

## Role of Oxidative and Nitrosative Stress in Pathophysiology of Toxic Epidermal Necrolysis and Stevens Johnson Syndrome—A Pilot Study

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### Abstract

**Background:** Oxidative and nitrosative stress caused by drug metabolism may be a trigger for keratinocyte apoptosis in the epidermis seen in toxic epidermal necrolysis (TEN) and Stevens Johnson syndrome (SJS). **Aims:** To estimate oxidative damage in the serum and to examine the role of nitric oxide in mediating epidermal damage in patients with TEN and SJS. **Materials and Methods:** A prospective study was conducted among TEN and SJS patients and controls in a tertiary care center between January 2006 and February 2010. Patients with a maculopapular drug rash without detachment of skin constituted the control group 1 (drug exposed). Patients without a drug rash constituted the control group 2 (drug unexposed). The serum values of protein carbonyls, malondialdehyde, conjugated diene and nitrates were measured. Two-group comparison with the non-parametric Mann-Whitney U test was used. Significance of differences if any was established using Pearson's Chi-square test. **Results:** Ten patients in the SJS-TEN group (study group), 8 patients in control group 1 and 7 patients in control group 2 were included. More than one drug was implicated in 4/10 patients in group 1 and 3/8 patients in group 2. SCORTEN of 0, 1 and 3 at admission were seen in 2, 6 and 2 patients, respectively. The serum values of protein carbonyls, malondialdehyde, conjugated diene and nitrates were not significantly increased in the study group when compared to the controls. **Conclusions:** There was no elevation of oxidative stress markers in patients with TEN and SJS as compared to the control population.

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### What was known?

Apoptosis is the cause of keratinocyte death in TEN. The oxidative stress in the keratinocytes caused by various triggers may lead on to apoptosis.

### Introduction

Toxic epidermal necrolysis (TEN) and Stevens–Johnson Syndrome (SJS) are severe cutaneous drug reactions of unknown mechanism. Toxic epidermal necrolysis represents the most severe variant of a disease spectrum that consists of bullous erythema multiforme (EM) and SJS and has a mortality rate of 30–40%.<sup>[1]</sup> Keratinocyte apoptosis has now been established as a major feature in TEN and SJS, with cell death resulting in detachment of the epidermis. Though studies have identified molecules such as the Fas ligand (FasL) and its receptor as well as tumor necrosis factor-alpha (TNF- $\alpha$ ) to be implicated in the process, the triggering event inducing apoptosis is currently unknown. FasL is a transmembrane protein from the TNF family that is expressed on the surface of cytotoxic T cells, NK cells, immune privileged cells

of the testes and eye, and keratinocytes. On activation of cytotoxic T cells, FasL is expressed on their surface and binds to its receptor on target cells, which activates intracellular caspases, leading to the controlled destruction of the target cell.<sup>[2]</sup> Other cytokines and molecules, such as TNF- $\alpha$  and nitric oxide (NO), have been implicated in the apoptosis of TEN.<sup>[3]</sup> Tumor necrosis factor- $\alpha$  has been shown to activate the “death receptor” TNF-R1, causing caspase activation and cell death.<sup>[3]</sup> NO has also been implicated in apoptosis. The role of NO in apoptosis is thought to be due to stimulation of the activity of caspases through the action of p53.<sup>[4]</sup> Recent studies have shown granulysin as the predominant trigger for apoptosis.<sup>[5]</sup> The combination of TNF- $\alpha$ , NO, and the resultant elevation of reactive oxygen species may also contribute to resultant oxidative stress and the disruption of the intracellular structures leading on to apoptosis.<sup>[6]</sup> TEN is initiated either by noncovalent, direct interaction of a drug with a specific MHC I allotype or by covalent binding of a drug metabolite to a cellular peptide, forming an immunogenic molecule. CD8 cells, activated by keratinocytes and APCs expressing specific MHC I and antigen, release INF- $\gamma$ , causing the activation of macrophages and keratinocytes and perforin/granzyme B and granulysin. Expression

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of glutathione-S-transferase pi (GST-pi), which is induced by oxidative stress, was found to be elevated in keratinocytes of TEN patients.<sup>[7]</sup> Oxygen-free radicals have also been shown to induce apoptosis in keratinocytes in culture. It is hypothesized that oxidative and nitrosative stress caused by drug metabolism is a trigger for keratinocyte apoptosis in the epidermis seen in TEN and SJS. This hypothesis was tested in patients with TEN and SJS by the pursuit of the following specific aims.

## AIMS

- To estimate oxidative damage in the serum in patients with TEN and SJS
- To examine the role of NO in mediating epidermal damage in TEN and SJS.

## Materials and Methods

A prospective study was conducted in a tertiary care center between January 2006 and February 2010. Patients with TEN, SJS and SJS-TEN overlap were included in the study group after obtaining an informed consent. To ascertain whether oxidative stress was unique to TEN, patients with a maculopapular drug rash without detachment of skin were included in the control group 1 (drug exposed). Patients undergoing minor dermatosurgical procedures and without a drug-induced rash were included in control group 2 (drug unexposed) and their serum was used for biochemical analysis. Patients who did not wish to be included in the study and patients with human immunodeficiency viral infection, hepatitis B and hepatitis C were excluded from the study as these infections are known to induce oxidative stress in tissue.

Protein carbonyls and lipid peroxidation parameters such as malondialdehyde and conjugated dienes and oxidative stress markers, levels of nitrate and nitrite, stable end products of NO were estimated in the serum from all patients. The alterations in protein carbonyl content and lipid peroxidation parameters, serum nitrite/nitrate levels, active metabolites of NO were compared between the groups. The study was approved by the institutional review board.

*Measurement of protein carbonyls in serum:* Samples were treated with 10mM 2,4-dinitrophenyl hydrazine dissolved in 2N HCl, followed by incubation at room temperature for 1 hour. Trichloroacetic acid (10% final concentration) was then added, followed by centrifugation to pellet the precipitated protein. To this pellet, an equal volume of 1:1 (v/v) ethyl acetate: Ethanol was added. Following centrifugation, the pellet was collected and dissolved in 1 ml of 6M guanidine HCl. Absorbance at 366 nm was then measured and was expressed as nmoles/ml serum.

*Measurement of malondialdehyde and conjugated dienes:* For measurement of malondialdehyde, samples (200ul)

were treated with 1.2 ml of 5% TCA and 0.4ml of 0.8% thiobarbituric acid. The samples were incubated at 80°C for 1.5 hours. The absorbance was then read at 532 nm, and amount of malondialdehyde formed was calculated from a standard curve prepared using 1, 19, 3, 39 tetramethoxypropane and was expressed as nmoles/ml serum. For conjugated diene measurement, total lipids were extracted from serum, dissolved in 1 ml heptane, and the absorbance was read at 233 nm in a spectrophotometer. Concentration was calculated using a molar absorption coefficient of  $2.52 \times 10^4$  and expressed as  $\mu$  mol/ml serum.

*Measurement of nitrite/nitrate:* For measurement of nitrite, nitrate in the samples was first reduced to nitrite, which was then measured by the Griess reaction. Reduction of nitrate to nitrite was carried out using a copper-cadmium alloy, which was prepared by mixing molten copper with cadmium in the ratio 1:9. Filings of the alloy were then prepared, followed by activation as follows: The copper-cadmium alloy filings were washed twice with 100 ml of de-ionized water, followed by two washes with 0.5N HCl. The activated filings were then washed with 0.1N HCl and stored in 0.1N HCl at 2–8°C until use. For analysis of nitrate/nitrite, samples were incubated with the alloy filings in carbonate buffer for 1 h at room temperature with shaking. The reaction was stopped by addition of 0.35M NaOH and 120mM ZnSO<sub>4</sub> solution, followed by vortexing. After standing for 10 minutes, the samples were centrifuged at 4000 g for 10 minutes. Aliquots of the clear supernatant are treated with 1% sulfanilamide and 0.1% N-naphthylethylenediamine. After 10 min, the optical density was read at 545 nm in a spectrophotometer.

## Statistical analysis

Two-group comparison with the non-parametric Mann-Whitney U test was used to compare the values in the groups. Significance of differences if any was established using Pearson's Chi-square test. SPSS Version 12 for Windows was used for all statistical analyses.

## Results

Fourteen patients with SJS/TEN (study group) and 10 patients with a maculopapular drug rash (control group 1) were included in the study. Blood samples of 10 patients who underwent minor dermatosurgery were taken as control. Four patients from the study group, two patients from control group 1 and three patients from control group 2 were excluded from the study as their blood samples were hemolysed. The study patients consisted of 10 patients in the SJS-TEN group (study group), 8 patients in control group 1 and 7 patients in control group 2. Figure 1 describes the case selection in a flow chart. Majority

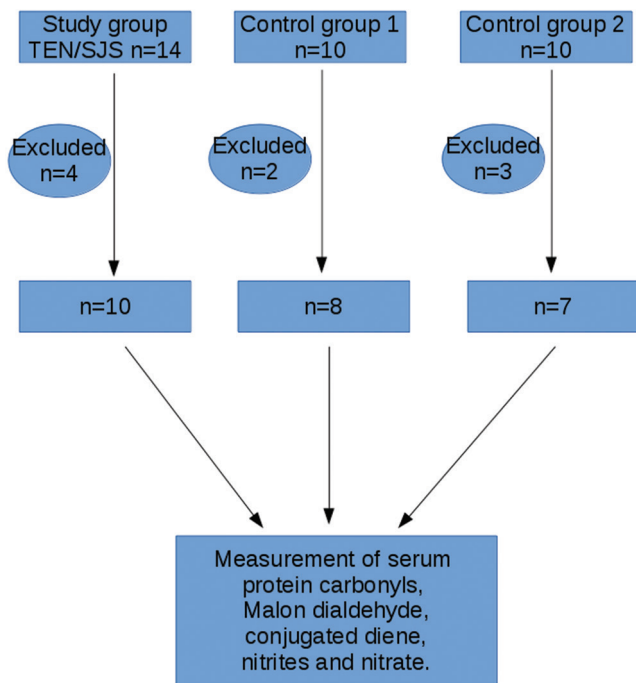


Figure 1: Flow chart of case selection

of patients in the study group (M: F 1:3) as well as in the control group 1 (M: F, 3:4) were females. More than one drug was implicated in 4/10 patients in group 1 and 3/8 patients in group 2. Carbamazepine was one of the most common drug implicated in 4/10 of study group and 2/8 of the control group patients. The drugs implicated in causing the TEN, SJS or drug rash is listed in Table 1. SCORTEN of 0, 1 and 3 at admission were seen in 2, 6 and 2 patients of the study group, respectively. Four patients had systemic steroid administration prior to sample collection.

The mean values of protein carbonyls, conjugated dienes, malondialdehyde and nitrate levels of all the patients included in the study is shown in Table 2. The mean value of malondialdehyde in the control group 2 was elevated as compared to the study group and control group 1. Two-group comparison with non-parametric Mann-Whitney U test showed that there is no statistically significant difference between the serum conjugated diene, nitrates, malondialdehyde and protein carbonyls between the TEN group and the control groups.

## Discussion

Toxic epidermal necrolysis and SJS are characterized by the rapid onset of keratinocyte cell death by apoptosis, a process that results in the separation of the epidermis from the dermis. An immune response to defective drug metabolism has been implicated in the process and studies examining apoptotic pathways in this context have identified interaction between Fas and FasL playing a role.<sup>[8]</sup> Other studies have shown soluble FasL (sFasL)

Table 1: Drugs implicated in the study and control group

Implicated drugs	Study Group (TEN, SJS) no.	Control Group 1 (drug exposed) no.
Carbamazepine	4	2
Phenytoin	0	3
Ciprofloxacin	2	0
Gentamicin	2	0
Diclofenac sodium	2	0
Paracetamol	2	0
Allopurinol	1	1
Pantoprazole	1	1
Dapsone	0	1
Brufen	0	1
Mefenemic acid	1	0
Amoxycillin	0	1
Penicillamine	1	0

TEN: Toxic epidermal necrolysis, SJS: Stevens Johnson syndrome

Table 2: Demography and mean biochemical parameter in each group

	Study (TEN and SJS)	Control Group 1 (drug exposed)	Control Group 2 (drug unexposed)
Number	10	8	7
Males	4	3	4
>1 drug implicated	4	3	NA
SCORTEN of 0,1,2,3	2,6,0,2	NA	NA
Protein carbonyl (nmoles/ml serum) (mean±SD)	93.2±43	124±36.6	111±36
Conjugated diene (µmoles/ml serum) (mean±SD)	3±0.9	3.6±0.7	4±0.5
Malondialdehyde (nmoles/ml serum) (mean±SD)	6.5±4.3	5.6±3	8.4±7.3
Nitrate (nmoles/ml serum) (mean±SD)	40.4±40.4	56.6±32	53.9±9

TEN: Toxic epidermal necrolysis, SJS: Stevens Johnson syndrome, SD: Standard deviation

secreted by peripheral blood mononuclear cells (PBMCs) and perforin and granzyme as playing a major role in apoptosis in SJS and TEN.<sup>[9-11]</sup> A recent study has shown secretory granulysin produced by the fully activated cytotoxic T lymphocytes (CTLs), natural killer (NK) cells and natural killer T (NK T) cells leads to widespread apoptosis in SJS-TEN.<sup>[5]</sup> Various new markers demonstrated in TEN include serum granulysin, high-mobility group box protein 1 (HMGB1), alpha defensins 1-3, thymus and activation regulated chemokine (TARC), Bcl-2 expression in dermal infiltrate and glutathione-S-transferase pi expression in keratinocytes.<sup>[12]</sup>

Another molecule which could play a role in apoptosis is NO whose role in cellular signaling is now well recognized. Low concentrations of NO generally have a beneficial effect in cellular systems, protecting against cell death and high NO concentrations can damage cellular components and induce apoptosis. The effects of NO on induction of apoptosis in keratinocytes have also been shown to be influenced by free radicals such as superoxide.<sup>[13]</sup> Toxic effects of NO are generally mediated through formation of reactive nitrogen species such as peroxynitrite, which can be formed in a redox environment with high NO concentrations.<sup>[14]</sup> NO is produced by one of the three isoenzymes of nitric oxide synthase (NOS). Activity of inducible form of NOS (iNOS) is independent of the calcium concentration and has the capacity to produce large quantities of NO. Expression of iNOS has been demonstrated in the lesional skin of patients with SJS and TEN.<sup>[15]</sup> It has been shown that activated T cells secrete high amounts of TNF- $\alpha$  and IFN- $\gamma$ , and that both cytokines lead to increased expression and activity of keratinocyte iNOS. The resulting increase in NO significantly upregulates keratinocyte FasL expression, resulting in Fas- and caspase-8-mediated keratinocyte cell death.<sup>[16]</sup>

It was found that activity of the anti-oxidant enzyme superoxide dismutase (SOD) was increased in all types of non-immediate reactions (including urticaria, maculopapular exanthema and toxic epidermal necrolysis), accompanied by elevations in lipid peroxidation in urticaria and maculopapular exanthema, and carbonyl groups in all types of reactions.<sup>[17]</sup> However, in the present study, the serum values of protein carbonyls, malondialdehyde, conjugated diene and nitrates were not significantly increased in the study group when compared to the controls. The biochemical values of patients who have received steroids were not different from the values of other patients; hence, exposure to steroids did not seem to have altered the values. The malondialdehyde levels in the control group were higher than the study group and control group 1. Studies have shown oxidative stress markers can be elevated both during surgery and in the immediate post-operative period.<sup>[18]</sup> This may be the reason for elevation of malondialdehyde in the control group 2. Verma *et al.* in their study on oxidative stress markers in cutaneous adverse drug reaction found that the mean malondialdehyde levels were raised in the study group as compared to the control group.<sup>[19]</sup> Fifteen percent of the study group were patients with SJS. Our study did not show elevated levels of protein carbonyl, conjugated dienes, malondialdehyde and nitrates in patients with SJS and TEN. As this was a pilot study with less number of patients, a definitive conclusion could not be drawn from the results of this study. Tissue levels of protein carbonyls, malondialdehyde, conjugated dienes and

nitrates would have given a better understanding of oxidative stress. Since a recent study demonstrated that treatment with the anti-oxidant *N*-acetyl cysteine, or its combination with infliximab, did not appear to reverse the evolving TEN process the role of oxidative stress in conditions such as TEN may be complex and need further detailed evaluation.<sup>[20]</sup> In our study population, oxidative stress markers were not elevated, hence a role of antioxidants in the treatment of TEN cannot be proposed. Studies with a larger group of patients examining alterations in skin biopsies may be required to ascertain the role of oxidative and nitrosative stress in pathophysiology of TEN and SJS.

## Conclusion

Immunopathogenesis of TEN is complex, the end result of which is apoptosis of keratinocyte. Oxidative stress may contribute to keratinocyte apoptosis. Our study could not demonstrate an elevation of oxidative stress markers in patients with TEN and SJS as compared to the control population.

### What is new?

In our pilot study, serum oxidative stress markers in patients with SJS and TEN were not elevated as compared to the controls. Other pathogenic markers or molecules need to be studied as an inducer of keratinocyte apoptosis in TEN.

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