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Research Article

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Autoantibodies Against ROS-Human Serum Albumin-A Potent Immunological marker in Depressed Individuals with Smoking History

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Abstract

***Background**: Depression is one of the significant problems in adults that accounts for up to five percent of cases worldwide. **Methods**: Volunteers were divided into eight groups, and their serum samples were tested for FBG, carbonyl contents, IFN-γ and TNF-α. Reactive oxygen species (ROS) modified human serum albumin (HSA) (ROS-HSA) was used as an antigen and levels of serum autoantibodies were estimated by direct binding and inhibition ELISA in all subjects. **Results**: Significant biophysical structural modifications were observed in ROS-HSA with increased carbonyl contents compared to native-HSA (N-HSA). Significantly high levels of carbonyl content (2.68 ± 0.33 nmol/mg protein; $p > 0.001$) and pro-inflammatory cytokines IFN-γ (7.4 ± 0.61 pg/ml; $p > 0.001$) and TNF-α (1.47 ± 0.23 pg/ml; $p > 0.001$) were detected in serum samples from F-D-S. Similarly, a high level of autoantibodies against ROS-HSA was observed in females who were depressed and smokers (F-D-S) group (0.89 ± 0.07; $p > 0.001$) compared to males who were both depressed and smokers (M-D-S) (0.66 ± 0.049). Furthermore, inhibition ELISA results exhibited high recognition of serum autoantibodies from F-D-S subjects (78.6 ± 5.7 mean maximum percentage inhibition MMPI) compared to M-D-S (58.8 ± 5.2 MMPI) subjects. **Conclusion**: Incoherence, long term unchecked chronic psychological stress may cause oxidation of blood proteins, which subsequently result in structural alterations of biomolecules, thus generating new-epitopes, capable of inducing autoantibodies specific for ROS-modified proteins. These autoantibodies may be a potential marker for subjects suffering from depression to understand the state of immune imbalance.*

***Keywords**: depression, proinflammatory cytokines, oxidative stress, autoantibodies, HSA*

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Introduction

Depression is one of the major problems in adults that accounts for up to 5 % of all adult cases worldwide (1). In the United States alone, about 17.3 million adults have reported major episodes of depression once in their lifetime (2). According to statistics, females had more depressive episodes than men (2). Depression changes the normal day to day function of individuals, which affects the quality of life (3). Mentally stressed

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individuals are more prone to suffer from diseases that are more common in the elderly such as heart-related diseases (4), diabetes mellitus (5), rheumatoid arthritis, obesity (6), and cancer (7). Multiple factors associated with depression include genetics, biological, physiological, and environmental factors.

Individuals under mental stress may show increased metabolic stress and cellular damage, which in turn may contribute to other associated diseases (8-10). Numerous previous studies suggest an association of depression with oxidative stress (11), which includes several molecular mechanisms that contribute to the aggregation of the disease. The activity of immune cells in the brain (11,12) actively contribute to the production of free radicals (10).

Reactive oxygen species are known to play essential roles in several physiological functions and defence against many microbes. However, normal equilibrium is disturbed with the excessive free radical production and an imbalance in oxidant inducing and antioxidant molecules can be associated with chronic diseases (13).

Oxidative stress with the excessive production of free radicals causes macromolecule (protein, lipids and nucleic acid) damage, which eventually leads to cell death. Blood proteins have an important physiological function *in vivo*. Oxidative stress can induce structural alterations in various proteins and hamper their biological functions (14). It is crucial to analyse pro- and antioxidant levels in subjects with psychological stress as this may help to manage stress and clinical depression.

A multitude of studies have shown that autoantibodies are associated with physiological conditions in depression (8,9,10,15). A higher amount of autoantibodies, specific to different antigens, were detected in chronic diseases with depression. Previously, our research group found increased levels of serum autoantibodies in various autoimmune conditions (systemic lupus erythematosus, rheumatoid arthritis, and type 1 diabetes), along with depression (9,10).

In light of this, our hypothesis in this study is to investigate the relationship of increased free radicals, structural alterations of proteins and immune imbalance in subjects with psychological stress. This hypothesis was tested by studying biophysical and biochemical changes in serum proteins and the existence of circulatory autoantibodies against oxidatively modified HSA in sera of subjects. These autoantibodies may serve as a potent marker for immune imbalance detection associated with individuals under psychological stress.

Materials and Methods

Reagents

HSA, diethylenetriaminepentaacetic acid (DETAPAC), superoxide dismutase (SOD) and catalase, p-nitrophenyl phosphate, guanidinum-HCl, dinitrophenyl hydrazine (DNPH), glycine, tri(hydroxymethyl)aminomethane (TRIS), Tween-20, ammonium persulphate, sodium dodecyl sulphate (SDS), goat anti-human IgG alkaline phosphatase conjugate (ALP), β-mercaptoethanol, and Coomassie brilliant blue-R250 (CBB) were sourced from Sigma (USA). Flat-bottom polystyrene microtiter (96 well) enzyme linked immunosorbent assay (ELISA) plates were purchased from NUNC (Denmark).

Human serum samples

Normal human sera were obtained from volunteers in Algomail-Clinic, Libya. Volunteers gave their full consent prior to the start of this study. This study was approved by the Institutional Ethics Committee, College of Medicine, UAA (Ref. No. uaa11-med239), AlGomail. The study was conducted over a period of twelve month. Volunteers (21-60 years) were divided into eight groups (n=25 for each group) depending on their psychological status as depressed or not, as well

as smokers and non-smokers. Descriptions of groups are as follows; males who were non-depressed and non-smokers (M), females who were non-depressed and non-smokers (F), males who were depressed but non-smokers (M-D), females who were depressed but non-smokers (F-D), males who were smokers but non-depressed (M-S), females who were smokers but non-depressed (F-S), males who were both depressed and smokers (M-D-S), and females who were depressed and smokers (F-D-S).

A modified version of the questionnaire (16) was applied to screen the volunteers and ascertain whether they were under depression or not. The basis for assessment of depression level included mood, a sense of hopelessness, sense of failure, dissatisfaction, feeling guilty, feeling punished, suicidal thoughts, increased unease, irritability, crying, social withdrawal, body image issues, hindrances in work, lack of sleep, restlessness, feeling tired, lack of appetite, and loss of weight (8-10). Subjects with comorbidities were not included in this study.

History of cigarette-smoking was assessed based on a self-reported questionnaire as published previously (9). All the samples were analysed for the estimation of fasting blood glucose (FBG) and glycated haemoglobin ($HbA1_C$) levels using fully automated Adams HA-8160 analyser (Arkray Inc, Kyoto, Japan) in the central clinical lab. Fresh blood samples were used in these assays. A well-established Trinder's method-based reaction, which includes glucose oxidase was used to estimate FBG. For HbA1c estimation, whole blood samples were automatically hydrolysed by the analyser and reverse phase cation exchange chromatography with colorimeter of dual wavelengths (samples measured at 415 nm while blank was used at 500 nm) was used.

Serum samples from all the subjects were stored at -20 °C and used in biochemical and immunological investigations.

HSA modification by Reactive Oxygen Species (ROS)

An aqueous preparation of HSA (10 mg/ml) in phosphate buffer saline, pH 7.4, at room temperature was irradiated (254 nm UV-light) for 20-30 minutes in the presence of hydrogen peroxide (H_2O_2) (10 mM). The hydroxyl radical ($^{\cdot}OH$) is the main radical generated. Extensive dialysis against phosphate buffer saline (PBS), pH 7.4 was used to remove unused H_2O_2 at the end of the reaction. Spectrophotometric (Shimadzu UV–1280, Kyoto, Japan) determination of protein concentration was by using E1%1cm = 5.3 $M^{-1}cm^{-1}$ at 280 nm (17).

Ultraviolet (UV) absorption spectroscopy

A Shimadzu UV–1280 spectrophotometer was used to record UV absorption spectra of native and modified HSA samples (100 µg/ml) using a wavelength range 260-360 nm.

Tryptophan fluorescence spectroscopy

A concentration of 100 µg/ml of protein samples in PBS (pH 7.4) was taken to determine tryptophan residue Trp-214 fluorescence in native and modified HSA at an excitation wavelength of 285 nm. Emission was recorded over 290-440 nm (18). All samples were measured at Hitachi model F2700 spectrofluorometer (Japan).

Gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted for native and modified HSA by a previously described method (19).

Analysis of Carbonyl Contents

A method published by Levine et al. was used to determine protein-bound carbonyl contents from the serum samples of volunteers from all groups, as well as in native and ROS-HSA samples (20). Calculations were carried out for the number of nanomole carbonyl groups/mg of protein; using

the equation: ε379 nm = 22,000 M^{-1} cm^{-1} (18). The effect of free radical damage of the proteins HSA was determined using various antioxidant compounds such as ascorbic acid, DETAPAC, SOD, and catalase. These antioxidants were co-incubated in the reaction of HSA modification with ROS.

Cytokine detection

Proinflammatory cytokines interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α) were detected in serum samples from the participants from all the groups using commercially available ELISA kits (R&D System, Minneapolis, MN, USA). Samples were assayed by automatic Accuris USA absorbance microplate Reader (MR9600-E) in triplicate.

Direct binding ELISA

Serum autoantibodies were detected using a direct binding ELISA. This assay was performed on 96-well polystyrene immunoplates (flat-bottomed), as described previously (8-10,21). Briefly, antigens (ROS-HSA or N-HSA) with the concentration of 10 μg/mL (100 μL) were coated on the immunoplates and incubated for 2 h at 25°C. Microplates were then washed 3-5 times using tris buffer saline (TBS) buffer including 0.05% Tween-20 (TBS-T). Unbound sites of the microplates were blocked using 2% of skimmed milk (150 μL/well) prepared in TBS (10 mM Tris, 150 mM NaCl, pH 7.4). The microplates were incubated for 4 – 6 h at 25°C. Microplates were subsequently washed 3 times with TBS-T buffer. After washing, diluted serum samples (1:100, in TBS buffer) were mixed (100 μL/well) into each well and incubated for 2 h at 25°C. Microplates were washed 3-5 times to remove unbounded antibodies. Anti-human IgG alkaline phosphatase conjugate was added to bond with the serum IgGs. The *p*-nitrophenyl phosphate was a substrate for the conjugated enzyme. Estimation of absorbance at 410 nm was performed on an automatic Accuris USA absorbance microplate Reader (MR9600-E).

Competition ELISA

Competition ELISA was used to determine antigenic specificity of ROS-HSA (8-10,21). At 25°C increasing concentrations (0–20 μg/mL) of ROS-HSA or N-HSA were incubated for 2-4 h with constant amount of autoantibodies in serum. After incubation, the antigen-antibody immune complexes were formed and were added to the microplate wells in spite of serum used in the above-mentioned direct binding ELISA. The remaining antibodies left in the mixture were bound to the coated antigens, and concentrations levels were estimated as given in the above-mentioned direct binding ELISA. The percentage inhibition of each sample was calculated as;

$$\text{Percent inhibition} = [1 - (A_{\text{inhibited}}/A_{\text{uninhibited}})] \times 100$$

Statistical evaluation

All the data are presented as mean ± standard deviation (SD). OriginPro 16 software (demo version) and student *t-test* were used for numerous data comparisons. The ***p***-values < 0.05 are considered statistically significant.

Results

Characterisation of ROS-HSA by biophysical studies

ROS induced modifications in HSA were characterised by biophysical studies. UV-spectral study of ROS-HSA showed a significant (56.8%; $p < 0.0001$) increase in hyperchromicity at the wavelength of 280 nm when compared to N-HSA (Figure 1). UV-spectra of ROS modified HSA exhibited unfolding of HSA molecules.

A single molecule of tryptophan residue is present in the HSA molecule. Study of tryptophan specific fluorescence provides important aspect of the site-specific structural changes due to ox-

idative stress. Tryptophan specific fluorescence for both types of HSA were recorded within the range of 290 – 430 nm, which exhibited a remarkable increase ($p < 0.0001$) in specific fluorescence for tryptophan in ROS modified HSA compared to native (Figure 2).

In vitro study of the effects of free radicals on the HSA molecules were further characterised by SDS-PAGE in the presence of denaturing reagent β-mercaptoethanol (Figure 3). A smear of the band with lower molecular weight ranges 65 to 14 kD (lanes 2) was observed after free radical treatment of HSA. An increase in electrophoretic mobility was observed. Results showed oxidative damage of HSA molecules and the presence of lower molecular weight peptides due to free radicals.

Biochemical analysis of native and ROS-HSA

Carbonyl groups bound to protein were also identified in *in vitro* modified HSA samples in the absence of or in the presence of free radical scavengers (Figure 4). Notably significant ($p < 0.0001$) quantities of protein-bound carbonyl

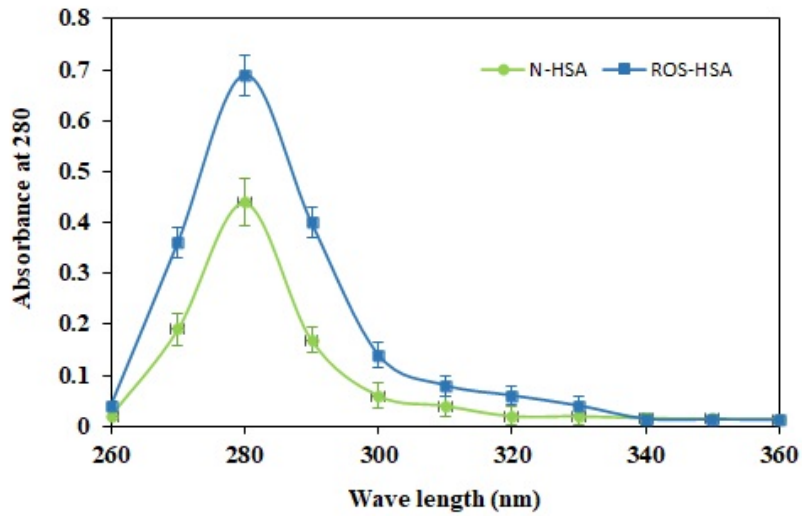

Fig. 1. UV spectra for native and modified HSA (100 µg/ml) were analysed at 280 nm.

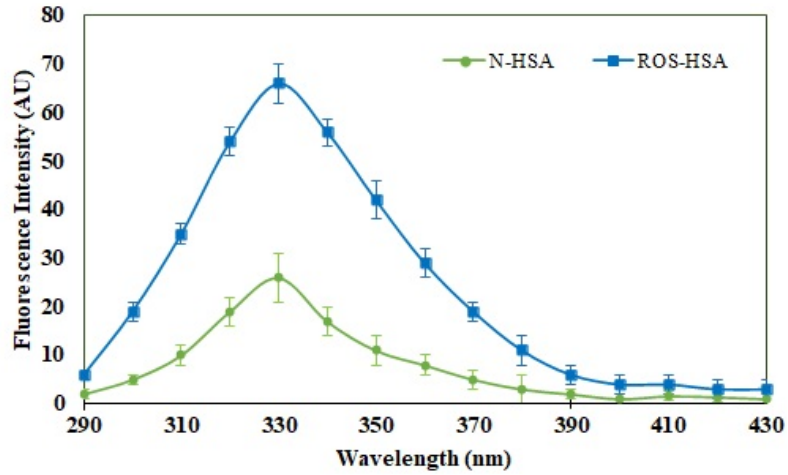

Fig. 2. Tryptophan fluorescence emission spectra of unmodified and ROS-HSA (100 µg/ml). All the samples underwent excitation at 285 nm and emission at 330 nm.

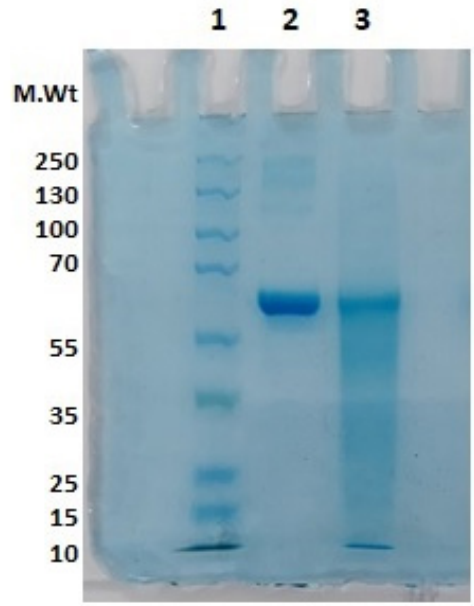

Fig. 3. SDS-PAGE of native HSA and ROS-HSA. Lane 1 molecular weight marker (10 – 250 KDa). Lanes 2 and 3 are native HSA and ROS-glycated HSA, respectively. The concentrations of proteins were 10 µg in each lane and the gel was stained using CBB R-250 dye.

groups were generated during ROS treatment of HSA as compared to the native protein. The carbonyl content of ROS-HSA samples was estimated to be 11.24 ± 0.9 moles/mol of HSA, whereas N-HSA showed the amount of 0.5 ± 0.2 moles/mole of HSA.

Concentrations of protein-bound carbonyls were estimated to understand the role of antioxidants

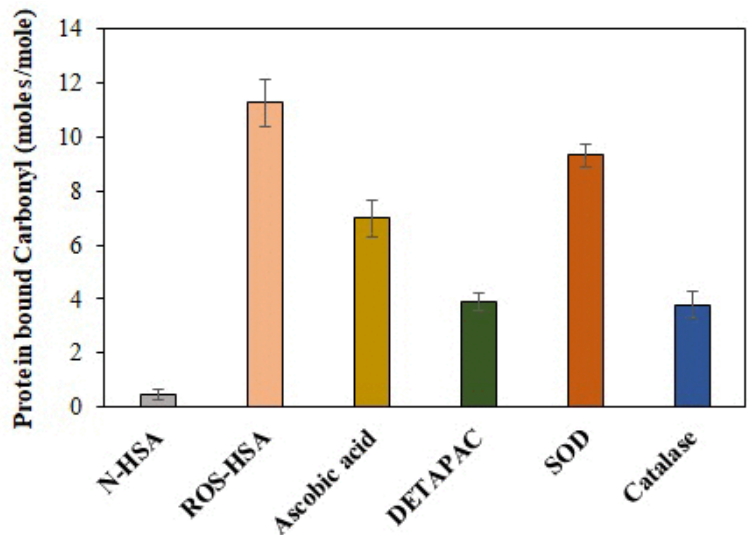

Fig. 4. Protein-bound carbonyl groups in native and ROS-HSA samples (150 µg/ml) and effect of antioxidants ascorbic acid (4 mM), DETAPAC (1 mM), SOD (200 units/ml) and catalase (200 units/ml). HSA without ROS or any other antioxidant served as control.

in inhibiting free radical formation. Antioxidants such as ascorbic acid, DETAPAC, SOD, and catalase were added to the reaction mixture of ROS and HSA. Catalase and DETAPAC exhibited almost similar percentage inhibition (66.2%; $p < 0.001$ and 65.3%; $p < 0.001$, respectively) in the formation of carbonyl content, followed by, ascorbic acid (37.9%; $p < 0.01$) and SOD (17.2%; $p < 0.05$). All the inhibition assays were conducted under similar conditions of temperature and pH.

Biochemical and immunological analysis in serum samples

Proinflammatory cytokines in serum samples

Proinflammatory cytokines IFN-γ and TNF-α were quantified in sera from all the individuals (Table 1) using commercially available ELISA kits (R&D System, Minneapolis, MN, USA). Both cytokines showed a significant increase in levels [IFN-γ (7.4 ± 0.61 pg/mL; $p < 0.001$) and TNF-α (1.47 ± 0.23 pg/mL; $p < 0.001$)] in female subjects who were smokers and depressed (F-D-S), compared to female subjects (F) who were either non-smokers and non-depressed, or female subjects who were depressed (F-D) or smokers (F-S). Moreover, these cytokine levels were also higher in F-D-S as compared to M-D-S (IFN-γ; 6.4 ± 0.53 pg/mL and TNF-α; 1.23 ± 0.18 pg/mL). Interestingly, higher concentrations of IFN-γ and TNF-α were found in F-D subjects compared to M-D subjects. However, elevated levels of IFN-γ and TNF-α were observed in M-D and F-D subjects compared to subjects from F and M groups, respectively.

Serum carbonyl contents

*P*rotein-bound carbonyl contents have been established as oxidative stress markers for protein oxidation. Protein carbonyl content was estimated by Dinitrophenylhydrazine (DNPH) reaction. Estimation of carbonyl content was conducted in all samples from each group (Table 1). Significantly higher amounts of carbonyl content (nmol/mg protein) were found in subjects from the F-D-S group (2.68 ± 0.33) followed by M-D-S (2.34 ± 0.31), F-D (2.31 ± 0.29), M-D (2.03 ± 0.17), M-S (1.27 ± 0.18), and F-S (1.11 ± 0.15). Lower amounts of carbonyl content were detected in male (0.69 ± 0.12) and female (0.64 ± 0.11) subjects who reported no depression or were non-smokers. The female subject group, who were depressed, and smokers exhibited the highest amount of carbonyl compounds as compared to other subjects who were depressed (F-D) or smokers (F-S). Similar trends of serum carbonyl content were observed in groups that included males. Moreover, female F-D-S sub-

Table 1. Clinical investigations and demographic characterization of all the subjects based on gender and smoking habits.

Groups (21 – 60 years) n= 25	Fasting blood glucose (mg/dl)	HbA1C (%)	IFN-γ (pg/mL)	TNF-α (pg/mL)	Duration of Smoking n (years ± SD)	Protein-bound carbonyl content (nmol/mg protein)
M	91.6 ± 4.1	5.5 ± 0.3	4.8 ± 0.61	0.97 ± 0.18	—	0.69 ± 0.12
F	89.9 ± 5.5	5.4 ± 0.3	4.7 ± 0.54	0.94 ± 0.16	—	0.64 ± 0.11
M-D	90.5 ± 4.7	5.5 ± 0.3	5.9 ± 0.57	1.13 ± 0.13	17.5 ± 6.1	2.03 ± 0.17**
F-D	92.1 ± 6.1	5.6 ± 0.4	6.3 ± 0.51	1.26 ± 0.16	11.9 ± 7.3	2.31 ± 0.29**
M-S	93.2 ± 3.8	5.5 ± 0.4	5.6 ± 0.65	1.11 ± 0.14	15.5 ± 4.1	1.27 ± 0.18*
F-S	95.8 ± 4.5	5.6 ± 0.4	5.4 ± 0.69	1.04 ± 0.17	6.5 ± 5.1	1.11 ± 0.15*
M-D-S	93.8 ± 4.1	5.4 ± 0.3	6.4 ± 0.53	1.23 ± 0.18	18.9 ± 5.7	2.34 ± 0.31***
F-D-S	94.1 ± 5.3	5.6 ± 0.3	7.4 ± 0.61	1.47 ± 0.23	8.9 ± 4.7	2.68 ± 0.33***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

jects exhibited a clear difference ($p < 0.01$) as compared to M-D-S subjects.

Recognition of ROS-HSA by serum autoantibodies

*T*he role of the oxidation of protein in different subject groups was evaluated by categorisation of subjects as normal individuals, psychologically depressed and either smokers or non-smokers. Sera samples from all eight different groups were tested against both N-HSA and ROS-HSA using immunoassay 'ELISA' (Figure 5). Group F-D-S showed highest recognition (OD 0.89 ± 0.07) of circulatory antibodies against ROS-HSA using direct binding ELISA, followed by M-D-S (OD 0.66 ± 0.049), F-D (OD 0.44 ± 0.042), M-D (OD 0.34 ± 0.033), M-S (OD 0.32 ± 0.038), and, lastly F-S (OD 0.28 ± 0.022). Subjects from groups M and F showed very low recognition with ROS-HSA. Conversely, with N-HSA, no appreciable bindings were observed in any of the groups.

These ELISA assay findings exhibited a significant increase in recognition of serum autoantibodies in F-D-S subjects as compared to F, F-D, and F-S subjects. Hence, simultaneous depression and smoking showed a synergistic effect in the production of circulatory autoantibodies against free radical modified HSA. Overall, an additive effect of depression and smoking was observed in F-D-S subjects.

Native and ROS-HSA were used as inhibitors to determine the binding specificity of free radical modified HSA and serum autoantibodies in inhibition ELISA (Table 2). Serum samples from all the groups were analysed by inhibition ELISA. For the non-depressed and non-smoker subjects from both males and females, an inhibition of less than 8% was detected against both the antigens i.e., N-HSA and ROS-HSA. All 25 sera samples from F-D-S showed inhibition toward ROS-HSA antigen, and only two serum samples were recognized by N-HSA. From M-D-S group, 21 subjects recognized ROS-HSA and one subject recognized N-HSA. The highest

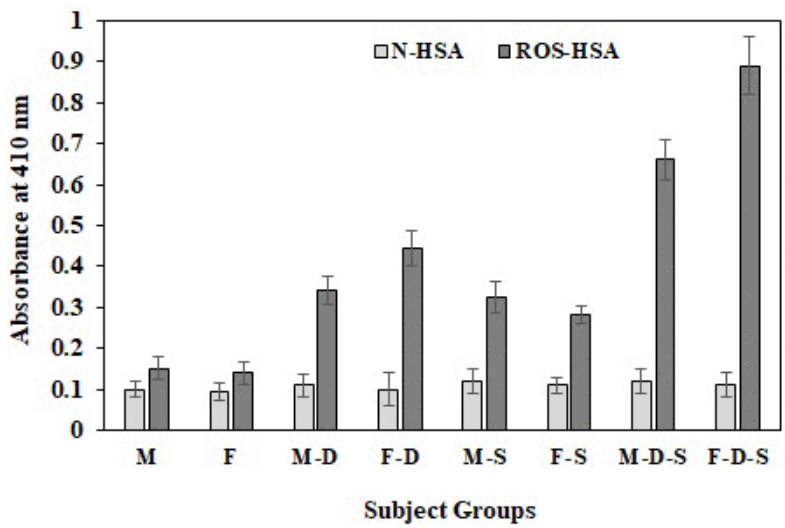

Fig. 5. Immunoassy; direct ELISA of sera (1:100 diluted) from eight different groups Sera samples were from all eight different groups (F, M, M-D, F-D, M-S, F-S, F-D-S and N-D-S) were tested against both N-HSA (□) and ROS-HSA (■). Native and ROS-HSA (10 µg/ml) were used to coat microtitre plates. Histogram for each group represents the mean (± SD) values of autoantibody levels against both the antigens. The number of sera in each histogram was 25. SD indicated by bars.

Table 2. Inhibition ELISA assay against N-HSA and ROS-HSA antigens.

Groups	Maximum percent inhibition at 20 µg/ml	
	Anti-N-HSA-Ab	Anti-ROS-HSA-Ab
M	6.9 ± 1.3	7.2 ± 1.1
F	7.7 ± 1.4	7.8 ± 1.2
M-D	5.9 ± 1.2	17* (39.3 ± 3.7)
F-D	6.3 ± 1.6	19* (47.1 ± 4.4)
M-S	7.9 ± 1.8	16* (35.5 ± 4.8)
F-S	7.7 ± 1.6	13* (31.4 ± 3.1)
M-D-S	1* (8.1 ± 2.3)	21* (58.8 ± 5.2)
F-D-S	2* (8.4 ± 2.4)	25* (78.6 ± 5.7)

* subject numbers in each group

mean maximum percentage inhibition (MMPI) was also observed in F-D-S (78.6 ± 5.7) subjects, followed by M-D-S (58.8 ± 5.2). On comparison with the inhibition results of the ELISA assay for ROS-HSA, the following results were observed. A greater number of subjects (n=19) with higher MPI (41.1 ± 4.4) were observed in F-D subjects as compared to M-D subjects (n=15; 37.3 ± 3.7 MPI). However, an increased number (n=18) of M-S subjects showed higher MPI (35.5 ± 4.8) as compared to F-S subjects (n= 16; 31.4 ± 3.1 MPI).

Correlation Analysis

The correlation between autoantibodies against ROS (ROS-HSA-Abs) versus FBG, serum levels of protein carbonyl content, IFN-γ and TNF-α was analysed (Table 3). No correlation could be established between FBG and any of the other groups. An increase in FBG levels with age was found, which is a normal age-related clinical change. Analysis showed the correlation of different groups (M-D, F-D, M-S, F-S, M-D-S, and F-D-S) with the levels of ROS-HSA-Abs and carbonyl content, IFN, and TNF. The highest level of correlation of carbonyl content, IFN-γ and TNF-α was observed in F-D-S group compared to the other group, followed by M-D-S, FD, MD, M-S, F-S. However, no correlation was found for FBG, carbonyl content, IFN-γ and TNF-α with ROS-HSA-Abs for groups F and M. Thus, depression and smoking habits correlate with oxidative stress autoantibody production and proinflammatory cytokines production. Hence, pathophysiological changes may be induced by the simultaneous effect of a multitude of factors.

Discussion

This research study investigated oxidative stress and autoantibodies generated against free radical modified antigen among eight different groups, comprising 25 subjects, each as defined earlier. The subjects were mainly middle-aged adults.

Table 3. Correlation analysis for the levels of ROS-HSA-Abs with FBG, carbonyl contents, and cytokine levels.

		M	F	M-D	F-D	M-S	F-S	M-D-S	F-D-S
		Correlation coefficient (r)							
ROS-HSA-Abs	FBG	−0.17	−0.14	0.05	0.11	0.08	0.09	0.13	0.11
	Carbonyl content	0.28	0.17	0.88**	0.92***	0.85**	0.81**	0.91***	0.99***
	IFN-γ	0.19	0.21	0.87**	0.91***	0.84**	0.80**	0.93***	0.97***
	TNF-α	0.24	0.28	0.86**	0.90***	0.82**	0.79**	0.91***	0.95***

** $p < 0.01$; *** $p < 0.001$

The biochemical, immunological, and psychological comparisons were analysed amongst the groups based on carbonyl content, proinflammatory cytokines (IFN-γ and TNF-α) and depression. Gender and smoking habits were also included. Blood protein HSA was modified *in vitro* with ROS, mainly hydroxyl radicals, and the changes in the protein were studied by biophysical and biochemical analysis. UV-spectroscopic studies revealed a significant increase in UV intensity in ROS-modified HSA. This change may be due to the exposure of chromophobic groups (tyrosine, tryptophan and dicystine disulphide bond) in globular protein (HSA) (22). HSA is a unique protein molecule that has a single tryptophan residue (214) which is present in the physiologically important subdomain 2A ligand binding site (23). Any change in tryptophan specific fluorescence provides a critical insight into the site-specific structural changes due to oxidative stress. A significant increase was observed in tryptophan specific fluorescence for ROS modified HSA as compared to the native protein. Thus, there is a possibility of modification in subdomain 2A due to free radical modification of HSA.

Electrophoretic results showed visible damage in native protein on exposure to free radicals, and a smear of low molecular weight peptides was seen in the gel. However, native HSA exhibited a single clear band at 65 kDa of molecular weight, which indicates that the protein is intact, and no visible damage or change was observed.

Protein-bound carbonyl content is an essential intermediate in oxidation reactions. Hence, higher amount of protein-bound carbonyl content was detected *in vitro* ROS modified HSA, which was remarkably inhibited by various free radical scavengers. Potent inhibition in the formation of this content was exhibited by DETAPAC and catalase. The $OH^{\bullet}$ were generated in the reaction that was used in *in vitro* modification of HSA protein. In previous studies, DETAPAC and catalase were found to inhibit or decrease the formation of $OH^{\bullet}$ (24). Hence, free radical induced stress and antioxidant imbalance in the body can play a considerable role in various disorders.

Various factors like the over-production of free radicals, as well as failure of removal of excessive free radicals due to compromised state of antioxidant defence mechanisms result in oxidative stress (25). The lipid rich brain environment and high oxygen consumption contribute to the pathogenesis of inflammatory psychiatric disorders caused by oxidative stress (26, 27), and has been linked to depression and anxiety (30, 31). Depression is linked to lowered concentrations of antioxidants like vitamin E, tryptophan, tyrosine, albumin, zinc, glutathione, and CoQ10 in plasma (30-32). Malondialdehyde (MDA) is a hallmark for oxidative stress. In a study, MDA concentration was considerably higher in patients with depression than in healthy individuals ($p < 0.0001$) (33, 34). Not only MDA, but even ascorbic acid and SOD concentrations were significantly decreased in individuals with major depression compared to healthy individuals ($p < 0.0001$) (33).

In our study, carbonyl content levels were significantly higher in subjects who were under depression. Furthermore, the gender difference is very much prevalent in depression; more females experience depression as compared to males (35). For the subjects who were under depression, results showed higher levels of carbonyl content detected in female subjects' serum samples as compared to male counterparts. Mood disorders were influenced by neuroendocrine, neurotransmitters as well as circadian cycle which are affected by the female sex hormones oestrogen and progesterone (36).

Oxidative stress can be increased due to smoking which leads to a decrease in the levels of antioxidants in platelets and red cells (37). Moreover, under oxidative/ nitrosative stress conditions, there is an up-regulation of HIF-1α gene expression in smokers, which may cause

transcription of eNOS and erythropoietin genes (37). These findings were consistent with our results; carbonyl content levels were significantly higher in female subjects with depression who were smokers compared to male subjects with depression and smokers ($p < 0.01$). Even female subjects with depression and smoking history exhibited higher carbonyl content than female subjects who were considered depressed or female subjects classified as smokers or female subjects who were non-depressed and non-smokers.

It was hypothesised that all the above-mentioned changes may occur in *in vivo* systems under psychological stress conditions and with excessive smoking. Thus, the levels of autoantibodies were estimated against *in vitro* ROS modified HSA in all the groups. Immunoassay direct binding ELISA was used to determine levels of anti-ROS-HSA antibodies. It was observed that depression plays a definitive role in the production of autoantibodies against ROS-HSA. Furthermore, smoking is also a contributing factor to the induction of these autoantibodies. Female subjects who were under severe depression, as well as smokers, exhibited the highest levels of these autoantibodies as compared to the rest of the groups. Subjects from males and females who were non-depressed and non-smokers showed no remarkable autoantibody titre.

Findings from direct binding immunoassays were further ascertained by another immunoassay i.e., inhibition ELISA. This assay also indicates the strong binding activity of serum autoantibodies with ROS-HSA. The MPI was achieved in samples from group F-D-S, followed by M-D-S, F-D, M-D, M-S, F-S.

Hence, depression and smoking habits may induce oxidative modification in blood proteins, such as HSA, causing structural alterations and the subsequent generation of new epitopes/antigens, making them immunogenic. This might be one of the reasons for the production of high levels of autoantibodies in subjects with depression and history of smoking. Indeed, gender is also a contributing factor. Future studies with detailed clinical and socio-economic data, including ethnicity of the volunteers could provide further insight.

Limitations of the study: The follow-up sample collection of the volunteers for this study was not maintained. Such information could have shed light on the relationship between psychological changes and immunological imbalance(s). Lack of socio-economic as well as detailed clinical data of the volunteers would have contributed to findings as well.

Conclusions

This study showed that depression and smoking habits might cause excessive oxidative stress, which leads to the damage of blood proteins and may induce the generation of autoantibodies against free radical modified proteins (ROS-HSA). These changes might exert influence on the levels of proinflammatory cytokines, exacerbating psychological stress. Females are more prone to depression, and with smoking habits showed highest levels of IFN-γ and TNF-α, as well as protein bound carbonyl compounds. Increased levels of autoantibodies against ROS-HSA were also observed in female subjects who were smokers. Thus, depressed female subjects who are smokers might be more prone to the immunological imbalances. Furthermore, the immune imbalance in depression and other clinical and demographic factors require further studies in this direction which could facilitate the diagnosis, treatment and management of the psychological disorders and their associated complications.

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Authors' Contributions

Conceptualisation, methodology, investigations, data curation, and resources were done by M.W.A.K. Formal analysis, S.S., M.W.A.K. Writing-original draft preparation, M.W.A.K. Writing-review and editing, S.S. All authors read and approved the final manuscript.

Conflicts of Interest

There is no conflict of interest.

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