https://doi.org/10.48047/AFJBS.6.5.2024.11638-11658

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Estimation of age and vitality of tissues in and around a burn lesion by studying inducible nitric oxide synthase (iNOS) and IL-6 proteins

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Volume 6, Issue 5, May 2024

Received: 15 Apr 2024

Accepted: 05 May 2024

Published: 28 May 2024

doi:10.48047/AFJBS.6.5.2024.11638-11658

ABSTRACT

Estimation of burn injury age and vitality both in living and dead is essential and pivotal issue in forensic pathology to evaluate accurately its causal relationship to death. Nitric oxide and interleukin-6 (IL-6) play an important role in skin burn healing. This study shows that afterburn injury, there was a strong increase inflammatory infiltrates in the 2nd and 3rdweekante-mortem burn and decrease in post-mortem burn. Our study demonstrated, there was a statistically significant difference in both iNOS and IL-6 expression between the different time periods of the ante-mortem burn and decreased remarkably in the post-mortem burn. The expression of iNOS and IL-6 proteins in skin burn injury healing in patients by immunohistochemistry for its forensic application in determination of skin burn age and vitality in addition to their possible role in differentiating between ante-mortem and post-mortem burn was identified. Further research on various burn types is recommended.

Key Words: Age of Burns, Vitality, Immunohistochemistry, IL-6, iNOS, Antemortem Burns, Postmortem Burns.

INTRODUCTION

A burn is an injury to the skin or other organic tissue primarily caused by heat or due to radiation, radioactivity, electricity, friction or contact with chemicals.

INTERNATIONAL STATUS

Burns are a global public health problem, accounting for an estimated 180 000 deaths annually. The majority of these occur in low- and middle-income countries and almost two thirds occur in the WHO African and South-East Asia regions. Non-fatal burns are a leading cause of morbidity, including prolonged hospitalization, disfigurement and disability, often with resulting stigma and rejection. Burns are among the leading causes of disability-adjusted life-years (DALYs) lost in low- and middle-income countries. In 2004, nearly 11 million people worldwide were burned severely enough to require medical attention.

NATIONAL STATUS

In India, over 1 000 000 people are moderately or severely burnt every year (who 2018). In India around 7 million people suffer from burn injuries each year with 1.4 lakh deaths and 2.4 lakh people suffer with disability.The National Programme for Prevention, Management and Rehabilitation of Burn Injuries (NPPMRBI) is an initiative by the Directorate General of Health Services, Ministry of Health and Family Welfare, Government of India to strengthen the preventive, curative and rehabilitative services for burn victims (nhp.gov.in).

Wound examination is an essential issue in forensic practice.Determination of wound age and vitality is a classic but still popular and pivotal issue in forensic pathology to evaluateaccurately its causal relationship to death (Kubo H*et al.,* 2014).Wound healing is a dynamic process consisting of four overlapping phases: 1. The Hemostasis phase: consists of vasoconstriction. 2. The inflammatory phase: including coagulation and inflammation. 3. The proliferative phase: consisting of angiogenesis, the formation of granulation tissue and re-epithelialization and 4. The maturation phase: involving matrix formation and tissue remodeling(Mendonça RJ*et al.,* 2009).

There are many studies using investigative procedures for determination of mechanically induced skin wounds by sharp or blunt objects using either animal experiments or samples from

cadavers(Takamiya M 2002, Kondo T2007, Kondo T*et al*., 2010). Burn injuries are usually considered a major cause of disability and death(Alsarhan A*et al.,* 2013). But limited information is available on the determination of human skin burn injury.To study the vitality and age of a burn injury, inflammatory mediators or cells and matrix proteins in injured tissue could be analyzed (Dong X*et al*., 2015).

Nitric oxide (NO) plays a crucial role as a signal molecule in the healing process of skin burn(Lakshmi RT*et al.,* 2011). It is synthesized during the conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS) family of enzymes(Speranza L*et al.,* 2012). Three NOS isoforms have been identified; two are constitutively expressed in cells including neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS). The third isoform is inducible nitric oxide synthase (iNOS) and is activated in response to various stimuli such as cytokines, endotoxins and physiopathological conditions(Feorstermann U*et al*., 2012).

Interleukin-6 (IL-6) is a multi-functional cytokine released by a variety of cells including macrophages, T cells, fibroblasts, keratinocytes and endothelial cells. It could regulate the inflammatory response of wound healing process(Abali AE*et al.,*2013). Dating of an injury depending on the subjective naked eye evaluation is highly variable. Therefore, it is important to study the injuries microscopically. Immunohistochemical staining supports the histological findings and makes observations and interpretation more effective(Kumar L*et al.,*2011). Estimation of vitality and age of burn injury in both the living and dead is essential in forensic practice and it helps to the investigating agencies and courts to solve legal issues. Recently, it has become clear that interleukin-6 (IL-6), earlier known as hybridoma growth factor, is involved in systemic changes associated with tissue injury and infection (Martin C et al 1997). IL-6 is released by a wide variety of lymphoid and non-lymphoid cells such as T cells, B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangium cells and several (various) tumour cells. The damaged tissues release IL-6 and it induces the synthesis of proteins in the liver (acute phase proteins) that protects the host against inflammatory reactions(Fey GH*et al.,*1990).

The cutaneous reaction to heat and flame leading to vital reaction (red-flare/red-line), vesication / blisters and microscopic examination of the tissues from the burnt area has been considered very

important(Vij K et al 2002). The initial changes were due to direct effects of thermal injury and the subsequent effects are the result of inflammatory response(SevittS*et a1*.,1957). In human burns, the earliest histological change in antemortem burn was leucocytic infiltration within 6 hours after burning(Vij K*et al.*, 2002).

Histopathological section of the affected burn tissue with adjoining intact skin will show evidence of congestion, small areas of hemorrhage and infiltration of polymorphonuclear cells. These characteristic changes will not be present in burns sustained after death. The only vital sign, detectable by conventional methods, is inflammatory reaction but this is not apparent to the naked eye until several hours after injury. The line of redness being a vital reaction in antemortem burns persists even after death(Mukherjee J B2011).

AIM AND OBJECTIVE

To investigate the expression of iNOS and IL-6 proteins during skin burn injury healing in patients by immunohistochemistry for its forensic application in determination of skin burn age, vitality of burns in addition to their possible role in differentiating between ante-mortem and post-mortem burn.

PLAN OF WORK, METHODS AND TECHNIQUES:

1. **STUDY DESIGN:** Prospective analytical study
2. **STUDY SETTING:** Study subjects were patients admitted with thermal burns and scaldscategories of antemortem burns and postmortem burns.
3. **METHOD:**

   Skin tissue was taken from the burnt and junctional area of burnt and unburnt region and were subjected to histopathological examination as described by Culling *et al.*, 1985.

   Informed consent was obtained from the patients or their relatives for participation in the study.

   Patients with thermal burns and scaldscategories were included and assessed for the severity of burn injury by calculating the percentage of total burnt body surface area (%TBSA)(Coleman DJ 2004).Clinical evaluation of burn wound extent was byWallace's rule of nine.A detailed clinical history was taken at the time of admission and physical examination was carried out.

The post burn period was divided into 4 stages of healing for the study purpose, which was as follows, 1. Hemostasis stage (0 – 12hours) 2. Inflammatory stage (12hours -3 days), 3. Proliferation stage (3-14 days) and 4. Remodeling stage (15-28 days).In case of post-mortem burns, samples were taken immediately after their arrival to the mortuary.

SAMPLE PREPARATION AND IMMUNOHISTOCHEMISTRY STAINING FOR iNOSAND IL-6:

Sharp tangential excision (STE) using a dermatome was the standard of care, with derma abrasion reserved for smaller areas and burns that are more superficial; avulsion debridement

was used for the deeper and more extensive burns. To assess the thoroughness and extent of our STE, we measured the thickness of the excised eschar and examine the excised tissue for the presence of healthy viable tissue (Can J Plast Surg. 2010).

Skin samples were taken from both the center and periphery of the burn. The dissected tissues were immersed in 10% formaldehyde solution, with a volume ten times to the volume of the tissues. Then, they were embedded in paraffin and cut into 5-micron sections for staining with hematoxylin and eosin(Bancroft JD*et al.*,2008, followed by immunohistochemical staining foriNOS and IL-6(Duraiyan J*et al.,* 2012).

In this technique, sections of skin samples were dewaxed in xylene and rehydrated in graded ethanol solutions. Endogenous peroxidase activity was blocked by 3% hydrogen peroxides. The specimens were permeabilized in phosphate buffered saline (PBS) for 10 min, blocked in 20% normal goat serum in 0.01 M PBS, and subjected to antigen retrieval in citrate buffered solution at 92ºC for 15 min. After being washed in PBS, the slides were incubated with the antibody. After washing in PBS, the tissues were incubated by use of biotin-conjugated secondary antibody for 1hr. Then the slides were incubated in streptavidin-biotin horseradishperoxidase complex.Immunoreactivity was visualized by exposing the specimens to diaminobenzidinetetra-hydrochloride (DAB). The sections were counter stained with hematoxylin and then rinsed and mounted.

TECHNIQUES USED: Histopathology, Immunohistochemical staining for iNOS and IL-6.

INCLUSION CRITERIA:

1. Patients admitted with definite history of thermal and scaldscategories antemortem burns and postmortem burns. 2. The cases with different duration and survival times were studied.

EXCLUSION CRITERIA:Patients with obvious infections at other sites, patients with a recent history of diseases such as malaria/typhoid/viral hepatitis/other infectious diseases, and patients with chronic diseases such as diabetes mellitus / tuberculosis / chronic obstructive pulmonary disease / malignancies etc.were not included.

Burn injuries due to electrocution, chemical and corrosives were not included.

Decomposed bodies and bodies with no specific history were not included.

ETHICAL CLEARANCE: The Prospective analytical study was approved and ethically cleared by the Scientific Committee of SriVenkateswara Medical College, Multi-Disciplinary Research Unit(MDRU- ICMR- DHR- Government of India),Tirupati, Andhrapradesh, India.All patient information was kept confidential.

STATISTICAL ANALYSIS:

Allthestudy data wereenterdintothecomputerdatabaseusing standard format, checkedforerrors and verified. Data maintained in thecomputersheetswereorganisedby SPSS version 20 software for Windows. Data will be peresented in aprropriateTablesbycaliculating of percentage, rate etc.

RESULTS:

A total number of 52 patients Skin burn wound samples from various body areas were included in this Prospective analytical studybetween 2019 to 2022. The ages of study groups ranged from 20 years to 60 years.

Table- 1: Age Distribution of the Skin Burns Patients

	20 – 40 years	40 - 60 years	Total
Number	36	16	52
Percentage	69.23%	30.77%	100%

The occurrence of the Skin Burns in 20-40 years age is (69.23%) was found to be higher compared to 40 -60 years age (30.77%) respectively. (Table -1).

Table2: Demographic Data of patients and types of burns

I. Gender: [Number (%)]

a. Male: 24 (46.15%)
b. Female 28 (53.85%)

II. Kind of Burn: [Number (%)

a. Flame: 46 (88.47%)
b. Scald: 06 (0.16%)

III. Percentage of Burn- (%)

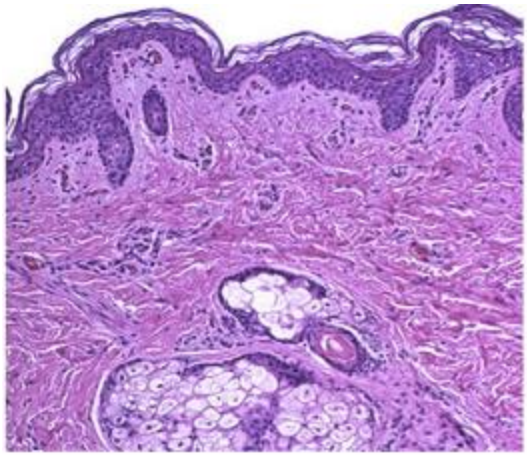
a. 10- 30 26 (50%)
b. 30 - 50 20 (38.46%)
c. >50 06 (11.54%)

IV. Out come: [Number (%)

d. Recovery: 26 (50%)
e. Partial recovery: 20 (38.46%)
f. Death: 06 (11.54%)

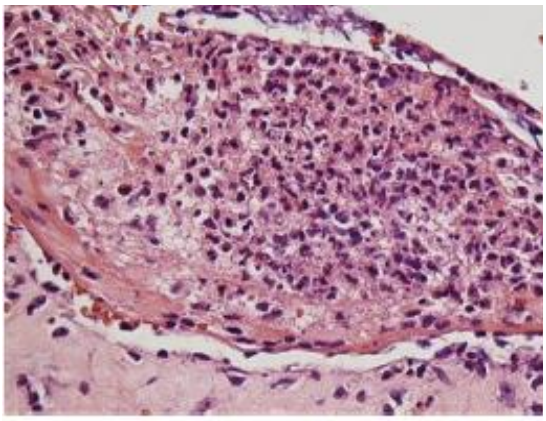
The Prospective analytical studyof 52 patients admitted to the Burns Unit between 2019 to 2022, 24 (46.15%) were male and 28 (53.85%) were female, 46 (88.47%) flame burns and 06(0.16%) were scalds burns(Table 2). Percentage of body surface area burns 10% -30% is 50% (N=26), 30% - 50% is 38.46% (N=20) and >50% is 11.54% (N= 06), the patients 50% (*N* = 26) were discharged after full recovery and 38.46% (*N* = 20) after partial recovery; 11.54% (*N* = 06) died (Table 2). Most of the patients who died were >50% body surface burns and elderly patients. Most of the patients who died had flame burns, followed by scald skin burns. Death is mainly due to infection, hypovolaemic shock and acute renal failure as per available recorded data.

HISTOPATHOLOGICAL EXAMINATION:



Examination of H&E stained sections at the normal skin revealed its characteristic epidermis and dermis (Figure.1) while at the burned area revealed deep second-degree burns involving mostof the dermis.

Figure.1. Normal skin (non burnt) showing normal epidermis and dermis



During the inflammatory stage, the most prominentchanges were infiltration of neutrophils and extensive edema and necrosis (Figure. 2).

Figure. 2. Burned skin during inflammatory stage (24 h post burn) showing acute inflammation, extensive edema and necrosis

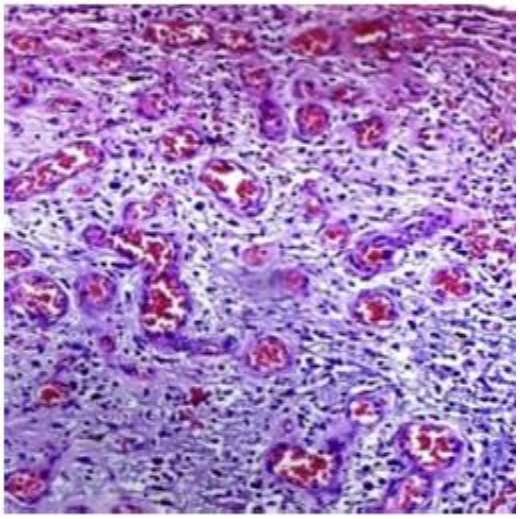
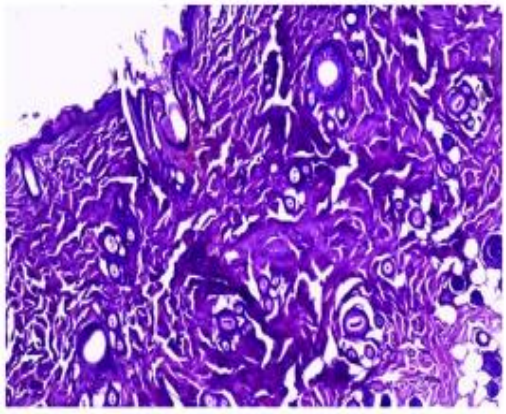


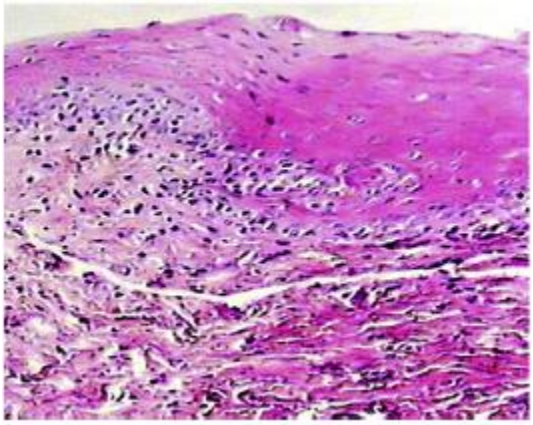
Figure. 3. Burned skin during proliferation stage -day 7, showing extensive acute inflammation, neovascularization

In the proliferation stage: by the day3, macroscopically, crust was formed from the necrotic tissues and microscopically this crust was rejected from the underlying viable tissues along with the zoneof neutrophils infiltration. By the day7, neutrophils were replacedlargely by macrophages with the early formation of granulation tissue and new blood vessels. By the day 9, the neovascularization reaches its peak with started scab formation (Figure. 3). By the end of inflammatory and starting of proliferative stage, decrease in the neovascularization with started deposition of few collagen fibers from accompanied fibroblastswasnoticed. The edema fluid started to decrease with increased infiltration with histiocytes, lymphocytes and plasma cells



In the remodeling stage -14 - 28 days, the number ofinflammatory cells decreased and collagenaccumulation and fibroblast proliferation was increased till all the burnt area started to be replaced by collagen at the end of the stage - Figure. 4.

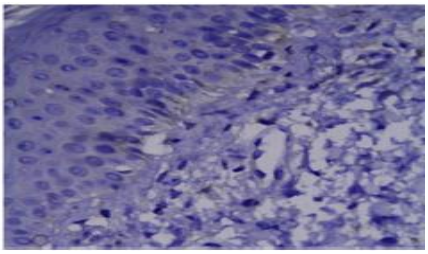
Figure. 4. Burned skin during remodeling stage -day 15, showing dense collagen deposition



At the post-mortem burns samples, the changes were less prominent in the form of few polymorphnuclear cells infiltrating the dermis with transudate fluid, which was seen at the site of burn (Figure. 5).

Figure. 5. Burned skin during postmortem stage with few changes in both epidermis and dermis with few neutrophils infiltrating the dermis and transudate fluid

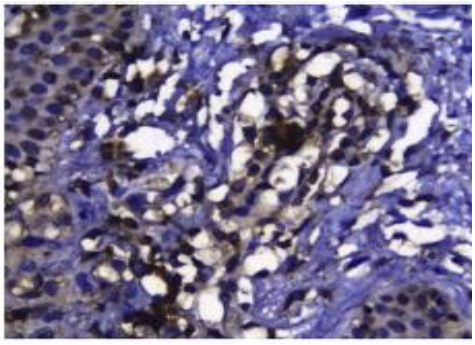
IMMUNOHISTOCHEMICAL RESULTS OF iNOS EXPRESSION



The iNOS protein expression was negative in the normal skin (unburnt area) Figure. 6

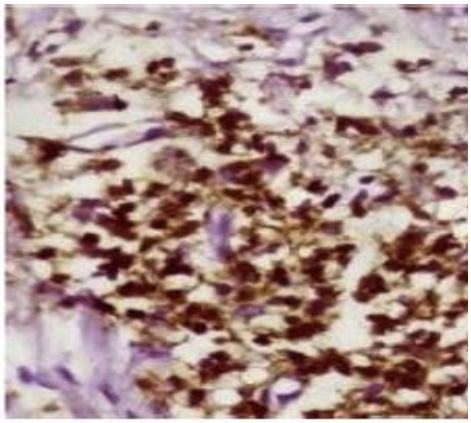
Figure. 6 negative iNOS expression

TIME SENSITIVE CHANGES OF iNOS PROTEIN EXPRESSION IN THE ANTE-MORTEM BURN



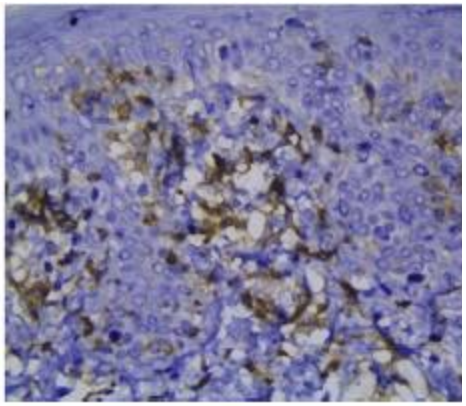
On 1st post burn day, the burned tissue showed iNOS positive staining in thesweat glands and neutrophils. (Figure.7)

Figure. 7 Positive iNOS expression in the cytoplasm of keratinocytes and inflammatory cells



During inflammatory stage the iNOS expression steadily increased and reached the high level.Thereafter, the number of positive cells declined from in the proliferative stage. The iNOS protein expression was observed in the inflammatory cells, fibroblasts and endothelial cells of the granulation tissue Figure.8

Figure. 8 positive iNOS expression in the cytoplasm of inflammatory cells

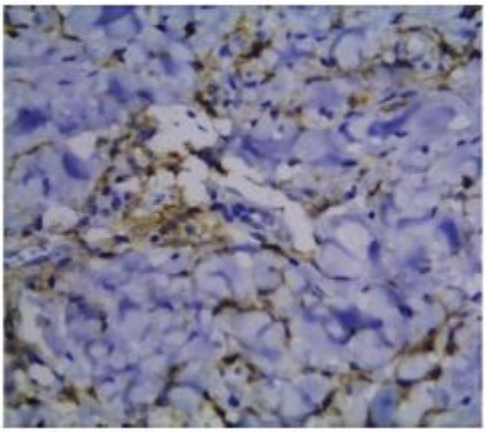


There was lower rates of iNOS protein expression detected during the remodeling stage of burnhealing. It was observed mainly in the macrophages whereas in the keratinocytes, iNOS expression was very low. Positive iNOSexpression was also detected in few histiocytes and fibroblasts. Figure. 9.

Figure. 9. Positive iNOS expression in the few histiocytes, fibroblasts and very low in theKeratinocytes

This shows a significant difference in theiNOS expression during the various skin burn healing stages with different time periods.

iNOS PROTEIN EXPRESSION IN THE POST-MORTEM BURN

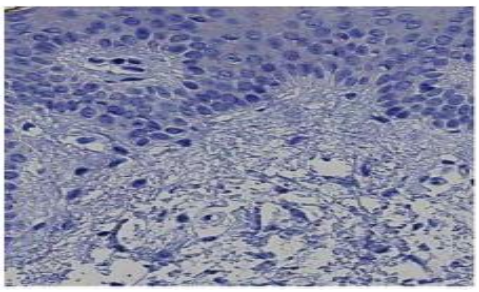


iNOS protein expression decreased in the cells including polymorphnuclear cells, keratinocytes and sweat glands Figure. 10.

Figure. 10 DecreasediNOS expression in the cytoplasm of inflammatory cells

TheiNOS positive staining expression was the lowestwith significant difference from all the ante-mortem healing stages were observed.

IMMUNOHISTOCHEMICAL RESULTS OF IL-6 PROTEIN EXPRESSION

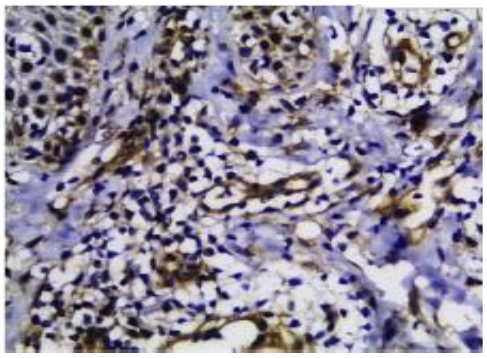


The samples taken from normal skin (unburnt skin) adjacent to the burn were completely negative for IL-6 expression during all the ante-mortem healing stagesstudied. Figure. 11

Figure. 11 negative expression of IL-6

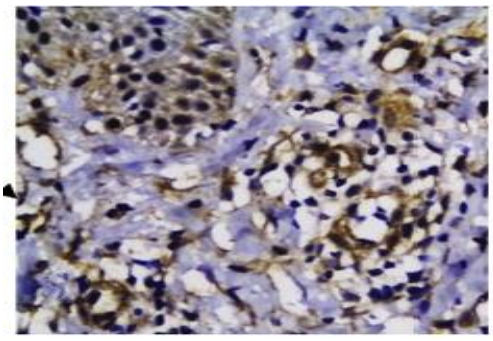
Burn injury caused elevation in the expression of IL-6 in samples collected from the burn injurysite throughout the various ante-mortem healing stages. It was expressed mainly in the cytoplasm of the inflammatory cells and granulation tissue fibroblastic and vascular endothelial cells.

IL-6 PROTEIN EXPRESSION IN DIFFERENT ANTE-MORTEM BURN HEALING STAGES



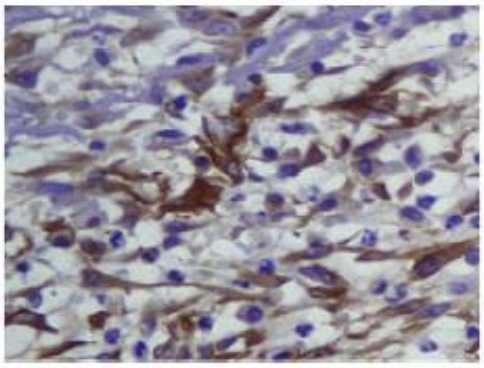
On 1st to 3rd post burn day, all the samples were positive for IL-6 expression. Figure. 12. This positive expression remained with high in inflammatory stage and was variedin proliferative stage.

Figure. 12 expression of IL-6 in the inflammatory and endothelial cells



From 9thday, negativeIL-6 expressionin samples was started to appear - Figure. 13. At the proliferative stage in collectively, the expression was high with the peak intensity seen at 5thday.

Figure. 13 Expression of IL-6 in the inflammatory and endothelial cells

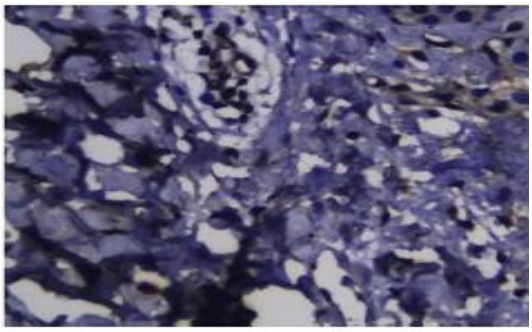


On 14thday, most of the samples were positive for IL-6 expression, then decreased on day 21. So, collectively during the remodeling stage, half of the cases were positive and the expression decreased gradually with the time. Figure. 14.

Figure. 14 Moderate to low expression of IL-6 in the fibroblasts

This shows a significant difference in the IL-6 expression during the various skin burn healing stages with different time periods.

IL-6 PROTEIN EXPRESSION IN THE POST-MORTEM BURN



Only 2 samples was positive for IL-6 expression, other samples were negative. Figure. 15

Figure. 15 Decreased expression of IL-6

Lowest IL-6 expression in postmortem samples with a significant difference from all the ante-mortem healing stagestime intervals.

There was a correlation between iNOS and IL-6 expressions during the ante-mortem healing stages revealed a significant positive association betweenthe two markers. Both increased gradually in inflammatory and in early proliferation stages and started to decrease gradually in late proliferative stage and remodeling stage while reaching the minimum or absent at the postmortem stage.

DISCUSSION

Determination of the vitality and age of burns by examining the order and time at which various cellular components of the healing process are present in burn wounds is a crucial medico-legal issue in the field of crime investigation in both the living and dead (Kondo T*et al.,* 2010)

Histopathological examination of the edge of a burn, including both the burnt area and adjacent grossly unburnt skin, may reveal vital reaction microscopically, consisting of acute inflammation, hemorrhage, edema, necrosis, and vertical streaming epidermal nuclei and homogenization of the dermal collagen. Skin shows petechial hemorrhages in deeper layers, epithelial cells are elongated, flattened and stained deeply with hematoxylin and eosin and vacuolization of epidermal and dermal layers is prominently seen. These findings help in differentiating antemortem burn from post-mortem burn (Cullings CFA1985, Foley FD. 1970 and Malik MOA 1971).

The present study was conducted on 52 patients admitted with thermal burns and scalds categories of antemortem burns and postmortem burns. In the patient's ante-mortem burns, our results indicate that IL - 6 expression was time sensitive. It started at 1st day of antemortemburnsand reached its highest point with highest intensity at day 3rd to 5th day. The positive IL-6 expression and its intensity decreased gradually from 7th day to until the end of Remodeling stage (28th day).

The studied samples were positive with a significant difference between all the studied periods. Significant enhancement of IL-6 was observed at wound sites during skin wound healing in patient samples (Sato Y*et al.,* 2000).Additionally, Biffl WL*et al* and Sasaki *et al*.suggested there is positive association between IL- 6 and burn-induced immune-inflammatoryresponse during the skin burn injury healing process.(Biffl WL1996 andSasaki JR 2011).

Increased levels of IL-6 within hours of thermal skin injury have been reported byModi2014 and Gebhard F. 2000.In addition, Patrick P.G.Mulder andGuoY,reported increased serum levels of IL-6 over a 3-week interval with highest concentrations reached during the first week after injury in a population of burn patients. (Patrick P.G.Mulder 2022 and GuoY 1990).Several workers have observed a positivecorrelation between circulating IL‑6 levels and the magnitude of burn injury.(Biffl WL 1996, Gebhard F 2000 and Yagmur Y 2005).High levels of IL‑6 are seen in various diseases, inpatients with multiple injuries, burns, septicaemia etc., Theimportant role of IL‑6 in inflammatory reactions led to study of its role in multi‑organ failure and sepsis.

(Cullings CFA*et al.,* 1985).Increased levels of IL‑6 within hours of thermal trauma has been reported by several workers. (Kowal‑Vern A1994and Nijsten MW 1991).We also found raised IL‑6 levels within hours of burn injury. All the burn patients showed initial elevation in IL‑6 levels. (Modi S*et al.,* 2014).

In the ante-mortem burned patient samples, our results indicate that iNOS expression was time sensitive. On 1stday of antemortemburn, it was high in burned skin sample and negative expression in the normal skin.The number of iNOS positive cells progressively increased in inflammatory stageand peaked at the 7thday. Then started to decline and reached the lowest level with the end of the remodeling stage. Additionally, iNOS protein was not only detected in the cytoplasm of keratinocytes, fibroblasts, endothelial cells and inflammatory cells but also in sweat glands and hair follicles in the burnt skin. This pattern of changes in iNOS expression coincided with that obtained by Zhao R, 2005who studied the time dependent changes of iNOS and eNOS protein expression in mice cutaneous incised wound healing. (Zhao R*et al.,*2005).

The observed cellular distributions of iNOS expression suggests that nitric oxide plays an essential role in all the sequences of wound healing; affecting inflammatory stage, adjusting cell proliferation, differentiation and apoptosis, forming granulation tissue and neo-vascularization and in tissue remodeling. The role of NO in the process of healing of burn injuries was proved ByWallace 2005, Takashi Kitano 2017and Lakshmi RT 2011.Additionally, Bakinam M.H. Tammam2023 and Oliveira 2004,found higher NO content in the skin of burnt rat compared to its levels in the plasma and visceral organs suggesting that burnt tissue may be an important site for the production of NO.Under normal conditions, iNOS is not usually active; it is activated in thermal injury dueto the effect of pro-inflammatory agents in affected tissues (Alsarhan A*et al.,* 2013) andproved the role of iNOS in the discontinuous synthesis of high amounts of NO in the burnt patients. (Filippou D*et al.,*2007).The results suggested that loss of iNOS retards reepithelialization by keratinocytes in the process of healing of an excision cutaneous injury. (Takashi Kitano*et al.,*2017).Our current immunohistochemical analysis further confirmed the histology findings; invasion of macrophages and myofibroblast appearance were both suppressed by lacking iNOS. (Cohen B. J*et al.,*1987).

It is worth to mention that Lakshmi RT *et al.,*2011, intheir trial of using low molecular weight heparin in the treatment of burn patients proved the role of iNOS and IL-6 in the process of burn

healing.Our study revealed that in the post-mortemburn injury the IL-6 expression was weakly positive andiNOS expression in the burnt skin was low and only in polymorphnuclear leucocytes with significant difference from the unburned skin and all the studied ante-mortem periods. (Bohnert M *et al.*, 2003).Thus both markers shall help indiagnosis of burn vitality.

Therefore, our research has focused on improved methods for distinguishing between antemortem burn and early post-mortemburn injury by analyzing burn tissue. This will decrease the problems associated with the diagnosis of age and vitality in burned bodies.

In conclusion, the present results indicate that both iNOS and IL-6 expression in ante-mortem burnt skin was time sensitive and significantly differed from post-mortem burn. Further research on various burn types is recommended and the use of these markers as objective methods for burn injury dating and vitality determination. In future, the iNOS and IL-6are pioneered therapeutics candidates for clinical application in burn wound healing.

ACKNOWLEDGMENTS

We would like to thank the Multi-Disciplinary Research Unit (MDRU) - Indian Council of Medical Research (ICMR)–DHR - Sri Venkateswara Medical College, Tirupati, India, for providing financial assistance for this study and like to acknowledge our second and third authors for carrying out manual statistical analyses of the data.

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