Toxicology in Vitro 30 (2015) 300–308

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/tiv

The effect of foetal bovine serum supplementation upon the lactate dehydrogenase cytotoxicity assay: Important considerations for *in vitro* toxicity analysis

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ARTICLE INFO

Article history:
Received 24 June 2015
Received in revised form 16 September 2015
Accepted 18 October 2015
Available online 21 October 2015

Keywords:
Assay interference
Cell viability
Foetal bovine serum
Heat inactivated
Lactate dehydrogenase
MTT assay

ABSTRACT

The lactate dehydrogenase (LDH) assay is a commonly-used tool for assessing toxicity *in vitro*. However, anecdotal reports suggest that foetal bovine serum (FBS) may contain LDH at concentrations significant enough to interfere with the assay and thus reduce its sensitivity. A series of experiments were performed to determine whether addition of FBS to culture medium significantly elevated culture media LDH content, and whether replacement of FBS with heat inactivated foetal bovine serum (HI-FBS) reduced LDH content and interfered with cell response to cytotoxic challenge. The addition of FBS at 5, 10 and 15% final concentrations increased culture medium LDH content in a dose-dependent manner. The substitution of HI-FBS for FBS reduced culture medium LDH content and increased the dynamic range of the assay. Cell viability of the SH-SY5Y human neuroblastoma and N27 rat mesencephalic neurone cell lines were significantly reduced as measured using the MTT reduction assay, whilst HI-FBS only affected toxicity response in a cell- and toxin-specific manner, although these effects were small. Hence, for cell lines with a high FBS requirement, the use of HI-FBS or alternative toxicity assays can be considered, or the use of alternative formulations, such as chemically-defined serum-free media, be adopted.

1. Introduction

The lactate dehydrogenase (LDH) assay is a commonly-used tool for determining *in vitro* cell toxicity and death. Functionally, the LDH assay determines toxicity by measuring the extent of LDH leakage from cells following plasma membrane rupture. This is achieved *via* the spectrophotometric measurement of the LDH-mediated production of a red formazan dye (Bopp and Lettieri 2008). The availability of the LDH assay in commercial formats and its scalability for high-throughput screening has made it a popular assay for assessing toxicity in cultured cell lines (Weyermann et al. 2005). However, significant drawbacks relating to its use can arise due to the variety of medium supplements commonly used in cell culture studies. Primary amongst these is the use of foetal bovine serum (FBS). FBS is used to provide cells with the nutrients, growth factors and hormones necessary for survival and proliferation (Wirthensohn and Barth 1985). FBS however also serves as an exogenous source of LDH, which may have significant consequences for toxicity analysis, in particular in those cell lines which are highly serum-dependent such as the SH-SY5Y human neuroblastoma cell-line, which routinely require 10–15% serum concentration (Parsons et al. 2011). The significant amount of LDH in FBS exceeds that released by test cells, thus reducing the sensitivity of the LDH assay. In order to circumvent these drawbacks, many researchers opt to reduce serum content (Decker and Lohmann-Matthes 1988; Bopp and Lettieri 2008). However, such reductions in serum may predispose cell cultures to lower viability prior to the introduction of toxins, which may compromise the cells' response to toxin challenge and creates the potential for false positive results. To address these concerns, we undertook a series of experiments designed to elucidate the applicability of lowering or replacing the FBS content in LDH toxicity analyses and the effects that such changes have upon cell viability and response to cytotoxic challenge using the SH-SY5Y and N27 cell lines.

2. Materials and methods

Unless otherwise stated, all reagents were obtained from Sigma (Poole, Dorset, UK) and were of the highest grade available.

2.1. Culture of SH-SY5Y and N27 cells

SH-SY5Y human neuroblastoma cells were cultured in 1:1 Dulbecco's Modified Eagle Medium:F12 medium supplemented with 15% FBS, 1× non-essential amino acids, 2 mM L-glutamine, 100 Units/mL penicillin and 100 μg/mL streptomycin (Invitrogen, Paisley, UK). SH-SY5Y were used in their undifferentiated state as differentiation protocols require the replacement of FBS with 1% heat inactivated FBS (HI-FBS)

Abbreviations: FBS, foetal bovine serum; HI-FBS, heat inactivated foetal bovine serum; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NH, norharman; MeN, 1-methylnorharman.

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http://dx.doi.org/10.1016/j.tiv.2015.10.007
0887-2333/

(Lopes et al. 2010). N27 rat mesencephalic neurones were cultured in AQmedia supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin and 100 U/mL streptomycin.

2.2. Linear absorbance range of the lactate dehydrogenase assay

Triplicate 50 μL samples of 10× LDH positive control (diluted in 1% bovine serum albumin-supplemented phosphate-buffered saline), obtained as part of the CytoScan® LDH Cytotoxicity assay (G-Biosciences, St. Louis, Missouri, USA), were added to the wells of a clear, flat-bottomed 96-well plate (Thermo Scientific, Loughborough, UK) as outlined in Fig. 1. The LDH assay was performed as previously described (Parsons et al. 2011), with absorbance measured at 490 nm. Results were expressed as absorbance units ± S.D. (n = 3) without subtracting the background absorbance (1% bovine serum albumin-supplemented phosphate-buffered saline). The dynamic range of the LDH assay (DRt) was subsequently calculated using the following formula:

$$DRt = A_{max} - A_{min}$$

and expressed as absorbance units ± SD.

2.3. Linear absorbance range of the MTT reduction assay

SH-SY5Y cells were seeded into the wells of a 96-well plate in quintuplicate at a density of 0–50,000 cells/well and allowed to settle overnight, after which 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was measured as previously described (Matharu et al. 2009). Results were calculated and expressed as absorbance units ± SD (n = 4), with DRt calculated as described for the LDH assay.

2.4. The effect of supplementation upon culture medium lactate dehydrogenase content

Supplements were added step-wise to culture media as detailed in Fig. 2. Triplicate 50 μL samples were transferred at each stage to a clear, flat-bottomed 96-well plate and assayed for LDH content using a Wallac Victor^2 Multilabel Photon Counter (ThermoFisher). Results were calculated and expressed as absorbance ± SD. The dynamic range (DR) of the assay for each supplement sample was calculated as described for DRt, after which the relative DR (rDR) was calculated and expressed as a percentage of DRt ± SD (n = 3) using the following formula:

$$rDR = 100 \times \frac{DR}{DRt}.$$

2.5. The effect of foetal bovine serum concentration and heat inactivated foetal bovine serum upon culture media lactate dehydrogenase content

Triplicate 50 μL samples of SH-SY5Y media supplemented with 0%, 5%, 10%, and 15% FBS were added to a clear, flat-bottomed 96-well plate, along with LDH positive control, after which the LDH assay was performed. Furthermore, triplicate samples of 15% HI-FBS-supplemented media (50 μL) were also analysed. Results were calculated and expressed as both absorbance ± SD and rDR ± SD.

2.6. The effect of foetal bovine serum & heat inactivated foetal bovine serum upon cell viability and cell death

SH-SY5Y and N27 cells cultured in both FBS and HI-FBS-supplemented media were seeded in quadruplicate into the wells of a 96-well plate at a density of 10,000 cells/well. The cells were then incubated for a further 48 h, after which cell viability and death were assessed using the MTT reduction and LDH release assays respectively. Results were calculated, cell viability was expressed as percentage cell viability compared to cells cultured in FBS-supplemented media ± SEM (n = 4), and cell death was expressed as percentage cell death ± SEM (n = 6).

2.7. Comparison of the effect of foetal bovine serum and heat inactivated foetal bovine serum upon the toxicity of norharman and 1-methylnicotinamide

SH-SY5Y and N27 cells were seeded as described above in quadruplicate and incubated with serial dilutions of norharman (NH) (0–800 μM) or 1-methylnicotinamide (MeN) (0–10 mM) for 48 h, after which cell viability and death were measured using the MTT reduction and the LDH release assays respectively. Results were calculated, cell viability was expressed as percentage cell viability compared to untreated cells ± SEM, and cell death was expressed as percentage cell death ± SEM. To calculate EC_{50} for each compound, concentration was log-transformed, and analysed using non-linear regression analysis using the following equation:

$$y = \text{minimum} + \frac{\text{maximum} - \text{minimum}}{1 + 10^{(\text{LogEC50} - x) * \text{hillslope}}}$$

using a constraint of y = 0 for the minimum.

2.8. Statistical analysis

All statistical analyses were undertaken using Prism 5 (GraphPad, San Diego, USA). In all instances, significance was taken as $p < 0.05$.

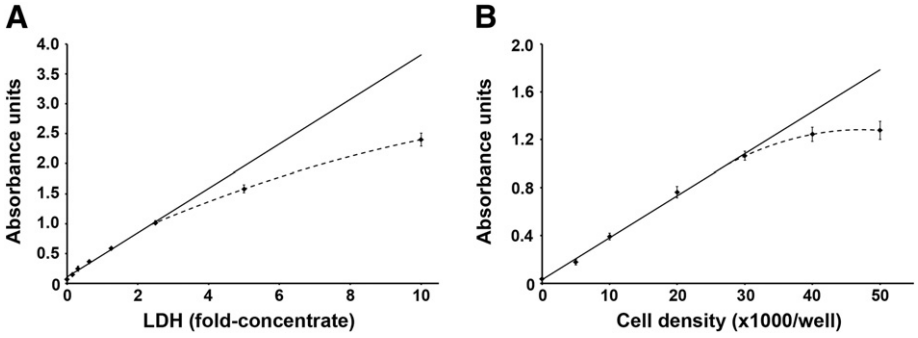

Fig. 1. Linearity of the lactate dehydrogenase and MTT reduction assays. (A) Lactate dehydrogenase assay. Dilutions of LDH positive control were assayed for LDH content. (B) MTT reduction assay. SH-SY5Y cells were seeded at the densities shown, allowed to settle for 24 h and MTT reduction measured. For both panels: results are expressed as absorbance units ± SD (n = 3 for LDH, n = 4 for MTT) without background being subtracted. The linear range was determined using linear regression analysis (solid lines), with deviation from linearity shown (dashed lines).