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Preclinical safety assessment of Superparamagnetic Iron Oxide Nanoparticles: exploring the *Pomacea canaliculata* as a suitable model for screening toxicity. Preliminary results

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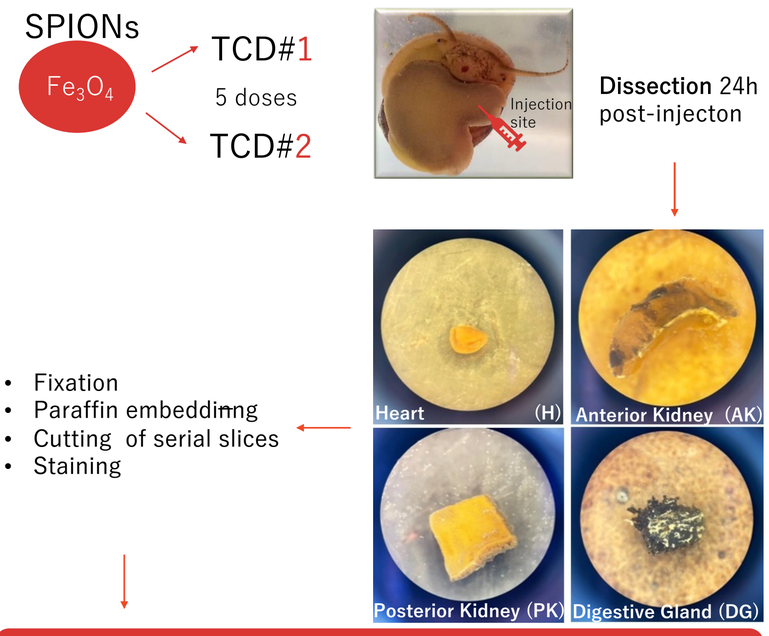
Introduction

The European Directive 2010/6/EU recalls the **3R Principle** (*Reduce*, *Refine* and *Replace*), and aims to implement the use of reliable alternatives to vertebrate models, improve laboratory animal conditions, reduce suffering, and increase the quality of animal studies. In this perspective, we have investigated the potential value of adopting the freshwater snail, ***Pomacea canaliculata***, as a model for the preliminary toxicity and accumulation screening of Superparamagnetic Iron Oxide Nanoparticles (**SPIONs**). *P. canaliculata* is easy to breed, manipulate, and it presents size, weight and longevity similar to mice; hence, we hypothesized that snails could be used as an alternative to these rodents when answering specific questions regarding organ toxicity and accumulation.
The study was carried out in a set of blind experiments using two differently coated preparations of SPIONs (labelled **TCD#1** and **TCD#2**). These nanoparticles were tested for their accumulation in the heart, anterior and posterior kidney, and the digestive gland. A workflow comparable to that used on mice and rats in SPION bioaccumulation protocols was used. Five different doses (from 7.5 to 60 mg Fe/kg) were injected into the snail foot and, after 24 hours, the animals were examined for SPION toxicity and microsurgical dissected for organ collection. Snail organs underwent standard histological preparation and the slides were stained with Perls or hematoxylin-eosin staining. Light microscope images were recorded for the subsequent analysis of tissue integrity and SPION accumulation. To achieve SPION semi-quantitative accumulation data, an *ad hoc* and semi-automatic data mining routine was developed in **MATLAB®** and focused on the detection of Perls-positive and cobalt blue pixelated section of each acquired images.

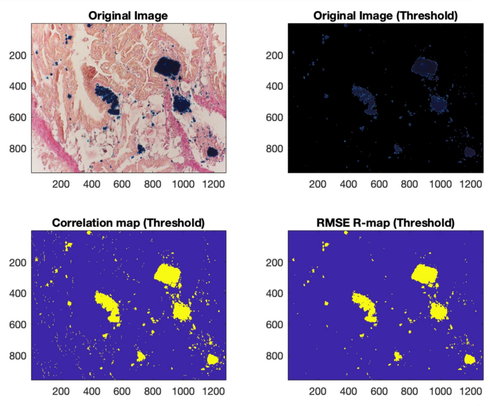
Materials and Methods



Perls' Prussian Blue

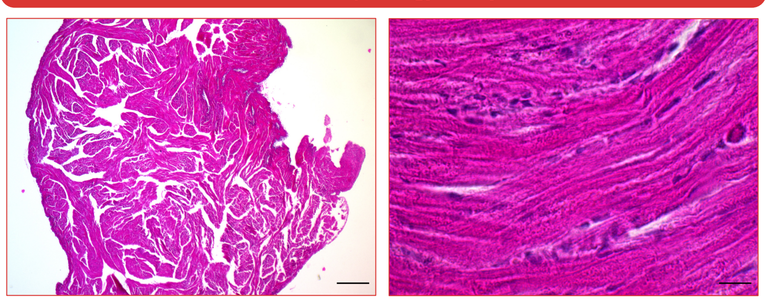
Semi-automatic quantification with MATLAB®

- Select the areas where **blue spots** are present
- Create two false colour images (not shown)
- Establish the threshold levels of correlation and error
- Apply the parameters over the images under the analysis, to obtain masked images
- Count pixels (in number and percentage) from the masked images
- Statistical analysis of collected data (here **ANOVA** was used)



Hematoxylin-Eosin (HE)

Tissue morphology analysis



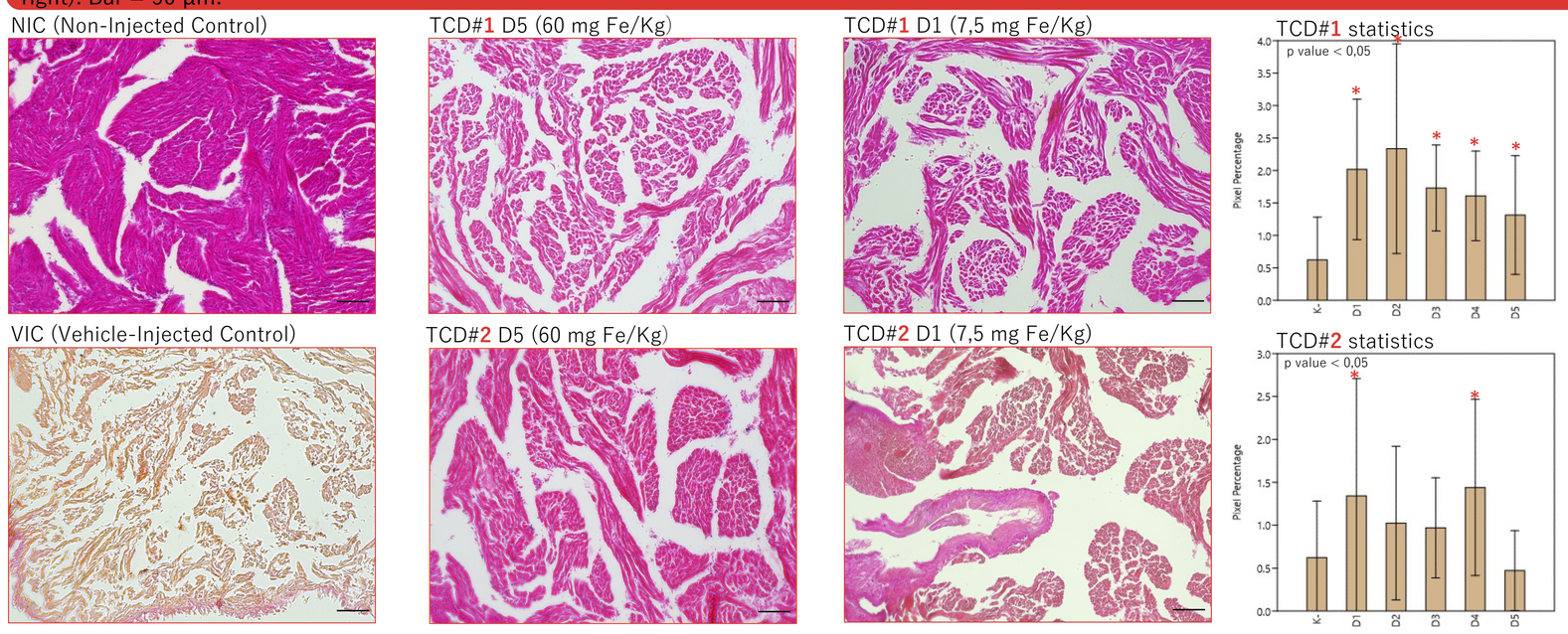
*Example of **H** sections from a Non-Injected Animal (NIC), stained with HE observed at 4X (left) and 100X (right) magnifications. Bar 50 µm.*

Conclusions

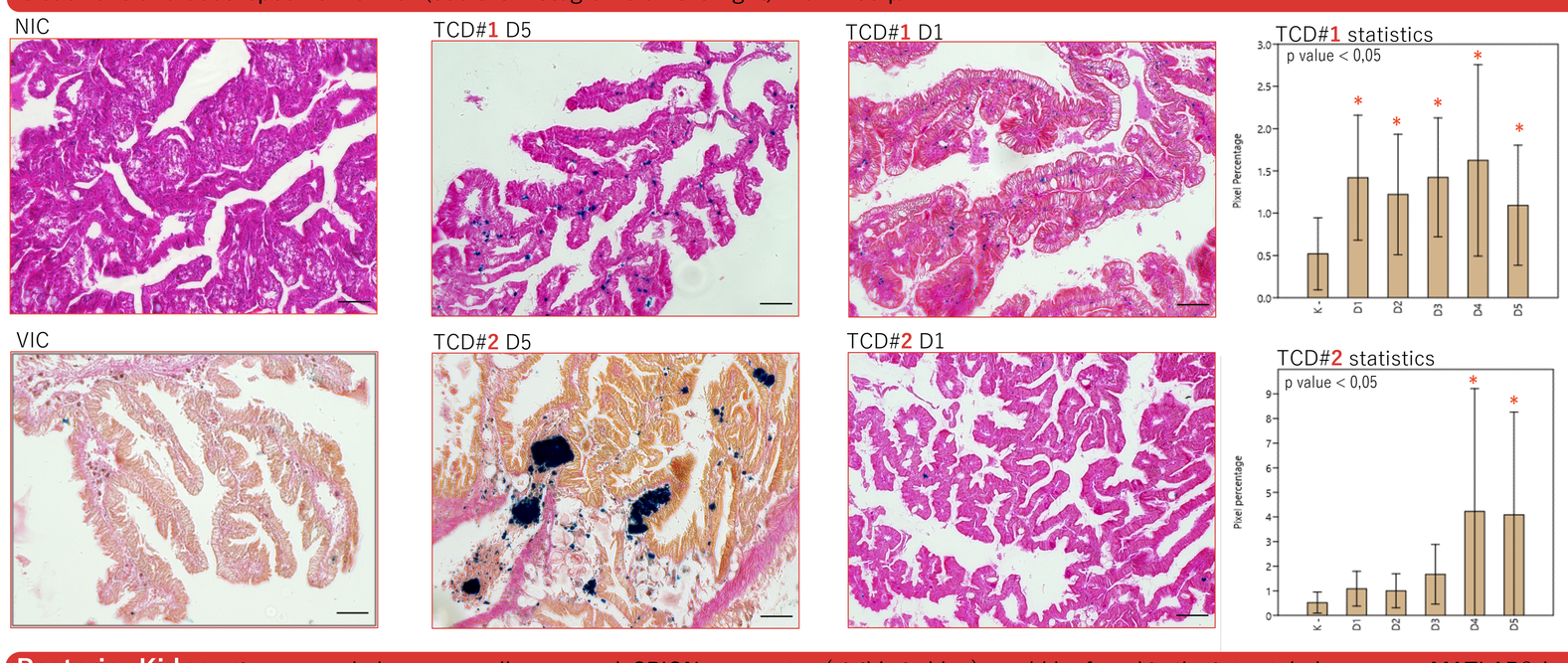
In this study, we investigated the potential of the snail species *P. canaliculata* to act as a model for iron accumulation and organ damage following injection with SPIONs. Across the study, all the snails survived, and no evidence of organ damage was accounted. By combining histochemical and image analysis methods, the protocol was reliable and repeatable. The quantification of iron content (indicative of SPIONs) in snail organs by **inductively coupled plasma-mass spectrometry**, gave results comparable to the semi-quantitative data collected after histochemistry. The SPIONs accumulated mostly in organs that receive more hemolymph (*i.e.*, the snail blood), but the differences observed between TCD#1 and TCD#2 - especially in the posterior and anterior kidney suggest that also the coating of SPIONs may influence their accumulation. This work will be compared to rodents to determine if *P. canaliculata* can act as a potential alternative for preliminary studies on bioaccumulation and biosafety of iron-based products.

Results

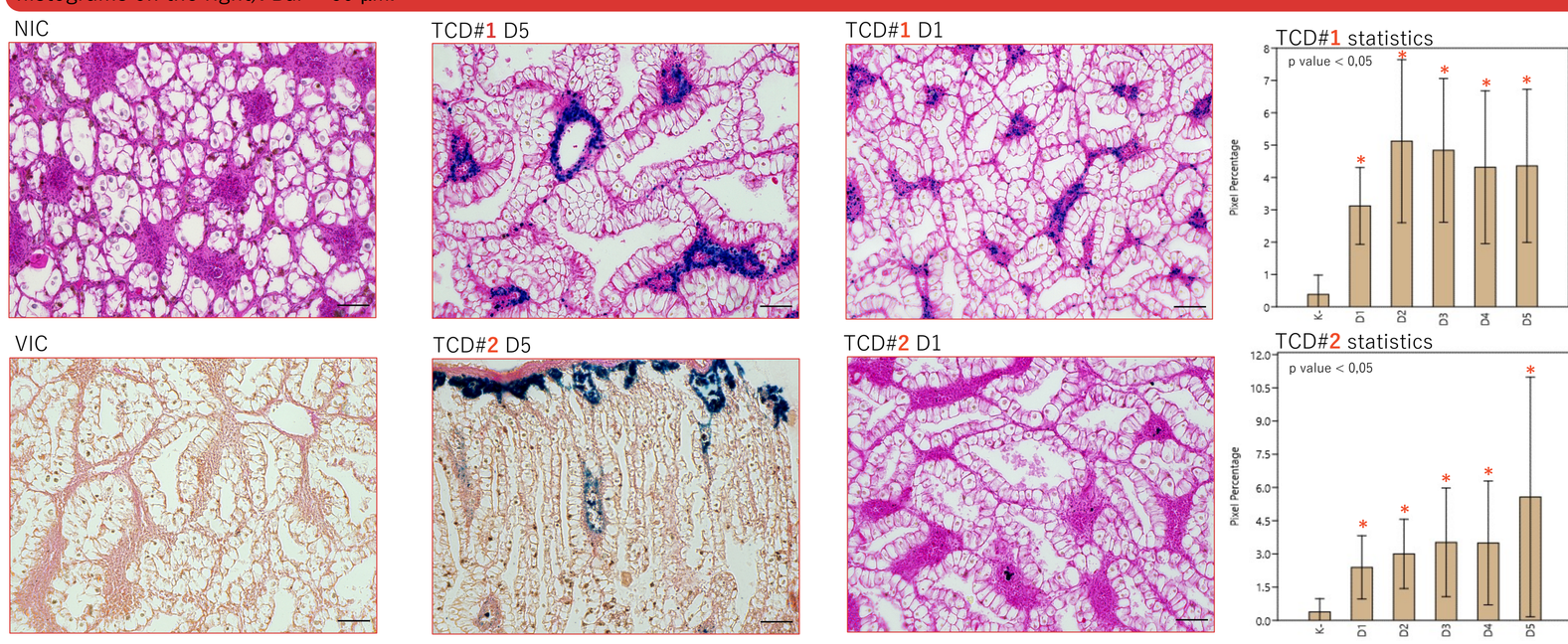
Heart tissue was intact and the morphology conserved among treatments. Histology revealed no relevant SPION accumulation although MATLAB®-based counting and successive ANOVA analysis could detect SPIONs and showed differences between control and treated specimens (see the histograms on the right). Bar = 50 µm.



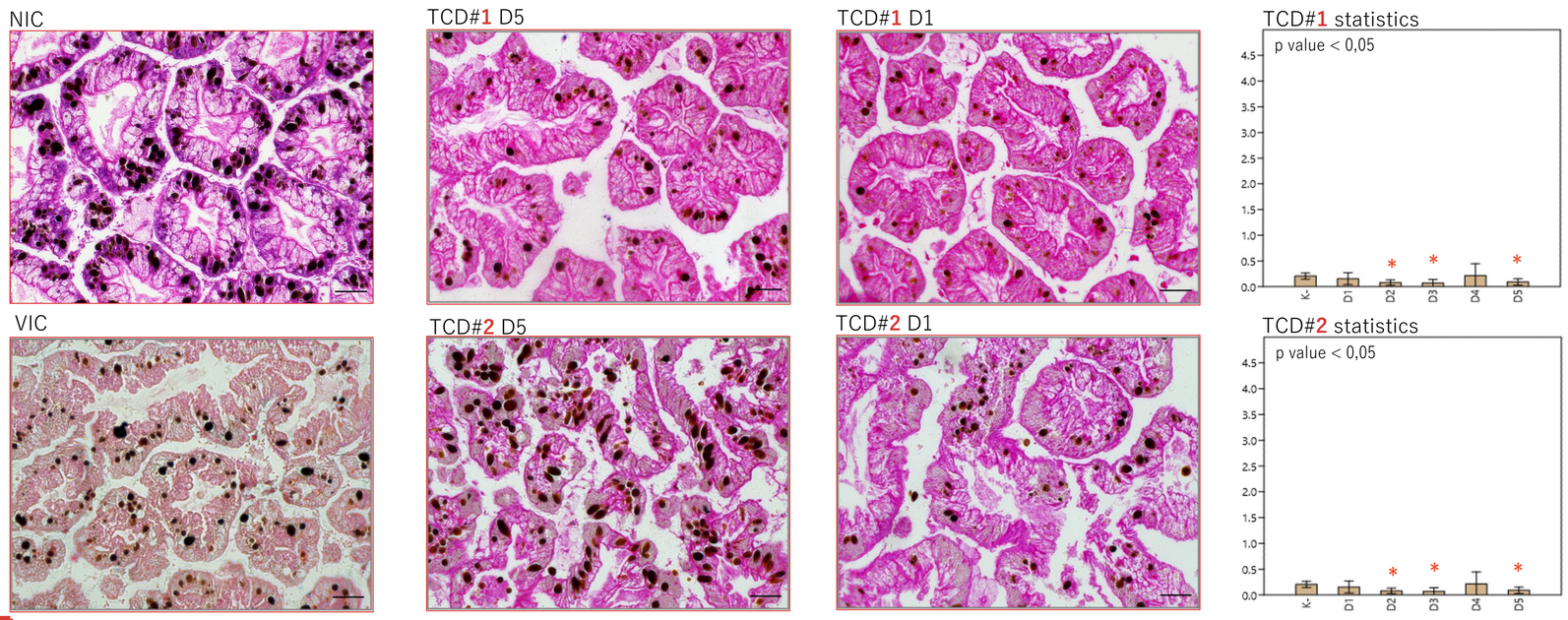
Anterior Kidney histology revealed no morphological alterations after treatments despite evident SPION presence. SPIONs accumulated in a treatment-and dose-specific manner (see the histograms on the right). Bar = 50 µm.

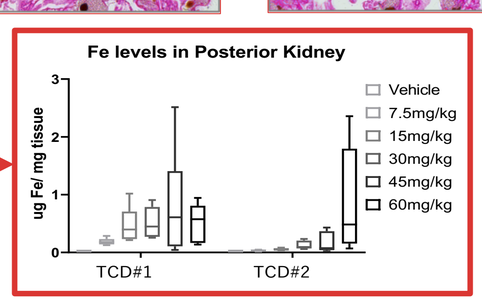


Posterior Kidney tissue morphology was well conserved. SPION aggregates (visible in blue) could be found in the inter-tubular spaces. MATLAB®-based counting and successive ANOVA analysis showed a different SPION accumulation between TCD#1 and TCD#2 with a dose-dependent trend for TCD#2 (see histograms on the right). Bar = 50 µm.



Digestive Gland tissue conformation was comparable to the NIC controls. SPIONs were not accumulated after either TCD#1 or TCD#2 injected specimens. Histological and computer-assisted analysis confirmed the rare presence of SPIONs (see histograms on the right). Bar = 50 µm.





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