

## Quantative Expression of Cell Death by LDH Activity

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**Abstract:** We hypothesize that a universal quantitative relation exists between the number of necrotic cells in culture and LDH activity in culture media. Previously this relation was found in immortalised cell lines. We hypothesized that similar correlation exists in normal cells of human origin. We investigated cultured human osteoblast-like cells. LDH activity in the media of cultured samples (n=8) was determined by 340 nm wavelength spectrophotometry. Cell viability was determined using automated cell counter. Significant and high correlation was found between LDH activity in culture media and the numbers of non-viable cells (n=8, CC – 0.95, power – 0.984, SE= 13,920, p< 0.001). These findings support the use of media LDH activity measurements for estimation of cell death rate in culture without the need of a major manipulation of the cultured cells.

**Keywords:** LDH, cell death, osteoblast, cell viability, cell number.

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### I. Introduction

The number of cells in in vitro culture is determined by the rate of cell proliferation and the rate of cell death. These parameters are usually measured in order to characterize a specific cell culture. The main obstacle in determining these parameters is the necessity to interfere with culture progress when manipulation of cells is required in different tests. In the case of the determination of cell death rate a “minimally invasive” method of measurement of LDH activity in culture media was suggested and established (Legrand et al. 1992). The rationale for this method originates from the fact that the intracellular enzyme *lactate dehydrogenase* (LDH) leaks out of the cells through damaged cell membrane of non-viable cells when the cell death progresses. Therefore, the measurement of LDH activity in culture media reflects the cell necrosis level of the cultures cells. Usually in order to quantify the rate of cell necrosis under specific conditions a comparison to control cultures exposed only to the vehicle of the tested culture conditions is utilized (Rosenberg et al. 2011; Rosenberg et al. 2013; Rosenberg et al. 2014). Much easier approach would be if a direct quantification of necrotic cells from the culture LDH activity would be possible. Although the initial report of this method presented a linear dependence between the number of necrotic cells in culture and the LDH activity in the culture media of BHK21/C13 cells (Legrand et al. 1992), that report has not been repeated yet on the same cells’ type or in other type of cells and therefore it is not apparent if an universal quantitative relation between the number of necrotic cells in culture and the LDH activity level in the culture media exists. Such knowledge might promote the ability to study the cell culture behavior without the necessity of major manipulation of the cultured cells.

In order further promote the use of the method of cell necrosis measurement we investigated the quantitative relation between cellular necrosis and LDH activity in culture media in different from the previously studied cell type (Legrand et al. 1992). i.e. we investigated cultures of human osteoblast-like cells, which are highly metabolically active mature cells. We hypothesized that a similar quantitative to the previously published relation between the studied parameters might be found in these cells. Such finding should indicate on a constant relation of these parameters in different cell types and will allow further confident use of measurement of LDH activity in culture media that should reflect a specific number of necrotic cells in a specific cell culture conditions.

### II. Methods

This experimental protocol was approved by the institutional Ethical Committee.

#### Cell Culture

In this study we used osteoblast-like cells of human origin originated from cancellous bone from distal femur. The cancellous bone samples were taken from the disposable bone tissue during osteoarthritic knee arthroplasty. Chips of cancellous bone, 2 – 3 grams in total, were incubated in DMEM with heat-inactivated fetal calf serum (10%), 20mM HEPES buffer, 2mM L-glutamine, 100µM ascorbate-2-phosphate, 10nM dexametasone, 50 U ml-ml penicillin, 150µ ml-ml streptomycin at 37°C in humidified atmospheric environment of 95% air with 5% CO<sub>2</sub> (v:v) for 20 days. Human osteoblast-like cells grew out from the chips as adherent to the plastic culture plates until confluence. The human bone cell cultures obtained by this method have been shown previously to express osteoblast-like characteristics (Gundle et al. 1998, Rosenberg et al. 2010). These

cells were passaged into 8 wells in plastic 24 well plate. Each well was seeded with equal number of cells ( $10^4$  cells) and cultured for 6 days in the above described osteogenic media with starting LDH activity in culture media of 15.5 U/L. On the 7<sup>th</sup> day of culture the media from all samples was collected for the LDH assay and the necrotic cells were counted by cytometric analysis.

#### LDH Activity in Culture Media

LDH activity in media of cultured samples was determined by 340 nm wavelength spectrophotometry of reduced nicotinamide adenine dinucleotide (NAD), which is directly proportional to LDH activity (Gay and Bowers 1968), i.e. measurement of oxidation of L- lactate to pyruvate at pH= 8.55 in Tris buffer 15.3 $\mu$ mol/L (Gay and Bowers 1968). The range of the assay was 0-600 U/l (spectrophotometer - Dimension AR IMT 110V/60HZ, Dade Behring, Inc. Newark, DE).

From the measured total LDH activity in all samples the basic activity of the LDH in the original media before its addition to cells was deduced, i.e. deduction of 15 U/l value from the final measurements of LDH activity, in order to measure the “true” LDH leak from damaged cells.

#### Cell number and Viability by Automatic Cytometry

Cell number and cell viability was determined using automated cell counter (1450101 TC20<sup>TM</sup>, Bio-Rad laboratories Inc. Hercules, CA, USA). Prior to counting the cells were re-suspended in 0.4% Trypan blue solution in order to distinguish between viable and non-viable cells (non-viable cells have permeable membranes and therefore stained by Trypan blue).

#### Statistical Analysis

The number of non-viable cells and the LDH activity in media were correlated by Pearson Product Moment correlation (Bourke et al. 1985). (Bourke et al. 1985). We used SigmaPlot 12.5 software (Systat Inc, USA) for the statistical calculations.

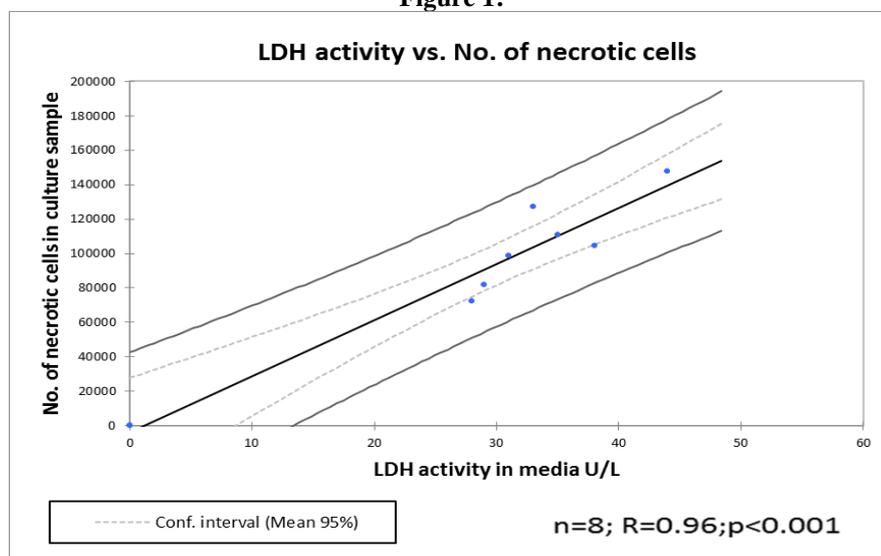
### III. Results

Significant and high correlation was found between LDH activity in culture media and the numbers of non-viable cells (n=8, correlation coefficient – 0.95, power – 0.984, SE= 13,920, p< 0.001, Pearson Product Moment Correlation, Fig. 1). This correlation shows that LDH activity of 10 U/L represents 28,677 $\pm$ 13,920 non-viable cells, i.e. between 15,000 – 42,597 non-viable cells.

### IV. Discussion

Our results show similar to the previously reported correlation between the number of the necrotic cells and LDH activity in culture media (1). Since we investigated normal human cells and reached similar quantitative results to the original report where malignant cell lines were investigated we can cautiously conclude on the relatively universal relation between the quantity of necrotic cells in culture and LDH activity in culture media. We think that this indication is of important practical use because it enables estimation of cell death rate in culture without the need of major manipulation of cultured cells.

Figure 1:



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