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In vitro Toxicology

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TU 11 : Pharmacology/Toxicology
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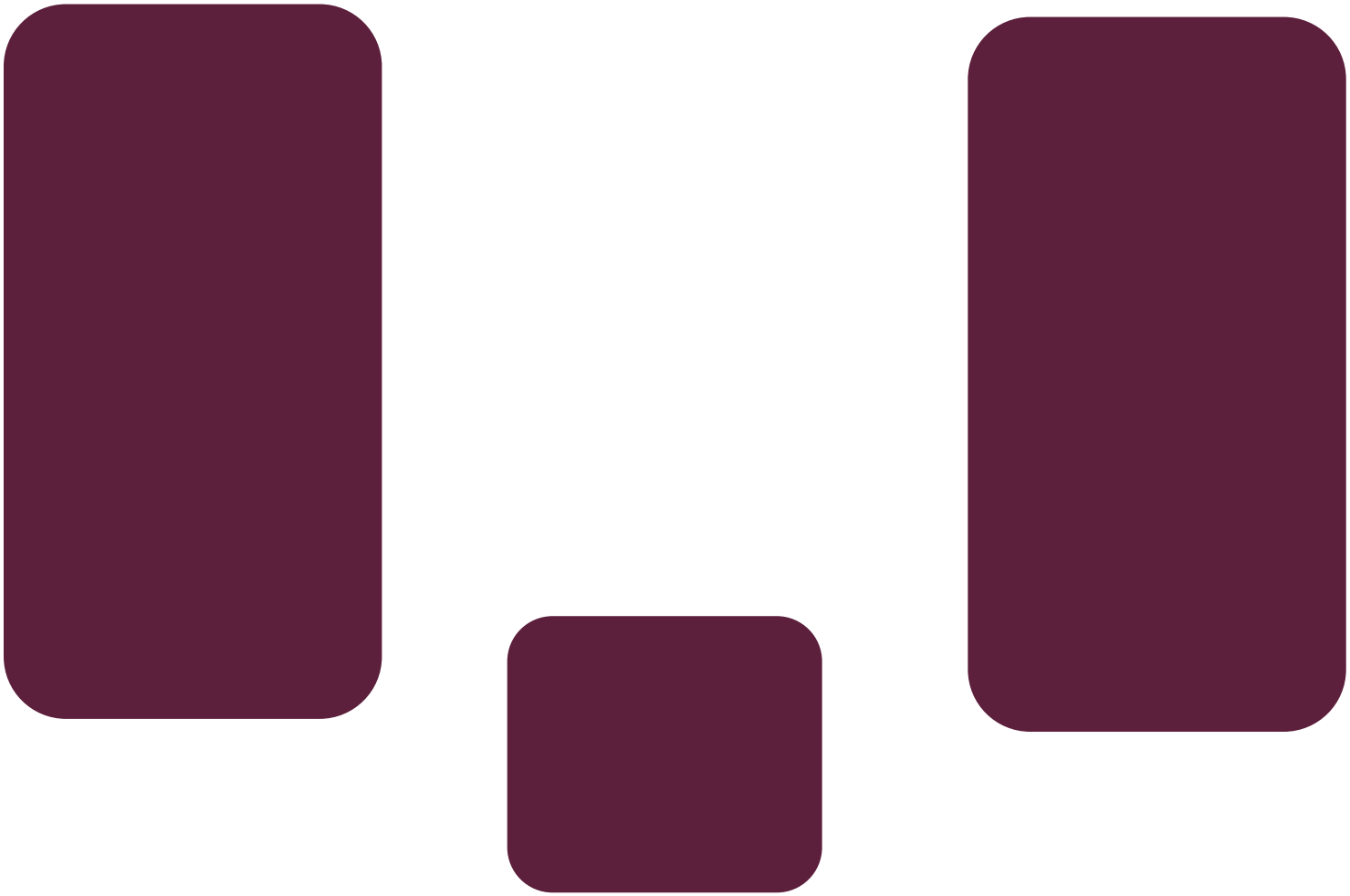
The Different Approaches Used in Toxicology

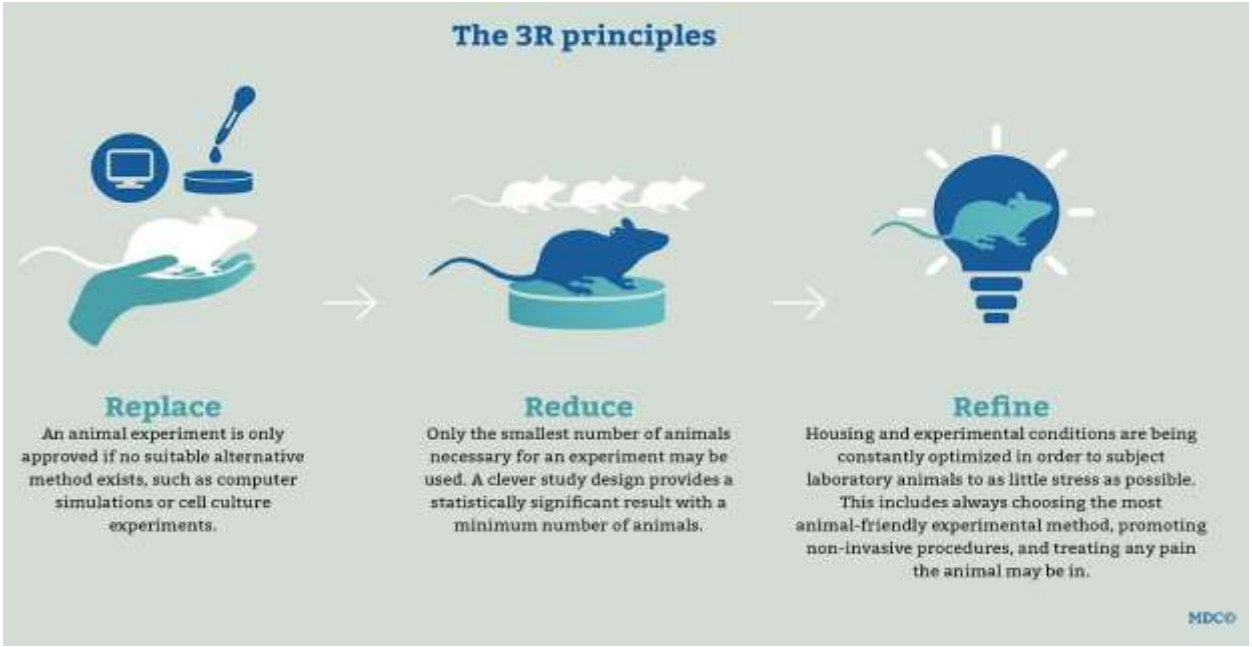


The Different Approaches Used in Toxicology

The 3Rs principles

After grading the suffering experienced by animals in experimentation in British laboratories, W.M.S. Russell and R.L. Burch developed, in 1959, a program for the establishment and development of "humane" guidelines, called the "3Rs rule," which includes the following points:

- **Reduce** the number of animals used.
- **Refine** experimental procedures to reduce animal stress and pain.
- **Replace** animal testing as soon as validated alternative methods are available.



- Cell culture is a laboratory technique that allows the multiplication of cells outside their original organism, under controlled conditions.

- It is widely used in biology, medicine, and toxicology to study cell behavior, test drugs, produce biomolecules, and more.

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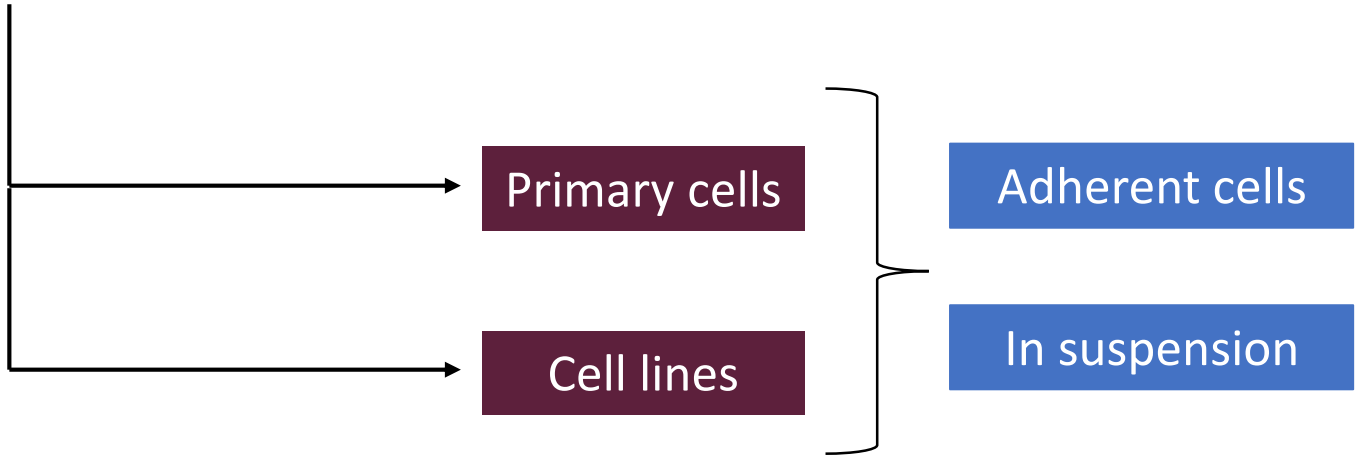
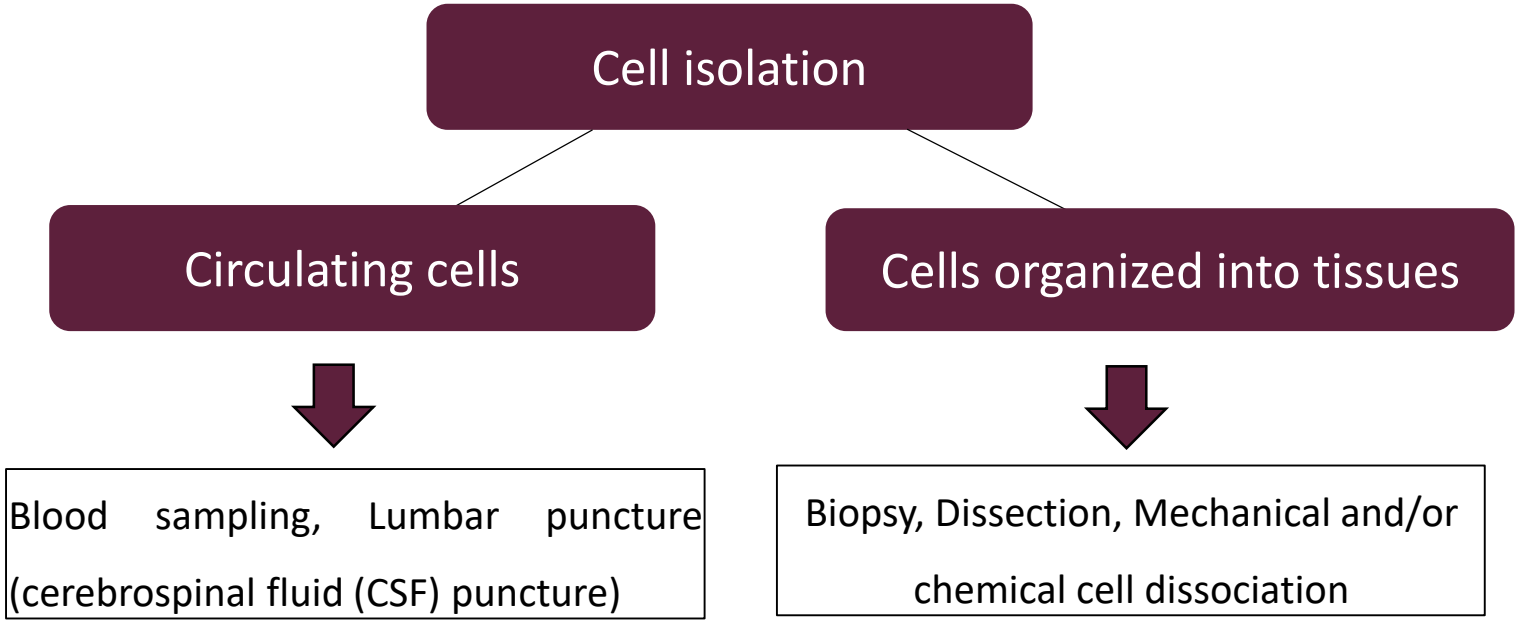


Primary cells

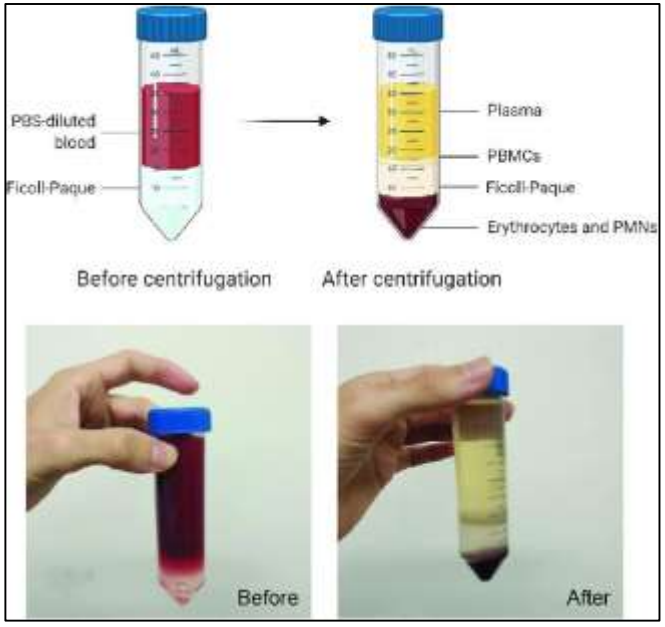
A primary cell is a cell that is directly isolated from a tissue or an organism and cultured in the laboratory.



Primary cells

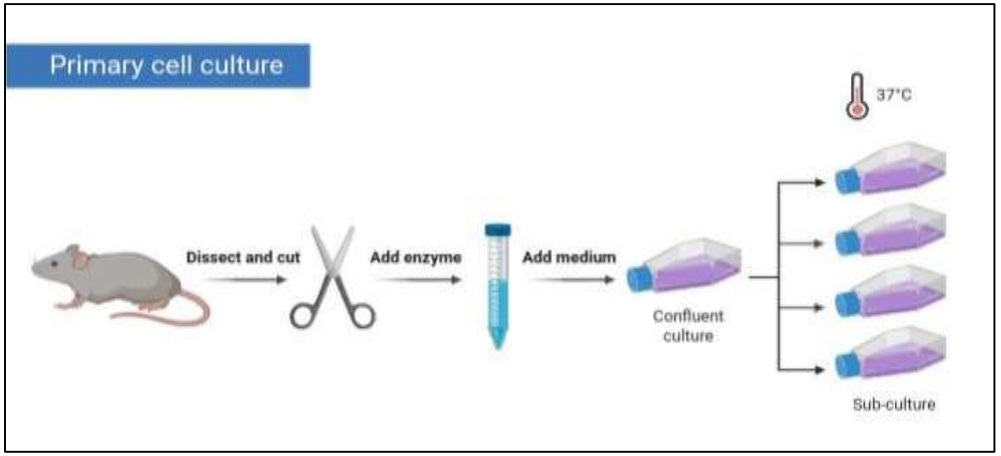
Cell isolation

Circulating cells



Isolation des PBMC (Peripheral Blood Mononuclear Cells) par gradient de Ficoll

Cells organized into tissues



Isolation de cellules par dissection de tissus animal

Primary cells

A primary cell is a cell that is directly isolated from a tissue or an organism and cultured in the laboratory.

Cell isolation

- **Circulating cells** → Blood sampling, Lumbar puncture (cerebrospinal fluid (CSF) puncture)
- **Cells organized into tissues** → Biopsy, Dissection, Mechanical and/or chemical cell dissociation

Primary cells in culture

Cell passage

Limited number of passage

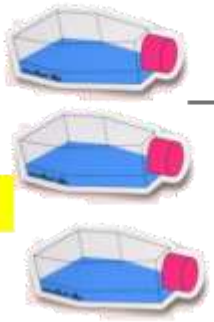
Cell subculturing

Main Cell culture flask

4-5 days depending of the cells type, number of cell seeded

Subculture

Cell counting!



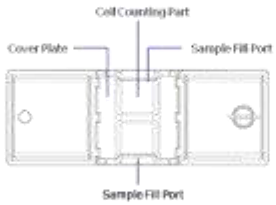
Subculture

Number of cells ↑

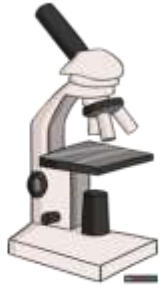
Confluence about 80%

Cellular suspension

Sample



Hemocytometer: Cell counting chamber



Cell line

A cell line is a group of cells derived from a tissue or an organism that has the ability to divide indefinitely in culture.

These cells have often undergone genetic modification or selection, allowing them to escape cell death (senescence).

Cell line

Cell isolation

Circulating cells

Blood sampling, Lumbar puncture (cerebrospinal fluid (CSF) puncture)

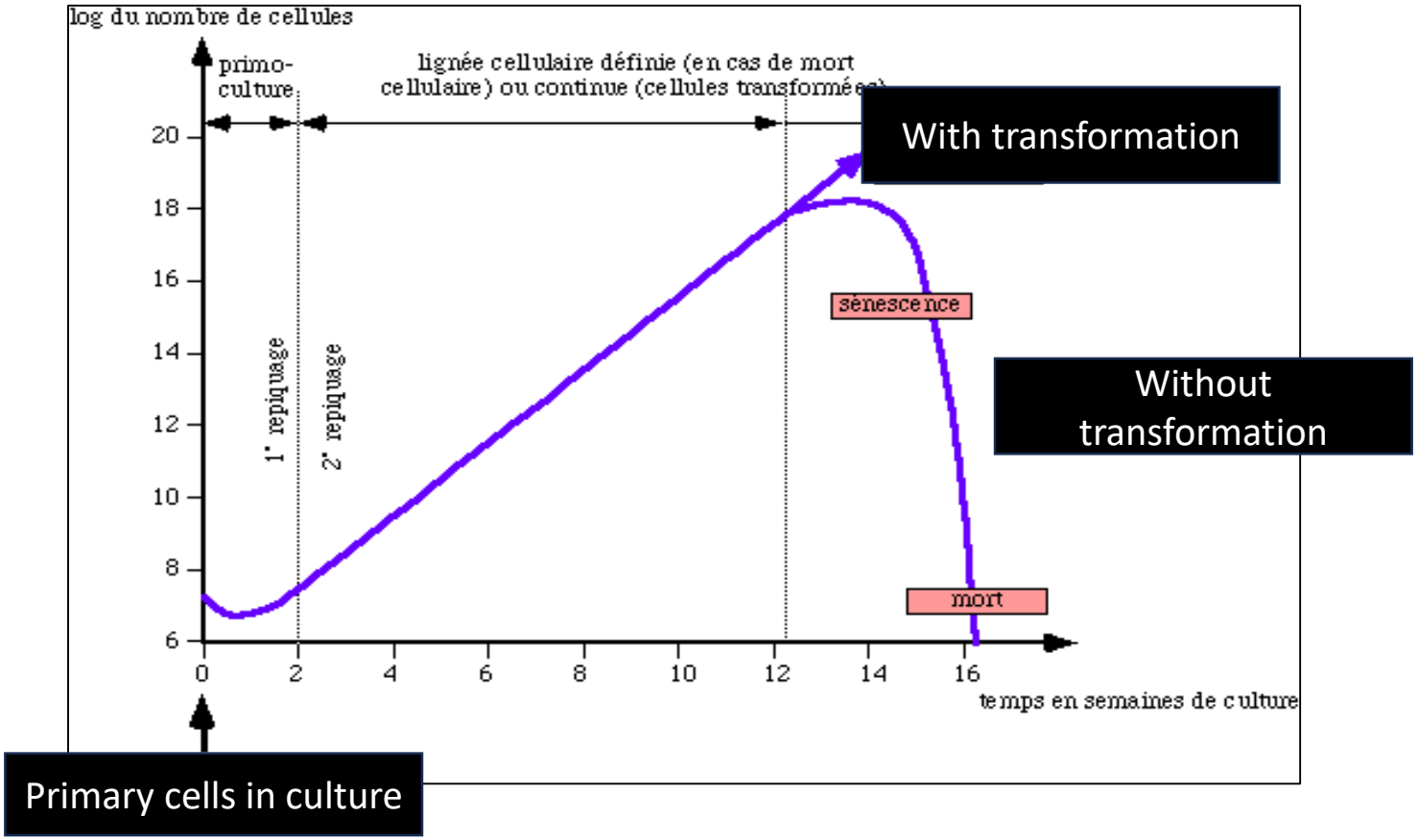
Cells organized into tissues

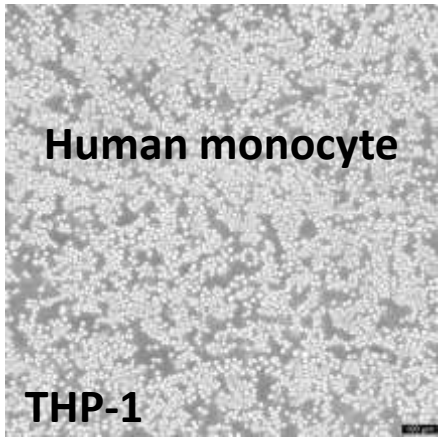
Biopsy, Dissection, Mechanical and/or chemical cell dissociation

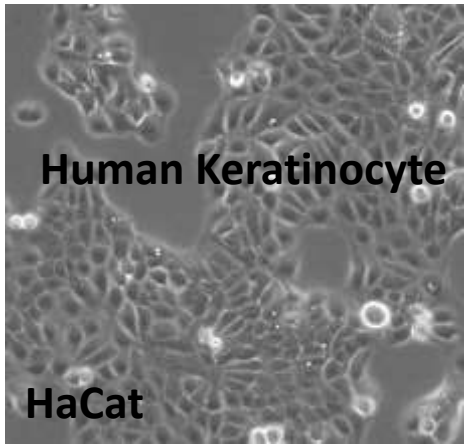
Genetic modifications

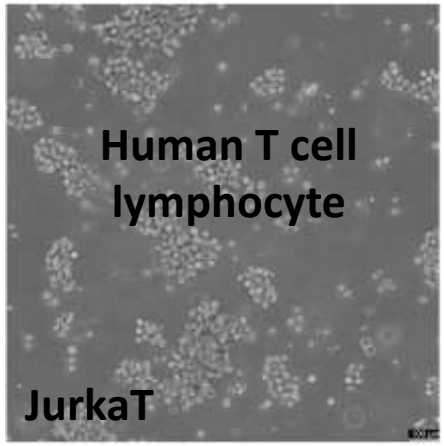
Primary cells in culture

Cell passage


log du nombre de cellules
primo-culture
lignée cellulaire définie (en cas de mort cellulaire) ou continue (cellules transformées)
1er repiquage
2e repiquage
sénescence
mort
20
18
16
14
12
10
8
6
0
2
4
6
8
10
12
14
16
temps en semaines de culture
With transformation
Without transformation
Primary cells in culture







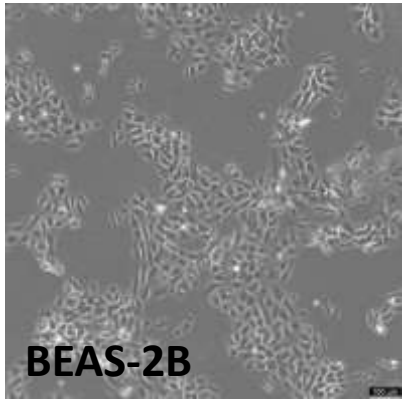
BEAS-2B: Normal Human Bronchial epithelial cells

- Immortalized via the expression of T-antigen of the virus SV40

- **Mechanism of action of SV40-T** :

- Inhibition of p53: p53 is a tumor suppressor protein that controls apoptosis and prevents the division of damaged cells.

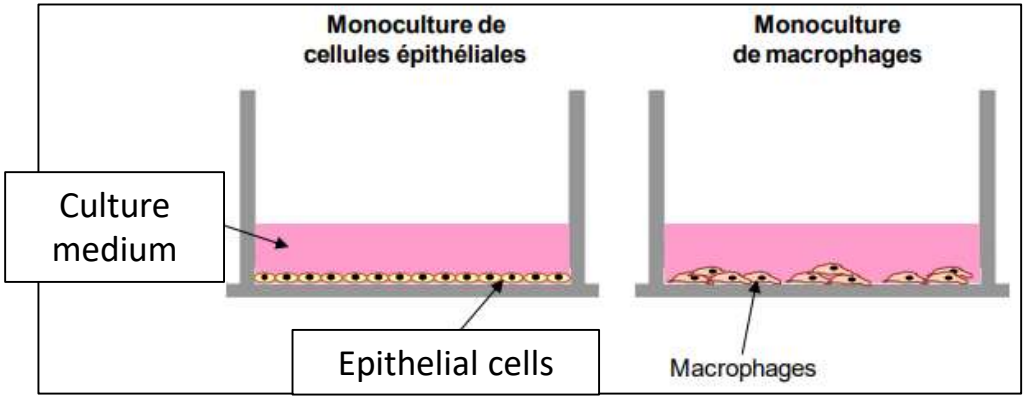
- Inhibition of the Rb protein (retinoblastoma protein): which regulates the transition from the G1 phase to the S phase of the cell cycle.



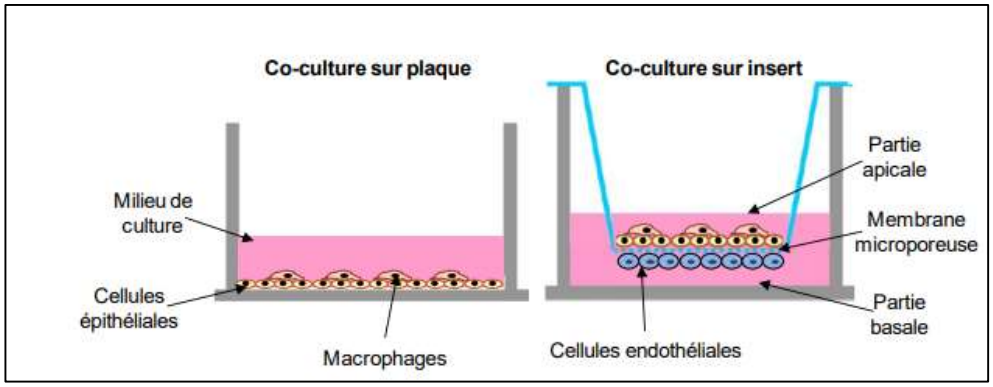
2D vs 3D models

2D: Classical culture – Submerged cells

Monoculture

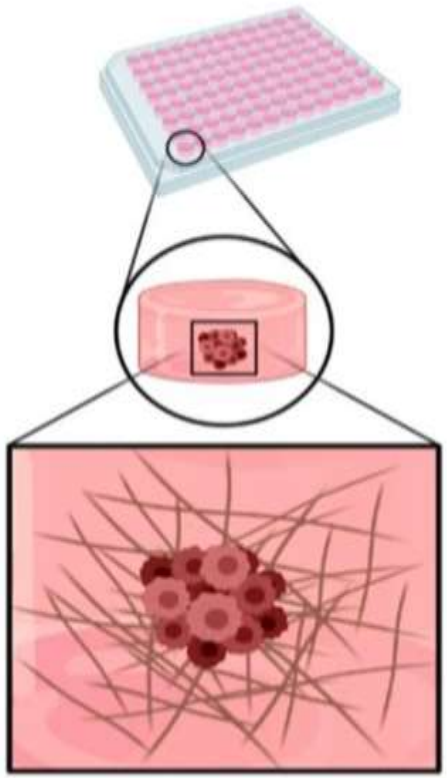


Co-culture



2D vs 3D models

3D model



2D vs 3D models

3D model

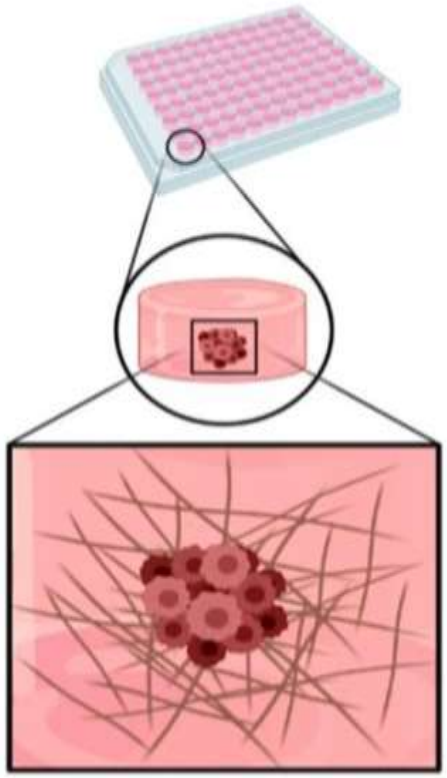
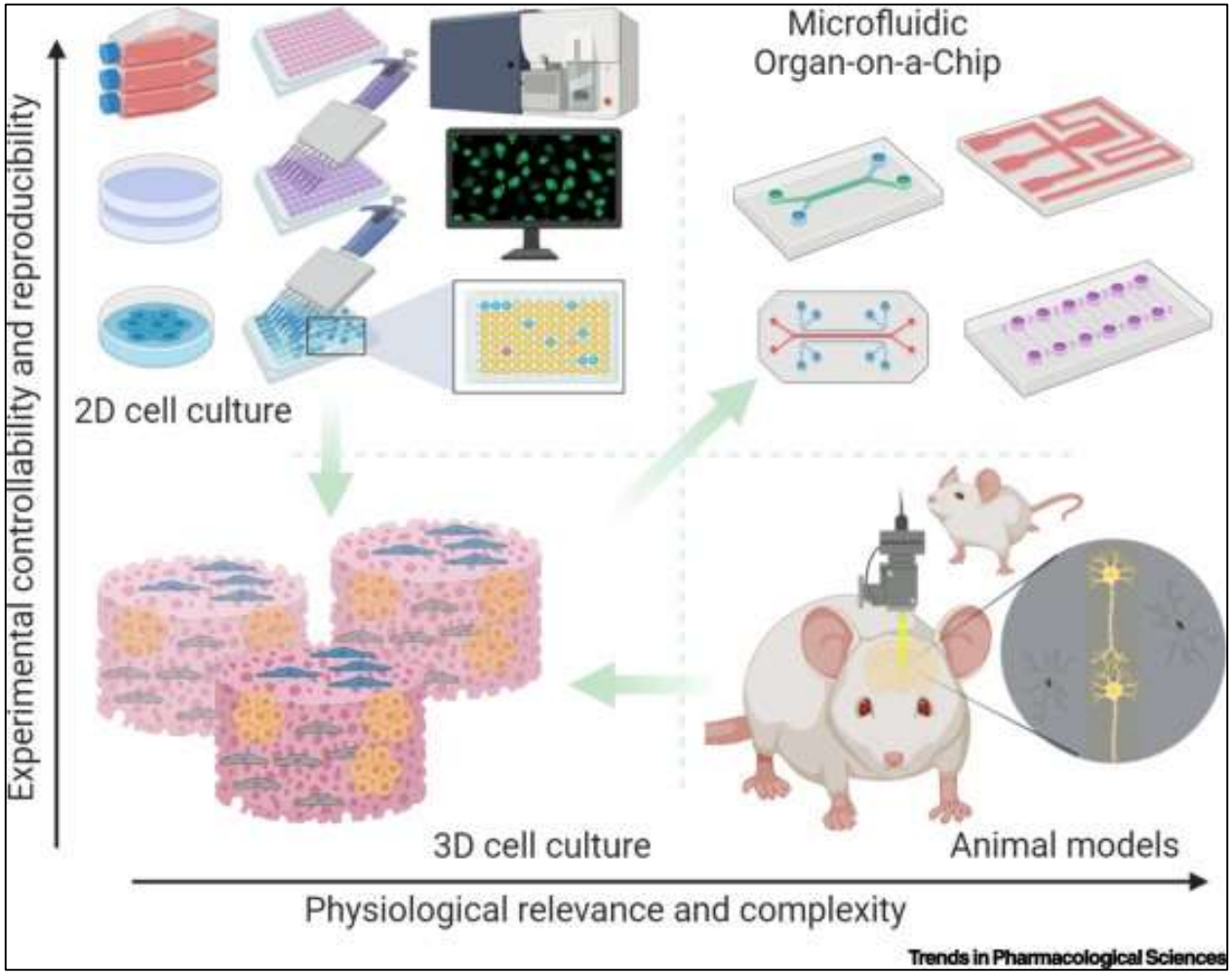


Table 1
Comparison of 2D and 3D culture systems. Despite the higher complexity of 3D systems, the benefit provided by 3D culture is overwhelming and promising.

	2D culture	3D culture	
+	Easier to prepare	Complex protocols	−
+	High reproducibility	Risk of low reproducibility	−
+	Easy use of a microscope	Imaging techniques require optimisation	−
−	Two-dimensional cell-cell contact, monolayer	Three-dimensional cell-cell contact, multilayer	+
−	Very low cell density	Cell density more similar to *in vivo*	+
−	Physical limitation for growing	Expanded possibility of grow	+
−	Prevailing contact with plastics	Contact with plastics minimized	+
−	Static medium	Possibility of flowing medium	+
−	Gradient of oxygen and other nutrients	Constant supply of oxygen and other nutrients	+
−	Lower *in vivo* like functionality	Similar *in vivo* like functionality	+
−	Limited co-culture opportunity	Easier co-culture systems	+

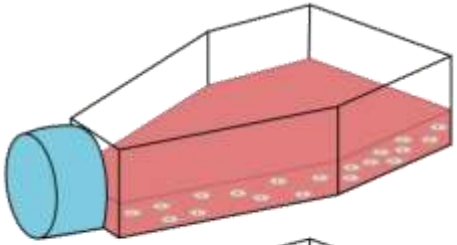
Reproducibility and Physiological Relevance.



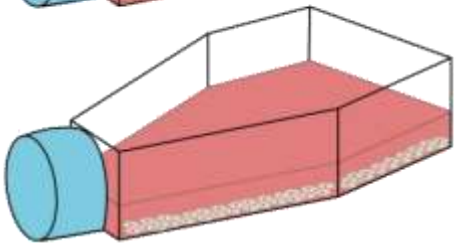
In vitro Toxicology

Cell Culture in Practice

Suspension Cell Culture



Adherent Cell Culture



Plastic surface

Collagen: Mimics the Extracellular Matrix (ECM)

Cell culture flasks: cell growth and multiplication

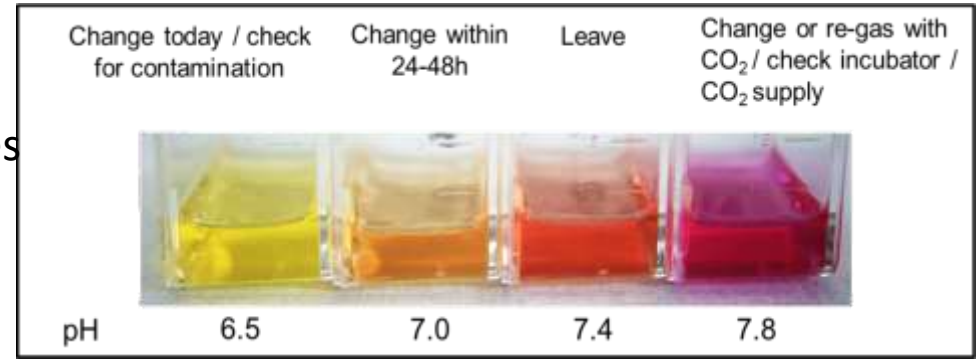
Cell culture plate: experimental analysis

Cell culture medium

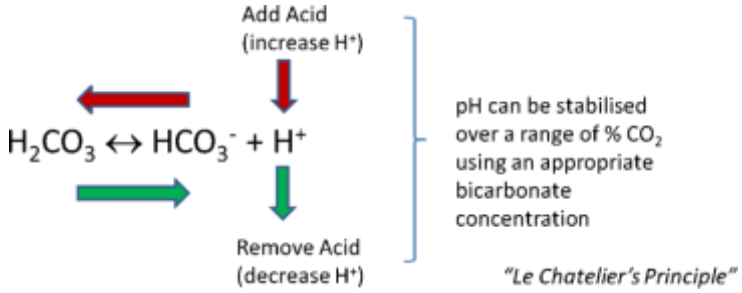
- **Mineral salts**: sodium, potassium, calcium, magnesium, phosphate, carbonate, chloride. Osmotic pressure, membrane transport, metabolism.
- **Sugars**: usually D-glucose (1 g/L).
- **Amino acids**: L-glutamine and other essential amino acids. Protein synthesis, regulation of enzymatic systems, maintenance of the cell cycle.
- **Vitamins**
- **pH**: between 7.2 and 7.4.

- PH indicator: Phenol Red

pH indicator for visual monitoring of pH changes

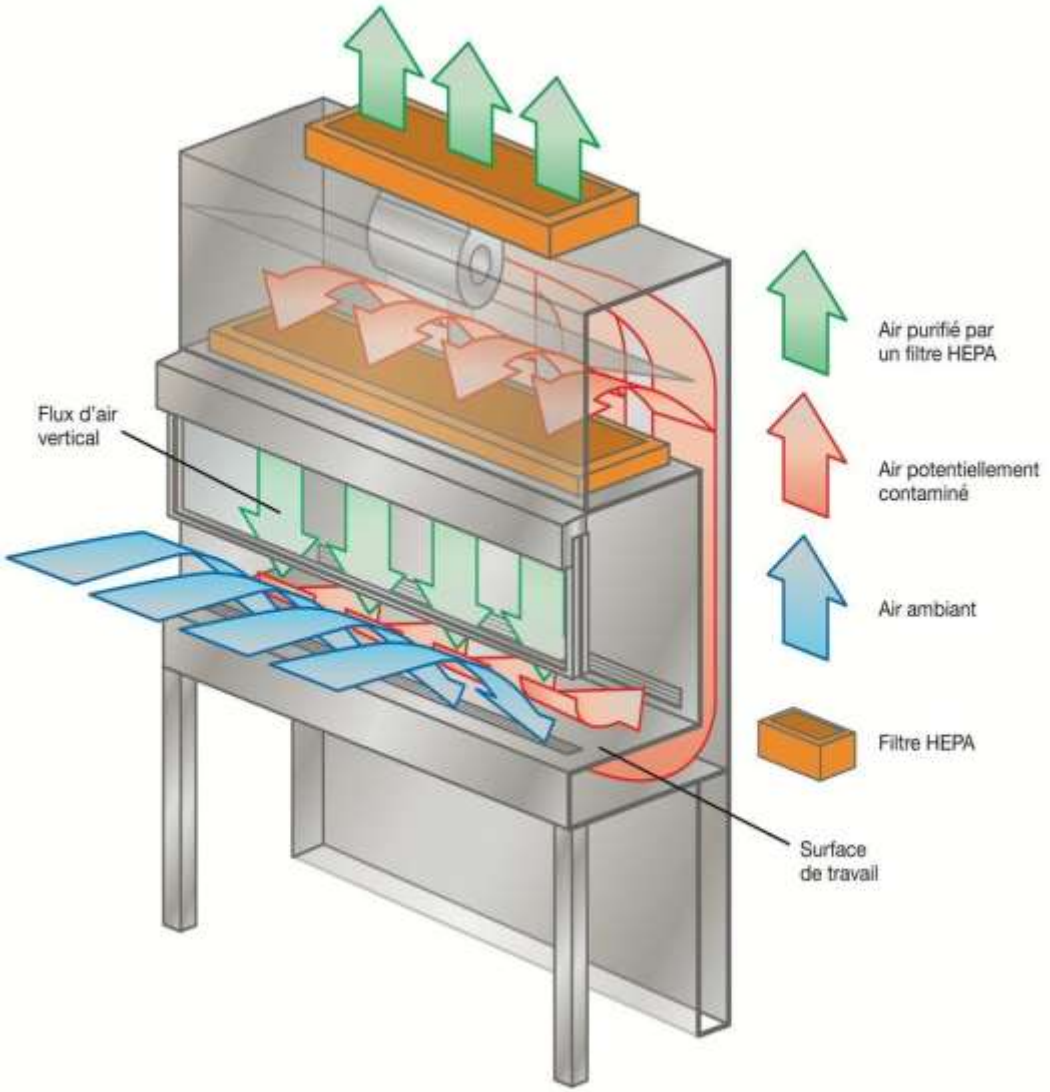


- Buffer systems: bicarbonate/phosphate:



- **FBS (Fetal Bovine Serum)** is a common supplement added to cell culture media to provide essential nutrients, growth factors, and hormones necessary for cell survival and proliferation.

Vertical luminaire flow hood: sterility



Incubators: controlled atmosphere

Atmosphère de culture : 37°C, 5% de CO_2, saturée en vapeur d'eau.

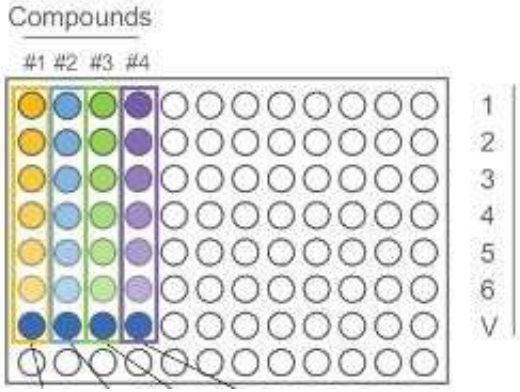
Cell culture : experimental conditions

Cell culture : experimental conditions

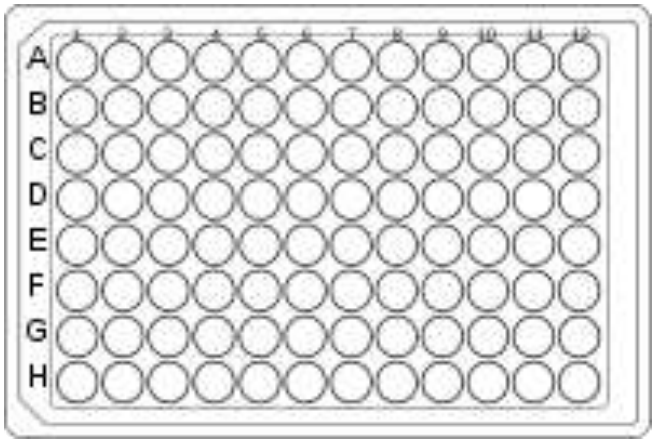
- **Cell type**
- **product solubilization medium,**
- **Concentration(s) of exposure (low or no toxic concentration to study cellular mechanisms)**
- **Exposure times**

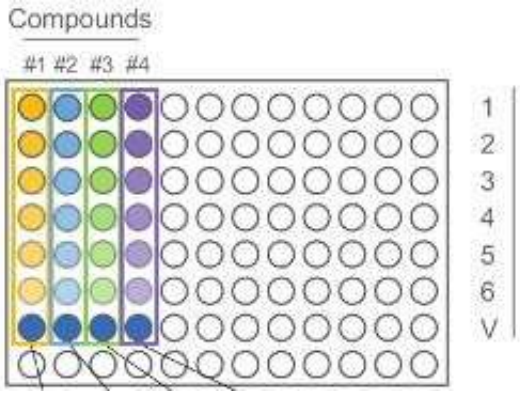
Experimental conditions: Concentrations and time of exposure

Cytotoxicity assays



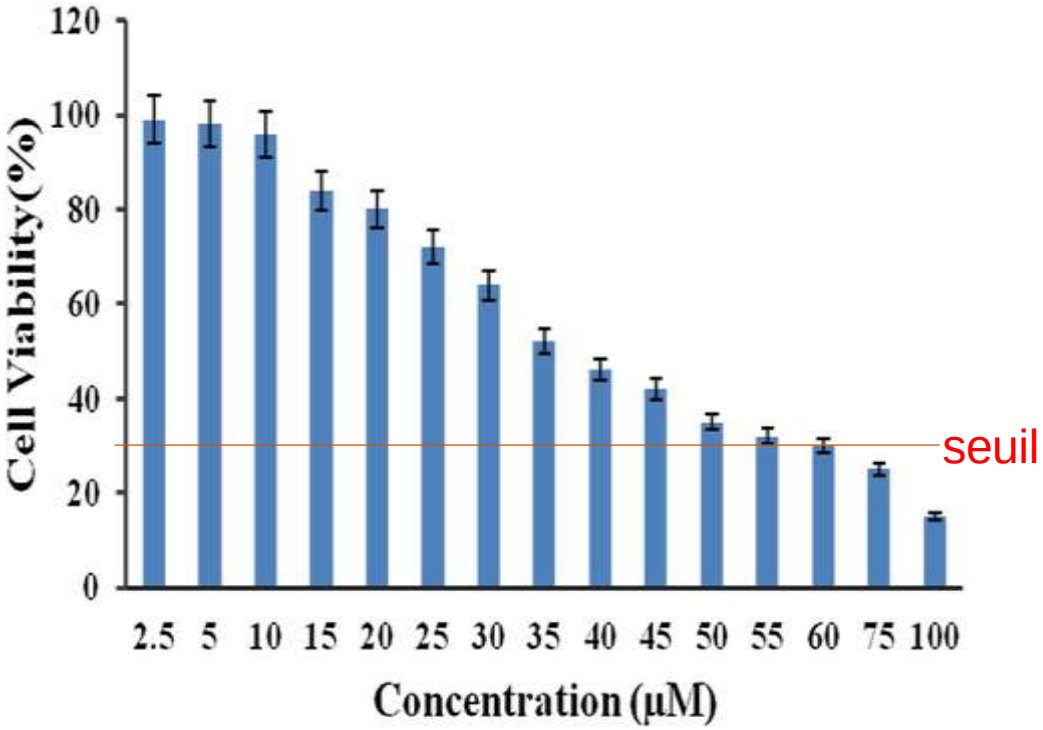
Time 1





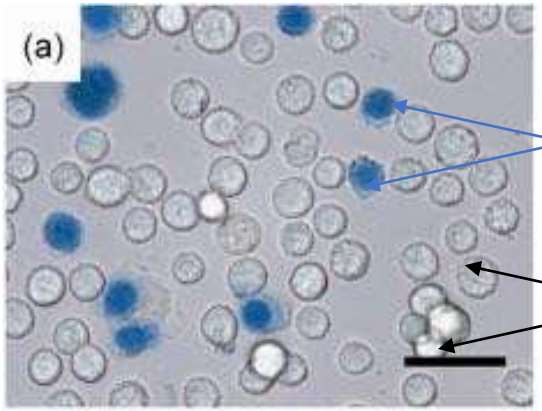
Time 2…

Cytotoxicity assays

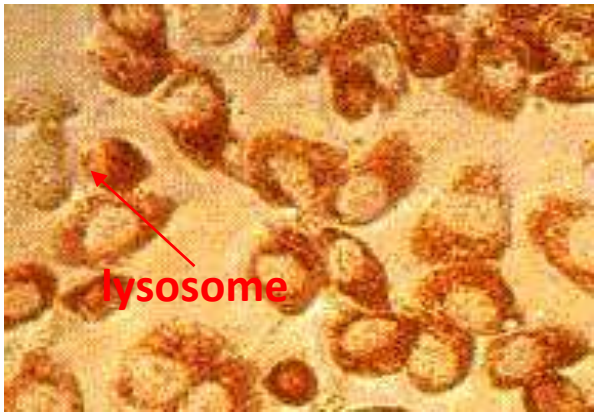


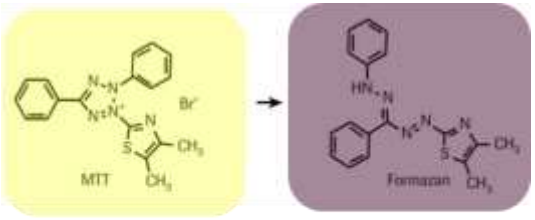
Cytotoxicity assays

Trypan Blue



Neutral red





MTT assay

- The MTT assay measures cell viability based on the reduction of MTT to purple formazan crystals by metabolically active cells: mitochondrial enzymes
- The amount of formazan formed is proportional to the number of viable cells and is quantified by measuring absorbance.

Increased number of cells

Exercice 1

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Toxicology and Applied Pharmacology

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Nickel (II)-induced cytotoxicity and apoptosis in human proximal tubule cells through a ROS- and mitochondria-mediated pathway

Yi-Fen Wang [a], Huey-Wen Shyu [a], Yi-Chuang Chang [b], Wei-Chang Tseng [a], Yeou-Lih Huang [c], Kuan-Hua Lin [a], Miao-Chen Chou [a], Heng-Ling Liu [a], Chang-Yu Chen [a,*]

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Mitochondria
HK-2 cells

ABSTRACT

Nickel compounds are known to be toxic and carcinogenic in kidney and lung. In this present study, we investigated the roles of reactive oxygen species (ROS) and mitochondria in nickel (II) acetate-induced cytotoxicity and apoptosis in the HK-2 human renal cell line. The results showed that the cytotoxic effects of nickel (II) involved significant cell death and DNA damage. Nickel (II) increased the generation of ROS and induced a noticeable reduction of mitochondrial membrane potential (MMP). Analysis of the sub-G1 phase showed a significant increase in apoptosis in HK-2 cells after nickel (II) treatment. Pretreatment with *N*-acetylcysteine (NAC) not only inhibited nickel (II)-induced cell death and DNA damage, but also significantly prevented nickel (II)-induced loss of MMP and apoptosis. Cell apoptosis triggered by nickel (II) was characterized by the reduced protein expression of Bcl-2 and Bcl-xL and the induced the protein expression of Bad, Bcl-Xs, Bax, cytochrome c and caspases 9, 3 and 6. The regulation of the expression of Bcl-2-family proteins, the release of cytochrome c and the activation of caspases 9, 3 and 6 were inhibited in the presence of NAC. These results suggest that nickel (II) induces cytotoxicity and apoptosis in HK-2 cells via ROS generation and that the mitochondria-mediated apoptotic signaling pathway may be involved in the positive regulation of nickel (II)-induced renal cytotoxicity.

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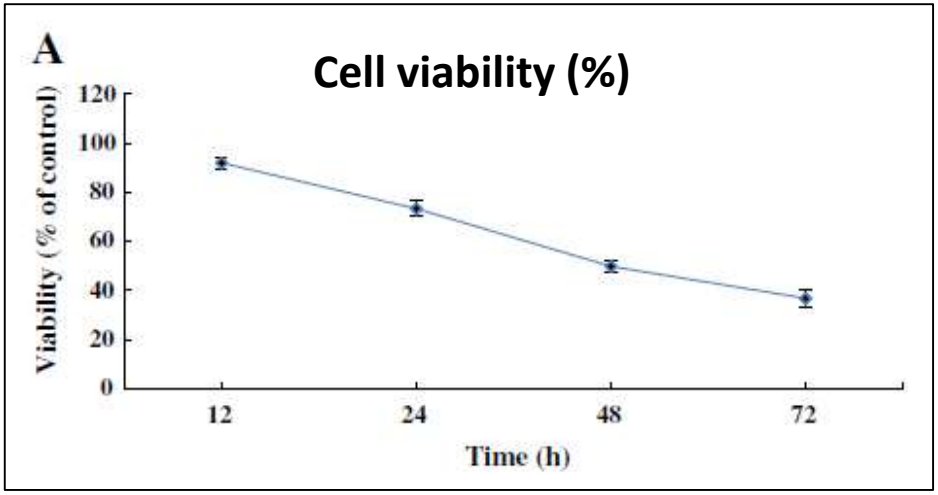
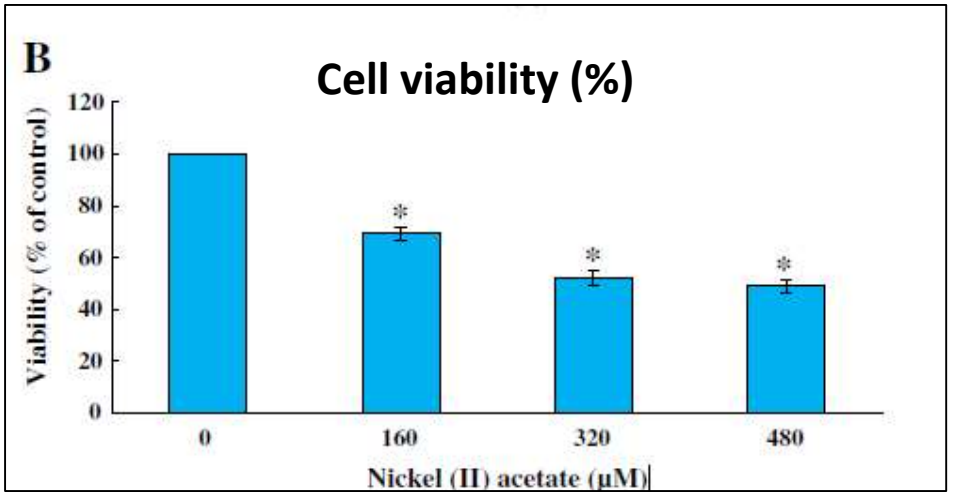
Why Nickel? Why HK-2 cells

Study of cell viability





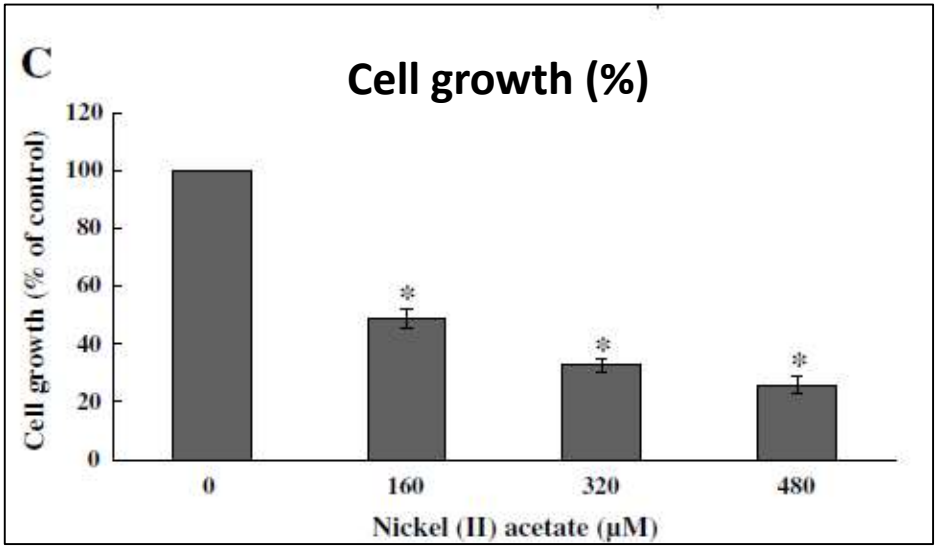


Fig. 1.
(A) HK-2 cells were treated with 480 μM nickel (II) (saline, as control) for 12, 24, 48 and 72 h.
(B) HK-2 cells were treated with 0 (saline, as control), 160, 320 or 480 μM nickel (II) for 48 h. Viability was assessed with the MTT assay.
(C) Colony formation of HK-2 cell was determined in clonogenic assay.
Data are presented as the mean±SD of 5 separate experiments.
***** Significantly different from control ($p<0.01$).

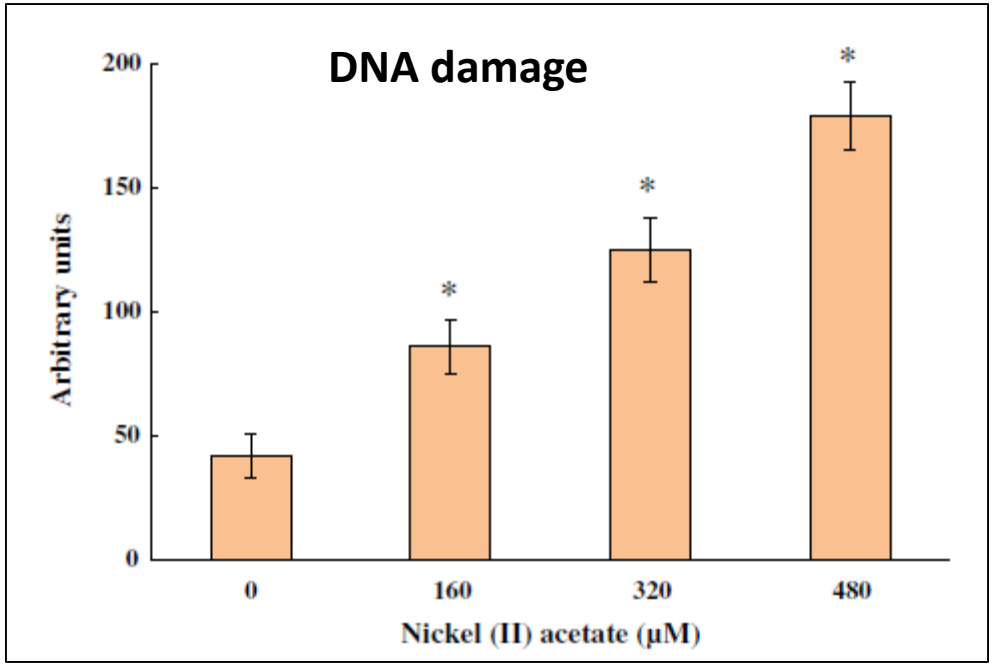


Fig. 2.

The DNA damage in arbitrary units would be calculated to 100 comets of each class when assessed visually.

Data are presented as the mean±SD of 5 separate experiments. * Significantly different from control ($p<0.01$).

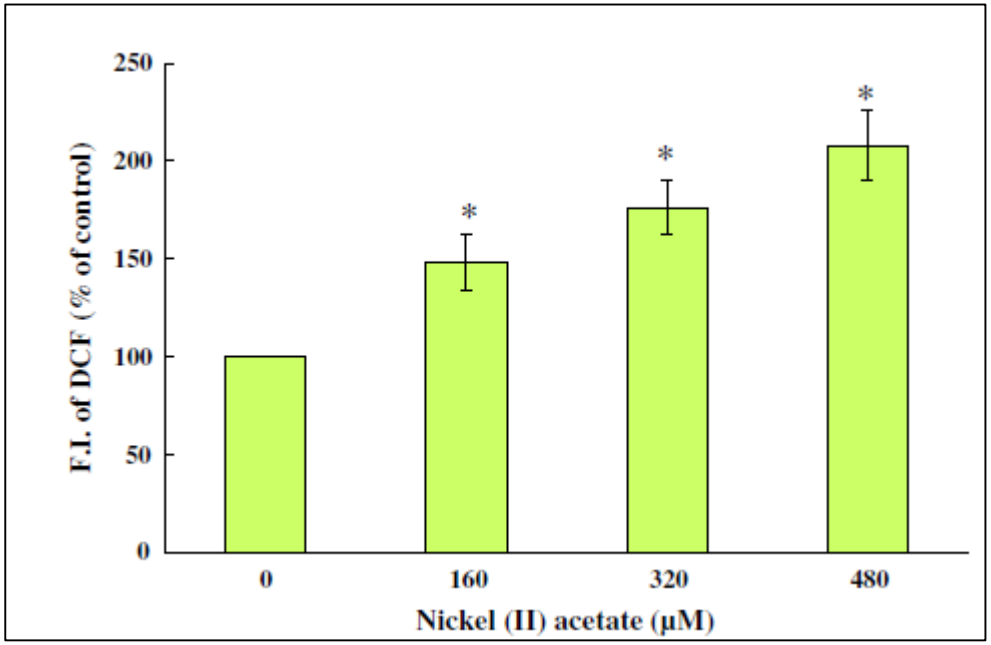


Fig. 3. HK-2 cells were treated with nickel (II) (0–480 µM) for 48 h.

The fluorescence intensity of DCF was determined by the fluorescent DCF assay with a FACS-Calibur flow cytometer. Data are presented as the mean±SD of 5 separate experiments.

* Significantly different from control ($p<0.01$).

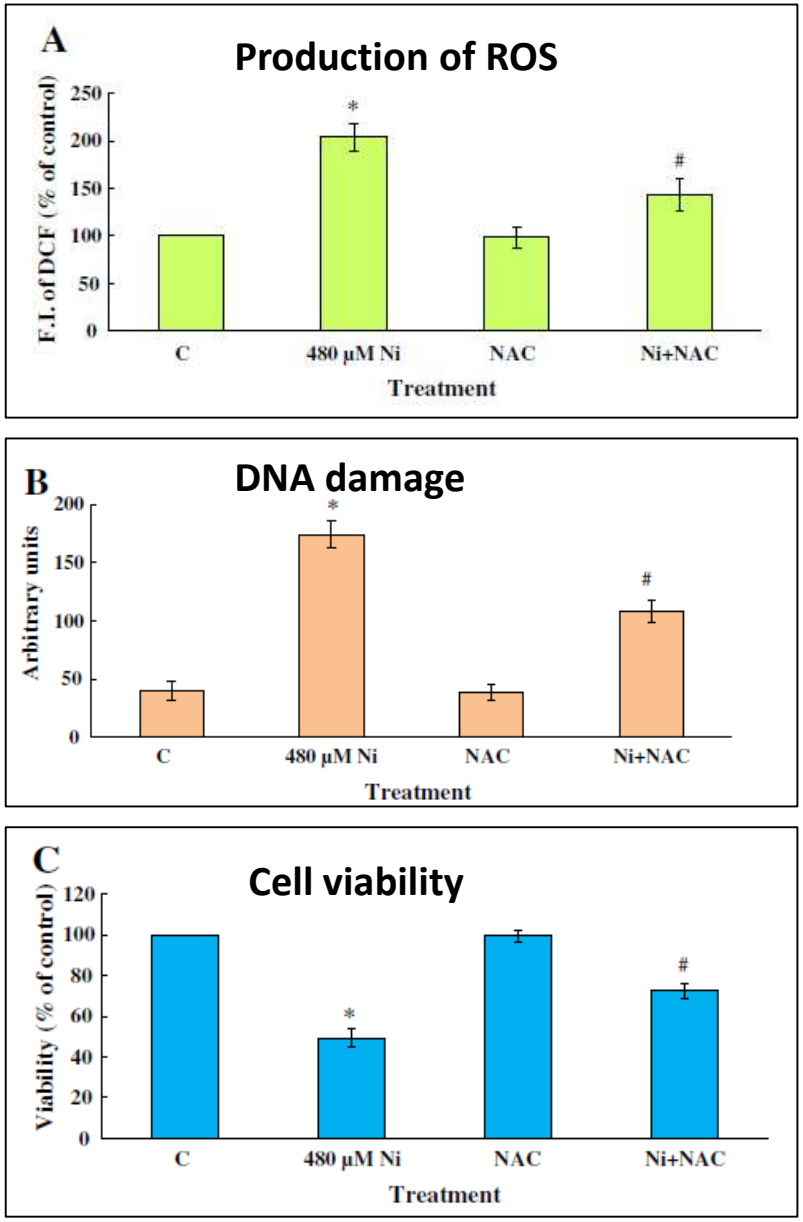


Fig. 4.

(A): ROS production; (B): DNA damage; (C): cell viability.

Cells were pre-incubated with N-acetylcystein (NAC, 5mM) for 1 h, followed by treatment with nickel (II) (480 µM) for 48 h.

The fluorescence intensity of DCF was determined by the fluorescent DCF assay.

Viability was assessed with The MTT assay.

DNA damage in arbitrary units was estimated by the comet assay.

Data are presented as the mean±SD of 5 separate experiments. * Significantly different from control ($p<0.01$). # Significantly different from nickel group ($p<0.01$).

Apoptosis

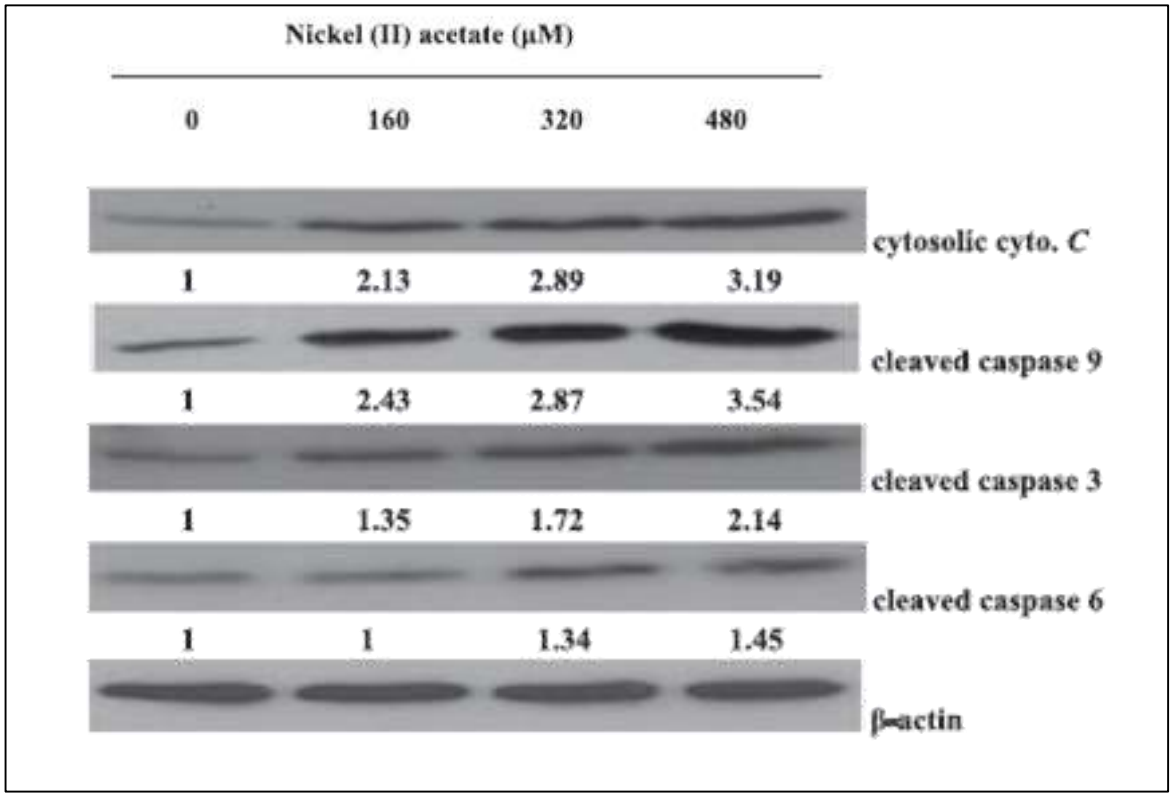
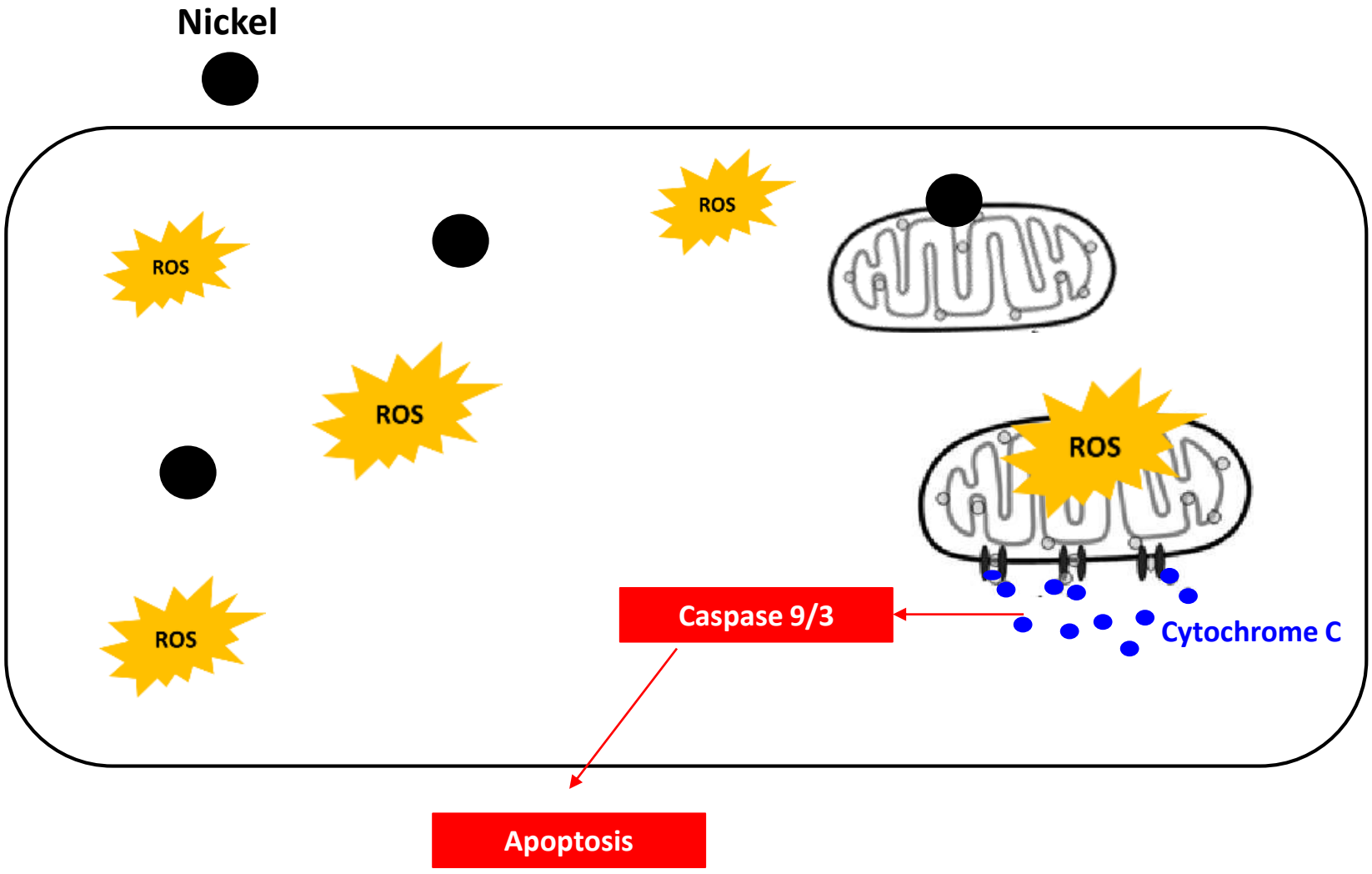


Fig. 5. Effects of nickel (II) acetate on cytochrome c and caspases 3, 6 and 9. Western blot analysis shows the levels of cytochrome c and caspases 3, 6 and 9 protein expression in HK-2 cells after treatment with nickel (II) (0–480 μM) for 48 h.

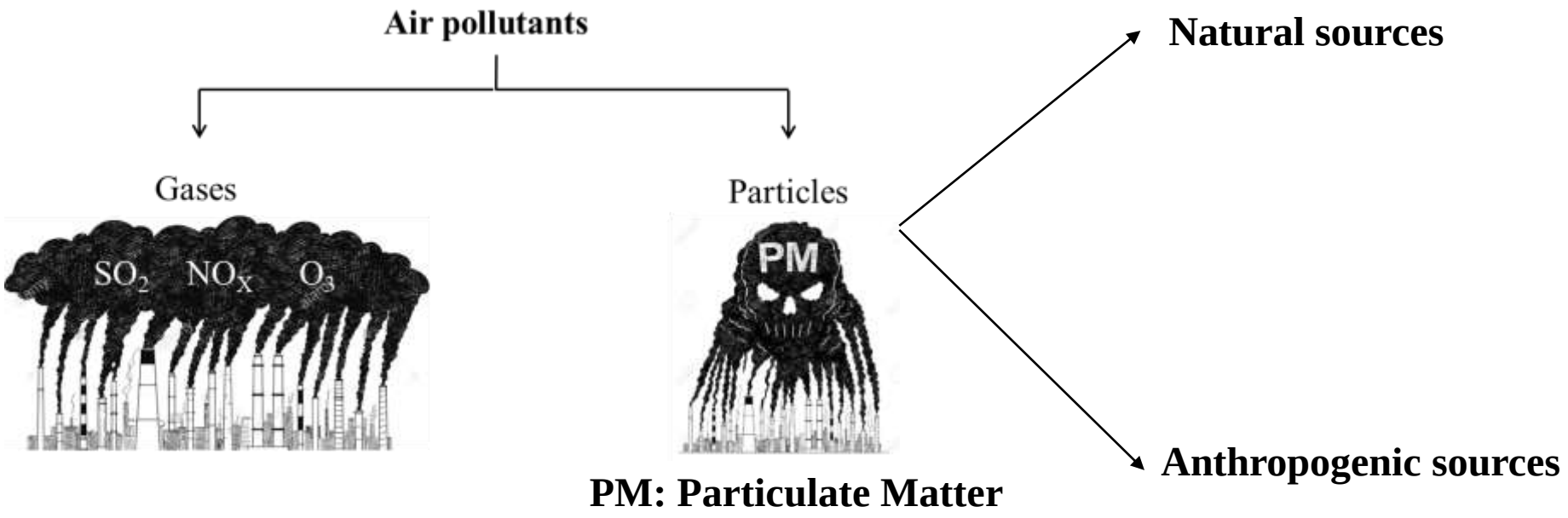

Nickel
ROS
ROS
ROS
ROS
ROS
Caspase 9/3
Cytochrome C
Apoptosis

Exercice 2

Air pollution represents the contamination of the indoor or outdoor environment by chemical, physical or biological agents that alter the natural characteristics of the atmosphere **(WHO, 2013).**

It is a major health problem, because world health organization guidelines **(WHO, 2018).**

In addition, this pollution is responsible of 7 millions premature deaths are related to air pollution each year **(WHO, 2018).**

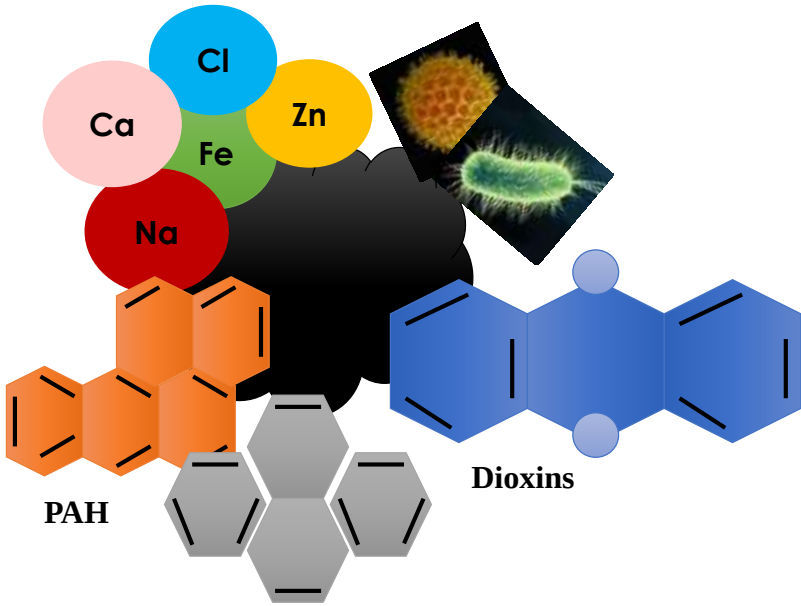


PM: Particulate Matter

Exercice 2

Mixture of :

- **Inorganic compounds** (e.g. metals, ions)
- **Organics** (e.g. polycyclic aromatic hydrocarbons: PAH)
- **Biological materials** (e.g. bacteria, fungi, pollen)



PM toxicity highly dependent on PM **composition**

↓

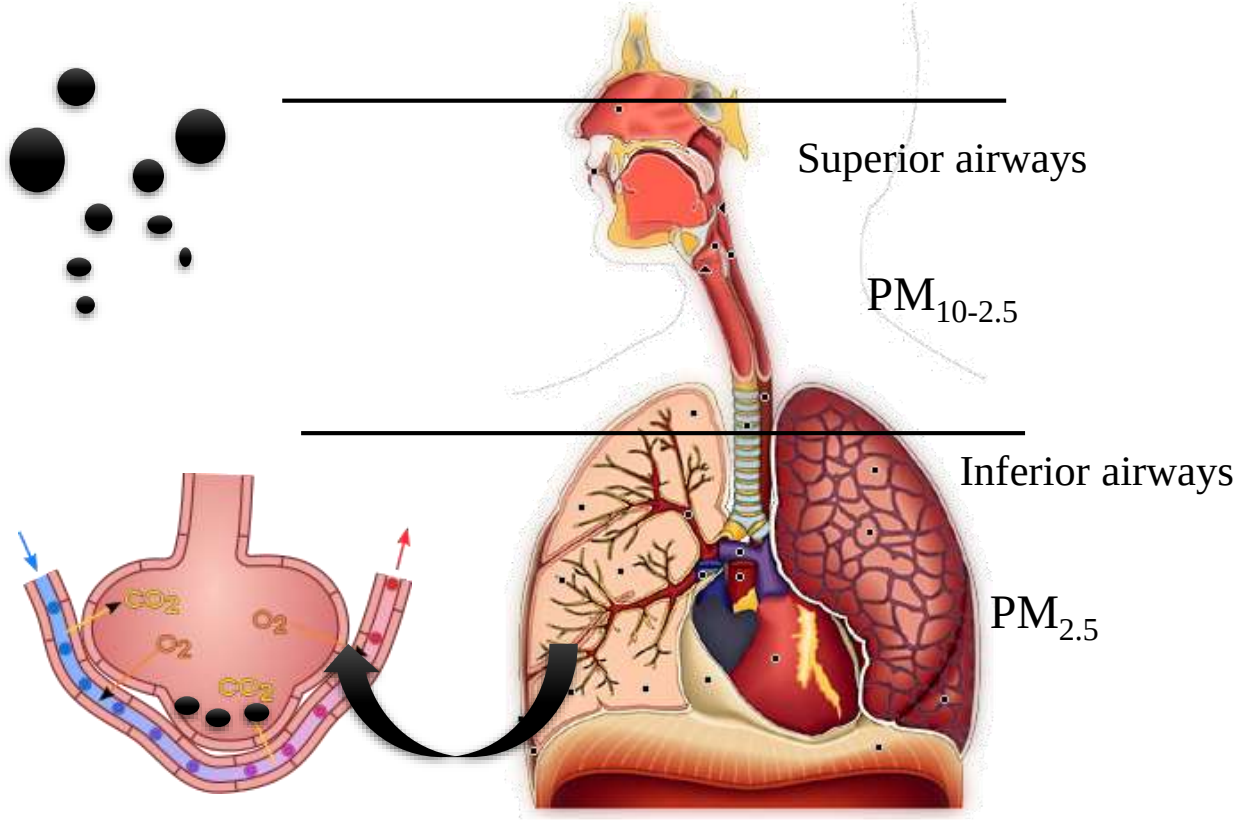
Varies according to the geographical location, contributing sources and several factors (climate…)

↓

Difficult to predict the toxic effects

Exercice 2

PM fractions		Equivalent aerodynamic diameter (EAD)
Coarse fraction	$PM_{10-2.5}$	2.5 < EAD < 10 µm
Fine fraction	$PM_{2.5}$	EAD < 2.5 µm
Ultrafine fraction	$PM_{0.1}$	EAD < 0.1 µm



Exercice 2

Research project: Evaluate the *in vitro* toxicity of atmospheric particles.