EFFECTS OF KANGLAITE, AN ANTICANCER HERBAL MEDICINE, ON BREAST CANCER CELL METABOLISM

by
Sarah A. Hillhouse

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2019

Approved by:

Advisor: Dr. Yu-Dong Zhou

Reader: Dr. Dale G. Nagle

Reader: Dr. John M. Rimoldi
DEDICATION

I would like to dedicate this Capstone Project to my loving parents, Becky and Timothy Hillhouse, and my sister, Kelsey Hillhouse. Without them, I would never have pushed myself to achieve my dreams.
ACKNOWLEDGEMENTS

I would like to thank Dr. Yu-Dong Zhou, my thesis advisor, for the guidance she has given me during my college years. I thank her for sharing her research ideas with me and for letting me be a part of her laboratory work. Thanks to her, I have gained invaluable research experience that I can now carry into my future career.

I would like to thank my professors in the Department of Biology as well as the faculty of the Sally McDonnell Barksdale Honors College for challenging me these past four years and providing me with the knowledge I need to succeed in my future endeavors.

Lastly, I would like to thank my family and friends for their love and support.
ABSTRACT

Sarah A. Hillhouse: Effects of Kanglaite, An Anticancer Herbal Medicine, on Breast Cancer Cell Metabolism (under the direction of Dr. Yu-Dong Zhou)

Kanglaite (KLT) is an anticancer herbal medicine developed in China that consists of mainly fatty acid triglycerides from *Coix lacryma-jobi* L. var. mayuen (Roman.) Stapf. In this study, we analyzed the effects of KLT on normal versus breast cancer cell metabolism using the C2C12, MCF-7, and MDA-MB-231 cell lines. Each cell line was divided into controls (n=3), cells with 1 µM of rotenone (n=2), or cells 0.3%, 1%, and 1.72% KLT concentrations. Cells were cultured in serum containing regular FCS and serum containing charcoal-stripped FCS in order to examine the full effects of KLT on the cell. Cell energy phenotype assays were conducted using the Seahorse XFe96 Analyzer, which simultaneously measured the cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). The data from the cell phenotype assay was then analyzed in order to determine the effect of KLT on breast cancer cell metabolism by comparing OCR and ECAR within and between cell lines and generating cell energy phenotypes. The results showed that KLT increased OCR and ECAR for C2C12 cells when cultured in charcoal-stripped fetal calf serum (FCS)-enriched media. The KLT increased OCR and ECAR in MCF-7 cells when cultured in regular FCS. The KLT increased OCR in MDA-MB-231 cells cultured in both serums and decreased ECAR in MDA-MB-231 cells cultured in regular FCS-enriched media.
# TABLE OF CONTENTS

List of Tables and Figures................................................................. viii

List of Abbreviations........................................................................ ix

1. Introduction.................................................................................. 1

   1.1 Breast Cancer Background.................................................... 1

   1.2 Metastasis............................................................................. 2

   1.3 Tumor Metabolism................................................................. 5

   1.4 Kanglaite (KLT) ................................................................... 7

2. Effects of Kanglaite, an Anticancer Herbal Medicine, on Breast Cancer Cell Metabolism.............................................................. 12

   2.1 Introduction.......................................................................... 12

   2.2 Methods............................................................................ 15

      2.2.1 Cell Lines and Cell Culture.......................................... 15

      2.2.2 Agilent Seahorse XF Cell Energy Phenotype Test Assay................................................................. 15

      2.2.3 Statistical Analysis......................................................... 16

   2.3 Results.................................................................................. 17

      2.3.1 Baseline Comparison..................................................... 17
2.3.2 OCR Between Conditions Over Time.........................23
2.3.3 ECAR Between Conditions Over Time.........................28
2.3.4 Cell Energy Phenotype Analysis..............................33

2.4 Conclusion................................................................38
2.5 Discussion................................................................40

List of References..........................................................42
# LIST OF TABLES AND FIGURES

Figure 1. Hallmarks of cancer.................................................................3
Figure 2. Sequential steps in the pathogenesis of cancer metastasis...........5
Figure 3. The Warburg effect in cancer................................................6
Table 1. Clinical trials for the study of safety and efficacy of KLTi in humans..8
Figure 4. Chemical structure of rotenone..............................................14
Figure 5. Baseline comparison of OCR and ECAR between conditions for C2C12 cell line.................................................................18
Figure 6. Baseline comparison of OCR and ECAR between conditions for MCF-7 cell line........................................................................19
Figure 7. Baseline comparison of OCR and ECAR between conditions for MDA-MB-231 cell line..........................................................20
Figure 8. OCR vs. time in minutes between conditions for C2C12 cell line.....24
Figure 9. OCR vs. time in minutes between conditions for MCF-7 cell line.....25
Figure 10. OCR vs. time in minutes between conditions for MDA-MB-231 cell line.................................................................26
Figure 11. ECAR vs. time in minutes between conditions for C2C12 cell line.........29
Figure 12. ECAR vs. time in minutes between conditions for MCF-7 cell line.....30
Figure 13. ECAR vs. time in minutes between conditions for MDA-MB-231 cell line...31
Figure 14. Cell energy phenotype analysis of C2C12 cell line........................34
Figure 15. Cell energy phenotype analysis of MCF-7 cell line........................35
Figure 16. Cell energy phenotype analysis of MDA-MB-231 cell line............36
LIST OF ABBREVIATIONS

ATP  Adenosine triphosphate
ECAR  Extracellular acidification rate
ETC  Electron transport chain
FCCP  Trifluoromethoxy carbonylcyanide phenylhydrazone
FCS  Fetal calf serum
KLT  Kanglaite
KLTi  Kanglaite injection
NADH  Nicotinamide adenine dinucleotide
TCA  Tricarboxylic acid
TNBC  Triple-negative breast cancer
NSCLC  Non-small-cell lung carcinoma
OCR  Oxygen consumption rate
CHAPTER I

Introduction

Sarah A. Hillhouse

1.1 Breast Cancer Background

One in eight women living in the United States will develop breast cancer in their lifetime.\(^1\) Breast cancer is the most common cancer among women worldwide, with higher incidence rates among more developed countries.\(^2\) Many factors can contribute to a higher risk of breast cancer, including alcohol use, obesity, late-onset menopause, age, and genetics.\(^3\) Breast cancer occurs when cells in the breast tissue proliferate uncontrollably, eventually forming large masses called tumors.\(^1\) Although early screening methods have improved over the past few decades, breast cancer prevalence has remained relatively stable since the initial increase seen when early screening was first developed.\(^1\)

The most effective early screening method is mammography, which is a procedure using X-ray photographs of the breasts to check for any abnormalities before it is possible to detect them in a clinical examination.\(^2,5\) This early detection is especially helpful for diagnosing breast cancer at early stages that have a higher survival rate and are, therefore, easier to treat.\(^2\) For countries with less developed health care and limited resources, other screening options include clinical breast examination for
lumps and the use of ultrasound; however, these screening options are not as effective as mammograms.\textsuperscript{2}

Treatment of breast cancer varies widely depending on the stage and localization of the cancer in the body. Often, women will have a mastectomy, which is a surgical removal of the breast, along with radiation therapy and a combination of drugs if the cancer is localized to the breast tissue.\textsuperscript{4} However, a more advanced and metastasized breast cancer often results in the affected women exploring other treatment options, such as chemotherapy with a single drug treatment plan, hormone therapy, immunotherapy, or targeted therapies.\textsuperscript{4} These therapies target different mechanisms by utilizing various drugs, such as estrogen hormone receptor blockers and monoclonal antibodies. Despite the wide array of therapies and treatment drugs, breast cancer is still highly prevalent world-wide with more than 3.1 million women with breast cancer in the United States as of January 2019 and an expectation of 41,760 deaths within the year.\textsuperscript{6} Therefore, new therapies and non-traditional treatments need to be explored in the future.

1.2 Metastasis

Ten hallmarks of cancer, represented in Figure 1, include uncontrollable cell proliferation, increased signaling for cell proliferation, formation of new capillary networks, cell resistance to apoptosis, cells evading tumor suppressors, avoidance of immune destruction, tumor-promoting inflammation, genome instability, deregulating cellular energetics, and activation of metastasis.\textsuperscript{7}
Metastasis is the spread and progressive growth of cells from a primary neoplasm to distant organs. This spread of cancer to distant organs is responsible for at least 90% of cancer-associated deaths and is a highly feared aspect of cancer. Many metastatic cancers frequently spread to the specific organs. For instance, metastatic breast cancer, also called systemic or stage IV breast cancer, most frequently metastasizes to the lungs, bones, liver, and brain. This specificity in the metastasis of cancer to distant organs shows that metastasis is not a random process. In 1889, Stephen Paget proposed the “seed and soil” hypothesis stating that metastasis depends...
on the interaction between cancer tumor cells, or the seeds, and the specific organ microenvironments, or the soil. In other words, breast cancer may not metastasize to particular organs because they lack the microenvironment needed for the cells to survive and spread, but it may metastasize to other organs containing a preferred microenvironment. This hypothesis still remains today and acts as a foundation for further studies on cancer metastasis.

Metastasis involves a multi-step process: invasion of the cancerous cell into surrounding tissue, intravasation (invasion into vasculature), survival in vasculature and translocation to distant tissues, extravasation (escape from vasculature to distant tissue), survival in the microenvironment of distant tissue, and adaptation to the new microenvironment that facilitates proliferation and colonization into a secondary neoplasm. In order for the cancer cells to metastasize, all conditions for each step must be met; otherwise, the cancer will not spread. While many steps are required for a cancer cell to metastasize, the treatment of a metastatic cancer is very difficult to achieve because of the invasive nature of metastasis, as well as the various mechanisms behind it.

Despite improvements in screening and advances in treatment therapies and surgeries, most cancer mortalities result from the invasive and progressive growth of metastases that are resistant to conventional therapies. For instance, metastatic cancer stem cells have been shown to have a heightened resistance to drug-induced death. Therefore, new treatment options and therapies need to be further explored for the treatment of metastatic cancers.
Figure 2. Sequential steps in the pathogenesis of cancer metastasis. Each step is regulated by modifications to cancer cell nucleic acids or proteins. Only cancer cells that fully accomplish each step (seen in number 1) will be competent metastatic cells. Metastasis can be blocked at any one of these sequential steps (as seen in 2-7). Reprinted from Nature Reviews Cancer, 3(6), Fidler, Isaiah J., The pathogenesis of cancer metastasis: the ‘seed and soil’ hypothesis revisited, page 456, Copyright (2002), with permission from Springer Nature.\(^9\)

1.3 Tumor Metabolism

Cancer cells in tumors have altered metabolic pathways that facilitate energy production and supply the cell with precursors for the formation of macromolecules in order to continuously grow and survive; therefore, one strategy for developing new treatment therapies for cancers is to research the mechanisms for these altered pathways and find ways to target them.\(^12\) One major pathway that is primarily studied is the Warburg effect, which is illustrated in Figure 3. This pathway enhances the
activity of glycolysis, in order to make energy in the form of ATP. Consequently, oxidative phosphorylation is impaired, even when oxygen is present. Because glycolysis is enhanced, cancer cells tend to have an overproduction of lactate, which acidifies the tumor microenvironment and facilitates the production of the enzyme lactate dehydrogenase. This enzyme oxidizes NADH into NAD+, thereby maintaining glycolysis activity.

Figure 3. The Warburg effect in cancer. A normal cell’s metabolism utilizes oxidative phosphorylation for basic functioning in the presence of oxygen. However, a proliferating cancer cell’s metabolism shifts to using glycolysis for energy and the formation of macromolecules. Reprinted from International Journal of Biological Sciences, 11(12), Zhang W. et. al., Targeting Tumor Metabolism for Cancer Treatment: Is Pyruvate Dehydrogenase Kinases (PDKs) a Viable Anticancer Target, page 1390, Copyright (2019), with permission from Ivyspring International Publisher.

Glycolysis also may provide precursors for other biosynthetic pathways needed for cell growth by producing pyruvate, which is needed along with oxaloacetate to
activate the Krebs cycle. The Krebs cycle, also known as the TCA cycle, normally produces CO$_2$, ridding the cell of carbon needed for macromolecules that could be used to develop cell growth. However, this cycle is weakened in cancer cells as intermediates formed during the cycle are exported and utilized for biosynthetic pathways to form macromolecules, such as proteins and fatty acids. Therefore, less of the final product, oxaloacetate, is created, resulting in a decrease in the activation of the Krebs cycle. As a result, more pyruvate is utilized to form lactate mentioned earlier instead of being incorporated into the Krebs cycle.

Another altered metabolic pathway that is a consequence of the Warburg effect is the Crabtree effect. This pathway involves the inhibition of oxidative phosphorylation and cellular respiration in cancer cells by the presence of glucose from increased glycolysis. This decrease in oxidative phosphorylation and oxygen consumption occurs because of the decrease in the use of the Krebs cycle to form the final product of carbon dioxide. Better understanding of these alternate metabolic pathways in cancer cells can lead to new discoveries and new targets for treatment therapies.

1.4 Kanglaite (KLT)

As the need for alternative medicines became more prevalent, a Chinese pharmacologist named Li Dapeng developed the drug Kanglaite, also known as KLT, from the seeds of a coix plant \([Coix lacryma-jobi L. var. mayuen (Roman.) Stapf.]\). Coix seeds had been previously used in traditional Chinese medicine for a number of functions including the stimulation of the function of the spleen and lung and the treatment of symptoms for diarrhea and arthritis. The KLT product is a new
anticancer drug first approved by the Chinese government in 1995 that can be administered intravenously, intra-arterially, or orally.\textsuperscript{16} The injected drug, known as KLTi, has been administered to more than 650,000 cancer patients in China.\textsuperscript{17}

The KLTi product has been proven to have a marked inhibitory effect on malignant tumors.\textsuperscript{16} Studies suggest that the KLTi mechanism of action for this marked inhibition is as follows: inhibit tumor cells mitosis in G2/M phase, inducing tumor cells apoptosis, altering genetic expression of tumor cells by up-regulating FAS/Apo-1 gene expression and down-regulating Bc1-2 gene expression, inhibition of new vasculature formation, counteracting cancer cachexia, and reversing tumor cell resistance to anticancer chemotherapeutics.\textsuperscript{16} Clinical studies of KLT have been conducted in China, Russia, and a current phase II clinical trial underway in the United States.\textsuperscript{17} Table 1 shows a few of clinical studies on KLT that verify an increase in therapeutic response of chemotherapy drugs when combined with KLTi, as well as an increase in the quality of life in the patients.

<table>
<thead>
<tr>
<th>Clinical Trial</th>
<th>Target</th>
<th>Dosage of KLTi</th>
<th>Findings</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I (USA)</td>
<td>Patients with solid tumors given KLTi to determine safety of the drug.</td>
<td>10, 20, 30, 40, and 50 grams (or 100-500 mL dosages)</td>
<td>KLTi reported to be safe and well tolerated. Maximum tolerated dose not reached.</td>
<td>16, 17</td>
</tr>
<tr>
<td>Phase I (Russia)</td>
<td>Patients with NSCLC given KLTi for determination of safety and effects of the drug in humans.</td>
<td>200 mL in a 90 minute intravenous infusion daily for days 1-21 and 29-49.</td>
<td>KLTi has good therapeutic effects and improves patient’s quality of life. Absence of toxicity for cytotoxic agents.</td>
<td>16, 17</td>
</tr>
<tr>
<td>Phase I (Russia)</td>
<td>Patients with lung cancer given KLTi to determine effect on immune function.</td>
<td>N/A</td>
<td>KLTi increased antigens CD95 and CD50, improving response of chemotherapy in patients.</td>
<td>16, 17</td>
</tr>
<tr>
<td>------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----</td>
<td>--------------------------------------------------------------------------------</td>
<td>-------</td>
</tr>
<tr>
<td>Phase II (USA)</td>
<td>To assess safety and efficacy of KLTi+gemcitabine combined therapy in patients with pancreatic cancer.</td>
<td>30 g/day (300 mL/day) and 50 g/day (500 mL/day) via a central venous line on days 1-5, 8-12, and 15-19 for each 28 day cycle.</td>
<td>Combined therapy had enhanced survival rates. Combined therapy with KLTi 30 g/day showed better results for survival and evidence for anti-neoplastic activity than the 50g/day dose of KLTi.</td>
<td>18</td>
</tr>
<tr>
<td>Phase II (China)</td>
<td>Efficacy of KLTi vs. chemotherapy in patients with primary lung carcinoma.</td>
<td>N/A</td>
<td>KLTi and chemotherapy drug had no significant difference in effective rate. KLTi had superior therapeutic results than chemotherapy. No adverse reactions in liver, kidney, or heart functions.</td>
<td>16</td>
</tr>
<tr>
<td>Phase II (China)</td>
<td>Efficacy of KLTi vs. chemotherapy in patients with NSCLC.</td>
<td>N/A</td>
<td>KLTi had significant difference in effective rate of 45% while chemotherapy had 22% effective rate.</td>
<td>16</td>
</tr>
<tr>
<td>Phase II (China)</td>
<td>Efficacy of KLTi vs. chemotherapy in patients with primary liver carcinoma.</td>
<td>N/A</td>
<td>KLTi and chemotherapy drug had no significant difference in effective rate. KLTi improves quality of life, symptoms, and immune functions. No adverse effects on bone marrow, hepatic, or renal functions.</td>
<td>16</td>
</tr>
<tr>
<td>-----------------</td>
<td>--------------------------------------------------------------------------</td>
<td>-----</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>Phase III (China)</td>
<td>Combined KLTi treatment with intervention therapy (IT) for patients with primary liver carcinoma and primary lung carcinoma.</td>
<td>N/A</td>
<td>Combined therapy (KLT+IT) was significantly more effective than IT alone.</td>
<td>16</td>
</tr>
<tr>
<td>Phase III (China)</td>
<td>Combined KLTi with radiotherapy vs. radiotherapy only treatments for carcinomas of lung, esophagus, nasopharynx, etc.</td>
<td>Combined: KLT intravenously injected 100 mL with 200cGy radiotherapy 5 days/week. Radiotherapy only: some radio-dose.</td>
<td>Combined therapy was significantly more effective than radiotherapy alone.</td>
<td>16</td>
</tr>
<tr>
<td>Phase III (China)</td>
<td>To observe efficacy, side effects, and impact on quality of life by KLTi combined with chemotherapy in the treatment of gastric cancer patients.</td>
<td>100 mL intravenously/day of KLTi in combined therapy group only</td>
<td>Combined therapy had significantly higher response rate but not a significantly different clinical benefit rate. KLTi enhanced efficacy, reduced side effects of</td>
<td>19</td>
</tr>
</tbody>
</table>
Table 1. Clinical trials for the study of safety and efficacy of KLTi in humans.

Overall, KLTi has increased therapeutic effects and quality of life in patients in these clinical trials. No maximum tolerated dosage (MTD) of KLTi in humans has been established.
CHAPTER II

Effects of Kanglaite, an Anticancer Herbal Medicine, on Breast Cancer Cell Metabolism

Yu-Dong Zhou, Sarah A. Hillhouse, and Dale G. Nagle

*SAH contributed to analysis of Seahorse XFe96 data

2.1 Introduction

Kanglaite (KLT), a Chinese herbal medicine, has been one of the top five anticancer drugs in China for the past decade. The KLT product is developed from coix seed oil into a micro-emulsion drug for intravenous use. This intravenous KLT drug (KLTi), is made up of 0.1 g coix seed oil per mL that consists of 20.8% 1,2,3-trioleylglycerol (C_{57}H_{104}O_{6}), 19.2% 2,3-dioleoyl-1-linoleylglycerol (C_{57}H_{102}O_{6}), 18.9% 1,2-dilinoleyl-3-oleylglycerol (C_{57}H_{100}O_{6}), 14.8% 2-linoleyl-3-oleyl-1-palmitoylglycerol (C_{55}H_{100}O_{6}), 11.8% 2,3-dioleoyl-1-palmitoylglycerol (C_{55}H_{102}O_{6}), 7.5% 1,2-dilinoleyl-3-palmitoylglycerol (C_{55}H_{98}O_{6}) and 7.0% 1,2,3-trilinoleylglycerol (C_{57}H_{98}O_{6}). Over the past twenty years, this drug consisting mainly of fatty acid triglycerides, has been successfully used by over one million patients in multiple countries and has a proven effect in the treatment of many cancers.
Various studies have shown that KLTi remarkably improves immune function, improves cachexia (a wasting syndrome) by providing high energy nutrients, increases efficacy and reduces toxicity of chemotherapy or radiation therapy treatment, improves quality of life, and improves survival in cancer patients.\textsuperscript{17} Based on studies in China, multiple mechanisms for the effect of KLT on cancer cells have been proposed. Some of these mechanisms include the inhibition of angiogenesis, the induction of cancer cell apoptosis, the inhibition of tumor cell mitosis, regulation of FAS/Apo-1 and Bcl-2 gene expression, and reversing cachexia by providing high energy nutrients.\textsuperscript{17} For our study, we are mostly concerned with the effect KLT on tumor cell metabolism in breast cancer cell lines. In the US, a pre-clinical study at Johns Hopkins University showed that KLT inhibited the growth of tumors from xenografted MDA-MB-231 breast cancer cells by over 50\%.\textsuperscript{17} We propose that one significant mechanism for this reduction in tumor growth is caused by the introduction of fatty triglycerides from KLT, resulting in an altered tumor cell metabolism.

In this study, Dr. Yu-Dong Zhou utilized the Seahorse XFe96 Analyzer to perform a cell energy phenotype assay measuring the OCR and ECAR simultaneously in living cells with control conditions ($n=3$), cells with 1 $\mu$M of rotenone ($n=2$), and cells with three different concentrations of KLT in both regular fetal calf serum (FCS)-enriched and charcoal-stripped FCS-enriched media. This allowed us to study the cell metabolism because their cellular oxygen consumption rate (OCR) is an indicator for oxidative phosphorylation, while their extracellular acidification rate (ECAR) is an indicator for glycolysis.\textsuperscript{21} A normal myogenic cell line C2C12 acted as a model control system, while a human breast adenocarcinoma cell line MCF-7 and a triple-negative
breast cancer (TNBC) cell line MDA-MB-231 acted as model breast cancer systems.\textsuperscript{22,23} The Seahorse Analyzer measured the OCR and ECAR both before and after the addition of a stressor containing Oligomycin+FCCP. Oligomycin and FCCP are compounds that disrupt oxidative phosphorylation in cells by inhibiting ATP synthase or by acting as a protonophoric uncoupler that dissipates the mitochondrial proton gradient, respectively.\textsuperscript{21} The combination of these compounds acts as a stressor on the cells to induce a demand for energy, increasing OCR and ECAR\textsuperscript{21}.

Rotenone (Figure 4) is a compound that inhibits the transfer of electrons from mitochondrial complex I to ubiquinone in the ETC, thus reducing the NAD oxidation and inhibiting cellular oxidative phosphorylation.\textsuperscript{24} Therefore, the rotenone condition ($n=2$) established a reduced cellular respiration both before and after the addition of the stressor compared to the normal cellular respiration seen in controls ($n=3$).

![Figure 4. Chemical Structure of Rotenone.](image)
By observing the OCR and ECAR rates in each set of conditions for each cell line, we expected to observe a stronger increase in OCR and ECAR for cells with KLT after the introduction of the stressor compounds compared to cells acting as controls or cells containing rotenone. This would support our hypothesis that adding fat in the form of KLT will change cancer cell metabolism and increase cellular respiration via oxidative phosphorylation.

2.2 Materials and Methods

2.2.1 Cell Lines and Cell Culture

Normal mouse myogenic C2C12 cells were purchased from ATCC and used as a model control cell line. Human breast cancer MCF-7 and MDA-MB-231 cells were purchased from ATCC and used as model breast cancer cell lines. Cells were cultured in DMEM/high glucose medium with 4 mM L-glutamine supplemented with 10% FCS [Hyclone, Cat# SH30910.03] with P/S [50 µg/mL penicillin, 50 µg streptomycin, Hyclone, Cat# GIBCO15140] or in DMEM/high glucose medium with 4 mM L-glutamine supplemented with 10% charcoal-stripped FCS [Hyclone, Cat# SH30068.02] with P/S [50 µg/mL penicillin, 50 µg streptomycin, Hyclone, Cat# GIBCO15140].

2.2.2 Agilent Seahorse XF Cell Energy Phenotype Test Assay

The C2C12 and MDA-MB-231 cells were seeded at the density of 2000 cells/well and MCF-7 cells were seeded at the density of 4000 cells/well into 96-well microplates in a volume of 80 µL/well of either DMEM/high glucose medium with 4 mM L-glutamine supplemented with 10% FCS [Hyclone, Cat# SH30910.03] with P/S [50 µg/mL penicillin, 50 µg/mL streptomycin, Hyclone, Cat# GIBCO15140] or in DMEM/high-
glucose medium with 4 mM L-glutamine supplemented with 10% charcoal-stripped FCS [HyClone, Cat# SH3006802] with P/S [50 µg/mL penicillin, 50 µg/mL streptomycin, HyClone, Cat# GIBCO15140]. Select cells in each cell line were exposed to 1 µM of rotenone \( (n=2) \) or KLT at 0.3%, 1%, and 1.72% concentrations. Then, the 96-well microplates were incubated in a humidified environment with 5% CO\(_2\) and 95% air at 37°C for 24 hours. A sensor cartridge was hydrated in double-distilled H\(_2\)O at 37°C in a non-CO\(_2\) incubator overnight prior to the day of the assay.

On the day of the assay, the 96-well microplates with the cells were washed with XF Base Medium [1 mM pyruvate, 2 mM glutamine, 10 mM glucose] to have a total well volume of 180 µL. The cells were then incubated at 37°C in a non-CO\(_2\) incubator for one hour prior to the assay. The sensor cartridge was taken out of the water bath and placed in a utility plate containing 200 µL of the pre-warmed XF Calibrant. This was then incubated at 37°C in a non-CO\(_2\) incubator for one hour prior to assay.

After an hour, the sensor plate was removed from the calibration plate and 20 µL of the stressor containing oligomycin and FCCP was injected into the port A of the sensory plate. The sensor plate was then loaded into the Seahorse XFe96 Analyzer along with the 96-well microplate containing the cells. The Seahorse XFe96 Analyzer was then used to calibrate the OCR and ECAR of the cells simultaneously before and after the stressor was added to the cells [1 µM Oligomycin, 1 µM FCCP].

2.2.3 Statistical Analysis

Standard deviations were determined using Excel for all control conditions and all rotenone treatment conditions for each cell line in the baseline comparison of OCR and ECAR.
2.3 Results

Control 1 was excluded from all data for the C2C12 cell line in charcoal-stripped FCS-enriched media because the OCR values were significantly higher than the OCR values in Controls 2 and 3. Control 2 was excluded from all data for the MCF-7 cell line in both regular and charcoal-stripped FCS-enriched media because no effect on OCR or ECAR was recorded after the addition of the Oligomycin+FCCP stress treatment, indicating the stressors were not mixed with the cells very well. All other conditions in the experiment were included in the following data analysis.

2.3.1 Baseline Comparison

Baseline measurements, obtained from the Seahorse XFe96 Analyzer, of OCR and ECAR for control (n=3), 1 µM of rotenone (n=2), 0.3% KLT concentration, 1% KLT concentration, and 1.72% KLT concentration conditions were compared in each cell line for both regular and charcoal-stripped FCS-enriched media before the addition of mitochondrial stress-inducing compounds, Oligomycin+FCCP. All baseline values were obtained from the third baseline measurement taken 13 minutes after the start of the cell energy phenotype assay. This is the last baseline measurement taken before the addition of stressor compounds, Oligomycin+FCCP, at 20 minutes. Standard deviations for the mean control condition and the mean rotenone condition for each serum in all cell lines are represented as error bars.
Figure 5. Baseline comparison of OCR and ECAR between conditions for C2C12 cell line. A) Mean baseline OCR levels (pmol/min) for C2C12 cells with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. B) Mean baseline OCR levels (pmol/min) for C2C12 cells with control ($n=2$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media. C) Mean baseline ECAR levels (mpH/min) for C2C12 cells with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. D) Mean baseline ECAR levels (mpH/min) for C2C12 cells with control ($n=2$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media.
Figure 6. Baseline comparison of OCR and ECAR between conditions for MCF-7 cell line. A) Mean baseline OCR levels (pmol/min) for MCF-7 cells with control (n=2), rotenone (n=2), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. B) Mean baseline OCR levels (pmol/min) for MCF-7 cells with control (n=2), rotenone (n=2), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media. C) Mean baseline ECAR levels (mpH/min) for MCF-7 cells with control (n=2), rotenone (n=2), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. D) Mean baseline ECAR levels (mpH/min) for MCF-7 cells with control (n=2), rotenone (n=2), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media.
Figure 7. Baseline comparison of OCR and ECAR between conditions for MDA-MB-231 cell line. A) Mean baseline OCR levels (pmol/min) for MDA-MB-231 cells with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. B) Mean baseline OCR levels (pmol/min) for MDA-MB-231 with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media. C) Mean baseline ECAR levels (mpH/min) for MDA-MB-231 cells with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in regular FCS-enriched media. D) Mean baseline ECAR levels (mpH/min) for MDA-MB-231 cells with control ($n=3$), rotenone ($n=2$), 0.3% KLT, 1% KLT, and 1.72% KLT in charcoal-stripped FCS-enriched media.
In Figure 5A, the mean OCR baseline for C2C12 cells with control conditions \( (n=3) \) was similar to that of the 1% and 1.72% KLT concentration baseline OCRs, all of them being between 350 and 400 pmols/min. However, the 0.3% KLT concentration baseline OCR was fairly low at about 248 pmols/min along with the OCR baseline of the average rotenone conditions \( (n=2) \) at 134 pmols/min. The OCR also noticeably increased in cells with KLT as the concentration of KLT increased, indicating higher amounts of fat increases cellular respiration. In Figure 5B showing the OCR values in charcoal-stripped FCS-enriched media, the baseline OCR values change drastically from those in the regular FCS-enriched media for cells with 0.3% KLT with a new increased baseline OCR of 388 pmol/min and for the mean control \( (n=2) \) with a new decreased baseline OCR value of 183 pmol/min. Because the basal OCR values increased for cells with KLT but decreased for control cells in charcoal-stripped FCS-enriched media compared to cells in regular FCS-enriched media, this indicates that the presence of fat in the cell causes increased cellular respiration. The baseline ECAR values for C2C12 cells was represented in Figures 5C and 5D, showing similar findings to OCR between conditions in each serum. In other words, the condition with the highest OCR baseline value had the highest ECAR baseline value. Cells with rotenone \( (n=2) \) had slightly increased ECAR but was still the lowest compared to the other conditions. These results indicate that the C2C12 cell line had relatively balanced cell energy pathways by utilizing both cellular respiration and glycolysis for functioning.

In Figure 6A, the highest baseline OCR value for MCF-7 cells in regular FCS-enriched media was the OCR for cells with 1.72% KLT; however, there was no pattern of increase in OCR as KLT concentration was increased. Cells with rotenone \( (n=2) \) had the
lowest OCR as expected. In Figure 6B with MCF-7 cells in charcoal-stripped FCS-enriched media, all conditions had lower OCR levels than in cells within the regular FCS-enriched media in Figure 6A. The highest baseline OCR values in Figure 6B consisted of cells containing KLT with the highest being cells with 0.3% KLT. This indicates that cells with fat had higher levels of cellular respiration than in control cells (n=2). ECAR values represented in Figure 6C and Figure 6D show relatively high mean ECAR values for cells with rotenone (n=2) in both serums, indicating increased glycolysis within those cells. Cells with KLT for all concentrations had slightly increased, if not similar, ECAR values compared to the mean control ECAR (n=2) in both serums. Therefore, the addition of fat in MCF-7 cells may also increase glycolysis as well.

Comparison of Figure 7A and Figure 7B shows similar results for the MDA-MB-231 cell line with decreases in baseline OCR values for all conditions in the charcoal-stripped FCS-enriched media compared to cells in regular FCS-enriched media with the cells containing some concentration of KLT having the highest OCR in both serums. Also, a pattern of increase in OCR as KLT concentration increased was observed in cells in charcoal-stripped FCS-enriched media (Figure 7B). This indicates that fat may increase cellular respiration within MDA-MB-231 cells. Figure 7C shows that cells with rotenone (n=2) in regular FCS-enriched media had the lowest ECAR and control cells (n=3) had the highest ECAR levels. Because the control cells had the highest rate of glycolysis here, this indicates that cells containing some concentration of KLT may have caused a very small decrease in glycolysis within the cancer cell. In Figure 7D, the ECAR decreased as the concentration of KLT increased within cells in charcoal-stripped FCS-enriched media.
2.3.2 OCR Between Conditions Over Time

Measurements of OCR in pmol/min obtained from the Seahorse XFe96 Analyzer were plotted over time in minutes for control ($n=3$), 1µM of rotenone ($n=2$), 0.3% KLT concentration, 1% KLT concentration, and 1.72% KLT concentration conditions in each cell line for both regular and charcoal-stripped FCS-enriched media. Baseline values of OCR can be seen from 0 to 20 minutes. At 20 minutes, the stressor compounds, Oligomycin+FCCP, were added to each well and mixed with the cells. Because these stressor compounds inhibit cellular respiration, a subsequent increase in OCR should be observed following 20 minutes as the need for cellular energy is induced. 21
Figure 8. OCR vs. time in minutes between conditions for C2C12 cell line. A) OCR vs. time in minutes from C2C12 cells in regular FCS-enriched media. B) OCR vs. time in minutes from C2C12 cells in charcoal-stripped FCS-enriched media.
Figure 9. OCR vs. time in minutes between conditions for MCF-7 cell line. A) OCR vs. time in minutes from MCF-7 cells in regular FCS-enriched media. B) OCR vs. time in minutes from MCF-7 cells in charcoal-stripped FCS-enriched media.
Figure 10. OCR vs. time in minutes between conditions for MDA-MB-231 cell line. 
A) OCR vs. time in minutes from MDA-MB-231 cells in regular FCS-enriched media. B) OCR vs. time in minutes from MDA-MB-231 cells in charcoal-stripped FCS-enriched media.

In all the figures above, rotenone had the lowest OCR values and showed no or a minimal increase in OCR after the stressor was introduced. In Figure 8A, the OCR
decreased at 20 minutes and slowly began to increase in the time following for Controls 1 and 3, as well as cells with KLT concentrations of 1% and 1.72%, meaning that cellular respiration was induced in these conditions. However, KLT concentrations of 0.3% and Control 2 only showed a small increase in OCR after the addition of the stressor. Figure 8B shows how KLT at all concentrations has a higher OCR both before and after the addition of the stressor compared to control conditions in the charcoal-stripped FCS-enriched media. Therefore, one could possibly infer that cells with KLT had fat that could act as an energy source to be utilized in cellular respiration unlike the control cells stripped of fat in Figure 8B.

Figure 9A shows a marked decrease in OCR in the MCF-7 cell line after the addition of the stressor compounds and a slight increase thereafter in all conditions except rotenone. This indicates that cellular respiration was induced in cells acting as a control (n=2) and cells with KLT at all concentrations. However, the MCF-7 cells with the highest OCR both before and after the addition of the stressor were cells with 1.72% KLT. Figure 9B shows similar effects seen in Figure 9A; however, the baseline OCR for all conditions is lower in Figure 9B with charcoal-stripped FCS-enriched media compared to that in Figure 9A with regular FCS-enriched media. This supports the findings for the possible effect of fat as an energy source for altered cell metabolism and increased cellular respiration.

Figure 10A shows similar effects of the stressor compounds on the OCR of MDA-MB-231 cells as seen in MCF-7 cells. The OCR in control cells (n=3) and cells with KLT at all concentrations slightly decreased until the addition of the stressor induced an increase in OCR after 20 minutes. In Figure 10B, cells with KLT at all conditions...
concentrations had a higher OCR compared to those with control conditions in the charcoal-stripped FCS-enriched media, further supporting the hypothesis that fat increases cellular respiration and alters cancer cell metabolism.

2.3.3 ECAR Between Conditions Over Time

Measurements of ECAR in mpH/min obtained from the Seahorse XFe96 Analyzer were plotted over time in minutes for control (n=3), 1µM of rotenone (n=2), 0.3% KLT concentration, 1% KLT concentration, and 1.72% KLT concentration conditions in each cell line for both regular and charcoal-stripped FCS-enriched media. Baseline values of ECAR can be seen from 0 to 20 minutes. At 20 minutes, the stressor compounds, Oligomycin+FCCP, were added to each well and mixed with the cells. Because these stressor compounds inhibit cellular respiration and induce a need for energy, an increase in ECAR should be observed following 20 minutes.21
Figure 11. ECAR vs. time in minutes between conditions for C2C12 cell line. A) ECAR vs. time in minutes from C2C12 cells in regular FCS-enriched media. B) ECAR vs. time in minutes from C2C12 cells in charcoal-stripped FCS-enriched media.
Figure 12. ECAR vs. time in minutes between conditions for MCF-7 cell line. A) ECAR vs. time in minutes from MCF-7 cells in regular FCS-enriched media. B) ECAR vs. time in minutes from MCF-7 cells in charcoal-stripped FCS-enriched media.
Figure 13. ECAR vs. time in minutes between conditions for MDA-MB-231 cell line.

A) ECAR vs. time in minutes from MDA-MB-231 cells in regular FCS-enriched media.

B) ECAR vs. time in minutes from MDA-MB-231 cells in charcoal-stripped FCS-enriched media.
In Figure 11A and Figure 11B, the ECAR for C2C12 cells in regular FCS-enriched media markedly increased at 20 minutes for all conditions, rotenone having a minimal increase, as the stressor compounds were mixed with the wells. However, the ECAR values slowly began to decrease in the time following for all conditions. This indicates that glycolysis was induced immediately after the stressor but began to decline as time progressed. In Figure 11A, controls 2 and 3 in the regular FCS-enriched media had the highest ECAR values. However, in Figure 11B, C2C12 cells with all KLT concentrations in the charcoal-stripped FCS-enriched media had the highest ECAR values immediately before and after the addition of the stressors compared to control cells (n=2) and cells with rotenone (n=2). Because cells containing KLT had the highest ECAR in charcoal-stripped FCS-enriched media and not in regular FCS-enriched media, it can be suggested that fat provides a source of energy for glycolysis, thereby increasing ECAR compared to cells without fat.

Figure 12A and Figure 12B show a marked increase in ECAR in the MCF-7 cell line after the addition of the stressor compounds and a slight increase thereafter in all conditions except rotenone, which had an ECAR that increased after the stressor but stabilized soon after. This marked increase in ECAR indicates that glycolysis was induced by the addition of the stressor compounds in all conditions in both regular and charcoal-stripped FCS-enriched media. In Figure 12A, all cells with KLT had higher ECAR values than control cells (n=2). However, the baseline ECAR in Figure 12B for cells with 1.72% KLT was less than the ECAR for Control 1 after the stressor was introduced than in the charcoal-stripped FCS-enriched media. These results suggest that
introducing a small amount of fat to MCF-7 cells may increase glycolysis and a large amount of fat may decrease glycolysis.

Figure 13A and Figure 13B show a steady increase of ECAR in MDA-MB-231 cells for all conditions except rotenone from baseline to the final measurement of the cell phenotype assay. The ECAR in rotenone for both figure remained relatively steady both before and after the addition of the stressors. Control 2 had the highest ECAR values in both figures; however, cells with 0.3% KLT increased to match that of Control 2 by the last measurement in the charcoal-stripped FCS-enriched media seen in Figure 13B. This suggests that fat introduced to cells in low amounts may be able to act as a source of energy to fuel glycolysis.

2.3.4 Cell Energy Phenotype Analysis

Baseline and stressed OCR in pmol/min and ECAR in mpH/min for all conditions in both regular and charcoal-stripped FCS-enriched media were obtained from the Seahorse XFe96 Analyzer after performing a cell energy phenotype assay. These baseline and stressed values taken immediately before and after the addition of the stressors are presented below in cell energy phenotype graphs for all three cell lines. The cell energy phenotype assay uses the Seahorse XFe96 Analyzer to simultaneously measure OCR and ECAR in order to determine the energy phenotype of a cell.\textsuperscript{21} There are four different cell energy phenotypes including, quiescent, aerobic, glycolytic, and energetic. A quiescent phenotype is characteristic of a cell that is not growing because it does not use either oxidative phosphorylation or glycolysis very well.\textsuperscript{21} An aerobic phenotype is characteristic of a normal cell that is using both metabolic pathways but relies mainly on
oxidative phosphorylation rather than glycolysis for energy.\textsuperscript{21} A glycolytic phenotype is characteristic of cancer cells, which rely on glycolysis for energy.\textsuperscript{13,21} Lastly, an energetic phenotype indicates that the cell is utilizing both oxidative phosphorylation and glycolysis to gain energy.\textsuperscript{21}

**Figure 14. Cell energy phenotype analysis of C2C12 cell line.** A) Cell energy phenotype analysis of C2C12 in regular FCS-enriched media. B) Cell energy phenotype analysis of C2C12 in charcoal-stripped FCS-enriched media. Baseline values are represented by open markers. Stressed values are represented by closed markers.
Figure 15. Cell energy phenotype analysis of MCF-7 cell line. A) Cell energy phenotype analysis of MCF-7 in regular FCS-enriched media. B) Cell energy phenotype analysis of MCF-7 in charcoal-stripped FCS-enriched media. Baseline values are represented by open markers. Stressed values are represented by closed markers.
Figure 16. Cell energy phenotype analysis of MDA-MB-231 cell line. A) Cell energy phenotype analysis of MDA-MB-231 in regular FCS-enriched media. B) Cell energy phenotype analysis of MDA-MB-231 in charcoal-stripped FCS-enriched media. Baseline values are represented by open markers. Stressed values are represented by closed markers.
In Figure 14A, controls 1 and 3, cells with rotenone (n=2), and cells with 1% KLT and 1.72% KLT had an aerobic phenotype at baseline but moved towards a glycolytic phenotype when under stress. Control 2 and cells with 0.3% KLT had less of an aerobic phenotype at baseline compared to controls 1 and 3, but they had a more energetic phenotype when under stress. In Figure 14B, all conditions moved towards a more glycolytic phenotype when under stress; however, all cells with KLT had the least glycolytic phenotype with a more aerobic or energetic phenotype than control cells (n=2) and cells with rotenone (n=2).

In Figure 15A, all conditions had a more glycolytic phenotype when under stress than at baseline. However, cells with 1.71% KLT had the highest OCR and ECAR of all the other conditions, leading to less of a glycolytic phenotype. Therefore, cells with 1.72% KLT had a higher energetic phenotype compared to controls (n=2) and cells with rotenone (n=2). In Figure 15B, cells with rotenone (n=2) had a more glycolytic phenotype when under stress than at baseline. However, the controls (n=2) and cells with KLT for all concentrations had an energetic phenotype when under stress with similar OCR and ECAR values. Because these cells had similar energetic phenotypes, no significant effect of KLT on the MCF-7 cells in charcoal-stripped FCS-enriched media can be made.

In Figure 16A, MDA-MB-231 cells with rotenone (n=2) had a more glycolytic phenotype, shown in rotenone 1 condition, and a more quiescent phenotype, shown in rotenone 2 condition, when under stress than at baseline. Control 1, Control 3, and all cells with KLT slightly had a more glycolytic phenotype when under stress than at baseline. Control 2 was the only condition with an energetic phenotype when stressed.
Cells with 1% KLT had a slightly less glycolytic phenotype and a more energetic phenotype compared to all other conditions. In Figure 16B, MDA-MB-231 cells with rotenone \((n=2)\) had a more glycolytic phenotype, shown in rotenone 1 condition, and a very slight increase in an energetic phenotype, shown in rotenone 2 condition, when under stress than at baseline. All controls \((n=3)\) and all cells with KLT had a more energetic phenotype when under stress than at baseline. Cells with KLT at any concentration analyzed had the highest energetic phenotype of all conditions, except for Control 2. This is because Control 2 has a higher OCR rate while under stress than the other conditions for the measurement used to determine the stressed phenotype. However, Control 2 has a decrease in OCR immediately after this initial spike in cellular respiration caused by the addition of the stressor, as can be seen in Figure 10B. Therefore, KLT has a more energetic phenotype than controls \((n=3)\) in MDA-MB-231 cells.

2.4 Conclusion

Comparison of baseline OCR and ECAR measurements in each cell line and in each serum showed the effects of KLT on cell metabolism without a stressor in place. In both FCS-enriched media and charcoal-stripped FCS-enriched media, the C2C12 cell line showed results that indicate increased cellular respiration and glycolysis occurred as fat concentration in the form of KLT increased. The breast cancer MCF-7 cell line showed results that indicate increased cellular respiration and glycolysis occurred for cells with 1.72% KLT compared to controls \((n=2)\) in regular FCS-enriched media, while increased cellular respiration and glycolysis occurred for cells with 0.3% KLT compared to
controls \((n=2)\) in charcoal-stripped FCS-enriched media. The breast cancer MDA-MB-231 cell line showed results that indicate increased cellular respiration for all cells containing KLT in both serums and a decrease in glycolysis for cells containing KLT at all concentrations in regular FCS-enriched media and at higher concentrations in charcoal-stripped FCS-enriched media.

Analyzing the OCR and ECAR over time in each cell line and in each serum showed the effects of KLT on cell metabolism after a stressor was introduced with OCR representing cellular respiration and ECAR representing glycolysis.\(^ {21} \) The C2C12 cell line showed no major or outlying effect of KLT on cellular respiration compared to controls \((n=3)\) in the regular FCS-enriched media but cells with KLT at all concentrations in charcoal-stripped FCS-enriched media had increased cellular respiration compared to controls \((n=2)\). The C2C12 cell line also showed similar results for ECAR with increased glycolysis in cells containing KLT compared to controls \((n=2)\) only apparent cells in the charcoal-stripped FCS-enriched media. The MCF-7 cell line showed increased cellular respiration in cells with 1.72% KLT only compared to controls \((n=2)\) in regular FCS-enriched media and no apparent difference in cellular respiration for cells with KLT compared to controls \((n=2)\) in charcoal-stripped FCS-enriched media. The MCF-7 cell line also showed increased glycolysis for all cells with KLT compared to controls \((n=2)\) in regular FCS-enriched media and no apparent difference in glycolysis for cells with KLT compared to controls \((n=2)\) in charcoal-stripped FCS-enriched media. The MDA-MB-231 cell line showed increased cellular respiration for cells with 1\% or 1.72\% KLT compared to controls \((n=3)\) in regular FCS-enriched media and increased cellular respiration for cells with 0.3\% and 1\% KLT compared to controls \((n=3)\) in charcoal-
stripped FCS-enriched media. The MDA-MB-231 cell line also showed a decrease in glycolysis for cells with 0.3% KLT and 1.72% KLT compared to controls (n=3) in regular FCS-enriched media and no apparent difference in the rate of glycolysis for cells with KLT when compared to all controls (n=3) in charcoal-stripped FCS-enriched media.

The cell energy phenotype assay showed the energy phenotypes for each condition within each cell line for both serums by comparing the OCR and ECAR both immediately before and after the stressor was introduced. The C2C12 cell line showed similar energy phenotypes for both cells with KLT and controls (n=3) in regular FCS-enriched media, but the energy phenotype for all cells with KLT in the charcoal-stripped FCS-enriched media was less glycolytic than the controls (n=2). The MCF-7 cell line showed a slightly less glycolytic phenotype for cells with 1.72% KLT compared to controls (n=2) in regular FCS-enriched media, while both controls (n=2) and all cells with KLT have similar energy phenotypes in charcoal-stripped FCS-enriched media. The MDA-MB-231 cell line showed very little differences in energy phenotypes between cells with KLT and controls (n=3) in regular FCS-enriched media; however, cells with 1% KLT had a slightly less glycolytic phenotype compared to controls (n=3) in regular FCS-enriched media. In charcoal-stripped FCS-enriched media, the MDA-MB-231 cells showed an energetic phenotype for both cells with KLT and controls (n=3) with cells with KLT having higher phenotypes than Controls 1 and 3.

2.5 Discussion

Based on the data from this experiment, KLT tends to increase cellular respiration and glycolysis within cells. The normal myogenic C2C12 cell line acting as a control cell
line had a stronger balance between oxidative phosphorylation and glycolysis than the two breast cancer cell lines. KLT in C2C12 cells had no significant effect on cellular respiration or glycolysis when fat was already present in the serum. Cells with KLT in charcoal-stripped FCS-enriched media resulted in increases in both metabolic pathways for this cell line. This indicates that KLT will only benefit normal cells if they are deprived of fat. The breast cancer cell lines should have altered metabolism resulting in decreased oxidative phosphorylation, and therefore cellular respiration, and increased glycolysis under normal conditions.\textsuperscript{13} This increase in glycolysis and subsequent decrease in cellular respiration is an adaptation cancer cells evolved to obtain enough energy for cell proliferation, in addition to cell functioning.\textsuperscript{14} We proposed that addition of fat to breast cancer cells may provide a source of energy for cancer cells to utilize cellular respiration once more. The data presented for the MCF-7 breast cancer cell line showed almost no significant effects of KLT on breast cancer; however, MCF-7 cells with 1.72% KLT in regular FCS-enriched media increased cellular respiration and glycolysis compared to the controls ($n=2$). The majority of the data for the MDA-MB-231 breast cancer cell line showed increases in cellular respiration and decreases in glycolysis with KLT. Because KLT was shown to increase cellular respiration in both breast cancer cell lines, it can be inferred that fat from KLT altered the cell metabolism by providing another source of energy. Further study on the effects of KLT and fat on cancer cell metabolism needs to be explored with a focus on the different cell energy phenotypes between cancer cell lines.
List of References

   Atlanta: American Cancer Society, Inc.


