

# **Tumour cell culture survival following glucose and glutamine deprivation at typical physiological concentrations**

**Edward Henry Mathews, PhD<sup>a</sup>, B. André Stander, PhD<sup>b</sup>,**

**Annie M. Joubert, PhD<sup>b</sup>, and Leon Liebenberg, PhD<sup>a,\*</sup>**

<sup>a</sup> Centre for Research and Continued Engineering Development, North-West University, Suite No 91, Private Bag X30, Lynnwood Ridge 0040, South Africa; and consultants to TEMM International (Pty) Ltd, and MCI (Pty)Ltd.

[EHMathews@researchtoolbox.com](mailto:EHMathews@researchtoolbox.com); [LLiebenberg@researchtoolbox.com](mailto:LLiebenberg@researchtoolbox.com)

<sup>b</sup> Department of Physiology, School of Medicine, Faculty of Health Sciences, University of Pretoria, Pretoria 0001, South Africa.

[Annie.Joubert@up.ac.za](mailto:Annie.Joubert@up.ac.za); [standerandre@gmail.com](mailto:standerandre@gmail.com)

**RUNNING HEAD: Glucose deprivation for cancer control?**

**\* Corresponding author:** Tel.: +27 12 809 0995; Fax: +27 86 661 8408

E-mail: [LLiebenberg@researchtoolbox.com](mailto:LLiebenberg@researchtoolbox.com) (L. Liebenberg)

WORD COUNT: **4949**

NUMBER OF FIGURES (AND TABLES): **7**

NUMBER OF REFERENCES: **37**

## ABSTRACT

**Objective:** Most glucose (and glutamine) deprivation studies of cancer cell cultures focus on *total* depletion, and are conducted over 24 h or longer. It is difficult to extrapolate findings from such experiments to practical anti-glycolytic treatments such as with insulin-inhibiting diets (with 10% - 50% carbohydrate dietary restriction) or with isolated limb perfusion (ILP) therapy (which usually lasts around 90 min). We aimed to obtain experimental data on the effect of *partial* deprivation of D-glucose and L-glutamine (to typical physiological concentrations) during 0 – 6 h exposures of HeLa cells.

**Methods:** HeLa cells were treated for 0 to 6 h with 6 mM D-glucose and 1 mM L-glutamine (normal *in vivo* conditions), 3 mM D-glucose and 0.5 mM L-glutamine (severe hypoglycaemic conditions), and 0 mM D-glucose and 0 mM L-glutamine ('starvation'). PlasDIC and phase contrast light microscopy were employed to investigate morphological changes.

**Results:** Reduction of glucose levels from 6 mM to 3 mM (and glutamine levels from 1 mM to 0.5 mM) brings about cancer cell survival of 73% after 2 h exposure and 63% after 4 h exposure. Reducing glucose levels from 6 mM to 0 mM (and glutamine levels from 1 mM to 0 mM) for 4 h resulted in 53% cell survival.

**Conclusion:** These data reveal that glucose (and glutamine) deprivation to typical physiological concentrations result in significant cancer cell killing after as little as 2 hours. This supports the possibility of combining anti-glycolytic treatment, such as a carbohydrate-restricted diet, with chemotherapeutics for enhanced cancer cell killing.

**Keywords:** Glucose deprivation; HeLa cell survival and morphology; Metabolic cancer control

## Introduction

Around 90% of cancer-associated deaths can be ascribed to highly glycolytic cancers and metastases (HGCM) [1]. *In vitro* tests have shown that increased glucose consumption in HGCM ensures that the metabolically inflexible cancer cells are more susceptible to glucose deprivation-induced cytotoxicity and oxidative stress than non-transformed cells [2-5].

Inverse HGCM comorbidity in people with certain complex disorders could provide *in vivo* proof of the beneficial effect of low blood glucose (BG) microenvironments [6]. The typical adverse response of chemotherapeutics on HGCM in rodents (with low BG microenvironments) but not in their human counterparts (with their high BG microenvironments) provides further *in vivo* evidence of the beneficial effects of a low BG microenvironment [7].

Recent evidence also links chronic hyperglycaemia to increased risk of most cancers [8]. Diabetogenic glucose concentrations ( $\approx 11$  mM) compared to physiological ones ( $\approx 5.5$  mM) lead to altered expression of genes that promote cell proliferation, migration and adhesion in tumour cells lines from several organs [9]. Adding insulin to the high-glucose medium further enhances proliferation rates by 20-40% [9] and promotes activation of the tumorigenic PI3K pathway [10]. Many cancer cells express insulin receptors (IR) and show hyperactivation of the insulin growth factor (IGF)-IR [8,10].

Early evidence also suggests that the anti-diabetic drug metformin is associated with lower risk of cancer [8,10,11]. Metformin decreases basal glucose by suppressing hepatic gluconeogenesis and glycogenolysis and by increasing glucose uptake in muscle tissue [8]. The lower circulating glucose levels lead to improved insulin sensitivity and decreased insulin levels, which are thought to diminish cancer growth [12,13].

Further *in vivo* proof of the benefits of low BG levels is provided by the fact that calorie restriction in animal models and in humans may be responsible, directly or indirectly, for their significantly prolonged survival compared to normally fed control animals or humans [5,9,15]. This inhibition of tumorigenesis is possibly due to reduced levels of IGF-1 [10,15]; stimulation of autophagy, mitophagy and the AMPK pathway [16];

and mTOR inhibition and its reduction of inflammatory cytokines [17]. Severe caloric restriction also lowers basal metabolic rates with a resultant decrease of mutagenic oxidative stress [18].

Whilst the high demand for carbon in proliferating tumour cells and associated fibroblasts is provided by glucose metabolism [19], the high demand for nitrogen is obtained from glutamine metabolism [5,11]. Co-regulation of pathways that govern glucose and glutamine uptake and utilization is therefore expected [20]. It has been shown that glucose deprivation indeed leads to a marked reduction in glutamine uptake [21]. Also, some HGCM have been shown to be totally dependent on glucose, not glutamine, for energy production and survival [22].

Glucose thus appears at the top of cancer cell (and cancer-associated fibroblast) metabolic hierarchy, followed by glutamine. Therefore, if cancer cells and associated fibroblasts can be deprived of glucose, the cancer cells would not only be less able to metabolise glutamine but would also be deprived of *de novo* produced lactate, fatty acids and ketone bodies. However, if simultaneous deprivation of glucose and glutamine can be performed, cancer cells would be dealt a severe metabolic blow. Valid predictive models for highly glycolytic cancers exposed to glucose (and glutamine) deprivation would be helpful in establishing appropriate *in vivo* glucose (and glutamine) concentrations to bring about maximum cancer cell killing with minimal ‘innocent bystander’ effects.

Most researchers show the effect of *total* glucose deprivation (i.e., ‘starvation’) on cancer cell survival. Typically, cancer cell lines are cultured in 25 mM glucose medium and then subjected to 0 mM glucose medium for 24 h [2,4]. It is difficult to extrapolate from here to more typical physiological blood glucose (BG) environments, such as 3 mM or 6 mM glucose [19], and for shorter deprivation periods (such as 2 h or 4 h). This may be important when translating *in vitro* findings to practical metabolic therapies including insulin-inhibiting diets [14], and *ex vivo* (extracorporeal) anti-glycolytic treatments such as with isolated limb perfusion (ILP) therapy of soft-tissue carcinoma that lasts in the vicinity of 90 min [23].

This paper intends to partially address the lack of such *in vitro* data. We investigate the effect of short-term exposure (2 to 6 h) of HeLa cells to varying D-glucose and L-glutamine concentrations that are similar to typical physiological values.

## **Materials and Methods**

### ***Cell line***

The effect of combined D-glucose and L-glutamine deprivation was conducted on HeLa (human epithelial cervix carcinoma) cells. The HeLa cell line was purchased through Sterilab Services (Pty) Ltd, Johannesburg, South Africa, from the American Tissue Culture Collection (ATCC), Maryland, USA.

### ***General reagents***

D-Glucose, L-glutamine and sodium pyruvate-free Dulbecco's Minimum Essential Medium Eagle (DMEM) as well as high D-glucose (25.52 mM, 4500 mg/L), L-glutamine (4 mM) and sodium pyruvate (1 mM, 110 mg/L) containing DMEM, bicarbonate, L-glutamine, D-glucose, Trypsin-EDTA, crystal violet, NaCl, KCl,  $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$  were supplied by Sigma Chemical Co. (St. Louis, MO, USA). Heat-inactivated fetal bovine serum (FBS), sterile cell culture flasks and plates were obtained through Sterilab Services (Johannesburg, South Africa). Penicillin, streptomycin and fungizone were purchased from Highveld Biological (Pty) Ltd. (Sandringham, South Africa).

### ***General cell culture procedures***

Cells were grown and maintained in 25 cm<sup>2</sup> tissue culture flasks in a humidified atmosphere at 37°C, 5% CO<sub>2</sub> in a Forma Scientific water-jacketed incubator (Ohio, United States of America). HeLa cells were cultured in DMEM with 25.52 mM D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Cells were harvested by trypsinization and counted by making use of a haemocytometer as described by Freshney (2008). Suspended cells (20 µl) were mixed with 80 µl FBS and 100 µl trypan blue to provide a

concentration of cells with a 10-times dilution factor. Dead cells absorb the dye and are consequently stained blue, which are then left uncounted. The number of *viable* cells per mL was determined by:

$$\text{Cells/ mL} = \text{Average count of } \textit{viable} \text{ cells in the corner squares} \times \text{dilution factor} \times 10^4$$

For experiments, cells were seeded in 6-well (60 000 cells per well, 500  $\mu\text{L}$ /well) tissue culture plates, or in 25  $\text{cm}^2$  tissue culture flasks (750 000 cells per flask). Cells were incubated for 24 hours after seeding to allow for attachment after which medium was removed; cells were rinsed twice with FBS and subsequently exposed to the various experimental conditions.

### ***Morphology: Light microscopy via polarization-optical differential interference contrast (PlasDIC) and phase contrast***

Polarization-optical differential interference contrast (PlasDIC) is a polarization-optical transmitted light differential interference contrast method from Zeiss where linearly polarized light is only generated after the objective lens [24]. PlasDIC and phase contrast light microscopy were employed to recognize and display morphological changes in relation to other cells in order to gain insight on the effects that various exposures have on the cells.

### ***Cell Survival Assay - Colony formation***

Cells were seeded at 60 000 cells per well in 6-well plates and cultured overnight to allow for attachment. After cell attachment, the medium was discarded and the cells were rinsed twice with FBS. Cells were exposed to the following conditions for 2 hours, 4 hours and 6 hours:

Control: DMEM with 25.52 mM D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100  $\mu\text{g/ml}$  streptomycin and fungizone (250  $\mu\text{g/l}$ ).

Experimental Condition 1: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100  $\mu\text{g/ml}$  streptomycin and fungizone (250  $\mu\text{g/l}$ ).

Experimental Condition 2: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Experimental Condition 3: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Experimental Condition 4: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 0 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Experimental Condition 5: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 0 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

Experimental Condition 6: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 0 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56 °C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l).

After exposure, cells were trypsinized, counted and reseeded in 6-well plates at 800 cells per well. Cells were cultured under normal growth conditions (DMEM with 25.52 mM D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate supplemented with 10% heat-inactivated FBS (56°C, 30 min), 100 U/ml penicillin G, 100 µg/ml streptomycin and fungizone (250 µg/l) for 7 days to form colonies.

Cell numbers were spectrophotometrically quantified with crystal violet as a DNA stain. Staining cell nuclei of fixed cells with crystal violet allows for rapid, accurate and reproducible quantification of cell number in cultures grown in culture plate [25,26]. After 7 days the medium was discarded and 600 µl of 1% glutaraldehyde (in FBS) was added to each well and incubated at room temperature for 15 min. Glutaraldehyde was discarded and 600 µl 0.1% crystal violet (in FBS) was added and left at room temperature for 30 min. Crystal violet was discarded, micro-titer plates were immersed in running tap water for 10 min and left overnight to dry.



Stained cells were solubilized by adding 1200  $\mu$ l 0.2% Triton X-100 and incubated at room temperature for 30 min. The solution 100  $\mu$ l (3 $\times$ ) was transferred to a clean 96-well micrometer plate and the absorbance was read at 570 nm with an EL<sub>x</sub>800 Universal Microplate Reader from Bio-Tek Instruments Inc. (Vermont, United States of America).

Quantification of fixated monolayer cells were spectrophotometrically determined by employing crystal violet as a DNA stain. Cell survival was expressed as a percentage of the control which was set as 100%.

### **Statistics**

Data from the crystal violet assay were obtained from three independent biological replicates and each biological replicate provided three technical replicates. The obtained data were statistically analyzed for significance using a two-tailed Student's t-test and two-way ANOVA and *P*-values < 0.05 were regarded as statistically significant. Cell survival of the control was set to 100% and the means of the experimental conditions were calculated relative to the control [27]. Means of the experimental conditions are represented on the graph with error-bars referring to standard deviation.

### **Results**

#### ***Polarization-optical differential interference contrast and phase contrast (PlasDIC) light microscopy***

PlasDIC and phase contrast light microscopy were used to recognize and display morphological changes in relation to other cells in order to gain insight on the effects that various exposures have on the cells. No major morphological observations of increased apoptosis, reduction in cell density or cell shrinking were observed in any of the experimental conditions after 2 h, 4 h or 6 h (Figs. 1, 2 and 3, respectively). However, 7 days after reseeding the various experimental conditions differences in cell density were observed, as well as the formation of debris as a result of detached dead cells.

HeLa cells that were exposed to 0 mM D-glucose, 0 mM L-glutamine, and 1 mM sodium pyruvate DMEM for 2 h, 4h and 6h (Figs. 4D, 5D and 6D, respectively), 3 mM D-glucose, 0.5 mM L-glutamine, and 0 mM sodium pyruvate for 2 h, 4 h and 6 h (Figs. 4F, 5F and 6F, respectively) and DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 0 mM sodium pyruvate for 2 h, 4 h and 6 h (Figs. 4G, 5G and 6G, respectively) all displayed a decrease in cell density compared to their controls (Figs. 4A, 5A and 6A, respectively). Also, an increase in the formation of cellular debris in these samples, particularly pronounced in cells treated with 0 mM D-glucose, 0 mM L-glutamine, and 0 mM sodium pyruvate for 6 h, was revealed (Fig. 6).

These results suggest that immediate morphological changes after 2-6 h exposure are not apparent with PlasDIC light microscopy. However, the experimental conditions where D-glucose and L-glutamine levels were below 6 mM and 1 mM for 2-6 h had a negative effect on colony formation and cell growth 7 days after reseeded. Debris formation is also an indication of an increase in cell death with these experimental conditions.

We also investigated the effect of HeLa cells to withdrawal of glucose/glutamine and the glycolytic product pyruvate, which may serve as an alternate substrate for the TCA cycle. Pyruvate withdrawal resulted in only a minor ( $\approx 10\%$ ) loss of cell viability. This is consistent with previous reports [2].

**Fig. 1.** PlasDIC light microscopy (40 $\times$ ) showing influence of a 2 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 0 h exposure, (B) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (C) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (D) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (E) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (F) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (G) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (H) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

**Fig. 2.** PlasDIC light microscopy (40×) showing influence of a 4 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

**Fig. 3.** PlasDIC light microscopy (40×) showing influence of a 6 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 6 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

**Fig. 4.** Phase contrast light microscopy (10×) showing influence of a 2 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

**Fig. 5.** Phase contrast light microscopy (10×) showing influence of a 4 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, D-glutamine or sodium pyruvate.

(A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 0 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

**Fig. 6.** Phase contrast light microscopy (10×) showing influence of a 6 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate.

(A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.

## Discussion

All of the experimental conditions resulted in a reduction in the number of surviving cells when compared to the control. This indicates that reduction in D-glucose and L-glutamine levels in a dose-dependent manner from 6 mM and 1 mM and lower, respectively, have a negative effect on cell survival (Fig. 7A). Complete removal of D-glucose and L-glutamine had the most significant adverse effect on cell survival (Fig. 7A) and an increase in the time of exposure to D-glucose and L-glutamine removal (E3 and E6) resulted in further decreases in cell survival (Figure 7B).

**Fig. 7.** Control: DMEM medium with 25.52 mM D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate;

E1: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 1 mM sodium pyruvate;

E2: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 1 mM sodium pyruvate;

E3: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 1 mM sodium pyruvate;

E4: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 0 mM sodium pyruvate;

E5: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 0 mM sodium pyruvate;

E6: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 0 mM sodium pyruvate.

**A)** For 2 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E2 vs E6, E1 vs E6, E3 vs E6 and E4 vs E6.

For 4 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E1 vs E5, E1 vs E6, E2 vs E3, E2 vs E4, E2 vs E6, E3 vs E4, E3 vs E6, E4 vs E5, E4 vs E6, and E5 vs E6.

For 6 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E1 vs E6, E2 vs E4, E2 vs E6, E3 vs E4, E3 vs E6, E4 vs E6, and E5 vs E6.

**B)** For E3; student *t*-test *P*-values less than 0.05 for 2 hours exposure vs 6 hours exposure.

For E6; student *t*-test *P*-values less than 0.05 for 2 hours exposure vs 4 hours exposure and 2 hours exposure vs 6 hours exposure. Two-way ANOVA analysis (*P*-values less than 0.05) suggested that an increase in the time of exposure to conditions E3 and E6 resulted in decreased cell survival.

The increased pro-oxidant status of cancer cells provides an opportunity for the selective killing of cancer cells [15]. Reactive oxygen species (ROS) and an intracellular pro-oxidant status above a certain level selectively activate pro-apoptotic SAPK pathways [28]. It is known that glucose deprivation is able to increase oxidative stress as early as 1 h after the onset of glucose deprivation [29]. Glucose deprivation results in increased formation of glutathione disulfide (GSSG), superoxide and hydrogen peroxide, resulting in pro-oxidant status [28,29].

Redox homeostasis abrogation in cancer cells is characterized by an intracellular pro-oxidant status as a result of the cumulative production of ROS and the diminished capacity of antioxidant systems to balance the excess production of ROS [28]. ROS activate pro-growth, pro-survival and proliferative signals through the RAS-RAF-ERK1/2, PKB/AKT and NF- $\kappa$ B pathways [30,31].

We argue that the initial 2-6 h glucose deprivation results in a pro-oxidant status in cancer cells. Since cancer cells are more susceptible to oxidative stress due to their increased oxidative state [28,31], this initial increase should result in oxidative damage to important biomolecules [32]. This initial damage possibly plays a causal role in the subsequent abrogation of cell growth we observed and we think this provides a basis for future research.

Figure 7A further reveals that a reduction in D-glucose and L-glutamine levels has an adverse effect on HeLa cell survival even after two hours of exposure. This demonstrates the possibility of killing cancer cells through glucose deprivation *in vitro*. Recapitulating this putative therapeutic effect is difficult by only cytotoxic means [33] due to, among others, the innocent bystander effect [15].

When depriving the whole body of glucose and glutamine, safe concentrations are typically 2 mM and 1 mM, respectively [19]. Extensive cancer cell killing is expected based on results from the present *in vitro* experiment. Preliminary data show that carbohydrate restriction diets resulting in moderate ketosis can result in stabilization or remission of cancer [14]. Such diet-induced suppression of glucose and insulin levels might be more effective when combined with pharmacological treatments such as metformin, but this remains to be proven [34].

Wu et al. (2012) demonstrated that lactic acidosis resulted in resistance to cell death in glucose-deprived cancer cells [35]. This suggests that disrupting lactic acidosis can be beneficial in conjunction with glucose-deprivation of cancer cells. Dichloroacetic acid is an inhibitor of pyruvate dehydrogenase kinase 2 and promotes the entry of pyruvate into oxidative phosphorylation, thus negatively affects fermentation and lactic acid formation [36]. To enhance cancer cell killing, it seems to be prudent to combine glucose/glutamine deprivation treatment, inhibitors of lactic acidosis such as DCA and other forms of chemoradiotherapy. Also, lower concentrations of cytotoxic agents would then be required, with lower adverse effects to surrounding healthy tissue, meriting further investigation.

## **Conclusion**

*In vitro* data reveal that glucose (and glutamine) deprivation to typical physiological concentrations result in significant HeLa cancer cell killing after as little as 2 hours. Further *in vivo* studies are warranted to investigate the possibility of combining glucose and glutamine deprivation with chemoradiotherapeutic treatment of highly glycolytic cancers for enhanced cancer cell killing.

## **Acknowledgements**

We acknowledge angel investor Dr Arnold van Dyk. TEMM International (Pty) Ltd, MCI (Pty) Ltd, and the University of Pretoria's Department of Physiology (School of Medicine) co-funded the project. We thank Prof. Eugene Fine (Albert Einstein College of Medicine), Prof. Thomas Seyfried (Boston College), Prof.

Douglas Spitz (University of Iowa), Dr. Rainer Klement (University Hospital of Würzburg), and Prof. Robert Gatenby (Moffitt Cancer Center) for proofreading the manuscript and for their valuable comments and suggestions.

### **Competing interests**

The authors declare that they have no competing interests.

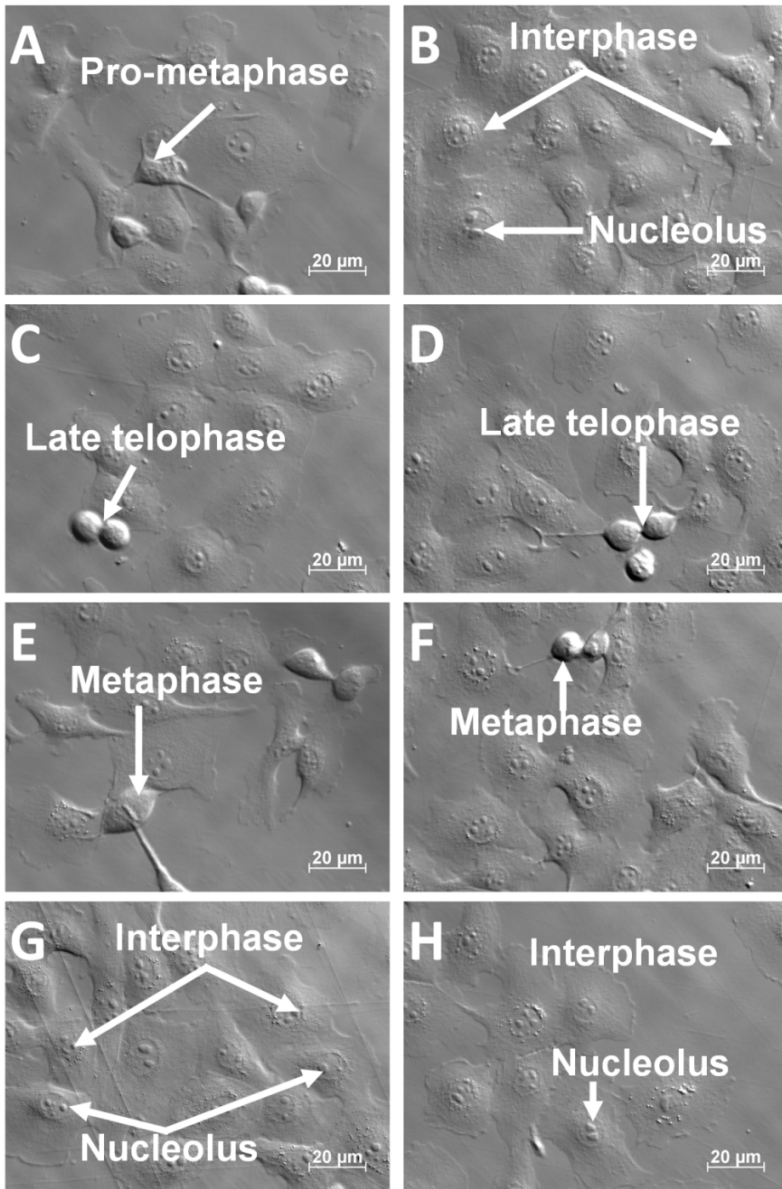


## References

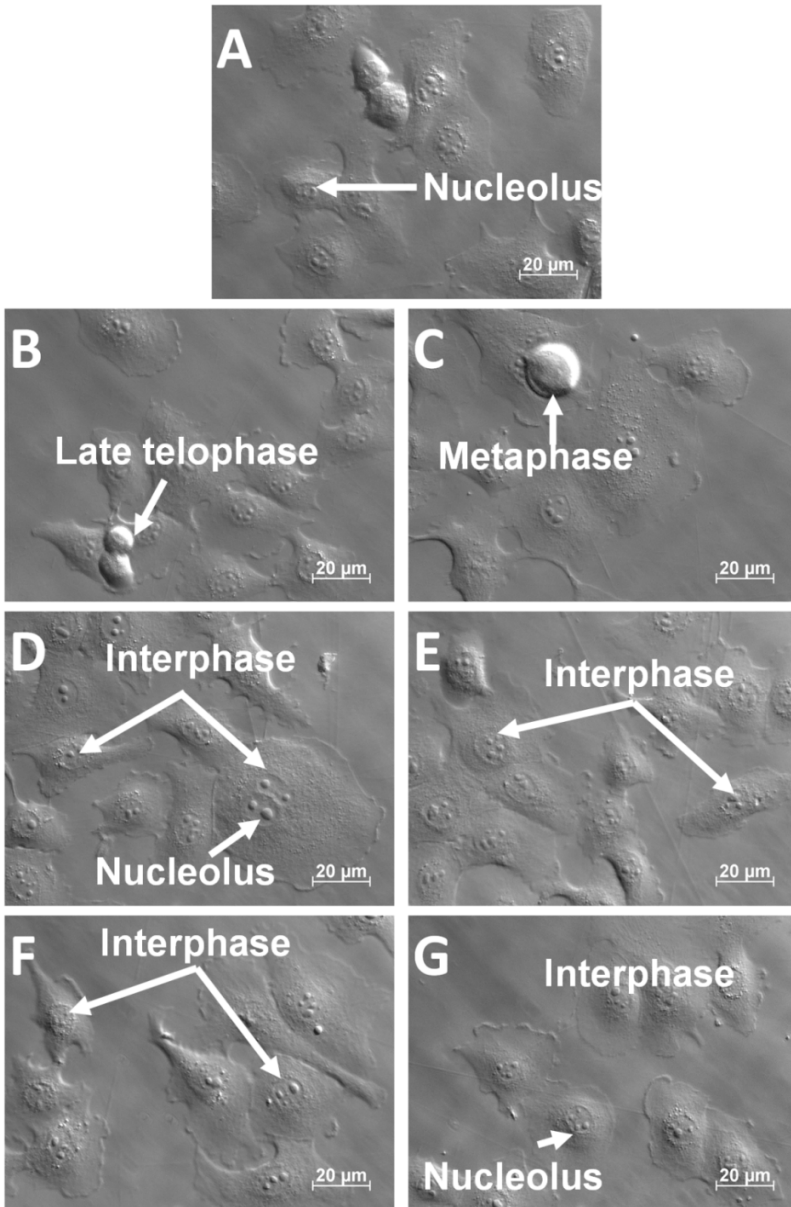
- [1] Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. *Science* 2011;331:435–39.
- [2] Graham NA, Tahmasian M, Kohli B, Komisopoulou E, *et al.* Glucose deprivation activates a metabolic and signaling amplification loop leading to cell death. *Mol Syst Biol* 2012;8:589.
- [3] El Mjiyad N, Caro-Maldonado A, Ramírez-Peinado S, Muñoz-Pinedo C. Sugar-free approaches to cancer cell killing. *Oncogene* 2011;30:253–64.
- [4] Priebe A, Tan L, Wahl H, Kueck A, *et al.* Glucose deprivation activates AMPK and induces cell death through modulation of Akt in ovarian cells. *Gynecol Oncol* 2011;122:389–95.
- [5] Seyfried TN, Shelton LM. Cancer as a metabolic disease. *Nutr Metab* 2010;7:7.
- [6] Mathews EH, Liebenberg L. Can successful cancer therapies build on what we learn from complex disorders? *Med Hypoth* 2012;78:687–9.
- [7] Mathews EH, Liebenberg L. Complexity of metabolic cancer control: can we exploit the superior metabolic position of glucose? *Cancer Biol Therap* 2012b;13(8):1.
- [8] Giovannucci EA, Harlan DM, Archer MC, Bergental RM, *et al.* Diabetes and Cancer, a consensus report. *Diab Care* 2010;33:1674–85.
- [9] Klement RJ, Kämmerer U. Is there a role for carbohydrate restriction in the treatment and prevention of cancer? *Nutr Metab* 2011;8:75.
- [10] Pollak M. Metformin and Other Biguanides in Oncology: Advancing the Research Agenda. *Cancer Prev Res* 2010;3(9):1060–5.
- [11] Vander Heiden MG, Lunt SY, Dayton TL, Fiske BP, *et al.* Metabolic pathway alterations that support cell proliferation. *Cold Spring Harbor Symposia on Quantitative Biology* LXXVI, 2012:10 pages.
- [12] Martinez-Outschoorn UE, Pestell RG, Howell A, Howell A, *et al.* Energy transfer in “parasitic” cancer metabolism. *Cell Cycle* 2011;10(24):4208–16.
- [13] Bost F, Saha IB, LeMarchand-Brustel Y, Tanti JF. Metformin and cancer therapy. *Curr Opin Oncol* 2012;24:103–8.

- [14] Fine EJ, Segal-Isaacson CJ, Feinman RD, Herszkopf S, *et al.* Targeting insulin inhibition as a metabolic therapy in advanced cancer: a pilot safety and feasibility dietary trial in 10 patients. *Nutrition* 2012;28:1028–35.
- [15] Dang CV. Links between metabolism and cancer. *Genes Devel* 2012;26:877-90.
- [16] Mihaylova MM, Shaw RJ. The AMPK signaling pathway coordinates cell growth, autophagy and metabolism. *Nat Cell Biol* 2011;13:1016–23.
- [17] Lee C, Longo VD. Fasting vs dietary restriction in cellular protection and cancer treatment: from model organisms to patients. *Oncogene* 2011;30:3305–16.
- [18] Colman RJ, Anderson RM, Johnson SC, Kastman EK, *et al.* Caloric restriction delays disease onset and mortality in rhesus monkeys. *Science* 2009;325:201–4.
- [19] Mathews EH, Liebenberg L, Pelzer R. High-glycolytic cancers and their interplay with the body's glucose demand and supply cycle. *Med Hypoth* 2011;76:157–65.
- [20] Berardi MJ, Fantin VR. Survival of the fittest: metabolic adaptations in cancer. *Curr Opin Gen Devel* 2011;21:59–66.
- [21] Sandulache VC, Skinner HD, Ow TJ, Zhang A, *et al.* Individualizing antimetabolic treatment strategies for head and neck squamous cell carcinoma based on TP53 mutational status. *Cancer* 2012;118:711–21.
- [22] Le A, Cooper CR, Gouw AM, Dinavahi R, *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci* 2010;107:2037–42.
- [23] Grabellus F, Kraft C, Sheu-Grabellus S-Y, Bauer S, *et al.* Tumor Vascularization and Histopathologic Regression of Soft Tissue Sarcomas Treated With Isolated Limb Perfusion With TNF- $\alpha$  and Melphalan. *J Surg Oncol* 2011;103:371–9.
- [24] Danz R, Vogelgsang A, Käthner R. PlasDIC—a useful modification of the differential interference contrast according to Smith/Nomarski in transmitted light arrangement. *Photonik* 2004;1:42.
- [25] Aykin-Burns N, Ahmad IM, Zhu Y, Oberley LW, *et al.* Increased levels of superoxide and hydrogen peroxide mediate the differential susceptibility of cancer cells vs normal cells to glucose deprivation. *Biochem J* 2009;418:29–37.

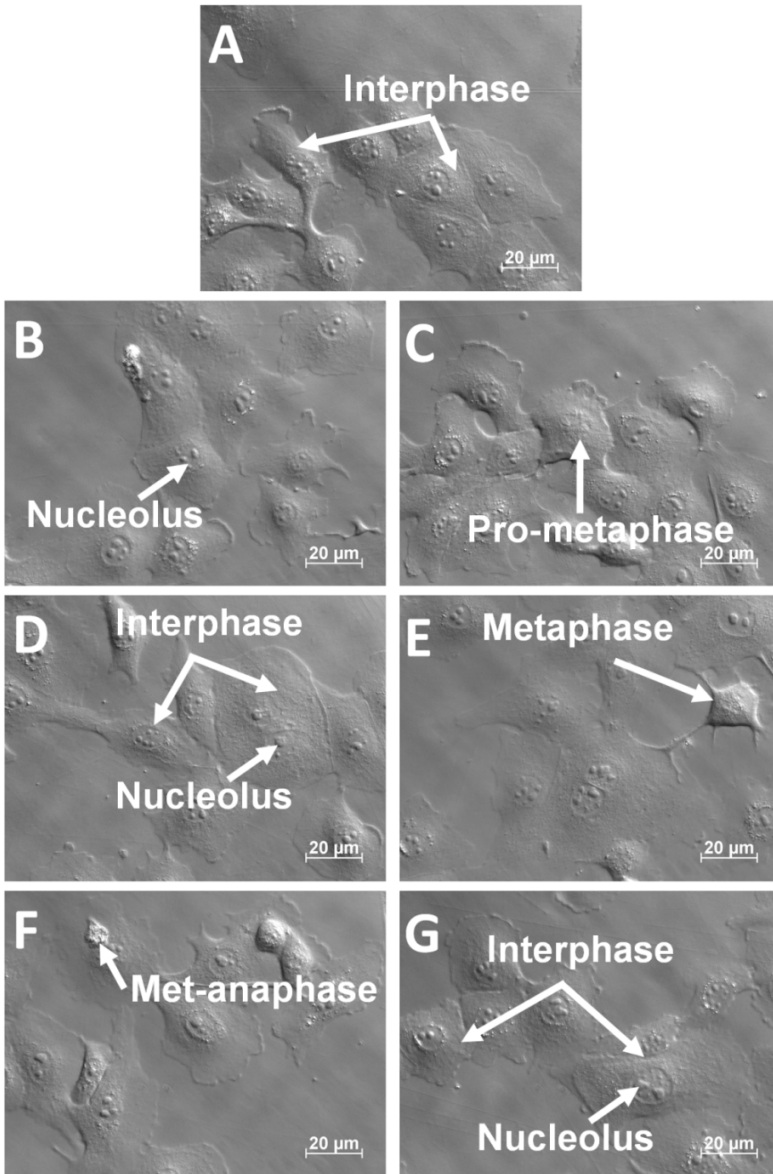
- [26] Liu H, Hu YP, Savaraj N, Priebe W, *et al.* Hypersensitization of tumor cells to glycolytic inhibitors. *Biochem* 2001;40:5542–7.
- [27] Freshney R. *Culture of Animal Cells: A Manual of Basic Technique*. New York: Wiley-Liss Ltd. 2008.
- [28] Circu ML, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Rad Biol Med* 2010;48(6):749–62.
- [29] Galaris D, Pantopoulos K. Oxidative stress and iron homeostasis: mechanistic and health aspects. *Crit Rev Clin Lab Sci* 2008;45:1–23.
- [30] Straussman R, Morikawa T, Shee K, Barzily-Rokni M, *et al.* Tumour micro-environment elicits innate resistance to RAF inhibitors through HGF secretion. *Nature* 2012; doi:10.1038/nature11183.
- [31] Valko M, Leibfritz D, Moncol J, Cronin MT, *et al.* Free radicals and antioxidants in normal physiological functions and human disease. *Int J Biochem Cell Biol* 2007;39:44–84.
- [32] Valko M, Rhodes CJ, Moncol J, Izakovic M, *et al.* Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 2006;160:1–40.
- [33] Bryson BD, White FM. Signaling for death: tyrosine phosphorylation in the response to glucose deprivation. *Mol Syst Biol* 2012;8:591.
- [34] Myers AP, Cantley LC. Sugar free, cancer free? *Nutrition* 2012;28:1036.
- [35] Wu H, Ding Z, Hu D, Sun F, Dai C, Xie J, *et al.* Central role of lactic acidosis in cancer cell resistance to glucose deprivation-induced cell death. *The Journal of Pathology* 2012;227(2):189–99.
- [36] Stacpoole PW, Lorenz AC, Thomas RG, Harman EM. Dichloroacetate in the treatment of lactic acidosis. *Annals of Internal Medicine* 1988;108(1):58–63.



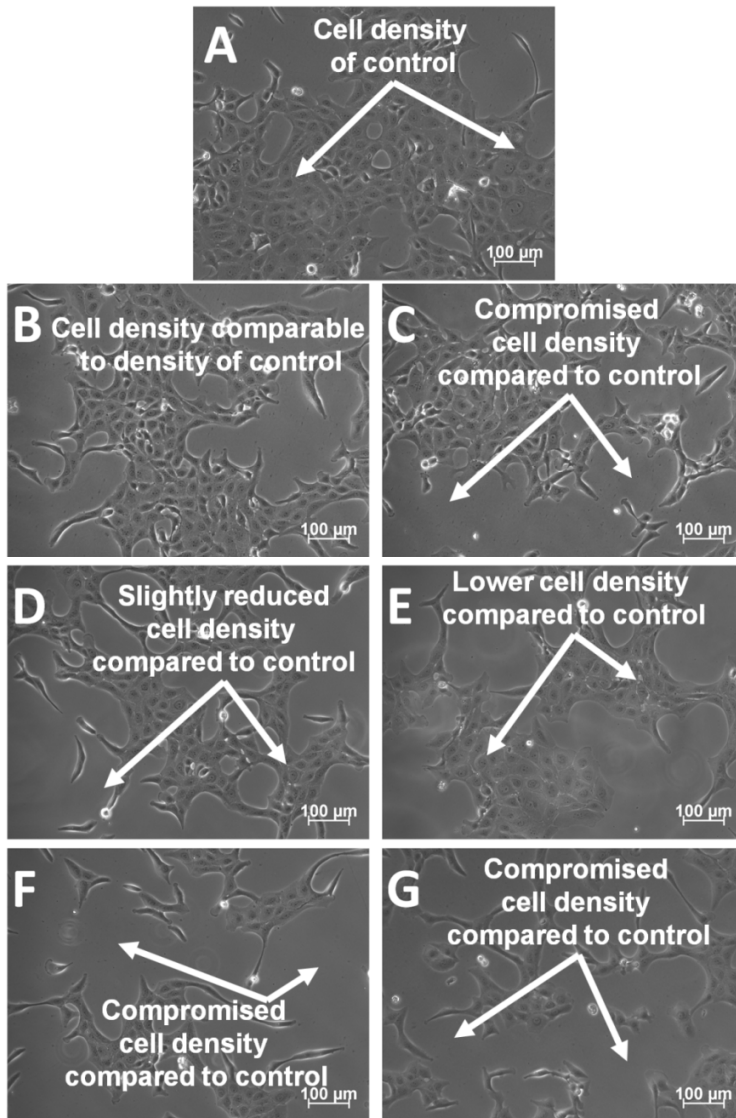
**Fig 1.** PlasDIC light microscopy (40 $\times$ ) showing influence of a 2 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 0 h exposure, (B) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (C) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (D) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (E) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (F) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (G) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (H) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.



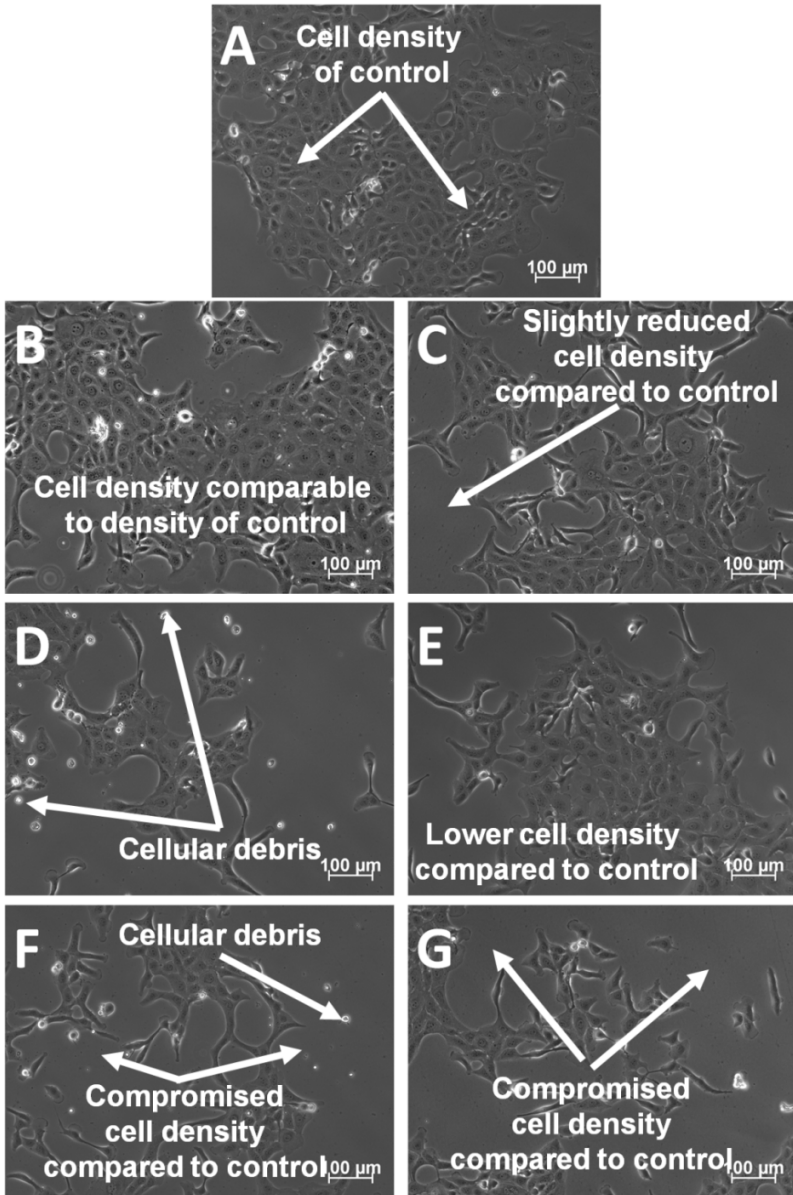
**Fig. 2.** PlasDIC light microscopy (40×) showing influence of a 4 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.



**Fig. 3.** PlasDIC light microscopy (40X) showing influence of a 6 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 6 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.



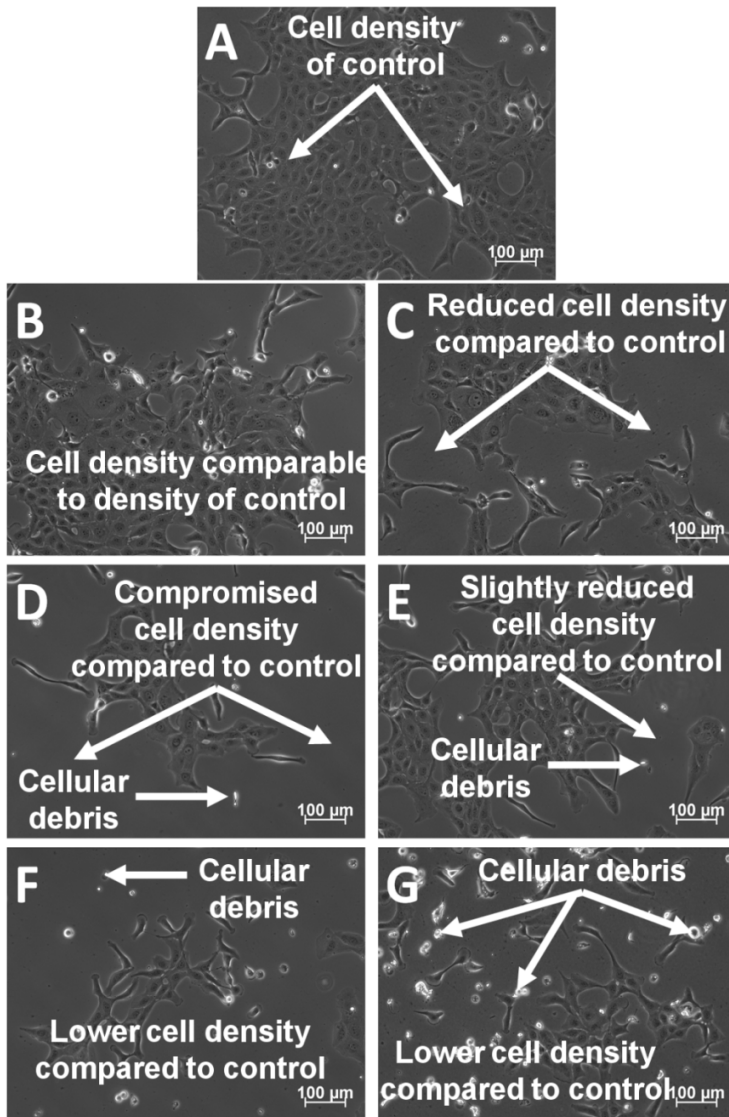
**Fig. 4.** Phase contrast light microscopy (10 $\times$ ) showing influence of a 2 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate. (A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.



**Fig. 5.** Phase contrast light microscopy (10×) showing influence of a 4 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, D-glutamine or sodium pyruvate.

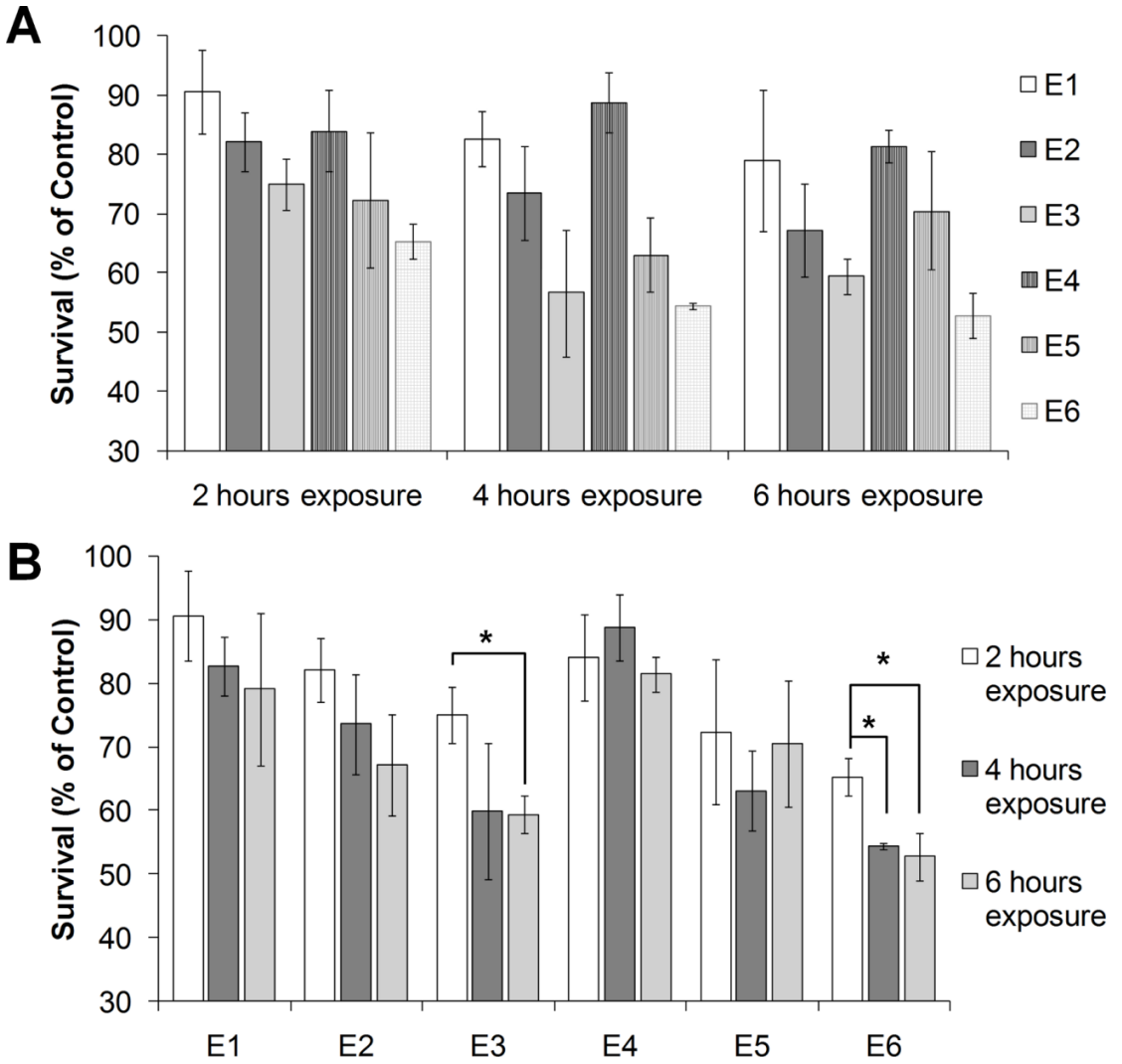
(A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 0 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.





**Fig. 6.** Phase contrast light microscopy (10×) showing influence of a 6 h exposure of HeLa cells to DMEM media containing various concentrations of D-glucose, L-glutamine or sodium pyruvate.

(A) 25.52 mM glucose, 4 mM glutamine, and 1 mM pyruvate, (B) 6 mM glucose, 1 mM glutamine, and 1 mM pyruvate, (C) 3 mM glucose, 0.5 mM glutamine, and 1 mM pyruvate, (D) 0 mM glucose, 0 mM glutamine, and 1 mM pyruvate, (E) 6 mM glucose, 1 mM glutamine, and 0 mM pyruvate, (F) 3 mM glucose, 0.5 mM glutamine, and 0 mM pyruvate, and (G) 0 mM glucose, 0 mM glutamine, and 0 mM pyruvate.



**Fig. 7.** Control: DMEM medium with 25.52 mM D-glucose, 4 mM L-glutamine, and 1 mM sodium pyruvate;

E1: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 1 mM sodium pyruvate;

E2: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 1 mM sodium pyruvate;

E3: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 1 mM sodium pyruvate;

E4: DMEM with 6 mM D-glucose, 1 mM L-glutamine, and 0 mM sodium pyruvate;

E5: DMEM with 3 mM D-glucose, 0.5 mM L-glutamine, and 0 mM sodium pyruvate;

E6: DMEM with 0 mM D-glucose, 0 mM L-glutamine, and 0 mM sodium pyruvate.

**A)** For 2 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E2 vs E6, E1 vs E6, E3 vs E6 and E4 vs E6.

For 4 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E1 vs E5, E1 vs E6, E2 vs E3, E2 vs E4, E2 vs E6, E3 vs E4, E3 vs E6, E4 vs E5, E4 vs E6, and E5 vs E6.

For 6 hours exposure; student *t*-test *P*-values less than 0.05 for E1 vs E3, E1 vs E6, E2 vs E4, E2 vs E6, E3 vs E4, E3 vs E6, E4 vs E6, and E5 vs E6.

**B)** For E3; student *t*-test *P*-values less than 0.05 for 2 hours exposure vs 6 hours exposure.

For E6; student *t*-test *P*-values less than 0.05 for 2 hours exposure vs 4 hours exposure and 2 hours exposure vs 6 hours exposure. Two-way ANOVA analysis (*P*-values less than 0.05) suggested that an increase in the time of exposure to conditions E3 and E6 resulted in decreased cell survival. \* indicates a *t*-test *P*-value of < 0.05.