Original Article

Long-Term Effects of Metabolic Stress on Human Malignant Melanoma Cell Line

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ABSTRACT

Introduction: Tumour is a heterogeneous tissue consisting of cells with different levels of metabolic activity. Often the outer layer cells of a tumour have more optimal conditions to grow/proliferate compared to the inner cells. **Objective:** The aim of the current study was to study the reactions of malignant melanoma A375 cells in exposure to different levels of metabolic stress and their ability to returning to life upon re-exposure to optimum nutritional conditions. **Methods:** A375 cells, at early plateau phase, were exposed to media containing 10% (control), 0.5, 0.25 and 0% serum for 1 to 6 days. At 24 h intervals, the cells were tested for morphology, cell cycle distribution, cell size changes, cell count, mitochondrial function and protein content. Also, after each day of starvation, the cells were re-exposed to optimum media (10% serum) and tested again. **Results:** The results showed that the cells made foci structures at long-term starvation. They primarily accumulated in G1-phase and at long-term starvation, most of them entered the apoptotic state. However, before death, cell count did not decrease, even though the mitochondrial function and protein content were less than the control cells. Releasing the cells in optimum conditions could trigger cells' proliferation ability, mitochondrial function and protein content. **Conclusion:** The results suggest that this model of malignant melanoma has a resistant feature against sub-optimal nutritional and metabolic status and the cells can return to life with even stronger internal engines, as evidenced by higher levels of mitochondrial function and protein content.

Key words: A375, Neoplasm, Serum, Starvation, Metabolic Stress.

INTRODUCTION

Cancer resistance against chemotherapy and its recurrence is still one of the biggest challenges in medicine.[1-4] There are many methods and chemotherapy regimens suggested, tested or proved for cancer treatment including surgery, radiation therapy, immunotherapy, targeted therapy, hormone therapy, stem cell transplant and precision medicine.[5,6] anti-angiogenic agents.[7-9] novel nanomedicines and nanotechnology-based[10-12] methods and etc. Besides all these methods and novel medicines,[3,6,13] there seems to be a general formula in the success rate of cancer treatment: The sooner the cancer is diagnosed, the higher rate of survival is achieved.

The question that arises here is why besides so many improvements and technologies, there is still this big challenge in fully recovering from cancer. The answer seems to be residing in tumour cells' biology. For years and years, scientists believed that cancer is a ball full of rapidly proliferating cells. It is now a known fact that a tumour is not a homogenous tissue and in recent years, many researchers have focused on the heterogeneity of the tumour tissue.[14-18] The outer layers of a tumour can proliferate easily. These cells have enough space to proliferate, and sufficient access to blood as a source of oxygen and nutrition and a readily accessible stream for getting rid of all the

Submission Date: 07-03-2018;
Revision Date: 13-04-2018;
Accepted Date: 17-05-2018

DOI: 10.5530/ijper.52.4.70

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metabolic wastes. However, not all the cancer cells are this lucky. The cells in the inner layers of a tumour, not only do not have such favourable nutritional access as the outer layer cells but also bear more physical stress. These cells enter seize proliferation while still living is an arrested state. That is why a tumour is composed of various types of cells with different metabolic activities.[16]

Melanoma is the fifth and sixth most common cancer in men and women, respectively. Malignant melanoma is the major cause of death from skin cancer although it accounts for a small percent of skin cancers (less than 1%). Based on the report by American Cancer Society (ACS), more than 9500 cases of melanoma are expected to occur only in the US in 2017.[19] Although there has been a great improvement in cancer and melanoma treatment, the possibility of recurrence for this disease still exists. Treatment of a recurred cancer is usually harder and different from primary cancer.

A375 is a widely used cell line as a model for human malignant melanoma for *in vitro* cancer studies.[20] In the current research, we aimed to study the biology of malignant melanoma *in vitro* at different metabolic states. In our previous research, we studied human lung carcinoma cell lines' reaction against metabolic stress.[21-23] To the best of authors' knowledge, this is the first time that this cancer cell line's behaviour at different metabolic states and its ability to come back to life following metabolic stress is studied.

METHODS

Materials

All the materials used in this study were purchased from Sigma, otherwise mentioned in the text. Human skin melanoma A375 (IBRCC10141) cell line was provided by the Iranian biological resource centre.

Cell culture methods

Cells were seeded at the density of 4000 cell/cm^2. Following reaching the plateau phase, the supernatant media was changed with media containing 10% (control), 0.5%, 0.25% and 0% serum (Gibco) to impose metabolic stress to the cells for 1 to 6 days. At 24-h intervals, the cells were tested as described below. Also, to study the ability of the cells to recover from the stress was evaluated by releasing the cells in optimum media (containing 10% serum). In other words, at 24-h intervals, when the cells had passed 1 to 6 days of starvation, they were exposed to optimum media and tested again.

Morphology studies

Cells morphology was studied using an inverted light microscope and images were taken with the installed Moticam Pro camera and Motic® software.

Cell cycle analysis

To study the cells' distribution in their cycle, a BD FACSCalibur™ was used. The cells were washed and exposed to the staining solution containing propidium iodide, RNase and Triton X-100. The results were analyzed using FlowJo® software, version 7.6.1.[21]

Cell size analysis

To measure average cells' size, the method described by Rathmell *et al.* was used.[24] Briefly, the geometrical mean of forward scattering laser was used as an indicator of cell size.

Proliferation assay

Cells' ability to proliferate was measured using trypan blue assay.[25] The cells were trypsinized and mixed with trypan blue dye in equal volumes. Cell count was performed under inverted light microscope.[26]

Mitochondrial function evaluation

The mitochondrial function of the cells was evaluated using MTT method.[27] Briefly, the cells were exposed to 10% v/v of MTT solution for 4 h in darkness. Later the supernatant was gently discarded and 200 µL of DMSO was added. The absorption was read at 570nm.

Protein content measurement

Cells' protein content was measured using the SRB method as previously described.[28,29] Briefly, the cells were fixed using cold trichloroacetic acid, washed, and exposed to Sulforhodamine B dye (0.4% v/w) for 30 minutes. Later the dye was washed off and 200 µL of tris buffer (10 mM, pH 10) was added to the cells. Following a 30-minute shake, the absorbance was read at 540 nm.

Statistical analysis

Two-way analysis of variance, repeated measurement was used for all the comparisons in GraphPad PRISM® version 5. The graphs were made in Microsoft Excel®, version 2013. The p-value of less than 0.05 was considered as the level of significate difference. The tests were performed in triplicate and the results are presented as the mean ± standard deviation.

RESULTS

Figure 1 shows the morphology of the A375 cells in 3 days of the study. As it is shown in the Figure,

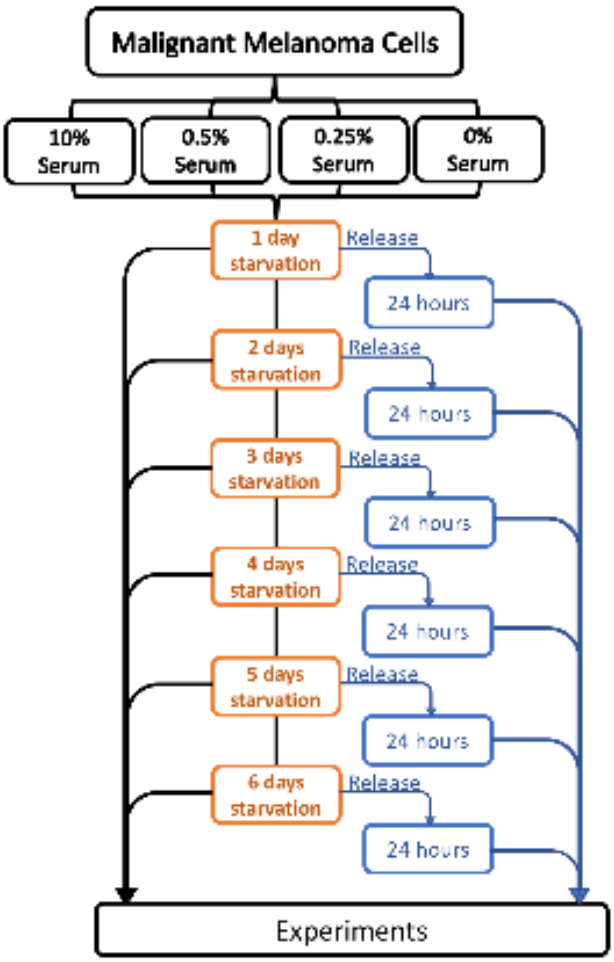

Figure 1: schematic steps undertaken to study the effect of metabolic stress on MCF-7 cell s.

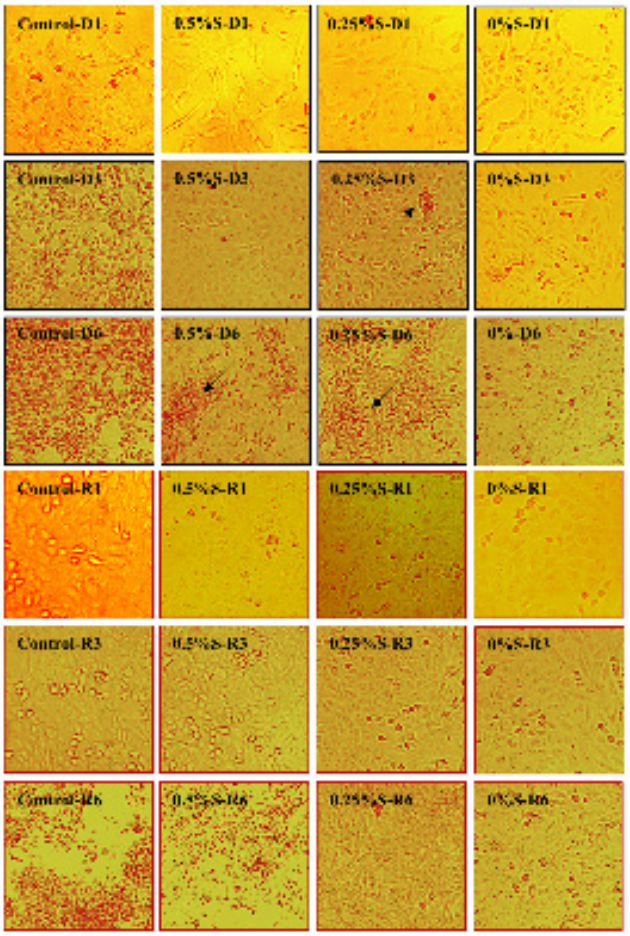

Figure 2: Morphology of A375 cell line following exposure to 10%, 0.5%, 0.25% and 0% serum for 1 to 6 days. D, S and R are abbreviations for day, serum and release, respectively.

control cells, which had favourable growth conditions, are slowly becoming smaller and rounder. This is in agreement with the other finding of this study.

As Figure 2 shows, control cells have actually stopped active proliferation from day 3 of the study and have entered the death phase of the growth curve, as live cells were significantly reduced. Also, Table 1 shows that cell size has a decreasing pattern, although statistics could not identify any significant differences in average cell size change. The cell cycle histograms also show that at the last days of the study, the cells are entering the sub-G_1 phase, which shows the apoptotic population.

This finding describes and explains the round and small size of the observed cells. The interesting observation was in starved cells. These cells showed increased cell population (Figure 1) and proliferation (Figure 2) in the first four days of the study. The cell population then neither decreased nor increased in the last 2 days of the study, as shown by statistical analysis ($p<0.05$). The other interesting observation was the existence of cellular foci, which are indicated in Figure 1.

Flow cytometric data (Figure 3) showed that serum starvation imposes cell accumulation in the G_1 phase of the cycle. This accumulation had an increasing trend and this increasing trend was more obvious as the serum concentration decreased. In other words, the increasing trend in G_1 cell population was observed for 2, 3 and 4 days for cells starved at 0.5, 0.25 and 0% serum, respectively. Following those days, the G_1 population decreased and cells tended to leave G_1 phase and enter in sub-G_1 or G_2/M phase of the cycle. These trends are in agreement with the fluctuations in average cell size.

The mitochondrial function of the cells was also studied. The results showed that serum starvation-induced some more mitochondrial function in the starved cells on the second day of the study. However, the general pattern of mitochondrial function had the same trend as in control cells (Figure 4). Releasing the cells, on the other hand, showed that the fresh media could increase

Table 1:Size distribution of A375 cell line following 1 to 6 days of starvation in media containing 0.5%, 0.25% and 0% serum, compared to control cells exposed to 10% serum. The table shows the mean cell size distribution of cells released for 24 hours following the determined starvation period. The test was performed in triplicate and data are presented as mean ± SD.

Status	Serum (%)	Time (day)					
		1	2	3	4	5	6
Starvation	10	419±2.8	312±18.4	290.5±20.5	311.5±23.3	261.5±16.3	256±7.1
	0.5	394.5±2.1	443±0.0	538.5±14.8	369.5±55.8	345.5±27.6	336.5±6.4
	0.25	403±0.0	420±2.8	504±14.1	587±60.8	358±19.8	387±5.6
	0	390.5±6.4	430±5.6	532.5±2.1	435.5±12.0	438.5±2.1	3776.5±2.5
Release	10	244±11.3	252.5±13.4	378.5±10.6	297±57.9	276±29.7	287±0.0
	0.5	3558.5±2.1	465±2.8	509.5±41.7	425.5±37.5	420.5±7.8	443.5±48.8
	0.25	350.5±0.7	442.5±3.5	465±1.4	441.5±19.1	377.5±1.2	449±2.82
	0	375.5±0.7	436±5.6	483.5±4.9	541.5±2.1	371.5±6.3	457.5±4.9

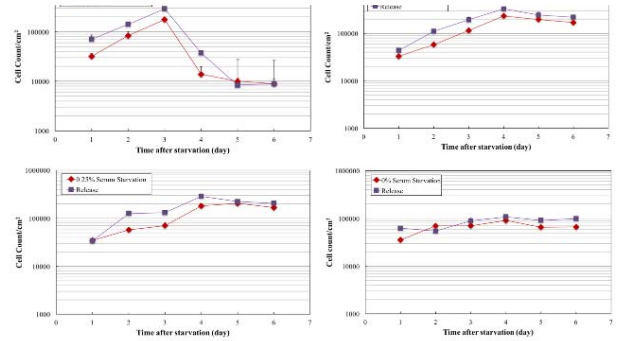

Figure 3: A375 cell counts per cm2 in a 6-day study, following exposure to 10%, 0.5%, 0.25% and 0% serum concentration, or release for 24 hours in a media containing 10% serum. The test was performed in triplicate and data are presented as mean ± SD.

mitochondrial function in the cells significantly ($p<0.05$), except the second day of the study (Figure 4).

Similarly, protein function of the cells was also studied as it is shown in Figure 5. Serum starvation induced an increased pattern of cell protein content in starved cells. This increasing trend was negligible in case of cells exposed to serum-free media. In other words, low serum exposure could induce an increased pattern of protein content in cells, while in case of exposure to a serum-free media, the cells kept the protein content at a steady state.

Releasing the cells in optimum media caused a significant increase in cells' intracellular protein content. Once again, in case of cells starved at 0% serum, although some increase in protein content is observed, this increase is not statistically significant.

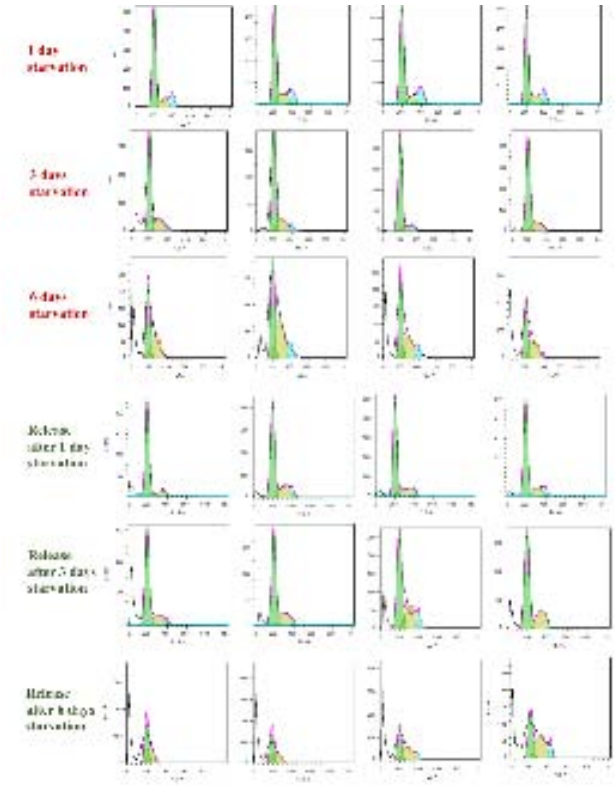

Figure 4: Histograms of A375 cell distribution in the cell cycle phases. The cells were kept at 10, 0.5, 0.25 and 0% serum for 1 to 6 days. S and R represent starvation and release status, respectively. Selected histograms on days 1, 3 and 6 of the study are presented here. S stands for starvations and R stands for release status.

DISCUSSION

The key challenge is cancer treatment is cancer cells' resistance to chemotherapy and cancer recurrence following recovery. One reason behind this barrier in successful treatment could be the heterogeneity in tumour cells and the metabolic status that they bear.[15,16] In the presented research, malignant melanoma cells' reaction towards different levels of metabolic stress is evaluated and studied. Cell lines, such as this A375 melanoma cell line, usually require a media supplemented with 10% serum. The serum is the source of different growth factors, hormones, amino acids and

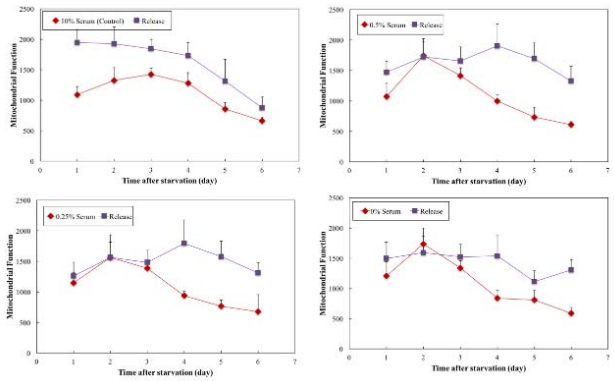

Figure 5: Mitochondrial function (absorption) in A375 cell line based on MTT assay in a 6-day study, following exposure to 10%, 0.5%, 0.25% and 0% serum concentration, and a 24-hour release in a media containing 10% serum. The test was performed in triplicate and data are presented as mean ± SD.

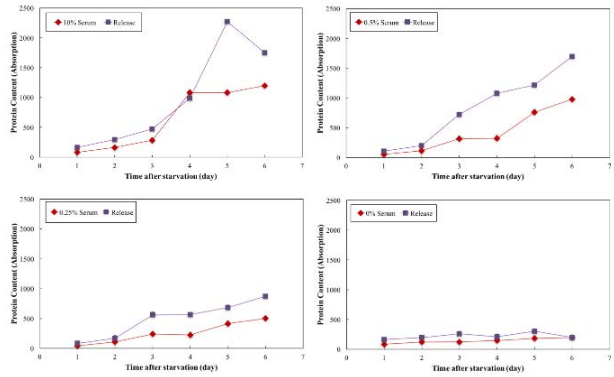

Figure 6: Protein content (absorption) in A375 cell line based on SRB assay in a 6-day study, following exposure to 10%, 0.5%, 0.25% and 0% serum concentration, and a 24-hour release in a media containing 10% serum. The test was performed in triplicate and data are presented as mean ± SD.

proteins, and etc. to help the cells survive and have a normal life pattern.[30, 31] To induce different levels of metabolic stress, the cells were exposed to 3 levels of low serum concentration (0.5%, 0.25% and 0% serum) and their biological reactions were compared to control cells that were grown at 10% serum. Also, cells ability to resist against metabolic starvation was evaluated within 6 days of the study and at 24-h intervals. To the best of author's knowledge, this is the first study to evaluate melanoma cells reaction towards such metabolic stress in a long-term study. Cells' biological reactions were evaluated in different aspects including morphological features, cell size changes, cell cycle distributions, cells' proliferation, cells' mitochondrial function and protein content. Furthermore, after 24, 48, 72, 96, 120 and 144 h of metabolic stress, the cells were re-exposed to fresh 10%-serum supplemented media for another 24 more h. In other words, following different periods of metabolic stress, the cells were given a second chance to be exposed to favourable conditions for 24 h and the biological reactions after this release was evaluated again.

The foci are multilayer aggregation of cancer cells and are suggestive of cell invasiveness.[32] The foci formation phenomenon could suggest that metabolic stress and subsequent exposure to optimal environmental conditions can transform certain cell lines into cells with more aggressive characteristics, a phenomenon and characteristic that can explain melanoma metastasis which is a very well-known feature of melanoma. Such intensity of foci formation was not observable in our other studied cell line, human lung carcinoma.[21] This seems to be a specific characteristic of this cell line. On the other hand, serum starvation has been known to induce G_1 arrest and accumulation. Arthur Pardee, in 1973, was the first to describe the effect of suboptimal nutritional conditions on normal cells cycle.[33] At this suboptimal condition, the cells escape the cycle at a certain point in G_1 phase, known as restriction point. The cells maintain a minimal metabolic status, known as quiescence, and wait for the optimal environmental conditions. Scientists believe that cancer cells have lost this control at the G_1 phase and hence, can continue their cycle and proliferate independently from external growth signals.[33,34] In the current study we have shown the dependence of G_1 accumulation on the serum concentration. In other words, the less the serum concentration, the longer the cells accumulated in the G_1 phase of the cycle. Although an increased sub-G_1 population was also observed. In our other study on human lung carcinoma cell line also we noticed a similar reaction.[21] The cells accumulated in G_1, even more than A375 cell line. These observations could imply that these cancer cells show different degrees of sensitivity towards suboptimal environmental conditions. Although, we have noticed that besides low serum concentration and excess metabolic waste, as evidenced by the yellowish colour of the supernatant media, these cancer cells still maintain the ability to proliferate to some levels higher than the control cells. This is an expression of cancer hallmarks such as self-sufficiency in growth signals, evading growth suppressors, and sustaining proliferative signalling.[34] As it is shown in the results, the cells which escape G_1 enter G_2M and S phase, and it seems like they are trying hard to survive.

Increased cell population was more obvious for cells grown at 0.5 and 0.25% serum, while cells grown at 0% serum kept a plateau-like state of cell count. This plateau-like state of these cells may not show the invasiveness as other groups of cells show, but it shows the resistance of these cells against dying. The question that arises here is how these cells can proliferate

in such harsh conditions when not enough growth signals and even amino acids and lipids are not available in the media. Otto Warburg was the first to describe the exceptional metabolism pathway in cancer cells. He introduced it as the "Warburg effect" and explained it as the high rate of aerobic glycolysis.[35,36] Glucose and glutamine are two major sources of catabolism in cancer cells. They provide sufficient carbon, nitrogen and energy for the cells. Some of the glucose is used in cells mitochondria to produce ATP and some is used to produce macromolecular precursors such as Acetyl Co-A for fatty acid production and ribose for nucleotide production and other precursors for amino acid production.[37] Glutamine is a good source of nitrogen and NADPH and in involved in a phenomenon known as "glutamine addiction".[38,39] Glutamine could also be a source for nucleotides and be connected to the mTOR pathway and hence affect the anti-oxidant, growth, nutritional and cycle of the cells.[38,40,41]

In this study, mitochondrial function and protein content of the cells was also measured. In this cell line, in contrary to lung carcinoma, the mitochondrial function did not show the increasing pattern. This is while lung cancer cell line, A549, showed increased levels of both mitochondrial functions. The protein content of the A375 cells had an increasing trend, yet this increase was in lower levels compared to the control cells. It seems that the decrease in mitochondrial function and protein content of the cells has a serum concentration-dependent manner. It can be concluded that this cell line has a weaker characteristic in harsh conditions compared to A549 cell line. These data in addition to the observed increase in cell count in the first days of the study could suggest the possibility of autophagy. Autophagy makes it possible for the cells to survive longer in a quiescence state.[42] This needs further tests to confirm the mechanism undergoing behind these observations.

The more interesting part of this study was the results of the release experiments. As mentioned earlier, following 24, 48, 72, 96, 120 and 144 h of serum starvation, the cells were released in media containing 10% serum. The idea behind this experiment was to model the situation when a tumour is shrunk following chemotherapy and the stressed cells are once again exposed to favourable conditions. In this study, replacing the supernatant media with fresh media, not only exposed the cells with sufficient serum and growth signals but also discarded the metabolic waste around the cells. So, the cells, even in confluent colonies, proliferated more and made denser colonies. This was accompanied by the cells leaving G_1 phase very slowly.

Also, releasing the cells could significantly increase the mitochondrial function and protein content of the cells. This shows that although the cells were resisting against harsh environmental conditions and poor nutritional status and some of them died at the long-term starvation, they could still get back to life and get fresh and re-energize the internal machines of the cell. This could explain the more invasive and resistant feature of the recurrent melanoma.

CONCLUSION

In conclusion, this is the first study to show the effects of long-term metabolic stress on human malignant melanoma cell line. The used model was to show the resistance characteristics of these cancer cells when exposed to sub-optimal nutritional conditions. At starvation status, A375 melanoma cell line did not show as strong features as the lung carcinoma cells in our other study. However, at release state, the cells could strongly be back to life and regenerate in the internal machines.

ACKNOWLEDGEMENT

The authors would like to express their appreciation from Pharmaceutical Sciences Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran and SBMU reference lab for their assistance in financial support and equipment to accomplish this project.

ABBREVIATIONS

American Cancer Society (ACS)

CONFLICT OF INTEREST

The authors declare no conflcit of interest.

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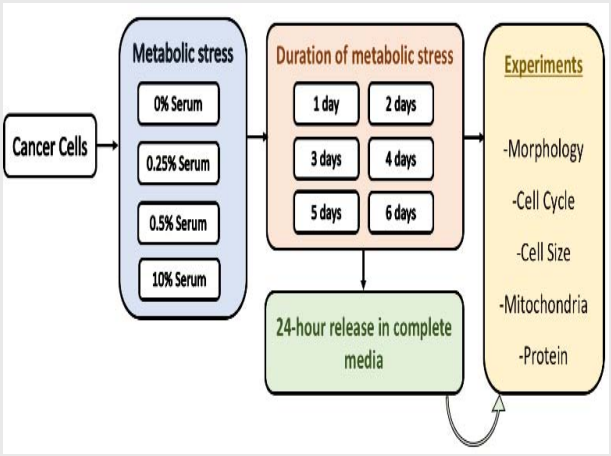
Cite this article: Nakhjavani M, Shirazi FH. Long-Term Effects of Metabolic Stress on Human Malignant Melanoma Cell Line. Indian J of Pharmaceutical Education and Research. 2018;52(4):602-9.

PICTORIAL ABSTRACT



SUMMARY

Melanoma is still one of the most common cancers in the world, while the 5-year relative survival rate of the disease has not had a dramatic increase over 30 years. Hence, the requirement to do more in-depth research for this cancer is still felt. Previously, we investigated the effect of metabolic stress on human lung and ovary and breast cancer cell lines. In the current study, the effects of different levels of sever and long-term metabolic stress was assessed on human melanoma cell line. It was found that this malignant cell line has a resistant characteristic towards metabolic stress and as a result, it is suggested that this should be taken into account while doing pre-clinical drug design and efficacy studies.

About Authors

Dr. Maryam Nakhjavani: A professional doctor of pharmacy, with more than 10 years of research in cancer, toxico/pharmacology, and more than 20 publications in peer reviewed journals and two book chapters. Her major research experience and focus include cancer cells' biology and reactions towards cytotoxic agents, bio-spectroscopic means of cancer detection and translational science in cancer treatment. Currently, she is doing cancer research in the University of Adelaide, Australia.

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