

A STUDY OF THE EFFECTS OF THERMAL INJURIES ON INTESTINE OF ADULT MALE ALBINO RATS

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ABSTRACT

Thermal injuries are one of the most common problems faced in the emergency room and can cause multi-organ failure distant from the original burn wound. Gastrointestinal system is one of the most affected systems in systemic inflammatory response to thermal injury. The purpose of this study was to evaluate the effects of scald injuries on the intestine (ileum) of adult male albino rats at 6, 12 & 24 hours post injury. Fifty-four adult male albino rats were equally divided into three groups: group I (negative control group), group II (sham group) and group III (scald injured group). Effects of scald injuries on intestine (ileum) at 6, 12 & 24 hours post injury were studied through: measuring of serum tumor necrosis factor alpha (TNF- α) level and histopathological examination using light microscope including H&E stained sections and immunohistochemical staining for detection of (TNF- α) activity. The results revealed that, in group III (scald injured group), Systemic inflammatory responses were demonstrated by significant increases in serum (TNF- α) levels at 6 h post injury and remained up regulated till 24h, histopathological changes were (shedding of epithelial cells, shortening and fusion of the villi and inflammatory cellular infiltrations in the connective tissue core of intestinal villi). Also, time dependent TNF- α activation was detected in the intestine at 6 hours post-injury and persisted till 24 hours. From the previous results it can be concluded that, scald injury induced marked systemic inflammatory responses, as characterized by significant increases in serum (TNF- α), histopathological changes in the intestine. Also, a time dependent TNF-activation was detected in the intestine of adult male albino rats.

Keywords: Thermal injury, multiple organ failure, intestine, TNF- α

INTRODUCTION

Thermal injuries and related trauma are a major cause of death and disability, especially in subjects under the age of 40. Even in developed countries, more than 2 million individuals annually are burned seriously and require medical care (1).

İseri et al. (2008) (2) discussed that thermal trauma causing damage to multiple organs, distant from the original burn wound and leading to multi-organ failure (MOF) continues to be a serious clinical problem. According to the clinical and experimental research findings, a local burn insult produces oxidant-induced organ changes as evidenced by increased lipid peroxidation in lung, liver and gut.

Gastrointestinal system is one of the most affected systems in burn patients (3). Actually, generalized tissue inflammation is present in uninjured organs within hours of thermal injury, even in the absence of shock.

Where circulating endotoxins become evident probably as a result of burn wound colonization and an early gut leak in experimental models of stress and injury were reported. Endotoxin and other bacterial by-products are potent activators of the macrophage and neutrophil. This leads to the release of massive amounts of oxidants, arachidonic acid metabolites, proteases, etc. which cause further local and systemic inflammation (2).

Furthermore, the increased production of cytokines by the "stressed" intestine is considered a critical component in MOF pathophysiology (4).

Wang et al., (2011) (5) demonstrated early appearance of inflammatory cytokines in the systemic circulation, including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6), following thermal injury both in humans and animals.

Tumor necrosis factor alpha (TNF- α) is supposed to be the initiating cytokine that promote a cascade of secondary cytokines and humoral factors which can conduct local and systemic sequelae. Furthermore, TNF- α is a potent mediator of the shock-like state associated with thermal injury and sepsis (6). This study aimed to evaluate the effects of scald burn injuries on intestine (ileum) of adult male albino rats at 6, 12 and 24 hours post injury through studying the following parameters; measuring serum TNF- α , histopathological examination by using hematoxylin & eosin (H&E) stained sections and immunohistochemical staining for detection of tumor necrosis factor alpha (TNF- α) activity.

MATERIAL AND METHODS

A-Material

1-Animals

Fifty-four adult male albino rats, weighing 150 -200 gm, were used in this study. The animals were kept in standard housing conditions and were freely supplied with food and water for one week before the experiment, for acclimatization.

2-Experimental Design

Each animal was anesthetized with intraperitoneal injection of ketamine hydrochloride (20 mg/kg) and diazepam (0.1 mg/kg). The back and flank skin of the rats were shaved. Rats were placed in supine position in a plaster cast with an area of their backs exposed through an opening in the cast, immersed in a hot water bath (100°C) for 10 seconds (7). This should produce non-lethal full thickness injury to the skin that covers 20 % of the total body surface area which were calculated by using the formula of Lee which is: Total body surface area (TBSA) = (body weight in grams x 0.78) + 148 (8). TBSA of a rat weighing 300 grams is 382 cm². Therefore, that 20% of TBSA equals 76.4cm².

Grouping of animals: the study was carried out on 54 adult male albino rats. They were divided into three equal groups, 18 rats in each group.

Group I (negative control group): Rats received only regular diet and tap water to measure the basic parameters.

Group II (sham group): Rats were anesthetized and given analgesic (with an intraperitoneal injection of ketamine hydrochloride 20 mg/kg and diazepam 0.1 mg/kg), and shaved. Then, they were put belly up in tap water for 10 seconds and removed.

Group III (scald-injured group): Rats were anesthetized and given analgesic (with an intraperitoneal injection of ketamine hydrochloride 20 mg/kg and diazepam 0.1 mg/kg), and shaved. Then, they were immersed in hot water bath (100°C) for 10 seconds to produce full thickness injury to the skin (7).

At the end of the specified durations (6, 12 & 24 hours), six rats for each time point from each group were anesthetized and blood samples were drawn for measuring of serum TNF- α level, then they were sacrificed, the intestine (ileum) was subjected to histopathological examination using H&E stained sections and immunohistochemical staining for TNF- α detection.

B-Methods

1- Biochemical Study:

Venous blood samples were collected from the retro-orbital plexus of the animals by capillary glass tubes according to procedure described by **Nemzek et al. (2001) (9)** for measuring of serum TNF- α by using commercially available kits. The principal of the method depends on Enzyme-Linked Immunosorbent Assay (ELISA), manufactured by (R&D Systems, USA), according to the instructions of the manufacturers. The technique involves simultaneous reaction of (TNF- α) present in the sample or standard with antibody directed against different epitopes on the TNF- α . One antibody (monoclonal) is coated onto the walls of the microtiter wells, and the other (polyclonal) is conjugated to the enzyme horseradish peroxidase. Any TNF- α present form a bridge between the two antibodies. 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 be then quantified photometrically. The absorbance was recorded at 450-nm wavelength with an automated enzyme-linked immunosorbent assay (ELISA) reader. Quantitative results were obtained in relation to standard curves with recombinant protein (10).

2- Histopathological Examination:

-Hematoxylin & Eosin stain:

The intestine specimens (ileum) from each group were fixed immediately in a 10% neutrally buffered formalin solution, and processed for light microscopic study to obtain paraffin sections of 5- μ m thickness. They were stained with H&E (11).

- Immunohistochemical study according to Happerfield et al. (1993) (12):

Tumor necrosis factor alpha was detected immunohistochemically. The primary antibodies were rabbit polyclonal antibody. The deparaffinized sections were incubated in hydrogen peroxide to block the endogenous peroxidase. Then, the slides were incubated with the primary antibodies (TNF- α), then the secondary anti-rabbit antibodies and peroxidase labelled streptavidin. Staining was completed by incubation with substrate chromogen (DAB). Sections from brain, intestine, lung and liver were used as negative control without using the primary antibodies. Mayer's hematoxylin was used as a counter stain. Immunostained slides were examined & the TNF- α positive staining was indicated.

3- Statistical Analysis:

For statistical analysis, statistical package for social sciences (SPSS) version 13.0 for windows program was used. Data was represented as means \pm SD. The differences were compared for statistical significance by analysis of variance (ANOVA). Difference was considered significant at $p \leq 0.05$ and Chi

square (X^2) was used to test the association between 2 variables for qualitative data (Number and percent).

Results

All the parameters of both control group and sham group were within normal and there were no statistically significant differences between them all over the periods of the study.

1- Biochemical studies (Table- 1).

There were significant increases in the concentrations of (TNF- α) in the serum of scald injured group from 6 hours up to 24 hours as compared with that of control groups (Table-1).

2- Histopathological study (Table- 2)

Hematoxylin-eosin (H & E) stained sections: Stained sections from intestine (ileum) of control groups (group I & II) showed normal intestinal mucosa (Fig.-2a). While, sections from intestine (ileum) of scald injured group (group III) revealed significant pathological changes as shedding of epithelial cells, shortening and fusion of the villi and inflammatory cellular infiltrations in the connective tissue core of intestinal villi (Fig.-2b,c,d).

3- Immunohistochemical study (Table- 3).

The immunohistochemical examination of intestine (ileum) of control groups showed negative immunoreactions for (TNF- α) (Fig. 3-a). While there were time dependent changes in (TNF- α) immuno-activation in the intestine of rats following scald injury. Significant increases in immunostaining (immunoreaction) were detected in the intestine of scald injured group at 6 hours post-injury and still significantly increased till 24 hours post injury (Fig. 3-b,c,d). Also, there was strong positive correlation between levels of TNF- α in serum and tissue (immunoreaction) in the scald injured group at the different time points of the study (6 h, 12 h and 24 h) (Table-4) (Fig. 1).

Table(1): Statistical comparison between sham, negative control group and scald injured group as regards serum TNF-α level at different time points of the study (6 h, 12 h and 24 h):

TNF- α	Sham group	Negative control group	scald injured group	P ¹
6 h	2.56 ± 0.85 ^a	2.67 ± 0.89 ^a	9.24 ± 2.43 ^b	<0.001**
12 h	2.90 ± 0.73 ^a	2.79 ± 0.82 ^a	14.6 ± 3.35 ^c	<0.001**
24 h	2.79 ± 0.82 ^a	2.87 ± 0.73 ^a	11.85 ± 2.85 ^d	<0.001**
P ²	>0.05 NS	>0.05 NS	<0.05*	

TNF-α: Tumor necrosis factor alpha

P¹: One-Way ANOVA test

P²: Two way ANOVA test NS: Non-significant

*: Significant (p<0.05) **: Highly significant (P<0.01) Groups with different letters are statistically significant.

Table (2): Statistical comparison between negative control group and scald injured group as regards histopathological changes of rat intestine (ileum) at the different time points of the study (6 h, 12 h and 24 h):

Histo-pathological changes	Negative control group (%) n= 6/ time point	Scald injured group (%) n= 6/ time point	P ^{\$1}			P ^{\$2}			P ^{\$3}	
	6 h	12 h	24 h	6 h	12 h	24 h				
Shedding of epithelial cells	1	0	0	3	5	6	<0.05*	<0.05*	>0.05 NS	
P ^{&4}	>0.05 NS	>0.05 NS								
Shortening and fusion of the villi	0	0	0	3	4	5	<0.05*	<0.05*	>0.05 NS	
P ^{&4}	>0.05 NS	>0.05 NS								
Cellular infiltrations	0	0	0	3	3	4	<0.05*	>0.05 NS	>0.05 NS	
P ^{&4}	>0.05 NS	>0.05 NS								

P^{\$}: Fisher exact test P[&]: McNamara test P¹: Comparison between two groups at 6 h P²: Comparison between two groups at 12 h P³: Comparison between two groups at 24 h

P⁴: Comparison between 6h,12h& 24h at the same group NS: Non-significant *: Significant (p<0.05)

Table (3): Statistical comparison between negative control group and the scald injured group as regards immunohistochemical reaction for expression of TNF-α (number of positive cells per mm²) of rat intestine at the different time points of the study (6 h, 12 h and 24 h):

	Negative Control group n= 6/ time point			Scald injured group n= 6/ time point			P ¹¹	P ¹²	P ¹³
	6 h	12 h	24 h	6 h	12 h	24 h			
TNF-α	4 ± 1.3	3.3 ± 1.1	3.9 ± 1.3	44 ± 1.3	74.71 ± 2.1	91.56 ± 1.9	<0.001**	<0.001**	<0.001**
P ⁴	>0.05 NS			<0.01**					

TNF-α: Tumor necrosis factor alpha

P¹: Independent t test P²: Two way ANOVA test NS: Non significant **: Highly significant

(P<0.01) P¹: Comparison between two groups at 6 h P²: Comparison between two groups at 12 h P³: Comparison between two groups at 24 h

P⁴: Comparison between 6h,12h & 24h at the same group

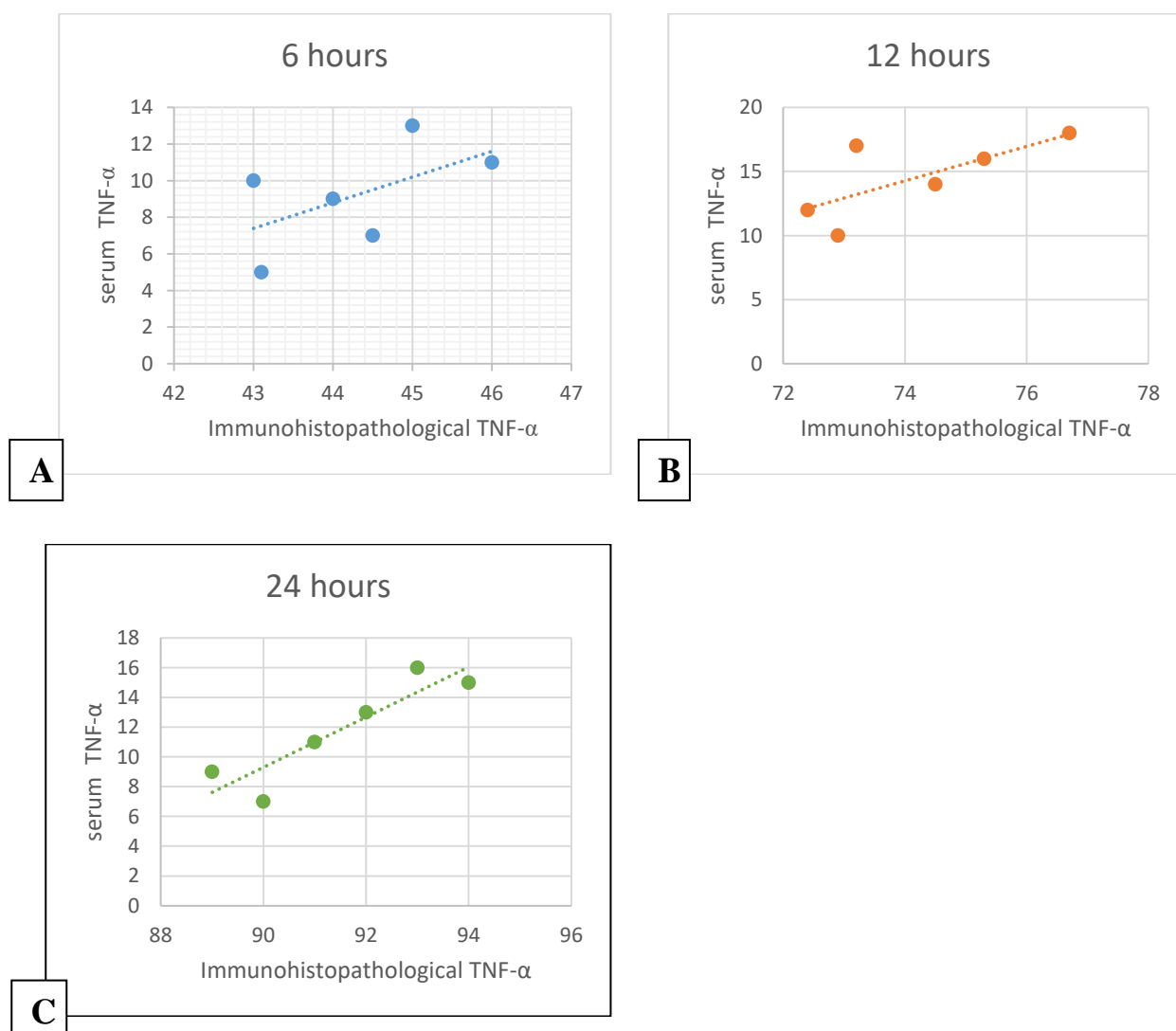
Table (4): Correlation between levels of TNF- α in serum and tissue (immunoreaction) in negative control group and the scald injured group at the different time points of the study (6 h, 12 h and 24 h):

Serum	Immune-histo-pathological TNF- α			
	Negative Control group (n=6)		Scald injured group (n=6)	
	r	P	r	P
TNF- α 6h	0.19	>0.05 NS	0.43	<0.01**
TNF- α 12h	0.06	>0.05 NS	0.59	<0.001**
TNF- α 24h	0.12	>0.05 NS	0.68	<0.001**

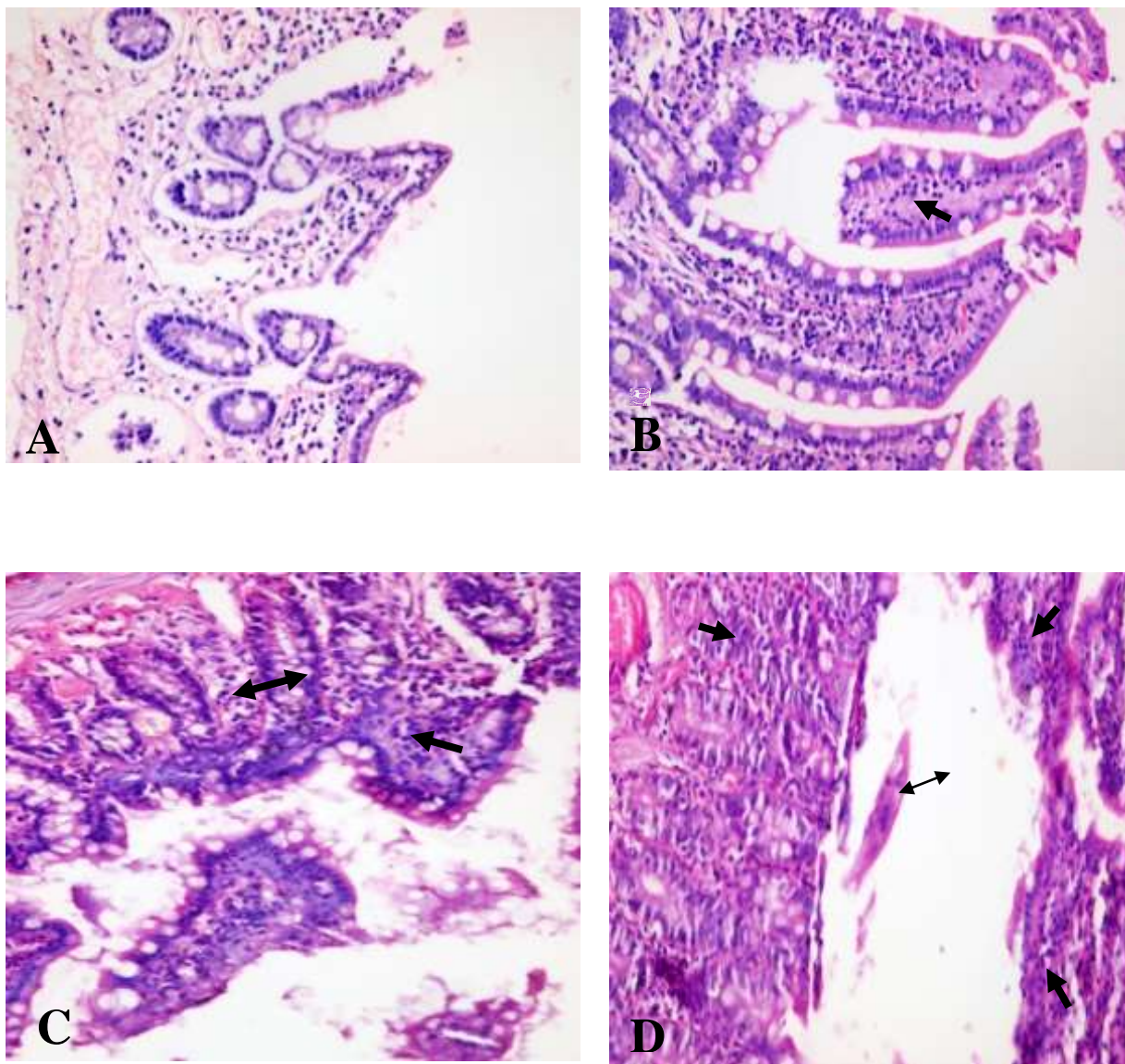
TNF- α : Tumor necrosis factor alpha

r: Pearson’s correlation coefficient NS:Non significant

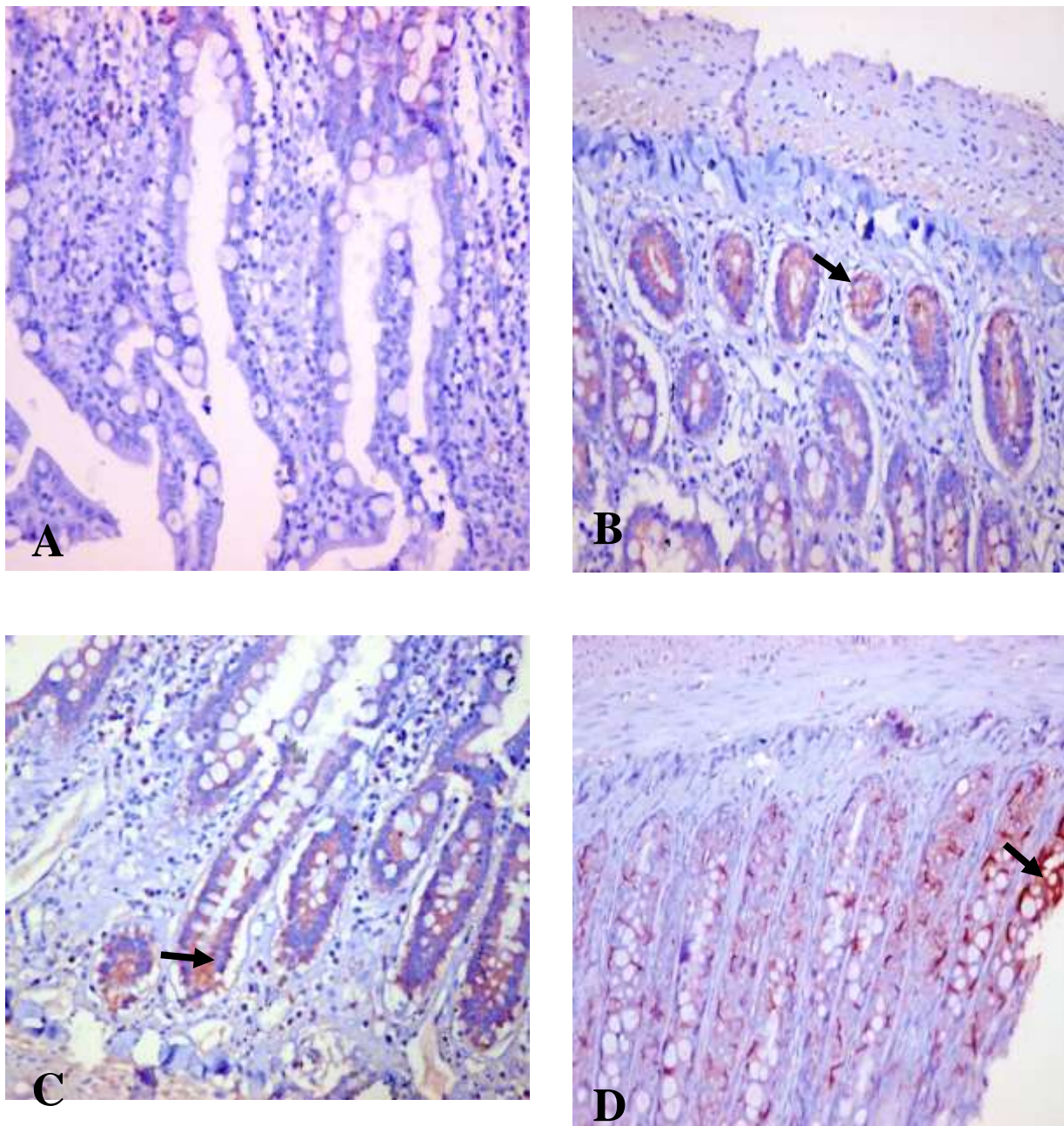
** :Highly significant (P<0.01)



Figure(1):Correlation between levels of tumor necrosis factor alpha (TNF- α) levels in serum and tissue (immunoreaction) in negative control group and the scald injured group : **A-**at (6 h) are positively correlated (r = 0.43). **B-** at (12 h) are positively correlated (r = 0.59). **C-** at (24 h) are positively correlated (r = 0.68).



Figure(2): A photomicrograph of a section in the intestine (ileum) obtained from an adult male albino rats showed **A-** Normal intestinal mucosa of control groups. **B-** Mild aggregates of inflammatory cells in the connective tissue core of intestinal villi (arrow) 6 hours after scald. **C-** Moderate aggregates of inflammatory cells (arrow), shortening and fusion of the villi (double head arrow) 12 hours after scald. **D-** Heavy aggregates of inflammatory cells in the lamina propria in the complete atrophy of villi (arrow) and shedding of epithelial cells (double head arrow) 24 hours after scald (H&E \times 200).



Figure(3): A photomicrograph of a section in the intestine (ileum) obtained from an adult male albino rats showed: **A-** control group showing negative immunoreaction for TNF- α . **B-** Mild staining for TNF- α 6 hours after scald (arrow). **C-** Moderate immunoreaction for TNF- α 12 hours after scald (arrow) **D-** Marked staining for immunoreaction for TNF- α 24 hours after scald (arrow) (Immunostain for TNF- α \times 200).

DISCUSSION

Clinical studies have shown that an uncontrolled and prolonged action of inflammatory cytokines, which is evidenced by a sustained release of acute phase proteins, may contribute to detrimental complications after thermal injury (13).

The result of the present study showed that the concentration of TNF- α in the serum of scald injured rats were significantly increased by 6 hours after scald injury as compared to that of control group and remained significantly elevated up to 24hours post-injury.

In accordance with findings of the present study, **Jeschke et al. (2008) (14)** who concluded that after a thermal injury, serum and hepatic concentration of pro-inflammatory cytokines such as IL-1 α/β , IL-6, and TNF- α are increased.

Tumor necrosis factor alpha is one of the mononuclear factors created earliest by macrophages after burn or trauma, which is involved in immune defence against infection. Appropriate quantity of TNF- α has protective effects, but the marked increased TNF- α production in macrophages after burn is harmful, which can cause organ injury and negative nitrogen balance (15).

The early appearance of inflammatory cytokines in the systemic circulation, including tumor necrosis factor-(TNF- α), interleukin-1 (IL-1), and interleukin-6 (IL-6), was demonstrated following thermal injury both in humans and animals (16).

The results of the present study also, revealed some pathological changes in the intestine as shedding of epithelial cells, shortening and fusion of the villi and inflammatory cellular infiltrations.

Similar results were demonstrated by **Berlanga et al. (2002) (17)** who stated that in rat burnt group, the intestinal damage was characterized by necrosis of the villi accompanied by partial or complete epithelial desquamation, which progressed to mucosal necrosis with areas of denudation and luminal hemorrhage.

Lutmer et al. (2013) (18) had concluded that the intestine is the primary seat of pathology after thermal injury. Also, impaired gut barrier function is a significant component of the multi organ dysfunction syndrome that accompanies thermal injuries.

A common mechanism explains intestinal histopathological lesions in thermal injury, is a decrease in the splanchnic perfusion resulting in a concomitant reduction in effective oxygen delivery to the intestinal mucosa and uptake by the local cells. Superoxide radicals and other oxygen reactive species are produced which may cause mucosal injury by lipid peroxidation and by damaging cell membranes system and the mitochondria. Other mediators/events, such as thromboxane release, nitric oxide over-production, complement activation and burn wound-derived pro-inflammatory and cytotoxic agents are involved in gut tissue injury and barrier failure pathogenesis (17).

In addition, immunohistochemical examination of intestinal tissue in this study showed time dependent changes in (TNF- α) activation of scald injured group. Significant up-regulation of immunostaining was detected in the intestine at 6 hours post injury and there were progress in the strength of the immunoreactions till 24 hours.

Production of pro-inflammatory mediators such as prostaglandin E2, reactive nitrogen intermediates, interleukin (IL)-6 and tumor necrosis factor (TNF- α), is markedly enhanced following thermal injury. There have been several reports indicating that circulating levels of IL-1h, IL-6 and TNF- α are increased in patients with burn injury (19). A possible mechanism elucidates this process is ischemia-reperfusion injury of the intestine. The reperfusion-injured gut serving as the motor of multi-organ dysfunction via release of pro-inflammatory mediators (20).

Many literatures established a link between splanchnic hypoperfusion and distant organ injury to rely on the liberation of arachidonic acid from the gut, with the attendant release of leukotrienes, prostaglandins, thromboxane, and platelet activating factor into the

mesenteric lymph. This phenomenon was later confirmed in a rat scald burn model, in which significant increases in lung permeability, pulmonary neutrophil sequestration, and alveolar apoptosis were prevented with division of mesenteric lymphatics (18).

CONCLUSION

From the previous results it can be concluded that, scald injuries induced a significant increases in serum (TNF- α) level. Significant histopathological changes in the intestine (ileum). Also, a time dependent up regulation of (TNF- α) immunoreactions was detected in intestine of adult male albino rats. In addition, biochemical, histopathological and immunohistochemical changes stated in this study suggest that scald injury can evoke a significant inflammatory response in the intestine, which led to gut structural alterations and barrier dysfunction and the detection of such changes could be valuable as one of the suggestive finding for ante mortem burn.

RECOMMENDATIONS

Further studies should be directed to detect other possible effects of scald injuries on remote organs at different time interval after injury in order to predict possible causes of mortality and disabilities of scald injury victims.

ACKNOWLEDGMENT

Sincere appreciation is expressed to to all members in forensic Medicine and Clinical Toxicology Department for their encouragement.

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دراسة تأثير الإصابات الحرارية على أمعاء ذكور الجرذان البيضاء البالغة

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لا تزال الإصابات الحرارية من أشهر المشاكل التي تواجهها مستشفيات الطوارئ وقد تصاحبها خلل وظيفي في العديد من أجهزة الجسم بعيداً عن مكان الحرق نفسه. وتهدف هذه الدراسة لبيان مدى تأثير الإصابات السمطية على أمعاء الجرذان البيضاء البالغة بعد مرور ٦ و ١٢ و ٢٤ ساعة على الإصابة. وقد تم تقسيم عدد أربع وخمسين جرذاً بالتساوي إلى ثلاث مجموعات: المجموعة الأولى (مجموعة المراقبة السلبية) والمجموعة الثانية (مجموعة صورية) والمجموعة الثالثة (المجموعة المصابة بالسمط) وقد تم دراسة آثار الحروق السمطية على أمعاء الجرذان عن طريق تحديد مستوى عامل النخر الورمي ألفا في مصل الدم كما تم أخذ عينات من الأمعاء لدراسة التغيرات الباثولوجية وتحديد عامل النخر الورمي ألفا بالفحص المناعي الكيميائي للأنسجة. تبين من النتائج أن في المجموعة الثالثة (مجموعة الإصابة بالسمط) وجد زيادة ذات دلالة إحصائية في عامل النخر الورمي ألفا في مصل الدم بعد ٦ ساعات من الإصابة ويستمر طوال مدة ٢٤ ساعة، كما لوحظ تغيرات باثولوجية ذات دلالة إحصائية في الأمعاء مثل انفصال في النسيج الطلائي، ارتشاحاً خلويًا في الخلايا والنسيج الضام مع انكسارات والتحامات في الخلايا، كما أظهر الكشف المناعي الكيميائي تغيرات زمنية ذات دلالة إحصائية في تنشيط عامل النخر الورمي ألفا في أنسجة الأمعاء بعد ٦ ساعات من الإصابة ويستمر طوال مدة ٢٤ ساعة. من النتائج السابقة يمكن استنتاج أن الإصابات السمطية تحدث استجابة التهابية في الأجهزة تتمثل في ارتفاع ذي دلالة إحصائية في مستوى عامل النخر الورمي ألفا في مصل الدم وتغيرات باثولوجية في الأمعاء كما تحدث تغييرات زمنية في تنشيط عامل النخر الورمي ألفا في أنسجة الأمعاء.