

Effect of hyperglycemia and hypoxia on proliferation of normal and cancer cell lines

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ABSTRACT

Recent reports indicate an increased incidence of cancer in type 2 diabetic patients. The mechanisms that promote the progression of cancer in such patients are not known but it is believed that hyperglycemia is the major cause. The objective of this study is to evaluate the effect of hyperglycemia and hypoxia in proliferating normal BHK21 and cancer H1299 cell lines. Cells were incubated for 18, 24 and 48 hours under normal, increased glucose concentrations (hyperglycemia) and decreased percentage of oxygen conditions (hypoxia).

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KEYWORDS

Hyperglycemia;
Hypoxia;
Cancer;
Proliferation;
Metabolism.

INTRODUCTION

Diabetes and Cancer are two heterogeneous, multi-factorial, severe and chronic diseases. Every year, 10.7 million new patients are diagnosed for cancer worldwide; 6.7 million people die because of the disease. It is also estimated that 250 million people are afflicted by type 2 diabetes (T2D). Because of their frequency, reciprocal influences, even if minor, may have a major impact. Though epidemiological studies clearly indicate that the risk for several types of cancer (including pancreas, liver, breast, colorectal, urinary tract and female reproductive organs) is increased in diabetic patients^{1,2}, they may be partially biased by relevant heterogeneity due to different study design (inclusion criteria), incomplete characterization of Diabetes mellitus, failure to consider potential confounders (obesity, diabetes duration and treatment) and also vari-

ably defined control population. However, the overall increased risk for the development of several types of cancer in diabetic patients must be considered well documented.

There are existing molecular links between cancer and T2D. A fatal glucose metabolism is a unique feature of both diseases. It appears evident that the abundance of glucose in extracellular fluids associated with T2D will support cell proliferation and represents a selective advantage for growth of cancer cells. Moreover, during the course of T2D relatively high concentration of insulin will be found either because of insulin resistance or due to iatrogenic application of insulin for the control of blood sugar –which will trigger PI₃K/Akt/mTOR signaling and promote the metabolic reprogramming that is characteristic of proliferating cancer cells^{3,4}. In many cancer cells, the ATP necessary for survival and proliferation is derived from glycolysis rather

than from oxidative phosphorylation (OXPHOS), even when oxygen supply would be adequate to sustain them. Increased conversion of glucose to lactic acid associated with decreased mitochondrial respiration and acidosis of the microenvironment is a unique feature of many tumors, known as Warburg effect. The PI₃K/Akt/mTOR pathway, HIF-1 α and Myc participate in the various facets of this metabolic phenotype, which provides cancer cells that are invasive or participate in metastasis formation a distinct competitive edge over normal cells^[5, 6].

Models have been successfully established which demonstrate that adaptation of initiated cancer cells to hypoxia and acidosis represents key events in transition from in situ to invasive cancer^[7, 8]. Hypoxia plays an important role in all diabetes complications. On the other side, it has been postulated that hyperglycemia by itself induces a pseudohypoxia state linked to increased flux of glucose via the sorbitol pathway; this theory is based on the finding that high glucose concentrations induce a high NADH⁺/NAD⁺ ratio in cells even when the oxygen tension is normal^[9]. More recently, it was reported that In Clear Cell Papillary Renal Cell Carcinoma, sorbitol, downstream fructose and sorbitol-6-phosphate metabolites were elevated; besides, sorbitol was found to induce HIF1-alpha protein expression under both normoxia and hypoxia in other three renal carcinoma cell lines^[10]. Sorbitol dehydrogenase concentrations and activity in colon adenomas and cancer cell lines are very increased^[11]. Hyperglycemia increases proliferation of both non-tumorigenic and malignant mammary epithelial cells and this is accomplished by increased leptin and pro-survival AKT/mTOR signaling. This represents at least two mechanisms by which diabetes results in worse cancer progression^[12].

Evidence indicates that hypoxia alters cellular proliferation by programmed cell death and by cell cycle arrest; most non-transformed hypoxic cells remain viable but are arrested in G1 and do not proliferate^[13]. Hypoxia is commonly found in solid tumors of various origins. Selection by hypoxia renders tumor cells resistant to hypoxia induced apoptosis. These cells with a reduced apoptotic potential may also explain the resistance of many solid

tumors to cancer treatment. In the hypoxic cell compartment, cells are in growth arrest; however, small numbers of proliferating cells could be seen ^[Reviewed in 14].

These reports indicate a clear relationship between cancer and diabetes. However, the cellular mechanisms by which hyperglycemia induces cancer development or apoptosis in cancer cell lines are not clear. In this work, the effect of hyperglycemia and hypoxia on proliferation and viability of a normal and a lung cancer cell lines was studied. Results indicate that hyperglycemia contributes notably to proliferation and survival of both cell lines in hypoxic conditions, favoring the transformation of normal cells and protecting cancerous cells.

MATERIALS AND METHODS

Cell culture: The cell lines used in this study were BHK21 (hamster kidney normal fibroblasts, purchased from the Hygiene National Institute "Rafael Rangel", Caracas, Venezuela) and H1299 (lung cancer epithelial cells, purchased from ATCC, USA). BHK21 cells were maintained in DMEM and H1299 cells in RPMI 1640 culture media (Invitrogen, USA) supplemented with 10% fetal bovine serum (Internegocios, Argentina), in the presence of penicillin (100 IU/ml) and streptomycin (100 μ g/ml) (Life Technologies, USA). Cultures were seeded at a density of 5.000 cells/well in 96 wells culture plates or 100.000 cells/well in 24 well plates, in 200 μ l or 1 ml volume, respectively, depending on the assay to be performed, and incubated for 24 hours to allow adherence and 80-90% confluence in standard culture conditions. In normoxia, cells were kept in an incubator (Nuair, class II type A/B3, Plymouth, MN, USA) at typical tissue culture conditions: 37°C; room air (21% O₂), 5% CO₂. After this time, the medium was replaced by one containing 0.5% fetal bovine serum in order to synchronize the culture and after a period of 18-24 hours this medium was removed and replaced by experimental medium containing normal physiological glucose (5 mM, 90 mg/dl) or hyperglycemic levels: 11 mM (198 mg/dl) and 21 mM (360 mg/dl). To reach these concentrations the medium was supple-

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mented using Glucose 45% (sterile, 2.500 mM, Cat. N° G8769- SIGMA-Aldrich, USA). Assays were done in quintuplicates. Glucose concentrations of 11 and 20 mM were chosen for hyperglycemia studies because they represent a physiologically relevant concentration of glucose commonly encountered in diabetic individuals and in order to determine whether hyperglycemia has a dose-dependent effect on cell proliferation. To minimize artifactual variations due to unequal growth rates and cell sizes, all assays were performed on confluent cells in fresh medium.

Hypoxia in cell culture

To expose cells to hyperglycemia and hypoxia, cultures in confluence and synchronized, were maintained in the respective experimental medium, as previously described, in the following oxygen pressures: 21% (159,44 mm Hg, considered normoxia), 6% (45,597 mm Hg) and 0.1% (0,759 mm Hg). To reach these oxygen levels, cell culture plates were placed into anaerobic jars for 18, 24 and 72 hours (Anaeropack for cell culture, Oxoid Ltd., Hampshire, England) as described^[15,16]. Briefly, the Anaeropack for Cell contains sodium ascorbate as the principal ingredient which absorbs oxygen and generates carbon dioxide by oxidative degradation. Magnesium hydroxide is used as a scavenger for carbon dioxide. These reagents are located in paper sachets and are placed into the jars (Campygen sachets~6% oxygen and Anaerogen sachets~0.1% oxygen). Controls were cultured in parallel under normal oxygen concentration of ~ 21%. Viability tests of cells in culture were performed by Trypan blue exclusion.

Cell proliferation assay

Cells were seeded into 24-well plates at a density of 1×10^5 cells per well (1 ml) and treated with each experimental medium and oxygen level. Samples from cultures in normal, normoglycemic-hypoxic conditions, hyperglycemic and hyperglycemic-hypoxic conditions, were taken at 18, 24 and 48 hours to measure cell proliferation. At the end of each incubation time cell viability was determined by Trypan blue dye exclusion assay. Triplicate wells of viable cells for each condition were counted on a

hemacytometer after trypsinization. Each well had three repeats of counting. Cell proliferation was expressed as the percentage of increase compared to the initial seeding. To determine cell viability the following formula was used: %viability= N° of viable cells / N° of total cells x 100.

MTS assays

Cells were seeded into 96-well plates at a density of 5.000 cells / well (100 μ l) and treated according to the different conditions described above, including controls. The cells were treated for 24 h, 48 h, or 72 h. For the MTS assay, the CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit was used following the manufacturer's instruction (Promega, Madison, WI, USA). Briefly, after the desired time points were reached, 20 μ l of the MTS reagent was added into each well and cells were incubated for four hours at 37°C in a humidified, 5% CO₂ atmosphere. The absorbance was detected at 490 nm with a Synergy HT Multi-mode microplate reader (Biotek, Vermont, USA). All the experiments were repeated three times in quintuplicates.

Lactate determinations

Lactate production was measured according to the method described by Hohorst^[17]. Briefly, the medium was taken from confluent cultures of cells that had been grown continuously for 18, 24 or 72 h, for each experimental condition. Lactate was measured by mixing 0.5 ml of culture medium with 0.5 ml of 10% HClO₄ in order to obtain extracts deprived of proteins. After 10 min at 0°C and centrifugation at 2.000 x g for 10 min, the supernatant was neutralized with 3 M KOH-KHCO₃. A second centrifugation was then performed to remove the KClO₄ precipitate. Ten μ l of the supernatant were placed in a reaction mixture containing 150 μ g of β -nicotinamide adenine dinucleotide (β -NAD, Sigma-Aldrich, USA), hydrazine-glycine buffer 0.4M (final concentration, Sigma-Aldrich, USA) and 50 μ l of distilled water. A blank was prepared in the same way but without sample and a tube containing medium culture as sample was included to measure the lactate level found in fresh medium (with 10% FCS). Absorbance was read at 340 nm and taken as time

zero value. The reaction was started by the addition of 2,6 Units/reaction of pure LDH (D-Lactic Dehydrogenase from *Lactobacillus leichmanii*, Sigma-Aldrich, USA) and changes in absorbance were recorded after 20 minutes of incubation. Lactate concentration was determined in quintuplicates by spectrophotometric measurement at 340 nm of the stoichiometric conversion of lactate to pyruvate with concomitant reduction of NAD by lactate dehydrogenase with a Synergy HT Multi-mode microplate reader (Biotek, Vermont, USA). Results are expressed as μmol lactate/ml of culture medium from the sample.

Protein concentrations

They were determined using a Coomassie Plus (Bradford) Protein Assay (Thermo Fisher Scientific, USA) and bovine serum albumin (Sigma-Aldrich, USA) as a standard.

Statistical analysis

All data are presented as means + SD. Results were evaluated by a one-way analysis of variance (ANOVA) and significant differences defined with

Tukey post-tests. Differences are considered significant when $p < 0.05$.

RESULTS

Cell proliferation

In vitro cell proliferation in normal and high glucose, under different oxygen concentrations, was measured using the Trypan Blue exclusion method in normal (BHK21) and cancer cells (H1299).

In normoxic conditions, normal cells, at 24 hours, showed an increase in proliferation compared to 18 hours, regardless of glucose concentration. At 48 hours, cells at normal glucose presented no change compared to 24 hours, but high glucose (11 and 20 mM) resulted in increased cell proliferation, being significant ($p < 0.05$) at 20 mM when compared to 5 mM Figure 1A. Cancer cells also showed an increase in proliferation at 24 hours when compared to 18 hours; however, there was a significant decrease ($p < 0.05$) between normal and 20 mM glucose concentration Figure 1Da. At 48 hours, a decrease in proliferation was observed compared to 24 hours,

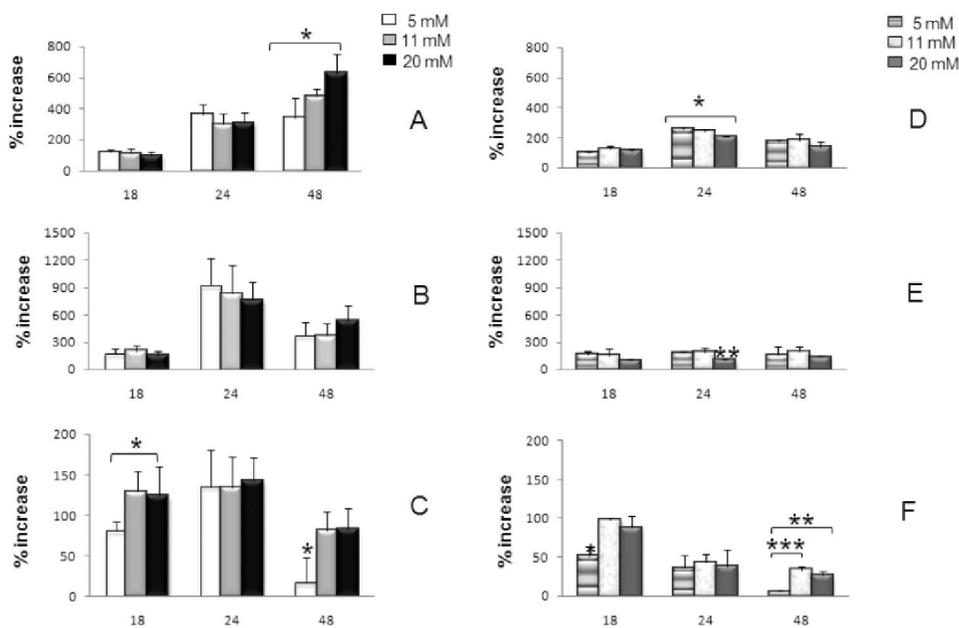


Figure 1 : Proliferation of BHK21 and H1299 cells cultured under increasing glucose (5, 11 and 20 mM, as described in the inserts) and decreasing oxygen concentrations, at the indicated periods of time: 18, 24 and 48 hours. BHK21 cells: Figures 1A, 1B and 1C correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively; H1299 cells: Figures 1D, 1E and 1F, correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively. Percentage of increase was determined by counting cells with the Trypan Blue method. \square : indicates association between two parameters. *: $p < 0.05$; **: $p < 0.01$; *: $p < 0.001$**

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though it was not significant between normal and high glucose concentrations. It is interesting to note that under normal glucose conditions, H1299 cells exhibited a lower growth rate compared to normal cells, condition that was more pronounced at 48 hours Figure 1D. Normal cells exhibited a greater rate of growth under hyperglycemia. Moreover, glucose appears to have a dose- and time-dependent effect on cell proliferation, because this was further enhanced at 20 mM compared to 11 mM glucose in normal cells when exposed for 48 hours, but not at 24 hours. The opposite effect was observed in lung cancer cells.

In mild hypoxic conditions (6% oxygen), normal cells, at 24 hours, showed a very significant increase in proliferation compared to 18 hours ($p < 0.001$), at any glucose concentration assayed; at 48 hours, cells presented a significant

Decrease, compared to 24 hours ($p < 0.01$), at any glucose concentration assayed; however, there was not a significant difference between the three glucose concentrations assayed, regardless of the incubation time Figure 1B. This suggests that high glucose concentrations do not affect the proliferation rate at 6% oxygen in normal cells. The proliferation rate was significantly higher at 24 hours of incubation in 6% oxygen ($p < 0.001$), when compared to the same time in normoxic conditions, regardless of glucose concentration.

Cancer cells incubated at 6% oxygen showed no significant changes at 5 and 11 mM, though there was a slight decrease in proliferation in time at 5 mM and an increase at 11 mM glucose. At 20 mM glucose, cells proliferated at a notably slower rate than at 5 and 11 mM, being significant at 24 hours ($p < 0.01$) Figure 1E. This shows that under mild hypoxic conditions cancer cells present a proliferation rate almost constant, regardless of glucose concentrations, though high concentrations do not help them to adapt to 6% oxygen. Again, normal cells exhibited a greater rate of growth under hyperglycemia.

Normal cells incubated at 0.1% oxygen showed a significant increase ($p < 0.05$) in proliferation at 11 and 20 mM glucose at 18 hours, when compared to 5 mM Figure 1C. At 24 hours there was not a sig-

nificant difference between cultures regardless of glucose concentration. At 48 hours, a significant increase in proliferation ($p < 0.05$) was observed at 11 and 20 mM glucose when compared to 5 mM, though the proliferation rate was lower than at 24 hours. This suggests that prolonged exposure to hyperglycemia allows normal cells to proliferate or survive under extreme hypoxia.

Cancer cells cultured at 0.1% oxygen showed a constant decrease in proliferation in time. At 18 hours, cells at 5 mM glucose proliferated significantly slower ($p < 0.05$) than at 11 and 20 mM Figure 1F. At 24 hours, regardless of glucose concentrations, the proliferation rate decreased significantly ($p < 0.01$) when compared to 18 hours. At 48 hours, proliferation rate was not significantly different compared to 24 hours at 11 and 20 mM glucose; however, cells cultured at 5 mM glucose showed a very significant decrease in proliferation ($p < 0.001$) when compared to 18 and 24 hours cultured at the same glucose concentration and also when compared to 11 and 20 mM glucose at 48 hours. At any time assayed, cells cultured in hyperglycemic conditions showed a higher rate of proliferation than at normoglycemic conditions. These results suggest that both normal and cancer cells increase proliferation in time when cultured at high glucose concentrations under extreme hypoxia; however, cancer cells show a lower, but stable, rate of proliferation at any oxygen and glucose condition and are more sensitive to hypoxia when compared to normal cells.

Cell viability

Viable cells present membrane integrity and normal cell functions. In normoxic conditions, BHK21 cells showed a high percentage of viable cells at any time and glucose concentrations tested. Though there was a tendency to increase viability with glucose concentrations, the differences were only statistically significant at 24 hours of incubation Figure 2A. H1299 cells also presented a high percentage of viability at any time and glucose concentration assayed. There was a slight but not significant tendency to diminish viability at 5 mM glucose from 18 to 48 hours of incubation, while at 11 mM glucose the viability was constant in time Figure 2D.

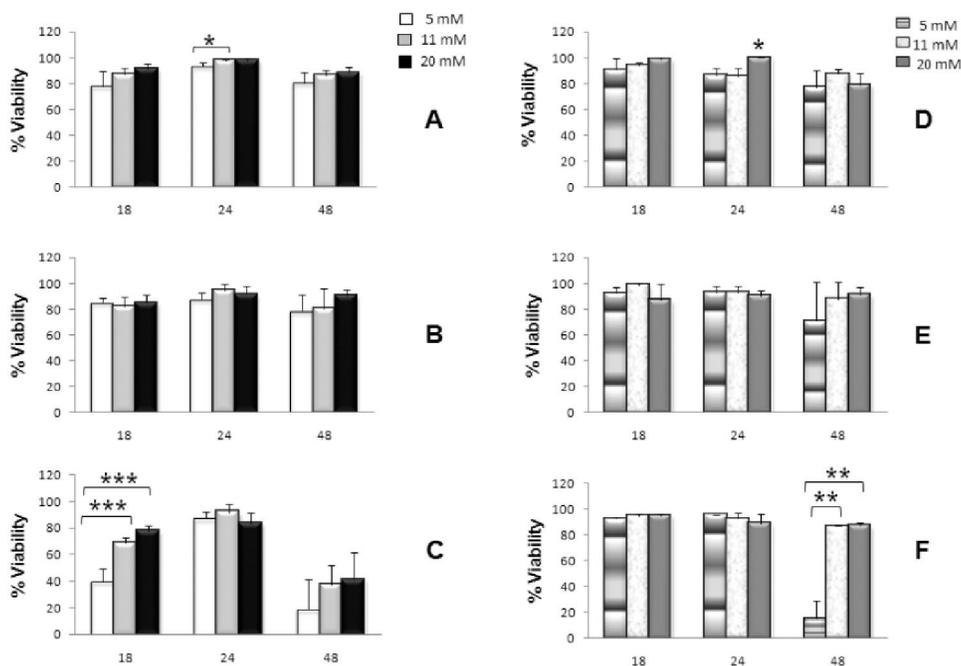


Figure 2 : Viability of BHK21 and H1299 cells cultured under increasing glucose (5, 11 and 20 mM, as described in the inserts) and decreasing oxygen concentrations, at the indicated periods of time: 18, 24 and 48 hours. BHK21 cells: Figures 1A, 1B and 1C correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively; H1299 cells: Figures 1D, 1E and 1F, correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively. Percentage of viability was determined by using the formula: %viability= N° of viable cells / N° of total cells x 100. : indicates association between two parameters. *: p<0,05; **: p<0,01; *: p<0,001**

Viability was higher at 20 mM glucose, being significant at 24 hours ($p<0.05$) when compared to 5 and 11 mM glucose. So, in normoxic conditions, the viability between both cell lines was not different in all glucose concentrations and time periods assayed.

When cultured at 6% oxygen, BHK21 cells presented a similar behavior to the normoxic conditions. A high viability was constant at any time and glucose concentration assayed, without statistically significant differences between conditions, though at 48 hours cells cultured at 20 mM glucose presented a tendency to increase viability Figure 2B. H1299 cells, like normal cells, showed a high and constant viability at any experimental condition assayed. Only at 48 hours of incubation, cells at 5 mM glucose showed a clear tendency, though not statistically significant, to diminish viability Figure 2E.

Normal cells cultured at 0.1% oxygen were significantly affected in a time- and glucose concentration-dependent manner (Figure 2C). At 5 mM glucose, cells viability was low; then significantly increased at 24 hours and diminished at 48 hours

($p<0.01$) to levels below the values found at 18 hours. Cells cultured at 11 mM presented a 70% viability at 18 hours of incubation; at 24 hours increased but at 48 hours decreased significantly to 38% ($p<0.01$). At 20 mM glucose, viability was high and constant during 24 hours, but diminished significantly ($p<0.01$) at 48 hours. Cells incubated at 11 and 20 mM glucose presented a significantly higher viability than at 5 mM at any time assayed. In the other hand, cancer cells showed a high and constant viability during 24 hours of incubation, at any glucose concentration assayed (Figure 2F). At 48 hours, cells cultured at 5 mM presented a very significant decrease in viability ($p<0.01$), compared to 18 and 24 hours and to the 11 and 20 mM glucose concentrations. Cells cultured at 11 and 20 mM glucose showed no statistically different values to the levels found at 18 and 24 hours. These results suggest that cancer cells are more adapted to survive under extreme hypoxic conditions when glucose concentrations are high, than normal cells, although proliferation is decreased under these conditions. The results point out to the possibility of a quiescent state

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of the cells under extreme conditions of oxygen, helped by high concentrations of glucose.

Cell metabolism

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay is a colorimetric method for determining the number of viable cells in proliferation. The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenases enzymes found in metabolically active cells. The quantity of formazan product as measured by the amount of 490nm absorbance is directly proportional to the number of living cells in culture. It is known that the reduction of the tetrazolium salt to formazan is influenced by the metabolic activity of the cells (18, 19). Besides, it has been reported, using MCF7 cells, that in the MTT assay the production of formazan is proportional to the energetic metabolism of the cells, which means that it is possible to detect metabolically active cells in the absence of proliferation (20). According to this, we decided to use this method as an indicator of cell metabolism.

In normoxic conditions, BHK21 cells showed a progressive increase of the metabolic activity in a time and glucose concentration dependent way, being significant the differences between glucose concentrations at 18 and 48 hours of incubation ($p < 0.05$ and $p < 0.01$, respectively, Figure 3A). The highest rate of metabolic activity was observed at 48 hours and 20 mM glucose ($p < 0.001$). The increase is proportional to glucose concentration and to the increase in proliferation observed in the same experimental conditions. This indicates that hyperglycemia increases the metabolic activity of normal cells. H1299 cells showed a significant higher rate of metabolic activity at normoglycemic conditions with respect to 11 and 20 mM glucose, independently of time ($p < 0.05$ at 18 and 48 hours and $p < 0.01$ at 24 hours). At 48 hours an important increase was observed at 11 and 20 mM glucose with respect to 18 and 24 hours, although lower than at 5 mM glucose (Figure 3D). The results indicate that cancer cells present a lower metabolic activity than normal cells in normoxic conditions and that hyperglycemia accentuates this behavior.

In mild hypoxic conditions (6% oxygen), nor-

mal cells cultured at 5 mM glucose showed a constant activity during the first 24 hours, but diminished significantly at 48 hours ($p < 0.01$, Figure 3B). Cultures at 11 and 20 mM glucose showed a significant decrease between 18 and 24 hours of incubation ($p < 0.05$), but then remained constant until 48 hours. Cultures at 11 and 20 mM glucose showed a significantly higher metabolic activity ($p < 0.01$) than at 5 mM regardless of incubation time. The behavior of the cells at 21% and 6% oxygen was similar but the metabolic activity rate was lower in mild hypoxic conditions.

Cancer cells incubated at 6% oxygen showed a significant increase of the metabolic activity in time when cultured at 5 and 11 mM glucose ($p < 0.01$ and $p < 0.05$, respectively, between 18 and 48 hours), while the cells cultured at 20 mM glucose presented a significant decrease of metabolic activity between 18 and 24 hours ($p < 0.01$), but then remained constant until 48 hours (Figure 3D). Metabolic activity was significantly higher ($p < 0.01$) at 5 mM when compared to 11 and 20 mM glucose, independently of the incubation time. At 20 mM glucose metabolic activity was very low. The data show that hyperglycemia (20 mM) acts in an opposite way in normal (increasing) and cancer cells (decreasing) at 6% oxygen, as in normoxic conditions, when compared to normoglycemic conditions.

Normal cells incubated at 0.1% oxygen showed a constant decrease of the metabolic activity from 18 to 48 hours of incubation (Figure 3C). Again, cells cultured at 20 mM glucose presented a significantly higher activity ($p < 0.001$) when compared to cells cultured at 5 and 11 mM, at any time assayed. The values obtained at 0.1% were not significantly different than those obtained at 6% oxygen. The data allow us to suggest that hyperglycemia (20 mM) induces a higher metabolic activity in normal cells under extreme hypoxia.

Cancer cells incubated at 0.1% oxygen in 5 and 11 mM glucose showed no significant changes at any time assayed. Cells incubated at 20 mM glucose, in the other hand, presented a significant decrease ($p < 0.05$) from 18 to 24 hours of incubation, but then remained stable (Figure 3F). At any time assayed, cultures at 5 mM glucose showed a signifi-

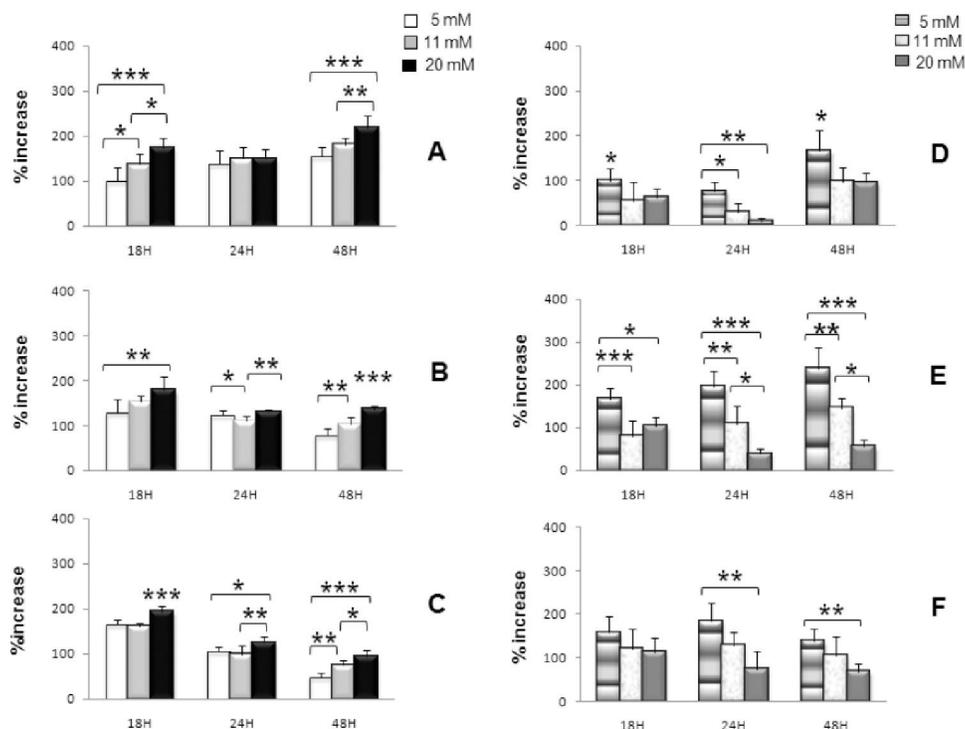


Figure 3 : % of increase of metabolic activity of BHK21 and H1299 cells cultured under increasing glucose (5, 11 and 20 mM, as described in the inserts) and decreasing oxygen concentrations, at the indicated periods of time: 18, 24 and 48 hours. BHK21 cells: Figures 1A, 1B and 1C correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively; H1299 cells: Figures 1D, 1E and 1F, correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively. Metabolic activity was determined with the MTS assay. : indicates association between two parameters. *: $p < 0,05$; **: $p < 0,01$; ***: $p < 0,001$

cantly higher metabolic activity ($p < 0.01$) when compared to results at 20 mM. The data, again, show that hyperglycemia (20 mM) acts in an opposite way in normal (increasing) and cancer cells (decreasing) at 0.1% oxygen, as occurs at 6% oxygen and in normoxic conditions, when compared to normoglycemic conditions.

Lactate production

Lactate production can be used as an indirect measure of the glycolytic rate of the cells. Normal cells cultured in normoxic conditions showed a different behavior depending on the glucose concentration (Figure 4A). At 5 mM, lactate production decreased significantly from 18 to 48 hours of culture ($p < 0.05$); at 11

mM increased significantly from 18 to 24 hours of culture ($p < 0.05$) but then remained stable; at 20 mM glucose there were not significant changes in time. Cancer cells cultured in normoxic conditions at 5 mM glucose showed a constant production of lactate during 24 hours; at 11 mM lactate production

presented a tendency to increase from 18 to 24 hours, though not statistically significant, while at 20 mM glucose only at 18 hours of incubation was detectable. At 48 hours, there was not detection of lactate production Figure 4D.

Normal cells cultured at 6% oxygen presented the same tendency and similar values of lactate production as in normoxic condition (Figure 4B), for 5 and 20 mM glucose concentrations. In the other hand, at 11 mM glucose concentration, there was a significant increase of lactate production at 18 hours, compared to normoxic conditions ($p < 0.01$), and from 18 to 24 hours of incubation at 6% oxygen ($p < 0.01$), but then there was a significant decrease at 48 hours ($p < 0.05$) when compared to 24 hours. Values at 48 hours of culture were similar to the levels in normoxic conditions for any glucose concentration assayed.

Cancer cells cultured at 6% oxygen presented no significant changes at any time and glucose concentration assayed Figure 4E. There was a tendency,

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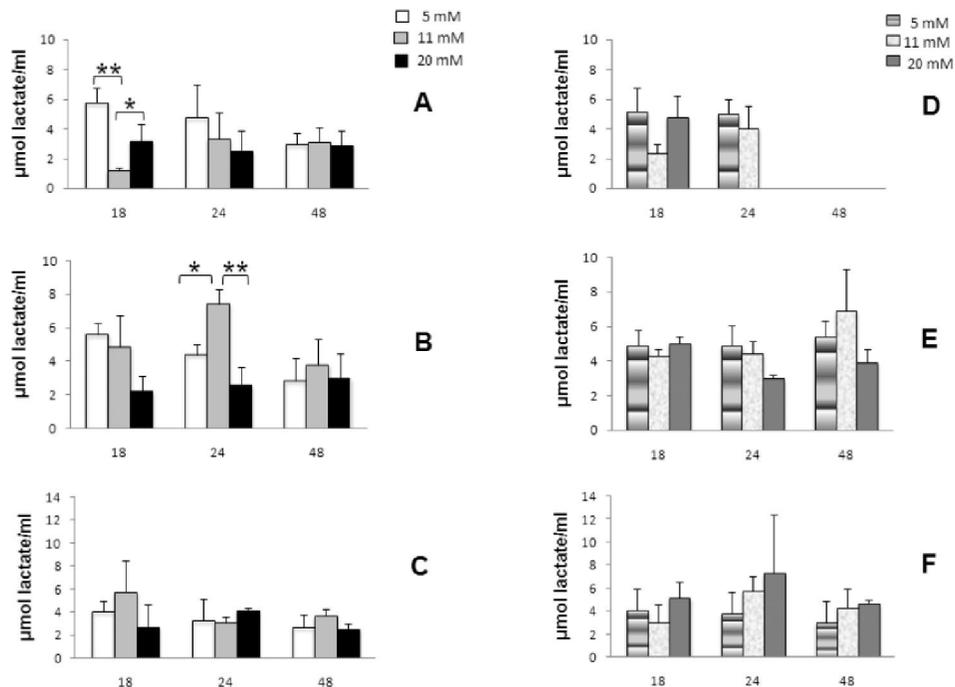


Figure 4 : Lactate concentration (released to the medium) of BHK21 and H1299 cells cultured under increasing glucose (5, 11 and 20 mM, as described in the inserts) and decreasing oxygen concentrations, at the indicated periods of time: 18, 24 and 48 hours. BHK21 cells: Figures 1A, 1B and 1C correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively; H1299 cells: Figures 1D, 1E and 1F, correspond to normoxia, 6% and 0.1% oxygen concentrations, respectively. Lactate production was measured according to the method described by Hohorst (see Methods). : indicates association between two parameters. *: $p < 0,05$; **: $p < 0,01$; *: $p < 0,001$**

though, to increase lactate production at 11 mM glucose concentration at 48 hours of incubation and a decrease at 20 mM at 24 hours of incubation.

Normal cells cultured at 0.1% oxygen showed no significant changes regardless of time and glucose concentrations assayed (Figure 4C). There was a change in tendency, however, at 11 mM glucose when compared to normoxic and 6% oxygen conditions. It is noteworthy that at 20 mM glucose, lactate production had no significant changes neither in tendencies nor values at any time and oxygen conditions assayed. Cancer cells cultured at 0.1% oxygen showed no significant changes between the different glucose concentrations assayed, though there was a clear tendency to increase lactate production at 20 mM when compared to 5 and 11 mM glucose at any experimental period of time.

DISCUSSION

Proliferation

In the present study we have found direct effects

of hyperglycemia alone and hyperglycemia and hypoxia combined, on basic aspects of cell survival: proliferation, viability and metabolism. In normoxic conditions, hyperglycemia alone significantly increased proliferation of normal cells at 48 hours and in cancer cells at 24 hours of culture, but proliferation rate was markedly higher in normal cells. Mild hypoxia promotes proliferation while severe hypoxia decreased it, an effect that was partially counteracted in hyperglycemic

Conditions, in both normal and cancer cells.

The mechanism by which hyperglycemia augments proliferation of BHK21 cells is not well understood, but it is known that hyperglycemia increases oxidative stress by increasing ROS, together with a decrease of antioxidants^[21], and a low concentration of H_2O_2 induces proliferation of BHK21 cells^[22]. Our results are in accord with an increase in proliferation after 48 hours of incubation of murine endothelial microvascular cells in 25 mM glucose^[23]. Hyperglycemia (25 mM) increased proliferation of non-tumorigenic breast epithelial cells

(MCF10A) in a time- and glucose concentration-dependent manner^[24].

H1299 cells showed a decrease in proliferation rate after 24 hours of incubation under hyperglycemic conditions. These results are opposite to an increase in proliferation induced by hyperglycemia in pancreas cancer^[25, 26] and MCF7 cells^[12, 27]. To our knowledge, no reports have described the effect of hyperglycemia in H1299 cells, but the increase in proliferation of cancer cells has been associated to a decrease in the expression of PKC β , and many NSCLC (non-small cell lung cancer) constitutively express PKC β ^[28]. It has also been reported that A549, H1299 and H460 cells express PKC α , a part of the PKC family highly related structurally to PKC β ^[29]. Besides, the loss of function of PTEN has been associated to the proliferative effect of hyperglycemia in cancer cells, and this phosphatase is constitutively expressed in H1299 cells^[30]. It can be suggested, then, that a decrease in the expression or function of PKC and an increase in the activity of PTEN could exert an inhibitory effect on proliferation in these cells.

For both BHK21 and H1299 cells, mild hypoxia (6% oxygen) does not represent an important restriction for proliferation; in fact, mild hypoxia corresponds to the highest values of proliferation rate in normal cells, independently of glucose concentration, but dependent on incubation time. These results are in agreement with previous studies in rat endothelial, rat smooth muscle and NIH3T3 cells, where 3% oxygen induced higher proliferation^[31, 32]. In cancer cells, instead, 20 mM glucose had a significant negative effect on proliferation, at any time assayed, and at 6% oxygen, proliferation rate was significantly lower at any time and glucose concentration assayed, when compared to normal cells.

In extreme hypoxia conditions, hyperglycemia contributes with survival instead of proliferation in both cell lines, suggesting that high glucose availability confers an advantage. The inhibitory effect of extreme hypoxia on proliferation of normal and cancer cell lines have been described before; severe hypoxia (1% oxygen) decreased proliferation in rat airway smooth muscle cells without affecting cells viability^[32]; culture at 0.2% oxygen decreased

survival of H1299 cells in 20% and anoxia decreased it in 97%^[33].

The characteristic hyperglycemic state of a non controlled diabetic can, under hypoxic conditions, promote the survival of organs and tissues during short periods of time. In longer exposition times, the antioxidant capacity of the cells would fail and oxidative stress could lead to apoptosis or necrosis of the more exposed cells of the tissue. Here, we show that hyperglycemia has similar protective effects on normal and cancer cells under extreme hypoxic conditions, but the results confirm that hyperglycemia appears to have the most profound impact on normal cells in normoxic conditions. Therefore, hyperglycemia particularly supports or enhances the growth of normal cells, which potentially translates to an increased risk of cancer in normal tissue or in premalignant lesions in diabetic patients not controlled, with glucose levels above 200 mg/dl. In contrast, in an already established cancer, a pro-proliferative effect seems to depend on hyperglycemia levels and in certain cell lines, like H1299, a concentration of 11 mM glucose is beneficial for survival and proliferation, but higher concentrations exert inhibitory effects, leading to a quiescent state. If the supply of glucose is adequate for the metabolic needs of the tumors, it remains a question whether growth alone can justify the large glucose consumption.

Viability

When the viability of the two cell lines, under the different conditions of glycemia and oxygen is compared, it was observed that at early stages of incubation (18 hours) there is a higher viability of cancer cells under moderate and extreme hypoxia in normoglycemic conditions. However, when time of incubation is increased, the viability is not significantly different between the two cell lines, suggesting that resistance to hypoxia of H1299 cells diminishes. At 11 and 20 mM glucose, there were not significant differences between the two cell lines in normoxia and moderate hypoxia; however, under extreme hypoxia, the viability of normal cells is significantly lower when compared to H1299 cells at 20 mM glucose. Our results indicate that H1299 are

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more resistant to hypoxia than BHK21 cells, and that high glucose concentrations contribute with this behavior. According to this, it has been described that hyperglycemia affects the cells viability depending of the cell type and time of incubation. The time-dependent effect could be a mechanism of metabolic adaptation. For example, in Sertoli cells from diabetic non obese mice, hyperglycemia (30 mM glucose) does not significantly affect the viability after 48 hours of incubation^[34]. In H5V cells, hyperglycemia (25 mM) augments viability after 24 hours of incubation^[23]. In contrast, it has been reported that in human retinal pericytes cells, hyperglycemia diminishes the viability of the cells and augments the release of TGF β 2 and VEGF^[35].

The diminishing effect on viability of BHK21 and H1299 cells after incubation under extreme hypoxia has been described in other cell lines. In Chinese hamster ovary cells (CHO) and human bladder cancer cells (MGH-UI), hypoxia (0.2% O₂) is highly toxic^[36]. In the glioma cancer cell lines LN-T229 and U87MG, hypoxia induced cell death and the inhibition of the EGFR activity, which protects against cell death induced by hypoxia in these cells^[37]. The cytotoxic effect of hypoxia has been associated to an increase of the oxidative stress^[38]. Hyperglycemia increases viability of both BHK21 and H1299 cell lines in extreme hypoxic conditions, with a higher effect on H1299 cells. These results are in agreement with a report that shows that hyperglycemia (25 mM glucose) significantly benefits survival when bovine vascular smooth muscle cells are cultured under hypoxia (2%)^[39]. This effect of high glucose concentration in cells is probably due to the fact that under hypoxia mitochondrial activity diminishes and anaerobic glycolysis augments. Cells depend on glucose as the main energy source; therefore, a higher availability of glucose should facilitate survival. Besides, in conditions of high glucose concentrations and hypoxia, the excess of glucose is diverted to the pentose phosphates pathway (PPP), increasing the availability of NADPH + H⁺ to be used by the anti-oxidant systems, reducing the harmful effect of free radicals^[40, 41]. Regarding this, it has been reported that glucose metabolism through PPP (but not through glycolysis) benefits the stabilization of

HIF1 α in hypoxic conditions in human mesangial MCDB131 and hepatocellular carcinoma Hep3B cells^[42].

Metabolic activity

Hyperglycemia augments metabolic activity of BHK21 cells while it diminishes it in H1299 cells, independently of oxygen concentration. In pioneer works, glucose consumption *in vivo* was found to be directly related to glycemia. In normoglycemic hosts the *in vivo* consumption of glucose by neoplastic tissues was found to be very high. In hyperglycemic hosts, the total consumption of glucose depended not only on the level of glycemia but also on its duration and the type of tumor. During the first hours of increased supply, tumors used more glucose than later; a plateau was eventually reached where the glucose consumption was maximal. It was found that the tumors grew less well in hyperglycemic hosts when consuming more glucose than normal^[43]. Hyperglycemia is known to depress tumor growth *in vivo*^[44]. More recently, clinical imaging with FdG-PET (fluoro-18-deoxyglucose-positron emission tomography) has demonstrated that the vast majority of human primary and metastatic cancers take up far more glucose than normal tissue reflecting the need to increase flux to compensate for diminished energy yield^[45].

MTT reduction depends on the activity of mitochondrial dehydrogenases and therefore, is a direct evidence of the Krebs cycle activity in cells. In BHK21 cells hypoxia stimulates the metabolic activity in early periods of incubation in normoglycemic conditions, but after 24 hours it was observed a lower metabolic activity in all cultures under hypoxic conditions, even if hyperglycemia augments metabolic activity when compared to normoglycemic conditions.

In H1299 cells, at a 5 mM glucose concentration, metabolic activity increased in time and was higher than in normal cells, regardless of oxygen concentration. At 11 mM, there was a tendency to increase at 6% and 0.1% oxygen when compared to normoxic conditions, but without significant differences between them; this is in accordance with the results reported with human lung alveolar epithelial

cancer A549 cells, where 0.1% oxygen increased mitochondrial oxidants production without affecting aconitase, NADH dehydrogenase and succinate dehydrogenase activities, meaning that metabolic activity is not altered^[46]. At 0.1% oxygen, 20 mM glucose partially helped maintain metabolic activity, which decreased in time. In accordance with our results using H1299 cells, it has been reported in MCF-7 and HCT116 cells that hypoxia (0.1% O₂) decreases the Krebs cycle, aconitase and Complex I of the electron transporter chain activities after 48 hours of incubation; the reduction of mitochondrial activity is accompanied by an increase of glycolysis^[47]. However, the comparison between glucose and oxygen consumed *in vivo* by transplanted tumors has confirmed what had already been shown *in vitro*, i.e., a large consumption of glucose contrasted to a small oxygen utilization. In hyperglycemic animals where the amount of glucose consumed showed to be larger than normal, the withdrawal of oxygen produced, most of the times, a sharp decrease of glucose consumption and lactate elimination. There was no indication that hypoxemia increased glucose consumption despite a large availability^[48].

Lactate

Interestingly, we observed that, when cultured in fresh medium at confluence, normal and cancer cell lines failed to induce the color shift. In normal cells, lactate production was dependent on glucose and oxygen concentrations. At 5 mM glucose, there were not significant differences regardless of oxygen levels. At 11 mM glucose, lactate concentration was higher at 6% oxygen, when compared to normoxic and extreme hypoxia conditions. At 20 mM glucose, lactate concentrations were similar regardless of oxygen concentrations. Taking into account that one of the consequences of hyperglycemia is an increment of OXPHOS^[49], it is expected that lactate production in normal cells does not vary/diminish in hyperglycemic conditions. In accordance to our results, it has been reported that in human endothelial cells^[50] and HUVEC, ECV304 and ECV304-GPX cell lines, 20 mM glucose does not affect significantly lactate production^[51].

In our work, H1299 cells augmented the excre-

tion of lactate in hypoxic conditions without significant differences between the glucose concentrations assayed. In agreement with this, it has been reported that JEG3 (human choriocarcinoma) cells exposed to 1% O₂ increased lactate production after 24 hours of incubation^[52]; in MCF7 and HCT116 (colorectal cancer) cells, hypoxia (0.1% O₂) decreased Krebs cycle, aconitase and Complex I of the electron transport chain activities after 48 hours of incubation, promoting anaerobic glycolysis and augmenting lactate production^[47].

It is important to note that even if it was expected a high lactate excretion in H1299 cultures, they did not present important variations of lactate production when increasing glucose concentrations, and we did not find significant differences between the two cell lines in most of the conditions assayed. This allows to suggest that H1299 cells are using a glucose pathway besides anaerobic glycolysis. In nonproliferating tissues such as heart or muscle, cellular bioenergetics are directed toward OXPHOS. In proliferating cancer cells, the majority of the pyruvate generated from glucose (>90%) is converted to lactate by lactate dehydrogenase (LDH-A), where it is readily secreted into the extracellular environment rather than oxidized to completion. By converting pyruvate to lactate, LDH-A recovers the NAD⁺ needed to maintain glycolysis. This step is critical for the maintenance of tumor proliferation *in vivo*^[53]. The remaining pyruvate from aerobic glycolysis that is not converted to lactate (10% of total) enters the mitochondria and is extruded from the TCA cycle at various steps for use in biosynthetic pathways. In hypoxic and hyperglycemic conditions, it has been reported an increment of glucose-6-phosphate dehydrogenase and the PPP activities^[41]. Besides, it has been found in human kidney HEK293 and mice myoblast C2C12 cells, that glucose-6-phosphate dehydrogenase associated to the mitochondrial matrix becomes the main source of NADPH in hyperglycemic conditions^[54]; the idea that mitochondria participate in hexose phosphate metabolism is completely novel. Recent literature has pointed to the ability of physiological concentrations of fructose phosphates to interfere with aerobic respiration^[55]. This suggests that the excess of glucose is

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been used to synthesize riboses phosphate, essentials for the synthesis of nitrogenous bases and NADPH, needed for proliferation.

The excess of glucose could also be addressed to OXPHOS, which is diminished in cancer cells^[56]. Regarding this, it has been reported that H1299, A549, H1975, H1650, H520, 786-0, H838 and U87MG cancer cell lines, exhibit different cellular respiration and glycolysis rates, presenting the H1299 cells the highest cell respiration rate and the lower lactate production, which oscillates between 2-4 mM^[57]; these values coincide with ours.

MCT1 transporter is highly related to the entry of lactate to skeletal muscle and its expression is negatively correlated with LDH activity^[58]; this transporter has been found to be over-expressed in cervical carcinoma cell lines during the invasive progression phase^[59] and in the lung cancer cell lines B203L, PC9, A110L, A549, QG56, SQ1, B1203L, PC10, 904L, PC1 and A529L^[60]. The expression of MCT1 is associated to lower levels of lactate in the culture medium, suggesting that these cells are not necessarily increasing glycolysis rate, but rather, they import lactate to be used as a fuel for the TCA cycle and OXPHOS^[59]. This mechanism has been named “inverse Warburg effect”: lactate, released as the end-product of glycolysis in the hypoxic tumor cell compartment, prominently fuels the oxidative metabolism of the oxygenated tumor cell subpopulation, thereby sparing glucose for glycolytic cells^[61] and supports a previous work that described lactate acting as an onco-paracrine metabolite, through a process that requires to augment cytosol-mitochondrial transfers and thereby increase ATP generation, without increasing glucose transport^[62].

Taken together, our results allow the conclusion that hyperglycemia affects the cells proliferation and viability depending of the cell type and time of incubation. Independently of the mechanism that cells utilize, hyperglycemia contributes notably to the survival of both cell lines in hypoxic conditions. Metabolic imbalances that mediate functional abnormalities associated with an increased NADH/NAD⁺ may differ in hypoxic and hyperglycemic tissues. This redox imbalance in tissues of diabetic individuals appears to result largely from an increased rate of

oxidation of sorbitol to fructose by SDH. In hypoxic tissues the same redox imbalance results from impaired mitochondrial oxidation of NADH to NAD⁺ because of decreased PO₂. Future studies will be aimed to determine the role of oxidative stress and intracellular signaling to promote this survival and malignant transformation in hyperglycemic/hypoxic conditions, proper of diabetes and solid tumors.

ACKNOWLEDGMENT

This work was supported by Instituto Venezolano de Investigaciones Científicas

(Project N/ 1142), República Bolivariana de Venezuela.

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