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Studijní podpora, předmět Precision medicine
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The extracellular lactate production

Theoretical part:

Glucose metabolism and the Warburg effect

Glucose serves a source for both catabolic and anabolic processes and is the main nutrient for cells, besides glutamine. Normal differentiated cells metabolize primarily glucose through mitochondrial oxidative phosphorylation up to carbon dioxide. **Oxidative phosphorylation is very effective process in which the cell can obtain up to 36 molecules of ATP per one molecule of glucose.** On the other hand, when oxygen is absent, e.g., in the muscles during exercise, glucose is converted to lactate in anaerobic glycolysis. Unlike normal differentiated cells, proliferative tissue, as well as cancer cells, prefer glucose conversion to lactate regardless of the presence of oxygen. This process is called aerobic glycolysis or the Warburg effect, since Otto Warburg first observed that cancer cells metabolize glucose in a distinct manner from normal differentiated cells. Originally, Warburg hypothesized that cancer cells have defective mitochondria. However, further studies revealed that most cancer cells do not show impaired mitochondrial function. The persuasive reason why cancer cells switch to a less efficient metabolism is still missing. The amount of ATP that is generated in the course of the **aerobic glycolysis** is low, **only four molecules of ATP** are generated per one molecule of glucose, but **the speed of reaction is much faster**. Therefore, the switch to aerobic glycolysis enables the cancer cell to overcome the rapidly reached saturation of ATP production when resources are abundant or when oxygen supply is limited and to compete efficiently with the other nearby fast-growing cancer cells.

The German physiologist Otto Heinrich Warburg was awarded the Nobel Prize in Physiology or Medicine in 1931 for the above-described discovery.

The principle of lactate bioluminescent assay

The Lactate-Glo assay couples lactate oxidation and NADH production with a bioluminescent NADH detection system. Lactate dehydrogenase uses lactate and NAD^+ to produce pyruvate and NADH. In

the presence of NADH a pro-luciferin reductase substrate is converted by reductase to luciferin, which is then used in a luciferin reaction to produce light. The Lactate-Glo assay contains an L-lactate selective dehydrogenase to confer specificity for L-lactate, the major stereoisomer found in mammalian cells.

Practical part:

A) Mammalian culture cells seeding

When working with mammalian culture cells, be careful as much as possible, since the cells and their medium are very sensitive to contaminations – wear gloves and a clean lab coat, do not touch nonsterile surfaces. Keep in mind that gloves and a lab coat are not sterile; therefore, spray your gloves with 70% ethanol just before starting work in the laminar flow hood and keep sterile surfaces clean!

1) Spray bottles containing culture medium, PBS and trypsin solution with 70% ethanol and place them in the biohazard laminar flow hood. Before work, all solutions must be placed for ~20 min in a water bath, which is set on 37°C.

2) The assistant provides you with two 10-cm plates – the first one with ovarian cancer cells (OV) and the second one with primary cells derived from retinal pigment epithelium (RPE). Both cell cultures are grown as a monolayer in a humidified incubator at 37°C, in 5% CO_2. Place the plate in the laminar flow hood.

3) Aspirate medium from the plate and wash the cells with **4 mL of PBS**.

4) Aspirate PBS and add **1 mL of trypsin solution** on the cells dropwise.

5) Place the plate for **5 min** in the humidified incubator.

6) Take the plate back to the laminar flow hood, add **4 mL of culture medium** and pipette the trypsinized cells up and down in order to tear the cells off from the plate and have a solution of single cells.

7) Pipette the cells out of the plate into the 50 mL tube.

8) **Cell counting:** Mix **10 µL of trypan blue** with **10 µL of cell suspension** in a 1.5 mL tube. Load 10 µL of the mixture onto a counting slide and insert it into the Biorad Cell Counter to obtain estimates of cell count in your cell suspension.

9) Dilute both cell lines with medium of two glucose concentrations (1 and 4.5 g/L) to the final concentration of **10 000 cells/100 µl** and pipette 100 µL of the cells into two wells in a 96-well plate (eight wells in total – four wells for lactate measurements and four wells for cell counting). **Add 2 µL of each medium into 98 µL of PBS** (two samples, which serve as a background control, since the medium contain serum with lactate) and keep them in the freezer until you measure lactate concentrations.

B) Sample collection and cell counting

The following day, count the seeded cells in order to interpret data properly and use these cell numbers for data normalization.

1) Count the numbers of cells that are present in four wells that represent the two cell lines grown in two conditions each. Carefully remove medium by pipetting, then add **25 µL of trypsin solution** and place the plate for **5 min** in the humidified incubator. Mix **10 µL of trypan blue** with **10 µL of cell suspension** in a 1.5 mL tube. Load 10 µL of the mixture onto a slide and insert it into the Biorad Cell Counter to obtain estimates of cell count in your cell suspension.

2) Sample collection – collect the samples of medium from another four wells **in the morning, in the afternoon and the third day in the morning** (prepare three samples of medium for each cell line and one condition, so **twelve samples in total**). Mix 2 µL of medium with 98 µL of PBS, label the samples and store the samples in the freezer until you measure lactate concentrations.

C) Lactate measurements

Let thaw the frozen samples on ice. Meanwhile, prepare the calibration curve for lactate.

1) The assistant provides you with an aliquot of **10 mM lactate solution**, from which you must prepare **200 µM lactate solution**, which is further diluted as specified below. Prepare solutions for the calibration curve as follows and count the final lactate concentrations:

Final lactate concentration (µM)					
200 µM lactate solution (µL)	1	5	25	50	100
PBS (µL)	99	95	75	50	0

2) Pipette **50 µL of prepared lactate solutions** with **50 µL of Lactate Detection Reagent** provided by the assistant into a white 96-well plate suitable for luminescent measurements.

3) Briefly shake the plate and incubate for **60 min at RT**.

4) Measure luminescence and prepare the calibration curve according to the assistant's instructions.

5) Measure your collected samples including the background samples – mix **50 µL of each sample** with **50 µL of Lactate Detection Reagent**, briefly shake and incubate for **60 min at RT**.

6) Calculate the lactate concentrations based on the calibration curve, subtract the respective background concentrations and normalize the data according to the cell numbers.