TIMING OF TRAUMATIC BRAIN INJURY AND ITS SYSTEMIC EFFECTS ON THE LUNGS OF ADULT MALE ALBINO RATS

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ABSTRACT

Objective: Timing of traumatic brain injury (TBI) is very common and important especially, in forensic autopsy cases with no signs of intracranial hemorrhage or contusion and with short time of survival following head injury. In such cases it is difficult to determine the cause of death. TBI results in systemic inflammatory responses that are complicated by dysfunction of peripheral organs such as the lungs, liver and intestine. The lung is especially critical in the setting of lung transplantation, where more than half of donor allografts are obtained postmortem from individuals with TBI. The aim of the present study was to assess post-intervals of traumatic brain injury and to evaluate the effects of TBI on lungs by measuring serum levels of High mobility group box 1 protein (HMGB1), performing quantitative real-time polymerase chain reaction (qPCR) analysis for gene expression and detecting any histopathological changes or immunohistochemical reaction of brain and lungs.

Materials and Methods: Fifty four adult male albino rats divided into in to 3 groups (18 rats each): Group I: Negative control: rats left without any intervention. Group II: (sham): rats were anesthetized with ether inhalation and left without TBI inflection. Group III (TBI): rats were anesthetized with ether inhalation then the head was placed on platform and brain injury was done by weight drop method. After TBI infliction, six rats from group III (TBI) at each time point (1st, 3rd & 7th day) and six rats from group I&II were anesthetized and blood samples were drawn for measuring serum level of (HMGB1).Then rats were sacrificed, specimens from contused cerebral cortex and lungs were subjected to quantitative real-time polymerase chain reaction (qPCR) analysis for gene expression of HMCB1, histopathology examination and immunohistochemical detection of HMGB1 immunoreaction. Results: Traumatic brain injury induced time dependent up-regulation of HMGB1 in serum and qPCR gene expression. Significant histopathological inflammatory changes and positive HMGB1 immunoreaction of brain and lung tissues of TBI (group III) were detected when compared with both control groups. Conclusion: It can be concluded that traumatic brain injury induced time dependent changes in serum and tissue levels of HMGB1, also histopathological changes in the brain and lungs of adult male albino rats. So, this marker can be useful for the diagnosis and timing of brain trauma and predict post TBI lung injury.

Keywords: Traumatic brain injury; systemic inflammatory responses; High mobility group box 1 protein; lung.
INTRODUCTION

A traumatic brain injury (TBI) primarily affects young people in industrialized countries. Approximately 25% of these injuries result in long-term disabilities and subsequently lead to familial, social and economic burden (Algattasaand Huang, 2013).

Inflammation is the main factor in pathophysiology of traumatic brain injury. The brain follow the same role of immune activation as well as the whole body organs in response to inflammation although of its almost complete isolation from the blood stream mediated by the blood–brain barrier (BBB) (Graber et al., 2015).

The post-traumatic local and systemic mediator release following TBI is suggested to cause secondary damage onto the injured brain and other organs such as cerebrovascular failure with capillary leak and edema, systemic inflammatory response syndrome and multiple organ failure (Arand et al., 2001).

Estimating post-intervals of traumatic brain injury (TBI) are very common and important in forensic practice (Tao et al., 2006). Accurate determination of the timing of injury in cases of inflicted TBI is often difficult but vital in identifying potential perpetrators and for determining if there has been a delay in seeking medical care especially in cases of suspected child abuse (Berger et al., 2006).

High-mobility group box-1 (HMGB1), a ubiquitous nuclear protein, serves as an early mediator of inflammation, is an alarm that can be released from immune cells through activation by lipopolysaccharides (LPS), tumor necrosis factor (TNF)-α, interleukin (IL)-1 and interferon (IFN)-γ and plays a key role in many pathogenic states, including TBI and ischemia-reperfusion injury (Andersson et al., 2011).

The presence of pulmonary dysfunction after brain injury is a very common event. Acute lung injury (ALI) occurs in 20% of patients with isolated brain injury and is associated with a poor outcome. The lung is the most vulnerable organ to the inflammatory cascade triggered by TBI (Araujo et al., 2014).

The inflammatory response to TBI has been noted as being considerably up-regulated, even months after injury. Preventing the inflammatory response is considered a high potential target for protection from detrimental consequences after TBI (VonderHaar et al., 2014).

Recent studies adopted neuroprotective strategies after brain trauma, may not only to save more lives by increasing available organs for transplantation but also to limit the progression of systemic manifestations that currently make TBI such a lethal process (Stein, 2011).

AIM OF THE WORK

The aim of the present study was to assess post-intervals of traumatic brain injury and to evaluate the effects of TBI on lungs by measuring serum levels of High mobility group box 1 protein (HMGB1), performing quantitative real-time polymerase chain reaction (qPCR) analysis for gene expression as well as detecting any histopathological changes or immunohistochemical reaction of brain and lung.
MATERIALS & METHODS

I- Animals:
The study was carried out on 54 adult male albino rats, their weight ranging from 180-200g. They were obtained from the breeding animal house, Faculty of medicine, Zagazig University. The rats were fed commercial rodent pellets and given water ad libitum throughout the experiment.

Ethical consideration of the study:
- All ethically approved conditions used for animal housing & handling were considered. The animals were acclimatized to experimental conditions prior to the start of study for a period of 14 days to ascertain their physical well-being and to exclude any diseased animals. Promotion of high standard of care & animal well-being at all times. Painless procedures were performed with appropriate sedation to avoid distress & pain.

Experiment design:
Traumatic brain injury by weight drop is a well-established animal model for brain trauma studies and was reported to generate a reproducible injury in the ipsilateral cortex (Zhanget al., 2006). In the present study, a modification of a well-described percussion trauma model was used (Marmarouet al., 1994). The traumatizing device consisted of a hollow tube 15 cm long. The device was kept vertical to the surface of the intact skull and guided a falling weight (iron road with a circular foot plate) weighting 200 g. The device was positioned on the right parietal bone anterior and lateral to the lambda (Flierl et al., 2009).

The rats were randomly assigned to three groups of eighteen animals each.
- Group I (negative control): Rats were considered as a negative control. They were left without handling.
- Group II (sham): Rats were anesthetized with ether inhalation but left without TBI infliction.
- Group III (TBI): Rats were served as traumatic brain injury (TBI) group. Each rat was anesthetized with ether inhalation. The head was placed on platform and brain injury was induced by dropping the previously described 200gm weight.

After TBI infliction, six rats from group III (TBI) at each time point (1st, 3rd & 7th day) and six rats from each control group (I &II) were anesthetized and blood samples were drawn for measuring serum level of High mobility group box 1 protein (HMGB1). Then rats were sacrificed, specimens from the brain (samples from contusion tissue with its border area sized 1 cm×1 cm×1 cm) and lungs were subjected to quantitative real-time polymerase chain reaction (qPCR) for HMGB1 gene expression, histopathological and immune-histochemical study.

General observation:
Rats in the sham control group usually recovered conscious ½ hour after anesthesia. Whereas rats in the TBI group were unconscious for 1-2hours post injury and recovered with normal eating behavior from 3-4hours after trauma.

II-Methods:
1- Biochemical Studies:
Venous blood samples (3cc) were collected from the retro-orbital plexus of the animals by capillary glass tubes according to procedure described by Nemzek et al.(2001). Blood samples
were incubated at 37 °C until blood is clotted and then centrifuged to separate the serum, for measuring serum levels of High mobility group box 1 protein (HMGB1) levels by using commercially available kits. The principal of the method depends on Enzyme Linked Immunosorbent Assay (ELISA), manufactured by (R&D Systems, USA). (Ueno et al., 2004).

**Principle of the assay:**

High mobility group box 1 protein (HMGB1) was measured in serum according to the instructions of the manufacturers. The technique involves simultaneous reaction of (HMGB1) present in the sample or standard with two antibodies directed against different epitopes on the (HMGB1). One antibody (monoclonal) is coated onto the walls of the microtiter wells, and the other (polyclonal) is conjugated to the enzyme horseradish peroxidase. Any HMGB1 present form a bridge between the two antibodies (Yamada et al., 2003). After removal of the unbound material by aspiration and washing, the amount of conjugate bound to the well is detected by reaction with a substrate specific for the enzyme, which yields a product in which the intensity of color is proportional to the amount of conjugate. This product can then be quantified photometrically. The absorbance was recorded at 450-nm wavelength with an automated ELISA reader. Quantitative results were obtained in relation to standard curves with recombinant protein (Boutté et al., 2016).

2- **Quantitative real-time polymerase chain reaction (qPCR) analysis for gene expression:**

Total RNA was extracted from brain and lung tissue homogenate using a Qiagen RNA isolation kit (RNeasy, Qiagen Ltd, Crawley, West Sussex, UK) according to the manufacturer’s protocol. The total RNA was quantified by the measured absorbance at 260 nm in a spectrophotometer. After that, RNA was converted into complementary DNA (cDNA) by reverse transcriptase (QuantiTect Reverse Transcription Kit, Qiagen, # 205310, Germany). This extracted RNA was used for HMGB1 gene expression. SYBR Green RT PCR amplification was carried out in 25-μL reaction volume containing 12.5μL of SYBR Green Real-time PCR Master Mix (Roche Diagnostics), 1μL each of the forward and reverse primer, 2μL of cDNA, and 8.5μL of distilled water. All samples were run in duplicates and PCR amplification consisted of 40 cycles of denaturation at 95 °C for 10 s, annealing at 55 °C for 10 s, and extension at 72 °C for 20 s. The primers were designed by the web based tool, Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) based on the published rat sequence. The sequence of the primers used for HMGB1 was as follow: 5’-ATGGGCAAAGGAGATCCTA-3’ and 5’-ATTCATCATCATCATCTTCTTCT-3’ (646 bp), the amount of cDNA in the reactions was normalized with an internal control, the constitutively expressed gene GAPDH. Relative gene expression was calculated as fold change from the formula: 2−ΔΔCT (Xiang et al., 2014).

3- **Histopathological study:**

Light microscope technique: The brain and lung specimens were fixed with 10% buffered formalin for 24 hours to prepare paraffin blocks and thick sections were stained with
Hematoxylin and eosin stain according to Bancroft and Gamble (2002).

4- Immunohistochemical study of HMGB1 immunoreaction:

The brain and lung sections were immunostained for HMGB1 using the streptavidin-biotin complex. All steps, including deparaffinization and counterstaining with hematoxylin, were performed in accordance with the manufacturer’s instructions. Non-specific binding of the antibodies was blocked by incubating them with 100 mL of 5% normal goat serum for 20 min. Then, the lung sections were incubated overnight at 4°C in the presence of mouse anti-rat HMGB1 polyclonal antibody (1:1000) as the primary antibody, washed with phosphate buffered saline, and incubated in the presence of anti-mouse IgG antibody (1:1000) as the secondary antibody. The number of brown granules in each high powered field (magnification: x 400) was quantified as the number of positively stained cells or nuclei. The measurements were expressed as the percentage of positively stained cells (Li et al., 2016).

Statistical analysis: For statistical analysis, SPSS 16.0.1 for windows programme was used. Data was represented as means ± SD. The differences were compared for statistical significance by independent t-test and f-test. Statistical difference between groups was calculated by LSD. Data for some groups at different time was compared using two way f-test. The descriptive data was analyzed by using chi-square (X²) test. Difference was considered significant at p<0.05 and highly significant at p<0.01.

RESULTS

- Processing the mean values of serum and tissue levels of HMGB1 (ng/mL) of the control groups negative & sham (I & II) were within normal values and there were no statistically significant differences (p>0.05) between them (Table-1, 3& 5). So, the results of the negative control group (I) were used for comparison with those of TBI group (III).

1- Biochemical Results:

Serum levels of High mobility group box 1 protein (HMGB1):

In TBI group (group III) there were significant increases in serum levels of HMGB1 during first day then decreased from day 3 till day 7 after TBI however still significantly elevated as compared with that of negative control group (group I) (Table -2).

2- qRT-PCR relative gene expression of High mobility group box 1 protein (HMGB1):

Gene expressions of HMGB1 were increased significantly in the TBI group (III) as compared to the control groups (I&II) in the 1st, 3rd and 7th day in both the brain and lungs (Tables-4,6).

Histopathological Results:

The light microscopic examinations of both control groups negative & sham (I&II) revealed similar results. Thus, group I was chosen as the control group in the figures.

-Brain;

Examination of sections from the brain tissue of negative control group (group I) showed normal neuronal structure formed of nerve cells with pale nuclei (Fig.1). While in TBI (group III) at the 1st day, sections revealed shrunken nerve cells with darkly stained pyknotic nuclei and neuropil oedema (Fig.2,3). At 3rd day, there were severe subarachnoid...
hemorrhage, congested blood vessels, neuropil edema, and nerve cells with darkly stained pyknotic nuclei, extensive neuronal loss and inflammatory cellular infiltration (Fig.4, 5a-b, 6). At the 7th day after TBI, there were numerous nerve cells with darkly stained pyknotic nuclei (Fig.7). Statistical analysis of histopathological changes of rat brain at the different time points of the study (1st, 3rd and 7th day) using chi-square ($X^2$) test revealed significant increase when compared to control group ($P < 0.05$). Moreover histopathological changes showed significant increase at 1st day then significant decrease at the 7th day when compared to the 3rd day (Table-7).

-Lung;

Lungs tissue of negative control group (group I) showed normal spongy histological structure and architecture of the lung with alveoli, alveolar sacs, thin and thick portions of interalveolar septa (Fig. 8). The alveoli appeared patent with thin interalveolar septa. In TBI group III at 1st day, there were severe alveolar congestion, prominent alveolar septal vessels, intraparenchymal congested blood vessels, marked infiltration of macrophages in alveolar lumen, intraparenchymal inflammatory infiltration and thickening of alveolar walls (Fig.9-a,b ,10). While, on 3rd day after TBI, there were gradual decrease in alveolar and blood vessel congestion, moderate thickening of alveolar walls with intraparenchymal cellular inflammatory infiltration (Fig.11-a,b,12). On the 7th day after TBI, the histopathological changes and intraparenchymal inflammatory infiltration became mildly detected (Fig. 13). Statistical analysis of histopathological changes of rat lung at the different time points of the study (1st, 3rd and 7th day) using chi-square ($X^2$) test revealed significant increase when compared to control group ($P < 0.05$). Moreover histopathological changes showed significant increase at 1st day then significant decrease at the 7th day when compared to the 3rd day (Table-8).

4-Immunohistochemical results:

-Immunohistochemistry was performed to ascertain the immunoreactivity of HMGB1 in brain and lung tissues. Positive immunoreaction means the number of brown granules in each high powered field that was quantified as the number of positively stained cells or nuclei in the nerve cells and alveolar epithelium. Control groups (I&II) showed a weak positive HMGB1 immunoreaction with scanty number of positive cells but significantly lower than group III (TBI) (Fig.14-a&15-a). TBI induced significant positive HMGB1 immunoreaction in brain and lung tissues at 1st, 3rd and 7th days of the study. Brain sections of group III (TBI) revealed moderate immunoreaction of HMGB1 in 1st day, strong positive on the 3rd day and became mild by reaching the 7th day (Fig14-b,d). While, lung sections of group III (TBI) revealed strong positive immunoreaction of HMGB1 in 1st day(Fig15-b), moderate reaction on the 3rd day then gradual decrease in intensity on the 7th day that showed mild immunoreaction (Fig.15-c,d).
Table (1): Comparison of mean values of serum levels of HMGB1 (ng/Ml) between negative and sham control groups (I&II) by using t-test and f-test.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Negative control group (group I)</th>
<th>sham group (group II)</th>
<th>t</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Serum levels of HMGB1 ng/mL</td>
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</tr>
<tr>
<td>1st day n=6</td>
<td>1.78 ± 0.59</td>
<td>1.70 ± 0.55</td>
<td>0.42</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td>3rd day n=6</td>
<td>1.83 ± 0.61</td>
<td>1.75 ± 0.58</td>
<td>0.40</td>
<td>0.69</td>
<td></td>
</tr>
<tr>
<td>7th day n=6</td>
<td>1.77 ± 0.57</td>
<td>1.81 ± 0.60</td>
<td>0.21</td>
<td>0.83</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.05</td>
<td>0.16</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.95</td>
<td>0.85</td>
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</tbody>
</table>

P value of >0.05 = Non-Significant. Values are expressed as mean ± standard deviation (SD)

Table (2): Comparison of mean values of serum levels of HMGB1 (ng/Ml) between negative control group I and group III (TBI) by using t-test and f-test.

<table>
<thead>
<tr>
<th>Day</th>
<th>Group</th>
<th>Negative control group (group II)</th>
<th>Traumatic Brain injury group (group III)</th>
<th>t-test</th>
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<tbody>
<tr>
<td></td>
<td>Serum levels of HMGB1 ng/mL</td>
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</tr>
<tr>
<td>1st day n=6</td>
<td>1.70 ± 0.55 a</td>
<td>7.51 ± 2.3 b</td>
<td>10.42</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>3rd day n=6</td>
<td>1.75 ± 0.58 a</td>
<td>5.61 ± 1.87 d</td>
<td>8.36</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>7th day n=6</td>
<td>1.81 ± 0.60 a</td>
<td>4.02 ± 1.34 f</td>
<td>6.39</td>
<td>&lt;0.001**</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.16</td>
<td>15.58</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.85</td>
<td>&lt;0.001**</td>
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</table>

Groups with the same letters are significantly indifferent (p >0.05) Values are expressed as mean ± standard deviation (SD) P value < 0.01 = Highly Significant (**)

Table (3): Comparison of mean values of qRT-PCR relative gene expression of HMGB1 in the brain tissues at different time intervals (1st, 3rd and 7th day) (ng/Ml) between negative and sham group (I&II) by using t-test.

<table>
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<tr>
<th>Day</th>
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<th>t</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>brain tissues levels of HMGB1 ng/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st day n=6</td>
<td>0.662±0.113</td>
<td>0.546±0.187</td>
<td>1.301</td>
<td>0.386</td>
<td></td>
</tr>
<tr>
<td>3rd day n=6</td>
<td>0.672±0.197</td>
<td>0.651±0.107</td>
<td>0.229</td>
<td>0.823</td>
<td></td>
</tr>
<tr>
<td>7th day n=6</td>
<td>0.598±0.136</td>
<td>0.526±0.139</td>
<td>0.907</td>
<td>0.223</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>0.414</td>
<td>1.234</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.668</td>
<td>0.319</td>
<td></td>
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</table>

P value of >0.05 = Non-Significant. Values are expressed as mean ± standard deviation (SD)
Table (4): Comparison of mean values of qRT-PCR relative gene expression of HMGB1 in the brain tissues at different time intervals (1\textsuperscript{st}, 3\textsuperscript{rd} and 7\textsuperscript{th} day) (ng/Ml) between negative control group I and group III (TBI) by using t-test and two way f-test.

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<td></td>
<td>brain tissues levels of HMGB1 (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} day n=6</td>
<td>0.662±0.113\textsuperscript{a}</td>
<td>2.341±0.265\textsuperscript{b}</td>
<td>12.549 &lt;0.001**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3\textsuperscript{rd} day n=6</td>
<td>0.672±0.197\textsuperscript{a}</td>
<td>3.775±0.276\textsuperscript{d}</td>
<td>12.381 &lt;0.001**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7\textsuperscript{th} day n=6</td>
<td>0.598±0.136\textsuperscript{a}</td>
<td>1.587±0.137\textsuperscript{f}</td>
<td>25.568 &lt;0.001**</td>
<td></td>
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<tr>
<td>F</td>
<td>0.414</td>
<td></td>
<td></td>
<td>134.629</td>
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</tr>
<tr>
<td>P</td>
<td>0.668</td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
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</tbody>
</table>

Groups with the same letters are significantly indifferent (p >0.05)  
Values are expressed as mean ± standard deviation (SD)  
P value < 0.01= Highly Significant (**).

Table (5): Comparison of mean values of qRT-PCR relative gene expression of HMGB1 in the lung tissues at different time intervals (1\textsuperscript{st}, 3\textsuperscript{rd} and 7\textsuperscript{th} day) (ng/Ml) between negative and sham group (I&II) by using t-test and f-test.

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<td>Lung tissues levels of HMGB1 (ng/mL)</td>
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<tr>
<td>1\textsuperscript{st} day n=6</td>
<td>0.661±0.102</td>
<td>0.527±0.177</td>
<td>1.391</td>
<td>0.195</td>
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<tr>
<td>3\textsuperscript{rd} day n=6</td>
<td>0.589±0.197</td>
<td>0.651±0.104</td>
<td>0.682</td>
<td>0.511</td>
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<tr>
<td>7\textsuperscript{th} day n=6</td>
<td>0.652±0.131</td>
<td>0.642±0.141</td>
<td>0.267</td>
<td>0.795</td>
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<tr>
<td>F</td>
<td>0.417</td>
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<td>1.387</td>
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</tr>
<tr>
<td>p</td>
<td>0.666</td>
<td></td>
<td></td>
<td>0.280</td>
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</table>

P value of >0.05 = Non-Significant.  
Values are expressed as mean ± standard deviation (SD)

Table (6): Comparison of mean values of qRT-PCR relative gene expression of HMGB1 in the lung tissues at different time intervals (1\textsuperscript{st}, 3\textsuperscript{rd} and 7\textsuperscript{th} day) (ng/Ml) between negative control group I and group III (TBI) by using t-test and f-test.

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<th>Traumatic Brain injury group (group III)</th>
<th>t-test</th>
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<td>Lung tissues levels of HMGB1 (ng/mL)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1\textsuperscript{st} day n=6</td>
<td>0.661±0.102\textsuperscript{a}</td>
<td>2.775±0.264\textsuperscript{d}</td>
<td>18.296 &lt;0.001**</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3\textsuperscript{rd} day n=6</td>
<td>0.589±0.197\textsuperscript{a}</td>
<td>1.978±0.245\textsuperscript{d}</td>
<td>10.822 &lt;0.001**</td>
<td></td>
<td></td>
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<tr>
<td>7\textsuperscript{th} day n=6</td>
<td>0.652±0.131\textsuperscript{a}</td>
<td>1.289±0.236\textsuperscript{b}</td>
<td>5.781 &lt;0.001**</td>
<td></td>
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</tr>
<tr>
<td>F</td>
<td>0.417</td>
<td></td>
<td></td>
<td>53.686</td>
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</tr>
<tr>
<td>P</td>
<td>0.666</td>
<td></td>
<td></td>
<td>&lt;0.001**</td>
<td></td>
</tr>
</tbody>
</table>

Groups with the same letters are significantly indifferent (p >0.05)  
Values are expressed as mean ± standard deviation (SD)  
P value < 0.01= Highly Significant (**).
Table (7): Statistical comparison between negative control group I and group III (TBI) as regards histopathological changes of rat brain at the different time points of the study (1st, 3rd and 7th day) using chi-square ($X^2$) test.

<table>
<thead>
<tr>
<th>Histo-pathological changes</th>
<th>Negative control group (%) n= 6/ time point</th>
<th>TBI group (%) n= 6/ time point</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
<td>3rd days</td>
<td>7th days</td>
<td>1st day</td>
<td>3rd days</td>
</tr>
<tr>
<td>Subarachnoid hemorrhage</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Pyknotic nuclei</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Shrunken neurons</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Congested blood vessels</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Neuropil edema</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>inflammatory cellular infiltration</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>6</td>
</tr>
</tbody>
</table>

P1: Day 1 control versus Day 1 TBI  
P2: Day 3 control versus Day 3 TBI  
P3: Day 7 control Versus Day 7 TBI  
P value < 0.01= Highly Significant (**)  
P value < 0.05= Significant (*)

Table (8): Statistical comparison between negative control group I and group III (TBI) as regards histopathological changes of rat lung at the different time points of the study (1st, 3rd and 7th day) using chi-square ($X^2$) test.

<table>
<thead>
<tr>
<th>Histo-pathological changes</th>
<th>Negative control group (%) n= 6/ time point</th>
<th>TBI group (%) n= 6/ time point</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st day</td>
<td>3rd days</td>
<td>7th days</td>
<td>1st day</td>
<td>3rd days</td>
</tr>
<tr>
<td>Alveolar congestion</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Prominent alveolar septal vessels</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Intraparenchymal congested blood vessels</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Inflammatory infiltration in alveolar lumen</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Macrophages in alveolar lumen</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Intraparenchymal inflammatory infiltration</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>Thickening of alveolar walls</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
</tr>
</tbody>
</table>

P1: Day 1 control versus Day 1 TBI  
P2: Day 3 control versus Day 3 TBI  
P3: Day 7 control Versus Day 7 TBI  
P value < 0.01= Highly Significant (**)  
P value < 0.05= Significant (*)
Table (9): Statistical comparison between negative control group and the TBI group as regards immunohistopathological reaction for expression of HMGB1 (number of positive cells per mm²) of rat brain and lung at the different time points of the study (1st, 3rd and 7th days).

<table>
<thead>
<tr>
<th>HMGB1 Immunoreaction</th>
<th>Negative Control group n= 6/ time point</th>
<th>TBI group n= 6/ time point</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>1st</strong> Day</td>
<td><strong>3rd</strong> Day</td>
<td><strong>7th</strong> Days</td>
<td><strong>1st</strong> Day</td>
<td><strong>3rd</strong> Day</td>
</tr>
<tr>
<td>Brain</td>
<td>4 ± 2.3</td>
<td>3.3 ± 1.7</td>
<td>3.9 ± 1.3</td>
<td>45 ± 1.3</td>
<td>70.71 ± 2.1</td>
</tr>
<tr>
<td>Lung</td>
<td>5 ± 1.2</td>
<td>4.9 ± 5.3</td>
<td>5 ± 2.1</td>
<td>90.25 ± 2</td>
<td>50 ± 2.4</td>
</tr>
</tbody>
</table>

P1: Day 1 control versus Day 1 TBI  
P2: Day 3 control versus Day 3 TBI  
P3: Day 7 control Versus Day 7 TBI  
P4: Day1 versus day 3 versus day 7  
P value < 0.01= Highly Significant (**)  
P value < 0.05= Significant (*)

(Figure 1). A photomicrograph of a section in the brain obtained from an adult male albino rat of control group I showing normal neuronal structure formed of nerve cells with pale nuclei (H&E x 400).

(Figure 2). A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 1st day after trauma showing Severe Shrunken neurons with darkly stained pyknotic nuclei (arrow) (H&E x 400).
A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 1st day after trauma showed Neuropil edema with pale stroma and intercellular spaces (arrow) (H&E x 400).

A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 3rd day after trauma showed Severe subarachnoid hemorrhage (arrow head), congested thick walled blood vessels (arrow) and shrunken neuron with darkly stained nuclei (double heads arrow) (H&E x 400).

A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 3rd day after trauma showed (A) inflammatory cell infiltration (arrow). (B) Extravasation (arrow head) , inflammatory cells infiltration (arrow) , shrunken neurons with pyknotic nuclei (double heads arrow) , edema and loss of nerve fibers (O) (H&E x 400).
(Figure 6). A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 3rd day after trauma showed Neuropil edema with nerve fibers loss (arrow head) (H&E x 400).

(Figure 7). A photomicrograph of a section in the brain obtained from an adult male albino rat of group III (TBI) at 7th day after trauma showed shrunken neurons with pyknotic nuclei (arrow) (H&E x 400).

(Figure 8). A Photomicrographs of lung from an adult male albino rat of control group I showed normal architecture of the lung with alveoli, alveolar sacs, thin and thick portions of inter alveolar septa. The alveoli appeared patent with thin inter alveolar septa (H&E x 200).
(Figure 9). A Photomicrographs of lung from an adult male albino rat of group III (TBI) at 1st day after trauma (A) & (B) showed marked thickening of alveolar septa(double head arrow), severe inflammatory infiltrate (arrow head) and congestion of blood vessels (arrow)(A)(H&E x 200) & (B) (H&E x 400).

(Figure 10). A Photomicrographs of lung from an adult male albino rat of group III (TBI) at 1st day after trauma showed massive inflammatory infiltrate( arrow head) and marked thickening of alveolar walls (arrow) (H&E x 200).

(Figure 11). A Photomicrographs of lung from an adult male albino rat of group III (TBI) at 3rd day after trauma showed (A) thickening of alveolar septa, moderate inflammatory infiltrate (arrow head) and congestion of blood vessels (arrow) (H&E x 200). (B) Blood vessel has thickened wall but not congested (arrow) (H&E x 400).
A Photomicrographs of lung from an adult male albino rat of group III (TBI) at 3rd day after trauma showed Thickening of alveolar wall (arrow) and moderate inflammatory cellular infiltrate (arrow head) (H&E x 200).

(Figure 12).

A Photomicrographs of lung from an adult male albino rat of group III (TBI) at 7th day after trauma showed thickening of alveolar septa (arrow) with mild inflammatory cells infiltrate (arrow head) (H&E x 400).

(Figure 13).

A photomicrograph of a section in the brain obtained from an adult male albino rat showing a weak positive immunoreaction of HMGB1 (arrow) in control group I (A). While, trauma group (TBI) showed positive immunoreaction which is moderate at the 1st day (B), strong at the 3rd day (C) and mild on the 7th day (D) (Immunostain for HMGB1 × 400).

(Figure 14).
DISCUSSION

There were many studies on estimation of traumatic brain injury time by using different markers. Most of which were studied on animal models and there were series of pathological, physiological and biochemical changes occurred after TBI (Tao et al., 2006).

Traumatic Brain Injury (TBI) mortality and morbidity is frequently caused by the neurological consequences of the brain injury. Also, non-neurological complications such as cardiovascular, respiratory, infections and others are frequent and may be the leading cause of death (Corral et al., 2012).

The result of the present study showed significant elevations of HMGB1 serum levels in injured rats (TBI) group as compared to those of control group. These significant increases of serum HMGB1 levels observed during first day after TBI then gradually decreases from day3 up to day7 after TBI however still significantly elevated as compared with that of (control group).

The result of the present study were in agreement with Weber et al. (2014) who found that TBI induced
significantly higher HMGB1 serum levels in wild-type mice compared to controls (4.1 ± 0.7 ng/ml, P < 0.01) at 24 hours after injury.

Also, the results of the current study are near to Su et al. (2011) who stated that the expression of HMGB1 was upregulated remarkably after TBI. And they observed that, traumatic brain injury induced inflammatory responses in the brain tissue, characterized by enhancing NF-κB activation, induced expression of pro inflammatory mediates, such as IL-1β, TNF-α, IL-6 and HMGB1.

The possible two mechanisms can produce the release of significant amounts of extracellular HMGB1 are that, HMGB1 is a non histone nuclear protein with dual function. Inside the cells, HMGB1 binds to DNA and plays a role in transcriptional regulation. Outside the cells, it serves as a cytokine-like mediator of inflammation (Lotze, and Tracey, 2005). HMGB1 can be actively or passively released from necrotic or dying cells (Scaffidi et al., 2002).

High mobility group box 1 protein serum level constitutes a novel marker in the diagnosis and prognosis of any traumatized patient especially with associated inflammation. HMGB1 is passively released from necrotic or damaged cells to act as a potent cytokine to trigger inflammation. Also, it is an identified inflammatory factor that is highly expressed in severe sepsis, acute pancreatitis, hemorrhagic shock, severe trauma and acute lung injury. And its expression is closely correlated with the severity of these symptoms and death due to its crucial role in endotoxin release (Wu et al., 2012). According to the results of the present study, the maximum level of HMGB1 (serum and tissues) reached at the 1st day and this reflect maximum destruction and tissue damages.

In the result of the present study, qRT-PCR relative gene analysis showed that HMGB1 mRNA Expression Was up-regulated Following TBI. Genes expressions of HMGB1 were increased significantly in TBI group (III) as compared to the control groups in the 1st, 3rd and 7th day.

The results of the present study were near to Kawabata et al. (2010) who observed that the level of expression of HMGB1 increased at 24 hours, and this increase persisted until 72 hours after injury.

Also, Su et al. (2011) stated that the mRNA expression of HMGB1 and TLR4 in the cortex surrounding to the contusion core were significantly increased after TBI compared with that of the Sham group (P<.01).

High mobility group box 1 protein is a pro inflammatory mediator can be actively released by cells of the immune and nervous systems and it is associated with injury or disease. HMGB1 activates inflammatory responses primarily through binding to receptor for advanced glycation end products (RAGE) and/or Toll-like receptors (TLRs), such as TLR4 through activation of nuclear factorkB (NF-kB). Both TLR4 and RAGE are expressed by many cell types including those in the lung. TBI cause disruption of the blood-brain barrier and the release of HMGB1 by injured neurons into the extracellular space which trigger an inflammatory cascade in tissues rich in RAGE receptorssuch as the lungs (Yang et al., 2013).

In the present study immunohistochemical examination revealed positive upregulation of
HMGB1 immunoreaction following TBI. Group III (TBI) showed significant increase in immunoreaction of HMGB1 in 1st and 3rd day in the lungs and brain tissues (consequently) then gradual decrease in intensity of immunoreaction till it became weak positive but still significant to control group by reaching the 7th day.

The results of the current study are in agreement with Kim et al. (2006) and Bianchi et al. (2007) who reported up-regulation of HMGB1 and they considered it as one of the Damage Associate Molecular Patterns (DAMPs) participating in many sterile inflammation diseases, such ischemia reperfusion injury and close trauma.

The results are near to Kawabata et al. (2010) who reported that numbers of HMGB1-positive cells were larger in injured rat spinal cord than in uninjured control rats. HMGB1-positive cells were maximal in number 48 hours after injury and persisted up to 72 hours after the injury.

Also, the results of the present study are similar to Su et al. (2011) who reported increased HMGB1 expression in brain rats after 24 hours of TBI infliction.

Andersson and Erlandsson-Harris (2004) and Wang et al. (2004) stated that HMGB1, a 30 kDa nuclear and cytosolic protein widely studied as a transcription factor and growth factor, has been identified as a cytokine mediator of lethal systemic inflammation (e.g., endotoxemia and sepsis), arthritis, and local inflammation. In such pathophysiological conditions, HMGB1 is secreted by activated monocytes and macrophages, and induces TNF- and IL-1 synthesis and secretion. It thus appears that HMGB1 could activate inflammatory reactions.

The results of the current study showed that TBI induced significant pathological changes in the brain as subarachnoid hemorrhage, congested blood vessels, neuropil edema, and shrunken nerve cells with darkly stained pyknotic nuclei, extensive neuronal loss and inflammatory cellular infiltration.

The pathological findings of brain in the present study were in accordance with Chen et al. (2003) who found shrunken and darkly stained neurons after 1 day. While 4 days after, there was an extensive loss of neuronal cells around the contusion edge. Also, Sönmez et al. (2007) demonstrated significant neuronal loss in ipsilateral cortices at the 7th day after injury.

Inflammatory reaction plays a key role in the pathophysiology of TBI, which was initiated by disrupting the blood brain barrier then edema formation and infiltration of inflammatory cells. Dying neurons signal the release of cytokines and chemokines. Proinflammatory cytokines can initiate the infiltration of inflammatory cells into the brain by activating adhesion molecules, which facilitate the adhesion of polymorphonuclear leukocytes (neutrophils) to endothelial cells that allow the leukocytes to infiltrate the brain. These cells then release proinflammatory cytokines, such as IL-1, which stimulate the accumulation of other inflammatory cells such as astrocytes. All these changes are called secondary changes and can do secondary injury to the brain (Grossman et al., 2004).

Also, there is a wealth of evidence to suggest that inflammation plays an
important role in the pathophysiology of TBI. TBI initiates the inflammatory response by disrupting the blood-brain barrier, creating edema, and infiltration of inflammatory cells (Morganti-Kossmann et al., 2001)

The result of the present study also revealed that there were severe pathological changes occurred in the lungs following TBI in the form of blood vessels congestion, macrophages infiltration in alveolar lumen, severe intraparenchymal inflammatory infiltration and thickening of alveolar walls. These changes were in its severe form in the first day after injury and at the 3rd day after TBI then gradual decreased in intensity till became mildly detected at the 7th day after TBI.

The mentioned results of the present study are in consistent with the study of Kalsotra et al. (2007) who stated that lung tissue sections of brain-injured rats showed thickening of the basement membrane with massive inflammatory cell infiltration around bronchioles, pulmonary blood vessels and alveoli at 24 hours post injury. This pronounced pulmonary inflammation started to disappear spontaneously by 3 days and the lung histology of 2 week injured rats was essentially similar to the control.

Pulmonary complications are common after TBI, occurring in up to 80% of cases. Many mechanisms are responsible for the development of acute lung injury after TBI, including pulmonary expression of a systemic inflammatory response, neurogenic pulmonary edema and as a consequence of pneumonia (Lim et al., 2007).

Moreover, Weber et al. (2014) stated that, mice developed alterations in lung architecture, such as alveolar hemorrhage, proteinaceous debris, and neutrophilic infiltration, after TBI infliction. Also, they demonstrated that the HMGB1-RAGE ligand-receptor pathway serves as a central signal transduction mechanism for pulmonary dysfunction and inducing pathology consistent with ALI after TBI. They concluded that these findings were RAGE-dependent because pulmonary functions and histology were restored almost completely to baseline in RAGE−/− mice subjected to TBI and HMGB1 serum concentration may be predictive of lung function after TBI.

Traumatic brain injury (TBI) results in systemic inflammatory responses that affect the lung. This is particularly relevant in the setting of lung transplantation, where between 40 and 70% of donor allografts are obtained postmortem from individuals with TBI. Of those evaluated, only about 15% are deemed suitable for transplant (Araujo et al., 2014 & Cypeland Keshavjee 2013).

However, recent studies on the role of inflammatory mediators after acute brain injury have yielded a commonly accepted theory is that, these mediators may produce opposing effects at different times after the brain injury happens. They can produce a neurotoxic function at the early stage (1-2 d) after TBI, while it can produce neuroprotective function in the later posttraumatic phase (2-4 weeks). So, it is reasonable to assume that an effective inhibition of inflammatory response at the acute period is beneficial to the recovery from TBI (Pan et al., 2007).
CONCLUSION

From the current study it was concluded that, traumatic brain injury induced time dependent changes in serum and tissue levels of HMGB1, also histopathological changes in the brain and lungs of adult male albino rats. So this marker can be useful for the diagnosis and timing of brain trauma and post TBI lung injury. Further studies at different time interval are recommended on ante-mortem and post-mortem human brain injured cases in order to detect different methods for diagnosis, timing and test the novel systemic treatment modalities of head trauma patients. Also, it is recommended to benefit from change in levels of HNGB1 in case of lung transplantation before occurrence of toxaemia and tissue death.

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الملخص العربي

تحديد زمن إصابات المخ وتأثيرها على الرئتين في ذكور الجرزان البيضاء البالغة

 Huey Sidh Metwally,nehal slym abu hashem* و مرأة حسن سليمان حيسين**

أسام الطب الشرعي و السموم الإكلينيكية، بالديولوجيا* و الكيمياء الحيوية الطبية **

كلية الطب البشري - جامعة الزقاق

المقدمة: يعتبر تطبيق عمر إصابات المخ من القضايا الطبية الشرعية الهامة خاصة في حالات الإصابات التي تحدث فيها الوفاة بعد فترة قصيرة من الإصابة و التي لم يصاب بها تزيف بالمخ أو كدمات. وفي مثل هذه الحالات من الصعب تحديد سبب الوفاة. كما أن إصابات المخ قد تحدث أثرًا إثراعياً شديداً في الرئتين و الكبد و الإعصاب. و تعتبر الرئة حالة خاصة في زراعة الأعضاء حيث أن أكثر من نصف الحالات من المتنازعات تكون بعد الوفاة المصاحبة لإصابات المخ. الأهداف من الدراسة: تهدف هذه الدراسة إلى تحديد عمر إصابات المخ وتأثير هذه الإصابات على عرض بروتين مربع المجموعة عالية التنقل 1 في مصل الدم و الأنسجة أيضاً قياس التحليل الكمي لتفاعل البلمرة المتسلسل في الوقت الحقيقي للتعبير الجيني و الكشف عن التغيرات الهيستوبولوبولوجية في المخ و الرئتين. المواد و الطرق المستخدمة: تم إجراء هذه الدراسة على عدد 54 جرذًا من ذكور الجرزان البالغة مقسمة إلى 3 مجموعات (18 جرذًا لكل مجموعة): المجموعة الأولى مجموعة ضابطة سلبية. المجموعة الثانية مجموعة ضابطة موجبة حيث تم تخدير كل جرذ منها دون احداث إصابات المخ. المجموعة الثالثة تم تخدير كل جرذ منها و احداث إصابات المخ بطريقة إسقاط وزن على رأس الجرذ. و بعد أحداث إصابات المخ تم أخذ 6 جرذًا عند كل فترة زمنية 1-3-7 أيام من المجموعة الثالثة وكذلك 6 جرذ (ابدأ إحداث إصابات) من كل مجموعة ضابطة حيث تم سحب عينات دم لقياس نسبة بروتين مربع المجموعة عالية التنقل 1 في مصل الدم ثم تم ذبح الجرزان وأخذ عينات من المخ و الرئتين لدراسة التحليل الكمي لتفاعل البلمرة المتسلسل في الوقت الحقيقي للتعبير الجيني و دراسة التغيرات الهيستوبولوبولوجية و تحديد بروتين مربع المجموعة عالية التنقل 1 بالفحص المناعي الكيميائي للنسبة. النتيجة: و قد أظهرت النتائج ارتفاع ذو دالة إحصائية في مستوى بروتين مربع المجموعة عالية التنقل 1 في مصل الدم و التحليل الكمي لتفاعل البلمرة المتسلسل في الوقت الحقيقي للتعبير الجيني و أيضاً ظهور تغيرات بالبيولوجيا ذات دالة إحصائية في نسج الدم و الرئتين في المجموعة الشعبية مقارنة بالمجموعة الضابطة. الخلاصة: من النتائج السابقة يمكننا الاستنتاج أن أحداث إصابات المخ في الجرزان يصاحبه تغيرات التهابية في المخ و الرئتين و يمكننا الاستدلال عليه من التغيرات الكيميائية الحيوية و التغيرات البوليولوجية. و يمكننا اعتبار مستوى بروتين مربع المجموعة عالية التنقل 1 عاملاً هاماً في تشخيص عمر إصابات المخ و توقع التلف المصاحب لهذه الإصابة على الرئتين.