

## ORIGINAL RESEARCH

# Burn-induced Oxidative Stress and Serum Glutathione Depletion; a Cross Sectional Study

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**Abstract:** **Introduction:** Several studies have shown the role of oxidative stress in pathophysiology of burn injuries. This study aimed to evaluate the changes of oxidant-antioxidant levels during the week following burn injuries and its correlation with grade of burn. **Methods:** In this prospective cross-sectional study, changes of total glutathione, reduced glutathione (GSH), oxidized GSH (GSSG), GSH/GSSG ratio, as well as Pro-oxidant-antioxidant balance (PAB) were investigated on the 1st, 2nd and 7th days of admission in patients with > 15% burns. **Results:** 40 patients with the mean age of  $21.1 \pm 14.5$  were studied (47.5% male). More than 50% of patients were in the 18 – 55 years age range and over 70% had 20% – 60% grade of burn. Total serum glutathione level and GSH had significant decreasing trends ( $P < 0.001$ ) and GSSG and GSH/GSSG ratio had increasing trends ( $p < 0.001$ ). No significant correlation was observed between serum GSH level and the total body surface area (TBSA) of burn injury ( $r = 0.047$ ;  $p = 0.779$ ). The evaluation of PAB and its correlation with TBSA showed a significant and direct association between them on the 1st (coefficient = 0.516;  $p = 0.001$ ), 2nd (coefficient = 0.62;  $p < 0.001$ ), and 3rd (coefficient = 0.471;  $p = 0.002$ ) day of follow up. **Conclusion:** According to this study, the redox perturbation occurred in burn injury which was measured and proved by decreased GSH/GSSG ratio as well as the shift of PAB in favour of oxidants. Besides, since PAB positively correlated with the severity of dermal damage, it might suggest the application of antioxidants as a part of therapeutic protocol for which the dosage should be proportionate to the surface area of the damaged skin.

**Keywords:** Oxidative stress; oxidants; antioxidants; Glutathione; Burns

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## 1. Introduction

Burn injury is a kind of inflammatory disease in which, besides the local dermal damages, systemic complications such as systemic inflammatory response syndrome, multiple organ failure and sepsis, which are the leading causes of morbidity and mortality, may occur (1, 2). Different factors such as inflammatory responses are involved in the pathophysiology of these systemic complications. Oxidative stress is

one of these factors, which happens due to the formation of free radicals at the area of injury due to intravascular stimulation of neutrophils and increased xanthine oxidase activity (3, 4). The subsequent transportation of these free radicals to distant parts through blood flow may explain one of the mechanisms by which other organs such as liver, heart and lungs can get damaged in patients with burn injury (5). LaLonde et al. even suggested a causal relationship between increased redox perturbation and inflammation (6). These damages are because of the high reactivity of free radicals, including both reactive oxygen species (ROS) and reactive nitrogen species (RNS), which react with and oxidize cellular molecules such as proteins, lipids and nucleic acids

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(7, 8) leading to a wide variety of diseases including cancers, autoimmune diseases, cardiovascular diseases, etc (9-14). On the other hand, the body is equipped with enzymatic as well as non-enzymatic antioxidants to recuperate the redox homeostasis (15-18). Glutathione, a tripeptide, is such a molecule, which plays an important role in providing and maintaining a reduced intracellular environment, which is necessary for cell survival (19). During oxidative stress, when pro-oxidant-antioxidant balance (PAB) is disrupted in favour of the former, reduced glutathione (GSH) is oxidized to the form of GSSG, and GSH/GSSG ratio, which is normally about 100/1 in cells, decreases to values of about 10:1 and even 1:1 (20-23). This study aimed to investigate if oxidative stress happens during burn injury, how it proceeds during the first week of injury and whether it correlates with the severity of dermal damage. For this purpose, two markers of oxidative stress were selected: the GSH/GSSG ratio and PAB. The latter is a newly-established method through which the whole redox status can be investigated using a simple method (24).

## 2. Methods

### 2.1. Study design and setting

This prospective cross-sectional study was conducted on burn patients who were hospitalized in Imam Reza Hospital, Mashhad, Iran, during a one-month period from February 2016 to March 2016. The changes of total glutathione, GSH, GSSG, as well as GSH/GSSG ratio were investigated on the 1st, 2nd and 7th days after a thermal burn injury. This project was ethically approved by the Research Council of Mashhad University of Medical Sciences (code: IR.MUMS.REC.1388.102), and an informed consent was provided by each patient.

### 2.2. Participants

Patients with over 15% burn of total body surface area (TBSA) of the second-degree were enrolled using non probability convenience sampling method. Those with known systemic diseases such as cardiovascular diseases, diabetes mellitus, smoking, opium addiction, etc. as well as patients with a recent history of consuming antioxidants such as Vitamin C and multivitamins in which the redox status could be affected were excluded from this study.

### 2.3. Measurements

After careful history taking and clinical examination, the protocol of study was explained to patients or relatives and an informed consent was obtained. Blood samples from each patient were collected three times: on the 1st day (during the first 6 hours after the injury), 2nd day (24 hours after the injury), and 7th day of the injury to study serum GSH in both oxidized and reduced forms as well as PAB. Blood sam-

ples were collected free of any anticoagulant, kept at room temperature for 30 minutes to clot. Thereafter, the samples were centrifuged at  $2000\times g$  for 15 min at  $4^{\circ}C$  to separate the serum, which was subsequently deproteinated by adding an equal volume of metaphosphoric acid 1 M in distilled water to the sample. After 5 minutes of incubation at  $25^{\circ}C$ , the mixture was centrifuged at  $3000\times g$  for 5 minutes and the supernatant was collected carefully. The concentration of serum GSH was measured by the method described before by Ellman, which is based on the reduction of 5,5'-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) by free thiols to 2-nitrobenzoic acid anion (TNB<sup>-</sup>), which is a yellowish compound (22, 27, 28). The absorbance of samples was measured spectrophotometrically at 412 nm, and the concentration of glutathione in each sample was calculated using a standard curve, which had been provided with different concentrations of glutathione (0-15  $\mu M$  in MES buffer). In order to measure the concentration of GSSG, excluding GSH, 10  $\mu l$  of 2-vinylpyridine 1 M in ethanol was added to 1 ml of deproteinated sample, incubated at  $25^{\circ}C$  for 60 min to block free thiols of GSH. Subtraction of GSSG level from total glutathione concentration equals to GSH level. Serum PAB was measured before deproteination as described before (24). In brief, it was performed by adding a cation to the TMB (3, 3', 5, 5'-Tetramethylbenzidine) solution by peroxidase enzyme. The cationized TMB solution (blue colored) was subsequently reduced by antioxidants present in the sample or standards, and converted to the reduced TMB (colorless). The higher the level of antioxidants in the sample was, the lower the density of blue color would be. For this purpose, the standard curve was prepared by mixing varying percentages of hydrogen peroxide 250  $\mu M$  and uric acid 3 mM in 10 mM of NaOH. The resulting absorption was read at a wavelength of 450 nm. The percentage of burnt TBSA was calculated using the law of Lound & Browder (25, 26). All materials and reagents were purchased from Sigma-Aldrich, Germany.

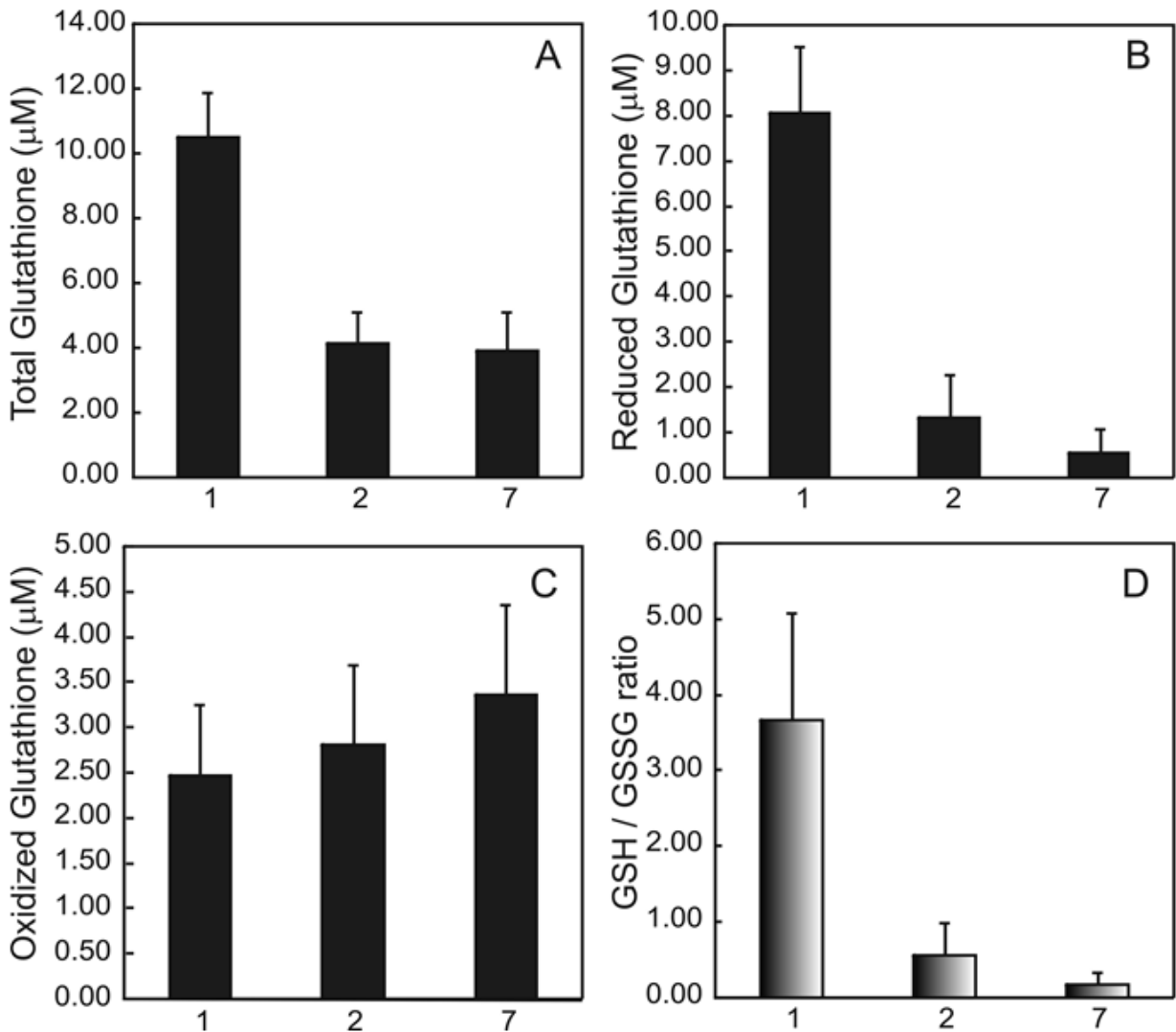
### 2.4. Data gathering

Demographic information (age, sex), vital signs (temperature, heart rate, respiratory rate), and the percentage of TBSA were recorded for all participants using a pre-design check list. There were no missing data. All data were collected prospectively by a trained nurse. Laboratory tests were performed by MSc students in Clinical Biochemistry.

### 2.5. Statistical Analysis

We have performed the Kolmogorov-Smirnov test to check the normality of data. Repeated measures ANOVA, paired sample t-test, and correlation coefficient tests were used to analyse the data. The level of significance was considered as  $p$ -value  $\leq 0.05$ . SPSS 11.0 software was used for statistical analysis and data are presented as Mean  $\pm$  standard deviation.





**Figure 1:** Serum glutathione changes following burn injury on the 1st, 2nd, and 7th day of admission.

tion (SD).

### 3. Results

#### 3.1. Baseline characteristics of studied patients

40 patients with the mean age of  $21.1 \pm 14.5$  (3 – 56) were studied (47.5% male). Table 1 shows the baseline characteristics of studied patients. More than 50% of patients were in 18 – 55 years age range and > 70% had 20% – 60% burn.

#### 3.2. GSH changes following burn injury

Figure 1 and table 2 summarize the changes of total serum glutathione level, GSH, GSSG, and GSH/GSSG ratio during the one week follow-up. Total serum glutathione level and GSH had significant decreasing trends ( $P < 0.001$ ) and GSSG

and GSH/GSSG ratio had increasing trends ( $p < 0.001$ ). No significant correlation was observed between serum GSH level and the TBSA of burn injury ( $r = 0.047$ ;  $p = 0.779$ ).

#### 3.3. PAB changes following burn injury

The evaluation of PAB and its correlation with TBSA showed a significant and direct association between them on the 1st (coefficient = 0.516;  $p = 0.001$ ), 2nd (coefficient = 0.62;  $p < 0.001$ ), and 3rd (coefficient = 0.471;  $p = 0.002$ ) day of follow up (figure 2).

### 4. Discussion

The aim of this study was to determine how the formation of reactive oxygen species and its subsequent oxidative stress



**Table 1:** Baseline characteristics of studied patients

Variables	Number (%)
<b>Sex</b>	
Male	19 (47.5)
Female	31 (52.5)
<b>Age (years)</b>	
< 18	16 (40.0)
18 – 35	12 (30.0)
35 – 55	11 (27.5)
≥ 55	1 (2.5)
<b>Burn grade* (%)</b>	
<20	3 (7.5)
20 – 40	26 (65.0)
40 – 60	3 (7.5)
≥ 60	8 (20.0)

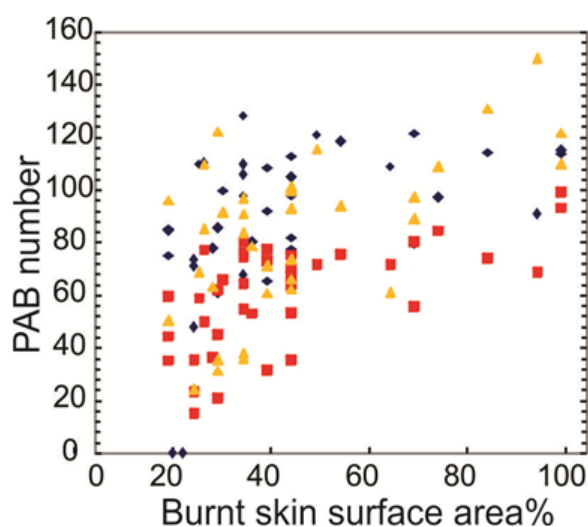
\*: Calculated using the law of Lound & Browder.

**Table 2:** Changes of total serum glutathione level, reduced glutathione (GSH), oxidized glutathione (GSSG), and GSH/GSSG ratio during the one week follow-up after burn injury among the studied patients

Variables	Values	P
<b>Total glutathione (<math>\mu\text{M}</math>)</b>		
1 <sup>st</sup>	10.52 ± 1.38	
2 <sup>nd</sup>	4.13 ± 0.96	< 0.001
7 <sup>th</sup>	3.91 ± 1.18	
<b>GSH</b>		
1 <sup>st</sup>	8.05 ± 1.46	
2 <sup>nd</sup>	1.32 ± 0.95	< 0.001
7 <sup>th</sup>	0.55 ± 0.51	
<b>GSSG</b>		
1 <sup>st</sup>	2.46 ± 0.81	
2 <sup>nd</sup>	2.81 ± 0.89	< 0.001
7 <sup>th</sup>	3.37 ± 1.01	
<b>GSH/GSSG ratio</b>		
1 <sup>st</sup>	3.66 ± 1.44	
2 <sup>nd</sup>	0.55 ± 0.44	< 0.001
7 <sup>th</sup>	0.17 ± 0.15	

Data are presented as mean ± standard deviation.

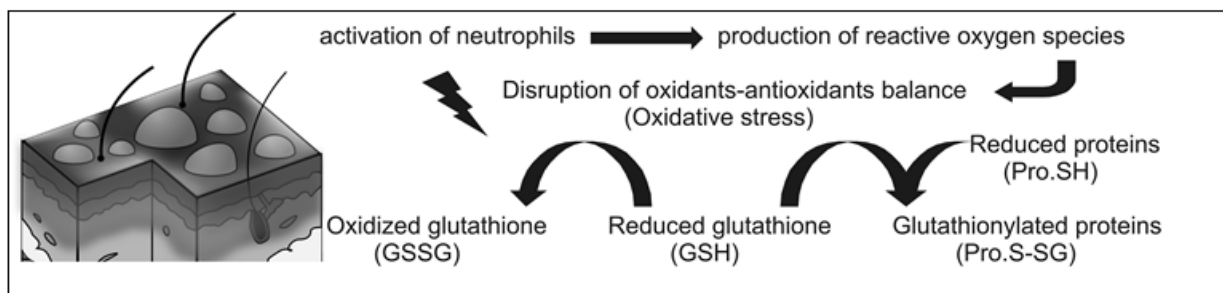
proceed during the first week of burn injury; for this purpose, GSH/GSSG ratio and PAB were chosen as the markers of the redox status. Based on this study, we observed time-dependent decreasing trends for both the serum total glutathione and GSH during the first week of burn trauma. However, an increasing trend was recorded for the serum oxidized glutathione within the first week of injury, which was time-dependent as well. Moreover, since the damaged body surface area is one of the factors involved in both the indication of hospitalization and prognosis, we planned to investigate the possible correlation between this factor and the above-mentioned redox markers. No significant correlation between serum glutathione level and the TBSA of

**Figure 2:** Correlation of pro-oxidant-Antioxidant Balance (PAB) with total body surface area (TBSA) burnt.

burn injury was observed in our study. However, PAB values showed a significant and direct association with burnt TBSA. The perturbation of redox homeostasis during burn injury has been addressed in a number of studies (4-6, 29, 30). In this study, we could also demonstrate a significant degree of redox perturbation, which was documented as the decreased level of serum total and reduced glutathione as well as GSH/GSSG ratio, which were accompanied with increased level of oxidized glutathione. The observed changes were time-dependent, which is supported by the results of Szczesny, B. et al (31).

However, as observed in this study, the magnitude of increase in serum oxidized glutathione (GSSG) was not as big as the decrease of serum glutathione (GSH) concentration. Considering this fact, along with decreased level of total serum glutathione, it might be suggested that GSH is probably recruited during burn injury to protect cellular and extracellular proteins from irreversible oxidations such as the formation of sulfinic and sulfonic acid (32) through glutathionylation of these proteins (figure 3) (33, 34). Since this post-translational modification is reversible, proteins can retain their activities after controlling the acute phase of injury (35-38). On the other hand, since the administration of ascorbic acid as a part of the routine therapeutic protocol in our hospital was started from the second day of hospitalization, the extent of oxidative damage as a consequence of burn injury could be much more significant if there were not any antioxidants in the drug regimen.

The more significant decrease of total glutathione and GSH, as well as the more dramatic decrease in GSH/GSSG ratio from the 1st day to 2nd day in comparison to changes from the 2nd day to 7th day could also be explained by the pro-



**Figure 3:** Possible mechanism and consequences of oxidative stress in burn injury. This schematic figure suggests two pathways of GSH consumption during burn injury including its oxidation to GSSG and its application in glutathionylation.

tective effects of ascorbic acid against oxidative damages. In this study, the first sample was taken during the first 6 hours of hospitalization, and the second sample was taken 24 hours later and before the administration of ascorbic acid, which is a part of the routine therapeutic protocol for burn injury in Imam Reza hospital, Mashhad, Iran. The third sample was taken on day 7, which means the serum redox status of patients was studied after the administration of a distinguished antioxidant for 6 days and daily administration of ascorbic acid. Since the administration of ascorbic acid could not be avoided due to ethical reasons, we could not study the changes of glutathione as well as PAB without use of any antioxidant in this group of patients. Performing the same study in animal models can be recommended to solve this limitation.

There are several studies showing the advantages of antioxidant therapy in management of burn injuries (39). N-acetylcysteine is an antioxidant and its effects on oxidative stress have been studied (8, 29). Ascorbic acid is another example of antioxidants that is shown to attenuate the oxidative stress (40). In a study by LaLonde et al., using rats as animal models, oral administration of a mixture containing glutathione, N-acetylcysteine and ascorbic acid showed a protective effect against burn-induced altered cell energetic (6). However, the severity of burn injury was not introduced as a factor for determination of the required dosage of antioxidants in any of these studies.

## 5. Limitation

The oxidative stress after burn is supposed to start quickly. Therefore, the first sample had to be taken as soon as possible. However, considering the time interval between injury and hospitalization and since our hospital is the referral center for a large area of Iran, more than 6 hours might have passed at from the trauma at the time of admission but decided to take the samples from the patients during the first 6 hours of admission anyway. For other samples, we should have ideally checked our patients on all days of hospitaliza-

tion; but financially we had limitations, which made us be restricted to the 2nd and 7th days.

## 6. Conclusion

According to this study, the redox perturbation in burn injury, which was measured as the PAB value positively correlated with the severity of dermal damage, which may suggest that the dosage of antioxidants should be proportionate to the surface area of skin that is damaged.

## 7. Appendix

### 7.1. Acknowledgements

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### 7.2. Author contribution

ABT, MS and SIH contributed to the conception and design of the study. RA, FSH and SIH contributed to the acquisition, analysis and interpretation of data. RA, FSH and SIH drafted the manuscript; ABT, MS and SIH revised it critically for content. All authors read and approved the final manuscript submitted for publication.

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### 7.4. Conflict of interest

Authors declare that there is no competing interest.

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