BIOCHEMICAL, HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMICAL STUDY OF BURN LESIONS IN PATIENTS ADMITTED TO BENHA EDUCATIONAL HOSPITAL: A PROSPECTIVE STUDY (2009-2010)

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ABSTRACT

Burn injury is a major public health issue in developing nations. The objectives of this work were to evaluate how the biochemical changes of liver enzymes and amylase can help in dating of burn injury and its relation with the severity and prognosis of burns. Also, this study aimed to determine the progressive skin changes in thermal burns, with serial biopsies and to investigate the time course of leucocytes and lymphocytes infiltrations, and finally, to study the effects of burn on CD4 and CD8 T cells in relation to time and severity of the injury. The study was conducted on 50 burned-patients admitted to the Burn Unit, Department of Plastic surgery, Benha Educational Hospital-Egypt; during the period from the 1st of September 2009 to the end of October 2010. Blood samples were taken at admission time, 5th and 10th post-burn days and skin biopsies were obtained at 3 days, 1 week, and 2 weeks post-burn from patients who gave informed consent to go through this study. Based on the severity of burns, burn patients were classified into 2 groups; 35 (70%) patients with moderate burn and 15 (30%) patients with severe burns. All patients enrolled in this study underwent blood samples for biochemical study and skin biopsy for histopathological and immunohistochemical evaluation. Fifteen clinically health individuals were submitted to the biochemical study as a control group, while the control group for burnt skin biopsy study was the nearby non-involved area taken from 15 patients. Biochemical results revealed no significant increase in liver enzymes and amylase levels on admission. On 5th and 10th post-burn day, liver enzymes and amylase showed a significant elevation in comparison with control group. Also, there was a non significant correlation between the severity of burn and liver enzymes and amylase at the time of admission and a significant correlation on 5th or 10th post-burn day. At the same time, it was found that there was a non-significant difference in the mean values of AST, ALT, ALP and amylase in surviving and dead cases. The examination of skin biopsy at 3 days, 1 week, and 2 weeks post-burn revealed progressive histopathological changes which can help in dating of burn and can be used as an indicator of burn severity. Immunohistochemical study revealed that burn injury was accompanied by marked immunodeficiency.
INTRODUCTION

Burn injury is a common type of traumatic injury, causing considerable morbidity and mortality. The after-effects of burns not only handicap the patient and leave psychological trauma on the victim's family and society in general (Yao et al., 2011). An extensive burn profoundly affects the patient's physique, psyche, financial situation and socio-cultural dynamics of the family. Patients with extensive burns frequently die, and for those with lesser injury, physical recovery is slow and painful as well (Shrivastava & Shrivastava, 2012).

The local and systemic inflammatory response to thermal injury is extremely complex, resulting in both local burn tissue damage and deleterious systemic effects on all other organ systems distant from the burn area itself. Thermal injury initiates systemic inflammatory reactions producing burn toxins and oxygen radicals and finally leads to peroxidation. The injured tissue initiates an inflammation-induced hyperdynamic, hypermetabolic state that can lead to severe progressive distant organ failure. Multiple organ failure (e.g., cardiac instability, respiratory or renal failure) and immune dysfunction remain major causes of burn morbidity and mortality (Çakir & Yeğen, 2004).

The forensic pathologist can be confronted with the estimation of wound age in association with murder, manslaughter, bodily harm with fatal consequences, accidents and further constellations. So, estimation of the age of human burn wounds has a medicolegal importance. New histochemical, immunohistochemical and biochemical techniques have lead to studies focusing on wound healing and timing (Cecchi, 2010). Routine histological examination allows the rough differentiation of cell types and tissue structures. Immigrating granulocytes, macrophages or fibroblasts can be frequently and easily detected. So, histopathology remains the gold standard for evaluation of burn depth, progression and healing (Hirth et al., 2012).

AIM OF THE WORK

The biochemical study aimed to determine the changes of some enzymes in burned patients through the first ten days and their relation with the severity and outcome of burns. The purpose of histopathological study was to determine progressive skin changes in burn injuries with serial biopsies up to the end of the 2nd week with particular interest of the timing of burn injury and also, to investigate the time course of infiltrated leucocytes and their relationship with injury severity, while the immunohistochemical study emphasized the post-burn effects on T lymphocyte subsets (CD4+ and CD8+ cells) in an attempt to reveal their role in immunosuppression and their relation to time and severity of injury.

SUBJECTS & METHODS:

A total of 50 burned patients {27 females and 23 males, mean age (± SE), 35.47 (±6.6) years} admitted to the Burn Unit, Department of Plastic Surgery, Benha Educational Hospital, Benha-Egypt, during the period from the 1st of September 2009 to the end of October 2010 were included in this clinical prospective study. Informed consent was obtained from all patients who fulfill the inclusion criteria.
Inclusion criteria:
1. Patients having age between 18 and 60 years.
2. Patients who consented to biopsy and blood sample (informed written consent).
3. Admission to hospital within 24 hours post-burn (recent dry burn).
4. Burn size of more than 10% total body surface area (TBSA) and not more than 65% TBSA, with at least 1% deep partial-thickness burn area which assessed by clinical manifestation of burn wound. Severity of burn was determined according to Yao et al. (2011).
5. Hospital staying period at least two weeks.

Exclusion criteria:
1. Patients having age 60 years or more.
2. Patients with any co-morbidity likely to affect the level of enzymes as hepatic or renal insufficiency.
3. Patients who had been admitted only for less than two weeks.
4. Any patient doesn't fulfill the inclusion criteria.

Based on severity of burn, two groups were made; 35 (70%) patients with moderate burn (11-30% TBSA) and 15 (30%) patients (10 patients with severe (31-50% TBSA) and 5 patients with extremely severe burn (51-65% TBSA). A total number of 9 (18%) patients out of 50 cases of the present study died after taking the samples, all the deaths occurred in patients with severe and extremely severe burn injury. All patients enrolled in this study underwent blood samples for biochemical study and skin biopsy for histopathological and immunohistochemical studies.

Biochemical study:-
Blood samples were obtained at time of admission; subsequently on 5th and 10th day after hospitalization, a control group composed of 15 clinically normal health individuals. The blood allowed to clot then serum was separated by centrifugation at 3000 rpm for 10 minutes. Aspartate amino transferase (AST) and alanine amino transferase (ALT) activities were measured according to the method of Reitman and Frankel (1957). Alkaline phosphatase (ALP) activity was measured by method described by King (1965) and serum amylase was measured according to the methods of Balsells et al. (1998).

1. Skin biopsy:-
Punch biopsies (6 mm) were obtained from the deep partial-thickness of involved area (50 biopsies) and a nearby non-involved area as a control sample (15 biopsies) at 3 days, (as the infiltrative reaction of leucocytes in burn wounds started at day 3 post-burn as mentioned by Peng et al. (2006), 1 week and 2 weeks post-burn. Biopsy samples were cut into two parts and processed for histological and immunohistochemical evaluation as described below.

2. Histopathological examination of skin biopsy:-
The specimens were fixed in 10% formalin solution and then dehydrated in ascending grades of alcohol and embedded in paraffin. Sections at 4 μm-thicknesses were taken, stained with hematoxylin and eosin (H&E) and examined under light microscope (Lamberg and Rothstein, 1978).

3. Immunohistochemical examinations of skin biopsy:-
Immunohistochemistry was performed on 6 μm paraffin sections for
CD4 and CD8 by using monoclonal antibodies; anti CD4 and anti CD8 (NeoMarkers, LABVISION, USA) by using Ultra-Vision system. The slides were visualized under light microscope and the extent of cell immune-positivity was assessed by presence of an insoluble brown reaction. The number of each cell type was determined by counting the number in each specimen in ten fields (magnification X400), followed by determination of the mean number of positive cells (Tingstedt et al., 2003).

**Statistical analysis:**

Data were expressed as means ± SEM. Statistical analysis was performed using a Student's *t*-test for the comparisons and Pearson's correlation coefficient (r" test). P value of <0.05 was considered to be statistically significant. The statistical analysis of data was done by using program statistical package for social science version 16 (SPSS, Inc, Chicago, IL).

**RESULTS**

**Biochemical results:**

At the time of admission, the levels of ALT, AST, ALP and amylase showed a non-significant difference when compared to control group. At 5\(^{th}\) and 10\(^{th}\) post-burn days, the mean values of ALT, AST, ALP and amylase were significantly elevated when compared with control group (table 1). At the same time, serum levels of AST, ALT, ALP and amylase showed significant increase at 5\(^{th}\) post-burn day when compared with their levels at the time of admission. The mean values of AST and ALT show increase till 5\(^{th}\) day then decline on 10\(^{th}\) post-burn day, whereas ALP and amylase levels continue to rise till 10\(^{th}\) post-burn day (figure 1). The correlation coefficient (r) values between extent of the burn (TBSA) and liver enzymes and amylase levels in burn patients at different periods of the study didn't show any significant correlation at the time of admission, whereas, there was a significant correlation at 5\(^{th}\) and 10\(^{th}\) post-burn days (table 2). It was also noticed that there was a non-significant difference in serum levels of ALT, AST, ALP and amylase in survived and deceased cases at different periods of the study (table 3).
Table (1): The activity of AST, ALT, ALP and amylase in burned patients and normal control at different period of the study.

<table>
<thead>
<tr>
<th></th>
<th>Control (15)</th>
<th>Cases (50)</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>On admission</td>
<td>5 days post-burn</td>
<td>10 days post-burn</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>50±3.1</td>
<td>51.76±1.1</td>
<td>74.9±4.0</td>
<td>62.1±2.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>23.1±1.6</td>
<td>24.8±1.7</td>
<td>46.4±3.6</td>
<td>31.9±1.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>118.9±3.4</td>
<td>132.1±5.4</td>
<td>148.3±4.5</td>
<td>170.1±7.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Amylase (U/l)</td>
<td>43.2±2.4</td>
<td>55.2±3.6</td>
<td>67.2±4.07</td>
<td>76.6±1.8</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.
Values are considered to be statistically significant at P< 0.05, t-test.
P1: Comparison between enzymes levels on burned patients on admission versus control.
P2: Comparison between enzymes levels on burned patients, 5th day post-burn versus control.
P3: Comparison between enzymes levels on burned patients, 10th day post-burn versus control.

Figure (1): Enzymes activity during different periods of the study (on admission, 5th, and 10th post-burn days). Values are expressed as mean± SEM.

*Significant difference between enzyme levels on 5th post-burn day versus on admission, P<0.0001 for ALT and AST & p<0.05 for ALP and amylase.
‡Significant difference between enzyme levels on 10th post-burn day versus on admission, P<0.001 for ALT, <0.01 for AST and <0.0001 for ALP and amylase.
† Significant difference between enzyme levels on 10th post-burn day versus on 5th post-burn day, P<0.01 for ALT, p<0.001 for AST and p<0.05 for ALP and amylase.
Table (2): Correlation coefficient "r" values between total body surface area (TBSA) and enzymes activity at different periods of the study.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>TBSA</th>
<th>&quot;r&quot;</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/l)</td>
<td>On admission</td>
<td>0.02936</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>0.267</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>0.436</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>On admission</td>
<td>0.139</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>0.37893</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>0.289</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>On admission</td>
<td>0.098</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>0.718797</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>0.723279</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Amylase (U/l)</td>
<td>On admission</td>
<td>0.184</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>0.701216</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>0.598867</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are coefficient of correlation "r" between burn size (TBSA) and serum enzyme levels. "r" measures the strength of a linear relationship.
Values are considered to be statistically significant at P< 0.05.

Table (3): Statistical comparison between survived and deceased patients with reference to different enzymes studied.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Outcome</th>
<th>Survived (n=41 cases)</th>
<th>Dead (n=9 cases)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT (U/l)</td>
<td>On admission</td>
<td>28.8±7.6</td>
<td>34±3.0</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>34.15±7.7</td>
<td>37.8±5.4</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>42.45±7.8</td>
<td>54.2±7.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>AST (U/l)</td>
<td>On admission</td>
<td>26.9±7.9</td>
<td>38±5.6</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>41.45±8.1</td>
<td>59.1±8.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>25.1±8.2</td>
<td>29.8±2.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ALP (U/l)</td>
<td>On admission</td>
<td>88.75±8.9</td>
<td>116.4±11.7</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>117.7±9.0</td>
<td>152.1±18.1</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>147.5±8.3</td>
<td>185.1±24.3</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Amylase (U/l)</td>
<td>On admission</td>
<td>49.25±8.3</td>
<td>72.8±11.5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>5th post-burn day</td>
<td>63.5±7.1</td>
<td>87.3±11.9</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td></td>
<td>10th post-burn day</td>
<td>61.65±8.7</td>
<td>88.3±12.3</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM.
Values are considered to be statistically significant at P< 0.05, t-test.
**Histopathological results:-**

Examination of sections of the skin from the control group revealed that the skin consists of a keratinized squamous epithelium (epidermis) and a fibroelastic connective tissue layer (dermis) separated by a basement membrane zone (dermal-epidermal junction). The dermis is attached to underlying loose connective tissue (subcutaneous layer or hypodermis) which contains primarily adipose tissue. The junction between the epidermis and dermis is characterized by downward folds of the epidermis called epidermal ridges or rete which interdigitate with upward projections of the dermis called dermal papillae. Several structures are embedded within the dermis, including epidermal appendages (sweat glands and hair follicles) as well as blood vessels and nerve endings (figure 2).

The skin biopsy, three days post-burn showed epidermal ulceration and superficial debridement of upper dermis. The deeper layers of dermis showed diffuse congested capillaries with tissue edema. Areas of loss of the original structure were noticed denoting tissue necrosis. A few tiny perivascular hemorrhages were present in the deep dermis and the collagen destruction was detected. The inflammatory cellular infiltrations with few polymorphonuclear leucocytes (neutrophils) near dermal capillaries were detected in moderately burned patients but with severely and extremely severe burned patients, there were very few leucocytes infiltration (figures 3-5).

At one week post-burn biopsy, the epidermis showed regeneration from basal layer. Congested blood vessels, collagen deposition and edema were detected in the dermis with appearance of several clusters of fibroblasts. Also, a large number of inflammatory cellular infiltrations in the form of polymorphonuclear leucocytes (neutrophils) in majority and a few mononuclear cells were noticed between necrotic and viable tissues which include lymphocytes, plasma cells and macrophages in moderate burn but in severe burn, only few number of neutrophils migrated into the wound (figures 6-7).

At two weeks post-burn, there was an epidermal growth, collagen fibers of dermis were more dense than normal, and there were numerous scattered fibroblasts. In samples of moderate burn, there were more complete, thicker, and more continuous zone of infiltrated leucocytes than before, and mononuclear cells (MNCs) were the main cellular components in the wounds, but after severe burn an obvious infiltrated leucocytes zone between necrotic tissue and normal tissue. The zone was only single and thin and included mostly PMNs and few MNCs. (figures 8-12).
**Figure (2):** A photomicrograph of normal skin (control group) showing normal appearance of epidermis (Ep). Dermis (D) containing blood vessels (BV), sebaceous gland (SG) and papillae (P) (H&E x 200).

**Figure (3):** A photomicrograph of skin biopsy, 3 days post moderate burn, showing a few lymphocytes infiltration (L) and epidermal ulceration (U) with tissue necrosis (N) (Hx& E x200).

**Figure (4):** A photomicrograph of skin biopsy, 3 days post moderate burn, showing congested capillary (cc), tissue edema (E) and a few lymphocytic infiltrations (L). (Hx& E x200).

**Figure (5):** A photomicrograph of skin biopsy, 3 days post severe burn, showing epithelial ulceration (U), hemorrhage (H) and necrosis (N) (Hx& E x200).
Figure (6): A photomicrograph of skin, 1 week post moderate burn, shows congested vascular channels (CV) and moderate infiltration of leucocytes (L) (H&E x 200).

Figure (7): A photomicrograph of skin, 1 week post severe burn, shows few leucocytes infiltration (L), fibrosis (F) and hemorrhage (H) (H&E x 200).

Figure (8): A photomicrograph of skin, 2 weak post moderate burns, shows diffuse leucocytes infiltration (L) and collagen deposition (CD) (H&E x 200).

Figure (9): A photomicrograph of skin, 2 weak post moderate burns with higher magnification, to show diffuse leucocytes infiltration with increased lymphocytes (L), macrophages (M) and plasma cells (P) and few PMNs (N) (H&E x 1000).
**Figure (10):** A photomicrograph of skin, 2 weeks post severe burn, shows diffuse PNL and lymphocytes (L) and congested vascular channels (CV) (H&E x 200).

**Figure (11):** A photomicrograph of skin, 2 week post moderate burn, shows fibrosis (F) and epidermal growth (G) (H&E x 200).

**Figure (12):** A photomicrograph of skin (deep dermis), 2 weeks post severe burn, showing increased collagen deposition (CD) and fibrous deposition (F) (H&E x 200).
Immunohistochemical results:-
This study compared the expression of CD4 and CD8 cells in the skin of moderate, severe and extremely severe burned patients and control healthy skin at 3 days, 1 week and 2 weeks post-burn. The expression of CD4+ T lymphocytes in the skin of moderate burned patient was significantly decreased as compared with the expression in healthy skin at 3 days and 1 week post-burn while the expression of CD4 at 2 weeks post-burn showed a non-significant difference when compared to control skin. At the same time, its expression was significantly decreased in severe and extremely severe burned skin at different periods of this study (table 4 & figure 13). Also, the expression of CD8+ T lymphocytes in lesional skin (epidermis and dermis) of moderate, severe and extremely severe burned patients was significantly decreased as compared with the expression in healthy skin at 3 days and 1 week post-burn but showed a significant elevation at 2 weeks post-burn (table 5 & figure 14). Table (6) shows a significant difference in the expression of CD4+ and CD8+ lymphocytes in severe and extremely severe burned patients when compared to the moderate burned patient along the periods of the study denoting a significant relation between the severity of burn and the absolute number of CD4+ and CD8+T lymphocytes.

Table (4): Expression of CD4+T lymphocytes in epidermis and dermis of burn patients compared to healthy control skin of control.

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Time of biopsy post-burn</th>
<th>Control skin (15 cases)</th>
<th>Burned skin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate burn (35 cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>54.2±1.6</td>
<td>39.9±1.2</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>54.2±1.6</td>
<td>49.1±1.2</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>54.2±1.6</td>
<td>52.03±1.1</td>
<td>&gt;0.05</td>
<td></td>
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<tr>
<td>Severe and extremely severe burn (15 cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>54.2±1.6</td>
<td>29.1±1.0</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>54.2±1.6</td>
<td>40.9±1.4</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>54.2±1.6</td>
<td>45.0±1.7</td>
<td>&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ±SEM.
Values are considered to be statistically significant at P< 0.05, t-test.

Table (5): Expression of CD8+T lymphocytes in epidermis and dermis of burn patients compared to healthy control skin of control.

<table>
<thead>
<tr>
<th>Study groups</th>
<th>Time of biopsy post-burn</th>
<th>Control skin (15 cases)</th>
<th>Burned skin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moderate burn (35 cases)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>33.3±1.2</td>
<td>29.3±0.5</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>1 week</td>
<td>33.3±1.2</td>
<td>31.2±0.4</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>33.3±1.2</td>
<td>38.6±0.5</td>
<td>&lt;0.0001</td>
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<tr>
<td>Severe and extremely severe burn (15 cases)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days</td>
<td>33.3±1.2</td>
<td>21.9±1.0</td>
<td>&lt;0.0001</td>
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<td>1 week</td>
<td>33.3±1.2</td>
<td>28.34±1.3</td>
<td>&lt;0.05</td>
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<tr>
<td>2 weeks</td>
<td>33.3±1.2</td>
<td>41.3±1.3</td>
<td>&lt;0.001</td>
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</table>

Values are expressed as mean ±SEM.
Values are considered to be statistically significant at P< 0.05, t-test.
Table (6): Statistical comparison between patients with moderate burn and those with severe and extremely severe burn regarding CD4 and CD8 positive cells

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time of biopsy post-burn</th>
<th>Moderate burn (35 cases)</th>
<th>Severe &amp; extremely severe burn (15 cases)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+cells</td>
<td>3 days</td>
<td>39.9±1.2</td>
<td>29.1±1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1 week</td>
<td>49.1±1.2</td>
<td>40.9±1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>52.0±1.1</td>
<td>45.0±1.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CD8+cells</td>
<td>3 days</td>
<td>29.3±0.5</td>
<td>21.9±1.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>1 week</td>
<td>31.2±0.4</td>
<td>28.3±1.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>2 weeks</td>
<td>38.6±0.5</td>
<td>41.3±1.3</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM.
Values are considered to be statistically significant at P< 0.05, t-test.

Figure (13): Significantly decreased number of CD4 positive cells (in brown) in burned skin (A). In contrast, there are abundant marked CD4 positive cells in the control skin (B). (Strept ABC x 400).
DISCUSSION

Biochemical study:

Various biological and metabolic alterations follow burn injury involves wide number of organs. Most vulnerable organs which succumb to these changes are liver, heart and pancreas (Bhagwat et al., 2007). After a thermal injury, a variable degree of liver injury is present and it is usually related to the severity of the thermal injury (Linares, 1996).

In the present study, it was found that the mean values of AST, ALT, ALP and amylase showed a non significant change as compared to control group at admission time while, there was a significant increase in these parameters on 5th and 10th post-burn days when compared to control group. Also, it was noticed that the levels of serum AST, ALT, ALP and amylase showed significant increases at 5th post-burn day when compared to their levels at the time of admission. The trends in the mean levels of serum AST and ALT showed increase till 5th day then decline on 10th day whereas ALP and amylase activities showed an increase from the baseline without any decline till 10th post-burn day.

These findings are in agreement with those of Bhagwat et al. (2007) who found that the mean value of AST, ALT and ALP on admission were not significantly different from control. Also, the study of Kumar et al. (1995) revealed the same results and stated that the increase in AST and ALT activities was not statistically significant on 2nd post-burn day while these activities were significantly raised on 5th and 10th post-burn days as compared with control patients.

The results of this study are supported by the findings of Jeschke (2009) who reported that serum transaminases and ALP are elevated from baseline when compared to normal levels. The serum levels of the hepatic enzymes were increased during the acute post-burn phase as thermal...
injury results in significant pathophysiologic changes by interplay of various mediators in early stages. These changes further exacerbate the whole body inflammatory response into a vicious cycle of accelerating organ dysfunction (Gibran & Heimbach, 2000; Jeschke et al., 2007).

On the contrary, Germann et al. (1997) observed that AST and ALT levels were elevated at time of admission as the hepatic dysfunction occurs early during the post injury course in approximately 60% of burn patients. This can be explained by decreased hepatic flow.

At the same time, Halkes et al. (2002) noticed that on the day of admission, AST and ALT showed enhanced activities which declined to a normal range within 8 or 12 days post-burn. The same findings were observed by Jeschke et al. (2004) and Bhagwat et al. (2007). In contrast, Mabrouk et al. (1997) stated that the ALT and AST were not significantly elevated in burned patients as compared to control at 4th post-burn day. Also, Latha et al. (1997) found that the serum enzymes levels were high initially and the increased levels were maintained even on 7th to 10th post-burn days. They also stated that the elevated levels of AST, ALT and ALP is due to acute liver damage resulted from toxic oxygen metabolites which produced by leucocytes and other cells in the course of inflammation and play an important role in tissue damage.

Pathological studies found that 10% to 15% of thermally injured patients have liver necrosis at autopsy and the damage of the liver may be associated with an increased hepatic edema formation immediately after burn (Jeschke et al., 2001). An increase in edema formation may lead to cell damage with the release of the hepatic enzymes (Jeschke et al., 2007). The mechanisms whereby a cutaneous burn induces programmed cell death in hepatocytes are not defined (Noda et al., 1998) but it has been suggested that, in general, the thermal injury causes liver damage by edema formation, hypoperfusion, proinflammatory cytokines, or other cell death signals with the release of the hepatic enzymes (Jeschke, 2009). Several experimental studies revealed that the thermal injury can result in increased lipid peroxidation mediated by free radicals (Nagane et al., 2003). Therefore, it is possible that the continuous rise in serum ALP and amylase is the direct consequence of the thermal trauma as the lipid peroxidation process continues leading to liver and pancreatic dysfunction (Bhagwat et al., 2007). Also, the elevation of serum amylase is due to its release into circulation from amylase-rich organs as pancreas (Odigie et al., 2002). Grewal et al. (1994) reported that the elevation of serum amylase is occurring due to post-burn pancreatitis which occurs with the onset of infection. It is possible that alterations in the local microcirculation of the pancreas could occur during infection, perhaps mediated by inflammatory cytokines, including interleukin-6, interleukin 1, or tumor necrosis factor-α. At the same time, Ryan et al. (1995) found the same result and observed that pancreatitis is a frequent complication after large burn injuries, and is more likely to occur in the presence of inhalation injury, deep burns, and associated trauma.

The results of the current study revealed a non significant correlation.
between the levels of AST, ALT, ALP and amylase with burn size (TBSA) at the time of admission of patients whereas there were a significant correlation at 5th or 10th post-burn days. These results indicate that the severity of thermal injury has no relation with serum enzyme levels at the onset whereas in the delayed phase, serum enzymes are better correlated with the severity of the injury.

The previous mentioned results coincided with results of Bhagwat et al. (2007) who reported that the serum enzyme levels on admission had poor correlation with burn size. This indicates that the severity of burn injury has no direct relationship with the serum enzyme levels on admission. It appears that there are no acute changes in the functions of liver or pancreas in early phase of thermal injury.

Similar results are reported by kraft et al. (2012) who found that the tremendous increase in enzyme level seen during the first week in patients with burn greater than 60% TBSA might be explained by severe tissue damage of the skin after burn injury and lipid peroxidation process. The process leads to liver and pancreatic dysfunction. Thus, monitoring of serum enzymes such as transaminases, ALP and amylase has an important prognostic value and clinical implications in the management of thermal injury (Bhagwat et al., 2007).

On the other hand, the findings of Mozingo et al. (2008) were partially in agreement with the findings of the current study, they found that an initial increase in the hepatic aminotransferase is common following burns of more than 50% TBSA. This is most likely due to the acute reduction in cardiac output, increased blood viscosity and associated splanchnic vasoconstriction that occur immediately following thermal injury. In contrast, Latha et al. (1997) stated that there were significant enzymatic changes but not related to the severity or nature of burns injury.

As regards the outcome of patients, it was also noticed that there was a non-significant difference in serum levels of ALT, AST, ALP and amylase in survived and deceased cases at different periods of the study. This result is in agreement to the study done by Mozingo et al. (2008), who reported that the magnitude of initial enzyme derangements has not been predictive of the outcome; however, the early onset of jaundice following thermal injury is associated with a poor prognosis, probably indicating pre-injury hepatic dysfunction or severely compromised hepatic perfusion during the resuscitative phase. On the other hand, Jeschke (2009) suggested that the liver, with its metabolic, inflammatory, immune and acute phase functions, plays a pivotal role in patient survival and recovery by modulating multiple pathways following thermal injury.

Histopathological study:

Determining age of burn from the forensic aspect can comprise an important part of the investigative process. It can be determined by studying how burn injury influences the inflammatory wound response at different times during the entire healing process. The histological manifestations of skin burn are firstly due to the response to the primary insult and then the inflammatory healing reaction. Therefore, it should be possible to estimate the age of a burn by examining the order and time at which components of the healing response are present in a
set of burns of known age, it may be possible to apply the information obtained to burns for forensic purpose (Tarran et al., 2006 and Schwacha et al., 2010).

In the present study, the microscopical examination of the skin, 3 days post-burn, revealed that the upper dermis showed ulceration and deep dermis showed formation of granulation tissues, congested capillaries and edema with areas of necrosis, hemorrhage and destructed collagen. As regards inflammatory cells, the skin of moderately-burned patients was infiltrated with neutrophils and a few number of macrophages but in severely-burned patients, a few leucocytes infiltration were detected.

The previous results were supported by the results of Mohajeri et al. (2011) who studied the experimental burn on the third day and concluded that the wound area was completely occupied by immature granulation tissue consisted of mainly fibroblasts and newly formed capillaries accompanied by hyperemia and focal hemorrhages. Simultaneously, presence of acute inflammatory cells in boundaries and surface of the wound was obvious. Also, Santos et al. (1996) reported that, burned skin shows the classic signs of epidermal necrosis, a diffuse perivascular infiltrate, and collagen degeneration at the papillary dermis.

In agreement with the present results, Tyler et al. (2001) and Peng et al. (2006) reported that in the moderate burn group, the infiltrative reaction of leucocytes in burn wounds started at 3rd day after burns, few leucocytes infiltrated out near the dermal capillary, but in severe burn group, there were no infiltrated leucocytes at the interface between the wound necrotic tissues and normal dermal tissues until 1 week after burn.

It was concluded that the neutrophils are the first immune cells to arrive and their primary role is wound debridement and protection against microbial infection (Schwacha et al., 2010). Nonetheless, activated neutrophils release proteases and reactive oxygen intermediates, which can lead to a significant damage (Moore, 1999). The influx of neutrophils into the burn injury is due to local increase in a neutrophil chemotactant. The delayed neutrophil infiltration in more severe burn patients would likely be related to the impaired production of local chemokines (Peng et al., 2006). In the present study, the biopsies of 1 week post-burn showed epidermal regeneration and the dermis revealed congested blood vessels, inflammatory cellular infiltration, collagen deposition, several clusters of fibroblasts and edema still present. In moderate-burned patients, polymorphonuclear leucocytes (PMNLs) were detected in the majority and few mononuclear cells (MNC) in the form of lymphocytes, plasma cells and macrophages but after severe burn, only few leucocytes were observed.

These results were in parallel with that of Mohajeri et al. (2011) and Oriana et al. (2012) who mentioned that that at 7 days after burn, biopsies showed that the surface of the burn was filled with inflammatory cells. Injured area was completely filled with immature granulation tissue and mild hyperemia in marginal vessels. Poor re-epithelialization and detachment of the epidermis from the dermis was detected. Also, Tarran et al. (2006) reported that the number of
macrophages was increased from 2nd post-burn day. They displayed a later rise in numbers than neutrophils, seen from day 2 onwards. There were some mid to late biopsy samples demonstrating a little or no increase of macrophages. This result is similar to the results obtained from the study done by Peng et al. (2006) who found that, at 1 week after burn, large numbers of leucocytes migrated from blood vessels into burn wounds and formed an obvious an infiltrated-leukocyte zone between necrotic and viable tissues. The cellular components in the zone consisted of PMNLs in the majority and a few lymphocytes and macrophages.

Linares (1996) found that low numbers of lymphocytes were anticipated in the early wounds with a trend for increasing numbers after about a week. Traditionally, forensic pathologists have used lymphocytes as a marker of older wounds. Migration of macrophages to the area of damage occurs at the same time as neutrophils, but their reduced motility delays their appearance. It was expected that macrophages would be present in increased numbers in the mid-aged burns and usually replace neutrophils as the predominant cell type at a burn site within a couple of days (Harding et al., 2002). Also, Tarran et al. (2006) observed that when macrophages predominate, the wound is probably a few days to weeks old.

After 2 weeks post burn, there was epidermal growth, dense collage fibers, scattered fibroblast were observed in the dermis. In moderate-burned patients, abundant mononuclear cellular infiltration while in patients with severe burn, there was a leukocyte infiltration and few mononuclear cells. These findings are in line with other studies of Tarran et al. (2006) and Peng et al. (2006) who observed formation of frank granulation tissues and proliferation of blood vessels by 10th to 14th post-burn day. They also recorded a shift towards predominantly mononuclear infiltrates.

Mohajeri et al. (2011) and Oriana et al. (2012) found the same results like that of the present study. On day 14 of experiment, biopsies showed complete re-epithelialization, with hypertrophic epidermis. Burn area was completely filled with mature granulation tissue consisting moderate deposition of delicate collagen fibers. Also, Peng et al. (2006) found that the infiltrated leukocyte zone included mostly PMNLs and few MNC formed at the interface between the wound necrotic tissues and normal tissues at 2 weeks post-burn but the burn patients with more severe injury had a more delayed infiltrate zone with less amounts of leucocytes but the burn infiltrative zone mainly consisted of numerous PMNLs at 7 days post-burn, and shifted to MNC at 14–21 days for moderate burn patients.

Also, Tarran et al. (2006) found that macrophages displayed a later rise in numbers than neutrophils, seen from day 2 onwards, with a possible trend to decrease in later samples from 15 days and onwards. The late persistence of macrophages in burns may be as consequence of an extended role for clearing wound debris in burns. After burn injury, macrophages are activated by various stimuli such as ischemia, necrotic tissue components, stress, infected bacteria and some cytokines derived from other inflammatory cells or macrophage itself. Activated macrophages then affect T or B lymphocytes either directly by...
intercellular contact or producing quantities of cytokines, chemokines and other bioactive substances such as interleukin-1 (IL-1), tumor necrosis factor-alpha (TNF-α), Monocyte chemoattractant protein-1 (MCP-1) and others, through which the whole immune system's function is impacted (Luo et al., 2005).

There is a lack of wound defense ability within the first or the second week after major burns. Also, a severe inflammatory response syndrome might affect the local cellular response of burn wound. In patients with more severe injury, acute inflammation of burn wound began later and continued longer, but acute inflammation of burn wound in patients with moderate injury occurred earlier, and changed into chronic inflammation more quickly (Peng et al., 2006).

**Immunohistochemical study:**

In burn injury, increased susceptibility to infection has been related to impaired immune response (Atiyeh & Al-Amm, 2001). In the present study, 3 days, 1 week and 2 weeks post-burn was chosen for specimen collection, it was found that there was a significant reduction in CD4 count in moderately-burned patients at 3 days and 1 week post-burn biopsies but this reduction was non-significant at 2 weeks post-burn biopsies and in severely-burned patients, CD4 count showed a significant decrease when compared with normal control skin. At the same time, CD8 count in moderate and severe burned patients showed a significant reduction in 3 days and 1 week post-burn biopsies and a significant elevation at 3 weeks post-burn.

**Buchanan et al. (2006)** examined the effect of burn injury on CD4 and CD8 T cell haemostatic proliferation after irradiation. They observed that CD8 T cells are more powerful than CD4 T cells in their proliferative response after injury. Another study showed that the burn injury induces a change in T cell homeostasis and affects mainly CD4 T cells after 10 days of thermal injury showing significant reduction in their absolute number. They interpreted this reduction in CD4 T lymphocytes, by the presence of high percentage of naive and effector/memory CD4 T activated lymphocytes ten days after burn injury, and added that these cells are effectively primed, as previously presented in an earlier study (Surh & Sprent, 2002).

The results of our study were consistent with the results of the study carried out at Ain-shams University by Mabrouk et al. (1997) who determined CD4+ and CD8+ T cells in patients with burn injury on the fourth day after burn injury; they recorded a significant decrease in absolute numbers CD4 and CD8+ T cells compared with controls. They explained the decline in these markers in the post-burn injury by immunosuppressive factors. One of these factors is a burn toxin that was characterized as a polymerized complex of cell membrane lipid proteins (lipid protein complex). This has been shown to inhibit the proliferation of normal T lymphocytes in response to stimulation.

Suppression in number of total lymphocyte population occurs with burn injury. In the early period of burn injury, activation of antigens on CD4 and CD8 cells are significantly depressed (Arturson, 1995). This is completely in agreement with the
results of the present work. Also, it is known that one of the outcomes of lymphocyte activation may be apoptosis, a phenomenon termed activation-induced cell death (AICD). Moreover, Teodorczyk-Injeyan et al. (1995) supposed that post-burn immunodeficiency may be caused by destruction of immune competent cells by the mechanism of AICD.

The decline in CD4+ and CD8+ T cells was explained as being mediated by corticosteroids on the basis of elevated levels of endogenous glucocorticoids and ACTH found in the first 48 hour after burn injury. This leads to massive apoptosis of lymphoid organs as thymus, spleen, lymph nodes and lymphoid tissue in the gastrointestinal tract. Apoptosis of the lymphocytes was the main cause of the decrease in the number of general pool lymphocytes, T-lymphocytes and T-helpers (a sub-group of lymphocytes) CD4 T cells. Also, many humoral factors, such as tumor necrosis factor-alpha (TNF-α) and tumor growth factor-beta (TGF-β) released after thermal injury induce apoptosis of the lymphocytes (Maekawa et al., 2002, Fazal et al., 2006 and Fayazov et al., 2009). Also, Rioja et al. (1993) concluded that severely burn injured patients are frequently reported to have immunological cell alterations as a result of induced prostaglandins and monocytes, activated T-suppressor (CD8 lymphocytes) arising from deficient production of interleukin-2. In addition, Sjöberg et al. (2004) reported that, heat-induced toxic lipid protein complex (LPC) is released into the circulation from the burnt skin, leading to inhibition of proliferation of normal T lymphocytes.

In the current study, data concerning the comparison between the mean values of CD4 T and CD8 T cells as regards the severity of burn injury revealed a significant decrease in CD4+ve and CD8+ve cells in severely and extremely severe-burned patients as compared to moderately-burned patients all over the periods of the study except the mean value of CD8+T lymphocytes at 2 post-burn weeks as its level showed a significant elevation.

The previous results were coincided with the results of Mabrouk et al. (1997) and Peng et al. (2006) who found that there was less T helper (CD4 T cells) and more T cytotoxic cells (CD8 T cells) in the wounds of more severe burn patients in comparison to patients with moderate burn. The study done by Hultman et al. (1995), reported that thermal injury with 20% or 40% TBSA were associated with significant inhibition and dysfunction of cytotoxic T lymphocytes (CTL) and there was a remarkable early depression of CD8 T cells after burn injury and returned to baseline after 7 to 10 days depending on severity of burn.

Consistent with our result, Maekawa et al. (2002) demonstrated that burn injury caused a reliable deficit in cytotoxic T lymphocytes (CD8 T cells) activity that was greatest at 3 days after burn injury and then gradually recovered to a normal level after 24 days. Also, Hunt et al. (1998) found that there was a rebound increase of CD8 T cells in burned cases due to increased proliferation.

**CONCLUSION**

The development of liver dysfunction seems to be as common as dysfunction in other organs and it can be interpreted as a part of the multiple
organ dysfunction syndrome (MODS) induced by burn. The current study revealed affection of all hepatic biomarkers by burn injury, thus the clinical implications of monitoring of these markers have important prognostic value in the management of the cases. Therefore, the attenuation of liver damage and restoration of liver function can improve the outcome of burned patients. Also, this study has addressed the role of post-burn histopathological changes in estimating injury age and severity. In addition, this current work concluded that the burn injury is accompanied by immunodeficiency, and T-lymphocyte subsets (CD4+ and CD8+ cells) are considered important parameters in immune-suppression and modulation of infection in burned patients; so, the results implies that a good understanding of these immune defects is essential for the development of new therapeutic approaches in the post-burn injury period and the restoration of normal immune function can prevent or minimize infection associated with burn injury.

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دراسة كيميائية حيّة، هستوباثولوجية و هستوكيميائية مناعية للإصابات الناتجة عن الحرق في المرضى المحتجزين بمستشفى بنها التعليمي: دراسة مستقبلية (2009- 2010)

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تعتبر الحروق من المشاكل الرئيسية التي تعاني منها الدول النامية. و هدفت هذه الدراسة إلى تقييم وظائف الكبد و إنزيم الأميليز وتحديد ما لهذا التقييم من قدرة على المساعدة في تحديد مدى إصابات الحرق و هل هناك علاقة بين النسبة المنوية لسواقة الجلد المصابة و مستوى الإنزيمات المختلفة على مدار مدة الدراسة و كذلك التنبؤ بتطور الحالة. أما الدراسة الهستوباثولوجية فكانت تحديد عمر الحرق عن طريق التغيرات التي تحدث في الخلايا و أيضاً عن طريق التحقق من وقت تسائل كريات الدم البيضاء و الخلايا الليفية في أماكن الإصابة و كذلك حاسة ما إذا كان لها علاقة مع شدة الإصابة. و أخيراً

دراسة الهستوكيميائية المناعية على عينات الجلد و ذلك باختبار تأثير الإصابة بالحرق على الخلايا المناعية (CD4 and CD8) و العلاقة بينها و شدة و عمر الحرق.

و قد أجريت هذه الدراسة على 50 مريضاً، كانوا يعانون حروقاً بالجد و قد تم إدخالهم وحدة الحروق بجامعة بنها وبعد أخذ مواقفهم عن المشاركة في هذا البحث، حيث تم تقييمهم إلى مجموعتين على حسب شدة الإصابة و قد تم حساب عينات الدم عند وقت دخول المصاب ثم عند اليوم الخامس و عند اليوم العاشر و ذلك لإجراء التحاليل اللازمة أما عينات الجلد فقد أخذت عند اليوم الثالث و بعد أسبوع ثم بعد أسبوعين على التوالي و ذلك للدراسة الهستوباثولوجية و الهستوكيميائية المناعية. و قد قدرت نتائج فحصات الدم للمريض بنقل الانتاج من 15 شخصاً لا يعانون أي إصابات.

و قد خلصت النتائج الدراسة الكيميائية إلى ارتفاع ليس له دلالة إحصائية في نسبة كل من إنزيمات الناقل الكبدية الفوسفات الفلوبي و الأميليز عند دخول المصاب بعد الإصابة مباشرة. أما العينات التي أخذت في اليوم الخامس أو العاشر فقد ارتفعت دون دلالة إحصائية في الناقل الكبدية و الفوسفات الفلوبي و الأميليز و ذلك عند مقارنتهما بالمجموعة الضابطة. كما لوحظ أن لا توجد علاقة بين ارتفاع إنزيمات الكبد و شدة الإصابة أو ما تنتهي إليه الإصابة حيث إن لا توجد فروق ذات دلالة إحصائية بين مستوى إنزيمات الكبد و الأميليز في أمثال المرضى الذين تم شفاءهم إذا ما قدرناوهم بذلك. أما الدراسة الهستوباثولوجية لعينات الجلد المصابة والتي أخذت من اليوم الثالث ثم بعد أسبوع و أخيراً بعد إسبوع و مقارنتها بين عينات من جلد غير مصاب من مصابي الحروق، حيث أظهرت العينات المصابة بالحرق تغيرات خالية تساعد في تحديد العمر الزمني للحرق و أيضاً قد تساعد في تحديد شدة الإصابة هذا وقد أثبتت الدراسة الهستوكيميائية المناعية أن الإصابة بالحروق لها تأثير متبع على الجهاز المناعي.