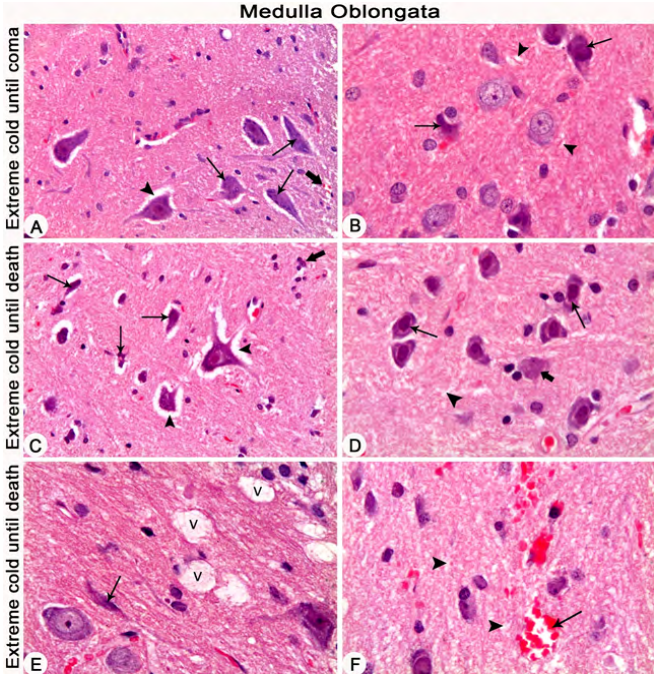


**Fig. III** (A-F). Medulla oblongata, rat. (A&B) Extreme cold until coma group. A) Showing neuronal necrosis (thin arrows), perineuronal edema (arrowhead), and haemorrhage (thick arrow). HE, X 40. B) Showing neuronal necrosis (arrows), edema in neuropil (arrowhead). HE, X 40. C, D, E & F) Extreme cold 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 haemorrhage (arrow), edema in neuropil (arrowheads). HE, X 40.

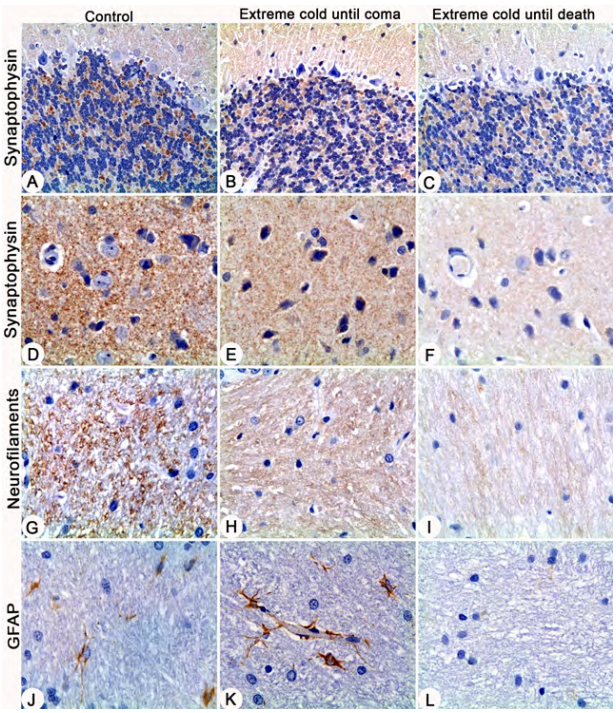


**Fig. IV** (A-L). Brain, rat, IHC, X 40. A, B & C) Cerebellum, synaptophysin IHC. Showing intensive, mild, and weak immunolabeling in control, extreme cold until coma, and extreme cold until death groups, respectively. D, E & F) Cerebrum, synaptophysin IHC. Showing intensive, mild, and weak immunolabeling 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.

## Discussion

The line between live and death in hypothermia is somewhat difficult to be determine. Postmortem biochemical and histopathological investigations are valuable in detecting the adaptive responses and metabolic changes that occur following cold exposure. In this study, we focus on some serum and brain biochemical changes, in addition to brain histopathological and immunohistochemical alterations in adult rats exposed to subfreezing temperature. In previous studies, 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 (Katoh et al. 1994; Miller & O'Callaghan 2002; Al-Ayadhi et al. 2006).

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 (Yuksel et al. 2002). In addition, acute cold stress (-10oC/ 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 (Al-Ayadhi et al. 2006).

Contrary to our findings, rats stressed in cold water at 15°C for 5 consecutive days showed increased serum protein levels (Jain et al. 1996). 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 till 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 produce heat are mainly fueled by carbohydrates and lipids (Vallerand et al. 1990; Jacobs et al. 1994; Vallerand et al. 1995).

Beside shivering thermogenesis, cold stress induces elevation in non-shivering thermogenesis by enhanced substrate combustion from lipid and carbohydrates with increased oxygen consumption and enhanced release of reactive oxygen species (Barja et al. 1991; Kovacs et al. 1996).

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 (Cannon & Nedergaard 2004). Adrenergic stimulation due to cold exposure enhances glucose uptake and glycolysis in brown adipose tissue with lowering in glucose level (Vallerand et al. 1990; Hao et al. 2015; Albert et al. 2016).

Exposure of wild mice to a cold environment (4oC) for 4 hours with or without prior fasting for 20 hours led to increase in glucose uptake, lipolysis and increase in serum non-esterified fatty acids (Syamsunarno et al. 2014). 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 (Lee et al. 2015). Therefore, reduction in serum glucose and cholesterol levels and an increase in serum triglyceride level in our study is logical.

Furthermore, thyroid hormones, in the presence of norepinephrine, are major determinants of thermogenic activity in muscle and liver of cold-acclimated rats (Zaninovich et al. 2003). 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 (Silva 1988; Nedergaard et al. 2001). In hyperthyroid state, synthesis of endogenous plasma triglycerides and free fatty acids was increased due to adipose tissue lipolysis (Nikkild & Kekki 1972). On the other hand, hyperthyroidism can be associated with hypocholesterolemia due to increase in cholesterol turnover (Rizos et al. 2011).

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 (Yamauchi et al. 2013).

Cortisol, adrenocorticotrophic hormone (ACTH) and adrenaline can be used as fatal hypothermia markers as hypothermia stimulates the hypothalamic–pituitary–adrenal axis (Castellani et al. 2002; Ma & Morilak 2005; Ishikawa et al. 2008; Banka et al. 2013; Elshama et al. 2016). In our study it was found that the serum cortisol was significantly increased in cold exposed groups than that of control group. Additionally, lipid peroxide levels were 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 cell DNA damage as evidenced by increase in tail length, DNA% in tail and tail moment in comet assay.

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 (Deb et al. 2010).

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 (Chen et al. 2000).

In this study, hypothermia induces necrosis and loss of Purkinje cells in cerebellum, neuronal degeneration/ necrosis and vasogenic perivascular edema in cerebrum and MO. These pathologic changes are increased in severity with time by hypothermia as result coincides with data reported previously (Elshama et al. 2016). Cold stress is associated with increasing ROS production which causing lipid peroxidation, membrane injury and disturbance in tissues in-

tegrity (Bagchi et al. 1999; Selman et al. 2000; Kausik & Kaur 2003; Sahin & Gumuslu 2004; Yuksel & Asma 2006).

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 that immuno-expression of synaptophysin has been decreased during hypothermia in brain hippocampus (Strijkstra et al. 2003). Synaptophysin is a pre-synaptic vesicles protein indicator for synaptic efficacy (Wiedenmann & Frank 1985). Its reduction during hypothermia indicates a decrease in synaptic efficacy (Strijkstra et al. 2003).

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 (Yuan et al. 2012). Decrease of neurofilament during hypothermia may indicates 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 (Feresten et al. 2013; Radomska et al. 2013). GFAP expression increases as a specific marker of astrocyte proliferation during severe hypothermia and decrease by the death of the animal.

In conclusion, during hypothermia, the body will try to maintain its energy but this will consume the main sources of energy like glucose and CHO and unfortunately this will increase some of the harmful elements such as TG and cortisol. MDA will be increased during hypothermia while the TAC will be decreased in brain tissues which facilitating nuclear degeneration and DNA lysis. Hypothermia induce neuronal damage such as edema, degeneration/necrosis or loss of neurons in cerebellum, cerebrum, hippocampus and MO. Hypothermia affect the function and structure of neurons by disrupting the expression of synaptophysin, neurofilaments and GFAP in brain tissue. Hypothermia can be resulting in several biochemical and structural changes, if adaptation fail to overcome these alterations, death will be the end result.

Conflicts of Interest, Source of Funding and Acknowledgement

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