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ANALYSIS OF SERUM PROTEINS AND ENZYMES LEVEL IN HUMAN SUBJECTS WITH OSTEOARTHRITIS

ANALIZA NIVOA PROTEINA I ENZIMA U SERUMU OSOBA OBOLELIH OD OSTEOARTRITISA

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Summary: The aim of the present study was to assess the serum proteins and enzymes level using polyacrylamide gel electrophoretic (PAGE) profiles in human subjects with osteoarthritis (OA). Forty-one subjects with confirmed OA were selected for the present study. Sera were collected from these individuals and loaded in equal amounts on native and denaturing PAGE separately. Software analysis of these profiles was done using Scion Imaging (Beta release-4, Scion Corporation) and GelPro (Media Cybernetics, USA) programs. To visualize esterases (Est) and lactate dehydrogenase (LDH) isoenzymes in the sera of these patients substrate specific staining was performed. Differences in the values of control and OA subjects were tested statistically. Software analysis of native-PAGE profiles revealed the presence of nineteen peptides in control and twenty one in OA subjects respectively. Two extra peptides were present in the β-globulins region of OA subjects. Significant decline from 42.77% to 34.72% in albumin levels (hypoalbuminemia) was observed in OA subjects with total albumin to globulin ratio 0.58. In SDS-PAGE, the difference in control and OA subjects was observed among eight peptides with molecular weight 25, 22 and 20 kDa (absent in OA) and five novel peptides 270, 125, 30, 21.36 and 18.4 kDa (absent in controls), while albumin retains the major activity. For enzymes, Est follow a relative order, BchEst (42.86%)> ArylEst (16.24%)>AchEst (6.85%) in OA subjects with the expression of a new BchEst isoform in 4.78% and two isoforms of ArylEst at 2.13 and 1.61% concentrations respectively. Significantly declined albumin esterase-like activity (AlbEst)

Kratak sadržaj: Cilj ove studije bio je da se pomoću profila PAGE odrede nivoi proteina i enzima u serumu subjekata sa osteoartritisom (OA). Za studiju je izabrana 41 osoba sa potvrđenom dijagnozom OA. Uzorci seruma su u jednakim količinama zasebno stavljeni na PAGE sa i bez denaturacije. Softverska analiza ovih profila obavljena je pomoću programa Scion Imaging (Beta release-4, Scion Corporation) and GelPro (Media Cybernetics, SAD). Radi vizualizacije esteraza i (Est) izoenzima laktat dehidrogenaze (LDH) u serumu ovih pacijenata izvršeno je bojenje specifičnim supstratom. Razlike u vrednostima kontrolnih i osoba sa OA su statistički testirane. Softverska analiza PAGE profila bez denaturacije otkrila je prisustvo devetnaest peptida u kontroli i dvadeset jednog u osobama sa OA. Dva dodatna peptida bila su prisutna u regionu gama-globulina kod osoba sa OA. Značajan pad sa 42,77% na 36,72 % nivoa albumina (hipoalbuminemia) primećen je kod osoba sa OA a odnos ukupnog albumina i globulina bio je 0,58. U SAD-PAGE, razlika između kontrola i osoba sa OA uočena je između osam peptida molekularne težine 25, 22 i 20 kDa (kojih nema u OA) i pet novih peptida od 270, 125, 30, 21,36 i 18,5 kDa (kojih nema kod kontrola), dok je albumin zadržao najveću aktivnost. Za enzime, Est prate relativni poredak BchEst (42,86%) > ArylEst (16,24%) > AchEst (6,85%) kod osoba sa OA uz ekspresiju nove izoforme BchEst sa 4,78% i dve izoforme ArylEst u koncentracijama od 2,13 i 1,61%. Esterazi slična aktivnost albumina (AlbEst) bila je značajno (P<0,05) snižena (34%) kod obolelih u poređenju sa kontrolom (47%). Značajan porast LDH-5 i pad LDH-1 i -2 izoenzima takođe su primećeni

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was observed (34%) ($P<0.05$) in diseased subjects compared with controls (47%). Significant increase in LDH-5 and decline in LDH-1 and -2 isoenzymes were also observed in the sera of OA subjects. However, the overall rank of LDH isoenzymes was similar in control and OA subjects. Our results demonstrate noticeable differences in the sera PAGE profiles and enzymes activity in control and OA subjects and provide evidence to select serum for its use in the search for suitable biochemical markers in osteoarthritis.

Keywords: protein profiling, PAGE, esterases, lactate dehydrogenase, osteoarthritis

u serumu osoba sa OA. Međutim, ukupni poredak LDH izoenzima bio je sličan kod kontrola i osoba sa OA. Naši rezultati ukazuju na primetne razlike u profilima PAGE i aktivnostima enzima u serumu kontrola i osoba sa OA i pružaju dokaze u korist izbora seruma za upotrebu u potrazi za odgovarajućim biohemijskim markerima osteoartritisa.

Ključne reči: profilisanje proteina, PAGE, esteraze, laktat-dehidrogenaza, osteoartritis

Introduction

Arthritis is a medical condition affecting joints due to degenerative processes and causing pain, swelling and stiffness, loss of mobility, and eventual destruction and deformity of the joints in knees, ankles, wrists, elbows or hand (1–2). Out of many, osteoarthritis and rheumatoid arthritis are the most common and widely studied conditions.

Rheumatoid arthritis (RA) is an autoimmune disease which causes chronic inflammation of joints, the surrounding tissues and organs (3), while osteoarthritis (OA) is a complex, heterogeneous joint disease characterized by the progressive depletion and destruction of articular cartilage due to loss of the anabolic and catabolic homeostasis (involvement of IGF-1) and ultimate formation of osteophytes (4–8). The degradative process is believed to be largely mediated by metalloproteinases (MMPs), particularly MMP-1 and MMP-13, since they directly degrade the components of the cartilage matrix, including aggrecan and collagen (9–11).

Previously, efforts have been made to determine the quantity and quality of various proteins and enzymes in OA subjects. It has been reported that during the initial years of establishment of knee osteoarthritis, levels of serum cartilage oligomeric matrix proteins (COMP) increase (12–13). Literature suggests that the synovial fluid of OA subjects is poor in total protein contents, specifically the high molecular weight proteins that range from 1 to 2 g% (14) with an albumin to globulin ratio of 2:1 (15). Contrary to this, Kim et al. (16) reported that the levels of β-globulin fraction increases significantly without affecting the total protein contents of synovial fluid in different types of arthritis. Attempts have also been made to determine glucose concentration, total protein contents, lactate dehydrogenase (LDH) activity, C-reactive protein levels, hyaluronic acid (HA) levels, and white/red blood cell counts in synovial fluid and sera of OA subjects to demonstrate their significance as diagnostic markers (6, 8, 17–19). Elevated levels of HA in sera were noted due to cartilage degradation and synovial inflammation in OA subjects (8), thus it has been suggested to serve as marker for OA (6).

Enzymes like esterases along with lactate dehydrogenase (LDH), glyceraldehyde 3-phosphate dehydrogenase (GAPD) and glucose 6-phosphate dehydrogenase (G6PD) have been found most active in OA patients (20–22). Lactate dehydrogenase (LDH, EC. 1.1.1.27) is an oxido-reductase that catalyses the interconversion of pyruvate to lactate and vice versa in the presence of NAD^+/NADH. Available literature suggests the effect on total LDH activity (TLDH) in osteoarthritic knee-joint effusions (23), its correlation with C-reactive protein (an acute-phase protein) with advanced state of inflammation of OA and RA (23, 24) and higher LDH levels in arthritic synovial fluid and raised serum isozyme-5 in some arthritic patients (25).

It appears that the assessment of biochemical parameters in sera is equally important as in synovial fluid and both are necessary to understand the pathogenesis of the disease (26–28). Since OA is the most common arthritic disease of developed or developing nations affecting a majority of the population irrespective of age or sex (27–28), it is of great importance to know the basic underlying biochemical mechanism at the initial stages of the disease. During the present investigations, we have attempted to demonstrate the variation in total protein repertoire of control and OA subjects. Moreover, we have also demonstrated the variations in esterases (Est) and lactate dehydrogenase (LDH) isoenzymes and report the expression of new isoforms of esterases in OA subjects.

Materials and Methods

Study subjects description

In the present study, human subjects suffering from osteoarthritis (OA) were selected on the basis of their diagnosis confirmed by a clinician. These patients approached the Out Patient Department (OPD), Orthopaedic section of the JNMC Hospital during investigation timings. The patients were well informed in advance regarding the purpose of blood sampling and their verbal consents were taken and

recorded in the confidential files. Blood from healthy individuals who were tested normal for their general blood picture and sera profiles by a clinician were included as control.

Collection of sera samples

Initially, the patients were asked some basic questions to know the history of the disease and it was assured that they had not been on any medication in the past two months. Blood was taken through venipuncture by a 2 mL sterilized syringe. It was transferred to sterilized vials and kept for a few hours to ooze out the sera. Fresh sera were collected by low speed centrifugation and analysed. Sera for further analysis were stored in equal amounts of glycerol at –20 °C.

Total Protein Estimation

Protein concentration in different sera samples of controls and OA subjects was estimated by Biuret test using bovine serum albumin (BSA) as the standard (29). The appearance of purple colour indicated the presence of protein in the sera which is directly proportional to the optical density (absorbance) taken at 540 nm on a *GENESYS* 10UV-Visible spectrophotometer.

Polyacrylamide gel electrophoresis (PAGE) of sera samples

For non-denaturing gels, essentially the protocol of Laemmeli (30) was followed with the modification that the gels were lacking SDS. Gels were 7.5% in acrylamide and 15% glycerol. Equal amounts of proteins were loaded on the polyacrylamide gels and the runs were made at 4°C for 5 hrs. Staining of gels was done in Coommassie Brilliant Blue for the identification of protein bands. For enzyme visualization native gels were used.

For denaturing PAGE the proteins, PA gels and running buffers had SDS in their described molarities (30). When the runs were over, the gels were washed overnight in acetic acid and stained in CBB.

Visualization of esterases (Est) and lactate dehydrogenase (LDH) enzymes

Electrophoretic runs were made according to the described protocol by Ahmad and Hasnain (31). For Est staining, β-naphthyl acetate and α-naphthyl acetate were used as substrates. Gels were incubated separately for LDH staining in reaction mixture containing substrates, intermediates and coenzymes in their specified concentrations (32). Brown bands were visualized in Est staining while purple to dark blue bands appeared in LDH staining of control and OA subjects.

Documentation

Stained gels were documented using SONY-digital camera (Zoom-10X, 8.2 Mega pixels) and later by SONY-CYBERSHOT (Zoom-4X, 12 Mega pixels). LDH gels were documented by scanning on an all-in-one HP Deskjet (F370) computer assembly. These documented gels were used for further analysis of the data.

Densitometry and quantitative assessment of PAGE profiles

Different PA gels were processed through Adobe Photoshop (version 7.0) to obtain the best contrast for densitometric analysis through software. Best photoscans were taken and their densitometry was done using Scion Imaging (Scion Corporation; Beta release, 4.0) and GelPro (Media Cybernetics, USA) software programs.

Statistical analysis

Chi-square (χ^2) test was applied to demonstrate the differences in the proteins and enzyme levels in the sera of control and OA subjects; differences were considered significant at $P=0.05$.

Results

The data presented here is based on the sera electrophoretic analysis of forty-one subjects who were diagnosed to suffer from osteoarthritis (OA). Another group was comprised of unrelated fifteen healthy individuals (Control) who were tested normal for their general blood picture and sera profiles by Clinician. On native-polyacrylamide gel electrophoresis (native-PAGE) software detected only nineteen peptides in control and twenty one in OA subjects respectively (*Figure 1A-B, Table I*). Two extra peptides were present in the β-globulin region of OA subjects (*Table I*). In almost all the peptides there was an insignificant difference obtained between control and OA subjects, except the band corresponding to albumin. It showed a significant decline from 42.77% to 34.72% in OA subjects ($P<0.05$; *Table II*). The software analysis of PAGE profiles indicated hypoalbuminemia in OA subjects with an albumin to globulins ratio of 0.58 (*Table II*). Densitograms of selected lanes of the control and OA subjects also corroborate the above results of quantitative assessment of albumin (*Figure 2A-B*).

In denaturing conditions (SDS-PAGE), represented patterns of sera protein profiles revealed the presence of twenty-three peptides in OA subjects compared to twenty-one in controls (*Figure 3A-B*). The difference in control and OA subjects was observed among eight peptides and out of these, three

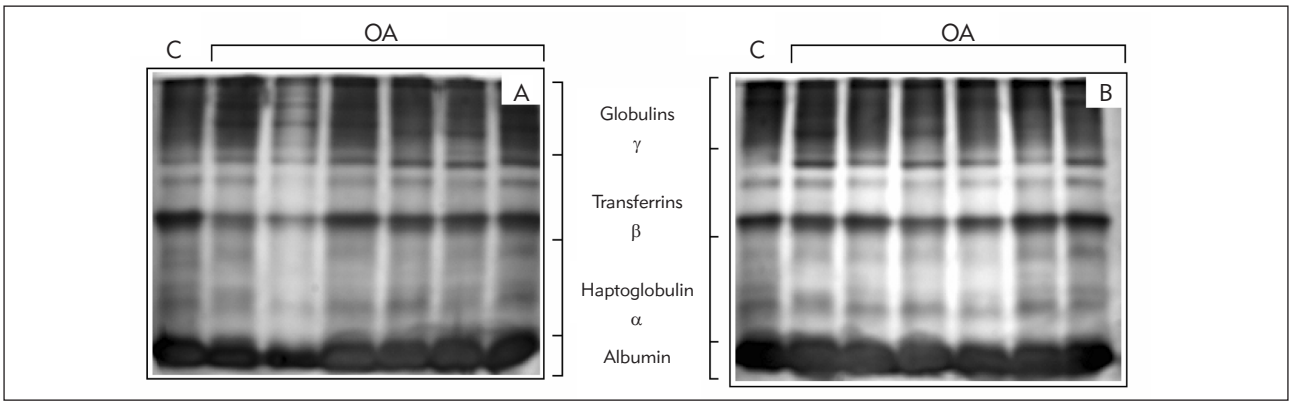

Figure 1 Typical sera PAGE profiles of Control (C) and human subjects with osteoarthritis (OA). Different fractions of human sera are labeled in the figure. Experimental conditions are described in Materials and Methods.

Table I Total protein fractions in control (healthy individuals) and OA subjects. α, β and γ are respective globulin fractions. αβ are the peptides sharing common occurrence in both the zones on PA gels.

Individuals	Pre-albumin	Albumin	Globulins				Total Protein fractions
			α	β	αβ	γ	
Control (n=10)	01	01	05	07	02	03	19
OA (n=41)	01	01	05	09	02	03	21

Table II Comparison of mean values for different sera protein fractions of control (healthy individuals) and OA subjects. Symbols represent the same fractions used in *Table I*.

Individuals	% Amount of protein fractions present in sera						Ratio			pA+A / G
	Pre-albumin (pA)	Albumin (A)	Globulins (G)							
			α	β	αβ	γ	A:α	A:β	A:γ	
Control (n=10)	1.73	42.77	23.30	27.82	1.99	2.60	1.83:1	1.53:1	16.45:1	0.79
OA (n=41)	1.91	34.72	24.97	35.01	1.80	1.59	1.39:1	0.99:1	21.83:1	0.58

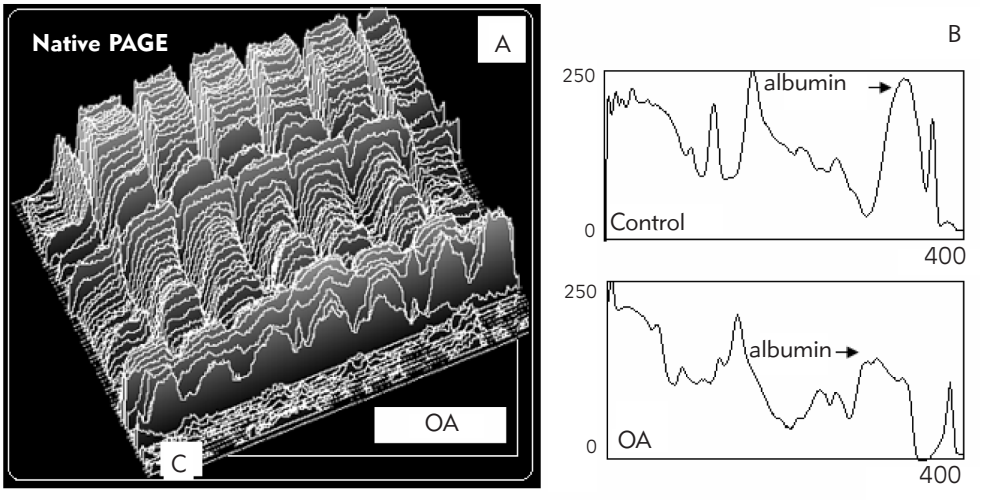

Figure 2 Densitograms of protein profiles of control (C) and OA subjects showing the differences in the quantity of peptide and reduced levels of albumin in OA subjects. (A) Densitogram of the whole PA gel; (B) Densitograms of representative lanes to show the difference in peaks. Arrow shows the differences in the levels of albumin.

Table III Differences in the average molecular weights of sera protein profiles in control and OA subjects as revealed from SDS-PAGE. Molecular weights are given in kDa. Novel peptides are marked in their respective lanes. (+ indicates the presence and -, absence of the corresponding peptide).

S. No.	Chicken actomyosin (MW marker, kDa)	Average molecular weight of peptides (kDa)	Control	OA
1		270.00	–	⊞
2		264.98	+	+
3		254.03	+	+
4		242.94	+	+
5		220.25	+	+
6	200.00		–	–
7		190.84	+	+
8		168.83	+	+
9	160.00		–	–
10		150.66	+	+
11	140.00	140.00	+	+
12		130.12	+	+
13		125.00	–	⊞
14		117.63	+	+
15	110.00		–	–
16		99.65	+	+
17		90.39	+	+
18		66.70	+	+
19		52.54	+	+
20		47.54	+	+
21	46.00		–	–
22		42.52	+	+
23	42.00		–	–
24	39.00	39.00	+	+
25		30.00	–	⊞
26	25.00	25.00	⊞	–
27	23.00		–	–
28		22.00	⊞	–
29		21.36	–	⊞
30		20.00	⊞	–
31	18.00	18.00	–	⊞
32	17.00		–	–
33		15.55	+	+
34	15.00		–	–

detected in controls were in the low molecular weight range of 25, 22 and 20 kDa and absent in OA subjects. Simultaneously, five novel peptides, 270, 125, 30, 21.36 and 18.4 kDa molecular weight respectively, showed considerable presence in OA subjects which were not observed in control individuals (*Table III*). Densitograms of selected SDS-PAGE profiles indicated albumin as the major fraction (*Figure 4A-B*).

Comparative analysis of the distribution of enzymes like esterases and lactate dehydrogenase was also made in control and OA subjects. Esterase zymograms showed the presence of butyrylcholinesterase (BchEst), paraoxonase/arylesterase (ArylEst) and acetylcholinesterase (AchEst). Albumin was also found to exhibit esterase-like activity (AlbEst) and was stained with α- and β-naphthylacetate. There were insignificant differences in the quantity of esterases except for one isoform of BchEst in 4.78% and two isoforms of ArylEst in 2.13 and 1.61% respectively, which showed their presence in the sera of OA subjects only (*Figure 5, Table IV*). Based on the quantity present in sera, the relative order of occur-

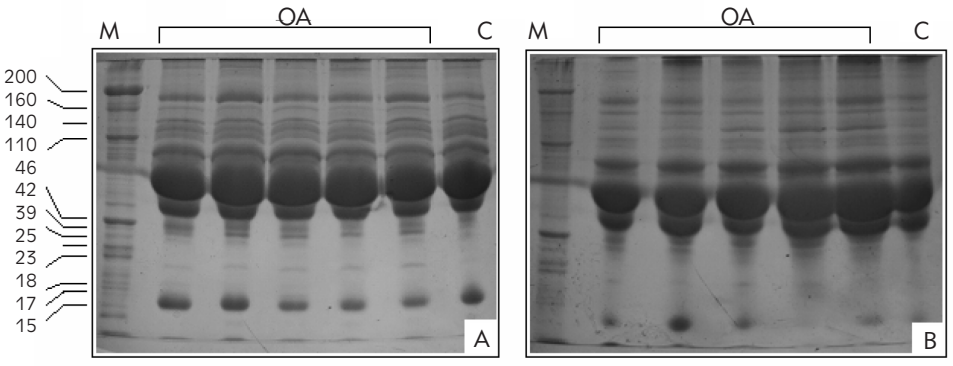

Figure 3 Sera PAGE profiles of control (C) and human subjects with osteoarthritis (OA) in denatured conditions.

M=molecular weight marker; C = control; OA = diseased subjects. Molecular weights are given in kDa. Experimental conditions are described in Materials and Methods.

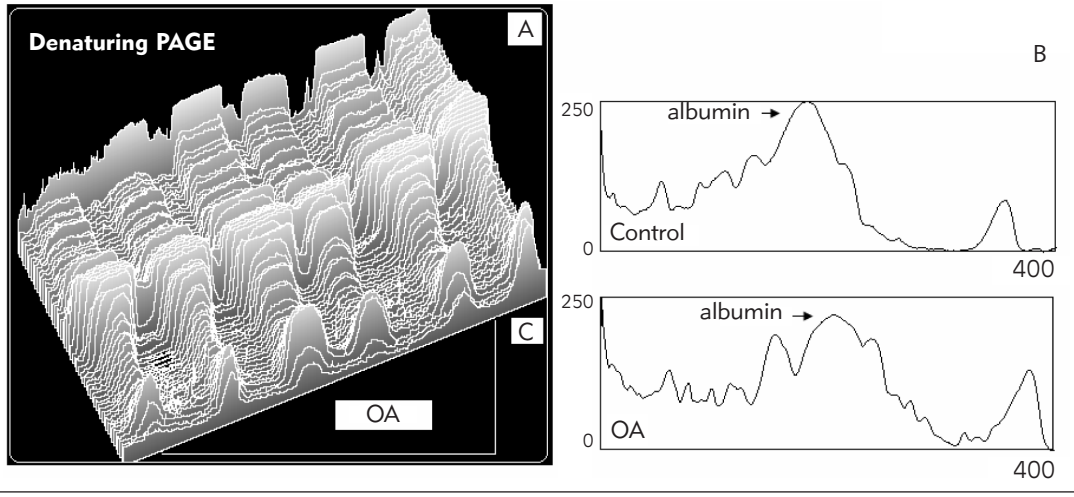

Figure 4 Densitograms of PAGE profiles (denatured) of control (C) and OA subjects (OA) showing differences in the molecular weights of different sera peptides. (A) Densitogram of the whole PA gel; (B) Densitograms of representative lanes of SDS-PA gel to show the differences in peaks. Arrow shows the differences in the levels of albumin.

Table IV Percent distribution of different esterases (Est) in the sera of control and OA subjects. Differences are based on the quantitative assessment of individual lane in AU.

Peptide #	Percent activity (AU) in control	Percent activity (AU) in OA subjects	Remarks
BchEst	–	4.78	Novel in OA
BchEst	36.52	38.00	
BchEst	–	2.13	Novel in OA
ArylEst	–	1.61	Novel in OA
ArylEst	4.20	5.01	
AchEst	5.33	6.09	
AchEst	6.70	6.85	
AlbEst	47.24	34.01	Reduced quantity

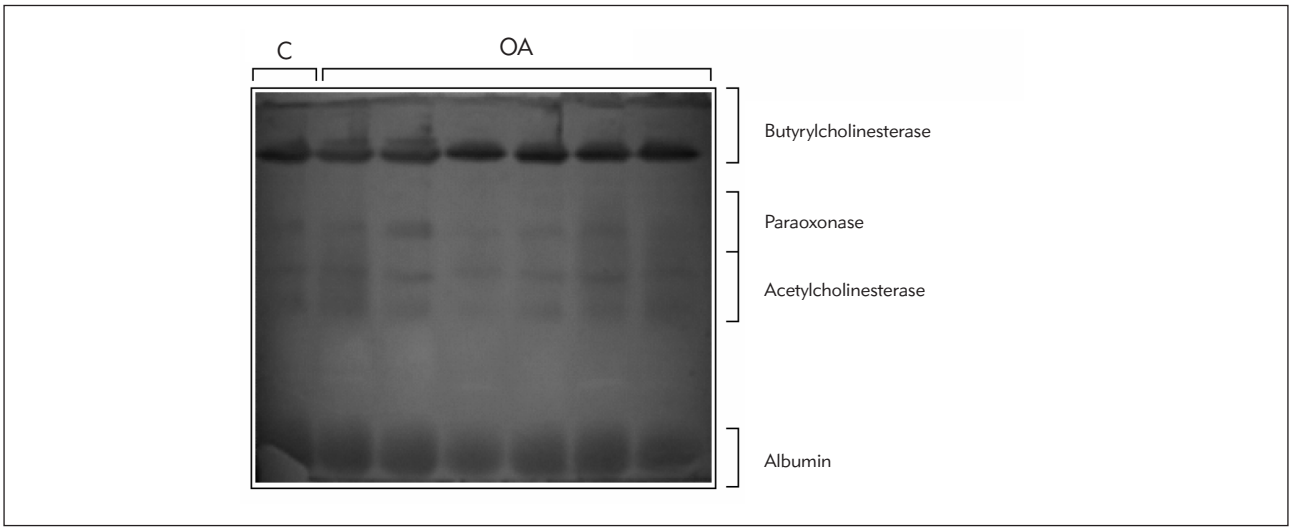

Figure 5 Zymograms showing the activity of different esterases in the sera of control (C) and OA subjects (OA). Details of experiments are given under Materials and Methods.

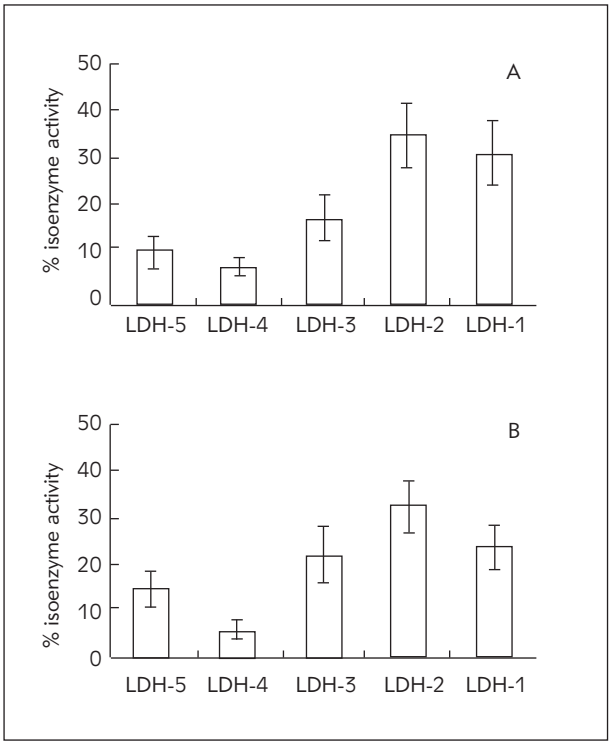

Figure 6 Quantitative differences in sera LDH isoenzymes in control (A) and OA subjects (B). Relative intensity of each band in individual PAGE lane is expressed and plotted as bars.

rence of esterases was BchEst (42.86%)>ArylEst (16.24%)>AchEst (6.85%) in the OA subjects. Significantly decreased albumin esterase-like activity was found in OA subjects (34%) compared to its activity in control sera (47%). Lactate dehydrogenase isoenzymes also showed remarkable changes with an overall shift in their levels. Significant increase in LDH-5 and decline in LDH-1 and -2 isoenzymes were observed in the sera of OA subjects compared with controls, however, an overall rank LDH-2>LDH-1>LDH-3>LDH-5>LDH-4 was found similar both in the sera of control and OA subjects (*Figure 6*).

Discussion

In arthritis, sera electrophoretic profiles may be of more value than other sources for diagnostic purposes. Available literature suggests that the synovial fluid protein profiles are most often fluctuating and could not be reproduced many times (33). However, the proteins present in synovial fluid and serum have been shown to be qualitatively identical (34). We have, therefore, selected sera as the potential source of changing proteomic profiles in subjects with osteoarthritis (OA). Various studies have identified potential biochemical markers as a means of diagnosing and monitoring OA, and investigators in some of these studies have reported radiographic assessment (35), hyaluronan (36), C-reactive proteins (37–38), enzymes (20, 22–23, 25, 39–41) and many more (42–46). Even the estimation of absolute protein values provides relatively better options and puts forward the marker value of many useful protein fractions (16, 33, 35, 47).

Previously, the sera protein fractions of arthritis patients have been demonstrated using disc electrophoresis or other means (16). During the present study we reported various protein fractions on native PAGE and determined their molecular weights on SDS-PA gels. Broadly, three activity zones were detected on native PAGE: pre-albumin, albumin and globulins (α, β, $\alpha\beta$, γ). A total of nineteen peptides were visible in the control group while in OA subjects there were twenty-one with two extra peptides in β-activity zone of OA subjects (*Table I*). Different activity

zones represented a considerable quantity of protein fractions, however albumin remained the main fraction in the sera of control as well as the subjects with OA. Published reports have shown the occurrence of twenty fractions in the synovial fluid of patients with osteoarthritis and demonstrated albumin as the major fraction (16, 33). The reason for the presence of an extra peptide in the β-globulin region may be either attributed to the sensitivity of the software used or the number of OA subjects analysed during the present investigation.

It has been argued that albumin is down-regulated while γ-globulins increase and the albumin to globulin ratio rises during arthritis (16, 48). Our results demonstrate a significant decline in albumin, from 42.77 to 34.72% in OA subjects (*Table II*) and hence, are in agreement with the above studies on hypoalbuminemia. Though γ-globulin levels increase in OA, the ratio of total albumin to globulin was observed to decline in the present study (*Table III*). This study on the decreased ratio of albumin to globulins is contrary to a few reports including those cited above. This may be due to the differences in tissue taken for estimation of protein content. Evidence suggests that the synovial fluid is poorer in protein repertoire than serum and may be rich in α- and β-globulins due to high contents of hexosamine (48–49).

In denaturing conditions (SDS-PAGE) five extra peptides with a molecular weight of 270, 125, 30, 21.36 and 18.4 kDa appeared in the sera of OA subjects but were not detected in controls, while three peptides, of 25, 22 and 20 kDa were observed only in control sera and could not be visualized in OA subjects (*Table III*). It appears that variation in peptides is more frequent in the low molecular weight (LMW) component of the sera of OA subjects. Earlier reports have also deduced that most of the variation in human proteome lies in the LMW region which is rich in cytokines, chemokines, peptide hormones and a few proteolytic fragments (35, 47, 50). We suggest further investigations in this direction to identify and characterize these novel LMW fractions present in the sera of OA subjects.

The present study also deals with the change in activities of two important enzymes, esterases (Est) and lactate dehydrogenase (LDH) in OA subjects. In compliance with reported literature, we observed butyrylcholinesterase, BchEst (EC 3.1.1.8); paraoxonase/arylesterase, ArylEst (EC 3.1.8.1); acetylcholinesterase, AchEst (EC 3.1.1.7) and the esterase-like activity of albumin (AlbEst) in control and OA subjects (20, 22, 39). However, a few novel isoforms of BchEst and ArylEst were detected in considerable amounts in the sera of OA subjects (*Figure 5*, *Table IV*). It may, therefore, appear that the expression of these isoforms is related to the pathophysiology of the disease and this needs to be evaluated further in a relatively larger sample size of the OA subjects. Evidence also suggest that lactate dehydrogenase has been very widely used as a marker of rheumatoid and osteoarthritis wherein an increase in LDH-4 and -5 and decrease in LDH-1 is reported (23, 25, 40–41). During the present investigation, we also observed a significant increase of 5% in the activity of LDH-5 ($P<0.05$) and a decrease of 7 and 2% in the activities of LDH-1 and -2 respectively in OA subjects compared with control ($P<0.05$). Specific isoenzyme elevations or decline in their level reflect the tissue source of the damaged cells, regardless of whether the damage was induced by inflammation or any other mechanism. In osteoarthritis the most affected tissue is the articular cartilage and may be the most probable source of isoenzyme leakage. Therefore, it is likely that, similar to other pathological states of sLDH elevation (23, 25, 40–41), the higher levels of LDH noted in our case are related to the state of inflammation in OA. Our results may therefore provide evidence to prefer serum over the conventional source, synovial fluid, for protein profiling and enzyme activity determination, and further recommend its use for the search of suitable molecular markers in case of osteoarthritis.

Conclusion

The present findings suggest that sera protein profiles and enzyme levels may be an additional tool to predict and identify the state of inflammation during osteoarthritis. Variation in sera low molecular weight peptides including hypoalbuminemia may offer their value as the biochemical marker for the diagnosis of osteoarthritis. Therefore, it is advised that in future more studies should be planned in this direction to further identify and characterize LMW fractions which are exclusively present in OA subjects. Due to accurate and reproducible results, the PAGE technique may be utilized for the routine monitoring of such protein and enzyme isoforms. For these purposes, the use of sera is strongly recommended to examine the activities of such molecules during the progress of osteoarthritis.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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