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Depression and its related parameters increased the production of autoantibodies against 16α-hydroxyestrone-albumin complex in systemic lupus erythematosus

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ABSTRACT

Depression is the common and early symptoms associated with early onset of SLE, 16α-hydroxyestrone (16α-OHE_1) levels were found to be significantly higher in serum and urine in patients with SLE. This study was carried out in order to know whether depression and its related parameters in the SLE patients enhanced the production of autoantibodies against 16α-OHE_1-albumin (A) complexes. The autoantibodies in the serum of 100 SLE [including 65 depressed SLE (DSLE)] patients and 37 control subjects were detected by using direct binding, inhibition ELISA and quantitative precipitin titration. Autoantibodies from DSLE patients (and also the patients who were taken anti-depressant and with neurological symptoms) showed high binding to 16α-OHE_1-A in contrast to SLE ($p < 0.05$) and control subjects ($p < 0.001$). Although, SLE sera showed high recognition to 16α-OHE_1-A in comparison to A ($p < 0.05$) or 16α-OHE_1 ($p < 0.001$). The affinity of autoantibodies for 16α-OHE_1-A was found to be high for DSLE (1.16×10^{-7} M) and SLE (1.24×10^{-7} M) patients as detected by Langmuir plot. The concentration of 16α-OHE_1 ($p < 0.05$) and inflammatory cytokines (IL-6, $p < 0.05$ and IL-17, $p < 0.001$) in the serum of SLE patients was found to be significantly higher than controls. Depression and its related parameters in SLE enhanced the production of autoantibodies against 16α-OHE_1-A through the generation of inflammatory conditions. Depression in SLE patients increased the release of pro-inflammatory cytokine (IL-6 and IL-17) that in turn generating more autoantibodies and showed strong recognition to 16α-OHE_1-A.

1. Introduction

Sex hormone known to influences the mammalian immune system and showed different effects on different autoimmune diseases [1]. The function of sex hormone may vary among patients with autoimmune disease that might contribute to the malfunction of immune system [2]. Sex hormone including estrogen metabolites somehow controls immune system by regulating the production of antibodies and other immune components [2].

Metabolism of estrogen within the body is complex and produces various unstable and active metabolites. Liver controls its metabolism through hydroxylation, methylation, glucuronidation, sulfation and finally excrete it in the urine and feces [3]. Cytochrome P-450 mediated the hydroxylation of estradiol and estrone. It takes place primarily at two sites on the mother compound, which is either at the carbon 2 (C-2) yielding 2-hydroxyestrone (2-OHE_1) or at the carbon 16 (C-16) yielding 16α-hydroxyestrone (16α-OHE_1). This enzyme also hydroxylate carbon 4 (C-4) yielding 4-hydroxyestrone (4-OHE_1) [4]. The 2-OHE_1 metabolite is termed as good estrogen because it has very week estrogenic activity. In contrast, the 16α-OHE_1 and 4-OHE_1 metabolites are active metabolites that persistently showed estrogenic activity and promote tissue proliferation [4,5].

The rate of occurrence of SLE is far higher in females than in males, especially during the reproductive phase. Animal models showed that estrogen causes acceleration of SLE by inducing anti-DNA antibodies [6]. The big question is to know whether serum or urinary level of estrogen is altered or not, in SLE. The conclusion is that estrogen serum level remains same as healthy controls in most of the SLE patients and the difference lying in the urinary level of estrogen. Earlier studies have shown an increased level of 16α-hydroxylated estrogens was detected

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in the urine of SLE patients compared to healthy controls [7,8]. Recently, these studies were confirmed in different SLE patients, where high levels of 16 α-hydroxyestrone in addition to low level of 2-hydroxylated estrogen, were found [9]. Because 16α-hydroxylated estrogens is an active metabolites that is mitogenic and proliferative and 2-hydroxylated form done the opposite function, so this altered pattern might induces various inflammatory responses. The 2-hydroxylated estrogens reported to be 10 times lower in SLE patients compared to healthy controls, whereas the ratio of 16 α-hydroxyestrone/2-hydroxyestrogens was 20 times higher in these patients [9]. In addition, elevated serum concentration of 16α-hydroxyestrone was found in SLE patients [8]. Also, peripheral estrogen hydroxylation was found to be increased in SLE patients, and estrogenic metabolites produces as a resulted of these processes, were reported to increase B cell differentiation and activate T cells [10]. In addition, our previous data strongly suggest that catecholestrogens (CE) appears to play an important role in antigen-driven induction of SLE autoantibodies [11,12]. Oxidation of estrogen to catecholestrogen quinone and semiquinone react with DNA, thus forming stable DNA adducts [13]. Even oxidation of catecholestrogen produces hydroxyl radical that damaged DNA, alter its antigenicity and produces high level of SLE autoantibodies [14].

Albumin is the most abundant serum protein found in the blood plasma, that has a variety of function including binding to various protein, molecules and drugs and also help in the their transport. As high concentration of 16α-OHE_1 was found in SLE patients and albumin is the most abundant protein in human plasma and both are making complex [15], so there is a good opportunity to use this complex as an immunological marker in the sera of these SLE patients. In the present study, we demonstrate the preferential recognition of 16α-OHE_1-A by autoantibodies from SLE patients. Sera from SLE (DSLE) were screened for the presence of autoantibodies against 16α-OHE_1-A to elucidate the potential antigenic role of this complex in SLE. Furthermore, 16α-OHE_1-A was also used as antigen to triggered antibodies in experimental animals that can be used as probe for the estimation of 16α-OHE_1 in the sera of SLE patients.

2. Material and methods

2.1. Patients and controls

Study SLE patients were diagnosed according to the American College of Rheumatology revised and SLICCS criteria for the diagnosis of SLE disease [16,17]. Blood samples were collected from 100 SLE patients (including 65 mentally depressed SLE) and 37 normal subjects, who served as negative controls. The socio-demographic date is given in Table 1. Twenty eight patients were untreated, 31 were treated with azathioprine (mean, 60 mg/day), 50 with prednisolone (9.4 mg/day), 25 with methotrexate (7.5 mg/week) and 19 with cyclophasphamide (100 mg/day), 60 with anti-depressant (Duloxetine, 60 mg/day). The dose of anti-depressant decreases with the time if the mood is stable and stop if no symptom of depression was observed. The Self-Rating Depression Scale questionnaire was used to screen the SLE patients and determine their level of depression. We used a modified version of the earlier used questionnaire [18] to screen the patient's level of depression. SLE patients gave their full consent and the study was approved by the Institutional Ethics Committee. All serum samples were decomplemented by heating to deactivate complement protein at 56 °C for 30 min and stored at −20 °C.

2.2. 16α-OHE_1-albumin (16α-OHE_1-A) complex formation

16α-OHE_1-A complex was formed as described by Bucala et al. [15] with slight modifications. 16 α-hydroxyestrone (1–10 mM) was incubated with 1 mg of albumin (human serum albumin, Sigma, USA) in 50 mM potassium phosphate pH 7.4. 1 μ mol of sodium cyanoborohydride was added and reaction mixture was incubated at 37 °C for 48 h with gentle shaking. 16α-OHE_1 was dissolved in ethanol such that the final concentration of ethanol in the reaction mixture was 0.1%. After incubation, the reaction mixture was dialyzed against PBS pH 7.4 to remove excess unbound 16 α-OHE_1 and albumin.

Table 1
Socio-demographic features and estimation of 16α-hydroxyestrone by anti-16α-OHE_1-A antibodies in different SLE patients.

Characteristics	SLE (n = 100)	Controls (n = 37)
Number and gender (M/F)	5/95	2/35
Age (mean ± SD)	48 ± 4.1	45 ± 6.3
Disease duration (years)	13 ± 3.2	_
Disease activity	4.8	_
Body mass index	24.3 ± 8.4	_
Pain score (median)	5.3	_
Marital status		
- Single	25 (25%)	10 (27%)
- Married	70 (70%)	25 (67%)
- Divorced	5 (5%)	2 (5%)
Education level		
- Primary	24 (24%)	9 (24%)
- Middle school	20 (20%)	9 (24%)
- High school	30 (30%)	10 (37%)
- College	26 (26%)	9 (24%)
Employed		
- Yes	45 (45%)	15 (41%)
- No	55 (55%)	22 (59%)
Race or ethnic group		
- White/Caucasian	52 (52%)	_
- Black/African	33 (33%)	_
- Asian	10 (10%)	_
- Others	5 (5%)	_
16α-Hydroxyestrone estimation in serum (n = 40) by[a]		
- Anti-16α-OHE_1-A antibodies	37.3 ± 8.9 pg/ml[c]	13.3 ± 4.8 pg/ml[h]
- Human 16α-hydroxyestrone ELISA Kit	38.4 ± 10.8 pg/ml	_
Inflammatory cytokines[b] estimation		
- IL-6	13.8 ± 4.3[d] pg/ml	8.3 ± 1.8 pg/ml
- IL-17	19.8 ± 5.9[e] pg/ml	10.1 ± 3.1 pg/ml
- IL-10	27.8 ± 5.2[f] pg/ml	5.4 ± 1.1 pg/ml
- BAFF	6.3 ± 1.2[g] ng/ml	1.8 ± pg/ml

[a] The amount of 16α-OHE_1 level was measured by ELISA and the values are presented in median ± SD. The concentrations of cytokines are given in mean ± SD and 35 SLE patients showing high titer antibodies have been reported. Age-match controls were taken for cytokines estimation.
[b] n = 35.
[c] $p < 0.05$. Correlation coefficient $r = 0.94$ ($p < 0.001$).
[d] $p < 0.05$.
[e] $p < 0.001$.
[f] $p < 0.01$.
[g] $p < 0.01$.
[h] n = 15.

2.3. Antibodies against 16α-OHE_1-A

Antibodies against this complex was induced in experimental animals (female rabbit, n = 5) as described earlier [19]. Antibodies against other antigens (16α-OHE_1, A) were also induced in the rabbits that served as negative controls.

2.4. Enzyme linked immunosorbent assay (ELISA)

Direct binding ELISA was performed to detect antibodies against this complex (16 α-OHE_1-A) in SLE/immunized sera, as mention earlier [11]. Competition ELISA was performed to detect the specific recognition of circulating SLE/immunized antibodies to 16α-OHE_1-A [11]. Briefly, 100 μl of 16α-OHE_1-A (2.5 μg/ml) was coated on the microtitre plate, initially for 2 h at room temperature and 4 °C overnight. The plates were washed with TBS-T (2.68 mM KCl, 20 mM Tris, 150 mM NaCl, pH 7.4, with 0.05% Tween 20) three times. The

unoccupied sites were then blocked by 1.5% BSA (150 µl) in TBS (150 mM NaCl, 10 mM Tris, pH 7.4). Immune complexes (100 µl) prepared already (by mixing 100 µl of 1:100 dilution of SLE/immunized sera with increasing amounts of antigen, 0–20 µg/ml, at 37 °C initially for 4 h and later overnight at 4 °C), was added to each well, wait for 2 h at 37 °C and overnight at 4 °C. The bound IgGs were assayed with anti-human IgG alkaline phosphatase conjugates followed by using their substrate (*p*-nitrophenyl phosphate). The reaction was developed and the reading was taken at 410 nm on to the microplate reader. The sensitivity and specificity for competition ELISA for SLE autoantibodies was 98.8% and 100% respectively. For estimation of 16α-OHE_1 in SLE sera we used "Human 16α-hydroxyestrone ELISA Kit" (Glory science Co. Lt., USA) (sensitivity 0.5 pg/ml, assay range 3–400 pg/ml). "Human IL-6 Quantikine ELISA Kit" (Minneapolis, USA) (sensitivity 0.7 pg/ml, assay range 3.1–300 pg/ml) and "Human IL-17 Quantikine ELISA Kit" (Minneapolis, USA) (sensitivity 5 pg/ml, assay range 10–700 pg/ml) were used for IL-6 and IL-17 estimation. "Human IL-10 Quantikine ELISA Kit" (Minneapolis, USA) (sensitivity 3.9 pg/ml, assay range 7.8–500 pg/ml) and "BAFF Human Instant ELISA Kit" (Thermo Fisher Scientific) (sensitivity 0.13 ng/ml, assay range 0.31–20 ng/ml) were used for IL-10 and BAFF estimation. The correlation coefficient between 16α-OHE_1 or cytokines (IL-6, IL-17, IL-10, BAFF) concentration and their expected values varies from 97.8 to 99.9 in all commercial immunoassays.

2.5. Purification of immunoglobulin G

Immunoglobulin G against 16α-OHE_1-A was isolated and purify from SLE patients (or immunized animal) on a Protein A-Agarose column as described previously [20]. Purity of the isolated IgG was checked on 7.5% SDS-PAGE and their concentration was determined by using the formula 1.40 OD_{280} = 1 mg/ml.

2.6. Formation and quantification of immune complexes against 16α-OHE_1-A in SLE patients

Formation and quantification of immune complexes against 16α-OHE_1-A in SLE patients was done as described earlier [21]. Briefly, increasing amount of various antigens (16α-OHE_1-A, 16α-OHE_1 and A) (0–40 µg) were incubated with constant amount of purified SLE IgG (100 µg) in an assay volume of 500 µl. Normal human IgG served as a negative control. The reaction mixture was incubated initially for 2 h at 37 °C and overnight at 4 °C. The immune complexes thus formed, were pelleted and washed with PBS pH 7.4, and finally dissolved in 1 N NaCl (250 µl). Complex bound antigen and free antigen was estimated by colorimetric methods [22]. The binding data were used to estimate antibody affinity by determining affinity constant [23].

2.7. Statistical analysis

Significant difference relative to controls was determined by using the Student's *t-test*. A $p < 0.05$ was considered statistically significant. The values are presented as mean ± SD, wherever presented.

3. Results

3.1. Characterization of 16α-OHE_1-A adducts

Fig. 1 showed the UV absorption spectra and electrophoretic pattern of 16 α-OHE_1-A, and A separately. Treatment of 16α-OHE_1 with A results in the formation of 16α-OHE_1-A complex as shown in Fig. 1 (Insert: Lane 2), that showed very thick band in the gel as compared to A (Fig. 1, Lane 1, 2). In addition, this complex showed about 38.5% UV hyperchromacity at 280 nm indicating the formation of high weight complex. The hydroxyl group adjacent to the C-17 carbonyl of 16α-OHE_1 permits initial Schiff base formation followed by Heyns rearrangement during the formation of 16α-OHE_1-A complexes [15]. Thermal stability of 16α-OHE_1-A and control showed that this complex (16α-OHE_1-A) have about 9 °C higher melting temperature compared to A. UV absorption and thermal denaturation studies have shown that the complex, formed between 16α-OHE_1 and A, is table and strong.

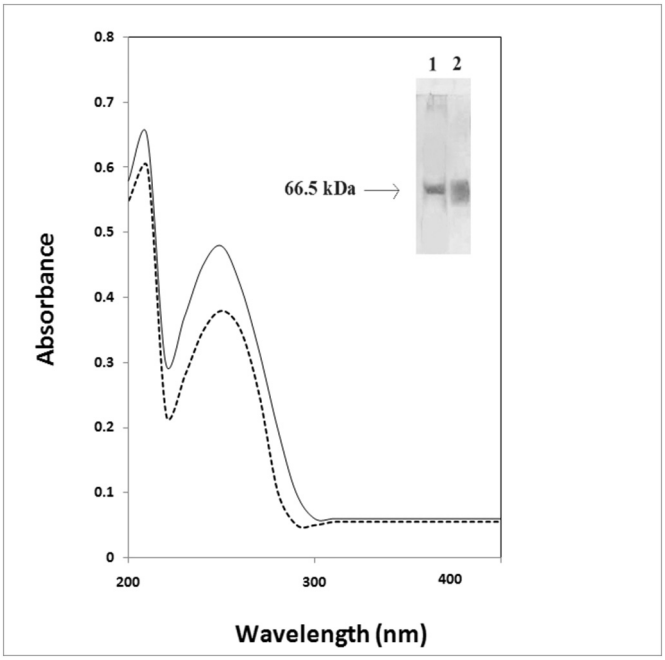

Fig. 1. UV absorption spectra of 16α-OHE_1-A complex (———) and A (·····). Insert: SDS–polyacrylamide gel electrophoresis of 16α-OHE_1-A complex and control. Lanes: 1- A, 2- 16α-OHE_1-ER complex.

3.2. Presence of anti-16α-OHE_1-A antibodies in the sera of SLE patients

SLE sera (n = 100) were screened for the presence of autoantibodies against 16α-OHE_1-A, 16α-OHE_1 and A by direct binding ELISA. We have chosen 100 sera out of 150 SLE patients that showed higher binding with this antigen. Almost all the chosen sera from the SLE patients showed higher binding with 16α-OHE_1-A in contrast to 16α-OHE_1 ($p < 0.001$) or A ($p < 0.05$) (Fig. 2). No specific binding were shown by sera from control subjects with either of the antigen. Binding was also checked with A and 16α-OHE_1, and it was observed that their binding is less in comparison to 16α-OHE_1-A. The recognition was highest for DSLE patients with 16α-OHE_1-A, which was even higher than for the overall SLE ($p < 0.05$). Specific recognition of SLE autoantibodies against this antigen was also checked by competition ELISA. 16α-OHE_1-A demonstrates maximum inhibition of about 60 ± 5.3% (range, 15.5%–75.5%) in the sera of 100 SLE patients, while for A and 16α-OHE_1, it was 35.3 ± 3.2% (range, 6.2%–58.3%) and 11.8 ± 3.2% (range, 1.8%–16.5%) respectively (Fig. 3a). DSLE patient's sera (n = 65) showed an inhibition of 64.7 ± 5.8 (21.8%-87.3%) with 16α-OHE_1-A as an inhibitor, which was higher than the SLE.

SLE IgG was purified on Protein A-Agarose column by affinity chromatography according to manual instructions given with the column (Sigma, USA). Purity of IgG from the column was checked on SDS-PAGE under non-reducing conditions, and it was found that IgGs showed single homogenous band on the gel and was also eluted as a single symmetrical peak on the column. In competition ELISA, SLE autoantibodies showed an inhibition of 69.8 ± 5.6% (range, 25.5%–88.3%) for 16α-OHE_1-A (Fig. 3b). A showed an inhibition of about 38 ± 3.6% (range, 11.9%–61.3%), while 16α-OHE_1 showed an inhibition of 15.3 ± 7.1%. We have also tested the inhibition values

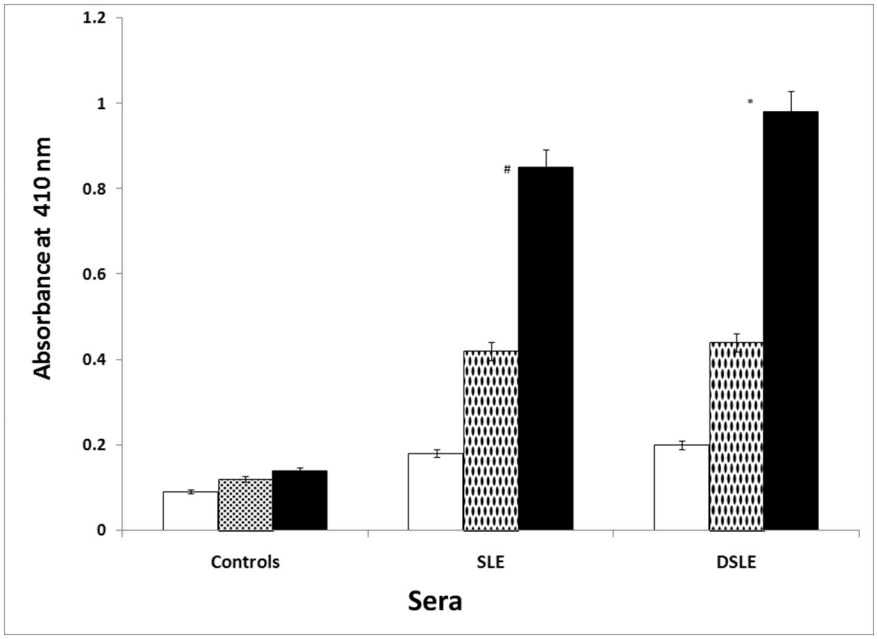

Fig. 2. Direct binding ELISA of control, SLE and DSLE patients. Direct binding enzyme-linked immunosorbent assay of control (n = 37), SLE (n = 100) and DSLE patient's antibodies (n = 65) to 16α-OHE_1-A (■), A (▒) and 16α-OHE_1 (□). Microtitre plates were coated with 100 µl of respective antigen (2.5 µg/ml). The reaction was developed with p-nitrophenyl phosphate as the substrate and the absorbance was recorded at 410 nm as describe in "Materials and methods". Each histogram represents the mean ± SD. $^{\#}p < 0.001$, significantly higher binding than normal sera, $^{*}p < 0.05$, significantly higher binding than SLE, $^{*}p < 0.001$, significantly higher binding than normal sera.

according to various clinical characteristics in these patients (Table 2). Among all, depressed SLE (DSLE) patients showed highest inhibition (i.e. 75.8 ± 8.3%), followed by the SLE patients who were anti-dsDNA positive (73.8 ± 5.9%), photosensitivity (72.5 ± 8.9%), anti-$R_{\bigcirc}$/La positivity (72.2 ± 8.9%), rash (71.3 ± 8.1) and mouth ulcers (70.4 ± 6.1). Inhibition values was more or less same in other group of SLE patients that were having fever, fatigue, weightloss, anti-phospholipid antibodies and smoking.

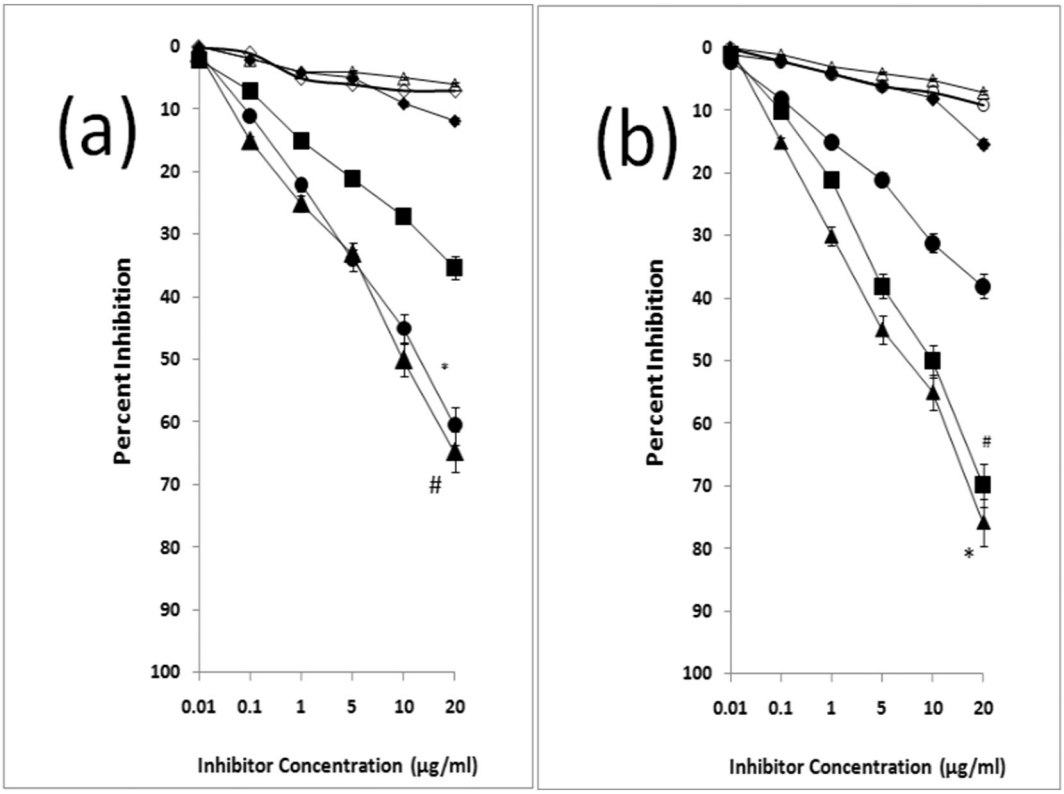

Fig. 3. Inhibition ELISA of control, SLE and DSLE patients. a) Inhibition ELISA of anti-(16α-OHE_1-A, A, 16α-OHE_1) SLE & DSLE (-●-, -■-, -◆- & -▲-) and Control (-Δ-, -◇-) sera with 16α-OHE_1-A, A. b) Inhibition of SLE & DSLE anti-(16α-OHE_1-A, A, 16α-OHE_1) IgG binding to 16α-OHE_1-A (-■- & -▲-), A (-●-), 16α-OHE_1 (-◆-). (-Δ-, -◯-) Represent the inhibition of normal anti-16α-OHE_1-A and A IgG binding to 16α-OHE_1-A and A. Microtitre plates were coated with respective antigens (2.5 µg/ml). Immune complexes were prepared by mixing 100 ml of 1:100 dilution of serum antibodies from SLE patients and control individuals, with the increasing amount (0–20 mg/ml) of respective antigens at 37 °C. *Note:* Inhibition values for control sera and IgG with 16α-OHE_1 were negligible and are not shown. $^{*\#}$Significantly higher inhibition than A ($p < 0.05$, $p < 0.05$) and 16α-OHE_1 ($p < 0.001$, $p < 0.001$). Each points represent mean ± SD for 100 SLE sera/IgGs, 65 DSLE sera/IgGs and 37 controls sera/IgGs.

Table 2
Clinical characteristics and immunological data of different SLE patients.

SLE patients (n = 100)*	Maximum percent (%) inhibition at 20 μg/ml		
	16α-OHE_1-ER[a]	A[b]	16α-OHE_1[c]
Overall	69.8 ± 5.6	38 ± 3.6	15.3 ± 7.1
Depressed (DSLE) patients (n = 65)	75.8 ± 8.3	39.8 ± 9.1	15.5 ± 4.1
Fever (n = 35)	68.4 ± 5.2	38.8 ± 3.1	13.2 ± 3.2
Fatigue (n = 40)	67.9 ± 4.7	37.7 ± 4.2	14.3 ± 2.1
Weight loss (*n* = 21)	68.9 ± 6.3	38.1 ± 3.4	15.8 ± 3.4
Rash (*n* = 31)	71.3 ± 8.1	39.1 ± 4.3	14.3 ± 8.1
Photosensitivity (*n* = 57)	72.5 ± 8.9	38.4 ± 5.1	14.9 ± 3.4
Anti-dsDNA (*n* = 61)	73.8 ± 5.9	40.8 ± 3.3	15.9 ± 3.2
Anti-Ro/La positivity (n = 21)	72.2 ± 8.9	38.4 ± 3.2	15.8 ± 4.1
Anti-phospholipid antibodies (*n* = 19)	69.8 ± 5.6	39.3 ± 5.2	14.8 ± 3.2
Smoking (*n* = 18)	68.4 ± 9.9	39.8 ± 3.9	11.4 ± 3.1
Mouth ulcer (n = 35)	70.4 ± 6.1	38.3 ± 4.2	11.8 ± 9.3

The experiments were carried out by incubating ELISA plate with 100 μl of different antigens (2.5 μg/ml) as described in "Materials and methods" section; mean ± SD.

* 16α-OHE_1-A vs A or 16α-OHE_1 ($p < 0.05$, $p < 0.001$).

[a] 16α-OHE_1-A as inhibitor.

[b] A as inhibitor.

[c] 16α-OHE_1 as inhibitor.

The inhibition values were also tested according to what they were taken as medication and secondary complications in these SLE patients. We have divided them into 6 groups based on what medications they have taken during the course of the study. Twenty eight of them remains untreated, 60 were taken anti-depressant (Duloxetine), 31 azathioprine, 50 prednisolone, 25 methotrexate and 19 cyclophasphamide (Fig. 4). Among the treat groups, SLE patients who were taken anti-depressant showed the highest inhibition (63.1 ± 8.9%), followed by those SLE patient who were taken cyclophasphamide (45.2 ± 6.9%), methotrexate (38.8 ± 5.8%), azathioprine (21.1 ± 9.8) and prednisolone (17.5 ± 7.8%). Untreated SLE patients showed an inhibition of about 61.9 ± 8.2%. The inhibition values were also tested according to various secondary complications in these SLE patients (Fig. 5). We again divided them into 10 groups based on what types of secondary complications they are having during SLE. Among them, those SLE patients that are having neurological symptoms showed highest inhibition (65.8 ± 7.1%) followed by alopecia areata (62.4 ± 6.9%), rheumatoid arthritis (61.3 ± 7.2%), kidney disease (60.5 ± 9.1), leukopenia (58.1 ± 4.6), serositis (57.9 ± 5.6%), anemia (53.3 ± 8.9%), pulmonary disease (51.9 ± 7.1%), thrombocytopenia (48.3 ± 6.5%) and thyroid pathology (41.1 ± 5.9%).

The antibody affinity against 16α-OHE_1-A and other antigen (16α-OHE_1 and A) was also evaluated by quantitative precipitation titration in SLE and DSLE patients. In this technique, increasing amount of various antigens (16α-OHE_1-A, 16α-OHE_1 and A) were incubated with the constant amount of SLE and DSLE IgG (100 μg, $n = 20$). Normal human IgGs, who were treated under identical condition with the same antigens, were served a negative controls. 26 μg of 16α-OHE_1-A was bound to about 73 μg of SLE IgG and 22 μg of 16α-OHE_1-A was bound to about 79 μg of DSLE patient's IgG. While, 40 μg of A was bound to about 61 μg of SLE IgG and about 46 μg of 16α-OHE_1 was bound to about 60 μg of SLE IgG. The apparent association constant was calculated from Langmuir plot to determine affinity of SLE IgG with 16α-OHE_1-A (Fig. 6). The affinity constant was found to be 1.24×10^{-7} M, 1.52×10^{-6} M and 1.18×10^{-6} M for 16α-OHE_1-A, A and 16α-OHE_1 with SLE IgG respectively. Again for DSLE patients, the constant was found to be highest with an order of 1.16×10^{-7} M, and showed maximum affinity with 16α-OHE_1-A.

3.3. Production and characterization of anti-16α-OHE_1-A antibodies

The antigenicity of 16α-OHE_1-A was determined by inducing antibodies against this complex. We have taken female rabbits to induced antibodies against 16α-OHE_1-A along with suitable controls. Their antigenicity was confirmed by direct binding and competition ELISA. 16α-OHE_1-A was found to be highly immunogenic, inducing high titer antibodies (≥1:25,600). In contrast, pre-immune sera did not show any appreciable titer and served as negative control. After immunization with A, the anti-serum showed almost same titer (but low) as compared to 16α-OHE_1-A. The titer shown by 16α-OHE_1 was found to be negligible. Competition ELISA was used to further validate specificity of induced antibodies. An inhibition of 75.3 ± 7.1% in antibody activity

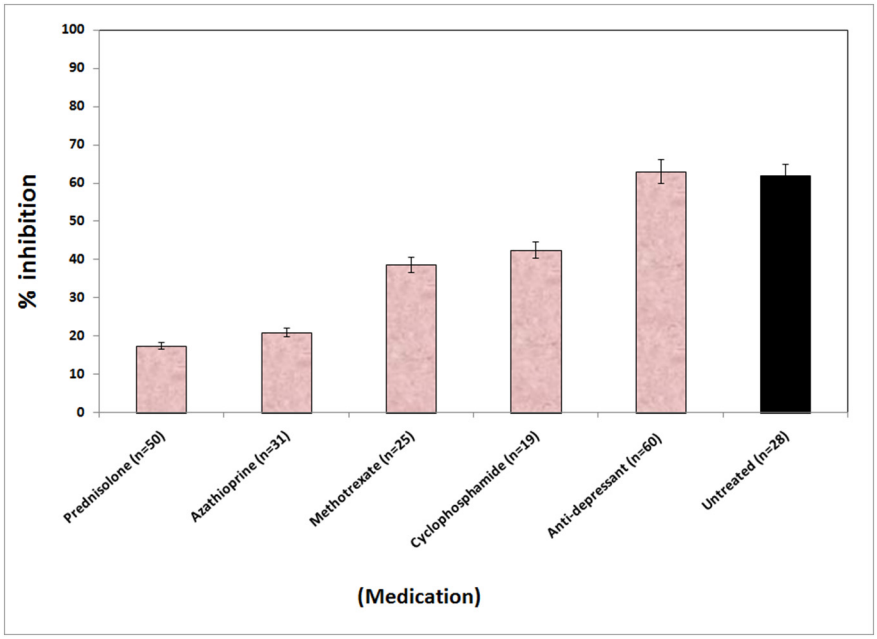

Fig. 4. Detection of autoantibodies according to medication in different SLE patients by competition ELISA. Microtitre plates were coated with respective antigens (2.5 μg/ml) and values are presented in % inhibition as describe in Material and methods.

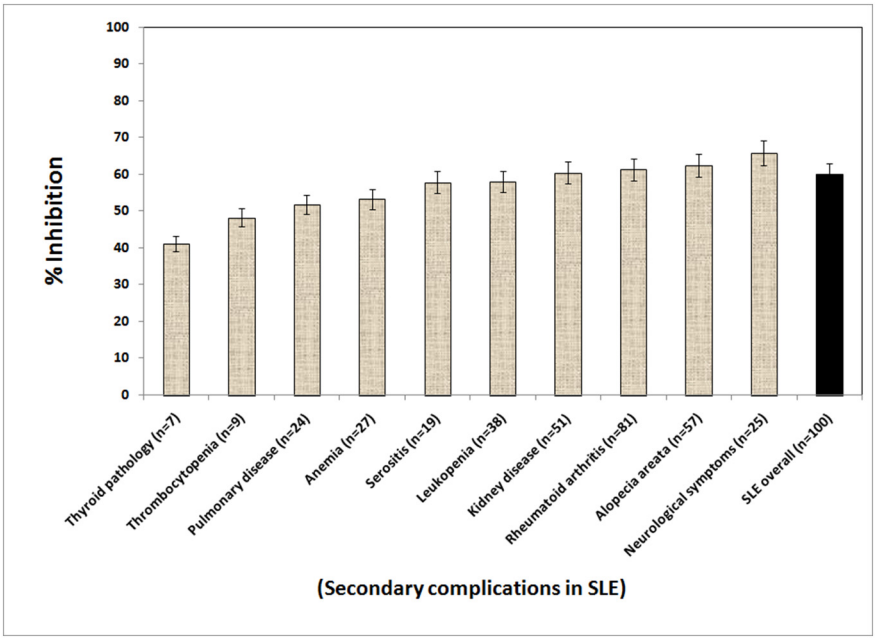

Fig. 5. Detection of autoantibodies according to various secondary complications in SLE patients by competition ELISA. Microtitre plates were coated with respective antigens (2.5 μg/ml) and values are presented in % inhibition as describe in Material and methods.

(anti-16α-OHE_1-A antibodies) was observed at 20 μg/ml and 50% inhibition was observed at only 4.9 μg/ml (Fig. 7a). In case of A, a maximum of about 70.3 ± 4.2% inhibition in the antibody activity was detected at an A concentration to 20 μg/ml and 50% inhibition was observed at 10.3 μg/ml of immunogen. While for 16α-OHE_1, an inhibition of 64.8 ± 5.1% in the antibody activity was observed at an immunogen concentration of 20 μg/ml and 50% inhibition was achieved at 13.1 μg/ml (Fig. 7a).

Immunized IgG was isolated and purified by affinity chromatography on Protein A-Agarose column (Sigma, USA). Direct binding ELISA demonstrates high binding of induced antibodies (anti-16α-OHE_1-A antibodies) with 16α-OHE_1-A. Pre-immune IgG showed negligible binding and served as a negative control. Competition ELISA was used to further confirm specificity of the induced IgG. In competition ELISA, the anti-16α-OHE_1-A antibodies showed an inhibition of about 94.5 ± 7.2% in antibody binding at 20 μg/ml of the immunogen concentration (Fig. 7b). Fifty percent inhibition was achieved only at 3.8 μg/ml. For A, a maximum of about 91.3 ± 6.5% inhibition in antibody was observed with the immunogen and 50% inhibition was achieved at 7.1 μg/ml. While for 16α-OHE_1, an inhibition of 83.8 ± 5.8% in antibody activity was observed with the immunogen as inhibitor. Fifty percent inhibition was achieved at 11.1 μg/ml (Fig. 7b).

The antigenic specificity of the induced antibodies (against 16α-OHE_1-A, A and 16α-OHE_1) was also checked by competition ELISA using 16α-OHE_1-A, 16α-OHE_1, A, BSA (bovine serum albumin), 4-OHE_1 (4-hydroxyestrone), 2-OHE_1 (2-hydroxyestrone) as inhibitor (Table 3). Anti-16α-OHE_1-A antibodies showed no binding toward all other antigen except 16α-OHE_1-A and 16α-OHE_1. However, anti16α-OHE_1 antibodies showed cross-reactivity toward 16α-OHE_1-A in addition to the binding shown toward its own immunogen (i.e. 16α-OHE_1). While anti-

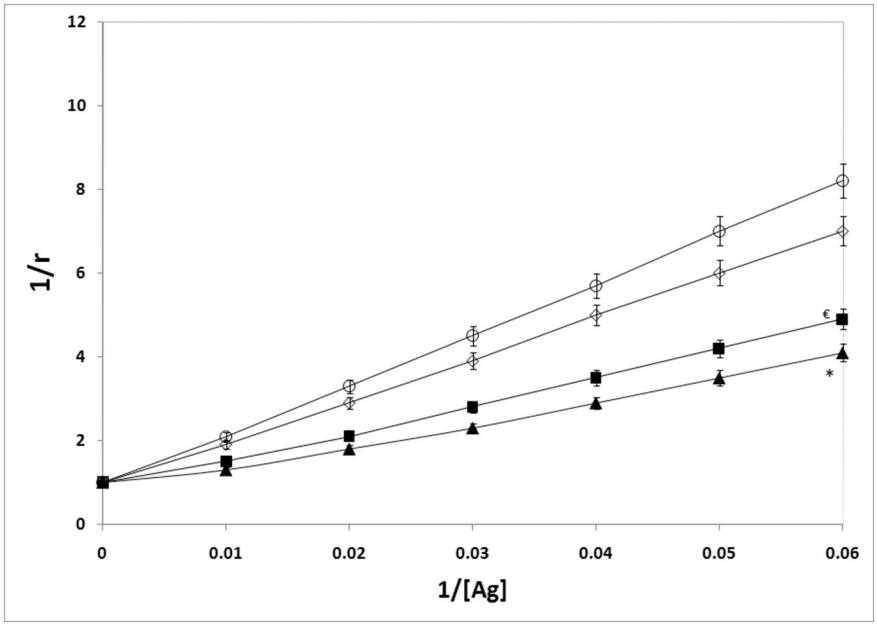

Fig. 6. Determination of an apparent association constant by Langmuir plot. Antigens were 16α-OHE_1-A (-■- & -▲-), A (-◇-) and 16α-OHE_1 (-◯-). Immune complexes were prepared by incubating 100 mg of IgG (SLE, DSLE and controls) with varying amount of different antigens (0–100 mg) in an assay volume of 500 μl for 2 h at room temperature and overnight at 4 °C. The binding data were analyzed for antibody affinity as described in Material and methods. €Significantly higher binding than A ($p < 0.05$) and 16α-OHE_1 ($p < 0.001$). *Significantly higher binding than A ($p < 0.05$) and 16α-OHE_1 ($p < 0.001$).

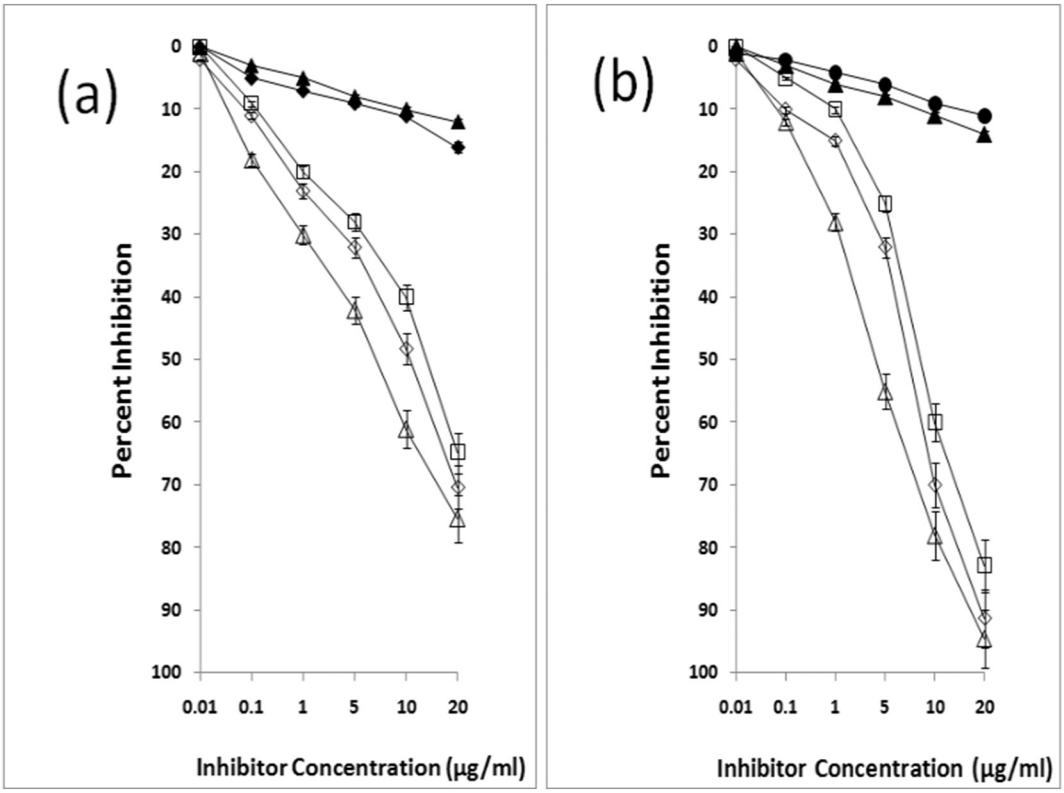

Fig. 7. Inhibition ELISA of immunized antigens. a) Inhibition ELISA of immune sera (-Δ-, -◇-, -□-) and pre-immune sera (-♦-, -▲-) pre-incubated with 16α-OHE_1-A, A, 16α-OHE_1, on binding to antigen coated plate. b) Inhibition ELISA of immune IgG pre-incubated with 16α-OHE_1-A (-Δ-), A (-◇-), 16α-OHE_1 (-□-), on the binding to antigen coated plates. (-▲-, -●-) represent the inhibition of pre-immune anti-16α-OHE_1-A and A IgG binding to 16α-OHE_1-A and A. The experiments were carried out by incubating ELISA plate with 100 µl of respective antigens (2.5 µg/ml) as described in "Materials and methods".

Table 3
Relative affinity of induced antibodies toward different inhibitors.

Inhibitors	Maximum % inhibition at 20 µg/ml	Concentration for 50% inhibition (µg/ml)	Percent relative affinity
Anti-16α-OHE_1-A antibodies			
- 16α-OHE_1-A	94.5	3.8	100
- A	21.8	_[b]	_
- 16α-OHE_1	70.5	6.1	62.2
- BSA[a]	17.9	_	_
- 2-OHE_1	13.5	_	_
- 4-OHE_1	12.3	_	_
Anti-A antibodies			
- A	91.3	7.1	100
- 16α-OHE_1-A	20.8	_	_
- 16α-OHE_1	12.5	_	_
- BSA	56.4	13.8	51.4
- 2-OHE_1	9.9	_	_
- 4-OHE_1	11.4	_	_
Anti-16α-OHE_1 antibodies			
- 16α-OHE_1	83.8	11.1	100
- 16α-OHE_1-ER70.1	14.1	78.7	
- A	9.3	_	_
- BSA	8.9	_	_
- 2-OHE_1	12.4	_	_
- 4-OHE_1	15.3	_	_

The experiments were carried out by incubating ELISA plate with 100 µl of different antigens (16α-OHE_1-A, A, 16α-OHE_1) at 2.5 µg/ml. Rest of the experimental conditions were explained in "Material and Methods".

[a] BSA: Bovine Serum Albumin, 2-OHE_1: 2-Hydroxyestrone, 4-OHE_1: 4-Hydroxyestrone.

[b] 50% inhibition was not achieved.

A antibodies showed some cross-reactivity with BSA (Table 3). Immunocross-reactivity of anti-16α-OHE_1-A antibodies toward 16α-OHE_1 allow us to use these antibodies as a probe to estimate 16α-OHE_1 in the serum of different SLE patients, which was further confirmed by commercially available kit (Human 16α-hydroxyestrone ELISA Kit). The mean value of 16α-OHE_1 was found to be 37.3 ± 8.9 pg/ml by anti-16α-OHE_1-A antibodies, which is comparable to the values obtained by using Human 16α-hydroxyestrone ELISA Kits (38.4 ± 10.8 pg/ml) (Table 1). In control subjects, the mean values were 13.3 ± 4.8 pg/ml. The concentration of 16α-OHE_1 in the serum of SLE patients was found to be significantly higher than controls ($p < 0.05$). The concentration of IL-6 and IL-17 in SLE patients was found to be 13.8 ± 4.3 pg/ml and 19.8 ± 5.9 pg/ml respectively. While in controls, it was 8.3 ± 1.8 pg/ml and 10.1 ± 3.1 pg/ml. The concentration of IL-6 and IL-17 in SLE patients was found to be significantly higher than controls ($p < 0.05$, $p < 0.001$). Concentration of IL-10 and BAFF (B-cell activating factor) in SLE patients was found to be 27.8 ± 5.2 pg/ml 6.3 ± 1.2 ng/ml, which was found to be significantly higher as compared to controls ($p < 0.01$, $p < 0.01$) (values are 5.4 ± 1.1 pg/ml and 1.8 ± 0.3 ng/ml, respectively).

4. Discussion

Systemic lupus erythematosus (SLE) is a chronic condition involving multiple organ systems, leads to functional disability and significant depressive symptom. These symptoms vary according to the disease activity and include fever, arthritis, fatigue, weight loss, autoantibodies and other clinical manifestations. There are various factors contributing to the depression and anxiety in SLE. These include socio-cultured and environmental factors, disease activity and its severity, age and sex [24]. SLE patients facing both physical and psychological symptoms during the disease. Due to large complexity in this disease, the causes of

depression are not known. Some biological mechanisms have been associated because depression and anxiety in lupus is linked with other complications such as CNS disease, higher disease activity and various clinical manifestations [25]. Examination of various factors including depression might give us some clues to underlying mechanism in the etiopathogenesis of SLE. In this study, the binding characteristics of autoantibodies from the sera of 100 SLE and 37 normal subjects to 16α-OHE_1-A, 16α-OHE_1 and A, were checked by direct binding, competition ELISA and quantitative precipitin titration. The 16α-OHE_1-A showed preferentially higher binding with SLE sera in comparison to A ($p < 0.05$) or 16α-OHE_1 ($p < 0.001$). The data showed that 16α-OHE_1-A can function as an effective inhibitor, showing a substantial difference in the recognition of 16α-OHE_1-A over A or 16α-OHE_1. The data shown here is same as the previous studies that showed enhanced recognition of estrogen modified antigen by SLE autoantibodies [11]. Formation of complex exposed unique epitopes on the molecules that were showing high binding to these SLE autoantibodies.

To further confirm the specificities of SLE autoantibodies against 16α-OHE_1-A, quantitative precipitin titration was performed. The affinity constant for 16α-OHE_1-A clearly indicates better recognition of this antigen over A or 16α-OHE_1. Higher affinity of 16α-OHE_1-A by SLE autoantibodies demonstrates the possible participation of 16α-OHE_1-A in SLE pathogenesis. Therefore, epitopes generated by formation of this adduct is of unique type, that was well recognized by SLE autoantibodies. We have also tested recognition of SLE autoantibodies according to various clinical characteristics and the data showed that the recognition was highest for DSLE patients followed by anti-dsDNA positive SLE patients, SLE with rush and mouth ulcer patients. Depression and anxiety are the common and early symptoms associated with early onset of SLE [26]. IL-6 was found to be significantly high in SLE patients compared to healthy controls [27]. This interleukin (i.e. IL-6) play an important role in acquired immune response by stimulation of antibody production and effector T-cell development [27]. These antibodies produce in response to IL-6 might bind to 16α-OHE_1-A and showed higher recognition in comparison to other antigens. We have also found that IL-10 and BAFF was significantly high in SLE patients compared to normal controls. Higher serum level of IL-10 and BAFF augment autoantibodies production and correlate with SLE disease activity [28,29]. High binding to this complex might be due to the autoantibodies production by IL-10 and BAFF in these SLE patients. Earlier studies have shown that elevated levels of prolactin are associated with increased production of antibodies in SLE and hyperprolactinemia is also associated with depression [30,31], so binding is more due to these two factors. All the other groups of SLE patients with different clinical characteristics might have high antibody response or affected by some autoimmune conditions, so might showed high binding with 16α-OHE_1-A. Effect of medication on the binding of SLE autoantibodies to 16α-OHE_1-A was also done. The antibodies showed maximum inhibition to those SLE patients that were taken anti-depressant followed by untreated patients, SLE patients on cyclophasphamide, methotrexate, azathioprine and prednisolone. Again, those SLE patients with anti-depressant might linked with depression which showed high binding just as DSLE patients. Although, untreated patients already have high levels of autoantibodies- these are not either suppressed or controlled by any drugs. Furthermore, the other groups showed lowered inhibition because all of these drugs linked with the suppression of immune system and somehow caused less autoantibodies production and that further showed lower inhibition.

We have also tested the binding according to various secondary complications they are having during SLE. Among them, those SLE patients that are having neurological symptoms showed highest inhibition followed by alopecia areata, arthritis, kidney diseases, leukemia, serositis, anemia, pulmonary disease, thrombocytopenia and thyroid pathology. Neurological manifestations have been well recognized and studied in SLE [32]. Although, multiple organs have been involved in SLE but the nervous system is commonly affected in both children and adults with SLE [33,34]. In addition, neurological manifestations had been associated with antiphospholipid antibodies [34]. The American College of Rheumatology (ACR) established nomenclature and case definitions for neuropsychiatric lupus syndromes [35]. Anti-cardiolipin and anti-glutamate receptor antibodies might play a role in cognitive dysfunction and psychiatric disease in patients with SLE [36,37]. Subsets of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in SLE patients [38]. These autoantibodies might also cross reacts with 16α-OHE_1-A and showed higher inhibition. Alopenia areata and arthritis occurs as a result of some autoimmune phenomena [39,40], that generate autoantibodies against various molecules. These autoantibodies might show high binding with 16α-OHE_1-A. As far as the high binding of kidney disease is concern, this disease in SLE patients might occur from various factors and one of the factors is glomerulonephritis: which is kidney inflammation. Again, high binding is due to the autoantibodies produced during inflammation in the kidney. All the other groups showed almost same or low binding as compared to SLE patients.

Quantitative precipitin titration was performed for the detection of interaction of SLE autoantibodies with 16α-OHE_1-A. The affinity constant of the order of 10^{-7} clearly indicates better recognition of 16α-OHE_1-A over A or 16α-OHE_1. The high binding of this complex by SLE autoantibodies clearly indicates the presence of this complex or some of its epitopic regions in the serum of these SLE patients. Earlier studies have shown that 16α-hydroxyestrone is formed in various tissues such as bile, urine and blood [41]. Albumin is the most abundant serum protein in the blood. Therefore, 16α-hydroxyestrone and albumin come in contact with each other and make a complex, which was highly recognized by SLE autoantibodies. The data shown here is exactly same as the previous study that showed high binding of catecholestrogen modified DNA by SLE autoantibodies [5,12]. Therefore, it could be possible that 16α-OHE_1-A adduct might be one of the contributing factors for the generation of SLE autoantibodies.

In conclusion, our data demonstrates that combined action of 16α-OHE_1 and A produces unique epitopes, which were efficiently recognized by SLE autoantibodies. Furthermore, DSLE/anti-depressant taken SLE patients/SLE with neurological manifestations are showing high binding with 16α-OHE_1-A as compared to other groups, indicating that these groups of patients recognizing 16α-OHE_1-A more efficiently. The possible mechanism involves the production of SLE autoantibodies due to the generation of pro-inflammatory cytokines (IL-6, IL-17). These cytokines act as mediator for inflammation [42,43] and higher in SLE patients. Inflammatory cytokines (IL-6, IL-17) causes the generation of SLE autoantibodies that showed stronger recognition to 16α-OHE_1-A adduct (Fig. 8). The roles of IL-10 and BAFF were also taken into consideration because these cytokines increased the production of autoantibodies in SLE patients, these autoantibodies might show strong recognition to this complex. High prolactin levels have been associated with increased production of antibodies in SLE and hyperprolactinemia is linked to depression. Binding might be high due to these factors also. This study showed the possible participation of 16α-OHE_1-A in generating neo-epitopes that might form one of the factors for the generation of SLE autoantibodies.

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Conflict of interest

All authors declare that they have no conflict of interest.

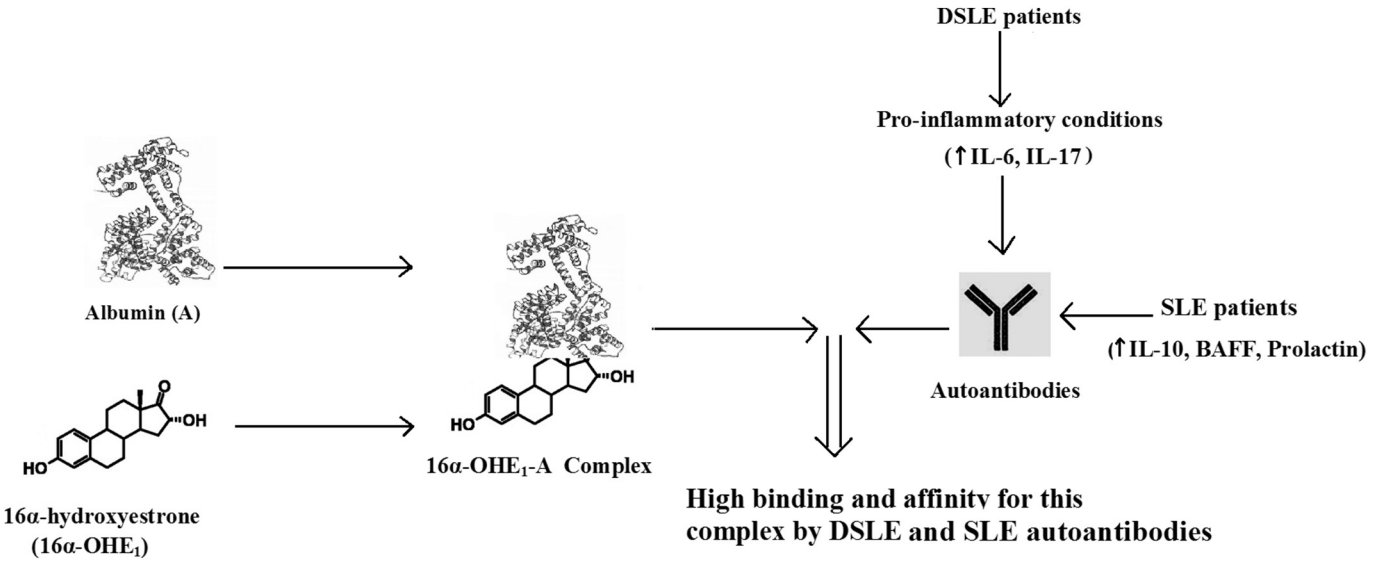

Fig. 8. The proposed mechanism for the generation of high affinity autoantibodies in DSLE and SLE patients.

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