

# Biochemical and Apoptotic Biomarkers as Indicators of Time Elapsed Since Death in Experimentally Induced Traumatic Brain Injury

Amany Abdel-Rahman Mohamed\*, Khlood M Elbohi, Nabela I El Sharkawy and Mona A Hassan

Department of Forensic Medicine and Toxicology, Zagazig University, Egypt

## Article Information

Received date: Aug 24, 2017

Accepted date: Oct 02, 2017

Published date: Oct 09, 2017

## \*Corresponding author

Amany Abdel-Rahman Mohamed,  
Department of Forensic Medicine  
and Toxicology, Faculty of Veterinary  
Medicine, Zagazig University, Zagazig  
- 44511, Egypt, Tel: 02/01205421116;  
Fax: 02/0552283683;  
Email(s): amanyrahman292@yahoo.com  
(or) aabdaziz@zu.edu.eg

**Distributed under** Creative Commons  
CC-BY 4.0

**Keywords** TBI; CSF; Blood; Bcl-2; P53;  
Histopathology

## Abstract

The present study declares the relationship between the cause of death and postmortem alterations in some body fluids (CSF, serum and plasma). Immuno-histochemical and micro architecture examination of brain tissue of White New Zealand rabbits were applied at different Postmortem Interval (PMI) after Traumatic Brain Injury (TBI). Thirty adult male White New Zealand rabbits were divided into two groups; the first group was killed by cervical dislocation and the second through head trauma. Each group was subdivided into three times of PMIs (zero, 6, and 12 hrs PM). CSF was used to detect the levels of K<sup>+</sup>, Na<sup>+</sup>, Ca<sup>++</sup>, and albumin. While, lactic acid, hypoxanthine, ammonia and uric acid concentrations were measured in plasma. Estimation of High Mobility Group Box-1 (HMGB1), Interleukin-1-beta (IL-1B) and Tumor Necrosis Factor-alpha (TNF-α) were assessed in serum. In addition, immunohistochemical observations of Bcl-2 and P53 apoptotic proteins in brain tissue. The results revealed that some of the examined markers as K<sup>+</sup>, Na<sup>+</sup>, albumin, ammonia, hypoxanthine and High Mobility Group Box-1(HMGB1) had the potential role in estimation of PMI at examined time periods in physical and traumatized death. Traumatic death induced severe cerebral hemorrhages and necrosis of cerebral parenchyma than physical death. Immunohistochemical results of P53 and Bcl-2 in brain tissue declared focal positive reactions of some neurons, astrocytes and microglia in different degrees with time since death. It was concluded that biochemical analysis of some body fluids, tissue pathological changes and apoptotic markers are applicable tools for assessing accurate PMI after traumatic brain injury and could have a crucial role in legal medicine.

## Introduction

Postmortem biochemistry for investigating the cause and process of death involves analyses of biomarkers of general conditions, systemic vital reactions and metabolic disorders to demonstrate deterioration of vitality, including circulation, respiration, metabolic homeostasis and Central Nervous System (CNS) with tissue-specific biomarkers to detect fatal damage to life-supporting organs, including heart, lungs and brain [1]. Postmortem examination of body fluids like blood, Cerebro-Spinal Fluid (CSF), the Vitreous Humor (VH), pericardial fluid (PF) and Synovial fluid (SF) can help forensic pathologists to estimate PMI with a reasonable accuracy [2] and have proved to be very useful in medicolegal investigations of death [3]. Traumatic Brain Injury (TBI) is one of the most common and important causes of violent death and therefore daily routine of medico legal work [4]. Systemic inflammation and immune responses are involved in deaths due to trauma. In forensic practice, one of the essential roles of legal medicine is to provide a different approaches to overcome difficulties that may face medicine and law as a forensic pathologist may face a lot of limitations regarding the precise diagnosis of traumatic deaths [5].

The essential social and academic tasks of forensic medicine, which involve medicolegal issues, including the cause and process of death, especially in cases of traumatic and unexpected sudden death. Postmortem biochemical analyses in combination with immunohistochemical markers have become ancillary procedures in determining the cause and time of death [6]. The postmortem level of brain-specific biomarkers in body fluids such as CSF and blood may be used to determine the severity of brain damage in consideration of potentially unspecific postmortem changes. In general, the benefit of forensic biochemistry is limited to cases in the early postmortem period before the appearance of decomposition and it is quite necessary to define own postmortem reference intervals for all relevant biomarkers, considering the agonal and postmortem changes [7].

Few reviews, case reports or published series in the veterinary literature discuss or describe the physical or clinical course in animals suffering from naturally occurring traumatic events. However, traumatic injury commonly occurs in veterinary medicine, no national database for data collection exists for veterinary patients [8]. This study aimed to spotlight on the correlation between the cause of death and some postmortem biochemical markers in CSF and blood, concomitant with

immunohistochemical and histopathological studies on the brain tissue of White New Zealand rabbits at different time intervals (zero, 6 and 12 hours postmortem) which may be valuable tools in PMI estimation.

## Materials and Methods

### Anesthetic compounds

Ketamine hydrochloride (Ketalar 5%) (Ketaject, Phoenix Pharmaceutical Inc., St. Joseph). Xylazine (Xyla-Ject Phoenix Pharmaceutical Inc., St. Joseph) 2%)

### Ethics statement

The procedure was carried out according to the general guidelines of the National Institutes of Health (NIH) for the Care and Use of Laboratory Animals in scientific investigations and approved by the ethics of animal use with a card number (IBR-3895) in research committee (EAURC), Zagazig University, Egypt.

### Animals and experimental design

Thirty adult male White New Zealand Rabbits weighing between 1800 g and 2000 g and had about 75 to 80 days of age were obtained from the Laboratory Animals' Farm Unit, Faculty of Veterinary Medicine, Zagazig University, Egypt. The animals were kept under hygienic conditions, housed in individual stainless steel cages for at least two weeks for accommodation under the suitable laboratory conditions as (good aeration, lightening and a constant room temperature about 24°C). The animals were allowed to access water and standard rabbit fodder (25.1% proteins, 3.8% fat, 18.05% cellulose). After the accommodation period, thirty rabbits were weighed and divided into two main equal groups, The rabbits of all groups (control and traumatized groups) were sedated by intramuscular injection of a mixture of ketamine hydrochloride (35 mg/kg of body weight) and xylazine (5 mg /kg of body weight) anesthetic agents [9] to minimize animal suffering. The first group served as a control (C) (killed via cervical dislocation), while the other group served as traumatized group (T) (killed through the weight drop method by specifically designed apparatus). Each group was classified into three subgroups

within three postmortem intervals (at time of death (zero hour), 6 and 12 h postmortem). The control group (C) contains three sub groups including (C0, C6, and C12). The traumatized group includes (T0, T6, and T12).

### Induction of traumatic death using weight drop method

The anesthetized rabbits have been secured and the head trauma was developed by a controlled drop of metallic spheres weighted (1 kg) released through a duct always from the same height (1 meter) on a platform that was designed (Figure 1) in order to maintain the experimental animal head partially fixed above rigidity bulkhead [10].

### Collection of biologics from rabbits

CSF is collected from the cisterna magna (foramen magnum) [11]. CSF samples were centrifuged directly after collection at 3000 round/ minute (RPM) for 10 min. The supernatant was immediately preserved at -20°C till analysis. Blood samples were collected by cardiac puncture and divided into two parts; the first part was collected with heparin for obtaining of plasma then stored at 4°C till biochemical analysis after 24 hrs. The second part was placed in centrifuge glass tubes for serum separation and rapidly stored at -20°C till used in the biochemical analysis.

### Estimation of potassium ions (K<sup>+</sup>), sodium ions (Na<sup>+</sup>), calcium ions (Ca<sup>++</sup>) and albumin in CSF

Potassium (K<sup>+</sup>) and Sodium (Na<sup>+</sup>) level in CSF was measured using Spectrum-Diagnostics Bioassay kits photometrically at 578 nm and at 405 nm, respectively. The Calcium concentration in CSF was measured using Spin react commercial kinetic UV bioassay kits. Albumin concentration in CSF was measured using Modified Bromocresol blue (BCG) calorimetric method photometrically at 605 nm [12].

### Determination of Total Leucocytic Count (TLC)

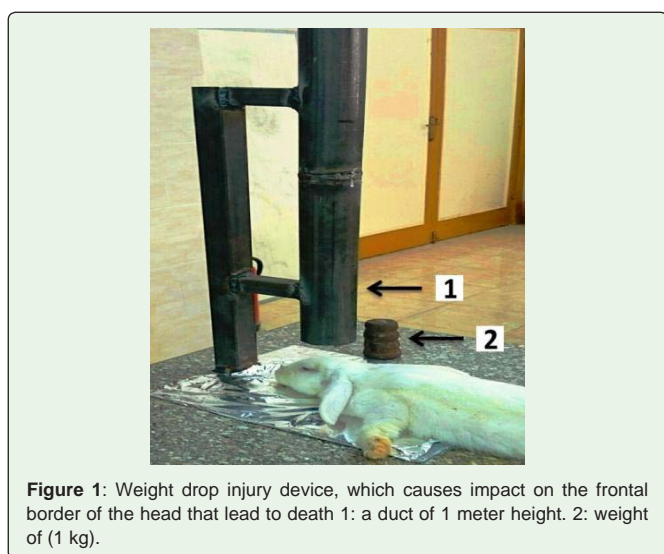
TLC was done using the Improved Neubaur Counting Chamber. The CSF sample was drawn in White Blood Cell (WBC) pipette up to 0.5 marks and diluted with the diluting fluid up to 11 marks on tube and mixed. Then the specimen was put at counting chamber and cells were counted in 4 squares, the number of cells counted equals the number of cells/mm<sup>3</sup> which is the equivalent of cells/UL.

### Biochemical analysis of lactic acid, hypoxanthine, ammonia and uric acid in plasma

Concentration of lactate in plasma was measured by Spectrum-Diagnostics Bioassay kits at 546 nm [13]. Hypoxanthine was determined in plasma samples using a commercial kit Amplex<sup>®</sup> Red Xanthine/Xanthine Oxidase Assay kits (Invitrogen). Ammonia and uric acid level in plasma samples was measured using Spin react bioassay kits, using Enzymatic-UV method at 340 nm [14] and at 520 nm [15], respectively.

### Determination of High Mobility Group Box-1 (HMGB1), Interleukin-1-beta (IL-1B) and Tumor Necrosis Factor-alpha (TNF-α)

The concentration of HMGB1 in serum was measured by an enzyme-linked immunosorbent assay using a rabbit ELISA kit of



**Figure 1:** Weight drop injury device, which causes impact on the frontal border of the head that lead to death 1: a duct of 1 meter height. 2: weight of (1 kg).

CusaBio, Catalog No. CSB-E08894Rb) at 540 nm. The cytokine IL-1 $\beta$  was determined in serum using rabbit IL-1 $\beta$  ELISA kits, (Ray Biotech, Catalogue Number: ELL-IL1 $\beta$ ) at 450 nm. TNF- $\alpha$  was measured by using rabbit TNF- $\alpha$  ELISA kits in serum, (Catalog No. CSB-E06998Rb, CusaBio Company Us and arrived through Medicomex, Egypt. (<http://indomedix.com/in-fertility-and-ivf-units-supplies/>). Absorbance at 450 nm.

### Histopathological and immunohistochemical investigation

Brain samples were fixed in 10% neutral buffered formalin, fixed specimens had dehydrated in a gradual ethanol concentration (70-100%), and cleared in the xylene, and embedded into paraffin. Five-micron thick paraffin sections were also prepared and then routinely were stained with hematoxylin and eosin (H&E) dyes for the histopathological examination under a light microscope [16]. Brain sections used were mounted on charged slides, deparaffinized in 4 changes of fresh xylene, and re-hydrated in graded ethanol (100%, 95% and 70%) then washed in PBS at pH 7.2 for 5 minutes. The sections were incubated with antisera containing the specific primary antibodies: anti-p53 antibody (Santa Cruz Biotechnology. Cat. No. sc- 6243 rabbit) and anti-Bcl-2 antibody (Santa Cruz Biotechnology. Cat. No. sc - 783 rabbit). The sections were incubated in a humidified chamber at room temperature overnight. Labeling antibody was added to each section. Diaminobenzidine was added as chromogen. Then the sections were counter stained, dehydrated in ascending grades of alcohol, mounted in Canada balsam and examined with a standard light microscope [17].

In order to assess the effect of trauma on some biochemical markers in blood, serum and CSF from time of death through 12 h after, repeated measures ANOVA was used with time interval (0 hr, 6 hr and 12 hrs) as the within-subjects factor while trauma, were considered as between-subjects factor, followed by Tukey's Honestly Significant Difference (Tukey's HSD) test as post hoc test. Analysis

**Table 1:** Comparison of CSF potassium, sodium, calcium, albumin levels and TLC between control groups (cervical dislocation) and traumatized groups (weight drop method) at 0, 6, and 12 hours PM.

| CSF Parameter       | Cause of death | Time after death (PMI)           |                                  |                                 |
|---------------------|----------------|----------------------------------|----------------------------------|---------------------------------|
|                     |                | 0 hr                             | 6 hrs                            | 12 hrs                          |
| Potassium (mmol/l)  | Control        | 6.32 $\pm$ 0.29 <sup>c</sup>     | 11.68 $\pm$ 0.62 <sup>b</sup>    | 16.71 $\pm$ 0.46 <sup>a</sup>   |
|                     | Traumatized    | 6.88 $\pm$ 0.47 <sup>c</sup>     | 12.37 $\pm$ 0.63 <sup>b</sup>    | 19.02 $\pm$ 0.25 <sup>a**</sup> |
| Sodium (mmol/l)     | Control        | 152.16 $\pm$ 0.48 <sup>a**</sup> | 144.24 $\pm$ 0.51 <sup>b**</sup> | 130.45 $\pm$ 0.37 <sup>c</sup>  |
|                     | Traumatized    | 149.34 $\pm$ 0.3 <sup>a</sup>    | 136.15 $\pm$ 0.38 <sup>b</sup>   | 129.92 $\pm$ 0.21 <sup>c</sup>  |
| Calcium (mg/dl)     | Control        | 8.17 $\pm$ 0.07 <sup>b</sup>     | 9.3 $\pm$ 0.21 <sup>b</sup>      | 13.7 $\pm$ 0.6 <sup>a**</sup>   |
|                     | Traumatized    | 11.11 $\pm$ 0.6 <sup>b**</sup>   | 14.6 $\pm$ 0.23 <sup>a**</sup>   | 10.2 $\pm$ 0.34 <sup>b</sup>    |
| Albumin (mg/dl)     | Control        | 6.27 $\pm$ 0.13 <sup>a</sup>     | 5.64 $\pm$ 0.18 <sup>a</sup>     | 3.97 $\pm$ 0.28 <sup>b</sup>    |
|                     | Traumatized    | 7.37 $\pm$ 0.12 <sup>a**</sup>   | 5.87 $\pm$ 0.13 <sup>a</sup>     | 2.42 $\pm$ 0.38 <sup>b</sup>    |
| TLC (cell/ $\mu$ l) | Control        | 3.4 $\pm$ 0.51 <sup>b</sup>      | 5.4 $\pm$ 0.51 <sup>b</sup>      | 10.8 $\pm$ 1.43 <sup>a</sup>    |
|                     | Traumatized    | 17.8 $\pm$ 0.86 <sup>c**</sup>   | 23 $\pm$ 0.71 <sup>b**</sup>     | 27.8 $\pm$ 0.86 <sup>a**</sup>  |

Means within the same row carrying different superscripts are sig. different at P < 0.05 based on Tukey's Honestly Significant Difference (Tukey's HSD) test. While <sup>a</sup>strikes indicate significance between Control and traumatized at each time point, <sup>b</sup>when (P < 0.05) and <sup>c</sup>when (P < 0.01).

was done using Statistical Package for Social Sciences version 24.0 (IBM Corp., Armonk, NY, USA). Results were reported in means  $\pm$  SEM (Standard Error of Mean). The value of P < 0.05 was used to indicate statistical significance.

## Results

### Biochemical analysis in CSF

The data in table 1 demonstrated a significant (P > 0.05) increase in the level of potassium ion (K<sup>+</sup>) in CSF of control groups (C0, C6 and C12) and traumatized groups (T0, T6 and T12) in ascending pattern with increasing PMI. At the same time, significant increase (P > 0.01) in K<sup>+</sup> concentration of traumatized rabbits than control at 12 hrs postmortem. The other intervals have no significant difference between two conditions of death. Na<sup>+</sup> in CSF of the control groups was declined along with the time interval passed since death. Moreover, Na<sup>+</sup> was less in 0 and 6 hrs time intervals of control groups than traumatized.

The results revealed an obvious increase in the level of calcium (Ca<sup>++</sup>) in the CSF of the control groups (C0, C6 and C12) with rising PMI. While Ca<sup>++</sup> concentration in CSF of traumatized groups was increased significantly after 6 hrs than at time of death, then, decreased at 12 hrs postmortem than both times. There was a significant (P > 0.01) difference (T0 and T6) and their respective controls. Albumin concentration in CSF of both control and traumatized groups, there was a considerable decline along with the time elapsed after death. Moreover, there was a difference (P > 0.05) between the traumatized group at zero time (T0) and its respective control (C0).

Our results clearly demonstrated that TLC was considerably higher in the C12 group than the previous two time intervals, without significant difference in its values between C0 and C6 groups. On the other hand, it showed highly significant (P > 0.05) increase in all traumatized groups (T0, T6 and T12) along with the time passed after death as shown in table 2. Generally there was a marked significant (P > 0.01) elevated TLC values in all traumatized rabbits regarding to its respective controls.

**Table 2:** Comparison of plasma lactic acid, ammonia, hypoxanthine and uric acid levels between control groups (cervical dislocation) and traumatized groups (weight drop method) at 0, 6, and 12 hours PM.

| Plasma Parameter     | Cause of death | Time after death (PMI)           |                                  |                                  |
|----------------------|----------------|----------------------------------|----------------------------------|----------------------------------|
|                      |                | 0 hr                             | 6 hrs                            | 12 hrs                           |
| Lactic acid (mmol/l) | Control        | 1.32 $\pm$ 0.23 <sup>c</sup>     | 7.29 $\pm$ 0.77 <sup>b</sup>     | 10.01 $\pm$ 0.37 <sup>a</sup>    |
|                      | Traumatized    | 2.33 $\pm$ 0.38 <sup>c*</sup>    | 9.04 $\pm$ 0.64 <sup>b</sup>     | 11.6 $\pm$ 0.44 <sup>a*</sup>    |
| Ammonia (mmol/l)     | Control        | 102.45 $\pm$ 1.11 <sup>c</sup>   | 113.86 $\pm$ 1.76 <sup>b</sup>   | 136.05 $\pm$ 3 <sup>a</sup>      |
|                      | Traumatized    | 125.38 $\pm$ 1.92 <sup>b**</sup> | 135.05 $\pm$ 1.82 <sup>b**</sup> | 160.96 $\pm$ 3.93 <sup>a**</sup> |
| Hypoxanthine (mg/dl) | Control        | 1.06 $\pm$ 0.24 <sup>c</sup>     | 3.63 $\pm$ 0.2 <sup>b</sup>      | 6.1 $\pm$ 0.26 <sup>a</sup>      |
|                      | Traumatized    | 1.07 $\pm$ 0.18 <sup>c</sup>     | 4.34 $\pm$ 0.22 <sup>b*</sup>    | 8.69 $\pm$ 0.42 <sup>a**</sup>   |
| Uric acid (umol/l)   | Control        | 1.26 $\pm$ 0.08 <sup>a</sup>     | 1.37 $\pm$ 0.08 <sup>a</sup>     | 1.52 $\pm$ 0.07 <sup>a</sup>     |
|                      | Traumatized    | 1.98 $\pm$ 0.09 <sup>c**</sup>   | 2.69 $\pm$ 0.04 <sup>b**</sup>   | 3.08 $\pm$ 0.1 <sup>a**</sup>    |

Means within the same row carrying different superscripts are sig. different at P < 0.05 based on Tukey's Honestly Significant Difference (Tukey's HSD) test. While <sup>a</sup>strikes indicate significance between Control and traumatized at each time point, <sup>b</sup>when (P < 0.05) and <sup>c</sup>when (P < 0.01).



## Biochemical analysis in plasma

Lactic acid in plasma of the present work was increased in both control and traumatized rabbits along with the time elapsed after death. At 6 hrs interval, there was non-significant difference between traumatized and control groups. The same for ammonia level and hypoxanthine in plasma all groups was markedly elevated along with the time passed after death. The recorded data in table 2 showed that there was a highly significant ( $P > 0.01$ ) increase in ammonia concentration in traumatized groups (T0, T6 and T12) in comparison with their controls. Uric acid levels in plasma of the control groups showed non-significant increase along with the time passed after death, but in traumatized groups it is not only significantly increased along with postmortem time, but also it is clearly elevated ( $P > 0.01$ ) in comparison with its corresponding control groups.

## Biochemical analysis in serum

High Mobility Group Box-1 (HMGB1) was detected in the serum of all groups; it revealed a very noteworthy increase with time in both control and traumatized rabbits. Our data showed a significant ( $P > 0.05$ ) difference at 6 and 12 hrs after death of traumatized groups than cervical dislocated rabbits. On the other hand, there was non-significant elevation in the levels of TNF- $\alpha$  in serum of both control and traumatized groups along with the time elapsed after death. But its level was higher in the traumatized than the corresponding control (Table 3). The concentration of IL-1 $\beta$  in serum, there was a slight increase (non-significant) with the time passed after death. A significant ( $P > 0.01$ ) increase was noticed in traumatized groups than controls at the different time intervals.

## Histopathological findings of brain

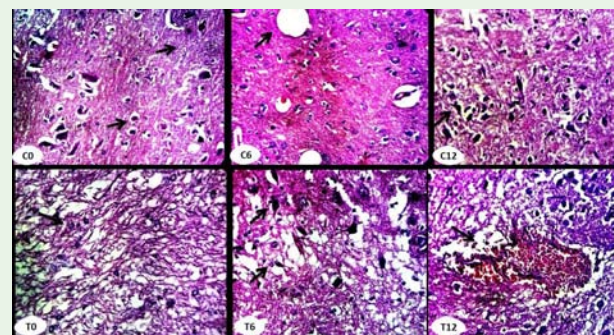
Examined sections (Figure 2) of the rabbit brain from the control group at the time of death (C0 group) revealed mild focal neuronal degeneration, perivascular and perineural edema and demyelination of some nerve fibers. T0 group revealed severe congestion of the meningeal and cerebral blood vessels with mild extravasation of the erythrocytes (recent hemorrhages) were detected.

Brain sections from C6 group revealed congestion of the cerebral blood vessels with perivascular edema. Diffuse cerebral and cerebellar

**Table 3:** Comparison of serum HMGB1, TNF- $\alpha$  and IL-1 $\beta$  levels between control groups (cervical dislocation) and traumatized groups (weight drop method) at 0, 6, and 12 hours PM.

| Serum Parameter       | Cause of death | Time after death (PMI)           |                                 |                                  |
|-----------------------|----------------|----------------------------------|---------------------------------|----------------------------------|
|                       |                | 0 hr                             | 6 hrs                           | 12 hrs                           |
| HMGB1 (ng/ml)         | Control        | 7.83 $\pm$ 0.29 <sup>c</sup>     | 168.96 $\pm$ 20.9 <sup>b</sup>  | 464.5 $\pm$ 19.82 <sup>a</sup>   |
|                       | Traumatized    | 11.42 $\pm$ 2.2 <sup>c</sup>     | 241.24 $\pm$ 7.77 <sup>b*</sup> | 537.16 $\pm$ 16.98 <sup>a*</sup> |
| TNF- $\alpha$ (pg/ml) | Control        | 8.4 $\pm$ 0.72 <sup>a</sup>      | 8.7 $\pm$ 0.83 <sup>a</sup>     | 10.42 $\pm$ 0.75 <sup>a</sup>    |
|                       | Traumatized    | 105.78 $\pm$ 1.92 <sup>a**</sup> | 106.34 $\pm$ 2.4 <sup>a**</sup> | 106.1 $\pm$ 1.65 <sup>a**</sup>  |
| IL-1 $\beta$ (pg/ml)  | Control        | 6.54 $\pm$ 0.73 <sup>a</sup>     | 6.58 $\pm$ 0.71 <sup>a</sup>    | 6.52 $\pm$ 0.6 <sup>a</sup>      |
|                       | Traumatized    | 179.12 $\pm$ 5.1 <sup>a**</sup>  | 175.32 $\pm$ 6 <sup>a**</sup>   | 177.14 $\pm$ 5.23 <sup>a**</sup> |

Means within the same row carrying different superscripts are sig. different at  $P < 0.05$  based on Tukey's Honestly Significant Difference (Tukey's HSD) test. While <sup>a</sup>strikes indicate significance between Control and traumatized at each time point, <sup>\*</sup>when ( $P < 0.05$ ) and <sup>\*\*</sup>when ( $P < 0.01$ ).

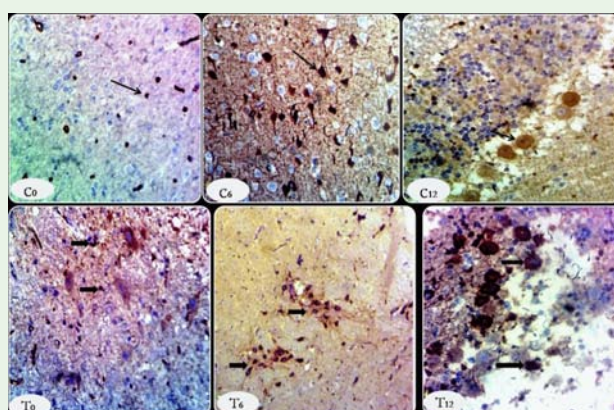


**Figure 2:** Indicative H&E stained sections of rabbit brain in C0 group showing perivascular and peri-neural edema together with demyelination of some nerve fibers and mild focal neuronal degenerations (arrows). T0 group showing congestion of the cerebral blood vessels together with neuronal degeneration, cytoplasmic vacuolation and perivascular and edema with marked axonal degenerations. C6 Group with mild neuronal degeneration with activated microglia and astrocytes and focal malacic areas. T6 Group: neuronal degeneration, activated, microglia with demyelination of some nerve fibers. C12 Group showed increased number of activated microglia and astrocytes (arrow). T12 Group showing multiple large and small old cerebral hemorrhages (arrow) X400.

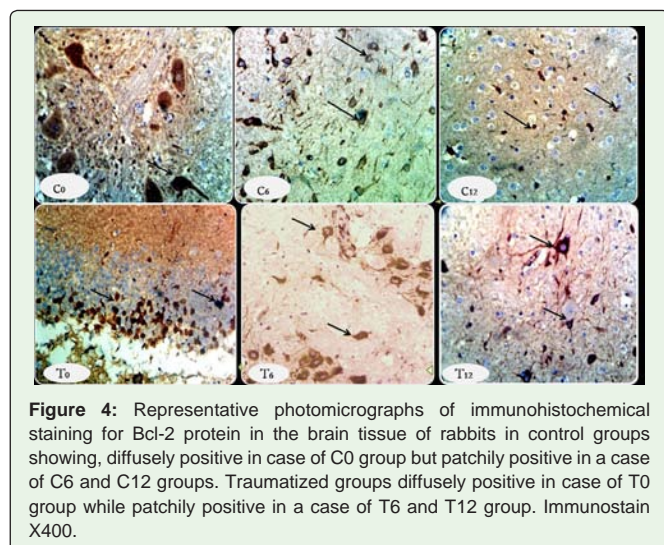
neuronal degeneration with a clear intracytoplasmic vacuolation accompanied by activated microgliosis and astrogliosis. While T6 group revealed multifocal old cerebral hemorrhages accompanied by necrosis of the surrounding tissue were observed.

In C12 group mild congestion of cerebral blood vessels with multiple focal malacic areas of variable shapes and sizes, neuronal degeneration in a moderate number of cells, perineural edema, and demyelination of the nerve fibers and increase in number of activated microglia and astrocytes were also detected. T12 group revealed multiple large and small old hemorrhages were seen in the cerebral parenchyma. Large and small malacic areas of different shapes and sizes were seen in figure 2.

## Immunohistochemistry (IHC)



**Figure 3:** Representative photomicrographs of immunohistochemical staining for p53 protein in the brain tissue of rabbits in control groups showing gradual positive reaction to p53 protein, weak reaction in C0, moderate in C6 while intense in case of C12. Traumatized groups (T0, T6 and T12) respectively showing positive reaction to p53 protein, weak reaction in T0, moderate in T6 while intense in case of T12. Immunostain X400.



**Figure 4:** Representative photomicrographs of immunohistochemical staining for Bcl-2 protein in the brain tissue of rabbits in control groups showing, diffusely positive in case of C0 group but patchily positive in a case of C6 and C12 groups. Traumatized groups diffusely positive in case of T0 group while patchily positive in a case of T6 and T12 group. Immunostain X400.

Immunohistochemical study of apoptotic inducer (p53) in brain tissue (represented by brown granules) in C0 group revealed about 5-10 of the brain cells are positive for the apoptotic protein (p53). C6 group showed multi focal strongly positive of a moderate number of neurons, microglia and astrocytes for the p53. In C12 group revealed multi focal strong positivity of a large number of neurons, microglia and astrocytes for the p53 (Figure 3). In traumatized group at the time of death (T0 group) there was focal neuronal and microglia cells are positivity for the apoptotic protein (p53). At 6 hrs PM, multi focal strong positivity of some neurons and a few microglia and astrocytes (5-10%). In T12 group, showed focal positivity of some neurons, astrocytes and microglia for the apoptotic protein.

Immunohistochemical findings of apoptotic inhibitor (Bcl-2) represented by brown granules in brain tissue of C0 group revealed positive reactions for most of the neurons and oligodendroglia cells. At 6 hrs after death bcl-2 stainability revealed a negative reaction for moderate number of neurons, astrocytes and microglial cells. At C12 group, most of the cerebral neurons, oligodendroglia were negatively reacted. T0 group revealed positive reaction for moderate number of the neurons, microglia and astrocytes. At 6 hrs PM bcl-2 stainability revealed that microglia cells in most parts are negatively stained except a few of them. At 12 hrs PM bcl-2 showed focal weak reaction of some of the cerebral neurons, astrocytes and microglial cells as shown in figure 4.

## Discussion

The Cerebro-Spinal Fluid (CSF) is the fluid of choice in cases of traumatic death. It is in direct contact with the extracellular matrix in the brain, and its composition reflects biochemical changes that occur in the brain [18].

In the present study, there is a significant increase in the  $K^+$  and  $Ca^{++}$  concentration with increasing PMI. Our results are in parallel with some previous studies On the same topic [19-21]. Similar results were obtained in CSF of dogs and donkeys [22,23]. On the other hand, CSF sodium and albumin levels significantly declined along with the time passed after death. The potassium levels were significantly elevated in traumatized rabbits than control at 12 hrs after death. On the other hand, CSF sodium levels were significantly declined

in traumatized rabbits at 0 and 6 hrs postmortem than control one. These changes may be attributed to the changes in the membrane potential that caused by transient cell membrane disruptions due to trauma [24]. It may be attributed to the massive release of glutamate from presynaptic terminals, which disrupts ionic equilibrium on postsynaptic membranes [25]. This decline in CSF albumin levels may be attributed to the postmortem degradation of albumin. The extensive biochemical changes taking place in CSF due to stoppage of oxygen circulation, cellular degradation, and altered enzymatic reaction cause a decline in albumin concentration after death [26].

In the current study, the Total Leucocytic Count (TLC) in CSF after death increased in the control groups with increasing PMI. These results agreed with the results obtained by many other authors [27-29]. Our results demonstrated that there was a highly significant increase of TLC in all traumatized rabbits regarding to their respective control, which may be due to the acute inflammatory response that occur due to TBI, including activation of the complement system which results in chemotaxis of neutrophils, and leukocyte activation [30]. Our results clearly showed that the traumatic groups having the highest values in lactic acid concentration than control groups, this may be attributed to the hemorrhagic shock that occurs after traumatic injury and it is one of the potential causes of elevated lactate in blood [31] or may be due multiple organ insufficiency following TBI that leads to high levels of lactate after delayed traumatic death [32]. This was confirmed by multifocal old cerebral hemorrhages which observed in our histopathological findings in brain tissue of the traumatized rabbits.

The current study showed that the hypoxanthine concentration in the blood of traumatized rabbits showed an apparent increase, other studies obtained parallel results who observed that the hypoxanthine was dramatically increased after TBI [33], this increase may be attributed to the breakdown of ATP, which present in brain tissue. When cells lack ATP, adenosine is metabolized to inosine by the action of adenosine deaminase (an enzyme present in large quantities in erythrocytes) and inosine is further metabolized to hypoxanthine [34]. Regarding the uric acid concentration in blood of traumatized groups was increased and there was a highly significant ( $P>0.01$ ) difference between traumatized groups in comparison with their respective controls, its concentration was increased in the blood of delayed traumatic deaths due to multiple organ insufficiency [35]. It has been proposed that the increase in uric acid concentration in traumatic deaths is a proinflammatory marker to indicate necrosis and death-induced inflammation, which confirmed by a significant increase in pro-inflammatory cytokines in our experiment. It has been bountifully high significant ( $P>0.01$ ) increase in the levels of  $TNF-\alpha$  and  $IL-1\beta$  in the serum of traumatized groups in our result, some studies attributed these results to cell destruction caused by inflammation resulting from traumatic injury [36].

High Mobility Group Box-1 (HMGB1) is a new postmortem marker and could be used as a new tool for the estimation of the PMI in the short- and long-term. In the present study, the postmortem serum levels of HMGB1 in control rabbits showed a time-dependent increase up to 12 hours postmortem, these results are in parallel with the results of a published work [35] in rats, in which serum HMGB1 level was increased up to seven days, the HMGB1 level peaked at day 2, decreased at day 3, and then became stable, this change was attributed to postmortem changes which induce necrosis and



necrotic cells release HMGB1, so detection of serum HMGB1 exuded from corpus necrotic tissue may be related to PMI. Moreover, our data showed that there were a significant ( $P>0.05$ ) differences at 6 and 12 hrs after death of traumatized groups than control, this may be attributed to traumatic brain injury which induce necrosis that occurs in response to severe mechanical or ischemic / hypoxic tissue damage and necrotic cells release HMGB1 [37]. Also, these observations were assured by axonal and neuronal degeneration and necrosis (Figures 2 and 3).

Concerning Immunohistochemistry (IHC) of rabbit brain tissue in control groups, our study revealed increase in the expression of apoptotic inducer (p53) with increasing PMI, while expression of apoptotic inhibitor (Bcl-2) was decreased in the same manner with more time postmortem, an author suggested that p53 and Bcl-2 parameters could be a good indicator for PMI [38]. Concerning the effect of trauma in the expression of p53 and Bcl-2 proteins, our results revealed an increase in p53 immunoreactivity and decreases in intracellular Bcl-2 immunoreactivity in injured brain regions may precede cell death following experimental brain trauma [39].

## Conclusion

It was concluded that biochemical analysis of some body fluids (CSF, Plasma and serum) could be used as key tools for assessment on accurate PMI. Herein, potassium, sodium, albumin and hypoxanthine were could play a key role in assessing accurate PMI. Moreover, IL-1 $\beta$ , TNF- $\alpha$ , ammonia and uric acid could be surprising tools in differentiation between control and traumatized deaths. Immunohistochemical results of p53 and Bcl-2 apoptotic markers in brain tissue declared that they could be good indicators for PMI but have very little difference between control and traumatized rabbits. Histopathological changes in the brain tissue were more severe in traumatic death, particularly meningeal and cerebral hemorrhages and necrosis of the surrounding tissues.

## Acknowledgments

The authors would like to thank Prof. Dr. El Sayed Rashad El Attar, Professor of Pathology, Faculty of Veterinary Medicine, Zagazig University, Egypt and Dr. Mohamed Gomaa Tantawy, Associate professor of Surgery, for their supportive contribution in this study.

## References

- Maeda H, Michiue T, Zhu BL, Ishikawa T, Quan L, Bessho Y, et al. Potential risk factors for sudden cardiac death: an analysis of medicolegal autopsy cases. *Leg Med*. 2009; 11: 263-265.
- Garg SDP, Arora A, Dubey BP. A study of serum enzymal changes after death and its correlation with time since death. *J Indian Acad Forensic Med*. 27: 16-18.
- Arikeri SM, Laveesh MR, Priyadarshini AV, Suja P, Farsana UK, Michael N, et al. Determination of time since death by estimating sodium and potassium levels in synovial fluid. *International Journal of Research in Pharmaceutical and Biomedical*. 2013; 4: 194-196.
- Asemota AO, George BP, Bowman SM, Haider AH, Schneider EB. Causes and trends in traumatic brain injury for United States adolescents. *J Neurotrauma*. 2013; 30: 67-75.
- Maeda H, Ishikawa T, Michiue T. Forensic biochemistry for functional investigation of death: concept and practical application. *Legal Med*. 2011; 13: 55-67.
- Zhu BL, Ishikawa T, Michiue T, Li DR, Zhao D, Quan L, et al. Postmortem serum catecholamine levels in relation to the cause of death. *Forensic Sci Int*. 2007; 173: 122-129.
- Palmiere C, Mangin P. Postmortem chemistry update part II. *Int J Legal Med*. 2012; 126: 199-215.
- Muir W. Trauma: physiology, pathophysiology, and clinical implications. *J Vet Emer Crit Care*. 2006; 16: 253-263.
- Hobbs BA, Rolhall TG, Sprenkel TL, Anthony KL. Comparison of several combinations for anesthesia in rabbits. *Am J Vet Res*. 1991; 52: 669-674.
- Silva Ddos S, Brito JN, Ibiapina JO, Lima MF, Medeiros AR, Queiroz BH, et al. Traumatic brain injury: clinical and pathological parameters in an experimental weightdrop model. *Acta Cir Bras*. 2011; 26: 94-100.
- Meredith A, Flecknell P. *BSAVA Manual of Rabbit Medicine and Surgery*. 2<sup>nd</sup> Edition. 2006; 224 pages.
- Doumas BT, Watson WA, Biggs HG. Albumin standards and the measurement of serum albumin with bromocresol green. *Clin Chim Acta*. 1971; 31: 87-96.
- Field M, Block JB, Levin R, Rall DP. Significance of blood lactate elevations among patients with acute leukemia and other neoplastic proliferative disorders. *Am J Med*. 1996; 40: 528-547.
- Fossati P, Prencipe L, Berti G. Use of 3,5-dichloro-2-hydroxybenzenesulfonic acid/4-aminophenazone chromogenic system in direct enzymic assay of uric acid in serum and urine. *Clin Chem*. 1980; 26: 227-231.
- Ng, Valerie. *Effects of Disease on Clinical Laboratory Tests*. 4<sup>th</sup> Edition. Vol. 1 and 2. Young DS, Friedman RB (Eds.). AACC Press, Washington, DC, USA. *Clinical Chemistry*. 2002; 48: 682-683.
- Bancroft JD, Layton C, Suvarna K. *Bancroft's theory and practice of histological techniques*. 7<sup>th</sup> Edition. Churchill Livingstone Elsevier. 2013; 6: 83-92.
- Bancroft JD, Gamble M. *Theory and Practice of histological techniques*. 6<sup>th</sup> Edition. Churchill, Livingstone, New York, London. 2008; 21: 440-450.
- Zetterberg H, Smith DH, Blennow K. Biomarkers of mild traumatic brain injury in cerebrospinal fluid and blood. *Nat Rev Neurol*. 2013; 9: 201-210.
- Fraschini F, Muller E, Zanoboni A. Postmortem increase of potassium in human cerebrospinal fluid. *Nature*. 1963; 198: 1208.
- Kärkelä JT. Critical evaluation of postmortem changes in human autopsy cisternal fluid. Enzymes, electrolytes, acid-base balance, glucose and glycolysis, free amino acids and ammonia. Correlation to total brain ischemia. *J Forensic Sci*. 1993; 38: 603-616.
- Yadav J, Deshpande A, Arora A, Athawal BK, Dubey BP. Estimation of time since death from CSF electrolyte concentration in Bhopal region of central India. *Leg Med (Tokyo)*. 2007; 9: 309-313.
- Schoning P, Straffuss AC. Determining time of death of a dog by analyzing blood, cerebrospinal fluid, and vitreous humor collected at postmortem. *Am J Vet Res*. 1980; 41: 955-957.
- Ashry KM. Postmortem Biochemical Changes in Canine Cerebrospinal Fluid and Relation to Time after Death. *Benha Vet Med J*. 2004; 15: 147-158.
- Katayama Y, Becker DP, Tamura T, Hovda DA. Massive increases in extracellular potassium and the indiscriminate release of glutamate following concussive brain injury. *J Neurosurg*. 1990; 73: 889-900.
- Andriessen TM, Jacobs B, Vos PE. Clinical characteristics and pathophysiological mechanisms of focal and diffuse traumatic brain injury. *J Cell Mol Med*. 2010; 14: 2381-2392.
- Palmiere C, Mangin P. Urea nitrogen, creatinine, and uric acid levels in postmortem serum, vitreous humor, and pericardial fluid. *Int J Legal Med*. 2015; 129: 301-305.
- Platt MS, McClure S, Clarke R, Spitz WU, Cox W. Postmortem cerebrospinal fluid pleocytosis. *Am J Forensic Med Pathol*. 1989; 10: 209-212.

**Citation:** Mohamed AAR, Elbohi KM, Sharkawy NIE and Hassan MA. Biochemical and Apoptotic Biomarkers as Indicators of Time Elapsed Since Death in Experimentally Induced Traumatic Brain Injury. *SM J Forensic Res Criminol*. 2017; 1(2): 1010.

28. Wyler D, Marty W, Bär W. Correlation between the post-mortem cell content of cerebrospinal fluid and time of death. *Int J Legal Med.* 1994; 106: 194-199.
29. Rajesh B. Evaluation of cerebrospinal fluid cells in postmortem period to estimate death interval. *J Indian Acad Forensic Med.* 2009; 31: 205-209.
30. Simpson SA, Syring R, Otto CM. Severe blunt trauma in dogs: 235 cases (1997-2003). *J Vet Emerg Crit Care (San Antonio).* 2009; 19: 588-602.
31. Akkose S, Ozgurur A, Bulut M, Koksall O, Ozdemir F, Ozguç H. Relationships between markers of inflammation, severity of injury, and clinical outcomes in hemorrhagic shock. *Adv Ther.* 2007; 24: 955-962.
32. Odom SR, Howell MD, Silva GS, Nielsen VM, Gupta A, Shapiro NI, et al. Lactate clearance as a predictor of mortality in trauma patients. *J Trauma Acute Care Surg.* 2013; 74: 999-1004.
33. Bell MJ, Kochanek PM, Carcillo JA, Mi Z, Schiding JK, Wisniewski SR, et al. Interstitial adenosine, inosine, and hypoxanthine are increased after experimental traumatic brain injury in the rat. *J Neurotrauma.* 1998; 15: 163-170.
34. Geiger JD, Parkinson FE, Kowaluk EA. Regulators of endogenous adenosine levels as therapeutic agents. *Purinergic approaches in experimental therapeutics.* 1997; 55-84.
35. Kikuchi K, Kawahara KI, Biswas KK, Ito T, Tancharoen S, Shiomi N, et al. HMGB1: A new marker for estimation of the postmortem interval. *Exp Ther Med.* 2010; 1: 109-111.
36. Mimasaka S, Ohtsu Y, Tsunenari S, Funayama M. Postmortem cytokine levels and severity of traumatic injuries. *Int J Legal Med.* 2006; 120: 265-270.
37. Werner C, Engelhard K. Pathophysiology of traumatic brain injury. *Br J Anaesth.* 2007; 99: 4-9.
38. Elias E, Osman K, Abdul Aziz S, Mansar A, Ibrahim F, Mohamed J. Determination of Time of Death Based on Basic Histological Stain and Immunostain Changes. *Journal Sains Kesihatan Malaysia.* 2004; 2: 63-70.
39. Raghupathi R, Strauss KI, Zhang C, Krajewski S, Reed JC, McIntosh TK. Temporal alterations in cellular Bax:Bcl-2 ratio following traumatic brain injury in the rat. *J Neurotrauma.* 2003; 20: 421-435.