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Morphological changes in subcutaneous white adipose tissue after severe burn injury

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Abstract

Introduction—Severe burn injury produces a plethora of metabolic abnormalities which contribute to the prolonged morbidity of burn survivors. We have recently demonstrated trans-differentiation of white adipose tissue following burn trauma, towards a more thermogenic phenotype. However, the impact of burn injury on subcutaneous WAT (sWAT) morphology in humans is unknown. Here, we studied the effect of severe burn injury on the architecture of sWAT.

Methods—sWAT was collected from eleven severely burned children (11 ± 3 yrs; $55 \pm 16\%$ total body surface area burned) and twelve non-burned healthy children (9 ± 3 yrs). Histology, electron microscopy, immunohistochemistry and immunofluorescence were performed on fixed adipose tissue sections. sWAT cytokine and collagen concentrations were measured by multiplex assay and sirius/fast green staining method respectively.

Results—sWAT histology demonstrated multiple fat droplets, significantly ($p < 0.05$) reduced mean cell size ($104 \pm 6 \mu\text{m}$ vs. $68 \pm 3 \mu\text{m}$) and higher collagen content (7 ± 0.8 vs 4 ± 0.4). sWAT from burn victims stained positive for CD68 suggesting infiltration of macrophages. Further, electron microscopic analysis showed multiple fat droplets and greater mitochondrial abundance in sWAT of burn survivors. In agreement with this, mitochondrial respiratory capacity in the leak and coupled state increased by 100% in sWAT of burned children from 1 to 3 week post injury. The cytokines IL-6, IL-8, IL-13, IL-1a, IL-1b), MCP-1 and TNF- α were all significantly greater in the sWAT of burned children vs. healthy children ($P < 0.05$). Furthermore, IL-6, IL-8, IL1-a, IL-1b and TNF- α significantly increased after injury in sWAT of burned children ($P < 0.05$).

Conclusions—This study provides the detailed evidence of morphological and functional changes in sWAT of burn survivors which was associated with tissue inflammation.

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Applicability of Research to Practice—A better understanding of morphological and functional changes in sWAT will help discern the mechanisms underlying hypermetabolism in burned patients.

Keywords

Burn Injury; White Adipose Tissue; Inflammation; Collagen; Hypermetabolism

Introduction

Severe burn injury is characterized by a hypermetabolic stress response that begins shortly after burn injury and can persist for up to two years post injury. This hypermetabolic response results in increased resting metabolic rate and substrate mobilization which leads to the erosion and remodeling of peripheral tissues such as striated muscle and adipose tissue (1; 2; 3; 4; 5; 6). A significant amount of subcutaneous white adipose tissue (sWAT) is lost after burn injury and the rest undergoes morphological and functional changes (8; 9). sWAT likely contributes to the stress response to burns, since burn injury and the massive catecholamine response that follows elevates the rate of triglyceride-fatty turnover (1).

In addition to hypermetabolism, burn injury is associated with systemic and tissue inflammation (10) (11; 12). While burn victims have diminished sWAT, remaining sWAT depot is thought to undergo morphological and functional alterations, and ultimately contribute to the prolonged stress response to burns. Indeed, the infiltration of inflammatory immune cells following burn trauma has been postulated to alter WAT morphology. Further, these changes in WAT morphology are thought to underlay altered WAT function following burn injury. However, to date, little data exists characterizing the impact of severe burn on human sWAT. Here, we set out to comprehensively detail the impact of severe burn injury on morphology and cytology of human sWAT. Our working hypothesis was that the marked adrenergic and inflammatory stress response would profoundly alter sWAT morphology and composition.

Material and Methods

Patients and sample collection

Eleven burned pediatric patients (11 ± 3 yrs; $>30\%$ total body surface area burned) and twelve non-burn children (9 ± 3 yrs) were included in this study (Table 1). Written patient ascent and/or parental consent were obtained where appropriate. The study was approved by the Institutional Review Board of the University of Texas Medical Branch at Galveston. sWAT samples were collected from severely burned patients under general anesthesia. sWAT samples from burned children were isolated from the exposed healthy subcutaneous WAT of the torso or limbs. sWAT from non-burned healthy children were extracted from the abdominal region doing elective surgery for non-metabolic diseases.

Histology, immunohistochemistry and immunofluorescence analysis

sWAT samples were dehydrated, embedded in paraffin and cut into $6\mu\text{m}$ sections. Sections were then stained with hematoxylin and eosin for histology. Morphometric analysis of

adipose tissue was performed in at least three images obtained at the same magnification from three different location of same tissue section. Immunostaining was performed in 6µm thick sections of sWAT according to a standard protocol for immunohistochemistry⁽¹³⁾ and immunofluorescence. Anti-rabbit CD68 antibody (Abcam, MA, USA), ABC kit, DAB kit and Texas Red labelled - anti-rabbit IgG (Vector laboratories, Inc, Burlingame, USA) were used. For imaging we used Olympus microscope (BX41C) with Olympus camera (U-TVO-63XC, Tokyo, Japan) and software such as cellSens Viewer 1.5 (Olympus Soft Imaging Solutions GmbH, Munster, Germany), Image J (National Institute of Mental Health, Bethesda, Maryland, USA) and adobe photoshop CC (Adobe Systems Inc, USA).

Transmission electron microscopy

For electron microscopy, fresh adipose tissue was fixed in 2.5% glutaraldehyde in sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA), and post fixed in 1% osmium tetroxide and embedded in an Epon-Araldite mixture. 2µm sections were then stained with toluidine blue. Ultrathin (80 nm) sections were obtained with an Ultracut E ultramicrotome (Leica Microsystems, Buffalo Grove IL) and post-stained with 3% uranyl acetate, followed by 0.4% lead citrate. The ultrathin sections were examined with a JEOL 100CX transmission electron microscope, images were recorded digitally.

High-resolution respirometry

Mitochondrial respiration was determined in approximately 50 mg of fresh sWAT. Immediately after collection, sWAT samples were submerged in a cold EGTA buffer (pH of 7.1) containing 10mM CaK₂-EGTA; 7.23mM K₂-EGTA; 20mM imidazole; 20mM taurine 50mM K-MES; 0.5mM dithithreitol; 6.56 MgCl₂; 5.77mM ATP and 15mM phosphocreatine. Approximately 50 mgs of sWAT was blotted on filter paper then weighed before being transferred into an Oxygraph-2k (O2K) respirometer (Oroboros Instruments, Innsbruck, Austria) containing 2mls of a pH adjusted (7.1) respiration buffer (0.5mM EGTA; 3mM MgCl₂; 60mM K-lactobionate; 20mM taurine; 10mM KH₂PO₄; 20mM HEPES; 110mM sucrose; and 1mg/ml essential fatty acid free bovine serum albumin). Temperature was maintained at 37°C during all mitochondrial respirometry experiments. Leak (state 2) respiration was determined after 1.5mM octanoyl-L-carnitine, 5mM pyruvate, 2mM malate and 10mM glutamate were titrated into the O2K chamber. Coupled (state 3) respiration was then determined following the titration of 5mM ADP.

Collagen content measurement

sWAT was stained by the sirius red/fast green collagen staining method. Sirius red specifically binds fibrillary collagen such as type I to V collagen. Fast green binds to non-collagenous protein. Tissue collagen content was semi-quantified spectrophotometer using assay kit (Chondrex Inc, Redmond, WA).

Tissue cytokines measurement

Tissue cytokines were measured in sWAT lysates using 'magnetic bead panel based multiplex' assay kit (Millipore, USA).

Statistical analysis

Patient characteristics have shown as group means \pm the standard deviation. All other data is reported as group means \pm standard error of the mean. The normality of biochemical outcome measures was tested with a Shapiro-Wilk normality test. Burn and healthy groups were compared by a Student t-test or Mann Whitney test or Wilcoxon matched-pair signed rank test. Statistical analysis was performed in GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA) or Sigma Plot 12 (Sigma Software, CA).

Results

Patients

The basic anthropometric and injury characteristics of burned and healthy children are presented in Table 1. We enrolled a total of eleven severely burn children admitted to Shriners Hospital for Children-Galveston, Texas, USA, between January 2012 and December 2014 for acute burn care. Twelve metabolically healthy children admitted over the same period to John Sealy Hospital at the University of Texas Medical Branch at Galveston scheduled for surgical procedures were recruited as healthy controls. Samples were collected up to 12 weeks post injury in burned children and categorized into two subgroup early burn (up to 2 weeks days post burn) and late burn (> 2 weeks days post burn) group.

Burn induced changes the architecture of adipose tissue

sWAT from healthy children typically exhibited a single large lipid droplet (unilocular). Adipocytes were uniformed in terms of their gross size and packed tightly in healthy sWAT (Fig 1A). In contrast, adipocytes in sWAT from burned children (burn subject-1: 19 days post burn, 43% TBSA, 18% 3rd degree burn and 2 yr old; subject-2: 32 days post burn, 70% TBSA, 65% 3rd degree burn and 15 yr old; subject-3: 45 days post burn, 80% TBSA, 80% 3rd degree burn, and 12 yr old) were shrunken and disarranged, with a wide range of diameters. Moreover, these cells were loosely packed with more intercellular space, extracellular matrix, loose connective tissue and extracellular infiltrate. We also observed arbitrarily distributed adipocytes with numerous small lipid droplets in the sWAT of burned children. Morphometric quantification of sWAT from burned and healthy children demonstrated a significant reduction of sWAT cell size ($104 \pm 6 \mu\text{m}$ vs $68 \pm 3 \mu\text{m}$; $p < 0.001$) following burn injury (Figure 1B). Electron-microscopic analysis of sWAT morphology provided further evidence of presence of multiple lipid droplets and an apparent greater mitochondrial abundance in sWAT of burned children (Figure 1C). In support of this, mitochondrial respiration in the leak state (state-2 0.9 ± 0.2 vs. 0.4 ± 0.06 pmol/sec/mg, $p < 0.05$) increased after burn injury. Similarly mitochondrial respiration in the coupled state (state-3 (1.33 ± 0.2 vs. 0.67 ± 0.15 pmol/sec/mg, $p < 0.01$) significantly increased in sWAT after week of burn injury (Figure 1D).

Both percentage collagen content (7 ± 0.8 vs 4 ± 0.4 ; $p < 0.05$; Figure 2A) and ratio of collagen to non-collagen protein (0.07 ± 0.01 vs 0.04 ± 0.0 ; $p < 0.05$; Figure 2B) were significantly increased in sWAT of burned compared to healthy children, as demonstrated by staining with picrosirius and fast green. Staining of sWAT from burn victims exhibited intense red

(collagen protein) and green (non-collagen protein) staining in comparison to sWAT from healthy children. Collagen staining in intracellular space and extracellular matrix of burn sWAT (Figure 2C) suggests significant fibrosis within the sWAT of burn survivors.

Burn injury increases the number of inflammatory immune cells within sWAT

Immuno-staining of sWAT from healthy and severely burned children with CD68 antigen (a marker of macrophages) demonstrated multiple brown or red spots in sWAT from burned children (Figure 3A). Healthy sWAT did not show any (or very few) CD68 stained macrophages (Figure 3A). By immuno-staining of sWAT from burned and healthy children with CD14 antigen (another marker of macrophages) we further confirmed the presence of macrophages in the sWAT of burn survivors (images not shown). These data were confirmed by immuno-fluorescence staining of sWAT with CD-68 (Figure 3B), providing further evidence of presence of higher number of macrophages in intercellular space of the sWAT of burn survivors.

Tissue cytokines were measured in the clear supernatant of sWAT homogenates following centrifugation at 10,000 g for 20 min. The cytokines IL-6 (710 ± 171 vs 1.7 ± 1 pg/mg; $p < 0.05$), IL-8 (1457 ± 563 vs 1.4 ± 1 pg/mg; $p < 0.05$), IL-13 (23 ± 7.3 vs 5.9 ± 2.4 pg/mg), IL-1a (305 ± 167 vs 44 ± 3.7 pg/mg), IL-1b (23 ± 8 vs 0 ± 0 pg/mg), MCP-1 (4474 ± 1162 vs 159 ± 59 pg/mg) and TNF- α (35 ± 11 vs 2.1 ± 1 pg/mg, $p < 0.05$) were increased in the sWAT of burned children when compared to sWAT from healthy children. However, IL-3 level (1.8 ± 0.5 vs 2.2 ± 0.2 pg/mg) was not changed in subcutaneous WAT from burn survivors. IL-6 (710 ± 171 vs 288 ± 169 pg/mg), IL-8 (1457 ± 563 vs 382 ± 170 pg/mg), TNF- α (35 ± 11 vs 10.6 ± 3.8 pg/mg), IL-1a (305 ± 167 vs 65 ± 15 pg/mg) and IL-1b (23 ± 8 vs 2 ± 1.0 pg/mg) were higher in the late group versus early group. IL-13 (23 ± 7 vs 13 ± 2 pg/mg), MCP-1 (4474 ± 1162 vs 3441 ± 988 pg/mg) and IL-3 (1.8 ± 0.5 vs 1.6 ± 0.2 pg/mg) were not different between the two groups. The increased level of cytokines IL-6, IL-8, TNF- α , IL-1a and IL-1b in sWAT from burn survivors suggests sWAT inflammation following burn injury.

Discussion

Burn injury results in a large and prolonged stress response, which is characterized by a hypermetabolic hypercatabolic phenotype. While whole body lipid turnover and adipose tissue catabolism are increased following burn trauma, likely to mobilize fatty acid and glycerol reserves, the impact of burn trauma on sWAT morphology remains largely unknown. Our hypothesis was that burn injury and the resultant adrenergic and inflammatory stress would alter adipose tissue morphology and function. We collected sWAT underneath the skin from abdominal region of healthy children. We also harvested sWAT underneath the skin from different regions of body (torso and limbs) of burned victims depending upon location of surgery. In our hands, the morphology of sWAT does not change considerably due to its anatomical location (i.e., the torso, limbs etc). We show for the first time in humans that severe burn trauma significantly alters the morphology and composition of sWAT. Specifically, cell size was significantly diminished following burn injury, while there is an appearance of small cells with numerous lipid droplets. Further, collagen deposition and an increase in organelles such as mitochondria and immune cells

such as macrophages occur within WAT after burn injury. Finally, the above morphological changes in sWAT were associated with and infiltration of inflammatory cytokines.

We found that sWAT from healthy children contained mostly uniformly shaped, large unilocular adipocytes which were packed tightly to each other (i.e., there was minimal extracellular space). In stark contrast, histology of sWAT from burned children showed loosely packed smaller adipocytes with greater intracellular and extracellular space, suggesting burn affects adipocyte arrangement and size. sWAT is a main storage depot of lipid *in vivo*, largely in the form of triglycerides. The catecholamine mediated stress response to burn trauma increases sWAT lipolysis ^(14; 15), which likely explains the reduction in adipocyte size observed in the current study. Furthermore, the appearance of adipocytes with several smaller lipid droplets (as apposed to one larger lipid droplet seen in healthy sWAT likely reflects chronic lipolysis following burn trauma. We have previously shown that post burn lipolysis is mediated by β_2 adrenergic receptors, and can be blocked with propranolol treatment ⁽¹⁴⁾. While the physiological role of increased lipid turnover post-burn remains unclear, our previous data suggest that a mis-match in glycerol and fatty acid release from adipose tissue lipolysis in burn victims ^(1; 15). Specifically, full triglyceride hydrolysis results in the release of one glycerol and three fatty acids. However, following burn injury, only 50% of these fatty acid are released into the systemic circulation, meaning there is a high rate of intracellular fatty acid cycling. This response may fulfill a number of physiological roles such as releasing glycerol and thus providing the liver with gluconeogenic precursors. Alternatively, it may provide fatty acids for intracellular oxidation within sWAT. In support of this notion, we see an abundance of mitochondria in the sWAT of burned children, but not the sWAT of healthy children. We also observed an increase in mitochondrial respiratory capacity in the sWAT of burn victims. Thus, reduced adipocyte size and increased mitochondrial abundance within sWAT following burn trauma supports the notion that sWAT oxidative capacity increases in response to burn injury.

Collagen is an extracellular matrix (ECM) protein located around adipocytes within adipose tissue. Its major function is to provide mechanical support to adipocytes. The upregulation of ECM causes adipose tissue fibrosis which is hallmark of metabolically challenged adipocytes ⁽¹⁷⁾. In this study we found that burn injury significantly increases collagen content in sWAT. Specifically, sWAT histology showed loose ECM with tissue infiltrate after burn injury, which may contain numerous cell types, such as macrophages, leukocytes, fibroblast, vascular endothelial cells etc. Indeed, it is known that fibroblast (also macrophages), secrete collagen ⁽¹⁸⁾. Recent studies have identified a link between adipose tissue inflammation and ECM ⁽¹⁷⁾. Macrophages also release various proinflammatory cytokines such as TNF- α , interleukin (IL) -6, IL-8, IL-13, MCP-1 ^(19; 20; 21). IL-6, a cytokine, is secreted by macrophages and T-cells to elicit an immune response. We found increased levels of IL-6 in sWAT of burned children when compared to healthy sWAT. The increased tissue levels of IL-6, IL8, IL1 α and TNF- α in sWAT of burned children suggests the presence of inflammation in sWAT, which is at least associated with morphological changes in sWAT of burn victims. Johnson *et al.*, reported that cytokines contribute to adipose tissue remodeling and metabolic abnormalities in HIV patients with lipodystrophy

syndrome⁽²²⁾, suggesting that inflammatory infiltrate, in addition to adrenergic stress, likely mediates the marked changes in adipocyte morphology within the sWAT of burn survivors.

To summarize, our current data highlights the impact of burn injury on adipose tissue. These results demonstrate morphological changes in sWAT with high inflammatory cytokine levels and fibrosis following burn injury, suggesting a potential linkage of morphological changes with thermogenesis and hypermetabolism of sWAT. A better understanding of morphological and functional changes in WAT will help discern the mechanisms underlying hypermetabolism in burned patients, which will provide the basis to improve the nutritional support of burn patients and to develop better interventions that attenuate the increase in metabolic rate. While the physiological significance of these adaptations require further investigation, we postulate that these morphological changes likely support greater lipid turnover and intracellular fatty acid oxidation following burn trauma. Although the current study was not designed to identify the detriments of altered sWAT morphology in response to burn trauma, our data suggest that infiltration of a plethora of inflammatory cells likely contribute to this phenomenon.

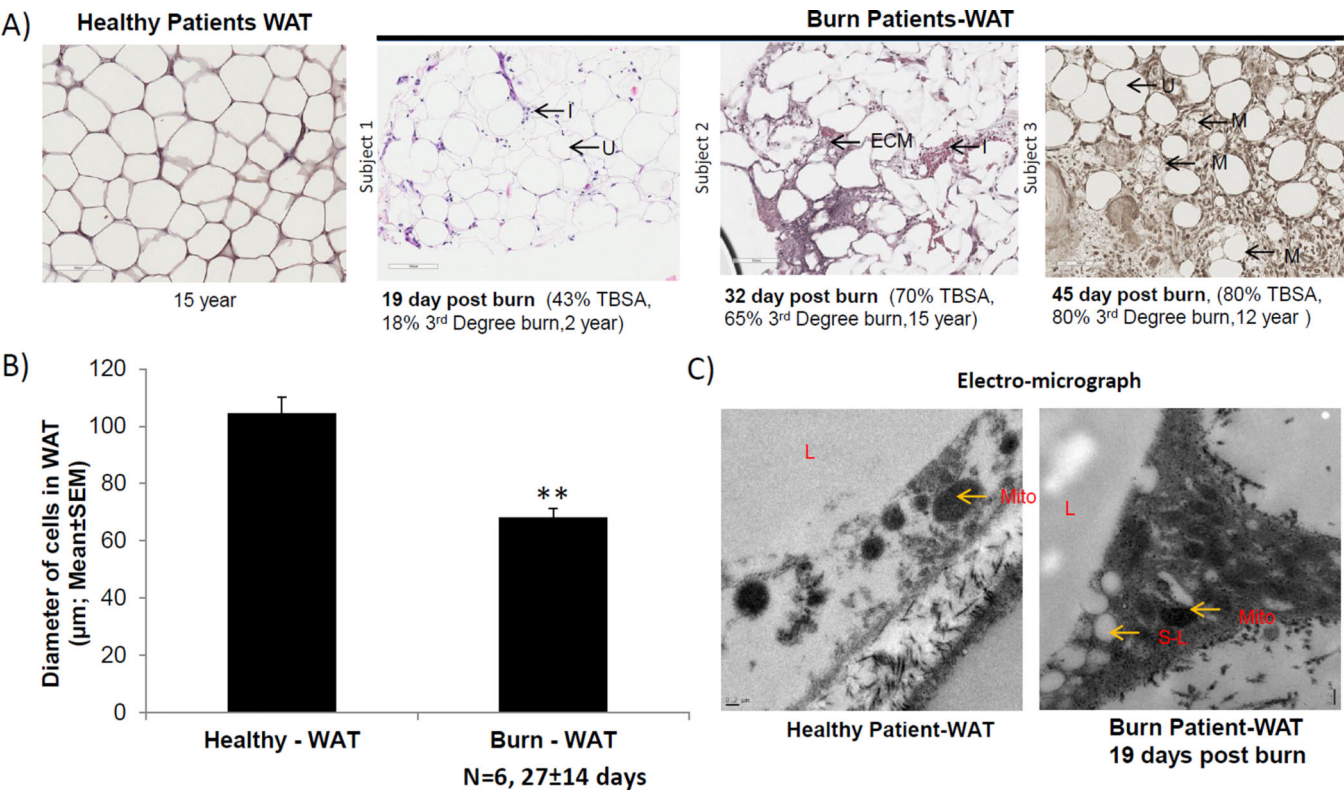
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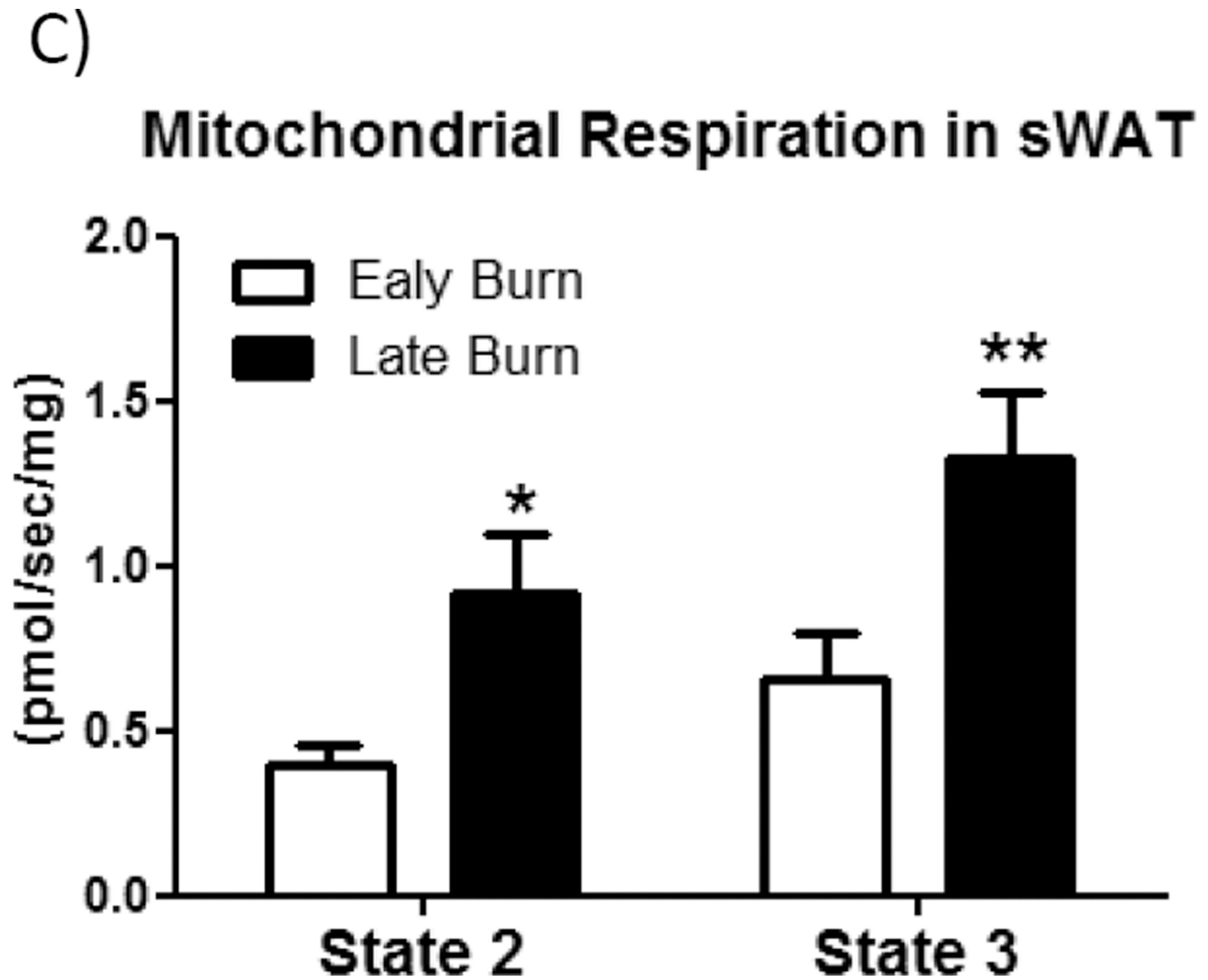


Figure 1. Impact of burn injury on adipocytes morphology

A) Hematoxylin and eosin stained sWAT from healthy and burn patients - demonstrated unilocular tightly packed adipocytes in sWAT from healthy patients. sWAT from burn patients (Subject-1 19 day post burn, 43% TBSA, 18% 3rd degree burn, age 2 year; Subject-2 32 day post burn, 70% TBSA, 65% 3rd degree burn, 15 year; Subject-3 45 day post burn, (80% TBSA, 80% 3rd degree burn, 12 year) exhibited many small unilocular cells (U) and few multi/paucilocular (M) cells with high extracellular matrix (ECM) and tissue infiltrate (I). **B)** Bar graph showed that the diameter of sWAT of burn patients (n=6; 27±14 days post burn, 63±15 % TBSA, 55±26 % 3rd degree burn, age 8±6 year) was smaller than sWAT of healthy patients (n=6; age 8±4 years). Student T-Test **P<0.001. **C)**

Electromicrograph displayed small (S-L) and big (L) lipid droplet and mitochondrial (Mito) in sWAT- from healthy (age 2 year) and burn (19 days post burn, 22 % TBSA, age 7 year) patients. **D)** Leak (state 2) (0.9±0.2 vs 0.4±0.06 pmol/sec/mg, p<0.05) and coupled (state-3) (1.33±0.2 vs 0.67±0.15 pmol/sec/mg, p<0.01) mitochondrial respiration were significantly increased in late group versus early group. Student Paired T-Test, n=7 per group.

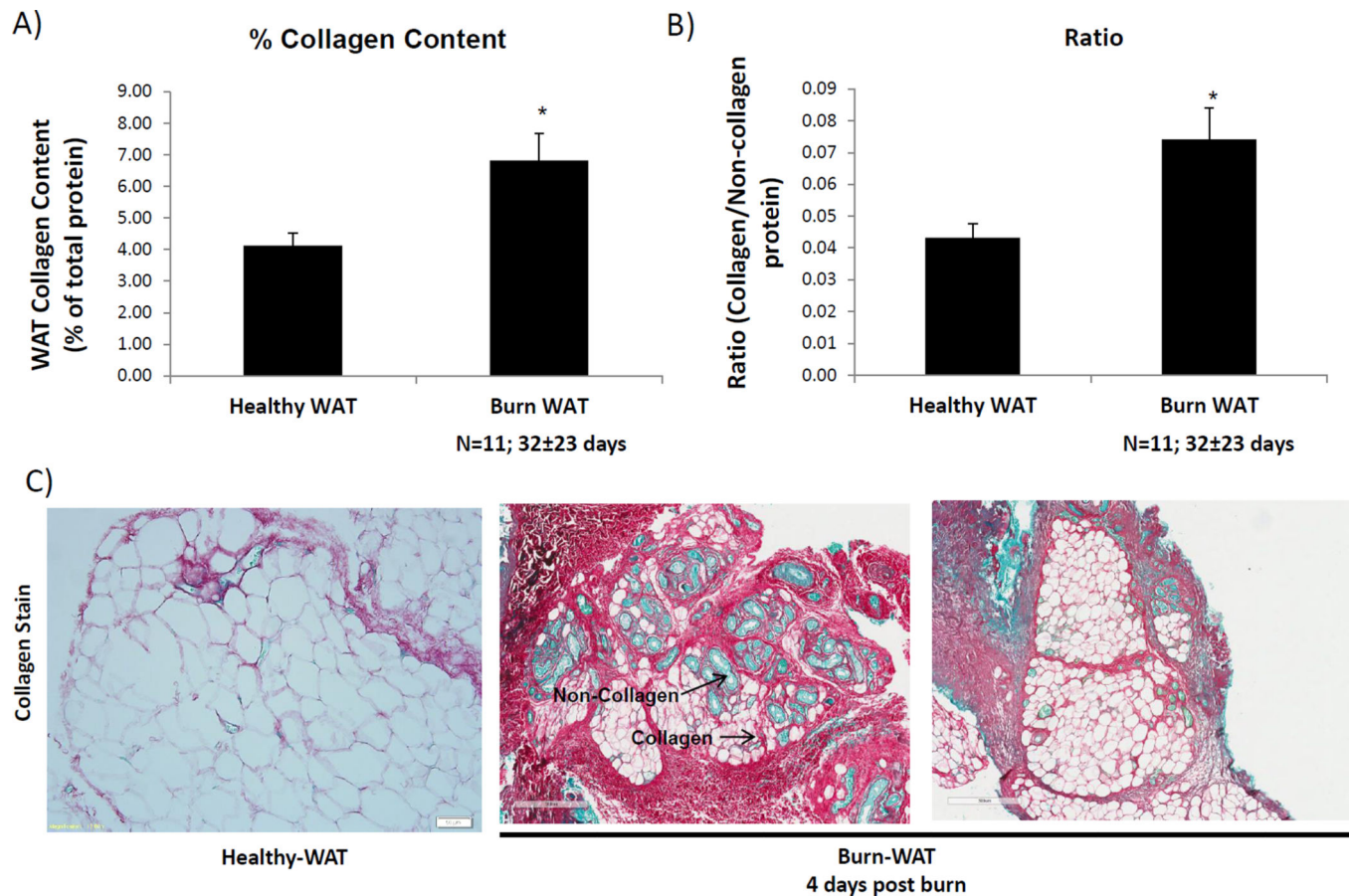
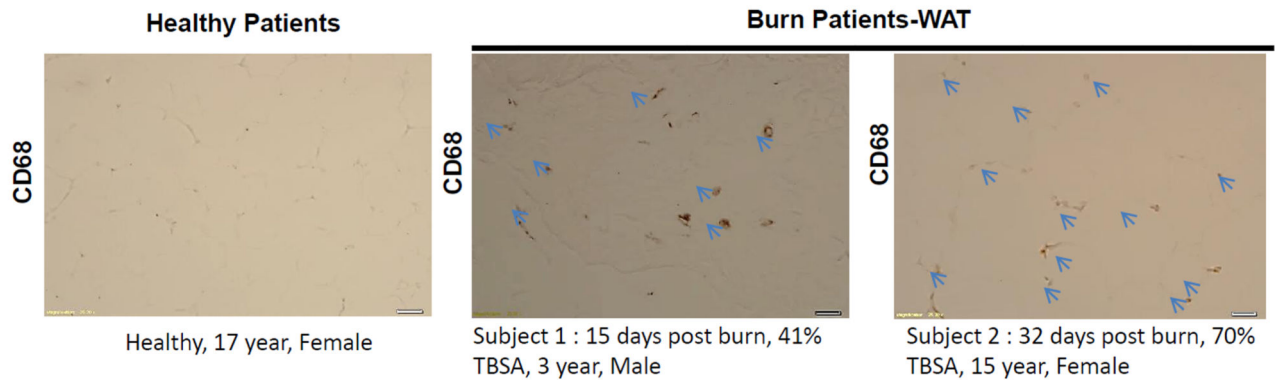


Figure 2. Burn injury causes adipose tissue fibrosis

Panel A and **B** show percentage of collagen content and ratio of collagen and non-collagen protein respectively. Both collagen content and ratio were significantly higher in sWAT of burn patients (n=11, 32±23 days post burn, 55±16% TBSA, 40±22 3rd Degree burn, age 12±2 year) as compared to sWAT of healthy person (n=12, 8±5 year). Student T-Test, *p<0.05. **C**) Sirius and fast green staining of WAT of healthy and burn patient exhibited intense red (collagen protein) and green (non-collagen protein) staining in sWAT of burn patient (43 % TBSA, 16% 3rd degree burn, age 16 year) at different location of tissue section.

A)



B)

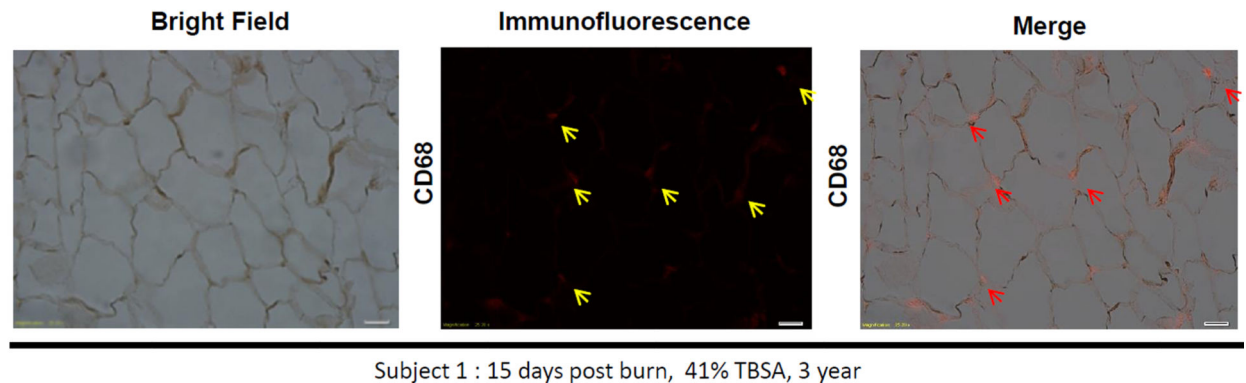
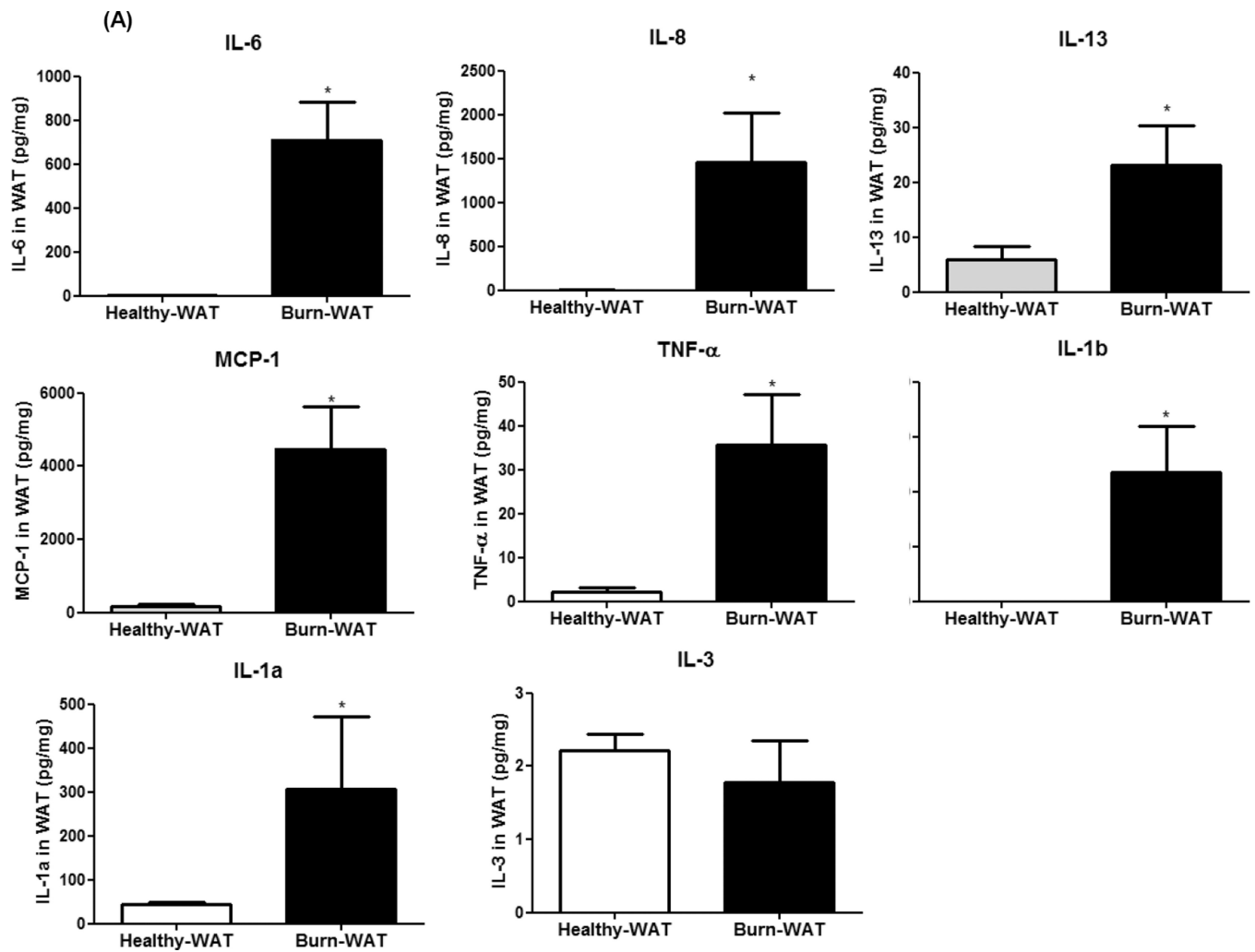


Figure 3. Burn injury results in macrophage infiltration of WAT

A) Immuno-staining of sWAT from burn and healthy patients with CD68 antigen (a marker of macrophages) demonstrated multiple brown spot (blue arrow) in sWAT of burn subject 1 (41% TBSA, 15 days post burn, 3 year, Male) and burn subject 2 (70% TBSA, 15 year, 32 days post burn, Female). However healthy -sWAT did not or rarely showed CD68 stained brown spot. **B)** Immunofluorescence staining of sWAT from burn subject 1 (41% TBSA, 15 days post burn, 3 year, Male) also displayed various red spot (yellow), located in intercellular space of adipocytes (red arrow).



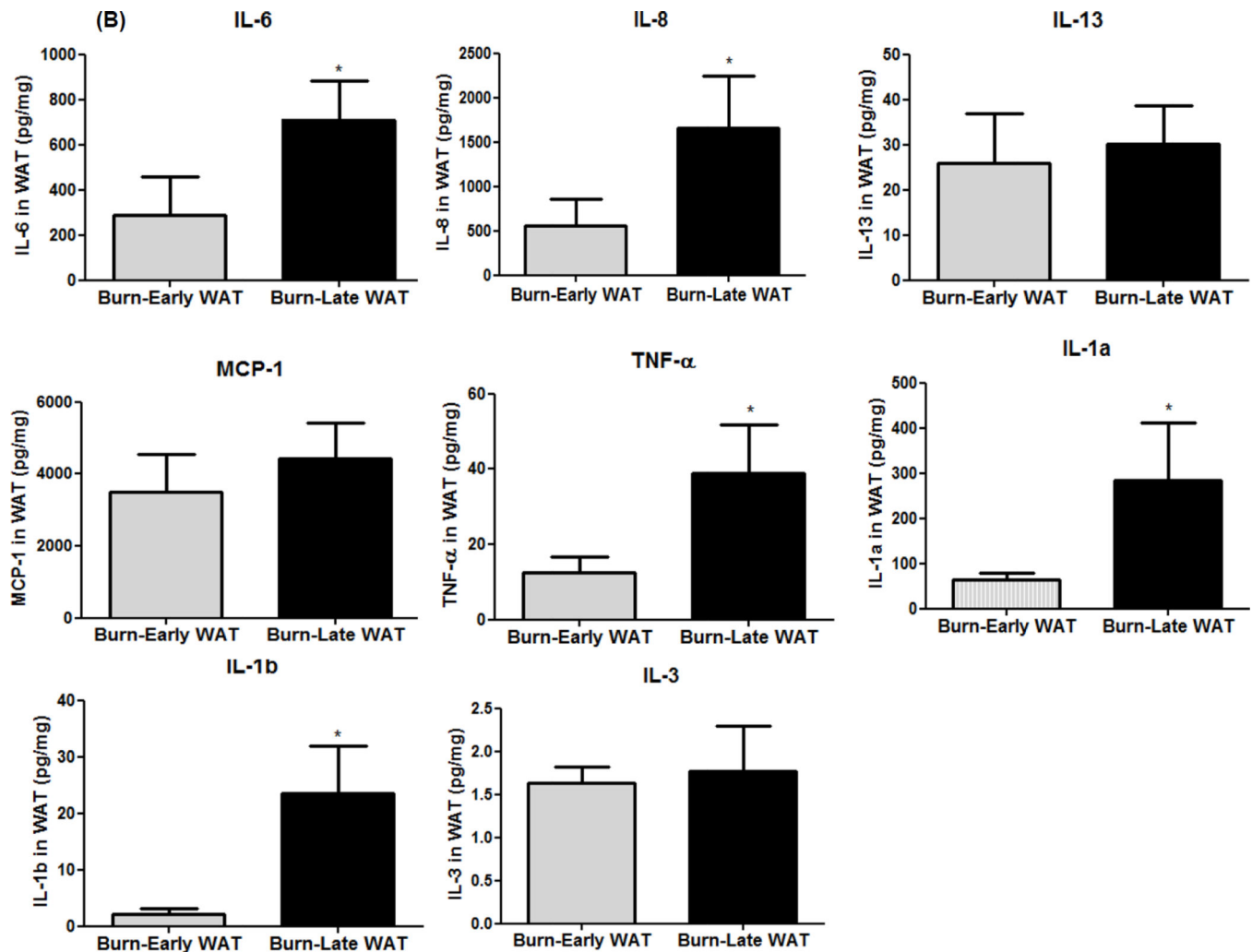


Figure 4. Burn injury results in inflammatory infiltration in WAT

A) IL6, IL8, IL-13, IL-1a, IL-1b, MCP1 and TNF- α were increased significantly ($p < 0.05$) in sWAT ($n=11$, > 14 days post burn) from burn patients as compared to sWAT ($n=10$) from healthy patients, suggesting the inflammation of sWAT in burn victims. However IL3 were not altered in burn sWAT. Mann Whitney test, $*p < 0.05$. **B)** IL6, IL-8, TNF- α , IL-a and IL-b were increased significantly in late sWAT ($n=11$, > 14 days post burn) as compared to early subcutaneous WAT ($n=11$, 1–14 days post burn). However IL-13 and IL-3 were not altered in late burn sWAT as compared to early burn sWAT. Wilcoxon matched-pair signed rank test, $*p < 0.05$. Mitochondrial respiration state-2 (0.9 ± 0.2 vs 0.4 ± 0.06 pmol/sec/mg, $p < 0.05$) and state-3 (1.33 ± 0.2 vs 0.67 ± 0.15 pmol/sec/mg, $p < 0.01$) were significantly increased in late group versus early group. Student Paired T-Test, $n=7$ per group.

Table 1

Characteristics of burned and non-burned control subjects

	Burn Patients	Healthy Patients	<i>p</i> value
<u>n (Male : Female)</u>	11 (8:3)	12 (5:7)	-
<u>% TBSA (mean \pm SD)</u>	55 \pm 16	-	-
<u>3rd Degree Burn (mean \pm SD)</u>	40 \pm 22		-
<u>Age (year; mean \pm SD)</u>	12 \pm 3	9 \pm 3	0.47
Post burn Days (mean \pm SD)	32 \pm 23	-	-
•Early burn	1–14 Days		
•Late burn	> 14 days		
<u>Height (cm; mean \pm SD)</u>	153 \pm 20	139 \pm 39	0.45
<u>Weight (kg; mean \pm SD)</u>	49 \pm 17	40 \pm 21	0.37