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Altered Tregs and oxidative stress in pregnancy associated lupus

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Abstract

Aim: SLE is a systemic autoimmune disease generally affecting woman in the reproductive age. It is associated with an altered level of Tregs and oxidative stress while an increase in Tregs, and different antioxidant mechanisms to combat oxidative stress are essential for successful pregnancy. Hence, this study aims to determine the level of $CD4^+$ and $CD8^+$ Tregs and oxidative stress in pregnant lupus patients.

Methods: Ten healthy and 10 pregnant lupus volunteers from the North Indian population, within the age group of 20–30 years were enrolled in the study. All the patients were non-smokers, non-alcoholics and were not associated or undergoing therapy for any other disease. They had a SLEDAI of 37.4 ± 7.32 with 5.2 ± 1.93 years of disease duration. Oxidative stress was determined by measuring the enzyme activity of anti-oxidant enzymes (catalase, superoxide dismutase and glutathione peroxidase) and the level of reduced glutathione and lipids peroxidised, spectrophotometrically. Flowcytometry was performed for immunophenotyping to determine $CD8^+$ and $CD4^+$ Tregs.

Results: Elevated $CD8^+$ Tregs and diminished $CD4^+$ Tregs were observed in pregnant lupus patients. Oxidative stress was significantly increased as the activities of anti-oxidant enzymes and level of reduced glutathione was considerably diminished. There was a substantial increase in the amount of lipids peroxidised.

Conclusion: Pregnant lupus patients undergo considerable level of oxidative stress in comparison to healthy pregnant woman. The decreased level of $CD4^+$ Tregs and an increase in $CD8^+$ Tregs might be another important factor responsible for pregnancy associated complications. Hence, lupus leads to alterations in the necessary conditions for a successful pregnancy, which might eventually cause higher mortality, morbidity and associated complications.

Keywords: Systemic lupus erythematosus, Oxidative stress, Pregnancy, $CD4^+$ Tregs, $CD8^+$ Tregs

Introduction

Systemic autoimmunity is primarily characterized by the loss of immunological tolerance and inability of the immune system to discriminate self antigens from non self antigens, hence rendering it a complex disease process. Systemic lupus erythematosus (SLE) is one such autoimmune disease characterized by the presence of high titer of auto-antibodies against nuclear antigens [1]. These auto-antibodies are produced against a broad range of antigens and as a consequence the manifestations of the disease are diverse. Wide arrays of factors are responsible for its etiology including genetic, hormonal and environmental triggers but the fundamental molecular mechanisms behind this systemic autoimmune response remain primarily indefinite. It involves multiple organ failure and is also harmful to the fetus during pregnancy. SLE mainly affects women in their reproductive years. Pregnancy in lupus patients often leads to higher maternal and fetal mortality, morbidity, pre-eclampsia and disease flares [2]. Higher risk of fetal loss, pre-term birth [3], intra-uterine growth restriction and neonatal lupus syndromes are major fetal complications during pregnancy in SLE patients [4].

A key issue in the pathogenesis of lupus is how intracellular antigens become exposed and targeted by the immune system. In this regard, excessive production of reactive oxygen species (ROS), altered redox state [5]

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and a defect in regulation of apoptosis [6] are considered as imperative factors. Despite the diverse clinical features, imbalance in oxidative state is considered to be a key feature in the development of SLE [7, 8]. Oxidative stress alters serum proteins, eventually leading to the establishment of autoimmunity and organ damage associated with lupus [9].

ROS target double bonds in polyunsaturated fatty acids of the cell membrane leading to lipid peroxidation (LPO) causing the associated complications of oxidative damage in SLE [5] Intracellular depletion of glutathione (GSH) levels are also one of the reason for the formation of ROS which causes oxidative damage and may be involved in deregulation of apoptosis in lupus. Delayed clearance of apoptotic cells may prolong interaction between ROS and apoptotic cells generating neoepitopes. Autoantibody are formed against these neoepitopes as well, eventually leading to the generation of a wide array of antibodies which causes tissue damage [9]. An increase in Malondialdehyde (MDA: a byproduct of lipid peroxidation), anti-superoxide dismutase (SOD) and anti-catalase (CAT) antibodies in the sera of SLE patients support a critical role for oxidative stress in disease development and progression. The positive relationships between oxidative stress markers and apoptosis marks the implications of oxidative stress in lupus [10].

Along with oxidative stress there has been growing evidence suggesting that infiltration of T-lymphocytes and other leukocytes into the sites of inflammation play a critical role in organ involvement during autoimmune diseases [11]. The altered functions of lymphocytes are a hallmark of SLE. T cells have been recognized to be crucial in the pathogenicity of SLE through their capabilities to communicate with and offer enormous help to B cells for driving autoantibody production [12]. Considerable attempts are focused on understanding the process by which self-reactive lymphocytes escape tolerance and induce autoimmune diseases. While it is well established that $CD4^+$ helper T cells play an important role in the process of B cell activation during antibody-mediated autoimmunity and in cell-mediated disease, the role of Tregs also holds enormous significance.

The aim of the study was to explore the state of oxidative stress, $CD4^+$ Tregs and $CD8^+$ Tregs in SLE, which may have further implications in better understanding of pathology of lupus and in the therapeutic management of the disease.

Materials and methods

Participants

Patients enrolled in the study were from Gynecology/Obstetrics Out-Patient Department of Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. The parameters considered for this study have been approved by Institutional Ethical Clearance Committee, PGIMER, Chandigarh (PGI/IEC/2015/911). A total of twenty female volunteers were enrolled in the study, within the age group of 20–30 years and a mean age 28.1 ± 2.33 years. Of the total twenty, ten were healthy pregnant females and remaining were pregnant females with SLE. Disease activity of SLE patients was determined using SLE Disease Activity Index (SLEDAI) score [13] (maximum score of 105; severe score > 20; moderate score 10–20; mild score < 10). All the patients enrolled in the present study were non-smokers and non-alcoholics, not associated with any other autoimmune disease and were not undergoing therapy for any other disease. The patients were allowed to continue their drug regimens during the period of study. The Laboratory findings of pregnant SLE patients and pregnant healthy controls are listed in Table 1.

Blood samples

Venous blood samples were obtained from patients and controls. Samples were collected into heparinized vacutainers (Becton Dickinson, USA). Heparinized blood samples were used for separation of plasma for the estimation of reduced glutathione (GSH), LPO, and antioxidant enzymes SOD, CAT and glutathione peroxidase (GPx) and for the isolation of peripheral blood mononuclear cells (PBMCs) for T cell profiling.

Cell surface staining

PBMCs were isolated using red blood cell (RBC) lysis buffer. T-lymphocytes in the PBMCs were labelled with antibodies against specific cell surface markers for immunophenotyping. CD3 FITC, CD8 PE-Cy5, CD4 FITC and CD25 PE (BD Biosciences) antibodies were added to the cell suspension and incubated in dark at room temperature for 45 min. Cells were washed and acquired on flowcytometer (BD FACS LSR, BD Biosciences, USA) and analysed using FACSDiva 6.1.3 software (BD Biosciences, USA).

Table 1 Demographic characteristics of pregnant SLE patients and pregnant healthy controls

Parameters	SLE patients	Controls
Age	28.1 ± 2.33	25.5 ± 0.84
Month of pregnancy	5.4 ± 3.02	5.1 ± 2.18
Duration of disease (years)	5.2 ± 1.93	NA
ESR	29 ± 4.11***	16.4 ± 5.05
ANA (positive/negative)	9/1	NA
dsDNA (positive/negative)	9/1	NA
SLEDAI score	37.4 ± 7.32	NA

***Erythrocyte sedimentation rate (ESR) of SLE patients was significantly higher than control ($p < 0.001$). 90% of the SLE patients enrolled in the study were positive for anti-nuclear antibodies (ANA) and ds-DNA antibodies, with high Systemic Lupus Erythematosus Disease activity Index (SLEDAI)

Biochemical analysis

Protein estimation

Plasma protein was estimated by the method of Lowry [14] using bovine serum albumin as standard. The protein concentration was determined by comparing the optical density of the test sera with the standard plot and the result was expressed as mg/ml.

Lipid peroxidation

Lipid peroxidation was quantified in the plasma samples by measuring the levels of a secondary product of lipid peroxidation, malondialdehyde (MDA) [15]. MDA thiobarbituric acid adducts formed were measured spectrophotometrically at 532 nm. The results were expressed as nmol MDA/mg protein using molar extinction coefficient of MDA–thiobarbituric chromophore ($1.56 \times 10^5\ M^{-1}\ cm^{-1}$).

Determination of SOD activity

The method is based on the determination of the rate of reduction of nitroblue tetrazolium (NBT) to blue formazan dye in the plasma [16]. The decrease in absorbance was followed for 3 min at 560 nm. The enzyme activity was expressed as Units (U)/mg protein, where one unit of enzyme activity was defined as the amount of enzyme required to inhibit the rate of formazan formation by 50%.

Determination of CAT activity

Catalase activity was evaluated in the plasma samples and the reaction analysed the decrease in absorbance at 240 nm for 3 min [17]. Results were expressed as amount of hydrogen peroxide (H_2O_2) decomposed per min/mg protein using molar extinction coefficient of H_2O_2 ($71\ M^{-1}\ cm^{-1}$).

Determination of GSH levels

GSH was measured determining the yellow coloured complex formed by the conversion of 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB) to 2-nitro-5-mercaptobenzoic acid in the plasma, which was measured spectrophotomterically at 412 nm [18]. The concentration was calculated against glutathione standard and was expressed as nmol GSH/mg protein.

Determination of GPx activity

GPx activity was assayed in the plasma samples. The assay determines the decrease in absorbance of the reaction mixture at 340 nm for 3 min [19]. Results were expressed as nmoles of NADPH oxidized per min per mg of protein, using molar extinction coefficient of NADPH ($6.22 \times 10^3\ M^{-1}\ cm^{-1}$).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (Graphpad Software version 5.01, San Diego, USA). Values were expressed as Mean ± SD. Two tailed Student's t-test was used to determine statistical difference between the groups. The p value of 0.05 or less was considered significant.

Results

Demographic profile of subjects

The clinical and demographic characteristics of pregnant patients with SLE and healthy controls are stated in Table 1. 4 pregnant SLE patients were found to have severe SLEDAI score while 6 patients were in the moderate score category. The erythrocytes sedimentation rate (ESR) was significantly higher in pregnant SLE patients in comparison to healthy pregnant individuals (Fig. 1). The patients suffered from general manifestations like fatigue, headache; skin manifestations like alopecia, photosensitivity; nausea and vomiting, muscle weakness, lachrymal gland enlargement, severe manifestations like thrombocytopenia, etc. Previous history of child loss due to heart block was also reported in a patient. The SLE patients were being treated with low dose glucocorticosteroids (60%), non-steroidal anti-inflammatory drugs (NSAIDs) (25%), hydroxychloroquine (52%) and methotrexate and cyclophosphamide (15%).

T cell profiling

The percentage of $CD3^+CD8^+$ cells and $CD3^+CD8^+CD25^+$ cells were assessed in both the groups. The percentage of $CD3^+CD8^+$ cells and $CD3^+CD8^+CD25^+$ cells increased remarkably in SLE patients (43.85 ± 9.58% and 56.21 ± 2.96%) in comparison to the control group (23 ± 5.85% and 27.45 ± 1.06% respectively) (p value = 0.0023) Fig. 2.

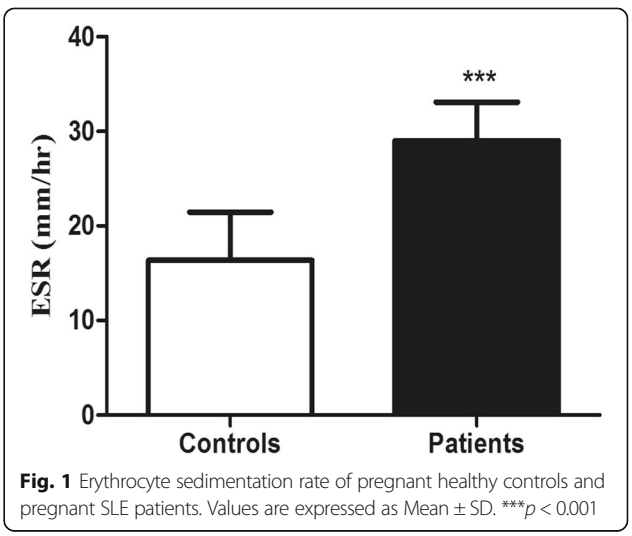

Fig. 1 Erythrocyte sedimentation rate of pregnant healthy controls and pregnant SLE patients. Values are expressed as Mean ± SD. ***$p < 0.001$

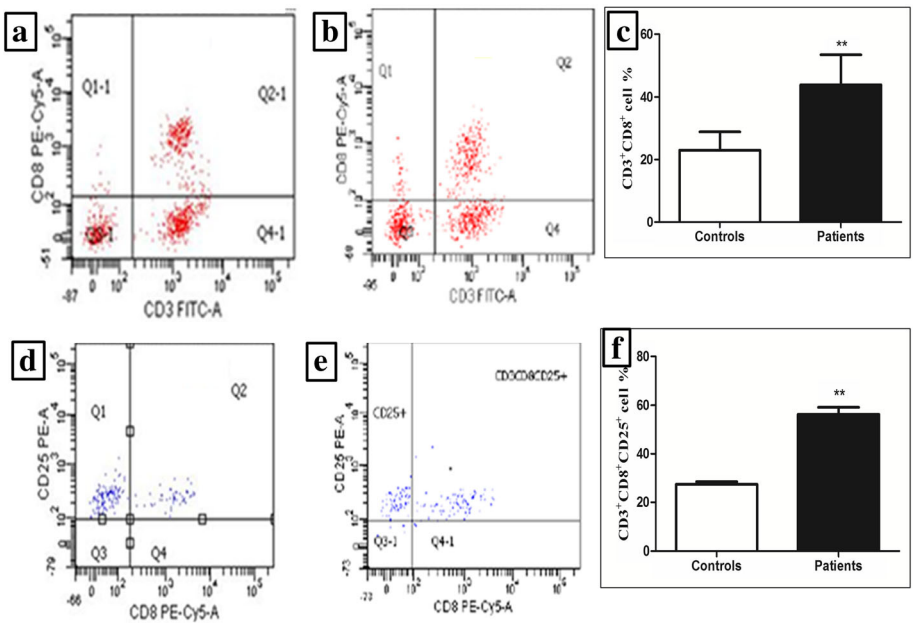

Fig. 2 a Dot plot of $CD3^+CD8^+$ T cells in controls (**b**) Dot plot of $CD3^+CD8^+$ T cells in patients (**c**) Statistical analysis of $CD3^+CD8^+$ T cells in controls and patients (**d**) Dot plot of $CD3^+CD8^+CD25^+$ T cells in controls (**e**) Dot plot of $CD3^+CD8^+CD25^+$ T cells in patients (**f**) Statistical analysis of $CD3^+CD8^+CD25^+$ T cells in controls and patients. All values are calculated as Mean ± SD. $^{**}p < 0.01$

Unlike the $CD3^+CD8^+$ T-cells levels, the level of $CD3^+CD4^+$ T-cells were significantly lower in pregnant SLE patient (15.75 ± 1.89%) in comparison to healthy pregnant women (26.36 ± 2.62%). Diminished levels of $CD3^+CD4^+CD25^+$ T-cells were observed in pregnant lupus patients as compared to healthy controls. The $CD3^+CD4^+CD25^+$ T cell levels in pregnant lupus patients were 15.39 ± 1.94% which was remarkably lower than healthy controls 27.43 ± 1.74% (p value = 0.0002) Fig. 3.

Plasma protein estimation

Plasma protein levels were determined as the plasma of pregnant lupus patients and compared to healthy pregnant controls. A significant ($p = 0.0003$) elevation was seen in the level of proteins in case of SLE patients (5.247 ± 0.38 mg/ml) as compared to controls (3.694 ± 0.086 mg/ml) Fig. 4a.

Estimation of oxidative stress markers

It has been found that the activities of antioxidant enzymes such as SOD, CAT, GPx and the level of GSH are significantly reduced in patients with SLE as compared to controls. SOD is an antioxidant enzyme that plays role in neutralizing superoxide radicals (O_2^-). SOD activity was assessed in both controls and patients and as shown in Fig. 4b, it was found that SOD activity in patients was 23.29 ± 1.602 U/mg protein whereas, in control samples the SOD activity was found to be 43.16 ± 2.260 U/mg protein. This clearly demonstrated diminished SOD activity in patients as compared to control. When the data was analysed statistically, the p value was found to be 0.0001 which depicts a significant difference between both the values.

Catalase is another antioxidant enzyme that is responsible for scavenging H_2O_2 from cellular systems and thus protecting against cellular damage. In the study control samples showed a specific activity of 4.34 ± 0.73 U/mg protein. On the other hand, in SLE patients the specific activity was found to be 1.64 ± 0.08 U/mg protein. Figure 4c shows a significant decrease in the catalase activity in patients as compared to controls with a p value of 0.0001.

GPx activity was measured in both controls as well as patient samples and as shown in Fig. 4d SLE patients showed an enzyme activity of 2.73 ± 0.01 U/mg protein whereas in control samples the enzyme activity was measured to be 6.24 ± 0.23 U/mg protein. Statistical analysis of the data revealed significant decrease in enzyme activity in SLE patients with a p value of 0.0015.

GSH is an antioxidant molecule which protects the body from oxidative damage by neutralizing free radicals produced during biological processes. As shown in Fig. 5a it was found that the amount of GSH levels in SLE patients was significantly reduced (9.87 ± 0.38 nmoles/mg protein) as compared to GSH levels in control samples (18.28 ± 0.67 nmoles/mg protein), with a p value of 0.0001.

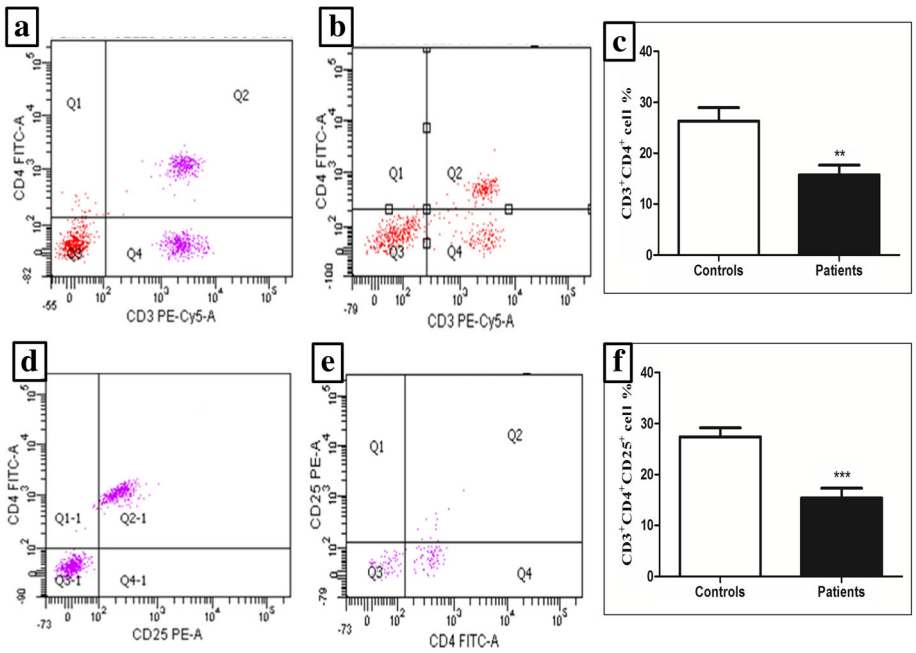

Fig. 3 a Dot plot of $CD3^+CD4^+$ T cells in controls (**b**) Dot plot of $CD3^+CD4^+$ T cells in patients (**c**) Statistical analysis of $CD3^+CD4^+$ T cells in controls and patients (**d**) Dot plot of $CD3^+CD4^+CD25^+$ T cells in controls (**e**) Dot plot of $CD3^+CD4^+CD25^+$ T cells in patients (**f**) Statistical analysis of $CD3^+CD4^+CD25^+$ T cells in controls and patients. All values are calculated as Mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$

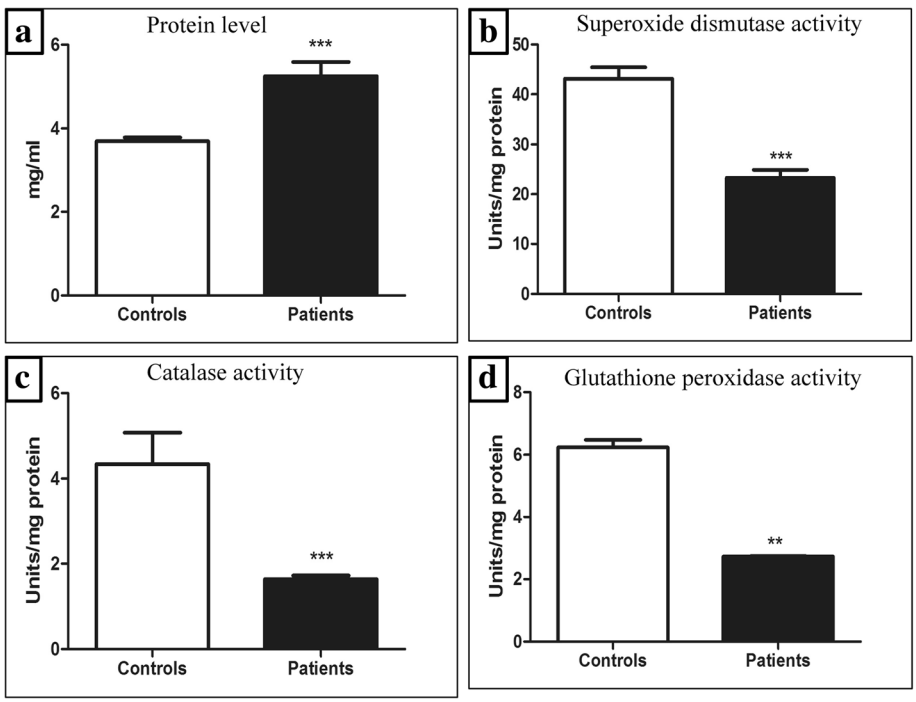

Fig. 4 Statistical analysis of (**a**) protein levels (**b**) activity of superoxide dismutase (**c**) activity of catalase (**d**) activity of glutathione peroxidase in plasma sample of controls and patients. All values are calculated as Mean ± SD. $^{**}p < 0.01$; $^{***}p < 0.001$

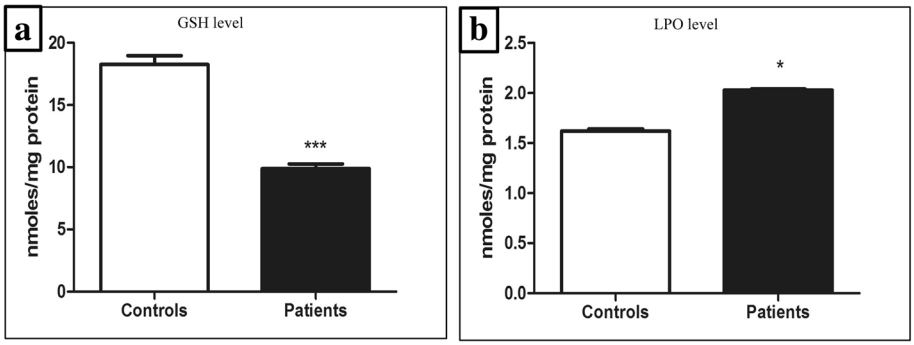

Fig. 5 Statistical analysis of the amount of (**a**) reduced glutathione (**b**) lipid peroxidation in plasma samples of controls and patients. All values are calculated as Mean ± SD. *$p < 0.05$; ***$p < 0.001$

The extent of lipid peroxidation was assessed by measuring the amount of MDA-thiobarbituric acid adduct formation. SLE patients showed MDA level of 2.03 ± 0.01 nmoles/mg protein whereas the controls measured 1.62 ± 0.02 nmoles/mg protein. As shown in Fig. 5b, the amount of MDA production in SLE patients is significantly higher than the amount of MDA production in controls with a p value of 0.0112.

Discussion

This study led to some intriguing observations which enhanced the understanding of the role of oxidative stress, and Treg cells in the pathogenesis of pregnancy associated systemic lupus erythematosus. Flow cytometry of labelled PBMCs in pregnant lupus patients showed diminished levels of $CD3^+CD4^+CD25^+$ Treg cells and a significant elevation of $CD3^+CD8^+CD25^+$ Tregs cells in comparison to healthy pregnant women.

Treg cells are regulatory cells which play a pivotal role in the maintenance of immune tolerance regardless of their origin [20]. $CD4^+CD25^+$ Treg cells have been implicated in peripheral tolerance and regulation of autoimmune response [21]. Their reduced levels in SLE have been reported and it has been documented that these reduced levels may be responsible for the loss of self-tolerance and generation of autoimmune response [22]. On the other hand a successful pregnancy has to ensure fetal tolerance for which Tregs are hastily employed to the uterus-draining lymph nodes for successful implantation on the embryo [23]. A decrease in the levels of Tregs may be associated with loss of fetal tolerance and a pre-term cessation of pregnancy [24]. Results from our study state that pregnant women with lupus are at a high risk of loss of fetal tolerance due to depletion of $CD4^+CD25^+$ Tregs.

While Tregs have garnered much thought for their role in the preservation of immune homeostasis, studies have shown that subset of cells called $CD8^+$ Tregs also play immune regulatory functions [25]. These cells are known for their role in preventing autoantibody production and decreasing accumulation of autoimmune cells in organ that face prime manifestations in lupus [26]. On the other hand, during pregnancy human placental trophoblasts recruit $CD8^+$ Tregs to prevent fetal rejection [27] and pregnancy is associated with an expansion in these cells. Our study witnessed a significant increase in the level of $CD8^+CD25^+$ Tregs in pregnant lupus patients in comparison to healthy pregnant women. The altered interleukin-2 profile during pregnancy activates $CD8^+$ Tregs more than $CD4^+$ Tregs [28], hence $CD8^+$ Tregs might be experiencing higher level of stimulation than $CD4^+$ Tregs to maintain pregnancy. The increase in their number can also be associated with the fact that these cells have the role of complementing the function of $CD4^+$ Tregs [29], which are low in number hence, the number of T suppressor cells increases to compensate this decrease to allow a successful pregnancy.

Oxidative stress is increased in systemic lupus erythematosus (SLE), and it contributes to immune system dysregulation, abnormal activation and processing of cell-death signals, autoantibody production and fatal co morbidities. Oxidative modification of self antigens triggers autoimmunity, and the degree of such modification of plasma proteins shows striking correlation with disease activity and organ damage in SLE [9]. In this study the activities of antioxidant enzymes- catalase, superoxide dismutase, glutathione peroxidase, the amount of antioxidant molecule reduced glutathione and the extent of lipid peroxidation was measured. A significant decrease in the activity of CAT, SOD and GPx was observed in SLE patients as compared to controls. In addition to this, our results show significantly increased levels of lipid peroxidation in SLE patients. Lipid peroxidation in mitochondrial, lysosomal and cell membranes by ROI generates reactive aldehydes including malondialdehyde and 4- hydroxy-2-nonenal (HNE), which can spread oxidative damage through circulation. MDA and HNE, two major lipid peroxidation products, have

extensively been used as the biomarkers of oxidative stress [30]. These products are highly reactive and can form adduct with proteins, making them highly immunogenic. Increased formation and subsequent accumulation of such aldehyde-modified protein adducts were found in various pathological states including autoimmune diseases like SLE [30]. Lipid peroxidation is associated with several pregnancy complications like preeclamsia [31]. Compiling these observations it can be stated that lupus is associated with increase in lipid peroxidation, hence, the pregnancy pathogenesis associated with lupus may also stem from the increase in lipid peroxidation.

Our observations also witnessed a decrease in enzyme activity of antioxidant enzymes like catalase, superoxide dismutase and glutathione peroxidase. Normal pregnancy is marked by an increase in oxidative state but this state is balanced by the increase in the enzyme activity of anti-oxidant enzymes for successful pregnancy [32]. As oxidative stress is associated with miscarriages and preeclampsia, it is very essential to combat oxidative stress [33]. On the other hand lupus is associated with a decrease in enzyme activities of catalase, superoxide dismutase, glutathione peroxidase and levels of reduced glutathione [34]. Pregnant lupus patients in our study manifested an increase in oxidative stress and the inability of the antioxidant defense mechanisms to combat this increased stress. These manifestations state that the various pregnancy complications like preeclampsia and miscarriages associated with lupus associated pregnancy may be due the inability of the anti-oxidative system to combat the increase in oxidative stress.

The role of oxidative stress and Tregs in lupus pathogenesis has been extensively researched. Findings suggest that lupus is associated with an increase in oxidative stress [35] and the associated mitochondrial hyperpolarisation [36]. The activation, proliferation and autophagy are T-cell is influenced by the state of ROS and ATP, which in turn is regulated by mitochondrial membrane potential [37]. Mitochondrial hyperpolarisation leads to a state of ATP depletion, which is sensed by FKBP12-rapamycin associated protein (FRAP, also known as mTOR or RAFT). mTOR is a member of phosphatidylinositol kinase-related kinase family and functions as a sensor to altered energy homeostasis and leads to the autophagy of T-cells during ATP depletion [38]. A similar state of increased ROS, mitochondrial hyperpolarisation, ATP depletion and T-cell apoptosis and necrosis has been reported in lupus patients [39].

Therapeutic interventions to regulate the altered state of oxidative stress and the associated T-cell autophagy in lupus have been given paramount interest by scientists. Studies have shown that the use of N-acetyl cysteine helps in reducing disease activity in lupus by inhibitor mTOR in T-cell [40]. NAC is a precursor of GSH and is hence a potent antioxidant whose potential has not only been proven in lupus but in idiopathic pulmonary fibrosis as well [41]. Another trial determining the effect of 6–15 ng/mL of sirolimus (Rapamycin) for 12 months on lupus patients documented an improvement in the state of T-cells [42]. Rapamycin is another mTOR inhibitor and has shown to improve the number of Tregs in lupus patients but this trial also manifested side effects such as nausea and infections [43].

Though the present finding has reported an increase in oxidative stress and a deregulating in the number of Tregs, but extending the above mentioned treatment regimens is another challenge. The beneficial effects of such treatments can only be extended to pregnant lupus patients after a thorough examination in pre clinical studies. Generating a suitable mouse model which replicates these manifestations is a challenge.

A remarkable elevation in the plasma protein concentrations were observed in pregnant SLE patients as compared to healthy controls. A high level of protein concentration, owing to the increase in interleukins, antibodies, Matrix metalloproteins, etc. has been reported earlier. Their association with organ damage has also been documented [44]. A similar finding in pregnant lupus patients hints to an increase in susceptibility to different organ complications, particularly to lupus nephritis.

Conclusion

Compiling the findings of this study it can be concluded that pregnancy associated with lupus is marked by an increase in plasma proteins, $CD8^+CD25^+$ Tregs, oxidative stress and a decrease in $CD4^+CD25^+$ Tregs. The two essential conditions for safe pregnancy i.e., low oxidative stress and an altered $CD4^+CD25^+$ Tregs are breached in pregnant lupus patients. Therefore, the patients are at a high risk of developing the associated complications i.e. preeclampsia and miscarriages. These findings state that there is potential for therapies targeting oxidative stress and Tregs in ameliorating the lupus associated complications in pregnancy.

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Authors' contributions

NP and AB have designed the work plan, RS and SS have executed the work, SC has provided the required samples and NP, RS and AB have drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The authors confirm that the data supporting the findings of this study are available within the article.

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the Institutional Ethical Clearance Committee of PGIMER, Chandigarh, India with the ethical clearance letter number of PGI/IEC/2015/911 and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication
Consent to publish was also obtained from all the participants included in the study.
Informed consent: An informed consent was obtained from all the participants included in the study.

Competing interests
The authors declare that they have no competing interests.

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