Original Article

How Human Lung Adenocarcinoma Cells React Towards Long-term Metabolic Stress; A Follow Up

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ABSTRACT

Introduction: A key challenge in fighting against cancer is cancer recurrence and resistance. Apart from the advances in the field of cancer treatment, the outcome is not still as high as expected. This may be partly due to the poor understanding about the true biology of a tumour. Tumour is a complex tissue and cells in its central parts bear nutrition deficiency and poor angiogenesis. **Objective:** The main aim of this study was to investigate the effect of long-term serum starvation on an *in vitro* model of human lung adenocarcinoma, A549 cell line. **Methods:** The cells bore serum starvation for 6 days and at 24-hour intervals, their proliferation, size, mitochondrial function and protein content was studied. Also, at 24-hour intervals and following at least 1 day of starvation, the cells were released in a 10% serum supplemented media and their reactions were studied as above. **Results:** The results demonstrated that despite the harsh conditions around the starved cells, they still proliferate and show increased mitochondrial function and protein content. Upon re-exposure to favourable conditions, this increase is more obvious. These observations were not recorded in control cells. **Conclusion:** It was concluded that following a long-term metabolic stress, these cells do not die, but become stronger and that can be a plausible explanation behind the difficulty in treatment of recurrent cancers.

Key words: Cancer, A549, Serum Starvation, Proliferation, MTT, SRB.

INTRODUCTION

Cancer is a challenging disease. There are many new protocols and improvements in the field of cancer protocols, treatments and regimens. For example, huge improvements have been made in developing many new anti-angiogenesis medicies have been developed[1-5] or nano technology has been involved in developing more selective medicines for cancer patients.[6-12]. However, the outcomes are not as predicted. In other words, no matter how novel the treatment protocol is, there are still lots and lots of cases of failure in cancer treatment. This failure is not always the primary result in cancer treatment. It usually happens when the cancer recurs either at the primary site or as a metastasis in another organ of the body.[13] The challenge with the treatment of this recurring cancer is that these secondary tumors are usually more resistant to chemotherapeutics and treatment protocols.[14] Usually the cancer patients cannot win against these secondary tumors. According to the American Cancer Society (ACS), lung cancer is still the leading cause of cancer death in both men and women, accounting for 1 out of 4 cancer death in patients.[15] Non-small cell lung carcinoma (NSCLC) is the most common type of this cancer and the leading cause of cancer death, many of which are due to cancer recurrence.[16-18] The current study focuses on lung cancer, specifically A549 cell line as a model for NSCLC model. Our previous publication on this cell line showed that severe serum starvation does not kill these

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cells significantly but mainly increases the percent of cells accumulated in G_1 phase of the cell cycle.[19] Based on those results, we became more determined to focus on other characteristics of such cancer cells. Hence, In the current study, for the first time, other specifications of these cells in such a long-term metabolic stress is investigated considering this enquiry that reported other specifications of these cells in such a long-term metabolic stress and how these cells can get back to life.

METHODS

Materials

All the materials used in this study were purchased from Sigma, otherwise mentioned in the text. A549 human lung adenocarcinoma cell line (IBRCC10080) was purchased from Iranian Biological Resource Center (IBRC).

Cell Culture Methods

Cancer cells, following thawing, were passaged for 3 times and were kept in a humid 37°C incubator with 5% CO_2. Cells were kept in RPMI-1640 media (Gibco BRL®), supplemented with 10% fetal bovine serum (FBS) (Gibco BRL®) and 1% penicillin/streptomycin (Gibco BRL®). Cells were seeded at the density of 4000 cells/cm^2 and the experiment started when the cells were at their early plateau phase. On the day of the experiment, the media was discarded and replaced with fresh media supplemented with 10% FBS (as control) and 0.5% and 0.25% FBS as test groups. The cells then underwent 1 up to 6 days of starvation. On 24-hour intervals the following tests were performed on these cells. Also, after the starvation period was over on each day, a fresh 10% FBS-supplemented media was exposed to the cells.

Cell Size Measurement

Cell size was measured by flow cytometry, based on the method previously described by Rathmell *et al.* [20]. Briefly, 1×10^6 cells per mL of A549 cell suspension was centrifuged and washed with cold Ca^{2+}/Mg^{2+} free phosphate buffered saline twice. Then following fixing the cells with cold 70% ethanol solution, the cells were analyzed for size with flow cytometry (BD FACSCalibur TM, USA). Geometrical mean forward scatter was used as a measurement of cell size.

Cell Count Method

Cell count was performed based on the trypan blue assay [21, 22]. At the specific time of the test, the supernatant was gently discarded, cells' surface was washed with sterile saline (0.9% w/v) and trypsin was added on the cells. Following detachment of the cells, complete media was added to the cells. A 1:1 ratio of cell suspension and trypan blue dye was prepared and 20 µL of this sample was placed on a hemocytometer. The number of live cells in 3 out of 9 squares in the hemocytometer were counted and averaged. Number of live cells was counted under an inverted microscope, based on the following formula;

$$cell\ count\ per\ cm^2 = Average\ counted\ cells\ under\ microscpe \times 2 \times 10^4$$

Mitochondrial Function Assessment

To assess mitochondrial function, the already well-established MTT assay was used.[23] As it is already known, oxidoreductase enzymes available in mitochondria of cells are responsible for converting MTT matter into formazan crystals,[24] hence, based on this conversion, the mitochondrial activity can be assessed. So, briefly, MTT (10 µL) was added to each well and kept in darkness for 4 hours. Later the supernatant was replaced with 200 µL of dimethyl sulfoxide. The plates were shaken for 30 minutes and the optical density was recorded at 570 nm.

Protein Content Assessment

For an accelerated assessment of cells' protein content, SRB method was applied based on the already described method [25]. Briefly, at the experiment day, the cells in multi-well plates were fixed with cold (20% w/v) trichloroacetic acid for 30 minutes in refrigerator, the plates were later washed and air dried and sulforhodamine B (0.4% w/v in 1% acetic acid- 50 µL) was added to the wells for 30 minutes, and later the extra dye was washed with 1% acetic acid. Following air-drying of the plates, 200 µL of tris buffer (10mM, pH 10) was added to each well and the absorbance was recorded at 540 nm.

Statistical Analysis

Cell size measurement was done in FLowJo® software, version 7.6.1. All the comparisons were done in GRAPHPAD PRISM® version 5 using two-way analysis of variance, repeated measurement. Level of significance was considered at $p<0.05$.

RESULTS AND DISCUSSION

As it is generally known, tumor is not a homogenous tissue. As the tumor grows rapidly, angiogenesis takes the responsibility of feeding the cells around the tumor. However, this phenomenon is not always a success. There are many parts in a tissue without sufficient angiogenesis and hence the cells suffer from nutrition and oxygen deficiency. Some cancer scientists believe that in a tumor, most of the cells are not active in their cell cycle.[26] This is while most of the chemotherapeutic drugs are active on cycling and rapidly proliferating cells

Table 1: Comparison of average A549 cell size in serum starved and released states, measured by flow cytometry. The cells were either kept in 10% serum (as control) or starved (in 0.5% and 0.25% serum contained media) for 1 up to 5 days and after each day they were released in media containing 10% serum. Experiments were performed in triplicate and results are shown as mean ± standard deviation.

Status	Serum%	Time (day)				
		1	2	3	4	5
Starvation	10%	480±2.8	397.5±0.7	423.5±9.2	287.5±2.1	301±2.1
	0.5%	442.5±0.7	378±9.9	429±0	438±1.4	440.5±20.5
	0.25%	468.5±2.1	376.5±7.8	405.5±2.1	450±5.7	450.5±0.7
Release	10%	433±32.5	362.5±0.7	386.6±2.1	380±4.2	368.5±20.5
	0.5%	415±1.4	314±45.2	396±2.8	437.5±0.7	442±3.8
	0.25%	416.5±0.7	404±0	444.5±2.1	478±8.5	452.5±3.5

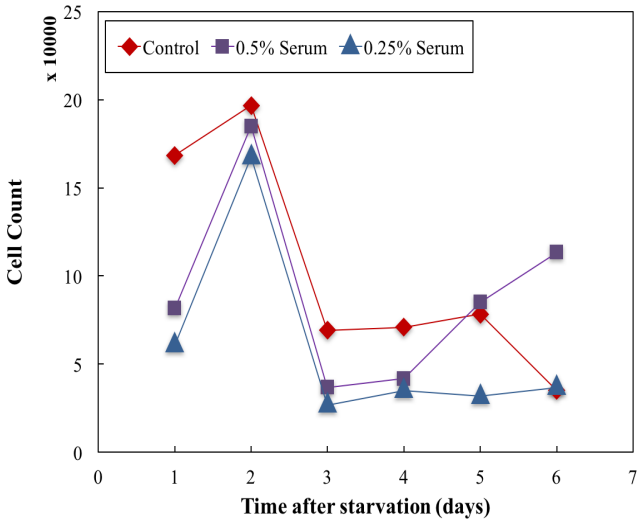

Figure 1: Dependence of A549 cell count/cm² on time measured by trypan blue assay in 3 different states; control (10% serum in media), 0.5% and 0.25% serum in media. Experiments were performed in triplicate and results are shown as mean ± standard deviation (which are small and embedded in curves' markers).

and *in vitro* cancer studies focuses on such cells which are in their exponential phase of growth. In the current study, we sought to focus on non-cycling cells and for the first time report the long-term effects of metabolic stress on cancer cells. Hence, we used the *in vitro* model for non-small cell lung carcinoma, A549 cell line. As it was previously shown,[19] the cells were study started when the cells were at plateau phase of the growth curve. We showed that the cells either at plateau (control) or the cells bearing metabolic stress accumulate in G_1 phase. This accumulation is more obvious in serum starved cells. Exposing the cells to the 10%-supplemented media could release the cells but this release was more obvious when the cells had borne short term starvation and also the control cells.[19] In the current study, we performed the same in terms of treating the cells and later tested their proliferation rate, mitochondrial function and protein content. The results showed that there were no statistically significant differences between the average A549 cell size during the starvation days or even following releasing (Table 1).

Reducing the serum concentration on this cell line caused the cells to go on with the proliferation at a lower basal rate. The interesting point is when the cells are at 0.5% serum, they start to increase cell population on the last 3 days of the study, while the cells grown in 0.25% serum keep the cell count (Figure 1). In other words, the trend of cell count for the cells treated with 0.5% serum is 36666.7± 8856.2, 41666.7±6568.5, 85666.7±5996.6 and 113333.4±75424.7 cell/cm² for the last four day of the study. At the same time, cells grown in 0.25% serum have 26666.7±9428.1, 35555.6±8856.2, 31666.7±6865.5 and 36666.7±2824.3 cells/cm². This result is consistent with the microscopic view of the cells and flow cytometry data in the study by Nakhjavani *et al.*[19] Releasing the cells in a favorable serum condition does not cause significant increase in cell count in the last days of the study (Figure 2). This could imply that the observed increase in cell count could be independent from the extracellular growth factors, an already known characteristic of cancer cells.[27] The results show that the cells can still be affected by the external growth factors while they can still actively proliferate (first day of the study). While long term metabolic stress is affecting cells, other (probably internal) mechanisms could drive cells' proliferation machine. Such increase in proliferation would definitely require more energy production. Hence, we checked the mitochondrial function of the cells.

As the results in Figure 3A show, in control cells which were exposed to favorable serum concentration and had slowly started to enter the death phase of the growth curve, the mitochondrial function also decreased slowly. For example, formazan absorbance of 817.1±54.2 in the first day of the study reaches to 224.8±24.9 in the last day of the study. In contrast, the cells that bore

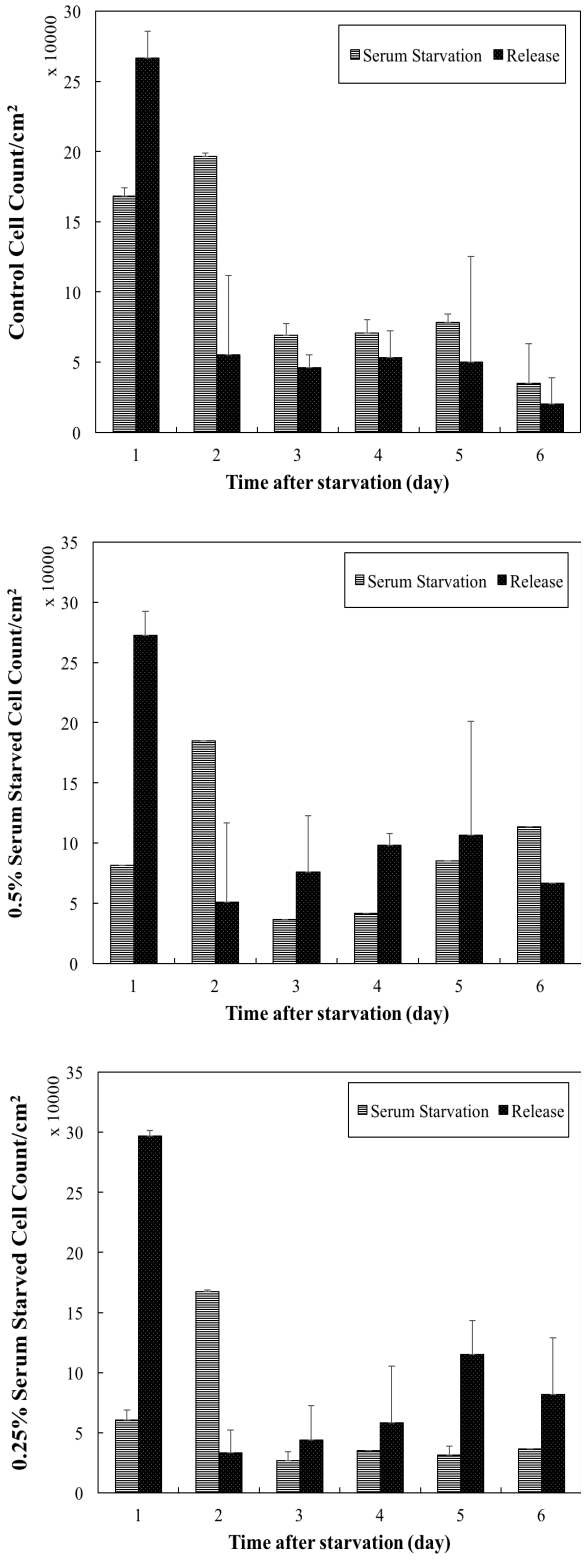

Figure 2: Comparison of A549 cell count/cm² in serum starved and released states, based on trypan blue assay. The cells were either kept in 10% serum (as control) or starved (in 0.5% and 0.25% serum contained media) for 1 up to 6 days and after each day they were released in media containing 10% serum. The results are shown for A) control cells, B) 0.5% serum starvation and C) 0.25% serum starvation. Experiments were performed in triplicate and results are shown as mean ± standard deviation.

serum starvation, had significantly higher levels of mitochondrial function. The highest absorbance in this period of study is for starved cells in 0.5% serum and is recorded at 941.5±46.4, 925.75±54.9 for days 2 and 3 of the study. At the same time, cells starved at 0.25% serum also show no significant difference with 0.5% serum starved cells in terms of absorbance. They show optical densities of 917.1±43.4 and 911.58±61.2 for these two days. This indicates that such harsh conditions can trigger higher energy production engine in the cells. A 24-hour release in media containing 10% serum increases this mitochondrial function (Table 2).

Cells protein content was also studied and the results showed that in control cells, protein content is at a stable level with a declining pattern on the final study day. Meanwhile, the serum starved cells show an increasing pattern of intracellular protein content, which could be a second witness for the resistant characteristic feature of these cells (Figure 4A). Protein content production also seems to be dependent on the extracellular growth signals, because following replacing the low-serum media with 10% serum media, protein content in cells increased dramatically (Table 2). This increase was more in starved cells rather than control cells. This could be interpreted as although extracellular growth signals do not promote cell proliferation, those signals can initiate higher energy production motors and higher protein content in this cell line. The other interesting point is when the average mitochondrial and protein content in each cell is calculated. As shown in Figure 3B and 4B, the less the serum concentration is, the higher mitochondrial function and protein levels become.

The increased cell size, increased cell proliferation, increased mitochondrial function and protein content in these cells show that although the cells are mainly actively proliferating as the fresh cells in their exponential phase of growth, harsh environmental conditions works as an attack towards the cells and makes then raise their defence mechanisms. The cells somehow become stronger. Also in a release, the cells seem to become stronger internally by increasing protein and mitochondrial content. This model could represent a possible mechanism on why there are so many cases of cancer recurrence even after tumor shrinkage. In fact, the possible explanation behind this could be that upon tumor shrinkage, the cells that lie inside a tumor, those that are not actively proliferating but also not necrotic too, can once again be exposed to favourable nutrition status (similar to the release step in our experiments). This happens while the cells have already become stronger.

This strong feature could explain the fact that cancer treatment in patients with a secondary tumour is usually

Table 2: Comparison of A549 cells' mitochondrial function and protein content in starved and released states. The cells were either kept in 10% serum (as control) or starved (in 0.5% and 0.25% serum contained media) for 1 up to 6 days and after each day they were released in media containing 10% serum. Experiments were performed in triplicate and results are shown as mean ± standard deviation.

Serum%	Time (day)	Mitochondrial Function		Protein Content	
		Starvation	Release	Starvation	Release
10	1	817.1 ± 54	941.75±55	1747.58±166	2321.75±87
	2	750.3 ± 34	1093±30	2030.08±115	2344.33±41
	3	587.8 ± 22	1136.75±48	2008.75±80	2520.25±112
	4	592.2 ± 32	1003.75±38	2030.42±89	2165.17±69
	5	479.9 ± 44	1011.17±41	1770.33±88	2200±117
	6	224.8±25	262.5±48	*n.d.	n.d.
0.5	1	817.5±54	1099.92±55	1193.25±108	2062.42±166
	2	941.5±46	1176.83±58	1186.25±132	2213.42±140
	3	925.7±55	1186.92±68	1428±201	2545.83±67
	4	816.1±34	1094.83±54	1889.92±95	2658.92±49
	5	767.3±44	1198.83±48	1791.5±160	2650.22±57
	6	755.7±23	1109±60	n.d.	n.d.
0.25	1	792±44	1041.5±41	1092±146	2008.17±195
	2	917.1±43	1123.33±67	1189.92±242	2389.66±277
	3	911.6±61	1104.75±73	1333.75±121	2563.42±89
	4	820±35	1111.12±75	1866.42±125	2629.75±80
	5	762±60	1102.58±60	1784.83±146	2639±93
	6	786.6±62	1082.67±52	n.d.	n.d.

*n.d. Not Determined

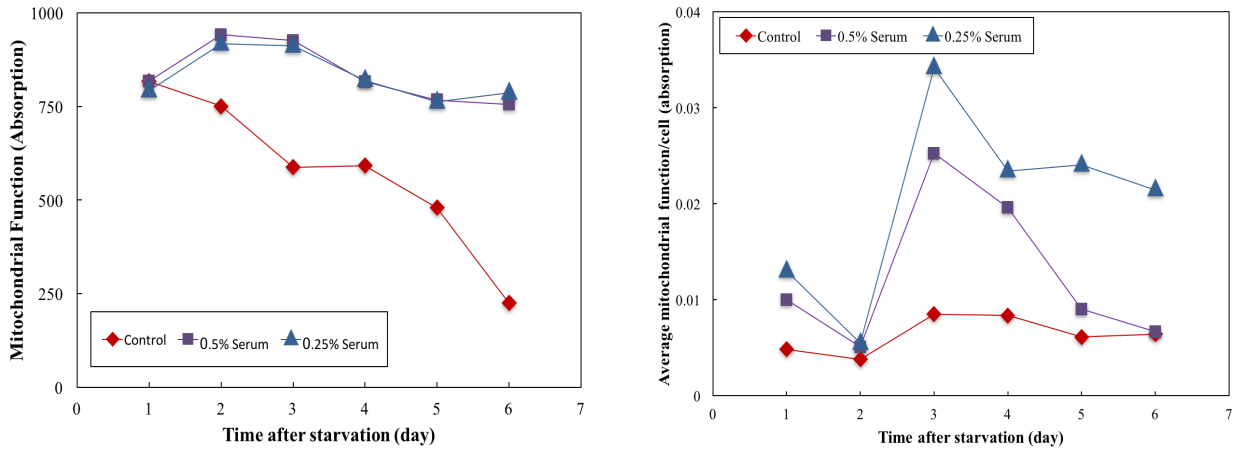

Figure 3: Mitochondrial function of A549 cells in serum starved and released states, based on MTT assay. The cells were either kept in 10% serum as control or starved for 1 up to 6 days in 0.5% and 0.25% serum contained media. The results are shown for A) mitochondrial function of all cells (measured by the plate reader), and B) mitochondrial function per cell (calculated). Experiments were performed in triplicate and results are shown as mean ± standard deviation.

harder. The underlying mechanisms behind these observations might be cancer specific and be different in each cell type. Otto Warburg is one of the first scientists who described the unique metabolic pathways in cancer cells [28] He showed that cancer cells rely on glycolysis for ATP production and even in the presence of sufficient oxygen, they use mitochondrial oxidative phosphorylation mechanisms to live. Glutamine, on the other hand, is an important source of nitrogen for the production of amino acids and NADPH. It also plays role in glutaminolysis, as a hallmark of cancer metabolism, and "glutamine addiction" [28-30]. Glutamine can also provide required nucleotides for DNA synthesis, is connected to the mTOR pathway and has roles in cell cycle control

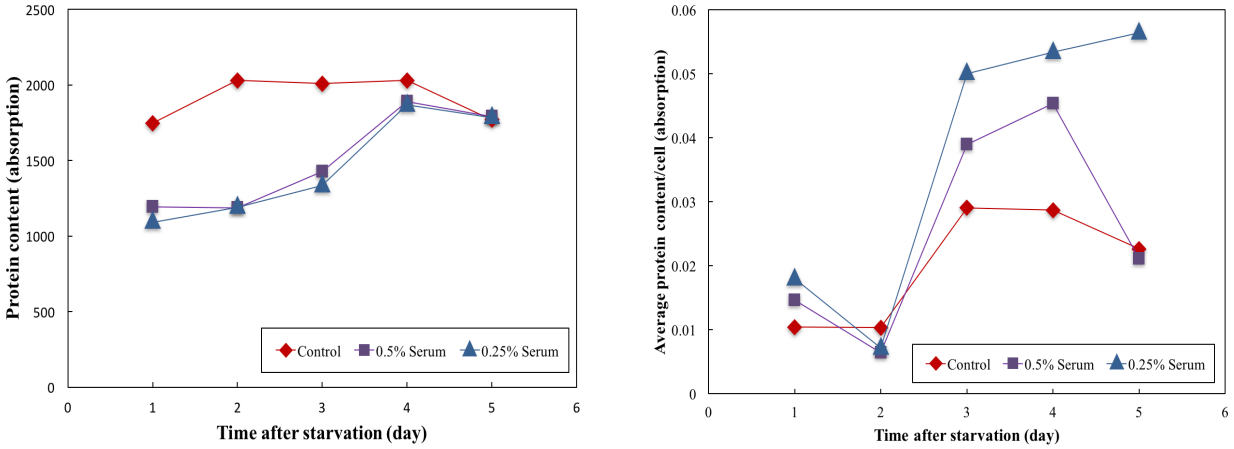

Figure 4: protein content of A549 cells in serum starved and released states, based on SRB assay. The cells were either kept in 10% serum as control or starved for 1 up to 6 days in 0.5% and 0.25% serum contained media. The results are shown for A) protein content of all cells (measured by the plate reader), and B) protein content per cell (calculated). Experiments were performed in triplicate and results are shown as mean ± standard deviation.

and nutrition status [30-32]. It has been shown that mTOR pathway also influences cells proliferation and growth. This pathway is dependent upon nutrients signals and growth factors and can initiate cells' autophagy.[33,34] Involvement of any of these pathways could possibly explain the observations in this study and open new doors for further drug design studies and cancer treatment.[35-40]

In conclusion, this study was the first study to observe the long-term effects of serum starvation on human lung adenocarcinoma model, A549 cell line. The results showed that although in such a long-term metabolic stress, cancer cells do not die, they become stronger. After 6 days of starvation, the cells are still functional, and upon exposure to a better metabolic status, the cells are even more active internally. This study could also explain the not very successful use of newly developed anti-angiogenic agents which restrict cells access to sufficient nutrition.

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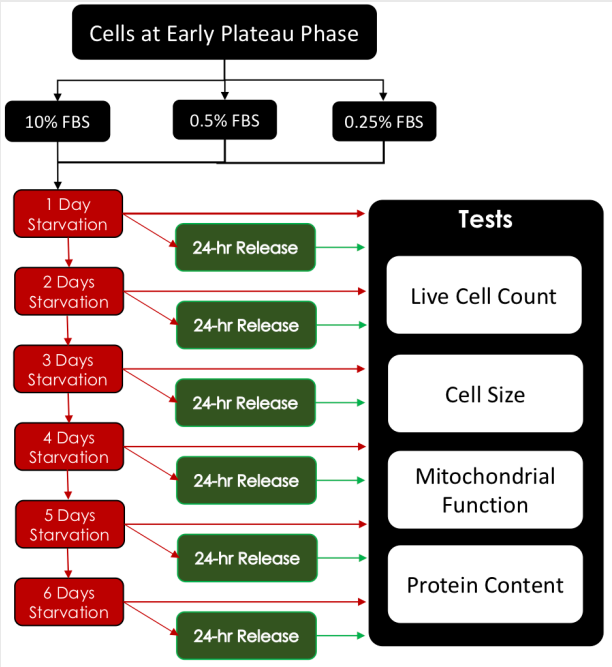
PICTORIAL ABSTRACT



SUMMARY

- Cancer cells are smart cells which can change their characteristics to adopt to the environment that they live in and somehow survive. In the current study, as follow-up to the previous research on human lung cancer, we have studied lung cancer cells reactions towards severe and long-term metabolic stress and found out that these cells have a resistant feature. This feature makes them resist against metabolic stress and upon re-exposure to optimum conditions, they start to re-grow. Hence, it is suggested that these characteristics be considered and studied at pre-clinical drug design and efficacy studies.

About Authors

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