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SIGNIFICANCE OF SERUM LACTATE DEHYDROGENASE AND ITS ISOENZYMES DURING POST-BURN FOLLOW-UP

ZNAČAJ LAKTAT DEHIDROGENAZE U SERUMU I NJENIH IZOENZIMA U PRAĆENJU OPEKOTINA

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Summary: The present study aims to evaluate the role of lactate dehydrogenase (LDH) isoenzymes in thermal burns. A total of 18 patients of both genders with 20 to 50% total burn surface area (TBSA), admitted to the Burn Ward of JN Medical College and Hospital was assessed. These patients were subjected to general and systemic examinations. The sera collected at day 1, 2, 5 and 10 during follow-up of burn patients were used for LDH quantitation. PAGE profiles showed significant differences in the levels of LDH isoenzymes in all the burn subjects (P=0.05). Software analysis of gel-scans showed the presence of five isoenzyme bands of which LDH-1 and -2 are the least contributors. During follow-up, it was observed that the ranking of LDH isoenzymes approaches control values at day 2 in 20% TBSA patients, while in the remaining cases it occurs at day 5. 3D-densitograms indicated high activity of LDH in 50% of TBSA patients even at day 10; however, the relative ranking of these isoenzymes was similar to control values (LDH-4>-5>-3>-1>-2). We were of the opinion that the high activity of LDH enzyme is due to the enzyme-immunoglobulin-G (LDH-IgG) complex, but surprisingly we did not observe this complex in 50% of burn patients at any of the durations. Therefore, it is suggested that LDH isoenzymes play a role in the pathophysiology of the disease and can be an asset to ascertain the invisible tissue damage. Moreover, the high activity of LDH in 50% of burns is due to some unknown mechanism and not due to the binding of LDH with IgG.

Keywords: enzyme-immunoglobulin complex, lactate dehydrogenase isoenzymes, polyacrylamide gel electrophoretic profiles, thermal burns

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Kratak sadržaj: Cilj studije je da se odredi uloga izoenzima laktat dehidrogenaze (LDH) u termičkim opekotinama. Analizirano je ukupno 18 pacijenata oba pola sa ukupnom površinom opekotina 20 – 50% koji su primljeni na Odeljenje za opekotine Medicinskog koledža i bolnice Džavaharlal Nehru. Pacijenti su podvrgnuti opštim i sistemskim ispitivanjima. Za kvantifikaciju LDH korišćeni su serumi uzeti posle 1, 2, 5 i 10 dana praćenja stanja pacijenata sa opekotinama. Profili PAGE pokazali su značajne razlike u nivoima izoenzima LDH kod svih pacijenata sa opekotinama (P=0,05). Softverskom analizom gel-skenova otkriveno je prisustvo pet nizova izoenzima, od kojih su LDH-1 i -2 dali najmanji doprinos. Tokom praćenja stanja uočeno je da se rangiranje izoenzima LDH približava kontrolnim vrednostima 2. dana kod pacijenata sa ukupnom površinom opekotina od 20%, dok se u preostalim slučajevima isto događa 5. dana. Trodimenzionalni densitogrami MERAč GUSTINE pokazali su visoku aktivnost LDH kod 50% pacijenata sa opekotinama čak i 10. dana, međutim, relativno rangiranje tih izoenzima bilo je slično kontrolnim vrednostima (LDH-4>-5>-3>-1>-2). Mišljenja smo da se visoka aktivnost enzima LDH javlja usled kompleksa enzim-imunoglobulin-G (LDH-IgG), ali začudo nismo uočili taj kompleks kod 50% pacijenata sa opekotinama u bilo kom trenutku. Stoga, verujemo da izoenzimi LDH učestvuju u patofiziologiji bolesti i mogu biti korisni za utvrđivanje nevidljivih oštećenja tkiva. štaviše, visoka aktivnost LDH kod 50% pacijenata sa opekotinama javlja se usled nekog drugog mehanizma a ne usled vezivanja LDH za IgG.

Ključne reči: kompleks enzim-imunoglobulin, izoenzimi laktat dehidrogenaze, elektroforetski profili na poliakrilamidnom gelu, termičke opekotine

List of abbreviations: bovine serum albumin (BSA), lactate dehydrogenase (LDH), polyacrylamide gel electrophoresis (PAGE), sera LDH activity (sLDH), total burn surface area (TBSA), total LDH activity (TLDH)

Introduction

The enzymes, also known as the reaction catalysts of biological systems, are the most remarkable and highly specialized proteins. These enzymes or their multiple forms together with other proteins have been shown as reliable markers to assess the type and extent of damage to many tissues under a variety of clinical conditions. Monitoring of these enzyme levels and their distribution in postburn subjects has valuable diagnostic and prognostic importance (1–4). Various metabolic and biological changes follow burn injury due to anomalies in a number of both serum and enzyme activities.

A vast number of studies have been done which bring out the enzymes' role and their importance in burns. In clinical subjects, many enzyme systems are normally involved, among which lactate dehydrogenase has found extensive use in diagnosis, either as a total activity or as an isoenzyme (2, 5–9).

Lactate dehydrogenase (LDH, EC 1.1.1.27), an oxidoreductase, is an important enzyme of glycolysis which catalyzes the interconversion of pyruvate to lactate and vice versa using NADH and NAD^+ as cofactors. On acrylamide gels LDH resolves into five isoenzyme bands i.e. LDH-1(H_4), LDH-2 (H_3M), LDH-3 (H_2M_2), LDH-4 (HM_3) and LDH-5 (M_4). These isoenzymes are found in different tissues of the body like heart, liver, brain, eye, kidney, ovary/testes etc. according to their metabolic preferences, e.g. in the skeletal muscle which is anaerobic in nature the 'M' subunit is present, while in the case of aerobic heart the 'H' subunit predominates (5–6, 10).

Under normal conditions, low levels of LDH are present in the blood. Any increase in the TLDH activity is an implication of tissue damage and therefore, release of LDH may be an asset in the diagnosis of several human ailments. In a variety of clinical conditions like myocardial infarction, hepatitis and muscle diseases, megaloblastic and hemolytic anaemia, leukemia and lymphoma, meningitis, acute pancreatitis, Small Cell Lung Cancer (SCLC), certain opportunistic or HIV infections, necrotic and obstructive jaundice, LDH has been reported to serve as biomarker (6–7, 11–13).

Work on the relevance of LDH isoenzymes in burn subjects has also been carried out (1, 4, 14, 15). However, reports that signify the importance of LDH during postburn follow-up and wound healing are still scarce. In patients with varying degrees of thermal burns management of disease is an important factor. Unnecessary laboratory examinations delay the diagnosis and further treatment of the stressed patients. Therefore, the present study was undertaken with the view to demonstrate the significance of LDH and its isoenzymes during postburn follow-up and to draw a relationship with the degree/intensity of burns.

Materials and Method

Patient description and collection of sera samples

The post-burn subjects were approached in the Burn Ward of the Department of Plastic Surgery, Jawaharlal Nehru Medical College and Hospital (JNMCH), Aligarh Muslim University, Aligarh. Patients with pre-burn injuries or on any other treatment regimen were not included here. The selection of patients was random as per their availability in the Burn Ward of the Hospital. A total of 18 patients included in the present study were of both genders with the mean age ranging from 19±5 to 35±7 years. They were admitted to the Emergency Ward within 3–5 hrs after the accident occurred. The details of the patients included in the present study are further summarized in *Table I*.

Prior to taking the blood samples, the patients were well informed regarding the purpose of sampling and their consents were recorded in their confidential files. Blood was taken through venipuncture by 2 mL sterilized syringes and transferred to eppendorff tubes. Sera were obtained from the collected blood within 24 hrs by low speed centrifugation. Sera from 5 healthy individuals with no record of pre-burn injury or treatment course served as control.

The blood samples were also collected from every burn patient at days 2, 5 and 10 during follow-up and the course of wound healing or recovery. Fresh sera samples were used for further analysis and experimental use.

Protein estimation

Protein concentration in the sera samples of burn subjects was estimated by Biuret test using bovine serum albumin (BSA) as the standard. The presence of violet color indicates a positive test, wherein copper (II) ion is reduced to copper (I) and forms a complex with the nitrogens and carbons of the peptide bonds at pH > 7.0. The absorption or color intensity was taken at 540 nm. Equal amounts of 20 μL of sera sample from each burn subject were loaded on polyacrylamide gels for LDH quantitation.

Polyacrylamide gel electrophoresis (PAGE) and histochemical staining of LDH isoenzyme bands

Screening of sera samples was initially done on 7.5% polyacrylamide vertical slab gels (10). Based on the obtained results, final runs were made in cooling on superimposed 10% polyacrylamide (PA) gels containing 10% glycerol using a vertical midi gel system (Bangalore-Genei Pvt. Ltd., India).

LDH isoenzyme bands were visualized by incubating the PA gels at 4 °C in a specific stain containing substrate, cofactors and intermediates in their specified concentrations as described previously (4). LDH iso-

enzymes appears as dark purple to blue bands. Stained gels were fixed in acetic acid.

Immunological approach to search the LDH-IgG complex

Ouchterlony double immuno-diffusion test was carried out to detect the LDH-IgG complex in the sera samples of burn subjects. For this purpose, 1% agarose gels were made in 0.1 mol/L phosphate buffer saline (PBS, pH = 7.1). Molten mixture was poured onto the pre-cleaned sterilized glass slide (6×5×1 mm). Wells were made at equal distances by puncturing the solidified agarose gel. Equal amounts of the sera samples of burn patients were loaded into the surrounding wells, while in the centre calibrated quantities of rabbit anti-human IgG (Chromous Biotech Pvt. Ltd., India; Conc. = 1 mg/mL) were filled. The slide was kept overnight in a wet chamber for the reaction to proceed and observations were made the next morning.

Agarose gel was later stained with Coomassie Brilliant Blue (CBB) for further identification of the cross-reactivity between the sera samples (antigens) and rabbit anti-human IgG (antibody).

Documentation

Stained LDH gels and agarose gels were documented by scanning on an all-in-one HP DeskJet (F370) computer assembly. They were used for further analysis of the data.

Statistical analysis

LDH-gel scans were processed through the software analyses. Densitometric tracing of gel-scans was made with Scion Imaging program (Beta release-4, Scion Corporation). Quantitative as well as qualitative analyses of gel-scans were made by the GelPro software program (Media Cybernetics, USA). Activity of total LDH or of an individual isoenzyme (in arbitrary units) was presented as mean ± s.d. Student's t-test was applied and the differences in the LDH tetramer activities were considered significant at a 5% level of significance.

Results

The description of burn patients included in the present study is summarized in *Table I*.

Figure 1 demonstrates typical polyacrylamide gel electrophoretic (PAGE) profiles of lactate dehydrogenase (LDH) isoenzymes in the sera of patients with varying degrees of thermal burns. Sera collected from healthy individuals (used as control) were also run and stained for LDH isoenzymes in similar conditions *(Figure 1D; lane, 3)*. The screening of sera samples was done at two concentrations of acrylamide, i.e. 7.5% and 10%. Crisp resolutions were obtained in 10% PA gels. Hence, the LDH isoenzymes profiles of 10% gels were taken as reference for further analysis of the data.

Analysis of sera LDH-PAGE profiles showed the presence of five isoenzymes in every sera sample

Table I Description of burn patients selected during the present study.

S. No.	No. of burn patients	Mean age (years)	Source of thermal burn	Total burn surface area (TBSA)	Classification
1.	7	33 ± 3	Oil burn	20	Second degree (superficial)
2.	6	19 ± 5	Oil burn	36	Second degree (deep)
3.	3	35 ± 5	Oil burn	45	Second degree (deep)
4.	2	35 ± 7	Oil burn	50	Second degree (deep)

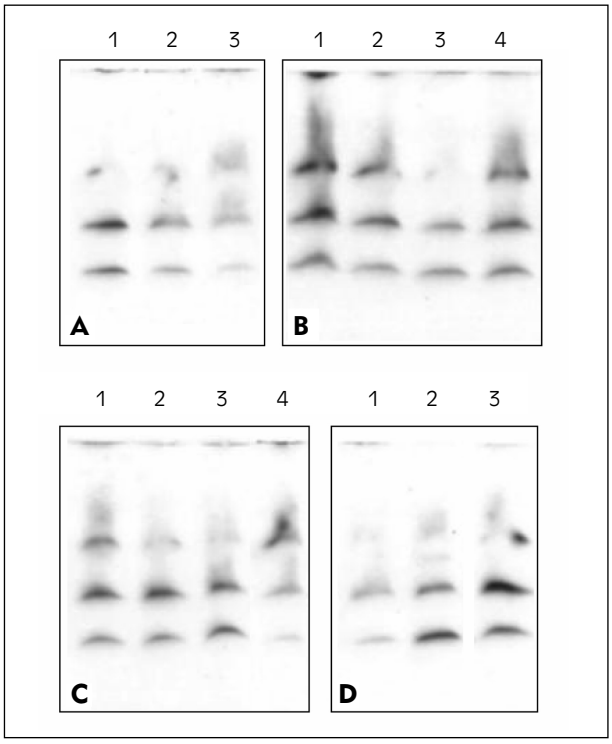

Figure 1 Polyacrylamide gel electrophoretic (10% acrylamide gels) profiles of lactate dehydrogenase (LDH) isoenzymes of burn subjects.

[A] 20% TBSA: Lanes 1–3 = Day-1, Day-5, Day-10
[B] 36% TBSA: Lanes 1–4 = Day-1, Day-2, Day-5, Day-10
[C] 45% TBSA: Lanes 1–4 = Day-1, Day-2, Day-5, Day-10
[D] 50% TBSA: Lanes 1–3 = Day-1, Day-5, Control (Healthy individual)

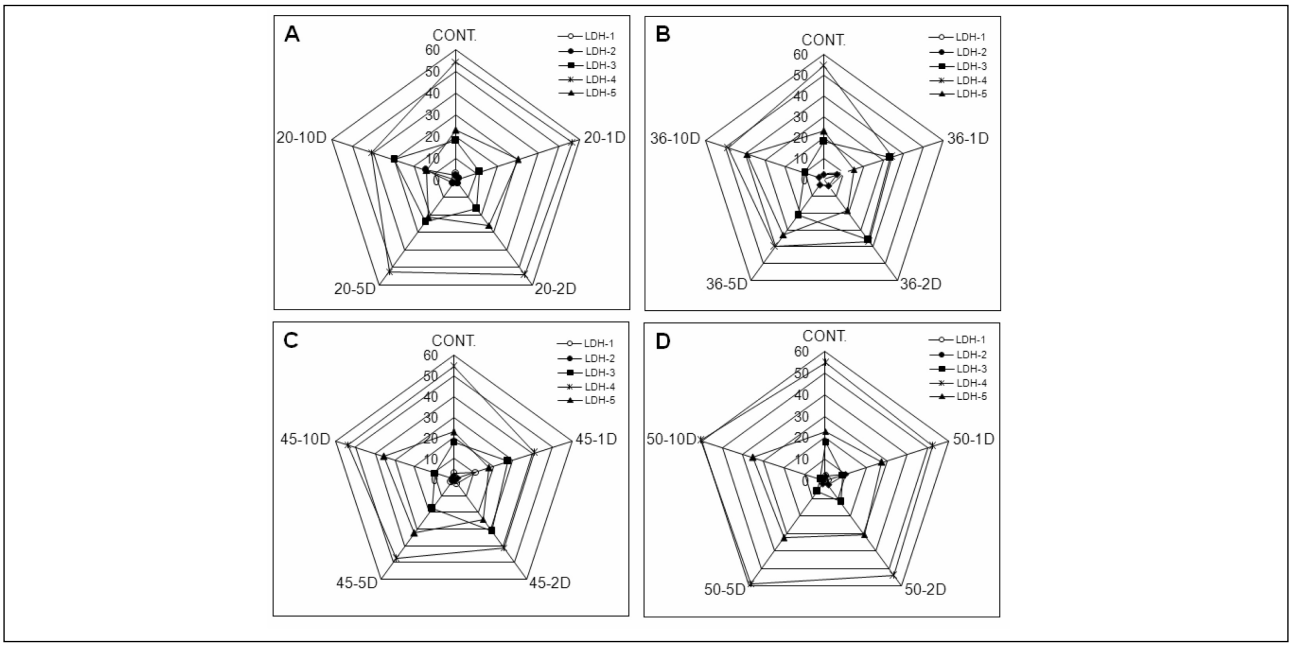

Figure 2 Radars showing the changes in sera LDH isoenzymes during post-burn follow-up. Relative intensity of each band (LDH tetramer) in individual PAGE lanes occupies different shells. Plotted values are the mean of total number of observations. CONT represents the isoenzyme levels in control (healthy individuals).

[A] 20% TBSA: 20-1D = day-1, 20-2D = day-2, 20-5D = day-5, 20-10D = day-10.
[B] 36% TBSA: 36-1D = day-1, 36-2D = day-2, 36-5D = day-5, 36-10D = day-10.
[C] 45% TBSA: 45-1D = day-1, 45-2D = day-2, 45-5D = day-5, 45-10D = day-10.
[D] 50% TBSA: 50-1D = day-1, 50-2D = day-2, 50-5D = day-5, 50-10D = day-10.

collected from burn subjects afflicted with 20%, 36%, 45% and 50% total burn surface area (TBSA) *(Figure 1)*. No apparent extra band was detected in either type of PA gels. LDH isoenzymes 1 and 2 were detected in traces in all the lanes. Software analysis of gel-scans also demonstrated the quantitative differences in LDH isoenzymes during the follow-up *(Figure 2)*. Remarkable quantitative differences were detected in LDH 3, 4 and 5 isoenzymes. In 20% of burn subjects LDH 3 decreases during the first day while LDH 4 and 5 increase; their level reaches to near normal values at day 10 post burn *(Figure 2A)*. In 36% of burn patients, elevated levels of LDH 1 and 2 were found during the first day post burn, which during the follow-up approach normal values. The maximum activity of LDH 3 was found at day 5 post burn that fell back in the normal range further with the process of wound healing. The activities of both LDH 4 and 5 decline during the first day post burn and then increase with the recovery in the burn subject *(Figure 2B)*. In 45% of burn patients, the trend of LDH isoenzyme activity adjustment was similar with that of 36% of burn patients at all the durations *(Figure 2C)*. No correlation between the activity change of LDH isoenzymes 1, 2 and 3 and the duration was observed in patients with 50% TBSA. LDH 4 and 5 isoenzymes were found elevated at all the durations up to day 10 in the patients with 50% burns. It appears that the activity of isoen-

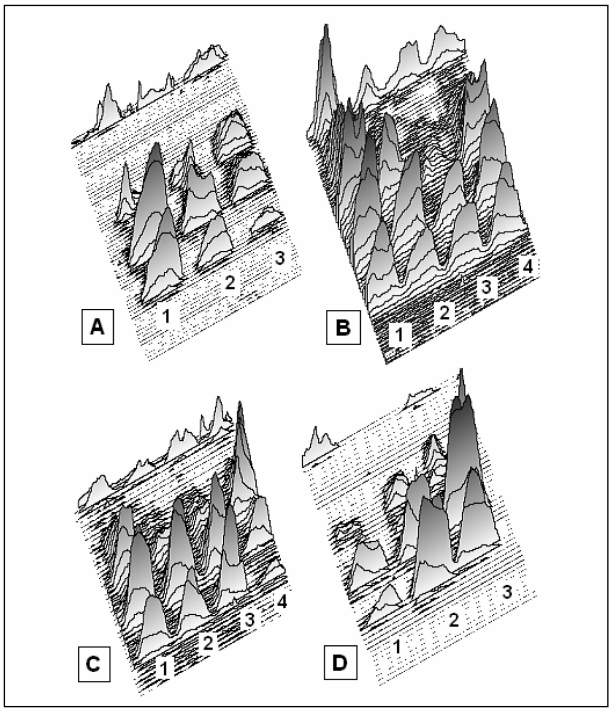

Figure 3 Enhanced imaging 3D-densitograms of the gels described in the legend of *Figure 1*. Individual peaks showing the existence of LDH isoenzymes in the sera of burn subjects and control sample.

Table II Apparent ranking of LDH tetramers in the sera samples of control (healthy individuals) and burn subjects during follow-up.

	1-Day	2-Day	5-Day	10-Day
Control	4>5>3>1>2	–	–	–
20%	4>5>3>2>1	4>5>3>2>1	–	–
36%	4>3>5>1>2	4>3>5>1>2	4>5>3>2>1	–
45%	4>3>5>1>2	4>3>5>1>2	4>5>3>1>2	–
50%	4>5>2>3>1	4>5>3>2>1	4>5>3>1>2	–

zymes in these patients remains sufficient even after day 10 of follow-up *(Figure 2D)*. The 3-D densitograms prepared from gel-scans also support and demonstrate noticeable differences in LDH isoenzymes during post burn follow-up *(Figure 3)*. As a whole, the ranking of LDH isoenzymes in patients with different degrees of TBSA is summarized in *Table II*. It was observed that, in patients with >20% TBSA, the LDH isoenzyme levels reach normal values by the end of day 5, while in case of 20% burn-patients the same was observed at day 2 *(Table II)*.

Statistical analysis using Student's t-test revealed significant differences in the LDH enzyme levels in burn patients compared with healthy individuals (control) during the post-burn follow-up and apparent recovery ($P = 0.05$; $df = 8$; $t = 1.86$).

We did not observe the cross reactivity signals between sera samples of burn patients and rabbit anti-human IgG antibody on 1% agarose gels. This indicates absence of the enzyme-immunoglobulin G complex (LDH-IgG) in the investigated burn patients.

Discussion

Burn injury is classified as a chronic disease. The patients after hospitalization immediately require supervised rehabilitation, reconstructive surgery and obviously strong psychological support. The clinical scope of a burn case basically involves electrolyte physiology, surgical infection, nutritional maintenance, cardiopulmonary support and wound care (16).

Burns are among the most devastating of all injuries, with outcomes resulting in a broad spectrum of consequences, from physical impairments and disabilities to emotional and mental trauma. Recent developments in the knowledge of burn pathophysiology, treatment and management of burn have led to a reduced mortality rate. At present, mortality in severely burned patients is either due to infection with virulent and resistant microbial agents which leads to septicemia and toxemia or a poor diagnostic approach. Initial diagnosis based on unnecessary laboratory tests delays the appropriate treatment of burn patients and puts extra burden on the stressed patient. Therefore, it becomes equally important to understand the underlying mechanism of the disease by using a suitable biochemical marker.

Extensive literature is available to suggest biochemical approaches as the most useful way to assess treatment strategy and management of the burn patients (4, 17–20). A few reports on burn subjects showed deficiencies in total circulating hemoglobin (21) and demonstrated reduced levels of γ-globulins by quantitative changes in serum electrophoretic profiles (22). Elevated serum elastase and its inhibitors levels (23) and pancreatitis have also been suggested as frequent complications after large burn injuries (11). In another study, relative protein deficiency in heavily burnt patients has been reported (24). It is suggested that due to a fall in the levels of non-essential amino acids the amount of total free amino acids in the plasma of burn subjects declines. A strong association has also been established between increased level of plasma phenylalanine and mortality of patients with burns (24).

Literature on burns is present in bulk but the diagnostic approaches are still inferior. Although wound healing in thermal burns is so obvious that apparently there is no need of any enzymatic diagnostic marker, the importance of a marker is realized during treatment or follow-up when the assessment is objective rather than subjective. In this way the present study becomes significant in documenting the lactate dehydrogenase (LDH) isoenzymes in the form of data to sort out medico-legal problems. In this context, the present study was undertaken with the aim to propose the significance of lactate dehydrogenase (LDH) enzyme data in thermal burn patients. As this is a very important enzyme of the last step of anaerobic glycolysis, its utility in terms of a metabolic indicator in a variety of animals cannot be ignored (4, 6, 8, 9, 13, 25, 26). Alterations in the level of LDH tetramers in the damaged tissues have wide applications in the management of various diseases, including burns (4, 27–29).

We assessed LDH isoenzymes in patients with varying degrees of burn injury by polyacrylamide gel electrophoresis (PAGE). For comparative analyses, gels with 10% acrylamide containing 10% glycerol proved better for observing the apparent quantitative as well as qualitative differences in sera LDH tetramers of burn subjects *(Figure 1)*. Nowadays, PAGE is a routine laboratory technique due to easy reproducibility of results, the least average cost, wide applicability and sensitivity (8, 9). Therefore, its use in laboratories to screen the sera samples for enzymatic or protein activity in burn subjects is strongly recommended. Platelets have been reported to interfere during the measurement of LDH

activity in the plasma of burn subjects. These platelets have been suggested rich in LDH heterotetrameric contents (4, 29, 30). Hence, during the present investigations use of sera is preferred over plasma to evaluate the LDH activity in burn subjects.

In thermal burns, the life span of RBCs is reduced (≤ 90 days, ref. range: 90–120 days) due to loss in their pliability. This results in increased destruction of these RBCs. Therefore, the alterations in sera LDH activity of burn patients also noted in this study are apparent. In superficial burns (up to 2nd degree) muscle is not directly involved, but may play a role indirectly as a result of stress or trauma. It appears that the direct contribution of muscle in the total LDH activity in superficial thermal burns is almost negligible. Patients included in this study were selected at random irrespective of their age, gender or socio-economic status. They were afflicted with 20, 36, 45 and 50% thermal burns. We observed noticeable differences in LDH isoenzyme levels of burn patients from the day 1 (admitted) up to day 10 during follow-up (recovery/ wound healing). Liu et al. (31) conducted a study on post-burn patients where abnormal values of the sera LDH isoenzymes were reported. Decrease in the activity of LDH 1, 2 and 3 and elevated levels of sera LDH 4 and 5 were shown in post-burn patients. Our findings based on a quantitative assessment of LDH isoenzymes are summarized below:

1. In 20% burns, LDH 3 decreases during day 1 and LDH 4 and 5 increase approaching the normal range by the end of day 2 *(Figure 1–3A)*.
2. In 36% burn cases, LDH 1, 2 and 3 increase during day 1, while LDH 4 and 5 decrease during day 1 and then increase further *(Figure 1–3B)*.
3. Like 36% of patients with 45% burns followed a similar trend in LDH isoenzymes and reached the normal range by the day 5 *(Figure 1–3C)*.
4. Unlike 36 to 45% burn subjects, in 50% burn patients elevated levels of LDH 4 and 5 were observed even at day 10 *(Figure 1–3D)*. However, the relative quantities of LDH isoenzymes fall back in the reference range by the day 5.

Therefore, our study does not fully comply with the previous report (31). This discrepancy in the subunit variation may be due to: (a) variation in the time of collection of blood, (b) area and intensity of burns *(Table I)* and (c) number of patients. The reliability of our data lies in the larger sample size. Software analysis of the LDH-gel scans reveals a significant increase in the sera TLDH activity of patients compared with control values (P=0.05). This is in agreement with the previous reports where an elevation in the sera TLDH activity of burns has been demonstrated (1, 32).

It is apparent that the nature and intensity of burns are the major factors upon which the activity of LDH (or its isoenzymes) depends. Previous report on burn patients with 30% TBSA demonstrated LDH 5 and 4 as the stable isoenzymes and suggested a diagnostic marker to assess the extent of damage and wound healing in postburns (4). Unlike our previous report, during the present investigation the isoenzyme ranking appears to approach the control values within 5 days *(Table II)*. This discrepancy in the stability of LDH may be either due to the intensity of burns or their nature *(Table I)*.

A few reports describe the presence of enzyme-immunoglobulin (LDH-IgG) complex, also known as macroenzyme in burns (14, 31, 32). Due to high LDH values in patients with 50% TBSA at day 10, we opined the presence of LDH-IgE complex in these patient. Surprisingly, LDH-IgG complex was not observed during PAGE in any of the patients throughout the follow-up. Therefore, Ouchterlony immuno-double diffusion test was performed utilizing rabbit anti-human IgG (antibody) against various sera samples (antigen) and control. Absence of cross-reactivity between the antigen present in the sera of burn patients and the commercially available antibody showed nonexistence of the LDH-IgG complex in these patients. Therefore, the high activity of LDH 4 and 5 in 50% postburns may be due to some unknown mechanism. Moreover, we are not sure of the binding specificity of LDH molecule to any other immunoglobulin.

Therefore, taking LDH activity as a whole (TLDH) or variation in isoenzymes, monitoring of initial treatment during post-burn follow-up may be done. It is suggested that such studies should be performed in future on a larger number of patients with increased follow-up duration to collect concrete evidence on the role of LDH isoenzymes in proper diagnosis, treatment monitoring and postburns management.

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