RESEARCH ARTICLE

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Agar gel electrophoretic monitoring of Lactate Dehydrogenase (LDH) in the testes of Swiss Albino Mice challenged by various doses of γ-radiation

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ABSTRACT

The present investigations highlight the effect of various doses of γ-radiations on the testicular LDH isoenzyme vis-à-vis control based on agar gel electrophoresis assay in the sexually mature adult male of Swiss albino mice. Six distinct bands of LDH isoenzymes were visually discerned in the testicular homogenate of mice not challenged by γ-radiation. Relative flow and quantification by densetemetric gel scanning showed their order of abundance to be LDH-5 ≥ LDH-2 ≥ LDH-3 ≥ LDH-4 ≥ LDH-1 ≥ LDH-X and the presence of LDH-X clearly showed that the testes were spermatogenetically active. Thus, in the present study it was observed that the percent activity of LDH isoenzyme fluctuates. On exposure to 0.2 Gy, the order of abundance was LDH-4 ≥ LDH-5 ≥ LDH-1 ≥ LDH-3 ≥ LDH-2. However, when exposed to 0.4 Gy the sequence of abundance was computed to be LDH-4 ≥ LDH-5 ≥ LDH-1 ≥ LDH-3 ≥ LDH-2. The order of abundance of LDH isoenzyme in testicular homogenate of mice treated by 0.6 Gy was LDH-4 ≥ LDH-3 ≥ LDH-1 ≥ LDH-5 ≥ LDH-2. Further changes in the order of abundance were observed in mice challenged by 0.8 Gy were LDH-3 ≥ LDH-5 ≥ LDH-4 ≥ LDH-2 ≥ LDH-1. The LDH–X was observed to be eliminated in all irradiated mice testes. This disturbance in the LDH isoenzyme may detrimentally affect the biochemical milieu of the testes. These aberrations are to support the suggestion that normal spermatogenesis is severely affected.

Key words: *γ-radiation, Swiss albino mice, testes, LDH-X, LDH-Isoenzyme*

INTRODUCTION:

Mammalian testes is a heterogeneous assemblage of germ cells e.g., spermatocytes, spermatids and sperms; endocrine cells *i.e.,* leydig cells and somatic cells e.g., sertoli cells. These cells differ structurally, metabolically and physiologically. Further, while hypothalamo-hypophyseal secretions precisely initiate, sustain and regulate events in germ and endocrine cells; other cell type of the testes are exempt from this coordinating and controlling functions of the former (Bloom and Fawcett 1975, Singh 1991, Rato et al. 2012). LDH form an important of class of oxido-reductase. This enzyme has been shown to be intimately linked with the events of development and maturation of germ cells in the testes (Mathur 1992). A variety of other oxido-reductases has also been histochemically demonstrated, biochemically quantitated, and electrophoretically assayed in the testes of several placentals (Montagna 1952, Turpeinen et al. 1962, Goldberg 1963, Tice and Barnett 1963, Zinkham et al. 1964, Blackshaw 1973, Swami and Lall 1979, Singwi and Lall 1979, Singwi and Lall 1982, Kaneko 1997). Experimental studies have shown that testicular cells are responsive to such physical factors as temperature and radiation. Radiosensetivity of pre-meiotic spermatocytes and differentiating spermatids has been documented by several investigators *i.e.*, Latailade (1991), Mamina and Sheiko (1993), Kangasniemi et al. (1996), Liu et al. (2006).

LDH is one of the key enzymes of glycolytic cycle which catalyses the reversible reaction lactate ↔ pyruvate in the presence of NAD which act as electron acceptor (Elkington et al. 1973), showed it is very active in the interstitium and absent in leydig cells, and exhibited relatively attenuated activity in the seminiferous tubules. Electrophoretic assay of this enzyme has manifested the occurrence of five distinct LDH bands (isoenzymes) in all

somatic cells of vertebrates recognized easily on the basis of their relative flow on gel column (agar, agarose and polyacrylmalide). These have been designated as LDH-1, LDH-2, LDH-3, LDH-4, LDH-5, a sixth band called LDH-X was electrophoretically observed in the spermatogenetically active testes of several mammals. Thus, in the mature and spermatogenically active testes of man, mouse, dog, guinea pig, ram and bull, this unique LDH-X isoenzyme has been persistently observed by Blackshaw (1973), Singwi and Lall (1982), Carra and Mulcahy (1991). Sperm cells also contain abundant LDH-X. Studies on the testes of all placentals which breed seasonally reveals that LDH-X, appears when sperm production occurs. In continuous breeders such as metatherians e.g., Wallaby and Wombat LDH-X is present throughout the year. It has been suggested that LDH-X can be used as a reliable "finger print" in delineating the intricate steps in spermatogenesis (Bishop 1961, Carra and Mulcahy 1991, Pant 1995).

Further it has been reported that all LDH isoenzymes are coded by distinct autosomal gene loci. Each of these isoenzymes performs a precise metabolic role (Markert and Möller 1959, Markert 1963). LDH-X is a tetramer composed of C-subunits; the other two tetramer of LDH are of A and B polypeptide subunits (Carra and Mulcahy 1991). The C-subunit has been shown to be encoded in separate gene loci from A and B (Blanco et al. 1964, Golberg 1971, Golberg 1973, Jagetia et al. 2000). Alteration in LDH isoenzymogram of mammalian testes in response of various forms and doses of radiation have not been adequately studied; no tangible and comparative information is available on gamma radiation induced alterations (dose related). In view of this, γ-radiation induced electrophoretic alterations in the testicular LDH were investigated in response to 0.2 Gy, 0.4Gy, 0.6Gy, 0.8Gy vis-à-vis controle using sexually mature spermatogenically active males of Swiss albino mice as a "model".

MATERIALS AND METHODS:

Procedure of radiation: The animals were restrained in position by tying rubber bands around the forelimb and hind limbs. They were exposed to single pulse of various doses of gamma radiation by Cobalt-60 camera. Radiation was applied to the abdominal region where the paired testes were located.

Sexually mature Swiss albino mice weighting 18±2gm were used in the present studies. Five groups were set up each having 5 mice.

Group 1: served as control, and were sham irradiated.
Group 2: were irradiated by 0.2 Gy of γ radiations
Group 3: were irradiated by 0.4 Gy of γ radiations
Group 4: were irradiated by 0.6 Gy of γ radiations
Group 5: were irradiated by 0.8 Gy of γ radiations

All experimental groups and control group sacrificed after 24 h after giving single dose of irradiation. These experiments were repeated twice.

Surgical process and preparation of Testicular homogenate:

Mice of control and experimental groups weighed before and after radiation .They were sacrificed by cervical dislocation after 24 h of radiation. Testes were surgically excised under aseptic conditions. They were freed off of excess of fascia and blood clots; rinsed several times in chilled physiologic saline (4 deg). After blotting the tissue the wet weight of each testes were separately recorded on monopan electric balance and pooled. They were homogenized in 0.1M phosphate buffer (pH 7.4); centrifuged at 3000 rpm to obtain 10% homogenate. The supernatant was decanted and stored at 4°C for subsequent quantitative and qualitative assay of LDH isoenzyme by agar gel electrophoresis performed according to the method of Wieme (1965) using microscopic slides (2.6 X 11.3 mm).

Preparation of working solutions

1. Tris-citrate buffer – 0.1M (pH 7.4)

2. Agar gel – 500 mg agar powder was dissolved in 50 ml tris buffer.
3. Incubating medium:
   Sodium D-L lactate – 200 mg
   NAD – 5mg
   NBT – Tris citrate 0.1 M (pH-7.4)
   The assay solution was made up to 50 ml in the incubation buffer and phenozine methosulphate (PMS) 1.0 mg was added into it.
4. Gel fixative
   A – Absolute alcohol – 70.0 ml
   B – Distilled water – 25.0 ml
   C – Acetic acid – 5.0 ml
   was mixed to form the fixative
   Electrophoresis was carried out an output voltage of 120v/12 slides and the correct 24mA/12 slides.

Procedure:

1. All the stock solutions were brought to room temperature before use.
2. Ultraclean chilled microslides were used to spread 0.1 % agar gel evenly on it.
3. Slides coated with agar gel were put in refrigerator for settling of gel.
4. After settling of gel, they were placed on electrophoretic apparatus. The tub of this was filled by 0.1 M tris buffer (pH 7.4).
5. Contact between the gel layer and the buffer was made by wet Whatman filter paper (no. 1) strips.
6. Microdepot (wells) were made in the agar gel and loaded with microdrop of testicular homogenate (10%) by a 1.0 c.c. hypodermic syringe.
7. A narrow filter paper strip (Whatman#1) dripped in Bromophenol blue solution was similarly placed just before the microdepot. This was used for determining the extent of electrophoretic run.
8. A current of 0.2 MA/slide and voltage 10v/slide was applied and maintained throughout the electrophoresis.
9. When the marker "bromophenol blue dye" reached almost up to the end of the gel column of the slide the electric supply was switched off.
   Slides were removed for LDH staining and subsequent fixation as follows:
   A – slides were incubated in the substrate medium at 37°C for 60 min in dark to develop the colour reaction (diformazon formation occurred which give violet blue colour)
   B – They were then transferred into fixative and kept in it for 30 min.

Slides were than washed in distilled water and dried, of such were used for densitometric scanning and photography as narrated below:

Scanning of gels:

1. The gel scanner was adjusted on to zero and left for some time for stabilizing it.
2. Yellow filter was selected.
3. "100" transmission was set with the help of '100 set knob'.
4. The slides were placed on the glass plate and inserted into the densitometer from the left side.
5. The percentile transmission of densitometer was recorded by turning the knob in the clock wise direction.

Determination of R_f values:

The bands of LDH activity were numbered 1 through 5 in order from anode to cathode.
Relative flow (R_f) of each band was determined by the following method.
R_f = Distance travelled by bands (LDH)/ Distance travelled by tracking dye

RESULTS:

A - Control (group 1): Six isoenzyme bands were clearly seen in the testicular homogenate based on their R_f values and staining intensities. These were designated as LDH-1, LDH-2, LDH-3, LDH-4, LDH-5 and LDH-X was interposed between LDH-3 and LDH-4.

The R_f values of LDH isoenzyme in control mice from LDH-1 to LDH-5 were 0.14, 0.48, 0.63, 0.73 and 0.95, respectively. Densitometric estimation of LDH isoenzymes revealed that the amount of LDH-1 was 6.84% and that of LDH-2, LDH-3, LDH-X, LDH-4, LDH-5 were 21, 12.86, 6.02, 9.41, 26.01%, respectively (Table 1 & 2).

Table 1: Quantitative analysis of the lactate dehydrogenase (LDH) banding patterns in testes of Swiss albino mice exposed to various doses of γ radiation as compare to control

Doses of γ radiation (Gy)	Band length(BL) Relative flow(R_f) % change	LDH-1	LDH-2	LDH-3	LDH-X	LDH-4	LDH-5
Control	BL(cm) R_f % change	0.40 0.14 –	1.45 0.48 –	1.90 0.63 –	2.05 0.68 –	2.20 0.73 –	2.85 0.95 –
0.2	BL(cm) R_f % change	0.30 0.12 14.28	0.65 0.26 45.83	0.95 0.38 39.68	Absent	1.60 0.64 12.32	2.10 0.84 11.68
0.4	BL(cm) R_f % change	0.20 0.06 57.14	0.80 0.30 37.5	1.55 0.51 19.04	Absent	2.10 0.70 4.10	2.60 0.86 9.47
0.6	BL(cm) R_f % change	0.40 0.14 0	0.65 0.23 52.08	0.85 0.30 37.5	Absent	1.65 0.58 20.54	1.90 0.67 29.47
0.8	BL(cm) R_f % change	0.45 0.20 42.85	0.75 0.34 29.16	1.45 0.65 3.01	Absent	1.85 0.84 15.06	2.10 0.95 0

Table 2: Testicular lactate dehydrogenase (LDH) isoenzyme activity (%) of Swiss albino mice challenged by various doses of γ- radiation

Doses of γ radiation (Gy)	LDH-1	LDH-2	LDH-3	LDH-X	LDH-4	LDH-5
Control	6.84	21.00	12.86	6.02	9.41	26.01
0.2 % change	14.44 111.11	11.93 43.19	13.61 5.83	– –	37.31 296.46	23.61 47.67
0.4 % change	21.00 207.01	12.77 39.19	23.02 78.22	– –	36.10 283.20	12.77 50.90
0.6 % change	16.26 137.71	11.09 47.19	20.00 55.52	– –	38.48 308.87	12.77 50.90
0.8 % change	10.16 48.53	14.45 31.19	42.35 227.30	– –	16.26 72.79	37.31 43.44

Experimental:

Group II (0.2 Gy): Exposure to 0.2 Gy of γ- radiation caused change in the R_f values for LDH-1 to LDH-5 were 0.12, 0.26, 0.38, nil, 0.64, 0.84, respectively. Densitometric estimates indicated the percentile activity of various isoenzymes from LDH-1 to LDH-5 to be 14.44, 11.93, 13.61, nil, 37.31, 23.61%, respectively (Table 1 & 2).

Group III (0.4 Gy): At the exposure of 0.4 Gy the R_f of bands was computed to be 0.06, 0.30, 0.51, 0.70, 0.86 from LDH-1 to LDH-5, and LDH-X was not observed. The percentile activity of LDH-1 to LDH-5 isoenzyme was 21, 12.77, 23.02, 36.10 and 12.77%, respectively (Table 1 & 2).

Group IV (0.6 Gy): The relative flow values of LDH isoenzyme was observed to be different and it was 0.14 for LDH-1, 0.23 for LDH-2, 0.30 for LDH-3, 0.58 for LDH-4 and LDH-5 was 0.67 and LDH-X nil. Although the percentile activity patterns were LDH-1 16.26%, LDH-2 and LDH-3 11.09% and 20%, LDH-X nil LDH-4 and LDH-5 were 38.48% and 12.77%, respectively (Table 1 & 2).

Group V (0.8 Gy): Observed R_f values of LDH isoenzymes from LDH-1 to LDH-5 were 0.20, 0.34, 0.65, 0.84 and 0.95, respectively. LDH –X was missing. The percentile activities were 10.16, 14.45, 42.35, 16.26 and 37.31%, respectively and LDH-X showed no activity as it was absent (Table 1 & 2).

DISCUSSION:

The present investigations highlight the effect of various doses of γ-radiations on the testicular LDH isoenzyme vis-à-vis control based on agar gel electrophoresis assay in the sexually mature adult male of Swiss albino mice.

Six distinct bands of LDH isoenzymes were visually discerned in the testicular homogenate of mice not challenged by γ- radiation. Relative flow and quantification by densetemetric gel scanning showed their order of abundance to be LDH-5 ≥ LDH-2 ≥ LDH-3 ≥ LDH-4 ≥ LDH-1 ≥ LDH-X and the presence of LDH-X clearly showed that the testes were spermatogenetically active.

The testes of mice irradiated by 0.2 Gy, displayed different R_f values for LDH isoenzymes. The R_f values for LDH-1 decreased up to 14.28% and for LDH-2, it was observed to be 45.83% R_f values for LDH-3, LDH-4 and LDH-5 were estimated to be decreased by 39.68, 12.32 and 11.68%, respectively. This computed percentile change was as compare to control, which was considered to be 100%. In the same group, the LDH-4 activity increased maximally and this percentile increment maximally and this percentile increment was 296.49%. This percentile increment in the values of LDH-1 (111.11%), LDH-2 (43.19%), LDH-5 (47.67%), and the minimum increase was noted for LDH-3 (5.83%). Thus their order of abundance was LDH-4 ≥ LDH-5 ≥ LDH-1 ≥ LDH-3 ≥ LDH-2.

In mice irradiated by 0.4 Gy, there was significant decrease in the R_f values of different LDH isoenzyme. Thus, for LDH-1 and LDH-2 these decremented values were very high *i.e.,* 57.14% and 37.5%, respectively. Comparatively less percentile decrement was observed for LDH-3 and LDH-5. It was 19.04 and 9.47%. However, very less declination in R_f value of LDH-4 was computed. It was only 4.10% vis-à-vis control (100%). In the group irradiated by 0.4 Gy, maximum percentile increment in the activities (283.20%) of LDH-4 and LDH-1 (207%) was noticed. However, this increase in other isoenzymes was substantially lower *viz.,* LDH-2 (39.19%), LDH-3 (78.22%). Percentile decrement in LDH-5 was 50.90% as compare to control (100%). There order of abundance was LDH-4 ≥ LDH-5 ≥ LDH-1 ≥ LDH-3 ≥ LDH-2, and LDH-X was observed to be absent.

With increased dose *i.e.,* 0.6 Gy, the percentile decrease in the R_f value of LDH-1 and LDH-3 was approximately same *i.e.,* 52.08 and 52.38%, while the percentile decrement for LDH-4 and LDH-5 were observed to be 20.45 and 29.47%. LDH-1 did not change as compare to control (100%). Decremental trend was also exhibited by LDH-2 and this was 47.19%. However, other isoenzymes showed maximum percentile increase e.g. LDH-4 (308.87%) and LDH-1 (137.71%). The percentile activity increased for LDH-3 (55.52%) but LDH-5 decreased by 50.90% as compare to control. There order of abundance was LDH-4 ≥ LDH-3 ≥ LDH-1 ≥ LDH-5 ≥ LDH-2.

In mice irradiated by 0.8 Gy, which was the maximum dose delivered in these studies, differential profile of R_f values for LDH-isoenzymes was observed. The R_f values for LDH-1 and LDH-3, LDH-4 increased and were 42.85, 3.01 and 15.06%, respectively as compare to control. The value of LDH-5 was not affected at all and it remained similar to control. However, LDH-2 showed percentile decrement of 29.16%. The percentile activity also changed differentially as compare to control. Thus maximum percentile increase was shown by LDH-3 (227.3%) rather than LDH-4 (72.79%). On other hand, LDH-2 isoenzyme activity declined by31.19%, LDH-1 and LDH-5 isoenzymes activity increased by48.53%

and 43.44%, respectively. Their order of abundance was LDH-3 ≥ LDH-5 ≥ LDH-4 ≥ LDH-2 ≥ LDH-1. The dose related changes in the LDH isoenzymograph clearly indicate that the testicular cells are biochemically intolerant to γ radiation irrespective of dose.

LDH-X has been related to active spermatogenesis in variety of placental and is reported to be severely impaired by γ radiation (Suzuki et al. 1990, Bansal et al. 1990, Latailade et al. 1991). These reports are compatible with the results obtained in the presented study and seem to indicate that while the pattern of effects induced by γ radiation may not be species specific yet significant alternations do occur.

LDH-4 and LDH-5 are considered to be thermo labile and unstable below 0°C. However, in the present study these enzyme showed incremental trends due to increase of temperature produced by γ radiations. Gamma radiation dose of 0.8 Gy caused significant elevation in LDH-5 and LDH-4. This result on healthy mice agrees with the work of different workers *viz.*, Klimov et al. (1989), Rai et al. (2008), who reported a somewhat similar LDH isoenzyme pattern in oncologic patients undergoing γ-radiation treatment chronically (Dohijyo 1971, Naphade and Naphade 2011). In this study in mice challenged by γ-radiation, isoenzyme that disappear or manifest a significant decrement trend may means that either coding mechanism of relevant genes is switched off or perhaps aberrations in the expression of such genes occur.

LDH is an important enzyme of the tricarboxylic cycle (kreb cycle). It catalyzes the interconversion of lactate to pyruvate and vice-versa with NAD serving as electron receptor in the glycolytic pathway (Naphade and Naphade 2011). LDH-X is a sperm specific isoenzymes of all mammals so far studied and can be used as a reliable "molecular marker" for active spermatogenesis (Schill et al. 1985, Carra and Mulcahy 1991, Hacker-Klom 2009).

LDH-X is a tetramer composed of C sub-units. The other two tetramers are composed of A & B sub units (Cahn et al. 1962, Ahmad 2008). The hetrerogenecity of LDH has been explained by Markert (1962) in terms of subunit hypothesis. Thus, the five isoenzyme units of LDH found in most vertebrate tissue were described as the product of two distinct genes coding for peptide A and B. These sub units combine randomly to form the enzymatically active tetemeric molecules (Appella and Markert 1961, Cahn et al. 1962, Markert 1962). The C (=x) sub-unit is enclosed in a separate gene from A and B of polypeptides (Blanco and Zinkham 1963, Blanco et al. 1964, Golberg 1971, Golberg 1973, Jagetia et al. 2000) postulated that because of the sperm specificity of LDH-X; this enzyme may be of considerable significance by providing a new approach to control male fertility showed in the mice (Hacker-Klom 2009), that the gene for LDH-X is activated at the time of spermatogenesis while at the other times it is in a suppressed state.

One or more LDH-X enzymes have been reported in the testes of many animals such as mouse, rat, guinea pig, ram, hamster, dog, bat, marsupial, Mongolian-gerbil by Goldberg (1963), Zinkham et al. (1964), Blanco et al. (1964). Other mammals like rat, bat and pig were reported to have more form of LDH-X, LDH-X represent 11% of the total LDH in human testes (Carra and Mulcahy, 1991). No such situation visualized in the present electrophoretic assay.

The concept relating multiple molecular form of enzyme is of great biological and evolutionary significance, since it may be used in resolving many intricate taxonomic problems. Further, it may have a critical role in deciding the mode of life of animals has proposed that LDH isoenzyme have became diversified by epigenetic mechanism. Thus, in the present study, it was observed that the percent activity of LDH isoenzyme fluctuates. On exposure to 0.2 Gy, the order of abundance was LDH-4 ≥ LDH-5 ≥ LDH-1 ≥ LDH-3 ≥ LDH-2. However, when exposed to 0.4 Gy the sequence of abundance was computed to be LDH-5 ≥ LDH-4 ≥ LDH-3 ≥ LDH-2 ≥ LDH-1. The order of abundance of LDH isoenzyme in testicular homogenate of mice treated by 0.6 Gy was LDH-5 ≥ LDH-4 ≥ LDH-2 ≥ LDH-3 ≥ LDH-1. Further changes in the order of abundance were observed in mice challenged by 0.8 Gy were LDH-5 ≥ LDH-4 ≥ LDH-3 ≥ LDH-2 ≥ LDH-1.

The LDH-X was observed to be eliminated in all irradiated mice testes. This disturbance in the LDH isoenzyme may detrimentaly effect the biochemical milieu of the testes. The study also appears to indicate that there may be not nexus between histological tolerance/resistance, biochemical sensitivity/tolerance in response of various doses of γ-radiation.

CONCLUSION:

The aberration in LDH isoenzyme pattern tends to support the suggestion that the normal spermatogenesis is severely affected which impaired the fertility of an individual exposed to gamma radiation. Thus, the current and rampant use of this radiation, therefore, warrants further in depth investigation in view of the long term hazards.

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