UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA


# LIVER REGENERATION AND TISSUE ENGINEERING

PEDRO MIGUEL ALMEIDA DE MATOS BAPTISTA

DOUTORAMENTO EM FARMÁCIA

(BIOTECNOLOGIA FARMACÊUTICA)

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**ABBREVIATIONS**

3D – Three-Dimensional
AFS - Amniotic Fluid Stem
anti-GFP – Anti-Green Fluorescent Protein
AVB – Acelular Vascularized Bioscaffold
BHA – Butylated Hydroxyanisole
DAB - 3,3' Diaminobenzidine
DMEM – Dulbecco's Modified Eagle's Médium
DMSO - Dimethyl Sulfoxide
ECM – Extra-cellular Matrix
EGM - Endothelial Cell Medium
FBS - Fetal Bovine Serum
hAFS – Human Amniotic Fluid Stem Cells
HDL - High Density Lipoprotein
HepG2 - Human Hepatocellular Liver Carcinoma Cell Line
hFL – Human Fetal Liver
HpSC – Hepatic Stem Cells
IL-1 – Interleukin-1
IL-2 - Interleukin-2
MDR – Multidrug Resistant
MS1 - Mouse Endothelial Cells
NGF - Nerve Growth Factor
NO – Nitric Oxide
PBS – Phosphate Buffered Saline
PI – Propidium Iodide
RER - Rough Endoplasmic Reticulum
SEC – Sinusoidal Endothelial Cells
VEGF - Vascular Endothelial Growth Factor
VLDL – Very Low-Density Lipoprotein
WHO – World Health Organization

**ABSTRACT**

Over 21 million people in the world are estimated to live with chronic liver disease, and about 800,000 expire annually, accordingly with WHO. Liver transplantation remains today the definitive treatment for end-stage liver failure. However, the gap between organ donation and the number of patients in the waiting list for a liver keeps widening. The shortage of organs has stimulated the research for alternatives in end-stage liver disease. Cell therapies with some degree of success are today a reality. Nevertheless, the goal of generating a whole liver *in vitro* remains elusive. The purpose of the work of this dissertation was to develop a new approach to liver tissue engineering that would allow the generation of significant mass tissue readily implantable and/or transplantable. Cell sources for tissue engineering applications are of vital importance. Our research focus on the use of hFL progenitor cells in combination with the liver bioscaffold and on a new promising source of fetal stem cells from amniotic fluid, in liver therapies and tissue engineering.

In our work, we were able to create a novel acellular liver derived bioscaffold with preserved vascular network. This bioscaffold could be efficiently re-cellularized and considerable mass tissue, displaying some hepatic functions, was generated. It was also successfully transplanted to living hosts and perfused with blood. Another goal attained in this work was that the combination of hFL progenitor cells with the liver bioscaffold produced cell engraftment, expansion and differentiation of the hFL cells. hAFS cells also showed engraftment and integration in injured livers, representing a new alternative for *in vivo* cell therapies and liver regeneration.

In conclusion, this doctoral dissertation clearly demonstrates the successful generation of 3D liver tissue with a novel acellular liver bioscaffold using different cell sources. This potentially represents a new hope for patients suffering of end-stage liver disease.

**SUMMARY**

The severity of end-stage liver disease is directly related with the vital role that the liver plays in systemic metabolic homeostasis. Organ transplantation remains today the definitive treatment for end-stage liver disease. Due to the shortness in organ availability, new alternatives have been sought in the last decade. With the dawn of regenerative medicine, cell transplantation using adult hepatic cells has emerged as a potential therapeutic option to treat various severe liver conditions. Although successful, therapies with adult hepatic cells usually fade within several months. The advancement of stem cell biology as also brought new opportunities in cell therapies with newly identified or differentiated stem/progenitor cells. It also increased the opportunities in finally generating a whole liver *in vitro* able to be readily implanted or transplanted into a host.

In this work, the generation of a novel liver derived biomaterial that could preserve its native vascular network was investigated. We attempted to improve the decellularization of thick tissues and solid organs employing some of the tissue decellularization techniques used for the generation of naturally derived scaffolds. We were succeeded in our attempts on perfusing decellularization solution through the liver vascular system instead of simply shaking the organ with the decellularization solutions relying only on reagent diffusion. The outcome was the generation of a novel acellular liver derived bioscaffold which preserves its native vascular network. This allows the perfusion of culture media and cell seeding through the decellularized organ vasculature reaching virtually every position in the thick 3D bioscaffold. Oxygen and nutrient diffusion limitations are now overcome by the use of the decellularized organ native vascular system, allowing *in vitro* generation of dense 3D tissue.

The optimization of the re-cellularization process was carried out by seeding the cells with culture media perfusion. The use of different perfusion flow rates allowed us to find the optimal

conditions to seed these liver bioscaffolds with different types of cells, which generate high cellular density 3D tissues expressing characteristic hepatic functions.

In order to further enhance hepatic tissue generation, we investigated the use of hFL progenitor cells in combination with the liver bioscaffold. The use of these liver progenitor cells are a valuable resource for liver tissue engineering and cell therapies. Preliminary experiments indicated that hFL progenitor cells seeded in bioscaffold disks engrafted, proliferated and differentiated in hepatocytic and biliary cell lineages.

Finally, we investigated the use of hAFS cells in several *in vivo* regeneration models. Our work confirmed the non-tumorigenic potential of these cells and demonstrated their integration and differentiation in injured muscle and response to angiogenic stimuli. Moreover, these cells were able to engraft in damaged livers. The *in vivo* multipotenty exhibited by hAFS cells is encouraging and confirms their potential in regenerative medicine applications.

Overall, this dissertation work emphasizes the relevance of a new organ decellularization method able to generate liver acellular bioscaffolds with preservation of a functional vascular network. This allows culture media perfusion with 3D tissue generation beyond the oxygen and nutrient diffusion limits. Furthermore, we present evidence that hFL progenitors in combination with the liver bioscaffold and hAFS cells offer new possibilities in liver tissue engineering and cell therapies of end-stage liver disease.

**RESUMO**

A gravidade das doenças hepáticas terminais está directamente relacionada com o papel vital que o fígado desempenha na homeostase metabólica sistémica. A transplantação de orgãos permanece ainda hoje como o único tratamento definitivo para a doença hepática terminal. Devido à escassez de orgãos, novas alternativas têm sido investigadas na última década. Com o despontar da medicina regenerativa, a transplantação de células hepáticas adultas tem emergido como um opção terapêutica válida no tratamento de várias patologias hepáticas graves. No entanto, embora com algum sucesso, o efeito terapêutico diminui e desaparece ao fim de alguns meses. Os recentes avanços no conhecimento da biologia das células estaminais, nomeadamente na identificação e caracterização, trouxeram novas oportunidades de terapêuticas celulares com recurso a populações de células estaminais/progenitoras. A possibilidade de finalmente gerar um fígado totalmente *in vitro* passível de ser facilmente implantado ou transplantado, também aumentou.

Neste trabalho, foi investigada a geração de um novo biomaterial derivado do fígado preservando intacta a sua rede vascular. Para tal, foram empregues algumas das técnicas de descelularização de tecidos, usadas na obtenção de matrizes naturais, e efectuada a optimização destes processos na descelularização de tecidos mais espessos e orgãos sólidos. O processo revelou-se eficaz quando soluções de descelularização foram perfundidas pelo sistema vascular do fígado em alternativa à simples agitação do órgão imerso nas soluções de descelularização e cujo princípio se baseia na difusão dos reagentes pelo órgão. O resultado foi a obtenção de uma nova matriz acelular derivada do fígado e que preserva o seu sistema vascular nativo. Esta matriz permite a perfusão de meio de cultura e de células através da rede vascular do orgão descelularizado, permitindo virtualmente atingir qualquer posição da espessa matriz 3D. As limitações de difusão de oxigénio e de nutrientes são assim ultrapassadas pela utilização da rede vascular nativa do orgão descelularizado com a geração de denso tecido 3D *in vitro*.

Foi também efectuada a optimização do processo de re-celularização, através da perfusão das células com meio de cultura pela rede vascular do orgão descelularizado. A utilização de diferentes velocidades de perfusão da suspensão celular permitiu determinar as condições ideais para a re-celularização destas matrizes acelulares com diferentes tipos de células. Isto permitiu também gerar tecido 3D densamente re-celularizado e com expressão de marcadores hepáticos característicos.

Para melhorar a geração de tecido hepático, foi investigado o uso de células progenitoras humanas obtidas a partir de fígados fetais em combinação com a matriz acelular. O uso destas células progenitoras constitui sem qualquer dúvida um recurso determinante na engenheria de tecidos do fígado e em terapias celulares. Ensaios preliminares revelaram que estas células progenitoras humanas cultivadas em discos de matriz acelular se estabeleceram, proliferaram e diferenciaram em células de linhagem hepática e biliar.

Finalmente, foi investigado o uso de células estaminais humanas obtidas a partir do fluído amniótico em vários modelos de regeneração *in vivo*. As experiências confirmaram a natureza não tumorigénica destas células e demonstraram a sua integração e diferenciação em modelos de lesão muscular e de angiogénese. Adicionalmente, estas células demonstraram capacidade para se fixar em fígados danificados. A plasticidade exibida *in vivo* por estas células é notável e confirma o seu potencial em medicina regenerativa.

Globalmente, nesta dissertação de doutoramento destaca-se a relevância de um novo método de descelularização de orgãos passível de gerar matrizes acelulares derivadas a partir do fígado com preservação de uma rede vascular funcional. A perfusão de meio de cultura e a geração de tecido 3D permite ultrapassar os problemas de difusão de oxigénio e de nutrientes. Por último, a

utilização de células progenitoras humanas, obtidas a partir de fígados fetais em combinação com a matriz acelular, e de células estaminais humanas, derivadas a partir do fluído amniótico, apresentam potencialmente novas oportunidades na engenharia de tecidos do fígado e nas terapias celulares na doença hepática terminal.