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The non-enzymatic glycation of LDL proteins results in biochemical alterations - A correlation study of Apo B_{100}-AGE with obesity and rheumatoid arthritis

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ABSTRACT

Advanced glycation end-products (AGEs) can aggregate amid incessant inflammation, as may be available in patients with rheumatoid arthritis. D-Ribose reacts more promptly than glucose monosaccharide to the proteins and forms heterogeneous group of products known as AGEs. Obesity includes persons with provocative joint inflammation with increased lipid profile. Immunogenic evidences recommend a cross-sectional relationship between glycated LDL-Apo B100 and inflammation.

The point of this examination was to look at the connection between D-ribose glycated ApoB100 (ApoB100-AGE) with obesity and rheumatoid arthritis. The binding specificity of auto-antibodies against ApoB100-AGE antigen present in obesity and rheumatoid arthritis patient's serum were inspected by direct binding and was further established by competitive inhibition ELISA. In the present study, hydroxyl radical, superoxide radical, ketoamine moieties, hydroxyl-methyl furfural (HMF) and carbonyl substances were evaluated in the patients' serum via respective specific methods. The prevalence of auto-antibodies against ApoB100-AGE antigen was recorded to be 58% and 52.86% from obese and rheumatoid arthritis patient respectively in contrast to its native analogue ($P < 0.001$). Moreover, the autoantibodies present in obese and arthritis patients were found to be highly specific towards ApoB100-AGE as confirmed by inhibition ELISA.

1. Introduction

Obesity is characterized by an increase in body weight by excessive fat accumulation [1] and has also been documented as a major fundamental factor in the pathogenesis of various diseases [2]. Rheumatoid arthritis (RA) is a long-lasting inflammatory autoimmune disorder which chronically interrupts the multiple systems. RA is described by invasion of provocative cells into the synovium and synovial hyperplasia which finally lead to the destruction of bone along-with articular cartilage. The occurrence of RA is around 1–2% of the world total population with women prevalence is more as compared to the men [3,4].

RA also links with altered body composition. The chronic inflammation of the disease, particularly trigger metabolic alterations [5], activation of the nuclear factor kappa-β (NF-κβ) pathway, leading to the degradation of lean tissue/muscle mass, a condition known as Rheumatoid Cachexia [6] which has received significant researchers attention [7].

Lipoproteins have a key role in various disease states and a rheumatoid arthritis risk in obese patients reported the positive association of induced LDL-cholesterol and inflammation [8,9].

The basic system forming LDL is identified with over production of apolipoprotein B100 (ApoB100) and restricted release of LDL by the LDL-receptor in which both peripheral and hepatic tissues participates [10]. ApoB100 of LDL might be impacted by harm caused by glycation, yet the quantitative measures of damage in healthy human population and obese or RA patients remain unclear. Moreover, the damage caused by AGE to the ApoB100 and other AGE-altered proteins are pro-inflammatory in nature [11].

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The pro-inflammatory properties of modified ApoB100 of LDL appear to be considerably enhanced as a consequence of the induced immunogenicity and its role in several disease states like, diabetes and its secondary complications [12,13].

D-ribose is a standout amongst the most accessible sugar which assumes a vital part in the glycation of biomolecules. Besides, the bioavailability of D-ribose makes this carbonyl species very reactive and harming, subsequently having direct ramifications in diseases and other complexities. As of late, our examination has worked on the role of D-ribose glycated LDL induced immunogenicity in diabetes and their associated secondary complications such as atherosclerosis [12,13]. Till now, no investigation conveyed the D-ribose glycated ApoB100 (D-ribose-ApoB100) role in obesity and RA. Hence, this investigation was done to investigate the role of D-ribose glycated ApoB100 in obese and RA by probing the prevalence of auto-antibodies against it.

2. Material and methods

2.1. Study population

This investigation contains total of 220 subjects in which there were 100 obese and 70 rheumatoid arthritis patients sera, as characterized by the World Health Organization (WHO) and American College of Rheumatology (ACR) guideline individually were selected from the Integral Institute of Medical Sciences and Research (IIMS&R), Lucknow, India and Rheumatology department of the King George Medical University (KGMU), Lucknow, India. In both gathering of patients, none of the patients were on lipid lowering or anti-inflammatory drugs. The samples of 50 healthy subjects were used as negative control.

2.2. Inclusion/exclusion criteria

2.2.1. Inclusion criteria

Following inclusion criteria were kept for patient recruitment.

A. Patients with obese and rheumatoid arthritis (based on WHO and ACR criteria).
B. Age group was kept 30–75 years.

2.2.2. Exclusion criteria

A. The subject should not be having chronic disease.
B. The subject should not be having acute infection.
C. The subject should not be on lipid lowering drugs.
D. Unstable disease indicated by a change of Disease Modifying Anti-Arthritic Drugs (DMAADS) in the previous 3 months or Surgery to the upper limb during the previous 6 months (in Arthritis case).

2.3. Collection of serum samples

Five milliliters of blood samples were collected from obese and rheumatoid arthritis patients attending the OPD in IIMSR and KGMU, Lucknow, India after the informed consent. The examination has been completed as per the declaration of Helsinki. The isolated bloods were kept at room temperature for clotting and sera were then isolated by spinning slowly for 3–5 min. The complement proteins present in the sera were de-complemented by heating it at 56 °C for 30 min. Post complementation the sera were then stored at −20 °C with 0.01% sodium azide as a preservative.

2.4. Biochemical studies

Every biochemical parameter lke plasma glucose, HbA1c, lipid profile, Erythrocyte Sedimentation Rate (ESR), Rheumatoid Factor (RF), Uric Acid (UA), Anti-Nuclear Antibody (ANA), Hemoglobin (Hb), High-Sensitivity C-Reactive Protein (hs-CRP) were collected from clinical laboratory of IIMS&R and KGMU.

2.5. Preparation of D-ribose-ApoB$_{100}$

ApoB$_{100}$ of isolated LDL from the healthy subjects was modified by using 80 mM concentrations of D-ribose as performed earlier [14]. In brief, ApoB$_{100}$ (62.5 μg/ml) was glycated with a pentose sugar, D-Ribose. The reaction was performed at 80 mM concentration of D-ribose and then incubated at 37 °C for three weeks. Upon reaction completion, the glycated samples were dialysed in 100 mM PBS, pH-7.4.

2.6. Estimation of superoxide and hydroxyl radical

The quantitation of $O_2^{\bullet -}$ ion in serum was performed by cytochrome-c reduction assay as described previously [15].

Detection of •OH radicals in serum samples were carried out by measuring thiobarbituric acid (TBA) reactive 2-deoxy-D-ribose oxidation products. The degradation of 2′deoxyribose was estimated by adding 1 ml of 2.8% (w/v) trichloro acetic acid, 1 ml of 1% (w/v) TBA followed by heating at 100 °C for 10 min. The absorbance was finally read at 532 nm.

2.7. Determination of protein-bound carbonyl groups

The carbonyl contents were quantified as described previously [16]. Briefly, carbonyl substance were estimated in healthy, obese and Rheumatoid patients' sera utilizing 2,4-dinitrophenylhydrazine. The absorbance was examined at 360 nm and the carbonyl substance was calculated by extinction coefficient (22,000 M^{-1} cm^{-1}).

2.8. NBT assay

The ketoamine moieties was calculated in healthy and patients' sera samples by nitroblue-tetrazolium reduction assay as represented earlier [12,13].

2.9. HMF estimation

Hydroxy-methyl-furfural (HMF) was calculated in patients sera as described previously [13]. The HMF (nmol/ml) was quantified using molar extinction coefficient (4×10^4 M^{-1} cm^{-1}) at 443 nm [13].

2.10. Direct binding enzyme linked immunosorbent assay (ELISA)

Direct binding ELISA was performed on polystyrene plates as published previously [17,18] with slight modifications. The results are pronounced as mean of absorbance of test sample minus mean of absorbance of the control sample.

2.11. IgG isolation

Immunoglobulin-G (IgG) was isolated from patients (obese and rheumatoid arthritis) and healthy control through a protein A-Agarose column as described previously [19,20].

2.12. Competitive inhibition ELISA

The specificity of antibodies was quantitated by competition inhibition ELISA [21,22]. Immune complexes were prepared as published previously [16], thus the immune complexes were then coated in the wells instead of serum and the left over stages were alike direct binding ELISA. The percent inhibition was estimated by following equation - Percent Inhibition $= 1 - (A_{inhibited}/A_{uninhibited}) \times 100$.

Table 1
General clinical and metabolic characteristic of the study objects.

Type of patient	Selected patients
Obese	Age: 25–70 years BMI: >25 kg/mt^2 WHR: >1.0 Glucose level: Fasting: <126 mg/dl Post Prandial (P.P.): <200 mg/dl HbA1c: <6.5%
Rheumatoid arthritis	Age: 25–70 years Erythrocyte Sedimentation Rate (ESR): >40 mm/hour Rheumatoid Factor (RF): >20 IU/ml Uric acid (UA): >7.4 mg/dl (male), > 5.8 mg/dl (female) Anti-Nuclear Antibody (ANA): >1:20 Hb: <13 g/dl (Male), <12 g/dl (Female) Joint swelling and redness hs-CRP: >3 mg/dl

2.13. Statistical analysis

Data are presented as mean ± SD and statistical significance of the data was determined by StatView 5.0.1. The statistical significance was considered at <0.5.

3. Results

3.1. Biochemical assessment in healthy and diseased person's sera

The clinical characteristics and various biochemical parameters for healthy control (HC), obese patients and Rheumatoid patients are given in Tables 1 and 2 respectively. Amongst the key parameters like, HbA1c, it was normal in HC and Rheumatoid patients but for obese it is near to borderline. TC, LDL and TG were higher in obese than Rheumatoid patients and HC, while HDL was moderately lower in obese than Rheumatoid and HC. The TC/HDL ratio in obese was moderately higher than Rheumatoid and HC. In addition, ESR, RF, UA, ANA and hs-CRP level was higher in Rheumatoid while its level was normal in obese and HC subjects (Table 2). Level of Hb was also moderately low in Rheumatoid in comparison to obese and HC (Table 2).

3.2. Determination of superoxide and hydroxyl radical generation

It has been reported that the glycation reaction first produces superoxide anion which later on converted to H_2O_2 and hydroxyl radical by a combination of Fenton-type reactions [15]. Estimated superoxide in obese and Rheumatoid patient's serum samples were 9.47 ± 1.37 nM ml^{-1} h^{-1} and 13.45 ± 1.02 nM ml^{-1} h^{-1} respectively which was much higher as compared to healthy control (1.2 ± 0.25 nM ml^{-1} h^{-1}) (Fig. 1). In addition, induced hydroxyl radical generation was also quantified in patients and healthy subject's serum samples by the estimation of TBARS formation. In the healthy subjects it was 2.2 ± 0.78 nmol TBARS ml^{-1} however; it was significantly enhanced in obese (16.37 ± 2.12 nmol TBARS ml^{-1}) and Rheumatoid patient's serum (17.23 ± 1.77 nmol TBARS ml^{-1}) which confirms the glycation induced hydroxyl radical generation measured in terms of TBARS.

3.3. Quantification of carbonyl contents in sera of normal and patients

Oxidation of $ApoB_{100}$ reasonably results in an augmentation in protein carbonyl compounds, which is a documented biomarker of oxidation leading to the oxidative burst. The standard carbonyls (±SE) of three autonomous assays of patient's serum (obese and Rheumatoid) were 19.5 ± 3.27 μmol mg^{-1} and 26.8 ± 2.56 μmol mg^{-1} protein, respectively (Fig. 2A) whereas healthy subject which is a control serum had just about insignificant level of carbonyl content (9.6 ± 1.47 μmol mg^{-1} protein).

3.4. NBT quantitation

The ketoamine moieties build-up in normal and obese and rheumatoid subjects were estimated colorimetrically by NBT method. Healthy human subjects demonstrated insignificant quantity of ketoamine (7.5 ± 1.46 μmol mg^{-1} protein) however; patients' samples had greatest ketoamine value. Mean ketoamines of 3 autonomous

Table 2
General clinical and metabolic characteristic of the study objects.

Characteristic	HC	Obese	Rheumatoid arthritis
N	50	100	70
Age (years)	32.5 ± 8.6	52.7 ± 18.5	60.3 ± 8.2
Gender (M/F)	25/25	42/58	11/59
BMI (kg/mt^{-2})	21.1 ± 1.7	29.56 ± 3.37	22.7 ± 2.1
WHR	0.7 ± 0.2	1.6 ± 1.1	0.8 ± .0.7
BP (SST) mm Hg	111.5 ± 16.4	142.6 ± 5.17	129.3 ± 13.3
BP (DST) mm Hg	82.2 ± 4.8	101.6 ± 5.9	98.8 ± 9.2
HbA1c (%)	4.3 ± 0.12	5.2 ± 4.2	4.29 ± 3.6
TC (mml^{-1})	152 ± 13.6	272 ± 12.6	171 ± 6.4
LDL (mml^{-1})	145 ± 4.21	248 ± 4.6	152.2 ± 9.2
HDL (mml^{-1})	48 ± 2.1	31 ± 9.6	42.2 ± 5.3
TG (mml^{-1})	178 ± 4.2	206 ± 6.4	179 ± 4.6
TC/HDL	3.33 ± 0.7	8.9 ± 3.7	3.65 ± 0.25
ESR	Male: 14.5±5.2 mm/hour Female: 19.6±5.6 mm/h	Men: 28.7±6.8 mm/hour Women: 32.5±7.3 mm/h	Men: 31.8±5.3 mm/hour Women: 37.5±11.5 mm/h
RF	9±6 IU/ml	17.4±5.9 IU/ml	70.7±34.6 IU/ml
UA	Male: 3.9±1.8 mg/dl Female: 4.2 mg/dl	Male:6.9±3.2 mg/dl Female: 5.9±1.8 mg/dl	Male:9.6±6.3 mg/dl Female: 9.3±4.2 mg/dl
ANA positivity (%)	No	13.5±7.5	45.8±12.2
Hb	Male: 14.7±2.1 g/dl Female: 13±2.8 g/dl	Male: 12.6±3.5 g/dl Female: 11.6.6±1.4 g/dl	Male: 11.6±4.8 g/dl Female: 10.6±4.6 g/dl
Join swelling and redness	No	No	No
hs-CRP	hs-CRP: 1.63±1.7 mmol/L	hs-CRP: 6.4±5.3 mmol/L	hs-CRP: 12.44±11.45 mmol/L

Values are expressed as mean ± SE.
HC: Healthy control; M/F: Male/Female; BMI: Body mass index; BP (SST): Blood pressure (systolic);BP (DST): Blood pressure (diastolic); DD: Duration of disease; HbA1c: Glycosylated hemoglobin; TC: Total cholesterol; LDL: Low density lipoprotein; HDL: High density lipoprotein; TG: Triglyceride; ESR: Erythrocyte sedimentation rate; RF: Rheumatoid factor; UA: Uric acid; ANA: Anti-nuclearantibody; Hb: Hemoglobin;hs-CRP: High sensitivity serum reactive protein.

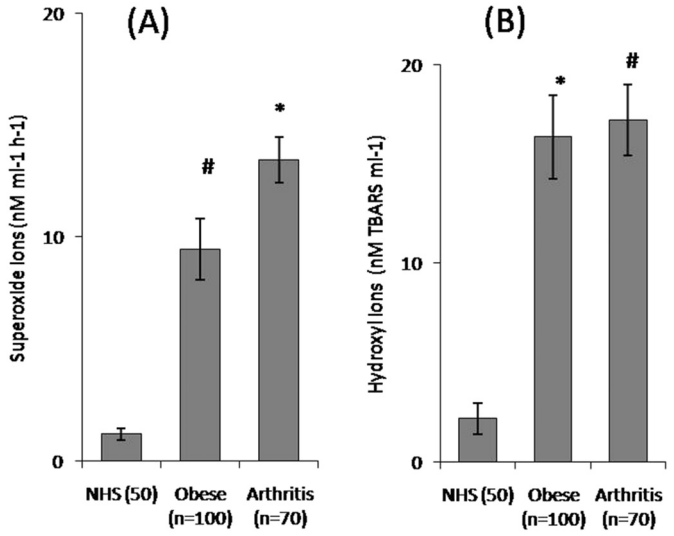

Fig. 1. Level of superoxide (A) and hydroxyl radicals (B) in serum sample of Obese and Rheumatoid patients as well as in normal healthy subjects. [*$p < 0.05$ (significant) healthy control vs other groups, #$p < 0.001$ (very significant) healthy control vs other groups].

measures in patient's serum of obese and rheumatoid were 48.6 ± 4.38 and 38.7 ± 2.94 μmol mg^{-1} protein, respectively (Fig. 2B).

3.5. HMF content estimation

The HMF which is formed in the early glycation of $ApoB_{100}$ was quantitated as thiobarbituric acid reactive substance. The HMF content in obese and Rheumatoid serum was 17.37 ± 2.45 and 10.36 ± 1.99 μmol mg^{-1} respectively, whereas healthy subjects had 4.67 ± 0.94 μmol mg^{-1} (Fig. 2C).

3.6. Binding of patient's sera antibodies with native and D-ribose-$ApoB_{100}$

The experimental examination was carried out to screen out the positive sera tests (sera indicating more the double binding with D-ribose modified ApoB100) from obese and Rheumatoid patient's subjects where patient's auto-antibodies demonstrated essentially higher specificity/binding against D-ribose-ApoB100 as compared to healthy control.

Our study comprised of total 220 serum samples of patients (obese patients: 100; Rheumatoid patients: 70) and 50 control serum tests from age and sex matched people were acquired from healthy subjects. All sera were diluted to 1:100 in TBS-T and subjected to direct binding ELISA with equal amounts of native and D-ribose-$ApoB_{100}$. Fifty eight samples out of 100 sera (58.0%) from obese patients and 37 sera from RA out of 70 sera samples (52.86%) (Fig. 3), demonstrated higher binding with the glycated $ApoB_{100}$ as compared to its native conformer.

3.7. Competitive inhibition ELISA

The competitive inhibition ELISA was done to evaluate the specificity of antibodies in the sera of patient to native and glycated $ApoB_{100}$. In the group of obese and rheumatoid, the observed maximum inhibition with glycated $ApoB_{100}$ was 87% and 64.29% respectively (Fig. 4), while with native $ApoB_{100}$ it was significantly less. Mean inhibition of the obese and rheumatoid samples tested with native $ApoB_{100}$ were 25.01 ± 4.39% and 25.38 ± 4.94%, while for glycated $ApoB_{100}$, it was 58.458 ± 6.59% and 62.47 ± 7.6, respectively. This indicated considerable binding of glycated $ApoB_{100}$ by antibodies in obese and rheumatoid arthritis subjects.

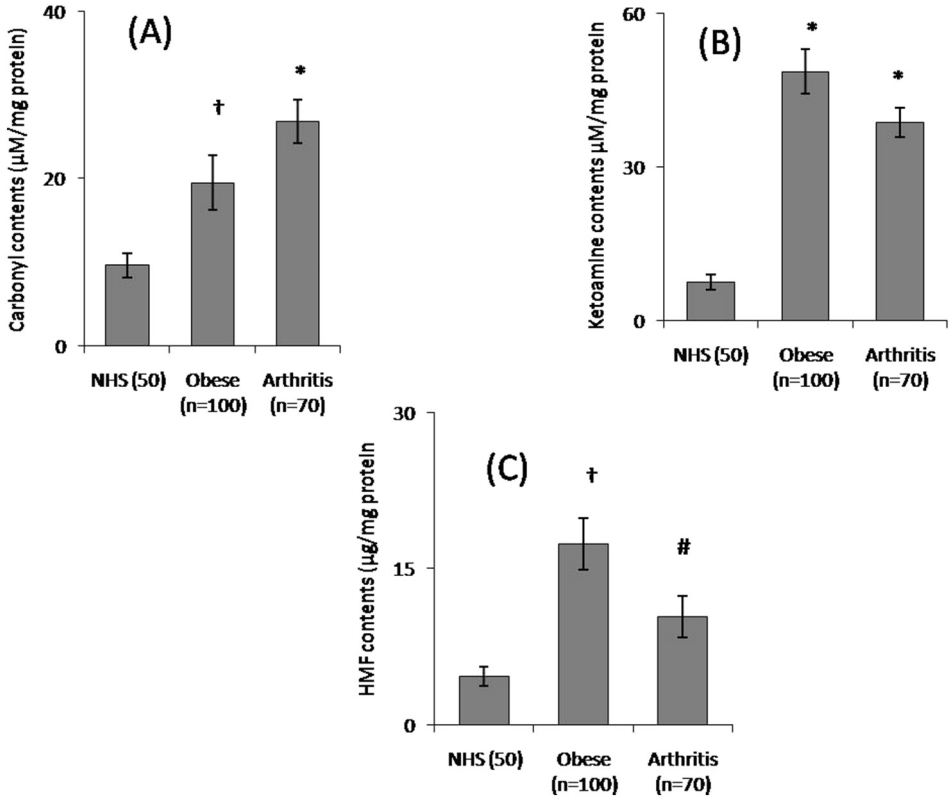

Fig. 2. Level of carbonyl content (A), ketoamine content (B) and HMF content (C) in serum sample of Obese and Rheumatoid patients as well as in normal healthy subjects. [*$p < 0.05$ (significant) healthy control vs other groups, †$p < 0.01$ (very significant) healthy control vs other groups, #$p < 0.001$ (extremely significant) healthy control vs other groups].

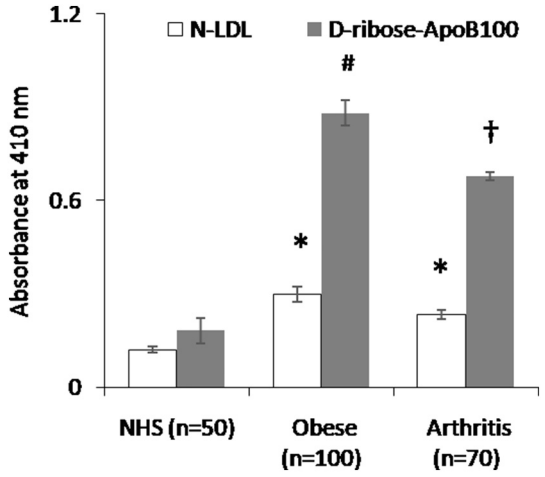

Fig. 3. Direct binding ELISA of serum antibodies from Obese and Rheumatoid patients to native $ApoB_{100}$ (□) and D-ribose glycated $ApoB_{100}$ (■). Serum from normal human subjects (NHS) served as control. The microtitre plates were coated with the respective antigens (10 μg/ml). [*$p < 0.05$ (significant) healthy control vs other groups, †$p < 0.01$ (very significant) healthy control vs other groups, #$p < 0.001$ (extremely significant) healthy control vs other groups].

4. Discussions

Obesity or overweight is expanding worldwide and now achieve close by 33% of the populaces in rising nations [23] Obesity additionally includes patients with rheumatoid arthritis and the effect of obesity on rheumatoid arthritis and rheumatic diseases is thus an essential issue [24] which is responsible for the hazardous increment in event of the sickness around the world. D-ribose, a characteristic pentose monosaccharide, shows in every living cell and is an imperative part of various biomolecules in numerous essential metabolic pathways. It also participates in the Maillard reaction of proteins producing AGEs that lead to cell dysfunction and death [25].

The findings of the study revealed that obese and RA patient's sera had significant concentration of glycation induced oxidative stress parameters and intermediate products (superoxide, hydroxyl ions, carbonyl, ketoamine and HMF contents), which corresponds to almost two to eight fold increase, while healthy control subjects showed negligible amount. Significant level of HMF content in patient's serum is in concurrence with the NBT result. Protein carbonyl content is utmost commonly used biomarker of protein oxidation and glycation adducts formation [26]. The principal reason of ketoamine development is its change to protein carbonyl moieties by means of a protein ene-diol producing superoxide radical [27].

In order to examine the task of D-ribose-$ApoB_{100}$ in producing immune response in Rheumatoid and obese patients, we examined total 100 samples of obese and 70 samples of RA. Precise binding of auto-antibodies in patients against native and ribosylated $ApoB_{100}$ was evaluated through competitive inhibition ELISA. The observed maximum inhibition in obese and Rheumatoid with the glycated $ApoB_{100}$ ranged from 44.8–79.6%, and 45.9–74.5% (Fig. 4). It is likely that the duration of the disease may be having effect on the quantity/titre of anti-$ApoB_{100}$ antibodies (auto-antibodies against D-ribose-$ApoB_{100}$).

Native $ApoB_{100}$ protein is moderately immunogenic as described in our previous published reports [28,29]. Induced immunogenicity of D-ribose-$ApoB_{100}$ leads to the generation of antibodies, immune complexes and inflammation which might be responsible for the cause of rheumatoid arthritis in obese subjects.

Glycation of ApoB100 of LDL rose as major pathogenic factors in inflammatory reactions. Altered variety of ApoB100-LDL (mApoB100-LDL) are pro-inflammatory by themselves, furthermore, AGE-product-modified $ApoB_{100}$ incites immune system reactions and induces autoimmune responses in humans.

The immune system reaction includes T cells in synovial liquid and synthesis of IgG antibodies. The IgG auto-antibodies that respond with mApo-B100-LDLs produce mApoB100-IgG immune complexes (IC), and these IC initiate the complement system and additionally phagocytic cells by means of the ligation of Fc receptors. Our examination has indicated before that D-ribose causes structural perturbations in ApoB100 of LDL macromolecule which causes the formation of neoantigenic epitopes wand are perceived as non-self, thus breaking the immune tolerance to self-antigens [14,28,29]. Already it has likewise been demonstrated that oxidized type of the LDL is highly immunogenic and shows potential recognition of auto-antibodies raised against altered ApoB100 [29,30].

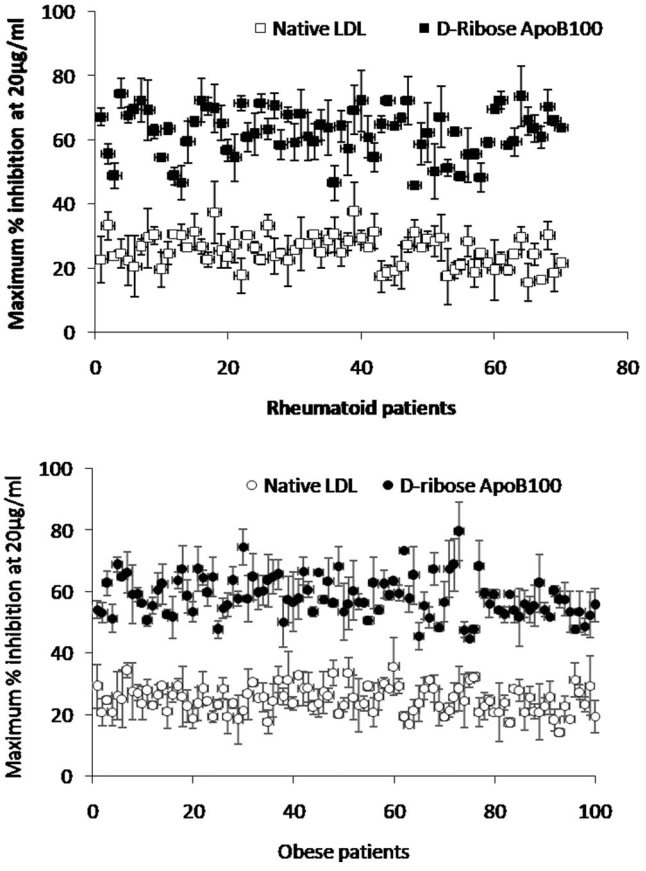

Fig. 4. Maximum percent inhibition of serum antibodies from Obese (A) and Rheumatoid patients at 20 μg/ml of each native $ApoB_{100}$ (□) and D-ribose glycated $ApoB_{100}$ (■). The microtitre plates were coated with the respective antigens (10 μg/ml). [$p < 0.05$ (significant)].

5. Conclusion

The current investigation may establish a basis for the early investigation of rheumatoid arthritis and obesity. Our earlier works on glycation study have proved the auto-antibodies against glycated biomolecules in hyperglycemic and hyperlipidemic conditions [31–33]. We believe our results would likewise help the route for the treatment of obese induced complications and Rheumatoid disease. Therefore, the prerequisite is to check the glycation mediated anomalies by novel therapeutic methodologies [34–36].

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Conflict of interest

The authors declare that they have no conflict of interests.

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