Effects of *p-cresol* on HK-2 renal cells. Pro-healing effects of polyphenols.

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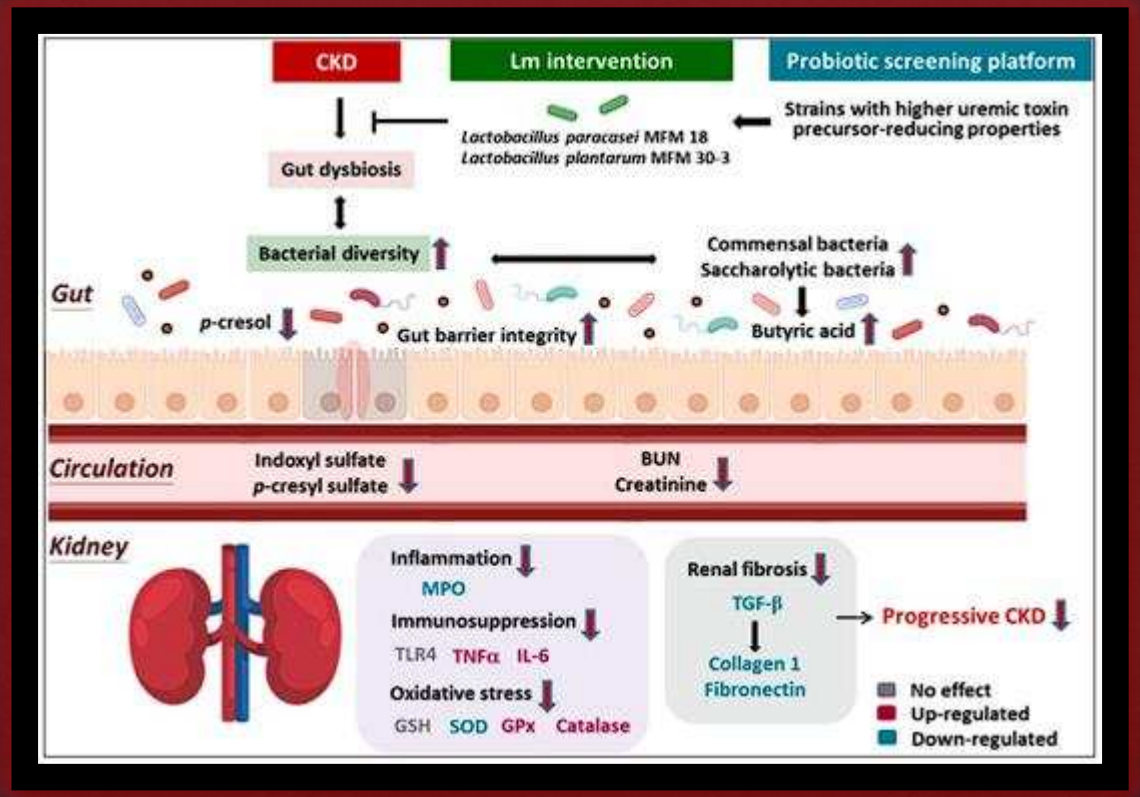

1. Introduction

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- The kidney is one of the organs that serve to filter the blood. Blood from the whole-body circulation enters Bowman's pouch, where filtration takes place and then through the proximal tubule, loop of Henle, distal tubule to the collecting duct, reabsorption, secretion and excretion. The whole process involves filtration of the blood, entry of pure blood into the circulation and final excretion of urea and other impurities.

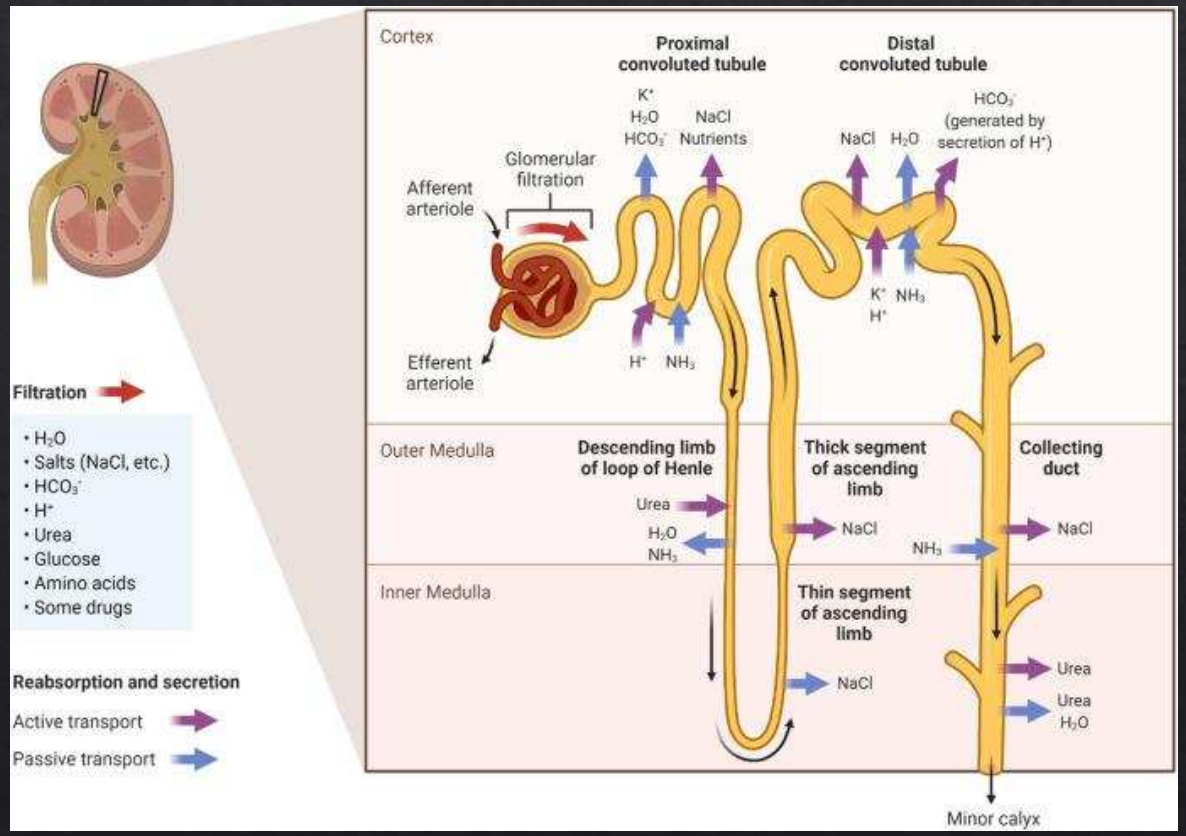

Figure 1. Blood filtration process in the kidney circuit.

1. Introduction

- The gut microbiota, serves to support the digestive process. Incorrect digestion caused by various factors e.g. (poor diet, illness, low immunity) can lead to inhibited albumin function so that harmful toxins can be released from our microbiota.
- Uremic Toxins (UTs) can be inactivated by filtration in the proximal tubule, but if it is not working properly then UTs are deposited in the cells leading to CKD.

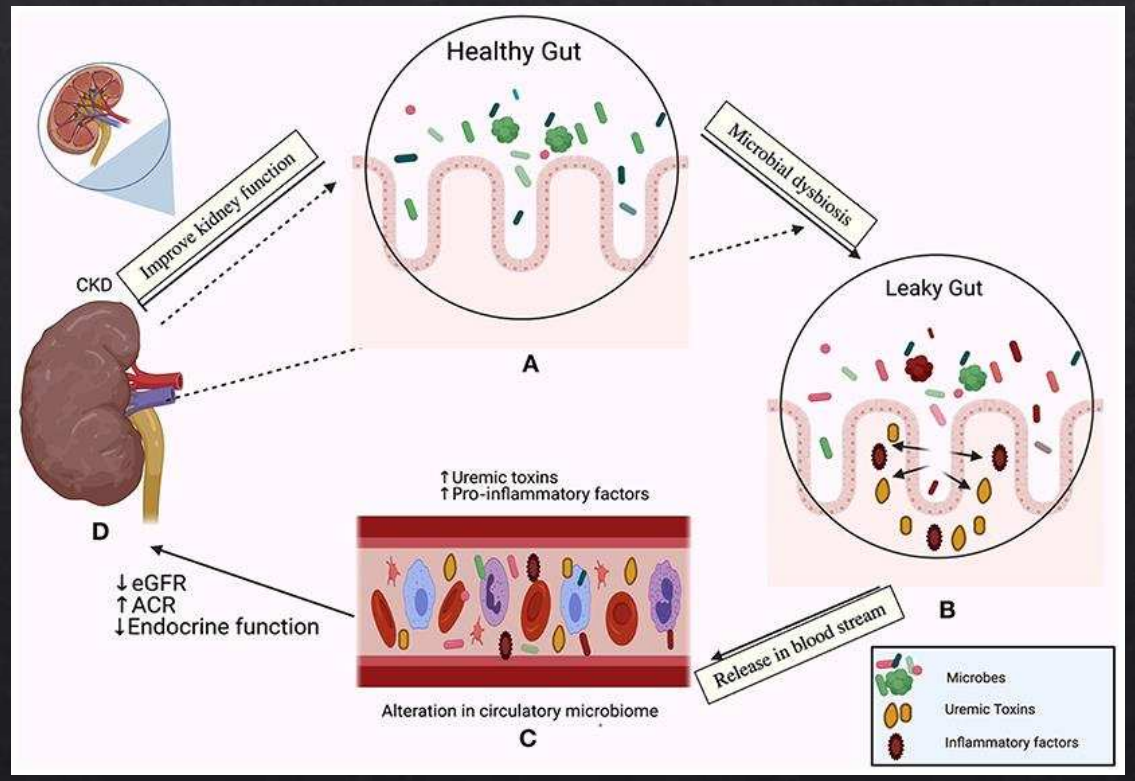

Figure 2. Uremic toxins from gut microbiota effects on kidney function.

2. Main Topic

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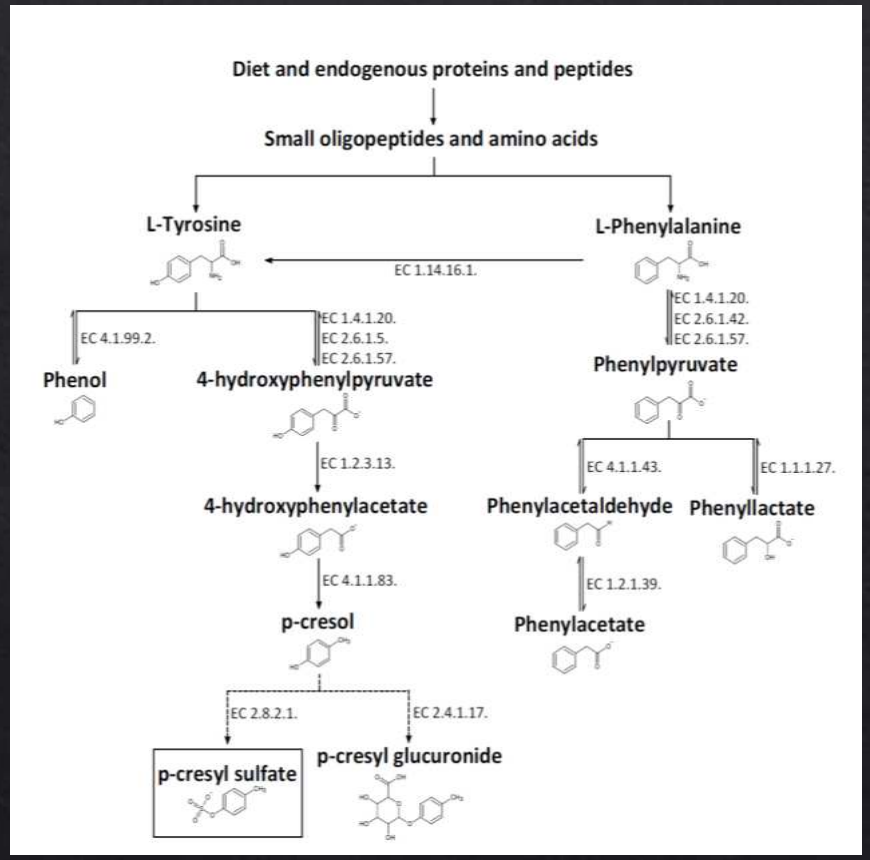
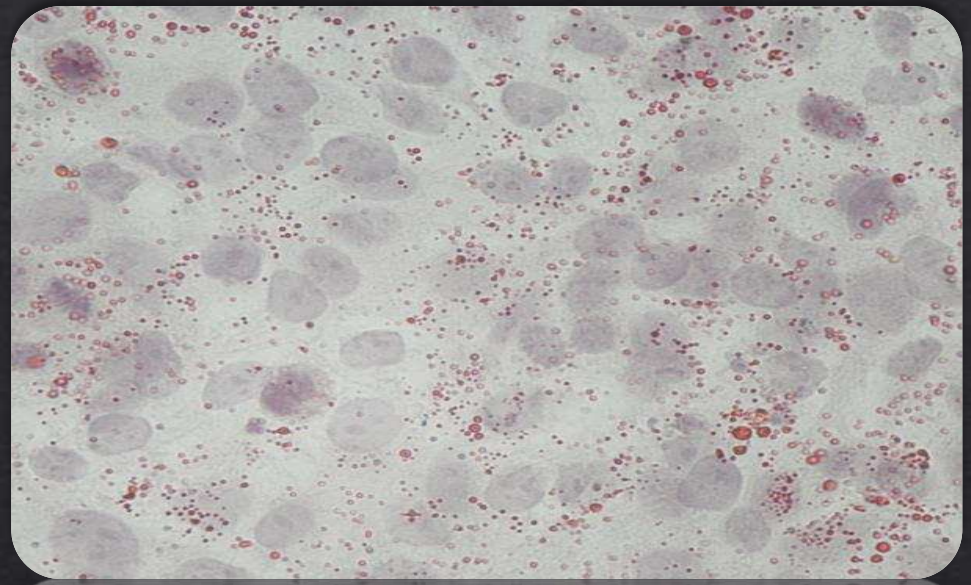


Figure 3. Peptides and protein metbolism scheme

- ***p*-cresol**, which is one of the uremic toxins, is produced from the intestinal microbiota as the result of L-tyrosine metabolism and is transported into the blood binded by albumin.
- *p*-cresol is subjected to sulfonation in the liver, then it's filtered into the kidney proximal tubule and excreted via the urine
- If *p*-cresol isn't metabolized properly, it accumulates into the proximal tubule cells, damaging them.



3. Experiments

3.1 Cell Culture Maintenance

- HK-2 cells (a human proximal tubule cell line) were kept under constant control, observation and change the medium to fresh.
- Cell growth takes place in a 25 cm^2 flask.
- When the cells have achieved sufficient growth, they can be seed.

3.2 Cells seeding

- It is important to remember to prepare the cell concentrations carefully so that growth is equal in each sample in order to obtain the most reliable result.
- Continue cell seeding until the aggregation is observed under the microscope.
- Then, when the concentration is reached, proceed with the treatments: HK-2 cells are treated with ***p*-cresol** and **natural extract** enriched in polyphenols.

3.3 Oil RED O staining

Lab protocol:

- Prepare Oil Red O solution : weight 0,2g of mix Sudan III and IV powder and dissolve in 100ml *Isopropanol*.
- Prepare of Oil Red O „Working Solution" : 30 ml of Oil Red solution mixed with 20 ml distilled water.
- Filtrate the dyes (Oil Red O and emallume).
- Fix cells by using 4% *Paraformaldehyde* (PFA) for 20 minutes.
- Wash them with PBS^{++} (3 times for 5 minutes).
- Wash it again by using 50% *isopropanol* for 5 minutes.
- Incubate in Oil Red "Working Solution" for 20 min.
- Wash it 3 times for 5 min with 50% *isopropanol*.
- Wash with distilled water.
- Stain cells with EMALLUME for 10 min.
- Wash it with PBS++ for 5 min.
- Mount the slides using MOWIOL o/n at room temperature.

3.3 Oil RED O staining

- When cells are treated with *p*-cresol (100μM/24H) and the lipid droplets are stained, it is possible to observe their significant increasing number.
- On top of this, the use of the natural extract together with the treatment with *p*-cresol, shows that the action of the polyphenols contained into the extract can decrease the number of lipid droplets.

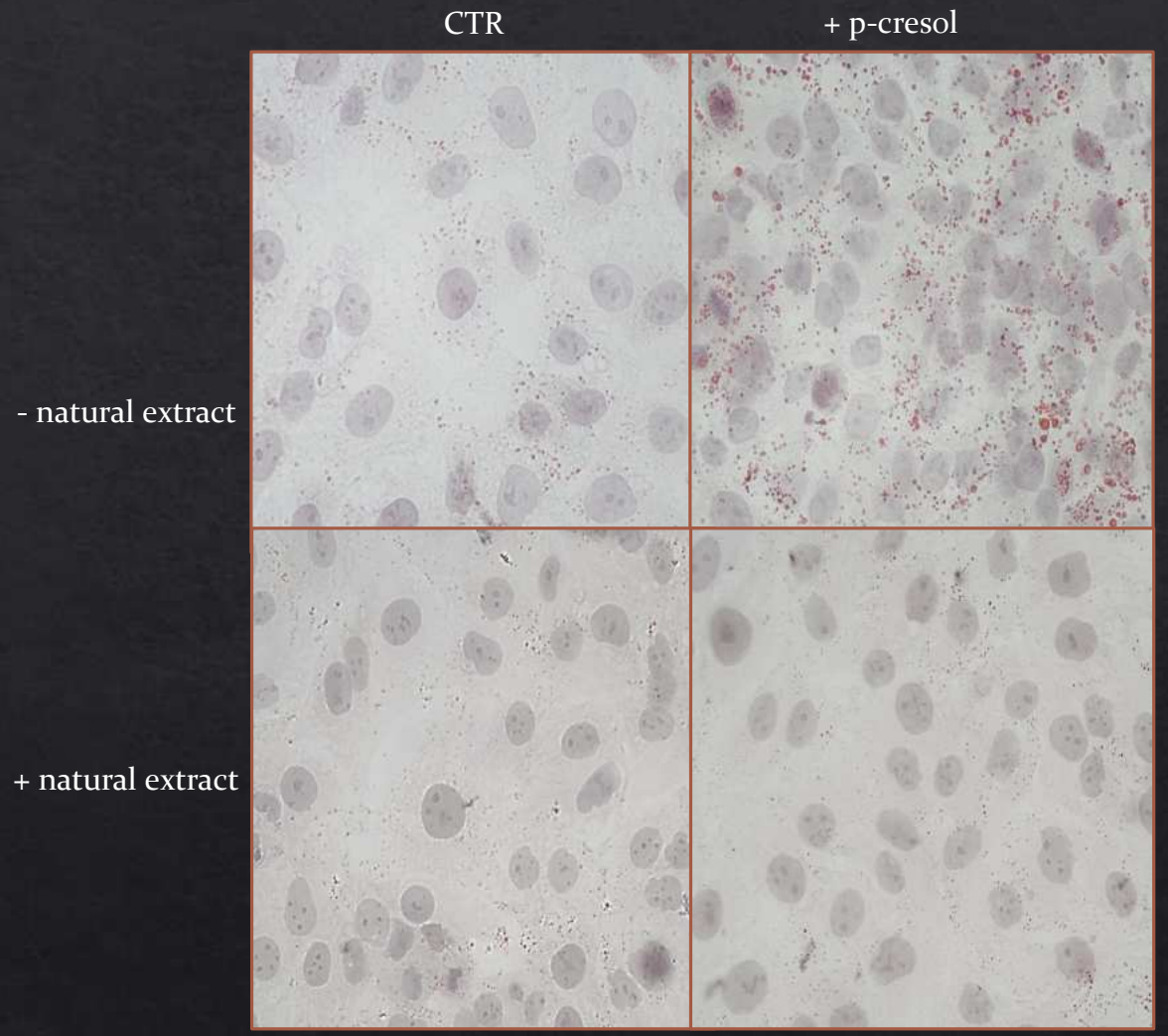


Figure 4. Oil red O staining.

3.4 Immunofluorescence

- Through actin staining, changes in the cytoskeleton can be seen during the action of *p*-cresol. The images, taken under a confocal microscope, show that the action of *p*-cresol leads to the cells stress and, probably, to dysfunctional activity.
- A significant difference can be observed between the effect of *p*-cresol on the cytoskeleton, without and with the addition of the natural extract to the tests. There is a huge improvement when HK-2 cells are treated with *p*-cresol/natural extract.

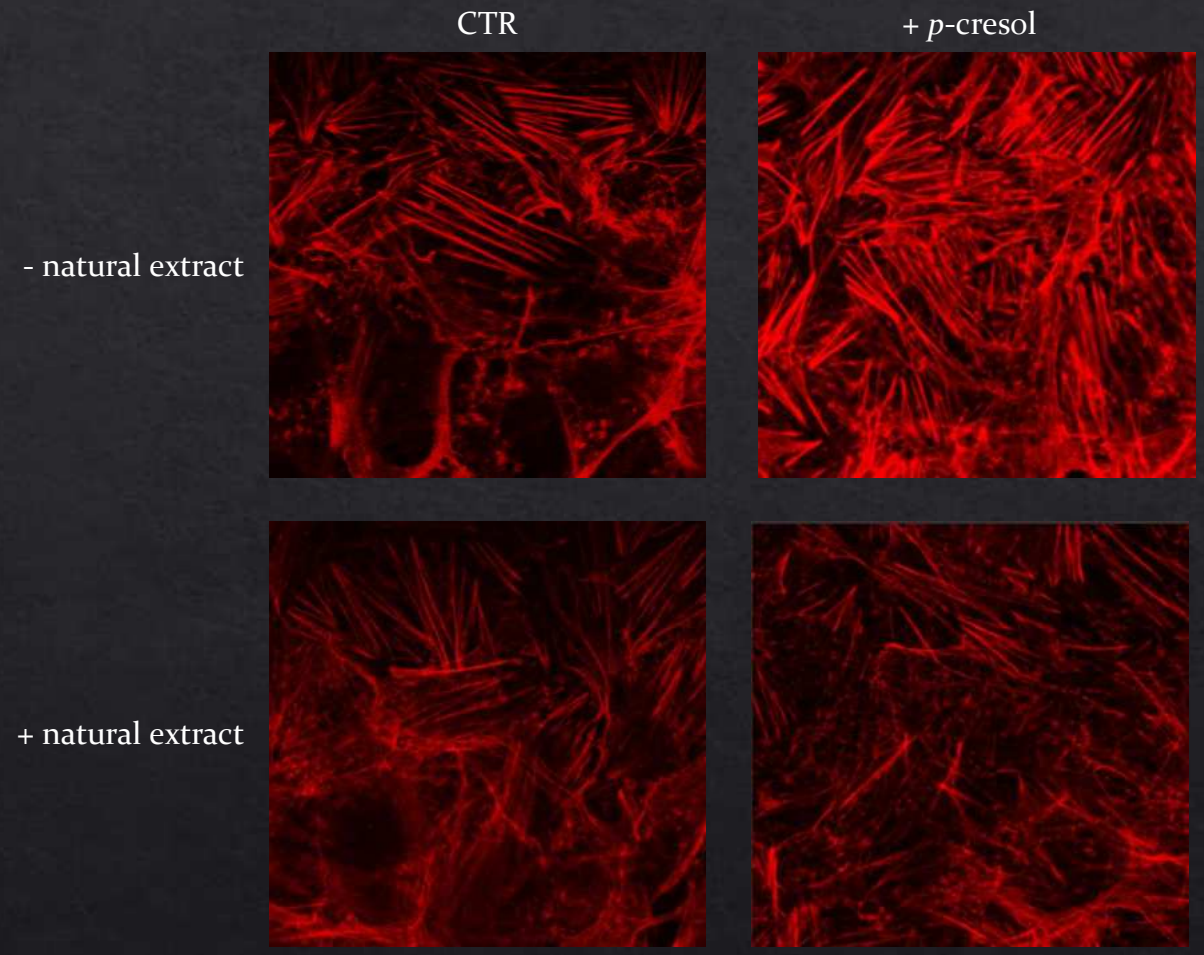


Figure 4. Actin staining seen through confocal microscopy.

3.5 ROS detection

- Reactive Oxygen Species (ROS) are unstable molecules, which contain oxygen as a byproduct of the natural metabolism of oxygen. These molecules are very reactive, they can damage DNA, RNA, proteins, and even cause cell death.
- The amount of ROS occurrence correlates with an increased chance of chronic kidney disease.

Radicals	Non-radicals
Superoxide: O_2^-	Hydrogen peroxide: H_2O_2
Hydroxyl: OH^-	Hypochlorus acid: HOCL
Peroxyl: RO_2^-	Hypobrromus acid: HOBr
Alkoxyl: RO^-	Ozone: O_3
Hydroperoxyl: HO_2^-	Singlet oxygen: Δg

Figure 5. Reactive Oxygen Species (ROS).

3.5 ROS detection

Lab protocol:

- Remove the medium from the wells.
- Add *Dihydrorhodamine 123* (DHR123) 10 µM in PBS++ to every well, then incubate at 37°C for 30 min.
- Remove DHR.
- Add complete medium to the wells and add to 3 wells the *tert-butyl hydroperoxide* (TBHP) 2 mM in complete medium mix for the positive controls ; then incubate in 37°C for 30 min.
- Remove solutions.
- Wash with PBS++.
- Remove PBS++.
- Add RIPA buffer, and transfer cell lysates into eppendorf.
- Vortex eppendorfs every 5 minutes for 30 minutes.
- Centrifuge at 12,000 rpm for 10 minutes at 4°C.
- Take the supernatant and transfer it to a new eppendorf.
- Add 3 samples of 100 µl each to 96 wells plate.
- Measure fluorescence under the detector.

3.5 ROS detection

- Studies on reactive oxygen species in HK-2 cells treated with *p*-cresol show that the uremic toxin significantly increases the level of ROS.
- The *p*-cresol/natural extract combined treatment effectively reduce the negative effect of *p*-cresol.

4. Summary

- The intestinal microbiota has major implications for our health.
- The accumulation of uremic toxins in our body threatens our kidneys functionality.
- *p*-cresol treatment leads to increasing lipid droplets formation that are known to cause renal cell injury
- The increased amount of ROS due to *p*-cresol's effects correlates with an increased chance of Chronic Kidney Disease (CKD)
- Polyphenols contained into the natural extract show effective activity as a drug against *p*-cresol's adverse effects

Thank you for your attention!