Doctoral Program in Bioengineering and Medical-Surgical Sciences (32th Cycle)

The use of Human Liver Stem cells- Extracellular Vesicles (HLSC-EVs) as a biological treatment to recondition marginal rat livers during *ex-vivo* normothermic perfusion (NMP)

Liver transplantation (LT) is the last therapy to end-stage liver diseases. However, a great number of potential recipients have not the possibility to access to liver transplant because of the lack of organ donors. To increase the pools of organs donors', marginal livers were taken in consideration for LT, such as the ones from donors after cardiac death (DCD). Nevertheless, the standard liver preservation technique, named Static Cold Storage (SCS), which consists in the slowing of hepatocellular metabolism, is imperfect in protecting marginal livers organs. In this context, the *ex-vivo* normothermic machine perfusion (NMP) could offer the possibility to recondition graft viability prior to implantation. Human Liver Stem Cells (HLSC) have been identified in the liver as a population of pluripotent resident cells expressing markers of the mesenchymal lineage together with hepatic markers, suggesting a partial hepatic commitment. The extracellular vesicles (EVs) from HLSC have been isolated and characterized, showing that they are able to mediate a regenerative effect mainly by horizontal transfer of specific mRNA and miRNA.

In this thesis, two models of marginal rat livers were reconditioned *ex-vivo* by NMP, testing the HLSC-EVs as a biological treatment to rescue injured livers, improving liver features and functionality.

The first marginal rat liver model consists of a hypoxic rat liver perfused *ex-vivo* by NMP for four hours with a low haematocrit (<10%), to obtain an ongoing and constant hypoxic injury. We compared the difference between the injured non-treated livers (control group, n=10) and the injured livers treated by HLSC-EVs (treated group, n=9). The uptake of HLSC-EVs was analysed by immunofluorescence, while tissue injury was analysed by haematoxylin-eosin (H&E) staining. Then, apoptosis was assessed by TUNEL assay. Total bile production was quantified and perfusate samples were collected hourly to measure cytolysis markers (AST, ALT, LDH). Finally, the expression of HIF-1α, and TGF-β1 was measured by RT-PCR. During the hypoxic NMP perfusion, livers were able to maintain homeostasis (pH, pO_2, pCO_2) and to produce bile. The HLSC-EVs were engrafted into the hepatic parenchyma. The HLSC-EVs treatment significantly reduced the Suzuki's score, the apoptosis index and the release of AST and LDH, already at third hour of perfusion. In addition, the HLSC-EVs treatment acted at a molecular level reducing the expression of HIF-1α and TGF-β1.

The second marginal rat liver model consists of 60 minutes warm ischemic rat liver (DCD) perfused *ex-vivo* by NMP for six hours. We tested two different doses of HLSC-EVs, EV1 and EV2, where

EV2 dose is 5 times greater than EV1 one. The rat livers were divided into four experimental groups: NMP, WI+NMP, WI+NMP+EV1 and WI+NMP+EV2. The NMP livers were subjected to ≈34 minutes of SCS + 6 hours of NMP; the WI+NMP livers were subjected to 60 minutes of warm ischemia + 6 hours of NMP. The WI+NMP+EV1 and WI+NMP+EV2 livers were subjected to 60 minutes of warm ischemia + 6 hours of NMP and treated with HLSC-EVs dose 1 or 2. During NMP, metabolic (phosphates, bile, pH, HCO_3^-) and hemodynamic (pressure, flow) parameters were measured; perfusate samples were collected hourly and the release of cytolysis enzymes (AST, ALT) were measured. Then, at the end of each perfusion, tissue samples were collected to verify the engraftment of HLSC-EVs (fluorescence analysis), to do histological evaluation (H&E, TUNEL assay, PCNA immunohistochemistry) and to measure RNA expression of adhesion molecules (ICAM-1, VCAM-1, Selectin-E, Selectin-P). The HLSC-EVs were engrafted in hepatic parenchyma. The EV2 dose of HLSC-EVs was able to significantly reduce hepatocytes necrosis. Although the apoptosis level was negligible compared to necrosis, the HLSC-EVs treated livers reported an inferior apoptosis level compared to the ischemic livers. In addition, the EV2 dose of HLSC-EVs was able to increase the proliferation capability of the liver, which was ablated by ischemia.

Both the two doses of HLSC-EVs were able to significantly reduce the release of the cytolysis enzymes AST and ALT at the sixth hour of perfusion and the EV2 dose decreased the AST level already at the fourth hour of perfusion. At a metabolic level, both the two doses EV1 and EV2 required a small amount of HCO^-_3 for pH regulation, and promoted the self-regulation of pH and the phosphates consumptions during perfusion. Only the EV2 dose succeeded in significant increase the amount of produced bile, compared to the control ischemic liver. The hemodynamic resistance in the perfusion circuit due to ischemia was significantly reduced by the EV2 dose. The injured ischemic hepatocytes expressed a low level of the analysed adhesion molecules, while the HLSC-EVs tended to rescue their expression in treated livers.

The HLSC-EVs treatment showed to be able to recover the hypoxic rat liver and the DCD rat liver respectively during the four and six hours *ex-vivo* NMP. Besides, between the two tested doses in DCD model, the EV2 dose could be more effective than the EV1 one. The association of HLSC-EVs to NMP in order to reduce liver injury is a promising strategy for *ex-vivo* graft reconditioning before transplant surgery although further investigations are required.