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Effect of azidothymidine on the radiation-induced LDH release in HeLa cells

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Treatment of HeLa cells with various concentrations of 0, 0.01, 0.1, 1, 10, and 100 μM AZT resulted in a concentration dependent elevation in the LDH release at 0, 0.5, 1, 2 and 4 h post-treatment. An elevation of 1.7 to 9.2 fold in LDH content was observed at 1 h post-treatment depending on the drug concentration. Similarly, treatment of HeLa cells with 0.1 μM AZT before irradiation caused an irradiation dose dependent increase in the LDH release in AZT + irradiation groups. This increase in LDH release was approximately two fold greater at 0 h post-irradiation in AZT + irradiation group, when compared with the PBS + irradiation group. This trend of elevation in LDH release continued up to 2 h, except 2 and 3 Gy, where it was 1.7 fold in the former group when compared with the latter. However, a peak level of LDH release was observed at 0 h post-irradiation.

1. Introduction

Azidothymidine (zidovudine, 3′-azido-3′-deoxythymidine, AZT), a thymidine analogue has been reported to be effective as an antiviral agent and has been found to be active against HIV-1 and other mammalian retroviruses [1, 2]. Currently, AZT is used in the treatment of acquired immunodeficiency syndrome (AIDS) and AIDS related complex (ARC) in humans [3].

Combination of various drugs with radiation has been used to enhance the effect of radiation [4, 5]. Nucleoside analogues like 5-fluorouracil [6], lonidamine, β-arabinofuranosyladenine, aphidicolin have been reported to enhance the effect of radiation [7-11]. The estimation of LDH in cell culture supernatant provides a quantitative basis for the loss of cell viability and cytotoxicity [12 to 16]. The effect of various concentrations of AZT or its combination with radiation on LDH release has not yet been studied. Therefore, the present study was undertaken to evaluate the effect of AZT alone or in combination with radiation on the LDH release by HeLa cells in the medium.

2. Investigations and results

The results are expressed as the amount of LDH released in the culture medium as U/L ± SEM (standard error of the mean) in Tables 1 and 2.

AZT treatment of HeLa cells caused a concentration dependent elevation in the LDH release. This increase in LDH contents was significantly higher after AZT treatment than that of non-drug treated controls (Fig. 1). Treatment of HeLa cells with 0.01, 0.1, 1, 10 and 100 μM AZT resulted in 1.7, 1.9, 2.7, 5.4 and 9.2 fold elevation in LDH release, respectively at 1 h post-treatment when compared to the non-drug treated control. The LDH release also increased with the assay time and the highest values were observed at 1 h post-treatment, thereafter the increase in LDH was less compared to 0.5 and 1 h post-treatment (Table 1).

The exposure of HeLa cells to different doses of gamma radiation resulted in an irradiation dose dependent elevation in LDH release at all the post-irradiation time periods in both the PBS + irradiation and AZT + irradiation groups (Fig. 2). The AZT pretreatment resulted in an approximately 2 fold elevation in the LDH contents at 0 h in the AZT + irradiation group compared to the PBS + irradiation groups, where the activity was highest. The amount of LDH accumulated in the AZT + irradiation group remained two fold higher at 0.5 and 2 h post-treatment, when compared with the PBS + irradiation group except 2 and 3 Gy irradiation, where it was 1.7 fold greater at 0.5 h post-irradiation. The LDH release declined with time and the lowest values were reported at 4 h post-irradiation. The increase in LDH release was linear for both PBS + irradiation and AZT + irradiation groups (Table 2).

3. Discussion

Cytotoxicity can be measured by several methods like clonogenic assay [17], radioisotope and fluorescing material release [18–20], dye exclusion [21], release of enzymes [22, 23] and MTT assay [24]. The LDH assay is a simple assay and can be used to evaluate the cytotoxicity of any treatment. Treatment of HeLa cells with various concentrations of AZT has resulted in a dose dependent elevation in the LDH release, where 1.7 to 9.2 fold elevation was observed depending on the AZT concentration at 1 h post-treatment. To the best of our knowledge reports regarding the LDH estimation after AZT treatment in cell systems are lacking. However, AZT maintenance therapy in patients suffering from immunodeficiency syndrome (AIDS) has been reported to increase serum LDH levels [25]. Other drugs like taxol and teniposide has been reported to induce a concentration dependent elevation in LDH release by V79 cells. [26, 27]. Similarly, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) has also been reported to increase the LDH contents in a concentration dependent manner in endothelial cells [28]. A dose dependent increase in LDH release has been observed in Sertoli cells treated with various doses of α-interferon [15]. A time dependent increase in LDH contents up to 1 h post-treatment was observed in the present study that declined thereafter.

Treatment of HeLa cells before exposure to various doses of radiation resulted in a significant elevation in LDH release when compared to the non-drug treated irradiated group. A similar effect has been reported earlier, where a dose dependent elevation in LDH release has been observed in V79 cells treated with teniposide before irradiation [29]. The highest elevation in LDH release was observed immediately after irradiation in both PBS + irradiation and AZT + irradiation groups, where the increase in LDH release was two fold greater in the latter group. This shows the enhancement of cellular damage by AZT. The greatest increase was at 0 h post-irradiation because the

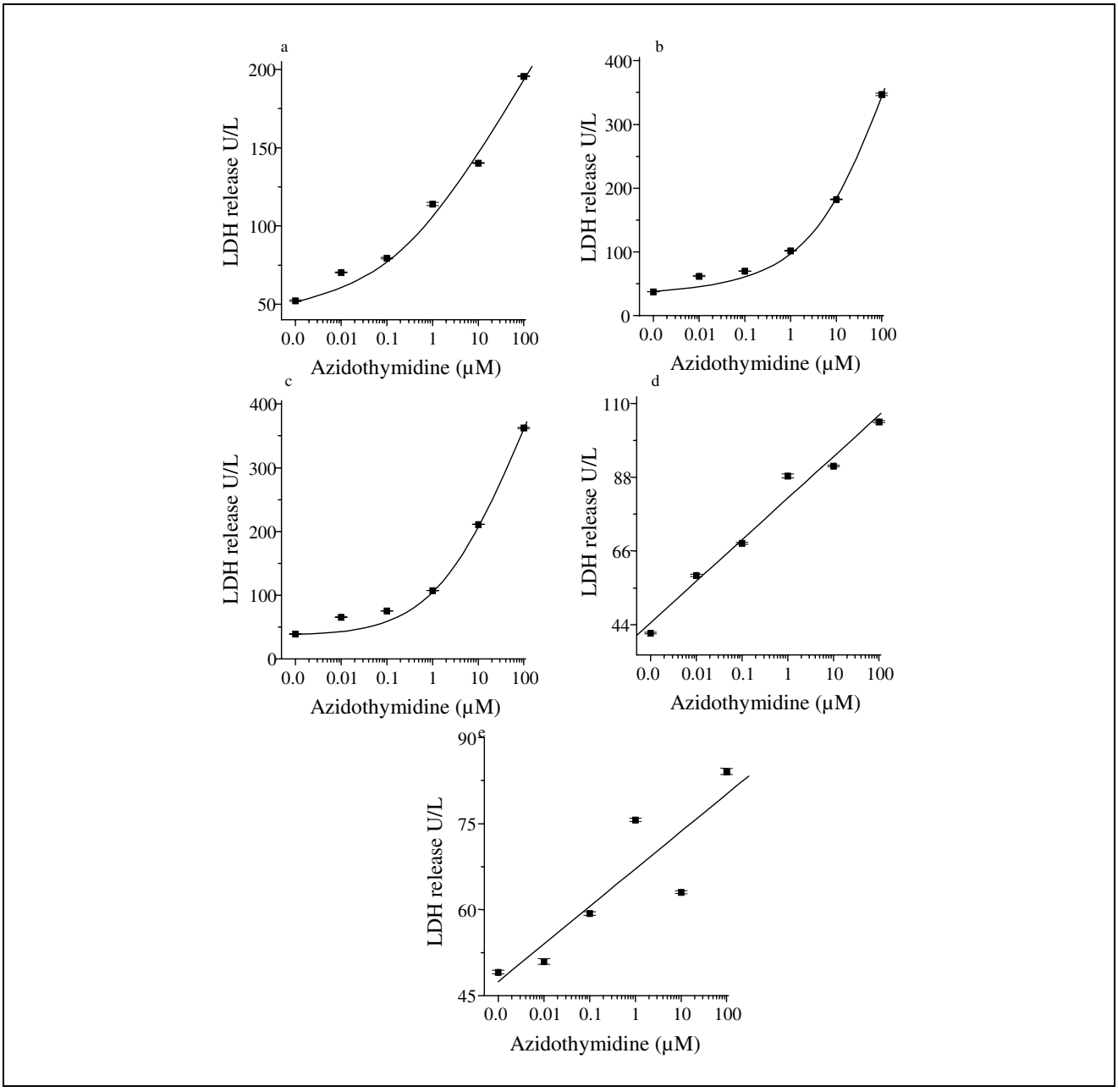

Fig 1: Effect of various concentrations of azidothymidine on the LDH release by HeLa cells at various time periods a) 0 h; b) 0.5 h; c) 1 h; d) 2 h and e) 4 h post-treatment.

cells were exposed up to 8 h of 0.1 μM AZT treatment before irradiation and the media was collected immediately after irradiation and LDH release measured. Therefore, it is an accumulation of LDH for 8 h, however for the sake of convenience it is considered 0 h post-irradiation. Various chemotherapeutic agents have been reported to elevate the LDH release in HepG2 cells in conjunction with irradiation [14]. The cytotoxic agents may be able to

Table 1: Influence of various concentrations of azidothymidine on the LDH release (U/L ± SEM) by HeLa cells at different post-treatment time periods

AZT	Post-treatment time (h)				
(μM)	0	0.5	1	2	4
0	52.24 ± 0.57	37.55 ± 0.23	39.41 ± 0.31	41.54 ± 0.25	49.13 ± 0.32
0.01	70.36 ± 0.53[a]	62.15 ± 0.87[a]	65.62 ± 0.24[a]	58.76 ± 0.33[a]	50.98 ± 0.50[a]
0.1	79.39 ± 0.42[a]	69.77 ± 0.24[a]	75.39 ± 0.25[b]	68.32 ± 0.30[a]	59.34 ± 0.30[a]
1	114.08 ± 0.99[a]	101.77 ± 0.72[a]	107.37 ± 0.40[a]	88.46 ± 0.60[a]	75.64 ± 0.27[a]
10	140.28 ± 0.24[a]	182.39 ± 0.68[a]	211.36 ± 0.25[a]	91.38 ± 0.26[a]	63.05 ± 0.27[a]
100	195.58 ± 0.23[a]	347.18 ± 1.73[a]	362.45 ± 0.93[a]	104.61 ± 0.30[a]	84.08 ± 0.53[a]

Level of significance a = $p < 0.0001$, b = $p < 0.003$ when compared to non-drug treated group.
Note: Each column shows average of 6 values at each data point

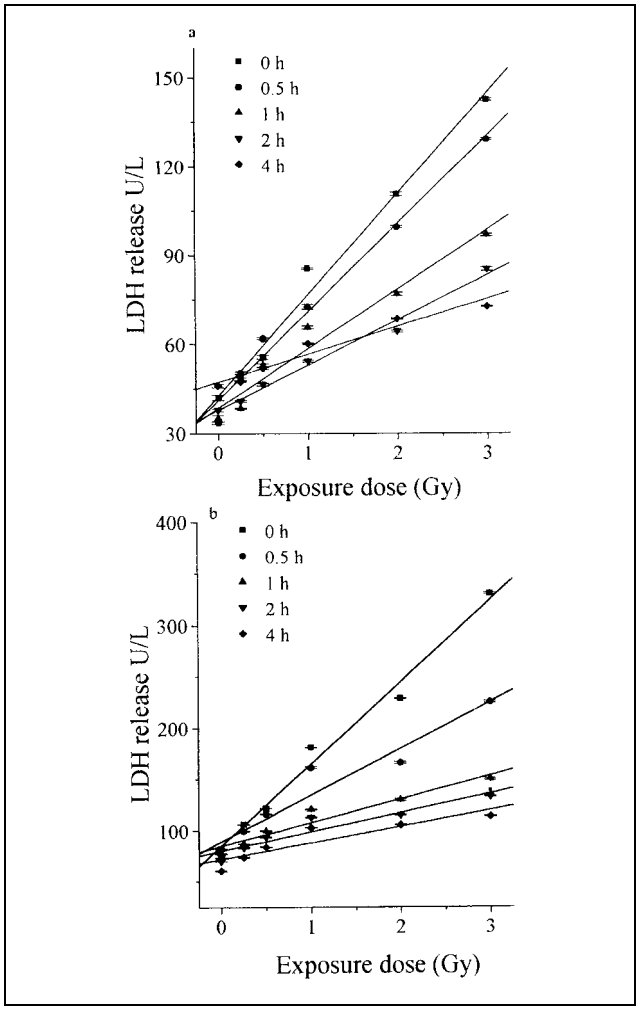

Fig 2: Alteration in the LDH release by HeLa cells treated with 0.1 μM AZT before exposure to different doses of γ-radiation. a) PBS + irradiation and b) AZT + irradiation groups.

bring about permeability changes in the cell membrane resulting in the leakage of LDH into the medium [30]. The dose dependent elevation of LDH in all three groups indicates the cytotoxic nature of various treatments. This is supported by our earlier study, where a concentration dependent reduction in cell survival and cell growth kinetics was observed after treatment with various concentration of AZT [31]. The pretreatment of HeLa cells with 0.1 μM AZT increased the radiation-induced decline in the cell survival [31]. Therefore, LDH assay can be a good-indicator of cytotoxicity as reported earlier [12–16, 32]. From our study it is apparent that AZT itself caused cytotoxic effects and its treatment before irradiation resulted in an enhancement of the effect of radiation.

4. Experimental

Appropriate amounts of azidothymidine (AZT) (Cipla Ltd., Bangalore, India) were dissolved in sterile double distilled water (DDW) and the drug was diluted to the required concentrations. A constant volume of 50 μl per 5 ml medium was added to individual cell cultures irrespective of the drug concentration. MEM, L-glutamine, gentamicin sulfate, fetal calf serum were procured from Sigma, Chemical Co., St. Louis, USA.

4.1. Cell line and culture

HeLa cells procured from National Centre for Cell Science, Pune, India, were used throughout this study. Cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal calf serum, 1% L-glutamine and 50 μg/ml gentamicin sulfate. Cells were routinely grown in 75 cm^2 flasks with loosened caps, and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air.

4.2. Experimental design

A fixed number of cells inoculated in several individual 25 cm^2 flasks were allowed to attain plateau phase. The plateau phase cells were divided into the following groups:
AZT treated group: The cells of this group were exposed to various concentrations viz. 0, 0.01, 0.1, 1, 10 and 100 μM of azidothymidine for 8 h [31].
PBS + irradiation: The cells of this group were treated with 10 μl/ml of sterile phosphate buffered saline (PBS) before irradiation.
AZT + irradiation: The cells of this group were treated with 0.1 μM of azidothymidine (AZT) before exposure to different doses of γ-radiation.

4.3. Irradiation

After 8 h of PBS or AZT (0.1 μM) treatment, the cells of PBS or AZT + irradiation group were exposed to 0, 0.25, 0.5, 1, 2 and 3 Gy γ-radiation from a ^{60}Cobalt therapy source (Gammatron, Siemens, Germany) at a dose rate of 1 Gy/min at a distance (SSD) of 54.5 cm. Triplicate cultures were used for each drug concentration or each radiation dose of PBS or AZT + irradiation group. The activity of LDH was estimated at 0, 0.5, 1, 2 and 4 h after drug treatment or post-irradiation as the case may be in the culture medum of all the three groups simultaneously. The estimation of LDH release in the culture media of those groups was carried out by the method described by Decker and Lohman Matthes [32] with minor modifications. The whole medium from the each cell culture of each group was removed and collected separately after 8 h of drug treatment or immediately after irradiation (within

Table 2: Alteration in the LDH (U/L ± SEM) release by HeLa cells treated or not with azidothymidine before exposure to various doses of γ-radiation

Exposure	Treatment	Post-irradiation time (h)				
Dose (Gy)		0	0.5	1	2	4
0	PBS + IR	41.92 ± 0.89	33.42 ± 0.44	34.83 ± 1.09	37.68 ± 0.44	45.90 ± 0.55
	AZT + IR	81.83 ± 0.34 [a]	72.83 ± 0.50 [a]	76.87 ± 0.48 [a]	69.36 ± 0.29 [a]	60.06 ± 0.52 [a]
0.25	PBS + IR	48.26 ± 0.44	50.27 ± 0.44	38.27 ± 0.22	40.75 ± 0.31	47.34 ± 0.26
	AZT + IR	105.79 ± 0.35 [a]	99.39 ± 0.42 [a]	86.79 ± 0.18 [a]	82.92 ± 0.65 [a]	73.59 ± 0.46 [a]
0.5	PBS + IR	55.53 ± 0.56	61.75 ± 0.32	52.84 ± 0.54	46.34 ± 0.45	51.79 ± 0.28
	AZT + IR	121.21 ± 0.34 [a]	115.94 ± 0.22 [a]	99.43 ± 0.42 [a]	93.07 ± 0.49 [a]	83.47 ± 0.48 [a]
1	PBS + IR	85.46 ± 0.23	72.58 ± 0.84	65.51 ± 0.58	54.24 ± 0.34	60.05 ± 0.30
	AZT + IR	180.86 ± 0.35 [a]	161.37 ± 0.89 [a]	120.35 ± 0.40 [a]	112.70 ± 0.58 [a]	102.62 ± 0.37 [a]
2	PBS + IR	110.52 ± 0.56	99.46 ± 0.38	76.83 ± 0.53	64.18 ± 0.30	68.43 ± 0.24
	AZT + IR	228.65 ± 0.62 [a]	165.88 ± 1.02 [a]	129.52 ± 0.89 [a]	114.85 ± 0.51 [a]	105.30 ± 0.34 [a]
3	PBS + IR	142.58 ± 0.42	129.11 ± 0.30	96.76 ± 0.45	85.23 ± 0.54	72.55 ± 0.20
	AZT + IR	330.54 ± 0.77 [a]	225.37 ± 0.88 [a]	149.86 ± 0.86 [a]	132.45 ± 0.60 [a]	113.47 ± 0.51 [a]
r	PBS + IR	0.99	0.99	0.98	0.99	0.97
	AZT + IR	0.99	0.96	0.96	0.93	0.90

Level of significance a=$p < 0.0001$ AZT + irradiation compared to corresponding PBS + irradiation group.
Note: Each column shows average of 6 values at each data point

5 min after irradiation) and was considered 0 h after treatment. The cells were fed with a fresh 5 ml medium and the above procedure (removal of media) was successively repeated at each assay period (i.e., 0.5, 1, 2 and 4 h) until termination of the experiment. Briefly, the tubes containing media were centrifuged and 50 µl of the medium was transferred to the individual tubes containing Tris-EDTA-NADH buffer followed by 10 min incubation at 37 °C and the addition of pyruvate solution. The absorbance was read at 340 nm on a photometer (Photometer 4010, Boehringer, Germany) and the values are expressed as units/liter (U/l). Significance of the treatment was determined using Student's "t" test. The experiments were repeated for confirmation of the results.

Acknowledgements: We are thankful to Dr. K. Koteshwar Rao, Professor and Head, and Dr. J. G. R. Solomon, Dept. of Radiotherapy and Oncology, Kasturba Medical College Hospital, Manipal, for providing the necessary irradiation facilities and for dosimetric calculations, respectively. The authors are grateful to ICMR, New Delhi, India, for providing financial assistance in the form of SRF to Mrs. Aruna to carry out this work. We are also grateful to CIPLA Ltd., Bangalore, India, for providing azidothymidine as a gift.

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Received March 10, 2000
Accepted April 25, 2000

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