RAW 264.7 CELLS AN ANTI-INFLAMMATORY MODEL: OPTIMIZATION OF CELL CULTURE CONDITIONS AND LPS CONCENTRATIONS

RAW 264.7 BUŇKY JAKO PROTIZÁNĚTLIVÝ MODEL: OPTIMALIZACE PODMÍNEK BUŇEČNÉ KULTURY A LPS KONCENTRACÍ

(Diplomová práce)

Ve spolupráci s Univesidade do Porto.

Vedoucí diplomové práce: Prof. Dr. Carmen Diniz,
Prof. Dr. Paula Fresco,
Prof. Mudr. Petr Višňovský, CSc.

Porto, Hradec králové 2007

Vendula Popešková
1. ACKNOWLEDGEMENTS

I would like to express my gratefulness and sincere thanks to:

Prof.ª Dr.ª Carmen Diniz,
Prof.ª Dr.ª Paula Fresco
and PhD student Ana Sofia Soares

for their valuable consultations and kind and friendly leading. Thanks a lot for all advices and help. I am further very obliged to all people from Department of Pharmacology.

My thanks belong Prof. MUDR. PETR VIŠNOVSKÝ, CSc., for his assistance.

This work became possible because of a financial support of the EU programme Socrates/Erasmus.
2. THE INTRODUCTION

The need to control inflammation is obvious because prolonged or inappropriate inflammatory responses contribute to the pathogenesis of many major diseases associated with inflammation. To harness the body's own natural ability to down regulate inflammation, there is a need to identify endogenous anti-inflammatory pathways. It is important to identify the molecules that can not only block inflammation but that also play a part in the physiological "sensory" mechanism that recognises when an inflammatory response is damaging rather than beneficial. [24] Adenosine has been described as playing a role in the control of inflammation, but it has not been certain which of its receptors mediate this effect. [27]

One potential candidate among many cyclic AMP elevating Gs protein receptors which may function as the sensory reporter of tissue damage is the A2A adenosine receptor coupled. It has been known for some time that inflammatory tissue damage is accompanied by the accumulation of extracellular adenosine in inflamed areas owing to its release into the extracellular space by a variety of cells; this is a consequence of local tissue hypoxia caused by inflammatory stimuli. Moreover, it is now well established that activation of A2A receptors on lymphoid cells induced by extracellular adenosine leads to inhibition of an inflammatory response, and this is due in large degree to its ability to induce accumulation of intracellular cAMP in activated immune cells. More recently, the use of A2AR deficient mice has illustrated that the A2AR is a critical inflammatory "OFF" button, which is necessary for the inhibition of inflammation and protection from tissue damage. A2ARs are promising targets in manipulating the inflammatory response. [24]

Based on pharmacologic studies, the G protein–coupled adenosine receptors were initially classified into adenylyl cyclase inhibitory (A1 and A3) and stimulatory (A2) categories. Further classification of A2 adenosine receptors (A2ARs) into subtypes 2A and 2B was determined by the presence of high-affinity A2AAR and low-affinity A2BAR binding sites. [27]

Adenosine is the preferred endogenous agonist at all adenosine receptors, but inosine can also activate the A3 receptors. The levels of adenosine seen under basal conditions are sufficient to cause some activation of all the receptors, even during ischemia. Under normal conditions, adenosine is continuously formed intracellularly as well as extracellularly. These receptors are integral membrane proteins with seven transmembrane domains made of hydrophobic amino acids units, each believed to constitute an α-helix of approximately 21 to
28 amino acids. The N-terminal of the protein lies on the extracellular side and the C-terminal on the cytoplasmatic side of the membrane. [20]

![Diagram of G Protein-coupled receptors](image)

**Figure 1:** Mechanism of G Protein coupling. Receptors coupled to heterotrimeric GTP-binding proteins (G proteins) are integral transmembrane proteins that transduce extracellular signals to the cell interior. G protein-coupled receptors exhibit a common structural motif consisting of seven membrane spanning regions. Receptor occupation promotes interaction between the receptor and the G protein on the interior surface of the membrane. This induces an exchange of GDP for GTP on the G protein α subunit and dissociation of the α subunit from the βγ heterodimer. Depending on its isoform, the GTP-α subunit complex mediates intracellular signaling either indirectly by acting on effector molecules such as adenylyl cyclase (AC) or phospholipase C (PLC), or directly by regulating ion channel or kinase function. [17]

It has been well established that pharmacological activation of A2A Rs on lymphoid cells stimulates an anti-inflammatory response. Investigations into the role of extracellular adenosine have shown that this physiologically abundant molecule triggers strong immunosuppressive responses in T cells, mediated by cAMP, by acting through the Gs protein coupled A2A adenosine receptors. In addition, the inhibitory effects of adenosine on the
secretion of proinflammatory cytokines by a variety of other cell types, most notably monocytes and macrophages, have been extensively documented.

The signal transduction pathways triggered by activation of A2A receptors are still not completely understood. A2A receptors are generally accepted to couple to the Gs-adenylate cyclase (AC)-protein kinase A (PKA) pathway, but may also couple to pathways involving G-proteins other than Gs (Golf, G\(\alpha_{516}\), Gi\(\alpha\)) or even to AC-cyclic adenosine-3',5'-monophosphate (cAMP)-PKA independent signal transduction pathways. [6]

This work aimed to optimize cell culture conditions and lipopolysaccharide (LPS) concentrations. As a cell culture has been using RAW 264.7 cell line (mouse monocyte macrophages). For obtaining suitable cell culture conditions were realized growth curve of RAW 264.7 cells.

LPS (endotoxin) triggers many biological responses such as fever, septic shock, and even death. Murine and human macrophages exhibit a particularly vigorous response to LPS, which induces a variety of inflammatory modulators such as nitric oxide (NO), interleukin-1b (IL-1b), TNF-a, IL-6, and prostaglandins. Among these, NO is a cytotoxic mediator and contributes to the anti-microbial, anti-tumor activity of these cells. [29]

NO is a free radical with multiple effects on various organ systems. The most prominent physiological actions of NO as a biological mediator include cGMP-dependent vasodilation, neural communication, and host defense. NO is produced in physiological and pathophysiological conditions by NO synthase (NOS). While eNOS and nNOS are constitutively expressed and regulated by Ca2+/calmodulin, iNOS is induced by inflammatory cytokines and/or bacterial lipopolysaccharide (LPS) in various cell types including macrophages. A large amount of NO, particularly synthesized by iNOS, induces an inflammatory response to inhibit the growth of invading microorganisms and tumor cells. This strong inflammatory response to foreign cells could also cause further damage for the neighboring cells and tissues of the host. Therefore, isozyme specific inhibitors of NOS are essential for therapeutic purposes and drugs that specifically inhibit iNOS could be useful in treating diseases mediated by NO overproduction.[29]
3. **THE THEORETICAL PART**

3.1. **Cell culture**

Cell culture has become one of the major tools used in life sciences today. Cell culture is the process by which either prokaryotic or eukaryotic cells are grown under controlled conditions. In practice the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a routine laboratory technique in the 1950s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century. [4]

New legislation enacted in many countries and regions of the world requires that laboratory animal use be reduced, refined and replaced wherever possible, for ethical and scientific reasons. There is a tendency to replace laboratory animals by alternative methods. [12]

Tissue Culture is the general term for the removal of cells, tissues, or organs from an animal or plant and their subsequent placement into an artificial environment conducive to growth. This environment usually consists of a suitable glass or plastic culture vessel containing a liquid or semisolid medium that supplies the nutrients essentials for survival and growth. The culture of whole organs or intact organ fragments with the intent of studying their continued function or development is called Organ Culture. When the cells are removed from the organ fragments prior to, or during cultivation, thus disrupting their normal relationships with neighboring cells, it is called Cell Culture. [3]

3.1.1. **The history**

The 19th-century English physiologist Sydney Ringer developed salt solutions containing the chlorides of sodium, potassium, calcium and magnesium suitable for maintaining the beating of an isolated animal heart outside of the body. In 1885 Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale University, published results of his experiments from 1907-1910, establishing the methodology of tissue culture. [8]
Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The Salk polio vaccine was one of the first products mass-produced using cell culture techniques. This vaccine was made possible by the cell culture research of John Franklin Enders, Thomas Huckle Weller, and Frederick Chapman Robbins, who were awarded a Nobel Prize for their discovery of a method of growing the virus in monkey kidney cell cultures. [21]

### 3.1.2. Primary and secondary cell cultures

There are two basic types of Cell Culture, primary and secondary cell culture (or cell line). Primary culture, cells are freshly isolated from a piece of tissue and they are placed in a suitable culture environment. Secondary culture is formed of cells taken from primary cell culture. Cells from secondary culture are immortal and have the ability to grow and divide indefinitely. The most normal cells can not grow indefinitely, but stop growing and die after 50 – 100 populations doublings. [21]

Primary cell culture is obtained by two basic methods – Explant Cultures or Enzymatic Dissociation. Small pieces of tissue are attached to a glass or treated plastic culture vessel and bathed in culture medium. After a few days, individual cells move from the tissue explant out into the culture vessel surface or substrate where they begin to divide and grow. This method is called Explant Cultures. The second, more widely used method, Enzymatic Dissociation, speeds up this process by adding digesting (proteolytic) enzymes, such as trypsin or collagenase, to the tissue fragments to dissolve the cement holding the cells together. This creates a suspension of single cells that are then placed into culture vessels containing culture medium and allowed to grow and divide. [3]

### 3.1.3. Cell culture systems

Two basic culture systems are used for growing cells. These are based primarily upon the ability of the cells to either grow attached to a glass or treated plastic substrate (Monolayer Culture Systems) or floating free in the culture medium (Suspension Culture Systems). Many cell lines, especially those derived from normal tissues, are considered to be anchorage-dependent, that is, they can only grow when attached to a suitable substrate. Some cell lines that are no longer considered normal (frequently designated as Transformed Cells) are frequently able to grow either attached to a substrate or floating free in suspension; they are
anchorage-independent. In addition, some normal cells, such as those found in the blood, do not normally attach to substrates and always grow in suspension. [21]

Some cell lines will eventually stop dividing and show signs of aging. These lines are called Finite. Other lines are, or become immortal; these can continue to divide indefinitely and are called Continuous cell lines. When a “normal” finite cell line becomes immortal, it has undergone a fundamental irreversible change or “transformation”. This can occur spontaneously or be brought about intentionally using drugs, radiation or viruses. Transformed cells are usually easier and faster growing, may often have extra or abnormal chromosomes and frequently can be grown in suspension. [4]

3.1.4. Morphology of culture cells

Culture cells are usually described based on their morphology (shape and appearance) or their functional characteristics. There are three basic morphologies:

1. Epithelial-like: cells that are attached to a substrate and appear flattened and polygonal in shape.

2. Lymphoblast-like: cells that do not attach normally to a substrate but remain in suspension with a spherical shape.

3. Fibroblast-like: cells that are attached to a substrate and appear elongated and bipolar, frequently forming swirls in heavy cultures.

It is important that the culture conditions play an important role in determining shape and that many cell cultures are capable of exhibiting multiple morphologies. It is also possible to obtain hybrid cells (called Hybridomas) by fusing related cells from two different parents.[21]
3.1.5. The subculturing of cells

Most animal cell lines and primary cultures grow as a single thickness cell layer or sheet attached to a plastic or glass substrate. When the cells in the culture vessel have grown and filled up all of the available culture substrate, they must be subcultured to give them room for continued growth. This is usually done by removing them as gently as possible from the substrate with enzymes. Thus, in order to keep the cells healthy and actively growing, it is necessary to subculture them at regular intervals. Usually, this subcultivation process involves breaking the bonds or cellular ‘glue’ that attaches the cells to the substrate and to each other by using proteolytic enzymes such as trypsin, dispase, or collagenase. Some cell lines can be harvested by gently scraping the cells off the bottom of the culture vessel. Once the available substrate surface is covered by cells (a confluent culture), growth slows and then ceases. Occasionally, these enzymes or dissociating agents are combined with divalent cation chelators such as EDTA (binds calcium and magnesium ions). The loosened cells are then removed from the culture vessel, counted, diluted and subdivided into new vessels. Cells then reattach, begin to grow and divide, and, after a suitable incubation period (depending on the initial inoculum size, growth conditions and cell line), again reach saturation or confluency. At this point, the subcultivation cycle can be repeated. Once a surplus of cells is available, they can be treated with suitable cryoprotective agents, such as dimethylsulfoxide (DMSO) or glycerol, carefully frozen and then stored at cryogenic temperatures (below -130°C) until they are needed. [26]

Counting the number of cells is a process used for determination of growth rates or setting up cultures at known concentrations. It is possible to count the cells using hemacytometer or electronic cell counting devices. The hemacytometer has the added advantages of both being less expensive and allowing cell viability determinations to be made during counting. [21]

A hemacytometer is a graduated counting chamber that can be viewed under a microscope to determine the concentration of cells in a suspension. Developed originally for counting blood cells, these chambers are widely used in cell culture to determine the concentration of cells in a suspension. There are a number of manufacturers of these types of chambers and some have slight variations in style. The description here is for a common “Neubauer” type chamber. The instrument is made of ground glass with a central area that is defined by a set of grooves that form an ‘H’ shape. Two counting areas with ruled grids are
separated by the horizontal groove of the H. The one of the ruled grids is shown and described here. The glass coverslip is held at 0.1 mm above the surface of the counting areas by ground glass ridges on either side of the vertical grooves of the H shape. In the example shown, the cells were resuspended in a total of 4 ml. The cell suspension was then diluted ½ with trypan blue and the chamber of the hemacytometer was loaded. [10]

Figure 1: Hemacytometer

Trypan Blue (TB) is a vital dye used to determine viable vs. non-viable cells. Determination of trypan blue viability is useful when seeding cells into culture that have just been thawed from cryopreservation or when performing subcultures. If only the total cell number is desired, as may be the case for the endpoint of an experiment. The reactivity of TB
is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is demaged. Therefore, all the cells which exclude the dye are viable. It is prepared a 1:2 dilution of the cell suspension in trypan blue. [5]

![Trypan Blue Structure](image)

**Figure 2:** Imagine of trypan blue

### 3.1.6. Contaminations

One of the most common problem when working with cells is cell contamination. Cell culture contamination can be chemical or biological. Chemical contamination is the most difficult to detect since it is caused by agents, such as endotoxins, plasticizers, metal ions or traces of chemical disinfectants, that are invisible. Biological contaminants form can be due to fast growing yeast, bacteria and fungi forms usually have visible effects on the culture (changes in medium turbidity or pH) and thus are easier to detect (especially if antibiotics are omitted from the culture medium). However, two other forms of biological contamination, mycoplasmas and viruses, are not easy to detect visually and usually require special detection methods. There are two major requirements to avoid contamination. First, good aseptic technique of the investigator. Second, properly designed, maintained and sterilized equipment, plasticware, glassware, media and all reagents. To avoid culture loss due to biological contamination is the careful and selective (limited) using of antibiotics. [28]

For maintaining aseptic conditions is used like a disinfectant solution 70% ethanol in the water, sterile gloves and laboratory coat for person who works with cells, and microbiological safety (the vertical hood). The exterior surface of each equipment or material before putting inside of the cabinet has to be washed by disinfectant solution. Its mode of activity is by dehydration and fixation. Ethanol is effective against bacteria, most viruses but not nonenveloped viruses. The vertical flow hood, also known as a biology safety cabinet, is best for working with hazardous organisms since the aerosols that are generated in the hood are filtered out before they are released into the surrounding environment. It has continuous displacement of air that passes through a HEPA (high efficiency particle) filter that removes
particulates from the air. In a vertical hood, the filtered air blows down from the top of the cabinet. The hoods are equipped with a short-wave UV light that can be turned on for a few minutes to sterilize the surfaces of the hood, but be aware that only exposed surfaces will be accessible to the UV light. The hoods should be turned on about 10-20 minutes before being used. Wipe down all surfaces with ethanol before and after each use. Keep the hood as free of clutter as possible because this will interfere with the laminar flow air pattern. [15]

3.1.6.1. Waste Disposal

Any employer has a ‘duty of care’ to dispose of all biological waste safely in accordance with national legislative requirements. Given below is a list of ways in which tissue culture waste can be decontaminated and disposed of safely. One of the most important aspects of the management of all laboratory-generated waste is to dispose of waste regularly and not to allow the amounts to build up. The best approach is ‘little and often’. Different forms of waste require different treatment.

- Tissue culture waste (culture medium) - Inactivate overnight in a solution of hypochlorite (10,000ppm) prior to disposal to drain with an excess of water
- Contaminated pipettes should be placed in hypochlorite solution (2500ppm) overnight before disposal by autoclaving and incineration.
- Solid waste such as flasks, centrifuge tubes, contaminated gloves, tissues etc. should be placed inside heavy duty sacks for contaminated waste and autoclaved prior to incineration.

If at all possible waste should be incinerated rather than autoclaved. [11]

3.1.7. Environmental requirements

Basic environmental requirements for „happy“ cells are controlled temperature (for mammalian cells 36 – 37°C), good substrate for cell attachment, appropriate medium and incubator that maintains the correct pH and osmolality.

3.1.7.1. The culture medium

The culture medium is the most important and complex factor to control in making cells “happy”. Besides meeting the basic nutritional requirement of the cells, the culture medium should also have any necessary growth factors, regulate the pH and osmolality, and provide essential gases (O₂ and CO₂). The ‘food’ portion of the culture medium consists of amino
acids, vitamins, minerals, and carbohydrates. These allow the cells to build new proteins and other components essential for growth and function as well as providing the energy necessary for metabolism. The growth factors and hormones help regulate and control the cells’ growth rate and functional characteristics. Instead of being added directly to the medium, they are often added in an undefined manner by adding 5 to 20% of various animal sera to the medium. Unfortunately, the types and concentration of these factors in serum vary considerably from batch to batch. This often results in problems controlling growth and function. When growing normal functional cells, sera are often replaced by specific growth factors. The medium also controls the pH range of the culture and buffers the cells from abrupt changes in pH. Usually a CO₂ bicarbonate based buffer or an organic buffer, such as HEPES, is used to help keep the medium pH in a range from 7.0 to 7.4 depending on the type of cell being cultured. When using a CO₂-bicarbonate buffer, it is necessary to regulate the amount of CO₂ dissolved in the medium. This is usually done using an incubator with CO₂ controls set to provide an atmosphere with between 2% and 10% CO₂ (for Earle’s salts-based buffers). Finally, the osmolality (osmotic pressure) of the culture medium is important since it helps regulate the flow of substances in and out of the cell. It is controlled by the addition or subtraction of salt in the culture medium. While Eagle’s original Minimal Essential Medium (E-MEM) is one of the most widely used media, it is often not the best for maximizing cell growth or production since it is a very simple “bare bones” formulation. For example it does not contain nonessential amino acids, such as praline which is required for growth of CHO cells. Richer media such as M199, F12, F12K, RPMI 1640, Dulbecco’s Modified Eagle’s Medium (D-MEM) or some of the fortified MEM formulations (i.e., Alpha MEM) will often work better and may require less serum supplementation. Simple side-by-side media and sera optimization experiments can be done with minimal effort and expense and may pay large benefits when it is time to scale-up. [3, 21, 7]

3.1.7.2. Vessels

Evaporation of culture media from open culture vessels (dishes, etc.) will rapidly increase the osmolality resulting in stressed, damaged or dead cells. To reduce contamination problems in CO₂ incubators the use of vented caps requiring gas exchange is highly recommended. [3]
Figure 3: Vented cap of flask. Green line means a flow of contaminants, Blue means a flow of Culture Medium and Red represents gas exchanging.

The characteristic of corning flasks where the cells grow:

Advantages

◦ Traditional and easy to use
◦ Good pipette access and easy to feed or harvest cells
◦ Cell growth can be quickly determined with a microscope
◦ Reduce media spills and culture contamination
◦ No additional equipment required

Disadvantages

◦ Labor intensive for large numbers
◦ Use more incubator space

Table 1: Basic characteristics of the corning flask. From a 100% confluent culture is a average yield of $1 \times 10^5$ cells/mL.

<table>
<thead>
<tr>
<th>flask</th>
<th>Average cell yields</th>
<th>Recommended medium volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>75 cm$^2$</td>
<td>$7.5 \times 10^6$</td>
<td>20 mL</td>
</tr>
</tbody>
</table>

The initial and most common suspects for cell culture problems are usually either the culture vessels or the medium being used. Virgin polystyrene, the resin used to manufacture most cell culture vessels, is hydrophobic in its untreated state. Protein attachment factors do not bind well to this natural surface resulting in poor cell adhesion and growth. This alteration of the plastic polymer (not a peelable coating) results in a hydrophilic surface with a net
negative charge that creates a surface suitable for cell attachment and growth. The culture vessels are then sterilized. If initial cell inoculum contains foam or bubbles, clear areas, often resembling single or small clusters of viral plaques, occur along the sides or in the middle of the vessels. Although they appear to float on top of the medium, bubbles also push below the surface, displacing the cell containing medium and reducing the cells available for attaching. Bubbles that occur during refeeding of cultures, but after cells have attached, may cut off the cells from the fresh medium. This will result in cell necrosis under those areas, if the bubbles last long enough. Bubbles can usually be avoided by careful attention to mixing and pipetting techniques. [7]

### 3.1.8. Characteristics of cells

„Happy“ cells are characterized by the morphology, the growth rate, the plating efficiency and the expression of specialized functions. The **Morphology** or cell shape is the easiest to determine but is often the least useful. While changes in morphology are frequently observed in cultures. The **Growth Rate** is sensitive to major changes in the culture environment. This allows the design of experiments to determine which set of conditions (culture media, substrate, serum, plasticware) is better for the cells, i.e., the conditions producing the best growth rate. **Plating Efficiency** is a testing method where small numbers of cells (20 to 200) are placed in a culture vessel and the number of colonies they form is measured. The percentage of cells forming colonies is a measure of survival, while the colony size is a measure of growth rate. The final characteristic, the **Expression of Specialized Functions**, is usually the most difficult to observe and measure. Usually biochemical or immunological assays and tests are used. While cultured cells may grow very well in suboptimal conditions, highly specialized functions usually require near perfect culture conditions and are often quickly lost when cells are placed in culture. [21]
3.1.9. **The application of cell lines**

Cell culture has become one of the major tools used in cell and molecular biology. Some of the important areas where cell culture is currently playing a major role like the model systems, in the toxicity testing, in the cancer research, virology, cell-based manufact, genetic engineering, gene therapy, drug screening and development.

**Model Systems**

Cell cultures provide a good model system for studying 1) basic cell biology and biochemistry, 2) the interactions between disease-causing agents and cells, 3) the effects of drugs on cells, 4) the process and triggers for aging, and 5) nutritional studies.

**Toxicity Testing**

Cultured cells are widely used alone or in conjunction with animal tests to study the effects of new drugs, cosmetics and chemicals on survival and growth in a wide variety of cell types. Especially important are liver- and kidney-derived cell cultures.

**Cancer Research**

Since both normal cells and cancer cells can be grown in culture, the basic differences between them can be closely studied. In addition, it is possible, by the use of chemicals, viruses and radiation, to convert normal cultured cells to cancer causing cells. Thus, the mechanisms that cause the change can be studied. cancer cells also serve as a test system to determine suitable drugs and methods for selectively destroying types of cancer.

**Virology**

One of the earliest and major uses of cell culture is the replication of viruses in cell cultures (in place of animals) for use in vaccine production. Cell cultures are also widely used in the clinical detection and isolation of viruses, as well as basic research into how they grow and infect organisms.

**Cell-Based Manufacturing**

While cultured cells can be used to produce many important products, three areas are generating the most interest. The first is the large-scale production of viruses for use in vaccine production. These include vaccines for polio, rabies, chicken pox, hepatitis B and measles. Second, is the large-scale production of cells that have been genetically engineered to produce proteins that have medicinal or commercial value. These include monoclonal antibodies,
insulin, hormones, etc. Third, is the use of cells as replacement tissues and organs. Artificial skin for use in treating burns and ulcers is the first commercially available product. However, testing is underway on artificial organs such as pancreas, liver and kidney. A potential supply of replacement cells and tissues may come out of work currently being done with both embryonic and adult stem cells. These are cells that have the potential to differentiate into a variety of different cell types. It is hoped that learning how to control the development of these cells may offer new treatment approaches for a wide variety of medical conditions.

Genetic Counseling

Amniocentesis, a diagnostic technique that enables doctors to remove and culture fetal cells from pregnant women, has given doctors an important tool for the early diagnosis of fetal disorders. These cells can then be examined for abnormalities in their chromosomes and genes using karyotyping, chromosome painting and other molecular techniques.

Genetic Engineering

The ability to transflect or reprogram cultured cells with new genetic material (DNA and genes) has provided a major tool to molecular biologists wishing to study the cellular effects of the expression of theses genes (new proteins). These techniques can also be used to produce these new proteins in large quantity in cultured cells for further study. Insect cells are widely used as miniature cells factories to express substantial quantities of proteins that they manufacture after being infected with genetically engineered baculoviruses.

Gene Therapy

The ability to genetically engineer cells has also led to their use for gene therapy. Cells can be removed from a patient lacking a functional gene and the missing or damaged gene can then be replaced. The cells can be grown for a while in culture and then replaced into the patient. An alternative approach is to place the missing gene into a viral vector and then “infect” the patient with the virus in the hope that the missing gene will then be expressed in the patient’s cells.

Drug Screening and Development

Cell-based assays have become increasingly important for the pharmaceutical industry, not just for cytotoxicity testing but also for high throughput screening of compounds that may have potential use as drugs. [3, 21]
3.1.10. Macrophage cell lines

The macrophage (MP) has become a major focus of many laboratories in the last decade. Its importance in immune regulation and possible role in tumor immunity has created a need for homogeneous cell populations and for large numbers of MPs. This has led to an interest in the development and use of macrophage cell lines to study problems in MP biology. Macrophage cell lines can be obtained in several ways. Some MP lines were derived from tumors or tumor-like cells. Macrophage hybridomas have been develop by fusing various kinds of immortalized cells with normal macrophages. Macrophage cell lines have also been developed by culturing bone marrow cells in the presence of macrophage colony-stimulating factor and maintaining the continuously dividing MPs. Once obtained, these MP lines have been or can be used to study macrophage differentiation, mediator production, accessory cell function, and phagocytosis. Furthermore, MP cell lines have been studied to determine their spontaneous and induced cytolytic activity, either by direct killing or via antibody-dependent cellular cytotoxicity (ADCC). The use of MP cell lines offers many advantages. One is the assurance that the population of cells is homogeneous. Even gradient-separated MP populations elicited in some manner in vivo contain contaminating cells that may affect results. The need to maintain a large mouse colony is unnecessary if one has a MP cell line that is functionally similar to in vivo-elicited MPs. This helps eliminate the worry about prevalent virus infections in the mouse colony, breeding, and cost incurred if purchasing mice. It is also of considerable importance that the development of in vitro systems to replace animal use is being encouraged to satisfy criticisms of politically powerful animal welfare groups. [22]

Cell lines could be found and bought in cell line banks and databases like for example: ECACC European Collection of Cell Cultures, ICLC Interlab Cell Line Collection, DSMZ German Collection of Microorganism and Cell Cultures, ATCC American Type Culture Collection, WFCC World of Culture Collections and JCRB Japanese Cancer Research Resources Bank.

There are many kinds of macrophage cell lines. To rapidly dividing murine macrophage cell lines belong RAW 264.7; P388D1 and J774.A1. P388D1 is a derivative of P388 selected for the high production of IL-1. The cells have a high effect or activity in antibody dependent cell mediated cytotoxicity. J774.A1 is derived from a tumour in a female BALB/c mouse. Cell line produces IL-5 and lysozyme. [9]
RAW 264.7 cell line (mouse monocyte macrophages) is a secondary cell culture, established from an ascites of a tumour induced in a male mouse by intraperitoneal injection of Abselon Leukaemia Virus (A-MuLV) and growing attached in a plastic flask. Cells will pinocytose neutral red and phagocytose zymosan. Cells capable of antibody dependent lysis of sheep erythrocytes and tumour targets. [11]

![Low Density](image1.png) ![High Density](image2.png)

**Figure 4:** Pictures of RAW 264,7 cell line. In the first picture there is a low density of cells and in the second there is a high density of the cells (confluent culture). The shape of the cells are spherical.

3.1.10.1. Lipopolysaccharide (LPS)-treated RAW 246,7 cell lines

Murine macrophages cells (RAW 264.7 cells) express adenosine receptors and play critical roles in the immune system and inflammatory response and, when activated, these cells secrete nitrogen intermediates and pro-inflammatory cytokines such as TNF-α, IL-1 or IL-6 and enzymes (COX-2 and iNOS) by activation of transcription factors. RAW 264.7 cells can be useful as a model for the study of the inflammatory process and therefore to test adenosine receptor ligands and evaluate their effects as pro- or anti-inflammatory drugs.

Stimulation of RAW 264,7 cells can be achieved using several agents such as LPS. Macrophages are extremely sensitive to LPS endotoxin from Gramnegative bacteria. LPS has a
major effects on macrophage phenotype and function, including adhesion. All solutions, buffers and media should be made with sterile, endotoxin-tested, destilled, deionized water.

Binding of LPS to the cell surface CD14 molecule triggers multiple intracellular biochemical cascades, including the phosphorylation of several proteins by either tyrosine or serine/threonine kinases. The protein tyrosine kinases that mediate LPS-initiated signal transduction are essential for the expression of LPS-induced macrophage functions. In addition to protein tyrosine kinases, exposure of macrophages to LPS activates protein kinases C (PKC), and experiments using various PKC inhibitors indicated that PKC activity is required for the expression of several macrophage functions, including TNF-α and IL-1 secretion, NO production, and tumoricidal activity. [25]

**Figure 5:** Activation of Protein Kinase C (PKC) via G-proteins. The activation of PKC is preceded by a number of steps, originating from the binding of an extracellular ligand that activates a G-protein on the cytosolic side of the plasma membrane. The G-protein, using GTP as an energy source, then activates PKC via the phosphatidylinositol bisphosphate (PIP2) intermediate, which is shown as the DAG/IP3 complex. [16]

Pro-inflammatory cytokines including TNF-α, interleukin (IL)-1, and IL-6 are generated in tissues infected by microbial pathogens as well as in tissues subjected to generalized trauma such as ischemia/reperfusion injury. Generation of inflammatory mediators serves a necessary function to facilitate wound healing, in part by recruiting various immune cell populations. Nevertheless, excessive or chronic inflammation can also lead to additional
tissue injury and is the target for therapy in a wide variety of diseases. In the context of microbial invasion and sepsis, pro-inflammatory cytokine expression is induced by engagement of Toll-like receptors (TLR) by bacterial components such as lipopolysaccharide (LPS). During generalized tissue injury, cytokine expression is induced by lipid mediators (e.g., leukotrienes, platelet-activating factor [PAF]), oxygen-derived free radicals, complement components, and possibly by activation of TLR or fMLP receptors activated by intracellular debris released from necrotic cells. [2]

3.1.10.2. Protein kinase C

PKC was first characterized as a Ca^{2+}-dependent and phospholipid-dependent protein serine/threonine kinase that requires diacylglycerol for activity. Subsequently, it has been established that PKC is not a single entity, but rather a family of closely related isoenzymes comprising at least 12 different members. Differences in their structure, requirement for activity, subcellular localization, and substrate specificity suggest that in a given cell, the various PKC isoenzymes may exert specific functions. Macrophages and monocytic cells express the Ca^{2+}-dependent isoenzymes, βI, and βII, the Ca^{2+}-independent isoenzymes δ and ε, and the atypical isoenzyme ξ. PKC-α activity regulates selective LPS-induced macrophage line RAW 264.7 functions involved in host defense and inflammation.[25]
3.2. THE GRIESS REACTION

Nitric oxide (NO) is an important physiological messenger and effector molecule in many biological systems, including immunological, neuronal and cardiovascular tissues. Due to its involvement in these diverse systems, interest in measuring NO in biological tissues and fluids remains strong. One means to investigate nitric oxide formation is to measure nitrite (NO$_2^-$), which is one of two primary, stable and nonvolatile breakdown products of NO. This assay relies on a diazotization reaction that was originally described by Griess in 1879.

Through the years, many modifications to the original reaction have been described. The Griess Reagent System is based on the chemical reaction shown in Figure 5, which uses sulphanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects NO$_2^-$ in a variety of biological and experimental liquid matrices such as plasma, serum, urine and tissue culture medium. [14]

![Chemical reactions involved in the measurement of NO$_2^-$ using the Griess Reagent System.](image)

The detection of azo compound is measured by spectrophotometers. One of the widely recommended microplate spectrophotometer is BioTek’s PowerWave™ XS spectrophotometer. It is designed for use in myriad applications, from direct quantitation of nucleic acids using automated pathlength correction to full-scale high throughput operations that demand speed, accuracy and reliability. With rugged hardware, proven optical performance and a very small footprint. Use PowerWave™ XS reader for applications requiring low UV measurements, such as direct DNA quantitation and purity testing, RNA
quantitation, as well as visible range measurements for basic ELISA’s, enzyme kinetics, colorimetric assays, and many other applications. [13]

Figure 7: BioTek’s PowerWave™ XS spectrophotometer

Adenosine and its receptor agonists enhance the production of nitric oxide (NO) in lipopolysaccharide (LPS)-treated RAW 264.7 cells. The enhancement of LPS-induced NO production by adenosine, as represented by the amount of its oxidation products, nitrite and nitrate, can be inhibited by adenosine uptake inhibitors, such as dipyridamole, NBTI and NBTG. These indicate that the uptake of adenosine by macrophages is a prerequisite for the enhancement of the effects observed. Adenosine uptake inhibitors have no effects on the enhancement activity of the adenosine receptor agonist. The enhancement effects of adenosine on NO production in macrophages could be mediated by the adenosine receptors as well as the downstream metabolites of adenosine. [1]

NO is an important molecule in many cells having different functions. NO is synthesized from molecular oxygen and one of the terminal guanino nitrogen atoms of L-arginine by nitric oxide synthase NOS with the contaminant production of citrulline. This family of enzymes are generally classified as constitutive, calcium dependent (neuronal NOS, nNOS, NOS1 and endothelial NOS, eNOS, NOS3) or inducible, calcium independent (inducible NOS, iNOS, NOS2). The constitutive isoforms release relatively low levels of NO, while iNOS is induced by inflammatory conditions and releases larger amounts of NO. [23]
Both eNOS and nNOS are constitutively expressed whereas the expression of iNOS requires cytokines or other stimuli. Upon exposure to inflammatory cytokines or LPS, iNOS is expressed in macrophages, hepatocytes, vascular endothelial and smooth muscle cells, chondrocytes, myocardium, and other tissue and cell types. Large amounts of NO formed by iNOS are responsible for the cytostatic and cytotoxic activity towards tumor cells and invasive microorganisms as well as pathogenesis of various immunologically-mediated disease. [1]

NOS require various co-factors to function, including calmodulin (CaM), FAD, FMN, and iron protoporphyrin IX (haem). Additionally, tetrahydrobiopterin (BH4) is required for NOS activity and is produced by the enzyme GTP-cyclohydrolase I (CHI). NO is able to inhibit protease release and chemokine (M Gilchrist, unpublished observations) and cytokine production by MC. NO can regulate autocrine apoptotic signals, and alter surface molecule expression, namely CD8. [23]

The diminished immune function associated with adenosine excess and dysfunction of adenosine receptors on lymphocytes from patients with systemic lupus erythematosus further support the hypothesis that adenosine is an important regulator of both immune and inflammatory responses. [1]

Cellular uptake of adenosine by macrophages was required for the enhancement effects of adenosine on LPS-induced NO release, the effects of the adenosine transport inhibitors, dipyridamole, NBTI and NBTG were studied.
3.3. **GROWTH CYCLE**

Generation of a growth curve can be useful in evaluating the growth characteristics of a cell line. From a growth curve, the lag time, population doubling time, and saturation density can be determined.

Clonal assay will not always detect insufficiencies in the amount of particular constituents. If the concentration of one or more amino acids is low, it may not affect clonal growth but could influence the maximum cell concentration attainable. A growth curve gives three parameters of measurement:

1. **the lag phase** before cell proliferation is initiated after subculture, indicating whether the cells are having to adapt to different condition

2. **the log phase** - the doubling time in the middle of the exponential growth, indicating the growth-promoting capacity

3. and **the plateau phase (stationary)** - the terminal cell density.

The log and plateau phases give vital information about the cell line, the population doubling time during log phase, the maximum cell density achieved in plateau (saturation density). Measurement of the population doubling time is used to quantify the response of the cells to different inhibitory or stimulatory culture conditions such as variations in nutrient concentration, hormonal effects, or toxic drugs. It is also a good monitor of the culture during serial passage and enables the calculation of the cell yields and the dilution factor required at subculture.

It must be emphasized that the population doubling time is an average figure and describes the net result of a wide range of division rates, including zero, within the culture.

Single time points are unsatisfactory for monitoring growth, without knowing shape of the growth curve. A reduced cell count after, 5 days could be caused by a reduced growth rate of some or all the cells, a longer lag period implying adaptation or cell loss (difficult to distinguish) or a reduction in saturation density. This is not to say that growth curves are of no value. They can be useful for a rapid screen, and once the response being monitored is fully characterized and the type of response predictable, e.g. an increased doubling time, then single time point observations may be sufficient. Growth curves are particularly useful for the
determination of saturation density. The preferred method for analyzing growth and survival at lower cell densities is by clonal growth analysis.

The preferred method for analyzing growth and survival at lower cell densities is by clonal growth analysis. This technique will reveal differences in growth rate within a population and will distinguish between alterations in growth rate (colony size) and survival (colony number). That cells may grow differently as isolated colonies at low cell densities. Fewer cells will survive even under ideal conditions, and all interaction, and all interaction is lost until the colony starts to form. Heterogeneity in clonal growth rates reflects differences in growth capacity between lineages within population, but these need not necessarily be expressed in an interaction monolayer at higher densities where cell communication is possible.

The population doubling time PDT derived from a growth curve should not be confused with the cell cycle or generation time. The PDT is an average figure for the population and subject to the reservations stated above. The cell cycle time or generation time is measured from one point in the cell cycle until the same point is reached again (see below) and refers only to the growing cells in the population, while PDT is influenced by nongrowing and dying cells.

A growth cycle is performed each time the culture is passaged and can be analyzed in more detail as described below.

The Lag Phase is the time following subculture and reseeding during which there is little evidence of an increase in cell number. It is a period of adaptation during which the cell replaces elements of the glyoxaldehyde lost during trypsinization, attaches to the substrate, and spreads out. During spreading the cytoskeleton reappears and its reappearance is probably an integral part of the spreading process. Enzymes, such as DNA polymerase, increase followed by synthesis of new DNA and structural proteins. Some specialized cell products may disappear and not reappear until cessation of the cell proliferation at high cell density.

The Log Phase is the period of exponential increase in cell number following the lag period and terminating one or two doublings after confluence is reached. The length of the log phase depends on the seeding density, the growth rate of the cells, and the density at which cell proliferation is inhibited by density. In the log phase the growth fraction is highly (usually 90–100%) and the culture is in its most reproducible form. It is the optimal time for sampling
since the population is and its most uniform and viability is high. The cells are, however, randomly distributed in the cell cycle and, for some purpose may need to be synchronized.

The Plateau Phase, toward the end of the log phase, the culture becomes confluent – i.e., all the available growth surface is occupied and all the cells are in contact with surrounding cells. Following confluence the growth rate of the culture is reduced, and in some cases, cell proliferation ceases almost completely after one or two further population doublings. At this stage, the culture enters the plateau (or stationary) phase, and the growth fraction falls to between 0 – 10%. The cells may become less motile; some fibroblasts become oriented with respect to one another, forming a typical parallel array of cells. „Ruffling“ of the plasma membrane is reduced, and the cell both occupies less surface area of substrate and presents less of its own surface to the medium. There may be a relative increase in the synthesis of specialized versus structural proteins and the constitution and charge of the cell surface may be changed.

The construction of a growth curve from cell counts performed at intervals after subculture enables the measurement of a number of parameters which should be found to be characteristics of the cell line under a given set of culture conditions. The first of these is the duration of the lag period obtained by extrapolation a line drawn through the points on the exponential phase until it intersects the seeding or inoculum concentration, and reading of the elapsed time since seeding equivalent to that intercept. The second is the doubling time, i.e., the time taken from the culture to increase two-fold in the middle of the exponential or log phase of growth. The last of the commonly derived measurements from the growth cycle is the plateau level or saturation density. The cell concentration depends on cell type and frequency of medium replenishment. It is difficult to measure accurately as a steady state is not achieved as easily as in the log phase. Ideally the culture should be perfused; but a reasonable compromise may be achieved by growing the cell on a restricted area. Medium limitation of growth is minimal, and the cell density exerts the major effect. Plateau represents a steady state where cell division is balanced by cell loss. [3, 4, 5]
4. MATERIALS AND METHODS

4.1. Materials

RAW 264,7 cell line was obtained from Sigma-Aldrich which has formed a working partnership with The European Collection of Cell Cultures (ECACC).

Reagents: Hanks’ balanced salt solution, Dulbecco’s Modified Eagle’s Medium, GlutaMax™ Gibco, Penicillin-Streptomycin Solution, Fetal Bovine Serum, lipopolysaccharide (LPS), destilled and deionized water, 70% ethanolum, hypochlorite, trypan blue, sulphanilamide, N-1-naphthylethylene diamine dihydrochloride (NED), phosphoric acid, dimethylsulfoxide (DMSO).

Materials: scrapers TPP 24cm, pipettes TPP 10 mL and 5 mL, Pasteur’s pipettes, centrifuge tubes 15mL and 50 mL, flasks 75 cm², tips MULTIGUARD (20µL, 250 µL, 500 µL, 1 mL), 6-well plates TPP, 24-well plates TPP, 96-well plates TPP, eppendorfs.

Equipments: biological safety cabinet BSC, vacuum suction bottle centrifuge EPPENDORF, microscope NIKON type 104, microplate spectrophotometer PowerWave™XS, incubator.

Table I: Components of Hanks’ balanced salt solution (BSS) – wash solution for cleaning cells

<table>
<thead>
<tr>
<th>components</th>
<th>[g/L]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCl</td>
<td>0.4</td>
</tr>
<tr>
<td>KH₂PO₄ (anhyd)</td>
<td>0.06</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>0.35</td>
</tr>
<tr>
<td>NaCl</td>
<td>8.0</td>
</tr>
<tr>
<td>Na₂HPO₃ (anhydrous)</td>
<td>0.04788</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>1.0</td>
</tr>
<tr>
<td>Phenol Red Na</td>
<td>0.011</td>
</tr>
<tr>
<td>components</td>
<td>[g/L]</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>inorganic salts</strong></td>
<td></td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0,269</td>
</tr>
<tr>
<td>Fe(NO₃)₃·9H₂O</td>
<td>0,0001</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>0,09767</td>
</tr>
<tr>
<td>KCl</td>
<td>0,4</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>3,7</td>
</tr>
<tr>
<td>NaCl</td>
<td>6,4</td>
</tr>
<tr>
<td>NaH₂PO₄</td>
<td>0,109</td>
</tr>
<tr>
<td><strong>amino acids</strong></td>
<td></td>
</tr>
<tr>
<td>L-Arginine·HCl</td>
<td>0,084</td>
</tr>
<tr>
<td>L-Cystine·2HCl</td>
<td>0,0626</td>
</tr>
<tr>
<td>Glycine</td>
<td>0,03</td>
</tr>
<tr>
<td>L-Histidine·HCl·H₂O</td>
<td>0,042</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>0,105</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0,105</td>
</tr>
<tr>
<td>L-Lysine·HCl</td>
<td>0,146</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0,030</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0,066</td>
</tr>
<tr>
<td>L-Serine</td>
<td>0,042</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>0,095</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0,016</td>
</tr>
<tr>
<td>L-Thyrosine·2Na·2H₂O</td>
<td>0,10379</td>
</tr>
<tr>
<td>L-Valine</td>
<td>0,094</td>
</tr>
<tr>
<td><strong>vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Choline Chloride</td>
<td>0,004</td>
</tr>
<tr>
<td>Folic Acid</td>
<td>0,004</td>
</tr>
<tr>
<td>myo-Inositol</td>
<td>0,0072</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>0,004</td>
</tr>
<tr>
<td>D-Pantothenic Acid·¹/₂Ca</td>
<td>0,004</td>
</tr>
<tr>
<td>Pyridoxine·HCl</td>
<td>0,004</td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0,0004</td>
</tr>
<tr>
<td>Thiamine·HCl</td>
<td>0,004</td>
</tr>
<tr>
<td><strong>other</strong></td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>4,5</td>
</tr>
<tr>
<td>Phenol Red·Na</td>
<td>0,015</td>
</tr>
<tr>
<td>Pyruvic Acid·Na</td>
<td>0,11</td>
</tr>
</tbody>
</table>

**GlutaMax™ Gibco (100 mL)** is a standard cell culture media that contain a stabilized form of L-Glutamine, the dipeptide L-alanyl-L-Glutamine, that prevents degradation and ammonia build-up even during long-term cultures. **Penicillin-Streptomycin Solution (100 mL)** consists of 10,000 units penicillin-G and 10 mg streptomycin per ml.
4.1.1. **Preparing of the culture medium**

*GlutaMax™ Gibco (100 mL)* were divided into 20 tubes (in each tube was 5 mL of *GlutaMax™ Gibco*). All tubes were frozen and stored in the freezer. The same process was done with *Penicillin-Streptomycin Solution*. *Fetal Bovine Serum* was the first inactivated at 56 °C for 30 minutes and were divided into tubes (in each tube was 50 mL of serum). All tubes were frozen and stored in the freezer. All tubes were labelled (name, date).

The Culture Medium was prepared by mixing of bottle of *DMEM-HG 5 mL of L-Glutamine*, 5 mL of *Penicillin-Streptomycin Solution* and 50 mL of *Fetal Bovine Serum*. Final volume of the medium was 500 mL. The culture medium was divided into ten 50 mL tubes for avoiding contamination and stored in the fridge. Whole process was done in the safety cabinet. *Fetal Bovine Serum*, *L-Glutamine* and *Penicillin-Streptomycin Solution* were thawed in a waterbath at 37 °C before mixing.
4.2. Methods

All works with cells were realized in a biology safety cabinet. For maintaining aseptic conditions was used 70% ethanol, sterile gloves and laboratory coat. The exterior surface of each equipment or material before putting inside of the cabinet had to be washed by 70% ethanol. Tissue culture waste was decontaminated and disposed of safely. It was sucked with a Pasteur's pipette by a vacuum suction bottle and then a solution of hypochlorite was sucked.

4.2.1. Incubation

Cell line was incubated in an incubator in flasks with area 75 cm$^2$ for maintaining and scaling up (for own experiments were cells incubated in different kind of plates). The incubator was permanently saturated of 95% of oxygen and 5% of carbon dioxide in 37°C.

4.2.2. Subculture of adherent cell lines

Subculture: Spliting at 70-80% confluency, approx. 1:2 to 1:8, seeding at 2-4x10$^4$ viable cells/cm$^2$ at 37°C and 5% CO$_2$. Remove cells mechanically. Cells are semiaherent and some become attached to culture surface and have a 'flattened' appearance. Cells should not be allowed to reach confluence as this can lead to loss of the flattened adherent cell characteristic.

It was important to examine the cell cultures daily and always prior to subcultivation (contamination, confluent cells). Using an light microscope (100 to 200x), quickly checked the general appearance of the culture and looked for signs of microbial contamination. Many cells rounded up during mitosis, forming very refractile (bright) spheres that might float free of the surface when the culture was disturbed. Dead cells often rounded up and became detached but were usually not bright or refractile.

If the signs of contamination was found. All the content of the flask was rejected. But if the cells were confluent and without contamination, cells were subcultured.

From time to time culture medium was removed and cells were washed with Hank’s Balanced Salt Solution (BBS) (half of the volume of culture medium – 10 mL). The fresh Dulbecco’s Modified Eagle’s Medium (D-MEM) was added and cells were mechanically deattached with the scraper. Then the cells were resuspended in the fresh culture medium, using a pipette (up and down). The cell suspension was centrifuged at 1300 rpm for 5 minutes, the supernatant was removed and fresh culture medium was added. Cells were well resuspended and counted. This process was repeated every two days or when necessary.
4.2.3. Counting the cell number

Cell suspension was vortexed. 100 μL of the cell suspension was placed in a vial. Than an equal volume of Trypan Blue 0.4% (dilution factor × 2) was added and mixed by gently pipetting. 20μL sample was withdrawn with a wide tip pipettor and a clean hemacytometer was carefully loaded. Both sides of the chamber was filled with cell suspension.

Figure 1: Hemacytometer. The number of viable (bright) and non-viable cells (stained blue) in A,C parts of each chamber was counted under light microscope. The medium of the numbers of the viable cells was calculate.

The number of a viable cells (bright cells) and non-viable (stained blue) cells was counted and the number of viable cells/mL was calculated:

\[
\text{Number of the viable cells} \times 2 \times (\text{TB}) \times 10^4 \ (\text{factor of hemacytometer})
\]

Then the required number of the cells was transferred to a new labelled flask containing pre-warming culture medium. The flask was incubated at appropriate temperature (37°C) and appropriate concentration of CO₂ in the atmosphere (5% CO₂). This process was repeated every two days or when necessary.

For maintaining cell line it was not necessary to count the cells. It was enough just to add a require volume of cell suspension (one third) into a new labelled flask with pre-warming culture medium.

The goal of the scale up process was to obtain more cells. From one flask it was possible to make up to four flasks.
Figure 2: Scaling up attachment-dependent cell line – RAW 246.7.

Excess cells were able to store frozen in vials at -76°C and when needed cells were thawed and used for experiments. Prior to freezing, the cells were maintained in an actively growing state to insure maximum health and a good recovery. Ideally, the culture medium was changed the previous day. The cooling rate used to freeze cultures had to be just slow enough to allow the cells time to dehydrate, but fast enough to prevent excessive dehydration damage. A cooling rate of -1°C to -3°C per minute was optimal. The cryoprotectant medium was composed of 10% of dimethylsulfoxide (DMSO) and 90% of Culture Medium. DMSO is toxic above 4 °C therefore it was essential that cultures were thawed quickly and diluted in culture medium to minimise the toxic effect.

For freezing cells, has been done a process similar to subcultivation cells (view of cells, exclusion of contamination, washing the cells, counting the cells: 2-4 x 10⁶ cells/mL was recommended by ECACC, centrifuging and resuspending with a fresh culture medium), has been done. The freeze medium was slowly added into cell suspension which was placed on the ice. 1 mL of the cell suspension was pipetted into labelled vials (cell line name, passage number and date). The vials were stored in a freezer at -32 °C for 1 hour and then removed into freezer at -76 °C. Frozen vials should be transferred to vapour phase of liquid nitrogen storage vessel.

When needed the cells could be thawed. A freezeed vial was allowed to thaw at room temperature for 1 minute and then it was transferred to a waterbath at 37 °C (1-2 minute). In the safety cabinet cells were placed into labelled flask with 20 mL of pre-warmed culture medium (diluted out the DMSO) and the flask was incubated. After 24 hours cells were examined, the old culture medium was removed and a new medium was added.
4.2.4. Plating of cells

Flasks needed for an experiment were checked under light microscope to access the cell density degree and to confirm the absence of bacterial and fungal contaminants. The spent culture medium was removed from each flask and the cells were washed by Hank’s Balanced Salt Solution (BBS) (half of the volume of culture medium – 10 mL). 6 mL of the fresh Dulbecco’s Modified Eagle’s Medium (D-MEM) was added and the cells were mechanically detached with the scraper, the cells were resuspended in the fresh culture medium, using a pipette (up and down). The cell suspension was centrifuged at 1300 rpm for 5 minutes, the supernatant was removed and 8 mL of the fresh culture medium was added, well resuspended. The cell suspension was vortexed. 100 μL of the cell suspension was placed in a vial. Than an equal volume of Trypan Blue (dilution factor × 2) was added and mixed by gently pipetting. 20μL sample was withdrawn with a wide tip pipettor and a clean hemacytometer was carefully loaded. Both sides of the chamber was filled with cell suspension. The number of a viable cells was counted and the cell concentration in one mL was calculated:

\[
\text{Number of the viable cells} \times 2 \times 10^4 \times \text{(factor of hemacytometer)}
\]

It was prepared a cell suspension with required concentration and plated in a suitable culture plate.
4.2.5. **Growth curve**

The cells were plated in a four 6-well culture plates, an area of each well was 9.3 cm², a volume of cell suspension in each well was 3 mL and recommended seeding of the cells in one cm² was $4 \times 10^4$ cells/cm² ($3.72 \times 10^5$ cells/well $= 1.24 \times 10^5$ cells/mL). The cells were allowed to adhere and to grow in the incubator.

![Diagram of cell suspension](image)

**Figure 3**: Plates with cell suspension. The first plate was made three times. Each condition was duplicated.

The cells were counted twice a day, in the morning and in the afternoon, for six days. The counting of the cells in each well was similar like the counting for subculturing or plating the cells. Spent culture medium was removed, cells were twice washed by 0.5 - 1 mL of BSS
and the fresh culture medium was added. The amount of the culture medium depended on the number of cells in a well. First days when cells didn’t grow properly, wells were filled with lower amount of culture medium (from 250μL till 500 μL). Then when the number of cells grew, the volume of culture medium added increased. The cells were mechanically detached with the scraper, cells were resuspended in the fresh culture medium, using a wide tip pipettor (up and down). Then an equal volume of Trypan Blue (dilution factor × 2) was added into 20 μL of the cell suspension in a vial and mixed by gently pipetting. 20μL sample was withdrawn with a wide tip pipettor and a clean hemacytometer was carefully loaded. One side of the chamber with one sample and the other with the second sample. Total cell number in a well was calculated.

After three days the culture medium was removed, the cells in each well were twice washed by BSS and the wells with cells were filled by 3 mL of the fresh culture medium.

Then a standard growth curve was constructed (cell number versus time).
4.2.6. Determination of nitric oxide produccion in lipopolysaccharide-treated RAW 264.7 cells

Cells were plated in 24-well culture plates (area \( \text{well} = 1.9 \text{ cm}^2 \)) and allowed to adhere for 2 hours in the incubator. Thereafter, medium was removed, cells were washed by BSS (250 \( \mu \text{L} \)) twice and filled with fresh culture medium (500\( \mu \text{L} \)). Cells were incubated with 20 \( \mu \text{L} \) of LPS for 24 hours.

Stock solution of LPS (50 \( \mu \text{L}, 1 \text{ mg/mL} \)) was freeze-dried in vials. To carry out the experiment, the required vials were thawed at room temperature. The process took few minutes. Then solution of LPS was prepared in the safety cabinet by diluting with culture medium.

**Table III:** Solutions of LPS for the first plate

<table>
<thead>
<tr>
<th>Solution</th>
<th>[Solution] (( \mu \text{g/mL} ))</th>
<th>Volume/well (( \mu \text{L} ))</th>
<th>Volume/experiment (( \mu \text{L} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(^a)</td>
<td>0.1</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>B(^b)</td>
<td>1</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>C(^c)</td>
<td>10</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\)\(V_1 = C_2 = V_2\)
\(V_1 = 20 \mu \text{L}; C_2 = 10 \mu \text{g/mL}; V_2 = 500 \mu \text{L} \) (volume/well)
\(C_1 = 250 \mu \text{g/mL}\)
1000 \( \mu \text{g/mL} / 250 \mu \text{g/mL} = 4 \)
Solution C: \((1:4) = 40 \mu \text{L} \) LPS (1 mg/mL) + 120 \( \mu \text{L} \) Culture Medium \((V_{\text{final}} = 160 \mu \text{L})\). Other solutions were prepared by diluting the previous solution.

\(^b\)Solution B: \((1:10) = 50 \mu \text{L} \) LPS (10 \( \mu \text{g/mL} \)) + 450 \( \mu \text{L} \) Medium \((V_{\text{final}} = 500 \mu \text{L})\)

\(^c\)Solution A: \((1:10) = 50 \mu \text{L} \) LPS (1 \( \mu \text{g/mL} \)) + 450 \( \mu \text{L} \) Medium \((V_{\text{final}} = 500 \mu \text{L})\)
Table IV: Solutions of LPS for the second plate

<table>
<thead>
<tr>
<th>Solution</th>
<th>[Solution] (µg/mL)</th>
<th>Volume/ well (µL)</th>
<th>Volume/ experiment (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A³</td>
<td>0.125</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>B⁵</td>
<td>0.25</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>C⁶</td>
<td>0.5</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>D⁷</td>
<td>1</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>E⁸</td>
<td>2</td>
<td>20</td>
<td>60</td>
</tr>
<tr>
<td>F⁹</td>
<td>8</td>
<td>20</td>
<td>60</td>
</tr>
</tbody>
</table>

³Solution F: (1:5) = 80 µL LPS (1 mg/mL) + 320 µL Culture Medium (V_{final}= 400 µL)
⁵Solution E: (1:4) = 80 µL LPS (8 µg/mL) + 240 µL Medium (V_{final}= 320 µL)
⁶Solution D: (1:2) = 80 µL LPS (2 µg/mL) + 80 µL Medium (V_{final}= 160 µL)
⁷Solution C: (1:2) = 80 µL LPS (1 µg/mL) + 80 µL Medium (V_{final}= 160 µL)
⁸Solution B: (1:2) = 80 µL LPS (0.5 µg/mL) + 80 µL Medium (V_{final}= 160 µL)
⁹Solution A: (1:2) = 80 µL LPS (0.25 µg/mL) + 80 µL Medium (V_{final}= 160 µL)
**Figure 4.**: Plating the cells with different concentration of LPS. The first plate: The cells were seeded $5 \times 10^5$ cells/500μL with a different concentration of LPS (0.1; 1; 10 μg/mL).

The second plate: The cells were seeded $4 \times 10^5$ cells/500μL with a different concentration of LPS (0.125; 0.25; 0.5; 1; 2; 4 and 8 μg/mL). Each condition was triplicated. Cells that were not incubated with LPS served as control and 3 wells of each plate were filled just with a culture medium like a Blank.

After 24 hours a supernatant of each well was collected, centrifuged at 1500 rpm for 5 minutes. 100 μL of cell free culture medium and standards were transfected to 96-well plate. NaNO₂ diluted in DMEM-HG was used as a standard.
Table V: Preparation of NaNO₂ solution. 0,069 g of NaNO₂ was measured and diluted in 10 mL of culture medium (S0 solution). S1 solution were prepared by mixing of 100 µL of S0 and 900 µL of MC and the other solutions by thinning of previous solutions except S8 which was only culture medium.

<table>
<thead>
<tr>
<th>Solution</th>
<th>[Solution] (µM)</th>
<th>Dilution</th>
<th>Volume final (mL)</th>
<th>Volume of well (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0⁴</td>
<td>1000</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>S1</td>
<td>100</td>
<td>1:10 [100 µL (S0) + 900 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S2</td>
<td>50</td>
<td>1:2 [500 µL (S1) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S3</td>
<td>25</td>
<td>1:2 [500 µL (S2) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S4</td>
<td>12,5</td>
<td>1:2 [500 µL (S3) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S5</td>
<td>6,25</td>
<td>1:2 [500 µL (S4) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S6</td>
<td>3,13</td>
<td>1:2 [500 µL (S5) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S7</td>
<td>1,56</td>
<td>1:2 [500 µL (S6) + 500 µL (MC)]</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>S8</td>
<td>0</td>
<td>1000 µL MC</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

⁴PM(NaNO₂) = 69 g/mol S0 = 1 mM V₉₈₀ = 10 mL
69 g .......... 1 M  0,069 g .......... 1000 mL
  x₁ .......... 0,001 M  x₁ = 0,069 g  x₂ .......... 10 mL  x₂ = 0,0069 g NaNO₂ + 10 mL
MC

1% Sulphanilamide in 5% H₃PO₄ was prepared by mixing 5,8 mL H₃PO₄ (86,1%) and 94,2 mL H₂O. To the acid was added 1 g of Sulphanilamide. 0,1% NED solution composed of 0,1 g of NED and 100 mL H₂O. Both solutions were stored in the fridge, NED solution protected from the light using a tinfoil. Before using the Griess solutions were mixed 1:1 (vol/vol).

Equal volume of Griess reagent was added into each well and mixed by gently tapping of the plate. The mixture was incubated at room temperature for 10 min to form a chromophore. The absorbance was read at 540 nm wavelength using a microplate reader. Measurement had to be realized until 30 min after adition Griess reagent to samples. Reasults would not be clear. A standard curve was constructed like a dependence of the absorption on the NO₂⁻ concentration. Concentration of NO was calculated from the regrese equation of Standard Curve.
5. RESULTS

5.1. Growth curve

The RAW 264.7 cells were plated in a four 6-well culture plates with recommended seeding of the cells $4 \times 10^4$ cells/cm² ($3.72 \times 10^5$ cells/well).

![Growth Curve Graph]

**Figure 1:** The Standard Growth Curve for RAW 264.7 cell line. The lag phase was defined from the seeding cells until 54 h. Cells adapted to different conditions and attached. First hours (till 22.5 h) the cell number decreased from beginning $3.72 \times 10^5$ to $3.12 \times 10^5$. Then the cell number began increasing a little. At 47 h the cell number was $1 \times 10^6$, but in 54 h the cell number decreased on $8.8 \times 10^5$. This situation could be explain like a not gently scraping and pro-long counting cells thanks of diluting cell suspension which was necessary for right counting cells. The cell number exponential increased from the 54 h until 94 h ($4.1 \times 10^6$ cells). This period was expressed like a log phase. At 101 h counting showed decreasing the cell number, because the culture became confluent and the growth rate of the culture was reduced.
5.2. Determination of nitric oxide production in lipopolysaccharide-treated RAW 264.7 cells

5.2.1. The first experiment

RAW 264.7 cells were treated with LPS. In the first experiment cells were stimulated with 0.1; 1 and 10 μM/mL of LPS. The first experiment was repeated three times (1, 2, 3) with the same conditions (seeding concentration: 5 × 10^5 cells/mL, 0.1; 1; 10 μM/mL of LPS).

Table I: Absorption at 540 nm in a standard and in RAW 264.7 cells stimulated by LPS (1).

<table>
<thead>
<tr>
<th></th>
<th>[NaNO₂] μM</th>
<th>A₅₄₀ (2)</th>
<th>A ± S.D.</th>
<th>A₅₄₀ (2)</th>
<th>A ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Curve</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of NO₂⁻</td>
<td>12.5</td>
<td>1.486</td>
<td>1.499</td>
<td>1.471</td>
<td>1.485 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>0.839</td>
<td>0.846</td>
<td>0.830</td>
<td>0.838 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>0.428</td>
<td>0.43</td>
<td>0.431</td>
<td>0.429 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>0.256</td>
<td>0.252</td>
<td>0.251</td>
<td>0.253 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.062</td>
<td>0.062</td>
<td>0.065</td>
<td>0.063 ± 0.001</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Table I: Absorption at 540 nm in a standard and in RAW 264.7 cells stimulated by LPS (1).

<table>
<thead>
<tr>
<th></th>
<th>[LPS] μg/mL</th>
<th>A ± S.D.</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells treated by LPS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0.067</td>
<td>0.065</td>
<td>0.066</td>
<td>0.066 ± 0.008</td>
<td></td>
</tr>
<tr>
<td>0.1</td>
<td>0.097</td>
<td>0.114</td>
<td>0.117</td>
<td>0.109 ± 0.009</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.175</td>
<td>0.192</td>
<td>0.186</td>
<td>0.184 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.174</td>
<td>0.181</td>
<td>0.172</td>
<td>0.176 ± 0.004</td>
<td></td>
</tr>
</tbody>
</table>

Table I: Absorption at 540 nm in a standard and in RAW 264.7 cells stimulated by LPS (1).

<table>
<thead>
<tr>
<th></th>
<th>[NaNO₂] μM</th>
<th>A₅₄₀ (2)</th>
<th>A ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard Curve</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of NO₂⁻</td>
<td>12.5</td>
<td>1.306</td>
<td>1.285</td>
</tr>
<tr>
<td></td>
<td>6.25</td>
<td>0.725</td>
<td>0.764</td>
</tr>
<tr>
<td></td>
<td>3.13</td>
<td>0.415</td>
<td>0.429</td>
</tr>
<tr>
<td></td>
<td>1.56</td>
<td>0.252</td>
<td>0.258</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.070</td>
<td>0.076</td>
</tr>
<tr>
<td>[LPS] μg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells treated by LPS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>0.075</td>
<td>0.068</td>
<td>0.073</td>
</tr>
<tr>
<td>0.1</td>
<td>0.083</td>
<td>0.109</td>
<td>0.108</td>
</tr>
<tr>
<td>1</td>
<td>0.141</td>
<td>0.154</td>
<td>0.176</td>
</tr>
<tr>
<td>10</td>
<td>0.154</td>
<td>0.163</td>
<td>0.155</td>
</tr>
</tbody>
</table>

(1) 2.6 x 10⁵ cells/cm² were incubated in 24-well plate (5 x 10⁵ cells/mL), final volume of cell suspension in each well is 500 μL. During 24 hours cells were incubated with different concentration of LPS.

(2) Absorption was measured at 540 nm using microplate reader after filling 96-well plate with standard (different concentration of Sodium Nitrite) or with samples (supernatant of treated cells with different concentration of LPS: 0, 1; 10 μM/mL, of cells and Culture Medium) and each condition was treated with Griess Reagent. Each condition was prepared with three different cultures initiated parallel. A ± S.D. is the mean of absorption A₅₄₀ of each triplicated condition and standard deviation.
Figure 1: Tree Standard Curves of NaNO₂ for tree versions (1st, 2nd, 3th) of the first experiment.

Linear regression was applied for standard curves. Regression equation and R-squared value were expressed for each standard curve.

Table II: Determination of concentration of NO in LPS treated RAW 264.7 cells for three times repeated experiment (1., 2., 3.).

<table>
<thead>
<tr>
<th>[LPS] µg/ml</th>
<th>[NO] µM⁵</th>
<th>[NO] µM⁶</th>
<th>[NO] µM⁴</th>
<th>[NO] µM ± S.D.⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.1</td>
<td>0.165</td>
<td>0.314</td>
<td>0.34</td>
<td>0.273 ± 0.077</td>
</tr>
<tr>
<td>1</td>
<td>0.849</td>
<td>0.997</td>
<td>0.945</td>
<td>0.930 ± 0.061</td>
</tr>
<tr>
<td>10</td>
<td>0.840</td>
<td>0.901</td>
<td>0.822</td>
<td>0.854 ± 0.034</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[LPS] µg/ml</th>
<th>[NO] µM⁵</th>
<th>[NO] µM ± S.D.⁶</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.1</td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>1</td>
<td>0.394</td>
<td>0.527 ± 0.148</td>
</tr>
<tr>
<td>10</td>
<td>0.527</td>
<td>0.619 ± 0.041</td>
</tr>
</tbody>
</table>

(3) Concentration of NO was calculated from the regression equation $y = 0.1142x + 0.0781$ of Standard Curve (Fig.1).
Concentration of NO was calculated from the regression equation $y = 0.1119x + 0.089$ of Standard Curve (Fig. 1).

Concentration of NO was calculated from the regression equation $y = 0.0977x + 0.1025$ of Standard Curve (Fig. 1).

Medium of NO concentration of each triplicated condition with the standard deviation.

Figure 2: Quantification of NO production in lipopolysaccharide treated RAW 264.7 cells with different concentration of LPS (0.1, 1, 10 μg/mL). First version of the experiment. Cells treated with 0.1 μg/mL of LPS released 0.273 μM of NO, with 1 μg/mL of LPS 0.93 μM of NO and with 10 μg/mL of LPS released 0.834 μM of NO.
Figure 3: Quantification of NO production in lipopolysaccharide treated RAW 264,7 cells with different concentration of LPS (0,1, 1, 10 μg/mL). Second version of the experiment. Cells treated with 0.1 μg/mL of LPS released 0.265 μM of NO, with 1 μg/mL of LPS 0.778 μM of NO and with 10 μg/mL of LPS released 0.893 μM of NO.

![Graph showing NO production with different LPS concentrations](image)

Figure 4: Quantification of NO production in lipopolysaccharide treated RAW 264,7 cells with different concentration of LPS (0,1, 1, 10 μg/mL). Third version of the experiment. Cells treated with 0.1 μg/mL of LPS did not release any NO, with 1 μg/mL of LPS 0.558 μM of NO and with 10 μg/mL of LPS released 0.561 μM of NO.
Table III: Summary of the experiment.

<table>
<thead>
<tr>
<th>[LPS] μM/mL</th>
<th>1.</th>
<th>2.</th>
<th>3.</th>
<th>A_{540}^{(1)}</th>
<th>[NO] μM^{(2)}</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.066</td>
<td>0.067</td>
<td>0.072</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.1</td>
<td>0.109</td>
<td>0.119</td>
<td>0.1</td>
<td>0.273</td>
<td>0.265</td>
</tr>
<tr>
<td>1</td>
<td>0.184</td>
<td>0.176</td>
<td>0.157</td>
<td>0.93</td>
<td>0.778</td>
</tr>
<tr>
<td>10</td>
<td>0.176</td>
<td>0.199</td>
<td>0.157</td>
<td>0.854</td>
<td>0.893</td>
</tr>
</tbody>
</table>

(1) Medium of absorbance at 540 nm wavelength of each triplicated condition
(2) Medium of NO concentration of each triplicated condition.

RAW 264,7 cells treated by 0.1 μg/mL of LPS produced three times less amount of NO than RAW 264,7 cells treated by 1 or 10 μg/mL (Fig. 2., Fig. 3.). In figure 4 there is no expressed NO production. Stimulated cells by 1 and 10 μg/mL showed us similar results, similar releasing of NO (Fig. 2., Fig. 3., Fig. 4.). In one case (first version, Fig.2) RAW 264,7 cells treated by 1 μg/mL of LPS produced higher amount of NO (0.93 μM) than cells treated by 10 μg/mL of LPS (concentration of NO was 0.854 μM). In the other case (third version, Fig.4) released amount of NO after treating with 1 μg/mL of LPS (0.558 μM) was nearly the same like after 10 μg/mL (0.561 μM).
5.2.2. The second experiment

RAW 264,7 cells in concentration $4 \times 10^5$ cells/mL were treated with different concentration of LPS (0,125; 0,25; 0,5; 2; 8 $\mu$M/mL).

Table IV: Absorption at 540 nm in a standard and in RAW 264,7 cells stimulated by LPS \(^{(1)}\).

<table>
<thead>
<tr>
<th>Standard Curve of NO\textsubscript{2}\textsuperscript{-}</th>
<th>[NaNO\textsubscript{2}] $\mu$M</th>
<th>$A_{540}^{(2)}$</th>
<th>$\bar{A} \pm S.D.$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12,5</td>
<td>1,298</td>
<td>1,309</td>
<td>1,332</td>
</tr>
<tr>
<td>6,25</td>
<td>0,647</td>
<td>0,771</td>
<td>0,771</td>
</tr>
<tr>
<td>3,13</td>
<td>0,448</td>
<td>0,453</td>
<td>0,458</td>
</tr>
<tr>
<td>1,56</td>
<td>0,29</td>
<td>0,293</td>
<td>0,283</td>
</tr>
<tr>
<td>0</td>
<td>0,12</td>
<td>0,115</td>
<td>0,143</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[LPS] $\mu$M/mL</th>
<th>Cells treated by LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,125</td>
<td>0,131</td>
</tr>
<tr>
<td>0,25</td>
<td>0,14</td>
</tr>
<tr>
<td>0,5</td>
<td>0,146</td>
</tr>
<tr>
<td>1</td>
<td>0,163</td>
</tr>
<tr>
<td>2</td>
<td>0,151</td>
</tr>
<tr>
<td>8</td>
<td>0,154</td>
</tr>
<tr>
<td>control</td>
<td>0,119</td>
</tr>
</tbody>
</table>

\(^{(1)}\) $2,11 \times 10^5$ cells/cm\textsuperscript{2} were incubated in 24-well plate ($4 \times 10^5$ cells/mL), final volume of cell suspension in each well is 500 $\mu$L. During 24 hours cells were incubated with different concentration of LPS. 

\(^{(2)}\) Absorption was measured at 540 nm using microplate reader after filling 96-well plate with standard (different concentration of Sodium Nitrite) or with samples (supernatant of treated cells with different concentration of LPS: 0,125; 0,25; 0,5; 2; 8 $\mu$M/mL, of cells and Culture Medium) and each condition was treated with Griess Reagent. Each condition was prepared with tree different cultures initiated parallel. $\bar{A}$ is a medium of absorption $A_{540}$ of each triplicated condition with standard deviations.

**Standard Curve**

\[
y = 0,0941x + 0,1415 \\
R^2 = 0,999
\]
Figure 5.: Standard curve of NaNO₂. Linear regression was applied for standard curve. Regression equation and R-squared value were expressed for this standard curve.

Table V.: Determination of concentration of NO in LPS treated RAW 264,7 cells.

<table>
<thead>
<tr>
<th>[LPS] µM/mL</th>
<th>[NO] µM(3)</th>
<th>[NO] µM(4) ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.25</td>
<td>0.000</td>
<td>0.154 ± 0.051</td>
</tr>
<tr>
<td>0.5</td>
<td>0.048</td>
<td>0.356 ± 0.153</td>
</tr>
<tr>
<td>1</td>
<td>0.101</td>
<td>0.345 ± 0.200</td>
</tr>
<tr>
<td>2</td>
<td>0.228</td>
<td>0.282 ± 0.253</td>
</tr>
<tr>
<td>8</td>
<td>0.133</td>
<td>0.590 ± 0.364</td>
</tr>
<tr>
<td>control</td>
<td>0.000</td>
<td>0.000 ± 0.000</td>
</tr>
</tbody>
</table>

(3) Concentration of NO was calculated from the regression equation $y = 0.0941x + 0.1415$ of Standard Curve (Fig.5).

(4) Medium of NO concentration of each triplicated condition with standard deviation.

Figure 6.: Quantification of NO production in lipopolysaccharide treated RAW 264,7 cells with different concentration of LPS (0.125; 0.25; 0.5; 1; 2; 8 µg/mL). Dose-dependent enhancement effects of LPS on production of NO. Cells treated with 0.125 µg/mL of LPS did not release NO. By stimulation cells with 0.25 µg/mL and higher doses of LPS was able to produce NO. 0.25 µg/mL expressed the lowest level of LPS (second condition) which stimulated cells to production of NO (0.051 µM). Two of this triplicated condition did not admit any NO production. Cells treated by 0.5 µg/mL of LPS released 0.14 µM of NO; by 1 µg/mL of LPS 0.2 µM of NO; by 2 µg/mL of LPS 0.253 µM of NO and by 8 µg/mL of LPS released 0.364 µM of NO. The control sample did not admit any contamination or NO production.
5.2.3. The third experiment

RAW 264.7 cells in concentration $4 \times 10^5$ cells/mL were treated with different concentration of LPS (0.125; 0.25; 0.5; 2; 8 μM/mL).

**Table VI.** Determination of concentration of NO in LPS treated RAW 264.7 cells.

<table>
<thead>
<tr>
<th>[LPS] μg/mL</th>
<th>[NO] μM&lt;sup&gt;(3)&lt;/sup&gt;</th>
<th>[NO] μM&lt;sup&gt;(4)&lt;/sup&gt; ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>0</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>0.000 ± 0.000</td>
</tr>
<tr>
<td>0.5</td>
<td>0.122</td>
<td>0.066 ± 0.041</td>
</tr>
<tr>
<td>1</td>
<td>0.069</td>
<td>0.090 ± 0.023</td>
</tr>
<tr>
<td>2</td>
<td>0.069</td>
<td>0.115 ± 0.035</td>
</tr>
<tr>
<td>8</td>
<td>0.282</td>
<td>0.246 ± 0.050</td>
</tr>
<tr>
<td>control</td>
<td>0</td>
<td>0.000 ± 0.000</td>
</tr>
</tbody>
</table>

<sup>(3)</sup> Concentration of NO was calculated from the regression equation $y = 0.0941x + 0.1415$ of Standard Curve (Fig. 5).

<sup>(4)</sup> Medium of NO concentration of each triplicated condition with standard deviation

**Figure 7.** Quantification of NO production in lipopolysaccharide treated RAW 264.7 cells with different concentration of LPS (0.125; 0.25; 0.5; 1; 2; 8 μg/mL). Cells treated with 0.125 and 0.25 μg/mL of LPS did not release NO. By stimulation cells with 0.5 μg/mL and higher doses of LPS was able to produce NO. 0.5 μg/mL expressed the lowest level of LPS (third condition) which stimulated cells to production of NO (0.066 μM). Two of this triplicated condition did not admit any NO production. Cells treated by 1 μg/mL of LPS released 0.090 μM of NO; by 2 μg/mL of LPS 0.115 μM of NO and by 8 μg/mL of LPS released 0.246 μM of NO. The control sample did not admit any contamination or NO production.
6. DISCUSSION

We have tried to optimise conditions for an evocation of an inflammation in LPS-treated RAW 264,7 cells. The inflammatory activity was tested by measuring of the nitric oxide production. LPS, a major component of the cell wall of Gram-negative bacteria, is one of the most potent and best characterized modulator of macrophage function.

We decided for RAW 264,7 cell line because of following reasons. The contamination and the mutation are not so usual in the secondary culture like in the primary. Cell lines are immortal, they can divide indefinitely. But big disadvantage of using cell lines is that cells in the primary culture more respond with cells in organism.

Generation of a growth curve of RAW 264,7 cells was referred as a good characteristic of cell line. It gave us informations about suitable period (doubling time) used to quantify the response of cells to different stimulatory culture conditions like a LPS-treating. The culture is in its most reproducible form; optimal for sampling, plating or subculturing. The culture is the most uniform viability is high.

It was made simillar experiment with seeding cell concentrations $1,8 \times 10^5$ cells/well = $6 \times 10^4$ cells/mL and $3,7 \times 10^5$ cells/well = $1,2 \times 10^5$ cells/mL for four days, which showed that four days are not enough to demonstrate all periods of growth standard curve (the plateau phase missed in both cases) and the growth cycle with the cell concentration $3,7 \times 10^5$ cells/well had a quicker process. That is why we decided to repeat the experiment only with $3,7 \times 10^5$ cells/well for 6 days.

The cells are randomly distributed in the cell cycle. The log period in our experiment was from 47.54.) hour (2. day) after seeding cells to 94. hour (4.days). See figure 1 in the results 4.1.

For determination of nitrite oxide production in lipopolysaccharide – treated RAW 264,7 cells we used two batches of LPS. First one was obtained and frozen on 23.11.2006 and the second on 15.2.2007. For the first and third experiment was used the batch of LPS froze on 23.11.2006 and for the second experiment the batch of LPS froze on 15.2.2007. This could be one of explanations for lower NO production in second experiment.

NOS activity in intact cells was monitored as nitrites released into the culture medium after exposure to the inducer. Cells that were not incubated with LPS served as control. NaNO$_2$ diluted in the culture medium was used as a standard. 100 µL of cell free
culture medium and standards were transfected to 96-well plate. Equal volume of Griess reagent (1% Sulphanilamide in 5% H$_3$PO$_4$ and 0,1% NED (H$_2$O)) was added into each well and mixed by gently tapping of the plate. The mixture was incubated at room temperature for 10 min to form a chromophore. The absorbance was read at 540 nm wavelength using a microplate reader. The NO$_2^-$ concentration in the culture medium was estimated by extrapolating from the sodium nitrite standard curve.

Figure 2.: The comparison of tree experiments for determination NO production in LPS-treated (two batches of LPS) RAW 264.7 cells. Tree times repeated first experiment with the same conditions ($5 \times 10^5$ cells/mL treated by 0,1; 1; 10 µg/mL of LPS) where cells released nearly the same amount of NO for each condition. Averages of tree results for each condition were calculated and compared with results of the second and third experiment ($4 \times 10^5$ cells/mL treated by 0,125; 0,25; 0,5; 2; 8 µg/mL of LPS).

The second and third exp. were done at the same time with the same conditions, but different batches of LPS. For the 3rd exp. was used the older batch. The results in the 3rd exp. are half as much than in the 2nd exp.

In the first experiment stimulation cells with 1 µg/mL of LPS showed almost the same result (released amount of NO was 0,755 µM) like treated cells with 10 µg/mL of LPS (amount
of NO was 0.759 μM). 0.1 μg/mL of LPS stimulated cells to 0.179 μM of NO production in the first experiment, but in the second cells began producing NO from 0.25 μg/mL of LPS (0.051 μM of NO) and in the third experiment from 0.5 μg/mL of LPS (0.066 μM of NO). The dose of LPS was more than once higher (2nd exp.) and five times higher (3rd exp.), but released amount of NO is five times lower than in the first experiment.

A clear difference is observed for the same condition, stimulation cells with 1 μg/mL of LPS. In the first experiment was released 0.755 μM of NO, but in the second 0.253 μM of NO (26.5%) and in the third 0.090 μM of NO (12%). Generally LPS-treated cells produced several times more NO in the first experiment than in the second and third. Differences between NO production in experiments decreased with higher dose of LPS. For example, stimulation cells with 8 μg/mL of LPS was expressed in the third exp. like 67% of NO production in the second exp., but stimulation cells with 0.5 μg/mL of LPS in the third exp. was produced only 47% of NO production in the second exp.

Noticed differences between these experiments with similiar conditions are in the cell concentration (5 × 10^5 cells/mL for the first exp. and 4 × 10^5 cells/mL for the second and third) and in different batch of LPS used in the first and third exp. and in the second exp, which can explain different amount of released NO for similiar conditions.

Especially from the first experiment we presume that 1-2 μg/mL of LPS is enough amount of LPS, which is able to induce an inflammation.

We could see decresing of LPS-activity in the time when LPS was stored in the freezer. We could compare the first and third exp. because of the same used batch of LPS. The LPS-activity in the 3rd exp. decreased on 12% of NO production in the 2nd exp. (1 μg/mL of LPS). We suppose that frozen vials with LPS degrade along the time. In our case it was during 3 months when the batch of LPS was frozen.

Other similar published works confirm that LPS-treated mouse macrophage cell lines induce inflammation which is measured like a amount of released nitric oxide. Stimulation of the mouse macrophage cell line J774.2 with LPS (1 μg/ml) caused a significant increase of 24-hr nitrite accumulation in culture medium (from 4.2 ± 0.7 to 34 ± 4 μM) (control cells were treated with vehicle, but not with LPS). Nitric oxide synthase (NOS-2) in macrophages was induced by lipopolysaccharide from Escherichia coli serotype 0127:B8 (LPS, 1 μg/ml). [19]

In the work of Tiina Salonen and his colleagues J774 cells were stimulated by LPS (10 ng/mL)and treated with increasing concentrations of RO318220, GÖ6976 or LY333531. NO
production in presence of PKC inhibitor (RO318220, LY333531, GÖ6976) was measured by Griess reaction in both works. It resulted in a inhibition of NO production in a dose-dependent manner. [18]

Other work engaged in the regulation of nitric oxide in LPS-treated RAW 246,7 cell line. They investigated an influence of NO production in presence of agonist/antagonist of adenosine receptors (for example: N^6^-2-(4-amino-phenyl)ethyladenosine (APNEA) or adenosine uptake inhibitor (dipyridamol, S(4-nitrobenzyl)-6-thioinosine (NBTI), and S(4-nitrobenzyl)-6-thioguanosine (NBTG) or inhibitor of adenosine deaminase ADA (erytho-9-(2-hydroxy-3-nonyl)adenine (EHNA)). [1]

Dose-dependent enhancement effects of APNEA were expressed as percentage of \([\text{NO}_2^-]\) in treated cells compared to cells treated with LPS (10 \(\mu\text{g/mL}\)) alone (100 \(\pm\) 1.5 \%, \(n=24\)). The effects of EHNA on the observed actions of adenosine on NO release were determined. This showed that the presence of ADA in the culture medium did affect the availability of adenosine and therefore reduced its enhancement potency at low concentrations. EHNA reduced the enhancement potency of adenosine at the higher concentrations of 500-1000 \(\mu\text{M}\). Nevertheless, the maximum enhancement effect of adenosine was actually observed in the absence of EHNA. Dipyridamole was actually able to reduce the enhancement effects of adenosine. The results demonstrated that 10 \(\mu\text{M}\) of dipyridamole managed to inhibit the enhancement effects of adenosine (1 \(\mu\text{M}\)) on LPS-stimulated \(\text{NO}_2^-\) production significantly by 53 \%. In addition, 10 \(\mu\text{M}\) of dipyridamole was able to inhibit the dose-dependent enhancement responses of adenosine (10 - 1000 \(\mu\text{M}\)). NBTI or NBTG (10 \(\mu\text{M}\)) alone had no effects on LPS-induced \(\text{NO}_2^-\) production, but were able to reduce the enhancement effects of adenosine. [1]

It was made a good model of LPS-treated RAW 264,7 cells, which will be used for the other investigation in the laboratory. Next works deal with activity analysis of adenosine receptor agonist/antagonists in the inflammatory response. They aim to develop new therapeutical strategies.
7. THE SUMMARY

This work deals with optimization of cell culture conditions of RAW 264.7 cells and optimization of LPS concentrations. We have been trying to form an anti-inflammatory model.

This work is composed of acknowledgements, the introduction, the theoretical part, materials and methods, results, the discussion, summary, abbreviations, references and the content.

In the theoretical part cell cultures are described; how to work with them, how to obtain them, common problems like contamination, environmental requirements like the culture medium and the application of cell lines. Furthermore this part is focused in macrophage cell lines, their roles in the immune system and inflammatory response. There are described the Griess reaction, which is used for determination of NO production, and the growth cycle of cells.

In the materials and methods all used materials, reagents, equipments and methods are described. The methodology is focus in conditions of incubation, subculturing of cells, counting of cells, plating of cells; growth cycle (counting of cells twice a day for six days) and determination of NO production in LPS-treated RAW 264.7 cells – Griess reaction. Griess reagents (NED and sulphonilamide in phosphoric acid) combined with NO$_2$· form a chromophore, which is detected by a spectrophotometer.

Cell line was incubated in an incubator in 37°C saturated by 95% of oxygen and 5% of carbon dioxide (CO$_2$). Every two days or if necessary were subcultured. Cells were washed by Hank’s Balanced Salt Solution (BBS). As a culture medium was chosen Dulbecco’s Modified Eagle’s Medium (D-MEM) mixed with serum, antibiotics to protect the cell culture against contamination, and glutamine. All works with cells were realized in a biology safety cabinet. For maintaining aseptic conditions was used 70% ethanol, sterile gloves and laboratory coat.

Results consist of two chapters: results of growth cycle and results of determination of NO production in LPS-treated RAW 246.7 cells. The growth cycle is characterized by the growth curve which has sigmoidal shape. The cells were plated and twice a day counted in the hemacytometer, in the morning and in the afternoon, for six days. From a growth curve, the lag time, population doubling time, and saturation density can be determined. [3, 4, 5] The lag phase was defined from the seeding cells untill 54 h ($8.8 \times 10^5$). The cell number exponential increased from the 54 h untill 94 h ($4.1 \times 10^6$ cells). This period was expressed like a log phase. At 101 h counting showed decreasing the cell number ($3.81 \times 10^5$), the plateau phase.
The anti-inflammatory activity was tested by measuring the production of nitric oxide (NO) (Griess reaction using spectrophotometer).

It was made tree experiments. In the first experiment cells were stimulated with 0,1; 1 and 10 \( \mu \text{M/mL} \) of LPS. The first experiment was repeated tree times (1., 2., 3.) with the same conditions (seeding concentration: \( 5 \times 10^5 \) cells/mL, 0,1; 1; 10 \( \mu \text{M/mL} \) of LPS). In the second and third experiment RAW 246,7 cells in concentration \( 4 \times 10^5 \) cells/mL were treated with LPS (0,125; 0,25; 0,5; 2; 8 \( \mu \text{M/mL} \)).

In the first experiment cells treated with 0,1 \( \mu \text{g/mL} \) of LPS released 0,179 \( \mu \text{M} \) of NO, with 1 \( \mu \text{g/mL} \) of LPS 0,755 \( \mu \text{M} \) of NO and with 10 \( \mu \text{g/mL} \) of LPS 0,759 \( \mu \text{M} \) of NO.

In the second experiment cells treated with 0,125 \( \mu \text{g/mL} \) of LPS did not release any NO. Cells treated by 0,25 \( \mu \text{g/mL} \) of LPS released 0,051 \( \mu \text{M} \) of NO, 0,5 \( \mu \text{g/mL} \) of LPS released 0,14 \( \mu \text{M} \) of NO; by 1 \( \mu \text{g/mL} \) of LPS 0,2 \( \mu \text{M} \) of NO; by 2 \( \mu \text{g/mL} \) of LPS 0,253 \( \mu \text{M} \) of NO and by 8 \( \mu \text{g/mL} \) of LPS released 0,364 \( \mu \text{M} \) of NO.

In the third experiment cells treated with 0,125 and 0,25 \( \mu \text{g/mL} \) of LPS did not release any NO. Cells treated by 0,5 \( \mu \text{g/mL} \) of LPS released 0,066 \( \mu \text{M} \) of NO, 1 \( \mu \text{g/mL} \) of LPS released 0,090 \( \mu \text{M} \) of NO; by 2 \( \mu \text{g/mL} \) of LPS 0,115 \( \mu \text{M} \) of NO and by 8 \( \mu \text{g/mL} \) of LPS released 0,246 \( \mu \text{M} \) of NO.
7. **SOUHRN**

Tato práce se zabývá optimalizací podmínek buněčné kultury RAW 264,7 a optimalizací LPS concentrací. Snažili jsme se vytvořit protizánětlivý model.

Tato práce je rozdělena do několika hlavních kapitol: poděkování, úvod, teoretická část, materiály a metody, výsledky, souhrn, zkratky, odkazy a obsah.

V teoretické části jsou popsány buněčné kultury; jak s nimi pracovat, jak je získat, společné problémy jako je kontaminace, environmentální požadavky – kultivační médium, a použití buněčných linií. Další část je zaměřena na buněčné linie makrofágů, jejich role v imunitním systému a v imunitní odpovědi. V teoretické části naleznete také popis Griessovy reakce, která slouží k determinaci NO produkce, a popis růstového cyklu buněk.

V části materiály a metody jsou popsány veškeré použité materiály, reagensia, přístroje a metody. Metodologie je zaměřená na podmínky inkubace, na kultivaci a počítání buněk; na růstový cyklus (počítání buněk dvakrát denně po dobu šesti dnů) a na determinaci NO produkce v LPS stimulovaných RAW 264,7 buňkách – Griessova reakce. Griessovy reagensia (NED a sulfonamid v kyselině fosforečné) reagují s NO₂⁻ a vytvářejí chromofory, které se detekují spektrofotometrem.

Buňky byly udržovány v inkubátoru při 37°C a při saturaci 95% kyslíku a 5% oxidu uhlíčitého. Každé dva dny nebo, pokud bylo nezbytné, byly rekultivovány. Buňky byly vyčištěny Hankovým vyváženým solným roztokem. Jako buněčné médium bylo vybráno Dulbecco modifikující Eagle médium se sérem, glutamátem a antibiotiky, která chránila buněčnou kulturu před kontaminací. Veškerá práce s buňkami byla realizována v biologicky bezpečném boxu. K udržení aseptických podmínek se používal 70% etanol, sterilní rukavice a laboratorní plášť.

Výsledky se skládají ze dvou částí: výsledky růstového cyklu buněk a výsledky determinace NO produkce v LPS stimulovaných RAW 264,7 buňkách. Růstový cyklus je charakterizován růstovou křivkou, která má sigmoidální tvar. Buňky byly kultivovány a dvakrát denně počítány v hemacytometru, ráno a odpoledne po šest dnů. Z růstové křivky byly detekovány tyto fáze: lag, doba zdvojení a saturační fáze. Lag fáze byla definována od počátku kultivace do 54 h (8,8 × 10⁵ buněk). Počet buněk stoupal od 54 h do 94 h (4,1 × 10⁶). Tato perioda byla vyjádřena jako log fáze. Ve 101 h počet buněk klesl na 3,81 × 10⁵, the plató fáze.
Protizánětlivá aktivita byla určena měřením NO produkce (Griessova reakce s využitím spektrofotometru). Byly provedeny tři experimenty. V prvním experimentu byly buňky stimulovány 0,1; 1 a 10 μM/mL LPS. První experiment byl opakován třikrát (1., 2., 3) ve stejných podmínkách (počáteční buněčná koncentrace: \(5 \times 10^5\) cells/mL, 0,1; 1; 10 μM/mL LPS). Ve druhém a třetím experimentu RAW 264,7 buňky o počáteční koncentraci \(4 \times 10^5\) cells/mL byly stimulovány LPS (0,125; 0,25; 0,5; 2; 8 μM/mL).

V prvním experimentu buňky léčené 0,1 μg/mL LPS uvolnily 0,179 μM NO, 1 μg/mL LPS 0,755 μM NO a 10 μg/mL LPS 0,759 μM NO.

Ve druhém experimentu buňky vystavené působení 0,125 μg/mL LPS neprodukovaly NO. Teprve až působením 0,25 μg/mL LPS na buňky se uvolnilo 0,051 μM NO, 0,5 μg/mL LPS stimulovalo buňky k 0,14 μM NO; 1 μg/mL LPS k 0,2 μM NO; 2 μg/mL LPS k 0,253 μM NO a 8 μg/mL LPS k 0,364 μM NO.

Ve třetím experimentu buňky vystavené působení 0,125 a 0,25 μg/mL LPS neprodukovaly NO. Až 0,5 μg/mL LPS stimulovalo buňky k produkci NO, a to k 0,066 μM, 1 μg/mL LPS k 0,090 μM NO; 2 μg/mL LPS k 0,115 μM NO a 8 μg/mL LPS vyvolalo uvolnění 0,246 μM NO.
8. ABBREVIATIONS

ADA  adenosine deaminase
ADCC  antibody-dependent cellular cytotoxicity
A-MuLV  Abelson Leukaemia Virus
ATCC  American Type Culture Collection
BSS  Hanks’ balanced salt solution
CO₂  carbon dioxide
DMEM-HG  Dulbecco’s Modified Eagle’s Medium
DMSO  dimethylsulfoxide
DSMZ  German Collection of Microorganism and Cell Cultures
ECACC  The European Collection of Cell Cultures
H₃PO₄  phosphoric acid
ICLC  Interlab Cell Line Collection
IL-1  interleukin 1
IL-6  interleukin 6
JCRB  Japanese Cancer Research Resources Bank
LPS  lipopolysaccharide
MC  culture medium
MP  macrophage
NaNO₂  sodium nitrite
NBTG  S(4-nitrobenzyl)-6-thioguanosine
NBTI  S(4-nitrobenzyl)-6-thioinosine
NED  N-1-naphthylethylenediamine dihydrochloride
NO  nitric oxide
NO₂⁻  nitrite
PAF  platelet-activating factor
PIP2  phosphatidylinositol bisphosphate
PKC  protein kinase C
TB  trypan blue
TLR  Toll-like receptors
TNF-α  tumor necrosis factor α
WFCC  World of Culture Collections
9. REFERENCES


18. Inhibition of classical PKC isoforms downregulates STAT1 activation and iNOS expression in LPS-treated murine J774 macrophages. T. Salonen, O. Sareila, U.


10. THE CONTENT

1. ACKNOWLEDGEMENTS 2

2. THE INTRODUCTION 3

3. THE THEORETICAL PART 6

3.1 Cell cultures 6

3.1.1. The history 6

3.1.2. Primary and secondary cultures 7

3.1.3. Culture systems 7

3.1.4. The morphology of cells 8

3.1.5. The subculturing of cells 9

3.1.6. Contaminations 11

3.1.6.1. Waste disposal 12

3.1.7. Environment requirements 12

3.1.7.1. The culture medium 12

3.1.7.2. Vessels 13

3.1.8. Characteristics of cells 15

3.1.9. The application of cell lines 16

3.1.10. Macrophage cell lines 18

3.1.10.1. LPS-treated RAW 246,7 cell lines 19

3.1.10.2. Protein kinase C 21

3.2. The Giess reaction 22

3.3. The growth cycle 25

4. MATERIALS AND METHODS 28

4.1. Materials 28

4.1.1. The preparing of the culture medium 30

4.2. Methods 31
4.2.1. The incubation
4.2.2. Subculture of adherent cells
4.2.3. The counting of cells
4.2.4. The plating of cells
4.2.5. The Growth curve
4.2.6. The determination of NO production in LPS-treated RAW 264.7 cells

5. RESULTS

5.1. The growth curve

5.2. The determination of NO production in LPS-treated RAW 264.7 cells

5.2.1. The first experiment
5.2.2. The second experiment
5.2.3. The third experiment

6. THE DISCUSSION

7. THE SUMMARY

8. ABBREVIATIONS

9. REFERENCES

10. THE CONTENT