Comparison of paraquat cytotoxicity in cell cultures: Caco-2 and RBE4

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"The purpose of our lives

is to be happy"

Dalai Lama
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RESUMO

O paraquato (PQ) é um herbicida amplamente usado, especialmente em países em desenvolvimento, desde da década de 1960. É altamente tóxico para os seres humanos, sendo responsável por um grande número de mortes, e há indícios de que poderá estar envolvido no desenvolvimento da Doença de Parkinson em casos de exposição crónica. O PQ exerce a sua toxicidade através do seu ciclo redox interferindo com a cadeia transportadora de elétrons, sendo reduzido ao aceitar um elétron e rapidamente reoxidado formando espécies reativas de oxigênio que vão, por sua vez, induzir stress oxidativo sobre as células.

A linha celular RBE4, um modelo in vitro de barreira hematoencefálica (BHE) de rato amplamente descrito e utilizado em estudos de farmacologia e toxicologia, foi referida como apresentado altos níveis de resistência à toxicidade induzida pelo PQ, por comparação com outras linhas celulares.

No presente estudo, a citotoxicidade do PQ em células RBE4 foi avaliada por exposição a diferentes concentrações (0.5, 1, 2.5, 5, 10, 15, 25, 50 mM) durante períodos de incubação de 24 e 48 h. Foram também utilizadas culturas em diferentes graus de confluência (2 a 6 dias após a realização da sementeira) para determinar a influência que este parâmetro detém sobre a resposta observada. Em paralelo, um estudo idêntico foi realizado em culturas da linha celular Caco-2 incubando as células, 3 dias após a realização da sementeira, com diferentes concentrações de PQ (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5 mM) durante 24 h. Para determinação das espécies reativas de oxigênio realizou-se também o ensaio do diacetato de diclorofluoresceína em células RBE4 e Caco-2 após exposição ao PQ. Os níveis de espécies reativas de oxigênio de culturas RBE4 expostas 5 dias após a sementeira e os observados ao nível de confluência de referência (3 dias) não apresentaram diferenças significativas, no entanto, a mesma comparação entre o 4º dia após a sementeira e o nível de confluência de referência apresentou diferenças pequenas, mas significativas.

Como resultados, observou-se que a resposta citotóxica das células Caco-2 ao PQ foi muito mais intensa do que a observada nas células RBE4, uma vez que as células RBE4 apresentaram um EC50 30 vezes superior ao das Caco-2. No entanto, apesar do facto de as células RBE terem sido expostas a concentrações de PQ 5-10 vezes superiores às das Caco-2, observaram-se valores de espécies reativas de oxigênio idênticos em ambas as linhas celulares quando exposta aos seus respetivos EC50 de PQ.

Concluindo, apesar da alta resistência das células RBE4 à toxicidade do PQ, o EC50 em cada linha celular produz os mesmos níveis de espécies reativas de oxigênio. Assim, parece estar presente um outro mecanismo de defesa contra o PQ em células RBE.

Palavras-chave: barreira hematoencefálica, células Caco-2, células RBE4, paraquato, espécies reativas de oxigênio
Paraquat (PQ) is an widely used herbicide, especially in developing countries, since the 1960's. It is highly toxic to humans and responsible to a great numbers of deaths. PQ has also been postulated to be involved in development of Parkinson's Disease (PD) in cases of chronic exposure to the herbicide. It exerts its toxicity through its redox cycle, where it interferes with the electron transport chain as it is reduced by accepting an electron (e⁻) and then quickly reoxidized, forming reactive oxygen species (ROS) in the process that in turn induce oxidative stress in cells.

The RBE4 cell line, which has been shown to provide an accurate and reliable rat blood-brain barrier (BBB) in vitro model, was reported to be highly resistant to PQ induced cytotoxicity when compared to several other cell lines.

In this study, PQ cytotoxicity on RBE4 cells was evaluated by incubation with eight different concentrations (0.5, 1, 2.5, 5, 10, 15, 25, 50 mM) over 24 and 48 h periods. Also, cultures of different degrees of confluence (2 to 6 days of culture after seeding) were used to assess whether this parameter would influence the response of RBE4 cells to PQ. A similar study was performed in Caco-2 cells with different concentrations (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5 mM) over 24h, 3days of culture after seeding. Furthermore, dichlorofluorescein diacetate assay, for reactive oxygen species determination was also performed in RBE4 and Caco-se cells, after paraquat incubation, for comparing oxidative stress levels.

Comparison between ROS levels of RBE4 cultures exposed 5 days after seeding and the reference confluence degree (3 days) show that there are no significant differences, however, small, but significant, differences were obtained by comparison between ROS levels of cultures exposed 4 days after seeding and the reference confluence degree.

As main results, it was observed that on Caco-2 cells, the measured cytotoxicity response after exposure to PQ was much lower than those observed for RBE4 cultures, as RBE4 cells presented and EC50 approximately 30 fold higher than Caco-2 cells. However, despite the fact that RBE4 cells were exposed to PQ concentrations 5-10 fold higher than Caco-2 cells, similar ROS levels were observed in Caco-2 cells and RBE4 cells, after incubation with EC50 of paraquat.

In conclusion, despite thereresistence of RBE4 cells to paraquat cytotoxicity, the EC50 in each cell culture model produce the same ROS levels. Thus, other mechanisms seem to be related to the paraquat cytotoxicity resistance in RBE4.

**Keywords:** blood-brain barrier, Caco-2 cells, paraquat, RBE4 cells, reactive oxygen species.
# INDEX

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>RESUMO</td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>v</td>
</tr>
<tr>
<td>INDEX</td>
<td>vi</td>
</tr>
<tr>
<td>ABBREVIATION LIST</td>
<td>viii</td>
</tr>
<tr>
<td>INDEX OF FIGURES</td>
<td>ix</td>
</tr>
<tr>
<td>INDEX OF TABLES</td>
<td>xi</td>
</tr>
<tr>
<td>Part I</td>
<td></td>
</tr>
<tr>
<td>General introduction</td>
<td></td>
</tr>
<tr>
<td>1. General considerations</td>
<td>3</td>
</tr>
<tr>
<td>2. Paraquat</td>
<td>6</td>
</tr>
<tr>
<td>2.1 Characteristics and metabolic pathway</td>
<td>6</td>
</tr>
<tr>
<td>2.2 Paraquat transport at the BBB</td>
<td>8</td>
</tr>
<tr>
<td>2.3 PQ penetration into the brain and neurotoxicity mechanisms (in vivo studies)</td>
<td>10</td>
</tr>
<tr>
<td>2.4 In vitro cytotoxicity</td>
<td>13</td>
</tr>
<tr>
<td>3. RBE4 cell line description</td>
<td>20</td>
</tr>
<tr>
<td>Part II</td>
<td>23</td>
</tr>
<tr>
<td>Scope and objectives of the thesis</td>
<td>25</td>
</tr>
<tr>
<td>Part III</td>
<td></td>
</tr>
<tr>
<td>Experimental</td>
<td>25</td>
</tr>
<tr>
<td>Chapter I</td>
<td>26</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>26</td>
</tr>
<tr>
<td>1. Material and methods</td>
<td>27</td>
</tr>
<tr>
<td>1.1 Materials and Reagents</td>
<td>27</td>
</tr>
<tr>
<td>1.2 RBE4 cell culture procedures</td>
<td>27</td>
</tr>
<tr>
<td>1.3 Caco-2 cell culture</td>
<td>28</td>
</tr>
<tr>
<td>1.4 Paraquat cytotoxicity profile on RBE4 cells</td>
<td>28</td>
</tr>
</tbody>
</table>
1.5. Paraquat cytotoxicity profile on Caco-2 cells 29
1.6. Dichlorofluorescein diacetate assay (reactive oxygen species determination) 30
1.7. Statistical analysis 31

Chapter II 32
Results and Discussion 32
  1. PQ-induced cytotoxicity in RBE4 cells 33
  2. PQ-induced cytotoxicity in Caco-2 cells 38
  3. ROS production in RBE4 and Caco-2 cells following PQ exposure 40

Chapter III 45
Conclusions and future perspectives 45

Part IV 48

References 48
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood-Brain barrier</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>B-FGF</td>
<td>Basic human fibroblast growth factor</td>
</tr>
<tr>
<td>DA</td>
<td>Dopamine</td>
</tr>
<tr>
<td>DAT</td>
<td>Dopamine Transporter</td>
</tr>
<tr>
<td>DCFH-DA</td>
<td>2',7'-Dichlorofluorescein diacetate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxorubicin</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HC-3</td>
<td>Hemicholinium-3</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal injection</td>
</tr>
<tr>
<td>MP</td>
<td>methylprednisolone</td>
</tr>
<tr>
<td>MPP⁺</td>
<td>1-methyl-4-phenyl-2,3-dipyridinium</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>MTT</td>
<td>methylthiazolylidiphenil-tetrazolium bromide</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's Disease</td>
</tr>
<tr>
<td>PQ</td>
<td>Paraquat</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>L-PGDS</td>
<td>Lipocalin-type prostaglandin D synthase</td>
</tr>
<tr>
<td>P-gp</td>
<td>P-glycoprotein</td>
</tr>
<tr>
<td>PUS</td>
<td>Pulmonary polyamine uptake system</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SLC</td>
<td>Solute Carrier</td>
</tr>
<tr>
<td>SNpc</td>
<td>Substantia nigra pars compacta</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
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</tbody>
</table>
Concentration-response curves were fitted using least squares as the fitting method and the comparisons in independent experiments, each performed in quadruplicate. Differences between the control and the increasing concentrations of \( \text{H}_2\text{O}_2 \) were estimated using one-way ANOVA followed by the Bonferroni’s multiple comparison post-hoc test. \( **P < 0.01, \quad ***P < 0.001 \) vs control (0 mM \( \text{H}_2\text{O}_2 \)).

Photograph of RBE4 cells at 70-80% confluence (10 x amplification). 

Photograph of Caco-2 cells at 80-90% confluence (20x amplification).

Mechanism of MPTP entry and toxicity [figure from [9]].

Chemical structures of MPTP and MPP+ [adapted from [7]].

Concentration-response curves of Paraquat-induced (PQ) cell death in RBE4 cells with different degrees of confluence (2-6 days after seeding) obtained 24 h after exposure to the herbicide. Results refer to 4-7 independent experiments, each performed in quadruplicate. Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the third day after seeding and all other confluence degrees curves (bottom, top and Log EC50) were made using the extra sum-of-squares \( F \) test. In all cases, \( P < 0.05 \) was considered statistically significant.

Concentration-response curves of Paraquat-induced (PQ) cell death in Caco-2 cells following 24 h of exposure to the herbicide, as assessed by the methylthiazolyldiphenil-tetrazolium bromide (MTT) reduction and the neutral red (NR) uptake assays. Results refer to the mean ± standard deviation (SD) of 1 experiment performed in quintuplicate. Concentration-response curves were fitted using least squares as the fitting method.

Concentration-response curves of Paraquat-induced (PQ) cell death in RBE4 cells with different degrees of confluence (2-6 days after seeding, a to e) respectively. Cells were exposed to PQ for 24 h and 48 h and cell death was assessed by the Neutral Red (NR) uptake assay. Results refer to 4-7 independent experiments, each performed in quadruplicate. Error bars refer to standard deviation (SD). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the 24 h and the 48 h incubation curves (bottom, top and \( \text{LOG EC50} \)) were made using the extra sum-of-squares \( F \) test. In all cases, \( P < 0.05 \) was considered statistically significant.

Concentration-response curves of Paraquat-induced (PQ) cell death in Caco-2 cells assessed by the DCFH-DA assay in 3/4/5 days after seeding cultures. Results refer to the mean ± standard deviation (SD) of 8-6 independent experiments, each performed in quintuplicate. Differences between the control and the different concentrations of PQ were estimated using one-way ANOVA followed by the Bonferroni’s multiple comparison post-hoc test. \( **P < 0.01, \quad ***P < 0.001 \) vs control (0 mM PQ). Differences between the...
control and the different degrees of confluence were estimated using two-way ANOVA followed by Bonferroni’s multiple comparison post-hoc test. $^5P=0.0136$ vs control (3 days after seeding cultures)......41

**Figure 15:** Paraquat (PQ)-induced reactive oxygen species (ROS) production in Caco-2 cells assessed by the DCFH-DA assay in 3/4/5 days after seeding cultures. Results refer to the mean ± standard deviation (SD) of 5-6 independent experiments, each performed in quintuplicate. Differences between the control and the different concentrations of PQ were estimated using one-way ANOVA followed by the Bonferroni’s multiple comparison post-hoc test. **P < 0.01, ***P < 0.001 vs control (0mM PQ). Differences between the control and the different degrees of confluence were estimated using two-way ANOVA followed by Bonferroni’s multiple comparison post-hoc test........................................................................................................42

**Figure 16:** Determined ROS levels of RBE4 and Caco-2 cells (3 days after seeding cultures) exposed to 30 mM and 1 mM of PQ, respectively, for a 24 h period. The PQ concentrations selected correspond to approximately to the EC50 value for PQ of each cell line. Data were analyzed using the t-test. No significant differences were found between the two groups.................................................................................................43
Table 1: Physical and chemical properties of paraquat ion (PQ2+) [adapted from[1]] .................................. 6

Table 2: RBE cell lines reviewed by Roux and Couraud (2005) ................................................................. 21

Table 3: Schematic of the timeline followed to perform cytotoxicity the experiments. RBE4 cells were exposed to different concentrations of paraquat (PQ), on different days after the seed and for different incubation periods. ...................................................................................................................................... 29

Table 4: Schematic of the timeline followed to perform the Dichlorofluorescein diacetate (DCFH-DA) on RBE4 and Caco-2 cells. On different days after the seed cells were exposed to different concentrations of paraquat (PQ) during a 24 h incubation period. .............................................................................................................. 30

Table 5: EC50 (half-maximum-effective concentration) values of the PQ concentration-response fitted curves after the 24 and 48 h incubation periods. ......................................................................................................................... 35

Table 6: EC50 (half-maximum-effective concentration) and bottom values of the PQ concentration-response fitted curves after the 24 h incubation period. .......................................................................................................... 37
This thesis will be divided in the following three parts:

- **Part I – General introduction**

- **Part II – Scope and objectives of the thesis**
  
  In this section the aims of this thesis will be presented and discussed.

- **Part III – Experimental**
  
  This section will be divided into three chapters:
  
  Chapter 1: Materials and methods
  
  Chapter 2: Results and discussion
  
  Chapter 3: Conclusions and future perspectives

- **Part IV - References**
Part I

General introduction
1. General considerations

Paraquat (PQ), or paraquat dichloride, 1,1'-dimethyl - 4,4'-bipyridinium dichloride (Figure 1), along with diquat, belongs to the bipyridylium quaternary ammonium herbicides and exert their toxicity through their redox properties [1]. PQ was first described in 1882 by Weidel et al. [1], and has been widely used in developed and developing countries since the 1960's [2]; [1]) However, it has been banned from the European Union since 2007 due to its toxicity to humans: considering an average adult (~70 Kg), ingesting as little as 20 mL of a 20% solution of PQ, corresponding to 55 mg/Kg, may be enough to cause death [3]. Even though it is responsible for a great number of deaths, mainly due to intentional or accidental ingestion, it is still used all over the world due to its properties that make it an excellent herbicide: it is very effective and starts killing the plants as soon as it touches the leaves; allows the roots to maintain their integrity and, consequently, enables them to prevent soil erosion; it is highly hydrophilic and thus is unable to be absorbed by intact skin; it is unable to reach humans alveoli due to the size of its aerosolized particles; it is rapidly and extensively inactivated once in contact with the soil; and also because it is a rather low cost herbicide [1]. These characteristics make PQ and excellent herbicide to be used especially in developing countries, with China being the largest producer and consumer of PQ [4], and the majority of cases of PQ poisoning being reported in Asiatic countries like China, South Korea or India.

Figure 1: Chemical structure of paraquat in ionic state (PQ2+). The two Cl- molecules represent its commercialized salt form (dichloride). [Adapted from [1]]
In humans, PQ exerts its toxicity mostly in highly irrigated organs [1], accumulating especially the lungs [5] due to the highly active polyamine uptake system, which is capable of transporting PQ against the concentration gradient [6].

The PQ relation to Parkinson's disease (PD) is of increasing interest, and there are epidemiological studies that relate the PQ's toxic effects and mechanisms with the pathophysiology of PD [7, 8]. PD is a neurodegenerative disease that leads to loss of control of motor skills, with tremors, loss of balance and muscle stiffness [7, 9], and may also lead to non-motor symptoms like hyposmia, constipation, depression, sleep disturbance or changes in cognitive function [9]. PD is characterized by the progressive degeneration of the dopaminergic neurons in the substantia nigra pars compacta (SNpc), leading to loss of neuronal connection between the substantia nigra (SN) and the striatum resulting in severe striatal dopamine deficiency [7, 9]; and also for the presence of eosinophilic cytoplasmic inclusions known as Lewy bodies (LBs) in some remaining neurons [7, 9]. However, if we consider PQ's high hydrophilicity we can easily assume it is not able to cross the Blood-Brain barrier (BBB) by itself in order to reach the brain and exert its toxicity, thus making the BBB an interesting subject of study.

The BBB is constituted by a monolayer of specialized endothelial cells that form the brain capillaries, a basement membrane (BM), the end-feet of astrocytes, and pericytes [10, 11]. These endothelial cells are polarized cells, with an apical membrane facing the blood (luminal membrane) and a basolateral membrane facing the brain tissue (abluminal membrane); and, morphologically, are long and spindle-shaped, making them very thin with a small cytoplasmic space between the luminal and abluminal membranes [10, 11]. Brain capillaries are surrounded by the basement membrane, which is complex in structure and provides external support for the endothelial cells, surrounds the endothelial cells, and mediates the contact of the pericytes, astrocytes and neurons with the endothelial cell (Figure 2) [10, 11]. One important physical characteristic of the BBB is the presence of the tight junctions between the endothelial cells that seal the paracellular space, and thus limiting the passage of hydrophilic molecules [10-12]. In addition, cerebral endothelial cells show low pinocytotic activity [11].

Another very important characteristic of the BBB is its ability to dynamically select which hydrophilic molecules enter the brain through the endothelial cells. This is only possible due to the presence of transmembrane proteins responsible for the uptake or excretion of endogenous or exogenous compounds called transmembrane transporters [12]. It is important to consider the role of these
proteins, not only when studying the efficacy of drugs in case of treatment, but also when evaluating the brain's correct homeostasis and function [12].

**Figure 2:** Morphology of brain capillaries. A - nucleus; B - Pericyte; C - Neuron; D - Astrocyte end foot; E - Tight Junctions; F - Basement membrane; G - mitochondrion; I - Endothelial cell. The basement membrane surrounds the entire endothelial cells mediating the interface between the endothelial cells and the pericytes, the neurons and the astrocytes end foot. [Adapted from [13]]
2. Paraquat

2.1 Characteristics and metabolic pathway
PQ, in its common state, is oxidized and carries two positive charges (PQ$^{2+}$), one on each of its nitrogen atoms, does not occur naturally and is manufactured as a salt, so it is highly hydrophilic, and almost insoluble in organic solvents. It is in its oxidized form that PQ is most prone to react and bind to an electron [7]. Table 1 resumes some of the chemical properties of PQ.

<table>
<thead>
<tr>
<th>Class</th>
<th>bipyridylium herbicide</th>
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<tbody>
<tr>
<td>Molecular formula</td>
<td>C$<em>{12}$H$</em>{14}$N$_2$</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>186.3 (ion), 257.2 (dichloride)</td>
</tr>
<tr>
<td>IUPAC name</td>
<td>1,1-dimethyl-4,4-bipyridilium</td>
</tr>
<tr>
<td>Specific gravity (20°C)</td>
<td>1.240–1.260 g/cm$^3$</td>
</tr>
<tr>
<td>Physical state</td>
<td>white (pure salts), yellow (technical products) crystalline, odorless, hygroscopic powders</td>
</tr>
<tr>
<td>Storage stability</td>
<td>Indefinitely long in original container</td>
</tr>
<tr>
<td>pH of liquid formulation</td>
<td>6.5–7.5</td>
</tr>
<tr>
<td>Decomposition</td>
<td>Thermal decomposition occurs at ~340°C with formation of poisonous vapors, and under UV radiation</td>
</tr>
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</table>

PQ exerts its phytotoxicity by inhibiting the photosynthesis, which it does by interfering with the electron transport chain, through a mechanism called the redox cycling (Figure 3) [1, 14]. This mechanism is transversal to other organisms, plants or mammalian, like humans.

Due to its redox potential of -0.446 V, PQ$^{2+}$ is readily reduced, forming PQ$^+$ and NAD$^+$ or NADP$^+$, by the NADPH-cytochrome P-450 reductase, nitric oxide synthase, xanthine oxidase [1, 14], and NADH-ubiquinone oxireductase [1], being immediately reoxidized to PQ$^{2+}$ in the presence of molecular oxygen (O$_2$) [1, 14], with a rate constant of $7.7 \times 10^8$ M$^{-1}$s$^{-1}$ [1], since the redox potential of O$_2$ is much lower [1, 14], forming a superoxide radical (O$_2^-$), which in turn will form reactive oxygen species (ROS) like hydrogen peroxide (H$_2$O$_2$), by dismutation of O$_2^-$, and also hydroxyl radicals [1, 14]. These ROS induce
oxidative stress and subsequent cell death, not only through inhibition of several important metabolic pathways but also by the disruption of lipids, proteins and DNA [7], and activating inflammatory pathways involving the nuclear factor kappa B (NK-κB) [15] and the activator protein 1 (AP-1) [16]. Also, PQ's redox cycling depletes the cell's supply of NADPH, which is important for the maintenance of normal cell metabolic processes and to enable the regeneration of reduced glutathione (GSH) by reduction of oxidized glutathione (GSSG) [7, 17]. GSH is very important as an antioxidant cell defense as it oxidized to GSSG during the detoxification of $\text{H}_2\text{O}_2$ and other peroxides [7, 17].

![Paraquat redox cycle](adapted from [7])

As mentioned previously, PQ exerts its toxicity in the entire human body, accumulating, particularly, in the lungs, more specifically in alveolar type I and II pneumocytes and in Clara cells, maybe due to PQ's similarity to some endogenous polyamines like, putrescine and cadaverin, which makes the pulmonary polyamine uptake system (PUS) recognize it as a substrate [1, 18]. Also, the lungs have high alveolar $\text{O}_2$ tension so they are even more exposed to PQ's toxicity mechanisms [1].

Most PQ ingested, in humans, is excreted in the feces and only 1-5%, over 1-6h period, is absorbed by the gastrointestinal tract [1], and ingested food decreases the amount of PQ absorbed. After absorption it is distributed throughout the body, accumulating in the lungs.
2.2. Paraquat transport at the BBB

It is generally accepted that PQ is not able to freely cross the BBB due to its high hydrophilicity, however its ability to cross this barrier altogether has been controversial, with different studies reporting different results [1, 19-22]. The well known neurotoxin 1-methyl-4-phenylpyridium (MPP+) (Figure 3), also known to be related to PD by inducing its disease mechanisms, is considered to be one of the best characterized toxicants at inducing PD-like symptoms in animal models, as it meets many of the criteria required for such role [7]. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Figure 4), the precursor to MPP+, due to its lipophilic nature, is able to freely cross the BBB [9]. Then, it is metabolized to MPP+ by monoamine oxidase (MAO)-B [19] in astrocytes [9], from where it is extruded by OCT3 and taken up into dopaminergic neurons, by the DA transporter, where it inhibits complex I of the mitochondria disrupting oxydative phosphorilation, ATP synthesis and inducing the formation of ROS, leading to cell death (Figure 5) [7, 9]. What is also important to know about the MPP+ is that it is similar to PQ, structurally speaking (Figure 4) [7, 20], which has been used as premise for many experimental studies, both with animal or in vitro models [20].

![Figure 4: Chemical structures of MPTP and MPP+](adapted from [7]).
As previously mentioned, the PUS recognizes PQ as a substrate transporting it across the cell membrane, however, the fact that this transport system is not expressed in the BBB [7, 21] suggests that other transporter family(ies) are responsible for this herbicide’s penetration in the brain. Several studies have proposed the neutral amino acid transport system of the BBB as possible transporter of PQ across this barrier with promising results [7, 21, 23], having shown that PQ’s penetration did not occur due to lesion of the endothelial cells by the herbicide [21], thus compromising the integrity of the barrier contradicting the postulations that this could be the mechanism of entry. These studies will be analyzed in more detail in the next chapters.

Another essential characteristic to consider when studying the entry of PQ in the CNS through the BBB is the existence of several efflux pumps [22], most from the ATP-binding cassette transporters superfamily (ABC transporters), like the P-glycoprotein (P-gp), which is known to transport PQ back to the circulation [24], which make the entry of PQ into the brain more difficult, and thus help with neuroprotection against toxicity from PQ.

Epidemiological studies seem to suggest that PQ and PD are related, however they have some limitations, since it is not always easy to clarify with certainty, which toxic pesticides the study subjects were exposed to [7, 20], and also, there are recent studies that disprove this hypothesis [25].
Concluding, the available data on PQ's ability to cross the BBB, postulated by a possible relation between exposure to PQ and PD and supported by the similarity of the chemical structure of PQ and MPP* and their cytotoxicity mechanisms, and its neurotoxic mechanisms is still not conclusive. Authors and studies results differ in conclusions, with some being convinced that PQ exposure is an etiological factor for PD, hence being able to cross the BBB [7, 21, 23, 26], while others are more skeptical or have different conclusions [19, 20, 25], but what does seem unanimous is the necessity of more studies, both in vitro or with animal models, in order to definitely conclude whether or not this relationship exists, and if so through which specific mechanisms and pathways [27].

An interesting point of view that is usually overlooked and lacks in studies is the PQ's ability to damage the BBB by exerting its cytotoxicity on the endothelial cells themselves [22].

2.3. PQ penetration into the brain and neurotoxicity mechanisms (in vivo studies)

There are a great number of studies on PQ's toxicity in the CNS, and more specifically in the brain, using animal models. This is of great interest, mainly, due to the suspicion that this herbicide, and others, might be an etiological factor to the development of PD, and also due to the previously mentioned controversy on this field of research.

In 2003, Shimizu et al. proposed that PQ exerts its neurotoxicity by activating the excitotoxic pathway, which is also considered to play an important role in the progressive degeneration of dopamine neurons in PD [28]. Excitotoxicity is a specific type of neurotoxicity mediated by glutamate and occurs through over stimulation of its neuronal receptors, like the N-methyl-D-aspartate (NMDA) receptor or the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor [29, 30]. This overstimulation leads to pathologically high intracellular levels of Ca^{2+}, through the NMDA channels, and depolarizing the cell, which in turn activates several degenerative pathways, including the neuronal nitric oxide synthase (NOS). NOS produces nitric oxide (NO), which reacts with the superoxide radicals to form the highly reactive peroxynitrite anion, thus inducing mitochondrial dysfunction [28, 29].
Part I: General introduction

In their study, Shimizu et al. subcutaneously administrated PQ into Wistar rats (8 weeks old, 210-260g) once a day for 5 days, and collected data suggesting that PQ is taken into the striatal tissue, at least partially, by the dopamine transporter (DAT), since GBR-12909, a specific inhibitor for DAT, significantly reduced PQ uptake. Also, a dose-dependent increase in extracellular glutamate was observed and, accordingly, extracellular levels of NO anions (NO$_2^-$ and NO$_3^-$) were slightly higher after PQ perfusion. They also observed long-lasting elevation of dopamine levels, following the previously described alterations, which they proposed to be the last step in PQ-induced cytotoxicity in dopaminergic neurons [28].

On another study, Bartlett et al. analyzed PQ penetration into the brain of four adult male rhesus macaques (10-13 Kg) [19]. To assess this they radiolabeled the eleventh carbon of paraquat ([$_{11}$C]paraquat) before systemically administrate it to the subjects, and then performed a 90 min dynamic PET scan. They observed that the BBB of these non-human primates was able to efficiently exclude the [$_{11}$C]paraquat from the brain (Figure 6), and that the only brain structures where PQ significantly penetrated were structures that lacked the BBB, namely the pineal gland and the lateral ventricles. Interestingly, even though these findings are contrary to the hypothesis that PQ induces PD or PD-like symptoms, the authors do not exclude this possibility by considering that chronic exposure to PQ might gradually compromise the BBB integrity and ability to keep protecting the brain from PQ toxicity, thus, eventually, accumulating in dopaminergic neurons to the point where neurodegeneration starts [19]. This last point of view underlies the previously mentioned importance in assessing the PQ's ability to damage the BBB.

On the study conducted by Rappold et al., two parallel experiments were conducted in order to assess the role of the DAT and the OCT3 (SLC22A3) in nigrostriatal damage induced by PQ. On one of them, the neurotoxicity study, Oct3$^{-/-}$ and Oct3$^{+/+}$ mice (10-12 weeks old) were injected intraperitoneally (i.p.) with 10mg/kg of PQ$^{2+}$ solution every second day for 20 days, and, after 7 days after the last injection, they were sacrificed and prepared for cell counting, striatal tyrosine hydroxylase (TH) immunoreactivity and DA levels measurement; and on the other experiment consisted of an in vivo microdialysis study, also using Oct3$^{-/-}$ and Oct3$^{+/+}$ mice (10-12 weeks old), using the microdialysis probe technique, which consists on inserting a probe, in this case to the brain, which enables continuous sampling of brain dialysates taken from the subjects, for posterior HPLC measurement of DA levels. On the
microdialysis experiment, mice were injected (i.p.) with 15 mg/kg of PQ$^{2+}$ and dialysates were taken every 30 min for 3 h after the injection. With their results, they were able to conclude that PQ$^+$ is a substrate of Oct3 and that it is taken up into DA neurons by Oct3 and DAT. They observed that PQ induces its neurotoxicity by oxidative stress due to ROS production during its redox cycling, since blocking DAT or NADPH oxidase, which is known to be present in microglia, astrocytes and DA neurons, lead to lower levels of PQ inside the cells and lower production of ROS. They also noticed an increase in ROS levels when PQ$^+$ was combined with DA, which suggests that DA might catalyze PQ toxicity in DA neurons. So, they propose that the mechanism by which PQ induces degeneration of DA neurons is mediated by NADPH oxidase, when it starts the PQ$^{2+}$ redox cycling, outside the DA neurons, producing PQ$^+$, which, in turn, is taken into DA neurons by DAT and Oct3 where it enters a new redox cycling and, again, produces ROS and leads to neurodegeneration [27].

Concluding, even though some studies have yielded promising results in terms of elucidating some of PQ's neurotoxic mechanisms and associate them with PD there is still major controversy in this field of research due to the contradictory observations in other studies, which makes some members of the scientific community skeptical to the importance of PQ exposure in PD in humans [20]. Also, it is still important to create better in vivo animal models in order to study the effects of PQ chronic exposure in brain and BBB function. Especially if we consider the hypothesis that chronic exposure to PQ might damage the endothelial cells of the BBB and, consequently, alter the herbicide's brain penetration over time.
Figure 6: Fused PET/MR images of [11C]Paraquat in adult male rhesus macaque brain displayed in axial (A) and sagittal (B) orientations. The arrows indicate the posterior lobes of the lateral ventricles (2A) and the pineal gland (2B). Note that most of the radioactivity is extra-cerebral (muscle, nasopharynx, etc.), indicating that most of 11C]Paraquat was excluded from the brain [Image taken from [19]].

2.4. In vitro cytotoxicity

In vitro studies on PQ are also very abundant, if not more, and are as important as in vivo studies. In vitro studies enable us to test hypothesis, or preliminary ideas, without sacrificing animal lives which, bioethically speaking and also from a personal point of view, is a serious advantage over in vivo models. Also, they allow us to experiment with much simpler models than is the case with animal models, which might be an advantage since we do not have to work and interpret through all the pathways and interactions the compose metabolism of living organisms. However, this can also be a disadvantage since it is not always easy to extrapolate the results to animals, and they do not always represent what happens when all variables in complete organisms are equated. Below, a few studies where cytotoxicity of PQ was evaluated on different cells lines will be briefly reviewed.

A very recent study, conducted in our laboratory, addressed the previously mentioned issue of the toxic effects of PQ on the endothelial cells that compose the BBB [22]. This study intended to assay the PQ toxicity profile on RBE4 cells, used as a rat BBB model, and the mechanisms that might be involved in its uptake and efflux. The transport mechanisms studied were chosen based on previously described transporters as being involved with PQ uptake, such as the large neutral amino acid transport system [21], the basic amino acid
transport system, the basic amino acid transport system [21], the polyamine uptake system [1] and the choline-uptake system [22]. The role of each of these transporters was tested by co-exposure of PQ and known substrates to the transporter under study, so that if PQ is transported by the same transporter as the substrate there should be a decrease in PQ uptake. PQ is known to be a substrate for P-gp, and its role in extruding PQ from inside the cell was also studied, this time with co-administration of PQ and elacridar (GF120918), a known third generation P-gp inhibitor. The first observation of this study was the extraordinary resilience RBE4 cells showed to PQ toxicity, as at 24h of exposure to 50 mM PQ only approximately 43% cells were dead, which is up to 10 fold higher than the reported viability in other cell lines (0.5 mM to 1 mM depending on the cell line). It was also observed that, from the tested transporters, PQ is taken up into RBE4 cells only by the choline-uptake system as the presence of hemicholinium- 3 (HC-3), an inhibitor for this transport system, increased the cell viability and the EC50 [EC50(PQ)=3.1 mM; EC50(PQ+HC-3)=4.2 mM] when exposed to the herbicide; and no differences were observed for the other transport systems. The study confirmed that P-gp is active in the RBE4 cell line and transports PQ out of these cells. A comparison on the sensitivity of the Neutral Red uptake assay (NR) and the MTT assay is also mentioned, and is of considerable importance to our study, as the NR assay is reported to have higher sensitivity, probably due to the fact that the enzymes responsible for the MTT reduction may be involved with the PQ toxic pathway [22].

In 2011, Silva et al. [31] performed a study seeking to clarify if P-gp induction by doxorubicin (DOX) would be reflected in lower cytotoxicity by PQ in Caco-2 cells. This study was performed based on the premises that P-gp is expressed in the apical surfaces of many epithelial tissues, like the BBB, kidneys or the gastrointestinal tract [32], and also that P-gp was effective at reducing PQ’s lung toxicity in rats [33]. Caco-2 cell line (Figure 7) is derived from human colorectal adenocarcinoma and its cells are generally accepted as an accurate model to study the drug absorption and excretion in the human gastrointestinal tract. Caco-2 cells are also known to express several transmembrane transporters, including P-gp in the apical membrane, in similar levels to those verified in normal human jejunum. First, DOX cytotoxicity was evaluated on this cell line (concentrations ranging from 0.1 to 100 µM), revealing that, at 96 h of exposure, the EC50 was around 100 µM, and that 20%, or more, cell death was observed only after exposure to 100 µM for a period of 24 h, or more. These cytotoxicity
studies were performed using the MTT reduction assay. Then, DOX capacity to induce P-gp expression and activity needed to be confirmed, and this was done by flow cytometry. Using the same DOX concentrations and the same exposure periods, significant increases in P-gp expression were observed, increases as high as, approximately, 350% of control levels shortly after 6h exposure. Significant increases in P-gp activity were also observed following DOX exposure, as not only the higher DOX concentrations considerably increased P-gp activity after a short 6h of exposure period, but also after longer exposure periods, like 48h, as little as 0.1 µM of DOX effectively increased P-gp activity by 136%. Following these determinations, PQ cytotoxicity on Caco-2 cells was evaluated by the MTT reduction assay, at the same time points (6, 12, 24, 48, 72 and 96 h) as for the previous assays, and concentrations ranging from 0.1 to 1000 µM. Here, Silva, et al. 2011 observed that after 12 h exposure to 500-1000 µM PQ cell viability had decreased to, approximately, 90%, and that the EC50 after 24 h and 48 h was just above the 1000 µM (1047 µM) and 500 µM, respectively. Then, after analyzing the described data the experiment was designed, according to the time line shown in Figure 8, with the chosen time-points and concentrations being: 24h exposure to 5-100 µM DOX, and 24 h exposure to 0-5000 µM PQ, followed by an MTT assay to test the cell viability. This study observed significant decreases in cell death following co-exposure with DOX and PQ when compared to exposure to PQ alone. This decrease in cell death with higher EC50 (at around 1800 µM) was not concentration-dependent [31].
Figure 7: Photograph of Caco-2 cells at 80-90% confluence (20x amplification)

Figure 8: Schematic representation of the sequence of experimental procedures when evaluating doxorubicin (DOX) protective effects against paraquat (PQ) cytotoxicity [from [31]]
In 2013, the same group performed an experiment related to the previously described study, where they intended to study whether the protective effect of DOX against PQ cytotoxicity remained if it was administered after PQ [34]. The protocols were identical, except in this case DOX exposure was conducted 6h after PQ exposure. Five concentrations of PQ (100, 500, 1000, 2500 and 5000 µM) and 10, 50 or 100 µM of DOX were tested. In this study, the EC50 was 1244 µM, which is slightly higher than the one observed in [31]. Also, a P-gp inhibitor (UIC2 antibody) was used in order to confirm the involvement of this transporter in DOX protective effects. Again, the involvement of P-gp in Caco-2 cytotoxicity was confirmed, by observing that when P-gp was inhibited maximal cell death increased and that cells treated with PQ+DOX+UIC2 the concentration-response curves fitted below (less cell death) the PQ+UIC2 curves and above (more cell death) the PQ+DOX curves. They also observed significantly less cell death with increasing concentrations of DOX following PQ exposure, with significantly lower TOPs for the concentration-response curves: 88.1%, 81.5%, 48.4% and 38.9% for 5, 10, 50, 100 µM respectively. To help confirming this hypothesis that the observed cell death was due to P-gp activity acting as an efflux pump for PQ, the intracellular levels of the herbicide were measured by gas chromatography-ion trap-mass spectrometry (GC-IT-MS) and significant lower levels of PQ were detected in cells exposed to PQ+DOX50 µM when compared to PQ alone, and higher levels of intracellular PQ in cells exposed to PQ+UIC2 (these differences were more clear at higher PQ concentrations, namely 2500 and 5000 µM). It is also important to mention that the presence of UIC2 did not, completely, cancel DOX protective effect, suggesting that P-gp activity is not the sole defensive mechanism in which DOX is involved. These two studies showed the protective role that DOX has in Caco-2 cells and the group proposes that these studies are taken further, to possible apply these principles in human treatments [34].

PQ cytotoxicity has also been studied in other cells lines, like is the case of the SH-SY5Y human neuroblastoma cell line. Several PQ cytotoxicity studies, and also in neurodegenerative diseases studies like, Alzheimer’s or PD, have been performed on this cell line [35-40], in order to get a better understanding on PQ neurotoxic mechanisms and whether they are related to the development of PD and its pathological pathways, as this neuron model expresses many of the phenotypes, biochemical and functional, of human dopaminergic neurons [35].

In 2012 Fujimori et al. [36] set out to study the role of lipocalin-type prostaglandin D synthase (L-PGDS) on PQ induced neuronal damage using the
SH-SY5Y cell line as the cell model. L-PGDS was first identified in the rat brain as a catalytic enzyme involved in prostaglandin D$_2$ (PGD$_2$) synthesis [41]. Also, L-PGDS has an homologue structure to the proteins of the lipocalin gene family, and some members of this family are postulated to be involved in oxidative stress protection [36]. In this rather complex study, they exposed SH-SY5Y cell to three different PQ concentrations for 24 h, namely 0.05, 0.1 and 0.5 mM. They also performed several other enzymatic and genetic assays, but what is important about this study to our study is that cytotoxicity levels of PQ on this cell line, with a cell viability of around 50% for the 0.5 mM concentration being reported, which is around 100x more susceptible than what was reported to the RBE4 cells in [22] (43% cell death with 50 mM PQ exposure).

On a different study, SH-SY5Y cells were exposed to 0.1, 0.5 and 1 mM of PQ for 24, 48 and 72h [39], and viability was measured by the lactate dehydrogenase (LDH) kinetic assay and the, already mentioned, MTT assay. This study reported a concentration-dependent susceptibility to PQ toxic effects where, at the 24 h time-point in the MTT assay, about 65-70% remained viable when incubated with 1 mM PQ. Even though these results showed a considerably higher resistance to PQ toxicity than the ones obtained by Fujimori et al. [36], it is still much lower than those observed with the RBE4 cells [22]. Furthermore, Martins et al. [39] did not observe differences, after 24h incubation with PQ, on the LDH assay, even though differences were visible with the MTT assay, leading the group to believe that PQ toxicity is initially observed in the disruption of the mitochondrial electron transport chain [39].

The A549 cell line was first described by Giard et al. in 1972 [42], and is now commercially available. This cell line is derived from lung tissue extracted from a 58 year-old man [42] (human lung adenocarcinoma epithelial cell line) and is used as a model for human alveolar type-II epithelial cells [3, 43]. In 2011, Zerin et al. [3] conducted a study where they took a look into the effects of methylprednisolone (MP) in PQ-treated A549 cells. MP is a corticosteroid drug used for different treatments like autoimmune diseases, bronchitis, arthritis, and others. This group hypothesized that it might also induce P-gp activity, which might be reflected in lower intracellular levels of PQ [3]. A549 cells were exposed to seven concentrations of PQ (100-700 µM) for 24 h, and to 300 µM during 6/12/18/24/48/72/120 h, and the cell viability was determined by the MTT assay. Their results suggested that MP does in fact have a protective role against PQ-induced cytotoxicity by inducing P-gp activity, as a significant decrease in cell death was observed in PQ-exposed cells. However, to
determine this MP-induced changes in the A549 cells when exposed to PQ, the group had to also determine their susceptibility to PQ. For this, they exposed A549 cells to seven concentrations of PQ (100-700 µM) for 24h; and to 300µM during 6/12/18/24/48/72/120 h, and used the MTT assay to determine the cell viability. They observed that at the 300 µM concentration, after 24 h, cell death was already rather significant, and that when treated with 700µM for 24h cell death was around 80% [3]. Again, A549 cells' susceptibility to PQ is much greater than the reported susceptibility of RBE4 cells [22].

This small review lets us conclude that in fact RBE4 cells are resistant to much higher level of PQ than other tested cell lines, which raises the question "why?".
3. RBE4 cell line description

For a long time, studies have been performed in order to create reliable *in vitro* models for BBB studies, since these models may provide us tools to assess the cellular events and restrictive properties of the BBB [44] without the sacrifice of animal lives. Several *in vitro* models have been created from cocultures between bovine, porcine, rodent, or human brain endothelial cells and rodent, or human astrocytes [45]. Noting that most *in vivo* studies are performed on small rodents, especially rats, the importance of a rat brain endothelial (RBE) cell model becomes greater. On the other hand, usually these mentioned *in vitro* models have some drawbacks like their high cost, both in terms time and money; the fact that they may de-differentiate in cell culture; or that, in primary cultures, it is difficult to eliminate all non-endothelial cells, which may prevent the characteristic monolayer from forming correctly, since endothelial cells appear to be unable to grow over other cells [45].

The first RBE cell line to be described was the RBE4 by (Roux et al., 1994) (Figure 9), however, the most recent review on the subject [45] mentions several other RBE cell lines listed in Table 2. These immortalized cell lines were selected based on a set of phenotypic criteria: exhibition of a nontransformed phenotype, expression of certain endothelial cell markers, and expression of specific BBB properties. Most of these RBE cell lines were immortalized by transfection.

The RBE4 cell line was immortalized by transfection with the pE1A/neo plasmid using the calcium phosphate coprecipitation procedure, which, basically, consists in binding the DNA plasmid to the insoluble calcium phosphate precipitate that adheres to the cell surface and the complex then enters the cell by endocytosis [46]. Even though this technique yields low efficiency it is relatively simpler, when compared to other methods of transfection [47]. The plasmid used in this procedure contains the E1A region of adenovirus 2 and a neomycin resistance gene, so, after the transfection procedure, cells were selected by culture in medium containing 300 µg/mL G418, which is an aminoglycoside antibiotic [45].
Table 2: RBE cell lines reviewed by Roux and Couraud (2005)

<table>
<thead>
<tr>
<th>Cell line name</th>
<th>Immortalized gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RBE4</td>
<td>Adenovirus E1A gene</td>
<td>[48]</td>
</tr>
<tr>
<td>GP8/3.9</td>
<td>SV-40 T-antigen</td>
<td>[49]</td>
</tr>
<tr>
<td>GPNT</td>
<td>SV-40 T-antigen</td>
<td>[50]</td>
</tr>
<tr>
<td>RBEC1</td>
<td>SV-40 T-antigen</td>
<td>[51]</td>
</tr>
<tr>
<td>TR-BBBs</td>
<td>SV-40 T-antigen</td>
<td>[52]</td>
</tr>
<tr>
<td>rBCEC4</td>
<td>Polyoma virus large T-antigen</td>
<td>[53]</td>
</tr>
</tbody>
</table>

As mentioned above, RBE4 cells have to meet some criteria in order to be considered an immortalized BBB in vitro model. Their nontransformed phenotype was confirmed by observing: the formation of contact-inhibited monolayers of cells; that the proliferation is dependent on the presence of specific cell substrates and serum components, and β-FGF; no growth in soft agar; and no formation of tumors after subcutaneous, or intrathecal, injection in athymic nude mice [45]. For a detailed description of the expressed endothelial cell markers on the several RBE cell lines the reader is referred to Table II of [45], but, shortly, these cell markers include morphological cell shape, intercellular adhesion molecules, adherens junctions, binding sites for lectin, endothelial origin and vasoactive substances, like some cytokines, that induce NO production. The other parameter that the cell lines need to meet is the accurate expression of specific BBB properties which include tight junctions cytoskeletal differentiation and tightness of the monolayer, γ-glutamyltranspeptidase and alkaline phosphatase (ALP) enzymes activity and membrane transporters activity, including the Pgp. For further description of these parameters for the different RBE cell lines the reader is referred to Table III of [45]. It is important to mention, also, that these cell lines, including the RBE4, do have some discrepancies when compared to in situ function of the BBB, such as lower activity levels of enzymes or transporters and higher paracellular permeability, which must be taken into account when performing studies using these models as tools transposing the results for in vivo or in situ models [45].
Figure 9: Photograph of RBE4 cells at 70-80% confluence (10 x amplification).
Part II
Scope and objectives of the thesis
SCOPE AND OBJECTIVES OF THE THESIS

PQ is a widely used herbicide due to its biochemical properties and low cost. Even though PQ is rapidly inactivated once it touches the ground, it is also responsible for a great number of fatal intoxications, mainly through accidental ingestion or suicide attempt. PQ exerts its toxicity in humans, in the most irrigated organs with lungs as the main site of accumulation, however there is an increasing interest on PQ's neurotoxicity and the development of PD-like symptoms. Unfortunately, there is still some controversy on this subject as some authors find evidence of a correlation between the neurotoxicity mechanisms of PQ and the pathological mechanisms leading to PD, while others not only do not find any correlation as even propose that PQ cannot cross the BBB.

Several studies have been performed in order to clarify PQ's neurotoxicity mechanisms, both in vivo and in vitro, however few information on the effects of PQ's cytotoxicity on the endothelial cells of the BBB, and on whether these cells have ways to cope with the stresses induced upon cell entry, is available. This is an important aspect to consider, since chronic exposure to PQ might, gradually or in the long-term, compromise the BBB ability to protect the CNS from PQ and thus leading to more severe neurodegeneration.

The aim of this thesis is, therefore, to evaluate the influence of the degree on confluence on the susceptibility of RBE cells to PQ exposure; to investigate the involvement of ROS in the PQ-induced toxicity in RBE4 cells; and to compare the ROS contribution to PQ-induced cytotoxicity in PQ-resistant RBE4 cells and PQ-sensitive Caco-2 cells.
1. Material and methods

1.1. Materials and Reagents

Minimal Essential Medium Eagle (MEM) (M0643), Nutrient Mixture F-10 HAM (HAM F-10), Dulbecco’s Modified Eagle’s Medium - high glucose (DMEM), Paraquat, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), neutral red (NR) solution, Neomycin trisulfate salt hydrate and β-FGF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Nonessential amino acids (NEAA), heat inactivated fetal bovine serum (FBS), 0.25% trypsin/1 mM EDTA (25200-072), antibiotic (10000 U/mL penicillin, 10000 μg/mL streptomycin), Fungizone (250 μg/mL amphotericin B), human transferrin (4 mg/mL), Collagen type I rat tail protein (3mg/mL) and HBSS (with or without Ca\(^{2+}/\text{Mg}^{2+}\)) were purchased from Gibco Laboratories (Lenexa, KS, USA). Ethanol absolute and glacial acetic acid were acquired from Panreac Química (Castellar del Vallès, Barcelona, Spain). Sodium bicarbonate and dimethyl sulfoxide (DMSO) were acquired from Merck Millipore (Darmstadt, Germany). HEPES was acquired from Fisher Scientific (Porto Salvo, Portugal) and 2’,7’-Dichlorofluorescein diacetate (DCFH-DA) was acquired from Fluka Biochemika.

1.2. RBE4 cell culture procedures

1.2.1. RBE4 cell culture

Immortalized rat brain microvessel endothelial cells [(Roux et al., (1994))] were grown in MEM/Ham's F10 (1:1) supplemented with 300 μg/ml neomycin, 10% FBS, 1 ng/ml β-FGF, 100 U/ml penicillin G, 0.25 μg/ml amphotericin B, 100 μg/ml streptomycin, 25mM sodium bicarbonate and 25mM HEPES. This mixture will be referred to as cell culture medium and was changed every 48 to 72 h. These cells (passages 70–85) were maintained in a humidified atmosphere of 5% CO\(_2\) at 37°C. For subculturing, the cells were washed/rinsed with HBSS-/ and then dissociated with 0.25% trypsin-EDTA, which was inactivated with culture medium, and subcultured in 75-cm\(^2\) flasks. For all experiments cells were seeded in 96-well plates at a density of 10 000 cells per well.
1.2.2. 96 well plate coating with collagen

96 well Falcon® plates were coated with 5 µg/cm² of type I rat tail collagen solution. Collagen stock solution was diluted in 0.02 M acetic acid before being used to coat the bottom of the wells and, after 15 min inside the laminar flux chamber without the lid, plates were stored at 4ºC until seed day. Before seeding the cells, the wells were rinsed with HBSS+/-, with a volume no smaller than the volume of collagen solution used during the coating process.

1.3. Caco-2 cell culture

Caco-2 cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% FBS, 1% NEAA, 1% antibiotic, 1% fungizone and 6 μg/ml transferrin. This mixture will be referred to as cell culture medium and was changed every 48 to 72 h. These cells (passages 75–90) were maintained in a humidified atmosphere of 5% CO₂ at 37°C. For subculturing, the cells were washed/rinsed with HBSS-/- and then dissociated with 0.25% trypsin-EDTA, which was inactivated with culture medium, and subcultured in 75-cm² flasks. For all experiments cells were seeded in 96-well plates at a density of 21 000 cells per well.

1.4. Paraquat cytotoxicity profile on RBE4 cells

Two, three, four, five and six days after seeding, cells were exposed to PQ (0.5 to 50 mM) and cytotoxicity was evaluated after 24 and 48 h using the NR uptake assay (Table 3). All PQ solutions were prepared through serial dilution with culture medium (without β-FGF) of a stock solution, obtained by dissolving the appropriate amount of PQ in culture medium (without FBS) in order to achieve a 100 mM concentration. 4-7 independent experiments were performed, each in quadruplicate. Cell death was expressed as a percentage of the control (untreated cells).
Table 3: Schematic of the timeline followed to perform cytotoxicity experiments. RBE4 cells were exposed to different concentrations of paraquat (PQ), on different days after the seed and for different incubation periods.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
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</thead>
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<td>2 day after seed</td>
<td>Seed</td>
<td>PQ exposure</td>
<td>NR (24h)</td>
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</tr>
<tr>
<td>3 day after seed</td>
<td>Seed</td>
<td>PQ exposure</td>
<td>NR (24h)</td>
<td>NR (48h)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>4 day after seed</td>
<td>Seed</td>
<td>Change medium</td>
<td>PQ exposure</td>
<td>NR (24h)</td>
<td>NR (48h)</td>
<td></td>
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<tr>
<td>5 day after seed</td>
<td>Seed</td>
<td>Change medium</td>
<td>PQ exposure</td>
<td>NR (24h)</td>
<td>NR (48h)</td>
<td></td>
<td></td>
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<tr>
<td>6 day after seed</td>
<td>Seed</td>
<td>Change medium</td>
<td>PQ exposure</td>
<td>NR (24h)</td>
<td>NR (48h)</td>
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</table>

1.4.1. Neutral Red uptake assay (lysosomal membrane integrity)
Cell viability was estimated according to the uptake of vital dye NR, as previously described [22]. At the end of each predefined time-point, cells were incubated for 90 min with NR (50 μg/ml in cell culture medium without β-FGF, at 37 °C and 5% CO₂). The dye retained in viable cells was extracted with solubilization solution (ethanol absolute/distilled water (1:1) with 5% glacial acetic acid) and measured at a test wavelength of 540 nm, and a reference wavelength of 690 nm, using a multi-well plate reader (Bio-Tek Instruments, Potton, Bedfordshire, UK).

1.5. Paraquat cytotoxicity profile on Caco-2 cells

1.5.1. Neutral Red uptake assay (lysosomal membrane integrity) and MTT reduction assay (mitochondrial enzymes activity)
Cell viability was also evaluated using the NR uptake and the MTT reduction assays according to the previously described protocols [34], with minor modifications. Three days after seeding cells were exposed to increasing concentrations of PQ (0.01, 0.05, 0.1, 0.5, 1, 2.5, 5, 7.5 mM) and incubated for 24 h. At the of the predefined time-point, 150 μl of 0.5 mg/ml MTT solution was added to each well, followed by incubation of the plates for 30 min (at 37 °C and 5% CO₂). After this incubation period cell culture medium was removed and the formed formazan crystals dissolved in 100% DMSO. The absorbance was measured at a test wavelength of 550 nm, and a reference wavelength of 690 nm, in a multi-well plate reader (Bio-Tek Instruments, Potton, Bedfordshire, UK).
1.6. Dichlorofluorescein diacetate assay (reactive oxygen species determination)

All PQ solutions were prepared through serial dilution with culture medium (without β-FGF) of a stock solution, obtained by dissolving the appropriate amount of PQ in culture medium (without β-FGF) in order to achieve: a 100 mM concentration in the case of the RBE4 cells, and 10 mM in the case of the Caco-2 cells.

Experiments were performed following a timeline schematized in Table 4. At the end of each predefined time-point cells were incubated with 150 μL DCFH-DA 10 μM for 1 h at 37ºC. After this incubation period, cells were exposed to increasing concentrations of PQ for 24 h. Following the exposure to PQ cell media was aspirated, 150 μL HBSS+/- were added and fluorescence measured (Excitation=485 nm; Emission=530 nm) using a multi-well plate reader (Bio-Tek Instruments, Potton, Bedfordshire, UK). ROS production levels were expressed as a percentage of control.

Table 4: Schematic of the timeline followed to perform the Dichlorofluorescein diacetate (DCFH-DA) on RBE4 and Caco-2 cells. On different days after the seed cells were exposed to different concentrations of paraquat (PQ) during a 24 h incubation period.

<table>
<thead>
<tr>
<th>Day</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 day after seed</td>
<td>Seed</td>
<td>PQ exposure</td>
<td>DCFH-DA (24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 day after seed</td>
<td>Seed</td>
<td>PQ exposure</td>
<td>DCFH-DA (24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 day after seed</td>
<td>Seed</td>
<td>PQ exposure</td>
<td>DCFH-DA (24h)</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: DCFH-DA stock solution should be aliquoted in volumes that do not require them to be frozen/unfrozen more than 8 to 9 times.
1.7. Statistical analysis

All statistical analysis was performed with GraphPad Prism software v5.03 (GraphPad Software, CA, USA).

Concentration-response curves for the NR uptake assay performed in RBE4 cells, with mean values ± SD, were fitted using the least squares as the fitting method and the comparisons between curves (Bottom, Top and Log EC50) were made using the extra sum-of-squares $F$ test. In all cases, $p$ values lower than 0.05 were considered statistically significant.

For the DCFH-DA assay obtained data was expressed as mean ± SD. Differences between the control and the different concentrations of PQ were estimated using one-way ANOVA followed by the Bonferroni’s multiple comparison post-hoc test. Statistical differences in ROS production between cultures with the same degree of confluence and at different confluence degrees were analyzed by a two-way ANOVA, followed by Bonferroni’s multiple comparison post-hoc test. In all cases, $p$ values lower than 0.05 were considered statistically significant.

A t test was performed on the mean values of ROS levels in RBE4 or in Caco-2, at the tested PQ concentration closest to the respective EC50 values, to check for statistically significant differences.
Chapter II
Results and Discussion
1. PQ-induced cytotoxicity in RBE4 cells

PQ cytotoxicity on RBE4 cells was evaluated by incubation with eight different concentrations (0.5, 1, 2.5, 5, 10, 15, 25, 50 mM) over 24 and 48h periods, and these parameters were selected based on a previous study [22]. Also, cultures of different degrees of confluence (days of culture after seeding) were used to assess whether this parameter would influence the response of RBE4 cells to PQ, and, if so, following what pattern. Also, the NR uptake assay was preferred over the MTT reduction assay since Vilas-Boas et al. (2014) observed it to be more sensitive in their study [22].

Looking at Figure 10, a clear difference in the susceptibility of RBE4 cells to PQ-induced cytotoxicity between the 24 and 48h incubation time-points is observed. At the 48h incubation time-point, the PQ-induced toxicity was more pronounced than after 24h of incubation, with the concentration-response curves at the latter time-point presenting significantly lower EC50 values (Table 5). In fact, EC50 values for the 24h time-point are 4 or 5 fold higher than the ones registered for the 48h time-point.

The reported EC50 for PQ-induced cytotoxicity in RBE4 cells after 48 h of incubation, by Vilas-Boas et al. (2014) [22], is of 3.1 mM and, as it is shown in Table 5, is of the same order of magnitude to the EC50 value obtained in this study (7.5 mM), so we considered cells' behavior to be similar in both studies.
Chapter II: Results and Discussion

2 days after seed 24/48H

3 days after seed 24/48H

4 days after seed 24/48H

5 days after seed 24/48H

6 days after seed 24/48H

- Cell Death (% control)
- PQ (mM)
Figure 10: Concentration-response curves of Paraquat-induced (PQ) cell death in RBE4 cells with different degrees of confluence (2-6 days after seeding, a) to e) respectively. Cells were exposed to PQ for 24 h and 48 h and cell death was assessed by the Neutral Red (NR) uptake assay. Results refer to 4-7 independent experiments, each performed in quadruplicate. Error bars refer to standard deviation (SD). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the 24 h and the 48 h incubation curves (bottom, top and LOG EC50) were made using the extra sum-of-squares $F$ test. In all cases, $P < 0.05$ was considered statistically significant.

Table 5: EC50 (half-maximum-effective concentration) values of the PQ concentration-response fitted curves after the 24 and 48 h incubation periods.

<table>
<thead>
<tr>
<th></th>
<th>2 days after seed</th>
<th>3 days after seed</th>
<th>4 days after seed</th>
<th>5 days after seed</th>
<th>6 days after seed</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 at 24h (mM)</td>
<td>20.6</td>
<td>31.6</td>
<td>30.8</td>
<td>34.4</td>
<td>30.9</td>
</tr>
<tr>
<td>EC50 at 48h (mM)</td>
<td>5.9</td>
<td>7.5</td>
<td>7.9</td>
<td>6.7</td>
<td>8.4</td>
</tr>
<tr>
<td>LogEC50 $P$ values (comparison between LogEC50 values)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Curve $P$ values (comparison between fitted curves)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Results refer to 4-7 independent experiments, each performed in quadruplicate. Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the 24 h and the 48 h incubation curves (bottom, top and LOG EC50) were made using extra sum-of-squares $F$ test. In all cases, $P < 0.05$ was considered statistically significant.

In addition, the SD values of the 48 h curves are higher compared to the ones of the 24 h curves (all SD values for the 24 h curves are between 0-10%), due to the high percentage of cell death observed after 48 h of incubation with PQ. In addition, the 6 days after seeding cultures were hyperconfluent, which may also contribute for the high SD values obtained (Figure 10). This was confirmed using a microscope, after having noticed that on this day, the measured cell response to PQ-induced toxicity was more erratic and less reliable.

A comparison between the concentration-response curves of all degrees of confluence cultures obtained at 24 h after incubation with PQ was performed (Figure 11). The curve obtained for the third day after seeding cultures was considered the reference concentration-response curve for the analysis in order to allow comparison of results with the previous work of Vila-Boas et al. (2014) [22]. The respective EC50 and bottom values are represented in Table 6.
Chapter II: Results and Discussion

Figure 11: Concentration-response curves of Paraquat-induced (PQ) cell death in RBE4 cells with different degrees of confluence (2-6 days after seeding) obtained 24 h after exposure to the herbicide. Results refer to 4-7 independent experiments, each performed in quadruplicate. Error bars refer to standard deviation (SD). Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the third day after seeding curve and all other confluence degree curves (bottom, top and Log EC50) were made using extra sum-of-squares $F$ test. In all cases, $P < 0.05$ was considered statistically significant.
Table 6: EC50 (half-maximum-effective concentration) and bottom values of the PQ concentration-response fitted curves after the 24 h incubation period.

<table>
<thead>
<tr>
<th></th>
<th>3 days after seeding</th>
<th>2 days after seeding</th>
<th>4 days after seeding</th>
<th>5 days after seeding</th>
<th>6 days after seeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC50 values at 24h (mM)</td>
<td>31.6</td>
<td>20.6</td>
<td>30.8</td>
<td>34.4</td>
<td>30.9</td>
</tr>
<tr>
<td>LogEC50 P values (comparison between LogEC50 values)</td>
<td>-</td>
<td>0.0078</td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
</tr>
<tr>
<td>Bottom values at 24 h (mM)</td>
<td>5.6</td>
<td>8.3</td>
<td>0.25</td>
<td>~ 2.220e-16</td>
<td>0.3577</td>
</tr>
<tr>
<td>Bottom P values (comparison between bottom values)</td>
<td>-</td>
<td>ns</td>
<td>0.0045</td>
<td>0.0003</td>
<td>0.0148</td>
</tr>
<tr>
<td>Curve P values (comparison between fitted curves)</td>
<td>-</td>
<td>&lt; 0.0001</td>
<td>0.0039</td>
<td>&lt; 0.0001</td>
<td>0.0196</td>
</tr>
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</table>

Results refer to 4-7 independent experiments, each performed in quadruplicate. Concentration-response curves were fitted using least squares as the fitting method and the comparisons between the third day after seeding curve and all other confluence degree curves (bottom, top and Log EC50) were made using extra sum-of-squares F test. In all cases, $P < 0.05$ was considered statistically significant.

Analyzing Table 6, we observed that the concentration-response curves obtained for each confluence degree culture were significantly different when compared to the concentration-response curve obtained for the 3 days after seeding culture (reference curve). No statistically significant differences were observed in the EC50 values, except for the second day after seeding culture curve (20.6 vs 31.6 mM). For the 4-, 5- and 6-days after seeding cultures, significant differences in the curves were obtained comparing with the 3 day after seeding cultures curve. This was due to the bottom values (minimum effect) of each curve, which were significantly different from the bottom value of the concentration-response curve of the reference confluence degree. Biologically, this means that cultures exposed to PQ 4, 5 and 6 days after seeding were more sensitive to lower concentrations of PQ than the cultures exposed 3 days after the seeding, even though no statistically differences between the respective EC50 values were obtained.
2. PQ-induced cytotoxicity in Caco-2 cells

For comparison with the cytotoxicity levels observed in RBE4 cells, and also with the cytotoxicity levels reported by Silva et al. [31, 34], an MTT and a NR uptake assays were performed on PQ-sensitive Caco-2 cells following exposure, 3 days after seeding and for a 24 h incubation period, to PQ concentrations ranging from 0.01 to 7.5 mM. Here, selected concentrations of PQ were based on previous works conducted in our lab [31, 34]. Results are shown in Figure 12.

![Figure 12: Concentration-response curves of Paraquat-induced (PQ) cell death in Caco-2 cells following 24 h of exposure to the herbicide, as assessed by the methylthiazolyldiphenil-tetrazolium bromide (MTT) reduction and the neutral red (NR) uptake assays. Results refer to the mean ± standard deviation (SD) of 1 experiment performed in quintuplicate. Concentration-response curves were fitted using least squares as the fitting method.](image)

Even though EC50 values between the NR uptake and MTT reduction assays are quite similar, during the NR assay, in some wells of the highest tested PQ concentrations, part of the viable cells that had incorporated the red dye detached from the bottom and were aspirated, hence losing part of the signal and rendering this assay unfit for comparison with the, already mentioned, previously reported work [31, 34]. On the other hand, the EC50 value of PQ obtained based on the MTT reduction assay (1204 μM) is very close the reported by Silva et al. (2013) (1244 μM) [34]. Due to the small gap of time between works, these observations were enough to consider that cells would have the same response patterns in both studies.
Our results have shown that RBE4 cells are extremely resistant to PQ, when compared with other cells, as we observed that the EC50 for PQ in RBE4 cells is approximately 30 fold higher than what observed in Caco-2 cells. This comes corroborate what has been previously observed in our lab.

Several studies have been reported on different cell lines’ susceptibility to PQ-induced cytotoxicity [3, 22, 31, 34, 36, 39, 54]. Zerin et al. (2012) reported that A549 cultures exposed to 0.3 mM of PQ, for 24 h, had their cell viability significantly decreased, and that after the same incubation period with 0.7 mM (the lowest concentration of PQ tested in RBE4 cells was 0.5 mM) had decreased cell viability by around 80% [3]. Fujimori et al. (2012) reported that SH-SY5Y cells exposed 0.5 mM PQ for 24h had their cell viability decreased to around 50% [36]. A different study, also on SH-SY5Y cells, reported that after a 24 h incubation period with 1 mM PQ cell viability had decreased to 65-70% [39], which, even though is slightly higher than what reported by Fujimori et al. (2012), is still much lower than what we observed for the RBE4 cells in this study. Janda et al. (2013) reported that 100 µM of PQ, for a 72 h incubation period, induced the death of over 60% of the U373 astroglial cells.

So far, no reports on similar resistance levels to PQ-induced cytotoxicity to those observed in RBE4 have been found in literature, suggesting that this cell line might have unique defensive mechanisms that allow them to cope with such incredible concentrations of PQ.
3. ROS production in RBE4 and Caco-2 cells following PQ exposure

We hypothesized that the huge difference observed in PQ-induced cytotoxicity between RBE4 and Caco-2 cells could be due to mechanisms that enable RBE4 cells to cope with high levels of ROS, which we expected to see following exposure to such high concentrations of PQ. To test this hypothesis, an experiment was performed in order to determine ROS levels in RBE4 cells following PQ exposure.

For this purpose, and also based on the results obtained on the NR uptake assay, 3, 4 and 5 days after seed RBE4 cells were exposed, for 24h, to 5 concentrations of PQ (10, 20, 30, 40, 50 mM) (Figure 14).

A preliminary experiment with hydrogen peroxide (H$_2$O$_2$), a positive control, was performed in order to validate the DCFH-DA assay. As expected, a concentration-dependent increase in ROS levels was observed in RBE4 cells exposed to H$_2$O$_2$ for 2 h (Figure 13).

![Figure 133: H2O2-induced ROS production on RBE4 cells. Three days after being seeded cells were exposed to 3 concentrations of H$_2$O$_2$ for 2 h. Results refer to the mean ± standard deviation (SD) of 1 experiment performed in quadruplicate. Differences between the control and the increasing concentrations of H$_2$O$_2$ were estimated using one-way ANOVA followed by the Bonferroni's multiple comparison post-hoc test. **P < 0.01, ***P < 0.001 vs control (0 mM H$_2$O$_2$)
Figure 144: Paraquat (PQ)-induced reactive oxygen species (ROS) production in RBE4 cells assessed by the DCFH-DA assay in 3/4/5 days after seeding cultures. Results refer to the mean ± standard deviation (SD) of 8-6 independent experiments, each performed in quintuplicate. Differences between the control and the different concentrations of PQ were estimated using one-way ANOVA followed by the Bonferroni’s multiple comparison post-hoc test. $^{**} P < 0.01$, $^{***} P < 0.001$ vs control (0 mM PQ). Differences between the control and the different degrees of confluence were estimated using two-way ANOVA followed by Bonferroni’s multiple comparison post-hoc test. $^{*} P = 0.0136$ vs control (3 days after seeding cultures).

On Figure 14, we can observe that RBE4 cells' exposure to PQ was accompanied by a concentration-dependent increase in ROS production. This pattern was similar in all cultures, independently of their degree of confluence. A significant difference in the ROS production between the 4 days after seeding and the 3 days after seeding cultures was detected ($p=0.0136$).

The ROS levels in cells incubated with a PQ concentration closest to the EC50 value (30 mM) were 112.7%, 121.6% and 113.8% of the control, in the 3, 4 and 5 days after seeding cultures, respectively. In addition, in the same cultures, the ROS production at the highest PQ used (50 mM) were 120.0%, 130.8% and 123.2%, respectively.
Chapter II: Results and Discussion

Then, in order to compare the ROS production between the PQ-resistant RBE4 cells and the PQ-sensitive Caco-2 cells, we proceeded to perform the DCFH-DA assay on the Caco-2 cells. The tested concentrations were the same used in the NR uptake assay performed on this cell line. The results are shown in Figure 15.

Figure 15: Paraquat (PQ)-induced reactive oxygen species (ROS) production in Caco-2 cells assessed by the DCFH-DA assay in 3/4/5 days after seeding cultures. Results refer to the mean ± standard deviation (SD) of 5-6 independent experiments, each performed in quintuplicate. Differences between the control and the different concentrations of PQ were estimated using one-way ANOVA followed by the Bonferroni's multiple comparison post-hoc test. **P < 0.01, ***P < 0.001 vs control (0mM PQ). Differences between the control and the different degrees of confluence were estimated using two-way ANOVA followed by Bonferroni's multiple comparison post-hoc test.
On Figure 15, we can observe that Caco-2 cells' exposure to PQ was accompanied by a concentration-dependent increase in ROS production. This pattern was similar in all cultures, independently of their degree of confluence. No significant differences in the ROS production between the different confluence degrees and the reference confluence degree were detected.

The ROS levels in cells incubated with a PQ concentration closest to the EC50 value (1 mM) were 116.0%. At the maximum PQ concentration used in this experiment, ROS levels were 124%, 119.2% and 117.4% 3, 4 and 5 days after seeding, respectively.

In order to compare the contribution of ROS production in the PQ-induced cytotoxicity in RBE4 and Caco-2 cells, the ROS levels of cells incubated with PQ concentrations corresponding to ~EC50 value of PQ for each cell line were determined [EC50(RBE4)= 31.6 mM - ROS levels measured at 30 mM; EC50(Caco-2)= 1.2 mM - ROS levels measured at 1 mM)]. The results are presented in Figure 16.

Despite the fact that RBE4 cells were exposed to a PQ concentration 30 fold higher than Caco-2 cells, similar ROS levels were observed in Caco-2 cells and RBE4 cells, after incubation with EC50 of PQ.

![Figure 16: Determined ROS levels of RBE4 and Caco-2 cells (3 days after seeding cultures) exposed to 30 mM and 1 mM of PQ, respectively, for a 24 h period. The PQ concentrations selected correspond to approximately to the EC50 value for PQ of each cell line. Data were analyzed using the t-test. No significant differences were found between the two groups.](image-url)
Comparison between ROS levels of RBE4 cultures exposed 5 days after seeding and the reference confluence degree showed no significant differences. However, small, but significant, differences were obtained by comparison between ROS levels of cultures exposed 4 days after seeding and the reference confluence degree.

On Caco-2 cells, the measured cytotoxicity response after exposure to PQ was, as expected and previously reported [31, 34], much lower than that observed for RBE4 cultures, as RBE4 cells presented an EC50 approximately 30 fold higher than Caco-2 cells. However, despite the fact that RBE4 cells were exposed to PQ concentrations 5-10 fold higher than Caco-2 cells, the same was not true for ROS levels in Caco-2 cells following exposure, where obtained results seemed to be around the same levels of those of the RBE4 cells, contrary to what we expected. A comparison between ROS levels in RBE4 and Caco-2 cells exposed 3 days after seed, at the PQ concentration closest to their respective EC50, showed no significant differences between the two cell lines.

These observations lead us to hypothesize that either PQ uptake in RBE4 cells is much lower than in Caco-2 cells, maybe due the presence of an efflux pump, or that RBE4 cells might have some kind of protection against the PQ cytotoxicity mechanisms, like inhibition of its characteristic, and well described, redox cycle.
Chapter III
Conclusions and future perspectives
This study confirmed the high resistance to PQ-induced cytotoxicity observed in RBE4 cells, when compared to Caco-2 cells, and that the EC50 value to remains identical throughout different confluence degrees (except on the 2 day after seeding, where The EC50 is significantly lower). It is important to consider, as mentioned in this work's introduction, that when comparing the measured cytotoxicity levels of PQ in RBE4 cells with the reported PQ-induced cytotoxicity for other cell lines, like SH-S5Y5 [36, 39] or A549 [3], we observe that RBE4 cells exhibit a much higher resistance profile to PQ. These lead us to hypothesize that this high resistance observed could be due to some kind of built-in mechanism in RBE4 cells that could protect them against the oxidative stress induced by the characteristic ROS formed due the PQ redox cycle. To investigate this possibility we determined the ROS production levels on RBE4 cells exposed to different concentrations of PQ over different confluence degrees.

Having set these parameters for RBE4 cells sought to compare them with the ones obtained from experiments performed on Caco-2 cells, having observed that following exposure to concentrations of PQ close to their respective EC50s values of PQ ROS levels in both cell lines were identical. Even if RBE4 cells were exposed to a concentration 30 fold higher than Caco-2, the ROS production was the same. This seems to suggest that either PQ is not entering in RBE4 cells at the same extent as it does in other cell lines, maybe due to the activity off an efflux pump, or that the RBE4 cells inhibit PQ's normal metabolic pathway to a point where it is preventing its characteristic redox cycle from producing the same ROS levels as in other cell lines.

For future works, it would be interesting to measure the PQ-induced cytotoxicity levels on Caco-2 cells of different confluence degrees, in order to determine whether the lack of difference in ROS production is accompanied by an identical cytotoxicity profile, or if, like RBE4 cells, PQ-induced cytotoxicity in Caco-2 cells varies with the confluence degree.

To further explore the RBE4 cell resistance mechanisms against PQ-induced cytotoxicity, a comparative study with Caco-2 cells on the activity and expression of efflux pumps would also be interesting. Based on the previous works of Vilas-Boas et al. (2014) and Silva et al. (2013) [22, 31, 34], P-gp seems to be a good starting point, as the induction of the activity of this transmembrane transporter has been confirmed increase cells resistance to PQ-induced cytotoxicity, and its inhibition to increase susceptibility. Measuring P-gp activity in RBE cells on different confluence degrees could help us understand if the differences in the observed susceptibility to PQ-induced cytotoxicity is accompanied by an altered P-gp activity, or if the transporter's activity remains the same suggesting that the change in cytotoxicity would be due the activity
of another transmembrane transporter, for example the choline uptake system which has also been involved in the PQ uptake in both cell lines [22, 31, 34], or due to metabolic differences between both cell lines.

Having established Caco-2 cells' PQ induced cytotoxicity profile over different confluence degrees, it might be worthwhile to also determine P-gp activity in cultures with those different confluence degrees.

Another approach to the matter, would be determining PQ intracellular levels in cultures with different confluence degrees, in order to help us understand if PQ uptake/efflux varies with the confluence degree. Since ROS levels roughly remained in similar orders of magnitude in cultures with different confluence degrees, an increase in PQ intracellular levels could suggest that RBE4 cells' unusual high resistance to the herbicide comes from metabolic defense mechanisms that inhibit, at least to some extent, the PQ's redox cycle. Also, comparing P-gp activity over different confluence degrees with PQ intracellular levels over those same confluence degrees could elucidate whether the observed difference, if there were any, was due to the activity of the P-gp or another transporter.

Noteworthy, is the fact that P-gp and the choline uptake system have both been shown to be involved with PQ transport across cells' membranes in both cell lines [22, 31, 34], which means that RBE4 cells extraordinary resistance to PQ-induced cytotoxicity can come from the interaction of more than one characteristic. And if shown that, for example, P-gp activity is increased, thus providing a protective role against cytotoxicity of the herbicide, it is still not out of the question that these cells might have some other kind of alternate pathway that helps RBE4 cells to cope with high levels of PQ, preventing it from forming ROS.

A comparative study between RBE4 and Caco-2 cells could, perhaps, provide us with clues to what is so unique to the RBE4 cell line that enables half the exposed cells to survive a concentration of PQ over 30mM, following a 24h incubation period.

Once the mechanisms are elucidated, human brain endothelial cells could be tested for the same defensive mechanisms and, if they are present, studies can be performed in order to find ways to modulate those defensive mechanisms.
Part IV

References


