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Abstract

Liver transplantation (LT) is the only curative therapy for severe end-stage liver disease. However, organ demand exceeds supply with a consequent increase in the mortality of patients on waiting lists. In the context of graft shortage, several strategies have been explored to increase the number of liver grafts available for transplantation. These include the use of marginal and living donors, split livers, and the improvement of marginal donor grafts (machine perfusion). However, recent advances in the understanding of liver organogenesis, stem cells, and matrix biology provide novel insights in tissue engineering. Today, the newest technologies and discoveries open the door to the development of new methods for organ implementation such as the recellularization of natural scaffolds, liver organoids, bio-printing, and tissue or generation of chimeric organs. These approaches have the potential to generate an unlimited source of grafts (allogenic or chimeric) which could be used in the near future for LT or as a temporary bridge toward LT. This qualitative review focuses on all methods of organ implementation and highlights the newest developments in tissue engineering and regenerative medicine.

Keywords	tissue engineering, extended criteria donors, machine perfusion, organoids, liver transplantation.

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Dear Editor-in-Chief,

We would like to submit as invited paper, by Cesaretti et al, entitled "**From deceased to bioengineered graft: new frontiers in liver transplantation"** for possible publication on the Transplantation Reviews journal.

One of the major challenges in the current transplantation era is organ shortage. Several procedures and pathways have been shown to provide practical and effective solutions to this crisis. These include the utilization of marginal and living donors, the improvement in surgical technique (split livers) and the development of a method able to improve quality of existing donor grafts (machine perfusion). However, recent advances in understanding of liver organogenesis, stem cell and matrix biology provide novel insights in tissue engineering. This one has the potential to generate an unlimited source of grafts (allogenic or chimeric) which could be used in the near future for liver transplantation or as a temporary bridge in liver transplantation. However these news strategies for graft implementation have to be summarized and clearly explained. To this end, in this qualitative review, we focus on all methods for organs implementations and highlighted the newest developments in techniques of tissue engineering and regenerative medicine.

We believe that the paper provides interesting information for the readers of your journal. At the same time we are ready to accept any suggestion that may improve the paper.

Sincerely yours,

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HIGHLIGHTS

- Imbalance between the number of potential recipients of liver transplantation and available donors will not be resolved within the next decades
- Marginal and living donors, split liver and machine perfusion are effective strategies for graft implementation.
- Technology and scientific accomplishments in the past two decades have contributed to the development of other promising methods to reduce organ shortage. Tissue engineering and regenerative medicine is a cross-cutting interdisciplinary field that applies the principle of engineering in life sciences to replace partial or whole damaged tissues or organs with organs that are created artificially in vitro.
- We offer a detailed overview of the current status of art of liver graft implementation focusing the newest developments in tissue engineering

TITLE PAGE

FROM DECEASED TO BIOENGINEERED GRAFT: NEW FRONTIERS IN LIVER TRANSPLANTATION

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Abstract

Liver transplantation (LT) is the only curative therapy for severe end-stage liver disease. However, organ demand exceeds supply with a consequent increase in the mortality of patients on waiting lists. In the context of graft shortage, several strategies have been explored to increase the number of liver grafts available for transplantation. These include the use of marginal and living donors, split livers, and the improvement of marginal donor grafts (machine perfusion). However, recent advances in the understanding of liver organogenesis, stem cells, and matrix biology provide novel insights in tissue engineering. Today, the newest technologies and discoveries open the door to the development of new methods for organ implementation such as the recellularization of natural scaffolds, liver organoids, bio-printing, and tissue or generation of chimeric organs. These approaches have the potential to generate an unlimited source of grafts (allogenic or chimeric) which could be used in the near future for LT or as a temporary bridge toward LT. This qualitative review focuses on all methods of organ implementation and highlights the newest developments in tissue engineering and regenerative medicine.

Key words: tissue engineering, extended criteria donors, machine perfusion, organoids, liver transplantation.

INTRODUCTION

Over the last two decades, major advances have been achieved in liver transplantation (LT) but access to allografts remains the main limitation [1]. This issue has led to the expansion of

deceased donor selection criteria and the utilization of extended criteria allografts which have been historically associated with a high risk of primary non-function (PNF) or delayed graft function (DGF) and consequent high recipient morbidity and mortality [2]. Extended criteria donors (ECD) or marginal livers include donors with steatosis, malignancies, viral infections, older or elderly donors, and donors after cardiac death (DCD). Despite recent encouraging literature resulting in the utilization of ECD allografts [3,4], the need remains and finding innovative ways to increase the pool of available grafts is mandatory. In parallel to the utilization of ECDs, living donors (LDs), and split donors (SDs), new strategies have been developed to improve the quality of existing donor grafts. This review surveys methods for organ implementation, providing an updated view of the current state of the art in the field of liver regenerative medicine. Advantages and limitations of the current approaches to liver tissue engineering are explored, with special emphasis on potential clinical applications.

STATE OF THE ART

An ideal or reference donor is currently defined as having the following criteria: age below 40 years, trauma as the cause of death, donation after brain death, hemodynamic stability at the time of procurement, no steatosis or any other underlying chronic liver disease, and no transmissible disease [5]. A reference donor implies a very low risk of PNF or DGF leading to death or requiring re-transplantation. However, prolonged cold ischemia time (CIT), or surgical technical variants (i.e. split liver allograft) may influence LT outcomes and, thus, an ideal allograft is different from an ideal donor. Contrarily to the ideal donor, at present, there is no precise definition of ECD that is defined as a donor without the reference criteria implying an increased incidence of poor allograft function, allograft failure, or transmission of a donor-derived disease [6].

Donors with abnormal liver function tests, fatty livers, elderly donors, donors with infections, donors with malignancy, and DCD are marginal donors that contribute to the graft pool implementation. Findings of an abnormal liver biochemistry *per se* do not preclude the acceptance of a graft for LT since it is often the result of hemodynamic instability (e.g., high levels of transaminases, which probably indicates a recent ischemic insult commonly due to hypoperfusion or hypoxia as is often seen in patients with cardiorespiratory arrest) [7]. However, underlying conditions such as steatosis may have a synergic effect on hypoperfusion, increasing graft damage [8]. There are no definite guidelines on the upper limit of acceptable abnormal biochemistry, therefore, a careful evaluation of liver function is important especially in the case of a severe pre-morbid donor history.

Among ECD, elderly and steatotic grafts are the most utilized and discussed. Steatosis is one of the most important factors affecting liver allograft function and given the worldwide increase in the prevalence of obesity, a further increase in the prevalence of fatty grafts is expected. Grafts with severe steatosis (>60%) are almost always discarded while the use of grafts with moderate steatosis (30–60%) remains a challenging issue [9]. Nonetheless, some authors from experienced LT centers consider moderate steatosis not a contraindication to graft acceptance [10]. Donor age has been reported to be the strongest factor associated with liver graft failure [5], however, using older livers is the most practical and frequent measure of increasing the liver pool, and thus to reduce waiting list mortality. Aging is characterized by normal progressive declines in functions that, cumulatively, diminish the capacity of cells and organs to respond to intrinsic and extrinsic stimuli. Furthermore, when other factors may impact liver graft function (CIT, cardiac arrest, and altered liver tests), good results can be achieved with these marginal donors [11].

Historically, human immunodeficiency virus (HIV), hepatitis B virus (HBV) and hepatitis C virus (HCV) were considered absolute contraindications for organ donation [12]. Donors with infections should be used where transmission to the recipient is possible (i.e., hepatitis B core antibody

positivity, hepatitis B surface antigen positivity, HCV antibody positivity, and other infections, e.g., human T-lymphotropic virus 1) or in certain circumstances (e.g., when the recipient is already infected with the same agent or the recipient has a critical need and is fully informed of the risks associated with the subsequent donor transmission). Since fibrosis and cirrhosis could develop in the recipient after LT if a small percentage of donors are infected with hepatitis viruses, accurate screening, liver graft biopsy, histology activity index inflammatory grade, and fibrosis scoring are helpful in decision-making [13].

Donors with a documented history of malignancy may play a role in expanding the donor pool. Low-grade malignancies treated many years before donation with a low risk of recurrence or donors with low-grade central nervous system tumors have a low risk of transmission to the recipients. However, any metastatic malignancy in the donor should exclude donation. Guidelines and practices can vary in different countries [14].

DCD is a promising way to increase organ supply [15]. Non-heart beating donors can be uncontrolled or controlled if death occurred or not at admission, respectively [15]. In early reports, a prolonged period of warm ischemia resulted in markedly increased early graft dysfunction in comparison with donation after brain death [16]. However, in the last decade, the improvement of preservation and procurement techniques, and judicious donor selection (i.e. donors age below 40 years and with no steatosis), the use of extracorporeal oxygenation, a short warm ischemia time (less than 15 minutes), and a short CIT have resulted in improved results with an incidence of PNF below 15% and a lower incidence of biliary complications [17, 18, 19].

Another way to expand the liver graft pool is the improvement in surgical techniques such as the use of LDs and SDs in LT. Liver anatomy allows the creation of partial liver grafts from either deceased or LDs. Although the transplant community has come to accept LD as a viable option for both pediatric and adult patients, several ethical issues related to LD still need to be resolved. Moreover, unlike deceased organ donation, LD involves significant risks to the donor. These

include physical consequences related to the partial loss of an organ and the risks associated with organ procurement surgery [20]. The procedure also involves psychological and emotional risks related to the recovery and aftermath of surgery, and its effects on the relationship between donor, recipient, and others [21, 22]. Moreover, death associated with liver donation is not so rare because approximately two in a thousand liver donors actually die [23, 24]. SD, both adult and pediatric, is associated with a significant graft failure because the lower volume of the graft compared with the standard liver volume of the recipient, increases biliary leakage, hepatic artery thrombosis, focal or outflow obstruction in comparison with whole organ transplantation and has higher CIT and technical requirements [25]. However, due to significant technical advances, SD LT currently gives excellent results and can be considered an available method to expand the donor pool [26].

Given the increased vulnerability of the marginal grafts and the potential injury incurred during procurement and storage/transportation, the development of a method able to improve the quality of existing donor grafts is mandatory. Machine perfusion (MP) is an alternative to static cold storage (SCS) (Fig.1). It has three major benefits: the capability to preserve donor organs while providing them with oxygen and nutrients at various temperatures (optimal and prolonged preservation); the ability to recondition and optimize the function of donor organs, particularly, extended criteria organs with, for instance, oxygen perfusion, de-fatting techniques for steatotic livers, and pharmaceutical intervention (organ resuscitation and function recovery); and lastly, to provide the possibility of testing the function and viability of the organ prior to transplantation (ex situ viability testing) [27]. In the last 10 years there has seen an incredible advancement in both experimental and clinical research into donor liver MP that can be divided into three major types: hypo-, subnormo-, or normothermic with temperatures between 0–10°C, 20–33°C, and 35–38°C, respectively. In hypothermic conditions, the rate of metabolism and enzymatic reactions in mammalian cells decrease to rates as low as 20% or even less minimizing preservation injury and improving organ viability [28, 29]. However, in this setting, there is a time-

dependent increase in vascular resistance, which leads to damage to the sinusoidal endothelium [30]. Normothermic MP maintains normal liver function throughout the period of preservation avoiding ischemic injury. Ideally, normothermic perfusion creates a paraphysiological environment but an oxygen carrier is necessary and the graft has to be exposed to cold storage [31, 32]. A subnormothermic environment reduces cell metabolism as well as oxygen consumption, and the low temperature (4°C) avoids damage to the liver [33]. Single or dual vessel perfusion (hepatic artery/portal vein), continuous or pulsatile flow, computerized or manually controlled systems are different technical aspects of MP. During conditioning in MP, viability and quality of the graft can be assessed. Viability evaluation based on lactate clearance criteria is assessed at 4 h. Defatting techniques, with the consequent increased rescue of steatotic livers, is the best result achieved by MP, because grafts treated with a cocktail (consisting of peroxisome proliferator-activated receptor (PPAR)α ligands GW7647 and GW501516, pregnane X receptor (PXR) ligand Hypericin, the constitutive androstane receptor (CAR) ligand Scorparone, the glucagon mimetic cyclic adenosine monophosphate [34] (cAMP) activator forskolin and the insulin-mimetic adipokine visfatin) results in a reduction of 18% in large droplet macrovesicular steatosis [35]. The advantages of MP over SCS in improving graft quality, prognosis, viability assessment, and graft conditioning has been largely reported. However, the ideal perfusion solution, temperature, optimal pressures, flow velocity, numbers of perfusion vessels, the ideal oxygen carrier, rewarming time, and perfusion protocols remain unknown.

PRESENT AND FUTURE ALTERNATIVES FOR LT

Alternatives to LT, such as liver support systems, including bioartificial livers, and hepatocyte transplantation have been extensively explored in the past but none have been adopted in clinical practice [36]. Tissue engineering and regenerative medicine (TERM) is a cross-cutting interdisciplinary field that applies the principle of engineering and life sciences to promote or

enhance the regeneration intrinsic capacity of an organism with the aim of restoring damaged tissue function and/or structure or even to replace partial or whole damaged tissues/organs with artificial organs created in vitro (Fig.2).

Scaffolds play an important role in tissue engineering. They mimic the tissues native environment and support the cells inside the construct. They are made from biomaterials that are fully biocompatible so that no adverse effects are observed once they are implanted in humans [37]. Scaffolds are mainly produced by three approaches: (i) decellularization of live tissues; (ii) synthetic production; and (iii) the 3D-printing/bio-printing of a scaffold or of a seeded tissue/organ from a computer-aided design model. Different approaches are used depending upon the final use of the tissue/organ. However, the scaffold interacts with other cells, supporting cells in a two or three-dimensional environment, by providing anchoring points and by allowing cells to proliferate and/or migrate, and thus requires bioactivity [38]. It would be highly advantageous if the materials can be modified by the cells themselves or by the environment. Biodegradable materials are degraded over time, to be replaced by the cells own extracellular matrix (ECM) [39] as in the case of absorbable sutures or prosthetic materials. Ideally, the scaffold mimics the ECM [40] of the tissue with different characteristics, including biophysical and biochemical properties. The ECM is capable of absorbing and maintaining amounts of water and hydrogels (hydrophilic cross-linked polymer networks). Therefore, they are of interest to different applications in the biomedical field, including soft tissue engineering. Biological hydrogels have been formed from agarose, alginate, chitosan, hyaluronan, fibrin, and collagen, as well as many other materials.

As scaffolds, hydrogels are used to provide bulk and mechanical constitution to a tissue construct, whether cells are adhered to or suspended within the 3D gel framework. When cellular adhesion directly to the gel is favored over suspension within the scaffold, the incorporation of various peptide domains into the hydrogel structure can dramatically increase

the tendency for cellular attachment. In hydrogels, peptides can be incorporated on the surface or throughout the bulk of the gel, and have shown enhanced cellular migration, proliferation, growth, and organization in tissue regeneration applications [41]. Another biophysical property, which can influence cellular behavior, is scaffold stiffness because cells are capable of 'sensing' the stiffness of their microenvironment and respond subsequently [42]. Mesenchymal stromal cells (MSCs), for example, can differentiate toward different cell lineages purely based on the stiffness or elasticity of the substrate [43]. Cells are also influenced by biochemical factors, such as growth factors and cytokines, that are produced and released by specific trigger responses. These molecules can influence cell survival, proliferation and/or differentiation so they are also important for tissue engineering purposes, as they can be used to either differentiate cells, stimulate cells to "recreate" a tissue [44], and/or to mimic the native tissue environment. In fact, the integration of angiogenic growth factors into implantable scaffolds could promote the recruitment of host vessels. For instance, preceding hepatocyte delivery with the implantation of scaffolds that release angiogenic vascular endothelial growth factor (VEGF) enhanced capillary density and improved engraftment in rat liver lobules [45]. Similarly, fibroblast growth factor 2 (FGF2) coated scaffolds served as a supportive environment for mouse ESC-derived hepatocyte inoculation in vivo in a mouse liver failure model [46].

In addition to vascular integration, an improved understanding of multicellular organization and morphogenesis in the liver could also aid in the formation of functional biliary transport systems. Various in vitro models have been developed that exhibit organized bile canaliculi [47] or artificial duct structures, but their incorporation into implantable systems has yet to be fully explored. Although early work demonstrated engrafted bile ducts in ectopic sites [48], the degree to which the biliary tree must be reconstructed has not yet been established; in ectopic cell transplantation experiments the hepatocytes do not appear cholestatic, and biliary products do appear to find their way to the digestive tract. One hypothesis is that the biliary products are redirected or 'leak' into the bloodstream where they circulate and are processed by the remnant

liver into bile. This scenario would argue against the removal of the diseased liver in the setting of transplantable tissue engineered constructs and is consistent with the functional outcome achieved in peritoneal transplantation of mature hepatocytes and hepatocyte-like cells that lacked biliary networks [49].

In the UK, over 40% of the grafts offered for LT are declined because of criteria or co-morbidities judged beyond marginal criteria [50]. This provides a major opportunity to explore alternative uses of human livers found to be unsuitable for LT following organ procurement. In particular, while cellular viability is easily compromised, ECM is better maintained in discarded livers and it may be used as scaffold for normal human liver cells and to recreate functional human liver tissue in vitro. A major advantage of using the liver ECM as a scaffold for tissue engineering purposes is that all structural and functional components of the ECM, which make liver micro-environments tissue-specific, are present within the scaffold [51]. The liver ECM can be obtained by decellularization (Fig. 3). During this process, all cellular components are removed from the ECM, without damaging the ECM itself. Decellularization of the left liver can be completed within 14 days of perfusion while 6 weeks are necessary for a whole human liver. Decellularization can be achieved using various methods. It has been done for murine [52], porcine [53], and human liver [54] and different decellularization protocols have been described but all use a combination of chemical and enzymatic methods. The decellularization chemical protocol, based on a retrograde perfusion through the hepatic venous system, is characterized by the combination of different cell-damaging factors (CDFs): i) mechanical cell-damaging (freezing/thawing) to favor cell destruction; ii) isotonic stress to allow cell lysis; iii) enzymes to allow cell detachment; iv) action of detergents to remove debris; and v) flow shear stress to allow penetration into the hepatic sinusoid leading to the detachment of cells and debris. Once decellularization is complete, human liver scaffolds can be dissected by scalpel cleavage to obtain liver cubes utilized for 3D-platform for biocompatibility and bioengineering studies. Tissues are

considered decellularized when no DNA fragment larger than 200 base pairs remain within the matrix. The resulting decellularized liver matrix is biocompatible and biodegradable but the most important advantage of utilizing the whole organ scaffold is that the architectural layout and ECM of other tissue types, such as the biliary tree and the vasculature network, are present avoiding the need to add them to the scaffold. Because of all these reasons, the liver ECM is the best scaffold for liver tissue engineering. A liver scaffold decellularized can be repopulated [55] (partially or completely) with functional cells using different methods and with different goals (2D coating, 3D hydrogel, or liver organoid proliferation). The latter could be used for an auxiliary partial LT [56]. Moreover, human hepatocytes could be added to a swine ECM resulting in the development of a chimeric liver (CL). In 2013, Hata et al. [57], investigated its feasibility, developing a rodent model of CL by repopulation of rat hepatocytes in a mouse and successfully transplanted the auxiliary CL into a rat recipient with vessel reconstruction. However, utilization of xenogeneic (porcine) scaffolds raise concerns about surgical technique and xenozoonosis such as porcine endogenous retrovirus [58]. Nevertheless, cautious and longer investigations to secure human patient safety are indispensable.

The recellularizing of a liver scaffold is a complex process. Cells have to migrate in the vascular tree and bile ducts without damaging the scaffold. While attempts using murine and porcine scaffold are ongoing, upscaling whole human organ scaffolds remains a challenge [52]. Moreover, ensuring that the cells end up in the right location is a further challenge. Hepatocytes, for example, may be injected in vessels into the empty ECM or as cholangiocytes via bile ducts. In the first case, clogging of the blood vessels with hepatocytes and consequent thrombosis after transplantation of the organoid may occur. The vascular system and biliary tree also need to be fully repopulated with a layer of endothelium and cholangiocytes, respectively. The integrity of the vessels barrier and biliary ducts is fundamental to separating the structures and to ensure functionality of the recellularized scaffold. Furthermore, different types of cells in specific ratios

are required. For example, hepatocytes are required in large quantities. The cells from organoids can either be differentiated toward hepatocytes or cholangiocytes in vitro before they are injected into the liver graft. However, they can be injected as undifferentiated organoids or as a mixture of both differentiated and undifferentiated cells with a certain ratio.

A hepatocyte transplantation involves the injection of cells obtained from healthy donors into the diseased liver of the recipient and the donor cells are maintained and expanded in vitro. However, since it is estimated that a human liver contains approximately 300 billion cells, the first challenge is to obtain a sufficient number of hepatocytes from healthy donors [59, 60]. Moreover, when hepatocytes are cultured on relatively hard plastic in vitro, they quickly dedifferentiate and lose their functionality [61]. Potential alternatives include pluripotent and adult stem cells with the potency to differentiate into functional hepatocytes. Induced pluripotent stem cells, which can potentially be differentiated into cells with hepatocyte-like morphology and function, are promising. However, these so-called iHEPs do not fully mimic all the characteristics of hepatocytes [62]. MSCs are another source of adult stem cells that may be used in an alternative transplantation approach and are known to prevent or reduce ischemia/reperfusion injury in donor livers [61]. MSCs show enough plasticity to be differentiated into hepatocyte-like cells [63] but, at present, they cannot be differentiated into fully functional hepatocytes [64].

Cells are paramount in creating functional tissue constructs in vitro. Since the liver is involved in a lot of complex functions such as homeostasis, glucose and lipid metabolism, detoxification, production of serum proteins, and secretion of bile, different cell types are present in the liver of an adult human. The majority of these cells are hepatocytes [65] (one-third of the liver in cell number and 70–85% of the liver volume). Non-parenchymal cells exist as cholangiocytes, endothelial cells (which create a barrier between the parenchyma and blood), Kupffer cells, and stellate cells [65] (important for liver immunity and its response to damage). In order to create a

functional tissue construct to replace the damaged liver, all cell types have to be obtained, expanded, and seeded within the scaffold material. To obtain a wide number of cells, different culture platforms are being developed [66]. These might increase the primary hepatocyte yield, however, these approaches are laborious and are still not able to supply sufficient numbers of functional cells. Therefore, other potential cell sources should be considered. Hepatic cell lines, (adult) stem cells and/or progenitor cells, are interesting alternatives that could be expanded in vitro and differentiate into all liver cell lines in order to replace primary hepatocytes, cholangiocytes, and other cell types of the human liver [67]. Although fewer cells are required to repopulate the graft, extensive proliferation and differentiation are still needed before they become functional hepatocytes. Recently, a new 3D culture method has been established for the long-term expansion of liver-derived stem cells. These stem cells self-organize into so-called liver organoids, which are transplantable structures because they retain many characteristics of the original epithelial architecture [68].

The first organoid culture system was developed almost ten years ago when a 3D long-term culture was established from murine small-intestinal stem cells, which closely resembled crypt-villus units. These intestinal stem cells were marked by the expression of the leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5). The Lgr5-positive cells were shown to be multipotent stem cells able to form all cell types of the intestinal epithelium by lineage tracing [69]. These adult stem cells were cultured in a specific mouse-derived hydrogel in vitro, which allowed cells to organize into 3D crypt-villus units containing both self-renewing stem cells and differentiated cells of all intestinal epithelial lineages. Adaptations to this culture method have allowed organoid cultures from many stem cell sources and, to date, several 3D cell culture systems are currently available to create liver organoids [69]. In general, these systems display better physiologic and metabolic aspects of intact liver tissue than 2D culture systems. However, none of these systems reliably mimic human liver development, including the parallel formation of hepatocyte and cholangiocyte anatomical structures. However, such models of tissue

development have important applications in the discovery and treatment of human diseases. For example, the Gunn rat model of inherited bilirubin-UGT deficiency, such as the Crigler–Najjar syndrome [70], and the inv-mouse (partial deletion of the inversin gene) model of biliary atresia [71] have been particularly helpful in the study of hepatic and biliary diseases, respectively. However, these models are not optimal for the study of human-specific congenital diseases and corresponding new therapeutic targets, due to differences in liver fetal development between species. Hepatocyte maturation is a dynamic process highlighted by changes in the levels of various cytokines and transcription factors associated with differentiation and maturation of hepatoblasts into hepatocytes. These disease models can be used to study disease mechanisms and discover new diagnostic, therapeutic, and prognostic approaches. Besides providing a better model for human liver development, the liver organoids may be used for drug development and toxicity screening applications [72].

Organoid cultures were also initiated from human adult liver tissue. They have an extensive proliferative capacity, having the potential to give rise to approximately one million cells from one single stem cell within two months. Secondly, they have proven to have great genetic stability as demonstrated by the karyotypic analysis of chromosome numbers and detailed sequencing of the whole genome which confirmed stability over time [73]. This is in contrast to other hepatocyte-like culture systems, such as induced pluripotent stem cells, which are prone to acquire genetic variations in vitro [74]. Thirdly, organoids are bipotent (hepatocyte and cholangiocyte cell lines). Differentiation in vitro requires a change in the composition of the medium, after which the cells differentiate toward either the hepatocyte or cholangiocyte fate. Upon hepatocyte differentiation, besides acquiring hepatocyte morphology and upregulation of classic hepatocyte markers, organoids gain some hepatocyte function as well. They were shown to take up glycogen and LDL and produce albumin and bile acid salts, although to a lesser extent than primary hepatocytes. Organoid differentiation toward cholangiocytes is less established. However, differentiation toward a cholangiocyte-like cell with corresponding phenotype and

function has been obtained with induced pluripotent stem cells. During differentiation, these cells are switched to organoid culture conditions to facilitate the final maturation toward cholangiocyte-like cells, suggesting that organoid-forming cells are quite capable of differentiating toward the cholangiocyte fate [75]. Finally, adult tissue-derived organoids retain more commitment to their tissue of origin. This is in contrast to embryonic-type and induced pluripotent stem cells which are omnipotent and not committed to a particular tissue or organ type.

Another method of bioengineering that works for solid tissues, such as the liver, is bio-printing. In the past 10 years, 3D printers have been actively adapted to be compatible with manipulation of living mammalian cells developing patterned 2D cultures and 3D tissue structures in which multiple distinct cell types can be organized in a space relative to each other per user specifications. The bio-ink material is crucial because it provides a spectrum of biochemical (i.e., chemokines, growth factors, adhesion factors, or signaling proteins) and physical (i.e., interstitial flow, mechanical and structural properties of extracellular matrix) cues which promote a favorable environment for cell survival, motility, and differentiation [76]. In TERM, scaffolds could be fabricated by biomaterials and serve as ECM. In an earlier work, a 3D hepatocyte/gelatin construct was printed from a 38-layer assembly [77]. The laminated hepatocytes remained viable and performed biological functions in the construct for more than two months. Recently, metabolically active, anatomical, 3D hepatic tissues have also been developed successfully [78]. However, organs such as the liver have highly complex architectures and properties and they may require a combination of several bio-printing techniques along with specifically designed bio-inks to introduce structural heterogeneity and functionality. Although it is attractive, bio-printing currently remains an arduous challenge but this technology has already demonstrated a remarkable potential for future development and the 3D scale-up of functional organs.

In conclusion, a worldwide shortage of liver grafts available for LT has led scientists to develop other promising therapies. ECDs and regenerative medicine are future solutions. In TERM, decellularized livers constitute a good option for obtaining a scaffold because the vascular and biliary architecture is well preserved. Moreover, in decellularized livers, the ECM maintains both viable and functional hepatocytes and cholangiocytes. This approach has the potential to generate an unlimited source of grafts (allogenic or chimeric), to provide patients with better timing for the procedure and to improve patient quality of life after surgery. Moreover, they could be used in the near future as a temporary bridge in LT (e.g., auxiliary partial orthotopic or heterotopic transplantation of the engineered liver graft) until an allograft becomes available. However, difficulties in engraftment and scaffold repopulation need to be resolved and problems with xenozoonosis and rejection still persist. Combined efforts in research from different specialists (surgeons, hepatologists, pathologists, and bioengineers) have the potential to achieve future clinical success.

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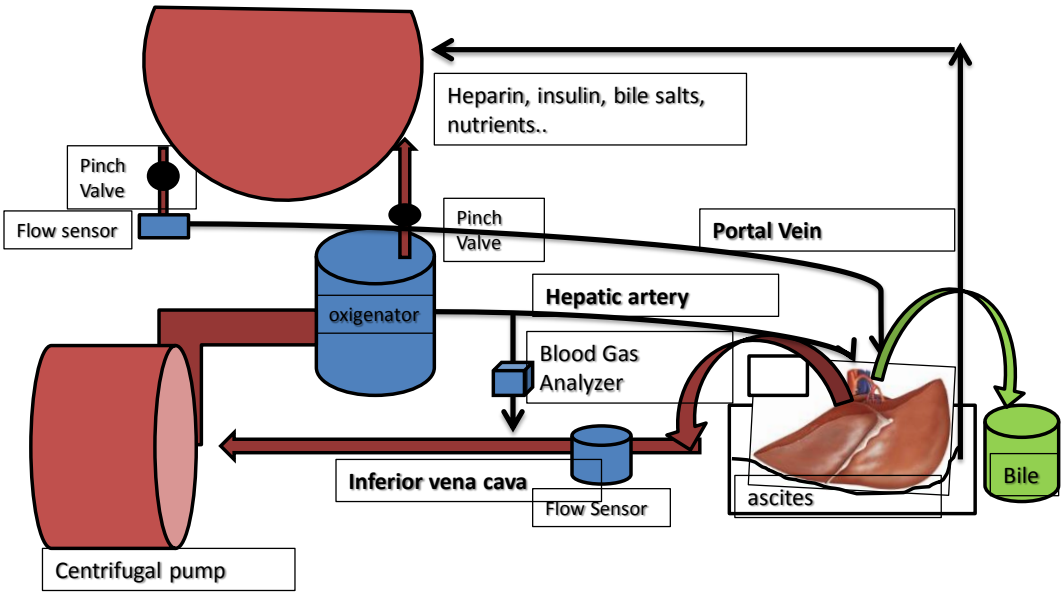
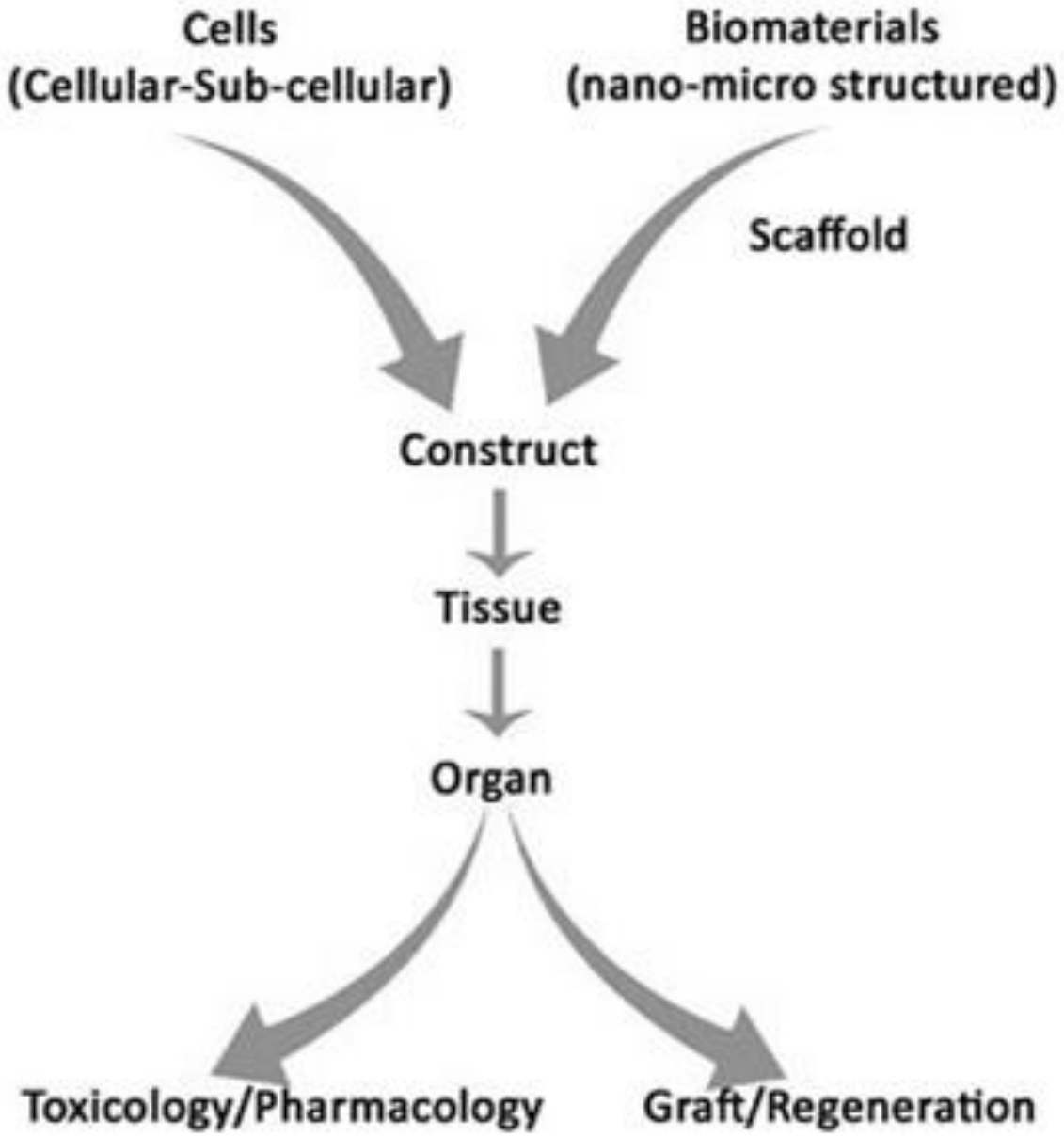
Fig.1 Schematic view of machine perfusion for liver graft.

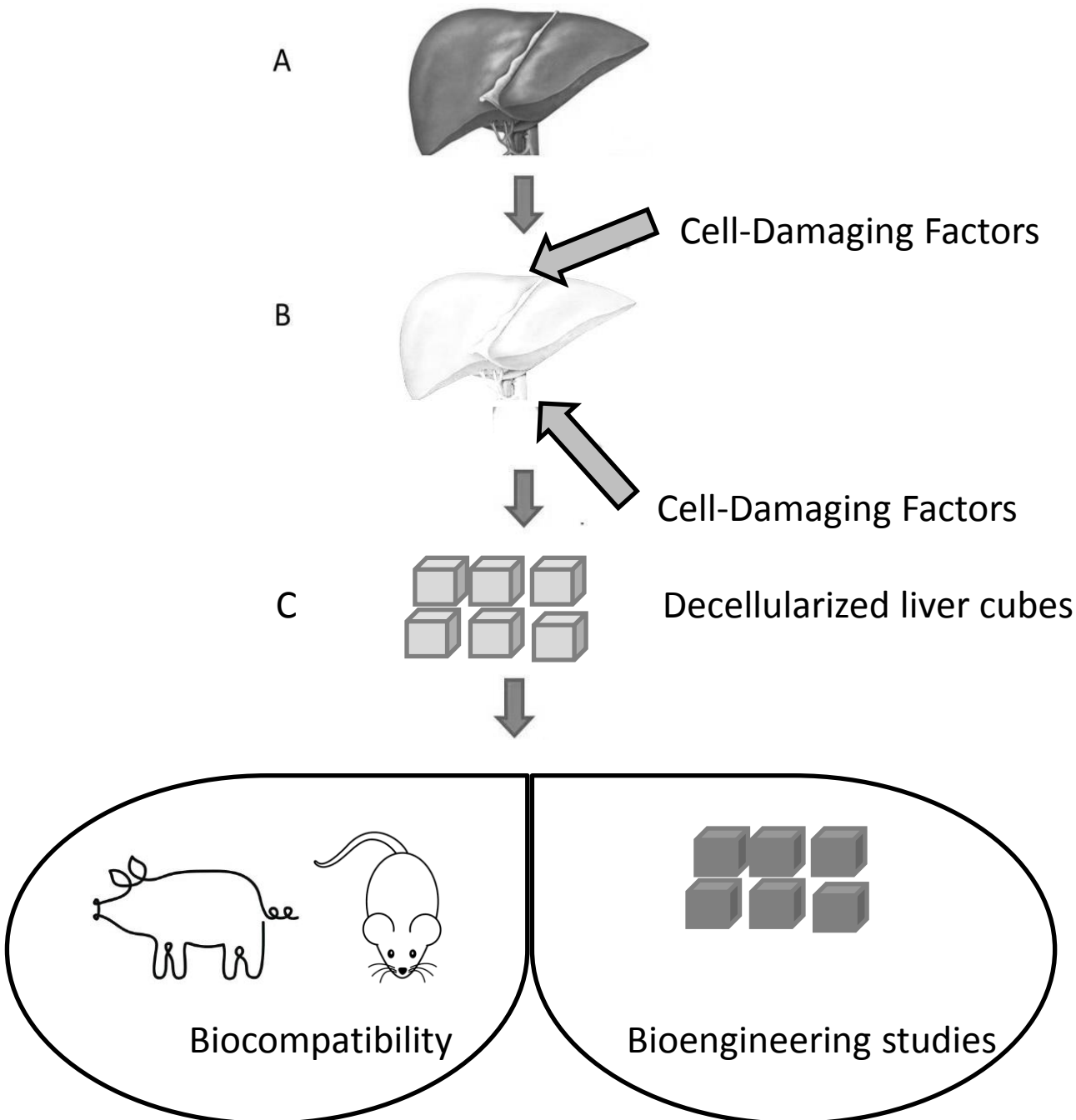
Fig.2 Schematic view of the tissue engineering and regenerative medicine (TERM) steps and possible finale use of the obtained construct, tissue or organ (Teodori_et_al Journal_of_Biophotonics 2017. Reproduced with permission [79]

Figure. 3 Decellularization of a whole liver (A). The decellularization chemical protocol, based on a retrograde perfusion through the hepatic venous system, was characterized by the

combination of the different Cell-Damaging Factors (B). Decellularized whole or split liver (B) is translucent because of the dissolution of cells. Once decellularization is completed, human liver scaffolds are dissected by scalpel cleavage to obtain liver cubes (C) utilized for 3D-platform for biocompatibility and bioengineering studies (D, E).


Heparin, insulin, bile salts, nutrients..
Pinch Valve
Flow sensor
Pinch Valve
Portal Vein
oxigenator
Hepatic artery
Blood Gas Analyzer
Inferior vena cava
Flow Sensor
ascites
Bile
Centrifugal pump


Cells
(Cellular-Sub-cellular)
Biomaterials
(nano-micro structured)
Scaffold
Construct
Tissue
Organ
Toxicology/Pharmacology
Graft/Regeneration


A
Cell-Damaging Factors
B
Cell-Damaging Factors
C
Decellularized liver cubes
Biocompatibility
Bioengineering studies